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ASPECTOS FISIOLÓGICOS E GENÉTICOS DO METABOLISMO DE AÇÚCARES UTILIZADOS COMO SUBSTRATOS PARA A PRODUÇÃO DE BIOETANOL PELA LEVEDURA INDUSTRIAL *BRETTANOMYCES BRUXELLENSIS*

Recife
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Tese apresentada ao Programa de Pós-Graduação em Genética da Universidade Federal de Pernambuco, como requisito parcial para obtenção do título de Doutora em Genética.

Área de concentração: Biologia Molecular.

Orientador: Dr. Will de Barros Pita

Coorientador: Dr. Marcos Antonio de Morais Junior

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À minha família, pelo incentivo e compreensão em toda minha trajetória.

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RESUMO

A capacidade da levedura *Brettanomyces bruxellensis* utilizar diferentes fontes de carbono é um dos fatores associados à sua alta adaptação aos ambientes industriais. Assim, o presente estudo teve como objetivo investigar a potencial aplicação de *B. bruxellensis* na fermentação alcoólica, avaliando aspectos fisiológicos e genéticos do metabolismo de açúcares utilizados como substratos no processo industrial. Para isso, foram realizadas triagens com diferentes isolados de vinho e etanol em sete açúcares industrialmente relevantes. A capacidade de assimilar e fermentar xilose, arabinose e galactose, bem como a influência da glicose no metabolismo dessas fontes de carbono também foi estudada. Os resultados mostraram a ampla diversidade de assimilação de açúcares por *B. bruxellensis*. Adicionalmente, foi observado que o efeito repressor da glicose é menos estrito em linhagens isoladas de etanol do que de vinho. Em ensaios com xilose e arabinose, ambas as pentoses foram convertidas a biomassa quando em condições aeróbicas. Em limitação de oxigênio, houve produção de etanol a partir de xilose, ao passo que a arabinose não foi utilizada. Análises de expressão gênica mostram que a glicose não exerce efeito repressor na linhagem isolada de etanol, JP19M. Finalmente, foi observado que JP19M é capaz de fermentar galactose e consumir simultaneamente glicose e galactose, apresentando metabolismo respiro-fermentativo. Em conjunto, os resultados mostram que a linhagem JP19M apresenta características relevantes para uma possível aplicação na produção de etanol a partir de açúcares encontrados em hidrolisados lignocelulósicos.

Palavras-chave: assimilação de açúcares; disponibilidade de oxigênio; expressão gênica; metabolismo do carbono; metabolismo respiro-fermentativo; repressão catabólica pela glicose.

ABSTRACT

The ability to assimilate a wide range of carbon sources is an important adaptation factor for the yeast *Brettanomyces bruxellensis* in industrial substrates. Therefore, the present study aimed to investigate the potential application of *B. bruxellensis* in alcoholic fermentation processes, by evaluating physiological and genetic aspects of the metabolism of sugars found in substrates used for bioethanol production. In this sense, we performed a screening with isolates from winemaking and ethanol production cultivated in seven industrially relevant sugars. We also assessed the capacity of some strains to assimilate and ferment xylose, arabinose and galactose, as well as the influence of glucose on the metabolism of these sugars and in the ethanol production. The results confirm the wide diversity of sugar assimilation in *B. bruxellensis*. Additionally, it was observed that Glucose Catabolite Repression is less strict in ethanol strains than in winemaking ones. In aerobic assays with xylose and arabinose, both pentoses were preferentially converted to biomass. In oxygen limitation, ethanol was produced from xylose, while arabinose was not consumed. Gene expression analyses showed that glucose did not impose a repressive effect in JP19M strain. Finally, it was observed that JP19M is able to ferment galactose and simultaneously consume glucose and galactose, in the respiro-fermentative metabolism. Altogether, the results show that JP19M strain has relevant characteristics for a possible industrial application in the ethanol production from sugars found in lignocellulosic hydrolysates.

Keywords: sugar assimilation; oxygen availability; gene expression; carbon metabolism; respiro-fermentative metabolism; glucose catabolite repression.

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LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

2-DG	2-deoxi-D-glicose
AA	Antimicina A
ADP	Adenosina difosfato, do inglês *Adenosine diphosfate*
ATP	Adenosina trifosfato, do inglês *Adenosine triphosfate*
C	Carbono
cDNA	DNA complementar
CO_2	Dióxido de carbono
CSRE	Elemento responsivo à fonte de carbono
g l^{-1}	Gramas por litro
GCR	Repressão Catabólica pela Glicose, do inglês, *Glucose Catabolite Repression*
L	Litro
mg	Miligrama
mL	Mililitros
NAD^+	Nicotinamida adenina dinucleotídeo (forma oxidada)
NADH	Nicotinamida adenina dinucleotídeo (forma reduzida)
$NADP^+$	Nicotinamida adenina dinucleotídeo fosfato (forma oxidada)
NADPH	Nicotinamida adenina dinucleotídeo fosfato (forma reduzida)
NCBI	National Center for Biotechnology Information
O_2	Oxigênio
OD	Densidade óptica, do inglês *optical density*
pH	Potencial hidrogeniônico
PKA	Proteína quinase A
PPP	Via das Pentoses-Fosfato, do inglês *Pentose Phosphate Pathway*
QR	Quantificação relativa
RNA	Ácido ribonucleico
RPM	Rotações por minuto
RT-qPCR	PCR quantitativa com transcrição reversa
SAGA	Complexo acetil-transferase Spt-Ada-Gcn5
TFIID	Fator geral de transcrição
UAS	Sequência ativadora à montante

URS	Sequência repressora à montante
YNB	*Yeast Nitrogen Base*
μ	Velocidade de crescimento específico (h^{-1})
μg	Micrograma

SUMÁRIO

1 INTRODUÇÃO

Brettanomyces bruxellensis é uma espécie de levedura relacionada a diferentes ambientes industriais, principalmente aos de fermentação alcoólica, sendo considerada a principal responsável por episódios de contaminação na indústria vinícola e da produção de etanol combustível em sua primeira geração. Esta observação pode ser explicada pela alta capacidade adaptativa exibida por diferentes linhagens de *B. bruxellensis* aos mais diversos substratos industriais. De fato, em destilarias de bioetanol, essa levedura pode ser isolada em diferentes setores do processo, variando desde a água de lavagem até o caldo misto. Nesse contexto, *B. bruxellensis* compete com *Saccharomyces cerevisiae* pelo substrato industrial e reduz a produtividade de etanol. Entretanto, na última década, o papel previamente atribuído a *B. bruxellensis* vem sendo substituído por um novo, no qual essa levedura possui potencial aplicação em processos fermentativos específicos. Essa mudança está relacionada principalmente a algumas características exibidas por *B. bruxellensis*, como sua capacidade de produzir etanol, desde que as condições apropriadas sejam fornecidas, e de resistir aos mais diferentes ambientes industriais.

Nesse sentido, pesquisas vem estudando os principais aspectos do metabolismo central de *B. bruxellensis*, no intuito de entender e aprimorar a sua capacidade fermentativa, visando (i) a diminuição dos problemas associados à sua presença ou (ii) a sua aplicação em outros setores industriais. Apesar de ser possível vislumbrar o possível uso, ainda que restrito, na indústria de etanol de primeira geração, *B. bruxellensis* possui o maior potencial de aplicação no contexto da produção de etanol de segunda geração. Isso se deve ao fato de que essa levedura é capaz de utilizar a ampla gama de fontes de carbono, incluindo açúcares derivados da hidrólise do material lignocelulósico (segunda geração). Além disso, *B. bruxellensis* apresenta requisitos necessários à sua aplicação nesse processo, tais como, resistência aos inibidores de fermentação gerados no pré-tratamento desse material, a altas concentrações de etanol e ainda a baixos valores de pH.

Essas características fenotípicas despertaram o interesse em estudos que identifiquem fatores ecológicos, fisiológicos e genéticos responsáveis pela alta capacidade adaptativa de *B. bruxellensis*, bem como sua aplicação em diferentes ambientes e substratos industriais. Nesse sentido, a presente tese de doutorado

investiga o potencial emprego de *B. bruxellensis* na indústria de fermentação alcoólica, especialmente a de etanol de segunda geração, analisando aspectos metabólicos envolvidos com a utilização de açúcares encontrados em substratos industriais. Assim, o primeiro capítulo da presente tese, intitulado "***Biological diversity of carbon assimilation among isolates of the yeast Dekkera bruxellensis from wine and fuel-ethanol industrial processes***", descreve a capacidade de assimilação de diversos açúcares por diferentes linhagens de *B. bruxellensis*, isoladas da produção de vinho e etanol combustível. Além disso, nós observamos a capacidade fermentativa de *B. bruxellensis* em celobiose (dissacarídeo resultante da hidrólise incompleta da celulose) e em sacarose (principal açúcar do caldo de cana) em condições de anaerobiose. Adicionalmente, a análise fenotípica sugeriu que a repressão pela glicose é menos estrita em *B. bruxellensis* do que em *S. cerevisiae*, ao passo que parece ser uma característica linhagem-específica, podendo permitir o co-consumo de açúcares em substratos industriais.

A triagem de linhagens de *B. bruxellensis* capazes de assimilar diferentes açúcares de interesse biotecnológico gerou as hipóteses que resultaram no segundo capítulo da presente tese de doutorado. O estudo, intitulado "***Fermentation profiles of the yeast Brettanomyces bruxellensis in D-xylose and L-arabinose aiming its application as a second-generation ethanol producer***" teve como foco a capacidade de utilização e fermentação de duas pentoses encontradas na produção de etanol de segunda geração. Nesse capítulo, nós verificamos que a capacidade de assimilação de xilose e arabinose também é um traço metabólico linhagem-dependente em *B. bruxellensis*. Além disso, descrevemos pela primeira vez dados fisiológicos relacionados à influência do oxigênio no crescimento e fermentação dessa levedura em meio com essas pentoses. Nesse contexto, nós observamos que, apesar de ser capaz de fermentar xilose, a linhagem JP19M de *B. bruxellensis* apresenta baixa produtividade de etanol, o que limita a sua pronta aplicação em substratos que contenham uma significativa proporção desse açúcar. Por fim, apresentamos dados sobre a influência exercida pela glicose no consumo das pentoses, confirmando que o efeito repressor da glicose não é uma característica universal em *B. bruxellensis* e que algumas linhagens não apresentam esse fenótipo de maneira rigorosa.

A confirmação de que o fenótipo da repressão pela glicose em *B. bruxellensis* não apresentava o rigor encontrado em *S. cerevisiae* nos levou ao conjunto de ensaios que resultaram no terceiro capítulo da presente tese. Esse capítulo, intitulado "***Brettanomyces bruxellensis JP19M strain does not respond to Glucose Catabolite Repression and might be a target for genetic engineering strategies aiming its application in second-generation ethanol industry*"**, revela que a linhagem JP19M de *B. bruxellensis* apresenta uma importante característica metabólica. Nossos dados mostraram que essa linhagem é capaz de assimilar simultaneamente glicose e xilose, uma vez que não é sujeita à Repressão Catabólica pela Glicose (GCR), um traço fisiológico associado a um importante background genético. Essa característica é rara entre leveduras e desejável para o micro-organismo produtor na indústria de etanol de segunda geração, a fim de aumentar a eficiência da utilização da mistura de açúcares presentes no substrato industrial.

Por fim, a diversidade fenotípica apresentada pelas linhagens de *B. bruxellensis*, mostradas no primeiro capítulo, também nos levou a investigar o perfil de assimilação e a capacidade fermentativa em galactose, de duas linhagens de *B. bruxellensis*. Os resultados gerados nesse estudo culminaram no quarto capítulo da presente tese, intitulado **"*Aerobic utilization of galactose by different strains of the yeast Brettanomyces bruxellensis directs pyruvate to distinct metabolic fates and impacts its fermentative capacity*"**. Nesse capítulo, nós descrevemos que diferentemente de GDB 248, a linhagem JP19M é capaz de produzir etanol tanto na presença de galactose como fonte única de carbono quanto na presença de glicose e galactose em meio misto. Para entender a base genética responsável pelos diferentes perfis encontrados, verificamos que a preferência entre o metabolismo oxidativo ou respiro-fermentativo é sustentada pelos perfis de expressão de genes envolvidos com o metabolismo energético. Por fim, mostramos que a ausência do fenótipo de repressão pela glicose em JP19M resulta na sua capacidade de co-assimilar glicose e galactose.

Diante dos resultados obtidos e descritos nos quatro capítulos que compõem o corpo da presente tese de doutorado, é possível concluir que a levedura *B. bruxellensis*, em especial a linhagem JP19M, possui características importantes para a indústria de etanol de segunda geração. O acúmulo desses conhecimentos auxilia na compreensão do metabolismo de fontes de carbono

encontradas em substratos industriais pela levedura *B. bruxellensis*, o que pode fortalecer a hipótese de sua aplicação nesses setores. Entretanto, nós observamos que a aplicação imediata de *B. bruxellensis* na produção de etanol de segunda geração ainda é limitada pela sua eficiência de fermentação em xilose. Apesar disso, estratégias de engenharia genética podem ser desenvolvidas, a fim de maximizar suas características metabólicas importantes e solucionar gargalos metabólicos que diminuem seu potencial de utilização industrial.

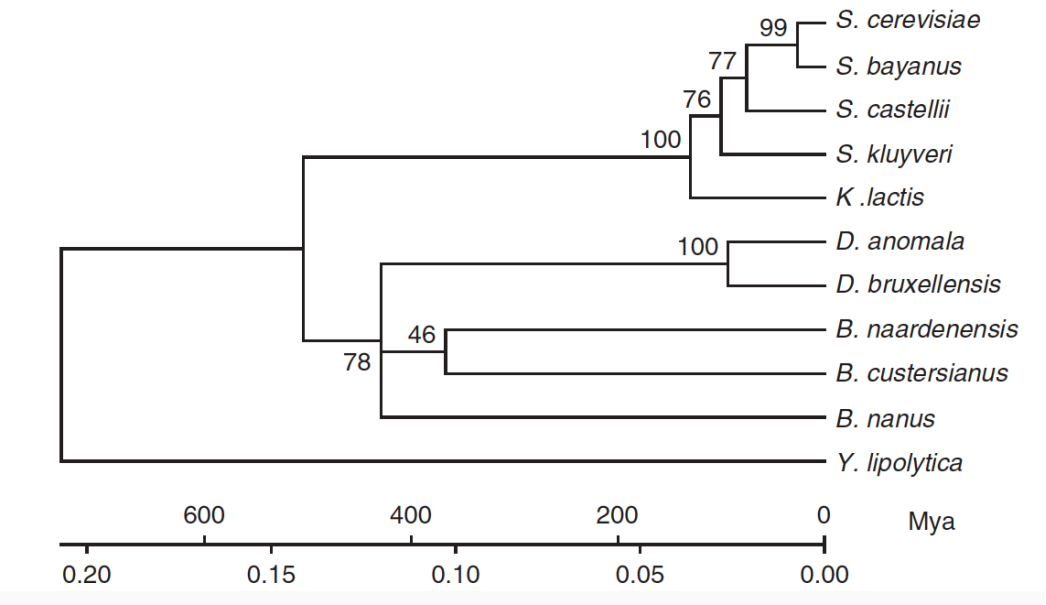
2 REVISÃO DA LITERATURA

2.1 CARACTERÍSTICAS GERAIS DE *BRETTANOMYCES/DEKKERA BRUXELLENSIS*

A levedura *Brettanomyces/Dekkera bruxellensis*, assim como *Saccharomyces cerevisiae*, pertence a família Saccharomycetaceae, ambos os gêneros constituindo parte do grupo dos hemiascomicetos (Kurtzman et al., 2011). A primeira menção ao gênero *Brettanomyces* foi feita em 1904, quando essa levedura foi isolada pela primeira vez a partir da fermentação secundária de cervejas britânicas, sendo caracterizada como “indispensável para a produção de cervejas Inglesas do tipo real”. Devido ao seu local de isolamento, essa levedura foi nomeada como *Brettanomyces* (fungo britânico) (Claussen, 1904). Posteriormente, uma levedura isolada da cerveja Lambic Belga foi também identificada como *B. bruxellensis* (Kufferath e van Laer, 1921).

O gênero *Brettanomyces* foi caracterizado em 1940, ao passo que o gênero *Dekkera* foi introduzido em 1964, após a observação da formação de ascósporos em algumas linhagens (van der Walt, 1964). Atualmente, ambos os nomes dos gêneros são utilizados na literatura como sinônimos e esse grupo compreende as espécies *B/D. bruxellensis*, *B/D. anomala*, *B. custersianus*, *B. nanus*, *B. naardenensis*, *B. acidodurans* sp. nov. (Kurtzman et al., 2011; Peter et al., 2017; Roach e Borneman, 2020) (Figura 1). Essa última espécie foi recentemente descrita para o gênero, e foi isolada a partir de azeite e azeite contaminado na Espanha e Israel, respectivamente (Peter et al., 2017). Análises comparativas dos genomas revelaram que essas espécies são geneticamente distantes e polifiléticas (Roach e Borneman, 2020).

Figura 1 – Árvore filogenética das espécies do gênero Brettanomyces/Dekkera baseada na análise de restrição da sequência de rDNA 26S nuclear. Mya = milhões de anos atrás.



Fonte: Rozpedowska et al. (2011).

Apesar de a descrição inicial desse gênero ter sido em cervejas britânicas, *B. bruxellensis* tem sido isolada de diversas outras bebidas fermentadas, tais como vinho, kombucha, kefir, tequila e cerveja Lambic (Lachance et al., 1995; Teoh et al., 2004; Gray et al., 2011; Albertin et al., 2014; Curtin et al., 2015; Spitaels et al., 2015; Longin et al., 2016). Dentre essas, *B. bruxellensis* é mais frequentemente encontrada na produção de vinho, principalmente nos barris de envelhecimento de vinhos tintos. Nesse cenário, essa espécie é considerada um contaminante devido a produção, em altas concentrações, de compostos fenólicos voláteis, como 4-etil-fenol e 4-etil-guaiacol, que alteram a composição do aroma e do sabor do vinho (Chatonnet et al., 1992; Crauwels et al., 2017). Além do papel deteriorante em vinhos, *B. bruxellensis* também foi identificada como o principal contaminante das destilarias de produção de etanol combustível no nordeste do Brasil (de Souza Liberal et al., 2007). Essa contaminação é favorecida por falhas nas etapas de esterilização, que favorecem o crescimento e a propagação de leveduras selvagens. De fato, *B. bruxellensis* tem sido isolada de diversos ambientes da destilaria, incluindo a água de lavagem, vinhaça e caldo misto (da Silva et al., 2016).

B. bruxellensis possui um extenso repertório de utilização de fontes de carbono, como pode ser observado na descrição fisiológica da espécie. Do ponto

de vista industrial, a utilização de diversos açúcares permite o emprego de *B. bruxellensis* em diferentes processos fermentativos que visam a produção de etanol. Entretanto, a capacidade de assimilar algumas dessas fontes é variável dentre as linhagens descritas (Kurtzmann et al., 2011; Crauwels et al., 2015). Algumas linhagens de *B. bruxellensis* são capazes de metabolizar manose, bem como alguns trissacarídeos e polissacarídeos (Galafassi et al., 2011; Crauwels et al., 2015). De maneira geral, quando as fontes de carbono são captadas pelas células, elas são direcionadas para o chamado Metabolismo Central do Carbono.

2.2 METABOLISMO CENTRAL DO CARBONO

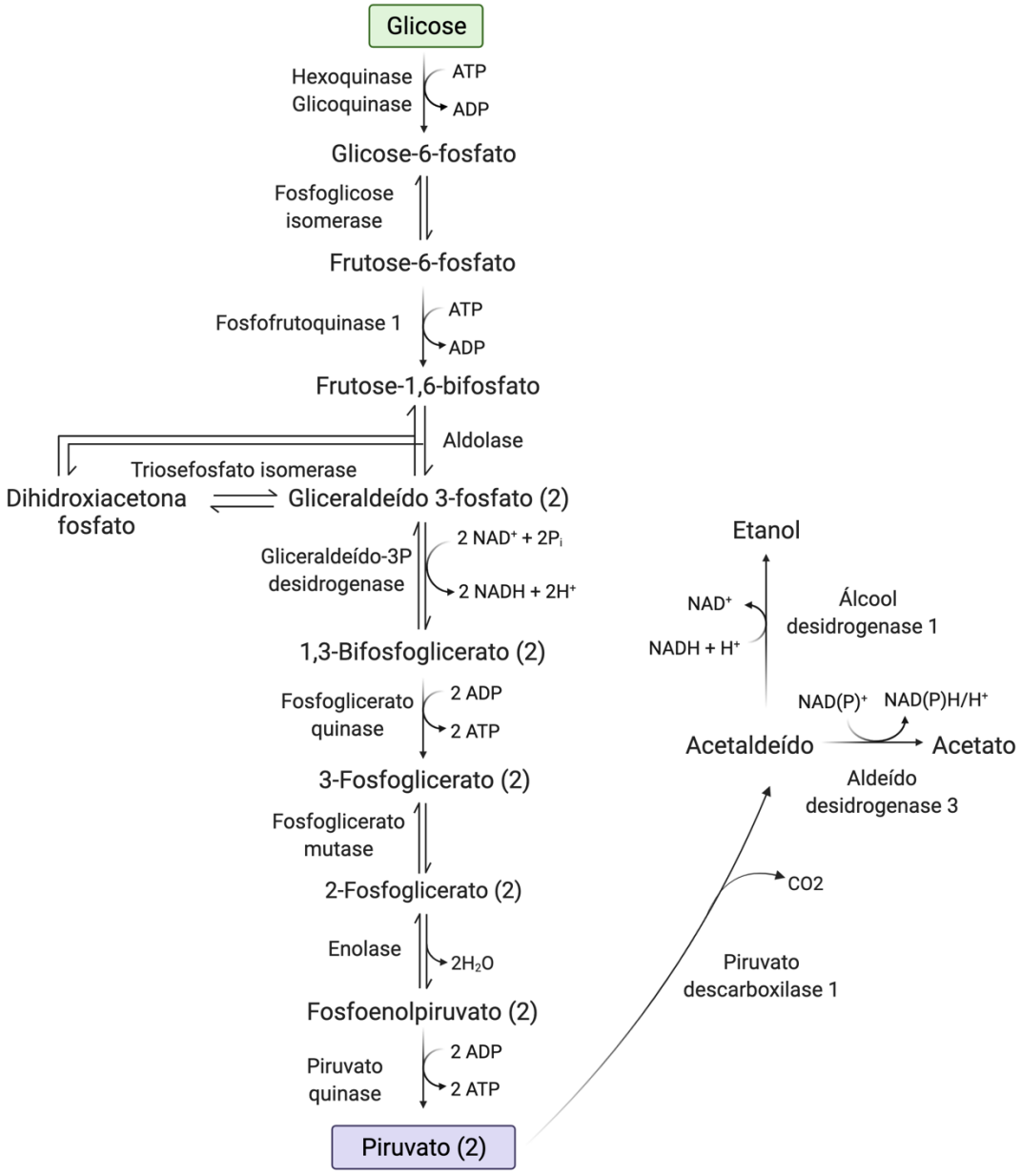
2.2.1 Via glicolítica

De modo geral, a oxidação de açúcares é realizada através da glicólise, na qual uma série de 10 reações levam à quebra da glicose em duas moléculas de piruvato. Durante esse processo, a célula produz ATP e NADH sem necessidade de oxigênio molecular (Piškur e Compagno, 2014). A primeira reação da glicólise compreende a hidrólise de uma molécula de ATP para formar glicose-6-fosfato (Figura 2). Em *S. cerevisiae*, esta reação é catalisada por três enzimas denominadas hexoquinases, que por sua vez são codificadas por três genes *HXK1*, *HXK2* e *GLK1* (glicoquinase). Nesse momento ocorre uma importante etapa de regulação do metabolismo da glicose que é exercida pela proteína Hxk2, envolvida no mecanismo de repressão catabólica pela glicose (GCR) (Peláez et al., 2010). Em seguida, a glicose-6-fosfato é isomerizada a frutose-6-fosfato pela enzima fosfoglicose isomerase, codificada pelo gene *PGI1*.

A terceira reação, envolve outra hidrólise de ATP para então formar a frutose-1,6-bifosfato, um intermediário central que desempenha um dos principais papéis na regulação do fluxo do carbono nas células (Peeters et al., 2017). A enzima chave que catalisa essa reação é a fosfofrutoquinase, codificada pelos genes *PFK1* e *PFK2*. De fato, essa etapa é o ponto principal de controle da glicólise, que pode ser observada pela forte indução da expressão desses genes na presença de glicose (Moore et al., 1991). A atividade da fosfofrutoquinase é inibida por ATP e citrato, e ativada por AMP e frutose-2,6-bifosfato (Piškur e Compagno, 2014). Em seguida, a frutose-1,6-bifosfato é convertida em duas moléculas de três

carbonos, gliceraldeído-3-fosfato e dihidroxiacetona fosfato, pela enzima frutose-1,6-bifosfato aldolase, codificada pelo gene *FBA1*. Esses dois produtos podem sofrer interconversão reversível pela enzima triosefosfato isomerase (*TPI1*).

Figura 2 – Vias glicolítica e fermentativa em *S. cerevisiae*. O número 2 entre parênteses, presente após a formação do gliceraldeído-3P e nas etapas subsequentes refere-se à quantidade de moléculas de cada composto por molécula de glicose que entra na via glicolítica.



Fonte: a autora (2021).

As duas moléculas de gliceraldeído-3-fosfato formadas são oxidadas a duas moléculas de 1,3-bifosfoglicerato, pela ação da enzima gliceraldeído-3-fosfato-desidrogenase, resultando na formação de dois NADH. Os equivalentes redutores, por sua vez, são posteriormente reoxidados pela cadeia transportadora de elétrons ou pela via fermentativa para manter o equilíbrio redox na célula. Em seguida, as moléculas de 1,3-bifosfoglicerato são convertidas a duas moléculas de 3-fosfoglicerato pela ação da fosfoglicerato quinase, com formação de dois mols de ATP, por meio da fosforilação de dois ADP (Nelson, 2019). Nessa etapa, a fosforilação não é oxidativa, pois não há transferência de elétrons, mas sim de fosfato, em nível de substrato. Logo após, ocorre a conversão das duas moléculas de 3-fosfoglicerato a duas moléculas de 2-fosfoglicerato, mediante ação da fosfoglicerato mutase. As últimas moléculas formadas sofrem desidratação por meio da ação da enolase, formando então, duas moléculas de fosfoenolpiruvato. Por fim, ocorre a transferência de fosfato a partir de cada fosfoenolpiruvato para cada ADP, através da enzima piruvato quinase, formando então duas moléculas de piruvato e dois mols de ATP. Desse modo, a produção líquida final da glicólise é igual a dois mols de ATP e duas moléculas de NADH. Como veremos na seção seguinte, o piruvato pode seguir dois caminhos distintos, entrar na mitocôndria ou continuar no citoplasma (Piškur e Compagno, 2014).

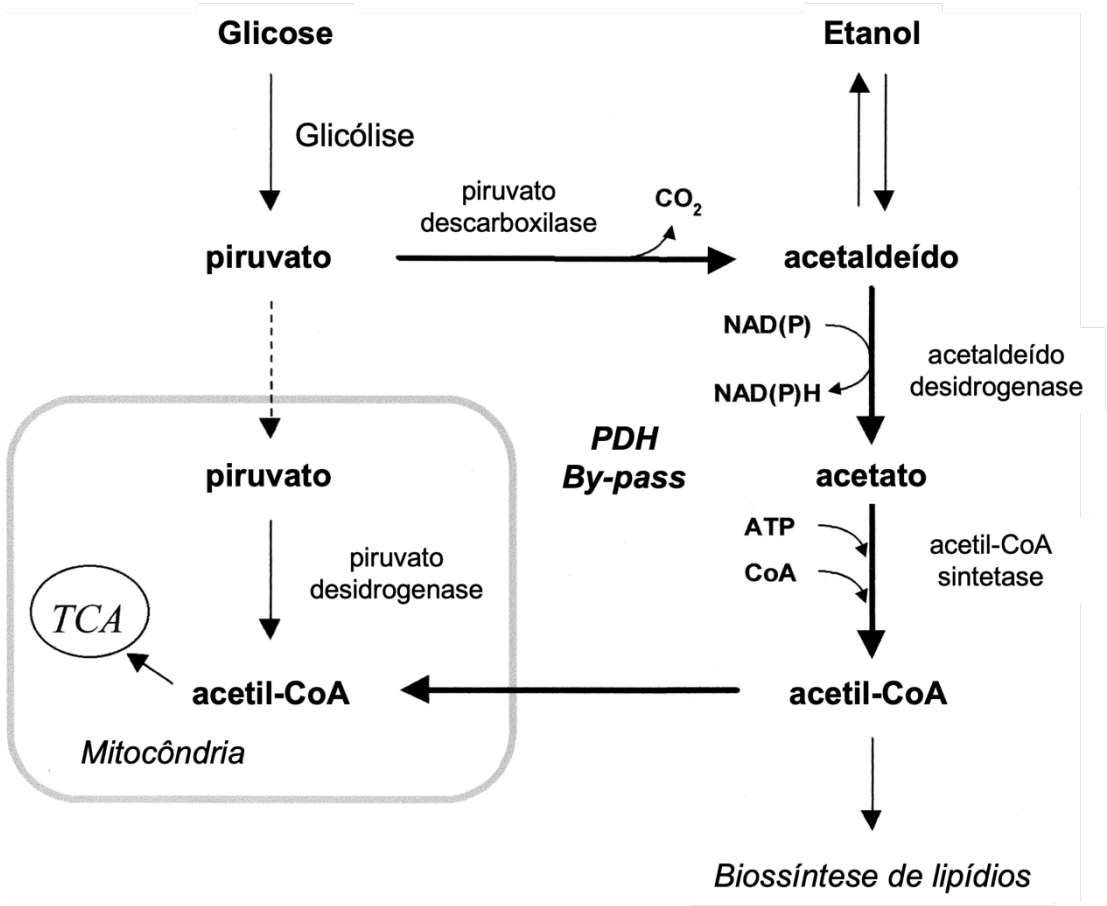
2.2.2 Respiração celular e fermentação

Em leveduras, o destino do piruvato depende da espécie e da condição de cultivo, podendo ser destinado à respiração celular ou à fermentação (Flores et al., 2000). Quando o destino é a respiração celular (mitocôndria), o piruvato é então convertido a acetil-CoA pela ação do complexo piruvato desidrogenase e segue para o ciclo de Krebs. Alternativamente, o piruvato pode ser convertido a acetil-CoA no citoplasma, via acetaldeído e acetato, pela chamada via *PDH-bypass* (Pronk et al., 1996; Remize et al., 2000) (Figura 3). No ciclo de Krebs, o acetil-CoA é oxidado a CO_2 com geração de NADH e $FADH_2$ que serão posteriormente reoxidados na cadeia respiratória para a produção de ATP (Piškur e Compagno, 2014). Por outro lado, quando destinado à fermentação alcoólica, o piruvato é descarboxilado pela enzima piruvato descarboxilase (codificada pelo gene *PDC1*) gerando acetaldeído e CO_2. Posteriormente, o acetaldeído é reduzido a etanol pela enzima álcool

desidrogenase (*ADH 1, 3, 4* e *5*), levando a reoxidação do NADH citosólico produzido pela glicólise (Figura 2) (Hohmann e Cederberg, 1990; Thomson et al., 2005).

Para a maioria dos organismos em condições aeróbicas, o piruvato é convertido a acetil-CoA e direcionado à respiração. Entretanto, em algumas leveduras, tais como *S. cerevisiae* e *B. bruxellensis*, o piruvato citoplasmático derivado da glicose entra na via de fermentação alcoólica mesmo quando o oxigênio está presente na célula (Rozpedowska et al., 2011). Esse fenótipo é denominado de efeito *Crabtree* positivo (Crabtree, 1929). Adicionalmente, o metabolismo fermentativo pode ocorrer concomitantemente à respiração em condições aeróbicas, constituindo o chamado metabolismo respiro-fermentativo, presente nas duas espécies mencionadas (Alexander e Jeffries, 1990; Pfeiffer e Morley, 2014). É importante ressaltar que o etanol só é produzido a partir de açúcares e, depende da produção de piruvato a partir de glicólise (Esposito e Azevedo, 2010). Isto implica que os polissacarídeos, tais como o amido, devem ser hidrolisados aos monossacarídeos constituintes e esses a algum metabólito intermediário da glicólise (Santana, 2007). Isso se aplica para outros açúcares não preferenciais, que devem ser metabolizados para gerar intermediários da via glicolítica. Pode-se citar como exemplos a galactose, que por meio da via de Leloir chega a glicose-6-fosfato, e D-xilose, que por meio da via das pentoses-fosfato gera frutose-6-fosfato e gliceraldeído-3-fosfato (Bhat e Murthy, 2001; Olofsson et al., 2008).

Figura 3 – Enzimas envolvidas na via *PDH* By-pass em *S. cerevisiae*. TCA: Ciclo do Ácido Tricarboxílico (Ciclo de Krebs).



Fonte: adaptado da figura 1 de Remize et al. (2020).

2.2.3 Via das pentoses-fosfato

A via das pentoses-fosfato (PPP) consiste em duas fases (oxidativa e não-oxidativa) e tem como principal função fornecer intermediários anabólicos para biossíntese e crescimento celular (Figura 4), tais como ribulose-5-fosfato, eritrose-4-fosfato e NADPH (Hahn-Hagerdal et al., 2007). Em leveduras, a primeira fase (oxidativa) engloba as enzimas glicose-6-fosfato desidrogenase (*ZWF1*, que converte glicose-6-fosfato a 6-fosfogliconolactona), 6-fosfogliconolactonase (*SOL*, que converte 6-fosfogliconolactona a 6-fosfogluconato), e 6-fosfogluconato desidrogenase (*GND1*, que converte 6-fosfogluconato a D-ribulose-5-fosfato) (Dickinson e Schweizer, 2004; Bertels et al., 2021). A primeira e terceira reação geram NADPH, necessários para reações de biossíntese (Piškur e Compagno, 2014).

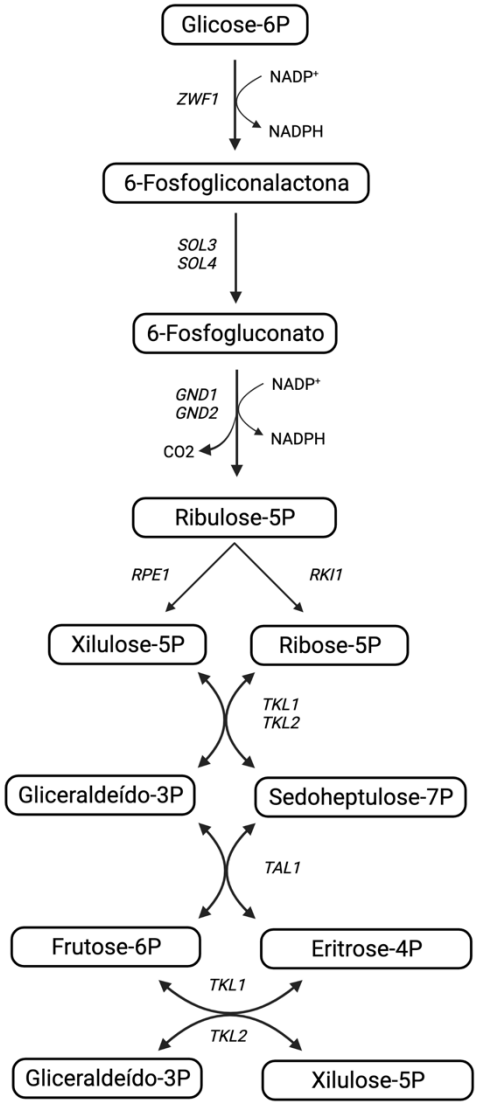
Na segunda fase (não-oxidativa), a D-ribulose-5-fosfato gerada é epimerizada a xilulose-5-fosfato através da enzima ribulose-5-fosfato 3-epimerase (*RPE1*) ou isomerizada a ribose-5-fosfato pela enzima ribose-5-fosfato cetol-isomerase (*RKI1)*. Em seguida, uma série de rearranjos de esqueleto de carbono são realizados mediante ação das enzimas transcetolase (*TKL1* e *TKL2*) e transaldolase (*TAL1*) (Flores et al., 2000). Desse modo, a xilulose-5P e a ribose-5P podem então atuar juntas em uma reação catalisada pela transcetolase para produzir gliceraldeído-3-fosfato e sedoheptulose-7-fosfato (Figura 4). O gliceraldeído-3P gerado pode entrar na glicólise/gliconeogênese ou sofrer reação com a sedoheptulose-7-fosfato para formar, por meio da ação transaldolase, frutose-6-fosfato e eritrose-4-fosfato. Nesse ponto, a transcetolase atua novamente a partir de eritrose-4-fosfato e xilulose-5-fosfato para formar frutose-6-fosfato e gliceraldeído-3-fosfato que seguem para via glicolítica (Dickinson e Schweizer, 2004; Jeffries, 2006).

Em *S. cerevisiae*, o fluxo pela via não-oxidativa das pentoses-fosfato é menor do que em outras leveduras. A baixa atividade dessa via pode ser interpretada como o resultado da sua domesticação para produção de etanol e gás carbônico a partir de hexoses (Hahn-Hagerdal et al., 2007). Entretanto, a atividade da PPP é crucial para metabolização de pentoses, visto que este é o único modo de introduzir a xilulose ao metabolismo central do carbono (Kotter e Ciriacy, 1993; Temer, 2014). Além disso, um estudo realizado por Jouhten et al., (2008) mostra que a atividade *in vivo* da PPP nesta espécie, relativa ao fluxo da via glicolítica, em condições anaeróbicas é menor do que em condições aeróbicas.

A análise do genoma de *B. bruxellensis* exibe genes que codificam enzimas da PPP, tal como a transcetolase (Godoy et al., 2017). O aumento da expressão desse gene, através de análise relativa, foi observado quando *B. bruxellensis* foi submetida a meio com glicose e nitrato sob limitação de oxigênio (de Barros Pita et al., 2013a). Dados do transcriptoma de *B. bruxellensis* em cultivo contínuo contendo glicose mostram que assim como *S. cerevisiae*, a expressão do gene que codifica a enzima glicose-6-fosfato desidrogenase (*ZWF1*) é significativamente mais baixa do que a expressão do gene que codifica a hexoquinase (*HXK*) (Tiukova et al., 2013). Isso indica que a glicólise representa o principal fluxo de carbono se comparado a via das pentoses-fosfato (Tiukova et al., 2013). Apesar desses achados, pouco se sabe sobre o comportamento fisiológico e genético dessa

espécie em diferentes condições de cultivo quando pentoses e hexoses são fornecidas juntamente como fontes de carbono.

Figura 4 – Via das pentoses-fosfato em *S. cerevisiae*.



Fonte: a autora (2021).

2.2.4 Metabolismo de pentoses

As pentoses resultantes da hidrólise do material lignocelulósico, xilose e arabinose, são inicialmente metabolizadas pela via das pentoses-fosfato para gerar intermediários da via glicolítica. Dessa forma, a compreensão acerca da PPP é de

grande interesse para a produção de etanol de segunda geração. Nesse contexto, os metabolismos da xilose e da arabinose em leveduras estão descritos a seguir.

2.2.4.1 Metabolismo de xilose

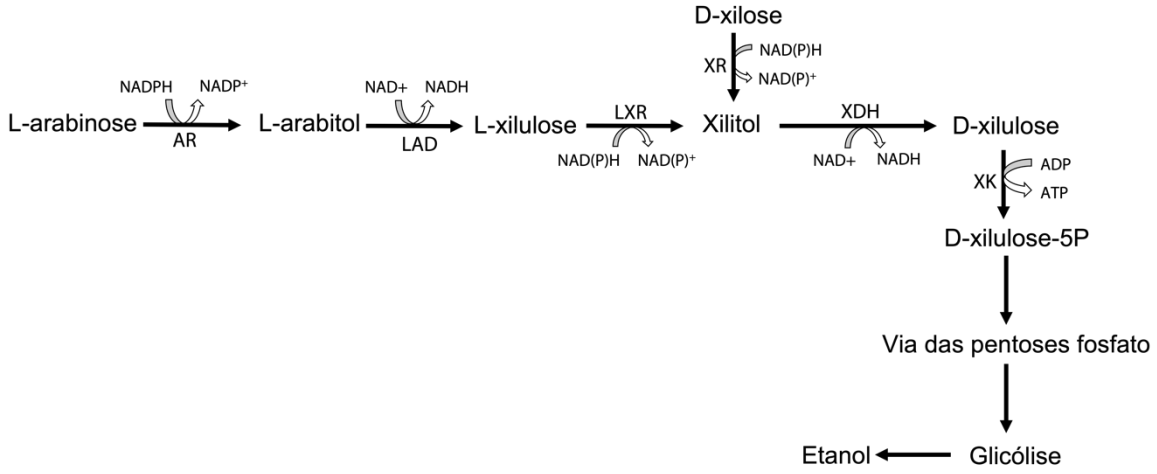
A D-xilose (ou simplesmente xilose) é a pentose mais abundante dentre os açúcares oriundos da biomassa lignocelulósica, sendo o principal derivado hemicelulósico do bagaço de cana de açúcar, com uma proporção de 12:1 em relação a arabinose (Rocha et al., 2015). Dentre as leveduras capazes de utilizar xilose como fonte de carbono, estima-se que apenas 1% seja capaz de utilizá-la na via fermentativa, com destaque para as espécies *Scheffersomyces (Pichia) stipitis*, *Spathaspora passalidarum* e *Candida shehatae* (Hou 2012; Yuvadetkun et al., 2017; Nakanishi et al., 2017). A xilose é internalizada nas células por permeases denominadas transportadores de hexoses (*HXT*), assim como a glicose em *S. cerevisiae*, porém com diferentes afinidades (Olofsson et al., 2008). Dentre os transportadores de hexoses que transportam xilose, *HXT4*, *HXT5*, *HXT7* e *GAL2* possuem maior afinidade. Experimentos com a superexpressão desses genes revelam que a internalização da xilose não necessariamente indica o aumento do seu fluxo, visto que não resulta no aumento da taxa de crescimento em condições aeróbicas e nem na produção de etanol em condições anaeróbicas (Hamacher et al., 2002).

Uma vez dentro da célula, em leveduras, a xilose é metabolizada por uma via denominada de via de oxirredução, constituída pelas enzimas xilose redutase (XR), xilitol desidrogenase (XDH) e xiluloquinase (XK) codificadas pelos genes *XYL1*, *XYL2* e *XKS1*, respectivamente (Jeffries et al., 2007). A xilose é reduzida a xilitol pela enzima XR com a utilização preferencial do cofator NADPH, em seguida o xilitol é oxidado a xilulose pela enzima XDH dependente de NAD^+ (Figura 5). A xilulose uma vez formada é então fosforilada a xilulose-5-fosfato pela enzima xiluloquinase e segue para a via das pentoses-fosfato para geração de intermediários da glicólise (Olofsson et al., 2008).

Linhagens selvagens de *S. cerevisiae*, principal levedura aplicada na produção de bioetanol, não são capazes de utilizar as pentoses resultantes da hidrólise da hemicelulose (Hahn-Hagerdal et al., 2007). Curiosamente, o genoma

de *S. cerevisiae* possui os genes da via de oxirredução da xilose, entretanto as enzimas são expressas em níveis baixos, incapazes de sustentar a utilização da D-xilose (Kuhn e Prior 1995; Richard et al., 1999). Portanto, vias heterólogas são inseridas nessas células com o objetivo de fermentar xilose ou co-fermentar D-xilose, L-arabinose e glicose (Wang et al., 2017; Huang et al., 2019). As modificações genéticas em *S. cerevisiae* são feitas pela construção de linhagens recombinantes através da inserção de genes de *S.* (*Pichia*) *stipis* que codificam as enzimas xilose redutase e xilitol desidrogenase (Scalcinati et al., 2012; Ha et al., 2013).

Figura 5 – Via de oxirredução da xilose e arabinose em linhagens modificadas de *S. cerevisiae*.



Fonte: adaptado da Figura 2 de Stambuk et al. (2008).

Assim como ocorre em *S. stipitis* e *B. naardenensis*, em linhagens modificadas de *S. cerevisiae*, o xilitol é considerado um coproduto da fermentação da xilose em condições anaeróbicas ou em limitação de oxigênio (Galafassi et al., 2011; Shin et al., 2019). Isso ocorre pelo desequilíbrio redox gerado pela preferência por diferentes cofatores das enzimas envolvidas nas reações, visto que a XR apresenta preferência pelo NADPH e a XDH é dependente de NAD^+. A explicação para esse gargalo está relacionada com a falha na reoxidação do NADH devido a ausência de oxigênio, resultando no seu acúmulo, enquanto o NADP+ gerado pode ser reduzido na PPP (Jeffries, 2006). Em condições de limitação de oxigênio, *S. cerevisiae* soluciona o acúmulo de NADH mediante formação de glicerol, entretanto isso não ocorre quando a xilose é única fonte de carbono

disponível para célula (Jeffries, 2006). Por outro lado, quando a D-xilulose é fornecida como fonte de carbono, linhagens selvagens de *S. cerevisiae* são capazes de fermentá-las, visto que a enzima xiluloquinase (XK) é funcional (Chiang et al., 1981; Hahn-Hagerdal et al., 2007). Além disso, tem sido observado que a capacidade de metabolizar xilose a etanol vai além da taxa de consumo, e está relacionada a expressão de genes da via não-oxidativa das pentoses-fosfato (Hahn-Hagerdal et al., 2007).

Diferentemente de *S. stipitis* e linhagens recombinantes de *S. cerevisiae* que necessitam de oxigênio para manter o equilíbrio redox, a levedura *S. passalidarum* mantém o equilíbrio redox mesmo em condições de anaerobiose e consequentemente apresenta menor acúmulo de xilitol (Hou, 2012). Isso ocorre porque a enzima xilose redutase desta levedura tem preferência pela forma reduzida do cofator NAD (NADH), ao passo que a xilitol desidrogenase utiliza preferencialmente sua forma oxidada (NAD^+). Portanto, o NAD^+ requerido pela enzima xilitol desidrogenase pode ser garantido pela reoxidação do NADH pela xilose redutase, sem haver a necessidade da presença de oxigênio (Hou, 2012). Outra maneira de evitar o acúmulo de xilitol consiste na inserção da enzima xilose isomerase (XI) de *Clostridium phytofermentans* ou *Piromyces*, que resulta na conversão de xilose diretamente a D-xilulose, sem a necessidade de cofatores (Figura 6) (Brat et al., 2009; Zhou et al., 2012). Entretanto, a taxa de consumo de xilose através da XI é normalmente menor que a das linhagens com a via XR-XDH (Demeke et al., 2013; Li et al., 2019).

Algumas linhagens selvagens de *B. bruxellensis* apresentam a capacidade de assimilar e fermentar xilose (Crauwels et al., 2015; Codato et al., 2018). Do ponto de vista genômico, *B. bruxellensis* possui genes que codificam enzimas da via de metabolização da xilose, tais como a D-xilose redutase, D-xilulose redutase, xiluloquinase, e genes da via das PPP, tal como transcetolase, o que explica a habilidade dessa levedura em consumir D-xilose e utilizá-la como fonte energética (Godoy et al., 2017). Entretanto, até o momento, não há dados sobre a preferência de cofatores para XR e XDH em *B. bruxellensis*. Em condições fermentativas contendo a mistura de xilose (40 g/L) e glicose (10 g/L), *B. bruxellensis* produziu 5,92 g/L de etanol, indicando que apesar de um baixo rendimento e produtividade, o etanol foi produzido a partir de xilose (Codato et al., 2018). Além disso, seu perfil

fermentativo pode variar de acordo com a concentração de xilose no meio (Codato et al., 2018).

2.2.4.2 Metabolismo da arabinose

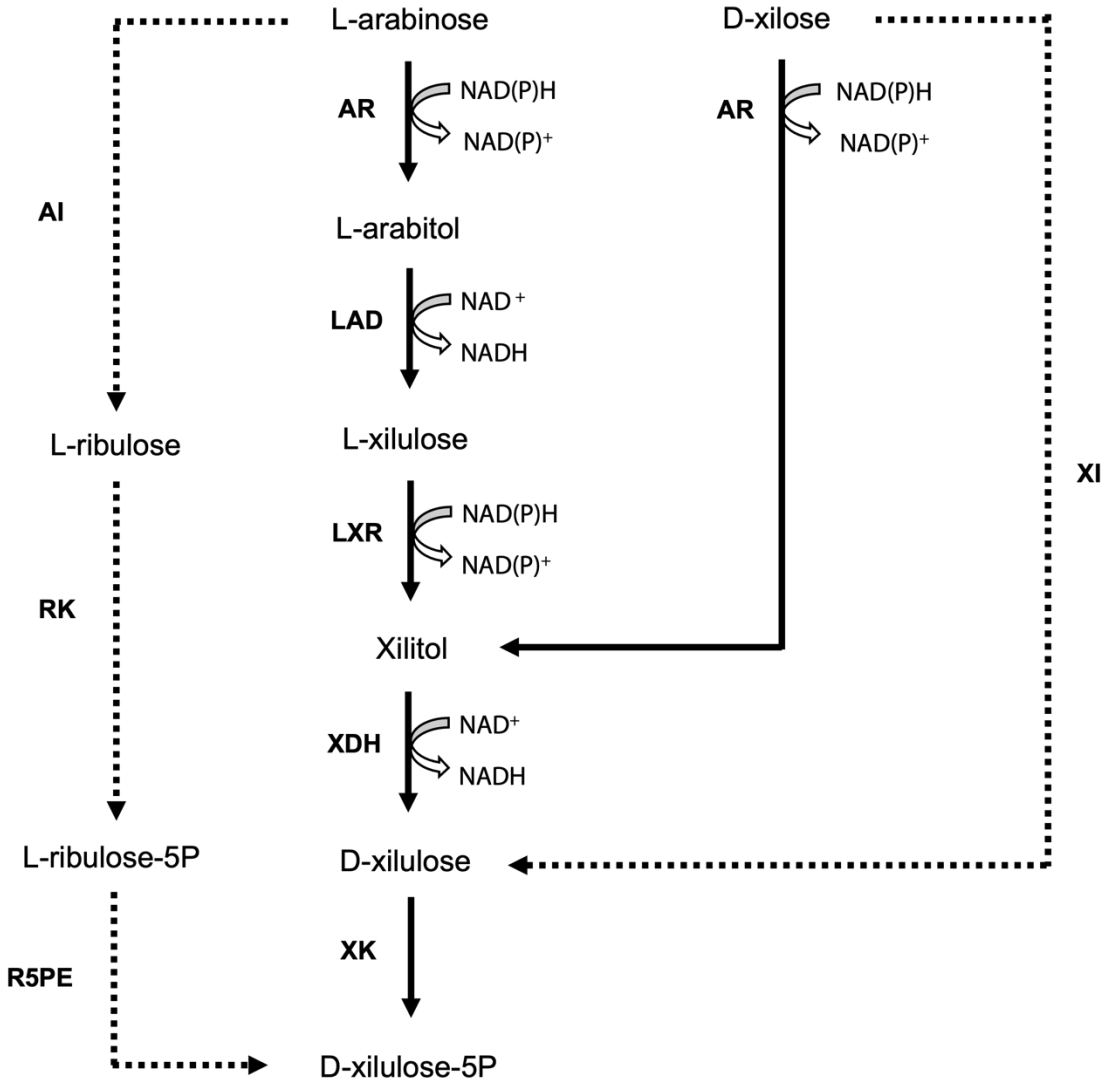
Como mencionado anteriormente, nos materiais lignocelulósicos, a concentração de L-arabinose (ou simplesmente arabinose) é menor em relação a de xilose (Rocha et al., 2015). Apesar disso, ambos os açúcares são as pentoses mais abundantes da biomassa vegetal (Passoth, 2014) e consequentemente a busca por micro-organismos capazes de utilizar essas fontes é de grande interesse na produção de etanol de segunda geração (Subtil e Boles, 2011). Em *S. cerevisiae*, a arabinose é internalizada pelos transportadores de galactose (Gal2p), porém, uma vez que linhagens selvagens não são capazes de utilizar esse açúcar (Hahn-Hagerdal et al., 2007), é necessária a inserção de vias heterólogas para a sua fermentação (Caballero e Ramos 2017; Wang et al. 2017; Wang et al., 2019).

Nesse sentido, para que a arabinose seja convertida a D-xilulose e entre na PPP, em leveduras, é necessária a presença de quatro enzimas: xilose (aldose) redutase (XR), L-arabitinol 4-desidrogenase (LAD), L-xilulose redutase (LXR) e xilitol desidrogenase (XDH) (Richard et al., 2003) (Figura 6). A XR, além de atuar na conversão da xilose em xilitol, também converte arabinose a arabitol (arabitinol) (Ye et al., 2019). As enzimas XR e LXR apresentam preferência pelo cofator NADPH, e as enzimas LAD e XDH e são NAD^+ específicas. Essa preferência diferencial por cofatores resulta no acúmulo de arabitol como coproduto em condições anaeróbicas ou em limitação de oxigênio, devido a um desbalanço redox similar ao observado no metabolismo da xilose (Hahn-hagerdal et al., 2007).

Alternativamente à via de oxirredução, a inserção da via de isomerização de bactérias em linhagens de *S. cerevisiae* resulta em xilulose-5-fosfato sem formação de coproduto (Hahn-Hägerdal et al., 2007). Esta via é considerada redox neutra (Figura 6) e consiste nas enzimas L-arabinose isomerase (AI), L-ribuloquinase (RK) e L-ribulose-5-P 4-epimerase (RE) (Ye et al., 2019). Diferentes estudos têm sido realizados com a expressão heteróloga em *S. cerevisiae* a partir de genes de *Bacillus subtilis*, *Escherichia coli* e *Lactobacillus plantarum* (Becker e Boles, 2003; Wang et al., 2013; Wang et al., 2019). A produção de etanol a partir das linhagens modificadas variarou entre 6 e 9 g/L a partir de 20 g/L de arabinose.

Além da inserção da via de metabolização da arabinose, tem sido mostrado que outras modificações genéticas, tais como a superexpressão de genes da fase não-oxidativa da PPP aumentam a capacidade de uso desse açúcar por *S. cerevisiae* (Wang et al., 2019). Dentre eles, o gene *TKL2* e o gene *TAL1* (para mais informações verificar o tópico “Via das pentoses-fosfato”) (Becker e Boles, 2003; Wiedemann e Boles, 2008; Wisselink et al., 2010). Ambos os estudos mostram que o alto fluxo da PPP é necessário para utilização de arabinose.

Figura 6 – Vias de utilização de arabinose e xilose em leveduras (setas contínuas), em bactérias e alguns fungos anaeróbicos (setas tracejadas).



Fonte: adaptado da figura 1 de Bettiga et al. (2009).

Em *B. bruxellensis*, apenas algumas linhagens selvagens são capazes de utilizar arabinose (Galafassi et al., 2011; Crauwels et al., 2015), o que pode ser justificado pela presença do gene da L-xilulose redutase, necessário para o catabolismo da arabinose (Woolfit et al., 2007). Além da assimilação de arabinose, algumas linhagens de *B. bruxellensis* são capazes de fermentar arabinose a etanol ainda que com baixos valores de rendimento e produtividade (Codato et al., 2018). Apesar disso, pouco se sabe sobre a capacidade de assimilação e fermentação de arabinose entre linhagens de *B. bruxellensis* isoladas de diferentes processos industriais.

2.2.5 Via de Leloir e metabolismo de galactose

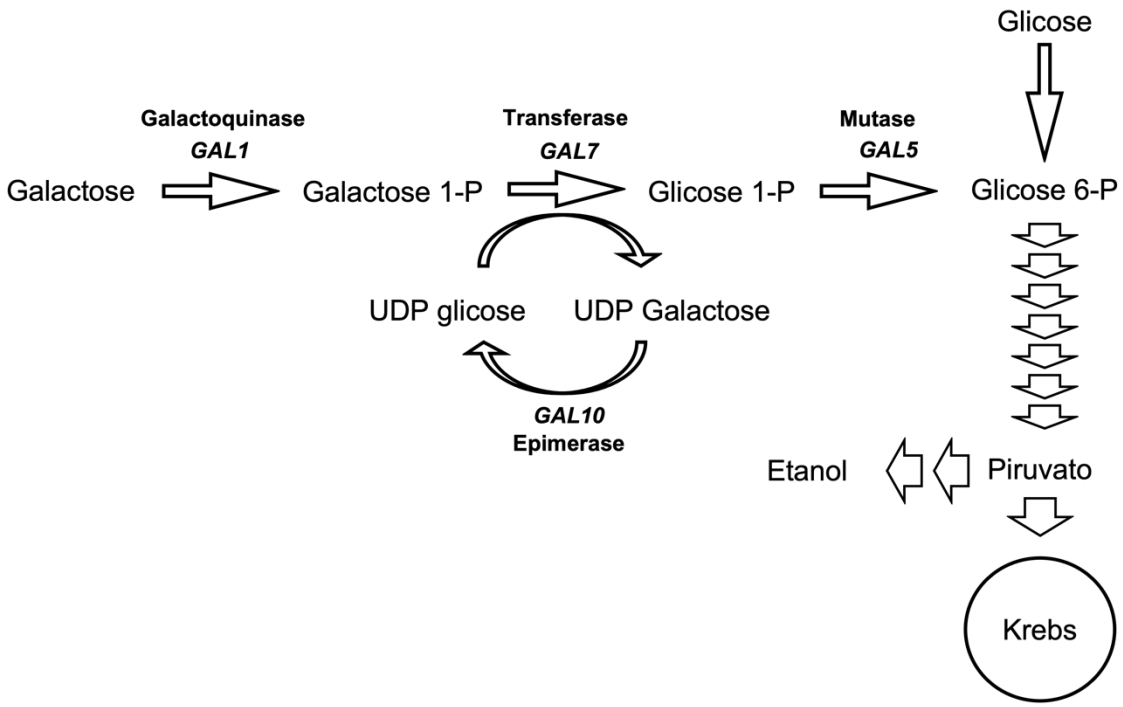
A galactose é uma hexose encontrada em diferentes substratos, tais como em laticínios (quebra da lactose), na parede celular de plantas (cana de açúcar) e em algas marinhas vermelhas, representando uma atrativa fonte de carbono industrial (Sellick et al., 2008; Packer, 2009). Na biomassa lignocelulósica, a galactose é encontrada na hemicelulose em conjunto com outros açúcares, e após a etapa de pré-tratamento, essa hexose é liberada para ser utilizada pelos micro-organismos presentes no processo (dos Santos et al., 2016). Apesar de a concentração da galactose na hemicelulose ser inferior à de glicose, xilose e arabinose, encontrar micro-organismos capazes de fermentar esse açúcar pode resultar no aumento da eficiência do processo fermentativo.

Como mencionado anteriormente, a galactose precisa ser convertida a um metabólito intermediário da glicólise para gerar piruvato, e isso ocorre por meio da via de Leloir. Em *S. cerevisiae*, a galactose é internalizada na célula através da permease Gal2p, codificada pelo gene *GAL2*, e então é epimerizada de β-D-galactose a α-D-galactose pela enzima galactose mutarotase (Figura 7). Depois, a α-D-galactose é fosforilada pela enzima galactoquinase 1 (*GAL1*) a galactose-1-fosfato. A galactose-1-fosfato é convertida a glicose-1-fosfato pela ação da galactose-1-fosfato-uridiltransferase (*GAL7*). A UDP-glicose necessária para essa reação é reabastecida pela conversão de UDP-galactose a UDP-glicose pela epimerase (*GAL10*). Por fim, a glicose-1-fosfato é convertida a glicose-6-fosfato

pela enzima fosfoglicomutase, *PGM* (*GAL5*) e é assimilada pela via glicolítica ou pela via das pentoses-fosfato (Bhat e Murthy, 2001; Bhat, 2008).

A utilização da galactose por *S. cerevisiae* é amplamente estudada devido sua importância como modelo para entendimento da regulação transcricional de genes em eucariotos (Bhat e Murthy, 2001; Bhat e Iyer, 2009). Os genes *GAL* são ativamente transcritos quando a fonte de carbono fornecida pela célula é a galactose. A transcrição dos genes da via de Leloir, tais como, *GAL1*, *GAL2*, *GAL7* e *GAL10* é induzida em mais de 1000 vezes quando a galactose está presente no meio (Johnston, 1987; Johnston et al., 1994). A indução da expressão dos genes *GAL* é rápida, ocorrendo dentro de 30 minutos após a adição da galactose no meio de cultura. Entretanto, quando as células são cultivadas em outras fontes de carbono, tais como glicerol ou rafinose, os genes *GAL* não são expressos, mas ficam “dispostos” para uma rápida ativação se a galactose for adicionada no meio (Sellick et al., 2008).

Figura 7 – Metabolismo da galactose em leveduras. *GAL1*: Galactoquinase; *GAL7*: Galactose 1-fosfato uridiltransferase; *GAL10*: UDP-glicose 4-epimerase; *GAL5*: fosfoglicomutase. Via de Leloir.



Fonte: adaptado da figura 2.2.4 de Bhat (2008).

As mudanças na expressão dos genes *GAL* em resposta ao ambiente são controladas principalmente pela interação entre três proteínas regulatórias, os

ativadores transcricionais codificados pelos genes *GAL3* e *GAL4* e o repressor, codificado pelo gene *GAL80* (Bhat e Murthy, 2001; Bhat e Iyer, 2009). A indução dos genes *GAL* pela galactose é dependente de Gal4, que atua na sequência ativadora a montante (UAS_{GAL}) de seus promotores através do recrutamento da maquinaria transcricional composta por, pelo menos, três complexos de proteínas, SAGA, TFIID e um mediador (Traven et al., 2006; Watson et al., 2015). A ligação do ativador Gal4 ao promotor pode ser impedida pelo repressor transcricional Gal80, mediante o bloqueio da superfície de interação de Gal4 com a maquinaria transcricional (Carrozza et al., 2002; Sellick et al., 2008). A expressão do repressor *GAL80* também depende do ativador Gal4, de modo que este último induz um ciclo de inibição de feedback autorregulado em resposta à galactose (Conrad et al., 2014). A proteína Gal3, pertencente ao regulon *GAL*, é responsável por aliviar Gal4 da inibição por Gal80. Isso ocorre através da interação de Gal3 com Gal80, o que torna o ativador Gal4 livre para atuar na UAS dos genes *GAL*, na presença de galactose e ausência de glicose (Li et al., 2010).

Embora a expressão do gene *GAL4* não seja ativada pela galactose, ele é responsivo a repressão pela glicose, assim como os outros genes *GAL* (Conrad et al., 2014). Na presença de glicose, a repressão dos genes *GAL* ocorre mesmo que a galactose esteja disponível para a célula, através do mecanismo de repressão pela glicose. A repressão desses genes é realizada pelo repressor transcricional Mig1 que se liga a sequência repressora a montante (URS) dos genes *GAL4* e *GAL1*, resultando na repressão dos genes *GAL* (Keleher et al., 1992; Nehlin et al., 1991; Treitel e Carlson, 1995). Uma vez que a glicose é exaurida do meio, a proteína quinase Snf1 fosforila Mig1, que por sua vez, é exportado do núcleo para o citoplasma, e a célula é liberada do estado de repressão pela glicose (Kayikci e Nielsen, 2015). Sem glicose e na presença de galactose, a expressão dos genes *GAL* é induzida, como mencionado no parágrafo anterior. O mecanismo de repressão pela glicose em leveduras será discutido na seção seguinte.

Do ponto de vista fisiológico, a depender da levedura, a galactose pode ser um açúcar preferencialmente respirável ou respiro-fermentável. Em *S. cerevisiae* CAT-1, a galactose é um açúcar respiro-fermentável, apesar de apresentar produtividade em etanol inferior a outras fontes de carbono, como glicose, frutose, sacarose e maltose (Nascimento e Fonseca, 2019). Em *B. bruxellensis* CBS 2499, a galactose é preferencialmente uma fonte de carbono respirável (Moktaduzzaman

et al., 2015). Apesar disso, tem sido observado que a variação intraespecífica em *B. bruxellensis* quanto a utilização de açúcares não permite concluir que o destino metabólico da galactose seja apenas a respiração.

2.3 REPRESSÃO CATABÓLICA PELA GLICOSE EM LEVEDURAS

A glicose é o açúcar preferencial para a maioria dos organismos. Em *S. cerevisiae*, quando a glicose é fornecida, o metabolismo fermentativo é acionado mesmo na presença de oxigênio. Com isso, genes relacionados à utilização de fontes alternativas de carbono, bem como genes de respiração e gliconeogênese são reprimidos (Rolland et al., 2002). Além da glicose, a frutose e a manose exercem papel semelhante (Broach, 2012). Esse efeito repressor, chamado de efeito da glicose ou repressão catabólica pela glicose (GCR), é transmitido para maquinaria celular por meio de interações regulatórias que são interligadas e por vias de sinalização. A atividade de repressão é coordenada, principalmente, a nível transcricional, mas pode ocorrer também a nível pós-transcricional e pós-traducional (Kayikci e Nielsen, 2015). A glicose pode interferir na atividade de enzimas através da diminuição dos níveis de mRNAs correspondentes, na diminuição da sua taxa de tradução ou aumentando a taxa de degradação da proteína. Com isso, os níveis de mRNAs dependeriam da taxa de transcrição do gene correspondente e da estabilidade do mRNA (Gancedo, 1998).

2.3.1 Detecção e sinalização da glicose

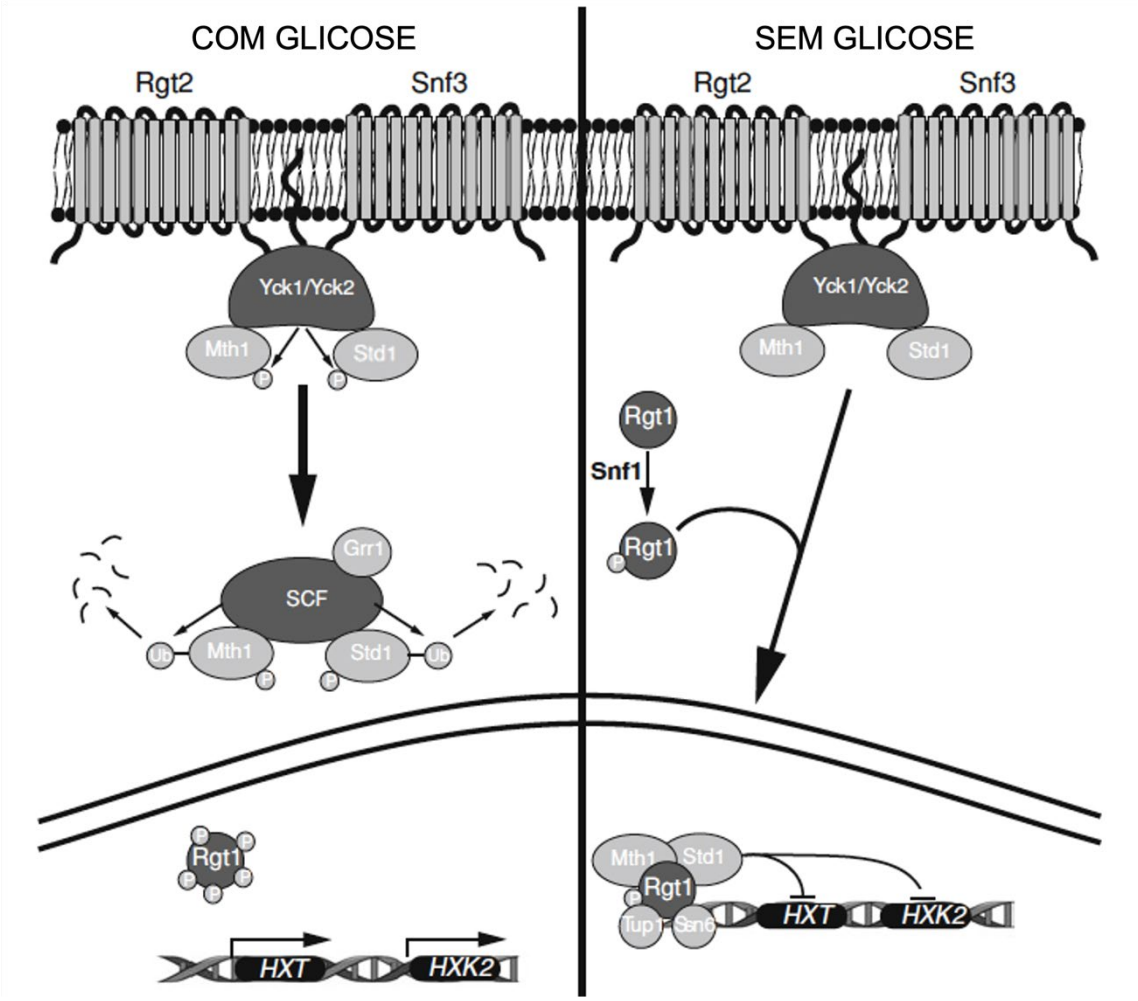
Em *S. cerevisiae*, a glicose é internalizada na célula por transportadores de hexoses codificados por genes da família *HXT* (Boles e Hollenberg, 1997). Esses transportadores apresentam diferentes afinidades e atividades catalíticas, podendo ter nível de expressão diferenciada a depender da concentração de glicose no meio (Ozcan e Johnston, 1995). Os transportadores de glicose de baixa afinidade (*HXT1*, *HXT3* e *HXT5*) são expressos quando a concentração de glicose está situada entre 9 e 100 mM, ao passo que os transportadores de alta afinidade (*HXT2*, *HXT4*, *HXT6*

e *HXT7*) são expressos quando a concentração de glicose é inferior a 5 mM (Reifenberger et al., 1997; Ozcan, 2002; Kayicki e Nielsen, 2015).

A via de sinalização Snf3/Rgt2 (Figura 8) desempenha um papel chave neste controle, pois é por meio dessa via que a célula detecta os níveis da glicose extracelular, e consequentemente regula a captação desse açúcar e ativa o mecanismo de repressão pela glicose (Kayikci e Nielsen, 2015). A proteína Snf3 apresenta alta afinidade, enquanto Rgt2 apresenta baixa afinidade pela glicose extracelular (Peeters e Thevelein, 2014). Ambas as proteínas possuem duas partes funcionais, uma transmembrana que se liga a glicose, e uma larga extensão citoplasmática que desencadeia um sinal intracelular para a maquinaria a jusante (Moriya e Johnston, 2004). O mecanismo preciso de como as proteínas Sfn3 e Rgt2 desencadeiam o sinal da glicose e a transdução para a maquinaria intracelular ainda não é conhecido (Peeters e Thevelein, 2014). Esse sinal parece estar relacionado com a fosforilação de duas outras proteínas transdutoras de sinal, Mth1 e Std1, mediante ação das proteínas caseínas quinases Yck1 e Yck2 (Moriya e Johnston, 2004).

A via de sinalização Snf3/Rgt2 está conectada com outras vias de sinalização. Foi observado que Rgt1 (restores glucose transport) pode atuar como repressor da expressão de *HXK2* (Figura 8). Hxk2 é uma hexoquinase responsável por fosforilar glicose a glicose-6-fosfato, além de ser necessária para repressão da glicose na principal via de repressão da glicose, a chamada Via de Repressão Catabólica (Palomino et al., 2005). A proteína quinase Snf1 (Sucrose nonfermenting), componente central da principal via de repressão, parece estar relacionada com esse processo através da fosforilação de Rgt1. Além disso, existe uma relação entre a principal via de repressão e a via de sinalização da glicose dependente de cAMP, da qual a subunidade catalítica da proteína quinase A (PKA), Tpk3, hiperfosforila Rgt1 e consequentemente alivia a repressão de Rgt1 em *HXK2* (Palomino et al., 2006).

Figura 8 – Via de Sinalização Snf3/Rgt2 em *S. cerevisiae*.

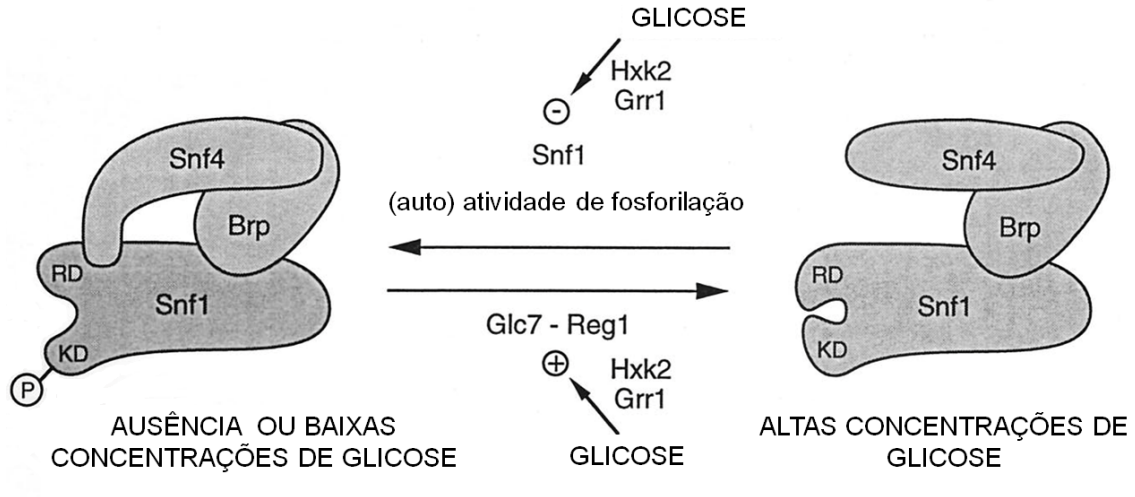


Fonte: adaptado da figura 2.1 de Peeters e Thevelein (2014).

Uma vez que a glicose é exaurida do meio, a célula muda seu metabolismo de fermentação para respiração. A proteína Snf1 apresenta papel chave para mudança do estado de repressão pela glicose para “desrepressão”, por meio da regulação de diferentes repressores e ativadores (Kayikci e Nielsen, 2015). Dependendo da concentração de glicose disponível no ambiente, o regulador Snf1 pode estar ativo ou não. Em níveis de glicose acima da concentração crítica, Snf1 é inativado (sendo removido do núcleo) por auto inibição gerada a partir da interação entre o seu domínio catalítico N-terminal e o domínio regulatório C-terminal (Celenza e Carlson 1989; Jiang e Carlson, 1996; Leech et al., 2003). Isso permite que o principal alvo a jusante, o repressor transcricional Mig1, não seja fosforilado e consequentemente permaneça no núcleo reprimindo a expressão de

seus genes alvo (Piskur e Compagno, 2014; Kayikci e Nielsen, 2015). Por outro lado, em limitação de glicose, a auto inibição de Snf1 é removida pela sua interação com Snf4 (Figura 9) que por sua vez, fosforila Mig1. Mig1 é então exportado do núcleo para o citoplasma, e os genes de utilização de açúcares não preferenciais, como sacarose e galactose, e de fontes de carbono não fermentáveis, como etanol e glicerol, podem ser expressos (Hedbacker e Carlson, 2008; Zaman et al., 2008; Peeters e Thevelein, 2014).

Figura 9 – Modelo para a regulação pela glicose do complexo Snf1 em *S. cerevisiae*. A proteína de ligação (Brp - bridging protein) conecta Snf1 a Snf4.



Fonte: adaptado da figura 3 de Gancedo (1998).

2.3.2 Efeitos transcricionais da glicose no metabolismo do carbono

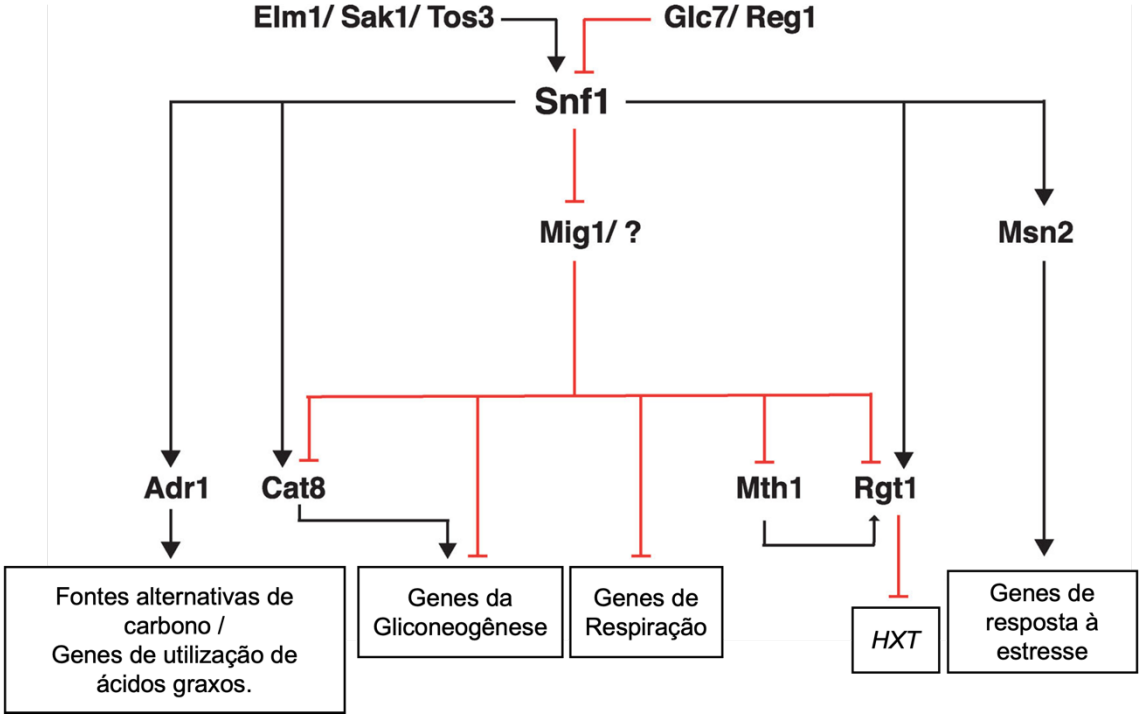
Em *S. cerevisiae*, Snf1 utiliza diferentes mecanismos para regular a expressão de vários genes, seja ativando-os ou reprimindo-os. O principal alvo da Snf1 é Mig1, um repressor transcricional que atua em associação a Hxk2 (Kayikci e Nielsen, 2015). Quando a glicose é abundante (Snf1 está inativa), Hxk2 interage com Mig1 na Ser^{311}, um sítio que também é alvo de fosforilação por Snf1. A ocupação desse sítio por Hxk2 previne a fosforilação por Snf1, e permite que os repressores permaneçam no núcleo (Peláez et al., 2010). Em contrapartida, na ausência ou em baixas concentrações de glicose, Snf1 fosforila Mig1 e Hxk2 (Ser^{14}) e previne a localização nuclear desses repressores. Essa ação evita a ligação dos repressores à região promotora de genes alvo, principalmente de genes de

utilização de fontes alternativas de carbono (Ahuatzi et al., 2004; Fernández-García et al., 2012).

Além de atuar como repressor de Mig1, Snf1 ativa fatores transcricionais tais como Cat8 e Sip4, responsáveis pela indução de genes da gliconeogênese durante o crescimento na ausência de glicose (Figura 10), através da ligação aos elementos responsivos a fonte de carbono, CSRE (Vincent e Carlson, 1999; Roth et al., 2004). Genes chaves da gliconeogênese, bem como genes do ciclo do glioxilato e da utilização de fontes alternativas de carbono, incluindo *FBP1*, *MSL1* e *ICL1*, dependem de Cat8 para regular sua transcrição (Randez-Gil et al., 1997; Tachibana et al., 2007; Biddick et al., 2008; Weinhandl et al., 2014). Na presença de glicose, quando Snf1 está inativo, o ativador transcricional Cat8 é expresso em níveis baixos, e as proteínas que se ligam a sequência ativadora a montante UAS1 e UAS2 do gene *FBP1* não são sintetizadas (Gancedo, 2008).

Desse modo, o complexo Mig1 se liga na sequência repressora a montante e, portanto, não há transcrição do gene *FBP1*. Quando a glicose está ausente, Snf1 está ativa, Mig1 é removido do núcleo e Cat8 é expressa (Gancedo, 2008). Adicionalmente, Snf1 regula a expressão de genes envolvidos na utilização de etanol e na β-oxidação de ácidos graxos através da modulação do fator transcricional Adr1. Assim como Cat8, Adr1 se liga a CSREs para desreprimir a expressão de genes chaves, tal como *ADH2* (Verdone et al., 2002; Tachibana et al., 2005). O gene *ADH2* codifica a enzima álcool desidrogenase 2 necessária para o catabolismo do etanol através da conversão de etanol a acetaldeído. Este gene é susceptível à repressão pela glicose (Walther e Schuller, 2001).

Figura 10 – A proteína Snf1 desempenha papel central na via de repressão pela glicose, atuando tanto na repressão quanto na ativação de fatores transcricionais. Linhas vermelhas representam repressão e linhas pretas a ativação de genes ou fatores transcricionais.



Fonte: adaptado da figura 2 de Kayikci e Nielsen (2015).

2.2.3 Outros efeitos da glicose no metabolismo celular

Quando as células são submetidas a condições desfavoráveis, o balanceamento dos níveis de energia celular é realizado mediante interações dinâmicas de Snf1 tanto em nível pós-transcricional quanto pós-traducional (Kayikci e Nielsen, 2015). Sob limitação de glicose, por exemplo, Snf1 regula negativamente a biossíntese de aminoácidos através da inibição da transcrição e da tradução do ativador transcricional *GCN4* (Broach, 2012). De modo contrário, quando Snf1 está inativo ou ausente (mutantes *snf1*), ocorre a indução de vários genes do metabolismo de aminoácidos que são regulados por Gcn4 (Ljungdahl e Daignan-Fornier, 2012; Dever et al., 2016).

Snf1 também coordena a disponibilidade de carbono e a energia celular por meio da sua influência sobre o metabolismo de lipídios. Durante a limitação de carbono, a célula trabalha em função de economizar energia, e por isso, a síntese de ácidos graxos deve ser minimizada (Klug e Daum, 2014). Quando a célula está

sob deficiência energética, Snf1 fosforila e inativa a enzima acetil-CoA carboxilase (Acc1) que catalisa a carboxilação do acetil-CoA citosólico para a forma malonil-CoA, primeira reação da biossíntese de ácidos graxos (Shi et al., 2014). Além disso, Snf1 promove a geração de energia estimulando a degradação de ácidos graxos por proliferação dos peroxissomos e β-oxidação (Usaite et al., 2009).

Além dos níveis de controle da regulação gênica mencionados anteriormente, Snf1 também atua a nível pós-transcricional, através do decaimento do mRNA de determinados genes (Kayikci e Nielsen, 2015). Embora o controle pós-traducional pela glicose seja raro, a glicose desencadeia a inativação e proteólise de alguns alvos, um efeito denominado de inativação catabólica, em analogia a repressão catabólica (Holzer, 1976; Lucero et al. 2002). Um exemplo de inativação é observado com a proteína frutose-1,6-bifosfatase (FbPase), codificada pelo gene *FBP1*, envolvida na gliconeogênese. A inativação é causada pela rápida fosforilação da FbPase e degradação proteolítica da enzima (Gancedo, 1971; Stein e Chiang, 2014). Dois mecanismos alternativos para proteólise dessa enzima foram descritos: o primeiro envolve a transferência da FbPase para o vacúolo e consecutiva degradação pelas proteases vacuolares (Chiang e Schekman, 1991; Shieh et al., 2001) e, o segundo envolve a ubiquitinação de FbPase e posterior degradação no proteassomo (Schork et al., 1994, 1995). A degradação no vacúolo requer a fosforilação da glicose, mas pode ocorrer na ausência de Hxk1 ou Hxk2 (Hung et al. 2004; Belinchón e Gancedo, 2007b), enquanto a degradação no proteassomo é dependente da ação de Hxk2 (Horak et al., 2002).

Embora o mecanismo de repressão pela glicose seja bem descrito em *S. cerevisiae*, pouco se sabe sobre a influência da glicose no metabolismo de diferentes açúcares em *B. bruxellensis*. Em *B. bruxellensis* GDB 248, a glicose está envolvida na repressão dos genes *FBP1* (frutose-1,6-bifosfatase) e *NTH1* (trealase neutra), assim como ocorre em *S. cerevisiae* (Leite et al., 2016; Stenger et al., 2020). Além disso, a glicose reprime os genes envolvidos na utilização da galactose em *B. bruxellensis* CBS 2499 (Moktaduzzaman et al., 2015). Apesar disso, o modo como diferentes linhagens respondem a presença de glicose e outras fontes alternativas ainda não é conhecido, devendo ser mais amplamente explorado em *B. bruxellensis*.

2.4 *B. BRUXELLENSIS* NO CONTEXTO DA PRODUÇÃO DE ETANOL COMBUSTÍVEL

2.4.1 Produção de etanol combustível

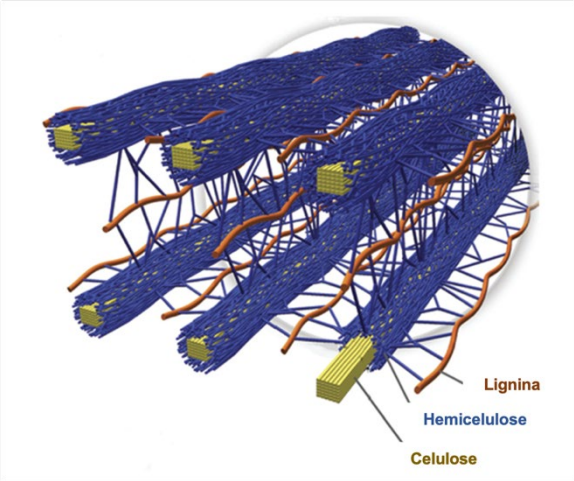
O sistema energético internacional é estritamente dependente de combustíveis fósseis, tais como o petróleo, carvão e gás, visto que cerca de 80% do consumo mundial de energia é oriundo dessas fontes (Ogeda e Petri, 2010). A procura por fontes renováveis de energia como alternativa ao uso de combustíveis fósseis, em conjunto com a conscientização e preservação ambiental promoveram o desenvolvimento dos biocombustíveis (Rosa e Garcia, 2009). Esse crescente interesse não é resultado apenas do esgotamento gradativo das reservas de combustíveis fósseis, mas também pelos efeitos negativos que eles causam ao meio ambiente (Vohra et al., 2014; Cunha et al., 2020). Por exemplo, a produção de etanol a partir da cana-de-açúcar apresenta taxa de redução entre 40 e 62% na emissão de gases do efeito estufa, em comparação com a gasolina (Wang et al., 2012).

O bioetanol é classificado de acordo com o substrato utilizado para a sua obtenção. O etanol de primeira geração envolve, principalmente, a fermentação de açúcares presentes no caldo da cana-de-açúcar (Brasil), bem como no milho (Estados Unidos), e na beterraba (Europa) (Amorim e Lopes, 2009). Atualmente, os EUA e o Brasil são os principais produtores de bioetanol, representando cerca de 85% da produção mundial (Bertrand et al., 2016; Jacobus et al., 2021). Em contrapartida, o etanol de segunda geração é resultante da hidrólise de biomassa lignocelulósica para geração de açúcares que são fermentados a etanol por micro-organismos, principalmente leveduras, um processo relativamente custoso (Robak e Balcerek, 2018). Adicionalmente, o etanol de terceira geração é obtido por meio da biomassa de micro-organismos, em especial microalgas, visto sua capacidade de armazenar carboidratos que podem ser utilizados para produzir o bioetanol (Tsigie et al., 2013; Silva e da Silva, 2019). A seguir, iremos focar na produção de etanol de segunda geração.

2.4.2 Produção de etanol de segunda geração

A biomassa vegetal é uma fonte renovável que apresenta alto potencial para geração de bioenergia (Guilherme et al., 2019). A biomassa lignocelulósica, por sua vez, é formada por celulose, hemicelulose e lignina (Figura 11), materiais que compõem as paredes celulares de plantas lenhosas, como árvores, arbustos e gramíneas (Brandt et al., 2013). No Brasil, o principal substrato para produção de etanol de segunda geração é o bagaço da cana-de-açúcar, através de etapas de pré-tratamento, hidrólise e fermentação (Guilherme et al., 2019). O pré-tratamento atua desorganizando a matriz lignocelulósica, reduzindo a quantidade de lignina e hemicelulose, e modificando a estrutura da celulose para torná-la mais propícia à hidrólise (Silverstein et al., 2007; Canilha et al., 2012). A hidrólise atua na conversão de celulose a moléculas de glicose, entretanto a hidrólise incompleta desse polímero resulta em celobiose (dissacarídeo formado por duas moléculas de glicose), ao passo que a quebra da hemicelulose gera D-glicose, D-manose, D-galactose, D-xilose e L-arabinose (Goldemberg, 2013; Cunha et al., 2020). A lignina presente na biomassa é utilizada para gerar eletricidade e calor para as biorrefinarias (Gamage et al., 2010).

Figura 11 – Arranjo espacial da biomassa lignocelulósica na parede celular vegetal.



Fonte: adaptado da figura 2 de Brandt et al. (2013).

Durante as etapas de pré-tratamento e hidrólise, inibidores do crescimento microbiano e da fermentação são gerados, o que inclui furanos (furfural e 5-

hidroximetilfurfural), ácidos carboxílicos (ácido acético) e compostos fenólicos (aldeídos, cetonas, ácidos p-cumárico e ferúlico) (Bušić et al., 2018). Este último interfere na função e na integridade da membrana celular (Palmqvist e Hahn-Hagerdal, 2000). Por outro lado, a etapa de fermentação envolve o emprego de micro-organismos capazes de realizar a fermentação alcoólica a partir da mistura de açúcares gerados durante a hidrólise (Robak e Balcerek, 2018). Para isso, os micro-organismos utilizados precisam atender a um conjunto de características chaves, tais como resistência aos inibidores gerados na hidrólise, a pH ácido, altas concentrações de etanol, bem como fermentar de maneira eficiente a mistura de açúcares disponíveis (Dien at al., 2003; Davison et al., 2016; Robak e Balcerek, 2018; Gao et al. 2019).

2.4.3 Características relevantes de *B. bruxellensis* para a indústria de fermentação alcoólica

A alta capacidade adaptativa de *B. bruxellensis* tem motivado diversos estudos com o objetivo de melhor compreender e explorar aspectos fisiológicos e genéticos que resultam na robustez metabólica de *B. bruxellensis*, visto que a exclusão dessa espécie nas plantas de etanol tem se mostrado improvável. Esses estudos incluem desde a capacidade de assimilar diversas fontes de carbono (Crauwels et al., 2015) e nitrogênio (de Barros Pita et al., 2011; Peña-Moreno et al., 2019), bem como a resposta a estresses associados ao processo fermentativo (Mukherjee et al., 2017). Adicionalmente, dados recentes comprovam que isolados dessa espécie tem a habilidade de produzir etanol com rendimentos atrativos e próximos aos apresentados por *S. cerevisiae* (Teles et al., 2018; Peña-Moreno et al., 2019; de Barros Pita et al., 2019). Além disso, apesar de serem consideradas parentes distantes, *B. bruxellensis* e *S. cerevisiae* compartilham diferentes aspectos fisiológicos, tais como anaerobiose facultativa, tolerância a etanol e o efeito *Crabtree* positivo (Rozpędowska et al., 2011).

Apesar do caráter contaminante, *B. bruxellensis* é considerada uma levedura promissora no contexto da produção de etanol de segunda geração, devido a capacidade de metabolizar açúcares resultantes da hidrólise do material lignocelulósico (Reis et al., 2014; Godoy et al., 2017; Codato et al., 2018; de Barros Pita et al., 2019). Além disso, a sua capacidade em resistir aos inibidores gerados

durante a hidrólise (Blomqvist et al., 2011; Tiukova et al., 2014), a ácidos fracos (Moktaduzzaman et al., 2015), a baixos valores de pH e a altas concentrações de etanol são características de interesse industrial (Rozpędowska et al., 2011; Bassi et al., 2013). Entretanto, apesar de *B. bruxellensis* apresentar um conjunto de características chaves para esse processo, pouco ainda é conhecido sobre sua capacidade fermentativa, por exemplo, quando em meio contendo D-xilose, galactose ou com uma mistura de diferentes açúcares oriundos da hidrólise do bagaço de cana de açúcar.

Nesse contexto, a presente tese de doutorado propõe um conjunto de ensaios fisiológicos e genéticos para investigar a capacidade de assimilação e fermentação de *B. bruxellensis* em diferentes açúcares encontrados em substratos industriais, tais como, glicose, sacarose, celobiose, xilose, arabinose e galactose. Os dados descritos nas próximas seções revelam que *B. bruxellensis* possui a capacidade de assimilar uma ampla gama de açúcares e de fermentar, em condições específicas, galactose e xilose. Além disso, *B. bruxellensis* (linhagem JP19M) é capaz de consumir simultaneamente outros açúcares ainda que na presença de glicose, uma característica linhagem-dependente rara em leveduras. Nossos dados sugerem que a linhagem JP19M de *B. bruxellensis* possui potencial de aplicação na produção de etanol de segunda geração, uma vez que o problema da eficiência de fermentação de xilose seja contornado.

3 OBJETIVOS

3.1 GERAL

Investigar o potencial de utilização de *B. bruxellensis* na indústria de fermentação alcoólica, avaliando os aspectos fisiológicos e genéticos do metabolismo e da sua capacidade fermentativa de açúcares utilizados como substratos para a produção de bioetanol.

3.2 ESPECÍFICOS

- Determinar a capacidade de assimilação de açúcares de interesse biotecnológico em diferentes linhagens industriais da levedura *B. bruxellensis*.
- Avaliar a influência do oxigênio no metabolismo e na capacidade fermentativa de *B. bruxellensis* em presença de xilose e arabinose, pentoses derivadas do material lignocelulósico.
- Investigar a influência da Repressão Catabólica pela Glicose na linhagem JP19M e seu efeito sobre a expressão de genes do metabolismo central do carbono e da xilose em *B. bruxellensis*.
- Determinar a capacidade fermentativa de *B. bruxellensis* em galactose, bem como os perfis transcricionais de genes envolvidos na fermentação e fosforilação oxidativa.

4 ARTIGO 1 - BIOLOGICAL DIVERSITY OF CARBON ASSIMILATION AMONG ISOLATES OF THE YEAST *DEKKERA BRUXELLENSIS* FROM WINE AND FUEL-ETHANOL INDUSTRIAL PROCESSES

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Abstract

Dekkera bruxellensis is considered a spoilage yeast in winemaking, brewing and fuel-ethanol production. However, there is growing evidence in the literature of its biotechnological potential. In this work, we surveyed 29 *D. bruxellensis* isolates from three countries and two different industrial origins (winemaking and fuel-ethanol production) for the metabolization of industrially relevant sugars. The isolates were characterized by the determination of their maximum specific growth rates, and by testing their ability to grow in the presence of 2-deoxy-D-glucose and antimycin A. Great diversity was observed among the isolates, with fuel-ethanol isolates showing overall higher specific growth rates than wine isolates. Preferences for galactose (three wine isolates) and for cellobiose or lactose (some fuel-ethanol isolates) were observed. Fuel-ethanol isolates were less sensitive than wine isolates to GCR induction by 2-deoxy-D-glucose. In strictly anaerobic conditions, isolates selected for having high aerobic growth rates were able to ferment glucose, sucrose, and cellobiose at fairly high rates without supplementation of casamino acids or yeast extract in the culture medium. The phenotypic diversity found among wine and fuel-ethanol isolates suggest adaptation to these environments. A possible application of some of the GCR-insensitive, fast-growing isolates in industrial processes requiring co-assimilation of different sugars is considered.

Keywords: anaerobic cultivation; carbon assimilation; *Crabtree* effect; disaccharides; glucose catabolite repression; selective adaptation

Introduction

As a member of the Saccharomycetaceae yeast family, *Dekkera bruxellensis* is a distant relative of the yeast *Saccharomyces cerevisiae* and they both share a physiological trait related to the capacity to convert sugars into ethanol even in the presence of oxygen, the '*Crabtree* effect'. It seems that this characteristic was acquired as a consequence of convergent evolution after the splitting of both lineages approximately 200 My ago, as part of a life strategy called 'make-accumulate-consume' (Rozpedowska *et al.* 2011). This strategy consists in the fast conversion of sugars into two-carbon molecules ('make'), which are produced even in the presence of oxygen ('accumulate'), and can be further catabolized as a carbon and energy source ('consume') when sugar becomes scarce.

It has been reported that strains of *Dekkera bruxellensis* can assimilate a variety of sugars including glucose, fructose, galactose, maltose, sucrose and trehalose (Conterno *et al*. 2006; Leite e*t al.* 2013; Crauwels *et al*. 2015). In addition, this yeast can consume and ferment cellobiose, although less efficiently than other disaccharides (Reis *et al.* 2014, 2016). However, it seems that the assimilation of cellobiose is strain-dependent, which is probably due to the huge genetic variability found among isolates from different industrial processes for wine or beer (Hellborg & Piskur *et al*. 2009; Vigentini *et al.* 2013). Historically, the anamorph *Brettanomyces bruxellensis* has been considered the most important spoilage agent in wineries, affecting the fermentation process or the product storage (Sangorrín *et al.* 2013). On the other hand, its presence is essential for the fermentation of Lambic beers. Whether the intraspecific variation of *Dekkera bruxellensis* is relevant to its adaptation to different industrial niches is still a matter of debate, but it may certainly influence its potential use as industrial yeast (Galafassi *et al*. 2011).

Two important aspects must be taken into consideration regarding the industrial use of this yeast. First, it is desirable that the yeast be able to efficiently utilize all the sugars present in the fermentation wort, which ultimately implies the ability for the co-assimilation of such components. It is well known that glucose is a preferential carbon source (C-source), and it exerts great influence on the assimilation of any other C-source in the wort by inducing the Glucose Catabolite Repression (GCR) regulatory mechanism, triggered by the intracellular

accumulation of glucose 6-phosphate and ultimately fructose 1,6-bisphosphate (Peeters *et al*. 2017). Whenever glucose is present in the medium in concentrations exceeding 5 mmol L^{-1}, GCR works by diminishing or preventing the use of other C-sources. The mechanism entails the repression of a set of genes and/or the allosteric inhibition of catabolic enzymes (Alexander and Jeffries 1990). Recently, we proposed that the GCR mechanism in *D. bruxellensis* is less tightly controlled than in *S. cerevisiae* (Leite *et al*. 2016). An easy way to study the influence of GCR on the assimilation of different C-sources is the use of 2-deoxy-D-glucose (2-DG), an analog of glucose that cannot be isomerized to fructose and therefore is not further metabolized in glycolysis. The phosphorylated form 2-DG-6P accumulates in the cells and keeps them in a permanent state of glucose repression (Pelicano *et al.* 2006; O'Donnell *et al*. 2015). In *S. cerevisiae* cells, the presence of 2-DG drastically reduces or blocks the assimilation of various C-sources, including disaccharides, or respirable C-sources like glycerol or ethanol (McCartney *et al*. 2014). 2-DG also works as a GCR inducer for these respirable C-sources in *D. bruxellensis* (Leite *et al*. 2016). The mechanism mediating the resistance or sensitivity of the yeast cells to 2-DG is related to the activation of the GCR pathway through the action of the regulatory proteins Snf1p and Mig1p (McCartney *et al*. 2014). Therefore, the genetic diversity of *D. bruxellensis* can be explored by using 2-DG to screen for '2-DG-resistant', GCR-relieved cells capable of co-assimilating different sugars.

A second aspect relevant to the industrial use of *D. bruxellensis* is the ability to ferment a certain sugar in strictly anaerobic conditions. This can be tested by cultivating the cells in the presence of the respiration inhibitor antimycin A (AA), a compound produced by *Streptomyces* bacteria that binds to cytochrome C reductase and inhibits the oxidation of ubiquinone at site III of the respiratory chain, resulting in blockage of respiration (Turrens 1997). Therefore, cells capable of growing exclusively by fermentation on a given C-source are tolerant to the toxic effects of AA.

D. bruxellensis has received increasing attention for its biotechnological potential in a variety of fermentation processes, because of its adaptability to industrial environments and its ability to grow in strictly anaerobic conditions, a rare feature among yeasts. In the present work, 29 isolates of *D. bruxellensis* from wine and fuel-ethanol industries were screened on several substrates of

industrial relevance in order to select strains with good industrial aptitude, that is, having the following three features: high growth rate in aerobiosis, ability of assimilating different C-sources and capacity of fermenting different sugars in strictly anaerobic conditions.

Material and Methods

Strains and maintenance

Twenty-nine isolates of *Dekkera bruxellensis* were used in this study, of which ten were isolated from fuel-ethanol fermentation processes in Brazil and 19 were isolated from wineries in Brazil, Argentina, and Chile (Table 1). All 29 isolates were identified by molecular analysis, as described by Basilio *et al*. (2008) and Silva *et al*. (2016). *Dekkera bruxellensis* GDB 248 (De Souza Liberal *et al.* 2007) and *Saccharomyces cerevisiae* JP1 (da Silva Filho *et al*. 2005), which were also isolated in a Brazilian fuel-ethanol industry, were used as reference strains in this work. Cells were freshly maintained in YPD medium (yeast extract 10 g L^{-1}; peptone 20 g L^{-1}; glucose 20 g L^{-1}; agar 20 g L^{-1}).

Table 1 – Isolates of *Dekkera bruxellensis* used in this study. BR: Brazil; AR: Argentina; CH: Chile

Isolate	Process	Localization	Year of isolation
GDB 248*	Fuel-ethanol	Paraíba—BR	2006
JP249A	Fuel-ethanol	Paraíba—BR	2013
TB457A	Fuel-ethanol	Paraíba—BR	2014
JP258A	Fuel-ethanol	Paraíba—BR	2013
JP19M	Fuel-ethanol	Paraíba—BR	2013
JP206M	Fuel-ethanol	Paraíba—BR	2013
JP287V	Fuel-ethanol	Paraíba—BR	2014
JP184V	Fuel-ethanol	Paraíba—BR	2014
TB259V	Fuel-ethanol	Paraíba—BR	2013
TB283V	Fuel-ethanol	Paraíba—BR	2014
L1359	Wine	Mendoza—AR	2003
L2480	Wine	Maypo Valley—CH	2005
L2552	Wine	Maypo Valley—CH	2005
L1400	Wine	Maule Valley—CH	1999
MRC181a	Wine	Santa Catarina—BR	2011
MRC180a	Wine	Santa Catarina—BR	2011
MRC172a	Wine	Santa Catarina—BR	2011
MRC177a	Wine	Santa Catarina—BR	2011
MRC140a	Wine	Santa Catarina—BR	2011
MRC190b	Wine	Rio Grande do Sul—BR	2011
MRC172b	Wine	Rio Grande do Sul—BR	2011
MRC178b	Wine	Rio Grande do Sul—BR	2011
MRC117b	Wine	Rio Grande do Sul—BR	2011
MRC78b	Wine	Rio Grande do Sul—BR	2011
MRC86b	Wine	Rio Grande do Sul—BR	2011
MRC180b	Wine	Rio Grande do Sul—BR	2011
MRC177b	Wine	Rio Grande do Sul—BR	2011
MRC181b	Wine	Rio Grande do Sul—BR	2011
MRC80b	Wine	Rio Grande do Sul—BR	2011

*Reference strain.

Aerobic micro-cultures

Aerobic micro-culture experiments were carried out in a Synergy HTX microplate reader (Biotek, Switzerland) using 96-well microplates incubated at maximal agitation and 30ºC. The micro-cultures were performed in synthetic medium YNB w/o amino acids (1.7 g L^{-1}) supplemented with ammonium sulphate (75 mM) and a carbon source (glucose, fructose, galactose, maltose, cellobiose, sucrose or lactose) at a concentration of 20 g L^{-1}. Cells of the *D. bruxellensis* isolates were pre-cultivated in YPD medium at 160 rpm and 30ºC. After 48 h, cells were collected by centrifugation, washed with sterile distilled water and suspended in sterile saline solution (NaCl 9 g L^{-1}) to an optical density of 0.1 units measured at 600 nm (OD600). In each well of the microtiter plate, 10 µL of the cell suspension were inoculated in 140 µL of the synthetic medium. Measurements of the optical density of each well were taken automatically every 30 minutes. Experiments were performed in triplicate.

Anaerobic micro-cultures

Anaerobic micro-cultures were carried out in a Biolector NA micro-fermenter (m2p Co., Germany) using an anaerobic chamber. Cells were pre-cultivated aerobically in YPD medium at 160 rpm and 30ºC for 24 h. Three mL of culture were collected, centrifuged, washed in saline solution and resuspended in 10 mL of synthetic YNB medium containing 20 g L^{-1} of glucose, sucrose or cellobiose and cultivated aerobically for another 48 h. For the anaerobic micro-culture experiments, 48-well Biolector flower-plates™ were filled with 1.4 mL of synthetic medium YNB containing glucose, sucrose or cellobiose as carbon sources and supplemented with 420 mg L^{-1} Tween 80 and 10 mg L^{-1} ergosterol (Parente *et al.* 2017). Each well was inoculated with 100 µL of the cell suspension pre-cultivated in the same carbon source. The plate was placed inside an anaerobic mini-chamber sparged with a constant flux of gaseous nitrogen (99.99% purity) and incubated at 800 rpm and 30ºC for 72h. Automatic readings of light scattering were recorded every 30 minutes and converted to optical density values (OD600) by means of a calibration curve. Experiments were performed in triplicate.

Maximum specific growth rates

The OD600 data recorded every 30 min from each aerobic or anaerobic micro-culture were plotted as ln(OD600) versus time, and the linear portion of the curve was identified by visual inspection. A linear regression was performed with the experimental points of that portion, and the maximum specific growth rate was calculated as the slope of the regression line, as exemplified in Supplementary Figure S1. Typically, the duration of the exponential growth phase was 6-8 h (12-16 experimental points). For each growth condition, the maximum specific growth rate reported in this work is the mean value of the maximum specific rates calculated with data from three independent experiments.

Significance test

The non-parametric Mann-Whitney U test was used to compare differences between the maximum specific growth rates of the fuel-ethanol and the wine groups of isolates. The test was performed using the STATISTICA software (StatSoft Inc., USA).

Principal Component Analysis

The maximum specific growth rate dataset from the aerobic micro-cultures of the 29 *D. bruxellensis* isolates on seven carbon sources was preprocessed by autoscaling: for each carbon source, the mean value of the specific growth rates was subtracted from each isolate's specific growth rate, and the result was divided by the standard deviation of the mean. The autoscaled dataset was then submitted to Principal Component Analysis using the PLS_Toolbox software (Eigenvector Research Inc., USA) which works within MATLAB® environment.

Plate tests in the presence of inhibitors

Cells of the *D. bruxellensis* isolates grown in YPD plates were inoculated in 1 mL YPD medium and incubated at 160 rpm and 30^{o}C. After six hours of incubation, cells were collected, washed and resuspended in saline solution. Five µL of each cell suspension were dropped onto three YNB Petri plates containing 20 g L^{-1} of a C-source, and either 1 µM Antimycin A (Sigma-Aldrich), 10 mM 2-deoxy-D-glucose (Sigma-Aldrich) or none of the inhibitors (reference plate). The carbon sources tested were glucose, fructose, galactose, maltose, cellobiose,

and sucrose. The plates were incubated for 72 hours at 30ºC. Relative growth was classified as ‘unaffected’ (growth similar to the reference condition), ‘reduced‘ or ’impaired’ (absence of growth).

Results and Discussion

Aerobic assimilation of mono- and disaccharides

D. bruxellensis isolates collected from wine fermentation in Southern Brazil, Chile and Argentina and from fuel-ethanol fermentation in North-eastern Brazil were tested for assimilation of disaccharides of industrial relevance, such as sucrose (for wine and first-generation ethanol), cellobiose (for second-generation ethanol), maltose (for beer) and lactose (for milk whey), as well as their constituent monomers glucose, fructose and galactose. The growth of the 29 *D. bruxellensis* isolates on each sugar was registered for 72 h by a microplate reader. A great diversity of growth profiles was found among the isolates (Supplementary Figure S1 available online). Only about half of the isolates were able to grow on all seven carbohydrates. Glucose, fructose, sucrose and maltose were the only sugars assimilated by all isolates.

The maximum specific growth rates of the 29 *D. bruxellensis* isolates were calculated from the growth curves on each carbon source (Supplementary Table S1 available online). For all sugars tested the growth rates of the fuel-ethanol isolates are significantly higher than the wine isolates (Table 2). The specific growth rate dataset was then submitted to principal component analysis (PCA) in order to get more insight into the differences among the *D. bruxellensis* isolates that could be ascribable to their sources of isolation. PCA is an exploratory technique of analysis that uses maximum variance to describe the dataset in a new space with reduced dimensionality. The original variables are combined in a linear way, leading to a new variable (Principal Component, PC) corresponding to a weight-average of the original ones. The method searches for a set of optimal weights (loadings) that will lead to a combined variable retaining most of the relevant information. In other words, PCA can be seen as a modeling activity, in which the model is the weight-averaged variable that best explains the variation in the whole dataset (Bro & Smilde, 2014). Principal components represent

sources of variation in the dataset, in which the first component explains the greater variability of the data, followed by the second, and so on.

Table 2 – Results of the Mann-Whitney U test for the comparison of maximum specific growth rates of fuel-ethanol and wine isolates of *D. bruxellensis* cultivated on different carbohydrates.

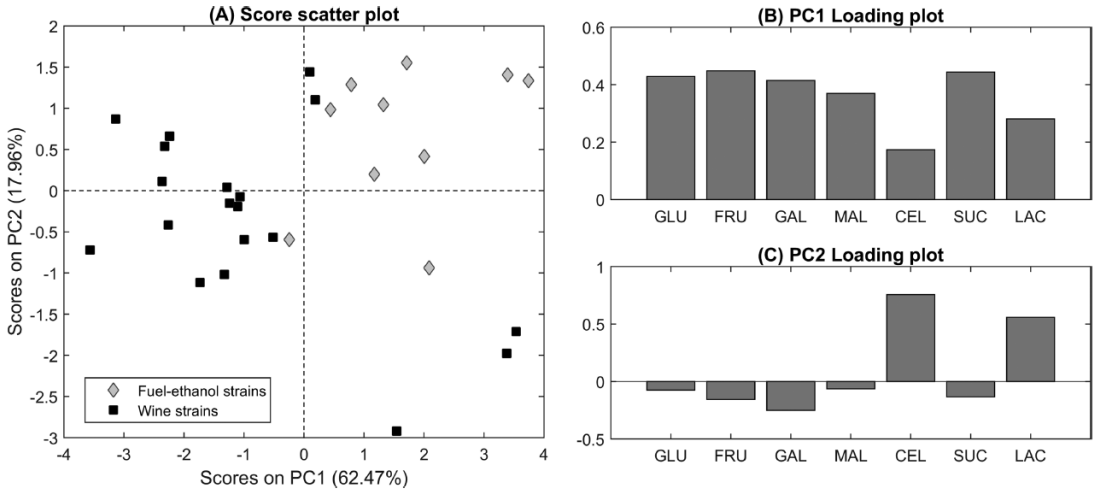
	Mean value of the maximum specific growth rates (h^{-1})		
C-source	Fuel-ethanol strains	Wine strains	P-value*
Glucose	0.234	0.114	0.004
Fructose	0.215	0.123	0.002
Galactose	0.126	0.088	0.040
Maltose	0.152	0.097	0.040
Cellobiose	0.134	0.067	0.000
Sucrose	0.202	0.108	0.002
Lactose	0.082	0.037	0.004

*Differences are significant at P-value <0.05.

As shown in Figure 1A, the first principal component (PC1) of the dataset explains 62.5% of the variation among the isolates, and it segregates fuel-ethanol and wine isolates in two almost distinct groups along the horizontal axis. Most of the fuel-ethanol isolates have positive scores on PC1 and hence, will have fairly high values on C-sources that have positive loadings in Figure 1B. Thus, for all C-sources, the fuel-ethanol isolates tend to have higher specific growth rates than the wine isolates, as indicated by the significance test in Table 2. However, three of the highest positive scores in PC1 (on the right-hand side of Figure 1A) are from wine strains, which is not evidenced by the univariate analysis.

The second principal component (PC2) explains 18% of the variation in the dataset (Figure 1A, vertical axis). It shows a moderate segregation of the fuel-ethanol isolates from the wine isolates, including the three wine isolates that have grouped with the fuel-ethanol isolates in PC1. The growth rates on cellobiose, lactose and galactose are the most relevant for the second component (Figure 1C). Again, positive loadings of cellobiose and lactose on PC2 show a tendency of fuel-ethanol isolates (most of which have positive scores) to grow faster than most of the wine isolates on these sugars. Galactose, however, has a negative loading on PC2, which is mainly due to the three wine isolates mentioned before, which grow on galactose faster than any other isolate.

Figure 1. Principal component analysis for the maximum specific growth rates of *D. bruxellensis* industrial isolates on different carbon sources. (A) Score scatter plot for PC1 and PC2, (B) loading plot of PC1 and (C) loading plot of PC2. Squares: wine isolates; diamonds: fuel-ethanol isolates. GLU: glucose; FRU; fructose; GAL; galactose; MAL; maltose; CEL: cellobiose; SUC: sucrose; LAC: lactose.



Differential ability to assimilate these sugars may reflect the adaptation to an ecological niche. β-glucosidase activity is required for assimilation of cellobiose, and it seems to be a common feature among fuel-ethanol isolates of *D. bruxellensis* (Reis *et al.* 2014; Reis *et al*. 2016). A higher conservation of this phenotype among isolates from the fuel-ethanol industry may indicate that at least trace amounts of cellobiose are released during sugarcane processing. Even in small amounts, the presence of cellobiose in these industrial broths may represent an ecological advantage for *D. bruxellensis* over *S. cerevisiae*, which is unable to assimilate it. Similarly, maceration of grapes in winemaking may induce glycosyl-hydrolases that break down pectin into galactose, as well as rhamnose, xylose and arabinose (Uenojo and Pastore 2007), although sucrose and fructose are still the most abundant sugars in grape musts (Nadal *et al*. 1999). Therefore, wine isolates are in permanent contact with galactose, which is not the case for fuel-ethanol isolates.

The maximum specific growth rates of the reference strain *D. bruxellensis* GDB 248 were calculated as 0.17 h^{-1} on sucrose, 0.16 h^{-1} on maltose, 0.14 h^{-1} for glucose and fructose, 0.05 h^{-1} on galactose, 0.06 h^{-1} on cellobiose and 0.04 h^{-1} on lactose. These values were similar to those reported by Leite *et al.* (2013) for the same strain and are used herein as reference values for comparison with

the industrial isolates. Some isolates stood out for presenting a very short or even absent lag phase and for having high specific growth rates in comparison to the reference strain GDB 248. The growth kinetics of these isolates are shown in Figs. 2 and 3. Among them, we highlight the performance of the Brazilian fuel-ethanol JP19M isolate, which grows at 0.28 h^{-1} on sucrose, 0.32 h^{-1} on glucose and 0.30 h^{-1} on fructose (Figs. 2A, 3A and 3B, respectively). On galactose, the maximum specific growth rates of the Chilean wine isolates L2552, L1400 and MRC 177b are the highest among all isolates, with values of 0.21-0.22 h^{-1} which are four times higher than the reference strain GDB 248 (Fig. 3C and Supplementary Table S1 available online) and three times higher than the type strain CBS 2499 (Moktaduzzaman *et al.* 2014). On maltose, two Brazilian fuel-ethanol isolates (JP258A and JP287V) and two Chilean wine isolates (L2552 and L1400) did not show lag phase and have the highest growth rates (Fig. 2B). On cellobiose, the Brazilian fuel-ethanol isolate TB283V had the highest specific growth rate (0.21 h^{-1}; Fig. 2C and Supplementary Table S1 available online), whereas most of the wine strains grew poorly on this sugar.

Lactose was hardly assimilated by wine isolates in the present study (Fig. 2D and Fig. S1 available online), even by those that were able to metabolize galactose efficiently (*e.g.* L2552 and L1400). However, a few fuel-ethanol isolates grew on lactose with a fairly high growth rate of 0.14-0.15 h^{-1} (*e.g.* JP206M and JP19M in Fig. 2D). *Dekkera bruxellensis* is generally considered as lactose-negative in the handbooks of yeast taxonomy, with some isolates showing faint growth on this sugar, including the reference strain GDB 248 (Leite *et al.*, 2013; Conterno *et al.*, 2006). Several hypotheses can be raised to explain the clearly positive phenotype for growth on lactose shown by some isolates in the present study. One of them is a horizontal transfer of a beta-galactosidase gene, as has been reported for some nutrient transporters in yeast (Coelho *et al*. 2013; Marsit *et al*. 2015).

Figure 2. Aerobic growth kinetics of *D. bruxellensis* isolates in synthetic medium containing one of the disaccharides sucrose (A), maltose (B), cellobiose (C) or lactose (D). Fuel-ethanol isolates: GDB 248 (open squares), JP 19M (open circles), TB 457A (closed triangles), TB 283V (grey circles), JP 287V (closed diamonds), TB 259V (grey triangles), JP 206M (grey squares), JP 258A (open triangles). Wine isolates: L 2552 (closed squares), L 1400 (open diamonds).

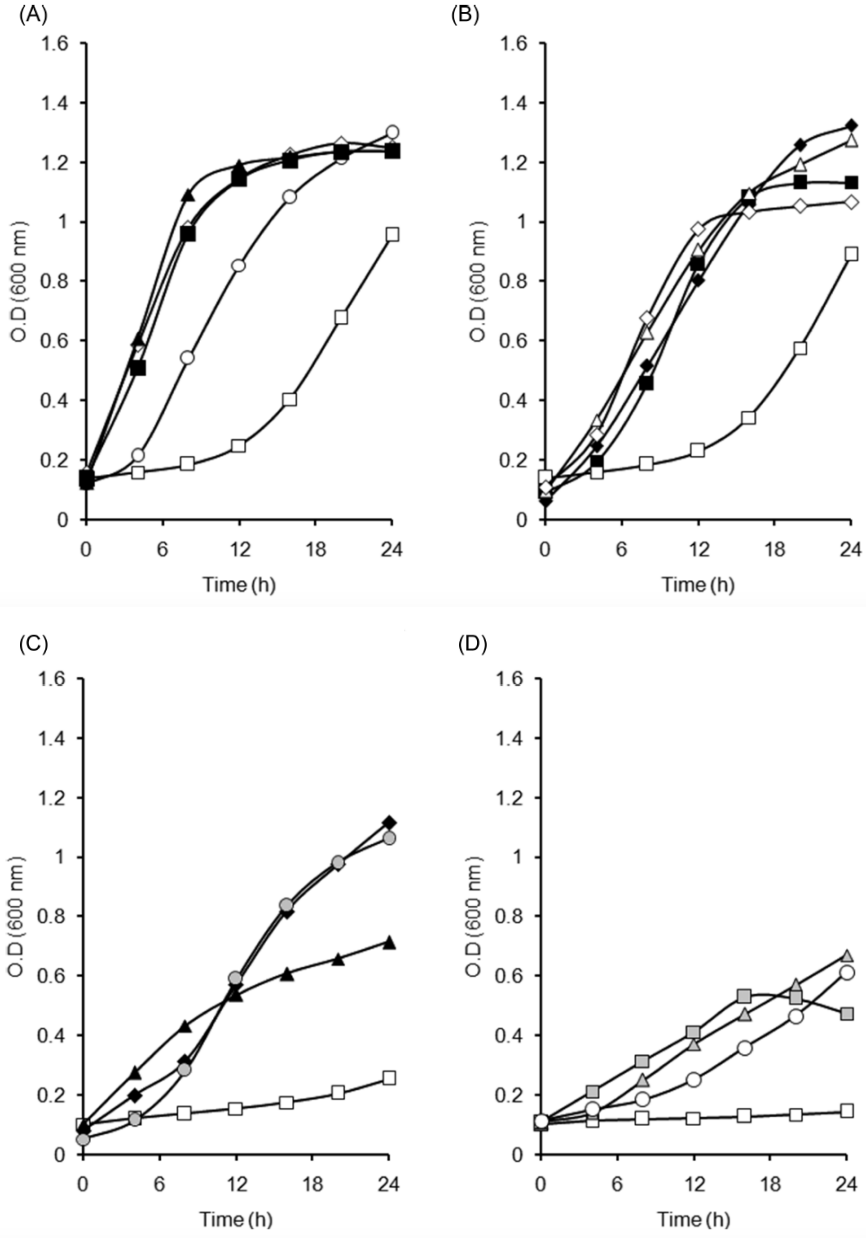
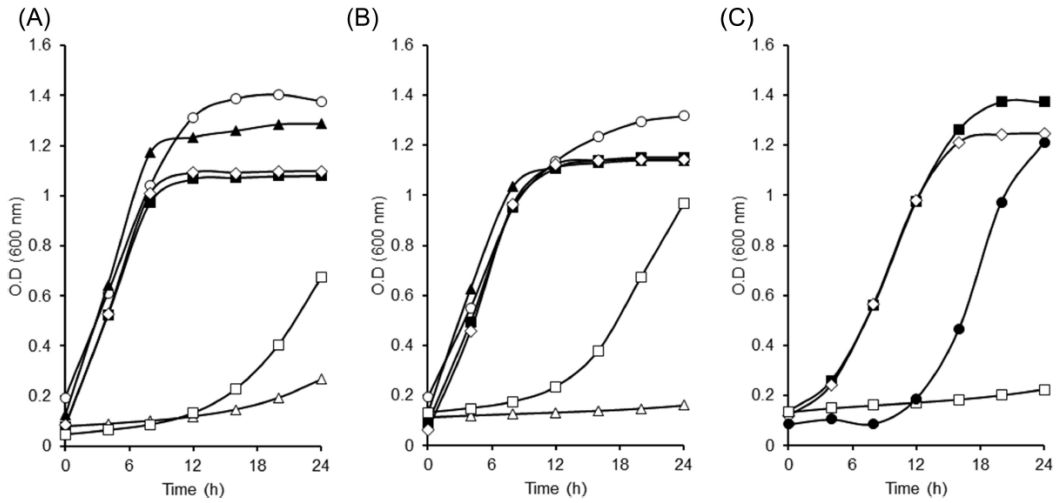


Figure 3. Aerobic growth kinetics of *D. bruxellensis* isolates in synthetic medium containing one of the monosaccharides glucose (A), fructose (B) or galactose (C). Fuel-ethanol isolates: reference strain GDB 248 (open squares); JP 19M (open circles); TB 457A (closed triangles). Wine isolates: L 2552 (closed squarse); L 1400 (open diamonds); MRC 177b (closed circles); MRC 140 (open triangles).



The ability of *Brettanomyces bruxellensis* to assimilate different carbon sources has been investigated by Crauwels *et al.* (2015). Seven strains isolated from different industrial processes of soft drink (one), beer (four) and wine (two) were tested for growth on several carbon sources, including monosaccharides, disaccharides, and polysaccharides. Regarding cellobiose and galactose, the ability to assimilate these sugars was quite segregated according to the origin of the strains, with only one of the beer isolates being able to assimilate galactose, whereas only the soft drink and wine isolates were positive for assimilation of cellobiose. In conclusion, the assessment of the variability of the carbon assimilation patterns contributes to the understanding of the phenotypic diversity of *D. bruxellensis*, which seems to be related to the ecological niche from which the lineage is isolated.

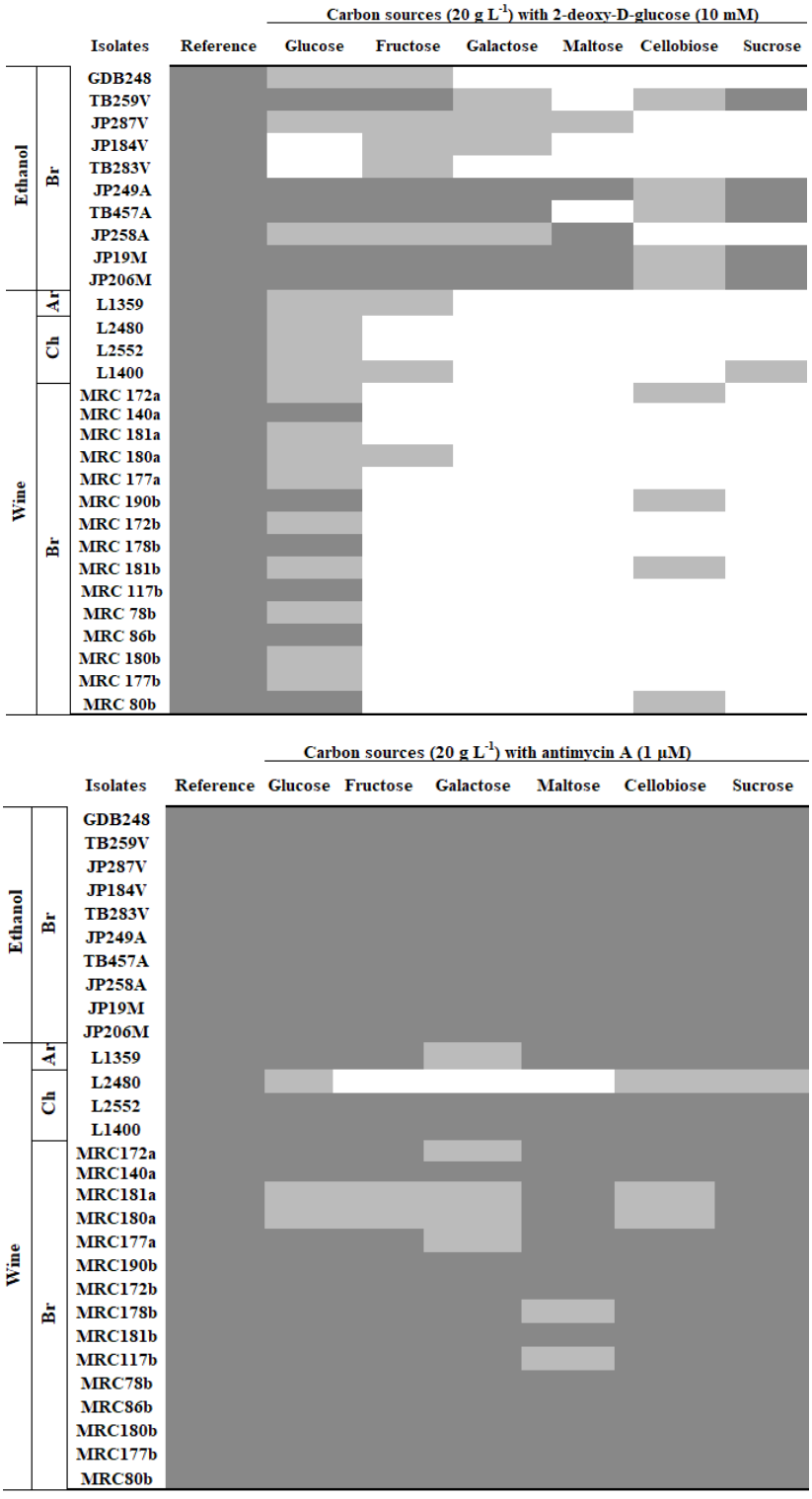
Glucose Catabolite Repression (GCR) among the industrial isolates of *D. bruxellensis*

We used 2-deoxy-D-glucose (2-DG) to investigate how *D. bruxellensis* isolates deal with GCR for the assimilation of other sugars, as well as for glucose itself. This aspect is of paramount importance, considering the complex composition of industrial substrates and the need for fast and efficient co-

metabolization of their sugar constituents. In preliminary tests, the growth of *S. cerevisiae* JP1 and *D. bruxellensis* GDB248 on glycerol was abolished by 2-DG (data not shown), attesting the efficacy of this compound to induce GCR in the test conditions utilized in this work. The results in Fig. 4A shows that most of the isolates had their growth on the different C-sources affected by 2-DG in different intensities, even when glucose was the carbon source. Isolates from wine were more sensitive to 2-DG than the fuel-ethanol isolates. Fuel-ethanol isolates that were insensitive to 2-DG on glucose and fructose were also insensitive on sucrose, but not necessarily on maltose or cellobiose.

The regulatory effect of GCR is based on the downregulation or repression of genes involved in the catabolism of carbon sources other than glucose, as well as inhibition of gluconeogenesis and transient mobilisation of reserve carbohydrates and activation of protein kinase A (PKA) regulatory pathway (Pautasso *et al*. 2016; Thevelein *et al*. 1994). In a previous work, we have proposed that GCR is alleviated in *D. bruxellensis* in relation to *S. cerevisiae* due to a higher basal activity of PKA, although its high repressive action on the expression of the gluconeogenic gene *FBP1* is still detected (Leite *et al*. 2016). The results of the present work show that there is a high intraspecific variation in GCR response, even among isolates from the same ecological niche. For example, the fuel-ethanol isolates JP249A, JP19M and JP206M showed remarkable tolerance to 2-DG even when cellobiose was the carbon source, while the fuel-ethanol TB283V isolate and the reference strain GDB 248 displayed sensitivity to the compound. The variations observed in this work regarding the tolerance to 2-DG may be linked to the already reported genetic variability among isolates and strains of *D. bruxellensis* (Conterno *et al.* 2006). It is worth noting that the JP19M isolate, which has shown the highest maximum specific growth rates for most of the sugars tested in this work, was also highly resistant to the GCR-inducer 2-DG.

Figure 4. Heat map of the relative growth of *D. bruxellensis* isolates on different C-sources in agar plates containing 2-deoxy-D-glucose (left-hand panel) or antimycin A (right-hand panel). The growth of each isolate on a given carbohydrate in the absence of inhibitors is taken as the reference condition. Dark grey bars refer to a growth similar to the reference ('unaffected growth'); light grey bars refer to a reduced growth as compared with the reference ('reduced growth'); white bars refer to the absence of growth ('impaired growth').



Fermentation of different sugars by *D. bruxellensis* isolates

The ability of the isolates to metabolize sugars when respiration is blocked was analyzed by the use of Antimycin A (AA), making possible the selection of isolates presenting the ability to ferment a specific substrate in a strictly anaerobic condition, which is a relevant feature for an industrial strain. The efficiency of AA in our test conditions was confirmed by the total growth impairment of *S. cerevisiae* JP1 strain on glycerol, a carbon source that can only be assimilated by respiration (data not shown). For all the tested C-sources, the growth of 21 out of the 29 industrial isolates was not affected by the presence of AA (Fig. 4B), meaning that these isolates can efficiently metabolize those mono- or disaccharides in fully anaerobic conditions, which is a quite rare feature among yeasts, being displayed by the species of the *Saccharomyces* stricto sensu complex (*S. cerevisiae, S. eubayanus, S. paradoxus, S. uvarum,* and their hybrids). It has been proposed that the convergent evolution of *D. bruxellensis* and the species of the *Saccharomyces* stricto sensu complex has enabled *D. bruxellensis* to produce *petite* mutants and survive in the absence of mitochondrial DNA just like *S. cerevisiae* (Procházka *et al.* 2010).

All eight isolates showing some sensitivity to AA were from wine. The most AA-sensitive isolate was the Chilean wine L2480 isolate, which was unable to ferment fructose, galactose, and maltose, and showed some impairment in fermenting glucose, sucrose, and cellobiose (Fig. 4B). The growth inhibition phenotype was mostly observed when galactose was the carbon source, being displayed by six out of the 29 isolates, while growth on sucrose was hardly affected. Overall, these results showed that most of the *D. bruxellensis* isolates are not subjected to the so-called '*Kluyver* effect' that represents the inability of the cells to assimilate galactose or disaccharides in anaerobiosis or in the absence of respiration (Sims and Barnett 1978). It is important to highlight that the *Kluyver* effect is strain-dependent even in the model yeast *Kluyveromyces lactis* (Fukuhara 2003). The introduction of galactose permease gene from *S. cerevisiae* can revert the *Kluyver*-positive phenotype of *K. lactis* for galactose, meaning that sugar transport is a crucial step in controlling the metabolic flux towards fermentation in yeast (Goffrini *et al*. 2002). In addition, Moktaduzzaman *et al.* (2015) reported that the ability of *D. bruxellensis* CBS 2499 to ferment galactose depended on the type of nitrogen source in the medium: in ammonium-

based media, cells tend to respire galactose, while nitrate-based media induce the respiro-fermentative metabolism of this sugar. All *D. bruxellensis* we isolated so far from fuel-ethanol plants were nitrate-positive (De Souza Liberal *et al*. 2007; Silva *et al*. 2016), while this phenotype is less spread among strains and isolates from wine (Crauwels *et al*. 2015; Galafassi *et al*. 2013). None of the Brazilian or Chilean wine isolates studied in this work was capable of growing in nitrate (data not shown). The relation between the ability to ferment galactose and the nitrogen source, including nitrate deserves further attention.

Anaerobic growth of selected industrial isolates

Taking into account the observed specific growth rates and the test results for the tolerance to 2-DG and AA, four fuel-ethanol isolates and one wine isolate were selected for anaerobic growth experiments using sugars relevant to fuel-ethanol production (glucose, sucrose, and cellobiose). These experiments were carried out in a 48-wells microplate inside an anaerobic chamber, with continuous monitoring of the cell growth. The anoxic environment in this apparatus has been confirmed by the lack of growth of *S. cerevisiae* JP1 strain on ethanol (data not shown). The strains *D. bruxellensis* GDB 248 and *S. cerevisiae* JP1 were used as a reference. All five selected isolates grew anaerobically on each of the three tested sugars in an ammonium-based synthetic medium (Fig. 5). These results contrast with the reports of Rozpedowska *et al*. (2011) and Blomqvist *et al*. (2011), who have found that *D. bruxellensis* cells require a supplementation of the culture medium with amino acids or yeast extract in order to grow anaerobically. Moreover, the strain *D. bruxellensis* CBS 11270 studied by Blomqvist *et al*. (2012) was not able to grow anaerobically on glucose, while the strain Y879 (CBS 2499) utilized by Rozpedowska *et al*. (2011) grew anaerobically on glucose with a maximum specific growth rate of 0.075 h^{-1}, a much lower value than any of those observed for our industrial isolates (0,15-0,44 h^{-1}, *cf.* Table 3). It can be seen that even within a limited number of isolates it is possible to observe a great phenotypic variability regarding the ability to assimilate these three sugars and the requirement of oxygen for their assimilation. The maximum specific growth rates calculated from the anaerobic growth curves in Figure 5 are shown in Table 3, together with the aerobic growth rates of the same isolates. The highest specific growth rates in anaerobiosis were observed on glucose and

sucrose for the fuel-ethanol isolates JP19M and TB457A, which have shown values exceeding 0.4 h^{-1}, which are comparable to or higher than those observed for the *S. cerevisiae* reference strain. The highest specific growth rates on cellobiose (>0.14 h^{-1}) were observed for the fuel-ethanol isolates JP19M and JP287V and for the reference strain GDB 248 (Figure 5; Table 3). The ability of strain GDB 248 to assimilate and ferment cellobiose was already reported (Leite *et al*. 2013; Reis *et al.* 2014; Reis *et al.* 2016).

Figure 5. Anaerobic growth kinetics of *D. bruxellensis* isolates in synthetic medium containing glucose (squares), sucrose (circles) or cellobiose (triangles) as carbon sources. Letters refer to reference strain GDB 248 (A), JP 287V (B), TB 457A (C), TB 283V (D), L 1400 (E), JP 19M (F) and *S. cerevisiae* JP1 (G).

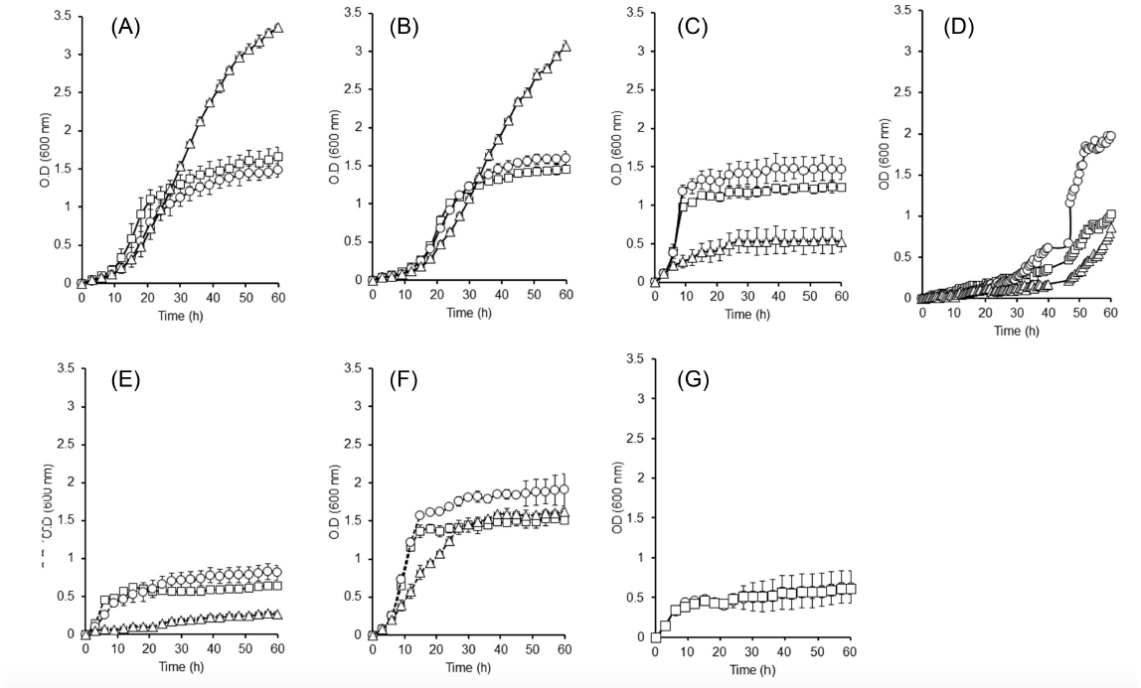


Table 3. Maximum specific growth rates (h−1) of selected isolates of *Dekkera bruxellensis* in different conditions.

	Glucose		Sucrose		Cellobiose	
Isolate	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
GDB 248	0.14 ± 0.00	0.20 ± 0.01	0.17 ± 0.00	0.17 ± 0.01	0.06 ± 0.00	0.15 ± 0.01
JP287V	0.28 ± 0.04	0.158 ± 0.01	0.19 ± 0.02	0.15 ± 0.01	0.18 ± 0.01	0.14 ± 0.00
TB283V	0.24 ± 0.02	0.15 ± 0.04	0.13 ± 0.01	0.09 ± 0.01	0.21 ± 0.01	0.10 ± 0.01
TB457A	0.33 ± 0.01	0.44 ± 0.02	0.31 ± 0.00	0.43 ± 0.02	0.09 ± 0.01	0.135 ± 0.00
L1400	0.28 ± 0.01	0.33 ± 0.02	0.27 ± 0.01	0.28 ± 0.03	0.11 ± 0.02	0.10 ± 0.02
JP19M	0.32 ± 0.01	0.40 ± 0.01	0.30 ± 0.00	0.41 ± 0.02	0.16 ± 0.01	0.26 ± 0.01
JP1[a]	0.38 ± 0.01	0.34 ± 0.02	0.34 ± 0.04	0.31 ± 0.02	N.A.[b]	N.A.[b]

[a] *Saccharomyces cerevisiae* JP1 industrial strain.
[b] Not applicable (N.A.).

In conclusion, the results of the present work confirmed the phenotypic plasticity of *Dekkera bruxellensis* regarding the capacity to assimilate and ferment mono- and disaccharides of industrial importance, as well as the capacity of cells isolated from the wine or fuel-ethanol industrial processes to display differential ability to assimilate sugars that are specific to those niches. In general, this yeast can use all the tested sugars when respiration is inhibited, and it seems, as we proposed recently, that GCR is less tightly regulated in this yeast than in *S. cerevisiae*, which may prove to be an interesting property for the efficient co-assimilation of sugars in complex industrial media. Among the 29 *D. bruxellensis* isolates screened, the fuel-ethanol JP19M isolate presented the highest specific growth rates, either in aerobic or in anaerobic conditions, and displayed a wider C-source assimilation range and a more relaxed GCR response (*i. e.* a higher tolerance to 2-DG). The JP19M isolate shall, therefore, be selected for further tests with industrial substrates. Other isolates in the present work represent interesting platforms for studying regulatory mechanisms of sugar assimilation in aerobic and anaerobic conditions.

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Conflict of interest

There is no conflict of interest.

References

Alexander MA, Jeffries TW. Respiratory efficiency and metabolite partitioning as regulatory phenomena in yeasts. *Enz Microb Technol* 1990; **12**: 2-19.

Basilio AC, de Araújo PR, de Morais JO *et al*. Detection and identification of wild yeast contaminants of the industrial fuel ethanol fermentation process. *Curr Microbiol* 2008; **56**:322-326.

Blomqvist J, South E, Tiukova I *et al.* Fermentation of lignocellulosic hydrolysate by the alternative industrial ethanol yeast *Dekkera bruxellensis*. *Lett Appl Microbiol* 2011; **53**:73–78.

Bro R, Smilde AK. Principal component analysis. *Analytical Methods* 2014; **6**:2812-2831.

Blomqvist J, Nogué VS, Gorwa-Grauslund M *et al*. Physiological requirements for growth and competitiveness of *Dekkera bruxellensis* under oxygen-limited or anaerobic conditions. *Yeast* 2012; **29**:265–274.

Carlson M. Glucose repression in yeast. *Curr Opin Microbiol* 1999; **2**:202-207.

Conterno L, Joseph CML, Arvik TJ *et al*. Genetic and physiological characterization of *Brettanomyces bruxellensis* strains isolated from wines. The *J. Enol. Vitic* 2006; **57**:139–147.

Crauwels S, Van Assche A, Jonge R *et al*. Comparative phenomics and targeted use of genomics reveal variation in carbon and nitrogen assimilation among different *Brettanomyces bruxellensis* strains. *Appl Microbiol Biotechnol* 2015; **99**:9123–9134.

da Silva Filho EA, de Melo HF, Antunes DF, Dos Santos SKB, do Monte Resende A, Simões, DA, de Morais Jr MA. Isolation by genetic and physiological characteristics of a fuel-ethanol fermentative *Saccharomyces cerevisiae* strain with potential for genetic manipulation. *Journal of Industrial Microbiology and Biotechnology* 2005; **32**:481-486.

da Silva TCD, Leite FCB, de Morais MA. Distribution of in a sugarcane-based fuel ethanol fermentation plant. *Lett Appl Microbiol* 2016; **62**:354-358.

de Barros Pita W, Leite FCB, de Souza Liberal AT *et al*. The ability to use nitrate confers advantage to *Dekkera bruxellensis* over *S. cerevisiae* and can explain its adaptation to industrial fermentation processes. *Anton Leeuw Int J G* 2011; **100**:99–107.

de Souza Liberal AT, Basılio ACM, do Monte Resende A *et al*. Identification of *Dekkera bruxellensis* as a major contaminant yeast in continuous fuel ethanol fermentation. *J Appl Microbiol* 2007; **102**:538–547.

Fukuhara H. The *Kluyver* effect revisited. MiniReview. *FEMS Yeast Res* 2003; **3**:327–31.

Galafassi S, Merico A, Pizza F *et al***.** *Dekkera/Brettanomyces* yeasts for ethanol production from renewable sources under oxygen-limited and low-pH conditions. *J Ind Microbiol Biotechnol* 2011; **38**:1079-1088.

Galafassi S, Capusoni C, Moktaduzzaman M *et al.* Utilization of nitrate abolishes the '*Custers* effect' in *Dekkera bruxellensis* and determines a different pattern of fermentation products. *J Ind Microbiol Biot* 2013; **40**:297–303.

Goffrini P, Ferrero I, Donnini C. Respiration-dependent utilization of sugars in yeasts: a determinant role for sugar transporters. *J Bacteriol* 2002; **184**:427–432.

Lagunas R, Dominguez C, Busturia A *et al*. Mechanisms of appearance of the Pasteur effect in *Saccharomyces cerevisiae*: inactivation of sugar transport systems. *J Bacteriol* 1982; **152:**19–25.

Leite FCB, Basso TO, de Barros Pita W *et al*. Quantitative aerobic physiology of the yeast *Dekkera bruxellensis*, a major contaminant in bioethanol production plants. *FEMS Yeast Res* 2013; **13**:34–43.

Li S, Du J, Sun J *et al*. Overcoming glucose repression in mixed sugar fermentation by co-expressing a cellobiose transporter and a β-glucosidase in *Saccharomyces cerevisiae*. *Mol BioSystems* 2010; **6**:2129-2132.

Loureiro V, Malfeito-Ferreira M. Spoilage yeasts in the wine industry. *Int J Food Microbiol* 2003; **86**:23–50.

Gonzáles-Siso MI. The Biotechnological utilization of cheese whey: a review. *Bioresour Technol* 1996; **57**:1-11.

Hellborg L and Piškur J. Complex Nature of the Genome in a Wine Spoilage Yeast, *Dekkera bruxellensis*. *Eukaryot Cell* 2009; **8**:1739-1749.

Kratky Z, Biely P, Bauer S. Mechanism of 2-deoxy-D-glucose inhibition of cell-wall polysaccharide and glycoprotein biosyntheses in *Saccharomyces cerevisiae*. *Eur J Biochem* 1975; **54**:459-467.

McCartney RR, Chandrashekarappa DG, Zhang BB *et al*. Genetic analysis of resistance and sensitivity to 2-deoxyglucose in *Saccharomyces cerevisiae*. *Genetics* 2014; **198**: 635-646.

Merico A, Sulo P, Piskur J *et al*. Fermentative lifestyle in yeasts belonging to the *Saccharomyces* complex. *FEBS J* 2007; **274**:976-989.

Metsalu T and Vilo J. Clustvis: a web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap. *Nucleic Acids Res* 2015; **43**: W566–W570.

Moktaduzzaman Md, Galafassi S, Capusoni C *et al*. Galactose utilization sheds new light on sugar metabolism in the sequenced strain *Dekkera bruxellensis* CBS 2499. *FEMS Yeast Res* 2015; **15**, 2.

Nadal D, Carro D, Fernandez-Larrea J *et al*. Analysis and dynamics of the chromosomal complements PF wild sparkling-wine yeast strains. *Appl. Environ. Microbiol* 1999; **65**:1688-1695.

O'Donnell AF, McCartney RR, Chandrashekarappa DG *et al*. 2-Deoxyglucose impairs *Saccharomyces cerevisiae* growth by stimulating Snf1-regulated and α-arrestin-mediated trafficking of hexose transporters 1 and 3. *Mol Cell Biol* 2015; **35**:939 – 955.

Parente DC, Cajueiro DBB, Moreno ICP *et al.* On the catabolism of amino acids in the yeast *Dekkera bruxellensis* and the implications for industrial fermentation processes. *Yeast* 2018; **35**:299-309.

Pautasso C, Reca S, Chatfield-Reed K *et al*. Identification of novel transcriptional regulators of PKA subunits in *Saccharomyces cerevisiae* by quantitative promoter-reporter screening. *FEMS Yeast Res* 2016; **16**: fow046.

Peeters K, Van Leemputte F, Fischer B *et al*. Fructose-1,6-bisphosphate couples glycolytic flux to activation of Ras. *Nat Commun*. 2017; **8**:922.

Pereira LF, Bassi AP, Avansini SH *et al*. The physiological characteristics of the yeast *Dekkera bruxellensis* in fully fermentative conditions with cell recycling

and in mixed cultures with Saccharomyces cerevisiae. *Anton Leeuw Int J G* 2012; **101**:529–539.

Pereira LF, Lucatti E, Basso LC *et al*. The fermentation of sugarcane molasses by *Dekkera bruxellensis* and the mobilization of reserve carbohydrates. *Anton Leeuw Int J G* 2014; **105**:481–489.

Procházka E, Poláková S, Piskur J *et al*. *Dekkera bruxellensis*—spoilage yeast with biotechnological potential, and a model for yeast evolution, physiology, and competitiveness. *FEMS Yeast Res* 2010; **10**:545-57.

Pronk JT, Yde Steensma H, Van Dijken JP. Pyruvate metabolism in *Saccharomyces cerevisiae*. *Yeast* 1996; **12**:1607-33.

Ramirez-Castrillon M, Mendes SDC, Valente P. South Brazilian wines: culturable yeasts associated to bottled wines produced in Rio Grande do Sul and Santa Catarina. *World J Microbiol Biotechnol* 2017; **33**: 77.

Rasimus-Sahari S, Mikkola R, Andersson MA *et al*. *Streptomyces* strains producing mitochondriotoxic antimycin A found in cereal grains. *Int J Food Microbiol* 2016; **21**: 78-85.

Reis ALS, Souza RFR, Torres RRNB *et al*. Oxygen-limited cellobiose fermentation and the characterization of the cellobiase of an industrial *Dekkera/Brettanomyces bruxellensis* strain. *SpringerPlus* 2014; **3**:38.

Rozpedowska E, Hellborg L, Ishchuk OP *et al*. Parallel evolution of the make–accumulate–consume strategy in *Saccharomyces* and *Dekkera* yeasts. *Nat Commun* 2011; **2**:302.

Rubio-Teixeira MAA. Comparative analysis of the *GAL* genetic switch between not-so-distant cousins – *Saccharomyces cerevisiae* versus *Kluyveromyces lactis*. *FEMS Yeast Res* 2005; **5**:1115-1128.

Sangorrín MP, García V, Lopes CA *et al*. Molecular and physiological comparison of spoilage wine yeasts. *J Appl Microbiol* 2013; **114**:1066–1074.

Stambuk BU, Silva MA, Panek AD *et al*. Active α-glucoside transport in *Saccharomyces cerevisiae*. *FEMS Lett* 1999; **170**:105-110.

Sims AP, Barnett JA. The requirement of oxygen for the utilization of maltose, cellobiose, and D-galactose by certain anaerobically fermenting yeasts (*Kluyver* Effect). *J Gen Microbiol* 1978; **106**:277-288.

Thevelein JM. Signal transduction in yeast. *Yeast* 1994; **10**:1753-90.

Turrens JF. Superoxide production by the mitochondrial respiratory chain. *Biosci Rep* 1997; **17**:3–8.

Uenojo M, Pastore GM. Pectinases: aplicações industriais e perspectivas. *Quím. Nova* 2007; **30**:388-394.

Vigentini I, Joseph CML, Picozzi C *et al.* Assessment of the *Brettanomyces bruxellensis* metabolome during sulphur dioxide exposure. *FEMS Yeast Res* 2013; **13**:597–608.

Woolfit M, Rozpedowska E, Piskur J *et al*. Genome survey sequencing of the wine spoilage yeast *Dekkera* (*Brettanomyces*) *bruxellensis*. *Eukaryot. Cell* 2007; **6**:721–733.

5 ARTIGO 2 - FERMENTATION PROFILES OF THE YEAST *BRETTANOMYCES BRUXELLENSIS* IN D-XYLOSE AND L-ARABINOSE AIMING ITS APPLICATION AS A SECOND-GENERATION ETHANOL PRODUCER

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Abstract

The yeast *Brettanomyces bruxellensis* is able to ferment the main sugars used in first generation ethanol production. However, its employment in this industry is prohibitive because the ethanol productivity reached is significantly lower than the observed for *Saccharomyces cerevisiae*. On the other hand, a possible application of *B. bruxellensis* in the second-generation ethanol production has been suggested because this yeast is also able to use D-xylose and L-arabinose, the major pentoses released from lignocellulosic material. Although the latter application seems to be reasonable, it has been poorly explored. Therefore, we aimed to evaluate whether or not different industrial strains of *B. bruxellensis* are able to ferment D-xylose and L-arabinose, both in aerobiosis and oxygen-limited conditions. Three out of nine tested strains were able to assimilate those sugars. When in aerobiosis, *B. bruxellensis* cells exclusively used them to support biomass formation, and no ethanol was produced. Moreover, whereas L-arabinose was not consumed under oxygen limitation, D-xylose was only slightly used, which resulted in low ethanol yield and productivity. In conclusion, our results showed that D-xylose and L-arabinose are not efficiently converted to ethanol by *B. bruxellensis*, most likely due to a redox imbalance in the assimilatory pathways of these sugars. Therefore, despite presenting other industrially relevant traits, the employment of *B. bruxellensis* in second-generation ethanol production depends on the development of genetic engineering strategies to overcome this metabolic bottleneck.

Keywords: fermentative capacity, industrial application, pentose metabolism, redox imbalance, second generation ethanol.

1 INTRODUCTION

The use of lignocellulosic biomass for second-generation ethanol production has strategic importance because it is a renewable and environment-friendly source because of the reduction of greenhouse gases (Nogueira et al., 2018). In Brazil, the main substrate used in this process is the sugarcane bagasse, which undergoes (i) pretreatment, (ii) hydrolysis and (iii) fermentation steps (Guilherme et al., 2019). Pretreatment works by disorganizing the lignocellulosic matrix, whereas hydrolysis acts on the conversion of (i) cellulose to D-glucose and

(ii) hemicellulose to D-glucose, D-mannose, D-galactose, D-xylose and L-arabinose (Goldemberg, 2013; Silverstein, Chen, Sharma-Shivappa, & Boyette, 2007). Although these compounds are further used in the fermentation step (Robak & Balcerek, 2018), the lignin present in biomass is generally used to produce electricity and heat for biorefineries (Gamage, Lam, & Zhang, 2010; Sousa-Aguiar, Appel, Zonetti, & Fraga, 2014).

In the mixture of sugars from lignocellulosic biomass, D-xylose is the most abundant pentose, being the main hemicellulosic derivative of sugarcane bagasse, with a ratio of 12:1 in comparison with L-arabinose (Rocha, Nascimento, Gonçalves, Silva, & Martín, 2015). Therefore, the ability to ferment D-xylose is an essential requirement in order to make the second-generation ethanol production a process economically viable (Rodrussamee, Sattayawat, & Yamada, 2018). In filamentous fungi and yeasts capable of using D-xylose, it is converted to xylulose-5-phosphate within the cells, which enters the pentose phosphate pathway (PPP) to generate glycolytic intermediates, which can be destined to fermentation or respiration (Dickinson & Schweizer, 2004; Jeffries, 2006). This conversion occurs in three sequential reactions catalysed by the enzymes (i) xylose reductase (XR), which reduces D-xylose to xylitol, (ii) xylitol dehydrogenase (XDH), which oxidizes xylitol to D-xylulose, and (iii) xylulokinase (XK), which phosphorylates D-xylulose to xylulose 5-phosphate (Olofsson, Bertilsson, & Lidén, 2008). Curiously, the ability to ferment D-xylose to ethanol is present only in approximately 1% of the yeasts able to use this sugar as a carbon source, especially *Scheffersomyces stipitis*, *Spathaspora passalidarum* and *Candida shehatae* (de Souza et al., 2018; Hou, 2012; Nakanishi et al., 2017; Yuvadetkun, Leksawasdi, & Boonmee, 2017). Similar to D-xylose, in order for L-arabinose to be converted to D-xylulose and to enter PPP, the presence of four enzymes is required: (i) XR, which reduces L-arabinose to L-arabitol, (ii) arabinitol 4-dehydrogenase (LAD), which oxidizes L-arabitol to Lxylulose, (iii) xylulose reductase (LXR), which reduces L-xylulose to xylitol, and (iv) XDH, which oxidizes xylitol to D-xylulose (Richard, Verho, Putkonen, Londesborough, & Penttila, 2003).

The yeast *Brettanomyces bruxellensis* is an industrial species presenting an extensive yet strain-dependent range of sugar utilization (Kurtzman, Fell, & Boekhout, 2011; Crauwels et al., 2015; da Silva et al., 2019). For example, fuel-ethanol isolates have a greater ability to consume cellobiose and lactose compared

with the winemaking ones (da Silva et al., 2019). Additionally, some *B. bruxellensis* strains are capable of metabolizing mannose as well as trisaccharides and polysaccharides (Crauwels et al., 2015; Galafassi et al., 2011). From an industrial point of view, the use of several substrates allows the utilization of *B. bruxellensis* in large ranges of fermentative processes (de Barros Pita et al., 2019). In fact, *B. bruxellensis* is able to ferment sugars used in first-generation ethanol production (de Barros Pita, Castro Silva, Simões-Ardaillon, Volkmar, & de Morais, 2013; Pereira et al., 2012). However, because the volumetric productivity achieved is lower when compared with *Saccharomyces cerevisiae*, its current utilization is not cost-effective (de Souza Liberal et al., 2007; de Barros Pita et al., 2013). In addition, B. bruxellensis presents a set of attractive physiological traits for second-generation ethanol production (de Barros Pita et al., 2019). First, this yeast is able to resist to inhibitors generated during the hydrolysis step (such as furfural and acetic acid), as well as to the fermentation environment itself (weak acids, low pH and high ethanol concentrations), which is valuable to this industry (Bassi, Silva, Reis, & Ceccato-Antonini, 2013; Blomqvist et al., 2011; Moktaduzzaman et al., 2015; Rozpędowska et al., 2011; Tiukova et al., 2014). Second, some strains of *B. bruxellensis* are able to natively use D-xylose and L-arabinose as carbon sources (Codato, Martini, Ceccato-Antonini, & Bastos, 2018; Crauwels et al., 2015). In this sense, it seems reasonable to point that *B. bruxellensis* could be employed in the second-generation ethanol production, as it was previously suggested (de Barros Pita et al., 2019). However, too little is known regarding the metabolic capacities of this yeast when in the presence of these pentoses (Codato et al., 2018; Crauwels et al., 2015).

Because the ability to efficiently ferment D-xylose and L-arabinose is critical for a microorganism to be successfully used in second generation ethanol production, we evaluated different industrial strains of *B. bruxellensis* for their fermentative capacity in the presence of these sugars. The results reported herein show that industrial strains of *B. bruxellensis* are able to use hemicellulose-derived pentoses, yet with low efficiency for ethanol production. In the following sections, we provide a detailed discussion of the metabolic aspects of *B. bruxellensis* that resulted in this phenotype. Despite that, our findings might help driving genetic engineering strategies for using this yeast in the second-generation ethanol industry by overcoming its metabolic limitation and taking advantage of its inherent tolerance to this environment.

2 MATERIAL AND METHODS

2.1 Yeast strains, maintenance and growth screening in different carbon sources

The strains of *B. bruxellensis* used in the present work were previously isolated from fuel ethanol production processes (Table 1) and identified by molecular biology tools (da Silva, Leite, & de Morais, 2016). Strains were maintained in yeast extract peptone dextrose (YPD) medium (10 g/L yeast extract, 20 g/L glucose, 20 g/L bacteriological peptone and 20 g/L agar) with constant transfers to new Petri dishes in order to keep cell colonies fresh (Leite et al., 2013). GDB 248 strain was used as a reference (de Barros Pita, Leite, de Souza Liberal, Simões, & de Morais, 2011). Cells from each strain were cultured in 2 ml microtubes containing 1 ml of YPD medium (as mentioned above) at 30 °C, 160 rpm for 6 h. Subsequently, cells were centrifuged and washed with sterile saline solution (8.5 g/L). To evaluate the assimilation capacity of sugars, 5 µl of the culture was added to Petri dishes containing 1.7 g/L yeast nitrogen base (YNB), 5 g/L ammonium sulphate, a carbon source (D-glucose, D-xylose or L-arabinose) at 20 g/L and agar at 20 g/L. Growth was evaluated after 72 h of incubation at 30 °C.

TABLE 1 Isolates of *Brettanomyces bruxellensis* used in this study

Strain	Process	Location	Year of isolation
GDB248	Feeding juice	Paraíba–BR	2006
TB457A	Washing water	Paraíba–BR	2014
JP258A	Washing water	Paraíba–BR	2013
JP19M	Feeding juice	Paraíba–BR	2013
JP206M	Feeding juice	Paraíba–BR	2013
JP287V	Vinasse	Paraíba–BR	2014
JP184V	Vinasse	Paraíba–BR	2014
TB259V	Vinasse	Paraíba–BR	2013
TB283V	Vinasse	Paraíba–BR	2014

2.1. Aerobic cultivations in flasks

Cells were precultured in 125-ml flasks containing 50 ml of YPD at 30 °C and 160 rpm for 48 h. Subsequently, cells were collected by centrifugation, washed with sterile distilled water for removal of culture medium traces and suspended in sterile saline solution (8.5 g/L) to a concentration corresponding to 0.1 unit of absorbance at 600 nm. Then, cells were transferred to 50-ml flasks containing 30 ml of YNB w/o ammonium sulphate and amino acid medium supplemented with 5 g/L of ammonium sulphate and 20 g/L from one of the following carbon sources: (i) D-xylose, (ii) D-glucose, (iii) L-arabinose or (iv) a combination of these sugars (20 g/L each). Cultures were maintained for 48 h at 30 °C and 160 rpm. Samples were collected at times 0, 2, 4, 6, 8, 24 and 48 h for absorbance verification (optical density 600 nm). Each experiment was carried out in biological duplicates, and optical density measurements were performed with two technical replicates.

2.3 Fermentation assays

Fermentation assays were performed under aerobic conditions and oxygen limitation. For the aerobic fermentation assay, cells were cultivated as described in the previous section, in order to evaluate respiro-fermentative metabolism in the presence of (i) D-xylose, (ii) D-glucose, (iii) L-arabinose or (iv) a combination of these sugars (20 g/L each). The combination of sugars under aerobic conditions was named mixed medium I. Cultures were maintained for 48 h at 30 °C and 160 rpm. Samples were taken at times 0, 8, 24 and 48 h for absorbance determination (optical density at 600 nm) and metabolites measurements (sugars, xylitol, ethanol, glycerol and acetate by high performance liquid chromatography [HPLC]).

In oxygen limitation, isolates were grown in YPD for 72 h, 30 °C and 160 rpm until biomass reached 10% cell w/v. Subsequently, cells were centrifuged for 5 min, 5,000 g at room temperature and washed with sterile saline (8.5 g/L). Cells were transferred to 15-ml tubes containing YNB medium (w/o ammonium sulphate and amino acids) supplemented with the carbon source (D-xylose, D-glucose, L-arabinose or a combination of these sugars) and 5 g/L of ammonium sulphate at 30 °C without agitation. Two groups of experiments were then carried out in order to analyse the influence of D-xylose concentration as well as the fermentation period on the ethanol production capacity of *B. bruxellensis*. The first set of experiments was performed with a concentration of 20 g/L of one of the sugars D-glucose, D-

xylose or L-arabinose, as well as the combination of the three sugars (20 g/L each), yielding 60 g/L (mixed medium II). The second set of experiments was performed with three different media, and the concentration of sugars was as follows: (i) D-xylose or (ii) D-glucose at 40 g/L and (iii) the combination of 40 g/L of D-xylose, 10 g/L of D-glucose and 7 g/L of L-arabinose in the mixed medium III. The mixed medium was prepared similarly to the concentrations found in the hemicellulose hydrolysis of sugarcane bagasse (Rudolf, Baudel, & Zacchi, 2008). Samples from the first experiment set were collected at times 0, 4, 8 and 48 h, and samples from the second set of experiments were collected at times 0 and 96 h. For each collected sample, the optical density (OD 600 nm) was measured to determine cell growth. All samples were centrifuged for 5 min, 10,000 g and 4 °C. The supernatant was used to determine the concentration of metabolites, as described below. Each experiment was carried out in biological duplicates.

2.2 Determination of extracellular metabolites by HPLC

The concentrations of ethanol, xylitol, acetate, glycerol and sugars (D-glucose, D-xylose and L-arabinose) were determined by HPLC in a Shimadzu system equipped with a quaternary pump coupled with a degasser, an oven for controlling column temperature, set at 60 °C, and a refractive index detector. The software used for data acquisition was LC Solutions, manufactured by Shimadzu Corporation (Kyoto, Japan). A 300 mm Å~ 7.8 mm ionic exchange column (Aminex® HPX- 87H, Bio-Rad, USA), with 9-mm particle size, was used. The mobile phase used was ultrapure water acidified with isocratic elution of H_2SO_4 5 mM at a flow rate of 0.6 ml/min. The correlation coefficients (R^2) of the calibration curves were higher than 0.999. All samples were run with two technical replicates.

2.3 Statistical analysis

Statistical tests were performed in PAST (version 2.17). Analysis of variance (ANOVA, p value ≤ 0.05) was performed for the following parameters: sugar consumed, biomass yield, ethanol yield and ethanol volumetric yield. PAST software 3.14 for Windows was used to determine the Tukey test ($p \leq 0.05$).

3 RESULTS AND DISCUSSION

3.1 Qualitative screening and aerobic growth profile of *B. bruxellensis* industrial strains in D-xylose and L-arabinose

Nine industrial strains of *B. bruxellensis* isolated from fuel ethanol production were qualitatively screened for their capacity to grow in D-xylose and/or L-arabinose. Only three out of these nine strains (TB457A, JP206M and JP19M) were able to grow in both sources and were, therefore, selected for further assays (Table 2). Moreover, none of our strains was able to grow exclusively in D-xylose or in L arabinose, meaning that either cell is able to grow on both sources or they are not able to use them whatsoever (Table 2). This observation is not surprising because the assimilatory pathways of these two pentoses are overlapping to some extent, sharing enzymes and xylitol as a common intermediate (Dien, Kurtzman, Saha, & Bothast, 1996; Hahn-Hägerdal, Karhumaa, & Jeppsson, 2007). A previous screening with different species of the genus *Brettanomyces* showed that none of 28 *B. bruxellensis* Strains analysed were able to grow on D-xylose and L-arabinose (Galafassi et al., 2011). Indeed, the ability to consume these pentoses seems to be a strain-specific trait in *B. bruxellensis*, as different studies show phenotypic variation between isolates detected in various industrial processes, such as wine, beer and soft drink production (Crauwels et al., 2015; Galafassi et al., 2011).

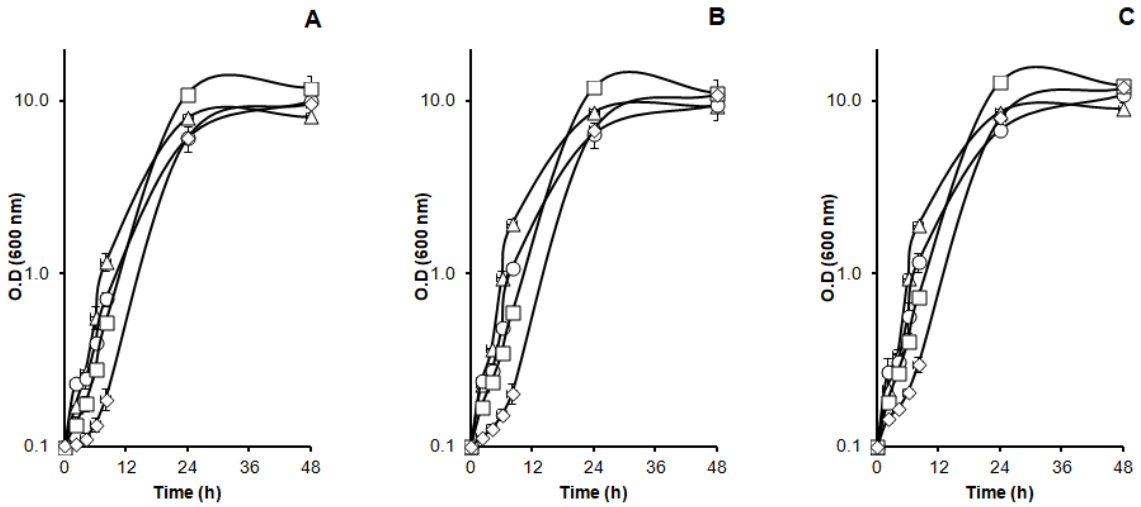
After our initial screening, TB457A, JP206M and JP19M strains were aerobically cultivated in order to determine the growth profile in D-glucose (as a reference), D-xylose, L-arabinose or the mixture of the three sugars (20 g/L each). As can be seen in Figure 1, all strains reached high final cell densities. Moreover, growth rates were superior in the mixed medium (0.39, 0.42 and 0.44 h^{-1} for TB457A, JP206M and JP19M, respectively), which is somehow expected because of its higher carbon input. In addition, cells presented high growth rates when D-xylose was the sole carbon source (0.32, 0.34 and 0.33 h^{-1}), similar to those previously described for *Meyerozyma guilliermondii* PYCC 3012 and *Candida arabinofermentans* PYCC 5603 (Fonseca, Spencer-Martins, & Hahn-Hägerdal, 2007). Growth rates in D-xylose for our strains exceeded even those found in glucose (0.27, 0.23 and 0.26 h^{-1}) and L-arabinose (0.14, 0.12 and 0.15 h^{-1}). In the following section, we provide an explanation for these findings.

TABLE 2 Screening *of Brettanomyces bruxellensis* industrial strains for growth in D-xylose and L-arabinose.

	Carbon sources (20 g/L)		
Isolates	Glucose	D-Xylose	L-Arabinose
GDB248	+	–	–
TB457A	+	+	+
JP258A	+	–	–
JP19M	+	+	+
JP206M	+	+	+
JP287V	+	–	–
JP184V	+	–	–
TB259V	+	–	–
TB283V	+	–	–

Note: Glucose was used as a reference. Symbols: (+) presence or (–) absence of colonies. NT, not tested.

FUGURE 1 Growth capacity in medium containing D-xylose, L-arabinose, glucose and the mixture of these sources by different *Brettanomyces bruxellensis* strains. Strains (a) TB457A, (b) JP206M and (c) JP19M. Glucose (square), mixed medium (triangle), L-arabinose (diamond) and D-xylose (circle).



3.2 Aerobic fermentative capacity of *B. bruxellensis* industrial strains in D-xylose and L-arabinose

The ability to ferment D-xylose and L-arabinose by *B. bruxellensis* strains was evaluated under aerobic conditions. In a glucose-based medium, the three strains presented a fermentative performance (ethanol yield and productivity, Figure 2 and Table 3) similar to previous studies (Galafassi et al., 2011; Peña-Moreno et al., 2019; Teles, da Silva, Mendonça, de Morais Junior, & de Barros, 2018). When D-xylose was the sole carbon source, *B. bruxellensis* completely consumed this sugar, yet none of the three strains was able to produce ethanol in this condition (Figure 2 and Table 3). Despite previous studies reporting ethanol production by *B. bruxellensis* from secondary carbon sources (Moktaduzzaman et al., 2015; Reis et al., 2014), our data indicate that carbon was preferentially directed to biomass formation (Yx/s 0.39, 0.46 and 0.48; Figure 2 and Table 3) and trace xylitol amounts. This is similar to those observed for *M. guilliermondii* PYCC 3012 and *C. arabinofermentans* PYCC 5603 (Fonseca et al., 2007). This observation might be related to the fact that, despite being a Crabtree positive yeast, *B. bruxellensis* has a preference for oxidative metabolism, with high biomass yield and low ethanol values in environments with high oxygenation (Leite et al., 2013; Teles et al., 2018). In fact, that is also the explanation for the differences in the growth rates for D-xylose and glucose found in the previous section.

Our strains were also able to metabolize L-arabinose under aerobic conditions, as previously observed for other yeasts, such as *M. guilliermondii* PYCC 3012, *C. arabinofermentans* PYCC 5603 and *S. stipitis* (Fonseca et al., 2007; Granados-Arvizu et al., 2019). However, similar to that in D-xylose, cells did not produce ethanol from L-arabinose, with a preferential targeting for biomass formation (Yx/s 0.54, 0.54 and 0.59; Figure 2 and Table 3), parallel to that observed for *S. passalidarum* CMUWF1–2 (Rodrussamee et al., 2018). Moreover, L-arabinose was consumed at a slower rate than D-xylose (Figure 2 and Table 3) yet similar to *S. passalidarum* CMUWF1–2, which, even in yeast peptone (YP) medium, consumed only 50% of the initial sugar after 60 h (Rodrussamee et al., 2018).

FIGURE 2 Sugar consumption and production of industrial metabolites by *Brettanomyces bruxellensis* isolates in aerobiosis. Closed diamond, glucose; closed square, D-xylose; closed circle, L-arabinose; open circle, OD (optical density); open triangle, ethanol; open square, acetate; open diamond, glycerol; asterisk, xylitol.

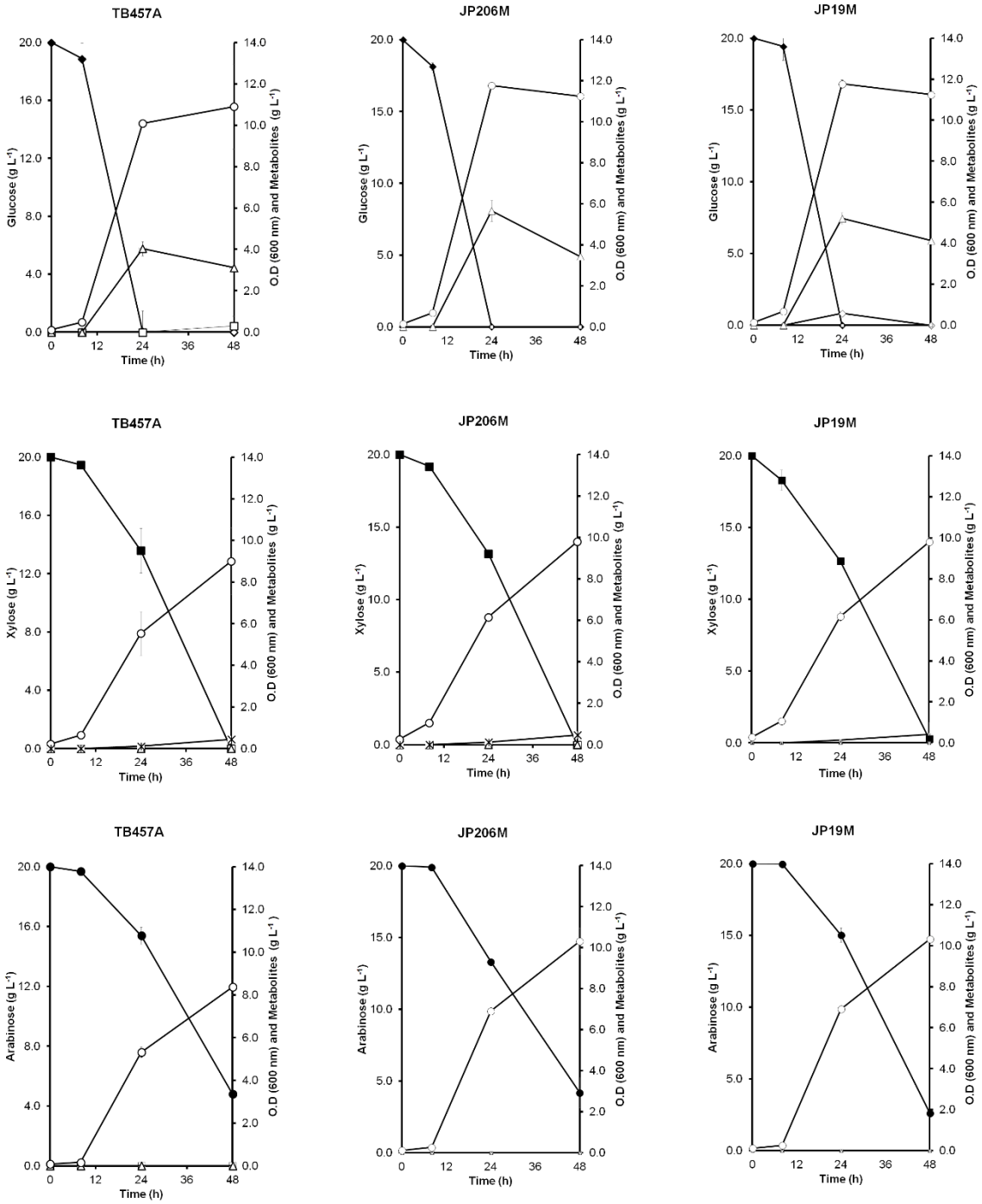


TABLE 3 Fermentative parameters of *Brettanomyces bruxellensis* industrial isolates in media containing glucose, D-xylose or L-arabinose under aerobic conditions and oxygen limitation.

Strains	Sugar in the medium	Oxygen availability	Glucose consumed (g/L)	D-Xylose consumed (g/L)	L-Arabinose consumed (g/L)	$Y_{x/s}$	$Y_{p/s}$ ethanol	Productiv. (g.l.h)
TB457A	Glucose	Aerobiosis	20 ± 0.00	–	–	0.51 ± 0.05	0.16 ± 0.01	0.065 ± 0.00
		O_2 limitation	20 ± 0.00	–	–	N/A	0.42 ±0.00	0.174 ± 0.00
	D-Xylose	Aerobiosis	–	20 ± 0.00	–	0.39 ± 0.00	0.00 ± 0.00	0.000 ± 0.00
		O_2 limitation	–	1.39 ± 0.13	–	N/A	0.11 ± 0.01	0.003 ± 0.00
	L-Arabinose	Aerobiosis	–	–	15.2 ± 0.22	0.54 ± 0.01	0.00 ± 0.00	0.000 ± 0.00
		O_2 limitation	–	–	0.00 ± 0.00	N/A	0.00 ± 0.00	0.000 ± 0.00
JP206M	Glucose	Aerobiosis	20 ± 0.00	–	–	0.50 ± 0.01	0.17 ± 0.01	0.071 ± 0.00
		O_2 limitation	20 ± 0.00	–	–	N/A	0.40 ± 0.00	0.167 ± 0.00
	D-Xylose	Aerobiosis	–	19.56 ± 0.62	–	0.46 ± 0.04	0.00 ± 0.00	0.000 ± 0.00
		O_2 limitation	–	1.18 ± 0.03	–	N/A	0.34 ± 0.02	0.008 ± 0.001
	L-Arabinose	Aerobiosis	–	–	15.81 ± 0.92	0.59 ± 0.00	0.00 ± 0.00	0.000 ± 0.00
		O_2 limitation	–	–	0.00 ± 0.00	N/A	0.00 ± 0.00	0.000 ± 0.00
JP19M	Glucose	Aerobiosis	20 ± 0.00	–	–	0.55 ± 0.00	0.21 ± 0.00	0.086 ± 0.00
		O_2 limitation	20 ± 0.00	–	–	N/A	0.41 ± 0.00	0.172 ± 0.00
	D-Xylose	Aerobiosis	–	19.71 ± 0.07	–	0.48 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
		O_2 limitation	–	1.28 ± 0.06	–	N/A	0.33 ± 0.03	0.009 ± 0.00
	L-Arabinose	Aerobiosis	–	–	17.38 ± 0.11	0.59 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
		O_2 limitation	–	–	0.00 ± 0.00	–	0.00 ± 0.00	0.00 ± 0.00

Note: $Y_{x/s}$, cell yield coefficient (g biomass produced/g substrate utilized); $Y_{p/s}$, product yield coefficient (g product/g substrate utilized); –, data not calculated; N/A, data not available; Productiv, volumetric productivity of ethanol.

3.3 Fermentative capacity of *B. bruxellensis* industrial strains in D-xylose and L-arabinose in oxygen limitation

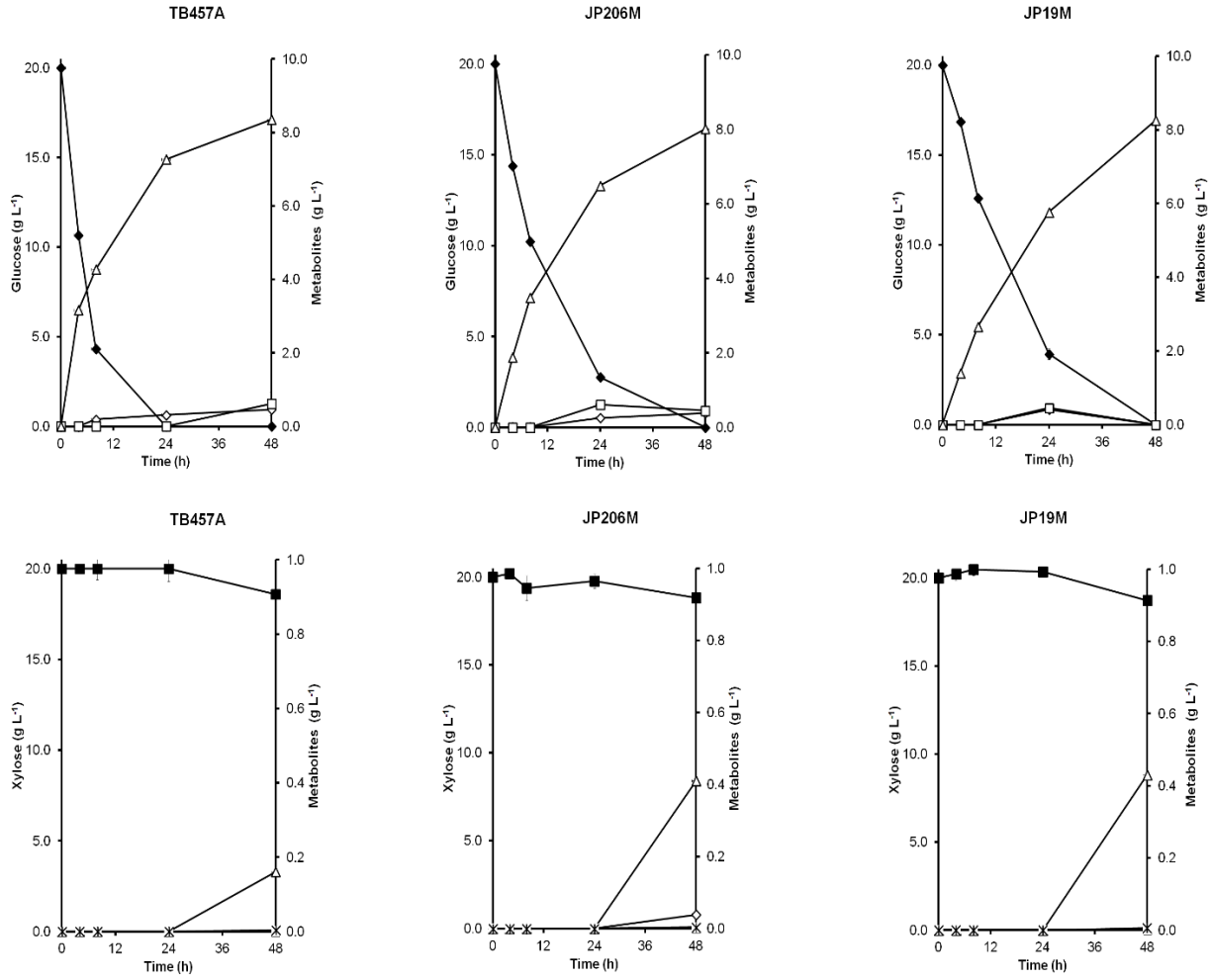
In order to test the capacity of *B. bruxellensis* to ferment D-xylose and L-arabinose in a scenario closer to that found in the industrial environment, we carried out fermentation assays also in oxygen limited conditions. When D-xylose was the carbon source, ethanol yields were higher (0.11, 0.34 and 0.33, Table 3) than the ones previously described for *B. bruxellensis* (Codato et al., 2018). However, the fermentative performance of our strains was significantly lower (ethanol yield and productivity, Table 3) than the observed for *S. passalidarum* and *S. stipitis*, yeasts

commonly used in second-generation ethanol production (Veras, Parachin, & Almeida, 2017). Additionally, we tested whether a higher sugar concentration (D-xylose at 40 g/L) and a longer period of fermentation (96 h) could influence the fermentative capacity of *B. bruxellensis*. Nonetheless, changing these parameters did not enhance the performance of our strains (data not shown), unlike a previous work with different *B. bruxellensis* isolates (Codato et al., 2018). In fact, although many yeast species are able to assimilate D-xylose, few are competent to natively ferment this sugar to ethanol (Rodrussamee et al., 2018). This is usually explained by the fact that XR and XDH, the first two xylose-assimilating enzymes, have a differential preference for cofactors (XR mainly for NADPH and XDH for NAD^+), which in oxygen limitation or anaerobiosis leads to a redox imbalance and ultimately results in xylitol accumulation (Jeffries et al., 2007; Shin et al., 2019). In the present work, xylitol was detected only in trace amounts, and the reasons for that observation are yet to be determined. However, because the NADPH supply can be restored by PPP/gluconeogenesis cycles, the redox imbalance is thought to be linked to the inability to regenerate NAD^+ in the absence of a functional respiratory chain. Our hypothesis is that oxygen limitation results in decreased uptake of D-xylose by *B. bruxellensis* cells (Figure 3) as it has been previously described for *S. stipitis* (Skoog & Hahn-Hägerdal, 1990). In our case, the lagging influx of D-xylose is eventually handled by XR and results only in low amounts of xylitol, which can be properly managed by XDH, as long as NAD^+ supply enables its reaction. Therefore, no significant xylitol accumulation is observed prior to the exhaustion of NAD+, when XDH stops working and D-xylose metabolism is completely halted. In fact, this is likely to be the explanation not only for the absence of xylitol but also for the little ethanol produced by our strains in oxygen limitation (Figure 3).

Our strains were not able to consume L-arabinose in oxygen limitation (data not shown). Similar to that with D-xylose, this is also likely to be due to the inability for cofactor regeneration within the L-arabinose metabolism pathway when the presence of oxygen or another electron acceptor is required (Hahn-Hägerdal, Galbe, Gorwa-Grauslund, Lidén, & Zacchi, 2006). In addition, it has been observed that a *S. cerevisiae* strain modified to metabolize L-arabinose produces $13_{1/3}$ (aerobically) and $1_{2/3}$ moles (anaerobically) of ATP from 1 mol of arabinose (Wisselink et al., 2007). This means that under anaerobic conditions (fermentation), an eightfold higher arabinose influx is required for ATP production to be similar to

aerobic conditions (respiration) (Wisselink et al., 2007). Therefore, the strains of *B. bruxellensis* isolated from first-generation ethanol production are not able to efficiently ferment the two major pentoses found in lignocellulosic hydrolysate. This output is a bottleneck that inhibits its applicability in the second-generation ethanol industry. However, because *B. bruxellensis* presents other physiological relevant traits, genetic engineering strategies should be encouraged in order to overcome this metabolic constraint.

FIGURE 3 Sugar consumption curves and industrial metabolite production by *Brettanomyces bruxellensis* isolates in oxygen limitation. Closed diamond, glucose; closed square, D-xylose; open circle, OD (optical density); open triangle, ethanol; open square, acetate; open diamond, glycerol; asterisk, xylitol.



3.4 Glucose influence on the capacity of *B. bruxellensis* strains to ferment D-xylose and L-arabinose

Because our strains were not able to efficiently ferment either D-xylose or L-arabinose individually, we aimed to test whether the presence of glucose might favour pentose consumption, as it was previously observed for D-xylose (Boles, Ller, & Zimmermann, 1996; Meinander, Boels, & Hahn-Hägerdal, 1999). Therefore, cells from TB457A, JP206M and JP19M strains were grown in different (i) concentrations of the three sugars, (ii) oxygen availability and (iii) cultivation times (see Section 2). In aerobiosis (reference), glucose is preferably consumed, followed by D-xylose, whereas L-arabinose was not significantly used (Figure 4). The preference for D-xylose over L-arabinose has already been described in a modified strain of *S. cerevisiae* (Wisselink, Toirkens, Wu, Pronk, & van Maris, 2009). In contrast, *Candida akabanensis* UFVJM-R131 is capable of using both sugars simultaneously, converting them to ethanol (de Matos, Souza, Santos, & Pantoja, 2018). Interestingly, in aerobiosis, ethanol yield and productivity values in the mixed medium were higher than those observed in glucose-based medium (Tables 3 and 4). For instance, strain TB457A presented an increase of 56% in ethanol yield (0.16 vs. 0.25) and a 2.3-fold increase in volumetric productivity (0.065 versus 0.150) in the mixed medium (Tables 3 and 4). One possible explanation for this observation is that D-xylose might have been diverted to biomass formation (as seen in the respective individual aerobic environment), whereas glucose was probably directed to fermentation. Thus, the combined effects of D-xylose and glucose increase the aerobic fermentative performance of *B. bruxellensis*. However, it was not possible to determine whether the additional ethanol produced in these assays was xylose driven because the values found (7 g/L) might be stoichiometrically linked solely to the presence of glucose in the medium (Table 4). In the scenarios in which oxygen was limited, we observed distinct fermentative parameters and opposite profiles in some cases. *B. bruxellensis* cells consumed more D-xylose in mixed media than in the individual medium (Figures 3 and 4 and Tables 3 and 4). Because L-arabinose was not significantly consumed under the conditions tested, it is likely that increasing the rate of D-xylose consumption is an effect of the glucose present in the medium (Figures 4 and Tables 3 and 4). This could be related to the fact that glucose is required for the production of metabolic intermediates for the initial reactions of D-xylose metabolism and PPP through the regeneration of NADPH, required for

conversion of D-xylose to xylitol (Meinander & Hahn-Hagerdal, 1997; Meinander et al., 1999). However, as oxygen availability is low in our case, the process to regenerate NAD+ (the cofactor for XDH) under these conditions has yet to be determined. In addition, when cells were grown in mixed medium III (D-xylose at 40 g/L and 96 h cultivation), the yields were higher than those in mixed medium II (D-xylose at 20 g/L and 48 h cultivation). In this case, we observed an increase in ethanol yield ranging from 19% to 47%, depending on the strain (0.25, 0.24 and 0.25 versus 0.17, 0.17 and 0.21, Table 4). On the other hand, the volumetric productivity was reduced by half in the same comparison (almost three times for JP19M strain), weakening the fermentation performance of our strains (0.042, 0.045 and 0.046 versus 0.103, 0.106 and 0.122, Table 4). Therefore, the presence of glucose did not significantly enhance the fermentative capacity of *B. bruxellensis* in D-xylose.

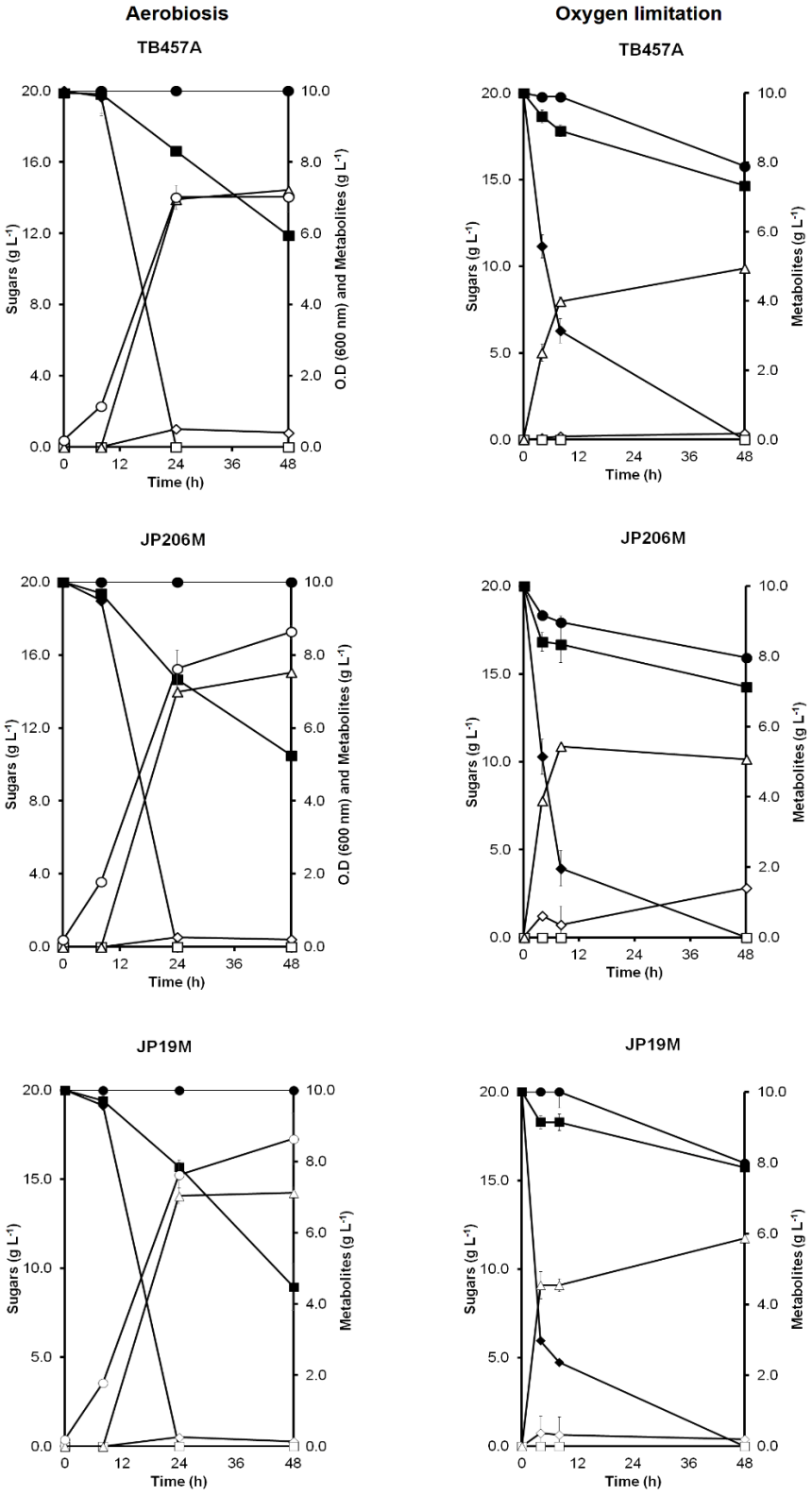
Finally, all strains started consuming D-xylose before the complete exhaustion of glucose in oxygen-limited conditions (Figure 4). It seems that glucose repression is less strict in our strains than it is in *S. cerevisiae*, as it was previously reported (da Silva et al., 2019; Leite, Leite, Pereira, de Barros, & de Morais, 2016), allowing both sugars to be co-consumed. This is an interesting result because the co-utilization of glucose and D-xylose is not common, but it is essential for the conversion of lignocellulose to ethanol to be economically viable (Rech et al., 2019). For example, genetically modified strains of *S. cerevisiae* are able to consume D-xylose only after glucose utilization because of its repressive effect on the metabolism of secondary carbon sources (Scalcinati et al., 2012). Similar to our results, glucose does not repress the use of D-xylose under aerobic conditions when a rich medium is supplied to *S. passalidarum*, allowing the co-consumption of these sugars (Hou, 2012; Rodrussamee et al., 2018). In this sense, this is another relevant metabolic trait presented by *B. bruxellensis* that could be industrially explored.

TABLE 4 Fermentative parameters of *Brettanomyces bruxellensis* industrial isolates in mixed media under aerobic conditions and oxygen limitation

Strains	Oxygen availability	Condition	Glucose consumed (g/L)	D-Xylose consumed (g/L)	L-Arabinose consumed (g/L)	$Y_{x/s}$	$Y_{p/s}$ ethanol	Productiv. (g.l.h)
TB457A	Aerobiosis	Mixed medium I	20.00 ± 0.00^{a}	8.11 ± 0.02^{a}	0.00 ± 0.00^{a}	0.25 ± 0.01^{a}	0.26 ± 0.00^{a}	0.150 ± 0.00^{a}
	O_2 limitation	Mixed medium II	20.00 ± 0.00^{a}	5.35 ± 0.01^{b}	4.23 ± 0.13^{b}	0.12 ± 0.01^{b}	0.17 ± 0.01^{b}	0.103 ± 0.00^{b}
		Mixed medium III	10.00 ± 0.00^{b}	6.35 ± 0.49^{b}	0.00 ± 0.00^{a}	0.36 ± 0.01^{c}	0.25 ± 0.02^{a}	0.042 ± 0.00^{c}
JP206M	Aerobiosis	Mixed medium I	20.00 ± 0.00^{a}	9.58 ± 0.06^{a}	0.00 ± 0.00^{a}	0.28 ± 0.01^{a}	0.25 ± 0.00^{a}	0.157 ± 0.00^{a}
	O_2 limitation	Mixed medium II	20.00 ± 0.00^{a}	5.74 ± 0.01^{b}	4.06 ± 0.11^{b}	0.05 ± 0.00^{b}	0.17 ± 0.01^{b}	0.106 ± 0.00^{b}
		Mixed medium III	10.00 ± 0.00^{b}	6.60 ± 0.28^{c}	1.39 ± 0.02^{c}	0.33 ± 0.02^{c}	0.24 ± 0.00^{a}	0.045 ± 0.00^{c}
JP19M	Aerobiosis	Mixed medium I	20.00 ± 0.00^{a}	11.16 ± 0.22^{a}	0.00 ± 0.00^{a}	0.24 ± 0.01^{a}	0.22 ± 0.01^{a,b}	0.148 ± 0.00^{a}
	O_2 limitation	Mixed medium II	20.00 ± 0.00^{a}	4.28 ± 0.08^{b}	4.01 ± 0.07^{b}	0.15 ± 0.00^{b}	0.21 ± 0.01^{a,b}	0.122 ± 0.01^{b}
		Mixed medium III	10.00 ± 0.00^{b}	6.25 ± 0.21^{c}	1.33 ± 0.06^{c}	0.27 ± 0.02^{a}	0.25 ± 0.01^{a}	0.046 ± 0.00^{c}

Note: Mixed I and mixed II containing 20 g/L from each source for 48 h; mixed III containing 10 g/L glucose, 40 g/L xylose and 7 g/L arabinose for 96 h. $Y_{x/s}$, cell yield coefficient (g biomass produced/g substrate utilized); $Y_{p/s}$, product yield coefficient (g product/g substrate utilized); Productiv, volumetric productivity of ethanol. Superscripts a, b and c: Tukey's test ($p < 0.05$) indicating whether or not differences among cultivation conditions are statistically significant. Comparisons were performed within a given strain, as shown by superscripts letters in the columns. Mean values with the same letter indicate no significant difference, whereas values with different superscript letters are significantly different.

FIGURE 4 Sugar consumption curves and industrial metabolite production by *Brettanomyces bruxellensis* isolates in aerobiosis and oxygen limitation in the mixed environment. Closed diamond, glucose; closed square, D-xylose; closed circle, L-arabinose; open circle, OD (optical density); open triangle, ethanol; open square, acetate; open diamond, glycerol; asterisk, xylitol.



4 CONCLUSION

The results reported in the present work show that, despite being able to use both D-xylose and L-arabinose as carbon source, *B. bruxellensis* does it preferentially via biomass formation to the detriment of fermentation when in aerobiosis. In oxygen limitation, their consumption is decreased and results in little or no ethanol. Moreover, even when ethanol was produced, the volumetric productivity was significantly lower than the ones found in other microorganisms used for second generation ethanol production. Therefore, despite showing industrially relevant traits, *B. bruxellensis* presents bottlenecks regarding the management of D-xylose and L-arabinose towards fermentative metabolism, which narrow its feasible utilization in second-generation ethanol production. On the other hand, we observed that D-xylose and glucose might be co-consumed, which is a desired trait for a microorganism to be employed in this industry. This latter finding, along with the fact that *B. bruxellensis* natively uses D-xylose and tolerates several industrial stresses, should drive future studies aiming to develop genetically modified strains able to overcome the metabolic constraints in pentose metabolism.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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REFERENCES

Bassi, A. P. G., Silva, J. C. G., Reis, V. R., & Ceccato-Antonini, S. R. (2013). Effects of single and combined cell treatments based on low pH and high concentrations of ethanol on the growth and fermentation of *Brettanomyces bruxellensis* and Saccharomyces cerevisiae. World

Journal of Microbiology and Biotechnology, 29, 1661–1676. https://doi.org/10.1007/s11274-013-1329-x

Blomqvist, J., South, E., Tiukova, L., Momeni, M. H., Hansson, H., Ståhlberg, J., … Passoth, V. (2011). Fermentation of lignocellulosic hydrolysate by the alternative industrial ethanol yeast *Brettanomyces bruxellensis*. Letters in Applied Microbiology, 53, 73–78. https://doi.org/10.1111/j.1472-765X.2011.03067.x

Boles, E., Ller, S., & Zimmermann, F. K. (1996). A multi-layered sensory system controls yeast glycolytic gene expression. Molecular Microbiology, 1996(19), 641 642. https://doi.org/10.1046/j.1365-2958.1996.t01-1-442924.x

Codato, C. B., Martini, C., Ceccato-Antonini, S. R., & Bastos, R. G. (2018). Ethanol production from *Brettanomyces bruxellensis* in synthetic media with pentose. Journal of Chemical Engineering, 35, 11–17. https://doi.org/10.1590/0104-6632.20180351s20160475

Crauwels, S., Van Assche, A., de Jonge, R., Borneman, A. R., Verreth, C., Troels, P., … Curtin, C. D. (2015). Comparative phenomics and targeted use of genomics reveals variation in carbon and nitrogen assimilation among different *Brettanomyces bruxellensis* strains. Applied Microbiology and Biotechnology, 91, 23–34. https://doi.org/10.1007/s00253-015-6769-9

da Silva, J. M., Silva, G. H. T. G., Parente, D. C., Leite, F. C. B., Silva, C. S., Valente, P., … de Morais Junior, M. A. (2019). Biological diversity of carbon assimilation among isolates of the yeast *Brettanomyces bruxellensis* from wine and fuel-ethanol industrial processes. FEMS Yeast Research, 19, 1–10. https://doi.org/10.1093/femsyr/foz022

da Silva, T. C., Leite, F. C., & de Morais, M. A. Jr. (2016). Distribution of *Brettanomyces bruxellensis* in a sugarcane-based fuel ethanol fermentation plant. Letters in Applied Microbiology, 62, 354–358. https://doi.org/10.1111/lam.12558

de Barros Pita, W., Castro Silva, D., Simões-Ardaillon, D., Volkmar, P., & de Morais, M. A. (2013). Physiology and gene expression profiles of *Dekkera bruxellensis* in response to carbon and nitrogen availability. Antonie Van Leeuwenhoek, 104, 855–868

de Barros Pita, W., Leite, F. C. B., de Souza Liberal, A. T., Simões, D. A., & de Morais, M. A. Jr. (2011). The ability to use nitrate confers advantage to *Brettanomyces bruxellensis* over *S. cerevisiae* and can explain its adaptation to industrial fermentation processes. Antonie Van Leeuwenhoek, 100, 99–107. https://doi.org/10.1007/s10482-011-9568-z

de Barros Pita, W., Teles, G. H., Peña-Moreno, I. C., da Silva, J. M., Ribeiro, K. C., & de Morais Junior, M. A. (2019). The biotechnological potential of the yeast *Brettanomyces bruxellensis*. World Journal of Microbiology and Biotechnology, 35, 103. https://doi.org/10.1007/s11274-019-2678-x

de Matos, J. P., Souza, K. R., Santos, A. S., & Pantoja, L. A. (2018). Fermentação alcoólica de hidrolisado hemicelulósico de torta de girassol por *Galactomyces geotrichum* UFVJM-R10 e *Candida akabanensis* UFVJM-R131. Química Nova, 41, 23–29. https://doi.org/10.21577/0100-4042.20170146

de Souza Liberal, A. T., Basílio, A. C. M., do Monte Resende, A., Brasileiro, B. T. V., da Silva-Filho, E. A., de Morais, J. O., … de Morais, M. A. Jr. (2007). Identification of *Dekkera bruxellensis* as a major contaminant yeast in continuous fuel ethanol fermentation. Journal of Applied Microbiology, 102, 538–547. https://doi.org/10.1111/j.1365-2672.2006.03082.x

de Souza, R. F. R., Dutra, E. D., Leite, F. C. B., Cadete, R. M., Rosa, C. A., Stambuk, B. U., … De Morais, M. A. Jr. (2018). Production of ethanol fuel from enzyme-treated sugarcane bagasse hydrolysate using D-xylose-fermenting wild yeast isolated from Brazilian biomes. 3 Biotech, 8, 312. https://doi.org/10.1007/s13205-018-1340-x

Dickinson, J. R., & Schweizer, M. (2004). The metabolism and molecular physiology of *Saccharomyces cerevisiae* (Second ed., Vol. 108) (p. 1105). London: Taylor & Francis ltd. https://doi.org/10.1017/S0953756204211169

Dien, B. S., Kurtzman, C. P., Saha, B. C., & Bothast, R. J. (1996). Screening for L-arabinose fermenting yeasts. Applied biochemistry and biotechnology 1996, Spring57-58, 233–242

Fonseca, C., Spencer-Martins, I., & Hahn-Hägerdal, B. (2007). L-arabinose metabolism in *Candida arabinofermentans* PYCC 5603 and *Pichia guilliermondii* PYCC 3012: Influence of sugar and oxygen on product

formation. Applied Microbiology and Biotechnology, 75, 303–310. https://doi.org/10.1007/s00253-006-0830-7

Galafassi, S., Merico, A., Pizza, F., Hellborg, L., Molinari, F., Piškur, J., & Compagno, C. (2011). *Dekkera/Brettanomyces* yeasts for ethanol production from renewable sources under oxygen-limited and low-pH conditions. Journal of Industrial Microbiology & Biotechnology, 38, 1079–1088. https://doi.org/10.1007/s10295-010-0885-4

Gamage, J., Lam, H., & Zhang, Z. (2010). Bioethanol production from lignocellulosic biomass, a review. Journal of Biobased Materials and Bioenergy, 4, 3–11. https://doi.org/10.1166/jbmb.2010.1071

Goldemberg, J. (2013). Sugarcane ethanol: Strategies to a successful program in Brazil. In J. Lee (Ed.), Advanced biofuels and bioproducts. New York, NY: Springer. https://doi.org/10.1007/978-1-4614-3348-4_2

Granados-Arvizu, J. A., Madrigal-Perez, L. A., Canizal-Garcia, M., González-Hernández, J. C., García-Almendárez, B. E., & Regalado-González, C. (2019). Effect of cytochrome bc1 complex inhibition during fermentation and growth of *Scheffersomyces stipitis* using glucose, xylose, or arabinose as carbon sources. FEMS Yeast Research, 19, 1–8. https://doi.org/10.1093/femsyr/foy126

Guilherme, A. D. A., Victor, P., Dantas, F., Eduardo, C., Padilha, D. A., Silvino, E., & de Macedo, G. R. (2019). Ethanol production from sugarcane bagasse: Use of different fermentation strategies to enhance an environmental-friendly process. Journal of Environmental Management,234, 44–51. https://doi.org/10.1016/j.jenvman.2018.12.102

Hahn-Hägerdal, B., Galbe, M., Gorwa-Grauslund, M.-F., Lidén, G., & Zacchi, G. (2006). Bioethanol—The fuel of tomorrow from the residues of today. Trends in Biotechnology, 24, 549–556. https://doi.org/10.1016/j.tibtech.2006.10.004

Hahn-Hagerdal, B., Karhumaa, K., & Jeppsson, M. (2007). Metabolic engineering for pentose utilization in *Saccharomyces cerevisiae*. Advances in Biochemical Engineering/Biotechnology, 108, 147–177. https://doi.org/10.1007/10_2007_062

Hou, X. (2012). Anaerobic xylose fermentation by *Spathaspora passalidarum*. Applied Microbiology and Biotechnology, 94, 205–214. https://doi.org/10.1007/s00253-011-3694-4

Jeffries, T. W. (2006). Engineering yeasts for xylose metabolism. Current Opinion in Biotechnology, 17, 320–326. https://doi.org/10.1016/j.copbio.2006.05.008

Jeffries, T. W., Grigoriev, I. V., Grimwood, J., Laplaza, J. M., Aerts, A., Salamov, A., … Richardson, P. M. (2007). Genome sequence of the lignocellulose-bioconverting and xylose-fermenting yeast *Pichia stipitis*. Nature Biotechnology, 25(3), 319–326. https://doi.org/10.1038/nbt1290

Kurtzman, C., Fell, J., & Boekhout, T. (2011). The yeasts: A taxonomic study. Amsterdam: Elsevier.

Leite, F. C., Leite, D. V., Pereira, L. F., de Barros, P. W., & de Morais, M. A. Jr. (2016). High intracellular trehalase activity prevents the storage of trehalose in the yeast *Brettanomyces bruxellensis*. Letters in Applied Microbiology, 63, 210–214. https://doi.org/10.1111/lam.12609

Leite, F. C. B., Basso, T. O., de Barros, P. W., Gombert, A. K., Simões, D. A., & de Morais Jr, M. A. (2013). Quantitative aerobic physiology of the yeast *Brettanomyces bruxellensis*, a major contaminant in bioethanol production plants. FEMS Yeast Research, 13, 34–43. https://doi.org/10.1111/j.1567-1364.2012.12007.x

Meinander, N. Q., Boels, I., & Hahn-Hägerdal, B. (1999). Fermentation of xylose/glucose mixtures by metabolically engineered *Saccharomyces cerevisiae* strains expressing XYL1 and XYL2 from *Pichia stipitis* with and without overexpression of TAL1. Bioresource Technology, 68, 79–87. https://doi.org/10.1016/S0960-8524(98)00085-6

Meinander, N. Q., & Hahn-Hagerdal, B. (1997). Influence of cosubstrate concentration on xylose conversion by recombinant, XYL1-expressing *Saccharomyces cerevisiae*: A comparison of different sugars and ethanol as cosubstrates. Applied and Environmental Microbiology, 63, 1959–1964. https://doi.org/10.1128/AEM.63.5.1959-1964.1997

Moktaduzzaman, M., Galafassi, S., Capusoni, C., Vigentini, I., Ling, Z., Piskur, J., & Compagno, C. (2015). Galactose utilization sheds new light on sugar metabolism in the sequenced strain *Brettanomyces bruxellensis* CBS

2499. FEMS Yeast Research, 15, 1–9. https://doi.org/10.1093/femsyr/fou009

Nakanishi, S. C., Soares, L. B., Biazi, L. E., Nascimento, V. M., Costa, A. C., Rocha, G. J. M., & Lenczak, J. L. (2017). Fermentation strategy for second generation ethanol production from sugarcane bagasse hydrolysate by *Spathaspora passalidarum* and *Scheffersomyces stipitis*. Biotechnology and Bioengineering, 114, 2211–2221. https://doi.org/10.1002/bit.26357

Nogueira, C. C., Padilha, C. E. A., Leit~ao, A. L. S., Rocha, P. M., Macedo, G. R., & Santos, E. S. (2018). Enhancing enzymatic hydrolysis of green coconut fiber—Pretreatment assisted by tween 80 and water effect on the post-washing. Industrial Crops and Products, 112, 734–740. https://doi.org/10.1016/j.indcrop.2017.12.047

Olofsson, K., Bertilsson, M., & Lidén, G. (2008). A short review on SSF—An interesting process option for ethanol production from lignocellulosic feedstocks. Biotechnology Biofuels, 1, 7. https://doi.org/10.1186/1754-6834-1-7

Peña-Moreno, I. C., Castro Parente, D., da Silva, J. M., Andrade Mendonça, A., Rojas, L. A. V., Morais, M. A. Jr., & de Barros, P. W. (2019). Nitrate boosts anaerobic ethanol production in an acetate dependent manner in the yeast *Brettanomyces bruxellensis*. Journal of Industrial Microbiology & Biotechnology, 46, 209–220. https://doi.org/10.1007/s10295-018-2118-1

Pereira, L. F., Bassi, A. P., Avansini, S. H., Neto, A. G., Brasileiro, B. T., Ceccato-Antonini, S. R., & de Morais, M. A. Jr. (2012). The physiological characteristics of the yeast *Dekkera bruxellensis* in fully fermentative conditions with cell recycling and in mixed cultures with *Saccharomyces cerevisiae*. Antonie Van Leeuwenhoek, 101, 529–539. https://doi.org/10.1007/s10482-011-9662-2

Rech, R. F., Fontana, C. R., Rosa, A. C., Camassola, M., Ayub, M., & Dillon, J. P. A. (2019). Fermentation of hexoses and pentoses from sugarcane bagasse hydrolysates into ethanol by *Spathaspora hagerdaliae*. Bioprocess and Biosystems Engineering, 42, 83–92. https://doi.org/10.1007/s00449-018-2016-y

Reis, A. L. S., de Souza, R. F. R., Torres, R. R. N. B., Leite, F. C. B., Paiva, P. M. G., Vidal, E. E., & de Morais Jr, M. A. (2014). Oxygen limited cellobiose

fermentation and the characterization of the cellobiase of an industrial *Dekkera/Brettanomyces bruxellensis* strain. Springer Plus, 3, 1–9. https://doi.org/10.1186/2193-1801-3-38

Richard, P., Verho, R., Putkonen, M., Londesborough, J., & Penttila, M. (2003). Production of ethanol from L-arabinose by *Saccharomyces cerevisiae* containing a fungal L-arabinose pathway. FEMS Yeast Research, 3, 185–189. https://doi.org/10.1016/S1567-1356(02)00184-8

Robak, K., & Balcerek, M. (2018). Review of second-generation bioethanol production from residual biomass. Food Technol Biotech, 56, 174–187. https://doi.org/10.17113/ftb.56.02.18.5428

Rocha, G. J. M., Nascimento, V. M., Gonçalves, A. R., Silva, V. F. N., & Martín, C. (2015). Influence of mixed sugarcane bagasse samples evaluated by elemental and physical–chemical composition. Industrial Crops and Products, 64, 52–58. https://doi.org/10.1016/j.indcrop.2014.11.003

Rodrussamee, N., Sattayawat, P., & Yamada, M. (2018). Highly efficient conversion of xylose to ethanol without glucose repression by newly isolated thermotolerant *Spathaspora passalidarum* CMUWF1–2. BMC Microbiology, 18, 1–11. https://doi.org/10.1186/s12866-018-1218-4

Rozpędowska, E., Hellborg, L., Ishchuk, O. P., Orhan, F., Galafassi, S., Merico, A., … Piškur, J. (2011). Parallel evolution of the make-accumulate-consume strategy in *Saccharomyces* and *Brettanomyces* yeasts. Nature Communications, 2, 302. https://doi.org/10.1038/ncomms1305

Rudolf, A., Baudel, H., & Zacchi, G. (2008). Simultaneous saccharification and fermentation of steam-pretreated bagasse using *Saccharomyces cerevisiae* TMB3400 and *Pichia stipitis* CBS6054. Biotechnology and Bioengineering, 99, 783–790. https://doi.org/10.1002/bit.21636

Scalcinati, G., Otero, J. M., van Vleet, J. R. H., Jeffries, T. W., Olsson, L., & Nielsen, J. (2012). Evolutionary engineering of *Saccharomyces cerevisiae* for efficient aerobic xylose consumption. FEMS Yeast Research, 12, 582–597. https://doi.org/10.1111/j.1567-1364.2012.00808.x

Shin, M., Kim, J., Ye, S., Kim, S., Jeong, D., Lee, D. Y., … Kim, S. R. (2019). Comparative global metabolite profiling of xylose-fermenting *Saccharomyces cerevisiae* SR8 and *Scheffersomyces stipitis*. Appl

Microbiol Biotechnol, 103, 5435–5446. https://doi.org/10.1007/s00253-019-09829-5

Silverstein, R. A., Chen, Y., Sharma-Shivappa, R. R., & Boyette, M. D. J. O. (2007). A comparison of chemical pretreatment methods for improving saccharification of cotton stalks. Bioresource Technology, 98, 3000–3011. https://doi.org/10.1016/j.biortech.2006.10.022

Skoog, K., & Hahn-Hägerdal, B. (1990). Effect of oxygenation on xylose fermentation by *Pichia stipitis*. Applied and Environmental Microbiology, 56, 3389–3394. https://doi.org/10.1128/AEM.56.11.3389-3394.

Sousa-Aguiar, E. F., Appel, L. G., Zonetti, P. C., & Fraga, A. C. (2014). Some important catalytic challenges in the bioethanol integrated biorefinery. Catalysis Today, 234, 13–23. https://doi.org/10.1016/j.cattod.2014.02.016

Teles, G. H., da Silva, J. M., Mendonça, A. A., de Morais Junior, M. A., & de Barros, P. W. (2018). First aspects on acetate metabolism in the yeast *Brettanomyces bruxellensis*: A few keys for improving ethanol fermentation. Yeast, 35, 577–584. https://doi.org/10.1002/yea.3348

Tiukova, I. A., de Barros, P. W., Sundell, D., Haddad Momeni, M., Horn, S. J., de Morais, M. A., & Passoth, V. (2014). Adaptation of *Brettanomyces bruxellensis* to lignocellulose-based substrate. Biotechnology and Applied Biochemistry, 61, 51–57. https://doi.org/10.1002/bab.1145

Veras, H. C. T., Parachin, N. S., & Almeida, J. R. M. (2017). Comparative assessment of fermentative capacity of different xylose-consuming yeasts. Microbial Cell Factories, 161, 1–8. https://doi.org/10.1186/s12934-017-0766-x

Wisselink, H. W., Toirkens, M. J., del Rosario Franco, B. M., Winkler, A. A., van Dijken, J. P., Pronk, J. T., & van Maris, A. J. A. (2007). Engineering of *Saccharomyces cerevisiae* for efficient anaerobic alcoholic fermentation of L-arabinose. Applied and Environmental Microbiology, 73, 4881–4891. https://doi.org/10.1128/AEM.00177-07

Wisselink, H. W., Toirkens, M. J., Wu, Q., Pronk, J. T., & van Maris, A. J. A. (2009). Novel evolutionary engineering approach for accelerated utilization of glucose, xylose, and arabinose mixtures by engineered *Saccharomyces cerevisiae* strains. Applied and Environmental Microbiology, 75, 907–914. https://doi.org/10.1128/AEM.02268-08

Yuvadetkun, P., Leksawasdi, N., & Boonmee, M. (2017). Kinetic modeling of *Candida shehatae* ATCC 22984 on xylose and glucose for ethanol production. Preparative Biochemistry & Biotechnology, 16, 268–275. https://doi.org/10.1080/10826068.2016.1224244

6 ARTIGO 3 - *BRETTANOMYCES BRUXELLENSIS* JP19M STRAIN DOES NOT RESPOND TO GLUCOSE CATABOLITE REPRESSION AND MIGHT BE A TARGET FOR GENETIC ENGINEERING STRATEGIES AIMING ITS APPLICATION IN SECOND-GENERATION ETHANOL INDUSTRY

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Significance and Impact of the Study

The present study reports the absence of Glucose Catabolite Repression in a specific strain of the yeast *Brettanomyces bruxellensis*, which results in the possibility to simultaneously consume glucose and xylose. This feature is of great interest to second-generation ethanol industry, since the full management of sugars present in lignocellulosic hydrolysates is essential to achieve high efficiency in the process. Our results show that *B. bruxellensis* JP19M strain is a clear target for the development of genetic engineering strategies that could bring this species to the frontline as an ethanol producer in this industrial sector.

Abstract

Second-generation ethanol production is a promising but still challenging industrial process, in which the microorganism of choice is essential to achieve high efficiency. *Brettanomyces bruxellensis* has been suggested as a potential candidate to be employed in this industry, since it presents relevant physiological features, especially regarding to its fermentative capacity and resistance to inhibitors present in the industrial substrate. Previous works have suggested that Glucose Catabolite Repression (GCR) is a strain-specific trait in *B. bruxellensis*, as well as less strict than in *Saccharomyces cerevisiae*. A less stringent GCR is important since glucose often inhibits the utilization of other carbon sources, decreasing the efficiency of the fermentative process. In this sense, we investigated whether glucose presents any repressive effect in the assimilation of xylose, the major pentose in lignocellulosic hydrolysate, in *B. bruxellensis* JP19M strain. By analysing both physiological and gene expression data, we were able to confirm that JP19M does not respond to GCR, which increases its list of industrially relevant traits. Our results might also enhance the potential interest in the application of *B. bruxellensis* in the second-generation ethanol production as well as boost the development of genetic engineering strategies aiming to overcome its poor fermentative capacity in xylose.

Keywords: xylose metabolism; fermentation; ethanol production; glucose repression; sugar co-consumption; gene expression.

Introduction

The yeast *Brettanomyces bruxellensis* can produce ethanol by fermentation from different carbon sources and under different oxygen availabilities, such as aerobiosis, oxygen limitation and anaerobiosis (de Souza Liberal *et al*. 2007; Pereira *et al*. 2012; de Barros Pita *et al.* 2011, 2013b; Crauwels *et al*. 2015; da Silva *et al*. 2019; Peña-Moreno *et al*. 2019). The adaptation to different industrial environments allows *B. bruxellensis* to compete with *Saccharomyces cerevisiae* for different substrates, as well as to use sugars not natively consumed by wild-type strains of *S. cerevisiae*, such as cellobiose, D-xylose and L-arabinose (Reis *et al*. 2014; Codato *et al*. 2018; da Silva *et al*. 2020). Besides a wide range of sugar assimilation and the ability to produce ethanol, the tolerance to fermentation inhibitors found in the lignocellulosic hydrolysate led *B. bruxellensis* to be recently suggested as a potential ethanol producer in second-generation ethanol industry (de Barros Pita *et al*. 2019). However, an important metabolic bottleneck was later reported, which ultimately hinders the immediate application of *B. bruxellensis* in second-generation ethanol plants. The restriction is related to a poor fermentative performance in D-xylose and L-arabinose, the major pentoses found in lignocellulosic hydrolysate, which are important to a full exploitation of the industrial substrate (Codato *et al.* 2018; da Silva *et al*. 2020).

In yeasts, xylose is internalized by hexose transporters, such as *HXT4*, *HXT5*, *HXT7* and *GAL2*, which have high affinity for this sugar (Hamacher *et al*. 2002). Once inside the cell, xylose is reduced to xylitol by the enzyme xylose reductase (XR), which is then oxidized to xylulose by xylitol dehydrogenase (XDH). Subsequently, xylulose is phosphorylated by xylulokinase (XK) to xylulose-5-phosphate, which proceeds to the pentose-phosphate pathway (PPP) for the generation of glycolysis intermediates fructose-6-phosphate and glyceraldehyde-3-phosphate (Jeffries *et al*. 2007; Olofsson *et al*. 2008). Due to a differential usage of cofactors between XR (which preferentially uses NADPH) and XDH (which uses NAD^+), the flux through the xylose assimilatory pathway leads to a redox imbalance when oxygen is a limiting factor (Hahn-Hagerdal *et al*. 2007). In fact, oxygen limitation or anaerobiosis decreases or even prevents NAD^+ regeneration, which leads to xylitol accumulation and subsequently to the interruption of xylose assimilation (Skoog and Hahn-Hagerdal 1990; Hahn-

Hagerdal *et al*. 2007). In this sense, the presence of oxygen is then required for the reoxidation of NAD^+ via Electron Transport Chain (ETC), in order to prevent the disruption of xylose metabolism (Hahn-Hägerdal *et al*. 2007). Therefore, the redox imbalance mentioned above is the major direct obstacle for the use of xylose by yeasts, such as *Scheffersomyces (Pichia) stipitis* and *B. naardenensis* (Galafassi *et al*. 2011, Hou 2012). On the other hand, both XR and XDH enzymes use NADH as cofactor in *Spathaspora passalidarum*, which favours the xylose consumption under oxygen-limited and anaerobic conditions (Hou 2012). This feature boosts this yeast to be the preferred microorganism in second-generation ethanol production plants (Hou 2012; Cadete *et al.* 2016; Yu *et al.* 2017). Nevertheless, crescent search for yeasts capable of consuming xylose is of great importance to ensure the possible application of different yeasts in the process.

Despite the redox imbalance, *B. bruxellensis* JP19M strain has been linked to a physiological trait that cannot go unnoticed, which is the ability to co-consume glucose and xylose, an aspect far from trivial when it comes to yeast (da Silva *et al*. 2020). This finding is quite important, especially for the fermentation industry since this ability ultimately allows the microorganism to a rapid and simultaneous conversion of different sugars to ethanol (Hua *et al*. 2019). In fact, the co-consumption of sugars when glucose is available is a rare trait in yeasts, since even low amounts are usually enough to trigger a metabolic effect often called “Glucose Catabolite Repression” (GCR) or “glucose effect” (Rettori and Volpe 2000; Stasyk and Stasyk 2019). Despite being a rather generic term, the GCR has a clear phenotype, which is the preference for glucose assimilation to the detriment of other carbon sources (Peeters and Thevelein 2014; Lane *et al*. 2018). In *S. cerevisiae*, the glucose effect has a well-documented origin, an intricate and coordinated network of proteins acting as key regulators, which differentially control the expression of several groups of genes (Kayikci and Nielsen 2015; Stasyk and Stasyk 2019). For instance, while the presence of glucose triggers the activation of fermentation-related genes, the ones involved in the respiratory metabolism are deactivated (Hagman and Piskur 2015). This genetic programming is one of the pillars of another metabolic trait, the so-called Crabtree effect. The Crabtree effect is the ability to ferment even in presence of oxygen, when glucose concentration reaches a specific threshold (de Deken 1966; Postma *et al.* 1989; Hagman and Piškur 2015). In addition, the

Crabtree effect improves cell adaptation to glucose metabolism through increased glycolytic ATP and ethanol production (Rothman *et al*. 2021). In *B. bruxellensis*, on the other hand, much less information is available in this regard. Previous studies with GDB 248 strain reported that *FBP1* gene (which encodes fructose-1,6-bisphosphatase, the key regulatory enzyme of gluconeogenesis) is downregulated after a glucose pulse (Leite *et al*. 2016). On the other hand, *TPS2* gene (which codes for the phosphatase subunit of the trehalose-6-P synthase/phosphatase complex) did not respond to glucose pulse, unlike *S. cerevisiae* (Winderickx *et al*. 1996; Leite *et al*. 2016). In addition, it was observed that *B. bruxellensis* strains isolated from fuel-ethanol plants are more resistant to glucose repression when compared to wine isolates, suggesting that GCR is a strain-specific trait in this yeast (da Silva *et al*. 2019). Finally, another major regulatory mechanism, the Nitrogen Catabolite Repression (NCR) is not as tightly controlled in *B. bruxellensis* than it is in *S. cerevisiae* (de Barros Pita *et al*. 2011; de Barros Pita *et al.* 2013b; Conrad *et al.* 2014; Peña-Moreno *et al*. 2019). For instance, in sugarcane juice, *B. bruxellensis* can simultaneously use ammonium and nitrate, primary and secondary nitrogen sources, respectively (de Barros Pita *et al*. 2011; Peña-Moreno *et al*. 2019).

Since the consumption of sugars other than glucose in the substrate might accelerate the industrial process and increase both yield and productivity, we aimed to further investigate the GCR effect in *B. bruxellensis* JP19M strain. Once it has been recently suggested that this strain might not be under a strict GCR control (da Silva *et al*. 2019; da Silva *et al.* 2020), we analysed whether glucose could trigger any repressive effect on the consumption of xylose by this strain, both in physiological and transcriptional levels. The results presented herein confirm that *B. bruxellensis* JP19M is not susceptible to glucose effect and continues to assimilate both glucose and xylose in mixed medium. This is possible because the presence of glucose does not halt the expression of genes expected to be under GCR control, such as the xylose-assimilatory ones. Altogether, our data pile up with other previously described advantageous traits presented by *B. bruxellensis* and point to a possible employment of JP19M strain in the second-generation ethanol industry, provided genetic engineering strategies are developed to bypass its metabolic bottleneck.

Results and Discussion

Glucose does not prevent the utilisation of xylose in *B. bruxellensis* JP19M under aerobic conditions

Since the glucose repression effect seems to be a strain-dependent trait in *B. bruxellensis* (da Silva *et al*. 2019), we aimed to investigate the real influence of glucose on the xylose metabolism in JP19M strain. In the present work, we evaluated how cells respond to the presence of a mixture of glucose and xylose in assays carried out aerobically for three hours (a time frame in which cells are adapted to the medium and both sugars are still available). In this sense, we attempted to determine whether any initial glucose concentration could trigger a repressive effect in JP19M. Therefore, we performed physiological analyses in samples collected from cultivations in which the glucose concentration ranged from 0.5 to 5 g l^{-1}, while xylose concentration was kept steady at 10 g l^{-1}. Our results showed that the presence of glucose did not inhibit xylose consumption by *B. bruxellensis* JP19M in aerobic conditions, in any of the concentrations tested (Table 1), similar to *S. passalidarum* (Hou 2012; Rodrussamee *et al.* 2018). This profile is in accordance with the assumption that glucose repression in some strains of *B. bruxellensis* is less tightly controlled than in *S. cerevisiae*, in which a concentration as low as 0.9 g l^{-1} of glucose can activate the glucose repression mechanism (Rettori and Volpe 2000; Leite *et al*. 2016; da Silva *et al*. 2019). The lack of an observable GCR phenotype in *B. bruxellensis* JP19M led us to increase glucose concentration up to 20 g l^{-1} (as in a standard growth assay), which did not interfere in the xylose consumption pattern for JP19M strain (data not shown).

The absence of GCR in a *B. bruxellensis* strain goes beyond a biological observation and has also a few implications, since glucose effect is an issue that inhibits the utilization, in industrial substrates, of sugars other than glucose, often decreasing fermentation efficiency (Lane *et al*. 2018). For instance, in order to the ethanol production from lignocellulosic hydrolysates be economically viable, co-consumption of glucose and xylose is necessary (Zha *et al*. 2014). Therefore, *B. bruxellensis* JP19M strain might be useful for fermentation processes involving a complex mixture of sugars. However, since *B. bruxellensis* do not efficiently ferment xylose, genetic engineering strategies must be developed prior to its

utilization (da Silva *et al*. 2020). Conversely, the presence of glucose at a low concentration (2 g l^{-1}) increased xylose consumption in *B. bruxellensis* JP19M (Table 1), suggesting that glucose either provides metabolic intermediates or at least favours the uptake of xylose in this strain. In fact, it has been observed that low glucose concentrations (~<2 g l^{-1}) improve xylose uptake and its subsequent conversion to ethanol in *S. cerevisiae* engineered strains (Pitkänen *et al*. 2003; Krahulec *et al*. 2010). This observation might seem contradictory, however, glucose induces the production of receptors for its own uptake, which can result, as a side effect, in an increase of xylose influx to the cell (Olofsson *et al*. 2008). Moreover, glucose can be directed to the pentose phosphate pathway (PPP) and produce NADPH, a cofactor required for xylose reductase, which reduces D-xylose to xylitol in the first reaction of xylose pathway (Meinander, Boels and Hahn-Hägerdal 1999; Jeffries *et al*. 2007).

Table 1 Sugar consumption by *B. bruxellensis* JP19M in medium containing a fixed concentration of xylose (10 g l^{-1}) and variable concentrations of glucose (0 to 5 g l^{-1}) after 3h of cultivation in aerobic conditions.

Initial glucose concentration (g l^{-1})	Glucose consumed (g l^{-1})	Xylose consumed (g l^{-1})
0	-	2.64 ± 0.05
0.5	0.39 ± 0.00	1.66 ± 0.06
1	0.60 ± 0.03	1.98 ± 0.10
2	1.11 ± 0.01	3.04 ± 0.00
3	1.30 ± 0.01	2.78 ± 0.10
4	1.56 ± 0.03	2.66 ± 0.09
5	1.63 ± 0.01	2.42 ± 0.04

B. bruxellensis JP19M is not susceptible to Glucose Catabolite Repression

Since glucose did not prevent the utilisation of xylose, we focused our attention to the elucidation of the genetic basis behind the observed absence of glucose repressor effect in JP19M strain. According to the GCR model for yeasts, rather well-established for *S. cerevisiae*, the presence of glucose should be able to block the expression of genes for the utilization of secondary carbon sources,

such as xylose (Lane *et al*. 2018). Moreover, GCR also hinders the expression of genes involved in some other cellular processes, such as gluconeogenesis and ATP synthesis, as well as glucose high-affinity transporters (Kayikci and Nielsen 2015; Pautasso *et al*. 2016). On the other hand, genes related to fermentative metabolism should be active in presence of glucose since fermentation is the major metabolic route for NADH reoxidation in this scenario (Bakker *et al*. 2001). Ultimately, this combination of regulatory actions is responsible for the establishment of the Crabtree effect, which directs yeast metabolism to fermentation rather than to respiratory metabolism (de Deken 1966; Hagman and Piškur 2015). The underlying mechanisms behind this physiological response are not fully understood, however, it is likely to be caused by the downregulation of respiratory genes (Hagman and Piškur 2015). In this sense, we analysed the expression of key genes involved in these important aspects of yeast central metabolism. The gene expression results presented below are consistent with the physiological findings that point out to JP19M as a non-responsive-to-GCR strain of *B. bruxellensis*, confirming that GCR is a strain-dependent trait in this species (da Silva *et al*. 2019).

First, we analysed the expression of genes that should be repressed by glucose, as expected for a yeast strain responsive to GCR. Therefore, we started our investigation by assessing the expression levels of *HXT6/7* gene, which codes for the high-affinity hexose transporter (K_m-values of 1-2 mM) and it is susceptible to GCR in *S. cerevisiae* (Walsh *et al*. 1994; Ozcan and Johnston 1999). Our results show that this gene was not influenced by the presence of glucose, remaining with stable expression, both in xylose-based and mixed media (Fig. 1A). *HXT6*, *HXT7* and *HXT6/7* are nearly identical genes that share >99% sequence identity in the coding regions, are similarly regulated and the coding sequence of the chimera (designated *HXT6/7*) differs from de *HXT6* and *HXT7* by two and three nucleotides, respectively (Liang and Gaber 1996). In *S. cerevisiae*, when the extracellular glucose concentration reaches 0,5% or higher, Snf3 (high-affinity extracellular glucose sensor) can generate a repression signal from the *HXT6* and *HXT7* genes (Liang and Gaber 1996; Ozcan 2002). Besides, despite being mostly related to the influx of glucose, *HXT7* is also involved with the uptake of xylose in *S. cerevisiae* (Hamacher *et al*. 2002; Alff-Tuomala *et al*. 2016; Cheng *et al*. 2018). Therefore, our results indicate not only that *HXT6/7*

does not respond to GCR but also that this gene is not directly involved with an increased xylose uptake when in presence of glucose (da Silva *et al*. 2020).

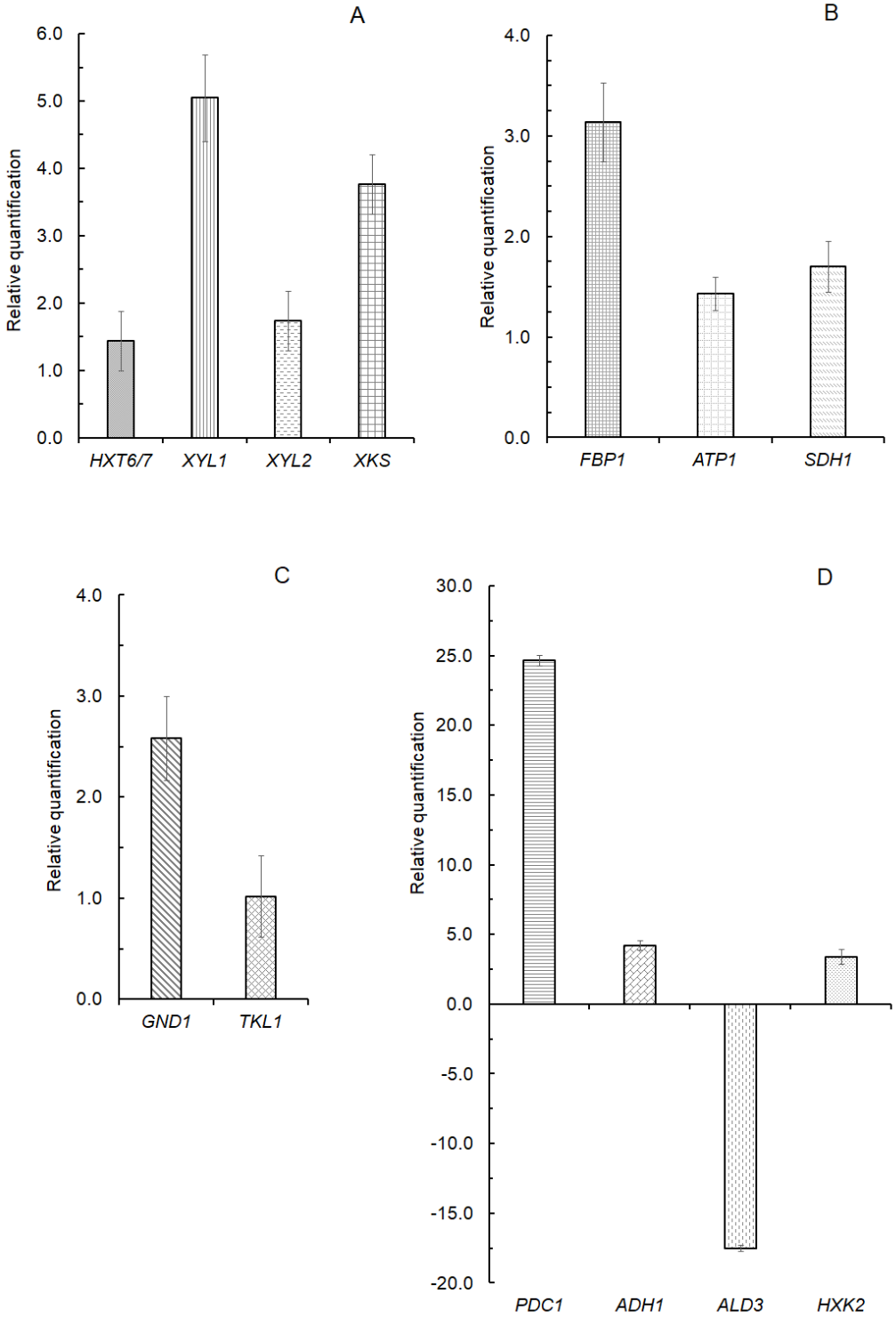
Further, we analysed the expression of genes involved in the early steps of xylose assimilation: *XYL1*, which encodes xylose reductase (converts xylose to xylitol), *XYL2,* which encodes xylitol dehydrogenase (converts xylitol to xylulose) and *XKS1*, which encodes xylulokinase (converts xylulose to xylulose-5P) (Olofsson *et al*. 2008). Again, different from the GCR model, yet in accordance with our physiological assays, xylose-assimilatory genes were not repressed by glucose in JP19M (Fig. 1A and 2), which enables this strain to co-consume xylose along with glucose. As pointed above, the co-consumption of sugars is not a common feature among yeasts. For instance, in an engineered strain of *S. cerevisiae* (WXY34) cultivated in mixed media (glucose and xylose), the transcripts levels of *XYL1* and *XYL2* increased only after total glucose consumption, whereas *XKS1* presented decreased expression levels throughout fermentation (Zhang *et al*. 2018). Moreover, in thermotolerant yeast *Kluyveromyces marxianus* DMKU3-1042 also cultivated in mixed medium containing glucose and xylose, the *XYL1* gene is repressed by glucose effect (Rodrussamee *et al*. 2011). As discussed in the previous section, the ability to co-consume xylose and glucose should be further explored so *B. bruxellensis* could rise as a second-generation ethanol producer, by exploring its other industrially relevant traits (Barros Pita *et al*. 2019). For instance, *B. bruxellensis* is resistant to high concentrations of ethanol, acidic pH, as well as inhibitors generated during hydrolysis step, common features of second-generation ethanol production (Dien *et al*. 2003; Davison *et al*. 2016; Robak and Balcerek 2018).

Since our initial analyses revealed that neither *HXT6/7* nor xylose-assimilatory genes were downregulated by glucose, we decided to expand our investigation by including other genes that should be controlled in a GCR-manner. In this sense, we investigated the expression of *FBP1* (coding for fructose-1,6-bisphosphatase, the key regulator of gluconeogenesis), *ATP1* (which codes for the alpha subunit of the ATP synthase complex) and *SDH1* (coding for the flavoprotein subunit of succinate dehydrogenase, which links the tricarboxylic acid cycle to ETC). Consistently with the data reported above, the presence of glucose did not trigger a repressive effect in their expression levels (Fig. 1B). In our assays, *FBP1* gene was upregulated even in presence of

glucose. This observation is different from the reports for *B. bruxellensis* GDB 248 (Leite *et al*. 2016) and *S. cerevisiae* (Rolland *et al*. 2002; Stenger *et al*. 2020), in which the *FBP1* gene is subjected to GCR. On the other hand, *ATP1* and *SDH1* remained with stable transcription levels. In *B. bruxellensis* GDB 248, *ATP1* gene expression is dependent on glucose concentration under oxygen limitation, unlike *SDH1* whose expression level remains unaltered (de Barros Pita *et al*. 2013a). In *S. cerevisiae*, glucose has a strong effect on destabilizing the mRNA that encodes succinate dehydrogenase subunits, by decreasing the molecule's half-life from more than 60 minutes to less than 10 minutes (Lombardo *et al*. 1992). In contrast, when xylose is the only carbon source for *S. cerevisiae*, *SDH2* gene, which codes for another subunit of succinate dehydrogenase complex is upregulated (Zha *et al*. 2014). Once again, our results clearly show the absence of glucose regulation for *FBP1*, *ATP1* and *SDH1* genes in JP19M strain.

Following our initial investigation in genes expected to be repressed by GCR, we were interested in analysing the expression pattern of two genes involved with PPP, since when glucose is internalized, it is phosphorylated to glucose-6P and might proceed either to glycolysis or PPP (Fig. 2). Our results showed that *GND1* gene (6-phosphogluconate dehydrogenase) was upregulated in mixed media, which might be related to an increased demand for NADPH in PPP, required for the conversion of xylose to xylitol by xylose reductase (Fig. 1A and 2). A similar result was found in *S. cerevisiae* SyBE005 for the *ZWF1* gene, which codes for glucose-6-phosphate dehydrogenase and it is involved in the production of NADPH in the oxidative phase of PPP (Zha *et al*. 2014). In addition, following xylulose-5P formation, the late steps of xylose assimilation take place at the non-oxidative phase PPP, a series of carbon rearrangements performed by the action of transketolases (*TKL1* and *TKL2*) and transaldolase (*TAL1*) (Flores *et al*. 2000). In our assays, *TKL1* gene remained with stable expression (Fig. 1A). In contrast, in *B. bruxellensis* GDB 248 the *TKL1* gene is upregulated in glucose or sucrose medium with sodium nitrate (de Barros Pita et al. 2013a). It has been observed in *S. cerevisiae* that the low expression of genes from the non-oxidative phase of PPP is one of the reasons for a slow xylose metabolism, while the overexpression of *TKL1*, *TAL1* and *RKI1* (ribose-5-phosphate ketol-isomerase) genes favour increased consumption of this pentose (Zha *et al*. 2014).

Figure 1 Relative transcription levels of genes involved in glucose and xylose transport and xylose metabolism (panel A); gluconeogenesis, ATP synthesis and TCA cycle (panel B); PPP (panel C); fermentative pathway and glucose repression (panel D) in *B. bruxellensis* JP19M in mixed medium (10 g l^{-1} xylose plus 5 g l^{-1} glucose) under aerobic condition. Genes: *HXT6/7*, hexose transporter; *XYL1*, aldose reductase; *XYL2*, xylitol dehydrogenase; *XKS*, xylulokinase; *GND1*, 6-phosphogluconate dehydrogenase; *TKL1*, transketolase; 2; *FBP1*, fructose-1,6-bisphosphatase; *ATP1*, ATP synthase 1; *SDH1*, succinate dehydrogenase; *PDC1*, pyruvate decarboxylase 1; *ADH1*, alcohol dehydrogenase 1; *ALD3*, aldehyde dehydrogenase; *HXK2*, hexokinase.



In the GCR model, genes involved in xylose metabolism, gluconeogenesis and respiration, such as the ones we analysed in the present study, are expected to be under control of glucose repression. On the other hand, genes coding for proteins involved with fermentation are usually upregulated in presence of

glucose, even under aerobic conditions (Hagman and Piškur 2015). Our results showed that glucose led to the upregulation of *PDC1* and *ADH1* genes, coding for pyruvate decarboxylase 1 and alcohol dehydrogenase 1, respectively (Figs. 1C and 2). Although no ethanol was detected in our assays (3 hours) with xylose and glucose (data not shown), a previous work reported that ethanol is produced by JP19M in aerobic conditions but with longer cultivation periods (between 8 and 24 h), and in oxygen limitation (starting 4 h) (da Silva et al. 2020). In contrast, the *ALD3* gene, coding for the cytoplasmic aldehyde dehydrogenase, was downregulated when glucose was present in the medium (Fig. 1C), which might explain the absence of acetate detection when *B. bruxellensis* JP19M is cultivated in a mixed medium (da Silva et al. 2020). It is interesting to note that, as a Crabtree positive yeast, *B. bruxellensis* is prone to fermentation once glucose is readily available (Rozpedowska et al. 2011). As previously discussed, this phenotype is granted by the combination of several regulatory mechanisms which maximizes fermentation to the detriment of respiration. However, despite the observation that fermentation genes are, in fact, upregulated in JP19M strain, respiratory genes are not repressed as it should be expected. This indicates that the regulatory problem in this strain is indeed related and restricted solely to the GCR control, not being extendable to other regulatory networks. This means that the absence of GCR control is likely to be the product of one or only a few mutations, which blocks the entire GCR mechanism but does not necessarily interfere with other pathways.

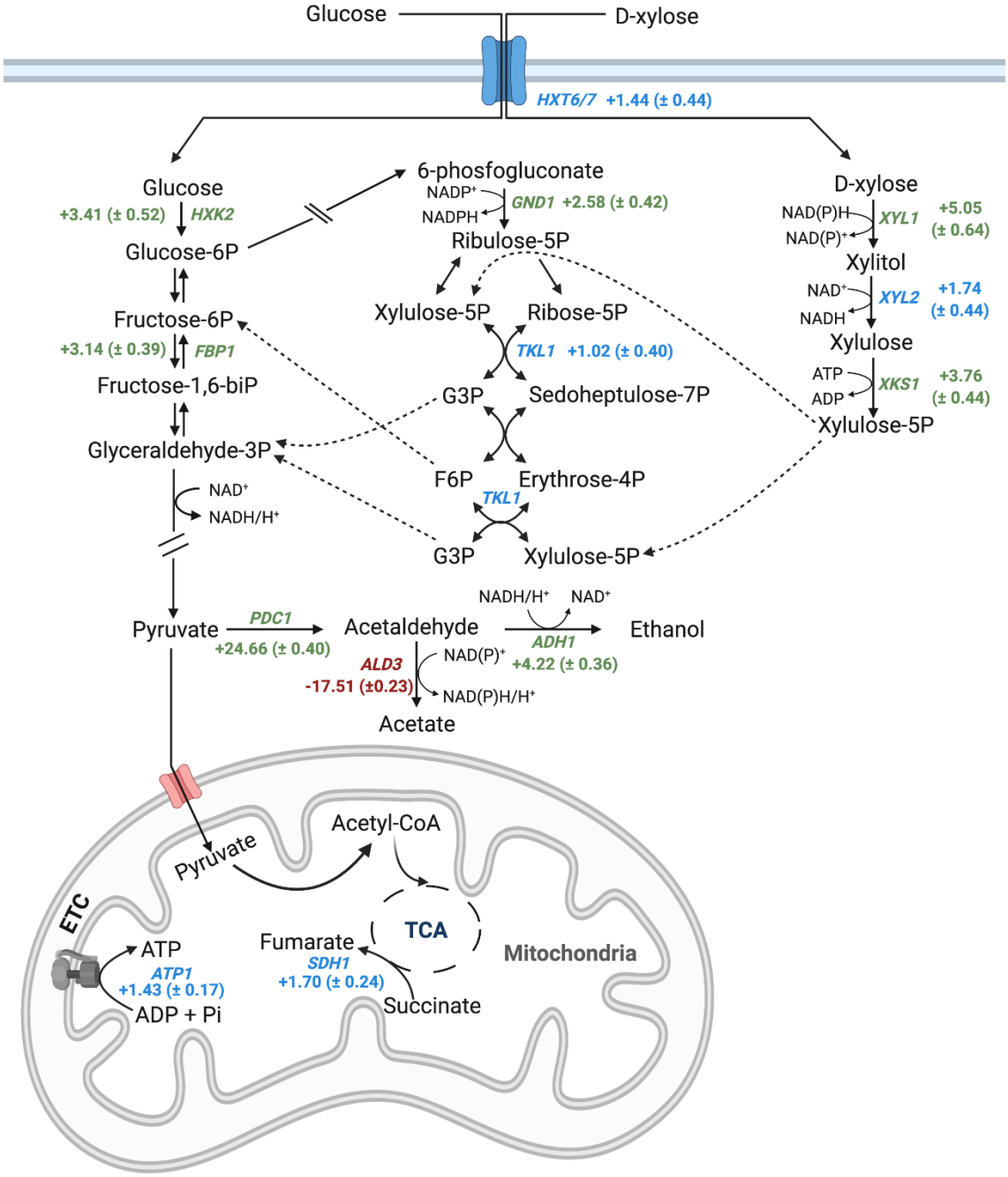
Finally, we observed that the gene coding for hexokinase 2 (*HXK*2) was upregulated in the mixed medium (Fig. 1C). In *S. cerevisiae*, the Hxk2p protein is a bifunctional enzyme involved in the phosphorylation of glucose to glucose-6-phosphate (the first reaction of glycolysis) and a key regulator of glucose repression signal in the nucleus (Ahuatzi *et al*. 2004; Busti *et al*. 2010). In the latter case, Hxk2p acts along with Mig1 the to downregulate genes responsive to glucose. Depending on the extracellular glucose concentration, the Snf1 complex (required for glucose-repressed gene transcription) together with other regulatory proteins, regulate the transcriptional repressor Mig1 (Peeters and Thevelein 2014). When glucose concentration is above the critical concentration, Snf1 is inactive, which allows Hxk2p to translocate to the nucleus along with transcriptional repressor Mig1 to act as a repressor complex for the expression

of gluconeogenesis and respiration genes (Kayikci and Nielsen 2015). On the other hand, when glucose is depleted, Snf1 is active and phosphorylates Mig1 at the Hxk2p binding site, preventing formation of the Mig1-Hxk2p complex. As a result, both proteins are reallocated to the cytosol, and the gluconeogenesis and respiration genes are derepressed (Fernandez-Garcia *et al*. 2012). Moreover, the deletion of the *HXK2* gene makes the Snf1 kinase complex trapped in the active conformation, which results in the absence of glucose repression (Sanz *et al*. 2000). The expression profile of *HXK2* and the other genes investigated in the present work, suggest that the absence of a clear GCR phenotype in *B. bruxellensis* JP19M. In addition, this behaviour is not directly involved with *HXK2*, and it is likely to be related to a downstream effector in the regulatory cascade or another regulatory pathway.

In conclusion, the data described in the present work confirms that Glucose Catabolite Repression is not a universal trait in *B. bruxellensis*. We were able to determine, with both physiological and gene expression data, that the presence of glucose did not inhibit neither the consumption nor the expression of xylose-assimilatory genes in *B. bruxellensis* JP19M. In addition, we found that several other genes involved with different aspects of yeast central metabolism did not respond to a repressive effect caused by glucose. As discussed in the previous sections, the absence of a GCR phenotype in *B. bruxellensis* JP19M might be faced as an opportunity to boost the development of genetic engineering strategies to outcome its poor fermentative performance in xylose. Once this bottleneck has been bypassed, *B. bruxellensis* JP19M might arise as potential producer microorganism in the second-generation ethanol industry.

Figure 2 Metabolic pathways and genes analysed in the present study. Glucose did not inhibit xylose consumption, nor did it repress xylose metabolism, gluconeogenesis or respiration genes. Genes labelled in green were upregulated, genes labelled in red were downregulated and genes labelled in blue presented stable expression (< 2.0). Values indicate the gene relative quantification (+) upregulated and (-) downregulated with standard deviation. Dotted arrows indicate interaction of the molecule in another metabolic pathway. Abbreviations: TCA, Tricarboxylic Acid Cycle; ETC, Electron Transport Chain. Genes: *HXT6/7*, hexose transporter; *XYL1*, aldose reductase; *XYL2*, xylitol dehydrogenase; *XKS*, xylulokinase; *GND1*, 6-phosphogluconate dehydrogenase; *TKL1*, transketolase; 2; *FBP1*, fructose-1,6-bisphosphatase;

ATP1, ATP synthase; *SDH1*, succinate dehydrogenase; *PDC1*, pyruvate decarboxylase 1; *ADH1*, alcohol dehydrogenase 1; *ALD3*, aldehyde dehydrogenase 3; *HXK2*, hexokinase.



Materials and Methods

Strain and cell maintenance

B. bruxellensis JP19M used in the present work was isolated from fuel-ethanol production processes and properly identified by molecular biology (da Silva *et al*. 2016). This strain was selected due to a previous indication that it can

co-consume glucose and xylose under oxygen limitation (da Silva *et al*. 2020). JP19M cells were freshly maintained in dishes of YPD medium (1% yeast extract, 2% peptone, 2% glucose and 2% agar) for 72 h at 30 ºC.

Influence of glucose in the assimilation of xylose in *B. bruxellensis* JP19M

Cells from *B. bruxellensis* JP19M were pre-cultivated in YNB medium w/o amino acids, supplemented with ammonium sulphate (5 g l^{-1}) and xylose (20 g l^{-1}) under agitation (160 r.p.m), at 30 ºC until reaching OD (600 nm) of 1.0. Then, cells were collected after centrifugation, washed and suspended in saline solution (NaCl 8.5 g l^{-1}) and added to 50 ml flasks containing 30 ml of YNB medium w/o amino acids, supplemented with ammonium sulphate (5 g l^{-1}), D-xylose (10 g l^{-1}) plus different glucose concentrations (0, 0.5, 1, 2, 3, 4 and 5 g l^{-1}). Samples were taken (i) immediately after the inoculation (t 0h) and (ii) after 3 hours of cultivation (t 3h), for sugar consumption analyses by HPLC and RNA extraction for gene expression assays. The cultures were carried out both in biological and technical duplicates.

Measurement of extracellular metabolites

Glucose or xylose concentrations were determined by high-performance liquid chromatography (HPLC) in a Shimadzu system equipped with a pump quaternary coupled to a degasser, an oven to control the column temperature, adjusted to 60 °C, and a refractive index detector (da Silva et al. 2020). The software LC Solutions, manufactured by Shimadzu Corporation (Kyoto, Japan) was used for data acquisition. A 300 mm x 7.8 mm ion exchange column (Aminex® HPX-87H, Bio-Rad, USA) with a particle size of 9 μm was used. Acidified ultrapure water with isocratic elution of H_2SO_4 5mM at a flow rate of 0.6 ml/min was used as mobile phase. The correlation coefficients (R^2) of the calibration curves were higher than 0.999. All samples were run with two technical replicates.

RNA extraction and cDNA synthesis

B. bruxellensis JP19M cells collected from the assays in xylose and mixed media (glucose and xylose) were used for RNA extraction by using Maxwell® 16 LEV simplyRNA Purification Kits (Promega, USA). RNA integrity and

quantification were determined by agarose gel (1%) electrophoresis and Nanodrop 2000 (Thermo Scientific, USA), respectively (de Barros Pita et al. 2012). Then, 1 µg of total RNA was converted to cDNA by using GoScript™ Reverse Transcriptase Kit (Promega, USA), according to the manufacturer's instructions. cDNA samples were stored at -20 ºC until RT-qPCR was performed.

Primer design

The nucleotide sequences for the target genes were obtained from *D. bruxellensis* CBS 2499 database (https://mycocosm.jgi.doe.gov/pages/search-for-genes.jsf?organism=Dekbr2), after analysis by tBlastx (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using corresponding orthologous sequences from the genome of *Saccharomyces cerevisiae* (https://www.ncbi.nlm.nih.gov/nuccore). Primers were designed using the Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) and evaluated by the OligoAnalyzer IDT tool (https://www.idtdna.com/pages/tools/oligoanalyzer). *TEF1* and *ACT1* were used as reference genes, as previously reported (Barros Pita *et al*. 2012). Target genes of interest are listed in Table 2 and S1.

Gene expression analyses by RT-qPCR

RT-qPCR analyses were performed using GoTaq® qPCR Master Mix kit (Promega, USA) in 96-well plates. The amplification reactions had a total volume of 10 µL per well, whose composition was 5 µL of GoTaq® qPCR Master Mix, 0.8 µL of primer (0.4 µL of forward primer plus 0.4 µL of reverse primer – 200 nmol l^{-1} final), 0.1 µL of supplemental CXR reference dye, 1 µl cDNA and 3.1 µL of nuclease free water. Cycling parameters were 95 °C for 10 min as hot start, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min in the ABI Prism 7300 (Applied Biosystems). Controls were performed on each plate to verify nonspecific amplifications (negative control) and contamination with genomic DNA (RT control), according to de Barros Pita *et al*. (2012). In order to assess the repressive influence of glucose on the xylose metabolism, we performed relative expression analyses for each target gene within samples of mixed media collected after 3 hours of cultivation and using xylose as reference (calibrator).

Data normalization, as well as the geNorm analyses and the determination of the relative quantification were carried out following the recommendations proposed by de Barros Pita *et al*. (2012; 2013) and MIQE Guidelines (Bustin *et al*. 2009). For each condition, two biological and three technical replicates were performed, in a total of 6 wells for each gene per sample. Figure 2 was created by using the online tool BioRender (BioRender.com).

Table 2 List of genes investigated in the present study.

Gene	Metabolic pathway	Name Description	Description*
ACT1	Reference genes	Actin	Structural protein involved in cell polarization
TEF1		Translation Elongation Factor	Translational elongation factor EF-1α
HXT 6/7	Transport	Hexose transporter	High affinity glucose and xylose transport
XYL1	Xylose metabolism	Xylose reductase	Reduces D-xylose to xylitol
XYL2		Xylitol dehydrogenase	Oxidizes xylitol to xylulose
XKS1		Xylulokinase	Phosphorylates xylulose to xylulose-5-phosphate
GND1	Pentose Phosphate Pathway	6-phosphogluconate dehydrogenase	Converts 6-phosphogluconolactone to D-ribulose-5-phosphate
TKL1		Transketolase	Converts xylulose-5P and ribose-5P to glyceraldehyde-P and sedoheptulose-7P
FBP1	Gluconeogenesis	Fructose-1,6-BisPhosphatase	Key regulatory enzyme. Dephosphorylates fructose-1,6-biphosphate to fructose-6-phosphate.
SDH1	Krebs cycle	Flavoprotein subunit of succinate dehydrogenase	Couples the oxidation of succinate to the transfer of electrons to ubiquinone.
ATP1	Electron Transport Chain	Alpha subunit of the F1 sector of mitochondrial F1F0 ATP synthase	Preserved enzyme complex for ATP synthesis
PDC1	Fermentative pathway	Pyruvate decarboxylase	Decarboxylates pyruvate to acetaldehyde
ADH1		Alcohol dehydrogenase	Reduces acetaldehyde to ethanol
ALD3		Cytoplasmic aldehyde dehydrogenase	Oxidizes acetaldehyde to acetate
HXK2	Glycolysis and Glucose Repression Pathway	Hexokinase	Phosphorylates glucose in cytosol and participates in the signal of glucose repression in the nucleus

*Description based on the information available on the *Saccharomyces* Genome Database (https://www.yeastgenome.org)

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Conflict of Interest

Authors declare that they have no conflict of interest.

References

Ahuatzi, D., Herrero, P., de la Cera, T., and Moreno, F. (2004) The glucose regulated nuclear localization of hexokinase 2 in *Saccharomyces cerevisiae* is Mig1-dependent. *J Biol Chem* **279**, 14440–14446.

Alff-Tuomala, S., Salusjärvi, L., Barth, D., Oja, M., Penttilä, M., Pitkänen, J.P., Ruohonen, L. and Jouhten, P. (2016) Xylose-induced dynamic effects on metabolism and gene expression in engineered *Saccharomyces cerevisiae* in anaerobic glucose-xylose cultures. *Appl Microbiol Biotechnol* **100**, 969-85.

Bakker, B.M., Overkamp, K.M., van Maris, A.J., Kotter, P., Luttik, M.A., van Dijken. J.P. and Pronk J.T. (2001) Stoichiometry and compartmentation of NADH metabolism in *Saccharomyces cerevisiae*. *FEMS Microbiol Rev* **25**, 15–37.

Busti, S., Coccetti, P., Alberghina, L. and Vanoni, M. (2010) Glucose signaling-mediated coordination of cell growth and cell cycle in *Saccharomyces cerevisiae*. *Sensors* **10**, 6195–6240.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., et al. (2009) The MIQE guidelines: minimum information for publication ofquantitative real-time PCR experiments. *Clin Chem* **55**, 611–622.

Cadete, R. M., Las Heras, A. M. de., Sandstrom, A.G., Ferreira, C., Gírio, F., Gorwa-Grauslund, M.F., Rosa, C. A. and Fonseca C. (2016) Exploring xylose metabolism in *Spathaspora* species: XYL1.2 from *Spathaspora passalidarum* as the key for efficient anaerobic xylose fermentation in metabolic engineered *Saccharomyces cerevisiae*. *Biotechnol Biofuels* **9**, 1-14.

Cheng, C., Tang, R.Q., Xiong, L., Hector, R.E., Bai, F.W. and Zhao, X.Q. (2018) Association of improved oxidative stress tolerance and alleviation of glucose repression with superior xylose-utilization capability by a natural isolate of *Saccharomyces cerevisiae*. *Biotechnol Biofuels* **11**, 1-19.

Codato, C. B., Martini, C., Ceccato-Antonini, S. R. and Bastos, R. G. (2018) Ethanol production from *Brettanomyces bruxellensis* in synthetic media with pentose. *J Chem Eng* **35**, 11–17.

Conrad, M., Schothorst, J., Kankipati, H.N., Van Zeebroeck, G., Rubio-Texeira, M. and Thevelein, J.M. (2014) Nutrient sensing and signaling in the yeast *Saccharomyces cerevisiae*. *FEMS Microbiol Rev* **38**, 254-99.

Crauwels S, Van Assche A, Jonge R et al. (2015) Comparative phenomics and targeted use of genomics reveal variation in carbon and nitrogen assimilation among different *Brettanomyces bruxellensis* strains. Appl Microbiol Biotechnol 99:9123–34.

da Silva JM, da Silva GHTG, Parente DC, Leite FCB, Silva CS, Valente P, Ganga AM, Simões DA, de Morais MA Jr. (2019) Biological diversity of carbon assimilation among isolates of the yeast Dekkera bruxellensis from wine and fuel-ethanol industrial processes. FEMS Yeast Res. 1;19(3):foz022. doi: 10.1093/femsyr/foz022. PMID: 30848782.

da Silva JM, Ribeiro KC, Teles GH, Ribeiro E, de Morais Junior MA, de Barros Pita W. (2020) Fermentation profiles of the yeast *Brettanomyces bruxellensis* in D-xylose and L-arabinose aiming its application as a second-generation ethanol producer. *Yeast* **37**, 597-608.

da Silva, T.C.D., Leite, F.C.B. and de Morais, M.A. (2016) Distribution of *Dekkera bruxellensis* in a sugarcane-based fuel ethanol fermentation plant. *Lett Appl Microbiol* **62**, 354-358.

Davison, S.A., den Haan, R. and van Zyl, W.H. (2016) Heterologous expression of cellulase genes in natural *Saccharomyces cerevisiae* strains. *Appl Microbiol Biotechnol* **100**, 8241–8254.

de Barros Pita, W., Castro Silva, D., Simões-Ardaillon, D., Volkmar, P. and de Morais M.A. (2013b) Physiology and gene expression profiles of *Dekkera bruxellensis* in response to carbon and nitrogen availability. *Antonie van Leeuwenhoek* **104**:855–868.

de Barros Pita, W., Leite, F.C.B., de Souza Liberal, A.T., Pereira, L.F., Carazzolle, M.F., Pereira, G.A. and de Morais, M.A.Jr. (2012) A new set of reference genes for RT-qPCR assays in the yeast *Dekkera bruxellensis*. *Can J Microbiol* **58**, 1362–1367.

de Barros Pita, W., Leite, F.C.B., de Souza Liberal, A.T., Simões, D.A. and de Morais, M.A.Jr (2011) The ability to use nitrate confers advantage to *Dekkera bruxellensis* over *S. cerevisiae* and can explain its adaptation to industrial fermentation processes. *Antonie Van Leeuwenhoek* **100**, 99–107.

de Barros Pita, W., Teles, G. H., Peña-Moreno, I. C., da Silva, J. M., Ribeiro, K. C. and de Morais Junior, M. A. (2019) The biotechnological potential of the yeast *Brettanomyces bruxellensis*. *World J Microbiol Biotechnol* **35**, 103.

de Barros Pita, W., Tiukova, I., Leite, F.C.B., Passoth, V., Simões, D.A. and de Morais, M.A. (2013a) The influence of nitrate on the physiology of the yeast *Dekkera bruxellensis* grown under oxygen limitation. *Yeast* **30**, 111–117.

de Deken, R.H. (1966) The Crabtree effect: a regulatory system in yeast. *J Gen Microbiol* **44**, 149–156.

de Souza Liberal, A.T., Basílio, A. C. M., do Monte Resende, A., Brasileiro, B. T. V., da Silva-Filho, E. A., de Morais, J. O., Simões, D.A. and de Morais, M. A. Jr. (2007) Identification of *Dekkera bruxellensis* as a major contaminant yeast in continuous fuel ethanol fermentation. *J Appl Microbiol* **102**, 538–547.

Dien, B.S., Cotta, M. and Jeffries, T.W. (2003) Bacteria engineered for fuel ethanol production: current status. *Appl Microbiol Biotechnol* **63**, 258–266.

Fernandez-Garcia, P., Pelaez, R., Herrero, P., and Moreno, F. (2012) Phosphorylation of yeast hexokinase 2 regulates its nucleocytoplasmic shuttling. *J Biol Chem* **287**, 42151–42164.

Flores, C.L., Rodríguez, C., Petit, T. and Gancedo, C. (2000) Carbohydrate and energy-yielding metabolism in non-conventional yeasts. *FEMS Microbiol Rev* **24**, 507–529.

Galafassi, S., Merico, A., Pizza, F., Hellborg, L., Molinari, F., Piškur, J. and Compagno, C. (2011) *Dekkera/Brettanomyces* yeasts for ethanol production from renewable sources under oxygen-limited and low-pH conditions. *J Ind Microbiol Biotechnol* **38**, 1079–1088.

Hagman, A. and Piškur, J. (2015) A study on the fundamental mechanism and the evolutionary driving forces behind aerobic fermentation in yeast. *PLoS One* **10**, e0116942.

Hahn-Hagerdal, B., Karhumaa, K., and Jeppsson, M. (2007). Metabolic engineering for pentose utilization in *Saccharomyces cerevisiae*. *Adv Biochem Engin/Biotechnol* **108**, 147–177.

Hamacher, T., Becker, J., Gárdonyi, M., Hahn-Hagerdal, B. and Boles, E. (2002) Characterization of the xylose-transporting properties of yeast hexose transporters and their influence on xylose utilization. *Microbiol* **148**, 2783-2788.

Hou X (2012) Anaerobic xylose fermentation by *Spathaspora passalidarum*. *Appl Microbiol Biotechnol* **94**, 205–214.

Hua, Y., Wang, J., Zhu, Y., Zhang, B., Kong, X., Li, W., Wang, D. and Hong, J. (2019) Release of glucose repression on xylose utilization in *Kluyveromyces marxianus* to enhance glucose-xylose co-utilization and xylitol production from corncob hydrolysate. *Microb Cell Fact* **18**, 24.

Jeffries, T.W., Grigoriev, I.V., Grimwood, J., Laplaza, J.M., Aerts, A., Salamov, A., Schmutz, J., Lindquist, E., Dehal, P., Shapiro, H., et al. (2007) Genome sequence of the lignocellulose-bioconverting and xylose-fermenting yeast *Pichia stipitis*. *Nat Biotechnol* **25**, 319–326.

Kayikci, Ö. and Nielsen, J. (2015) Glucose repression in *Saccharomyces cerevisiae*. *FEMS Yeast Res* **15**, fov068.

Krahulec, S., Petschacher, B., Wallner, M., Longus, K., Klimacek, M. and Nidetzky, B. (2010) Fermentation of mixed glucose-xylose substrates by engineered strains of *Saccharomyces cerevisiae*: role of the coenzyme specificity of xylose reductase, and effect of glucose on xylose utilization. *Microb Cell Factories* **9**, 16.

Lane, S., Xu, H., Oh, E. J., Kim, H., Lesmana, A., Jeong, D., Zhang, G., Tsai, C.S., Jin, Y. S. and Kim, S. R. (2018) Glucose repression can be

alleviated by reducing glucose phosphorylation rate in *Saccharomyces cerevisiae*. *Sci Rep* **8**, 2613.

Leite, F.C., Leite, D.V., Pereira, L.F., de Barros Pita, W. and de Morais, M.A.Jr. (2016) High intracellular trehalase activity prevents the storage of trehalose in the yeast *Dekkera bruxellensis*. *Lett Appl Microbiol* **63**, 210-4.

Liang, H. and Gaber, R.F. (1996) A novel signal transduction pathway in *Saccharomyces cerevisiae* defined by Snf3-regulated expression of *HXT6*. *Mol biol cell* **7**, 1953–1966.

Lombardo, A., Cereghino, G.P. and Scheffler, I.E. (1992) Control of mRNA turnover as a mechanism of glucose repression in *Saccharomyces cerevisiae*. *Mol Cell Biol* **12**, 2941–2948.

Meinander, N.Q., Boels, I. and Hahn-Hägerdal, B. (1999) Fermentation of xylose/glucose mixtures by metabolically engineered *Saccharomyces cerevisiae* strains expressing *XYL1* and *XYL2* from *Pichia stipitis* with and without overexpression of *TAL1*. *Bioresour Technol* **68**, 79-87.

Olofsson, K., Bertilsson, M., Lidén, G. (2008) A short review on SSF - An interesting process option for ethanol production from lignocellulosic feedstocks. *Biotechnol Biofuels* **1**, 7.

Ozcan, S. (2002) Two different signals regulate repression and induction of gene expression by glucose. *J Biol Chem* **277**, 46993–7.

Ozcan, S. and Johnston, M. (1999) Function and regulation of yeast hexose transporters. *Microbiol Mol Biol Rev* **63**, 554-69.

Pautasso, C., Reca, S., Chatfield-Reed, K., Chua, G., Galello, F., Portela, P., Zaremberg, V. and Rossi, S. (2016) Identification of novel transcriptional regulators of PKA subunits in *Saccharomyces cerevisiae* by quantitative promoter-reporter screening. *FEMS Yeast Res* **16**, fow046.

Peeters, K. and Thevelein, J.M. (2014) Glucose Sensing and Signal Transduction in *Saccharomyces cerevisiae* ed. Piškur, J. and Compagno, C. pp 21-56. Molecular Mechanisms in Yeast Carbon Metabolism. Springer-Verlag Berlin Heidelberg.

Peña-Moreno, I.C., Castro Parente, D., da Silva, J.M., Andrade Mendonça, A., Rojas, L.A.V., Morais, M.A.Jr. and de Barros, P.W. (2019) Nitrate boosts anaerobic ethanol production in an acetate dependent manner in the

yeast *Brettanomyces bruxellensis*. *J Ind Microbiol Biotechnol* **46**, 209–220.

Pereira, L.F., Bassi, A. P. G., Avansini, S. H., Neto, A. G. B., Brasileiro, B. T. R. V., Ceccato-antonini SR, de Morais MA (2012) The physiological characteristics of the yeast *Dekkera bruxellensis* in fully fermentative conditions with cell recycling and in mixed cultures with *Saccharomyces cerevisiae*. *Antonie Van Leeuwenhoek* **101**, 529–539.

Pitkänen, J.P., Aristidou, A., Salusjärvi, L., Ruohonen, L. and Penttilä, M. (2003) Metabolic flux analysis of xylose metabolism in recombinant *Saccharomyces cerevisiae* using continuous culture. *Metab Eng* **5**, 16–31.

Postma, E., Verduyn, C., Scheffers, W.A. and Van Dijken, J.P. (1989) Enzymic analysis of the crabtree effect in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*. *Appl Environ Microbiol* **55**, 468–477.

Reis, A. L. S., de Souza, R. F. R., Torres, R. R. N. B., Leite, F. C. B., Paiva, P. M. G., Vidal, E. E., & de Morais Jr, M. A. (2014) Oxygen-limited cellobiose fermentation and the characterization of the cellobiase of an industrial *Dekkera/Brettanomyces bruxellensis* strain. *Springer Plus* **3**, 1–9.

Rettori, D. e Volpe, P.L.O. (2000) Microcalorimetria: uma técnica aplicável ao estudo do diauxismo da *Saccharomyces cerevisiae*. Química Nova **23**, 257-261.

Robak, K. and Balcerek, M. (2018) Review of second-generation bioethanol production from residual biomass. *Food Technol Biotech* **56**, 174–187.

Rodrussamee. N., Lertwattanasakul, N., Hirata, K., Suprayogi, Limtong, S., Kosaka, T. and Yamada, M. (2011) Growth and ethanol fermentation ability on hexose and pentose sugars and glucose effect under various conditions in thermotolerant yeast *Kluyveromyces marxianus*. *Appl Microbiol Biotechnol* **90**, 1573-86.

Rodrussamee. N., Sattayawat, P. and Yamada, M. (2018) Highly efficient conversion of xylose to ethanol without glucose repression by newly isolated thermotolerant *Spathaspora passalidarum* CMUWF1–2. *BMC Microbiol* **18**: 73, 1-11.

Rolland, F., Winderickx, J. and Thevelein, J.M. (2002) Glucose-sensing and -signalling mechanisms in yeast. *FEMS Yeast Res* **2**, 183–201.

Rothman, D.L., Stearns, S.C. and Shulman, R.G. (2021) Gene expression regulates metabolite homeostasis during the Crabtree effect: Implications for the adaptation and evolution of Metabolism. *Proc Natl Acad Sci U S A* **118**, e2014013118.

Rozpedowska, E., Hellborg, L., Ishchuk, O.P., Orhan, F., Galafassi, S., Merico, A., Woolfit, M., Compagno, C. and Piskur, J. (2011) Parallel evolution of the make-accumulate-consume strategy in *Saccharomyces* and *Dekkera yeasts*. Nat Commun 2:302.

Sanz, P., Alms, G.R., Haystead, T.A. and Carlson, M. (2000) Regulatory interactions between the Reg1-Glc7 protein phosphatase and the Snf1 protein kinase. *Mol Cell Biol* **20**, 1321–1328.

Skoog, K. and Hahn-Hagerdal, B. (1990) Effect of oxygenation on xylose fermentation by *Pichia stipitis*. *Appl Environ Microbiol* **56**, 3389–3394.

Stasyk, O.G. and Stasyk, O.V. (2019) Glucose Sensing and Regulation in Yeasts. In: Sibirny A. (eds) Non-conventional Yeasts: from Basic Research to Application. Springer, Cham.

Stenger, M., Le, D.T., Klecker, T. and Westermann, B. (2020). Systematic analysis of nuclear gene function in respiratory growth and expression of the mitochondrial genome in *S. cerevisiae*. *Microbial cell* **7**, 234–249.

Winderickx, J., de Winde, J.H., Crauwels, M., Hino, A., Hohmann, S., Van Dijck, P. and Thevelein, J.M. (1996) Regulation of genes encoding subunits of the trehalose synthase complex in *Saccharomyces cerevisiae*: novel variations of STRE-mediated transcription control? *Mol Gen Genet* **252**, 470–482.

Yu, H., Guo, J., Chen, Y., Fu, G., Li, B., Guo, X. and Xiao, D. (2017). Efficient utilization of hemicellulose and cellulose in alkali liquor-pretreated corncob for bioethanol production at high solid loading by *Spathaspora passalidarum* U1-58. *Bioresour Technol* **232**, 168-175.

Zha, J., Shen, M., Hu, M., Song, H. and Yuan, Y. (2014) Enhanced expression of genes involved in initial xylose metabolism and the oxidative pentose phosphate pathway in the improved xylose-utilizing *Saccharomyces cerevisiae* through evolutionary engineering. *J Ind Microbiol Biotechnol* **41**, 27-39.

Zhang, X., Wang, J., Zhang, W., Hou, J.Y., Xiao, W. and Cao, L. (2018) Optimizing the coordinated transcription of central xylose-metabolism genes in *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* **102**, 7207-7217.

7 ARTIGO 4 - AEROBIC UTILIZATION OF GALACTOSE BY DIFFERENT STRAINS OF THE YEAST *BRETTANOMYCES BRUXELLENSIS* DIRECTS PYRUVATE TO DISTINCT METABOLIC FATES AND IMPACTS ITS FERMENTATIVE CAPACITY

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Abstract

The yeast *Brettanomyces bruxellensis* has been recently suggested as a potential microorganism in the second-generation ethanol production, since it presents metabolic traits essential to achieve a high fermentation efficiency, such as the ability to ferment a wide range of sugars. Since galactose is a hexose found in hemicellulose, which once fermented, might increase the ethanol production, we intended to determine the fermentative capacity of two strains of *B. bruxellensis* in presence of this sugar. Furthermore, we examined the probable preferred metabolic routes to pyruvate fate and the reoxidation of NADH, by connecting physiological profiles and transcriptional analyses of genes related to fermentation and oxidative phosphorylation. Moreover, since lignocellulosic hydrolysate is a complex mixture of sugars, we analysed the possible effects of glucose in these parameters. Our results showed GDB 248 strain used galactose to produce biomass and acetate and transcriptional data revealed its preference to reoxidize catabolic NADH through respiration. This profile changed when glucose was available and cells produced ethanol, shifting to respiro-fermentative metabolism. Moreover, we observed that GDB 248 is responsive to Glucose Catabolite Repression, using galactose only after glucose concentration is depleted. On the other hand, JP19M strain produced ethanol and biomass from galactose and mixed media, suggesting a respiro-fermentative metabolism, which was confirmed by transcriptional data. Finally, we found that JP19M co-consumes glucose and galactose, a particularly valuable metabolic aspect, especially in complex substrates. Altogether, our results build-up to previous data in which *B. bruxellensis* JP19M strain presents important physiological traits for the second-generation ethanol production.

Key words: Alcoholic fermentation; oxidative metabolism; respiro-fermentative metabolism; sugars co-consumption; transcriptional ratio.

Introduction

Different types of biomasses have the potential to be used as raw material to produce bioethanol, such as sugarcane, molasses, whey, corn, sugar beet, lignocellulosic residues and others (Mussatto et al. 2010). In Brazil, the first-generation ethanol production process is carried out mainly through sugarcane juice and the lignocellulosic residue generated can be used as substrate to produce second-generation ethanol (Kang et al. 2014; de Araujo Guilherme et al. 2019). Lignocellulosic biomass is composed of cellulose (20–50%), hemicellulose (20–35%), and lignin (10–35%), as well as other components present in trace amounts (Knauf and Moniruzzaman 2004; Gamage et al. 2010). This biomass is subjected to pre-treatment and hydrolysis of cellulose and hemicellulose, which then releases glucose, xylose, arabinose, galactose and cellobiose (derived from incomplete hydrolysis of hemicellulose), used by microorganisms to produce ethanol (dos Santos et al. 2016). On the other hand, lignin might be used to produce electricity and energy in the biorefineries (Gamage et al. 2010). The search for microorganisms capable of efficiently ferment these sugars is crucial to ensure the feasibility of the process (Rech et al. 2019). In this context, the yeast *Brettanomyces bruxellensis* has been pointed as a possible ethanol producer, due to its ability to consume different carbon sources, such as glucose, galactose, sucrose, cellobiose, xylose, arabinose and others (Reis et al. 2014; Crauwels et al. 2015; Codato et al. 2018; da Silva et al. 2019, 2020). In addition, *B. bruxellensis* is able to co-consume xylose and glucose, as well as to resist to inhibitors generated during the hydrolysis process, weak acids, low pH and high concentrations of ethanol (Bassi et al. 2013; Tiukova et al. 2014; da Silva et al. 2020). Altogether, these physiological traits suggest a potential application of this yeast in the second-generation ethanol production (de Barros Pita et al. 2019).

Although the concentration of galactose generated by the hydrolysis of hemicellulose is lower than glucose, xylose and arabinose, finding microorganisms capable of co-consuming these sugars can result in increased efficiency of the fermentation process (van Maris et al. 2006). The simultaneous consumption of different sugars when glucose is present is uncommon in yeasts due to a repressive effect, triggered by glucose, on the assimilation of other carbon sources (Conrad et al. 2014). For instance, wild strains of *S. cerevisiae*

and *B. bruxellensis* (CBS 2499) exhibit a pattern of preferential use of glucose over galactose in cultures with a mixture of these sugars (Rettori and Volpe 2000; Moktaduzzaman et al. 2015). Therefore, in yeast species that present this pattern, galactose metabolism is activated only when glucose is absent (or present in low concentration), and galactose is available in the medium (Rettori and Volpe 2000). In turn, when activated, galactose assimilation involves its conversion to an intermediate metabolite of glycolysis to generate pyruvate through the Leloir pathway (Sellick et al. 2008). In *Saccharomyces cerevisiae*, Leloir pathway proceeds through the influx of galactose to the cell (via Gal2p permease), which is then epimerized from □-D-galactose to □-D-galactose (by galactose mutarotase). Further, □-D-galactose is phosphorylated to galactose-1-phosphate (by galactokinase 1), which is subsequently converted to glucose-1-phosphate (by galactose-1-phosphate-uridyltransferase). The UDP-glucose required for this reaction is replenished by the conversion of UDP-galactose to UDP-glucose (by UDP-glucose 4-epimerase). Finally, glucose-1-phosphate is converted to glucose-6-phosphate (by phosphoglucomutase), which enters either in the glycolytic or pentose-phosphate pathway (Bhat and Murthy, 2001; Bhat 2008; Sellick et al. 2008).

Depending on the yeast species, the reoxidation of the NADH derived from sugar metabolism is achieved through one out of three possibilities. Cells might resort to (i) respiration, (ii) fermentation or (iii) a combination of these two pathways, in the so-called respiro-fermentative metabolism (Pfeiffer and Morley 2014). In fermentation, pyruvate from glycolysis proceeds through a non-oxidative decarboxylation reaction catalysed by the enzyme pyruvate decarboxylase (coded by *PDC1* gene) and it is converted to acetaldehyde (Pronk et al. 1996). Depending on the cell requirements, acetaldehyde then might be a target of one out of two enzymes, with different fates and impact in the redox state of the cells (Medina et al. 2016). On one hand, if acetaldehyde is targeted by the enzyme alcohol dehydrogenase (coded by *ADH1* gene), it is reduced to ethanol, which is the major pathway to reoxidize the glycolytic NADH in cells with active fermentation (van Dijken and Scheffers 1986). On the other hand, if acetaldehyde is a target for the enzyme aldehyde dehydrogenase (coded by *ALD3* gene), this results in its oxidation to acetate, which consumes additional NAD^+ and generates NADH, with a direct impact in the redox balance (Rodrigues

et al. 2006). When cells present an active respiratory metabolism, pyruvate enters the mitochondria and it is further oxidized in the TCA cycle, generating additional NADH (as well as $FADH_2$), which together with glycolytic and acetate-derived NADH are reoxidized into the electron transport chain (Pronk et al. 1996). The ATP synthase complex, in turn, is the endpoint for the work of the electron carriers and the factory that produces the vast majority of the cell energy (Rodrigues et al. 2006). The respiro-fermentative metabolism represents the functioning of both fermentation and respiration simultaneously and yeasts that present this metabolism are denominated Crabtree-positive, which means that they have the capacity to produce ethanol even in the presence of oxygen (De Deken 1966; Postma and Verduyn 1989; Van Urk et al. 1990).

In *S. cerevisiae* CAT-1 strain, galactose is a respiro-fermentable sugar, despite its lower ethanol productivity than other carbon sources, such as glucose, fructose, sucrose and maltose (Nascimento and Fonseca 2019). On the other hand, in *B. bruxellensis* CBS 2499, the ability to ferment galactose was related to the nitrogen source available in the medium, in which the presence of ammonium sulphate led to a metabolism strictly respiratory (Moktaduzzaman et al. 2015). However, since *B. bruxellensis* presents a high intraspecific variation (phenotypical and genotypical), its sugar assimilation profile is far from stringent, and it is mostly strain-dependent (Conterno et al. 2006; Crauwels et al. 2015; Avramova et al. 2018). In fact, a previous screening revealed that *B*. *bruxellensis* JP19M and GDB 248 strains present different growth profiles and the first grows in galactose with a higher rate than the latter in aerobic microcultures (da Silva et al. 2019). Moreover, in that study, it was suggested that JP19M could be able to co-consume glucose and galactose, different from the observed for GDB 248 (da Silva et al. 2019). In the present work, we investigated the major physiological parameters as well as the transcription profile of genes involved in fermentation and oxidative phosphorylation in *B. bruxellensis* GDB 248 and JP19M strains in response to galactose. Our results outline the fermentative capacity and the preferred pathway to reoxidize the catabolic NADH derived from galactose in the two strains. Moreover, we evaluated the influence of glucose on the metabolic response of cells in the presence of galactose.

Materials and methods

Yeast strains, cell maintenance and growth assays

Two *B. bruxellensis* strains were used in the present work, GDB 248 (de Souza Liberal et al. 2007) and JP19M (da Silva et al. 2016), which were isolated from Brazilian fuel-ethanol distilleries and identified by molecular biology. Cells were conserved in Petri dishes containing YPD medium (1% yeast extract, 2% peptone, 2% glucose and 2% agar) with constant plating to keep cell colonies fresh. *B. bruxellensis* strains JP19M and GDB 248 were pre-cultivated in 250 mL flasks containing 100 mL of YNB medium w/o amino acids, supplemented with glucose (20 g/l) and ammonium sulphate (5 g/l) for 48 h, at 30 ºC, under agitation (160 rpm). Cells were collected by centrifugation (10,000 *g* for 5 minutes), washed and suspended in sterile saline solution (NaCl 8.5 g/l) for an initial cell concentration measured as 0.1 optical density (OD 600 nm). Then, the cells were inoculated in 50 ml flasks containing 30 ml of YNB medium w/o amino acids, supplemented with ammonium sulphate (5 g/l) and (i) galactose (20 g/l) or (ii) combination of galactose and glucose (10 g/l each). Cultivation was carried out under agitation (160 rpm) at 30 ºC for 48 h with samples collected at times 0, 3, 6, 12, 24 and 48 h for OD measurements and supernatant analyses by HPLC. Cells were collected after 3 hours from galactose-based and mixed media for RNA extraction. The cultures were carried out in biological duplicates and technical duplicates.

Residual sugars and determination of extracellular metabolites

Ethanol, acetate, glycerol and residual sugar (glucose and galactose) concentrations were determined in a high-performance liquid chromatography system (Shimadzu Corporation, Japan) using an ion exchange column (Aminex® HPX-87H, Bio-Rad, USA), as previously described (da Silva et al. 2020). The mobile phase used was acidified ultrapure water with isocratic elution of 5 mM H_2SO_4 at a flow rate of 0.6 mL/min. The correlation coefficients (R^2) of the calibration curves were greater than 0.999. All samples were run with two technical replicates.

RNA extraction, cDNA synthesis, oligonucleotides and RT-qPCR assays

Cells of *B. bruxellensis* JP19M and GDB 248 were collected after 3 hours of cultivation, from growth assays and were used for RNA extraction using a Maxwell® 16 LEV simplyRNA Purification Kit. RNA integrity and quantification were analysed using an agarose gel (1%) and Nanodrop 2000 (Thermo Scientific, USA), respectively (de Barros Pita et al. 2012). Then, 1 µg of total RNA was converted to cDNA using the GoScript™ Reverse Transcriptase Kit according to the manufacturer's instructions. cDNA samples were stored at -20 ºC until RT-qPCR assays were performed.

RT-qPCR assays were performed in order to determine the transcription profile of genes involved with fermentative pathway and oxidative phosphorylation and, therefore, to evaluate the preferred pathway to reoxidize the catabolic NADH in our two strains grown in galactose. Oligonucleotides for the target genes *PDC1*, *ADH1*, *ALD3* and *ATP1* were previously described (de Barros Pita et al. 2013a; Teles et al. 2018) and *ACT1* was used as reference gene as previously reported (Nardi et al. 2010; Barros Pita et al. 2012). RT-qPCR assays were performed using GoTaq® qPCR Master Mix kit (Promega, USA) on 96-well plates. The amplification reactions had a total volume of 10 □L per well, whose composition was 5 □L of GoTaq® qPCR Master Mix, 0.8 □L of oligonucleotides (0.4 □L each, 200 nmol l^{-1} final), 0.1 µL of supplemental CXR reference dye, 1 µl cDNA and 3.1 µL of nuclease free water. Cycling parameters were 95 °C for 10 min as a hot start, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min in the ABI Prism 7300 (Applied Biosystems, USA). Quality controls were included in each plate in order to investigate nonspecific amplifications and contamination with genomic DNA (de Barros Pita et al. 2012). Experiments were carried out following the recommendations proposed by MIQE Guidelines (Bustin et al. 2009) and de Barros Pita et al. (2012). For each condition, two biological and three technical replicates were performed, totalling 6 wells for each gene per sample.

Transcript quantification and ratios for target genes

The transcript quantities of target genes were determined by using the $2^{-\Delta Cq}$ method, a variation of the $2^{-\Delta\Delta Cq}$ gene expression quantification method, previously described by Livak et al. (2001). The $2^{-\Delta Cq}$ is determined from the ΔCq

parameter obtained in the RT-qPCR assays, which is calculated from subtracting Cycle Quantification (Cq) values of the reference gene from the Cq values of a target gene in a given sample (Livak et al. 2001). The ΔCq value is subsequently applied in the formula $2^{-\Delta Cq}$ to provide the Normalized Transcript Quantity (NTQ), which is an arbitrary unity that estimates the transcript quantity of a gene within a particular sample relative to the reference gene. Therefore, NTQ values are useful to compare the transcript levels of different genes in a given sample. Once NTQ ($2^{-\Delta Cq}$) values are obtained for each target gene, it is possible to calculate the transcript ratios between different genes, which might point to a possible metabolic direction. Standard deviations were calculated from the average of the six values obtained for each gene in each sample, as described in the previous section.

Results and discussion

Fermentative capacity of *B. bruxellensis* JP19M and GDB 248 strains in galactose-based and in mixed glucose/galactose media

Since *B. bruxellensis* is able to assimilate the main sugars present in the lignocellulosic hydrolysate, such as glucose, cellobiose, xylose and arabinose, we aimed to investigate its fermentative performance in galactose, another sugar found in this industrial substrate. In this sense, we analysed growth profiles as well as sugar consumption and metabolites production by two strains of *B. bruxellensis*, GDB 248 and JP19M, grown in galactose and in a mixture of galactose and glucose (mixed medium). Our results showed that JP19M strain did not present a lag phase and the growth rate in galactose was equal to 0.15 h^{-1} (Fig.1a and Table 1). Moreover, JP19M consumed 80% (16 g/l) of the galactose available after 24 hours of cultivation, preferentially by converting it to biomass and ethanol, *i.e.*, in the respiro-fermentative metabolism (Fig.1a). Similar to JP19M, *S. cerevisiae* KL17 presents respiro-fermentative metabolism in galactose-based medium. However, in that strain, galactose is rapidly consumed (20 g/l in ~15 h of cultivation), which results in high ethanol yield (0.44 g) (Kim et al. 2014). The highest ethanol yield (0.25 g/g) in JP19M was observed within 24 hours of cultivation (Table 1). Although these parameters are lower than those

observed for *S. cerevisiae* CAT-1 (0.28 h^{-1}, 0.34 g/g) and *Kluyveromyces marxianus* 6C17 (0.34 g/g), they are higher than the ones found in *B. bruxellensis* CBS 2499 (Moktaduzzaman et al. 2015; Beniwal et al. 2017; Nascimento and Fonseca 2019).

On the other hand, GDB 248 presented a large lag phase, with a slow-start in the 12-24 hours interval and entering the exponential growth phase only after 24 hours of cultivation (Fig.1b). The growth rate observed for that strain was low (0.07 h^{-1}) similar to *B. bruxellensis* CBS 2499 (Moktaduzzaman et al. 2015). The slow growth performance resulted in a poor assimilation of galactose (less than 14% or 2.77 g/l after 24 hours), which was preferentially directed towards biomass formation and small amounts of acetate (48h), with no ethanol produced (Fig.1b and Table 1). In *B. bruxellensis* CBS 2499, the galactose consumed (20 g/l after 140 hours of cultivation) is also directed to biomass production, without ethanol or acetate detected (Moktaduzzaman et al. 2015). The absence of detectable ethanol is compatible with a preference for the respiratory metabolism to the detriment of fermentation in GDB 248 and it is similar to the profile observed for *B. bruxellensis* CBS 2499 strain (Moktaduzzaman et al. 2015).

In the mixed medium, we observed that JP19M strain also did not present a lag phase and the growth rate was 0.14 h^{-1} (Fig.1c and Table 1). The presence of glucose resulted both in a similar growth rate and biomass yield, when compared with galactose-based medium (Table 1). Moreover, JP19M simultaneously consumed glucose and galactose (~38% and ~24%, respectively, in the first six hours of cultivation) and both sugars were completely exhausted within the 24-hours interval (Fig.1c and Table 1). Co-consumption of glucose and galactose in qualitative assays (da Silva et al. 2019) as well as glucose and xylose in fermentation assays (da Silva et al. 2020) have been previously reported for JP19M. This phenotype is achieved due to the absence of Glucose Catabolite Repression (GCR) in this strain and represents an important physiological trait for the second-generation ethanol industry (manuscript in preparation). It is important to note that *S. cerevisiae* KL17 strain can also simultaneously consume glucose and galactose (100% and 39%, respectively, within six hours) with ethanol yield of 0.47 g/g and no acetate detected (Kim et al. 2014). Our data showed that JP19M presents a physiological profile compatible with respiro-fermentative metabolism, in which the utilization glucose

and galactose resulted both in biomass and ethanol (Fig.1c, d and Table 1). Moreover, the ethanol yield observed for JP19M in the present work (0.32 g/g) is comparable to the data reported for *K. marxianus* 6C17 (0.30 g/g) in similar conditions (Beniwal et al. 2017). On the other hand, the presence of glucose in the mixed medium decreased the lag phase observed for GDB 248 in galactose-based medium and the growth rate calculated was 0.17 h^{-1} (Fig.1d and Table 1). It is interesting to note that the availability of glucose resulted both in increased growth rate and biomass yield when compared with galactose-based medium (Table 1). Moreover, the consumption of galactose in GDB 248 was possible only after glucose exhaustion, which is compatible with a yeast strain under GCR control (Fig.1d and Table 1), similar to *S. cerevisiae* CAT-1, *K. marxianus* 6C17 and *B. bruxellensis* CBS2499 (Moktaduzzaman et al. 2015; Beniwal et al. 2017; Nascimento and Fonseca 2019). We also observed a profile compatible with respiro-fermentative metabolism for GDB 248 in mixed medium, in which the assimilation of both sugars resulted in biomass (0.54 g/g), as well as similar acetate and ethanol yields (0.10 g/g) after 24 hours of cultivation. However, after 48 hours, acetate yield was higher than the ethanol one, as observed by Leite et al. (2013) in aerobic C-limited chemostat pulsed with glucose. Moreover, the shift from respiratory to respiro-fermentative metabolism in response to the presence of glucose in the mixed medium is also observed in *B. bruxellensis* CBS 2499 (Moktaduzzaman et al. 2015).

Fig. 1 Sugar consumption and metabolite production of *B. bruxellensis* JP19M (A and C) and GDB 248 (B and D) under aerobic conditions in galactose-based (A and B) and mixed media (C and D). Symbols: closed square, glucose; closed circle, galactose; open triangle, O.D. (600 nm); open diamond, ethanol; open circle, acetate.

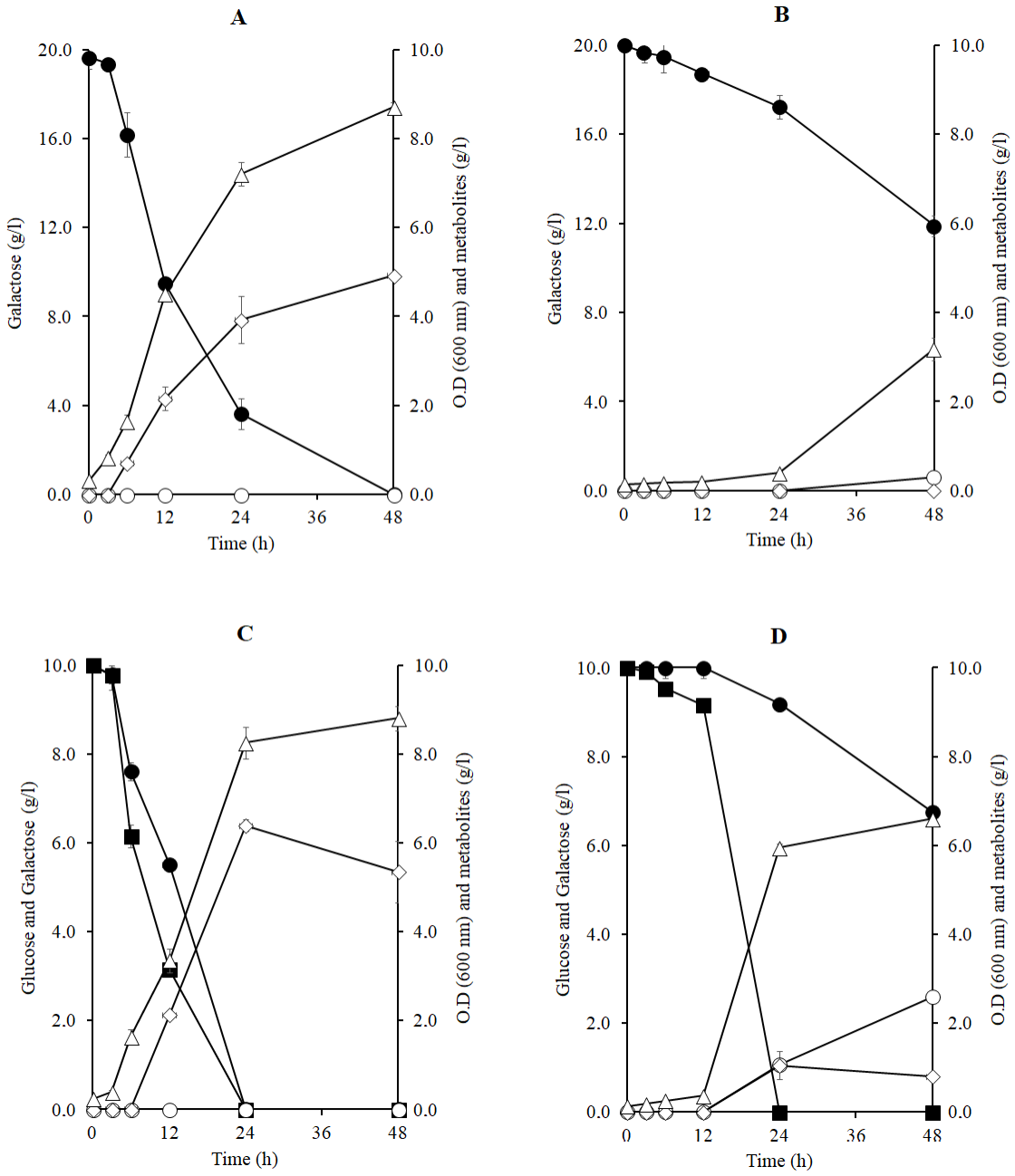

Table 1 Fermentative parameters of *B. bruxellensis* GDB 248 and JP19M under aerobic growth assays containing galactose or a mixture of galactose and glucose.

Strain	Medium	Time (h)	Growth rate (h-1)	Residual Glucose (g/l)	Residual Galactose (g/l)	$Y_{P/S}$ ethanol (g/g)	$Y_{P/S}$ acetate (g/g)	$Y_{P/S}$ CO_2 (g/g)	$Y_{X/S}$ biomass (O.D/g)	Mass balance (%)
GDB 248	Galactose	6	0.07 ± 0.00	-	19.49 ± 0.72	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.03 ± 0.00	3.04 ± 0.13
		12			18.71 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.04 ± 0.00	3.62 ± 0.82
		24		-	17.22 ± 0.51	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.09 ± 0.02	9.46 ± 2.49
		48		-	11.88 ± 0.46	0.00 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	0.39 ± 0.03	45.06 ± 3.54
JP19M		6	0.15 ± 0.02	-	16.19 ± 1.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.38 ± 0.04	38.47 ± 3.96
		12			10.15 ± 0.29	0.21 ± 0.03	0.00 ± 0.00	0.20 ± 0.02	0.41 ± 0.01	82.98 ± 4.36
		24		-	3.63 ± 0.69	0.25 ± 0.03	0.00 ± 0.00	0.23 ± 0.03	0.43 ± 0.02	91.07 ± 4.80
		48		-	0.00 ± 0.00	0.25 ± 0.01	0.00 ± 0.00	0.24 ± 0.00	0.43 ± 0.01	91.81 ± 1.22
GDB 248	Mixed medium	6	0.17 ± 0.00	9.53 ± 0.09	10.00 ± 0.58	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.26 ± 0.04	25.76 ± 4.59
		12		8.95 ± 0.09	10.00 ± 0.43	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.22 ± 0.01	21.94 ± 1.42
		24		0.00 ± 0.00	9.18 ± 0.11	0.10 ± 0.00	0.10 ± 0.01	0.14 ± 0.03	0.54 ± 0.01	89.86 ± 0.08
		48		0.00 ± 0.00	6.75 ± 0.01	0.06 ± 0.00	0.20 ± 0.00	0.18 ± 0.00	0.49 ± 0.00	88.73 ± 0.16
JP19M		6	0.14 ± 0.01	6.15 ± 0.25	7.61 ± 0.21	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.22 ± 0.02	22.33 ± 2.20
		12		3.15 ± 0.03	5.50 ± 0.14	0.19 ± 0.00	0.00 ± 0.00	0.18 ± 0.00	0.27 ± 0.02	64.04 ± 1.74
		24		0.00 ± 0.00	0.00 ± 0.00	0.32 ± 0.00	0.00 ± 0.00	0.30 ± 0.00	0.40 ± 0.03	102.92 ± 0.99
		48		0.00 ± 0.00	0.00 ± 0.00	0.27 ± 0.03	0.00 ± 0.00	0.24 ± 0.01	0.43 ± 0.02	93.94 ± 2.11

Transcriptional ratio of target genes of *B. bruxellensis* strains in galactose-based and mixed media

Since GDB 248 and JP19M presented contrasting phenotypes regarding the production of metabolites, we analysed the expression pattern of fermentation genes, *PDC1*, *ADH1* and *ALD3*, and gene of oxidative phosphorylation, *ATP1* aiming to determine a possible link between mRNA levels and metabolite production in our strains. In order to investigate (i) the fate of pyruvate, (ii) the preferable pathway for reoxidizing the catabolism-derived NADH and (iii) the fate of acetaldehyde, we determined *PDC1/ATP1*, *ADH1/ATP1* and *ADH1/ALD3* transcript ratios, respectively. *PDC1* gene codes for pyruvate decarboxylase, an enzyme that converts pyruvate in acetaldehyde, which further acts as a branchpoint to ethanol or acetate production (Pronk et al. 1996). The direction of this metabolism is dependent on a few factors and relies mostly on the action of either alcohol dehydrogenase (Adh1, which reduces acetaldehyde to ethanol*)* or aldehyde dehydrogenase (Ald3, which oxidizes acetaldehyde to acetate). On the other hand, *ATP1* gene codes for alpha subunit of the F1 sector of mitochondrial F1F0 ATP synthase, a representant of respiratory metabolism (Takeda et al. 1986).

In galactose-based medium, *PDC1/ATP1* ratio for JP19M was ~1 (near to stability), meaning that both pathways (fermentation and respiration) should be active (Table 2). In addition, *ADH1/ATP1* ratio was ~2, which means that *ADH1* is twice as transcribed as *ATP1*, suggesting that JP19M does not present a clear preference for a pathway to reoxidize catabolism-derived NADH in galactose. Finally, *ADH1/ALD3* ratio was ~4, which means that *ADH1* transcript in this condition is almost four times more abundant than *ALD3* and suggests a preference for reducing acetaldehyde to ethanol. In fact, we were able to detect only ethanol in our assays with JP19M. Nevertheless, it is important to note that acetate is essential for cell metabolism and despite its absence in our samples, it should be produced in small amounts in order to meet yeast requirements (Van Rossum et al. 2016). Altogether, our data point to a pattern compatible with respiro-fermentative metabolism in JP19M, in accordance with the data observed in our physiological assays. Similar to JP19M, *S. cerevisiae* CEN.PK 113-7D also exhibits respiro-fermentative metabolism in galactose based-medium, presenting low acetate formation (Ostergaard et al. 2000b). On the other hand, *PDC1/ATP1* ratio for GDB 248 was ~0.25, which means that *ATP1* is ~4 times more transcribed than *PDC1*

(Table 2). Moreover, *ADH1/ATP1* ratio was only ~0.08, meaning that *ATP1* is ~12 times more transcribed than *ADH1*, suggesting that GDB 248 present a preference for reoxidizing NADH through respiratory chain when in galactose. Increased levels of transcripts of genes involved in respiratory pathway and TCA genes are observed in the *B. bruxellensis* CBS 2499 strain, which exhibits respiratory metabolism in galactose-based medium (Moktaduzzaman et al. 2015). Finally, *ADH1/ALD3* transcript ratio in GDB248 was ~14, despite the lack of detectable ethanol in our samples. Curiously, in spite of the higher *ADH1/ALD3* ratio for GDB 248 than JP19M, the relative abundance of *ADH1* transcripts was 6 times lower in GDB 248 than in JP19M, which might explain the large difference regarding ethanol production between our two strains. Moreover, in *B. bruxellensis* CBS 2499 strain, Adh1 enzyme activity was similar in galactose or in glucose, yet with no ethanol production in galactose-based medium, which means that even with Adh1 enzyme active, *B. bruxellensis* cells do not necessarily produce ethanol (Moktaduzzaman et al. 2015). Altogether, our results point to a profile compatible with a respiratory metabolism for GDB 248, also in accordance with our physiological data. It is also interesting to note that the relative abundance of *ATP1* transcripts was ~4 times higher in GDB 248 than in JP19M, reinforcing the oxidative profile of GDB 248 when grown in galactose. This observation might be related to its low consumption of galactose, which leads to slow flow through the glycolytic pathway and the oxidation of pyruvate through tricarboxylic acid cycle (Ostergaard et al. 2000a).

In mixed medium, *PDC1/ATP1* ratio was ~0.30 in JP19M, which means that *ATP1* is approximately three times more transcribed than *PDC1* in this condition (Table 2). In addition, *ADH1/ATP1* ratio was 0.21, meaning that *ATP1* is ~5 times more transcribed than *ADH1*. An increased transcription rate, in presence of glucose, for a gene involved with oxidative phosphorylation, such as *ATP1*, indicates that respiration is working along with fermentation. Moreover, this fact is likely to be related to the absence of GCR control in JP19M, different from the observed for *S. cerevisiae* (Rettori and Volpe 2000; da Silva et al. 2019; manuscript in preparation). Finally, *ADH1/ALD3* transcript ratio in mixed medium was only ~1.2, which means that *ADH1* transcript in this condition is slightly more abundant than *ALD3,* yet only ethanol was detected in our assays for this strain. Once again, it is likely that acetate is produced in small amounts (undetectable) to fulfil the metabolic requirements of the cells. It is interesting to note that the presence of glucose shifts the ratios in

favour of *ATP1* transcription (towards respiration) in JP19M. However, the detection of *ADH1* transcripts and ethanol in our assays points to a profile compatible with a respiro-fermentative metabolism, which is the usual response in *B. bruxellensis* when glucose is available (Rozpędowska et al. 2011; de Barros Pita et al. 2013a, 2013b; Leite et al. 2013). On the other hand, in GDB 248, *PDC1/ATP1* transcript ratio was ~5.25, which means that, in principle, cells are fermentation prone (Table 2). However, *ADH1/ATP1* transcript ratio was and ~0.5, *i.e*., that *ATP1* is twice as transcribed as *ADH1* in this condition and that both pathways are likely to be active. Finally, *ADH1/ALD3* transcript ratio was ~23, meaning that *ADH1* gene is largely more transcribed than *ALD3* gene. Similar to galactose-based medium, in spite of a higher *ADH1/ALD3* ratio for GDB 248, the relative abundance of *ADH1* transcripts was 7.3 times higher in JP19M than in GDB 248. It is interesting to note that the presence of glucose triggers the respiro-fermentative metabolism in GDB 248, as previously reported (de Barros Pita et al. 2013a, 2013b; Leite et al. 2013), since we observed a shift in the transcript ratios (however, towards fermentation, Table 2).

Table 2 Transcript ratio of genes involved with fermentative pathway and oxidative phosphorylation in *B. bruxellensis* JP19M and GDB 248 strains grown in galactose-based and mixed media. Genes: *ATP1*, alpha subunit of the ATP synthase complex; *PDC1*, pyruvate decarboxylase 1; *ADH1*, alcohol dehydrogenase 1; *ALD3*, cytosolic aldehyde dehydrogenase. The transcript ratios were calculated for each strain after the determination of the normalized transcript quantity in each medium as described in the Materials and Methods section. Standard deviations are not shown as they were lower than 10% of the mean values.

	B. bruxellensis JP19M		*B. bruxellensis* GDB 248	
Gene	Galactose	Galactose + Glucose	Galactose	Galactose + Glucose
PDC1/ATP1	1.04	0.31	0.25	5.26
ADH1/ATP1	2.05	0.21	0.08	0.49
ADH1/ALD3	4.34	1.17	13.96	23.5

Conclusion

Our results showed that *B. bruxellensis* GDB 248 and JP19M strains present different metabolic profiles, as observed from our physiological and genetic data. In the present work, we showed that galactose assimilation by GDB 248 resulted only

in biomass and acetate production, suggesting a respiratory-prone metabolism. Transcription profiles of genes related to fermentation and ATP synthesis led us to conclude that, in fact, GDB 248 strain used preferentially the respiratory chain to reoxidize the catabolism derived NADH, when galactose was the sole carbon source. The presence of glucose in the mixed medium led GDB 248 cells to also produce ethanol yet with a low yield, indicating a shift to the respiro-fermentative metabolism, which was confirmed by our transcriptional analyses. Moreover, the consumption of galactose in the mixed medium by GDB 248 was possible only when glucose was depleted, indicating that this strain is subjected to the repressive effect of glucose. Different from GDB 248, JP19M strain was able to produce biomass and ethanol from galactose and in the mixture of glucose and galactose, suggesting a respiro-fermentative metabolism in both conditions. Transcription ratios obtained from fermentation and ATP synthesis (respiration) genes confirms that JP19M presented, in fact, both pathways active. Moreover, JP19M consumes glucose and galactose simultaneously, a useful physiological trait, mainly in substrates that are often a complex mixture of sugars. In summary, our results confirm that *B. bruxellensis* JP19M strain presents important metabolic characteristics, such as fermentation of galactose and co-consumption of sugars, which are beneficial and might be further explored in the fermentation industry, especially in the second-generation ethanol production.

References

Avramova M, Cibrario A, Peltier E et al (2018) *Brettanomyces bruxellensis* population survey reveals a diploid-triploid complex structured according to substrate of isolation and geographical distribution. Sci Rep 8:4136. DOI: https://doi.org/10.1038/s41598-018-22580-7

Bassi APG, Silva JCG, Reis VR, Ceccato-Antonini SR (2013) Effects of single and combined cell treatments based on low pH and high concentrations of ethanol on the growth and fermentation of *Dekkera bruxellensis* and *Saccharomyces cerevisiae*. World J Microbiol Biotechnol 29:1661–1676. DOI: 10.1007/s11274-013-1329-x

Beniwal A, Saini P, Kokkiligadda A, Vij S (2017) Physiological growth and galactose utilization by dairy yeast *Kluyveromyces marxianus* in mixed sugars and whey during fermentation. Biotech 7:349. DOI: 10.1007/s13205-017-0985-1

Bhat PJ (2008) Galactose Regulon of Yeast. Springer-Verlag, Berlin Heidelberg, 219 pp.

Bhat PJ, Murthy TV (2001) Transcriptional control of the *GAL*/*MEL* regulon of yeast *Saccharomyces cerevisiae*: mechanism of galactose-mediumted signal transduction. Mol Microbiol 40:1059–66. DOI: 10.1046/j.1365-2958.2001.02421.x

Bustin SA, Benes V, Garson JA et al (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55:611-22. DOI: 10.1373/clinchem.2008.112797

Codato CB, Martini C, Ceccato-Antonini SR, Bastos RG (2018). Ethanol production from *Brettanomyces bruxellensis* in synthetic medium with pentose. J Chem Eng 35:11–17. DOI: https://doi.org/10.1590/0104-6632.20180351s20160475

Conrad M, Schothorst J, Kankipati HN, Van Zeebroeck G, Rubio-Texeira M, Thevelein JM (2014) Nutrient sensing and signaling in the yeast *Saccharomyces cerevisiae*. FEMS Microbiol Rev 38:254-99. DOI: 10.1111/1574-6976.12065

Conterno L, Joseph CML, Arvik TJ, Henick-Kling T, Bisson LF (2006) Genetic and Physiological Characterization of *Brettanomyces bruxellensis* Strains Isolated from Wines. Am J Enol Vitic 57:139-147. DOI: 10.1016/j.ijfoodmicro.2009.01.025

Crauwels S, Van Assche A, de Jonge R et al (2015) Comparative phenomics and targeted use of genomics reveals variation in carbon and nitrogen assimilation among different *Brettanomyces bruxellensis* strains. Appl Microbiol Biotechnol 91:23–34. DOI: https://doi.org/10.1007/s00253-015-6769-9

da Silva JM, da Silva GHTG, Parente DC, Leite FCB, Silva CS, Valente P, Ganga AM, Simões DA, de Morais MA Jr (2019) Biological diversity of carbon assimilation among isolates of the yeast *Dekkera bruxellensis* from wine and fuel-ethanol industrial processes. FEMS Yeast Res 19:foz022. DOI: 10.1093/femsyr/foz022

da Silva JM, Ribeiro KC, Teles GH, Ribeiro E, de Morais Junior MA, de Barros Pita W (2020) Fermentation profiles of the yeast *Brettanomyces bruxellensis* in D-xylose and L-arabinose aiming its application as a second-generation ethanol producer. Yeast 37:597-608. DOI: 10.1002/yea.3519

da Silva TC, Leite FC, De Morais MA Jr (2016) Distribution of *Dekkera bruxellensis* in a sugarcane-based fuel ethanol fermentation plant. Lett Appl Microbiol 62:354-8. DOI: 10.1111/lam.12558

de Araujo Guilherme A, Dantas PVF, Padilha CEA, Dos Santos ES, de Macedo GR (2019) Ethanol production from sugarcane bagasse: Use of different fermentation strategies to enhance an environmental-friendly process. J Environ Manage 234:44-51. DOI: 10.1016/j.jenvman.2018.12.102

de Barros Pita W, Leite FC, de Souza Liberal AT, Pereira LF, Carazzolle MF, Pereira GA, de Morais MA Jr (2012) A new set of reference genes for RT-qPCR assays in the yeast *Dekkera bruxellensis*. Can J Microbiol 58:1362–1367. DOI: 10.1139/cjm-2012-0457

de Barros Pita W, Silva DC, Simões DA, Passoth V, de Morais MA Jr (2013b) Physiology and gene expression profiles of *Dekkera bruxellensis* in response to carbon and nitrogen availability. Antonie Van Leeuwenhoek 104:855-68. DOI: 10.1007/s10482-013-9998-x

de Barros Pita W, Teles GH, Peña-Moreno IC, da Silva JM, Ribeiro KC, de Morais Junior MA (2019) The biotechnological potential of the yeast *Dekkera bruxellensis*. World J Microbiol Biotechnol 35:103. DOI: 10.1007/s11274-019-2678-x

de Barros Pita W, Tiukova I, Leite FC, Passoth V, Simões DA, de Morais MA Jr (2013a) The influence of nitrate on the physiology of the yeast *Dekkera bruxellensis* grown under oxygen limitation. Yeast 30:111-7. DOI: 10.1002/yea.2945

De Deken, RH (1966). The Crabtree effect: a regulatory system in yeast. J Gen Microbiol 44:149–156. DOI: https://doi.org/10.1099/00221287-44-2-149

de Souza Liberal AT, Basılio ACM, do Monte Resende A et al (2007) Identification of *Dekkera bruxellensis* as a major contaminant yeast in continuous fuel ethanol fermentation. J Appl Microbiol 102:538–47. DOI: 10.1111/j.1365-2672.2006.03082.x

dos Santos LV, de Barros Grassi MC, Gallardo JCM, et al (2016) Second-Generation Ethanol: The Need is Becoming a Reality. Ind Biotechnol 12:40–57. DOI: https://doi.org/10.1089/ind.2015.0017

Gamage J, Lam H, Zhang Z (2010) Bioethanol production from lignocellulosic biomass, a review. J Biobased Mater Bioenergy 4:3–11. DOI: https://doi.org/10.1166/jbmb.2010.1071

Kang Q, Appels L, Tan T, Dewil R (2014) Bioethanol from lignocellulosic biomass: current findings determine research priorities. Sci World J 2014:1-13. DOI: 10.1155/2014/298153

Kim JH, Ryu J, Huh IY, Hong SK, Kang HA, Chang YK (2014) Ethanol production from galactose by a newly isolated *Saccharomyces cerevisiae* KL17. Bioprocess Biosyst Eng 37:1871-8. DOI: 10.1007/s00449-014-1161-1

Knauf M, Moniruzzaman M (2004) Lignocellulosic Biomass Processing: A Perspective. Int Sugar J 106, 147-150.

Leite FCB, Basso TO, de Barros PW, Gombert AK, Simões DA, de Morais Jr MA (2013) Quantitative aerobic physiology of the yeast *Dekkera bruxellensis*, a major contaminant in bioethanol production plants. FEMS Yeast Res 13:34–43. DOI: https://doi.org/10.1111/j.1567-1364.2012.12007.x

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-8. DOI: 10.1006/meth.2001.1262

Medina K, Boido E, Fariña L, Dellacassa E, Carrau F (2016) Non-*Saccharomyces* and *Saccharomyces* strains co-fermentation increases acetaldehyde accumulation: effect on anthocyanin-derived pigments in Tannat red wines. Yeast 33:339-43. DOI: 10.1002/yea.3156

Moktaduzzaman M, Galafassi S, Capusoni C, Vigentini I, Ling Z, Piškur J, Compagno C (2015) Galactose utilization sheds new light on sugar metabolism in the sequenced strain *Dekkera bruxellensis* CBS 2499. FEMS Yeast Res 15:fou009. DOI: 10.1093/femsyr/fou009

Mussatto SI, Dragone G, Guimarães PMR et al (2010) Technological trends, global market, and challenges of bio-ethanol production. Biotechnol Adv 28:817–30. DOI: 10.1016/j.biotechadv.2010.07.001

Nardi T, Remize F, Alexandre H (2010) Adaptation of yeasts *Saccharomyces cerevisiae* and *Brettanomyces bruxellensis* to winemaking conditions: a comparative study of stress genes expression. Appl Microbiol Biotechnol 88:925–937. DOI: 10.1007/s00253-010-2786-x

Nascimento VM, Fonseca GG (2019) Effects of the carbon source and the interaction between carbon sources on the physiology of the industrial *Saccharomyces cerevisiae* CAT-1. Prep Biochem Biotechnol 50:349-356. DOI: 10.1080/10826068.2019.1703192

Ostergaard S, Olsson L, Johnston M, Nielsen J (2000b) Increasing galactose consumption by *Saccharomyces cerevisiae* through metabolic engineering of the *GAL* gene regulatory network. Nat Biotechnol 18:1283-6. DOI: 10.1038/82400

Ostergaard S, Roca C, Rønnow B, Nielsen J, Olsson L (2000a) Physiological studies in aerobic batch cultivations of *Saccharomyces cerevisiae* strains harboring the *MEL1* gene. Biotechnol Bioeng 68:252-9. DOI: 10.1002/(sici)1097-0290(20000505)68:3<252::aid-bit3>3.0.co;2-k

Pfeiffer T, Morley A (2014) An evolutionary perspective on the Crabtree effect. Front Mol Biosci 1:17. DOI: https://doi.org/10.3389/fmolb.2014.00017

Postma E, Verduyn C, Scheffers WA, Van Dijken JP (1989) Enzymic analysis of the Crabtree effect in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*. Appl Environ Microbiol 55: 468–477. DOI: https://doi.org/10.1128/aem.55.2.468-477.1989

Pronk JT, Yde Steensma H, van Dijken JP (1996) Pyruvate metabolism in *Saccharomyces cerevisiae*. Yeast 12:1607–1633. DOI: 10.1002/(sici)1097-0061(199612)12:16<1607::aid-yea70>3.0.co;2-4

Rech RF, Fontana CR, Rosa AC, Camassola M, Ayub M, Dillon JPA (2019) Fermentation of hexoses and pentoses from sugarcane bagasse hydrolysates into ethanol by *Spathaspora hagerdaliae*. Bioprocess Biosyst Eng 42:83–92. DOI: 10.1007/s00449-018-2016-y

Reis ALS, Souza RFR, Torres RRNB et al (2014) Oxygen-limited cellobiose fermentation and the characterization of the cellobiase of an industrial *Dekkera/Brettanomyces bruxellensis* strain. SpringerPlus 3:38. DOI: 10.1186/2193-1801-3-38

Rettori D, Volpe PLO (2000) Microcalorimetria: uma técnica aplicável ao estudo do diauxismo da *Saccharomyces cerevisiae*. Química Nova 23:257-261.

Rodrigues F, Ludovico P, Leão C (2006) Sugar Metabolism in Yeasts: an Overview of Aerobic and Anaerobic Glucose Catabolism. In: Péter G., Rosa C. (eds)

Biodiversity and Ecophysiology of Yeasts. The Yeast Handbook. Springer, Berlin, Heidelberg. https://doi.org/10.1007/3-540-30985-3_6

Rozpędowska E, Hellborg L, Ishchuk OP, Orhan F, Galafassi S, Merico A, Woolfit M, Compagno C, Piskur J (2011) Parallel evolution of the make-accumulate-consume strategy in *Saccharomyces* and *Dekkera* yeasts. Nat Commun 2:302. DOI: 10.1038/ncomms1305

Sellick CA, Campbell RN, Reece RJ (2008) Galactose Metabolism in Yeast—Structure and Regulation of the Leloir Pathway Enzymes and the Genes Encoding Them. Int Rev Cell Mol Biol 269:111-150. DOI: 10.1016/S1937-6448(08)01003-4

Takeda M, Chen WJ, Saltzgaber J, Douglas MG (1986) Nuclear genes encoding the yeast mitochondrial ATPase complex. Analysis of *ATP1* coding the F1-ATPase alpha-subunit and its assembly. J Biol Chem. 261:15126-33.

Teles GH, da Silva JM, Mendonça AA, de Morais Junior MA, de Barros Pita W (2018) First aspects on acetate metabolism in the yeast *Dekkera bruxellensis*: a few keys for improving ethanol fermentation. Yeast 35:577–584. DOI: 10.1002/yea.3348

Tiukova IA, de Barros Pita W, Sundell D, Haddad Momeni M, Horn SJ, de Morais MA, Passoth V (2014) Adaptation of *Dekkera bruxellensis* to lignocellulose-based substrate. Biotechnol Appl Biochem 61:51–57. DOI: 10.1002/bab.1145

Van Dijken JP, Scheffers WA (1986) Redox balances in the metabolism of sugars by yeasts. FEMS Microbiol Rev 32:199–224. DOI: https://doi.org/10.1111/j.1574-6968.1986.tb01194.x

Van Maris AJ, Abbott DA, Bellissimi E et al (2006) Alcoholic fermentation of carbon sources in biomass hydrolysates by *Saccharomyces cerevisiae*: current status. Antonie Van Leeuwenhoek 90:391-418. DOI: 10.1007/s10482-006-9085-7

Van Rossum HM, Kozak BU, Pronk JT, van Maris AJA (2016) Engineering cytosolic acetyl-coenzyme A supply in Saccharomyces cerevisiae: Pathway stoichiometry, free-energy conservation and redox-cofactor balancing. Metab Eng 36:99-115. DOI: 10.1016/j.ymben.2016.03.006

Van Urk H,Voll WS, Scheffers WA and Van Dijken JP (1990) Transient-state analysis of metabolic fluxes in Crabtree-positive and Crabtree-negative yeasts. Appl Environ Microbiol 56:281–287. DOI: 10.1128/aem.56.1.281-287.1990

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Declarations

Conflict of interest

The authors declare that there are no conflicts of interest.

Ethical approval

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

8 DISCUSSÃO GERAL

Apesar de a levedura *B. bruxellensis* estar relacionada a episódios de contaminação nos processos de produção de vinho e etanol de primeira geração, um novo destaque vem sendo atribuído a esse micro-organismo, agora na indústria de etanol de segunda geração (de Souza Liberal et al., 2007; de Barros Pita et al., 2019). Esse destaque se deve ao fato de *B. bruxellensis* apresentar características fisiológicas e genéticas que possuem relevância para esse segmento produtivo, como a possibilidade de utilizar açúcares presentes na biomassa lignocelulósica, porém não metabolizados por *Saccharomyces cerevisiae* (Reis et al., 2014; Crauwels et al., 2015; Codato et al. 2018). Dessa forma, para avaliarmos a hipótese de que *B. bruxellensis* possui potencial de atuar na indústria de etanol de segunda geração, nós investigamos algumas das suas características metabólicas de maior relevância para esse setor industrial.

Inicialmente, a fim de entender a diversidade fenotípica entre linhagens de *B. bruxellensis* no que concerne à utilização de açúcares, realizamos uma triagem com 29 isolados, sendo 19 provenientes de vinícolas e 10 de destilarias de etanol combustível. A partir dessa triagem foi possível observar características contrastantes entre linhagens dos dois ambientes industriais, como o melhor desempenho dos isolados de etanol em celobiose e lactose em relação aos de vinho. Além disso, diferentemente dos isolados de vinho, algumas linhagens de etanol foram capazes de crescer na presença da 2-deoxiglicose (2DG), um repressor catabólico análogo à glicose. Esses fenótipos contrastantes sugeriram que o mecanismo de repressão catabólica pela glicose é mais aliviado em *B. bruxellensis* do que em *S. cerevisiae*. Em *S. cerevisiae*, a 2-DG é internalizada e fosforilada a 2-DG-6P, contudo, devido a ausência do oxigênio no C'2 desse composto, a 2-DG-6P não é isomerizada a frutose-6-fosfato e, com isso, é acumulada na célula mantendo o estado de repressão pela glicose ativo (McCartney et al., 2014; O'Donnel et al., 2015). Por outro lado, nos isolados de etanol JP19M, TB457A, JP206M e JP249A, a presença dessa molécula repressora não inibiu o crescimento celular sugerindo uma capacidade de co-consumir glicose e outras fontes de carbono. A habilidade de consumir simultaneamente diferentes açúcares é uma das características desejáveis para aplicação dos micro-

organismos na produção de etanol a partir da biomassa lignocelulósica e é rara em leveduras (Gao et al., 2019).

A diversidade fenotípica encontrada em *B. bruxellensis* nos levou a estudar o desempenho de três linhagens em xilose e arabinose, pentoses derivadas da hemicelulose e encontradas no material lignocelulósico. Apesar de esses açúcares não terem sido originalmente incluídos na lista de assimilados pela espécie (Kurtzman et al., 2011), estudos posteriores mostraram que a habilidade de assimilar xilose e arabinose é uma característica linhagem-específica em *B. bruxellensis* (Galafassi et al., 2011; Crauwels et al., 2015; Codato et al., 2018). Pela importância e abundância relativa no substrato, verificamos inicialmente a habilidade de diferentes linhagens de *B. bruxellensis* para a assimilação e fermentação de xilose e arabinose. Nós observamos que apenas três entre os nove isolados de etanol analisados foram capazes de assimilar ambas as pentoses, confirmando a característica linhagem-específica sugerida previamente (Crauwels et al., 2015). Curiosamente, estas mesmas três linhagens foram capazes de crescer na presença de 2-DG, TB457A, JP206M e JP19M. Em condições aeróbicas, TB457A, JP206M e JP19M converteram xilose e arabinose à biomassa, sem detecção de etanol ou acetato, semelhante a *M. guilliermondii* PYCC 3012 e *C. arabinofermentans* PYCC 5603 (Fonseca et al., 2007). Entretanto, sob limitação de oxigênio, nós observamos uma mudança nos perfis de consumo de açúcares e produção de metabólitos. Nesse cenário, nenhuma das três linhagens foi capaz de consumir arabinose, enquanto fermentaram xilose a etanol, ainda que com baixo rendimento e produtividade. Apesar disso, os rendimentos em etanol encontrados foram superiores aos apresentados para outras linhagens de *B. bruxellensis* na presença de xilose (Codato et al., 2018). A ausência de consumo de arabinose e a baixa velocidade de consumo de xilose está possivelmente atrelada ao desequilíbrio redox gerado pela limitação de oxigênio (Hahn-Hagerdal et al., 2007). Com isso, para o potencial emprego de *B. bruxellensis* na produção de etanol de segunda geração é necessário superar esse gargalo metabólico.

Além de fermentar pentoses a etanol, o micro-organismo candidato à produção de etanol de segunda geração precisa co-consumir os açúcares gerados da hidrólise da celulose e da hemicelulose (Rech et al., 2019). Esse requisito é uma das características relevantes de *B. bruxellensis*, pois as linhagens TB457A, JP19M e JP206M foram capazes de consumir xilose mesmo com a presença de

glicose em concentrações elevadas. Além disso, sob condições aeróbicas e em meio contendo a mistura de glicose e xilose, nós observamos que a presença de glicose, ainda que em baixas concentrações, favoreceu o consumo de xilose na linhagem JP19M. De fato, a presença de glicose aumenta a disponibilidade do NADPH (através da fase oxidativa da PPP) necessário para a redução de xilose a xilitol, reação catalisada pela xilose redutase (Meinander et al., 1999; Jeffries et al., 2007). Embora o co-consumo de glicose e fontes não preferenciais seja raro, a capacidade de co-consumir glicose e xilose sob condições aeróbicas também foi observada na levedura *S. passalidarum* (Hou, 2012; Rodrussamee et al., 2018). Ainda, em JP19M, os genes relacionados ao metabolismo de xilose, tais como *XYL1*, *XYL2*, *XKS1* não tiveram sua expressão reprimida pela presença da glicose, diferentemente de leveduras que respondem à repressão catabólica, tais como *K. marxianus* e linhagens modificadas de *S. cerevisiae* (Rodrussamee et al., 2011; Zhang et al., 2018). Além disso, genes associados à respiração celular (*SDH1*, *ATP1*), gliconeogênese (*FBP1*) e transporte de glicose de alta afinidade (*HXT6/7*) também não tiveram sua expressão reprimida pela glicose, enquanto o gene *HXK2* estava induzido. Em *S. cerevisiae*, Hxk2p está envolvida na repressão de genes de respiração, da gliconeogênese e de fontes alternativas de carbono (Conrad et al., 2014; Peeters e Thevelein, 2014). Entretanto, em JP19M, a indução do gene *HXK2* não resultou no fenótipo associado à GCR. É importante ressaltar que a ausência da GCR nesta linhagem permitiu o consumo simultâneo de açúcares, uma característica crucial para o emprego de micro-organismos na produção de etanol a partir de uma mistura de açúcares.

A busca por uma linhagem que reúne as melhores características para uma possível aplicação na produção de etanol nos levou a avaliar a capacidade de utilização aeróbica da galactose em duas linhagens de *B. bruxellensis*, GDB 248 e JP19M. As análises fisiológicas e genéticas apontaram para diferentes padrões de utilização de galactose entre essas linhagens. A galactose direcionou o metabolismo de GDB 248 para respiração, sem formação de etanol, semelhantemente ao que ocorre com *B. bruxellensis* CBS 2499 (Moktaduzzaman et al., 2015). Por outro lado, a galactose encaminhou JP19M para o metabolismo respiro-fermentativo com formação de etanol, assim como em *S. cerevisiae* KL17 e *K. marxianus* 6C17 (Kim et al., 2014; Beniwal et al., 2017). Levando em consideração a presença da glicose nos açúcares gerados pela hidrólise da

biomassa lignocelulósica, analisamos a sua influência sobre o metabolismo da galactose em ambas as linhagens. Observamos que, assim como sugerido no primeiro artigo (da Silva et al., 2019), a glicose inibiu o consumo de galactose por GDB 248. Apesar disso, o metabolismo dessa linhagem mudou de respiratório para respiro-fermentativo devido a presença de glicose, o que está relacionado ao efeito *Crabtree* positivo (Rozpędowska et al., 2011; Leite et al., 2013). Interessantemente, a glicose não exerceu efeito repressor sobre o metabolismo da galactose em JP19M, o que permitiu o consumo simultâneo de glicose e galactose, bem como a produção de etanol por essa linhagem, semelhante a *S. cerevisiae* KL17 (Kim et al., 2014). A análise de transcritos para os genes *ATP1*, *PDC1*, *ADH1* e *ADL3* sugerem que, em galactose, GDB 248 preferiu reoxidar o NADH derivado do catabolismo via respiração, ao passo que a adição de glicose mudou as razões de transcritos em favor da fermentação. Em *B. bruxellensis* CBS 2499, a presença de galactose induz a expressão de genes da cadeia transportadora de elétrons e do ciclo de Krebs (Moktaduzzaman et al., 2015). Por outro lado, em JP19M, os níveis de transcritos para esses genes sugerem que não houve uma preferência clara entre respiração e fermentação, apresentando a utilização de ambas as vias (metabolismo respiro-fermentativo). A presença de glicose, em JP19M, alterou os níveis de transcritos em favor de *ATP1*, contudo as células continuaram produzindo etanol com rendimentos maiores do que os apresentados em meio contendo apenas galactose.

Diante do exposto, os resultados gerados na presente tese de doutorado indicam que a linhagem JP19M de *B. bruxellensis* reúne um conjunto de características metabólicas relevantes para a indústria de fermentação alcoólica, principalmente para a produção de etanol de segunda geração. Dentre essas características, destacamos a capacidade de consumo simultâneo de glicose e outros açúcares, como a xilose e a galactose, resultado da ausência de controle transcricional pela GCR. Além disso, a linhagem JP19M também foi capaz de fermentar esses açúcares a etanol, porém, no caso da xilose, com baixos rendimento e produtividade. Por outro lado, considerando que a utilização eficiente de um substrato industrial complexo, como o hidrolisado lignocelulósico, requer a exploração da maior parte dos açúcares presentes, o desempenho fermentativo de JP19M em xilose ainda é um entrave para sua utilização nesse setor industrial. Entretanto, há a possibilidade do desenvolvimento de estratégias de engenharia

genética no intuito de superar os gargalos metabólicos de JP19M, que diminuem significativamente a sua performance fermentativa em xilose.

9 CONCLUSÕES

- As linhagens de *B. bruxellensis* apresentam ampla diversidade de assimilação de açúcares, com altas taxas de crescimento em condições aeróbicas e anaeróbicas.
- Os isolados de etanol combustível, JP19M, TB457A, JP206M e JP249A possuem a habilidade de assimilar fontes alternativas de carbono mesmo na presença de um repressor catabólico análogo a glicose. Esse perfil fenotípico sugere que essas linhagens podem co-consumir diferentes açúcares.
- As linhagens JP19M, TB457A e JP206M são capazes de assimilar as pentoses encontradas em substratos como o hidrolisado lignocelulósico, mas direcionam majoritariamente para a produção de biomassa.
- Enquanto as linhagens JP19M, TB457A e JP206M não são capazes de fermentar a arabinose, a eficiência de fermentação da xilose por essas linhagens é baixa, o que impede a aplicação imediata de *B. bruxellensis* na indústria de produção de etanol de segunda geração.
- *B. bruxellensis* JP19M é capaz de consumir simultaneamente glicose e xilose, mesmo em concentrações elevadas de glicose, o principal repressor catabólico.
- O co-consumo de açúcares mesmo em presença de glicose resulta da não susceptibilidade de JP19M ao mecanismo de GCR, resultando no alívio da repressão naturalmente imposta pela glicose e na expressão de genes de assimilação de fontes secundárias.
- *B. bruxellensis* JP19M é capaz de fermentar galactose a etanol, ao passo que GDB 248 utiliza galactose para produção de biomassa e acetato. Adicionalmente, JP19M é capaz de consumir glicose e galactose simultaneamente, enquanto a glicose exerce o seu efeito repressor sob o metabolismo da galactose em GDB 248.
- *B. bruxellensis* JP19M e GDB 248 apresentam diferentes perfis no que concerne às vias preferidas para reoxidação do NADH derivado do catabolismo. GDB 248 utiliza o metabolismo respiratório em presença de galactose e muda para o metabolismo respiro-fermentativo em meio misto.

Por outro lado, JP19M apresenta utiliza o metabolismo respiro-fermentativo em ambas as condições.

- Os resultados apresentados nesta tese apontam a linhagem JP19M como promissora para indústria de etanol de segunda geração, desde que gargalos relacionados a fermentação de pentoses sejam solucionados, a fim de melhorar sua performance fermentativa.

REFERÊNCIAS

Ahuatzi D, Herrero P, de la Cera T and Moreno F (2004) The glucose-regulated nuclear localization of hexokinase 2 in *Saccharomyces cerevisiae* is Mig1-dependent. J Biol Chem 279: 14440–14446.

Albertin W, Panfili A, Miot-Sertier C, Goulielmakis A, Delcamp A, Salin F, Lonvaud-Funel A, Curtin C and Masneuf-Pomarede I (2014) Development of microsatellite markers for the rapid and reliable genotyping of *Brettanomyces bruxellensis* at strain level. Food Microbiol 42:188–195.

Alexander MA and Jeffries TW (1990) Respiratory partitioning in yeasts efficiency and metabolize as regulatory phenomena. Enzyme Microb Technol 12:1-19.

Amorim HV e Lopes ML (2009) Tecnologia sobre processamento de leveduras vivas, inativas e seus derivados: conceitos básicos. In: Congresso Internacional Sobre uso da Levedura na Alimentação Animal. Campinas, São Paulo, Brazil: CBNA:5–20.

Bassi APG, Silva JCG, Reis VR and Ceccato-Antonini SR (2013) Effects of single and combined cell treatments based on low pH and high concentrations of ethanol on the growth and fermentation of *Dekkera bruxellensis* and Saccharomyces cerevisiae. World J Microbiol Biotechnol 29:1661–1676.

Becker J and Boles E (2003) A modified *Saccharomyces cerevisiae* strain that consumes L-Arabinose and produces ethanol. Appl Environ Microbiol 69:4144-4150.

Belinchón MM and Gancedo JM (2007a) Different signalling pathways mediate glucose induction of *SUC2*, *HXT1* and pyruvate decarboxylase in yeast. FEMS Yeast Res 7: 40–47.

Beniwal A, Saini P, Kokkiligadda A and Vij S (2017) Physiological growth and galactose utilization by dairy yeast *Kluyveromyces marxianus* in mixed sugars and whey during fermentation. Biotech 7:349.

Bertels LK, Fernández Murillo L and Heinisch JJ (2021) The Pentose Phosphate Pathway in Yeasts-More Than a Poor Cousin of Glycolysis. Biomolecules. 11(5):725.

Bertrand E, Vandenberghe LPS, Soccol CR, Sigoillot JC and Faulds C (2016) First generation bioethanol. In: Soccol CR, Brar SK, Faulds C, Ramos LP (eds) Green fuels technology. Switzerland: Springer International Publishing 175–212.

Bettiga M, Bengtsson O, Hahn-hägerdal B and Gorwa-grauslund MF (2009) Arabinose and xylose fermentation by recombinant *Saccharomyces cerevisiae* expressing a fungal pentose utilization pathway. Microb Cell Fact 12:1–12.

Bhat PJ (2008) Galactose Regulon of Yeast. Springer-Verlag, Berlin Heidelberg, 219 pp.

Bhat PJ and Iyer RS (2009) Epigenetics of the yeast galactose genetic switch. J Biosci 34:513–522.

Bhat PJ and Murthy TV (2001) Transcriptional control of the GAL/MEL regulon of yeast *Saccharomyces cerevisiae*: mechanism of galactose-mediated signal transduction. Mol Microbiol 40:1059–66.

Biddick RK, Law GL and Young ET (2008) Adr1 and Cat8 mediate coactivator recruitment and chromatin remodeling at glucose regulated genes. PLoS One 3:e1436.

Blomqvist J, South E, Tiukova L, Momeni MH, Hansson H, Ståhlberg J, Horn SJ, Schnürer J, Passoth V (2011) Fermentation of lignocellulosic hydrolysate by the alternative industrial ethanol yeast *Dekkera bruxellensis*. Lett Appl Microbiol 53:73–78.

Boles E and Hollenberg CP (1997) The molecular genetics of hexose transport in yeasts. FEMS Microbiol Rev 21:85–111.

Brandt A, Gräsvik J, Hallett JP and Welton T (2013) Deconstruction of lignocellulosic biomass with ionic liquids. Green Chemistry 15:550-583

Brat D, Boles E and Wiedemann B (2009) Functional expression of a bacterial xylose isomerase in *Saccharomyces cerevisiae*. Appl Environ Microbiol 75:2304-11.

Broach JR (2012) Nutritional control of growth and development in yeast. Genetics 192:73–105.

Bušić A, Marđetko N, Kundas S, et al (2018) Bioethanol Production from Renewable Raw Materials and Its Separation and Purification: A Review. Food Technol Biotechnol 56:289-311.

Caballero A and Ramos JL (2017) Enhancing ethanol yields through D -xylose and L -arabinose co-fermentation after construction of a novel high efficient L -arabinose-fermenting *Saccharomyces cerevisiae* strain. Microbiology (Reading) 163:442–452.

Canilha L, Kumar Chandel A, dos Santos Milessi TS, Fernandes Antunes FA, da Costa Freitas WL, das Graças Almeida Felipe M and da Silva SS (2012) Bioconversion of sugarcane biomass into ethanol: an overview about composition, pretreatment methods, detoxification of hydrolysates, enzymatic saccharification, and ethanol fermentation. J Biomed Biotechnol 2012:1-15.

Carrozza MJ, John S, Sil AK, Hopper JE and Workman JL (2002) Gal80 confers specificity on HAT complex interactions with activators. J Biol Chem 277:24648–24652.

Celenza JL and Carlson M (1989) Mutational analysis of the *Saccharomyces cerevisiae* SNF1 protein kinase and evidence for functional interaction with the SNF4 protein. Mol Cell Biol 9:5034–44.

Chatonnet P, Dubourdie D, Boidron J and Pons M (1992) The origin of ethylphenols in wines. J Sci Food Agric 60:165–178.

Chiang HL and Schekman R (1991) Regulated import and degradation of a cytosolic protein in the yeast vacuole. Nature 350: 313–318.

Chiang L, Gong C, Chen L and Tsao GT (1981) D-Xylulose Fermentation to Ethanol by *Saccharomyces cerevisiae*. Appl Environ Microbiol 42:284–289.

Claussen NH (1904) On a method/or the application of Hanseris pure yeast system in the manufacturing of well-conditioned English stock beers. J Inst Brew 10:308–331.

Codato CB, Martini C, Ceccato-Antonini SR and Bastos RG (2018) Ethanol production from *Dekkera bruxellensis* in synthetic media with pentose. J Chem Eng 35:11–17.

Conrad M, Schothorst J, Kankipati HN, Van Zeebroeck G, Rubio-Texeira M and Thevelein JM (2014) Nutrient sensing and signaling in the yeast *Saccharomyces cerevisiae*. FEMS Microbiol Rev 38:254-99.

Crabtree HG (1929) Observations on the carbohydrate metabolism of tumours. Biochem J 23:536–545.

Crauwels S, Van Assche A, de Jonge R, Borneman AR, Verreth C, Troels P, De Samblanx G, Marchal K, Van de Peer Y, Willems KA et al (2015)

Comparative phenomics and targeted use of genomics reveals variation in carbon and nitrogen assimilation among different *Brettanomyces bruxellensis* strains. Appl Microbiol Biotechnol 91:23-34.

Crauwels S, Van Opstaele F, Jaskula-Goiris B, Steensels J, Verreth C, Bosmans L, Paulussen C, Herrera-Malaver B, de Jonge R, De Clippeleer J et al (2017) Fermentation assays reveal differences in sugar and (off-) flavor metabolism across different *Brettanomyces bruxellensis* strains. FEMS Yeast Res 17:1–10.

Cunha JT, Soares PO, Baptista SL, Costa CE and Domingues L (2020) Engineered *Saccharomyces cerevisiae* for lignocellulosic valorization: a review and perspectives on bioethanol production. Bioengineered 11:883-903

Curtin C, Varela C and Borneman A (2015) Harnessing improved understanding of *Brettanomyces bruxellensis* biology to mitigate the risk of wine spoilage. Aust J Grape Wine Res 21:680–692.

da Silva TCD, Leite FCB and de Morais MA (2016) Distribution of *Dekkera bruxellensis* in a sugarcane-based fuel ethanol fermentation plant. Lett Appl Microbiol 62:354-358.

Davison SA, den Haan R and van Zyl WH (2016) Heterologous expression of cellulase genes in natural *Saccharomyces cerevisiae* strains. Appl Microbiol Biotechnol 100:8241–8254.

de Barros Pita W, Leite FCB, de Souza Liberal AT, Simões DA and de Morais MA Jr (2011) The ability to use nitrate confers advantage to *Dekkera bruxellensis* over *S. cerevisiae* and can explain its adaptation to industrial fermentation processes. Antonie Van Leeuwenhoek 100:99–107.

de Barros Pita W, Teles GH, Peña-Moreno IC, da Silva JM, Ribeiro KC and de Morais Junior MA (2019) The biotechnological potential of the yeast Dekkera bruxellensis. World Journal of Microbiology and Biotechnology 35:1-9.

de Barros Pita W, Tiukova I, Leite FC, Passoth V, Simões DA and de Morais MA Jr (2013a) The influence of nitrate on the physiology of the yeast *Dekkera bruxellensis* grown under oxygen limitation. Yeast 30:111-7.

de Souza Liberal AT, Basílio ACM, do Monte Resende A, Brasileiro BTV, da Silva-Filho EA, de Morais JO, Simões DA and de Morais MA Jr (2007)

Identification of *Dekkera bruxellensis* as a major contaminant yeast in continuous fuel ethanol fermentation. J Appl Microbiol 102:538–547.

Demeke MM, Dietz H, Li Y, Foulquié-Moreno MR, Mutturi S, Deprez S, Den Abt T, Bonini BM, Liden G, Dumortier F et al (2013) Development of a D-xylose fermenting and inhibitor tolerant industrial *Saccharomyces cerevisiae* strain with high performance in lignocellulose hydrolysates using metabolic and evolutionary engineering. Biotechnol Biofuels 6:89.

Dever TE, Kinzy TG and Pavitt GD (2016) Mechanism and Regulation of Protein Synthesis in *Saccharomyces cerevisiae*. Genetics 203:65-107.

Dickinson JR and Schweizer M (2004) The Metabolism and Molecular Physiology of *Saccharomyces cerevisiae*. Second edition. Taylor & Francis Ltd – London, 480 pp.

Dien BS, Cotta M and Jeffries TW (2003) Bacteria engineered for fuel ethanol production: current status. Appl Microbiol Biotechnol 63:258–266.

dos Santos LV, de Barros Grassi MC, Gallardo JCM, Pirolla RAS, Calderón LL, de Carvalho-Netto OV, Parreiras LS, Camargo ELO, Drezza AL, Missawa SK et al (2016) Second-Generation Ethanol: The Need is Becoming a Reality. Ind Biotechnol 12:40–57.

Esposito EO e Azevedo JL (2010) Fungos: uma introdução à biologia, bioquímica e biotecnologia. 2rd edition. Educs, Caxias do Sul, 510 pp.

Fernández-García P, Peláez R, Herrero P and Moreno F (2012) Phosphorylation of yeast hexokinase 2 regulates its nucleocytoplasmic shuttling. J Biol Chem 287:42151–64.

Flores CL, Rodríguez C, Petit T and Gancedo C (2000) Carbohydrate and energy-yielding metabolism in non-conventional yeasts. FEMS Microbiol Rev 24:507–529.

Fonseca C, Spencer-Martins I and Hahn-Hägerdal B (2007) L-arabinose metabolism in *Candida arabinofermentans* PYCC 5603 and *Pichia guilliermondii* PYCC 3012: Influence of sugar and oxygen on product formation. Appl Microbiol Biotechnol 75:303–310.

Galafassi S, Merico A, Pizza F, Hellborg L, Molinari F, Piškur J and Compagno C (2011) *Dekkera/Brettanomyces* yeasts for ethanol production from renewable sources under oxygen-limited and low-pH conditions. J Ind Microbiol Biotechnol 38:1079–1088.

Gamage J, Lam H and Zhang Z (2010) Bioethanol Production from Lignocellulosic Biomass, A Review. J Biobased Mater Bio 4:3–11.

Gancedo C (1971) Inactivation of fructose-1,6-diphosphatase by glucose in yeast. J Bacteriol 107:401–405.

Gancedo JM (1998) Yeast Carbon Catabolite Repression. Microbiol Mol Biol Rev 62:334–361.

Gancedo JM (2008) The early steps of glucose signalling in yeast. FEMS Microbiol Rev 32:673-704.

Gao M, Ploessl D and Shao Z (2019) Enhancing the Co-utilization of Biomass-Derived Mixed Sugars by Yeasts. Front Microbiol 22, 9:1-21.

Godoy L, Silva-Moreno E, Mardones W, Guzman D, Cubillos FA and Ganga A (2017) Genomics perspectives on metabolism, survival strategies, and biotechnological applications of *Brettanomyces bruxellensis* LAMAP2480. J Mol Microbiol Biotechnol 27:147–158.

Goldemberg J (2013) Sugarcane Ethanol: Strategies to a Successful Program in Brazil. In: Lee J. (eds) Advanced Biofuels and Bioproducts. Springer, New York, pp 13-20.

Gray SR, Rawsthorne H, Dirks B and Phister TG (2011) Detection and enumeration of *Dekkera anomala* in beer, cola, and cider using real-time PCR. Lett Appl Microbiol 52:352–359.

Guilherme ADA, Victor P, Dantas F, Eduardo C, Padilha DA, Silvino E and de Macedo GR (2019) Ethanol production from sugarcane bagasse: Use of different fermentation strategies to enhance an environmental-friendly process. J Environ Manage 234:44–51.

Ha SJ, Kim SR, Kim H, Du J, Cate JHD and Jin YS (2013) Continuous co-fermentation of cellobiose and xylose by engineered *Saccharomyces cerevisiae*. Bioresour. Technol 149:525–531.

Hahn-Hagerdal B, Karhumaa K and Jeppsson M (2007) Metabolic Engineering for Pentose Utilization in *Saccharomyces cerevisiae*. Adv Biochem Engin/Biotechnol 108:147–177.

Hamacher T, Becker J, Gárdonyi M, Hahn-Hagerdal B and Boles E (2002) Characterization of the xylose-transporting properties of yeast hexose transporters and their influence on xylose utilization. Microbiology 148: 2783-2788.

Hedbacker K and Carlson M (2008) SNF1/AMPK pathways in yeast. Front Biosci 13:2408–2420.

Hohmann S and Cederberg H (1990) Autoregulation may control the expression of yeast pyruvate decarboxylase structural genes *PDC1* and *PDC5*. Eur J Biochem 188:615–621.

Horak J, Regelmann J and Wolf DH (2002) Two distinct proteolytic systems responsible for glucose-induced degradation of fructose-1.6-bisphosphatase and the gal2p transporter in the yeast *Saccharomyces cerevisiae* share the same protein components of the glucose signaling pathway. J Biol Chem 277:8248–8254.

Hou X (2012) Anaerobic xylose fermentation by *Spathaspora passalidarum*. Appl Microbiol Biotechnol 94:205–214.

Huang S, Liu T, Peng B and Geng A (2019) Enhanced ethanol production from industrial lignocellulose hydrolysates by a hydrolysate-cofermenting *Saccharomyces cerevisiae* strain. Bioprocess Biosyst Eng 42:883-896.

Hung GC, Brown CR, Wolfe AB, Liu J and Chiang HL (2004) Degradation of the gluconeogenic enzymes fructose-1, 6-bisphosphatase and malate dehydrogenase is mediated by distinct proteolytic pathways and signaling events. J Biol Chem 279: 49138–49150.

Jacobus AP, Gross J, Evans JH, Ceccato-Antonini SR and Gombert AK (2021) *Saccharomyces cerevisiae* strains used industrially for bioethanol production. Essays Biochem PMID: 34156078.

Jeffries TW (2006) Engineering yeasts for xylose metabolism. Current Opinion in Biotechnology 17:320–326.

Jeffries TW, Grigoriev IV, Grimwood J, Laplaza JM, Aerts A, Salamov A, Schmutz J, Lindquist E, Dehal P, Shapiro H et al (2007) Genome sequence of the lignocellulose-bioconverting and xylose-fermenting yeast *Pichia stipitis*. Nat Biotechnol 25:319-26.

Jiang R, Carlson M (1996) Glucose regulates protein interactions within the yeast SNF1 protein kinase complex. Gene Dev 10:3105–15.

Johnston M (1987). A model fungal gene regulatory mechanism: The GAL genes of *Saccharomyces cerevisiae*. Microbiol Rev 51:458–476.

Johnston M, Flick JS and Pexton T (1994) Multiple mechanisms provide rapid and stringent glucose repression of *GAL* gene expression in *Saccharomyces cerevisiae*. Mol Cell Biol 14:3834–3841.

Jouhten P, Rintala E, Huuskonen A, Tamminen A, Toivari M, Wiebe M, Ruohonen L, Penttilä M and Maaheimo H (2008) Oxygen dependence of metabolic fluxes and energy generation of *Saccharomyces cerevisiae* CEN.PK113-1A. BMC Syst Biol 2:60.

Kayikci Ö and Nielsen J (2015) Glucose repression in *Saccharomyces cerevisiae*. FEMS Yeast Res 15(6):fov068.

Keleher CA, Redd MJ, Schultz J, Carlson M, Johnson AD (1992) Ssn6-Tup1 is a general repressor of transcription in yeast. Cell 68:709–719.

Kim JH, Ryu J, Huh IY, Hong SK, Kang HA, Chang YK (2014) Ethanol production from galactose by a newly isolated Saccharomyces cerevisiae KL17. Bioprocess Biosyst Eng 37:1871-8.

Klug L and Daum G (2014) Yeast lipid metabolism at a glance. FEMS Yeast Res 14:369–88.

Kotter P and Ciriacy M (1993) Xylose fermentation by *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 38:776.

Kufferath H and van Laer M (1921) Études sur les levures du Lambic. Bull Soc Chim Belg 30:270–276.

Kuhn A and Prior BA (1995) Purification and Partial Characterization of an Aldo-Keto Reductase from *Saccharomyces cerevisiae*. Appl Environ Microbiol 61:1580–1585.

Kurtzman CP, Fell JW and Boekhout T (2011) The yeasts, a taxanomic study. 5th edition. Elsevier, London, 2354 pp.

Lachance MA (1995) Yeast communities in a natural tequila fermentation. Antonie Van Leeuwenhoek 68:151–160.

Lee DW, Hong YH, Choe EA, Lee SJ, Kim SB, Lee HS, Oh JW, Shin HH and Pyun YR (2005) A thermodynamic study of mesophilic, thermophilic, and hyperthermophilic L-arabinose isomerases: the effects of divalent metal ions on protein stability at elevated temperatures. FEBS Lett 579:1261-1266.

Leech A, Nath N, McCartney RR, Schmidt MC (2003) Isolation of mutations in the catalytic domain of the snf1 kinase that render its activity independent of the snf4 subunit. Eukaryot Cell 2:265–73.

Leite FC, Leite DV, Pereira LF, de Barros Pita W and de Morais MAJr (2016) High intracellular trehalase activity prevents the storage of trehalose in the yeast *Dekkera bruxellensis*. Lett Appl Microbiol 63:210-4.

Leite FCB, Basso TO, de Barros PW, Gombert AK, Simões DA and de Morais Jr MA (2013) Quantitative aerobic physiology of the *yeast Dekkera bruxellensis*, a major contaminant in bioethanol production plants. FEMS Yeast Res 13:34–43.

Li X, Chen Y and Nielsen J (2019) Harnessing xylose pathways for biofuels production. Curr Opin Biotechnol 57:56-65.

Li Y, Chen G and Liu W (2010) Multiple metabolic signals influence *GAL* gene activation by modulating the interaction of Gal80p with the transcriptional activator Gal4p. Mol Microbiol 78: 414–428.

Ljungdahl PO and Daignan-Fornier B (2012) Regulation of amino acid, nucleotide, and phosphate metabolism in *Saccharomyces cerevisiae*. Genetics 190:885–929.

Longin C, Degueurce C, Julliat F, Guilloux-Benatier M, Rousseaux S and Alexandre H (2016) Efficiency of population-dependent sulfite against *Brettanomyces bruxellensis* in red wine. Food Res Int 89:620–630.

Lucero P, Moreno E and Lagunas R (2002) Catabolite inactivation of the sugar transporters in *Saccharomyces cerevisiae* is inhibited by the presence of a nitrogen source. FEMS Yeast Res 1:307-14.

McCartney RR, Chandrashekarappa DG, Zhang BB and Schmidt MC (2014) Genetic analysis of resistance and sensitivity to 2-deoxyglucose in *Saccharomyces cerevisiae*. Genetics 198:635–46.

Meinander NQ, Boels I and Hahn-Hägerdal B (1999) Fermentation of xylose/glucose mixtures by metabolically engineered *Saccharomyces cerevisiae* strains expressing *XYL1* and *XYL2* from *Pichia stipitis* with and without overexpression of *TAL1*. Bioresour Technol 68:79-87.

Moktaduzzaman Md, Galafassi S, Capusoni C, Vigentini I, Ling Z, Piskur J and Compagno C (2015) Galactose utilization sheds new light on sugar

metabolism in the sequenced strain *Dekkera bruxellensis* CBS 2499. FEMS Yeast Res 15:1–9.

Moore PA, Sagliocco FA, Wood RM and Brown AJ (1991) Yeast glycolytic mRNAs are differentially regulated. Mol Cell Biol 1:5330–5337.

Moriya H and Johnston M (2004) Glucose sensing and signaling in *Saccharomyces cerevisiae* through the Rgt2 glucose sensor and casein kinase I. Proc Natl Acad Sci USA 101:1572–1577.

Mukherjee V, Radecka D, Aerts G, Verstrepen KJ, Lievens B and Thevelein JM (2017) Biotechnology for Biofuels Phenotypic landscape of non - conventional yeast species for different stress tolerance traits desirable in bioethanol fermentation. Biotechnol Biofuels 10:1–19.

Nakanishi SC, Soares LB, Biazi LE, Nascimento VM, Costa AC, Rocha GJM and Lenczak JL (2017) Fermentation strategy for second generation ethanol production from sugarcane bagasse hydrolyzate by *Spathaspora passalidarum* and *Scheffersomyces stipitis*. Biotechnol Bioeng 114:2211–2221.

Nascimento VM and Fonseca GG (2019) Effects of the carbon source and the interaction between carbon sources on the physiology of the industrial *Saccharomyces cerevisiae* CAT-1. Prep Biochem Biotechnol 50:349-356.

Nehlin JO, Carlberg M, Ronne H (1991) Control of yeast GAL genes by Mig1 repressor: A transcriptional cascade in the glucose response. EMBO J 10:3373–3377.

Nelson, DL (2019) Princípios de Bioquímica de Lehninger. 7 ed. Porto Alegre: Artmed/Panamericana Editora Ltda, 1312 pp.

O'Donnell AF, McCartney RR, Chandrashekarappa DG, Zhang BB, Thorner J and Schmidt MC (2015) 2-Deoxyglucose impairs *Saccharomyces cerevisiae* growth by stimulating Snf1-regulated and α-arrestin-mediated trafficking of hexose transporters 1 and 3. Mol Cell Biol 35:939–55.

Ogeda TL, Petri DFS (2010) Hidrólise enzimática de biomassa. Quim. Nova 33:1549-1558.

Olofsson K, Bertilsson M and Lidén G (2008) A short review on SSF - An interesting process option for ethanol production from lignocellulosic feedstocks. Biotechnol Biofuels 1:7.

Ozcan S (2002) Two different signals regulate repression and induction of gene expression by glucose. J Biol Chem 277:46993–7.

Ozcan S and Johnston M (1995) Three different regulatory mechanisms enable yeast hexose transporter (*HXT*) genes to be induced by different levels of glucose. Mol Cell Biol 15:1564–1572.

Packer M (2009) Algal capture of carbon dioxide; biomass generation as a tool for greenhouse gas mitigation with reference to New Zealand energy strategy and policy. Energ Policy 37:3428–37.

Palmqvist E and Hahn-Hägerdal B (2000) Fermentation of lignocellulosic hydrolysates. II: Inhibitors and mechanisms of inhibition. Bioresour Technol 74:25–33.

Palomino A, Herrero P and Moreno F (2005) Rgt1, a glucose sensing transcription factor, is required for transcriptional repression of the *HXK2* gene in *Saccharomyces cerevisiae*. Biochem J 388 (Pt 2):697–703.

Palomino A, Herrero P and Moreno F (2006) Tpk3 and Snf1 protein kinases regulate Rgt1 association with *Saccharomyces cerevisiae HXK2* promoter. Nucleic Acids Res 34(5):1427–1438.

Passoth V (2014) Molecular Mechanisms in Yeast Carbon Metabolism: Bioethanol and Other Biofuels. In Piskur J and Compagno C (eds) Molecular Mechanisms in Yeast Carbon Metabolism. 2014th edition. Springer-Verlag Berlin Heidelberg, pp 217-259.

Peeters K and Thevelein JM (2014) Glucose Sensing and Signal Transduction in *Saccharomyces cerevisiae* ed. Piškur, J. and Compagno, C. pp 21-56. Molecular Mechanisms in Yeast Carbon Metabolism. Springer-Verlag Berlin Heidelberg

Peeters K, Van Leemputte F, Fischer B, Bonini BM, Quezada H, Tsytlonok M, Haesen D, Vanthienen W, Bernardes N, Gonzalez-Blas CB et al. (2017) Fructose-1,6-bisphosphate couples glycolytic flux to activation of Ras. Nature Communications 8:1-15.

Peláez R, Herrero P and Moreno F (2010) Functional domains of yeast hexokinase 2. Biochem J 432:181–90.

Peña-Moreno IC, Castro Parente D, da Silva JM, Andrade Mendonça A, Rojas LAV, Morais MA Jr and de Barros Pita W (2019) Nitrate boosts anaerobic ethanol

production in an acetate-dependent manner in the yeast Dekkera bruxellensis. J Ind Microbiol Biotechnol 46:209-220.

Peter G, Dlauchy D, Tobias A, Fulop L, Podgorsek M, Cadez N (2017) *Brettanomyces acidodurans* sp. nov., a new acetic acid producing yeast species from olive oil. Antonie Van Leeuwenhoek 110:657–64.

Pfeiffer T and Morley A (2014) An evolutionary perspective on the Crabtree effect. Front Mol Biosci 1:1-6.

Piškur J and Compagno C (2014) Molecular Mechanisms in Yeast Carbon Metabolism. Heidelberg, Springer, 334 pp.

Pronk JT, Yde Steensma H, van Dijken JP (1996) Pyruvate metabolism in *Saccharomyces cerevisiae*. Yeast 12:1607–1633

Randez-Gil F, Bojunga N, Proft M and Entian KD (1997) Glucose derepression of gluconeogenic enzymes in *Saccharomyces cerevisiae* correlates with phosphorylation of the gene activator Cat8p. Mol Cell Biol 17:2502–10.

Rech RF, Fontana CR, Rosa AC, Camassola M, Ayub M and Dillon JPA (2019) Fermentation of hexoses and pentoses from sugarcane bagasse hydrolysates into ethanol by *Spathaspora hagerdaliae*. Bioprocess Biosyst Eng 42:83–92.

Reifenberger E, Boles E and Ciriacy M (1997) Kinetic characterization of individual hexose transporters of *Saccharomyces cerevisiae* and their relation to the triggering mechanisms of glucose repression. Eur J Biochem 245:324–33.

Reis ALS, de Souza RFR, Torres RRNB, Leite FCB, Paiva PMG, Vidal EE and de Morais Jr MA (2014) Oxygen-limited cellobiose fermentation and the characterization of the cellobiase of an industrial *Dekkera/Brettanomyces bruxellensis* strain. Springer Plus 3:1–9.

Remize F, Andrieu E and Dequin S (2000) Engineering of the pyruvate dehydrogenase bypass in *Saccharomyces cerevisiae*: role of the cytosolic Mg (2+) and mitochondrial K (+) acetaldehyde dehydrogenases Ald6p and Ald4p in acetate formation during alcoholic fermentation. Appl Environ Microbiol 66:3151-3159.

Richard P, Toivari MH and Penttila M (1999) Evidence that the gene YLR070c of *Saccharomyces cerevisiae* encodes a xylitol dehydrogenase. FEBS Letters 457:455–458.

Richard P, Verho R, Putkonen M, Londesborough J and Penttila M (2003) Production of ethanol from L -arabinose by *Saccharomyces cerevisiae* containing a fungal L -arabinose pathway. FEMS Yeast Res 3:185–189.

Roach MJ and Borneman AR (2020) New genome assemblies reveal patterns of domestication and adaptation across *Brettanomyces* (*Dekkera*) species. BMC Genomics 21:194.

Robak K and Balcerek M (2018) Review of second-generation bioethanol production from residual biomass. Food Technol Biotech 56:174–187.

Rocha GJM, Nascimento VM, Gonçalves AR, Silva VFN and Martín C (2015) Influence of mixed sugarcane bagasse samples evaluated by elemental and physicalchemical composition. Ind Crop Prod 64:52–58.

Rodrussamee N, Lertwattanasakul N, Hirata K, Suprayogi, Limtong S, Kosaka T, Yamada M (2011) Growth and ethanol fermentation ability on hexose and pentose sugars and glucose effect under various conditions in thermotolerant yeast *Kluyveromyces marxianus*. Appl Microbiol Biotechnol 90:1573-86.

Rodrussamee N, Sattayawat P and Yamada M (2018) Highly efficient conversion of xylose to ethanol without glucose repression by newly isolated thermotolerant *Spathaspora passalidarum* CMUWF1–2. BMC Microbiol 18: 73.

Rolland F, Winderickx J and Thevelein JM (2002) Glucose-sensing and signalling mechanisms in yeast. FEMS Yeast Res 2:183–201.

Rosa SES e Garcia JLF (2009) O etanol de segunda geração: limites e oportunidades. BNDES 32:118- 156.

Roth S, Kumme J and Schuller HJ (2004) Transcriptional activators Cat8 and Sip4 discriminate between sequence variants of the carbon source-responsive promoter element in the yeast *Saccharomyces cerevisiae*. Curr Genet 45: 121–128.

Rozpędowska E, Hellborg L, Ishchuk OP, Orhan F, Galafassi S, Merico A, Woolfit M, Compagno C and Piškur J (2011) Parallel evolution of the make-accumulate-consume strategy in *Saccharomyces* and *Dekkera* yeasts. Nat Commun 2:302.

Santana NB (2007) Eficiência da hidrólise de amido de mandioca por diferentes fontes de enzimas e rendimento da fermentação alcoólica para produção

de etanol. 115 f. Dissertação. Universidade Federal de Viçosa, Viçosa – MG.

Scalcinati G, Otero JM, van Vleet JRH, Jeffries TW, Olsson L and Nielsen J (2012) Evolutionary engineering of *Saccharomyces cerevisiae* for efficient aerobic xylose consumption. FEMS Yeast Res 12:582–597.

Schork SM, Bee G, Thumm M and Wolf DH (1994) Catabolite inactivation of fructose-1,6-bisphosphatase in yeast is mediated by the proteasome. FEBS Lett 349:270–274.

Schork SM, Thumm M and Wolf DH (1995) Catabolite inactivation of fructose-1,6-bisphosphatase of *Saccharomyces cerevisiae*. Degradation occurs via the ubiquitin pathway. J Biol Chem 270:26446–26450.

Sellick CA, Campbell RN and Reece RJ (2008) Galactose Metabolism in Yeast—Structure and Regulation of the Leloir Pathway Enzymes and the Genes Encoding Them. Int Rev Cell Mol Biol 269:111-150.

Shi S, Chen Y, Siewers V and Nielsen J (2014) Improving production of malonyl coenzyme A-derived metabolites by abolishing Snf1-dependent regulation of Acc1. mBio 5:e01130–14.

Shieh HL, Chen Y, Brown CR adn Chiang HL (2001) Biochemical analysis of fructose-1,6-bisphosphatase import into vacuole import and degradation vesicles reveals a role for UBC1 in vesicle biogenesis. J Biol Chem 276: 10398–10406.

Shin M, Kim JW, Ye S, Kim S, Jeong D, Lee DY, Kim JN, Jin YS, Kim KH, Kim SR (2019) Comparative global metabolite profiling of xylose-fermenting *Saccharomyces cerevisiae* SR8 and *Scheffersomyces stipitis*. Appl Microbiol Biotechnol 103:5435-5446.

Silva BM and Silva WSD (2019) Um panorama da implantação do etanol de 3ª geração como uma fonte de energia sustentável. An overview of the deployment of 3rd generation ethanol as a sustainable energy source. Engevista 21:176-192.

Silverstein RA, Chen Y, Sharma-Shivappa RR and Boyette MDJO (2007) A comparison of chemical pretreatment methods for improving saccharification of cotton stalks. Bioresour Technol 98:3000–3011.

Spitaels F, Wieme AD, Janssens M, Aerts M, van Landschoot A, de Vuyst L and Vandamme P (2015) The microbial diversity of an industrially produced

lambic beer shares members of a traditionally produced one and reveals a core microbiota for lambic beer fermentation. Food Microbiol 49:23–32.

Stein K and Chiang HL (2014) Exocytosis and Endocytosis of Small Vesicles across the Plasma Membrane in *Saccharomyces cerevisiae*. Membranes (Basel) 4:608-29.

Stenger M, Le DT, Klecker T and Westermann B (2020) Systematic analysis of nuclear gene function in respiratory growth and expression of the mitochondrial genome in *S. cerevisiae*. Microbial cell 7:234–249.

Subtil T and Boles E (2011) Improving L-arabinose utilization of pentose fermenting *Saccharomyces cerevisiae* cells by heterologous expression of L-arabinose transporting sugar transporters. Biotechnol for Biofuels 4:38.

Tachibana C, Biddick R, Law GL and Young ET (2007) A poised initiation complex is activated by *SNF1*. J Biol Chem 282:37308–15.

Tachibana C, Yoo JY, Tagne JB, Kacherovsky N, Lee TI and Young ET (2005) Combined global localization analysis and transcriptome data Identify genes that are directly coregulated by Adr1 and Cat8. Mol Cell Biol 25:2138–46.

Teles GH, da Silva JM, Mendonça AA, de Morais Junior MA and de Barros Pita W (2018) First aspects on acetate metabolism in the yeast *Dekkera bruxellensis*: a few keys for improving ethanol fermentation. Yeast 35:577–584.

Temer B (2014) Expressão da xilose isomerase de *Propionibacterium acidipropionici* em *Saccharomyces cerevisiae* visando a produção de etanol de segunda geração. 85 f. Dissertação. Universidade de Campinas, Campinas – SP.

Teoh AL, Heard G and Cox J (2004) Yeast ecology of Kombucha fermentation. Int J Food Microbiol 95:119–126.

Thomson JM, Gaucher EA, Burgan MF, De Kee DW, Li T, Aris JP and Benner SA (2005) Resurrecting ancestral alcohol dehydrogenases from yeast. Nat Genet 37:630–635.

Tiukova IA, de Barros Pita W, Sundell D, Haddad Momeni M, Horn SJ, de Morais MA and Passoth V (2014) Adaptation of *Dekkera bruxellensis* to lignocellulose-based substrate. Biotechnol Appl Biochem 61:51–57.

Tiukova IA, Petterson ME, Tellgren-roth C, Bunikis I, Eberhard T, Pettersson OV and Passoth V (2013) Transcriptome of the Alternative Ethanol Production

Strain *Dekkera bruxellensis* CBS 11270 in Sugar Limited, Low Oxygen Cultivation. PLoS one 8:1–8.

Traven A, Jelicic B and Sopta M (2006) Yeast Gal4: a transcriptional paradigm revisited. EMBO Rep 7: 496–499.

Treitel MA and Carlson M (1995) Repression by SSN6-TUP1 is directed by Mig1, a repressor/activator protein. Proc Natl Acad Sci USA 92:3132–3136.

Tsigie AY, Wua CH, Huynh LH, Ismadji S and Ju YH (2013) Bioethanol production from *Yarrowia lipolytica* Po1g biomass. Bioresour Techno 145:210–216.

Uenojo M and Pastore GM (2007) Pectinases: aplicações industriais e perspectivas. Quím Nova 30:388–94.

Usaite R, Jewett MC, Oliveira AP, Yates JR 3rd, Olsson L and Nielsen J (2009) Reconstruction of the yeast Snf1 kinase regulatory network reveals its role as a global energy regulator. Mol Syst Biol 5:1–12.

van der Walt J (1964) *Dekkera*, new genus of Saccharomycetaceae. Anton Van Lee J M S 30:273–280.

Veras HCT, Parachin NS and Almeida JRM (2017) Comparative assessment of fermentative capacity of different xylose-consuming yeasts. Microb Cell Fact 16:153.

Verdone L, Wu J, van Riper K, Kacherovsky N, Vogelauer M, Young ET, Grunstein M, Di Mauro E and Caserta M (2002) Hyperacetylation of chromatin at the *ADH2* promoter allows Adr1 to bind in repressed conditions. EMBO J 21:1101–11.

Vincent O and Carlson M (1999) Gal83 mediates the interaction of the Snf1 kinase complex with the transcription activator Sip4. EMBO J 18: 6672–6681.

Vohra M, Manwar J, Manmode R, Padgilwar S and Patil S (2014) Bioethanol production: feedstock and current technologies. J Environ Chem Eng 2:573–584.

Walther K and Schuller HJ (2001) Adr1 and Cat8 synergistically activate the glucose-regulated alcohol dehydrogenase gene *ADH2* of the yeast *Saccharomyces cerevisiae*. Microbiol Read Engl 147:2037–44.

Wang C, Shen Y, Zhang Y, Suo F, Hou J and Bao X (2013) Improvement of L-arabinose fermentation by modifying the metabolic pathway and transport in *Saccharomyces cerevisiae*. Biomed Res Int 2013:461204.

Wang C, Zhao J, Qiu C, Wang S, Shen Y, Du B, Ding Y and Bao X (2017) Coutilization of D-Glucose, D-Xylose, and L-Arabinose in *Saccharomyces cerevisiae* by coexpressing the metabolic pathways and evolutionary engineering. BioMed Res 2017:1–8.

Wang M, Han J, Dunn JB, Cai H and Elgowainy A (2012) Well-to-wheels energy use and greenhouse gas emissions of ethanol from corn, sugarcane and cellulosic biomass for US use. Environ Res Lett 7:045905:1-13.

Wang X, Yang J, Yang S and Jiang Y (2019) Unraveling the genetic basis of fast L-arabinose consumption on top of recombinant xylose-fermenting *Saccharomyces cerevisiae.* Biotechnol Bioeng 116:283-293.

Watson JD, Baker TA, Bell SP, Gann A, Levine M, Losick R (2015) Biologia Molecular do Gene. 7° edição. Editora Artmed, Porto Alegre, 912 pp.

Weinhandl K, Winkler M, Glieder A, Camattari A (2014) Carbon source dependent promoters in yeasts. Microb Cell Fact 13:5.

Wiedemann B and Boles E (2008) Codon-optimized bacterial genes improve L-arabinose fermentation in recombinant *Saccharomyces cerevisiae*. Appl. Environ. Microbiol 74:2043-2050.

Wisselink HW, Cipollina C, Oud B, Crimi B, Heijnen JJ, Pronk JT and van Maris AJ (2010) Metabolome, transcriptome and metabolic flux analysis of arabinose fermentation by engineered *Saccharomyces cerevisiae*. Metab Eng 12:537-551.

Woolfit M, Rozpedowska E, Piskur J and Wolfe KH (2007) Genome survey sequencing of the wine spoilage yeast *Dekkera (Brettanomyces) bruxellensis*. Eukaryot Cell 6:721–733.

Ye S, Kim JW and Kim SR (2019) Metabolic Engineering for Improved Fermentation of L-Arabinose. J. Microbiol. Biotechnol 29:339–346.

Yuvadetkun P, Leksawasdi N and Boonmee M (2017) Kinetic modeling of *Candida shehatae* ATCC 22984 on xylose and glucose for ethanol production. Prep Biochem Biotechnol 16:268-275.

Zaman S, Lippman SI, Zhao X and Broach JR (2008) How *Saccharomyces* responds to nutrients. Annu Rev Genet 42:27–81.

Zhang X, Wang J, Zhang W, Hou JY, Xiao W, Cao L (2018) Optimizing the coordinated transcription of central xylose-metabolism genes in *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 102:7207-7217.

Zhou H, Cheng J, Wang BL, Fink GR and Stephanopoulos G (2012) Xylose isomerase overexpression along with engineering of the pentose phosphate pathway and evolutionary engineering enable rapid xylose utilization and ethanol production by *Saccharomyces cerevisiae*. Metab Eng 14:611–22.

APÊNDICE A – ARTIGO 1 DA TESE

APÊNDICE B – ARTIGO 2 DA TESE

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RESEARCH ARTICLE

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Fermentation profiles of the yeast Brettanomyces bruxellensis in D-xylose and L-arabinose aiming its application as a second-generation ethanol producer

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Abstract

The yeast Brettanomyces bruxellensis is able to ferment the main sugars used in first-generation ethanol production. However, its employment in this industry is prohibitive because the ethanol productivity reached is significantly lower than the observed for Saccharomyces cerevisiae. On the other hand, a possible application of B. bruxellensis in the second-generation ethanol production has been suggested because this yeast is also able to use D-xylose and L-arabinose, the major pentoses released from lignocellulosic material. Although the latter application seems to be reasonable, it has been poorly explored. Therefore, we aimed to evaluate whether or not different industrial strains of B. bruxellensis are able to ferment D-xylose and L-arabinose, both in aerobiosis and oxygen-limited conditions. Three out of nine tested strains were able to assimilate those sugars. When in aerobiosis, B. bruxellensis cells exclusively used them to support biomass formation, and no ethanol was produced. Moreover, whereas L-arabinose was not consumed under oxygen limitation, D-xylose was only slightly used, which resulted in low ethanol yield and productivity. In conclusion, our results showed that D-xylose and L-arabinose are not efficiently converted to ethanol by B. bruxellensis, most likely due to a redox imbalance in the assimilatory pathways of these sugars. Therefore, despite presenting other industrially relevant traits, the employment of B. bruxellensis in second-generation ethanol production depends on the development of genetic engineering strategies to overcome this metabolic bottleneck.

KEYWORDS

fermentative capacity, industrial application, pentose metabolism, redox imbalance, second-generation ethanol

1 | INTRODUCTION

The use of lignocellulosic biomass for second-generation ethanol production has strategic importance because it is a renewable and environment-friendly source because of the reduction of greenhouse gases (Nogueira et al., 2018). In Brazil, the main substrate used in this process is the sugarcane bagasse, which undergoes (i) pretreatment, (ii) hydrolysis and (iii) fermentation steps (Guilherme et al., 2019). Pretreatment works by disorganizing the lignocellulosic matrix, whereas hydrolysis acts on the conversion of (i) cellulose to D-glucose and (ii) hemicellulose to D-glucose, D-mannose, D-galactose, D-xylose and L-arabinose (Goldemberg, 2013; Silverstein, Chen, Sharma-Shivappa, &

APÊNDICE C – MATERIAL SUPLEMENTAR DO ARTIGO 1

Biological diversity of carbon assimilation among isolates of the yeast *Dekkera bruxellensis* from wine and fuel-ethanol industrial processes

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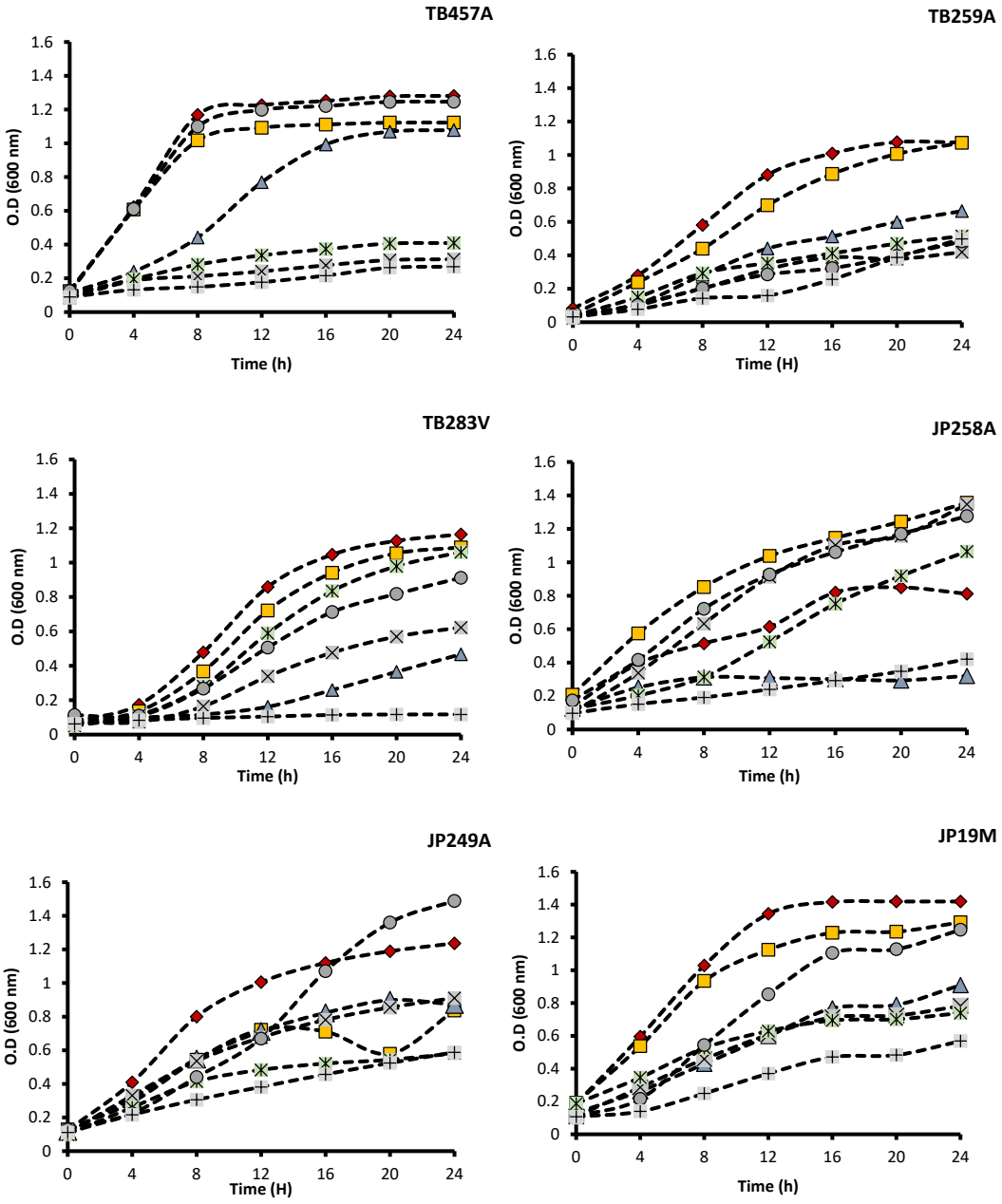
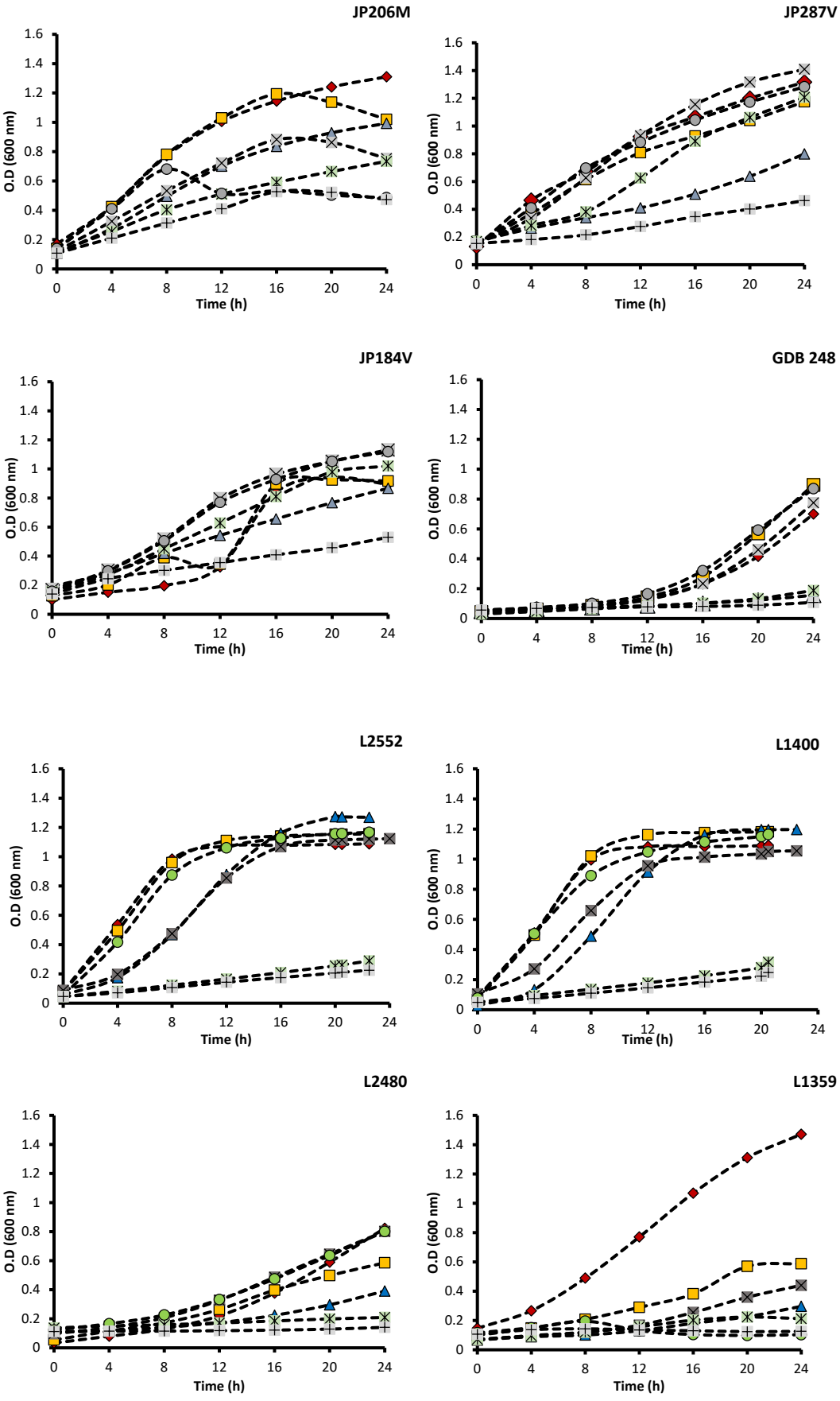
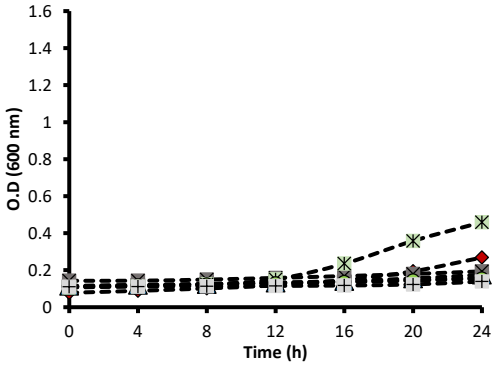
Figure S1: Aerobic cultures in synthetic medium containing different carbon sources of isolates of *D. bruxellensis* collected from fuel-ethanol process in Brazil and from wineries in Brazil, Argentina and Chile. Carbon sources: glucose (red diamond -♦-); fructose (yellow square -■-); galactose (blue triangle-▲-); maltose (green x -✕-); cellobiose (pink asterisk -✳-); sucrose (green circle -●-); and lactose (positive sign -✚-).

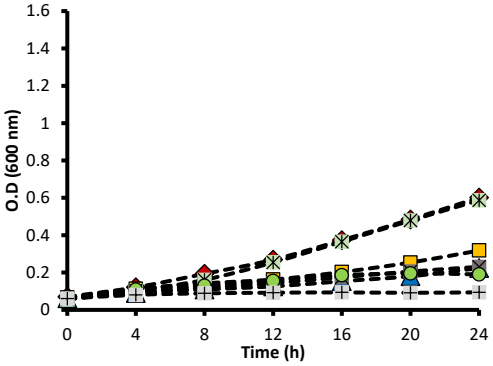



JP206M
JP287V
JP184V
GDB 248
L2552
L1400
L2480
L1359
O.D (600 nm)
Time (h)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
0
4
8
12
16
20
24

MRC 140a



MRC 177a



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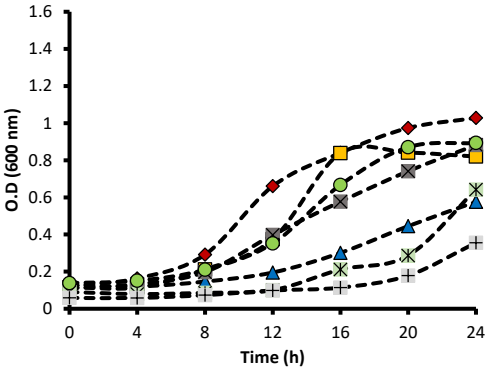
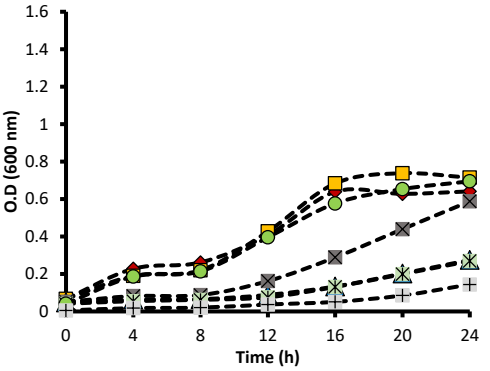


MRC 172a



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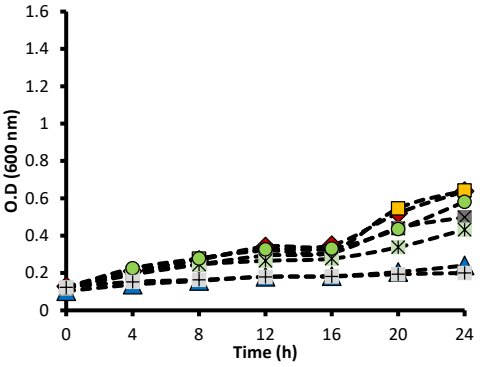
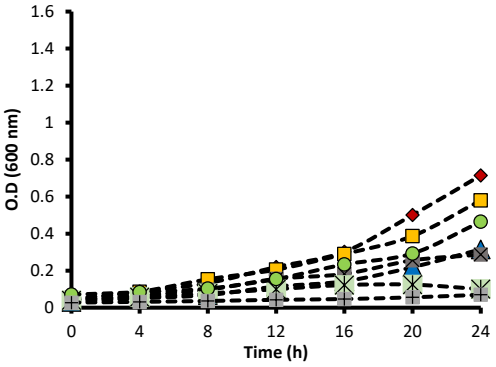
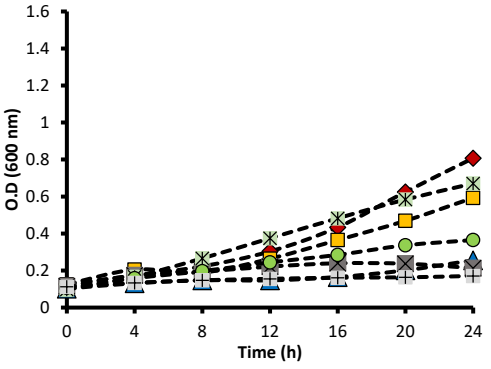


MRC 80b



MRC 117b



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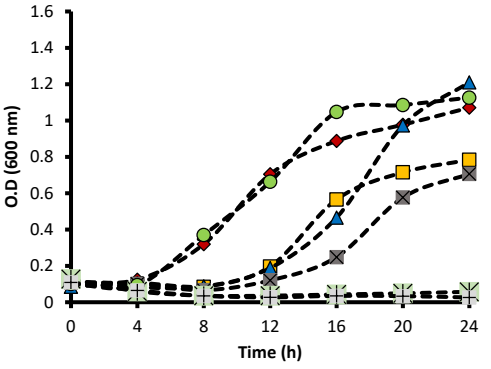

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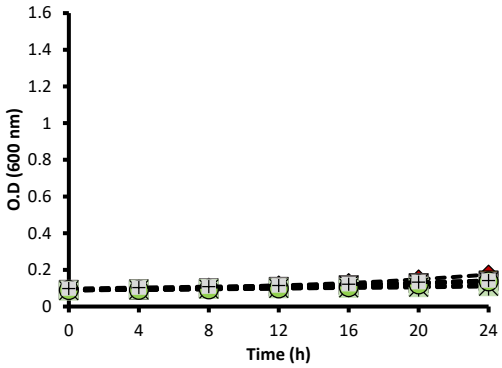

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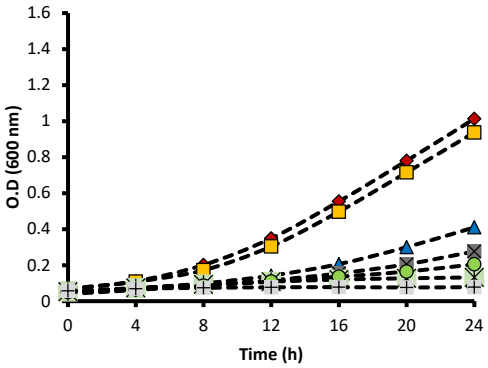
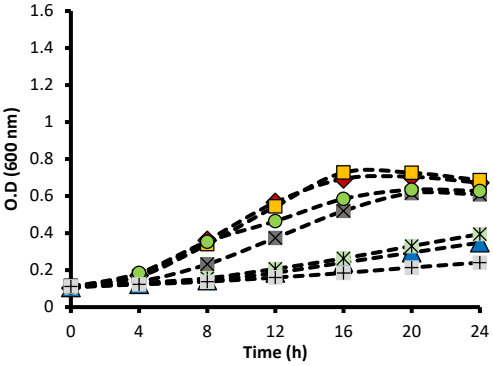
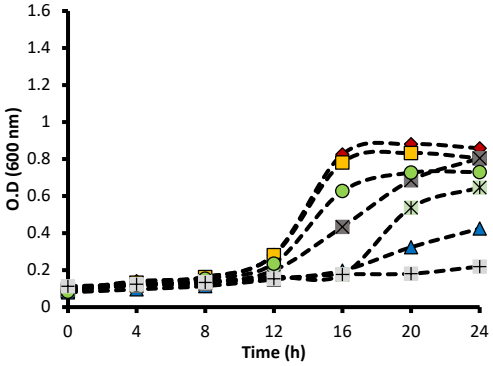


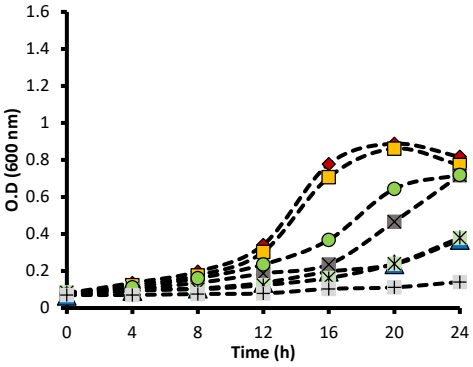
MRC 172b



MRC 86b



MRC 78b



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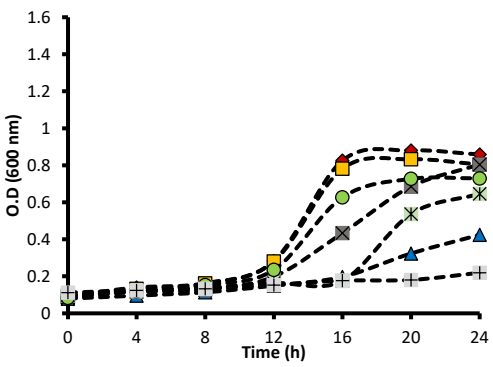

MRC 181b

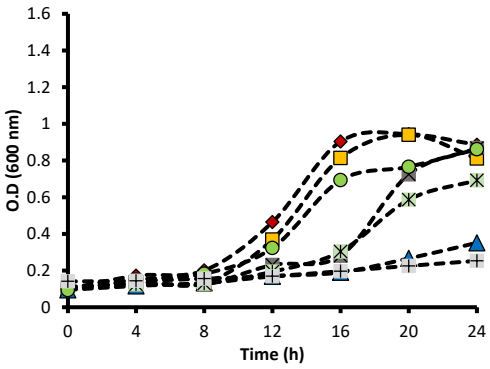

Figure S2: Determination of the maximum specific growth rates. The linear portion of the curve utilized for the linear regression is indicated by the vertical lines.

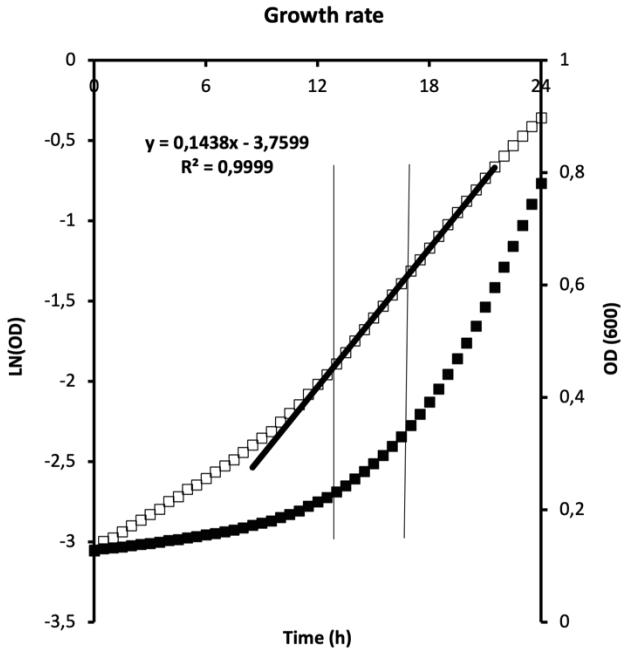

Table S1: Maximum specific growth rates of different *D. bruxellensis* isolates from fuel-ethanol and wine distillery in different carbon sources in aerobiosis.

		Specific growth rates μ (h-1)						
Substrate	**Strains**	**Glucose**	**Fructose**	**Galactose**	**Maltose**	**Cellobiose**	**Sucrose**	**Lactose**
Fuel ethanol	**GDB 248**	0.14 ± 0.004	0.17 ± 0.001	0.05 ± 0.004	0.17 ± 0.004	0.06 ± 0.000	0.17 ± 0.002	0.03 ± 0.010
	TB457A	0.33 ± 0.007	0.29 ± 0.004	0.16 ± 0.007	0.03 ± 0.006	0.09 ± 0.002	0.31 ± 0.001	0.05 ± 0.008
	JP249A	0.28 ± 0.020	0.17 ± 0.004	0.17 ± 0.010	0.18 ± 0.020	0.14 ± 0.009	0.19 ± 0.010	0.07 ± 0.010
	JP258A	0.15 ± 0.005	0.23 ± 0.007	0.10 ± 0.001	0.20 ± 0.009	0.12 ± 0.004	0.20 ± 0.005	0.06 ± 0.007
	TB283V	0.24 ± 0.020	0.24 ± 0.020	0.11 ± 0.010	0.18 ± 0.030	0.21 ± 0.006	0.13 ± 0.010	0.03 ± 0.005
	JP184V	0.12 ± 0.020	0.13 ± 0.010	0.15 ± 0.030	0.15 ± 0.001	0.15 ± 0.000	0.17 ± 0.004	0.10 ± 0.010
	TB259V	0.20 ± 0.040	0.15 ± 0.003	0.14 ± 0.020	0.08 ± 0.010	0.10 ± 0.000	0.10 ± 0.008	0.12 ± 0.001
	JP287V	0.27 ± 0.050	0.17 ± 0.004	0.07 ± 0.005	0.21 ± 0.010	0.17 ± 0.020	0.19 ± 0.020	0.09 ± 0.006
	JP19M	0.32 ± 0.010	0.31 ± 0.005	0.14 ± 0.003	0.14 ± 0.001	0.16 ± 0.010	0.28 ± 0.000	0.14 ± 0.005
	JP206M	0.29 ± 0.020	0.29 ± 0.005	0.17 ± 0.010	0.19 ± 0.001	0.15 ± 0.020	0.28 ± 0.020	0.15 ± 0.020
Wine	**L1359**	0.16 ± 0.010	0.09 ± 0.020	0.06 ± 0.010	0.10 ± 0.003	0.08 ± 0.008	0.07 ± 0.007	0.04 ± 0.003
	L2552	0.31 ± 0.009	0.31 ± 0.005	0.22 ± 0.004	0.21 ± 0.001	0.05 ± 0.001	0.30 ± 0.030	0.04 ± 0.000
	L1400	0.32 ± 0.002	0.30 ± 0.005	0.22 ± 0.020	0.21 ± 0.008	0.06 ± 0.002	0.30 ± 0.010	0.05 ± 0.002
	L2480	0.13 ± 0.001	0.07 ± 0.005	0.07 ± 0.060	0.09 ± 0.002	0.02 ± 0.000	0.10 ± 0.001	0.02 ± 0.004
	MRC 140a	0.12 ± 0.004	*No Growth*	*No Growth*	0.02 ± 0.003	0.12 ± 0.001	0.01 ± 0.000	0.01 ± 0.000
	MRC 180a	0.10 ± 0.010	0.08 ± 0.004	0.04 ± 0.010	0.09 ± 0.000	0.06 ± 0.005	0.04 ± 0.000	0.01 ± 0.002
	MRC 181a	0.13 ± 0.020	0.16 ± 0.020	0.11 ± 0.000	0.11 ± 0.020	0.16 ± 0.010	0.11 ± 0.010	0.08 ± 0.010
	MRC 177a	0.12 ± 0.007	0.06 ± 0.004	0.04 ± 0.010	0.04 ± 0.004	0.12 ± 0.000	0.05 ± 0.002	0.02 ± 0.004
	MRC 172a	0.13 ± 0.010	0.17 ± 0.008	0.10 ± 0.002	0.11 ± 0.003	0.10 ± 0.001	0.13 ± 0.010	0.12 ± 0.002
	MRC 190b	0.04 ± 0.001	0.02 ± 0.000	0.01 ± 0.000	0.02 ± 0.000	0.01 ± 0.001	0.04 ± 0.020	0.02 ± 0.000
	MRC 80b	0.11 ± 0.010	0.11 ± 0.005	0.08 ± 0.000	0.10 ± 0.004	0.07 ± 0.003	0.11 ± 0.005	0.05 ± 0.010
	MRC 78b	0.12 ± 0.008	0.11 ± 0.000	0.08 ± 0.010	0.10 ± 0.010	0.07 ± 0.010	0.10 ± 0.020	0.04 ± 0.020
	MRC 178b	0.14 ± 0.001	0.14 ± 0.001	0.09 ± 0.000	0.08 ± 0.001	0.03 ± 0.000	0.06 ± 0.002	0.03 ± 0.001
	MRC 172b	0.13 ± 0.004	0.13 ± 0.010	0.06 ± 0.008	0.15 ± 0.006	0.06 ± 0.009	0.18 ± 0.005	0.03 ± 0.004
	MRC 180b	0.14 ± 0.040	0.10 ± 0.001	0.07 ± 0.009	0.10 ± 0.002	0.07 ± 0.020	0.08 ± 0.010	0.04 ± 0.002
	MRC 117b	0.09 ± 0.007	0.09 ± 0.001	0.06 ± 0.003	0.03 ± 0.000	0.10 ± 0.003	0.04 ± 0.005	0.01 ± 0.001
	MRC 181b	0.06 ± 0.003	0.05 ± 0.005	0.06 ± 0.003	0.05 ± 0.000	0.09 ± 0.006	0.04 ± 0.010	0.04 ± 0.010
	MRC 86b	0.20 ± 0.020	0.06 ± 0.006	0.10 ± 0.009	0.06 ± 0.000	0.05 ± 0.005	0.13 ± 0.020	0.04 ± 0.010
	MRC 177b	0.22 ± 0.020	0.29 ± 0.010	0.21 ± 0.010	0.19 ± 0.010	*No Growth*	0.17 ± 0.050	*No Growth*

APÊNDICE D – MATERIAL SUPLEMENTAR DO ARTIGO 3

Table S1 Oligos used for qRT–PCR assays and their respective amplicon size and T_m for melting curve analysis.

Oligo	Sequence	Amplicon size	Amplicon T_m
HXT 6/7F	GAAGCTCCTGTTGAACCAGTG	107	80.095
HXT 6/7R	CAACCGAAAACAAAACCACCG		
XYL1F	AGATGGGCAACGCAGAATGA	96	81.001
XYL1R	CGTCAAGTCAAAGGAGTCCGA		
XYL2F	CAAGTCCACCGAAGATTTGGC	94	81.268
XYL2R	GCAGACTGGAGCACCTGTAG		
XKS1F	ACGGGTCCGCAAATAAGGAA	141	81.650
XKS1R	ACACGCATCCGCTTCATCTA		
HXK2F	CGTTCTTGGGAGCACTCTGT	80	81.972
HXK2R	ATATCTCGCTTGCCACACCG		

APÊNDICE E – ARTIGO PUBLICADO EM COLABORAÇÃO

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DOI: 10.1002/yea.3348

RESEARCH ARTICLE

WILEY Yeast

First aspects on acetate metabolism in the yeast *Dekkera bruxellensis*: a few keys for improving ethanol fermentation

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Abstract

Dekkera bruxellensis is continuously changing its status in fermentation processes, ranging from a contaminant or spoiling yeast to a microorganism with potential to produce metabolites of biotechnological interest. In spite of that, several major aspects of its physiology are still poorly understood. As an acetogenic yeast, minimal oxygen concentrations are able to drive glucose assimilation to oxidative metabolism, in order to produce biomass and acetate, with consequent low yield in ethanol. In the present study, we used disulfiram to inhibit acetaldehyde dehydrogenase activity to evaluate the influence of cytosolic acetate on cell metabolism. *D. bruxellensis* was more tolerant to disulfiram than *Saccharomyces cerevisiae* and the use of different carbon sources revealed that the former yeast might be able to export acetate (or acetyl-CoA) from mitochondria to cytoplasm. Fermentation assays showed that acetaldehyde dehydrogenase inhibition re-oriented yeast central metabolism to increase ethanol production and decrease biomass formation. However, glucose uptake was reduced, which ultimately represents economical loss to the fermentation process. This might be the major challenge for future metabolic engineering enterprises on this yeast.

KEYWORDS

acetaldehyde dehydrogenase, acetyl-CoA, carbon distribution, *Dekkera bruxellensis*, disulfiram, ethanol fermentation

1 | INTRODUCTION

In the past decade, several studies have been performed regarding the biology of the yeast *Dekkera bruxellensis*, the teleomorph of *Brettanomyces bruxellensis*. These reports ranged from identification as contaminant in fermentation processes to the perception of its potential to produce bio-compounds (Blomqvist & Passoth, 2015; Radecka, Mukherjee, Mateo, et al., 2015; Steensels, Daenen, Malcorps, et al., 2015). In the case of fuel-ethanol fermentation, it has been revealed that *D. bruxellensis* has high adaptability and capacity to replace the initial population of *Saccharomyces cerevisiae*, as well as potential to produce ethanol at high yields (De Souza Liberal, Basílio, Do Monte Resende, et al., 2007; Passoth, Blomqvist, & Schnurer, 2007). This profile places *D. bruxellensis* within a particular group of fermenting yeasts of the Saccharomycetacea family. Evolutionary analyses estimated that *D. bruxellensis* and *S. cerevisiae* diverged from a common ancestor around 200 million years ago (Rozpedowska, Hellborg, Ishchuk, et al., 2011). However, they have converged to unique characteristics that enables the production of ethanol: (a) both are *Crabtree* positive, that is, at high concentrations of glucose they can perform the fermentative metabolism even in aerobic condition; (b) they exhibit tolerance to high ethanol content in the medium; and (c) both can survive without mitochondrial DNA (petite positive phenotype), meaning that it can grow anaerobically (Piskur, Rozpedowska, Polakova, et al., 2006). However, *D. bruxellensis* is also considered as acetogenic yeast with the production of acetic acid and ethanol at similar yields in aerobic conditions (Leite, Basso, De barros Pita, et al., 2013). This physiological trait is related to the so-

APÊNDICE F – ARTIGO PUBLICADO EM COLABORAÇÃO

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https://doi.org/10.1007/s10295-018-2118-1

GENETICS AND MOLECULAR BIOLOGY OF INDUSTRIAL ORGANISMS - ORIGINAL PAPER

Nitrate boosts anaerobic ethanol production in an acetate-dependent manner in the yeast *Dekkera bruxellensis*

Irina Charlot Peña-Moreno[1] · Denise Castro Parente[1] · Jackeline Maria da Silva[1] · Allyson Andrade Mendonça[1] · Lino Angel Valcarcel Rojas[3] · Marcos Antonio de Morais Junior[1] · Will de Barros Pita[2,4]

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Abstract
In the past few years, the yeast *Dekkera bruxellensis* has gained much of attention among the so-called non-conventional yeasts for its potential in the biotechnological scenario, especially in fermentative processes. This yeast has been regarded as an important competitor to *Saccharomyces cerevisiae* in bioethanol production plants in Brazil and several studies have reported its capacity to produce ethanol. However, our current knowledge concerning *D. bruxellensis* is restricted to its aerobic metabolism, most likely because wine and beer strains cannot grow in full anaerobiosis. Hence, the present work aimed to fulfil a gap regarding the lack of information on the physiology of *Dekkera bruxellensis* growing in the complete absence of oxygen and the relationship with assimilation of nitrate as nitrogen source. The ethanol strain GDB 248 was fully capable of growing anaerobically and produces ethanol at the same level of *S. cerevisiae*. The presence of nitrate in the medium increased this capacity. Moreover, nitrate is consumed faster than ammonium and this increased rate coincided with a higher speed of glucose consumption. The profile of gene expression helped us to figure out that even in anaerobiosis, the presence of nitrate drives the yeast cells to an oxidative metabolism that ultimately incremented both biomass and ethanol production. These results finally provide the clues to explain most of the success of this yeast in industrial processes of ethanol production.

Keywords Ethanol · Acetate metabolism · Anaerobic growth · Energetic demand · Nitrogen catabolite repression

Introduction

Dekkera bruxellensis is a yeast species known for its association with fermentation processes for fuel-ethanol production in USA and Canada [1], Europe [8], Brazil [11, 16] and Sweden [23]. Its potential to become an industrial microorganism for production of bio-ingredients has recently being reported [26, 29, 33]. Many works have shown its capacity to produce first-generation ethanol from sugar cane juice [24] and molasses [25], and second-generation ethanol from hydrolysates of sugar cane bagasse [30] and sorghum [31], as well as oat straw [35]. Its potential to be used as ethanol-producing yeast comes from the high fitness observed for different *D. bruxellensis* strains, which is likely due to (1) a better management and response to stressful conditions and tolerance to inhibitors [2, 7, 35] or to (2) a high ability to assimilate different nutrients available in the fermentation substrate [9, 22]. In the latter scenario, the assimilation of nitrate as a nitrogen source found in industrial substrates such as sugar cane juice is a clear advantage over *Saccharomyces cerevisiae* [12]. Therefore, only those yeasts capable of using this mineral form of nitrogen could prevail whenever the stock of widely used nitrogen sources in the substrate (ammonium, urea or amino acids) becomes scarce in a competitive environment, such as the open fermentation process. Some physiological and genetics aspects of nitrate assimilation have been reported in conditions of full or limited supply of oxygen [3, 6, 12, 14, 17, 21]. Two previous works in which *D. bruxellensis* cells were grown anaerobically used medium supplemented with amino acids

APÊNDICE G – ARTIGO PUBLICADO EM COLABORAÇÃO

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REVIEW

The biotechnological potential of the yeast *Dekkera bruxellensis*

Will de Barros Pita[1] · Gilberto Henrique Teles[2] · Irina Charlot Peña-Moreno[2] · Jackeline Maria da Silva[2] · Karol Cristianne Ribeiro[2] · Marcos Antonio de Morais Junior[2]

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Abstract
Dekkera bruxellensis is an industrial yeast mainly regarded as a contaminant species in fermentation processes. In winemaking, it is associated with off-flavours that cause wine spoilage, while in bioethanol production this yeast is linked to a reduction of industrial productivity by competing with *Saccharomyces cerevisiae* for the substrate. In spite of that, this point of view is gradually changing, mostly because *D. bruxellensis* is also able to produce important metabolites, such as ethanol, acetate, fusel alcohols, esters and others. This dual role is likely due to the fact that this yeast presents a set of metabolic traits that might be either industrially attractive or detrimental, depending on how they are faced and explored. Therefore, a proper industrial application for *D. bruxellensis* depends on the correct assembly of its central metabolic puzzle. In this sense, researchers have addressed issues regarding the physiological and genetic aspects of *D. bruxellensis*, which have brought to light much of our current knowledge on this yeast. In this review, we shall outline what is presently understood about the main metabolic features of *D. bruxellensis* and how they might be managed to improve its current or future industrial applications (except for winemaking, in which it is solely regarded as a contaminant). Moreover, we will discuss the advantages and challenges that must be overcome in order to take advantage of the full biotechnological potential of this yeast.

Keywords Non-conventional yeast · Industrial application · Industrially relevant metabolites · Second-generation ethanol · Strain improvement

Dekkera bruxellensis and fermentative processes: for better or worse, a close relationship

Dekkera/Brettanomyces bruxellensis (currently, both terms are used as synonyms and we shall adopt the name *Dekkera* henceforth) is a yeast species found in close contact with industrial fermentative processes, especially in winemaking, brewing and in bioethanol production plants (de Souza Liberal et al. 2007; Roder et al. 2007; Oelofse et al. 2008). In fact, *D. bruxellensis* passed through the twentieth century as the main cause for wine spoilage worldwide, mostly by producing volatile phenolic compounds involved with unpleasant aromas ("horse sweat", "corral", "wet animals", etc) associated with the "Brett" character (Chatonnet et al. 1992; Licker et al. 1998; Lentz and Harris 2015; Crauwels et al. 2017; Felipe-Ribeiro et al. 2018). In spite of being mostly related with winemaking, its first report dates back to early 1900s, when a new species was found in British beers and the term *Brettanomyces* was proposed as a generic name in reference to its origin, a "British brewing fungus" (Claussen 1904). In the following decades, a yeast isolated from Belgian Lambic beer was then classified as *Brettanomyces bruxellensis* and the genus *Dekkera* was later introduced to describe strains able to produce ascospores (Kufferath and van Laer 1921; van der Walt 1964). After some reclassifications, currently, only five species are recognized in the *Dekkera/Brettanomyces* group: *D. bruxellensis*, *D. anomala*, *B. custersianus*, *B. nanus* and *B. naardenensis* (Kurtzman et al. 2011). Since *D. bruxellensis* is considered a major contaminant in wine industry, its presence, role and importance has been subject of previous review articles and readers should be referred to these works for additional information (Smith and Divol 2016; Agnolucci et al. 2017; Berbegal et al. 2018). Moreover, other reviews dealt with different

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