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**Engenharia de uma endopoligalacturonase por inserção de
diferentes CBMs (*Carbohydrate Binding Modules*): Quimerogênese
e efeito de proximidade**

Sibeli De Carli

Tese apresentada à Faculdade de Filosofia,
Ciências e Letras de Ribeirão Preto da USP,
como parte das exigências para a obtenção
do título de Doutora em Ciências, Área:
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Engenharia de uma endopoligalacturonase por inserção de diferentes CBMs (*Carbohydrate Binding Modules*): Quimerogênese e efeito de proximidade

Engineering of an endopolygalacturonase by insertion of different Carbohydrate Binding Modules (CBMs): Chimerogenesis and proximity effects

Sibeli De Carli

Orientador: Prof. Dr. Richard John Ward

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*Aos meus pais, Irineu e Acileide, que
são um grande exemplo de vida e sempre
me forneceram apoio incondicional,
incentivo, carinho e amor, dedico este
trabalho.*

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“Faça as coisas o mais simples que você puder, porém não se restrinja às mais simples”

Albert Einstein

RESUMO

O grande excedente de biomassa proveniente da agroindústria pode ser aproveitado para a produção de bioprodutos com alto valor agregado. Entre as estratégias para melhorar o aproveitamento e reduzir os custos relacionados com a hidrólise enzimática destes resíduos lignocelulósicos incluem-se a identificação e expressão heteróloga de enzimas com propriedades catalíticas interessantes, a utilização de técnicas de engenharia de proteínas, para melhorar as características das enzimas já identificadas e por fim, a formulação de coquetéis enzimáticos mais eficientes. A desconstrução precisa desta matriz lignocelulósica é bastante desafiadora e constitui uma barreira importante para alcançar uma produção razoável de bioprodutos a preços atraentes. Neste sentido, é conhecido que a presença de pectinas, um dos principais componentes da lamela média nas plantas, dificulta a hidrólise da parede celular vegetal e impede a liberação eficiente de açúcares, sendo então, necessário o desenvolvimento de tecnologias visando otimizar a hidrólise deste polissacarídeo. O presente trabalho visou avaliar as propriedades bioquímicas e cinéticas de uma endopoligalacturonase de *Chondrostereum purpureum* (EndoPGCp) expressa em *Pichia pastoris* e em *Escherichia coli*, bem como avaliar o efeito da imobilização sobre a atividade da enzima. Além disso, foi proposto o uso da quimerogênese para avaliar o efeito da proximidade de diferentes polissacarídeos presentes na parede celular vegetal sobre a atividade de uma EndoPGCp_bac, engenheirada pela adição de um módulo de ligação a carboidrato (CBM), bem como o potencial de aplicação na suplementação de um coquetel enzimático comercial para hidrólise de diferentes resíduos lignocelulósicos. A enzima expressa em *P. pastoris* apresentou uma massa molecular de cerca de 60,0 kDa, com um teor estimado de carboidratos de 67,0% (m/m). A temperatura ótima e o pH da catálise foram 60 - 70 °C e pH de 4,5, respectivamente. A enzima foi altamente estável em pH 6,0 - 8,0 e reteve cerca de 60% de sua atividade inicial após incubação a 70 °C por 30 min. A enzima hidrolisou pectina cítrica com $V_{\max}$ e K_M de $4947,10 \pm 393,63 \text{ U.mg}^{-1}$ e $2,45 \pm 0,23 \text{ mg.mL}^{-1}$, respectivamente, e apresentou eficiência catalítica de $2052,90 \pm 193,54 \text{ mL.mg}^{-1} \text{ s}^{-1}$. Além disso, a enzima foi eficiente na clarificação de sucos de maçã e maracujá. Após a imobilização em nanopartículas ferromagnéticas, a enzima não apresentou alterações significativas em suas características bioquímicas e cinéticas e manteve cerca de 65% da atividade inicial após 7 ciclos de reuso. Na suplementação do coquetel comercial Celluclast, a EndoPGCp_Pp aumentou em 1,8 vezes a liberação de açúcares redutores de resíduos de laranja. Além disso, as enzimas expressas em *E. coli* foram reenoveladas a partir dos corpos de inclusão e processo de quimerogênese com os CBMs não afetou as propriedades da enzima. As enzimas hidrolisaram a pectina cítrica com $V_{\max}$ e K_M muito semelhantes. No entanto, significativo aumento foi observado no k_{cat} , uma vez que as quimeras 3N, 44N e 77N apresentaram valores 1,4, 1,6 e 1,3, vezes maiores, respectivamente, que o obtido para o domínio catalítico isolado. Como resultado, a eficiência catalítica (k_{cat}/K_M) para a hidrólise da pectina de citros foi cerca de 60, 75 e 30% maior para as quimeras 3N, 44N e 77N, respectivamente, em comparação ao obtido para a EndoPGCp_bac. Quando aplicadas na suplementação de um coquetel comercial para hidrólise de resíduos lignocelulósicos, os resultados indicaram que as pectinases podem melhorar a eficiência da sacarificação da biomassa pela remoção da pectina que impedem a ação de outras enzimas. Além disso, a maior eficiência na hidrólise das enzimas quiméricas pode estar relacionada à ação dos CBMs, que podem aumentar muito a concentração da enzima nas proximidades do substrato, levando ao aumento na hidrólise da pectina e, conseqüentemente, diminuem a recalcitrância da parede celular vegetal à hidrólise enzimática.

Palavras chaves: Endopoligalacturonase; Módulos de ligação a carboidratos; quimerogêneses; efeito de proximidade; bioenergia.

ABSTRACT

The large biomass surplus from agribusiness activity can be used to produce bio-products with high added value. Strategies to improve utilization and reduce costs related to the enzymatic hydrolysis of these lignocellulosic residues include the identification and heterologous expression of enzymes with interesting catalytic properties, the use of protein engineering techniques, to improve the characteristics of the enzymes already identified and finally, the formulation of more efficient enzyme cocktails. The controlled deconstruction of the lignocellulosic matrix is technically challenging and constitutes major barrier for a reasonable production of bioproducts at attractive prices. It is known that the presence of pectins, one of the main components of the medium lamella in plants, hinders the hydrolysis of the plant cell wall and prevents the efficient release of sugars, so it is necessary to develop technologies to optimize the hydrolysis of this polysaccharide. The present work aimed to evaluate the biochemical and kinetic properties of an endopolygalacturonase from *Chondrostereum purpureum* (EndoPGCp) expressed in *Pichia pastoris* and *Escherichia coli*, as well as to evaluate the effect of immobilization on the activity of the enzyme. In addition, the use of chimerogenesis was proposed to evaluate the effect of the proximity of different polysaccharides present in the plant cell wall on the activity of an EndoPGCp_bac, engineered by the addition of carbohydrate binding modules (CBM), as well as the potential for application in the supplementation of a commercial enzymatic cocktail for hydrolysis of different lignocellulosic residues. The enzyme expressed in *P. pastoris* had a molecular mass of about 60.0 kDa, with an estimated carbohydrate content of 67.0% (w/w). The optimum temperature and pH of the catalysis were 60 - 70 ° C and pH 4.5, respectively. The enzyme was highly stable at pH 6.0 - 8.0 and retained about 60% of its initial activity after incubation at 70 ° C for 30 min. The enzyme hydrolyzed citric pectin with $V_{\max}$ and K_M of $4947.10 \pm 393.63 \text{ U.mg}^{-1}$ and $2.45 \pm 0.23 \text{ mg.mL}^{-1}$, respectively, and presented catalytic efficiency of $2052.90 \pm 193.54 \text{ mL.mg}^{-1}.\text{s}^{-1}$. In addition, the enzyme was effective in clarifying apple and passion fruit juices. After immobilization on ferromagnetic nanoparticles, the enzyme showed no significant changes in its biochemical and kinetic characteristics and maintained approximately 65% of the initial activity after 7 cycles of reuse. In supplementing the commercial cocktail Celluclast, EndoPGCp_Pp increased the release of sugars reducing of orange residue by 1.8 times. In addition, the enzyme expressed in *E. coli* was refolded from the inclusion bodies and the process of chimerogenesis with CBMs did not affect the properties of the enzyme. The enzymes hydrolyzed the citrus pectin with very similar $V_{\max}$ and K_M . However, a large increase was observed in the k_{cat} , since the chimeras 3N, 44N and 77N showed values 1.4, 1.6 and 1.3, times higher, respectively, than that obtained for the isolated catalytic domain. As a result, the catalytic efficiency (k_{cat}/K_M) for the hydrolysis of citrus pectin was about 60, 75 and 30% higher for chimeras 3N, 44N and 77N, respectively, compared to that obtained for EndoPGCp_bac. When applied in the supplementation of a commercial cocktail for hydrolysis of lignocellulosic residues, the results indicated that pectinases can improve the efficiency of the biomass saccharification by removing the pectin that prevent the action of other enzymes. In addition, the improved hydrolytic efficiency by chimeric enzymes may be related to the action of CBMs, which can greatly increase the concentration of the enzyme in the vicinity of the substrate, leading to an increase in pectin hydrolysis and, consequently, a decrease in the recalcitrance of the plant cell wall to enzyme hydrolysis.

Key words: Endopolygalacturonase; Carbohydrate binding modules; chimerogenesis; proximity effect; bioenergy

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LISTA DE ABREVIATURAS E SIGLAS

A₄₂₀: Absorbância à 420 nm

AB-NTA: Sal dissódico N- (5-amino-1-carboxipentil) iminodiacético monohidratado

AIR: *Alcohol insoluble residue*

Avicel: Celulose microcristalina (Fluka Chemical Co., Seelze, Alemanha)

BCIP: Fosfato de 5-bromo-4-cloro-3-indolil

BSA: Soralbumina bovina

CAZy: Carbohydrate Active enZyme

CBD: *Cellulose Binding Domain*

CBMs: *Carbohydrate Binding Modules*

CD: *Circular dichroism*

CHAPS: 3-[(3-colamidopropil)-dimetil-amônio]-1-propano-sulfonato

CMC: Carboximetilcelulose

CPEC: *Circular polymerase extension cloning*

DNS: Ácido dinitrosalicílico

dNTP Mix: Solução contendo 2 mmol.L⁻¹ de cada um dos dNTP

DMSO: Dimetil sulfóxido

DO: Densidade óptica

EDTA: Ácido etileno diaminotetracético

GH: Glicosil hidrolases

Hepes: Ácido 4-(2-hidroxietil)-1-piperazinetanolsulfônico

IMAC: *Immobilized metal-ion affinity chromatography*

IPTG: Isopropil-β-D-tiogalactopiranosídeo

K_M: Constante de Michaelis–Menten

NBT: Nitroazul de tetrazólio

NCBI: *National Center for Biotechnology Information*

Ni-NTA: *Nickel-nitrilotriacetic acid*

NPMs: Nanopartículas paramagnéticas

PAGE: Eletroforese em gel de poliacrilamida

PDB: *Protein Data Bank*

SEC: *Size Exclusion Chromatography*

SDS: Dodecil sulfato de sódio

T₆₆₀: Transmissão à 660 nm

Tris: Tris(hidroximetil)aminometano

U: Unidade de atividade enzimática

UFC: Unidades formadoras de colônias

V_{max}: Velocidade máxima

YNB: Base nitrogenada para leveduras sem aminoácidos e sulfato de amônio

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Capítulo 1

Introdução

1.1. A biomassa lignocelulósica

A preocupação da sociedade atual com as questões ambientais e a sustentabilidade tem aumentado muito nas últimas décadas. A substituição de combustíveis fósseis por biomassa lignocelulósica, bem como de resíduos da agroindústria, como matéria-prima bruta para a produção de produtos químicos e combustíveis é uma opção interessante para responder a essas preocupações (ADILETTA et al., 2020; CHEN; LIU, 2017; KAYALVILI, 2017; KENNES et al., 2016; KUMAR et al., 2016; SAINI; SAINI; TEWARI, 2015) e representa a força motriz no fortalecimento do conceito emergente de biorrefinaria (ADILETTA et al., 2020; SINGHVI; GOKHALE, 2019).

Isso porque, a utilização da biomassa lignocelulósica, no conceito de biorrefinaria, tem sido a alternativa mais promissora, considerando sua grande disponibilidade e preço atraente (ADILETTA et al., 2020; ZOGHLAMI; PAES, 2019; SINGHVI; GOKHALE, 2019; AREVALO-GALLEGOS et al., 2017; KAYALVILI, 2017; KUMAR et al., 2016). De modo geral, a biomassa lignocelulósica é composta principalmente por lignina, celulose, hemiceluloses, pectinas (Fig. 1), além de ceras, sais minerais e proteínas. No entanto, a proporção e ocorrência destes compostos pode variar com o tipo de planta, tecido vegetal ou até mesmo com a idade da planta (LOIX et al., 2017).

Em um tecido vegetal, quando as células vegetais ainda estão se alongando, elas são envoltas pela parede celular primária, cujo principais componentes são polissacarídeos - microfibrilas de celulose associadas à hemiceluloses e pectinas (Fig. 1, A). Uma vez que o alongamento celular cessa, uma parede secundária é depositada em alguns tipos de células. As paredes secundárias também consistem em polissacarídeos - principalmente celulose, hemiceluloses e pectina) – além da presença de lignina (Fig. 1, B) (LOQUÉ; SCHELLER; PAULY, 2015).

Figura 1. Modelos esquemáticos de paredes celulares vegetais.

A ênfase é colocada na composição da parede ao invés da arquitetura. (A) Paredes celulares primárias. (B) Paredes celulares secundárias. Figura adaptada de Lóque e colaboradores (2015).

A lignina é um heteropolímero aromático que se encontra associado aos polissacarídeos presentes na parede celular vegetal, conferindo rigidez, impermeabilidade e resistência a ataques microbiológicos e mecânicos aos tecidos vegetais. É um heteropolímero amorfo muito complexo e constituído por unidades de fenilpropano, sintetizadas a partir de três álcoois *p*-hidróxi-cinâmicos precursores: *p*-cumarílico, coniferílico e sinapílico (SINGHVI; GOKHALE, 2019; TOUSHIK et al., 2017; ZOGHLAMI; PAËS, 2019).

A celulose é o principal componente (40 a 60% em massa) da parede celular vegetal. Este polímero é constituído por uma cadeia não ramificada de monômeros de D-glicose unidos entre si por ligações O-glicosídicas do tipo β -1,4 (SINGHVI; GOKHALE, 2019; SUN et al., 2016; ZOGHLAMI; PAËS, 2019). As cadeias de celulose, paralelas entre si, organizam-se em microfibrilas por meio de ligações de hidrogênio, formando uma estrutura supramolecular cristalina, de alta resistência tensional e com poucas regiões amorfas (SHARMA; PATEL; SUGANDHA, 2017; SUN et al., 2016; ZOGHLAMI; PAËS, 2019). A união de várias microfibrilas dão origem às fibrilas, as quais são incorporadas em uma matriz amorfa, constituída por polissacarídeos não celulósicos, em especial as hemiceluloses, além de lignina (DAMM et al., 2016; LOQUÉ; SCHELLER; PAULY, 2015; SHARMA; PATEL; SUGANDHA, 2017; ZOGHLAMI; PAËS, 2019).

As hemiceluloses representam cerca de 20 a 35%, em massa da parede celular vegetal, e constituem um grupo heterogêneo de biopolímeros, estruturalmente diversos que se associam à celulose por diferentes interações químicas, formando também ligações covalentes com a lignina e com as pectinas, favorecendo a adesão e coesão das fibras, a manutenção da integridade da parede celular vegetal e a proteção da celulose contra a ação de celulasas (BEG et al., 2001; DAMM et al., 2016; JORDAN et al., 2012; SHARMA; KUMAR, 2013; SINGHVI; GOKHALE, 2019; TOUSHIK et al., 2017). Sua cadeia principal é composta por pentoses (principalmente D-xilose, L-arabinose) e/ou hexoses (D-manose, D-glicose ou D-galactose) unidas entre si por ligações glicosídicas do tipo β -1,4 e frequentemente apresentam ramificações (DEUTSCHMANN; DEKKER, 2012; LOQUÉ; SCHELLER; PAULY, 2015). As ramificações podem conter ácidos urônicos (ácidos α -D-glucurônico, α -D-4-O-metil-galacturônico e α -D-galacturônico), ácido ferúlico e outros açúcares, como arabinose e galactose. Além disso, monossacarídeos da hemicelulose podem ser

parcialmente substituídos por grupos acetil (DEUTSCHMAN e DEKKER, 2012; SUN et al., 2016).

As pectinas representam um grupo de polissacarídeos estruturalmente heterogêneos amplamente distribuídos nas paredes celulares primárias e na lamela média de plantas superiores (DRANCA; OROIAN, 2018). Este polissacarídeo apresenta três tipos principais (EDWARDS; DORAN-PETERSON, 2012; MOHNEN, 2008):

- 1- A homogalacturonana, principal tipo de pectina, é um homopolímero constituído por longas cadeias de unidades de ácido galacturônico unidos por ligações α -(1,4), que podem estar parcialmente esterificadas, razão pela qual são mais resistentes à hidrólise. Unidades de ramnose podem ser encontradas ocasionalmente neste polímero (BRETT et al., 1990; WANG et al., 2018; WILLATS; KNOX; MIKKELSEN, 2006).
- 2- A ramnogalacturonana do tipo I é o segundo polissacarídeo mais importante das pectinas e consiste em uma cadeia principal formada por repetições de unidades de dissacarídeos de ácido galacturônico e ramnose. As cadeias laterais são formadas por diferentes açúcares, principalmente arabinose e galactose, ligados diretamente às unidades de ramnose (ALBUQUERQUE et al., 2016; CARPITA; GIBEAUT, 1993).
- 3- A ramnogalacturonana do tipo II é um polissacarídeo complexo de baixa massa molar e representa cerca de 10% das pectinas (O'NEILL et al., 2004). Formada principalmente por uma cadeia principal de ácido galacturônico unidos por ligações do tipo α -(1,4), com ramificações de diferentes açúcares, como ramnose e galactose, além de alguns açúcares incomuns como apiose e ácido acérico (VIDAL et al., 2000).

Figura 2. Estrutura esquemática das pectinas.

A abundância relativa dos diferentes tipos de pectina varia, mas a homogalacturonana e a ramnogalacturonana do tipo I são considerados os componentes principais, enquanto a ramnogalacturonana II aparece em menor proporção. Figura adaptada de Albuquerque et al., 2016.

Uma particularidade na estrutura das substâncias pécicas é a esterificação por grupos metil (na posição C-6) e/ou grupos acetil (em O-2 e/ou O-3) de resíduos de ácido galacturônico na cadeia contínua de ácido galacturônico da homogalacturonana (DRANCA; OROIAN, 2018). Além disso, o grau de esterificação (GE) é frequentemente usado para classificar as pectinas em duas categorias: pectinas com alto teor de metoxilas (ATM, GE > 50%) e pectinas com baixo teor de metoxila (BTM, GE < 50%). O GE é um parâmetro importante, pois influencia no mecanismo de gelificação, nas condições de processamento e nas propriedades da pectina (DRANCA; OROIAN, 2018; NOREEN et al., 2017).

Além das pectinas representarem uma classe de polissacáridos responsáveis pela adesão das células vizinhas, estes carboidratos também controlam a penetração de enzimas na biomassa, uma vez que as pectinas são responsáveis pela determinação da porosidade da parede celular (BARON-EPEL et al., 1988), limitando o tamanho e as dimensões das enzimas com capacidade de penetrar na parede

(LATARULHO et al., 2016). Esses fatores têm estimulado o recente interesse na remoção de pectina para converter a biomassa vegetal em açúcares fermentáveis para a síntese de bioprodutos.

1.2. As enzimas envolvidas na hidrólise das pectinas

Como consequência da complexidade do substrato polissacarídico, a hidrólise enzimática da pectina envolve um conjunto de múltiplas enzimas, conforme esquematizado na Figura 3.

Figura 3. Estrutura esquemática das pectinas e locais de ação das enzimas pectinolíticas.

Enzimas envolvidas na hidrólise da homogalacturonan e rhamnogalacturonan I. A rhamnogalacturonan II não é apresentada, uma vez que não é conhecido ser um substrato para a degradação microbiana. Figura adaptada de Benz e colaboradores (2014).

No caso da homogalacturonana, de acordo com o modo de ação e a preferência do substrato, as pectinases são classificadas em dois grupos, nomeadamente (i) esterases de pectina (EC - *Enzyme Commission Numbers*: 3.1.1.11) e (ii) despolimerases de pectina, sendo este último grupo dividido em poligalacturonases (EC 3.2.1.15, EC 3.2.1.67 e EC 3.2.1.82) e liases (EC 4.2.2.2 e EC 4.2.2.10) (ABDULRACHMAN et al., 2017; BENZ et al., 2014; GARG et al., 2016; SISTA KAMESHWAR; QIN, 2018).

As poligalacturonases representam a maior classe com função hidrolítica e pertencem à família 28 das glicosil hidrolases (GH), de acordo com o sistema de classificação das GHs, disponível no banco de dados Cazy (*Carbohydrate-Active enzymes*, <http://www.cazy.org>) (LOMBARD et al., 2014). Essas enzimas catalisam a hidrólise da ligação glicosídica α -1,4 entre dois resíduos de ácido galacturônico (ABDULRACHMAN et al., 2017; BENZ et al., 2014; LATARULLO et al., 2016; SHARMA; PATEL; SUGANDHA, 2017; SISTA KAMESHWAR; QIN, 2018) e podem apresentar ação endo- (EPG, EC 3.2.1.15), hidrolisando as ligações internas dentro de moléculas de homogalacturonanas de maneira randômica, originando oligogalacturonídeos de tamanhos diferentes, ou ação exo- (EC 3.2.1.67 e EC 3.2.1.82), que atuam sobre a extremidade não redutora do polímero, produzindo o ácido monogalacturonídeo (SISTA KAMESHWAR; QIN et al., 2018; SHARMA et al., 2017; LATARULLO et al., 2016).

As enzimas da família GH28 catalisam a hidrólise das ligações glicosídicas da cadeia principal da homogalacturonana com inversão da configuração do carbono anomérico. Dois resíduos de aspartato adequadamente posicionados no sítio ativo estão diretamente envolvidos no mecanismo de catálise (Fig. 4) (DAVIES; HENRISSAT, 1995; MASSA et al., 2007). Neste tipo de mecanismo, a cadeia lateral do primeiro resíduo de aspartato, que se encontra desprotonada, atua como aceptor de H^+ , abstraindo um próton de uma molécula de água que ataca o carbono anomérico da molécula de substrato. Simultaneamente, o segundo resíduo de aspartato, inicialmente protonado, doa um H^+ para o oxigênio glicosídico do substrato e inverte a estereoquímica no carbono anomérico.

Figura 4. Mecanismo de reação das poligalacturonases da família GH28.
Adaptado de Davies e Henrissat (1995).

Na literatura, é possível encontrar diferentes EPGs produzidas por fungos, bactérias, plantas, insetos e nematoides (ANISA; GIRISH, 2014; GARG et al., 2016; LATARULLO et al., 2016). No entanto, os organismos mais estudados são fungos e plantas, sendo somente as EPGs fúngicas estruturalmente caracterizadas (VAN POUDEROYEN et al., 2003; VAN SANTEN et al., 1999).

Dentre as enzimas fúngicas, destaca-se a EPG de *Chondrostereum purpureum* (EndoPGCp) (SHIMIZU et al., 2005; SHIMIZU; MIYAIRI; OKUNO, 2000), que foi originalmente isolada do extrato bruto da linhagem fúngica ASP-4B (HASUI et al., 1998) e sua sequência de nucleotídeos foi clonada e expressa como uma proteína heteróloga em *Escherichia coli* (SHIMIZU et al., 2005) e *Pichia pastoris* (OGAWA et al., 2009). A estrutura tridimensional das enzimas nativas e recombinantes foi resolvida (Fig. 5) (SHIMIZU et al., 2005; SHIMIZU et al., 2001, 2002), e os locais de glicosilação e o padrão das pontes dissulfeto foram determinados (OGAWA et al., 2010; SHIMIZU; MIYAIRI; OKUNO, 2000). No entanto, uma caracterização bioquímica mais detalhada não foi descrita, e as potenciais aplicações desta enzima em bioprocessos não foram avaliados.

Figura 5. Representação em fita da estrutura tridimensional da EndoPGCp de *C. purpureum* (código PDB: 1K5C).

1.3. Fontes de pectina e aplicação industrial das pectinases

As paredes celulares de materiais lignocelulósicos ricos em pectina contêm cerca de 12 a 35%, do seu peso seco, de pectina (EDWARDS; DORAN-PETERSON, 2012; LATARULHO et al., 2016). Em comparação, outros vegetais que são caracterizados pelo baixo conteúdo de pectinas, como milho, gramíneas e biomassa lenhosa, contêm entre 2 a 10% de pectinas (EDWARDS; DORAN-PETERSON, 2012; LATARULLO et al., 2016; MOHNEN, 2008). A Figura 6 compara a composição de materiais ricos em pectina com outros tipos de biomassa.

Resíduos ricos em pectinas notavelmente possuem menos lignina em comparação com outros tipos de biomassa lignocelulósica. Aproximadamente 2% do peso seco dos resíduos cítricos e beterraba são de lignina, um teor muito menor quando comparado a cerca de 20 a 25% presente na cana-de-açúcar e no capim-elefante. Isso é significativo porque, em materiais ricos em pectina, a recalcitrância a hidrólise enzimática dos polissacarídeos presentes na parede celular vegetal é modulada pela pectina (EDWARDS; DORAN-PETERSON, 2012; LATARULHO et al., 2016).

Figura 6. Comparação da composição de biomassas ricas em pectina e outras biomassas lignocelulósicas.

Adaptado de Edwards e Doran-Peterson (2012).

No setor industrial, as pectinas são muito utilizadas na indústria alimentícia, como agente gelificante, espessante, emulsificante e estabilizante, nas indústrias de processamento de frutas, na produção de doces e confeitos, em confeitaria industrial, na indústria láctea, além da indústria de bebidas (DRANCA; OROIAN, 2018). Atualmente, a produção comercial de pectinas está limitada a duas fontes principais, bagaço de maçã (14,0%) e casca de frutas cítricas (85,5%). Ambas as fontes de extração representam subprodutos das operações de fabricação de suco (DRANCA; OROIAN, 2018; NOREEN et al., 2017).

Já em relação ao uso de pectinases no setor industrial, globalmente, o comércio de enzimas movimentou, em 2017, cerca de US\$ 6,3 bilhões e somente o mercado da indústria alimentícia, segmento com maior uso de pectinases, movimentou cerca US\$ 2 bilhões no mesmo ano. As pectinases, especificamente, equivalem a aproximadamente 10% da produção total de enzimas (PRIYADHARSHINI, 2012). Estas enzimas são empregadas em uma diversidade de segmentos industriais e o

primeiro relato de sua utilização em escala comercial foi em 1930 na preparação de sucos e vinhos (KASHYAP et al., 2001). As principais aplicações das pectinases no âmbito industrial são apresentadas na Tabela 1.

Tabela 1. Aplicações das pectinases em diferentes segmentos industriais

Segmento industrial	Aplicação
Sucos e vinhos	Melhora na extração de sucos, clarificação, pigmentos, além da liberação de aroma e outros compostos.
Chá e café	Melhora na fermentação
Alimentos	Produção de polpas de frutas, compostos prebióticos e ração animal
Óleos	Melhora na extração de óleos essenciais de cítricos e na extração de agentes antioxidantes
Biocombustíveis	Degradação de material biomassa lignocelulósica e liberação de açúcares para produção de bioetanol
Têxtil	Tratamento das fibras
Papel e celulose	Branqueamento da polpa <i>kraft</i>

Adaptado de Santi et al. (2014).

Dentro da indústria alimentícia, o uso das pectinases é focado principalmente na produção de sucos. O processo de extração de sucos inicia-se com a maceração das frutas, o que acarreta um aumento da viscosidade e de partículas em suspensão, dificultando a extração e a filtração, principalmente em frutos ricos em pectina. Assim, as pectinases são adicionadas para diminuir a viscosidade e a turbidez, auxiliando no aumento da extração, da pigmentação e na clarificação do suco, podendo reduzir em até 50% o tempo de filtração do produto final (KASHYAP et al., 2001; KHAN; NAKKEERAN; UMESH-KUMAR, 2013; SAXENA et al., 2008; SOUZA et al., 2003).

Na produção de bioetanol, o uso das pectinases tem ganhado grande atenção, isso porque, as pectinas contribuem para a recalcitrância da parede celular (BONNIN; GARNIER; RALET, 2014; CAFFALL; MOHNEN, 2009; WANG et al., 2018) e dificultam o acesso das enzimas hidrolíticas criando uma barreira física (TAVARES; DE SOUZA; BUCKERIDGE, 2015).

1.3.1. Estratégias para o uso de enzimas recombinantes

Nos últimos séculos, houve um notável aumento na aplicação de enzimas em diversos setores da indústria. Isso ocorre porque as enzimas podem ser utilizadas como um substituto para alguns produtos químicos tóxicos que estavam anteriormente envolvidos nos processamentos industriais.

No entanto, a produção em larga escala de enzimas por organismos nativos é, na maioria das vezes, inconveniente, devido principalmente a baixa produtividade (LUO et al., 2015). Além disso, as enzimas existentes muitas vezes não apresentam características adequadas para aplicações nos processos industriais, como estabilidade em determinadas condições de temperatura e pH, boa atividade em presença de altas concentrações de substrato ou produto, etc (BÖTTCHER; BORNSCHEUER, 2010; RIBEIRO; RIBEIRO, 2013).

Como alternativa prática, o desenvolvimento de técnicas de biologia molecular possibilitou a análise e transferência de um único gene, um conjunto de genes (*cassette*) ou um *cluster* para um hospedeiro geneticamente tratável, permitindo obter enzimas heterólogas em grande escala. Aliada às técnicas de engenharia de proteínas, foi possível obter estruturas modificadas que apresentam características mais adequadas para o desempenho de papéis específicos em diferentes processos industriais (ARRIZUBIETA; POLAINA, 2000; BÖTTCHER; BORNSCHEUER, 2010).

A quimerogeneses ou engenharia de enzimas multidomínios cria proteínas por meio da união de duas ou mais genes que originalmente codificados para proteínas separadas. Esta técnica tem sido uma estratégia potencial para reduzir os custos envolvidos com a produção de enzimas em processos industriais. Além disso, quando as enzimas são fusionadas, a proximidade física entre os domínios proteicos pode aumentar a velocidade das reações, e frequentemente essas enzimas podem apresentar um melhor desempenho em comparação com as atividades das enzimas parentais (ADLAKHA et al., 2011; BALABANOVA et al., 2015; REDDY CHICHILI; KUMAR; SIVARAMAN, 2013).

A criação de quimeras pode ser feita utilizando-se duas estratégias principais, como o desenho racional e por fusões *end-to-end*. No desenho racional são definidos sítios na superfície da proteína que sejam mais favoráveis para a inserção de um outro domínio proteico, a fim de minimizar perturbações nas estruturas parentais, evitar o

impedimento estérico e orientações não-favoráveis dos domínios proteicos na quimera (FURTADO et al., 2013; RIBEIRO et al., 2011). Já na estratégia de fusão *end-to-end* a extremidade C-terminal de uma proteína é unido a extremidade N-terminal de outra proteína (KIM; LEE; KIM, 2000; STROHL, 2017). Frequentemente os dois domínios são unidos por meio de uma sequência conectora ou *linker*. O *linker* pode ser rígido (rico em resíduos de prolina) para impedir interações indesejadas ou efeitos estéricos entre os domínios ou flexível (rico em resíduos de glicina) para conectar os domínios sem interferir na função de cada domínio na quimera (REDDY CHICHILLI et al., 2013).

Associado às técnicas de engenharia de proteínas, a escolha de um sistema de expressão para a produção de alto nível de enzimas recombinantes é fundamental e depende de muitos fatores. Estes fatores incluem características de crescimento celular, níveis de expressão, expressão intracelular e extracelular, modificações pós-tradução e atividade biológica da proteína de interesse (BROADWAY, 2012; LIU et al., 2013). As enzimas recombinantes podem ser expressas em *E. coli*, bactérias do ácido láctico (por exemplo, *Lactobacillus lactis*), fungos filamentosos (por exemplo, *Aspergillus*) e leveduras (por exemplo, *Pichia pastoris*) (BANEYX, 1999; LIU et al., 2013; PUNT et al., 2002; WARD, 2012). A Tabela 2 resume as principais vantagens e desvantagens de alguns dos sistemas de expressão de proteínas comumente usados.

Tabela 2. As principais características de alguns sistemas de expressão de enzimas heterólogas

Sistema de expressão	Vantagens	Desvantagens
<i>E. coli</i>	<ul style="list-style-type: none"> - Níveis elevados de expressão; - Condições simples de cultivo; - Crescimento rápido; - Protocolos simples de transformação; - Muitos parâmetros podem ser alterados para otimizar a expressão. 	<ul style="list-style-type: none"> - Formação ineficiente de pontes dissulfeto (algumas cepas, como Origami, podem minimizar esse problema); - Formação de corpos de inclusão; - Enovelamento ineficiente de proteínas; - Protocolos de reenovelamento demorados e ineficientes; - Endotoxinas; - Modificações pós-traducionais mínimas; - Ausência de códons raros presente em eucariotos (algumas cepas como Rosetta e CodonPlus podem minimizar esse problema).
<i>S. cerevisiae</i>	<ul style="list-style-type: none"> - Bons níveis de expressão - Baixo custo - Condições simples de cultivo - Capaz de executar a maioria das modificações pós-traducionais eucarióticas - Enovelamento eficiente de proteínas - Livre de endotoxinas 	<ul style="list-style-type: none"> - Menores níveis de expressão e secreção quando comparada a <i>P. pastoris</i> - Tendência a produzir proteínas hiperglicosiladas - Estruturas de N-glicano consideradas alergênicas
<i>P. pastoris</i>	<ul style="list-style-type: none"> - Secreção eficiente de proteínas - Permite purificação simples - Extensa modificação pós-traducional - Enovelamento eficiente de proteínas - N-glicosilação mais parecida com eucariotos superiores quando comparada à de <i>S. cerevisiae</i> - Livre de endotoxinas 	<ul style="list-style-type: none"> - O uso do metanol como indutor é perigoso em grande escala - O padrão de glicosilação ainda é diferente quando comparado à glicosilação presente em células de mamíferos

Tabela 2. As principais características de alguns sistemas de expressão de enzimas heterólogas

(final)

Sistema de expressão	Vantagens	Desvantagens
Fungos filamentosos	<ul style="list-style-type: none"> - Alto nível de expressão - Bom sistema de secreção - Modificação pós-tradução - Meio de cultura barato 	<ul style="list-style-type: none"> - Vetor comercial não disponível - Maiores tempos de cultura - Esporos são preocupações para problemas de saúde
Células animais	<ul style="list-style-type: none"> - Bons níveis de expressão - Células adaptadas para facilitar o aumento de escala - Enovelamento eficiente de proteínas - Bom para proteínas secretadas - Todas as modificações pós-traducionais - Livre de endotoxinas 	<ul style="list-style-type: none"> - Meios de cultura caros
Células de insetos infectadas com baculovírus	<ul style="list-style-type: none"> - Bons níveis de expressão (especialmente para proteínas intracelulares) - Crescimento relativamente rápido - Enovelamento de proteínas eficiente - Extensa modificação pós-traducional de proteínas - A glicosilação é mais parecida com a de células de mamífero - Proteínas podem ser facilmente deglicosiladas enzimaticamente (bom para o estudo da estrutura) - Livre de endotoxinas 	<ul style="list-style-type: none"> - Meios de cultura caros - Grandes volumes de vírus necessários para o aumento de escala - A glicosilação ainda difere daquela das células de mamíferos - A infecção viral conduz à lise das células e potencial degradação das proteínas expressas

Tabela adaptada de Broadway (2012).

Os sistemas de expressão bacteriana (Tabela 2) são muito atraentes devido à sua capacidade de crescer rapidamente e em alta densidade em substratos baratos, sua genética bem caracterizada e a disponibilidade de um número cada vez maior de vetores de clonagem e cepas hospedeiras mutantes (TERPE, 2006). No entanto, apesar do amplo conhecimento da genética e biologia molecular da *E. coli*, nem todos os genes podem ser expressos de forma eficiente neste organismo.

As proteínas recombinantes geralmente requerem a ajuda de moduladores de enovelamento, como proteínas chaperonas, durante a expressão em *E. coli*. Neste sistema de superexpressão, a taxa de agregação de proteínas é frequentemente muito maior do que a do dobramento adequado e as proteínas chaperonas são rapidamente utilizadas (BANEYX; MUJACIC, 2004; YAMAGUCHI; MIYAZAKI, 2014). Esses agregados de proteínas são chamados de corpos de inclusão. Como as proteínas alvo são geralmente purificadas a partir de frações solúveis em lisados de células de bactérias, a formação de corpos de inclusão é muitas vezes uma desvantagem no uso de sistemas de expressão de *E. coli* (YAMAGUCHI; MIYAZAKI, 2014). Alterar as condições de crescimento, como temperatura de crescimento, concentração de indutor e tempo de indução, muitas vezes pode ajudar a diminuir a formação de corpos de inclusão.

Embora a produção de uma proteína alvo inativa na forma de corpos de inclusão seja uma desvantagem importante, ela também tem várias vantagens, como o alto grau de pureza da proteína alvo na fração de agregado e a maior proteção contra degradação proteolítica em comparação com a fração solúvel (VALLEJO; RINAS, 2004; YAMAGUCHI; MIYAZAKI, 2014). Como os corpos de inclusão contêm proteínas recombinantes relativamente puras e intactas, várias abordagens foram relatadas para recuperar essas formas agregadas como proteínas biologicamente ativas (SINGHVI; GOKHALE, 2019). Em um procedimento típico, as formas agregadas são desnaturadas e dissolvidas com uma alta concentração de desnaturante, como uréia, cloreto de guanidínio ou detergentes iônicos, como N-lauroilsarcosina (SINGHVI et al., 2020; YAMAGUCHI; MIYAZAKI, 2014). Esses reagentes químicos são usados para diminuir as interações não covalentes entre as moléculas de proteínas. O reenovelamento de proteínas desnaturadas em proteínas nativas ocorre pela remoção do desnaturante (SINGH et al., 2015; SINGHVI et al.,

2020). A eficiência de reenovelamento da proteína pode ser estimada pela atividade biológica, como a atividade enzimática.

O procedimento de remoção do agente desnaturante das proteínas desenoveladas é uma etapa fundamental na recuperação eficiente das proteínas (SINGHVI et al., 2020; YAMAGUCHI; MIYAZAKI, 2014). Portanto, várias abordagens foram relatadas para reenovelar uma proteína inativa em uma proteína ativa, como diluição direta, remoção controla do agente desnaturante por diálise e métodos cromatográficos com base na cromatografia de exclusão por tamanho e reenovelamento assistido por matriz (VALLEJO; RINAS, 2004). Embora esses métodos funcionem bem para muitas proteínas de corpos de inclusão e proteínas modelo desnaturadas, na maioria dos casos há uma quantidade significativa de precipitação de proteínas, resultando em um baixo rendimento de recuperação. Portanto, o procedimento de reenovelamento de proteína ainda é realizado com uma série de experimentos de tentativas e erro (SINGH et al., 2015; SINGHVI et al., 2020; YAMAGUCHI; MIYAZAKI, 2014). Por outro lado, a aplicação de enzimas em processos industriais depende em grande parte do custo de produção das enzimas e a escolha em usar enzimas solúveis ou enzimas na forma insolúvel é um ponto crucial (SINGH et al., 2013). As enzimas na forma insolúvel são essencialmente uma forma especializada de catálise heterogênea, em que o catalisador pode ser recuperado e reutilizado, geralmente mantendo a atividade por longos períodos de tempo e sendo aplicáveis a uma variedade de formatos de processo (BASSO; SERBAN, 2019).

A imobilização de uma enzima significa combinar a seletividade, estabilidade e cinética dessa enzima com as propriedades físicas e químicas do suporte em uma formulação que tem como papel principal maximizar a estabilidade física e catalítica da enzima (Fig. 7).

Figura 7. Fatores que afetam a imobilização enzimática.

Figura adaptada de Basso e Serban, 2019.

Existem muitos métodos diferentes usados para imobilização de enzimas, mas a indústria tem preferência por métodos simples e econômicos. Os métodos mais utilizados baseiam-se na imobilização física (adsorção ou aprisionamento físico) e na imobilização química (ligação covalente e reticulação) (CHAPMAN; ISMAIL; DINU, 2018; SINGH et al., 2013). A Tabela 3 reúne as principais vantagens e desvantagens do uso de enzimas imobilizadas.

Tabela 3. Vantagens e desvantagens das enzimas imobilizadas em processos industriais

Vantagens	Desvantagens
<ul style="list-style-type: none"> - Fácil separação de biocatalisador; - Custos reduzidos de processamento <i>downstream</i>; - Uso múltiplo de biocatalisador (reciclagem); - Melhor estabilidade, especialmente em relação a solventes orgânicos e temperaturas mais altas; - Possibilidade de co-imobilização com outras enzimas. 	<ul style="list-style-type: none"> - Atividade enzimática mais baixa em comparação com a enzima nativa; - Custos adicionais para produção de suporte e imobilização; - Taxas de reação mais baixas em comparação com enzimas nativas; - Eliminação de enzima imobilizada exaurida (incineração).

Tabela adaptada de Basso e Serban, 2019.

No geral, a relevância industrial das enzimas imobilizadas é principalmente direcionada à aplicação, nas vantagens oferecidas pelo biocatalisador em relação às enzimas solúveis (BASSO; SERBAN, 2019; SINGH et al., 2013). Como tal, a imobilização eficiente de uma enzima depende criticamente de técnicas que proporcionam altos graus de estabilidade e capacidade de reutilização, bem como funcionalidade adicional sem obstáculos significativos para a atividade da enzima e seletividade do produto (BASSO; SERBAN, 2019; CHAPMAN; ISMAIL; DINU, 2018).

1.4. Carbohydrate Binding Modules (CBMs)

Muitas enzimas que atuam sobre polissacarídeos possuem módulos não-catalíticos de ligação a carboidratos (*Carbohydrate Binding Modules* - CBMs) que reconhecem estes carboidratos. Os CBMs são formados por uma sequência contínua de aminoácidos ligada a uma enzima, além de possuírem uma estrutura terciária e afinidade de ligação independente da catálise (BORASTON et al., 2004a; CANTAREL et al., 2009). Primeiramente, foram caracterizados como tendo alta afinidade por celulose e assim denominados de CBD (*Cellulose Binding Domain*) (BORASTON et al., 2004a; CARRARD; LINDER, 1999). No entanto, com a descoberta de outros

módulos de ligação à diferentes carboidratos houve a necessidade de uma nomenclatura mais abrangente (BORASTON et al., 2004a; LYND et al., 2002).

Os CBMs são divididos em famílias de acordo com a similaridade de suas sequências de aminoácidos. Atualmente existem 87 famílias de CBMs e mais de 231.274 sequências conhecidas ou previstas no banco de dados CAZy, que apresentam uma ampla variedade de especificidade de ligação, e capacidade de reconhecimento aos mais diferentes tipos de carboidratos como celulose cristalina e amorfa, quitina, quitosana, β -glucano, xilana e pectina, sendo muito frequente casos de interações duplas, em que um mesmo CBM pode reconhecer e se ligar a outros polissacarídeos com diferentes graus de afinidade. Desta forma, estas proteínas são um excelente modelo para o estudo dos mecanismos da interação proteína-carboidrato (BORASTON et al., 2004).

Os CBMs são ainda classificados em 3 grandes grupos de acordo com o reconhecimento dos substratos (Fig. 8): os CBMs do grupo A apresentam seu sítio de ligação em uma topologia hidrofóbica e planar, e interagem com substratos insolúveis e cristalinos (Fig. 8, A); os módulos do grupo B contém o sítio de ligação em forma de fenda, de profundidade variável, que é rica em resíduos aromáticos capazes de reconhecer cadeias de polissacarídeos mais solúveis (Fig. 8, B); já os CBMs do grupo C, também chamados de “*lectin-like*”, podem se ligar apenas à mono, di ou trissacarídeos, em consequência das restrições estéricas ao redor do sítio de ligação (Fig. 8, C) (GUILLÉN; SÁNCHEZ; RODRÍGUEZ-SANOJA, 2010).

Figura 8. Diferentes tipos estruturais de CBMs.

A) CBM do tipo A de uma cellobiohidrolase I de *Trichoderma reesei* (código PDB: 1CBH). B) CBM do tipo B de uma endo- β -1,4-glucanase de *Cellulomonas fimi* (código PDB: 1GU3). C) CBM do tipo C de uma xilanase da família 10 de *Thermotoga maritima* (código PDB: 1I82). Os carboidratos estão representados como modelos na forma de bastão. Figura modificada de Guillen e colaboradores (2010).

1.4.1. CBMs com afinidade de ligação à pectina

Somente dois CBMs com afinidade por pectina foram descritos até o momento, um CBM da família 32 e outro da família 77 (ABBOTT; HRYNUIK; BORASTON, 2007; VENDITTO et al., 2016). O CBM 32 de *Yersinia enterocolitica* (YeCBM32) foi o primeiro descrito com afinidade de ligação a polissacarídeos pécticos, como homogalacturonana e a rhamnogalacturonana (Abbott et al., 2007) e possui uma propriedade incomum entre os CBMs, uma vez que não estão associados à um domínio catalítico (ABBOTT; HRYNUIK; BORASTON, 2007). Além disso, este CBM possui aproximadamente 20% de identidade com outros membros caracterizados na família 32. Uma análise das posições deste CBMs no genoma da enterobactéria *Y. enterocolitica* revelou que as ORFs que codificam o YeCBM32 estão posicionadas imediatamente a jusante de uma pectato liase da família 2 e dentro de “operons” supostamente regulados por proteínas denominadas de KDGR (reguladores negativos de genes metabólicos), envolvidos na despolimerização de pectina (RODIONOV; GELFAND; HUGOUVIEUX-COTTE-PATTAT, 2004). O YeCBM32 adota uma arquitetura de oito hélices em “ β -sanduíche” constituída por cinco cadeias em folha- β antiparalelas opostas a outras três cadeias, também em folha- β antiparalelas (Fig. 9). Uma pequena seção de α -hélice separa as folhas- β 1 e 2 (ABBOTT; HRYNUIK; BORASTON, 2007). O sítio de ligação aos oligossacarídeos de ácido galacturônico é constituído por três resíduos aromáticos de triptofano (W44, W83 e W124), sendo a localização deste sítio conservada entre outros CBM da mesma família (ABBOTT; HRYNUIK; BORASTON, 2007). A estrutura do YeCBM32 foi resolvida por cristalografia de raio-x e está depositada no PDB (*Protein Data Bank*) sob o número de acesso 2JD9.

Figura 9. Representação em fita da estrutura tridimensional do YeCBM32.

O CBM da família 77 de *Ruminococcus flavefaciens* (*RfCBM77*) é componente de um complexo que contém dois módulos catalíticos, pertencentes às famílias 1 e 9 de pectato liases (PL1 e PL9), além de uma dockerina na porção C-terminal (VENDITTO et al., 2016), conforme esquematizado na Figura 10. Este CBM possui afinidade por homogalacturonana, interagindo com 7 ou 8 resíduos de ácido galacturônico. Sua estrutura também adota uma arquitetura “ β -sanduíche”, em que quatro cadeias em folha- β antiparalelas opõem-se a cinco cadeias em folha- β antiparalelas e não apresenta nenhum sítio de ligação a metais. Em sua superfície, três resíduos de aminoácidos básicos (K32, K47 e K102) são responsáveis pela interação com a pectina (VENDITTO et al., 2016). O modo de interação de *RfCBM77* com o polissacarídeo é distinto do observado para outras famílias de CBMs, em que os resíduos de aminoácidos aromáticos dominam o reconhecimento de carboidratos (VENDITTO et al., 2016), mas se assemelha ao modo de interação de proteínas de ligação a glicosaminoglicanas, em que o reconhecimento também é mediado por resíduos básicos. A estrutura do *RfCBM77* foi resolvida por cristalografia e está depositada no PDB sob o número de acesso 5FU5.

Figura 10. Estrutura do RfCBM77.

(A) Gene do domínio que contém o RfCBM77 (verde), duas pectato liases (azul) e uma dockerina (azul claro). (B) Representação em fita da estrutura tridimensional do RfCBM77.

1.4.2. Os CBMs com afinidades a outros polissacarídeos

O microrganismo *Hungateiclostridium (Clostridium) thermocellum* sintetiza um complexo de hidrolases extracelular de alta massa molecular, denominado de celulosoma, o qual é composto por vários domínios catalíticos, tais como celulasas e hemicelulasas, e por proteínas de ação não catalítica, como os CBMs (ARAI et al., 2003; FERNANDES et al., 1999). Dentre os CBMs já caracterizados para este organismo, destacam-se os CBMs das famílias 3, 6, 11, 22 e 44.

Uma das proteínas que compõe o celulosoma de *H. thermocellum* é a proteína estrutural formada por vários domínios, denominada de CipA (proteína A integradora do celulosoma), um tipo de “scaffoldin” que inclui nove módulos de coesinas de ligação a enzimas, uma dockerina e um CBM da família 3 (HtCBM3), o qual apresenta alta afinidade por celulose cristalina, conforme esquematizado na figura 11 (TORMO et al., 1996; YANIV et al., 2013). Este CBM está localizado entre as coesinas 2 e 3 e é

separado destes domínios por regiões de ligação ricas em resíduos de treonina e prolina. Sua estrutura tridimensional é conhecida como “*jellyroll*” e corresponde a um “sanduíche” formado por dois conjuntos de nove fitas- β antiparalelas com um íon Ca^{2+} estabilizando a dobra (TORMO et al., 1996; YANIV et al., 2013). Em sua superfície, existem resíduos de aminoácidos conservados em duas faces definidas localizadas em lados opostos da proteína. Uma destas faces é dominada por uma fita linear e planar de resíduos aromáticos e polares, que são responsáveis por interagir com a celulose cristalina (TORMO et al., 1996). A estrutura do *HtCBM3* também foi resolvida por cristalografia de raio-x e está depositada no PDB sob o número de acesso 1NBC.

Figura 11. Estrutura do *HtCBM3*.

(A) *Scaffoldin* CipA incluindo os nove módulos de coesinas, uma dockerina e o *HtCBM3*. (B) Representação em fita da estrutura tridimensional do *HtCBM3*.

O CBM da família 6 de *H. thermocellum* (*HtCBM6*) faz parte do gene *xynV*, que também codifica a xilanase XylV (Fig. 12) (FERNANDES et al., 1999). Este CBM possui alta afinidade por xilana e, assim como o *HtCBM3*, exibe uma estrutura em forma de “*jellyroll*”, predominantemente constituída por cinco cadeias β antiparalelas em uma face e quatro cadeias β antiparalelas na outra face (CHARNOCK et al., 2000; CZJZEK et al., 2001; HENSHAW et al., 2004). Na sua superfície, há uma pequena fenda rasa, revestida por dois resíduos aromáticos (Y92 e Y34) e um resíduo de

asparagina, na posição 120, responsáveis pela interação com o polissacarídeo (CHARNOCK et al., 2000; CZJZEK et al., 2001). Dois sítios de ligação a íons também estão presentes no *HtCBM6*, sendo um sítio de ligação ao cálcio e outro, supostamente, de ligação ao sódio, embora o significado biológico deste íon metálico não esteja claro (CZJZEK et al., 2001). A estrutura do *HtCBM6* foi resolvida por cristalografia de raio-x e está depositada no PDB sob o número de acesso 1GMM.

Figura 12. Estrutura do *HtCBM6*.

(A) Gene do domínio que contém o *HtCBM6* (verde), a xilanase GH11 (laranja) e sinal de nodulação - NodB (azul claro). (B) Representação em fita da estrutura tridimensional do *HtCBM6*.

Um membro de CBM da família 11 faz parte do gene *celH* de *H. thermocellum* (*HtCBM11*) (YAGÜE; BÉGUIN; AUBERT, 1990). Este gene é constituído por dois módulos catalíticos, a β -1,3-1,4-glucanase, uma enzima da família GH26 localizada na porção N-terminal e a endo- β -1,4-glucanase interna, da família GH5, do *HtCBM11* e de uma dockerina C-terminal (Fig. 13).

Este CBM é caracterizado por se ligar a cadeias isoladas de polissacarídeos (VIEGAS et al., 2013) e apresenta alta afinidade por polímeros que contém ligações β -1,3-1,4 como β -glucano e liquenana, menor afinidade por ligações β -1,4 como celulose cristalina, e nenhuma afinidade para ligações β -1,3 (HORNAK et al., 2006).

Além disso, apresenta uma estrutura tridimensional em forma de rocambole (*jellyroll*), onde dois conjuntos de seis fitas- β antiparalelas adquirem um formato de “sanduíche”. Na superfície há uma fenda rica em aminoácidos hidrofóbicos incluindo sete fenilalaninas e seis triptofanos, responsáveis pela ligação ao polissacarídeo. Três resíduos de tirosinas (Y22, Y53 e Y129) presentes nesta fenda são conservados nos membros da família e exercem papel fundamental no reconhecimento e ligação à face apolar da cadeia do carboidrato (CARVALHO et al., 2004; VIEGAS et al., 2013). Existem ainda dois sítios de ligação ao cálcio que se encontram distantes do sítio de ligação ao polissacarídeo e acredita-se que estes sítios apresentem uma função estrutural, elevando a estabilidade da proteína e auxiliando no seu enovelamento (CARVALHO et al. 2004). A estrutura do *HtCBM11* foi resolvida por cristalografia e está depositada no PDB sob o número de acesso 1V0A.

Figura 13. Estrutura do *HtCBM11*.

(A) Gene do domínio que contém o *HtCBM11* (azul), as enzimas das famílias GH26 e GH5 (laranja) e uma dockerina (amarelo). (B) Representação em fita da estrutura tridimensional do *HtCBM11*.

O gene *xyn10B* de *H. thermocellum* possui duas cópias de CBMs da família 22 (CHARNOCK et al., 2000), sendo uma localizada na região N-terminal do polipeptídeo (*HtCBM22-1*) e outra numa posição mais interna (*HtCBM22-2*), e ambos flanqueiam

um domínio xilanase da família 10 (GH10) e na porção C-terminal do gene há ainda uma dockerina e uma feruloil esterase (Fig. 14).

O *HtCBM22-2*, assim como os outros CBMs, possui uma estrutura tridimensional com um enovelamento clássico de “*jellyroll*” com dois conjuntos de quatro fitas- β antiparalelas em ambas as faces da proteína (CHARNOCK et al. 2000). Na face côncava se forma um sulco raso, constituindo o sítio de ligação ao polissacarídeo, onde três resíduos aromáticos de triptofano (W53, W103 e W134), altamente conservados em CBMs desta família, fazem a interação com o polímero, que são compostos formados principalmente de cadeias de xilose ramificadas. Este CBM também contém um sítio de ligação ao cálcio, localizado no fim da fita- β 1 (XIE et al., 2001). A estrutura da *HtCBM22-2* foi resolvida por cristalografia de raio-x e está depositada no PDB sob o código de acesso 1DYO.

Figura 14. Estrutura do *HtCBM22*.

(A) Gene do domínio que contém os *HtCBM22* 1 e 2 (verde), a xilanase GH10 (laranja), a dockerina (rosa) e a feruloil esterase (azul). (B) Representação em fita da estrutura tridimensional do *HtCBM22-2*.

Por fim, o CBM da família 44 (*HtCBM44*) encontra-se como um domínio da proteína CelJ de *H. thermocellum*. Além este CBM, esta proteína também contém um CBM da família 30 na porção N-terminal e duas endoglucanases das famílias GH9 e

GH44 na porção interna do complexo (NAJMUDIN et al., 2006). Na porção C-terminal é encontrado o *HtCBM44*, o qual é adjacente à um domínio conhecido como PKD (domínio identificado em um glicoproteína de superfície celular relacionada à doença renal policística - *polycystic kidney disease*), conforme é ilustrado na figura 15.

Este CBM também apresenta uma estrutura no formato “*jellyroll*” com dois conjuntos de cinco fitas- β antiparalelas, formando duas faces na proteína, uma côncava e outra convexa. O sítio de ligação fica localizado em uma fenda rica em resíduos de aminoácidos hidrofóbicos com cinco fenilalaninas e três triptofanos (W189, W194 e W198) que exercem um papel importante no reconhecimento e ligação dos polissacarídeos que interagem com este CBM. O *HtCBM44* apresenta afinidade por polímeros com cadeia principal formada por resíduos de glicose em ligação β -1,4, como o xiloglucano, β -glucano, liquenana e a hidroxietilcelulose. Esta proteína também contém um sítio de ligação ao cálcio na própria estrutura do CBM, e um outro sítio é encontrado no domínio PKD (NAJMUDIN et al. 2006).

O domínio PDK também apresenta uma estrutura em formado de “ β -sanduiche” e ainda não se conhece sua real função dentro do polipeptídeo codificado pelo gene *celJ*, uma vez que foi demonstrado que sua ausência não afeta a capacidade de reconhecimento dos polissacarídeos do *HtCBM44* e nem a afeta a atividade catalítica da endoglucanase GH44 (NAJMUDIN et al. 2006). A estrutura da *HtCBM44* foi resolvida por cristalografia de raio-x juntamente com o domínio PKD e está depositada no PDB sob o número de acesso 2C26.

Figura 15. Estrutura do *HtCBM44*.

(A) Gene do domínio que contém o *HtCBM44* (rosa), *HtCBM30* (azul claro), e as duas endoglucanase GH9 (laranja) e GH44 (amarelo), além do domínio PKD (cinza). (B) Representação em fita da estrutura tridimensional do domínio PKD (esquerda) e do *HtCBM44* (direita).

1.5. Domínios catalíticos e CBMs: desconstrução da parede celular vegetal

As funções detalhadas dos CBMs ainda não estão completamente elucidadas. Acredita-se que o seu principal papel seja o de auxiliar na catálise, se ligando ao polissacarídeo e aproximando-o do domínio catalítico e assim, aumentar a concentração de enzima na superfície do substrato para melhorar a eficiência catalítica (LE COSTAOUËC et al., 2013; VÁRNAI; SIIKA-AHO; VIHKARI, 2013).

Estudos demonstraram que os CBMs, que aparentemente exibem as mesmas especificidades contra polissacarídeos estruturais isolados, podem exibir um reconhecimento diferencial com polissacarídeos localizados nas paredes celulares (BLAKE et al., 2006; HERVÉ et al., 2010; MCCARTNEY et al., 2006). A análise da importância funcional dos CBMs na ação enzimática tem sido limitada à exploração de seu papel *in vitro* contra substratos purificados ou altamente processados, em que a integridade da parede celular foi afetada (BLACK et al., 1996; BOLAM et al., 1998, 2001; HERVE et al., 2010). A utilização de tais abordagens não esclarece a especificidade dos CBMs ou a sua capacidade para aumentar a atividade catalítica em paredes celulares intactas. Por exemplo, é possível que os CBMs que interagem

com a celulose só possam beneficiar hemicelulases ou pectinases quando a estrutura da parede estiver intacta. Também não está claro quando os CBMs de substrato-alvo beneficiam os seus módulos catalíticos. Por exemplo, os CBM específicos para xilana só podem beneficiar as suas enzimas cognatas, uma vez que o processo de degradação tenha sido iniciado pela remoção das cadeias laterais das estruturas hemicelulósicas.

Hervé e colaboradores (2009), utilizando técnicas de imunofluorescência, demonstraram que as xilanases das famílias GH 10 e GH 11 das glicosil hidrolases apresentaram eficiências contrastantes na degradação da xilana de paredes de células secundárias intactas (quantificadas em termos de detecção de epítomos) em comparação com as suas atividades *in vitro* contra polímeros isolados. Tais estudos indicam que as paredes celulares intactas com arquiteturas variadas, podem fornecer contextos estruturais distintos e limitações específicas ao acesso das enzimas e dos CBMs aos polissacarídeos da parede (HERVÉ et al., 2009). O efeito de CBMs com afinidades a celulose e xilana ligados a diferentes enzimas também foi avaliado por este mesmo grupo de pesquisa (HERVE et al., 2010). Os resultados obtidos demonstram que CBMs, com afinidades a polissacarídeos diferentes do substrato-alvo, podem aumentar a atividade catalítica de uma enzima em paredes celulares intactas. As ações de direcionamento dos CBMs têm, portanto, fortes efeitos de proximidade dentro das estruturas das paredes celulares, explicando porque, por exemplo, CBMs com afinidade a celulose são adicionados às enzimas que não possuem atividade sobre este polissacarídeo.

Neste trabalho se propõe avaliar o efeito de diferentes CBMs com afinidades à celulose, xilana, β -glucano, xiloglucano e pectina sobre a atividade de uma endopoligalacturonase na desconstrução da parede celular vegetal, empregando-se a metodologia de quimerogênese. A contribuição destas construções na suplementação de coquetéis enzimáticos visando à hidrólise eficiente de materiais lignocelulósicos também será avaliada.

Capítulo 5

Conclusões

5.1. Conclusões

- A subclonagem da sequência codificadora da EndoPGCp no vetor pPIC9kf1CT para a expressão da enzima em *P. pastoris* foi realizada com sucesso;
- A expressão da enzima recombinante EndoPGCp_Pp foi otimizada e a máxima expressão ocorreu após 3 dias de cultivo em meio MM induzido com 1% (v/v) de metanol a cada 24 hrs;
- A enzima purificada por cromatografia de afinidade em coluna de níquel apresentou cerca de 60 kDa e um conteúdo de carboidratos estimado em 67% (m/m);
- A EndoPGCp_Pp foi imobilizada com sucesso em nanopartículas ferromagnéticas e não apresentou alterações significativas em suas características bioquímicas e cinéticas;
- Quatorze quimeras foram eficientemente construídas pela fusão de um domínio CBM ao domínio catalítico da EndoPGCp;
- Quatro enzimas quiméricas expressas em *E. coli* foram reenovadas a partir dos corpos de inclusão;
- O processo de quimerogênese com os CBMs não afetou as propriedades do domínio catalítico. As enzimas hidrolisaram a pectina cítrica com V_{max} e K_M muito semelhantes;
- Quando aplicadas na suplementação de um coquetel comercial para hidrólise de resíduos lignocelulósicos, as pectinases melhoraram a eficiência da sacarificação da biomassa;
- Maiores rendimentos na hidrólise das enzimas quiméricas estão relacionados à ação dos CBMs;
- As propriedades bioquímicas das EndoPGCp recombinantes a tornam particularmente atraente para compor coquetéis enzimáticos para a hidrólise de biomassa lignocelulósica.

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Anexos

Figura A. Mapa físico do vetor pPIC9kf1CT.

Amostra **pPIC9kf1CT** Restriction Map

Figura B. Mapa dos sítios múltiplos de clonagem do vetor pPIC9kf1CT

A sequência mostra detalhes dos sítios múltiplos de clonagem (SMC) que contém sítios de restrição para as enzimas *XhoI*, *EcoRI*, *AvrII*, *NotI* e *SnaBI*.

sintetizado	-----	0
original	ATGACTTCCTTCTCGTCTCTGTTAAACTCCTGATTTGGCGTCTGCCACTGCCGCGACGA	60
sintetizado	-----GCTAGC-----	54
original	CCATCTAAACGTGCTACTTGCACAGTCAAGTCTGTCGATGATGCCAAGGACATTGCCGCGC * * * * *	120
sintetizado	TGTTCCGCGTTACGTTGAACGGATTACGGTTCCAGCCGGTAATACACTTGTCTGAAT	114
original	TGCAGTGCGGTCACTCTCAACGGATTCACTGACCTGCTGGAAATACCTTGTGTTGAAT * * * * *	180
sintetizado	CCCGATAAAGGCGCCACGGTTACAATGGCGGGTGATATTACCTTCGCTAAGACCATTTA	174
original	CCCGACAAGGCGCTACCGTCAACATGGCTGGCGACATTACATTGCAAGACCACATTG * * * * *	240
sintetizado	GACGGGCCCTTATTCACCATTGACGGTACTGGGATTAACCTTTGTTGGTGAGATCACATC	234
original	GACGGTCCCTCTTCACTATTGACGGGACGGTATCAACTTCGTTGGAGCCGACACATA * * * * *	300
sintetizado	TTTGATGGGAACGGGCTCTGTATTGGGACGGGAAGGGGACTAACAAATGGAACCCATAAA	294
original	TTGATGGGAACGGCGCTTTGTATTGGGATGGCAAGGGAACCAAAATGGCACTCACAAAG * * * * *	360
sintetizado	CCGCACCCCTTTCTTAAAAATTAAGGGGTCGGGTACTTATAAGAAATTCGAAGTTTAAAT	354
original	CCGCACCCGTTCTCAAGATCAAAGGATCGGGGACCTACAAGAAATTCGAAGTTCTCAAT * * * * *	420
sintetizado	TCCCCGCTCAAGCTATTTGCGTTGGTCTACAGACGCTCACCTTACTTTGGATGGGATC	414
original	AGTCTGCACAAGCAATATCGTTGGCCCTACGACGACACCTTACCTTCGATGGGATC * * * * *	480
sintetizado	ACGGTTGACGATTTGCCGGTGATACTAAAAATCTGGGACACAACACGGAGGTTTGAT	474
original	ACTGTCGATGATTTTGGGGAGACACAAGAACTGGGGCACAACTGACGGCTTCGAC * * * * *	540
sintetizado	GTAAGCGCCAACAACGTAACCTATCCAAAATGTATCGTCAAAAACCAAGATGACTGCATT	534
original	GTCCTGCCAACCAATGTAACCATCCAGAACTGATTGTCAAGAACCAAGGACGACTGCTTC * * * * *	600
sintetizado	GCAATCAATGATGGAAATAATTCGCTTTGAAATAATCAATGCTCAGGGGGCCACGGG	594
original	GCAATCAATGACGGAAATAACATTGCTTTGAGAATAACCAAGTCTCGAGGGCCACGGG * * * * *	660
sintetizado	ATCTCTATCGGCTCAATCGCAACCGGTAAGCACGTGTCCAATGTCGTAATTAAAGGGAAAT	654
original	ATTTCATTGGCTCAATCGCACTGGCAAGCACGTCTCAACGTTGTCATCAAGGGTAAC * * * * *	720
sintetizado	ACAGTTACGCGTCTGATGACGGGGTTCGTATCAAAGCACAGCTACAGCGAGCTCTGCA	714
original	ACGGTCACCCGTAGCATGTACGGTGTCCGCATCAAAGCCAGCGACGGCTACCTCTGCC * * * * *	780
sintetizado	TCGGTCTCCGGTGTACATACGATGCTAACACAATTAGCGGTATCGCCAAATATGGGGTT	774
original	TCTGTGTCGGCGTGACCTACGACGCAACACGATCTCTGGAAATGCTAAATACGGTGTC * * * * *	840
sintetizado	CTTATCTCTCAAAGCTACCTGACGATGTAGGGAATCAAGGTACGGGTGCCCGTTCTCA	834
original	CTCATTTACAGTCTTACCTGACGATGTTGGAACCTGGGACTGGAGCCCCCTTTTCT * * * * *	900
sintetizado	GATGTCAACTTTACAGGTGGCGCCACGACCATCAAGGTCAACAATGCTGCTACGCGCGTA	894
original	GATGTCAACTTTACTGGAGGTGCACTACAATCAAGGTCAATAATGCAGCAACACGGGTG * * * * *	960
sintetizado	ACTGTTGAATGTGGTAACTGTTCTGGGAATTGGAATGGAGTCAAGTAACTGTTACTGGG	954
original	ACTGTAGAATGCGGAACTGCTCTGGAAATTGGAATTGGTCCCACTCACTGTCACCGGT * * * * *	1020
sintetizado	GGAAAGCGGGGACGATCAAAATCGGACAAGGCAAAAAACCCGGGGACAGTATTTGTAA	1014
original	GGAAAGGCTGGTACAATTAAAGAGTGATAAGGCCAAGATCACTGGTGGCCAGTACTTGGA * * * * *	1080
sintetizado	GGATCC-----	1020
original	GATCAGCCAGCGAGTAACGACATAGAAGAGATGCCGGCTCAGGACCCATATGACCAGAG *	1140
sintetizado	-----	1020
original	GACCAGATACTGCTATGCAAGAGGCTGAGGCAGAAGAGGCTGCTGACGGGAATCCACT	1200
sintetizado	-----	1020
original	ACTTCCGGGTGA	1212

Figura C. Alinhamento entre as sequências de nucleotídeos da EndoPGCp nativa e sintetizada.

Na sequência sintetizada foram excluídos os nucleotídeos 1-70, que correspondem a um peptídeo sinal, e os nucleotídeos 1016-1212, que correspondem a uma pro-sequência C-terminal que inativa a enzima. Os asteriscos indicam resíduos idênticos; os nucleotídeos destacados indicam os sítios de restrição de *NheI* e *BamHI*.

sintetizado	-----ATCTVKSVDDAKDIAGCSAVTLNGFTVPAGNTLVLN	36
original	MTSFSSLFKLLILASATAAAPSkratCTVKSVDDAKDIAGCSAVTLNGFTVPAGNTLVLN	60

sintetizado	PDKGATVTMAGDITFAKTTLDGPLFTIDGTGINFVGADHIFDGNALYWDGKGTNNGTHK	96
original	PDKGATVTMAGDITFAKTTLDGPLFTIDGTGINFVGADHIFDGNALYWDGKGTNNGTHK	120

sintetizado	PHPFLKIKSGTYKKFEVLNSPAQAISVGPTDAHLTLDGITVDDFAGDTKNLGHNTDGF	156
original	PHPFLKIKSGTYKKFEVLNSPAQAISVGPTDAHLTLDGITVDDFAGDTKNLGHNTDGF	180

sintetizado	VSANNVTIQNCIVKNQDDCIAINDGNNIRFENNQCSGGHGISIGSIATGKHVSNVVIKGN	216
original	VSANNVTIQNCIVKNQDDCIAINDGNNIRFENNQCSGGHGISIGSIATGKHVSNVVIKGN	240

sintetizado	TVTRSMYGVRIKAQRTATSASVSGVTYDANTISGIakyGVLIQSYPDDVGNPGTGAPFS	276
original	TVTRSMYGVRIKAQRTATSASVSGVTYDANTISGIakyGVLIQSYPDDVGNPGTGAPFS	300

sintetizado	DVNFTGGATTIKVNNAATRVTVCEGNCsgnwnwSQLTVTGGKAGTIKSDKAKITGGQYL -	335
original	DVNFTGGATTIKVNNAATRVTVCEGNCsgnwnwSQLTVTGGKAGTIKSDKAKITGGQYLA	360

sintetizado	-----	335
original	DQPASNDIEEMPAQDPNDPEDPDTAMQEAEAEAAAAGNSTTSG	403

Figura D. Alinhamento entre as sequências de aminoácidos da EndoPGCp nativa e sintetizada.

Na sequência sintetizada foram excluídos os resíduos 1-24, que correspondem a um peptídeo sinal, e os resíduos 336-403, que correspondem a uma pro-sequência C-terminal que inativa a enzima. Os asteriscos indicam resíduos idênticos.

Tabela A. Propriedades moleculares e bioquímicas de endopoligalacturonases fúngicas expressas heterologicamente em *Pichia pastoris*

Fungo / Enzima	MM (kDa)	Glicosilação (%)	Atividade ótima		Referência
			pH	Temperatura (°C)	
<i>Chondrostereum purpureum</i>/ EndoPGCp	60	67%	4,5	60 - 70	Neste trabalho
<i>Aspergillus luchuensis</i> /	40	-	4,5	40	Tan et al., 2019
PgaB					
<i>Talaromyces cellulolyticus</i> /	43	-	5,0	70	Peng et al., 2019
TcPG					
<i>Rhizoctonia solani</i> /	45	-	5,0	50	Chen et al., 2017
RsPG2					
<i>Penicillium oxalicum</i> /	37	-	5,5	55	Cheng et al., 2017
PoxaEnPG28A					
<i>Aspergillus niger</i> /	41	10%	4,5	40	Wang et al., 2017
Pga-zj5a					
<i>Aspergillus aculeatus</i> /	38,7	-	5,0	50	Abdulrachman et al., 2017
EndoPG					
<i>Talaromyces leycettanus</i> /	38	-	3,5	70	Li et al., 2017
endo-TePG28b					
<i>Achaetomium</i> sp./	~38 - 42	13%	5,5 - 6,0	45 - 55	Tu et al., 2015
PG8fn					
<i>Neosartorya fischeri</i> /	37	-	4,5	70	Li et al., 2015
NfPG5					
<i>Penicillium oxalicum</i> /	48	33%	5,0	50	Tu et al., 2014
S6PG					

Tabela A. Propriedades moleculares e bioquímicas de endopoligalacturonases fúngicas expressas heterologamente em *Pichia pastoris* (final)

Fungo / Enzima	MM (kDa)	Glicosilação (%)	Atividade ótima		Referência
			pH	Temperatura (°C)	
<i>Aspergillus niger</i> / rePgaA	40	0	5,0	50	Liu et al., 2014
<i>Achaetomium</i> sp./ endo-PG I	40	13	6,0	45	Tu et al., 2013
<i>Galactomyces citri-aurantii</i> / PG1 e PG2	35 e 38	-	PG1: 4,0 - 5,0 PG2: 4,0	50	Cho et al., 2012
<i>Penicillium</i> sp./ endo-PG I	37	0	3,5	40	Yuan et al., 2011
<i>Rhizopus oryzae</i> / 15 EPGs	52 - 60	~37%	4,0 - 5,0	30 - 40	Mertens e Bowman, 2011
<i>Bispora</i> sp./ endo-PGA1	70	50	3,5	50	Yang et al., 2011

- Dado não apresentado

Tabela B. Propriedades bioquímicas de endopoligalacturonases fúngicas expressas heterologicamente em *Pichia pastoris*

Fungo / Enzima	Estabilidade térmica	Estabilidade ao pH	Efeito de íons		Referência
			Inibição	Estimulação	
<i>Chondrostereum purpureum</i>/ EndoPGCp	Mantém 60% da atividade máxima à 70°C por 30 min	6,0 - 8,0	Ca ²⁺ , Fe ²⁺ , K ⁺ , Mg ²⁺ , Mn ²⁺ (a)	β- mercaptoetanol (a)	Neste trabalho
<i>Aspergillus luchuensis</i> / PgaB	Mantém 40% da atividade máxima à 60°C por 7 h	-	SDS, EDTA, Zn ²⁺ , Ni ²⁺ , Cd ²⁺ , Ca ²⁺ , Mn ²⁺ , Co ²⁺ , Ba ²⁺ , Cr ²⁺ , Cu ²⁺ , Cb ²⁺ (b)	K ⁺ (b)	Tan et al., 2019
<i>Talaromyces cellulolyticus</i> / TcPG	-	-	SDS, EDTA, Ca ²⁺ , Mn ²⁺ , Cu ²⁺ (c)	Na ⁺ , Mg ²⁺ , Zn ²⁺ , Fe ²⁺ (c)	Peng et al., 2019
<i>Penicillium oxalicum</i> / PoxaEnPG28A	T _{1/2} de 25 min a 50 °C	3,0 – 10,0	Cu ²⁺ , Mn ²⁺ (b)	Co ²⁺ (b)	Cheng et al., 2017
<i>Aspergillus niger</i> / Pga-zj5a	T _{1/2} de 1 h a 45 °C	2,0 – 6,0	SDS, CTAB, EDTA, Zn ²⁺ , Ni ²⁺ , Cd ²⁺ , Ca ²⁺ , Mn ²⁺ , Mg ²⁺ , Co ²⁺ , Pb ²⁺ (b)	-	Wang et al., 2017
<i>Aspergillus aculeatus</i> / EndoPG	Mantém 60% da atividade máxima à 40°C por 3 h	2,0 – 6,0	Mn ²⁺ , Zn ²⁺ (b)	Cu ²⁺ , Fe ²⁺ (b)	Abdulrachman et al., 2017

Tabela B. Propriedades bioquímicas de endopoligalacturonases fúngicas expressas heterologicamente em *Pichia pastoris* (continuação)

Fungo / Enzima	Estabilidade térmica	Estabilidade ao pH	Efeito de íons		Referência
			Inibição	Estimulação	
<i>Talaromyces leycettanus</i> / endo-TePG28b	T _{1/2} de 25 min a 70 °C	2,0 – 8,0	-	-	Li et al., 2017
<i>Neosartorya fischeri</i> / NfPG5	T _{1/2} de 20 min a 60 °C	2,0 – 11,0	EDTA, SDS, Mg ²⁺ , Ag ⁺ , Pb ²⁺ , Ca ²⁺ , Li ²⁺ (b)	β-mercaptoetanol, K ⁺ , Na ⁺ , Mn ²⁺ , Cr ²⁺ , Cu ²⁺ , Zn ²⁺ , Fe ²⁺ (b)	Li et al., 2015
<i>Penicillium oxalicum</i> / S6PG	Mantém 100% da atividade máxima à 40°C por 1 h	2,0 – 6,0	EDTA, SDS, Mg ²⁺ , Mn ²⁺ , Co ²⁺ , Ni ²⁺ , Zn ²⁺ , Cr ³⁺ , Ag ⁺ , Na ⁺ , K ⁺ , Pb ²⁺ , Ca ²⁺ , Fe ³⁺ , Cu ²⁺ , Li ²⁺ (b)	-	Tu et al., 2014
<i>Aspergillus niger</i> / rePgaA	T _{1/2} de 5 min a 55 °C	5,0 – 9,0	-	-	Liu et al., 2014
<i>Achaetomium</i> sp./ endo-PG I	T _{1/2} de 40 min a 55 °C	2,0 – 8,0	EDTA, Ag ⁺ , Ca ²⁺ , Mg ²⁺ , Cu ²⁺ , Cr ³⁺ (b)	β-mercaptoetanol (b)	Tu et al., 2013
<i>Galactomyces citri-aurantii</i> / PG1 e PG2	PG1: T _{1/2} de 1 h a 40 °C PG2: T _{1/2} de 1 h a 20 °C	PG1: 4,0 – 7,0 PG2: 4,0 – 6,0	Ca ²⁺ , Cu ²⁺ , Fe ³⁺ , Zn ²⁺ (b)	Mn ²⁺ (b)	Cho et al., 2012

Tabela B. Propriedades bioquímicas de endopoligalacturonases fúngicas expressas heterologicamente em *Pichia pastoris* (final)

Fungo / Enzima	Estabilidade térmica	Estabilidade ao pH	Efeito de íons		Referência
			Inibição	Estimulação	
<i>Penicillium</i> sp./ endo-PG I	Mantém 60% da atividade máxima à 50°C por 1h	4,0 - 6,0	Co ²⁺ , Mn ²⁺ , Ni ²⁺ , Cu ²⁺ , Ca ²⁺ , Pb ²⁺ , Cr ³⁺ , Fe ³⁺ , Zn ²⁺ , SDS (a)	β-mercaptoetanol (a)	Yuan et al., 2011
<i>Bispora</i> sp./ endo-PGA1	T _{1/2} de 20 min a 60 °C	2,0 - 7,0	Cu ²⁺ , Co ²⁺ , Mn ²⁺ , Ni ²⁺ , Fe ³⁺ , Mg ²⁺ , Zn ²⁺ , Ca ²⁺ , Cr ²⁺ , Hg ²⁺ , Pb ²⁺ e SDS (a)	β-mercaptoetanol (a)	Yang et al., 2011

- Dado não apresentado

(a) Concentração dos íons = 5 mmol.L⁻¹

(b) Concentração dos íons = 1 mmol.L⁻¹

(c) Concentração dos íons = 10 mmol.L⁻¹

Tabela C. Endopoligalacturonases imobilizadas covalentemente à suportes ativados via glutaraldeído

Enzima	Suporte	Rendimento (%)	Eficiência (%)	Referência
EndoPGCp-NPMs	Nanopartículas ferromagnéticas revestidas com quitosana	20	100	Neste trabalho
Pectinase de <i>Aspergillus aculeatus</i>	Esferas de Alginato-montmorilonita	85	-	Mohammadi et al., 2019
Poligalacturonase de <i>Aspergillus niger</i>	Glioxil-agarose modificada com etilenodiamina	65	15	Dal Magro et al., 2019
Pectinase comercial	Nanopartículas ferromagnéticas revestidas com dióxido de silício	73	-	Fang et al., 2016
Pectinase de <i>Aspergillus aculeatus</i>	Florisil (silicato de magnésio)	82	10	Alagöz et al., 2016
	Nanosílica	86	75	
Pectinase de <i>Aspergillus niger</i>	Quitina revestida com quitosana	80	82	Ramirez et al., 2015
Pectinase de <i>Penicillium oxalicum</i>	Microesferas magnéticas de amido de milho	86	-	Wang et al., 2013
Pectinase comercial	Nanopartículas magnéticas revestidas com sílica	98	89	Seenuvasan et al., 2013
Poligalacturonase de <i>Aspergillus niger</i>	Esperas de Nylon-6	70	40	Shukla et al., 2010
Poligalacturonase de <i>Aspergillus niger</i>	Matriz de polietileno	37	-	Saxena et al., 2008
Pectinase comercial	Alginato de sódio	66	-	Li et al., 2007
Endopoligalacturonase de <i>Aspergillus ustus</i>	Sílica gel	20	85	Rao et al., 2000

- Dado não apresentado

Tabela D. Propriedades bioquímicas de endopoligalacturonases imobilizadas covalentemente à suportes ativadas via glutaraldeído

Enzima	Temperatura ótima (°C)	pH ótimo	Estabilidade térmica	Reuso	Referência
EndoPGCp livre	60 – 70	4,5	Retém 25% da atividade após incubação a 70°C por 30 min		Neste trabalho
EndoPGCp-NPMs	60 – 70	4,0 – 5,5	T _{1/2} de 30 min a 70 °C	Retém 65% da atividade após 7 usos	
Pectinase livre	40	5,5	T _{1/2} de 100 min a 60 °C		Mohammadi et al., 2019
Pectinase imobilizada	40	5,0	T _{1/2} de 60 min a 60 °C	Retém 53% da atividade após 6 usos	
Poligalacturonase livre	50	4,0	T _{1/2} de 10 min a 50 °C		Dal Magro et al., 2019
Poligalacturonase imobilizada	60	4,0	Retém 80% da atividade após incubação a 50°C por 3 h	Retém 90% da atividade após 7 usos	
Pectinase livre	50	8,5	-		Fang et al., 2016
Pectinase imobilizada	55	8,5	-	Retém 65% da atividade após 7 usos	

Tabela D. Propriedades bioquímicas de endopoligalacturonases imobilizadas covalentemente à suportes ativadas via glutaraldeído *continuação*

Enzima	Temperatura ótima (°C)	pH ótimo	Estabilidade térmica	Reuso	Referência
Pectinase livre	35	5,0	T _{1/2} de 10,8 h a 50 °C		
Pectinase imobilizada em Florisil	35	5,0	T _{1/2} de 12,8 h a 50 °C	Retém 76% da atividade após 10 usos	Alagöz et al., 2016
Pectinase imobilizada em nano sílica	35	5,0	T _{1/2} de 14,8 h a 50 °C	Retém 80% da atividade após 10 usos	
Pectinase livre	40	5,0	T _{1/2} de 14 min a 50 °C		Ramirez et al., 2015
Pectinase imobilizada	50	4,5	T _{1/2} de 40 min a 50 °C	Retém 70% da atividade após 15 usos	
Pectinase livre	50	4,0	Retém 80% da atividade após incubação a 60°C por 1 h		Wang et al., 2013
Pectinase imobilizada	40	4,0	Retém 70% da atividade após incubação a 60°C por 1 h	Retém 60% da atividade após 8 usos	
Pectinase livre	50	4,0	-		Seenuvasan et al., 2013
Pectinase imobilizada	50	4,0	-	Retém 70% da atividade após 7 usos	

Tabela D. Propriedades bioquímicas de endopoligalacturonases imobilizadas covalentemente à suportes ativados via glutaraldeído *final*

Enzima	Temperatura ótima (°C)	pH ótimo	Estabilidade térmica	Reuso	Referência
Poligalacturonase livre	45	4,5	Retém 15% da atividade após incubação a 45°C por 2 h		Shukla et al., 2010
Poligalacturonase imobilizada	50	5,0	Retém 73% da atividade após incubação a 45°C por 2 h	Retém 17% da atividade após 7 usos	
Poligalacturonase livre	45	4,8	Retém 15% da atividade após incubação a 45°C por 1 h		Saxena et al., 2008
Poligalacturonase imobilizada	45	4,8	Retém 34,3% da atividade após incubação a 45°C por 1 h	Retém 50% da atividade após 3 usos	
Pectinase livre	40	3,0	Retém 20% da atividade após incubação a 50°C por 2 h		Li et al., 2007
Pectinase imobilizada	40	3,5	Retém 60% da atividade após incubação a 50°C por 2 h	Retém 80% da atividade após 11 usos	

- Dado não apresentado

Apêndices

Appendice A

Biochemical and kinetic characterization of the recombinant GH28 *Stereum purpureum* endopolygalacturonase and its biotechnological application

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ABSTRACT

Endopolygalacturonase (EndoPG) from *Stereum purpureum* was expressed as a soluble protein in *Pichia pastoris* GS115, where after 3 days methanol induction the enzyme activity in the culture supernatant was 40 U mL⁻¹. After purification by IMAC, SDS-PAGE analysis showed that the molecular weight of EndoPG was approximately 60.0 kDa. The carbohydrate content of the recombinant enzyme was estimated to be 67.0% (w/w). The optimum temperature and pH of catalysis were 60–70 °C and pH of 4.5, respectively. The enzyme was highly stable over the pH range 5.0–8.0 and retained approximately 60% of its initial activity after incubation at 70 °C for 30 min. The enzyme showed a specific activity of 5940.0 ± 217.1 mg⁻¹ and hydrolyzed citrus pectin with V_{max} and a K_m of 6947.10 ± 393.63 U mg⁻¹ and 2.45 ± 0.23 mg mL⁻¹, respectively, and showed a catalytic efficiency of 2052.99 ± 103.54 mL mg⁻¹ s⁻¹. EndoPG alone reduced the viscosity of papaya juice by 20% after 30 min, and increased its transmittance about 50% with a concomitant reduction of the color by about 55% after 3 h of enzymatic treatment. For apple juice, the relative reduction of viscosity was 30% after 5 h, and the reduction of the color was 30% with a 12% increase in transmittance. Supplementation of a commercial enzymatic cocktail for lignocellulose saccharification with EndoPG increased total reducing sugar release by 0.6 ± 2.1% against sugarcane bagasse, indicating improved access of the cellulosytic enzymes to the biomass polysaccharides.

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1. Introduction

Pectin is a complex polysaccharide present in plant cell walls composed mainly of three polysaccharides: homogalacturonan, a linear polymer of partially acetylated and/or methylated α -(1-4)-D-galacturonic acid (GalpA) present within the middle lamella [1–4], and rhamnogalacturonans types I and II. Rhamnogalacturonan type I is composed of a main of GalpA residues interrupted by L-rhamnose units composing an alternating structure of α -(1-4)-D-GalpA- α -(1-2)-L-Rha [1,2,4]. The GalpA backbone residues are rarely esterified with methyl groups, but may be acetylated at the O-2 and/or O-3 positions [1,5]. Rhamnogalacturonan II can be considered as a substituted galacturonan and has a main chain of α -(1,4) linked GalpA with branching chains of arabinans, galactans, and arabinogalactans [1,2]. The homogalacturonan sugars are covalently linked to rhamnogalacturonans I and II, and are also presumed to be covalently crosslinked to other wall polymers, thereby contributing to the recalcitrance of the cell wall [1,3,5] and hindering access to hydrolytic enzymes by forming a physical barrier [6]. These factors have stimulated

the recent interest in the removal of pectin in order to convert the vegetal biomass into fermentable sugars for the production of bioproducts.

As a consequence of the complexity of polysaccharide substrate, the enzymatic hydrolysis of pectin involves multiple enzymes. According to the mode of action and the cleavage site, pectinases are classified into two broad groups: the pectin esterases (EC 3.1.1.11) and pectin depolymerases, which are further divided into polygalacturonases (EC 3.2.1.15, EC 3.2.1.67 and EC 3.2.1.82) and lyases (EC 4.2.2.2 and EC 4.2.2.10) [2,5]. The polygalacturonases represent the most numerous class and catalyze the hydrolysis of the α -(1-4)-glycosidic bond between neighboring galacturonic acid residues [2,7]. These enzymes may have either an endo-action (EC 3.2.1.15), hydrolyzing the internal bonds within homogalacturonan molecules in a random manner by an inversion mechanism and generating galacturonic acid oligomers of different sizes, or an exo-action (EC 3.2.1.67 and EC 3.2.1.82) by acting on the non-reducing end of the polymer to give the monogalacturonic acid product [2,8].

The endopolygalacturonases (EPGs) produced by fungi, bacteria, plants, insects and nematodes [2,9] have been characterized, although to date only 3D-structures from fungal EPGs have been determined [10,11]. The fungal EPGs include the EndoPG from *Stereum purpureum* [12,13], which was originally isolated from a crude extract of the fungal isolate ASP-4B culture [14] and its nucleotide coding sequence was

cloned and expressed as a heterologous protein in *Escherichia coli* [13] and *Pichia pastoris* [15]. The three-dimensional structure of the native and recombinant enzymes has been resolved [13,16,17], and the glycosylation sites and disulfide bridge pattern have been determined [12,18]. However, a more detailed biochemical characterization has not been described, and the potential applications of this enzyme in bioprocesses have not been evaluated. In this work, we report the heterologous expression of this enzyme in *P. pastoris*, together with a detailed biochemical and kinetic characterization and report the results of experiments for possible biotechnological applications in lignocellulose saccharification and clarification of fruit juices.

2. Material and methods

2.1. Subcloning of the endoPG gene from *Stereum purpureum*

A circular polymerase extension cloning (CPEC) strategy [19] was used to subclone the *S. purpureum* endoPG gene. The nucleotide sequence encoding EndoPG was synthesized from the sequence deposited in the National Center for Biotechnology Information (Access Key: D45072.1) and was amplified by PCR using Pfu DNA polymerase (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions, with the following CPEC forward and reverse primers, respectively: 5' - GAGAAAGAGAGAGCTGAAGCTTACATGGCTAGCGCTA - 3' and 5' - TGAGATGAGTTTGTTCCTAGGCAAACTACTGTCCCC - 3'. The resulting PCR product was purified from the agarose gel using Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, Madison, WI, USA) and added to a CPEC reaction mixture in an equimolar ratio with 100 ng of the pPC98T/CT plasmid DNA linearized with enzymes AvrII (Thermo Scientific) and SnaBI (Thermo Scientific), 400 μ M deoxynucleoside triphosphates (dNTPs), and 1 U Pfuusion polymerase (Thermo Scientific). The reaction mixture was heated in a thermocycler to 98 °C for 3 min, followed by 30 cycles of 98 °C for 30 s, 55 °C for 30 s, and 72 °C for 6 min, and the reaction was completed with a 10-min incubation at 72 °C. Five microliters of this reaction mixture were transformed into *Escherichia coli* DH10Q (Novagen, Madison, WI, USA). The resulting recombinant plasmid, pPC98T/CT_endoPG, was validated by DNA nucleotide sequencing.

2.2. Construction of *Pichia pastoris* strain

P. pastoris GS115 was transformed as previously described [20]. Briefly, the pPC98T/CT_endoPG was linearized with the restriction enzyme SnaI (Thermo Scientific) and was used to transform *P. pastoris* GS115 cells by electroporation. The linearized construct pPC98T/CT_endoPG was inserted into the HIS4 gene by homologous recombination and selected by histidine auxotrophism on MD agar plates (3.4 g L⁻¹ yeast nitrogen base without amino acids and ammonium sulfate (YNB - Sigma-Aldrich Chemical Company, St. Louis, MO, USA), 10 g L⁻¹ ammonium sulfate, 0.0004 g L⁻¹ biotin, 0.1 mol L⁻¹ potassium phosphate buffer, pH 6.0, 20 g L⁻¹ dextrose, 1.5% (w/v) bacteriological agar).

Positive transformants were screened based on endopolygalacturonase activity in liquid medium. Single colonies of *P. pastoris* GS115/pPC98T/CT_endoPG were inoculated into 125 mL Erlenmeyer flask containing 10 mL of MG medium [3.4 g L⁻¹ YNB, 10 g L⁻¹ ammonium sulfate, 0.0004 g L⁻¹ biotin, 1% (v/v) glycerol, 100 mmol L⁻¹ potassium phosphate buffer, pH 6.0]. The culture was incubated at 30 °C for 16 h in an orbital shaker at 250 rpm. The cells were then harvested by centrifugation at 4000 rpm for 5 min, and the pellet resuspended in 0.5 mL of MM medium (MG medium, in which glycerol was substituted with 1% (v/v) methanol) and transferred to 125 mL Erlenmeyer flasks containing 10 mL of MM media and cultured in an OD₆₀₀ 1.0. Absolute methanol (1% (v/v) final concentration) was added to the cultures at 24 h periods to induce endoPG expression and the pH was corrected daily by addition of 100 μ L of 1 mol L⁻¹ potassium

phosphate buffer, pH 8.0. Pectinase activity was monitored in the supernatant of all cultures over a period of 7 days and the clones that showed increased activity were selected.

2.3. Heterologous expression in *P. pastoris* on a large scale and purification of EndoPG

The transformant with the highest pectin activity was inoculated into 1 L-Erlenmeyer flask containing 100 mL of MG medium. The culture was incubated at 30 °C for 16 h in an orbital shaker at 250 rpm. The cell pellet was then harvested by centrifugation at 4000 rpm for 5 min, and resuspended in 5 mL of the corresponding medium. The cells grown were transferred to three 1 L-Erlenmeyer flasks containing 100 mL of MM media to an OD₆₀₀ 1.0. Absolute methanol (1% (v/v) final concentration) was added to the cultures at 24 h periods and the pH was corrected daily by addition of 1 mL of 1 mol L⁻¹ potassium phosphate buffer, pH 8.0. The enzyme expression occurred in these conditions for 3 days, after which the cells were centrifuged (4000 rpm, 20 min) and the supernatant was stored at 4 °C until processed further.

The recombinant EndoPG was purified from the culture supernatant by immobilized metal affinity chromatography (IMAC). After adjustment to pH 8.0, the supernatant was applied to 1 mL of a nickel affinity chromatography resin (Promega Co.) previously equilibrated with 50 mmol L⁻¹ HEPES buffer, pH 8.0, containing 500 mmol L⁻¹ NaCl. The resin was washed with the same buffer containing increasing concentrations of imidazole until protein was no longer detected (A₂₈₀) in the eluate. The EndoPG was eluted in the same buffer containing 250 mmol L⁻¹ imidazole.

2.4. Enzymatic assays

Unless otherwise stated, the endopolygalacturonase activity was assayed at 60 °C in McIlwaine buffer [21], pH 4.5, containing 0.5% (w/v) citrus pectin (Sigma-Aldrich Chem. Co.) in a final volume of 600 μ L. The hydrolysis of β -glucan extract from barley [22], beechwood xylan (Sigma-Aldrich Chem. Co.), low viscosity CMC (Sigma-Aldrich Chem. Co.) and microcrystalline cellulose (Avicel® PH, Fluka Chemical Co., Seelze, Germany), all at a final concentration of 1% (w/v), was assayed using the same method as described for the citrus pectin. The total released reducing sugars were quantified using the dinitrosalicylic acid (DNS) method [23].

The experimental conditions (reaction times, enzymatic units) employed were adjusted to guarantee the reliable estimation of initial velocities from the linear response of product formation in respect to reaction time. One unit of enzyme activity (U) was defined as the amount of enzyme that catalyzes the formation of 1 μ mol of product per min under its optimal catalytic conditions and specific activity was defined as U per mg of protein (U mg⁻¹). Controls with heat inactivated enzyme were included in all enzymatic assays, and all experiments were performed in triplicate. The final values of activity were presented as the mean $\pm$ SD.

2.5. Estimation of protein and neutral carbohydrates

Protein concentration was estimated according to Read and Northcote [24], using bovine serum albumin (BSA, Sigma-Aldrich Chem. Co.) as the standard. Total neutral carbohydrates of pure EndoPG was determined according to the procedure described by Dubois et al. [25], using mannose as the standard.

2.6. Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli [26] in 10% acrylamide slab gels. The protein bands were revealed with silver

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ntrate [27] or Coomassie Brilliant Blue R (Sigma-Aldrich Chem. Co., St Louis-MO, USA).

2.7. Deglycosylation of EndoPG by endoglycosidase H

The pure denatured EndoPG (10 µg) was deglycosylated with endoglycosidase H (Endo H - New England Biolabs, Ipswich, MA, USA) at 37 °C for 24 h according to the manufacturer's instructions. The sample was subsequently analyzed by SDS-PAGE.

2.8. Effect of temperature and pH on EndoPG activity and stability

The optimum catalytic temperature was determined over the temperature range of 40–85 °C in McIlvaine buffer [21], pH 4.5. The thermal stability was evaluated by incubating the purified EndoPG diluted in Millipore MilliQ ultrapure water for 30 min at temperatures from 50 to 80 °C. After cooling in an ice bath for 1 min, the residual endopolysaccharuronase activity was estimated at 60 °C as described in Section 2.4. The effects of pH on activity of EndoPG were determined at 60 °C in McIlvaine buffer [21] in the range 3.0–8.0. The pH stability was studied by incubating pure EndoPG at 4 °C in McIlvaine buffer [21], pH 3.0–8.0 for 24 h, before determination of the residual endopolysaccharuronase activity under the standard conditions (60 °C, pH 4.5).

2.9. Effect of ions and other agents on the enzymatic activity

The effect of various salts (KCl and NaCl), divalent metal ions (CaCl₂, FeCl₃, MnCl₂ and MgCl₂) and other reagents (ethylenediamine tetraacetic acid (EDTA, Sigma-Aldrich Chem. Co.), β-mercaptoethanol (Sigma-Aldrich Chem. Co.), Sodium dodecyl sulfate (SDS, Sigma-Aldrich Chem. Co.), Tween-20 (Sigma-Aldrich Chem. Co.), and Triton X-100 (Sigma-Aldrich Chem. Co.)) on pectinase activity of the pure enzyme was evaluated under standard assay conditions as described in Section 2.4.

2.10. Determination of kinetic parameters for citrus pectin hydrolysis by EndoPG

The kinetic parameters V_{max} (maximum velocity) and K_m (Michaelis-Menten constant) for the hydrolysis of citrus pectin (concentrations ranging from 0.1 to 10 mg mL⁻¹) were calculated by non-linear regression using the Sigafit software [28]. The kinetic experiments were repeated using three independent purified EndoPG preparations, where enzymatic assays were performed in duplicate. The values of the kinetic parameters presented are given as the mean ± SD for the three different EndoPG preparations ($n = 3$).

2.11. Supplementation of a commercial enzyme cocktail by EndoPG in the hydrolysis of sugarcane cell wall fraction

The sugarcane bagasse was prepared following De Souza et al. [29]. Briefly, culms were disk-milled to a 0.5 mm particle size, and prepared with successive extractions with 80% (v/v) ethanol at 80 °C. The alcohol insoluble residue (AIR) was washed with distilled water and dried at 60 °C for 24 h. The hydrolysis of AIR was carried out at 45 °C and 200 rpm in hermetically closed tubes containing 50 mmol L⁻¹ sodium acetate buffer, pH 5.0; 10 mmol L⁻¹ sodium azide, and 5% (w/v) suspension of substrate in a final volume of 1 mL. The reaction was initiated by the addition of an aliquot either of purified EndoPG, or of a cellulase-rich enzyme cocktail (Celluclast 1.5 L, Novozymes A/S, Denmark) or of a mixture of both enzyme preparations. After desired time intervals, aliquots of the reaction medium were centrifuged at 10,000 ×g for 5 min, and analyzed for total reducing sugars by the DNS method [23]. The filter paper activity of Celluclast 1.5 L extract was assayed in the same condition of the hydrolysis experiments, using a strip (1 × 3 cm)

of Whatman no. 1 filter paper as substrate and quantifying the released reducing sugars. In all experiments, the enzyme loading was constant, being 7.5 FPU g⁻¹ filter paper to Celluclast 1.5 L and 240 U g⁻¹ substrate to EndoPG. Each hydrolysis experiment was repeated three times using three independently purified EndoPG preparations. Each experimental point was assayed in triplicate.

2.12. Application of EndoPG in the clarification of fresh papaya and apple juices

The juices clarification assay was performed as described by Tu et al. [30]. Papaya and apple juices were prepared by macerating 200 g of fruit in 0.25% (w/v) ascorbic acid in a domestic liquidizer followed by filtration through four layers of gauze cloth. The pH and density of the fresh juices at room temperature were determined to be 4.6 and 2.0 g mL⁻¹, respectively. For the clarification of the papaya juice, 50 mL of juice were mixed with 100 U of EndoPG in incubated at 60 °C for 5 h, and after desired time intervals, aliquots of the reaction medium were analyzed. For apple juice, 20 mL of juice were mixed with 100 U of EndoPG and maintained under the same conditions. The same volume of heat-inactivated EndoPG was incubated with the same amount of papaya or apple juices as the control. The relative reduction in juice viscosity was measured using an Oswald viscometer. The light transmittance and the color by absorbance of the juice supernatant were determined at 660 and 420 nm, respectively. All determinations were carried out as triplicate and the averages reported.

3. Results and discussion

3.1. Expression and purification of EndoPG

The endog DNA coding sequence (without the native signal peptide [31]) and without the native pro-sequence in the C-terminal region previously identified as an inactivator of the protein [18]) was successfully expressed in *P. pastoris* GS115 in an active secreted form. Transformants showing endopolysaccharuronase activity were screened in MM media, where enzymatic activity of up to approximately 40 U mL⁻¹ was measured after methanol induction for 3 days. The EndoPG was purified from culture supernatants by Ni-affinity chromatography with a yield of approximately 70% and the purified enzyme presented a specific activity of 3040.0 ± 217.3 U mg⁻¹. SDS-PAGE analysis (Fig. 1a) revealed a single protein band of approximately 60 kDa, which is significantly higher than the theoretical molecular mass of 37 kDa. After treatment with Endo H, pure EndoPG presented an apparent molecular weight of the deglycosylated enzyme was approximately 37 kDa (Fig. 1b) indicating that the EndoPG is glycosylated. The NetNGlyc 1.0 program (<http://www.cbs.dtu.dk/services/NetNGlyc/>) was used to analyze the amino acid sequence and was identified five putative N-glycosylation sites in the EndoPG amino acid sequence (N⁶⁶NGT⁶⁷, N¹⁶⁷NVT¹⁶⁸, N²³⁹NT²⁴⁰, N²⁸³NCST²⁸⁴, N³⁰⁹NWST³¹⁰). The sixth possible glycosylation site described by Haul et al. [14] is present in the C-terminal region of the native enzyme which was deleted from the expressed coding sequence. The estimated carbohydrate content of EndoPG by Dubois et al. [25] method was of 67.0% (w/w), higher than that determined for the native EndoPG from *S. purpureus* by analyze in SDS-PAGE after deglycosylation treatment with Endo H [14] and by electrospray ion mass spectrometry [12]. Glycosylation is one of the most common post-translational modifications of proteins and has been reported in several fungal endopolysaccharuronases from *Aschersonium* sp. [30], *Penicillium oxalicum* [32], *Rhizopus oryzae* [33] and *Bispora* sp. [34] which were also expressed heterologous form in *P. pastoris*.

Fig. 1. Analysis of the purified EndoPG by SDS-PAGE, showing the purified enzyme before (lane a) and after (lane b) Endo H digestion. Total protein loading was 1 µg per lane.

3.2. Effect of temperature and pH on the activity and stability of the EndoPG

The purified recombinant EndoPG exhibited an enzymatic activity that gradually increased above 40 °C, reaching a plateau of maximum activity at 60–70 °C and abruptly decreased at higher temperatures (Fig. 2a). This result is similar to that observed for the enzyme expressed in *Escherichia coli*, where the optimum temperature was 60 °C [35]. EndoPG expressed in *P. pastoris* retained about 60% of its initial activity after incubation at 70 °C for 30 min (Fig. 2b). In contrast, the enzyme expressed in *E. coli* presented about 10% of the maximal activity under the same condition [35]. Endopolysaccharuronases from mesophilic fungi expressed in *P. pastoris* have optimal temperature between 40 and 50 °C, including those from *Aspergillus ochraceus* [7], *P. oxalicum* [32], *R. oryzae* [33], *Aschersonium* sp. [36], *Aspergillus niger* [37], only the EndoPG investigated in this study and the endopolysaccharuronase of *Jukavomyces leucostomus* [38] are optimally active at around 70 °C.

The optimal activity of endopolysaccharuronase was observed at pH 4.5, and is similar to the same enzyme expressed in *E. coli* [35]. Enzymes obtained from both these expression systems retained more than 80% of their maximal activities at a pH 4.0 or 5.0, however a marked decrease in enzyme activity was observed above pH 6.0 (Fig. 3a). These observations are consistent with numerous reports demonstrating that EPGs present greatest activities in acidic or neutral conditions [7,32,33,36–38]. Furthermore, the enzyme was stable between pH values of 6.0–8.0 and presented a loss of approximately 50% of the control activity when incubated in pH 3.0 to 5.0 for 24 h (Fig. 3b). In contrast, the enzyme expressed in *E. coli* presented pH stability over a range of 2.0–10.0 [35].

High temperatures and acid pH are required in some industrial processes, such processes of saccharification of lignocellulosic biomass, fruit juice clarification or processing of lignocellulose fibers in the textile industry [34,38]. In this context, EndoPG has interesting properties for a component in enzyme mixtures for application in biotechnological processes.

Fig. 2. Optimal temperature (a) and thermal stability (b) for the pectinase activity of EndoPG. (a) The activity was assayed at different temperatures in McIlvaine buffer, pH 4.5, using 0.5% (w/v) citrus pectin as the substrate. (b) For thermal stability, samples of pure enzyme diluted in water were incubated at 50 °C, 60 °C, 70 °C and 80 °C for 30 min. Residual activity was estimated at 60 °C in McIlvaine buffer, pH 4.5, using 0.5% (w/v) citrus pectin as the substrate. One hundred percent specific activity corresponded to 4000.4 ± 388.3 U mg⁻¹. The values shown represent means ± SD from triplicate experiments ($n = 3$) carried out with three independently prepared purified enzyme samples.

3.3. Effect of ions and effectors on the endopolysaccharuronase activity of EndoPG

The effect of several ions and effectors on the enzymatic activity of EndoPG is presented in Table 1. The enzyme was tolerant to the presence of Mg²⁺ and Na⁺ at the concentration tested. In contrast, the presence of Mn²⁺ and K⁺ ions had a weak inhibitory effect (17 and 21%, respectively) on pectinase activity. In addition, the enzyme was highly sensitive to Ca²⁺ and Fe³⁺, with residual activity at 18% and 0%, respectively. EDTA presented a weak inhibitory effect on the enzymatic activity, suggesting that the EndoPG is not a metalloenzyme. In contrast, β-mercaptoethanol at a concentration of 5 mmol L⁻¹ stimulated the enzymatic activity by 25%. In contrast to that observed for EndoPG, the EPGs produced by *A. aculeatus* [7], *P. oxalicum* [32] and *Bispora* sp. [34], were not inhibited by K⁺ in the same concentration tested in this study, but were sensitive to inhibition by Mn²⁺.

3.4. Substrate specificity of EndoPG

The specificity of the EndoPG was evaluated using various polysaccharide and natural substrates. The enzyme showed a specific activity of 49942 ± 202.4 U mg⁻¹ against citrus pectin and no activity was detected against CMC, Avicel®[®], β-glucan, xyloglucan and xylan. This

Sibeli Carli and Luana P. Meleiro conceived, designed and performed the experiments; Sibeli Carli, Luana P. Meleiro and Richard J. Ward analyzed the data; Richard J. Ward contributed reagents, materials and analysis tools; Sibeli Carli and Luana P. Meleiro wrote the paper.

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Apêndice B

Immobilization of a β -glucosidase and an endoglucanase in ferromagnetic nanoparticles: A study of synergistic effects

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ABSTRACT

Nanoparticles can act as support materials for enzymatic immobilization, introducing a balance of characteristics that modulate the efficiency of biocatalysis, such as specific surface area, resistance to mass transfer and effective enzymatic loading. Magnetic nanoparticles can be easily separated using an external magnetic field, and in this work two recombinant enzymes, the β -glucosidase from *Hammaria insulens* (Hgl) and the endoglucanase from *Syzygium thermophilum* (Egt) were immobilized on synthesized Fe₃O₄ nanoparticles derivatized with chitosan/glutaraldehyde/N-(5-amino-1-carboxy-pentyl) iminodiacetic acid and functionalized with NiCl₂. The immobilization yields were about 20% for Hgl and Egt with efficiencies of 122% and 115%, respectively. The two enzymes were also co-immobilized with yield was about 49%. The optimal temperatures of the immobilized enzymes were 70 °C and 50 °C for Egt and Hgl, respectively. Egt hydrolyzed CMC in the presence of 4 mM NaCl with $V_{max} = 625.0 \pm 6.7$ U mg⁻¹ and $K_m = 6.4 \pm 0.5$ mg mL⁻¹ resulting in a catalytic efficiency (k_{cat}/K_m) of 107.4 ± 5.4 mg⁻¹ s⁻¹ mol⁻¹. Hgl hydrolyzed pNP-Glc with $V_{max} = 52.7 \pm 2.7$ U mg⁻¹ and $K_m = 0.23 \pm 0.01$ mM resulting in a catalytic efficiency (k_{cat}/K_m) of 214.3 ± 10.2 s⁻¹ mol⁻¹. The individually immobilized enzymes when combined showed a synergistic effect on the substrates tested and a very similar action when compared to the co-immobilized enzymes, suggesting excellent potential for application in biotechnological processes.

1. Introduction

Lignocellulosic ethanol is viewed as a promising alternative to the use of fossil fuels, and can contribute to reduce global dependence on non-renewable energy resources [1]. Cellulose is the most abundant polysaccharide in lignocellulosic biomass and can be transformed into biofuel via depolymerization of the polysaccharide to glucose, which can be easily fermented to ethanol [1–3]. Of the various strategies available for the hydrolysis of cellulose, the enzymatic methods are the most efficient and least environmentally invasive. The enzymes involved in cellulose depolymerization are divided into 3 groups which include endoglucanases (EC 3.2.1.4), which randomly cleave the β -1,4 glycosidic linkages of cellulose, cellobiohydrolases (EC 3.2.1.91) that

attack cellulose chain ends to produce cellobiose and β -glucosidases (EC 3.2.1.21) that hydrolyze cellobiose into two molecules of glucose [1,3–5].

The development of efficient an enzyme-based technology for the conversion of lignocellulosic biomass to sugars is limited by the hydrolysis step, since during the process most enzymes are inhibited by the accumulation of their reaction products [6]. Overcoming this inhibition is a major challenge, since the alternative is the use of high enzyme loading, which increases the cost of production and impacts on the economic viability of the process. Therefore, the use of enzymes that are thermostable, tolerant to hydrolysis products and that are amenable to reuse may have significant economic and ecologic potential in the development of sustainable bioprocesses.

Abbreviations: Hgl, recombinant β -glucosidase from *Hammaria insulens*; Egt, recombinant endoglucanase from *Syzygium thermophilum*; MNPs, functionalized paramagnetic nanoparticles; Bis-Tris, 2-Bis(2-hydroxyethyl)amino-2-(hydroxymethyl)-1,3-propanediol; pNP-Glc, p-nitrophenyl β -D-glucopyranoside; CMCase, carboxymethylcellulase; CMC, carboxymethylcellulose; DNS, dinitrosalicylic acid; Ea, activation energy; V_{max} , maximum velocity; K_m , Michaelis-Menten constant; AB, alcohol insoluble residue; DMSO, dimethyl sulfoxide; TLC, thin layer chromatography; TDS, total reducing sugar; IEM, Scanning Electric Microscopy; AB-NTA, N-(5-amino-1-carboxy-pentyl) iminodiacetic acid

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Reuse of immobilized enzymes is an effective way of reducing the quantity of enzyme in a given process [7]. Immobilization methods are divided into three main categories: (1) self-immobilization, (2) surface immobilization and (3) entrapment, in which different materials (organic or inorganic) can be used as the support [7–10]. However, the ease of separating the support from the reaction products is a key factor to the immobilization efficiency and magnetic nanoparticles (MNPs) are promising in terms of their high surface area, leading to a higher load of enzymes per unit mass of support, and the ease of separating them from mixtures using a magnetic field [7,11–13].

Recent improvements have been reported in the methodology for the preparation of Fe₃O₄ nanoparticles derivatized with chitosan/glutaraldehyde/N-(5-amino-1-carboxy-pentyl) iminodiacetic acid (AB-NTA) [13]. These ferromagnetic nanoparticles functionalized with NiCl₂ were successfully applied for immobilization of histidine-tagged proteins and proved to be a suitable support in multiple cycles of enzyme use and washing with minimal loss of catalytic activity [13]. In this present study we have investigated the immobilization on ferromagnetic nanoparticles functionalized of a recombinant endoglucanase from *Syzygium thermophilum* (Egt) [14] and a recombinant β -glucosidase from *Hammaria insulens* (Hgl) [15]. These enzymes combine interesting characteristics for application in biotechnological processes. Egt was expressed in *Pichia pastoris* and presented optima of pH and temperature of reaction of pH 5.5 and 60–70 °C, respectively. The enzyme presented a half-life of 100 min at 65 °C and was highly stable at pH 3.0–8.0. Moreover, Egt showed good tolerance to reaction products [14]. Hgl was expressed in soluble form in *Escherichia coli* and presented optimum temperature and pH of 60 °C and 5.0–7.0, respectively. The recombinant enzyme was stable for 1 h at 50 °C and pH 4.5 to 8.0. Hgl was stimulated about 2-fold by glucose and xylose [15]. In addition, the three-dimensional structure of the Hgl was solved [16], and the enzyme was engineered for studies about the mechanism of stimulation of GH1 β -glucanases by the reaction product [17].

Therefore, the biochemical and kinetic characteristics these enzymes were analyzed against several natural substrates using both individually immobilized and co-immobilized preparations.

2. Materials and methods

2.1. Expression and purification of recombinant β -glucosidase and endoglucanase

The recombinant β -glucosidase from *H. insulens* (Hgl) was over-expressed in *Escherichia coli* BL21 (DE3) after transformation with the plasmid pET28-Hgl containing the Hgl coding sequence [15]. The cells were grown in HDM medium (yeast extract 25 g L⁻¹, tryptone 15 g L⁻¹, and MgSO₄ 10 mM) supplemented with specific antibiotics at 37 °C and grown to an OD₆₀₀ of approximately 0.6. Protein expression was induced by addition of isopropyl β -D-thiogalactopyranoside to the culture medium at a final concentration of 1 mM. After 5 h induction at 37 °C, the cells were collected by centrifugation at 5000 $\times$ g and 4 °C for 20 min. The pellet was resuspended in lysis buffer (50 mM HEPES buffer, pH 8.0, containing 500 mM NaCl and 1% (v/v) Triton X-100) and disrupted by sonication. Cell debris was removed by centrifugation, and the recombinant protein was purified from the supernatant by nickel affinity chromatography (HisLink[®], Promag[®], according to the manufacturer's instructions).

The recombinant endoglucanase from *S. thermophilum* was over-expressed in *Pichia pastoris* GS115 previously transformed with linearized pPIC9K/CT_{gpt} as previously described by Meleiro and collaborators [14]. Briefly, a single colony of *P. pastoris* GS115/pPIC9K/CT_{gpt} was inoculated into 100 mL of BMGY medium (3.4 g L⁻¹ yeast nitrogen base without amino acids and ammonium sulfate, 10 g L⁻¹ yeast extract, 20 g L⁻¹ peptone, 10 g L⁻¹ ammonium sulfate, 0.0004 g L⁻¹ biotin, 1% (v/v) glycerol, 100 mM potassium phosphate buffer, pH 6.0) and grown at 30 °C in an orbital shaker until

the culture reached an OD_{600 nm} of 3. Cells were harvested by centrifugation at 4000 rpm for 5 min, and the cell pellet was resuspended to an OD_{600 nm} of 1 in 100 mL BMGY medium (BMGY medium in which glycerol was substituted with 1% (v/v) methanol) and the culture was grown for 3 days at 30 °C. Every 24 h methanol was added, and at the end of the culture, the cells were centrifuged (4000 rpm, 20 min) and Egt was purified from the supernatant by nickel affinity chromatography.

The protein concentrations of the purified enzymes were determined according to Bradford and Northcutt [18] using bovine serum albumin as standard.

2.2. Preparation of functionalized paramagnetic nanoparticles (MNPs) and enzyme immobilization

The Fe₃O₄ nanoparticles derivatized with chitosan/glutaraldehyde/N-(5-amino-1-carboxy-pentyl) iminodiacetic acid (AB-NTA) and functionalized with NiCl₂ were prepared as previously described [13]. Egt and Hgl were immobilized on the MNPs to produce Egt-MNPs and Hgl-MNPs. About 100 μ g of each of the pure enzymes in 100 mM sodium phosphate buffer containing 30 mM NaCl in a final volume of 100 μ L were incubated separately with 100 μ L of a MNPs suspension at 4 °C for 16 h. The supernatants were removed and the MNPs were washed 4 times with 1.0 mL of wash buffer (100 mM sodium phosphate, pH 8.0, 150 mM NaCl) to remove unbound protein. During washing the nanoparticles were separated from the solution using a neodymium magnet of 11 mm diameter and 3 mm thickness. The MNPs were resuspended in 200 μ L of the same buffer, and the amount of protein retained on the support was measured by the difference between the quantity of offered protein and the protein in the supernatant and wash fractions. The immobilization yields and efficiencies of the immobilized enzymes were calculated according to Equations (1) and (2), respectively.

$$\text{Amount of protein retained on the support (mg protein)} = \text{Amount of offered protein to immobilization (mg protein)} \quad (1)$$

$$\text{Specific activity of immobilized enzyme } \left(\frac{\text{U}}{\text{mg}} \right) = \frac{\text{Specific activity of free enzyme } \left(\frac{\text{U}}{\text{mg}} \right) \times 100}{\text{Specific activity of free enzyme } \left(\frac{\text{U}}{\text{mg}} \right)} \quad (2)$$

The co-immobilization of Egt and Hgl into MNPs were performed as described above with 50 μ g of each enzyme to produce Egt-Hgl-MNPs. The total amount of protein retained on the support after separation of the supernatant and four washes with buffer was measured as described above. The amount of Hgl retained on the support in co-immobilization was estimated by the difference between the initial activity of enzyme offered for co-immobilization and the activity measured in the wash supernatants. After determination of the amount of Hgl retained on the support, the amount of Egt retained was estimated from the difference between the total retained protein and the amount of retained Hgl. The co-immobilization yield was calculated according to equation (1), however the co-immobilization efficiency was not calculated since the product of the Egt serves as the substrate for the Hgl.

2.3. Enzymatic assays

The pNP-glucosidase activity of Hgl was measured at 50 °C in 50 mM 2-Bis(2-hydroxyethyl)amino-2-(hydroxymethyl)-1,3-propanediol (Bis-Tris, Sigma-Aldrich Chem. Co, St. Louis, MO, USA) buffer, pH 6.0, containing p-nitrophenyl β -D-glucopyranoside (pNP-Glc, Sigma-Aldrich Chem. Co.) as substrate in a final volume of 0.6 mL, as previously described [15].

The carboxymethylcellulase (CMCase) activity of Egt was estimated at 60 °C was assayed at 60 °C in 50 mM sodium acetate buffer, pH 5.5, containing 1% (w/v) medium viscosity carboxymethylcellulose

(CMC, Sigma-Aldrich Chem. Co.) and 4 mM MnCl_2 in a final volume of 0.6 mL, as previously described [14]. Released reducing sugars were quantified using the dimethyl sulfoxide (DNS) method [19].

For all activity measurements, the experimental conditions (enzyme units, reaction times) were adjusted to ensure the reliable estimation of initial velocities. One unit (U) was defined as the amount of enzyme that releases 1 μmol of product per min. Specific activity was defined as U mg protein^{-1} (U mg^{-1}).

2.4. Effect of temperature on immobilized BglII and Egt

The optimum temperature of the catalytic activities of BglII-MNPs and Egt-MNPs were determined in the range from 35 to 65 °C in 50 mM Bis-Tris buffer, pH 6.0, using pNP-Glc (2 mM) as substrate for BglII-MNPs and in the range from 40 to 80 °C in 50 mM sodium acetate buffer, pH 5.5, containing 4 mM MnCl_2 and 1% (w/v) CMC as substrate for Egt-MNPs.

The activation energies (Ea) for the hydrolysis of pNP-Glc and CMC substrates by BglII-MNPs and Egt-MNPs, respectively, were determined using the Arrhenius equation as described by Segel [13]. The activation energies for the hydrolysis of pNP-Glc and CMC by the free BglII and Egt were determined based on previously data measured at optimum catalytic temperatures [14,15].

2.5. Estimation of kinetic parameters and data fitting

Maximum velocities (V_{max}) and Michaelis-Menten constants (K_m) for the hydrolysis of pNP-Glc by BglII-MNPs were estimated using the SigmaPlot software [20] which fits experimental data to the Hill equation using non-linear regression. The V_{max} and K_m for the hydrolysis of CMC by Egt-MNPs were estimated by linear regression using the Hanes-Woolf graphical treatment as described by Segel [13]. The enzyme expression/purification/immobilization protocols were repeated three times, resulting in three independent preparations of immobilized enzymes. The experimental kinetic curves were repeated three times, using each pure enzyme preparation, and each curve included at least 15 experimental points assayed in duplicate. The kinetic parameters presented in this study are the calculated values of the mean $\pm$ SD for the three different enzyme preparations ($n = 3$). Data fitting and statistical analyses were carried out using the OriginPro 8.5.0 software package (OriginLab Corp., Northampton, MA, USA).

2.6. Sugarcane cell wall preparation and fractionation

Two pretreated fractions sugarcane designated as alcohol insoluble residue (AIR) and the dimethyl sulfoxide (DMSO) extracted biomass were prepared exactly as described previously [14].

2.7. Synergic action of BglII and Egt on the hydrolysis of CMC, β -glucan and pretreated fractions sugarcane biomass

The enzymatic assays were conducted at 45 °C and 180 rpm in 50 mM Bis-Tris buffer, pH 6.0 for a period of 6 h in hermetically closed conic plastic tubes containing either 1% (w/v) CMC or β -glucan extract from barley [19] or for a period of 24 h with 5% (w/v) AIR or DMSO extracted biomass. The reaction was initiated by the addition of 3 U of Egt and/or 1 U of BglII in following preparations: (i) free form, (ii) immobilized or (iii) co-immobilized.

After defined time intervals, 20 μL aliquots of the reactions containing CMC or β -glucan were mixed with 20 μL of 20% (w/v) TCA and centrifuged for 5 min at 10,000 $\times$ g. The products present in 10 μL of the supernatants were analyzed by thin layer chromatography (TLC) on silica-gel G-60 plates, as detailed by Carli and collaborators [21]. The total reducing sugars (TRS) released were quantified using DNS method [19]. The degree of synergism was defined as the ratio of the TRS released by the action of both enzymes together to the sum of the TRS

released by each enzyme individually.

2.8. Scanning Electron Microscopy (SEM) of β -glucan samples before and after treatment with Egt-BglII-MNPs

The enzymatic assays were conducted as described above using 1% (w/v) β -glucan as substrate and Egt-BglII-MNPs (equivalent to 3 U Egt and 1 U BglII). A control reaction was performed under the same conditions with MNPs without enzymes. The reactions were interrupted by heating in boiling water for 5 min, followed by centrifugation for 10 min at 10,000 $\times$ g. About 100 μL of each of the samples were transferred to glass coverlips, dried at 50 °C and then coated with gold using a sputter coater (BAL-TEC, mod. SCD 050, Formentor, Liechtenstein). The samples were examined in a scanning electron microscope (Zeiss mod. EVO 50, Cambridge, UK) with an acceleration voltage of 20 kV. The secondary electron detector was used for micrograph acquisition.

2.9. Reuse of BglII-MNPs, Egt-MNPs and Egt-BglII-MNPs

The reusability of the immobilized enzymes was evaluated as described in item 2.3. After each reaction cycle, the immobilized enzymes were magnetically separated and washed with 1.0 mL of wash buffer. A new cycle was initiated using the washed immobilized enzymes by addition of fresh reaction medium.

3. Results and discussion

3.1. Immobilization efficiency and yields

The morphological and chemical characterization of the functionalized paramagnetic nanoparticles during the sequential modification of the nanoparticle surface have been previously described [13]. In the present study we have immobilized heterologous proteins by the coordination of the imidazole group of a 5-histidine tag added to either the N-terminal (in the case of BglII) or C-terminal (in the case of Egt) of the protein with the Ni^{2+} coordinated with NTA group of the nanoparticles.

The immobilization efficiencies of BglII and Egt were 132% and 115% with 21% and 20% immobilization yields, respectively. Lower immobilization efficiencies were reported to other cellulases immobilized on nanomaterials [22,23]. The maintenance of enzymatic activity is one of the advantages of affinity immobilization, where only minimal conformational changes in the immobilized protein are observed [24]. Relatively high immobilization efficiencies can be attributed to modifications in the structure of the proteins after the immobilization process by the polyhistidine tag, causing an improvement in the catalytic activity.

The co-immobilization of BglII and Egt to produce Egt-BglII-MNPs was successful with 49% yield. In addition, every 100 μL of Egt-BglII-MNPs contains about 3 U of Egt and 1 U of BglII. This ratio was obtained indirectly because the product of the Egt serves as the substrate for the BglII (as described in section 2.2 in Material and Methods).

3.2. Effect of temperature on the activity of the immobilized enzymes

The optimum catalytic temperature of the BglII-MNPs was 55 °C and at 60 °C and 65 °C the activity decreased to 76% and 14% of the maximum, respectively (Fig. 1a). A similar activity profile was previously described for the free BglII, which presents a maximum activity at 60 °C [15]. In the case of the Egt-MNPs, greater than 80% of the catalytic activity was maintained between 55 and 80 °C (Fig. 1b). A very similar plateau in the temperature dependence of the activity was previously observed for free Egt [14]. The optimal temperatures of Egt-MNPs and BglII-MNPs are compatible for application in industrial processes for the saccharification of lignocellulosic materials.

Fig. 3. Influence of temperature on the enzymatic activity of immobilized (□) and free (●) BglII (a) and Egt (b). Arrhenius plots for the effect of temperature on the enzymatic activity of immobilized (□) and free (●) BglII (c) and Egt (d).

The Arrhenius plots for the effect of temperature on the hydrolysis of pNP-Glc by BglII (both free and immobilized) and CMC by Egt (both free and immobilized) are shown in Fig. 1c and d, respectively. The values of the E_a estimated from the Arrhenius plots are $49.8 \pm 2.3 \text{ kJ mol}^{-1}$ (free BglII) and $49.6 \pm 1.8 \text{ kJ mol}^{-1}$ (BglII-MNPs) for pNP-Glc and $13.1 \pm 0.8 \text{ kJ mol}^{-1}$ (free Egt) and $32.2 \pm 1.7 \text{ kJ mol}^{-1}$ (Egt-MNPs) for CMC. The E_a was unchanged in the case of β -glucosidase suggesting that the temperature dependence of the catalytic reaction rate was maintained between the free and immobilized enzyme. In contrast, the convex curve observed with the Egt-MNPs may indicate a temperature dependent activation of the immobilized enzyme. A previous report comparing E_a of the reactions performed with free and immobilized enzymes on nanoparticles [25] has reported a decrease of about 1.4-fold in E_a for β -glucosidase from sweet almond immobilized in magnetic Fe_3O_4 nanoparticles coupled with agarose. These variable results on E_a reflect the differing effects that immobilization may have on an enzyme.

3.3. Kinetic parameters

Table 1 summarizes the kinetic parameters for the hydrolysis of pNP-Glc and CMC by BglII-MNPs and Egt-MNPs, respectively, together with the parameters previously reported for the free enzymes. The synthetic substrate pNP-Glc was hydrolyzed by the immobilized BglII with a specific activity ($V_{\text{max}} = 52.7 \pm 2.1 \text{ U mg}^{-1}$) about 1.4-fold higher and similar K_m value as the free BglII [15]. For Egt, the V_{max} of the immobilized enzyme ($625.02 \pm 19.12 \text{ U mg}^{-1}$) was slightly higher than that of the free form and the K_m value was about 20% lower. V_{max}

K_m values showed that catalytic efficiencies of both immobilized enzymes were higher than those of free enzymes. It is known that immobilization of enzymes can affect their kinetic parameters, probably as a consequence of changes in their flexibility or deformation of the enzyme structure on interaction with the support [24–26]. Furthermore, the access of the substrate to the active site may be altered, resulting in changes in the catalytic activity of the enzymes. In both cases (BglII-MNPs and Egt-MNPs) the immobilization via histidine tags probably appears not to have significantly affected this access.

The β -glucosidase from sweet almond also showed positive variations in the kinetic parameters for hydrolysis of pNP-Glc when compared to the free form [25]. In contrast, the adsorption of cellulase from *Aspergillus niger* on TiO_2 nanoparticles caused an increase in the affinity of the enzyme for the substrate but a decrease in V_{max} when compared to the free enzyme [26].

3.4. Mode of action of Egt-MNPs, BglII-MNPs and Egt-BglII-MNPs against barley β -glucan and CMC

The mode of action of the immobilized enzymes against CMC (Fig. 2) and barley β -glucan (Fig. 3) were investigated through the analysis of the reaction products by TLC and were compared to the mode of action of the free enzymes. TLC analysis revealed that both free (Figs. 2a and 3a) and Egt-MNPs (Figs. 2b and 3b) hydrolyzed barley β -glucan and CMC to produce oligosaccharides with different degrees of polymerization (DP) of between 2 and 5 glycosyl units as the predominant products, confirming that the immobilization process had no effect on the mode of action of the enzyme. As reported by Meleiro and

Table 1
Kinetic parameters for pNP-Glc and CMC hydrolysis by free and immobilized BglII and Egt.

Substrate	K_m (mM, mg mL^{-1})	V_{max} (U mg^{-1})	V_{max}/K_m ($\text{U mg}^{-1} \text{ mM}^{-1}$, $\text{U mg}^{-1} \text{ mg mL}^{-1}$)	Reference
Free BglII	0.26 ± 0.02^a	36.8 ± 1.4	142.3^a	[15]
BglII-MNPs	0.23 ± 0.01^a	52.7 ± 2.1	229.1^a	This work
Free Egt	7.78 ± 2.82^b	615.02 ± 23.96	79.1^b	[14]
Egt-MNPs	6.32 ± 0.60^b	625.02 ± 19.12	98.1^b	This work

The kinetic parameters are presented as the mean $\pm$ SD of the values calculated from data from three separate experiments ($n = 3$).

Fig. 2. Time course analysis of the hydrolysis of CMC by free Egt (a), Egt-MNPs (b), free BgH (c), BgH-MNPs (d), free Egt plus BgH (e), Egt-MNPs plus BgH-MNPs (f), Egt-BgH-MNPs (g). The reaction times were 0 (lanes t0), 10 min (lanes t1), 20 min (lanes t2), 30 min (lanes t3), 60 min (lanes t4), 120 min (lanes t5) and 6 h (lanes t6). Standards: G1, glucose; G2, cellulose; G3, cellulose; G4, cellulose.

collaborators [14] the migration pattern of the oligosaccharides formed after the hydrolysis of these substrates with both free and Egt-MNPs is characteristic of endo-hydrolytic mode of action. O mesmo padrão de hidrólise foi observado para a GHS Endoglucanase from *Vibrio parvulus* [27]. In addition, in relation to β -glucan hydrolysis pattern, we can suggest the formation of oligosaccharides containing β -1,3-type binding [14].

The BgH in both the free (Figs. 2c and 3c) and immobilized (Figs. 2d and 3d) form does not result in the significant release of glucose or oligosaccharides even after long reaction times, confirming that the enzyme alone has no action against either of the polysaccharides. Similar results were reported for other GH1 β -glucosidase [1,28–30]. However, when both enzymes were combined, either in the free form (Figs. 2e and 3e) or as a mixture of separately immobilized enzymes (Figs. 2f and 3f), the TLC profile revealed a synergistic effect against both substrates. The oligosaccharides generated by the action of the endoglucanase were rapidly consumed and an increase in the glucose product was observed over the course of the reaction times analyzed. The degree of synergism for immobilized enzymes were estimated as 1.2 and 3.7-fold using CMC and β -glucan, respectively, and is higher than those estimated for free enzymes (1.1 and 2.7-fold using CMC and β -glucan, respectively). It is noteworthy that the Egt-BgH-MNPs (Figs. 2g and 3g) showed the same catalytic performance by rapidly converting the oligosaccharides to glucose, confirming that the synergistic effect of the enzymes was maintained in the co-immobilization process, where the degree of synergism for co-immobilized enzymes were estimated as 1.2 and 3.3-fold using CMC and β -glucan, respectively.

3.5. Analysis of the treatment of β -glucan with Egt-BgH-MNPs by SEM

The treatment of β -glucan with Egt-BgH-MNPs was analyzed by

SEM (Fig. 4). Treatment of the substrate with the co-immobilized enzymes (Fig. 4b) led to a disintegration and fragmentation of the material when compared to treatment with nanoparticles without immobilized enzymes (Fig. 4a). Differences were observed in the size distribution and shape of the particles indicating that co-immobilization did not hinder the access of the enzymes to the substrate, raising the possibility that a similar effect may occur with more complex substrates such as lignocellulosic biomass.

3.6. Synergic action of Egt MNPs, BgH-MNPs and Egt-BgH MNPs on the hydrolysis of pretreated sugarcane bagasse

The total reducing sugar released by incubation of Egt-MNPs alone, a Egt-MNPs and BgH-MNPs mixture and Egt-BgH-MNPs with DMSO treated sugarcane were about 1.9-, 3.0- and 2.3-fold higher than that released from AIR (Fig. 5). BgH-MNPs alone released limited amounts of reducing sugars due to low activity against polysaccharides (see section 3.4). The higher amounts of sugars released from the DMSO-treated fraction can be explained by the higher exposure of cellulose and β -glucan in this material [31,32]. The degree of synergism of the immobilized enzymes was estimated as 1.6-fold against pretreated sugarcane bagasse.

3.7. Recycling of the immobilized enzymes

The cost of enzymes is still a major barrier to their application in industrial processes [33]. Regarding the many advantages of immobilized enzyme systems, the possibility of reuse is among the most important economically [34,35]. The reusability of the immobilized and co-immobilized enzymes is shown in Fig. 6. BgH-MNPs retained about 50% of the initial activity after three reuse cycles. Moreover, even after the fifth washing cycle, the immobilized enzyme still

Fig. 3. Time course analysis of the hydrolysis of barley β -glucan by free Egt (a), Egt-MNPs (b), free BgH (c), BgH-MNPs (d), free Egt plus BgH (e), Egt-MNPs plus BgH-MNPs (f), Egt-BgH-MNPs (g). The reaction times were 0 (lanes t0), 10 min (lanes t1), 20 min (lanes t2), 30 min (lanes t3), 60 min (lanes t4), 120 min (lanes t5) and 6 h (lanes t6). Standards: G1, glucose; G2, cellulose; G3, cellulose; G4, cellulose.

Fig. 4. Scanning electron micrographs of the treatment of β -glucan without (a) and with Egt-BgH-MNPs (b).

Fig. 5. Hydrolysis of sugarcane bagasse AIR (blue bars) and bagasse pretreated with DMSO (red bars) with Egt-MNPs (a), BgH-MNPs (b), Egt-MNPs plus BgH-MNPs (c) and Egt-BgH-MNPs (d).

Fig. 6. Reusability of Egt-MNPs (■), BgH-MNPs (■) and Egt-BgH-MNPs (▲) determined by repetition of the hydrolysis assay.

retained 40% of the initial activity. With Egit-MNPs, the activity during reuse was lower and about 45% of the activity was maintained after the second use and about 10% after the fifth use. Surprisingly, when the enzymes were co-immobilized, the reusability was improved and Egit-BglII-MNPs retained about 80% of its initial activity even in the fifth reuse. It is noteworthy that the reuse of the immobilized enzymes by a magnetic field was facilitated by the paramagnetic property of the support [13]. The fast and effective immobilization and reuse methods of individual and co-immobilized enzymes and has potential for reducing costs in biotechnological processes.

Honda and collaborators [35] investigated the reusability of co-immobilized endoglucanase and β -glucosidase (EG/BG-MNPs) on a paramagnetic support and demonstrated that about 70% of the initial activity was maintained after 5 cycles of reuse. Previous studies using a cellulase enzyme complex co-immobilized on magnetic particles indicates that the enzymatic activity dropped to 20–50% after five reuse cycles [36,37].

4. Conclusions

Here we have demonstrated the use of functionalized magnetic nanoparticles to individually immobilize a β -glucosidase, an endoglucanase and to co-immobilize these two enzymes. The immobilized enzymes showed a significant synergistic effect in the hydrolysis of CMC, barley β -glucan and pretreated fractions sugarcane both when combined individually and applied in a co-immobilized form. The improvement of the catalytic activity, high optimum temperature, together with reusability with easy recovery using the magnetic field from a permanent magnet makes them attractive for industrial applications.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Apêndice C

Overexpression of a Cellobiose-Glucose-Halotolerant Endoglucanase from *Scytalidium thermophilum*

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Abstract Enzyme reaction products and by-products from pretreatment steps can inhibit endoglucanases and are major factors limiting the efficiency of enzymatic lignocellulosic biomass hydrolysis. The gene encoding the endoglucanase from *Scytalidium thermophilum* (egst) was cloned and expressed as a soluble protein in *Pichia pastoris* GS115. The recombinant enzyme (Egst) was monomeric (66 kDa) and showed an estimated carbohydrate content of 53.3% (w/w). The optimum temperature and pH of catalysis were 60–70 °C and pH of 5.5, respectively. The enzyme was highly stable at pH 3.0–8.0 with a half-life in water of 100 min at 65 °C. The Egst presented good halotolerance, retaining 84.1 and 71.4% of the control activity in the presence of 0.5 and 2.0 mol L⁻¹ NaCl, respectively. Hydrolysis of medium viscosity carboxymethylcellulose (CMC) by Egst was stimulated 1.77-, 1.84-, 1.64-, and 1.8-fold by dithiothreitol, β-mercaptoethanol, cysteine, and manganese at 10, 10, 10, and 5 mmol L⁻¹ concentration, respectively. The enzyme hydrolyzed CMC with maximal velocity and an apparent affinity constant of 432.10 ± 16.76 and 10.5 ± 2.53 mg mL⁻¹, respectively. Furthermore, the Egst was tolerant to reaction products and able to act on pretreated fractions

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sugarcane bagasse demonstrating excellent properties for application in the hydrolysis of lignocellulosic biomass.

Keywords Endoglucanase · GH5 · Halotolerance · *Pichia pastoris* · *Scytalidium thermophilum*

Introduction

Lignocellulosic agroindustrial residues are abundant and widespread renewable biomass resources and are the favored raw material for the sustainable production of biofuels and for biorefinery. The conversion of lignocellulose into biofuels can be accomplished with a low environmental impact by enzymatic hydrolysis of the polysaccharides present in these materials to release reducing sugars, which may subsequently be used in microbial fermentations to produce alcohols such as ethanol or butanol [1, 2].

Enzymatic hydrolysis of cellulose, the main component of lignocellulosic residues, is promoted by the synergistic action of enzymes of at least three classes: endoglucanases (endo-1,4-β-glucosidases, EC 3.2.1.4), exoglucanases or cellobiohydrolases (exo-1,4-β-glucosidases, EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21) [1, 3]. Endoglucanases play an important role in the initiation of hydrolysis by acting on amorphous regions in the cellulose fibrils to release cello-oligosaccharides and cellobiose. In addition to cellulose hydrolysis, endoglucanases also participate in the hydrolysis of β-glucan present in hemicellulose [4]. In contrast, cellobiohydrolases CBHI and CBHII release cellobiose from the reducing and non-reducing ends of cellulose chains in the crystalline regions. Some CBHII also displayed some endo character [5]. The β-glucosidases hydrolyze short cello-oligosaccharides and cellobiose to glucose [1, 3, 6].

One current limitation for the enzymatic hydrolysis of lignocellulosic biomass for bioethanol production is enzyme inhibition by reaction products. High enzyme loading is required in order to obtain acceptable yields during biomass saccharification, which not only increases process costs but also produces high concentrations of reducing sugars. The accumulation of the hydrolysis products cellobiose and glucose results in inhibition of the enzymes involved in cellulose degradation [1–3, 7]. In addition, inhibition of the lignocellulolytic enzymes by products of biomass pretreatment steps may also result in significant reduction of catalytic activity. The extremes in pH used in acid and alkaline pre-treatments generate large amounts of salt after neutralization, and there is a growing interest in the identification of enzymes tolerant to high salt concentrations [8–10].

Recently, an endoglucanase (Celulase II) produced from thermophilic fungus *Scytalidium thermophilum* CBS 619.91 has been purified and characterized [11]. The enzyme presents good thermostability and tolerance to both cellobiose (70 mmol L⁻¹) and to glucose (140 mmol L⁻¹). In the present paper, we report the cloning and heterologous expression of this enzyme in *Pichia pastoris* along with a detailed biochemical and kinetic characterization of the recombinant enzyme.

Material and Methods

Microorganism and Culture Conditions

The *S. thermophilum* (CBS 619.91) strain was kindly provided from Dr. G. Straatsma (Mushroom Experimental Station, The Netherlands), and stocks were maintained at 40 °C

on slants of solid medium containing 4% (w/v) oatmeal baby food. For enzyme induction, conidia were inoculated into a 250-mL Erlenmeyer flask containing 50 mL of liquid medium as previously reported [11], using microcrystalline cellulose (Avicel®, particle size 20–100 µm, Sigma-Aldrich Chem. Co., St Louis, MO, USA) as the carbon source. After cultivation for 120 h at 40 °C and 130 rpm, the mycelium was harvested by centrifugation. All solutions were prepared using Millipore MilliQ (EMD Millipore Co., Billerica, MA, USA) ultrapure water, and all reagents were of the highest purity commercially available.

Cloning of the *egst* Gene from *S. thermophilum*

Approximately 80 mg of fungal mycelia were frozen in liquid nitrogen and crushed to a fine powder. Total RNA was extracted and purified using RNeasy Mini Kit® (Qiagen Science, Germantown, MD, USA) and used to generate cDNA with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Wilmington, DE, USA) according to the manufacturer's instructions. The *egst* gene from *S. thermophilum* was amplified by PCR with the cDNA template using *Pfu* DNA polymerase (Thermo Scientific) and the primers 5'-GGGCTCAGAGAAA GAGAGGCTGAACAGGGCGGTGCATGG-3' (forward) and 5'-GGGGAATTC TGGC ACGTATTTCTTGAGAAG-3' (reverse), which introduced restriction sites for the *XhoI* and *EcoRI* enzymes, respectively (underlined sequences). The resulting PCR product was digested with the appropriate restriction enzymes, cloned into the pPIC9K/CT plasmid in-frame with an C-terminal polyhistidine tag, and transformed into *Escherichia coli* DH5α (Novagen, Madison, WI, USA). The resulting recombinant plasmid, pPIC9K/CT-*egst*, was validated by DNA nucleotide sequencing.

Construction of *Pichia pastoris* Strain

The pPIC9K/CT-*egst* was linearized with the restriction enzyme *SnaI* (Thermo Scientific) and was used to transformation of *P. pastoris* GS115 by electroporation. The linearized construct pPIC9K/CT-*egst* was inserted into the HIS4 gene by homologous recombination and selected by histidine auxotrophism on MD agar plates (3.4 g L⁻¹ yeast nitrogen base without amino acids and ammonium sulfate (YNB), 10 g L⁻¹ ammonium sulfate, 0.0004 g L⁻¹ biotin, 100 mmol L⁻¹ potassium phosphate buffer, pH 6.0, 20 g L⁻¹ dextrose, 1.5% (w/v) bacteriological agar).

Heterologous Expression in *P. pastoris* and Purification of Egst

Single colonies of *P. pastoris* GS115/pPIC9K/CT-*egst* were inoculated into 1-L Erlenmeyer flask containing 100 mL of BMGY medium (3.4 g L⁻¹ YNB, 10 g L⁻¹ yeast extract, 20 g L⁻¹ peptone, 10 g L⁻¹ ammonium sulfate, 0.0004 g L⁻¹ biotin, 1% (v/v) glycerol, 100 mmol L⁻¹ potassium phosphate buffer, pH 6.0). The culture was incubated at 30 °C in an orbital shaker for 12 h. Cells were harvested by centrifugation at 4000 rpm for 5 min, and the cell pellet was resuspended in 5 mL of the corresponding medium. The cells grown were transferred to two 1-L Erlenmeyer flasks containing 100 mL of BMMY media (BMGY medium, in which glycerol was substituted with 1% (v/v) methanol) to an OD₆₀₀ 1.0. Methanol (1%, v/v) was subsequently added to the cultures at 24 h periods and the pH was corrected daily by addition of 1 mL of 1 mol L⁻¹ potassium phosphate buffer, pH 6.0. The enzyme expression occurred at 30 °C, 250 rpm for 3 days, after which the cells were centrifuged (4000 rpm, 20 min) and the supernatant was stored at 4 °C until processed further.

The recombinant Egst was purified from the culture the supernatant containing the recombinant Egst by immobilized metal affinity chromatography. After adjustment to pH 8.0, the

supernatant was applied to 1 mL of a nickel affinity chromatography resin (Promega, Madison, USA) previously equilibrated with 50 mmol L⁻¹ HEPES buffer, pH 8.0, containing 500 mmol L⁻¹ NaCl and 10 mmol L⁻¹ imidazole. The resin was washed with the same buffer until protein was no longer detected (A₂₈₀) in the eluate. The Egst was eluted in the same buffer containing 300 mmol L⁻¹ imidazole.

Enzymatic Assays

Unless otherwise stated in the text, the carboxymethylcellulase (CMCase) activity was assayed at 60 °C in 50 mmol L⁻¹ sodium acetate buffer, pH 5.5, containing 1% (w/v) medium viscosity carboxymethylcellulose (CMC, Sigma-Aldrich Chem. Co.) and 4 mmol L⁻¹ MnCl₂, in a final volume of 0.6 mL. The hydrolysis of β-glucan extract from barley [12], birchwood and beechwood xylan (Sigma-Aldrich Chem. Co.), low viscosity CMC (Sigma-Aldrich Chem. Co.), filter paper, microcrystalline cellulose (Avicel®, Fluka Chemical Co., Seelze, Germany) and starch at 1% (w/v) final concentration, was assayed as above, in the absence of MnCl₂. Released reducing sugars were quantified using the dinitrosalicylic acid (DNS) method [13].

Enzymatic activity against the synthetic substrate was determined under the same conditions with 4-nitrophenyl-β-D-cellobioside (pNP-C) at 5 mmol L⁻¹ final concentration. The reactions were initiated by the addition of the enzyme at a convenient dilution and were interrupted after determined time intervals by the addition of 1.0 mL of a saturated sodium tetraborate solution. The hydrolysis rates were determined by quantifying the release of the p-nitrophenolate ion ($\epsilon_{410\text{nm pH } 12} = 17,500 \text{ mol}^{-1} \text{ L cm}^{-1}$), as described by Souza et al. [14]. The hydrolysis of cellobiose (at 10 mmol L⁻¹ final concentration) was assayed under the same conditions by quantification of the released glucose as previously described [14].

The experimental conditions (reaction times, enzymatic units) employed were adjusted to guarantee the reliable estimation of initial velocities from the linear response of product formation in respect to reaction time. One enzyme unit (U) was defined as the amount of enzyme that releases 1 µmol of product per min and specific activity was defined as U mg protein⁻¹ (U mg⁻¹). Controls with heat inactivated enzyme were included in all enzymatic assays, and all enzymatic assays were performed in triplicate.

Estimation of Protein and Neutral Carbohydrate

Protein concentrations were estimated according to Read and Northcote [15], using bovine serum albumin as the standard. Total neutral carbohydrate was determined by the method of Dubois et al. [16], using mannose as the standard.

Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 10% acrylamide slab gels, according to Laemmli [17]. Protein bands were revealed with Coomassie Brilliant Blue R.

Deglycosylation of Egst by Endoglycosidase H

Purified Egst was deglycosylated by denaturing the protein at 100 °C for 10 min. After cooling, the protein sample was incubated with endoglycosidase H (Endo H, New England

Biolab, Ipswich, MA, USA) at 37 °C for 24 h according to the manufacturer's instructions. The sample was subsequently analyzed by SDS-PAGE.

Effect of Temperature and pH on Egst Activity and Stability

The optimum temperature for the enzymatic activity was determined over the range 50–80 °C in 50 mmol L⁻¹ acetate buffer, pH 5.5. The thermal stability of Egst was evaluated by incubating the purified enzyme diluted in Millipore MilliQ ultrapure water for different time intervals at temperatures from 45 to 65 °C. After cooling in an ice bath for 1 min, the residual CMCase activity was estimated at 60 °C as described in the "Enzymatic Assays" section. The optimum pH of activity was determined at 60 °C in McIlvaine buffer [18] in the range 3.0–8.0. The pH stability was determined by incubating Egst at 4 °C in McIlvaine buffer, pH 3.0–8.0, for 24 h. The residual CMCase activity was also estimated at 60 °C in 50 mmol L⁻¹ acetate buffer, pH 5.5 as described in the "Enzymatic Assays" section.

Effect of NaCl on Egst Activity and Stability

The effect of NaCl on the CMCase activity was evaluated at 60 °C in 50 mmol L⁻¹ acetate buffer, pH 5.5, containing NaCl (0–2.0 mol L⁻¹). To measure halostability of Egst, the purified enzyme was incubated with solution of NaCl up to 2 mol L⁻¹ at 25 °C for 24 h. The residual activities were measured at 60 °C as described in the "Enzymatic Assays" section.

Effect of Ions and Other Agents on the Enzymatic Activity

The effect of various salts (NH₄Cl, NaBr, NaCl, CoCl₂, CuSO₄, MnCl₂, KCl, CaCl₂, BaCl₂, MgCl₂ and HgCl₂), ethylenediamine tetraacetic acid (EDTA, Sigma-Aldrich Chem. Co.), dithiothreitol (DTT, Sigma-Aldrich Chem. Co.), cysteine and β-mercaptoethanol (Sigma-Aldrich Chem. Co.) on the CMCase activity of Egst was determined as described in "Enzymatic Assays" section. The different effectors were added to the reaction medium at final concentrations of 1.0, 5.0 and 10.0 mmol L⁻¹.

The effect of stimulation of the CMCase activity by Mn²⁺, cysteine, and DTT was analyzed with an increase in effector concentration, in the range from 0.1 to 20 mmol L⁻¹. The enzymatic activities were measured as described in the "Enzymatic Assays" section and the kinetic parameters V_{max} and K_M for the CMC hydrolysis by Egst were calculated by non-linear regression using the SigrafW software [19]. The experimental kinetic curves were repeated three times, with three different enzyme preparations. The enzymatic assays were performed in quadruplicate and the values of the kinetic parameters presented are calculated values and are given as the mean ± SD for three different Egst preparations (n = 3).

Effect of Glucose and Cellobiose on Egst Activity

The effect of glucose and cellobiose on the endoglucanase activity of Egst was evaluated by both hydrolysis of cellulose-azure (Sigma Chem. Co., USA) and by the reduction in viscosity of a carboxymethyl cellulose (CMC) solution. In both assays, reactions were performed at 60 °C in 50 mmol L⁻¹ acetate buffer, pH 5.5, 4 mmol L⁻¹ MnCl₂, containing 1% (w/v) of substrate (cellulose-azure or medium viscosity CMC) with glucose or cellobiose at known

concentrations, in a final volume of 2.0 mL. In the cellulose-azure hydrolysis assay, the reaction was stopped after determined time intervals by the addition of 4.0 mL of an alcohol reagent solution (ARS—0.5 mL saturated calcium chloride solution in methanol plus 99.5 mL ethanol). The mixture was centrifuged at 10,000g, and the absorbance of the limped supernatant was measured at 575 nm. Controls with heat-inactivated enzyme were included in the mixture after addition of ARS to quantify the non-enzymatic hydrolysis of the substrate. In the viscosity reduction assay, the activity was expressed as the relative reduction in viscosity as described by Silva et al. [11]. The enzymatic assays were performed in five replicates and the values presented are the calculated values of the mean ± SD for three separate Egst preparations (n = 3).

Determination of Kinetic Parameters for CMC Hydrolysis by Egst

The kinetic parameters V_{max} (maximum velocity) and K_M (Michaelis-Menten constant) for the hydrolysis of CMC (in the absence or in the presence of 4 mmol L⁻¹ MnCl₂) were calculated by linear regression using the Hanes-Woolf graphical treatment as described by Segel [20]. The kinetic experiments were repeated using three separate Egst preparations, where enzymatic assays were performed in duplicate. The values of the kinetic parameters presented are given as the mean ± SD for the three different Egst preparations (n = 3).

Thin Layer Chromatography (TLC) Analysis of the Reaction Products of the Hydrolysis of CMC, Avicel, and Barley β-Glucan by Egst

The enzymatic assays were conducted at 50 °C and 200 rpm in hermetically sealed conical plastic tubes. The reaction conditions were 50 mmol L⁻¹ acetate buffer, pH 5.5, containing 1% (w/v) medium viscosity CMC or low viscosity CMC or Avicel or barley β-glucan and 10 U of Egst in a final volume of 1.0 mL. After suitable time intervals, the reactions were interrupted by the addition of 1.0 mL of 20% (w/v) trichloroacetic acid. The samples were kept at room temperature for 1 h and centrifuged for 10 min at 10,000 g. For each assay, a 10 µL aliquot of the supernatant containing the reaction products was analyzed by TLC on silica-gel G-60 plates (10 × 20 cm, DC-Alufolien Kieselgel 60, Merck, Darmstadt, Germany), as described by Carli et al. [9].

Sugarcane Cell Wall Preparation, Fractionation, and Enzymatic Hydrolysis

The sugarcane bagasse culms were hammer-milled to a 0.5 mm particle size, and prepared with successive extractions with 80% (v/v) ethanol at 80 °C, as described by De Souza et al. [4]. The alcohol insoluble residue (AIR) was washed with distilled water and dried at 60 °C for 24 h. The AIR was subjected to two consecutive extractions with 25 mL of 0.5 M ammonium oxalate pH 7.0 at 80 °C for 3 h according De Souza et al. [4]. Approximately 4 g of oxalate extracted sugar-cane bagasse was subjected to two DMSO extractions followed a previously reported protocol [21]. The solid fraction containing the DMSO extracted biomass was recovered and washed with ethanol and dried.

The activity of Egst against the AIR and the DMSO extracted biomass was performed using 5% (w/v) suspension of these substrates prepared in 50 mmol L⁻¹ acetate buffer, pH 5.5, 480 µg of Egst at 50 °C for 48 h in an orbital shaker at 200 rpm. The release of total reducing sugars was measured by the DNS method [13].

Results and Discussion

Cloning and Sequence Analysis of Egst

Three tryptic peptides from the glucose-cellobiose-tolerant cellulase from *S. thermophilum* (cellulase II) have been sequenced by mass spectrometry [11]. These peptides share 92% sequence similarity with the Endoglucanase 3 from *S. thermophilum* CBS 625.91 (GeneModelID: Scyth2p4_001262, <https://www.fungalgenomics.ca/>). The design of the oligonucleotides used for cloning the Egst coding sequence (*egst*) were based on the Endoglucanase 3 coding sequence and excluded the predicted peptide signal (Met¹-Ala¹⁶). The nucleotide sequencing of *egst* revealed 98% sequence identity with the nucleotide sequence of Endoglucanase 3 (Supplemental data Fig. S1), in which a 1119 bp nucleotide sequence (including stop codon) encodes a protein of 372 amino acids. Conserved domain database searches identified a cellulase catalytic domain belonging to glycosyl hydrolase family 5 (GH5, pfam00150) and a cellulose binding domain (CBM1, pfam00734). Egst also showed high similarity to GH5 cellulases from *Hemicella grisea* var. *thermoidea* (GenBank: BAA12676.1, 99% similarity), *Thermothelomyces thermophila* ATCC 42464 (NCBI Reference Sequence: XP_003659014.1, 71% identity), *Thielavia terrestris* NRRL 8126 (NCBI Reference Sequence: XP_003657015.1, 72% similarity) and *Chaetomium thermophilum* var. *thermophilum* DSM 1495 (NCBI Reference Sequence: XP_006695916.1, 71% similarity) (Supplemental data Fig. S2). The catalytic residues Glu¹⁰⁹ and Glu³⁰⁶ (indicated as arrows in Supplemental data Fig. S2) in Egst are fully conserved among all GH5 enzymes [22].

Expression and Purification of Egst

The *egst* gene was successfully expressed in *P. pastoris* to produce a heterologous active secreted enzyme that accumulated in the culture broth. Egst was purified from the culture supernatant by Ni-affinity chromatography with a final concentration of 1.4 mg protein mL⁻¹, with about 60% yield. The SDS-PAGE analysis (Fig. 1a) revealed a single protein band, corresponding to an apparent molecular mass of about 66 kDa, higher than the theoretical

Fig. 1 Analysis of the purified Egst SDS-PAGE, showing the purified enzyme before (lane a) and after (lane b) Endo H digestion. Total protein loading of 5 µg per lane

Fig. 2 Optimal temperature (a), optimal and stability to pH (b), and thermal stability (c) for the CMCase activity of Egst. **a** The activity was assayed at different temperatures in 50 mmol L⁻¹ acetate buffer, pH 5.5, using 1% (w/v) medium viscosity CMC as the substrate and 4 mmol L⁻¹ MnCl₂. **b** The optimal pH (●) was estimated at 60 °C in McIlvaine buffer, pH 3.0–8.0, using 1% (w/v) medium viscosity CMC as the substrate and 4 mmol L⁻¹ MnCl₂. For pH stability (□), the pure enzyme was incubated in McIlvaine buffer (pH 3.0–8.0) at 4 °C for 24 h. **c** For thermal stability, samples of pure enzyme diluted in water were incubated at 45 °C (□), 50 °C (●), 55 °C (▲), 60 °C (Δ) and 65 °C (○). Residual activity was estimated at 60 °C in 50 mmol L⁻¹ acetate buffer, pH 5.5, using 1% (w/v) medium viscosity CMC as the substrate and 4 mmol L⁻¹ MnCl₂. One hundred percent specific activity corresponded to 333.1 ± 20.7 U mg⁻¹. The values shown represent means ± SD from triplicate experiments (n = 3) carried out with three separate preparations of pure enzyme

molecular weight of 41 kDa. After Egst was treated with Endo H and analyzed by SDS-PAGE (Fig. 1b), the apparent molecular mass of the deglycosylated enzyme was approximately 42 kDa, indicating that the Egst is a glycoprotein. Amino acid sequence analysis by the NetNGlyc 1.0 program (<http://www.cbs.dtu.dk/services/NetNGlyc/>) identified three putative N-glycosylation sites in the Egst amino acid sequence (⁷⁸NQS⁷⁸, ¹³⁸NLT¹⁴¹, ²⁴³NVT²⁴⁵). The estimated carbohydrate content of Egst was of 53.3% (w/w), as compared with 93% (w/w) determined for the native cellulase II from *S. thermophilum* [11]. The difference between the carbohydrate content of the native and recombinant enzymes can be attributed to the different glycosylation patterns between *P. pastoris* and the *S. thermophilum*. Over the last years, several genes encoding fungal GH5 endoglucanases have been cloned and expressed successfully as active proteins in *P. pastoris* [23–34].

Effect of Temperature and pH on the Activity and Stability of the Egst

Egst showed a plateau of maximal catalytic activity between 60 and 70 °C, and maintained > 80% of the maximal activity at 50 °C and 75 °C (Fig. 2a). Furthermore, the enzyme retained about 70% of the maximum activity at 80 °C. The optimum pH of CMCase activity was pH 5.5, and the enzyme presented 80 and 75% of the maximal activity at pH 4.0 and 7.0, respectively. However, a pronounced decrease in activity was observed below pH 4.0 (Fig. 2b). Nevertheless, the enzyme was stable over the entire pH range that was analyzed (Fig. 2b). Full catalytic activity was maintained at 45 °C and the enzyme retained up to 83% of maximal activity at 50 and 55 °C for 120 min. Furthermore, at 65 °C the half-life time was 100 min. It is noteworthy that after incubation for 60 and 120 min at 60 °C the enzyme retained about 80 and 70% of the initial activity, respectively (Fig. 2c).

Although slightly less thermostable than the native Cellulase II, the heterologous Egst presented interesting catalytic properties with respect to reaction temperature and pH when compared to the native enzyme [11]. Saccharification of lignocellulosic biomass is commonly conducted in the pH range of 4.5 to 5, and temperatures between 45 and 50 °C [35]. Therefore, Egst meets the criteria for inclusion in enzyme cocktails for saccharification of biomass. Indeed, optimal temperatures and pH very similar to those found for Egst were observed for other thermophilic fungi endoglucanases [28, 36–38].

Effect of NaCl on the activity and stability of Egst

The effect of NaCl on the Egst activity is shown in Fig. 3. The CMCase activity gradually decreased with increasing salt concentration, reaching about 71.4% of the control activity in

Fig. 3 Effect of NaCl on the activity and stability at 25 °C of Egt. The Egt activity in the presence of increasing concentrations of NaCl (●) was assayed at 60 °C in 50 mmol L⁻¹ acetate buffer, pH 5.5, 4 mmol L⁻¹ MnCl₂, containing 1% (w/v) medium viscosity CMC as the substrate, and NaCl in increasing concentrations as described in Material and Methods. The activities were expressed as percentages of the control activity. The halotolerance of Egt (○) was evaluated after 24-h incubation at 25 °C in solutions of NaCl (0–2.0 mol L⁻¹) in water. Residual activities were assayed at 60 °C in 50 mmol L⁻¹ acetate buffer, pH 5.5, 4 mmol L⁻¹ MnCl₂, and containing 1% (w/v) medium viscosity CMC as the substrate. One hundred percent specific activity (control) corresponded to 340.1 ± 47.9 U mg⁻¹. The values shown represent means ± SD from triplicate experiments (*n* = 3) carried out with three separate preparations of pure enzyme

the presence of 2.0 mol L⁻¹ NaCl. Furthermore, at 0.5 mol L⁻¹ NaCl, equivalent to the seawater salinity (0.46 mol L⁻¹) [39], about 84.1% of the control activity was maintained. The enzymatic activity remained almost constant after 24 h incubation of Egt at 25 °C in the presence of NaCl in the range 0–2.0 mol L⁻¹ (Fig. 3). Taken together, these results are evidence that Egt is a halotolerant enzyme.

Halotolerance is a highly desirable feature for enzymes that are used in processes aimed at the saccharification of lignocellulosic biomass using seawater rather than fresh water, or in processes involving biomass pretreatments that result in high saline concentrations [9]. Recently, other halotolerant endoglucanases have been described [40–44], including the GH5 from soil metagenome [45] and *Bacillus subtilis* BEC-1 [46].

Effect of Other Ions and Effectors on the CMCase Activity of Egt

The effect of several ions and effectors on the CMCase activity of Egt is presented in Table 1. The enzymatic activity was stimulated by several ions and agents such as DTT (177.1% at 10 mmol L⁻¹), β-mercaptoethanol (184.1% at 10 mmol L⁻¹), cysteine (164.2% at 10 mmol L⁻¹) and especially Mn²⁺ (180.1% at 5 mmol L⁻¹). It is also noteworthy that most of the tested agents, such as NH₄⁺, Na⁺, K⁺, Ba²⁺, Ca²⁺, and Mg²⁺ at all concentrations had little or no effect on CMC hydrolysis by Egt. In contrast, Co²⁺ had essentially no effect on the activity at the lowest concentration, although showed a strong inhibitory effect at 10 mmol L⁻¹, while Cu²⁺ presented a slight inhibitory effect, with residual activity of about 90% at the lowest concentration tested. In addition, the enzyme was highly sensitive to Hg²⁺, with no residual activity observed with 1 mmol L⁻¹ concentration. EDTA had no effect on the enzymatic activity, suggesting that Egt is not a metalloenzyme.

The stimulatory effect observed with DTT, β-mercaptoethanol and cysteine suggests that either thiol groups are important for catalysis or that the maintenance of SH groups in their

Table 1 Effect of ions and additives on the CMCase activity of Egst

Effector	Relative activity (%)		
	1 mmol L ⁻¹	5 mmol L ⁻¹	10 mmol L ⁻¹
Control ^a	100.0 ± 13.1	100.0 ± 13.1	100.0 ± 3.7
NH ₄ Cl	106.6 ± 14.9	100.4 ± 7.4	107.4 ± 13.8
NaBr	103.9 ± 12.2	99.3 ± 6.8	108.6 ± 14.4
NaCl	93.8 ± 8.8	98.9 ± 7.4	89.8 ± 7.7
CoCl ₂	104.3 ± 11.6	75.8 ± 4.2	49.2 ± 1.1
CuSO ₄	91.4 ± 3.3	NA	NA
MnCl ₂	144.6 ± 14.0	180.1 ± 24.2	175.1 ± 17.1
KCl	91.5 ± 8.3	98.1 ± 10.5	90.4 ± 7.8
CaCl ₂	92.2 ± 13.6	99.3 ± 13.1	102.6 ± 15.6
BaCl ₂	101.5 ± 10.7	92.2 ± 14.7	108.3 ± 16.1
MgCl ₂	112.5 ± 5.9	98.5 ± 16.3	115.5 ± 16.6
EDTA	101.9 ± 14.5	112.2 ± 14.2	92.0 ± 9.1
β-mercaptoethanol	143.2 ± 12.5	182.5 ± 15.9	184.1 ± 18.9
Cysteine	114.4 ± 13.9	143.8 ± 10.4	164.2 ± 17.8
DTT	150.0 ± 13.6	170.2 ± 17.8	177.1 ± 19.6
HgCl ₂	0	0	0

One hundred percent specific activity corresponded to 183.6 ± 5.5 U mg⁻¹. Values are the means ± SD of four different experiments (n = 4)

NA not evaluated due to interference in the colorimetric method employed for the enzymatic assays

reduced form is important for the native conformation of the enzyme. The stimulatory effect of Mn²⁺ may be due to the participation of this ion in the stabilization of the enzyme structure. The stabilizing effect of Mn²⁺ has also been observed for other endoglucanases [31, 47–51], including the two native cellulases from *S. thermophilum* [11].

Effect of Increasing Concentrations of Mn²⁺, Cysteine, and DTT on the Activity of Egst

The effect of increasing concentrations of Mn²⁺ and other stimulating agents on the enzymatic activity of Egst is shown in Supplemental data Fig. S3, and the kinetic parameters are summarized in Table 2. The CMCase activity of all the stimulating agents was analyzed up to concentrations where saturating conditions were observed. The highest maximum velocities were obtained for Mn²⁺ ($V_{max} = 368.9 \pm 15.1$ U mg⁻¹), followed by cysteine ($V_{max} = 331.6 \pm 13.9$ U mg⁻¹) and DTT (317.1 ± 20.5 U mg⁻¹). Egst showed an apparent affinity for DTT about 2.68- and 1.6-fold higher than those estimated for cysteine and Mn²⁺, respectively.

Table 2 Kinetic parameters for the stimulation of Egst CMCase activity by Mn²⁺, cysteine, and DTT

Agent	V_{max} (U mg ⁻¹)	K_M (mmol L ⁻¹)
Mn ²⁺	368.9 ± 15.1	1.55 ± 0.01
Cysteine	331.6 ± 13.9	2.66 ± 0.02
DTT	317.1 ± 20.5	0.99 ± 0.01

The kinetic parameters are presented as the mean ± SD of the values calculated from data from four different experiments (n = 4)

Substrate Specificity of Egst and Analysis of Hydrolysis Products

The specificity of the Egst was evaluated using various polysaccharide and natural substrates (Table 3). The enzyme showed the highest activity with barley β-glucan, followed by low viscosity CMC, medium viscosity CMC and filter paper. In contrast, Egst did not hydrolyze Avicel®, beechwood and birchwood xylan, starch, cellobiose and pNP-C. This indicated high specificity for the β-anomeric configuration of the glycosidic linkage of glucose residues in polysaccharide substrates. The absence of activity against Avicel®, cellobiose and pNP-C, further suggested the absence of exocellulase and β-glycosidase activity. Similar catalytic properties have been described for other endocellulases of fungal origin [31, 37], including the native enzyme [11].

The mode of action of Egst against barley β-glucan, CMC (low and medium viscosity), and Avicel® were investigated through the analysis of the reaction products by TLC (Fig. 4). TLC analysis showed that Egst hydrolyzed barley β-glucan and low and medium viscosity CMC to produce oligosaccharides with different degrees of polymerization (DP2-DP4, based on the migration pattern of the standards) as the predominant products, confirming its endo-hydrolytic mode of action. In addition, the absence of hydrolysis products was confirmed when Avicel® was used as substrate (Fig. 4d). Regarding the action of Egst against β-glucan, we can suggest the formation of oligosaccharides containing β-1,3-type binding.

Kinetic Parameters

The effect of increasing concentrations of medium viscosity CMC on Egst activity was evaluated in the absence and presence of manganese ions. The kinetic parameters were estimated by the Hanes-Woolf linearization method (See supplemental data Fig. S4) and are presented in Table 4. The hydrolysis of medium viscosity CMC by Egst in the absence of Mn²⁺ occurred with a V_{max} of 432.10 ± 16.76 U mg⁻¹ and K_M of 10.50 ± 2.53 mg mL⁻¹, resulting in a catalytic efficiency (k_{cat}/K_M) of 45.27 ± 3.45 mL mg⁻¹ s⁻¹. In presence of 4 mmol L⁻¹ Mn²⁺, the CMCase activity was stimulated approximately 1.4-fold, reaching a specific activity of 615.53 ± 25.90 U mg⁻¹. The K_M value obtained

Table 3 Substrate specificity of the Egst

Substrate	Specific activity (U mg ⁻¹)
Barley β-glucan	248.2 ± 15.3
CMC low viscosity	246.8 ± 13.2
CMC medium viscosity	192.4 ± 6.2
Filter paper	0.888 ± 0.062
Avicel®	ND
Beechwood xylan	ND
Birchwood xylan	ND
Starch	ND
Cellobiose	ND
pNP-C	ND

The activity was assayed at over a period of 2 h at 60 °C using a substrate concentration of 1% as described in Material and Methods. Values are means ± SD of five separate experiments (n = 5)

ND activity not detected after 2 h reaction using 0.1 U CMCase activity

Fig. 4 Time course analysis of the hydrolysis of **a** medium viscosity CMC, **b** low viscosity CMC, **c** barley β -glucan, and **d** Avicel® by Egst. The reaction times were 0 (lanes t0), 5 min (lanes t1), 10 min (lanes t2), 30 min (lanes t3), 60 min (lanes t4), and 120 min (lanes t5). Standards: G1, glucose; G2, cellobiose; G3, cellobiose; G4, cellobiose

in presence of Mn^{2+} was about 1.3-fold lower, resulting in a catalytic efficiency about 1.9-fold higher.

Compared to the native enzyme [11], the recombinant enzyme hydrolyzed CMC with eight- and 4.7-fold higher V_{max} and apparent affinity, respectively. Thus, the enzyme expressed in *P. pastoris* presented altered recognition and interaction with the substrates that affected the velocity constants of one or more catalytic steps.

Table 4 Kinetic parameters for the hydrolysis of medium viscosity CMC by Egst in the absence and presence of $4 \text{ mmol L}^{-1} \text{ Mn}^{2+}$

Parameter	Experimental condition	
	Control	$4 \text{ mmol L}^{-1} \text{ Mn}^{2+}$
V_{max} (U mg^{-1})	432.10 ± 16.76	615.53 ± 25.90
K_{Mapp} (mg mL^{-1})	10.50 ± 2.53	7.78 ± 2.87
k_{cat} (s^{-1})	475.31 ± 17.83	677.08 ± 19.23
k_{cat}/K_{Mapp} ($\text{mL mg}^{-1} \text{ s}^{-1}$)	45.27 ± 3.45	87.03 ± 6.57

The kinetic parameters are presented as the mean $\pm$ SD of the values calculated from data from four separate experiments ($n = 4$)

Effect of glucose and cellobiose on the endoglucanase activity of Egst

Using cellulose-azure as substrate, it was observed that the activity of Egst was inhibited by 20% and 8% in the presence of 1% (w/v) of cellobiose and glucose, respectively (Fig. 5a). At the highest concentration of effectors (2.0%, w/v), residual activities corresponded to 58 and 81% for cellobiose and glucose, respectively. Moreover, in the viscosity assays (Fig. 5b), the CMCase activity was stimulated about 1.6-fold by glucose at 30 mmol L^{-1} concentration and slightly inhibited by cellobiose (approximately 33%) at 50 mmol L^{-1} . The characteristic of

Fig. 5 Effect of glucose (□) and cellobiose (■) on CMCase activity of Egst. Potential product inhibition was analyzed by the methods of cellulose-azure hydrolysis (a) and medium viscosity reduction (b), as described in Material and Methods. In the viscosity reduction assays, the control (100%) was estimated in the absence of the effectors, and corresponded to the specific viscosity of the reaction media containing thermo-inactivated crude filtrate. The values shown represent means $\pm$ SD from triplicate experiments ($n = 5$) carried out with three separate preparations of pure enzyme

Fig. 6 Hydrolysis of pretreated fractions sugarcane bagasse with Egs1. The hydrolysis of the AIR (a) and the DMSO (b) extracted biomass was performed using 5% (w/v) suspension of these substrates prepared in 50 mmol L⁻¹ acetate buffer, pH 5.5, 480 µg of Egs1 at 50 °C for 48 h.

reaction product tolerance is highly desirable for the application of cellulases in the hydrolysis of the biomass.

Hydrolysis of Pretreated Fractions Sugarcane Bagasse with Egs1

The total reducing sugar released by Egs1 from the sugarcane fraction after treatment with DMSO was about 2.7 times higher than that released from AIR (Fig. 6), suggested that Egs1 is a good candidate to compose enzymatic cocktails for biomass hydrolysis. The treatment of the sugarcane bagasse with ethanol and water (to leave the AIR) remove only the soluble sugars and other soluble components, practically not affecting the recalcitrance of the material. Proteins, pectin, and 70–80% of the soluble cell wall xylan are eliminate after fractionation with ammonium oxalate and DMSO [4, 21]. The greater action of Egs1 against sugarcane fraction after treatment with DMSO can be explained by the access of the enzyme to the cellulose and the β-glucan more exposed in this fraction when compared with the AIR.

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Authors' Contributions Luana P. Melo, Sibeli Carli and Rosa P. M. Furiel conceived and designed the experiments; Luana P. Melo, Sibeli Carli, Raquel F. Maldonado, Marcelo S. Torricillas and Ana L. R. L. Zanbardi performed the experiments; Raquel F. Maldonado and Richard J. Ward conducted the cloning and expression experiments; Luana P. Melo, Sibeli Carli and Marcelo S. Torricillas analyzed the data; Richard J. Ward, Rosa P. M. Furiel and João A. Jorge contributed reagents, materials and analysis tools; Luana P. Melo and Sibeli Carli wrote the paper.

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Compliance with Ethical Standards

Competing Interests The authors declare that they have no conflict of interest.

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Apêndice D

A halotolerant bifunctional β -xylosidase/ α -L-arabinofuranosidase from *Colletotrichum graminicola*: Purification and biochemical characterization

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ABSTRACT

A β -xylosidase from *Colletotrichum graminicola* (Bgx) was purified. The enzyme showed high halotolerance, retaining about 65% of the control activity in the presence of 2.5 mol L⁻¹ NaCl. The presence of NaCl has not affected the optimum reaction temperature (65 °C), but the optimum pH was slightly altered (from 4.5 to 5.0) at high salt concentrations. Bgx was fully stable at 50 °C for 24 h and over a wide pH range even in the presence of NaCl. In the absence of salt, Bgx hydrolyzed *p*-nitrophenyl (β -D)-xylopyranoside with maximum velocity of 348.8 $\pm$ 11.5 U mg⁻¹ and high catalytic efficiency (1432.7 $\pm$ 47.3 L mmol⁻¹ s⁻¹). Bgx revealed to be a bifunctional enzyme with both β -xylosidase and α -L-arabinofuranosidase activities, and hydrolyzed xyloglucan oligosaccharides containing up to six pyranose residues. The enzyme showed high synergistic effect (3.1-fold) with an endo- α -glucanase for the hydrolysis of birchwood xylan, whereas in the absence of presence of 0.5 mol L⁻¹ NaCl, and was tolerant to different organic solvents and surfactants. This is the first report of a halotolerant bifunctional (β -xylosidase/ α -L-arabinofuranosidase) from *C. graminicola*, and the enzyme showed attractive properties for application in lignocellulosic hydrolysis, particularly under high salinity and/or in the presence of residues of pretreatment steps.

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1. Introduction

Over the last few years the fast economic development of many countries has increased the interest in new renewable energy resources, mainly due to the depletion of oil reserves and growing concerns about climate changes [1,2]. This has generated a great interest in the use of lignocellulosic biomass as a feedstock for bioethanol production [2,3].

Lignocellulose is mainly composed by three polymers, cellulose, hemicellulose and lignin, arranged in a complex three-dimensional matrix within which the polysaccharides are protected against chemical and biological degradation [4,5]. Xylans are the major form of hemicellulose found in agro-industrial residues; they consist of a backbone of β -1,4-linked α -xylopyranoside residues which may be branched, showing different substituents in the side chain such as α -arabinofuranose,

glucuronic acid and/or 4-O-methyl-glucuronic acid, ferulic acid and acetyl groups [6–8].

Due to the structural diversity of xylans, their complete degradation requires different hemicellulases synergistically acting on the main chain and the branches [9–10]. The depolymerization of the main chain involves the action of the xylosidases, endoxylanases (endo- β -1,4-xylan xylohydrolases, EC 3.2.1.8), which randomly act on the homopolymeric backbone liberating xyloglucan oligosaccharides, xylobiose and xylose [8,11], and β -D-xylosidases [β -D-xyloside xylohydrolases, EC 3.2.1.37], which act on short xylooligosaccharides and xylobiose releasing xylose [6,9].

As a result of the recalcitrance of the lignocellulosic materials, different pretreatment processes are often required to increase the accessibility of the target polymers to hydrolytic and non-hydrolytic enzymes. However, some chemicals that remain at residual levels or accumulate in the biomass after pretreatment, such as solvents or salts, can inhibit the enzymatic action in the subsequent hydrolysis stage, demanding the use of high enzyme loads or enzymes with lower sensitivity to these contaminants [12,13].

Recently, a xylanase (Exg1) produced by the mesophilic fungus *Colletotrichum graminicola* has been purified and characterized [11].

The enzyme presented good thermal stability and halotolerance, retaining about 65% and 30% of the control activity in the presence of 0.5 mol L⁻¹ and 3.0 mol L⁻¹ NaCl, respectively. In the present work we report the purification of β -D-xylosidase (Bgx) from the same microorganism along with a detailed biochemical and kinetic characterization of the enzyme, both in the presence and absence of salt. The emergence of Bgx and Exg1 for xylan hydrolysis was also evaluated.

2. Materials and methods

2.1. Organism, culture conditions and preparation of crude extracts

The *C. graminicola* strain was isolated and classified as described by Zimbaridi et al. [14]. The isolated strain was subcultured periodically on Potato Dextrose Agar (PDA, Oxoid, UK) medium and maintained at 25 °C. For the production of β -xylosidase the fungus was cultivated in 250 mL Erlenmeyer flasks at 70% constant humidity and under conditions previously optimized (8 days, 25 °C, 5 g dry wheat bran, 4.7% (w/w) yeast hulls and 10 mL of deionized water) [14]. After growth, the culture medium was suspended in 25 mL cold water, homogenized, filtered through a nylon sieve, and centrifuged at 10,000 $\times$ g and 4 °C for 30 min. The supernatant was maintained at 4 °C for up to 30 days without appreciable loss of activity and used as *C. graminicola* β -xylosidase-rich crude extract.

2.2. Enzymatic assays

The β -xylosidase activity was routinely assayed at 65 °C, in 50 mmol L⁻¹ sodium acetate buffer, pH 4.5, containing 5 mmol L⁻¹ *p*-nitrophenyl (β -D)-xylopyranoside (pNP-Xyl; Sigma-Aldrich Chem. Co., St. Louis, MO, USA) as substrate in a final volume of 0.6 mL, as described by Souza et al. [15]. Enzymatic activity against other synthetic substrates (Sigma-Aldrich Chem. Co.) [*p*-nitrophenyl (β -D)-galactopyranoside, α -nitrophenyl (β -D)-galactopyranoside, *p*-nitrophenyl (β -D)-fucopyranoside, *p*-nitrophenyl (β -D)-glucopyranoside, *p*-nitrophenyl (β -D)-mannopyranoside, *p*-nitrophenyl (α -D)-arabinopyranoside, *p*-nitrophenyl (α -D)-sorbitol furanoside, *p*-nitrophenyl (α -D)-xylopyranoside and *p*-nitrophenyl (α -D)-mannopyranoside] was estimated as above, at 5 mmol L⁻¹ final concentrations.

The hydrolysis of cellulose (5 mmol L⁻¹) was estimated under the same conditions by quantifying the glucose released using the method of glucose oxidase [15]. The hydrolysis of birchwood, beechwood and oat spelt xylans (Sigma-Aldrich Chem. Co.), carboxymethylcellulose (CMC, Sigma-Aldrich Chem. Co.) and Avicel® (Sigma-Aldrich Chem. Co.), at 1% (w/v) final concentration, was assayed in the same conditions by quantifying the reducing sugars released, using the dinitrosalicylic acid (DNS) method [16]. The hydrolysis of xylobiose (5 mmol L⁻¹) was estimated under the same conditions, but the xylose released was quantified using the o-Xylose Assay Kit (Megazyme International Ireland Ltd., Co. Wicklow, Ireland) according to the manufacturer's instructions.

The experimental conditions (reaction times, enzymatic units) employed in all experiments were adjusted to guarantee the estimation of initial velocities. All enzymatic assays were performed in duplicate. One enzyme unit (U) was defined as the amount of enzyme that releases 1 μ mol product/min. The specific activity was defined as units per milligram total protein (U mg⁻¹).

2.3. Purification of Bgx

The β -xylosidase-rich crude extract from *C. graminicola* was adjusted to pH 5.0 with 1 mol L⁻¹ sodium acetate buffer and applied onto a CM-Cellulose column (10.0 $\times$ 7.0 cm) equilibrated and eluted with 10 mmol L⁻¹ sodium acetate buffer, pH 5.0 (Buffer A). Fractions (1.0 mL) were collected and analyzed for protein (A_{280nm}) and β -xylosidase activity. The active fractions were pooled and applied onto

a DMAE-Fractogel column (13.0 $\times$ 2.0 cm) equilibrated in 10 mmol L⁻¹ sodium acetate buffer, pH 5.5 (Buffer B) and eluted with a linear gradient of NaCl (0–500 mmol L⁻¹) in the same buffer. Fractions (1.0 mL) were collected and analyzed for β -xylosidase activity and those with higher β -xylosidase activity were pooled and concentrated in centrifugal concentrators with a 10-kDa cutoff polyethersulfone membrane (Vivaspin 20, GE Healthcare, Buckinghamshire, UK). The concentrated sample was applied onto a Sepharyl 5–200 column (12.0 $\times$ 2.0 cm), equilibrated and eluted with buffer B containing 150 mmol L⁻¹ NaCl at a flow rate of 15 mL h⁻¹. The fractions (1.0 mL each) with the highest β -xylosidase activity were pooled and stored at 4 °C for further analysis. After standardization of the methodology, the purification protocol was repeated 3-fold using three different crude culture extracts from *C. graminicola*, resulting in three separate preparations of pure enzyme.

2.4. Polyacrylamide gel electrophoresis

Non-denaturing polyacrylamide gel electrophoresis (PAGE) was carried out in 7% acrylamide slab gels according to Davis [17]. Sodium dodecyl sulfate (SDS)-PAGE was carried out in 10% acrylamide slab gels as described by Laemmli [18]. The protein bands were revealed using Coomassie Brilliant Blue R-250 (Sigma-Aldrich Chem. Co.).

The β -xylosidase activity on the gel, following PAGE, was determined using 4-methylumbelliferyl (β -D)-xylopyranoside as substrate. Once the electrophoresis was finished, the gel was immersed in 50 mmol L⁻¹ sodium acetate buffer, pH 5.0, at room temperature (25 °C) for 30 min. Thereafter, the gel was incubated with 1% (v/v) 4-methylumbelliferyl (β -D)-xylopyranoside in 50 mmol L⁻¹ sodium acetate buffer, pH 5.0, at 25 °C for 10 min. Finally, the gel was exposed to ultraviolet light to visualize eventual bands of β -xylosidase activity, revealed by the fluorescence of 4-methylumbelliferone.

2.5. Determination of the isoelectric point of Bgx by two-dimensional gel electrophoresis

An aliquot of Bgx (200 μ g) was precipitated using 15% (w/v) trichloroacetic acid (TCA) at 4 °C for 24 h, centrifuged at 10,000 $\times$ g at 4 °C for 20 min, washed twice with chilled acetone, and centrifuged at 10,000 $\times$ g at 4 °C for 20 min. The pellets were solubilized in 300 μ L of rehydration buffer, containing 8 mol L⁻¹ urea, 1 mg dithiothreitol (DTT), 2% (w/v) CHAPS, 0.002% (w/v) bromophenol blue and 2% (v/v) IPG buffer (pH 3–11; GE Lifesciences). For the first dimension, the sample was applied to 7 cm immobilized pH 3–10 gradient strips (GE Lifesciences). After rehydration (15 h/20 °C), isoelectric focusing was performed in IPGphor system (GE Lifesciences) under the following conditions: 300 V (10 min), gradient 1000 V (10 min), gradient 5000 V (1 h and 20 min), 5000 V (25 min) and 50 V for 24 h. The focused strips were equilibrated for 15 min in an equilibrium buffer: 50 mmol L⁻¹ Tris buffer, 30% (v/v) glycerol, 6 mol L⁻¹ urea, 10 mg mL⁻¹ DTT, 2% (w/v) SDS, and 0.002% (w/v) bromophenol blue. For the second dimension, the strips were transferred to 12% SDS-PAGE as described in item 2.4. The isoelectric point was obtained by gel image analysis using the Image Master 6.0 2D software (GE Lifesciences).

2.6. Quantification of protein and neutral carbohydrates

Protein concentrations were determined according to Read and Northcote [19] using bovine serum albumin (Sigma-Aldrich Chem. Co.) as standard. Total neutral carbohydrates were quantified by the method described by Dubois et al. [20] using nanomose as standard.

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2.7. Estimation of the native molecular mass of the purified enzyme

The apparent native molecular mass of Bcgl was estimated by high performance liquid chromatography (HPLC) gel filtration using a Bio-Sil SEC-400 column (Bio-Rad Laboratories, Hercules, CA, USA) according to Souza et al. [15]. The molecular mass markers used were thyroglobulin, bovine γ -globulin, ferritin myoglobin, and ovalbumin (Sigma-Aldrich Chem. Co.).

2.8. Deglycosylation of Bcgl

The purified enzyme (200 μ g) was deglycosylated using Peptide N-Glycosidase F (PNGase F; Sigma-Aldrich Chem. Co.). The treatment with PNGase F followed the supplier recommendations and the deglycosylated enzyme was analyzed by SDS-PAGE as described above.

2.9. Characterization of Bcgl by mass spectrometry (MS) analysis

The purified deglycosylated β -xylosidase was subjected to SDS-PAGE and the excised band was submitted to *in situ* trypsin digestion followed by MS analysis as described by Souza et al. [15]. The analysis was carried out using an Axima Performance MALDI-TOF/TOF mass spectrometer (Shimadzu-Kioto, Shimadzu Corp., Kyoto, Japan).

2.10. Effect of NaCl on the β -xylosidase activity and stability

The effect of NaCl on Bcgl activity was evaluated under the same conditions described in the item 2.2. The salt was added to the reaction medium at final concentrations of 0.5 mmol L⁻¹ to 3 mmol L⁻¹. The half-stability of Bcgl was estimated by incubating the enzyme at room temperature (25 °C) for 24 h in NaCl aqueous solution at final concentrations of 0.5 mmol L⁻¹ to 3.0 mol L⁻¹. The residual activity was determined as described in the item 2.2, in the absence of NaCl.

2.11. Optimal temperature and pH of Bcgl

The optimum temperature for the β -xylosidase activity was determined in the range 50–80 °C in 50 mmol L⁻¹ sodium acetate buffer, pH 4.5, using pNP-Xyl (5 mmol L⁻¹) as substrate. The optimum pH of activity was determined at 65 °C in McIlwaine buffer [21] adjusted for pH 3.0 to 6.0, containing 5 mmol L⁻¹ pNP-Xyl. The effect of NaCl on optimum pH and temperature of Bcgl was evaluated under the same conditions, but in the presence of NaCl at final concentrations of 0.5 or 2.5 mol L⁻¹ in the reaction medium.

2.12. pH and thermal stabilities of Bcgl

The pH stability was evaluated by incubating Bcgl at 4 °C in McIlwaine buffer, pH 3.0 to 6.0, for 24 h. The residual activity was determined in 50 mmol L⁻¹ sodium acetate buffer, pH 4.5, as described in the item 2.2. The thermal stability of Bcgl was evaluated by incubating the purified enzyme conveniently diluted in Millipore MilliQ (EMD Millipore Co., Billerica, MA, USA) water for different time intervals at temperatures ranging from 50 to 75 °C. After cooling on ice bath (1 min), the residual activity was determined at 65 °C as described in the item 2.2. The effect of NaCl on pH and thermal stabilities of Bcgl was evaluated as described above, except that the enzyme was incubated under the different pH and temperature conditions tested in the presence of NaCl at final concentrations of 0.5 or 2.5 mol L⁻¹.

2.13. Determination of the kinetic parameters and data fitting

The maximum velocities (V_{max}) and Michaelis-Menten constants (K_m) were calculated by non-linear regression using the SigmaPlot software [22]. The inhibition constant (K_i) of the β -xylosidase activity by xylitol was estimated using Dixon plots, according to Segel [23]. Data

fitting and statistical analysis were carried out using the OriginPro 8.5.0 software package (OriginLab-Corp., Northampton, MA, USA). All experimental kinetic curves were repeated at least three times, using three different pure enzyme preparations, and every experimental point was averaged in duplicate. The kinetic parameters are given as the mean $\pm$ SD of the values calculated for three different experiments ($n = 3$).

2.14. Effect of salts, organic solvents, surfactants and other effectors on the activity of Bcgl

The effect of various salts (KCl, NaCl, NH₄Cl, MnCl₂, MgCl₂, HgCl₂, CoCl₂, CaCl₂, ZnCl₂, SrCl₂, FeCl₃, Pb(NO₃)₂, AgNO₃, Cr(NO₃)₃, Cu(SO₄)₂ ethylenediamine tetraacetic acid (EDTA, Sigma-Aldrich Chem. Co.), SDS, β -mercaptoethanol (Sigma-Aldrich Chem. Co.) on β -xylosidase activity of the pure enzyme was evaluated under the same conditions described in the item 2.2. The effectors were added to the reaction medium at final concentrations of 1 and 10 mmol L⁻¹.

The effect of carbohydrates (glucose, xylitol, galactose, maltose, sucrose, mannitol, fructose and alicofose) on the enzymatic activity was evaluated under the same conditions, but the sugars were added to the reaction medium at final concentrations of 10 and 50 mmol L⁻¹.

The effect of different organic solvents (ethanol, methanol, butanol, propanol, glycerol, toluene and acetone), triton X-100, tween 80, tween 20, sodium acetate and the ionic liquid 1-ethyl-3-methylimidazolium acetate ([Emim][Ac]; Sigma Aldrich Chem. Co.) was evaluated under the same conditions, except that the experiments were conducted at 50 °C in hermetically closed tubes. The organic solvents and surfactants were added to the reaction medium at 5% (v/v) final concentration; sodium acetate was added at the final concentrations of 50 and 200 mmol L⁻¹ and [Emim][Ac] was added at the final concentrations of 1 and 5% (w/v).

2.15. Hydrolysis of xylooligosaccharides by Bcgl

The xylooligosaccharide mixture was obtained by treatment of beechwood xylan with the purified xylanase from *C. graminicola* [Bcgl] [11]. The reaction assay was conducted in hermetically closed conic plastic tubes containing 1% (w/v) beechwood xylan and 5 U of Bcgl in 50 mmol L⁻¹ sodium acetate buffer, pH 5.0, at 50 °C and 180 rpm for 15 min. Thereafter, the reaction mixture was heated in boiling bath for 10 min, centrifuged for 5 min at 10,000 $\times$ g, and the supernatant was used as xylooligosaccharide mixture in subsequent experiments. The hydrolysis of xylooligosaccharides by Bcgl was conducted at 50 °C and 180 rpm in hermetically closed conic plastic tubes containing xylooligosaccharide mixture in 50 mmol L⁻¹ sodium acetate buffer, pH 5.0, in a final volume of 0.5 mL, either in the absence or in the presence of 0.5 mol L⁻¹ NaCl. The reactions were initiated by the addition of 1 U of Bcgl and, after adequate time intervals, interrupted by the addition of 0.5 mL of 20% (w/v) TCA to precipitate protein and long-chain carbohydrates. After standing at room temperature for 30 min, the reaction mixtures were centrifuged for 5 min at 10,000 $\times$ g. The products present in the supernatants (5 μ L) were analyzed by thin layer chromatography (TLC) on silica-gel G-60 plates [10 $\times$ 20 cm, DC-Alukolen Kieselgel 60, Merck, Darmstadt, Germany] as described by Carli et al. [11].

2.16. Synergic action of Bcgl and Bcgl1 on the hydrolysis of beechwood xylan

The enzymatic assays were conducted at 50 °C and 180 rpm in hermetically closed conic plastic tubes containing 1% (w/v) beechwood xylan in 50 mmol L⁻¹ sodium acetate buffer, pH 5.0, in a final volume of 1.0 mL, either in the absence or in the presence of 0.5 mol L⁻¹ NaCl. The reaction was initiated by the addition of 1 U of Bcgl, 5 U of Bcgl1, or a mixture of 5 U of Bcgl and 1 U of Bcgl1. After desired time intervals,

Table 1
Purification of Bcgl.

Step	Total activity (U)	Total protein (mg)	Specific activity (U mg ⁻¹)	Yield (%)	Purification (fold)
Crude extract	830.1	65.5	12.7	100	1
CM-Sepharose	340.2	28.4	28.4	41	2.2
DEAE-Sepharose	287.7	2.97	97.0	34.4	7.6
Superdex 5-200	282.3	0.87	324.8	24.2	23.8

The purification procedure was repeated three fold. Representative data obtained from one of the purification procedures are presented.

0.5 mL aliquots of the reaction medium were withdrawn, added to 0.5 mL of 20% (w/v) TCA and centrifuged for 5 min at 10,000 $\times$ g. The products present in the supernatants (5 μ L) were analyzed by TLC on silica-gel G-60 plates as described above. The concentrations of xylitol released were determined using the α -Xyloside Assay Kit (Megazyme International Ireland Ltd.), according to the supplier's recommendations. The synergism degree was defined as the ratio of the xylitol concentration released by the action of both enzymes to the sum of the xylitol concentrations released using each enzyme individually.

3. Results

3.1. Enzyme purification and molecular properties

Bcgl was purified from the crude culture extract from *C. graminicola* by three sequential chromatographic steps (Table 1). The enzyme was purified 23.8-fold with a yield of 24.2%, reaching a specific activity of 324.8 U mg⁻¹. The activity of the purified enzyme remained unaltered for at least 6 months when stored in water at 4 °C.

The homogeneity of the purified enzyme was confirmed by the single protein band (Fig. 1, line a) revealed after PAGE analysis, coincident with a single β -xylosidase activity band (Fig. 1, line b). A single protein band was also revealed after SDS-PAGE analysis (Fig. 1, line c),

corresponding to an apparent molecular mass of about 137 kDa, confirming the homogeneity of the preparation. A similar apparent molecular mass (128.6 kDa) was estimated by gel filtration, suggesting that the native enzyme has a monomer. Many fungal β -xylosidases are monomers with molecular masses above 100 kDa [24–27].

A single spot was revealed after two-dimensional electrophoresis (Fig. S1; Supplementary material), and the estimated pI of Bcgl was 4.2. The carbohydrate content of the enzyme was estimated to be 54 $\pm$ 4.1% (w/w), and SDS-PAGE analysis after deglycosylation revealed a single protein band corresponding to an apparent molecular mass of approximately 92 kDa (data not shown). In general, fungal β -xylosidases exhibit acidic isoelectric points, in the range 4.0–5.0 [25,27–29], and most are glycosylated, with carbohydrate content in the range 40–60% (w/w) [24,28,30,31].

Bcgl was characterized by mass spectrometry after enzymatic hydrolysis using trypsin. Four tryptic peptides were detected in the spectrum of MALDI-TOF-MS, and their respective precursor ions and amino acid sequences were: m/z 1040.6 (AAQGVILK); m/z 2366.1 (YVSEVPMIDMGHPSDGNPK); m/z 1650.8 (SGDYWIPNNPPK) and m/z 1902.8 (HFTGYDMENWGNR). The alignment in database revealed that the peptides identified by MS shared 73.2% sequence similarity and covered about 4.8% of a GH3 family glycosyl-hydrolase from *Colletotrichum graminicola* M1.001 (NCBI Reference Sequence XP_008096402.1, Fig. S2; Supplementary material), suggesting that Bcgl may be categorized in the GH3 family. To date, there are no reports in the literature of GH3 β -xylosidases from *C. graminicola*.

3.2. Effect of NaCl on the activity and stability of Bcgl

The activity of Bcgl gradually decreased with increasing NaCl concentrations (Fig. 2), reaching about 63% of the control activity in the presence of 3.0 mol L⁻¹ NaCl. However, the pure enzyme retained about 80% of the control activity at 0.5 mol L⁻¹ NaCl (concentration close to that usually found in the sea water, 0.46 mol L⁻¹) [32]. Moreover, Bcgl remained stable when incubated in the presence of NaCl (0.25–3.0 mol L⁻¹) for 24 h at 25 °C, maintaining at least 90% of the

Fig. 1. Analysis of the purified Bcgl by PAGE (a and b) and SDS-PAGE (c). Lanes a and b, Coomassie blue staining for protein; lane b, xylosidase activity; lane c, molecular mass markers. Total protein loading was 15 μ g per lane.

Fig. 3. Effect of NaCl on the activity and stability of Bxg. The activity of Bxg (■) was measured at 40 °C in 50 mmol L⁻¹ sodium acetate buffer, pH 4.5, using 5 mmol L⁻¹ pNP-Xyl as substrate. In the presence of increasing concentrations of NaCl (0–3.0 mol L⁻¹), the halotolerance of Bxg (□) was evaluated after 24 h incubation at 20 °C in substrate of NaCl (9–1.8 mol L⁻¹); residual activities were assayed as described above. One hundred percent specific activity (control) corresponded to 206.0 ± 6.8 U mg⁻¹. The values shown represent means ± SD from triplicate experiments (n = 3); carried out with three separate preparations of pure enzyme.

control activity. Altogether, these properties indicate that Bxg is a halotolerant enzyme. Similar results were described for the halotolerant β -xylosidase from *Aspergillus oryzae* [33], which maintained about 60% of the control activity in the presence of 1.0 mol L⁻¹ NaCl. Although few studies have addressed the halotolerance of β -xylosidases, this property has been described for several other fungal and bacterial glycosylhydrolases, such as xylanases, cellobioses and β -glucosidases [11,34–36]. Similarly, as Bxg, the halotolerant xylanase from *C. graminecola* [11] maintained about 50% of the control activity in the presence of 3.0 mol L⁻¹ NaCl, showing good stability when incubated at 25 °C in NaCl solutions from 0.5 to 3.0 mol L⁻¹.

High salt concentrations critically affect the activity and stability of proteins, and most known enzymes are severely inhibited or inactivated under these conditions [37,38]. Thus, Bxg halotolerance represents a desirable property for application in lignocellulose saccharification processes, bypassing washing steps, since neutralization steps in some biomass pretreatments lead to salt accumulation. In addition, halotolerant enzymes are very interesting for application in industrial processes conducted in sea water, reducing costs and preserving fresh water reserves [11,32].

3.3. Effect of temperature and pH on the activity and stability of Bxg in the presence and absence of NaCl

The presence of salt had no effect on the temperature-activity profile of Bxg (Fig. 3a). The enzymatic activity gradually increased above 50 °C, reaching a maximum at 70 °C and abruptly decreasing at higher temperatures. In general, the optimal temperature of the β -xylosidases from mesophilic fungi is in the range 60–70 °C [24–26,30,35].

In the absence of salt, the optimum pH for the β -xylosidase activity of Bxg was 4.0–4.5, and about 70% of the maximal activity was observed in the presence of NaCl 0.5 mol L⁻¹, but the maximum activity occurred at pH 4.3. In contrast, a very different profile was observed in the presence of 2.5 mol L⁻¹ NaCl; the optimum pH was 5.0 (Fig. 3b), and the enzymatic activity was severely impaired at acidic pH, resulting in total depletion at pH 4.0. Furthermore, under these conditions the enzyme had 60% of the maximum activity at pH 6.0, a much higher percentage than that observed in the absence of salt (37%).

Fig. 4. Optimal temperature (a) and pH (b) for the β -xylosidase activity of Bxg in the absence and presence of NaCl. (a) The activity was determined at different temperatures in 50 mmol L⁻¹ sodium acetate buffer, pH 4.5, using 5 mmol L⁻¹ pNP-Xyl as substrate. In the absence of salt (■) or in the presence of NaCl at 0.5 mol L⁻¹ (□) or 2.5 mol L⁻¹ (▲). Final concentrations, specific activities of 100% under these conditions corresponded to 309.0 ± 10.2 U mg⁻¹, 272.7 ± 9.9 U mg⁻¹ and 203.8 ± 18.1 U mg⁻¹, respectively. (b) The activity was assayed at 40 °C in Mclintock buffer (pH 3.0–8.0) containing pNP-Xyl as substrate. In the absence of salt (■) or in the presence of NaCl at 0.5 mol L⁻¹ (□) or 2.5 mol L⁻¹ (▲). Final concentrations, specific activities of 100% under these conditions corresponded to 284.0 ± 14.1 U mg⁻¹, 301.9 ± 10.7 U mg⁻¹ and 176.9 ± 7.9 U mg⁻¹, respectively. The values shown represent means ± SD from triplicate experiments (n = 3); carried out with three separate preparations of pure enzyme.

In the absence of salt, Bxg was stable over a wide pH range (4.0 to 7.0), with residual activities of about 50% and 72% at pH 3.0 and 8.0, respectively (Fig. 4a). The presence of salt at high concentrations (0.5 mol L⁻¹ and 2.5 mol L⁻¹) severely impaired the stability of the enzyme at acidic pH, and residual activities of about 8% were estimated at pH 3.0 at both conditions tested. In contrast, high salt concentrations slightly improved enzyme stability at pH 8.0 and about 97% of the control activity was detected at this pH at the two concentrations tested (Fig. 4a). Similarly to Bxg, the optimum pH of most fungal β -xylosidases is in the range 4.0–4.5 [24–26,31] and several of them were stable in the acidic pH range (3.0–6.0) [24,25,31,40].

To date, no study has addressed the optimum pH and pH stability of β -xylosidases in the presence of NaCl. However, the results observed for Bxg were very similar to those described for the halotolerant Bxgl [11]. In contrast, the optimal pH of a halophilic β -glucosidase from *Bacillus* sp. S-10 [41] and an endoglucanase from *Vibrio* sp. G21 [33] were not affected by the presence of NaCl in the reaction medium. The changes in the pH-activity profiles of enzymes in the presence of NaCl

Fig. 8. pH stability (a) and thermal stability (b) of Bxg. (c) pH stability and (d) thermal stability of Bxg in the presence of NaCl. The activity of Bxg (■) was measured at 4 °C in Mclintock buffer (pH 3.0–8.0) for 24 h in the absence of salt (■) or in the presence of NaCl at 0.5 mol L⁻¹ (□) or 2.5 mol L⁻¹ (▲). Final concentrations, specific activities of 100% under these conditions corresponded to 283.1 ± 12.1 U mg⁻¹, 283.1 ± 12.1 U mg⁻¹ and 283.1 ± 12.1 U mg⁻¹, respectively. (b) Thermal stability of Bxg in water at 40 °C in the absence (■) or in the presence of NaCl at 0.5 mol L⁻¹ (□) or 2.5 mol L⁻¹ (▲). Final concentrations, specific activities of 100% under these conditions corresponded to 283.1 ± 12.1 U mg⁻¹, 283.1 ± 12.1 U mg⁻¹ and 283.1 ± 12.1 U mg⁻¹, respectively. (c) Thermal stability of Bxg in water at 40 °C in the absence (■) or in the presence of NaCl at 0.5 mol L⁻¹ (□) or 2.5 mol L⁻¹ (▲). Final concentrations, specific activities of 100% under these conditions corresponded to 283.1 ± 12.1 U mg⁻¹, 283.1 ± 12.1 U mg⁻¹ and 283.1 ± 12.1 U mg⁻¹, respectively. The values shown represent means ± SD from triplicate experiments (n = 3); carried out with three separate preparations of pure enzyme.

at high concentrations has been attributed to changes in the pKa values of the carboxyl groups of the side chains of catalytic aspartate and glutamate residues conserved within all glycosyl hydrolases of the GH3 family [9–11]. Likewise, the differences in pH stability of Bxg observed in the presence of NaCl may be tentatively attributed to changes in the pKa of ionizable groups in the side chains of amino acid residues present on the protein surface, favoring the neutral forms and therefore disrupting structurally relevant ionic interactions [11,42].

Bxg was fully stable in water for 120 min at 60 and 65 °C, with a half-life of about 30 min at 70 °C. At 75 °C, activity steadily decreased with time and residual activities of about 3% were estimated after 10 min (Fig. 4b). The thermal stability of Bxg was increased at high salt concentrations. In the presence of 2.5 mol L⁻¹ NaCl, the pure enzyme retained about 67% of the control activity after 120 min at 70 °C (Fig. 4c) and a half-life of 25 min was determined at 75 °C (Fig. 4c). Moreover, Bxg was stable for up to 24 h at 50 °C, either in the absence or presence of NaCl (at final concentrations of 0.5 mol L⁻¹ or 2.5 mol L⁻¹), maintaining about 90% of the control activity (Fig. 5). Supplementary material. Bxg was found to be more thermostable than the purified β -xylosidase from *A. oryzae* [25], a fungus [43] and *A. niger* [44]. No report was found regarding the effect of NaCl on the thermal stability of β -xylosidases. However, similar positive effects were described for xylanases from *C. graminecola* [11] and *Zizosporangium profluvium* [36], and a halophilic β -glucosidase from *Bacillus* sp. S-10 [41]. The physical-chemical mechanism underlying these

effects are still not well understood [11,45]. Taken together, the optimum pH and temperature values, as well as the good thermal and pH stability of Bxg are valuable properties for biotechnological applications.

3.4. Enzyme specificity

Bxg showed high specificity for pNP-Xyl (Table 2) but hydrolyzed other synthetic substrates such as pNP- β -D-glucopyranoside, pNP- β -D-galactopyranoside and pNP- β -D-galactopyranoside. Moreover, the enzyme hydrolyzed pNP- α -D-arabinofuranoside and pNP- α -D-arabinopyranoside with activities about 4.2 and 5.2-fold lower, respectively, as compared to that determined for pNP-Xyl. In contrast, Bxg showed undetectable activity on pNP- α -D-xylopyranoside and pNP- α -D-mannopyranoside, suggesting that it is capable of hydrolyzing substrates with α -glycosidic linkages only with α -arabinose. The pure enzyme also hydrolyzed xylanase with high specificity, but polymer substrates (beechwood, birchwood and oat spelt xylan) were poorly hydrolyzed. Moreover, the hydrolytic activity against CMC and Avicel was undetectable. These results indicate that Bxg is a bifunctional β -xylosidase- α -arabinosidase. Similarly to Bxg, α -arabinofuranosidase activity has been described for various fungal β -xylosidases [24,26,38–40]. The bifunctionality of Bxg, which can be attributed to the spatial similarity between β -xylosyltransferase and α -

Table 3
Substrate specificity of Bxg^a.

Substrate	Activity (U mg ⁻¹)
Synthetic substrates	
pNP- β -D-glucopyranoside	264.9 $\pm$ 5.2
pNP- β -D-galactopyranoside	18.3 $\pm$ 9.1
pNP- β -D-glucopyranoside	19.8 $\pm$ 5.4
pNP- β -D-galactopyranoside	4.3 $\pm$ 0.9
pNP- β -D-glucopyranoside	1.4 $\pm$ 0.2
pNP- β -D-galactopyranoside	0.10 $\pm$ 0.03
pNP- α -D-glucopyranoside	47.7 $\pm$ 9.8
pNP- α -D-galactopyranoside	54.7 $\pm$ 8.7
pNP- α -D-glucopyranoside	160
pNP- α -D-galactopyranoside	160
Natural substrates	
Xylobiose	122.8 $\pm$ 2.3
Beechwood xylan	6.60 $\pm$ 0.03
Bamboo xylan	6.63 $\pm$ 0.03
Oat spelt xylan	0.47 $\pm$ 0.07
Cellobiose	160
CMC	160
Avicel	160

ND: activity not detected after 2 h reaction employing 0.1 U of xylobiose activity. Values are means $\pm$ SD of three different experiments ($n = 3$).

^a The activity was assayed at 50 °C in 50 mmol L⁻¹ sodium acetate buffer, pH 4.5, containing the different substrates at 5 mmol L⁻¹ concentration, except for beechwood xylan, bamboo xylan, oat spelt xylan, CMC and Avicel, employed at 10 (w/v) concentration.

arabinofuranose [9,45,46], is highly desirable for the hydrolysis of substituted xyans [9,28].

3.5. Kinetic parameters

In the absence of salt, the hydrolysis of pNP-Xyl by Bxg occurred with V_{max} of 348.8 $\pm$ 11.5 U mg⁻¹ and K_m of 0.52 $\pm$ 0.02 mmol L⁻¹, resulting in a catalytic efficiency (k_{cat}/K_m) of 1432.7 $\pm$ 47.3 L mmol⁻¹ s⁻¹ (Table 3). Similar kinetic parameters were determined in the presence of 0.5 mol L⁻¹ NaCl. In contrast, a V_{max} about 1.5-fold lower was determined in the presence of 2.5 mol L⁻¹ NaCl, resulting in a catalytic efficiency near 1.8-fold lower, as compared to that determined in the absence of salt. Likewise, the kinetic parameters determined for the hydrolysis of beechwood xylan by the halotolerant xylanase from *C. graminicola* were only slightly altered by the presence of salt, which was attributed to conformational alterations of the enzyme, hindering enzyme-substrate interactions and/or the proper positioning of the catalytic groups [11]. Even in the presence of salt, the catalytic efficiency of Bxg for the hydrolysis of pNP-Xyl may be considered high, when compared to those reported for other fungal β -xylosidases [26,29–31].

3.6. Effect of ions and other effectors on the activity of Bxg

The activity of the pure enzyme was insensitive to the presence of K⁺, Na⁺, Mn²⁺, Mg²⁺, Co²⁺, Ca²⁺, Zn²⁺, at both concentrations tested (Table 4). In contrast, Ni²⁺ and Sr²⁺ exerted little effect on the β -xylosidase activity at the lowest concentration, but had an inhibitory effect of about 27% at 10 mmol L⁻¹ concentration. In addition, Bxg was highly sensitive to Ag⁺ and Hg²⁺, which completely depleted the activity

Table 4
Effect of ions and other effectors on β -xylosidase activity^a of Bxg.

Effectors	Enzymatic activity (%)	
	1 mmol L ⁻¹	5 mmol L ⁻¹
Control	100 ^b	100 ^b
KCl	101.2 ± 1.5	106.4 ± 2.2
NaCl	98.3 ± 1.2	101.5 ± 1.8
NiCl ₂	98.2 ± 1.3	12.7 ± 1.3
MnCl ₂	98.3 ± 1.2	119.9 ± 1.1
MgCl ₂	108.2 ± 1.5	89.8 ± 1.3
HgCl ₂	0.5 ± 1.0	0
CaCl ₂	99.8 ± 1.2	91.2 ± 1.5
CdCl ₂	103.9 ± 2.5	84.1 ± 1.3
CoCl ₂	98.2 ± 1.2	123.5 ± 1.1
FeCl ₂	98.3 ± 1.3	123.5 ± 0.8
Pb(NO ₃) ₂	98.3 ± 1.4	100.5 ± 1.0
AgNO ₃	21.2 ± 1.5	0
Cu(NO ₃) ₂	92.8 ± 1.3	98.5 ± 1.3
CaSO ₄	79.3 ± 1.2	5.4 ± 0.2
EDTA	102.7 ± 1.7	93.2 ± 1.2
SDS	91.2 ± 1.4	0
β -D-glucopyranoside	104.5 ± 2.3	103.0 ± 1.0

Values are means $\pm$ SD of three different experiments ($n = 3$).

^a The activity was assayed at 50 °C in 50 mmol L⁻¹ sodium acetate buffer, pH 4.5, using 5 mmol L⁻¹ pNP-Xyl as substrate.

^b One hundred percent specific activity (control) estimated in the absence of metal ions and other effectors, corresponding to 287.3 $\pm$ 11.1 U mg⁻¹.

at 5 mmol L⁻¹ concentration. A strong inhibitory effect (about 80%) was also observed in the presence of 5 mmol L⁻¹ Fe²⁺ or Cu²⁺. EDTA and β -mercaptoethanol had no effect on β -xylosidase activity. The inhibitory effect of Hg²⁺, Fe²⁺, Ag⁺, Pb²⁺ and Cu²⁺ has been reported for several fungal β -xylosidases [28,31,43,44].

3.7. Effect of carbohydrates on the activity of Bxg

The activity of the pure enzyme was virtually insensitive to glucose, galactose, maltose, sucrose, mannose, fructose and arabinose, even at 50 mmol L⁻¹ concentration (Table 5). In contrast, xylose exerted a strong inhibitory effect (70%) at the same concentration, and was shown to be a competitive inhibitor of the hydrolysis of pNP-Xyl by Bxg, with $K_i = 3.3$ mmol L⁻¹ [Fig. S4; Supplementary material]. Similarly, most fungal β -xylosidases are strongly inhibited by xylose, with K_i values in the range 2 to 5 mmol L⁻¹ [27,39,60,64].

3.8. Analysis of xylooligosaccharide hydrolysis products by TLC

In the absence of salt, Bxg completely hydrolyzed xylobiose (X2) after short reaction times, with release of xylose (X1) (Fig. 5a). After longer reaction times (9 h), Bxg also hydrolyzed xylofuranose (X3),

Table 5
Effect of carbohydrates on β -xylosidase activity^a of Bxg.

Carbohydrates	Enzymatic activity (%)	
	10 mmol L ⁻¹	50 mmol L ⁻¹
Control	100 ^b	100 ^b
Glucose	91 ± 1	94 ± 3
Xylose	67 ± 9	29 ± 3
Galactose	91 ± 2	95 ± 2
Maltose	97 ± 1	95 ± 4
Sucrose	96 ± 2	87 ± 3
Mannose	105 ± 9	88 ± 4
Fructose	103 ± 3	89 ± 2
Xylofuranose	96 ± 2	82 ± 3

Values are means $\pm$ SD of three different experiments ($n = 3$).

^a The activity was assayed at 50 °C in 50 mmol L⁻¹ sodium acetate buffer, pH 4.5, using 5 mmol L⁻¹ pNP-Xyl as substrate.

^b One hundred percent specific activity (control) estimated in the absence of carbohydrates, corresponding to 287.3 $\pm$ 11.1 U mg⁻¹.

Fig. 5. Thin layer chromatography (TLC) analysis of the hydrolysis of xylooligosaccharides by Bxg in the absence (a) and presence of 0.5 mol L⁻¹ NaCl (b). The reaction assay was conducted at 50 °C in 50 mmol L⁻¹ sodium acetate buffer, pH 4.5, containing a mixture of xylooligosaccharides and 1 U of Bxg. Samples were taken after different times were incubated by addition of X2A and analyzed by TLC. Standards: P: xylose X1, xylobiose X2, xylofuranose X3, xylofuranose with a substitution of 4-O-methyl- β -D-glucoside and X3-O-MeGlc, xylofuranose X4, xylofuranose X5, P⁺: X1 (xyl, 100 mmol L⁻¹) and X2 (2 μ L, 15 mmol L⁻¹).

xylofuranose with a branching of 4-O-methyl- β -D-glucoside (X3-O-MeGlc) and xylofuranose (X4). Finally, after 24 h, complete hydrolysis of xylofuranose (X5) and xylofuranose (X6) was observed. The purified enzyme appears to act exclusively by removing xylose from xylooligosaccharides, indicating that it is a true β -xylosidase [3,28,44]. Similar hydrolysis patterns have been described for some β -xylosidases [28,39], while others hydrolyzed only xylooligosaccharides up to X3 or X4 [31,44]. Finally, a very similar hydrolysis profile was observed for reactions performed in the presence of 0.5 mol L⁻¹ NaCl (Fig. 5b), reaffirming the potential of Bxg application in biotechnological processes conducted at high salinities.

3.9. Synergistic action of Bxg and Exg1 on the hydrolysis of beechwood xylan

The synergism between Bxg and Exg1 for the hydrolysis of beechwood xylan was investigated by TLC (Fig. 6). Using Exg1 alone, X2, X3-O-MeGlc and traces of X1, X4 and X5 were observed after 24 h (Fig. 5a), with release of 1.29 mg mL⁻¹ of xylose. Using Bxg alone, only traces (0.31 mg mL⁻¹) of X1 were produced after 24 h (Fig. 5b). When both enzymes were combined, however, the TLC profile revealed a remarkable synergistic effect (Fig. 6c). After 1 h, X1 and xylooligosaccharides from X2 to X5 were observed as the main products. After 9 h, X2 and larger xylooligosaccharides were hydrolyzed, except for X3-O-MeGlc, resulting in increased X1 concentration. At higher reaction times, however, X1-O-MeGlc was also hydrolyzed, and after 24 h, all the xylooligosaccharides were completely hydrolyzed, yielding 5.01 mg mL⁻¹ of xylose. The degree of synergism was estimated as 3.1-fold. A similar hydrolysis profile (Fig. 6d) and a similar degree of synergism (3-fold) were observed for reactions performed in the presence of 0.5 mol L⁻¹ NaCl, increasing the evidence of the good potential of application of Bxg in biotechnological processes carried out under conditions of high salinity or using seawater.

It is well known that the synergism of β -xylosidases and other xylosidase enzymes is essential for a complete and efficient hydrolysis of hemicelluloses, since the action of β -xylosidases on xylooligosaccharides and xylobiose releases the endo-xylosidases from product inhibition [9,47–49]. The synergistic action of

several β -xylosidases and endo-xylosidases on the enzymatic degradation of xylan is well documented [8,47–50]. In particular, results similar to those obtained with Bxg and Exg1 were reported when mixtures of a β -xylosidase, an endo-xylosidase and an α -arabinofuranosidase from *A. niger* [31] were employed for the hydrolysis of arabinoxylan in the presence of 1.0 mol L⁻¹ NaCl.

3.10. Effect of salts, organic solvents, surfactants and other effectors on the activity of Bxg

The purified enzyme was tolerant to butanol, glycerol, toluene, acetone, methanol and propanol at 5% (v/v) concentration, maintaining at least 70% of the control activity (Fig. 7). In contrast, ethanol had an inhibitory effect of about 42%. Bxg was also tolerant to Triton X-100, Tween 80 and Tween 20 at the same concentration. Sodium acetate did not affect Bxg activity at low concentration and the enzyme maintained about 60% of control activity at the highest concentration tested (0.200 mol L⁻¹). Furthermore, in the presence of 1.0% (v/v) [Emul][Ac], an ionic liquid commonly used in some biomass pretreatment processes, Bxg maintained about 60% of control activity. However, [Emul][Ac] had a strong inhibitory effect (about 70%) at 5% (v/v) concentration. To date, the tolerance of fungal β -xylosidases to ionic liquids, surfactants and organic solvents used for biomass pretreatment has not been investigated. The good tolerance to various organic solvents and surfactants in residual levels is an attractive property of Bxg for application in the saccharification of pretreated biomass, reducing costs and water consumption in washing steps.

4. Conclusions

A β -xylosidase from *C. graminicola* was purified and characterized. Bxg presented high halotolerance and thermal stability, as well as good stability over a wide pH range and tolerance to residual levels of various organic solvents and surfactants commonly used in pretreatment processes of lignocellulosic biomass. The enzyme hydrolyzed pNP-Xyl with high catalytic efficiency either in the absence

Fig. 6. Time course analysis of the hydrolysis of beechwood xylan by Bxg1 (a, b) and Bxg2 (c, d) in the absence (a, c) and presence (b, d) of 0.5 mM NaCl. The reaction was conducted at 30 °C in 50 mM Tris-HCl buffer, pH 5.0, containing 10 mM beechwood xylan, employing 5 U of Bxg1 (a, b) or 5 U of Bxg2 (c, d) as a mixture of 3 U of Bxg1 and 2 U of Bxg2 in the absence (a, c) and in the presence (b, d) of 0.5 mM NaCl (a, d). Samples withdrawn after different times were inactivated by addition of TCA and analyzed by HPLC. Standards: P (pinose), X1 (xylotriose), X2 (xylobiose), X3 (xylotriose with a modification of 4-O-methyl glucosyl), X4 (xylohexose), X5 (xylobiose), X6, P, X1 (14, 100 mM), X2 (24, 20 mM), X3 (24, 20 mM), X4 (24, 20 mM), X5 (24, 20 mM), X6 (24, 20 mM).

or in the presence of NaCl. In addition, Bxg2 showed a bifunctional character of β -xylosidase (α -arabinofuranosylase and efficiently hydrolyzed xyloglucan derivatives from xylobiose up to xylohexose, including xylohexose with a 4-O-methyl glucosyl branching. Furthermore, Bxg2 and Bxg1 had a remarkable synergistic effect on the hydrolysis of beechwood xylan either in the absence or in the presence of NaCl. Taken together, these properties have suggested that Bxg2 has attractive properties for application in saccharification processes of lignocellulosic biomass, pretreated or untreated, particularly under high salinity conditions or using sea water. Finally, to our best knowledge, this is the first halotolerant bifunctional β -xylosidase (α -arabinofuranosidase from fungal origin described to date.

Conflict of interest

The authors declare that they have no conflict of interest.

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Fig. 7. Effect of salts, organic solvents, surfactants and other effectors on the activity of Bxg2. The activity was determined at 30 °C in 50 mM Tris-HCl buffer, pH 5.0, using 50 U of Bxg2 in the reaction. The organic solvents and surfactants were added to the reaction mixture at 50 mM final concentration. Saline acetate was added at the final concentrations of 50 and 200 mM/L. [Emulsi]Ac was added at 1 and 5% (v/v) final concentrations. One hundred percent specific activity corresponded to 117.2 $\pm$ 2.2 U mg⁻¹.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijbiomac.2018.03.111>.

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Apêndice E

1. Introduction

β -glucosidases (β -D-glucoside glucosylhydrolase, EC 3.2.1.21) are ubiquitous enzymes found in ARCHAEA, BACTERIA, and EUKARYOTES (Kusuda, Gomes and Eari, 2010), and are involved in diverse cellular functions. This heterogeneous family of enzymes can hydrolyze glycosidic bonds of alkyl-, amino-, or aryl- β -D-glucosides, cyanogenic glucosides, disaccharides and short oligosaccharides. Under certain conditions, the synthesis of a glycosyl bond between different molecules can occur via reverse hydrolysis or transglycosylation (Mhais et al., 2002). In bacteria and fungi β -glucosidases hydrolyze short cellulosaccharides and cellulose (Devorak, Gomes and Eari, 2010) and in plants, these enzymes can act in the breakdown of glycolipids, in lipidification processes as a defense against phytopathogens, in the activation of phytochemicals, in cell wall catabolism in the ripening of fruits, emission of fragrances in flowers and are involved in plant-microorganism and in plant-insect interactions (Singh et al., 2015). In humans, β -glucosidases perform the hydrolysis of glycosyl ceramides (glucosylceramides) and other conjugated glycosides (Diao and Galante, 2002). Due to this substrate diversity and their ability to both hydrolyze and synthesize glycosidic bonds, β -glucosidases have found a broad range of applications (Mhais et al., 2002), and this highlights the importance of studies aimed at understanding the catalysis and regulation of these enzymes.

The isolation and biochemical characterization of β -glucosidases from a wide variety of organisms have been reviewed (Mhais et al., 2002; Kusuda, Gomes and Eari, 2010; Kusuda, Gomes and Eari, 2010; Kusuda and Viljamaa, 2017; Marcano et al., 2017; Singh et al., 2015, 2013), and it is widely appreciated that the activity of many β -glucosidases is inhibited by the reaction product glucose. A reduced catalytic activity caused by this inhibitory effect may result in significant limitations to the application of β -glucosidases and has encouraged the search for enzymes with kinetic properties that are compatible with the accumulation of high glucose concentrations. Over the previous two decades, there has been a steady increase in the number of reports describing glucose tolerant and glucose stimulated β -glucosidases, and here it has been reviewed this accumulated body of knowledge which provides valuable insights as to the kinetics of these enzymes, the structural basis underlying the mechanism of the glucose tolerant/stimulated effect and a range of possible applications of these enzymes in several areas of biotechnology. It also has been attempted to cover evolutionary aspects, such as the origin and evolution of glucose tolerant/stimulated β -glucosidases. The level of knowledge of these enzymes as reviewed here opens up possibilities for engineering these proteins for the creation of more robust enzymes adapted to a variety of processes of interest.

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2. Classification of beta-glucosidases

Two classification systems for β -glucosidases have been described (Singh et al., 2016; Singh et al., 2013). The first is based on the substrate specificity of the enzyme, and in this system β -glucosidases are divided into three groups: (1) aryl- β -glucosidases, which preferentially cleave aryl-glucosides, such as *p*-nitrophenyl β -D-glucopyranoside (pNP-Glc), (2) near cellulases, which hydrolyze cellulose to glucose, or (3) enzymes with broad substrate specificity, which act on a wide variety of substrates. The majority of characterized β -glucosidases are assigned to the last group. The second classification system is based on both similarity of the nucleotide sequences and the 3D-structures of the enzymes (Devorak, 1991) and is accessed through the Carbohydrate Active enZYme (CAZy) Web site (<http://www.cazy.org>, as of May 2018) (Cassand et al., 2016). According to this classification, the glycosyl hydrolases (GH) are currently divided into 153 families, where the β -glucosidases are primarily found in families GH1 and GH2, with lesser representation in GH families 2, 5, 9, 30, 39 and 116 (Singh et al., 2016; Tregias and Viljamaa, 2013).

Although convenient and widely used, these existing classification systems do not take into consideration many important functional aspects of β -glucosidases, such as glucose-tolerance and glucose-stimulation. An attempt to categorize these enzymes based on the effect of glucose on their catalytic activity has been previously proposed by Cao et al. (2015), and for the purpose of this review, it has been expanded this functional classification of the β -glucosidases into four classes: (I) β -glucosidases strongly inhibited by low concentrations of glucose, (II) β -glucosidases tolerant to glucose, (III) β -glucosidases stimulated by low glucose concentration and inhibited by high glucose concentrations, and (IV) β -glucosidases not inhibited by glucose at high concentrations. The experimental curves typically observed for the effect of increasing glucose on the enzymatic activity of these four classes of enzymes is shown schematically in Fig. 1.

Class I β -glucosidases strongly inhibited by low concentration of glucose: The enzymes assigned to this class are those that present K_i values for glucose of less than 0.1 M. Although most known GH1 β -glucosidases are included in this class (Devorak et al., 2013; Chaves et al., 2010; Bredet et al., 2001, 2009; Hu et al., 2011; Haraguchi et al., 2009; Karsauer et al., 2012; Singh et al., 2010; Meleiro et al., 2014; Seidel et al., 2004; Tregias and Viljamaa, 2013; Xia et al., 2010; Yoon et al.,

2000), some GH1 β -glucosidases have also been reported (Pan et al., 2011; Fries-Pass et al., 1990; Toyama et al., 2018; Vallanarajan et al., 2007; Wierzbicka-Was et al., 2013) and several β -glucosidases that have not been allocated to current CAZy groups are also included in this class of enzymes (Ali et al., 2012; Bredet et al., 2010; Seidel et al., 2017; Issa et al., 2017; Chaturvedi et al., 1994; Chel et al., 2017; Tiedje et al., 2001; Kato et al., 2011; Katschinski et al., 2007; Rasmussen et al., 2009; Mollat-Fabiani and Bolyard, 2016; Ng et al., 2012; Park et al., 2012; Barnett et al., 2015; Rhee et al., 1998; Yoo et al., 1996; Zhang et al., 2011; Zou et al., 2013).

Class II β -glucosidases tolerant to glucose: The enzymes belonging to this group present K_i values for glucose higher than 0.1 M. In the Supplementary material has been presented a table summarizing the main characteristics of these enzymes, presented in chronological order of their discovery. It is noteworthy that the overwhelming majority of the glucose tolerant β -glucosidases that have been characterized to date belong to the GH1 family, and to the best of the knowledge, the only exception is the GH1 β -glucosidase from *Mucor circinellus* (Zhang et al., 2014). These enzymes are isolated from a wide variety of sources including bacteria, fungi, animals and from screening metagenomic DNA libraries. In general, the glucose tolerant β -glucosidases show maximum catalytic activity between 40 and 65 °C, and between pH 4.5 and 6.5, although optimal conditions have been observed outside these ranges. Moreover, many glucose tolerant β -glucosidases preferentially hydrolyze the synthetic substrate 4-nitrophenyl- β -D-glucopyranoside (pNP-Glc) rather than the natural substrate cellulose, and particularly high catalytic efficiency coefficient values (k_{cat}/K_m) were reported with pNP-Glc for the β -glucosidases from *Mucor circinellus* (Zhang et al., 2017) and *Neosartorya fischeri* (Chaturvedi et al., 2012).

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.biortech.2018.07.137>.

Class III β -glucosidases stimulated by low glucose concentration and inhibited by high glucose concentration: These enzymes present increasing activity over a range of glucose concentrations as compared with the enzymatic activity in the absence of glucose. The maximum increase in glucose stimulated activity is conveniently expressed as the stimulation factor (SF), which is the ratio between the activity in the presence of glucose and activity in the absence of glucose and varies from 1.1 to 4.5. The glucose concentration at which the maximum stimulation occurs (MC_{max}) varies depending on the individual β -glucosidase and is typically in the concentration range of 20–500 mM. The values of the SF and MC_{max} together with the other biochemical characteristics of the enzymes of this class are presented in the Supplementary material in chronological order of discovery.

This group of β -glucosidases are predominantly found in the GH1 family, with only two representatives from the GH2 family. Although these enzymes have frequently been classified as only tolerant to glucose, a more thorough collation of the data in the literature reveals a range of glucose concentrations over which a stimulatory effect is observed. It is noteworthy that the activity of the majority of the enzymes was characterized using the synthetic substrate pNP-Glc. In a directed evolution study performed with the β -glucosidase from *Hanicola isolens* (Rhee) the mutants N226S, A141T/N225S and D237V exhibiting a glucose-stimulated pNP-glucosidase activity, however these same mutants did not show the same effect when the natural substrate cellulose was used, and highlights the importance of studies using the natural substrate (Meleiro et al., 2017).

Class IV β -glucosidases not inhibited by glucose or high concentrations: The catalytic activity of these enzymes in the presence of glucose is always greater than the enzymatic activity in the absence of glucose over the entire range of glucose concentrations tested. The concentration of glucose where activity is stimulated can be in the molar range, for example the β -glucosidases from *Bacillus halodurans* (Dai et al., 2011) and from a microbial metagenome (Lohmeyer et al., 2013) and a soil metagenome (Nishimura and Yano, 2010) were stimulated by 1 M glucose, while the β -glucosidases from an agricultural soil metagenome

Fig. 1. Schematic curves for the effect of glucose on the enzymatic activity of the β -glucosidases. Class I β -glucosidases strongly inhibited by low concentration of glucose (orange line); class II β -glucosidases tolerant to glucose (yellow); class III β -glucosidases stimulated by low concentration of glucose and inhibited by high concentration of glucose (green); class IV β -glucosidases are not inhibited by glucose up to high molar concentration (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and from a halophilic bacteria *Halobacterium salinarum* were stimulated by 2.5 M glucose (Bhore et al., 2014; Law et al., 2010). In an extreme example, the activity of the β -glucosidase from *Acetivibrio* sp. D73-1 was stimulated by glucose up to 5 M, and moreover, the enzyme retained 93% and 43% of the relative activity in 10 and 15 M glucose, respectively (Chen et al., 2016).

3. Enzyme mechanism, tolerance and stimulation in β -glucosidases

All the glucose-tolerant and/or glucose-stimulated β -glucosidases discovered to date are members of the GH1 and GH3 families. Enzymes in these families present a catalytic cycle that occurs in two distinct glycosylation and deglycosylation steps, with a double displacement mechanism involving two active site carboxyl groups (either glutamate or aspartate residues) separated by approximately 5.5 Å, resulting in retention of the configuration of the anomeric carbon in the glucose product (Molina, 2008; Tschel and Withers, 2000). In the glycosylation step, one of the carboxyl groups acts as an acid, donating a proton to the oxygen in the acetate O-glycosidic linkage with the concomitant cleavage of the glycosidic bond, formation of a glycosyl-enzyme covalent intermediate and liberation of the aglycone product. The second active site carboxyl group acts as a nucleophile, attacking the anomeric carbon to form a glycosyl-enzyme intermediate. In the deglycosylation step the carboxyl group that previously acted as an acid, now acts as a base and sequesters the proton from a nucleophilic group of an acceptor molecule located in the region of the active site. This nucleophile could be water (hydrolysis) or another acceptor such as a monosaccharide, disaccharide, aryl, alkyl alcohol or monoterpene alcohol (transglycosylation) that attacks the glycosyl-enzyme intermediate restoring the free enzyme with the concomitant liberation of a second product, thereby completing the catalytic cycle (Molina, 2008; Meleiro et al., 2017).

The term “enzyme modifier” was introduced in 1953 (Gott and Monod, 1953) and describes those substances that are distinct from the enzyme itself, are not substrates and that can act as either enzyme inhibitors or activators. Enzyme modifiers that increase the reaction velocity when included in an enzymatic reaction medium, are known as “enzyme activators” (Biochemistry: Glossary of the International Union of Biochemistry (IUB), 1983; Segel, 1975). For the purposes of this review, an activator (A) acts by favouring the productive interaction between enzyme and substrate to form the enzyme-substrate complex (ES) by causing an enzyme-substrate-activator complex, (ESA), which presents an increased reaction velocity and in this case the activator is deemed to act as a “positive modifier”. If the reaction only occurs in the presence of the activator it is called “essential”, and if the reaction occurs in the absence of the activator it is called “non-essential” (Fornes et al., 2000; Segel, 1975). Using this nomenclature, the glucose stimulation of β -glucosidases is an example of non-essential enzyme activation.

The first approach to proposing a kinetic model and a mechanism for a glucose-stimulated β -glucosidase was reported in 2013 (Gómez et al., 2013) and was based on the general scheme for non-essential enzyme activation previously proposed by Segel (1975). Based on kinetic studies with the purified native GH1 β -glucosidase from *H. insolens* (HglB), a model for the activation of the enzyme by either glucose or xylitol was proposed and was used to analyse the experimental results. On formation of the binary ES complex in the absence of glucose, the enzyme displayed non-Michaelis kinetics (GH1 coefficient $n = 1$) with apparent values of V_{max} and K_m that were designated as V_1 and K_{m1} , respectively. In the presence of saturating concentrations of the glucose activator, the ternary ESA complex was favoured and the enzyme continued to present non-Michaelis kinetics, but with altered apparent values of V_{max} and K_m that were designated as V_2 and K_{m2} , respectively. At any intermediate concentration of glucose, the enzyme displayed apparent values of V_{max} between V_1 and V_2 (Fornes et al., 2000). The non-Michaelis kinetics of HglB indicated an allosteric

regulation of the enzyme with an apparent positive cooperativity ($n > 1$) in the presence of glucose and suggests that the enzyme has a regulatory binding site for glucose that is distinct from the active site (Fornes et al., 2000).

In general terms, enzyme activation may occur by increasing the affinity of substrate binding (K_m), and this may be numerically described by the factor α (resulting in the value αK_m), where values of $\alpha < 1$ are observed in cases of enzyme activation. Alternatively, the value of the catalytic constant (k_c) may also be modulated and can be described by the factor β (resulting in the value βk_c), where values of $\beta > 1$ are observed for enzyme activation (Salvo, 2009). Mixed-type systems, where $\alpha > 1$ and $\beta > 1$, are also possible (Segel, 1975) and in the presence of glucose the HglB presents a value of $\alpha = 2.13$ and of $\beta = 2.25$. Since the enzyme has $\alpha > 1$ and $\beta > 1$ (Gómez et al., 2013), glucose in this case is classified as an activator at high substrate concentrations. The effect of glucose on the enzyme activity was confirmed experimentally where at low concentrations, V_2 is lower than V_1 , confirming that glucose acts as an inhibitor. At increased glucose concentration and the same substrate concentration, V_2 is greater than V_1 , demonstrating that under these conditions glucose acts as an activator (see Fig. 2) (Meleiro et al., 2017). The concentration of substrate where the V_2 is equal to V_1 is denominated as S_0 , which is the substrate concentration where the reaction rate is unaffected by the modifier (Fornes et al., 2000). Therefore, when $[S] < S_0$, the glucose acts as an inhibitor and under conditions where $[S] > S_0$, glucose acts as an activator. When $[S] = S_0$, glucose has no effect in the reaction rate. Therefore, glucose acts as a nonessential activator for the HglB and depending on the substrate concentration ($[S] = S_0$) can act as an inhibitor or an activator.

4. Three-dimensional structure of the glucose-stimulated β -glucosidases

Comparative analysis of the 3D-structures of GH1 β -glucosidases reveal the α -[β]-TBM barrel architecture that is typical of this enzyme family (Barnett et al., 1993). The -1 glycosyl binding region and active site residues lie at the bottom of a deep surface pocket and using the HglB as a model structure (4H0C), this region includes residues Q17, H120, W121, N140, E166 (the catalytic acid/base), Y308, E377 (the catalytic nucleophile), W434, W435 and P443. These residues are structurally conserved in other bacterial and fungal GH1 β -glucosidases

Fig. 2. The effect of enzyme modifier concentration on the catalytic activity (v) as a function of substrate concentration ($[S]$). The blue line represents the activity in the absence of modifier ($[M] = 0$), the yellow line the activity at low concentration of modifier ($[M] = 0.1$) and the green line at high modifier concentration ($[M] = 0.5$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and participate in hydrogen bonding interactions between polar and charged amino acid side chains with equatorial hydroxyl groups of the glycosyl group, and in hydrophobic stacking interactions with the pyranose ring. Immediately adjacent to the glycosyl site, the $+1$ and $+2$ aglycone sites are characterized by a broadening of the pocket to form an antechamber that is continuous with the protein surface and active site, and previous x-ray diffraction studies have identified ligands bound in this region in several GH1 β -glucosidases (De Giuseppe et al., 2014; Fornes et al., 2007).

The topology of the $+1$ and $+2$ aglycone binding sites of the GH1 β -glucosidases is more variable, and with the aim of understanding the structural basis of glucose tolerance this region in the glucose inhibited enzymes from *Paenibacillus polymyxa* (D39P), *Hypocrea jeikei* (J34N9), and *Hypocrea diut* (D4N9), has been compared with the glucose stimulated enzymes from a metagenomic sample (BWH5), from *Neococcus kushanensis* (GN10) and the HglB. It has been previously suggested that the bulky side chains W168 and L173 in the HglB cause a narrowing of the $+2$ aglycone site, restricting access of saccharides to the active site (De Giuseppe et al., 2014; Fornes et al., 2016). Although the analogous positions to W168 and L173 in the HglB are not conserved in the other glucose stimulated enzymes, comparison of the protein surface topology of the $+1$ and $+2$ aglycone sites reveals that the narrowing observed in the HglB is also observed in the glucose tolerant Td2F2. In contrast, the HglB, TrglB2 and HglB structures present a broader cleft in this region, and all three enzymes are inhibited by glucose in the millimolar concentration range. The L167 and F172 residues in TrglB2 are structurally analogous to positions 168 and 173 in the HglB, and the site directed double mutant L167W/F172L in the TrglB2 results in a glucose tolerant and glucose stimulated enzyme (Gao et al., 2014). Although these observations support the suggestion that a narrowing of the antechamber in the $+2$ aglycone region may result in glucose tolerance, it is noteworthy that the glucose tolerant Td2F2 presents a broad cleft in the $+1/+2$ aglycone binding region. This may indicate that glucose affinity to the $+1/+2$ aglycone binding site may be important, rather than a simple occlusion, and the results of further studies are needed to further understand the details of the molecular mechanisms of glucose tolerance.

A detailed comparison of 3D structures has led to the compilation of an online public database (<http://bioinformatics.bioinformatics.org/>) with twenty-one GH1 and two GH3 3D structures of the glucose-stimulated β -glucosidases (Molina et al., 2017). The authors proposed that the number of contacts of each residue made in the substrate inside the catalytic pocket might be a metric and suggest mutations in other non-tolerant/stimulated β -glucosidases. Taken together, these data will facilitate comparisons when new β -glucosidase structures become available.

5. Transglycosylation activity in glucose stimulated β -glucosidases

Variable transglycosylation products of HglB have been demonstrated by TLC and mass spectrometry (Meleiro et al., 2017), and it was proposed that accommodation of glucose in various orientations at the $+1$ aglycone allows the formation of β -1,2, β -1,3, or β -1,4 glycosidic bonds between the acceptor glucose and glycosyl-enzyme intermediate (De Giuseppe et al., 2014). In addition, the formation of a glycosyl-xylose transglycosylation product has been demonstrated, in which the acceptor was xylitol (Meleiro et al., 2017). Therefore, the model proposed for HglB stimulation was revised and it was proposed that enzyme stimulation occurs via glucose binding to a subsite on the protein resulting in the modification of activity to favour transglycosylation rather than hydrolysis (Yang et al., 2015a). This mechanism is compatible with the glucose stimulated enzymes that have previously been described (Gao et al., 2013; Villalba et al., 2011; Uchiyama et al., 2013), and has been experimentally evaluated using the GH1 β -glucosidase Td2F2 isolated from a metagenomic library (Uchiyama et al., 2013).

The wild-type Td2F2 presents a pronounced transglycosylation activity in the presence of high glucose concentrations to form laminaribiose and sophorose. The amino acid Asn223 is located between subsites $+1$ and $+2$ (Matsuoka et al., 2016) and substitutions at this position result in variable proportions of the major transglycosylation products sophorose (β -1,2-glucobiose), laminaribiose (β -1,3-glucobiose) and gentiobiose (β -1,6-glucobiose). Furthermore, analysis of the pNF⁺ and glucose release by HglB variants showed that the mutants H307Y, D257Y and N223S modulate the hydrolysis to transglycosylation ratio in the absence of the activator xylitol. These three mutations are clustered close the $+1/+2$ glucose binding site, and it was proposed that the topology of the aglycone binding site determines the transglycosylation activity and level of stimulation by monosaccharide activators in the HglB (Meleiro et al., 2017).

Glucose-stimulated enzymes that do not present transglycosylation activity have also been characterised (Liu et al., 2017; Uchiyama et al., 2015). In addition, several GH1 and GH3 β -glucosidases have been reported that show transglycosylation of cellobiose (Dhandapani et al., 2017; Chantakaprasit et al., 1994; Katsukawa et al., 2009; Malik-Fakhreddin and Bright, 2016; Pérez-Pérez et al., 1994; Yan et al., 1998), pNP-Glc (Seidle et al., 2004) and 4-methylumbelliferyl glucoside (MUG) (Zou et al., 2001), and which are inhibited by low glucose concentrations. This suggests that generalization of the mechanism of tolerance and stimulation through transglycosylation might be an oversimplification, and recently these diverse effects have been explained by an elegant proposal involving the overlapping binding of substrates and inhibitors in the active site cleft (Kusák and Viljanen, 2017). Since GH1 enzymes tend to have less flexible structures that show only local active-site movements on substrate binding, the traditional structural explanation for allosteric effects involving significant structural changes appears to be unlikely (Fornes et al., 2013).

The mechanism of O-glycosidic bond cleavage in β -glucosidases occurs in two steps: glycosylation (formation of glycosyl-enzyme intermediate) and deglycosylation (disruption of this intermediate) (Molina, 2008; Matsuoka et al., 2016; Meleiro et al., 2017; Tschel and Withers, 2000). It is noteworthy that the overall k_c of this double-displacement mechanism includes not only the rate of formation of the glycosyl-enzyme intermediate and liberation of the first product (the glucose or aglycone moiety), but also the rate of cleavage of the glycosyl-enzyme intermediate and second product release (either glucose or the product of transglycosylation). It has been suggested that activation of β -glucosidase by glucose is by the regulation of the rate-limiting steps in the catalytic cycle (Viviani and Viljanen, 2017). For example, if the rate-limiting step is formation of the glycosyl-enzyme intermediate, enzyme activation could be explained by the binding of glucose to other subsites (for example $+2$) thereby impeding non-productive substrate binding and orientating the substrate to bind in a productive manner in the -1 or $+1$ subsites. In the case of deglycosylation as the rate-limiting step, enzyme activation could be explained by the preferred use of an acceptor other than water to cleave the glycosyl-enzyme intermediate, resulting in transglycosylation. It is noteworthy that both these effects may be involved in enzyme activation when the glycosylation and deglycosylation steps make significant contributions to the reaction rates (Kusák and Viljanen, 2017). Although many studies conclude with speculation as to the roles of substrate binding and transglycosylation (Gao et al., 2013; Fornes et al., 2016; Fang et al., 2016, 2010; Gao et al., 2016; Meleiro et al., 2015; Pérez-Pérez et al., 1999; Sathre et al., 2017; Yang et al., 2015b; Zhang et al., 2017b; Zhao et al., 2017), further detailed kinetic studies of the type recently reported are necessary to understand the mechanism of enzyme stimulation by the reaction product (Matsuoka et al., 2016; Meleiro et al., 2017).

A recent comparison of 21 glucose tolerant enzymes led to the proposal that the amino acids responsible for regulating the glucose tolerance are located in the walls of the deep pocket that leads to active site (Molina et al., 2017). The enzymes present a highly conserved

Fig. 9. Current view on the enzymatic degradation of cellulose. GH1 and LPMOs cleave internal cellulose chains releasing chain ends that are targeted by GH2. LPMO cleave cellulose chains by using an oxidative mechanism at the glucose active site and may generate partially amorphous regions with oxidized chain ends that become points of attachment for hydrolytic enzymes such as GH1 and GH2. GH1 generates cellobiose in oxidized cellulose that are subsequently hydrolyzed by GH2, releasing glucose or oxidized glucose. GH2 or monooxygenase enzymes, such as ascorbic acid, gallic acid and reduced glutathione, provide electrons to LPMO. GH2 act by breaking the hydrogen bonds between cellulose microfibrils and other polysaccharides without hydrolytic action. BGL, β -glucosidase, GH1 cellobiohydrolase, GH2 cellobiohydrolase, GH3 cellobiohydrolase, GH4 cellobiohydrolase, GH5 cellobiohydrolase, GH6 cellobiohydrolase, GH7 cellobiohydrolase, GH8 cellobiohydrolase, GH9 cellobiohydrolase, GH10 cellobiohydrolase, GH11 cellobiohydrolase, GH12 cellobiohydrolase, GH13 cellobiohydrolase, GH14 cellobiohydrolase, GH15 cellobiohydrolase, GH16 cellobiohydrolase, GH17 cellobiohydrolase, GH18 cellobiohydrolase, GH19 cellobiohydrolase, GH20 cellobiohydrolase, GH21 cellobiohydrolase, GH22 cellobiohydrolase, GH23 cellobiohydrolase, GH24 cellobiohydrolase, GH25 cellobiohydrolase, GH26 cellobiohydrolase, GH27 cellobiohydrolase, GH28 cellobiohydrolase, GH29 cellobiohydrolase, GH30 cellobiohydrolase, GH31 cellobiohydrolase, GH32 cellobiohydrolase, GH33 cellobiohydrolase, GH34 cellobiohydrolase, GH35 cellobiohydrolase, GH36 cellobiohydrolase, GH37 cellobiohydrolase, GH38 cellobiohydrolase, GH39 cellobiohydrolase, GH40 cellobiohydrolase, GH41 cellobiohydrolase, GH42 cellobiohydrolase, GH43 cellobiohydrolase, GH44 cellobiohydrolase, GH45 cellobiohydrolase, GH46 cellobiohydrolase, GH47 cellobiohydrolase, GH48 cellobiohydrolase, GH49 cellobiohydrolase, GH50 cellobiohydrolase, GH51 cellobiohydrolase, GH52 cellobiohydrolase, GH53 cellobiohydrolase, GH54 cellobiohydrolase, GH55 cellobiohydrolase, GH56 cellobiohydrolase, GH57 cellobiohydrolase, GH58 cellobiohydrolase, GH59 cellobiohydrolase, GH60 cellobiohydrolase, GH61 cellobiohydrolase, GH62 cellobiohydrolase, GH63 cellobiohydrolase, GH64 cellobiohydrolase, GH65 cellobiohydrolase, GH66 cellobiohydrolase, GH67 cellobiohydrolase, GH68 cellobiohydrolase, GH69 cellobiohydrolase, GH70 cellobiohydrolase, GH71 cellobiohydrolase, GH72 cellobiohydrolase, GH73 cellobiohydrolase, GH74 cellobiohydrolase, GH75 cellobiohydrolase, GH76 cellobiohydrolase, GH77 cellobiohydrolase, GH78 cellobiohydrolase, GH79 cellobiohydrolase, GH80 cellobiohydrolase, GH81 cellobiohydrolase, GH82 cellobiohydrolase, GH83 cellobiohydrolase, GH84 cellobiohydrolase, GH85 cellobiohydrolase, GH86 cellobiohydrolase, GH87 cellobiohydrolase, GH88 cellobiohydrolase, GH89 cellobiohydrolase, GH90 cellobiohydrolase, GH91 cellobiohydrolase, GH92 cellobiohydrolase, GH93 cellobiohydrolase, GH94 cellobiohydrolase, GH95 cellobiohydrolase, GH96 cellobiohydrolase, GH97 cellobiohydrolase, GH98 cellobiohydrolase, GH99 cellobiohydrolase, GH100 cellobiohydrolase.

consensus sequence of 22 amino acids (GHNSENWCLHSEANYYTNER-EWF) in the cellulose binding region, and due to the high degree of amino acid conservation this region in the GH1 β -glucosidase from *Thermoanaerobacter brockii* was used as the consensus sequence (A complete alignment of the glucose tolerant β -glucosidases and glucose stimulated β -glucosidases with deposited database sequence was provided in Supplementary material). Using computer-aided docking methods, the highly conserved residues W122, N166, E167, C170, L174, Y299, W402, E490, and W410 were identified as forming contacts with the ligand cellulose. The occurrence of C170 in the consensus sequence appears to be correlated with the presence of W169 and L174 at the entrance to the active site in the tolerant enzymes, and the substrate docking studies suggested that these residues made fewer contacts with glucose (Mariano et al., 2017). In *R. ruber* BglII the residues W169 and L173 (homologous to the W169 and L174 in *T. brockii*) may explain the tolerance of the enzyme to high glucose concentrations (De Giuseppe et al., 2014). Furthermore, the double mutant L167W/P172L in the GH1 β -glucosidase BGLI/CelIIA from *Trichoderma reesei* transformed the enzyme into a highly glucose tolerant and glucose stimulated enzyme (Gao et al., 2016). It is noteworthy that this enzyme is known to exhibit transglucosylation activity (d-Gugary et al., 1989; Kufner, 1987), however the increased activity of the double mutant in the presence of glucose was not associated with an altered pattern transglucosylation products. It is noteworthy that many of GH1 β -glucosidases present transglucosylation at glucose concentrations that are approximately 3 orders of magnitude less than the concentration of water. Whereas the water concentration is 55.5 M, glycosylation is observed at glucose concentrations of about 50–100 mM (Supplementary material) (Matsushima and Yoda, 2016; Melero et al., 2017; Soria et al., 2015, 2010), and these enzymes apparently can exclude water from the active site preferring to use a saccharide as an acceptor.

6. Applications

Due to the diversity of their substrates and their ability to both hydrolyze and synthesize glycosidic bonds, β -glucosidases have found a very broad range of applications (Bhatia et al., 2002). Industrial applications include aroma improvement of juices and wine, hydrolysis of soybean isoflavone glycosides, toxicity reduction of animal feed, production of anti-cancer compounds, and cellulose degradation for lipase/acidolysis. Human conversion in biolact production (Bhatia et al., 2002; Costa et al., 2015; Marcano et al., 2017; Raghavakumar et al., 2017). The availability of glucose-tolerant and/or glucose-stimulated β -glucosidases creates opportunities for additional and novel applications in

many of the processes cited above. For example, the hydrolysis of soybean isoflavone glycosides results in the accumulation of glucose, which hinders efficient conversion of the substrate. In this case, a β -glucosidase from a soil metagenomic library (BglI209) with a $K_i = 4.28$ M for glucose demonstrated improved hydrolysis of soybean isoflavone glycosides, and 0.6 units of enzyme could completely convert both daidzein and genistein (0.5 mg mL⁻¹) to daidzein and genistein, respectively, at 40 °C in 30 min (Guo et al., 2013). In another example, the glucose-stimulated variant A28S/T297Y obtained by semi-rational engineering of the β -glucosidase from a marine microbial metagenomic library (BglIIA) achieved faster conversion from isoflavone glycosides to aglycones (10 min reaction at 25 °C) and hydrolysis yields of 99% for daidzein and 98% for genistein (Fang et al., 2016).

The application of glucose tolerant and/or glucose stimulated β -glucosidase to release aromatic compounds from flavonoid glycoside precursors in wine processing is a promising approach. In this case the enzymes should be stable at low pH (pH between 2.5 and 3.8), maintain activity at a 10–20% glucose concentration and retain activity in the presence of 10–15% ethanol. The glucose-tolerant enzyme from *Aperghia arylae* released flavor related compounds, such as geraniol, terpenol, and linalol, from a grape must at pH 2.9 and a glucose titer of 90 g L⁻¹ (Gao et al., 1990). Linalol is one of the main volatile terpenes that confer a fresh and fruity aroma to wines, and enzymatic treatment of white wine (variety Niagara) with a crude extract from *Sporobolus parvus* containing a glucose-tolerant β -glucosidase showed an increase in linalol levels from 1.0 to 22.1 mg mL⁻¹ after 12 h at 18 °C (Bohl et al., 2011). The purified β -glucosidase from *S. parvus* showed a reduced hydrolysis of aroma precursors in comparison with the crude extract, not only suggesting additional factors are important for observed effect, but also circumventing the need for expensive protein purification procedures (Bohl et al., 2011).

As increase in global energy demand together with depletion of fossil fuels and concerns over climate change has led to intensive studies of the use of glucose-tolerant and/or stimulated β -glucosidases for the production of biofuels. In particular, ethanol produced by fermentation of the reducing sugars resulting from enzymatic decomposition of lignocellulose (so-called second generation, or 2G ethanol) has received considerable attention due to the abundance and low cost of agro-industrial byproducts and residues. Product inhibition of cellulases has long been recognized as a major factor limiting the efficiency of cellulose hydrolysis (Fig. 3), and the concentrations of glucose and xylose in enzymatic hydrolyses of lignocellulose at high solids concentrations may reach 600–1000 mM and 300–400 mM, respectively (Bhatia et al., 2013). The use of glucose-tolerant and/or stimulated

enzymes can substantially increase the yield of the process, and the efficiency of using glucose-tolerant enzymes in the hydrolysis of cellulose in the presence of 6% w/w glucose has been demonstrated (Sato and Bhatia, 1996). Furthermore, supplementation of an enzymatic cocktail for the hydrolysis of sugarcane bagasse with the glucose-stimulated β -glucosidase from *Syntherisma thermophilum* increased the release of reducing sugar by approximately 50% (García et al., 2010). In addition, the glucose-tolerant β -glucosidase from *Neutrophil* *halophilus* was more effective than a commercial β -glucosidase preparation from *Aspergillus niger* for releasing reducing sugars from Avicel® when mixed with a commercial enzyme cocktail (Chahine et al., 2012). It has also been demonstrated that the application of glucose-tolerant β -glucosidases enhanced the methanification efficiency of steam-exploded bagasse, increasing the total sugar yield by 20% in 52 h (Rajasekhar et al., 2013). Similar results were obtained with the β -glucosidase from *Thermoanaerobacterium saarnense* (Yang et al., 2015a). Supplementation of crude cellulases from *T. reesei* PC3-7 with the glucose-stimulated β -glucosidase identified in a *Xanadu* grass metagenome resulted in a 2-fold increase of release of glucose from cellulose-treated rice straw as compared to the crude cellulases alone, without the accumulation of cellobiose (Eichengrün et al., 2013). Similar results for rice straw hydrolysis were obtained with the saccharose-stimulated β -glucosidase from a soil metagenomic library (Matsushima and Yoda, 2016). Supplementation of a commercial enzyme cocktail with the glucose-stimulated β -glucosidase from *Bacillus subtilis* B410 resulted in a 1.34-fold increase in glucose release from pretreated paddy straw (Tiwari et al., 2017). Finally, supplementation of a commercial cocktail with the glucose-stimulated β -glucosidase from *Anoxybacillus flavithermus* subsp. *parvus* E13 (BglI) enhanced the hydrolysis of sugarcane bagasse by 3.4- and 3.0-fold in presence of initial concentrations of 500 mM glucose and 200 mM xylose, respectively (Liu et al., 2017). Although not covered in this review, xylose tolerant and/or stimulated β -glucosidases are also of interest in the application of lignocellulose hydrolysis, since the current consensus is that the economic viability of lignocellulose ethanol production depends on the efficient, low-cost hydrolysis of both cellulose and hemicelluloses with subsequent fermentation of the released hexoses and pentoses (Koppram et al., 2013; Melero et al., 2015). In this respect, it is noteworthy that several of the enzymes discussed here are also stimulated by xylose (Costa et al., 2015; Gargioli et al., 2016; Liu et al., 2017; Li et al., 2015; Melero et al., 2017, 2015; Nakamura et al., 2010; Soria et al., 2013, 2010; Zambelli et al., 2004; Zhao et al., 2012).

7. Origin and evolution of glucose stimulated β -glucosidases

As of the moment of preparation of this review, the vast majority of glucose activated enzymes are β -glucosidases, although the glucose activation of a GH13 type II α -glucosidase has been reported (Li et al., 2016). This characteristic appears to be a feature of the β -glucosidase class of enzymes and raises the question as to what is the advantage for an organism to produce enzymes that are activated by their own reaction product? From a broader perspective, it should be appreciated that the biosphere is generally oligotrophic (nutrient poor) and it has been proposed that the natural physiological state of microorganisms in response to the oligotrophic state is a starvation-survival lifestyle (Morris, 1997, 1998). Microorganisms adapt to this lifestyle by either reduced activity, arrested activity, or by entering a dormant state. However, even in the low activity state microorganisms maintain the biochemical capacity for a rapid response to exogenous changes in substrate levels (Hobbs and Holmbe, 2012; White, 2010). The concentrations of sugars in the biosphere tend to be very low with reports of 54 μ M glucose in agricultural soils from North Wales and 45 mM glucose in temperate forest plantation soil (Gill et al., 2008; Johnson and Peggins, 2007). Microbial uptake is by far the dominating process determining the destiny of sugars in soils (García and Rodríguez, 2015), and the uptake of monosaccharides from soils by

microorganisms varies from seconds to minutes (Hobbs and Holmbe, 2012; Jaron and Murphy, 2007). In planktonic aquatic systems, the concentration of individual sugars are typically <5 mM, although glucose concentrations of 2–15 mM were observed in surface waters in the Gulf of Mexico (Ritchman, 2000; Sling et al., 1999), and concentrations in the range of 20–60 mM have been reported in the equatorial Pacific Ocean (Bohl et al., 1996). As a consequence of the low availability of free glucose in the biosphere, organisms producing glucose tolerant or stimulated enzymes could gain an evolutionary advantage. For example, in the presence of a source of polysaccharides, the metabolic capability to rapidly hydrolyze this substrate and use the glucose for energy and respiration would be advantageous. Under these circumstances, the local concentration of glucose where the enzyme is located would rise rapidly and inhibit the growth of organisms containing enzymes that are not glucose tolerant. Furthermore, although the hydrolysis of cellulose by β -glucosidase remains a limiting step in cellulose degradation, the glucose tolerant or stimulated enzymes would still be active under saturated substrate conditions and at high glucose concentrations.

An analysis of 5123 annotated bacterial genomes revealed that only 24% contain genes for both cellulases and β -glucosidases, and are classified as potential organisms that degrade cellulose, whereas 56% contain genes encoding β -glucosidases but no cellulases and are classified as potential opportunistic strains (Dierckmann and Murphy, 2013). The transglucosylation activity of β -glucosidases could represent an additional metabolic adaptation for the rapid synthesis of oligosaccharides, thereby depriving monosaccharides from potential opportunistic strains in the vicinity and reducing competition from other microorganisms. Furthermore, products of transglucosylation reactions can be used by fungi as signaling molecules in the expansion of other enzymes. For example, the disaccharide sophorose is considered to be a strong inducer of cellulase expression in *T. reesei* and is synthesized during cellulose hydrolysis in a transglucosylation reaction by BGLI/CelIIA, an extracellular β -glucosidase inhibited by low concentrations of glucose (García et al., 2014; d-Gugary et al., 1989; Kufner, 1987).

The first reported GH1 glucose-stimulated β -glucosidase from the mesophilic fungus *Neurospora crassa* (GH1-1, NCU901C10) (Melero et al., 2015), is an intracellular enzyme induced by Avicel® and involved with the cellulase induction (Gargioli et al., 2016; Wu et al., 2012). When the wild type *N. crassa* was grown in Avicel®, the expression levels of cellulases were increased but with cellulase lower cellulase levels were observed (Zamora et al., 2012). In contrast, after the triple deletion of the β -glucosidases (ΔBGLI of GH1-1 (ΔNCU901C10), GH3-3 (ΔNCU08755) and GH3-4 (ΔNCU94952) *N. crassa* showed an increase in the expression of cellulases when grown with cellulose. It was proposed that this triple deletion enabled induction of transcription and the eventual secretion of the complete repertoire of cellulases when grown in the presence of cellulose. The level of cellulase expression in ΔBGLI on cellulose were similar to that observed when the wild type strain was grown in Avicel®. Moreover, the authors discovered that *N. crassa* cellulases were not induced by sophorose and propose that cellulose, or a modified version of cellulose is the major cellulase inducer in *N. crassa* (Zamora et al., 2012). The major function of GH1-1 appears to be to cleave the intracellular cellulose to release glucose in the fungal cell (Zamora et al., 2012). The GH1-1 shares 73.6% amino acid sequence identity with BGLI/CelIIA from *T. reesei*, which is also intracellular, and transglucosylation activity was observed in both enzymes (Liu et al., 2011; Salas et al., 2002; Wu et al., 2012). As previously noted, the BGLI/CelIIA shares 73% amino acid sequence identity with BglII from *N. insolens* and was transformed into a highly glucose tolerant-stimulated β -glucosidase by the introduction of just two mutants, L167W and P172L (Gao et al., 2016).

The regulation of a biochemical pathway such as cellulose degradation, usually involves sophisticated and complex mechanisms (Puri, 2012). Regulatory mechanisms include alteration in the amount

of enzyme present by induction/repression of gene expression, reversible inhibition by substrate and/or products, protein post-translational modifications, allosteric activation or inhibition and proteolytic activation of a zymogen (Corrêa *et al.*, 2012). During the course of evolutionary processes diverse molecular mechanisms and driving forces have been active at different times and have contributed to the content and regulation of the genomes in current organisms (Pawl, 2013). The appearance of new metabolic abilities is the consequence of gene duplications and divergence (Pawl, 2012; Martinez *et al.*, 2015). Moreover, new catalytic characteristics can be derived from the pre-existent catalytic activity of enzymes, whose variability in the catalytic efficiency of an enzyme against related substrates that can be modulated by few mutations, allowing selective pressure to diversify the catalytic repertoire encoded in a given genome (Martinez *et al.*, 2015). In the specific case of cellulose degradation, it has been proposed that at least two complementary enzyme systems have evolved to completely degrade cellulose (Tan *et al.*, 2016). Comparison of the cellulase and xylanase families shows that the conserved amino acids in the active sites are mainly located in the $-2/+1$ subsites (Yoon *et al.*, 2015). In contrast, the conserved amino acids in the active sites of β -glucosidases are concentrated at the -1 subsite. It has been noted that from an engineering perspective it would be difficult to transfer a cellulase into a β -glucosidase, because too few positions are common to both enzymes (Tan *et al.*, 2016). Further studies focusing on the comparative analysis of genes, genomes and the structure of enzymes among Archaea, Bacteria, and Eukaryotes could shed a light on the origin and evolution of these enzymes.

8. Future perspectives

The β -glucosidases are versatile enzymes that capable of both hydrolysis and transglycosylation, and it may be expected that the number and scope of the applications of these enzymes will increase. Their successful application depends on biochemical characterization of specific enzymes and in this review has been focused on kinetic and structural aspects of glucose tolerant and / or stimulated enzymes isolated from fungi, bacteria and metagenomic libraries. The traditional approach of classic biochemistry for identification of novel β -glucosidases should continue to provide insight as to these enzymes, as well as provide a source of new *Caly*-enzymes stimulated by reaction products. However, the trend toward isolation of β -glucosidases from metagenomic sources is likely to grow. Advances in the structural characterization of the β -glucosidases together with bioinformatics techniques such as substrate docking and a range of mutagenic studies is opening possibilities for enzyme engineering. Studies of key amino acids have been crucial for the understanding the structural basis for enzyme glucose tolerance/stimulation (Gao *et al.*, 2015; Gao *et al.*, 2016; Martinez *et al.*, 2016; Veng *et al.*, 2015a). These methods have already yielded novel insights as to the structure/function relationship of these enzymes and will contribute to future progress in the understanding of the regulation and catalytic properties of the β -glucosidases. As one of the regulatory mechanisms for glucose tolerance and stimulation are elucidated, it should become more feasible to engineer more robust enzymes that are suitable for a wide range of applications. In addition to the possibilities for enzyme engineering, genetic modification of bacteria and fungal host cells for the production of β -glucosidases is an emerging trend (Singhania *et al.*, 2017), and further progress in systems biology can be expected to facilitate engineering of microbial strains for the production of β -glucosidases for specific functions. Finally, an understanding of the evolution of these enzymes is also important because such studies can lead to an understanding of the advantage of an organism to incorporate glucose stimulated enzymes, and takes the study of glucose tolerant and/or stimulated β -glucosidases beyond the phenomenon itself. It must always be remembered that nothing in biology makes sense except in the light of evolution (Dobzhansky, 1973).

9. Conclusions

It has been present a comprehensive review of a novel class of β -glucosidases with the intriguing characteristic of being tolerant and/or stimulated by glucose. Over the last decades there has been an increase in research effort to characterize β -glucosidases, culminating in recent reports of enzymes with kinetic properties compatible with the accumulation of high glucose concentrations. Due to the ongoing interest in these enzymes, here it has been summarized this accumulated body of knowledge which provides valuable insights as to the kinetics, structure and regulation of glucose tolerant and glucose stimulated β -glucosidases.

Competing interests

The authors declare no conflict of interest.

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Apêndice F

Use of Algae Biomass Obtained by Single-Step Mild Acid Hydrolysis in Hydrogen Production by the β -Glucosidase-Producing *Clostridium beijerinckii* Br21

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Abstract

Macroalgae biomass is a potential feedstock for fermentative H_2 production: it has high carbohydrate concentration and is lignin-free. Here, we optimize a mild acid treatment of *Kappaphycus alvarezii* biomass by an experimental design 2^4 . The optimal acid treatment conditions were 90 °C, HCl concentration of 55.9 mmol/L, 0.375 g of algae mass, and 8 h of treatment. Under these conditions, the hydrolysate presented mono-, but also di- and oligosaccharides. We used this hydrolysate as substrate for fermentative hydrogen (H_2) production by the *Clostridium beijerinckii* Br21 grown in two different conditions: (1) in medium containing galactose and no β -glucosidase activity; (2) in medium with cellobiose to stimulate β -glucosidase activity, which was 0.33 U/mL. The fermentative assay conducted in the presence of the *C. beijerinckii* Br21 with β -glucosidase activity provided higher H_2 concentration and yield as compared to the assay accomplished in the presence of the inoculum with no β -glucosidase activity—171.76 $\pm$ 2.10 and 140.95 $\pm$ 10.92 mL of H_2 /L, and 70.3 $\pm$ 0.9 and 62.7 $\pm$ 4.9 mL of H_2 /g of dry algae, respectively. Therefore, a fermentative β -glucosidase-producing organism such as *C. beijerinckii* Br21 can potentially complete biomass saccharification for later renewable H_2 production.

Keywords *Kappaphycus alvarezii* · Acid treatment · Factorial design · Biohydrogen

Statement of Novelty

Kappaphycus alvarezii biomass has never been explored as feedstock for H_2 production. A biomass hydrolysate containing mostly di- and oligosaccharides and only small monosaccharide concentrations was used as substrate for fermentative H_2 production by *Clostridium beijerinckii* Br21. The fermentation inoculum grown in cellobiose presented β -glucosidase activity of a *C. beijerinckii* strain, which has been described for the first time in this work.

β -glucosidase activity stimulation in the inoculum helped *C. beijerinckii* Br21 to produce higher H_2 concentration per unit of algae biomass as compared to the inoculum grown in galactose with no β -glucosidase activity. Biomass hydrolysis to the monosaccharide level was not necessary because the H_2 -producing microorganism can produce hydrolases that help to consume higher-molecular-weight saccharides.

Introduction

Hydrogen (H_2) is a clean fuel; its burning produces water only [1–3]. However, current strategies to produce H_2 demand high amounts of energy or rely on fossil fuel-based methods [4]. H_2 can also originate from renewable fermentation of carbohydrate-rich materials, such as wastewater, wastes, and biomasses, which is a more sustainable and economically viable process [3]. Various renewable biomasses can be used as low-cost substrate for fermentative H_2 production, which significantly reduces production costs [5, 6].

In the last years, algae biomass has become a promising feedstock to produce biofuels, including biohydrogen, the so-called third-generation H_2 [3–10]. As a substrate for fermentative biofuel production, algae biomass offers many advantages: it sequesters high CO_2 amounts, grows at high rate, and does not require arable land. In addition, algae biomass lacks hemicellulose and lignin, which facilitates access to its carbohydrates [5, 8]. Consequently, algae biomass pretreatment to convert polymeric carbohydrates into monomeric sugars for further fermentation calls for milder conditions as compared to lignocellulose pretreatment [5–8].

According to the literature, brown macroalgae like *Laminaria japonica* [7] and red microalgae like *Gelidium acerosum* [9] have high carbohydrate content and therefore display the greatest potential for application as feedstock for biohydrogen production [10]. The red macroalgae *Kappaphycus alvarezii* also has high carbohydrate content (ca. 50% d.w) and has already been employed as substrate for bioethanol production [11]. Nevertheless, there are no studies on the use of this macroalgae as substrate for fermentative H_2 production. *K. alvarezii* biomass contains mainly heterogeneous polysaccharides known as carrageenans, which consist mostly of α -galactose and 3,6-anhydro- α -galactose (AHG) with different degrees of sulfate groups, linked by β -1,4 and α -1,3 bonds [10, 12].

Although algae biomass does not contain lignin, it still has a complex structure that requires a pretreatment step necessary [6]. Additionally, fermentative bacteria usually have low hydrolytic activity, so pretreatment releases organic substances from the algal cells and makes them readily biodegradable. Several pretreatment technologies that have been developed to treat lignocellulosic biomasses, such as milling, heating, and ultrasound, acid, basic, ozone, and enzymatic treatments, can also be employed to treat algae biomass [6].

Because acid pretreatment is easy to accomplish, is inexpensive, and can be applied on an industrial scale, it has been the most commonly used technology to pretreat biomasses for fermentable sugar production from carrageenan-rich biomasses [13, 14]. The hydrolysis reaction conditions, including acid concentration, temperature, and reaction time, vary depending on the carrageenan type [10, 13]. The acid and temperature conditions employed during algae biomass hydrolysis may result not only in monosaccharides, but also in sugar degradation products. Indeed, dehydrated galactose, like AHG from carrageenan, is more susceptible to degradation as compared to the α -galactose hydrated form. AHG can be promptly converted into 5-hydroxymethylfurfural (HMF), a well-known fermentation inhibitor [10, 15]. Therefore, mild acid hydrolysis conditions is more suitable to saccharify algae biomass for further fermentation [9, 10].

Here, we optimize acid hydrolysis conditions for *K. alvarezii* biomass for later application of this biomass in fermentative H_2 production, and we employ *Clostridium beijerinckii*

Br21 (GenBank accession number KT626859), isolated in our laboratory, as H_2 -producing organism to ferment the algae acid hydrolysate. This strain can consume a wide scope of substrates and has a large number of genes encoding for glycosyl hydrolases [16]. Hence, we hypothesize that the *C. beijerinckii* Br21 enzymes can help to complete the hydrolysis of the mild acid pretreated algae biomass.

Materials and Methods

Macroalgae Biomass

The *K. alvarezii* macroalgae biomass was obtained from the Fisheries Institute, Ubatuba, São Paulo, Brazil (GPS coordinates 23°27'S, 45°02'W). This seaweed is cultivated in an open raft system. The macroalgae biomass was collected and dried under solar light before being sent to the lab. In the lab, the seaweed biomass was washed three times in distilled water, to remove salt and impurities, and dried in an oven with air re-circulation at 60 °C for 14 h. After drying, the seaweed was milled in a knife mill (Solab model SL 31, Brazil) and sieved at #35 mesh, to standardize it for the acid hydrolysis assays.

Seaweed Biomass Chemical Composition Analysis

The total solids, moisture, and ash contents in the macroalgae biomass were analyzed according to the methodology proposed by the National Laboratory of Renewable Energies—NREL, from the US Department of Energy [17]. The total carbohydrate content in the algae was determined according to the NREL methodology [18], modified by addition of concentrated H_2SO_4 to hydrolyze the biomass before the total sugars content was quantified. This methodology has also been described for analysis of the carbohydrate content in lignocellulosic materials [19]. The protein content was determined by the Lowry method modified by Hartree [20]. Sulfate was quantified by the methodology described by Hayashi [21]. First, the biomass was digested by addition of 0.1 mL of ethanol 92% and 1.0 mL of HCl 0.5 mol/L to 0.05 g of algae (dry mass basis), which was followed by autoclaving at 120 °C for 15 min. After that, a reaction with the gelatin reagent + BaCl₂ was performed, and the absorbance was read at 550 nm [21].

All the analytical quantifications were conducted at least in triplicate.

Surface Response Methodology (SRM) for Algae Biomass Acid Hydrolysis Optimization

Surface-response methodology (SRM) and multi-response analysis [22] helped to optimize the experimental conditions [temperature (X1), time (X2), algae mass (X3), and

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acid concentration (X_4) that led to the highest total reducing sugars (TRS) concentration possible. A 2^4 full factorial central composite design (star configuration) aided in this purpose (Table 2). The experimental intervals of the independent variables analyzed in the SMR experiments (Table 2) were chosen on the basis of literature [13, 14, 23] and of previous experiments performed in the laboratory with the one factor at a time (OFAT) methodology, which provided the highest TRS concentration and the lowest HMF concentration possible. The preliminary tests showed the optimum hydrolysis conditions were temperature between 50 and 90 °C, algae mass between 0.075 and 0.375 g, HCl concentration between 11.2 and 55.9 mmol/L, and treatment time between 7 and 11 h. The effect of temperature, time, algae mass, and acid concentration on TRS concentration was determined with the aid of STATISTICA® software 7.0. The data were fit to a second-order equation (Eq. 1) as a function of the independent variables at 90% confidence level, and an analysis of variance (ANOVA) was carried out to evaluate the significance of this mathematical model ($p < 0.05$).

$$Y_i = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{44}X_4^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{34}X_3X_4 + b_{123}X_1X_2X_3 + b_{124}X_1X_2X_4 + b_{134}X_1X_3X_4 + b_{234}X_2X_3X_4 + b_{1234}X_1X_2X_3X_4 \quad (1)$$

where b_0 corresponds to constant regression coefficients; Y_i refers to the dependent variables TRS; and X_1 , X_2 , X_3 , and X_4 are the coded independent variables temperature, HCl concentration, time, and algae mass, respectively.

After the surface-response results were obtained, the process conditions were optimized by using the software Excel® 2010, with the algorithm generalized reduced gradient (GRG).

Finally, hydrolysates prepared in the optimal conditions helped to validate the optimized conditions provided by response surface methodology (RSM). The validation experiments were accomplished in triplicate, and the resulting hydrolysates were characterized with respect to TRS. Comparison of the values predicted by the model with the actual values was made through the relative error generated by the complete model obtained by the coded values (Eq. 2).

$$\bar{\epsilon}_{\text{RSM}} = \left(\frac{\sum_{i=1}^n \epsilon_i}{n} \right) \times 100 \quad (2)$$

where $\bar{\epsilon}_{\text{RSM}}$ = relative error of the complete model; Y = experimental value; Y_{MC} = value predicted by the complete model.

Acid Hydrolysis Assays

All the hydrolysis assays were conducted in 30 mL of HCl solution in a 125-mL Erlenmeyer; the temperature was controlled with a water bath. The acid treatment conditions were tested according to a 2^4 full factorial central composite design (star configuration, Table 2). The acid treatment was interrupted by cooling the mixture in an ice bath, which was followed by filtration through Whatman® filter paper number 1 under vacuum. The residual solid algae retained in the filter paper was washed with distilled water, and the liquid was collected in the same volumetric flask (50 mL) as the hydrolysate, to ensure that all the solubilized sugars were removed from the residual algae and to standardize the final volume of the liquid hydrolysate for the chemical analyses (TRS, monosaccharides, and HMF). Before the flask volume reached 50 mL, pH was adjusted to neutral with sodium hydroxide 5 mol/L. The acid hydrolysis assays were carried out at least in duplicate. Hydrolysis under the optimal conditions was accomplished in triplicate.

Hydrolysate Chemical Analysis

The TRS concentration in the algae hydrolysate was determined by the 3,5-dinitrosalicylic acid (DNS) method described by Miller [24]. The length of the carbohydrate products arising after algae acid treatment optimization was analyzed by thin layer chromatography (TLC). Aliquots of the filtered samples were treated with 10% trichloroacetic acid for 30 min, to precipitate proteins and long-chain carbohydrates. The samples were centrifuged at 10,000g for 15 min, and the supernatants (10 µL) were analyzed by TLC on silica-gel G-60 plates (10 × 15 cm, DC-Alufolien Kieselgel 60, Merck, Darmstadt, Germany) [25]. The monosaccharides (D-glucose and D-galactose) and HMF in the hydrolysates were quantified by high performance liquid chromatography (HPLC). To this end, 500 µL of the sample was injected into a high performance liquid chromatograph (Shimadzu, Japan) equipped with an Aminex HPLC-87H Ion Exclusion Column (300 × 7.8 mm) from Bio Rad. The mobile phase consisted of sulfuric acid 5 mM in milli Q water at a flow rate of 0.6 mL/min (Pressure = 68 kgf/cm²). The detector was a diode array spectrophotometer (Model: SPD10A-VP) operating at 190, 205, and 276 nm with Refractive Index (Model: RID-10A). The carbohydrates were measured with a refraction index detector (RID), and 5-HMF was analyzed by the diode array spectrophotometer at 280 nm.

Fermentative Assays for H₂ Production

Culture Medium and Inoculum

Clostridium beijerinckii Br21, which was isolated in our laboratory and can consume the main monosaccharides from algae biomass (galactose, glucose, and mannose) was employed to produce H₂ by fermentation [22]. The microorganism was cultivated in a culture medium designated CH medium [26] at pH 7.0 and 35 °C for 48 h, under anaerobiosis. The CH medium consisted of glucose (5.0 g/L), yeast extract (1.0 g/L), Na₂HPO₄ (3.0 g/L), KH₂PO₄ (1.0 g/L), NaCl (1.0 g/L), MgSO₄·7H₂O (0.1 g/L), FeSO₄ (0.025 g/L), and a trace elements solution (2.0 mL/L) containing H₃BO₃ (2.86 g/L), MnSO₄·4H₂O (2.03 g/L), and FeCl₃ (0.1 g/L). All the chemicals were analytical grade.

The *C. beijerinckii* Br21 inoculum used in the H₂ fermentative assays was prepared by two different approaches. In the first approach, *C. beijerinckii* Br21 was cultivated in CH medium containing galactose instead of glucose as carbon source. In the second approach, *C. beijerinckii* Br21 was grown in cellobiose as carbon source to stimulate intracellular β-glucosidase production.

β-Glucosidase Activity Assays

The *C. beijerinckii* Br21 cultures harvested in galactose or in cellobiose were employed as crude extract that might contain β-glucosidases. The *Clostridium* cells were disrupted by sonication; the cell debris was removed by centrifugation, and the supernatant was used in the enzymatic assays.

The β-glucosidase activity was assayed by using p-nitrophenyl-β-D-glucopyranoside (pNP-Glc; Sigma-Aldrich Chem. Co., St. Louis, MO, USA) as substrate. pNP-Glc hydrolysis was estimated discontinuously, at 35 °C [27]. The standard assay conditions were bis-tris buffer 50 mmol/L, pH 6.0 containing 2 mmol/L pNP-Glc in a final volume of 0.6 mL. All the enzymatic assays were performed in duplicate. One enzyme unit was defined as the amount of enzyme that released 1 µmol of product/min.

Fermentation Assay

The hydrolysate obtained after algae biomass acid treatment under optimal conditions was employed as the only carbon source in the fermentative assays. The algae hydrolysate (14 mL) was supplemented with the CH medium nutrients described above (item 2.4.1), filtered under sterile conditions through a cellulose acetate filter (0.22-µm), and added to pre-sterilized 50-mL flasks. One milliliter of

the *C. beijerinckii* Br21 inoculum prepared by one of the two different approaches described in “Culture Medium and Inoculum” section was added to the fermentation flask. The inoculum had optical density of 0.1 at 600 nm, which corresponded to 6×10^7 cells/mL, according to a previously established correlation [22]. After this procedure, the tubes were bubbled with argon gas for 3 min (to establish anaerobiosis), closed with a rubber cap and an aluminum seal, and incubated at 35 °C and 200 rpm. All the fermentative assays were conducted in triplicate.

Sugar consumption during fermentation was determined by collecting liquid samples at the beginning and at the end of the fermentative assays to measure TRS.

The gas composition was also analyzed by periodically withdrawing 100 µL of gas from the headspace of the fermentation vials with a gas-tight syringe. The gas composition was determined on a Shimadzu GC (Japan) GC 2014 gas chromatograph equipped with a thermal conductivity detector (TCD). The column consisted of molecular sieves 5A (2 m × 4.7 mm); argon at a flow rate of 30 mL/min was used as the carrier gas. The injector, column, and detector temperatures were 80, 50, and 100 °C, respectively. The H₂ volume was determined by the total volume (mL) of the headspace multiplied by the percentage of H₂ analyzed in the headspace gas.

To calculate the product (H₂) conversion factor in relation to the substrate (TRS) consumed in the fermentation assays, the final H₂ volume was transformed (in mmol) and divided by the substrate (in mmol) consumed during the test (Eq. 3). The TRS concentration was converted into millimoles of glucose by dividing it by the glucose molecular weight (MW = 180 g/mol):

$$Y_{P/S} = \frac{\Delta P}{\Delta S} \quad (3)$$

where $Y_{P/S}$ = yield or factor of substrate (TRS in glucose) conversion into product (H₂); ΔS = Difference between the number of mmol of substrate at the beginning and at the end of the test; ΔP = mmol of H₂ formed during the fermentative assay.

The H₂ yield relative to the amount of seaweed biomass was also calculated. The H₂ volume (in mL) was divided by the seaweed initial dry mass (in grams) (Eq. 4).

$$Y_{P/M} = \frac{\Delta P}{M} \quad (4)$$

where, $Y_{P/M}$ = product yield in relation to the algae mass; ΔP = milliliter of H₂ formed in the fermentative assay; M = dry algae biomass employed in the fermentative assays.

The fermentation assay kinetic parameters were statistically compared by Tukey's test with 95% confidence with STATISTICA® software 7.0.

Table 1 *Agarophyton abarenz* biomass chemical composition

Compound	(%)
Total solids	97.8 ± 2.1
Ash ^a	11.1 ± 0.6
Carbohydrates ^a	51.3 ± 2.1
Protein ^a	4.1 ± 0.03
Sulphate ^a	13.6 ± 2.2
Other ^a	24

^aPercentage in dry mass basis

Results and Discussion

Algae Biomass Chemical Composition

The *K. abarenz* biomass chemical composition, depicted in Table 1, was similar to the composition of different *K. abarenz* strains, i.e. between 54.6 and 55.8% carbohydrates, 1.1% ash, and 10.1% mean sulfate concentration [28]. Abd-Rahim et al. [13] described the *K. abarenz* chemical composition as 19.70%, 59.58%, and 5.74% ash, total carbohydrates, and proteins, respectively. The sulfate amount in *K. abarenz* varies depending on the strain and on the carrageenan type and content. Commercial carrageenans contain between 22 and 38% sulfate [29]. However, the sulfate content in

Table 2 Central composite design matrix with the variables temperature, HCl concentration, algae mass, and, time, as well as the response for total reducing sugars (TRS) in the algae hydrolysates

Assay	X ₁ (temperature (°C))	X ₂ (HCl (mmol/L))	X ₃ (algae mass (g))	X ₄ (time (h))	TRS (g/L)	HMF ^a (mg/L)	Glucose ^a (mg/L)	Galactose ^a (mg/L)
1	−1(60)	−1(22.4)	−1(0.15)	−1(8)	0.86	0	0	0
2	−1(60)	−1(22.4)	−1(0.15)	+1(16)	0.94	0	0	0
3	−1(60)	−1(22.4)	+1(0.3)	−1(8)	1.32	0	0	0
4	−1(60)	−1(22.4)	+1(0.3)	+1(16)	1.49	0	0	0
5	−1(60)	+1(44.7)	−1(0.15)	−1(8)	1.01	0	0	0
6	−1(60)	+1(44.7)	−1(0.15)	+1(16)	1.00	0	0	0
7	−1(60)	+1(44.7)	+1(0.3)	−1(8)	1.93	0	0	0
8	−1(60)	+1(44.7)	+1(0.3)	+1(16)	1.71	0	0	0
9	+1(80)	−1(22.4)	−1(0.15)	−1(8)	1.24	0	0	0
10	+1(80)	−1(22.4)	−1(0.15)	+1(16)	1.30	56.32	26.02	20.08
11	+1(80)	−1(22.4)	+1(0.3)	−1(8)	2.44	58.35	6.04	54.23
12	+1(80)	−1(22.4)	+1(0.3)	+1(16)	2.49	54.00	26.95	21.31
13	+1(80)	+1(44.7)	−1(0.15)	−1(8)	1.38	33.66	20.38	28.47
14	+1(80)	+1(44.7)	−1(0.15)	+1(16)	1.38	28.95	20.32	17.44
15	+1(80)	+1(44.7)	+1(0.3)	−1(8)	2.72	55.02	20.99	15.76
16	+1(80)	+1(44.7)	+1(0.3)	+1(16)	2.52	82.64	17.83	8.91
17	−2(50)	0(33.5)	0(0.225)	0(9)	1.01	0	21.82	0
18	+2(90)	0(33.5)	0(0.225)	0(9)	2.03	307.5	6.61	6.32
19	0(70)	−2(12)	0(0.225)	0(9)	1.49	8.19	0	5.87
20	0(70)	+2(55.9)	0(0.225)	0(9)	1.77	13.42	0	0
21	0(70)	0(33.5)	−2(0.075)	0(9)	0.61	2.47	0	0
22	0(70)	0(33.5)	+2(0.375)	0(9)	2.18	10.99	20.47	9.56
23	0(70)	0(33.5)	0(0.225)	−2(7)	1.38	3.60	14.27	9.49
24	0(70)	0(33.5)	0(0.225)	+2(11)	1.80	9.60	14.29	11.09
25 (C)	0(70)	0(33.5)	0(0.225)	0(9)	1.58	8.84	21.00	6.73
26 (C)	0(70)	0(33.5)	0(0.225)	0(9)	1.64	12.14	0	0
27 (C)	0(70)	0(33.5)	0(0.225)	0(9)	1.59	11.38	0	0
28 (C)	0(70)	0(33.5)	0(0.225)	0(9)	1.69	8.58	0	0

^aVariables not considered in the optimization

X₁, X₂, X₃, and X₄ are the *ex* coded variables

Zero(0) means that the concentration is under the method detection limit

the whole macroalgae biomass has been described to range between 8 and 12% [28].

Regarding the use of this macroalgae biomass for fermentative purposes, its total carbohydrate content is higher than 50% and is in the same order of magnitude of the total carbohydrate content of other macroalgae biomasses that have been studied as substrates for H₂ production; e.g., *G. assamii* [9] and *L. japonica* [7], 67.3 and 59.7% total carbohydrate content, respectively. Therefore, the algae we investigate here is a potential feedstock for H₂ production.

Acid Hydrolysis Optimization

Table 2 lists the TRS, HMF, D-glucose, and D-galactose concentrations in the algae submitted to acid treatment carried out according to the conditions (temperature, HCl concentration, algae mass, and time) established by the 2⁴ experimental design matrix.

On the basis of the results displayed in Table 2, acid treatment of 0.3 g of algae biomass at 80 °C in HCl 44.7 mmol/L for 8 h (assay 15) affords the highest TRS concentration.

In all the assays, the glucose and galactose concentrations range from 6.04 to 26.95 mg/L and from 5.87 to 54.23 mg/L, respectively. Hence, the mild acid treatment conditions applied here generate monosaccharides at very low concentrations, so that the TRS concentration is probably represented by di-, tri-, or oligosaccharides. Thin layer chromatography of the hydrolysate (TLC; Fig. 2) confirms this effect. Because HMF originates from hexoses dehydration, it also emerges at low concentration (between 0 and 307.5 mg/L) albeit its concentration is sometimes higher than the monosaccharide concentrations. Tests accomplished at the highest temperatures, 80 and 90 °C, give the highest HMF concentrations, 82.64 and 307.5 mg/L, respectively (Table 2). According to Yun et al. [30], acid treatment is the method that is the most commonly employed to hydrolyze carrageenan-containing algae biomass, but it requires high temperature, large acid concentration, and long time to yield monosaccharides. Nevertheless, these severe acid hydrolysis conditions degrade D-galactose and 3,6-anhydro-D-galactose (AHG), which are carrageenan monosaccharides, to HMF [30]. Park et al. [9] reported that *G. assamii* hydrolysis with 2% sulfuric acid at 121 °C for 59 min produces D-galactose and AHG. However, AHG rapidly degrades to HMF, levulinic acid, and formic acid, which are fermentation inhibitors. We did not target AHG in our chromatographic analyses, but it may have been readily converted into HMF, which could be the reason why HMF concentration is sometimes as high as or even higher than the monosaccharide concentrations.

We used the TRS concentration and not the monosaccharide concentrations to optimize the algae acid treatment because the H₂-producing *C. beijerinckii* Br21 employed

here can ferment different types of sugars [22]. Equation 5 refers to the mathematical model we used to determine the TRS concentration in the hydrolysates after statistical analyses of the results. The linear parameters of temperature (X₁) and algae mass (X₂) and the interaction between these variables (X₁X₂) influence the TRS values more than the other variables (p < 0.1).

$$\begin{aligned} \text{TRS} = & 1.620 + 0.296X_1 - 0.025X_2^2 + 0.077X_3 \\ & + 0.490X_3 + 0.030X_1^2 + 0.130X_1X_2 \\ & - 0.031935X_1X_2 - 0.038X_2X_3 \end{aligned} \quad (5)$$

Analysis of variance (ANOVA) shows that the model we used to calculate the TRS concentration in the hydrolysates (Eq. 5) is statistically significant and predictive at a confidence level of 90% (p < 0.1) because F_{calculated} > F_{table} and their F ratio (F_{calculated}/F_{table}) is higher than the corresponding F_{table}. We generated the response-surface curves for TRS for further fermentation (Fig. 1).

According to the surface-response curves, the TRS concentration increases with increasing HCl concentration and temperature (Fig. 1a). Simultaneous rise in temperature and algae mass also increases TRS concentration (Fig. 1b). The algae mass limits ART concentration. Concerning the variables time and temperature, the maximum ART concentration is reached at the highest temperatures (Fig. 1c). At higher temperatures, longer times do not boost the ART concentration in the algae hydrolysate. Therefore, the shorter times proposed in the experimental design are sufficient to reach high TRS concentrations (Fig. 1c).

On the basis of Eq. 5, the optimal conditions to reach the highest TRS concentration are 90 °C, 55.9 mmol of HCl/L, 0.375 g of algae, and 8 h, which theoretically afford 3.94 g of TRS/L. Algae acid hydrolysis conducted in the optimal conditions, in triplicate, helped to validate the optimization methodology. The relative deviation value revealed that the predicted and experimental values correlate well (Table 3).

The algae hydrolysate TRS concentration (3.5 g/L) is slightly lower than the predicted TRS concentration. The hydrolysate also contains HMF at 0.28 g/L. We performed the assays under low algae biomass load (0.375 g in 50 mL), to obtain enough sugar concentration and small amounts of inhibitors in the hydrolysate for further fermentation. Low inhibitor concentrations eliminate the need for a detoxification step. Higher algae biomass loading (about 10%) leads to higher TRS concentration in the hydrolysate, but also to higher concentration of fermentation inhibitors, which are beyond the lower TRS extraction yield. Meinita et al. [31] found optimal conditions for acid *K. abarenz* hydrolysis when they used HCl as catalyst and optimized the temperature, reaction time, algae concentration, and acid concentration as 130 °C, 15 min,

Fig. 1 TRS concentration surface response as a function of acid concentration versus temperature (A), algae mass versus temperature (B), time versus temperature (C)

Table 1 Predicted and experimental TRS concentration responses in the optimal pretreatment conditions (90 °C, HCl at 55.9 mmol/L, 0.375 g of algae, and 8 h)

Response	Predicted	Experimental	RDP (%)
TRS (g/L)	3.94	3.50	− 7.0

*Relative deviation (RD): $[(\text{experimental value} - \text{predicted value}) / \text{experimental value}] \times 100$

10 g of substrate/100 mL, and HCl 0.2 M, respectively. This optimal condition produced 22.68 g/L of TRS in the hydrolysate, but fermentation inhibitors also arose: HMF and levulinic acid at 0.8 and 2.8 g/L, respectively. HMF and levulinic acid concentrations of about 0.5 and 1.5 g/L, respectively, inhibit 50% of H_2 production by a mixed culture [35, 32]. Here, we carried out algae hydrolysis under the optimized conditions at 0.7% (w/v) algae loading, which yielded a TRS concentration of 3.5 g/L, and an

HMF concentration of 0.28 g/L in the hydrolysate. These values do not represent potential inhibition. Interestingly, low HMF concentrations, ca 0.25 g/L, have been considered to stimulate fermentation [32].

The presence of low concentrations of monosaccharides and of their degradation derivative (HMF) in the liquid does not mean that the structure of the algae polysaccharide remained intact, but that the hydrolysis conditions probably sufficed to release oligosaccharides only, as demonstrated by TLC (Fig. 2). Controls C1 and C2 in Fig. 2 correspond to algae and water only and showed that untreated algae biomass has high-molecular-weight polysaccharides that are retained at the bottom of the TLC (Fig. 2). On the other hand, α -glucose and α -galactose, which have the lowest molecular weight, migrate to the top of the TLC plate, followed by the disaccharide cellobiose, which stops just below the monosaccharides. The algae hydrolysate (TRS; Fig. 2) displays stains mainly at the same height as the disaccharide

Fig. 2 TLC of the assayed samples. Glu glucose, Cel cellobiose, Gal galactose, C control (algae and water), TRS hydrolysate obtained at the optimized conditions (90 °C, HCl at 55.9 mmol/L, algae mass of 0.375 g, and 8 h)

as well as below, which indicates that TRS also refer to oligosaccharides.

The low degree of hydrolysis we achieved for the *K. alvarezii* biomass hydrolysate could avoid prompt dehydrated galactose degradation under acid conditions. Nevertheless, successful employment of such hydrolysate as substrate will depend on the fermentative microorganism ability to convert higher sugars into biofuels.

Fermentative Assays for H_2 Production with Algae Hydrolysate

We employed the hydrolysate obtained from the algae subjected to acid treatment under optimal conditions, which yielded the highest TRS concentration, and the TRS were the only carbon source in the fermentative assays for H_2

Fig. 3 H_2 concentration as a function of time in the fermentative assays conducted with inoculum prepared under different conditions: condition 1—inoculum grown in galactose and presenting no β -glucosidase activity; condition 2—inoculum grown in cellobiose and presenting 0.33 U/mL of initial β -glucosidase activity

production. Although the sugars present in the hydrolysate consist mostly of oligosaccharides, *C. beijerinckii* Br21 bears a high amount of genes encoding for glycosyl hydrolases [16], which could help in the final algae biomass hydrolysis step. Here, the *C. beijerinckii* Br21 cell extract cultivated in galactose or in cellobiose exhibits no or β -glucosidase 0.33 U/mL activity, respectively. Albeit low, we have succeeded in detecting β -glucosidase activity by a *C. beijerinckii* strain for the first time.

Figure 3 presents the H_2 concentration measured during the fermentative assays that employed the algae hydrolysate as substrate and the inoculum cultivated in galactose or in cellobiose, named conditions 1 and 2, respectively. The H_2 concentration obtained with *C. beijerinckii* displaying β -glucosidase activity (condition 2, 171.76 ± 2.10 mL of H_2 /L) is significantly higher ($p < 0.05$) as compared to the inoculum without initial β -glucosidase activity (condition 1, 140.95 ± 10.92 mL of H_2 /L). β -Glucosidases hydrolyze short-chain oligosaccharides and cellobiose, to yield glucose, which is fermentable. Therefore, β -glucosidase may help to increase the content of fermentable sugars, such as glucose, in biomass hydrolysates, and consequently raise the amount of generated biohydrogen [33]. Glucose assimilation is usually preferred over assimilation of other carbohydrates and, depending on the glucose concentration, the catabolic repression effect may emerge; i.e., the presence of glucose inhibits assimilation of other sugars [34]. Galactose and glucose (the main red macroalgae cell wall monosaccharides) are hexoses, but the bacterial cell takes them up differently. Galactose first needs to be converted into glucose by the Leloir pathway. In this process, galactose is converted into glucose-1-phosphate, which consumes more ATP than glucose conversion into this same molecule. For this reason, galactose as substrate leads to lower fermentation yields

Table 4 Maximum H_2 concentration, Y_{H_2} and Y_{H_2} from the fermentative assays conducted in the presence of *C. beijerinckii* Br21 cultivated in galactose (1) or in cellobiose (2)

Inoculum condition	H_2 max (ml. of H_2/L)	Y_{H_2} (mmol of H_2 /mmol of ART as glucose) ^a	Y_{H_2} (ml. of H_2/g of dry algae)
1	140.95 ± 10.92	0.76 ± 0.05	62.7 ± 4.9
2	171.76 ± 2.10	0.87 ± 0.09	70.3 ± 0.9

^aConverted considering TRS as glucose; molecular weight = 180 g/mol

[22]. Moreover, disaccharides such as cellobiose as well as small oligosaccharides are only used if the fermentation microorganism can hydrolyze these molecules to their respective monosaccharides [33].

Apart from higher H_2 concentration, the assay accomplished with *C. beijerinckii* presenting β -glucosidase activity (Fig. 3, condition 2) gives significantly higher H_2 yield relative to the amount of seaweed biomass employed in the fermentation as compared to the assay performed with the inoculum grown in galactose (Table 4). This result confirms that the β -glucosidase activity in the *Clostridium* grown on cellobiose improves the use of the algae hydrolysate.

As for the ratios of conversion of TRS as glucose to H_2 (Y_{H_2}), they were 0.76 and 0.87 mmol of H_2 /mmol of TRS as glucose for conditions 1 and 2, respectively, which represents 19 and 22% if we consider the maximum H_2 stoichiometric yield from glucose as 4 mol of H_2 /mol of glucose, respectively.

When it comes to pretreated red algae as feedstock, the yield 70.3 mL of H_2 /g of dry algae is very promising as compared to other literature results. Only one paper has reported a much higher yield, 159.6 mL of H_2 /g of dry weight of *L. japonica* pretreated at 4.8% HCl and 93 °C for 23 min and loaded onto an anaerobic sludge [35]. Our values are higher than the data obtained by Liu and Wang [36], which was 43.65 mL of H_2 /g of dry algae when they employed *L. japonica* treated with HCl 1 mol/L for 30 min loaded at 1% onto an anaerobic sludge. Our values were also higher than the 53.5 mL of H_2 /g of dry algae obtained from a *G. sinensis* hydrolysate in the presence of a mixed culture at 150 °C for 15 min [9]. In the latter article, fermentation was only possible after a detoxification step with activated carbon [9].

Finally, our results show that, although the algae hydrolysate we obtained from a mild acid treatment presents low monosaccharide content, it can be employed as substrate for H_2 production by *C. beijerinckii* Br21. Indeed, stimulating β -glucosidase production by *C. beijerinckii* Br21 improves H_2 production and yield. This enzyme can hydrolyze cellobiose and other di- and oligosaccharides present in humans hydrolysates [33] and could thus generate more

fermentable sugars as compared to the assay where there was no β -glucosidase activity stimulation.

Conclusions

The factorial design we employed here showed that the variables temperature and algae mass are the most significant when it comes to obtaining high TRS concentrations from *K. alvarezii* biomass. Mild acid treatment conditions mainly provide di- and oligosaccharides as well as low monosaccharide and HMF concentrations. Here, we have described β -glucosidase activity for the *C. beijerinckii* strain cultivated in cellobiose for the first time. This activity helped to increase fermentative H_2 production from the *K. alvarezii* hydrolysate.

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Apêndice G

RESEARCH

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Lignocellulose binding of a Cel5A-RtCBM11 chimera with enhanced β -glucanase activity monitored by electron paramagnetic resonance

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Abstract

Background: The *Bacillus subtilis* endo- β -1,4-glucanase (BxCe5A) hydrolyzes β -1,3-1,4-linked glucan, and the enzyme includes a family 3 carbohydrate-binding module (CBM3) that binds β -1,4-linked glucan.

Methods: Here we investigate the BxCe5A β -1,3-1,4-glucanase activity after exchanging the CBM3 domain for the family 11 CBM from *Ruminococcium thermocellum* celH (RtCBM11) having β -1,3-1,4-glucan affinity.

Results: The BxCe5A-RtCBM11 presents a 50.4% increase in V_{max} , a 10% reduction in $K_{0.5}$, and a 2.1-fold increase in catalytic efficiency. Enzyme mobility and binding to barley β -1,3-1,4-glucan and pre-treated sugarcane bagasse were investigated using Electron Paramagnetic Resonance (EPR) with Site-Directed Spin Labeling (SDSL) of the binding site regions of the CBM3 and RtCBM11 domains in the BxCe5A-CBM3 and BxCe5A-RtCBM11, respectively. Although higher mobility than the RtCBM11 was shown, no interaction of the spin-labeled CBM3 with β -1,3-1,4-glucan was observed. In contrast, a K_d value of 0.22 mg/ml, was estimated from titration of the BxCe5A-RtCBM11 with β -1,3-1,4-glucan. Enzyme binding as inferred from altered EPR spectra of the BxCe5A-RtCBM11 was observed only after xylan or lignin extraction from sugarcane bagasse. Binding to xylan- or lignin-free lignocellulose was correlated with a 4.5- to 5-fold increase in total reducing sugar release as compared to the milked intact sugarcane bagasse, suggesting that xylan impedes enzyme access to the β -1,3-1,4-glucan.

Conclusions: These results show that the non-specific binding of the BxCe5A-RtCBM11 to the lignin component of the cell wall is minimal, and represent the first reported use of EPR to directly study the interaction of glycoside hydrolytic enzymes with natural insoluble substrates.

Keywords: Electron paramagnetic resonance, Site-directed spin labeling, Lignocellulose binding, Enzyme engineering

Background

Cellulose is the major component of the plant cell wall, and is integrated with various other polysaccharides and lignin to form a complex polymer matrix. Cellulose forms

linear chains comprising glucose monomers linked by β -1,4-glycosidic bonds [1]. Abundant hydrogen bonding both within and between the cellulose polymers imposes a largely crystalline structure that is recalcitrant to degradation [2]. The enzymatic hydrolysis of cellulose requires a consortium of glycoside hydrolases initiated by the action of endoglucanases (endo-1,4- β -glucosidases; EG; EC 3.2.1.4) that cleave β -1,4-glycosidic bonds in the regions of the cellulose fibers showing low crystallinity,

which results in the release of oligosaccharides, cellobiose, and glucose. Subsequently, exoglucanases or cellobiohydrolases (exo-1,4- β -glucosidases; CBH; EC 3.2.1.91) act on the reducing and non-reducing extremities in crystalline regions of the cellulose fibers to release glucose and cellobiose, and finally β -glucosidases hydrolyze short-cell oligosaccharides and cellobiose to release glucose [3]. This synergistic hydrolysis of cellulose contributes to carbon recycling in the biosphere [3–5], and has been extensively studied from the viewpoint of biotechnological applications in the biofuels, animal feed, pulp bleaching, wastewater treatment, and paper bioreconversion industries [6, 7]. The use of enzymes can reduce energy costs and avoid the use of chemical processes that could ultimately harm the natural environment [8].

Endo- and exoglucanases frequently comprise two or more domains that display multi-functional properties, where a catalytic domain (CD) is responsible for the hydrolytic activity, and non-catalytic domains enhance catalysis or perform structural functions [9]. The non-catalytic modules include the Carbohydrate-Binding Modules (CBMs) [10], which are coupled to the catalytic domains via flexible polypeptide linkers [11]. It is widely recognized that the CBM domains of cellulases improve the efficiency of enzymatic hydrolysis of insoluble substrates [12] as a result of their functions in polysaccharide recognition and binding [13]. The CBMs are currently grouped into 81 families (CAZy database, <http://www.cazy.org>) that display variable binding specificities against different plant cell wall polysaccharides such as crystalline or amorphous cellulose, β -glucans, xylans, laminarins, mannans, galactans, xyloglucans, arabinans, and others.

Electron Paramagnetic Resonance (EPR) spectroscopy is a well-established technique, which targets defined paramagnetic centers [14] and is not subject to interference from signal scattering resulting from the presence of particulate material [15]. EPR spectroscopy together with site-directed spin labeling (SDSL) has been used to investigate structural and conformational changes of proteins [16–18]. The use of EPR is well suited to study the binding of spin-labeled proteins to lignocellulosic biomass, and in contrast to Nuclear Magnetic Resonance (NMR) and X-ray crystallography, the technique can be applied under conditions found in nature [19] or under high solids loading. In SDSL, an extrinsic spin probe is covalently attached to the thiol side chain in a native or site-directed mutated Cys residue [16], and analyses of the EPR spectrum can provide information with respect to protein chain dynamics, solvent accessibility, local polarity, and protein interactions under the chosen experimental conditions [14, 20–24]. Furthermore, when multiple probes are introduced into a protein, EPR-SDSL can be used to

measure distances between the spin probes within the polypeptide chain [25, 26].

The thermostable *Bacillus subtilis* cellulase 5A (BxCe5A) is a well-characterized endo- β -1,4-glucanase, and comprises a family 3 glycoside hydrolase (GH5) catalytic domain fused to a family 3 carbohydrate-binding module (CBM3) [27]. The CBM3 family has an extended planar binding surface, and has been proposed to interact mainly with insoluble substrates such as crystalline cellulose [10]. Biochemical characterization of BxCe5A showed a maximum enzymatic activity at pH 6 and 60 °C, and a high thermal stability [27]. Although the BxCe5A hydrolyzes the β -1,4 linkages both in soluble carboxymethylcellulose (CMC) and in crystalline cellulose, the enzyme has a 5-fold higher catalytic efficiency against barley β -glucan and lichenan [27], which are soluble polysaccharides composed of β -1,3- β -1,4 mixed linked glucose monomers [28].

The celH of *Ruminococcium thermocellum* (previously *Clavibacterium thermocellum*) is a multidomain protein [29], which encodes an N-terminal GH26 β -1,3-1,4-glucanase catalytic domain, a second catalytic GH5 β -1,4-cellulase domain, a family 11 carbohydrate-binding module (RtCBM11), and a dockerin domain at the C-terminus [29, 30]. Since the RtCBM11 domain has high affinity for β -glucan polymers, we surmised that its fusion with the catalytic domain of BxCe5A could increase the β -1,3-1,4-glucanase activity of the enzyme. Here we report the application of EPR-SDSL to investigate the dynamics and binding characteristics of the BxCe5A catalytic domain fused with the carbohydrate-binding modules CBM3 and RtCBM11 in the presence of the natural substrate barley β -glucan and several types of pre-treated sugarcane bagasse.

Methods

Cloning, expression, and purification

Expression systems in *E. coli* using the pET28a vector have been previously described for the *Bacillus subtilis* BxCe5A-CBM3 [27] and the RtCBM11 from the celH of *R. thermocellum* [31]. The RtCBM11 nucleotide sequence cloned in the pET28a plasmid was used as template for amplification with oligonucleotides CBM_Bsp_F (containing restriction site BspI) and the T7 terminator sequence (containing restriction site BamHI). The amplified fragment was purified from 1% agarose gels using the GeneJET kit (Fermentas, Vilnius, Lithuania), and digested with restriction enzymes BspI and BamHI. Digestion of the BxCe5A-CBM3 pET28a construct with the same two restriction enzymes liberates the sequence encoding the CBM3 domain, and generates compatible cohesive ends for ligating the digested RtCBM11 PCR fragment. Following ligation, the integrity of the

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BsCel5A-RtCBM11_pET28a construct was confirmed by automated nucleotide sequencing.

Competent *E. coli* Rosetta BL21 (DE3) cells were transformed with either the BsCel5A-CBM3 or the BsCel5A-RtCBM11. After regeneration, the transformed cells were cultured in 10 mL of selective LB liquid at 37 °C for 16 h. These cultures were used to inoculate 1 L of selective LB liquid medium, and after an appropriate cell density had been reached ($OD_{600} = 0.7$) protein expression was induced by the addition of 0.5 mM IPTG (isopropyl- β -D-thiogalactopyranoside) and run for 6 h under the same culture conditions. Subsequently, the cells were harvested by centrifugation, and lysed by sonication in buffer containing 40 mM HEPES, 300 mM NaCl, pH 7.5, and 40 mM imidazole. After centrifugation to clear cell debris, the supernatant was incubated with 2 mL of Ni-NTA Superflow resin (Qiagen) in an orbital shaker at 4 °C at 50 rpm for 60 min, and packed into a suitable chromatographic column. After passing 40 mL of wash buffer (60 mM Imidazole, 100 mM HEPES, 30 mM NaCl, pH 7.5), the recombinant proteins were recovered with elution buffer (100 mM Imidazole, 100 mM HEPES, 300 mM NaCl, pH 7.5). Eluted protein samples were concentrated and further purified by gel filtration (Superdex 200, 5 $\times$ 150 mm column) equilibrated with 25 mM HEPES, 250 mM NaCl, pH 6, with a solvent flow rate of 0.5 mL/min.

Circular dichroism (CD)

Far-UV (190–250 nm) CD measurements were performed with a Jasco J-815 CD Spectrometer (JASCO Corporation, Japan) using a 1-mm path length quartz cell using protein samples at a final concentration of 2 μ M in 10 mM of sodium phosphate buffer, pH 8.0. The spectra were recorded at a scan speed of 50 nm/min and at time response of 1 s. An average of 9 spectra were recorded, the buffer baseline was subtracted and after Savitzky-Golay smoothing using the CDTools software [32], the processed spectra were deconvoluted by the Contin software [33] with database 4 [34] available in the DichroWeb analysis server [35]. The normalized root mean-square deviation (NRMSE) goodness-of-fit parameter was < 0.15, suggesting that the calculated spectra are a good approximation of the experimental data [36].

Enzymatic assays and determination of the kinetic parameters

The hydrolysis of carboxymethylcellulose (CMC; Sigma-Aldrich Chem. Co.) and barley β -glucan [28] was assayed at 50 °C in Bis-Tris buffer, pH 6.0, in a final volume of 0.6 mL. The reducing sugars released were quantified using the dinitrosalicylic acid (DNS) method [37]. Controls with heat-inactivated enzyme were included in all

enzymatic assays. The experimental conditions (reaction times, enzymatic units) were adjusted to guarantee the estimation of initial velocities. One enzyme unit (U) was defined as the amount of enzyme that releases 1 μ mol of product per min. The specific activity was defined as the enzyme units per milligram of total protein (U mg⁻¹). All enzymatic assays were performed in duplicate. Maximum velocities (V_{max}) and apparent affinity constants (K_{app}) for the hydrolysis of CMC and β -glucan by BsCel5A-RtCBM11 and BsCel5A-CBM3 were estimated using the SigmaPlot Software [38]. All experimental kinetic curves were repeated three times using different recombinant enzyme preparations, where each experimental point was assayed in duplicate. The kinetic parameters presented are calculated values and are given as the mean $\pm$ SD of the three replicates ($n = 3$).

Site-directed spin labeling and EPR measures

The purified proteins were transferred to labeling buffer (40 mM Hepes, 300 mM NaCl, pH 8.0), and immediately incubated with a 5-fold molar excess of the spin-labeled 2,2-dihydro-2,2,5,5-tetramethyl-1-[[[methylsulfonyl]thio]methyl]-1H-pyrrol-1-yl-xyloxy (MTSSL) for 24 h at 25 °C. The modified protein was separated from excess free spin label with a Superdex 200 column (5 $\times$ 150 mm) eluted with 40 mM HEPES, 300 mM NaCl, and pH 8.0 buffer. The spin-labeled proteins were concentrated to 200 μ M by ultrafiltration.

Continuous wave EPR spectroscopy was performed at room temperature using a JEOL JE5-FA200 equipped with temperature control and a cylindrical cavity operating in X band mode. Spectral analyses were performed according to empirical parameters as previously described (Columbus and Hubbell, 2004). The M_s mobility parameter was calculated as function of the central line width as $M_s = (\delta_{1/2}^2 - \delta_{1/2}^2) / (\delta_{1/2}^2 - \delta_{1/2}^2)$, where $\delta_{1/2}$ is the value of the central line width of the MTSSL-labeled protein. The $\delta_{1/2} = 8.4$ G and $\delta_{1/2} = 2.1$ G are the values of the greatest degree of either immobilization or mobility of sites observed in a protein, respectively, as obtained in studies with rhodopsin [14, 18].

Solutions containing labeled proteins free in solution, and in the presence of either purified β -glucan or pre-treated sugarcane cell wall samples were prepared and drawn into capillary tubes. The final protein concentration was 100 μ M, and the β -glucan concentration was varied from 0.1 to 5 mg/mL. The sugarcane cell wall preparations were used at a concentration of 1 mg/mL. The titration curve was fitted to a sigmoidal Hill function by non-linear regression using the program Microcal Origin version 8.0, to yield estimates of the association constant (K_a).

Sugarcane cell wall preparation, fractionation, and enzymatic assay

Five hundred milligrams of sugarcane bagasse culms were hammer-milled to a 0.5 mm particle size, and extracted 6 times with 25 mL of 80% (v/v) ethanol at 80 °C for 20 min to remove sugars and other soluble compounds. The alcohol insoluble residue (AIR) was washed with distilled water and dried at 60 °C for 24 h [39]. The AIR was subjected to two consecutive extractions with 25 mL of 0.5 M ammonium oxalate pH 7.0 at 80 °C for 3 h with continuous stirring. The solid oxalate-extracted residue was recovered by centrifugation at 13,000g for 30 min and dried at 60 °C for 24 h [39]. Subsequent extraction with DMSO followed a previously reported protocol [40], where approximately 4 g of oxalate-extracted sugarcane bagasse was subjected to two DMSO extractions using a ratio of 14 mL DMSO per gram of biomass at room temperature with continuous stirring at 20 rpm at 70 °C for 2 h. The solids fraction containing the DMSO-extracted biomass was recovered and washed with ethanol and dried [40]. Biomass delignification was performed as previously described [41] where approximately 10 g of milled sugarcane bagasse was suspended in 100 mL of water to which was added 4 g NaClO₂, followed by the addition of 500 mL of glacial acetic acid. The mixture was incubated at 60 °C for 3 h. At hourly intervals, 4 g of NaClO₂ was added until a total of 0.80 g of NaClO₂/g of biomass was reached. The mixture was vacuum filtered and the solid fraction was washed with deionized water and dried at 50 °C for 24 h [41, 42].

The activity of the enzymes against AIR and all the treated lignocellulosic substrates was performed using a 1% w/v suspension of the treated substrate prepared in 50 mM Bis-Tris buffer, pH 6.0, buffer, 100 μ M of either BsCel5A-CBM3 or BsCel5A-RtCBM11 at 50 °C for 24 h in an orbital shaker at 200 rpm. The release of total reducing sugars was measured by the DNS method [37].

Results and discussion

Exchanging the CBM3 domain of the BsCel5A for RtCBM11 increases catalytic efficiency

The cysteine mutants of the BsCel5A-CBM3 and BsCel5A-RtCBM11 nucleotide coding sequences encode polypeptides of 494 and 514 amino acids, respectively, and were successfully expressed as soluble proteins in *E. coli* Rosetta (DE3). The molecular masses of the cysteine mutant proteins BsCel5A-CBM3 and BsCel5A-RtCBM11 were 54,787.9 and 56,897.3 Da, respectively, including the polyhistidine sequence added to the N-termini derived from the pET28a plasmid. The relative molecular mass of both enzymes estimated by gel filtration was approximately 55 kDa (data not shown), and it was therefore assumed that both enzymes are monomeric. The far-UV CD spectra of the pure enzymes show a maximum at approximately 195 nm, a minimum around 215 nm, and a shoulder at 225 nm (Additional file 1: Figure S1) that are characteristic of spectra from proteins with a mixed α -helix and β -strand secondary structure content. The secondary structure content (inset to Additional file 1: Figure S1) estimated by deconvolution of the CD spectra of the BsCel5A-CBM3 was 26.4% α -helix and 20.7% β -strand and for the BsCel5A-RtCBM3 was 26.6% α -helix and 22.3% β -strand. These values are consistent with the sum of the secondary structure assignments from the crystallographic structure of the BsCel5A catalytic domain (PDB 3PZV, [27]) with either the CBM3 (PDB 2L8A [27], sum of 22.5% α -helix and 21.5% β -strand) or the RtCBM11 (PDB 1Y0A [43], sum of 21.2% α -helix and 27.0% β -strand).

The endoglucanase activity with different concentrations of either β -glucan or CMC substrates (see Fig. 1) was higher against the β -glucan for both chimeric enzymes, confirming that the catalytic domain prefers the natural β -glucan as a substrate. Analysis of

Fig. 1 The catalytic activity of the cysteine mutants BsCel5A-CBM3 and BsCel5A-RtCBM11 against (a) barley β -glucan and (b) CMC

Table 1 Kinetic parameters for β -glucan and CMC hydrolysis by cysteine mutants BcCel5A-CBM3 and BcCel5A-CBM11 enzymes

Enzyme	Substrate	V_{max} (U/mg ⁻¹)	K_{m1} (mg mL ⁻¹)	K_{cat} (s ⁻¹)	K_{cat}/K_{m1} (mL mg ⁻¹ s ⁻¹)
BcCel5A-CBM3	β -glucan	408.1 $\pm$ 20.1	2.90 $\pm$ 0.05	472.54 $\pm$ 20.4	164.0
	CMC	218.7 $\pm$ 10.4	0.97 $\pm$ 0.11	188.70 $\pm$ 10.4	19.65
BcCel5A-RtCBM11	β -glucan	983.7 $\pm$ 40.2	2.60 $\pm$ 0.04	938.72 $\pm$ 40.2	351.8
	CMC	251.3 $\pm$ 9.6	0.98 $\pm$ 0.22	238.49 $\pm$ 9.6	24.16

the kinetic parameters for CMC and β -glucan hydrolysis (see Table 1) reveals that the V_{max} and k_{cat} values for β -glucan hydrolysis were almost 2-fold higher for the BcCel5A-RtCBM11 as compared to the BcCel5A-CBM3 enzyme, and was accompanied by a slight decrease in the K_{m1} (from 2.88 to 2.66 mg/mL) of the BcCel5A catalytic domain when the CBM3 was replaced by RtCBM11. The catalytic efficiency (k_{cat}/K_{m1}) of β -glucan hydrolysis was 53% greater for BcCel5A-RtCBM11 as compared to BcCel5A-CBM3. Both enzymes presented similar K_{m2} values for CMC hydrolysis, suggesting that the catalytic domain can maintain hydrolytic function independent of the associated CBM. This result is in agreement with the previously known affinities of the CBMs for the polysaccharide used in this study, where RtCBM11 shows a higher affinity for β -glucan as compared to CBM3, and neither CBM3 nor RtCBM11 presents significant binding to CMC [44].

Binding of the BcCel5A-RtCBM11_{Y151C} to β -glucan is confirmed by EPR-SDSL

Experimental strategies used to study polysaccharide binding to CBMs include affinity electrophoresis, insoluble polysaccharide pull-down, and NMR [45]. Here we have introduced a novel and complementary method to investigate CBM binding dynamics based on spin labeling electron paramagnetic resonance (SDSL-EPR) experiments. We have produced single cysteine mutants in the carbohydrate-binding regions of the CBM domains in both the BcCel5A-CBM3 and BcCel5A-RtCBM11, and modified these cysteines with the spin probe MTSSL. The labeled proteins were used to analyze changes in the dynamics of both enzymes in the presence of the natural substrate β -glucan and several pre-treated biomass samples.

Type A CBMs interact with the smooth surface of crystalline cellulose with a planar hydrophobic carbohydrate-binding site [10, 12]. Type B CBMs bind within soluble extended polysaccharide chains (*owlo*-binding) in a cleft on the protein structure, whereas Type C CBMs have shortened pockets that bind to the termini of the polysaccharides (*exo*-binding) [46]. The crystal structures of the Type A *Bacillus subtilis* CBM3 (BcCBM3) and the Type

B *Ruminiclostridium thermocellum* CBM11 (RtCBM11) are presented in Fig. 2. The CBM3 domain from BcCel5A presents a β -sandwich fold, with a core formed by two β -sheets of four and five β -strands, and the flat surface typical of Type A CBMs comprises the four β -strands that includes the charged and polar residues that make contacts with the surface of aggregated cellulose [39]. In contrast, the RtCBM11 has a "jelly roll" architecture consisting of two six-stranded anti-parallel β -sheets, which form a curved surface that defines a single ligand-binding cleft that is typical for Type B CBMs [43].

The CBM3 domain from BcCel5A and the RtCBM11 structures presented in Fig. 2 also highlights the residues labeled with the MTSSL probe in the spin-labeling EPR experiments. The crystal structure of the wild-type CBM3 domain from BcCel5A (PDB code 2LRA) reveals two unpaired cysteine residues (C405 and C412). Residue C412 in the BcCel5A-CBM3 was mutated to alanine resulting in a protein containing the single cysteine C405. The wild-type RtCBM11 does not include any cysteine residues, and analysis of the crystal structure (PDB code 1V0A) reveals that the Y151 is located in the substrate binding cleft, which is convenient for reporting local changes by SDSL-EPR. This residue was mutated in the BcCel5A-RtCBM11 to generate the Y151C single cysteine mutant. Both single cysteine mutant proteins were successfully labeled with the MTSSL spin probe to generate the BcCel5A-RtCBM11_{Y151C} and BcCel5A-CBM3_{C405} and used in SDSL-EPR experiments.

An empirical analysis of the mobility of the MTSSL probes exploits the fact that the EPR spectra are doubly integrated, resulting in spectra that are normalized with respect to total area between samples with an equal number of spin labels [14]. In the case of nitroxide radicals such as MTSSL tumbling free in solution, the EPR spectrum is defined by three narrow well-resolved resonance lines. When the nitroxide radicals are covalently linked to cysteine side chains in a protein, the line shape of the EPR spectra is modified according to the dynamics of the region, where dynamically restricted labeled side chain show less intense and broadened lines [14, 18, 47]. Figure 3 presents the EPR spectra of BcCel5A-RtCBM11_{Y151C} and BcCel5A-CBM3_{C405} in the presence and

Fig. 2 Ribbon representations of the structures of the single-cysteine mutants of (a) BcCBM3 and (b) RtCBM11 created by SVES-MODEL using the PDB file 2LRA and 1V0A, respectively, as templates. The residues modified by the MTSSL probe for EPR studies are depicted as dark gray spheres and the residues involved in carbohydrate binding are indicated by black sticks

Fig. 3 Measured EPR spectra of (a) BcCel5A-CBM3_{C405} and (b) BcCel5A-RtCBM11_{Y151C} both free in solution and in the presence of β -glucan. The spectra are superimposed and normalized to the total area to emphasize the difference in line width and shape. The arrows highlight the increase in the out-peak separation of the spectrum due to β -glucan binding and immobilization of the spin-labeled side chain

absence of β -glucan. All spectra show three prominent narrow lines typical of nitroxide spin probes (Fig. 3a) that are typical of highly mobile probes. The EPR spectra of BcCel5A-CBM3_{C405} are not significantly altered in the

presence of β -glucan, demonstrating that the dynamics of the labeled residue is unchanged and suggesting the absence of binding of β -glucan by CBM3 domain. In contrast, the EPR spectrum of BcCel5A-RtCBM11_{Y151C}

in the absence of β -glucan is typical of a probe showing intermediate dynamics (Fig. 3b). In the presence of β -glucan, the EPR spectra show significant changes with a clear increase in the out-peak separation (arrows in Fig. 3b) suggesting a more immobilized nitroxide radical. Therefore, the spectral changes promoted by the presence of β -glucan indicate that the labeled residue experiences a restriction in mobility due to the interaction of the RCBM11 domain with β -glucan.

EPR-SDSL can measure the binding affinity of BcCel5A-RCBM11_{V111R} to β -glucan

To estimate the degree of mobility changes of the labeled side chains, the value of the M_s parameter was calculated from the nitroxide EPR spectra as the inverse of the central line width [14, 18]. An increase of the M_s value is directly correlated with the increase in the mobility experienced by the labeled side chain. The M_s values

Table 2 M_s parameter estimation from EPR spectra of labeled proteins

Labeled protein	M_s parameter
BcCel5A-RCBM11 _{V111R} in solution	1.22
BcCel5A-RCBM11 _{V111R} in solution	0.49
BcCel5A-RCBM11 _{V111R} + milled sugarcane	0.49
BcCel5A-RCBM11 _{V111R} + AD residual fraction	0.45
BcCel5A-RCBM11 _{V111R} + AIR fraction	0.47
BcCel5A-RCBM11 _{V111R} + DMSO residual fraction	0.39
BcCel5A-RCBM11 _{V111R} + delignified fraction	0.29

for BcCel5A-RCBM11_{V111R} and BcCel5A-RCBM11_{V111R} in the absence of β -glucan (see Table 2) are 1.22 and 0.49, respectively, which is consistent with the surface exposure and higher mobility of the spin label in the BcCel5A-RCBM11_{V111R}.

The titration of BcCel5A-RCBM11_{V111R} with β -glucan was accompanied by EPR spectroscopy (Fig. 4a). An increase in β -glucan concentration results in an increase of the out-peak separation with a corresponding broadening of the spectral line shape, as indicated by the decrease in M_s values (see Fig. 4b). The vertical lines in Fig. 4a are included as a guide to assist the accommodation of the spectral features resulting from the immobilization of the spin probe on association of the protein with the β -glucan substrate. From the sigmoidal transition in M_s values, the β -glucan shows cooperative binding to the BcCel5A-RCBM11_{V111R} (Fig. 4b), and reveals a saturating interaction at concentrations above 0.5 mg/mL of β -glucan with an association constant (K_a) of 0.23 ± 0.01 mg/mL. This value is consistent with the previously reported K_a value of 0.24 mg/mL for cooperative binding of the RCBM11 protein to β -glucan as estimated from isothermal titration calorimetry experiments [43].

Comparison of the titration results from EPR with the results from reducing sugar release reveals that the RCBM11 binding is saturated at β -glucan concentrations of less than 0.5 mg/mL, whereas the measured catalytic activity of the BcCel5A-RCBM11 continues to increase even at a β -glucan concentration of 15 mg/mL. The high-affinity association of the Type B RCBM11 within the β -glucan might be expected to facilitate the binding of

the BcCel5-RCBM11 and enhance catalysis by the active site domain. After hydrolysis, we propose that a reduced affinity of the RCBM11 *exo*-binding at the polysaccharide termini generated by hydrolysis favors dissociation even at high β -glucan concentrations, thereby liberating the enzyme for high-affinity *endo*-binding to the substrate. We note that no significant change was observed in the K_{cat} between the BcCel5A-RCBM11 and BcCel5A-RCBM11 constructs, further indicating that the catalytic and carbohydrate-binding domains can function independently. It has previously been observed that a xyloglucan-specific endo- β -1,4-glucanase from *Aspergillus niger* (XegA) fused to a xyloglucan-specific CBM44 domain from the Cell of *R. thermocellum* (RtCBM44) exhibits a 30% increase in the catalytic efficiency for xyloglucan as compared to the parental enzyme [6]. It was proposed that the CBM in the XegA-RtCBM44 chimera alters the topology of the carbohydrate interaction in the active site of the XegA-RtCBM44 chimera thereby promoting a favorable conformation of the substrate in the catalytic domain [6].

The EPR technique is not only robust, but also has the advantage of selectively targeting defined paramagnetic centers [14] and is not subject to interference from scattering due to the presence of particulate material [15]. The use of EPR is therefore well suited to study the binding of spin-labeled proteins to lignocellulosic biomass. The lignocellulose composition of the sugarcane cell wall is 38–48% cellulose and β -glucan, 25–32% hemicelluloses (mainly xylan, xyloglucan, and arabinoxylan), and

17–24% lignin [46, 49]. The EPR spectrum of BcCel5A-RCBM11_{V111R} in the presence of untreated milled sugarcane bagasse (Fig. 5) presents no significant changes as compared to the protein in solution, indicating that there is no interaction with this material. This is consistent with the intrinsic recalcitrance of untreated lignocellulose to enzymatic hydrolysis [50], which is a consequence of the high level of complexity of the plant cell wall [39] that restricts enzyme access to the constitutive polysaccharides.

Lignocellulose binding of BcCel5A-RCBM11_{V111R} measured by EPR-SDSL correlates with reducing sugar release

The different components of the cell wall lignocellulose can be selectively extracted with alternative chemical treatments that break or rearrange the covalent and non-covalent linkages between polysaccharide structures [51]. These treatments reduce the polysaccharide crystallinity and increase the surface area, which facilitates enzyme access resulting in enhanced hydrolysis [52, 53]. Here we have performed a hierarchical cell wall deconstruction with chemical treatments that sequentially modifies the polysaccharide and lignin content of the biomass.

The first treatment is with ethanol and water to remove the soluble sugars and other soluble components to leave the alcohol insoluble residue (AIR). The EPR spectra of the BcCel5A-RCBM11_{V111R} in the presence of this material (Fig. 5) did not present significant differences in the M_s parameter as compared to the free protein control

Fig. 4 **a** Measured EPR spectra of the BcCel5A-RCBM11_{V111R} in solution in the absence and in the presence of increasing concentrations of β -glucan. The dashed lines indicate the spectral lines in BcCel5A-RCBM11_{V111R} spectra that are indicative of the immobilized protein population. **b** The M_s parameter from spectra of the BcCel5A-RCBM11_{V111R} is a function of β -glucan concentration. The light gray line shows the fitted curve which yields a K_a value of 0.23 mg/mL

Fig. 5 **a** Measured spectra of BcCel5A-RCBM11_{V111R} in solution, and in the presence of different fractions of milled sugarcane bagasse or pre-treated fractions. The dashed lines indicate the spectral lines in BcCel5A-RCBM11_{V111R} spectra that are indicative of the immobilized protein population. **b** Total reducing sugar release after treatment of milled sugarcane bagasse or pre-treated fractions with the cysteine mutant BcCel5A-RCBM11

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Apêndice H

RESEARCH ARTICLE

Engineering the GH1 β -glucosidase from *Humicola insolens*: Insights on the stimulation of activity by glucose and xylose

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Abstract

The activity of the GH1 β -glucosidase from *Humicola insolens* (BgH1) against *p*-nitrophenyl- β -D-glucopyranoside (*p*NP-Glc) and cellobiose is enhanced 2-fold by glucose and/or xylose. Kinetic and transglycosylation data showed that hydrolysis is preferred in the absence of monosaccharides. Stimulation involves allosteric interactions, increased transglycosylation and competition of the substrate and monosaccharides for the +1 glycone and the +1/+2 aglycone binding sites. Protein directed evolution has been used to generate 6 mutants of BgH1 with altered stimulation patterns. All mutants contain one of three substitutions (N235S, D237V or H307Y) clustered around the +1/+2 aglycone binding sites. Two mutants with the H307Y substitution preferentially followed the transglycosylation route in the absence of xylose or glucose. The strong stimulation of their *p*NP-glucosidase and cellobiose activities was accompanied by increased transglycosylation and higher monosaccharide tolerance. The D237V mutation favoured hydrolysis over transglycosylation and the *p*NP-glucosidase activity, but not the cellobiose activity, was stimulated by xylose. The substitution N235S abolished the preference for hydrolysis or transglycosylation; the cellobiose, but not the *p*NP-glucosidase activity of the mutants was strongly inhibited by xylose. Both the D237V and N235S mutations lowered tolerance to the monosaccharides. These results provide evidence that the fine modulation of the activity of BgH1 and mutants by glucose and/or xylose is regulated by the relative affinities of the glycone and aglycone binding sites for the substrate and the free monosaccharides.

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Introduction

β -glucosidases (β -D-glucoside glucosylhydrolases [E.C.3.2.1.21]) catalyze the hydrolysis of alkyl- and aryl- β -D-glucosides, cyanogenic glucosides, disaccharides and short oligosaccharides. Found in all domains of living organisms, they are represented in glycosyl hydrolase (GH) families GH1, GH2, GH3, GH5, GH9, GH30, GH39 and GH116 [1–4].

The β -glucosidases have attracted considerable attention due to their potential application in biotechnological processes, particularly the production of bioethanol from lignocellulosic biomass, where they are considered key enzymes for the complete hydrolysis of cellulose [4]. The β -glucosidases play a dual role by catalyzing the hydrolysis of cellobiose and soluble cellobioses to glucose, and by reducing the reaction product inhibition of endo- and exoglucanases [5]. A major limitation for their application in saccharification of lignocellulosic biomass is that many known β -glucosidases are strongly inhibited by glucose, with K_i values in the low millimolar range [1,5–7].

In the current consensus, the enzymatic hydrolysis of cellulose for second generation ethanol production would be performed using high initial solid loading in order to reduce water usage and costs. This results in accumulation of polysaccharide hydrolysis products, where the concentration of glucose may reach hundreds of millimolar [5,6,9]. There is therefore a growing interest in enzymes that are glucose tolerant, and recently β -glucosidases tolerant to, or even stimulated by glucose have been described [8,9–20]. However, the majority of these studies have been conducted with the exclusive use of the synthetic substrate *p*-nitrophenyl β -D-glucopyranoside (*p*NP-Glc), where the tolerance/stimulation by glucose was investigated not by following the liberation of free glucose, but by release of *p*-nitrophenolate [9,12,16–18,20–23].

Most known glucose tolerant or stimulated enzymes belong to the GH1 family. In the retaining catalytic mechanism (Fig 1), one of the two glutamate residues in the GH1 β -glucosidase active site (Fig 1A) acts as an acid/base catalyst and the other acts as a nucleophile [2,29]. The cleavage of the glycosidic bond occurs in two steps, denominated as glycosylation and deglycosylation, respectively. Glycosylation involves the formation (Fig 1, step 1) of a covalent glucosyl-enzyme intermediate (Fig 1B), which is subsequently deglycosylated in a hydrolysis reaction (Fig 1, step 2) through the attack of a water molecule, thereby liberating glucose and restoring the free enzyme (Fig 1C). Under certain conditions, however, the attack on the glucosyl-enzyme intermediate may be carried out not by water, but by another acceptor nucleophile such as a monosaccharide, disaccharide, aryl-, alkyl-alcohol or monoterpene alcohol (Fig 1D), resulting in a transglycosylation reaction (Fig 1, step 3) [30,31].

Although the catalytic mechanism of the GH1 β -glucosidases has been confirmed experimentally [32,33] only recently have the concurrent reactions of hydrolysis and transglycosylation been recognized as being crucial for the understanding of the glucose stimulation of the GH1 β -glucosidases [18,36,27], a property which had previously been attributed to allosteric effects [5,13–15,34] or to a reduction in substrate inhibition [28]. The topology of the active site has also been associated with the tolerance of some enzymes to high concentration of glucose [28,29,35]. Furthermore, a recent theoretical study raised the possibility that competition of inhibitors with the non-productive binding of substrate may be a possible mechanism of glucose/xylose stimulation of β -glucosidases that have more than one glycosyl residue binding subsite in the aglycone binding site [36]. It has been recently proposed that the relative binding affinity/preference by sites at the entrance and middle of the substrate channel regulate the effects of glucose [37].

Filamentous fungi of the genus *Humicola* are excellent producers of various cellulolytic enzymes, including several β -glucosidases [38]. To date, an intracellular GH1 β -glucosidase (BgH1) [14], an extracellular GH3 β -glucosidase (BgH2) [39] and three heterologous GH5 β -glucosidase isoenzymes from *Humicola insolens* expressed in *Pichia pastoris* (HbBg13A, HbBg13B and HbBg13C) [40] have been characterized. With the exception of the BgH2, these

Fig 1. Catalytic reaction mechanism of the retaining β -glucosidase. After the formation of the glucose-enzyme intermediate (step 1), the entry of a water molecule leads to hydrolysis (step 2) and the entry of a sugar leads to transglycosylation (step 3).

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enzymes present high glucose tolerance [14,39,40]. We have recently described the catalytic properties of the Bglh1, which in addition to high glucose and thermal tolerance was also shown to have high catalytic efficiency for cellobiose hydrolysis. Furthermore, catalytic activity against both pNP-Glc and cellobiose was stimulated 2-fold by glucose and/or xylose [14,34], suggesting that this β -glucosidase is therefore an interesting candidate for application in industrial processes for the hydrolysis of lignocellulosic materials. The recombinant Bglh1 enzyme expressed in *Escherichia coli* maintained both an elevated catalytic efficiency for cellobiose hydrolysis and glucose and xylose tolerance/stimulation [15]. In addition, the three-dimensional structure of the Bglh1 has been solved [29], and the enzyme has been useful to study the mechanism of stimulation of GH1 β -glucosidases by the reaction product.

In the present study, a directed evolution approach using error-prone PCR has been applied to create a series of Bglh1 mutants with altered catalytic properties with respect to glucose/xylose stimulation and/or tolerance. The characterization of these mutants, particularly with respect to their action on the natural substrate cellobiose, has contributed to elucidate the structural determinants and the dynamics of the phenomenon of glucose and xylose stimulation of this GH1 β -glucosidase.

Materials and methods

Construction of a random mutant library

The construction of the pET28₊Bglh1 plasmid containing the Bglh1 has been previously described [15], and random mutagenesis of the Bglh1 coding sequence was performed by

error-prone PCR (epPCR) using the pET28₊Bglh1 plasmid as template. The reaction mixture (100 μ L) contained 7 mM MgCl₂, 200 μ M dNTPs, 100 pmol of each universal DNA primers (T7promoter and T7terminator, complementary to the 5' and 3' regions flanking the Bglh1 coding sequence in the pET-28a(+) vector), 200 ng template, 100 μ M MnCl₂ and 5 units (U) of Taq polymerase (Thermo Scientific, Wilmington, USA) and its respective reaction buffer. Thermocycling involved a single initial heating to 95°C for 2 min, followed by 30 cycles of 94°C for 1 min, 45°C for 1 min and 72°C for 3 min and a final heating to 72°C for 10 min. The PCR products were separated by agarose gel electrophoresis and purified by Wizard® SV Gel Extraction kit (Promega, Madison, USA). The extracted DNA fragments were digested with restriction enzymes *Nde*I and *Bam*HI (Thermo Scientific, Waltham, MA, USA), and ligated into the pET-28a(+) vector (Novagen, Madison, WI, USA) linearized with the same enzymes. The random mutant library (denominated pET28₊mut) was used to transform *E. coli* BL21 (DE3) by electroporation. The recombinant plasmid pET28₊Bglh1 was also used to transform *E. coli* BL21 (DE3) as a control for all random mutant library screening steps.

Random mutant library screening

The *E. coli* cells transformed with either the pET28₊mut library, or the pET28₊Bglh1 as positive control, were plated on Luria-Bertani broth (LB)-agar plates containing 40 μ g mL⁻² kanamycin and 34 μ g mL⁻¹ chloramphenicol. After incubation at 37°C for approximately 16 h, individual colonies were transferred to 96-well microplates containing 200 μ L LB medium supplemented with the same antibiotics using an automated colony picker (model K6, Khiosystems, Basildon, Essex, UK). The 96-well plates were incubated at 37°C for 16 h, and replicated onto 250 $\times$ 250 mm bioassay plates containing LB-agar including the same antibiotics, 50 μ mol L⁻¹ isopropyl- β -D-1-thiogalactopyranoside (IPTG), 1 g L⁻¹ esculin and 0.3 g L⁻¹ ferric chloride. After incubation of the plates at 37°C for 16 h, the clones expressing β -glucosidase activity were identified by the formation of dark halos surrounding the colonies. These colonies were manually transferred to two separate 96-well microplates containing 200 μ L LB-medium, specific antibiotics and IPTG at the same concentrations. After incubation of the plates at 37°C for 16 h, one of the plates was supplemented with 20% (v/v) glycerol and stored at -80°C. The other plate was centrifuged at 4000 $\times$ g for 15 min, and the supernatants were used for enzymatic assays in 96-well plates at 50°C. After the extended time of expression, spontaneous cell lysis releases proteins that can be detected in the culture supernatant. After adequate dilution, ten-microliter aliquots of each culture supernatant were mixed with 80 μ L of a reaction medium containing 2 mM pNP-Glc in 50 mM Bis-Tris buffer, pH 6.0, in the presence or absence of 100 mM glucose or xylose. After 5 min the reactions were interrupted by the addition of 160 μ L of a saturated sodium tetraborate solution and the pNP-glucosidase activities were determined (see the "Enzymatic assays" section). For each selected clone, two stimulation factors (SF) were calculated from the measured activities: SF_{glu}, which was defined as the ratio of the activity in the presence of 100 mM glucose to the activity without glucose; and SF_{xy}, which was defined as the activity at 100 mM xylose to the activity without xylose. The wild type Bglh1 (SF_{glu} = 1.8, SF_{xy} = 2.0) was employed as a control throughout the screening procedure, and those clones with an SF lower than 1.3 or greater than 2.2 were selected and the mutations were identified by nucleotide sequencing of the plasmid DNA. Each enzymatic assay was performed in duplicate and the experiments were repeated three times with each monosaccharide (glucose and xylose). The SF values presented in the accompanying tables are the mean $\pm$ SD for these triplicate measurements (n = 3).

Site-directed mutagenesis

The single mutation H307Y was generated by PCR based site-directed mutagenesis [41], where the PCR reaction contained 10 ng of plasmid pET28_{hglhi} as template, 30 pmol of the primers 5'-GGCATGAACTACTACACGCGCACTACATCAAGCAG-3' (forward) and 5'-CGTGTAGTAACTTATGCGGTAGAACTGCTTGAAGCC-3' (reverse), 200 μ M dNTPs and 2.5 U of *Pfu* DNA polymerase (Thermo Scientific, Waltham, MA, USA) and its respective buffer. The PCR was performed with initial denaturation of the template DNA at 95°C for 3 min, followed by 30 cycles of 95°C for 1 min, 50°C for 1 min, 72°C for 1 min and a final extension step at 72°C for 10 min. After PCR, the product was incubated with 2 U of *DpnI* (Thermo Scientific, Waltham, MA, USA) at 37°C for 3 h to digest the methylated template DNA. One microliter of the *DpnI* digested sample was used to transform *E. coli* DH5 α . After nucleotide-sequencing confirm the mutation, the plasmid (denominated pET28_{hglhi}) was subsequently used to transform *E. coli* BL21 (DE3) for the expression of the recombinant enzyme (see the "Protein expression and purification" section).

Protein expression and purification

The *hglhi* and the selected mutants were overexpressed in *E. coli* BL21 (DE3) after transformation with the plasmids pET-28a(+) containing the respective coding sequences. The cells were grown in HDM medium (25 g L⁻¹ yeast extract, 15 g L⁻¹ tryptone and 10 mM MgSO₄) supplemented with specific antibiotics at 37°C to an OD₆₀₀ of approximately 0.6. Protein expression was induced by adding IPTG to the culture media at a final concentration of 1 mM. After 5 h induction at 37°C, the cells were collected by centrifugation at 5000 $\times$ g for 20 min at 4°C. The pellets were resuspended in lysis buffer (50 mM HEPES buffer, pH 8.0, containing 500 mM NaCl and 1% (v/v) Triton X-100) and disrupted by sonication. Cell debris were removed by centrifugation and the recombinant proteins were purified from the supernatant using nickel affinity chromatography (HisLink[®], Promega), according to the manufacturer's instructions. The protein concentrations were determined according to Read and Northcote [42], using bovine serum albumin as the standard.

Electrophoresis

Electrophoresis under denaturing conditions (SDS-PAGE) was performed in 10% acrylamide slab gels, according to Laemmli [43], using a pre-stained molecular weight marker (6.1–204.3 kDa, Sigma-Aldrich Chem. Co., St Louis, MO, USA). The DNA analyses were performed in 1% (w/v) agarose gels (Agarose, Madrid, Spain) containing 0.02% SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA), and the bands were visualized under UV light.

Enzymatic assays

The pNP-glucosidase activity of *hglhi* and mutants was defined as the rate of liberation of *p*-nitrophenolate ion (pNP) and was assayed spectrophotometrically as previously described [44]. The first reaction step (formation of the glycosyl-enzyme intermediate, Fig 1) releases *p*-Nitrophenol, and is a product that is common to both the hydrolysis and the transglycosylation routes. Unless otherwise stated, the pNP-glucosidase activity was determined at 90–95% saturating substrate concentrations, which were determined for each enzyme (2 mM for *hglhi*, N89Y/H307Y and H307Y; 1.5 mM N235S, A141T/N235S and D237V/P389H/E395G/K475R, and 3 mM for D237V). Standard assays conditions were 50°C and 50 mM Bis-Tris buffer, pH 6.0, for *hglhi*, N89Y/H307Y and H307Y; 50°C and McIlwaine buffer [44], pH 5.5 or 5.0, for the

N235S and A141T/N235S mutants, respectively; 45°C and 50 mM sodium acetate buffer, pH 5.5 or 5.0, for D237V/P389H/E395G/K475R and D237V, respectively.

The cellobiose activity was defined as the rate of free glucose liberation with cellobiose as substrate, where a free glucose molecule is the first product released in the glycosylation step of the catalytic cycle (Fig 1 step 1), and an additional free glucose is generated by the hydrolysis of the glycosyl-enzyme adduct (Fig 1 step 2). When transglycosylation occurs (Fig 1 step 3) oligosaccharides are generated, resulting in reduced free glucose/cellobiose ratios and the accumulation of cello-oligosaccharides. Unless otherwise stated, the cellobiose activity was assayed under the same pH and temperature conditions as described for the pNP-glucosidase activity and at 90–95% saturating cellobiose substrate conditions (5 mM for *hglhi*; 40 mM for N89Y/H307Y, 70 mM for H307Y and D237V; 1 mM for N235S; 2 mM for A141T/N235S and 10 mM for D237V/P389H/E395G/K475R). The reactions were interrupted by boiling for 10 min and the free glucose concentration was quantified using the glucose assay kit GAHK20 (Sigma-Aldrich Chem. Co., St Louis, MO, USA) according to the manufacturer's instructions.

The experimental conditions (reaction times, enzymatic units) for the activity measurements were adjusted for each mutant to guarantee the estimation of initial velocities (linear response of product formation with the reaction time). One enzyme unit (U) was defined as the amount of enzyme that releases 1 μ mol of product per min. Specific activity was defined as U per milligram total protein (U mg⁻¹).

Determination of kinetic parameters

The kinetic parameters for the release of pNP from pNP-Glc and the release of free glucose from cellobiose by *hglhi* and mutants were calculated using the SigafW Software which fits experimental data to the Hill equation using non-linear regression [45]. For all enzymes, values were estimated for the V_{max} (maximum velocity), $K_{M,pNP-Glc}$ (enzyme-substrate apparent dissociation constant with the pNP-Glc substrate), $K_{M,cellobiose}$ (enzyme-substrate apparent dissociation constant with the cellobiose substrate), $K_{0.5}$, (the glucose concentration needed for half-maximal activation of the enzyme), $K_{0.5x}$ (the xylose concentration needed for half-maximal activation of the enzyme) and n_H (the Hill coefficient). The k_{cat} was defined as the number of molecules of pNP or free glucose liberated from pNP-Glc or cellobiose, respectively, by one molecule of enzyme per second. The IC_{50} was defined as the concentration of glucose or xylose that inhibited 50% of the control activity determined at 90–95% saturating substrate concentration and in the absence of glucose or xylose, and was determined graphically from plots of activity as a function of glucose or xylose concentration.

All experimental kinetic curves were repeated three times using different pure recombinant enzyme preparations, where each experimental point was assayed in duplicate. The kinetic parameters presented are calculated values and are given as the mean $\pm$ SD of the three replicates ($n = 3$). Data fitting and statistical analyses were carried out using the OriginPro 8.5.0 software package (OriginLab Corp., Northampton, MA, USA).

Determination of the ratios of pNP-Glc hydrolysis and transglycosylation

The determination of the rates of pNP and free glucose release from pNP-Glc allows the estimation of the probabilities of the hydrolysis and the transglycosylation reactions catalyzed by a given mutant enzyme under particular experimental conditions. These probabilities are expressed as the fractions of reactions resulting in hydrolysis (%H) or transglycosylation (%T) [46].

To determine the effect of xylose on the values of %H and %T, enzymatic reactions were performed (see the "Enzymatic assays" section) in the absence or presence of xylose at a concentration that resulted in maximal stimulation of the enzymatic activity (MC_{max}), which

corresponded to 100 mM for BglhI, 150 mM for the N89Y/H307Y and H307Y mutants, 200 mM for the D237V mutant, and 80 mM for the N235S, A141T/N235S and D237V/P389H/E395G/K475R mutants. For each enzyme, total enzyme units and time intervals were adjusted to achieve approximately 5% substrate consumption by the end of the experimental reaction (in the absence of xylose). The reactions were interrupted by boiling for 10 min and the sample volume reduced from 600 μ L to 100 μ L using a vacuum concentrator (Concentrator Plus 5301, Eppendorf Inc., NY, USA) at 45°C. The concentration of pNP in each reaction medium was determined spectrophotometrically according to Souza et al. [16] while free glucose was quantified using the glucose assay kit GAHK20 (Sigma-Aldrich) according to the manufacturer's instructions. The enzymatic assays were repeated six times and the %H and %T values are given as means $\pm$ SD of the six calculated values ($n = 6$).

Analysis of the reaction products of BglhI and mutants

The reaction products resulting from the action of the wild type BglhI and mutants against cellobiose were analyzed by thin layer chromatography (TLC) and mass spectrometry (MS). The reactions were performed at 90–95% saturating cellobiose concentration in the absence of xylose and glucose. Subsequently reactions were performed under various conditions as follows: i) in the presence of xylose or glucose at MC_{max} (100 mM for BglhI, 200 mM for the N89Y/H307Y mutant and 500 mM for H307Y); ii) in the presence of xylose or glucose at the maximal concentration that did not apparently inhibit the enzymatic activity (maximum tolerance, MT), corresponding to 30 mM for D237V/P389H/E395G/K475R, 300 mM for D237V, 50 mM for A141T/N235S and 30 mM for N235S. The reactions were performed as described in the "Enzymatic assays" section, except that the reaction buffer was 10 mM ammonium acetate, pH 5.5. The total enzyme units of all enzymes were adjusted to achieve 5% substrate consumption after 5 min on the basis of glucose liberation in the absence of xylose. The time course of the formation of hydrolysis and transglycosylation products was followed from 10 min to 24 h.

TLC analyses were carried out on silica gel plates (DC-Alufolien Kieselgel 60, Merck, Darmstadt, Germany) at room temperature using ethyl acetate/acetic acid/formic acid/water (9:3:1:4, v/v/v/v) as the mobile phase. Volumes of the samples were spotted on the TLC plates, along with glucose (G_1), cellobiose (G_2), cellobiose (G_3), cellobiose (G_4), xylose (X_1), xylobiose (X_2), gentiobiose (G_6) and an equimolar mixture of sophorose (S) and cellobiose (G_2) as standards. After two runs, the products were detected by spraying the plates with 0.4% (w/v) orcinol in sulfuric acid/ethyl alcohol (1:9 v/v) followed by heating at 150°C to reveal the individual spots.

MS analyses were performed on a Xevo® TQ-S instrument (Waters Corporation, Milford, MS, USA) with an electrospray ionization source operated in positive mode. Five μ L aliquots of the reaction samples were introduced into the mass spectrometer by direct insertion in acetonitrile/formic acid 0.1% (50% v/v) at a flow rate of 0.1 mL min⁻¹. The ESI interface conditions were as follows: capillary voltage 3.2 kV, cone voltage 40 V, Z-spray source temperature 150°C, desolvation gas (N_2) temperature 250°C, desolvation gas flow 600 L h⁻¹ (mass range from m/z 100 to 1200). The experiments of tandem mass spectrometry (MS/MS) on precursor ions of interest were performed by collision-induced dissociation (CID) using argon gas as the collision gas. The collision energy was over the range from 10 to 30 eV. Data acquisition and processing were performed using the MassLynx V4.1 (Waters Corporation).

Results

Screening of random mutant library

Approximately 4,400 bacterial colonies were screened for β -glucosidase activity, and 418 clones that expressed active enzymes were identified. Screening of these 418 clones identified

Table 1. Characterization of the selected mutants.

Mutant	Mutation	Amino acid	Substitution	SF	
				Glucose	Xylose
N235S	754 A $\rightarrow$ G	235	Asn $\rightarrow$ Ser	1.16	1.96
	717 C $\rightarrow$ T	*	*		
A141T/N235S	421 Q $\rightarrow$ A	141	Ala $\rightarrow$ Trp	1.12	1.05
	754 A $\rightarrow$ G	235	Asn $\rightarrow$ Ser		
D237V	710 A $\rightarrow$ T	237	Asp $\rightarrow$ Val	1.28	1.18
D237V/P389H/E395G/K475R	710 A $\rightarrow$ T	237	Asp $\rightarrow$ Val	1.17	1.16
	679 C $\rightarrow$ T	*	*		
	1146 G $\rightarrow$ A	389	Pro $\rightarrow$ His		
	1184 A $\rightarrow$ G	395	Glu $\rightarrow$ Gly		
N89Y/H307Y	1424 A $\rightarrow$ G	475	Lys $\rightarrow$ Arg		
	265 A $\rightarrow$ T	89	Asn $\rightarrow$ Tyr	2.51	2.71
	919 C $\rightarrow$ T	307	His $\rightarrow$ Tyr		

* Silent mutations

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mutants showing pronounced differences in the stimulation of the pNP-glucosidase activity by glucose and xylose as compared to the wild type BglhI. Five mutants exhibiting SF_{glc} and/or SF_{xy} <1.3 or >2.2 were selected (Table 1). Nucleotide sequence analysis of these 5 mutants showed a mutation rate of 1.7 ± 0.5 bp per kb with amino acid substitutions ranging from one to four per mutant.

Structural mapping, overexpression and purification of BglhI and its mutants

The positions of the amino acid substitutions were mapped onto the BglhI crystal structure (PDB 4MDD; [20]) (Fig 2). All selected mutants showed at least one mutation in the active site region of BglhI, and additional peripheral substitutions occurred in mutants D237V/P389H/E395G/K475R, N89Y/H307Y and A141T/N235S. The mutant D237V showed a single substitution that was identical to that found in the active site region of D237V/P389H/E395G/K475R, while N235S showed the same substitution present in the active site region of A141T/N235S. The single mutant H307Y was constructed by site-directed mutagenesis to contain the same substitution present in the active site region of N89Y/H307Y. The wild type BglhI, and the mutants N89Y/H307Y, H307Y, D237V/P389H/E395G/K475R, D237V, A141T/N235S and N235S were overexpressed in *E. coli* BL21 (DE3) as soluble proteins and purified to homogeneity (S1 Fig).

Biochemical characterization and kinetic analysis of BglhI and mutants using pNP-Glc as substrate

The pNP-glucosidase activity of BglhI and mutants was analyzed between 30 to 65°C and over the pH range 4.0 to 8.0 (S2 Fig). The BglhI activity gradually increased from 30 to 55°C, reaching a maximum at 60°C and abruptly decreasing at higher temperatures. Similar profiles were observed for the H307Y, A141T/N235S and N235S mutants, although with the maximum activity at 55°C. In contrast, alterations in the temperature/activity profiles were observed for the other mutants, with plateaus of maximum catalytic activity from 50–60°C, 40–50°C and 35–50°C for N89Y/H307Y, D237V/P389H/E395G/K475R and D237V, respectively. Similar

Fig 2. Positions of the mutations inserted in BglhI in each mutant. The 3D structures of (a) N235S, (b) A141T/N235S, (c) D237V/P389H/E395G/K475R, (d) D237V, (e) N89Y/H307Y and (f) H307Y were modeled using the crystal structure of BglhI (PDB code: 4MDC) as template with the MODELLER 9.10 software. The amino acid substitutions identified by random mutagenesis and functional selection are indicated as yellow spheres in Fig 2. The catalytic residues (E166 and E377), the glycone and the aglycone-binding sites are shown as red, green and blue spheres, respectively. The inset shows a ribbon representation of the +1/+2 aglycone binding region of the wild-type BglhI (in green) showing the catalytic residues (E166 and E377), together with the mutants N235S (in magenta), D237V (in light blue) and H307Y (in yellow). The highly conserved positions of the main chain atoms resulted in the mottled appearance of the main chain after superposition of the different structures.

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profiles were observed for the pH optima for BglhI, N89Y/H307Y and H307Y, with maximum activity plateaus from pH 5.0 to 7.0. However, catalytic activity profiles of as a function of pH were more restricted for D237V/P389H/E395G/K475R, D237V, A141T/N235S and N235S, with maximal activities at pH 5.5, 5.0, 5.0 and 5.5, respectively.

The kinetic parameters for the effect of pNP-Glc on the pNP-glucosidase activity of BglhI and mutants in the absence or in the presence of xylose or glucose are presented in Fig 3A and Table 2. In the absence of monosaccharides, the stimulation of the wild type BglhI and all mutants occurred as single curves following non-Michaelis-Menten kinetics ($n_H > 1$). Similar maximum velocities were observed for BglhI, N89Y/H307Y, H307Y, A141T/N235S and

Fig 3. Effect of increasing concentrations of pNP-Glc, glucose and xylose on the pNP-glucosidase activity. (a) Bglh, (b) N89Y/H307Y, (c) H307Y, (d) D237V/P389H/E395G/K475R, (e) D237V, (f) A141T/N235S, (g) N235S. (A) Effect of increasing concentrations of pNP-Glc on the pNP-glucosidase activity in the absence (black lines and dots) or in the presence of glucose (red lines and dots) or xylose (blue lines and dots) of fixed concentrations equal to $K_{M,glucose}$. (B) Effect of increasing concentrations of glucose (red lines and dots) or xylose (blue lines and dots) on the pNP-glucosidase activity of each mutant enzyme at 90–95% saturating concentrations of pNP-Glc (2 mM for Bglh, N89Y/H307Y and H307Y, 1.5 mM for D237V/P389H/E395G/K475R, A141T/N235S and N235S; 3 mM for D237V). All experiments were repeated three times using three separate pure enzyme preparations. Each point represents the mean of duplicate assays $\pm$ SD (error bars are not evident, as they lie within the area of the symbols).

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N235S. In contrast, V_{max} values about 1.5-fold higher than the Bglh were determined for D237V/P389H/E395G/K475R and D237V. With the exception of D237V, which showed a $K_{M,pNP-Glc}$ value about 1.3-fold higher, all other enzymes showed lower $K_{M,pNP-Glc}$ values, as compared to the wild type Bglh. The efficiency of substrate utilization ($k_{cat}/K_{M,pNP-Glc}$) determined for A141T/N235S was similar to that for the Bglh. In contrast, catalytic efficiencies 1.8, 1.2-, 2.1-, 1.2- and 2.1-fold higher were estimated for N89Y/H307Y, H307Y, D237V/P389H/E395G/K475R, D237V and N235S, respectively.

Fig 3B shows the effect of increasing glucose or xylose concentrations over the range 0–1000 mM on the pNP-glucosidase activity of wild type Bglh and mutants at 90–95% saturating concentrations of substrate for each enzyme. In agreement with previous studies, the

Table 2. Kinetic parameters for the stimulation of the pNP-glucosidase activity of Bglh and mutants against pNP-Glc in the absence or presence of fixed concentrations of glucose or xylose.

Enzyme	(Monosaccharide) (mM)	V_{max} (μ mol min ⁻¹)	$K_{M,pNP-Glc}$ (mM)	$k_{cat}/K_{M,pNP-Glc}$ (s ⁻¹ mM ⁻¹)	n_H
Bglh	0	37.3 $\pm$ 1.8	0.22 $\pm$ 0.01	158.53 $\pm$ 15.89	1.2
	Glucose (50)	72.1 $\pm$ 2.9	0.50 $\pm$ 0.02	154.83 $\pm$ 11.57	1.6
	Xylose (100)	77.3 $\pm$ 3.9	0.54 $\pm$ 0.03	133.84 $\pm$ 15.17	1.6
N89Y/H307Y	0	34.0 $\pm$ 1.7	0.11 $\pm$ 0.01	289.00 $\pm$ 43.55	1.1
	Glucose (100)	83.7 $\pm$ 3.7	0.43 $\pm$ 0.02	203.74 $\pm$ 18.74	1.1
	Xylose (100)	110.9 $\pm$ 3.3	0.37 $\pm$ 0.02	181.91 $\pm$ 12.62	1.1
H307Y	0	30.9 $\pm$ 1.6	0.16 $\pm$ 0.01	192.26 $\pm$ 22.85	1.1
	Glucose (100)	103.1 $\pm$ 6.1	0.56 $\pm$ 0.03	172.14 $\pm$ 20.78	1.2
	Xylose (100)	103.2 $\pm$ 7.2	0.57 $\pm$ 0.04	169.29 $\pm$ 25.34	1.3
D237V/P389H/E395G/K475R	0	54.7 $\pm$ 3.8	0.15 $\pm$ 0.01	340.96 $\pm$ 49.64	1.1
	Glucose (50)	58.3 $\pm$ 2.8	0.50 $\pm$ 0.03	109.02 $\pm$ 12.80	1.1
	Xylose (80)	77.7 $\pm$ 4.7	0.36 $\pm$ 0.02	191.18 $\pm$ 23.13	1.2
D237V	0	55.5 $\pm$ 3.3	0.28 $\pm$ 0.02	195.33 $\pm$ 25.84	1.2
	Glucose (100)	73.0 $\pm$ 5.1	0.35 $\pm$ 0.07	71.85 $\pm$ 11.03	1.4
	Xylose (200)	89.6 $\pm$ 6.3	0.52 $\pm$ 0.04	161.11 $\pm$ 25.37	1.3
A141T/N235S	0	27.1 $\pm$ 1.3	0.16 $\pm$ 0.01	158.37 $\pm$ 18.71	1.1
	Glucose (30)	36.7 $\pm$ 2.3	0.31 $\pm$ 0.02	116.72 $\pm$ 15.47	1.1
	Xylose (80)	37.6 $\pm$ 2.2	0.40 $\pm$ 0.02	87.89 $\pm$ 10.20	1.1
N235S	0	35.9 $\pm$ 2.8	0.10 $\pm$ 0.01	355.67 $\pm$ 64.90	1.3
	Glucose (30)	47.4 $\pm$ 3.8	0.22 $\pm$ 0.02	201.45 $\pm$ 36.86	1.3
	Xylose (80)	54.9 $\pm$ 3.8	0.32 $\pm$ 0.02	163.41 $\pm$ 22.60	1.1

Glucose and xylose concentrations (in brackets) are equal to the MC_{50} presented in Table 3. Each experimental kinetic curve was repeated three times, using three separate preparations of the pure enzyme. Each activity assay was performed in duplicate. The kinetic parameters are the means $\pm$ SD of the values calculated for each repetition ($n = 3$).

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Table 3. Glucose- and xylose-stimulation of the pNP-glucosidase activity of Bglh and mutants.

Enzyme	[pNP-Glc] (mM)	Monosaccharide	$K_{M,glucose}$ or $K_{M,xylose}$ (mM)	SF (fold)	$MC_{50\%}$ (mM)	MT (mM)	n_H
BglH	2	Glucose	10.9 ± 0.5	1.9	50	~ 400	1.2
		Xylose	17.8 ± 1.1	2.1	100	~ 750	1.2
N89Y/H307Y	2	Glucose	28.5 ± 1.7	2.8	100–200	~ 1000	1.2
		Xylose	31.1 ± 2.5	3.3	150–200	~ 1000	1.4
H307Y	2	Glucose	42.1 ± 2.1	3.1	100–200	~ 1000	1.2
		Xylose	29.7 ± 1.6	3.1	150–200	> 1000	1.2
D237V/P389H	1.5	Glucose	–	–	50	–	–
Xylose		16.7 ± 0.9	1.4	80	~ 340	1.1	
E395G/K475R	3	Glucose	18.7 ± 1.1	1.3	80–100	300	1.3
		Xylose	36.2 ± 2.9	1.6	150–200	~ 500	1.4
D237V	1.5	Glucose	3.4 ± 0.2	1.4	30	150	1.2
		Xylose	8.7 ± 0.3	1.4	50–80	500	1.3
A141T/N235S	1.5	Glucose	5.8 ± 0.3	1.3	20–30	150	1.2
		Xylose	6.7 ± 0.1	1.5	30–40	500	1.1

Each experimental curve was repeated three times, using three separate preparations of the pure enzyme under 90–95% saturating substrate; each activity assay was performed in duplicate. The kinetic parameters ($K_{M,glucose}$, $K_{M,xylose}$ and n_H) were determined at increasing concentrations of glucose or xylose within the stimulatory range ($0-MC_{50}$), and are given as means $\pm$ SD of the values calculated for each repetition ($n = 3$).

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release of pNP by Bglh was stimulated up to 1.9- and 2.1-fold by glucose and xylose, respectively, with MC_{50} values for glucose and xylose of 50 and 100 mM, respectively (Table 3). However, different profiles for the stimulation of pNP liberation by glucose or xylose were observed for the mutant enzymes. The pNP-glucosidase activity of the N89Y/H307Y and H307Y mutants was stimulated up to 2.8- and 3.1-fold by 100–200 mM glucose, and up to 3.3- and 3.1-fold by 150–200 mM xylose. In contrast, the other mutants presented lower SF values for both monosaccharides as compared to the wild type Bglh. Remarkably, although the D237V/P389H/E395G/K475R mutant was not stimulated by glucose, this enzyme showed a SF₂₀₀ of about 1.4. Furthermore, the D237V/P389H/E395G/K475R, D237V, A141T/N235S and N235S mutants presented high variability of the MC_{50} for glucose and xylose (Table 3).

In addition to the differences in the stimulatory effects, the mutant enzymes also showed variable tolerance to xylose and glucose, as reflected by their MT values (Table 3). While the N89Y/H307Y and H307Y mutants presented increased tolerance to both monosaccharides as compared to Bglh, the other mutants showed significantly reduced tolerance, particularly to glucose. This corroborates with a quantitative analysis of glucose tolerance based on the IC_{50} values determined for each enzyme, which revealed values of 780 mM for Bglh, and > 1 M for N89Y/H307Y and H307Y, contrasting with 400, 580, 800 and 450 mM for D237V/P389H/E395G/K475R, D237V, A141T/N235S and N235S, respectively. For xylose, however, the IC_{50} values were > 1 M for Bglh, N89Y/H307Y and H307Y, and 790, 870, 1000 and 920 mM for D237V/P389H/E395G/K475R, D237V, A141T/N235S and N235S, respectively.

The kinetic analysis of the effects of increasing concentrations of glucose and xylose over the stimulatory range (i.e. $0-MC_{50}$) on the activity of Bglh and mutants revealed that the stimulation occurred through non-Michaelis-Menten kinetics ($n_H > 1$, Table 3). The values of $K_{M,glucose}$ and $K_{M,xylose}$ determined for the N89Y/H307Y and H307Y mutants were about 2.0 to 4.0-fold higher than those estimated for the wild type Bglh. In contrast, the A141T/N235S and N235S mutants showed reduced $K_{M,glucose}$ and $K_{M,xylose}$ values as compared to Bglh. Remarkably, the

Fig 6. Effect of increasing concentrations of cellobiose and xylose on the cellobiase activity. (a) BglhI, (b) N89Y/H307Y, (c) H307Y, (d) D237V/P389H/E395G/K475R, (e) D237V, (f) A141T/N235S, (g) N235S. (A) Effect of increasing concentrations of cellobiose on the cellobiase activity. In the absence (black line and dots) or in the presence of fixed concentrations of xylose (blue lines and dots). The fixed concentrations of xylose were equal to $MC_{50\%}$ (BglhI, N89Y/H307Y and H307Y) or MT (D237V/P389H/E395G/K475R, D237V, A141T/N235S and N235S). (B and C) Effect of increasing concentrations of xylose on the cellobiase activity of each enzyme at 90–95% saturating concentrations of cellobiose (5 mM for BglhI, 40 mM for N89Y/H307Y, 70 mM for H307Y and D237V, 10 mM for D237V/P389H/E395G/K475R, 2 mM for A141T/N235S and 1 mM for N235S). The red asterisks (*) in Fig C indicate the MT concentrations. The experiments were repeated three times using three separate pure enzyme preparations. Each point represents the mean of duplicate assays $\pm$ SD (note that the error bars are not visible, as they lie within the area of the symbols).

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D237V/P389H/E395G/K475R mutant showed a similar K_{cat} as compared to BglhI but apparently does not present a stimulatory glucose binding site, while the D237V mutant presented K_m values about 2.6-fold higher for both monosaccharides.

Fig 5A shows the effect of increasing concentrations of pNP-Glc on the activity of BglhI and mutants in the presence of fixed concentrations of glucose or xylose corresponding to $MC_{50\%}$ (except for D237V/P389H/E395G/K475R, where the glucose concentration was equal to MT), and the kinetic parameters are summarized in Table 2. The presence of either monosaccharide resulted in increased values of K_{cat}/K_m in all mutants (Table 2), indicating that even at a concentration in which their stimulatory effect is maximal, both glucose and xylose compete with

Table 4. Kinetic parameters for the stimulation of the cellobiase activity of BglhI and mutants by cellobiose in the presence or absence of fixed concentrations of xylose.

Enzyme	[Xylose] (mM)	V_{max} (U mg ⁻¹)	$K_{m,xylose}$ (mM)	$K_{cat}/K_{m,xylose}$ (s ⁻¹ mM ⁻¹)	n_H
BglhI	0	213.5 $\pm$ 6.4	0.53 $\pm$ 0.02	375.29 $\pm$ 19.67	1.4
	100	499.5 $\pm$ 15.1	1.75 $\pm$ 0.05	265.36 $\pm$ 16.64	1.5
N89Y/H307Y	0	184.9 $\pm$ 11.1	2.41 $\pm$ 0.15	72.03 $\pm$ 9.38	0.6
	200	314.6 $\pm$ 18.8	5.92 $\pm$ 0.31	56.83 $\pm$ 7.63	1.1
H307Y	0	175.4 $\pm$ 12.3	5.11 $\pm$ 0.36	32.34 $\pm$ 4.94	0.6
	500	348.2 $\pm$ 24.1	12.0 $\pm$ 0.86	27.13 $\pm$ 4.07	1.1
D237V/P389H/E395G/K475R	0	37.1 $\pm$ 3.3	2.23 $\pm$ 0.21	15.77 $\pm$ 3.05	1.2
	20	37.1 $\pm$ 3.4	5.44 $\pm$ 0.48	6.42 $\pm$ 1.24	1.1
D237V	0	85.6 $\pm$ 4.3	3.01 $\pm$ 0.15	28.74 $\pm$ 2.85	1.1
	300	87.2 $\pm$ 4.4	10.12 $\pm$ 0.51	8.07 $\pm$ 0.87	1.1
A141T/N235S	0	83.1 $\pm$ 3.6	0.34 $\pm$ 0.02	173.53 $\pm$ 22.09	1.6
	50	95.4 $\pm$ 4.6	0.85 $\pm$ 0.06	71.94 $\pm$ 10.84	1.6
N235S	0	72.5 $\pm$ 3.3	0.32 $\pm$ 0.01	225.96 $\pm$ 17.39	2.0
	30	72.8 $\pm$ 2.9	0.52 $\pm$ 0.03	131.08 $\pm$ 13.66	1.5

Xylose concentrations correspond to $MC_{50\%}$ (BglhI, N89Y/H307Y and H307Y) or MT (D237V/P389H/E395G/K475R, D237V, A141T/N235S and N235S). Each experimental kinetic curve was repeated three times, using three separate preparations of the pure enzyme, where each activity assay was performed in duplicate. The kinetic parameters are given as means $\pm$ SD of the values calculated for each repetition ($n = 3$).

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pNP-Glc for binding to the catalytic site. Despite these common properties, the patterns of competition between the substrate and the monosaccharides were different for each mutant.

Kinetic analysis of BglhI and mutants using cellobiose as substrate

The cellobiase activity of BglhI was stimulated by cellobiose through non-Michaelis-Menten kinetics (Fig 4 and Table 4). Apparent positive cooperativity was observed for the stimulation of BglhI, and the A141T/N235S and N235S mutants ($n_H > 1$), while an apparent negative cooperativity was seen for the stimulation of the N89Y/H307Y and H307Y mutants (Table 4), possibly reflecting the accumulation of transglycosylation products that resulted in a gradual increase of the rate of free glucose liberation as the substrate concentration increased. The V_{max} for cellobiase activity of the N89Y/H307Y and H307Y mutants was essentially unaltered as compared to the wild type BglhI. In contrast, all other mutants presented lower V_{max} values. Furthermore, the $K_{m,xylose}$ values obtained for the A141T/N235S and N235S mutants were ~ 1.6 -fold lower as compared to the BglhI. In contrast, the N89Y/H307Y, H307Y, D237V/P389H/E395G/K475R and D237V mutants showed higher $K_{m,xylose}$ than BglhI (Table 4).

The effect of increasing xylose concentrations (0–1000 mM) on the cellobiase activity of BglhI and mutants was also investigated (Fig 4B and 4C). Xylose stimulated the release of free glucose by BglhI, N89Y/H307Y and H307Y by 230%, 170% and 200%, respectively, with $MC_{50\%}$ values of 100, 200 and 500 mM, respectively. Above the $MC_{50\%}$, a decreased specific activity was observed, and in the presence of 1000 mM xylose BglhI exhibited 76% of the control activity, while the N89Y/H307Y and H307Y mutants maintained activities above the control (Fig 4B). Although glucose liberation by the other mutants was not increased in the presence of xylose (Fig 4B and 4C), tolerance to the monosaccharide was maintained, and the activities of the D237V/P389H/E395G/K475R, D237V, A141T/N235S and N235S mutants remained almost constant up to xylose concentrations of ~ 30 , 300, 50 and 30 mM, respectively

Table 5. Rates of pNP and glucose liberation and percentages of hydrolysis (%H) and transglycosylation (%T) of pNP-Glc by BglhI and mutants.

Enzyme	No xylose				MC ₅₀₀			
	pNP (nmol)	Glucose (nmol)	%H	%T	pNP (nmol)	Glucose (nmol)	%H	%T
BglhI	12.9 ± 1.1	11.1 ± 1.2	85.5 ± 7.1	14.5 ± 7.1	24.9 ± 2.1	8.1 ± 0.7	32.4 ± 2.5	67.6 ± 2.5
N89Y/H307Y	12.2 ± 1.2	3.8 ± 0.2	30.9 ± 2.9	69.1 ± 2.9	31.5 ± 2.9	6.3 ± 0.6	30.0 ± 1.8	69.0 ± 1.8
H307Y	12.8 ± 1.0	4.3 ± 0.2	33.2 ± 2.7	66.8 ± 2.7	33.7 ± 2.8	7.3 ± 0.8	21.7 ± 1.9	78.3 ± 1.9
D237V/P389H/E395G/K475R	11.0 ± 0.9	10.9 ± 0.8	90.5 ± 8.5	9.5 ± 8.5	15.9 ± 1.1	10.9 ± 1.0	68.8 ± 5.4	31.2 ± 5.4
D237V	8.8 ± 0.6	8.4 ± 0.4	96.0 ± 8.9	2.0 ± 8.9	12.9 ± 1.0	5.5 ± 0.2	42.8 ± 3.7	57.2 ± 3.7
A141T/N235S	8.4 ± 0.5	2.9 ± 0.1	34.7 ± 2.2	65.3 ± 2.2	11.1 ± 0.9	1.8 ± 0.1	16.1 ± 1.1	83.9 ± 1.1
N235S	10.2 ± 0.9	5.0 ± 0.1	48.8 ± 3.5	51.2 ± 3.5	14.5 ± 1.1	4.5 ± 0.2	31.0 ± 2.1	69.0 ± 2.1

The assays were repeated 6 times. pNP and glucose are expressed as total quantity (in nmols) released after 5 minutes. The %H and %T are given as the means ± SD of the values calculated for each assay (n = 6).

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(MT values are indicated by the red asterisks in Fig 4C). Above the MT, however, the activities of the D237V/P389H/E395G/K475R, A141T/N235S and N235S mutants decreased rapidly with IC₅₀ values of ~200, 270 and 170 mM xylose, which are significantly lower than those determined using pNP-Glc as substrate. The D237V mutant presented 70% of the control activity in the presence of 1000 mM xylose.

The stimulation of the cellobiose activity of BglhI, N89Y/H307Y and H307Y by xylose within the stimulatory range (0–MC₅₀₀) and at 90–95% saturating concentrations of cellobiose occurred through non-Michaelis-Menten kinetics ($n_H > 1$). The values of K_{0.5M} determined for BglhI and the N89Y/H307Y and H307Y mutants were 39.5 ± 0.87 mM, 63.3 ± 3.2 mM and 76.7 ± 4.1 mM, respectively, which is consistent with the higher tolerance of the mutant enzymes to this monosaccharide.

The effect of increasing concentrations of cellobiose on the cellobiose activity of BglhI and mutants in the presence of fixed concentrations of xylose corresponding to the MC₅₀₀ (BglhI, N89Y/H307Y and H307Y) or MT (D237V/P389H/E395G/K475R, D237V, A141T/N235S and N235S) is shown in Fig 4A. Non Michaelis-Menten behavior was clearly observed for the stimulation of activity of BglhI and the A141T/N235S and N235S mutants by cellobiose ($n_H > 1$). The V_{max} values for liberation of free glucose from cellobiose in the presence of xylose for BglhI and the N89Y/H307Y and H307Y mutants increased by 2.3-, 1.7- and 2.0-fold, respectively, as compared to those in absence of the monosaccharide (Table 4). In contrast, V_{max} values determined in the presence or absence of xylose for the other mutants were essentially unchanged. However, as observed for pNP-Glc, the values of K_{0.5M} for all enzymes were significantly enhanced in the presence of xylose (see Table 4), suggesting that xylose competes with the natural substrate for binding to the active site.

Investigation of transglycosylation activity

The fractional hydrolysis (%H) and transglycosylation (%T) activities of the BglhI and mutants at 90–95% saturating concentrations of pNP-Glc in the absence or presence of xylose at MC₅₀₀ are presented in Table 5, together with the quantity of pNP and glucose released after 5 min reaction times. In the absence of xylose, the wild type BglhI and the D237V/P389H/E395G/K475R and D237V mutants predominantly followed the hydrolysis route, as demonstrated by the similar rates of pNP and glucose release and the low %T values. In the presence of xylose, increased %T values for these enzymes indicated a significant increase in the fraction of the glucosyl-enzyme intermediates following the transglycosylation route, resulting in

Fig 5. Time-course analysis of the reaction products formed by BglhI and mutants against cellobiose. (a) BglhI, (b) N89Y/H307Y, (c) H307Y, (d) D237V/P389H/E395G/K475R, (e) D237V, (f) A141T/N235S, (g) N235S. The reactions were performed at 90–95% saturating concentrations of cellobiose (see legend to Fig 4) in the absence (indicated as "control") or presence of glucose (indicated as "Plus G, 1" or xylose (indicated as "Plus X, 1"). The final concentrations of each monosaccharide were equal to MC₅₀₀ (BglhI, N89Y/H307Y and H307Y) or MT (D237V/P389H/E395G/K475R, D237V, A141T/N235S and N235S). The reaction times were 0 (lanes 1), 5 min (lanes 2), 10 min (lanes 3) and 24 h (lanes 4). Standards: G1, glucose; G2, cellobiose; G3, cellobiose; G4, cellobiose; G5, cellobiose; G6, cellobiose; G7, cellobiose; G8, cellobiose. The volumes of each aliquot applied to the TLC plates were adjusted among the best visualization of the products.

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higher rates of pNP liberation as compared to glucose. In contrast, high %T values were also determined for the N89Y/H307Y, H307Y, A141T/N235S and N235S mutants in the absence of xylose, demonstrating that these mutations have increased transglycosylation activity as compared to BglhI. In these mutants, the presence of xylose only slightly increased the %T.

The products formed by the BglhI and mutants with the cellobiose substrate in the absence and presence of xylose or glucose at MC₅₀₀ (BglhI, N89Y/H307Y and H307Y) or MT (D237V/P389H/E395G/K475R, D237V, A141T/N235S and N235S) were investigated by TLC (Fig 5). Although the wild type BglhI predominantly formed G1 in the absence of either glucose or xylose, faint stains corresponding to G2 were visible in the TLC experiments (Fig 5A, control). In contrast, transglycosylation products were clearly observed when the reaction was performed in the presence of 100 mM xylose (Fig 5A, Plus X₁), with the preferential formation of diaccharides. After long reaction times, glucose was the main reaction product either in the absence (Fig 5A, control, lane 1) or presence of xylose (Fig 5A, Plus X₁, lane 4), indicating

that over longer reaction times the BglhI hydrolyzed previously formed transglycosylation products. Some transglycosylation products were also observed when glucose was added to the reaction medium (Fig 3A, Plus G₁), however the high sample viscosity and intense glucose spot prevented their reliable separation and visualization.

In contrast to the wild type BglhI, transglycosylation products (mostly G₁ and G₂) were clearly observed with the N89Y/H307Y (Fig 5B, control) and H307Y (Fig 5C, control) mutants in the absence of either glucose or xylose. In the presence of xylose, low levels of G₁ and two other transglycosylation products (presumably disaccharides, one of them corresponding to S) were clearly observed (Fig 5B and 5C, Plus X₁) and amplified region of Fig 5C). Thus, although the TLC pattern resembled that observed for BglhI in the presence of xylose (Fig 3A, Plus X₁), the stained regions were more intense. As observed with the BglhI, G₁ was the main product detected after long reaction times for the N89Y/H307Y and H307Y mutants, both in the absence (Fig 5B and 5C, control, line t₀) or the presence of xylose (Fig 5B and 5C, Plus X₁, line t₁). Although the high sample viscosity in the presence of glucose impaired the visualization of spots located between G₁ and G₂ (Fig 5B and 5C, Plus G₁), spots were observed corresponding to G₂ after 5 and 10 min (Fig 5B and 5C, Plus G₁, lines t₁ and t₂) and G₂ after 24 h (Fig 5B and 5C, Plus G₁, line t₃).

In the absence of glucose or xylose, the D237V/P389H/E395G/K475R (Fig 5D, control) and D237V (Fig 5E, control) mutants predominantly followed the hydrolysis route releasing G₁ and only low levels of G₂ (Fig 5A, control). Furthermore, G₁ was clearly the main product even when the reaction was performed in the presence of xylose (Fig 5D and 5E, Plus X₁), although some weak stains corresponding to transglycosylation products (disaccharides and G₂) were observed for both mutants under this condition. In all experiments after long incubation times, glucose was essentially the only product observed (Fig 5D and 5E, line t₄).

The A141T/N235S (Fig 5F, control) and N235S (Fig 5G, control) mutants preferentially followed the hydrolysis route in the absence of xylose and glucose, with G₁ as the only reaction product. Some disaccharide transglycosylation products were observed in the presence of xylose (Fig 5F and 5G, Plus X₁), although at reduced levels as compared to the N89Y/H307Y and H307Y mutants. Under all experimental conditions, G₁ was essentially the only product released by A141T/N235S (Fig 5F, lanes t₁) and N235S (Fig 5G, lanes t₁) mutants after prolonged reaction times. The MS/MS analysis of the reaction products of BglhI and mutants against cellobiose, both in the absence and presence of glucose, revealed the formation of trisaccharides and tetrasaccharides constituted by units of glucose (Fig 6B and 6C). In contrast, in the presence of xylose, an ion of *m/z* 335 was detected in positive ionization mode, and MS/MS analysis indicated that this corresponded to a glucopyranosyl-xylose disaccharide (GX, Fig 6A). Based on this result and on the migration pattern of the standards used in the TLC analyses, we assigned a spot in the TLC to GX (see the amplified region of Fig 3C). No evidence was found for the formation of trisaccharides or tetrasaccharides containing xylose units by BglhI or the mutant enzymes.

Discussion

The crystal structure of the BglhI (PDB 4MDO, [29]) conserves many features typical of the GH1 family β -glucosidases. The -1 glycone binding region together with the catalytic residues E166 (the catalytic acid/base) and E177 (the catalytic nucleophile) lie at the base of a deep pocket lined with polar and non-polar residues (see Fig 2). These residues optimize both specific hydrogen bonding interaction with equatorial hydroxyl groups of the glycosyl group, and aromatic-sugar stacking interactions with the pyranose ring. Immediately adjacent to the glycone site, the +1 and +2 aglycone binding sites are characterized by a broadening of the

xylose with pNP-Glc as substrate, the BglhI preferentially followed the hydrolysis route (%H of ~85%) as shown by the similar release rates of pNP and glucose (Table 3). However, in the presence of xylose at MC_{max} , the 30% decrease in the rate of free glucose release as compared to pNP suggests that transglycosylation was favoured. This may be the result of xylose acted as an acceptor at high concentration thereby favouring transglycosylation and enhancing the rate of formation of the glucosyl-enzyme intermediate in response to its increased consumption in the transglycosylation step. It is worthy of note that allosteric modulation of the rates of steps 1 and 2 (Fig 1) by xylose may also occur. The %T of about 19% in the absence of xylose further indicated that pNP-Glc (or free glucose released from it) also acted as an acceptor in transglycosylation reactions, suggesting that besides binding to the -1 glycone/+1 aglycone sites, pNP-Glc may also bind to the +1/+2 aglycone sites. The monosaccharides simultaneously compete with pNP-Glc for binding to the -1 glycone and/or the +1/+2 aglycone sites, as confirmed by the increased $K_{app,pNP-Glc}$ determined in the presence of xylose or glucose at the MC_{max} (Table 3). Above the MC_{max} , the gradual decline of the pNP-glucosidase activity as the xylose or glucose concentration increases results from increasing competition of the monosaccharides with pNP-Glc for binding to the -1 glycone/+1 aglycone sites. At the MT, the stimulating effect of xylose or glucose is exactly counteracted by the reduced substrate binding to the enzyme. The lower value of K_{app} as compared to K_{app} (Table 3) further suggests that glucose binds to the -1 glycone and +1/+2 aglycone sites of BglhI with higher affinity than xylose, and is consistent with the lower MC_{max} value determined for glucose as compared to xylose. Thus, the fine modulation of the pNP-glucosidase activity of BglhI by glucose and/or xylose may be attributed to competition of the substrate and the modulation for the -1 glycone and the +1/+2 aglycone binding sites, and is regulated by the relative affinities of these sites for the glucose and *p*-nitrophenol moieties of the substrate, and for the free monosaccharides.

With cellobiose as substrate (Fig 5), the main reaction product in the absence of xylose was free glucose (released at steps 1 and 2, Fig 1), with low levels of G_2 derived from transglycosylation (Fig 1, step 3) reactions in which cellobiose acted as a glucose acceptor. In contrast, transglycosylation products (mostly disaccharides) were abundant in the presence of xylose at MC_{max} , indicating stimulation of the transglycosylation step. This in turn enhances the rates of the preceding glycosyl-enzyme formation (Fig 1, step 1) and hydrolysis steps (Fig 1, step 2), resulting in a net increase in the rate of free glucose liberation. With cellobiose as substrate, xylose may act as both a glucose acceptor and an allosteric effector, stimulating step 1 (and eventually step 2). As proposed for pNP-Glc, the cellobiose may bind to either the -1/+1 sites or the +1/+2 sites of BglhI, and xylose at concentrations up to the MC_{max} stimulates the rate of glucose release by acting as a glucose acceptor at the +1 aglycone site and/or an allosteric modulator at other aglycone sites. Simultaneously, xylose competes with cellobiose for binding to the -1 and/or the +1/+2 sites, as shown by the increased $K_{app,cellobiose}$ determined in the presence of xylose at MC_{max} (Table 3). The gradual decrease of the activity at xylose concentrations above MC_{max} may result from increasing competition with the substrate at the -1/+1 sites, and at MT the stimulating effect of xylose is compensated by less effective cellobiose binding to the enzyme. As with the pNP-Glc, the fine modulation of cellobiose activity of BglhI by xylose derives from the competition between cellobiose and xylose for binding to the -1 glycone and the +1/+2 aglycone sites, and depends on the relative affinities of these sites for the free monosaccharide and the cellobiose.

Various effects contribute to the xylose/glucose stimulation of the wild type BglhI, including allosteric interactions, competition between the substrate and the monosaccharides for binding to different sites on the enzyme molecule, and stimulation of the transglycosylation activity. Although it has been proposed that these modulatory mechanisms may act either separately [19,20, 26,34,36, 37] or together [24] to stimulate the pNP-glucosidase activity of

GH1 β -glucosidases, this is the first study of the stimulation mechanisms of the cellobiose activity. Results from the BglhI mutants N235S, A141T/N235S, D237V and D237V/P389H/E395G/K475R, indicate that the glucose/xylose stimulation of pNP liberation from pNP-Glc is not always correlated with stimulation of the glucose release from cellobiose. Crucially, this demonstrates that measurement of the pNP-glucosidase activity is not reliable for evaluating the potential for stimulation of cellobiose hydrolysis by β -glucosidases. Our results strongly suggest that an accurate description of the modulatory mechanisms of GH1 β -glucosidases depends on studies performed using the natural substrate.

All three single BglhI mutations (H307Y, D237V and N235S) presented altered patterns of glucose or xylose stimulation and all these substitutions are located in a cluster close to the +1/+2 aglycone binding sites. These mutations directly affected the balance between the reaction rates of transglycosylation and hydrolysis in the absence of xylose, with either pNP-Glc or cellobiose as substrate. This indicates that the topology and surface properties of the +1/+2 aglycone binding sites are critical determinants of the reaction route followed by the wild type BglhI. Nevertheless, in all mutant enzymes, allosteric effects, competition between the substrate and the monosaccharides for binding to different sites on the enzyme molecule, and stimulation of the transglycosylation activity all contribute to the xylose/glucose modulation of the pNP-glucosidase and cellobiose activities.

In the absence of xylose and with pNP-Glc as substrate, the N89Y/H307Y and H307Y mutants showed similar V_{max} values to the wild type BglhI (Table 2), yet both mutants presented significantly increased rates of transglycosylation and decreased rates of hydrolysis (Table 3). These mutants also presented lower $K_{app,pNP-Glc}$ values (Table 2), and since the substrate can bind to either the -1 glycone/+1 aglycone sites or the +1/+2 aglycone sites, an increased affinity for the substrate at the +1/+2 aglycone sites may contribute to the reduced $K_{app,pNP-Glc}$. In agreement with their preference for transglycosylation, xylose further increased transglycosylation in the N89Y/H307Y and H307Y mutants, and stimulation of the pNP release apparently occurred through mechanisms similar to those for the wild type BglhI. As compared to the wild type BglhI, the higher $K_{app,Glc}$, $K_{app,Xyl}$, MC_{max} and MT values determined for the N89Y/H307Y and H307Y mutants (Table 3) support the proposal that these mutants present higher affinity for pNP-Glc at the +1/+2 aglycone binding sites, since higher concentrations of xylose and glucose are needed to efficiently compete with the substrate. These mutants reveal that the relative affinities of a GH1 β -glucosidase for xylose, glucose and the substrate at the -1 glycone, and the +1/+2 aglycone binding sites are crucial to determine the range of stimulatory concentrations and the tolerance to the monosaccharides.

With the cellobiose substrate, the N89Y/H307Y and H307Y mutants present significantly higher transglycosylation in the absence of xylose as compared to the wild type BglhI (Fig 5). The rapid formation of cellobioses with 3 to 5 glucose units corroborates with an increased affinity for saccharides at the +1/+2 aglycone binding sites. Although the V_{max} for the cellobiose activity was almost unaltered, the mutants showed significantly higher $K_{app,cellobiose}$ values as compared to the wild type BglhI (Table 3). Apparently, the mutants present similar affinities for cellobiose at the -1 glycone/+1 aglycone sites and the +1/+2 aglycone sites, leading to an equal distribution of the substrate between these binding sites. Therefore, since cellobiose hydrolysis only occurs in molecules bound to the -1 glycone/+1 aglycone sites, an apparent negative cooperativity was observed for the stimulation of N89Y/H307Y and H307Y by cellobiose. Furthermore, the products of transglycosylation are also substrates for the mutant enzymes, and low affinity binding of these oligosaccharides might also contribute to a higher apparent $K_{app,cellobiose}$. Xylose promoted a reduction in the levels of G_2 , G_3 and disaccharides transglycosylation products formed by the N89Y/H307Y and H307Y mutants, and it appears that in spite of the increased affinity of the +1/+2 aglycone binding sites, oligosaccharides are displaced by xylose at MC_{max} . The higher $K_{app,Glc}$, MC_{max} and MT values

determined for the N89Y/H307Y and H307Y mutants (Table 3) as compared to the wild type BglII further supports the suggestion of higher affinity of the mutants for cellobiose at the +1/+2 aglycone sites. The formation of GX unequivocally demonstrates that xylose stimulated transglycosylation activity by binding to the +1 aglycone site, but the increased rate of glucose release in the presence of xylose at MC_{max} indicates that xylose also stimulates steps 1 and/or 2 (Fig 1) of the reaction. The rapid formation of isophorone (or other glucose dimers) in the presence of xylose at MC_{max} further suggests that the +1 aglycone binding site of the mutant enzymes has a significantly higher affinity for glucose, as compared to xylose. Importantly, this is the first report of the formation of GX by a GH-1 β -glucosidase in a transglycosylation reaction.

In the absence of xylose, the D237V/P389H/E395G/K475R and D237V mutants exclusively followed the hydrolysis route with the pNP-Glc substrate (Table 3). The higher V_{max} for the pNP-glucosidase activity of these mutants, as compared to the wild type BglII (Table 2), may reflect a stabilization of the transition state of the first step of the catalytic cycle (Fig 1, step 1) probably due to stronger interaction of the pNP moiety at the +1 aglycone site. Xylose at the MC_{max} substantially increased the %T values for the D237V/P389H/E395G/K475R and D237V mutants, and the stimulation of transglycosylation apparently occurred through mechanisms similar to those proposed for the wild type BglII, resulting in increased pNP-glucosidase activities. The higher values of K_{cat/K_m} , K_{cat/K_m} , and MC_{max} determined for the D237V mutant (Table 3), as compared to wild type BglII, are consistent with a reduced binding affinity of xylose and glucose to the +1 aglycone site, while the lower MT values observed for both monosaccharides may reflect an increased affinity at the -1 glycone site. The lower K_{cat/K_m} , as compared to K_{cat/K_m} , and the much higher K_{cat/K_m} determined in the presence of glucose at MC_{max} also reflect a higher affinity for glucose binding at the -1 glycone sites of D237V as compared to xylose. Taken together, these data suggest that the D237V mutation reduced affinity of the +1 aglycone sites more for glucose than for xylose. This effect was even more apparent in the D237V/P389H/E395G/K475R mutant, where the pNP-glucosidase activity was not stimulated by glucose but increased 1.4-fold with xylose. Nevertheless, glucose efficiently competed with the substrate for binding to the -1 glycone site of the D237V/P389H/E395G/K475R mutant, as shown by the increased K_{cat/K_m} value in the presence of glucose at MC_{max} (Table 3), and strong glucose inhibition of enzyme activity at concentrations above 50 mM. It is noteworthy that a previous site directed mutagenesis study of two metagenome derived β -glucosidases have shown that residues such as aspartic acid with hydrogen bonding potential at the position homologous to 237 in BglII retain glucose stimulated activity [37]. In contrast, residues with side-chains having no hydrogen bonding potential, such as valine, lose their glucose stimulation [37]. Taken together, the results from the D237V/P389H/E395G/K475R and D237V mutants further corroborate the hypothesis that the affinities of the GH1 β -glucosidases for xylose, glucose or the substrate at the glycone and aglycone binding sites are important determinants of monosaccharide tolerance.

Hydrolysis of the cellobiose substrate was strongly favoured in the D237V/P389H/E395G/K475R and D237V mutants in the absence of xylose, which may be attributed to a decreased affinity for the non-reducing glucose residue of cellobiose at the +1 aglycone site of the mutants. In striking contrast with pNP-Glc as substrate, the V_{max} values for the cellobiose activity of both mutants were much lower than for the wild type BglII (Table 3), possibly due to an increased activation energy of step 1 (Fig 1). Both mutants also showed significantly increased K_{cat/K_m} as compared to BglII, possibly reflecting a lower cellobiose affinity at the +1 aglycone site. The cellobiose activity of the D237V/P389H/E395G/K475R mutant was not stimulated by xylose, and the low levels of transglycosylation products formed are possibly the result of a combination of low affinity for xylose at the +1 aglycone site associated with a higher affinity for xylose at the -1 glycone site, which is also in accord with the very low MT value. In

contrast, although the cellobiose activity of the D237V mutant was also not stimulated by xylose, some transglycosylation products (mainly disaccharides) were formed at low levels in the presence of xylose at the MT (Fig 3). As observed for the D237V/P389H/E395G/K475R mutant, xylose strongly competed with the substrate, as revealed by the much higher K_{cat/K_m} determined in the presence of xylose at MT (Table 3). However, in the D237V mutant the monosaccharide apparently binds with considerably higher affinity at the +1 aglycone site, as compared to D237V/P389H/E395G/K475R, and with lower affinity at the -1 glycone sites, as revealed by the 10-fold higher MT.

The A141T/N235S and N235S mutants showed 50–85% transglycosylation with the pNP-Glc substrate (Table 3) in the absence of xylose, indicating that the N235S reduced the preference for the hydrolysis seen in the wild type BglII, and indicate a higher pNP-Glc affinity at the +1/+2 sites. The reduced K_{cat/K_m} observed in the A141T/N235S and N235S mutants (Table 2) may also reflect an increased affinity for the substrate at the -1 glycone site. Similar V_{max} values were observed for the pNP-glucosidase activities of these mutants and the wild type BglII, indicating that the rate of pNP release was not altered. This is in contrast with the increased rate of transglycosylation and decreased rate of glucose release. In the presence of xylose, the %T determined for both mutants were slightly increased and the pNP release was apparently stimulated by a mechanism similar to that proposed for the wild type enzyme. The reduced K_{cat/K_m} , K_{cat/K_m} , MC_{max} and MT values determined for the A141T/N235S and N235S mutants (Table 3) as compared to BglII are consistent with the binding of the monosaccharides at the -1 glycone and +1/+2 aglycone sites with higher affinities, as confirmed by the increased K_{cat/K_m} determined in the presence of xylose or glucose at MC_{max} (Table 3).

Transglycosylation products at appreciable levels were not formed with cellobiose as substrate (Fig 5) by the A141T/N235S and N235S mutants in the absence of xylose, suggesting that the N235S mutation does not alter the affinity of the +1/+2 aglycone binding sites for the natural substrate. The lower K_{cat/K_m} determined for the mutants, as compared to BglII, are consistent with higher affinities for the substrate at the -1 glycone/+1 aglycone sites. The cellobiose activity of the A141T/N235S and N235S mutants was not stimulated by xylose, and only low levels of transglycosylation products were formed in the presence of xylose at MT, which is consistent with reduced affinity for the monosaccharide at the +1 aglycone site of the mutants. The lower MT values reflect higher apparent affinities for xylose at the -1 glycone site, as compared to the wild type BglII.

Conclusions

Using the BglII in a combined protein engineering, kinetic and transglycosylation study, we have investigated the mechanisms of glucose/xylose stimulation of the cellobiose activity of a GH1 β -glucosidase, which has yielded novel insights as to the stimulation of pNP-glucosidase activity. Our results suggest that allosteric interactions, increased transglycosylation and competition of the monosaccharides glucose and xylose with the substrate at the -1 glycone and the +1/+2 aglycone binding sites all play a role in the stimulation mechanism. A directed protein evolution strategy has created a series of BglII mutants with altered catalytic properties with respect to glucose/xylose stimulation and/or tolerance. Our data suggest that the fine modulation of the activity of the BglII and mutants by glucose and/or xylose is regulated by the relative affinities of the glycone and aglycone binding sites for the substrate and the five monosaccharides. It is proposed that changes in the topology and physicochemical properties of the +1/+2 aglycone sites of the mutants underlie the altered kinetic and transglycosylation activities of the enzyme.

Supporting information

S1 Fig. Analysis of purified Bglh1 and mutants by SDS-PAGE.
(DOC)

S2 Fig. Effect of temperature (A) and pH (B) on pNP-glucosidase activity of Bglh1 and mutants. (a) Bglh1, (b) N89Y/H307Y, (c) H307Y, (d) D237V/I589H/E395G/K475R, (e) D237V, (f) A141T/N235S, (g) N235S. The optimum catalytic temperatures were estimated in 50 mM Bis-Tris buffer, pH 6.0, containing 2 mM pNP-Glc. The optimum catalytic pH were estimated in McIlwaine buffer at the optimum temperatures determined for each enzyme, using 2 mM pNP-Glc as substrate. The values shown represent means $\pm$ SD from triplicate assays ($n = 3$) carried out with three separate preparations of pure recombinant enzymes (error bars are not evident, as lie within the area of the symbol).
(DOC)

S1 File. Detailed kinetic analysis of the stimulation of Bglh1 using pNP-Glc as substrate.
(PDF)

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Apêndice I

Article

A High Redox Potential Laccase from *Pycnoporus sanguineus* RP15: Potential Application for Dye Decolorization

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Abstract: Laccase production by *Pycnoporus sanguineus* RP15 grown in wheat bran and corn cob under solid-state fermentation was optimized by response surface methodology using a Central Composite Rotational Design. A laccase (Lacps1) was purified and characterized and the potential of the pure Lacps1 and the crude culture extract for synthetic dye decolorization was evaluated. At optimal conditions (eight days, 26 °C, 18% (w/w) milled corn cob, 0.8% (w/w) NH₄Cl and 50 mmol L⁻¹ CuSO₄, initial moisture 4.1 mL g⁻¹), the laccase activity reached 138.6 ± 13.2 U g⁻¹. Lacps1 was a monomeric glycoprotein (67 kDa, 24% carbohydrate). Optimum pH and temperature for the oxidation of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) were 4.4 and 74.4 °C, respectively. Lacps1 was stable at pH 3.0–8.0, and after two hours at 55–60 °C, presenting high redox potential (0.747 V vs. NHE). ABTS was oxidized with an apparent affinity constant of 147.0 ± 6.4 μmol L⁻¹, maximum velocity of 413.4 ± 21.2 U mg⁻¹ and catalytic efficiency of 3140.1 ± 149.6 L mmol⁻¹ s⁻¹. The maximum decolorization percentages of bromophenol blue (BPPB), remazol brilliant blue R and reactive blue 4 (RB4), at 25 or 40 °C without redox mediators, reached 90%, 80% and 60%, respectively, using either pure Lacps1 or the crude extract. This is the first study of the decolorization of BPPB and RB4 by a *P. sanguineus* laccase. The data suggested good potential for treatment of industrial dye-containing effluents.

Keywords: *Pycnoporus sanguineus*; solid state fermentation; response surface methodology; laccase; purification; characterization; decolorization; remazol brilliant blue R; Reactive blue 4; bromophenol blue

1. Introduction

Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) are multicopper oxidases broadly distributed in plants, insects, fungi and bacteria [1–3]. Most of them, blue colored, catalyze the direct oxidation of a wide variety of phenolic compounds [3,4]. Some yellow laccases, able to oxidize both

phenolic and non-phenolic compounds, have also been described [5]. Besides the ability to oxidize a multitude of substrates, the laccases do not require hydrogen peroxide for the oxidation reaction; moreover, they use molecular oxygen as an electron acceptor, generating water. Therefore, they are considered ideal “green catalysts”, and are among the most promising oxidoreductases for application in different biotechnological processes in food, textile, pulp and paper, pharmaceutical and other industries [2,6].

The laccases are monomeric, dimeric or tetrameric proteins that contain four copper atoms per monomer, distributed in three redox sites named T1, T2 and T3 [5]. The redox potential (E°) of the T1 copper site is characteristic of each enzyme, ranging from 0.4 to 0.8 V vs. normal hydrogen electrode (NHE). According to it, the laccases are classified into three groups: low- (0.4–0.5 V), medium- (0.5–0.6 V) and high- (0.7–0.8 V) redox potential [7,8]. The E° is one of the most relevant characteristics of a laccase, since the redox potential difference between the T1 copper site and the substrate is one of the major factors that affect the oxidation rate. Furthermore, the E° of a laccase restrains the substrates that it may oxidize to those with lower E° . This limitation may be overcome by using redox mediators, which increase the oxidative capacity of the enzyme towards the oxidation of higher E° compounds; however, the industrial use of mediators is still hampered by their prohibitive cost and toxicity [4,7–9]. Therefore, high-redox potential laccases are more attractive for biotechnological purposes.

The increasing volume and chemical complexity of synthetic dye-rich industrial effluents, particularly from textile, tannery and pulp and paper plants, represent a serious environmental problem today [3,10,11]. Synthetic dyes often have low biodegradability, several are toxic, carcinogenic and mutagenic and it is not uncommon that their degradation products show similar properties. Furthermore, the absorption and reflection of the sunlight by dyes in surface waters interferes with bacterial and plant growth, disturbing the ecological balance [9,11,12]. As a result, there is a great interest in the development of efficient treatments for the removal of synthetic dyes from wastewaters.

Various physicochemical methods for dye removal are available, but they are costly, poorly efficient and not applicable for a variety of compounds. Microbiological processes are relatively inexpensive, in contrast, and environmentally friendly; however, their use depends on the identification of suitable microbial strains and the establishment of adequate conditions for dye degradation, often requiring previous physical and/or chemical steps. Furthermore, numerous synthetic dyes are resistant to microbiological attack. Thus, the use of oxidative enzymes is now considered the most promising alternative for the removal of dyes from industrial effluents [9–11,13,14]. The large-scale use of laccases for wastewater treatments is, however, severely limited by their high production costs. This has lately generated several studies on the optimization of the culture conditions for laccase production by different fungi in low-cost media [14–16].

The fungi from the phylum Basidiomycota are among the most efficient laccase producers known [1,3,4]. Among them, the white-rot fungus, *Pycnoporus sanguineus*, has attracted considerable attention in recent years due to its ability to produce high levels of high redox potential laccases as the main ligninolytic enzymes [7,17–19]. Furthermore, laccases from different *P. sanguineus* strains revealed good potential for application in industrial dyes bioremediation, detoxification and decolorization processes [7,17,19,20].

Several authors have studied the production of laccases by different strains of *P. sanguineus* under submerged fermentation (SmF) conditions [21–23]. In the last decade, however, solid state fermentation (SSF) processes were consolidated as an interesting alternative to SmF for the production of industrial enzymes, particularly from fungal origin [24–27]. The main advantages of SSF include higher yields and the production of enzymes that are not produced under SmF, and are attributed to the conditions in which the microorganisms are cultivated, similar to those of their natural environment [24,26]. Furthermore, SSF processes have several potential economic and environmental advantages: different agro-industrial by-products and residues may be used as solid substrates, lowering the production costs and avoiding pollution issues related to waste disposal; the effluent generation and the demands

on energy and sterile water are lower; the enzymes are obtained at higher concentrations, reducing downstream processing [24,26].

In spite of that, few studies have investigated the production of laccases by *P. sanguineus* under SSF conditions, employing the agro-residues sago “hampas”, rubber wood sawdust and oil palm frond parenchyma tissue (OPFT), abundant in Malaysia [28–30]. A recent study has also investigated laccase production using *Eucalyptus grandis* bark shavings, an abundant residue in Uruguay, but in this case the fungus was cultivated under semi-solid-state fermentation (semi-SSF) conditions, a variety of SSF with higher free liquid content in the culture medium [31].

In this study, we investigated the potential of different agro-industrial residues and by-products that are abundant in Brazil as carbon sources for the production of laccases by the newly isolated strain *P. sanguineus* RP15 under SSF conditions. The culture conditions for enzyme production were optimized using response surface methodology (RSM) and a major laccase (Lacps1) was purified and characterized in detail. The potential of the laccase-rich crude culture extract and the pure enzyme for the decolorization of the synthetic dyes remazol brilliant blue R (RBBR), reactive blue 4 (RB4) and bromophenol blue (BFB) was evaluated.

2. Results and Discussion

2.1. Preliminary Screening for the Best Carbon Source, Inducer and Supplementary Nitrogen and Carbon Sources for the Production of Laccases by *P. sanguineus* RP15

The best carbon source for laccase production by *P. sanguineus* RP15 was wheat bran, reaching 29.8 ± 1.9 U g^{-1} (Table 1). Negligible enzyme levels were produced in peanut hulls and rice husks, and the organism was not able to grow on corn husks. Laccase activity was undetectable in the crude extracts obtained after *P. sanguineus* culture in all other carbon sources tested. Under 5mF conditions, wheat bran was a poor carbon source for laccase production by *P. sanguineus* CS2 [21]. In contrast, the lignocellulosic agro-residues sago “hampas”, rubber wood sawdust and oil palm frond parenchyma were good carbon sources for the production of laccases by *P. sanguineus* CY788 under SSF conditions [28–30], while *Eucalyptus* bark shavings were revealed to be a good solid substrate for enzymatic production by a Uruguayan strain of *P. sanguineus* under semi-SSF conditions [31]. Similarly, a variety of lignocellulosic waste materials or by-products derived from food and agricultural processing industries have been successfully used as solid substrates for the production of laccases by other white-rot basidiomycetes under SSF conditions [32–36]. As observed for *P. sanguineus* RP15, wheat bran has been successfully employed as the main or sole carbon source for the production of laccases by *Fomes sclerodermeus* [37], *Ganoderma* sp. [38] and *Fomes fomentarius* [39] under SSF conditions, possibly due to the high content of cellulose, hemicelluloses and lignin as well as growth factors, vitamins and proteins [40,41].

Table 1. Effect of different carbon sources and supplementary carbon and nitrogen sources on the production of laccases by *P. sanguineus* RP15.

Carbon Source	U g^{-1}	U mL^{-1}
Wheat bran	29.8 ± 1.9	5.9 ± 0.4
Steam-exploded sugarcane bagasse	ND ^a	ND
Raw sugarcane bagasse	ND	ND
Peanut hull	2.1 ± 0.2	0.40 ± 0.03
Rice husk	1.9 ± 0.1	0.40 ± 0.02
Milled corn-cob	ND	ND
Milled Soybean	ND	ND
Sugarcane trash	ND	ND

Table 1. Cont.

Carbon Source	U g ⁻¹	U ml ⁻¹	
Carbon Supplement (1% w/w)	U g ⁻¹	%	
None	30.1 ± 1.8	100.0	
Peanut hull	31.9 ± 1.4	105.8	
Rice husk	29.6 ± 1.1	98.2	
Soybean meal	23.7 ± 1.1	78.7	
Corn husk	25.5 ± 1.3	84.6	
Sugarcane trash	30.5 ± 1.7	101.3	
Glucose	28.2 ± 1.1	93.7	
Milled corn-cob	35.5 ± 1.7	117.8	
Nitrogen Supplement	% (w/w)	U g ⁻¹	%
None	—	33.5 ± 1.7	100.0
Asparagine	1	35.3 ± 1.5	105.6
Casam	1	37.3 ± 1.6	111.2
Soybean meal	1	26.9 ± 1.2	80.4
Glycine	1	29.2 ± 1.3	87.4
Peptone	1	46.0 ± 2.4	137.4
Yeast extract	1	35.8 ± 1.9	107.0
Malt extract	1	39.0 ± 1.9	116.4
NH ₄ NO ₃	0.8	31.0 ± 1.7	92.8
KNO ₃	0.8	33.6 ± 1.5	100.5
NaNO ₃	0.8	17.6 ± 0.9	52.2
(NH ₄) ₂ SO ₄	0.8	29.4 ± 1.3	87.7
NH ₄ Cl	0.8	36.1 ± 2.7	107.6
Urea	0.8	35.9 ± 2.0	107.3

Pyrenopeziza sanguineus RP15 was cultured for 192 h at 25 °C in 5 g dry carbon source (or a mass occupying maximally 1/3 of the total volume of the culture flask) and deionized water (2 mL g^{-1} dry substrate). Supplementary carbon and nitrogen sources were added to dry wheat bran at the indicated concentrations. The enzymatic assays were performed in duplicate; each experiment was repeated three fold ($n = 3$). Data are presented as means $\pm$ SD. ^a ND: undetectable by the methods used.

The addition of veratryl alcohol (0–10.0 mmol L^{-1}) to wheat bran slightly inhibited the laccase production by *P. sanguineus* RP15 with respect to the control (wheat bran and water only). The inhibition was concentration-dependent and a decrease of about 23% occurred at 10 mmol L^{-1} veratryl alcohol. In contrast, the addition of CuSO₄ (0–100 mmol L^{-1}) resulted in enhanced laccase production, reaching 37.2 ± 2.1 U g^{-1} (17% above the control) at 50 mmol L^{-1} . At higher concentrations, however, a steady decrease of the enzymatic production occurred and 66% lower levels, as compared to the control, were obtained at 100 mmol L^{-1} CuSO₄. The production of fungal laccases is often influenced by a series of inducers, which apparently regulate the enzyme synthesis at transcriptional level. Aromatic and phenolic compounds, especially those structurally related to lignin, and metallic ions are known inducers of laccase expression. However, the effect of each compound/ion and the optimum concentration for laccase production are highly variable among different fungal strains [1,4,36,42]. Veratryl alcohol and Cu²⁺ are among the most common inducers of fungal laccases, and their effect on the enzymatic production by various strains under 5mF conditions is well characterized [4,42]. Few studies, however, addressed this point under SSF conditions. Contrasting to our results, the production of laccases by *Pyrenopeziza cinnabarinus* ss3 [43] and *Trametes versicolor* [16] cultured in sugarcane bagasse and horticultural waste, respectively, was increased about nine- and three-fold, respectively, by supplementation with veratryl alcohol. Furthermore, laccase production levels by *Fomes fomentarius* WRF-1 [39] and *Ganoderma* sp. [38] cultured in wheat bran were highly enhanced in response to the addition of CuSO₄. Slight inhibition was observed, however, when cultures of *Fomes sclerodermeus* in wheat bran were supplemented with micromolar concentrations of CuSO₄ [37].

The supplementation of wheat bran with 1% (w/w) milled corn cob resulted in an increase of about 18% of the laccase production by *P. sanguineus* RP15 (Table 1). With the exception of soybean meal and corn husks, which slightly reduced the production, all other supplementary carbon sources tested were practically without effect. Up to date, the use of corn cob as the main or sole carbon source for laccase production by *Pycnoporus sanguineus* has not been investigated. Furthermore, few authors have studied the use of this cheap agro-residue for laccase production by other basidiomycetes, although it should provide conditions for enzyme induction due to its lignocellulosic nature [44]. Low production levels in media containing corn cob as the carbon source have been described for *Pleurotus florida* EM 1303 [44], *Lentinula edodes* CCB-42 [45] and *Trametes versicolor* IBL-04 [46].

The best supplementary nitrogen sources for the production of laccases by *P. sanguineus* RP15 decreased in the following order: NH_4Cl > peptone > malt extract > casein > urea > yeast extract > asparagine > KNO_3 > NH_4NO_3 > $(\text{NH}_4)_2\text{SO}_4$ > NaNO_3 . The production was increased about 37% by 1.0% (w/w) peptone and 68% by 0.8% (w/w) NH_4Cl , while it was inhibited about 50% by 0.8% (w/w) NaNO_3 (Table 1). Thus, the quality of the nitrogen source has strongly influenced the laccase production in wheat bran, a complex substrate that presents high nitrogen levels [47]. Furthermore, when NH_4Cl was added to wheat bran at variable concentrations (0.0%–5% w/w), the highest laccase levels were produced by *P. sanguineus* RP15 at 0.8%–1.0% (w/w). Contrasting to our results, the highest laccase production by *Pleurotus florida* under SSF in wheat straw was obtained with a mixture of 9% (w/w) NH_4Cl and 5% (w/w) malt extract as nitrogen sources [48].

2.2. Optimization of Laccase Production by *P. sanguineus* RP15 Using RSM

After preliminary studies to determine the experimental ranges for each independent variable (culture time, initial moisture of the medium, temperature and milled corn cob concentration) the culture conditions for the production of laccase were optimized using a 2^4 full-factorial Central Composite Rotational Design (CCRD) (Table 2). The maximum and minimum laccase levels were, respectively, $137.2 \pm 14.2 \text{ U} \cdot \text{g}^{-1}$ (run 26) and $29.4 \pm 2.4 \text{ U} \cdot \text{g}^{-1}$ (run 21).

Table 2. Experimental conditions and results of the statistical experimental design for the production of laccases by *P. sanguineus* RP15.

Run	Real (Coded) Values				Laccase ($\text{U} \cdot \text{g}^{-1}$)
	Time	Initial Moisture	Temperature	Milled Corn cob	
	(Days)	($\text{mL} \cdot \text{g}^{-1}$)	($^{\circ}\text{C}$)	(%, w/w)	
1	7 (−1)	3 (−1)	20 (−1)	10 (−1)	47.5 ± 3.9
2	9 (+1)	3 (−1)	20 (−1)	10 (−1)	56.7 ± 4.1
3	7 (−1)	5 (+1)	20 (−1)	10 (−1)	36.9 ± 5.1
4	9 (+1)	5 (+1)	20 (−1)	10 (−1)	42.9 ± 4.3
5	7 (−1)	3 (−1)	30 (+1)	10 (−1)	49.1 ± 5.7
6	9 (+1)	3 (−1)	30 (+1)	10 (−1)	47.6 ± 5.3
7	7 (−1)	5 (+1)	30 (+1)	10 (−1)	68.2 ± 6.1
8	9 (+1)	5 (+1)	30 (+1)	10 (−1)	59.7 ± 6.3
9	7 (−1)	3 (−1)	20 (−1)	20 (+1)	73.9 ± 6.9
10	9 (+1)	3 (−1)	20 (−1)	20 (+1)	77.2 ± 7.4
11	7 (−1)	5 (+1)	20 (−1)	20 (+1)	45.7 ± 3.9
12	9 (+1)	5 (+1)	20 (−1)	20 (+1)	48.6 ± 4.6
13	7 (−1)	3 (−1)	30 (+1)	20 (+1)	65.7 ± 5.9
14	9 (+1)	3 (−1)	30 (+1)	20 (+1)	74.7 ± 8.4
15	7 (−1)	5 (+1)	30 (+1)	20 (+1)	112.9 ± 10.9
16	9 (+1)	5 (+1)	30 (+1)	20 (+1)	95.1 ± 9.7
17	6 (−2)	4 (0)	25 (0)	15 (0)	115.2 ± 10.6
18	10 (+2)	4 (0)	25 (0)	15 (0)	119.8 ± 12.3
19	8 (0)	2 (−2)	25 (0)	15 (0)	33.1 ± 2.1
20	8 (0)	6 (+2)	25 (0)	15 (0)	38.3 ± 6.4

Table 2. Cont.

Run	Real (Coded) Values				Laccase ($\text{U} \cdot \text{g}^{-1}$)
	Time	Initial Moisture	Temperature	Milled Corn cob	
	(Days)	($\text{mL} \cdot \text{g}^{-1}$)	($^{\circ}\text{C}$)	(%, w/w)	
21	8 (0)	4 (0)	15 (−2)	15 (0)	29.4 ± 2.4
22	8 (0)	4 (0)	35 (+2)	15 (0)	54.6 ± 4.8
23	8 (0)	4 (0)	25 (0)	5 (−2)	51.6 ± 4.1
24	8 (0)	4 (0)	25 (0)	25 (+2)	119.5 ± 12.5
25	8 (0)	4 (0)	25 (0)	15 (0)	136.4 ± 14.7
26	8 (0)	4 (0)	25 (0)	15 (0)	137.2 ± 14.2
27	8 (0)	4 (0)	25 (0)	15 (0)	135.8 ± 13.1

The microorganism was cultured in 5 g wheat bran containing 50 mmol L^{-1} CuSO_4 and 0.0% (w/w) NH_4Cl . The experiments were conducted in triplicate ($n = 3$) and each enzymatic assay was carried out in duplicate. The average of the laccase production ($\text{U} \cdot \text{g}^{-1}$) was used as the response value. Data are presented as mean ± SD.

From the results of the CCRD, the variables that exerted significant effects on laccase production at 95% confidence level ($p < 0.05$) were identified. The linear terms of temperature and milled corn cob concentration, and the quadratic terms of culture time, temperature and initial moisture of the medium, exerted statistically significant effects on the enzymatic production. The interaction between the initial moisture of the medium and the temperature also had a significant effect, but not the other interactions between the independent variables tested. An ANOVA analysis was performed considering only the significant effects and the regression coefficients obtained are presented in Table 3. The quadratic effects of the initial moisture of the medium and the temperature were the most important factors that affected the production of laccases.

Table 3. Analysis of variance (ANOVA) for the second-order polynomial models and coefficient values for the production of laccases by *P. sanguineus* RP15.

Effect	Coefficient	p-Value
Intercept	124.33	0.000000
(x) Time (Q)	−12.54	0.001921
(y) Initial Moisture (Q)	−23.42	0.000002
(z) Temperature (L)	9.72	0.011855
(z) Temperature (Q)	−20.04	0.000014
(w) Milled Corn cob (L)	8.10	0.031957
Zy	11.25	0.016384
Source	SS ^a	Df ^b
Regression	24,581.59	6
Residual	3923.10	20
Total	30,504.69	26

$R^2 = 0.80$; F tested (5% = 2.60); ^a SS: sum of squares; ^b Df: degrees of freedom; ^c MSq: mean square.

The second-order model that describes the production of laccases by *P. sanguineus* as a function of the independent variables tested was expressed by the equation:

$$\text{Lac} \left(\text{U} \cdot \text{g}^{-1} \right) = \left[124.33 + 9.72 x + 8.10 w - 12.54 x^2 - 23.42 y^2 - 20.04 z^2 + 11.25 zy \right]$$

Based on the F test, the model was predictive of the production of laccases as a function of culture time, culture temperature, medium initial moisture and milled corn cob concentration, since the calculated F value was higher than the listed one. The R^2 coefficient of 0.80 confirmed the goodness of the model and indicated that it could explain 80% of the response variability.

The statistical analysis showed that in a medium composed of wheat bran as the carbon source supplemented with 0.8% (w/w) NH_4Cl and 50 $\text{mmol} \cdot \text{L}^{-1}$ CuSO_4 , the maximum laccase production occurred after 8.19 culture days at 26.2 °C, with initial moisture of 4.10 $\text{mL} \cdot \text{g}^{-1}$ and addition of 17.98% (w/w) milled corncob as the supplementary carbon source. The maximum predicted laccase production was 140.78 $\text{U} \cdot \text{g}^{-1}$, about 4.7-fold higher than that obtained in wheat bran and water ($29.8 \pm 1.9 \text{ U} \cdot \text{g}^{-1}$).

For the experimental validation of the optimized culture conditions, the fungus was grown for eight days at 26 °C in wheat bran supplemented with 0.8% (w/w) NH_4Cl , 50 $\text{mmol} \cdot \text{L}^{-1}$ CuSO_4 and 18% (w/w) milled corncob, with initial moisture of 4.1 $\text{mL} \cdot \text{g}^{-1}$, and the mean value determined for the laccase activity was $138.6 \pm 13.2 \text{ U} \cdot \text{g}^{-1}$, in very good correlation with the value predicted by the model, confirming its validity. The response surfaces for the factors that affected the production of laccases by *P. sanguineus* RP15 are presented in Figure 1. The analysis of the curves revealed that the best conditions for the enzymatic production are in the ranges of 7.5–9.0 culture days, temperature of 25.5–27.5 °C, medium initial moisture of 3.5–4.5 $\text{mL} \cdot \text{g}^{-1}$ and milled corncob concentration of 15%–25% (w/w).

Figure 1. Response surface plots showing the interactive effects of initial moisture and milled corncob concentration (a), and temperature and culture time (b), on the production of laccases by *P. sanguineus* RP15.

The relatively short culture period, the low-cost salts employed as the supplementary nitrogen source (NH_4Cl) and inducer (CuSO_4), and the use of about 20% milled corncob as the supplementary carbon source constitute definite advantages of the culture medium employed for laccase production

by *P. sanguineus* RP15. In particular, corncobs constitute one of the most abundant and cheap residues of the agricultural industry around the world [49] and the carbon source employed in a certain enzyme production process constitutes about 40%–60% of the total costs [15].

Few authors have investigated the production of laccases by *P. sanguineus* strains under conditions of SSF [28–30] and semi-SSF [31]. The main carbon sources used in these studies were lignocellulosic wastes and/or agro-industrial by-products, but only Annuaiz *et al.* [30] and Gioia *et al.* [31] performed the statistical optimization of the enzymatic production. Gioia *et al.* [31] employed ABTS to estimate the laccase activity, and under optimal conditions their *P. sanguineus* strain produced 106 $\text{U} \cdot \text{g}^{-1}$ solid substrate, about 25% less than *P. sanguineus* RP15. Furthermore, in spite of the low cost of the culture medium employed (bark shavings with the addition of salts, thiamine and yeast extract), the culture period for maximal enzyme production (14 days) was much higher than that determined for *P. sanguineus* RP15, increasing the production costs.

In contrast to *P. sanguineus*, the production of laccases by other white-rot fungi species under SSF conditions has been investigated by several authors. From three- to twenty-fold lower production levels, as compared to that obtained for *P. sanguineus* RP15, were reported for different species of the genus *Phanerochaete* [33,34,50,51] and for *T. versicolor* [16,51]. In great contrast, a 72-fold higher production, as compared to that obtained in this study, was described for an isolate of *Ganoderma* sp. [38]. Levels about two- and six-fold higher were also found for strains of *F. sclerotiorum* [37] and *Phanerochaete chrysosporium* [15], respectively. However, these higher productivities were obtained in more expensive culture media, as compared to that of *P. sanguineus* RP15, and/or the culture times were much higher (from two- to three-fold), contributing to an increase in the production costs.

2.5. Laccase Purification and Molecular Properties

LaccaseI was purified by a simple two-step method from the crude culture extract from *P. sanguineus* RP15 cultivated under optimized conditions for laccase production (Table 4). The enzyme was purified about 24.5-fold with an overall yield of 30%, reaching a specific activity of 411.0 $\text{U} \cdot \text{mg}^{-1}$. When maintained in water at 4 °C, the activity of LaccaseI remained constant for at least 60 days.

Table 4. Purification of LaccaseI.

Step	Total Activity (U)	Total Protein (mg)	Specific Activity ($\text{U} \cdot \text{mg}^{-1}$)	Yield (%)	Purification (Fold)
Crude extract	548.0	32.70	16.8	100	1
60% $(\text{NH}_4)_2\text{SO}_4$	344.7	13.10	26.3	63	1.6
Phenyl-Sepharose	164.4	0.40	411.0	30	24.5

The purification protocol was repeated five-fold. Representative data from one of the purification procedures are presented.

Non-denaturing polyacrylamide gel electrophoresis (PAGE) of the purified LaccaseI revealed a single laccase activity band (Figure 2a), coincident with a single band after Coomassie Blue staining (Figure 2b), confirming the homogeneity of the preparation. A single protein band corresponding to 65 kDa was also revealed after sodium dodecyl sulfate-PAGE (SDS-PAGE) analysis (Figure 2c). The apparent molecular mass of LaccaseI estimated by gel filtration was 67 kDa, in good agreement with that estimated by SDS-PAGE, suggesting that the native enzyme was a monomer. Similar apparent molecular masses were reported for several laccases from other *P. sanguineus* strains [7,17–19,52–56] and other *Pyrenopeziza* species [7,20].

Figure 2. Analysis of the purified laccase by PAGE (lanes a and b) and SDS-PAGE (lane c). Lane a, laccase activity after PAGE, using 1 mmol L⁻¹ 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) as substrate; lanes b and c, protein staining. Total protein loading was 25 µg per lane. M, molecular mass markers.

Most extracellular fungal laccases are glycoproteins, and their carbohydrate content usually ranges from 10% to 30%. Laccases with higher saccharide content, however, have been described [2,4,7]. Lacps1 showed a carbohydrate content of about 24%, close to those determined for two laccases from *P. sanguineus* CS43 [19], but about three-fold higher than those estimated for the laccases from *P. sanguineus* BRFM66 and *P. sanguineus* BRFM902 [7]. The enzyme-bound carbohydrate moieties of fungal laccases greatly affect their properties, such as proteolytic susceptibility, copper retention ability, kinetic parameters, optimum pH and thermal stability [19,56].

Lacps1 was characterized by mass spectrometry (MS) after trypsinolysis. Three tryptic peptides were detected, and their respective precursor ion masses and amino acid sequences were: *m/z* 1,242.682 (SPGTTAADLAVIK), *m/z* 1718.75 (SAGSSEYNYDNPIER) and *m/z* 1992.69 (ANPSFGNTCFAGGINSAILR). The results of the mass-spectrometry analysis and database search demonstrated that the identified peptides shared 100% sequence identity to peptides of two laccases from *Pycnoporus coccineus* M85-2 (UNIPROT: Q96TR6 and Q96VA5) [57]. The amino acid sequences of the tryptic peptides covered about 9.3% of the amino acid sequences of both *P. coccineus* laccases.

2.4. Effects of pH and Temperature on Laccase Activity

The effects of pH and temperature on the enzymatic activity of Lacps1 were evaluated using a CCD and RSM analysis. The experimental conditions and the results of the experimental design are summarized in Table 5.

The statistical analysis of the data revealed extremely low *p*-values for the linear and quadratic effects, and also for the interaction between the pH and temperature, indicating their significant effects on the enzymatic activity (Table 6).

The quadratic model that describes the laccase activity of Lacps1 as a function of the reaction pH and temperature was expressed by the equation:

$$U \cdot \text{mg}^{-1} = [396.25 - 47.99 \text{ pH} - 20.24 T - 135.57 \text{ pH}^2 - 89.72 T^2 + 18.23 \text{ pH} \cdot T]$$

The ANOVA for the model (Table 6) revealed that, based on the *F* test, it was predictive of the laccase activity, since the calculated *F* value was much higher than the listed *F* value. The *R*² coefficient (0.99) confirmed the goodness of the model.

Table 5. Experimental conditions and results of the statistical experimental design for the laccase activity.

Run	Real (Coded) Values		U · mg ⁻¹
	T (°C)	pH	
1	70 (−1)	4.0 (−1)	258.4 ± 11.9
2	80 (+1)	4.0 (−1)	176.8 ± 8.5
3	70 (−1)	5.0 (+1)	123.6 ± 6.9
4	80 (+1)	5.0 (+1)	114.9 ± 7.8
5	75 (0)	3.5 (−1.41)	193.8 ± 9.7
6	75 (0)	5.5 (+1.41)	91.4 ± 4.7
7	68 (−1.41)	4.5 (0)	248.0 ± 12.4
8	82 (+1.41)	4.5 (0)	197.9 ± 9.9
9	75 (0)	4.5 (0)	396.1 ± 19.8
10	75 (0)	4.5 (0)	395.4 ± 19.5
11	75 (0)	4.5 (0)	397.1 ± 20.8

The experiment was repeated three times and different pure enzyme preparations were employed for each replicate (*n* = 3). The enzymatic assays were carried out in duplicate, and the response values presented for each run were the means of the three experiments.

Table 6. Coefficients values and ANOVA for laccase activity.

Effects	Coefficient	p-value
Intercept	396.25	0.0000002
pH (L)	−47.99	0.000039
pH (Q)	−135.57	0.000007
T (L)	−20.24	0.000024
T (Q)	−89.72	0.000036
pH·T	18.23	0.000546

Source	SS ^a	Df ^b	MSq ^c	F
Regression	141,950.64	5	28,390.12	—
Residual	106.22	5	21.24	1336.63
Total	142,056.81	10	14,205.68	—

*R*² = 0.99; *F* limit 5% = 5.05; ^a SS = sum of squares; ^b df = degrees of freedom; ^c MS = mean square.

According to the statistical analysis, the optimum pH and temperature for the laccase activity were 4.4 and 74.4 °C, respectively, with a maximum predicted value of 402.4 U · mg⁻¹. A very close value was determined in the experimental validation (397.1 ± 20.8 U · mg⁻¹), confirming the validity of the model. The response surface curve for the effects of pH and temperature on the laccase activity of Lacps1 is illustrated in Figure 3a; useful pH and temperature working ranges corresponded to 4.0–4.75 and 70–79 °C, respectively.

Among the purified laccases from *P. sanguineus* described to date, most presented optimum pH for the oxidation of ABTS in the range of 2.0–3.0 [17,19,53,54,58], and similar values were determined for two laccases from *Pycnoporus* sp. 5YBC [20]. However, the enzymes from *P. sanguineus* BRFM66 and *P. sanguineus* BRFM902 [7] exhibited maximum activity at pH 4.0–5.0, similarly as observed for Lacps1. In general, the laccases from other white-rot basidiomycetes exhibit pH optima for the oxidation of ABTS in the acid range (2.2–5.0) [18,59–66].

The optimum temperature for ABTS oxidation by Lacps1 was somewhat higher than those reported for the laccases from other *P. sanguineus* strains, that varied from 50 to 70 °C [17,19,53,54], which is an attractive feature for application in some biotechnological processes. Optimal temperatures in the range 40–70 °C were found for laccases from other white-rot fungi [59–66].

Figure 3. Response surface curve for the effects of pH and temperature on laccase activity (a), and thermal stability of the purified Lacps1 (b). In thermal stability studies (b), samples of the purified laccase in water were incubated at 55 °C (□), 60 °C (●), 65 °C (▲), 70 °C (◇) or 75 °C (■), and the residual activities were determined at 65 °C in McIlvaine buffer, pH 4.5, containing 1.0 mmol·L⁻¹ ABTS. One hundred percent specific activity corresponded to 401.4 ± 17.9 U·mg⁻¹. The values presented correspond to means ± SD from triplicate experiments (n = 3) performed with three separate preparations of pure Lacps1.

2.5. Thermal and pH Stabilities of Lacps1

Lacps1 was fully stable over the pH range from 3.0 to 8.0, with residual activities of about 57% and 64% at pH 2.0 and 10.0, respectively. Furthermore, the pure enzyme maintained full activity for 120 min at 55 and 60 °C, and retained about 64% of the initial activity after 120 min at 65 °C. Half-lives of 45 min and 16 min were determined at 70 and 75 °C, respectively (Figure 3b).

In contrast to the wide pH stability of Lacps1, two laccases from *P. sanguineus* CS43 were stable only in the range from pH 6.0 to 8.0 [19], while a laccase from a Chinese isolate of *P. sanguineus* showed good stability only in the acidic pH range (2.0–5.0) [17]. Likewise, the laccases from *Pleurotus* sp. [60] and *Gomphidia lucidum* [61] were stable in a restrict pH range (3.0–6.0). Similarly to Lacps1, various laccases from different *P. sanguineus* strains show good thermal stability [7,53,54], although some less stable laccases from this fungus have been described [17,19,55]. The good thermal stability and wide pH tolerance of Lacps1 are valuable properties for biotechnological applications, since at certain stages of some industrial processes the enzymes may be, even temporarily, exposed to harsh conditions, such as elevated temperatures and/or pH conditions far from those required for their maximal activity and stability.

2.6. Kinetic Properties and the Effects of Metals and Inhibitors on the Laccase Activity

The stimulation of the activity of Lacps1 by ABTS followed Michaelian kinetics, with a maximum velocity (V_M) of 413.4 ± 21.2 U·mg⁻¹. The Michaelis-Menten constant (K_M) value corresponded to 147.0 ± 7.4 μmol·L⁻¹ and the turnover number (k_{cat}) value was 462.4 ± 2.4 s⁻¹, resulting in a catalytic efficiency (k_{cat}/K_M) of about 3140.1 ± 149.6 L·mmol⁻¹·s⁻¹. Markedly different parameters were determined for a laccase produced by the same strain in wheat bran and water only, which oxidized ABTS with a K_M about 3.5-fold higher and a 10-fold lower k_{cat} value, at 25 °C, resulting in a catalytic efficiency around 88 L·mmol⁻¹·s⁻¹ [67]. Lower k_{cat} values (1.15 to 236.9 s⁻¹), as compared to that determined for Lacps1, were also found for the laccases from other *P. sanguineus* strains [7,17,53–56] and other white-rot fungi [61–63,68]. Remarkable exceptions are, however, two laccases from *P. sanguineus* CS43 [19], one from *Pycnoporus* sp. SYBCL1 [20] and two from different *Trametes* species [64,65], with similar or higher k_{cat} values. The K_M values for the oxidation of ABTS by the laccases from different *P. sanguineus* strains are highly variable (12 to 238 μmol·L⁻¹) [7,17,19,53–56]. The value estimated for Lacps1 lies in the upper half of this range, resulting in a relatively low catalytic efficiency for ABTS oxidation, as compared to some other enzymes.

The activity of Lacps1 was practically unaffected by the presence of Ca²⁺, Sr²⁺, Co²⁺, Zn²⁺, Na²⁺, Mg²⁺ and Na⁺ at 1 and 5 mmol·L⁻¹ concentrations. In contrast, the enzyme was highly sensitive to Hg²⁺, Ag⁺ and Fe²⁺, with residual activities of 17.0%, 31.6% and 22.4%, respectively, at 1 mmol·L⁻¹ concentration and total depletion at 5 mmol·L⁻¹ concentration. In addition, Pb²⁺ completely abolished the activity, at both concentrations tested. Similar inhibition patterns by divalent metal ions were observed for the laccases from *Pycnoporus* sp. SYBCL1 [20] and other white-rot fungi [60,69].

Differently, Cu²⁺ stimulated about 12.7% and 31.5% the activity of Lacps1, at concentrations of 1 and 5 mmol·L⁻¹, respectively. Similarly to our results, stimulation by Cu²⁺ has been described for two laccases from *Pycnoporus* sp. SYBCL1 [20], as well as for the laccases from some other white-rot fungi [39,66,70]. The stimulation by Cu²⁺ has been attributed to the filling of the T2 copper sites on the enzyme molecules [66]. However, some Cu²⁺-inhibited laccases have also been described [69].

Lacps1 was fairly resistant to SDS: residual activities of 78% and 17.3% were estimated in the presence of the anionic detergent at 1 and 5 mmol·L⁻¹ concentrations, respectively. In contrast, most laccases from white-rot fungi were strongly inhibited by SDS [20,60]. Ethylenediaminetetraacetic acid (EDTA) and sodium azide completely depleted the activity of Lacps1 at 1 mmol·L⁻¹ concentration, and a residual activity of about 10% was observed in the presence of 1 mmol·L⁻¹ dithiothreitol (DTT): the reducing agent completely inhibited the activity at a 5 mmol·L⁻¹ concentration. Inhibition by thiol compounds, azide and EDTA has been extensively described for white-rot fungi laccases [1,20,60,66,69,71], including two enzymes from *P. sanguineus* strains [17,52]. However, some EDTA- and DTT-insensitive laccases have been identified [20,60,69,71]. Azide is considered a true laccase inhibitor and seems to bind to the T2 and T3 copper sites, interfering with the internal electron transfer and decreasing the catalytic activity [69,71]. The inhibition of laccases by DTT is less understood. According to some authors, it results from the coordination of the thiol reagent to the copper atoms at the active site [69]. However, there is also some evidence that the inhibition could be a secondary effect, derived from a reduction of the reaction product, ABTS[•], by DTT [71].

2.7. UV-Vis Absorption Spectrum of Lacps1

Concentrated solutions of Lacps1 exhibited the typical color of the blue laccases. In agreement, the absorption spectrum exhibited a peak around 600 nm, indicating the presence of a T1, or blue copper, atom (Figure 4a). The shoulder seen in the spectrum at around 300 nm suggested the presence of a T3 copper site in the enzyme molecule (Figure 4b). Highly similar absorption spectra have been obtained for other blue laccases from white-rot fungi [19,69].

Figure 4. UV-Vis spectrum of pure Lacps1 (a), and detail of the range from 300–400 nm (b). The experiment was repeated three-fold with three separate preparations of pure Lacps1; representative spectra are presented.

2.8. Determination of the Redox Potential of the T1 Copper Site of Lacps1

At the concentration of $0.2 \text{ mmol} \cdot \text{L}^{-1}$, the mediator $\text{Fe}(\text{dipyridyl})_2\text{Cl}_2$ has not significantly interfered with the “blue” band of the copper II ion at the T1 site of Lacps1. As the concentration of $\text{Fe}(\text{dipyridyl})_2\text{Cl}_2$ was increased, the reduction of the copper II was evidenced by the progressive disappearance of the “blue” absorbance band over 550–800 nm, and from the resulting Nernst plot a one-electron reduction was observed with an E° of 0.747 V vs. NHE for the T1 copper site. Thus, Lacps1 belongs to the group of high-redox potential laccases, an attractive feature for different biotechnological applications. Comparable E° values (0.72 – 0.75 V vs. NHE) have been determined for laccases from other *P. sanguineus* strains [7] and also from *P. coccineus* BRPM 938 [7] and some *Trametes* strains [72].

2.9. Dye Decolorization by Lacps1

The ability of the pure Lacps1 and the laccase-rich crude extract from *P. sanguineus* RP15 to degrade RBBR, RB4 and BPB was analyzed at 25 and 40°C (Figure 5), temperatures in which preliminary experiments revealed that the enzyme showed good levels of activity on the dyes and excellent thermal stability. The temperature exerted little effect on the maximum levels of decolorization, when the pure enzyme was employed (Figure 5a,c). After 120 min reaction with an enzyme load of $5 \text{ U} \cdot \text{mL}^{-1}$, the percentages of decolorization reached about 90%, 80% and 60% for BPB, RBBR and RB4, respectively, either at 25°C (insets to Figure 5a,c) or 40°C . However, at 40°C the maximum decolorization of BPB and RBBR was attained after 30 min (Figure 5a), while 60 min were required at 25°C (inset to Figure 5a). In contrast, RB4 was maximally decolorized after 30 min at both temperatures. The reaction temperature also affected the enzyme load needed to attain the maximum level of decolorization after 120 min (Figure 5c). While at 40°C it corresponded to $0.5 \text{ U} \cdot \text{mL}^{-1}$ for BPB, $0.1 \text{ U} \cdot \text{mL}^{-1}$ for RBBR and $1.0 \text{ U} \cdot \text{mL}^{-1}$ for RB4, at 25°C about $1.0 \text{ U} \cdot \text{mL}^{-1}$ was required for BPB and RBBR, and $2.0 \text{ U} \cdot \text{mL}^{-1}$ for RB4 (inset to Figure 5c).

Figure 5. Effects of the reaction time and the laccase activity load on the decolorization of BPB, RB4 and RBBR by the pure Lacps1 (a,c), or the crude culture extract from *P. sanguineus* RP15 (b,d), at 40°C . Dyes final concentrations were $100 \text{ mg} \cdot \text{L}^{-1}$ (RBRR and RB4) or $25 \text{ mg} \cdot \text{L}^{-1}$ (BPB), and the reactions were carried out in McIlvaine buffer, pH 4.5. (a,b). The enzymatic load was $5 \text{ U} \cdot \text{mL}^{-1}$ (c,d); the reaction time was 120 min. Insets: Effects of the reaction time (a,b), and the laccase activity load (c,d), on dye decolorization by the crude extract (b,d), or the pure Lacps1 (a,c), at 25°C . Data are presented as means $\pm$ SD from triplicate experiments ($n = 3$) performed with three separate preparations of crude extract or pure Lacps1 (error bars are not evident, as they lie within the area of the symbol). Symbols: BPB (■), RBRR (●) and RB4 (□).

BPB is a triphenylmethane dye extensively used in textile, paper, leather, food and cosmetic industries [73]. Among the different dye groups, the triphenylmethane dyes are particularly resistant to enzymatic treatments and long reaction times are usually required for, sometimes, even a partial decolorization. To the best of our knowledge, this is the first study on the decolorization of BPB by the action of a laccase from *P. sanguineus*, either in pure or crude form. In great contrast to our results, a maximum decolorization level of BPB around 20% was obtained using $8 \text{ U} \cdot \text{mL}^{-1}$ of a pure commercial laccase from *T. versicolor* after three hours at 35°C [74], while maximum levels of about 90% were obtained with $6.5 \text{ U} \cdot \text{mL}^{-1}$ of a pure laccase from *Trametes troglit* 463 at 30°C , but after 24 h [75]. However, similarly as observed for Lacps1, a decolorization level of about 90% was achieved after treatment of BPB with $5 \text{ U} \cdot \text{mL}^{-1}$ of a pure laccase from *Lentinula edodes* for 90 min at 30°C [70].

RB4 and RBRR are typical reactive anthraquinonic dyes, widely used in the textile industry. The dyes of this class are especially resistant to degradation due to their fused aromatic rings, and most are toxic, carcinogenic and mutagenic. Furthermore, under usual dyeing conditions, up to 50% of the reactive dye initially present is discharged, generating highly colored effluents [12,20,76,77]. In spite of its unquestionable interest, the degradation of RB4 by other crude or pure laccases from *P. sanguineus* has not been investigated to date. However, similar to our results, maximum levels of RB4 decolorization of about 69% were attained after 30 min at 30°C using a laccase-rich crude extract from *T. troglit* SYBC-LZ at an enzyme load of $1.1 \text{ U} \cdot \text{mL}^{-1}$ [78].

In great contrast, the degradation of RBBR by purified laccases has been under intense investigation in the last years. Considering the enzymes produced by different species or strains of *Pycnoporus*, maximum levels of decolorization comparable to those determined for Lacps1 were obtained with a laccase from a Chinese isolate of *P. sanguineus*, under similar conditions of enzyme load, reaction time and temperature [17]. In contrast, a maximum decolorization of 20% was reached under the same conditions when a laccase from *Pycnoporus* sp. SYBC-L1 was employed [20], and 52 h were necessary to attain around 90% decolorization using each of three purified laccases from *P. coccineus*, at an enzyme load of 0.1 U·mL⁻¹ [7]. Among the enzymes from other white-rot fungi, similar maximum percentages of decolorization, as compared to Lacps1, were obtained with the purified laccases from *Trametes* sp. SQ01 [79] and *Cerrena* sp. HYB07 [80], under comparable reaction conditions. Maximum decolorization levels of 80%–90% have also been obtained with the laccases from *Lentinus* sp. [13], *T. versicolor* [74] and *Armillaria* sp. F022 [81], but much higher enzyme loads or reaction times were required. In contrast, a maximum decolorization of about 15% was obtained with a laccase from *Gonolobus* sp. rckk-02, even after 12 h with an enzyme load of 10 U·mL⁻¹ [82]. Taken together, these comparisons reveal that Lacps1 is among the most efficient white-rot fungi laccases known to date for the decolorization of BPB, RBBR and RB4.

The patterns of decolorization of BPB, RBBR and RB4 obtained using the laccase-rich crude extract from *P. sanguineus* RP15 (Figure 5b,d) were similar to those obtained with the pure Lacps1. The reaction temperature exerted little effect on the maximum levels of decolorization, which were close to those obtained with the pure enzyme: after 120 min with 5 U·mL⁻¹ of laccase activity the percentages of decolorization reached 80%–90% for BPB, 70%–80% for RBBR and 50%–60% for RB4, both at 25 °C (insets to Figure 5b,d) and 40 °C. At 40 °C, however, the maximum decolorization of RBBR and RB4 was achieved after 45 min (Figure 5b), while about 90 min and 75 min were needed at 25 °C, respectively (inset to Figure 5b). Furthermore, the percentage of decolorization of BPB did not reach a well-defined maximum after 120 min, at both temperatures tested (Figure 5b and inset to Figure 5b). Similarly, the percentages of decolorization of the three dyes have not reached maxima at 5 U·mL⁻¹ (Figure 5d), suggesting that higher decolorization efficiencies could be achieved using higher laccase loads, both at 25 °C (inset to Figure 5d) and 40 °C. Similar to our results, maximal decolorization of RBBR and BPB (around 80%) occurred after 30 min at 30 °C using 6.5 U·mL⁻¹ of a purified laccase from *T. ingii* BAF6 463 while a three-fold higher enzyme load was required to obtain a similar level of decolorization using the crude culture extract [75].

A comparison of the results obtained with the pure Lacps1 and the crude extract strongly suggested that the decolorization of BPB, RBBR and RB4 by the crude extract could be mostly attributed to the action of Lacps1, although other oxidases and natural redox mediators could have been present and contributed to the degradation, as proposed by other authors [75,83]. Furthermore, the excellent potential of the *P. sanguineus*-RP15 crude extract for application in dye decolorization processes was undoubtedly demonstrated. Although somewhat higher enzyme loads and reaction times may be needed to attain the same decolorization efficiencies obtained with the pure enzyme, the use of crude laccase preparations at relatively low temperatures (25–40 °C) is much more economical for practical applications, minimizing energy consumption and avoiding expensive and time-consuming steps of enzyme purification [75,83]. In spite of all these considerations, however, the success of a practical application of either the pure Lacps1 or the crude culture extract from *P. sanguineus* RP15 for the treatment of industrial effluents containing BPB, RBBR and/or RB4 depends on the results of a toxicity test of the degradation products of these dyes. In fact, the identification of the metabolites produced during the decolorization and/or biodegradation of a certain dye, as well as the evaluation of their toxicity, are essential steps for an eventual application of a laccase, assuring the safety of the treated effluents [11,36,84,85].

3. Materials and Methods

3.1. Organism and Strain Maintenance

The *Pycnoporus* strain was isolated from basidiocarps developing on dead fallen logs at the Campus of the University of São Paulo, at Ribeirão Preto (São Paulo, Brazil), and classified as *Pycnoporus sanguineus* according to morphologic and microscopic features as described by Ryvarden [86] and Teixeira [87,88]. The isolated strain (*P. sanguineus* RP15) was maintained on potato dextrose agar (PDA) medium, at 25 °C, and subcultured periodically.

3.2. Preliminary Screening for the Best Carbon Source, Inducer and Supplementary Nitrogen and Carbon Sources for the Production of Laccases by *P. sanguineus* RP15 under SSF Conditions

Mycelial sections (0.25 cm²) taken from 8-day-old cultures of *P. sanguineus* in PDA medium were inoculated into a sterile solid state medium (5 g of a dry agro-industrial by-product or residue as carbon source, 10 mL deionized water) in 250 mL Erlenmeyer flasks. The flasks were incubated for 192 h at 25 °C and 70% humidity, monitored by a thermo hygrometer MT-240 (Minipa, São Paulo, Brazil). After the determination of the best carbon source, the effect of supplementation with various nitrogen and carbon sources on the production of laccases was examined, under the same culture conditions. The effects of the addition of the presumed laccase inducers CuSO₄ (0.0–100 mmol·L⁻¹) and veratryl alcohol (0.0–10 mmol·L⁻¹) to the best carbon source were also investigated, under equal conditions. All the experiments were conducted in triplicate (n = 3), and the enzymatic assays were carried out in duplicate.

3.3. Response Surface Methodology for the Optimization of the Production of Laccases

After choosing the best carbon source, inducer, and supplementary nitrogen and carbon sources, the culture conditions for the production of laccases by *P. sanguineus* RP15 were optimized using a CCD and RSM. The levels of the independent variables (culture time, medium initial moisture, temperature and milled corncob concentration) were defined according to a 2⁴ full-factorial central composite design (star configuration) with 8 axial and 3 central points (triplicate in the central point) which resulted in 27 experiments. The experimental ranges of each independent variable were previously determined using the OFAT (one factor at a time) methodology, and corresponded to 6–10 culture days, 2.0–6.0 mL deionized H₂O per gram dry carbon source, 15–35 °C and 5.0%–25.0% (w/w) milled corncob. Sun-dried corncobs were milled at 30 mesh (0.595 mm) using a knife mill SL31 (Solab, Piracicaba, Brazil). The experiment was repeated three-fold (n = 3) and each enzymatic assay was carried out in duplicate. The average of the laccase production (U·g⁻¹) was used as the response value. Three cultures of *P. sanguineus* under optimized conditions for the production of laccases were performed to validate the optimization.

3.4. Enzyme Extraction

After growth, the *P. sanguineus* culture medium was suspended in 30 mL cold water and filtered through a nylon sieve after gentle homogenization in an ice bath. The suspension was centrifuged at 4 °C and 10,000 × g for 15 min and the supernatant (crude extract) was maintained at 4 °C without appreciable loss of laccase activity for at least 60 days.

3.5. Enzymatic Assays

Unless otherwise stated, the laccase activity was determined at 65 °C in McIlvaine buffer [89], pH 4.5, containing 1.0 mmol·L⁻¹ 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS, Sigma-Aldrich Chem. Co., St. Louis, MO, USA) as substrate in a final volume of 1.0 mL. The reactions were initiated by the addition of the enzyme to the reaction medium and the oxidation of ABTS to ABTS^{•+} (ε₄₂₀, pH 4.5 = 3.6 × 10⁴ mol·L⁻¹·cm⁻¹) was monitored continuously under magnetic stirring for 1 min at 420 nm in a Carry 60 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara,

CA, USA) equipped with thermostated cell holders. The experimental conditions (reaction times, enzymatic units) employed in all activity measurements were adjusted to guarantee the estimation of initial velocities, and controls without added enzyme were included in each experiment to quantify the non-enzymatic oxidation of the substrate. One enzyme unit was defined as the amount of enzyme that catalyzes the oxidation of 1 μmol ABTS per min. The specific activity was defined as units per milligram total protein. All enzymatic assays were carried out in duplicate.

3.6. Purification of Lacps1

Pycnoporus sanguineus RP15 was cultivated in 250 mL Erlenmeyer flasks at 70% humidity and under optimized conditions for laccase production (8.0 days, 25 °C, 5.0 g dry wheat bran, 0.8% (w/w) NH_4Cl , 50 mmol L^{-1} CuSO_4 , 18% (w/w) dry milled corn cob, initial moisture of 4.12 mL g^{-1}). The laccase-rich crude extract was adjusted to 60% saturation with solid $(\text{NH}_4)_2\text{SO}_4$ under stirring and after standing overnight at 4 °C the precipitate was collected by centrifugation at 10,000 $\times$ g and 4 °C for 15 min, dissolved in a small volume of McIlvaine buffer, pH 4.5, and exhaustively dialyzed against the same buffer. The dialyzed sample was adjusted to 2 mol L^{-1} $(\text{NH}_4)_2\text{SO}_4$ and applied onto a Phenyl-Sepharose CL-4B column (10.0 cm $\times$ 2.0 cm) equilibrated and eluted with McIlvaine buffer, pH 4.5, containing 2 mol L^{-1} $(\text{NH}_4)_2\text{SO}_4$ at a flow rate of 120 mL/h. Fractions of 1.0 mL were collected and analyzed for protein (A_{280}) and laccase activity. A single activity peak was eluted and the most active fractions were pooled and washed with Millipore Milli Q (EMD Millipore Co., Billerica, MA, USA) ultrapure water in centrifugal concentrators with a 10-kDa cutoff polyethersulfone membrane (Vivaspin 20, GE Healthcare, Buckinghamshire, UK) for salt removal. The purified enzyme (Lacps1) was stored at 4 °C for further use. The purification protocol was repeated 5-fold using five different crude culture extracts from *P. sanguineus*, resulting in five separate preparations of pure Lacps1.

3.7. Estimation of Protein and Neutral Carbohydrates

Protein concentrations were determined as described by Read and Northcote [90] using bovine serum albumin (Sigma-Aldrich Chem. Co., St. Louis, MO, USA) as standard. Total neutral carbohydrates were quantified by the method described by Dubois *et al.* [91], using mannose as standard.

3.8. Polyacrylamide Gel Electrophoresis

PAGE was carried out in 7% acrylamide slab gels according to Davis [92]. SDS-PAGE was carried out in 10% acrylamide slab gels as described by Laemmli [93]. The protein bands were revealed using Coomassie Brilliant Blue R-250 (Sigma-Aldrich Chem. Co., St. Louis, MO, USA). The bands with laccase activity after PAGE were visualized by incubating the gel at 65 °C for 30 min in McIlvaine buffer, pH 4.5, containing 1 mmol L^{-1} ABTS.

3.9. Estimation of the Apparent Molecular Mass of the Native Laccase

The apparent molecular mass of Lacps1 in the native state was estimated by high performance liquid chromatography (HPLC) gel filtration, using a Bio-Sil SEC 400 column (Bio-Rad Laboratories, Hercules, CA, USA) according to Souza *et al.* [94]. The molecular mass markers used were bovine myoglobin, ovalbumin and bovine γ -globulin (Sigma-Aldrich Chem. Co. Sigma-Aldrich Chem. Co., St. Louis, MO, USA).

3.10. Characterization of the Purified Lacps1 by Mass Spectrometry Analysis

The purified laccase was subjected to SDS-PAGE and the excised band was submitted to *in situ* trypsin digestion followed by MS analysis [94]. The analysis was carried out using an Axima Performance MALDI-TOF/TOF mass spectrometer (Shimadzu-Kratos, Shimadzu Corp., Kyoto, Japan). The peptide mass fingerprint was obtained and the sequences of the tryptic peptides were deduced

from a series of b- and y-ion fragments produced by high-energy collision-induced dissociation (CID-MS/MS). The CID spectra were directly submitted to MASCOT (<http://matrixscience.com>) against NCBI/nr database (nonredundant database at the National Center for Biotechnology Information, USA, <http://www.ncbi.nlm.nih.gov/>).

3.11. Effects of Temperature and pH on the Enzymatic Activity of the Purified Lacps1

The optimum pH and temperature for the activity of Lacps1 were determined using RSM. The levels of the independent variables were defined according to a 2^2 full-factorial CCD, comprising 11 experimental runs, including 4 axial and 3 central points (triplicate in the central point) (Table 2). The experiment was repeated three times and different pure enzyme preparations were used for each replicate ($n = 3$); the laccase activity was determined in duplicate. The average of the specific laccase activities determined at each pH and temperature condition was used as the response value. The optimization was validated by a triplicate determination of the laccase activity under the optimized conditions.

3.12. Thermal and pH Stabilities of the Lacps1

The thermal stability was evaluated by incubating Lacps1 diluted in Millipore Milli Q water for different time intervals at temperatures from 55 to 75 °C. After cooling on ice (1 min), the residual activities were determined at 65 °C, as described above. The pH stability was evaluated by incubating Lacps1 at 4 °C in McIlvaine buffer, pH 2.0–8.0, or glycylglycine buffer, pH 8.5–10.0, for 24 h. The residual activities were estimated in McIlvaine buffer pH 4.5, as described above. The experiments were repeated three-fold with different pure Lacps1 preparations ($n = 3$) and the enzymatic assays were performed in duplicate.

3.13. Experimental Design, Statistical Analysis and RSM Modeling

The software Statistica, version 11.0 (StatSoft Inc., Tulsa, OK, USA), was employed for the experimental designs (CCRD), and to obtain the ANOVA and the regression coefficients, which were used to derive mathematical models from the experimental data. The variables that exerted significant effects on the response of interest at 95% confidence level ($p < 0.05$) were identified. The quality of fit of the models was expressed by the R^2 coefficients and the statistical significance of the models was checked by the F-test using the same software.

3.14. Determination of the Kinetic Parameters and Data Fitting

The values of V_M , K_M and the Hill coefficient (n_H) for the oxidation of ABTS by Lacps1 at 65 °C were calculated using the SigrafW software, which fits experimental data to the Hill equation using non-linear regression [95]. All experimental kinetic curves were repeated three times, using different pure enzyme preparations, and every experimental point was assayed in duplicate. The kinetic parameters are given as the mean $\pm$ SD of the values calculated for three different experiments ($n = 3$). Except for the RSM experiments, data fitting and statistical analyses were carried out using the OriginPro 8 SRO software package (OriginLab Corp., Northampton, MA, USA).

3.15. UV-Vis Absorption Spectrum of Lacps1

The absorption spectrum of the enzyme in McIlvaine buffer, pH 4.5, was recorded from 300 to 800 nm, at 25 °C, using a Carry 60 UV-Visible spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) and a 1.0 cm light path quartz cuvette. The experiment was repeated three-fold with different enzyme preparations.

3.16. Determination of the Redox Potential of Lacps1

The determination of the E° of the Lacps1 T1 center was carried out by following a procedure of protein redox titration employing $\text{Fe}(\text{dipyridyl})_2\text{Cl}_3/\text{Fe}(\text{dipyridyl})_2\text{Cl}_2$ as mediators [96]. The E° for the couple $\text{Fe}(\text{dipyridyl})_2\text{Cl}_3/\text{Fe}(\text{dipyridyl})_2\text{Cl}_2$ lies at 0.780 V vs. NHE [97], which is appropriate for high-redox potential laccases. The components of the redox couple were prepared by mixing FeCl_2 or FeCl_3 with 2,2'-dipyridyl in water at a molar ratio of 1:2. As a control, the absorption spectrum of pure Lacps1 (10 mg mL⁻¹ in 0.1 mol L⁻¹ phosphate buffer, pH 6.0) was recorded under anaerobic conditions in the range 550–800 nm, corresponding to the typical absorption band of the T1 copper site. Subsequently, spectra of Lacps1 in the presence of a fixed concentration (0.2 mmol L⁻¹) of the electron donor, $\text{Fe}(\text{bipyridyl})_2\text{Cl}_2$, and increasing concentrations of the electron acceptor $\text{Fe}(\text{dipyridyl})_2\text{Cl}_3$ (0.01–0.2 mmol L⁻¹) were recorded under the same conditions after reaching the equilibrium, monitoring the concentrations of the oxidized (copper II) and reduced (copper I) states.

The value of the T1 E° for Lacps1 was obtained from a Nernst plot of $\log [(A_0 - A)/A]$ vs. the potential of the redox couple $[\text{Fe}(\text{dipyridyl})_2\text{Cl}_3/\text{Fe}(\text{dipyridyl})_2\text{Cl}_2]$ vs. NHE in Volts, where A_0 and A corresponded to the absorbances of the solution at 740 nm before and after the reaction equilibrium. The potential of the redox couple was calculated from the Nernst equation associated with the redox species involved ($E = E^\circ + 0.058 \log \{[\text{Fe}(\text{dipyridyl})_2\text{Cl}_3]/[\text{Fe}(\text{dipyridyl})_2\text{Cl}_2]\}$), in which the concentrations of each species were obtained from the initial values considering the concentration changes due to the interaction with the enzyme after the equilibrium.

3.17. Decolorization of Synthetic Dyes by the Laccase-Rich Crude Extract and Pure Lacps1

Decolorization experiments were performed employing either the laccase-rich crude extract from *P. sanguineus* RP15 or the pure Lacps1 and three different dyes as substrates: the anthraquinone dyes RBBR and RB4, and the triphenylmethane dye BFB (Sigma-Aldrich Chem. Co.). The reaction mixtures contained the dye at final concentrations of 100 mg L⁻¹ (RBBR and RB4) or 25 mg L⁻¹ (BFB) in McIlvaine buffer, pH 4.5, in a final volume of 1 mL. The reactions were performed under magnetic stirring at 25 or 40 °C and initiated by the addition of the enzyme to the reaction media. The enzymatic load was varied from 0.1 to 5.0 U mL⁻¹ and reaction mixtures containing aliquots of boiled crude extract or pure enzyme were used as negative controls. Dye decolorization was followed continuously in a Carry 60 UV-Visible spectrophotometer (Agilent Technologies) equipped with thermostated cell holders by monitoring the decrease of the absorbance at the wavelength of maximum absorbance of each dye (596 nm for RBBR, 592 nm for BFB and 601 nm for RB4). The percentage of decolorization was calculated according to the equation: Decolorization (%) = $[A_i - A_t/A_i] \times 100$, where A_i is the initial absorbance of the reaction mixture and A_t is the absorbance after incubation for t minutes [83]. The experiments were repeated three times using three different crude extract or pure enzyme preparations for each replicate ($n = 3$). The enzymatic assays were carried out in duplicate.

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Apêndice J

A novel thermostable and halotolerant xylanase from *Colletotrichum graminicola*

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ABSTRACT

A novel endoxylanase from *Colletotrichum graminicola* (Exg1) was purified. Similar apparent molecular masses were estimated by gel filtration (17.3 ± 3.6 kDa) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (20.0 ± 2.4 kDa), suggesting that Exg1 is monomeric. The enzyme showed good halotolerance, retaining about 80% and 50% of the initial activity in the presence of 0.5 mol L⁻¹ and 3.0 mol L⁻¹ NaCl, respectively. The optimum temperature of Exg1 (65 °C) was not affected by NaCl, but the optimum pH rose from 5.5 in the absence and presence of 0.5 mol L⁻¹ NaCl to 6.0, in 2.5 mol L⁻¹ NaCl. Exg1 was highly thermostable at 50 °C, with half-lives around 48 h in either water or 0.5 mol L⁻¹ NaCl and a residual activity of 75% at 2.5 mol L⁻¹ NaCl. Exg1 was fully active at pH 3.0–10.0 in the absence of salt, and from pH 4.0–10.0 in the presence of 0.5 mol L⁻¹ and 2.5 mol L⁻¹ NaCl. The enzyme hydrolyzed beechwood xylan with maximal velocity and apparent affinity constant of 481.3 ± 34.6 U mg⁻¹ and 3.7 ± 0.3 mg mL⁻¹, respectively. Similar kinetic parameters were obtained in the presence of 0.5 mol L⁻¹ NaCl, but a maximum velocity about 34% lower was determined in 2.5 mol L⁻¹ NaCl. Exg1 was tolerant to various organic solvents at a concentration of 5% (w/v) and also to sodium acetate up to 200 mmol L⁻¹. Xylobiose and xylofuranose with a 4-O-methylglucuronic acid branching were the main products of beechwood xylan hydrolysis, and the time course of hydrolysis was not affected by NaCl 0.5 mol L⁻¹ or sea water. To the best of our knowledge, this is the first report of a halotolerant endoxylanase from fungal origin. The properties of Exg1 suggested good potential for use in lignocellulosic saccharification processes particularly using sea water or under high salt conditions, or in the presence of residues and/or byproducts of pretreatment steps, contributing to improve the economic viability of 2G ethanol production.

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1. Introduction

The use of biofuels has attracted considerable attention around the world in the last years, and much research effort has been devoted to the development of industrial processes for the production of 2nd generation ethanol (2G ethanol) from agro industrial residues [1,2]. Presently, the main challenges for the 2G ethanol comprise giving the technological leap to a large scale production

with highly competitive prices compared to the current, and overcoming the barrier of the recalcitrance of lignocellulosic biomass [3,4].

Lignocellulose is primarily composed of cellulose, hemicellulose and lignin disposed in a complex three-dimensional matrix within which the polysaccharides are protected against chemical and biological degradation [4,5]. Xylan, the main hemicellulose in agro industrial residues [5,6], is predominantly composed of xylose residues. The xylose backbone can be substituted with arabinose, glucuronic acid and/or methylglucuronic acid, which affect some important physico-chemical characteristics of the polymer as solubility, conformation and reactivity [4,7,8].

Although chemical methods are available, the enzymatic hydrolysis of lignocellulosic biomass is a more environmentally

friendly alternative [3,9,10]. Furthermore, there is a consensus in the scientific community that the production of 2G ethanol efficiently and at a competitive price depends on the efficient hydrolysis of cellulose and hemicellulose to fermentable sugars (pentoses and hexoses), and fermentation of these sugars, as well as the use of lignin [2,10].

The complete hydrolysis of xylan requires a complex enzymatic system synergistically acting on the main chain and the branches [11]. The main enzymes involved in the hydrolysis are the endo-β-1,4-xylanase (β-1,4-xylan-xylohydrolase, EC 3.2.1.8), which releases xylooligosaccharides, xylobiose and xylose from the main chain [11,12], and the β-D-xylosidase (β-D-xyloside xylohydrolase, EC 3.2.1.37), which hydrolyzes xylooligosaccharides and xylobiose to xylose [6,13]. Some accessory enzymes work in branches, contributing to the complete depolymerization of xylan [8,13–15].

Different pretreatment steps are used to reduce the recalcitrance of the lignocellulosic biomass, enabling the action of hydrolytic enzymes on polysaccharides and increasing the efficiency of hydrolysis. For the production of 2G ethanol, some mild alkaline pretreatments and the organosolv pretreatment are interesting, since lignin is removed with low loss of cellulose and hemicellulose [3,4]. However, residual solvents, as well as salts at high concentrations and some byproducts of the alkaline pretreatment, such as acetate, are potential inhibitors of subsequent steps [16,17]. Thus, washing steps of the pretreated biomass are needed, greatly increasing the water consumption in ethanol 2g industrial plants [18].

Due to the global concern about the shortage of potable water, the possibility of using sea water for the washing steps of the pretreated biomass and even for the enzymatic hydrolysis is attracting considerable interest lately [19]. However, the salts in sea water can also inhibit most lignocellulosidic enzymes, including xylanases, generating a growing interest in the identification of enzymes tolerant to high concentrations of salts as well as organic solvents.

In nature, a wide variety of microorganisms are able to produce xylanases, including bacteria, yeasts and filamentous fungi [12,13,20]. However, filamentous fungi are known to produce extracellular xylanases at higher levels compared to yeasts and bacteria, making them attractive for the production of these enzymes on industrial scale [6,13]. The filamentous fungus *Colletotrichum graminicola* proved to be an excellent producer of an extracellular thermostable xylanase when grown under solid-state fermentation (SSF) in culture medium composed of wheat bran, milled corn cobs and ammonium sulfate [21]. The aim of this study was the purification and characterization of an endo-xylanase from *C. graminicola* (Exg1), with emphasis in the tolerance of the purified enzyme to elevated concentrations of NaCl and organic solvents.

2. Materials and methods

2.1. Organism and strain maintenance

The *Colletotrichum* strain was isolated and classified as described by Zimbardi et al. [21]. The fungus was maintained on Potato Dextrose Agar (PDA, Oxoid, UK) medium, at 25 °C, and subcultured periodically.

2.2. Culture conditions and preparation of crude extracts

Colletotrichum graminicola was grown under solid-state fermentation (SSF) conditions in 250 mL Erlenmeyer flasks, as described by Zimbardi et al. [21]. Sections (0.50 cm²) of mycelium from 8-day-old cultures on PDA medium were inoculated into sterile solid medium composed of 5.0 g wheat bran supplemented with 0.9% (w/w) milled corn cob and 0.1% (w/w) ammonium sulfate, with

initial moisture of 2.0 mL water per g dry bean. The flasks were incubated for 8 days at 25 °C under 70% constant humidity, monitored by a thermo hygrometer MT-240 (Minipa, São Paulo, Brazil).

After growth, the culture was suspended in 25 mL cold water and gently homogenized with a glass rod maintaining the flask in an ice bath. The resulting suspension was filtered through a nylon sieve and centrifuged at 10,000 × g and 4 °C for 20 min. The supernatant (xylanase-rich crude extract) was kept at 4 °C for up to 30 days without substantial loss of activity.

2.3. Enzymatic assays

Unless otherwise stated, the xylanase activity was assayed at 65 °C in McIlwaine buffer [22], pH 5.5, containing 1% (w/v) beechwood xylan (Sigma-Aldrich Chem. Co., St. Louis, MO, USA), in a final volume of 0.6 mL. The hydrolysis of birchwood xylan (Sigma-Aldrich Chem.), carboxymethylcellulose (CMC, Sigma-Aldrich Chem. Co.) and microcrystalline cellulose (Avicel[®], Fluka Chemical Co., Seelze, Germany), at 1% (w/v) final concentration, was assayed as above. The reducing sugars released were quantified using the dimethylglycolic acid (DNS) method [23]. The activity on beechwood xylan was also determined in sea water. The assay conditions were the same as described above but all reagents were prepared in sea water (32‰ salinity) from the Ubatuba Bay (23° 30' S, 45° 08' W), São Paulo State, Brazil.

The β-xylosidase activity was determined under the same conditions using p-nitrophenyl-β-D-xylopyranoside (pNP-Xyl) (Sigma-Aldrich Chem. Co.) as substrate, at 2.0 mmol L⁻¹ final concentration. The reactions, initiated by the addition of the enzyme at a convenient dilution, were interrupted after convenient time intervals by the addition of 1.0 mL saturated sodium tetraborate solution. The hydrolysis rates were determined by quantifying the release of the p-nitrophenolate anion ($K_{\text{a}}(\text{pNP-Xyl}) = 17,500 \text{ mol}^{-1} \text{ Lcm}^{-1}$), as described by Souza et al. [24].

Controls with heat inactivated enzyme were included in all enzymatic assays to quantify the non-enzymatic hydrolysis of the substrates. The experimental conditions (reaction times, enzymatic units) employed were adjusted to guarantee the estimation of initial velocities (linear response of product formation in respect to reaction time). One enzyme unit (U) was defined as the amount of enzyme that releases 1 μmol of product per min. The specific activity was defined as units per milligram total protein (U mg⁻¹). All enzymatic assays were performed in duplicate.

2.4. Purification of Exg1

The xylanase-rich crude extract was adjusted to 1.0 mol L⁻¹ NaCl and applied onto a Phenyl Sepharose CL-4B column (10.0 × 1.0 cm) equilibrated and eluted with 10.0 mmol L⁻¹ sodium acetate buffer, pH 5.0, containing 1.0 mol L⁻¹ NaCl (buffer A). The column was washed with buffer A until protein ($A_{280\text{nm}}$) and/or xylanase activity were no longer detected in the eluate. The most active fractions (1.0 mL each) were pooled and concentrated in a vacuum concentrator (Concentrator Plus/Vacufuge[®] plus, Eppendorf, New York, USA). The concentrated sample was desalted using a Sephadex G-25 column (35.0 × 2.0 cm) equilibrated and eluted with 10.0 mmol L⁻¹ Tris buffer, pH 9.0 (buffer B). Fractions showing xylanase activity were pooled and applied onto a DEAE-Fractogel column (10.0 × 2.0 cm) equilibrated and eluted with buffer B. The column was washed with buffer B until protein and/or xylanase activity were no more detected in the eluate, and the most active fractions (1.0 mL each) were pooled, aliquoted and stored at 4 °C. This purification procedure was repeated 5-fold using five different

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xylanase-rich crude extracts from *C. griseicola*, resulting in five different preparations of the pure enzyme (ExoG1).

2.5. Polyacrylamide gel electrophoresis and isoelectric focusing

Non-denaturing polyacrylamide gel electrophoresis (PAGE) was carried out according to the method of Bensfield et al. [25] in 7% acrylamide slab gels. Sodium dodecyl sulfate (SDS)-PAGE was carried out in 10% acrylamide slab gels as described by Laemmli [26]. The protein bands were revealed with silver nitrate [27].

Isoelectric focusing (IEF) of ExoG1 was carried out at 500 V for 5 h in 6% acrylamide rod gels (0.6 × 13 cm) containing 5% (v/v) carrier ampholyte (Pharmalyte, Sigma-Aldrich Chem. Co.), pH 8.0–10.0, according to Venturi et al. [28]. The protein bands were revealed with Coomassie Brilliant Blue R-250 (Sigma-Aldrich Chem. Co.).

2.6. Determination of neutral carbohydrates and protein

Total neutral carbohydrates of ExoG1 were determined according to the procedure described by Dubois et al. [29], using glucose as standard. Protein concentrations were estimated by the method of Read and Northcote [30], using bovine serum albumin (BSA, Sigma-Aldrich Chem. Co.) as standard.

2.7. Estimation of the apparent molecular mass of the purified enzyme

The apparent molecular mass of the ExoG1 was estimated by SDS-PAGE as described above, using a prestained protein molecular mass marker (6–204 kDa) (Bio-Rad, Laboratories, Hercules, CA, USA).

The apparent native molecular mass of the ExoG1 was estimated by high-performance liquid chromatography (HPLC) gel filtration, using a Bio-Sil SEC 400 column (Bio-Rad) according to Souza et al. [24]. The molecular mass markers used were bovine γ -globulin (158 kDa, Bio-Rad), BSA (67 kDa, Sigma-Aldrich Chem. Co.), ovalbumin (44 kDa, Bio-Rad) and equine myoglobin (17 kDa, Bio-Rad).

2.8. Characterization of the purified ExoG1 by mass spectrometry analysis

ExoG1 was subjected to trypsin digestion followed by MS analysis as described by Souza et al. [24]. The analysis was performed using an Axima Performance matrix-assisted laser desorption/ionization-time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF-MS) (Shimadzu-Kratos, Shimadzu Corp., Kyoto, Japan). The mass profile of the tryptic peptides (MS fingerprint) was obtained and the sequences of these peptides were deduced based on the fragmentation pattern of b- and y-ions of some selected peptides in high-energy collision-induced dissociation experiments (CID-MS/MS). The CID spectra were directly submitted to comparative analysis with the NCBI database (nonredundant database at the National Center for Biotechnology Information, USA, <http://www.ncbi.nlm.nih.gov/>) using the MASCOT algorithm (<http://matrixscience.com/>). Comparison of the sequenced peptides with the database containing the genome of *C. griseicola* was performed using the Blastp tool available at the NCBI platform (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

2.9. Effect of pH on ExoG1 activity and stability

The optimum pH for xylanase activity was determined at 65 °C in McIlvaine buffer adjusted for pH 3.0–8.0 containing 1% (w/v) beechwood xylan, either in the absence or in the presence of NaCl at 0.5 and 2.5 mol L⁻¹ concentrations. The pH stability was evaluated by incubating ExoG1 for 24 h at 4 °C in McIlvaine buffer adjusted for

pH 2.0–8.0, or glycylglycine buffer adjusted for pH 8.5–10.0, in the absence or in the presence of NaCl at final concentrations of 0.5 and 2.5 mol L⁻¹. The residual activities were estimated as described in the item 3.3.

2.10. Effect of temperature on ExoG1 activity and stability

The optimum temperature for the xylanase activity was determined in the range from 50 to 85 °C, in the absence and in the presence of NaCl at concentrations of 0.5 or 2.5 mol L⁻¹. The reaction medium consisted of McIlvaine buffer containing 1% (w/v) beechwood xylan. The buffer was adjusted for pH 5.5 in the absence of salt and in the presence of 0.5 mol L⁻¹ NaCl, and for pH 6.0 in the presence of 2.5 mol L⁻¹ NaCl. The thermal stability was evaluated by incubating ExoG1 in Millipore MilliQ (EMD Millipore Co., Billerica, MA, USA) ultrapure appropiate water for different time intervals at temperatures ranging from 50 to 65 °C, in the absence and in the presence of NaCl at final concentrations of 0.5 and 2.5 mol L⁻¹. After cooling in an ice bath for 1 min, the residual activities were determined at 65 °C as described in the item 3.3. The same procedure was employed to evaluate the thermal stability of the enzyme at 50 °C in water containing 1% (w/v) beechwood xylan, in the absence and in the presence of 0.5 and 2.5 mol L⁻¹ NaCl.

The halo-stability at room temperature was examined by incubating ExoG1 at 25 °C for 48 h in water or NaCl solutions at final concentrations in the range 0.5 mol L⁻¹ to 3.0 mol L⁻¹. The residual activities were determined as described in the item 3.3.

2.11. Determination of the kinetic parameters

Maximum velocities (V_{max}) and Michaelis-Menten constants (K_m) for the hydrolysis of beechwood xylan by ExoG1 in the presence and absence of NaCl were calculated using the program Sigafit, which fits the experimental data to the Hill equation by nonlinear regression [31]. Three repetitions of the purification procedure (item 3.4) were performed, from three different xylanase-rich crude extracts, resulting in three separate preparations of pure ExoG1. The kinetic experiments were repeated 3-fold, using each pure ExoG1 preparation, and each enzymatic assay was performed in duplicate. The kinetic parameters are presented as the mean $\pm$ SD of the values calculated for the three replicates ($n = 3$).

2.12. Effect of ions, organic solvents and sodium acetate on the enzymatic activity

The effect of various salts (Pb(NO₃)₂, CuSO₄, HgCl₂, AgCl, NiCl₂, ZnCl₂, MnCl₂, MgCl₂, CoCl₂, CaCl₂, Cr(NO₃)₃, KCl, NaCl, SrCl₂, FeCl₃ and AgNO₃), ethylenediamine tetraacetic acid (EDTA, Sigma-Aldrich Chem. Co.) and β -mercaptoethanol (Sigma-Aldrich Chem. Co.) on the activity of ExoG1 against beechwood xylan was determined as described in the item 3.3. The different effectors were added to the reaction medium at final concentrations of 1.0 and 10.0 mmol L⁻¹.

The effect of different organic solvents (ethanol, methanol, butanol, propanol, glycerol, toluene and acetone) and sodium acetate on the activity of ExoG1 was also assessed under the conditions described above, except that the experiments were conducted at 50 °C, in hermetically closed tubes. The different solvents were added to the reaction medium to a final concentration of 5% (v/v); sodium acetate was added at final concentrations of 50 and 200 mmol L⁻¹.

Table 1

Purification of ExoG1.

Step	Total activity (U)	Total protein (mg)	Specific activity (U mg ⁻¹)	Yield (%)	Purification (fold)
Crude extract	811.7	346	23.5	100.0	1.0
Phenyl-Sepharose	572.4	8.8	64.2	70.5	1.5
Sepharose G-25	441.6	5.6	78.9	94.4	1.4
DEAE-Fractogel	276.0	1.0	276.0	35.0	1.0

The purification procedure was repeated fivefold. Representative data obtained from one of the purification procedures are presented.

Fig. 3. Analysis of the purified ExoG1 by PAGE (A), SDS-PAGE (B) and isoelectric focusing (IEF) (A and B) silver staining; (C) Coomassie Blue staining. Total protein loading: 5 μ g (A) and 10 and 40 μ g (C).

2.13. Thin layer chromatography (TLC) analysis of the reaction products of the hydrolysis of beechwood xylan by ExoG1

The enzymatic assays were conducted at 50 °C and 180 rpm in hermetically closed conic plastic tubes. The reaction conditions were McIlvaine buffer, pH 5.5, containing 1% (w/v) beechwood xylan and 5 U of ExoG1 in a final volume of 1.0 mL, in the absence and in the presence of 0.5 mol L⁻¹ NaCl or in natural sea water (32‰ salinity). After adequate time intervals the reactions were interrupted by the addition of 1.0 mL of 20% (w/v) trichloroacetic acid to precipitate proteins and long-chain carbohydrates. After standing at room temperature for 30 min, the reaction mixtures were centrifuged for 5 min at 10,000 $\times$ g. The products present in the supernatants (5 μ L) were analyzed by TLC on silica-gel G-60 plates (10 $\times$ 20 cm, DC-Alufolien Kieselgel 60, Merck, Darmstadt, Germany). An aliquot of 2 μ L of a mixture containing xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5) and xylohexaose (X6) (Sigma-Aldrich Chem. Co.) at its final concentration was employed as standard. The mobile phase was composed of ethyl acetate/acetic acid/formic acid/Millipore MilliQ water (9:3:1:4, v/v/v/v). After two runs, the products were detected by spraying the plates with 0.4% (w/v) orcinol in sulfuric acid/ethyl alcohol (1:9 v/v) followed by heating at 150 °C until the appearance of stains.

2.12. Characterization of the hydrolysis products by mass spectrometry

The hydrolysis of beechwood xylan was carried out as described in the item 3.1.3 except that the buffer was 10 mmol L⁻¹ ammonium acetate buffer, pH 5.5. In addition, the reaction time was 48 h, and the reactions were interrupted by heating in boiling water for 5 min, followed by centrifugation for 10 min at 10,000 $\times$ g. The hydrolysis products were analyzed by direct insertion mass spectrometry (MS-IE) on a Xevo[®] TQ-S instrument (Waters Corporation, Milford, MA, USA) with an electrospray ionization source operated in positive mode. Aliquots of 5 μ L of the reaction samples were introduced into the mass spectrometer by direct insertion into a flow of 0.1% formic acid and acetonitrile (0.1% (v/v) formic acid set at a flow rate

of 0.1 mL min⁻¹). The ESI interface conditions were as follows: capillary voltage 3.2 kV, cone voltage 40 V, Z-spray source temperature 150 °C, desolvation gas (N₂) temperature 250 °C, desolvation gas flow 900 L h⁻¹ (mass range from m/z 100–1200). The experiments of MS/MS were performed by CID using argon gas as the collision gas for the precursor ions of interest. The collision energy was over the range from 10 and 50 eV. Data acquisition and processing were performed using the Masslynx V4.1 (Waters Corporation).

3. Results and discussion

3.1. Enzyme purification and molecular properties

ExoG1 was purified by a simple two-step method comprised of Phenyl-Sepharose and DEAE-Fractogel column chromatography (Table 1). The enzyme was purified 5.0-fold, with a yield of about 34%, reaching a specific activity of 276.0 U mg⁻¹. The activity of purified ExoG1 remained unaltered for at least 6 months when it was maintained in 10 mmol L⁻¹ Tris buffer, pH 9.0, at 4 °C.

A single protein band was revealed when the purified xylanase was submitted either to PAGE (Fig. 1A) or SDS-PAGE (Fig. 1B), indicating the homogeneity of the preparation. The apparent molecular mass of ExoG1 estimated by gel filtration was 17.3 $\pm$ 1.9 kDa, in good agreement with that estimated by SDS-PAGE (20.0 $\pm$ 2.4 kDa, Fig. 1B), suggesting that the native enzyme was a monomer. Most known xylanases from mesophilic endophytic fungi are monomeric, with molecular weights in the range 8–45 kDa [11,13,32–40]. A single protein band was also revealed after IEF in a narrow pH range (8.0–10.0), with an estimated pI of 9.20 $\pm$ 0.02 (Fig. 1C).

ExoG1 was heavily glycosylated, with an estimated carbohydrate content of 97 $\pm$ 3.7% (w/w). Thus, the relatively poor focusing of the enzyme after IEF could be due to differential glycosylation [41,42], although the narrow pH range combined with a considerably long gel may have contributed to a wider band. Many of the known xylanases from mesophilic endophytic fungi are glycoproteins, including some with a carbohydrate content above 60% (w/w) [32–36].

Fig. 2. Effect of NaCl on the activity and stability of ExgI. The activity of ExgI in the presence of increasing concentrations of NaCl (●) was assayed at 55 °C in McIlwaine buffer, pH 5.5, containing 15 (w/v) birchwood xylan. The activities were expressed as percentages of the control activity. The halotolerance of ExgI (○) was evaluated after 48 h incubation at 25 °C in solutions of NaCl (0–3.0 mol L⁻¹) in water. Residual activities were assayed at 55 °C in McIlwaine buffer, pH 5.5, containing 15 (w/v) birchwood xylan. One hundred percent specific activity (control) corresponded to 380 ± 150 U mg⁻¹. The values shown represent means ± SD from triplicate experiments (n = 3) carried out with three separate preparations of pure enzyme.

ExgI was subjected to tryptic hydrolysis and characterized by mass spectrometry. Three peptides were detected in the spectrum of MALDI-TOF/MS, and their respective precursor ions and amino acid sequences were: m/z 1,762.8 (SYINDYNDLSATYAK); m/z 2,157.0 (YVAVWVVEHNEHEDGSMR) and m/z 2,265.1 (GHTTYVWH-SQPSWVSSITDK). A database search revealed that these peptides shared 77.4% sequence similarity and covered about 12.4% of a family GH10 glycosyl-hydrolase from *C. graminicola* M1.001; CLRG_08914.1 (NCBI Reference Sequence: XP_008097790.1). Furthermore, about 60% of the substitutions found in the peptides were conservative. Taken together, these data suggest that ExgI may be categorized in the family GH10. Although most xylanases from this family present molecular masses above 30 kDa [43] several exceptions to this general pattern have been reported [44].

3.2. Effect of increasing concentrations of NaCl on the activity and stability of ExgI at 25 °C

The xylanase activity of ExgI gradually decreased as the salt concentration in the reaction medium increased (Fig. 2), reaching about 50% of the control activity (absence of salt) in the presence of 3.0 mol L⁻¹ NaCl. However, at 0.5 mol L⁻¹ NaCl, a concentration close to that usually found in sea water (0.46 mol L⁻¹) [19], about 85% of the control activity was maintained. Similarly, the xylanase activity determined in natural sea water (32‰ salinity) corresponded to about 85% of the control activity. The enzymatic activity remained almost constant after 48 h incubation of ExgI at 25 °C in the presence of NaCl in the range 0–3.0 mol L⁻¹ (Fig. 2). Taken together, these results characterized ExgI as a halotolerant enzyme.

To the best of our knowledge, this is the first report on a fungal halotolerant xylanase. However, this interesting feature has been investigated in bacterial xylanases. The results observed for ExgI were very similar to those described for the halotolerant xylanases from *Moraxella* sp. [45] and *Glaciecola mesophila* KMM241 [46], which maintained respectively about 60% and 50% of the control activity in the presence of 3.0 mol L⁻¹ NaCl and about 85–90% in 0.5 mol L⁻¹ NaCl. In contrast, a xylanase from *Bacillus* sp. SNS retained 75% of the control activity at 3.0 mol L⁻¹ NaCl, further

presenting a stimulation of about 25% in 0.5 mol L⁻¹ NaCl [47]. Similarly as observed for ExgI, the xylanases from *Bacillus halodurans* [20,48] presented good stability in the presence of salt in a wide concentration range, at 25 °C.

Most known enzymes are severely inhibited or inactivated in the presence of salts at high concentrations. Apparently, this is essentially due to the sequestration of water molecules by the ions, limiting the free water molecules available for the enzyme hydration. In addition, the ions unsettle the hydration shells that surround the hydrophobic regions of the protein surface and disturb electrostatic interactions between adjacent charged groups. As a consequence, the structure and the function of the enzymes are drastically affected, as well as the solubility and stability [18,49]. Otherwise, the halotolerant enzymes compete with the ions for the water molecules, preserving the structure and activity. Apparently, this is related to a higher content of charged amino acid residues and a lower content of amino acid residues with bulky hydrophobic side chains at the protein surface, enabling the hydration of the molecule [40].

The halotolerance is a very interesting feature for a possible application of ExgI in lignocellulosic biomass saccharification processes using sea water to replace fresh water, or in processes in which the pre-treated biomass has high concentrations of salts.

Fig. 3. Optimal pH (A) and temperature (B) for the xylanase activity of ExgI in the absence and presence of NaCl. (A) The activity was estimated at 55 °C in McIlwaine buffer, pH 3.0–8.0, in the absence of salt (●) and in the presence of 0.5 mol L⁻¹ or 3.0 mol L⁻¹ NaCl. Specific activities of 100 U under these conditions corresponded to 280 ± 15 U mg⁻¹, 247 ± 13 U mg⁻¹, and 172 ± 10 U mg⁻¹, respectively. (B) The activity was assayed at different temperatures in McIlwaine buffer, pH 5.5, in the absence of salt (●) and in the presence of 0.5 mol L⁻¹ NaCl (○) or 3.0 mol L⁻¹ NaCl (■). Specific activities of 100 U under these conditions corresponded to 302 ± 20 U mg⁻¹, 252 ± 13 U mg⁻¹, and 198 ± 11 U mg⁻¹, respectively. The values shown represent means ± SD from triplicate experiments (n = 3) carried out with three separate preparations of pure enzyme.

Fig. 4. Stability to temperature and pH of the ExgI in the absence and presence of NaCl. (A) Thermal stability of ExgI in water at 50 °C (●) and 55 °C (■) and 0.5 mol L⁻¹ NaCl. One hundred percent specific activity corresponded to 309 ± 23 U mg⁻¹. (B) Thermal stability of ExgI at 50 °C in the absence of salt (●) and in the presence of 0.5 mol L⁻¹ (○) and 2.5 mol L⁻¹ (■) NaCl. One hundred percent specific activity corresponded to 305 ± 21 U mg⁻¹. (C) pH stability of ExgI in the absence of salt (●) and in the presence of 0.5 mol L⁻¹ (○) and 2.5 mol L⁻¹ (■) NaCl. One hundred percent specific activity corresponded to 284 ± 19 U mg⁻¹. The values shown represent means ± SD from triplicate experiments (n = 3) carried out with three separate preparations of pure enzyme.

3.3. Effect of pH and temperature on the xylanase activity of ExgI

In the absence of salt ExgI showed a plateau of maximum activity from pH 4.5–6.0 (Fig. 3A). A sharper pH-activity profile was observed in the presence of 0.5 mol L⁻¹ NaCl, with maximal activity at pH 5.5 and a steep decrease at more acidic pH, reaching about 75% and 20% of the maximal activity at pH 4.5 and 4.0, respectively. Above pH 5.5, however, the decrease of the enzymatic activity was similar in the presence and in the absence of salt. A very different profile was observed in the presence of 2.5 mol L⁻¹ NaCl. The optimum pH was 6.0, and below this value the activity sharply decreased, reaching 55% and 6% of the maximum at pH 5.0 and 4.5, respectively. Above the optimum pH, however, the xylanase activity decreased gradually to reach 45% of the maximum at pH 8.0.

The different pH-activity profiles observed for ExgI in the presence of NaCl may tentatively be attributed to changes in the pKa values of the catalytic groups of the catalytic glutamate residues, conserved in all xylanases from families GH10 and GH11 [6,11]. The altered pKa possibly reflect changes in the microenvironment of the catalytic residues in response to conformational changes in the enzyme molecules associated to the presence of high ion concentrations.

The optimal pH of the xylanases from mesophilic fungi are generally in the range 4.0–6.5 [13,32–38,40]. In contrast, most known bacterial halotolerant xylanases present optimum pH in the alkaline range (7.0–12.0). However, some recently described enzymes showed optimum pH close to that estimated for ExgI, such as the xylanases from *Moraxella* sp. BHM26 [50], *Bacillus subtilis* [51] and *Bacillus* sp. [52]. The optimum pH of about 5.0 determined for ExgI in the presence of 0.5 mol L⁻¹ NaCl is a very interesting feature for a possible inclusion in enzyme cocktails for saccharification of biomass using sea water.

At both salt concentrations tested, the temperature-activity profiles were similar to that observed in the absence of salt, with a gradual increase of the activity above 50 °C, reaching a maximum at 65 °C and decreasing above 70 °C (Fig. 3B). Furthermore, the salt had little effect on the activity of ExgI at 50 °C, which corresponded to about 60–70% of the maximal.

The optimal temperatures of the xylanases from mesophilic fungi are usually in the range 40–65 °C [13,32–40]. In contrast, some bacterial halotolerant xylanases show elevated optimal temperatures (90–70 °C) as determined for ExgI [20,45,48,51–53]. However, some halotolerant enzymes with maximal activity in the range 35–45 °C have also been described [40,47,50].

3.4. Effects of temperature and pH on the stability of ExgI

ExgI was fully stable at 50 °C and 55 °C for 120 min in water and half-lives of about 90.0 ± 6.3 and 25.0 ± 1.7 min were determined at 60 °C and 65 °C, respectively (Fig. 4A). Most xylanases from mesophilic endophytic fungi are stable in the range of 35–55 °C [32–37], with few exceptions showing good stability at temperatures up to 65 °C [38–40]. Thus, ExgI is among the most thermostable xylanases produced by the microorganisms of this group. The use of thermostable enzymes in industrial processes has a number of well-known advantages, contributing to their economic stability [54–56].

Table 2

Substrate specificity of the purified Exg1

Substrate	Relative activity (%)
Beechwood xylan	100 ^a
Birchwood xylan	88 ± 1
Avicel [®]	ND
CMC low viscosity	ND
CMC high viscosity	ND
pNP-Xyl	0.290 ± 0.001

ND: activity not detected after 2 h reaction employing 0.110 U xylanase activity. The activity was assayed at 65 °C in McIlwaine buffer, pH 3.5, containing the different substrates at 1% (w/v) concentrations, except for pNP-Xyl, employed at 3 mmol L⁻¹ concentration.

Values are means ± SD of five different experiments (n = 5).

^a One hundred percent specific activity corresponded to 100 ± 0.5 U mg⁻¹.

High salt concentrations further increased the thermal stability of Exg1 at 50 °C. In the presence of 2.5 mol L⁻¹ NaCl a residual activity of 75% was determined after 48 h, much higher than those observed in the absence of salt (52%) and in the presence of NaCl at 0.5 mol L⁻¹ concentration (46%) (Fig. 4B). Moreover, Exg1 was fully stable at 50 °C for 48 h in the presence of 1% beechwood xylan either in the absence or in the presence of NaCl at both concentrations tested (data not shown), suggesting that the substrate has a stronger protective effect on the enzyme activity, as compared to the salt. Similarly as observed for Exg1, a xylanase from *Zinogwangia profunda* exhibited increased thermal stability in the presence of high salt concentrations [57].

While increasing the thermal stability of an enzyme in the presence of salt would be desirable for a biotechnological application, the physico-chemical mechanisms underlying this effect are not well understood. Apparently, the stabilization of protein active conformations by reduction of the repulsion between charged groups and/or enhancement of hydrophobic interactions may be involved [58].

In the absence of salt Exg1 was fully stable in a wide pH range, from 3.0 to 10.0 (Fig. 4C). The presence of NaCl exerted a minor effect on the enzyme stability in the range of pH 4.0–10.0, but it was severely impaired at pH 3.0 and 3.5 at both salt concentrations tested, with residual activities of about 54–64% at pH 3.5 and 5–20% at pH 3.0. There is ample evidence that the pKa of ionizable side chains of amino acid residues on the surface of the protein are modified by high concentrations of salt, favoring the neutral form [59]. The lower stability of Exg1 at pH 3.0 and 3.5 can then be tentatively attributed to increased pKa of the side chains of acid residues, disrupting structurally relevant ionic interactions. In contrast to our results most known bacterial halotolerant xylanases are stable in a narrow pH range, more often in the alkaline region [20,47,48,53].

3.5. Substrate specificity of Exg1

The best substrates for Exg1 were beechwood xylan and birchwood xylan (Table 2). Further, the enzyme hydrolyzed the synthetic substrate pNP-Xyl with low activity. In contrast, it was unable to hydrolyze CMC and Avicel[®], indicating high specificity for the β -anomeric configuration of the glycosidic linkage of xylene residues. Similar properties have been described for other fungal xylanases

[34,60,61]. These results suggested that Exg1 is an endo-xylanase free of cellulase activity.

3.6. Kinetic parameters

In the absence of salt the hydrolysis of beechwood xylan by Exg1 occurred with V_{max} of 481.3 ± 34.0 U mg⁻¹ and K_m of 3.7 ± 0.3 mg mL⁻¹, resulting in a catalytic efficiency (k_{cat}/K_m) of 36.9 ± 1.5 mL s⁻¹ mg⁻¹ (Table 3). Similar parameters were determined in the presence of 0.5 mol L⁻¹ NaCl, but at a salt concentration of 2.5 mol L⁻¹ the V_{max} was about 34% lower, while the K_m was about 50% higher. As a consequence, a reduction of about 56% in the catalytic efficiency for the hydrolysis of beechwood xylan occurred at the highest salt concentration.

Few authors have performed the kinetic characterization of the hydrolysis of beechwood xylan by halotolerant xylanases. In the absence of salt, the enzymes from *Mucilaginibacter* sp. [50] and *Halobacter* sp. [52] showed apparent affinities for the substrate about 2.6- and 1.8-fold lower, respectively, as compared to Exg1. Furthermore, the hydrolysis occurred with lower V_{max} , resulting in catalytic efficiencies 7- and 3.2-fold lower, respectively. In contrast, although lower V_{max} were determined for the xylan hydrolysis by the xylanases from *Mucosaccus* sp. [45], *G. mesophilus* KM241 [46] and *Halobacter* sp. [47], the catalytic efficiencies were higher than those determined for Exg1.

The marked differences observed in the kinetic parameters determined for xylan hydrolysis by Exg1 in the presence of 2.5 mol L⁻¹ NaCl as compared to those obtained in the absence of salt, may be attributed to conformational changes in the enzyme molecule that may hinder the enzyme-substrate interactions and the adequate positioning of the catalytic groups, thus resulting in decreased apparent affinity and V_{max} . Up to date, a single similar study has compared the kinetic parameters for the hydrolysis of beechwood xylan by the halophilic xylanase from *Z. profunda* in the absence and in the presence of salt. In contrast to our results, increases of about 2.6- and 4.4-fold were observed in the values of K_m and k_{cat} , respectively, in the presence of 3 mol L⁻¹ NaCl [57].

3.7. Analysis of the xylan hydrolytic products

The mode of action of Exg1 on beechwood xylan was investigated through the analysis of the reaction products by TLC (Fig. 5A). Before 30 min reaction, only high-molecular-weight xylooligosaccharides (degree of polymerization (DP) > 5) were produced (not shown). After 30 min, the main products were X2, X3, X4, traces of X5 and X6, and two species near X3 and X4 that possibly corresponded to branched forms of X3 (X3B) and X4 (X4B). After 1 h, stains corresponding to X2, X3, X3B and X4B were seen, but after longer times (3–15 h) X3 and X4B, but not X3B, were gradually hydrolyzed, with accumulation of X2 and liberation of minor amounts of X1. Finally, after 48 h the only products were X2, X3B and traces of X1. The hydrolysis profiles obtained from reactions conducted in the presence of 0.5 mol L⁻¹ NaCl (Fig. 5B) and in 32% salinity natural sea water (not shown) were entirely similar to that obtained in the absence of salt, reaffirming the potential of

Fig. 5. Time course analysis of the hydrolysis of beechwood xylan by Exg1 in the absence (A) and presence (B) of 0.5 mol L^{-1} NaCl. Standards: X1: xylose; X2: xylobiose; X3: xylofuranose; X4: xylofuranose; X5: xylopentose; X6: xylohexose.

application of Exg1 in biotechnological processes performed at high salinities.

The MS-ED analysis (Fig. 6A) of the 48 h reaction mixture confirmed the presence of three major peaks of m/z 168, 305, 627, consistent with the adducts $[X1 + NH_4]^+$, $[X2 + Na]^+$ and $[X3 + 4-O-Me-GlcA + Na]^+$. CID-MS/MS experiments further indicated that $[X2 + Na]^+$ comprised two pentoses (Fig. 6B) while $[X3 + 4-O-Me-GlcA + Na]^+$ comprised three pentoses and one 4-O-methyl-D-glucuronic acid (Fig. 6C). These results suggested that Exg1 is an endo-xylanase that is not able to hydrolyze the glycosidic bonds of the main chain near a branch point, as described for other endo-xylanases [62–64]. Furthermore, the major final products formed reinforce the categorization of Exg1 as a xylanase from the GH10 family, according to the classification proposed by [65].

3.8. Effect of ions and other effectors on the xylanase activity of Exg1

The activity of Exg1 was insensitive to the presence of Ph^{3+} , Ni^{2+} , Zn^{2+} , Mn^{2+} , Mg^{2+} , Co^{2+} , Ca^{2+} , K^+ , Na^+ and Sr^{2+} , at both concentrations tested (Table 4). In contrast, Cu^{2+} , Ag^+ , Cr^{3+} , Fe^{3+} and Ag^+ exerted little effect on the activity at the lowest concentration, but had a strong inhibitory effect (47–97%) at 10 mmol L^{-1} concentration. Exg1 was less sensitive to the presence of Mn^{2+} , Co^{2+} , Zn^{2+} , Ni^{2+} and Ag^+ than other halotolerant xylanases [45–48,50]. In contrast, the enzyme showed greater sensitivity to Cu^{2+} and Fe^{3+} , as compared to some of these enzymes [46,50]. Furthermore, Exg1 was highly sensitive to Hg^{2+} at 1 mmol L^{-1} concentration, with a residual activity of only 13.6%, which was consistent with data obtained for most halotolerant xylanases [45,47,48,50]. EDTA had little effect on the enzymatic activity, suggesting that Exg1 is not a metalloenzyme. In contrast, β -mercaptoethanol at a 10 mmol L^{-1} concentration stimulated the enzymatic activity by 39%, as described for other xylanases [32,34,66], including the halotolerant ones [45,50]. The effect of the reducing agent may be attributed to the protection of thiol groups of cysteine residues, preventing its oxidation [34].

Table 4
Effect of ions and additives on the xylanase activity of Exg1.

Effector	Residual activity (%)	
	1 mmol L ⁻¹	10 mmol L ⁻¹
Control	100 ^a	100 ^a
Ph(NO ₃) ₃	101.2 ± 1.1	72.8 ± 1.3
NiCl ₂	94.4 ± 2.2	99.1 ± 2.2
ZnCl ₂	97.1 ± 2.3	98.1 ± 1.2
MnCl ₂	100.4 ± 1.1	107.7 ± 4.3
MgCl ₂	107.1 ± 2.2	94.3 ± 2.2
CaCl ₂	96.5 ± 2.2	92.9 ± 3.8
CoCl ₂	102.3 ± 2.8	95.5 ± 1.9
KCl	101.7 ± 1.4	94.5 ± 2.3
NaCl	101.0 ± 2.1	109.6 ± 2.3
LiCl	114.1 ± 4.1	106.9 ± 1.2
CuSO ₄	70.4 ± 1.3	5.0 ± 0.8
AgCl	92.8 ± 3.3	3.6 ± 0.7
Cr(NO ₃) ₃	96.1 ± 1.2	53.1 ± 2.0
FeCl ₃	103.1 ± 2.9	23.4 ± 1.3
AgNO ₃	95.2 ± 1.6	9.8
HgCl ₂	11.8 ± 1.3	1.6 ± 0.4
EDTA	102.4 ± 1.8	80.9 ± 2.8
β-mercaptoethanol	106.2 ± 1.8	138.2 ± 6.1

NA: not evaluated due to interference in the colorimetric method employed for the enzymatic assays.

Values are the means ± SD of five different experiments (n = 5).

3.9. Effect of different organic solvents and acetate on the xylanase activity of Exg1

The effects of different solvents used in biomass pretreatment processes, as well as acetate, a byproduct of alkali pretreatments, on the xylanase activity of Exg1 were evaluated (Fig. 7). The enzyme was highly tolerant to acetate up to 200 mmol L^{-1} . Propanol and toluene have not affected the enzymatic activity, while a slight stimulation (about 20%) by acetone was observed. Furthermore, the enzyme was highly tolerant to glycerol, methanol, ethanol and butanol, with residual activities from 70 to 90%.

To date, few studies have evaluated the tolerance of fungal xylanases to organic solvents. A xylanase from *Aspergillus awamori* WRTS-F312 [67] was slightly inhibited (14–37%) by

Table 3
Kinetic parameters for the hydrolysis of beechwood xylan by Exg1.

Addition	K_m (mg mL ⁻¹)	V_{max} (U mg ⁻¹)	k_{cat} (s ⁻¹)	k_{cat}/K_m (mL s ⁻¹ mg ⁻¹)
–	3.7 ± 0.3	481.3 ± 34.0	176.4 ± 9.2	30.0 ± 1.5
NaCl (0.5 mol L ⁻¹)	3.8 ± 0.3	403.0 ± 21.8	113.3 ± 7.8	37.8 ± 1.8
NaCl (2.5 mol L ⁻¹)	5.0 ± 0.5	318.0 ± 38.2	90.3 ± 9.4	18.1 ± 0.8

The kinetic parameters are presented as the means ± SD of the values calculated from data from three different experiments (n = 3).

Fig. 6. (A) MS spectrum of the 48 h reactive mixture derived from the hydrolysis of benzofused xylem by Enx1; (B) CID-MS/MS spectrum of the peak of *m/z* 303 ($[C_8H_8O_2 + Na]^+$); (C) CID-MS/MS spectrum of the peak of *m/z* 327 ($[C_8H_8O_2 + Na]^+$).

different organic solvents, such as ethanol, butanol, methanol, propanol and acetone, at a final concentration of 30% (v/v). In contrast, a xylanase from *Aspergillus niger* [68] was stable for 1 h at 40 °C in the presence of 1% (v/v) ethanol, butanol, methanol, propanol, glycerol, and acetone.

4. Conclusions

A new endo-xylanase from *C. grammicola* was purified and characterized. To the best of our knowledge, this is the first halotolerant endo-xylanase from fungal origin described to date. In addition, the enzyme was thermostable and tolerant to a wide pH range, as well as organic solvents in residual levels and sodium acetate. Furthermore, the thermal stability of the endoxylanase was improved by

Fig. 7. Effect of organic solvents and sodium acetate on the activity of EupI. The solvents were added to the reaction mixture at 5% (v/v) final concentration. Symbols: (a) control; (b) 50 mmol L⁻¹ sodium acetate; (c) 200 mmol L⁻¹ sodium acetate; (d) ethanol; (e) propanol; (f) butanol; (g) acetone; (h) toluene; (i) glycerol; (j) methanol. One hundred percent specific activity (control) was determined in the absence of any additive and corresponded to 302 ± 22 IU mg⁻¹.

high concentrations of NaCl. Exclg1 hydrolyzed beechwood xylan with good catalytic efficiency and high V_{max} either in the absence or presence of 0.5 mol/L NaCl, and a V_{max} about 144-fold lower was determined in the presence of 2.5 mol/L NaCl. The main products of the hydrolysis of beechwood xylan by Exclg1 were xylofuranose and xylose with a molar ratio of 4:6. In hydrochloric acid and in a natural course of the hydrolysis was similarly similar in 32% salinity natural sea water and in the absence or presence of 0.5 mol/L NaCl. Taken together, these results suggested that Exclg1 is a good candidate to compose enzymatic cocktails for biomass hydrolysis, particularly using sea water or under high salt conditions, or in the presence of residues and/or byproducts of pretreatment steps, potentially contributing to improve the economic viability of 2G ethanol production.

Conflicts of interest

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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References

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