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IRINA CHARLOT PEÑA MORENO

Regulação do metabolismo de nitrato na levedura industrial *Dekkera bruxellensis*

Recife

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Tese de doutorado apresentada ao Programa de Pós-Graduação em Genética da Universidade Federal de Pernambuco como parte dos requisitos exigidos para obtenção do título de Doutora em Genética.

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Orientador: Prof. Dr. Marcos Antonio de Morais Junior

Co-orientador: Prof. Dr. Will de Barros Pita

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BANCA EXAMINADORA:

_____

Porof. Dr. Marcos Antonio de Morais Junior (Orientador)

Universidade Federal de Pernambuco

_____

Prof. Dr. Terciílio Calsa Junior (Examinador Interno)

Universidade Federal de Pernambuco

_____

Prof^{a}. Dr^{a}. Elaine Malosso (Examinadora Interna)

Universidade Federal de Pernambuco

_____

Prof. Dr. Thiago Henrique Napoleão (Examinador Externo)

Universidade Federal de Pernambuco

_____

Prof$^{a.}$Dr$^{a.}$ Sandra Regina Ceccato Antonini

Universidade Federal de São Carlos

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“Confio que, a partir dessa nova realidade, extrairemos lições valiosas, entre elas que a ciência será reconhecida de maneira real e determinada para enfrentar as adversidades”

(José Luján Alcaraz)

RESUMO

A levedura *Dekkera bruxellensis* tem capacidade de assimilar nitrato como única fonte de nitrogênio, uma vantagem adaptativa em meio industrial. Vários estudos mostraram os aspectos metabólicos da assimilação aeróbia do nitrato, mas pouco é conhecido sobre sua assimilação anaeróbia, algo relevante nos processos fermentativos industrias. O presente trabalho teve como objetivo preencher este vazio biológico. Na primeira etapa mostramos que esta levedura é capaz de crescer anaerobicamente em nitrato e produzir etanol no mesmo nível de *Saccharomyces cerevisiae*. Mesmo sem oxigênio, o padrão de expressão gênica mostrou indução do metabolismo oxidativo e de produção de ATP para suprir a alta demanda energética gerada pelo nitrato. Na segunda etapa mostramos que este alto crescimento celular foi induzido pelo acúmulo de proteínas da via glicolítica, dos processos de transcrição gênica e de síntese protéica, de purinas e de ATP. Já na presença de oxigênio, o proteoma revelou intensa produção de proteínas que respondem a estresse oxidativo e danos no DNA. Como conclusão, este trabalho mostra que o aumento da produção de etanol em nitrato em anaerobiose coincide com maior demanda energética observada nos altos níveis de expressão dos genes do metabolismo fermentativo e energético. Além disso, o nitrato pode ser considerado fonte primária de nitrogênio e que a deficiência no crescimento aeróbio se dá não por dificuldades na assimilação de nitrato, mas pelo intenso estresse oxidativo que é gerado pela sua assimilação.

Palavras-chave: Anaerobiose. Expressão gênica. Fermentação alcoólica. Fontes de nitrogênio. Proteômica. Regulação metabólica. Via TOR.

ABSTRACT

The yeast *Dekkera bruxellensis* has the capacity to assimilate nitrate as the sole source of nitrogen, an adaptive advantage in an industrial environment. Several studies have shown the metabolic aspects of aerobic assimilation of nitrate, but little is known about its anaerobic assimilation, something relevant in industrial fermentation processes. The present work aimed to fill this biological gap. In the first stage we show that this yeast is capable of growing anaerobically in nitrate and producing ethanol at the same level as *Saccharomyces cerevisiae*. Even without oxygen, the pattern of gene expression showed an induction of oxidative metabolism and ATP production to supply the high energy demand generated by nitrate. In the second stage, we show that this high cell growth was induced by the accumulation of proteins from the glycolytic pathway, the processes of gene transcription and protein synthesis, purines and ATP. In the presence of oxygen, the proteome revealed intense production of proteins that respond to oxidative stress and DNA damage. In conclusion, this work shows that the increase in ethanol production in nitrate in anaerobiosis coincides with the higher energy demand observed in the high levels of expression of the fermentative and energetic metabolism genes. In addition, nitrate can be considered a primary source of nitrogen and the deficiency in aerobic growth occurs not because of difficulties in the assimilation of nitrate, but because of the intense oxidative stress that is generated by its assimilation.

Key words: Anaerobiosis. Gene expression. Alcoholic fermentation. Nitrogen sources. Proteomics. Metabolic regulation. TOR pathway.

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LISTA DE ABREVIATURAS E SIGLAS

ACDH	Acetaldeído desidrogenase
ADH1	Alcool desidrogenase
2D-PAGE	Eletroforese bi dimensional em gel de poliacrilamida na presença de dodecilssulfato de sodio
2-OG	2-oxoglutarate
CNM	central nitrogen metabolism
cDNA	DNA complementar
DAP	Peptideos diferencialmente acumulados
DO	Densidade óptica
DTT	Ditiotreitol
FBP1	Fosfofruto quinase
GOGAT	Glutamine/2-oxoglutarate amino transferase
GAAC	Via geral de controle dos aminoácidos
GS	Glutamine synthase
IEF	Focalização isoelétrica
HPLC	Cromatografia líquida de alto desempenho
MALDI TOF	Matrix associada a laser para ionização por dessorção
MW	Peso molecular
mRNA	RNA mensageiro
N	Nitrogen
NADH	Nicotinamida adenina dinucleotídeo (forma reduzida)
NADPH	Nicotinamida adenina dinucleotídeo fosfato (forma reduzida)
NAD+	Nicotinamida adenina dinucleotídeo (forma oxidada)
NADP+	Nicotinamida adenina dinucleotídeo fosfato (forma oxidada)
NADPH-Gdh	NADPH-dependent glutamate dehydrogenase
NAD-Gdh	NAD^+-dependent glutamate dehydrogenase
NCBI	National Center for Biotechnology Information
NCR	Repressão catabolica do nitrogênio
NR	Nitrato redutase
NMR	Repressão metabolica do nitrogênio

PDH	Piruvato desidrogenase
PI	Ponto isoelétrico
PMF	Peptide mass fingerprinting
qPCR	PCR quantitativa
QR	Quantificação Relativa
rDNA	Ácido desoxirribonucleico ribossomal
ROS	Espécies reativas de oxigênio
RNA	Ribonucleic acid / Ácido ribonucléico
SD	Synthetic defined medium
RT-qPCR	PCR quantitativa com transcrição reversa
TCA	Citric Acid Cycle
THF	Tetrahidrofolato
UV	Ultravioleta
YNB	Yeast Nitrogen Base
YPD	Extrato de levedura, glicose e peptona bacteriana

SUMARIO

1 INTRODUÇÃO

Dekkera bruxellensis é uma espécie de levedura encontrada na produção de cerveja, vinhos e em destilarias de etanol combustível. Na maioria desses processos, incluindo a produção de bioetanol, é considerada uma levedura contaminante, competindo com *Saccharomyces cerevisiae* pelos substratos industriais. Por outro lado, é capaz de produzir etanol em rendimentos semelhantes aos calculados para *S. cerevisiae*, embora sejam baixos os valores de produtividade volumétrica. Nesse cenário, a disponibilidade de nitrogênio é um dos principais fatores limitantes do crescimento microbiano e tem papel fundamental na competitividade de *D. bruxellensis* no meio industrial. Estudos prévios do nosso grupo de pesquisa têm mostrado que *D. bruxellensis* possui a habilidade de assimilar nitrato, uma fonte secundária de nitrogênio, apontada como um importante fator de adaptação desta levedura no ambiente de fermentação alcoólica industrial. Essa habilidade permite sua multiplicação nos substratos industriais em ausência de oxigênio mesmo quando fontes de nitrogênio mais convencionais, como amônio e aminoácidos, se tornam escassos. No entanto, a presença de oxigênio no substrato produz um efeito contrário, diminuindo a produção de biomassa e de etanol quando nitrato é a fonte de nitrogênio. O paradoxo entre esses dois perfis fisiológicos revela grandes diferenças nos mecanismos regulatórios, e os respectivos aspectos fisiológicos e genéticos, envolvidos no metabolismo do nitrato que podem contribuir para o esclarecimento da alta competitividade exibida por *D. bruxellensis* no cenário industrial. Além disso, a utilização do nitrato em associação com amônia poderia levar ao aumento na produtividade volumétrica e específica de etanol a partir do caldo de cana. Deste modo, o presente trabalho teve como objetivo preencher uma lacuna referente à regulação do nitrogênio em *D. bruxellensis*, especialmente referente ao nitrato com e sem disponibilidade de oxigênio, sua fisiologia e sua resposta genética nas vias metabólicas mais importantes, resposta na biossíntese de proteínas e sua resposta à adição de um inibidor da via TOR, responsável pela regulação da assimilação do nitrogênio. A linhagem de etanol GDB 248 foi capaz de crescer anaerobicamente e produzir etanol no mesmo nível de *S. cerevisiae* e, a presença de nitrato no meio aumentou essa capacidade. Nossos resultados de expressão genica e proteômica mostram a tendência de *D. bruxellensis* pelo metabolismo oxidativo e aumento do

metabolismo glicolítico, principalmente na presença de nitrato em anaerobiose. Além disso, ao comparar o perfil proteico gerado pelo nitrato com ou sem presença de oxigênio, podemos inferir que na ausência de oxigênio, o nitrato poderia torna-se melhor assimilável, melhorando tanto o crescimento celular quanto para a produção de etanol em nossa linhagem industrial. De igual forma encontramos que em anaerobiose, o nitrato aumenta o fluxo glicolítico, a fim de aumentar equivalente redutores NADP(H) necessários para a assimilação desta fonte, através do metabolismo oxidativo e do metabolismo do GTP. Finalmente, foi observado que nossa linhagem de *D. bruxellensis,* é capaz de suportar concentrações maiores de rapamicina comparado com *S. cerevisiae* e que o nitrato ajuda a aumentar essa capacidade de resistência. Esses resultados finalmente fornecem pistas para explicar em parte o sucesso dessa levedura nos processos industriais de produção de etanol.

1.1 OBJETIVOS

1.1.1 Objetivo geral

Investigar os mecanismos regulatórios envolvidos com o metabolismo central do nitrato na levedura *Dekkera bruxellensis*.

1.1.2 Objetivos específicos

1. Avaliar os principais efeitos do nitrato no perfil fisiológico e genético de *D. bruxellensis* cultivada em anaerobiose

2. Avaliar a resposta proteômica de *D. bruxellensis* cultivada em nitrato na presença ou ausência de oxigênio

2 REVISÃO BIBLIOGRÁFICA

2.1 ASPECTOS GERAIS DA LEVEDURA *DEKKERA BRUXELLENSIS*

A levedura *D. bruxellensis* faz parte da família Saccharomycetacea (Molina et al., 1993) e a origem etimológica do gênero ocorreu no século passado, uma vez isolada em cervejas britânicas, sendo então chamada de *Brettanomyces* (grego: "*brettano*" significa cervejaria britânica, "*myces*": fungo) (Claussen, 1904). No entanto, em 1921, Kufferath e Van Laer isolaram uma cepa de levedura de cervejas lambic belgas com as mesmas características descritas por Claussen e classificaram como *Brettanomyces bruxellensis* (Custers, 1940). Desde sua primeira descrição, a taxonomia desta levedura tem sido assunto do debate e houve muitas reclassificações ao longo dos anos. O nome *Dekkera* foi escolhido em homenagem a Nellie Margaretha Stelling-Dekker, por sua contribuição a taxonomia de leveduras (Van Der Walt, 1964). Curiosamente, os dois gêneros ainda são utilizados de maneira intercambiável. Além da *D. bruxellensis,* outras 4 espécies foram reconhecidas até o presente, sendo elas (i) *D. anomala,* (ii) *B. custersianus,* (iii) *B. nanus* e (iv) *B. naardenensis* (Mitrakul *et al.,* 1999; Roder *et al*., 2007). A espécie *D. bruxellensis* já foi identificada em vários outros processos fermentativos, como na produção de vinho, cidra, kombucha, quefir, etc (Crauwels et al., 2015). No Brasil, esta levedura vem sendo consistentemente associada à episódios de contaminação da produção de bioetanol (De Souza Liberal et al., Basílio et al., 2007; De Barros Pita et al., 2011).

A morfologia de *D. bruxellensis* é variada (Figura 1), encontrando-se frequentemente na forma ogival ou ainda elipsoidal, esférica, cilíndrica e alongada e de tamanho menor ao se comparar com leveduras como *Saccharomyces cerevisiae* (Van Der Walt, 1964).

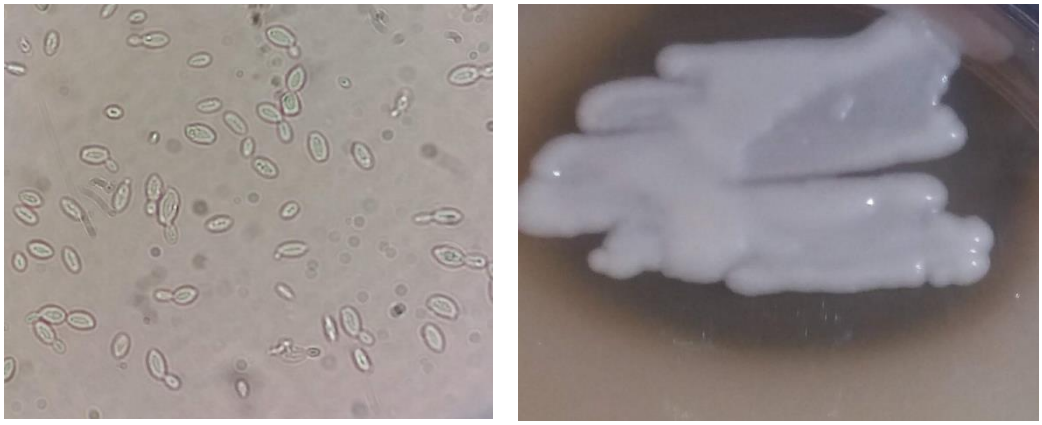

Figura 1: Linhagem GDB248 da espécie *Dekkera bruxellensis*. (A) Microscopia de contraste de fase 40X (B) Crescimento em placa de petri em meio YPD com carbonato de cálcio (Peña-Moreno et al, 2019).

Com relação às suas características metabólicas, *D. bruxellensis* possui anaerobiose facultativa, é *petite* positiva (capaz de sobreviver sem DNA mitocondrial) e *Crabtree* positiva, ou seja, apresenta metabolismo fermentativo quando altas concentrações de glicose estão presentes no meio mesmo em condições aeróbicas (Piskur et al., 2006; Woolfit et al., 2007; De Barros Pita et al., 2013). Assim como *S. cerevisiae, D. bruxellensis* apresenta crescimento em altas concentrações de etanol, valores de pH elevados e fontes de nitrogênio secundárias (Rozpedowska et al., 2011). Além disso, é capaz de metabolizar diferentes fontes de carbono dentre as quais glicose, frutose, sacarose e etanol são importantes no cenário industrial (Conterno et al., 2006). As principais fontes de nitrogênio utilizadas por *D. bruxellensis* são comuns à outras leveduras, com a assimilação preferencial de íons amônio (Magasanik, 2002). Entretanto, outras fontes de nitrogênio podem ser utilizadas, tais como aminoácidos e ureia (Conterno et al., 2006). Notavelmente, *D. bruxellensis* é capaz de utilizar nitrato como fonte de nitrogênio, uma vez que essa espécie possui em seu genoma os genes que codificam as proteínas da via de assimilação deste composto nitrogenado (Woolfit et al., 2007; Piskur et al., 2012).

2.2 FISIOLOGIA DE *DEKKERA BRUXELLENSIS*

D. bruxellensis apresenta características metabólicas e genéticas valiosas para o cenário industrial que já tem sido bem estudada (Borneman et al 2014, Avramova et al 2018). Esta levedura se adapta às tensões que normalmente ocorrem em

ambientes fermentativos, como altas concentrações de nutrientes, pH, temperatura, fontes de nitrogênio disponíveis, entre outros.

Com relação às fontes de carbono, está levedura apresenta preferência pela assimilação de glicose, frutose e sacarose, açúcares capazes de apoiar altas taxas de crescimento. Essas fontes apresentam relevância industrial por fazerem parte da composição de substratos, como o caldo-de-cana e o melaço (De Souza Liberal et al., 2007). Além disso, *D. bruxellensis* também é capaz de utilizar galactose, maltose, celobiose e trealose (Conterno et al., 2006). Linhagens com potencial de serem empregadas na produção de etanol de segunda geração apresentam habilidade de assimilar celobiose, mas não xilose e arabinose (Galafassi et al., 2011). O metabolismo do carbono em *D. bruxellensis* consiste primariamente na assimilação de açúcares, que como a glicose, são divididos em moléculas menores para se tornar uma fonte de energia e/ou síntese de outras moléculas. A glicólise (Figura 2) é o principal processo de degradação do açúcar, na qual uma molécula de glicose é sequencialmente oxidada em duas moléculas de piruvato. Dependendo da condição de crescimento, as leveduras podem direcionar o piruvato para fermentação ou para a respiração (Schifferdecker et al., 2014).

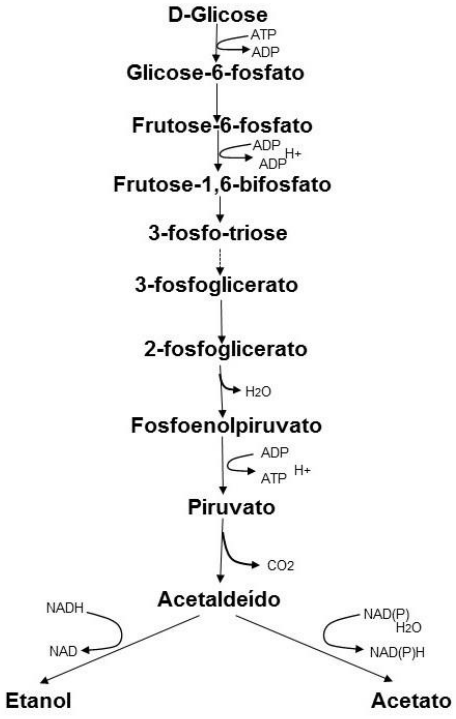

Figura 2: Via glicolítica com duas possíveis vias: via fermentativa para produção de etanol ou via oxidativa para produção de acetato e ciclo de Krebs.

Uma vez que a respiração de açúcares é energeticamente mais favorável do que a fermentação, a maioria dos organismos utiliza a fermentação apenas quando a respiração é prejudicada, por exemplo, quando a disponibilidade de oxigênio diminui. Em outras espécies de leveduras, como *D. bruxellensis* e *S. cerevisiae*, o destino metabólico do piruvato formado em taxas altas, passa da respiração para a fermentação, mesmo quando o oxigênio é abundante (Rozpedowska et al., 2011). O efeito *Crabtree* (Figura 3) é a capacidade de se iniciar a imediata produção de etanol, ou fermentação etanólica, assim que as células se encontrem em ambiente com abundância de glicose mesmo na presença de oxigênio (Piskur et al., 2006; Woolfit et al., 2007; de Barros Pita et al., 2013). Este efeito teve como base a aquisição de diversas características genéticas que resultaram em uma estratégia evolutiva denominada "*make-accumulate-consume*" ("fazer-acumular-consumir"), na qual as leveduras possuem (i) grande capacidade de assimilação de açúcares, (ii) elevada taxa de produção, (iii) alta capacidade de acúmulo e tolerância ao etanol, (iv) bem como seu posterior consumo (Rozpedowska et al., 2011).

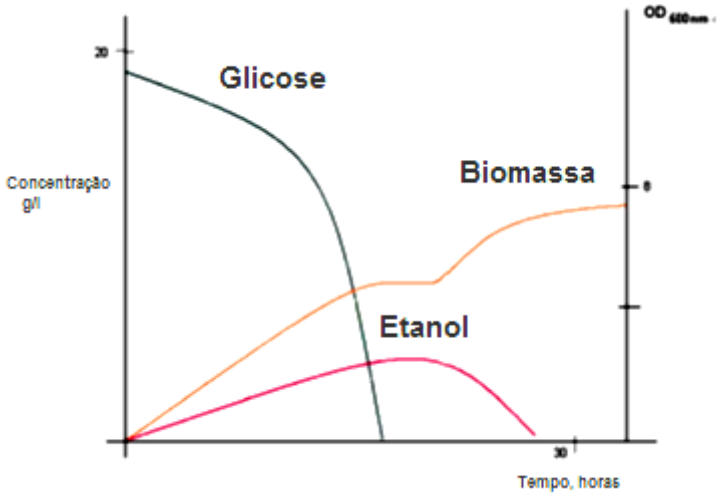

Figura 3: Efeito *"Crabtree positivo"* de *D. bruxellensis* Y879 (CBS 2499) em condições aeróbias (Rozpedowska et al, 2011)

Nesse contexto, o etanol produzido seria uma reserva de carbono e de energia para quando a glicose for exaurida do ambiente. O resultado fisiológico da aquisição desta estratégia (Efeito *Crabtree*) é o favorecimento da fermentação em detrimento da respiração, sendo esse efeito frequentemente associado à saturação da

capacidade celular em re-oxidar o NADH resultante da glicólise (Procházka *et al.,* 2010; Rozpedowska et al., 2011). Essa saturação faz com que o piruvato seja direcionado ao metabolismo fermentativo, possibilitando que o NADH seja re-oxidado via fermentação alcóolica. Apesar de a respiração apresentar maior eficiência energética, a estratégia "*make-accumulate-consume*" é vantajosa, pois proporciona um melhor desempenho competitivo frente a outras espécies (Rozpedowska et al., 2011). Esse é uma característica quase que restrita ao conjunto de leveduras que componhem o chamado grupo Cerevisiae senso estrito (*S. cerevisiae, S. uvarum, S. bayanus, S. eubayanus, S. paradoxos, S. pastorianus, S. cariocanus, S. manacensis* e *S. carlsbergensis*) envolvido na produção industrial de pães e de bebidas fermentadas e destiladas. Nesse grupo, se enquadram algumas espécies de *Dekkera/Brettanomyces*, com destaque para *D. bruxellensis*. No entanto, apesar de seu fenótipo Crabtree positivo, essa espécie tem a particularidade de se caracterizar pela tendência na produção de biomassa e pela produção de acetato (ambos produtos de metabolismo oxidativo) como importante produto de fermentação (Leite et al., 2013).

Como mencionado acima, durante o crescimento aeróbio, as células de levedura podem produzir energia na forma de ATP tanto pelo direcionamento do piruvato para a cadeia respiratória, na mitocôndria, quanto pela via fermentativa (Rozpedowska et al., 2011; Pereira et al., 2012). A preferência por um ou outro mecanismo depende, entre outros aspectos, da espécie e das condições nutricionais do meio, sendo que nesses casos, em geral, a produção de energia não é um fator limitante do crescimento celular (Curtin et al., 2014). Por outro lado, na ausência de oxigênio, um dos principais problemas enfrentados pelas leveduras é o baixo crescimento, devido à ausência de aceptores finais de elétrons na cadeia respiratória (Weusthuis et al., 1994; Curtin et al., 2014). Nesse cenário, a produção de ATP é restrita à glicólise. Por sua vez, a glicólise utiliza o cofator NAD^+ para a oxidação de intermediários da via, gerando NADH (a forma reduzida do cofator), que deve ser posteriormente reoxidado (Magasanik B & Kaiser CA, 2002). Na ausência de oxigênio, a fermentação é a principal via utilizada pelas leveduras para restaurar o balanço redox (Schifferdecker et al, 2014). Devido à menor eficiência energética da fermentação, o crescimento em anaerobiose é geralmente menor quando comparado à aerobiose (Estela-Escalante et al., 2014).

Outra característica fisiológica marcante de *D. bruxellensis* é o chamado efeito *Custers*, no qual ocorre a inibição temporária da fermentação alcóolica com a diminuição da disponibilidade de oxigênio (Custers, 1940; WIJSMAN *et al.,* 1984). Este efeito, ainda pouco entendido, parece ser ocasionado pela baixa capacidade de produção de glicerol como forma de restaurar o balanço redox celular causado pelo excesso de NADH gerado pelo fluxo glicolítico (Scheffers, 1966; Wijsman et al., 1984; De Barros Pita et al, 2013b). Soma-se a isso a escassez de NAD^+ causada pelo seu uso na oxidação do acetaldeído a acetato e consequente diminuição da razão NAD^+/NADH, resultando num processo de retroinibição do fluxo glicolítico e maior conversão de piruvato a acetil-CoA e não a acetaldeído (Wijsman et al., 1984).

2.3 GENÉTICA DE *DEKKERA BRUXELLENSIS*

D. bruxellensis possui alta variabilidade genotípica entre linhagens e consequentemente características fenotípicas específicas para cada uma delas. Isto pode ser explicado pela diversidade de ambientes fermentativos e substratos onde está levedura pode ser encontrada, gerando uma grande relevância industrial dessa espécie de levedura (Avramova et al 2018., Conterno et al 2006., Crauwels et al 2015., Hellborg e Piskur 2009).). Assim, apenas estudos focados na relação genótipo-fenótipo gerados nos últimos anos, permitem ter maior clareza sobre as variações genotípicas e seu comportamento nos diferentes ambientes em que a levedura é encontrada. No entanto, a diversidade intraespecífica entre as linhagens de *D. bruxellensis* dificulta a previsão geral dos nichos dessa levedura (Conterno et al., 2006, Curtin et al 2007). No entanto, no nível filogenético, *D. bruxellensis* é um parente distante de *S. cerevisiae* e ambas são encontradas frequentemente nos mesmos habitats e compartilham várias características fisiológicas relacionadas com nutrição, produção de níveis elevados de etanol e capacidade de crescer sem oxigênio (Piskur et al., 2012). Apesar das semelhanças, o sequenciamento do seu revelou que *D. bruxellensis* é filogeneticamente mais próxima a *Pichia (Komagataella) pastoris*, uma levedura conhecida pela baixa produção de etanol em aerobiose (Piskur et al., 2012). Neste sentido, três eventos cruciais não foram detectados em *D. bruxellensis* e que ocorreram durante o curso evolutivo de *S. cerevisiae*, (i) a duplicação total do genoma, seguida de (ii) perda massiva de blocos gênicos e enriquecimento de genes *ADH* e

(iii) a transferência horizontal do gene *FUR1*, responsável pela síntese *de novo* de pirimidinas em anaerobiose (Piskur & Langkjaer, 2004; Piskur et al., 2006). Análises bioinformáticas têm colocado a *D. bruxellensis* como membro de um grupo evolutivo "intermediário" compreendido por espécies como *Komagetalla pastoris*, *Kuraishia capsulata* e *Ogataea polymorpha* que parece ter divergido de um clado progenitor contendo um ancestral comum com *S. cerevisiae* (Curtin & Pretorius 2014). Embora não tenha sido encontrado um evento de duplicação completa do genoma em *D. bruxellensis*, em comparação com espécies próximas, dados genômicos inferem outras adaptações próprias de *D. bruxellensis* não compartilhadas com *S. cerevisiae* que podem aumentar a sobrevivência no ambiente de fermentação, como genes que codificam permutas de aminoácidos, além de álcool e aldeído desidrogenases (Curtin et al., 2012; Piskur et al., 2012).

O primeiro estudo de sequenciamento do genoma em *D. bruxellensis* foi realizado em 2007 com a linhagem CBS2499 (Woolfit et al 2007). Aproximadamente 3000 genes foram identificados usando principalmente o banco de dados de *S. cerevisiae* para identificação de proteínas em *D. bruxellensis* e o banco de dados de proteínas não redundantes para identificar outras proteínas. Os resultados desse primeiro sequenciamento mostraram 50% dos genes identificados com alta similaridade aos ortólogos de *S. cerevisiae.* Além disso, um gene da adenil-desaminase semelhante ao encontrado em *Burkholderia* (Woolfit et al., 2007) e genes que conferem grande vantagem adaptativa e não presentes em *S. cerevisiae* (Woolfit et al. 2007, Barros Pita et al. Até 2013a). Em 2012, foi realizado um novo sequenciamento em paralelo do genoma, que incluía duas linhagens (CBS2499 e AWRI1499) confirmando sua alta variabilidade intraespecífica (Piskur et al., 2012; Curtin et al., 2012). A linhagem de *D. bruxellensis* AWRI1499 isolada de vinícolas australianas, possui genoma triploide com aproximadamente 5000 genes e 12,7 Mb de tamanho e provavelmente surgiu a partir de uma hibridização de duas espécies proximamente relacionadas possuindo consequentemente um genoma triploide (Curtin et al., 2012). O genoma dessa linhagem é constituído de um "núcleo diploide", com dois conjuntos cromossômicos heterozigotos e um terceiro conjunto haploide divergente (Curtin et al., 2012). A outra linhagem, CBS2499, apresenta genoma com 13,4 Mb de tamanho e aproximadamente 5600 genes (Piskur et al., 2012). Na atualidade, além dessas duas linhagens, outros quatro genomas já foram

sequenciados: AMAP2480 (Valdéz et al, 2014) AWRI1608, AWRI1613 (Borneman et al 2014), ST05.12 / 22 (Crlauwels et al 2014). Esses estudos foram capazes de confirmar a variação genética de *D. bruxellensis* que apresentam genoma poliploide, contendo entre quatro e nove cromossomos (Hellborg & Piskur 2009., Avramova et al 2018) dependente de deformação, processos de hibridação, aberrações cromossômicas como aneuploidia, inserções entre linhagens específicas, deleções e perda de heterozigosidade por conversão gênica. (Borneman et al., 2014, Crauwels et al., 2014). Esses eventos podem estar associados às características de adaptação a ambientes e crescimento em condições de estresse (Borneman et al 2014, Avramova et al 2018). A partir do sequenciamento completo do genoma, estudos sobre caracterização cromossômica, expressão gênica e até abordagens de técnicas proteômicas, podem ser realizados utilizando os bancos de dados específicos para esta levedura (Hellborg & Piskur, 2009; Tiukova et al 2013, Barbosa- Neto et al. al 2014, de Barros Pita et al 2013a eb, Peña-Moreno et al 2019)).

Estudos de expressão genica de *D. bruxellensis* vem sendo conduzidos em diferentes grupos de pesquisa do mundo, no intuito de verificar as capacidades metabólicas de *D. bruxellensis* em resposta à diferentes condições, com foco em seus traços nutricionais, resposta a estresse, entre outros (NARDI et al., 2011; De Barros Pita et al., 2011; De Barros Pita et al., 2013a,b). Por exemplo, a resposta a estresse e adaptação de *D. bruxellensis* ao ambiente de produção de vinho são devidas a mecanismos únicos para esta levedura (NARDI et al., 2011). Ainda, um trabalho de expressão gênica de *D. bruxellensis* identificaram dois genes *ARO10*, codificantes da enzima fenilpiruvato descarboxilase no genoma da levedura, uma característica única entre os hemiascomicetos (De Souza Liberal et al., 2012).

Um estudo de transcriptômica global por RNA-Seq revelou a expressão do complexo respiratório I de NADH-ubiquinona embora *D. bruxellensis* seja uma levedura *Crabtree positiva*. A alta expressão de enzimas geradoras de NADH comparado como a geração de enzimas NAD^+ pode ser a razão para o desequilíbrio NADH anteriormente observado e o resultante *efeito Custer* em *D. bruxellensis* (Tiukova et al., 2013). Também foi achado que o alto grau de expressão de genes transportadores de açúcar é consistente com a hipótese de que a competitividade de

D. bruxellensis é devido a uma maior afinidade para o substrato limitante (Tiukova et al., 2013).

2.4 PROTEÔMICA DE *D. BRUXELLENSIS*

Em geral, as células regulam a atividade de fatores de transcrição especializados e, em resposta, ativam ou reprimem a expressão de conjuntos específicos de genes, sendo o mecanismo responsável por impedir ou reduzir a capacidade sintética das células para a formação de enzimas e permeases para a utilização de fontes secundárias, quando uma fonte preferencial está disponível (Magasanik & Kaiser, 2002).

Além das técnicas de PCR, técnicas proteômicas como sequenciamento *de novo* de peptídeos por espectrometria de massa de ionização por laser, assistida por matriz (MALDI-TOF/TOF), poderiam ser usadas para identificação e caracterização de microrganismos, particularmente microrganismos não-sequenciados ou com genomas parcialmente sequenciados. O estudo de proteínas, com uma abordagem proteômica, é uma estratégia importante para ampliar o conhecimento sobre a biologia de leveduras, onde o objetivo é estudar a função/comportamento dos genes com base nas identificações das proteínas por eles expressas. Com esta ferramenta é possível não somente determinar o conjunto de proteínas que estão sendo produzidas por um organismo em determinada condição, o que já é bastante desafiador, mas também caracterizar as inúmeras e comumente presentes isoformas das proteínas, produtos de modificações pós-traducionais sofridas e determinar o papel dessas moléculas no funcionamento celular, sua interação com outras proteínas e consequentemente, como o organismo se adapta ao meio onde estiver presente (Krizanovic et al 2018).

O proteoma é representado pelo conjunto de proteínas expressas pelo genoma de organismos, que juntam proteínas intracelulares (proteoma intracelular) e de proteínas extracelulares que foram secretadas ou que podem estar presentes no meio extracelular devido a lise celular (proteoma extracelular) (Godoy et al 2017). No caso de *D. bruxellensis*, ainda pouca estudada, a proteômica contribui para o conhecimento sobre as proteínas envolvidas nos sistemas fisiológico e biológicos e contribui para uma maior compreensão desta levedura instalada no ambiente

industrial (Krizanovic et al 2018). Assim, a espectrometria de massas (MS) é uma tecnologia indispensável para a interpretação do proteoma que utiliza bancos de dados para a identificação de peptídeos de duas formas: o primeiro é usar a informação relativa à massa molecular dos peptídeos procedentes da digestão enzimática (*Peptide Mass Fingerprint* – PMF), enquanto o segundo faz uso de resultados obtidos pela fragmentação de peptídeos individuais previamente detectados.

Em *D. bruxellensis* tem sido conduzido estudos proteômicos principalmente para linhagens desta levedura associadas com a produção de vinho (Carmona, et al; Krizanovic et al., 2018). A maioria dos resultados mostram proteínas que podem ser usadas como biomarcadores em *D. bruxellensis* (Krizanovic et al 2018), dentro das quais proteínas envolvidas no metabolismo de carboidratos, de biossíntese, e de estresse, foram encontradas como resposta à fenóis voláteis, ácidos graxos e alta concentração de etanol presente na fermentação dos vinhos (Carmona et al 2016; Krizanovic et al 2018). Igualmente foi descrito que três das sete linhagens sequenciadas LAMAP2480, CB2499 e AWRI1499, de *D. bruxellensis* (De Barros Pita et al 2019), conservam 1034 proteínas em comum (Godoy et al 2017). Entretanto, existem várias proteínas presentes na linhagem vinícola *B. bruxellensis* LAMAP2480 que não possuem genes paralelos nas outras duas linhagens, como por exemplo, o gene LAMAP2480_6256 que codifica um transportador de UDP-galactose ou várias proteínas associadas à proteína de montagem do citocromo C oxidase (Godoy et al 2017).

No caso da linhagem industrial brasileira GDB248 de *D. bruxellensis,* Barboza-Neto et al (2014) realizou o primeiro estudo de proteômica comparativa por análise de MS-ToF/ToF usando amônio ou nitrato como fontes de nitrogênio em anaerobiose. Desta forma já foi possível observar trinta e quatro proteínas super-produzidas em nitrato de relacionadas com a síntese de ATP e PPP, TCA, via glicolítica que apoio a a alta demanda de energia e a disponibilidade de NADH para a assimilação de nitrato e via fermentativa (Barboza-Neto et al 2014). Isto permitiu gerar o primeiro panorama de proteínas na nossa linhagem brasileira.

2.5 O PAPEL INDUSTRIAL DA LEVEDURA *DEKKERA BRUXELLENSIS*

D. bruxellensis é uma levedura referenciada principalmente em dois processos fermentativos industriais. Por um lado, leveduras pertencentes ao gênero *Dekkera* são geralmente consideradas o principal microrganismo em deterioração na indústria do vinho, uma vez que sua presença altera as características organolépticas do vinho, gerando perdas econômicas significativas em todo o mundo (Wedral et al., 2010). Esta levedura já foi reconhecida na indústria do vinho por conferir características olfativas indesejáveis semelhantes a lã úmida, camundongo, odores medicinais, plástico queimado ou cavalo molhado (característica *Brett*), que se devem ao aumento da concentração de fenóis voláteis no perfil aromático da bebida (Woolfit et al., 2007). Esses aromas particulares são produzidos a partir de compostos fenólicos presentes em uvas e mostos, chamados ácidos hidroxicinâmicos (AHC) (Abramovic, 2014) e gerando compostos voláteis como o ácido acético, bem como etilfenóis, como 4-etilfenol e 4-etilguaiacol (Chatonnet et al., 1995; 1997). Uma forma de se tentar conter a contaminação do vinho por *D. bruxellensis* é utilizando o metabisulfito de potássio (Bassi et al., 2015).

De outro lado*, D. bruxellensis* exibe um papel importante no processo de produção de cervejas de estilos belgas onde também utiliza compostos hidroxicinâmicos para a formação de aromas durante a segunda fermentação de alguns estilos de cerveja (lambic, gueze, coolship ale); entretanto, nesse contexto, *D. bruxellensis* contribui positivamente (Vanderhaegen et al., 2003*). D. bruxellensis* já foi encontrada em outros processos fermentativos como na produção de cidra, kombucha e kefir (Morrissey et al., 2004, Verce et al., 2019).

Outro papel relevante estudado recentemente em *D. bruxellensis* é seu potencial para a produção de etanol de segunda geração, ao ser capaz de assimilar a celobiose embora com menor rendimento (64,5%) e produtividade de etanol (62,5%) comparado com a assimilação de sacarose, quando o oxigênio é limitado, semelhante às condições industriais (Reis et al. 2014; Blomqvist et al. 2011; Reis et al. 2014).

No Nordeste do Brasil *D. bruxellensis* representa o principal contaminante da produção de bioetanol (De Souza Liberal et al., 2007; Basílio et al., 2007). Nesse cenário, quando as contagens celulares de *D. bruxellensis* estão aumentadas, observa-se uma diminuição da produtividade volumétrica do etanol, acompanhada de significativo prejuízo econômico (De Souza Liberal et al., 2007). É importante salientar

que o papel contaminante desempenhado por esta levedura na produção de etanol combustível, que ocorre por competição pelo substrato, principalmente os açúcares presentes no caldo-de-cana e as fontes de nitrogênio com *S. cerevisiae* (Chatonnet et al., 1995; 1997; De Souza Liberal et al., 2007).

No entanto, vários trabalhos apresentaram a capacidade fermentativa desta levedura ao produzir etanol em rendimentos semelhantes àqueles alcançados por *S. cerevisiae* (Blomqvist et al. 2010; Rozpedowska et al., 2011; Peña-Moreno et al 2019). O grande problema observado é a sua reduzida produtividade que decorre da assimilação mais lenta dos açúcares, e em traços de ácido acético encontradona produção pelo desvio na assimilação desses nutrientes o que resulta em baixas produtividades volumétricas e atrasos na produção e no rendimento diário do processo (Basílio *et al.,* 2008; Leite et al., 2012; de Barros Pita et al 2011; Pereira et al., 2012; Meneguine et al., 2013).

Além da característica Crabtree positiva, *D. bruxellensis* apresenta outras semelhanças fisiológicas com *S. cerevisiae*. Essa levedura pode crescer em condições que poderiam ser impeditivas para outras leveduras, tais como em meio mais acidificado (pH 3) ou em temperatura de 37^{o}C, ou ambas as condições (Galafassi et al 2013), ou mesmo quando combinadas a diminuição do pH do meio com o aumento do etanol (Bassi et al., 2013). No entanto, há que se considerar que a grande variabilidade entre as linhagens e isolados de *D. bruxellensis* entre processos industriais de diferentes regiões (Pereira et al 2012) e mesmo entre diferentes processos industriais (Da Silva et al 2019). Mas, em geral, observa-se que essa levedura apresenta características genéticas e fisiológicas muito relevantes para seu emprego industrial (De Barros Pita et al., 2020).

Apesar da sua importância industrial, vários aspectos do metabolismo de *D. bruxellensis* ainda precisam ser esclarecidos gerados dados fisiológicos e juntar ferramentas moleculares que ajudem à adquisição de dados genéticos e pós-transcricionais sobre esta levedura para a compreensão do seu metabolismo e estabelecimento no ambiente industrial. Nesse sentido, trabalhos filogenéticos, fisiológicos e genéticos já publicados que identificam e descrevem esta levedura como contaminante (De Souza Liberal et al., 2007, de Barros Souza et al 2012) precisam ser complementados com novos estudos fisologicos, genéticos e proteômicos em

condições similares às encontradas na fermentação industrial para explicar como *D. bruxellensis* também pode ser aproveitada nestes processos de produção.

2.6 IMPACTO DO OXIGÊNIO NA PRODUÇÃO DE BIOMASSA E ETANOL

Em princípio, todas as leveduras fermentadoras (facultativas) são capazes de gerar ATP por fosforilação ao nível do substrato e, portanto, não dependem da respiração para dirigir reações que requerem energia. Entretanto, a função fisiológica do oxigênio não é limitada ao seu papel como um aceptor de elétrons na respiração mitocondrial. O oxigênio é essencial para biossíntese de ergosterol, componente da membrana plasmática das células, indispensável para o crescimento celular (Dijken et al., 1993). Mesmo com a adição do ergosterol no meio de cultivo, o crescimento anaeróbico é um atributo de poucas leveduras. Um estudo que avaliou a capacidade de crescimento anaeróbico de 75 espécies de leveduras em meio complexo e em meio mineral mostrou que apenas 17 foram capazes de crescer nessa condição, dentre elas *D. bruxellensis*, entretanto apenas *S. cerevisiae* atingiu taxa de crescimento específico superior a 0,10 h^{1} (Visser et al., 1990).

Quando cultivos feitos em quimiostato com *S. cerevisiae* sob limitações de açúcar e com concentrações de biomassa e velocidades de agitação constantes foi alimentado com altas concentrações de oxigênio, o metabolismo da glicose por *S. cerevisiae* foi totalmente respiratório e a fermentação alcoólica não foi observada. Quando a alimentação do oxigênio foi diminuída, a fermentação alcoólica e respiração ocorreram simultaneamente, e foi possível também observar crescimento celular. Em tais níveis de glicose e oxigênio, um aumento da concentração de biomassa poderia ser percebido aumentando a concentração de glicose (o que leva também a aumento fermentação alcoólica) ou aumentando a disponibilidade de oxigênio (o que permite mais respiração e, consequentemente, um maior crescimento celular). (Dijken et al., 1993).

Em *D. bruxellensis,* segundo Leite et al. (2013), em cultivos aerados feitos em quimiostato sob limitação de glicose ou sacarose, não foi detectado metabólitos extracelulares como etanol, glicerol e acetato, apresentando assim um metabolismo completamente respiratório, e o açúcar foi totalmente convertido em biomassa e CO_2.

Nesse caso, o rendimento em biomassa foi 0,62 gDW gS^{-1} para essas fontes de carbono, esse valor é 20% maior do que o rendimento apresentado por *S. cerevisiae* nas mesmas condições de cultivo. Este maior rendimento de biomassa de *D. bruxellensis* é acompanhado por uma menor produção de CO_2 em ambos os substratos em comparação com culturas de quimiostatos de *S. cerevisiae* em glicose na mesma taxa de diluição. O fato de *D. bruxellensis* ser mais eficiente do que *S. cerevisiae* na conversão de glicose em biomassa pode ser explicado por diferenças na estrutura do aparelho respiratório. Nesse caso, mais ATP pode ser produzido a partir da oxidação de um equivalente redutor na cadeia respiratória de *D. bruxellensis*, implicando que menos glicose deve ser oxidada para produzir a energia necessária para o crescimento celular. A produção de etanol nesse experimento só foi observada após um pulso de glicose de 50 mmol. Indicando que a célula passou do metabolismo completamente respiratório para o metabolismo respiro-fermentativo, característico de leveduras *Crabtree*-positivas (Leite et al., 2013).

Em limitação de oxigênio, o rendimento em biomassa por *D. bruxellensis* é maior em relação a *S. cerevisiae* (Blomqvist, Johanna et al., 2010; De Souza Liberal, A. et al., 2007; Pereira et al., 2012; Pereira et al., 2014). Entretanto, os autores discordam quanto ao rendimento em etanol e produção de glicerol. A maioria dos trabalhos mostra que o rendimento em etanol é semelhante ou maior que em *S. cerevisiae* e o rendimento em glicerol é menor. Por outro lado, de acordo com Pereira et al (2012) rendimento em etanol foi menor do que em *S. cerevisiae* e não foi detectada produção de glicerol. A produtividade em etnaol também é menor do que *S. cerevisiae* (Basilio et al., 2008). Nesse caso, o menor rendimento em etanol por *D. bruxellensis* pode ter sido em decorrência da grande aeração do meio (160 rpm). Apesar da maioria dos trabalhos relatarem altos rendimentos em etanol, o maior problema desta levedura é o maior tempo que leva para converter o açúcar em etanol em relação a *S. cerevisiae* (Blomqvist et al., 2010; Leite et al., 2012; Pereira et al., 2012).

2.7 METABOLISMO CENTRAL DO NITROGÊNIO

O nitrogênio é um nutriente essencial do metabolismo celular devido ao seu papel constitutivo construção celular como parte constituinte de blocos de construção celulares, como aminoácidos e ácidos nucléicos. Consequentemente, células e

organismos se adaptam precisamente à disponibilidade de nitrogênio em seu entorno. Neste sentido, as leveduras possuim a habilidade de assimilar uma grande quantidade de fontes de nitrogênio, o que torna amplo o espectro de ambientes capazes de suportar o crescimento destes organismos (Siverio, 2002). Igual a outras respostas à escacez de nutrientes como o carbono, quando leveduras estão em um meio com pouca disponibilidade compostos nitrogenados passam por processos regulatorios de transcrição, tradução e pós-tradução que diminuem a biogênese do ribossomo, tradução e induzem a parada do ciclo celular e consequentemente a reduução do tamanho da célula (Siverio, 2002). Além disso, para compensar a perda, induzem processos catabólicos que permiten reciclar o nitrogênio a partir da quebra gradual de macromoléculas não essenciais que contêm nitrogênio, sintetizando assim proteínas mais importantes (Siverio, 2002)

Os processos principais pelo qual os compostos nitrogenados são incorporados ao interior das células de levedura são a redução de fontes nitrogênadas à amônio (NH_4^+) e a assimilação de nitrogênio diretamente como NH_4^+ ou glutamato encontrados no meio (Conrad et al 2014). Em leveduras como *D. bruxellensis* o transporte de fontes de nitrogênio para dentro da célula geralmente se dá através de um sistema de transportadores e proteínas transmembrana, como as permeases induzidas por substratos específicos ou de especificidade ampla e facilitam principalmente a captação de metabólitos nas células (Ljungdahl & Daignan-Fornier 2012). Uma vez internalizados, os compostos nitrogenados podem ser usados pelas células diretamente em processos biossintéticos, sendo desaminados para gerar amônio ou como substratos de transaminases que transferem grupos amino para 2-oxoglutarato para formar glutamato e glutamina. (Magasanik 1992; Magasanik e Kaiser 2002). Estas reações coordenadas de amônio, glutamato e glutamina formam a via central do Metabolismo do Nitrogênio (Figura 4) (Magasanik & Kaiser, 2002, Magsanik, 2003) e servem como doadores de nitrogênio que permitem às células realizar suas reações catabólicas e de biosíntese de aminoácidos anabolizantes e nucleotídeos que funcionam de forma paralela (Ljungdahl & Daignan-Fornier 2012). Nas células cultivadas com glicose, o amônio pode ser assimilado por duas reações anabólicas, ou seja, a síntese de glutamato de amônio e 2-oxoglutarato catalisada pela glutamato desidrogenase dependente de NADPH (Gdh1) (Figura 4) e a síntese de glutamina de amônio e glutamato por glutamina sintetase (Gln1). Em células

cultivadas em etanol como fonte de carbono, uma isozima Gdh1 codificada por *GDH3* é expressa e contribui para a assimilação de amônio (Avendano et al. 1997; DeLuna et al. 2001). Quando a glutamina é a única fonte de nitrogênio, a enzima glutamato sintase dependente de NADH (Glt1) é necessária para catalisar a síntese de glutamato (Ljungdahl & Daignan-Fornier 2012).

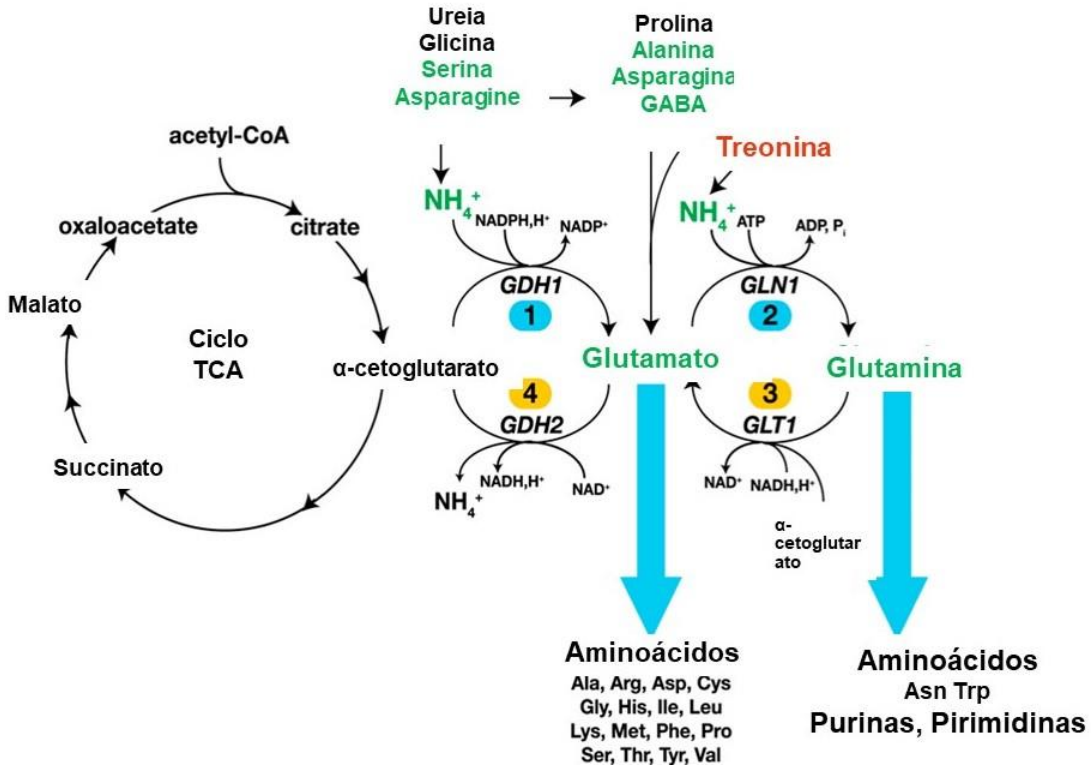

Figura 4: Metabolismo Central do Nitrogênio. Compostos nitrogenados são sintetizados a partir de glutamato ou glutamina. A principal via para a síntese de glutamato é a combinação de amônia e 2-oxoglutarato (α-cetoglutarato), sintetizado a partir de acetil-CoA no ciclo TCA (Daignan-Fornier et al, 2012).

Os compostos nitrogenados retornam ao meio quando os organismos morrem, na forma de aminoácidos ou no caso do amônio sendo sequencialmente oxidados em NO_3^- por uma via dependente de oxigênio conhecida como nitrificação. Além disso, o NO_3^- pode ser usado por alguns microrganismos como um aceitor de elétrons durante a respiração anaeróbica (Ljungdahl & Daignan-Fornier 2012, Magasanik & Kaiser, 2002).

Um requisito para a coordenação do metabolismo é a capacidade de monitorar concentrações de nutrientes no ambiente extracelular e dentro das células (Ljungdahl & Daignan-Fornier 2012). As leveduras têm a capacidade de usar uma variedade de compostos de nitrogênio com fontes únicas para seu crescimento e reagem a sua disponibilidade no ambiente, controlando a captação da fonte de nitrogênio que garantam altas taxas de crescimento e biossíntese de glutamato e aminoácidos, para regular seus processos catabólicos e anabólicos (Rodkaer 2014). Neste contexto, as fontes de nitrogênio são classificadas como *preferenciais* ou *primarias* e não *preferenciais*, *secundarias* ou *alte*rnativas, de acordo à facilidade com que elas são assimiladas. Compostos nitrogenados prontamente utilizáveis proporcionam taxas mais de crescimento mais altas do que fontes que necessitam de reações adicionais e com maior demanda energética para serem assimiladas (Conterno et al., 2006; Godard et al., 2007)

Entretanto essa classificação não é absoluta e seus efeitos repressivos podem variar significativamente entre origens de diferentes tipos de leveduras (Ljungdahl & Daignan-Fornier 2012). Em *D. Bruxellensis* por exemplo, já foi observado que a hieraquia na assimilação de fontes de nitrogênio depende entre outras coisas, da disponibilidade de oxigênio e incluem como fontes preferenciais a glutamina e amônio em aerobiose (Ter Schure, 1998; Magasanik & Kaiser, 2002; Magasanik, 2003; Boer et al., 2007, Parente et al 2017) e aspartato, glutamato, glutamina e amônio em anaerobiose (Parente et al 2017). Também foi reportado que *D. bruxellensis* consegue assimilar de forma concomitante duas fontes de nitrogênio (de Barros Pita et al, 2011). Fontes inorgânicas de nitrogênio, tais como nitrato e nitrito, têm sido classificadas como secundárias e sua capacidade de assimilação é restrita a algumas poucas espécies (Siverio, 2002).

De maneira geral, a limitação da disponibilidade de fontes primarias de nitrogênio em ambientes fermentativos é limitado para leveduras (Attfield, 1997; Pretorius, 2000). No caso da produção de etanol combustível no Brasil, a presença fontes preferenciais e a capacidade de assimilação por parte das leveduras como *S. cerevisiae* e *D. bruxellensis* confere uma vantagem para a adaptação, pois o suco de cana-de-açúcar pode conter quantidades de amônio e nitrato em maior proporção e

aminoácidos em menor quantidade derivados da cana, fertilizantes de campo e/ou do metabolismo das bactérias do solo. Por tanto, as leveduras adaptaram seu metabolismo de acordo às condições ambientais, permitindo principalmente a assimilação de fontes que supram as necessidades mais rapidamente. A Repressão Catabólica do Nitrogênio (NCR) é um mecanismo de regulação transcricional que serve para regular se fontes secundarias de nitrogênio devem ser utilizadas ou não (Cooper,2002). Assim, durante a presença de fontes preferenciais de nitrogênio, genes para a formação de enzimas e permeases necessárias para a utilização de fontes alternativas são reprimidos ou diminuída sua expressão (Cooper, 2002; Marzluf, 1997; Magasanik & Kaiser, 2002).

Entretanto, existem outros mecanismos de regulação do nitrogênio além da NCR, como o sistema Ssy1-Ptr3-Ssy5 (SPS), via de sinalização retrógrada (RTG) e controle geral de aminoácidos (GAAC) (Ljungdahl & Daignan-Fornier, 2012; Conrad et al, 2014). Em leveduras como *D. bruxellensis* proteínas transmembrana como as permeases codificadas pelos genes *GAP1*, *MEP1* e *PUT4* estão sob regulação de nitrogênio do tipo NCR (De Barros Pita et al, 2013b; Marini et al., 2000; Magasanik & Kaiser, 2002; Cain & Kaiser, 2011). Outras permeases e proteínas de transporte por exemplo aquelas codificadas pelos genes *GNP1* e *PTR2* estão controladas transcricionalmente pelo Sensor SPS de aminoácidos extracelulares (Ljungdahl & Daignan-Fornier 2012). O transportador de nitrato *YNT1* (de Barros Pita et al 2011; Galafassi et al 2013) que será descrito no próximo item do metabolismo do nitrato e encontrado em algumas leveduras como *Hansenula polymorpha, Hansenula anomala* e *D. bruxellensis,* tem controle parcial da NCR de acordo às condições do meio (Ávila et al, 1995; de Barros Pita et al, 2011, 2013a).

A transcrição dos genes reprimidos por nitrogênio é inibida por dois fatores de transcrição GATA, Dal80 e Deh1 e desreprimida por fatores GATA Gln3 e Gat1 (Rodkaer 2014). Fatores do tipo GATA são proteínas reguladoras que compartilham um ou dois domínios de ligação ao DNA tipo “zinc finger” (Cys-X2-Cys-X17-Cys-X2-Cys) que reconhecem sequências 5'-GATAA-3 'de regiões 5 'não codificação de certos genes e que estão presentes em organismos que variam de levedura a seres humanos (Rodkaer 2014). Além desses quatro fatores transcricionais, a expressão gênica relacionada ao catabolismo ao nitrogênio é regulada negativamente pelo produto do

gene ScURE2 (ScUre2). Isso é evidente nos mutantes Δ*ure2* (Rodkaer 2014; Ljungdahl & Daignan-Fornier 2012). A regulação desses fatores será discutida durante a regulação da via TOR.

2.8 METABOLISMO DO NITRATO

O nitrato é a principal fonte de nitrogênio assimilável para a maioria das algas e plantas vasculares superiores. Nos processos de fermentação industrial para produção de bioetanol combustível, o nitrato pode estar presente na composição do caldo de cana de açúcar ou no solo como resultado de irrigação por vinhaça (de Barros Pita et al., 2011). O metabolismo do nitrato em leveduras tem recebido pouca atenção principalmente pelo fato de que as principais leveduras estudadas, *S. cerevisiae* e *Schizosaccharomyces pombe,* não possuem a capacidade de assimilar esse nutriente (Marzluf, 1997; Siverio, 2002). No entanto, o primeiro sequenciamento do genoma de de *D. bruxellensis* revelou a presença de cinco genes envolvidos na metabolização dessa fonte de nitrogênio (Woolfit et al. 2007), embora a grande maioria das linhagens dessa espécie caracterizadas nos bancos genéticos tais como NRRL (EUA), CBS (Holanda), DSMZ (Alemanha) e JCM (Japão) não apontam a capacidade desta espécie em utilizar nitrato como única fonte de nitrogênio. Por outro lado, fenótipo está bastante disseminado entre os isolados de *D. bruxellensis* de destilarias de etanol no Brasil (De Barros Silva et al., 2011, 2013b; Da Silva et al., 2019)

O metabolismo de assimilação de nitrato por leveduras começou a ser estudado pela espécie *Hansenula polymorpha*, e parece seguir o padrão comum ao que acontece em plantas e fungos filamentosos (Ávila et al., 1995). Foram clonados os genes *YNT1* (codificador da permeasse de nitrato), *YNR1* (codificador de nitrato redutase, responsável pela primeira reação de conversão de nitrato a nitrito) e *YNI1* (codificador de nitrito redutase, responsável pela segunda reação de conversão de nitrito a amônio). Em *Hansenula polymorpha*, estes genes estão localizados em um cluster gênico e apresentam co-regulação induzida por nitrato (Figura 5). Esse mesmo agrupamento de genes é encontrado no genoma de *D. bruxellensis*, adicionado de dois genes (*YNA1* e *YNA2*) que parecem codificar fatores de transcrição que regulariam a expressão dos três genes estruturais (Woolfit et al., 2007). Dessa maneira, o processo de assimilação do nitrato é iniciado pela entrada do ânion NO_3^-

por meio do transportador de alta afinidade Ynt1p. Uma vez dentro das células, esse ânion é convertido em amônia por duas reações de redução consecutivas catalisadas pelas enzimas nitrato redutase (que converte NO_3^- a NO_2^-) usando um mol de NAD(P)H, seguido pela redução de NO_2^- a NH_4^+ pela nitrito redutase que utiliza três moles de NAD(P)H (Ávila et al., 1995; Siverio, 2002).

Esse gasto de equivalente redutor torna esse processo bastante custosos do ponto de vista energético. Soma-se a isso, mais um mol de NADPH que utilizado para a fixação do amônio em glutamato a partir de 2-oxoglutarato (Figura 4). Portanto, a equação global da assimilação metabólica do nitrato seria:

1 mol NO_3^- + 5 moles NAD(P)H + 1 mol 2-oxalacetato = 1 mol glutamato + 5 moles $NAD(P)^+$

Após essa descoberta, estudos fisiológicos e genéticos sobre a assimilação de nitrato em *D. bruxellensis* começaram a ser realizados. Inicialmente, foi mostrado que as células da linhagem GDB 248 era capaz de utilizar nitrato como única fonte de nitrogênio em meio de cultura sintético, e que o nitrato poderia ser co-assimilado com o amônio disponível no meio, tanto de laboratório quanto do caldo de cana, aumentando a capacidade da levedura de produzir etanol (Barros Pita et al., 2011).

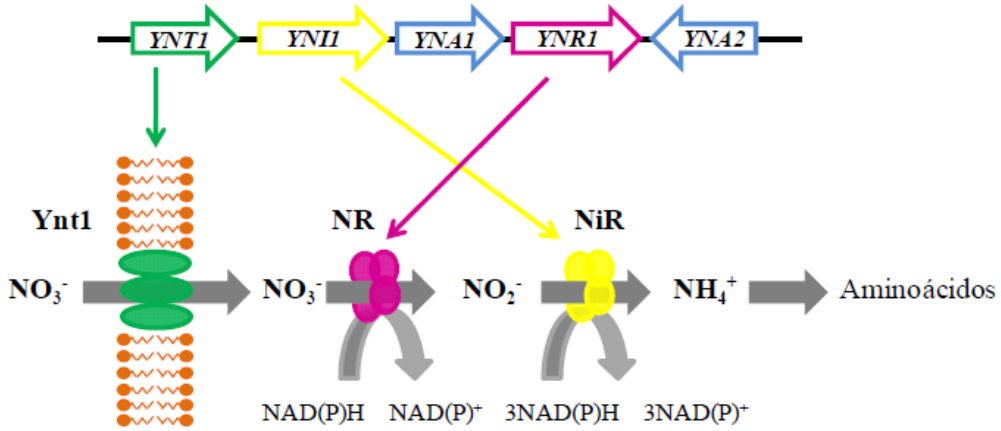

Figura 5: Metabolismo do nitrato. Acima estão representados os cinco genes e abaixo a função de suas proteínas correspondentes. O gene *YNT1* codifica a permease que permite a entrada de nitrato na célula. O gene *YNR1* codifica a enzima nitrato redutase que converto o nitrato a nitrito. E o gene *YNI1* codifica a enzima nitrito redutase que converte o nitrito a amônio, o qual é assimilado pela via central do nitrogênio. Os genes *YNA1* e *YNA2* codificam prováveis proteínas reguladoras da expressão dos três genes anteriores.

Isto foi possível pela foato que os três genes estruturais *YNT1, YNR1* e *YNI1* não são reprimidos pela presença do amônio, embora apresentassem diferentes níveis de expressão. Isto levou a formulação da hipótese de que a capacidade de assimilar nitrato seria uma vantagem adaptativa de *D. bruxellensis* na população de leveduras do processo industrial. (De Barros Pita et al., 2011). Em seguida, esses resultados foram corroborados a partir de estudos que mostraram que o nitrato poderia aliviar o efeito Custer, aumento a produção de etanol em anaerobiose, já que o excedente de NADH glicolítico poderia ser reoxidado por nitrato redutase (Galafassi et al., 2013). No entanto, a utilização dessa fonte de nitrogênio parece ser pouco eficiente para o crescimento celular, diminuindo bastante a velocidade de crescimento da cultura em aerobiose em diferentes fontes de carbono (De Barros Pita et al., 2013b). Isso indicaria que o nitrato é uma fonte não preferencial de nitrogênio.

De maneira geral, a transcrição dos genes da via de assimilação de nitrato responde a dois sinais essenciais, o primeiro, a ausência de fontes preferidas de nitrogênio (sinal de desrepressão) e no segundo a presença de nitrato (sinal de indução). Entretanto, estudos em *D. bruxellensis* usando nitrato como fonte única ou combinada com amônio reportaram que o gene YNT1 não respondem de forma coordenada com os outros genes da assimilação do nitrato quando o oxigênio não está presente no meio (De Barros Pita et al., 2011; Cajueiro et al., 2017). O primeiro desses sinais é de natureza geral, pois não apenas desreprime a expressão de genes necessários para a assimilação de nitrato, mas também de outras fontes alternativas de nitrogênio, aumentando as chances de assimilação de nutrientes presente no meio. O segundo sinal é específico, uma vez que apenas genes responsáveis pela assimilação do nitrato e formação de glutamato a partir de amônio respondem à indução gerada pela presença de nitrato no meio (Cooper, 2002; Marzluf, 1997; Siverio, 2002). O sinal de desrepressão é regulado por RCN e por sua vez, a RCN é mediada por fatores transcricionais do tipo GATA e proteínas que interagem com eles e modulam sua atividade (Crawford & Arst, 1993; Magasanik, 2002; Marzluf, 1997; Siverio, 2002; Wong, 2008).

2.9 REGULAÇÃO DO NITROGÊNIO PELO ALVO DA RAPAMICINA (TOR)

Como foi mencionado acima, o principal controle da regulação no consumo de nitrogênio envolve a participação das quinases TOR (Target of Rapamicy). As quinases TOR são quinases de serina e treonina altamente conservadas, de leveduras a mamíferos, que desempenham um papel central no controle do crescimento de células eucarióticas (Wullschleger et al, 2005). Elas são membros da superfamília da 3-fosfatidil inositol (PI-3K) quinase, que coordenam o crescimento e a diferenciação celular em respota à disponibilidade de nutrientes (Ljungdahl & Daignan-Fornier 2012; Conrad et al 2014), respondendo predominantemente à quantidade e qualidade das fontes de nitrogênio presentes no meio, provavelmente através da detecção de níveis intracelulares de aminoácidos.

Nas leveduras TOR (Target of Rapamycin) é codificada por dois genes, TOR1 e TOR2 (Yerlikaya et al 2015*)* e cada uma forma complexos de multiproteínas funcionalmente e estruturalmente denominado complexo TOR 1 (TORC1) e complexo TOR 2 (TORC2) respetivamente (Yerlikaya et al 2015*).* De um lado TORC1 controla o crescimento celular através de uma cascata de sinalização sensível à rapamicina que regula a transcrição, tradução, consumo de nutrientes, biogênese dos ribossomos e a autofagia, enquanto que TORC2 controla a organização da actina, endocitose e biossíntese lipídica através de uma cascata de sinalização insensível à rapamicina (Wullschleger et al, 2005; Yerlikaya et al 2015). A atividade do TORC1 diminui após falta de nitrogênio e aumenta com o aumento do nitrogênio (Yerlikaya et al 2015; Molinet et al 2019). Em *S. Cerevisiae* a atividade do complexo TORC1 é afetada pelo macrolídeo lipofílico Rapamicina, produzido por *Streptomyces hygroscopicus*, que inibe sua função e consequentemente bloqueia o crescimento celular (Yerlikaya et al 2015) ao se ligar à proteína FKBP12, inativando TORC1 e desta forma reduz os níveis de fosforilação de Gln3, que esta envolvido com seu direcionamento nuclear (Yerlikaya et al 2015; Conrad et al 2014).

Entre as alterações transcricionais induzidas por TOR está a indução da expressão de genes envolvidos no uso de fontes alternativas de nitrogênio (Conrad et al 2014; Ljungdahl & Daignan-Fornier 2012). A atividade do TORC1 diminui após a falta de nitrogênio e aumenta com o aumento do nitrogênio (Ljungdahl & Daignan-Fornier 2012) (Figura 6).

Em *S. cerevisiae,* TORC1 é capaz de detectar nutrientes e utiliza preferencialmente os ativadores GATA Gln3p e Gat1p para regular a transcrição em resposta à baixas concentrações de carbono e nitrogênio (Beck & Hall, 1999; Conrad et al 2014). Esses ativadores e repressores GATA Dal80p e Ure2p, são membros principais da via regulatória do metabolismo do nitrogênio. A transdução de sinal, desencadeada em resposta à nutrição de nitrogênio e detectada pelas proteínas Tor, opera através de uma via regulatória envolvendo o fator citoplasmático Ure2p (Magasanik & Kaiser, 2002; Cooper, 2002). Quando o carbono e o nitrogênio são abundantes, o Ure2p fosforilado ancora o Gln3p e Gat1p fosforilados no citoplasma, impedindo a ativação da transcrição dos genes regulados por esses fatores (genes para fontes secundárias de nitrogênio, etc). Quando o carbono e o nitrogênio são escassos, Gln3p e Nil1p defosforiladas entram no núcleo e promovem a transcrição dos genes importantes no metabolismo de fontes secundárias de nitrogênio.

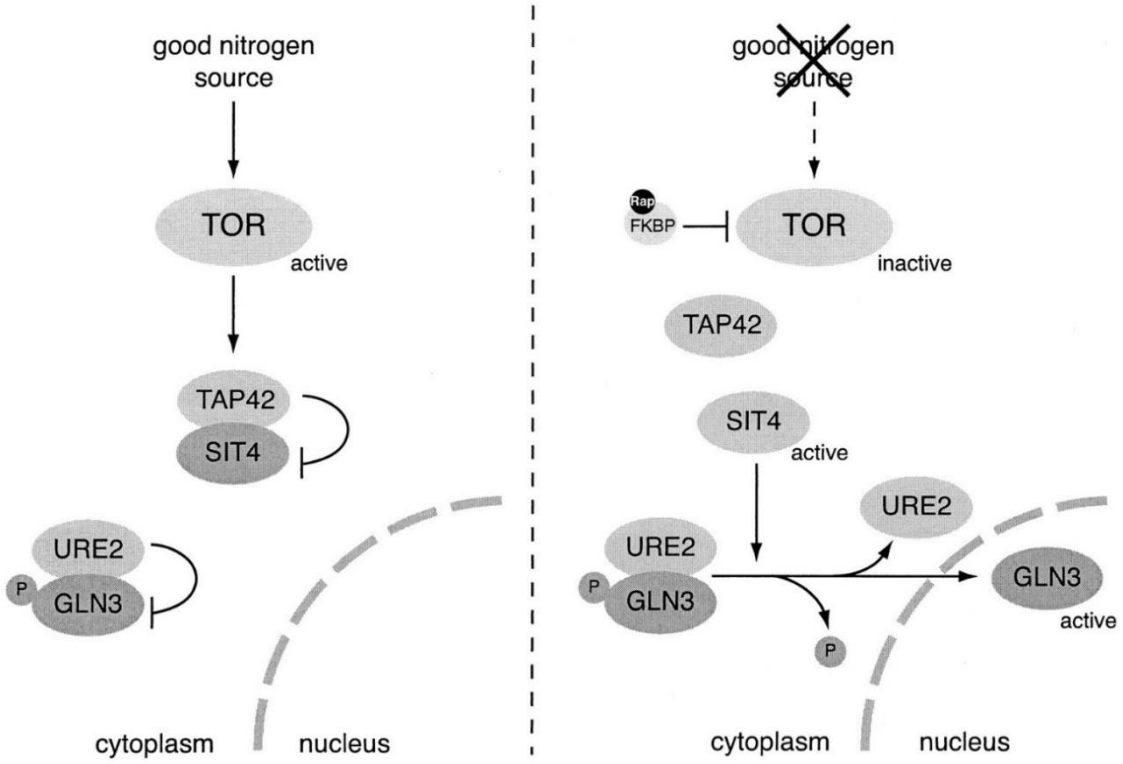

Figura 6: Via de sinalização TORC1 respondendo à disponibilidade e qualidade de fontes de nitrogênio (Crespo & Hall, 2002)

Além dos fatores GATA, dois reguladores principais funcionam como intermediários entre a atividade do TORC1 e vários componentes celulares que afetam o crescimento e o metabolismo: a quinase Sch9 e o complexo Tap42-PP2A. Desta forma, TORC1 modula regula a biogênese e a tradução ribossômica e para inibir a resposta ao estresse, que é incompatível com o crescimento celular e normalmente é induzida em células quiescentes (Conrad et al 2014., Molinet et al 2019).

Embora já se tenha bastante informação sobre a regulação do metabolismo do nitrogênio pela via TOR em *S. cerevisiae*, existem poucas informações sobre como o TORC1 afeta o perfil de consumo de nitrogênio em diferentes linhagens. E mais importante ainda, nada se sabe sobre a ação desta via em *D. bruxellensis*, especificamente quando se avalia o metabolismo de assimilação de nitrato que é específico desta levedura.

2.10 CONSIDERAÇÕES FINAIS E MOTIVAÇÃO

Os trabalhos com *D. bruxellensis* foram iniciados pelo nosso grupo em no ano de 2005, com a identificação desta levedura como importante constituinte da população de leveduras do processo industrial de fermentação de etanol combustível na região Nordeste do Brasil (De Souza Liberal et al., 2007; Basílio et al 2008). A partir desse ponto foram iniciados uma série de trabalhos genéticos e fisiológicas desta levedura em aerobiose ou condição limitante de oxigênio. No estudo de Parente et al. (2017) iniciamos essas análises com a utilização diferencial de fontes organicas de nitrogênio (aminoácidos) e no presente estudo complementamos as análises estudando o efeito da ausência de oxigênio no metabolismo do nitrato, como uma fonte de nitrogênio muito importante que confere uma vantagem adaptativa desta levedura para processos industriais.

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3 ARTIGO I

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] and Will de Barros Pita[2*]

[1]Department of Genetics, [2]Department of Antibiotics and [3]Department of Nuclear Energy, Federal University of Pernambuco, Recife, PE 50760-901, Brazil.

Running head: Nitrate induces ethanol production by D. bruxellensis in anaerobiosis.

*Correspondence to:

Will de Barros Pita

Departamento de Antibióticos – Universidade Federal de Pernambuco

Avenida Professor Artur de Sá, Departamento de Antibióticos, Cidade Universitária

50740520 - Recife, PE - Brasil

E-mail: will.barros.pita@gmail.com

Phone: 55-81-21268347

Keywords: Ethanol. Acetate metabolism. Anaerobic growth. Energetic demand. Nitrogen Catabolite Repression.

3.1 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 produce 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.

3.2 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 [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 (i) a better management and response to

stressful conditions and tolerance to inhibitors [2, 7, 35] or to (ii) 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 (either ammonium, urea or amino acids) become 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 [4, 17], a condition appropriated for beer or wine but not for fuel-ethanol production. To fulfil this gap, the present work evaluated the main aspects regarding the effect of nitrate under full anaerobic condition in the physiology of *D. bruxellensis* industrial strain GDB248 and consequently, its high potential in ethanol production when oxygen is absent.

3.3 MATERIAL AND METHODS

3.3.1 Yeast strain and maintenance

Yeast cells of industrial strain *D. bruxellensis* GDB248 were used in the present work [12, 14, 15, 16]. *Saccharomyces cerevisiae* JP1 strain isolated from distillery was used for comparison [24]. Cells were maintained in solid YPD (containing 2% agar) with constant re-inoculations. Cultivations were performed in YPD (1% yeast extract, 2% glucose, 2% bacteriological peptone) medium at 30 ^{o}C in orbital shaker at 150 rpm.

Glucose pulse assays

Seed cultures were prepared by cultivating yeast cells in YPD for 24 h at 30 ^{o}C/150 rpm in the presence of ampicillin (100 μg ml^{-1}) to prevent bacterial contamination. After this time, fresh medium was added to the cultures for a new cycle of cultivation. This was repeated until reaching an amount of biomass sufficient for fermentation assays. Afterwards, the cells were centrifuged (5 min, 9,000 rpm, room temperature) and washed with saline solution (0.85% sodium chloride). The sediment

of biomass was transferred to 50 ml of synthetic defined (SD) medium (Yeast Nitrogen Base w/o amino acids and ammonium sulphate at 1.7 g l^{-1}) supplemented with ammonium sulphate to 75 mmol of nitrogen l^{-1} and glycerol 20 g l^{-1} to initial cell concentration of 10% (w/v) and cultivated for additional 24 h in a strictly respiratory metabolism. These respiratory de-repressed cells were recovered and washed as above and resuspended to 50 ml of the SD fermentation media containing glucose at 50 mmol l^{-1} and one of the following N-sources (at final 75 mmol N l^{-1}): (1) ammonium sulphate, (2) sodium nitrate and (3) the mixture of both N-sources. Tubes were incubated at 30 ^{o}C without agitation and samples were taken at the beginning and after 120 min. After centrifugation, the supernatants were recovered, filtered through a 0.22 μm sterile filter and frozen at -20 ^{o}C until analysis for metabolites content. Cells were used for biomass quantification by dry cell weight determination according to [18].

3.3.2 Growth profile during anaerobic cultivations

Seed yeast cells were pre-grown in SD medium containing glucose (110 mmol l^{-1}) and ammonium sulphate (75 mmol N l^{-1}) as above. Then, cells were harvested by centrifugation (5 min, 5,000 g, room temperature) and suspended to 0.1 OD_{660nm} in a final volume of 1.5 ml of specific media. Each specific medium contained a mixture of glucose as carbon source (as described for the pre-growth) and one of the following combinations of nitrogen sources: (i) ammonium sulphate, (ii) sodium nitrate or (iii) both sources. All media presented final nitrogen concentration of 75 mmol l^{-1}. Yeast cells were cultivated in anaerobiosis in BioLector microfermentation device (m2p Labs, Germany) flushed with ultrapure gaseous nitrogen (O_2 less than 1 ppm). Tween 80 and ergosterol were added to fulfil lipid requirement [22]. Cultivations were performed at 800 rpm (equivalent to 180 rpm in rotatory shaker), 30 °C and 85% humidity with automatic measurements of light scattering variation every 30 min. This measurement was converted to absorbance at 600 nm by the off-line defined calibration factor. Each specific medium was run in biological duplicates and seven technical replicates. The complete absence of oxygen was attested by the failure of *S. cerevisiae* JP1 strain to grow in SD medium containing glycerol (data not shown), which is strictly metabolised by respiration. Data were recovered in xls format and processed in Microsoft® Excel® 2010 worksheet to generate growth curves. Specific growth rates were calculated from

the linear portion of exponential phase as defined by [18]. After defining growth profiles in each nitrogen source, experiments were repeated and ended in the time of sampling defined in the Results and Discussion section. Cells were harvested by centrifugation as above and flash-frozen in liquid nitrogen and stored at -80 ^{o}C until RNA extraction. Supernatants were prepared as above for metabolite analyses.

3.3.3 Determination of extracellular metabolites

Supernatants from fermentation and growth experiments were thawed and analysed by HPLC for the quantification of glucose, ethanol, glycerol and acetate. These were separated by the Aminex HPX-87H BioRad column at 60 ^{o}C using 8 mM H_2SO_4 as the mobile phase at the flow rate of 0.6 ml min^{-1} and detected by a UV detector connected in series to an index detector of refraction. Total nitrogen concentration was determined by colorimetric assays. Ammonia was measured following the salicylate method for total ammonia [27] at a wavelength of 640 nm. Analysis for nitrate determination was performed by using a flow-injection analyser FIAlab-2500 system (*FIAlab® Instruments*) according to manufacturer's instructions and using the optical channel 3 at a wavelength of 620 nm. All samples were diluted 500 times and used a final volume of 5 ml for both methods.

3.3.4 RNA isolation and cDNA synthesis

Cells from anaerobic cultivations in Biolector were thawed, harvested by centrifugation and suspended in 480 μl of lysing solution [13] and the lysates were submitted to total RNA extraction using Maxwell 16 LEV simply RNA Kit in Maxwell™ 16 device (Promega, USA), according to manufacturer's instructions. RNA quantification and purity ratios determination were performed in Nanodrop (Thermo Fischer Scientific, USA) and RNA integrity was evaluated by agarose gel electrophoresis (1%) in TBE buffer. Samples were stored at -80 °C until use. cDNA synthesis was performed within 24 hours using ImProm-II™ Reverse Transcription System Promega II Kit (Promega, USA), according to manufacturer's instructions. RNA

input was established as 0.5 µg of total RNA for each reverse transcription tube (20 µl) and cDNA was stored at -20 ºC. Every step of RNA isolation and cDNA synthesis followed the recommendations proposed by the MIQE Guidelines [5].

3.3.5 Gene expression analysis

To determinate the connection between metabolic profiles and the expression of genes involved in the metabolic pathways of uptake and biosynthesis, the level of mRNA of key genes of *D. bruxellensis* were determined by Real Time PCR (RT-qPCR) assays in ABI Prism 7300 platform (Applied Biosystems, USA), using SYBR Green PCR Master Mix as detection chemistry (Life Technologies, USA). Negative PCR controls and negative RT controls were run in parallel for internal control and reactions were carried out as previously described [13]. Target genes were involved in the assimilation of nitrate (*YNT1, YNR1, YNI1*) and ammonium (*MEP1, GDH1, GLT1*), fermentation (*ADH1, PDC1, ALD3, ALD5*), TCA cycle (*SDH1*) and ATP production from the respiratory chain (*ATP1)* [10]. Data normalization for each sample was performed by normalization factors calculated from the geometric mean of the reference genes *TEF1* and *ACT1* as indicated by geNorm [36] and as previously stablished for *D. bruxellensis* [13]. All experiments were performed in biological duplicates and technical triplicates and gene expression analyses were conducted in Microsoft Excel 2010, and the relative expression for each gene in the different media was calculated using the transcript levels of those genes at the beginning of cultivation as reference. Every step of RT-qPCR assays followed the recommendations indicated by the MIQE Guidelines [5].

Ordination analysis was performed with the use of ClustVis online web tool at (https://biit.cs.ut.ee/clustvis/) [20] using values of gene expression in different cultivation conditions regarding the N source. Cq values at 24 h and 40 h were relativized by the Cq values at the beginning of cultivation. Yeast isolates were clustered by co-relation distance and the three were ordered in both axes by the tightest clustering values of the orthogonal analysis of gene expression.

3.4. RESULTS AND DISCUSSION

3.4.1 Increased anaerobic ethanol production in nitrate is connected to acetate metabolism

The fermentative capacity of *D. bruxellensis* GDB248 strain was evaluated when cells growing under full respiratory metabolism in glycerol as carbon source were submitted to sudden exposure to glucose to 50 mM and incubated without agitation for two hours. In this condition, it was expected that the high initial cell density (10% w/v) could rapidly consume the oxygen dissolved in the medium leading to an anoxic environment. The yeast *S. cerevisiae* industrial strain JP1 was used as reference. The results showed that ethanol yield in the reference JP1 strain practically achieved the maximal theoretical (Table 1), attesting the reliability of the procedure. Ethanol was immediately produced by *D. bruxellensis*, confirming the canonical definition of a short-term Crabtree pattern reported for this yeast [28]. This phenomenon was previously reported when *D. bruxellensis* cells cultivated in C-limited conditions were suddenly exposed to glucose pulse [18]. In static synthetic defined (SD) medium with ammonium (SD-ammonium), ethanol yield was higher than that observed for agitated sugarcane juice medium and ethanol was the only fermentation product in both cases (Table 1) [24].

This was in contrast with a condition in which oxygen was minimally supplied by agitation of cultures in SD-ammonium that also produced acetate as end-fermentation product (Table 1) [34]. When nitrate was the N-source, ethanol yield in SD-nitrate was higher than in SD-ammonium (Table 1) and approached that obtained in sugarcane molasses (Table 1) [25]. Acetate might be produced as indication of an oxidative metabolism triggered in the cells even in fermentative condition. In the case of molasses, it is the consequence of the presence of oxidant molecules in this industrial substrate [25]. High acetate yield at the same level of ethanol yield was obtained when oxygen was abundantly supplied to cultures of *D. bruxellensis* (Table 1) [18]. Therefore, it can be concluded that nitrate promotes metabolic re-orientation similar to those triggered by oxidant molecules, either oxygen in agitated cultures or furaldehydes or other oxidants in the case of molasses. In this case, nitrate induces *D. bruxellensis* cells to produce acetate during fermentation. It might be highlighted that,

despite acetate production, ethanol yield was increased by the presence of nitrate (Table 1), probably as a consequence of an impairment of the Custer effect, as previously reported [12, 17]. The Custer effect is a temporary halt in fermentation due to the absence of oxygen, mainly associated to a limited ability to re-oxidise the NAD(P)H produced from glycolysis [37]. In our scenario, the reduction of nitrate to ammonium led to the re-oxidation of NAD(P)H, decreasing the influence of Custer effect [12, 17]. We had shown that the use of nitrate as N-source in SD medium significantly reduced growth rate and final biomass of *D. bruxellensis* as well as diminished ethanol production when oxygen is available [15]. Lastly, the presence of ammonium in SD-nitrate medium drastically reduced acetate production while keeping the high ethanol yield (Table 1). All these findings helped to explain the contradictory industrial data in which some fermentations processes contaminated by *D. bruxellensis* at high counts have a drop in the ethanol production [16], while other processes remained with normal ethanol production when nitrate was present in the substrate [12].

Table 1: Effect of nitrate and oxygenation on the fermentation of sugar to ethanol and acetate by *Dekkera bruxellensis* GDB248 strain.

	Substrate	C-source	N-source	Condition	Y_{etoh} (g g^{-1})	Y_{ace} (g g^{-1})	Reference
S. cerevisiae JP1	YNB	Glucose	NH_4^+	Static (O_2-limited)	0.50	0.00	This work
D. bruxellensis GDB248	YNB	Glucose	NH_4^+	Static (O_2-limited)	0.35	0.00	This work
	YNB	Glucose	NO_3^-	Static (O_2-limited)	0.45	0.05	This work
	YNB	Glucose	NH_4^+ + NO_3^-	Static (O_2-limited)	0.46	0.00	This work
	YNB	Glucose	NH_4^+	Air flushing (O_2-supplied)	0.17	0.20	Leite et al (2013)
	YNB	Glucose	NH_4^+	Agitated flasks	0.39	0.02	De Barros Pita et al (2013a)
	YNB	Glucose	NO_3^-	Agitated flasks	0.35	0.02	De Barros Pita et al (2013a)
	YNB	Glucose	NH_4^+	Agitated flasks	0.33	0.03	Teles et al (2018)
	Cane juice	Sucrose	NH_4^+	Agitated flasks	0.22	0.00	Pereira et al (2012)
	Cane molasses	Sucrose	NH_4^+	Agitated flaks	0.45	0.02	Pereira et al (2014)

3.4.2 Nitrate stimulates ethanol production during anaerobic growth

Following the analysis of short-term fermentation, which is hardly connected to biomass formation, we performed anaerobic cultivations of *D. bruxellensis* cells in SD medium containing ammonium, nitrate or a mixture of these N-sources and analysed physiological and genetic parameters. The complete absence of oxygen in the cultivations was attested by the absence of growth of *S. cerevisiae* JP1 in SD medium with glycerol as fully respiratory C-source, as previously reported [22]. In SD-ammonium, yeast cells grew at the rate 0.175 h^{-1} (Table 2) after a lag time of 8 h and entered stationary phase after 50 hours of cultivation (Fig. 2). In SD-nitrate, the lag time was extended to 12 h followed by the exponential growth at the same rate observed for SD-ammonium (Fig. 2). Overall, growth in SD-nitrate was at least twice higher in anaerobiosis (Table 2) than that calculated for aerobiosis [6, 14]. When ammonium was present in SD-nitrate (SD-mix), lag phase lasted for 8 h, followed by an exponential growth at the same rate as in SD-ammonium (Fig. 2). Unexpectedly, in medium containing nitrate, alone or in combination with ammonium, it was observed two exponential growth phases intercalated by a diauxic-like pause that lasted for four hours (Fig. 2), which was not observed when cells were grown in SD-nitrate in aerobiosis [6]. So far, the meaning of that pause in cell growth is not clear. Therefore, samples were collected in the middle of each exponential growth phase, at 24 h (henceforth defined as 1^{st} exp) and 40 h (henceforth defined as 2^{nd} exp) of cultivation as representative of those putative different metabolic states. Results showed that in both exponential growth phases, cell growth rate was higher in SD-nitrate while biomass yield was higher in SD-ammonium (Table 2). In SD-mix, the growth rate was higher as in SD-nitrate while biomass yield was higher as in SD-ammonium. These results corroborated the previous findings in which that co-assimilation of ammonium and nitrate present in the industrial substrate could provide a selective advantage for *D. bruxellensis* over *S. cerevisiae* in the strictly anaerobic industrial fermentation, as originally proposed by [12].

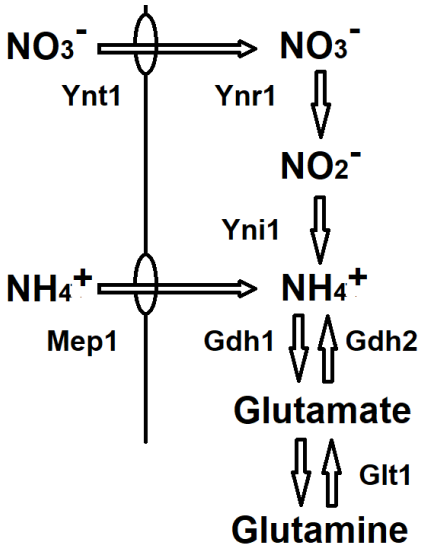

Figure 1: Scheme of nitrate (NO_3^-) and ammonium (NH_4^+) assimilation pathways representative of the main nitrogen metabolism pathway in the yeast *Dekkera bruxellensis*. Ynt1: nitrate permease product of *YNT1* gene; Ynr1: nitrate reductase product of *YNR1* gene; Yni1: nitrite reductase product of *YNI1* gene; Mep1: ammonium permease product of *MEP1* gene; Gdh1: NADPH-dependent glutamate dehydrogenase product of *GDH1* gene; Glt1: glutamine/2-oxoglutarate transaminase product of *GLT1* gene.

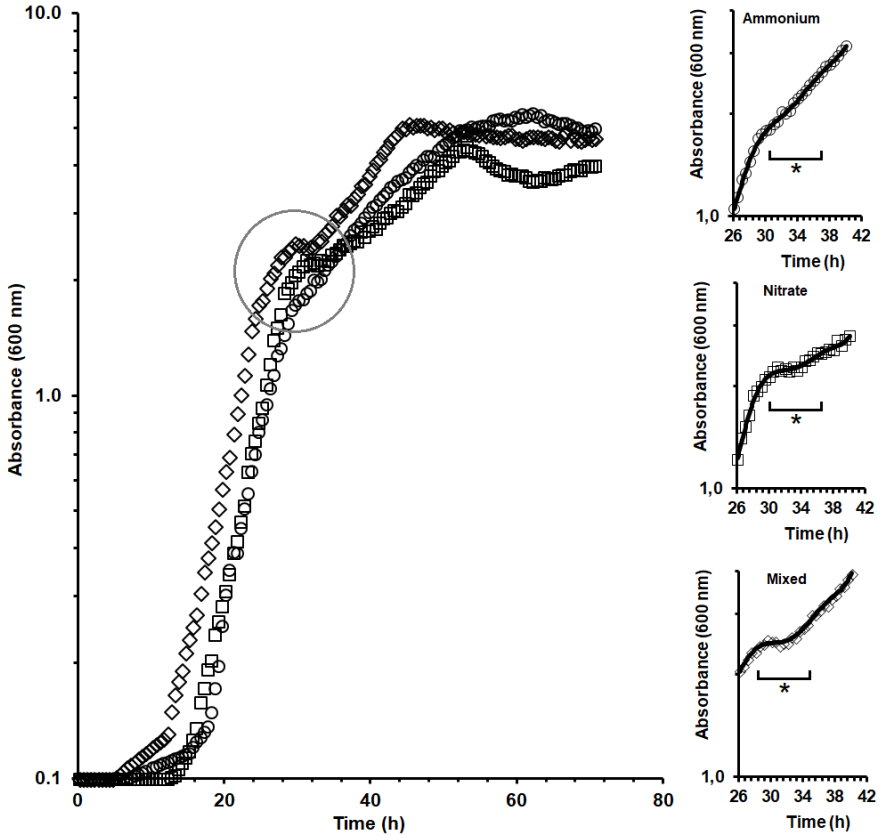

Figure 2: Anaerobic growth curves of *Dekkera bruxellensis* GDB 248 in presence in synthetic defined medium containing glucose and one of three different combinations of nitrogen sources as ammonium (circle), nitrate (square) or the equimolar mixture of both N-sources (diamond). The right-

hand inserts show the extended growth kinetics in the interval of 26 h to 42 h of cultivation in ammonium (up), nitrate (middle) or the mixture (down), highlighting the diauxic-like changes in growth profile (*) in nitrate-containing media.

Since biomass yield was lower in nitrate, it can be assumed that carbon from glucose could be dissimilated for the biosynthesis of fermentation products. Glucose consumption rate in the first exponential phase was calculated as 3.75 $mmol.L^{-1}.h^{-1}$ for both media containing nitrate, alone or in combination with ammonium. It was 42% higher than 2.63 $mmol.L^{-1}.h^{-1}$ calculated for SD-ammonium. Proportionally, ethanol production rate of 1.56 $mmol.L^{-1}.h^{-1}$ for both media containing nitrate was 48% higher than 1.05 $mmol.L^{-1}.h^{-1}$ calculated for SD-ammonium. In the second exponential phase these rates increased by at least two times in the three media, achieving the highest values in SD-nitrate, followed by SD-mix and SD-ammonium (Table 2). Therefore, the overall analysis led to the conclusion that the metabolic flux through the central carbon metabolism was more accelerated in nitrate than in ammonium. This profile was similar to that previously described by [17]. However, in the present work we report higher values for growth rate and ethanol yield and lower biomass yield induced by nitrate in anaerobiosis than in that report in the literature. Yet another major difference between the present work and that of Galafassi [17] is the fact that our strain is capable of growing anaerobically with nitrate as sole N-source. The presence of ammonium in nitrate medium increased biomass yield, slightly decreased ethanol yield and delayed production of acetate (Table 2). Thus, it might be concluded that in anaerobiosis, the presence of ammonium drives the glycolytic carbon towards a more assimilatory metabolism (biomass formation) in the opposite direction of the dissimilatory metabolism (by-products formation) induced by nitrate.

The end product of glycolytic/EMP pathway is pyruvate that afterwards takes two major pathways, being converted to mitochondrial acetyl-CoA by pyruvate dehydrogenase (PDH) or to cytosolic acetaldehyde by pyruvate decarboxylase (PDC), also known as PDH-bypass [10]. Acetaldehyde can be further reduced to ethanol by NADH-dependent alcohol dehydrogenase (ADH) or oxidised to acetate by $NAD(P)^{+}$-dependent acetaldehyde dehydrogenase (ACDH). Therefore, synthesis of ethanol is connected to consumption of reduced coenzymes while synthesis of acetate is

connected to production of reduced coenzymes. In aerobically-growing *D. bruxellensis* cells, acetaldehyde is mostly equally distributed to both by-products in ammonium [18] or in nitrate media [17]. Only ethanol was detected when cells were grown anaerobically in SD-ammonium, while acetate appeared together with ethanol when nitrate was present in the medium (Table 2). These results are in accordance to previous reports [17, 32]. Nitrate assimilation is dependent on the supply of NAD(P)H for its conversion to nitrite by nitrate reductase (NR) and then to ammonium by nitrite reductase (NiR). Therefore, the demand for NAD(P)H for nitrate assimilation explains this production of acetate in anaerobiosis. Galafassi [17] performed enzymatic analysis to conclude that the reaction catalysed by NR in anaerobic nitrate assimilation might preferable use NADPH generated by ACDH activity, resulting in acetate production, while the second reaction of NiR might preferable use NADH generated by assimilatory reactions. Therefore, the demand for NADPH imposed by NR might result in an increased flux towards the PDH bypass while the reaction by NiR would solve the anaerobic redox imbalance of NAD^+/NADH and allows *D. bruxellensis* cells to produce more ethanol [12, 17]. Thus, that industrial advantage pointed out for *D. bruxellensis* when nitrate was present in the substrate is explained by the metabolic change imposed by the demand for reduced cofactors, leading to both ethanol and acetate production. Once again, it explains why fermentation processes contaminated with *D. bruxellensis* can still yield ethanol at high levels when nitrate is present in the substrate [12].

Table 2: Physiological parameters of the anaerobic growth of *Dekkera bruxellensis* GDB248 in glucose synthetic mineral medium containing different nitrogen sources from samples taken in the middle of first (24 h) and second (40 h) exponential growth phases.

	Ammonium		Nitrate		Mix	
Parameters[a]	24 h	40 h	24 h	40 h	24 h	40 h
Growth rate (h^{-1})	0.172±0.003	0.178±0.003	0.187±0.002	0.230±0.008	0.187±0.004	0.232±0.002
Biomass yield ($g.g^{-1}$)	0.061±0.004	0.073±0.001	0.045±0.002	0.063±0.000	0.076±0.001	0.078±0.001
Ethanol yield ($g.g^{-1}$)	0.418±0.008	0.438±0.001	0.418±0.002	0.456±0.003	0.428±0.001	0.438±0.001
Acetate yield ($g.g^{-1}$)	0.00	0.00	0.025±0.001	0.045±0.002	0.00	0.025±0.001

CO_2 yield ($g.g^{-1}$)[b]	0.510	0.512	0.499	0.429	0.496	0.440
Mass balance (%)	98.9	100.0	98.7	101.0	100.0	98.8

[a]All experiments were performed in biological triplicates.

[b]Data calculated from the stoichiometry of mean biomass, ethanol and acetate production using the biomass equation (Leite et al 2013).

3.4.3 *GDH1/GLT1* cross-regulation is the sensor for the anaerobic metabolism of nitrate

Afterwards, we aimed to understand how this metabolic picture is influenced by changing the profile of genes involved in these metabolic pathways in the middle of each exponential growth phase of cells cultivated in the three media relative to the beginning of cultivations. The first set of genes referred to those involved in the metabolism of ammonium and nitrate, which are ultimately connected (Fig. 1). As we showed for cells growing aerobically [6], transcript level of *YNT1* (coding nitrate permease) was up-regulated by SD-nitrate and not repressed by the presence of ammonium in SD-mix at 24 h of cultivation (Fig. 4). The drop in its transcript level in SD-mix at 40 h of cultivation might be related to assimilation of nitrate during cell growth, with loss of its inductive capacity. In fact, in nitrate-based medium and in mixed medium, nitrate concentrations are 40 and 24 mM (Fig. 3), respectively (a 40% reduction). It showed that *YNT1* gene is solely submitted to inductive regulation by nitrate. On the other hand, the transcript level of *YNR1* (coding nitrate reductase) and *YNI1* (coding nitrite reductase) were up-regulated by nitrate and down-regulated by the presence of ammonium in SD-mix at 24 h of cultivation (Fig. 4). It attested the dual regulatory control of these genes also in anaerobiosis, with up- 7regulation by nitrate and down-regulation by ammonium.

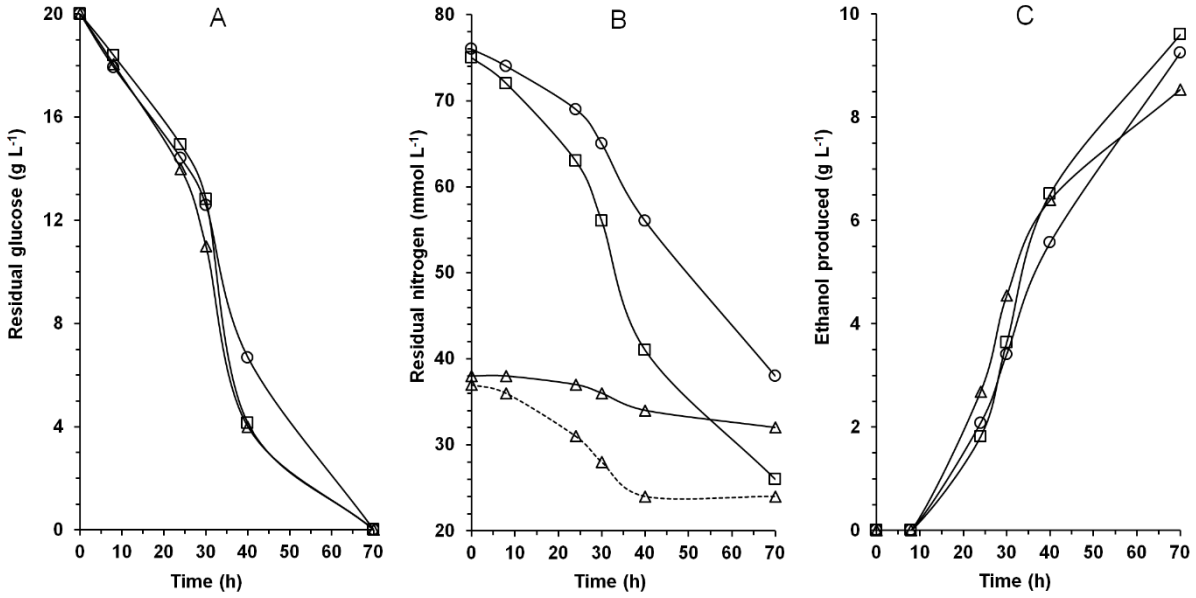

Figure 3: Consumption of carbon (panel A) and nitrogen (panel B) and production of ethanol (panel C) during growth of Dekkera bruxellensis GDB 248 in synthetic defined medium under strict anaerobic condition. Nitrogen was provided in three different combinations as ammonium (circle), nitrate (square) or the equimolar mixture of both N-sources (triangle). Nitrogen in mixed condition in panel B (triangles) were measured as ammonium (straight line) and nitrate (dotted line)

Following the metabolic pathway in which nitrate is converted to ammonium, we analysed the expression of genes involved in ammonium assimilation (Fig. 1). The transcript level of *MEP1* (encoding ammonium permease) in the three media did not change at 24 h of cultivation (Fig. 4), probably due to the presence of enough assimilable N sources (Fig. 3). However, this gene was upregulated at 40 h of cultivation specially in the presence of nitrate (Fig. 4). At this point, concentration of assimilable nitrogen is low enough to de-repress this gene, yet high enough to promote cell growth at high rate (Table 2). Indeed, ongoing experiments revealed that *D. bruxellensis* cells can keep high growth rates even at limiting concentration (<10 mM) of nitrogen in the medium (unpublished results). This is an indicative that this permease-encoding gene might solely respond to the limitation of assimilable nitrogen rather than the type of N source, like observed when the cells were cultivated in proline in aerobiosis [15]. In terms of enzyme-encoding genes, it was observed the expected opposite expression profile of *GDH1* and *GLT1* in anaerobiosis (Fig. 4), similar to what was reported for aerobiosis [14].

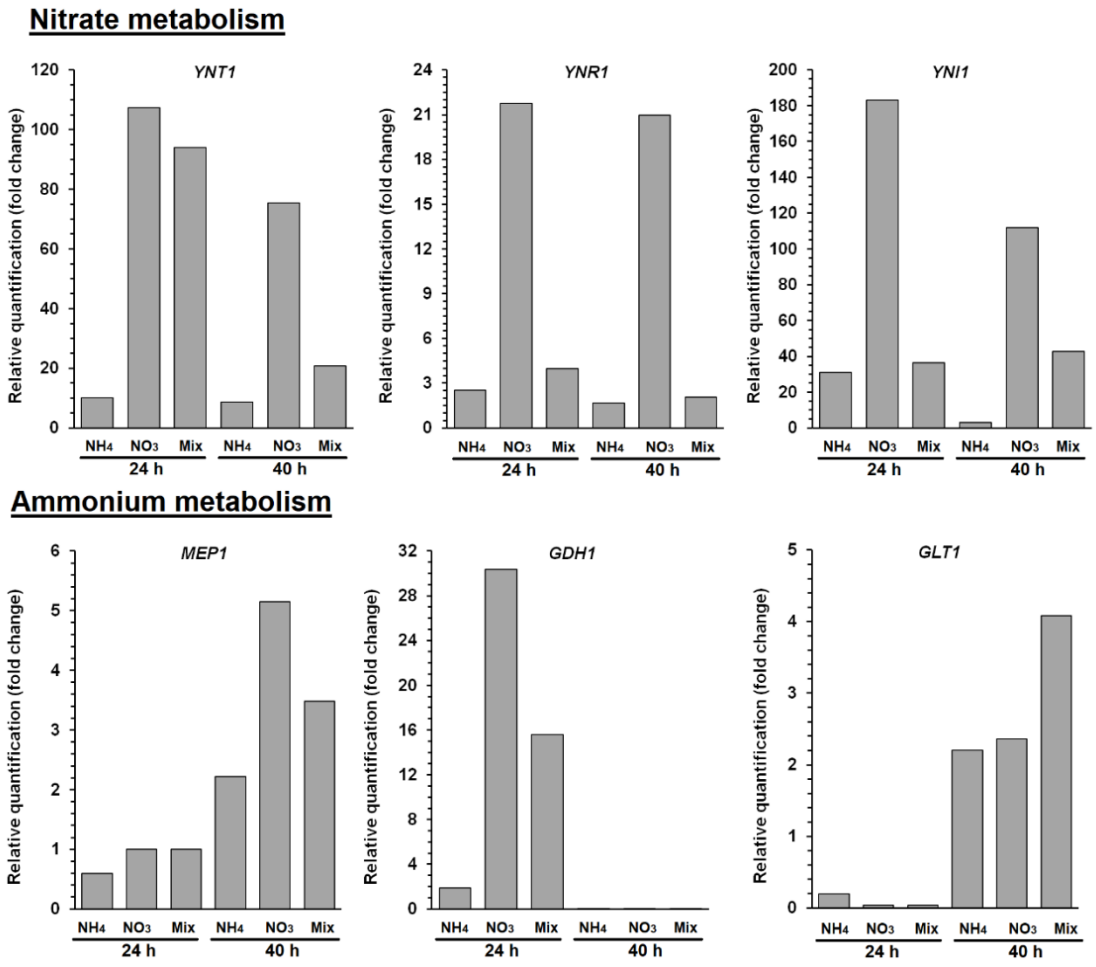

Figure 4. Transcript levels of genes involved in nitrate metabolism (YNT1, YNR1 and YNI1) and ammonium (MEP1, GDH1 and GLT1) at 24 h or 40 h of cultivation in synthetic defined medium containing glucose and one of three different combinations of nitrogen sources as ammonium (NH4), nitrate (NO3) or the equimolar mixture of both N-sources (Mix) relative to the beginning of cultivation in the same medium. Standard deviations were lower than 5% of mean values.

Surprisingly, *GDH1* was upregulated in nitrate-containing media at 24 h while the level of its transcript dramatically dropped at 40 h in all three media (Fig. 4). So far, it is not clear the regulatory mechanism acting on *GDH1* since no conventional NCR-related TF-binding motif was found in the promoter region of this gene [6]. In that work, we related the unchanged profile of *GDH1* in aerobiosis growing cells. Regarding to *GLT1,* it was observed a slight upregulation of this gene at 40 h phase independent of the nitrogen source (Fig. 4). We assumed that this profile is most probably related the lower level of assimilable nitrogen in the media at that time, as discussed above in the upregulation of *MEP1*. At this point, cells should change the way of ammonium assimilation from energetic neutral Gdh1p pathway to ATP-demanding glutamine synthetase. It confirmed that *GLT1* gene product plays a secondary role in the

glutamate biosynthetic pathway in this yeast [19]. Besides, the change in the pattern of glutamate production might impose energetic consequences to the yeast cells. To produce glutamate via Glt1p, cells have to first aminate glutamate to glutamine in an ATP-requiring reaction by glutamine synthetase. Afterwards, glutamine is transaminated with 2-oxoglutarate via Glt2p to produce two molecules of glutamate. This energetic requirement indicated by upregulation of *GLT1* coincided with the higher upregulation of *ATP1* gene at 40 h as discussed below.

Taking together the gene expression profile (Fig. 3) and the physiological data (Table 2), it can be concluded that nitrate assimilation uses the low affinity pathway by NADPH-dependent Gdh1p (when at high nitrogen concentration in the medium). This profile might be changed to a high-affinity pathway of ATP-demanding Glt1p (when at low nitrogen concentration). Under anaerobic condition, this ATP is provided by increased fermentative metabolism (Table 2).

3.4.4 The increased ethanol production by nitrate coincides with higher energetic demand

Besides genes of nitrogen metabolism, it was also assessed the expression of key genes of the fermentative pathway *(PDC1, ADH1, ALD3* and *ALD5*) and genes associated to energetic metabolism (*SDH1* and *ATP1*). In this regard, it was observed that the transcript level of either *PDC1* or *ADH1* genes were high and did not change along the anaerobic cell cultivation in both exponential growth phases irrespective to the N source in the media (Fig. 5). Therefore, the higher transcript levels of these two genes allow the higher ethanol yield in anaerobiosis in any source of nitrogen (Table 2). This result differed from a previous report in which we showed that *ADH1* gene was downregulated in SD-nitrate in aerobiosis, which coincided with the lower ethanol yield in that condition [14]. We also reported the coincidence between high ethanol yield and increased transcription level of *ADH1* and *PDC1* when *D. bruxellensis* cells were treated with disulfiram [34], a drug that impairs the activity of ACDH and reduces cytosolic acetate production.

Regarding the genes involved in acetate production, significant increase in the up-regulation of *ALD3* gene was observed in nitrate-containing media at 40 h

compared to 24 h (Fig. 5). This coincided with the detection of acetate in the supernatant of these media (Table 2). This gene encodes the cytosolic $NADP^+$-dependent ACDH responsible for providing acetate for the biosynthesis of cytosolic acetyl-CoA required for anabolic reactions. Hence, the upregulation of this gene when nitrate was in the medium seemed a clear cellular response to the metabolic demand for NADPH imposed by the reaction catalysed by NR encoded by *YNR1* gene. Alternatively, the ACDH isoform encoded by *ALD5* supplies the mitochondrial requirement for acetyl-CoA. This gene followed the same pattern of transcript level of *ALD3,* with the exception that its expression at 40 h was also observed in SD-ammonium (Fig. 5). Therefore, we propose that the acetate detected in the supernatant of 40 h in SD-nitrate or in SD-mix was the result of the metabolic excess of reaction catalysed by cytosolic ACDH encoded by *ALD3.* Yet, the acetate produced by the mitochondrial Ald5p might be entirely converted to acetyl-CoA to fulfil the functioning of the mitochondria in anaerobiosis, most probably connected to the energetic demand for ATP as stated above.

In view of this, we analysed the relative expression of genes involved in the TCA cycle and ATP production from the respiratory chain as representative of the mitochondrial functioning in anaerobiosis. Transcript level of *SDH1* gene, which encodes the succinate dehydrogenase, was high at 24 h and even higher at 40 h of cultivation independent of the N source (Fig. 5). This enzyme constitutes the so-called site II of the phosphorylation chain and its upregulation corroborated the assumption that the mitochondrial functioning was much more required by the cells of *D. bruxellensis* even in the absence of oxygen at the second exponential growth phase. At that moment, concentrations of both C and N-sources should be low in the media. The expression of this gene, and other TCA genes, remained unaltered high during aerobic cultivation of *D. bruxellensis* cells in primary (glutamate) or poor (proline) N sources [15]. Therefore, it seemed a particular response to the absence of oxygen and not to the type of N source in the medium. Similarly, The *ATP1* gene was also upregulated at 24 h, with substantial increase in its transcript level at 40 h especially in nitrate-containing media (Fig. 5).

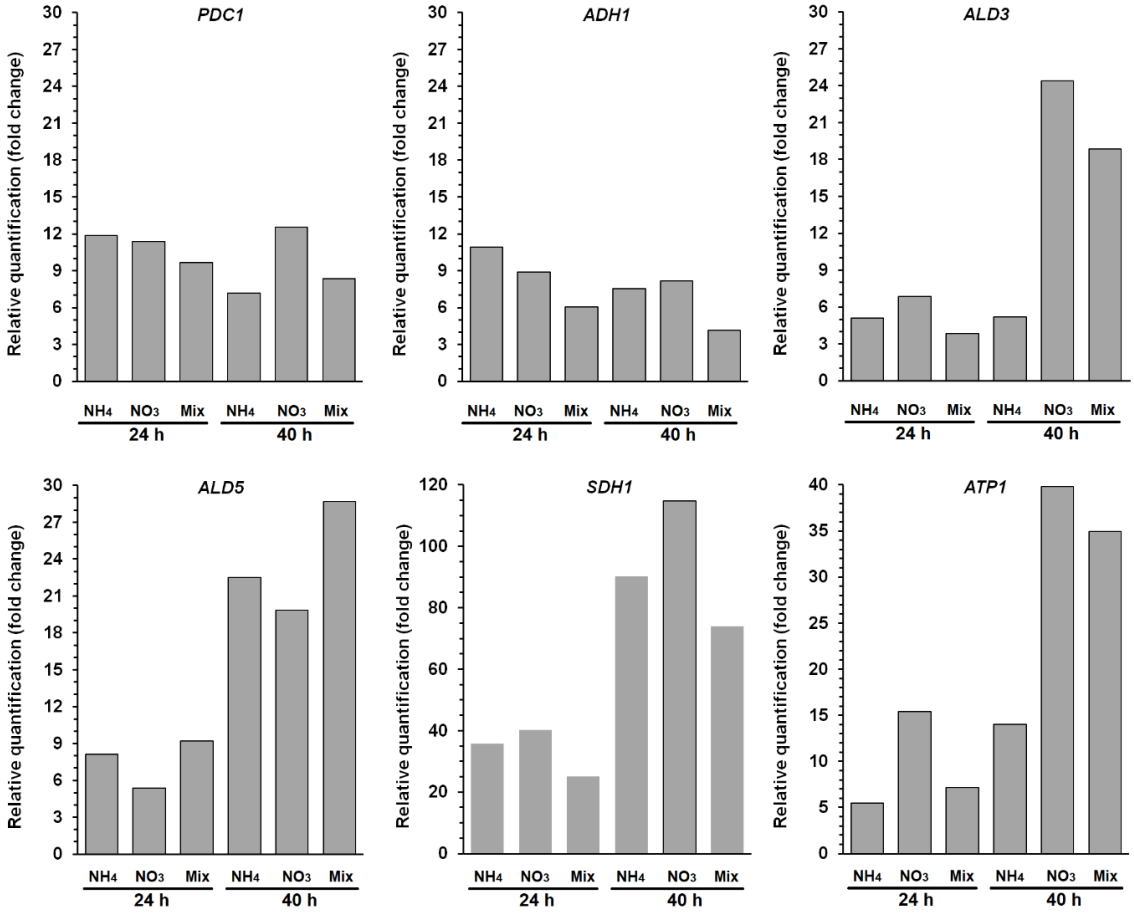

Figure 5: Transcript levels of genes involved in ethanol (*PDC1* and *ADH1*) and acetate (*ALD3* and *ALD5*) biosynthesis and representative of mitochondrial functioning (*SDH1* and *ATP1*) at 24 h or 40 h of cultivation in synthetic defined medium containing glucose and one of three different combinations of nitrogen sources as ammonium (NH4), nitrate (NO3) or the equimolar mixture of both N-sources (Mix) relative to the beginning of cultivation in the same medium. Standard deviations were lower than 5% of mean values.

This gene encodes the α subunit of the F1 sector of mitochondrial F1F0 ATP synthase and its expression is directly linked to energy demand of the cells. This gene was also upregulated by nitrate when *D. bruxellensis* cells were cultivated in the presence of oxygen [14], confirming the high demand for ATP imposed by the metabolisation of nitrate [15]. This high demand for ATP might also be caused by the change in the glutamate production from Gdh1p to Glt1p reaction at 40 h, as showed above. The upregulation of *ATP1* observed in anaerobiosis (Fig. 5) was similar to that reported for the yeast cells treated with disulfiram [34], both conditions resulting in high ethanol yields (Table 1). In the presence of this inhibitor, we proposed that

mitochondrial production of acetate might impose a high metabolic activity of this organelle.

3.4.5 Co-regulation patterns of genes for nitrogen metabolism and energetic metabolism

Clustering analysis was performed in order to identify similar patterns of expression (co-expression) among the genes analysed in the present study according to the nitrogen sources in the medium (Fig. 6). As the first component of the similarity matrix it was clearly observed that N availability was paramount for the pattern of gene expression, with the genes being clustered by the availability of ammonium as N-source, followed by the limitation of nitrate at the second exponential growth phase and then by the abundance of nitrate at the first exponential growth phase (Fig. 6). In the second component of this analysis, genes were separated in three metabolic clusters. The first cluster was related to nitrate assimilation composed by *YNT1, YNR1* and *YNI1,* that ultimately converts nitrate to ammonium, and by *GDH1* that uses this ammonium to produce glutamate (Fig. 1). This observation differs from a previous work in aerobiosis in which *GDH1* is not co-regulated with nitrate genes [6]. That disconnection, could partially explain the lower aerobic growth rate in nitrate [13]. The second cluster was composed by *MEP1* and *GLT1*, which might correspond to the metabolic reorientation in the ammonium assimilation pathway, as discussed above. These genes grouped with *PDC1* and *ADH1* that did not respond to nitrogen availability, but to the absence of oxygen. This observation also points towards the new metabolic profile, in which cells have an increased ATP demand. In this scenario, ATP production is boosted by glycolysis and, consequently, fermentation (Table 2). And the third cluster included genes that might respond to the high cellular energetic demand induced by Glt1p pathway in anaerobiosis, composed by ACDH-encoding genes whose products lead to acetyl-CoA production and by *SDH1* and *ATP1* genes whose products are somehow involved in the mitochondrial metabolisation of acetyl-CoA.

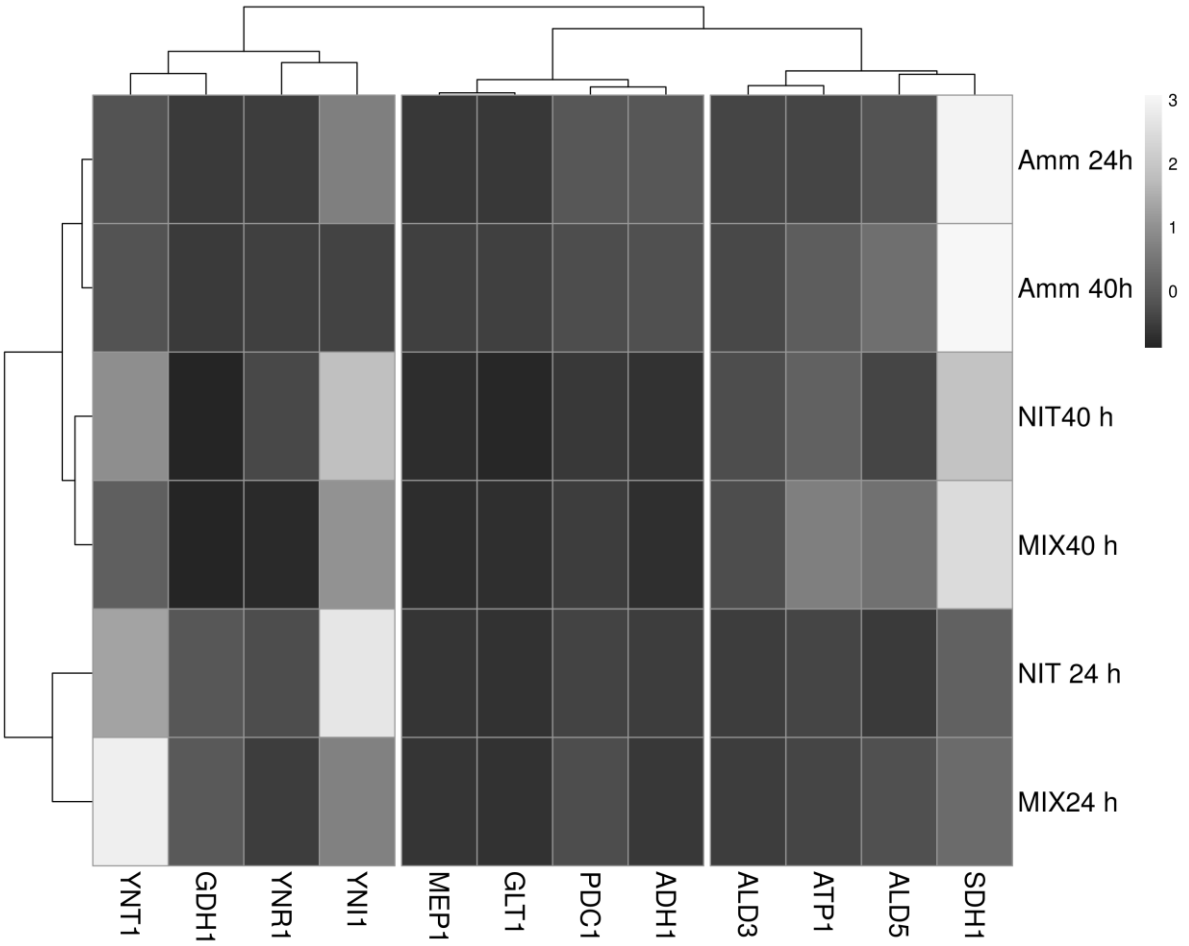

Figure 6: Clusterisation of the genes analysed in this work by their co-regulation profile regarding their expression at 24 h or 40 h of cultivation in synthetic defined medium containing glucose and one of the three different combinations of nitrogen sources as ammonium (Amm), nitrate (NIT) or the equimolar mixture of both N-sources (MIX) relative to the beginning of cultivation in the same medium. The tree was constructed by ordering the genes in both axis by the tightest clustering values of the orthogonal analysis of gene expression using on-line ClustVis tool.

3.5 CONCLUSION

The results in the present work definitely prove that *D. bruxellensis* is capable of producing ethanol at the same yield as *S. cerevisiae* when oxygen is absent, which is rather incremented by the presence of nitrate in the substrate. Nitrate also promoted higher growth rate in this condition, by increasing the demand for ATP which lead to increased glycolytic flux. Altogether, these findings explain the success of *D. bruxellensis* in the industrial environment as well as reinforces its potential for other biotechnological processes.

Compliance with ethical standards

Funding

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4. ARTIGO II

Proteomic analyses uncover the metabolic aspects of anaerobic nitrate assimilation by the industrial yeast *Dekkera bruxellensis*

Irina Charlot Peña-Moreno[1], Denise Castro Parente[1], Karolini Miranda[1], Elton Pedro Nunes Pena[2], Fabiana Aparecida Cavalcante Silva[2], Tercilio Calsa Junior[2], Will de Barros Pita[3] and Marcos Antonio de Morais Junior[1*]

[1]Laboratory of Microbial Genetics and [2]Laboratory of Plant Genomics and Proteomics, Department of Genetics and [3]Laboratory of Bioprocesses, Department of Antibiotics. Federal University of Pernambuco, Recife, PE 50760-901, Brazil.

Corresponding author:
Marcos Antonio de Morais Jr
Departamento de Genética, Universidade Federal de Pernambuco
Av. Moraes Rego, 1235. Cidade Universitária, Recife PE, 50.670-901, Brasil.
Phone/Fax: 00-55-81-21268522
E-mail: marcos.moraisjr@ufpe.br
ORCID: 0000-0002-9727-0300

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4.1 ABSTRACT

The yeast *D. bruxellensis* is well known for its adaptation to industrial ethanol fermentation processes, which can be further increased if nitrate is present in the substrate. To date, the assimilation of nitrate was considered inefficient by the apparent energy cost imposed to cell metabolism. However, recent research showed that nitrate promotes high growth rate and ethanol yield when oxygen is absent in the environment. In this sense, the present work aimed to identify the biological mechanisms behind this physiological behaviour. Proteomic analyses comparing four contrasting growth conditions revealed some clues on how nitrate can be used as primary nitrogen source by *D. bruxellensis* cells in anaerobiosis. The superior anaerobic growth in nitrate seems to be a consequence of increased cell metabolism (glycolytic pathway, production of ATP and NADPH and anaplerotic reactions providing metabolic intermediates) regulated by balanced activation of TORC1 and NCR de-repression mechanisms. On the other hand, the poor growth observed in aerobiosis is likely to de due to an intense oxidative stress triggered by nitrate. These results represent a milestone regarding the knowledge on nitrate metabolism and might be explored for future usage of *D. bruxellensis* as an industrial yeast.

SIGNIFICANCE: the present work finally uncovered the metabolic aspects of the paradox stablished upon the observation that *D. bruxellensis* grows and ferments very well anaerobically when nitrate is the nitrogen source in the medium, opposite to what happens in the presence of oxygen. Besides, it provides a reliable explanation for the physiological advantages of *D. bruxellensis* relative to *S. cerevisiae* when the industrial fermentation substrate contains ammonium and nitrate. Since nitrate is a cheaper and easily available source of nitrogen, these results could help in the development of novel industrial bioprocesses in which *D. bruxellensis* could be used as fermenting yeast or host cells for the production of proteins and metabolites of biotechnological interest.

4.1 INTRODUCTION

The yeast *Dekkera bruxellensis* is known for its emergence and maintenance in industrial fermentative processes for the production of fuel ethanol. Initially placed as a contaminant, recent studies have revealed a set of physiological characteristics that show its biotechnological potential (Blomqvist et al. 2010; Leite et al. 2013; Castro-Parente et al. 2015; Reis et al 2016). A striking feature in the yeast *D. bruxellensis* is its ability to assimilate nitrate, a source of nitrogen present in the sugarcane juice and which is not used by *Saccharomyces cerevisiae* (de Barros Pita et al.201, 2013; Pereira et al 2012,2014). Our recent results have shown for the first time that *D. bruxellensis* strains isolated from sugarcane juice fermentation can grow anaerobically with nitrate as the sole source of nitrogen (N), producing ethanol with yields similar to *S. cerevisiae* (Peña-Moreno et al. 2019). This is a particular feature of bioethanol strains since their wine counterparts can only grow in anaerobiosis in the presence of nitrate if at least traces of ammonium or amino acids are present in the medium (Galafassi et al. 2013; Tiukova et al.2013). Unlike the observed in aerobiosis, nitrate increases both cell growth and ethanol production at high levels (Peña-Moreno et al 2019).

The limited capacity to grow in nitrate has been related to the energetic cost of the assimilatory reactions, in which four mols of NAD(P)H are necessary for the conversion of one mol of nitrate to ammonium (Siverio 2002; Galafasssi et al. 2013).

To fulfil this requirement, *D. bruxellensis* cells produce acetate by the so-called PDH-bypass pathway, from which the pyruvate is converted to acetaldehyde by pyruvate decarboxylase and this molecule further converted to acetate by $NADP^+$-dependent acetaldehyde dehydrogenase (Van Rossum et al. 2016). This last reaction competes with NADH-dependent alcohol dehydrogenase in a way that the more acetate is produced the less ethanol is generated (de Barros Pita et al. 2013). Actually, this yeast is classified as acetogenic due the constitutive production of acetate even in ammonium (Leite et al. 2013; Teles et al. 2018). This physiological characteristic seems to be the cause of the Custer effect, defined as negative effect on glycolysis caused by the shortage of NAD^+ cofactor by ACDH reaction (van Dijken et al. 1986). A previous proteomic analysis of *D. bruxellensis* cells growing aerobically with nitrate as N source showed overproduction of the pentose-phosphate pathway enzymes, probably as a provider of NADPH for nitrate assimilation, besides ACDH reaction, as well as proteins of the TCA and ATP synthesis pathways to provide energy and anabolic metabolites for biomass formation (Neto et al. 2014). These results were in agreement with gene expression profiles under the same conditions (de Barros Pita et al. 2013).

Our recent data on the high growth rate of *D. bruxellensis* in nitrate, when oxygen is completely absent, raised questions over the real extension of the energetic cost for nitrate assimilation (Peña-Moreno et al.2019). Another relevant issue in this matter is the paradox created when considering previous classification of nitrate as a non-preferential or even poorly assimilable N source in the presence of oxygen (Parente et al. 2017; Cajueiro et al. 2017). So, the aim of the present work was to identify genetic and metabolic factors leading to the distinct physiological profiles observed in *D. bruxellensis* in different N sources, depending on the oxygen availability. Therefore, we performed differential proteomics in nitrate-based media both in aerobiosis and anaerobiosis. In addition, we compared proteomic profiles of *D. bruxellensis* cells in anaerobiosis when ammonium or nitrate was used as N source, in a feasible attempt to resemble the industrial environment. Overall, the results uncovered some relevant physiological aspects of nitrate assimilation in anaerobiosis, and also pointed the major regulatory metabolism behind the cellular conversion of nitrate to ammonium in *D. bruxellensis* in presence or absence of oxygen.

4.2 MATERIAL AND METHODS

4.2.1 Yeast strain and preculture conditions

D. bruxellensis GDB 248 was used in the present work. The cells were maintained in solid YPD medium (10 g L^{-1} yeast extract, 20 g L^{-1} glucose, 20 g L^{-1} peptone and 20 g L^{-1} agar). For the pre-inoculum, cells were cultivated in flasks containing 50 mL liquid YPD at 30ºC for 48 h in rotatory shaker at 160 rpm. Then, cells were centrifuged and resuspended in liquid synthetic defined SD medium (1.7 g L^{-1} Yeast Nitrogen Base w/o amino acids and ammonium sulphate, 20 g L^{-1} glucose; 5 g L^{-1} ammonium sulphate) at 30 °C and 160 rpm for 24 h. These were the seed cells used in the experiments with different growth conditions.

4.2.2 Media composition and cultivation procedures

Microfermentation experiments used specific SD medium containing sucrose at 110 mmol L^{-1} (20 g L^{-1}) as carbon source and ammonium sulphate or sodium nitrate at 75 mmol of nitrogen L^{-1} as nitrogen (N) source. Yeast seed cells were used to inoculate 1.5 mL of specific SD in FlowerPlates™ for initial absorbance of 0.1 units at 600nm (A_{600nm}). For aerobic assays, yeast cells were cultivated in BioLector NA device (m2p Labs, Germany) at 30 ºC, 85% humidity and 800 rpm (equivalent to 180 rpm in rotatory shaker), with constant flux of sterile air. For anaerobic growth, specific SD media were supplemented with Tween 80 to 420 mg L^{-1} and ergosterol to 10 mg L^{-1} to meet the lipid requirement in anaerobiosis (Jouhten et al. 2008; Blomqvist et al. 2012; Peña-Moreno et al. 2019). Cultivations were performed at the same conditions as in aerobioses, replacing air by ultrapure gaseous nitrogen (O_2 < 1 ppm). The complete absence of oxygen was attested by the failure of *S. cerevisiae* JP1 strain to grow in SD-ammonium medium containing glycerol as carbon source (data not shown), which is strictly metabolised by respiration. Automatic readings of light scattering were recorded every 30 minutes for 24 h and converted to optical density values (A600) by means of a calibration curve. Data were recovered in .xls format and processed in Microsoft® Excel® 2010 worksheet to generate growth curves. Specific growth rates were calculated from the linear portion of exponential phase as previously reported (Peña-Moreno et al. 2019). All experiments were performed in biological triplicate. For

protein extraction, experiments were repeated and samples were collected from ten wells when the cultures reached around 0.5 A_{600nm}, pooled to increase the number of cells, centrifuged and the cell sediment was frozen in liquid nitrogen.

4.2.3 Growth conditions and physiological comparisons

Four growth conditions were tested in the present work. In condition #1 yeast cells were cultivated in SD medium containing nitrate as N source in anaerobiosis (NO_3^-/N_2); in condition #2 yeast cells were cultivated in SD medium containing nitrate as N source in aerobiosis (NO_3^-/O_2); in condition #3 yeast cells were cultivated in SD medium containing ammonium as N source in anaerobiosis (NH_4^+/N_2); in condition #4 the yeast cells were cultivated in SD medium containing ammonium as N source in aerobiosis (NH_4^+/O_2). This last one was only used as control quality parameter to evaluate growth profile [5,18]. From these conditions, two physiological comparisons were performed. In comparison [A], protein profile of condition #1 was compared to condition #2 to describe how cells respond to the availability of oxygen when nitrate is used as N source. In comparison [B], protein profile of condition #1 was compared to condition #3 to describe how the cells respond to different N sources in the absence of oxygen.

4.2.4 Total protein extraction

Cells collected in the mid log phase for each of the four growth conditions were thawed on ice and proteins were extracted as previously described (Neto et al. 2014). Cell sediments were washed with 2 mL of sterile deionised water and resuspended in 200 µL of sterile deionised water. Then, 200 µL of 600 mM NaOH was added and the tubes were incubated for five minutes at room temperature. Cells were recovered by centrifugation (5 min, 10,000 rpm at 4ºC), re-suspended with 200 µL SDS Sample Buffer (60 mM tris-HCL pH 6.8, 5% glycerol, 4% β-mercaptoethanol, 2% SDS, 0.0025% bromophenol blue) and lysed by boiling for three minutes. The lysates were centrifuged and the cell free extracts transferred to new tubes. The sediments were re-

suspended with 200 µL SDS Sample Buffer and glass beads (425-600 µm Ø, Sigma-Aldrich) were added to equivalent of the buffer volume. The tubes were submitted to fives cycles of 30 sec in vortex and 30 sec on ice bath to breakdown the remaining intact cells. After further centrifugation, the second cell-free extract was pooled with the first cell-free extract and kept at 4ºC for protein extraction [16].

Afterwards, cell-free extracts were mixed with 1 mL extracting solution (100 mM ammonium acetate in methanol), incubated at -20ºC for 30 minutes and centrifuged (20 min, 17000 rpm, 4ºC). One mL of cold 80% acetone was carefully added to protein precipitates, the tubes gently inverted and centrifuged again. This procedure was repeated twice and then repeated once with 1 mL cold 70% ethanol. After centrifugation, the sediments were dried at room temperature for 15 min, solubilized with 200 µL solubilizing buffer (8 M urea and 2 M thiourea) and stored at -80ºC. Total proteins were quantified by the Bradford method (Bradford et al. 1976) using Coomassie Brilliant Blue G-250. Bovine serum albumin (BSA) was used for the establishment of the standard curve.

To verify the integrity of the extracted proteins, electrophoresis was performed in 12% SDS-PAGE in tris-glycine buffer at 150 V and 17 mA. A 14-97 kDa molecular weight marker (GE Healthcare) was used to estimate protein size. The gels were stained with Coomassie Brilliant blue and later digitized on an ImageScanner III transparency scanner (GE LifeSciences).

4.2.5 **Two-dimensional electrophoresis**

Isoelectric focusing (IEF) was performed on 11 cm/pH 3-10 IPG drystrips (GE LifeSciences) for each of three biological replicates from each treatment. Approximately 200 µg of protein were dissolved in rehydration solution (7 M, urea, 2 M thiourea, 2% CHAPS (m / v), 19.4 mM DTT, IPG buffer / pH 4-7 non-linear 1.0% (GE LifeSciences) and 0.005% bromophenol blue (w / v). Rehydration step was made in the IEF dispositive (IPG-Box, GE Life Sciences) at room temperature for 18 h. Proper IEF was performed on Multiphor II Electrophoresis Unit (GE Life Sciences) and

conducted at 10° C in three stages i) 300 V–30 V.h^{-1}; ii) 3500 V–2900 V.h^{-1}; and iii) 3500 V–6170 V.h^{-1}, with constant current (2 mA) and power (5 W) conditions. After IEF, strips were stored at −80 °C until second dimension (Neto et al. 2014). For this, the strips were equilibrated in a solution containing 50 Mm Tris-HCI (pH 8,8), 6 M urea, 30% (v/v) glycerol, 2% (m/v) SDS, 1% DTT, and 2.5% IAA; the last two were added separately in two steps of 20 min under orbital rotation. In the second dimension, the proteins were resolved via 12.5% SDS-PAGE using a DGV-20 system (Biosystems®) at 30 mA/gel for 15 min, 40 mA/gel for 30 min, and then 50 mA/gel for 3 hours. After electrophoresis, the 2D gels were stained in 0.08% w/v colloidal Coomassie blue G-250. 2D gels images were obtained by digitalization in ImageScanner III (GE Life Sciences) at 300 dpi, calibrated for optical density, with program LabScan 6.0 m (GE Life Sciences).

4.3.5 Two- Dimensional Gels Image Analysis

Differentially accumulated proteins/peptides (DAPs) were selected through comparative analysis of 2D-PAGE digital images, by using Image Master 2D Platinum v7.05 software (IMP, GE Life Sciences), according to previous protocols (Pacheco et al., 2013; Neto et al., 2014). Three biological replicates were considered for each condition. Replicates presenting a correlation coefficient (R) equal to or higher than 0.7 were considered for analysis. Based on normalization and statistical analyses performed in IMP, it was possible to verify the differential accumulation of spots, as well as exclusive spots, between treatments. Differential spots that presented a percent volume (%vol) ratio ≥ 1.5 and ANOVA test value ≤ 0.05 were considered DAPs and selected for identification.

4.3.6 DAPs identification by mass spectrometry and putative annotation

Selected spots were manually excised from gels and digested with trypsin [22]. Resulting peptides were elutedin solution containing 5% trifluoroacetic acid and 50%

acetonitrile, concentrated in Speed-vac (Eppendorf) for 30 min and stored at -20ºC until mass spectrometry (MS) analysis.

The mass spectra (MS) of the peptides originating from samples digestion were obtained in the Autoflex III MALDI-ToF / ToF analyzer (Bruker Daltonics), using an α-cyano-4-hydroxycinnamic acid matrix. The equipment was calibrated using a peptide 158 mixture [M + H]+ ions for standard calibration. The resulting *m/z* ratio comprises the specific molecular mass of the sample peptides, and is then named peptide mass fingerprint (PMF method). Further, the acquisition of the MS/MS spectrum was achieved by collision energy of 1 kV, first in negative and then in positive ion mode using argon collision gas. The same precursor ions generated by the negative ion MS were analysed in negative and positive MS/MS. The peak processing of LC1 MALDI MS was controlled by the so-called peak selection method throughout the work. Peak selection was done automatically to analyse MS spectra and acquire MS / MS data for selected peaks. Peak lists files were generated using the Flex Analysis 3.4 software (Bruker Daltonics).

4.3.7 Protein identification

The identification of the spectra was performed through the MASCOT online software (http://www.matrixscience.com), initially by the public access version, based in peptide mass fingerprinting method (PMF) for MS spectra and MS/MS ion search method for MS/MS spectra, against the sub-banks *D. bruxellensis* and *S. cerevisiae* from NCBIprotein and SwissProt database. Subsequently, additional identification was conducted through a private version of the MASCOT program, kindly made available for access in collaboration with the Center for Advanced Proteomics of the University of Washington (Seattle, Washington, USA (http://proteomicsresource.washington.edu/), against the sub-database *D. bruxellensis.* The experimental error used for mass values was as follows: i) to MS spectra analysis: up to 1.2 Da of tolerance; ii) to MS/MS spectra: 100 to 200 ppm and 0.2 to 1.2 Da (for parental ion), and 100 to 200 ppm and 0.2 to 0.6 Da (for ion fragment). Another search parameters were set in both methods as following: carbamidomethylation of cysteines as fixed modification and methionine oxidation as

variable modifications, allowing up to one missed cleavage. Protein identification was performed using the Mascot search probability-based molecular weight search (MOWSE) score. Only the identifications with calculated probability score equal or greater than to the cut-off value were considered significant. The score equals -10.log (P), where P is the probability that the similarity found is random. Peptides with a Mascot Score exceeding the threshold value corresponding to <5% false positive rate, calculated by the Mascot procedure.

Additional searches were performed with SearchGUI v.3.2.20 software using xml files obtained after analysis via MALDI-ToF/ToF that were individually converted to mzXML format through the FlexAnalysis software tool (Bruker Daltonics), and submitted to the presumptive identification (Carmona et al. 2016), whose results were visualized using the program 1PeptideShaker v.1.16.15 (de Groot et al. 2007). The following parameters were used to search the SearchGUI: tolerance m/z of the parental ion: 0.25 Da; ion fragments m/z tolerance: 0.25 Da; charge of the parental ion: 1-1; isotope: 0-1; maximum missed cleavages: 1; fixed modifications: carbamidomethylation (C); variable modifications: oxidation (M). The other parameters remained in the default software mode. Those proteins identified with the SwissProt database were compared by BLASTp against the NCBI database for *Brettanomyces bruxellensis* AW.

4.3.8 Ontology and protein-protein interaction (PPI) analysis

Initially, the Uniprot ID of previously identified sequences from *B. bruxellensis* database were aligned through *BLASTP* (https://blast.ncbi.nlm.nih.gov) to find the protein accession statistical significance of matches against *S. cerevisiae* proteome. These accesses served to find the names of correspondents for each protein identified using UniProt (http://www.uniprot.org/) and reconfirmed in the *S. cerevisiae* database (http://yeastgenome.com/). For gene ontology Yeastmine software (https://yeastmine.yeastgenome.org) was used considering the biological process at each of the four conditions. The Holm-Bonferroni correction test was used and a maximum p-value of 0.5. The most prominent gene clusters were included in our study.

The PPI analyses were predicted using StringApp from Cytoscape (version: 3.8.0) (Szklarczyk et al. 2016). In software was imported network from STRING database v. 11.0 (https://string-db.org/) using the following of parameters: i) Data Source: STRING: protein query, ii) Specie: *Saccharomyces cerevisiae* and iii) cutoff: medium confidence (0.4). The StringApp obtained PPI through different prediction methods, such as conserved neighborhood, co-occurrence, fusion, co-expression, experiments, databases and text mining. Additionally, was imported the table with ratio data of the treatments. Each treatment was identified by three different colors and each protein mapped was differentiated by nodes size gradient.

4.3.9 Gene expression.

RT-qPCR experiments were performed to transcriptionally validate the proteomics results. Aliquots of frozen cells collected from each of four growth conditions were used for RNA extraction and cDNA synthesis (de Barros Pita et al. 2012; Peña-Moreno et al. 2019). Primer design and amplification reactions were carried out by using the SYBR Green PCR Master Mix kit in ABI Prism 7300 detection system were performed (Applied Biosystems, USA) (de Barros Pita et al. 2012; Peña-Moreno et al. 2019). For relative expression analysis, condition #2 (NO_3/O_2) was used as a reference for comparison [A] and condition #4 (NH_4/N_2) was the reference for comparison [B]. A third analysis expressed the ratio of relative expression of key gene in comparison [A] by comparison [B] to evaluate the amplitude of gene expression in nitrate/anaerobiosis condition.

4.4 RESULTS

4.4.1 Absence of oxygen speeds up D. bruxellensis growth in nitrate

It has been very well reported that *D. bruxellensis* grows slowly when nitrate is available in the medium as the sole nitrogen (N) source, which led us to place this N source as non-preferential for the yeast metabolism (Jouhten et al 2008; de Barros Pita et al. 2011, 2013; Parente et al. 2017). However, this nutrient was proposed to be

an important adaptive factor for this yeast in industrial fermentation processes using sugar cane juice as substrate, being co-assimilated with ammonium (de Barros Pita et al. 2011). Recently, we showed that yeast growth rate in nitrate is practically the same as that in ammonium when oxygen is absent (Peña-Moreno et al. 2019). Therefore, it was concluded that the assimilatory pathway for nitrate is very efficient and that this N source can be re-classified as preferential in anaerobic growth condition. Hence, what are the biological constraints that impairs yeast growth in nitrate when oxygen is present?

To address this question, we performed growth experiments in four different conditions (Fig. 1). Based the growth curves (Fig. 1), specific growth rates were calculated as 0.21 h^{-1}, 0.20 h^{-1} and 0.22 h^{-1} for conditions #1 (NO_3^-/N_2), #3 (NH_4^+/N_2) and #4 (NH_4^+/O_2), respectively. It confirmed that ammonium and nitrate are similarly assimilated by the yeast cells in the absence of oxygen, and in these conditions the cells grew at the same growth rate of that calculated for ammonium-based medium in the presence of oxygen. Noteworthy, these high growth rate values were achieved in medium containing sucrose as sole carbon (C) source. It was already reported that growth rate in sucrose is higher than in glucose (Leite et al. 2013; da Silva et al. 2019). In the present work, sucrose was chosen as C source because it is the major sugar constituent of sugar cane juice, used for industrial fermentation towards bioethanol. In contrast, the growth rates of 0.13 h^{-1} were calculated for condition #2 (NO_3^-/O_2), confirming that nitrate restricted cell growth in the presence of oxygen. Based on the physiological features described above, the protein profile of this yeast was investigated in order to identify potential functional biomarkers that could explain such observations.

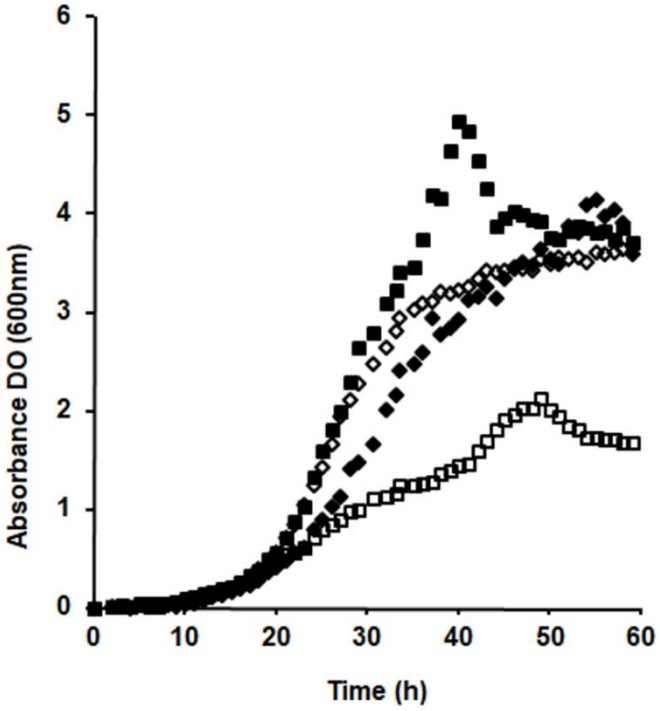


Figure 1: Growth profile of *Dekkera bruxellensis* GDB 248 cultivate in the presence (open symbols) or absence (closed symbols) in synthetic defined medium containing sucrose as carbon source and ammonium (diamond) or nitrate (square)) as a nitrogen source. The results represent the average of two independent experiments.

4.4.2 Overall proteomic analysis

In order to find the panel of proteins that might help in the understanding of nitrate metabolism by the yeast *D. bruxellensis,* proteomic analysis was performed in two different comparisons. Comparison [A] had the aim of identifying how the cells respond to the availability of oxygen when nitrate is used as N source (condition #1 vs. condition #2). Comparison [B] was defined to identify how the cells respond to different N sources in the absence of oxygen (condition #1 vs. condition #3). To normalise the analyses, proteins were extracted when the yeast cultures reached the same point in the growth curve (0.5 A_{600nm}). A total of twelve protein extracts were used for 2D electrophoresis with previous quality control of total protein extraction (Fig. 2a), producing 344 spots for comparison [A] (Fig. 2b) and 357 spots for comparison [B] (Fig 2c). Subsequently, DAP spots were assigned as (i) exclusive if they were detected only in a specific growth condition or (ii) prevalent when its relative amount in one condition

was ≥1.5 higher than in other growth condition, with a 99.99% confidence level (p-value ≤0,0001). Consequently, 95 and 91 DAPs were validated for comparison [A] and [B], respectively. DAPs were submitted to GO analysis for the identification of the major biological processes and cellular mechanisms that might be more active in each of the growth conditions (Fig. 3).

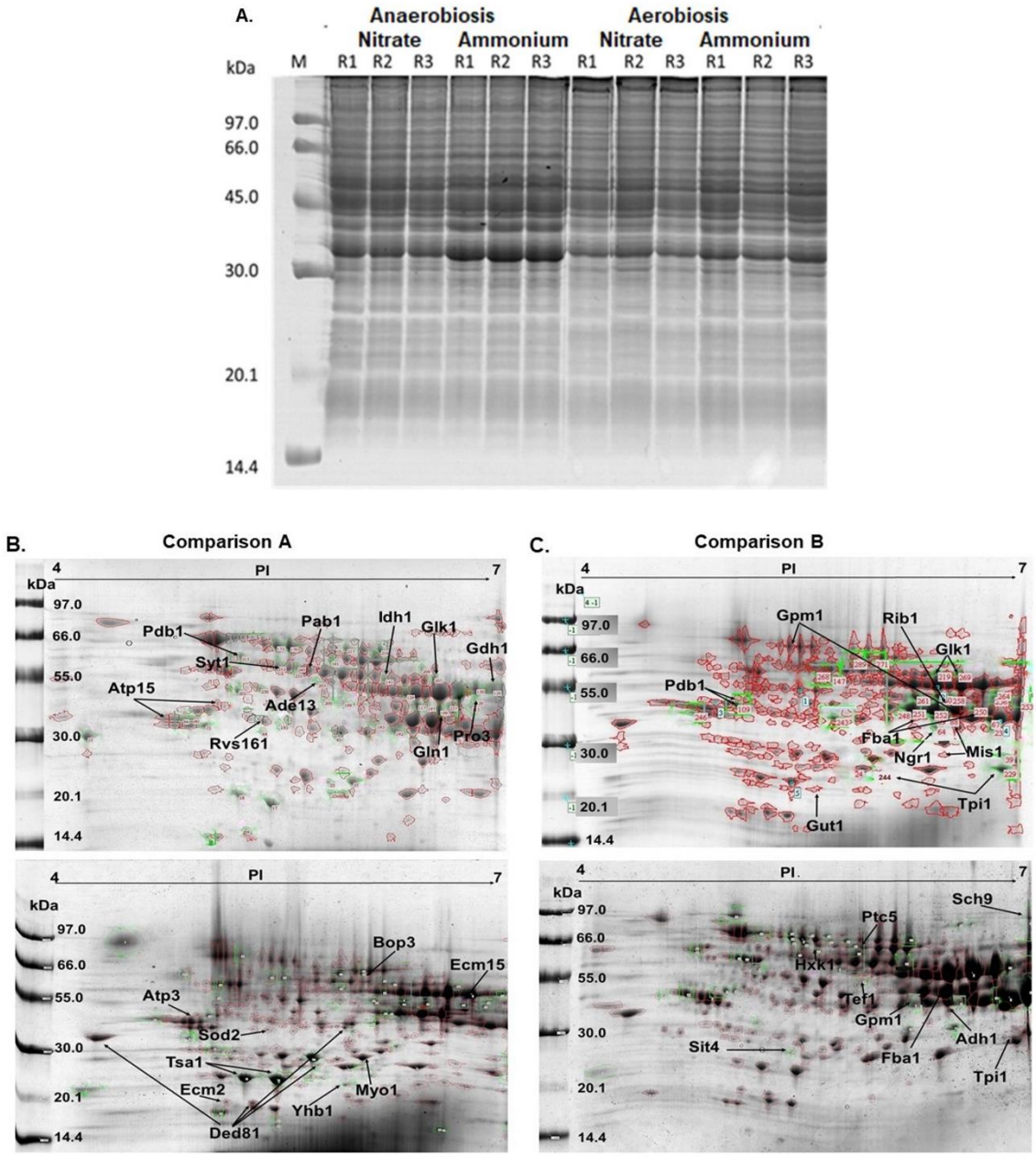
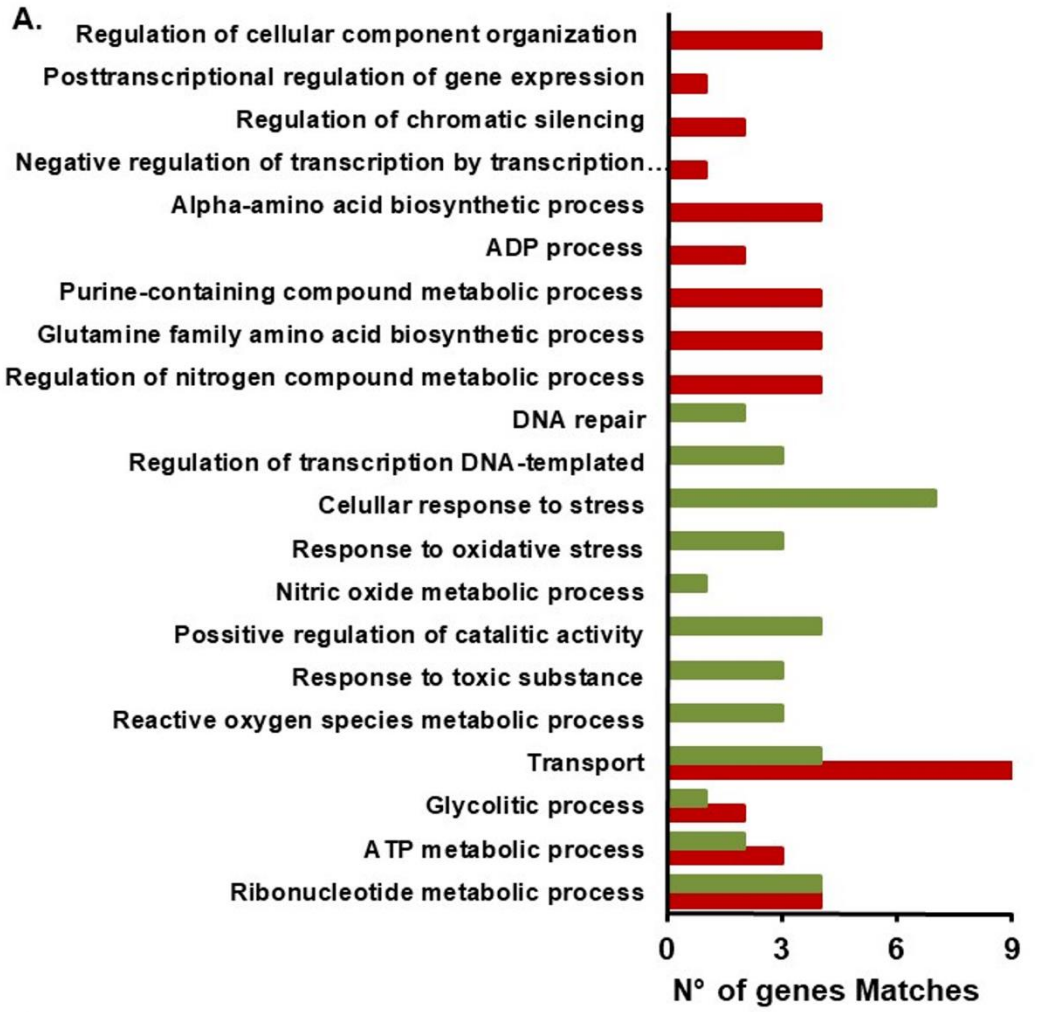


Figure 2: Differential proteome analysis of *D. bruxellensis* GDB248 cells cultivated in ammonium or nitrate as a nitrogen source and in the presence or absence of oxygen. (Panel A) Protein profile of three independent cell extracts (R1-R3) separated in 12% SDS-PAGE. (M) Molecular weight marker is shown. (Panel B) Isoelectrical Focusing for the identification of differentially accumulated proteins (DAPs) in the comparison [A] between nitrate/anaerobiosis (upper gel) and nitrate/aerobiosis (lower gel). (Panel C) Isoelectrical Focusing for the identification of DAPs in the comparison [B] between

nitrate/anaerobiosis (upper gel) and ammonium/anaerobiosis (lower gel) 2D-PAGE. The indicated spots were excised from the gels and peptides identified by PMF and MS/MS MALDI-ToF analyses.

4.4.2 Protein synthesis and cell progression are activated in anaerobic nitrate growth

In comparison [A], peptides of *D. bruxellensis* cells that accumulated in condition #1 over condition #2 represented the metabolic difference regarding the presence of oxygen in the medium. These DAPs corresponded to central nitrogen metabolism, oxidative metabolism and biosynthesis of ATP, amino acid and purine nucleotides (Fig. 3A; Table 1). Among these DAPs it could be highlighted three proteins of the central sugar metabolism, glucokinase (Glk), triophosphate isomerase (Tpi) and pyruvate dehydrogenase component E1 beta subunit (Pdb1). Several DAPs identified as subunits of ATP synthase complex accumulated in both condition #1 (Table 1) and condition #2 (Table 2), confirming the nitrate increases the oxidative metabolism independent of oxygen. Besides, the ATP synthase of the F1 subunit epsilon complex (Atp15) exclusively accumulated in condition #1, confirming the high demand for ATP imposed by nitrate metabolism (de Barros Pita et al. 2013; Galafassi et al. 2013; Peña-Moreno et al. 2019). The key protein in central nitrogen metabolism, glutamate dehydrogenase (Gdh1), was overproduced in condition #1, while one spot corresponding to glutamine synthetase (Gln1) was exclusively found in this condition (Table 1). Two DAPs involved in the amino acid biosynthesis identified as Delta-1-pyrroline-5-carboxylase reductase (Pro3) and $NADP^+$-dependent mitochondrial isocitrate dehydrogenase (Idh1p) were detected in this condition. Additionally, the adenilosuccinate liase (Ade13) and Arf guanyl-nucleotide exchange factor (Syt1) involved in the purine synthesis were exclusively found in condition #1, while the protein Asparaginyl-tRNA synthetase (Ded81) involved in protein translation was found in condition #1 and 2. However, one higher weight isoform of Ded81p was found in condition #1 that could represent the highest phosphorylation level, while three lower weight isoforms were found in condition #2 which may represent less phosphorylated forms of the protein in the five phosphorylation sites identified.


A.
Regulation of cellular component organization
Posttranscriptional regulation of gene expression
Regulation of chromatic silencing
Negative regulation of transcription by transcription...
Alpha-amino acid biosynthetic process
ADP process
Purine-containing compound metabolic process
Glutamine family amino acid biosynthetic process
Regulation of nitrogen compound metabolic process
DNA repair
Regulation of transcription DNA-templated
Celullar response to stress
Response to oxidative stress
Nitric oxide metabolic process
Possitive regulation of catalitic activity
Response to toxic substance
Reactive oxygen species metabolic process
Transport
Glycolitic process
ATP metabolic process
Ribonucleotide metabolic process
0
3
6
9
N° of genes Matches

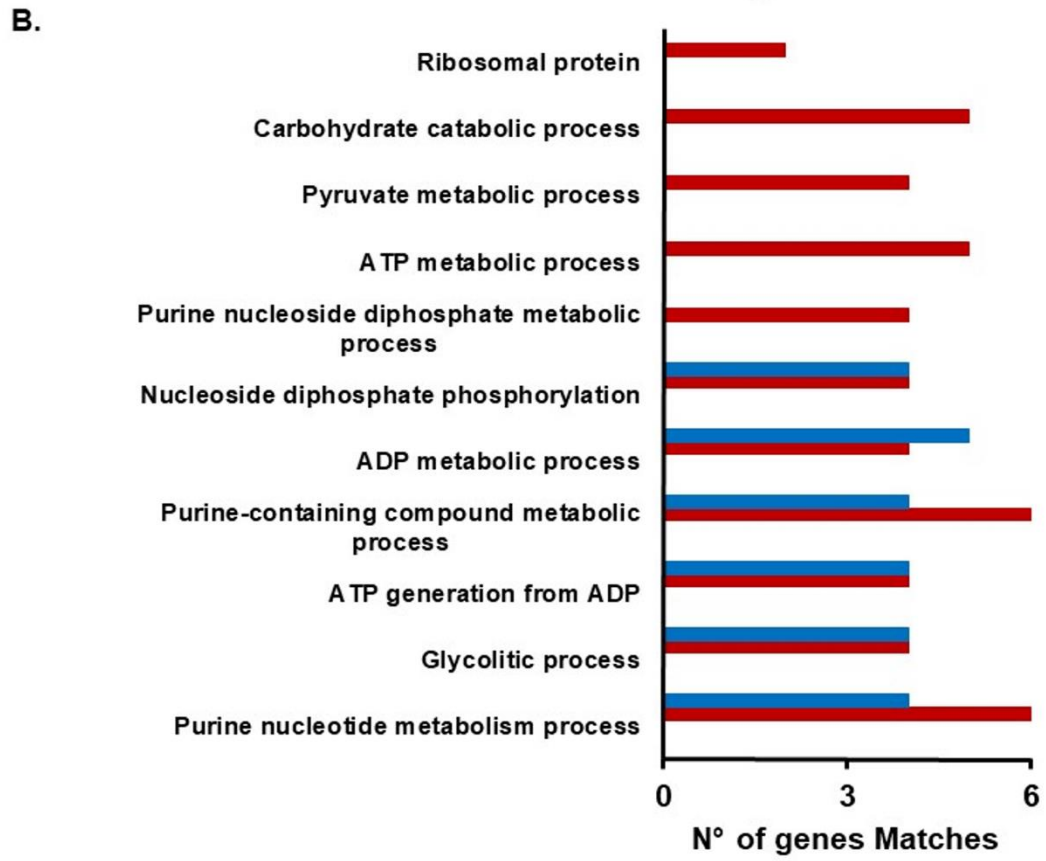

B.
Ribosomal protein
Carbohydrate catabolic process
Pyruvate metabolic process
ATP metabolic process
Purine nucleoside diphosphate metabolic process
Nucleoside diphosphate phosphorylation
ADP metabolic process
Purine-containing compound metabolic process
ATP generation from ADP
Glycolitic process
Purine nucleotide metabolism process
0
3
6
N° of genes Matches

Figure 3: Gene ontology analysis of biologicals processes of differentially accumulated proteins in the proteome of *Dekkera bruxellensis* GDB248 cells in the comparison [A] (Panel A) and the comparison [B] (Panel B). Red bars represent nitrate anaerobiosis, green bars nitrate aerobiosis, blue bars ammonium anaerobiose

In addition, proteins involved in the regulatory processes of gene expression were identified (Fig. 3A). The polyadenylate-binding protein 2 (Pab1) that is part of the translation initiation was exclusively accumulated in anaerobic nitrate, while the pre-mRNA SLT11 factor-splicing (Ecm2) involved in post-transcription modification accumulated more in aerobiosis. Transcription initiation factor IIb (Cti6) was present as different high and low weighted isoforms in anaerobiosis and aerobiosis, respectively. At last, two proteins involved in cytoskeleton organization and cell progression were exclusively observed in condition #1, such as a Rho GTPase activating protein (Bem2) and an amphiphysin-like lipid raft protein (Rvs161).

4.4.3 Oxidative stress is promoted in aerobic nitrate growth

Proteins of *D. bruxellensis* cells that accumulated in condition #2 over condition #1 corresponded were almost exclusively involved in oxidative stress responses (Fig. 3A), such as manganese-dependent superoxide dismutase (Sod2), thioredoxin peroxidase (Tsa2) and flavohaemoprotein (Yhp1) with nitric oxide oxidoreductase activity (Table 2). Proteins involved in nitric oxide metabolism, as well as in cellular response to stress, were detected in this condition (Table 2). This indicates that cells cultivated in condition #2 are submitted to intense oxidative stress, which might limit cell growth as reported above.

4.4.3 Glycolytic flux is enhanced in nitrate-based medium

In comparison [B], peptides of *D. bruxellensis* cells accumulated in condition #1 over condition #3 represented the metabolic difference regarding the type of N source in the medium. These DAPs corresponded again to enzymes from the glycolytic

pathway such as glucokinase (Glk), phosphoglycerate-mutase (Gpm1), triose phosphate isomerase (Tpi1) and pyruvate dehydrogenase component E1 beta subunit (Pdb1), an indicative of intense sugar metabolism in anaerobiosis (Fig. 3B). Besides, a glycerol kinase (Gut1), involved in the re-assimilation of the glycerol produced in anaerobiosis, was found exclusively in condition #1. Proteins of the oxidative metabolism appeared more accumulated in this condition (Table 3). GTP cyclohydrolase II (Rib1) involved in the formation of folate (tethahydrofolate -THF - in its active form) from GTP was exclusive of condition #1, while the mitochondrial tetrahydrofolate synthetase (Mis1) was present in condition #1 and #3 in different isoforms. Ribosomal proteins S2 (Rps2) and S3 (Rps3) of the 40s ribosome subunit were found only in condition #1. Paradoxically to the high growth rate observed for condition #1, a DAP spot identified as the negative growth regulator Ngr1p was more accumulated in nitrate than in ammonium (Table 1). As discussed later in this paper, the overproduction of Ngr1p might be part of the remodelling program of the yeast cells to increase the levels of fermentation-based cell growth in the absence of oxygen and in the presence of nitrate.

4.4.4 TORC1-dependent regulation of anaerobic growth in ammonium

Peptides of *D. bruxellensis* that accumulated in condition #3 compared to condition #1 corresponded to proteins that respond to the complex 1 TOR signalling regulatory mechanism (TORC1), which adjusts the cell growth to the availability of a primary nitrogen source, such as ammonium. The S6-like serine/threonine kinase Sch9, involved in regulation of ribosome biogenesis and translation initiation, was more produced in condition #3, while the type 2A-related serine/threonine phosphatase activator Sit4 was exclusive of this condition (Table 4). The comparison of GO metabolic processes in this second comparison revealed that the six most representative biological processes in anaerobic ammonium are also common in anaerobiosis nitrate (Fig. 3B). However, the metabolism of pyruvate seemed to be strongly associated with anaerobic nitrate. Therefore, when focus was given to interactions among the largest number of significantly responsive proteins, two prominent groups could be found: proteins that respond to signalling by TOR, and

proteins that help in the cell cycle, which respond to the presence of ammonium in anaerobiosis. It suggests that when nitrate is the sole N source in the medium, TOR-dependent nitrogen catabolite repression (NCR) regulatory mechanism is less relevant for cell growth.

4.4.5 Gene expression induced by nitrate

Genes that encode some of the identified proteins in addition to others studied in previous works of gene expression in the condition of nitrate and ammonium, were chosen to evaluate relevant metabolic pathways in our results and to validate the data of the proteomic profiles. In comparison [A] we detected upregulation of *PFK1* (phosphofructokinase in the glycolytic pathway) and *ATP1* (alpha subunit of the F1 sector of mitochondrial F1F0 ATP synthase), indicating the higher glycolytic flux associated with production of energy (Fig. 4A). In addition, the upregulation of *GDH1* (NADPH-dependent glutamate dehydrogenase) confirmed that glutamate biosynthesis was boosted to provided amino acids for biomass formation (Fig. 4A). Moreover, upregulation of *FKS1* (catalytic unit of beta glucan synthase) was detected in anaerobic condition (Fig. 4A) as indicative that the cells are in active growth state. On the other hand, the expression of *CTT1* (cytosolic catalase) was downregulated in condition #1 (Fig. 4A), meaning that it is more expressed in nitrate/aerobiosis. This protein plays an important role against oxidative damage by peroxide and responds to oxidative stress. This confirmed that aerobic growth in nitrate induces intense oxidative stress to *D. bruxellensis*.

Regarding comparison [B], relative gene expression data showed the overexpression of genes of the glycolytic pathway, oxidative/energy metabolism and glutamate biosynthesis in condition #3 over condition #1 (Fig. 4B), indicating that ammonium still more inductive of the central metabolism than nitrate in anaerobiosis. A slight upregulation of *ADH1* was detected in nitrate, even though its protein was more accumulated in ammonium (Fig. 4B). This indicated some sort of compensatory mechanism that ensures the high flux in the fermentation metabolism, since ethanol is equally produced in anaerobiosis both in nitrate and in ammonium (Peña-Moreno et al. 2019). In an overall analysis of gene expression, it can be seen that both high

growth rate and ethanol production in nitrate/anaerobiosis were ensured by the differential expression of genes of central carbon and nitrogen metabolisms, energy production and ethanol production (Fig. 4C).

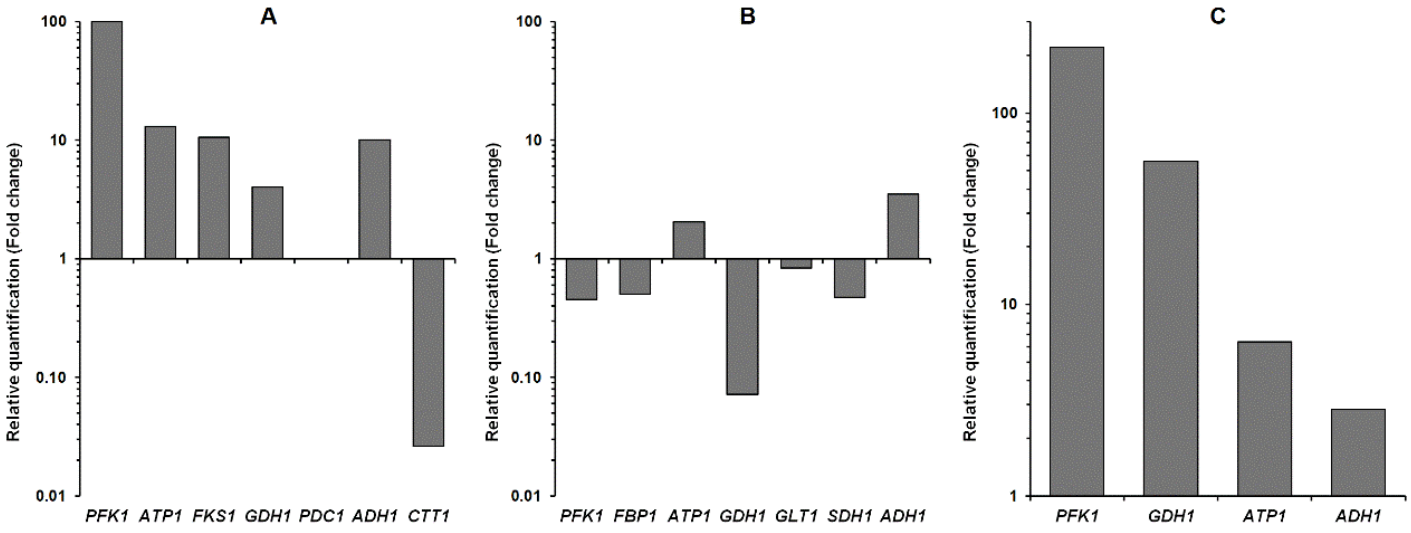


Figure 4: Relative expression of genes of glycolysis, cell progression and synthesis of ATP and glutamate in *D. bruxellensis* GDB248 in the comparison [A] between nitrate/anaerobiosis and nitrate/aerobiosis conditions (Panel A) and in the comparison [B] between nitrate/anaerobiosis and ammonium/anaerobiosis conditions (Panel B). Induction ratio of key genes was calculated by dividing their relative expression in the comparison [A] by their relative expression in the comparison [B] (Panel C).

4.5 DISCUSSION

4.6 Oxidative stress is the limiting factor for D. bruxellensis to grow in nitrate

The capacity of *D. bruxellensis* strain GDB 248 to assimilate nitrate as the sole N source has already been described (de Barros Pita et al. 2011; 2013; Cajueiro et al. 2017). Likewise, previous results have established that, in the absence of oxygen, nitrate becomes a more assimilable source for this yeast, capable of increasing both the rate of cell growth and ethanol yield (Peña-Moreno et al 2019). These findings contrasted with the previous reports showing the negative effect of nitrate on both physiological parameters when in aerobiosis (de Barros Pita et al. 2011; 2013; Cajueiro et al. 2017). The values of specific growth rate of 0.21 h^{-1} calculated in the present

work for cultivation in sucrose/ammonium was in the range of those reported in previous works, and higher than those calculated when its monosaccharides glucose and fructose were used as carbon source (Leite et al. 2013; Parente et al. 2017; Cajueiro et al. 2017; Peña-Moreno et al. 2019). Besides, the fact that *D. bruxellensis* grew in sucrose/nitrate at the same rate of sucrose/ammonium when oxygen is absent indicated that nitrate can be considered a primary N source for yeast metabolism, which complements the classifications established by (Parente et al. 2017) for different N sources in *D. bruxellensis* anaerobic metabolism. The lower growth rate observed when nitrate was aerobically assimilated has been always referred to the high demand for reduced cofactors required for its complete conversion to ammonium. It requires 4 mols of NAD(P)H for each mol of nitrate assimilated by the two consecutive redox reactions of nitrate reductase and nitrite reductase, plus one mol of NADPH to convert ammonium to glutamate by glutamate dehydrogenase (Siverio 2002). In principle, this high energy demand indicated that anaerobic growth would be much less supported, unless the limited number of reduced cofactors produced in the glycolytic pathway and its derivatives, the pentose-phosphate pathway and the PDH-bypass pathway, fulfils this requirement. In addition, high level of expression has been reported for nitrate assimilation pathway genes *YNR1, YNI1* and *YNT1* both in aerobic (de Barros Pita et al. 2011; 2013; Cajueiro et al. 2017) and in anaerobiosis (Peña-Moreno et al. 2019). These observations lead to the conclusion that the lower yeast growth rate in aerobiosis extensively reported in our previous works should be related neither to deficiency in the nitrate assimilation pathway nor to the high demand for reduced cofactors by the redox reactions in this pathway. Therefore, it could only be explained by the action of some indirect metabolic burden that makes difficult the cell cycle and progression in nitrate when oxygen is present.

Cell cultivation in aerobiosis using nitrate as N source (condition #2) resulted in high level of Tsa1p and Sod1p compared to growth in anaerobiosis. These proteins play role as antioxidant enzymes, indicating that *D. bruxellensis* cells should be under oxidative stress condition when nitrate is assimilated in the presence of oxygen. Apparently, there is a contradiction with the work of Barbosa-Neto et al (2014) in which a lower Tsa1p accumulation was reported in *D. bruxellensis* cultivated in nitrate medium. However, it has to be pointed out that the comparison made in that work was between aerobic growth in ammonia and in nitrate. In that case, the results showed

that the amount of Tsa1p was higher in ammonium medium. It has been reported that the metabolism of *D. bruxellensis* is preferentially oxidative in any combination of sugar and nitrogen sources (Leite et al. 2013; Parente et al. 2017; da Silva et al. 2019). Hence, we propose that oxidative stress does occur during aerobic growth of *D. bruxellensis* in ammonium, which seems to be much higher when nitrate is used. This assumption was confirmed by downregulation of *CTT1* gene encoding cytosolic catalase in comparison [A] (Fig. 3A), meaning that this gene is more expressed when nitrate is assimilated aerobically than anaerobically. Besides the direct damages to cell molecules, oxidative stress increases the demand for NADPH to detoxify the produced reactive oxygen species (ROS) (Steensels et al. 2015; Vigentini et al. 2013). In the detoxification pathway, the anti-oxidant defence molecules (mainly containing sulfhydryl groups) reduce the oxidants while becoming oxidised. These are reduced again by mean of NAPDH. In view of this, two non-exclusive possibilities arise: limited production of cytoplasmic sulfhydryl groups for detoxification and/or the competition between two metabolic pathways (nitrate assimilation and oxidative stress response) for the reducing power NADPH. Hence, the cell cannot overcome this oxidative problem and its growth is ultimately compromised. Two recent results come from a study of *D. bruxellensis* tolerance to oxidative stress support this assumption (manuscript in preparation). First, the level of intracellular thiols seemed indeed lower in *D. bruxellensis* biomass than in *S. cerevisiae.* Second, aerobic growth in nitrate can achieve the same level as ammonium when reduced glutathione (GSH) is added to the medium. This demand re-directs the central metabolism of *D. bruxellensis* to pyruvate be converted to acetaldehyde and this to acetate through the PDH-bypass of the $NADP^+$-dependent acetaldehyde dehydrogenase, which provides two mols of NADPH per mol of acetate produced. This explains why acetate is highly detected in the supernatants of nitrate-based media (Leite et al. 2013).

Another DAP that was exclusive for nitrate/aerobic condition #2 was Yhb1p, a flavohemoglobin-like protein that provides protection against nitric oxide (NO) through its C-terminal domain using NADPH as electron donor for detoxification reactions ;9 Gardner 2012; Mukai et al. 2001). In *S. cerevisiae,* the expression of *YHB1* gene is enhanced when the respiration chain is compromised, the cells are exposed to oxidative stress or when the oxidative stress response is deficient (Zhao et al. 1996). NO can be produced in plants as partially reduced intermediate of the conversion of

nitrate to ammonium by the action of nitrate/nitrite reductase (Corpas 2020). The fact that we had found high levels of flavohemoglobin Yhb1p would be indicative that NO can also be produced in the course of aerobic assimilation of nitrate by *D. bruxellensis*, damaging the cells and reducing growth rate. Proteins of oxidative stress have already been reported in *D. bruxellensis* in the presence of p-coumaric acid, inducing similar metabolic responses in order to obtain the necessary energy to support the energy demand caused by growth under this condition of stress (Carmona et al 2016).

Interactome analysis performed by combining DAPs from conditions #1 and #2, showing proteins connecting with at least two other proteins (Fig. 5A). In aerobiosis it can be seen the connection between Yhb1p, Tsa1p and a specific isoform of Tpi1p (Fig. 5A) that together with the overexpression of *CTT1* gene (Fig. 4) represents the cytoplasmic response to oxidative stress. This subnet is linked from one side to the cluster Hsp10 (mitochondrial chaperonin)/Mrp1/Mrpl10 (mitochondrial ribosomal proteins) that is indicative of mitochondrial stress. In the other side, this subnet is connected to Apn1p (AP endonuclease) and Rad17p (DNA damage checkpoint protein) that is indicative of oxidative stress caused by nitrate on nucleotide bases to form 8-oxoguanosine.

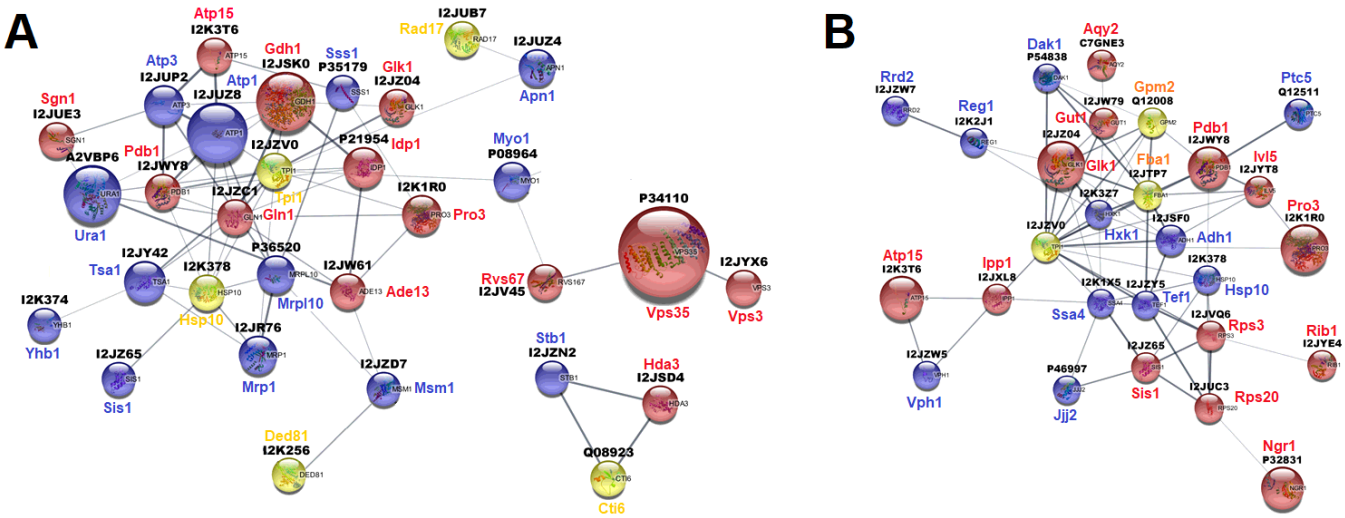


Figure 5: Interactome of *Dekkera bruxellensis* GDB248 based on the profile of differentially accumulated proteins (DAPs). (Panel A) Cells cultivated in synthetic defined medium containing sucrose and nitrate in presence or absence of oxygen. Nodes represent the proteins more or exclusively accumulated in anaerobiosis (red) or in aerobiosis (blue) or present in both conditions in different isoforms (yellow). The length of nodes represents the amount of DAPs, and the width of connecting lines represents the strength of connection. (Panel B) Cells cultivated in synthetic defined medium containing sucrose and nitrate or ammonium in absence of oxygen. Nodes represent the proteins more

or exclusively accumulated in nitrate (red) or in ammonium (blue) or present in both conditions in different isoforms (yellow). The length of nodes represents the amount of DAPs and the width of connecting lines represents the strength of connection.

Despite the higher expression of *ATP1* gene in nitrate/anaerobiosis (Fig. 4; Peña-Moreno et al. 2019), the protein Atp1 is more accumulated in nitrate/aerobiosis together with Atp3 (Table 2; Fig. 5A). Since both proteins compose the F1F0 ATP synthase complex, it makes clear and emphasizes the distinction between the metabolic signalling for energy production by expression of the genes in anaerobiosis and the functionality of the complex in aerobiosis.

4.5.2 Nitrate demands more ATP than ammonium for cell growth

The accumulation of proteins involved in glycolysis confirms that the assimilation of nitrate by *D. bruxellensis* in anaerobiosis increases the glycolytic flow to generate sufficient reduced equivalents to support the intense redox metabolism and to increase ATP production at substrate level that is independent of the mitochondrial respiratory chain (de Groot et al 2007; de Barros Pita et al. 2011; Galafassi et al 2013; Neto et al. 2013; Peña-Moreno et al 2019;). It explains the intensive production of ethanol, reaching yields that are similar to those found with ammonium [9]. In line with the low levels of F1F0-ATPase detected in *S. cerevisiae* cell extract from anaerobic cultures (de Groot et al. 2013), accumulation of these proteins was detected in protein extracts of *D. bruxellensis* in nitrate/aerobiosis (Fig. 5A). It corroborates previous studies reporting that assimilation of nitrate is a high-energy demanding process (de Barros Pita et al. 2013; Peña-Moreno et al. 2019). F1F0 ATP synthase complex is the major ATP producer of ATP, using the proton gradient force, while the epsilon subunit Atp15p of *S. cerevisiae* prevents the hydrolysis of that ATP produced (Pagadala et al. 2011) In the present work it is showed that the orthologous Atp15p is accumulated in *D. bruxellensis* cells cultivated in condition #1, which complements the upregulation of *ATP1* gene in the same condition (Peña-Moreno et al. 2019). These findings confirmed the re-orientation of the yeast metabolism for increasing ATP production in different ways, by overproducing

mitochondrial proteins, even in the absence of oxygen, and by increasing the glycolytic flux (Fig. 6).

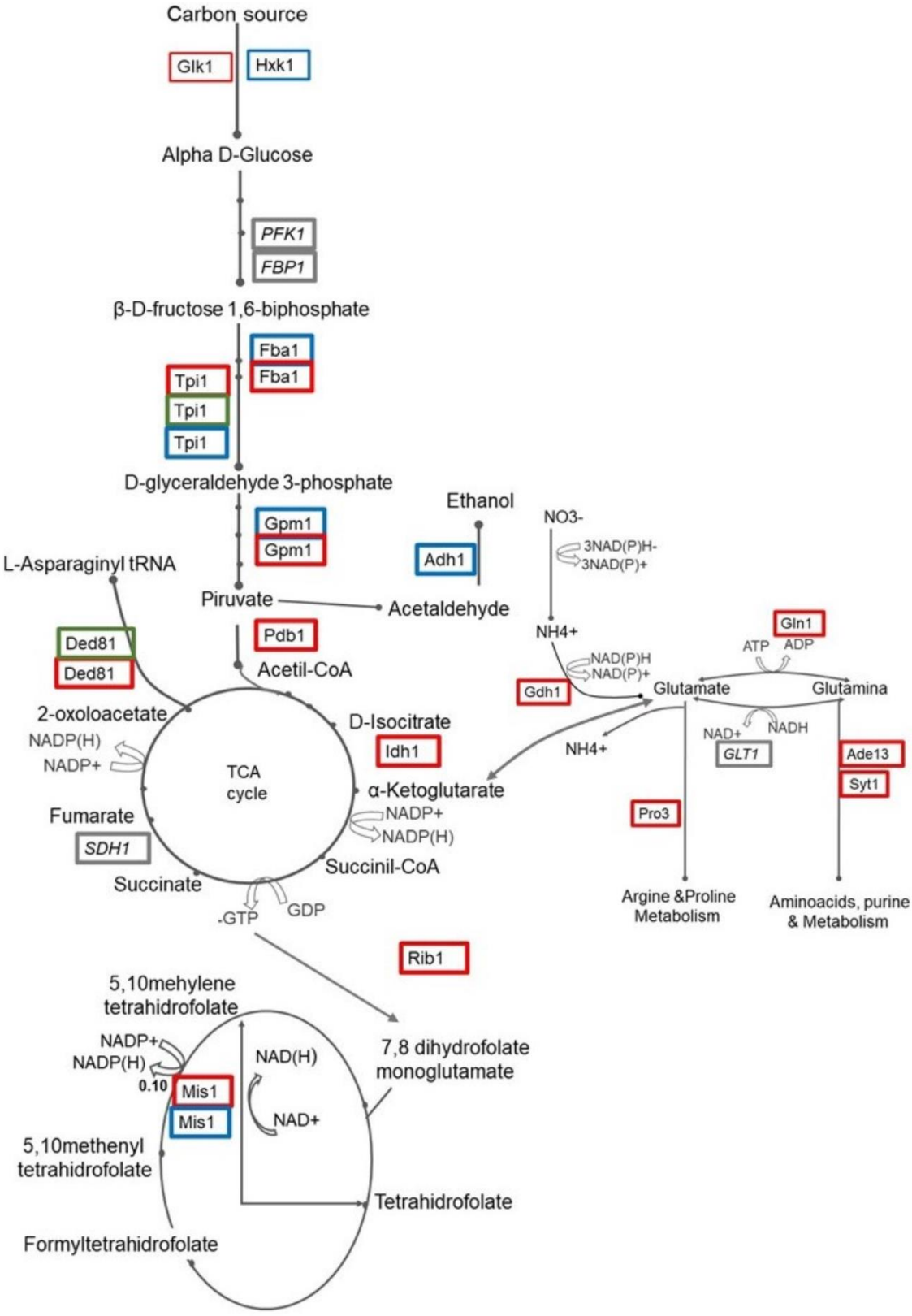


Figure 6: General scheme of the central metabolism of *Dekkera bruxellensis* connecting glycolysis, TCA cycle, nitrogen assimilation and mitochondrial tetrahydrofolate (THF) pathways, as putatively depicted from differential proteome analyses. The red boxes represent proteins found in nitrate anaerobiosis, green boxes proteins found in nitrate aerobiosis and blue boxes proteins found in ammonia anaerobiosis. The grey boxes represent some of the genes evaluated in expressed in the present work.

4.5.3 Protein synthesis rate is accelerated to allow growth in nitrate

Besides the increment in sugar assimilation and ATP formation, the assimilation of nitrate in absence of oxygen induced the accumulation of proteins involved in various steps of protein synthesis, from amino acids production to transcription/translation machinery. Verduyn et al (1990) calculated the energetics of *S. cerevisiae* cultivation in anaerobiosis and concluded that amino acids synthesis and polymerisation reactions account for half of ATP (energy) requirement during biomass formation. Hence, to support high growth rate in nitrate the cells have to increase the glycolytic flux to produce more ATP as stated above. It also explains why proteins involved in purine biosynthesis accumulated more in the condition #1 than in conditions #2 and #3.

In addition, DAPs related to cytoskeleton remodelling during cell cycle progression and division were also identified in condition #1, indicating that the machinery for growth is highly increased in this condition (Fig. 1). Hence, the low growth observed in aerobiosis seems only to be the consequence of the oxidative stress rather than the reduced capacity to assimilate nitrate into yeast biomass. It also explains the significant growing of *D. bruxellensis* populations in some ethanol fermentation processes, in which oxygen is absolutely absent and nitrate can be present together with ammonium (de Souza Liberal et al. 2007; Passoth et al 2007; de Barros Pita, et al. 2011; de Souza et al. 2012; Pereira et al. 2012). In these conditions, no oxidative stress is produced and the cell metabolism is accelerated.

High amounts of Gdh1p and Gln1p, responsible for the biosynthesis of glutamate and glutamine, respectively, were detected in the growth condition #1 of this work. The overproduction of Gdh1 protein (Table 1) fits very well with the upregulation *GDH1* gene in anaerobiosis when nitrate is present in the medium (Fig. 3; Peña-

Moreno et al 2019). This increment in gene expression and protein production were not observed in the presence of oxygen (Neto et al. 2014; Cajueiro et al. 2017). In *S. cerevisiae* the expression of *GDH1* and *GLN1* genes is subjected to regulation by Gln3p transcriptional activator that responds to TORC1 signalling pathway and to the availability of 2-oxoglutarate from the TCA cycle (Bertram et al. 2000; Conrad et al 2013). Contrary to its orthologous in *S. cerevisiae*, *GDH1* gene in *D. bruxellensis* was described as non-responsive to Nitrogen Catabolite Repression (NCR) and its constitutive expression profile together with the lack of regulatory motifs in its promotor region indicated that it is not subjected any regulatory mechanism under aerobic condition either in ammonium or nitrate as N source (Cajueiro et al. 2017). However, Parente et al (2017) showed upregulation of this gene when cells were cultivated in the presence of all 20 amino acids in aerobiosis or in glutamate in anaerobiosis. The fact that *GDH1* is upregulated by amino acids or nitrate in anaerobiosis, but not by nitrate in aerobiosis, lead to the hypothesis that it might be subjected to an alternative regulatory circuit, which is less dependent on the N source and more dependent on the oxygen. Both Gdh1p and Gln1p showed a central position in the interactome analysis, with the second presenting the highest number of connections (Fig. 5a). Both proteins connect with Idp1p, a mitochondrial isoform of isocitrate dehydrogenase responsible for 2-oxoglutarate formation in that compartment. Therefore, nitrate metabolism might provide enough intracellular ammonium that is assimilated 2-oxoglutarate by Gdh1 to glutamate, and glutamate is further aminated to glutamine by Gln1 (Fig. 6). This glutamine ultimately signals for TORC1 to trigger cells growth. We have proposed that glutamine is the internal sensor of nitrogen availability in *D. bruxellensis* (Cajueiro et al. 2017; Parente et al. 2017).

The expression of genes whose proteins control the formation of 2-oxoglutarate is subjected to the retrograde signalling pathway (RTG) that is also controlled by TORC1 (Conrad et al. 2013; Butow et al. 2004). RTG is defined as cellular responses to changes in the functional state of the mitochondria to compensate the lack of oxygen by inducing the expression of genes whose products function in the anaplerotic pathways, refilling the mitochondrial TCA cycle with oxaloacetate and acetyl-CoA (Butow et al. 2004). Under nitrate-based oxygen limitation condition we reported the overexpression of *IDH1* gene whose protein accounts for the direct production of 2-oxoglutarate from isocitrate in the TCA cycle (de Barros Pita et

al.2013). By providing enough 2-oxoglutarate, glutamine is produced as state above and the mechanisms for cell cycle progression and protein synthesis are triggered (Fig. 6). This relationship between RTG and TORC1 is observed in *S. cerevisiae* (Butow et al. 2004).

Additional interplay between TORC1 and RTG regulatory pathways came from the set of proteins involved in cytoskeleton structure. Rvs161p and Bem2p are related to cytoskeleton organization and cell morphology, which are important to cell cycle progression and connects TORC1 pathway to membrane trafficking in *S. cerevisiae* (Aronova et al. 2017). It can be seen from interactome analysis a subnet of proteins Rvs167, Vps35 and Vps3 involved in cytoskeleton and in retrograde and vacuolar transport. These results indicated that in the absence of oxygen, when the *D. bruxellensis* cells are exclusively fermenting, the presence of nitrate as sole N source induces high cell growth by the command of TOR and triggers RTG, through TORC1, for the production of metabolic intermediates of the TCA cycle.

The linkage between the metabolisms of sugar and nitrogen arose from the overproduction of the hexokinase Hxk1p in condition #3. This is the major kinase that converts glucose to glucose-6-phosphate in the glycolytic pathway. Nevertheless, its orthologous in *S. cerevisiae* have a second function of regulating the translocation of Gln3p transcription factor to the nucleus to promote the transcription of genes under the nitrogen catabolite repression (NCR) mechanism (Conrad et al. 2013; Rødkaer and Faergeman 2014). This might be similar in *D. bruxellensis* cultivated in ammonium. On the other hand, the use of nitrate as N source promoted the accumulation of the glucokinase Glk1p, which was exclusive in anaerobiosis relative to aerobiosis (Tables 1 and 2). In addition, two spots (isoforms) were identified in nitrate/anaerobiosis, one exclusive of this condition and a second shared with the proteome of ammonium/anaerobiosis (Tables 3 and 4). *GLK1* gene is subjected to Glucose Catabolite Repression (GCR) in *S. cerevisiae* (Rodriguez et al. 2001), meaning that it is not expressed when glucose is present at high level. These results showed that *GLK1* is being expressed in *D. bruxellensis* in ammonium despite the presence of glucose, in line to the lower strength of GCR proposed to this yeast (Leite et al. 2013). Therefore, in nitrate it seems that Glk1p has two modifications forms: one that accumulates at the same amount in nitrate, higher than in ammonium, and a second

that is exclusive for nitrate/anaerobiosis. Since, *GLK1* gene is expressed in non-fermentable carbon source, in oxidative metabolism (Rodriguez et al. 2001) the overproduction of Glk1p fits the assumption that nitrate triggers an oxidative metabolism in *D. bruxellensis*, as reported (de Barros Pita et al 2013; Neto et al. 2014; Peña-Moreno et al. 2019). It explains that overproduction in nitrate/anaerobiosis of the glycerol kinase Gut1p (Table 3), an enzyme involved in the reabsorption of glycerol under oxidative metabolism.

It is noteworthy that Ubc6p, the ATP-dependent ubiquitin sputtering enzyme E2 protein, was more accumulated in condition #1 (Table 1). This protein performs the second step in the ubiquitination reaction that directs a protein for degradation through the proteasome required for protein turnover during intensive growth. Protein degradation in anaerobic growth has been reported for *S. cerevisiae* (Haugen et al. 2004; de Groot et al. 2007), but the biological function of this mechanism remains unclear to date. However, it might represent the accelerated rate of synthesis/turnover of the *D. bruxellensis* proteome in face of the accelerated growth induced in this condition. In addition, it was detected the accumulation of ribosomal proteins Rps2p and Rps3p in that condition. In *S. cerevisiae* these proteins take part of a complex that forms the small ribosomal subunit 40S and they accumulate in response to both PKA and TORC1 regulatory mechanisms, connecting cell growth to the availability of sugar and nitrogen in the medium (Conrad et al. 2013). It also pointed for the intensive protein synthesis allowing cells to accelerate their growth in nitrate.

4.5.4 GTP metabolism supplements the requirement for energy and reduced cofactor to support anaerobic growth

High growth rate is also a high nucleotide-demanding process and this feature explains the accumulation of DAPs involved in purine biosynthesis. In *S. cerevisiae,* the anaerobic production of nucleotide is subjected to, among others, TORC1-independent activity of Gnc4p transcription activator (Conrad et al. 2013; Kolkman et al. 2006). The data from comparison [B] led us to propose that such TORC1-independent nucleotide production also takes place in *D. bruxellensis* in anaerobic cultivation with nitrate.

In the re-organization of the yeast metabolism promoted by the presence of nitrate and absence of oxygen, it was proposed that the TCA cycle is enhanced not only by the increased sugar consumption via glycolysis but also by the refilling anaplerotic reactions, very much necessary for the synthesis of 2-oxoglutarate that is aminated to glutamate. Even in anaerobiosis, the functioning of TCA provides GTP generated by means of succinate dehydrogenase. It was proposed by [7] that reported the upregulation of TCA genes in nitrate. In addition to its role as energy-provider, GTP is important for the synthesis of folate (tetrahydrofolate, or THF, in biologically active form) in *S. cerevisiae* and THF can then be converted to one of the various 1C unit-carrying folate cofactors (Denis et al. 1998; Grawert et al. 2013). Hence, a connection can be established between TCA cycle and folate pathway (Fig. 6). In the course of these transformations from THF, a series of reactions catalysed by the mitochondrial C1-tetrahydrofolate synthase Mis1p produce NADPH and ATP (Bradshaw 2019). Hence, the mitochondrial synthesis of these two molecules seems very stimulated when *D. bruxellensis* cells are cultivated under anaerobic condition independent of the N source used (Tables 3 and 4; Fig. 6). As stated above, NAD(P)H is highly required during the two stages of the nitrate assimilation pathway (Leite et al. 2013; de Barros Pita et al. 2013). It has already been pointed out that the production of acetate in cultures of *D. bruxellensis* with nitrate in anaerobiosis provides NAD(P)H to assimilate this source of nitrogen through the PDH-bypass (Leite et al. 2013; de Barros Pita et al. 2013; Peña-Moreno et al. 2019). It can be speculated that induction of the THF pathway could also be the response of yeast cells for the high demand of NADPH in the course of nitrate assimilation and biomass formation.

Paradoxically to all these evidences for growth stimulation, the proteome of condition #1 showed accumulation of Ngr1p over the condition #2. In *S. cerevisiae*, *NGR1* (*RBP1*) gene is annotated as negative regulator of cell growth and its deletion stimulates intense glucose consumption even at limiting concentration of assimilable nitrogen in the medium (Gardner et al. 2005). Besides, Ngr1p (Rbp1p) binds to poly-A tail of *POR1* transcript, decreasing its translation rate and increasing its degradation (Lee et al. 1998). *POR1* gene encodes a mitochondrial voltage-dependent anion channel (VDAC) responsible for membrane osmotic stability and permeability, and its deletion reduces growth on non-fermentable carbon source (Ronne et al. 1991). Hence, overproduction of Ngr1p would lead to depletion of Por1p, and then could

mimicking *POR1* deletion. In this case, the cell metabolism might be driven towards a fermentative/reductive metabolism rather than respiratory/oxidative metabolism, as it has been stated above for the increasing of glycolytic activity connected with an increase of ethanol production for *D. bruxellensis* cultivated in nitrate in the absence of oxygen.

4.5.5 Differences in the regulation by TORC1 on the assimilation of ammonium and nitrate

Contrary to what is observed in aerobiosis, *D. bruxellensis* cells grow at high growth rate in nitrate when oxygen is absent, in the same range of ammonium-based medium (Peña-Moreno et al. 2019). It means that nutrient sensor mechanisms are recognizing the presence of readily assimilable nitrogen in the medium. Therefore, and as already state above, nitrate can be considered a preferential N source for *D. bruxellensis* anaerobic growth. Control of cell growth is very dependent of sensing mechanisms that evaluate that availability of assimilable carbon (PKA regulatory pathway) and nitrogen (TOR regulatory mechanism) in the medium. Indeed, we observed several proteins involved in protein synthesis and anabolic reactions accumulated in nitrate/anaerobiosis condition #1 that are subjected to control by TORC1 regulatory pathway as presented above. The accumulation of some of those proteins was observed in comparison [B] in ammonium over nitrate, meaning that ammonium strongly triggers TORC1 mechanism as it would be expected from the model regulatory pathway in *S. cerevisiae.* Nevertheless, the proteomic data revealed that the signalling through TORC1 mechanism seems to take different pathways regarding the N source. In ammonium it was observed the nine-times accumulation of Sch9p kinase and the exclusive production of Sit4p 2A-related serine-threonine phosphatase (Table 4). In *S. cerevisiae,* Sch9p is phosphorylated and activated by TORC1 in response to adequate nutrient supply and its activated form positively regulates ribosome biogenesis to promote high protein synthesis, adjusts cell size control to cell cycle and division and inhibits stress response that is incompatible with growth (Conrad et al. 2013; Haugen et al. 2004). In parallel, TORC1 phosphorylates and activates Sit4p that, in turn, mediate the de-phosphorylation and inactivation of

Gln3, which acts as the transcriptional activator of genes that encodes proteins for the assimilation of alternative nitrogen sources (Bertram et al. 2000; Butow et al. 2004). Therefore, yeast cells turn-on the assimilation of preferential N source (ammonium or glutamate) and turn-off the assimilation of non-preferential N sources (amino acids) (Fig. 7A). However, we demonstrated that NCR might be also alleviated in *D. bruxellensis* that allow, for example, nitrate be co-assimilated with ammonium (de Barros Pita et al. 2013; Parente et al. 2017; Cajueiro et al. 2017).

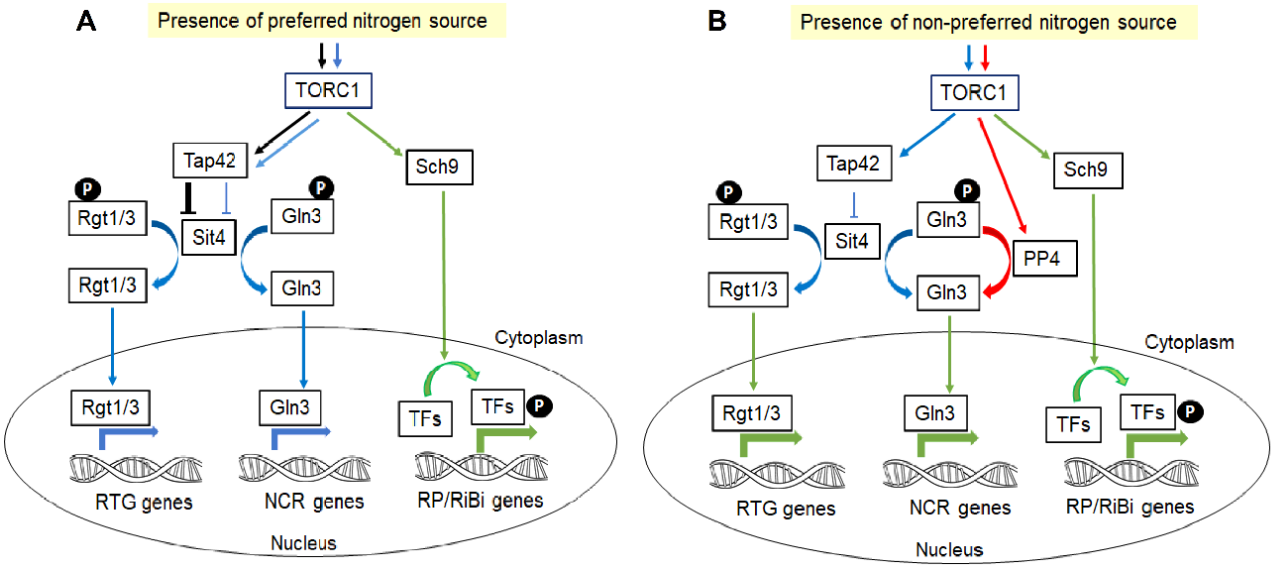


Figure 7: Regulatory network commanded by Target of Rapamycin Complex 1 (TORC1) in response to the availability of nitrogen in the environment. (Panel A) Comparative network for the presence of ammonium as nitrogen source between *Saccharomyces cerevisiae* (black symbols) and *Dekkera bruxellensis* (blue symbols) or for both yeasts (green symbols). (Panel B) Comparative network for *Dekkera bruxellensis* cultivated in ammonium (blue symbols) or nitrate (red symbols) or for both nitrogen sources (green symbols). Straight arrow represent activation and T-shaped lines represent inhibition. Regulatory proteins are represented in the black boxes. P in black circles represent phosphate residues and TFs mean transcription factor. Retrograde (RTG), nitrogen catabolite repression (NCR), ribosomal proteins (RP) and ribosomal biogenesis (RiBi) genes are the target of the regulatory networks. Bended arrows represent dephosphorylation and L-shaped arrows represent gene transcription.

It explains the expression of genes ou production of proteins that would be subjected to NCR (Fig. 7A). In the case of nitrate/anaerobiosis condition, it was observed accumulation of proteins involved in ribosome biogenesis, protein synthesis and cell morphology, which orthologous in *S. cerevisiae* are subjected to regulation by TORC1, despite the lack of accumulation of Sch9p and Sit4p that was observed in

ammonium/anaerobiosis. It was assumed that these proteins were equally produced at lower amounts in nitrate, independent of oxygen. Hence, an alternative mechanism of activation might be operational in nitrate as it has been proposed (Parente et al. 2017; Cajueiro et al. 2017). Another target of TORC1 in *S. cerevisiae* are the type 2A phosphatase complex, involved in cytoskeleton organization, bud morphogenesis and cell growth (Ronne et al. 1991) and the PP4 phosphatase complex that activates Gln3p to alleviate NCR (Bertram et al. 2000). In this case, nitrate assimilation in anaerobiosis could produce an intracellular amount of ammonium that could be enough to trigger Sch9-independent TORC1-mediated signalling for cell growth, but below the threshold for activation of NCR. It would open the possibility for the yeast cells to scavenge others N sources in the environment. Hence, in the case of nitrate/anaerobiosis condition, NCR might be even more alleviated by the cooperative action of Sit4 and PP4 phosphatases that release the expression of RTG and NCR genes (Fig. 7B), which result in an intense signalling for cell growth as reported (Fig 1; (Peña-Moreno et al. 2019).

4.6 CONCLUSION

The first relevant outcome of this work is the perception that nitrate is a readily assimilable source of nitrogen by *D. bruxellensis* in anaerobiosis, which impairs its up to date classification as poorly assimilated N source in presence of oxygen. This does not translate into aerobic cell growth due to the intense oxidative stress produced in the course of cell metabolism. Thus, the anaerobic growth in nitrate is ensured by the activation of the glycolytic pathway, production of ATP and NADPH and by the anaplerotic reactions that provide the intermediates required for cell growth, with balanced activation of the TORC1 pathway and NCR de-repression. These results represent a milestone regarding to the knowledge on the metabolism of nitrate that can be explored in future towards the use of *D. bruxellensis* as an attractive industrial yeast.

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Author contributions:

ICPM: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Supervision, Validation, Visualization, Writing-original draft, Writing - review & editing **DCP:** Conceptualization, Investigation, Methodology, Software, Writing- original draft **KM**: Investigation, Methodology, Software **Elton Nunes**: Investigation, Methodology, Software, Data curation **FCS:** Methodology, Software, Data curation **TCJr:** Resources, Funding acquisition, Formal analysis, Writing-review & editing **WBP**: Visualization, Formal analysis, Funding acquisition, Writing - review & editing **MAMJr:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Resources, Supervision.

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Table 1: Proteins of Dekkera bruxellensis more accumulated in the condition of anaerobic nitrate in comparison I. The DAPs annotations were obtained by mass spectrometry - MALDI ToF/ToF and identified by the methods PMF, MS/MS and Search Gui. *Common spots in both conditions

Spot	-O2:O2 Ratio	ANOVA	Protein identified	ID UniProt	*Gene Blast* in *S. cerevisiae*	Score	% coverage	MM Cal/Obs	PI Cal/Obs	Organism Reference
128*	5.1049	2.73E-02	Glutamate dehydrogenase	I2JSK0	*GDH1*	78	15	50395/ 47103	7.01/ 6.76	*B. bruxellensis AWRI1449*
129*	4.29	3.60E-02	Rad 17p	I2JUB7	*RAD17*	50	9	58962/ 46558	4.93/ 6.38	*B. bruxellensis AWRI1449*
136*	3.82	2.12E-04	Protein csf1	I2JUT8	*CSF1*	50	6	77519/ 41990	6.21/ 6.83	*B. bruxellensis AWRI1449*
148*	1.61	1.30E-03	Delta 1-pyrroline-5-carboxilase redutase	I2K1R0	*PRO3*	8.31E+04	1.17	76670/ 43362	/ 6.85	*B. bruxellensis AWRI1449*
170*	10.72	4.43E-02	Vacuolar protein sorting-associated protein 35	P34110	*VPS35*	1.83E+04	1.06	10907/ 72133	/ 5.57	*S. cerevisiae*
172*	5.2241	2.57 E-02	Asparaginyl-tRNA synthetase (Fragment)	I2K256	*DED81*	49	10	51332/ 71829	8.07/ 5.49	*B. bruxellensis AWRI1449*
191*	1.83	6.40E-03	Isocitrate dehydrogenase (NADP) mitochondrial	P21954	*IDH1*	1.92E+04	1.87	48160/ 51996	/ 6.21	*S. cerevisiae*
192*	Exclusive-ANERO	4.49E-05	K (+) H (+) antiporter	I2JU43	*KHA1*	9.30E+03	1.17	76370/ 34675	/ 6.3	*B. bruxellensis AWRI1449*
193	Exclusive-ANERO	1.08E-03	Transcription initiation factor iib	I2JR92	*SUA7*	5.08E+04	2.79	30660/ 39370	6.46/ 6.42	*B. bruxellensis AWRI1449*
197	Exclusive-ANERO	4.70E-02	Putative gtp exchange factor for arf	I2JSA2	*ARF1*	1.58E+04	0.8	12680/ 42026	/ 6.35	*B. bruxellensis AWRI1449*
198	Exclusive-ANERO	2.79E-04	Glutamine synthetase	I2JZC1	*GLN1*	93	20	41701/ 41339	6.46/ 6.42	*B. bruxellensis AWRI1449*
202	Exclusive-ANERO	1.69 E-02	Rad 17p	I2JUB7	*RAD17*	50	8	58962/ 44629	4.93/ 5.28	*B. bruxellensis AWRI1449*
203	Exclusive-ANERO	4.20E-02	Adenylosuccinate lyase	I2JW61	*ADE13*	50	9	55232/ 56546	6.01/ 6.31	*B. bruxellensis AWRI1449*
205	Exclusive-ANERO	6.52E-03	Phosphotransferase	I2JZ04	*GLK1*	74	26	53261/ 51791	5.8/ 6.39	*B. bruxellensis AWRI1449*
209*	3.92	4.06E-04	DNA damage checkpoint control protein RAD17	I2JUB7	*RAD17*	50		58962/ 59573	4.93/ 4.79	*B. bruxellensis AWRI1449*
212	Exclusive-ANERO	3.30E-02	Arf guany-nucleotide exchange factor	I2JSI3	*SYT1*	55	11	96145/ 59242	7.87/ 5.4	*B. bruxellensis AWRI1449*
215	Exclusive-ANERO	4.16E-02	Pyruvate dehydrogenase E1 component subunit beta	I2JWY8	*PDB1*	73	29	36443/ 60663	4.81/ 5.19	*B. bruxellensis AWRI1449*

235	Exclusive-ANERO	1.64E-05	DNA damage checkpoint control protein RAD17	I2JUB7	*RAD17*	50	8	58962/ 53403	5.65/ 4.93	*B. bruxellensis AWRI1449*
240	Exclusive-ANERO	7.40E-03	Polyadenylate-binding protein 2	I2JUE3	*PAB1*	7.66E+04	4.32	26120/ 59242	/ 5.51	*B. bruxellensis AWRI1449*
248	Exclusive-ANERO	9.43E-03	Ubiquitin-conjugating enzyme involved in ERAD	I2K419	*UBC6*	52	10	28842/ 24715	8.24/ 5.78	*B. bruxellensis AWRI1449*
249	Exclusive-ANERO	9.43E-03	Triosephosphate isomerase	I2JZV0	*TPI1*	51	25	26938/ 26821	6.21/ 5.81	*B. bruxellensis AWRI1449*
250	Exclusive-ANERO	9.44E-03	Para-hydroxybenzoate-mitochondrial	I2JYX6	*VPS3*	1.14E+04	9.3	9440/ 26896	/ 5.76	*B. bruxellensis AWRI1449*
269	Exclusive-ANERO	5.10E-03	Subunit of a possibly tetrameric trichostatin a-sensitive class ii histore deacetylase complex	I2JSD4	*HDA1*	56	11	44858/ 24776	7.22/ 7	*B. bruxellensis AWRI1449*
302	Exclusive-ANERO	1.60E-02	tRNA-specific adenosine deaminase subunit TAD2	TAD2_YEAST	*TAD2*	53	15	28633/ 21471	6.71/ 5.9	*S. cerevisiae*
318	Exclusive-ANERO	4.45E-06	Actin-associated subunit of a complex (Rvs161p-rvs167p)	I2JV45	*RVS161*	50		66102/ 37461	8.69/ 5.37	*B. bruxellensis AWRI1449*
323	Exclusive-ANERO	1.27E-04	Mitochondrial atp synthase epsilon chain domain-containing protein	I2K3T6	*ATP15*	51		7707/ 40397	9.4/ 5.15	*B. bruxellensis AWRI1449*
324	Exclusive-ANERO	1.21E-05	Mitochondrial atp synthase epsilon chain domain-containing protein	I2K3T6	*ATP15*	52		7707/ 39294	9.4/ 4.57	*B. bruxellensis AWRI1449*
329	Exclusive-ANERO	2.00E-02	Histone deacetylase complex subunit CTI6	CTI6_YEAST	*CTI6*	54		57417/ 42562	4.81/ 6.07	*S. cerevisiae*
393	Exclusive-ANERO	1.78E-05	Rho Gtpase-activating protein	I2K1N6	*BEM2*	52		96112/ 67881	7.71/ 6.13	*B. bruxellensis AWRI1449*
417	Exclusive-ANERO	1.70E-02	10 kDa heat shock mitochondrial	I2K378	*HSP10*	55	32	11184/ 65594	9.6/ 5.99/	*B. bruxellensis AWRI1449*

Table 2: Proteins of *Dekkera bruxellensis* more accumulated in the condition of aerobic nitrate in comparison I. The DAPs annotations were obtained by MALDI ToF/ToF mass spectrometry and identified by the PMF, MS/MS and Search Gui methods. *Common spots in both conditions

Spot	O2: -O2 Ratio	ANOVA	Protein identified	ID UniProt	*Gene Blast* in *S. cerevisiae*	Score	% coverage	MM Cal/Obs	PI Cal/Obs	Organism Reference
6*	2.57	1.6E-02	Asparaginyl-trna synthetase (Fragment)	I2K256	*DED81*	52	16	51332/ 18228	8.07/ 5.30	*Brettanomyces bruxellensis AWRI1449*
8*	4.30	3.5E-02	Pre-mRNA-splicing factor SLT11	P38241	*ECM2*	4.89E+04	2.2	36320/ 19615	/ 5.12	*Saccharomyces cerevisiae*
9*	7.29	3.1E-02	Uncharacterized protein	I2JXP4		56	19	25325/ 20884	6.09/ 5.93	*Brettanomyces bruxellensis AWRI1449*
23*	2.29	1.8E-04	Peroxiredoxin tsa1	I2JY42	*TSA1*	59	25	21743/ 23484	5.23/ 5.46	*Brettanomyces bruxellensis AWRI1449*
24*	2.96	3.0E-02	Peroxiredoxin tsa1	I2JY42	*TSA1*	65	29	21743/ 23907	5.23/ 5.25	*Brettanomyces bruxellensis AWRI1449*
30*	7.09	1.7E-02	Dihydroorotate dehydrogenase (quinone)	A2VBP6	*URA1*	60	18	48784/ 27861	9.24/ 5.23	*Brettanomyces bruxellensis AWRI1449*
33*	2.03	1.7E-02	Asparaginyl-trna synthetase	I2K256	*DED81*	52	12	51332/ 27932	8.07/ 5.86	*Brettanomyces bruxellensis AWRI1449*
37*	1.86	3.0E-02	Mitochondrial peroxiredoxin prx1	P08964	*MYO1*	77	19	25902/ 28219	5.9/ 6.03	*Saccharomyces cerevisiae*
46*	2.18	2.7E-02	Subunit of the nuclear pore complex	I2JW84	*NUP188*	2.27E+04	2.06	43800/ 31080	/ 4.91	*Brettanomyces bruxellensis AWRI1449*
57*	2.39	4.6E-02	Asparaginyl-trna synthetase (Fragment)	I2K256	*DED81*	50	11	51332/ 32793	8.07/ 4.27	*Brettanomyces bruxellensis AWRI1449*
60*	7.87	1.1E-02	ATP synthase subunit alpha	I2JUZ8	*ATP1*	52	9	59240/ 34455	9.15/ 5.75	*Brettanomyces bruxellensis AWRI1449*
64*	2.18	2.6E-02	Mn-superoxide dismutase	I2JR76	*SOD2*	51	12	18893/ 34721	9.21/ 5.29	*Brettanomyces bruxellensis AWRI1449*
69*	2.43	3.9E-02	Asparaginyl-trna synthetase (Fragment)	I2K256	*DED81*	52	12	51332/ 33635	8.07/ 5.36	*Brettanomyces bruxellensis AWRI1449*
75*	1.78	9.9E-03	Dna-(Apurinic or apyrimidinic site) lyase	I2JUZ4	*APN1*	53	38	15017/ 35668	9.76/ 5.91	*Brettanomyces bruxellensis AWRI1449*
90*	2.04	2.3E-02	54S ribosomal protein L10, mitochondrial	P36520	*MRPL10*	2.62E+04	2.8	40900/ 36647	/ 4.93	*Saccharomyces cerevisiae*
94*	2.48	1.4E-02	ATP synthase subunit gamma	I2JUP2	*ATP3*	52	19	31745/ 37384	/ 8.88	*Brettanomyces bruxellensis AWRI1449*
142*	2.29	2.4E-05	Protein ecm15	I2JWH0	*ECM15*	51		12246/ 42494	9.1/ 6.68	*Brettanomyces bruxellensis AWRI1449*
195	Exclusive-AERO	4.3E-04	Protein with role in regulation of MBF-specific transcription at Start	I2JZN2	*STB1*	8.87E+04	3.77	22550/ 36065	/ 5.55	*Brettanomyces bruxellensis AWRI1450*

259	Exclusive-AERO	2.1E-02	10kDa heat shock mitochondrial	I2K378	*HSP10*	61	32	11184/ 22033	9.6/ 4.15	*Brettanomyces bruxellensis AWRI1451*
260	Exclusive-AERO	3.5E-03	Triosephosphate isomerase	I2JZV0	*TPI1*	51	25	26938/ 28332	6.21/ 6.85	*Brettanomyces bruxellensis AWRI1452*
261	Exclusive-AERO	6.1E-03	Uncharacterized protein	I2JZD7		8.62E+04	3.88	23200/ 30149	/ 5.59	*Brettanomyces bruxellensis AWRI1453*
285*	2.02	1.4E-02	Bop3p (unknow protein involved in oxidative stress)	I2JX60	*BOP3*	52		18908/ 59574	8.74/ 5.99/	*Brettanomyces bruxellensis AWRI1449*
426	Exclusive-AERO	1.7E-03	Flavohemoprotein	I2K374	*YHB1*	52	16	37241/ 22893	6.68/ 5.91	*Brettanomyces bruxellensis AWRI1450*
430	Exclusive-AERO	7.7E-04	Ph domain protein	I2JYH7	*RGC1*	52	10	57387/ 24587	6.45/ 6.09	*Brettanomyces bruxellensis AWRI1449*
431	Exclusive-AERO	5.6E-05	DNA damage checkpoint control protein RAD17	I2JUB7	*RAD17*	54		58962/ 23222	4.93/ 5.65	*Brettanomyces bruxellensis AWRI1450*
439	Exclusive-AERO	5.5E-05	Proteasome component c11	I2K0G5	*PRE1*	9.56E+04	4.32	20920/ 30903	/ 6.25	*Brettanomyces bruxellensis AWRI1451*
440	Exclusive-AERO	1.9E-02	Protein phosphatase regulatory (glycogenesis)	I2JSA8	*GLC8*	53	23	25373/ 34626	5.12/ 5.96	*Brettanomyces bruxellensis AWRI1449*
442	Exclusive-AERO	5.9E-05	ative hsp40 family chaperone	I2JZ65	*HSP40*	54		38206/ 43549	6.21/ 6.06	*Brettanomyces bruxellensis AWRI1449*
445	Exclusive-AERO	7.05E-05	Protein transport protein SSS1 (transporter)	SC61G_YEAST	*SSS1*	53		8995/ 52267	9.48/ 5.02	*Saccharomyces cerevisiae*
455	Exclusive-AERO	2.9E-02	Gtpase-activating protein	I2K1N6	*IRA1*	54		96112/ 36326	7.71/ 6.05	*Saccharomyces cerevisiae*
463	Exclusive-AERO	3.4E-03	Zinc finger protein ypr022c	I2K0D6	*SDD4*	53	9	61838/ 9422	8.81/ 5.05	*Brettanomyces bruxellensis AWRI1449*
467	Exclusive-AERO	4.1E-04	Histone deacetylase complex subunit CTI6	CTI6_YEAST	*CTI6*	59		57417/ 16208	4.81/ 7.00	*Saccharomyces cerevisiae*

Table 3: Proteins of *Dekkera bruxellensis* more accumulated in the condition of anaerobic nitrate in comparison II. The DAPs annotations were obtained by MALDI ToF/ToF mass spectrometry and identified by the PMF, MS/MS and Search Gui methods. *Common spots in both conditions

Spot	NO3:NH4 Ratio	ANOVA	Protein identified	ID UniProt	*Gene Blast* in *S. cerevisiae*	Score	% coverage	MM Cal/Obs	PI Obs/Cal	Organism Reference
7*	4.13	5.5E-03	Mitochondrial atp synthase epsilon chain domain-containing protein	I2K3T6	*ATP15*	51		7707/ 39504	9.4/ 4.53	*Brettanomyces bruxellensis AWRI1449*
24 *	2.56	3.7E-03	Suppressor of DNA polymerase and splicing mutations	I2JZ59	*PSP2*	4.56E+04	2.18	72300/ 24352	/ 5.85	*Brettanomyces bruxellensis AWRI1499*
64*	3.84	2.5E-02	Negative growth regulatory protein	P32831	*NGR1*	2.67E+04	1.19	74980/ 36407	/ 6.27	*Saccharomyces cerevisiae*
49*	1.72	5.8E-03	C-1 tetrahydrofolate mitochondrial	I2K3P9	*MIS1*	50	6	10643/ 30964	9.97/ 6.47	*Brettanomyces bruxellensis AWRI1449*
67*	1.85	3.7E-02	Cbs and pb1 domain-containing protein	I2JUJ0	*PEP7*	50	8	50931/ 35718	8.99/ 6.60	*Brettanomyces bruxellensis AWRI1499*
77*	2.28	1.3E-04	Pyruvate dehydrogenase E1 component subunit beta	I2JWY8	*PDB1*	68		36443/ 40307	4.81/ 4.95	*Brettanomyces bruxellensis AWRI1449*
88*	3.84	2.50E-02	Uncharacterized protein	I2JUV4		49	14	28415/ 36226	5.12/ 6.33	*Brettanomyces bruxellensis AWRI1499*
109*	3.69	4.9E-02	Pyruvate dehydrogenase E1 component subunit beta	I2JWY8	*PDB1*	88	26	36443/ 39621	4.81/ 4.99	*Brettanomyces bruxellensis AWRI1499*
136*	1.91	1.2E-03	Phosphoglycerate mutase 2	PMG2_YEAST	*GPM1*	55		36336/ 70277	6.15/ 5.39	*Saccharomyces cerevisiae*
159*	4.97	4.8E-04	Delta 1-pyrroline-5-carboxilase reductase	I2K1R0	*PRO3*	8.31E+04	1.17	76670/ 44475	6.85/ 6.83	*Brettanomyces bruxellensis AWRI1449*
162*	1.66	9.6E-03	Protein ecm15	I2JWH0	*ECM15*	53		12246/ 31116	9.1/ 6.16	*Brettanomyces bruxellensis AWRI1499*
178*	4.08	1.1E-03	tRNA-specific adenosine deaminase subunit TAD2	TAD2_YEAST	*TAD2*	51	15	28633/ 21730	6.71/ 6.37	*Saccharomyces cerevisiae*
219*	4.90	3.0E-02	Phosphotransferase	I2JZ04	*GLK1*	135	31	53261/ 52339	5.8/ 6.26	*Brettanomyces bruxellensis AWRI1499*
229	Exclusive-NO3	1.1E-03	Triosephosphate isomerase	I2JZV0	*TPI1*	99	25	26938/ 23917	6.21/ 6.84	*Brettanomyces bruxellensis AWRI1499*
239	Exclusive-NO3	9.1E-03	40s ribosomal protein s3	I2JVQ6	*RPS3*	111	40	28400/ 33515/	7.77/ 6.62	*Brettanomyces bruxellensis AWRI1499*
241	Exclusive-NO3	1.2E-02	tRNA-specific adenosine deaminase subunit TAD2	TAD2_YEAST	*TAD2*	55	15	28633/ 22241	6.71/ 5.22	*Saccharomyces cerevisiae*
243	Exclusive-NO3	4.9E-04	Inorganic pyrophosphatase	I2JXL8	*IPP1*	54	17	32068/ 36056	5.54/ 5.61	*Brettanomyces bruxellensis AWRI1499*

244	Exclusive-NO3	5.2E-04	Triosephosphate isomerase	I2JZV0	*TPI1*	52	20	26938/ 25101	6.21/ 5.99	*Brettanomyces bruxellensis AWRI1449*
246	Exclusive-NO3	2.6E-04	quitin-conjugating enzyme e2g 2	I2JUN2	*UBC7*	51		19125/ 30105	6.9/ 5.01	*Brettanomyces bruxellensis AWRI1499*
249	Exclusive-NO3	3.4E-03	C-1 tetrahydrofolate mitochondrial	I2K3P9	*MIS1*	52	7	10643/ 34549	9.97/ 6.56	*Brettanomyces bruxellensis AWRI1449*
250	Exclusive-NO3	7.9E-03	Fructose-bisphosphate aldolase	I2JTP7	*FBA1*	70	22	39786/ 38789	6.08/ 6.49	*Brettanomyces bruxellensis AWRI1499*
251	Exclusive-NO3	4.6E-03	Fructose-bisphosphate aldolase	I2JTP7	*FBA1*	76	24	39786/ 38425	6.08/ 6.1	*Brettanomyces bruxellensis AWRI1499*
252	Exclusive-NO3	7.9E-05	40s ribosomal protein s20	I2JUC3	*RPS2*	50	32	12926/ 38065	9.48/ 6.24	*Brettanomyces bruxellensis AWRI1499*
253	Exclusive-NO3	1.5E-02	Acetohydroxyacid reductoisomerase	I2JYT8	*ILV5*	55	18	42113/ 40376	9.01/ 6.79	*Brettanomyces bruxellensis AWRI1499*
255	Exclusive-NO3	1.2E-04	Phosphoglycerate mutase 2	PMG2_YEAST	*GPM1*	49	8	57207/ 37986	5.89/ 6.6	*Brettanomyces bruxellensis AWRI1499*
256	Exclusive-NO3	2.90E-04	ative hsp40 family chaperone	I2JZ65	*HSP40*	50		38206/ 37798	9.48/ 5.22	*Brettanomyces bruxellensis AWRI1499*
256	Exclusive-NO3	3.8E-04	Uncharacterized protein	I2JQL7		7.21E+04	3.39	27730/ 41829	/ 6.62/	*Brettanomyces bruxellensis AWRI1499*
260	Exclusive-NO3	2.3E-03	Gtp cyclohydrolase ii	I2JYE4	*RIB1*	53	18	35610/ 42126	5.96/ 6.27	*Brettanomyces bruxellensis AWRI1499*
262	Exclusive-NO3	4.6E-07	C-1 tetrahydrofolate mitochondrial	I2K3P9	*MIS1*	55	7	10643/ 38784	9.97/ 5.22	*Brettanomyces bruxellensis AWRI1449*
269	Exclusive-NO3	5.9E-03	Phosphotransferase	I2JZ04	*GLK1*	74	26	53261/ 51791	5.8/ 6.39	*Brettanomyces bruxellensis AWRI1499*
271	Exclusive-NO3	1.7E-02	Aquaporin-like protein 2	C7GNE3	*AQY2*	52	69	17455/ 57259	4.86/ 6.04	*Saccharomyces cerevisiae*
273	Exclusive-NO3	1.2E-05	Protein ecm15	I2JWH0	*ECM15*	51		12246/ 39545	9.1/ 6.68	*Brettanomyces bruxellensis AWRI1499*
278	Exclusive-NO3	4.1E-02	Histone deacetylase complex subunit CTI6	CTI6_YEAST	*CIT6*	54		57417/ 42920	4.81/ 6.07	*Saccharomyces cerevisiae*
301	Exclusive-NO3	2.2E-02	Uncharacterized protein	I2JY35		50		22676/ 65545	9.97/ 6.3	*Brettanomyces bruxellensis AWRI1499*
313	Exclusive-NO3	2.7E-03	Phosphoglycerate mutase 2	PMG2_YEAST	*GPM1*	53		57207/ 53189	5.89/ 5.26	*Brettanomyces bruxellensis AWRI1499*
324	Exclusive-NO3	2.0E-03	Glycerol kinase	I2JW79	*GUT1*	53		28713/ 21696	9.39/ 5.59	*Brettanomyces bruxellensis AWRI1449*

Table 4: Proteins of *Dekkera bruxellensis* more accumulated in the condition of anaerobic ammonium in comparison II. The DAPs annotations were obtained by MALDI ToF/ToF mass spectrometry and identified by the PMF, MS/MS and Search Gui methods. *Common spots in both conditions.

Spot	NO3:NH4 Ratio	ANOVA	Protein identified	ID UniProt	*Gene Blast* in *S. cerevisiae*	Score	% coverage	MM Cal/Obs	PI Cal/Obs	Organism Reference
9*	2.08	9.6E-03	Phosphoglycerate mutase 2	PMG2_YEAST	*GPM1*	50	7	57207/ 37016	5.89/ 6.19	*Brettanomyces bruxellensis AWRI1499*
39*	5.38	1.8E-02	Triosephosphate isomerase	I2JZV0	*TPI1*	140	51	26938/ 25522	6.21/ 6.83	*Brettanomyces bruxellensis AWRI1499*
81*	1.84	1.2E-02	Amphiphysin-like lipid raft protein (response to osmotic stress)	I2JZ66	*RVS161*	52		30111/ 38110	7.62/ 6.99	*Brettanomyces bruxellensis AWRI1499*
89*	1.85	2.7E-02	Alcohol dehydrogenase	I2JSF0	*ADH1*	49	25	37665/ 38243	6.65/ 6.38	*Brettanomyces bruxellensis AWRI1499*
118*	2.24	5.6E-03	Very-long-chain 3 oxoacyl- CoA reductase	MKAR_YEAS1	*SCRG_02811*	58	14	39014/ 57522	9.47/ 5.42	*Saccharomyces cerevisiae*
141*	2.31	9.80E-03	Serine threonine protein kinase	I2JRJ0	*SCH9*	52		51520/ 74414	9.58/ 7.01	*Brettanomyces bruxellensis AWRI1499*
173*	1.57	3.7E-03	Pyruvate dehydrogenase (acetyl tranferase) phosphatase 1	pdp1_yeast	*PTC5*	42	4	63801/ 60134	8.7/ 5.83	*Saccharomyces cerevisiae*
198*	3.94	1.8E-04	C-1 tetrahydrofolate mitochondrial	I2K3P9	*MIS1*	56		10643/ 38310	9.97/ 6.74	*Brettanomyces bruxellensis AWRI1499*
239	Exclusive-NH4	2.7E-02	tRNA-specific adenosine deaminase subunit TAD2	TAD2_YEAST	*TAD2*	53	15	28633/ 21507	6.71/ 5.91	*Saccharomyces cerevisiae*
297	Exclusive-NH4	1.1E-03	Mediator of RNA polymerase II transcription subunit 10	I2JWI4	*NUT2*	58	44	16245/ 28449	5.36/ 6.84	*Brettanomyces bruxellensis AWRI1499*
299	Exclusive-NH4	3.4E-02	J protein JJ2	P46997	*JJJ2*	2.97E+04	1.37	57350/ 36575	/ 5.84	*Saccharomyces cerevisiae*
302	Exclusive-NH4	1.7E-06	Fructose-bisphosphate aldolase	I2JTP7	*FBA1*	56	19	39786/ 40583	6.08/ 6.36	*Brettanomyces bruxellensis AWRI1499*
303	Exclusive-NH4	3.9E-02	Dihydroxyacetone kinase 1	P54838	*DAK1*	3.22E+04	1.37	62170/ 47138	/ 5.99	*Saccharomyces cerevisiae*
308	Exclusive-NH4	1.9E-02	Phosphotransferase	I2K3Z7	*HXK1*	124	25	54447/ 60696	5.59/ 5.54	*Brettanomyces bruxellensis AWRI1499*
367	Exclusive-NH4	1.42 E -02	Uncharacterized protein	I2K0B2		50	21	21358/ 67236	8.91/ 5.71	*Brettanomyces bruxellensis AWRI1499*
317	Exclusive-NH4	8.31E-03	10kDa heat shock mitochondrial	I2K378	*HSP10*	50	32	11184/ 70756	9.6/ 6.76	*Brettanomyces bruxellensis AWRI1499*
318	Exclusive-NH4	5.90E-03	Dihydroxyacetone kinase 1	P54838	*DAK1*	3.22E+04	1.37	62170/ 71748	/ 5.69	*Saccharomyces cerevisiae*

374	Exclusive-NH4	2.5E-04	Heat shock protein 70	I2K1X5	*SSA4*	50	13	71860/ 81132	5.26/ 5.14	*Brettanomyces bruxellensis AWRI1499*
322	Exclusive-NH4	3.2E-02	Proteinase inhibitor i2b	I2JZW5	*VPH1*	8.92E+04	9.3	9440/ 90061	/ 5.43	*Brettanomyces bruxellensis AWRI1499*
336	Exclusive-NH4	1.3E-02	Elongation factor 1-alpha	I2JZY5	*TEF1*	50		50098/ 44643	9.38/ 5.85	*Brettanomyces bruxellensis AWRI1499*
340	Exclusive-NH4	3.8E-03	Serine/threonine-protein phosphatase 2A activator	I2JZW7	*SIT4*	54		36694/ 26118	6.17/ 5.37	*Brettanomyces bruxellensis AWRI1499*
341	Exclusive-NH4	1.7E-03	tRNA-specific adenosine deaminase subunit TAD2	TAD2_YEAST	*TAD2*	60	13	28633/ 68197	6.71/ 5.63	*Saccharomyces cerevisiae*
355	Exclusive-NH4	8.7E-04	Ubiquitin-conjugating enzyme E2 13	UBC13_YEAST	*UBC13*			17514/ 53618	5.31/ 6.13	*Saccharomyces cerevisiae*
357	Exclusive-NH4	6.1E-03	C-1 tetrahydrofolate mitochondrial	I2K3P9	*MIS1*	50	8	10643/ 55524	9.39/ 5.06	*Brettanomyces bruxellensis AWRI1499*
364	Exclusive-NH4	3.0E-05	Protein ecm15	I2JWH0	*ECM15*	53		12246/ 65259	9.1/ 5.46	*Brettanomyces bruxellensis AWRI1499*
371	Exclusive-NH4	2.1E-02	RSC functions in transcriptional regulation and elongation	I2K1G5	*RSC58*	50	27	23415/ 68472	4.64/ 6.16	*Brettanomyces bruxellensis AWRI1499*
376	Exclusive-NH4	7.6E-05	tRNA-specific adenosine deaminase subunit TAD2	TAD2_YEAST	*TAD2*	57	15	28633/ 82369	6.71/ 6.5	*Saccharomyces cerevisiae*
382	Exclusive-NH4	1.2E-02	Hydantoinase (enzymes for the production of optically pure D- and L-amino acids)	I2JYV2		52	18	23438/ 32393	5.15	*Brettanomyces bruxellensis AWRI1499*
384	Exclusive-NH4	1.3E-02	Uncharacterized protein	I2K2J1		52	18	32885/ 59377	4.72	*Brettanomyces bruxellensis AWRI1499*

5 DISCUSSÃO GERAL

Vários trabalhos anteriores apresentaram dados sobre a fisiologiaa e o padrão de expressão gênica em *D. bruxellensis* em condições de crescimento aeróbio ou de fermentação em alta concentração de açúcar e alta densidade celular (Leite et al., 2013; Pereira et al, 2012, 2014; De Barros Pita et al., 2013a; Reis et al., 2014; Reis et al., 2016). A partir daí foi possível mostrar que a assimilação de nitrato pode ser considerada uma vantagem adaptativa desta levedura sobre *S. cerevisiae* (De Barros Pita et al., 2011), embora sua utilização na presença de oxigênio resulte em menor crescimento celular, menor produção de biomassa e menor rendimento em etanol (De Barros Pita et al., 2013b; Cajueiro et al., 2017). Isto fez com que o nitrato sempre fosse considerado uma fonte não-preferencial de nitrogênio. Os dados de expressão gênica (De Barros Pita et al., 2013a, 2013b; Cajueiro et al., 2017) e de proteômica (Barbona-Neto et a., 2017) apontavam para a indução do metabolismo oxidativo, com indução dos processos de respiração, ciclo de Krebs e síntese de ATP ao invez do metabolismo fermentativo. Apenas recentemente foram analisados os dados fisiológicos e de expressão gênica desta levedura em crescimento em plena ausência de oxigênio (Parente et al., 2018), revelando aspectos importante sobre os mecanismos regulatórios da utilização de diferentes fontes de nitrogênio. No entanto, ainda faltava se estudar especificamente os efeitos do oxigênio na assimilação do nitrato, um aspecto fundamental para a concepção desta levedura como agente de processos biotecnológicos industriais que utiliza fontes bartas e acessíveis de carbono e nitrogênio para seu metabolismo. Este foi então o objetivo do presente trabalho.

Inicialmente, nosso primeiro objetivo foi avaliar a capacidade fermentativa desta levedura tanto em ensaios fermentativos quanto cinéticas de crescimento em condições de anaerobiose plena, uma característica importante na produção de etanol biocombustível. Embora já tenham sido reportados trabalhos anteriores em que as células de *D. bruxellensis* foram cultivadas anaerobicamente (Galafassi et al 2013; Parente et al 2017) ambos estudos foram suplementados com aminoácidos, fontes de nitrogênio encontradas em maior abundância em processos fermentativos de cerveja ou vinho.

Nossos resultados em fermentação confirmaram o padrão de *Crabtree* positivo de curto prazo para *D. bruxellensis* GDB 248 (Leite et al 2013), que conseguiu produzir etanol imediatamente após as células passarem do metabolismo respiratório para o metabolismo fermentativo na presença de glicose, como observado no Capítulo 1 desta tese. De igual forma, conseguimos observar que o rendimento de etanol melhorava quando os ensaios de fermentação eram colocados em meios contendo nitrato de forma exclusiva ou combinado com amônio, como acontece em outros estudos usando nitrato (de Barros Pita et al 2013a). A partir daqui vemos que o estado de anaerobiose permitiu às células de *D. bruxellensis* atingir melhor rendimento na produção de etanol, sendo o produto final em meios com amônio como acontece no trabalho de Barros Pita et al (2013) ou em contrapartida com nitrato presente nos meios que aumentou o rendimento de acetato, porém sem afetar em grande proporção e rendimento de etanol (Peña-Moreno et al 2019), atribuído a uma diminuição do efeito Custer, como relatado anteriormente (de Barros Pita et al 2011; Galafassi et al 2013). De igual forma, os resultados da cinética de crescimento de *D. bruxellensis GDB 248* em anaerobiose testando três condições, amônio, nitrato ou uma mistura dessas fontes de nitrogênio, mostraram que os crescimentos em nitrato

foram pelo menos duas vezes maiores do que para a aerobiose (de Barros Pita et al 2013a; Cajueiro et al 2017) e que as paradas temporárias achadas nas curvas de crescimento com meios contendo nitrato, poderiam representar uma reorientação metabólica das células induzida pela assimilação de nitrato e sua conversão intracelular em amônio observada apenas na ausência de oxigênio.

Resultados de sobre expressão de genes *PDC1*, *ADH1* e do metabolismo energético *ATP1* mostradas no Capítulo 1 e de igual forma o acúmulo de proteínas envolvidas na glicólise e na F1F0 ATPase (Atp1p, Atp15p, Atp3p) no Capítulo 2, especialmente quando o nitrato esteve presente, confirmam o aumento o fluxo glicolítico em *D. bruxellensis* na anaerobiose, principalmente para gerar equivalentes reduzidos suficientes e apoiar o metabolismo redox intenso e aumentar a produção de ATP no nível do substrato, independente da cadeia respiratória mitocondrial (Groot et al 2007; de Barros Pita et al 2011; Galafassi et al 2013; Barbosa-Neto et al 2014; Peña-Moreno et al 2019). Além de explicar a produção intensiva de etanol, atingindo rendimentos semelhantes aos encontrados com amônio (Peña-Moreno et al 2019). A superprodução de ATPases pode ser uma resposta regulatória do metabolismo da levedura para alta produção de ATP. No entanto, não pode ser descartada a possibilidade de que um receptor final alternativo de elétrons seja usado em algum tipo de respiração anaeróbica. Isso corrobora estudos anteriores que relatam que a assimilação de nitrato é um processo que exige elevada disponibilidade metabólica de poder redutor (De Barros Pita et al 2013a; Peña-Moreno et al 2019). O complexo F1F0 ATP sintase é o principal produtor de ATP, utilizando a força do gradiente de prótons, enquanto a subunidade epsilon Atp15p de *S. cerevisiae* impede a hidrólise do ATP produzido (Pagadala et al 2011). Estes resultados confirmaram a reorientação do metabolismo da levedura para aumentar a produção de ATP de diferentes

maneiras, superproduzindo proteínas mitocondriais, mesmo na ausência de oxigênio, e aumentando o fluxo glicolítico.

Resultados fisiológicos e de expressão gênica do Capítulo 1 permitiram direcionar nossas análises apontando o nitrato como uma fonte capaz de favorecer a produção de etanol quando o oxigênio não este presente no meio. Ensaios fermentativos como crescimentos anaeróbicos mostraram que o rendimento da biomassa foi menor em nitrato, podendo concluir que o nitrato é muito eficiente na condição de crescimento anaeróbico apontando mais para o metabolismo dissimilatório (formação de subprodutos), enquanto que a presença de amônio direciona o carbono glicolítico para um metabolismo mais assimilatório (formação de biomassa). Além disso, os resultados do Capítulo 2 apresentaram acúmulo diferencial de proteínas envolvidas em vários processos metabólicos e fisiológicos, desde a biossíntese de aminoácidos até amaquinaria de transcrição/tradução, mais induzidas quando o nitrato estava presente e em ausência de oxigênio. Isto já foi observado em *S. cerevisiae,* no qual há demanda energética para síntese de proteinas e as células precisam aumentar o fluxo glicolítico para produzir mais ATP (Verduyn et al 1990). Os resultados gerados em anaerobiose direcionaram o Capítulo 2 e, assim, entender quais são as restrições biológicas que prejudicam o crescimento de leveduras no nitrato quando o oxigênio está presente e como são regulados os mecanismos pós-transcricionais e mesmo pós-traducionais em *D. bruxellensis*.

Um dos resultados mais relevantes encontrado no segundo capítulo foi apontar o estresse oxidativo como o fator limitante para o crescimento de *D. bruxellensis* em nitrato, e não necessariamente a deficiência na via de assimilação de nitrato nem à alta demanda de cofatores reduzidos pelas reações redox nessa via

como sempre foi referida (de Barros Pita et al 2013a; Parente et al 2017). A assimilação de cada mol de nitrato exige 4 mols de NAD(P)H pelas duas reações redox consecutivas da nitrato redutase e da nitrito redutase, mais um mol de NADPH para converter amônio em glutamato pela glutamato desidrogenase (Gdh1) (Siverio 2002). Resultados no Capítulo 1 indicaram a produção de NA(P)H quando o acetato é gerado em anaerobiose (Peña-Moreno et al 2019), NADH e NADPH é produzida na via de pentose-fosfato (Barbosa-Neto et al 2014), e ocorre a supervia do folato (tetra-hidrofolato ou THF, na forma biologicamente ativa), que em nitrato-anaerobiose fornece NAP(H) (Bradshaw et al 2019; Grawert et al 2013), conforme comparação [B] do Capítulo 2. Isto indicaria que essa demanda por equivalentes redutores seria superada por esta levedura quando em anaerobiose. De fato, nos resultados fisiológicos do capítulo 2, *D. bruxellensis* cresceu em sacarose/nitrato na mesma taxa de sacarose/amônio quando o oxigênio está ausente indicaram que o nitrato pode ser considerado uma fonte primária de N para o metabolismo da levedura, o que atualiza as classificações estabelecidas por Parente et al (2018) para diferentes fontes de N no metabolismo de *D. bruxellensis*. Além disso, foi relatado alto nível de expressão dos genes da via de assimilação de nitrato *YNR1*, *YNI1* e *YNT1*, tanto em aeróbicos (De Barros Pita et al 2011, 2013; Cajueiro et al 2017) como em anaerobiose (Peña-Moreno et al 2019).

O elevado acúmulo des proteínas que atuam como enzimas antioxidantes Tsa1p e Sod1p foi observado quando as células cresceram em aerobiose em comparação com o crescimento em anaerobiose, contrastando com outros estudos nos quais havia baixa produção de Tsa1p cultivada em meio com nitrato comparado com amônio (Barbosa-Neto et al 2014). Entretanto, vários estudos mostraram que o metabolismo de *D. bruxellensis* é preferivelmente oxidativo em qualquer combinação

de fontes de açúcar e nitrogênio (De Barros Pita et al 2013; Leite et al., 2013; Da Silva et al 2018). De igual forma, a regulação negativa do gene *CTT1* que codifica a catalase citosólica envolvida contra o dano oxidativo por peroxido encontrado na comparação [B], significando que esse gene é mais expresso quando o nitrato é assimilado aerobicamente do que anaerobicamente. Outro resultado que reforça nossa hipótese foi o DAP Yhb1p, uma proteína semelhante à flavohemoglobina que fornece proteção contra óxido nítrico (NO), através de seu domínio C-terminal usando o NADPH como doador de elétrons para reações de desintoxicação (Mukai et al 2001) e que foi encontrado na condição de nitrato-anaerobiose na comparação [A]. Em *S. cerevisiae*, o gene *YHB1* é mais expresso quando a cadeia respiratória é comprometida, as células são expostas ao estresse oxidativo ou quando a resposta ao estresse oxidativo é deficiente (Zhao et al 1996). O NO pode ser produzido em plantas como intermediário parcialmente reduzido da conversão de nitrato em amônio pela ação da nitrato/nitrito redutase (Corpas e Palma 2018). Os altos níveis de flavohemoglobina Yhb1p seriam indicativo de que o NO também pode ser produzido durante a assimilação aeróbica de nitrato por *D. bruxellensis*, danificando as células e reduzindo a taxa de crescimento como também foram reportados em outros estudos utilizando ácido *p*-coumárico, fator de estresse celular (Carmona et al 2016).

Outros DAPs na comparação [B] permitiram entender como na reorganização do metabolismo da levedura principalmente pela presença do nitrato, o ciclo do TCA não era apenas estimulado pelo aumento do consumo de açúcar via glicólise, mas também pelas reações anapleróticas de reabastecimento necessárias para a síntese de 2-oxoglutarato que é aminado ao glutamato. Mesmo na anaerobiose, o funcionamento do TCA fornece GTP gerado por meio da succinato desidrogenase (de Barros Pita et al (2013). GTP além de forrnecer energia, é importante para a síntese

de folato e no curso dessas transformações a partir do THF, uma série de reações catalisadas pela C1-tetra-hidrofolato-sintase mitocondrial Mis1p produz NADPH e ATP (Grawert et al 2013) necessário durante os dois estágios da via de assimilação do nitrato (De Barros Pita et al 2013a; Galafassi et al 2013) como mencionado acima.

O controle do crescimento celular é dependente de vias regulatórias que respondem à disponibilidade de carbono assimilável (via reguladora da PKA) e nitrogênio (mecanismo regulador da TOR) no meio (Conrad et al 2014). O acúmulo de algumas proteinas sujeitas a controle pela via reguladora do TORC1 foram observadas em amônio na comparação [B], desencadeando fortemente o mecanismo TORC1, como foi descrito em *S. cerevisiae* (Conrad et al 2014). Os dados proteômicos indicam que a sinalização de TORC-1 parece seguir caminhos diferentes em relação à fonte de N. Em amônio, observou-se o acúmulo de Sch9p quinase que é fosforilada e ativada pelo TORC1 em resposta ao suprimento adequado de nutrientes e sua forma ativada regula positivamente a biogênese do ribossomo para promover alta síntese protéica, ajusta o controle do tamanho da célula ao ciclo e divisão celular e inibe a resposta ao estresse que é incompatível com o crescimento (Conrad et al 2014; Aronova et al 2007). Paralelamente, outra proteína, Sit4p fosfatase tipo 2A, é fosforilada por TORC1 que, por sua vez, medeia a defosforilação e a inativação do Gln3, que atua como ativador da transcrição de genes que codificam proteínas para a assimilação de fontes alternativas de nitrogênio (Bertram et al 2000; Rodkaer et al. 2014). Este comportamento já foi observado em *D. bruxellensis* cultivada em amônio, mas não em nitrato (De Barros Pita et al 2013; Cajueiro et al 2017; Parente et al 2018). No caso de nitrato/anaerobiose, foram acumuladas proteínas envolvidas na biogênese do ribossomo, síntese de proteínas e morfologia celular que estão sujeitos à regulação pelo TORC1, apesar da falta de acúmulo de Sch9p e Sit4p. Portanto, um mecanismo

alternativo de ativação pode estar operacional no nitrato, conforme proposto (Cajueiro et al 2017; Parente et al 2018). Nesse caso, a assimilação de nitrato na anaerobiose pode produzir amônio intracelular a uma concentração que aciona a sinalização mediada por TORC1 independente de Sch9 para o crescimento celular, enfraquecendo o NCR para abrir a possibilidade de células de levedura buscarem outras fontes de N no ambiente.

Desta forma, trabalhos futuros em outras plataformas ômicas e também em abordagens funcionais deverão complementar os resultados das análises já realizadas em nível transcricional e traducional, de modo a enriquecer a ideia central de que a capacidade de assimilar nitrato representa diretamente uma vantagem seletiva para as células de *D. bruxellensis* sobre *S. cerevisiae* no substrato de ambiente industrial (De Barros Pita et al 2011).

6 CONCLUSÃO GERAL

O presente trabalho mostrou que a levedura *Dekkera bruxellensis* pode utilizar nitrato da mesma forma que o amônio, como uma fonte primaria de nitrogênio, quando em cultivos na ausencia de oxigênio. Isso requer uma reorientação metabólica, resultante de rearranjos na regulação da expressão gênica, que envolve aumento do fluxo glicolítico e da produção de energia e cofatores e a atividade da via central de assimilação de nitrogênio e da capacidade de síntese proteica. Essas alterações metabólicas têm como consequência não apenas o aumento na velocidade de crescimento mais também na capacidade de produção de etanol, tornando *D.*

bruxellensis uma levedura fermentadora industrial. No entanto, as alterações metabólicas induzidas por nitrato em aerobiose levam a uma intensa produção de estresse oxidativo que perturba significativamente as funções moleculares de maneira a comprometer os processos biológicos das células. Esses resultados representam um marco no conhecimento do metabolismo do nitrato que pode ser explorado para uso futuro de *D. bruxellensis* como levedura industrial, não apenas para a produção de etanol como para a produção de produtos biotecnológicos de maior valor agregado em cultivos anaeróbios usando substratos simples e baratos como sacarose e nitrato.

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APENDICE A

Ensaios fisiológicos de *Dekkera bruxellensis* em duas fontes de nitrogênio sob efeito da Rapamicina atuando como agente inibidor do complexo TORC1

Resumo:

O presente trabalho foi pensado para complementar os nossos dados fisiológicos, de expressão gênica e de proteômica sobre regulação do mecanismo central de assimilação de fontes de nitrogênio pela levedura industrial *Dekkera bruxellensis,* comparando-se a eficiência de assimilação de amônio e de nitrato na presença ou ausência de rapamicina, um inibidor da via TOR que comanda a expressão de genes e atividades enzimáticas da assimilação de nitrogênio e controle do crescimento celular. O estágio internacional realizado pela aluna, ajudou a unir as especialidades de ambos os grupos de pesquisa em uma análise sistêmica mais ampla da transcriptômica (USACH) e proteômica (UFPE) que pode resultar na compreensão do potencial fermentativo de *D. bruxellensis* e do papel do nitrato com controle do fluxo metabólico central, que poderia ser mais explorado em atividades biotecnológicas para a formação de bioprodutos. Os resultados gerados mostram diferenças no perfil de crescimento da linhagem brasileira GDB248 (isolada de destilarias de etanol) e da linhagem chilena L2480 (isolada de vinícolas) em resposta a rapamicina. As análises transcriptômicas comparativas entre essas linhagens contrastantes vai permitir a identificação das diferenças metabólicas entre elas. Adicionalmente, o estágio permitiu a continuidade da parceria entre os dois grupos de pesquisa da UFPE e da USACH.

1. INTRODUÇÃO E JUSTIFICATIVA

Dekkera bruxellensis é uma levedura principalmente relacionada ao ambiente industrial, sendo encontrada nos processos fermentativos de produção de vinhos e cervejas. No Brasil, esta levedura é reconhecida como a principal contaminante nos processos industriais de produção de bioetanol ao competir com *Saccharomyces cerevisiae* pelos substratos encontrados no caldo de cana. Entretanto, o potencial de *D. bruxellensis DGB 248* já foi apresentado, uma vez consegue superar os valores de rendimento de etanol superando às vezes a população da levedura padrão (Peña-

Moreno et al 2019). Uma explicação para esta característica adaptativa em processos industriais tem relação com a assimilação de nutrientes. Neste sentido, o metabolismo de compostos nitrogenados é relevante em *D. Bruxellensis* isolada em processos de produccção de bioetanol uma vez apontada sua capacidade de assimilar nitrato como única fonte de nitrogênio (de Barros Pita et al 2011). *D. bruxellensis* pode, assim como *S. cerevisiae,* reconhecer a natureza e disponibilidade de compostos nitrogenados e consequentemente ajustar seus mecanismos transcricionais e biossintéticos. Quando o nitrogênio é limitado ou difícil de assimilar pelo custo metabólico que gera, as células crescem de forma lenta, reduzindo sua tradução ribossômica e alongando seu ciclo celular (Ljungdah Po & Daignan-Fornier B 2012; Molinet et al 2019). Enquanto que, quando há depleção de nitrogênio, o crescimento celular é parado. Por tanto, a capacidade sintética da taxa de cresciemento celular depende da quantidade e qualidade da fonte (Magasanik and Kaiser 2002). Neste contexto, as fontes de nitrogênio são classificadas como preferenciais e não preferenciais, porem essa classificação não é absoluta e seus efeitos repressivos podem variar significativamente entre origens de diferentes tipos de leveduras (Georis et al 2009a). Em *D. bruxellensis* por exemplo, já foi observado que a hieraquia na assimilação de fontes de nitrogênio depende entre outras coisas, da disponibilidade de oxigênio (Peña-Moreno et al 2019; Parente et al 2017). Em geral, a regulação do nitrogênio é um processo estritamente regulado por um mecanismo de controle transcricional conhecido como Repressão Catabólica do Nitrogênio (NCR). O NCR funciona principalmente para garantir que as células usem seletivamente fontes preferenciais de nitrogênio quando disponíveis e desreprime genes regulados por NCR em ausência de uma fonte preferencial de nitrogênio, evitando que as células eliminanem essas fontes alternativas (Magasanik & Kaiser, 2002; Georis et al 2009a). No cenário de *D. bruxellensis*, na assimilação de nitrato, considerada como uma fonte secundária, partcipam cinco genes que compõem a via de assimilação de nitrato (Piskur et al., 2012; de Barros Pita et al, 2013a,b). Para aproveitar aminoácidos e outros compostos nitrogenados, *D. bruxellensis* possui múltiplas permeases para facilitar o transporte desses compostos através da membrana plasmática *(*Ljungdah Po & Daignan-Fornier B 2012).

De outro lado, é conhecido que a sinalização por TOR, regula diretamente genes de NCR controlando a retenção citoplasmática do ativador e respressor GATA

Gln3 e Ure2 respectivamente que regulam a transcrição em resposta à baixas concentrações de carbono e nitrogênio e são membros principais da via regulatória do metabolismo do nitrogênio *(*Beck & Hall, 1999; Bertram et al, 2002*).* TOR (Target of Rapamycin) é uma proteína quinase que forma um complexo de regulação na assimilação de nutrientes celulares (Beck and Hall, 1999; Bertram et al, 2002*)*. Nas leveduras TOR (Target of Rapamycin) é uma proteína quinase codificada por dois genes, TOR1 e TOR2 (Yerlikaya et al 2015). As quinases TOR formam dois complexos distintos de multiproteínas funcionalmente e estruturalmente denominado complexo TOR 1 (TORC1) e complexo TOR 2 (TORC2) que de forma geral regulam a assimilação de nutrientes e crescimento celular (Beck and Hall, 1999; Bertram et al, 2002, Yerlikaya et al 2015*).* A atividade do TORC1 diminui após a falta de nitrogênio e aumenta com o aumento do nitrogênio (Molinet et al 2019). Também em *S. cerevisiae* o complexo TORC1 é afetado pelo tratamento de Rapamcina, um antifúngico que inibe sua função e consequentemente bloqueia o crescimento celular (Crespo and Hall 2002; Yerlikaya et al 2015). Rapamicina liga-se à proteína FKBP12, inativando TOR e desta forma reduz os níveis de fosforilação de Gln3, que está envolvido com seu direcionamento nuclear. Em aparente apoio deste modelo, a fosfatase regulada negativamenre por TORC1 Tap42-Sit4, influencia a fosforilação de Gln3, assim como acontece quando o nitrogênio está em níveis baixos no meio (Crespo and Hall 2002, Ljungdah Po & Daignan-Fornier B 2012).

Já foi apontado que *D. bruxellensis* cultivada em metionina sulfoximina (inibe a glutamine sintetase, liberando as células de NCR) em aerobiose, o nitrato libera o mecanismo NCR e desta forma confirma que os genes de assimilação desta fonte estão mais sujeitos a um mecanismo indutivo por nitrato do que a um mecanismo repressivo por amônio ou glutamato/glutamina (Cajueiro et al 2017; Parente et al 2017). De igual forma, a regulação de genes que respondem à NCR em cultivos de amônio, parece ser constitutiva. De forma oposta, foi proposto no Capítulo 2 desta tese que em anaeorbiose a assimilação de nitrato pode produzir uma quantidade intracelular de amônia suficiente para ativar a sinalização mediada por TORC1, independente de Sch9, para crescimento celular, mas abaixo do limiar para a ativação de NCR. Isso abriria a possibilidade de células de levedura utilizarem outras fontes de N do ambiente. Por tanto, é necessário entender como é a resposta ao nível transcricional do nitrato mediado por TORC1.

Diante o exposto, o presente trabalho foi pensado para complementar os nossos dados fisiológicos, de expressão genica e proteômica, e assim entender a regulação de forma global de fontes de nitrogênio ao nível trancricional dependente de TOR, utilizando Rapamicina como inibidor desta proteína quinase, grande regulador de assimilação de nutrientes na célula. Os resultados gerados a partir daqui, serviram para complementar as especialidades de dois grupos de pesquisa importantes nesta área (LAMAP e LGM) resultando na compreensão do potencial fermentativo de *D. bruxellensis* e do papel do nitrato que poderia ser mais explorado em atividades biotecnológicas para a formação de bioprodutos.

2. MATERIAIS E MÉTODOS

2.1 Microrganismos e condições de pré-cultivo

Inicialmente foi utilizada a linhagem industrial de *Dekkera bruxellensis,* GDB248 isolada de uma destilaria de etanol combustível no nordeste do Brasil. As células foram pre-inoculadas em fracos de vidro contendo 30 ml de meio sintético YPD (1% extrato de levedura, 2% glicose, 2% peptona) por 48h em agitação constante e 30°C em incubador tipo shaker até sua inoculação nas condições testes.

2.2 Determinação da concentração de rapamicina

Células pré-cultivadas em meio YPD foram coletadas, lavadas e utilizadas para inocular meio sintético (YNB na concentração de 0,17%) contendo glicose (2%) e sulfato de amônio (0,5%) na ausência (condição de referência) ou presença de rapamicina em diferentes concentrações desde 10 ng/ml até 160 ng/ml, seguindo o protocolo de Lee et al (2017). Os cultivos foram realizados como descrito acima. A cinética de crescimento celular em cada condição será avaliada por meio de curvas de crescimento utilizando multileitor para placa de ELISA que contem 96 poços com capacidades de 300 ul de meio e com medição de absorbância de 600 nm. A concentração de rapamicina a ser escolhida para os ensaios posteriores foi aquela suficiente para não inibir completamente a via TOR, permitindo crescimento celular relativo a 50% do crescimento na condição de referência. Esta concentração se ré designada como dose sub-MIC. Para atestar a significância dos resultados, os cultivos serão realizados em triplicata biológica independentes.

2.3 Cultivos em fontes de nitrogênio com e sem rapamicina

Células pré-cultivadas em meio YPD foram coletadas, lavadas e utilizadas para inocular meio sintético (YNB na concentração de 0,17%) contendo glicose (2%) e duas fontes de nitrogênio: amônio como fonte preferencial e nitrato como fonte secundária, na concentração de 75 mM de nitrogênio. Para cada fonte de nitrogênio, foi determinada a concentração de 60 ng/ml de rapamicina, de maneira que os crescimentos foram feitos na presença ou ausência deste composto. Inicialmente os cultivos foram realizados em placas estéreis de microtitulação com medição de absorbância a cada 30 minutos em equipamento de leitor de ELISA. A cinética de crescimento celular em cada condição foi dada por meio de curvas de crescimento (DO_{600nm} x tempo). Para atestar a significância dos resultados, os cultivos foram realizados em triplicata biológica independentes.

2.4 Ensaios de crescimento para extração de RNA TOTAL

A partir desta etapa e para atingir a quantidade de células necessarias para extração de material genético, foram realizados crescimentos em frasco com 50 ml de meio específico YNB (sem aminoácidos e sem amônio) com suplementação de glicose 110 mM e utilizando três condições: NH4, NO3 e NH4, na ausência ou presença de rapamicina 60 ng/ml. Todas as fontes de nitrogênio foram iniciadas em concentração de nitrogênio de 75mM. Os crescimentos foram mantidos até 30 horas realizando quatro pontos de coleta de células: T0 (referência), T10, T20 e T30 horas. Os pre-inóculos foram realizados como se indicado item *2.2* desta sessão. Uma vez terminados os ensaios, as células foram centrifugadas em 6000 rpm durante 5 minutos a 25 °C, e os sobrenadantes foram separados das células. Estes pontos foram tomados para obtenção de sobrenadante e quantificação por HPLC de glicose consumida. Todos os experimentos foram realizados em triplicata biológica e duplicata técnica.

6.5 Quantificação de açucares

Ao final de cada experimento, amostras foram coletadas e centrifugadas e o sobrenadante foi filtrado em filtro 0,22 mm para determinação da quantidade de açúcar residual por cromatografia líquida de alto desempenho (HPLC). Para isso, foi utilizada coluna de permuta iônica HPX-87H (BioRad, EUA) e solução de 5 mM de

H_2SO_4 como fase móvel no fluxo de 600 µl/min. O volume de amostra injetado foi 20 µL e a temperatura do forno foi 35 °C. A quantidade de glicose residual foi identificada considerando os tempos de retenção relativos e quantificados com base na comparação direta utilizando uma curva padrão, entretanto, os valores mínimos de detecção da coluna são de 2g/L, impedindo observar valores pequenos de etanol. Os resultados de HPLC foram realizados em triplicata biológica e duplicata técnica

6.6 Extração de RNA TOTAL

As células cultivadas como descrito no item 2.4 desta sessão, foram coletadas após observação do inicio da fase exponencial para cada uma das condições, centrfugadas a 3500 rpm por 10 minutos, descartado o sobrenadante e ressuspendido o sedimento em 200 µL de RNA Buffer e 400 µL de fenol ácido. O protocolo de extração de RNA TOTAL, purificação de RNA e armazenagem de amostras foi de acordo ao protocolo utilizado por Godoy et al (2016).

7 RESULTADOS

3.1 Perfil de crescimento de *D. bruxellensis* sob varias concentrações de Rapamicina

Para observar o efeito da rapamicina na assimilação de fontes de nitrogênio, foram realizados ensaios de crescimento em sulfato de amônio ou nitrato de sodio (dois substratos presentes no caldo de cana) e suplementado com rapamicina em várias concentrações em ng/ml como descrito por Lee et al (2017). Entretanto, diferente do que acontece com *S. cerevisiae*, que mostrou inibição do crescimento com Rapamicina em 20 ng/ml (Lee et al 2017), nossos resultados mostram que *D. bruxellensis* é mais resistente à várias concentrações independentemente da fonte de nitrogênio utilizada (Fig 1a, 1b). Entretanto, chamou a atenção o efeito da rapamicina nas duas fontes. No caso dos crescimentos com amônio, o crescimento celular observado pela densidade óptica começou a diminuir em 20 ng/ml. O crescimento celular nesta condição caiu à metade a partir de 60 ng/ml (Fig 1a). De forma oposta, nos ensaios com nitrato, os crescimentos celulares de *D. bruxellensis* aumentaram quando a Rapamicina estava em concentrações de 20 até 60 ng/ml comparado com a condição controle (sem suplementação de rapamicina) como observado na Figura

1b. Só apartir de 80 ng/ml há inibição no no perfil de crescimento desta levedura. Nesse cenério, a sinalização pelo nitrato poderia não ser necessariamente dependente de TORC1.

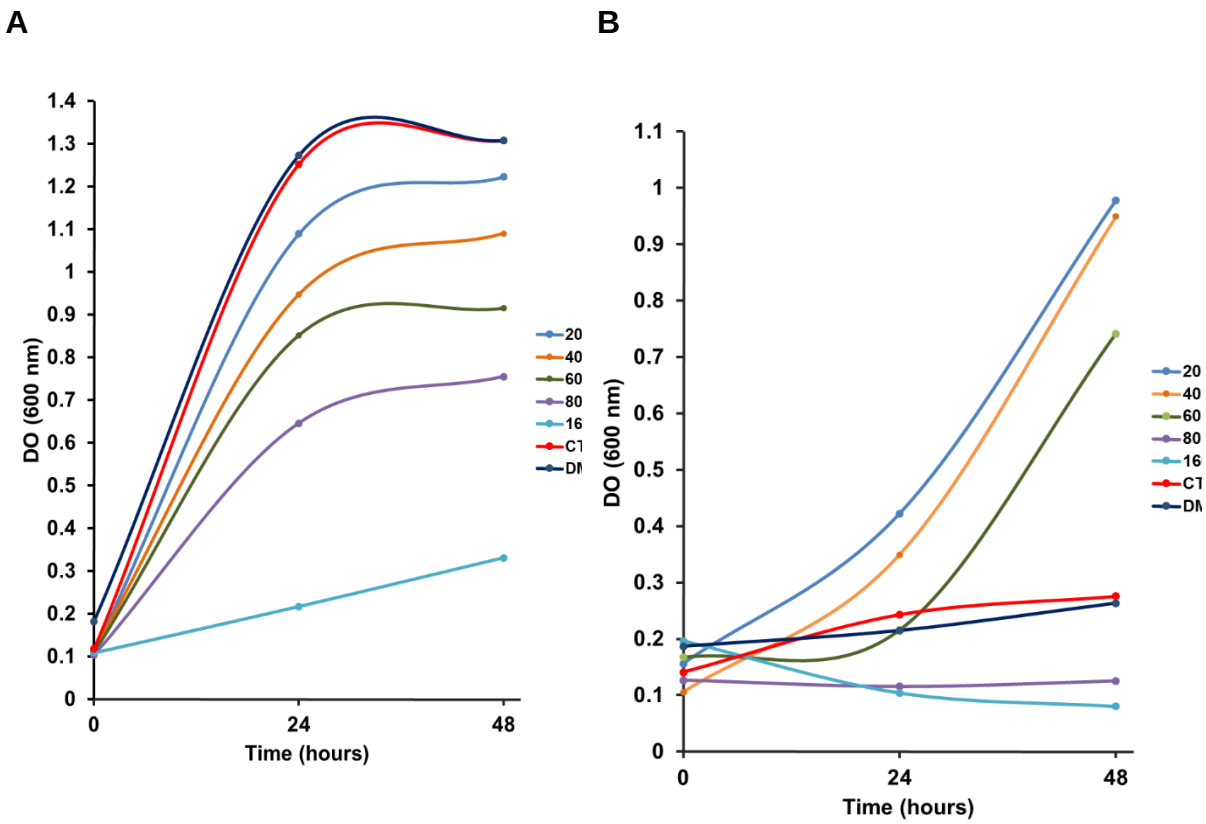

Figura 1: Cinética de crescimento de *Dekkera bruxellensis* GDB 248 em meio YNB contendo glicose e duas fontes de nitrogênio, suplementado com rapamicina em várias concentrações ng/ml. A. Sulfato de amônio (NH4) e B. Nitrato de sódio (NO3).

A partir desses perfis foi estabelecido que a concentração 60 ng/ml de rapamicina seria utilizada devido a sua inibição no crescimento celular reduzido à metade em meios contendo amônio, realizando testes em três condições: i) NH4, ii) NO3 e iii) NH4 + 60 ng/ml de rapamicina. Desta forma nós vamos conseguir estabelecer diferenças na regulação do nitrogênio ao nível transcricional em uma fonte primaria, uma fonte secundaria e uma fonte preferencial com o inibidor da TORC1. Assim, os ensaios de crescimento foram acompanhados durante 100 horas, permitindo assim acompanhar a cinética de crescimento nas três condições (Fig 2)

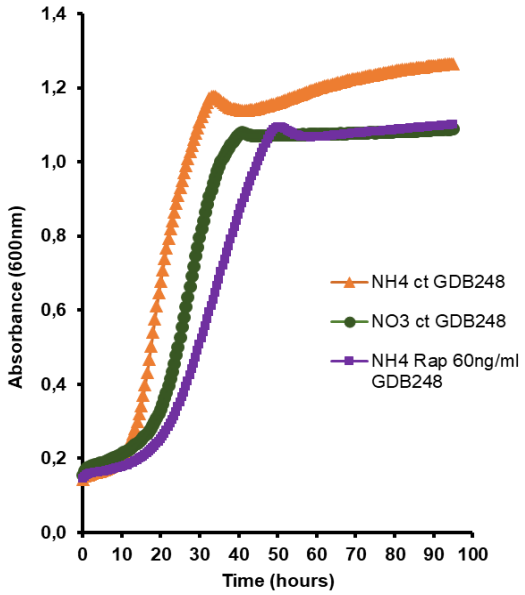

Figura 2: Perfil de crescimento da linhagem *D. bruxellensis, GDB248.* Células inoculadas em meios específicos contendo uma das três condições: NH4, NO3 e NH4+ Rapamicina 60ng/ml. Crescimentos mantidos durante 8 dias aproximadamente

3.2 Determinação de pontos de coleta das condições testes

A resposta fisiológica de *D. bruxellensis* GDB 248 submetida em três condições diferentes, permitiu observar diferenças nas cinéticas de crescimento, tanto em frascos como em microplacas. Assim, durante os ensaios realizados em frascos contendo 50 ml de meio em cada uma das condições, coletamos células em 4 tempos (0, 10, 20, 30h) para quantificar o consumo de glicose como observados na Figura 3. Com estes resultados comparamos parâmetros cinéticos encontrados nas curvas de crescimento e na quantificação em cada condição teste.

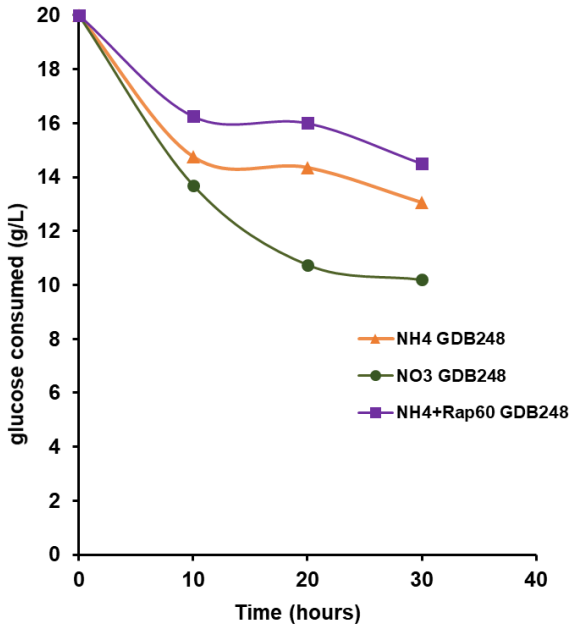

Figura 3: Quantificação por HPCL de quantidade de glicose consumida por *D. bruxellensis* GDB248, partindo de 20 g/L (110 mM).

Além disso, a contagem de unidades formadoras de colônias (UFC) foi realizada para cada condição nos tempos 6, 17 e 24 horas, partindo de um pre-inóculo de 48 h em YPD, no qual foi observado que na DO 0.1 havia $1.5x10^5$ células viáveis de *D. bruxellensis* (Fig 4).

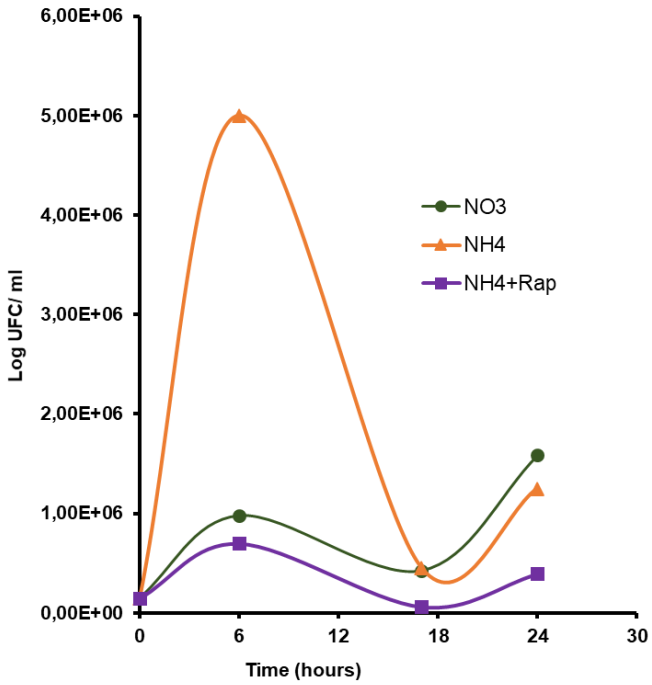

Figura 4: Quantificação por HPCL de quantidade de glicose consumida por *D. bruxellensis* GDB248, partindo de 20 g/L (110 mM).

A partir dessas comparações estabelecemos pontos de coleta para cada condição teste que correspondem ao tempo em que a levedura completa duas horas após o término da fase *lag* e desta forma evitar o enriquecimento da amostra em genes relacionados à adaptação geral e aumentar a probabilidade de observação de transcritos relacionados ao nitrogênio (NH4, NO3 e sua regulação). Foi descrito que o consumo dessas fontes de nitrogênio está no início de seu crescimento (de Barros Pita et al 2011; Galafassi et al 2013; Peña-Moreno et al 2019), razão pela qual também descartamos as amostras na metade da fase exponencial, onde as células já têm uma resposta aprendida e a possibilidade de encontrar informação nova sobre regulação é menor. Desta forma os pontos de coleta para cada condição foram i) 8 horas no meio contendo exclusivamente amônio, ii) 14 horas nos meios NO3 e NH4 + Rap (Fig 5).

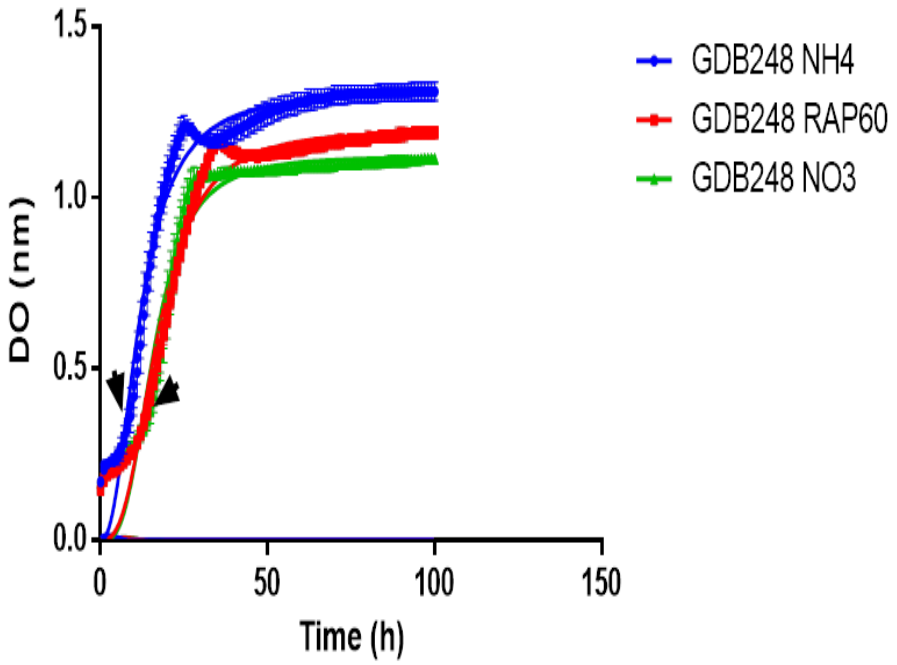

Figura 5. Curva de crescimento de *D. bruxellensis* linhagem GDB248 com definição de pontos de coleta de células para extração de RNA total

Após a determinação dos pontos de coleta na triagem inicial, os ensaios de crescimento foram realizados para permitir que as amostras fossem coletadas em momentos específicos para cada condição. Assim, para cada um dos três meios específicos (NH_4^+, NO_3-, NH_4^+ rap 60 ng/ml) foram realizados três novos ensaios de crescimento, totalizando 9 amostras. Uma vez coletadas as células, o protocolo de extração de RNA total, purificação e concentração do RNA Clean & Concentrator ™ - 51 foi adaptado para *D. bruxellensis* no laboratório da Universidade Católica de Chile. Foi também realizada uma quantificação em Nanodrop e eletroforese em gel de agarose a 1,5% para verificar a integridade em cada uma das amostras (Fig. 6).

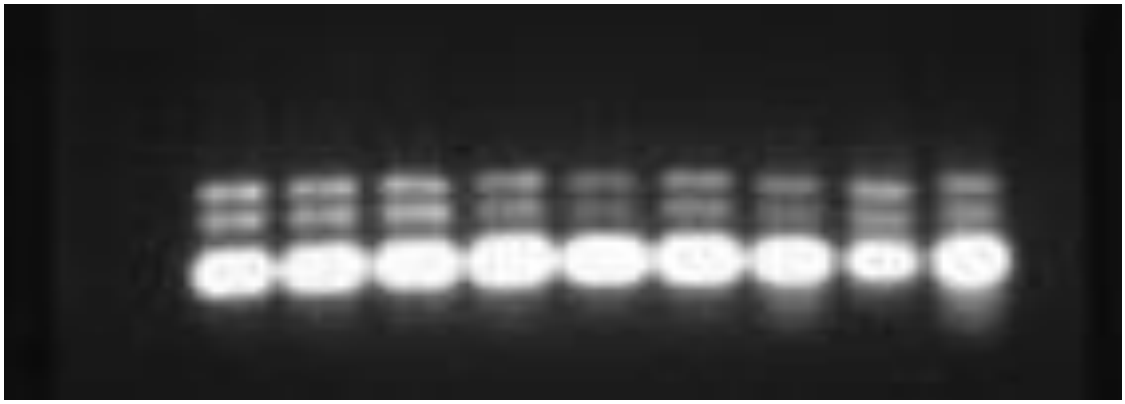

Figura 6: Eletroforese em gel de agarose 1,5% de amostras de RNA total *de D. bruxellensis GDB 248* em três condições (cada condição encontra-se em triplicata biológica.

8 PERSPECTIVAS DE FINALIZAÇÃO

Nossos resultados da transcriptômica permitirão estabelecer um painel genes em duas condições diferentes de nitrogênio e mais uma condição com rapamicina que avalia a regulação por TOR em *D. bruxellensis.* Desta forma integraremos informações de regulação transcricional de eventos regulatórios liderados por TORC1 e se o papel da Rapamicina é similar ao observado quando uma fonte de nitrogênio como nitrato está presente no meio.

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ANEXO A

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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] and Marcos Antonio de Morais Junior[2]

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Will de Barros Pita

will.barros.pita@gmail.com

[1]Department of Antibiotics, Federal University of Pernambuco, Recife, PE 50760-91, Brazil
[2]Department of Genetics, Federal University of Pernambuco, Recife, PE 50760-901, Brazil.

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.

D. 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 1900's, 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 aspects of *D. bruxellensis* (Curtin et al. 2014; Blomqvist and Passoth, 2015; Radecka et al. 2015; Steensels et al. 2015). Herein, we shall focus on the role of this yeast in other industrial sectors, as well as its potential application in these processes.

The production of Lambic beer is, for instance, an industrial field in which *D. bruxellensis* plays an important role, especially during the maturation stage, being essential for the flavour composition (Spitaels et al. 2014; de Roos and de Vuyst, 2018). Moreover, several studies have consistently reported the presence of different strains of *D. bruxellensis* with particular contributions to sensorial properties of many other fermented beverages, such as cider, kombucha, kefir and tequila (Lachance et al. 1995; Teoh et al. 2004; Gray et al. 2011; Albertin et al. 2014; Spitaels et al. 2015; Curtin et al. 2015; Longin et al. 2016). In the past decade, *D. bruxellensis* has also drawn attention due to its involvement in bioethanol production (de Souza Liberal et al. 2007; Passoth et al. 2007). In the latter scenario, this yeast competes with *Saccharomyces cerevisiae* for the sugar present in the industrial substrate and it is mostly faced as a contaminant micro-organism (de Souza Liberal et al. 2007; de Barros Pita et al. 2011). It is interesting to note that despite being regularly pointed as a contaminant, *D. bruxellensis* has also the potential to be industrially employed (de Souza Liberal et al. 2007;

Passoth et al. 2007; de Barros Pita et al. 2011). This apparent contradiction lies on the fact that this yeast presents useful physiological and genetic traits, not only for fermentative processes but also for other industries. In fact, *D. bruxellensis* is able to produce ethanol by alcoholic fermentation, as well as other industrially relevant compounds (de Souza Liberal et al. 2007; de Barros Pita et al. 2011; Rozpedowska et al. 2011; de Barros Pita et al. 2013a). For instance, as we will see in the following sections, its acetogenic profile might be exploited in a large scale for acetic acid production. Moreover, its capacity to assimilate sugars from lignocellulosic material leads the way to a possible use in second-generation ethanol production. These features, once properly explored, might increase both the contribution and the industrial role of *D. bruxellensis*. Finally, strain improvement strategies are underway, aiming to enhance favourable properties and to overcome its drawbacks, in order to take advantage of the full biotechnological potential of this yeast.

Current industrial applications of *D. bruxellensis*: advantages, challenges and perspectives

S. cerevisiae is the leading industrial microorganism, while the non-*Saccharomyces* (and non-*Schizosaccharomyces*) species are often referred to as non-conventional yeasts (NCY), since they have not been largely explored by humans (Wolf, 1996). The presence of a given NCY is usually reported in specific processes, for example the spontaneous fermentations that produce the Belgian Lambic beers (Bokulich and Bamforth, 2013; Spitaels et al. 2014). In these beverages, acetic acid in proper amounts (0.4 g/L to 1.2 g/L) is crucial to the final taste and *D. bruxellensis* has an important role in this process (Gamero et al. 2014; Spitaels et al. 2014). In fact, *D. bruxellensis* is distinguished by the production and release of acetic acid, higher alcohols and several esters, such as ethyl acetate, ethyl caprate, ethyl caprylate and ethyl lactate, contributing to floral or fruity characteristics of these beers (Verachtert et al. 1992; Crauwels et al. 2015a; Cortés-Diéguez et al. 2015; Parente et al. 2015; Basso et al. 2016). Moreover, this yeast also participates in the production of other acidic beverages, such as American coolship ales inspired by Lambic beers, some Belgian Trappist, as well as Berlin style wheat beers (Martens et al. 1997; Annemüller et al. 2008; Steensels and Verstrepen 2014). *D. bruxellensis* is also related with the production of Kombucha, in association with bacteria from *Acetobacter*, *Gluconobacter* and *Lactobacillus* genera (Teoh et al. 2004), as well as the production of cider, together with *Hanseniaspora*, *Kloeckera* sp. and *S. uvarum*, composing the beverage flavour (Morrissey et al. 2004).

As mentioned above, in spite of being regarded as NCY, some features presented by *D. bruxellensis* are rather interesting, especially in bioethanol production processes. For instance, under typical industrial conditions, such as high sugar concentrations in fermentation

tanks, *D. bruxellensis* has the capacity to produce ethanol even in presence of oxygen. This observation reflects the so-called Crabtree effect, which is certainly its most advantageous trait (van Dijken and Scheffers, 1986; Piskur et al. 2006; Procházka et al. 2010). In fact, *D. bruxellensis* frequently presents ethanol yields similar to those of *S. cerevisiae.* However, the major problem associated with *D. bruxellensis* in bioethanol production plants is a decreased volumetric productivity (Table 1). This issue mostly relies on a slow sugar consumption rate, a fact that has also been reported even when fermentation assays lasted for long periods (Basílio et al. 2008; Blomqvist et al. 2010; Galafassi et al. 2011; Pereira et al. 2012). For instance, in sugarcane molasses *D. bruxellensis* presents a sucrose consumption rate 50% slower than *S. cerevisiae* (Table 1; Pereira et al. 2014). Since *D. bruxellensis* competes with *S. cerevisiae* for the industrial substrate, this feature ultimately leads to large economic losses (de Souza Liberal et al. 2007; Basílio et al. 2008). Therefore, understanding how *D. bruxellensis* handles industrial sugars is an important challenge in order to explore its maximum fermentative capacity and increase the volumetric productivity.

Oxygen availability also seems to influence the fermentative capacity of *D. bruxellensis.* While oxygen limitation seems to favour alcoholic fermentation (Table 1), under aerobic conditions, *D. bruxellensis* presents tendency to an oxidative metabolism, which increases acetic acid production to levels closer to those of ethanol (Freer, 2002; Aguilar Uscanga et al. 2003; Dequin et al. 2003; Rozpędowska et al. 2011; Leite et al. 2013; Teles et al. 2018). However, since acetic acid has a great importance as a chemical compound, the acetogenic profile of *D. bruxellensis* might be used in this process, an alternative that has not yet been explored. This possibility also relies on the fact that this yeast is able to endure the stressful industrial conditions, such as pH variations (Freer et al. 2003). The metabolic potential of *D. bruxellensis* makes it capable of being used also in pharmaceutical and food industries. In this sense, a new application field for this yeast is related to the extraction of bioactive compounds, such as resveratrol (Kuo et al. 2017).

Table 1 Ethanol production parameters of *D. bruxellensis* (Db) and *S. cerevisiae* (Sc) in laboratory media and industrial substrates

Condition	Observation	Yeast	Y_{etoh} (g g^{-1})	Volumetric productivity (g l^{-1} h^{-1})	Reference
Oxygen-limited fermentation of synthetic medium (glucose as carbon source unless otherwise stated)	none	Sc	0.50	1.10	Peña-Moreno et al. (2019)
		Db	0.45**	0.89	
		Sc	0.43	0.25	Blomqvist et al. (2012)
		Db	0.44**	0.14	
		Db	0.45	0.15	Blomqvist et al. (2010)
		Db	0.44	0.08	Galafassi et al.(2011)
		Db	0.33	1.56	Teles et al.(2018)
		Db	0.37**	0.22	de Barros Pita et al. (2013a)
	sucrose replacing glucose as carbon source	Db	0.43**	0.56	
	aldehyde dehydrogenase blocked by Disulfiram	Db	0.49	1.49	Teles et al.(2018)
	sucrose replacing glucose as carbon source	Sc	0.32	19.24*	de Souza Liberal et al. (2007)
	sucrose replacing glucose as carbon source	Db	0.32	9.45*	
	sucrose replacing glucose as carbon source	Sc	0.47	18.73*	da Silva Filho et al. (2005)
Oxygen-limited fermentation of industrial substrates	sugar cane molasses	Sc	0.44	9.00	Pereira et al.(2014)
		Db	0.45	4.50	
	sugar cane juice	Sc	0.48	5.83	Pereira et al.(2012)
		Db	0.45	1.58	
Moderate aeration in continuous cultivation in glucose	synthetic medium	Db	0.41	0.06	Blomqvist et al. (2012)
		Sc	0.41	0.06	
Aerobic batch cultivations in the (glucose)	defined minimal medium	Db	0.32	0.04	Rozpędowska et al.(2011)
	synthetic minimal medium	Db	0.13**	0.04	Galafassi et al. (2013)
Aerobic C-limited chemostat	pulse with glucose	Db	0.16	0.25	Leite et al.(2013)
Anaerobic batch cultivations in glucose	synthetic minimal medium	Db	0.35**	0.04	Galafassi et al. (2013)
	synthetic mineral medium	Db	0.45**	0.22	Peña-Moreno et al. (2019)
	synthetic mineral medium	Db	0.43**	0.21	

	defined minimal medium	Db	0.34	0.05	Rozpędowska et al. (2011)

In this case, *D. bruxellensis* presented the highest bioconversion rate of this compound (through the specific activity of β-glucosidase enzyme) in comparison with the utilization of commercial enzyme or acid hydrolysis (Kuo et al. 2017). The use of resveratrol is based on several biological activities, such as antimicrobial, antiviral, cancer cell inhibition, antioxidant, menopause-relieving and anti-aging properties (Kaeberlein, 2010; Baur et al. 2006). Moreover, *D. bruxellensis* is linked to the production of natural sweeteners from mogrosids extracted from *Siraitia grosvenorii*. This yeast is capable of converting mogroside V to siamenoside I, considered to be the sweetest and most preferred natural sweetener among mogrosides (Wang et al. 2018). This application might decrease the use of artificial ones, which have been reported to cause type 2 diabetes (Fagherazzi et al. 2013) and cancer (Soffritti et al. 2006).

As discussed above, *D. bruxellensis* is part of several industrial processes, even though presenting either a supporting or detrimental role in most cases. Yet, bioethanol production seems to be one of the most interesting fields in which this yeast might be explored, as long as metabolic bottlenecks are overcome. Interestingly, the participation of *D. bruxellensis* in bioethanol production is regarded in two main aspects. The first one, already presented, is related to a decrease in overall productivity. The second aspect is, in fact, a promising industrial application related to the second-generation ethanol production from lignocellulosic substrates, which we will detail later.

Industrially relevant metabolic traits and genetic improvement of strains

The availability of genome sequencing data shed some light on the genetic background for the metabolic capacity of several strains of *D. bruxellensis* and revealed a high genotypic and ploidy variation (Woolfit et al. 2007; Piskur et al. 2012; Curtin et al. 2012; Valdes et al. 2014; Borneman et al. 2014; Crauwels et al. 2015b; Fournier et al. 2017; Avramova et al. 2018; Tiukova et al. 2019). These genomic analyses also showed that *D. bruxellensis* presents particular characteristics and possible adaptive advantages to industrial substrates, such as a wide capacity to assimilate nutrients. For instance, this yeast harbours genes coding for enzymes involved with the utilization of xylose, L-arabinose and cellobiose, which are not naturally fermented by *S. cerevisiae* (Woolfit et al. 2007; Curtin et al. 2012; Godoy et al. 2017). We will detail the importance of these sugars in the following section. Moreover, *D. bruxellensis* presents a gene cluster involved in the assimilation of nitrate, a nitrogen source often found in sugarcane juice (Woolfit et al. 2007; de Barros Pita et al. 2011). Since *S. cerevisiae* cannot use this compound, this was an interesting discovery and, in fact, nitrate was later pinpointed as an adaptation factor for *D. bruxellensis* in a competition with *S. cerevisiae* in fuel ethanol

plants (de Barros Pita et al. 2011), as it was previously observed (Abbott et al. 2005; de Souza Liberal et al. 2007; Passoth et al. 2007). Nitrate was also involved with an increased fermentative capacity of *D. bruxellensis*, especially when associated with ammonium and limiting or absent oxygen (Table 1), possibly by eliminating the Custer effect (de Barros Pita et al. 2011; Galafassi et al. 2013; Peña-Moreno et al. 2019). In spite of that, it is important to state that nitrate assimilation is not found in all strains, as well as it was not crucial for CBS11270 fermentative performance (Blomqvist et al. 2012; Borneman et al. 2014).

As we discussed earlier, *D. bruxellensis* is a Crabtree positive yeast, which is an important physiological trait that results in the so-called "*make-accumulate-consume*" strategy (Rozpedowska et al. 2011). The primary physiological result of this strategy is the preference for fermentation rather than respiration and leads to a high capacity to produce, tolerate and subsequently use ethanol (Procházka et al. 2010; Rozpedowska et al. 2011; Pfeiffer and Morley et al. 2014). In the industrial scenario, this is an interesting feature, since it increases the fermentative capacity of the yeast (Piskur et al. 2006; Rozpedowska et al. 2011). In fact, it has been described that *D. bruxellensis* responds to the definition of a Crabtree positive yeast in a similar way to *S. cerevisiae,* i.e., by producing ethanol immediately after a sudden exposure to glucose (Peña-Moreno et al. 2019). Therefore, with proper screening and selection methods, genetic modification or even by taking advantage of its own metabolism, it is possible to improve the fermentative capacity of *D. bruxellensis*. In this sense, a recent approach has been proposed to increase its ethanol production and involved the use of Disulfiram, a drug that inhibits the cytosolic acetaldehyde dehydrogenase (Teles et al. 2018). In this study, enzyme inhibition resulted in a diverted traffic of cytosolic acetaldehyde exclusively to ethanol production.

Since *D. bruxellensis* already presents genetic characteristics that are valuable to several industrial sectors, how can this species be further explored? Strategies aiming to develop genetically modified strains are emerging, yet in an early stage, in order to allow or improve the function of its metabolic pathways. Methods such as electroporation, lithium acetate/PEG (LiAc/PEG) and spheroplast transformation have been adapted for *D. bruxellensis*, with efficiency similar to *S. cerevisiae*, yet with random and unaddressed integration (Miklenic et al. 2013; Miklenic et al. 2015). Fortunately, experiments joining the treatment with LiAc and electroporation (with a control in key steps) increased the transformation efficiency (Miklenić et al. 2015). Later on, gene transformation cassettes for *D. bruxellensis* have also been developed, providing greater efficiency in strain transformation by achieving antibiotic resistant and fluorescent protein markers insertion (Varela et al. 2018). Moreover, cloning and overexpressing *ADH3* gene (coding for alcohol dehydrogenase) resulted in a higher ethanol yield than the wild-type strain under anaerobic conditions, probably

by inducing a faster consumption of glucose and alleviating the Custer effect (Schifferdecker et al. 2016). Interestingly, to date, no CRISPR approach has been proposed to *D. bruxellensis*.

In fermentation industries, such as in bioethanol production plants, maximizing industrial parameters (production, yield and efficiency) is the ultimate goal and this could be achieved, for instance, by improving the process or the micro-organisms employed. In spite of being an interesting way to take advantage of the full biotechnological potential of *D. bruxellensis*, there is still a large field to be explored in genetic engineering for this yeast and more advances are necessary to reach proper strain improvement. However, it is clear that with the accumulation of knowledge regarding its metabolic capabilities and the development of modern genetic tools, *D. bruxellensis* will emerge as an important industrial microorganism.

Second-generation ethanol production by *D. bruxellensis*: a real chance to reach the spotlight?

Lignocellulosic material is a renewable natural resource available in large quantities and low costs, which has a significant importance for bioenergy production and environmental issues, such as the reduction of polluting gases released by fossil fuels (Kang et al. 2014; Wang et al. 2014; Lynd 2017). Second-generation ethanol production is associated with the use of substrates derived from lignocellulosic hydrolysates (notably hemicellulose and cellulose) by microorganisms that are able to ferment the sugars released in this process (Bušić et al. 2018). While the lignin is used to generate electricity and heat to biorefinery, the hydrolysis of hemicellulose and cellulose leads to the release of D-glucose, D-mannose, D-galactose, D-xylose, L-arabinose and D-cellobiose (Gamage et al 2010; Bušić et al. 2018). These sugars could be further fermented to ethanol, however, only genetically modified strains of *S. cerevisiae* are able to use cellobiose, D-xylose and L-arabinose, which account for a large portion of sugars released in the process (Ha et al. 2011, 2013; Caballero and Ramos, 2017; Wang et al. 2017). Moreover, in order to achieve a high sugar conversion efficiency, it is necessary to insert several pathways in *S. cerevisiae*, which could lead to a metabolic imbalance (Fazzini et al. 2010; Chen et al. 2018).

On the other hand, *D. bruxellensis* strains might be able to assimilate cellobiose, since its genome harbours the *BGL* gene, coding for β-glucosidase (E.C. 3.2.1.21), which represents an advantage in a potential biotechnological use for second-generation ethanol production (Blomqvist et al. 2011; Curtin et al. 2012; Reis et al. 2014). In fact, it has been reported that this yeast is able to use this sugar, regardless the oxygen availability, with growth rates similar to those observed in glucose (Leite et al. 2013; da Silva et al. 2019). Moreover, *D. bruxellensis*

was able to ferment cellobiose in oxygen limitation (similar to industrial conditions), even though with lower efficiency (21% lower) when compared to sucrose (Reis et al. 2014). In *D. bruxellensis*, □□-glucosidase acts mostly intracellularly, which is a useful feature since cellobiose is a potent inhibitor of cellobiohydrolases and when in excess, this disaccharide leads to a decrease in the hydrolysis rate (Reis et al. 2014; Gruber et al. 2007). Therefore, *D. bruxellensis* could be used in the process of simultaneous saccharification and fermentation (SSF), in which, even without the addition of β-glucosidase to the cellulolytic cocktail, the accumulation of cellobiose could be avoided.

Some *D. bruxellensis* strains are also able to use pentoses, such as D-xylose and L-arabinose under fermentative conditions, with maximum ethanol yields of 0.29 g g^{-1} and 0.34 g g^{-1}, respectively (Codato et al. 2018). This is possible since its genome harbours genes coding for xylose-metabolizing enzymes, such D-xylose reductase, D-xylulose reductase, xylulokinase and transketolase (Godoy et al. 2017). In yeasts such as *B. naardenensis* and engineered strains of *S. cerevisiae*, the use of xylose under oxygen-limited conditions results in the accumulation of xylitol (Galafassi et al. 2011; Shin et al. 2019). This is caused by a redox imbalance generated by a preference for different cofactors of xylose reductase (NADPH) and xylitol dehydrogenase (NAD^+) (Hahn-hägerdal et al. 2007). Currently, this is still unknown for *D. bruxellensis* and represents an open field for research. The ability to ferment xylose is relevant since this is the most abundant pentose of the lignocellulosic material. Therefore, the economic viability of the process depends on the maximum assimilation of available sugars (Rech et al. 2019).

The steps of pre-treatment and hydrolysis of lignocellulosic material also generates fermentation inhibitors, such as furans (furfural and 5-hydroxymethylfurfural), carboxylic acids (acetic acid) and phenolic compounds, that might negatively interfere in the fermentation process (Palmqvist and Hahn-Hagerdal, 2000; Bušić et al. 2018). Therefore, besides being able to ferment the mixture of sugars released, key microorganisms also need to be resistant to these inhibitors (Robak and Balcerek 2018). In this regard, *D. bruxellensis* presents resistance to fermentation inhibitors when cells are initially adapted during batch cultivation, while unadapted cells have a more extended lag phase and lower ethanol yield (Tiukova et al. 2014). The mechanisms by which *D. bruxellensis* resists to these inhibitors are still uncertain, whereas in *S. cerevisiae* this has been associated with increased expression of the *ADH7* (alcohol dehydrogenase) and *ARI1* (aldehyde reductase intermediate) genes (Sehnem et al. 2013). Moreover, the tolerance to weak acids, low pH values and high ethanol concentration are important adaptation factors to this process (Dien et al. 2003; Davison et al. 2016). In this sense, the resistance of *D. bruxellensis* at low pH values (in the range of pH 1.5-2.0) is similar

to *S. cerevisiae*. Still, at high ethanol concentrations (9%), both species are resistant, but show a decreased growth (Bassi et al. 2013).

The search for yeasts with key characteristics for the second-generation ethanol production aims to increase both ethanol yield and productivity from lignocellulosic materials. In this sense, *D. bruxellensis* meets the major requirements to become an ethanol-producing yeast, allowing its application in biotechnological processes, such as SSF. In addition, genetically modified strains of *D. bruxellensis* could be developed in order to increase the ethanol production from the fermentation of lignocellulosic-derived sugars.

Concluding remarks

D. bruxellensis is a yeast species well adapted to most substrates in fermentation processes. In spite of being mostly known for its role as a contaminant in winemaking, this species presents physiological traits that might be explored in other industrial fields. While this yeast is part of the production process of several fermented beverages, especially the Belgian beers, its contribution is mostly secondary. On the other hand, in bioethanol production, *D. bruxellensis* might have a larger participation, once problems such as a reduced volumetric productivity are solved. In addition, in second-generation ethanol production, this yeast might assume a major role, since it is able to use sugars released from lignocellulosic material. Moreover, its acetogenic profile opens the possibility to use this yeast to produce acetic acid in industrial scale. Finally, *D. bruxellensis* might also be employed in pharmaceutical and food industries. Therefore, the industrial fitness and the presence of highly desired metabolic traits pushes *D. bruxellensis* towards the centre of the so-called non-conventional yeasts. While strain improvement is a promising endeavour, currently, genetic modification is still a challenge that must be overcome in order to maximize the industrial employment of this yeast.

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ANEXO B

Article

Biodiversity among *Brettanomyces bruxellensis* Strains Isolated from Different Wine Regions of Chile: Key Factors Revealed about its Tolerance to Sulphite

Camila G-Poblete [1], Irina Charlot Peña-Moreno [2], Marcos Antonio de Morais Jr [2], Sandra Moreira [1] and María Angélica Ganga [1,*]

[1] Departamento de Ciencia y Tecnología de los Alimentos, Facultad Tecnológica, Universidad de Santiago de Chile, 9170020, Chile; camila.gonzalezpo@usach.cl (C.G-P.); sandra.moreira.r@gmail.com (S.M.)

[2] Department of Genetics, Biosciences Center, Universidade Federal de Pernambuco, 50670-901, Brazil; irina.penamoreno@gmail.com (I.C.P.-M.); marcos.moraisjr@ufpe.br (M.A.d.m.Jr.)

* Correspondence: angelica.ganga@usach.cl

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Abstract: *Brettanomyces bruxellensis* is regarded as the main spoilage microorganism in the wine industry, owing to its production of off-flavours. It is difficult to eradicate owing to its high tolerance of adverse environmental conditions, such as low nutrient availability, low pH, and high levels of ethanol and SO_2. In this study, the production of volatile phenols and the growth kinetics of isolates from various regions of Chile were evaluated under stressful conditions. Through randomly amplified polymorphic DNA (RAPD) analysis, 15 strains were identified. These were grown in the presence of *p*-coumaric acid, a natural antimicrobial and the main precursor of off-flavours, and molecular sulfur dioxide (mSO_2), an antimicrobial synthetic used in the wine industry. When both compounds were used simultaneously, there were clear signs of an improvement in the fitness of most of the isolates, which showed an antagonistic interaction in which *p*-coumaric acid mitigates the effects of SO_2. Fourteen strains were able to produce 4-vinylphenol, which showed signs of phenylacrylic acid decarboxylase activity, and most of them produced 4-ethylphenol as a result of active vinylphenol reductase. These results demonstrate for the first time the serious implications of using *p*-coumaric acid, not only for the production of off-flavours, but also for its protective action against the toxic effects of SO_2.

Keywords: *Brettanomyces bruxellensis*; sulphur dioxide; *p*-coumaric acid; spoilage microorganisms

1. Introduction

Yeasts belonging to the *Brettanomyces* genus are generally regarded as the main spoilage microorganisms in the wine industry as their presence alters the organoleptic characteristics of the wine, and thus leads to significant economic losses throughout the world [1]. *Brettanomyces bruxellensis*, also known as the teleomorph *Dekkera bruxellensis*, is one of the species recognized in the wine industry for conferring unwanted olfactory characteristics similar to wet wool, musty and medicinal odours, burned plastic or wet horsehair (Brett characters) through the production of volatile off-flavours in the winemaking process [2]. These particular aromas are produced from phenolic compounds that are present in the grape and musts that are called hydroxycinnamic acids (AHC) [3]. The compounds, which

include *p*-coumaric, ferulic and caffeic acids, are endogenous components of grapes and have been described as preservatives of natural foods [4] because of their antimicrobial activity [5,6]. *Brettanomyces bruxellensis* is able to metabolize AHCs during the winemaking by means of a phenylacrylic acid decarboxylase [7], and convert them to hydroxystyrenes (vinylphenols), which are then reduced to ethyl derivatives by a NADH-dependent vinyl phenol reductase to produce ethylphenols [8,9].

Due to its harmful effects on wine quality, the elimination of *B. bruxellensis* from the fermentation processes is very important. However, this has proved to be a difficult task on account of its tolerance to adverse environmental conditions such as low nutrient availability, low pH and high levels of ethanol [10–12]. However, there are several techniques that can be employed to restrict or prevent the growth of this yeast in the wine, such as the addition of sulphur dioxide (SO_2), in the form of potassium metabisulphite (PMB), which is the chemical antimicrobial agent that is most widely used in the control of unwanted microorganisms [13]. Additionally, molecular SO_2 (mSO_2) is an oxidizing agent that is used in winemaking for controlling and stabilizing the end product. Sulphurous anhydride is generally added to musts and wines as an aqueous solution in concentrations ranging from 0.3 to 0.8 mg L^{-1} in the red wine technology [14]. SO_2 in sufficient inhibitory concentration is capable of inhibiting enzymes such as glyceraldehyde-3-phosphate dehydrogenase, ATPase, alcohol dehydrogenase, aldehyde dehydrogenase and NAD^+-dependent glutamate dehydrogenase, which can affect key metabolic processes and lead to cell death [15]. Despite this, a high SO_2 concentration can lead to altered sensory characteristics of the wine. However, numerous authors have stated that the use of increasing concentrations should take account of the specific physiological response to the genetic constitution of *B. bruxellensis* strains and the tolerance of these strains to this agent [12]. This is a very serious matter as wine-producing regions can harbour strains of different clonal origins that, as a result of variations in genetic constitution and metabolic profiling, can vary in their levels of tolerance, as well as their capacity to produce off-flavours. Chile is an important wine producer in the world, and its grapes are grown in several regions and fermented in different winemaking conditions. In light of this, the aim of this study was to investigate the genetic diversity, the physiological characteristics and the growth fitness in the presence of the combination of the antimicrobials SO_2 and *p*-coumaric acid of 15 isolates of *B. bruxellensis* collected from fermentation processes in various regions of Chile. The evaluation of the yeast response to the inhibitors allowed understanding of the complexity of the yeast resistance and their influence on the production of aromatic compounds.

2. Materials and Methods

2.1. Strain Selection and Cell Maintenance

The strains stored in the Laboratory of Biotechnology and Applied Microbiology of the University of Santiago de Chile (LAMAP) were used in this study. The isolated strains were selected from wine fermentation processes from several regions in Chile. Initially the cells were activated in selective medium for *B. bruxellensis*, corresponding to solid YPD with calcium carbonate (yeast extract 10 g L^{-1}, peptone 20 g L^{-1}, glucose 20 g L^{-1}, agar 20 g L^{-1}, $CaCO_3$ 5 g L^{-1}) [7]. The cells were incubated in Petri dishes for 6–10 days at 28 °C.

The origin of *B. bruxellensis* strains correspond to: L-2472, L-2474, L-2476, L-2480 and L-2478 from Alto Jahuel (33°44′01″ S; 70°41′03″ O), L-2570, L-2482, L-2755 and L-2597 from Rengo (34°24′23″ S; 70°51′30″ O), L-2676, L-2679 and L-2690 from Molina (35°21′12.13″ S; 70°54′34.34″ O) and L-2731, L2742, L-2759 and L-2763 from Nancagua (34°40′3.94″ S; 71°11′30.98″ O).

2.2. Molecular Identification

Yeast cells were cultivated in synthetic medium consisting of 2% glucose and 6.7 g L^{-1} yeast nitrogen base (YNB) (Difco Laboratories, Detroit, USA) and distilled water to pH 6.0 [7]. These assays were done under constant agitation (120 r.p.m.) at 28 °C for 7 days (aerobic condition). Genomic DNA

extraction was performed using Wizard® Genomic DNA Purification Kit with modifications (Promega, Wisconsin, USA).

The extracted DNA was analysed by PCR amplification using ITS1 (5 'TCCGTAGGTGAACCTGCGG 3') and ITS4 (5 'TCCTCCGCTTATTGATATGC3 ') primers [16]. The reaction mixture contained 1X buffer ABM, 1.5 mM $MgSO_4$, 0.2 mM of each dNTP, 0.5 µM of each primer, 1.25 U Taq DNA polymerase (ABM, Richmond, Canada) and 80 ng of DNA template. Amplification reactions were carried out in a Peltier Thermal Cycler (PT−100) under the following conditions: denaturation at 94 °C for 5 min followed by 30 cycles of amplification with denaturation of 94 °C for 1 min, annealing at 55 °C for 1 min and extension of 72 °C for 2 min, with a final extension at 71 °C for 10 min. PCR products were visualized by means of electrophoresis using 1.5% agarose gel at 90 V for 60 min in 0.5× TBE buffer.

The RAPD-PCR technique was used for clonal discrimination between the yeast isolates. Three primers from the OPA series were used: OPAE09 (5'-TGCCACGAGG−3'), OPAD08 (5'-GGCAGGCAAG-3') and OPAE12 (5'-CCGAGCAATC-3') (Operon Technologies, CA, USA), which were previously selected in LAMAP-USACH as being suitable for *B. bruxellensis* strain discrimination (unpublished data). PCR was performed using a final volume of 20 µL, containing 1X buffer ABM, 1.5 mM $MgSO_4$, 0.2 mM of each dNTP, 0.4 µM of each primer, 1.25 U Taq DNA polymerase (ABM) and 100 ng of DNA template. The amplification reactions consisted of initial denaturation at 94 °C for 2 min followed by 25 cycles of amplification with denaturation of 94 °C for 1 min, annealing at 35 °C for 45 s and extension of 72 °C for 1 min, with a final extension at 71 °C for 10 min. The amplicons were subjected to electrophoresis in 5% polyacrylamide gel matrix (5% acrylamide-bisacrylamide 3008 in TBE 0.5× containing 20% ammonium persulfate and 50% tetramethylethylenediamine (TEMED)) at 70 volts for 130 min. The size of the bands was detected by using Quantity One software (Bio-Rad, California, USA).

2.3. Growth Kinetics Using Micro-Cultures in the Presence of p-Coumaric Acid (APC) and Sulphur Dioxide (SO_2)

Yeast cells were activated by inoculation in 5 mL of liquid YPD minimum medium (1% yeast extract, 2% glucose and 2% peptone) for 8 days at 28 °C with constant agitation [17]. Subsequently, 100 µL of each culture was transferred to 5 mL of synthetic medium and incubated for 8 days at 28 °C. These were used as seed cultures for the tests in the presence of inhibitors. This involved diluting seed cells in synthetic media to 5×10^5 cells mL^{-1} to 200 µL final volume. Six different conditions were tested: a) medium without inhibitor (control), b) medium containing potassium metabisulfite (mSO_2) as a producer of sulphur dioxide at 0.3 mg L^{-1}, c) medium containing potassium metabisulfite (mSO_2) as a producer of sulphur dioxide at 0.6 mg L^{-1}, d) medium containing *p*-coumaric acid at 100 mg L^{-1}, e) medium containing 100 mg L^{-1} of *p*-coumaric acid and sulphur dioxide at 0.3 mg L^{-1}, f) medium containing 100 mg L^{-1} of *p*-coumaric acid and sulphur dioxide at 0.6 mg L^{-1}. The pH of the culture media was adjusted to 3.5 with HCl 1 M (average value for pH generally encountered in winemaking conditions) [18]. The kinetics of the cell growth of each strain in these conditions was evaluated by means of growth curves and using sterile microplates containing 96 wells, with absorbance measurements made every 30 min in a TECAN microplate reader. Absorbance measurements were taken from the microplates (OD 600 nm × time) [18] with agitation at 120 (r.p.m) before each reading, in biological triplicate and technical triplicate. The experiments were carried out for 10–25 days at 28 °C.

2.4. Quantification of Volatile Phenols

Volatile phenol concentration was quantified using the method described by [19]. For this, we utilized the HPLC technique (Shimadzu Corporation, Kyoto, Japan). The column used was C18 Shimadzu reverse phase (150 × 4.6 mm). The solvent system corresponded to a water/formic acid gradient (90:10% *v*/*v*) with methanol. Standard curves were prepared for *p*-coumaric, 4-vinilphenol and 4-etilphenol (Sigma Aldrich, St. Louis, USA) in the range of 0.5–100 mg L^{-1}.

All of the strains were cultivated in synthetic medium (YNB) supplemented with 100 mg L^{-1} *p*-coumaric acid. Two milliliters of the culture media of all strains, which reached an OD 600 nm of 0.8, was centrifuged at 16.000× *g* for 10 min at 20 °C. The supernatant was collected and refrigerated at 4 °C until the analysis.

2.5. Principal Component Analysis and Clustering

Relative values regarding the growth rate and lag phase were calculated for each treatment condition used. This was achieved by dividing the absolute value of the physiological parameterwhen the cells were cultivated in one of the treatment conditions (combinatory effect of *p*-coumaric acid and mSO_2)—by the absolute value in the reference condition. Consumption of *p*-coumaric acid was defined as the percentage of the acid consumed at the end of the cultivation period. The production of off-flavours was recorded as the absolute concentration of the product at the end of the cultivation. All of the data were recorded in a CSV format for the ExcelTM worksheet and uploaded to ClustVis web tool. The clustering analysis followed the instructions available online by using the default parameters. The principal component analysis (PCA) used the singular value decomposition (SVD) method for single imputation, with unit variance scaling applied to rows, and data clustering based on correlations.

3. Results

3.1. Molecular Characterization of Yeast Strains

In the first screening, the yeast DNA was submitted to amplification of the ITS1-5.8S-ITS2 rRNA region and the results showed an amplicon of approx. 485 bp for all the yeast isolates (Figure 1). This specific species band pattern corresponded to what was reported for the specified *B. bruxellensis* [16]. After this, intraspecific characterization by RAPD using three OPA primers was performed and the resulting amplification profiles of each primer were combined to produce the strain-specific fingerprinting for each of the 16 isolates. The total number of bands obtained and the polymorphic bands were used to calculate the cophenetic correlation coefficient for the distance correlation defined by the binary indicator matrix (Table 1). With regard to this, the group with the highest coefficient is expected to be the best for describing the natural grouping of the matrix that is entered [20]. When the coefficient of cophenetic correlation is greater than 0.9, it can be interpreted as a very good fit, that is, there is a clear hierarchical structure between the objects, while values between 0.8 and 0.9 are considered to be good. On the other hand, values less than 0.8 or 0.7 are poor or very poor and suggest there is a clear disparity between the similarities and/or initial dissimilarities and those resulting from the graphic representation. This means that the data displayed in Table 1 are in an optimal range and show that the primers used were able to represent the similarity and/or dissimilarity between the RAPD patterns.

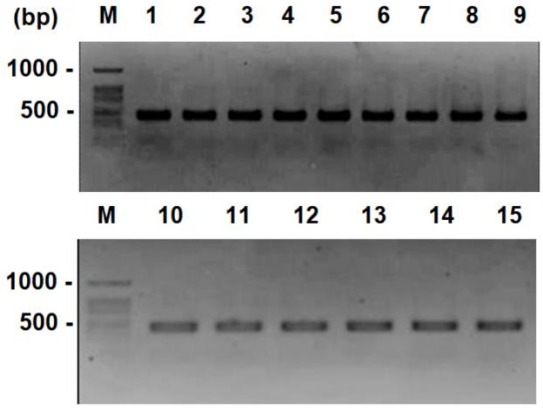

Figure 1. Amplification of the ITS1-5.8S-ITS2 segment from the DNA of *Brettanomyces bruxellensis* isolated from winemaking regions in Chile. Strains: L-2472 (lane 1), L-2474 (lane 2), L-2476 (lane 3), L-2480 (lane 4), L-2482 (lane 5), L-2570 (lane 6), L-2597 (lane 7), L-2676 (lane 8), L-2679 (lane 9), L-2690 (lane 10), L-2731 (lane 11), L-2742 (lane 12), L-2755 (lane 13), L-2759 (lane 14) and L-2763 (lane 15). 100 bp molecular weight was used (lanes M).

Table 1. Numbers and percentages of polymorphism of amplification products based on the different RAPD primers.

Primers	Total Number of Bands	Number of Polymorphic Bands	Polymorphism (%)	Cophenetic Correlation Coefficient
OPAD08	23	20	86.9	0.897
OPAE09	21	17	80.8	0.945
OPAE12	17	11	64.7	0.927
Total bands	61	48	77.5	

A cluster analysis of each isolate studied was conducted to assess whether there are differences between the strains with regard to the origin of isolation, and this was based on the primer with the highest percentage of polymorphism (OPAD08). This involved relying on the Jaccard similarity coefficient to cluster the isolates by means of the unweighted pair group method with arithmetic mean (UPGMA). The resulting dendrogram showed the differentiation of 15 isolates of *B. bruxellensis* (Figure 2). In this case, L-2763 and L-2731, which were isolated from the same winemaking process, showed full phenetic similarity and were considered to be isolates from the same clonal origin. For practical purposes, only L-2731 was used for the forthcoming experiments and comprised what will hereafter be called yeast genetic strains.

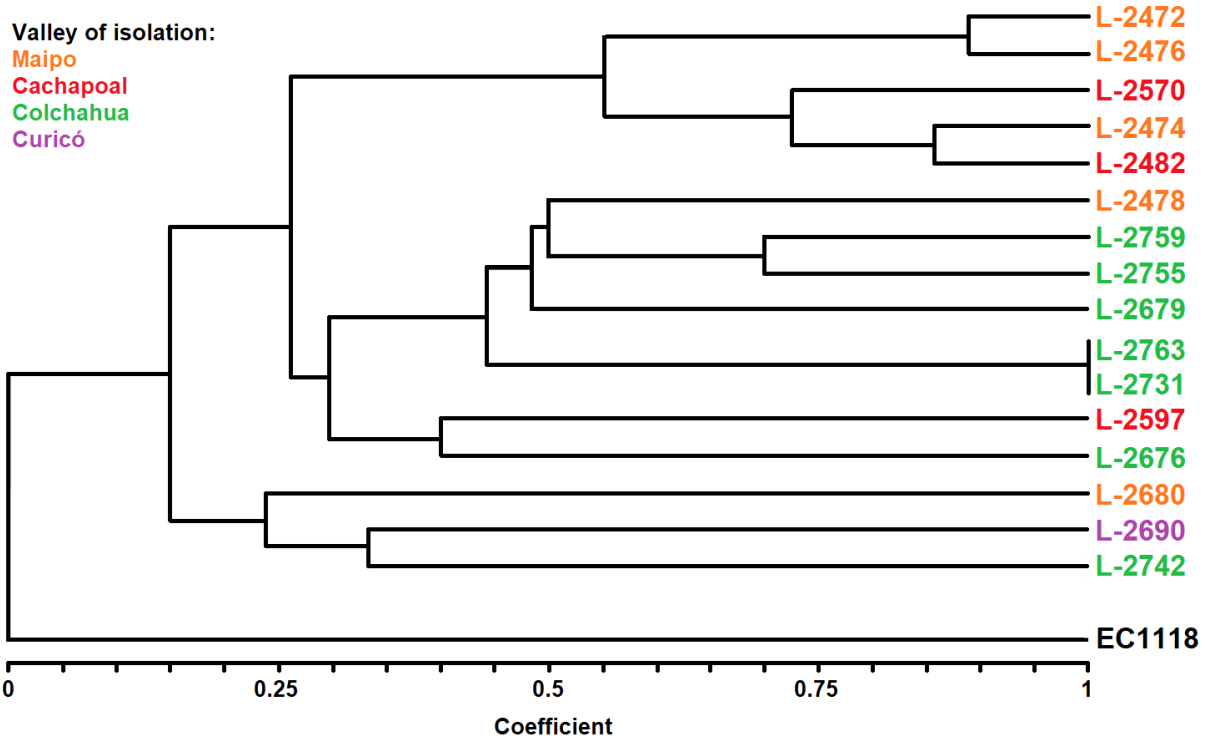

Figure 2. Dendrogram of genetic similarity among wine strains of *Brettanomyces bruxellensis* from Chile obtained by means of DNA fingerprinting patterns produced from RAPD technique using OPA series primers. The colors indicate the valley of origin of each strain.

3.2. Yeast Response to Inhibitors

Small-scale cultures were carried out with 15 strains in either the absence or presence of mSO_2, *p*-coumaric acid or a combination of both. In addition to their genetic differences, the 15 strains also had a similar growth profile in the presence of inhibitors, which confirms the degree of strain-specific sensitivity within the *B. bruxellensis* species (Figure 3). The first features that should be noted are the basic physiological characteristics of the wine isolates of *B. bruxellensis* in synthetic medium, which is designated the reference condition (Table 2). The very long lag phase varied from 19.5 h to 92.6 h (mean = 46.7 h; median = 38.9 h) while the very low growth rate varied from 0.003 h^{-1} to 0.035 h^{-1} (mean = 0.013 h^{-1}; median = 0.011 h^{-1}). Four out of 15 strains (L–2472, L–2474, L-2570 and L-2755) were very sensitive to mSO_2 and their growth was completely inhibited at 0.3 mg L^{-1} (Figure 3; Table 2). The strain L-2482 started growing in the presence of 0.6 mg L^{-1} of mSO_2 but only after a very long lag phase 83.3 h at an extremely low specific growth rate of 0.004 h^{-1} (Figure 3; Table 2). For this reason, most of the strains showed an increase in the lag phase in the presence of mSO_2 that corresponded to the metabolic adaptation to this inhibitor.

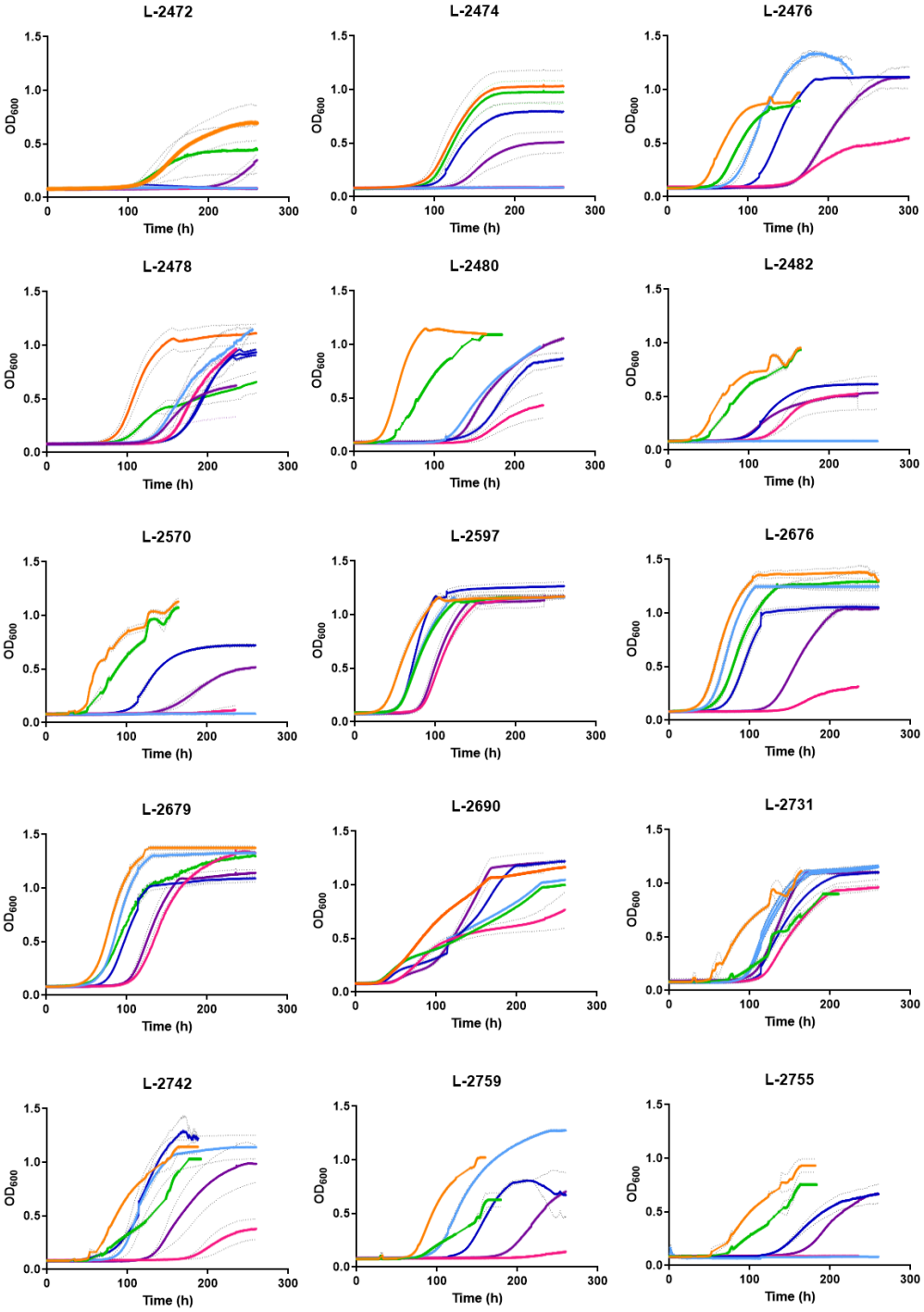

Figure 3. Growth curves of wine strains of *Brettanomyces bruxellensis* from Chile in synthetic (YNB) medium (orange curves),synthetic media supplemented with *p*-coumaric acid (100 mg L^{-1}) (green curves), SO_2 (0.3 mg L^{-1}) (light blue curves), SO_2 (0.6 mg L^{-1}) (Pink), SO_2 (0.3 mg L^{-1}) and *p*-coumaric acid (100 mg L^{-1}) (blue curves) and SO_2 (0.6 mg L^{-1}) and *p*-coumaric acid (100 mg L^{-1}) (purple curves).

Table 2. Growth rate and extension of lag phase of *Brettanomyces bruxellensis* strains used in the study isolated from several wine-producing regions of Chile cultivated in synthetic medium in the absence or presence of *p*-coumaric acid and sulphite.

		No Inhibitor		***p*-coumaric Acid (100 mg L^{-1})**		**SO2 (0.3 mg L^{-1})**		**SO2 (0.3 mg L^{-1}) + *p*-coumaric Acid (100 mg L^{-1})**		**SO2 (0.6 mg L^{-1})**		**SO2 (0.6 mg L^{-1}) + *p*-coumaric Acid (100 mg L^{-1})**	
Strain	**Valley**	**Lag (h)**	**μ (h^{-1})**	**Lag (h)**	**μ (h^{-1})**	**Lag (h)**	**μ (h^{-1})**	**Lag (h)**	**μ (h^{-1})**	**Lag (h)**	**μ (h^{-1})**	**Lag (h)**	**μ ($^{h-1}$)**
2472	Maipo	92.7 ± 2.5	0.005 ± 0.001	101.6 ± 4.6	0.002 ± 0.001	0	0	0	0	0	0	191.3 ± 2.499	0.003 ± 0.004
2474	Maipo	86.3 ± 1.6	0.013 ± 0.001	93.1 ± 3.5	0.014 ± 0.004	0	0	95.8± 5.648	0.010 ± 0.005	0	0	86.3 ± 3.22	0.003 ± 0.001
2476	Maipo	39.0 ± 1.3	0.016 ± 0.002	54.8 ± 2.6	0.013 ± 0.003	87.2 ± 3.0	0.022 ± 0.004	111.4 ± 8.562	0.017 ± 0.004	135.6 ± 4.9	0.002 ± 0.001	139.6 ± 4.441	0.011 ± 0.003
2478	Maipo	84.2 ± 0.7	0.018 ± 0.001	44.0 ± 1.7	0.003 ± 0.001	125.4 ± 5.6	0.009 ± 0.002	159.5 ± 3.885	0.011 ± 0.002	148.2 ± 3.7	0.011 ± 0.003	108.4 ± 2.554	0.005 ± 0.001
2680	Maipo	31.6 ± 2.2	0.035 ± 0.002	35.3 ± 2.4	0.010 ± 0.002	96.4 ± 4.9	0.010 ± 0.003	138.0 ± 2.445	0.008 ± 0.003	123.9 ± 4.2	0.003 ± 0.001	121.8 ± 2.645	0.009 ± 0.002
2482	Cachapoal	18.5 ± 1.7	0.011 ± 0.004	22.0 ± 3.6	0.008 ± 0.002	0	0	85.4 ± 4.533	0.007 ± 0.003	86.3 ± 2.5	0.004 ± 0.001	42.3 ± 3.441	0.003 ± 0.004
2570	Cachapoal	31.5 ± 1.4	0.014 ± 0.006	38.4 ± 3.7	0.014 ± 0.004	0	0	75.6 ± 2.114	0.009 ± 0.001	0	0	114.0 ± 2.566	0.003 ± 0.006
2597	Cachapoal	46.1 ± 2.4	0.021 ± 0.002	62.1 ± 3.1	0.019 ± 0.005	54.9 ± 4.3	0.022 ± 0.003	57.0 ± 2.159	0.029 ± 0.002	82.1 ± 4.3	0.019 ± 0.003	81.9 ± 1.823	0.022 ± 0.002
2676	Colchahua	49.7 ± 5.0	0.003 ± 0.002	67.9 ± 4.2	0.01 ± 0.002	51.8 ± 4.5	0.027 ± 0.004	71.8 ± 3.299	0.021 ± 0.002	139.4 ± 4.8	0.003 ± 0.002	125.4 ± 4.411	0.012 ± 0.003
2679	Colchahua	21.7 ± 3.2	0.008 ± 0.001	57.6 ± 2.9	0.014 ± 0.003	68.5 ± 2.4	0.026 ± 0.002	77.3 ± 4.558	0.021 ± 0.002	109.6 ± 2.6	0.016 ± 0.003	104.9 ± 2.956	0.019 ± 0.002
2731	Colchahua	35.3 ± 1.5	0.010 ± 0.003	79.0 ± 4.2	0.007 ± 0.001	94.5 ± 4.3	0.017 ± 0.002	105.9 ± 6.05	0.013 ± 0.002	112.0 ± 5.3	0.011 ± 0.003	107.3 ± 2.461	0.018 ± 0.004
2742	Colchahua	43.8 ± 2.4	0.011 ± 0.004	46.7 ± 4.2	0.009 ± 0.002	95.5 ± 2.3	0.018 ± 0.003	84.3 ± 3.24	0.016 ± 0.004	124.6 ± 4.6	0.024 ± 0.002	127.6 ± 3.224	0.010 ± 0.001
2755	Colchahua	34.0 ± 2.7	0.007 ± 0.001	59.3 ± 3.3	0.008 ± 0.001	0	0	111.9 ± 4.66	0.005 ± 0.002	0	0	145.8 ± 2.163	0.005 ± 0.001
2759	Colchahua	66.3 ± 3.6	0.014 ± 0.004	78.3 ± 2.6	0.007 ± 0.002	93.2 ± 4.3	0.013 ± 0.001	131.2 ± 3.685	0.013 ± 0.002	0	0	176.9 ± 4.998	0.007 ± 0.003
2690	Curicó	19.5 ± 2.8	0.009 ± 0.002	24.111 ± 2.4	0.004 ± 0.001	23.0 ± 2.5	0.005 ± 0.001	41.9 ± 4.627	0.007 ± 0.001	28.5 ± 1.4	0.006 ± 0.002	80.7 ± 1.437	0.012 ± 0.003

When observing the growth kinetics of the strains studied only in the presence of 100 mg L^{-1} of *p*-coumaric acid, all showed an increase in the adaptation phase, varying from 21,998 h to 101,563 h (mean = 57.6 h; median = 57.6 h). Of the 15 haplotypes, five isolates were the only ones that presented an increase in their specific growth rate (L 2474, L 2597, L 2676, L 2679 and L 2763), of which the isolated L 2679 stands out, increasing this parameter by 75% from 0.008 (h^{-1}) to 0.014 (h^{-1}).

3.3. Response of Yeast Strains to p-coumaric Inhibitors Acting Simultaneously with mSO_2

The combined effects of mSO_2 and *p*-coumaric acid were tested to evaluate the effectiveness of mSO_2 as a contaminant-controller when there is a significant concentration of *p*-coumaric acid in the grape must. Overall, the presence of *p*-coumaric in the medium increased the lag time and reduced the growth rate by 30% in both cases. Despite the expected dose-dependent inhibitory effects of increasing concentrations of mSO_2, an unexpected and significant protective effect of *p*-coumaric acid was observed on the mSO_2 toxicity. For example, at the mean value for the 15 strains, the cells grew in the presence of 0.6 mg L^{-1} mSO_2 at 39% of the growth rate of the reference condition. This was increased to 77% of the reference growth rate when *p*-coumaric acid was also present in the medium. Similarly, the extension of the lag phase obtained by the mSO_2 dosage was reduced by *p*-coumaric from 135 h to 113 h as a mean value. It should be noted that the L−2474, L-2570 and L-2755 strains showed no growth in the presence of mSO_2 at 0.3 or 0.6 mg L^{-1}, but their growth was restored when the medium was supplemented with *p*-coumaric acid (Table 2). This means that, for the first time, we can show an antagonistic interaction, in which *p*-coumaric acid decreases the toxic effect of SO_2. Furthermore, it has important implications for the winemaking process, where the grape must is used with high concentrations of *p*-coumaric contaminated by *B. bruxellensis*.

3.4. Metabolic Products in the Yeast Cultures

In the light of the results discussed above, *p*-coumaric acid turns out to be significant not only because it produces off-flavours, but also on account of its protective action against the toxic effects of SO_2. Thus, there was a need to test the capacity of the yeast strains to consume and metabolise this molecule. All the *B. bruxellensis* strains were cultivated in synthetic medium in the presence of *p*-coumaric acid (100 mg L^{-1}) as a precursor of the biosynthesis of the off-flavour volatile phenols 4-vinylphenol (4-VP) and 4 ethylphenol (4-EP). These three molecules form a part of the same pathway in which *B. bruxellensis* first converts *p*-coumaric acid to 4-VP via phenylacrylic acid decarboxylase (*PAD*) and then 4-VP is reduced by vinyl phenol reductase (VPR) to 4-EP. The average rate of *p*-coumaric consumption among the strains was 63.9% of its initial concentration, ranging from 15.5% for the L-2742 strain to 100% in the cases of L-2570 and L-2679 (Figure 4A). With regard to their resulting metabolization, the production of 4-VP ranged from zero to 94 mg L^{-1}, while the production of 4-EP ranged from zero to 78 mg L^{-1}. Most of the strains converted 100% of the consumed *p*-coumaric acid to at least one of these phenyl-derivatives, with variations among the strains (Figure 4B). In most of the strains, the percentage of 4-VP that was released to the medium was higher than the percentage of 4-EP, which means that the VPR enzyme is only partially working in these strains. This might be the result of enzyme deficiency or a lack of reducing power (NAD(P)H) for the reductive reactions. On the other hand, L-2570 converted 100% of *p*-coumaric to 4-EP (Figure 4B). Interestingly, the L-2679 strain consumed 100% of the *p*-coumaric acid added to the medium without producing any of the phenyl derivatives. This means that there might be another metabolic route for *p*-coumaric metabolization that does not convert its carbon chain to either 4-VP or 4-EP. In the same area, it can also be concluded that 34% of the *p*-coumaric acid consumed by the L-2759 strain was metabolised by this alternative pathway (Figure 4B).

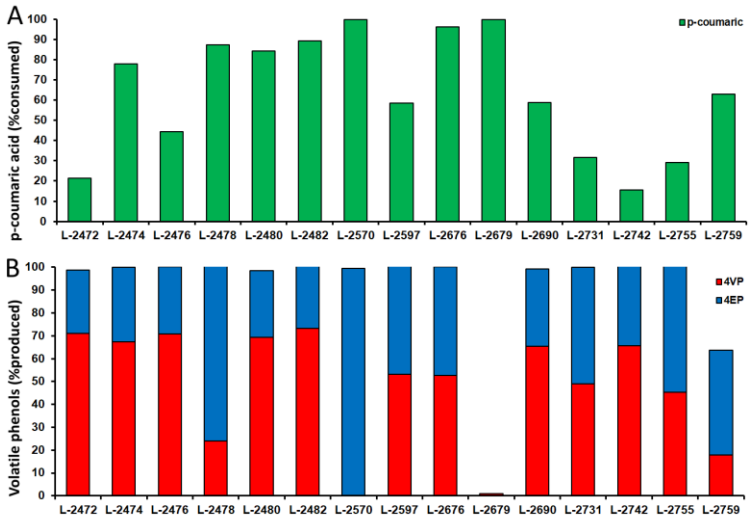

Figure 4. Relative consumption of *p*-coumaric acid (panel **A**) and relative production of volatile phenols 4-vinylphenol (4-VP) (red columns) and 4-ethylphenol (4-EP) (blue columns) (panel **B**) in cultures of wine strains of *Brettanomyces bruxellensis* from Chile.

3.5. Cluster Analysis of the Brett Wine Strains

Multivariate analysis was conducted based on four physiological conditions: extension of lag phase, growth rate, *p*-coumaric acid consumption and the production of volatile phenols. A relative index was used for the first two parameters, which means the ratio between the absolute value for each culture in treatment and the absolute value of the reference condition. The principal component analysis showed that the first component was responsible for 34.9% of the total variation and the second component for 22.3% (Figure 5A). Most of the strains showed a physiological profile that fluctuated around the median value of all 15 strains, with the exception of L-2676 and L-2679 strains (Figure 5A). By analyzing the data shown in Table 2, these strains showed a growth rate that was higher in all inhibitor-supplemented media than in the reference condition . Hence, these strains can be classified as highly tolerant to both agents (SO_2 and *p*-coumaric acid). Overall, these strains only converted 50% of *p*-coumaric acid to 4-EP (Figure 4B), which revealed that this is not connected to sulphite tolerance. In addition, data clustering revealed some interesting features in the interaction between physiological parameters and yeast biodiversity (Figure 5B). First, there was the clustering of the set of data regarding the growth rate in the presence of inhibitory agents, together with the capacity of the strains to consume *p*-coumaric acid. However, this did not correlate with their ability to convert this substrate to phenyl derivatives, since L-2679 consumed all the *p*-coumaric acid provided and did not lead to any of these products (Figure 4). In fact, the ability to produce 4-VP and 4-EP was not related to the growth rate or to the extension of the lag phase (Figure 5B), which suggests that it is an independent variable with regard to sulphite tolerance. Moreover, the clustering analysis showed that there is no geographical distribution of the yeast strains, which is evidence that the ecological and industrial conditions of the different regions did not exert enough selective pressure to influence the physiological characteristics of the native strains. This result is in accordance with the genetic fingerprinting analysis that showed a greater diversity among the strains.

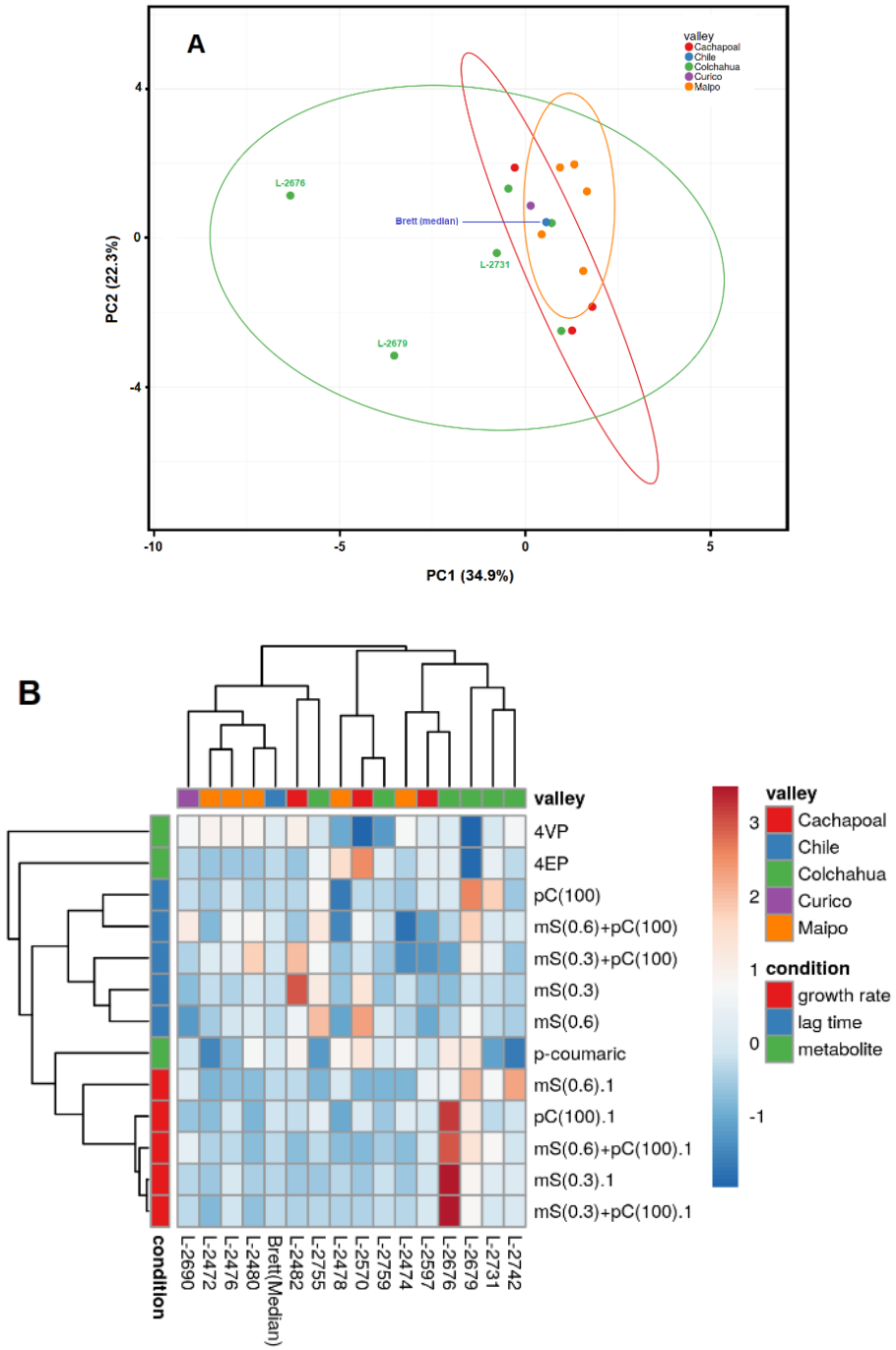

Figure 5. Multivariate analysis conducted on the basis of four physiological datasets of wine strains of *Brettanomyces bruxellensis* from Chile. Yeasts were cultivated in synthetic (YNB) medium supplemented with *p*-coumaric acid at 100 mg L^{-1} for evaluation of *p*-coumaric acid consumption (*p*-coumaric) (dataset 1) and production of 4-vinylphenol (4-VP) and 4-ethylphenol (4-EP) (dataset 2). Yeast cells were cultivated in synthetic media supplemented with *p*-coumaric acid 100 mg L^{-1} (pC(100)), SO_2 0.3 mg L^{-1} (mS(0.3)), SO_2 0.6 mg L^{-1}(mS(0.6)), SO_2 0.3 mg L^{-1} and *p*-coumaric acid 100 mg L^{-1} (mS(0.3) + pC(100)) or SO_2 0.6 mg L^{-1} and *p*-coumaric acid 100 mg L^{-1} (mS(0.3) + pC(100)). Values were calculated for the extension of lag phase (dataset 3) and exponential growth rate (dataset 4) and reported as relative values to cultivation in medium without inhibitors. **A**: Principal Component; **B**: Clustering.

4. Discussion

Sixteen strains of *B. bruxellensis* that were isolated from fermentation processes in various regions of Chile were used to assess their genetic diversity and physiological characteristics with regard to their tolerance to *p*-coumaric acid and metabisulphite. Phenetic discrimination of the strains was carried out by RAPD, using three primers of the OPA series (OPAE09, OPAD08 and OPAE12) and this showed a high degree of discrimination with 15 separate fingerprints. Previous studies have demonstrated the importance of the choice of primers and the need to employ the technique to obtain optimal results [21,22]. For example, Miot-Sertier employed a method of typing to distinguish between different isolates of *B. bruxellensis* by RAPDS, by using four different primers (including OPAE09). The results showed that there was low heterogeneity in each group, in addition to a limited number of specific patterns per strain [23]. In contrast, Mitrakul showed that the PCR profiles generated by the OPAE09 primer were sufficient to type the *B. bruxellensis* strains studied [21,24]; this demonstrates that conditions for this experiment must be strictly standardized to ensure good reproducibility and accurate profiles.

By grouping the isolates from the same valley, each belonged to a different group, which means that, although this technique allows the strains to be genetically differentiated, they cannot be grouped by region or province [25]. The yeast strains that were isolated from the same wine region belonged to different phenetic groups. Hence, it is clear that this technique was sufficiently robust for the individual discrimination of the Brett isolates, since it succeeded in separating those that are different and grouping those that represent the same biological entity. However, this technique cannot be used to predict the physiological characteristics of a given isolate. This fact was previously described by Godoy [26], who, when differentiating 12 strains of *B. bruxellensis* using RAPD, did not find any relationship between the patterns of the isolates and their growth rates or enzymatic activities. For example, the phenetic group formed by L-2472 and L-2476 also yielded similar physiological data with regard to tolerance to the inhibitors. On the other hand, L-2480 shared a similar response to L-2472 and L-2476 with regard to the effect of inhibitors on the growth rate and only showed 15% of phenetic similarity to these strains. A similar situation was observed for L-2570 and L-2759, which were closely related in physiology, but remained unrelated by RAPD.

The strains identified by RAPD were physiologically characterized, and their ability to produce volatile phenols was evaluated, in addition to their growth and survival under stressful conditions. In assessing the ability of the different phenetic groups to produce volatile phenols, the culture medium was supplemented with 100 mg L^{-1} of *p*-coumaric acid, since the aromatic compounds (4-VP and 4-EP) are produced as a result of the metabolization of this hydroxycinmic acid [7]. Fourteen out of fifteen strains were capable of producing 4-VP, which is evidence that they all have phenylacrylic acid decarboxylase activity [27]. Moreover, most of them produced 4-VP as the result of active vinylphenol reductase [26]. The exception was the L-2679 strain that consumed all the *p*-coumaric acid without converting it to phenyl derivatives. This strain also had a high tolerance to SO_2. Further experiments should be carried out to disclose this alternative pathway of *p*-coumaric acid metabolization.

The concentrations of volatile phenols were heterogeneous in the set of strains tested, without being correlated with their place of origin. This feature indeed showed that the capacity of producing off-flavours does not seem to be linked to the ecological and industrial conditions of the winemaking processes, even when the strains are genetically close [28].

Additionally, the effect of *p*-coumaric acid on the fitness of the different strains was evaluated. This substrate is commonly found in grape musts, so that its influence on the yeast performance might be an important feature in yeast adaptation and yeast spoiling activity. All of the samples showed the same general trend—they experienced a delay in the adaptation phase. This is mainly due to the inhibitory effect of hydroxycinnamic acids, which are weak organic acids that alter the intracellular pH and cellular metabolism [7]. In *B. bruxellensis*, it was found that the H^+-ATPase pump (homologous to Pma1p in *S. cerevisiae*) plays a key role in reducing the concentration of intracellular protons during its adaptation to *p*-coumaric acid [17], which can be observed in changes in its growth kinetics. However, the data so far obtained cannot establish a direct connection between the presence of *p*-coumaric acid,

and yeast tolerance to sulphite, which has serious implications for cytoplasmic acidification and ATP demand.

In the wine industry, SO_2 is one of the most widely used additives due to its antimicrobial and antioxidant properties [29]. In general, the concentration of mSO_2 used to prevent the growth of *B. bruxellensis* ranges from 0.2 to 0.8 mg L^{-1} [30,31]. The kinetic response of the fifteen identified strains was evaluated in synthetic minimum (YNB) and synthetic media supplemented with 0.3 or 0.6 mg L^{-1} of mSO_2. In the case of most of the isolates, the presence of SO_2 led to an increase in the adaptation phase, observed as the extension of the lag phase, as its concentration in the medium increased. However, the values obtained for the specific growth rate showed variations between the strains tested, regardless of the concentration of SO_2 added. Five out of 15 strains tested were susceptible to the lowest SO_2 concentration (0.3 mg L^{-1}). One of the most widely known mechanisms of SO_2 tolerance involves the activity of the SSU1 sulphite pump [32,33], a member of the Tellurite-resistance/dicarboxylate transporter (TDT) family. Diversity in sulphite tolerance has been found in strains from different geographical areas and with varying physiological patterns of behaviour [34,35], which suggests that this characteristic can be determined by quantitative factors. This is very important information since it is strongly recommended that mSO_2 should be used when less tolerant genotype groups are included in the process [36]. However, this cannot apply to processes contaminated with L-2676 or L-2679 clonal groups. The existence of allotriploidy and genotype diversity in *B. bruxellensis* has been linked to high tolerance to SO_2 [31–38], and has been attributed to the selection that occurs when adding this antimicrobial to the wide range of fermented beverages. If allotriploidy is the key factor in these two strains, this is a matter that requires further investigation.

The final quality of the wine results from a complex combination of factors, and these may be influenced by the type of hydroxycinnamic acid present in the grape must and the presence of residual SO_2 and spoiling *B. bruxellensis* cells [39]. It is thus of great importance to evaluate the combined effect of these antimicrobial compounds on the fitness of the spoiling yeast. According to the results of Table 2 and Figure 4, tolerance to mSO_2 and consumption of *p*-coumaric acid, including its conversion to volatile phenols, depend on both the substrate and the strain [39]. Potassium metabisulphite, which is one of the most widely used microbial contamination controllers during wine fermentation [40], can either kill the cells or simply impair cell growth. This last condition is described as a viable but not a cultivable state (VBNC) after sulphite stress [40]. In this scenario, it is essential to determine what kind of situations can interfere with this condition to ensure the full inhibitory activity of sulphite is retained, and to prevent its use at a higher concentration that can lead to a selection of tolerant strains and/or harm the sensory characteristics of the end product. To the best of our knowledge, this study has shown for the first time that *p*-coumaric acid can alleviate the inhibitory effect of sulphite for some strains, and thereby improving their fitness. Previous studies have revealed that the effect of different antimicrobial compounds against *Saccharomyces cerevisiae* present an unexpected variety of cellular responses when used in different concentrations and combinations [41], making it clearly complex to determine compound–compound interactions and the cellular response. In this sense, the improvement in yeast growth when exposed to both compounds could be due to more efficient responses by the cell, such as defense mechanisms (changes in metabolism, absorption and excretion of compounds, etc.) that allow it to survive more easily, compared to the mechanisms that are activated when the compounds are separated. We believe this effect should be the aim of future investigations in the field to ensure better control of spoiling yeast in winemaking processes.

5. Conclusion

This study confirmed that there is genetic diversity among yeast isolates of *B. bruxellensis* in different wine-producing regions of Chile, which is also represented by variability in the physiological characteristics regarding *p*-coumaric consumption and metabolization and sulphite tolerance. Despite the limitations with regard to the data that establishes a direct involvement of *p*-coumaric metabolization and sulphite tolerance, our multivariate analysis strongly suggested that this feature is

also influenced by biological variables other than the SSU1 sulphite pump activity. In light of this, the L-2676 and L-2679 strains offer a suitable biological platform for carrying out in-depth studies on sulphite tolerance. In addition, the observed effects of *p*-coumaric in alleviating sulphite toxicity are of paramount importance. This is because it can provide a useful means of establishing the mechanisms that allow *B. bruxellensis* to tolerate different stressors, and thus forms the basis for future research into ways of determining the effects, occurrence and distribution of this industrially important yeast.

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ANEXO C

Selection of *Saccharomyces cerevisiae* isolates for ethanol production in the presence of inhibitors

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Keyla Tortoló Cabañas[a], Irina Charlot Peña Moreno[c], Denise Castro Parente[c], Antonio Bell García[a], Roxana García Gutiérrez[b], Marcos Antonio de Morais Jr[c*].

[a]Department of Alcohol, Yeasts and Animal Food and [b]Department of Microbiology, Cuban Research Institute of Sugar Cane Byproducts (ICIDCA), P. O. Box 4026, San Miguel del Padrón- 11 000, La Habana, Cuba.

[c]Department of Genetics, Federal University of Pernambuco (UFPE), Av. Moraes Rego, 1235 Cidade Universitária 50670-901, Recife, PE, Brazil.

*Author for correspondence:

Marcos A Morais Jr (ORCID 0000-0002-9727-0300)

Departamento de Genética – Universidade Federal de Pernambuco

Av. Moraes Rego, 1235, Cidade Universitária 50670-901 Recife PE Brasil. E-mail: marcos.moraisjr@ufpe.br Phone: 55-81-21267817 Fax: 55-81-21268522

Abstract

Eight yeast isolates identified as Saccharomyces cerevisiae were recovered from molasses-using Cuban distilleries and discriminated by nucleotide sequence analysis of ITS locus. The isolates L/25-7-81 and L/25-7-86 showed the highest ethanol yield from sugarcane juice, while L/25-7-12 and L/25-7-79 showed high ethanol yield from sugarcane molasses. The isolate L/25-7-86 also displayed high fermentation capacity when molasse was diluted with vinasse. In addition, stress tolerance was evaluated on the basis of growth in the presence of inhibitors (acetic acid, lactic acid, 5-hydroxymethylfurfural and sulfuric acid) and the results indicated that L/25-7-77 and L/25-7-79 congregated the highest score for cross-tolerance and fermentation capacity. Hence, these isolates, especially L/25-7-77, could serve as potential biological platform for the arduous task of fermenting complex substrates that containing inhibitors. The use of these yeasts was discussed in the context of second-generation ethanol and the environmental and economic implications of the use of vinasse, saving the use of water for substrate dilution.

Keywords: fermentation capacity; 5-HMF; stress resistance; vinasse; yeast selection; weak organic acid

Introduction

Lignocellulosic ethanol offers the opportunity to revalorize low-cost resources in addition to conventional feedstock used for first generation fermentations (Naik et al. 2010). Biomass pre-treatment stage represents the biggest challenge for this technology due to, among other issues, the formation of compounds that hampers a key microbial cell factory of the Saccharomyces cerevisiae yeast (Naik et al. 2010).

There are several metabolites that present such inhibitory activity which can be mostly divided in three groups. First, there are the aromatic aldehydes like furfural (2-furaldehyde) and 5-hydroxymethylfurfural (5-hydroxymethyl-2-furaldehyde or HMF) formed by dehydration of pentoses and hexoses, respectively, during thermal-acid treatment of lignocellulose (Liu et al. 2009). Their concentrations in the hydrolysates can reach up to 6 g/L depending on the source of biomass (Larson et al. 1999; Aguiar et al. 2002). This type of molecules can also be found in industrial substrates like molasses, although at lower concentrations, as by-product of sugar dehydratation during milling process (García and Otero 2015). They affect growth and fermentation capacity of the yeast cells by inhibiting key enzymes of the central metabolism, interfering with the rate of protein synthesis and/or increasing the energy demand as ATP and NAD(P)H for repairing damages to cellular structures (Sanchez and Bautista 1988; Modig et al. 2002; Almeida et al. 2008). NADPH-dependent oxo-reductases are very important for the detoxification of these inhibitors (Almeida et al. 2008; Liu et al. 2009; Sehnem et al. 2013).

The second group of molecules comprises the weak organic acids, as lactic and acetic acids, also produced from the pre-treatment of the biomass and/or are produced by the contaminant yeasts and lactic acid bacteria (Makanjuola et al. 1984; Bischoff et al. 2009; Beckner et al. 2011; Basso et al. 2014). These molecules in the environment with low pH (below 4.0) are mostly protonated and enter to the cells by diffusion. Once inside at neutral pH of the cytoplasm (pH 6.8), the molecules dissociate to H+ and the anions lactate or acetate that lead to disturbance of cellular homeostasis. Under this condition, yeast cells are required to use energy to drive the efflux pumps to get rid of these inhibitors. Again, both growth and fermentation capacity are compromised.

Lastly, the most common practice of biomass pre-treatment involves the heating the lignocellulose suspended in acid solution (usually diluted sulfuric acid solution). Hence, the produced hydrolysate, despite of the already commented inhibitors, also presents the acidic characteristic that imposes acid stress to the yeast cells (De Melo et al. 2010). Even that such stressing condition is not so detrimental to the fermentation capacity, it can compromise cell viability and its recycling during the harvest season.

Therefore, the search for strains that deal with this type of stress is also relevant in the context of production of second-generation ethanol.

Cuban distilleries have the characteristics of almost exclusively use molasses as substrate for fermentation, since sugar mills are mostly oriented for the production of sugar. As consequence, the molasses might be exhausted in terms of sugar content while concentrated in various molecules like those mentioned above. The yeast populations in the processes have been poorly characterized, as well as the physiological profile of the S. cerevisiae population (both the commercial and the native strains). Hence, the yeasts could exhibit distinct traits associated to local fermentation stimuli and should be very adapted to hard conditions as consequence of the continuous selective process accumulated along the decades of fermentations. Therefore, this kind of process could be considered an interesting source of naturally evolved strains with relevant capacities to produce ethanol even in unfavorable conditions. In the present study, we have surveyed a group of eight local industrial S. cerevisiae strains regarding ethanol yield in four different substrates and their resistance to inhibitors and revealed relationships between these parameters. The quantification of this cross-analysis allowed us to identify isolates with superior performance for their use to ferment substrates of hard condition.

Materials and Methods

Sampling, cell maintenance and molecular identification

Seven industrial must samples were collected directly from fermentation vessels and as a single, one from fermenter´s slurry in six bio-ethanol distilleries located along Cuba Island, at different crop harvesting periods. All distilleries produce ethanol from sugar cane molasses, diluted to approximately 120 g of total inverted sugar per liter of substrate. Samples were diluted in sterile saline and plated on Wallerstain Nutrient medium (WLN) (Da Silva-Filho et al. 2005a) to produce approximately 10 CFU/plate. Colonies showing prospective Saccharomyces spp. cellular and cultural WLN-morphotype were isolated and named as L/25-7-12, L/25-7-77, L/25-7-79, L/25-7-80, L/25-7-81, L/25-7-82, L/25-7-86 and L/25-7-90. Stock cultures were maintained in glycerol at -80°C.

Molecular identification was performed by sequencing the ITS1-5.8S-ITS2 rDNA locus (Da Silva-Filho et al. 2005a; Basilio et al. 2008) using an ABI prisma 3500 device (Applied Biosystems), previous cells culture in Yeast Extract-Peptone-Dextrose (YPD) broth. Total DNA was extracted, checked for quality and purity and the concentration evaluated (Basilio et al. 2008). The ITS locus was amplified with the use of the primers ITS4 (5´-TCCTCCGCTTATTGATATGC-3´) and ITS5 (5´-GGAAGTAAAAGTCGTAACAAGG-3´) according to Basilio et al. (2008) and sequenced for both strands (two times each) in ABI prisma 3100 platform (Applied Biosystems, USA). Sequence output files were analyzed by BioEdit v7.0 package and submitted to BLAST analysis in the NCBI nucleotide database (http//www.ncbi.nlm.nih.gov). Yeast identification was assumed if the query sequence

showed >99%identity with DNA sequences from yeast type strain deposited at NCBI (Basilio et al. 2008). The industrial Saccharomyces cerevisiae strain JP-1 (Da Silva-Filho et al. 2005a, b) was used as reference for molecular characterization. Nucleotide sequences of 140 bp of ITS1 locus were recovered and analyzed in Bioedit™ software and aligned with ClustalW tool (Hall et al. 1999). Clustering analysis was performed in MEGA v7.0 software using UPGMA method (Kumar et al. 2016). For comparison, nucleotide sequences from S. cerevisiae S288c (internal reference) and Candida glabrata NRRL Y-68 (phylogenetically closed to Saccharomyces group outgroup control) were recovered from NCBI/Genebank and included.

Fermentation trials

Three sugarcane byproducts-based fermentation medium (FM) were defined: FM1 with sugar cane juice, FM2 with sugar cane molasses and FM3 consisting of a mix formulation with sugar cane molasses and distillery vinasse. All substrates were provided by the Sugar Cane Experimental Station of Pernambuco (EECA-PE), Brazil, centrifuged to remove insoluble solids and adjusted to 13(±1) °Brix with distilled water (FM1 and FM2) or distillery vinasse (FM3) and to pH 4.8 whenever necessary. These industrial substrates were prepared just before the experiments and used without sterilization to mimic the industrial process. A control fermentation medium mimicking industrial C:N ratio was prepared with YNB (1.6 g/L), sucrose (120 g/L) and ammonium sulphate (1.0 g/L), adjusted to pH 4.8 and sterilized by filtration with sterile 0.22 □m filters.

Yeast seed cultures were prepared by cultivating the cells on YPD broth for 48 hours at 30ºC followed further growth for 24 h after adding fresh YPD sterile medium. Cells were collected by centrifugation, twice sterile-washed and 4°C-stored cell cultures until use. One gram of cells was transferred to 20x180-mm tubes closed with a small off gas tubing containing 15 mL of fermentation media. Fermentation trials were conducted in three biological replicates as previously described (Dutra et al. 2013). Samples were taken at the beginning and the tubes were incubated at 33°C without agitation. Total suspended solid was monitored by manual refractometer for °Brix decay for eight hours, after which samples were taken at the final fermentation time.

Samples from the start and end of fermentation were centrifuged to separate cells from supernatant, which were used for determination of residual sugar and production of ethanol (E), glycerol (Gly) and acetate (Ace). For this purpose, samples were prepared by filtration through a sterile 0.22 µm filter and freezing at -20°C until analysis. Samples were separated by HPLC Agilent device in Aminex HPX-87H Biorad column at 60°C, using 8 mM H_2SO_4 solution as mobile phase at a flow rate of 0.6 mL/min. Detection of metabolites was performed by UV and by refractive index type detectors. The following parameters were determined:

Ethanol yield ($Y_{p/s}$) = $(\Delta E)/(\Delta °Bx * 14.5)$ expressed as [g/g] Eq.1

Specific ethanol productivity (Q_p) = $(\Delta E)/(Q_w * t)$ expressed as [g/(L. h)] Eq.2

Ratio of glycerol per ethanol (K_{Gly}) = $(\Delta Gly)/(\Delta E)$ Eq. 3

Where ∆E, ∆Gly and ∆Brix were the difference between final and initial concentration of ethanol, glycerol and ºBrix, respectively; Qw was the final volume of fermentation wort with cell; t was the fermentation time and the constant 14.5 value was the fixed coefficient which correlate reduction of ºBrix and glucose depletion (unpublished results).

Yeast tolerance to fermentation inhibitors

Synthetic growth medium consisted of YNB (0.67 g/L), glucose (20 g/L) and ammonium sulphate (5 g/L) with initial pH of 5.5, supplemented or not with one of the inhibitors. The inhibitors acetic acid and lactic acid were added to synthetic medium to concentrations of 5 g/L or 15 g/L, while 5-hidroxymethyl furfural (5-HMF) was added to 5 g/L or 6 g/L (Taherzadeh et al. 2000; Sehnem et al. 2013). In addition, synthetic medium was adjusted to initial pH of 2.0 with sulfuric acid to mimic stress by inorganic acid (10 mM dissociated H+) in the industrial processes. Moreover, the protective action of magnesium (Barros de Souza et al. 2015) was tested by adding magnesium sulfate (7 H2O) to 0.5 g/L of synthetic medium, with or without the inhibitors. That concentration referred to the Mg2+ cation. All media were sterilized by filtration with 0.22 µm sterile Millipore membranes. For all these combinations, the volume of 135 µL of synthetic media were distributed in sterile 96-well microtiter plates.

Seed cultures were prepared by cultivating yeast strains in 5 mL of YPD broth at 30°C during 16 hours at 170 rpm and 15 µL of the cells were used to inoculate media combinations above, for the total volume of 150 µL. These plates were incubated in Sinergy HT Microplate Reader (Biotek, Switzerland) at full speed and 30°C during 24 hours with continuous automated monitoring of absorbance at 660 nm (A660) every hour. All the experiments were carried out in biological replicates (n=2) with technical triplicate for each condition. Data were recovered as xls files and processed in Excel™ worksheet to prepare graphics of growth curves. Yeast growth rates were calculated from the slope of exponential growth phase by plotting Ln(A660) versus time. Net increments in biomass (ΔX) were determined as the difference between A660 at the beginning and at the end of cultivations and used to estimate the level of tolerance of the strains to the inhibitors relative to reference synthetic medium without inhibitors and Mg+2.

Statistical Analyses

Tukey's test was used to compare means and differences with a p value < 0.05 were considered significant.

Results and Discussion

Molecular yeast identification

Since the fermentation media are not sterilized prior to fermentation, microbial contaminants are continuously introduced to the distillery environment, resulting in a dynamic competition between the desired inoculated strain, native yeast strains and

bacteria (Da Silva-Filho et al. 2005a; Basilio et al. 2008; Lucena et al 2010; Beckner et al. 2011; Basso et al. 2014). Ethanol fermentation industry relies only on a slight portion of yeasts diversity (Della-Bianca et al. 2013; Steensels et al. 2014), sometimes overlooking the potential of native strains to overcome harsh environments with high ethanol yields (Da Silva-Filho et al. 2005b; Basso et al. 2008; Lancheros et al. 2015). The huge physiological diversity among the clonal strains, consequence of an intense process of adaptation to a specific type of substrate, stimulate the search for the most adequate strain for specific substrates. In this context, eight distillery yeast strains recovered from Cuban ethanol industry were identified as S. cerevisiae using molecular markers and tested for the first time screened for their profiles of fermentation capacity and tolerance to inhibitors. DNA extracted from eight representative yeast colonies recovered from molasses-using Cuban distilleries samples was submitted to DNA sequencing of the ITS1-5.8S-ITS2 rDNA region. All strains produced an amplicon of 850 bp (data not shown), similar to JP-1 S. cerevisiae strain (Basilio et al. 2008). All DNA sequences obtained from yeast strains presented >99% identity to S. cerevisiae sequences in the NCBI database (data not shown) and confirmed the identity of the yeast strains (Basilio et al. 2008). Furthermore, nucleotide sequences of 140 bp region of ITS1 locus were used to verify the intraspecific variability among the isolates by clustering analysis. The results showed that all isolates were separated due to the presence of polymorphic positions in nucleotide sequences (Fig. 1), characterizing their distinct clonal origin. Therefore, the isolates were genetically different and in the henceforth analysis were performed to test whether these differences also reflected in physiological traits.

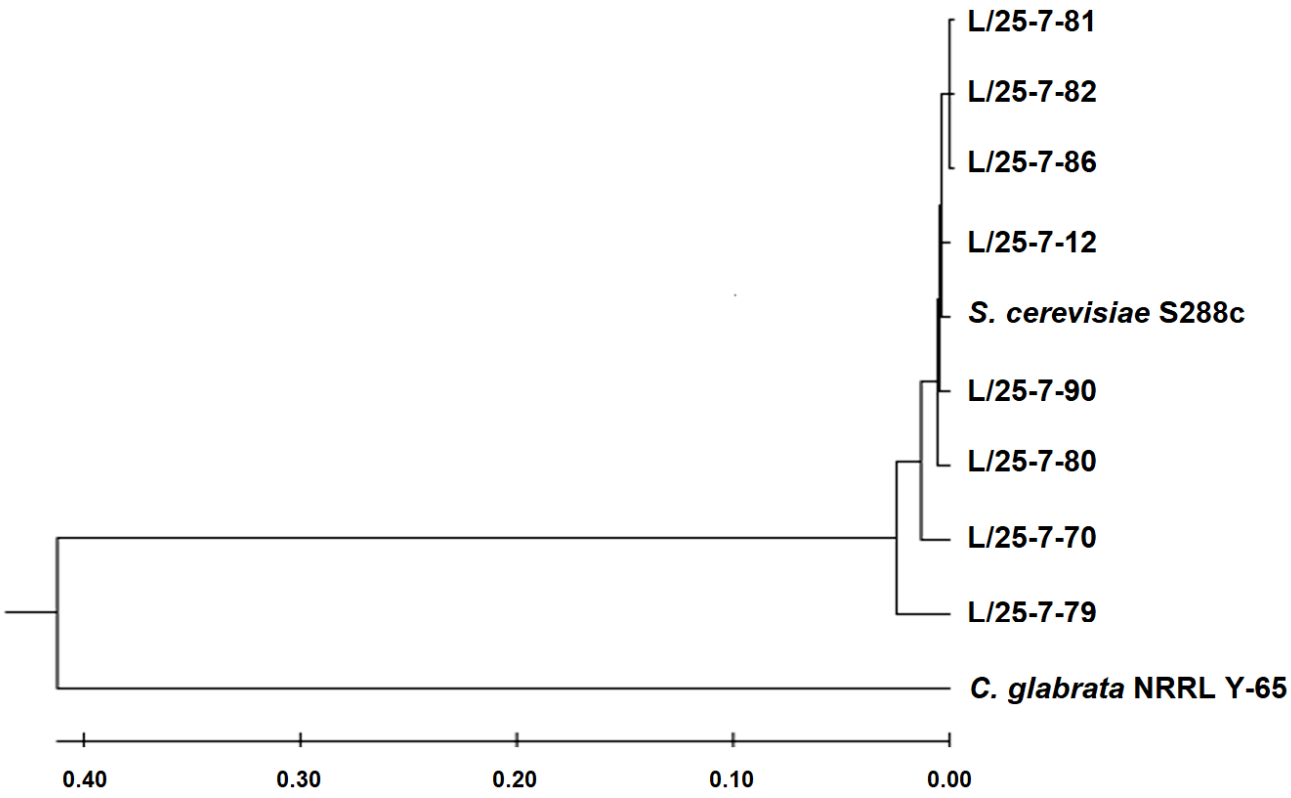

Figure 1. Clustering analysis using UPGMA method of the eight industrial isolates of Saccharomyces cerevisiae from partial sequence of ITS1 locus. Nucleotide sequences from S. cerevisiae S288c and Candida glabrata NRRL Y-68 were recovered from NCBI/Genebank and used as internal reference and outgroup control, respectively.

Fermentation trials

Fermentation experiments performed herein used the synthetic minimal medium as reference for the comparison with three industrial compositions of sugarcane derivatives. In general, the industrial plants work with substrates containing total assimilable sugars in the range of 120 to 160 g/L, which means that the crude substrates (either the juice or its molasses) are normally diluted with water to reach that range of concentration. However, as the use of water is becoming restrictive and since the constant use of vinasse in the soil has bringing environmental concerns (Christofoletti et al. 2013), some productive areas of the globe decided for the recycling of vinasse for the dilution of the fermentation substrates.

Fermentation experiments were carried out with each one of the eight strains in four different media conditions named as reference medium (synthetic minimal medium - REF) and three industrial compositions of sugarcane juice diluted with water (FM1), sugarcane molasses diluted with water (FM2) or sugarcane molasses diluted with vinasse (FM3). Therefore, eight data were produced for each substrate, while four data were produced for each strain. First, we analyzed the general aspects of fermentation considering each wort by the mean values of the fermentative parameters from all strains (Table 1). Sugar consumption by the yeast cells was significantly higher in sugarcane juice (FM1) and similar between reference medium (REF) and molasses (FM2). The presence of vinasse lowered sugar uptake from molasses (FM3). This byproduct presents chemical composition that can be detrimental not only for the soils but for the microorganisms as well (Christofoletti et al. 2013). Ethanol production followed the same pattern, with the difference that it was significantly higher in reference medium than in molasses. Hence, ethanol yield was similarly higher in REF and FM1 and lower in FM2 or FM3 (Table 1). This result agrees with previous report showing better fermentation efficiency in sugarcane juice than in molasses (Kaushal and Phutela 2015). The quality of molasses also influences microbial growth (García and Otero 2015). On the other hand, the presence of vinasse in molasses did not potentiate this lower fermentability of molasses. This was the clear indication that, whatever might be present in vinasse composition, its composition turned difficult the uptake of sugars but stimulated its conversion to ethanol by imposing some sort of redox imbalance. This last result was very interesting since there is an increasing concern on the use of vinasse for field fertilization due to environmental concerns in some world's agricultural regions. This means that industries will have to find an efficient means of using this by-product. In the case of some industrial plants located in Valle del Cauca, Colombia, they use vinasse to dilute molasses instead of water. In Cuba, vinasse is used to dilute molasses for cultivation of Candida utilis as single cell protein. Hence, the isolation of vinasse-tolerant strains like those in this work seems a relevant subject for such areas.

Table 1. Statistical analysis of the fermentation variables regarding the mean values of all industrial Cuban isolates of *Saccharomyces cerevisiae* in a pairwise comparison of fermentation conditions using four substrates as reference medium (REF), sugarcane juice (FM1), sugarcane molasses (FM2) and molasses diluted with fermentation sludge (FM3).

	Ref x FM1		Ref x FM2		Ref x FM3		FM1 x FM2		FM2 x FM3		FM1 x FM3	
Parameter	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
ºBrix consumed (1)	7.15		7.15		7.15		7.17		7.17		9.02	
ºBrix consumed (2)	9.02		7.17		6.37		9.02		6.37		6.37	
p value	>0.001		0.945		0.059		>0.001		0.023		>0.001	
$F_{calculated}$	23.39		0		4.23		31.99		6.48		59.14	
$F_{critical}$	4.60		4.60		4.60		4.60		4.60		4.60	
Ethanol produced (1) (g/L)	1.915		1.915		1.915		1.279		1.279		2.432	
Ethanol produced (2) (g/L)	2.432		1.279		1.208		2.432		1.208		1.208	
p value	0.022		0.003		>0.001		>0.001		0.479		>0.001	
$F_{calculated}$	6.62		12.90		19.66		54.83		0.52		81.95	
$F_{critical}$	4.60		4.60		4.60		4.60		4.60		4.60	
Ethanol yield (1)	0.269		0.269		0.269		0.177		0.177		0.269	
Ethanol yield (2)	0.269		0.177		0.190		0.269		0.190		0.190	
p value	1.000		0.001		0.002		>0.001		0.237		>0.001	
$F_{calculated}$	0		16.99		14.18		48.38		1.52		51.93	
$F_{critical}$	4.60		4.60		4.60		4.60		4.60		4.60	

Afterwards, each strain was analyzed in all four media to identify the most appropriate yeast for a specific substrate. Yeast strains can diverge regarding the capacity to assimilate sugars and convert them to ethanol, as well as for their adaptability to specific type of substrate. Da Silva-Filho et al. (2005a) reported the isolation of strains genotypes mostly observed in sugarcane juice than in molasses, and vice-versa, from Brazilian industries. In addition, the authors showed that those strains also differed in terms of ethanol yield and glycerol production (Da Silva-Filho et al. 2005b). Later on, other reports also showed the diversity of the Brazilian strains regarding the substrate of isolation (Basso et al. 2008; Della-Bianca et al. 2013). In the present work, we tested the capacity of the Cuban isolates to assimilate sugars present in industrial substrates (Fig. 2). The strain L/25-7-79 displayed the best performance in FM2 while the strain L/25-7-77 exhibited the highest sugar consumption in both FM1 and FM3 (Fig. 2, data statistically significant at $p<0.05$ with HSD test).

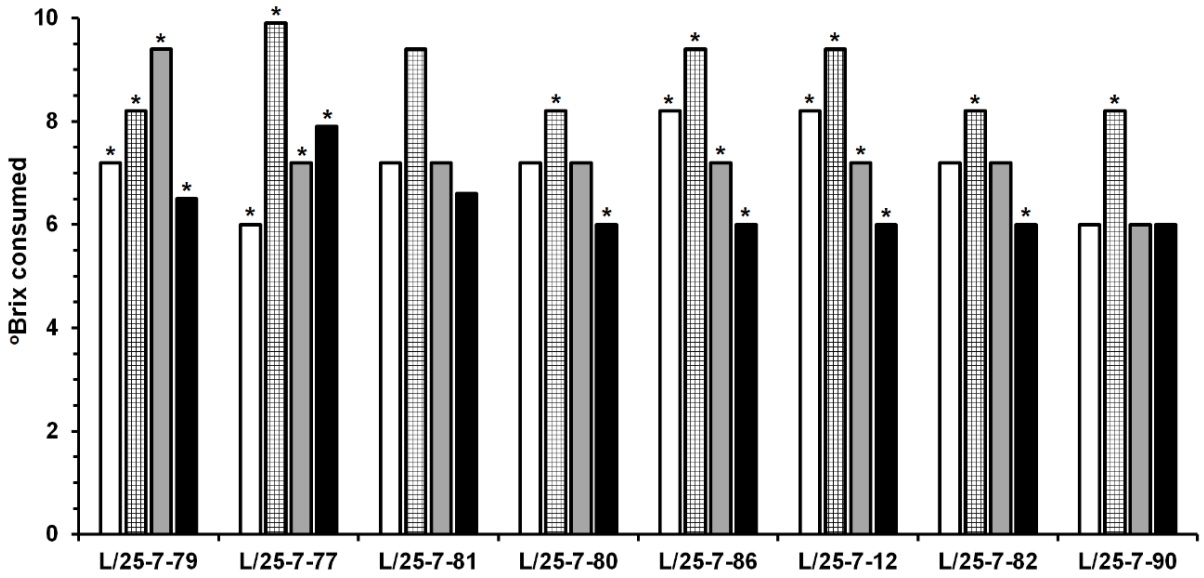

Figure 2. Comparative analysis of sugar consumption among eight isolates of Saccharomyces cerevisiae isolated from Cuban distilleries in synthetic reference medium (white columns), sugarcane juice (white hatched columns), sugarcane molasses (grey columns) or molasses diluted with vinasse (black columns). All values are presented as means of biological triplicates (CV< 20%). Asterisks represent significative differences ($p<0.05$) for assimilation of sugars in the different substrates for each isolate.

Calculation on ethanol yield showed that L/25-7-81 and L/25-7-86 more efficiently converted sucrose from FM1 towards ethanol, while L/25-7-12 and L/25-7-79 were more efficient when FM2 was the substrate (Table 2). This substrate specificity has been reported, with the strain JP1 to sugarcane juice while P6 and PE-2 strains were more adapted to molasses (Da Silva-Filho et al. 2005a; Della-Bianca et al. 2013). Interestingly, L/25-7-86 showed the best efficiency when fermenting FM3. It indicates that this isolate might be very tolerant to oxidants and excess of minerals present in the vinasse. Regarding specific ethanol productivity (Qp), L/25-7-81 and L/25-7-79 were the best performing strains in this parameter using FM1 and FM2, respectively

(Table 2). Again, L/25-7-86 showed remarkable Qp value when fermenting FM3 (Table 2). And lastly, we calculated the production of the by-product glycerol relative to the main fermentation product ethanol (KGly). Significant differences ($p \leq 0.05$) between strains fermenting in all the natural sources were detected despite the synthetic formulation with sucrose. The results highlighted the strains L/25-7-77 and L/25-7-79 with significant reduced glycerol biosynthesis per ethanol generated in FM1 and FM2, respectively. In this case, L/25-7-79 was more efficient in avoiding glycerol production when fermenting FM3 (Table 2). By scoring all these parameters for all four media, the results showed that the strain L/25-7-81 presented the highest performance for fermentation of FM1, L/25-7-79 for FM2 and L/25-7-86 for FM3.

Table 2. Fermentative parameters of eight industrial isolates of *Saccharomyces cerevisiae* isolated from Cuban distilleries in different substrates.

Fermentation medium	**Isolate**	**Parameter**		
		Yp/s [g/g]	Qp [g/(L.h)]	K_{Gly}
Reference (REF)	L/25-7-80	0.38±0.15^{a}	4.81±1.89^{a}	0.15±0.06^{a}
	L/25-7-81	0.28±0.09^{a}	3.53±1.19^{a}	0.15±0.00^{a}
	L/25-7-82	0.31±0.02^{a}	3.95±0.29^{a}	0.20±0.01^{a}
	L/25-7-86	0.21±0.03^{a}	3.08±0.49^{a}	0.19±0.01^{a}
	L/25-7-90	0.23±0.00^{a}	2.48±0.04^{a}	0.16±0.00^{a}
	L/25-7-12	0.21±0.04^{a}	3.05±0.64^{a}	0.18±0.04^{a}
	L/25-7-77	0.25±0.04^{a}	2.76±0.44^{a}	0.15±0.04^{a}
	L/25-7-79	0.28±0.04^{a}	3.51±0.50^{a}	0.12±0.01^{a}
FM1	L/25-7-80	0.27±0.02^{a}	3.93±0.30^{a}	0.17±0.01^{a,b}
	L/25-7-81	0.32±0.05^{a}	5.15±0.86^{a}	0.11±0.02^{b,c}
	L/25-7-82	0.24±0.04^{a}	3.46±0.52^{a}	0.19±0.04^{a}
	L/25-7-86	0.30±0.02^{a}	4.94±0.28^{a}	0.12±0.01^{b,c}
	L/25-7-90	0.25±0.00^{a}	3.59±0.03^{a}	0.14±0.00^{a,b,c}
	L/25-7-12	0.26±0.06^{a}	4.26±0.92^{a}	0.13±0.02^{b,c}
	L/25-7-77	0.26±0.03^{a}	4.79±0.52^{a}	0.10±0.00^{c}
	L/25-7-79	0.25±0.03^{a}	4.02±0.47^{a}	0.13±0.01^{a,b,c}
FM2	L/25-7-80	0.15±0.03^{a}	3.60±0.40^{b}	0.26±0.05^{a}
	L/25-7-81	0.16±0.02^{a}	2.02±0.20^{b}	0.23±0.03^{a,b}
	L/25-7-82	0.16±0.02^{a}	2.06±0.25^{b}	0.28±0.03^{a}
	L/25-7-86	0.16±0.02^{a}	2.02±0.29^{b}	0.20±0.03^{a,b,c}
	L/25-7-90	0.17±0.03^{a}	1.86±0.36^{b}	0.19±0.03^{a,b,c}
	L/25-7-12	0.21±0.02^{a}	2.61±0.21^{a,b}	0.13±0.00^{b,c}
	L/25-7-77	0.20±0.03^{a}	2.58±0.37^{a,b}	0.14±0.02^{b,c}
	L/25-7-79	0.21±0.00^{a}	3.00±0.03^{a}	0.13±0.01^{c}
FM3	L/25-7-80	0.18±0.01^{a}	1.97±0.11^{a}	0.22±0.01^{a}
	L/25-7-81	0.19±0.02^{a}	2.18±0.89^{a}	0.18±0.04^{a}
	L/25-7-82	0.22±0.04^{a}	2.43±0.40^{a}	0.20±0.05^{a}

L/25-7-86	0.22±0.01[a]	2.40±0.08[a]	0.15±0.01[a,b]
L/25-7-90	0.18±0.01[a]	1.96±0.14[a]	0.15±0.02[a,b]
L/25-7-12	0.20±0.00[a]	2.19±0.05[a]	0.10±0.00[b]
L/25-7-77	0.18±0.02[a]	2.13±0.53[a]	0.09±0.01[b]
L/25-7-79	0.19±0.03[a]	2.26±0.41[a]	0.09±0.00[b]

Values are presented as mean ± standard deviation of three biological replicates. Homologies between groups (HSD, Tukey test for a 95 % of confidence) are represented by the same letters. Groups were considered by the comparison of isolates in a defined fermentation medium. $Y_{p/s}$ ethanol yield in grams per gram of sugar consumed, Qp ethanol volumetric productivity, K_{Gly} ratio of glycerol per ethanol produced.

Response of the yeast strains to fermentation inhibitors

Growth kinetics analyses were carried out only in REF medium due to difficult in reading absorbance in the industrial substrates. In general, it was observed a huge variation in growth rates from 0.09 h-1 to 0.24 h-1. Besides, strains could be divided into two groups: the first group composed by strains L/25-7-81 and L/25-7-90 displaying a short exponential growth phase and early entry to stationary phase, and the second group composed by the other six strains that showed slower exponential growth phase (Fig. 2). Afterwards, we tested the growth profile of these strains in the presence of inhibitors molecules, such as those found in processes using sugarcane juice (lactic acid produced by lactic acid bacteria) (Beckner et al. 2011; Basso et al. 2014) or lignocellulosic hydrolysates (acetic acid or 5-HMF) (Jönsson and Martín 2016).

Strategies are being increasingly sought to increase tolerance of yeasts to different forms of stress in a way to increase ethanol production from non-conventional substrates (Deparis et al. 2017). Molasses also presents molecules with oxidant potential, like 5-HMF, due to thermal-induced transformation of hexoses during sugar milling (García and Otero2015). Besides, acidic stress can also be trigged when yeast biomass is being treated with diluted sulfuric acid during the recycles to control bacterial population (De Melo et al. 2010). The presence of acetic acid at mild concentrations as 5 g/L did not affect growth kinetics of all isolates. Only isolates L/25-7-81 and L/25-7-90 showed abrupt drop in cell density of the cultures after 15 hours of cultivation at this concentration. Moreover, 5-HMF at 5 g/L did not influence cell growth as well. Previous work reported that 5-HMF added up to 4 g/L reduced CO2 evolution rate but did not impair cell growth unless in the presence of furfural (Taherzadeh et al. 2000).

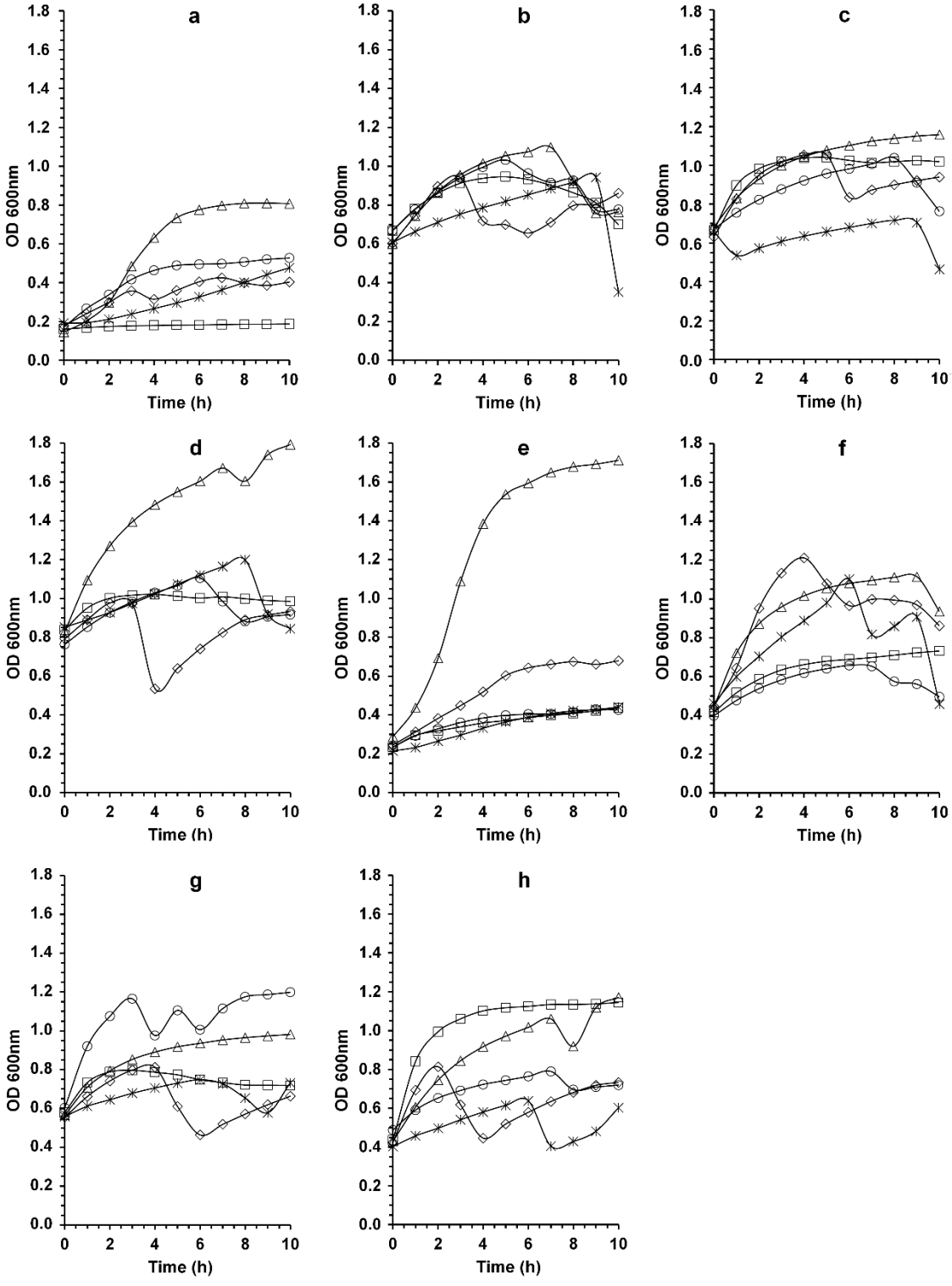

Figure 3. Growth profiles of eight S. cerevisiae isolates collected from Cuban ethanol-producing distilleries: (a) L/25-7-81, (b) L/25-7-82, (c) L/25-7-86, (d) L/25-7-90, (e) L/25-7-80, (f) L/25-7-12, (g) L/25-7-77 and (h) L/25-7-79. Symbols refer to the following growth conditions: reference medium without inhibitor at pH 4.5 (squares), in the presence of acetic acid at 15 g/L and pH 4.5 (open circles), in the presence of 5-HMF at 6 g/L and pH 4.5 (crosses), in the presence with lactic acid at 15 g/L and pH 4.5 (triangles) and in the absence of inhibitors and pH adjusted to 2.0 with H2SO4 (closed circles). Means of biological triplicates (n=3) with CV< 20% were only represented.

Acidic stress was analyzed using inorganic (sulfuric) or organic (lactic and acetic) acids. The effect of inorganic acid was tested by adjusting pH of the reference medium to pH 2.0 with sulfuric acid, which represented 10 mM of dissociated H+ in the medium. In this condition, cell growth of all isolates did not differ from those displayed by the cells in unadjusted medium (pH 4.5) (Fig. 2). Besides, isolates L/25-7-82, L/25-7-12 and L/25-7-79 stood out as isolates with the best performance in this harsh condition. This result showed that Cuban isolates were perfectly adapted to acidic environment. This is a quite interesting result since yeast isolates, even industrial ones like JP1 (De Melo et al. 2010), are very difficult to grow in synthetic medium adjusted to such low pH. However, this acidic environment seemed hardly affect fermentation efficiency of the cells (De Melo et al. 2010). Only when the cells were submitted to adaptive selection approach it was possible to select a derivative mutant strain JP1M that grew in synthetic reference medium at pH 2.0 adjusted with sulfuric acid (De Melo et al. 2010). Again, the results showed that Cuban isolates herein are the product of an adaptive selection process along the years of permanence in those distilleries that lead to metabolic re-organization to stress tolerance.

In the presence of lactic acid at 15 g/L, the isolates L/25-7-82, L/25-7-80 and L/25-7-77 showed a growth kinetic profile very close to the reference medium, while L/25-7-81 was highly sensitive for this inhibitor (Fig. 2). Moreover, the isolates L/25-7-86, L/25-7-12 and L/25-7-79 behaved like L/25-7-80 when in the presence of this inhibitor. Moreover, lactic acid seemed to be used as carbon co-substrate for the isolates L/25-7-82, L/25-7-86, L/25-7-12, L/25-7-77 and L/25-7-79 in reference medium, with a two-times increasing in biomass production of L/25-7-79 after 24 hours of cultivation. It is plausible to assume that these isolates submitted to permanent exposure to sub-toxic doses of inhibitors (lactic acid plus furfurals) could evolve to a de-repressed metabolic condition that lead to release of stress tolerance as well as residual lactate co-consumption with glucose, with increase tolerance to lactate as consequence. Acetic acid at 15 g/L was very toxic for all eight isolates, including those tolerant to 5-HMF and lactic acid. In this study, a high sensitivity to acetic acid at 15.0 g/L was evident for all isolates. Referencing results were obtained by Guo and Olsson (2014) which conducted a physiological study with S. cerevisiae CEN.PK 113-7D strain. This type strain coped concentrations up to 13.0 g/L with prolonged latency phase, but with a reduced maximum specific growth rate.

Another class of inhibitors includes furfurals and furaldehydes produced from oxidation of sugars and aromatic compounds present in sugarcane juice during sugar milling or from thermal-acid treatment of lignocellulosic plant biomass. Among them, 5-hydroxymethyl furfural (5-HMF) is considered strong inhibitor of yeast growth and fermentation capacity (Taherzadeh et al. 2000). Reduced growth was observed for all isolates when in presence of 5-HMF at 6 g/L, with decreased cell density by 10 to 15 hours of cultivation, followed by restoration of growth thereafter. Growth data showed a remarkable performance of L/25-7-82 and L/25-7-77 isolates that could be assigned as tolerant for this oxidant molecule (Fig. 2bg). Pereira et al. (2016) reported that S. cerevisiae IMS0351 strain displayed tolerance growing at concentrations up to 1.5 g/L of 5-HMF. Moreover, no growth was observed for any of the eight isolates in the present study when 5-HMF was added to 15 g/L (data not shown) just like what was reported for S. cerevisiae NRRL Y-12632 and ATCC211239 (Liu 2006). Previous work

reported that the fuel-ethanol industrial strain JP1 was able to growth in the presence of 5-HMF at 3.5 g/L, while its derivative mutant strain P18R selected from adaptive selection experiments grew at 5 g/L (Sehnem et al. 2013). On the other hand, the fuel-ethanol strain P6 collected from molasses-based industry (Da Silva-Filho et al. 2005a) was already tolerant to this condition. When submitting P6 to the adaptive selection approach, a derivative mutant strain P6H9 arise that tolerated 5-HMF even at 6 g/L. These natural strains JP1 and P6 diverge regarding their substrate of selection: JP1 was selected from distillery that uses sugarcane juice while P6 was selected from molasses-using distillery (Da Silva-Filho et al. 2005a, b). Altogether, these results indicated that cells pre-adapted in molasses environment that contains residual concentration of furfurals, such as P6 or the Cuban isolates selected here (that already tolerate 5-HMF at 5 g/L), are more adequate to fermentation of plant hydrolysates that contains these inhibitors at higher concentration. The problem is that such metabolic re-organization in the adapted cells that leads to 5-HMF tolerance was accompanied by a decrease in ethanol yield in natural tolerant molasse strain P6 and even more in the adapted derivative P6H9 when fermenting synthetic reference medium (Sehnem et al. 2013). Indeed, we observed that all the eight Cuban isolates tested here that were naturally tolerant to 5-HMF at 5 g/L also presented lower ethanol yield (Table 2) compared to other industrial isolates from sugarcane juice (Sehnem et al. 2013; Da Silva-Filho et al. 2005a,b).

The protective effect of magnesium ion against copper toxicity has been reported by our previous work (De Barros Souza et al. 2015) and hence we tested whether it could protect the yeast cells against fermentation inhibitors. Its addition to synthetic media at 0.5 g/L did not exhibited significant improvements regarding isolate tolerance for any of the inhibitory conditions assessed. In overall terms, it was observed that L/25-7-82 and L/25-7-77 showed the best performance among the isolates when in the presence of these inhibitors independent of Mg2+, which indicated that these isolates evolved for naturally tolerant yeasts.

With all these physiological data, we attempted to select high performance isolates that converge high fermentative capacity and stress tolerance. For this purpose, each isolate received a score (one to eight) and were ordered for both parameter (Table 3). Regarding fermentative capacity, means of brix consumption (from Fig. 1) and of ethanol yield, ethanol productivity and relative glycerol production (from Table 2) were calculated for each isolate in all four fermentation conditions. Then, the isolates were classified from highest (score eight) to lowest (score one) performance for the first three parameters, and highest (score one) to lowest (score eight) glycerol production. These partial four scores were summed and then the isolates were again classified from eight (higher sum result) to one (lowest sum result) to give the final physiological score (FC parameter in Table 3). Similar approach was used for stress tolerance score (ST parameter in Table 3), in which the isolates were classified from the highest (score eight) to the lowest (score one) tolerance for each of the four stressors. The sum of the four tolerance scores produced the ST parameter. Table 3 summarized the ranking of the isolates firstly based on the highest fermentation capacity, showing that isolates S. cerevisiae L/25-7-77 and L/25-7-79 outstand as converging the performance of converting sugar for any of the fermentation conditions tested towards ethanol with high yield in shorter time with prominent tolerance to fermentation inhibitors. Besides,

two contrasting isolates were also revealed from this analysis: L/25-7-81 with high fermentation capacity and stress sensitivity phenotype, and L/25-7-82 with the opposite profile (Table 3). It is well known that strains with higher tolerance to stressing agents are normally less efficient in fermentation, as we reported for tolerance to acid stress (Melo et al. 2010). Therefore, the major challenge in this kind of strategy is now to combine these characteristics (each one depending of quantitative heritage) in a single strain.

Table 3. Score of *Saccharomyces cerevisiae* industrial isolates distillery isolates based on parameters of fermentation capacity and stress tolerance.

Isolate	Fermentative capacity (FC)	Stress tolerance (ST)	Final score (FC+ST)
L/25-7-77	7	7	14
L/25-7-79	8	5	13
L/25-7-82	2	8	10
L/25-7-12	3	6	9
L/25-7-81	6	1	7
L/25-7-80	5	2	7
L/25-7-86	4	3	7
L/25-7-90	1	4	5

Conclusion

In this study, the fermentation capacity and tolerance to inhibitors of eight S. cerevisiae isolates from Cuban distilleries were studied. Comparing these isolates, S. cerevisiae L/25-7-77 showed considerable tolerance to typical fermentation inhibitors and significantly best fermentation parameters in sugar cane byproducts-based media among the isolates tested. Based on the results, we concluded that relationship between stress tolerance and fermentative performance is specific trait in S. cerevisiae isolates from molasses-based distilleries. Quantitative physiology assessment must be considered as an important tool in searching local ethanol producer yeasts. The study leads to further investigation toward these industrial S. cerevisiae isolates could be prospect to 2nd ethanol generation approaches.

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Compliance with ethical standards

Ethical approval

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

Conflict of interest

The author declares no conflict of interest.

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