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ESTUDO DO METABOLISMO E INFLUÊNCIA DE FONTES DE NITROGÊNIO NA FISIOLOGIA E EXPRESSÃO GÊNICA DA LEVEDURA *DEKKERA BRUXELLENSIS*

Will de Barros Pita

Recife

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Tese de doutoramento apresentada ao Programa de Pós-Graduação em Ciências Biológicas do Centro de Ciências Biológicas da Universidade Federal de Pernambuco para obtenção do título de Doutor em Ciências Biológicas, na Área de concentração Biotecnologia.

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Resumo

A levedura *Dekkera bruxellensis* é consistentemente associada a contaminações de processos de fermentação alcoólica industrial. Na produção de vinhos, esta levedura é responsável pela produção de aromas indesejáveis, enquanto que na produção de etanol combustível, *D. bruxellensis* compete com *Saccharomyces cerevisiae* pelo substrato industrial. Apesar de compartilhar alguns fenótipos com *S. cerevisiae*, *D. bruxellensis* apresenta características peculiares, como por exemplo, a capacidade de utilizar nitrato como única fonte de nitrogênio. No ambiente industrial, as quantidades de açúcares são elevadas e, nesses casos, o fator limitante do crescimento é geralmente a disponibilidade de nitrogênio, um nutriente essencial para todas as formas de vida. O objetivo do presente trabalho foi investigar o metabolismo de diferentes fontes de nitrogênio e determinar a influência da natureza e da concentração destas fontes na fisiologia e expressão gênica de *D. bruxellensis*, em busca de potenciais fatores positivos de adaptação para esta levedura. Os resultados mostraram que a assimilação de nitrato pode favorecer *D. bruxellensis* no ambiente industrial, pois fornece o nitrogênio necessário para manter o crescimento desta levedura mesmo após a depleção da amônia no caldo de cana. Adicionalmente, a escassez de nitrogênio diminui a taxa de crescimento, consumo de açúcares e produção de etanol em *D. bruxellensis*. No entanto, a limitação de carbono é ainda mais drástica para o metabolismo celular, ocasionando redução significativa dos principais parâmetros fisiológicos. Com relação à assimilação de fontes de nitrogênio, as enzimas glutamato desidrogenase e glutamato sintase podem trocar de papéis como principal via de biossíntese de glutamato e que genes codificantes de permeases de nitrogênio estão sob rígido controle transcricional. Além disso, *D. bruxellensis* apresenta preferência pela utilização do metabolismo respiratório em detrimento da fermentação em condições limitantes de crescimento. Finalmente, um novo grupo de genes de referência para ensaios de expressão gênica em *D. bruxellensis* foi estabelecido. A partir dos resultados gerados no presente trabalho, é possível entender as repostas metabólicas de *D. bruxellensis* em diferentes fontes de nitrogênio, o que pode auxiliar na identificação de novos fatores de adaptação para esta levedura, que permitem o seu estabelecimento e manutenção no ambiente indutrial.

Palavras-chave: *Dekkera bruxellensis*; metabolismo do nitrogênio; assimilação de amônia; produção de glutamato; expressão gênica; fermentação alcoólica.

Abstract

Dekkera bruxellensis is repeatedly linked to contaminations in industrial processes. In winemaking, this yeast is regarded as spoiler microganism, producing unpleasant off-flavor compounds. In ethanol production plants, *D. bruxellensis* is able to outcompete *Saccharomyces cerevisiae*, becoming the major yeast populations in the final steps of fermentation. In spite of sharing some phenotypes with *S. cerevisiae*, *D. bruxellensis* show particular charcateristics, such as the ability to use nitrate as sole nitrogen source. In the industrial envrionemnt, sugars are present in large amounts and the growth is often limited by the availability of nitrogen, an essential nutrient for all living organisms. The aim of the presente work was to evaluate the metabolism of different nitrogen sources and determine their influence on the physiology and gene expression profile in *D. bruxellensis*, in order to reveal potential adaptation factors for this yeast. The results showed that nitrate might favor *D. bruxellensis* in the industrial environment, since it provides the nitrogen required to support the growth, even when ammonia is depleted in sugarcane juice. Moreover, the lack of nitrogen impairs growth, sugar consumption and etanol production in *D. bruxellensis*. However, the scarcity of carbon is more severe to the cell metabolism, since the physiological parameters presented an even larger reduction. Regarding to nitrogen sources assimilation, the enzymes glutamte dehydrogenase and glutamate synthase may switch roles as the major pathway for glutamate biosynthesis and nitrogen permeases are under strict transcriptional control. Additionally, *D. bruxellensis* prefers the repsiratory metabolism over the fermentation under poor growth conditions. Finally, a new set of reference genes to gene expression assays was established. Our results help to understand the metabolic response of *D. bruxellensis* in different nitrogen sources, which might lead to the identification of new adaptation factors for this yeast.

Key words: *Dekkera bruxellensis*; nitrogen metabolism; ammonia assimilation, glutamate production, alcoholic fermentation.

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Lista de Abreviações

CO_2	Dióxido de carbono
cDNA	DNA complementar
DNA	Deoxyribonucleic acid/Ácido Desoxirribonucléico
Mb	Megabases
ml	Mililitro
mRNA	RNA mensageiro
µM	Micromolar
µg	Micrograma
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)
NCBI	National Center for Biotechnology Information
nm	Nanômetro
nM	Nanomolar
NO_2-	Íon nitrito
NO_3-	Íon nitrato
NH_4^+	Íon amônio
pb	Pares de base
PFGE	Pulsed field gel electrophoresis/Eletroforese em campo pulsátil
pH	Potencial hidrogeniônico
qPCR	PCR quantitativa
QR	Quantificação Relativa
RCN	Repressão Catabólica do Nitrogênio
rDNA	Ácido desoxirribonucleico ribossomal
RPM	Rotações por minuto

RNA	Ribonucleic acid / Ácido ribonucléico
RT-qPCR	PCR quantitativa com transcrição reversa
Tm	Temperatura de Dissociação
UV	Ultravioleta
YNB	Yeast Nitrogen Base

SUMÁRIO

INTRODUÇÃO

A levedura *Dekkera bruxellensis* é constantemente identificada como principal contaminante em diversas destilarias de álcool combustível no Nordeste do Brasil e em vinícolas de todo o mundo. Nestes ambientes, o principal fator limitante do crescimento celular é a disponibilidade de fontes de nitrogênio, visto que os açúcares são encontrados em grandes concentrações nestes substratos. Além da presença dos compostos nitrogenados, a qualidade destas fontes, ou seja, a capacidade e a velocidade com as quais são assimiladas pelos microrganismos, regulam quais espécies são capazes de crescer. Entre as fontes presentes no caldo de cana, o nitrato, que é assimilado apenas por *D. bruxellensis*, pode ser um fator nutricional importante na competição com *Saccharomyces cerevisiae* pelo substrato industrial, pois é capaz de fornecer subsídios para que *D. bruxellensis* continue seu ritmo de crescimento, mesmo após o esgotamento da amônia e aminoácidos.

A hipótese de que o nitrato pode atuar como um fator positivo para o estabelecimento de *D. bruxellensis* nos ambientes industriais destaca a importância de fontes de nitrogênio para o metabolismo celular. Entre os compostos nitrogenados mais utilizados pelas leveduras, a amônia é considerada uma fonte importante, pois se localiza no ponto central do metabolismo do nitrogênio e é rapidamente assimilada para a produção de glutamato e glutamina, dois dos mais importantes aminoácidos para o metabolismo sintético celular. Além de componentes proteicos, estes aminoácidos atuam como doadores de nitrogênio para todos os outros compostos nitrogenados da célula. Tendo em vista que em *S. cerevisiae*, a natureza e a disponibilidade de fontes de nitrogênio interferem em diversos aspectos fisiológicos e genéticos, o entendimento acerca de como estes compostos são metabolizados em *D. bruxellensis* pode ajudar a compreender os mecanismos que tornam esta levedura um organismo modelo na competição industrial.

O estabelecimento e a manutenção de *D. bruxellensis* nos processos industriais vêm sendo alvo de investigações que priorizam elucidar como este microrganismo se tornou a primeira levedura descrita como capaz de superar as qualidades industriais de *S. cerevisiae*, sendo em alguns casos, a principal população de leveduras nesses processos. Os primeiros trabalhos publicados associando *D. bruxellensis* a contaminações industriais foram relacionados a mudanças nos aromas de vinhos, devido à produção de compostos voláteis indesejáveis. Posteriormente, *D. bruxellensis* passou a ser descrita também em fermentações para produção de etanol combustível, sendo associada à queda nos rendimentos em etanol nas destilarias. A partir desses trabalhos de identificação, *D. bruxellensis* passou a ter lugar de destaque em diversos grupos de pesquisa no mundo. No entanto, os trabalhos que abordam esta levedura podem ser classificados em dois períodos distintos. O primeiro envolve publicações que se dedicaram ao desenvolvimento de métodos moleculares de detecção e quantificação de linhagens de *D. bruxellensis*. Este período perdurou até o sequenciamento parcial

do genoma de *D. bruxellensis*, em 2007, que gerou as informações necessárias para o ponto de partida para estudos que associaram a fisiologia à genética desta levedura. A partir de então, o número de publicações dedicadas ao entendimento dos mecanismos que controlam o metabolismo de *D. bruxellensis* cresceu de forma exponencial.

Neste contexto, os principais grupos de pesquisa em *D. bruxellensis* no mundo, notadamente o grupo da Universidade Federal de Pernambuco, coordenado pelo Profº Marcos Morais, e os grupos sediados na Suécia e Austrália, realizaram estudos que começaram a caracterizar a fisiologia e a genética desta levedura. Entre esses estudos, o segundo capítulo do presente trabalho, intitulado "The ability to use nitrate confers advantage to *Dekkera bruxellensis* over *S. cerevisiae* and can explain its adaptation to industrial fermentation processes", foi dedicado a entender o papel que o nitrato pode desempenhar como fator de adaptação favorecendo *D. bruxellensis* em sua competição com *S. cerevisiae* no ambiente industrial. Apesar de associar no nitrato como fator diferencial e possível explicação para o sucesso competitivo de *D. bruxellensis*, ainda era necessário estabelecer os efeitos do nitrato no metabolismo desta levedura. Para isso, o terceiro capítulo do presente estudo, intitulado "The influence of nitrate on the physiology of the yeast *Dekkera bruxellensis* grown under oxygen limitation", traz dados fisiológicos e genéticos que comprovam a alta demanda energética exigida pela assimilação de nitrato, o que interfere em aspectos fisiológicos importantes.

No entanto, outras fontes de nitrogênio podem desempenhar papéis importantes no metabolismo celular e o entendimento de como os principais compostos nitrogenados influenciam diversos aspectos metabólicos é fundamental para associá-los a um mais alto desempenho competitivo de *D. bruxellensis*. O quarto capítulo do presente trabalho intitulado "Physiological and transcriptional response of *Dekkera bruxellensis* to different carbon and nitrogen supplies", apresenta dados de como diferentes fontes de nitrogênio são utilizadas, além de investigar a influência da limitação de carbono e nitrogênio no metabolismo energético e na produção de glutamato. Por fim, a necessidade de estabelecer e validar genes de referência para estudos de expressão gênica por PCR em Tempo Real gerou o quinto capítulo deste trabalho, intitulado "A new set of reference genes for RT-qPCR assays in the yeast *Dekkera bruxellensis*". No presente trabalho, foram iniciados os primeiros estudos acerca do metabolismo de fontes de nitrogênio, um nutriente essencial para todos os organismos. Os resultados gerados no presente trabalho auxiliam o entendimento de como *D. bruxellensis* se comporta em face de diferentes condições ambientais, o que pode levar a identificação de potenciais fatores de adaptação para esta levedura ao ambiente indutrial.

OBJETIVOS

OBJETIVO GERAL

Investigar o metabolismo e as consequências fisiológicas e genéticas de diferentes fontes de nitrogênio na levedura *Dekkera bruxellensis.*

OBJETIVOS ESPECÍFICOS

1. Investigar o papel do nitrato na competição entre *D. bruxellensis* e *Saccharomyces cerevisiae* no ambiente industrial;

2. Determinar a influência do nitrato na fisiologia e expressão gênica de *D. bruxellensis*;

3. Estudar o perfil fisiológico e transcricional da levedura *D. bruxellensis* em resposta a disponibilidade de glicose e fontes de nitrogênio;

4. Estabelecer um banco de genes de referência para ensaios de expressão gênica por PCR quantitativa em Tempo Real.

CAPÍTULO I

REVISÃO BIBLIOGRÁFICA

1. *Dekkera bruxellensis*: aspectos gerais

A levedura *Dekkera bruxellensis* (Figura 1) é a forma produtora de ascosporos (teleomórfica) de *Brettanomyces bruxellensis*, pertencente ao grupo dos hemiascomicetos (MOLINA et al., 1993). A presença de *B. bruxellensis* em cervejarias inglesas, nas quais sua participação está relacionada a características aromáticas, consistiram nas primeiras descrições da espécie em processos fermentativos (CLAUSSEN, 1904). Posteriormente, as leveduras do gênero *Brettanomyces* foram isoladas em diversos outros processos relacionados à produção de bebidas fermentadas, tais como o vinho, cidra e cerveja (LOUREIRO & MALFEITO-FERREIRA, 2003; IBEAS et al., 1996, COTON et al., 2006). *D. bruxellensis* está inserida na família Saccharomycetacea, como um parente distante de *Saccharomyces cerevisiae*. Análises de rDNA mitocondrial 15S e nuclear 26S indicaram que a divergência entre as duas espécies pode ter ocorrido há pelo menos 200 milhões de anos (ROZPEDOWSKA et al., 2011).

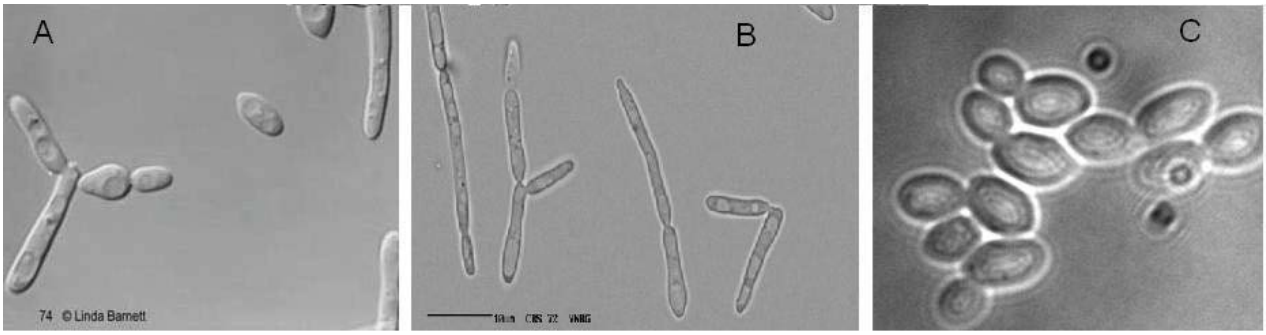

Figura 1. Linhagens da espécie *Dekkera bruxellensis*. (A) CBS 74; (B) CBS 2499; (C) GDB 248 (industrial) em meio YPD (Microscopia de contraste de fase). Leite, 2012.

O gênero *Brettanomyces* foi descrito em 1940 (CUSTERS et al., 1940). Porém, em 1964, o gênero *Dekkera* foi inserido para descrever linhagens de *Brettanomyces* capazes de formar ascósporos (VAN DER WALT, 1964). A validação do gênero *Dekkera*, realizada a partir das análises de restrição do DNA ribossomal, estabeleceu a equivalência entre os gêneros *Brettanomyces* (anamorfo) e *Dekkera* (teleomorfo) e suas espécies (MOLINA et al., 1993). Apesar de diferentes espécies de *Dekkera/Brettanomyces* terem sido previamente relatadas,

apenas cinco são reconhecidas até o momento: *D. bruxellensis*, *D. anomala, B. custersianus, B. nanus* e *B. naardenensis* (MITRAKUL et al., 1999; RODER et al., 2007). A morfologia das células de *D. bruxellensis* é bastante diversificada, podendo apresentar-se na forma elipsoidal a esférica, frequentemente ogival, e ainda cilíndrica a alongada (Figura 1). Além disso, frequentemente é observada a formação de pseudomicélio (VAN DER WALT, 1964).

D. bruxellensis é capaz de metabolizar diversas fontes de carbono e nitrogênio (CONTERNO et al., 2006). Dentre as fontes de carbono, glicose, frutose, sacarose e etanol são importantes no cenário industrial, enquanto que entre as fontes de nitrogênio já descritas como assimiláveis por esta levedura, encontramos amônia, prolina, arginina e nitrato (CONTERNO et al., 2006). Além disso, assim como *S. cerevisiae, D. bruxellensis* é tolerante a etanol, 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). *D. bruxellensis* é também conhecida por produzir uma grande variedade de metabólitos, sendo os mais significantes o ácido acético e etilfenóis, como 4-etilfenol e 4-etilguaiacol.

2. *Dekkera bruxellensis*: contaminante industrial

S. cerevisiae é considerado o principal microrganismo fermentador nos processos industriais, porém outras espécies já foram identificadas e algumas destas podem agir como contaminantes (BASÍLIO et al., 2008). Essas leveduras podem ser provenientes do mosto de alimentação ou podem ser ainda residentes do processo, localizando-se na tubulação, nos trocadores de calor e na água de lavagem da cana e de diluição do mosto (SILVA-FILHO, 2003). Sherata (1960) reportou pela primeira vez a presença de 14 espécies de levedura do caldo de cana que pertenciam aos gêneros *Candida, Endomyces, Hansenula, Kloekera, Pichia, Saccharomycodes, Schizosaccharomyces* e *Torulopsis* (SHERATA, 1960). Mais tarde, espécies de *Debaromyces, Rhodotorula* e *Cryptococcus* foram também identificadas neste substrato (DE AZEREDO et al., 1998). Nos últimos anos, mais espécies dos gêneros *Candida, Hanseniaspora, Kloekera, Kluyveromyces, Schizosaccharomyces* e *Pichia* têm sido identificadas no processo fermentativo industrial tanto para a produção de etanol combustível quanto para a produção de cachaça (GOMES et al., 2002; OLASUPO et al., 2003). Análises moleculares para tipagem e identificação de leveduras mostraram a presença de mais de 30 espécies no processo fermentativo em várias destilarias da região Nordeste, dentre as quais as mais importantes foram *D. bruxellensis*, *Candida tropicalis* e *Pichia galeiformis* (BASÍLIO et al., 2008).

Na indústria vinícola, a presença de compostos voláteis, como o 4-etilfenol e 4-etilguaiacol está relacionada especificamente a atividade de *D. bruxellensis* e aos aromas desagradáveis encontrados em vinhos contaminados (CHATONNET et al., 1995; CHATONNET et al., 1997). Além disso, o ácido acético produzido por *D. bruxellensis* inibe o crescimento e diminui a capacidade fermentativa de *S. cerevisiae*, sendo associado a perdas econômicas significativas (CHATONNET et al., 1995; LEMA et al., 1996). O estudo conduzido por Liberal e colaboradores, em 2007, representou o ponto de partida dos trabalhos envolvendo *D. bruxellensis* como contaminante industrial no Brasil. Neste estudo, que descreveu espécies contaminantes não-*Saccharomyces* em amostras de fermentação de destilarias da região Nordeste, a linhagem GDB 248 foi isolada como apresentando alta adaptabilidade ao processo industrial quando comparada a células de *S. cerevisiae* (DE SOUZA LIBERAL et al., 2007). Ainda neste trabalho, *D. bruxellensis* foi identificada como a principal levedura contaminante dos sistemas de fermentação para a produção de etanol combustível, em que a subpopulação de *D. bruxellensis* substitui a de *S. cerevisiae*, mesmo com repetidas ocasiões de troca da biomassa celular total (DE SOUZA LIBERAL et al., 2007).

Por causa desta habilidade de substituir a população de *S. cerevisiae*, *D. bruxellensis* representa um dos contaminantes mais significativos em destilarias que fermentam o caldo da cana em sistemas contínuos. Na produção de etanol combustível, quando as contagens 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). Além disso, a maioria dos estudos realizados tem mostrado que *D. bruxellensis* é a espécie prevalente em eventos de contaminação de produtos de fermentação (RODER et al., 2007; MIOT-SERTIER & LONVAUD-FUNEL, 2007). Embora haja a percepção de que *D. bruxellensis*, principalmente na sua forma anamorfa *B. bruxellensis*, representa um contaminante industrial, dados recentes mostram que esta levedura é capaz de produzir etanol em rendimentos muito próximos àqueles apresentados por *S. cerevisiae* (DE SOUZA LIBERAL et al., 2007; PASSOTH et al. 2007; BLOMQVIST et al. 2010). No entanto, o problema causado pela presença desta levedura em altas contagens no meio é o maior tempo de fermentação que é necessário para converter o açúcar em etanol (DE SOUZA LIBERAL et al., 2007), o que provoca atraso na produção e no rendimento diário do processo. Paralelamente, em um estudo desenvolvido na Suécia, Passoth e colaboradores identificaram a participação de *D. bruxellensis* em um consórcio com *Lactobacilus vini*, no qual foram observadas alta estabilidade e produtividade do processo, além de alta qualidade do produto (PASSOTH et al., 2007). Os autores ainda sugerirem estas duas espécies como potenciais organismos comercialmente produtores de etanol Estes dados reforçam a necessidade de estudos mais detalhados acerca dos principais aspectos do metabolismo de *D.*

bruxellensis, na busca por fatores determinantes do sucesso competitivo desta levedura.

3. Características fisiológicas de *Dekkera bruxellensis*

Apesar de assimilar diversas fontes de carbono, *D. bruxellensis* apresenta preferência por glicose, frutose e sacarose, açúcares capazes de apoiar altas taxas de crescimento para a levedura. Além disso, *D. bruxellensis* também é capaz de utilizar galactose, maltose, celobiose e trealose (CONTERNO et al., 2006). Dentre estas, glicose, frutose e sacarose apresentam relevância industrial, por fazerem parte da composição de substratos, como o caldo-de-cana e o melaço. Galafassi e colaboradores (2011) analisaram cerca de 50 linhagens pertencentes ao gênero *Dekkera/Brettanomyces* quanto à assimilação e/ou fermentação de celobiose, xilose e arabinose, em face do crescente interesse em utilizar hidrolisados lignocelulósicos para geração de etanol de 2ª geração (GALAFASSI et al., 2011). Como resultado, os autores relataram a habilidade de linhagens de *D. bruxellensis* em assimilar celobiose, mas não xilose e arabinose (GALAFASSI et al., 2011). Com o mesmo intuito, Blomqvist e colaboradores (2010) realizaram experimentos em condições limitantes de oxigênio e demostraram que a linhagem de *D. bruxellensis* CBS 11269 é capaz de assimilar e fermentar a celobiose, porém mais lentamente e menos eficientemente que glicose (BLOMQVIST et al., 2010). Os autores ainda calcularam rendimentos em etanol menores em celobiose do que em glicose [0,29 g (g celobiose)$^{-1}$ e 0,41 g (g glicose)$^{-1}$].

Um importante aspecto fisiológico apresentado por algumas leveduras é o chamado efeito *Crabtree*, que indica a capacidade de degradar açúcares a compostos formados por dois carbonos, em especial o etanol, mesmo em presença de oxigênio (PISKUR et al., 2006; WOOLFIT et al., 2007). O efeito *Crabtree* em *S. cerevisiae* e outras leveduras é frequentemente associado à saturação da capacidade celular em re-oxidar o NADH resultante da glicólise (PROCHÁZKA et al., 2010). Esta saturação faz com que o piruvato seja direcionado ao metabolismo fermentativo, possibilitando que o NADH seja re-oxidado via fermentação alcóolica (PRONK et al., 1996). Esta característica é a base para a estratégia "*Make-Accumulate-Consume*" (fazer-acumular-consumir), na qual as espécies apresentam rápido consumo de açúcares além de alta capacidade em produzir, acumular e tolerar etanol, o que resulta em um melhor desempenho competitivo frente a outras espécies. Um estudo recente revelou que ainda que os grupos *Dekkera/Brettanomyces* e *Saccharmoyces* tenham divergido antes do surgimento desta estratégia, a evolução deste mecanismo ocorreu de forma paralela nos dois grupos, há cerca de 100 milhões de anos (ROZPEDOWSKA et al., 2011). Os dados encontrados por estes autores indicam que a

evolução paralela pode ter acontecido devido a pressões seletivas semelhantes para os dois organismos. O desenvolvimento evolutivo desta estratégia parece estar relacionado à presença do motivo AATTTT conservado nas regiões promotoras dos genes associados ao rápido crescimento enquanto que, em genes relacionados à respiração, este motivo não se apresenta numa região conservada (ROZPEDOWSKA et al., 2011).

Outro mecanismo comumente observado em *D. bruxellensis* é o chamado efeito *Custers*, em que ocorre a inibição temporária da fermentação alcóolica, como resultado da ausência de oxigênio (CUSTERS, 1940; WIJSMAN et al., 1984). Este efeito é ocasionado pela tendência das células de *D. bruxellensis* em produzir acetato, além da baixa capacidade de produção de glicerol, uma alternativa capaz de restaurar o balanço redox celular (SCHEFFERS, 1966; WIJSMAN et al., 1984). A escassez de NAD^+ causada pelo seu uso na oxidação do acetaldeído a acetato e consequente diminuição da razão NAD^+/NADH, resulta em diminuição do fluxo glicolítico. Em células de *D. bruxellensis*, o efeito *Custers* pode ser abolido pela adição de oxigênio ou um aceptor orgânico de elétron, por exemplo, acetoína, ao meio, possibilitando que o NADH formado via glicólise e formação de acetato seja re-oxidado via cadeia respiratória (CARRASCOSA et al., 1981). No que concerne à produção de glicerol, Galafassi e colaboradores (2011) relataram que a enzima glicerol 3-fosfato desidrogenase apresenta atividade apenas em condições limitantes de oxigênio.

A influência da disponibilidade de oxigênio, temperatura e pH também foram descritas em *D. bruxellensis* (AGUILAR USCANGA et al., 2003; BRANDAM et al., 2007; BLOMQVIST et al. 2010). A concentração de oxigênio tem um efeito sobre a drenagem do substrato para a produção de etanol ou ácido acético. Assim, quanto mais aerado for o sistema de cultivo, as células de *D. bruxellensis* tendem a drenar mais carbono para a produção de ácido acético do que para etanol (AGUILAR USCANGA et al., 2003). Esta mudança é um fenômeno progressivo no qual o aumento no consumo de oxigênio e a diminuição na produção de etanol são compensados pela síntese de ácido acético (AGUILAR USCANGA et al., 2003). Em um recente estudo, Blomqvist e colaboradores observaram que a faixa de pH (3 a 5) e temperatura (25 a 37^o C) testados não afeta a taxa de crescimento e o rendimento em etanol para uma linhagem industrial de *D. bruxellensis* (BLOMQVIST et al., 2011). Apesar de diversos estudos abordarem diferentes aspectos da fisiologia de *D. bruxellensis* nos últimos anos, o metabolismo do nitrogênio permanece desconhecido nesta levedura, com apenas alguns trabalhos mostrando a sua capacidade de assimilar um determinado composto, porém sem avaliar as consequências fisiológicas e genéticas da assimilação destas fontes (CONTERNO et al., 2006; GODARD et al., 2007).

4. Aspectos genéticos de *Dekkera bruxellensis*

D. bruxellensis tem atraído o interesse de diversos grupos de pesquisa em diferentes partes do mundo, principalmente devido ao seu potencial industrial. A importância crescente resultou em um grande número de estudos fisiológicos e genéticos nos últimos anos (LIBERAL et al., 2007; WOOLFIT et al., 2007; PASSOTH et al., 2007; HELLBORG & PISKUR 2009; NARDI et al., 2010; BLOMQVIST et al. 2010; PEREIRA et al., 2012; CURTIN et al., 2012; PISKUR et al., 2012). O genoma de *D. bruxellensis* foi sequenciado apenas em 2007, porém, devido à sua natureza incompleta, o conteúdo e a organização genômica permaneceram desconhecidos (WOOLFIT et al., 2007). Posteriormente, um estudo conduzido por Hellborg & Piskur (2009), descreveu que diversas linhagens de *D. bruxellensis* apresentavam genoma poliploide, contendo entre quatro e nove cromossomos (HELLBORG & PISKUR, 2009). Nas linhagens isoladas da fermentação industrial de caldo de cana no Brasil, este número pode variar entre 4 a 6 cromossomos, que possuem tamanhos entre 0,67 Mb e 5,7 Mb (DE SOUZA LIBERAL, 2010), comprovando a grande diversidade cromossômica da espécie.

No entanto, o genoma completo de *D. bruxellensis* foi sequenciado apenas em 2012, por dois grupos de pesquisa que conduziram paralelamente o sequenciamento genômico das linhagens AWRI1499 e CBS2499 (PISKUR et al., 2012; CURTIN et al., 2012). A linhagem isolada de vinícolas australianas, *D. bruxellensis* AWRI1499 possui genoma triploide com aproximadamente 5000 genes e 12,7 Mb de tamanho. Em comparação com espécies próximas, possui enriquecimento de genes que podem aumentar a sobrevivência no ambiente da produção de vinho, tais como genes codificantes de permeases de aminoácidos, além de álcool e aldeído desidrogenases (CURTIN et al., 2012). Esta linhagem provavelmente surgiu a partir de uma hibridização de duas espécies proximamente relacionadas, uma diploide e outra haploide, possuindo consequentemente um genoma triploide (CURTIN et al., 2012) A linhagem *D. bruxellensis* CBS2499 apresenta genoma com 13,4 Mb de tamanho e aproximadamente 5600 genes. Assim como a linhagem AWRI1499, a CBS2499 possui genes ADH (álcool desidrogenase) duplicados (PISKUR et al., 2012).

Diferenças no conteúdo gênico podem explicar as variações de comportamento fisiológico entre *D. bruxellensis* e outras leveduras. A partir do sequenciamento do genoma de *D. bruxellensis*, proteínas ortólogas em ascomicetos que participam de várias vias metabólicas foram descritas, várias destas ausentes em *S. cerevisiae*. No entanto, os fatores genéticos correspondentes a produção de etanol e a capacidade de sobreviver na ausência de oxigênio são bem conhecidos em *S. cerevisiae*, mas não em *D. bruxellensis* (PISKUR & LANGKJAER, 2004). Por exemplo, a duplicação total do genoma (WGD, do inglês, *whole genome duplication*), a

transferência horizontal do gene *URA1*, codificante da diidroorotato desidrogenase, envolvida síntese *de novo* de pirimidinas, além da duplicação de genes ADH, codificantes de álcool desidrogenases, são pelo menos parcialmente responsáveis pelo desenvolvimento da alta capacidade fermentativa e/ou propriedade anaeróbicas de *S. cerevisiae* (PISKUR & LANGKJAER, 2004; PISKUR et al., 2006). O evento de WGD, que aconteceu há aproximadamente 100 milhões de anos foi crucial para a adaptação de *S. cerevisiae* a fermentação, devido a diversos fatores, entre eles a duplicação de genes da via glicolítica (PISKUR et al., 2006). A análise do genoma de *D. bruxellensis* em busca de resquícios de um evento massivo de duplicação mostrou que *D. bruxellensis* apresenta um baixo número de regiões duplicadas, se comparado com *S. cerevisiae* e o nível de segmentos duplicados está dentro do esperado para espécies que não sofreram eventos de duplicação do genoma (PISKUR et al., 2012). O baixo número de genes duplicados pode ser uma das razões pelas quais *D. bruxellensis* possui capacidade fermentativa mais baixa do que *S. cerevisiae* (ROZPEDOWSKA et al., 2011).

5. Expressão Gênica em *Dekkera bruxellensis*

Apesar dos diferentes projetos de sequenciamento de *D. bruxellensis*, poucos trabalhos foram dedicados ao estudo da expressão gênica nesta levedura (NARDI et al., 2010; DE SOUZA LIBERAL et al., 2012). Nardi e colaboradores avaliaram a expressão de genes relacionados à resposta a estresse e adaptação de *D. bruxellensis* e *S. cerevisiae* ao ambiente de produção de vinho. Neste trabalho, os autores verificaram que a sobrevivência de *D. bruxellensis* é devida a mecanismos únicos para esta levedura (NARDI et al., 2010). Outro estudo de expressão gênica envolvendo *D. bruxellensis* mostrou a presença de dois genes *ARO10*, codificantes da enzima fenilpiruvato descarboxilase no genoma da levedura, uma característica única entre os hemiascomicetos (LIBERAL et al., 2012). Os estudos realizados indicam que análises da expressão de genes importantes do metabolismo podem ajudar a identificar potenciais fatores genéticos de adaptação, que favorecem *D. bruxellensis* em sua competição com *S. cerevisiae* no ambiente industrial.

A técnica de PCR quantitativa (qPCR) ou em Tempo Real tornou-se um padrão para a quantificação da expressão gênica. Desde o seu advento (HIGUCHI et al., 1992) tem sido largamente utilizada permitindo uma quantificação rápida, precisa e sensível dos níveis de RNA mensageiro (SCHEFE et al., 2006). A qPCR oferece a oportunidade de observar a cinética de amplificação da PCR em “tempo real” via acúmulo e detecção de sinais específicos de fluorescência a cada ciclo (HIGUCHI et al.,1992; SCHEFE et al., 2006). Entretanto, o uso da qPCR para análises de expressão gênica requer diversos controles internos, utilizados para

assegurar que as quantificações são confiáveis (LIVAK & SCHMITTGEN 2001; VANDESOMPELE et al., 2002; TESTE et al., 2009). Entre os principais controles, os genes de referência são usados para normalizar a quantificação do mRNA e evitar quantificações imprecisas. Contudo, visto que pequenas variações na expressão dos genes de referência podem levar a dados errôneos, a validação dos genes de referência é essencial para determinar se a expressão dos genes de referência é de fato estável e também para normalizar suas possíveis variações. O princípio matemático baseado na análise comparativa de Ciclos de quantificação (Cqs) tem sido utilizado para quantificação relativa da expressão gênica. Contudo, este método requer que genes alvo e de referência tenham eficiências de amplificação similares (LIVAK & SCHMITTGEN 2001). Para contornar o problema, Vandesompele e coloaboradores desenvolveram o *geNorm*, um algorítmo de quantificação da expressão gênica que leva em consideração a média geométrica das quantidades relativas dos genes de referências empregados para a normalização (VANDESOMPELE et al., 2002). Este método foi recentemente utilizado em *S. cerevisiae* e os genes mais comumente utilizados como referência nos estudos de expressão gênica, foram rejeitados após a utilização do geNorm (TESTE et al., 2009). Este resultado ressalta a necessidade de validação dos genes de referência para as análises de expressão gênica para evitar dados de quantificação imprecisos.

6. Metabolismo Central do Nitrogênio

O nitrogênio é um dos nutrientes essenciais para todas as formas de vida. Em geral, as leveduras podem utilizar diversos compostos nitrogenados distintos, incluindo amônia, aminoácidos, uréia e bases nitrogenadas (CONTERNO et al., 2006; GODARD et al., 2007). Além disso, são capazes de sintetizar todas as moléculas nitrogenadas essenciais à sua vida. Estas características são bem descritas em *S. cerevisiae*, levedura na qual o crescimento e a expressão gênica são dependentes da natureza e da concentração destas fontes (TER SCHURE et al., 1998; TER SCHURE et al., 2000). Os compostos nitrogenados são geralmente transportados para dentro da célula através de proteínas transmembrana, como as permeases codificadas pelos genes *GAP1*, *MEP1* e *PUT4* (MARINI et al., 2000; MAGASANIK & KAISER, 2002; CAIN & KAISER, 2011). Em princípio, uma vez dentro das células, todas as fontes de nitrogênio assimiladas devem gerar amônia intracelular para a produção de glutamato e, posteriormente glutamina. Estes dois aminoácidos servem como doadores de nitrogênio para todos os outros compostos nitrogenados da célula e, juntamente com a amônia, formam a via do Metabolismo Central do Nitrogênio (MAGASANIK & KAISER, 2002, MAGSANIK, 2003). As moléculas nitrogenadas entram na

célula através de permeases e são direcionadas para reações de biossíntese ou catabolizadas para a liberação de nitrogênio na forma de amônia (via desaminação), glutamato (via transaminação) ou ambos, sendo estes dois posteriormente condensados para formar a glutamina (HORAK, 1997; MAGASANIK & KAISER, 2002; MAGASANIK, 2003).

Em *S. cerevisiae*, quando o ambiente possui uma fonte abundante de amônia e glicose, a enzima glutamato desidrogenase dependente de $NADP^+$, produto do gene *GDH1* (NADP-GDH, EC 1.4.1.4) é responsável pela síntese de glutamato (Figura 2, reação 1) através da combinação do íon amônio com o α-cetoglutarato (MAGASANIK & KAISER, 2002). Subsequentemente, o glutamato é convertido à glutamina pela incorporação de outro íon amônio, pela glutamina sintetase (GS, EC 6.3.1.2), produto do gene *GLN1* (Figura 2, reação 2), com gasto de ATP (MAGASANIK & KAISER, 2002; MAGASANIK, 2003). Por outro lado, durante o crescimento em glutamato como única fonte de nitrogênio, este é degradado a α-cetoglutarato e NH_4^+ pela enzima glutamato desidrogenase (NADH-GDH, EC 1.4.1.3), codificada pelo gene *GDH2* (Figura 2, reação 3) para produzir amônia para a síntese de glutamina (VALENZUELA et al., 1995; TER SCHURE et al., 2000; DE MORAIS JR, 2003). A glutamina sintetase é a única forma pela qual glutamina é sintetizada e células carentes desta enzima necessitam de glutamina para crescer. Por outro lado, a perda de *GDH1* apenas reduz a taxa de crescimento em amônia (MAGASANIK & KAISER, 2002; MAGASANIK, 2003). A habilidade de mutantes *gdh1* crescerem em amônia é devida a existência da enzima glutamato sintase (GOGAT, EC 1.4.1.14), produto do gene *GLT1* (Figura 2, reação 4) (MILLER & MAGASANIK, 1990; MAGASANIK & KAISER, 2002).

Apesar de assimilarem uma ampla variedade de fontes de nitrogênio, as leveduras apresentam preferência na utilização de determinados compostos nitrogenados (MAGASANIK & KAISER, 2002). A existência de fontes preferenciais de nitrogênio ocorre devido às diferentes taxas de crescimentos proporcionadas por estas fontes ao serem assimiladas. Compostos nitrogenados prontamente utilizáveis proporcionam taxas mais de crescimento mais altas do que fontes que necessitam de reações adicionais para serem assimiladas (CONTERNO et al., 2006; GODARD et al., 2007). Portanto, as fontes de nitrogênio podem ser classificadas em fontes “preferenciais” ou “primárias” e em “não-preferenciais” ou “secundárias”, de acordo com a taxa de crescimento que elas suportam (MAGASANIK & KAISER, 2002). Neste contexto, amônia, glutamato, glutamina e asparagina são fontes preferenciais de nitrogênio para *S. cerevisiae* e a maior parte das leveduras, enquanto leucina, prolina e metionina são fontes não preferenciais (TER SCHURE, 1998; MAGASANIK & KAISER, 2002; MAGASANIK, 2003; BOER et al., 2007).

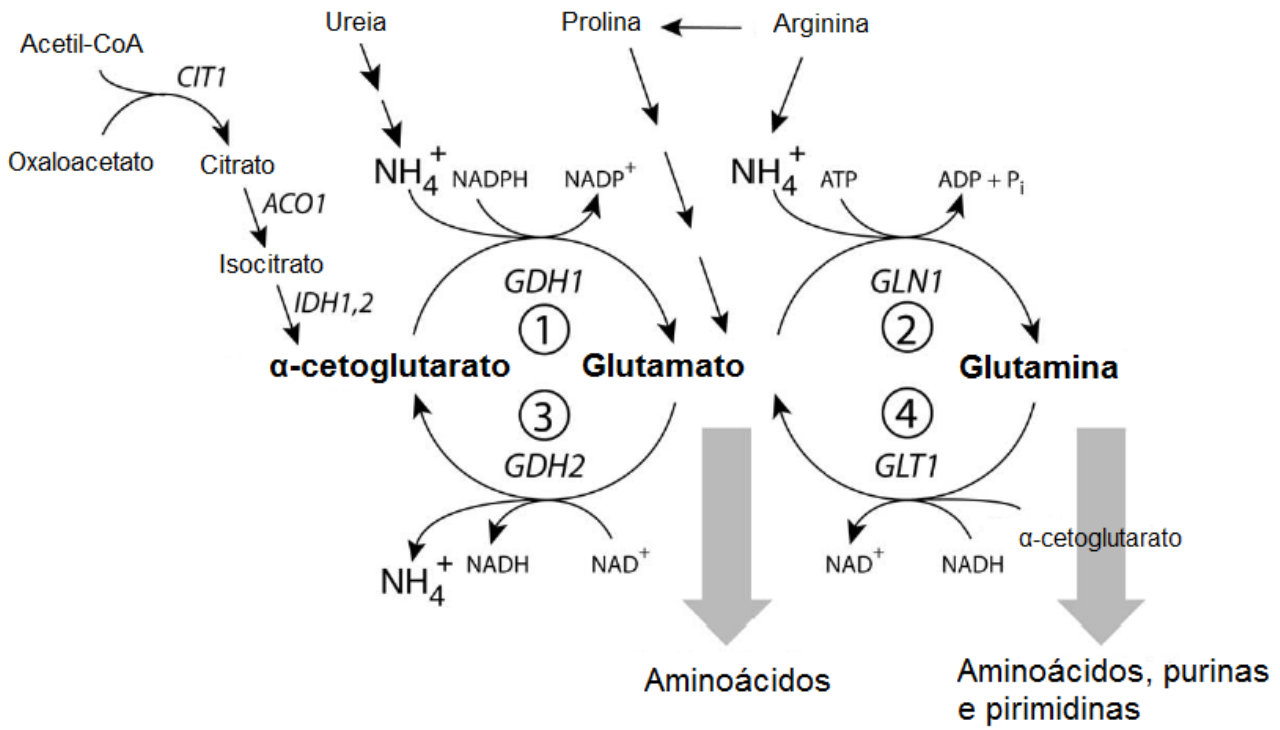

Figura 2. Metabolismo Central do Nitrogênio. Os compostos nitrogenados são sintetizados a partir de glutamato ou glutamina. A principal via para a síntese de glutamato é através da combinação de amônia com o α-cetoglutarato, que é sintetizado a partir de acetil-CoA nos passos iniciais do ciclo do ácido cítrico. A glutamina é produzida pela combinação da amônia com glutamato. Adaptado de Magasanik & Kaiser, 2002.

As fontes secundárias de nitrogênio podem ser utilizadas a partir do aumento da expressão genes codificantes de enzimas envolvidas na síntese de glutamato e glutamina, além de aumento na atividade de permeases responsáveis pela captação de aminoácidos (MAGASANIK & KAISER, 2002). O crescimento celular em *S. cerevisiae* é frequentemente limitado pela disponibilidade de nitrogênio (ATTFIELD, 1997; PRETORIUS, 2000) e, para se adaptar as várias condições ambientais, esta levedura desenvolveu um complexo sistema regulatório para garantir um eficiente controle do metabolismo do nitrogênio (COOPER, 2002; MAGASANIK & KAISER, 2002). Em culturas com diferentes substratos, *S. cerevisiae* exibe um uso sequencial de fontes preferenciais e não-preferenciais de nitrogênio (COOPER, 2002; MAGASANIK & KAISER, 2002). Este controle, que assegura que a "melhor" fonte de nitrogênio disponível será utilizada é coletivamente chamado de regulação do nitrogênio. Este mecanismo é 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 (MARZLUF, 1997; MAGASANIK & KAISER, 2002). Este controle é realizado por um mecanismo de regulação transcricional conhecido como Repressão Catabólica do Nitrogênio (RCN) e consiste na indução de genes necessários e na repressão de genes dispensáveis em determinado momento (COOPER, 2002; MAGASANIK &

KAISER, 2002). O sinal intracelular responsável pelo controle exercido pela RCN é a concentração de glutamina (MAGASANIK & KAISER, 2002; COOPER, 2002; MAGASANIK, 2003).

A base genética para a regulação do nitrogênio é bem descrita em diversos organismos. Em *S. cerevisiae*, a expressão de diversos genes cujos produtos são responsáveis pela utilização de compostos nitrogenados é ativada por duas proteínas do tipo "dedos de zinco" (*zinc finger*). Estas proteínas reconhecem a sequência GATAAG localizada à montante destes genes (MARZLUF, 1997; MAGASANIK & KAISER, 2002). A proteína Gln3p, produto do gene *GLN3*, é um desses ativadores, e possui atividade antagonizada pela proteína *URE2* em resposta a um aumento no nível intracelular de glutamina. O segundo ativador, produto do gene *GAT1* (*NIL1*) possui um motivo *zinc finger* homólogo ao de Gln3p e ambas as proteínas são capazes de compartilhar a ativação de alguns dos mesmos genes. No entanto, a atividade de Gat1p (Nil1p) é antagonizada pela proteína Nil2p, produto do gene *NIL2*, em resposta ao aumento intracelular de glutamato (MARZLUF, 1997; MAGASANIK & KAISER, 2002). Como resultado, a transcrição de um gene suscetível a este controle é ativada quase que exclusivamente por Gln3p durante crescimento em glutamato e quase que exclusivamente por Gat1p durante crescimento em amônia ou uréia e não é ativada durante crescimento em meio contendo glutamina (MAGASANIK & KAISER, 2002). A região *zinc finger* de Gln3p possui homologia com os fatores de transcrição GATA de fungos filamentosos, como NIT2 e AREA, de *Neurospora crassa* e *Aspergillus nidulans*, respectivamente. Outra proteína com um domínio *zinc finger* proximamente relacionado à Gln3p foi descoberta por seu efeito inibitório na expressão de alguns, mas não de todos os genes regulados pelo nitrogênio. Esta proteína, Dal80p é o produto do gene DAL80 (MAGASANIK, 2003).

O estímulo que causa a geração de um sinal intracitoplasmático é transduzido para os fatores de transcrição responsáveis pela ativação da expressão gênica. No caso da RCN, o estimulo responsável pela incapacidade de Gln3p ativar a expressão é a presença de uma fonte preferencial de nitrogênio no meio de crescimento (STANBROUGH & MAGASANIK, 1995; COFFMAN et al., 1996). Além disso, a presença de glutamato é o estímulo que resulta na incapacidade de Gat1p, mas não de Gln3p para ativar a expressão (STANBROUGH & MAGASANIK, 1995; ROWEN et al., 1997). O sinal gerado pela presença de fontes preferenciais de nitrogênio no meio de crescimento é transduzida para Gln3p através de Ure2p (MARZLUF, 1997; MAGASANIK & KAISER, 2002). Este sinal é aparentemente um aumento na concentração intracelular de glutamina, que bloqueia a habilidade de Ggln3p ativar a expressão gênica. Aparentemente, a atividade de Gln3p é inversamente proporcional a concentração intracitoplasmática de glutamina, que é alta em células crescidas em um meio contendo glutamina, baixa em meio contendo amônia e ainda mais

baixa em meio contendo glutamato (STANBROUGH & MAGASANIK, 1995; COFFMAN et al., 1996).

Em células crescidas com glutamina como fonte de nitrogênio, Gln3p está presente no citoplasma em uma forma fosforilada complexada com Ure2p. É provável que o agente responsável pela fosforilação de Gln3p seja uma TOR quinase (BERTRAM et al., 2000). A inativação de proteínas TOR quinase impede a fosforilação de Gln3p e resulta na ativação da Sit4p fosfatase, que pode ser o agente responsável pela defosforilação de Gln3p. A defosforilação de Gln3p parece ser essencial para sua translocação do citoplasma para o núcleo, visto que o agente responsável por esta translocação através do poro nuclear, a Srp1p é capaz de ligar apenas a Gln3p não-fosforialdo (CARVALHO et al., 2001). A glutamina intracelular parece ser o agente responsável pela habilidade de Ure2p ligar a Gln3p fosforilada. De acordo com esta visão, uma queda na concentração intracelular de glutamina resultaria na mudança conformacional do complexo Gln3p-Ure2p, que leva a liberação de Gln3p fosforilado, que seria então defosforilado no citoplasma, permitindo sua ligação a Srp1p e a translocação ao núcleo. Existe alguma evidência para a visão que glutamina interage com Ure2p, uma interação que pode ser responsável pelo aumento da afinidade de Ure2p para Gln3p (MAGASANIK, 2003).

7. Metabolismo do Nitrato

Apesar de as leveduras serem capazes de utilizar diferentes compostos nitrogenados, a assimilação de nitrato e/ou nitrito é restrita a apenas algumas poucas espécies (SIVERIO, 2002). O fato de leveduras pertencentes ao gênero *Saccharomyces* serem incapazes de utilizar estas fontes pode justificar parcialmente a escassez de informações encontradas acerca do metabolismo do nitrato em leveduras, em comparação aos fungos filamentosos (MARZLUF, 1997). A clonagem do gene *YNR1*, codificante da enzima nitrato redutase de *H. polymorpha* (ÁVILA et al., 1995) iniciou a fase de estudos moleculares da assimilação de nitrato em leveduras.

Em leveduras capazes de assimilar nitrato, sua utilização ocorre como em outros organismos. O NO_3^- entra na célula através de transportadores de alta afinidade e então é convertido em amônia via duas reduções consecutivas catalisadas por nitrato redutase e nitrito redutase (SIVERIO, 2002). A primeira reduz NO_3^- a NO_2^- e a segunda reduz NO_2^- a NH_4^+ (Figura 3).

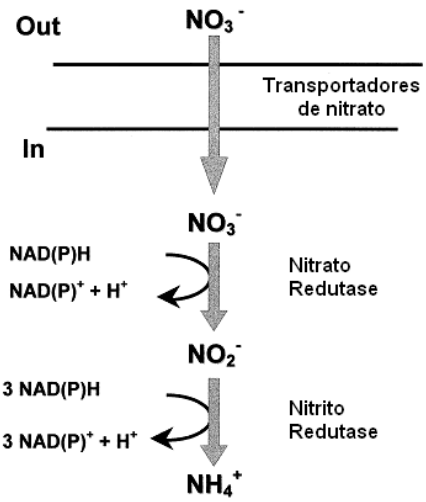

Figura 3. Via de assimilação do nitrato em leveduras. Adaptado de Siverio, 2002.

Na maioria dos organismos capazes de assimilar nitrato, este processo requer dois tipos diferentes de sinais: o primeiro é um sinal global indicando ausência de fontes preferenciais de nitrogênio (amônia, glutamato, glutamina); a segunda é uma via específica que indica a presença de nitrato (MARZLUF, 1997). Em *A. nidulans* e *N. crassa*, a indução por nitrato é mediada pelos reguladores positivos específicos NirA e NIT-4 (BURGER et al., 1991; FU et al., 1995), respectivamente. A repressão por amônia envolve um mecanismo mais complexo, que age regulando diversas vias específicas requeridas para a utilização de fontes secundárias de nitrogênio. Os ativadores globais de ligação ao DNA, AreA de *A. nidulans* e NIT-2 de *N. crassa* são fatores de transcrição Zinc-finger do tipo GATA que possuem papel central nestes mecanismos (MARZLUF, 1997). Em adição, os reguladores negativos NmrA e Nmr1 ligam a AreA e NIT2, respectivamente, para auxiliar a modulação de suas atividades e a repressão catabólica do nitrogênio (ANDRIANOPOULOS et al., 1998). Em *H. polymorph*a, a assimilação do nitrato também é sujeita a controle duplo: RCN, desencadeada por fontes reduzidas de nitrogênio e mecanismos de indução específicos, conduzidos pelo nitrato (PIGNOCCHI et al., 1998; SERRANI et al., 2001). A indução por nitrato está associada aos fatores de transcrição descritos *YNA1* e *YNA2* (SIVERIO, 2002), enquanto que a repressão por amônia é relacionada aos genes *NMR1*, *NMR2* e *NMR4* (ROSSI et al., 2005). Mutações no lócus *NMR1* desta levedura causam desrepressão da assimilação do nitrato em *H. polymorpha* na presença de glutamato, mas não da glutamina, sugerindo que um circuito de sinalização dependente de glutamina possa coexistir com um dependente de glutamato. O lócus *NMR2* é possivelmente um membro do mecanismo de sinalização dependente de glutamina e *NMR4* parece estar envolvido diretamente na percepção e/ou transporte de amônia (SERRANI et al., 2001).

Em *D. bruxellensis*, a identificação de genes envolvidos com o metabolismo do nitrato (WOOLFIT et al., 2007) veio comprovar o fato de que *D. bruxellensis* pode assimilar este nutriente (CONTERNO et al. 2006). A organização destes genes foi largamente estudada em *H. polymorpha*. Os trabalhos com este organismo revelaram que os genes *YNT1*, que codifica a proteína transportadora, *YNR1*, codificante da enzima nitrato redutase e o *YNI1*, que codifica a nitrito redutase estão situados em um único cluster (SIVERIO, 2002). Posteriormente, dois outros genes (*YNA1* e *YNA2*) codificantes de fatores de transcrição do tipo $Zn(II)_2Cys_6$ foram localizados neste mesmo cluster (ÁVILA et al., 1998; ÁVILA et al., 2002). Estes ativadores possuem domínio de ligação ao DNA compartilhado com o bem conhecido Gal4p (PARTHUN & JAEHNING, 1990) de *S. cerevisiae* e pelos bem caracterizados NirA de *A. nidulans* e NIT4 de *N. crassa*, envolvidos especificamente no metabolismo do nitrato (BURGER et al., 1991; FU et al., 1995). Devido à natureza fragmentada do seqüenciamento do genoma de *D. bruxellensis*, ainda não é possível determinar o grau de clusterização destes genes nesta espécie. Entretanto, os dados apresentados mostram claramente que os cinco genes não ocorrem em um cluster ininterrupto e que as posições relativas e orientações dos genes diferem entre *D. bruxellensis* e *H. polymorpha* (WOOLFIT et al., 2007) (Figura 4).

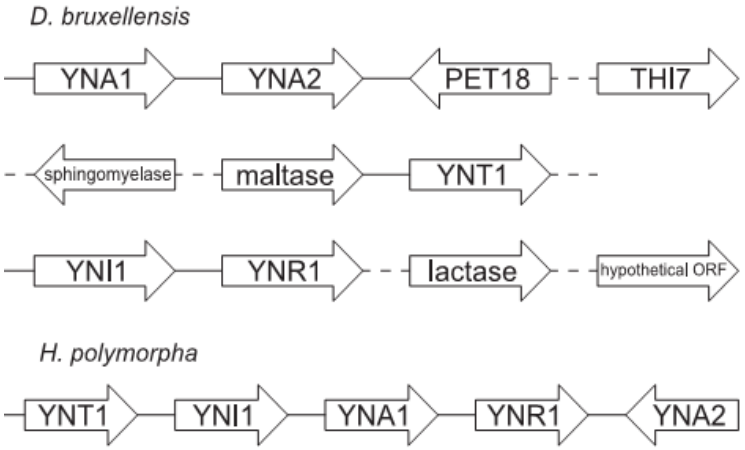

Figura 4. Comparação entre a organização dos genes da via de assimilação do nitrato em *D. bruxellensis* e *H. polymorpha*. Woolfit et al., 2007.

A vantagem adaptativa observada em *D. bruxellensis* em relação a *S. cerevisiae* no ambiente industrial demonstra sua elevada adaptação a estes ambientes. Para se tornar predominante na população de leveduras, *D. bruxellensis* deve crescer a uma taxa mais alta do que *S. cerevisiae* nas mesmas condições. Uma hipótese sugerida é que *D. bruxellensis* possui maior resistência ao etanol do que *S. cerevisiae*, conseguindo desta forma, superar a população desta última nas fases finais da fermentação (RENOUF et al., 2006). Adicionalmente, em um sistema de fermentação contínua com

reciclagem de células, altas densidades celulares são atingidas e o suprimento de nutrientes para o crescimento celular torna-se limitado. Nestes ambientes, a competição é determinada principalmente pela habilidade de utilizar o fator limitante do crescimento. Se *D. bruxellensis* é capaz de metabolizar um nutriente disponível mais eficientemente, ou se for capaz de captá-lo com maior afinidade do que *S. cerevisiae*, sua taxa de crescimento pode ser bem maior nesta condição particular. Dentre as evidentes diferenças metabólicas entre *S. cerevisiae* e *D. bruxellensis* destaca-se a capacidade desta última de assimilar nitrato e nitrito como fontes de nitrogênio, característica que é utilizada na taxonomia bioquímica do gênero (BARNETT et al., 2002). O caldo de cana pode apresentar concentrações significativas de nitrato oriundo do processo de adubação do campo com fertilizantes a base de nitrato (nitrato de cálcio, nitrato de magnésio ou mesmo nitrato de amônia), muito utilizado nos canaviais. Desta forma, o nitrato poderia constituir uma fonte de nitrogênio que seria assimilada por células de *D. bruxellensis* para seu crescimento. Como *S. cerevisiae* não assimila nitrato, então isso explicaria a diferença na velocidade de crescimento calculada para as duas leveduras no processo industrial (DE SOUZA LIBERAL et al., 2007).

Desta forma, entender o papel que diferentes fontes de nitrogênio desempenham no metabolismo de *D. bruxellensis* é fundamental para a identificação de fatores nutricionais que levaram esta levedura a se tornar um microrganismo tão bem adaptado ao ambiente de fermentação alcoólica industrial, sendo capaz de, em alguns casos, superar *S. cerevisiae*, assumindo o lugar de principal população de leveduras nesses processos. O presente trabalho apresenta dados fisiológicos e genéticos que avaliam a influência de fontes primárias e secundárias de nitrogênio no crescimento, produção de metabólitos e na expressão gênica de *D. bruxellensis*.

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CAPÍTULO II

The ability to use nitrate confers advantage to *Dekkera bruxellensis* over *S. cerevisiae* and can explain its adaptation to industrial fermentation processes

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ORIGINAL PAPER

The ability to use nitrate confers advantage to *Dekkera bruxellensis* over *S. cerevisiae* and can explain its adaptation to industrial fermentation processes

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Abstract The yeast *Dekkera bruxellensis* has been regarded as a contamination problem in industrial ethanol production because it can replace the originally inoculated *Saccharomyces cerevisiae* strains. The present study deals with the influence of nitrate on the relative competitiveness of *D. bruxellensis* and *S. cerevisiae* in sugar cane ethanol fermentations. The industrial strain *D. bruxellensis* GDB 248 showed higher growth rates than *S. cerevisiae* JP1 strain in mixed ammonia/nitrate media, and nitrate assimilation genes were only slightly repressed by ammonia. These characteristics rendered *D. bruxellensis* cells with an ability to overcome *S. cerevisiae* populations in both synthetic medium and in sugar cane juice. The results were corroborated by data from industrial fermentations that showed a correlation between high nitrate concentrations and high *D. bruxellensis* cell counts. Moreover, the presence of nitrate increased fermentation efficiency of *D. bruxellensis* cells in anaerobic conditions, which may explain the maintenance of ethanol production in the presence of *D. bruxellensis* in industrial processes. The presence of high levels of nitrate in sugar cane juice may be due to its inefficient conversion by plant metabolism in certain soil types and could explain the periodical episodes of *D. bruxellensis* colonization of Brazilian ethanol plants.

Keywords *Dekkera bruxellensis* · Ethanol fermentation · Nitrate assimilation · Sugarcane · Industrial adaptation

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Introduction

Dekkera bruxellensis (anamorph name *Brettanomyces bruxellensis*), a hemiascomycete yeast, is well known as the main cause of wine spoilage worldwide (Loureiro and Malfeito-Ferreira 2003). Recently, *D. bruxellensis* has also been identified as a major contaminant yeast in the fuel ethanol fermentation process in northern Brazil where its presence can result in reduction of ethanol productivity, with consequent industrial economic loss (Liberal et al. 2007).

Despite this observation, the species has also been described as a bioethanol fermenting yeast, possibly as part of a consortium of species including the bacterium *Lactobacillus vini*, as has been reported in a study involving a Swedish Ethanol plant (Passoth et al. 2007). Indeed, it has been stated that *Dekkera bruxellensis* has the potential to be a production yeast due to its high ethanol yield (Liberal et al. 2007; Blomqvist et al. 2010).

Its competitive success compared to *S. cerevisiae* in an industrial environment as found both in Brazil and Sweden could be explained by physiological adaptations such as higher ethanol (Medawar et al. 2003) or organic acid (Abbott et al. 2005) tolerance, or by the possibility of utilizing alternative nutrients, or even the ability to scavenge limiting nutrients present in the industrial substrate. Nitrate could be one of these adapting factors, since *D. bruxellensis* is able to use this compound as sole nitrogen source (Conterno et al. 2006), whereas *S. cerevisiae* cannot.

From the sequenced *D. bruxellensis* genome, Woolfit et al. (2007) reported the presence of a nitrate assimilation gene cluster that contains the genes encoding for the nitrate assimilation pathway: the nitrate transporter (*YNT1*); nitrite reductase (*YNI1*); and nitrate reductase (*YNR1*). This cluster also contains two Zn(II)2Cys6 type transcription factors specifically involved in nitrate induction.

Nitrate assimilation by *D. bruxellensis* cells may follow the same pathway described for filamentous fungi. First, it is transported into the cells by the nitrate transporter encoded by *YNT1* gene and subsequently converted to ammonium by two reductions catalyzed respectively by nitrate reductase encoded by *YNR1* gene and nitrite reductase encoded by *YNI1* gene (Siverio 2002). The expression of these structural genes is under the control of two transcription factors encoded by *YNA1* and *YNA2* genes. All five *D. bruxellensis* genes are orthologous to those described for *Hansenula anomala* and *H. polymorpha* (Avila et al. 1998; Garcia-Lugo et al. 2000). Usually, nitrate will not be utilized as nitrogen source unless the medium is depleted of a preferred nitrogen source, such as ammonium, glutamate or glutamine (Siverio 2002). This restricted use of nitrate is the result of nitrogen metabolite repression (NMR), the mechanism that regulates gene expression according to the availability of nitrogen sources (Magasanik 2003).

Since these species compete in an industrial environment, the presence of nitrate could provide an advantage for *D. bruxellensis* adaptation, since sugar cane juice may contain significant amounts this nutrient derived from soil bacteria metabolism and/or field fertilizers. Therefore, the expression of those genes in the industrial environment may confer on *D. bruxellensis* cells the ability to grow after exhausting preferred nitrogen sources, such as ammonium and amino acids. Under these circumstances only *D. bruxellensis* could take advantage of the nitrate present in the medium.

In view of this physiological capacity, we formulated a hypothesis in which the presence of nitrate in the industrial substrate could, at least in part, explain how *D. bruxellensis* can be successful in competing with *S. cerevisiae* in an industrial environment involving ethanol fermentation.

Materials and methods

Growth and fermentation experiments

The industrial strain *D. bruxellensis* GDB248 (Liberal et al. 2007) was cultivated in synthetic complete (SC) medium (1.7 g YNB, 5 g ammonium sulfate, 20 g glucose per liter) for pre-cultures and the cells were inoculated in 500 ml flasks containing 150 ml of the following media: (i) SC, (ii) SC containing sodium nitrate (6.43 g l^{-1}) as sole nitrogen source, (iii) SC medium containing both nitrogen sources, and (iv) diluted filter-sterilized sugar cane juice (with sucrose at 120 g l^{-1}) from a contaminated process that contained ammonium and nitrate at initial concentrations of 216 and 242 mg l^{-1}. Ammonium and nitrate concentrations were measured using enzymatic kits from Sigma–Aldrich (cat. nr. 23479 and AA0100, respectively). Yeast cells at an initial optical density of around 0.1 (OD_{600nm}) were grown at 30°C in a rotator shaker at 180 rpm and all experiments were performed in triplicate. Two samples were withdrawn at defined periods of time for OD determination and the mean values were plotted in semi-log graphics for the calculation of specific growth rate ($\mathbf{l}$) according to the equation: $\mathbf{l} = [\mathrm{Ln}X - \mathrm{Ln}X_0]/\mathrm{Dt}$, whereas X_0 and X represent initial and final cell biomass, respectively, in a certain interval of time (Dt) at the exponential growth phase.

Competition experiments

Successive batch recycling fermentation experiments were designed in which both *S. cerevisiae* JP1(Silva-Filho et al. 2005) and *D. bruxellensis* GDB 248 cells were pre-cultivated in SC medium and then mixed 90 and 10% of cell population, respectively, in different fermentation media to 10% (w/v) of initial biomass (approx. 5 x 10^8 cells ml^{-1}). *D. bruxellensis* cells were added at low cell count to mimic the initial stages of industrial yeast contamination (Liberal et al. 2007). Flasks of 500 ml contained 150 ml of the following fermentation media: (i) SC-high ammonium containing 5 g l^{-1} ammonium sulfate and 6.43 g l^{-1} sodium nitrate, (ii) SC-low ammonium containing 0.5 g l^{-1} ammonium sulfate and 6.43 g l^{-1} sodium nitrate and (iii) diluted filter-sterilized sugar cane juice. All fermentation media contained sucrose at 140 g l^{-1}. The cultures were incubated for 24 h at 30°C under slow agitation and samples were diluted and spread onto WLN medium (for total yeast colony counts) and WLN + cycloheximide (1.0 g l^{-1}) (for *D. bruxellensis* colony counting only) (Liberal et al. 2007; Basílio et al. 2008). The remaining cultures were centrifuged. The supernatant was used for ammonium and nitrate quantification using enzymatic kits and ethanol determinations by GC analysis (Liberal et al. 2007), and the cells suspended in the same volume of fresh media for a further 24 h incubation. Successive batches were repeatedly performed for 15 recycles. The differential growth rates of the two yeast subpopulations were calculated from the slope of a straight line by plotting $\mathrm{Ln}[(1/F_{Db})-1]$ against time, where F_{Db} means the fraction of the *D. bruxellensis* subpopulation (see appendix at Liberal et al. 2007 for calculations).

Industrial analysis

Industrial samples of the feeding substrate were collected from the distilleries Japungu Agroindustrial SA and Miriri Food and Energy SA during the harvesting season 2009–2010 in periods during which both processes were contaminated with *D. bruxellensis*. Ammonium and nitrate concentrations were determined using enzymatic kits, whereas the presence of *D. bruxellensis* was confirmed by spreading on WLN + cycloheximide followed by molecular analysis of the yeast colonies (Liberal et al. 2007; Basílio et al. 2008).

In view of the high concentration of nitrate in cane juice, we further investigated the origin of this nutrient as well as the physical–chemical characteristics of the fermentation substrates and the soil. Soil and cane juice samples from two farms that supplied cane for the Miriri distillery were analyzed for their mineral concentrations by Central Analitica Ltd. (Maceio, Brazil), a facility which provides physi- cal–chemical analysis for several agro-industries in northeastern Brazil. Healthy cane from Farm no. 1 had not been associated with fermentation problems at the distillery, whereas cane from Farm no 2 had been consistently associated with drops in ethanol production and yeast contamination (unpublished industrial data).

Gene expression analysis

Yeast cells were collected after 6 h of cultivation (see Fig. 1) by centrifugation from the growth experiments in synthetic and cane juice media and immediately frozen in liquid nitrogen and stored at −80°C. Total RNA was isolated using NucleoSpin® RNA II kit according to manufacturer's instructions (Macherey–Nagel, USA) and cDNA was synthesized using 1 µg of total RNA for each reverse transcription tube with ImProm-II™ Reverse Transcription System Promega II kit (Promega, USA) following the manufacturer's instructions. Nucleotide sequences of *D. bruxellensis YNR1*, *YNI1* and *YNT1* genes were obtained from Genbank database (accession numbers: EF364428; EF364429; EF364426). *TEF1* and *ACT1* were used as candidate reference genes, as proposed by Nardi et al. (2010). The oligonucleotides for RT-qPCR were designed by Primer Express® software (Applied Biosystems, USA). The following best pairs of primer for each gene were obtained: YNR1F (5´-TGCTGG AAACAGGCGTAAAGA-3´) and YNR1R (5´-TCC CAGCAGAACCCCAATT-3´), YNI1F (5´-AAAAGC TGAGCATCGTTGTG-3´) and YNI1R (5´-TTGTT CTGTTCCTGGTCCAA-3´), YNT1F (5´-CAGATAC GGTCCACGTTGGG-3´) and YNT1R (5´-ACGGCA TAAACGCTGTAGGG-3´). Standard curves were determined for all pairs of primers in order to assess their amplification efficiencies. All primers showed a variation of less than 5% between replicates, with R^2 above 0.99 and slope in the range of −3.4, indicating the high precision for Cq values obtained in these experiments. Normalization was performed on both

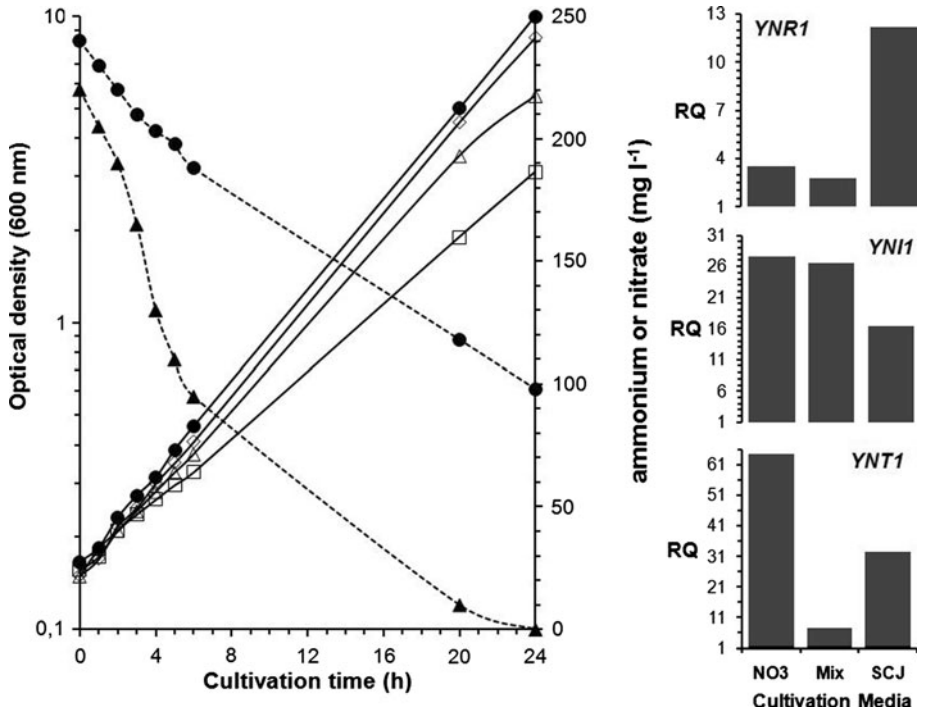

Fig. 1 Growth curves of *Dekkera bruxellensis* cells in SC medium containing ammonium (triangle), nitrate (square) or both N-sources (diamond) or in sugar cane juice (dark circle), where consumption of nitrate (dark circle with dotted line) and ammonium (dark triangle with dotted line) were followed in sugar cane juice. Relative Quantification (RQ) of the nitrate assimilation genes *YNR1*, *YNI1* and *YNT1* was evaluated in SC nitrate-based (NO_3), SC ammonium-nitrate (Mix) and in sugar cane juice (SCJ) relative to their expression in cells cultivated on the reference SC medium containing ammonium as N-source. Cells were collected after 6 h of cultivation

reference genes using the geNorm applet (Vandesompele et al. 2002). RT-qPCR assays were performed with SYBR Green PCR Master Mix (Applied Biosystems, USA), optimized for maximum efficiency for the lowest threshold cycle and higher DRn. The specificity of the reaction was evaluated by analyzing the melting curve profile. The amplification reactions were composed of 12.5 µl SYBR Green master mix, 1.0 µl each primers (200 nmol l^{-1} final), 2.5 µl cDNA and 8 µl H_2O. The cycling parameters were 95°C for 10 min as hot-start, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min in an ABI Prism 7300 device (Applied Biosystems). Amplification curves were analyzed via software SDS v.2.0 (Applied Biosystems). Negative PCR controls (for unspecific amplification without template) and negative RT controls (for genomic DNA contamination without reverse transcriptase) were run in parallel for quality control. The estimate of contamination by genomic DNA was determined as $[1/2^{Cq\ RNA\ sample\ -\ Cq\ cDNA\ sample}]$. The amplification results were analyzed by SDS 2.0 software (Applied Biosystems, USA) and the relative quantification calculation was performed in Microsoft Excel® 2007. Relative quantification of the reference genes was compared between the different cultures and a variation of less than 0.5 Cq was observed for all conditions tested. All amplification experiments were performed at least three times for each sample of duplicated cell cultures, totaling six samples for each growth medium.

Results

The specific growth rates of GDB 248 cells were calculated as 0.09, 0.14 and 0.16 h^{-1} for the cultivation in SC media containing nitrate, ammonium and mixed N-sources, respectively (Fig. 1). A similar result was observed when sucrose was used as carbon source (data not shown). In sugar cane juice that contains both N-sources, under the conditions used in the present work, *D. bruxellensis* cells grew at 0.18 h^{-1} (Fig. 1)—higher than in ammonium-nitrate

mixed synthetic medium. The hypothesis that nitrate assimilation confers an advantage to *D. bruxellensis* depended on the capacity of the yeast cells to assimilate this nitrogen source even in the presence of ammonium. The measurements of nitrate assimilation in sugar cane juice medium showed that it was co-consumed with ammonium (Fig. 1). Upon ammonium depletion, nitrate continued to sustain cell growth, even when the growth rate in the reference SC medium started to diminish (Fig. 1).

Nitrate co-assimilation was possible due to the high expression of the nitrate assimilation genes, even in presence of ammonium (Fig. 1). The *YNR1* gene seemed to be more affected by ammonium repression since, in sugar cane juice with very low ammonium, its expression was four times higher than in SC mixed medium. On the other hand, expression of *YNI1* gene seemed to be dependent on the nitrate concentration in the medium since it was two times lower in sugar cane juice that contains lower nitrate than in SC mixed medium. Moreover, the *YNT1* gene displayed an intermediary form of regulation in which repression by ammonium was counterbalanced by the presence of nitrate (Fig. 1).

In order to mimic industrial processes, yeast cells were submitted to fermentation recycling experiments with low growth rates due to the initial high cell density, limited concentration of N-source and absence of aeration. In SC mixed medium with higher concentration of ammonium, *S. cerevisiae* populations remained dominant during the fermentation cycles (Fig. 2a), taking advantage of its known higher growth rate on ammonium. Ethanol concentration at the end of each cycle ranged from 50 to 60 g l^{-1}. However, when ammonium concentration was lower in SC mixed medium, *D. bruxellensis* populations increased in proportion throughout the recycling periods to reach and eventually surpass *S. cerevisiae* population levels (Fig. 2b).

The fluctuations observed in that medium can be explained by the re-feeding with ammonium at the beginning of every recycle, which may benefit *S. cerevisiae* cells. In those experiments, no significant interference on ethanol concentration was observed even when the culture was overloaded with *D. bruxellensis* cells. If sugarcane was used as a growth substrate, with both ammonium and nitrate at low concentrations, the population of *S. cerevisiae* experienced limited growth as observed by the cell count at the end of each batch, while the percentage of the *D. bruxellensis* population increased much faster. Thus *D. bruxellensis* equaled *S. cerevisiae* population levels and remained high from a certain point during the recycling (Fig. 2c). Once again, no influence on ethanol production was detected. From these data, it was possible to calculate the difference between the yeast growth rates of 0.013 and 0.014 h^{-1} in favor of *D. bruxellensis* during the growth period using sugar cane juice (Fig. 2c) or synthetic medium with high nitrate/low ammonium (Fig. 2b), respectively.

A further challenge for this "nitrate hypothesis" was to connect the laboratory observations to data from

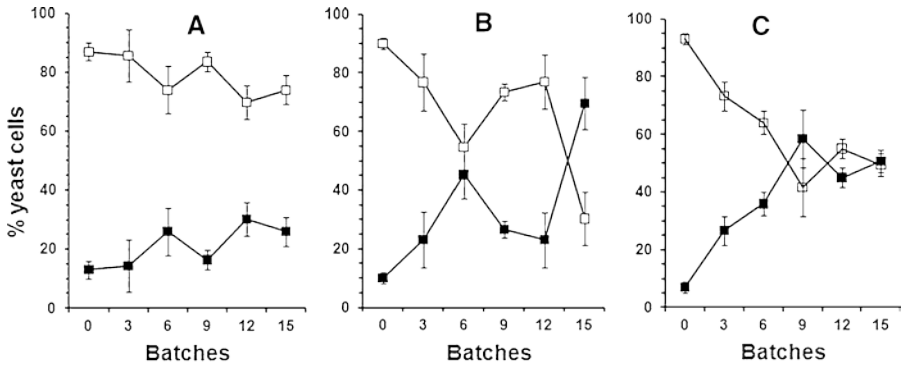

Fig. 2 Growth competition experiment in which cells of *Saccharomyces cerevisiae* (square) and *Dekkera bruxellensis* (dark square) were mixed to 9:1 initial proportion in SC medium containing high ammonium (panel a: ammonium at 5 g/l and nitrate at 6.43 g/l) or low ammonium (panel b: ammonium at 0.5 g/l and nitrate at 6.43 g/l) or in sugar cane juice (panel c: ammonium at 0.2 g/l and nitrate at 0.24 g/l). Successive batches of 24 h were performed and the results represent the quantification of each yeast population at the end of each fermentation cycle. Diferential growth calculations used data from cycles 1–6 (panel b) and 1–9 (panel c)

industrial processes. Thus, we analyzed the fermentation process at the Miriri and Japungu distilleries during the harvest season 2009–2010 when both distilleries were constantly affected by *D. bruxellensis* contamination (Liberal et al. 2007; Basílio et al. 2008). The results showed that *D. bruxellensis* populations increased to almost 50% in the fermented must when nitrate concentration in the substrate was significantly higher, and that it decreased to less than 5% of the total yeast when nitrate concentration fell in the feeding substrate (Fig. 3).

However, it was intriguing why the increase of *D. bruxellensis* cells did not interfere in ethanol production, either in laboratory experiments or in the industrial process, as it has been reported that this yeast ferments more slowly than *S. cerevisiae* cells (Liberal et al. 2007; Basílio et al. 2008; Blomqvist et al. 2010). Batch fermentation experiments were performed in SC media containing both N-sources. The results showed that ethanol production was lower when nitrate is the only N-source when compared to medium containing ammonium (Fig. 4), which can be explained by the high energy demand for nitrate assimilation. On the other hand, the presence of nitrate increased ethanol production by 27% in ammonium-based medium, and this coincided with the faster sucrose consumption (Fig. 4).

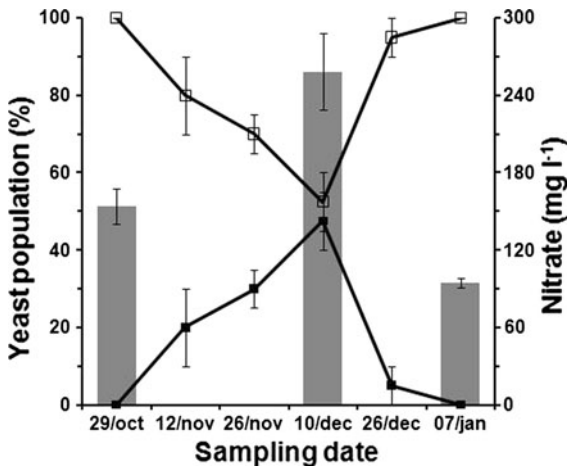

Fig. 3 Relation between cell concentration of *Saccharomyces cerevisiae* (square) and *Dekkera bruxellensis* (dark square) cells and the nitrate concentration (grey bars) in the fermented must of two different distilleries collected along the harvesting season 2009–2010. The results represent the mean value (± sd) of two samples of each distillery in the indicated sampling dates

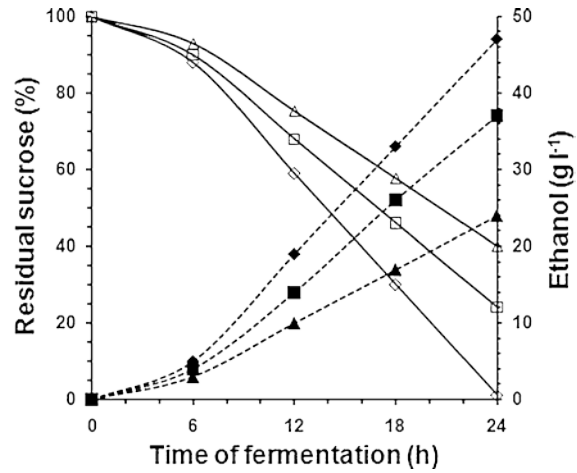

Fig. 4 Kinetics of sucrose fermentation by Dekkera bruxellensis cells in SC medium containing ammonium (square symbols), nitrate (triangle symbols) of both N-sources (diamond symbols) as measured by sugar consumption (straight lines) and ethanol production (dashed lines)

The origin of nitrate in the fermentation process was investigated in samples of sugar cane juice from the farms that supplied the distilleries during the periods analyzed. Nitrate concentrations were higher in cane juice from the farm no 2 (243 mg l^{-1}) that mostly supplied Miriri distillery during the contamination episodes than in cane juice from farm no 1 (128 mg l^{-1}) that mostly supplied that distillery in a period with no contamination episodes.

Since molybdenum is essential for nitrate metabolism, we analyzed its availability in the sugar cane field. It is noteworthy that molybdenum concentration was 32% lower in the juice from cane of farm no. 2 [9.53 ± 0.07 mg l^{-1} in farm no. 1 and 6.42 ± 0.09 mg l^{-1} in farm no. 2], despite a 35% higher concentration in the soil of farm no. 2 [12.23 ± 0.3 mg kg^{-1}] compared to farm no. 1 [7.95 ± 0.7 mg kg^{-1}]. The low assimilation of molybdenum, and accumulation in the soil, was due to the observed higher potential acidity of 7.1 mEq ($H^+ + Al^{+3}$)/ 100 ml observed for the soil of farm no. 2 compared to 3.4 mEq ($H^+ + Al^{+3}$)/100 ml of the soil in farm no. 1.

Discussion

Dekkera bruxellensis has been reported to out-

compete *S. cerevisiae* in bioethanol production plants in Brazil and Sweden (Liberal et al. 2007; Passoth et al. 2007). The slow growth of *D. bruxellensis* cells in laboratory media was always observed in our laboratory and the lag phase of the cultures was very long, as recently presented by Blomqvist et al. (2010). We confirmed that the low growth rate of *D. bruxellensis* in laboratory media seems to be a physiological trait of this species, whatever strain is used. Thus, since nitrate assimilation increased the specific growth rate in ammonium-rich media, it may be one of the physiological traits that ensures the high fitness of *D. bruxellensis* in industrial environments that naturally contain both nitrogen sources.

Usually, nitrate is not utilized as a nitrogen source unless the preferred nitrogen source, such as ammonium, glutamate or glutamine, is depleted from the medium (Siverio 2002). This restricted use of nitrate is the result of nitrogen metabolite repression (NMR), the mechanism that regulates gene expression according to the availability of nitrogen sources (Magasanik 2003). In the present case, nitrate was co-consumed with ammonium by *D. bruxellensis* (Fig. 1). This was possibly due to the expression of nitrate assimilation genes even in the presence of ammonium (Fig. 1). This partial ammonium repression resembles that presented by *H. anomala* (García-Lugo et al. 2000), but differed from the strong repression observed for *H. polymorpha* (Avila et al. 1998) and *Arxula adeninivorans* (Böer et al. 2009). When nitrate was the only N-source, the *YNT1* gene was the most induced gene in *D. bruxellensis* (Fig. 1), while *YNR1* gene was the most induced gene in *H. polymorpha* (Avila et al. 1998) and *YNI1* gene was the most induced gene in *A. adeninivorans* (Böer et al. 2009).

Another remarkable difference was the strong repression of *YNT1* gene in *D. bruxellensis* whereas this gene was weakly repressed in *H. polymorpha* (Avila et al. 1998). In sugar cane juice with both ammonium and nitrate at low concentration the high level of expression of the three genes in *D. bruxellensis* may allow enough nitrate uptake capacity for the yeast cells and the higher growth rate in this substrate. All the expression data were relative to ammonium-based medium. In nitrate-based medium we observed almost four times more *YNR1* gene transcripts than in ammonium, and that expression was even higher in sugar cane with low nitrogen concentration. Thus, we can conclude that expression of this gene depends more on the nitrogen availability than the source of nitrogen and any expression may ensure a certain enzyme level to metabolize nitrate to nitrite. Since the present results on gene expression are only corroborative of the physiological data on nitrate assimilation, we are not going into a detail on the regulation of the nitrate assimilation gene cluster. That regulation is currently being studied in our laboratory.

Given the fact that the presence of nitrate in cane juice is stimulatory of *D. bruxellensis* cell growth, we performed growth competition experiments mimicking the industrial process. Under the successive recycling regime in high cell density mixed cultures (Fig. 2), it was clearly demonstrated that the *D. bruxellensis* population outgrew *S. cerevisiae* when the ammonium concentration was low and nitrate was present in the media. This result resembles the population profile observed for industrial processes contaminated by *D. bruxellensis* (Liberal et al. 2007).

In the present work it is shown that the presence of nitrate in ammonium-based synthetic medium increased *D. bruxellensis* growth and that yeast growth in sugar cane juice was higher than in synthetic media. It could be speculated that nitrate assimilation produced nitrite which is very toxic for *S. cerevisiae*. This might be the case at high nitrate concentrations, but it is not the case for sugar cane. Besides, if it were the case, then the ability to assimilate nitrate, whether for cell growth or to produce inhibitory compound, would still be an advantage of *D. bruxellensis* over *S. cerevisiae*.

It has previously been demonstrated that any increment in the growth rate in the range of 0.014 h^{-1} might favor *D. bruxellensis* over *S. cerevisiae* in the cane juice industrial fermentation process (Liberal et al. 2007). In the present work we showed that this difference between the yeast growth rates can be reproduced under laboratory conditions in successive batches using sugar cane juice or synthetic medium with high nitrate/low ammonium, respectively. It fitted to the previous calculations on industrial data (Liberal et al. 2007). It was also possible to corroborate the laboratory findings with industrial data that showed the correlation between the increase in *D. bruxellensis* cell populations and the increasing of nitrate in the fermentation substrate (Fig. 3). Therefore, taking into account all the results presented above, we propose that nitrate assimilation is an important adaptation factor for *D. bruxellensis* cells to grow and to establish themselves in the industrial fermentation process.

However, it is intriguing why the increasing of *D. bruxellensis* cells did not interfere in ethanol production, either in laboratory experiments or in the industrial process, as it has been reported that this yeast ferments slower than *S. cerevisiae* cells (Liberal et al. 2007; Basílio et al. 2008; Blomqvist et al. 2010). The species *D. bruxellensis* is one of the model yeasts used to study the Custer effect (Barnett and Entian 2005) in which fermentation is stuck by the limitation of oxygen. This explanation is based on the lower capacity of those cells to re-oxidize NADH produced by the glycolytic pathway. In the present case, the presence of nitrate could also provide an alternative redox sink for the excess of NADH, since assimilation of 1 mol of nitrate consumes 4 mol of NAD(P)H (Siverio 2002). Indeed, this proved to be the case since nitrate increased not only sucrose assimilation but also the production of ethanol (Fig. 4). We can speculate that in mixed N-source medium NADPH should be preferentially used by glutamate dehydrogenase for ammonium assimilation while the glycolytic NADH could be used for nitrate assimilation, helping the cells maintain redox balance. This result could explain why under industrial conditions there is no clear correlation between *D. bruxellensis* contamination and drop in ethanol yield (Liberal et al. 2007). We have additional data showing that ethanol yield started to drop only when *D. bruxellensis* cells represented more than 40% of the yeast population (unpublished data). The role of nitrate in abolishing the Custer effect is currently under investigation in our laboratory.

If the "nitrate hypothesis" is accepted as one important factor for *D. bruxellensis* settlement at the industrial process, then the episodes of yeast contamination might be linked to the availability of this nitrogen source in the fermentation must during the harvesting season, as observed in the Fig. 3. Nitrate is produced in the soil by nitrifying bacteria (Nitrosomonas and Nitrobacter) or added by fertilization and is the preferred nitrogen source for most crops (Burger and Jackson 2004; Hirel et al. 2007). Once absorbed by plant cells, nitrate is converted to nitrite by nitrate reductase, and nitrite is converted to ammonium ion by nitrite reductase (Burger and Jackson 2004). It is noteworthy that nitrate reductase is highly dependent on molybdenum in that low uptake of this mineral, and consequently its lower concentration in the plant, may lead to accumulation of nitrate by the plant (Kaiser et al. 2005). We detected accumulation of molybdenum in the soil of cane plantation associated with contamination by *D. bruxellensis*. This shortage of molybdenum may lower metabolic conversion of nitrate by the plant, accounting for its periodical higher content in the cane juice. This could also explain the population dynamics periodically observed in the distilleries affected by *D. bruxellensis* contamination.

The present study provides strong indications that the capacity of *D. bruxellensis* to assimilate the small amounts of nitrate in sugar cane juice used for ethanol fermentation, even in the presence of ammonium, is one important factor in the competitiveness of this yeast against *S. cerevisiae*. Although we are still considering whether nitrate assimilation is the only explanation for industrial fitness, it opens an interesting field on the study on the *D. bruxellensis* metabolism and also on the ecological interactions between microorganisms involved in industrial fermentation processes.

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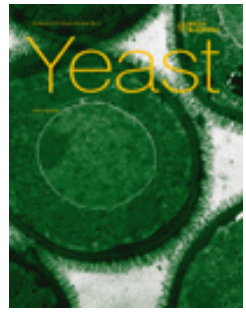
CAPÍTULO III

The influence of nitrate on the physiology of the yeast *Dekkera bruxellensis* grown under oxygen limitation



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The influence of nitrate on the physiology of the yeast *Dekkera bruxellensis* grown under oxygen limitation

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Abstract

A previous study showed that the use of nitrate by *Dekkera bruxellensis* might be an advantageous trait when ammonium is limited in sugarcane substrate for ethanol fermentation. The aim of the present work was to evaluate the influence of nitrate on the yeast physiology during cell growth under oxygen limitation in different carbon sources. If nitrate was the sole source of nitrogen, *D. bruxellensis* cells presented slower growth, diminished sugar consumption and growth-associated ethanol production, when compared to ammonia. These results were corroborated by the increased expression of genes involved in pentose-phosphate pathway, tricarboxylic acid cycle and ATP synthesis. The presence of ammonium in the mixed medium restored most parameters to the standard conditions. This work may open up a line of investigations to establish the connection between nitrate assimilation and the energetic metabolism in *D. bruxellensis* and their influence on its fermentative capacity in oxygen-limited or oxygen-depleted conditions.

Keywords: nitrate assimilation; energetic metabolism; ethanol production; gene expression; RT-qPCR.

Introduction

The yeast *Dekkera bruxellensis* has been isolated from ethanol fermentation, in which it is able to outgrow *Saccharomyces cerevisiae* populations (Passoth *et al.* 2007; de Souza Liberal *et al.* 2007; Basílio *et al.* 2008). This is an interesting feature, since it shows a very low specific growth rate in laboratory conditions (de Souza Liberal *et al.* 2007; Blomqvist *et al.* 2010). In the light of this trait, this yeast is likely to be physiologically adapted to the industrial environment and may be pointed as potential industrial yeast. Thus, a growing number of genetic and physiological studies were performed in the past few years (de Souza Liberal *et al.* 2007; Woolfit *et al.* 2007; Passoth *et al.* 2007; Hellborg and Piskur 2009; Nardi *et al.* 2010; Blomqvist *et al.* 2010; de Barros Pita *et al.* 2011; Pereira *et al.* 2012; Curtin *et al.* 2012; Blomqvist *et al.* 2012; Piskur *et al.* 2012; de Souza Liberal *et al.* 2012; de Souza *et al.* 2012; Leite *et al.* 2012; de Barros Pita *et al.* 2012), which has now led to a new field of possible metabolic studies.

D. bruxellensis possesses the gene cluster (NIT genes) encoding for nitrate assimilation pathway: nitrate transporter (*YNT1*); nitrate reductase (*YNR1*); nitrite reductase (*YNI1*) and two Zn(II)2Cys6 type transcription factors (*YNA1* and *YNA2*) that are specifically involved in gene regulation (Woolfit et al., 2007). Nitrate assimilation in *D. bruxellensis* is likely to follow the same pathway described for *Ogataea parapolymorpha* (formerly *Hansenula polymorpha*) and filamentous fungi (Siverio 2002). Since nitrate might be one important factor in industrial adaptation, understanding its influence on *D. bruxellensis* metabolism can provide valuable information on how this species can outcompete *S. cerevisiae* in industrial fermentation processes (de Souza Liberal *et al.* 2007; Passoth *et al.* 2007; de Barros Pita *et al.* 2011; Blomqvist *et al.* 2012).

Recently it has been demonstrated that an industrial *D. bruxellensis* isolate is unable to grow with nitrate as sole nitrogen source under anaerobic conditions (Blomqvist et al. 2012). On the other hand, stimulation of *D. bruxellensis* growth and competitiveness by nitrate has been shown in aerobic cultivations (de Barros Pita et al. 2011). To understand the influence of nitrate on the metabolism under conditions relevant

for industrial cultivations, this study investigates growth and ethanol production, as well as expression of key genes in the energy metabolism with ammonium and nitrate as nitrogen sources on different sugars.

Materials and Methods

Yeast strains and growth conditions

Dekkera bruxellensis GDB 248 industrial strain (de Barros Pita et al., 2011) was used in this study. Cells were pre-grown in synthetic minimal medium – SC (Yeast Nitrogen Base w/o amino acids and ammonium sulfate at 1.7 g l^{-1}) supplemented with ammonium sulphate and glucose to 38 mmol l^{-1} and 110 mmol l^{-1}, respectively, at 30°C and 150 rpm in a rotator shaker for 24 hours. Cells were harvested by centrifugation (5 min, 5,000 g, room temperature) and suspended to 0.1 OD_{660nm} in a final volume of 200 ml of SC medium, containing 110 mmol l^{-1} of carbon source and 75 mmol l^{-1} of nitrogen source ($(NH_4)_2SO_4$, $NaNO_3$ or both). Cultures were prepared in 250 ml flasks to obtain oxygen limitation as previously described (Blomqvist *et al.* 2010). Cells were cultivated as above and the growth rate was calculated from optical density of the cultures (de Barros Pita *et al.* 2011).

Biomass and metabolite analysis

Samples for metabolites quantification were collected after 24 hours of cultivation, which corresponded to glucose depletion in ammonium-based medium (reference medium), so all measurements could be standardized to the reference. Samples were collected in triplicates and prepared as described by Blomqvist *et al.* (2010). Glucose, sucrose, fructose, ethanol, acetate and glycerol concentrations were measured by HPLC on Agilent 1100 Series platform (Agilent, USA). Biomass was determined from optical density of the cultures, using a previous standard curve. Mass balance was

calculated using the value of CO_2 yield based on the stoichiometry for ethanol and acetate production (Blomqvist et al., 2010).

RNA isolation and cDNA synthesis

Cells were cultivated to 1.0 OD_{660nm}, which took different times for different carbon-nitrogen combinations, and cells were collected for total RNA extraction (de Barros Pita et al., 2011). This ensured that gene expression analyses were performed from cells in the same physiological state and could be compared to our previous work. Samples were processed immediately after cells had been collected. RNA integrity was evaluated using RNA Nano Chips on Agilent 2100 Bioanalyzer (Agilent Technologies, USA) and samples were quantified on NanoDrop 2000 (Thermo Scientific, USA). RNA samples were stored at -80 ºC and cDNA was synthesized employing oligo(dT) as primer and using 1 µg of total RNA for each reverse transcription tube with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, USA), following the manufacturer's instructions. cDNA was stored at -20 ºC until RT-qPCR assays.

Gene expression analysis

Nucleotide sequences for *D. bruxellensis* genes of interest (GOI) *ACO1* (aconitase), *MDH1* (mitochondrial malate dehydrogenase), *GND1* (6-phosphogluconate dehydrogenase), *TKL1* (transketolase) *ATP1* (alpha subunit of ATP synthase complex) and *ADH1* (alcohol dehydrogenase) were obtained from Genbank (EI016687.1, EI026362.1, EI025873.1, EI015058.1, EI014661.1 and EI016249.1, respectively). Sequences of NIT genes have been previously examined (de Barros Pita et al., 2011). Oligonucleotides were designed by Primer Express software (Life Technologies, USA) (Supporting information – Table S1). References genes *EFA1* and *ACT1* were used as candidate reference genes (Nardi *et al.* 2010; de Barros Pita *et al.* 2012). Amplification efficiencies and standard curves were performed for each pair of primers (de Barros Pita *et al.* 2012). Data normalization in each sample was performed by using both reference

genes in geNorm applet (Vandesompele et al., 2002). RT-qPCR assays were performed with IQ^{TM} SYBR Green Supermix (BIO-RAD, USA).

The reaction specificity was evaluated by analyzing the melting curve profile. Amplification reactions were composed of 12.5 µl SYBR Green master mix, 1.0 µl each primer (final concentration 200 nmol l^{-1}), 2.5 µl cDNA and 8 µl H_2O. Cycling parameters were 95 ºC for 10 min as hot-start, followed by 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min in the CFX96^{TM} Real-Time System, C1000 Thermal^{TM} Cycler (BIO-RAD). Negative PCR controls (for unspecific amplification) and negative RT controls (for genomic DNA contamination) were run in parallel for internal control (de Barros Pita *et al.* 2012). The amplification results were analyzed by Bio-Rad CFX Manager (BIO-RAD) and relative quantifications were conducted in Microsoft® Excel® 2007. All the amplification experiments were carried out at least three times for each sample of duplicated cell cultures, making a total of six samples for each growth medium. The stages of the RT-qPCR assays and analysis followed the MIQE guidelines (Bustin *et al.* 2009) and the allegiance to those parameters has been reported (de Barros Pita *et al.* 2012).

Results and Discussion

Influence of nitrate on *D. bruxellensis* physiology in different C-sources

Yeast cells were cultivated under oxygen limitation, characterized by small amounts of acetate production by *D. bruxellensis* (Aguilar Uscanga, Délia, & Strehaiano, 2003; Blomqvist et al., 2010; Pereira et al., 2012). Indeed, low acetate yield in the range of 0.02 g (g sugar)$^{-1}$ was observed in the present work (Table 1). In ammonium-based medium lower growth rate was observed for maltose while higher growth rate was observed for sucrose as previously reported (Blomqvist *et al.* 2010; de Barros Pita *et al.* 2011). This indicates the metabolic difference in the assimilation of these disaccharides. The efficiency in sucrose metabolism by *D. bruxellensis* was recently attested in chemostat cultures (Leite *et al.* 2012).

Table S1. Oligos used for RT-qPCR assays and their respective amplicon size, Tm for melting curve analysis and amplification efficiency.

Oligos	Sequences	Amplicon Size	Amplicon Tm	Eff. (%)
ACO1F	CATGGTCCACATGCATTTGC	72	80	97
ACO1R	TGAGCGTGATGGATTCCTTCA			
MDH1F	CGTGGCAAGACCGTCTGATT	71	81	95
MDH1R	ATCTTTCGCACATCCCAACAA			
ATP1F	CCCATACGCTGTTGAGGAAGAG	73	81	93
ATP1R	GGCAGGGAACTTATCGAGGAAA			
GND1F	ATGGTTGGATTGGCGGTGAT	82	80	95
GND1R	GCCGATTAAAGCAGGCAACTG			
TKL1F	GCGAGATGCCCTCCATCAT	72	82	95
TKL1R	CGGACTTCGTGGTGTCTGACA			
ADH1F	CTGGCTCAGGTTGCACCAA	74	81	98
ADH1R	GCACGCAAGTCTGCAGTCTTC			

Table 1. Physiological parameters of the oxygen-limited shake flask cultivation of *Dekkera bruxellensis* GDB 248 in minimal medium containing different carbon and nitrogen sources, after 24 hours cultivation*.

	Ammonium				Nitrate				Mix			
Parameters	Glucose	Fructose	Sucrose	Maltose	Glucose	Fructose	Sucrose	Maltose	Glucose	Fructose	Sucrose	Maltose
μ (h^{-1})	0.14 (±0.003)	0.13 (±0.003)	0.15 (±0.002)	0.08 (±0.002)	0.08 (±0.001)	0.07 (±0.001)	0.07 (±0.002)	0.04 (±0.002)	0.15 (±0.001)	0.13 (±0.002)	0.15 (±0.003)	0.09 (±0.002)
Sugar consumed (%)	100.0 (±0.000)	99.0 (±0.4)	83.0 (±1.65)	82.0 (±1.8)	6.20 (±0.4)	5.10 (±0.4)	5.40 (±0.3)	3.10 (±0.1)	94.00 (±0.8)	95.00 (±1.05)	82.00 (±0.7)	84.00 (±1.4)
Ethanol yield (g/g)	0.39 (±0.003)	0.43 (±0.003)	0.48 (±0.004)	0.40 (±0.002)	0.35 (±0.002)	0.44 (±0.001)	0.45 (±0.001)	0.00 (±0.000)	0.37 (±0.001)	0.43 (±0.003)	0.43 (±0.002)	0.34 (±0.001)
Glycerol yield (g/g)	0.04 (±0.002)	0.04 (±0.001)	0.04 (±0.001)	0.02 (±0.002)	0.04 (±0.001)	0.04 (±0.001)	0.04 (±0.000)	0.00 (±0.000)	0.04 (±0.001)	0.05 (±0.001)	0.04 (±0.001)	0.05 (±0.002)
Acetate yield (g/g)	0.02 (±0.001)	0.02 (±0.001)	0.00 (±0.000)	0.00 (±0.000)	0.02 (±0.002)	0.02 (±0.000)	0.00 (±0.000)	0.00 (±0.000)	0.00 (±0.000)	0.00 (±0.000)	0.00 (±0.000)	0.00 (±0.000)
Biomass (g/g)	0.08 (±0.002)	0.07 (±0.002)	0.08 (±0.001)	0.04 (±0.001)	0.04 (±0.001)	0.04 (±0.001)	0.04 (±0.001)	0.02 (±0.001)	0.08 (±0.001)	0.07 (±0.001)	0.08 (±0.002)	0.05 (±0.001)
CO_2 yield (g/g)**	0.38 (±0.002)	0.42 (±0.003)	0.46 (±0.004)	0.38 (±0.002)	0.34 (±0.003)	0.43 (±0.001)	0.43 (±0.001)	0.98*** (±0.000)	0.35 (±0.000)	0.41 (±0.002)	0.41 (±0.001)	0.32 (±0.001)
Mass balance (%)	89.2	97.4	105.1	83.9	79.9	96.6	95.5	100.1	84.2	95.8	95.9	76.1

*the results represent the average of biological duplicates, with technical triplicates each.
**Calculated on the basis of stoichiometry of ethanol and acetate production.
***CO_2 produced from the sugar consumed considering low biomass and no ethanol or acetate production.

When nitrate was the sole nitrogen source, the specific growth rate was 45% lower than in ammonium in medium containing glucose (Fig. 1A), as well as all the other sugars tested (Table 1). The lower growth rate was in agreement with the low sugar consumption of nitrate-growing cells, which represented only 3-6% of initial sugar in the media (Table 1; Fig. 1B).

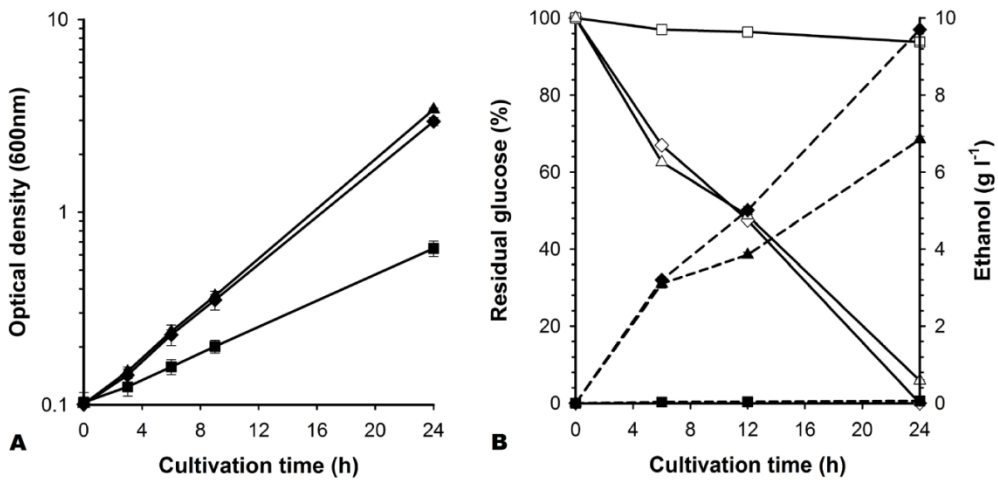

Figure 1. Oxygen-limited flask batch experiments of Dekkera bruxellensis grown in glucose and in different nitrogen sources combinations: ammonium (diamond), nitrate (square) or both N-sources (triangle). (Panel A) Growth curves. (Panel B) Residual glucose (straight lines) and ethanol production (dashed lines). Standard deviations are low and not visible for most of the points.

In mixed N-source media, the growth rate and sugar consumption were restored to the level of the ammonium-based condition (Table 1; Fig. 1A, B). Co-assimilation of ammonium and nitrate was previously reported in agitated flask cultures (de Barros Pita

et al. 2011). The ethanol production was reduced in nitrate-based media and the presence of ammonia in the mixed N-source cultivations did not fully restore this parameter to the standard levels (Table 1 and Fig. 1A, B). This effect was the consequence of lower sugar consumption observed for nitrate media. Nevertheless, when considering the yield, which represents the amount of assimilated sugar converted to ethanol, it was observed the reduction of this parameter under oxygen limitation. Even though the growth and ethanol production in nitrate media was lower than in ammonia, it is important to note that, since *S. cerevisiae* cannot use nitrate as N-source, even these reduced parameters might keep *D. bruxellensis* on the right track to outcompete *S. cerevisiae* in the industrial process upon ammonium depletion (de Barros Pita et al., 2011).

In a recent investigation on physiological studies, it was observed that *D. bruxellensis* can very efficiently use the available oxygen for biomass generation so that the energetic efficiency of this yeast is higher than *S. cerevisiae* (Blomqvist *et al.* 2010; Blomqvist *et al.* 2012; Leite *et al.* 2012). It was noteworthy that mass balance did not close neither for glucose nor maltose using the expected CO_2 yield calculated from the stoichiometry of ethanol and acetate production (Table 1). This putative CO_2 underestimation coincided with the lower ethanol yield observed, and could be suggestive of increased flows through the TCA and PP pathways that might release some CO_2 not connected to ethanol production (Smaczynska-De Rooij *et al.* 2004; Gorsich *et al.* 2006).

Nitrate influence on *D. bruxellensis* gene expression in different C-sources

The expression of genes encoding for nitrate assimilatory pathway, alcohol dehydrogenase and enzymes of the PP pathway and tricarboxylic acid cycle were quantified (Table 2). In nitrate media, NIT genes were induced in all C-sources tested. *YNT1* gene showed the highest induction levels in all carbon-sources (Table 2), as it has been observed for mineral medium and sugarcane juice (de Barros Pita *et al.* 2011), while *YNA1* did not vary its expression. When both ammonium and nitrate were present in the medium, nitrogen metabolic repression on NIT genes was observed for all, but glucose media (Table 2).

Upon assimilation, nitrate is reduced to ammonium in a two-step reaction catalyzed by Nitrate Reductase (NR) and Nitrite Reductase (NiR) (Siverio 2002), involving the consumption of eight electrons coupled in four NAD(P)H molecules (Siverio 2002). These molecules could be, otherwise, diverted to energy and biomass production. In the yeast *H. polymorpha* and most fungi species, NADPH is the preferred cofactor for NR and NiR enzymes (Siverio 2002). NADPH is mainly produced from glucose in the PP pathway after two oxidoreductive reactions catalyzed by glucose-6P dehydrogenase (*ZWF1* gene) and 6-phosphogluconate dehydrogenase (*GND1* gene) (Gorsich et al., 2006). No significant difference was observed for the expression of *GND1* gene in whatever conditions tested (Table 2).

Table 2. Relative expression of genes involved in nitrate assimilation, ethanol production, tricarboxylic acid cycle, pentose-phosphate pathway and ATP synthesis in mineral medium containing different carbon and nitrogen sources in *Dekkera bruxellensis.*

	Glucose		Fructose		Sucrose		Maltose	
	NO_3	Mix	NO_3	Mix	NO_3	Mix	NO_3	Mix
YNA1	+2.40 (±0.29)	-1.30 (±0.50)	-1.14 (±0.22)	-1.86 (±0.23)	+2.40 (±0.26)	-1.62 (±0.35)	-1.48 (±0.31)	-1.66 (±0.45)
YNT1	+19.14 (±0.48)	+3.01 (±0.66)	+14.50 (±0.25)	-2.00 (±0.24)	+20.65 (±0.25)	-1.30 (±0.85)	-1.84 (±0.40)	-1.64 (±0.25)
YNR1	+10.06 (±0.25)	+1.20 (±0.08)	+5.20 (±0.13)	-1.10 (±0.35)	+6.40 (±0.22)	-2.20 (±0.37)	+2.50 (±0.14)	+1.05 (±0.28)
YNI1	+19.10 (±0.46)	-1.52 (±0.93)	+4.44 (±0.13)	-1.31 (±0.27)	+12.62 (±0.10)	-1.45 (±0.52)	-1.70 (±0.47)	-1.41 (±0.53)
ADH1	-2.60 (±0.15)	-1.20 (±0.15)	-5.30 (±0.14)	-1.10 (±0.14)	-2.90 (±0.13)	-1.08 (±0.16)	-3.66 (±0.12)	+1.30 (±0.17)
MDH1	+3.00 (±0.36)	-1.27 (±0.17)	+1.56 (±0.30)	1.42 (±0.25)	+2.12 (±0.30)	-1.02 (±0.16)	-2.17 (±0.16)	-1.50 (±0.09)
ACO1	+1.89 (±0.25)	-1.37 (±0.17)	+1.16 (±0.23)	-1.02(±0.20)	+1.48 (±0.24)	-1.07 (±0.16)	+1.49 (±0.17)	+1.01 (±0.24)
GND1	+2.05 (±0.27)	+1.13 (±0.20)	-1.06 (±0.20)	+1.30 (±0.20)	+1.70 (±0.30)	+1.40 (±0.22)	+1.34 (±0.18)	+1.58 (±0.26)
TKL1	+5.40 (±0.59)	+1.26 (±0.32)	+2.20 (±0.35)	+1.06 (±0.21)	+3.50 (±0.41)	-1.15 (±0.20)	-1.08 (±0.09)	+1.16 (±0.14)
ATP1	+3.86 (±0.27)	+1.11 (±0.16)	+2.39 (±0.15)	-1.08 (±0.16)	+4.35 (±0.37)	+1.29 (±0.30)	+2.69 (±0.17)	-1.10 (±0.18)

However, up-regulation of the *TKL1* gene encoding for a transketolase responsible for interconverting xylulose-5P and ribose-5P to sedoheptulose-7P and glyceraldehyde-3P in the PP pathway was observed (Table 2). Thus, in nitrate-based medium, cell growth was low and the increase in *TKL1* transcription may be part of the metabolic response to offset the energy deficit. Disruption and overexpression of *TKL1* had a pronounced effect on the efficiency of glycolysis and the growth of *S. cerevisiae* on fermentable C-sources (Sundström, Lindqvist, Schneider, Hellman, & Ronne, 1993). The inductive effect was eliminated by the presence of ammonium (Table 2). Alternatively to the PP pathway, the cytosolic aldehyde dehydrogenase VI (Ald6p) could provide enough NADPH for anabolic reactions in the absence of Zwf1p (Minard and McAlister-Henn 2005). In the present study, the increased demand for NADPH production might not be dependent on the transcription level of the *GND1* gene (Table 2) or there was no increase in NAD(P)H production, which could explain the slower growth observed on nitrate. This demand also does not seem to be associated with the activity of Ald6p since no increase in acetate production was detected (Table 1). Furthermore, two genes of the TCA cycle were investigated. *MDH1* gene, which in *S. cerevisiae* encodes for malate dehydrogenase, was also up-regulated when nitrate was the sole N-source (Table 2), while the transcription level of aconitase encoding gene, *ACO1,* remained unaltered in all the C- and N-sources tested (Table 2). On the other hand, the results showed that *ADH1* gene was down-regulated in nitrate-based media and that such negative effect was suppressed by the presence of ammonium (Table 2).

The slower growth on nitrate can most probably be attributed to the high energy cost that requires extra ATP synthesis. This might explain the up-regulation of the *ATP1* gene in nitrate in combination with all carbon sources tested (Table 2). This gene codes for the α subunit of the mitochondrial F1F0 ATP synthase complex. When ammonium was present in the medium, the inductive effect of nitrate on *ATP1* gene expression disappeared (Table 2). As nitrate transport in *D. bruxellensis* is likely to be energy-dependent, similar to what has been found for other organisms (Galvan and Fernández 2001), in oxygen-limitation and in the presence of ammonium, all NIT genes including

YNT1 are down-regulated (Table 1). It might save energy that could be used to promote growth. This repressive effect was not observed under conditions where oxygen was not a limiting factor (de Barros Pita et al., 2011).

Thus *D. bruxellensis* cells activate metabolic flows through aerobic energy providing pathways in order to produce more ATP to offset the energetic deficit caused by nitrate assimilation. This implies a diminished ethanol production. Indeed, the down-regulation of the *ADH1* gene (Table 2) corroborates with this change in the metabolic flux from fermentation to respiration. Moreover, nitrate assimilation decreases cell growth, sugar consumption and ethanol production when used as sole N-source. Besides, since sugarcane juice in Brazilian distilleries is likely to contain both ammonium and nitrate (de Barros Pita *et al.* 2011), it is important to note that the presence of ammonium in mixed synthetic media restored most of the physiological parameters to the standard condition. Our data confirms the previous hypothesis on the role of nitrate during industrial ethanol fermentation. In the first stage, when both ammonia and nitrate are available in the medium, *S. cerevisiae* and *D. bruxellensis* compete for the substrate and the first yeast remains as the major fermenting organism. Afterwards, ammonia is depleted and *S. cerevisiae* stops growing, so nitrate can support a continuous, even though slower, growth and ethanol production by *D. bruxellensis* (de Barros Pita *et al.* 2011). Therefore, nitrate can provide means to *D. bruxellensis* keep the pace in which it will become the major population in contaminated industrial processes (de Souza Liberal *et al.* 2007; de Barros Pita *et al.* 2011).

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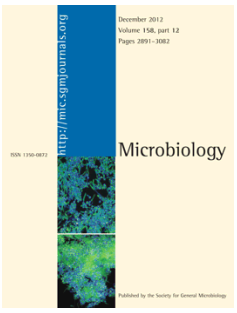
CAPÍTULO IV

Physiological and transcriptional response of *Dekkera bruxellensis* to different carbon and nitrogen supplies.

Trabalho a ser submetido à revista *Microbiology*



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Physiological and transcriptional response of *Dekkera bruxellensis* to different carbon and nitrogen supplies.

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Running headline: Nitrogen Regulation in *Dekkera bruxellensis*

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Abstract

The assimilation of nitrate, a nitrogenous compound, was previously described as an important factor favoring *D. bruxellensis* in the competition with *S. cerevisiae* for the industrial sugar cane substrate. Since nitrogen is an essential nutrient for all living organisms, understanding the mechanisms related to the utilization of these compounds is important to reveal the main aspects of the metabolism and highlights potential adaptation factors for *D. bruxellensis*. In the present study, we evaluated the physiological and transcriptional profile of *D. bruxellensis* in response to nutrient limitation. *D. bruxellensis* cells were grown in different carbon and nitrogen supplies in order to determine their influence on growth, sugar consumption and ethanol production. Besides, the expression of genes coding for permeases of nitrogen sources, biosynthesis of glutamate and energetic metabolism was investigated under these conditions. Our data show that genes involved with nitrogen uptake in *D. bruxellensis* are under control of nitrogen catabolite repression. Moreover, we provide indications that glutamate dehydrogenase and glutamate synthase may switch roles as the major pathway for glutamate biosynthesis in *D. bruxellensis*. Finally, our data show that in non-optimal growth conditions, *D. bruxellensis* leans towards the respiratory metabolism. The results presented herein show that *D. bruxellensis* and *S. cerevisiae* share some metabolic traits, but *D. bruxellensis* also presents particular characteristics, which might explain its adaptation to the industrial environment.

Keywords: nutrient limitation, glutamate biosynthesis; gene expression; energetic metabolism; ethanol production.

Introduction

Dekkera bruxellensis, a yeast species classified into the hemiascomycetous group, has been regularly charged as a contaminant microorganism in industrial fermentation processes, such as winemaking and bioethanol production (Loureiro & Malfeito-Ferreira, 2003; Passoth *et al.*, 2007; de Souza Liberal *et al.*, 2007). However, it is important to note that *D. bruxellensis* plays different roles as contaminant yeast in these two industrial processes. In winemaking, this yeast produces off-flavor compounds, which is responsible for wine spoilage (Chatonnet *et al.*, 1995; 1997). On the other hand, in bioethanol production, *D. bruxellensis* reduces the ethanol yield by outcompeting *S. cerevisiae* populations, leading to economic losses (Passoth *et al.*, 2007; de Souza Liberal *et al.*, 2007; Basílio *et al.*, 2008). In any case, *D. bruxellensis* is regarded as carrying genetic and physiological adaptations that could grant its establishment, maintenance and high fitness into the industrial environment. A previous work suggested nitrate as a potential factor favoring *D. bruxellensis* in the competition with *S. cerevisiae* in Brazilian distilleries (de Barros Pita *et al.*, 2011). In that report, we highlighted the importance attributed to the nitrogen into the cell metabolism.

Nitrogenous compounds are usually transported into the cell through transmembrane proteins, such as the ones coded by *GAP1*, *MEP1*, and *PUT4* genes (Marini *et al.*, 2000; Magasanik & Kaiser, 2002; Cain & Kaiser, 2011). Once into the cell, the final fate of most nitrogen sources is (i) the release of ammonia via deamination or (ii) production of glutamate via transamination (Magasanik & Kaiser, 2002; Magasanik, 2003). In most yeasts, the nitrogen sources are sorted into different categories based on its “quality”, which is related to the growth they can support (Magasanik & Kaiser, 2002; Conterno *et al.*, 2006; Godard *et al.*, 2007). In general, ammonia, glutamate and glutamine are commonly able to keep fast and optimal growth rates and are regarded in the literature as “preferential”, “primary” or “good” nitrogen sources. On the other hand, proline, urea and nitrate can only support a slow and delayed growth, and therefore are considered as “non-preferential”, “secondary” or “poor” nitrogen sources (Magasanik & Kaiser, 2002; Boer *et al.*, 2007). This brings to the light a complex mechanism responsible to determine which one and at what moment a nitrogen source should be used. This is achieved by a transcriptional control known as Nitrogen Catabolite Repression (NCR), which regulates the expression of most genes involved in the transport and metabolism of secondary nitrogen sources (Magasanik & Kaiser, 2002). This tight control avoids the utilization of these compounds when a primary nitrogen source is available in the medium (Cooper, 2002; Magasanik & Kaiser, 2002).

The Central Nitrogen Metabolism (CNM) is fairly well described in *S. cerevisiae* and it seems that ammonia assimilation and the biosynthesis of glutamate are controlled by the

concentration of both ammonia and glucose (Magasanik, 2003). In conditions in which these sources are abundant, the ammonia previously released by a nitrogen source is assimilated mostly by incorporation to the 2-oxoglutarate, in a reductive amination catalyzed by the $NADP^+$-dependent glutamate dehydrogenase (NADP-GDH; EC 1.4.1.4), coded by *GDH1* gene (DeLuna *et al.*, 2001; Riego *et al.*, 2002). On the other hand, when ammonia becomes limiting, the NAD^+-dependent glutamate synthase (GOGAT; EC 1.4.1.14), coded by *GLT1* gene, plays the key role in ammonia assimilation (Magasanik & Kaiser, 2002; Magasanik, 2003). In this case, glutamate biosynthesis occurs in a two-step reaction, in which GOGAT acts in a complex together with glutamine synthetase (GS; EC 6.3.1.2) in the GS-GOGAT pathway (ter Schure *et al.*, 1998; Magasanik & Kaiser, 2002). In the first step, glutamate is condensed with ammonia by glutamine synthetase (GS), coded by *GLN1* gene, in order to produce glutamine (terSchure *et al.*, 1998; Magasanik & Kaiser, 2002). Then, the amide group from glutamine is transferred to 2-oxglutarate, generating two glutamate molecules (Magasanik & Kaiser, 2002; Magasanik, 2003). Alternatively, in cells growing in glutamate as sole nitrogen source, NAD^+-dependent glutamate dehydrogenase (NAD-GDH; EC 1.4.1.3), coded by *GDH2*, catalyzes the breakdown of glutamate into 2-oxoglutarate and ammonia (Magasanik & Kaiser, 2002).

Although most of the yeast species studied so far have both GDH and GOGAT enzymes, the major mechanism for ammonia assimilation is not universal. In *Kluyveromyces lactis*, both pathways are important (Romero *et al.*, 2000), while in *Schizosaccharomyces pombe, Candida albicans* and *Kluyveromyces marxianus* the GS-GOGAT pathway seems to be the main responsible for glutamate biosynthesis (Barel & MacDonald, 1993; Holmes *et al*., 1989; de Morais Jr, 2003). Since *D. bruxellensis* presents growing industrial importance, it is fundamental to understand the mechanisms related to the main aspects of its metabolism. Therefore, investigations concerning to the nitrogen metabolism are central for understanding how *D. bruxellensis* can emerge as the first yeast described as able to surpass *S. cerevisiae* as a standard fermenting organism in industrial processes (de Souza Liberal *et al*., 2007; Passoth *et al.*, 2007; de Barros Pita *et al*., 2011).

Methods

Yeast strains and growth conditions

The industrial strain *Dekkera bruxellensis* GDB 248 (de Barros Pita *et al.*, 2011) was used in this study. Cells were pre-grown in synthetic minimal medium – SC (Yeast Nitrogen Base w/o amino acids and ammonium sulfate at 1.7 g l^{-1}) supplemented with ammonium sulfate and glucose to 38 mmol l^{-1} and 110 mmol l^{-1}, respectively, at 30°C and 150 rpm in a rotator shaker for 24 hours.

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 100 ml in each of the specific media. Each specific medium contained a mixture of glucose as carbon source either at 666 mmol C l^{-1} (carbon-rich) or 66.6 mmol C l^{-1} (carbon-limited) and one of the following nitrogen sources: ammonium sulfate, glutamate, glutamine or proline either at 75 mmol N l^{-1} (nitrogen-rich) or 7.5 mmol N l^{-1} (nitrogen-limited). As an internal control for NCR, urea was used at 75 mmol N l^{-1} as nitrogen source. The media were sorted into four groups based on the glucose and nitrogen availability as follows: (M1) carbon-rich/nitrogen-rich, (M2) carbon-rich/nitrogen-limited, (M3) carbon-limited/nitrogen-rich and (M4) carbon-limited/nitrogen-limited. Cultures were prepared in 250 ml flasks and the growth conditions were the same as the pre-growth. Growth rate was calculated from optical density of the cultures, measured at every hour of cultivation (de Barros Pita *et al.* 2011).

Biomass and metabolite analysis

In order to investigate the influence of the carbon and nitrogen source availability in the production of metabolites in *D. bruxellensis*, samples were collected in triplicates along the growth curve for physiological analysis or when cultures reached 1 OD_{660nm} for RT-qPCR analysis. Samples were prepared as described by Blomqvist *et al.*, (2010) by filtering the supernatant through a 0.22 μm sterile filter and freezing at -20ºC until analysis. Glucose, ethanol, glycerol and acetate concentrations were measured on Agilent 1100 Series platform (Agilent, USA). The biomass was determined from the optical density of the cultures, using a previous standard curve. The mass balance was calculated using the value of CO_2 yield based on the stoichiometry for ethanol and acetate production (Blomqvist *et al*., 2010).

RNA isolation and cDNA synthesis

RNA extraction was performed when yeast cultures reached 1.0 OD_{660nm} (de Barros Pita *et al.*, 2012), which ensured that gene expression analyses were performed from cells in the same physiological state and could be compared to our previous works. It is important to note that RNA isolation for 1.0 OD_{660nm} occurred at different times, depending on the growth rate resultant of the different carbon-nitrogen combinations in the cultivation media. Samples were processed immediately after cells were collected (de Barros Pita *et al.*, 2012). RNA integrity was evaluated using RNA Nano Chips on Agilent 2100 Bioanalyzer (Agilent Technologies, USA) and samples were quantified on NanoDrop 2000 (Thermo Scientific, USA). RNA samples were stored at -80 ºC and cDNA was synthesized employing oligo(dT) as primer and using 1 μg of total RNA for each

reverse transcription tube with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, USA), following the manufacturer's instructions. cDNA was stored at -20 ºC until RT-qPCR assays.

Gene expression analysis

In order to determine the mRNA levels of genes involved in the uptake of nitrogen sources, glutamate biosynthesis, Tricarboxylic Acid Cycle (TCA) and ATP synthesis in response to changes in the quality and availability of the carbon and nitrogen source in *D. bruxellensis*, gene expression quantification assays were performed by using the real time reverse transcription quantitative PCR (RT-qPCR). Nucleotide sequences for the genes of interest (GOI) were obtained from tBlastx tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using *S. cerevisiae* orthologous coding sequences, as query in the *D. bruxellensis* Genome Survey Sequences Database (http://www.ncbi.nlm.nih.gov/nucgss). Gene description and Genbank identifiers of the best hit sequences are described in Table 1. Oligonucleotides were designed by Primer Express software (Life Technologies, USA) (Supporting information – Table S1). *EFA1* and *ACT1* were used as reference genes as previously described (de Barros Pita *et al*., 2012). Amplification efficiencies and standard curves were performed for each pair of primers (de Barros Pita *et al.*, 2012). Data normalization in each sample was performed by using the reference genes in geNorm applet (Vandesompele *et al.*, 2002), as previously reported (de Barros Pita et al. 2012). RT-qPCR assays were performed with SYBR Green PCR Master Mix (Applied Biosystems, USA). 96-wells plates were used and the amplification reactions were composed of 12.5 µl SYBR Green master mix, 1.0 µl each primer (200 nmol l^{-1} final), 2.5 µl cDNA and 8 µl H_2O. Cycling parameters were 95 ºC for 10 min as hot-start, followed by 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min in the ABI Prism 7300 (Applied Biosystems).

Table 1. Gene description and Genbank IDs.

Gene	Genbank ID	Description
MEP1	EI017852	Ammonium permease.
GAP1	EI011930	General amino acid permease.
PUT4	EI016538	Proline permease, required for high-affinity transport of proline.
GAT1	EI013453	Transcriptional activator of genes involved in nitrogen catabolite repression.
GDH1	EI014207	$NADP^+$-dependent glutamate dehydrogenase, synthesizes glutamate from ammonia and alpha-ketoglutarate.
GLT1	EI014757	NAD^+-dependent glutamate synthase (GOGAT), synthesizes glutamate from glutamine and alpha-ketoglutarate.
GDH2	EI017486	NAD^+-dependent glutamate dehydrogenase, degrades glutamate to ammonia and alpha-ketoglutarate.
SDH1	EI011791	Flavoprotein subunit of succinate dehydrogenase.
ACO1	EI016687	Aconitase.
MDH1	EI026362	Mitochondrial malate dehydrogenase.
IDH1	EI013718	Subunit of mitochondrial NAD^+-dependent isocitrate dehydrogenase.
IDP1	EI023548	Mitochondrial NADP-specific isocitrate dehydrogenase.
IDP2	EI024011	Cytosolic NADP-specific isocitrate dehydrogenase.
ATP1	EI014661	Alpha subunit of the F1 sector of mitochondrial F1F0 ATP synthase.

Supporting information

Table S1. Oligos used for RT-qPCR assays and their respective amplicon size, Tm for melting curve analysis and amplification efficiency.

Genes	Oligos	Sequences	Amplicon size	Amplicon Tm	Amplification Eff. (%)
MEP1	MEP1F MEP1R	TTAAGTGCTTGCGACGCTTTC GGACTATGCCGGTGGTGGT	92	80	97
GAP1	GAP1F GAP1R	CCCAAGTGAACACCGACGAC GCTTTGCTTCTTGGCTGCTTC	89	80	99
GDH1	GDH1F GDH1R	GATTCACGGTTGGGTGCAAA TGAGATGCAGGTGAATCACGG	92	81	99
GDH2	GDH2F GDH2R	AAGGCCTTCTTCACGGGAAA ACGCACACTCAACGTGGTCA	81	82	98
GLT1	GLT1F GLT1R	AGATGAGAATGTGGTCCGCCT TGTCGGTGAAACTCGTGTCC	84	82	98
GAT1	GAT1F GAT1R	TTGCACAAACTGCCACACCA TTCAGAAACAAGCCGCACG	90	82	97
SDH1	SDH1F SDH1R	CACTGGTGGTTATGGTCGTGC CTCCAGAGGAAAACCGGCA	100	80	94

IDH1	IDH1F IDH1R	CCATGGTTCCGCTCCTGATAT GCCCATGTGTCTGAGCATCAT	91	81	97
IDP1	IDP1F IDP1R	CACCTCCGTCCTTTGGAGTGT GGTGACCAGTACAAGGCCACA	82	80	97
IDP2	IDP2F IDP2R	GATGGAAAAGGCTTCGAGGCT TGGAAGCAATGGAGTTTGTGG	103	80	97
MDH1	PUT4F PUT4R	CCGGTAAGATGTTCGGCAAA TCCTGCCCAACATCACGTTT	72	80	94
ACO1	PFK1F PFK1R	AAGCTCCGATGGTGTTGTCC CGGCAAGTACCAGCATTTGAC	86	83	98
ATP1	ATP1F ATP1R	CCCATACGCTGTTGAGGAAGAG GGCAGGGAACTTATCGAGGAAA	73	81	93
PUT4	PUT4F PUT4R	CCGGTAAGATGTTCGGCAAA TCCTGCCCAACATCACGTTT	72	80	94

Negative PCR controls (for unspecific amplification) and negative RT controls (for genomic DNA contamination) were run in parallel for internal control (de Barros Pita *et al.*, 2012). The reference sample for gene expression analyses was the medium containing glucose and ammonium sulfate at 666 mmol C l^{-1} and 75 mmol N l^{-1}, respectively (carbon-rich/nitrogen-rich). Amplification curves were analyzed by using SDS v.2.0 software (Applied Biosystems) and relative quantifications were conducted in Microsoft® Excel® 2010. The reaction specificity was evaluated by analyzing the melting curve profile. All the amplification experiments were carried out at least three times for each sample of duplicated cell cultures, making a total of six samples for each growth medium. For the quantification of gene expression, the Normalization Factors obtained from geNorm applet were applied. For *GDH1*, *GLT1* and *GDH2* analyses, the quantification method was different. In order to determine which gene presented the highest expression for each condition and therefore could play the major role in glutamate metabolism, the quantification data were normalized by reference genes and compared among them. The gene showing the lower Cq (highest expression) was set as presenting the Relative Quantity (RQ) equals 1. Subsequently, the expression of the two remaining genes was established by using the following equation: $[2^{(Cq2-Cq1)} x -1]$, in which *Cq2* represents the Cq value of the gene analyzed and *Cq1* represents the Cq value for the most expressed gene (RQ = 1). The stages of the RT-qPCR assays and analysis followed the MIQE guidelines (Bustin *et al.*, 2009) and the reliability of those parameters has been reported (de Barros Pita *et al.* 2011; de Barros Pita *et al.*, 2012).

Results

Impact of glucose and nitrogen concentration on growth and production of metabolites

The physiological response to different carbon and nitrogen supplies, as well as the influence of the nitrogen source quality in *D. bruxellensis* was determined. Yeast cells were grown in the specific media, which were sorted in four major groups based on the glucose and nitrogen source availability. This provided different combinations of either carbon-nitrogen abundance or limitation. No significant glycerol was detected in any condition tested. Moreover, acetate production present in samples in which glucose concentration was not limiting, although it was lower than ethanol production (Table 2). Since glycerol is produced by *D. bruxellensis* only under oxygen limited condition and acetate is produced in aerated process

(Leite *et al.*, 2012), the cultivations performed in the present work can be characterized as not oxygen-limited.

D. bruxellensis presented differential growth, responding either to carbon or nitrogen concentration in the medium (Figure 1). In M1 condition *D. bruxellensis* reached its highest growth rates and no significant difference was observed for the three preferential nitrogen sources used, ammonia, glutamate and glutamine (Figure 1A). On the other hand, proline (Figure 1) and urea (data not shown) supported only a slow and delayed growth. Moreover, in this condition glucose was consumed faster than in the other conditions, reaching levels near to zero after 24 hours of cultivation, except for proline, in which we detected about 60% of the initial glucose (Figure 1B). Additionally, it was also observed the peak of ethanol production, once again with no significant difference among the preferential nitrogen sources tested (Figure 1B). On the other hand, when grown in proline, *D. bruxellensis* cells produced much less ethanol, probably as a consequence of the lower consumption of glucose (Figure 1B).

In the samples providing nitrogen limitation (M2 condition), it was observed that *D. bruxellensis* presents slower growth compared to non-limited condition (0.16 h^{-1} in M1 *vs.* 0.08 h^{-1} in M2) for all preferential N-sources tested (Figure 1C).

Table 2. Physiological parameters of the shake flask cultivation of *Dekkera bruxellensis* GDB 248 in minimal medium containing different carbon and nitrogen supplies, after cell culture reaches 1.0 OD_{600nm} 24 hours cultivation*.

Cultivation media	μ (h^{-1})	Glucose consumed (%)	Ethanol yield (g/g)	Glycerol yield (g/g)	Acetate yield (g/g)	CO_2 (g/g)**	Biomass (g/g)	Mass Balance (%)
M1 (ammonium)	0.16 (± 0.012)	57.8% (± 1.21)	0.40 (± 0.012)	0.0 (± 0.000)	0.08 (± 0.008)	0.44 (± 0.014)	0.09 (± 0.009)	101
M1 (glutamate)	0.16 (± 0.014)	59.7% (± 1.37)	0.40 (± 0.016)	0.0 (± 0.000)	0.09 (± 0.007)	0.45 (± 0.016)	0.09 (± 0.008)	103
M1 (glutamine)	0.16 (± 0.011)	92.0% (± 2.78)	0.40 (± 0.011)	0.0 (± 0.000)	0.09 (± 0.008)	0.44 (± 0.012)	0.09 (± 0.010)	102
M1 (proline)	0.08 (± 0.009)	99.0% (± 2.61)	0.32 (± 0.013)	0.0 (± 0.000)	0.07 (± 0.006)	0.36 (± 0.008)	0.04 (± 0.006)	79
M2 (ammonium)	0.08 (± 0.013)	59.2% (± 1.48)	0.35 (± 0.010)	0.0 (± 0.000)	0.08 (± 0.007)	0.39 (± 0.009)	0.04 (± 0.007)	86
M2 (glutamate)	0.08 (± 0.013)	57.1% (± 1.39)	0.33 (± 0.009)	0.0 (± 0.000)	0.09 (± 0.010)	0.38 (± 0.009)	0.04 (± 0.006)	84
M2 (glutamine)	0.08 (± 0.014)	98.2% (± 2.14)	0.34 (± 0.010)	0.0 (± 0.000)	0.08 (± 0.010)	0.38 (± 0.008)	0.04 (± 0.004)	84
M2 (proline)	0.05 (± 0.007)	99.3% (± 2.21)	0.25 (± 0.008)	0.0 (± 0.000)	0.09 (± 0.011)	0.31 (± 0.007)	0.03 (± 0.004)	68
M3 (ammonium)	0.07 (± 0.006)	60.2% (± 1.56)	0.0 (± 0.000)	0.0 (± 0.000)	0.0 (± 0.000)	96.0*** (± 0.000)	0.04 (± 0.006)	100
M3 (glutamate)	0.07 (± 0.007)	56.7% (± 1.13)	0.0 (± 0.000)	0.0 (± 0.000)	0.0 (± 0.000)	97.0*** (± 0.000)	0.03 (± 0.005)	100

Cultivation media	μ (h^{-1})	Glucose consumed (%)	Ethanol yield (g/g)	Glycerol yield (g/g)	Acetate yield (g/g)	CO_2 (g/g)**	Biomass (g/g)	Mass Balance (%)
M3 (glutamine)	0.07 (± 0.006)	98.7% (± 1.82)	0.0 (± 0.000)	0.0 (± 0.000)	0.0 (± 0.000)	96.0*** (± 0.000)	0.04 (± 0.004)	100
M3 (proline)	0.05 (± 0.007)	99.1% (± 2.04)	0.0 (± 0.000)	0.0 (± 0.000)	0.0 (± 0.000)	97.0*** (± 0.000)	0.03 (± 0.006)	100
M4 (ammonium)	0.05 (± 0.007)	56.4% (± 1.09)	0.0 (± 0.000)	0.0 (± 0.000)	0.0 (± 0.000)	97.0*** (± 0.000)	0.03 (± 0.004)	100
M4 (glutamate)	0.05 (± 0.008)	57.7% (± 1.17)	0.0 (± 0.000)	0.0 (± 0.000)	0.0 (± 0.000)	97.0*** (± 0.000)	0.03 (± 0.05)	100
M4 (glutamine)	0.05 (± 0.006)	99.0% (± 2.14)	0.0 (± 0.000)	0.0 (± 0.000)	0.0 (± 0.000)	97.0*** (± 0.000)	0.03 (± 0.006)	100
M4 (proline)	0.04 (± 0.007)	99.4% (± 2.26)	0.0 (± 0.000)	0.0 (± 0.000)	0.0 (± 0.000)	98.0*** (± 0.000)	0.02 (± 0.004)	100

*the results represent the average of biological duplicates, with technical triplicates each.

**Calculated on the basis of stoichiometry of ethanol and acetate production.

***CO_2 produced from the sugar consumed considering low biomass and no ethanol or acetate production.

Once again, proline (Figure 1C) and urea (data not shown) could only support an even slower growth. In such nitrogen limitation, *D. bruxellensis* consumed less glucose than in conditions in which carbon and nitrogen were abundant (Figure 1D). The residual glucose after 24 hours of cultivation was about 50% in the samples containing preferential nitrogen sources and 75% in proline (Figure 1D). The ethanol production was also decreased in *D. bruxellensis* grown in nitrogen limitation. Ammonia, glutamate and glutamine samples presented an average reduction of about 60% in the ethanol concentration after 24 hours (Figure 1D). Interestingly, when proline was used as nitrogen source, the ethanol production was decreased only by 35% (Figure 1D). It is important to note that, in spite of the decline in the ethanol production is likely to be a consequence of the lower glucose consumption, the ethanol yield was also decreased in nitrogen limitation (Table 2).

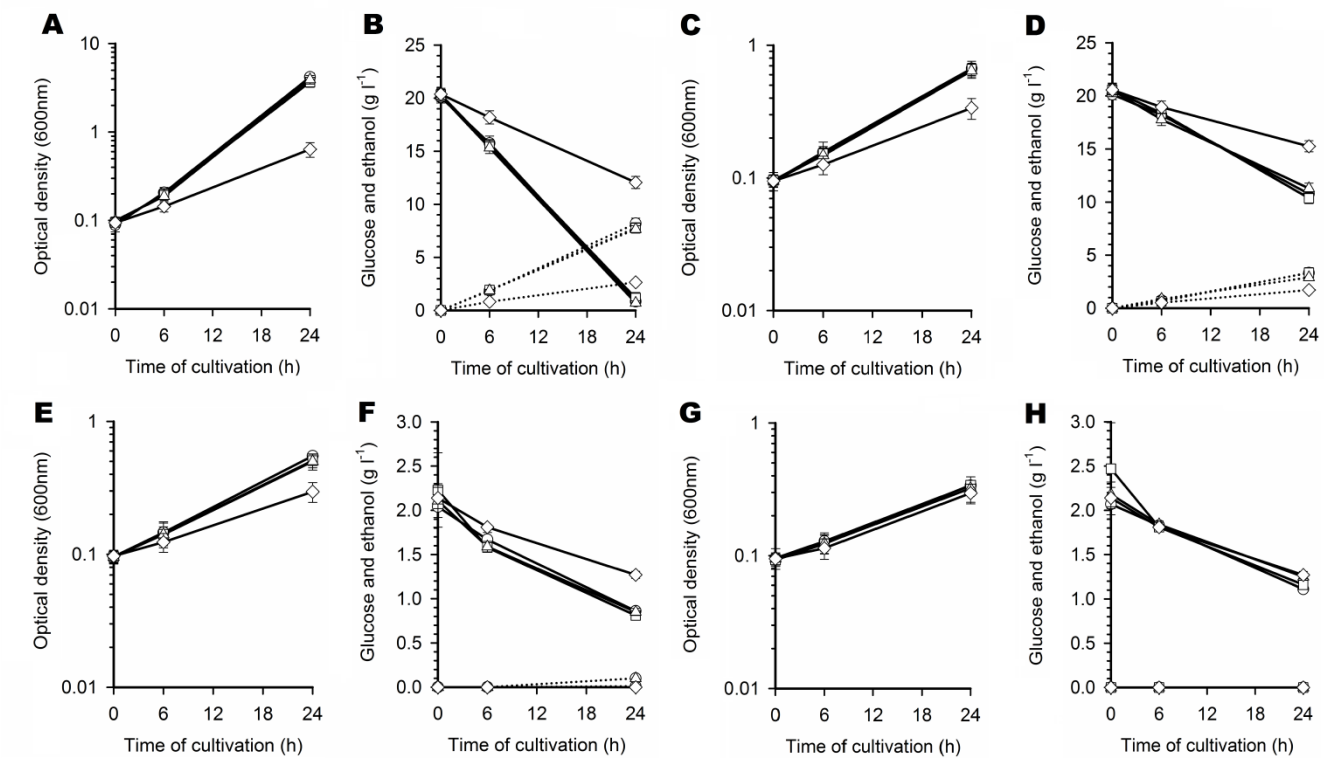

Figure 1. Physiological parameters of *Dekkera bruxellensis* grown in glucose as carbon source and ammonium sulfate (circle), glutamate (square), glutamine (triangle) or proline (diamond) as nitrogen source. Carbon-rich/nitrogen-rich media: Panel A (growth) and B (glucose, straight lines and ethanol, dotted lines). Carbon-rich/nitrogen-limited media: Panel C (growth) and D (glucose, straight lines and ethanol, dotted lines). Carbon-limited/nitrogen-rich media: Panel E (growth) and F (glucose, straight lines and ethanol, dotted lines). Carbon-limited/nitrogen-limited media: Panel G (growth) and H (glucose, straight lines and ethanol, dotted lines). Standard deviations are low and not visible for some points.

D. bruxellensis presented different physiological responses when grown in carbon limitation (M3 and M4 conditions) (Figure 1E-H). While the growth rates between M2 condition and M3 condition samples were fairly similar (Figure 1C, E), the ethanol production was severely affected by carbon scarcity (Figure 1F, H). In conditions in which nitrogen was abundant and the glucose supply was low (M3 condition), the residual glucose was about 40% in preferential nitrogen sources and 65% in proline after 24 hours of cultivation (Figure 1F). In limitation of both carbon and nitrogen sources (M4 condition), *D. bruxellensis* showed even lower glucose consumption, with an average residual glucose of about 60% for growth in preferential nitrogen sources (Figure 1H). Interestingly, in carbon limitation *D. bruxellensis* did not accumulate ethanol (neither acetate nor glycerol) in concentrations above the limit of detection for any nitrogen source tested (Figure 1H). Thus, in such condition the metabolism is fully respiratory.

MEP1, *GAP1*, *PUT4* and *GAT1* expression are under Nitrogen Catabolite Repression

The expression of three genes coding for nitrogen permeases was investigated in different cultivation conditions tested and the results showed that they were all induced when preferential nitrogen sources were limiting or when a secondary nitrogen source was available in the medium (Table 3).

Table 3. Relative expression of *Dekkera bruxellensis* genes involved in the uptake of nitrogen sources, glutamate biosynthesis, tricarboxylic acid cycle and ATP synthesis in mineral medium containing different carbon and nitrogen supplies. Ammonia (Amm), glutamate (Glu), glutamine (Gln), proline (Pro) and urea (Ure).

Genes	M1 (Amm)	M1 (Glu)	M1 (Gln)	M1 (Pro)	M1 (Ure)	M2 (Amm)	M2 (Glu)	M2 (Gln)	M2 (Pro)	M3 (Amm)	M3 (Glu)	M3 (Gln)	M3 (Pro)	M4 (Amm)	M4 (Glu)	M4 (Gln)	M4 (Pro)
MEP1	1.00	1.65	1.68	3.87	9.36	16.72	6.29	5.19	4.12	-2.10	1.47	1.36	3.23	11.40	4.78	6.32	3.67
	(±0.21)	(±0.48)	(±0.30)	(±0.46)	(±1.55)	(±2.62)	(±0.27)	(±0.51)	(±0.44)	(±0.67)	(±0.48)	(±0.37)	(±0.28)	(±1.98)	(±0.27)	(±0.36)	(±0.28)
GAP1	1.00	1.42	1.33	8.89	11.92	12.99	6.72	6.4	8.19	1.34	1.17	1.78	10.40	9.31	4.08	6.45	14.29
	(±0.36)	(±0.44)	(±0.23)	(±1.30)	(±1.39)	(±2.18)	(±0.43)	(±1.48)	(±1.42)	(±0.29)	(±0.46)	(±0.41)	(±1.78)	(±1.66)	(±0.42)	(±0.48)	(±1.63)
PUT4	1.00	1.67	-3.41	3.39	1.98	3.07	4.19	3.56	21.42	1.57	1.53	-1.24	3.44	3.75	3.97	3.79	19.63
	(±0.32)	(±0.45)	(±0.32)	(±0.46)	(±0.26)	(±0.69)	(±0.51)	(±0.22)	(±0.90)	(±0.39)	(±0.66)	(±0.24)	(±0.70)	(±0.40)	(±0.59)	(±0.18)	(±0.83)
GAT1	1.00	1.06	1.52	2.81	5.98	4.03	3.50	3.00	4.74	1.18	1.13	1.39	6.02	3.53	2.73	4.9	7.40
	(±0.38)	(±0.39)	(±0.46)	(±0.40)	(±0.46)	(±0.67)	(±0.48)	(±0.52)	(±0.52)	(±0.50)	(±0.60)	(±0.41)	(±0.66)	(±0.32)	(±0.71)	(±0.44)	(±0.61)
GDH1	1.00	-13.38	-12.16	-2.67	-3.42	-7.82	-11.45	-4.93	-3.71	-9.28	-10.58	-8.71	-6.06	8.69	-9.57	-13.2	-11.07
	(±0.17)	(±1.40)	(±0.73)	(±0.54)	(±0.48)	(±0.68)	(±0.62)	(±0.51)	(±0.42)	(±0.49)	(±0.53)	(±0.67)	(±0.51)	(±0.39)	(±0.43)	(±0.62)	(±0.54)
GLT1	-13.22	-16.72	1.00	1.00	1.00	1.00	-12.29	1.00	1.00	1.00	-9.23	1.00	1.00	1.00	-8.22	1.00	1.00
	(±0.78)	(±1.12)	(±0.21)	(±0.13)	(±0.17)	(±0.24)	(±0.23)	(±0.15)	(±0.18)	(±0.22)	(±0.84)	(±0.14)	(±0.11)	(±0.09)	(±1.04)	(±0.23)	(±0.17)
GDH2	-32.48	1.00	-26.12	-18.20	-16.13	-7.56	1.00	-14.7	12.23	-17.39	1.00	-18.52	-15.87	-9.39	1.00	-11.16	-10.37
	(±3.46)	(±0.12)	(±2.88)	(±1.95)	(±2.16)	(±0.83)	(±0.18)	(±1.48)	(±1.08)	(±2.40)	(±0.15)	(±3.65)	(±2.41)	(±1.13)	(±0.11)	(±2.10)	(±1.49)
SDH1	1.00	-1.10	-1.18	-1.08	-1.02	-1.14	1.05	-1.51	1.35	2.20	2.57	1.41	1.24	1.82	1.77	1.43	1.09
	(±0.19)	(±0.20)	(±0.24)	(±0.11)	(±0.13)	(±0.18)	(±0.16)	(±0.18)	(±0.17)	(±0.28)	(±0.28)	(±0.21)	(±0.17)	(±0.21)	(±0.27)	(±0.14)	(±0.16)

ACO1	1.00	1.52	1.29	2.84	1.92	-1.63	2.16	1.20	3.59	-2.01	2.93	1.77	3.70	-1.24	2.83	1.89	3.38
	(±0.12)	(±0.14)	(±0.13)	(±0.12)	(±0.10)	(±0.23)	(±0.14)	(±0.10)	(±0.16)	(±0.09)	(±0.18)	(±0.15)	(±0.20)	(±0.09)	(±0.22)	(±0.10)	(±0.16)
MDH1	1.00	2.61	2.16	3.81	2.33	-1.57	3.28	1.76	3.36	-1.39	1.18	2.90	2.83	3.12	2.69	4.04	3.79
	(±0.11)	(±0.18)	(±0.19)	(±0.14)	(±0.12)	(±0.10)	(±0.17)	(±0.15)	(±0.16)	(±0.12)	(±0.12)	(±0.19)	(±0.18)	(±0.19)	(±0.22)	(±0.14)	(±0.17)
IDH1	1.00	-1.06	1.94	1.40	2.10	1.03	-1.24	1.43	2.19	-1.55	1.88	-1.18	2.85	3.34	3.88	2.92	4.52
	(±0.18)	(±0.16)	(±0.32)	(±0.13)	(±0.15)	(±0.18)	(±0.16)	(±0.14)	(±0.19)	(±0.14)	(±0.20)	(±0.15)	(±0.22)	(±0.26)	(±0.33)	(±0.16)	(±0.23)
IDP1	1.00	-2.97	-2.48	-4.50	-3.92	-4.09	1.09	-3.46	-1.42	-1.01	-3.87	1.74	-2.27	-3.15	1.04	4.92	1.60
	(±0.11)	(±0.07)	(±0.38)	(±0.30)	(±0.40)	(±0.08)	(±0.10)	(±0.07)	(±0.08)	(±0.12)	(±0.08)	(±0.15)	(±0.08)	(±0.08)	(±0.13)	(±0.04)	(±0.10)
IDP2	1.00	2.32	1.08	1.43	-1.11	1.77	3.46	1.50	2.18	4.27	3.82	3.23	3.72	6.85	3.52	3.62	4.80
	(±0.26)	(±0.40)	(±0.29)	(±0.19)	(±0.17)	(±0.34)	(±0.41)	(±0.25)	(±0.37)	(±0.55)	(±0.47)	(±0.41)	(±0.42)	(±0.60)	(±0.59)	(±0.32)	(±0.42)
ATP1	1.00	1.33	1.47	2.67	2.83	2.74	2.42	2.59	2.90	2.82	3.11	3.02	3.43	3.27	3.42	3.12	3.32
	(±0.28)	(±0.13)	(±0.19)	(±0.31)	(±0.22)	(±0.16)	(±0.23)	(±0.32)	(±0.27)	(±0.14)	(±0.13)	(±0.18)	(±0.32)	(±0.21)	(±0.28)	(±0.24)	(±0.12)

The genes *MEP1* and *PUT4*, which in *S. cerevisiae* code for high-affinity ammonium and proline permeases, respectively, showed higher level of relative expression in low ammonium and low proline concentrations than for other N-sources (Table 3). On the other hand, *GAP1* gene coding for the general amino acid permease was induced by proline in both low and high concentrations (Table 3). At last, *GAT1* gene coding for the transcriptional activator of genes under NCR was particularly induced in proline and urea (Table 3).

Transcriptional control and interchange of roles between *GDH1* and *GLT1* as the major pathway for glutamate biosynthesis

The expression of genes involved in ammonia assimilation and glutamate biosynthesis in *D. bruxellensis* was investigated according to the availability of glucose and the quality of the nitrogen sources: *GDH1*, coding for the $NADP^+$-dependent glutamate dehydrogenase; *GLT1*, which codes for the NAD^+-dependent glutamate synthase (GOGAT); and *GDH2*, coding for the NAD^+-dependent glutamate dehydrogenase. *GDH1* presented the highest expression level among all three genes in high concentrations of glucose and ammonia (Table 3). *GLT1* showed low expression in glutamate (low or high concentrations) and in high amounts of ammonia while *GLT1* was the most expressed gene in proline, glutamine and in all limiting conditions (either carbon or nitrogen), except when glutamate was the nitrogen source (Table 3). Regarding to *GDH2*, it was the most expressed gene when glutamate was the N-source and also presented high expression levels in low concentrations of ammonia (Figure 2). However, its expression was low in glutamine, proline and in ammonium-rich media (Table 3).

Expression of genes related to TCA cycle and ATP biosynthesis

The expression of genes involved in TCA and ATP synthesis was also determined, in order to investigate a connection between poor growth conditions and a preference for the respiratory metabolism in *D. bruxellensis*. *SDH1*, coding for the flavoprotein subunit of succinate dehydrogenase, and *ACO1*, which codes for aconitase, did not change their expression levels in any conditions used (Table 3). On the other hand, *MDH1* and *IDH1*, coding for the mitochondrial malate dehydrogenase and for a subunit of mitochondrial NAD^+-dependent isocitrate dehydrogenase, respectively, showed increased expression in conditions in which carbon and nitrogen was limiting as well as in proline-based media (Table 3). *IDP1*, coding for mitochondrial NADP-specific isocitrate dehydrogenase, was induced in higher concentrations of ammonia and low amounts of glutamate (Table 3). This gene showed low expression levels in limiting

concentrations of ammonia and in higher amounts of glutamate (Figure 2). On the other hand, *IDP2*, coding for cytosolic NADP-specific isocitrate dehydrogenase, showed induced expression in glucose-limited media (Figure 2). Finally, the *ATP1* gene, coding for the alpha subunit of ATP synthase complex, was induced in conditions in which carbon or nitrogen was limiting (Figure 2).

Discussion

Yeast physiology under nutrient limitation

In the present study we performed the first investigation approaching the physiological and transcriptional profile of *D. bruxellensis* in response to the availability of glucose and nitrogen sources. In *S. cerevisiae*, the concentration and the quality of the nitrogen source are well known as able to influence the growth rate (Usaite *et al.*, 2006; Mendes-Ferreira *et al.*, 2007). Accordingly, ammonia, glutamate and glutamine are classified as preferential nitrogen sources, supporting fast growth rates (Magasanik & Kaiser, 2002; Boer *et al*., 2007). Our results show that when glucose is abundant and one of these nitrogen sources is available, *D. bruxellensis* reaches high and similar growth rates, indicating that they are equally assimilated by this yeast. On the other hand, proline and urea did not support high growth rates in agreement with classification as secondary nitrogen sources (Magasanik & Kaiser, 2002; Boer *et al*., 2007). The growth rate was roughly reduced by half when *D. bruxellensis* was submitted to either nitrogen or carbon limitation, indicating that the stringency of one of these nutrients is enough to disturb the growth in this yeast, as previously reported for *S. cerevisiae* (Usaite *et al.*, 2006; Mendes-Ferreira *et al.*, 2007).

Unlike the influence observed on growth, carbon and nitrogen limitation presented distinct metabolic effects in *D. bruxellensis*. Nitrogen shortage resulted in a moderate decrease of the glucose consumption and the ethanol production. However, the ethanol yield was also decreased, indicating that some of the carbon was diverted from fermentation to other metabolic processes, most probably respiration (Leite *et al.* 2012). In *S. cerevisiae*, the growth and the ability to break down sugars and produce ethanol is decreased in low concentrations of nitrogen (Mendes-Ferreira *et al.*, 2006). On the other hand, when *D. bruxellensis* cells were grown in carbon limitation, no ethanol accumulation was detected along the cultivations, regardless the nitrogen source available. Our data show that the low concentrations of carbon are far more severe for fermentation in *D. bruxellensis* than the nitrogen limitation. In *S. cerevisiae*, the lack of glucose may cause a derepression of genes involved in stress response and respiration, including TCA and respiratory chain-related genes (Gancedo, 1998; Carlson, 1999; Magasanik, 2003). Therefore, one might suggest a similar mechanism for glucose repression/derepression in *D. bruxellensis* since our recent

data show that in low glucose concentrations *D. bruxellensis* would rather use the respiratory over the fermentative metabolism (Leite *et al*., 2012). Additionally, in the present study we observed similar results when *D. bruxellensis* was grown in limitation of both carbon and nitrogen. Altogether, our physiological data indicate that, in adverse conditions, *D. bruxellensis* can rearrange its metabolism towards a more efficient way to produce energy, as it has been suggested for nitrate-growing cells, (manuscript in preparation). Moreover, we did not observe any significant glycerol accumulation in our experiments. The low production of glycerol is a characteristic of *D. bruxellensis* when grown in the presence of oxygen (Pereira *et al.*, 2012; Leite *et al*., 2012).

MEP1, *GAP1*, *PUT4* and *GAT1* are under NCR mechanism in *Dekkera bruxellensis*

The expression of genes coding for proteins involved with the uptake of nitrogen sources and the transcriptional activation of secondary nitrogen sources assimilation pathways was also investigated. Our data show that *MEP1*, *GAP1* and *PUT4* coding for permeases and *GAT1* coding for a regulatory protein are under NCR control in *D. bruxellensis*. *MEP1* was induced in condition of low nitrogen concentration, in particular ammonia, while repressed under abundance of preferential nitrogen sources. It is consistent with the described role for Mep1p as a high-affinity ammonia permease in *S. cerevisiae* (Marini *et al.*, 1994, 1997). Similar profile was observed for *GAP1* gene that codes for a non-specific amino acid transporter. This gene is under NCR control in *S. cerevisiaie* (Jauniaux & Grenson, 1990; Stanbrough and Magasanik, 1995; ter Schure *et al*., 1998). Additionally, transcription of *GAP1* genes was relatively higher than *MEP1* gene in *D. bruxellensis* cells when proline was the N-source. *PUT4* gene was down-regulated when ammonia and glutamine were abundant (ter Schure *et al*., 1998) and up-regulated in low concentrations of proline (Table 2). Thus, the co-expression of *GAP1* and *PUT4* genes might grant a high-affinity transport for this amino acid, as described for *S. cerevisiae* (Jauniaux & Grenson, 1990; Regenberg *et al.*, 1999). Similarly, *GAT1* genes induced in conditions that simulate nitrogen limitation (Table 3). This gene encodes for a transcriptional activator that positively induces the expression of genes subjected to NCR in *S. cerevisiae* (Coffman *et al.*, 1996; Magasanik & Kaiser, 2002). When the cells are under nitrogen limitation or when the nitrogen source is poor, Gat1p is dephosphorylated and enters the nucleus to up-regulate NCR-sensitive genes (Magasanik & Kaiser, 2002). This behavior may be similar in *D. bruxellensis*. Thus, a combined action of *GAT1* gene induction and Gat1p activation may ensure the complete activation of NCR-sensitive genes such as *GAP1* and *PUT4*.

Transcriptional control and interchange of roles between *GDH1* and *GLT1* as the major pathway for glutamate biosynthesis

Ammonia assimilation and further glutamate biosynthesis is one of the most important aspects of the cell metabolism. In the present study, we provide evidences from gene expression analyses that *GDH1* (coding for the $NADP^+$-dependent glutamate dehydrogenase) and *GLT1* (coding for NADH-dependent glutamate synthase - GOGAT) may switch roles as the main responsible for the biosynthesis of glutamate in *D. bruxellensis*. Moreover, this interchange is dependent on the nature and the availability of carbon and nitrogen in the medium. *GDH1* was highly expressed in conditions in which glucose and ammonia are exceeding and seems to play the major role for glutamate biosynthesis as it is observed for *S. cerevisiae*, converting 2-oxoglutarate and ammonia in glutamate (DeLuna *et al*., 2001; Riego *et al.*, 2002; Magasanik & Kaiser, 2002; Magasanik, 2003). On the other hand, *GLT1* was low expressed in that condition, apparently playing a secondary function on ammonia assimilation as in *S. cerevisiae* (Valenzuela *et al*., 1998; Magasanik & Kaiser, 2002; Magasanik, 2003). However, for other primary sources or when carbon and nitrogen are scarce, it was observed a switch in the gene expression pattern, with *GLT1* becoming more expressed and perhaps assuming the control over the ammonia assimilation, while *GDH1* is down-regulated (Table 3).

A similar mechanism also occurs in *S. cerevisiae*, in which the concentration of the nitrogen source seems to control the pathway chosen for glutamate biosynthesis (Magasanik & Kaiser, 2002; Magasanik, 2003), and different to *Kluyveromyces marxianus* that presents GOGAT pathway as the main mechanism for ammonia assimilation (de Morais Jr, 2003). In glucose limiting conditions, a drop in the intracellular content of 2-oxoglutarate might support an interchange between *GDH1* and *GLT1*, since glutamate synthase presents higher affinity for 2-oxoglutarate (Magasanik, 2003). Additionally, *GLT1* is low expressed in glutamate, suggesting a glutamate-mediated repression, as it has been described for *S. cerevisiae* (Valenzuela *et al*., 1998). *GLT1* seems to be also the major source of glutamate when *D. bruxellensis* is grown in proline and glutamine. Its higher expression in glutamine meets the necessity for the amide group in this amino acid to be transferred to 2-oxoglutarate, in order to generate two glutamate molecules, in a reaction catalyzed by GOGAT in most organisms (Valenzuela *et al*., 1998; Magasanik & Kaiser, 2002). When *D. bruxellensis* is grown in glutamate, the expression of *GDH2* is induced, indicating that the deamination of glutamate is required, in order to release the amino group essential for glutamine biosynthesis (Miller & Magasanik, 1990; Magasanik & Kaiser, 2002; Magasanik, 2003). However, it is important to note that, since the expression of both *GDH1* and *GLT1* genes were detected in all

samples, even though at different extents, we can assume that their expression are required for a plain biosynthesis of glutamate in *D. bruxellensis.*

Growth on limiting conditions increases the expression of TCA-related genes

Recent studies have described that the energetic efficiency of *D. bruxellensis* is higher than in *S. cerevisiae*, since *D. bruxellensis* is more capable of using the available oxygen for biomass generation (Blomqvist *et al.*, 2010; Blomqvist *et al.*, 2012; Leite *et al.*, 2012). A previous work suggested that *D. bruxellensis* have a tendency to a respiratory metabolism over the fermentative pathway when grown under oxygen-limited conditions and using nitrate, a secondary nitrogen source with a high energy-demand (manuscript in preparation). In order to determine whether the quality and availability of the nitrogen source is able to point the metabolism of *D. bruxellensis* towards respiration, the expression of genes involved in TCA and ATP synthesis was investigated. *ACO1* showed a slight induction in proline, presenting no alterations in the other conditions. Our previous data showed that *ACO1* was not induced in nitrate as sole nitrogen source, a condition requiring a high energetic supply (manuscript in preparation). On the other hand, *MDH1*, *IDH1*, *IDP2* and *ATP1* presented high expression in limiting conditions. It is well documented that *S. cerevisiae* growing on limiting concentrations of glucose might relieve the catabolite repression over genes involved in the assimilation of alternative carbon sources, stress response and respiration, including the TCA and respiratory chain-related genes (Gancedo *et al.*, 1998; Carlson, 1999; Magasanik, 2003). *IDP1* was highly expressed in glutamate and proline starvation and also when ammonia was abundant. This might suggest that, as in *S. cerevisiae*, *IDP1* might be related to a diversion of alpha-ketoglutarate to glutamate biosynthesis over the TCA flow (Haselbeck & McAlister-Henn, 1993). Finally, the expression of *SDH1* did not change in all tested samples, suggesting that it might be used as candidate reference gene for future RT-qPCR studies in *D. bruxellensis*. Altogether, the gene expression data show that *D. bruxellensis* increases the fluxes through the TCA and ATP synthesis, a strategy adopted to produce energy more efficiently.

In conclusion, our data show that either carbon or nitrogen availability can interfere in the growth, production of metabolites, such as ethanol, and the gene expression in *D. bruxellensis*. The carbon limitation more severely affected ethanol production, leading to no significant accumulation of this compound in the medium. Nitrogen limitation can also decrease growth and ethanol yield, but it occurs in a moderate extent. Our results also show that the genes coding for permeases in *D. bruxellensis* are susceptible to NCR and their expression are activated/deactivated in a coordinated profile. Moreover, an interchange of roles for glutamate biosynthesis seems to take place in *D. bruxellensis*, since the expression of *GDH1* and *GLT1* genes present opposite directions, depending

on the nature and concentration of the nitrogen source. Finally, *D. bruxellensis* shows a preference for respiration over fermentation in conditions of non-optimal growth, such as carbon or nitrogen limitation or even a nitrogen source with high-energy demand.

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CAPÍTULO V

A new set of reference genes for RT-qPCR assays in the yeast *Dekkera bruxellensis*

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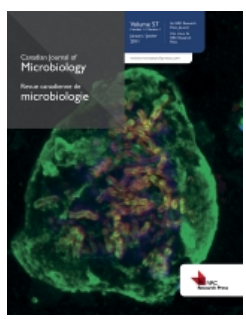

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A new set of reference genes for RT-qPCR assays in the yeast *Dekkera bruxellensis*

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Abstract: The yeast *Dekkera bruxellensis* has been recently regarded as an important microorganism for bioethanol production owing to its ability to convert glucose, sucrose, and cellobiose to ethanol. The aim of this work was to validate a new set of reference genes for gene expression analysis by quantitative real-time PCR in *D. bruxellensis* and compare the influence of the method of choice for quantification of mRNA levels with the reliability of our data. Three candidate reference genes, *DbEFA1*, *DbEFB1*, and *DbYNA1*, were used in a quantitative analysis of 4 genes of interest, *DbYNR1*, *DbTPS1*, *DbADH7*, and *DbUBA4*, based on an approach for calculating the normalization factors by means of the geNorm applet. Each reference gene was also individually used for a $2^{-\Delta\Delta C_q}$ (comparative C_q method) calculation of the relative expression of genes of interest. Our results showed that the 3 reference genes provided enough stability and were complementary to the normalization factors method in different culture conditions. This work was able to confirm the usefulness of a previously reported reference gene, *EFA1*/*TEF1*, and increased the set of possible reference genes in *D. bruxellensis* to 4. Moreover, this can improve the reliability of the analysis of the regulation of gene expression in the industrial yeast *D. bruxellensis*.

Key words: Dekkera bruxellensis, gene expression, geNorm, reference genes, RT-qPCR.

Résumé : La levure *Dekkera bruxellensis* a été récemment considérée comme un microorganisme important dans la production de bioéthanol à cause de sa capacité de convertir le glucose, le sucrose et le cellobiose en éthanol. Le but de ce travail était de valider un nouvel ensemble de gènes de référence pour l'analyse de l'expression génique par RT-qPCR chez *D. bruxellensis*, et de comparer l'influence de la méthode choisie pour quantifier les niveaux d'ARNm avec la fiabilité de nos données. Trois gènes de référence candidats *DbEFA1*, *DbEFB1* et *DbYNA1* ont été utilisés dans une analyse quantitative de quatre Gènes d'Intérêt (GI), *DbYNR1*, *DbTPS1*, *DbADH7* et *DbUBA4*, basée sur une approche de calcul des Facteurs de Normalisation (FN) à l'aide de l'applet geNorm. Chaque gène de référence a aussi été utilisé individuellement pour un calcul $2^{-\Delta\Delta C_q}$ de l'expression relative des GI. Nos résultats ont montré que les trois gènes de référence fournissaient suffisamment de stabilité et étaient complémentaires à la méthode NF dans différentes conditions de culture. Ce travail peut confirmer l'utilité d'un gène de référence antérieurement, *EFA1*/*TEF1*, et augmente à quatre la série des gènes de référence possibles chez *D. bruxellensis*. De plus, ceci peut améliorer la fiabilité de l'analyse de la régulation de l'expression génique chez la levure industrielle *D. bruxellensis*.

Mots-clés: Dekkera bruxellensis, expression génique, geNorm, gènes de référence, RT-qPCR.

[Traduit par la Rédaction]

Introduction

Dekkera bruxellensis (the teleomorph of *Brettanomyces bruxellensis*), a hemiascomycete yeast, is regarded as an important yeast found in different industrial processes, such as a contaminant of winery and bioethanol production (Abbott et al. 2005; Snowdon et al. 2006; de Souza Liberal et al. 2007; Basílio et al. 2008). However, it has also been recommended as fermenting yeast for bioethanol production (Passoth et al. 2007). The capacity of *D. bruxellensis* to surpass *Saccharomyces cerevisiae*

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cells in industrial fermentation has proved to be beyond expectations, partly on account of its very low specific growth rate in laboratory media and conditions (de Souza Liberal et al. 2007; Blomqvist et al. 2010). Given the fact that *S. cerevisiae* is the fermenting yeast and that no other yeast has surpassed its qualities in any other industrial process, *D. bruxellensis* has some physiological adaptations to the industrial environment that makes it a very attractive object for physiological and genomic studies (de Barros Pita et al. 2011). Our laboratory is now committed to understanding the mechanisms that control the genetic regulation of the central metabolism and its response to environmental stresses. This is valuable information for the molecular mechanism behind the industrial adaptation of *D. bruxellensis* (Blomqvist et al. 2010; de Barros Pita et al. 2011).

Currently, quantitative real-time PCR (RT-qPCR) is being used as the preferred method for mRNA quantification and gene expression. Its use requires several internal controls to ensure that the calculations are reliable (Livak and Schmittgen 2001; Ståhlberg et al. 2008; Teste et al. 2009; Vandesompele et al. 2002). Reference genes are used to normalize mRNA quantification and avoid imprecise quantification data. However, since small variations in expression levels of a reference gene may lead to inaccurate quantification data, validation experiments of candidate reference genes are essential to determine if their expression is in fact stable and also to normalize their possible variations. The mathematical approach based on the comparative C_q ($2^{-\Delta\Delta Cq}$) model has been used for a relative quantification of gene expression, but it is based on the assumption that target and reference genes provide both optimal and identical PCR efficiency (Livak and Schmittgen 2001). To overcome these problems with normalization, Vandesompele et al. (2002) employed a statistical algorithm called geNorm to evaluate the expression stability of the candidate reference genes. This approach was recently adopted to outline the use of *ACT1* and *TEF1* genes, encoding actin and EF1-α transla- tional elongation factor, respectively, as a set of reference genes for RT-qPCR in *D. bruxellensis* (Nardi et al. 2010). However, a recent work has shown that many genes that have been traditionally used as a reference point for *S. cerevisiae*, are not completely unchangeable (Ståhlberg et al. 2008; Teste et al. 2009). This makes the inclusion of additional genes imperative to extend the RT-qPCR analysis to *D. bruxellensis* genes in various culture conditions.

In the present work, the stability of the *TEF1* gene was reexamined (which from this point onwards will be referred to as *DbEFA1*), together with 2 other genes, *DbEFB1* (encoding EF1β translational elongation factor) and *DbYNA1* (encoding transcriptional regulatory factor of nitrate assimilation). The importance of the candidate reference genes was tested by the relative quantification of 4 unrelated *D. bruxellensis* genes of interest (GOI), each representing a different metabolism: nitrate assimilation (*DbYNR1*, encoding *Hansenula polymorpha* orthologous nitrate reductase), fermentation process (*DbADH7*, encoding *S. cerevisiae* orthologous NADPH-dependent medium chain alcohol dehydrogenase), storage carbohydrate (*DbTPS1*, encoding the *S. cerevisiae* orthologous synthase subunit of trehalose-6-phosphate synthase – phosphatase complex), and oxidative stress and redox metabolism (*DbUBA4*, encoding the *S. cerevisiae* orthologous thiosulfate sulfurtransferase).

The results confirmed the stability and reliability of these genes for studies on the regulation of some metabolic pathways related to carbon and nitrogen assimilation and of stress response genes in *D. bruxellensis*. As a result of this study, the list of reference genes in *D. bruxellensis* was increased to allow the application of relative gene expression analysis in this yeast.

Materials and methods

Yeast strains and growth conditions

Dekkera bruxellensis GDB248 is an industrial isolate (de Barros Pita et al. 2011) that has been used as a reference strain in our laboratory. Cells were pre-grown at 30 °C and 150 r·min^{-1} (1 r = 2 π rad) in a rotator shaker for 24 h in minimal synthetic (SC) medium (yeast nitrogen base at 1.6 g·L^{-1}) containing glucose (20 g·L^{-1}) and ammonium sulfate (5 g·L^{-1}). Following this, cells were transferred to 500 mL flasks containing 150 mL of the following media: (*i*) SC medium with glucose or sucrose (20 g·L^{-1}) (reference condition), (*ii*) SC medium with sodium nitrate (6.4 g·L^{-1}) and glucose or sucrose (test condition), and (*iii*) SC with ethanol (16 g·L^{-1}) and ammonium or nitrate (test condition). Cultures of an initial OD (optical density) of 0.1 at 660 nm were incubated in the same condition outlined above to a final OD of around 1.0 (full exponential growth phase). Cells were harvested at 4 °C for 2 min at 2500*g* and submitted to total RNA extraction. Technical triplicates were collected from 2 independent cultivations. The growth media supported different types of metabolisms: respiratory growth (ethanol) versus respiro-fermentative growth (glucose and sucrose), and high (nitrate) versus low (ammonium) redox demand for nitrogen assimilation.

RNA isolation and cDNA synthesis

For each culture, yeast total RNA was isolated using a NucleoSpin RNA II kit (Macherey-Nagel, USA), which includes a DNase digestion step, according to the manufacturer's instructions. The RNA was quantified by means of a spectrophotometric method (Nanovue, GE HealthCare), and its integrity was evaluated by agarose gel 1% electrophoresis. RNA was stored at −80 °C until cDNA synthesis. An amount of 500 ng of total RNA was used for each reverse transcription reaction tube (40 µL) using ImProm-II Reverse Transcription System Promega II kit with oligo(dT) and in compliance with the manufacturer's instructions (Promega, USA). cDNA was stored at –20 °C until the qPCR assays.

Primer design

Sequences of the *D. bruxellensis DbEFA1*, *DbYNR1* (yeast nitrate reductase) and *DbYNA1* (yeast nitrate assimilation) genes are publicly available at the GenBank Nucleotide database. The sequence of the target genes *DbEFB1*, *DbADH7*, *DbUBA4*, and *DbTPS1* were obtained from the restricted *D. bruxellensis* database (http://www.lge.ibi.unicamp.br/dekkera) after tBLASTx analysis by using their orthologous genes in the *S. cerevisiae* genome from the SGD database (http://www.yeastgenome.org/). The primers were designed with the aid of the Primer Express software (Applied Biosystems, Foster City, California, USA) and are described in Table 1.

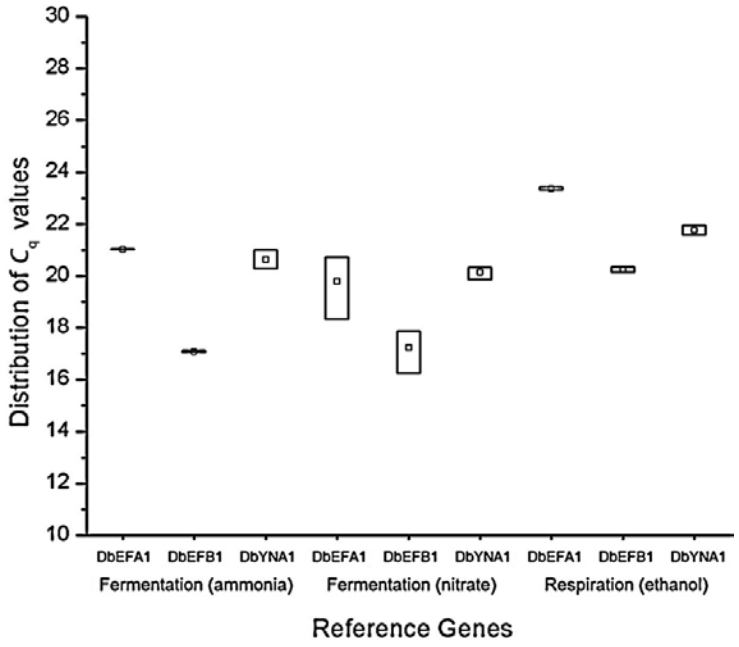
qPCR Assays

Experiments were optimized at a minimum material cost and a maximum efficiency by means of an experimental design, using SYBR Green PCR Master Mix (Applied Biosystems, USA).

Table 1. Primers used for quantitative real-time PCR assays

Name	Nucleotide sequences 5′–3′	Amplicon size (bp)	Acc No.
DbEFB1F	CATGGGATGACGAGACCGAT	71	—
DbEFB1R	CACCAAACCGTCCATCTTGAT		
DbYNA1F	TTTATATGGCGGGCATTGTA	109	EF364427
DbYNA1R	CCGTGAGCATTCAAGACATC		
DbEFA1F	GGATGGTCAGACCAGACAGCA	71	EF552481
DbEFA1R	TGACGGCAACAATAAGCTGC		
DbYNR1F	TGCTGGAAACAGGCGTAAAGA	71	EF364428
DbYNR1R	TCCCAGCAGAACCCCAATT		
DbTPS1F	GCTCTTCCGTATTCCACGGA	71	—
DbTPS1R	AAGTGAGTTTGCTGGTGCAGC		
DbADH7F	GGAAAGTGCTACAGGTGCAA	103	HQ693756
DbADH7R	ATCCTCCCTGAGTTTTGGTG		
DbUBA4F	TGATGGCTGTGGAGGCATTA	57	—
DbUBA4R	AGCAAATCCCGAGTAAAGCG		

Fig. 1. Global distribution of C_q values for candidate reference genes. Boxplot representation of raw C_q values obtained from amplification curves, according to Teste et al. (2009). The mean C_q values are indicated by the squares inside the boxes. Standard deviations are low and not visible for most of the points.

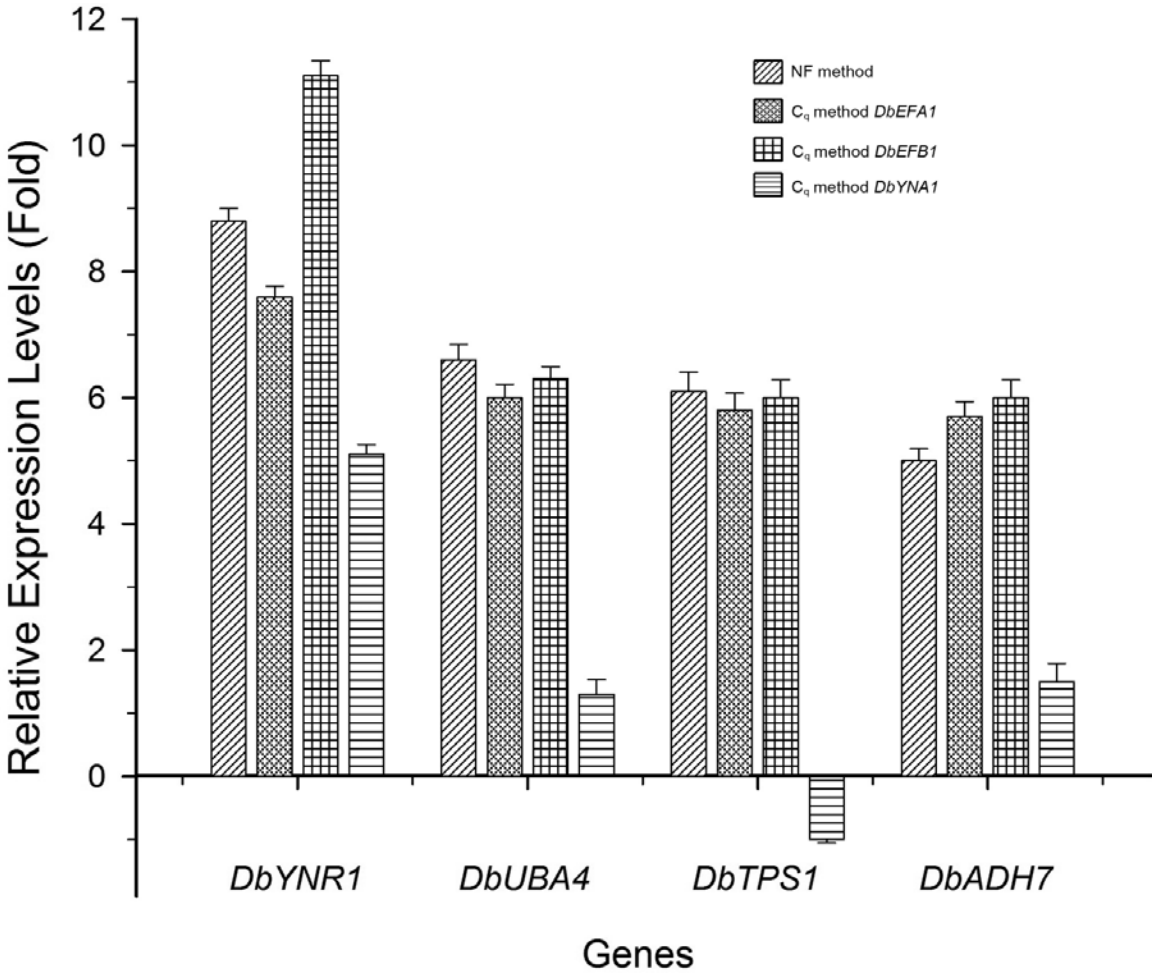


96-wells plates were used and the parameters were as follows: SYBR Green (5 µL), primers 200 nmol·L^{-1} (0.4 µL each), H_2O (3.2 µL) and cDNA (1 µL). The temperature–time profile (95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min) was optimized for ABI Prism 7300 (Applied Biosystems) and Melting Curves were included at the end of each reaction. The amplification curves were analyzed with the aid of SDS v.2.0 software (Applied Biosystems). Negative PCR control (no template) and negative RT control were run for quality control. All the samples were run in technical triplicates for each biological duplicate of cell cultivation, making a total of 6 samples for each growth medium. The standard $2^{-\Delta\Delta C_q}$ approach was calculated on the basis of a previous report (Livak and Schmittgen 2001).

geNorm analysis

The mean cycle threshold (C_q) values for each growth medium were then plotted in Microsoft Excel 2007 worksheets so that a suitable input file could be compiled for geNorm, in accordance with the recommendations of Vandesompele et al. (2002) (GeNorm tool (http://medgen.ugent.be/~jvdesomp/genorm/). The first step in the geNorm analysis was to determine the stability measurement (termed "*M*") for each candidate gene. The *M* value represents the gene expression stability as the average

Fig. 2. Effect of the normalization strategy on the expression ratios of *DbYNR1*, *DbUBA4*, *DbADH7*, and *DbTPS1* genes of *Dekkera bruxellensis*. The fold induction was calculated by comparing the test and reference media for the following genes: DbYNR1 (nitrate versus ammonium), DbUBA4, DbADH7, and DbTPS1 (ethanol versus sugar). The relative expression levels were calculated by means of the normalization factor (NF) approach or by the comparative (Cq; $2^{-\Delta\Delta Cq}$) method, using single reference genes *DbEFA1*, *DbEFB1*, or *DbYNA1*.



pairwise variation for that gene out of all the other tested references (Vandesompele et al. 2002). Stepwise exclusion of the gene with the highest *M* value allows the tested genes to be ranked according to their expression stability (Vandesompele et al. 2002). geNorm is also able to provide the optimal number of reference genes needed to obtain precise normalization. This number represents the pairwise variation values ($V(n/n + 1)$) between each combination of sequential normalization factors (NF). A cut-off value at 0.15 is recommended, as if it is below this, the inclusion of an additional gene does not result in a significant improvement of the normalization.

Results

Stability of expression levels of the candidate reference genes

The steps for RT-qPCR for reference genes followed the recommendations of the Minimum Information for Publication of Quantitative Real Time PCR Experiments (MIQE) guidelines described by Bustin et al. (2009). The efficiency of the amplification was 99.2% (slope of –3.34 and R^2 ranging from 0.991 to 0.998), and the optimal concentration was 200 nmol·L^{-1} for all primer pairs. Data from C_q were first analyzed by making a direct comparison to determine the transcript abundance of the reference genes in the different culture conditions. This comparison is based on the assumption that C_q is equal to the transcript number, since all the RT-qPCR were performed with an equal amount of total RNA. The C_q values ranged from 20 to 25, except for the *DbEFB1* gene, which showed lower C_q values than the other 2 genes for all of the conditions tested (Fig. 1). The dispersion levels were very low for all the genes, except for *DbEFA1* and *DbEFB1* in nitrate-containing medium (<1 C_q). For respiro-fermentative metabolism in ammonium, all 3 genes *DbEFA1*, *DbEFB1*, and *DbYNA1* were considered important ($V_{2/3} = 0.163$) to normalize the expression levels of the target genes. For respiro-fermentative metabolism in nitrate, the *DbEFB1* gene seemed to be unnec-

essary for the NF calculations, and it was possible to exclude it in the subsequent analyses ($V_{2/3} = 0.05$). Similarly, for respiratory metabolism, the gene *DbYNA1* was not necessary for NF calculations, while *DbEFA1* and *DbEFB1* were the best reference genes ($V_{2/3} = 0.057$).

Expression analysis of *D. bruxellensis* target genes

Following the parameters described by the MIQE guidelines, expression of GOI were tested using the reference genes above and comparing 2 methods. In a shift from respiro-fermentative to respiratory growth, the *DbUBA4*, *DbTPS1*, and *DbADH7* genes showed 5- to 6-fold induction when using NFs calculated from the geometric mean in the geNorm algorithm (Fig. 2). Similar results were observed when the $2^{-\Delta\Delta C_q}$ method was applied, using *DbEFA1* or *DbEFB1* as single genes (Fig. 2). On the other hand, the induction of these 3 GOI was not observed when *DbYNA1* was used as a unique reference gene (Fig. 2). By adopting the NF approach, the expression of *DbYNR1* gene increased by 9 times when the yeast cells were changed from ammonium to nitrate medium (Fig. 2). It is noteworthy that different expression levels of the *DbYNR1* gene were observed when using the 3 single reference genes independently. In this case in particular, the use of NF seemed to be essential when measuring the expression level of nitrate assimilation genes. Similar results were found regarding the need for an NF approach to the 3 reference genes when *D. bruxellensis* cells were submitted to the following conditions: (*i*) cultivation in phenylalanine as a single source of nitrogen, (*ii*) incubation at different temperatures for thermal stress analysis, and (*iii*) analysis of response to oxidative stress in the presence of hydrogen peroxide (data not shown).

Discussion

In this work, 3 candidates of reference genes for RT-qPCR in the yeast *D. bruxellensis* were tested. In general, both the genes encoding translation elongation factors, *DbEFA1* and *DbEFB1*, showed enough stability to be used as reference genes in the NF approach, as well as single genes by the $2^{-\Delta\Delta C_q}$ method. The *DbEFA1*/*TEF1* gene has previously been selected because of its high stability and has been used together with the *ACT1* gene to calculate the expression of stress resistance genes in *D. bruxellensis* (Nardi et al. 2010). In addition, we have shown that *DbYNA1* gene was an important auxiliary reference gene when included in an analysis of target gene expression under conditions of high growth rates in medium containing glucose, but not for ethanol or when using nitrogen sources other than ammonium. The C_q values of *DbYNA1* gene ranged from 20 to 22 in all the conditions tested. This means that it is constitutively expressed even in nitrate-containing medium. Taking this into account, the constitutive expression of the *DbYNA1* gene could offset any putative effects arising from a high growth rate in the expression of growth-related reference genes, and thus, possibly allow the relative expression analysis to be more robust. Al- though *DbEFA1* and *DbEFB1* genes are functionally related, it should be possible to use one of them together with 2 other genes (*DbYNA1* and *ACT1*) in different culture conditions, as they produced different fold change in the case of the *DbYNR1* target gene. This increases the range of candidate genes thatcan be considered for relative quantification of target genes in *D. bruxellensis*. The use of these genes as reference points was tested with 4 target genes belonging to different metabolic pathways. The results showed that choosing between the NF approach and the $2^{-\Delta\Delta C_q}$ method does not affect the relative expression of *DbADH7*, *DbTPS1*, and *DbUBA4* genes. This means that both methods can be used to normalize gene expression data, but NF seems to be more suitable, since there is a risk that using only 1 reference gene might lead to a misinterpretation of the data due to any possible change in its expression.

In conclusion, in addition to the previously discussed *ACT1* and *DbEFA1*/*TEF1*, 2 other genes, *DbEFB1* and *DbYNA1*, showed enough stability and provided different combinations of reference genes to allow normalization by the NF method. This occurred in a way that made it possible to overcome the variations normally observed during cell growth.

Acknowledgements

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CONCLUSÃO

- A utilização de nitrato, uma fonte de nitrogênio secundária encontrada no caldo de cana, interfere no metabolismo de *Dekkera bruxellensis* diminuindo a taxa de crescimento, o consumo de açúcares e a produção de etanol nesta levedura;

- Apesar de alterar diversos aspectos da fisiologia de *D. bruxellensis*, o nitrato presente no caldo de cana pode atuar como fator positivo na adaptação desta levedura, fornecendo a fonte de nitrogênio necessária para *D. bruxellensis* continuar crescendo, mesmo quando a amônia presente no caldo de cana é completamente utilizada e *S. cerevisiae* não possui mais recursos para crescer;

- A limitação de nitrogênio interfere no crescimento, consumo de açúcares e produção de etanol em *D. bruxellensis*;

- A limitação de carbono é uma condição ainda mais severa para *D. bruxellensis*, visto que o crescimento celular é reduzido e não há acúmulo detectável de etanol ou acetato;

- As enzimas glutamato desidrogenase e glutamato sintase podem alternar os papéis como principal via responsável pela biossíntese de glutamato em *D. bruxellensis*;

- Os genes codificantes de permeases de fontes de nitrogênio estão sob rígido controle da Repressão Catabólica do Nitrogênio em *D. bruxellensis*;

- *D. bruxellensis* apresenta preferência pelo metabolismo respiratório em detrimento da fermentação em condições limitantes de crescimento, como a escassez de carbono ou nitrogênio;

- Os genes *DbTEF1*, *DbEFB1* e *DbYNA1* foram validados em um novo grupo de genes de referência para ensaios de expressão gênica por PCR em tempo real em *D. bruxellensis*;

- Os resultados obtidos neste trabalho contribuem para o entendimento acerca das repostas metabólicas de *D. bruxellensis* ao nitrogênio, um dos principais nutrientes para a célula. O conhecimento destes mecanismos pode ajudar a identificar fatores de adaptação compatíveis com o sucesso competitivo desta levedura no ambiente industrial.

ANEXOS OBRIGATÓRIOS

Anexo 1: Yeast – Author Guidelines

Manuscript Submission. Preferred formats for the text and tables of your manuscript are DOC (.doc) or RTF (.rtf). Figures should be provided in TIFF (.tiff) or EPS (.eps) format.

Manuscript style. The language of the journal is English. 12-point type in one of the standard fonts: Times, Helvetica, or Courier is preferred. It is not necessary to double-line space your manuscript. Tables must be on separate pages after the reference list, and not be incorporated into the main text. Figures should be uploaded as separate figure files.

During the submission process you must enter the full title, short title of up to 70 characters and names and affiliations of all authors. Give the full address, including email, telephone and fax, of the author who is to check the proofs. Enter an abstract of up to 250 words for all articles. An abstract is a concise summary of the whole paper, not just the conclusions, and is understandable without reference to the rest of the paper. It should contain no citation to other published work. Include up to six keywords that describe your paper for indexing purposes. The text should generally be divided into: Introduction; Materials and Methods; Results and Discussion; References; Tables, Figure Legends. Please avoid section numbering.

Reference style. References should be quoted in the text as name and year within square brackets and listed at the end of the paper alphabetically. For reviews only, references should be cited by number in the text. The reference list should be ordered alphabetically and numbered in sequence. Hence references in the text will not be in numerical sequence. All references must be complete and accurate. Online citations should include date of access. References should be listed in the following style:

Cooper TG, Sumrada R. 1975. Urea transport in Saccharomyces cerevisiae. J Bacteriol121: 571-576.

Illustrations. Supply each illustration on a separate page, with the lead author's name, the figure number and the top of the figure indicated. Tints are not acceptable; lettering must be of a reasonable size that would still be clearly legible upon reduction, and consistent within each figure

and set of figures. Where a key to symbols is required, please include this in the artwork itself, not in the figure legend. Supply artwork at the intended size for printing.

Anexo 2: Microbiology – Author´s guideline

Microbiology publishes high-quality research papers and reviews on all aspects of microbiology, with a strong emphasis on fundamental studies on the biology of prokaryotic and eukaryotic micro-organisms; papers elucidating molecular mechanisms are particularly welcome. Papers published must make an original and significant contribution to the field and should be of interest to a general readership. Papers should be written concisely and contain only figures and tables that are essential for conveying the results. As a guide, research papers published in Microbiology should normally contain no more than approximately 6000 words (including the figure and table legends, in-text citations and any appendices, but not including the abstract, acknowledgements or reference list) and up to eight figures and tables combined.

Submission as separate word-processor and image files. Most standard word-processor files (including .docx files produced in Word 2007 or 2010) will convert successfully to PDF. Times, Times New Roman, Courier, Helvetica and Arial, and the Symbol font for special characters, are the recommended fonts. Other fonts are not guaranteed to convert successfully to PDF. Tables for the main paper must be prepared as part of the word-processor file; they must not be supplied as images or Excel files. (Excel files are, however, acceptable for supplementary data).

The paper must be written in clear and concise English, normally in the past tense, and should normally comprise: (a) Title page; (b) Summary; (c) Introduction; (d) Methods; (e) Results; (f) Discussion, with Conclusions if appropriate; (g) Acknowledgements; (h) References; (i) Tables; (j) Figures, with legends. A Theory section and Appendices may be included. Where appropriate, the Results and Discussion may be combined. Figures and tables should only be used to illustrate points that cannot easily be described in the text. Please check the length guidelines before submitting your manuscript and check that your paper is an appropriate length.

Title page.

• The title of the paper. A good title is very important. It will attract readers and facilitate retrieval by online searches, thereby helping to maximize citations. The title should include topical keywords and allude to the interesting conclusions of the paper. A title that emphasizes the main conclusions,

or poses a question, has more impact than one that just describes the nature of the study. A short 'running title', of not more than 55 characters (including spaces), for use as a headline. The names of the authors. The author for correspondence must be clearly indicated. The name and address of the laboratory or laboratories where the work was done, and present addresses of authors who have since moved. An e-mail address and telephone and fax numbers for the corresponding author. The number of words in (i) the summary and (ii) the main text (including the figure and table legends and any appendices, but not the title page, summary, acknowledgements, table bodies and footnotes, or references) and the number of tables and figures. If appropriate, a footnote defining any non-standard abbreviations. Guidance on abbreviations not requiring definition is given in the Abbreviations section.

The summary will be read by more people than the full paper. It must therefore be clear and comprehensible in its own right. References should not be cited, and any abbreviations used must be defined. The summary should if possible introduce the subject in the first sentence and present the main conclusion in the last sentence: when someone is skimming a block of text, the first and last sentences receive the most attention. The recommended maximum length for the summary is 250 words.

Introduction. This should state the objectives of the work, but should not contain a detailed summary of the results. Authors should not assume that all readers will know why an area is worth studying; they should briefly make this clear. Previous relevant work should be sufficiently cited but this should not constitute a full review.

Methods. Sufficient detail should be provided to allow the work to be repeated. The suppliers of chemicals and equipment should be indicated if this may affect the results. Suppliers' addresses should not be given unless this is considered essential for a particular reason. A source (name and brief address) or reference should be given for each strain used. Authors are encouraged to deposit important strains in a recognized culture collection and to refer to the collection and strain number in the paper.

Results. There should be sufficient subheadings to make clear how the work was organized, what the key questions being addressed were, how one experiment led to another, and perhaps what conclusions were reached. A reader should gain a clear picture of the work from the subheadings.

Discussion. This should not recapitulate the results, and should not be too long. Excessive discussion of few facts often gives an impression of poor science. Subheadings should be used where appropriate, to highlight the points under discussion. It may be helpful to list the main

conclusions at the end. A combined Results and Discussion section is encouraged where appropriate.

References. References in the text should be cited as follows: two authors, Smith & Jones (1996) or (Smith & Jones, 1996); three or more authors, Smith et al. (1996) or (Smith et al., 1996). References to papers by the same author(s) in the same year should be distinguished in the text and the reference list by the letters a, b, etc. (e.g. 1996a or 1996a, b).

For references with ten or fewer authors, give the names of all authors in the form "Surname, Initials". For references with more than ten authors, list the first nine followed by "& other authors".

Sample journal references:

Cerdà-Cuéllar, M., Rosselló-Mora, R. A., Lalucat, J., Jofre, J. & Blanch, A. (1997). Vibrio scophthalmi sp. nov., a new species from turbot (Scophthalmus maximus). Int J Syst Bacteriol 47, 58–61.

Tomb, J.-F., White, O., Kerlavage, A. R., Clayton, R. A., Sutton, G. G., Fleischmann, R. D., Ketchum, K. A., Klenk, H.-P., Gill, S. & other authors (1997). The complete genome sequence of the gastric pathogen Helicobacter pylori. Nature 388, 539–547.

Tables. These should be broadly comprehensible without reference to the text, but it is not necessary to repeat detailed descriptions of methods, etc. The symbols * † ‡ § ‖ ¶ # should be used for footnotes, rather than superscript letters or numbers. When results are expressed as percentages, the absolute value(s) corresponding to 100% must be stated. Statements of reproducibility should be included (see above). Tables should not be used to present results that can be described by a brief statement in the text.

Figures. This section outlines journal policy on figures. See these links for advice on preparing figures for inclusion as a PDF for submission and on the source files needed for publication. Figures should not be used to present results that can be described by a brief statement in the text. The points outlined above for tables regarding comprehensibility, relative values and reproducibility also apply to figures and their legends. The inclusion of large amounts of tabular data in figures is discouraged and authors may be asked to move such data to the text or a separate table. Authors should be aware that after publication, tabulated data within figures are not accessible via online text searching.

ANEXOS COMPLEMENTARES

Anexo complementar 1 - Trabalhos desenvolvidos em coautoria:

1 - "Quantitative aerobic physiology of the yeast *Dekkera bruxellensis*, a major contaminant in bioethanol production plants", trabalho desenvolvido através de parceria com integrantes do grupo de pesquisas coordenado pelo profº Marcos Antonio de Morias Junior. O trabalho foi publicado na revista *FEMS Yeast Research*, sob o doi: 10.1111/1567-1364.12007, Epub, outubro de 2012.

2 **-** "Yeast transcriptomics in sugarcane fermentation", trabalho desenvolvido através de parceria com integrantes do grupo de pesquisas coordenado pelo profº Marcos Antonio de Morias Junior. Submetido à revista *Journal of Industrial Microbiology and Biotechnology* em 27 de novembro de 2012.

3 - "Catabolite repression in the yeast *Dekkera bruxellensis*", estudo em desenvolvimento através de parceria com integrantes do grupo de pesquisas coordenado pelo profº Marcos Antonio de Morais Junior.

4 - "Adaptation of *Dekkera bruxellensis* to the lignocellosic substrate", estudo em desenvolvimento através de parceria com o grupo de pesquisas coordenado pelo profº Volkmar Passoth, do departamento de Microbiologia da Universidade de Ciências Agrícolas da Suécia, na cidade de Uppsala, Suécia.

5 - "5-hydroxymethylfurfural induces *ADH7* and *ARI1* expression in tolerant industrial *Saccharomyces cerevisiae* strain P6H9", estudo em desenvolvimento através de parceria com o grupo de pesquisas coordenado pelo profº Marco Antônio Záchia Ayub, do Laboratório de Biotecnologia a Engenharia Bioquímica da Universidade Federal do Rio Grande do Sul.

Anexo complementar 2 – Formação complementar

1 – Estágio no Exterior – Estágio de doutorado desenvolvido sob supervisão do profº Dr. Volkmar Passoth, no Departamento de Microbiologia da Universidade de Ciências Agrícolas da Suécia, na cidade de Uppsala, Suécia. Período: Abril a Dezembro de 2011.

Anexo complementar 3 – Trabalhos apresentados em congressos

1. de Barros Pita, W; Leite, F. C. B.; Simões, D. A.; Morais JR., M. A. “Nitrate assimilation is able to change gene expression profile in *Dekkera bruxellensis*”. In: XIX Encontro de Genética do Nordeste, 2012, Petrolina. Anais do XIX Encontro de Genética do Nordeste, 2012.

2. de Barros Pita, W; Leite, FCB; Liberal, A. T. S.; Simões, D. A.; Morais JR., M. A. “Nitrate as a potential adaptation factor to *Dekkera bruxellensis*”. In: Non-Conventional Yeasts in the Postgenomic Era, 2011, Lviv, Ucrânia. Non-Conventional Yeasts in the Postgenomic Era Abstract Book, 2011.

3. Leite, F. C. B.; de Barros Pita, W; Morais JR., M. A. “Estudo da Repressão Catabólica por glicose na levedura *Dekkera bruxellensis* por PCR em Tempo Real”. In: 27ª Reunião de Genética de Microrganismos, 2010, Guarujá. Anais da 27ª Reunião de Genética de Microrganismos, 2010.