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Use of surfactants as a strategy to improve the processing of sugarcane bagasse aiming at the production of biopigments by *Monascus ruber*

Uso de surfactantes como estratégia para melhorar o processamento do bagaço de cana-de-açúcar visando a produção de biopigmentos por *Monascus ruber*

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Tese apresentada à Escola de Engenharia de Lorena da Universidade de São Paulo para obtenção do título de Doutor em Ciências do Programa de Pós-Graduação em Biotecnologia Industrial na área de concentração de Biotecnologia Industrial.

Orientador: Prof. Dr. Silvio Silvério da Silva

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Thesis presented to the Escola de Engenharia de Lorena of Universidade de São Paulo to obtain the degree of Doctoral in Science issued by the Programa de Pos-graduação em Biotecnologia Industrial in the field of Industrial Biotechnology Area.

Advisor: Prof. Dr. Silvio Silvério da Silva

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Thesis dedicated to my family

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RESUMO

SÁNCHEZ-MUÑOZ, S. **Uso de surfactantes como estratégia para melhorar o processamento do bagaço de cana-de-açúcar visando a produção de biopigmentos por *Monascus ruber***. 2022. 159p. Tese (Doutorado em Ciências) - Escola de Engenharia de Lorena, Universidade de São Paulo, Lorena, 2022.

Para viabilizar a produção industrial de pigmentos é fundamental desenvolver alternativas de processo que possam ser aplicadas em larga escala no conceito de biorrefinaria. Nesse contexto, foi proposto a utilização de tensoativos nas diferentes etapas do processo da produção de pigmentos utilizando o bagaço de cana-de-açúcar como biomassa modelo, tais como pré-tratamento, hidrólise enzimática e fermentação. Diferentes estratégias de aplicação de surfactantes foram estudadas durante o processamento da produção de pigmentos pelo fungo filamentoso *Monascus ruber*. Primeiramente, no processo de hidrólise enzimática da celulignina do bagaço de cana-de-açúcar (obtida a partir de um tratamento ácido diluído) feito em frascos *Erlenmeyer*. Um experimento de Delineamento Composto Central Rotacional (DCCR) foi realizado para determinar a influência de diferentes proporções (0,5-2,5%) de tensoativos não iônicos, de forma a melhorar sequencialmente a produção de açúcares de 2ª geração e biopigmentos. Os resultados obtidos proporcionaram uma melhora de 1,77 vezes na liberação de açúcares monoméricos em relação aos controles sem surfactante. Experimentos empregando a formulação otimizada de tensoativos (*SOF-Surfactant optimized formulation*) (Tween 20-PEG) mostraram um efeito positivo na carga enzimática, com resultados semelhantes empregando dosagens de 2,5 FPU/g de biomassa (adição de *SOF*) e 10 FPU/g de biomassa (sem *SOF*). Além disso, sob a *SOF*, foi determinado que a estabilidade da enzima foi mantida em altas temperaturas e forças de cisalhamento. Também foi observado que a produção de biopigmentos foi 5 vezes maior em meio à base de glicose. Finalmente, sob a hidrólise e fermentação separadas (SHF) e sacarificação e fermentação semi-simultânea (SSSF) a produção máxima de biopigmentos foi de 10 UA_{510nm}/mL e 1,5 UA_{510nm}/mL, respectivamente. No processo SSSF, o pigmento adsorvido na biomassa atingiu valores de aproximadamente 9 UA_{510nm}/mL durante a primeira extração (realizaram-se 4 extrações). Paralelamente, foi realizado um segundo estudo utilizando o hidrolisado hemicelulósico obtido a partir da hidrólises ácida do bagaço da cana-de-açúcar para a produção de pigmentos de *Monascus*. Inicialmente, diferentes tensoativos não iônicos foram testados separadamente e em mistura para avaliar um possível sinergismo e para potencializar a liberação de pigmentos extracelulares durante a etapa de fermentação. Após esta avaliação inicial, Tween 80 e Triton X-100 foram selecionados, e um experimento DCCR foi realizado para encontrar a melhor formulação para produzir pigmentos de *Monascus* a partir de um meio rico à base de xilose. Os resultados mostraram um incremento de quatro vezes na produção de biopigmentos vermelhos quando a *SOF* obtida foi utilizada na fermentação de hidrolisados hemicelulósicos de subprodutos da cana-de-açúcar. No foi detectada a produção de citrinina, e os pigmentos produzidos também demonstraram alta estabilidade térmica. Por fim, uma última estratégia no uso de surfactantes foi estudada na etapa de pré-tratamento. O Tween 80 foi selecionado após alguns estudos preliminares e um pré-tratamento alcalino assistido por surfactantes e cavitação hidrodinâmica para bagaço de cana-de-açúcar foi avaliado. Um novo experimento DCCR foi realizado para encontrar a melhor relação entre NaOH:Tween 80 para aumentar a produção de açúcares monoméricos na sacarificação enzimática do bagaço de cana-de-açúcar. Na condição otimizada, foi relatado um incremento de 40% na remoção de lignina em relação ao controle. A biomassa pré-tratada também foi utilizada para produzir pigmentos de *Monascus* mediante uma estratégia SSSF em reator de leito fluidizado, obtendo uma produção máxima de 3,86 UA_{510nm}/mL. Em todas as etapas avaliadas, os tensoativos se mostraram como moléculas-chave para a conversão do bagaço da cana-de-açúcar em produtos de alto valor agregado como os pigmentos de *Monascus*, fato que se destaca por sua possível integração a estratégias de intensificação de processos em um conceito de biorrefinaria.

Palavras-chave: Biopigmentos. Hidrólise Enzimática. Fermentação. Pré-tratamento. Surfactantes

ABSTRACT

SÁNCHEZ-MUÑOZ, S. **Use of surfactants as a strategy to improve the processing of sugarcane bagasse aiming at the production of biopigments by *Monascus ruber***. 2022. 159p. Thesis (Doctoral of Science) - Escola de Engenharia de Lorena, Universidade de São Paulo, Lorena, 2022.

To make industrial pigment production viable, it is essential to develop process alternatives that can be applied on a large scale in a biorefinery concept. In this context, it was proposed the use of surfactants in different stages of the pigment production process such as pretreatment, enzymatic hydrolysis and, fermentation using sugarcane bagasse as model biomass. Different surfactant application strategies were studied during the processing of pigment production by the fungus *Monascus ruber*. First, in the enzymatic hydrolysis process of sugarcane bagasse cellulignin (obtained from a diluted acid treatment) conducted in Erlenmeyer flasks, a Central Composite Rotatable Design (CCRD) was performed to determine the influence of different ratios (0.5-2.5%) of non-ionic surfactants to sequentially improve the production of 2nd generation sugars and *Monascus* pigments. The results obtained yielded an improvement of 1.77 times, considering the release of monomeric sugars, compared to controls without surfactant. Experiments employing the obtained surfactant optimized formulation (SOF) (Tween 20-PEG) showed a positive effect on the enzymatic loading, showing similar results employing dosages of 2.5 FPU/g of biomass (SOF addition) and 10 FPU/g of biomass (without SOF). Also, under the SOF, it was reported that the enzyme stability is maintained at high shear force stress and temperatures. Additionally, it was observed that the production of biopigments was 5-fold higher in glucose-based medium. Finally, under separate hydrolysis and fermentation (SHF), and semi-simultaneous saccharification and fermentation (SSSF) the maximum biopigments production were of 10 AU_{510nm}/mL and 1.5 AU_{510nm}/mL, respectively. In the SSSF process, pigment adsorbed in the biomass was found with values of approximately 9 AU_{510nm}/mL during the first extraction (4 extractions were carried out). In parallel, a second study was conducted using the hemicellulosic hydrolysate obtained from the acid hydrolysate of sugarcane bagasse to produce *Monascus* pigments. Initially, different non-ionic surfactants were tested in separate and in blend to evaluate a possible synergism between them to enhance the release of extracellular pigments during the fermentation stage. After this initial evaluation, Tween 80 and Triton X-100 were selected and a CCRD experiment was performed to find the better formulation to produce *Monascus* pigments from a rich xylose-based medium. The results showed a four-fold increment in red biopigments production when the SOF was used in the fermentation of hemicellulosic hydrolysates of sugarcane by-products. The produced pigments also demonstrate high thermal stability and absence in citrinin production. Finally, a last strategy in the use of surfactants was studied at the pretreatment stage. Tween 80 was selected after some preliminary studies and a hydrodynamic cavitation surfactant-assist alkaline pretreatment for sugarcane bagasse was evaluated. A new CCRD experiment was performed to find the best NaOH: Tween 80 relation to enhance the production of monomeric sugars in the enzymatic saccharification of sugarcane bagasse. In the optimized condition, a 40% increment in the removal of lignin, compared to the control was reported. The pretreated biomass was also used to produce *Monascus* pigments with an SSSF strategy in a fluidized bed reactor, with a maximum production of 3.86 AU_{510nm}/mL. At all stages evaluated, surfactants were key molecules for the conversion of sugarcane bagasse to produce high value-added products as *Monascus* pigments, fact that is highlighted for its possible integration to intensification processes strategies in a biorefinery concept.

Keywords: Biopigments. Enzymatic Hydrolysis. Fermentation. Pretreatment. Surfactants.

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SUMMARY

CHAPTER I

Introduction, thesis overview and objectives

1.1 Introduction, thesis overview and objectives

Color is strong factor in the acceptability of different items, and it plays a decisive role in the purchase of quality products. Moreover, color affects the perception of materials, foods, cosmetics, along with flavor, texture, acceptance as well. In nature, the products have a very striking natural color, so during its processing, it is sought to preserve the pigments that generally have functional properties. However, traditional processing technologies usually affect the color integrity, being necessary to add synthetic pigments.

Synthetic colorants have been facing market resistance by multiple reasons, including allergenicity, toxicity, teratogenicity, and carcinogenicity problems (Lemoine et al., 2020; Sen et al., 2019). The toxicity of azo dyes directly depends on the structure of molecule and on mechanism of degradation during the processing/storage, resulting mostly aromatic amines with different structures, e.g., benzidine which is carcinogen for the human urinary bladder (Gičević et al., 2020). Other example is Allura Red (E129) a synthetic red pigment that is extensively used in beverages, candies, cereal, and cosmetics. However, considering the trend to consume fewer synthetic products, the development of natural pigments through biotechnological processes is relevant. Among all microbial sources, *Monascus* species are well known products of several pigments and they have been used as food coloring agents in several East Asian countries (Tallapragada et al., 2017). *Monascus* pigments (MPs) are a complex mixture of red colorants (rubropunctamine and monascorubramine), orange colorants (rubropunctatin and monascorubrin) and yellowish colorants (monascin and ankaflavin).

In the last years, the interest of research about the *Monascus* pigment has been increased mainly due to functional properties as immunomodulatory, anti-inflammatory properties and cancer-chemopreventive activity (Akihisa et al., 2005; Choe et al., 2020; Wu et al., 2020). In Figure 1, it can be observed the increase on number of publications about *Monascus* pigment in the SCOPUS (2022) database, where about 85% correspond to original research papers.

Aiming to increase the pigment production, strategies as ATP-citrate lyase (ACL) overexpression has been reported, once the ACL plays a key role in the acetyl-CoA formation which is a precursor for *Monascus* pigment biosynthesis, e.g., gene *acl1* and *acl2* was over-expressed in *M. ruber* CICC41233, increasing in 92% and 112% the total pigment production respect to wild-type strain, respectively (Long et al., 2019). In the selection, mutagenesis, genetic recombination, and genetic engineering tools has been used currently

to develope high pigment producer strain (Shi et al., 2022). Moreover, establishment optimal parameters (N_2 source, O_2, light, pH, temperature) and additives (tyrosol, salicylic acid, methyl jasmonate, cyclic adenosine monophosphate, linoleic acid, and surfactants) or precursors (e.g., caprylic acid, acetic acid) can be used for MPs production.

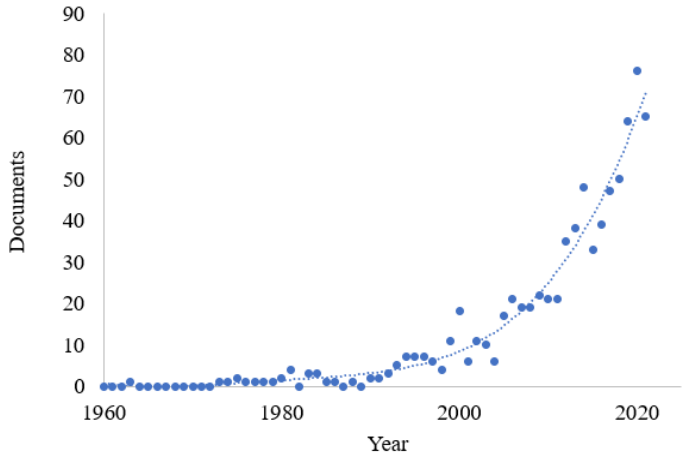

Figure 1. Number of publications by year of index in SCOPUS database.
Research Key: "Monascus AND pigment".

In this way, research works were also focused to use low-cost substrate as agro-industrial residues. Lignocellulosic biomass such as rice straw (Liu et al., 2020; Zhang et al., 2022), sugarcane bagasse (Hilares et al., 2018), potato pomace (Chen et al., 2021) and wheat bran (Das et al., 2022) have been used to produce MPs.

Lignocellulosic biomass has been considered the most important raw material for biorefineries to produce biofuels and bioproducts, in addition to being the most abundantly available bioresource, with a global generation of up to 1.3 billion tons per year (Parnthong et al., 2017; Zheng et al., 2017; Baruah et al., 2018). Lignocellulosic materials have a highly complex structure composed of cellulose, hemicellulose and lignin, and the presence of lignin makes its structure highly resistant to solubilization and chemical or enzymatic attack, thus hindering the process of hydrolysis of cellulose and hemicellulose into monomeric sugars, which is a significant challenge for its use as carbon source in bioprocesses (Zhao et al., 2008; Zheng et al., 2017; Baruah et al., 2018). This fact has been the subject of extensive research in the development of various pretreatment techniques, using different physical, chemical, physicochemical, and biological approaches, specifically adapted to the biomaterial and its subsequent application (Baruah et al., 2018). Therefore, pretreatment is a fundamental step for the saccharification of lignocellulosic biomass, which is recalcitrant to biodegradation by enzymatic and microbial attacks. Additionally, pretreatment is one of

the main cost contributors to biorefineries, affecting the efficiency of bioconversion and downstream processes (Zheng et al., 2017). Additionally, the high cost of the enzymes used in enzymatic hydrolysis and the low efficiency in the use of C5 sugars (obtained from the hemicellulosic fraction) are also limiting factors in the industrialization of molecules generated from the fermentation of lignocellulosic biomass (Zhao et al., 2008).

Among all strategies described to increase the production of MPs, using molecular tools, or optimizing physicochemical and biochemical parameters during fermentation, coupled to the use of alternative carbon sources as lignocellulosic biomass to enhance the process. Other strategies as the use of additives during the different stages of the bioprocess has been gained attention. Surfactants are described as versatile molecules for a wide range of applications. In the context of biorefineries, for pretreatments, surfactants have hydrophilic and hydrophobic properties that can reduce the surface tension between two liquid phases during the process and can extract hydrophobic compounds forming an emulsion, making them unavailable for repositioning on the surface of the biomass and improving the yield in pretreatments as diluted acid and alkaline ones (Pandey and Negi, 2015). Additionally, for enzymatic hydrolysis, a large amount of work has shown that the addition of non-ionic surfactants enhances the conversion of pretreated lignocelluloses into monomeric sugars, and many mechanisms has been described (Alkasrawi et al., 2003; Yan et al., 2015; Muñoz et al., 2022). Finally, in the case of fermentation stage, surfactants enhance membrane permeability and nutrient uptake of cells, allowing better production and release of molecules, with emphasis on Monascus pigments (Chen et al., 2018; Muñoz et al., 2022).

It is worth mentioning that, although there are studies on the use of surfactants in the production of pigments, the novelty of this thesis can be highlighted, since no studies were found in the literature that use mixtures between different surfactants or make processes in sequence with the use of surfactant remanence between the main steps in the conversion of lignocellulosic biomass (sugarcane bagasse), or its use in the fermentation process with hemicellulosic hydrolysates, or their application in emerging pretreatment processes (e.g., hydrodynamic cavitation). Therefore, this thesis is focused on the development of new approaches related to the use of non-ionic surfactants in the key steps of bioprocess as pretreatment, enzymatic hydrolysis, and fermentation. Besides this introductory chapter, as an overview of the whole work, this thesis has been divided in four other chapters. Chapter II addresses a deep discussion about the main mechanisms and role of surfactants as key molecules in various steps of biorefinery processes. In the chapter III, the application of a

surfactant optimized formulation to enhance sequentially the enzymatic hydrolysis of sugarcane bagasse cellulignin and Monascus pigment production is discussed. In the subsequent chapter IV, a new surfactant formulation was optimized to enhance the production of *Monascus* pigments using a xylose-based medium and sugarcane bagasse and straw hemicellulosic hydrolysates. In chapter V, the evaluation of hydrodynamic cavitation and Tween 80 to assist an alkaline pretreatment of sugarcane bagasse as strategy to produce *Monascus* pigments was also discussed. Finally, the chapter VI corresponds to the future works and conclusion of this thesis.

1.2 Main aims

To evaluate the use of non-ionic surfactants to produce biopigments during fermentation in semi-synthetic medium by the filamentous fungus *Monascus ruber*

To evaluate the use of non-ionic surfactants to produce biopigments during fermentation in sugarcane by-products hemicellulose hydrolysates by the filamentous fungus *Monascus ruber*

To optimize the concentrations of different non-ionic surfactants to improve the enzymatic hydrolysis of cellulignin of sugarcane bagasse SHF and SSSF process (Separate Hydrolysis and Fermentation, and Semi-Simultaneous Saccharification and Fermentation) to produce biopigments by *Monascus ruber*.

To study the effect of non-ionic surfactants in an alkaline pretreatment with NaOH assisted by hydrodynamic cavitation to improve sugarcane bagasse digestibility of sugarcane bagasse for the subsequent use of hydrolysates in the production of biopigments by *Monascus ruber*.

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CHAPTER II

Surfactants in biorefineries: Role, challenges & perspectives

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ABSTRACT

The use of lignocellulosic biomass (LCB) as feedstock has received increasing attention as an alternative to fossil-based refineries. Initial steps such as pretreatment and enzymatic hydrolysis are essential to breakdown the complex structure of LCB to make the sugar molecules available to obtain bioproducts by fermentation. However, these steps increase the cost of the bioproduct and often reduces its competitiveness against synthetic products. Currently, the use of surfactants has shown considerable potential to enhance lignocellulosic biomass processing. This review addresses the main mechanisms and role of surfactants as key molecules in various steps of biorefinery processes, viz., increasing the removal of lignin and hemicellulose during the pretreatments, increasing enzymatic stability, and enhancing the accessibility of enzymes to the polymeric fractions, and improving the downstream process during fermentation. Further, technical advances, challenges in application of surfactants, and future perspectives to augment the production of several high value-added bioproducts have been discussed.

Keywords: Surfactants. Biomass-pretreatment. Enzymatic-hydrolysis. Fermentation. Bioproducts

2.1 Introduction

Surfactants are amphipathic molecules comprised of a polar (hydrophilic) and a non-polar (hydrophobic) component (Nitschke and Pastore, 2002). Due to the dual characteristic of their structure, these compounds can distribute themselves in an orderly way on surfaces with different polarities to allow the interaction between two phases (oil in water; air and water). It is due to their ability to reduce the surface or interfacial tension between two fluids with different degrees of polarity. This property of reducing surface tension enables the use of surfactants in a wide range of industrial applications involving detergency, emulsification, lubrication, solubilization, and phase dispersion (de Oliveira and de Cassia, 2017).

Due to the varied applications of surfactants in industrial products, such as personal care, cleaning products, oil dispersants, among others, it can be observed that their production is constantly increasing, reaching between US $40-52 billion from 2021 to 2025 (Markets and Markets, 2021). The growing demand for development of sustainable processes in order to minimize environmental impacts and to comply with new environmental control legislation is leading to search for biotechnological processes for the generation of value added bioproducts from renewable sources such as plant biomass (Jahan et al., 2020). In recent years, bioprocesses have attracted attention due to their low toxicity, compared to chemical synthesis, high ecological acceptability, etc. (da Silva et al., 2018a; 2019). Additionally, bioprocesses with renewable biomass reduce the costs of the production process, since the cost on carbon source can account up to 30% of the final value of the products (Fontes et al., 2008).

Lignocellulosic biomasses (LCB) have great potential as a low-cost raw material for different industrial processes, for example, to produce fuels, chemical inputs, enzymes, food and feed, in addition to their potential for energy generation (Latif and Rojoka, 2001). These materials are mainly constituted by complex matrices of cellulose and hemicellulose, surrounded by lignin, forming a highly stable plant structure. Thus, to hydrolysate the carbohydrate fractions (cellulose and hemicellulose) into fermentable sugars, a pretreatment of biomass is required (Dias et al., 2013). Among different methods of pretreatment, *viz*., chemical, physical, and biological methods, the enzymatic hydrolysis is widely used to cleave polymeric fractions of carbohydrates from LCB in monomeric sugars (Antunes et al., 2018). Concomitant to the development of technology for obtaining sugars, the selection of microorganisms and as well as optimum process conditions are essential for the fermentation of LCB as raw materials in industrial processes (da Silva et al., 2018a; 2019).

Taking these considerations into account, the use of different classes of surfactants, e.g., Tween-80, dodecylbenzene sulfonic acid, polyethylene glycol (PEG) 4000 (Qing et al., 2010) and others, in the three major steps, namely pretreatment, enzymatic hydrolysis and fermentation, is a promising approach to maximize the efficiency and profitability of biorefineries. Several authors have reported that surfactants can assist in removing or solubilizing interference compounds that could physically impair the efficiency of the pretreatment step (Mesquita et al., 2015; Nargotra et al., 2019). Likewise, the use of surfactants in the enzymatic hydrolysis aided in increasing the yield due to enhancement of enzyme stability and enzyme-substrates interplay (Kim et al., 2007). Furthermore, the use of surfactants improved the efficiency of the fermentation process with LCB hydrolysates as substrate. It was observed that the use of surfactants increased the product release during extractive fermentation strategies, and thus minimized the stages of downstream process (Kang et al., 2013). This review is focused on the role, mechanisms of action, current and future strategies of using surfactants in the various steps of LCB processing to generate biofuels and high-value molecules in a biorefinery concept.

2.2 Current challenges of lignocellulosic biomass processing to generate biofuels and high-value molecules: key steps

LCB is a material abundantly available worldwide that offers several opportunities for the production of different value-added products; however, currently, the success of using LCB in industrial processes is far way below potential. For example, for cellulosic ethanol, despite the great efforts last decades, there are only a few industrial-scale facilities to produce it globally. Several issues need to be addressed in biorefineries so that inefficient or complex pretreatment and hydrolysis technologies become more efficient from an economic, energetic, and technical viewpoint. Relevant drawbacks include an irregular biomass supply chain (from the point of generation of the feedstock until it gets into a processing facility) and scale-up challenges leading to high capital and operating expenditures (Usmani et al., 2021).

In the pretreatment step, different physicochemical, chemical, and biochemical methods have been extensively explored. However, there are drawbacks when using traditional pretreatment techniques on large-scale processes, and developed new technologies have low technical maturity. Another drawback most developed methods present is the difficulty to adapt to continuous or even semi-continuous processes (Hilares et al., 2019; 2020). High

cost, demand for state-of-the-art reactors, high inhibitory by-products production, long process time, and requirement of high temperatures are the main disadvantages of chemical methods like ionic liquids (ILs), deep eutectic solvent, organic solvent, alkalis, or acids (Ning et al., 2021). For example, pretreatment using peracetic acid showed 90% of lignin remotion from the biomass with negligible loss of carbohydrates, but it requires a long process time (5h) at 90°C (Kundu et al., 2021).

Among the potential methods, the steam explosion is currently used in second-generation ethanol facilities (Beta renewable – Italy or Raizen -Brazil) due to its effectiveness and economic aspects (Chandel et al., 2021). Liquid hot water pretreatment also is used in several industries (e.g., Granbio-Brazil). Other pretreatment options such as steam explosion, wet oxidation, ammonia fiber expansion (AFEX), irradiation (microwave and ultrasound), and hydrodynamic cavitation combined with alkalis also have been explored for LCB pretreatment (Hilares et al., 2020). Nevertheless, more studies on those pretreatments are required to become an industrial reality.

Most of the key factors influencing the LCB pretreatment at a large scale are solid loading, continuous operation, low chemical catalyst addition, minimum loss of sugars, and minimum inhibitors generation. In addition, there are just a few studies on pretreatments such as steam explosion (Monschein and Nidetzky, 2016), deep eutectic solvent-mediated extrusion (Ai et al., 2020), isothermal (Pérez-Pimienta et al., 2020), and hydrodynamic cavitation (Hilares et al., 2020) pretreatments operated in a semi-continuous or continuous process.

The hydrolysis step, also called saccharification, is another key factor in LCB conversion to ethanol and value-added products. This step also has challenges as the development of new enzyme cocktails to improve the extraction and hydrolysis of carbohydrates at low enzyme loading (Usmani et al., 2021). The main drawback in this step is the enzyme cost, which contributes to nearly 25% of the overall production cost (Rocha-Martín et al., 2017), thus making necessary a development of optimized process to achieve high conversion of carbohydrate to fermentable sugars. Another challenge in enzymatic hydrolysis corresponds to unproductive enzyme adsorption on residual lignin that may not necessarily present accessibility hindrance but can competitively absorb the enzyme (Zheng et al., 2013). Some proposed alternatives correspond to the use of surfactants (Tween 20, Tween 80, and PEG) or non-catalytic proteins (whey and soy proteins, BSA- bovine serum albumin). In this case, the hydrophobic interaction of surfactants or other additives with the lignin present on the lignocellulosic surface is promoted, and it releases non-specific bounded enzymes (Eriksson

et al., 2002). The effects and benefits of using surfactants in the hydrolysis process are following discussed in section 3.

The use of high solid loading is also a challenge once >20% significantly reduces the water in the reactor, avoiding an adequate mass and heat transference. To overcome this problem, the configuration of the process (operation mode) can improve the hydrolysis efficiency. For example, a fed-batch process was performed to hydrolyze corncobs using high solid loading (25%) and Cellic CTec2 cellulases preparation (Novozymes, Copenhagen, Denmark) at 12 mg/g of biomass (7.3 FPU/g) (Cai et al., 2021).

Finally, the released sugars are converted into ethanol or high-value products in the fermentation step. Fermentation also includes challenges that require further study since few microorganisms can simultaneously convert all sugars available in the hydrolysate. In this way, strategies, as nanofiltration to separate xylose from glucose, were explored (Mah et al., 2019). Moreover, strategies focused on the microorganism were also used, such as the identification and isolation of wild strains able to metabolize the two sugars (glucose and xylose) and genetically engineered microorganisms (Komesu et al., 2020; Sun and Jin, 2021). There are other bottlenecks in the fermentation stage, as the growth inhibition due to the production of several intracellular products (e.g., lactic acid). This phenomenon could influence the transmembrane parameters (e.g., pH gradient) and decreases the energy available for cell growth. Another bottleneck is the high energy cost of recovering metabolites of interest from the fermentation broth. Those fermentative problems can be avoided by extractive fermentation techniques, described in the following sections (Dhamole et al., 2012).

2.3 Surfactants applied to bioprocesses: general panorama

Surfactants are amphipathic molecules whose structures have regions of varying polarities: a hydrophilic head and a hydrophobic tail. The hydrophobic groups are responsible for the solubility in non-polar substances, are composed of a linear or branched hydrocarbon chain, which can be a fatty acid, paraffin, an olefin, an alkylbenzene, alcohol, or an alkylphenol, commonly obtained from petrochemical derivatives. On the other hand, hydrophilic groups, crucial for the solubilization of surfactants in water, may contain ionizable or non-ionizable groups in water (Daltin, 2011). These compounds are classified according to the nature of hydrophilic groups. They can be non-ionic, anionic (negatively charged), cationic (positively charged), and zwitterionic (negatively and positively charged)

surfactants (Massarweh and Abushaikha, 2020). Due to their intrinsic characteristics, surfactants can interact with substances of different polarities, which gives them the ability to influence the surface tension of fluids and form micelles (Penteado et al., 2006).

These compounds accumulate at the fluid interface, promoting the reduction of surface and interfacial tensions. The structural and functional properties of surfactants allow increasing the solubility, mobility, bioavailability, and biodegradability of hydrophobic organic compounds (Singh et al., 2007). In today's society, besides surfactants utilization in industrial bioprocesses (Table 1), these compounds are also present in different products (Johnson et al., 2020). The first use of surfactants in history occurred in soap and detergent formulations for laundry and cleaning (Falbe, 2012). After their advent, surfactants were used as an active ingredient in different products, with an increase in the number of industries producing surfactants last century. Concomitantly, there was an increase in the possibilities of using these compounds in various industrial sectors, such as in agriculture, pharmaceutical, and cosmetic industries (Janshekar and Fiechter, 1983; Singh et al., 2007).

In addition to being applied in different industrial sectors, surfactants can also be used as tools in bioprocesses (Hamel and Hunter, 1990). The production process of various biomolecules presents many challenges, such as low productivity and low recovery at the downstream stage. Thus, there is a constant search for procedures or compounds that would reduce these problems. Many researchers are carrying out their works to verify the possible use of surfactants in bioprocesses and the advantages of such applications.

When incorporated into fermentative processes, surfactants can induce an increase in the production of extracellular products, act in the recovery processes of intracellular products by facilitating and promoting cell lysis, and also can reduce foam formation (Hamel and Hunter, 1990; Singh et al., 2007).

For decades, studies have shown the use of surfactants to obtain enzymes (Dekker, 1990; Hammel and Hunter, 1990). Reese and Maguire (1969) showed an introduction of Triton-X increased extracellular cellulase activity. After, Suha Sukan et al. (1989) performed an evaluation of the effects of oils and surfactants (Tween 80) on cellulase production by *Trichoderma reesei* and *Sporotrichum pulverulentum*, showing that emulsification by surfactant led to increased cellulase activity in the culture medium (Suha Sukan et al., 1989). In more recent work, da Silva et al. (2019) investigated the production of lignin peroxidase. This production occurred by the fungus *Pleurotus ostreatus* using low-cost agro-industrial residues supplemented with the surfactants Tween 80 and sodium dodecyl sulfate (SDS),

showing maximum lignin peroxidase activity in fermentation using jatropha supplemented with SDS (da Silva et al., 2019).

Table 1. Types and applications of surfactants

Types	Structural characteristic	Example	Application
Anionic	Negatively charged hydrophilic group	- Sodium lauryl ether sulfate - Lignosulfonate - Alkylbenzene sulphonates - Sodium dodecyl sulfate	- Cleaning products - Shampoos - Wetting agent - Concrete plasticizer - Pretreatment
Cationic	Positively charged hydrophilic group	- Benzalkonium chloride - Cetylpyridinium chloride - Cetrimonium chloride - Cetyltrimethyl ammonium bromide	-Antimicrobial, antifungal agent. - Cleaning products - Pretreatment
Zwitterionic	Hydrophilic group with opposite charges	- Cocamidopropyl betaine	- Latex paints - Membrane solubilization
Non-ionic	It has no charged groups in the hydrophilic region	- Span - Triton X-100 - Tween-80 - Tween-20 - Polyethylene glycol 4000 - Polyethylene glycol 2000	- Food additive; - Cleaning products; - Pretreatments - Enzymatic saccharification - Extractive fermentation

Source: Preté et al.; 2002b; Daltin, 2011; Xiong et al., 2015; Zhang et al., 2021b; Farias et al., 2021.

The incorporation of surfactants in bioprocesses was also related to microbial growth and metabolite production. For example, in filamentous fungi, these compounds can change the fungal mycelial morphology (Matošić et al., 1998). Matošić et al. (1998) observed that the addition of Pluronik surfactant, a polyethoxypolypropoxy polymer, caused an increase in alkaloid biosynthesis by immobilized *Claviceps paspali* mycelia. Moreover, surfactants can increase the permeability of the mycelium cell wall, increasing the excretion of the products, in addition to the capacity to prolong mycelium growth, keeping the mycelium structure intact. Furthermore, the presence of surfactants increases the rate of glucose consumption by the microorganism (Zhang et al., 2021a).

In 1999, the surfactant Span 20 was used in the production of β-carotene by the fungus *Blaskesla trispora*. The surfactant coupled with controlled aeration and dissolved oxygen reduced foaming associated with the fermentation process (Kim et al., 1999). Furthermore, some works describe that the addition of surfactants can favor the release of pigments

obtained by microorganisms in the extracellular medium. In the work of Hu et al. (2012), pigment fermentation by *Monascus* was carried by adding non-ionic surfactant Triton-X 100. The intracellularly synthesized pigment was exported to the extracellular environment and later extracted from the non-ionic surfactant micelles. In addition to facilitating recovery, this study showed an increase in the cell density and final pigment concentration when the processes were carried out in the presence of the surfactant. The final biomass reached about 28 g/L of DCW, with extracellular concentrations of pigments yellow, orange, and reds of 130, 84, and 47 AU, respectively (Hu et al., 2012).

Besides, studies show that surfactants can be applied in steps before fermentation in biorefineries (Vallander and Eriksson, 1990). The pretreatment and enzymatic hydrolysis have been enhanced the performance by the addition of surfactants. These compounds favor the release of fermentable sugars during the pretreatment of biomass, in addition, to help the recovery of the formed bioproducts (Zhang et al., 2021a).

In 1981, Castanon and Wilke observed an increase of 33% in enzymatic hydrolysis yield and enzyme recovery in the presence of Tween 80 surfactant (Castanon and Wilke, 1981). In another study, Kim et al. (1982) stated that the increase in saccharification performance by using surfactants occurred due to a decrease in the inactivation of enzymes in solution (Kim et al., 1982). In 2010, in the work of Ouyang et al. (2010), during hydrolysis, the addition of polyethylene glycol 4000 (PEG 4000) increased about 91% the conversion of microcrystalline cellulose, in addition to observing an increase in cellulase enzyme activity from Celluclast 1.5L. In the study of the Cao and Aita (2013), the use of different compounds with amphipathic characteristics, such as Tween 80, Tween 20, PEG 4000, and PEG 6000, was evaluated in the saccharification of sugarcane bagasse. The results showed higher cellulose digestibilities (62%, 66%) and ethanol yields (73%, 69%) by using PEG 4000 and Tween 80. It is suggested that this difference occurs due to the structural characteristics of these compounds and the condition used (Cao and Aita, 2013). Studies performed by Zhang et al. (2018) with sugarcane bagasse showed the addition of the surfactant Tween 80, reduces the hydrolysis time and the enzyme loading by 50%, resulting in an enzymatic hydrolysis yield of glucan of 93,8 %.

The yield of sugars obtained in enzymatic hydrolysis is dependent on the type of biomass, type of surfactant, and conditions employed in hydrolysis (Chen et al., 2018b), but also is strongly dependent on the used pretreatment technique. Regarding pretreatment, the use of surfactants has also been shown as an attractive strategy, as following discussed. Also,

sections 5 and 6 give a more detailed discussion about the use of surfactants in enzymatic hydrolysis and fermentation.

With the need to include green chemistry in the world scenario, there is an increase in research for tools that replace petroleum-derived products used in the bioprocesses to reduce the generation of harmful residues to the environment and impacts. Therefore, studies are observed aiming at the applications of biosurfactants, natural surface-active molecules, as an additive in bioprocesses to replace the uses of synthetic surfactants (Chang et al., 2016).

2.4 Effect of Surfactants in biomass pretreatment

As previously highlighted, pretreatment is considered a pivotal step for the technological and economic development of bioprocesses using LCB as raw material. Thus, energy costs and chemical requirements must be taken into account (Baral and Shah, 2017), and the pretreatment must be efficient enough to increase the yield of subsequent enzymatic hydrolysis without imposing high costs to biorefineries. Some chemicals compounds can be used as catalysts or additives in pretreatments to optimize the processes, facilitating the delignification or degradation of the principal fractions (Kumar & Wyman, 2009). Amongst, surfactants have been studied to enhance several types of pretreatments, obtaining better fractionation of the main components of LCB (Cao and Aita, 2013; Nasirpour et al., 2014; Mesquita et al., 2015).

2.4.1 Mechanisms of action of surfactants in the pretreatment step

The presence of lignin in several stages of bioprocesses (e.g., enzymatic hydrolysis) results in non-favorable effects. Those effects are the loss of desirable substances, absorption of other undesirable molecules to the process, interference in actions of elements decreasing the bioprocess yield, and others (Qing et al., 2010). For these problems, several types of reagents and additives are already tested in pretreatments to find a better form of lignin, organic and inorganic contaminants removal according to each bioprocess (Thite and Nerurkar, 2019). An alternative found was the use of surfactants as an additive in some pretreatments, which results in a significant increase in solubility of elements that affect the process and to the environment, making its removal more effective during the pretreatment (Qing et al., 2010).

Pretreatments using surfactants as additives take advantage of their hydrophobic and hydrophilic properties. In addition to other functions, surfactants can cause a decrease in the surface tension of two liquid phases, improving the removal of elements with hydrophobic characteristics. Surfactants can also modify the structure and the surface portion of biomass, increasing its solubility and improving the mass transfer capacity (Qing et al., 2010).

An important value correlated with the effect of the surface-active property of surfactants is an index called hydrophile-lipophile balance (HLB). HLB is a mathematically calculated surfactant value based on the chemical structure of the molecules associating their hydrophilic and lipophilic proportions moieties. This value can be calculated considering many experimental procedures that include quantitative determinations (e.g., quantitative structure-property relationship, titration methods, etc.) of target chemical or physical properties (e.g., solubility) (Roelants et al., 2019). Also, HLB is a property used for classifying surfactants by their level of solubility and their behavior related to the emulsification process, promoting several characteristics, such as enhancing the accessibility of many components (e.g., reagents, enzymes, etc.) to the biomass fibers (Liu et al., 2019). Surfactants that have a high HLB are more efficient in assisting pretreatments removing products with hydrophobic characteristics of lignin and hemicellulose, which is the focus and purpose of several pretreatments (Cao and Aita, 2013).

In the last few years, scientific reports focused their attention on different types of surfactants to assist pretreatments. Those molecules are effective for lignin and hemicellulose removal (as shown in Table 2). For example, Sindhu et al. (2013) were the first to report an ultrasound pretreatment of sugarcane tops assisted by surfactants. In this research, different surfactants such as Tween-80, Tween-40, Tween20, Triton X-100 (non-ionic), PEG 6000, PEG 8000, SDS, and CTAB showed great effectiveness. Tween surfactants obtained the highest results for the lignin and hemicellulose solubility during pretreatment (about 34% of mass remotion).

Non-ionic surfactants have a high potential for delignification, considering they have a high HLB value and represent a group without influence in the environment because of their lack of ionization when dissolved in water (Daltin, 2011). Among all surfactants, Tween 20 and Tween 80 are non-ionic molecules studied for their delignification potential in pretreatments. Those two surfactants are chemically similar with different HLB values (both high), giving them a high solubility in water, an advantage beyond the pretreatment stage (e.g., possible remaining action in enzymatic hydrolysis) (Tu and Saddler, 2010). It was reported that Tween 80 derived from polyethoxylate sorbitan and oleic acid significantly

increased delignification by 52% in the acid pretreatment (140 °C, 1% H_2SO_4), and in hot water treated biomass (30 min, 220 ºC) increased the delignification by 114% (Qing et al., 2010). Tween 80 also showed good performance in a metal-salt catalyzed pretreatment by removing hemicellulose (maximum remotion of 93.8%) and lignin (maximum remotion of 37.9%) (Zhang et al., 2021b). Tween 20 is another non-ionic surfactant (derived from polyoxyethylene sorbitan monolaurate) studied in different pretreatments, such as dilute acid and ionic liquid (IL) pretreatments (Kim et al., 2007; Nargotra et al., 2019). The addition of Tween 20 to the IL pretreatment helps to prevent the lignin redeposition on the biomass surface. Tween-20 assisted [Emim][MeSO3] pretreatment decreased the lignin content of biomass to 13%, and this non-ionic surfactant could also act in subsequent steps of the bioprocess (e.g., enzymatic saccharification) (Nargotra et al., 2019).

PEG surfactants are other non-ionic surfactants (e.g., PEG 4000 and PEG 6000) already used in pretreatments of LCB (Eriksson et al., 2002). One disadvantage in their use is that they can cost up to six times more than the Tweens (Tu and Saddler, 2010). However, research has shown its potential use when combined with NaOH. For example, in the pretreatment hardwood birch, where surfactants (e.g., PEG) significantly increased lignin removal levels (about 28.5%) in the first step (pretreatment) of the process and further improved the next ones (e.g., enzymatic hydrolysis and fermentation) (Mohsenzadeh et al., 2012).

In addition, it is good to emphasize that not only non-ionic surfactants have been used in pretreatments. Chang et al. (2016) reported the increase of delignification efficiency in an IL pretreatment of rice straw biomass assisted by anionic (SDS- 49.38% of lignin remotion) and cationic (CTAB- 34.76% of lignin remotion) surfactants. According to Daltin (2011), both surfactants act on the surface of biomass, extinguishing the hydrophobic bonds of lignin and increasing its degree of removal. In the case of the ionic liquid pretreatment, it has been reported that they can effectively solubilize different lignocellulosic fractions (e.g., lignin or hemicellulose) from several biomass, such as, switchgrass (lignin reduction from 22.4%, with 13.7%), sugarcane bagasse (63% of lignin solubilization), and others (Li et al., 2010; Pin et al., 2020). Nevertheless, the residual lignin and hemicellulose in the IL-pretreated lignocellulosic biomass significantly affect enzymatic hydrolysis. Surfactant-assisted IL-pretreatment results in lesser utilization of IL and also improves enzymatic hydrolysis efficiency which is critically important keeping in view of the high cost of IL (Sharma et al., 2019; Chang et al., 2017).

Table 2. Surfactant-assisted pretreatments for lignin and hemicellulose removal

Biomass	Biomass Characterization (untreated) (%)	Pretreatment + surfactant concentration (w/w)	Lignin removal (%)	Hemicellulose removal (%)	Reference
Sugarcane Bagasse	41% cellulose 24 % hemicellulose 23% total lignin	Dilute ammonia	14	25	Cao and Aita, 2013.
		Dilute ammonia + 3% Tween 20	18	--	
		Dilute ammonia + 3% Tween 80	37	44	
		Dilute ammonia + 3% PEG 6000	32	--	
		Dilute ammonia + 3% PEG 4000	47	--	
Sugarcane Bagasse	44.85 % cellulose 31.25% hemicellulose 23.90% total lignin	Ionic liquid	16.7	83.2	Nasirpour et al., 2014.
		Ionic liquid + 3% PEG 4000	26.52	90.36	
		Ionic liquid + 5% Tween 80	27.40	90.56	
Pine fallen foliages	30.91% cellulose 22.04% hemicellulose 32.04% total lignin	Acid pretreatment + 1% surfactant*	59.53	54.46	Pandey and Negi, 2015
		Alkali pretreatment + 1% surfactant*	73.47	52.23	
Rice Straw	35.36% cellulose 24.15% hemicellulose 25.98% total lignin	Ionic liquid	24.98	~ 8.11	Chang et al., 2016
		Ionic liquid + 1% CTAB	34.76	~ 5.54	
		Ionic liquid + 1% SDS	49.38	~ 15.48	
Green coconut fiber	32.80% cellulose 15.90% hemicellulose 35.70% total lignin	Diluted alkaline	29.13	--	Nogueira et al., 2017.
		Diluted alkaline + 3% Tween 80	25.21	--	
Miscanthus sinensis	42% cellulose 28% hemicellulose 20% lignin	Alkaline pretreatment	~ 15	~ 25	Xu et al., 2021
		Alkaline pretreatment + 1.0% Tween 40	~ 20	~ 32	
Rice Straw	31.74% cellulose 23.21% hemicellulose 15.79% lignin	Diluted acid	9.6	42.5	Wang et al., 2020
		Diluted acid + 0.33% SDBS	19.1	54.7	
Wheat straw	26% cellulose 21% hemicellulose 27.1% lignin	Diluted acid + humic acid (10 g/L)	40.0	96.2	Tang et al., 2021

[a] The appropriate surfactant for enhancing acid and alkali pretreatment efficiency were selected by supplementing the acid and alkali pretreatment with different surfactants such as Tween-20, Tween-80, Triton-X100, PEG-6000, PEG-20000, C-TAB and SDS at 1% (w/w) concentration (Pandey and Negi, 2015)

In another work, Fang et al. (2014) reported a cationic surfactant assisted (CTAB) microwave pretreatment method using peanut shells as biomass. The results showed an increment of about 60% of total reducing sugar release.

In many scientific findings involving surfactant-assisted pretreatments, the remaining surfactant was beneficial for the enzymatic hydrolysis and fermentation, reducing the amount of energy required in the wool process (Qi et al., 2010).

2.5 Effect of surfactants in enzymatic saccharification: main mechanisms

Enzymes can be applied in many bioprocesses of industrial interest, catalyzing chemical transformations of complex macromolecules to be performed under mild conditions and with a high degree of substrate specificity (Brena et al., 2013). Despite all the improvements in biorefineries for enzyme application (e.g., cellulases), some challenges are still to be overcome, principally those related to the high cost of enzymes needed to achieve satisfactory hydrolysis (Li et al., 2015). To make the cellulolytic cocktails economically viable for industrial applications, many efforts have been made to develop strategies to achieve better performance during enzymatic processes (Ning et al., 2021). Those studies aim to make the process easier, more practical, and efficient to be applied in biorefinery (Chandrasekaran and Bahkali, 2013). One of the alternatives to overcome these barriers is to use additives, such as surfactants, in biomass saccharification. As before described, surfactants are a key molecule for different steps in the biorefinery concept.

Many advantages have been described for the addition surfactants in the enzymatic hydrolysis step. Zhang et al. (2018) reported a 50% reduction in enzyme loading when adding Tween 80 to a sugarcane bagasse saccharification process. Ding et al. (2019) also report a 33% reduction in enzyme loading when adding BSA as an additive in the enzymatic hydrolysis of fresh poplar wood. In a more recent work, Lu et al. (2020) performed an economic analysis comparing the enzymatic hydrolysis process of a pretreated biomass (NaOH/O2) and the same process with the addition of surfactants (PEG 3000) in the enzymatic hydrolysis. The results showed a 30 % reduction in total costs for the first case and 56 % for the second one. Thus, the addition of surfactants can reduce the amount of enzyme loading and the total cost of the process.

In this section, we discuss mechanisms of action of surfactants to improve enzymatic saccharification, and some challenges still encountered in using these additives.

2.5.1 Description of the main mechanisms of surfactants to enhance enzymatic hydrolysis

In enzymatic saccharification, cellulases, mainly endoglucanases, need to bind to cellulose to catalyze its hydrolysis. However, these enzymes can also be bound through hydrophobic, electrostatic, or hydrogen bonding interactions to other surfaces, such as the lignin remaining from the biomass pretreatment (Agrawal et al., 2017). This inappropriate

enzyme-lignin binding is called unproductive adsorption, because it reduces the number of enzymes available to adsorb on cellulose and consequently reduces the efficiency of enzymatic saccharification (Pandey and Negi, 2015). The phenomenon of unproductive adsorption is illustrated in Figure 2. The use of additives like surfactants can help to reduce the unproductive adsorption of cellulolytic enzymes by different mechanisms. Some of those mechanisms are: (1) increasing the availability of the cellulose surface by altering the substrate structure and exposition of cellulase adsorption sites, (2) acting as a competitor for enzymes by binding to lignin, (3) increasing enzyme stability in the saccharification (Agrawal et al., 2017).

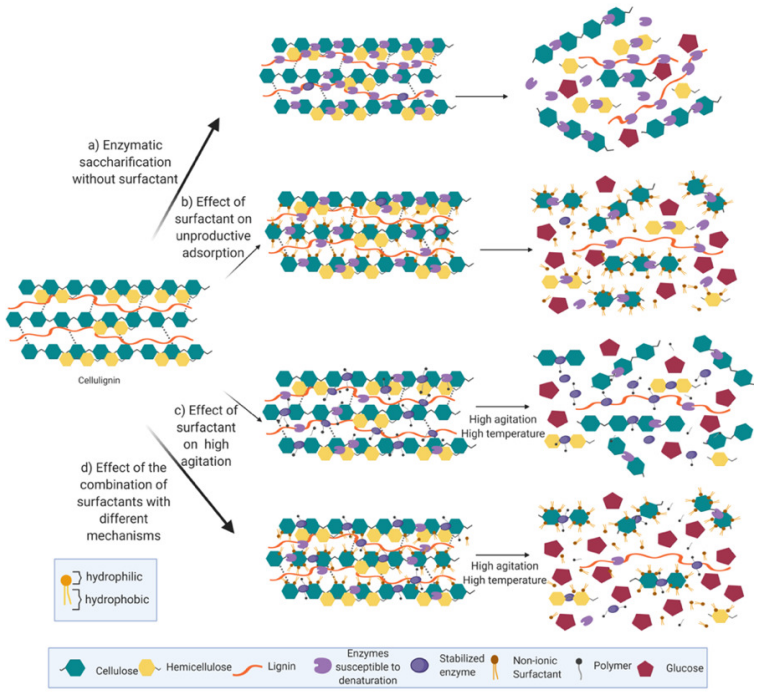

Figure 2. Mechanisms involved in the improvement of the enzymatic hydrolysis of lignocellulosic biomass by adding surfactants. (a) enzymatic saccharification without surfactants - the unproductive adsorption of enzymes occurs intensively, decreasing the enzymatic hydrolysis efficiency and subsequently the release of monomeric sugars; (b) the surfactants added to lignocellulosic biomass act as a competitor for cellulases in lignin, reducing the enzyme-lignin interaction and increasing the glucose yield; (c) the interaction of the hydrophilic part of the surfactant with water creates a barrier in the cellulase, strengthening the intermolecular forces, reducing the shear force, and enhancing enzyme stability, enabling changes in temperature and agitation conditions to increase sugar conversion; (d) the combined effect of different types of additives enables the contribution of several mechanisms, thus, while some surfactants compete with cellulases for lignin, reducing unproductive adsorption, others stabilize enzymes, avoiding their denaturation (Created with BioRender.com).

The addition of surfactants to the pretreated LCB with high lignin content is used to reduce the hydrophobicity of the residual lignin in the substrate. When surfactants are added to pretreated biomass, they form hydrophobic interactions with the residual lignin, resulting in a competition between the surfactant and cellulase to bind to lignin. Therefore, surfactants

help to reduce the affinity between cellulases and lignin, and there will be an increase in cellulase desorption in the presence of the surfactant, decreasing the unproductive adsorption and increasing the hydrolysis performance (Eriksson et al., 2002; Lin et al., 2019). Thus, the addition of surfactants in the enzymatic hydrolysis of specific biomass with low lignin content and high cellulose crystallinity may not be an efficient strategy (Chen et al., 2018b).

There are different types of surfactants (e.g., cationic, anionic, and non-ionic ones), but not all of them can improve the enzymatic saccharification step. For example, anionic and cationic surfactants may even reduce the hydrolysis efficiency of cellulose (Holmberg, 2018). The hydrophobic carbon chains and charged groups present in these surfactants can form deleterious hydrophobic and electrostatic interactions with cellulase, affecting the enzyme activity. On the other hand, non-ionic surfactants have several characteristics that can help to improve enzymatic saccharification.

Non-ionic surfactants have high values of hydrophilic-lipophilic balance and modify the characteristics of the environment in which they are introduced. In the hydrolysis of LCB, these compounds can change the structure of the pretreated substrate, facilitating the accessibility of enzymes (Mesquita et al., 2015). One of the most widely applied non-ionic surfactants is Tween 20. This surfactant cause changes in the hydrophobicity and surface charges of lignin, improving the saccharification of different types of biomasses. Literature reports a 50% increase in the conversion of cellulose in the hydrolysis of pretreated sugarcane bagasse, boosting the saccharification yield of steam-exploded hippophae, pretreated wheat straw, among another biomass (Mesquita et al., 2015; Chen et al., 2018b).

Tween 80 is another non-ionic surfactant widely used to improve enzymatic hydrolysis. It is attractive for having a lower cost when compared to other compounds used to improve the enzymatic saccharification, such as BSA and PEG (Ling et al., 2021). Promising results were obtained with the addition of Tween 80 in biomass hydrolysis, with 98% yield of hexoses after enzymatic hydrolysis of the steam-exploded straw with the addition of 1% Tween 80. This surfactant was also used to improve the saccharification of sugarcane bagasse pretreated with different strategies, such as pretreatment with metal-salt catalyst and ferric chloride-organosolv catalyst, increasing the glucose content released, reducing the hydrolysis time and enzyme dosage. The glucose yield of 82.9% was obtained after six hours of saccharification of sugarcane bagasse pretreated with ferric-organosolv chloride catalyst, using 20 FPU/g substrates and without surfactant. Whereas, by adding 150 mg of Tween 80 per g of substrate, the glucose yield increased to 92.5%. Also, in only six hours of enzymatic hydrolysis with Tween 80, it was possible to obtain the same level of

glucose as after 72 h of hydrolysis without this surfactant. In addition, the glucose yield of 91.7% was obtained with half the amount of enzyme used previously (10 FPU/g substrates) when adding 150 mg of Tween 80 per g of the substrate (Zhang et al., 2018a). Regarding the saccharification of metal-salt-catalyzed pretreated sugarcane bagasse, when pretreatment with CuCl2 was implemented, about 67.3% of glucose yield was obtained after 72h of enzymatic hydrolysis, and with the addition of Tween 80, the glucose yield reached 71.1% after 24h of hydrolysis. Thus, the addition of Tween 80 increased the glucose yield and reduced the enzymatic hydrolysis time (Zhang et al., 2021b).

Other compounds can also be used to improve enzymatic saccharification. For example, humic acid has some surfactant characteristics and can act in the delignification of pretreated biomass. Another example is the non-catalytic protein BSA, which has a high affinity for lignin and can also act as a competitor for the enzyme, reducing the unproductive adsorption of cellulases (Tang et al., 2020; Ding et al., 2019). However, some of these compounds signify an increment in costs to the process.

The application of surfactants in enzymatic hydrolysis not only acts in the interaction between the biomass and enzymes. Also, some surfactants can improve the stability of enzymes and make them more hydrophilic, facilitating their interaction with cellulose, enhancing digestibility, and reducing the enzyme loading required to obtain a large glucose yield (Cao and Aita, 2013; Eriksson et al., 2002). The increase in enzyme stability occurs due to the interaction of the hydrophilic part of the surfactant that creates a barrier in the cellulase through hydrogen bonds with water, strengthening the intermolecular forces and reducing the shear force under agitation (Figure 2c) (Lou et al., 2018). The addition of surfactants allowed using only half of the enzyme loading to obtain a glucose yield similar to the control without surfactants (Zhang et al., 2018b).

As described before, surfactants classified as non-ionic are more effective in reducing unproductive adsorption. Some other surfactants also show this property, like PEG. These surfactants act similarly to non-ionic surfactants due to their hydrophobic portions (Lin et al., 2019). PEG also improves the activity of cellulase in high agitation processes, helping to decrease the liquefaction time of biomass under low agitation conditions (Ouyang et al., 2010). However, its effect on the stability of the enzymes is even more important, preventing the denaturation of enzymes. The enzymatic hydrolysis of sugarcane straw for 72h at 50 °C, 150 rpm, with addition of 9 mg protein/g glucan and 1g/L of PEG400 resulted in an increase of about 7% in glucose released due the improvement of activity, stability, and/or availability of enzymes (Rocha-Martín et al., 2017). In addition, PEG can make hydrophobic and

hydrogen bonds with lignin, forming a layer on the lignin surface that hinders the unproductive adsorption of cellulase (Lai et al., 2017). Different types of this polymer have already been applied in the literature to improve enzymatic saccharification (Ling et al., 2021; Lin et al., 2019). For example, PEG 4600 was added during the enzymatic hydrolysis of Avicel to enhance the interactions between the air-liquid interface. The presence of this polymer also reduced the deactivation of cellulase caused by the shear force, increasing the sugar conversion yield from 36.0% to 89.5% (Lou et al., 2018). PEG6000 was also studied in separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) processes of pretreated wheat straw. During the hydrolysis in both fermentation strategies SHF and SSF, there was reported an increment in glucose release of about 16 and 14%, respectively (Kadhum et al., 2019). Other surfactants studies reporting the use of surfactants in the hydrolysis stage are described in Table 3.

In summary, the addition of surfactants in enzymatic hydrolysis can decrease the denaturation of enzymes in high agitated systems, increase the efficiency of biomass conversion into fermentable sugars due to the reduction of unproductive adsorption of cellulases by hydrophobic interactions formed between surfactants and lignin. Thus, the use of additives to improve enzymatic hydrolysis is a strategy that achieves higher conversion yields of monomeric sugars, which impact mainly on the fermentation step and the reduction of bioprocess costs.

Table 3. Effect of several surfactants on the enzymatic saccharification of pure cellulose and lignocellulosic materials

Surfactant	Type of surfactant	Conditions	Saccharification yield (%)	Reference
Tween 80	N	Concentration of 2% (w/v) sugarcane bagasse pretreated with $AlCl_3$ catalyzed was hydrolyzed with 20 FPU g^{-1} of cellulase, and 150 mg of surfactant per gram of pretreated substrate for 72h	87.7	Zhang et al., 2021b
PEG 4600	N	Avicel Solid loading of 2 wt % was hydrolyzed with commercial *Trichoderma reesi* cellulase cocktail loading of 5 FPU per gram of glucan, and concentration of 0.005 g of surfactant per gram of biomass for 72 h	27.9	Lou et al., 2018
Tween 80	N	Steam-pretreated spruce (50 g/L) was hydrolyzed using cellulase activity of 0.66 FPU ml^{-1} (Celluclast 2L) with β-glucosidase activity (Novozyme) added at 0.81 IU ml^{-1} and surfactants concentrations of 2.5 g l^{-1} for 24 h	45.5	Eriksson et al., 2002
Agrimul NRE 1205	N		33.3	
Triton X-100	N		48.5	
Triton X-114	N		45.5	
HM-EOPO			33.3	
Sodium dodecylsulphate (SDS)	A		42.4	
Dodecyltrimethyl-ammonium bromide (DoTAB)	C		15.2	

continue

concluded

Tween 20	N	5% (m/v) of alkaline-sulfite chemithermomechanical pretreated sugarcane bagasse was hydrolyzed with 20 FPU of enzyme preparation produced by *Trichoderma reesei* per gram of biomass, and 2.5 g/L of surfactant (5 % m/m) for 48h	20.0	Mesquita et al., 2015
Tween 80	N		9.5	
PEG 4000	N	Avicel solids loading of 20 % was hydrolyzed with 9 mg protein per gram of glucan of *M. thermophila* C1 cocktail, and 5 g/L of surfactant for 72 h	15.6	Rocha-Martín et al., 2017
Tween 80	N	Concentration of 2% (w/v) sugarcane bagasse pretreated with NaOH-catalyzed organosolv was hydrolyzed with 20 FPU g^{-1} of active cellulase Novozyme, and 150 mg surfactant per gram of dry pretreated substrate for 72 h	93.0	Zhang et al., 2021b
		Concentration of 2% (w/v) pretreated sugarcane bagasse with 5% (w/w) NaOH, and 60% ethanol solution was hydrolyzed with 20 FPU g^{-1} of active cellulase Novozyme, and 150 mg surfactant per gram of dry pretreated substrate for 72 h	88.2	
PEG 6000	N	Concentration of 5% (w/v) sugarcane bagasse pretreated with 5% (w/w) ChCl-FA was hydrolyzed with 20 FPU g^{-1} of cellulase Novozyme, and 100 mg of surfactant per gram of pretreated substrate for 72 h	47.9	Ling et al., 2021
Tween 80	N		71.0	
PEG 6000 (D-34)	N	Concentration of 5% (w/v) acid-pretreated bamboo residues was hydrolyzed with 20 FPU g^{-1} of cellulase CTec2 per gram of glucan, and 0.4 g/L of surfactant for 48h	72.0	Lin et al., 2019
PEG 40000	N		51.0	
PEG 6000 (D-34)	N	Concentration of 5% (w/v) Avicel was hydrolyzed with 20 FPU g^{-1} of cellulase CTec2 per gram of glucan, and 0.4 g/L of surfactant for 48h	74.9	

[a] N – non-ionic; A – anionic; C- cationic surfactants

2.6 Use of surfactants in fermentation: extractive fermentation, membrane permeation and other techniques

Metabolic inhibition or inhibition of microbial growth are the main issues in bioprocesses, especially in long-term or continuous processes. These inhibitions can be caused by several physical factors such as pH, temperature, or by chemical factors as metabolites present in the medium (Banik et al., 2003). As an alternative to overcome these challenges, "*in situ* product recovery" (ISPR) techniques come to the scene. These techniques promote the recovery or separation of products during the production process without interruption. (Banik et al., 2003; Kaur et al., 2015; Outram et al., 2016).

Among the different ISPR techniques, extractive fermentation, also known as “perstraction” (Yang and Lu, 2013), is a process used to minimize those existing barriers in fermentation processes, especially product inhibition and product recovery (Banik et al., 2003; Kaur et al., 2015). This type of fermentation has also proved to be advantageous when considering yield, productivity, and concentration of the final product, three factors of great industrial importance that are major pivotal when related to the use of microorganisms in larger production scales (Huang and Tang, 2007; Iyyappan et al., 2020).

The most important and remarkable advantage of extractive fermentation is that products are being simultaneously extracted and separated from broth, integrating fermentation and extractive process in only one stage (Huang et al., 2010; Yang & Lu, 2013).

Thus, this technique brings multiple advantages as: Avoids product, substrate, or metabolite inhibition, increases volumetric productivity, enhances production yields, reduces time and cost of downstream, and reduces time and cost of global processes (Banik et al., 2003; Yang and Lu, 2013; Huang et al., 2019; Kadhum et al., 2019).

Extractive fermentation is primarily based on the aqueous two-phase systems application into a fermentation process (Banik et al., 2003). One of those phases is the culture medium, and the other one is an extractive solution or substance. The extractive solution recovers the product during the fermentation process, integrating a crucial downstream process step (product extraction) into fermentation (Banik et al., 2003; Yang and Lu, 2013). Aqueous two-phase systems approximately contain from 80 to 90% of water-based phase (Banik et al., 2003), and, although extractive fermentations are inspired in these systems, there have been tested different relations of water-based phase: extractive phase to obtain the best results possible using perstraction (Dhamole et al., 2012; Xiong et al., 2015; Zheng & Gao, 2016; Morales-Oyervides et al., 2017; Combes et al., 2021).

Solvents that are biocompatible with the microorganisms used in the process should be employed in the extraction phase. In this way, organic solvents are commonly used. This extractive phase may avoid metabolic inhibition by separating the product, substrate, or metabolite from the aqueous broth (Yang and Lu, 2013). This separation is based on chemical compatibility and chemical affinity between molecules; it also can occur with micelles formations around product molecules (Banik et al., 2003).

Different research has been done in order to evaluate extractive fermentation and its capacity, as well as the toxicity of the extractors. Kollerup and Daugulis (1986) analyzed more than 1300 solvents and found only nineteen of them appropriate to achieve a continuous extractive fermentation for ethanol production. This research generated a database of solvents and their effects, highlighting the importance of choosing biocompatible solvents for each microorganism and process (Kollerup and Daugulis, 1986). More recently, researchers pointed out that there should be a higher amount of solvent to maximize the extraction efficiency (Lemos et al., 2017).

Alcohol production is an example of extractive fermentation applications. Thinking about the fact that accumulated ethanol in fermentation broth inhibits the microbial metabolic activity, directly affecting the final product concentration (Dias et al., 2013),

extractive fermentation has been widely associated with the production of alcohol and with the biorefineries concept (Kollerup and Daugulis, 1986; Dhamole et al., 2012; Lemos et al.,2017). Studies show that this type of fermentative process decreases the inhibition of alcohol (Lemos et al.,2017) and can improve profitability due to the product separation from de water-based medium (Dhamole et al., 2012). But extractive fermentation can be used for other purposes, in addition to ethanol production.

It has been found that the use of extractive fermentation associated with thermosetting polymers (EOPO and Ucon HTF 14) can enhance the production of lipases by the bacterium *Burkholderia cepacian* by 22%. In addition, it could optimize the purification in a single step with the recycling and direct recovery of the polymer and separation cells, which leads to a reduction in operating costs (Show et al., 2012), benefit found in other works as well (Kollerup and Daugulis, 1986).

Given the characteristics of surfactants, data has shown promising results related to their use associated with extractive fermentation (Kollerup and Daugulis, 1986; Dhamole et al., 2012; Xiong et al., 2015; Lemos et al., 2017). Surfactants have also been reported to act as solvents, adding in the extraction of cellular products (Duan et al., 2015., Wu et al., 2017; Kadhum et al., 2019). The use and role of surfactants in extractive fermentation will be following described.

2.6.1 Surfactants: why are they used in fermentation?

The formation of micelles by surfactants is an attractive aspect of bioprocesses. Surfactants can solubilize reagents, even being used to cap particles to improve their transport into cells (Raj et al., 2019). Moreover, surfactant micelles can alter the solute distribution and the surface tension of fluids (Hu et al., 2012; Zheng and Gao, 2016). These characteristics of surfactants can enhance the utilization of substrates of low solubility by the microorganisms; improve cell nutrition and, probably, metabolite production (Hu et al., 2012; Zheng and Gao, 2016). Besides entail the reduction of harmful impacts as metabolic inhibition caused by some substances or products present in the culture medium during bioprocesses (Dhamole et al., 2012).

These effects are possible because the aqueous phase decreases its metabolite and product saturation due to the capacity of micelles to ‘capture’ molecules into their matrix (Banik et al., 2003). So, micelles are able to perform both functions, solubilize nutrient

molecules to let the cell get them easier (delivery), and separate molecules from the aqueous phase, thus avoiding product repression/ inhibition.

Surfactants also have the ability to modify the microorganisms' cell wall structure and thus, increase the permeability of the membrane, enhancing the secretion of intracellular content (Chen et al., 2018a; Morales-Oyervides et al., 2017). This characteristic has been used to subserve fermentative processes, being non-ionic surfactants the most used in this case. This predilection is due to the fact that they do not carry electric charges and are relatively non-toxic (Sonia and Sharma, 2014).

However, an important fact to be observed when using surfactants is that the ideal concentration for each process must be accounted for even though these substances are efficient in extractive fermentation. Optimal conditions and concentrations for different bioprocesses using surfactants as an extractive phase has been an important research topic. In Table 2.4 are exposed some examples of the use of principal surfactants in industrially relevant processes as alcohol, enzymes, and pigment production. Research cited got high extracellular concentration of product, from 20 to >400 % increase during extractive fermentation using surfactants as extractive phase. Moreover, it can be noticed the time of addition of the surfactant during the process, which is a determinant parameter to be explored. This research is necessary to improve surfactant-extractive fermentation and get advantageous bioprocesses.

Some authors report better results when adding the surfactant at the beginning of the process for the production of butanol, kitamycin or some enzymes (Ooi et al., 2011; Dhamole et al., 2012; 2015; Xiong et al., 2015; Zheng and Gao, 2016; da Silva et al., 2018b; Badhwar et al., 2019). On the other hand, it is also shown in Table 2.4 some research with better results when surfactants are added at different times during the process (Zhang et al., 2013; Duan et al., 2015; Kadhum et al., 2019; Li et al., 2020). For example, Morales-Oyervides et al. (2017), reported a concentration increase of butanol produced by *Talaromyces spp*, adding Triton X-100 after 120 hours of fermentation. These results evidence the importance of studying not only different surfactants or concentration of them, but also different times of addition to the process to improve the product extraction.

On the other hand, very large or specific concentrations or the use of particular surfactants can cause microorganism cellular damage, inhibition, or lysis, and consequently impair the bioprocess yield, as reported in some works (Kollerup & Daugulis, 1986; Ding et al., 2010; Dhamole et al., 2012; Xiong et al., 2015; Lemos et al., 2017; Wu et al., 2017; Huang et al., 2019). For example, it was reported that tea-saponin and Brij 58 surfactants

caused an inhibitory effect in cell growth in a process for lipids production by *Cryptococcus curvatus* (Huang et al., 2019). Moreover, when using L62 above 6%, a decrease in butanol production was found (Dhamole et al., 2012). In other work, by increasing concentrations of Triton X-100, the conversion of red pigments increased significantly with the decrease in orange pigments, the latter being the target in that research (Xiong et al., 2015).

Regarding alcohol inhibition in microbial metabolism, by associating cloud point in extractive fermentation with the use of 6% of surfactant L62, it has been shown that the yield of butanol doubled, and 95% of the alcohol was recovered in the rich phase of surfactant. 30 g/L of butanol was obtained adding L62, compared to 5 g/L without surfactant addition (Table 2.4). Furthermore, the biocompatibility factor with the bacteria was found to be positive. In this case, L62 could be used without causing further damage to the cell (Dhamole et al., 2012). The association of cloud point in extractive fermentation has been extensively studied in other processes. As the optimization of pigment production by pigments producers as filamentous fungus, especially of the genus *Monascus.*

Another interesting example of the application of surfactants in fermentation is the use of Triton X-100 associated with extractive fermentation, which has resulted in a considerable increase in the production of red pigments. Using the MSG medium with Triton X-100 with 50 g/L for eight days, biomass volume appears to be reduced while improving the intracellular and extracellular concentration of *Monascus anka* pigments, with a significant increase in the red pigment production (Xiong et al., 2015). Chen et al. (2018a), by adding surfactant at 40 g/L, observed pigment secretion was 30 times higher at 470 nm and reached 9 AU at 410 nm, with only 11% pigments staying in the mycelia.

As observed in Table 2.4, the surfactant concentration in the extractive phase is very variable according to each process, microorganism, and surfactant used. It is important to take into account the biocompatibility of each surfactant with the microorganism that is being used for each process (Li et al., 2020; Morales-Oyervides et al., 2017). For example, it has been suggested that surfactants as Triton X-100 and Tween 80, may present a higher biocompatibility with certain microorganisms. This biocompatibility can facilitate membrane modifications and metabolic alterations as stress signalling and transport. (Li et al., 2020; Morales-Oyervides et al., 2017). On the other hand, these surfactants can be more aggressive to the cell, as consequence, less concentration is needed.

Moreover, the potential of using surfactants in extractive fermentation can increase the concentration of the product in the medium (Xiong et al., 2015; Kadhum et al., 2019). Extractive fermentation has been proved in different types of microorganisms like bacteria,

yeast, and filamentous fungus, with promising results in diverse kinds of processes of industrial relevance, such as butanol, antibiotics, and pigments production (Hu et al., 2012; Dhamole et al., 2015; Zheng and Gao, 2016; Lemos et al., 2017; Badhwar et al., 2019). Thus, surfactant extractive fermentation has shown up as a promising technique for several bioprocesses.

Table 4. Application of surfactants in extractive fermentation as extractive phase

Surfactants	Concentration (g/L)	Addition time (h)	Microorganisms	Product	Extracellular product (concentration) increase (%)	Reference
L62	60	0	*Clostridium pasteurianum*	Butanol	225 (yield)	Dhamole et al., 2012
	3				95	
L62D	30	0	*Clostridium acetobutylicum*		48	Dhamole et al., 2015
Triton X-100	50	0	*Monascus anka*	Red pigment	300	Xiong et al., 2015
	35	120	*Talaromyces spp.*	Pigments	27.7	Morales-Oyervides, et al., 2017
	25	36	*Shiraia bambusicola*	Hipocrelina A (HA)	439.8	Li et al., 2020
	5	40	*Escherichia coli*	Pullulanase	86	Duan et al., 2015
Tween 80	1	0	*Streptomyces kitasatoensis*	Kitasamycin (antibiotic)	23	Zheng and Gao, 2016
	1,1	72	*Antrodia camphorata*	Antrodin C	98	Zhang et al., 2013
Tween 85	0.1	0	*Streptomyces kitasatoensis*	Kitasamycin (antibiotic)	22	Zheng and Gao, 2016
SDS	0.5	0			55	
PEG 6000	20	12	*Saccharomyces cerevisiae*	Ethanol	19.2	Kadhum et al., 2019
PEG 8000 / Dextran T500	96/10 *	0	*Burkholderia pseudomallei*	Lipase	92.1	Ooi et al., 2011
PEG 8000 + citrate	240 + 200*	0	*Aspergillus tamarii*	Protease	98.4	da Silva et al., 2018b
PEG4000/ Dextran T5OO	69,3/60,5*	0	*Aureobasidium pullulans*	Pullulan	95	Badhwar et al., 2019

[a]* g/kg

The mechanisms of action of surfactants during extractive fermentation are not totally known yet, but their research has been boosted during the last years. Surfactant-

membrane interactions are the main issue on the way to understand how they work in extractive fermentation.

2.6.2 How do surfactants act during fermentation?

In fermentation, the cell membrane exports metabolic products to the extracellular medium (Figure 3a). The enhancement of this process is an advantageous characteristic of extractive fermentation. This phenomenon can be observed with the use of surfactants as extractive phase (Hu et al., 2012; Duan et al., 2015; Morales-Oyervides et al., 2017; Kadhum et al., 2019), with the 'plus' advantage of the enhancement of medium components solubilization (Zheng and Gao, 2016). However, surfactants can be used in the final stages or after fermentation to improve the extraction of products (Duan et al., 2015; Wu et al., 2017). Even though the function of surfactants is not only limited to separate or solubilize molecules or products in the culture medium (Banik et al., 2003).

Some authors claim to get production increase by using surfactants in fermentation. However, the product increase in broth could be the consequence of an extraction mechanism of surfactants and not due to an increased production rate, even though there is still a lack of metabolic analysis to confirm those facts. More studies are needed to determine whether the product in the extracellular broth is a consequence of annulation of metabolic inhibition or if it is due to extraction mechanisms not described yet. In this section, possible extractive mechanisms will be discussed based on current scientific evidence.

Now it is known that the interactions may be as plenty as kind of surfactant–kind of microorganism interactions we could make, in addition with experimental conditions as another factor. However, with available data, we can find some trends:

2.6.2.1 Improvement of membrane permeability

It is well-known that surfactants act at surface level, so exportation, solubilization, and extraction of fermentation products become easier thanks to the improvement of molecular interactions when superficial and interfacial tension are weakened by their presence in the culture medium (Show et al., 2012; Banik et al., 2013). As well, surfactants can also act at the cell membrane level, decreasing superficial tension, depending on their concentration, structure, and experimental conditions during fermentation. As superficial

tension decreases, permeability can be enhanced, improving solubilization and extraction of products (Hu et al., 2012, Manaargadoo-Catin et al., 2016).

The possible first mechanism improves membrane permeability affecting molecular interactions and tension. On its own, it does not alter cell membrane structure or composition, which makes it a harmless process for cell integrity (Duan et al., 2015). Thanks to their properties, surfactants can induce hydrophobicity alterations (Li and Zhu., 2012) and modify the Zeta potential of the cell surface (electrostatic repulsion force between the cell surface and other molecules or particles), producing a change in cell membrane 'chemical' permeability (Domingues et al., 2014). As part of the process, the improvement of cell surface hydrophobicity and Zeta potential can boost the affinity between some substrates and the cell and can facilitate the secretion of intracellular molecules and products (Chen et al., 2018a; Morales-Oyervides et al., 2017).

Thus, hydrophobic and hydrophilic interactions are in part responsible for surfactant-membrane interactions. For example, cationic surfactant (CTAB) was reported to have hydrophobic interactions with bacterial cell surface proteins (Cortez et al., 2012). These interactions are regularly realized through electrostatic attraction (Pan et al., 2018), as well micelles formation and their affinity with cell membranes are modulated by ionic interactions (Manaargadoo-Catin et al., 2016).

2.6.2.2 Membrane reorganization

Due to their characteristics and structural similarity with cell membrane lipids (Singer, 1972), surfactants also have the capacity to interact with the cell wall and membrane components (Manaargadoo-Catin et al., 2016; Pan et al., 2018; Wu et al., 2017). Consequently, they can alter membrane structure and arrangement, hydrophobicity, fluidity, permeability, and integrity (inducing membrane lysis) (Kang et al., 2013; Manaargadoo-Catin et al., 2016; Morales-Oyervides et al., 2017; Pan et al., 2018; Zhao et al., 2021).

Electrostatic interactions may lead to a partial or total absorption and incorporation of surfactant molecules into cell surface (Manaargadoo-Catin et al., 2016). This process could be considered as another mechanism or stage of the action of surfactants. As a surfactant penetrates the cell surface, it leads to a reorganization of membrane proteins and lipids, also interfere with their interactions as shown in Figure 3 (Manaargadoo-Catin et al.,

2016); consequently, there will be structural and shape changes of membranes (Manaargadoo-Catin et al., 2016; Morales-Oyervides et al., 2017).

Membrane reorganization by the incorporation of surfactants can lead as a consequence to the removal of membrane components (lipids, proteins, etc.) and their incorporation to surfactant micelles (Manaargadoo-Catin et al., 2016). During this process, the product can be extracted by the extractive phase according to the product-surfactant affinity, shown in Figure 3b (Banik et al., 2003; Wu et al., 2017; Chen et al., 2018a; Huang et al., 2019). This process is also called "membrane solubilization" and has been reported when used zwitterionic surfactants (Preté et al.; 2002b), non-ionic surfactants as Triton X-100 (Preté et al., 2002a), and alkyl ethers (Domingues et al., 2008) in model membrane cells (Manaargadoo-Catin et al., 2016). Membrane solubilization can be also used as an extractive method after bioprocess (Wu et al., 2017).

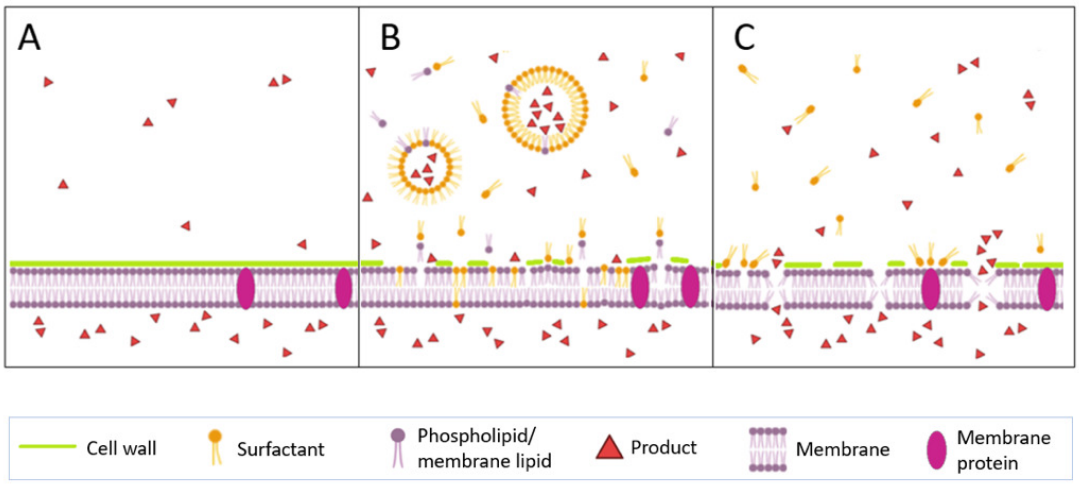

Figure 3. Mechanisms of action of surfactants in the cellular surface. A) Exportation of the product without surfactant B) Reorganization of membrane, shape modification, membrane solubilization, and formation of mixed micelles C) Cell wall disruption and formation of membrane pores (Created with BioRender.com).

2.6.2.3 Formation of membrane and trans-membrane pores

The extraction of lipids and proteins from cell membranes can lead to another linked mechanism: the increased porosity by trans-membrane or membrane pores formation (Figure 2.2c). Those pores can be repaired by the cell during the fermentative process (Manaargadoo-Catin et al., 2016), but if not, they may act as channels from the cytoplasm to the periplasm and extracellular medium (Nalini and Parthasarathi, 2014), increasing the exportation of molecules (King et al., 1991).

Moreover, all the above alterations mentioned can lead to an osmotic response (Manaargadoo-Catin et al., 2016), triggering transmembrane ion fluxes (Seeman et al., 1974) that can also cause morphological changes by the entrance and export of ions and water, besides product and metabolites (Nalini and Parthasarathi, 2014).

2.6.2.4 Metabolic alterations

Surfactants promote a conformational change in membrane proteins; this mechanism, added to the other discussed alterations, can cause leakage of intracellular substances crucial for metabolisms, such as proteins and amino acids, as a consequence, an increase in the production of a molecule concerning another can be observed (Chen et al., 2018a). Thus, according to the purpose of the process, surfactants can cause a positive or negative impact on the production of some metabolites.

On the other hand, cell surface alterations by surfactants and other interactions with the cell could trigger metabolic disturbances in the production of membrane components (Ding et al., 2010). This mechanism is considered an internal alteration as it involves metabolic machinery, differently from the other mechanisms explained before. E.g., there is evidence suggesting that non-ionic surfactant Tween 60 could inhibit the synthesis of fatty acids of cell membranes in experiments with *B. lactofermentum* (Takinami et al., 1965). This mechanism would highlight the importance of working with an optimal concentration of surfactant to avoid cell damage or death. Furthermore, it means an indirect way for cell surface alteration by inhibition or affecting metabolism. Nevertheless, it does not necessarily imply a direct interaction with the surface, thus deserving to be studied more deeply as an isolated phenomenon and for extractive fermentation.

In another research in 2020, it was found a cascade effect through nitric oxide production during extractive fermentation of Hypocrellin A. The researchers pointed to a metabolic relation between the addition of Triton X-100 and the regulation of expression of transporter genes for Hypocrellin A by Shiraia spp (Li et al., 2020).

Anyhow, scientific evidence does not separate the mechanisms mentioned before nor disclaim the action of different mechanisms simultaneously. Surfactants can be interacting with the cell surface in one or more different ways simultaneously, and also signaling and trigger metabolism reactions, even though it can be inferred that, according to their concentration and interaction, a ‘logical’ sequence, as shown before, would be expected in

most or at least some of the cases. Further studies must be done to affirm or discriminate a mechanism of action in each microorganism and process.

2.7. Research gaps and future perspectives of surfactants in biorefinery

The current world scenario requires attention on the growing energy demand and the consequent environmental impacts of using fossil fuels. This has resulted in a surge to research the production of clean energy sources from renewable raw materials and industrial wastes (Li and Zheng, 2017). LCB is one of the important alternatives for biotechnological applications; especially for the generation of renewable energy and value-added bioproducts. However, for use of LCB as feedstock to generate value-added products, it is important the application of essential processes (e.g., pretreatment, enzymatic hydrolysis, and fermentation). In this context, there has been a trend to develop new strategies to apply additives as surfactants to the main steps processes in biorefinery to improve their production yields. According to SCOPUS database (2021), from the last 5 years, 87 patents related to pretreatments (key-words: Surfactants; Pretreatments; Lignocellulosic; Biorefinery) and 842 for enzymatic hydrolysis (key-words: Surfactants; Enzymatic Hydrolysis; Lignocellulosic) were found for different areas of interest. All those findings show the potential of surfactants and the gap in literature to consider them key molecules for bioprocess. For example, many of those documents describe methods for the recovery of molecules (US-20210246608A1), pretreatments (US-20200207925), and enhanced saccharification of lignocellulose (US-10968322) (Casad et al., 2021; Chengyu et al., 2020; Satlewal et al., 2021).

a) Pretreatments

Lignin interferes and hinders the bioprocess in various manners and increases the cost of the process due to the requirement of a greater quantity of enzymes to facilitate hydrolysis (Singvi et al., 2014). More research has been done worldwide for a better understanding of the mechanisms of lignin interactions with the reagents and with enzymes in order to minimize the negative effects of lignin in all steps (Thite and Nerurkar, 2019). It has been observed that one of the most efficient ways for the removal of lignin from the LCB is by the addition of surfactants during pretreatment (Qing et al., 2010). It has been clearly demonstrated that surfactants use during initial stages of pretreatments significantly increased lignin removal and also improved the results of the next stages of the pretreatment

processes, viz., microwave-NaOH pretreatment, ionic-liquid pretreatment, hydrodynamic cavitation assisted-pretreatment, dilute ammonia pretreatment, etc. (Eriksson et al., 2002; Tu and Saddler, 2010; Cao, 2012; Sindhu et al., 2013; Nasirpour et al., 2014; Mesquita et al., 2015; Hilares et al., 2020; Nogueira et al., 2017).

Even with significant progress in pretreatment technology, several obstacles and challenges still remain to be overcome to increase the commercial viability of biorefineries. Further research on the use of different types of surfactants in pretreatment processes will help to optimize the process cost and as well minimize the negative environmental impacts of pretreatment steps.

b) Enzymatic hydrolysis (Saccharification):

Various studies have recorded the economic and technical benefits of surfactants in the saccharification of LCB. A simple economic analysis of enzyme cost savings indicated that the addition of Tween 80 during the hydrolysis could save 60% of the total enzyme cost. For example, the addition of Tween in the production of ethanol from lodgepole pine could reduce the material cost by 24% per 1 gal of product, and the ethanol production cost could be reduced by 8.6% (Tu and Saddler (2010). In another techno-economical analysis, it was indicated that the addition of 2% of PEG6000 resulted in a ROI (Return Over Investiment) of 3.29% (Kadhum et al., 2018).

Although various studies have demonstrated the utility of surfactants in the enzymatic saccharification of LCB, further research is needed to optimize the conditions of their use, like surfactant loading rate and the step of the process for the addition of the surfactants. The increase in the surfactant loading rate did not always increase the final glucose yield. It is observed that the addition of Tween 80 in excess during the enzymatic saccharification of sugarcane bagasse pretreated with metal salts, decreased the glucose yield (Zhang et al., 2018a; 2021b). Some studies have reported that the action of surfactants on pure cellulose did not enhance the yield of enzymatic hydrolysis (Lou et al., 2014; Zhou et al., 2015). In this case, the structure of the substrate, as the high crystallinity of the pure cellulose, and the high load of surfactants (about 5 g/L) can impair the pure cellulose conversion at the late phase of hydrolysis (Zhou et al., 2015). Even non-ionic surfactants do not consistently improve the hydrolysis of pure cellulose. The process with surfactants depends on several factors, and the interaction between surfactants, enzymes, and pure

cellulose substrate needs further study to achieve higher enzymatic hydrolysis of pure cellulose (Agrawal et al., 2017).

In addition, it has been observed that the type of pretreatment can also affect the yield of enzymatic saccharification, the addition of EOPO5 surfactant was shown to have a significant effect on improving enzymatic saccharification of wheat straw treated by steam-explosion than the wheat straw pretreated with dilute acid. Chemical differences in the lignin structure after each pretreatment have to be taken into account. For example, the lignin pretreated with steam-explosion had more hydrophobic characteristics due to the lower amount of carboxylic acid groups and higher amount of phenolic hydroxyl groups than the lignin obtained from acid pretreatment, which may increase protein adsorption. Also, it was observed a lower content of aliphatic hydroxyl groups in steam lignin, which may increase the surface hydrophobicity and probably explains the higher adsorption capacity of steam lignin than acid lignin for enzymes (Agrawal et al., 2017). However, more research is needed in terms of improving their stability, functionality, and process conditions for treating different types of lignocellulosic feedstocks.

c) Fermentation

As described before, the use of surfactants during the first stages of the process (e.g., pretreatment and enzymatic hydrolysis) facilitated that the surfactants remain and play a role in fermentation. Recently, different kinds of strategies have been discussed, viz., SHF, SSF, and pre-hydrolysis followed by simultaneous saccharification and fermentation (PH-SSF). More studies on the remaining surfactant effect on the sequential stages of bioprocess need to be analyzed. However, it is seen that remained surfactants from the previous stage of enzymatic hydrolysis improved the fermentation and could help to reduce the global production cost of the bioprocess.

2.8. Conclusions

In the search for sustainable, economical, and technically viable industrial processes, many strategies have been analyzed to improve the yield and economic cost of bioprocesses. One of the strategies that have attracted attention recently is the use of additives to improve the performance of biorefineries. Evidence from various studies has been discussed. The positive effect of many classes of surfactants in pretreatments, enzymatic saccharification,

and fermentation has been reported along with the limitations encountered in these processes. Surfactants are emerging as important molecules for improvement and wide-scale application of the biorefinery processes to recover energy/ value-added bio-products from the LCB.

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CHAPTER III

Non-ionic surfactant formulation sequentially enhances the enzymatic hydrolysis of cellulignin from sugarcane bagasse and the production of *Monascus ruber* biopigments.

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ABSTRACT

The effect of a non-ionic surfactant optimized formulation (SOF) obtained from an experimental design was evaluated for different influencing variables in the processing of sugarcane bagasse cellulignin to produce biopigments. The major findings in the saccharification stage using the SOF point that at same enzyme loading the sugar conversion is 2-fold higher; the enzyme loading could be 4-fold lower to achieve similar yield compared to control; 15% (m/v) of total solids loading maintained the yield in fed-batch configuration; the enzyme stability is maintained at high shear force stress and temperatures. Besides, it was observed that the production of biopigments was 5-fold higher in glucose-based medium. Finally, under separate and semi-simultaneous hydrolysis and fermentation the maximum biopigments production were of 10 AU510nm/mL and 17.84 AU510nm/mL, respectively. The SOF used in this study was found to be a promising additive either in a single or sequential steps to produce biopigments in biorefineries.

Keywords: Non-ionic surfactants. Formulation. Cellulignin. *Monascus ruber*. Biopigments

3.1 Introduction

Lignocellulosic biomass has been investigated as a promising source to produce second-generation fuels and value-added products (e.g., ethanol and biopigments) (Hilares et al., 2018). World energy sustainability demand and the complexity and recalcitrance of lignin from the lignocellulosic biomass have driven technical and economic efforts to enhance the conversion processes of this biomass through different strategies (Yoo *et al*., 2020; Sheng *et al*., 2021). Enzymatic and chemical methods to broke down the lignocellulosic biomass to release monomeric sugars have been extensively studied (Zhang *et al*., 2012).

Enzymatic hydrolysis of lignocellulosic biomass to produce second-generation sugars remains as a critical step in bioprocesses because of the many bottlenecks that can impact negatively the saccharification yield (Zheng *et al*., 2013; Bello *et al*., 2021). One of the main factors that hamper the enzymatic degradation is the non-productive binding and inactivation of cellulases onto cellulose and lignin (Converse *et al*., 1988; Djajadi *et al*., 2018). Non-productive binding onto lignin have been reported by several researchers as a cause of hydrophobic, electrostatic, and hydrogen bonding between enzymes and different surfaces, such as lignin (Agrawal et al., 2017; Djajadi *et al*., 2018; Huang *et al*., 2022).

Lignin, which accounts for 15–30% of lignocellulosic biomass could be enriched to over 40% after some pretreatment methods, e.g., dilute acid or steam explosion (Pan *et al*., 2005). Those pretreatments are important due to the obtention of hemicellulosic hydrolysates rich in pentoses (e.g., xylose). However, the solid biomass produced (known as cellulignin) after this kind of pretreatment has increased lignin content, impairing the attack of enzymes to this feedstock, consequently. To mitigate the negative effects of lignin on enzyme performance, different types of pretreatments as well as a deep understanding of how and where lignin binds to enzymes are prerequisites to choose the correct strategy to enhance the bioprocess (Huang *et al*., 2022).

Besides pretreatments, other alternative strategy that have been used in the last years is the use of surfactants as additives in several key steps of bioprocesses *viz*., pretreatment, enzymatic hydrolysis, and fermentation (Muñoz *et al*., 2022). Surfactants are liquid soluble, surface-active agents that reduce the surface and interfacial tensions that cause the adsorption of enzymes at interfaces (Menegol *et al*., 2014). The addition of surfactants in the process is one of the most effective optimization approaches for the enzymatic hydrolysis step (Huang *et al*., 2011; Olsen *et al*., 2011). Several primary mechanisms of the effects of

surfactants have been suggested: (a) The hydrophobic tail of surfactants tends to adsorb on the hydrophobic surface of lignin and reduce the unproductive binding of enzymes, (b) Surfactants lubricate the access of cellulase to cellulose through the pores and cracks on initial hydrolysis, (c) Surfactants combined with the free chemical groups of lignin prevent cellulase adsorption to lignin on subsequent hydrolysis, (d) The hydrophilic head of the adsorbed surfactant molecule that interacts with water makes lignin more soluble in water, (e) Many research works reported that the addition of non-ionic surfactants (Tween 20 and Tween 80) and poly-ethylene glycol (PEG) in enzymatic hydrolysis could increase the hydrolysis rate of enzyme and promote the removal of amorphous cellulose (Eriksson *et al*., 2002; Alkasrawi *et al*., 2003; Parnthong *et al*., 2017; Li *et al*., 2012). Also, surfactants positively affect cellulase activity and enzyme stability by reducing cellulase deactivation caused by the shear force and air-liquid interface that occurs in agitated systems (Eriksson *et al*., 2002; Yang *et al*., 2015; Kaar *et al*., 1998; Zhang *et al*., 2011).

Besides the effect of surfactants in the saccharification step, they have been also described as additives to enhance the release of high value-added products in fermentation process. Among all diversity of byproducts, the enhancement of biopigments production by fungus as of the genus *Monascus* is of great interest, mainly considering their properties: anti-inflammatory, anti-obesity, anti-hypercholesterolemic, anti-cancer, anti-diabetic supplements (type II diabetes), food coloring and essential fatty acids for human health (Moharram *et al*., 2012; Chen *et al*., 2015; Kim and Ku, 2018). Surfactants can act in the membrane of microorganisms, enhancing its permeability, reorganizing some components (e.g., lipids), and forming membranes and *trans* membrane pores (Muñoz *et al*., 2022). Those modifications have been studied in *Monascus* species associated to improve the release and production of biopigments (Yang *et al*., 2019).

In despite of there are many studies about the application of surfactants to enhance the enzymatic hydrolysis of lignocellulosic biomass, and some reports regarding to their use in fermentation step of the *Monascus* biopigments production, the integrated use of blends of non-ionic surfactants sequentially in hydrolysis and fermentation steps was not previously reported, representing a new strategy proposed in this work.

In this work, aspects such as enzyme stability, saccharification yield, high solid content, and production of biopigments from sugarcane bagasse in Separated Hydrolysis and Fermentation (SHF) and in Semi-simultaneous Saccharification and Fermentation (SSSF) strategies, were studied using an optimized formulation of non-ionic surfactants by a Central Composite Rotatable Design (CCRD). Sugarcane bagasse was chosen as model of study due

to its abundance in many countries, as Brazil, thus representing a biomass with high potential to be used as raw material in biorefineries.

3.2 Materials and Methods

3.2.1 Biomass

Sugarcane bagasse (SCB) was kindly donated by Ipiranga Agroindustrial (Descalvado, São Paulo, Brazil). When received, the SCB has initially allowed to sun-dry for 2 days. It was then ground using a knife mill Marconi model MA 680 (Marconi Ltda., São Paulo, Brazil) and passed through a 2 mm sieve. Before use, SCB was screened again to a final size of around 0.841 mm using a US mesh No. 20 sieve.

3.2.2 Pretreatment and chemical characterization of biomass

The sugarcane bagasse was subjected to dilute-acid hydrolysis with 1.0% (w/v) H2SO4 in a 350 L steel reactor at 121°C for 30 min at a 1:10 solid/liquid ratio to partially remove the hemicellulosic and lignin fractions, thus allowing the enzyme access to the cellulose. After pretreatment, the cellulose-rich solid phase was neutralized with tap water and stored at 4 ºC. Moisture of lignocellulosic biomass was individually determined using a fast dry weight scale with a UV chamber at 105 °C. Lignocellulosic biomass was characterized in terms of structural macromolecular fractions, ash, and extractives, before and after the pretreatment process, based on the analytical procedures established by the National Renewable Energy Laboratory - NREL (Sluiter et al., 2011).

3.2.3 Experimental design for enzymatic hydrolysis optimization assisted by non-ionic surfactants

Enzymatic hydrolysis of the pretreated SCB (cellulignin) was performed in 50 mL Erlenmeyer flasks, with a working volume of 20 mL in a rotary shaker set at 50 °C and 200 rpm. Initially, the pretreated SCB was mixed with appropriate volumes of 50 mM sodium citrate buffer (pH 4.8), which resulted in a 10% solid loading. Cellulase, enzyme blend (Sigma-Aldrich®, US) was added at an enzyme dosage of 10FPU/g of SCB biomass. All hydrolysis experiments were carried out for 24 h, at the end of which solids and liquids were separated by centrifuging at 10,000 rpm (6,738 *g*) for 10 minutes. The supernatants were

stored at -20ºC before total reducing sugar (TRS) analysis by dinitrosalicylic acid (DNS) method, and sugar monomer analysis by HPLC, respectively. Design Expert 7.0 (Stat-Ease, MN, USA), was used to design experiments, analyze experimental results, and to identify optimum conditions. Initially, a CCRD was created from a three-level factorial design augmented with center and star points. The CCRD for this experiment included a total of 17 experimental runs with triplicate at the center point. 17 sample runs were carried out according to CCRD based on varying percentages (0-2.5 %) of each of the three non-ionic surfactants: Tween 20, PEG 400, and Triton X100. Response surface graphs were plotted using STATISTICA (StaSoft, Inc., OK, USA). Answer variable was the total reducing sugars (TRS) hydrolysis yield, calculated as the ratio between the obtained and the theoretical TRS concentration, this last calculated based on reducing sugars which could be released from cellulosic and hemicellulosic fractions of the pretreated sugarcane bagasse.

3.2.4 Effect of different influent variables on the enzymatic hydrolysis of pretreated biomass under optimized surfactant formulation

3.2.4.1 Effect of the optimized surfactant formulation on the enzyme loading

At the optimum surfactant formulation condition that was identified in the previous section, enzyme loadings were investigated to determine if the formulation of non-ionic surfactants could decrease the enzyme dosage. Accordingly, different volumes of Cellulase, enzyme blend (Sigma-Aldrich®, US) ranging from 2.5 to 10 FPU/g of biomass were applied to pretreated SCB in 50 mL Erlenmeyer flasks under similar conditions to the described in section 3.2.3. The optimum enzyme dosage was determined based on HPLC results and overall glucose and xylose hydrolysis yields.

3.2.4.2 Effect of the high solids loading in different enzymatic hydrolysis configurations

At the optimum surfactant formulation condition (determined in section 3.2.3), different enzymatic hydrolysis tests were performed varying the total solids loading (from 5% -15%), and the surfactant optimized formulation and enzyme addition. Reactions were performed in Erlenmeyer flasks, under conditions to the above described (section 3.2.3). Experiments were performed in fed-batch operation mode, with biomass, surfactant and enzyme feeding. In this way, at the beginning of the process, Erlenmeyer flasks were loaded

with 5% of biomass, and the corresponding surfactant formulation (at previously optimized conditions) and enzyme dosages. Then, at each 12h, additional biomass correspondent to a loading of 2.5% was added in the flasks, with some experiments also added with more surfactant and biomass, according to Table 5, until 48h of process. The total process time was 96h, and the response was the glucose conversion yield. Each run was performed in triplicate

Table 5. Experimental configuration to evaluate the total solid loading effect in enzymatic hydrolysis, using the surfactant optimized formulation. Components added at each process time: SOF: Surfactant Optimized Formulation, B: Biomass (pretreated SCB), E (Enzyme – 10FPU/g of loaded biomass)

Test/time (hours)	0	12	24	36	48	72	96
1	SOF + E + B	B	B	B	B	--	--
2	SOF + E + B	SOF + E + B	SOF + E + B	SOF + E + B	SOF + E + B	--	--
3	SOF + E + B	E + B	E + B	E + B	E + B	--	--
4	SOF + E + B	SOF + B	SOF + B	SOF + B	SOF + B	--	--
5*	E+B	B	B	B	B	--	--

*Control experiment

3.2.4.3 Analysis of the thermostability and shear force stress of enzymes

For thermostability evaluation, experiments were performed in Erlenmeyer flasks (conditions previously described in section 3.2.3), using the surfactant optimized formulation and different temperatures (50, 60 and 70 ºC).

For the evaluation of the shear stress effect, hydrolysis experiments were performed in a 1.5 L bench scale stirred tank reaction BIOFLO III (New Brunswick Scientific, Edison, NJ, USA). The reactor was loaded with a working volume of 800 mL, with the reaction medium composed by 50 mM sodium citrate buffer (pH 4.8), pretreated biomass (10% solid loading), surfactant optimized formulation, and Cellulase, enzyme blend (10FPU/g of biomass). Temperature of the process was kept at 50°C and runs with different stirring rate (200 rpm and 800 rpm) were performed. For both experiments, each run was performed in triplicate, and 72 hours samples were taken to analyze the glucose and xylose hydrolysis yield.

3.2.5 Effect of the surfactant optimized formulation on biopigment production

3.2.5.1 Microorganism

Monascus ruber Tieghem IOC 2225 was kindly donated by the Culture Collection of Filamentous Fungi (CCFF) – Oswaldo Cruz Foundation (IOC/FIOCRUZ) (Rio de Janeiro, Brazil). The stock culture was maintained in Petri plates containing potato dextrose agar (PDA) at 5 °C (Cho et al., 2002).

3.2.5.2 Fermentation assays with commercial glucose-based medium

Fermentation assays to evaluate the effect of the surfactant optimized formulation on red pigment production by *M. ruber* Tieghem IOC 2225 were performed in 125-mL Erlenmeyer flasks containing 60 mL of glucose-based medium composed of ($g.L^{-1}$): glucose 20, yeast extract 2.5, malt extract 2.5, peptone 2.5, K_2HPO_4 5, $CaCl_2 \cdot 2H_2O$ 0.1, $MgSO_4 \cdot 7H_2O$ 0.5, $FeSO_4 \cdot 7H_2O$ 0.01, $ZnSO_4 \cdot 7H_2O$ 0.01 and $MnSO_4 \cdot 7H_2O$ 0.03. The initial pH of the medium was adjusted at 5.5 (Hilares *et al*., 2018). For the inoculum, 2 mycelial agar discs (0,00252 g) for each 60 mL of medium were punched out with a sterilized self-designed cutter (8 mm) from a 7–10 days old culture. Samples were periodically taken to monitor substrate consumption, surface tension and biopigments production. Final fungal cell biomass was collected for scanning electron microscope analysis.

3.2.5.3 Fermentation assays using pretreated sugarcane bagasse: SSSF and SHF strategies

Fermentation strategy was carried out by separated hydrolysis and fermentation (SHF) and by semi-simultaneous saccharification and fermentation (SSSF).

3.2.5.3.1 Separated hydrolysis and fermentation

SHF strategy was performed in 125 mL Erlenmeyer flasks with 60 mL of work volume. The hydrolysis stage was performed with 10% of total solids in 50 mM sodium citrate buffer pH 4.8, at 50º C and 200 rpm for 48 hours. The optimized surfactant formulation was used, and the enzyme dosage used was 10 FPU/g of pretreated sugarcane bagasse. After hydrolysis, the residual biomass was separated by centrifugation at 4000 rpm

(2860 *g*) and the enzymatic hydrolysate was supplemented the same nutrients added to the semi-synthetic medium and inoculated with *Monascus ruber* mycelium (medium and inoculum described in section 3.2.5.2). Fermentation was performed for 10 days.

3.2.5.3.2 Semi-simultaneous Saccharification and Fermentation

For the SSSF strategy, 125 mL Erlenmeyer flasks were used with 60 mL of work volume, loaded with 50 mM sodium citrate buffer pH 4.8, optimized surfactant formulation, and the enzyme dosage used was 10 FPU/g of pretreated sugarcane bagasse. In the pre-hydrolysis phase, the medium temperature was maintained at 50 °C. After 48 hours, the medium temperature was adjusted to 37 °C and maintained at during the following SSF phase; all flasks were supplemented with the same nutrients added to the semi-synthetic medium and inoculated with *Monascus ruber* mycelium (medium and inoculum described in section 3.2.5.2). During all the process, the agitation rate was maintained in 200 rpm. After inoculation of the fungus, the process was carried out for 16 days.

For both, SHF and SSSF, samples were periodically taken to analyze sugars concentration, biopigment production, and surface tension.

3.2.6 Analytical methods

3.2.6.1 Total reducing sugars and monomeric sugar quantification.

Total reducing sugars (TRS) were analyzed by colorimetric 3,5-dinitrosalicylic acid (DNS) method (Miller et al., 1959). Glucose and xylose concentrations were analyzed by High Performance Liquid Chromatography (HPLC) Agilent 1200 series (Agilent Technologies, Inc., USA) equipped with a Refractive index detector RID-6A and HPX-87H (300x7.8mm) column (Bio-Rad, USA). Conditions used in the analysis were as following: 45°C column temperature, 0.01N H_2SO_4 as the mobile phase, 0.6 mL/min flow rate, and 20 µL injection volume (Ahmed *et al*., 2017).

3.2.6.2 Analysis of extracellular red biopigment production

Samples taken from the fermentation medium, were centrifuged at 10,000×rpm (6,738 *g*) for 10 min to separate the cells from the supernatant containing the biopigments.

The concentration of the biopigments in the liquid fraction after biomass removal was estimated by the measurement of absorbance at 510 nm for red color pigments using an Eppendorf biospectrophotometer (Eppendorf AG, Germany), and the result was multiplied by the respective dilution factor (Vendruscolo *et al*., 2016).

3.2.6.2.1 Analysis of sugarcane bagasse adsorbed red biopigment production in SSSF strategy

After fermentation assays, residual sugarcane bagasse was collected under vacuum on Whatman No. 1 paper using Büchner funnel. Pigmented sugarcane biomass was soaked in 25 mL of a 70% (v/v) ethanol aqueous solution. Extraction was carried out on an orbital shaker Excella E24 (New Brunswick Scientific, CT, USA) at 200 rpm at 30 °C for 1 h. All process was repeated 4 times. In the liquid mixture obtained in each extraction procedure, the pigments were analyzed by spectrophotometry, as above described.

3.2.6.3 Analysis of the surface tension

To determine the surface tension (TS) of the samples, a Sensadyne QC6000 Tensiometer (Braseq, Ltda., SP, Brazil) will be used. The device was initially calibrated with water (high calibration) and ethanol (low calibration), and then the surface tension of the samples was assessed.

3.2.6.4 Fungal cell membrane analysis by SEM

After fermentation fungal biomass was harvest under vacuum on Whatman No. 1 paper using Büchner funnel. Any changes on the surface of the recuperated mycelium were evaluated using Scanning Electron Microscope HITACHI flex-SEM 1000 (Tokyo, Japan) with acceleration voltage of 15.0 kV. The samples with and without the surfactant optimized formulation were studied.

3.2.7 Statistical analysis

Data were analyzed using STATISTICA (StaSoft, Inc., Oklahoma, USA), and were presented as mean value ± standard deviation (SD). Means were tested for significant

differences through a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. In this study, the level of significance was settled at $p<0.05$.

3.3 Results and Discussion

3.3.1 Optimization of the Enzymatic hydrolysis assisted by surfactants

After the diluted acid hydrolysis, 60.5% of the hemicellulose fraction was removed, as a result, the studied cellulignin of sugarcane bagasse was composed by 47.7 ± 0.78% glucan, 9.47 ± 0.42% xylan, 31.7 ± 0.42% total lignin, and 3.32 ± 0.01% ashes. Extractives were found in negligible amounts.

The pretreated biomass was used to perform the enzymatic hydrolysis, conducted in Erlenmeyer flasks according to the CCRD (Table 6). The response was the yield of total reducing sugars after 24 hours of enzymatic hydrolysis (TRS-Y_{24}). The variability of the experiments in hydrolysis can be observed with data ranging from 30.0 to 47.8 % of TRS-Y_{24}, showing the influence of the different non-ionic surfactants studied in this experimental design.

Data of Table 6 shows that TRS yields from sugarcane bagasse enzymatic hydrolysis assisted by surfactants were affected by surfactant concentration, kind of surfactant, and relationship between them. For instance, it was observed higher yields when the surfactant formulation between Tween 20 and PEG400 was used, with values around 47% of TRS-Y_{24} (runs 1, 2 and 11).

For the response variable yield of total reducing sugars (TRS-Y_{24}), it was possible to obtain a satisfactory reduced quadratic model (Equation 3.1), which was significant ($p<0.05$), with no significant lack of fit ($p>0.1$) and with an R^2 value of 0.78 (Table 3.3). The model was reduced by excluding low significant coefficients.

Table 6. Results of CCRD carried out to optimize the non-ionic surfactants formulation to assist pretreated sugarcane bagasse enzymatic hydrolysis

Run	*Tween 20 (%)**	*Triton X-100 (%)**	*PEG400 (%)**	*TRS-Y_{24} (%)*
1	0.51 (-1)	0.51 (-1)	0.51 (-1)	46.3
2	2.00 (+1)	0.51 (-1)	0.51 (-1)	47.8
3	0.51 (-1)	2.00 (+1)	0.51 (-1)	30.2
4	2.00 (+1)	2.00 (+1)	0.51 (-1)	44.8
5	0.51 (-1)	0.51 (-1)	2.00 (+1)	40.8
6	2.00 (+1)	0.51 (-1)	2.00 (+1)	39.6
7	0.51 (-1)	2.00 (+1)	2.00 (+1)	30.0
8	2.00 (+1)	2.00 (+1)	2.00 (+1)	33.3

continue

concluded

9	0.00 (-α)	1.25 (0)	1.25 (0)	38.7
10	2.51 (+α)	1.25 (0)	1.25 (0)	37.8
11	1.25 (0)	0 (-α)	1.25 (0)	47.4
12	1.25 (0)	2.51 (+α)	1.25 (0)	40.7
13	1.25 (0)	1.25 (0)	0 (-α)	36.9
14	1.25 (0)	1.25 (0)	2.51 (+α)	36.5
15	1.25 (0)	1.25 (0)	1.25 (0)	41.8
16	1.25(0)	1.25 (0)	1.25 (0)	43.1
17	1.25 (0)	1.25 (0)	1.25 (0)	38.7

*Coded valued in parenthesis; TRS-Y_{24} (Total reducing sugars yield after 24 hours); PEG400 (Poli-ethylene glycol 400); α value: 1.68

Table 7. Analysis of variance (ANOVA) for the adjusted quadratic model for pretreated sugarcane bagasse enzymatic hydrolysis assisted by non-ionic surfactants.

Source	Sum of squares	Difference	Degree of Freedom	F-Value	*p*-Value
Model	358.31	7	51.19	4.50	0.0202
A-Tween 20	20.03	1	20.03	1.76	0.2171
B-Triton X100	165.72	1	165.72	14.58	0.0041
C-PEG	49.52	1	49.52	4.36	0.0665
AB	38.94	1	38.94	3.43	0.0972
AC	23.84	1	23.84	2.10	0.1814
A^2	24.83	1	24.83	2.19	0.1735
C^2	48.06	1	48.06	4.23	0.0699
Residual	102.29	9	67.98		
Lack of fit	92.33	7	124.33	4.83	0.3013
Pure error	9.96	2	25.72		
Total	460.60	16			
$R^2 = 0.78$					

Equation 3.1. $TRS\text{-}Y_{24}(\%) = 41.19 + 6.94272\ A - 9.65246\ B + 10.24731\ C + 3.96955\ AB - 3.10621\ AC - 2.55139\ A^2 - 3.54916\ C^2$

Where: Y_{24} is the response variable "total reducing sugars yield after 24 hours", and A, B, and C correspond to the actual values of independent variables "concentration of: Tween 20, Triton X-100, and PEG" respectively.

The adjusted model was used to compose response surface graphs, as shown in Figure 4 (a, b, c). As can be seem, when a high concentration of Triton X-100 (2.5% m/v) was used, a high concentration of Tween 20 is required to achieve a maximum yield of about 45% (Figure 4-c, 4-f). However, the absence of Triton X-100 (Figure 4-a, 4-d) allows a positive synergism between Tween 20 and PEG400, maximizing the yield up to about 50% in the conversion of total reducing sugars, using concentrations below the center point level used in the design (1.25%) for both surfactants.

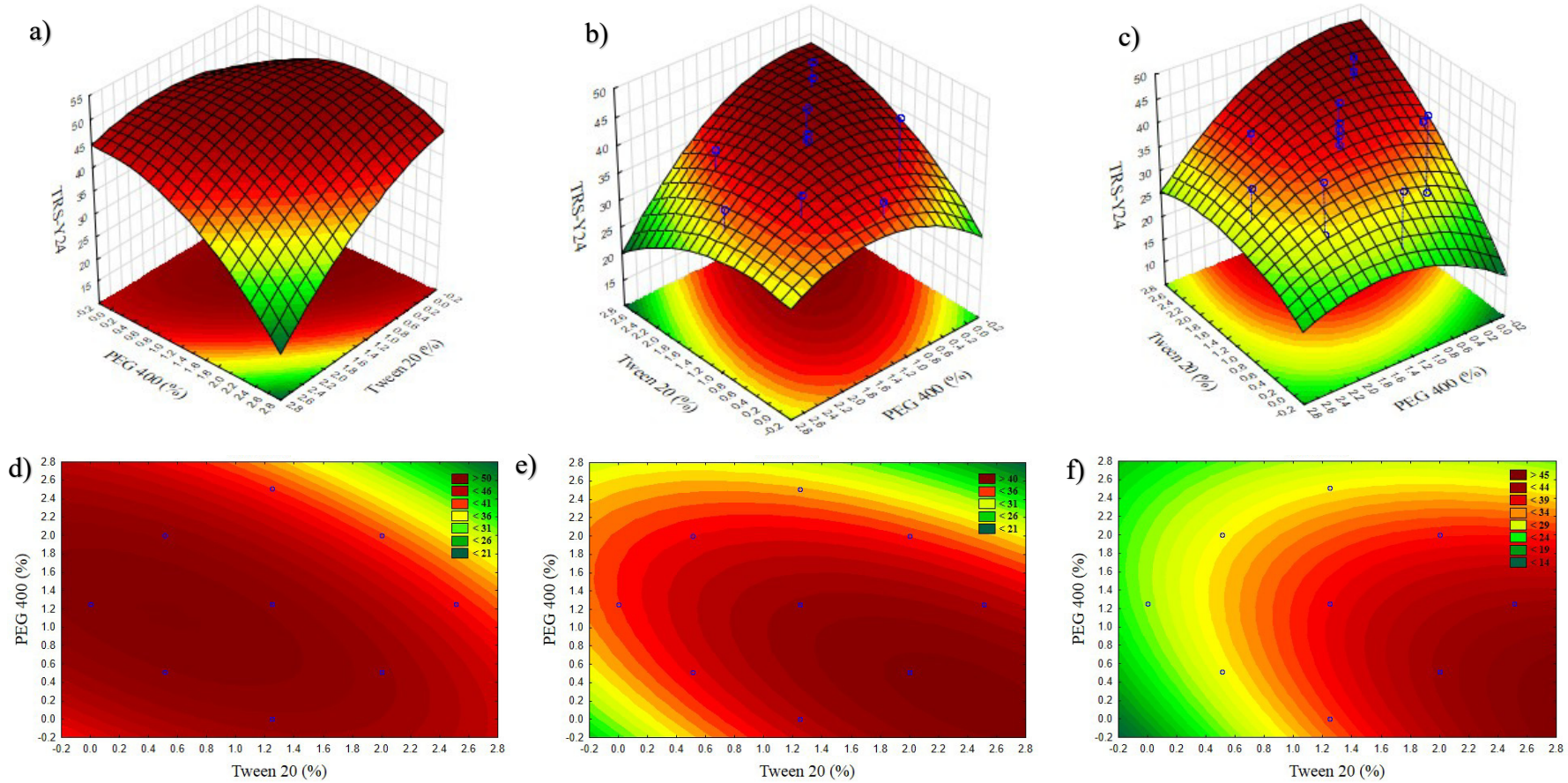

Figure 4. Response surfaces (a, b, c) and contour plots (d, e, f) for the enzymatic hydrolysis yield of the carbohydrate fractions of sugarcane bagasse pretreated with dilute H2SO4, in function of the variables: concentration of PEG and Tween 20. Triton X-100 Concentration: 0% (a, d); 1.25% (b, e); 2.5% (c, f).

The surfactant compounds used in this study consist of a mixture of non-ionic (Tween-20) and polymeric (PEG 400) surfactants. There are reports that non-ionic surfactants, such as Tween 20, are used in the extraction of hydrophobic degradation products (Kurakake *et al*., 1994). In addition, these compounds can modify the cell walls of the biomass, promoting pore formation for greater accessibility of the sugar polymers by the enzyme (Seo *et al*., 2011; Agrawal *et al*., 2017). On the other hand, polymers, such as PEG, are a precursor of non-ionic surfactants and used to modify interfaces in biological systems. This polymer performs PEG-lignin interactions to form hydrogen bonds or hydrophobic interactions, promoting the blocking of non-productive adsorption of the enzyme with lignin (Kristensen *et al*., 2007; Araújo *et al*., 2007). Furthermore, studies suggest that due to the high molecular mass of these compounds, they improve cellulase mobility over biomass (Kristensen *et al*., 2007) and catalytic activity (Sánchez-Trasviña *et al*., 2015; Nogueira *et al*., 2022). Therefore, considering the yield and cost of the process, it is advantageous to use surfactants during the saccharification steps to reduce the amount of enzyme used.

By using the numerical optimization tool of the Design-Expert software and placing as criteria the maximization of the yield of total reducing sugars, the following conditions were obtained: concentration of Tween 20 of 0.66% (v/v) and 1.16% (v/v) of PEG400 (this solution obtained for maximization does not include the addition of Triton X-100).

The model predicted a 49.39% yield in the release of TRS-Y_{24} with a confidence interval (95% CI) ranging from 43.48% to 55.31%. Aiming at confirming the model obtained for the TRS-Y_{24}, a new experiment was performed in the optimized condition, obtaining an TRS-Y_{24} of 43.85 ± 1.34 (mean ± standard deviation), this result within the values of confidence interval (95% CI), thus confirming the adjusted model. The optimized conditions were then employed in the sequence of experiments, as reported in the following section.

3.3.1.1 Analysis of enzyme loading effect on pretreated sugarcane bagasse hydrolysis

Considering the data obtained in the CCRD for the optimal condition of the formulations with surfactants (Tween 20 of 0.66 % (m/v) and 1.16 % (m/v) of PEG400), an evaluation of the effect of the enzyme loading in the presence of surfactant in the saccharification process was studied.

Thus, experiments were carried out with different initial load of enzyme (2.5, 5, and 10 FPU/g biomass) in Erlenmeyer flasks (Figure 5).

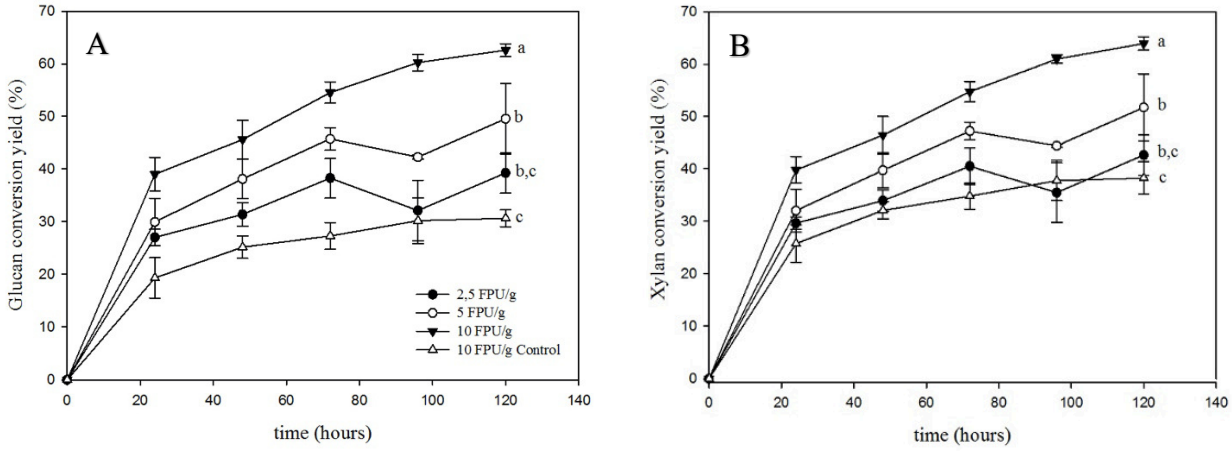

Figure 5. Effect the surfactant optimized formulation at different enzymes loadings on pretreated sugarcane bagasse hydrolysis. A- Glucan conversion yield; B- Xylan conversion yield. Letters indicate significant differences, according to Tukey's test ($p<0.05$).

Considering the data obtained, better results corresponded to yields for glucose and xylose, respectively, of 62.5% and 63.9%, in 120 h, using loading of 10 FPU/g and the surfactant optimized formulation. It is suggested that the surfactant addition reduces the unproductive adsorption of the cellulase enzyme, thus promoting the greater conversion of the cellulose polymer into fermentable sugars (Okino *et al*., 2013; Agrawal *et al*., 2017). Also, for both, glucose and xylose, there was a significant difference, when optimized formulation is used, between experiments using enzyme loading of 10 and 5 FPU/g, with results also statistically different when compared to the control assay (10 FPU/g, without surfactants addition). No significant difference was observed between experiments with 5 and 2.5 FPU/g of biomass. Furthermore, it is noteworthy that, in this study, it was observed the yield of sugars released (Glucose and xylose) using a lower enzyme dosage (2.5 FPU) combined with the surfactant formulation was not significantly different from the yield obtained by the control (10 FPU/g without surfactant).

The enzyme loading commonly used to obtain high hydrolysis yields ranges from 10 – 20 FPU/g of biomass (Gregg and Saddler, 1996; Balat, 2011). In this study, it was possible to show that the addition of surfactants promoted a greater release of sugars during the saccharification process using surfactants and low loadings of enzymes, with 2.5 FPU/g of biomass of enzyme loading in the presence of the optimized surfactant formulation resulting in similar statistical results to the obtained when 10 FPU/g of biomass without surfactant were used.

3.3.1.2 Evaluation of the thermostability and the effect of shear force stress on hydrolysis yield

Enzymatic hydrolysis is affected at high agitation and temperature due to the easy deactivation or denaturation of enzymes (e.g., cellulases), leading to the reduced speed of enzymatic reaction, and the final sugar released concentration is affected as a consequence (Lou *et al*., 2018; Guo *et al*., 2015; Taneda *et al*., 2012; Han *et al*., 2012). Taking these phenomena into account, shear force stress and thermostability of enzymes were analyzed using the chosen surfactant formulation described in previous sections. Shear force stress was studied in a bench-scale stirred tank reactor at 200 rpm, a stirring already considered as high or excessively high by some authors for cellulases (Lou *et al*., 2018; Guo *et al*., 2015). Also, an even higher stirring of 800 rpm in presence of surfactant was used to test enzymatic protection by surfactants during hydrolysis. Indeed, agitation higher than 100 rpm has been reported as significantly negative to enzymatic hydrolysis efficiency and as an impediment to reduce enzyme loading, especially for cellulases (Lou *et al*., 2018; Guo *et al*., 2015; Taneda *et al*., 2012).

At 200 rpm in control conditions (without surfactants), the assays resulted in glucose and xylose conversion yield of 42.7 and 48.6 %, respectively, lower values compared to those ones obtained in assays with surfactants, which resulted in conversion yields of 55.4 and 60.2 % for glucose and xylose, respectively. This behavior agrees with the reported by Lou et al. (2018), which showed an increase of 50% in enzyme activity by the addition of non-ionic surfactants at 200 rpm.

The results obtained in the present work also indicate that surfactants improved enzyme stability and decreased shear force stress effects at higher agitation, even at extremely high agitation (800 rpm). Figure 6 shows no significant difference between 200 rpm and 800 rpm on the sugar conversion yield using the optimized surfactant formulation, for xylose and glucose release. It has been proposed that non-ionic surfactants as PEG and Tween can reduce the exposition of enzymes to air-liquid interface and, as consequence, enhance enzymatic hydrolysis by reducing enzymatic deactivation by shear force (Lou *et al*., 2018).

For thermostability analysis, saccharification was performed in Erlenmeyer flasks at temperatures of 50, 60 and 70 °C. Stability of enzymes was affected above 60 °C (Fig. 6B), as a consequence, sugar conversion yield decreased for both glucose and xylose at 70 °C.

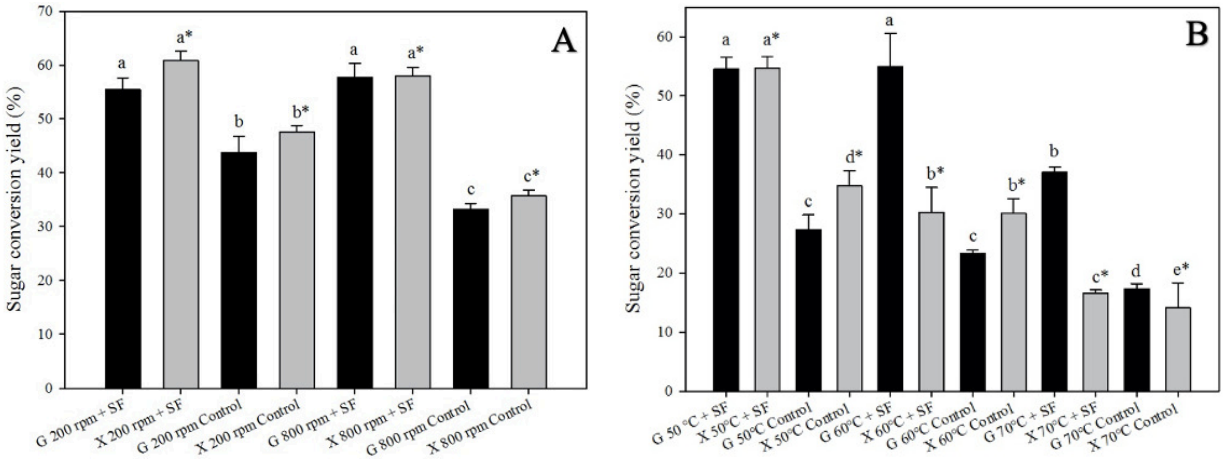

Figure 6. Effect of shear force (A) and temperature (B) on sugar conversion yield of pretreated sugarcane bagasse when the optimized surfactant formulation is used as additive. Glucose (G) conversions in black bars and xylose (X) in grey bars. SF indicates the runs with addition of optimized surfactants formulation. Values were represented as average ± standard deviation. Different letters indicate significant differences according to Tukey's test ($p<0.05$), with the xylan conversion yields labeled with *.

Eriksson et al. (2002) reported a weak effect of surfactants on temperature stability of cellulases. Nevertheless, in the present work, results showed that, when the optimized surfactant formulation was added to the reaction, sugar conversion yield was higher than the control at all temperatures tested. As shown in Fig. 6B, glucose conversion yield was maintained higher than 50% in 50 and 60 °C, respectively. Further, in 70 °C, the sugar conversion remained higher than 35%, which exceeds the saccharification rate obtained in control conditions (50 °C, without surfactants). In the case of xylose released, it was found that temperature increase affected sugar conversion even in surfactant presence, even though surfactants favored a higher xylose release in 50 and 70 °C.

3.3.1.3 High solids effect in different enzymatic hydrolysis configurations

The glucose conversion yield was evaluated during fed-batch enzymatic hydrolysis with gradual solids addition, concomitant with enzyme and/or surfactants addition (Figure 7).

High solids enzymatic hydrolysis can benefit the economic aspects of converting lignocellulosic biomass processes into bioproducts by reducing operating costs (da Silva *et al*., 2020). However, some technical difficulties are accentuated due to the use of high solids loadings, such as the increase of non-productive link between enzyme-lignin due to the higher amount of lignin which results in inefficient enzymatic action, and a significant

reduction in the water content that hinders homogenization of the system at the beginning of the process and avoiding an adequate transfer of mass and heat (Kadhum *et al*., 2019; Xu *et al*., 2019). Enzymatic hydrolysis mediated by surfactants is improved by different mechanisms, such as decreasing the viscosity of the reaction as a result of the surface tension reduction. Moreover, surfactants avoid unproductive enzyme adsorption acting as competitors for enzymes by binding to lignin, increasing enzyme availability. Both effects impact high solids loading hydrolysis, resulting in better mass transfer, higher productivity, and economic benefits (Gabelle *et al*., 2012; Muñoz *et al*., 2022).

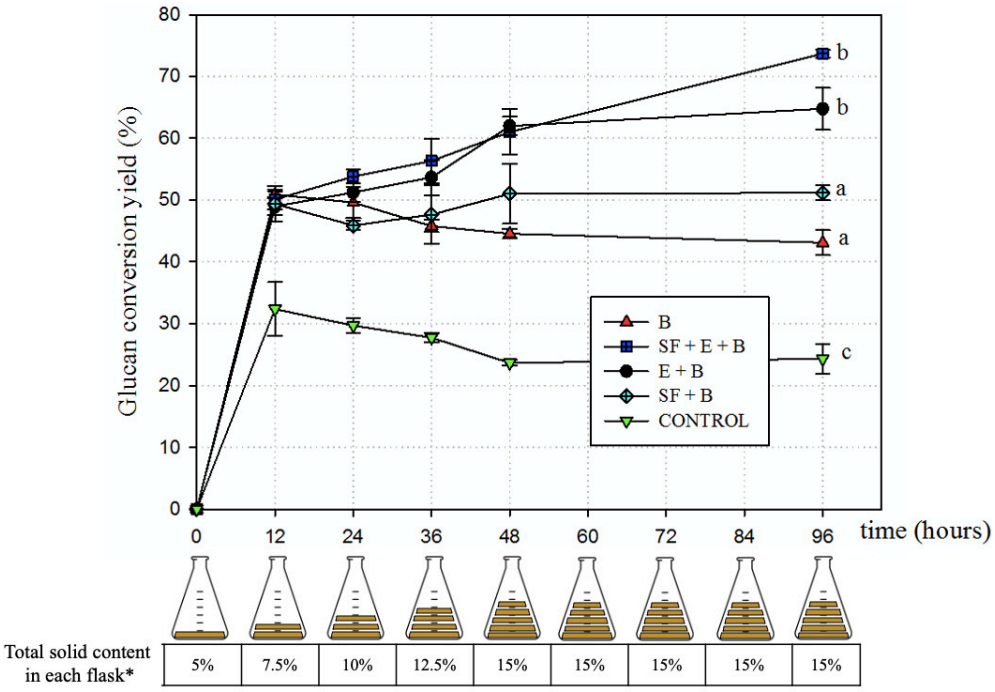

Figure 7. Glucose conversion yield from fed-batch enzymatic hydrolysis with gradual solids addition, concomitant with enzyme and/or surfactants addition. At 0h of process, all experiments were added with enzymes (10 FPU/g) and biomass; besides, except for control, all experiments were also added with optimized surfactants formulation. SF, B and E indicates experiments added, respectively, with optimized surfactants formulation, biomass, and Enzyme (addition of 10 FPU/g of loaded solids), at 12h, 24h, 36h, and 48h. Different letters indicate significant differences according to Tukey's test ($p<0.05$). *Each bar in the flask represents the loaded biomass, starting with 5% of total solids at 0 hours with an increment of 2.5% at each 12 hours, up to 48 hours (15% of total solids loaded).

As can be observed in Figure 7, after 96h of enzymatic hydrolysis with 15% of total solids (adding enzyme, biomass, and surfactant at 12, 24, 36, and 48h - SF+E+B), it was possible to obtain a glucose conversion yield from bagasse cellulignin of approximately

73.7%. It is noteworthy that the gradual addition of surfactant, enzyme, and biomass during enzymatic hydrolysis resulted in an increase of around 1.7 folds more in the glucose conversion yield compared to the enzymatic hydrolysis performed with the addition of surfactant only at the beginning of the process and biomass throughout the process. Besides, this strategy of adding surfactant, enzyme, and biomass during enzymatic hydrolysis also increases 3.04 folds the glucose conversion yield when compared to the hydrolysis performed without the addition of surfactant, only with the addition of biomass throughout the process (control). In agreement with these results, other studies also demonstrated the high solids enzymatic hydrolysis improvement by adding surfactants. For example, the conversion yield of Avicel at high solids loading enzymatic hydrolysis (15% w/v glucan) with low enzyme concentration (approximately 2.5 FPU/g glucans) doubled when added Tween 20 (Bhagia et al., 2018). Cheng *et al*. (2020) also confirm the efficiency of adding surfactants to improve enzymatic hydrolysis of hydrothermal pretreated sorghum, showing that it was possible to increase glucose and xylose yields by about 10% by adding a dosage of 2% PEG 4000 to the reaction with 50% solids loading from hydrolysis without the addition of surfactant. In addition, they also observed that fed-batch enzymatic hydrolysis added with surfactant improved mass transfer and increased final sugar yields by 14% (64.89% glucose yield and 64.66% xylose sugar yield) from batch hydrolysis.

3.3.2 Effect of surfactant formulation on biopigment production and morphological cell wall structural changes in semi-synthetic medium

Fermentation was performed in semi-synthetic based medium, as described in section 3.2.5.2 The kinetic of biopigments production was evaluated in the presence of the optimized surfactants formulation, with control runs with each of non-ionic surfactant in separate.

Non-ionic surfactants can lead to the cell wall structure modification and reorganization. This modification in the cell surface can be a consequence of the extraction or solubilization of membrane or cell wall elements, which is shown in the structure and shape change (Manaargadoo-Catin *et al*., 2016; Morales-Oyervides, *et al*., 2017; Yang *et al*., 2018). These changes in the structure can be noticed in the form of cavities or pores that can connect the intracellular with the extracellular medium (Nalini & Parthasarathi 2014). Consequently, all the cell surface modifications can lead to a product exportation to the culture medium (Yang *et al*., 2019; Wang *et al*., 2020).

Figure 8-B shows morphological changes in the cell surface of the fungus *M. ruber* grown in semi-synthetic medium with the surfactant optimized formulation (Tween 20: PEG). It can be observed a modification of the surface texture (roughness) of the mycellium and the formation of microcavities when compared to control without surfactants (Figure 8-A). These structure modifications could be enhancing the extraction of red pigments and the increase of their presence in the culture medium, as reported by Yang et al. (2019).

Also, Figure 8-C shows the biopigments production kinetics in semi-synthetic medium with and without the surfactant optimized formulation. As expected, the results shown an increment of around 5 folds in the release of red pigment (~10UA_{510nm}/mL) when the formulation was used in the fermentation process. Other non-ionic surfactants, such as, Triton X-100, Span 40-80, Tween 20-80 have been reported by Wang et al. (2013) as additives with an effect to enhance the production of biopigments, but in *Monascus purpureus*.

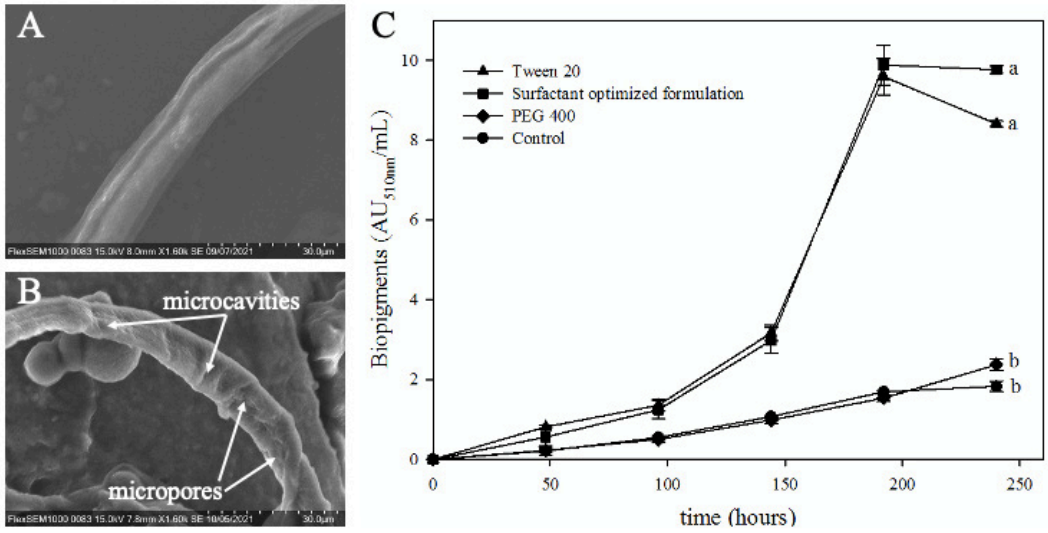

Figure 8. Effect of optimized surfactant formulation on biopigments production and mycelium morphology after 10 days of fermentation. SEM images: (A) without surfactants, (B) with surfactant optimized formulation, (C) Kinetics of the surfactant optimized formulation and each non-ionic surfactant, in separate, on biopigment production. Different letters indicate significant differences according to Tukey's test ($p<0.05$).

As can be observed in Figure 8-C, the optimized formulation of surfactants can have its advantageous effect in the production of biopigments by *M. ruber* attributed mainly to the presence of tween 20, without significant difference compared to the fermentation with this surfactant alone. Also, when alone, PEG 400 was not beneficial to the process, although

its effect together with Tween 20 was not negative. Anyway, as previously discussed, PEG 400 is important in the formulation for the enzymatic hydrolysis of the biomass.

3.3.3 Effect of surfactant formulation optimized condition in SHF and SSSF process strategies

The integration of consecutives operational stages in a process by incorporating the key steps all-in one, and the use of additives in the early steps of the process that may be remaining in subsequent steps such as fermentation, were studied in this work. Different strategies for enzymatic hydrolysis and fermentation (SHF and SSSF) were used to evaluate the production of biopigments from the pretreated sugarcane bagasse by *Monascus ruber* when the optimized surfactant formulation was added to the process. The production of pigments, substrate consumption, and surface tension were evaluated during SHF and SSSF processes.

As shown in Figure 9-B, in the SHF process, around 10 UA_{510nm}/mL of extracellular biopigments were obtained after 240 h of fermentation. The sugar consumption achieved was 73.4 and 76.7% for glucose and xylose, respectively. No reports were found in literature using sugarcane bagasse and surfactants to produce biopigments with *Monascus ruber*, thus turning difficult to compare the obtained values with other works. Anyway, it is important to note the obtained biopigments production was similar to the above reported for semi-synthetic medium using the optimized surfactant formulation (Figure 8-C).

For the SSSF strategy, the graph (Figure 9-A) is separated in three stages: I - pre-hydrolysis, II – beginning of the fermentation (after fungus inoculation), III - biopigments production. In contrast to the SHF strategy, during SSSF (Figure 9-A) the consumption of sugars (glucose and xylose) and the extracellular pigment production (around 1.5 AU_{510nm}/mL) was lower. However, after 12 days of fermentation, it was observed that the residual sugarcane bagasse in the medium began to be pigmented (Figure 9-C). Then, after fermentation, the bagasse was recuperated by filtration and the absorbed biopigments were extracted with 70% of ethanol. As shown in Figure 9-A, after the first extraction, 9.33 ± 1.49 AU_{510nm}/mL was obtained in the liquid mixture (a value similar to the obtained in SHF). After second, third, and forth extractions, those values were 4.6 ± 0.80 AU_{510nm}/mL, 1.22 ± 0.95 AU_{510nm}/mL, and 1.19 ± 0.68 AU_{510nm}/mL, respectively. This phenomenon in SSSF strategy could be due to the nature of the *Monascus* species that normally grow fixed to a

matrix, such as in solid state fermentation. As described, most of the pigments were adsorbed by the sugarcane biomass present in the SSSF process in a late stage of the fermentation.

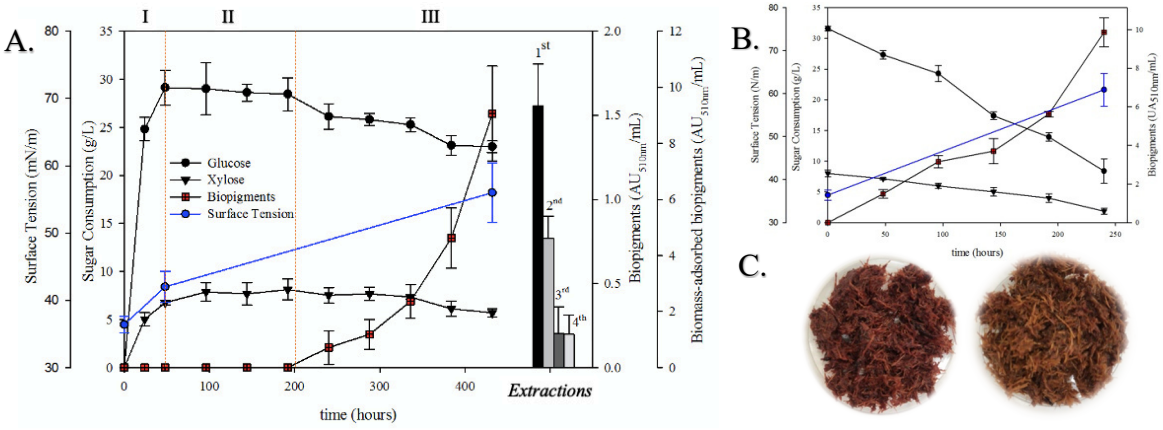

Figure 9. Fermentative strategies used to sequentially enhance enzymatic hydrolysis and production of biopigments. A. Semi-simultaneous Saccharification and Fermentation; B. Separated Hydrolysis and Fermentation. C. Biomass-absorbed biopigments before and after four washes with ethanol 70%.

For both strategies, the surface tension was analyzed at the beginning and at the end of the fermentation. In all cases, the values increased along the process, which could be an indicative of reduction of the quantity of surfactants in the medium. This could be due to the adsorption of the non-ionic surfactants onto the lignin during the hydrolysis stage thus reducing the non-productive adsorption of the enzymes (Chen *et al*., 2018). Besides, during fermentation different mechanisms could explain the consumption of the surfactants and the enhancement of the biopigments production, such as the possible partial or total absorption and incorporation of surfactant molecules into cell surface, reorganizing and removing membrane proteins and lipids (Manaargadoo-Catin *et al*., 2016; Wu *et al*., 2017). All before described could increase the porosity by trans-membrane or membrane pores formation, causing a facility transportation of ions, water, and important products as biopigments (King *et al*., 1991; Nalini and Parthasarathi, 2014; Manaargadoo-Catin *et al*., 2016).

3.4 Conclusions

The optimized surfactants formulation showed its potential in reducing the enzyme dosage, enhancing the enzyme stability at high shear force stress and temperature, and increasing the sugar conversion yield in high solids loading of a cellulignin with high lignin

content. Additionally, the optimized surfactant formulation showed positive effect in the production of biopigments from pretreated sugarcane bagasse in SHF and SSSF processes. The obtained results confirmed that surfactants are key additives in the bioprocessing of high lignin content lignocellulosic biomass for biopigments production using SHF and SSSF processes, which are interesting alternatives that can be incorporated in a biorefinery concept.

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CHAPTER IV

Sugarcane by-products hemicellulosic hydrolysates as potential source for biopigment production by *Monascus ruber* assisted by a non-ionic surfactant formulation

ABSTRACT

Sugarcane hemicellulosic hydrolysates by-products rich in C5 carbon sugars demonstrated their potential to be used as a source to produce biopigments by *Monascus ruber*. The influence of a surfactant optimized formulation (SOF), composed by Triton X-100 and Tween 80 enhanced the production of biopigments produced from sugarcane bagasse and straw hemicellulosic hydrolysates (SCBHH and SCSHH) by 4-fold (18.81 and 20.65 AU_{510nm}/mL, respectively) when compared to the control without these additives. The produced pigments derived from SCBHH and SCSHH supplemented with the SOF also showed high thermal stability (7.213 and 14.76 $Kcal.mol^{-1}$, respectively), and it is also highlighted the absence of citrinin derived from the fermentation. Thus, turning these pigments an interesting option for biorefineries, and the feasibility to apply these high valued-added products in several areas.

Keywords: Non-ionic surfactants. Formulation. Hemicellulosic Hydrolysate. *Monascus*-pigments. Thermal-stability

4.1 Introduction

The concerns about climate change and the scarcity of petroleum-based raw materials have driven researchers to seek alternative ways to produce the chemicals and materials required by society. The application of lignocellulosic biomass (LCB) as raw materials in the production of fuels, chemicals, materials, and energy is an attractive alternative source to petroleum utilization, duo to their composition and large availability worldwide (Hingsamer & Jungmeier, 2019). LCB are mainly composed by cellulose, hemicellulose, and lignin, which can be converted into several value-added products through biochemical and thermochemical processes (Menon & Rao, 2012). Besides, the utilization of these low-cost feedstocks can reduce environment pollution and can also make bioprocesses cost effective (Panesar et al., 2015).

Sugarcane bagasse and straw are LCB generated during sugarcane processing. While bagasse is mainly incinerated to coproduce electric and thermal energy, straw has been destined to soil nutrition improvement (Carvalho et al., 2017; Varanda et al., 2019). Considering the abundance of these by-products generated every year, part of them can be destined to the production of value-added products in a biorefinery context, thus contributing to the valorization of these feedstocks and allowing the expansion/diversification of sugarcane agro-industry product portfolio (Bozell, 2008; Lago et al., 2012).

The microbial conversion of cellulose and hemicellulose to different bioproducts is a route commonly performed to value LCB. In the case of C5 hemicellulosic sugars, which can represent about one third of the total mass of sugarcane bagasse and straw (Kumar et al., 2021). By that, researchers have reported the production of a large variety of products, such as xylitol, ethanol, 2,3-butanediol, poly-hydroxy butyric acid, organic acids, single cell protein, and so on (Chandel et al., 2018). Biopigments production by microorganisms has received increasing attention due to the need to replace synthetic pigments commonly used in food industry, as many of these synthetic molecules are considered as potential colon carcinogens and can also be associated to allergy and attention-deficit/hyperactivity disorders in children (Hilares et al., 2018). Different types of biopigments can be produced by bacteria, yeasts, and fungi depending upon their substrate.

Species of the fungal genus *Monascus*, like *Monascus ruber*, produce azaphilone pigments with three color variants, yellow, orange, and red, that are used for centuries in the eastern culture as food colorants (Silveira et al., 2013). Recently, production of biopigments from agro-industrial by-products has gained attention as these raw materials are cheap

substrates that can increase the cost-attractiveness of the bioprocess (Hilares et al., 2018; Panesar et al., 2015; Silveira et al., 2013).

Solid-state fermentation is usually performed to obtain pigments from *Monascus*, although submerged fermentations can also be used (Silveira et al., 2013). In both scenarios, the addition of non-ionic surfactants to fermentation medium can be performed to improve pigments production. Some of these surfactants are biocompatible and their addition in fermentation medium can favor both growth and final pigment concentration (Hu et al., 2012). This pigment production improvement is a result from shift in the equilibrium between intracellular pigment formation, degradation, and exportation to extracellular broth by the addition of non-ionic surfactants (Chen, 2007). In this case, intracellular pigment is easily exported to extracellular medium and can be extracted from the surfactant micelles (Hu et al., 2012). Henceforth, the production of intracellular *Monascus* pigments can be performed as an extracellular production. Besides, non-ionic surfactants addition to broth facilitates pigment recovery, improving downstream processing (Hu et al., 2012).

In spite of the above-described potential advantages, there are no previous reports in literature regarding the production of biopigments from *Monascus* in hemicellulosic hydrolysates, mainly considering the use of *Monascus ruber* to produce biopigments from sugarcane by-products in surfactant-assisted processes. Thus, this work dealt with the evaluation of the potential of two C5 rich carbon hydrolysates obtained from sugarcane by-products to produce *Monascus* pigments. A surfactant-optimized formulation to enhance *Monascus* pigments production and stability was obtained, and it was used to assist the fermentative process.

4.2 Materials and Methods

4.2.1 Microorganism

Monascus ruber Tieghem IOC 2225 was kindly donated by the Culture Collection of Filamentous Fungi (CCFF) – Oswaldo Cruz Foundation (IOC/FIOCRUZ) (Rio de Janeiro, Brazil). The stock culture was maintained in Petri plates containing potato dextrose agar (PDA) at 5 °C (Cho et al., 2002).

4.2.2 Effect of the addition of different non-ionic surfactants on the production of pigments by Monascus ruber

4.2.2.1 Fermentation assays with commercial xylose-based medium to produce biopigments

Fermentation assays to evaluate the effect Tween 20, Tween 80, PEG400, and Triton X100 in two different concentrations (10 and 20 g/L) on biopigment production by *M. ruber* Tieghem IOC 2225 were performed in 125-mL Erlenmeyer flasks containing 60 mL of xilose-based medium composed of ($g.L^{-1}$): xylose 20, yeast extract 2.5, malt extract 2.5, peptone 2.5, K_2HPO_4 5, $CaCl_2 \cdot 2H_2O$ 0.1, $MgSO_4 \cdot 7H_2O$ 0.5, $FeSO_4 \cdot 7H_2O$ 0.01, $ZnSO_4 \cdot 7H_2O$ 0.01 and $MnSO_4 \cdot 7H_2O$ 0.03. The initial pH of the medium was adjusted at 5.5 (Hilares et al., 2018). For the inoculum, 2 mycelial agar discs (total dry mass of 0,00252 g) for each 60 mL of medium were punched out with a sterilized self-designed cutter (8 mm) from a 7–10 days old stock culture. Samples were periodically taken to monitor biopigments production. Control experiments were performed without the addition of surfactants.

4.2.2.2 Synergic effect of non-ionic surfactants with Triton X100 on the fermentation of commercial xylose based medium to produce biopigments

After the individually evaluation of the effect of each non-ionic surfactant, a synergic study between Triton X100 in blend with other non-ionic surfactants were performed. The mixtures were composed by 10 g/L of Triton X100 and 10 g/L of each non-ionic surfactant (Tween 20, Tween 80, and PEG400). All fermentative conditions and medium were used as described in the section 4.2.2.1.

4.2.2.3 Experimental design for the optimization of the biopigment production assisted by non-ionic surfactants

According to the results obtained in the previous sections, a Central Composite Rotatable Design (CCRD) was used to optimize a non-ionic surfactant formulation to enhance the production of biopigments from xylose-based medium. Design Expert 7.0 (Stat-Ease, MN, USA), was used to design experiments, analyze experimental results, and to identify optimum conditions. Initially, a CCRD was created from a factorial design augmented with center and star points. The CCRD for this experiment included a total of 11 experimental runs with triplicate at the center point. 11 sample runs were carried out

according to CCDR based on varying concentrations (0-30 g/L) of each of the two non-ionic surfactants: Tween 80 and Triton X-100. Response surface graphs were plotted using STATISTICA (StaSoft, Inc., OK, USA). Answer variable was the biopigment production analyzed at 510nm.

4.2.3 Surfactant-assisted production of pigments by Monascus ruber from hemicellulosic hydrolysates of sugarcane bagasse and straw

4.2.3.1 Lignocellulosic Biomass

Sugarcane bagasse (SCB) and straw (SCS) were kindly donated by Ipiranga Agroindustrial (Descalvado, São Paulo, Brazil). When received, the biomass has initially allowed to sun-dry for 2 days until about 10% of humidity. It was then ground using a knife mill Marconi model MA 680 (Marconi Ltda., São Paulo, Brazil) and passed through a 2 mm sieve. Before use, SCB and SCS were screened again, using the fraction that passed through a standard US mesh No. 20 sieve.

4.2.3.2 Preparation of hemicellulosic hydrolysates and characterization of biomass

The sugarcane bagasse and straw were subjected, in separate, to dilute-acid hydrolysis with 1.0% (w/v) H_2SO_4 in a 350 L steel reactor at 121°C for 30 min at a 1:10 solid/liquid ratio to remove most of the hemicellulosic fraction, obtaining the hydrolysates, and separate them from the residual biomass by centrifugation. After the hydrolysis, the lignocellulosic-rich solid phase was neutralized with tap water and stored at 4 ºC prior chemical characterization.

4.2.3.2.1 Concentration and detoxification of the hemicellulosic hydrolysates

The hemicellulosic hydrolysates were concentrated (about 6 times) in a vacuum concentrator at 70°C (Rodrigues et al., 2003). The chemical characterization of hemicellulosic hydrolysates was performed by high performance liquid chromatography (HPLC) quantifying the concentrations of different components glucose, xylose, arabinose, furans (furfural, hydroxymethyl-furfural), and acetic acid.

The sugarcane bagasse and straw hemicellulosic hydrolysates of (SCBHH and SCSHH, respectively) were also submitted to a chemical detoxification process to remove

non-desire compounds that can inhibit microbial growth and to decrease the color of the hydrolysate, as proposed by Marton et al. (2006). Briefly, the pH of the hydrolysate was first raised to 7.0 with the gradual addition of sodium hydroxide (NaOH) microbeads, then, the pH was reduced to 2.5 with concentrated phosphoric acid (H_3PO_4). Subsequently, 1.0% (m/v) of activated carbon was added to the hydrolysate and incubated on a rotary shaker (100 rpm) at 60 °C for 30 min. Between the steps, the hydrolysate was filtered under vacuum, to finally be centrifuged at 3000 rpm (2560 *g*) for 15 min to remove activated carbon and finally sterilized (121 ºC for 15 min) for later use.

4.2.3.2.2 Biopigments production using the hemicellulosic hydrolysates of sugarcane bagasse and straw assisted by the surfactant optimized formulation

The fermentation of sugarcane bagasse and straw hemicellulosic hydrolysates was performed in 125 mL Erlenmeyer flasks, with a working volume of 60 mL in a rotary shaker set at 30 °C and 200 rpm. The inoculum preparation and medium supplementation was performed as described in section 4.2.2.1.

4.2.4. Evaluation of Pigments Stability

Pigments stability was evaluated following the method of Perumal et al. (2009) with modifications. First set of test tubes containing 1 mL of pigments (around 10AU/mL) were incubated in a ramp in which each temperature value (40, 50, 60, 70, 80, 90, and 100 °C) was kept for 10 minutes, using a hot water bath. Second set of test tubes containing 1 mL of pigments were adjusted to pH 1, 3, 5, 7, 9, 11, and 13 and incubated at room temperature (around 25ºC) for 24 hours. Third set of test tubes containing 2 mL of pigments were incubated in different light stress conditions (white light for 12 hours, sunlight for 2 h, and under UV light for 2 h). After each condition, the pigments were centrifugated (5 minutes at 10,000 rpm) and analyzed by spectrophotometry. The absorbance was measured using spectrophotometer and pigments stability, S (%), was calculated as described in Equation 4.1.

$$S\ (\%) = \{1-[(A_0-A)/A_0]\} \times 100 \qquad \text{(Eq. 4.1)}$$

where, A_0 is the absorbance before treatment, and A is for absorbance after different treatments. Absorbance was measured at 510, 470, and 430 nm to observe red, orange, and yellow pigments stability, respectively.

In the case of the pH effect, the color space indexes L* a* b* of the samples were measured using a colorimeter (Colormate, Scinco Co., LTD, São Paulo, Brazil). The colorimeter was calibrated with a standard white plate WHITE-100063 (Sinco Co. Ltd., São Paulo, Brazil) at the start of each batch of analysis.

4.2.4.1 Thermal stability of red pigment produced in SCBHH and SCSHH

After the first set of experiments to evaluate the stability of the pigments produced by *Monascus ruber* in SCBHH and SCSHH using the surfactant optimized formulation, a deeper experiment was performed to analyze the thermal stability of the red pigments produced in the hemicellulosic hydrolysates. The thermal stability was expressed in function of the thermal degradation constant (Dk, h^{-1}), and half-life time ($t_{1/2}$, h) of the red pigment produced in both hemicellulosic hydrolysates, and they were evaluated at different temperatures (60, 80, and 100 °C). For the experimental runs, the produced pigments were set to an optical density (510nm) of 1.0, expressed as 1 UA_{510nm}. The thermal treatment of the red pigment solution (2 mL) was performed in test tubes of 5 mL of total volume, which were incubated in a water bath during 2.5 h. Samples were taken every 30 minutes for pigment analysis at 510 nm.

The obtained results were used to determine the thermal degradation kinetics for the red pigment produced in SCBHH and SCSHH according to the model of Eq. 4.2, which can be integrated considering A_0 as the initial concentration of red pigment (AU_{510nm}), resulting in the Eq. 4.3:

$$\frac{dA}{dt} = -D_k \,.\, A \qquad \text{Eq. 4.2} \qquad\qquad \ln\left(\frac{A}{A_0}\right) = -D_k \,.\, t \qquad \text{Eq. 4.3}$$

where A is the concentration of pigment (AU_{510nm}) in a specific time (t), t is the incubation time (h), and D_k is the thermal degradation constant (h^{-1}).

Also, the half-life time was determined using the equation 4.4 as follows:

$$t_{1/2} = \frac{\ln 2}{D_k} \qquad \text{Eq. 4.4}$$

The temperature dependence of the color degradation of the two pigments produced by M. ruber in SCBHH and SCSHH under the surfactant optimized formulation was expressed by Arrhenius model as Eq. 4.5, which can be linearized to obtain Eq. 4.6

$$D_k = D_0 e^{-\frac{Ea}{RT}} \qquad \text{Eq. 4.5} \qquad \ln(D_k) = lnD_0 - \frac{Ea}{RT} \qquad \text{Eq. 4.6}$$

where Ea is the activation energy (kcal mol−1), D_0 is the pre-exponential factor, R is the universal gas constant (1.987 cal mol^{-1} K^{-1}) and T is the absolute temperature (K).

4.2.5 Analytical Methods

4.2.5.1 Biomass Characterization

Prior the chemical characterization, the moisture of lignocellulosic biomass was individually determined using a fast dry weight scale with a UV chamber at 105 °C. Lignocellulosic biomass were characterized in terms of structural macromolecular fractions, ash, and extractives, before and after the pretreatment process, based on the analytical procedures established by the National Renewable Energy Laboratory - NREL (Sluiter et al., 2011).

4.2.5.2 Analysis of extracellular biopigments production

Samples taken from the fermentation medium, were centrifuged at 10,000 rpm (6,738 g) for 10 min to separate the cells from the supernatant containing the biopigments. The concentration of the biopigments in the liquid fraction after biomass removal was estimated by the measurement of absorbance at 430, 470, and 510 nm for yellow, orange, and red color pigments respectively, using an Eppendorf biospectrophotometer (Eppendorf AG, Germany), and the result was multiplied by the respective dilution factor (Vendruscolo et al., 2016).

4.2.5.3 Carbohydrates, furans, and phenolic compounds analysis

Monomeric sugars concentration was analyzed by High Performance Liquid Chromatography (HPLC) Agilent 1200 series (Agilent Technologies, Inc., USA) equipped with a Refractive index detector RID-6A and HPX-87H (300x7.8mm) column (Bio-Rad, USA). Conditions used in the analysis were as following: 45°C column temperature, 0.01N H2SO4 as the mobile phase, 0.6 mL/min flow rate, and 20 µL injection volume (Ahmed et al., 2017).

The analysis of furans (furfural and 5-HMF) and phenolics (4-hydroxybenzoic acid, ferulic acid, gallic acid, p-coumaric acid, vanillic acid, pyrocatechol, syringaldehyde and vanillin) was performed as established by Skendi et al. (2017) using a Zorbax C18 column (Agilent, Santa Clara, CA) at 30 °C with a gradient of 1% acetic acid in water (A), acetonitrile (B) and methanol (C) at a flow rate of 1.3 $mL.min^{-1}$. In 0 min, 90% of A and 10% of C were used, this proportion being changed to 80% of A, 4% of B and 16% of C in 10 min, which was kept fixed until 14 min. Then, 100% B was set for column cleaning and in 18 min the initial conditions were resumed for column equilibration. The detection of the analyzed compounds was performed by UV detector, being furfural, 5-HMF, gallic acid, pyrocatechol, vanillin and syringaldehyde detected at 280 nm, 4-hydroxybenzoic acid and vanillic acid detected at 260 nm and p-coumaric acid and ferulic acid at 320 nm.

4.2.5.3 Thin Layer Chromatography (TLC) for biopigments and citrinin

The pigments obtained from the fermentation of SCBHH and SCSHH by *Monascus ruber* after 10 days of fermentation at 30 °C were separated by TLC following the methods of Babitha et al. (2006) for biopigments and Kang et al. (2014) for citrinin. The mobile phases used were chloroform: methanol: water (90:25:4, v/v) and chloroform: methanol: acetic acid (285:21:9, v/v), respectively. Four microliters of the extracellular pigments were applied on TLC plate (Silica gel 60 UV_{254} TLC plate (MACHEREY-NAGEL)), air dried, and placed in TLC development chamber for around 20 min. The pigments were detectable under visible light, but other molecules, as citrinin were detectable under ultraviolet light (UV at 365 nm) only.

4.2.6 Statistical analysis

Data were analyzed using STATISTICA (StaSoft, Inc., Oklahoma, USA), and were presented as mean value ± standard deviation (SD). Means were tested for significant differences through a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. In this study, the level of significance was settled at $p<0.05$.

4.3 RESULTS AND DISCUSION

4.3.1 Effect of the addition of different non-ionic surfactants on the production of pigments by Monascus ruber

The addition of surfactants in the submerged fermentation process is an efficient method to improve the production of extracellular *Monascus* pigments. Some reports indicate that the phenomenon occurs when intracellular hydrophobic pigments that are distributed in the mycelia during conventional fermentation, have their transport behavior across the cell membrane into the broth facilitated by the presence of surfactants (Hu et al., 2012; Chen et al., 2018). This may be due to different mechanisms in which surfactants are involved, such as facilitated transport, formation of pores in the cell membrane etc. (Muñoz et al., 2022).

In this study, the highest production of pigments (orange and red) was obtained when the surfactant Tween 20 was added to the fermentation broth in a concentration of 10 g/L and Tween 80 at 20 g/L with values approximately 4.5 times higher when compared to the control (Figure 10 A and B). In the case of yellow pigments PEG400 and Tween 80 in concentrations of 10 and 20 g/L, respectively, showed the highest production when they were added to the fermentation. The study of surfactants at different concentrations (10 and 20 g/L) showed variation in biocompatibility. When Tween 20 was used at a concentration of 10 g/L, the highest red pigment production was observed; however, the increase in the concentration of this same surfactant resulted in a decrease of about 50% in the production of red pigments. Otherwise, Tween 80 and Triton X-100 results show that increase in concentration does not affect the fermentative process, thus maintaining or enhancing the biopigment production.

In the case of Triton X-100, its concentration has been correlated directly to the production of *Monascus* pigments. Chen et al. (2018) described that when a micellar solution of Triton X-100 (40 g/L) is used, an extraction of around of 50% of the intracellular *Monascus* pigments is performed without harming the cell growth. Yang et al. (2019) found that different nonionic surfactants can improve cell membrane permeability and cell storage capacity by modifying the cell walls of *Monascus* mycelium at different degrees depending on their hydrophile–lipophile balance value (HLB) and cloud point temperature, thus enhancing biopigment production/extraction. Thus, it could support the fact about the different biocompatibility of Triton X-100 and other surfactants in this fermentative process.

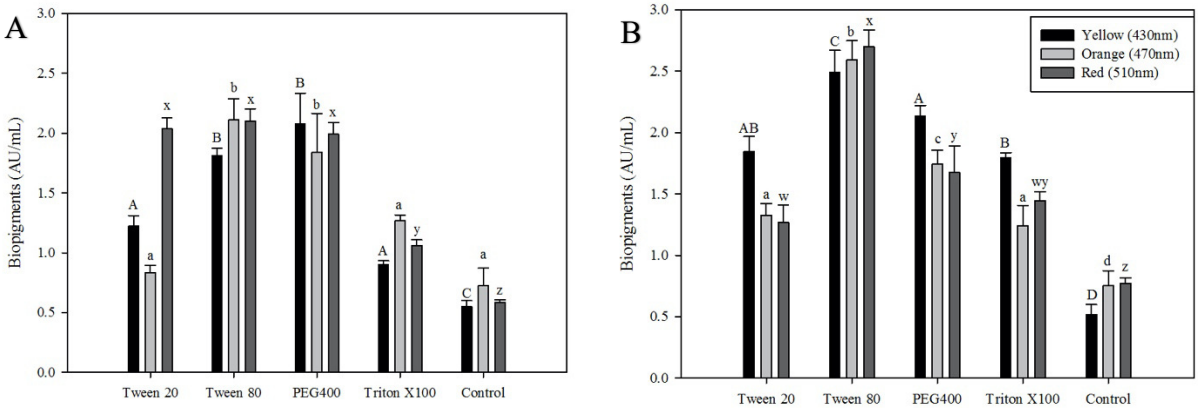

Figure 10. Effect of the addition of different non-ionic surfactants (Tween 20, Tween 80, PEG400 and Triton X100) on the production of biopigments by *Monascus ruber*. Graphs A and B show the results of pigment production when surfactants are found at a concentration of 10 g/L, and 20 g/L, respectively. Different letters in each graph indicate significant differences according to Tukey's test ($p<0.05$) for yellow, orange, and red pigments, respectively.

4.3.2 Effect of the synergism of Triton X-100 with other non-ionic surfactants on the fermentation of Monascus ruber to produce biopigments

It has been reported that Triton X-100 concentration is closely correlated to the production of *Monascus* pigments (Chen et al., 2018). Additionally, Wang et al. (2013), describes that the underlying mechanism of Triton X-100 on *Monascus* pigment production is driven by the degree of unsaturated lipids in cell membrane, improving its fluidity and permeability, facilitating intracellular pigment secretion, and increasing pigment production. Chen et al. (2018) also describes that Triton X-100 can modify cell membrane permeability by altering the ion channel and conformation of cell membrane proteins to facilitate trans-membrane transport of intracellular pigments. All these mechanisms make Triton X-100 a target molecule to analyze its synergistic effect with other non-ionic surfactants to enhance production of pigments of the fungus *Monascus ruber*. Then, experiments were performed to evaluate the combined effect of Triton X-100 with each of the non-ionic surfactants which isolated effect was above studied.

After the addition of non-ionic surfactants in mixture with Triton X-100, the pigment production increased significantly, as shown in Figure 11, except for the mixture with PEG400.

The phenomenon of synergism could be demonstrated in several of the tests carried out to produce pigments, particularly in the mixture Triton X-100-Tween 20 and Triton X-100-Tween 80, where the production of the red pigment (AU_{510nm}/mL) was increased by

about 5.15 times and 4.49 times respectively, compared to the control (5.57 AU_{510nm}/mL). However, in the case of Triton X-100-PEG mixtures, an antagonistic effect was presented between the surfactants with respect to pigment production, showing no significant difference between the mixture and the control (Figure 11). Similar effects were shown for yellow and orange pigments. This antagonistic effect was not expected, considering the results observed using PEG400 alone (Figure 10). When in mixture, the decrease of the cloud point of Triton X-100 (a non-ionic surfactant) in presence of a polymeric glycol as PEG400 (Mahajan et al., 2004; Hossain et al., 2020), could impair the beneficial effect of the surfactants, by precipitating them.

It is necessary to emphasize that the mixture of different surfactants is a poorly studied area for improving the release of intracellular metabolites and its use can opens possible alternatives to improve the downstream processes of biopigments. In the following section, a selected mixture was optimized using a CCRD design to improve the production of biopigments.

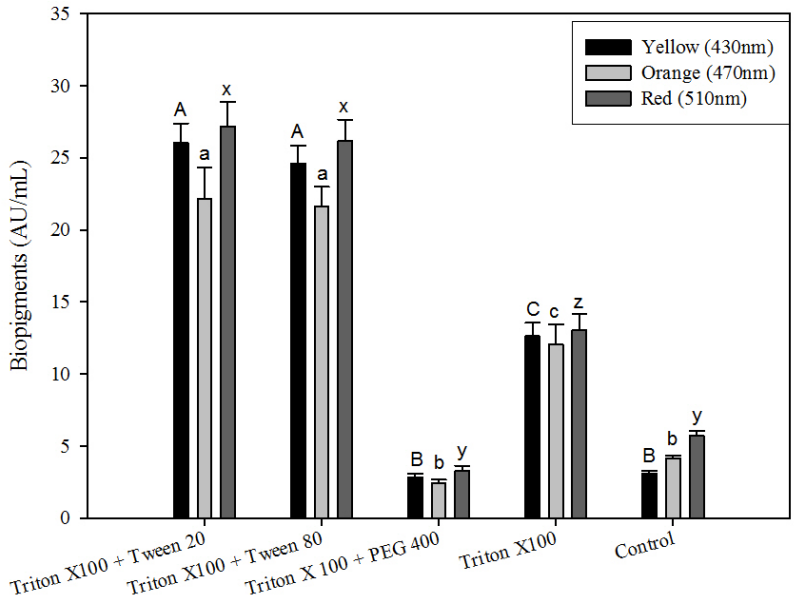

Figure 11. Synergistic effect of mixing Triton X-100 with other surfactants (Tween 20, Tween 80, PEG400) on the production of biopigments by *Monascus ruber*. Different letters indicate significant differences according to Tukey's test ($p<0.05$) for yellow, orange, and red pigments, respectively.

4.3.3 Optimization of the biopigments production assisted by non-ionic surfactants

According to the previous results, it a CCRD was used to optimize a surfactant formulation to enhance the biopigment production from a xylose-based medium.

Triton X-100 has been described with high potential for using in bioprocesses, with a well elucidated mechanisms described in literature. Moreover, as previously shown (section 4.3.2), Tween 20 and Tween 80 showed good compatibility with Triton X100. Thus, also considering that a high concentration of Tween 20 could have a negative influence on the production of *Monascus ruber* biopigments (section 4.3.1), the mixture of Triton X-100 and Tween 80 was selected for optimization experiments.

Results of the CCRD performed to evaluate the influence of different concentrations of Triton X-100 and Tween 80 in the production of biopigments (analyzed at 510 nm) are shown in Table 8. As can be seem, the runs number 2 and 3 are examples of the good biocompatibility of these surfactants to enhance the production of Monascus pigments, reaching values of 51.2 and 39.34 $UA_{510nm.}$ These values are around 2-folds higher when compared to the synergic results of the same mixture (Triton X-100: Tween 80) shown in section 4.3.2.

Table 8. Results of CCDR carried out to optimize the non-ionic surfactants formulation to assist the fermentation of sugarcane bagasse and straw hemicellulosic hydrolysates to produce biopigments

Run	*Triton X-100 (g/L) **	*Tween 80 (g/L) **	*AU_{510nm}*
1	4.39 (-1)	4.39 (-1)	18.5
2	25.61 (+1)	4.39 (-1)	51.2
3	4.39 (-1)	25.61 (+1)	39.34
4	25.61 (+1)	25.61 (+1)	3.1
5	0.00 (-α)	15.00 (0)	17.3
6	30.00 (+α)	15.00 (0)	11.49
7	15.00 (0)	0.00 (-α)	31.5
8	15.00 (0)	30.00 (+α)	15.2
9	15.00 (0)	15.00 (0)	30.23
10	15.00 (0)	15.00 (0)	31.2
11	15.00(0)	15.00 (0)	35.17

*Coded valued in parenthesis; AU_{510nm} (Absorbance Units at 510nm after 10 days of fermentation); α value: 1.68

In this experiment, for the response variable biopigments production at 510nm (AU_{510nm}), it was possible to obtain a satisfactory reduced quadratic model (Equation 4.7), which was significant ($p<0.05$), with no significant lack of fit ($p>0.1$) and with an R^2 value

of 0.89 (Table 9). The model was reduced by excluding the non-significant coefficients, except if necessary to model hierarchy.

Table 9. Analysis of variance (ANOVA) for the adjusted quadratic model for biopigments production (AU510nm) as a function of the concentration of non-ionic surfactants

Source	Sum of squares	Difference	Degree of Freedom	F-Value	*p*-Value
Model	1742.34	4	435.84	12.32	0.0047
A-Triton X100	17.29	1	17.29	0.49	0.5107
B-Tween 80	316.35	1	316.35	8.94	0.0243
AB	1188.35	1	1188.35	33.58	0.0012
A^2	221.35	1	221.35	6.29	0.0465
Residual	212.3	6	35.38		
Lack of fit	198.6	4	49.65	7.25	0.1249
Pure error	13.7	2	6.85		
Total	1955.65	10			
$R^2 = 0.89$					

$$AU_{510nm} = 30.19 - 1.47\ A - 6.29\ B - 17.24\ AB + 5.98\ A^2 \quad \text{(Eq. 4.7)}$$

Where: AU_{510nm} is the response variable "Absorbance units at 510nm", and A and B correspond to the actual values of independent variables "concentration of: Triton X-100 and Tween 80", respectively.

The adjusted model was used to plot response surface graphs, as shown in Figure 12. As can be seem, when a high concentration of Triton X-100 was used, a low concentration of Tween 80 is required to achieve a maximum yield of about 50 UA_{510nm}. However, when Triton X-100 and Tween 80 were used in high concentrations, a negative effect in the production of biopigments can be observed. By using the numerical optimization tool of the Design-Expert software and placing as criteria the maximization of the biopigment production (AU_{510nm}), the following optimized formulation was obtained: 25.61 and 4.39 g/L of Triton X-100 and Tween 80, respectively.

Under the optimized conditions, the model predicted a 46.26 AU_{510nm}/mL of biopigment production after 10 days of fermentation with a confidence interval (95% CI) ranging from 34.96 to 57.56 AU_{510nm}/mL. Aiming at confirming the model obtained for the AU_{510nm}, a new experiment, in triplicate, was performed in the optimized condition,

obtaining a production of 49.16 ± 0.91 AU_{510nm} (mean ± standard deviation), this result within the values of confidence interval (95% CI), thus confirming the model prediction. The surfactant optimized formulation (SOF) was used for further assays to produce biopigments from alternative carbon substrates as SCBHH and SCSHH.

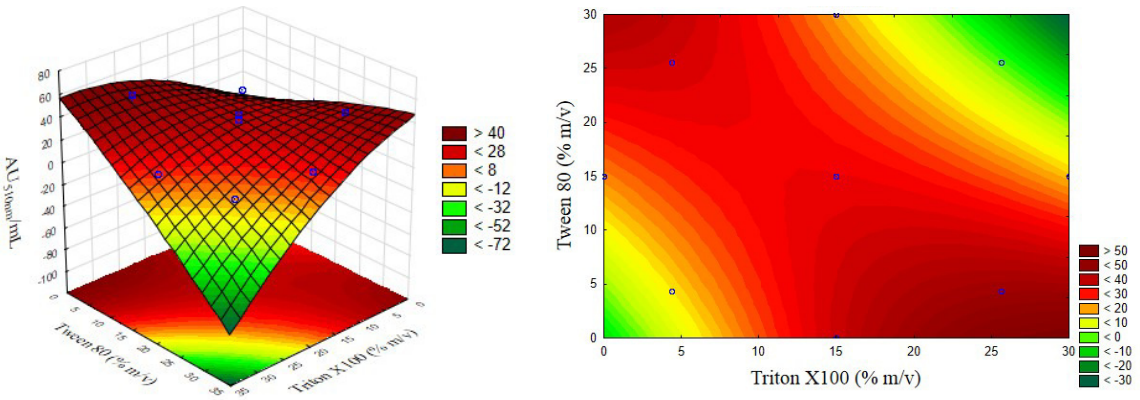

Figure 12. Response surface (A) and contour plot (B) of the production of biopigments (UA_{510nm}/mL) as a function of the variables: concentration of Triton X-100 and Tween 80.

4.3.4 Effect of surfactant formulation on biopigment production in SCBHH and SCSHH by Monascus ruber

Monascus pigments are traditionally produced by solid-state cultivation on rice. Submerged fermentation has been employed to produce these pigments using glucose or rice powder as the main carbon source (Zhou et al., 2014). To reduce the cost of raw material, different types of substrates have already been used, such as potato pomace, rice straw, and corn bran (Liu et al., 2020; Chen et al., 2021; de Almeida et al., 2021). However, there are just a few reports on the use of sugarcane hydrolysates to produce pigments by *Monascus* sp. (Teran-Hilares et al., 2018; Silbir and Goksungur, 2019). In the present study, the hemicellulosic hydrolysate of sugarcane bagasse (SCBHH) and hemicellulose hydrolysate of sugarcane straw (SCSHH) were used to produce biopigments by *Monascus ruber*.

Firstly, acid pretreatments were performed to release fermentable sugars from the lignocellulosic biomass. After dilute acid pretreatment, the greatest reduction in xylan content was found in SCB biomass (about 60%), followed by SCS (about 30%). Consequently, the remaining biomass of the acid pretreatment showed higher glucan contents, corresponding to 47.7 and 51.13 for BCA and PCA, respectively. Thus, this

residual cellulignin can be used for other processing steps, as enzymatic hydrolysis to obtain glucose-enriched hydrolysates.

The liquid streams from the acid pretreatments, corresponding to the hemicellulose hydrolysates, were then characterized, before and after the concentration and detoxification steps, regarding to the concentrations of xylose, glucose, arabinose, and acetic acid. Acid hydrolysis allowed obtaining a concentration of xylose about 8-fold greater than that of glucose in the case of SCBHH, and about 5-fold higher in the case of SCSHH, confirming the greater reduction of the xylan fraction content in BCA biomass.

The initial concentration of xylose in the hydrolysates was adjusted to a value of 30 g/L in the fermentation process. At this point, it was found the presence of different concentrations (in g/L, for SCBHH and SCSHH, respectively) of furan and phenolic compounds such as furfural (non-detected and 0.038), 5-Hydroximethyl furfural (5-HMF) (0.01 and 0.03), gallic acid (0.012 and 0.013), pyrocatechol (0.016 and 0.026), 4-hydroxybenzoic acid (0.08 and 0.011), vanillic acid (0.012 and 0.019), vanillin (0.010 and 0.027), syringaldehyde (0.014 and 0.023), p-coumaric acid (0.008 and 0.030), and ferulic acid (0.020 and 0.050). Despite of the presence of those molecules could have negative effects on the production of Monascus pigments, it has been described that some of them (furfural and 5-HMF) could also regulate the conversion of pigment components (Zhang et al., 2022).

As presented in Figure 13, the obtained results show the potential of sugarcane by-products hemicellulosic hydrolysates to produce *Monascus* pigments.

Regarding to the consumption of sugars, the present glucose in SCBHH and SCSHH (3.4 and 5.6 g/L, respectively) was completely consumed in the first 48h in all cases. Also, the rate of consumption of xylose was similar for both hydrolysates (Figure 13-B). However, the addition of the surfactant optimized formulation (SOF) in the process showed a significant difference on the final xylose consumption values, which were of 94.57 and 84.69% for SCSHH, and 95.33 and 88.02% for SCBHH, with and without the addition of SOF, respectively. This possible difference is attributed to the effect of non-ionic surfactants in enhancing the permeability of cell membranes and the contact between the medium and the cell, so raising the uptake of nutrients (Tu et al., 2015; Li et al., 2018).

Regarding to the production of biopigments, even with different compositions between the SCBHH and SCSHH, the values obtained for both hemicellulosic hydrolysates had no significant difference, remaining around 5.43 and 7.73 AU_{510nm}/mL, respectively, without SOF addition. According to Silbir and Goksungur (2019), the highest pigment

production of 15.35 UA_{500nm} was obtained in the fermentation medium containing BSG hydrolysate (Brewer's Spent Grain) with 2% (m/v) sulfuric acid. When the acid concentration was increased, a gradual decrease in pigment formation was observed. The decrease in pigment formation observed under extreme hydrolysis conditions was likely the result of the production of inhibitory compounds such as hydroxymethylfurfural and furfural, which are known to have negative effects on *Monascus* pigments when they are found in concentrations higher than 0.7 and 2.0 g/L for 5-HMF and furfural, respectively (Zhang et al., 2022).

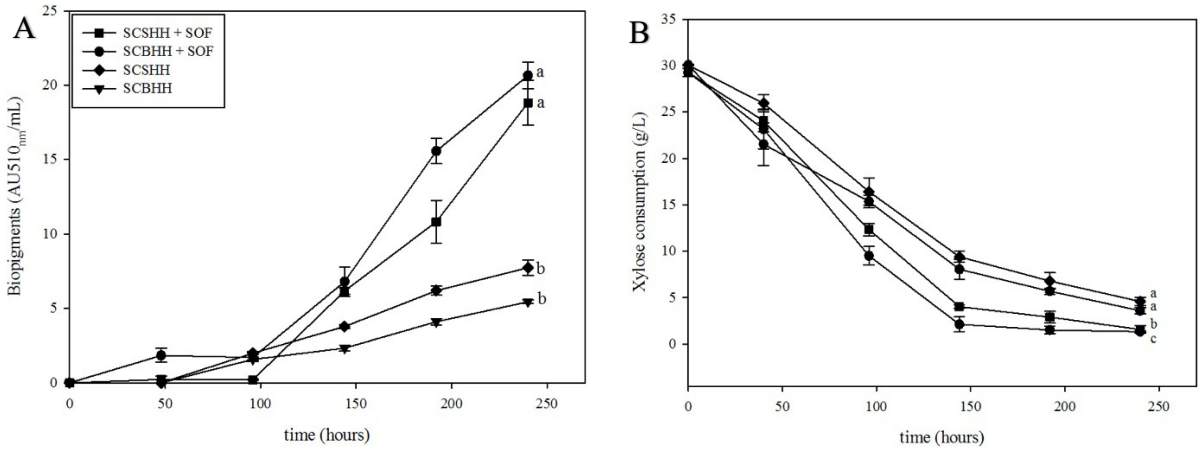

Figure 13. Effect of optimized surfactant formulation on biopigments production (A), and on the xylose consumption (B) in SCBHH and SCSHH. Different letters indicate significant differences according to Tukey's test ($p<0.05$) performed for the results at 240h of fermentation. *SCBHH- Sugarcane Bagasse Hemicellulosic Hydrolysate; SCSHH- Sugarcane Straw Hemicellulosic Hydrolysate; SOF- Surfactant optimized condition

In addition, as previously noted from cultures in a semi-synthetic medium, the addition of the non-ionic surfactant optimized formulation also increased biopigment production in the fermentative process using hemicellulose hydrolysates (Figure 13). From the SCBHH and SCSHH with the surfactant optimized formulation addition, the production of biopigment was 4-fold higher (18.81 and 20.65 AU_{510nm}/mL, respectively) than from the fermentation without these additives.

Some mechanisms involved in the effect of adding surfactants to improve pigment production by *Mona*

scus species have already been described. For example, Triton X100 can effectively alter the cell membrane permeability of *Monascus* due to the increased degree of unsaturation of fatty acids, improving the secretion of metabolites, as well as reducing the feedback inhibition by the product (Chen et al., 2017; Wang et al., 2013).

Each surfactant may have different effects on fermentation and production of pigments, so it becomes interesting to use a formulation of these additives to obtain synergetic mechanisms that will improve the yield of the process. The use of surfactant formulations in the fermentation process by *Monascus* sp. from lignocellulosic biomass has not been widely explored, hindering the comparison of the results obtained with other studies. Recently, the addition of surfactant formulation in separate and semi-simultaneous hydrolysis and fermentation by *Monascus ruber* was demonstrated to increase the biopigments production, reaching the maximum production of 10 AU_{510nm}/mL and 17.84 AU_{510nm}/mL (Sánchez-Muñoz et al., 2022).

4.3.5 Evaluation of the stability of the produced biopigments

It is of paramount concern to evaluate the stability of natural colorants to enable their application in several industrial processes in which they are likely to be subjected to different physico-chemical conditions such as temperature and light (Tirumale and Wani, 2018; Velmurugan et al., 2011). Within this context, Table 10 discloses the thermal stability, read in (%) with respect to control, of the most produced biopigments by *M. ruber* under different temperatures, type, and duration of light exposition.

Table 10. Temperature and light stability of *Monascus ruber* pigments produced from SCBHH and SCSHH and other general properties. Different letters indicate significant differences according to Tukey's test ($p<0.05$) for yellow (430nm), orange (470nm), and red (510nm) pigments, for temperature, white light, sunlight, and UV light test, separately.

Parameter		**Properties**					
Water Solubility		Soluble (both pigments)					
Color (λ max)		510nm (red); 470nm (orange); 430nm (yellow)					
Hue		Dark Red (both pigments)					
Stability (%) of the produced biopigments by *Monascus ruber*							
Based medium		SCBHH	SCSHH	SCBHH	SCSHH	SCBHH	SCSHH
λ max		510nm	510nm	470nm	470nm	430nm	430nm
Temperature (ºC)	50	95.36±3.04^{a}	97.16±2.40^{a}	96.98±3.84^{a}	97.31±2.13^{a}	97.12±1.26^{aeg}	91.18±0.11^{abdefg}
	60	91.19±3.50^{a}	92.92±2.58^{a}	96.35±6.51^{a}	89.27±7.31^{ab}	93.17±2.74^{aefg}	80.74±7.58^{bcfg}
	70	93.89±0.02^{a}	98.89±4.01^{a}	96.49±4.57^{a}	86.11±6.03^{ab}	92.23±5.56^{abefg}	77.01±7.19^{cf}
	80	91.09±2.85^{a}	92.26±4.82^{a}	92.99±2.04^{ab}	85.68±0.65^{ac}	92.50±4.72^{abefg}	89.87±1.84^{abefg}
	90	93.11±1.50^{a}	90.42±3.98^{a}	93.33±3.40^{ac}	79.84±1.09^{b}	94.84±3.55^{efg}	84.03±1.41^{fg}
	100	91.78±4.67^{a}	94.73±2.20^{a}	89.45±2.23^{ab}	82.00±2.62^{bc}	92.75±3.40^{g}	87.40±2.27^{abcefg}

continue

concluded

White light 12 hours	75.04±4.26[a]	84.44±0.99[b]	87.85±4.96[a]	94.29±1.41[a]	84.11±4.11[a]	86.80±1.58[a]
Sunlight 2 hours	55.88±2.00[a]	54.67±1.64[a]	58.85±3.03[a]	59.29±2.75[a]	55.15±3.07[a]	57.71±3.21[a]
UV light 2 hours	87.55±1.07[a]	101.87±1.62[b]	85.25±1.81[a]	102.66±1.83[b]	87.55±0.72[a]	101.97±1.23[b]

*SCBHH- Sugarcane Bagasse Hemicellulosic Hydrolysate; SCSHH- Sugarcane Straw Hemicellulosic Hydrolysate

It can be observed in Table 10 that *Monascus* red pigments (510 nm) produced with SCBHH and SCSHH attains, approximately, 90 to 97% of stability in the ramp of temperature without showing any significant difference. Whereas for the orange and yellow pigments derived from SCSHH exhibited lower stability at higher temperatures (90 and 100 ºC) stability. Moreover, the distinct susceptibility of the natural pigments to be degraded may vary according to the pigment color and their chromophores functional groups which can be subdivided into atoms, electron donors and receptor radicals (Pina et al., 2012; de Oliveira et al., 2022).

Indeed, the heat can induce pigment degradation due to destabilization of the molecules and the bonds among them. For red and orange pigments, extracted from *M. ruber*, high temperatures have already been reported to promote their degradation (Vendruscolo et al., 2013). However, in this study the SCBHH-derived orange and red pigments and SCSHH-derived red pigment showed remarkable stability at all temperatures tested.

Kinetic models of pigment stability are also a useful tool to evaluate the pigment behavior when exposed to extreme conditions, thereby, assessing feasible solutions to enhance their stability (Liu et al., 2022). In fact, in a recent study, by applying a response surface methodology, the authors have observed higher stability of *Monascus*-derived pigments in lower temperatures (60˚C - 75˚C) unless the pH was adjusted to 7 which enables the pigment stabilization in higher temperature range (75˚C - 88˚C) (Abdollahi et al., 2021). Furthermore, other researchers have evaluated the color stability of *Monascus*-derived pigments produced in conventional synthetic medium and it was found that no substantial stability was obtained after 25 h of incubation under 100˚C (Carvalho et al., 2005).

By comparison to this current study, it is displayed that no further significant losses in pigment intensity was encountered even in high temperature range (80˚C - 100˚C), thereby, the utilization of lignocellulosic hydrolysates may presumably provide stabilizing factors such as organic volatile acids, proteins and ions which favor the preservation of color in the natural pigment. In fact, it was previously stated that the utilization of sugarcane bagasse enzymatic hydrolysate-based medium for *M. ruber* markedly enhanced the thermal stability of the red pigment (Hilares et al., 2018).

Nevertheless, the addition of surfactants to produce biopigments could possibly serve as a protective molecule against harsh conditions. The surfactant-promoted molecular stability may be due to the physicochemical properties of surfactants to self-assemble into micelles which encapsulate the pigment molecules by chemical interactions to form a thick and steric barrier and hence, hindering the oxidation of the pigments (McClements et al., 2016; Kargar et al., 2012; Elias et al., 2008). Recently, the role of natural surfactants to improve thermal stability of carotenoids has been investigated and presented promising results with respect to their antioxidant and protective properties (Guo et al., 2022).

Analogously, by analyzing the stability of the pigments under light exposure (white light and UV light) it was also observed its low degradation which can be associated with the addition of the surfactants. Some surfactants have the potential to scavenge free radicals and serve as chelating agents, these characteristics may assist in the UV-protection of the pigments (Jacobsen, 2015; Uluata et al., 2015). Nonetheless, it was not observed in assays performed with sunlight (Table 10) exposure from which it was attained approximately half of the stability in comparison to the control.

As aforementioned, to be useful for different applications, microbial pigments are requested to have some specific properties, as stability in variety of environmental factors such as light, temperature, and pH (Sen et al., 2019). The pH stability of the pigments produced using the hemicellulosic hydrolysates of sugarcane bagasse and straw were studied in a range from 1 to 13. Different responses were analyzed during the experiment such as L*, a*/b* and AU_{510nm}. L* indicates lightness from 0 (black) to 100 (white). The a* and b* coordinates of the CIELAB represent the color positions between red and green as well as between yellow and blue, respectively. Positive and negatives values of a* indicate red and green, respectively, whereas negative and positive values of b* correspond to blue and yellow, respectively. This indicates that positive values of the ratio a*/b* correspond to predominantly red coloring, as desired for *Monascus* pigments (Jung et al., 2003; Teixeira et al., 2012).

The Figure 14 (heatmap) represents the maximum and minimum values according to the data obtained for all responses. L* values for both pigments (from SCBHH and SCSHH) indicates that the lightness value was recorded in pH 13, showing the absence of the dark red hue of the *Monascus* pigments obtained. In contrast, the darkest value obtained was at pH 5 (control experiment) for both biopigments. In the case of a*/b* values, where red color is predominant the range between pH 5-9 showed the maximum values for SCSHH and between pH 5-7 for SCBHH. These results indicate that in acidic or alkaline conditions the

dark red color hue produced by *Monascus ruber* with the addition of the surfactant optimized formulation was less stable. Nevertheless, as shown in Figure 14 (C and D) the variety of colors in all pH range show the color versatility of *Monascus* pigments, from light yellow to dark red hue, thus exhibiting promising application properties.

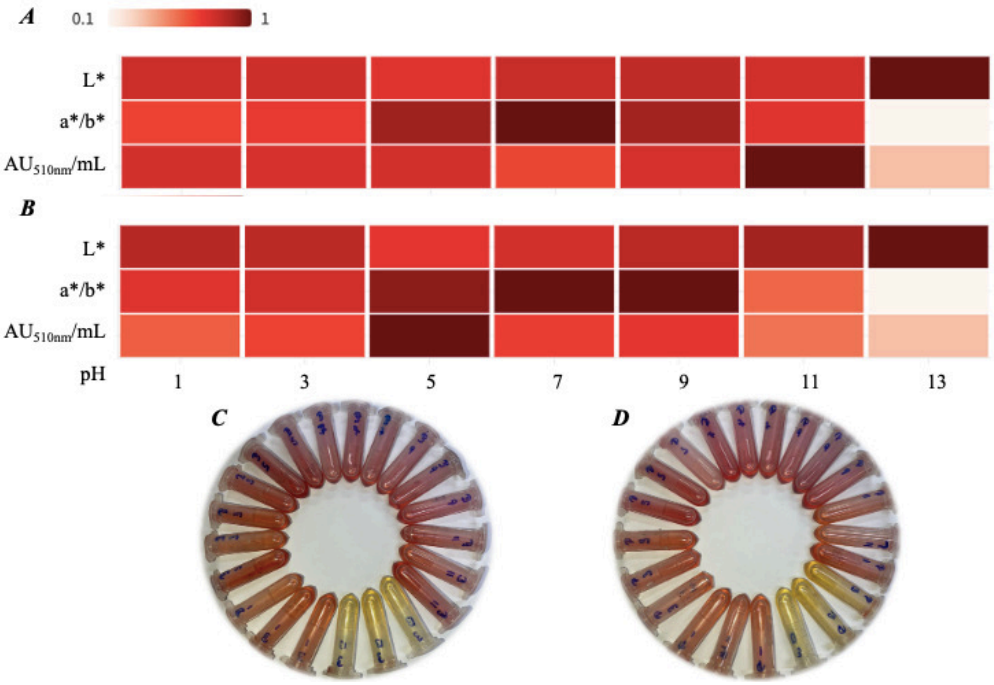

Figure 14. Heat map of the pH stability of *Monascus ruber pigments* produced from SCBHH (A) and SCSHH (B); color variability at different pH values for biopigments produced from SCBHH (C) and SCSHH (D).

4.3.5.1 Thermal stability of red pigment produced in SCBHH and SCSHH

As reported by Hilares et al. (2018) and Vendruscolo et al. (2013) a heat treatment on *Monascus* pigments shows significant difference in color degradation. Thus, to make a deeper analysis in the thermal stability of the pigments produced from the hemicellulosic hydrolysates, it was performed a new experiment, increasing the heating temperature and time of treatment. As shown in Figure 15, the degradation profile was adjusted to a first kinetic model with good regression coefficients ($0.87<R^2<0.96$).

Thermal degradation constant, half-life time and activation energy of red pigment produced by *Monascus ruber* in both hemicellulosic hydrolysates with the SOF are presented in Table 11. The lowest degradation constant was achieved at 60ºC for both hydrolysates, with values of 0.054 and 0.012 h^{-1} for SCBHH and SCSHH, respectively. These values are consequently related to the longest half-life time of 12.84h for SCBHH and 57.76 h for SCSHH. Hilares et al. (2018), reported 18.33 h of half-life time when red biopigments were

produced in sugarcane bagasse enzymatic hydrolysate, under thermal treatment of 50ºC. In the same study, it was reported a thermal treatment of 90 ºC with a half-time value of 2.3 h, that is 1.71-folds and 2.3 folds lower than the values found in the present work for red biopigments produced in SCBHH and SCSHH, respectively, with a thermal treatment of 100 ºC.

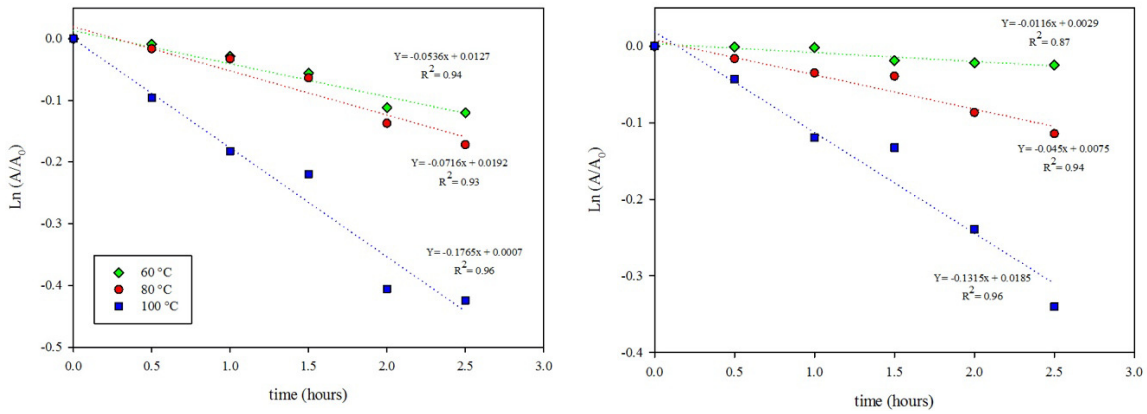

Figure 15. Kinetic of the degradation of red pigment produced by Monascus ruber in SCBHH (A) and SCSHH (B). Both produced pigments were submitted to thermal treatments of 60, 80, and 100 ºC for 2.5 hours.

The activation energies of the red pigments produced in both hemicellulosic hydrolysates were calculated based on linear regression analyses of the natural logarithms of the degradation constants, against the reciprocal of the absolute temperature (1/T) in the range from 60 to 100 °C. The activation energy values obtained in SCSHH derived pigments are similar to the obtained by Hilares et al. (2018) in sugarcane bagasse hydrolysate of 12.04 kcal.mol^{-1} in pH 5.5. In contrast, SCBHH derived pigments show lower susceptibility to thermal degradation with an activation energy value of 7.213 kcal.mol^{-1}. Also, de Almeida et al. (2021) reported higher activation energies of 10.19 Kcal.mol^{-1} of corn bran derived biopigments using *Monascus purpureus*. In comparison with other common pigments (e.g., carotenoids) used in several industries, SCBHH derived pigments demonstrate higher stability at high temperatures, because of the fact of high activation energy values indicates heat sensitivity of color degradation during pigment processing (Kardile et al., 2020). For example, the activation energy of the carotenoids of mixed juices was reported around 90 kJ.mol^{-1} (21 kcal.mol^{-1}) (Kardile et al., 2020).

Table 11. Thermal degradation constant (D_k), half-life time ($t_{1/2}$) and activation energy of red pigment produced by *Monascus ruber* in sugarcane hemicellulosic hydrolysates under different temperatures.

Medium	Temperature (ºC)	Thermal degradation constant (Dk, h−1)	Half-life time (t½, h)	Activation energy (Ea, kcal.mol^{-1})
SCBHH	60	0.054	12.84	7.213
	80	0.072	9.63	
	100	0.176	3.94	
SCSHH	60	0.012	57.76	14.76
	80	0.045	15.40	
	100	0.131	5.29	

*SCBHH- Sugarcane Bagasse Hemicellulosic Hydrolysate; SCSHH- Sugarcane Straw Hemicellulosic Hydrolysate

4.3.6 Thin Layer Chromatography analysis for biopigments and citrinin detection

Since *Monascus* pigments are composed by a mixture of different molecules, a thin layer chromatography was performed to analyze the different molecules presented on the pigments produced using SCBHH and SCSHH. Figure 16 (A and B) shows the presence of around ten pigmented molecules observed under visible and UV light for each hydrolysate, from which six are red coloring molecules, presumably. Under UV 365 nm (Figure 16-B), it was possible to identify two molecules with fluorescence properties in the channel number 1 that corresponds to the semi-synthetic medium based-xylose, and one fluorescent molecule for SCBHH and SCSHH, respectively. Other fluorescent molecules (e.g., Monasfluore A and Monasfluore B) have been reported in literature derived from *Monascus* metabolism (Huang et al., 2008).

Also, it was analyzed the presence of citrinin. Citrinin is a nephrotoxic and hepatotoxic mycotoxin that could be produced for some *Monascus* strains (Blanc et al., 1995). The absence of this mycotoxin is of great interest for the application of high value-added products produced by *Monascus* strains in different areas (Kang et al., 2014). The obtained results (Figure 16-D) showed that no citrinin spot was detected when *Monascus ruber* Tieghem IOC 2225 was grown in xylose-based media as well as in both hemicellulosic hydrolysates after 240 hours of fermentation.

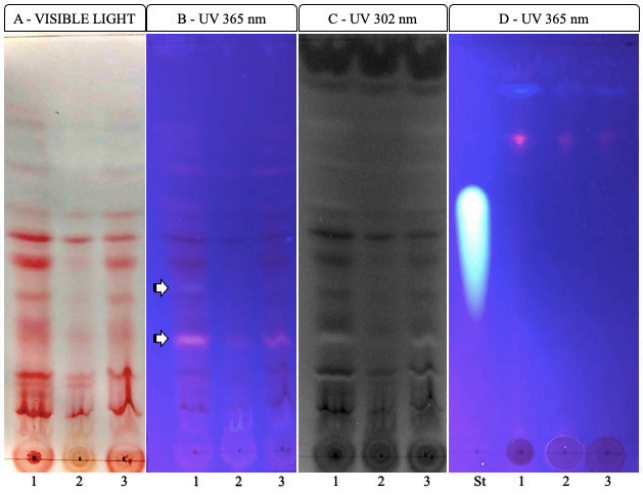

Figure 16. Thin Layer Chromatography of the produced biopigments (A) original picture under visible light; (B) under UV at 365 nm; C) under UV 302 nm C) citrinin detection under UV 365nm. St- Standard Citrinin;1- xylose-based medium; 2- SCBHH; 3; SCSHH

4.4 Conclusion

The production of pigments derived from de fermentation of hemicellulosic hydrolysates of sugarcane by-products by *Monascus ruber* was influenced using a surfactant optimized formulation of two non-ionic surfactants: Triton X-100 and Tween 80. Although, the produced pigments from the hemicellulosic hydrolysates in presence of the SOF demonstrate to be stable in thermal treatments. Another interesting finding was the absence of citrinin which increase the options for the application of the produced pigments. By harnessing the hemicellulosic fraction to produce high value-added products broaden the revenue of technological prospections to the operation of different configuration in biorefineries.

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CHAPTER V

Hydrodynamic cavitation surfactant-assisted alkaline pretreatment of sugarcane bagasse and semi-simultaneous saccharification and fermentation as alternative processes to produce *Monascus pigments*

ABSTRACT

Non-ionic surfactants are important and versatile molecules with a high range of applications in biorefineries. Surfactants have been studied as additives to enhance the major steps of the bioprocess as pretreatment, enzymatic hydrolysis, and fermentation. The effect of the hydrodynamic cavitation and Tween 80 were evaluated to assist an alkaline pretreatment for sugarcane bagasse. The effects of the concentration of NaOH and Tween 80 on the pretreatment of sugarcane bagasse to release of monomeric sugars were optimized by a Central Composite Rotatable design. Under the optimized conditions, the lignin removal efficiency increased about 40% when compared to the control without the addition of Tween 80. The pretreated biomass was used to perform a semi-simultaneous saccharification and fermentation strategy to produce Monascus pigments, achieving a maximum value of 3.86±0.57 AU_{510nm}/mL. The combinatory effect of hydrodynamic cavitation and surfactants to process lignocellulosic biomass is a potential strategy to be considered in biorefineries.

Keywords: Akaline-Pretreatment. Tween 80. Hydrodynamic-Cavitation. M*onascus*-pigments

5.1 Introduction

Lignocellulosic biomass (LCB) is the most abundant, inexpensive, and renewable source to obtain high value-added molecules (e.g., biopigments) produced through LCB conversion to fermentable sugars (Li et al., 2010; Lui et al., 2018; Hilares et al., 2018). Due to the complex and recalcitrant structured compounds presented in LCB, pretreatment methods are required to feasible modify and then transform polymers into fermentable sugars (Baral and Shah, 2017; Hassan et al., 2018). Pretreatment step is presented as the most critical bottleneck to biomass utilization in bioprocesses due to its importance and economical value-added to the process (Zheng et al., 2017). Pretreatments have been categorized as biological (e.g., white, brown, and soft rot fungi, and bacteria such as *Bacillus circulans*), chemical (e.g., acid, alkaline, ionic liquid methods), physical (e.g., steam explosion, liquid hot water, and extrusion), or physicochemical (hydrodynamic cavitation) (Kumar and Sharma, 2017; Zheng et al., 2017; Terán-Hilares et al., 2020). In this context, hydrodynamic cavitation has been gained attention due to the assisted intensification of this device on different chemical pretreatments for lignocellulosic biomass, besides of its high energy-efficiency and feasible scaling-up. Hilares et al. (2016; 2017a; 2017b; 2018; 2019; 2020) has been studied several parametric (e.g., temperature, residence time, and pressure), geometric (e.g., number and diameter of holes in the orifice plate of the cavitation device), chemical (e.g., NaOH, KOH, Na_2CO_3, $Ca(OH)_2$) and process configuration (e.g., batch, semi-continuous, and continuous) strategies to enhance the pretreatment of sugarcane bagasse assisted by hydrodynamic cavitation devices, demonstrating that the HC process is an emerging technology to valorize lignocellulosic biomass.

Among all studies involved onto the use of hydrodynamic cavitation, additives as surfactants have been also highlighted to assist pretreatment methods (Muñoz et al., 2022). Surfactants are molecules with a polar (hydrophilic) and a non-polar (hydrophobic) component in their structure, this characteristic makes them a versatile molecule that can act between two phases. This property gives them the potential to reduce the surface tension and enables them to be applied in a wide range of areas. In the case of pre-treatments, the reduction in the surface tension during the process enables the extraction of hydrophobic compounds forming emulsions and making them unavailable for repositioning on the surface of the biomass and improving the yield of pre-treatments to remove the principal target fractions (e.g., lignin and hemicellulose) with acid and alkaline treatments (Pandey and Negi, 2015). Surfactants can also modify the structure and disposition of surfaces making them

more feasible to be attacked for chemicals (e.g., alkaline reagents as NaOH) and biochemical (e.g., enzymes as cellulases) tools (Qing et al., 2010). In addition, surfactants have been described as key additives in pretreatment-subsequent steps as enzymatic hydrolysis and fermentation (Muñoz et al., 2022). All those advantageous characteristics, makes surfactants an interest molecule to produce high value-added products as biopigments in a biorefinery concept.

This is the first report about the use of non-ionic surfactants and hydrodynamic cavitation to assist alkaline pretreatments. In this work, it has been studied the effect of Tween 80 as additive to enhance the alkaline pretreatment of sugarcane bagasse assisted by hydrodynamic cavitation. Also, it has been described the potential of the pretreated biomass and Tween 80 in a semi-simultaneous saccharification and fermentation strategy to produce red pigments by *Monascus ruber*.

5.2 Material and Methods

5.2.1 Biomass

Sugarcane bagasse (SCB) was kindly donated by Ipiranga Agroindustrial (Descalvado, São Paulo, Brazil). When received, the SCB has initially allowed to sun-dry for 2 days. It was then ground using a knife mill Marconi model MA 680 (Marconi Ltda., São Paulo, Brazil) and passed through a 2 mm sieve. Before use, SCB was screened again using a US mesh No. 16 sieve. The content of glucan, xylan, and lignin were determined to be 36.07, 23.98, and 20.09%, respectively.

5.2.2 Effect of the addition of Tween 20 and Tween 80 in the alkaline pretreatment of Sugarcane bagasse: preliminary study

The pre-treatment with sodium hydroxide (2% m/v) was carried out using a 1:10 proportion between the sugarcane bagasse and the volume of the alkaline solution. This solution was supplemented with the addition of different non-ionic surfactants: Tween 20 and Tween 80 at fixed a concentration of 3% (w/w). The experiments were taken to the autoclave at 121 °C for 1 hour. After the reaction, the residual solid material was recovered by filtration and washed with tap water until neutral pH and dried at 45 °C. After drying, enzymatic tests were performed in 50 mL Erlenmeyer flasks, with a working volume of 20 mL in a rotary shaker set at 50 °C and 200 rpm. Initially, the pretreated SCB was mixed with

appropriate volumes of 50 mM sodium citrate buffer (pH 4.8), which resulted in a 10% solid loading. Cellulase, enzyme blend (Sigma-Aldrich®, US) was added at an enzyme dosage of 10FPU/g of SCB biomass. All hydrolysis experiments were carried out for 48 h, at the end of which solids and liquids were separated by centrifuging at 10,000 rpm (6,738 *g*) for 10 minutes. The supernatants were stored at -20ºC before monomeric sugar analysis by HPLC, respectively.

5.2.3 Hydrodynamic cavitation surfactant-assisted alkaline pretreatment of SCB

For the pretreatment of the sugarcane biomass, a batch hydrodynamic cavitation device based on an orifice plate (16 holes with a diameter of 1 mm) with 2.5 L of working volume will be used. The fluid content was recirculated in the system for 10 min at 60 ºC as described by Hilares et al (2020, and its composition was performed according to the central composite rotatable design (CCRD) created from a three-level factorial design augmented with center and star points. The CCRD for this experiment included a total of 11 experimental runs with triplicate at the center point. The sample runs were carried out according to CCRD based on varying concentrations of NaOH (0-2.35 M) and the selected non-ionic surfactant (0-5.93% m/m). For each pretreatment experiment, 25g of biomass was stored in the cylindrical wire line. Then, the pretreated samples were used to performed enzymatic hydrolysis tests (conditions as described in the section 2.2) to evaluate their feasible to be converted in monomeric sugars (glucose and xylose) and quantified by HPLC. The answer variable was the sum between glucose and xylose after 72 hours of enzymatic hydrolysis. The optimized pretreated biomass was characterized in terms of structural macromolecular fractions, ash, and extractives, before and after the pretreatment process, based on the analytical procedures established by the National Renewable Energy Laboratory - NREL (Sluiter *et al*., 2011).

5.2.4 Fermentation assay using the optimized pretreated sugarcane bagasse with semi-simultaneous saccharification and fermentation (SSSF) strategy

5.2.4.1 Microorganism

Monascus ruber Tieghem IOC 2225 was kindly donated by the Culture Collection of Filamentous Fungi (CCFF) – Oswaldo Cruz Foundation (IOC/FIOCRUZ) (Rio de

Janeiro, Brazil). The stock culture was maintained in Petri plates containing potato dextrose agar (PDA) at 5 °C (Cho et al., 2002).

5.2.4.2 Semi-simultaneous Saccharification and Fermentation

For the SSSF strategy, a 1.0 L fluidized bed reactor was used with 500 mL of work volume, loaded with 450 mL sodium citrate buffer pH 4.8, the optimized Tween 80 concentration, and the enzyme dosage used was 10 FPU/g of pretreated sugarcane bagasse. In the pre-hydrolysis phase, the medium temperature was maintained at 50 °C. After 48 hours, the medium temperature was adjusted to 37 °C and maintained at during the following SSF phase; the reactor was supplemented with (g/L): yeast extract 2.5, malt extract 2.5, peptone 2.5, K_2HPO_4 5, $CaCl_2 \cdot 2H_2O$ 0.1, $MgSO_4 \cdot 7H_2O$ 0.5, $FeSO_4 \cdot 7H_2O$ 0.01, $ZnSO_4 \cdot 7H_2O$ 0.01 and $MnSO_4 \cdot 7H_2O$ 0.03. For the inoculum, 2 mycelial agar discs (0,00252 g) for each 50 mL of medium were punched out with a sterilized self-designed cutter (8 mm) from a 7–10 days old culture. The fluidized bed reactor agitation was fluidized by adding oxygen with the aeration rate of 0.2 vvm (air volume per volume of culture medium, $minute^{-1}$). Samples were periodically taken to monitor substrate consumption, surface tension and biopigments production. After inoculation of the fungus, the process was carried out for 16 days.

5.2.5 Analytica Methods

5.2.5.1 Monomeric sugar quantification

Glucose and xylose concentrations were analyzed by High Performance Liquid Chromatography (HPLC) Agilent 1200 series (Agilent Technologies, Inc., USA) equipped with a Refractive index detector RID-6A and HPX-87H (300x7.8mm) column (Bio-Rad, USA). Conditions used in the analysis were as following: 45°C column temperature, 0.01N H_2SO_4 as the mobile phase, 0.6 mL/min flow rate, and 20 µL injection volume (Ahmed *et al*., 2017).

5.2.5.2 Analysis of extracellular red biopigment production

Samples taken from the fermentation medium, were centrifuged at 10,000×rpm (6,738 *g*) for 10 min to separate the cells from the supernatant containing the biopigments.

The concentration of the biopigments in the liquid fraction after biomass removal was estimated by the measurement of absorbance at 510 nm for red color pigments using an Eppendorf biospectrophotometer (Eppendorf AG, Germany), and the result was multiplied by the respective dilution factor (Vendruscolo *et al*., 2016).

5.2.5.3 Analysis of the surface tension

To determine the surface tension (TS) of the samples, a Sensadyne QC6000 Tensiometer (Braseq, Ltda., SP, Brazil) will be used. The device was initially calibrated with water (high calibration) and ethanol (low calibration), and then the surface tension of the samples was assessed.

5.2.6 Statistical analysis

Data were analyzed using STATISTICA (StaSoft, Inc., Oklahoma, USA), and were presented as mean value ± standard deviation (SD). Means were tested for significant differences through a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. In this study, the level of significance was settled at $p<0.05$.

5.3 Results

5.3.1 Hydrodynamic cavitation surfactant-assisted pretreatment of sugarcane bagasse

It was compared the effect of two non-ionic surfactants (Tween 20 and Tween 80) in the alkaline pretreatment, and it was showed that the non-ionic surfactant Tween 80 was the compound that most helped to the saccharification process. In the enzymatic hydrolysis, it was reported an increment of about 2-folds and 1-fold more released sugar for Tween 80 and tween 20, respectively, when compared to the control without the addition of surfactants. Cao and Aita, Nasirpour et al., and Nogueira et al. (2013; 2014; 2017, respectively) also reported better sugar release when Tween 80 was used in different pretreatments methods. According to the literature and these preliminary results, the non-ionic surfactant Tween 80 was selected to be used as additive in the alkaline pretreatment of sugarcane bagasse assisted by hydrodynamic cavitation.

In this context, a CCRD was performed to optimize the best conditions to pretreat sugarcane bagasse in presence of Tween 80. The biomass in all experiments was pretreated

at 3 bar of inlet pressure in the cavitation zone and the experimental parameters for sodium hydroxide and Tween 80 pretreatment, along with results of enzymatic hydrolysis obtained from the sum between glucose and xylose are shown in Table 12.

Table 12. CCRD for optimization of alkaline pre-treatment assisted with surfactants for sugarcane bagasse. Dependent variables: Concentration of Glucose and Xylose (g/L), after 72 hours of enzymatic hydrolysis using the pre-treated material, independent variables: concentration: Tween 80 (m/m) and NaOH (m/v).

Run	NaOH (%) (M) *	Tween 80 (%) (m/m) *	Glucose + Xylose (g/L)
1	0.30 (-1)	0.50 (-1)	29.48
2	2.00 (+1)	0.50 (-1)	24.26
3	0.30 (-1)	5.00 (+1)	33.19
4	2.00 (+1)	5.00 (+1)	36.06
5	0.00 (-α)	2.75 (0)	36.66
6	2.35 (+α)	2.75 (0)	32.79
7	1.15 (0)	0.00 (-α)	20.92
8	1.15 (0)	5.93 (+α)	34.51
9	1.15 (0)	2.75 (0)	32.97
10	1.15 (0)	2.75 (0)	29.14
11	1.15 (0)	2.75 (0)	33.12

*Coded valued in parenthesis; α value: 1.68

For the response variable sum of Glucose and Xylose released sugars after 72 hours of enzymatic hydrolysis ($Glu+Xyl_{72hours}$), it was possible to obtain a satisfactory reduced quadratic model (Equation 1), which was significant ($p<0.05$), with no significant lack of fit ($p>0.1$) and with an R^2 value of 0.87 (Table 13). The model was reduced by excluding low significant coefficients.

The adjusted model was used to plot response surface graphs, as shown in Figure 17. As can be seem, when a high concentration of Tween 80 was used, a high concentration of Tween 20 is required to achieve a maximum yield of about 40 g/L of the sum of glucose and xylose. However, the absence of Tween 80 (run 7-Table 5.1) shows a decrease in monomeric sugar release of about 1.5-fold when compared to its similar with different concentrations of NaOH, demonstrating that with at least a minimal concentration of the surfactant during the pretreatment, the saccharification process is enhanced.

Table 13. Analysis of variance (ANOVA) for the adjusted quadratic model for the summatory of glucose and xylose released from the pretreated sugarcane bagasse after 72 hours of enzymatic hydrolysis ($Glu+Xyl_{72\ hours}$) as a function of the concentration of NaOH and Tween 80

Source	Sum of squares	Difference	Degree of Freedom	F-Value	*p*-Value
Model	207.64	4	51.91	10.02	0.0080
A-NaOH	7.38	1	7.38	1.42	0.2778
B-Tween 80	162.54	1	162.54	31.36	0.0014
AB	16.36	1	16.36	3.16	0.1259
B^2	40.87	1	40.87	7.89	0.0308
Residual	31.1	6	5.18		
Lack of fit	20.92	4	5.23	1.03	0.5474
Pure error	10.18	2	5.09		
Total	238.74	10			
$R^2 = 0.87$					

$$Glu + Xyl_{72hours} = 33.06 - 0.97\ A + 4.71\ B + 2.02\ AB - 2.85\ B^2 \qquad \text{(Eq. 5.1)}$$

Where: $Glu + Xyl_{72hours}$ *is the response variable "summatory of glucose and xylose released after 72 hours of enzymatic hydrolysis", and A and B correspond to the actual values of independent variables "concentration of: NaOH and Tween 80", respectively.*

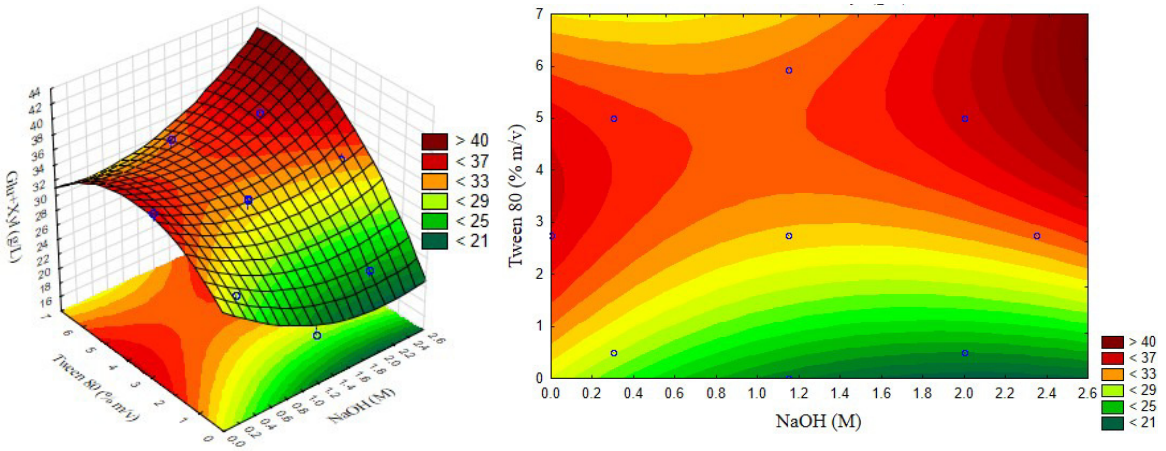

Figure 17. Response surface (A) and contour plot (B) of the released glucose and xylose sum after 72 hours of enzymatic hydrolysis ($Glu+Xyl_{72hours}$) as a function of the variables: concentration of NaOH and Tween 80.

Additionally, by using the numerical optimization tool of the Design-Expert software and placing as criteria the maximization of the sum of glucose and xylose released, the following conditions were obtained: concentration of NaOH of 2.0 (M) and 5.0 % (m/m) of Tween 80.

The model predicted a 35.96 g/L of the summatory of glucose and xylose released after 72 hours of enzymatic hydrolysis with a confidence interval (95% CI) ranging from 31.62 to 40.29 g/L. Aiming at confirming the model obtained for the glucose and xylose releasing sugars, a new experiment was performed in the optimized condition, obtaining 37.16 ± 0.919 (mean ± standard deviation), this result within the values of confidence interval (95% CI), thus confirming the adjusted model. The optimized conditions were then employed in the sequence of experiments, to evaluate the saccharification and delignification potential of the optimized conditions, including the synergy of the formulation and the separated effect of each active component.

The results showed in Figure 18, showed an increment of 1.35-fold and 4.94-fold in the glucose conversion yield for the sugarcane bagasse pretreated under optimized conditions when compared to the alkaline experiment without Tween 80 and the experiment with Tween 80 as sole reagent in the cavitation device, respectively. In the case of the lignin removal a value of 29.04% (m/m) was obtained in the optimized condition, followed by the alkaline condition without the presence of surfactant of 20.39%. No lignin removal was observed when the cavitation device was used only in the presence of Tween 80. It is important to highlight that it was found a decrease in the surface tension of the liquor (65.1 mN/m), when Tween 80 was added to the hydrodynamic cavitation process (Figure 18). The values for sugar conversion yield, lignin removal associated to the surface tension has been described by some authors. Wang et al. (2020), reported that the addition of surfactants could reduce the surface tension, thus increasing the dilution of some components (e.g., hydrogen ions) enhancing the lignin and hemicellulose dilution. Qing et al. (2010), described that some surfactants could enhance the removal of lignin, and as a result this effect could decrease the unproductive adsorption of enzymes (e.g., cellulases), increasing their potential to attack the carbohydrate fractions. Other authors also reported the increment in lignin removal when Tween 80 was applied to different pretreatment methods. Nasirpour et al. (2014) reported an increment of 12.5% when Tween 80 was used to assist the pretreatment of sugarcane bagasse with ionic liquids. Also, Zhang et al. (2021) showed that the addition of Tween 80 in metal-salt catalyzed pretreatment enhanced the hemicellulose and lignin removal of sugarcane bagasse. For example, when the metal-salt catalyzed pretreatment with

$CuCl_2$ was assisted by Tween 80, the lignin removal increased from 19.4 to 32.1%. In addition, the use of Tween 80 with other biomass such as bamboo were recorded by Li et al., 2016, with increments in lignin removal of about 6% when the surfactant was used in alkaline pretreatments with NaOH.

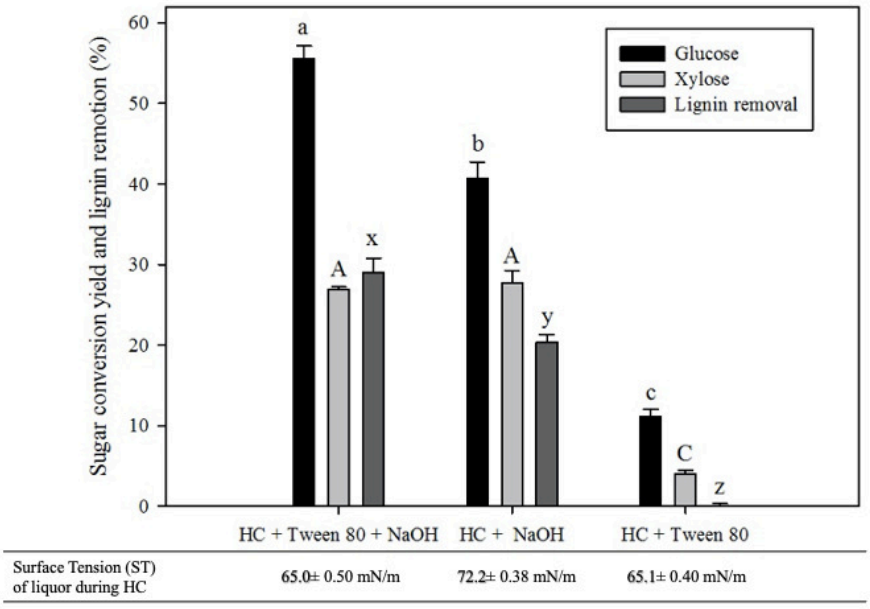

Figure 18. Sugar conversion yield (72 hours) and lignin removal analysis of the pretreated sugarcane bagasse under the optimized condition. Different letters indicate significant differences according to Tukey's test ($p<0.05$). HC – Hydrodynamic Cavitation

5.3.2 Semi-simultaneous Saccharification and Fermentation using the optimized pretreated biomass to produce Monascus red pigments

As aforementioned, the pretreated biomass obtained from the optimized condition, was used to perform a SSSF strategy in a fluidized bed reactor to produce Monascus pigments. Figure 19 shows the kinetics of the process from the pretreatment to the fermentation process for sugar consumption, red biopigment production and surface tension analysis. The results showed a maximum red biopigment production of 3.86±0.57 AU_{510nm} after 432 hours of fermentation, and a started production time after 192 hours of fermentation. As observed in Figure 19 the glucose and xylose were released in higher rate during the first 96 hours of the process. The maximum recorded sugar conversion yield was of 69.6±5.6 and 43.3±2.4% after 288 and 384 hours for glucose and xylose, respectively.

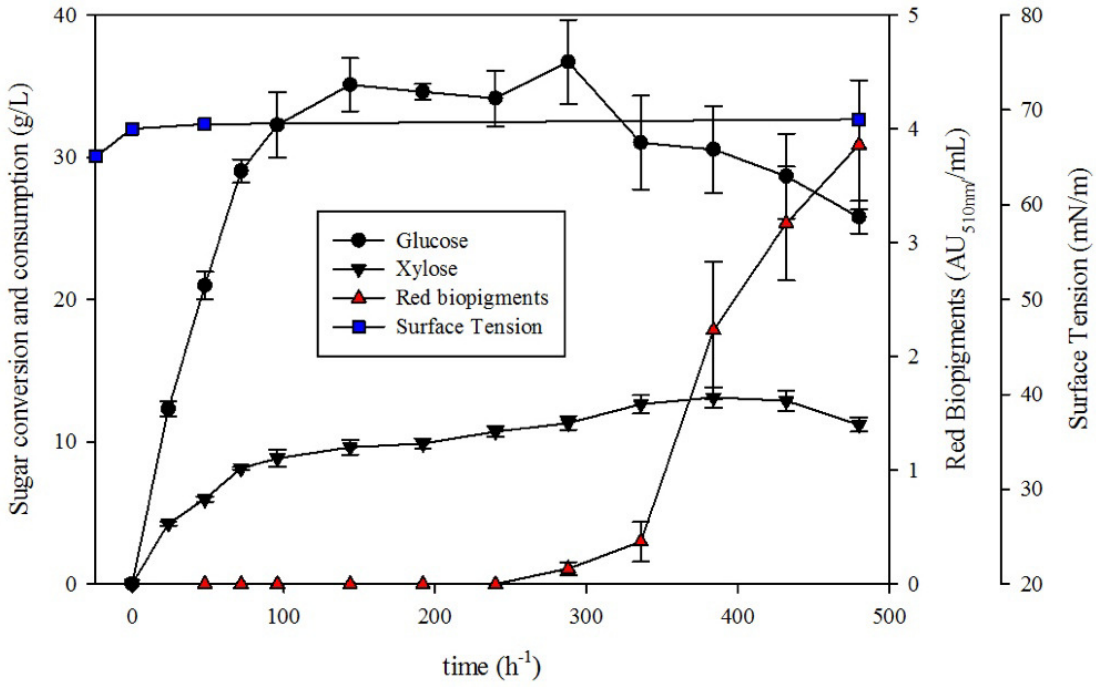

Figure 19. Semi-simultaneous Saccharification and Fermentation to produce red biopigments using the pretreated sugarcane bagasse under the optimized condition.

It can be also observed that the production of *Monascus* pigments is associated to the higher glucose consumption rate compared to the xylose one, as can be seen in the Figure 10 after 288 hours of process. Hilares et al. (2018), reported a similar consumption rate when *Monascus ruber* was used to produce red pigments in an enzymatic hydrolysate from an alkaline pretreated sugarcane bagasse in separate hydrolysis and fermentation. In this work was analyzed the surface tension during the whole process, to associate the surface tension with the extracellular products released during the process. However, no difference was detected between the initial and final surface tension in SSSF process. In overall, the SSSF strategy in fluidized bed reactor is an interesting option to produce high value-added products as biopigments. Nevertheless, further studies must be made to optimize the uptake of the carbon sources during the process and increase the productivity of the target molecule.

5.4. Conclusion

Non-ionic surfactants are versatile molecules that could be applied in different important steps in biorefineries such as pretreatments. The addition of Tween 80 during alkaline pretreatments has positive effect on the saccharification of sugarcane bagasse. In addition, under optimized conditions, the hydrodynamic cavitation surfactant-assisted

alkaline pretreatment showed an increment of about 40% in lignin removal and monomeric sugar release, respectively, for sugarcane bagasse. The proposed process is a potential strategy to produce Monascus pigments, and the presence of surfactants during the process make feasible the release, separation, and production of different molecules in the key steps of lignocellulosic biomass management.

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CHAPTER VI

Conclusions, Future works, and List of publications

6.1 Conclusions

The main conclusions are listed as following:

The non-ionic surfactant optimized formulation between Tween 20 and PEG 400 (0.66 % m/v and 1.16 % m/v, respectively) enhances the enzymatic hydrolysis of sugarcane bagasse celullignin, and it could also reduce about four times the enzyme dosage, maintaining its saccharification yield.

The same formulation also promotes enzyme stability in higher temperatures and shear force stress and enhances *Monascus* pigments production. It was also reported that the use of the surfactant optimized formulation in SHF and SSSF strategies were established for *Monascus ruber* pigments production.

The non-ionic surfactant optimized formulation between Triton X-100 and Tween 80 (25.61 and 4.39 g/L, respectively) increases the *Monascus ruber* pigment production when added to the fermentation of sugarcane by-products hemicellulosic hydrolysates.

Those derived sugarcane bagasse and sugarcane straw hemicellulosic hydrolysates pigments produced by *Monascus ruber* were also reported with high thermostability with activation energy values of 7.2 and 14.7 Kcal.mol^{-1}, respectively.

The optimized condition for an alkaline pretreatment assisted by hydrodynamic cavitation and Tween 80 (60º C, 10 min, 2M of NaOH, and 5.0 % (m/m) of Tween 80) increases the saccharification potential and lignin removal of sugarcane bagasse about 40% when compared to the control without the surfactant addition.

Surfactants are versatile molecules that could assist separated and sequential key steps (e.g., pretreatments, enzymatic hydrolysis, and fermentation) processes when a lignocellulosic biomass is used (e.g., sugarcane bagasse) to produce high value-added products as biopigments.

6.2 Suggestions for future works

To evaluate process intensification strategies to produce biofuels and biopigments from a C6 and C5 sugars enzymatic hydrolysate of sugarcane bagasse.

To evaluate the stability of cellulases and oxidative enzymes using a surfactant formulation in a hydrodynamic cavitation system for a sequential pretreatment and saccharification strategy to make feasible the production of high value-added products as biopigments.

To evaluate the possible applications of *Monascus* pigments produced from sugarcane byproducts hemicellulosic hydrolysates in cosmetics and textile areas.

6.3 List of publications

1. **Muñoz, S. S**., Balbino, T. R., Alba, E. M., Barbosa, F. G., de Pier, F. T., de Almeida, A. L. M., Balan-Zilla, A. H., Antunes, F. A. F., Hilares, R. T., Balagurusamy, N., Dos Santos, J. C., & da Silva, S. S. (2022). Surfactants in biorefineries: Role, challenges & perspectives. **Bioresource Technology**, 345, 126477. https://doi.org/10.1016/j.biortech.2021.126477
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