Wesley Leoricy Marques

Metabolic engineering of *Saccharomyces cerevisiae* aimed at improving the energetic yield of sucrose metabolism

Master thesis submitted to Programa de Pós-Graduação Interunidades em Biotecnologia USP/Instituto Butantan/IPT for the degree of Master in Science - Biotechnology.

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Candidato(a):	Wesley Leoricy Marques.
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To my parents, Maria Inês and José Marques; to my sister Wânia and to my brothers Walcirlei and Wagner. Also, to my precious Ana, Marina, Gabriel and Vinícius.

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“The key is man’s power of accumulative selection: nature gives successive variations; man adds them up in certain directions useful to him. In this sense he may be said to make for himself useful breeds”.

Charles Darwin. “The Origin of Species” 1859

“Não convém a gente levantar escândalo de começo; só aos poucos é que o *escuro é claro*”.

Guimarães Rosa. “Grande Sertão: Veredas” 1956

ABSTRACT

MARQUES, W. L. **Metabolic engineering of *Saccharomyces cerevisiae* aimed at improving the energetic yield of sucrose metabolism**. 2014. 56 p. Master's thesis (Biotechnology) – Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2014.

Industrial production of organic acids using the yeast *Saccharomyces cerevisiae* is still an expensive process compared to petrochemical routes, mainly due to costs related to bioreactor aeration. On the other hand, anaerobic production of organic acids in yeast is hampered by insufficient free-energy conservation for growth and acid export. In this work, an engineered *S. cerevisiae* strain was constructed, aiming at creating a biological platform for fully anaerobic organic acid production. The strategy adopted was based on the use of sucrose as the carbon source, due to its low cost and wide availability in Brazil. The native route for sucrose metabolism (extracellular hydrolysis coupled to facilitated uptake of the resulting monosaccharides) was replaced by facilitated sucrose transport (using *Phaseolus vulgaris* sucrose facilitator 1, *PvSUF1*) coupled to intracellular sucrose phosphorolysis (using *Leuconostoc mesenteroides* sucrose phosphorylase, *LmSPase*). With this strategy, it is expected that free-energy conservation in yeast under anaerobic conditions increases by 25% (from 4 to 5 moles of ATP per mole of sucrose consumed), which would in theory allow for grow and organic acid production under anaerobiosis. To test this concept, genes encoding native sucrose transporters and hydrolases were deleted. The knockout strain showed no sucrose-transport activity and a low residual sucrose hydrolytic activity (0.17 mmol mg protein^{-1} min^{-1}). Expression of *LmSPase* and *PvSUF1* resulted in slow growth aerobically (0.04 h^{-1}) and no growth anaerobically. An evolutionary strategy was pursued using sequential batch cultivations, during which the oxygen availability was systematically lowered until fully anaerobic conditions were achieved. At the end of this project, a yeast population that grows anaerobically (0.12 h^{-1}) was obtained. This population will be used futurely to characterize single-colony isolates in terms of anaerobic free-energy conservation. If a mutant is obtained displaying increased ATP yield, it can become a platform for fully anaerobic production of organic acids in yeast, an organism that presents several desirable traits for such processes (e.g. simple nutritional requirements, good growth at low pH, robustness towards harsh industrial conditions).

Keywords: *Saccharomyces cerevisiae*. Metabolic engineering. Bioenergetics. Sucrose phosphorylase. Sucrose Facilitator. Evolutionary engineering.

RESUMO

MARQUES, W. L. **Engenharia metabólica de *Saccharomyces cerevisiae* para o aumento do rendimento energético do metabolismo da sacarose.** 2014. 56 f. Dissertação (Mestrado em Biotecnologia) – Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2014.

A produção industrial de ácidos orgânicos através da levedura *Saccharomyces cerevisiae* ainda é um processo caro se comparado com a obtenção por rota petroquímica, principalmente em função da necessidade de aeração de biorreatores. Por outro lado, a produção de ácidos orgânicos em anaerobiose não ocorre devido à insuficiente produção de energia-livre celular, a qual também é necessária para a manutenção e crescimento celulares. Nesse trabalho, foi construída uma linhagem transgênica de *S. cerevisiae* com o objetivo de criar uma plataforma biológica para a produção de ácidos orgânicos em anaerobiose. A estratégia adotada se baseia no uso de sacarose como fonte de carbono dado seu baixo custo e vasta disponibilidade no Brasil. O metabolismo nativo da sacarose (hidrólise extracelular acoplada ao transporte facilitado dos monossacarídeos liberados) foi substituído pelo transporte facilitado desse dissacarídeo (usando o facilitador de sacarose de *Phaseolus vulgaris* 1, *PvSUF1*) além da fosforilação intracelular do açúcar (usando a sacarose fosforilase de *Leuconostoc mesenteroides*, *LmSPase*). Através dessa estratégia, é esperado que, em anaerobiose, a energia-livre celular aumente em 25% (de 4 para 5 moles de ATP por mol de sacarose consumida), o que, em teoria, seria suficiente para permitir crescimento celular e produção de ácido orgânico em anaerobiose. Nesse contexto, genes relacionados com o transporte e hidrólise de sacarose foram deletados resultando em uma linhagem incapaz de transportar sacarose e com baixa atividade de hidrólise desse açúcar (0.17 mmol mg proteína^{-1} min^{-1}). A expressão de *LmSPase* e *PvSUF1* restituíram o crescimento em sacarose, porém apenas em aerobiose e com uma velocidade específica baixa (0.04 h^{-1}). Foi então realizada engenharia evolutiva através de cultivos em bateladas sequenciais, no qual a disponibilidade de oxigênio foi sistematicamente reduzida até que condições de completa anaerobiose foram atingidas. No final desse projeto, uma população que cresce em anaerobiose (0.12 h^{-1}) foi obtida. Ela servirá para o isolamento de mutantes cuja produção de energia-livre em anaerobiose será quantificada. Uma vez constatado aumento da produção celular de ATP, ter-se-á uma alternativa para a produção de ácidos orgânicos em anaerobiose *usando S. cerevisiae*, um organismo que apresenta várias características desejáveis para esse tipo de processo (ex: requerimentos nutricionais simples, bom crescimento em baixo pH e robustez frente a estresses inerentes ao processamento industrial).

Palavras-chave: *Saccharomyces cerevisiae*. Engenharia metabólica. Bioenergética. Fosforilase de sacarose. Facilitador de sacarose. Engenharia evolutiva.

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LIST OF ABBREVIATIONS AND ACRONYMS

2 µm ori – plasmid 2 µm replication origin

ABC – ATP Binding Cassette

ADP – Adenosine Diphosphate

AmpR – Ampicillin resistance gene

ATP – Adenosine Triphosphate

cv – cultivar

DNA – Deoxyribonucleic acid

FAO – Food and Agriculture Organization of the United Nations

Fru – Fructose

Glc – Glucose

Glc-1P – glucose-1-phosphate

Glc-6P – glucose-6-phosphate

PCR – Polymerase chain reaction

pH – Hydrogen potential

Pi – Inorganic phosphate

PLA –Polylatic acid

P_{PMA1} – Promoter of gene *PMA1* (“Plasma Membrane ATPase”)

P_{TDH3} – Promoter of gene *TDH3* (glyceroldehyde-3-phosphate dehydrogenase)

SUC – Sucrose

SUF – Sucrose Facilitator

T_{ADH1} – Terminator of gene *ADH1* (alcohol dehydrogenase)

TAE – buffer Tris-Acetate-EDTA

T_{CYC1} – Terminator of gene *CYC1* (cytochrome c1)

T_{TDH3} – Terminator of gene *TDH3* (glyceroldehyde-3-phosphate dehydrogenase)

UNICA – Brazilian Sugarcane Industry Association

$V_{MÁX}$ – maximum reaction velocity

LIST OF SYMBOLS

$ – USA dollar

> – less than the following value

± – positive or negative variation of the following value

× g – earth gravitational force times the value before

≈ – approximately

°C – degrees Celsius

μ or μ_{max} – maximum specific growth rate

μm – micrometer(s)

g – gram(s)

g l^{-1} – gram(s) per liter

g/L/h – gram(s) per liter per hour

H^+ – hydrogen ion

Kcat – turnover number

kJ/mol – kilojoule(s) per mol

Km – Michaelis-Menten kinetics

kt – kiloton(nes)

M – molar

m/v – concentration: mass per total volume

mg l^{-1} – milligram(s) per liter

min – minute(s)

ml min^{-1} – milliliter(s) per minute

mM – millimolar

nm – nanometer(s)

rpm – rotation(s) per minute

s – second(s)

U – units of enzyme activity

v/v – concentration: volume per total volume

W – watts

$\Delta G'_0$ – Gibbs free energy variation at pH7

ΔG_0 – Gibbs free energy variation at standard conditions (e.g.: pH0)

SUMMARY

1 INTRODUCTION

There are commercially relevant molecules, such as carboxylic acids, that require aerated reactors to be produced by *Saccharomyces cerevisiae*, a well-known cell factory microorganism. Financial and environmental benefits can be achieved if the production of these compounds could be performed anaerobically (ABBOTT et al., 2009). The emerging problem is that, without respiratory metabolism, *S. cerevisiae* cannot produce enough ATP to support cell maintenance and product export (DE KOK et al., 2011; VAN MARIS et al., 2004). For that reason, increasing free-energy conservation (ATP) under anaerobic conditions in *S. cerevisiae* is the goal of this work.

This work was inspired by a previous work from our Dutch collaborators that describes an increase in ATP conservation from maltose metabolism in yeast, through the replacement of maltose hydrolysis by phosphorolysis (DE KOK et al., 2011). However, this increase in free-energy conservation on maltose, from 3 ATP to 4 ATP moles per mole of maltose consumed, is still not enough to allow for homofermentative organic acids production under anaerobiosis. This is because, different from ethanol, which is exported from the cells via simple diffusion, the export of organic acids requires ATP. Thus, more than 4 ATP moles are necessary to provide enough energy for the cells to grow and to export the organic acid produced under anaerobiosis. The authors already pointed out that, to accomplish this goal, the replacement of the active substrate uptake system by a passive mechanism could be a solution.

The work presented in this thesis focused on sucrose consumption, instead of maltose, due to the importance of this substrate for Brazilian industrial biotechnology. The native route for sucrose catabolism in yeast (extracellular hydrolysis and passive uptake of the resulting monosaccharides) was replaced by passive sucrose uptake and intracellular phosphorolysis, which has the potential to increase free-energy conservation from 4 to 5 ATP moles per mole of sucrose consumed anaerobically. The yeast strain obtained should be a suitable platform to produce lactic or other organic acids without medium aeration.

The project idea was conceived jointly by Dr. Ton van Maris (Delft University of Technology, The Netherlands) and Dr. Andreas K. Gombert (formerly at the University of São Paulo and currently at the University of Campinas). The transgenic

strains were constructed in Brazil and in The Netherlands, while the characterizations were performed at TU Delft. Besides Dr. Ton van Maris, Prof. Jack Pronk and the Ph.D. student Robert Mans have also been involved in this work.

2 LITERATURE REVIEW

2.1 The relevance of organic acids

Decreasing oil reserves and concerns on climate change represent major drivers for the development of new, non-petrochemical production routes for bulk chemicals that are based on renewable feedstocks. Industrial biotechnology and, in particular, the microbial fermentation of carbohydrate feedstocks is one of the alternative approaches that are currently under development. Many petroleum-derived chemicals can be directly or functionally substituted with chemicals from renewable feedstocks. Among these compounds, several organic acids — specially carboxylic acids, such as fumaric, malic, succinic and lactic acids — may fulfill a role as platform molecules using their (multiple) functional groups as targets for enzymatic or chemical catalysis (ABBOTT et al., 2009; JANG et al., 2012).

In 2004, the Department of Energy from the United States of America identified 10 organic acids as key chemical building blocks that can potentially be derived from plant biomass (WERPY; PETERSEN, 2004). Similarly, in 2006, the European focus group BREW identified 21 key compounds that can be produced from renewable feedstocks, a number of which are organic acids (PATEL et al., 2006). These studies reaffirm the great economic potential of microbially produced organic acids (SAUER et al., 2008).

Lactic acid is an example of a compound for which production via renewable technology is already commercial. The forecast for the annual lactic acid market growth is 9% per year until 2017 (IHS, 2013). Due to its chemical properties (acidulant, flavoring agent, pH buffering agent, preservative, biodegradable solvent, moisturizer agent, *etc*), lactic acid is used in diverse industrial segments such as beverages, food, cosmetics and pharmaceuticals. Polymerization of lactic acid produces a biodegradable polymer — polylactic acid (PLA) — which is used in food packaging including rigid containers, shrink-wrap and short shelf-life trays, as well as mulch films and rubbish bags. PLA is the major current use of lactic acid and projections point to an expressive increase in its demand in the coming years (Figure 1). Almost all lactic acid produced globally (300-400 kt) is manufactured by fermentation routes due to their low production costs and market drivers for

biorenewable products. The market price varies between $1300 and $1600 per tonne (HIGSON, 2011).

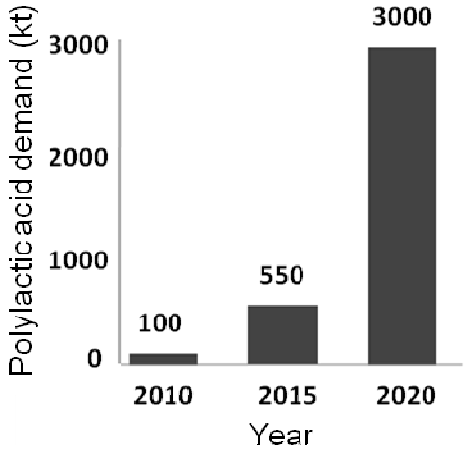

Figure 1: Global polylactic acid demand projection for 2015 and 2020 (CSM, 2012).

Differently from lactic acid, succinic acid is obtained from petroleum (16 kt per year); it has a smaller market size and Reverdia, a joint venture between DSM and Roquette Frères, is the only company already operating a commercial scale plant for succinic acid production, whose activities started late in 2012 based on yeast fermentation. With a competitive price (≈ $550 per tonne), succinic acid could replace petroleum-derived maleic anhydride, which has a market volume of 213 kt per year (SAUER et al., 2008) that is predicted to increase around 10% per year (HIGSON, 2010; XU; GUO, 2010). Nevertheless, multinational corporations like BASF, DSM and Roquette already announced plans of installing succinic acid production plants based on carbohydrate fermentation in Brazil (MYRIANT, 2012; REVERDIA, 2012).

2.2 Organic acids production: bacteria *vs* yeast

In this section, lactic acid production will be taken as an example — because its production through microbial fermentation is already in commercial scale — but the principles here discussed can also be applied for the production of other organic acids.

Lactic acid is produced by bacterial fermentation of starch. *Lactobacillus acidophilus* and *Streptococcus thermophilus* are the prokaryotic species used most widely in this industry. After fermentation has been concluded, evaporation is carried out to concentrate the final product. Yields of lactic acid in commercial operations are typically above 90% of the maximum theoretical yield, which is 1 gram of product per

gram of substrate consumed (Litchfield, 1996; VAN MARIS 2004a). These data show that bacterial fermentation allows for high substrate conversions into product; however, some biological bottlenecks inherent to the use of prokaryotes represent expensive costs in the process (LEE et al., 2011; ZHAO et al., 2013). Two bottlenecks will be highlighted:

- expensive culture medium: in general, bacteria require additional nitrogen and vitamins to grow properly (MORISHITA et al., 1981; TARANTO et al., 2003; WEE et al., 2006; ZHAO et al., 2013);
- low pH intolerance: due to organic acid production and export to the extracellular medium, the culture pH decreases during fermentation. Because bacteria cannot tolerate acidic conditions, the reactor pH has to be controlled through external addition of base (e.g. KOH or $Ca(OH)_2$). The added base reacts with the organic acid and is converted to the corresponding salt. Previously to product recovery, a cationic exchange operation is carried out to convert the salt back into the free acid. Bozell and Petersen (2010) report that this final conversion produces one tonne of by-product (e.g. gypsum, $CaSO_4$) per tonne of recovered acid. The proper disposal of this by-product represents an additional concern; and pH regulation, an additional process cost.

In this context, it is necessary to search for microorganisms that can produce organic acids at more competitive costs. An option pointed by the scientific community (VAN MARIS et al., 2004) is *Saccharomyces cerevisiae* due to its cheap nutritional requirements and its preference for acidic environments (pH 4 - 6), which dispenses reactor pH control and some product-recovering steps (SMIDT, 2011).

2.3 Microbial export of organic acids

Use of yeasts for organic acids production exempts the process from pH control but prejudices acid excretion. The reason for that relies on yeast cytosolic pH, which is close to 7. Intracellularly, organic acids exist dissociated — lactic acid pKa, for instance, is 3.86 (THE MERCK INDEX, 1989). In this situation, protons have to be exported against their concentration gradient; the extracellular pH is acidic, as discussed above (VAN MARIS et al., 2004b; KONINGS et al., 1995). Besides protons, the corresponding acid anion also has to be transported against an intense and increasing concentration gradient (since the product accumulates extracellularly

along the process). It is important to keep in mind that organic acids production is only economically viable when the product concentration is close to or higher than 100 g l^{-1} (VAN MARIS et al., 2004b and ZHAO et al., 2013). To sum up, cells can only transport these ions against their concentration gradients by using energy.

Diverse organic acids carriers can be found in several organisms, from bacteria to humans (KONINGS et al., 1995). There are proton symporters that utilize energy from anion export to expulse proton concomitantly; but it works only while extracellular product concentration is low (MICHELS et al., 1979; KONINGS et al., 1997). There are also antiporters that use energy from substrate uptake for product export: see malate/lactate antiport in malolactic bacteria (LOUBIERE et al., 1992; POOLMAN et al., 1991). Another example are primary transporters, in which energy from a chemical reaction is converted into driving force for product export, such as ATP-Binding Cassette (ABC) transporters (HIGGINS, 1992).

However, in *S. cerevisiae*, several acid exporters are unknown. For lactic acid, as an example, some candidate genes/proteins were already assayed, without success (e.g. *JEN1*, *MCH1* to *-5*; MAKUC et al., 2001). Van Maris et al. (2004b) propose that ATP-Binding Cassettes are the most likely mechanism for lactic acid export. With this mechanism, the energy released from the hydrolysis of one ATP mole would be enough to export one mole of the acid, resulting in the extracellular accumulation of cost-effective quantities of product.

2.4 Aerobiosis *vs* anaerobiosis

The additional energy required for acid excretion does not impair cell growth and acid production when yeast cultivation is performed in aerated fermenters, because, under these conditions, enough ATP is produced by oxidative phosphorylation. However, aeration of industrial scale reactors might render organic acid production economically non-competitive, because of the low commercial value of organic acids and due to the costs involved in fermenter stirring, cooling and air compression. Besides, respiratory metabolism decreases the theoretical substrate conversion into product due to carbon lost as CO_2 gas (DE KOK et al., 2011).

Therefore, recent efforts have focused on organic acids production by yeast in anaerobic reactors. The multinational company DSM, for instance, describes in a patent from 2012 a *S. cerevisiae* transgenic strain able to produce succinic acid in

anaerobiosis (0.21 g/l/h) (JANSEN et al., 2012). However, ethanol is concomitantly produced (0.28 g/l/h) decreasing the succinic acid yield. However, this ethanol formation is necessary in DSM's strain because it guarantees enough cellular energy for acid excretion and cell maintenance. The productivity values reported in this patent are rather low, since PATEL et al. (2006) estimate that an industrial process for carboxylic acid production only becomes economically viable when productivities of 2.5 g/l/h are achieved.

2.5 ZERO-ATP pathway

Through the classic glycolytic pathway (Embden-Meyerhof), glucose conversion into lactic acid, for instance, produces 2 ATP and 2 lactic acid molecules per hexose consumed, in a redox neutral process. However, the export of both acid molecules consumes 2 ATP (Figure 2). This is a "zero-ATP pathway" example that impairs cell maintenance and growth due to lack of cellular free-energy (ATP) (VAN MARIS et al., 2004a).

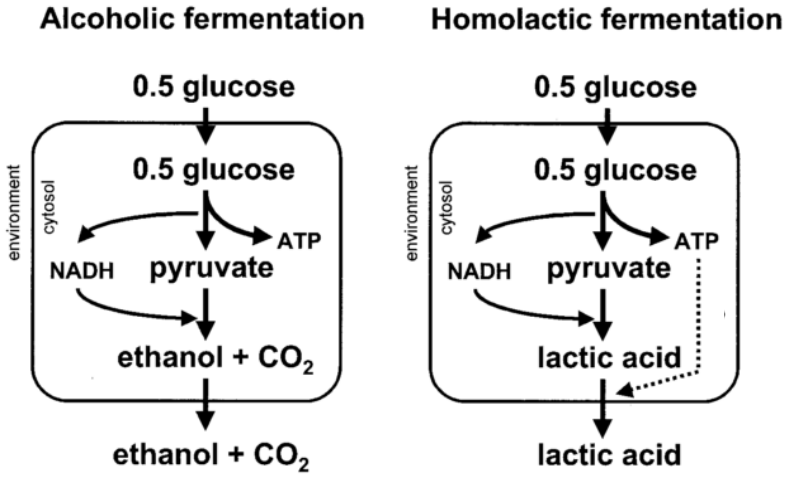

Figure 2: Ethanol and lactic acid metabolism in *Saccharomyces cerevisiae* under anaerobic conditions. In the left, ethanol passively diffuses through cell membranes and the only ATP molecule produced from 0.5 glucose oxidation can be utilized for cell maintenance and growth. In the right, lactic acid export consumes the only ATP molecule produced resulting in a "zero-ATP pathway", which impairs cell viability in anaerobic conditions. Adapted from Van Maris et al. (2004b).

Increasing free-energy (ATP) conservation from substrate catabolism is a suitable alternative to enable organic acids production under anaerobic conditions (DE KOK et al., 2011). In this context, this project aimed at improving the energetic yield of sucrose metabolism by *Saccharomyces cerevisiae* under anaerobiosis. Sucrose was chosen as the substrate, since it is the main sugar present in sugar-

cane-based raw materials, which are abundant and cheap in Brazil.

2.6 Sucrose

Sucrose — α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside — is the most available low molecular weight carbohydrate in the world (PETERS et al., 2010). The main sources of sucrose are sugar-cane and sugar-beet (ARD, 2013). Brazil is the main sugar-cane producer in the world with 588 million tons harvested in the crop year 2012/2013, which is two times more than India, the second largest producer globally (BRAZILIAN SUGARCANE INDUSTRY ASSOCIATION, 2013a; FOOD AND AGRICULTURE ORGANIZATION, 2013). Brazilian sugar-cane costs 31.1 USD/tonne (crop year 2012/2013), which is less than the costs in China, Australia or USA, for instance (UNICA, 2013b and FAO, 2013).

Concerns about sugar-cane use for biofuels/biochemicals instead of its usage for food manufacture are still actual and polemic. However, it is important to highlight that land availability for sugar-cane plantation is huge in Brazil and can accommodate cultures for food and also for biofuels/biochemical, at least when the domestic demands are considered. Currently, only 1.1% (9 million hectares) of the Brazilian territory accounts for sugar-cane culture (UNICA, 2013b) and these plantations can be expanded to 74 million hectares, without compromising the Amazon forest or other protected biomes, according to the last sugar-cane agro-ecological zoning (MANZATTO et al., 2009). Besides this, yeast fermentation can also be performed using a by-product of edible sugar production, namely molasses, which consists of a highly concentrated sucrose solution that remains after the sugar crystallization process (AMORIM et al., 2011)

2.7 Sucrose phosphorolysis *vs* hydrolysis

Saccharomyces cerevisiae hydrolyzes sucrose extracellularly through invertase (Reaction 1 and Figure 3), encoded by the *SUC* genes. This protein is mainly secreted from the cells, but a tiny fraction remains in the intracellular environment being responsible for a low, but constitutive intracellular invertase activity (CARLSON; BOTSTEIN, 1983). In this work, an alternative to conserve the energy dissipated during sucrose hydrolysis was explored. It involves the replacement of sucrose hydrolysis by phosphorolysis. In the phosphorolytic cleavage, one inorganic

phosphate is added during sucrose conversion, resulting in glucose 1-phosphate and fructose (reaction 2) (GOEDL et al., 2007).

$$\text{sucrose} + H_2O \xrightarrow{\text{extracellular}} \text{fructose} + \text{glucose} \quad (1)$$

$$\text{sucrose} + Pi \xrightarrow{\text{intracellular}} \text{fructose} + \text{glucose-1-phosphate} \quad (2)$$

Using the phosphorolytic pathway, one extra ATP molecule can be conserved, when compared to conventional invertase-driven sucrose hydrolysis, because hexokinase activity for one of the hexose sugars becomes needless (Figure 3b). Hexokinase is the first enzyme in glycolysis and consumes one ATP for glucose phosphorylation; differently from phosphorylase that uses inorganic phosphate for that purpose (ZHANG; LYND, 2005). In nature, this elegant strategy is ubiquitous for polysaccharide intracellular catabolism, while hydrolases perform extracellular cleavages (MADIGAN et al., 2012).

An important detail of phosphorolytic activity is the site used for phosphorylation. While hexokinase inserts a phosphate group at glucose carbon “6”, phosphorolysis inserts a phosphate at carbon “1”. Because of that, α-glucose-1-phosphate isomerisation to α-glucose-6-phosphate is necessary. Phosphoglucomutases (Pgm1p and Pgm2p) are native yeast enzymes that can perform this isomerisation (BOLES et al., 1994). Special attention needs to be taken when sugars different from sucrose are used as substrate. During maltose phosphorolysis, for instance, β-glucose-1-phosphate is produced instead of α-glucose-1-phosphate. In such cases, the native α-phosphoglucomutases are inefficient due to their inability to convert compounds in the “β” configuration. In this scenario, a heterologous β-phosphoglucomutase, e.g. from *Lactococcus lactis*, can be recruited (DE KOK et al., 2011).

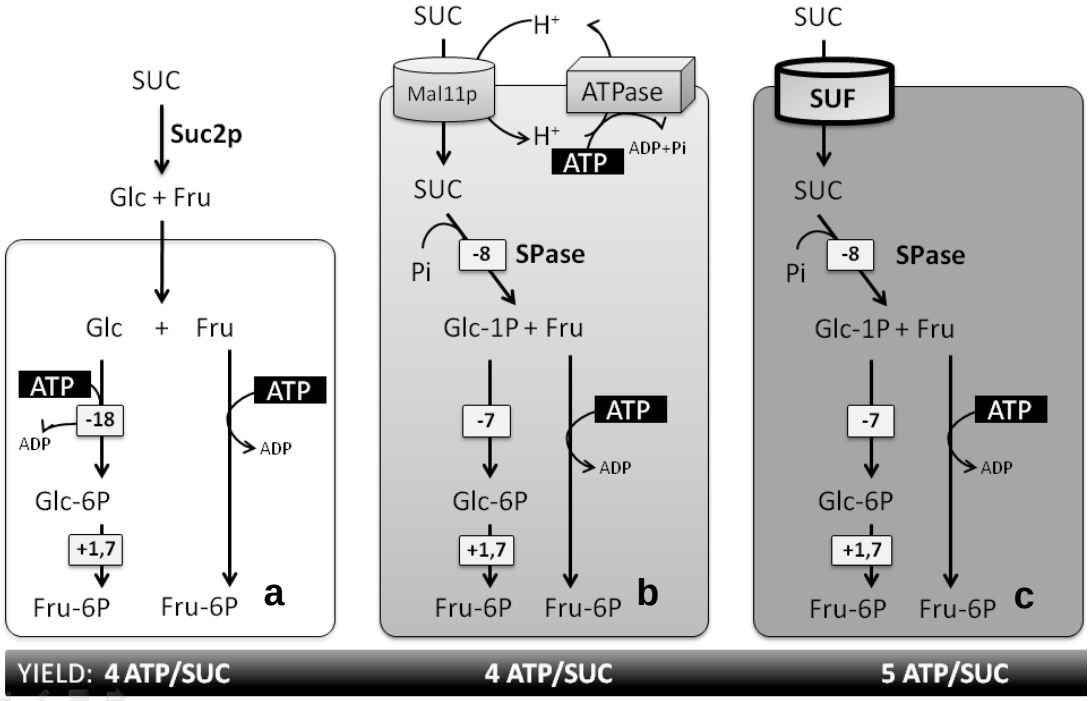

Figure 3: Schematic representation of the strategy used in this study to increase ATP conservation in *Saccharomyces cerevisiae*. **a**: wild type strain; **b**: expression of sucrose phosphorylase (SPase) replacing the native sucrose hydrolase; **c**: replacement of the active sucrose uptake by a passive one using plant sucrose facilitators. Numbers inside squares indicate standard Gibbs free-energy differences of the chemical reactions (ΔG_0', in kJ mol^{-1}). SUC: sucrose; Fru: fructose; Glc: glucose, SUF: sucrose facilitator; Pi: inorganic phosphate.

2.8 *Leuconostoc mesenteroides* sucrose phosphorylase

In the scientific literature, phosphorolytic enzymes that act on sucrose, maltose, cellobiose, trehalose and lactose were already described (ALEXANDER, 1968; BELOCOPITOW; MARECHAL, 1970; DE GROEVE et al., 2009; DOUDOROFF, 1955; KOGA et al., 1991). Recently, DE KOK et al. (2011) increased free-energy conservation by 1 ATP in *S. cerevisiae* through heterologous expression of a maltose phosphorylase from *Lactobacillus sanfransiscensis*. In a similar manner, in the present study we expressed sucrose phosphorylase (SPase, EC 2.4.1.7) in *S. cerevisiae* aimed at improving cell free-energy conservation under anaerobic conditions from 4 to 5 ATP moles per mole of sucrose consumed (Figure 3b).

SPase from diverse microorganisms were already characterized — e.g. *Bifidobacterium adolescentis* (VAN DEN BROEK et al., 2004), *Pelomonas saccharophila* (SILVERSTEIN et al., 1967; WEIMBERG; DOUDOROFF, 1954) and *Streptococcus mutans* (FERRETI et al., 1988; ROBESON et al., 1983; RUSSEL et al., 1988) —. In this work the SPase from *Leuconostoc mesenteroides* (*LmSPase*)

was chosen for heterologous expression in yeast because: *i*) its optimum pH and temperature are 7 and 30 ºC, respectively, i. e., the conditions found in the yeast cytosol; and *ii*) this is a monomeric enzyme, i.e., the chance of folding errors is minimized (GOEDL et al., 2007; 2010; KAWASAKI et al., 1996; LEE et al., 2006; 2008).

There are several *L. mesenteroides* strains and their SPase properties vary. In this scenario, *L. mesenteroides* ATTC 12291 SPase was chosen due to its higher kcat/Km value compared to SPases from other strains (AERTS et al., 2011), e.g. *L. mesenteroides* NRRL B1149 (LEE et al., 2008), and *L. mesenteroides* NRRL B1355 (AERTS et al., 2011).

Besides kinetic parameters, thermodynamics is also relevant in this study. Replacement of sucrose hydrolysis ($\Delta G'_0$ = -26 kJ/mol) by phosphorolysis ($\Delta G'_0$ = -8 kJ/mol) shouldn't represent a thermodynamic concern because Gibbs free-energy changes will be maintained negative, which indicates a tendency for sucrose consumption rather than the opposite reaction (GOLDBERG et al., 1989).

Before LmSPase expression in yeast can be performed, a sucrose knockout strain is necessary, i.e. a strain that cannot grow on sucrose. For that purpose, native genes that cleave sucrose were previously deleted by De Kok et al. (unpublished) in the CEN.PK strain background and kindly provided for our work. The deleted genes are: *SUC2* (invertase); *MAL12*, *MAL22* and *MAL32*, maltases that also act on sucrose (BROWN et al., 2010; VOORDECKERS et al., 2012).

Another prerequisite for ATP conservation through successful expression of *LmSPase* in yeast is related to sucrose uptake. Differently from a wild-type strain, yeasts here constructed metabolize sucrose in the cytosol (Figure 3). In such a case, sucrose uptake is carried out by proton symporters, such as Mal11p (also known as Agt1p), Mal21p and Mal31p (CHOW et al., 1989). Consequently, in order to maintain cytosolic pH homeostasis, protons are excreted through plasma H^+-ATPases with a stoichiometry of 1 ATP consumed per proton transported (Figure 3b). For that reason, the SPase strategy — that should lead to 1 extra ATP conservation — fails, because the only ATP conserved has to be expended to transport one proton out of the cell. However, this issue can be avoided if sucrose transport is carried out by a sucrose facilitator instead of an active transporter. In this way, the final ATP conservation can be increased from 4 to 5 ATP moles per mole of sucrose consumed (Figure 3c).

2.9 Plant sucrose facilitators

Sucrose permeases can be found in plant tissues. Chen et al. (2012) identified sucrose facilitators in *Arabidopsis thaliana* (e.g. *AtSWEET12*) and in *Oryza sativa* (e.g. *OsSWEET11*). Overvoorde (1996) expressed a sucrose facilitator in *S. cerevisiae* but no significant sugar uptake was detected. On the other hand, Zhou et al. (2007) successfully expressed, in *S. cerevisiae*, sucrose facilitators from pea (*Pisum sativum sucrose facilitator 1* and *4*, *PsSUF1* and *4*) and French bean (*Phaseolus vulgaris sucrose facilitator 1*, *PvSUF1*) and characterized their transport properties. In the present work, *PsSUF1*, *PsSUF4* and *PvSUF1* were chosen to promote sucrose uptake via facilitated diffusion. These genes were individually expressed in strains lacking other potential sucrose transporters genes (*MAL11*, *MAL21*, *MAL31*, *MPH2* and *MPH3*) (DE KOK et al., 2011).

3 OBJECTIVE

The objective of this work is to obtain a *S. cerevisiae* strain successfully expressing a sucrose phosphorylase and a sucrose facilitator. In such a strain, it is expected that the energetic yield of anaerobic sucrose catabolism will be 5 ATP moles per mole of sucrose consumed, i.e. 25% higher than the corresponding yield in a wild-type strain.

4 MATERIALS AND METHODS

4.1 Strains and maintenance

The *S. cerevisiae* strains used and constructed in this study (Table 1) are congenic members of the CEN.PK family (ENTIAN; KOTTER, 1998; VAN DIJKEN et al., 2000). Strains were received in the laboratory in dried filter membranes and immediately grown at 30 °C on an appropriate solid medium, according to each strain's nutritional requirements, i. e., synthetic medium supplemented with uracil (30 μg/ml) and/or leucine (60 μg/ml), when necessary. Medium preparation was performed according to Verduyn et al. (1992), with 20 g l^{-1} initial glucose, which was the single carbon source. Next, single colony isolates were cultivated in shake-flasks containing 100 ml of the same medium used before. After overnight growth, 20% (v/v) glycerol was added and 1 ml aliquots were stored at −80 °C.

4.2 Gene deletions and plasmid construction

The target genes of this study, plasmids and primers are listed in tables 2, 3 and 4, respectively. Three main genetic modifications required in this work are detailed below:

i) knockout of native genes involved in sucrose uptake and hydrolysis. This task was performed in a previous work by De Kok et al. (unpublished) and involved sucrose transporters (*MAL11, MAL21, MAL31, MPH2* and *MPH3*) and sucrose hydrolytic enzymes (*MAL12, MAL22, MAL32* and *SUC2*);

ii) genome integration of sucrose transporter expression cassettes. Plasmid pUDI035 (DE KOK et al., 2011) was used for *MAL11* expression downstream of the *TDH3* promoter. *PsSUF1* and *PsSUF4* coding sequences were kindly donated by professor Dr. John W. Patrick (School of Environmental & Life Sciences, The University of Newcastle, Australia) in the plasmid pDR196 (ZHOU et al., 2007). From pDR196, these genes were cloned into pUDI35 via the XbaI and SalI sites resulting in pUDI085 and pUDI086, respectively (table 3). A synthetic *PvSUF1* was purchased from GenScript USA Inc. (New Jersey, USA) on its codon optimized version for expression in *S. cerevisiae* (OptimumGene™, GenScript USA Inc., New Jersey,

USA). From pUC57, the plasmid in which *PvSUF1* was delivered, it was cloned into pUDI035 via the SpeI and SalI restriction sites resulting in pUDI087 (table 3).

Table 1: *Saccharomyces cerevisiae* strains used in this study.

Strain	Relevant genotype	Source
CEN.PK113-7D	MATa *MAL1x MAL2x MAL3x MAL4x LEU2 URA3 MAL2-8^C*	P.Kötter, Germany
IMK291	MATa *leu2-112 ura3-52 MAL2-8^C Mal11-mal12::loxP mal21-mal22::loxP mal31-mal32::loxP mph2::loxP mph3::loxP suc2::loxP-KANMX-loxP*	Stefan de Kok (TU Delft)
IMX470	IMK291 pUDI084 (*LEU2*)	This study
IMU051	IMK291 pUDI084 (*LEU2*) pUDE260 (*URA3*)	This study
IMU054	IMK291 pUDI084 (*LEU2*) pUDE44 (*URA3 MAL12*)	This study
IMU052	IMK291 pUDI084 (*LEU2*) pUDE262 (*URA3 LmSPase*)	This study
IMX469	IMK291 pUDI35 (*LEU2 MAL11*)	This study
IMU048	IMK291 pUDI35 (*LEU2 MAL11*) pUDE260 (*URA3*)	This study
IMU055	IMK291 pUDI35 (*LEU2 MAL11*) pUDE44 (*URA3 MAL12*)	This study
IMU049	IMK291 pUDI35 (*LEU2 MAL11*) pUDE262 (*URA3 LmSPase*)	This study
IMX467	IMK291 pUDI086 (*LEU2 PsSUF4*)	This study
IMU042	IMK291 pUDI086 (*LEU2 PsSUF4*) pUDE262 (*URA3 LmSPase*)	This study
IMX466	IMK291 pUDI085 (*LEU2 PsSUF1*)	This study
IMU041	IMK291 pUDI085 (*LEU2 PsSUF1*) pUDE262 (*URA3 LmSPase*)	This study
IMX468	IMK291 pUDI087 (*LEU2 PvSUF1*)	This study
IMU056	IMK291 pUDI087 (*LEU2 PvSUF1*) pUDE44 (*URA3 MAL12*)	This study
IMU043	IMK291 pUDI087 (*LEU2 PvSUF1*) pUDE262 (*URA3 LmSPase*)	This study

Table 2: Genes expressed in *Saccharomyces cerevisiae* in this study.

Gene	GenBank	Organism
α-glucoside transporter (*MAL11*)	AJ012752.1	*S. cerevisiae* CEN.PK113-7D
α-D-glucosidase (*MAL12*)	853209	*S. cerevisiae* CEN.PK113-7D
Sucrose phosphorylase (*LmSPase*)*	D90314.1	*Leuconostoc mesenteroides* ATCC 12291
Sucrose facilitator (*PsSUF1*)	DQ221698.1	*Pisum sativum* (cv BC3)
Sucrose facilitator (*PsSUF4*)	DQ221697.2	*Pisum sativum* (cv BC3)
Sucrose facilitator (*PvSUF1*)*	DQ221700.1	*Phaseolus vulgaris* (cv Redland Pioneer)

*Codon optimized gene.

iii) *episomal expression of sucrose metabolizing enzymes*. Vector pUDE044, constructed by De Kok et al. (2011) (Table 3) was used in this study for *MAL12*

expression. It is an episomal multicopy plasmid that contains *MAL12,* amplified from *S. cerevisiae* CEN.PK113-7D genome, under control of the strong and constitutive promoter *TDH3*. *Leuconostoc mesenteroides sucrose phosphorylase* gene (*LmSPase*) was ordered as a synthetic gene from Baseclear B.V. (Leiden, The Netherlands). It was codon-optimized for expression in *S. cerevisiae* according to Grote et al. (2005) and delivered within pUD155. The BamHI and SalI restriction sites were used to clone *LmSPase* from pUD155 behind *TDH3* promoter (from pUDE63), an episomal multicopy plasmid, resulting in pUDE262 (Table 3).

Table 3: Plasmids used in this study.

Plasmid	Characteristics	Reference
pDR196-PsSUF1	2 µm ori, *URA3*, P_{PMA}-*PsSUF1*-T_{ADH}	Zhou et al., 2007
pDR196-PsSUF4	2 µm ori, *URA3*, P_{PMA} -*PsSUF4*-T_{ADH}	Zhou et al., 2007
pUC57-PvSUF1	Synthetic gene, pUC57, SpeI:*PvSUF1*:SalI, AmpR	GenScript USA Inc
pUD155	pUC57, attB1-*LmSPase*-attB2, AmpR	Baseclear B.V. NL
pUDI035	Integration plasmid, *LEU2*, P_{TDH3}-*MAL11*-T_{CYC1}	de Kok et al., 2011
pUDI084	Integration plasmid, *LEU2*, P_{TDH3}-T_{CYC1}	This study
pUDI085	Integration plasmid, *LEU2*, P_{TDH3}-*PsSUF1*-T_{CYC1}	This study
pUDI086	Integration plasmid, *LEU2*, P_{TDH3}-*PsSUF4*-T_{CYC1}	This study
pUDI087	Integration plasmid, *LEU2*, P_{TDH3}-*PvSUF1*-T_{CYC1}	This study
pUDE063	2 µm ori, *URA3*, P_{TDH3}-*pgmβ*-T_{ADH1}	de Kok et al., 2011
pUDE260	2 µm ori, *URA3*	This study
pUDE044	2 µm ori, *URA3*, P_{TDH3}-*Mal12*-T_{ADH1}	de Kok et al., 2011
pUDE262	2 µm ori, *URA3*, P_{TDH3}-*LmSPase*-T_{ADH1}	This study

4.3 Strain construction

Transformations of *S. cerevisiae* were carried out using the protocols of Gietz and Woods (2002). The sucrose knockout strain (IMK291, Figure 4) was constructed through the deletion of the *SUC2* gene from strain IMK289, which is a maltose knockout strain constructed by De Kok et al. (2011). *SUC2* removal was performed by de Kok et al. (unpublished) using the loxP-marker-loxP/ Cre recombinase system (GUELDENER et al., 2002). Gene knockout was confirmed by PCR in this work using primers *SUC2 Ctrl-F* and *SUC2 Ctrl-R* (Table 4).

Plasmids pUDI084, pUDI085, pUDI086, pUDI087, and pUDI035 were linearized with BstEII and transformed into IMK291, resulting in IMX470, IMX466,

IMX467, IMX468 and IMX469, respectively (Figure 4). Then, pUDE260 was transformed into IMX469 and IMX470, resulting in IMU048 and IMU051, respectively. pUDE044 was transformed into IMX468, IMX469 and IMX470, resulting in IMU056, IMU055 and IMU054, respectively. pUDE262 was transformed into IMX466, IMX467, IMX468, IMX469 and IMX470, resulting in IMU041, IMU042, IMU043, IMU049 and IMU052, respectively.

Table 4: Primers used in this study.

Name	Sequence (5'→3')	Purpose
SUC2 Ctrl-F	GTTGTTGTCCTAGCGTAGTTC	knockout confirmation
SUC2 Ctrl-R	GATTGGAGTTCCTTCGTTTC	knockout confirmation
PsSUF1-F	GATCTAGAATGGATAATCCTTC	cloning with XbaI site
PsSUF1-R	CAGTCGACCTAATGAAATCCAC	cloning with SalI site
PsSUF1-Seq	ATATCGGTCACTCATTCG	sequencing
PsSUF4-F	GATCTAGAATGCCGAATCCCGACTCTTC	cloning with XbaI site
PsSUF4-R	GTGTCGACTCATACTGGGTTTCTAGGC	cloning with SalI site
PsSUF4-Seq	TGGATGTTGCTAATAATGTC	sequencing
PvSUF1opt-F	ATCCCCAACAAAGCCTATC	sequencing
PvSUF1opt-R	TTAATGAAAACCACCTGCTAC	sequencing
LmSPase Seq1	CTACAAGAGAAAGGACAAGG	sequencing
LmSPase Seq2	GTGCTAACGTTAAGAAGAC	sequencing
pUDI035 Seq-F	ACCTTCTATTACCTTCTGC	sequencing
pUDI035 Seq-R	CGTTCTTAATACTAACATAAC	sequencing
pUDE063 Seq-F	CCTTCTATTACCTTCTGCTC	sequencing
pUDE063 Seq-R	CAATAAGAGCGACCTCATG	sequencing

Underlined nucleotides correspond to restriction sites.

4.4 Molecular biology techniques

PCR amplification was performed using Taq Phusion® Hot Start High-Fidelity DNA Polymerase (Thermo Scientific-Finnzymes, Espoo, Finland) according to manufacturer's instructions in a Veriti™ 96-Well Thermal Cycler (Applied Biosystems, Califórnia, EUA). DNA fragments were separated on a 1% (w/v) agarose (Sigma, St. Louis, USA) gel in 1X TAE (40mM Tris-acetate pH 8.0 and 1mM EDTA). Isolation of fragments from gels was performed with the illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Bio-Sciences Corp, New Jersey, EUA). Restriction endonucleases (Thermo Scientific-Fermentas, Texas, EUA), T4 DNA ligase

(Promega Corporation, Wiscosin, EUA) and RNase A from bovine pancreas (Sigma, St. Louis, EUA) were used according to manufacturer's instructions. Transformation and amplification of plasmids was performed in *E. coli RR1* (*Δ(gpt-proA)62, leuB6, thi-1, lacY1, hsdS$_B$20, rpsL20 (Str^r), ara-14, galK2, xyl-5, mtl-1, supE44, mcrB$_B$*) (BOLIVAR et al., 1977). Plasmids were isolated from *E. coli* with the illustra plasmid Prep Mini Spin Kit (GE Healthcare Bio-Sciences Corp, New Jersey, EUA). DNA constructs were sequenced by Instituto de Biociências from University of São Paulo (São Paulo, Brazil). Yeast genomic and plasmid DNA were extracted with ZR Fungal/Bacterial DNA MicroPrep™ and Zymoprep™ Yeast Plasmid Miniprep II (Zymo Research, California, EUA), respectively.

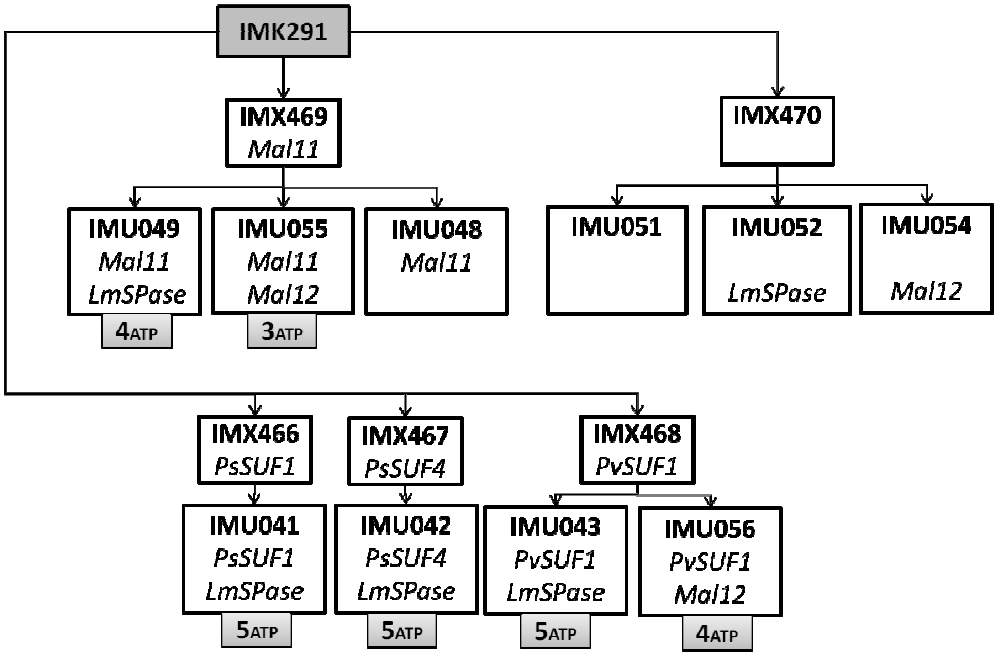

Figure 4: Strain construction pipeline. Each square represents one strain and clarifies about the genes being expressed. ATP values in grey boxes refer to the energy yield from one sucrose molecule consumed under anaerobiosis. All "IMU" strains are prototrophic due to the insertion of expression and/or empty vectors; IMU051, for instance, received two empty vectors to become prototrophic for uracil and leucine.

4.5 Cultivations

Medium composition: shake-flask cultivations were performed with synthetic medium containing per liter 20 g sucrose (Sigma, St. Louis, EUA), 5 g $(NH_4)_2SO_4$, 3 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, trace elements and vitamins as described previously by Verduyn et al. (1992). pH of the medium was set to 5.0 with 2 M KOH and 2 M H_2SO_4

prior to filter sterilization. For anaerobic cultures, medium was supplemented with the anaerobic growth factors ergosterol (10 mg l^{-1} final concentration) and Tween 80 (420 mg l^{-1} final concentration) dissolved in ethanol.

Cultivation apparatus and conditions:

a) the cultivation inocula were prepared transferring 1 ml of a frozen stock culture into 20 ml of the synthetic medium (as described before) containing glucose 2% (w/v). The inocula were grown overnight in 50 ml shake-flasks in an Innova incubator shaker (New Brunswick Scientific, Edison, USA) at 200 rpm and 30 ºC;

b) cultivations in glass test-tubes (20 mm diameter and 150 mm height) were performed with 4 ml synthetic medium containing sucrose 8% (w/v) as the sole carbon source in an incubator shaker (New Brunswick, Edison, USA) at 30 ºC and 200 rpm;

c) aerobic growth assays were prepared by inoculating 100 ml of medium in a 500 ml shake-flask, with an amount of the pre-culture to achieve an initial culture absorbance of 0.1 at 660 nm. The flask was subsequently incubated in an Innova incubator shaker (New Brunswick Scientific, Edison, USA) at 200 rpm and 30 ºC. Absorbance at 660 nm was measured with a Libra S11 spectrophotometer (Biochrom, Cambridge, UK);

d) for anaerobic characterization, 50 ml flasks containing 20 ml medium were incubated in an Bactron X anaerobic chamber (Shell Lab, Cornelius, OR, USA) at 30 ºC and 220 rpm (Heidolph Unimax 2010 shaker; Heidolph, Schwabach, Germany);

e) sequential batch cultivations were performed in shake-flasks and in reactors (Sequential Batch Reactors, SBRs). SBRs were carried out at 30 ºC in fermenters Infors HT Multifors 2 (Infors AG, Switzerland) with 100 ml working volume (500 ml total volume). This smaller working volume was possible due to manufacturer's special modifications on vessel and jacket size. The same medium as described above for anaerobic essays was used and additionally supplied with antifoam Pluronic® PE 6100 (BASF, USA). The antifoam was autoclaved separately (120 ºC) as a 0.15 g ml^{-1} solution and added to a final concentration of 0.15 g l^{-1}. The culture pH was maintained at 5.0 by automatic addition of 2 M KOH. Each fermenter was fed using a feed pump controlled by an electric level sensor. When the carbon and energy source was depleted, as indicated by a drop in the CO_2 level in the exhaust gas, a new cycle of batch cultivation was initiated by either manual or automated

replacement of *ca.* 90% of the culture with fresh medium. In order to select for anaerobically growing cells, the reactor oxygenation was lowered in each batch. This was achieved by variation of the following parameters: pressurized air/nitrogen mix on inlet gas; air flux (from 50 ml min^{-1} to 20 ml min^{-1}) and stirrer (from 1200 rpm to 250 rpm). For cycles in which exponential growth was observed, the maximum specific growth rate (μ_{max}) was estimated from the CO_2 profile. To minimize diffusion of oxygen, the bioreactors were equipped with Norprene tubing and Viton O-rings.

4.6 Sucrose determination

Sucrose concentration was analyzed via an enzymatic assay (10716260035, Boehringer Mannheim, Mannheim, Germany) according to manufacturer's instructions.

4.7 Enzyme activity measurements

For preparation of cell extracts, culture samples (around 62.5 mg cell dry weight) were harvested from exponentially growing shake flask cultures on 20 g l^{-1} of the appropriate carbon source. *LmSPase* constitutive expressing strains were grown on glucose due to their slow or inexistent growth on sucrose; CEN.PK113-7D was grown on sucrose; *MAL12* constitutive expressing strains were grown on ethanol instead of glucose to avoid any glucose catabolic repression effect. In the particular case of sucrose hydrolase activity determination from strain IMU048 (*malΔ mphΔ suc2Δ MAL11)*, sucrose was also added in the ethanol containing media to act as an inducer. Samples were harvested by centrifugation, washed twice with 10 mM potassium phosphate buffer (pH 7.5) containing 2 mM EDTA, and stored at - 20°C. Before cell breakage, samples were thawed at -4 ºC, washed and resuspended in 100 mM potassium phosphate buffer (pH 7.5) containing 2 mM $MgCl_2$ and 2 mM dithiothreitol. Extracts were prepared by sonication with 0.7 mm glass beads at 0°C for 2 min at 0.5 min intervals with an MSE sonicator (150 W output; 8 µm peak-to-peak amplitude). Unbroken cells and debris were removed by centrifugation (4 °C, 20 min, 36.000 × g). The supernatant was used as the cell extract for enzyme assays. Protein levels in cell extracts were determined using the Lowry assay (Lowry et al., 1951). Sucrose hydrolytic activity was measured at 30 ºC by monitoring the reduction

of $NADP^+$ at 340 nm in a 1 ml reaction mixture containing 50 mM imidazole-HCl (pH 7.0), 1 mM NADP+, 12.5 mM $MgCl_2$, 1 mM ATP, 3.5 units hexokinase, 3.5 units glucose-6-phosphate dehydrogenase and 1–40 µl cell extract. The reaction was started by the addition 100 mM sucrose (protocol adapted from de Kok et al., 2011). Sucrose phosphorylase activity was measured at 30 ºC by monitoring the reduction of $NADP^+$ at 340 nm in a 1 ml reaction mixture containing 200 mM potassium phosphate buffer (pH 7.0), 10 mM EDTA, 10 mM $MgCl_2$, 10 mM α-D-glucose 1,6-bisphosphate, 2 mM $NADP^+$, 2.65 U phosphoglucomutase, 5.25 U glucose 6-phosphate dehydrogenase and and 1–40 µl cell extract. The reaction was also started by the addition 100 mM sucrose. Protocol adapted from Goedl et al. (2007). An extinction coefficient of 6.3 mM^{-1} was assumed for NADPH.

5 RESULTS & DISCUSSION

5.1 Characterization of the sucrose knockout strain

To determine whether the *S. cerevisiae* genome encodes additional sucrose transporters and/or catalytic enzymes, growth tests were employed with strains derived from IMK291 (*malΔ mphΔ suc2Δ*). To analyze eventual residual activities of sucrose transport or sucrose hydrolysis, the sucrose hydrolase-encoding *MAL12* gene, the sucrose phosphorylase (*LmSPase*) gene and the α-glucoside active transporter *MAL11* (also known as *AGT1*) were re-introduced into IMK291, either alone or in combination. *MAL11* and *MAL12* were originally described as coding for a maltose transporter and a maltase, respectively, however, they also act on sucrose (BROWN et al., 2010; VOORDECKERS et al., 2012). The resulting prototrophic strains were tested in aerated shake flasks with sucrose as the sole carbon source. Under these conditions, CEN.PK113-7D (Suc^+, μ_{MAX} = 0.45 ± 0.01 h^{-1}) and IMU055 (*malΔ mphΔ suc2Δ MAL11 MAL12,* μ_{MAX} = 0.19 ± 0.01 h^{-1}) grew (Figure 5 and Table 5).

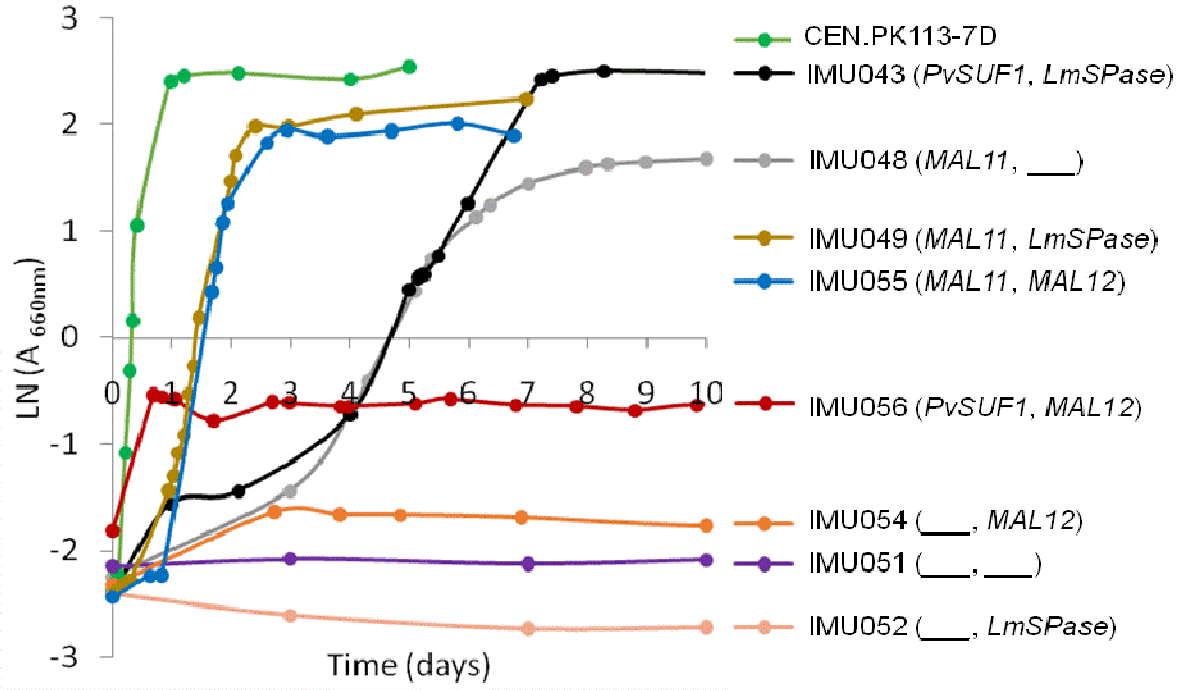

Figure 5: Growth curves of *Saccharomyces cerevisiae* strains obtained from shake flask cultivation under aerobic conditions. CEN.PK113-7D: *S. cerevisiae* reference strain; *MAL11*: α-glucoside active transporter; *PvSUF1*: *Phaseolus vulgaris* sucrose facilitator 1; *MAL12*: α-glucosidase (hydrolase); *LmSPase*: *Leuconostoc mesenteroides* sucrose phosphorylase. Cultivations were performed in synthetic media containing sucrose 2% (w/v) initial concentration.

IMU051 (*malΔ mphΔ suc2Δ*), IMU052 (*malΔ mphΔ suc2Δ SPase*) and IMU054 (*malΔ mphΔ suc2Δ MAL12*) did not show any growth during 10 days (μ_{MAX} <0.001 h^{-1}) (the slight initial growth observed for IMU054 and also for IMU056 was probably caused by the consumption of glucose that could be still present in the inocula) (Figure 5). These results give some indication that all sucrose transport activity was removed when the following genes were deleted: *MAL11*, *MAL21*, *MAL31*, *MPH2* and *MPH3*.

Under the same conditions, IMU048 (*malΔ mphΔ suc2Δ MAL11*) grew slowly (μ_{MAX} = 0.04 ± 0.01 h^{-1}) and the culture didn't get as turbid as the reference strain culture. This suggests that a low residual sucrose-hydrolyzing activity was still present in the sucrose knockout strain.

Some μ_{MAX} values couldn't be precisely determined using at least five points from the exponential phase (Supplemental Table 1 in the Appendix; Figures 5 and 8). However, this doesn't compromise the conclusions drawn, due to the large difference between the growth rates calculated.

Table 5: Aerobic and anaerobic growth rates and sucrose phosphorylase and sucrose hydrolase activities of *Saccharomyces cerevisiae* strains carrying different combinations of sucrose transporters and catalytic enzymes. Averages and mean deviations were obtained from duplicated experiments.

Strain	Relevant genotype	Growth rate [a] (h^{-1})		Enzyme activity (μmol mg protein^{-1} min^{-1})	
		Aerobic	Anaerobic	Sucrose phosphorylase	Sucrose hydrolase
CEN.PK113-7D	*MALxx SUC2*	0.45 ± 0.01	0.29 ± 0.01	<0.01 [s]	0.43 ± 0.01 [s]
IMU051	*malΔ mphΔ suc2Δ*	<0.001	N.D.	<0.01 [g]	N.D.
IMU052	*malΔ mphΔ suc2Δ +LmSPase*	<0.001	N.D.	5.54 ± 0.15 [g]	N.D.
IMU054	*malΔ mphΔ suc2Δ +Mal12*	<0.001	N.D.	N.D.	2.04 ± 0.07 [e]
IMU048	*malΔ mphΔ suc2Δ +Mal11*	0.04 ± 0.01	<0.001	N.D.	0.17 ± 0.01 [e,s]
IMU055	*malΔ mphΔ suc2Δ +Mal11+Mal12*	0.19 ± 0.01	≈0.04 [b]	N.D.	1.67 ± 0.08 [e]
IMU049	*malΔ mphΔ suc2Δ +Mal11+LmSPase*	0.17 ± 0.01	≈0.04 [b]	5.66 ± 0.49 [g]	N.D.
IMU056	*malΔ mphΔ suc2Δ +PvSUF1+Mal12*	<0.001	<0.001	N.D.	2.10 ± 0.08 [e]
IMU043	*malΔ mphΔ suc2Δ +PvSUF1+LmSPase*	0.04 ± 0.01	<0.001	1.89 ± 0.06 [g]	N.D.

N.D.= not determined.

[a] Growth rates were based on absorbance measurements in shake flasks at pH 5.0 and with 20 g l^{-1} sucrose. A growth rate of <0.001 h^{-1} means that no growth was detected during 10 days.

[b] Growth rate estimative based in 3 data points collected during the exponential growth.

[s, g, e] Some strains were grown with a carbon source other than sucrose, specifically for enzymatic assays. Letters "s", "g" and "e" specify the carbon source utilized. s: sucrose; g: glucose; e: ethanol; e,s: ethanol plus sucrose. 20 g l^{-1} of each carbon source was used, even in the case of two carbon sources (e.g. "e,s" means 20 g l^{-1} of ethanol plus 20 g l^{-1} of sucrose).

To quantify the remaining sucrose-hydrolysing activity in the sucrose knockout strain background, cell extracts were prepared from IMU048 (*malΔ mphΔ suc2Δ*

MAL11). Because IMU048 only grew very slowly on sucrose (μ_{MAX} = 0.04 ± 0.01 h^{-1}), a different carbon source was chosen that supports faster growth. Considerations in choosing another carbon source were that sucrose metabolism is repressed by glucose and induced by sucrose (DYNESEN et al., 1998). Therefore, cells extracts were prepared from an IMU048 culture grown on the non-repressing carbon source ethanol (2% w/v) in the presence of the inducer sucrose (2% w/v). This revealed a sucrose-hydrolysing activity of 0.17 ± 0.01 µmol mg $protein^{-1}$ min^{-1} for IMU048 (*malΔ mphΔ suc2Δ MAL11*), which represents around 10% of the hydrolase activity of a *MAL12*-expressing strain, such as IMU055 (*malΔ mphΔ suc2Δ MAL11 MAL12,* 1.67 ± 0.08 µmol mg $protein^{-1}$ min^{-1}) (Table 5).

The remaining sucrose hydrolase activity might originate from (an) unknown enzyme(s) or from specific hydrolysis by enzymes that are involved in the hydrolysis of other sugars, such as isomaltose (Ima1-5p) (BROWN et al., 2010; VOORDECKERS et al., 2012), glycogen (Gdb1p) (TESTE et al., 2000), trehalose (Nth1p and Ath1p) (ALIZADEH; KLIONSKY, 1996; KOPP et al., 1993) or cell wall oligosaccharides (e.g. Cwh41p) (ROMERO et al., 1997). This remaining activity can be an obstacle to gain the maximum benefit from the phosphorolytic pathway, as proposed in our work. It underlines the need for complete removal of sucrose hydrolytic activity. In this context, *IMA1-5* genes should be the first deletion targets due to their already proved activity on sucrose (VORDECKERS et al., 2012). This was not pursued in the present work and should be the aim of future projects.

It is important to mention that the internal sucrose-hydrolysing activity in the CEN.PK113-7D strain is rather low — 0.43 ± 0.01 µmol mg $protein^{-1}$ min^{-1} — because this reaction takes place mainly extracellularly through Suc2p activity (BASSO et al., 2011).

5.2 Successful expression of *Leuconostoc mesenteroides* sucrose phosphorylase

The expression of a functional sucrose phosphorylase (*SPase*) in a yeast background expressing the transporter Mal11p should increase the free-energy conservation in 1 ATP per sucrose compared to IMU055 (*malΔ mphΔ suc2Δ MAL11 MAL12*). In literature, several sucrose phosphorylase genes have been cloned and characterized (GOEDL et al., 2010); however there is no data about expression in

yeast. The SPase from *L. mesenteroides* ATCC 12291 was chosen because of its appropriate optimal pH and temperature (7 and 30 ºC, respectively) and also because of its high turnover number when compared to SPases from other *L. mesenteroides* strains (GOEDL et al., 2007). The *L. mesenteroides SPase* (*LmSPase*) gene was cloned behind the strong constitutive *TDH3* promoter on the multicopy plasmid pUDE262. The resulting *LmSPase* expressing strain IMU049 (*malΔ mphΔ suc2Δ MAL11 LmSPase*) displayed a high sucrose phosphorylase activity (5.66 ± 0.49 µmol mg protein^{-1} min^{-1}) while the sucrose phosphorylase activity of the reference strain CEN.PK113-7D was below the detection limit (<0.01 µmol mg protein^{-1} min^{-1}) (Table 5). IMU049 (*malΔ mphΔ suc2Δ MAL11 LmSPase*) and IMU055 (*malΔ mphΔ suc2Δ MAL11 MAL12*) growth rates were very similar, µ = 0.17 ± 0.01 h^{-1} and µ = 0.19 ± 0.01 h^{-1}, respectively (Table 5). This result together with enzymatic data clearly show that *LmSPase* is functional in *S. cerevisiae* and also that the phosphorolytic pathway for sucrose cleavage can substitute the natural hydrolytic pathway in this organism.

5.3 Successful expression of plant sucrose facilitators in *S. cerevisiae*

In strain IMU049 (*malΔ mphΔ suc2Δ MAL11 LmSPase*), sucrose is cleaved through phosphorolysis instead of hydrolysis. Compared to IMU055 (*malΔ mphΔ suc2Δ MAL11 MAL12*), IMU049 may yield one extra ATP from sucrose consumption in anaerobiosis. However, compared to a wild *S. cerevisiae* – in which sucrose hydrolysis takes place extracellularly – there is no free-energy conservation improvement in IMU049. In order to increase free-energy conservation, the active transporter *MAL11* was replaced by a facilitator. Zhou et al. (2007) characterized, in yeast, two facilitators from pea (*Pisatum sativum sucrose facilitator 1* and *4, PsSUF1* and *4*) and one from French bean (*Phaseolus vulgaris sucrose facilitator 1*, *PvSUF1*). In this work, each one of these genes was expressed separately in yeast behind the strong constitutive *TDH3* promoter. This was performed through single copy integration of the corresponding DNA construct at the *LEU2* locus in the yeast genome.

IMU043 (*malΔ mphΔ suc2Δ PvSUF1 LmSPase*, µ = 0.04 ± 0.01 h^{-1}) grew and achieved high absorbance value in sucrose cultures (Figure 5 and Table 5). Compared to strains expressing no transporters, IMU052 (*malΔ mphΔ suc2Δ*

LmSPase) and IMU054 (*malΔ mphΔ suc2Δ Mal12*) — that did not grow — this result suggests that *PvSUF1* can transport sucrose, which is in accordance with the work of Zhou et al. (2007).

Differently from the results with *PvSUF1*-expressing strains, IMU041 (*malΔ mphΔ suc2Δ PsSUF1 LmSPase*) and IMU042 (*malΔ mphΔ suc2Δ PsSUF4 LmSPase*) did not grow on sucrose even after 10 days of cultivation. Compared to *PvSUF1* (Km = 27.9 ± 1.7 mM), *PsSUF1* and *PsSUF4* have higher Km values for sucrose, 99.8 ± 10 mM and 37. 8 ± 5.5 mM, respectively (GOEDL et al., 2007). In this context, a growth assay using a medium containing higher initial sucrose concentration (8% w/v instead of 2% w/v) was carried out. In this situation, both strains grew and IMU052 (*malΔ mphΔ suc2Δ LmSPase*) did not (Figure 6). This shows that *PsSUF1* and *PsSUF4* can also transport sucrose, which is in agreement with Zhou et al. (2007). Besides this, the fact that only *PvSUF1* was codon-optimized for expression in yeast might have some contribution to the better results obtained with the expression of this gene, when compared to the other facilitators. To test this hypothesis, codon-optimization of *PsSUF1* and *PsSUF4* could be explored futurely.

A B C D E

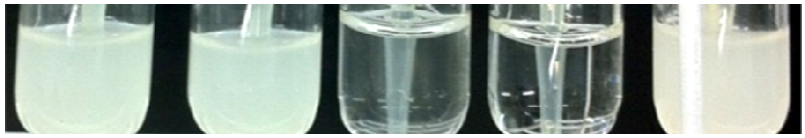

Figure 6: Cultures of *Saccharomyces cerevisiae* strains expressing sucrose facilitator genes from pea in 4 ml synthetic medium containing sucrose 8% (w/v) as the sole carbon source. **A**: strain IMU041 (*malΔ mphΔ suc2Δ PsSUF1 LmSPase*); **B**: IMU042 (*malΔ mphΔ suc2Δ PsSUF4 LmSPase*); **C**: IMU052 (*malΔ mphΔ suc2Δ LmSPase)* **D**: IMU051 (*malΔ mphΔ suc2Δ*) and **E**: *S. cerevisiae* CEN.PK113-7D (reference strain). *PsSUF1*: *Pisum sativum* sucrose facilitator 1; *PsSUF4*: *Pisum sativum* sucrose facilitator 4; *LmSPase*: *Leuconostoc mesenteroides* sucrose phosphorylase. The picture was taken after 8 days of cultivation. In each test tube a pipette tip can be observed, which was used to transfer cells from a single colony on a plate to the tube.

5.4 Sucrose passive uptake in a strain expressing the Mal12p maltase

Divergently from IMU043 (*malΔ mphΔ suc2Δ PvSUF1 LmSPase*), which can consume sucrose, IMU056 (*malΔ mphΔ suc2Δ PvSUF1 MAL12*) cannot, even after 10 days of culture (Figure 3). Because the only difference between them is the catalytic enzyme, our hypothesis is that LmSPase allows for a higher glycolytic flux than Mal12p does (Figure 7). Besides our results, this hypothesis is also supported

by enzymatic parameters already published: Mal12p kcat/Km for sucrose is around 2.26 mM^{-1} min^{-1} (VOORDECKERS et al., 2012) while LmSPase kcat/Km is close to 1736.84 mM^{-1} min^{-1} (GOEDL et al., 2007).

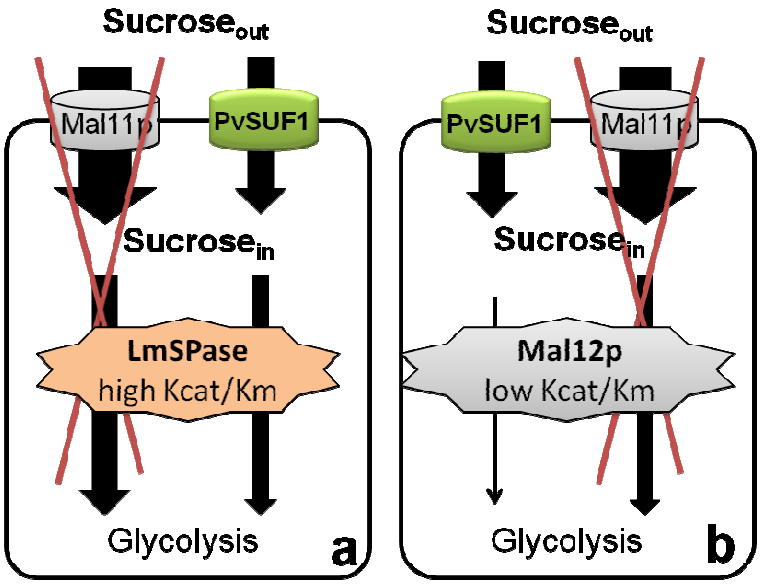

Figure 7: A model for sucrose uptake and cleavage by different combinations of transporters (active *vs* passive) and catalytic enzymes (hydrolysis *vs* phosphorolysis). In both scenarios shown, *MAL11* was deleted. In "**a**", LmSPase cleaves sucrose with sufficient flux to support cell growth. In "**b**", Mal12p conversion of sucrose is not enough to feed glycolysis. Arrow thickness is proportional to the carbon flux. Mal11p: α-glucoside active transporter; PsSUF1: *Pisum sativum* sucrose facilitator 1; LmSPase: *Leuconostoc mesenteroides* sucrose phosphorylase; Mal12p: α-glucosidase.

Due to the low MAL12p kcat/Km index, it wouldn't be expected that a *MAL12*-expressing strain, such as IMU055 (*malΔ mphΔ suc2Δ MAL11 MAL12*), grows on sucrose. The reason why IMU055 can grow on this sugar is probably related to transport kinetics. The active substrate uptake performed by MAL11p in IMU055 probably creates an intracellular sucrose concentration that is high enough to enable sufficient flux through Mal12p (Figure 7).

5.5 Anaerobic behaviour of *S. cerevisiae* expressing different sucrose transporters and catalytic enzymes.

The main goal of this work is to increase ATP conservation during anaerobic sucrose catabolism in yeast. The biomass yield on substrate ($Y_{X/S}$) is a physiological parameter that directly reflects this ATP conservation, if we may consider that the ATP requirement for biomass formation is a constant value. Verduyn et al. (1991) determined a Y_{ATP} value of 16 g biomass mol ATP^{-1} for *S. cerevisiae* growing

anaerobically on glucose as the sole carbon-source. The $Y_{X/S}$ parameter can be accurately determined through anaerobic chemostat cultivation (VERDUYN; POSTMA, 1990). Based on these assumptions, the IMU043 strain (*malΔ mphΔ suc2Δ PvSUF1 LmSPase*), should yield 25% more biomass than the reference strain (*S. cerevisiae* CEN.PK113-7D), during anaerobic cultivation on sucrose. Anaerobic chemostats with this reference strain were performed previously in our group (BASSO et al., 2011) with a dilution rate close to 0.1 h^{-1}, which is a value commonly used for physiological studies with yeast. For that reason, to allow for proper phenotypic comparisons, it is desired to run chemostats with the IMU043 strain at the same dilution rate of 0.1 h^{-1}. However, IMU043 cannot grow without oxygen (Figure 8).

Besides IMU043, all strains constructed in this work didn't grow or grew marginally under anaerobic conditions, compared to the reference strain. *MAL11*-expressing strains — IMU49 (*malΔ mphΔ suc2Δ MAL11 LmSPase*) and IMU055 (*malΔ mphΔ suc2Δ MAL11 MAL12*) — grew slowly, $\mu = 0.04 \pm 0.01$ h^{-1} and the highest absorbance value measured for these cultures was drastically lower than for the reference strain culture (Table 5 and Figure 8). This shows that, in most of the strains here constructed, the carbon flux during anaerobic sucrose metabolism is not enough to support fast growth. A hypothesis that can explain this scenario is based on sucrose uptake. The major difference between strains here constructed and the wild-type strain is the site of sucrose cleavage. While in wild-type yeast it takes place extracellularly, in the strains here constructed it occurs in the cytosol. For this reason, it is likely that the necessity of sucrose transport before its cleavage represents a bottleneck for the growth of the constructed strains under anaerobic conditions.

Another difference between the transgenic strains generated in this work and the wild-type strain relates to enzyme kinetics. While CEN.PK113-7D expresses the native invertase (*SUC2;* kcat/Km ≈ 5415.79 mM min^{-1}; LAFRAYA et al., 2011), the other strains hydrolyse sucrose using Mal12p or *LmSPase*. MAL12p kcat/Km is low for sucrose (≈ 2.26 mM min^{-1}) and this can explain why *MAL12*-expressing strains have marginal growth without aeration. However, some growth is expected from *LmSPase*-expressing strains because LmSPase kcat/Km (≈ 1736.84 mM min^{-1}) is not as low as MAL12p kcat/Km (see discussion above). In conclusion, it is possible that enzyme kinetics explains part of the behavior observed under anaerobiosis, but the sucrose uptake mechanism is probably the main cause.

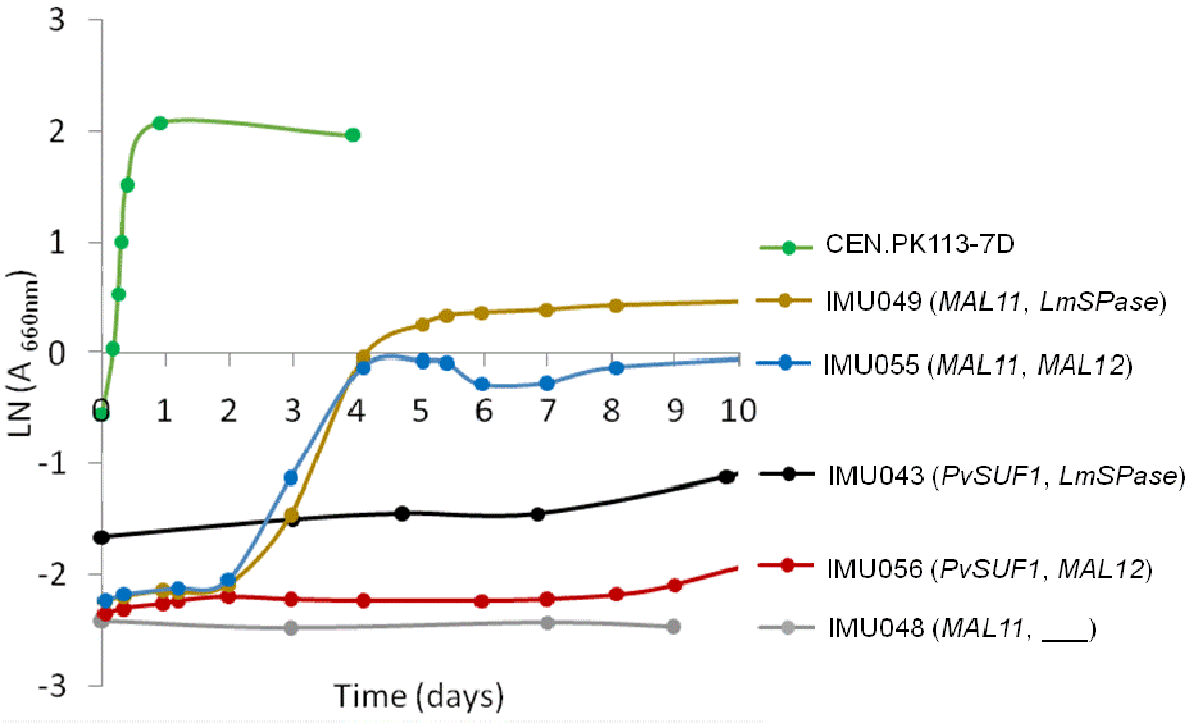

Figure 8: Growth curves of *Saccharomyces cerevisiae* strains obtained from shake flask cultivation under anaerobic conditions. Final culture absorbance at 660nm is shown above the corresponding curves, for three selected strains. CEN.PK113-7D: *S. cerevisiae* reference strain; *MAL11*: active α-glucoside transporter; *PvSUF1*: *Phaseolus vulgaris* sucrose facilitator 1; *MAL12*: α-glucosidase (hydrolase); *LmSPase*: *Leuconostoc mesenteroides* sucrose phosphorylase. Cultivations were performed in a synthetic medium containing sucrose 2% (w/v) initial concentration and the anaerobic growth factors: ergosterol (10 mg l-1) and Tween 80 (420 mg l-1), dissolved in ethanol.

5.6 Evolutionary engineering

The fact that IMU043 (*malΔ mphΔ suc2Δ PvSUF1 LmSPase*) does not grow without oxygen impairs the quantification of free-energy conservation under anaerobic conditions. To overcome this, an evolutionary approach based on sequential batch cultivations was pursued. This strategy was chosen for two main reasons: *i*) genome mutations occur during cell division and *ii*) a mutant with a higher specific growth rate will reach a higher proportion in the population during a submerged cultivation (CAKAR et al., 2012 and DRAGOSITS et al., 2013).

Initially, the evolution of the IMU043 strain was pursued through serial shake-flask cultivations in aerobic conditions. To avoid lag and stationary phases, the initial and final absorbance of each batch were fixed to 0.4 and 5, respectively — this is important because mutations happen more often during DNA replication. Each new cycle was started by inoculation of the grown culture in fresh medium. After eight

transfers (around 15 days), shake flasks were transferred to an anaerobic chamber and seven additional transfers were carried out (around 20 days). Unfortunately, by that point, bacterial contamination was detected through microscopy. Because of that, evolved cultures were spread on the surface of solid media in order to isolate non-contaminated yeast cells. After three transfers of isolated cells on solid media, it was possible to obtain colonies free of contamination that were used to start evolution in sequential batch reactors (SBRs).

SBRs offers lots of advantages compared to shake-flask transfers: *i*) in reactors, contaminations are less frequent; *ii*) it is possible to control parameters such as pH and aeration and *iii*), each medium exchange cycle can be done automatically based on the exhaust CO_2 profile, which, besides this, allows for the estimation of the specific growth rate in each batch cycle. SBRs were conducted with four independent replicates (each one corresponds to a different fermenter) named RW009, RW011, RW013 and RW014. All of them were inoculated with a different IMU043 colony pre-evolved in shake flasks. The exception is RW009, which was inoculated with a non-evolved IMU043 culture.

Because the last shake-flask cultivations were performed inside the anaerobic chamber, SBRs were already started under anaerobiosis. However, only a very slow and tiny production of CO_2 was observed under these conditions, which impaired the direct adoption of anaerobic SBR's (data not shown). Instead, a strategy was pursued in which the gas mixture bubbled in the fermenters was changed from 100% nitrogen to 100% air. In this way, a fully aerobic batch was achieved and became the beginning of SBR evolution, which was divided into three different steps. The first phase started with fully aerated batches and slowly migrated to an oxygen restricted cycle (it took around 25 days; Figure 9). This was possible because the fermenters used offer the option to mix pressurized air with nitrogen. Then, in each batch, the proportion of pressurized air was lowered. In the cycle that started after around 190 hours of cultivation only 3% of the sparged gas should correspond to pressurized air. However, due to equipment limits, only pure nitrogen was sparged, resulting in no or low growth (see constant CO_2 signal between 190 h and 300 h in Figure 9). Because of that, the proportion of pressurized air in the inlet gas mixture was fixed at 5% and other parameters were decreased, such as stirring speed (from 1200 rpm to 250 rpm) followed by gas flow (from 50 ml min^{-1} to 20 ml min^{-1}). As a consequence, an evolved

culture was obtained that could grow under oxygen restricted conditions (5% pressurized air, 20 ml min^{-1} inlet gas and stirring at 250 rpm).

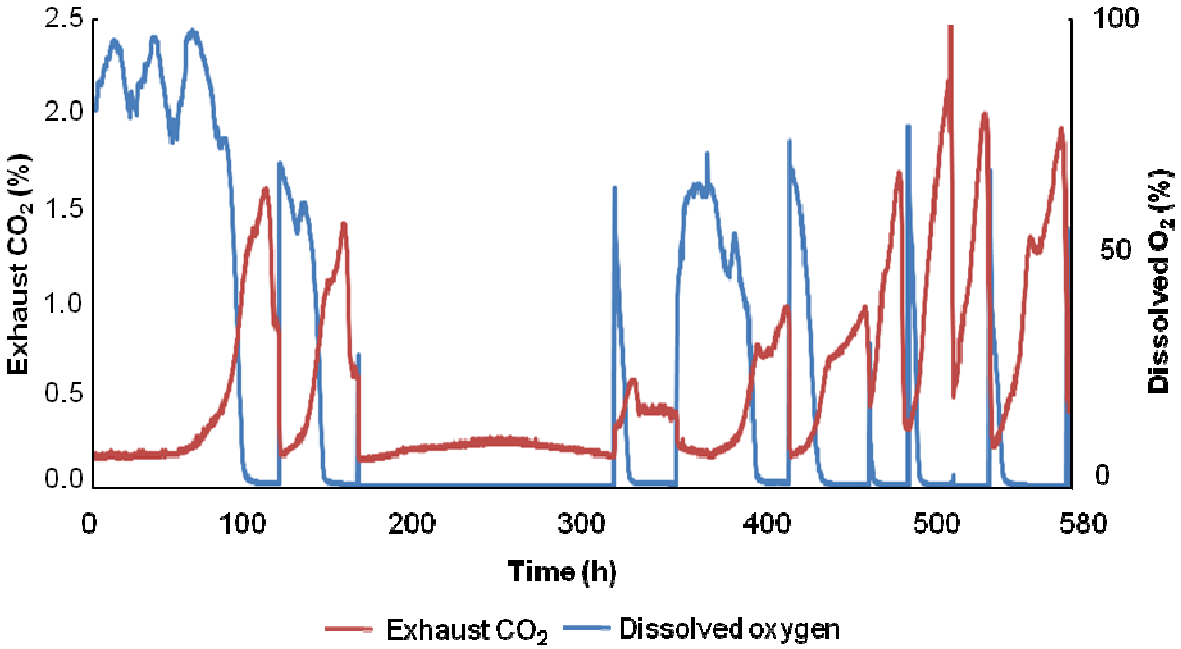

Figure 9: CO_2 in exhaust gas and dissolved O_2 profile during initial sequential batch cultivations of *S. cerevisiae* strain IMU043 in synthetic medium with 2% (w/v) sucrose. After each CO_2 peak, a new batch was initiated through replacement of about 90% of the medium with fresh medium. The first cycle was fully aerated and the last one is oxygen restricted. Between those, aeration parameters were changed in the following sequence: gas inlet composition (from 100% to 5% pressurized air, mixed with nitrogen); stirring (from 1200 rpm to 250 rpm) and gas flow (from 50 ml min^{-1} to 20 ml min^{-1}). This graph represents the experimental replicate RW011. The other replicates have similar profiles.

In the last evolution cycles (shown in Figure 9), it can be seen that all oxygen provided is consumed by the culture (dissolved O_2 value close to zero). Additionally, because CO_2 production varies during the batch and O_2 availability is constant, it can be concluded that cell metabolism is not only respiratory; fermentation is also being carried out by the yeast cells.

The first SBR phase mentioned above resulted in an evolved population that grows under oxygen restricted conditions. Subsequently, a second SBR step was carried out with this low oxygen availability in order to obtain faster growing mutants. The latter would then subsequently be submitted to evolution under fully anaerobic conditions. Figure 10 shows the culture specific growth rate (μ_{max}) increasing after each batch. In the end, when a μ_{max} close to 0.18 was achieved (after around 17 days), the third SBR phase was initiated. In this last evolution setup, fully anaerobic batches were conduced (Figure 11) (100% nitrogen gas inlet with 50 ml min^{-1} flow rate and 500 rpm stirring). Stirring was not raised back to 1200 rpm due to a

mechanical problem in the fermenter. However, this does not represent an obstacle in this project because 500 rpm is expected to be sufficient for the perfect mixing of 100 ml of medium. Evolving anaerobically, the culture μ_{max} raised from less than 0.02 h^{-1} to 0.12 h^{-1} after around 21 cycles (50 days; Figure 11).

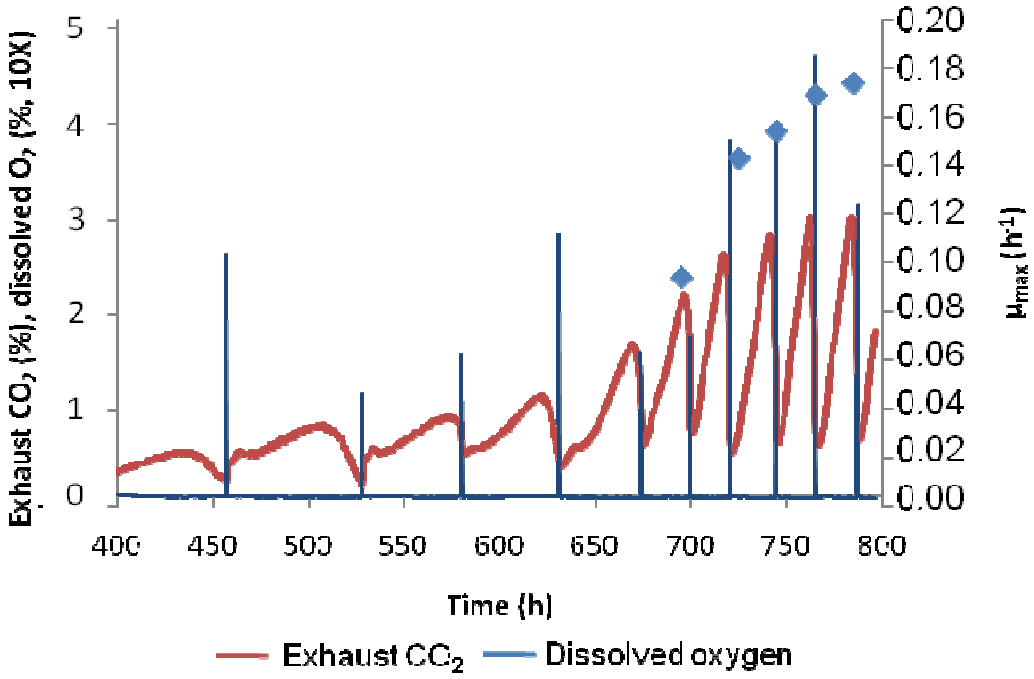

Figure 10: Specific growth rate, CO_2 in exhaust gas and dissolved O_2 profile during an oxygen restricted SBR fermentation of *S. cerevisiae* strain IMU043 in synthetic medium with 2% (w/v) sucrose. Each lozenge represents the specific growth rate estimated from the CO_2 profile (red lines) during exponential growth. The blue line represents the dissolved oxygen concentration and its peaks, in the beginning of each batch, which corresponds to the oxygen dissolved in the fresh medium added. Fermentation conditions: 5% pressurized air mixed with 95% nitrogen gas is the composition of the gas inlet, which has 20 ml min^{-1} flow rate; 250 rpm of stirring. This graph represents the experimental replicate RW014. The other replicates have similar profiles.

Because the final μ_{max} achieved after the 3-phase evolutionary engineering strategy is higher than 0.1 h^{-1}, it will be possible to obtain a single-cell isolate from the evolved culture that can be cultivated in anaerobic chemostats at a dilution rate of 0.1 h^{-1} with sucrose as the sole carbon-source. This will allow for a proper quantitative assessment of $Y_{X/S}$, which in turn can be used as a measure for free-energy conservation, as discussed above. A biomass yield on substrate ($Y_{X/S}$) close to 42 g mol $sucrose^{-1}$, 25% higher than the corresponding value for the CEN.PK113-7D strain — which was evaluated previously in our group by Basso et al. (2011) — is expected. This task will be pursued in the future (Ph.D. of Wesley Marques, to start in March, 2014), together with whole genome resequencing and reverse engineering of

some mutations in the reference CEN.PK113-7D strain, in order to identify which DNA alterations were important to make IMU043 able to grow anaerobically on sucrose, with increased free-energy conservation.

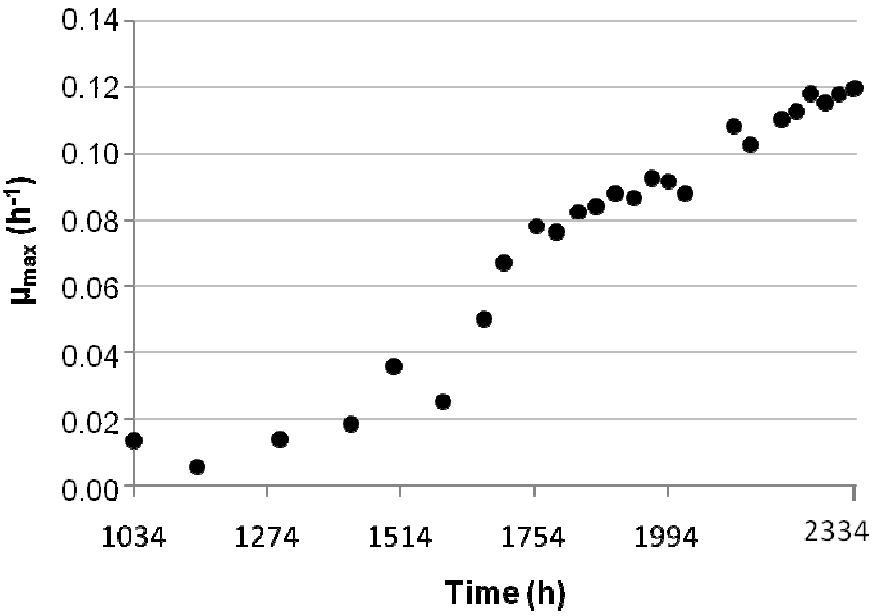

Figure 11: Specific growth rate evolution during anaerobic SBR fermentations of *S. cerevisiae* strain IMU043 in synthetic medium with 2% (w/v) sucrose (initial concentration for each batch). The specific growth rate was estimated from the CO_2 profile during exponential growth. Fermenter conditions: 100% nitrogen gas inlet with 50 ml min^{-1} flow rate and 500 rpm stirring. This graph represents the experimental replicate RW014. The other replicates have similar profiles.

6 CONCLUSIONS

- Additional deletions besides the *MALx2* gene family are necessary to completely abolish sucrose cleavage in *S. cerevisiae* CEN.PK113-7D;
- *Leuconostoc mesenteroides* sucrose phosphorylase was successfully expressed in *Sacharomyces cerevisiae*;
- Facilitators from French bean (*PvSUF1*) and pea (*PsSUF1, PsSUF4*) can transport sucrose in *S. cerevisiae* as previously shown by Zhou et al. (2011);
- The sucrose hydrolysis rate by native Mal12p is insufficient to support *S. cerevisiae* growth when substrate uptake is passive in 2% sucrose medium (w/v), even in aerobic conditions;
- Combined sucrose passive uptake and phosphorolysis cannot support *S. cerevisiae* growth under anaerobiosis. However, anaerobically growing mutants can be obtained by evolution through Sequential Batch Cultivations.

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APPENDIX - Supplemental Table 1

Exponential phase interval and number of points used to estimate μ_{MAX} from shake flasks cultures.

Strain and condition	Exponential phase interval (h)	Number of points used to estimate μ_{MAX}
IMU043 aerobiosis	96 - 143	5
IMU048 aerobiosis	71 - 146	4
IMU049 aerobiosis	28,8 - 35	4
IMU055 aerobiosis	20 - 46	5
CEN.PK113-7D aerobiosis	2,33 - 10	5
CEN.PK113-7D anaerobiosis	4,2 - 9,5	4