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SUÉLLEN PATRICIA HELD AZAMBUJA DA SILVA

SELEÇÃO DE LINHAGENS DE *Saccharomyces cerevisiae* CAPAZES DE PRODUZIR E TOLERAR *n*-BUTANOL

SCREENING OF *Saccharomyces cerevisiae* STRAINS CAPABLE OF PRODUCING AND TOLERATE *n*-BUTANOL

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"O reino dos céus é semelhante ao fermento, que uma mulher toma e introduz em três medidas de farinha, até que tudo esteja levedado." (Mateus 13.33)

Porque Dele e por Ele, e para Ele, são todas as coisas. (Romanos 11:36)

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RESUMO

O *n*-butanol é um álcool que pode ser utilizado como solvente ou biocombustível produzido naturalmente por microrganismos da espécies de *Clostridium*. No entanto, devido à problemas inerentes às fermentações empregando este microrganismo, a levedura *Saccharomyces cerevisiae* tem sido alvo de estudos para a produção de *n*-butanol. Em face disto, esta tese de doutorado teve como objetivo selecionar linhagens selvagens da levedura *S. cerevisiae* com potencial para produção de *n*-butanol a partir do aminoácido glicina como co-substrato, avaliando a relação entre a produção deste álcool e outros produtos metabólitos; bem como submeter as linhagens JAY270 e UFMG-CM-Y267 à edição por CRISPR/Cas9 para superexpressão do gene glicina oxidase; além disso selecionar linhagens capazes de tolerar a presença de *n*-butanol no meio e evoluir as linhagens CAT-1 e X2180-1B para se tornarem mais tolerantes. Dentre todas as linhagens estudadas, 48% delas foram capazes de produzir *n*-butanol em concentrações detectáveis, utilizando glicose como única fonte de carbono e o aminoácido glicina como co-substrato. As linhagens avaliadas mostraram grande diversidade de perfis de produção de metabólitos, produzindo em média 3,9 mg/L de *n*-butanol, tendo se destacado a linhagem UFMG-CM-Y267 com a maior produção de *n*-butanol (11,5 mg/L), e concentrações de isobutanol, etanol e glicerol de em média 32,8 mg/L, 5,1 mg/L e 1,9 mg/L, respectivamente. As linhagens modificadas mostraram maior atividade da glicina oxidase do que as linhagens parentais, com aumentos na atividade de 53% até mais de 200%. No entanto, a linhagem modificada SAJgox (background JAY270) destacou-se não só por apresentar uma atividade de glicina oxidase até 200% superior à linhagem parental, mas também por ter sido afetada positivamente em termos de produção de *n*-butanol, uma vez que foi capaz de produzir cerca de 300% a mais desse álcool. Em termos de tolerância à butanol, a linhagem industrial brasileira CAT-1 foi capaz de crescer com 93 e 72% de sua capacidade na ausência do álcool, quando cultivada em 1 e 2% de *n*-butanol, respectivamente. Desta forma, as linhagens selecionadas podem ser utilizadas para estudos que visam o aumento na produção e tolerância a *n*-butanol a partir de ferramentas de engenharia molecular e evolutiva.

Palavras-chave: *Saccharomyces cerevisiae*, Butanol, Glicina Oxidase, Tolerância

ABSTRACT

n-Butanol is an alcohol that can be used as a solvent or biofuel produced naturally by microorganisms of *Clostridium* species. However, due to problems inherent to fermentations using this microorganism, the yeast *Saccharomyces cerevisiae* has been the subject of studies for the production of *n*-butanol. In view of this, this doctoral thesis aimed to select wild-type strains of the yeast *S. cerevisiae* with potential for the production of *n*-butanol from the amino acid glycine as a co-substrate, evaluating the relationship between the production of this alcohol and other metabolite products; as well as submitting the JAY270 and UFMG-CM-Y267 strains to editing by CRISPR/Cas9 for overexpression of the glycine oxidase gene; in addition to selecting strains capable of tolerating the presence of *n*-butanol in the medium and evolving the CAT-1 and X2180-1B strains to become more tolerant. Among all the strains studied, 48% of them were able to produce *n*-butanol in detectable concentrations, using glucose as the only carbon source and the amino acid glycine as a co-substrate. The evaluated strains showed great diversity of metabolite production profiles, producing an average of 3.9 mg/L of *n*-butanol, with the UFMG-CM-Y267 strain standing out with the highest *n*-butanol production (11.5 mg/L), and concentrations of isobutanol, ethanol and glycerol averaged 32.8 mg/L, 5.1 mg/L and 1.9 mg/L, respectively. The modified strains showed greater glycine oxidase activity than the parent strains, with increases in activity from 53% to over 200%. However, the modified strain SAJgox (background JAY270) stood out not only for presenting a glycine oxidase activity up to 200% higher than the parental strain, but also for having been positively affected in terms of *n*-butanol production, since was able to produce about 300% more alcohol. In terms of butanol tolerance, the Brazilian industrial strain CAT-1 was able to grow to 93 and 72% of its capacity in the absence of alcohol, when grown in 1 and 2% *n*-butanol, respectively. In this way, the selected strains can be used for studies aimed at increasing *n*-butanol production and tolerance using molecular and evolutionary engineering tools.

Keywords: *Saccharomyces cerevisiae*, Butanol, Glycine Oxidase, Tolerance

SUMÁRIO

CAPÍTULO 1

INTRODUÇÃO GERAL, OBJETIVOS E ESTRUTURA DA TESE

INTRODUÇÃO

Os biocombustíveis, produzidos a partir de fermentação microbiana, representam uma importante e promissora opção para servir como substitutos dos derivados do petróleo. Neste sentido, a demanda por etanol cresceu rapidamente, passando a ser o biocombustível mais produzido nos dias de hoje e o mais utilizado como substituto da gasolina (Atsumi et al. 2008; Choi et al. 2014). No entanto, o etanol ainda não é o melhor substituto dos combustíveis fósseis, sendo o butanol considerado como um substituto mais adequado devido às suas propriedades físicas serem superiores às do etanol e comparáveis às da gasolina (Choi et al. 2014; Sakuragi et al. 2015).

O butanol é um álcool de quatro carbonos que apresenta quatro formas isoméricas, sendo o *n*-butanol um dos isômeros que têm sido alvo de muitos estudos. Este álcool apresenta muitas vantagens como um biocombustível, quando comparado ao etanol, como maior densidade energética, menor higroscopicidade, menor pressão de vapor, deste outras (ATSUMI et al., 2008; SCHADEWEG; BOLES, 2016). Além disso, o butanol possui boa parcela no mercado químico como solvente ou co-solvente (Mascal 2012; Mariano et al. 2013).

Espécies de *Clostridium*, em especial *Clostridium acetobutylicum*, são os mais utilizados para produção de *n*-butanol, podendo atingir até 13 g/L (Schadeweg and Boles 2016a). Entretanto, esta espécie é geneticamente complexa, não existem ferramentas genéticas para sua manipulação, apresenta baixa velocidade de crescimento, formação de esporos, baixa tolerância ao *n*-butanol, dentre outros problemas que interferem diretamente no uso destes organismos para produção industrial. Por estas razões, outros organismos mais comumente utilizados em escala industrial, têm sido modificados geneticamente para a produção de *n*-butanol, como *Escherichia coli* e *Saccharomyces cerevisiae* (Steen et al. 2008a).

S. cerevisiae é uma levedura largamente utilizada na indústria de alimentos e para produção de combustíveis, sendo o principal micro-organismo produtor de etanol de primeira geração no Brasil e na América do Norte (Beato et al. 2016). Devido à capacidade de adaptação em condições industriais e a gama de ferramentas genéticas existentes para este organismo, *S. cerevisiae* tornou-se objeto de estudo também quanto à sua capacidade de produzir *n*-butanol. Para isto, duas são as estratégias atualmente estudadas para a produção deste álcool por *S. cerevisiae*: expressão heteróloga da via do *Clostridium* e as vias de assimilação de aminoácidos (Kuroda. and Ueda 2015).

As concentrações de butanol produzidas por linhagens de *S. cerevisiae* ainda são muito baixas, quando comparadas à produção de etanol. Além disto, esta levedura ainda enfrenta outro desafio, sua baixa tolerância a este álcool. Uma vez que os produtos metabólicos começam a ser formados, como álcoois e ácidos, o microrganismo deve ser capaz de tolerar o acúmulo destes produtos no meio, caso contrário pode haver diminuição da velocidade de crescimento, até morte celular. A baixa tolerância dos microrganismos aos produtos metabólicos afeta os custos de recuperação do produto e dificultam a produção em escala industrial destes metabólitos (Liu and Qureshi 2009). Linhagens de *S. cerevisiae* são capazes de tolerar não mais que 2% (v/v) de butanol no meio, assim como linhagens de *Clostridium* (Knoshaug and Zhang 2009a). Desta maneira, o aumento da tolerância à butanol em linhagens de *S. cerevisiae* também tem sido estudado através de modificação genética ou engenharia evolutiva.

Diante do exposto acima, esta tese de doutorado teve como objetivos identificar linhagens da levedura *S. cerevisiae* com potencial para produção de *n*-butanol e submetê-las à modificação genética com a inserção do gene *goxB* (*glicina oxidase*) para aumentar as concentrações de *n*-butanol produzido, utilizando como estratégia a via de assimilação do aminoácido glicina. Em paralelo, submeter as linhagens que apresentaram maior e menor tolerância ao butanol à duas técnicas de evolução adaptativa em laboratório (do inglês *Adaptive Laboratory Evolution*, ALE) a fim de avaliar a capacidade adaptativa destas linhagens em butanol.

Para isso, esta tese foi dividida em quatro partes (capítulos 2 a 5) para melhor entendimento, sendo cada um destes capítulos um artigo publicado ou em fase de submissão. No **Capítulo 2** é apresentado um artigo de revisão que aborda as principais características do butanol e o porquê deste álcool ser um bom substituto da gasolina. Além disso, este artigo detalha as duas estratégias normalmente estudadas para a produção de butanol por *S. cerevisiae* (expressão heteróloga da via do *Clostridium* e as vias de assimilação de aminoácidos); e encerra um levantamento sobre a tolerância desta levedura ao butanol. Iniciando os trabalhos experimentais, o **Capítulo 3** apresenta o *screening* inicial de 48 diferentes linhagens de *S. cerevisiae*, dentre elas linhagens selvagens, laboratoriais e comumente utilizadas na produção de etanol, para avaliar a capacidade de utilização de glicina como co-substrato na produção de butanol e a relação entre a produção deste álcool e de outros metabólitos. A partir deste *screening* inicial, foi possível elencar candidatos para serem submetidos à inserção do gene *goxB*, resultados estes apresentados no **Capítulo 4**, a fim de avaliar se haveria aumento na produção de butanol. E, para encerrar, o **Capítulo 5**

apresenta um novo *screening* realizado com as mesmas 48 linhagens iniciais, agora com o objetivo de avaliar a capacidade de tolerância ao butanol. A partir disto, duas linhagens foram selecionadas para serem submetidas à duas estratégias de ALE e averiguar se as mesmas foram capazes de aumentar sua tolerância ao butanol.

CAPÍTULO 2

BUTANOL PRODUCTION BY *Saccharomyces cerevisiae*: PERSPECTIVES, STRATEGIES AND CHALLENGES

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Butanol production by *Saccharomyces cerevisiae*: perspectives, strategies and challenges

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Abstract

The search for gasoline substitutes has grown in recent decades, leading to the increased production of ethanol as viable alternative. However, research in recent years has shown that butanol exhibits various advantages over ethanol as a biofuel. Furthermore, butanol can also be used as a chemical platform, serving as an intermediate product and as a solvent in industrial reactions. This alcohol is naturally produced by some *Clostridium species*; however, *Clostridial* fermentation processes still have inherent problems, which focuses the interest on *Saccharomyces cerevisiae* for butanol production, as an alternative organism for the production of this alcohol. *S. cerevisiae* exhibits great adaptability to industrial conditions and can be modified with a wide range of genetic tools. Although *S. cerevisiae* is known to naturally produce isobutanol, the *n*-butanol synthesis pathway has not been well established in wild *S. cerevisiae* strains. Two strategies are most commonly used for of *S. cerevisiae* butanol production: the heterologous expression of the *Clostridium* pathway or the amino acid uptake pathways. However, butanol yields produced from *S. cerevisiae* are lower than ethanol yield. Thus, there are still many challenges needed to be overcome, which can be minimized through genetic and evolutive engineering, for butanol production by yeast to become a reality.

Keywords: ABE fermentation; amino acid pathway; butanol production; butanol tolerance; *Saccharomyces cerevisiae*.

Introduction

Biofuels, produced from microbial fermentation, represent an important and promising option for gasoline substitution. This lead to the demand for ethanol increasing rapidly; becoming the biofuel most produced today and the most used as a substitute for gasoline (Atsumi et al. 2008; Choi et al. 2014). However, ethanol is still not considered the best substitute for fossil fuels, with butanol being a more suitable alternative due to its physical properties; which are superior to that of ethanol and comparable to those of gasoline (Choi et al. 2014; Sakuragi et al. 2015).

Butanol is a four-carbon alcohol having four isomeric forms, with *n*-butanol being (one of the isomers) subject to many studies. Butanol has many advantages as a biofuel when compared to ethanol; such as, higher energy density, lower hygroscopicity, lower vapor pressure, and being an important solvent (Sakuragi et al. 2015; Chen and Liao 2016).

Clostridium species, especially *C. acetobutylicum*, *C. beijerinckii*, *C. saccharoperbutylacetonicum* and *C. saccharobutylicum* are the most used for *n*-butanol production (Kushwaha et al. 2019). However, this species is genetically complex and presents problems that directly interfere with industrial production, such as: low growth speed, formation of spores and several by-products, low tolerance to *n*-butanol, and phage contamination, besides being strictly anaerobic. For these reasons, other organisms more commonly used on an industrial scale have been genetically modified for the production of *n*-butanol, such as *Escherichia coli* and *Saccharomyces cerevisiae* (Steen et al. 2008).

S. cerevisiae is a yeast widely used in the food industry and also for fuel production, and is the main microorganism producing first generation ethanol in Brazil and North America (Beato et al. 2016). Due to the adaptability under industrial conditions and the range of genetic tools available for this organism, *S. cerevisiae* has also become the subject of study for its ability to produce *n*-butanol.

However, the low tolerance of microorganisms to metabolic products affects the recovery costs of the product and hinders the industrial scale production of these metabolites (Liu and Qureshi 2009). Once metabolic products begin to form, such as alcohols and acids, the microorganism must be able to tolerate the accumulation of these products in the medium, otherwise there may be a decrease in growth rate until cell death.

S. cerevisiae is able to tolerate no more than 2% (v/v) of *n*-butanol in the medium, as well as *Clostridium* strains. Thus, increased tolerance to butanol in *S. cerevisiae* strains has been studied through genetic modification or evolutionary engineering. Tools and

methodologies have already been developed, in addition to the genetic improvement and construction of strains capable of producing and tolerating higher concentrations of butanol.

Currently, the production of butanol by *S. cerevisiae* has been studied using two strategies: heterologous expression of the *Clostridium* pathway and the pathways of amino acid assimilation (Kuroda. and Ueda 2015). However, while promising, butanol production by *S. cerevisiae* still faces many challenges.

Butanol as a biofuel

In the last decades, many studies have been carried out in the search to find for green and new energy to replace classical energy. In this way, many studies have achieved excellent results, with the development of technologies and microorganisms capable of producing large amounts of ethanol, mainly from *S. cerevisiae* (Kuroda. and Ueda 2015). In addition, there is a growing search for microorganisms capable of using lignocellulosic biomass for the production of this biofuel, called second generation ethanol (2G) (Brethauer and Studer 2015). However, when compared to *n*-butanol, ethanol is still not the best substitute for gasoline (Hong and Nielsen 2012).

Butanol has advantages as biofuel compared to ethanol. The energy density of butanol is higher than that of ethanol, and comparable to gasoline (Si et al. 2014a). Butanol has low hygroscopicity, making it less corrosive (Swidah et al. 2015; Schadeweg and Boles 2016a), and therefore, can be transported through the pipeline infrastructure already in place for gasoline (Si et al. 2014a). Butanol has lower vapor pressure and is also safer to handle. Ethanol can be mixed with gasoline in up to 85% of the volume, while butanol can be mixed at any ratio or used pure (Dürre 2007). Even more, butanol is less water soluble compared to ethanol, making the butanol-gasoline mixture less susceptible to phase separation (Amiri and Karimi 2019). Table 1 presents a comparison of butanol, ethanol and gasoline properties.

Production of *n*-butanol can be accomplished through two methods, petrochemical or biological. When compared, the petrochemical method has the great advantage of being carried out in only one step. From this, butanol can be produced from ethanol, in the presence of catalysts, in a step involving three consecutive reactions, namely: dehydrogenation, aldol condensation and hydrogenation. In this sense, research involving the petrochemical route has focused on finding suitable catalysts, which can increase the yield in butanol (Ndaba et al. 2015).

Table 1. Physicochemical properties of *n*-butanol, ethanol and gasoline

Propertie	*n*-Butanol	Ethanol	Gasoline
Energy density (MJ/L)	29.2[a]	21.2[a]	32.5[a]
Fusion point (ºC)	-89.3[b]	-114[c]	-40[c]
Boiling point (ºC)	117.7[b]	78[c]	27-225[c]
Auto-ignition temperature (ºC)	385[d]	423[d]	257[c]
Density (g/mL)	0.8098[b]	0.79[c]	0.69-0.79[c]

[a](Si et al. 2014a)); [b](Lee et al. 2008)); [c](Yüksel and Yüksel 2004)); [d](Zhang et al. 2012))

Through the biological method, butanol (also called bio-butanol) begins to be produced naturally by organisms of the *Clostridiaceae* family, through the so-called acetone-butanol-ethanol (ABE) fermentation process, with the production of these three compounds in the proportion of 3:6:1, respectively (Kuroda. and Ueda 2015). The first report of biological butanol production was published by the famous French scientist Louis Pasteur in 1861. He reported the production of C4-alcohol in a culture named by him, *Vidrion butyrique*. However, it was probably a mixed culture containing organisms similar to *Clostridium* (Dürre 2008; Sauer et al. 2016). In the early 1900s, there were companies and universities interested in studying the production of synthetic rubber. Among the researchers involved in these studies was the chemist Chaim Weizmann, who concluded that synthetic rubber should be produced from butanol or isoamyl alcohol obtained by fermentation. Weizmann then isolated and studied an organism called BY, which was later named *Clostridium acetobutylicum* (Jones and Woods 1986). From that moment, *Clostridium* species began to be more expressively studied and used for industrial production of butanol, among other solvents.

Butanol produced by *Clostridium* species

Few species of bacteria produce butanol as a major product, with the anaerobic bacterium *Clostridium acetobutylicum* being the most used species to obtain butanol (Dürre 2008). Since the first isolation of a *Clostridium* species, there has been a rise in solvent production through ABE fermentation, which is considered one of the oldest industrial scale solvent productions (Jones and Woods 1986; Schiel-Bengelsdorf et al. 2013). *Clostridium*

species also produces butyrate, in addition to acetone and ethanol, as co-products, resulting in a lower final butanol concentration, mostly less than 20 g/L, in the traditional method of production. Which also adds to the maximum level of toxicity to the bacteria. Due to low butanol yields, biological production has a high cost for butanol recovery (Ndaba et al. 2015). In addition, *Clostridiaceae* family organisms present low growth rates and spore formation, which causes problems on an industrial scale (Atsumi et al. 2008).

However, in addition to the limitations of using these species to obtain butanol, the progression of the petrochemical industry also led to the decline of the industrial production of this alcohol via fermentation, between 1950 and 1960; whereas the production of butanol via microorganisms is not yet economically viable through the petrochemical method (Gu et al. 2011; Jiang et al. 2015). On the other hand, due to the fluctuation of oil prices, ABE fermentation is still of growing interest among researchers (Gu et al. 2011).

In the text published by Amiri and Karimi (2019), the authors describe and classify the existing obstacles to the traditional manufacture of butanol into three categories: substrate problems, process limitations, and strain shortcomings. When dealing with problems with substrate, one of the studied alternatives is the use of byproducts, lignocellulosic materials and syngas as low-cost substrates. In addition, in recent years the use of genetic engineering has grown, including the use of CRISPR/Cas9 technique, to obtain more robust *Clostridium* strains, capable of producing butanol with higher yields and productivity (Cheng et al. 2019), and with the development of specific tools for *Clostridium* species such as ClosTron (Heap et al. 2007) and flow-cytometric techniques (Tracy et al. 2008). In order to further minimize production costs, the use of genetically modified *Clostridium* strains have been studied in conjunction with simultaneous butanol extraction technologies during cultivation (Lee et al. 2016).

In the work carried out by Huang et al. (2019), the authors demonstrated the use of three strategies capable of solving the obstacles described by Amiri and Karimi (2019), that is, use of low cost substrate (cassava bagasse hydrolysate), a genetically modified strain (*Clostridium tyrobutyricum* overexpressing an aldehyde/alcohol dehydrogenase gene, adhE2), and a different fermentation process (a repeated-batch fermentation with cells immobilized in a fibrous-bed bioreactor). In this scenario, the strain was able to produce butanol with titer greater than 15 g/L, yield of 0.30 g/g and productivity of 0.3 g/L.h. In the end, the authors also carried out an economic analysis and showed that the use of cassava bagasse as a low-cost substrate is economically competitive with traditional food-based production.

Recent research has shown that it is possible to increase the concentration of butanol obtained by *Clostridium* species through the integration of butanol recovery technologies during fermentation. An example of this was demonstrated by Chen et al. (2019), in which the authors used the *Clostridium* sp. strain CT7 capable of using glycerol as a carbon source and producing butanol. The strain was cultured in a bioreactor initially with culture medium containing 60 g/L glycerol and three feeding operations with 20 g/L glycerol in fed-batch with membrane coupled pervaporation process and reached a final concentration of 41.9 g/L of butanol.

In this way, the obstacles that still exist in the traditional production of butanol by *Clostridium* species, have also motivated the study of the use of industrially friendlier organisms, which can be genetically modified for industrial *n*-butanol production, as potential butanol producing organisms. In the last decades, the interest in studying two potentially favorable and well-known organisms for the production of this alcohol has increased, these are *E. coli* and the yeast *S. cerevisiae* (Schadeweg and Boles 2016a).

Butanol production by *Saccharomyces cerevisiae*

S. cerevisiae is a microorganism widely used as a model for studies of other eukaryotic organisms. It is also called by several authors as a microbial cell factory (Si et al. 2014a) and was the first eukaryotic organism to be fully sequenced. This cellular species has been the most used cellular organism in the last decades for the industrial production of several bioproducts, as it is considered a robust organism and well adapted to industrial conditions. In addition, several specific platforms for the *S. cerevisiae* species have been developed to allow the production of new chemicals and fuels (Hong and Nielsen 2012). Within this category of new fuels, butanol has taken place in view of several research groups, which are in search of strategies that make *S. cerevisiae* able to produce large amounts of this alcohol.

It is known that, in addition to ethanol, *S. cerevisiae* strains are capable of naturally producing isobutanol (one of the four isomers of butanol) by the synthesis pathway of 2-ketoisovalerate, an intermediate of the biosynthesis of the amino acid valine. Since 2-ketoisovalerate is synthesized by the cell, it is converted to isobutanol via the Ehrlich pathway and, for this reason, isobutanol is considered a byproduct of valine synthesis (Generoso et al. 2015; Kuroda. and Ueda 2015). On the other hand, there was still some disagreement among

authors about the existence of a wild-type pathway of *n*-butanol production in *S. cerevisiae* (Si et al. 2014a).

In view of the growing interest in the production of *n*-butanol by *S. cerevisiae* and the possibility of an endogenous metabolism of this yeast that is capable of producing this alcohol, many researchers have spared no effort to understand the cellular metabolism of this species in terms of fermentative capacity for *n*-butanol production. A consensus exists among researchers in saying that it is possible to explore two metabolic pathways for *n*-butanol production by *S. cerevisiae*. The first of these is the heterologous expression of the *Clostridium n*-butanol pathway and the second through amino acid assimilation pathways (Si et al. 2014a; Schadeweg and Boles 2016a); or even, the combination of the two strategies (Fig 1).

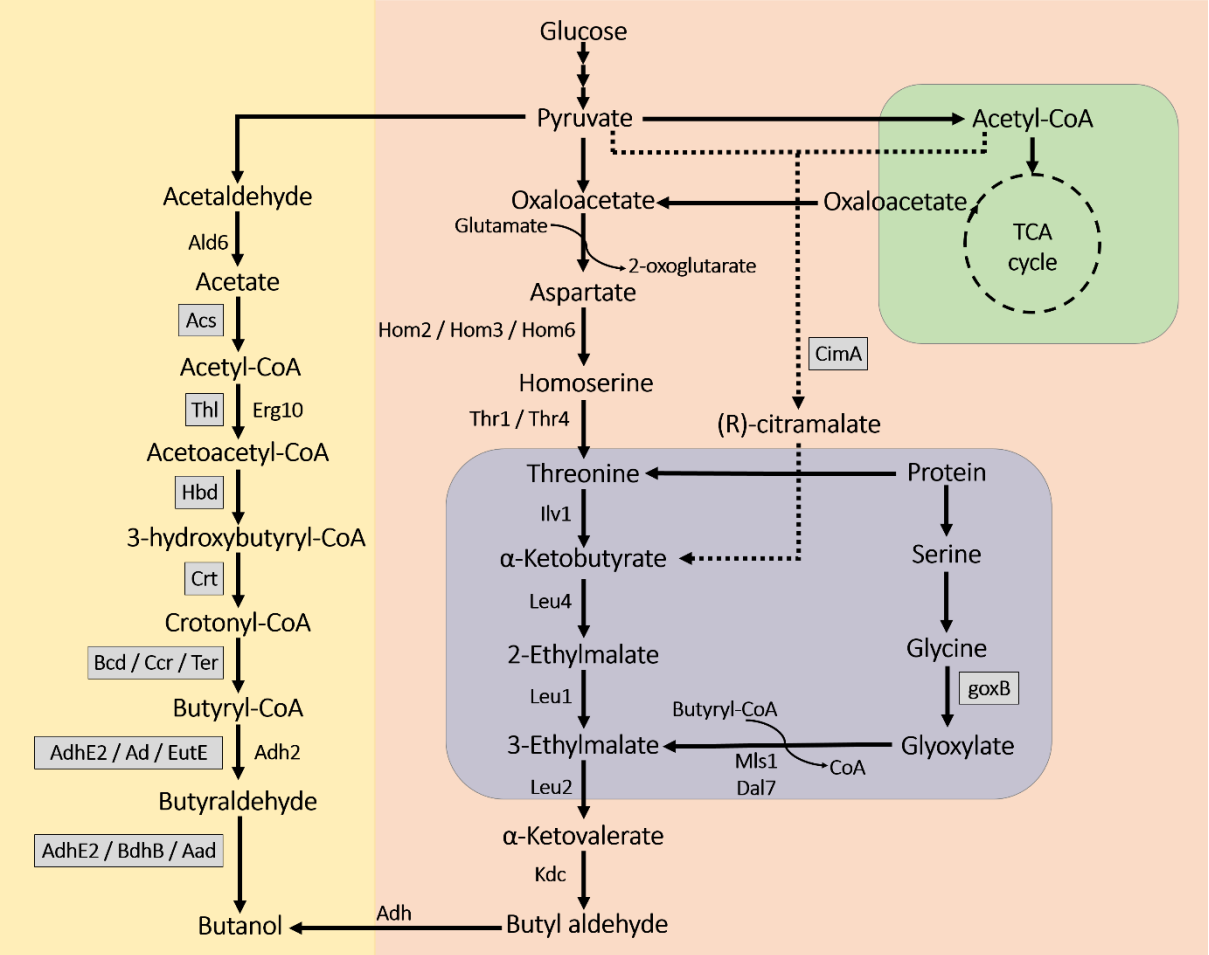

Fig 1 Simplified endogenous and exogenous metabolic pathways for butanol production in S. cerevisiae. Only genes from the relevant steps for butanol production were shown, including heterologous genes (gray background box). Co-factors are omitted for simplicity. The information of biochemical pathways and enzyme locations is from literature (Steen et al.

2008; Branduardi et al. 2013; Krivoruchko et al. 2013; Lian et al. 2014; Si et al. 2014; Swidah et al. 2018; Sakuragi et al. 2015; Swidah et al. 2015; Schadeweg and Boles 2016a, b; Shi et al. 2016). Sequential arrows indicate contraction of the glycolysis pathway. Dotted lines refer to the strategy used by Shi et al. (2016) with the introduction of a citramalate synthase (CimA) gene

Heterologous expression of the *Clostridium n*-butanol pathway in *S. cerevisiae*

The heterologous expression pathway is based on the idea of reconstructing a pathway by inserting the enzymes responsible for the *Clostridium n*-butanol production pathway into *S. cerevisiae* strains with a clean background. The first authors to report the construction of this pathway were Steen et al. (2008). In this work, the authors tested the insertion of several isoenzymes (Table 2) that catalyzed different reactions in the metabolic pathway for the production of *n*-butanol in *S. cerevisiae* BY4742 (strain derived from S288C). Using this strain as background, the authors observed a concentration of 2.5 mg/L *n*-butanol, with the best modified strain, from galactose (2%) as the sole carbon source (Table 3). From this work, others came up with the proposal to use the same strategy, but in different strains and culture media, besides the insertion and deletion of different enzymes to increase *n*-butanol concentrations (Table 2 and 3).

In the study by Krivoruchko et al. (2013), the authors demonstrated that, in addition to the pathway reconstruction, the improved flux towards cytosolic acetyl-CoA (the precursor metabolite for 1-butanol biosynthesis) is of utmost importance for increasing the final concentration of *n*-butanol. Initially, the authors inserted the same enzymes as the study carried out by Steen et al. (2008), however they were unable to detect butanol production in this strain. Then, the authors proposed replacing the crotonyl-CoA reductase (ccr) gene with a NADH-dependent crotonyl-CoA-specific trans-enoyl-CoA reductase (ter) from *Treponema denticola*, resulting in a strain capable of producing 2.1 mg/L butanol, a concentration comparable to the previous work. From this, the authors also proposed deletions in the CIT2 and MLS1 genes to reduce the use of acetyl-CoA via the glyoxylate pathway and noted that these deletions contributed to cytosolic acetyl-CoA supply. After several modifications, the best producer strain (Table 3) had a final *n*-butanol concentration of 16.3 mg/L, an increase of 6.5-fold when compared to the strain of work performed by Steen et al. (2008).

Lian et al. (2014) developed a *S. cerevisiae* strain capable of increasing levels of acetyl-CoA by combining the insertion of heterologous genes and deletion of competitive

pathways (ADH1 and ADH4 for ethanol production and GPD1 and GPD2 for glycerol production) (Table 2). After different combinations, the optimized strain (Table 3) was able to produce nearly 120 mg/L butanol.

In the work conducted by Sakuragi et al. (2015) the authors also confirmed that the deletion of the GPD1 and GPD2 genes reduces the production of glycerol and consequently increases the final concentration of butanol. Furthermore, in this same work the authors demonstrated that the use of the trans-enoyl-CoA reductase gene, in the construction of the *Clostridium* pathway in *S. cerevisiae*, increases the production of *n*-butanol, as well as performed by Krivoruchko et al. (2013). For this construction, the strain was able to produce 14.1 mg/L *n*-butanol after 48 h of cultivation (Table 3). However, the authors did not construct a strain containing the trans-enoyl-CoA reductase gene in conjunction with the deletion of genes from the glycerol pathway. In this sense, it is worth mentioning the strain constructed by Swidah et al. (2015), which was able to produce 300 mg/L *n*-butanol, in complex medium containing 2% glucose (Table 3).

Schadeweg and Boles (2016a) and Schadeweg and Boles (2016b) published two complementary works on the insertion of the *Clostridium* pathway in *S. cerevisiae*. In the first one, the authors started by testing several genes and obtained a production of 15 mg/L *n*-butanol. Then, the implementation of different strategies led to the final production of 120 mg/L *n*-butanol, under anaerobic conditions (Table 2 and 3), which are: increased CoA synthesis by overexpression of the pantothenate kinase coaA gene; pantothenate supplementation in the culture medium; deletion of the ADH1-6 and GPD2 genes to reduce the formation of ethanol and glycerol; and expression of an ATP independent acetylating acetaldehyde dehydrogenase to converting acetaldehyde into acetyl-CoA. In this first study, the authors showed that the VSY10 strain was able to produce *n*-butanol, but only half of the glucose was consumed, due to the inefficiency of NADH re-oxidation. Thus, in the next study, Schadeweg and Boles (2016b) repeated the cultivation of this same strain, however under more aerobic conditions, reaching final concentration of 235 mg/L *n*-butanol. From this, different strategies were used to construct a strain capable of producing the highest concentration among the published works, up until that time by *S. cerevisiae*, the VSY19 strain (Table 3) with 859.05 mg/L *n*-butanol, via a synthetic ABE pathway.

In summary, the authors have observed that one of the remaining challenges is that the mechanisms of *n*-butanol production still compete strongly with the formation of other primary metabolites, such as ethanol and glycerol. Therefore, in addition to the insertion of different enzymes from the *Clostridium* pathway, researchers still need to delete pathways

responsible for the production of these primary metabolites and further increase the availability of coenzyme A and cytosolic acetyl-CoA.

Amino acid assimilation pathway in *S. cerevisiae*

Production of *n*-butanol via the amino acid uptake pathway (Fig. 1) is based on the idea that the degradation of intermediates, such as keto acids in biosynthesis and amino acid degradation pathways, may result in the formation of alcohols by *S. cerevisiae* yeast. In a study by Villas-Bôas et al. (2005), the authors confirmed the existence of a metabolic pathway in *S. cerevisiae* for glyoxylate synthesis from glycine, which has not yet been fully described. In addition, the authors detected α-ketovalerate formation as one of the intermediates of this pathway, which is the precursor of *n*-butanol (Shen and Liao 2008). Knowing this information, Branduardi et al. (2013) hypothesized and biochemically demonstrated the production of *n*-butanol through the degradation of the amino acid glycine.

The authors (Branduardi et al. 2013) used the *S. cerevisiae* CEN.PK102-5B strain as background and, to verify if the cell was capable of producing *n*-butanol. The strain was cultivated in synthetic medium and observed good growth, but did no *n*-butanol production when ammonium sulfate was used as a source of nitrogen. On the other hand, when the same strain was cultivated with glycine as the only nitrogen source, they observed the production of 92 mg/L *n*-butanol. In this experiment the authors used glucose (20 g/L) and glycine (15 g/L) and observed the consumption of both substrates, proving that *S. cerevisiae* is able to produce n-butanol from these substrates.

To confirm the hypothesized metabolic pathway, Branduardi et al. (2013) performed a step by step study, verifying the influence and presence of each enzyme and intermediates of the pathway. The major obstacle in this study was the first reaction of the pathway, i.e., conversion of glycine to glyoxylate. However, a gene coding for this enzyme has not yet been described in the metabolism of *S. cerevisiae*. To prove the method proposed by them and to assume the existence of a native gene in *S. cerevisiae*, the authors suggested the use of the *goxB* gene of *Bacillus subtilis* that codes for a glycine oxidase, and that can catalyze the same reaction. In conclusion, they described as existing the proposed pathway for *n*-butanol production from glycine as a nitrogen source in *S. cerevisiae*. In addition, they speculated that *n*-butanol is derived from butyryl-CoA and that glycine acts as a metabolic flux driver, and is called a co-substrate of the reaction.

Table 2. List of genes inserted in *S. cerevisiae* strains for *n*-butanol production

Gene	Host	Function	Reference
Aad	*C. acetobutylicum*	Aldehyde-alcohol dehydrogenase	Sakuragi et al. (2015)
Acs	*Salmonella enterica*	Acetyl-CoA synthetase	Krivoruchko et al. (2013) and Lian et al. (2014)
Ad	*C. acetobutylicum*	Aldehyde dehydrogenase	Sakuragi et al. (2015)
Adh2	*S. cerevisiae*	Alcohol dehydrogenase	Krivoruchko et al. (2013)
AdhE2	*C. acetobutylicum*	Alcohol dehydrogenase	Schadeweg and Boles (2016) and Schadeweg and Boles (2016b)
AdhE2	*C. beijerinckii*	Alcohol dehydrogenase	Steen et al. (2008), Krivoruchko et al. (2013) and Swidah et al. (2015)
Ald6	*S. cerevisiae*	NADP-dependent aldehyde dehydrogenase	Krivoruchko et al. (2013)
BdhB	*C. acetobutylicum*	NADH-dependent butanol dehydrogenase B	Lian et al. (2014)
Ccr	*Streptomyces collinus*	Crotonyl-CoA reductase	Steen et al. (2008) and Krivoruchko et al. (2013)
Crt	*C. acetobutylicum*	3-hydroxybutyryl-CoA dehydratase	Sakuragi et al. (2015), Schadeweg and Boles (2016) and Schadeweg and Boles (2016b)
Crt	*C. beijerinckii*	3-hydroxybutyryl-CoA dehydratase	Steen et al. (2008), Krivoruchko et al. (2013), Lian et al. (2014) and Swidah et al. (2015)
Erg10	*S. cerevisiae*	Acetyl-CoA C-acetyltransferase	Steen et al. (2008), Krivoruchko et al. (2013), Swidah et al. (2015), Schadeweg and Boles (2016) and Schadeweg and Boles (2016b)
EutE	*E. coli*	Butyraldehyde dehydrogenase	Lian et al. (2014), Schadeweg and Boles (2016) and Schadeweg and Boles (2016b)
Hbd	*C. acetobutylicum*	3-hydroxybutyryl-CoA dehydrogenase	Lian et al. (2014), Sakuragi et al. (2015), Schadeweg and Boles (2016) and Schadeweg and Boles (2016b)
Hbd	*C. beijerinckii*	3-hydroxybutyryl-CoA dehydrogenase	Steen et al. (2008), Krivoruchko et al. (2013) and Swidah et al. (2015)
Ter	*Treponema denticola*	Trans-enoyl-CoA reductase	Krivoruchko et al. (2013), Lian et al. (2014), Sakuragi et al. (2015), Swidah et al. (2015), Schadeweg and Boles (2016) and Schadeweg and Boles (2016b)

Thl	*Candida tropicalis*	Thiolase	Sakuragi et al. (2015)
Thl	*C. acetobutylicum*	Thiolase	Lian et al. (2014)

Table 3. *S. cerevisiae* strains constructed for *n*-butanol production by heterologous expression of the *Clostridium* pathway

Strain	Characteristics	Butanol production (mg/L)	Reference
ESY7	MATα-his3Δ1-leu2Δ0-lys2Δ0-ura3Δ0: pESC-ERG10-hbd-crt + pESC-ccr-adhe2	2.5	Steen et al. (2008)
AKY3	MATa-SUC2-MAL2-8c-ura3-52-his3-D1-cit2D: pAK01-adhE2-ter-crt-hbd + pIYC08-acs^{L641P}-ALD6-ERG10-ADH2	16.3	Krivoruchko et al. (2013)
JL0534	MATa-his3D1-leu2-3-112-ura3-52-trp1-289-MAL2-8c-SUC2-ΔGPD1–ΔGPD2–ΔADH1–ΔADH4: pRS426-CaThl-CaHbd-CbCrt-TdTer-EcEutE-CaBdhB + pRS414-EcLpdA-EcAceE-EcAceF + pRS425-SeAcs^{L641P}Opt	120	Lian et al. (2014)
Strain #4	MATa-ade2-1-his3-11,15-leu2-3,112-trp1-1-ura3-1-can1-100: pRS406-thl-hbd-crt + pRS403-ter + pRS405-ad-aad	14.1	Sakuragi et al. (2015)
A6A2 B^{R} adh1Δ +5g	MATa-ade2-1-his3-11,15-leu2-3,112-trp1-1-ura3-1-can1-100: TRP1-Acs2-Ald6-Erg10-TRP1 + Integ-Adhe2-Bcd-Hbd-Crt-Ter-Integ*	300	Swidah et al. (2015)
VSY10	MATa-ura3-52-trp1-289-leu2-3,112-his3Δ1-MAL2-8C-SUC2 adh1::loxP; adh3::loxP; adh5::loxP; adh4Δ::loxP; adh2Δ::LEU2; sfa1Δ:adhE$^{A267T/E568K}$; adh6Δ:coaA; gpd2Δ: ERG10-hbd-crt-ter-adhE2-eutE	130	Schadeweg and Boles (2016)
VSY19	MATa-ura3-52-trp1-289-leu2-3,112-his3Δ1-MAL2-8C-SUC2 adh1::loxP; adh3::loxP; adh5::loxP; adh4Δ::loxP; adh2Δ::LEU2; adh6Δ::coaA, loxP; sfa1Δ::adhE$^{A267T/E568K/R577S}$, loxP; pFMS1Δ::HIS3, pADH1; ald6Δ; gpd2Δ: ERG10-hbd-crt-ter-adhE2-eutE + pRS62H_ter	859.05	Schadeweg and Boles (2016b)

* The site of integration was not specified in the reference

After this work, other studies were carried out to verify the production of *n*-butanol by *S. cerevisiae* from the amino acid degradation pathway. Si et al. (2014) studied the production of *n*-butanol via the degradation pathway of the threonine amino acid from overexpression of the proposed enzymes and elimination of the ethanol, glycerol, valine, leucine and isoleucine production pathways. Using *S. cerevisiae* YSG50 as background, the modified strain was capable of producing 242.8 mg/L *n*-butanol. In this work, the authors studied carbon marked in glycine ($_{L}$-glycine-2-^{13}C) and glucose ($_{D}$-glucose-$^{13}C_6$) and observed that all carbons of *n*-butanol formed were derived only from glucose and not from glycine. This confirms the hypothesis raised by Branduardi et al. (2013) of glycine as a co-substrate.

In another study on the role of the amino acid degradation pathway for butanol production, Shi et al. (2016) implemented a synergistic pathway with the endogenous threonine pathway and the introduced citramalate pathway in *S. cerevisiae*, besides overexpression of keto-acid decarboxylases (KDC) and alcohol dehydrogenase (ADH), and co-expression of LEU genes. The final strain was able to produce 835 mg/L *n*-butanol in anaerobic glass tubes. When cultivated in bioreactor, under micro-anaerobic condition, the same strain produced a final concentration of 1.05 g/L *n*-butanol.

Recently, a study by Swidah et al. (2018) demonstrated the contributions of the two strategies (heterologous expression and amino acid pathways) in the production of butanol by *S. cerevisiae*, called the combination of endogenous and exogenous pathways (Fig. 1). For this, they used a strain already constructed in a previous study containing the ABE pathway and the ADH1 deletion of the ethanol production pathway (Swidah et al. 2015). At the end of this study, the authors suggested that the exogenous pathway is responsible for most of the butanol produced. In addition, the presence of the amino acid glycine and the deletion of ADH1 demonstrated that the endogenous pathway is also responsible for part of the production of butanol, and the optimization of these combined strategies is a promising step for synthetic biotechnology.

Although *S. cerevisiae* is still capable of producing a much lower titer of *n*-butanol, the production of this alcohol by *Clostridium* species becomes industrially complicated due to the characteristic of these organisms to be strictly anaerobic. In addition, ABE fermentation generates other byproducts, reducing yields in butanol, since this type of fermentation occurs in two phases (acidogenesis and solventogenesis). As well as ethanol production by *S. cerevisiae* still going through to changes and improvements, even though it is a very well consolidated process, the butanol production by this yeast is a process with the potential to become economically viable. However, as it is a topic recently addressed in the literature, it

still requires a lot of study, with the results obtained until today being the beginning of a long work.

Butanol tolerance by *S. cerevisiae*

Once an organism begins to form metabolic products, the cells must be able to tolerate these compounds in the medium. The mechanism of microorganism tolerance to butanol is very similar to ethanol tolerance (Liu and Qureshi 2009). *S. cerevisiae* is an organism capable of tolerating up to 18% of ethanol in media, depending on the conditions of cultivation (Pereira et al. 2011; Della-Bianca and Gombert 2013; Ishmayana et al. 2017), however, this yeast is not able to tolerate more than 2% butanol (Knoshaug and Zhang 2009). Ishmayana et al. (2017) reported that, although butanol tolerance is related to membrane fluidity, different strains may present different behaviors due to the intrinsic properties of each strain.

Gonzalez-Ramos et al. (2013) analyzed the butanol tolerance of *S. cerevisiae* BY4741 and CEN.PK 113-7D strains in synthetic medium containing different concentrations of *n*-butanol (0 to 1.9%) in sealed 96-well plates to prevent transfer and oxidation of butanol. The authors observed that the strains grew 50% and 30%, respectively, slower in 1% of *n*-butanol and the growth of both was drastically reduced; not being able to grow in concentrations of *n*-butanol above 1.45% (BY4741) and 1.57% (CEN.PK 113-7D). From genomic-scale analyses, the authors identified mutations in three genes that encode transcription factors, showing that *n*-butanol tolerance in *S. cerevisiae* is related to protein degradation.

Knoshaug and Zhang (2009) performed a screening for tolerance at different concentrations of butanol in non-*Saccharomyces* and *S. cerevisiae* strains in microplates containing YPD (yeast-peptone-dextrose) media. Among the 10 strains evaluated, only one was not able to grow in 1% of *n*-butanol, while the others presented relative growth (RG%) of around 60%. However, in the presence of 2% *n*-butanol, only three *S. cerevisiae* strains (ATCC26602, ATCC20252 and Fali) were able to grow, with RG% between 10 and 20%.

Genetic and evolutionary engineering are the most widely used laboratory strategies to develop both the consumption ability and tolerance of a certain substrate or product and, when combined, can yield very efficient results (Mans et al. 2018). Tolerance to a particular product, such as butanol, laboratory evolution has been very useful, especially for a better understanding of the mechanisms that perform tolerance by identification of gene targets that improve alcohol tolerance through inverse metabolic engineering (Hong et al. 2010).

In the study by Ghiaci et al. (2013) an industrial *S. cerevisiae* strain was evolved from 30 sequential batches with a gradual increase of 2-butanol to make this strain more tolerant. At the end of the study, the authors observed that the evolved strain was more tolerant not only to 2-butanol, but also to 1-butanol, isobutanol, ethanol and propanol. In addition to laboratory evolution, which usually occurs by successive passages under selection pressure, the use of mutagenic agents can also be used to achieve a desired phenotype known as random mutagenesis (Teoh et al. 2015). Finally, it is believed that more butanol-tolerant strains may be able to produce higher titers of this alcohol (Crook et al. 2016).

Conclusions

The production of *n*-butanol by *S. cerevisiae* still faces many challenges, however, much remains to be studied. It is important to point out that all studies developed up to now have used laboratory strains of *S. cerevisiae* yeast as background. In addition, none of these researchers carried out studies on the bioprocess involved in the production of *n*-butanol. The production of butanol has still been studied mainly on synthetic media, so that the mechanisms of production are understood and the butanol yields increased. However, as well as in the production of second-generation ethanol, these butanol-producing *S. cerevisiae* strains will also need to be analyzed for the ability to convert sugars from other sources, such as lignocellulosic biomass. In terms of tolerance, the final concentration of butanol produced by *S. cerevisiae* is still much lower than the toxic concentration for the cell. However, evolution studies in *S. cerevisiae* aiming to increase butanol tolerance are important because tolerant strains may be able to produce more. In addition, tolerance information can be extremely important in order to also understand the mechanisms of *n*-butanol production.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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CAPÍTULO 3

ANALYSIS OF METABOLITE PROFILES OF *Saccharomyces cerevisiae* STRAINS SUITABLE FOR BUTANOL PRODUCTION

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Analysis of metabolite profiles of *Saccharomyces cerevisiae* strains suitable for butanol production

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ABSTRACT

Butanol has advantages over ethanol as a biofuel. Although butanol is naturally produced by some *Clostridium* species, clostridial fermentation has inherent characteristics that prevent its industrial application. Butanol-producing *Saccharomyces cerevisiae* strains may be a solution to this problem. The aim of this study was to evaluate the ability of wild-type and industrial Brazilian strains of *S. cerevisiae* to produce *n*-butanol using glycine as co-substrate and evaluate the relationship between the production of this alcohol and other metabolites in fermented broth. Of the 48 strains analyzed, 25 were able to produce *n*-butanol in a glycine-

containing medium. Strains exhibited different profiles of *n*-butanol, isobutanol, ethanol, glycerol and acetic acid production. Some wild-type strains showed substantial *n*-butanol production capability, for instance, UFMG-CM-Y267, which produced about 12.7 mg/L of butanol. Although this concentration is low, it demonstrates that wild-type *S. cerevisiae* can synthesize butanol, suggesting that selection and genetic modification of this microorganism could yield promising results. The findings presented here may prove useful for future studies aimed at optimizing *S. cerevisiae* strains for butanol production.

Keywords: *Saccharomyces cerevisiae*; butanol; strains; glycine; heat map; *z*-score.

INTRODUCTION

The current massive use of petroleum fuels and the dependence of many sectors of society on petroleum-derived materials stimulate the production of fuels and chemicals from renewable sources (Chen and Liao 2016). Bioethanol has received widespread attention in recent years, but another alcohol has several advantages over ethanol as a biofuel: butanol. This four-carbon alcohol has higher energy density, lower hygroscopicity, and lower vapor pressure than ethanol (Nanda et al. 2017), which are desirable characteristics for a biofuel. Furthermore, similar to ethanol, butanol can be used as a starting material or solvent in industrial reactions (Branduardi et al. 2014).

Butanol is naturally produced by some *Clostridium* species. However, clostridial fermentation has inherent problems, namely, the need for strictly anaerobic conditions, low microbial growth, and spore formation. Because of this, the yeast *Saccharomyces cerevisiae* has been studied as an alternative microorganism for butanol production (Schadeweg and Boles 2016). The metabolic pathways of isobutanol production by *S. cerevisiae* have been described (Milne *et al.* 2016), but the biosynthesis of the isomer *n*-butanol is less well established. Amino acid uptake pathways have been investigated to provide information on how to increase *n*-butanol production by *S. cerevisiae* (Kuroda and Ueda 2015). A study found that *S. cerevisiae* can synthesize *n*-butanol using a nitrogen source and glycine as co-substrate (Branduardi *et al.* 2013).

Microorganisms used in industrial fermentation processes must be tolerant to the metabolic products they release into the medium, such as alcohols and acids; otherwise, cell viability might be compromised. The susceptibility of microorganisms to metabolic products affects recovery costs and is an obstacle to the large-scale production of metabolites (Liu and Qureshi 2009). *S. cerevisiae* strains tolerate a maximum of 2% (v/v) butanol, as do *Clostridium* species (Knoshaug and Zhang 2009). Their butanol tolerance is lower than their

ethanol tolerance, but this has not been a problem under experimental conditions, as no *S. cerevisiae* strain has been reported to produce such high butanol concentrations. Poor tolerance and low production titers are challenges that must be addressed to make butanol production by *S. cerevisiae* economically feasible. Information on the metabolite production profile of wild-type and commercial strains, that is, the relationship between concentrations of butanol, isobutanol, and other metabolites (ethanol, glycerol, and acids) released into the medium, might prove useful to solve these problems.

Most studies analyzing *S. cerevisiae* for butanol production were carried out using laboratory strains and engineering techniques (Krivoruchko *et al.* 2013; Lian *et al.* 2014). Strains with wild-type or industrial genetic background are expected to exhibit greater tolerance to stress than laboratory background strains as a result of natural selection under environmental conditions (i.e., biomes or ethanol industries) (Pereira *et al.* 2014). We speculate that if a strain is naturally more conducive to butanol production, it is more likely to have a suitable genetic background for engineering high butanol-producing strains. This study aimed to screen wild-type and industrial Brazilian strains of *S. cerevisiae* for the ability to synthesize *n*-butanol using glycine as co-substrate. Another aim was to determine their metabolite production profiles for use in future studies.

MATERIAL AND METHODS

Yeast strains

Forty-eight *S. cerevisiae* strains were analyzed (Table 1). CEN.PK113-7D and X2180-1B (alpha SUC2 mal mel gal2 CUP1) are laboratory strains. JAY270 is a haploid of the industrial strain PE-2. Strains numbered 4–9 are industrial strains used in Brazilian ethanol production plants. Strains coded UFMG were isolated from tree bark samples collected from

different Brazilian ecosystems (Barbosa *et al.* 2016). ATCC strains were obtained from the American Type Culture Collection. Cells were stored in glycerol at −80 °C until use.

Table 1 *Saccharomyces cerevisiae* strains tested in this study

#	Strain	#	Strain	#	Strain
1	CEN.PK113-7D (Control)	**17**	UFMG-CM-Y455[d]	**33**	IZ0677
2	JAY270	**18**	UFMG-CM-Y266[d]	**34**	IZ0684
3	X2180-1B	**19**	UFMG-CM-Y267[d]	**35**	IZ1215
4	BG-1	**20**	UFMG-CM-Y643[a]	**36**	IZ1348
5	CR-1	**21**	UFMG-CM-Y259[a]	**37**	IZ1349
6	SA-1	**22**	UFMG-CM-Y260[c]	**38**	IZ1350
7	FT858	**23**	UFMG-CM-Y636[d]	**39**	IZ1351
8	CAT-1	**24**	IZ0137	**40**	IZ1716
9	NAD	**25**	IZ0310	**41**	IZ1832
10	UFMG-CM-Y254[a]	**26**	IZ0651	**42**	IZ1833
11	UFMG-CM-Y255[b]	**27**	IZ0658	**43**	IZ2003
12	UFMG-CM-Y256[a]	**28**	IZ0659	**44**	IZ2004
13	UFMG-CM-Y257[a]	**29**	IZ0662	**45**	ATCC4125
14	UFMG-CM-Y262[c]	**30**	IZ0669	**46**	ATCC4132
15	UFMG-CM-Y263[d]	**31**	IZ0671	**47**	ATCC24858
16	UFMG-CM-Y264[d]	**32**	IZ0672	**48**	ATCC26785

1–5: Bioprocess and Metabolic Engineering laboratory (LEMeB), Brazil.

6–9: Chemical, Biological, and Agricultural Pluridisciplinary Research Center (CPQBA), Brazil.

10–23: Collection of Microorganisms and Cells of the Federal University of Minas Gerais, Brazil.

24–44: Luiz de Queiroz College of Agriculture (ESALQ), Brazil.

45–48: American Type Culture Collection, USA.

Place of isolation. a) Quercus rubra (Red oak or Carvalho), Santuário do Caraça (Jardins), Minas Gerais, Brazil. b) Non-identified tree, Santuário do Caraça (Trilha da Cascatona), Minas Gerais, Brazil. c) Tapirira guianensis (Tapirirá), Taquaruçu (Mata ripária do Córrego Buritizal), Tocantins, Brazil. d) Tapirira guianensis (Tapirirá), Taquaruçu (Mata ripária do Córrego Bela Vista), Tocantins, Brazil.

Cultivation conditions and production of *n*-butanol and other metabolites

Strains were reactivated in solid YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, and 20 g/L agar) and incubated at 30 °C until the appearance of isolated colonies. One colony from each plate was inoculated in a modified synthetic medium (pH 6) containing the following components (per liter): 3 g KH_2PO_4, 6.6 g K_2SO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 2.3 g NH_2CONH_2, 20 g glucose, and trace elements and vitamin solution

(Verduyn *et al.* 1992). Cells were incubated at 30 °C and 200 rpm for 24 h. After this period, cells were centrifuged at 5000 *g* for 5 min, washed twice with sterile water, and resuspended in fresh medium to obtain a standardized cell suspension with an optical density (600 nm, OD_{600}) of 1 (spectrophotometer 4001/4 Genesys 20, Thermo Fisher Scientific, USA).

The standardized cell suspension (10 μL) was added to the wells of a 96-well plate and received the addition of 90 μL of fresh synthetic medium (20 g/L glucose) supplemented with 15 g/L glycine, resulting in an initial OD_{600} of 0.1. The plate was sealed with PCR sealing film (676040 Ampliseal, Greiner Bio-One GmbH, Kremsmünster, Austria) to reduce oxygen levels and was incubated at 30 °C and 198 rpm for 72 h on a 96-well plate reader (Infinite® M200, Tecan, Männedorf, Switzerland). The OD_{600} of each well was measured every 1 h during this period.

Metabolite determination

At the end of the 72 h incubation period, samples were filtered through a Millex® GV Durapore® PVDF membrane (0.22 μm, Millipore, Massachusetts, USA) for *n*-butanol, isobutanol, ethanol, glycerol, and acetic acid concentration analysis. Samples were quantified on an HPLC (Thermo Fischer Scientific, USA) system equipped with an infrared detector (Thermo Fischer Scientific, USA) at 50 °C and a HyperREZ XP column at 60 °C, using 5 mM H_2SO_4 as mobile phase at 0.6 mL/min. Calibration curves were constructed using analytical standard solutions (Sigma–Aldrich, USA) at concentrations of 5–50 mg/L for *n*-butanol and isobutanol and concentrations of 0.1–10 g/L for ethanol, glycerol, and acetic acid.

Hierarchical cluster analysis

The metabolite production data from the 48 strains were *z*-score transformed using Eqs. 1 and 2:

$$x = \frac{x_{\mathrm{i}}}{x_{\mathrm{i,control}}} \quad (1)$$

$$z_{\mathrm{i}} = \frac{x - \mu}{\sigma} \quad (2)$$

where x_{i} is the metabolite concentration produced by a strain, $x_{\mathrm{i,control}}$ is the metabolite concentration produced by the control strain (CEN.PK113-7D), z_{i} is the *z*-score for a specific metabolite, μ is the mean of the population, and σ is the standard deviation of the population.

CEN.PK113-7D was chosen as a control because it is widely used in laboratory experiments. Hierarchical clustering based on the Euclidean distance between *z*-scores was performed and a heat map was generated using Morpheus (https://software.broadinstitute.org/morpheus, Broad Institute, USA).

Statistical analysis

Analysis of variance (ANOVA) was carried out using Statistica® 5.5 (StatSoft, USA). Tukey's test was used to determine differences at $p < 0.05$.

RESULTS AND DISCUSSION

Influence of nitrogen source on *n*-butanol production

A strategy widely used in recent years to obtain *n*-butanol by non-clostridial fermentation is the use of keto acids. Keto acids are intermediates of the synthesis and degradation of amino acids via the Ehrlich pathway (Shen and Liao 2008). In order to evaluate the influence of the amino acid glycine as a nitrogen source on *n*-butanol production, we carried out preliminary experiments with CEN.PK113-7D (control) and JAY270 using glycine (15 g/L), urea (2.3 g/L), or a combination of both as nitrogen sources under the conditions described in the Methods section for cultivation and production of *n*-butanol and other metabolites. The glycine concentration was based on the results of Branduardi *et al.* (2013), and the urea concentration is the molar equivalent to the concentration of ammonium

sulfate used by Verduyn *et al.* (1992). The mixture of the two nitrogen sources contained the same final concentration of each component and not their molar equivalents. The preliminary results revealed that CEN.PK113-7D produced detectable concentrations of butanol (9.9 mg/L) in medium containing a mixture of glycine and urea but not in medium supplemented with only one of the nitrogen sources. On the other hand, JAY270 was able to produce *n*-butanol in medium containing urea (8.4 mg/L *n*-butanol) or urea and glycine (8.6 mg/L *n*-butanol) but not in medium containing glycine as the sole source of nitrogen.

Butanol is produced mainly by cells in the stationary phase. Urea reduces acidification of the medium during growth and increases biomass production (van Leeuwen *et al.* 2009), whereas glycine participates in the production of butanol. The control strain may not have been able to produce detectable concentrations of butanol in medium containing urea as the sole nitrogen source because of the lack of a nitrogen flow for butanol production, such as that provided by glycine. In medium containing glycine as the only nitrogen source, the control strain was not able to produce butanol probably because of the low cell concentration. The 72 h incubation period may not have been sufficiently long. On the other hand, JAY270, which is derived from a robust industrial strain (PE-2), was able to grow and produce butanol in medium containing urea as the sole nitrogen source. These preliminary results highlight that different strains from the same species may have different metabolite production profiles under the same conditions.

Branduardi *et al.* (2013) cultivated CEN.PK102-5B in synthetic medium (Verduyn *et al.* 1992) containing 5 g/L ammonium sulfate or 15 g/L glycine as nitrogen source and concluded that glycine is required for *n*-butanol production. In a study carried out with carbon-labeled glycine (L-glycine-2-^{13}C) and glucose (D-glucose-$^{13}C_6$), Si *et al.* (2014) observed that *n*-butanol was formed entirely from glucose, not from glycine, and argued that the presence of exogenous glycine reduces the conversion of threonine to glycine, which

increases the conversion of threonine to butanol. This corroborates the hypothesis that glycine acts as a co-substrate (Branduardi *et al.* 2013). As our results suggested that glycine enhances *n*-butanol production by *S. cerevisiae*, we tested the *n*-butanol production ability of the other strains in a synthetic medium containing both urea and glycine.

Metabolite production profile of *S. cerevisiae* strains

We evaluated *n*-butanol production by *S. cerevisiae* strains cultivated in medium containing glucose (20 g/L) as the sole carbon source and urea (2.3 g/L) and glycine (15 g/L) as nitrogen sources under oxygen-limiting conditions (Fig. 1). Of the 48 analyzed strains, 25 had a negative result for *n*-butanol production. This does not mean that these strains are not able to produce butanol but that butanol concentrations were below the detection limit of the method (5 mg/L) or that the alcohol was oxidized by dehydrogenase enzymes (Si *et al.* 2014). UFMG-CM-Y267, UFMG-CM-Y264, and UFMG-CM-Y259 were the major *n*-butanol producers, yielding a final butanol concentration of 12.7, 11.2, and 11.5 mg/L, respectively (Fig. 1).

S. cerevisiae strains produced low *n*-butanol concentrations under oxygen-limiting conditions. These results were expected, as the strains were not selected or genetically modified for expression or overexpression of genes associated with *n*-butanol production. Previous studies have also reported that butanol production is much lower than ethanol production, regardless of strain or culture conditions (Steen *et al.* 2008; Branduardi *et al.* 2013; Schadeweg and Boles 2016). In the current study, the mean concentration of ethanol was about 600-fold higher than that of *n*-butanol. For instance, UFMG-CM-Y267 (the strain with the greatest butanol production ability), reached a butanol yield of 0.63 mg/g glucose, which corresponds to only 0.15% of the theoretical maximum of 0.41 g butanol/g glucose

(Generoso *et al.* 2015); on the other hand, its ethanol yield was 0.26 g/g glucose, 51% of the theoretical maximum.

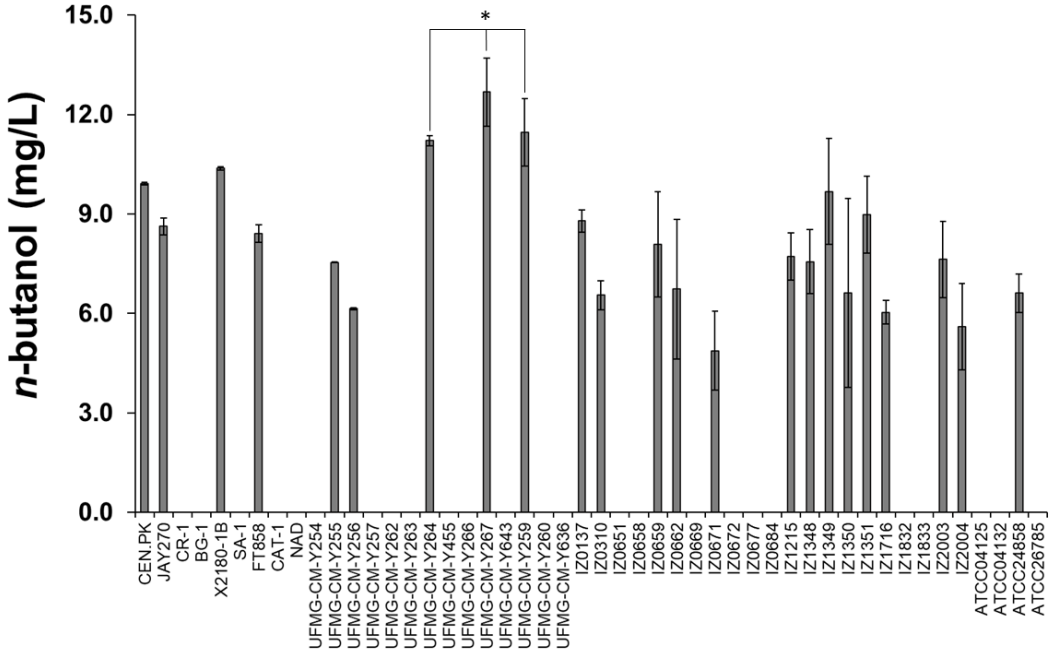

Figure 1. *n*-Butanol production by 48 strains of *Saccharomyces cerevisiae* cultivated in defined medium containing glucose (20 g/L), urea (2.3 g/L), and glycine (15 g/L) at 30 °C and 198 rpm for 72 h. Columns and error bars represent respectively the mean and stand ard deviation of three replicates. * Values do not differ significantly ($p < 0.05$) according to Tukey's test.

In addition to low concentrations of *n*-butanol, we also found low concentrations of isobutanol in the cultured broths. The mean isobutanol production was 11 mg/L. Of the 48 strains analyzed, only 6 were not able to produce detectable concentrations of this alcohol. The UFMG-CM-Y267 strain produced the highest concentration of isobutanol, 32.8 mg/L, which equates to a yield of 1.64 mg isobutanol/g glucose (0.4% of the theoretical maximum). Researchers have attempted to improve *n*-butanol and isobutanol yields of *S. cerevisiae* using metabolic engineering techniques, such as expression of complete metabolic pathways or overexpression of specific genes (Branduardi *et al.* 2013; Hammer and Avalos 2017).

Under oxygen-limited conditions, ethanol is the main (but not the sole) end product of glucose metabolism in *S. cerevisiae*. Pyruvate, a key molecule in yeast metabolism, can originate other alcohols and acids (Fiechter and Seghezzi 1992), as shown in the simplified metabolic flow chart of Fig. 2A. The chart shows the connection between ethanol, glycerol, and acetic acid production pathways and the metabolic routes for the formation of *n*-butanol and isobutanol using glycine as co-substrate. Fig. 2A also illustrates that, depending on the strain and reaction conditions, a metabolite may be preferentially produced instead of the others.

To evaluate the relationship between the production of *n*-butanol and other metabolites, we analyzed the main fermentation products of *S. cerevisiae*, namely, ethanol, glycerol, acetic acid, and isobutanol. The metabolite profile of the CEN.PK113-7D strain was used as control (Fig. 2C). Metabolite concentration data were *z*-score normalized and presented as a heat map for easy interpretation (Fig. 2B). Previous studies used *z*-score heat maps to analyze phenotypic data of *S. cerevisiae* strains (Mello *et al.* 2019) and changes in the expression of genes linked to diseases in humans (Xiong *et al.* 2019).

The mean ethanol production of the 48 strains was 2.6 g/L. CEN.PK113-7D (control) showed the highest ethanol production, 5.9 g/L (Fig. 2B). Ethanol was the main fermentation product of the control strain, followed by glycerol, acetic acid, isobutanol, and *n*-butanol. This pattern holds true for almost all strains analyzed. Were the heat map generated from non-normalized data, all strains would show an intense red color for ethanol (high production), an intense blue color for butanol (low production), and intermediate colors for the other metabolites.

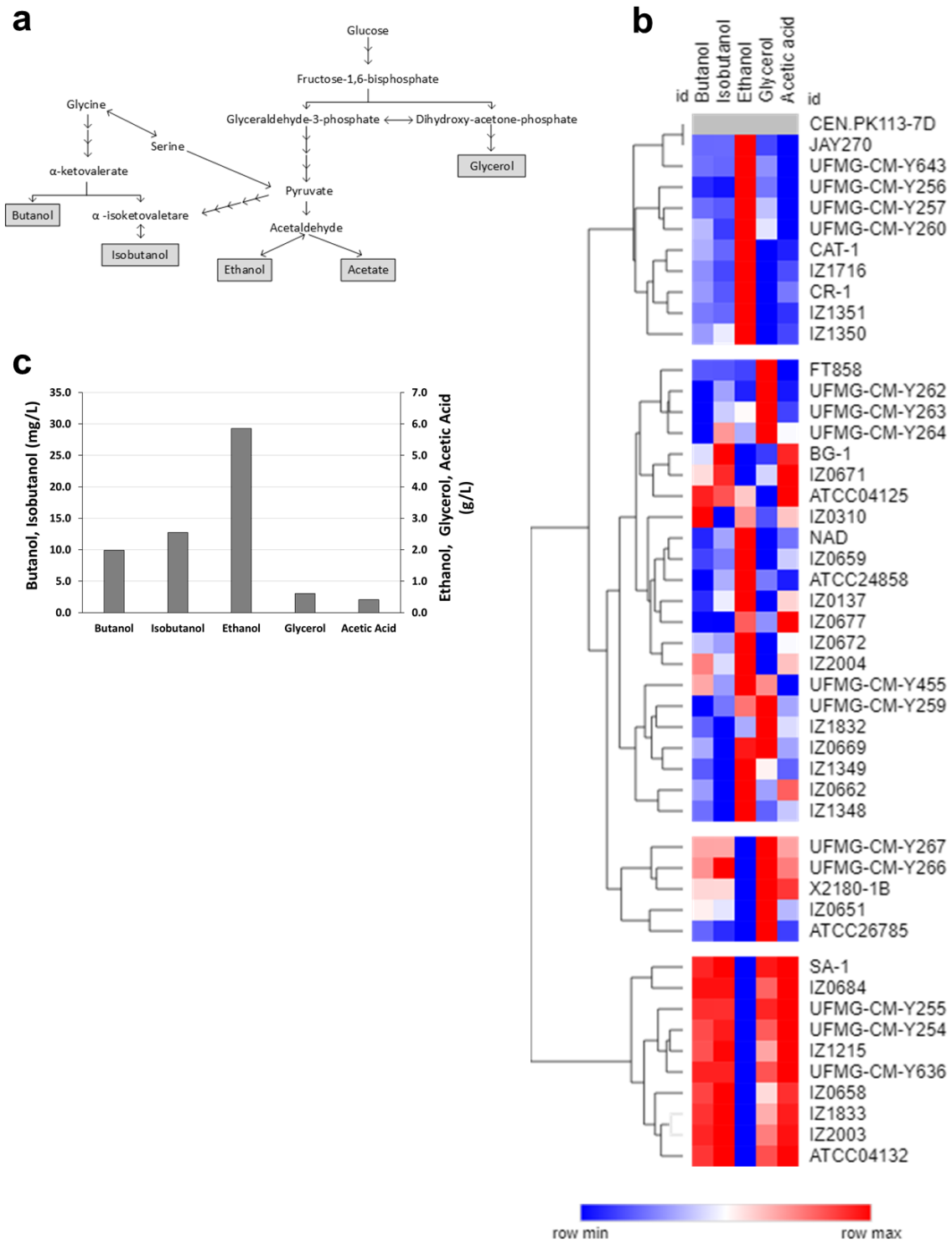

Figure 2. Metabolite production profile of 48 strains of *Saccharomyces cerevisiae* cultivated in defined medium containing glucose (20 g/L), urea (2.3 g/L), and glycine (15 g/L) at 30 °C and 198 rpm for 72 h. **(a)** Metabolic pathways for ethanol, glycerol, acetate, butanol, and isobutanol production using glucose as substrate and glycine as co-substrate. **(b)** Concentration of metabolites produced by CEN.PK113-7D (control strain). **(c)** Hierarchical cluster analysis of S. cerevisiae strains and heat map illustrating z-scores for metabolite production. Values are color-coded from dark blue (lowest) to dark red (highest).

Hierarchical cluster analysis organized the data into four clusters (Fig. 2B). The first cluster was formed by high ethanol-producing strains. The high *z*-score for ethanol of strains in the first cluster shows that their ethanol production is higher than the mean of the population. The second cluster consisted of strains with large variability in metabolite production. The third cluster was composed of strains with above average glycerol production. During anaerobic fermentation, glycerol plays an important role in the oxidation of NADH to NAD^+ in the cytosol. However, excessive glycerol production is undesirable because it reduces the availability of carbon for butanol formation (Ansell *et al.* 1997). Strains with the lowest *z*-scores for ethanol production were grouped in the fourth cluster.

The heat map (Fig. 2B) clearly shows that *S. cerevisiae* strains differ greatly in metabolite production. These microorganisms include industrial strains of *S. cerevisiae* used for first-generation ethanol production in Brazil, strains isolated from different trees species in Minas Gerais and Tocantins, Brazil, and strains used by the wine industry and molasses distilleries in Holland (Table 1). Our results suggest high genetic variability, selectivity for specific metabolic pathways, and genetic and phenotypic differences between wild-type and industrial strains, probably as a result of natural selection under environmental or industrial conditions. Gallone *et al.* (2016) also observed high genetic variability among *S. cerevisiae* strains.

CONCLUSIONS

In this study, we reported the ability of 48 strains of *S. cerevisiae* to produce *n*-butanol and other metabolites. Ethanol, glycerol, and acetic acid were the major fermentation products of all strains. Higher alcohols, such as *n*-butanol and isobutanol, were produced in low concentrations. Twenty-three strains were able to produce *n*-butanol using glycine as co-substrate without requiring genetic modification for expression or overexpression of genes

responsible for butanol production. The strains also varied in their ability to produce ethanol, glycerol, acetic acid, and isobutanol. These different metabolite profiles indicate differences in genetic background. Future studies can use the information presented here as a starting point for optimizing *S. cerevisiae* strains for *n*-butanol production or for the production of other metabolites.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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CAPÍTULO 4

BUTANOL PRODUCTION BY *Saccharomyces cerevisiae* OVEREXPRESSING GLYCINE OXIDASE GENE BY CRISPR/CAS9

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Article in progress

Butanol production by *Saccharomyces cerevisiae* overexpressing glycine oxidase gene by CRISPR/Cas9

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Abstract

Butanol production by *S. cerevisiae* strains has been the subject of studies in the last decade. These studies use two strategies to achieve this goal, the heterologous expression of the *Clostridium* pathway and the amino acid uptake pathway. The aim of this study was to obtain *S. cerevisiae* strains modified from the insertion of the gene that encodes the enzyme glycine oxidase (*goxB*) by CRISPR/Cas9 edition and to verify the influence of overexpression of this gene on *n*-butanol production by the glycine amino acid uptake pathway. For this, the laboratory strain JAY270 and the wild-type strain UFMG-CM-Y267 were selected. Both modified strains showed higher glycine oxidase activity than the parental strains, with increases in activity from 53% to just over 200%. However, the modified strain SAJgox (from the JAY270 background) stood out not only for presenting a glycine oxidase activity up to

200% higher than the parental strain, but also for having been positively affected in terms of *n*-butanol production, since which was able to produce around 300% more of this alcohol. Although the final concentration of *n*-butanol obtained was still very low (around 15 mg/L), the use of CRISPR/Cas9 as a genetic modification approach proved to be efficient, and the SAJgox strain can be used to intensify future studies.

Keywords: Butanol Production; CRISPR/Cas9; Glycine Oxidase Activity; *S. cerevisiae*.

Introduction

The generation of biofuels emerged as a challenging proposal to overcome the climate problems of recent decades. In addition to the environmental appeal, the production of fuels and chemicals from renewable sources has become a trend due to the constant increase and volatility in the price and supply of oil (García et al. 2011). One of the answers to this is the production of butanol, a biofuel that has gained visibility. Butanol is a superior alcohol that has physicochemical characteristics more similar to gasoline, compared to ethanol, and has been considered as a better substitute for fossil fuels (Gottumukkala et al. 2017; Nanda et al. 2017).

Traditionally, butanol is produced by the acetone-butanol-ethanol (ABE) fermentation process by strains of *Clostridium* species (Lan and Liao 2013). However, this production route still has some disadvantages such as low yield, high level of bacterial toxicity and high cost (Atsumi et al. 2008; Schiel-Bengelsdorf et al. 2013). In view of this, in recent years the yeast *S. cerevisiae* has been the subject of studies as a butanol-producing microorganism, because it is more easily adapted to industrial conditions and because it has a range of genetic tools that help in this process.

It has already been proven that the yeast *S. cerevisiae* is capable of naturally producing butanol, however, even at very low concentrations (Branduardi et al. 2013; Azambuja et al. 2019a). Thus, most studies have focused on carrying out genetic modifications in this yeast, using two strategies. The first one is heterologous expression of the *Clostridium* pathway, through the insertion of *Clostridium* pathway genes in *S. cerevisiae* strains, which is a very complex strategy. The second strategy is based on the idea that the amino acid uptake pathway by *S. cerevisiae* can result in the formation of higher alcohols (Villas-Bôas et al. 2005).

Branduardi et al. (2013) were the first authors to biochemically demonstrate the route and production of *n*-butanol from glycine as a co-substrate. The authors used a plasmid containing the coding gene for the enzyme glycine oxidase (*goxB*) from *Bacillus subtilis*, inserted it into a laboratory strain of *S. cerevisiae*, and described the metabolic pathway of *n*-butanol production from glycine acting as a metabolic flow driver. However, in the work carried out by Branduardi et al. (2013), the *goxB* gene was inserted using a plasmid, requiring the maintenance of selection markers. Therefore, in this work, the objective was to obtain modified *S. cerevisiae* strains with the insertion of the *goxB* gene by CRISPR/Cas9 edition and to verify the activity of this enzyme and the ability to produce *n*-butanol by the modified strains, in comparison with the parental strains.

Materials and methods

Strains, cultivation, and maintenance conditions

S. cerevisiae strains used in this work are presented in Table 1 and were stored in 20% glycerol at -80°C and reactivated in solid Yeast-Peptone-Dextrose (YPD) medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, and 20 g/L agar) at 30°C for further use. The main cultivations were carried out in a modified synthetic (MS) medium containing the following components (per liter, pH 6): 3 g KH_2PO_4, 6.6 g K_2SO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 2.3 g NH_2CONH_2, 20 g glucose, and trace elements and vitamin solution (Verduyn et al. 1992), and added glycine 15 g/L when necessary. *Escherichia coli* strain DH5α was used for plasmid propagations and vectors storage, grown in LB medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl) at 37ºC. Plasmid and primers used in this study are listed in Tables 1S and 2S, in the supplementary material.

Table 2. *Saccharomyces cerevisiae* strains used in this study

Strain	Description	Source
Saccharomyces cerevisiae JAY270	Haploid of the Brazilian ethanol-producing strain Pedra-2 (PE-2)	Argueso et al. 2009
Saccharomyces cerevisiae UFMG-CM-Y267	Strain isolated from Tapirira guianensis (Tapirirá) tree at Taquaruçu (Mata ripária do Córrego Bela Vista), Tocantins, Brazil	Barbosa et al. 2016
Saccharomyces cerevisiae SAJgox	*Saccharomyces cerevisiae* JAY270 genetically modified with *goxB* gene	This study
Saccharomyces cerevisiae SAYgox	*Saccharomyces cerevisiae* UFMG-CM-Y267 genetically modified with *goxB* gene	This study

Genetic Engineering via CRISPR/Cas9

Guide RNA (gRNA)

CRISPR guide RNA (gRNA) used in this work (pGS_004.29) contained the regulatory elements for editing. The gRNA sequence targeted locus *HO* in *S. cerevisiae* strains.

Donor DNA synthesis

Glycine oxidase (*goxB* gene) was amplified from the plasmid pYX212*goxB* opt, kindly provided by Professor Paola Branduardi from the University of Milano-Bicocca (Italy). The sequence of the gene is founded on Table 3S – supplementary material. The donor DNA synthesis was performed by amplifying the *goxB* gene sequence with PCR reaction using Phusion High Fidelity DNA Polymerase® (ThermoFisher). The PCR product was treated with the restriction enzyme dpnI® (ThermoFisher).

Yeast strains construction by CRISPR/Cas9

The strains *Saccharomyces cerevisiae* UFMG-CM-Y267 and JAY270 were independently co-transformed with the gRNA (pGS_004.29) and donor DNA (*goxB* gene). The co-transformation method employed was described by Gietz and Schiestl (2007), using Lithium Acetate (LiAc), salmon sperm DNA as carrier, and polyethylene glycol (PEG). In summary, were used 1000 ng of total donor DNA, 0.1 mg of SS carrier DNA (ThermoFisher®), 100 µL of yeast previously prepared and 900 µl of PEG/LiAc solution (40% PEG 4000, 1X TE, 1X LiAc). After the homogenization, yeast cells were maintained under agitation for 1h at 30ºC, then DMSO was added, and the yeast cells were submitted to a thermal shock (15 min at 42ºC). Plasmids pGS_004.1 and pGS_005.0 were also transformed as control of the procedure. The transformants were selected in YPD agar plates with G418. After the selection, yeast genomic DNA was extracted with phenol and treated with RNAse A, and the edition was confirmed by PCR.

Glycine oxidase activity

Wild-type and modified strains were evaluated for glycine oxidase activity in the presence of glycine as a co-substrate in the culture medium. For this, a colony was pre-inoculated in liquid YPD medium at 30°C and 200 rpm for 15h. The cell suspension obtained was transferred to 125 mL Erlenmeyer flask containing 50 mL of MS medium and incubated at 30°C and 200 rpm for 24 hours. The previously washed and centrifuged cells served as inoculum for 50 mL Falcons containing 30 mL of MS medium (15 g/L of glycine) with an initial optical density of 0.5, at 600 nm (OD_{600}, spectrophotometer 4001/4 Genesys 20, Thermo Fisher Scientific, USA), and incubated at 30°C and 200 rpm. The cell extracts and enzymatic activities were carried out in Falcons removed at 24, 48, 72, 96 and 120 h of culture.

Cell extracts were prepared according to the method by (Postma et al. 1989) with modifications. Samples were centrifuged for 10 min at 5000 rpm at 4°C, washed once with ice-cold freeze buffer (10 mM potassium phosphate, pH 7.5, and 2 mM EDTA), washed once with ice-cold sonication buffer (100 mM potassium phosphate, pH 7.5, and 2 mM $MgCl_2$), and resuspended in 4 mL of sonication buffer and 40 µL of 1,4-Dithiothreitol (DTT). Cell disruption was performed with the addition of approx. 3 g of glass beads of 425-600 µm (Sigma-Aldrich, Missouri, USA) in vortex for 1 min followed by resting on ice for 1 min, this cycle being repeated 5 times. Sample was centrifuged and the supernatant was used as the cell extract for glycine oxidase activity.

Glycine oxidase activity was evaluated spectrophotomically and expressed in U/mg of total proteins by the method described by Branduardi et al. (2013).

Cultivation conditions for n-butanol production

To evaluate the butanol production capacity of the wild-type and modified strains, one colony from each plate strain was inoculated in MS medium at 30 °C and 200 rpm for 24 h. Then, cells were centrifuged at 5000 g for 5 min, washed twice with sterile water, and resuspended in fresh medium to obtain a standardized cell suspension with an OD_{600} of 1.

Ten microliters of the standardized cell suspension and 90 µL of fresh MS medium (20 g/L glucose) supplemented with 15 g/L glycine were added to the wells of a 96-well plate, resulting in an initial OD_{600} of 0.1. The plate was sealed with PCR sealing film (676040 Ampliseal, Greiner Bio-One GmbH, Kremsmünster, Austria) and incubated at 30 °C and 198 rpm for 72 h on a 96-well plate reader (Infinite® M200, Tecan, Männedorf, Switzerland), with OD_{600} measurement every 1 h. At the end of the 72 h incubation period, samples were filtered through a Millex® GV Durapore® PVDF membrane (0.22 µm, Millipore, Massachusetts, USA) and sent for the *n*-butanol quantification.

Metabolite determination

The samples were analyzed by gas chromatography with a flame ionization detector by direct injection by Central Analítica (Institute of Chemistry/UNICAMP, Campinas, Brazil).

Statistical analysis

Analysis of variance (ANOVA) was carried out using Statistica® 5.5 (StatSoft, USA). Tukey's test was used to determine differences between samples at the significance level of 5%.

Results and Discussion

In previous studies by Azambuja et al. (2019), different strains of *S. cerevisiae* yeast, including wild-type and laboratory strains, were tested for their ability to produce butanol against other secondary metabolites. In this work, the strain UFMG-CM-Y267 was able to produce 12.7 mg/L of butanol in a culture medium containing glycine as a co-substrate. Now, in the present work, this strain was selected as background for the insertion of the glycine oxidase gene (*gox*B). The hypothesis is that *goxB* gene insertion results in an increase of butanol production capacity, once this gene encodes for an enzyme with ability to generate glyoxylate from glycine deamination and consequent formation of α-ketovalerate, a compound that can be converted into butanol. In addition, we decided to also incorporate as a background a strain commonly used in the industrial production of ethanol in Brazil, JAY270 strain, derived from Pedra-2 (PE-2).

For CRISPR/Cas9 edition approach, preliminary tests (data not shown) were conducted to determine the specific HO locus position for genetic modification. HO locus is often chosen to receive gene insertion in *S. cerevisiae* strains because it is considered a neutral

site for genetic edition. These tests showed that the same position in the HO locus could be used for both strains, industrial and laboratorial, for *goxB* gene insertion using the same guide RNA (pGS_004.29 gRNA). *gox*B gene – donor DNA – was ampliflied from the plasmid pYX212*goxB* opt background and to ensure that no methylated DNA was present in the donor DNA, the product of the amplification was treated with dpnI restriction enzyme. Thus, both strains were independently cotransformed.

To confirm the insertion of the gene of interest, transformed colonies were subjected to DNA extraction with phenol and PCR tests using primers GGO_001 and GGO_004. Colonies carrying the *goxB* gene were expected to have a PCR product of 3803 bp, referring to the sum of the size of the donor cassette *goxB* (2926 bp, Fig. 1a) and the external primers GGO_001 and GGO_004 (877 bp). As the objective of this step was to obtain the transformed strains, the work of confirming the insertions was carried out only until a first colony of each strain was identified with the gene of interest, thus obtaining the modified strains SAJgox and SAYgox (Fig. 1b and c).

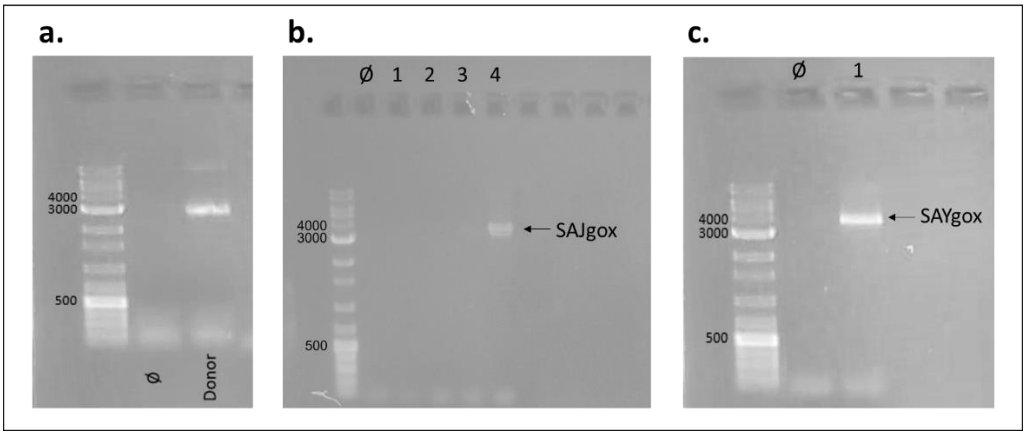

Figure 3. CRISPR/Cas9 editing. **a.** Donor cassette amplification; Control reaction Ø; Primer set: OSA_006 and OSA_007; Expected PCR product: 2926 bp. **b.** JAY270 confirmation colony; Control reaction Ø; Negative colonies #1, 2 and 3; Positive colony #4; Primer set: GGO_001 and GGO_004; Expected PCR product: 3803 bp. **c.** UFMG-CM-Y267 confirmation colony; Control reaction Ø; Negative colony #1; Positive colony #1; Primer set: GGO_001 and GGO_004; Expected PCR product: 3803 bp.

After confirming the presence of the *goxB* gene in the colonies, the now proven modified strains (*S. cerevisiae* SAJgox and SAYgox) and their respective parental strains were grown in a culture medium containing glycine for 72 h to evaluate the glycine oxidase activity every 24 h (Fig. 2 and 3). For JAY270 background, it can be observed that, at all times of analysis, the glycine oxidase activity of the modified strain (SAJgox) was higher than its parental strain, being a non-significant increase only for the time of 48 h. Although at 48 h the increase in activity was not significantly different, this increase was 53%. In contrast, for 24 and 72 h, there was a very significant increase in enzyme activity of 200 and 171%, respectively, by the modified strain. As for UFMG-CM-Y267 background, at all times of analysis there was a statistically significant increase when comparing the modified strain (SAYgox) with its parental strain, showing percentage increases of 148, 232 and 169%, respectively. This proves that, for both strains, the gene of interest was inserted into the yeast genome, and they began to overexpress the enzyme glycine oxidase, when cultivated in the presence of glycine in the medium.

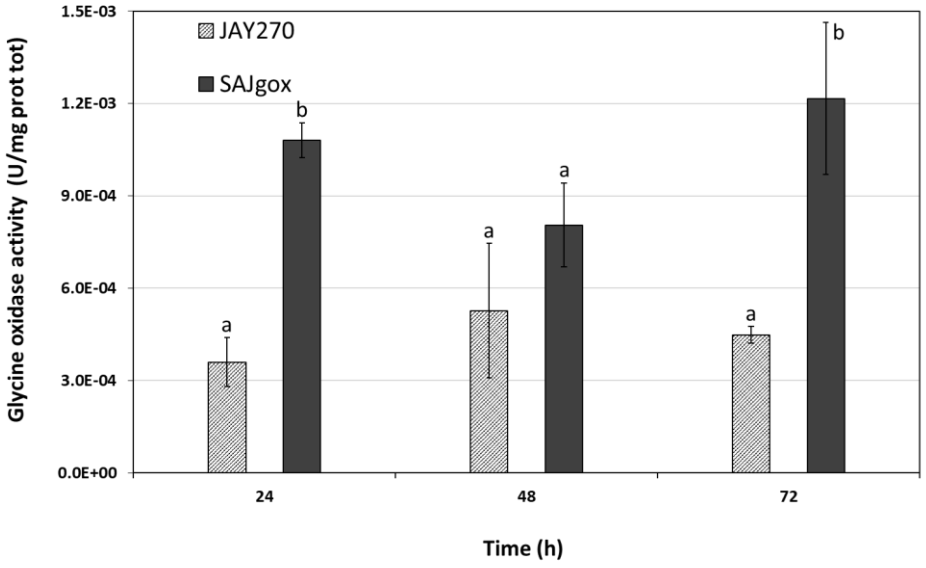

Figure 4. Glycine oxidase activity of *S. cerevisiae* JAY270 and SAJgox strains grown in medium containing glycine for 72 h. Columns and error bars represent respectively the mean and standard deviation of three replicates. Columns followed by the same letter, in the same analysis time (24, 48 or 72 h), were not significantly different from each other ($p < 0.05$) according to the Tukey's test.

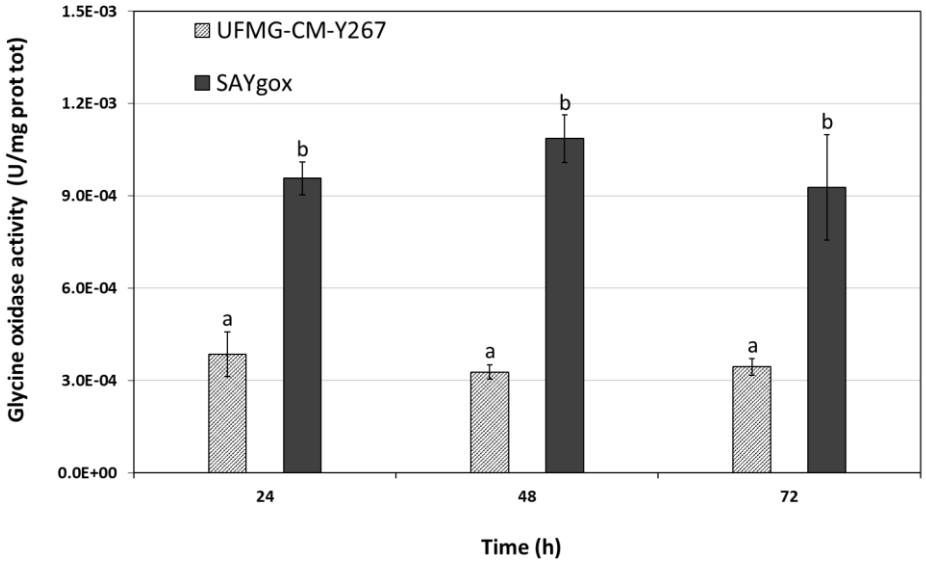

Figure 5. Glycine oxidase activity of *S. cerevisiae* UFMG-CM-Y267 and SAYgox strains grown in medium containing glycine for 72 h. Columns and error bars represent respectively the mean and standard deviation of three replicates. Columns followed by the same letter, in the same analysis time (24, 48 or 72 h), were not significantly different from each other ($p < 0.05$) according to the Tukey's test.

However, overexpression of the glycine oxidase gene by yeast may not necessarily lead to an increase in butanol production. Thus, the parental and modified strains were cultivated in a medium containing glycine and evaluated for *n*-butanol production after 72 h of cultivation (Table 2).

Table 2. *n*-Butanol production of parental e modified strains after 72 h of cultivation in MS medium containing 15 g/L of glycine

Strain	***n*-Butanol (mg/L)**
JAY270	$< 3^a$
SAJgox	13 ± 1^b
UFMG-CM-Y267	15 ± 1^b
SAYgox	9 ± 1^c

Averages followed by the same letter, were not significantly different from each other ($p < 0.05$) according to the Tukey's test.

Based on the results presented in Table 2, it is evident that for JAY270 background, the insertion of the glycine oxidase gene (*goxB*) significantly affected not only the activity of

this enzyme, but also increased its *n*-butanol production capacity by around 300%. On the other hand, for UFMG-CM-Y267 background, the insertion of the gene negatively affected the production of butanol by the modified strain, being able to produce approximately 40% less of its capacity without overexpression of the gene.

To demonstrate the production of *n*-butanol through the degradation of the amino acid glycine, Branduardi et al. (2013) modified the *S. cerevisiae* CEN.PK102-5B to overexpressing the *goxB* gene, and the yeast presented 1.5-fold higher glycine oxidase activity and was capable of producing 30% more butanol when compared to the parental strain. Compared to the present work, the wild-type strains JAY270 and UFMG-CM-Y267 showed average glycine oxidase activity during the 72 h of cultivation (4.4E-04 and 3.5E-04 U/mg prot, respectively) very similar to *S. cerevisiae* CEN.PK102-5B, also around 4.0E-04 U/mg prot. On the other hand, when comparing the modified strains, SAJgox and SAYgox showed higher activity of this enzyme, especially SAJgox with 1.0E-03 U/mg prot. The main difference between those modified strains (CEN.PK102-5B and those presented in this work) is the genetic modification approach. Branduardi et al. (2013) inserted *goxB* gene using a plasmid and this work modified the genome of JAY270 and UFMG-CM-Y267 strains by CRISPR/Cas9 edition. As CRISPR/Cas9 is a novel, innovative, effective, and efficient technique that provide new strains without the need of selection markers maintenance, feature that is not found for plasmid modifications that can undergo to changes in the actitivity of the inserted gene. For this reason, both strains SAJgox and SAYgox are considered robust strains applicable to different environments.

Other studies carried out for *n*-butanol production from pure amino acids and genetically modified strains of *S. cerevisiae*, observed the production of *n*-butanol at concentrations higher than those observed in this work. Si et al. (2014) investigated the production of *n*-butanol from the threonine amino acid degradation pathway, reaching a titer

of 242.8 mg/L of this alcohol. Shi et al. (2016) made improvements in the threonine degradation pathway and obtained a final concentration of 835 mg/L of *n*-butanol.

Is important to observe that the production of *n*-butanol by SAJgox strain (13 mg/L) was not statistically different from wild-type strain UFMG-CM-Y267 (15 mg/L), showing that the latter already has naturally a greater production capacity of this alcohol. The overexpression of only one gene of the amino acid degradation pathway proved to be effective only for JAY270 strain, but perhaps new insertions of genes responsible for other steps in this pathway could improve the performance of this strain and positively affect UFMG-CM-Y267 strain.

Another possibility, also being studied by other research groups, would be the combination of the amino acid assimilation pathway and the ABE (acetone-butanol-ethanol) heterologous pathway. As performed by Swidah et al. (2018), in which the authors proposed the combination of the heterologous ABE pathway and the endogenous glycine pathway of *S. cerevisiae* and showed that by supplementing the medium with glycine and α-ketovalerate, *n*-butanol titers reached 380 and 2400 mg /L, respectively, with the production of *n*-butanol being higher in α-ketovalerate since it is known that the addition of keto acids in the fermentation medium leads to the formation of higher alcohols in amounts directly proportional to the concentrations of keto acids added (Ayrapaa 1971).

Conclusions

Here, the use of the CRISPR/Cas9 genomic editing tool for overexpression of the coding gene for the enzyme glycine oxidase in *S. cerevisiae* was demonstrated. The modified strains showed glycine oxidase activity up to 200% higher than the parental ones. Only the modified strain SAJgox was positively affected in terms of *n*-butanol production capacity, starting from a butanol concentration by the parental strain of less than 3 mg/L to 13 mg/L by

the modified strain, representing an increase of 300%. Although this *n*-butanol concentration is still very small, this increased capacity of the SAJgox strain to produce *n*-butanol makes it a promising strain for future studies, from the insertion or deletion of specific genes, improvement of the bioprocess and culture media, to the use of raw material composed of different amino acids, since the use of pure amino acids may not be economically viable.

Author contribution

SPHA performed all the experiments, collected and analyzed the data, and written and reviewed the manuscript. MACS performed the experiments, collected and analyzed the data, and written and reviewed the manuscript. CAR, GST, and RG contributed to study design, protocol development, and reviewed the manuscript. All authors read and approved the final manuscript.

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Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Supplementary Material

Table 1S. List of plasmids used in this study

Plasmid name	Relevant feature	Source
pGS_004.0	CRISPR/cas9 background. Backbone used to assemble the guide RNAs pTEF1-Cas9-tCYC1; pSNR52-sgRNA-tSUP4	Research group
pGS_004.29	gRNA sequence targeting *HO* with the accessory elements necessary for CRISPR edition	Research group
pGS_004.1	CRISPR/cas9 background for URA3 deletion	Research group
pGS_005.0	Plasmid with resistance to Geneticin	Research group

Table 2S. List of primers used in this study

Primer name	Sequence	Purpose
OSA_006	CTTACGGTTTGTGACGATCACGTTCCGCTATCATGGTTATGGCAGGAACT	*goxB* gene amplification from pXY212
OSA_007	ATCTCCATAATGAAGCCTTACATGTTTGGCACGGTAAAACGACGGCCAGT	*goxB* gene amplification from pXY212
GGO_001	CAGCAATGTCAGACGCTTGA	primer HO *locus* for *goxB* confirmation
GGO_004	TCTGTGAAGTTGTTCCCCCA	primer HO *locus* for *goxB* confirmation

Table S3. Sequence of optimized *goxB* gene

ATGAAGAAACACTACGACACTGCAGTTATAGGTGGAGGGATCATTGGTTGTGCGATATCGTA
CGAATTGGCCAAAACTCAACAGAAGGTTGTCCTGCTAGAAGCTGGAGAAGTAGGTAGAAAGA
CTACTAGTGCTGCTGCTGGAATGCTTGGAGCTCATGCCGAATGCGAAAACAGGGATGCTTTC
TTTGACTTTGCCATGCACTCACAAAGGCTTTATGAACCAGCAGGGCAAGAATTGGAAGAAGC
ATGTGGTATTGATATTAGACGTCATAATGGCGGAATGTTGAAGTTAGCCTATACGGAAGAGG
ATATTGCCTGTTTAAGAAAGATGGATGATTTACCTAGCGTTACCTGGTTGTCTGCTGAAGAT
GCATTGGAGAAGGAACCTTATGCATCGAAAGACATACTAGGTGCATCCTTTATAAAAGATGA
TGTGCACGTAGAACCGTATTATGTCTGCAAAGCCTACGCTAAAGGGGCTAGGAGATATGGTG
CTGACATTTACGAACACACACAAGTCACCTCAGTGAAAAGAATGAACGGAGAGTATTGCATC
ACAACATCAGGTGGAGATGTTTATGCCGACAAGGTTGCAGTTGCTTCTGGTGTATGGTCTGG
TCGTTTCTTTTCCCAGTTAGGTTTAGGTCAACCATTCTTTCCAGTAAAAGGCGAGTGTTTGA
GTGTTTGGAATGACGATACCCCATTAACCAAGACTCTTTACCATGACCATTGTTACGTGGTT
CCAAGAAAGTCCGGCAGATTGGTCATTGGTGCCACTATGAAACATGGTGATTGGTCTGATAC
ACCTGACATTGGTGGCATTGAAGCTGTGATTGGTAAGGCGAAAACGATGCTACCAGCAATTG
AGCACATGAAAATCGATAGATTTTGGGCGGGTTTAAGACCGGGAACAAGAGATGGCAAACCC
TTCATTGGGAGACATCCCGAAGATAGCGGCATAATCTTTGCAGCCGGTCATTTCAGAAATGG
CATACTGCTGGCTCCTGCAACAGCTGAAATGGTCAGAGACATGATCTTGGAACGTCAGATAA
AACAAGAGTGGGAAGAGGCATTTAGGATCGATAGAAAAGAGGCGGTTCATATCTAA

CAPÍTULO 5

PERFORMANCE OF *Saccharomyces cerevisiae* STRAINS AGAINST THE APPLICATION OF ADAPTIVE LABORATORY EVOLUTION STRATEGIES FOR BUTANOL TOLERANCE

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Article under submission process

Performance of *Saccharomyces cerevisiae* strains against the application of adaptive laboratory evolution strategies for butanol tolerance

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Abstract

Although the industrial production of butanol has been carried out for decades by bacteria of the *Clostridium* species, recent studies have shown the use of the yeast *Saccharomyces cerevisiae* as a promising alternative. While the production of *n*-butanol by this yeast is still very far from its tolerability (up to 2% butanol), the improvement in the tolerance can lead to an increase in butanol production. The aim of the present work was to evaluate the adaptive capacity of the laboratory strain X2180-1B and the Brazilian ethanol-producing strain CAT-1 when submitted to two strategies of adaptive laboratory Evolution (ALE) in butanol. The strains were submitted, in parallel, to ALE with successive passages or with UV irradiation, using 1% butanol as selection pressure. Despite initially showing greater tolerance to butanol,

the CAT-1 strain did not show great improvements after being submitted to ALE. Already the laboratory strain X2180-1B showed an incredible increase in butanol tolerance, starting from a condition of inability to grow in 1% butanol, to the capacity to grow in this same condition. With emphasis on the X2180_*n100*#28 isolated colony that presented the highest maximum specific growth rate among all isolated colonies, we believe that this colony has good potential to be used as a model yeast for understanding the mechanisms that involve tolerance to alcohols and other inhibitory compounds.

Keywords: Tolerant Yeast; ALE; Evolved Colony; Successive Passages; UV Irradiation

Introduction

Although *Clostridium* species are traditionally used for butanol production, the yeast *S. cerevisiae* has been shown, for the last 10 years, as a more suitable organism option to produce this alcohol. Over the decades, *S. cerevisiae* has been accumulating advantages that make it one of the microorganisms most widely studied and used industrially, with a range of tools and genetic platforms available, making it the best organism model eukaryote for studies of bioprocesses, food production, drugs, in addition to the well-established ethanol production process (Nevoigt 2008; Çakar et al. 2012; Ryu 2021). Several studies have already demonstrated that *S. cerevisiae* yeast can produce butanol naturally (Branduardi et al. 2013; Si et al. 2014a; Shi et al. 2016; Swidah et al. 2018; Azambuja et al. 2019b) or from genetically modified strains (Steen et al. 2008; Krivoruchko et al. 2013; Lian et al. 2014; Sakuragi et al. 2015; Swidah et al. 2015; Schadeweg and Boles 2016a; Schadeweg and Boles 2016b), still in small concentrations, but with great potential.

Often, the production of a certain cellular metabolite is directly linked to its ability to tolerate the presence of the metabolite intra or extracellularly, so that there is no inhibition by the product. Comparable with *Clostridium* strains, in terms of butanol tolerance, the yeast *S. cerevisiae* can tolerate a maximum of 2% (v/v – or 20 g/L) in the medium (Knoshaug and Zhang 2009). Although the maximum concentration of butanol obtained until today from *S. cerevisiae* is still lower than its tolerance capacity – 859.05 mg/L (Schadeweg and Boles 2016b) – a more tolerant yeast may be able to present higher yields and final concentration of butanol (Knoshaug and Zhang 2009). This same phenomenon is already observed in industrial yeasts producing ethanol and, since the mechanisms of tolerance to ethanol by *S. cerevisiae* appear to be the same for butanol, it is necessary to search for more knowledge about how this mechanism of tolerance works and how yeast can be more tolerant to butanol (Nevoigt 2008; Liu and Qureshi 2009), this information is often obtained from adaptive laboratory evolution

(ALE) of strains subjected to a certain selection pressure to obtain an expected phenotype (Lee and Kim 2020).

Adaptive laboratory evolution is a technique capable of imitating, in the laboratory, an environment of natural evolution, applying certain selective pressure to the organism studied. ALE came about as part of inverse metabolic engineering to complement traditional metabolic engineering since it does not demand much prior knowledge for its realization and provides important information for subsequent genetic manipulation (Nevoigt 2008; Çakar et al. 2012; Winkler and Kao 2014; Lee and Kim 2020; Mavrommati et al. 2021). In addition to being simple, ALE is a very versatile approach, which can be applied to obtain different phenotypes, such as: to increase the consumption of substrates, resistance to inhibitors and adverse environmental conditions, and tolerance to metabolic products (Winkler and Kao 2014; Zhu et al. 2018).

There are several possible ways to perform ALE, however, Lee and Kim (2020) listed 3 factors that researchers need to take into account for satisfactory results to be achieved: the mode of cultivation, the appropriate selective pressure, and the time span. The most used ALE model is from successive batch cultivations. In this model, the cells are exposed to the desired selective pressure, and after a certain time span, the cells are transferred - repeatedly - to a fresh culture medium, under the same conditions. In this cultivation mode, ALE can be performed from a microscale, as in 96-well plates, to bioreactors. There is also the possibility of using chemical and physical mutagenic agents as a selective pressure in evolutionary engineering experiments. This technique can be used solo or to increase the frequency of mutation initially or during ALE (Winkler and Kao 2014; Zhu et al. 2018; Ryu 2021). One of the great issues of ALE is the third factor, the time span. As already reported, ALE has great advantages, but they are known as time-consuming experiments. One of the great challenges of this technique is to know when to stop (Lee and Kim 2020). Thinking about these issues,

our work aimed at the parallel application of two ALE strategies - with successive passages and UV irradiation as a mutagenic agent - in two different *S. cerevisiae* strains (laboratorial and Brazilian ethanol-producing strain), using 1% *n*-butanol as selective pressure, to evaluate the adaptive capacity of these strains in butanol.

Materials and methods

Strains and maintenance conditions

The 48 strains of *S. cerevisiae* initially screened are presented in Table 1 (Barbosa et al. 2016; Azambuja et al. 2019), as well as the pool of cells and selected evolved colonies. Yeast cells were maintained in 30% glycerol at -80 ºC until use. For the preparation of the standardized cell suspension, the strains were reactivated in solid YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, and 20 g/L agar) and incubated at 30 ºC for 48 h. Liquid cultures were carried out in synthetic medium contained the following, per liter (pH 6): 3 g KH_2PO_4, 6.6 g K_2SO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 2.3 g NH_2CONH_2, 20 g glucose, trace elements, and vitamins solution (Verduyn et al. 1992).

Screening for n-butanol tolerance

Cells from an isolated colony were incubated in synthetic medium at 30 °C and 200 rpm for 24 h. After this period, cells were resuspended in a fresh synthetic medium to obtain a standardized cell suspension with an optical density (OD_{600}) of 1 in a spectrophotometer at 600 nm (4001/4 Genesys 20, Thermo Fisher Scientific, USA). The standardized cell suspension (10 µL) was placed into a 96-well plate and received the addition of 90 µL of fresh synthetic medium containing different concentrations of *n*-butanol (0, 1, or 2% v/v), resulting in an initial OD_{600} of 0.1. The plate was sealed with PCR sealing film (676040 Ampliseal, Greiner Bio-One GmbH, Kremsmünster, Austria) and incubated at 30 °C and 198

rpm until cells reached the stationary phase. The OD_{600} of each well was measured every 30 min using the Infinite® M200 plate reader (Tecan, Männedorf, Switzerland). The relative growth (%RG) of cells was calculated according to Eq. 1:

$$\%RG = \frac{OD_{max} \text{ of cultivation with butanol}}{OD_{max} \text{ of cultivation without butanol}} \times 100 \quad (1)$$

Where OD_{max} is the maximum optical density reached in the stationary phase of cultivation. Cultivations were carried out in triplicate for each *n*-butanol concentration.

Strategy 1: adaptive laboratory evolution with successive passages in n-butanol

S. cerevisiae strains CAT-1 and X2180-1B were selected and submitted to two different ALE strategies. For the first strategy, strains were subjected to successive passages as described by de Melo et al. (2020), with the necessary adaptations for this work. A colony of solid YPD was grown in a 50 mL Falcon containing 30 mL of synthetic medium at 30 ºC and 200 rpm for 24 h. Cells were resuspended in a 50 mL Falcon containing 30 mL of fresh synthetic medium (1% *n*-butanol) with an initial OD_{600} of 0.5 and incubated at 30 ºC and 200 rpm for 24 h. After the incubation time, the cells were resuspended in a 50 mL Falcon containing 30 mL of fresh synthetic medium (1% *n*-butanol) with an initial OD_{600} of 0.5 and incubated at 30 °C and 200 rpm for 24 h. This step was performed daily until the strains reached 100 generations (*n100*), that is, 53 and 58 days for CAT-1 and X2180-1B, respectively. The final number of generations was estimated cumulatively by the number of generations at each successive passage, considering OD_{600} as the cell parameter measured at the end of each successive passage, using Eq. 2.

$$OD_f = OD_0 \times 2^n \quad (2)$$

Where OD_f is the optical density measured at the end of each successive passage, $OD_0 = 0.5$, and *n* is the number of generations.

Strategy 2: adaptive laboratory evolution with UV irradiation

For the second strategy, *S. cerevisiae* strains CAT-1 and X2180-1B were also subjected to successive passages in 1% *n*-butanol, but now with previous exposure of the cells to UV irradiation, in each cycle. A colony of solid YPD was grown in a 50 mL Falcon containing 30 mL of synthetic medium at 30 ºC and 200 rpm for 24 h. Cells were resuspended in sterile distilled water and inoculated with OD_{600} required for growth of approximately 200 colonies in a Petri dish (90 x 15 mm) containing solid synthetic medium (2% glucose and 2% agar). The cells in the Petri dish were exposed to UV light (NIS Germicida 15W G15T8) at a fixed height of 24.5 cm for 20 s (CAT-1) and 35 s (X2180-1B) and incubated at 30 ºC for 72 h. Five mL sterile distilled water were transferred to the Petri dish to obtain a cell suspension with the colonies. The cells were centrifuged (5000 rpm for 5 min), washed, resuspended in a 50 mL Falcon containing 30 mL of fresh synthetic medium (1% *n*-butanol) with an initial OD_{600} of 0.5, and incubated at 30 ºC and 200 rpm for 24 h. After the incubation time, the OD_{600} of the culture was measured, the cells were centrifuged, washed, and again exposed to UV light (~200 cells). Every 96 h, a new cycle of passage with UV light was performed, and this procedure was repeatedly performed during 53 and 58 days for CAT-1 and X2180-1B, respectively.

Selection of evolved colonies

The cell suspensions (pool) obtained at the end of the two strategies were subjected to a sequence of analyzes to select possible evolved colonies. The pool obtained from strategy 1,

which reached 100 generations (*n100*), was called CAT_*n100* and X2180_*n100*; and the pool of strategy 2 was called CAT_*UV* and X2180_*UV* (Table 1).

Qualitative analysis of evolved colonies

Was transferred 100 µL of the cell suspension to a 50 mL Falcon containing 3 mL of synthetic medium and incubated at 30 °C and 200 rpm for 24 h. After the incubation time, the cell suspension was inoculated into 10 Petri dishes (90 x 15 mm) containing solid synthetic medium (1% *n*-butanol) with OD_{600} standardized for the growth of approximately 200 colonies per plate. The plates were incubated at 30 °C until the colonies appeared. During the incubation of the plates, growth was observed concerning the size and speed of appearance of the colonies, which were selected for quantitative analysis, totaling 87 colonies of each strain.

Quantitative analysis of evolved colonies

The 87 selected colonies (from each strain) were transferred, separately, in a 96-well microplate with each well containing 100 µL of synthetic medium (absence of *n*-butanol), sealed with PCR film (676040 Ampliseal, Greiner Bio-One GmbH, Kremsmünster, Austria), and incubated at 30 °C and 198 rpm for 24 h in a microplate reader (Infinite® M200, Tecan, Männedorf, Switzerland). The controls used for this stage were the wild-type (parental) strain. The colony's suspensions were replicated using a Replica Plater for 96-well plate (R2508, 8×12 array, Sigma–Aldrich, USA) in a Petri dish (150 x 15 mm) containing synthetic medium (1% *n*-butanol) and incubated at 30 °C for 48 h. The plates were photographed in a photodocumenter and the ImageJ software was used to convert the size of the colonies into pixels, as well as performed by de Melo et al. (2020).

Maximum specific growth rate (μ_{max}, h^{-1}) evolved colonies

The 5 selected colonies were incubated, separately, in a 50 mL Falcon containing 3 mL of synthetic medium at 30 °C and 200 rpm for 24 h. The cells were incubated with an initial OD_{600} of 0.1 in a 96-well plate containing 100 µL of synthetic medium (1% *n*-butanol). Cell growth was monitored by OD_{600} in a microplate reader (Infinite® M200, Tecan, Männedorf, Switzerland) at 30 °C and 198 rpm until reaching the stationary phase.

The maximum specific growth rate (μ_{max}, h^{-1}) was obtained from the plot of the natural logarithm of optical density (ln OD_{600}) versus time (h), using only the experimental points referring to the exponential growth phase. The μ_{max} corresponded to the slope of the line obtained by linear regression. The relative μ_{max} ($\%\mu_{max}$) of cells was calculated according to Eq. 3:

$$\%\mu_{max} = \frac{\mu_{max} \text{ of colony in 1\% } n\text{-butanol}}{\mu_{max} \text{ of wild-type strain in 1\% } n\text{-butanol}} \times 100 \qquad (3)$$

Where μ_{max} is the maximum specific growth rate (μ_{max}, h^{-1}) calculated. Cultivations were carried out in triplicate for each strain or colony.

Phenotypic characterization of selected colonies (growth kinetics)

The 3 colonies selected in the previous stage (of each strain) were characterized in terms of growth profile, glucose consumption, and metabolite production. For this, the cells were incubated in a 50 mL Falcon containing 5 mL of synthetic medium at 30 °C and 200 rpm for 24 h. The cells were incubated with an initial OD_{600} of 0.1 in 500 mL Erlenmeyer containing 100 mL of synthetic medium (1% *n*-butanol) at 30 °C and 200 rpm until reaching the phase stationary. Cell growth was monitored by OD_{600} (4001/4 Genesys 20, Thermo Fisher Scientific, USA) every 1 h and aliquots were taken to quantify glucose and metabolites.

Samples were filtered through a Millex® GV Durapore® PVDF membrane (0.22 μm, Millipore, Massachusetts, USA) for glucose, ethanol, and glycerol concentration analysis. Samples were quantified on an HPLC (Thermo Fischer Scientific, USA) system equipped with an infrared detector (Thermo Fischer Scientific, USA) at 50 °C and a HyperREZ XP column at 60 °C, using 5 mM H_2SO_4 as mobile phase at 0.6 mL/min. Calibration curves were constructed using analytical standard solutions (Sigma–Aldrich, USA) at concentrations of 0.1 to 10 g/L.

Statistical analysis

Analysis of variance (ANOVA) was carried out using Statistica® 5.5 (StatSoft, USA). Tukey's test was used to determine differences at $p<0.05$.

Table 2 *Saccharomyces cerevisiae* strains tested for *n*-butanol tolerance

Strain	**Relevant information / Place or plant of isolation**
CEN.PK113-7D	MATa URA3 LEU2 MAL2-8C SUC2
JAY270 (haploid PE-2) BG-1 CR-1 SA-1 FT858 CAT-1 NAD	Brazilian ethanol production industry
UFMG-CM-Y254 UFMG-CM-Y256 UFMG-CM-Y257 UFMG-CM-Y643 UFMG-CM-Y259	*Quercus rubra* (Red oak or Carvalho), Santuário do Caraça (Jardins), Minas Gerais, Brazil
UFMG-CM-Y255	Non-identified tree, Santuário do Caraça (Trilha da Cascatona), Minas Gerais, Brazil
UFMG-CM-Y262 UFMG-CM-Y260	*Tapirira guianensis* (Tapirirá), Taquaruçu (Mata ripária do Córrego Buritizal), Tocantins, Brazil
UFMG-CM-Y263 UFMG-CM-Y264 UFMG-CM-Y455	*Tapirira guianensis* (Tapirirá), Taquaruçu (Mata ripária do Córrego Bela Vista), Tocantins, Brazil

UFMG-CM-Y266 UFMG-CM-Y267 UFMG-CM-Y636	
ATCC 4125	Molasses distillery yeast, the Netherlands
ATCC 4132	Molasses distillery yeast
ATCC 24858	Wild-type diploid, high ethanol-tolerance
ATCC 26785	Ferments apple juice
X2180-1B IZ0137 IZ0310 IZ0651 IZ0658 IZ0659 IZ0662 IZ0669 IZ0671 IZ0672 IZ0677 IZ0684 IZ1215 IZ1348 IZ1349 IZ1350 IZ1351 IZ1716 IZ1832 IZ1833 IZ2003 IZ2004	Luiz de Queiroz College of Agriculture (ESALQ), Brazil
CAT_*n100*	Pool of CAT-1 cells with 100 generations, after successive passages in 1% *n*-butanol (strategy 1)
CAT_*n100* #29 CAT_*n100* #40 CAT_*n100* #45 CAT_*n100* #70 CAT_*n100* #73	Selected colony of CAT_*n100*
X2180_*n100*	Pool of X2180-1B cells with 100 generations, after successive passages in 1% *n*-butanol (strategy 1)
X2180_*n100* #22 X2180_*n100* #28 X2180_*n100* #39 X2180_*n100* #83 X2180_*n100* #85	Selected colony of X2180_*n100*
CAT_*UV*	Pool of CAT-1 cells, after successive passages in UV light and 1% *n*-butanol (strategy 2)

CAT_*UV* #1 CAT_*UV* #24 CAT_*UV* #38 CAT_*UV* #39 CAT_*UV* #49	Selected colony of CAT_*UV*
X2180_*UV*	Pool of X2180-1B cells, after successive passages in UV light and 1% *n*-butanol (strategy 2)
X2180_*UV* #28 X2180_*UV* #60 X2180_*UV* #63 X2180_*UV* #68 X2180_*UV* #85	Selected colony of X2180_*UV*

Results

Evaluation of the 48 strains showed different tolerance capacities to n-butanol

Fermentative microorganisms release metabolic products in the medium and must be capable of tolerating these compounds. To evaluate the capacity of strains to tolerate butanol in the medium, the 48 *S. cerevisiae* strains (Table 1) were cultivated in defined medium containing *n*-butanol at different concentrations (0, 1, or 2% v/v), as shown in Fig. 1. The studied strains showed several *n*-butanol tolerance profiles with relative growth (%RG) ranged from 50 to 93% in 1% *n*-butanol and from 19 to 72% in 2% *n*-butanol.

X2180-1B strain showed the lowest resistance to 1% *n*-butanol (50.2%). Notably, the highest relative growths in 1% *n*-butanol were achieved by the Brazilian industrial strain CAT-1 (Usina Catanduva) and the strain IZ0658, with a %RG of 93 and 91.6%, respectively. The %RG of these two strains in 1% *n*-butanol did not differ significantly. However, when grown in 2% *n*-butanol, IZ0658 exhibited the lowest %RG of the 48 strains (19.1%). CAT-1 had a %RG of 72.4 in 2% *n*-butanol, being the most tolerant strain among the 48 studied, probably because of the natural evolution of this strain in alcohol, throughout its industrial application in the production of ethanol.

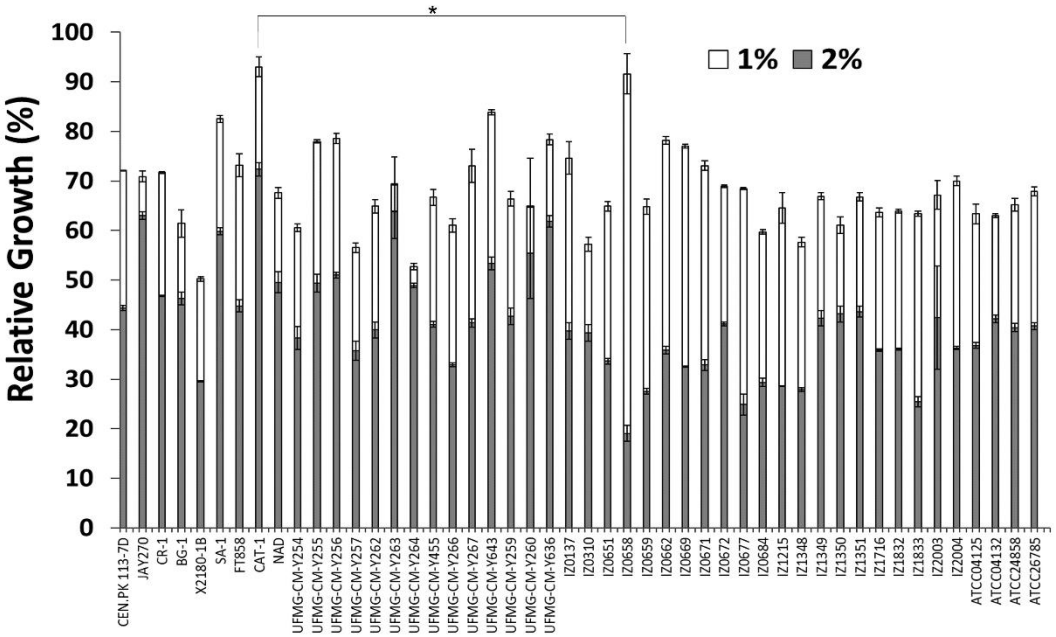

Fig. 1 Relative growth of 48 *Saccharomyces cerevisiae* strains cultivated in defined medium containing glucose (20 g/L) and urea (2.3 g/L) at 30 °C and 198 rpm until reaching the stationary phase. Columns and error bars represent respectively the mean and standard deviation of three culture replicates. * Values for 1% *n*-butanol do not differ significantly ($p < 0.05$) according to Tukey's test

One of the ways to obtain strains capable of tolerating a given cellular product is through ALE. Two of the 48 strains initially evaluated were selected to be submitted to the two strategies of ALE, being the least resistant strain (X2180-1B) and the most resistant strain (CAT-1), according to the growing conditions proposed in this study. In addition to being distinct in terms of their ability to tolerate butanol, these strains are also distinct in terms of their place of origin (Table 1). CAT-1 strain was isolated from an ethanol-producing plant, whereas X2180-1B is a laboratory strain; and in this way, it is possible to evaluate the strategy 1/strategy 2, less/more resistant and industrial/laboratory strain relations.

Selection of colonies possibly evolved from ALE with successive passages

To determine the time span for strategy 1, it was defined that ALE with successive passages would be performed until the cell population reached the number of 100 generations (called *n100*), since we believe it is sufficient for the purpose of the work. The strains showed

a difference of only 5 days to reach 100 generations, 53 days for CAT-1 and 58 days for X2180-1B strains, probably since the latter already showed an initial lower tolerance to butanol and, consequently, needed longer adaptation time. In the work carried out by Brennan et al. (2015), the yeast *S. cerevisiae* S288C was subjected to successive passages for 52 days in a synthetic medium containing increasing concentrations of limonene until the cell population reached 200 generations. When analyzing the isolated colonies between 100 and 200 generations, the authors obtained better results with the isolated colony in 120 generations and did not observe a significant increase in the growth of isolated colonies with more number of generations.

After the time of successive passages, an important and difficult part of ALE begins: the identification of the cells that were effectively evolved during the process. A cell pool (or cell population) is, by nature, phenotypically heterogeneous and, especially when exposed to stress conditions in adaptive laboratory evolution, this population is composed of cells that may or may not be evolved (Tibayrenc et al. 2011; Barrick and Lenski 2013; Winkler and Kao 2014). Therefore, it is not ideal for the cell pool to be evaluated, but to look for ways to identify the individual evolved cells. In cases of ALE involving tolerance improvement, evolved cells can be more easily identified by exposing the cell pool to selection pressure, followed by selection of surviving cells that show better growth (Steensels et al. 2014). In this sense, we decided to submit the population *n100* to different methods, qualitative and quantitative, to identify the cells that have possibly evolved. Each strain was subjected, separately, to an identification process that started with the observation of approximately 2000 single-cell colonies isolated in solid medium, passing through a tapering and final selection of 3 colonies for the phenotypic characterization.

For the first selection step, during the growth of the 2000 colonies, the colonies that grew first on the agar and the largest ones were selected. Fig. 2a and 2c show the quantitative

selection of 87 colonies by observing the size of the colony in a solid medium with 1% *n*-butanol, using a pixel scale, since it is believed that the size of the colony is directly linked to the growth capacity in the medium containing butanol. CAT-1 and X2180-1B wild-type strains, and the population *n100* obtained were used as controls. The measure of pixels of the controls were between the maximum and minimum measures of the colonies and this demonstrates, and this demonstrates how heterogeneous the pool of cells is. Then, the 5 colonies of each strain that had the highest pixel values were chosen as possible evolved colonies (highlighted in Fig. 2). For identification and distinction purposes, in this step, the selected colonies were named CAT_*n100* or X2180_*n100* with their respective number (*#number*).

Fig. 2b and 2c show the relative maximum specific growth rate (%μ_{max}) of the 5 previously selected colonies. For CAT-1 strain, two colonies stand out for presenting %μ_{max} greater than 100% (CAT_*n100*#29 and #40) and were promptly selected. As the objective of this stage was the selection of 3 colonies, it was decided to choose the CAT_*n100*#45 colony for presenting the highest relative growth, among the remaining colonies, even though its performance was lower in the presence of butanol (95%). The same approach was used for colonies X2180-1B, in which case, the 5 previously selected colonies showed %μ_{max} greater than 100% and the 3 colonies with the highest relative growths were selected (X2180_*n100*#28, #83 and #85).

Selection of colonies possibly evolved from ALE with UV irradiation

The use of UV irradiation has been shown to be a great ally for obtaining mutants with a certain phenotype. In the work carried out by Watanabe et al. (2011), the authors subjected the yeast *Pichia stipitis* NBRC1687 to UV light radiation at a distance of 50 cm for 30 s and obtained colonies capable of growing better than the parental strain in medium containing 5%

ethanol, in addition to having been better ethanol producers from xylose as a carbon source. Also in this work, the best ethanol producing colony was subjected to microaerobic cultures with increasing concentration of ethanol (5 to 7%) for 20 cycles, which resulted in a mutant capable of growing in medium containing 10% ethanol and producing ethanol in higher concentration than the parental strain and the UV-irradiated colony. Thus, the work demonstrated that UV irradiation and successive cultivations techniques, when used together, can result in mutants with greater capacity for tolerance and ethanol production.

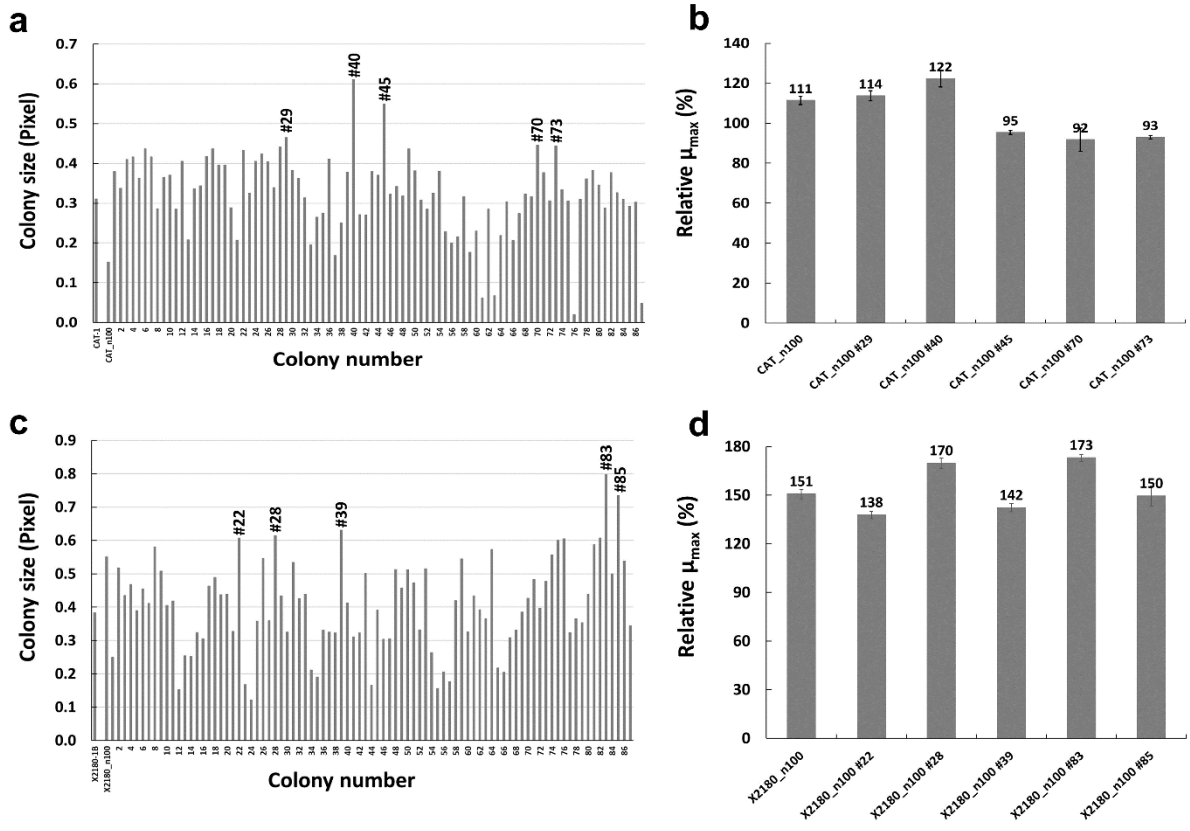

Fig. 2 Selection of evolved colonies step after application of strategy 1 (ALE with successive passage). (a) and (c) colony size image converted into pixels using ImageJ software of colonies, cultivated in Petri dish containing synthetic medium (1% *n*-butanol), at 30 °C for 48 h. (b) and (d) relative maximum specific growth rate μ_{max} of 5 selected colonies, cultivated in sealed 96-well plate containing synthetic medium (1% *n*-butanol), at 30 °C and 198 rpm until reaching the stationary phase. (b) and (c) data columns and error bars represent respectively the mean and standard deviation of three culture replicates

In parallel with strategy 1, the same strains were subjected to a slightly different approach, called strategy 2. The use of mutagens has already been demonstrated as an alternative to increase mutation rates and the initial genetic variability, but, although in theory it can be used before or during successive passages, studies carried out use this technique only at the beginning of successive passages, to initiate ALE with a mutagenized cell population. To study the use of a mutagenic agent during ALE, we decided to add a step of exposing cells to UV light during successive passage experiment. To compare whether there would be differences between the two strategies, the strains were subjected to tests by the same time span. Since strategy 1 was defined to be carried out until the evolved population reached 100 generations, we decided to conduct strategy 2 for the same number of days (53 days for CAT-1 and 58 days for X2180-1B).

To increase ethanol production from the yeast *Kluyveromyces marxianus*, Pang et al. (2010) used a strategy of cycles also containing growth and UV irradiation. For this, the GX-15 strain was exposed to two treatment cycles composed of UV irradiation, growth in a Petri dish, treatment with nitrosoguanidine and again growth in a Petri dish and UV irradiation. At the end of the work, the authors identified the GX-UN120 mutant that was able to produce and tolerate ethanol at higher concentrations than the parental strain, demonstrating that UV irradiation can cause desirable mutations in yeast and, the use of multiple mutagenic agents generate more efficient mutants.

Before starting the actual experiments, it was necessary to assess the time required for exposure to UV light for each strain. For this, the strains were inoculated with OD_{600} required for the growth of approximately 200 colonies in Petri dishes and exposed to different UV exposure times (data not shown). As expected, the cell viability of the strains decreased with increasing exposure time and, for standardization, the exposure time for each strain was defined as the time needed to reach 10% cell viability, being 20 s (CAT-1) and 35 s (X2180-

1B). There is no ideal percentage of viable cells that should be considered for adaptive laboratory evolution experiments using mutagenic agents, other authors have used different percentages of yeast cell viability, such as 2% (González-Ramos et al. 2016), 10% (Turanlı-Yıldız et al. 2017), 25% (Teunissen et al. 2002), and 50% (Stanley et al. 2010).

Knowing the necessary UV exposure time, the strains were then submitted to strategy 2 and, in the end, the selection of colonies possibly evolved began, using the same methodology applied to strategy 1, starting with the selection of 2000 up to 3 colonies. Fig. 3a and 3c show the colony size converted into pixels and, to strategy 2 the heterogeneity of the evolved population is once again clear, with colonies showing growth above and below the controls. The 5 colonies with the largest pixels were then selected and evaluated for relative μ_{max} (Fig. 3b and 3d). Finally, 3 colonies from each strain were selected for the phenotypic characterization step, CAT_*UV*#38, #39 and #49, and X2180_*UV*#28, #60 and #63.

Discussion

Butanol tolerance is associated with membrane fluidity, and different strains may present different behaviors because of properties intrinsic to each strain (Ishmayana et al. 2017). In the first part of the study carried out by Gonzalez-Ramos et al. (2013), the butanol tolerance of *S. cerevisiae* BY4741 and CEN.PK113-7D was analyzed in synthetic medium containing different concentrations of *n*-butanol (0 to 1.9%) and demonstrated that *S. cerevisiae* tolerance to *n*-butanol is associated with protein degradation. From the OD_{660} values reported by the authors, the BY4741 and CEN.PK113-7D strains presented a %RG (1% *n*-butanol) of approximately 55 and 70%, respectively. The %RG (1% *n*-butanol) of BY4741 was similar to the result of this work for X2180-1B strain with %RG of 50.2%, and CEN.PK113-7D strain showed similar %RG (around 70%) of the present work.

The authors of Knoshaug and Zhang (2009) screened non-*Saccharomyces* and *S. cerevisiae* strains for tolerance to butanol at different concentrations, growing them in microplates containing YPD (yeast peptone dextrose) medium. Of the 10 *S. cerevisiae* strains evaluated, only one was not able to grow in 1% *n*-butanol, whereas the other strains had a %RG of approximately 60%. These data reported by the authors are similar to those of the present study, in which several strains had %RG close to or equal to 60%, such as, BG-1 (61.5%), UFMG-CM-Y254 (60.6%), IZ1350 (61%), among others. The authors showed that only three *S. cerevisiae* strains were able to grow in the presence of 2% *n*-butanol (ATCC26602, ATCC20252, and Fali), having a %RG between 10 and 20%.

The mechanisms of yeast tolerance to butanol closely resemble those of ethanol tolerance (Liu and Qureshi 2009). *S. cerevisiae* strains can tolerate up to 18% ethanol in the medium, depending on growth conditions (Della-Bianca and Gombert 2013). On the other hand, this yeast is not capable of tolerating more than 2% butanol (Knoshaug and Zhang 2009).

Comparative phenotypic characterization of selected evolved colonies

Although ALE is a simple and low-cost strategy, one of the factors that need to be analyzed is the cost and time linked to the human labor required for this (Ryu 2021). Furthermore, in addition to the human time spent, one must consider the amount of manipulation to which the strains are being subjected, which can cause contamination (Winkler and Kao 2014). In the strategies applied in this work, it is already possible to identify a great gain in terms of reducing the number of manipulations and, consequently, reducing the human time spent. During the application of strategy 1, the strains were resuspended in fresh medium every 24 h, that is, 1 handling per day. In this sense, the strains were manipulated daily. On the other hand, the addition of the UV exposure step by strategy

2, reduced the number of manipulations by 50%, with 2 manipulations every 96 h. Thus, the use of a mutagenic agent, under the conditions proposed in this work, could not only be able to increase the incidence of mutations, but also reduced the handling time of the strains by half.

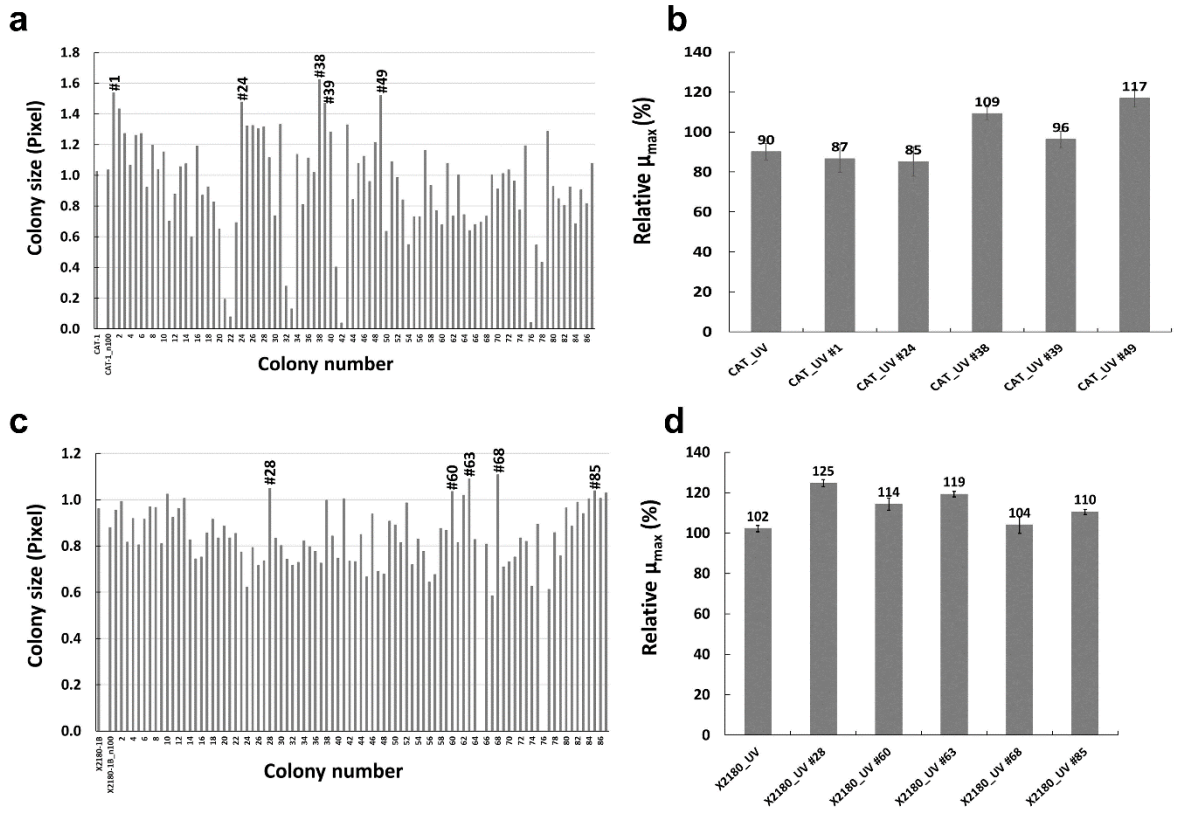

Fig. 3 Selection of evolved colonies step after application of strategy 2 (ALE with UV irradiation). (a) and (c) colony size image converted into pixels using ImageJ software of colonies, cultivated in Petri dish containing synthetic medium (1% *n*-butanol), at 30 °C for 48 h. (b) and (d) relative maximum specific growth rate μ_{max} of 5 selected colonies, cultivated in sealed 96-well plate containing synthetic medium (1% *n*-butanol), at 30 °C and 198 rpm until reaching the stationary phase. (b) and (c) data columns and error bars represent respectively the mean and standard deviation of three culture replicates

However, there will be no value in a 50% reduction in the number of manipulations by strategy 2, if this does not generate results at least similar to those obtained by strategy 1. Therefore, it is important now to carry out a comparative analysis between wild-type strains and their respective evolved colonies. When subjected to excessive stress, microbial cells can

enter a phase of cell death, on the other hand, if stimulated under milder conditions, cells can change specific genes that provide defense mechanisms and consequently can also present physiological changes (Ryu 2021). More simply, these changes can be observed from growth kinetics and their phenotypic characterization. Furthermore, in ALE experiments, it is common to use growth criteria as a parameter for selection (Shepelin et al. 2018). The purpose of this step was to observe cell growth, substrate consumption, and the formation of two metabolites commonly produced by the yeast *S. cerevisiae* (ethanol and glycerol), in medium containing 1% *n*-butanol (Table 2). All selected colonies, of both strains, and two strategies were able to grow and completely deplete the carbon source (Fig. 4 and 5).

For a better visualization and comparison of the differences between the strains before and after the ALE experiments, Figures 6 and 7 bring together the phenotypic characterization of the wild-type strains CAT-1 and X2180-1B in the presence or absence of butanol, as well as one evolved colony of every strain and strategy that were considered more robust, assuming that the colonies with the highest μ_{max} are those that best adapt to the applied selection pressure. According to Bruggeman et al. (2020), one of the evolutionary processes is called 'pruning', in which there is a phenotypic improvement of the microorganism, leading to a faster adaptation to a given external change and a consequent increase in the growth rate.

By observing the results obtained for strategy 1, CAT_*n100* colonies showed OD_{600} maximum between 1.7 and 2.4, but with depletion of carbon source between 16 and 18 h. On the other hand, the X2180_*n100* colonies were able to deplete glucose in less time (12 h) but showed lower growth. In terms of ethanol production (the most produced and studied alcohol by *S. cerevisiae* strains), colonies X2180_*n100*#83 and #85 stood out, with a final ethanol concentration of 10.6 and 10.8 g/L, respectively, while the CAT_*n100*#29 produced a maximum ethanol concentration of 8 g/L. Contrary to this, in strategy 2, CAT_*UV* colonies depleted the carbon source of the medium more quickly, between 15 h, with a slightly less

marked growth; on the other hand, X2180_*UV* colonies were able to deplete the carbon source slower, with time between 19.5 and 22 h, but showing very similar growth.

Table 3. Growth and production parameters of wild-type strains and colonies evolved in medium with no butanol or 1% addition *n*-butanol (experimental duplicate average)

	Maximum OD_{600}	**Glucose Depletion Time (h)**	**Maximum Ethanol (g/L)**	**Maximum Glycerol (g/L)**	**μ_{max} (h^{-1})**
Wild-type strains (no butanol)					
CAT-1	5.0	12.0	7.0	0.5	0.35
X2180-1B	3.8	12.0	7.5	0.9	0.32
Wild-type strains (1% butanol)					
CAT-1	2.1	16.5	8.8	0.6	0.21
X2180-1B	0.5	n.d.	n.d.	n.d.	n.d.
CAT-1 evolved colonies (1% butanol)					
CAT_*n100* #29	2.4	17.5	8.0	0.5	0.22
CAT_*n100* #40	1.7	16.0	7.7	0.8	0.22
CAT_*n100* #45	2.3	18.0	7.9	0.6	0.24
CAT_*UV* #38	1.7	15.0	8.3	0.5	0.25
CAT_*UV* #39	1.5	15.5	8.5	0.4	0.21
CAT_*UV* #49	1.6	15.5	8.3	0.5	0.23
X2180-1B evolved colonies (1% butanol)					
X2180_*n100* #28	1.8	12.0	8.7	1.1	0.34
X2180_*n100* #83	1.7	12.0	10.6	0.9	0.25
X2180_*n100* #85	1.8	12.0	10.8	0.9	0.17
X2180_*UV* #28	1.9	19.5	8.9	0.6	0.17
X2180_*UV* #60	1.8	19.5	8.8	0.4	0.16
X2180_*UV* #63	1.7	22.0	8.6	0.6	0.14

n.d. - not detected

At first, when comparing wild-type strains, grown with no butanol, the greater cell growth capacity of the CAT-1 strain is already clear. The X2180-1B strain when grown in the absence of butanol was able to deplete the carbon source and produce concentrations of ethanol and glycerol very close to those of the CAT-1 strain, although its cell concentration

and μ_{max} were lower. On the other hand, if compared when grown in 1% butanol, the growth of CAT-1 strain is much higher than X2180-1B strain, since the latter was not able to grow under these conditions.

And, again, this legitimizes the hypothesis that the higher butanol tolerance capacity of the CAT-1 strain may come from a natural adaptation to high concentrations of ethanol. CAT-1 is one of the most used strains as starter culture in most distilleries in Brazil and responsible for the annual production of billions of gallons of fuel ethanol (Basso et al. 2011; Babrzadeh et al. 2012; Beato et al. 2016). Furthermore, during the industrial ethanol production process, the *S. cerevisiae* strains are subjected to several cell recycling with diluted sulfuric acid at low pH, imposing a selective pressure and consequent adaptation to high stress conditions (Basso et al. 2008).

In the work carried out by Della-Bianca and Gombert (2013), the authors cultivated different industrial and laboratory strains under the same conditions as the present study and observed μ_{max} of 0.345 h^{-1}, including the industrial CAT-1 strain. In addition, the authors also submitted these same strains to several classical and ethanol industry stress factors and observed that industrial strains, in general, show better growth performance under stress conditions and they are more tolerant, when compared to laboratory strains. These results also indicated that the strains of *S. cerevisiae* currently used in Brazil for ethanol production have become more tolerant over time and, due to the drastic conditions to which they are submitted, having therefore undergone an adaptation and natural selection.

From Table 2, analyzing the selected colonies, it is still possible to note that CAT-1 evolved colonies, for both strategies, were able to grow with μ_{max} and metabolites production equal to or greater than the wild-type strain, with emphasis on CAT_*UV*#38 that showed the highest growth rate (0.25 h^{-1}). However, this increase in their growth capacity was not so accentuated. On the other hand, X2180-1B strain surprises with its results achieved for both

strategies. The evolved colonies were not only able to better tolerate butanol addition in the medium, but the colony X2180_*n100*#28 also presented a relative μ_{max} of 106% in relation to the parental strain in the absence of butanol. In this way, this colony was able to grow in a condition where the parental strain was not able (1% butanol), with the same carbon source depletion time, even with lower OD_{600}. Further, it is interesting to highlight that this colony had an increase in glycerol production of 22%, compared to wild-type (no butanol), probably as a response to stress (Ghiaci et al. 2013). In addition to having the highest μ_{max} in 1% butanol, X2180_*n100*#28 also reached growth rate very close to the CAT-1 strain without butanol, which was initially considered the most tolerant strain to this alcohol.

When comparing the OD_{600} values reached at the end of the cultivations in 1% of *n*-butanol by the parental and evolved ones, it is very evident that the evolution occurred by the X2180-1B strain was extremely superior to that of the CAT-1. For example, wild-type CAT-1 achieved an OD_{600} growth (1% butanol) equal to 2.1 and the CAT_*n100*#29 strain 2.4, representing an increase in biomass production capacity of only 14%. In contrast, from parental X2180-1B to evolved X2180_*n100*#28 there was an increase in biomass production, indirectly measured by OD_{600}, of 288%.

Although tolerance to alcohols by *S. cerevisiae* is difficult to understand phenotypically, and the production of *n*-butanol is still very far from its tolerability, the obtaining of strains that are even more tolerant to butanol is an important step, since the level of toxicity limits the production of higher final butanol concentrations. The nature of this butanol tolerance improvement has yet to be clarified and our research group plans to carry out genome sequencing of the best isolated colony.

Conclusion

The industrial strain CAT-1, despite initially having shown to be more tolerant to butanol, did not show great improvements after being submitted to ALE. The CAT-1 colonies selected for both strategies showed growth capacity, glucose consumption and metabolite production very similar to the parental strain, in 1% butanol. On the other hand, the laboratory strain X2180-1B showed an incredible increase in butanol tolerance, starting from a condition of inability to grow in 1% butanol (wild-type strain), to the capacity to grow in this same condition (X2180_*n100*#28) with the highest μ_{max} among all selected colonies. Regarding the type of ALE to be used, strategy 1 proved to be more advantageous than strategy 2, despite the addition of UV radiation reducing handling time by 50%. In addition, the use of strategy 1 eliminates the need for possible exposure of the researcher to UV radiation and the increased cost associated with obtaining UV light. Other studies have already shown that strains evolved in certain alcohols also show greater tolerance to other compounds, therefore, we believe that the X2180_*n100*#28 colony has good potential to be used as a model for the study of mechanisms linked to tolerance, not only to butanol, but also to various alcohols and other inhibitory compounds.

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DECLARATIONS

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Conflicts of interest

The authors declare that they have no conflict of interest.

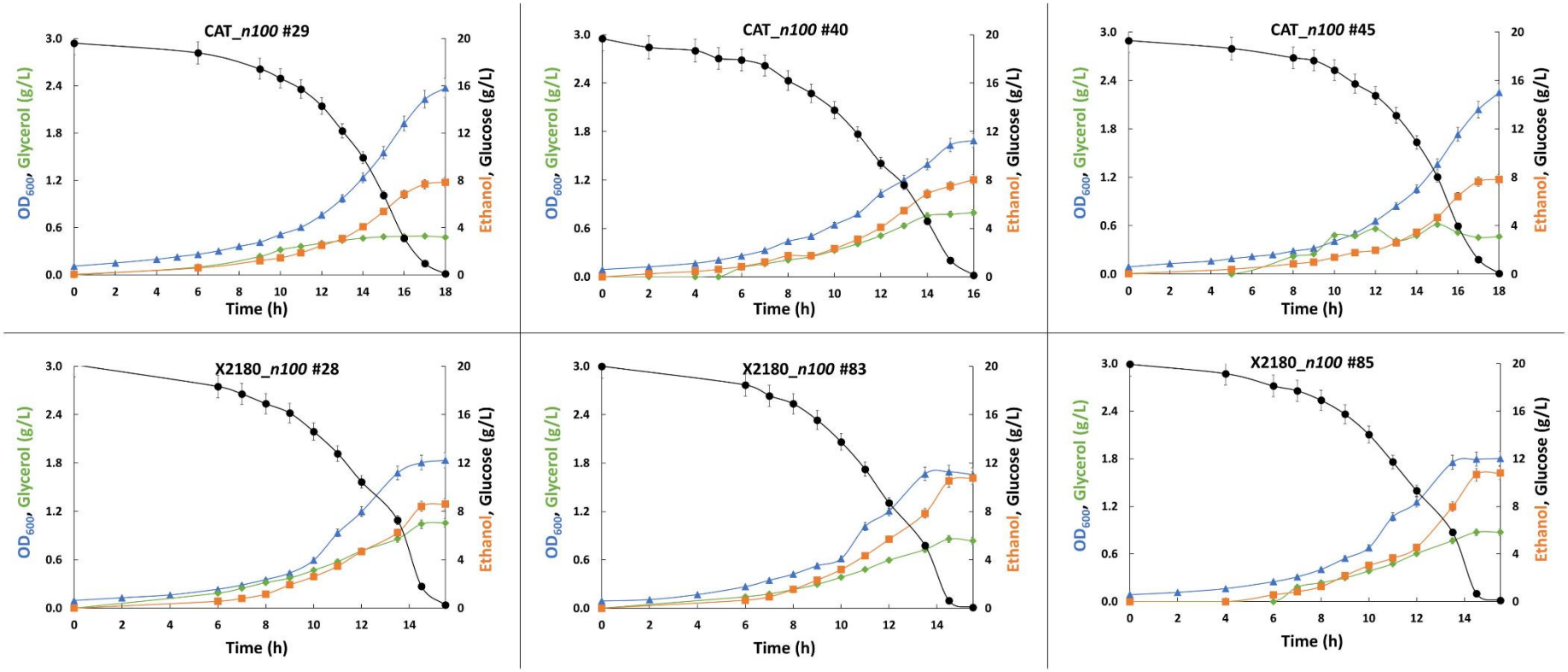

Fig. 4 Phenotypic characterization of selected colonies CAT_*n100* (#29, #40, and #45) and X2180_*n100* (#28, #83, and #85), after application of strategy 1 (ALE with successive passage), cultivated in defined medium containing glucose (20 g/L) and urea (2.3 g/L) at 30 °C and 200 rpm, in shaken bottle. Data corresponds to the mean of two culture replicates and standard deviation of 5%

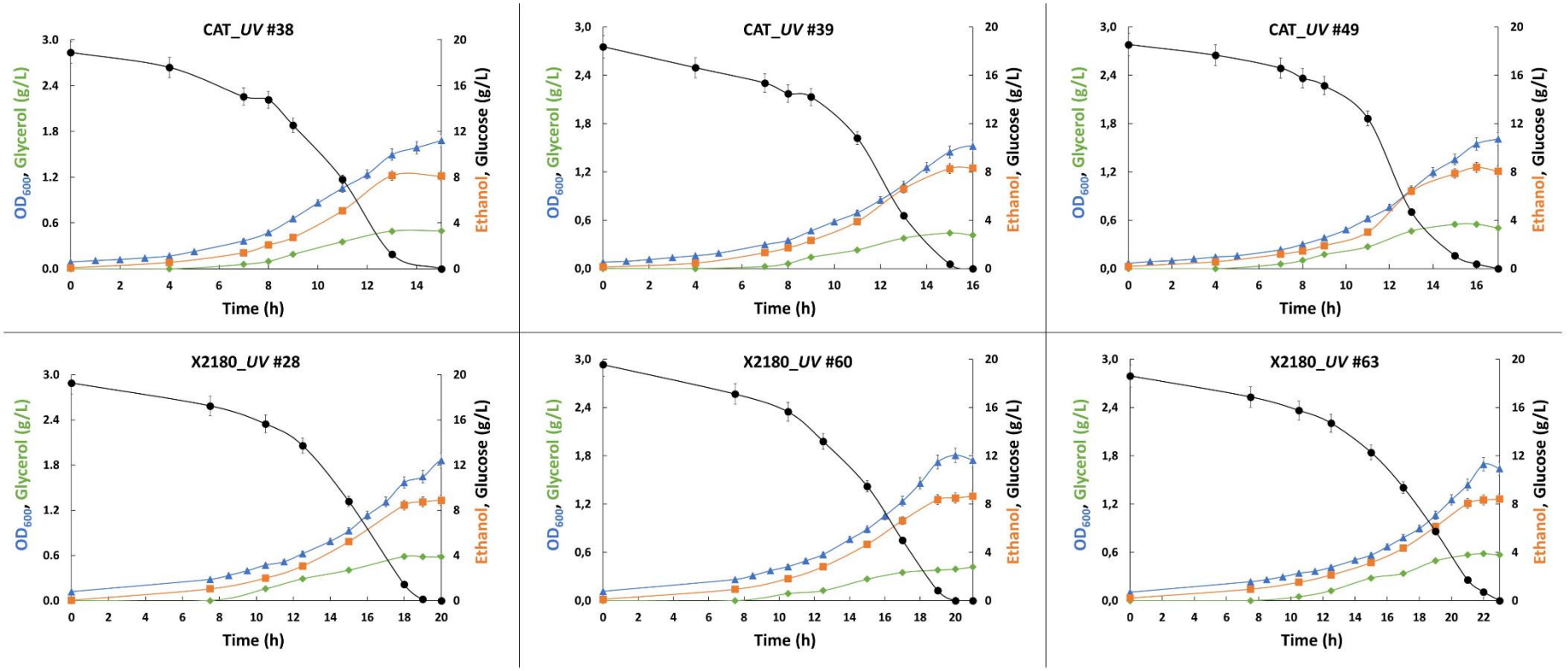

Fig. 5 Phenotypic characterization of selected colonies CAT_*UV* (#38, #39, and #49) and X2180_*UV* (#28, #60, and #63), after application of strategy 2 (ALE with UV irradiation), cultivated in defined medium containing glucose (20 g/L) and urea (2.3 g/L) at 30 °C and 200 rpm, in shaken bottle. Data corresponds to the mean of two culture replicates and standard deviation of 5%

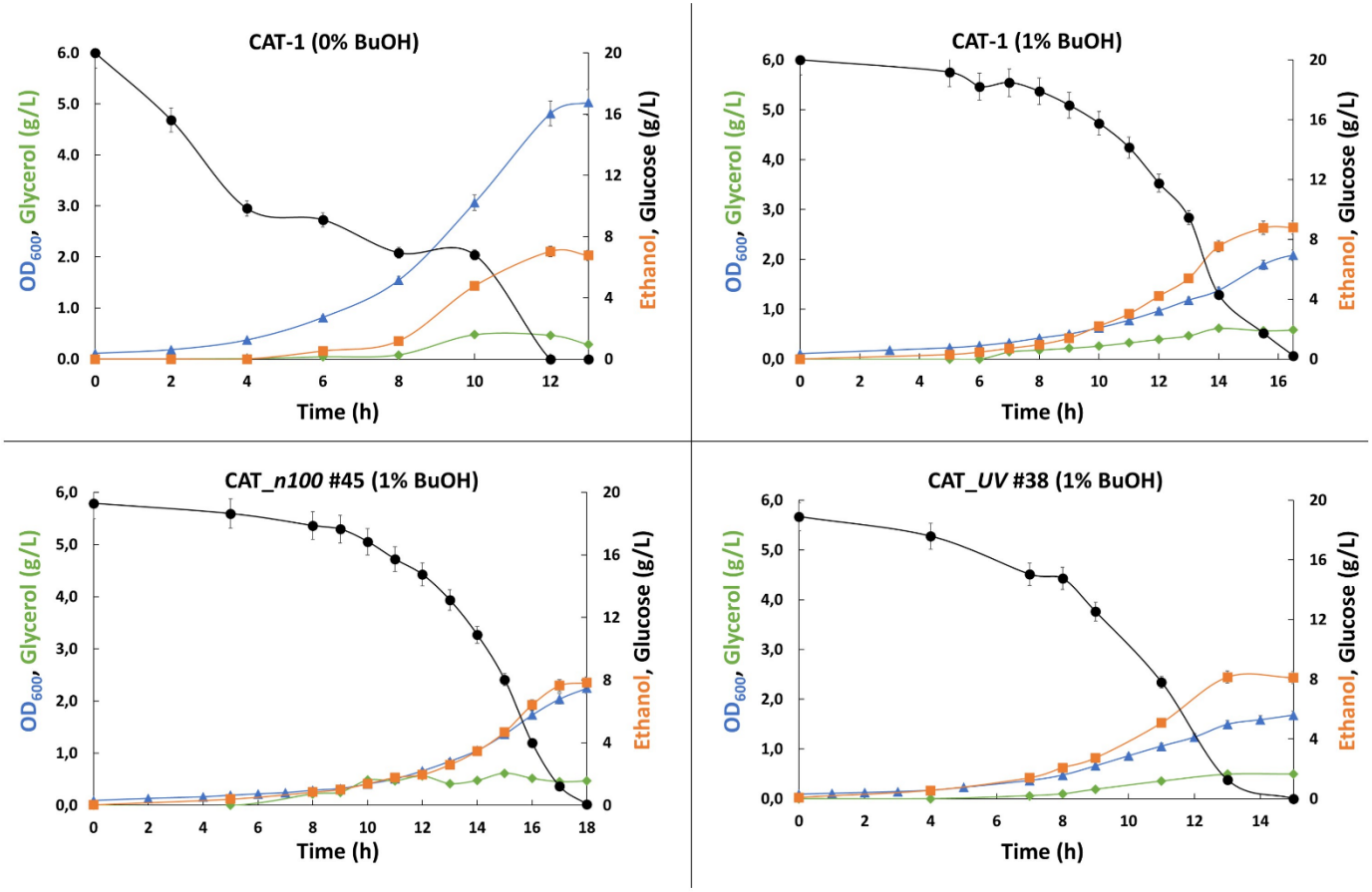

Fig. 6 Phenotypic characterization of CAT-1 wild-type strain in the absence of butanol or presence of 1% butanol, and selected colonies CAT_*n100*#45 and CAT_*UV*#38 in 1% butanol, cultivated in defined medium containing glucose (20 g/L) and urea (2.3 g/L) at 30 °C and 200 rpm, in shaken bottle, until reaching the stationary phase. Data correspond to the mean of two culture replicates and standard deviation of 5%. BuOH: *n*-butanol

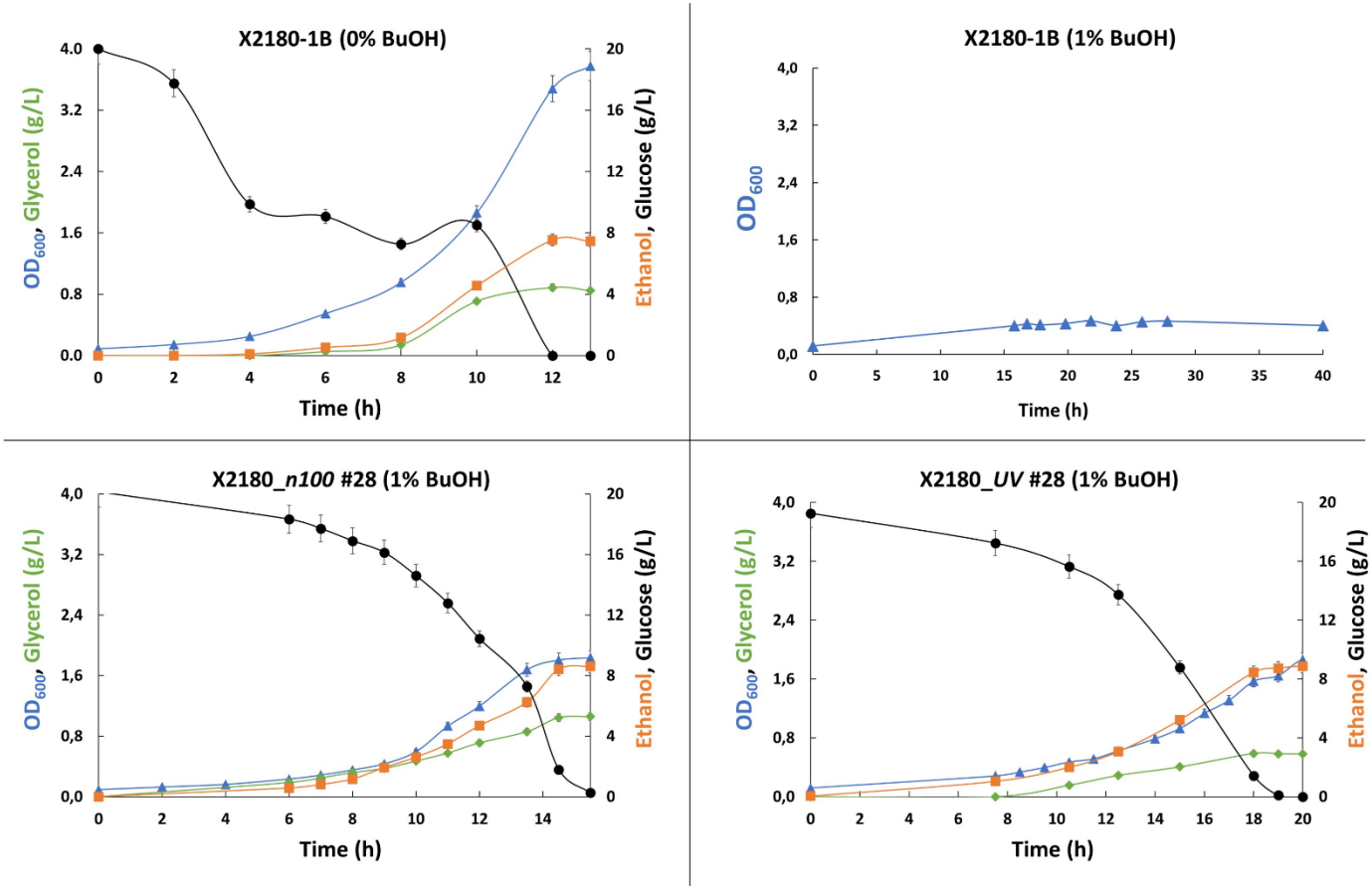

Fig. 7 Phenotypic characterization of X2180-1B wild-type strain in the absence of butanol or presence of 1% butanol, and selected colonies X2180_*n100*#28 and X2180_*UV*#28 in 1% butanol, cultivated in defined medium containing glucose (20 g/L) and urea (2.3 g/L) at 30 °C and 200 rpm, in shaken bottle, until reaching the stationary phase. Data correspond to the mean of two culture replicates and standard deviation of 5%. BuOH: *n*-butanol

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CAPÍTULO 6

DISCUSSÃO E CONCLUSÃO GERAIS

DISCUSSÃO GERAL

A busca por novos biocombustíveis que possam servir como substitutos da gasolina impulsionou os estudos quanto à obtenção de butanol a partir de microrganismos. Dentre uma das frentes possíveis, diversos grupos de pesquisas em todo o mundo voltaram esforços para entender a capacidade da levedura *S. cerevisiae* em produzir este álcool. Duas estratégias são comumente estudadas para a produção de butanol por S. cerevisiae: a expressão heteróloga da via do *Clostridium* ou a via de utilização dos aminoácidos. Dentre ambas as estratégias, existe uma diversidade de teorias que foram levantadas e comprovadas, levando a concentrações finais de butanol ainda muito baixas que variam desde 2,5 mg/L (STEEN et al., 2008) até apenas 1,05 g/L (SHI et al., 2016).

Neste sentido, **no Capítulo 3** foi realizada uma busca dentre 48 diferentes *S. cerevisiae*, englobando linhagens selvagens, industriais e laboratoriais, a fim de avaliar a capacidade das mesmas em produzir naturalmente *n*-butanol utilizando glicina como um co-substrato. Esta etapa fez-se importante para averiguar a capacidade natural destas linhagens em produzir o álcool, uma vez que elas podem ser utilizadas como background para trabalhos futuros. Sabe-se que, além de butanol, a levedura *S. cerevisiae* é capaz de produzir uma gama de metabólitos, principalmente o etanol. Desta forma, foi avaliada a relação entre a produção de butanol frente à outros metabólitos.

As linhagens estudadas produziram concentrações de butanol muito inferiores ao etanol, como também demonstrado em outros estudos (STEEN et al. 2008; BRANDUARDI et al. 2013; SCHADEWEG; BOLES 2016). No presente estudo, a média de *n*-butanol produzido foi aproximadamente 600 vezes menor que a produção de etanol (com média de 2,6 g/L). No entanto, ainda que em baixas concentrações, a linhagem selvagem UFMG-CM-Y267, destacou-se apresentando a maior produção de *n*-butanol (12,7 mg/L), o que

corresponde à 0,15% do máximo teórico de 0,41 g butanol/g glicose (GENEROSO et al. 2015). Além de *n*-butanol, as linhagens também apresentaram baixas concentrações de isobutanol – um isômero do butanol – com média de 11 mg/L. Assim, pesquisadores tentam melhorar os rendimentos de *n*-butanol e isobutanol de *S. cerevisiae* usando técnicas de engenharia metabólica, como a expressão de vias metabólicas completas ou superexpressão de genes específicos (BRANDUARDI et al. 2013; HAMMER; AVALOS 2017).

Em termos de superexepressão de genes, Branduardi et al. (2013) foram os primeiros pesquisadores a demonstrar bioquimicamente a rota de produção de *n*-butanol a partir de glicina como um co-substrato pela levedura *S. cerevisiae*. Os autores inseriram na levedura um plasmídeo contendo o gene que codifica para a enzima glicina oxidase (*goxB*), uma vez que esta enzima catalisa a formação de glioxilato a partir de glicina, levando à geração de α-ketovalerate, um composto que pode ser convertido em butanol. Para trazer uma nova abordagem a este trabalho, o **Capítulo 4** mostra a utilização da ferramenta de edição genética CRISPR/Cas9 para obtenção de linhagens que superexpressem o gene *goxB* sem a necessidade da manutenção de marcadores de seleção, característica que não é encontrada para modificações a partir de plasmídeos que podem sofrer alterações na atividade do gene inserido.

A partir dos estudos do Capítulo 3, foi possível selecionar a linhagem selvagem UFMG-CM-Y267 como background para a inserção do gene *goxB*, uma vez que esta foi destaque, frente às outras linhagens estudadas, quanto à produção de *n*-butanol. Além disso, decidiu-se também incorporar como background uma linhagem comumente utilizada na produção industrial de etanol no Brasil, a linhagem JAY270, derivada da Pedra-2 (PE-2). Após confirmação da inserção do gene nos backgrounds UFMG-CM-Y267 e JAY270, obteve-se as linhagens modificadas chamadas, respectivamente, de SAYgox e SAJgox. As linhagens modificadas SAYgox e SAJgox apresentaram atividade de glicina oxidase superior

às parentais em todos os tempos de cultivo. Isso comprova que, para ambas as linhagens, o gene de interesse foi inserido no genoma da levedura, e elas passaram a superexpressar a enzima glicina oxidase, quando cultivadas na presença de glicina no meio. No entanto, a superexpressão do gene da glicina oxidase pela levedura pode não necessariamente levar a um aumento na produção de butanol. Assim, as linhagens parentais e modificadas foram cultivadas em meio contendo glicina e avaliadas quanto à produção de *n*-butanol após 72 h de cultivo, a fim de verificar a influência do gene *goxB*.

Com base nos resultados apresentados no **Capítulo 4**, ficou evidente que para o background JAY270, a inserção do gene *goxB* afetou significativamente não apenas a atividade dessa enzima, mas também aumentou sua capacidade de produção de *n*-butanol em cerca de 300%. Por outro lado, para o background UFMG-CM-Y267, a inserção do gene afetou negativamente a produção de butanol pela cepa modificada, uma vez que foi capaz de produzir aproximadamente 40% menos butanol, quando comparada à sua parental. No trabalho realizado por Branduardi et al. (2013), a levedura por eles modificada (via plasmídeo contando o gene *goxB*), apresentou níveis inferiores à SAJgox, com atividade de glicina oxidase 1,5 vezes maior que o background e produção de butanol 30% superior. Apesar de um aumento muito significativo (300%), a linhagem SAJgox ainda apresenta concentrações de *n*-butanol muito inferiores à de outros autores de também estudaram a produção de butanol via rota de degradação dos aminoácidos, como 242,8 mg/L (SI et al. 2014) e 835 mg/L (SHI et al. 2016). No entanto, esta linhagem mostrou-se promissora para futuros estudos.

Uma das frentes bastante estudadas para aumentar a produção de *n*-butanol por *S. cerevisiae* é entender e ampliar a capacidade de tolerância à este álcool. Entende-se que, uma vez que um organismo começa a formar produtos metabólicos, as células devem ser capazes de tolerar esses compostos no meio. O mecanismo de tolerância ao butanol é muito semelhante à tolerância ao etanol (LIU; QURESHI 2009). *S. cerevisiae* é um organismo capaz

de tolerar até 18% de etanol em meio, dependendo das condições de cultivo (PEREIRA et al. 2011; DELLA-BIANCA; GOMBERT 2013; ISHMAYANA et al. 2017), porém, esta levedura não é capaz de tolerar mais de 2% de butanol (KNOSHAUG; ZHANG 2009). Ishmayana et al. (2017) relataram que, embora a tolerância ao butanol esteja relacionada à fluidez da membrana, diferentes cepas podem apresentar comportamentos diferentes devido às propriedades intrínsecas de cada cepa. Assim sendo, o **Capítulo 5** inicia com a avaliação das mesmas 48 linhagens do **Capítulo 3**, agora com foco na capacidade de tolerância destas linhagens quando cultivadas em meio contendo diferentes concentrações de *n*-butanol (0, 1, ou 2% v/v), a fim de selecionar candidatas para serem submetidas a duas estratégias de "Adaptive Laboratory Evolution" (ALE, tradução livre: evolução laboratorial adaptativa).

O estudo apresentado no **Capítulo 5** apresentou diferentes perfis de crescimento relativo (relação do crescimento na ausência e presença de *n*-butanol) pelas linhagens avaliadas, variando de 50 a 93% em 1% de *n*-butanol e de 19 a 72% em 2% de *n*-butanol. A linhagem X2180-1B mostrou menor resistência em 1% e 2% de *n*-butanol (50,2% e 28%, respectivamente), em contraste com a linhagem industrial CAT-1 (Usina Catanduva) com a maior tolerância (93% e 72,4%, respectivamente). Em outros estudos, diferentes linhagens da levedura S. cerevisiae também apresentaram crescimento relativo similares aos do presente estudo, de 55 e 70% para BY4741 e CEN.PK113-7D, respectivamente (GONZALEZ-RAMOS et al., 2013), em média de 60 % para 9 diferentes linhagens de não-*Saccharomyces S. cerevisiae* (KNOSHAUG; ZHANG, 2009).

Uma das maneiras de obter linhagens capazes de tolerar certo metabólito celular, é a partir de ALE. Por terem sido consideradas as linhagens com menor e maior tolerância ao *n*-butanol, X2180-1B e CAT-1 foram selecionadas para serem submetidas às estratégias de ALE, e verificar as mudanças de comportamento das mesmas frente ao *n*-butanol. As linhagens selecionadas, além de serem distintas em termos de sua habilidade em tolerar o

butanol, também se distinguem quanto ao local de origem. A linhagem CAT-1 foi isolada de uma planta produtora de etanol, enquanto X2180-1B é uma linhagem laboratorial. Após aplicadas as duas estratégias de ALE (passagens sucessivas e radiação por luz UV), as colônias evoluídas da linhagem X2180-1B destacaram-se por apresentar maior capacidade de crescimento na presença de butanol, para as duas estratégias, em relação à linhagem parental. As colônias evoluídas não só foram capazes de tolerar melhor a adição de butanol no meio, como a colônia X2180_*n100*#28 também apresentou um $\mu_{máx}$ relativo de 106% em relação à sua parental na ausência de butanol. Desta forma, esta colônia foi capaz de crescer em uma condição na qual a linhagem parental não foi capaz (1% butanol), com o mesmo tempo de consumo total da fonte de carbono, ainda que com menor densidade celular.

CONCLUSÕES GERAIS

Os resultados obtidos no **Capítulo 3** claramente mostraram que diferentes linhagens da mesma levedura, com diferentes locais de isolamento, diferem grandemente quanto à capacidade de produção dos diversos metabólitos. Butanol e isobutanol foram os compostos produzidos em menor concentração, em contrapartida com etanol, glicerol e ácido acético sendo produzidos em níveis muito superiores. Das 48 linhagens estudadas, quase 50% foram capazes de produzir *n*-butanol utilizando glicina como co-substrato, sem a necessidade de modificação genética para expressão ou superexpressão de genes responsáveis pela produção de butanol. Os resultados sugerem alta variabilidade genética, seletividade para vias metabólicas específicas e diferenças genéticas e fenotípicas entre linhagens selvagens e industriais, provavelmente como resultado da seleção natural sob condições ambientais ou industriais.

Dando seguimento ao estudo, no **Capítulo 4** foi demonstrado o uso da ferramenta de edição genômica CRISPR/Cas9 para superexpressão do gene codificador da enzima glicina

oxidase em *S. cerevisiae*. As linhagens modificadas apresentaram atividade da glicina oxidase até 200% maior que as parentais. Apenas a linhagem modificada SAJgox foi afetada positivamente em termos de capacidade de produção de *n*-butanol, partindo de uma concentração de butanol pela linhagem parental inferior a 3 mg/L para 13 mg/L pela SAJgox, representando um aumento de 300%. Embora essa concentração de *n*-butanol ainda seja muito baixa, esse aumento da capacidade da linhagem SAJgox em produzir *n*-butanol a torna uma linhagem promissora para estudos futuros, desde a inserção ou deleção de genes específicos, melhoria das condições e meio de cultivo, até a utilização de matéria-prima composta por diferentes aminoácidos, uma vez que a utilização de aminoácidos puros pode não ser economicamente viável.

Em termos da capacidade de tolerância da levedura *S. cerevisiae* frente ao *n*-butanol, o **Capítulo 5** mostrou que a linhagem industrial CAT-1, apesar de inicialmente ter se mostrado mais tolerante ao butanol, não apresentou grandes melhoras após ser submetida à ALE. As colônias CAT-1 selecionadas para ambas as estratégias apresentaram capacidade de crescimento, consumo de glicose e produção de metabólitos muito semelhantes à cepa parental, em 1% de butanol. Por outro lado, a cepa de laboratório X2180-1B apresentou um aumento incrível na tolerância ao butanol, partindo de uma condição de incapacidade de crescer em 1% de butanol (linhagem selvagem), para a capacidade de crescer nessa mesma condição (X2180_*n100*#28) com o maior $\mu_{máx}$ dentre todas as colônias selecionadas. Em relação ao tipo de ALE a ser utilizado, a estratégia 1 se mostrou mais vantajosa que a estratégia 2, apesar da adição de radiação UV reduzir o tempo de manuseio em 50%. Além disso, o uso da estratégia 1 elimina a necessidade de uma possível exposição do pesquisador à radiação UV e o aumento do custo associado à obtenção da luz UV. Outros estudos já demonstraram que linhagens evoluídas em determinados álcoois também apresentam maior tolerância a outros compostos, portanto, acredita-se que a colônia X2180_*n100*#28 tem

potencial para ser utilizada como modelo para o estudo de mecanismos ligados à tolerância, não apenas à butanol, mas também a vários álcoois e outros compostos inibidores. Por fim, estudos futuros podem usar o conjunto de informações apresentadas nesta tese como ponto de partida para otimizar linhagens de *S. cerevisiae* para produção e tolerância ao *n*-butanol ou outros metabólitos.

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ANEXOS

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