UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE BIOCIÊNCIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS
TESE DE DOUTORADO

GILBERTO HENRIQUE TELES GOMES DA SILVA

ASPECTOS FISIOLÓGICOS E GENÉTICOS DA PRODUÇÃO DE ACETATO E ETANOL NA REGULAÇÃO DO METABOLISMO RESPIRO-FERMENTATIVO DA LEVEDURA INDUSTRIAL *DEKKERA BRUXELLENSIS*

Recife, 2022

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Tese de doutoramento apresentada ao Programa de Pós-Graduação em Ciências Biológicas do Centro de Biociências da Universidade Federal de Pernambuco, como requisito parcial para obtenção do título de Doutor em Ciências Biológicas, na área de concentração biotecnologia e bioinformática.

Orientador: Profº Dr. Marcos Antonio de Morais Junior

Coorientador: Profº Dr. Will de Barros Pita

Recife, 2022

Catalogação na Fonte:
Bibliotecário Bruno Márcio Gouveia, CRB4/1788

Silva, Gilberto Henrique Teles Gomes da
Aspectos fisiológicos e genéticos da produção de acetato e etanol na regulação do metabolismo respiro-fermentativo da levedura industrial *Dekkera Bruxellensis* / Gilberto Henrique Teles Gomes da Silva. – 2022.

172 f. : il.

Orientador: Prof. Dr. Marcos Antonio de Morais Junior.
Coorientador: Prof. Dr. Will de Barros Pita.
Tese (doutorado) – Universidade Federal de Pernambuco. Centro de Biociências. Programa de Pós-graduação em Ciências Biológicas, Recife, 2022.
Inclui referências e apêndices.

1. Leveduras (Fungos). 2. Fermentação. 3. Microbiologia industrial. I. Morais Júnior, Marcos Antonio de (orientador). II. Pita, Will de Barros (coorientador). III. Título.

579.5 CDD (22.ed.) UFPE/CB – 2022-116

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Aprovada em 30/03/2022

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“Um livro, uma caneta, uma criança e um professor podem mudar o mundo”.

Malala Yousafza

AGRADECIMENTOS

Toda minha família, em especial minha mãe Luiza e noiva Luciana pelo amor e carinho em todos os momentos.

Meu Pai, Ibernon Gomes, e meu tio, Carlos Alberto, motivos da minha força e dedicação (in memoriam).

Aos meus orientadores Marcos Antonio de Morais Junior e Will de Barros Pita por todo o conhecimento nessa jornada, confiança, entusiasmo para que tudo corra bem e, sobretudo correções que irão contribuir significativamente para minha vida pessoal e profissional. Agradeço muito pela oportunidade de ter convivido com grandes profissionais, muito obrigado!

Universidade Federal de Pernambuco e Universidade de Pernambuco pelo suporte na realização da tese.

Ao grupo de pesquisa em biologia molecular e engenharia metabólica por todo apoio para realização da tese, em especial para Jackeline e Mariana.

Aos meus verdadeiros amigos por atos e palavras em todos os momentos.

Aos órgãos que contribuíram financeiramente com a tese, em especial a Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE) pela bolsa e a Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) por todo o suporte.

Agradeço aos professores que fizeram parte de todo o processo, da educação básica ao ensino superior.

Agradeço as professoras Monica Lomeli e Tereza Correia e os professores Boris Stambuk e Leucio Duarte pelas contribuições concedidas para o trabalho.

RESUMO

A levedura *Dekkera bruxellensis* tem sido apresentada recentemente com grande potencial industrial. Essa levedura adquiriu características primordiais para seu estabelecimento e manutenção nas dornas de fermentação alcoólica, como a habilidade diferencial em assimilar o nitrato como fonte de nitrogênio. O objetivo do presente estudo foi investigar fatores fisiogenômicos associados à produção de acetato e etanol, com ênfase no desvio da produção de acetil-CoA citosólico (*Pdh bypass*). Os resultados foram obtidos a partir de medidas fisiológicas e de expressão gênica em cultivos em meio sintético definido e na presença ou ausência do inibidor bioquímico da enzima acetaldeído desidrogenase (ald3p), dissulfiram (DSF). Em geral, a presença de DSF diminuiu o crescimento de *D. bruxellensis* em várias condições de cultivo, enquanto promoveu aumento na eficiência fermentativa da levedura. A eficiência na conversão em etanol na presença do inibidor ultrapassou 96% em alguns casos, representando incremento de 33% em relação à condição de referência. Esse resultado fisiológico foi acompanhado por um nível geral de aumento da expressão gênica relativa dos genes alvos (*PFK1, FBP1, PDC1, ADH1, ALD3, ALD5, IDH1, SDH1 e ATP1*). Os dados indicam que o acetato pode fluir da mitocôndria e restabelecer as necessidades citosólicas quando a enzima Ald3p é bloqueada. A galactose foi metabolizada exclusivamente pela via respiratória em *D. bruxellensis* e a utilização dessa fonte de carbono na presença de nitrato indica que o desvio metabólico da produção de acetil-CoA (*Pdh bypass*) é o principal caminho nesta levedura para o aporte de equivalentes redutores NAPDH para a redução do nitrato a amônio. Adicionalmente, a eficiência fermentativa de *D. bruxellensis* aumentou quando a sacarose foi utilizada como fonte única de carbono, independentemente da fonte de nitrogênio. Possivelmente a frutose liberada de sua hidrólise deve estar exercendo um efeito regulatório importante no metabolismo da levedura, principalmente no desvio do carbono pela via das pentoses fosfato. Outros aspectos genéticos e fisiológicos de *D. bruxellensis* são discutidos e contribuem para o entendimento de fatores diferenciais para a manutenção industrial e capacidade fermentativa desse microrganismo.

Palavras-chave: *Dekkera bruxellensis***;** acetato; fermentação alcoólica; respiro-fermentativo; bypass PDH; acetaldeído desidrogenase.

ABSTRACT

The yeast *Dekkera bruxellensis* has recently been presented with great industrial potential. This yeast acquired essential characteristics for its establishment and maintenance in alcoholic fermentation vats, such as the differential ability to assimilate nitrate as a source of nitrogen. The aim of the present study was to investigate physiogenomic factors associated with the production of acetate and ethanol, with emphasis on the pathway of cytosolic acetyl-CoA production (Pdh bypass). The results were obtained from physiological measurements and gene expression in cultures in synthetic media defined and in the presence or absence of the biochemical inhibitor of enzyme acetaldehyde dehydrogenase (Ald3p), disulfiram (DSF). In general, the presence of DSF decreased growth *of D. bruxellensis* under various culture conditions, while promoting an increase in yeast fermentative efficiency. The efficiency of conversion to ethanol in the presence of the inhibitor exceeded 96% in some cases, representing an increase of 33% in relation to the reference condition. This physiological result was accompanied by a general level of increase in the relative gene expression of the target genes (*PFK1, FBP1, PDC1, ADH1, ALD3, ALD5, IDH1, SDH1 e ATP1*). The data show that acetate can flow out of mitochondria and replenish cytosolic needs when the enzyme Ald3p is blocked. Galactose was metabolized exclusively by the respiratory route in *D. bruxellensis* and the use of this carbon source in the presence of nitrate indicates that the metabolic deviation of acetyl-CoA production (Pdh bypass) is the main pathway in this yeast for the contribution of NAPDH reducing equivalents for the reduction of nitrate to ammonium. Additionally, the fermentative efficiency of *D. bruxellensis* increased when sucrose was used as the sole carbon source, regardless of the nitrogen source. Possibly, the fructose released from its hydrolysis must be exerting an important regulatory effect on yeast metabolism, mainly in the shift of carbon to the pentose phosphate pathway. Other genetic and physiological aspects of *D. bruxellensis* are discussed and contribute to the understanding of differential factors for the industrial maintenance and fermentative capacity of this microorganism.

Keywords: *Dekkera bruxellensis*; acetate; alcoholic fermentation; respiro-fermentative; Pdh bypass; acetaldehyde dehydrogenase.

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LISTA DE ABREVIATURAS E SIGLAS

Acs	Acetil-CoA sintase
Adh	Álcool desidrogenase
Ald3	Acetaldeído desidrogenase/isoforma citosólica
Ald6	Acetaldeído desidrogenase/isoforma citosólica
Ald4	Acetaldeído desidrogenase/isoforma mitocondrial
Ald5	Acetaldeído desidrogenase/isoforma mitocondrial
ATP	Adenosina trifosfato
CO_2	Dióxido de carbono
cDNA	DNA complementar
cAMP	Adenosina 3',5'-monofosfato cíclico
Dak1	Dihidroxiacetona quinase
Dak2	Dihidroxiacetona quinase
DSF	Dissulfiram
DHAP	Dihidroxicetona fosfato
EMP	Embden-Meyerhof-Parnas
Eno	Enolase
FAD^+	Dinucleótido de flavina e adenina/forma oxidada
FADH2	Dinucleótido de flavina e adenina/forma reduzida
Fba	Aldolase
Fps1	Aquagliceroporina
G3P	Gliceraldeído-3-fosfato
Gapdh	Gliceraldeído-3-fosfato desidrogenase

GCR	Glucose catabolite repression
Gcy1	Glicerol desidrogenase
Glk	Glicoquinase
Gpd1	Glicerol-3-fosfato desidrogenase
Gpd2	Glicerol-3-fosfato desidrogenase
Gpm	Fosfoglicerato mutase
Gpp1	Glicerol-3-fosfato fosfatase
Gpp2	Glicerol-3-fosfato fosfatase
Gut1	Glicerol quinase
Gut2	Glicerol-3-fosfato desidrogenase
Hxk1	Hexoquinase
Hxk2	Hexoquinase
mM	Milimolar
MPC	Transportador mitocondrial de piruvato
µM	Micromolar
NAD^+	Nicotinamida adenina dinucleotídeo/forma oxidada
NADH	Nicotinamida adenina dinucleotídeo/forma reduzida
$NADP^+$	Nicotinamida adenina dinucleotídeo fosfato/forma oxidada
NADPH	Nicotinamida adenina dinucleotídeo fosfato/forma reduzida
NCY	Non-conventional yeasts
Pda	Piruvato desidrogenase
Pdc	Piruvato descarboxilase
Pfk	Fosfofrutoquinase
Pgi	Fosfoglicose isomerase

Pgk	Fosfoglicerato quinase
PKA	Proteína quinase A
Pyk	Piruvato quinase
qPCR	PCR quantitativa
TCA	Ciclo do ácido cítrico
Tpi	Triose fosfato isomerase
YNB	Yeast Nitrogen Base
YPD	Yeast peptone dextrose

SUMÁRIO

1 INTRODUÇÃO

A levedura *Dekkera bruxellensis* é um microrganismo muito adaptado e de grande importância nos processos fermentativos industriais. Nestes ambientes, a levedura é capaz de contaminar quimicamente na produção do vinho, substituir as populações iniciais de *Saccharomyces cerevisiae* e interferir nos rendimentos na indústria de bioetanol. No entanto, também possui papel positivo e preponderante na caracterização sensorial durante a fermentação espontânea para produção da cerveja Belga Lambic. A capacidade em produzir etanol mesmo em condições de aerobiose em cultivos com alta concentração de açúcar, denominado efeito *crabtree* positivo, além da habilidade em suportar o elevado teor alcoólico dos processos industriais, são características fundamentais de *D. bruxelllensis.*

Ao longo dos anos, a ciência buscou entender outros aspectos metabólicos de *D. bruxellensis* nesses variados processos, tanto do ponto de vista fisiológico quanto genético. Por exemplo, sabe-se que na indústria vinícola a contaminação da levedura está associada à produção de fenóis voláteis, tais como o *4*-etil-fenol e o 4-etil-guaiacol, compostos que conferem sabores e odores desagradáveis ao vinho, comprometendo o processo industrial. Na produção de bioetanol, a levedura é capaz de metabolizar o nitrato como fonte de nitrogênio e se beneficiar adaptativamente frente a *S. cerevisiae,* afetando a fermentação etanólica da principal levedura do processo. Já na produção da cerveja Lambic, a habilidade em produzir ácido acético é fundamental para a composição do painel sensorial desse produto industrial. Adicionalmente, *D. bruxellessis* é considerada uma levedura em potencial para produção de etanol de segunda geração. As características que favorecem a inserção, manutenção e capacidade biotecnológica de *D. bruxellensis* nos ambientes industriais são apresentadas no primeiro artigo da presente tese intitulado: **“The biotechnological potential of the yeast *Dekkera bruxellensis*”**.

A habilidade de *D. bruxellensis* em produzir acetato está relacionada ao efeito *Custer*, mecanismo que é caracterizado pela diminuição da coenzima NAD^+ que foi utilizada para oxidar o acetaldeído a acetato durante as reações do chamado bypass PDH (rota ao nível do piruvato). Com isso, as células da levedura possuem dificuldade em reoxidar o NADH resultante, afetando negativamente o fluxo pela via glicolítica, o que promove uma parada no metabolismo fermentativo e por

consequência afeta a produção de etanol. Buscando entender novos aspectos deste metabolismo em *D. bruxellensis,* o segundo artigo da presente tese refere-se ao trabalho intitulado: **“First aspects on acetate metabolism in the yeast *Dekkera bruxellensis*: a few keys for improving ethanol fermentation”**, o qual relata características deste importante metabólito produzido pela levedura e como sua disponibilidade é considerado um elemento chave para os rendimentos em etanol pela levedura. Nesse caso, foi utilizado o inibidor bioquímico da enzima acetaldeído desidrogenase (Ald), o dissulfiram (Dsf), composto presente no tratamento do alcoolismo crônico e já avaliado em cultivos com a levedura *S. cerevisiae*. Os resultados mostraram que existe uma escassez de acetil-CoA quando ocorre a inibição da Ald de *D*. *bruxellensis* e *S. cerevisiae,* que a adição de acetato melhora os parâmetros fisiológicos apenas de S. cerevisiae e que a utilização do Dsf melhora a eficiência fermentativa de *D. bruxellensis*.

O terceiro artigo da tese, intitulado: **“Metabolic and biotechnological insights on the analysis of Pdh bypass and acetate production in the yeast *Dekkera bruxellensis*”** aborda resultados de ensaios aeróbicos de *D. bruxellensis* utilizando, além da glicose, a fonte de carbono sacarose, açúcar industrialmente relevante. Além disso, o nitrato também foi adicionado nos cultivos, para avaliar as diversas respostas celulares com essa fonte de nitrogênio frente a inibição da enzima acetaldeído desidrogenase. Os resultados são discutidos com ênfase na importância do controle exercido pelo Pdh bypass para o metabolismo respiro-fermentativo da levedura e mostraram que a inibição da enzima acetaldeído desidrogenase aumenta a produção e rendimento em etanol da levedura *D. bruxellensis* mesmo em condições de aerobiose. Além disso, a Sacarose melhora parâmetros respiro-fermentativos de *D*. *bruxellensis* e o nitrato exerce efeito benéfico quando associado com essa fonte de carbono.

Para continuar caracterizando o metabolismo respiro-fermentativo da levedura *D. bruxellensis*, os parâmetros de crescimento e produção de metabólitos foram avaliados em condições aeróbicas, agora na presença de outras fontes de carbono. Para isso, o quarto artigo da tese é intitulado**: “The metabolism of respiring carbon sources by *Dekkera bruxellensis* and its relation with the production of acetate**”, que buscou entender aspectos da fisiologia do crescimento e produção de metabólitos utilizando agora fontes de carbono oxidativas, bem como na presença do inibidor bioquímico da produção de acetato citosólico. Parâmetros como a

assimilação de carbono, taxa de crescimento, direcionamento do carbono e expressão de genes chaves por PCR em tempo real foram avaliados. No presente artigo, concluímos que a galactose é uma fonte de carbono oxidativa no metabolismo de *D*. *bruxellensis* e que a utilização do antioxidade N-acetilcisteína melhora os crescimentos da levedura. Os resultados obtidos neste trabalho fornecem novas características do metabolismo respiro-fermentativo da levedura *D. bruxellensis* e pode ajudar no desenvolvimento de estratégias para potencialização na capacidade industrial da levedura.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Determinar a distribuição do carbono no metabolismo central de *Dekkera bruxellensis* em função do funcionamento da via de produção de acetato citosólico em diferentes combinações de fontes de carbono e de nitrogênio

2.2 OBJETIVOS ESPECÍFICOS

1. Determinar os parâmetros fisiológicos da produção de acetato e etanol por *D. bruxellensis* em condição de fermentação utilizando glicose e inibindo a enzima acetaldeído desidrogenase.

2. Analisar o perfil de expressão dos genes envolvidos na via glicolítica, fermentação etanólica, respiração celular e síntese de acetato na levedura *D. bruxellensis* em condição fermentativa.

3. Avaliar os parâmetros fisiológicos de *D. bruxellensis* em cultivos de crescimento na presença de glicose, sacarose, amônio e nitrato, inibição bioquímica de acetato citosólico e analisar o perfil de expressão gênica nessas condições.

4. Analisar parâmetros fisiológicos de *D. bruxellensis* utilizando diferentes fontes de carbono respiráveis, inibição da enzima acetaldeído desidrogenase e o antioxidante N-acetilcisteína.

5. Analisar o perfil de expressão de genes do metabolismo respiro-fermentativo de *D. bruxellensis* em resposta as condições de cultivo utilizando fontes respiráveis.

3 FUNDAMENTAÇÃO TEÓRICA

3.1 *DEKKERA BRUXELLENSIS*: CLASSIFICAÇÃO

A forma teleomórfica da levedura *Brettanomyces bruxellensis* foi descrita inicialmente pelo pesquisador Van Der Walt, quando, baseado nas suas observações de formas ascospóricas e guiado pelo código internacional de nomenclatura botânica em 1959 classificou a levedura *Dekkera bruxellensis,* representada celularmente abaixo (Figura 1), como o estado perfeito (produtor de ascósporos) da levedura *B. bruxellensis.* Esta nova nomenclatura foi uma homenagem ao Dr. N. M. Stelling-Dekker, o qual teve contribuição significativa na identificação de formas ascosporógenas de diversas leveduras (VAN DER WALT, 1964).

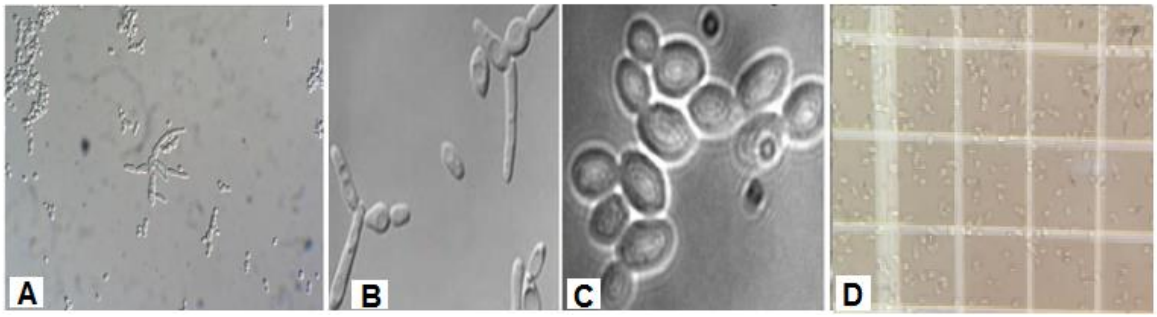
A forma anamorfa, *B. bruxellensis*, foi descrita por N. Hjelte, que estudara essa levedura durante um processo de degradação de cerveja na cervejaria Carlsberg (CLAUSSEN, 1904). Atualmente, cinco espécies do gênero *Dekkera/Brettanomyces* são aceitas: *Brettanomyces custersianus* (Van der Walt 1961); *Dekkera bruxellensis* (Van der Walt-1964); *Dekkera naardenensis* (Kolfschoten & Yarrow-1970); *Dekkera anomala* (M. Th. Smith & van Grinsven-1984) e *Brettanomyces nanus* (M. Th. Smith, Batenburg-van der Vegte & Scheffers M. Th. Smith, Boekhout, Kurtzman & O'Donnell-1994). Além disso, os gêneros *Dekkera/Brettanomyces* são utilizados como sinônimos, pois a distinção muitas vezes não é observada (OELOFSE et al, 2008). Taxonomicamente, a classificação é estabelecida da seguinte maneira: Reino *Fungi*, Filo *Ascomycota*, Subfilo *Saccharomycotina*, Classe *Saccharomycetes*, Ordem *Saccharomycetales*, Familia *Saccharomycetaceae*, Gênero *Dekkera*, Espécie *Dekkera bruxellensis* (BARNETT et al, 1990).

3.2 ASPECTOS GERAIS DA FISIOLOGIA DE *DEKKERA BRUXELLENSIS*

A levedura *D. bruxellensis* apresenta características fisiológicas similares a *Saccharomyces cerevisiae*, a principal levedura dos processos industriais. Apesar da divergência evolutiva a cerca de 200 milhões de anos (ROZPEDOWSKA et al, 2011), três aspectos das suas fisiologias convergem para a caracterização industrial

dessas leveduras no que diz respeito a produção de etanol. *D. bruxellensis e S. cerevisiae* São (I) *crabtree* positivas, fisiologia que favorece a produção de etanol mesmo em condições de aerobiose, quando o ambiente apresenta altas concentrações de açúcar; (II) conseguem suportar o alto teor alcoólico do processo fermentativo; (III) *petite* positivas, ou seja, conseguem sobreviver sem DNA mitocondrial (PISKUR et al, 2006). As pressões seletivas semelhantes fizeram com que essas leveduras compartilhassem essas características e o efeito *crabtree* é considerado a base necessária para a estratégia fazer-acumular-consumir, fundamental em habitats naturais (ROZPEDOWSKA et al, 2011). Outras características importantes que leva *D. bruxellensis a* possuir ampla utilização do ponto de vista industrial é sua capacidade de sobreviver a variações no pH (3-5) e temperatura (25-37°C). Esses parâmetros são fundamentais na competição que a levedura exerce nos ambientes industriais (BLOMQVIST et al, 2010). Apesar da faixa de pH, de maneira isolada a linhagem CBS2499 conseguiu sobreviver em uma condição com pH 2,3 (ROZPĘDOWSKA et al, 2011).

Figura 1 - Representação celular da Levedura *Dekkera bruxellensis.* A. Observação da formação de pseudomicélio na linhagem CBS11269. B. Linhagem AWRI1499. C. Estirpe industrial GDB248 em meio YPD (Yeast extract-Peptone-Dextrose) representada em microscopia de contraste de fase.D. Linhagem industrial GDB248 em meio YNB (Yeast nitrogen base) em microscopia óptica.



Fontes. (A) Blomqvist, 2011; (B) Diark.org/Brettanomyces_bruxellensis_AWRL1499; (C) Leite, 2012; (D) Teles, (2022).

Sob o ponto de vista metabólico, *D. bruxellensis* posssui a capacidade de assimilar uma viariedade de fontes de carbono, tais como: glicose, frutose, galactose, maltose, sacarose e trealose (CONTERNO et al, 2006; LEITE et al, 2016). A celobiose também é um dissacarídeo que pode ser assimilado e fermentado pela levedura (REIS et al, 2014). No ambiente industrial, uma característica importante para levedura é sua capacidade em assimilar ou co-assimilar açúcares no mosto de

fermentação, no entanto, sabe-se que de maneira geral *D. bruxellensis* reprime a utilização de outras fontes de carbono quando a glicose está presente no meio, mecanismo denominado repressão catabólica pela glicose (GCR), caracterizado pelo acúmulo dos componentes da via glicolítica: glicose-6-fosfato ou frutose-1,6-bisfosfato (PEETERS et al, 2017). No entanto, estudos mostraram que esse mecanismo de repressão se apresenta mais aliviado em *D. bruxellensis*, em comparação a *S. cerevisiae* (LEITE et al, 2016; TELES et al, 2018). Além disso, existe uma diversidade na capacidade de assimilação de fontes de carbono entre diversas linhagens de *D. bruxellensis*, e a velocidade assimilatória é maior em cepas provenientes da produção industrial de bioetanol, em comparação as isoladas na indústria vinícola (SILVA et al, 2019). Estes e outros aspectos relacionados à ampla capacidade de *D. bruxellensis* no ambiente industrial serão abordados no artigo sobre a biotecnologia da levedura: The biotechnological potential of the yeast *Dekkera bruxellensis.*

3.3 METABOLISMO CENTRAL DO CARBONO EM LEVEDURAS

A levedura *S. cerevisiae* é bastante utilizada como modelo para o estudo do metabolismo dependente do carbono. A capacidade em crescer rapidamente em uma variedade de fontes disponíveis é um dos fatores essenciais (BARNETT et al, 1983). De acordo com essa habilidade, as reações envolvidas no metabolismo celular estão relacionadas à conversão de recursos e energia para biossíntese de compostos celulares. Os fluxos, por exemplo, retratam a atividade metabólica da rede em questão, sendo influenciados por vários aspectos, tais como parâmetros transcricionais, traducionais, pós-traducionais e interações entre proteínas (NIELSEN, 2003).

A principal via que fornece energia para a maioria dos organismos é a via glicolítica ou via de Embden-Meyerhof e Parnas (EMP) (Figura 2). No catabolismo da glicose, é necessário que ocorra uma velocidade de fluxo adequada para que compostos intermediários não se acumulem, acúmulo esse que leva a taxas baixas de crescimento, desequilíbrio na produção de ATP e pode ocasionar morte celular (PLANKÉ et al, 2014; HUANG et al, 2015; TEUSINK et al, 2000).

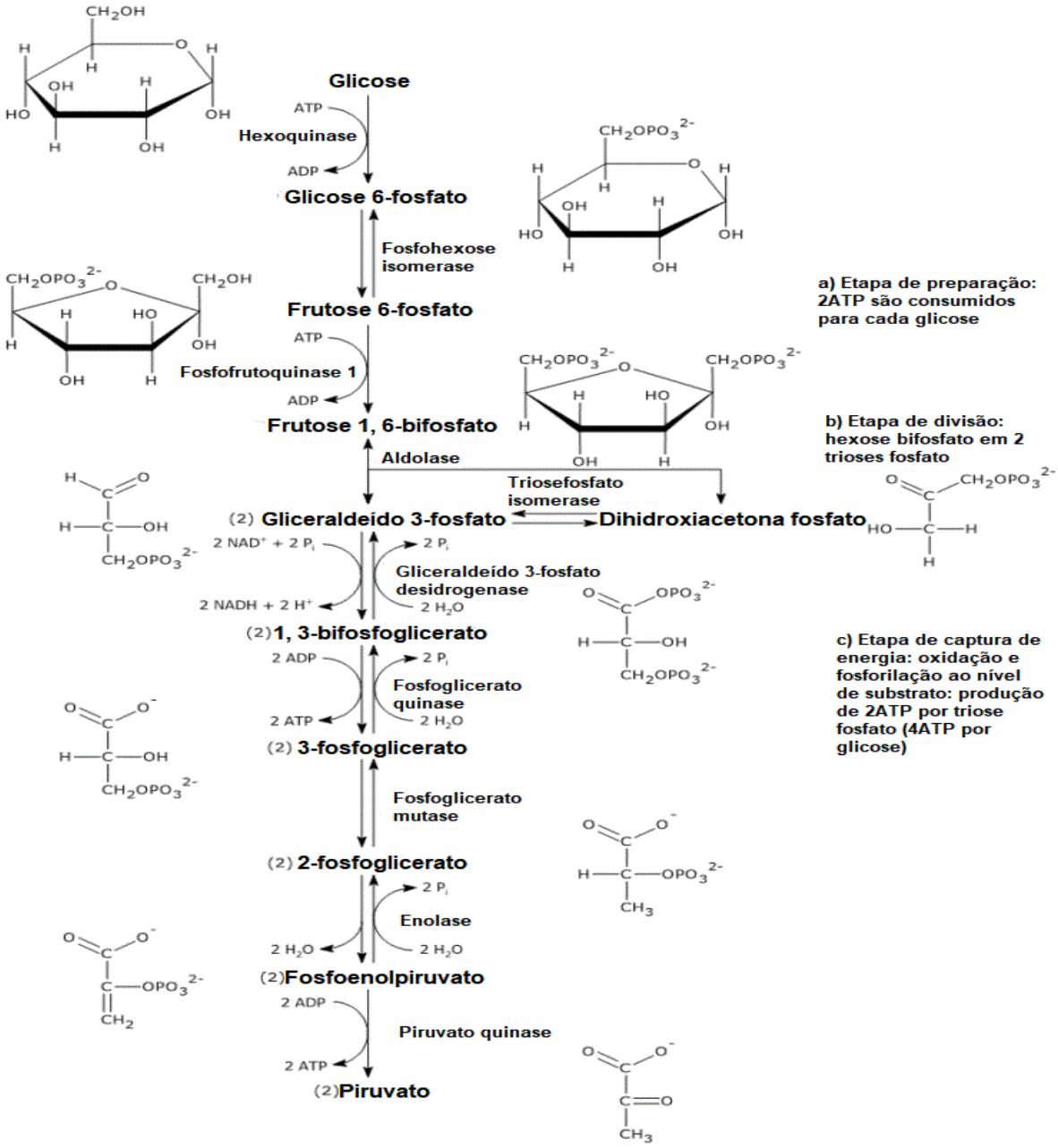
A etapa inicial da glicólise é caracterizada pela fosforilação da glicose, que pode ser catalizada por três enzimas: glicoquinase (Glk1) e hexoquinase 1 (Hxk1),

com os genes reprimidos em altas concentrações de glicose, e hexoquinase 2 (Hxk2), com alta expressão gênica nessas condições (VAN DE BRINK et al, 2008). Posteriormente, a fosfoglicose isomerase (Pgi) converte glicose-6-fosfato em frutose-6-fosfato e este é convertido em frutose-1-6-bifosfato em uma reação irreversível catalisada pela enzima fosfofrutoquinase (Pfk1), que foi caracterizada como importante controladora do fluxo glicolítico (VAN DE BRINK et al, 2008). A frutose-1-6-bifosfato agora é clivada em dihidroxicetona fosfato (DHAP) e gliceraldeído-3-fosfato (G3P) pela enzima aldolase (Fba) e a DHAP então é isomerizada em gliceraldeído-3-fosfato (G3P) pela triose fosfato isomerase (Tpi) (HARRIS; HARPER, 2015).

As etapas seguintes são caracterizadas pela formação de importantes intermediários: 1-3-bifosfoglicerato, 3-fosfoglicerato, 2-fosfoglicerato e fosfoenolpiruvato, através das enzimas gliceraldeído-3-fosfato desidrogenase (Gapdh), fosfoglicerato quinase (Pgk), fosfoglicerato mutase (Gpm), e enolase (Eno), respectivamente. Por fim, o produto da via glicolítica, o piruvato, é formado através da atividade da enzima piruvato quinase (Pyk) (figura 2) (VAN DE BRINK et al, 2008). Além disso, nessa etapa, denominada de estágio de captura de energia, a oxidação do gliceraldeído-3-fosfato gera NADH e a fosforilação ao nível de substrato a partir do 1-3-bifosfoglicerato e do fosfoenol piruvato produz ATP (HARRIS; HARPER, 2015). Portanto, a equação química da glicólise é a seguinte: Glicose + 2 NAD^+ + 2 ADP + 2 Pi --> 2 Piruvato + 2 NADH + 2 H^+ + 2 ATP + 2 H_2O.

Com a produção de piruvato, o fluxo pode seguir dois principais caminhos: em condições limitantes de glicose e aerobiose a maioria sofre oxidação a acetil-CoA pela piruvato desidrogenase (Pda1) (FLIKWEERT et al, 1999). Já em condições de excesso de glicose e anaerobiose, o piruvato é majoritariamente descarboxilado a acetaldeído através da atividade da enzima piruvato descarboxilase (Pdc1) (VAN DE BRINK et al, 2008).

Figura 2 - Etapas da via glicolítica: preparação, divisão e captura de energia. ADP: adenosina difosfato; ATP: adenosina trifosfato; NAD^+: nicotinamida adenina dinucleotídeo (forma oxidada); NADH: nicotinamida adenina dinucleotídeo (forma reduzida).



Fontes: adaptado de Harris; Harper, 2015 e Tazzini, 2018.

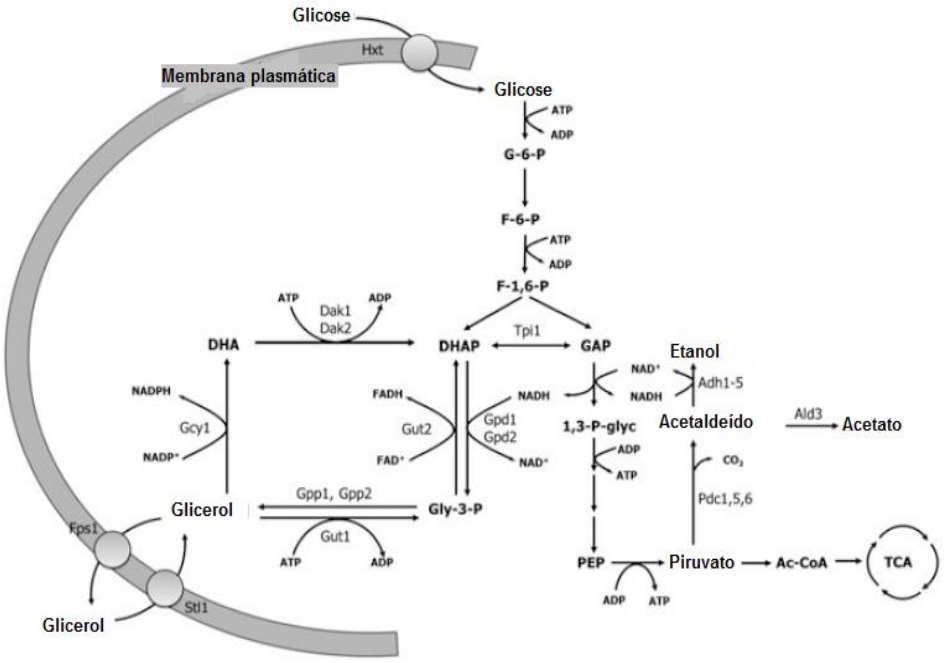
3.4 METABOLISMO FERMENTATIVO E PRODUÇÃO DE GLICEROL

O processo biológico realizado por leveduras, bactérias ou outros microrganismos que degrada o açúcar e converte em etanol e dióxido de carbono é denominado fermentação alcoólica (Figura 3). Esse processo pode ser relizado tanto

em aerobiose (fermentação aeróbia) como em anaerobiose (fermentação anaeróbia) (SABLAYROLLES, 2009; STANBURY et al, 2013).

Na degradação da glicose, sucessivas reações catabolizam a glicose em duas moléculas de ácido pirúvico (piruvato) com posterior redução e produção de duas moléculas de etanol e formação de $2CO_2$ (HUANG et al, 2015). O rendimento do processo fermentativo é de 2 mols de ATP por mol de glicose e o balanço redox é restabelecido através da regeneração do NAD^+ durante o processo redutivo do acetaldeído para etanol, catalisado pela enzima álcool desidrogenase (figura 3) (Adh) (CIANI et al, 2008).

Figura 3 - Fermentação alcoólica e metabolismo do glicerol em *S. cerevisiae.* G-6-P glicose-6-fosfato; F-6-P frutose-6-fosfato; F-1,6-P frutose-1,6-bisfosfato; DHAP dihidroxiacetona fosfato; DHA dihidroxiacetona; GAP gliceraldeído-3-fosfato; G-3-P glicerol-3-fosfato; 1,3-P-glyc 1,3-bisfosfoglicerato; PEP fosfoenolpiruvato; Ac-CoA acetil coenzima A; TCA ciclo do ácido tricarboxílico; Tpi1 triose fosfato isomerase; Adh1-5 Álcool desidrogenases; Pdc1,5,6 piruvato descarboxilases; Ald3 aldeído desidrogenase; Gpd1, Gpd2 glicerol-3-fosfato desidrogenases; Gpp1, Gpp2 glicerol-3-fosfato fosfatases; Gut1 glicerol quinase; Gut2 glicerol-3-fosfato desidrogenase; Gcy1 glicerol desidrogenase; Dak1, Dak2 dihidroxiacetona quinase; Hxt, Stl1, Fps1 Transportadores de membrana.



Fonte: adaptado de Semkiv et al, 2017.

Em *S. cerevisiae,* algumas situações podem fazer com que exista a necessidade de regenerar o NAD^+ alternativamente através da produção de glicerol (Figura 2), entre elas a indisponibilidade de acetaldeído, a baixa atividade da enzima piruvato descarboxilase (Pdc) ou a alta atividade da enzima acetaldeído desidrogenase (Ald) (CIANI et al, 2008). A via fermentativa ou a via de produção de glicerol dependem da produção de dois produtos intermediários, respectivamente: gliceraldeído-3-fosfato (GAP) e a diidroxiacetona fosfato (DHAP) (figura 3) (SEMKIV et al, 2017). No caso da síntese de glicerol, ela é realizada através de duas reações a partir da molécula diidroxiacetona fosfato, envolvendo as enzimas glicerol-3-fosfato desidrogenase (Gpd) e glicerol-3-fosfato fosfatase (Gpp), sendo a primeira responsável pela regeneração do NAD^+ (SEMKIV et al, 2017).

3.5 VIA DA PROTEÍNA QUINASE A E METABOLISMO RESPIRATÓRIO

A proteína quinase A (PKA) é considerada a mais conservada proteína responsiva ao transdutor de sinal adenosina 3',5'-monofosfato cíclico (cAMP) nas células dos eucariotos, de maneira que sua ligação a subunidade reguladora de PKA promove uma cascata de reações que fosforila centenas de alvos (STEWART-ORNSTEIN et al, 2017). Em *S. cerevisiae*, PKA é uma holoenzima tetramérica e possui subunidades regulatórias e catalíticas. O gene BCY1 codifica a subunidade reguladora, e os genes TPK1, TPK2 e TPK3 a subunidade catalítica (TODA et al, 1987a, b).

Em leveduras, a glicose extracelular, aspectos do crescimento e condições de estresse exercem papel fundamental na regulação dos níveis de cAMP nas células (ZAMAN et al, 2009). Portanto, a regulação da atividade do complexo PKA está diretamente relacionado às variações ambientais que modificam os níveis de cAMP (ZAMAN et al, 2009). PKA fosforila diretamente proteínas relacionadas à maquinaria mitocondrial, proteínas de autofagia, via glicolítica e uma variedade de fatores de transcrição (PTACEK et al. 2005).

Quando os recursos nutricionais estão em grandes concentrações na célula, a elevação nos níveis de cAMP cíclico promove a ativação da via PKA que auxilia na melhora do fluxo glicolítico, promovendo imediato crescimento celular (STEWART-ORNSTEIN et al. 2017). Por outro lado, quando o aporte nutricional se apresenta

escasso, a diminuição no cAMP inibe a via PKA, que entre outras respostas resulta em ativação de fatores relacionados ao estresse, como é o caso do fator de trancrição Msn2 (STEWART-ORNSTEIN et al. 2017). Com o auxílio da proteína Ras, no entanto, a atividade da PKA pode se recuperar mesmo em condições desfavoráveis (NIKAWA et al. 1987a). Portanto, a via Ras/cAMP/PKA exerce papel preponderante no controle do metabolismo celular das leveduras, resistência a estresse e proliferação celular (THEVELEIN, 1994; THEVELEIN et al, 2000).

3.5.1 Fisiologia mitocondrial

As mitocôndrias desempenham importantes funções, sendo reconhecidas não apenas como a força motriz celular, mas também relacionadas com diversos eventos fisiológicos, tais como: sinalização celular, proliferação, geração de espécies reativas de oxigênio, envelhecimento, doença e morte (PICARD et al, 2016). A fisiologia mitocondrial está intimamente relacionada com a dinâmica nutricional da célula e suas vias de sinalização, de maneira que o controle das mitocôndrias se adapta as diferentes variações intra e extracelulares, promovendo mudanças funcionais e estruturais na organela (CHANDEL, 2014; YUN E FINKEL, 2014).

Na levedura *S. cerevisiae,* a disponibilidade de oxigênio possui forte influência na morfologia mitocondrial (AUNG-HTUT et al, 2013), uma vez que em aerobiose ocorre o aumento substancial das suas estruturas tubulares (ROGOV et al, 2018). Além disso, a fase de crescimento celular e a fonte de carbono utilizada também estabelece influência morfológica nas mitocôndrias (LAM et al, 2011; AUNG-HTUT et al, 2013). Por outro lado, a fragmentação da organela pode ocorrer quando as células das leveduras estão sob estresse oxidativo (LEFEVRE et al, 2012; NGUYEN et al, 2014). Neste caso, se o estresse for um evento grave, as mitocôndrias emitem a chamada comunicação retrógrada, que pode promover a degradação em um processo denominado de mitofagia (KANKI et al, 2015; WEI et al, 2015).

O ATP (adenosina trifosfato) é produzido pelas mitocôndrias através do sistema de fosforilação oxidativa (OXPHOS), localizado na membrana mitocondrial interna. Esse processo oxidativo ocorre por meio de quatro complexos que permitem transferências de elétrons através dos substratos nicotinamida adenina dinucleotídeo (NADH) e flavina adenina dinucleotídeo ($FADH_2$), com o aceptor final dos elétrons sendo o O_2, gerando H_2O. A porção fosforiladora do processo é

realizada através do complexo ATP sintase, que utiliza o gradiente de prótons e produz ATP (BOUCHEZ; DEVIN, 2019).

S. cerevisiae não possui o complexo I da cadeia respiratória (BAKKER et al. 2001), no entanto, estão presentes desidrogenases na membrana mitocondrial interna (DE VRIES, 1987). O resultado é que em *S. cerevisiae* o bombeamento de prótons é realizado apenas pelos complexos III e IV (BOUCHEZ; DEVIN, 2019). Na fosforilação oxidativa, aproximadamente 10 prótons são bombeados pela oxidação do NADH e 6 pelo $FADH_2$ e os elétrons presentes nos equivalentes redutores são transferidos pelos complexos respiratórios (NATH; VILLADSEN, 2015). Essa quantidade de prótons é capaz de gerar no máximo 2,5 ATP para cada NADH e 1,5 ATP para cada $FADH_2$ (ZHANG; LIN, 2020).

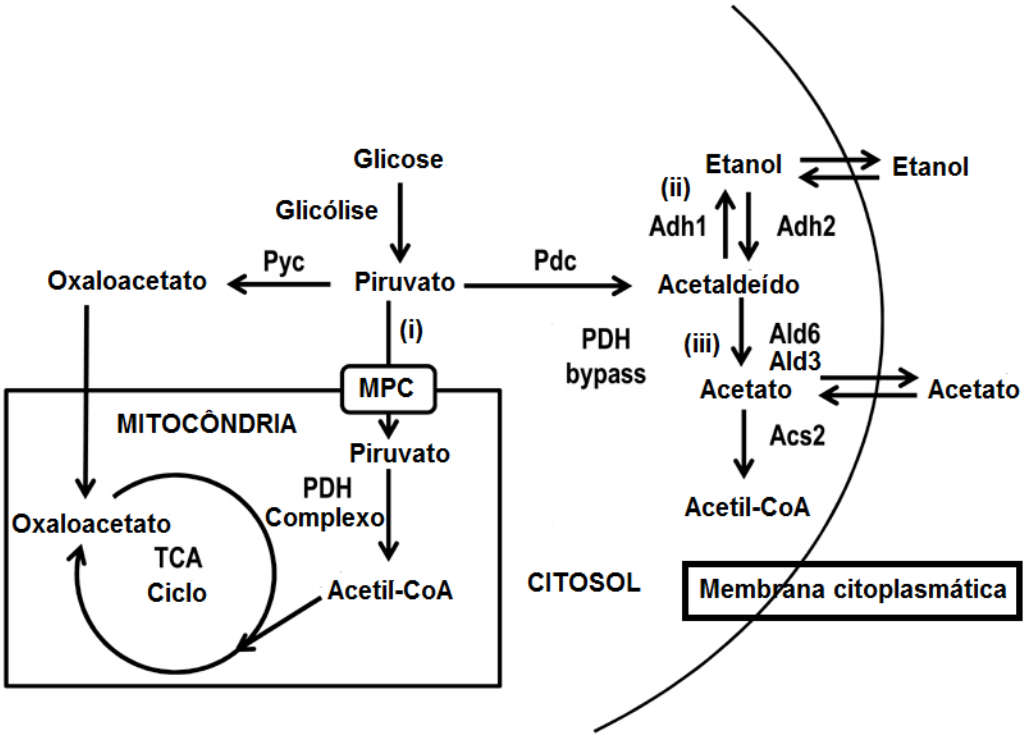
3.6 METABOLISMO DO ACETATO EM LEVEDURAS

O ácido acético é um importante produto químico e sua produção industrial é realizada principalmente por bactérias do gênero *Acetobacter (*FREER, 2002). Sob condições aeróbicas*,* a levedura *D. bruxellensis* geralmente possui a capacidade de produção de ácido acético e isso está relacionado principalmente ao efeito Custers (CUSTERS, 1940), caracterizado por uma parada no metabolismo fermentativo da levedura devido à tendência em utilizar o NAD^+ para oxidar o acetaldeído a acetato (Figura 4) (SCHEFFERS, 1966; WIJSMAN et al, 1984). Outras leveduras conseguem se organizar do ponto de vista redox e reoxidam o NADH resultante através da produção de glicerol, no entanto, a produção desse composto é insuficiente para fechar o balanço redox em células de *D. bruxellensis* (VAN DIJKEN; SCHEFFERS, 1986; PRONK et al, 1996). É importante salientar que a quantidade de produção de acetato por *D. bruxellensis* se enquadra como linhagem-dependente, bem como de acordo com a fonte de carbono utilizada (FREER, 2002).

Em S. cerevisiae, a produção de ácido acético normalmente é realizada no início da fermentação alcoólica da produção vinícola (ALEXANDRE et al,1994), sendo afetada por diferentes fatores, tal como a disponibilidade de nitrogênio (BARBOSA et al. 2009; VILANOVA et al, 2007), composição do mosto de uva (DELFINI E COSTA 1993), concentração de açúcar (RADLER, 1993), bem como a linhagem utilizada (ERASMUS et al, 2004; TORRENS et al, 2008). Além da

produção de acetato via oxidação do acetaldeído pela acetaldeído desidrogenase (Ald) (figura 4) (FLORES et al, 2000), as leveduras também podem utilizar o acetato como única fonte de carbono para geração de energia e biomassa celular em condições aeróbicas (Figura 5) (BARNETT et al, 1990; SCHÜLLER, 2003).

Figura 4 **-** Vias metabólicas a partir da utilização da glicose. O esquema representa a (i) descarboxilação oxidativa do piruvato a acetil-CoA, (ii) redução do acetaldeído a etanol e (iii) oxidação do acetaldeído a acetato. Acs, acetil-CoA sintase; Adh, álcool desidrogenase; Ald, aldeído desidrogenase; MPC, transportador mitocondrial de piruvato; Pdc, piruvato descarboxilase; PDH, piruvato desidrogenase; Pyc, piruvato carboxilase.



Fonte: adaptado de Orlandi et al. 2014

Como é uma fonte não fermentativa, o acetato entra na célula e é convertido em acetil-CoA, que pode entrar no ciclo do ácido cítrico (TCA) ou no ciclo do glioxilato (FLORES et al, 2000). Além disso, na presença de acetato como única fonte de carbono, as leveduras expressam genes envolvidos na biogênese mitocondrial, tais como os relacionados à formação e estabilização de complexos

oxidativos, bem como alguns genes que codificam proteínas ribossômicas (PAIVA et al, 2004).

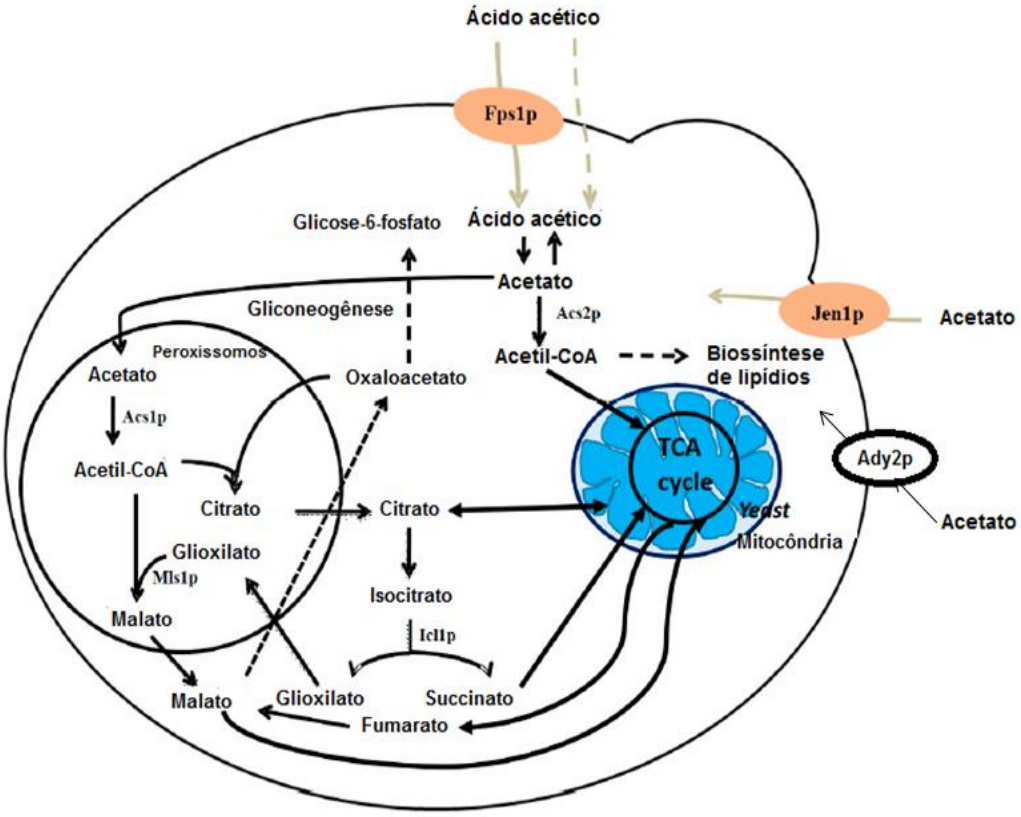
O transporte de acetato pode ser realizado em *S. cerevisiae* por uma permease de fontes respiráveis, codificado pelo gene JEN1 (Figura 5). Além de acetato, o transportador Jen1p pode realizar sua função quando a levedura cresce em piruvato, lactato ou propionato como fonte única de carbono (CASAL et al,1999). Nesta levedura, o transporte é dependente da GCR, no entanto, em cultivos em quimiostato com a presença de glicose e ácido acético, quando a primeira fonte de carbono se encontra em condições mínimas no meio, a repressão é aliviada e poderá ocorrer co-consumo (DOS SANTOS et al, 2003).

Adicionalmente, foi identificada a proteína de membrana Ady2p, dita como essencial ao transporte ativo de acetato em células de *S. cerevisiae* (PAIVA et al, 2004), e os fatores de transcrição Msn2p/Msn4p com sítios de ligação ao promotor do gene ADY2 (ZHANG et al, 2017). Estes fatores de transcrição estão associados à resposta geral ao estresse (MARTINEZ-PASTOR et al, 1996), o que indica a relação responsiva de ADY2 ao estresse ácido (ZHANG et al, 2017). Em *S. cerevisiae* sujeita a repressão pela glicose, a entrada de ácido acético na célula também pode ocorrer por difusão simples (CASAL et al, 1996) (figura 5), além disso, a forma não-dissociada pode ser mediada pela aquagliceroporina Fps1p (VILELA-MOURA, 2010).

Em relação a informação genética, a internalização de acetato em *S. cerevisiae* pode ser realizada pelos transportadores codificados pelos genes FPS1, JEN1 e ADY2 (SIMPSON-LAVY; KUPIEC, 2019). Os principais genes do metabolismo do acetato dentro das células de *S. cerevisiae* codificam as enzimas necessárias para produção do composto tanto no citoplasma celular como nas mitocôndrias. Os genes ALD2, ALD3 e ALD6 codificam as isoformas citoplasmáticas e os genes ALD4 e ALD5 as isoformas mitocondriais das acetaldeído desidrogenases (ARANDA; OLMO, 2003). Além disso, recentemente foi identificado que o gene ACH1 produz a transferase que converte acetyl-CoA em acetato também nas mitocôndrias (CHEN et al, 2015).

Figura 5 **-** Metabolismo do acetato em leveduras a partir das formas de transporte para o interior celular. As setas internas representam as vias metabólicas e as setas com início extracelular

representam sistemas de transporte. Fps1p: aquagliceroporina responsável pelo transporte da forma não-dissociada de ácido acético pela membrana celular. Linha marrom pontilhada: entrada de ácido acético por difusão simples. Jen1p: transportador de lactato que possui a capacidade de transportar acetato em *S. cerevisiae*. Ady2p: proteína responsável pelo transporte ativo de acetato em células de *S. cerevisiae*.



Fonte: adaptado de Vilela-Moura, 2010.

3.6.1 Inibição da produção de acetato citosólico pelo dissulfiram

O Dissulfiram (DSF) é um fármaco utilizado no tratamento do alcoolismo crônico em humanos que impede a conversão de acetaldeído em ácido acético através da inibição da enzima acetaldeído desidrogenase (Aldh, EC 1.2.1.10) (MIREK et al, 2012). Em estudos anteriores, os níveis de atividade da enzima Aldh em cultivos com a cepa *Pythium insidiosum* caiu de 443.2 nmol NADH/min/ml para 6.8 nmol NADH/min/ml quando na presença do DSF *(*KRAJAEJUN et al, 2019*).*

A utilização do DSF é um mecanismo complexo e com vários efeitos, como na atuação adicional como inibidor da enzima superóxido dismutase (Sod) (HEIKKILA et

al, 1976; MARIKOVSKY et al, 2003; KELNER e ALEXANDER, 1986), ocasionando elevação de radicais superóxidos em células humanas (Cen et al, 2002). Além disso, as enzimas urease (Urea), carbamato quinase (Ck) e dopamina beta-hidroxilase também são inibidas (Dbh) (GALKIN et al, 2014; DÍAZ-SÁNCHEZ et al, 2016; GAVAL-CRUZ; WEINSHENKER, 2009).

Em *S. cerevisiae*, o DSF promove uma série de efeitos celulares e fisiológicos, entre eles, desintegração mitocondrial, diminuição no conteúdo intracelular de glutationa reduzida (GSH) e diminuição na atividade metabólica das células da levedura (MIREK et al, 2012). Além disso, a cepa mutante para o gene SOD é mais sensível aos efeitos do DSF em comparação a linhagem selvagem (MIREK et al, 2012). Os microrganismos *Staphylococcus aureus* (LONG, 2017), *Pseudomonas aeruginosa* (VELASCO-GRACÍA et al. 2003), *Giardia Lamblia* (GALKIN et al, 2014), *Mycobacterium tuberculosis* (HORITA et al, 2012) e non-tuberculous *mycobacteria (*DAS et al*, 2019)* também já foram inibidos pelo DSF. Quando na presença de GSH, o DSF é convertido em S-metil-N,N-dietiltiocarbamato (MeDTC) sulfóxido, que é considerado o metabólito ativo do composto (PIKE et al, 2001). A presente tese mostra efeitos fisiológicos e genéticos da inibição da enzima acetaldeído desisdrogenase na levedura industrial *D. bruxellensis.*

4 ARTIGO I - THE BIOTECHNOLOGICAL POTENTIAL OF THE YEAST *DEKKERA BRUXELLENSIS*

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Abstract

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

discuss the advantages and challenges that must be overcome in order to take advantage of the full biotechnological potential of this yeast.

Keywords: non-conventional yeast; industrial application; industrially relevant metabolites; second-generation ethanol; strain improvement.

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

Dekkera/Brettanomyces bruxellensis (currently, both terms are used as synonyms and we shall adopt the name *Dekkera* henceforth) is a yeast species found in close contact with industrial fermentative processes, especially in winemaking, brewing and in bioethanol production plants (de Souza Liberal et al. 2007; Roder et al. 2007; Oelofse et al. 2008). In fact, *D. bruxellensis* passed through the twentieth century as the main cause for wine spoilage worldwide, mostly by producing volatile phenolic compounds involved with unpleasant aromas (“horse sweat”, “corral”, “wet animals”, etc) associated with the “Brett” character (Chatonnet et al. 1992; Licker et al. 1998; Lentz and Harris, 2015; Crauwels et al. 2017; Felipe-Ribeiro et al. 2018). In spite of being mostly related with winemaking, its first report dates back to early 1900’s, when a new species was found in British beers and the term *Brettanomyces* was proposed as a generic name in reference to its origin, a “British brewing fungus” (Claussen, 1904). In the following decades, a yeast isolated from Belgian Lambic beer was then classified as *Brettanomyces bruxellensis* and the genus *Dekkera* was later introduced to describe strains able to produce ascospores (Kufferath and van Laer, 1921; van der Walt, 1964). After some reclassifications, currently, only five species are recognized in the *Dekkera*/*Brettanomyces* group: *D. bruxellensis*, *D. anomala*, *B. custersianus*, *B. nanus* and *B. naardenensis* (Kurtzman et al. 2011). Since *D. bruxellensis* is considered a major contaminant in wine industry, its presence, role and importance has been subject of previous review articles and readers should be referred to these works for additional information (Smith and Divol, 2016; Agnolucci et al. 2017; Berbegal et al. 2018). Moreover, other reviews dealt with different aspects of *D. bruxellensis* (Curtin et al. 2014; Blomqvist and Passoth, 2015; Radecka et al. 2015; Steensels et al. 2015). Herein, we shall

focus on the role of this yeast in other industrial sectors, as well as its potential application in these processes.

The production of Lambic beer is, for instance, an industrial field in which *D. bruxellensis* plays an important role, especially during the maturation stage, being essential for the flavour composition (Spitaels et al. 2014; de Roos and de Vuyst, 2018). Moreover, several studies have consistently reported the presence of different strains of *D. bruxellensis* with particular contributions to sensorial properties of many other fermented beverages, such as cider, kombucha, kefir and tequila (Lachance et al. 1995; Teoh et al. 2004; Gray et al. 2011; Albertin et al. 2014; Spitaels et al. 2015; Curtin et al. 2015; Longin et al. 2016). In the past decade, *D. bruxellensis* has also drawn attention due to its involvement in bioethanol production (de Souza Liberal et al. 2007; Passoth et al. 2007). In the latter scenario, this yeast competes with *Saccharomyces cerevisiae* for the sugar present in the industrial substrate and it is mostly faced as a contaminant micro-organism (de Souza Liberal et al. 2007; de Barros Pita et al. 2011). It is interesting to note that despite being regularly pointed as a contaminant, *D. bruxellensis* has also the potential to be industrially employed (de Souza Liberal et al. 2007; Passoth et al. 2007; de Barros Pita et al. 2011). This apparent contradiction lies on the fact that this yeast presents useful physiological and genetic traits, not only for fermentative processes but also for other industries. In fact, *D. bruxellensis* is able to produce ethanol by alcoholic fermentation, as well as other industrially relevant compounds (de Souza Liberal et al. 2007; de Barros Pita et al. 2011; Rozpedowska et al. 2011; de Barros Pita et al. 2013a). For instance, as we will see in the following sections, its acetogenic profile might be exploited in a large scale for acetic acid production. Moreover, its capacity to assimilate sugars from lignocellulosic material leads the way to a possible use in second-generation ethanol production. These features, once properly explored, might increase both the contribution and the industrial role of *D. bruxellensis*. Finally, strain improvement strategies are underway, aiming to enhance favourable properties and to overcome its drawbacks, in order to take advantage of the full biotechnological potential of this yeast.

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

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

As mentioned above, in spite of being regarded as NCY, some features presented by *D. bruxellensis* are rather interesting, especially in bioethanol production processes. For instance, under typical industrial conditions, such as high sugar concentrations in fermentation tanks, *D. bruxellensis* has the capacity to produce ethanol even in presence of oxygen. This observation reflects the so-called Crabtree effect, which is certainly its most advantageous trait (van Dijken and Scheffers, 1986; Piskur et al. 2006; Procházka et al. 2010). In fact, *D. bruxellensis* frequently presents ethanol yields similar to those of *S. cerevisiae.* However, the major problem associated with *D. bruxellensis* in bioethanol production plants is a decreased volumetric productivity (Table 1). This issue mostly relies on a slow sugar consumption rate, a fact that has also been reported even when fermentation assays lasted for long periods (Basílio et al. 2008; Blomqvist et al. 2010; Galafassi et al. 2011; Pereira et al. 2012). For instance, in sugarcane molasses *D. bruxellensis* presents a sucrose consumption rate 50% slower than *S. cerevisiae* (Table 1; Pereira

et al. 2014). Since *D. bruxellensis* competes with *S. cerevisiae* for the industrial substrate, this feature ultimately leads to large economic losses (de Souza Liberal et al. 2007; Basílio et al. 2008). Therefore, understanding how *D. bruxellensis* handles industrial sugars is an important challenge in order to explore its maximum fermentative capacity and increase the volumetric productivity.

Oxygen availability also seems to influence the fermentative capacity of *D. bruxellensis.* While oxygen limitation seems to favour alcoholic fermentation (Table 1), under aerobic conditions, *D. bruxellensis* presents tendency to an oxidative metabolism, which increases acetic acid production to levels closer to those of ethanol (Freer, 2002; Aguilar Uscanga et al. 2003; Dequin et al. 2003; Rozpędowska et al. 2011; Leite et al. 2013; Teles et al. 2018). However, since acetic acid has a great importance as a chemical compound, the acetogenic profile of *D. bruxellensis* might be used in this process, an alternative that has not yet been explored. This possibility also relies on the fact that this yeast is able to endure the stressful industrial conditions, such as pH variations (Freer et al. 2003). The metabolic potential of *D. bruxellensis* makes it capable of being used also in pharmaceutical and food industries. In this sense, a new application field for this yeast is related to the extraction of bioactive compounds, such as resveratrol (Kuo et al. 2017). In this case, *D. bruxellensis* presented the highest bioconversion rate of this compound (through the specific activity of β-glucosidase enzyme) in comparison with the utilization of commercial enzyme or acid hydrolysis (Kuo et al. 2017). The use of resveratrol is based on several biological activities, such as antimicrobial, antiviral, cancer cell inhibition, antioxidant, menopause-relieving and anti-aging properties (Kaeberlein, 2010; Baur et al. 2006). Moreover, *D. bruxellensis* is linked to the production of natural sweeteners from mogrosids extracted from *Siraitia grosvenorii*. This yeast is capable of converting mogroside V to siamenoside I, considered to be the sweetest and most preferred natural sweetener among mogrosides (Wang et al. 2018). This application might decrease the use of artificial ones, which have been reported to cause type 2 diabetes (Fagherazzi et al. 2013) and cancer (Soffritti et al. 2006).

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

Condition	Observation	Yeast	Y_{etoh} (g g^{-1})	Volumetric productivity (g L^{-1} h^{-1})	References
Oxygen-limited fermentation of synthetic medium (glucose as carbon source unless otherwise stated)	None	Sc	0.50	1.10	Peña-Moreno et al. (2019)
		Db	0.45[b]	0.89	
		Sc	0.43	0.25	Blomqvist et al. (2012)
		Db	0.44[b]	0.14	
		Db	0.45	0.15	Blomqvist et al. (2010)
		Db	0.44	0.08	Galafassi et al. (2011)
		Db	0.33	1.56	Teles et al. (2018)
		Db	0.37[b]	0.22	de Barros Pita et al. (2013a)
	Sucrose replacing glucose as carbon source	Db	0.43[b]	0.56	
	Aldehyde dehydrogenase blocked by disulfiram	Db	0.49	1.49	Teles et al. (2018)
	Sucrose replacing glucose as carbon source	Sc	0.32	19.24[a]	de Souza Liberal et al. (2007)
	Sucrose replacing glucose as carbon source	Db	0.32	9.45[a]	
	Sucrose replacing glucose as carbon source	Sc	0.47	18.73[a]	da Silva Filho et al. (2005)
Oxygen-limited fermentation of industrial substrates	Sugar cane molasses	Sc	0.44	9.00	Pereira et al. (2014)
		Db	0.45	4.50	
	Sugar cane juice	Sc	0.48	5.83	Pereira et al. (2012)
		Db	0.45	1.58	
Moderate aeration in continuous cultivation in glucose	Synthetic medium	Db	0.41	0.06	Blomqvist et al. (2012)
		Sc	0.41	0.06	
Aerobic batch cultivations in the (glucose)	Defined minimal medium	Db	0.32	0.04	Rozpedowska et al.. (2011)
	Synthetic minimal medium	Db	0.13[b]	0.04	Galafassi et al. (2013)
Aerobic C-limited chemostat	Pulse with glucose	Db	0.16	0.25	Leite et al. (2013)
Anaerobic batch cultivations in glucose	Synthetic minimal medium	Db	0.35[b]	0.04	Galafassi et al. (2013)
	Synthetic mineral medium	Db	0.45[b]	0.22	Peña-Moreno et al. (2019)
	Synthetic mineral medium	Db	0.43[b]	0.21	
	Defined minimal medium	Db	0.34	0.05	Rozpedowska et al. (2011)

[a]Fermentation assays with high initial cell densities

[b]Fermentation assays in which nitrate was used as sole nitrogen source or combined with ammonium

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

Industrially relevant metabolic traits and genetic improvement of strains

The availability of genome sequencing data shed some light on the genetic background for the metabolic capacity of several strains of *D. bruxellensis* and revealed a high genotypic and ploidy variation (Woolfit et al. 2007; Piskur et al. 2012; Curtin et al. 2012; Valdes et al. 2014; Borneman et al. 2014; Crauwels et al. 2015b;

Fournier et al. 2017; Avramova et al. 2018; Tiukova et al. 2019). These genomic analyses also showed that *D. bruxellensis* presents particular characteristics and possible adaptive advantages to industrial substrates, such as a wide capacity to assimilate nutrients. For instance, this yeast harbours genes coding for enzymes involved with the utilization of xylose, L-arabinose and cellobiose, which are not naturally fermented by *S. cerevisiae* (Woolfit et al. 2007; Curtin et al. 2012; Godoy et al. 2017). We will detail the importance of these sugars in the following section. Moreover, *D. bruxellensis* presents a gene cluster involved in the assimilation of nitrate, a nitrogen source often found in sugarcane juice (Woolfit et al. 2007; de Barros Pita et al. 2011). Since *S. cerevisiae* cannot use this compound, this was an interesting discovery and, in fact, nitrate was later pinpointed as an adaptation factor for *D. bruxellensis* in a competition with *S. cerevisiae* in fuel ethanol plants (de Barros Pita et al. 2011), as it was previously observed (Abbott et al. 2005; de Souza Liberal et al. 2007; Passoth et al. 2007). Nitrate was also involved with an increased fermentative capacity of *D. bruxellensis*, especially when associated with ammonium and limiting or absent oxygen (Table 1), possibly by eliminating the Custer effect (de Barros Pita et al. 2011; Galafassi et al. 2013; Peña-Moreno et al. 2019). In spite of that, it is important to state that nitrate assimilation is not found in all strains, as well as it was not crucial for CBS11270 fermentative performance (Blomqvist et al. 2012; Borneman et al. 2014).

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

ethanol production and involved the use of Disulfiram, a drug that inhibits the cytosolic acetaldehyde dehydrogenase (Teles et al. 2018). In this study, enzyme inhibition resulted in a diverted traffic of cytosolic acetaldehyde exclusively to ethanol production.

Since *D. bruxellensis* already presents genetic characteristics that are valuable to several industrial sectors, how can this species be further explored? Strategies aiming to develop genetically modified strains are emerging, yet in an early stage, in order to allow or improve the function of its metabolic pathways. Methods such as electroporation, lithium acetate/PEG (LiAc/PEG) and spheroplast transformation have been adapted for *D. bruxellensis*, with efficiency similar to *S. cerevisiae*, yet with random and unaddressed integration (Miklenic et al. 2013; Miklenic et al. 2015). Fortunately, experiments joining the treatment with LiAc and electroporation (with a control in key steps) increased the transformation efficiency (Miklenić et al. 2015). Later on, gene transformation cassettes for *D. bruxellensis* have also been developed, providing greater efficiency in strain transformation by achieving antibiotic resistant and fluorescent protein markers insertion (Varela et al. 2018). Moreover, cloning and overexpressing *ADH3* gene (coding for alcohol dehydrogenase) resulted in a higher ethanol yield than the wild-type strain under anaerobic conditions, probably by inducing a faster consumption of glucose and alleviating the Custer effect (Schifferdecker et al. 2016). Interestingly, to date, no CRISPR approach has been proposed to *D. bruxellensis*.

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

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

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

On the other hand, *D. bruxellensis* strains might be able to assimilate cellobiose, since its genome harbours the *BGL* gene, coding for β-glucosidase (E.C. 3.2.1.21), which represents an advantage in a potential biotechnological use for second-generation ethanol production (Blomqvist et al. 2011; Curtin et al. 2012; Reis et al. 2014). In fact, it has been reported that this yeast is able to use this sugar, regardless the oxygen availability, with growth rates similar to those observed in glucose (Leite et al. 2013; da Silva et al. 2019). Moreover, *D. bruxellensis* was able to ferment cellobiose in oxygen limitation (similar to industrial conditions), even though with lower efficiency (21% lower) when compared to sucrose (Reis et al. 2014). In *D. bruxellensis*, □□-glucosidase acts mostly intracellularly, which is a useful feature since cellobiose is a potent inhibitor of cellobiohydrolases and when in excess, this disaccharide leads to a decrease in the hydrolysis rate (Reis et al. 2014; Gruber et al. 2007). Therefore, *D. bruxellensis* could be used in the process of simultaneous saccharification and fermentation (SSF), in which, even without the addition of β-glucosidase to the cellulolytic cocktail, the accumulation of cellobiose could be avoided.

Some *D. bruxellensis* strains are also able to use pentoses, such as D-xylose and L-arabinose under fermentative conditions, with maximum ethanol yields of 0.29

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

The steps of pre-treatment and hydrolysis of lignocellulosic material also generates fermentation inhibitors, such as furans (furfural and 5-hydroxymethylfurfural), carboxylic acids (acetic acid) and phenolic compounds, that might negatively interfere in the fermentation process (Palmqvist and Hahn-Hagerdal, 2000; Bušić et al. 2018). Therefore, besides being able to ferment the mixture of sugars released, key microorganisms also need to be resistant to these inhibitors (Robak and Balcerek 2018). In this regard, *D. bruxellensis* presents resistance to fermentation inhibitors when cells are initially adapted during batch cultivation, while unadapted cells have a more extended lag phase and lower ethanol yield (Tiukova et al. 2014). The mechanisms by which *D. bruxellensis* resists to these inhibitors are still uncertain, whereas in *S. cerevisiae* this has been associated with increased expression of the *ADH7* (alcohol dehydrogenase) and *ARI1* (aldehyde reductase intermediate) genes (Sehnem et al. 2013). Moreover, the tolerance to weak acids, low pH values and high ethanol concentration are important adaptation factors to this process (Dien et al. 2003; Davison et al. 2016). In this sense, the resistance of *D. bruxellensis* at low pH values (in the range of pH 1.5-2.0) is similar to *S. cerevisiae*. Still, at high ethanol concentrations (9%), both species are resistant, but show a decreased growth (Bassi et al. 2013).

The search for yeasts with key characteristics for the second-generation ethanol production aims to increase both ethanol yield and productivity from lignocellulosic materials. In this sense, *D. bruxellensis* meets the major requirements to become an ethanol-producing yeast, allowing its application in biotechnological

processes, such as SSF. In addition, genetically modified strains of *D. bruxellensis* could be developed in order to increase the ethanol production from the fermentation of lignocellulosic-derived sugars.

Concluding remarks

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

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5 ARTIGO II - FIRST ASPECTS ON ACETATE METABOLISM IN THE YEAST *DEKKERA BRUXELLENSIS*: A FEW KEYS FOR IMPROVING ETHANOL FERMENTATION

Running head: Acetate metabolism in *Dekkera bruxellensis*

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Keywords: *Dekkera bruxellensis*; acetaldehyde dehydrogenase; acetyl-CoA; carbon distribution; disulfiram; ethanol fermentation.

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Abstract

Dekkera bruxellensis is continuously changing its status in fermentation processes, ranging from a contaminant or spoiling yeast to a microorganism with potential to produce metabolites of biotechnological interest. In spite of that, several major aspects of its physiology are still poorly understood. As an acetogenic yeast, minimal oxygen concentrations are able to drive glucose assimilation to oxidative metabolism, in order to produce biomass and acetate, with consequent low yield in ethanol. In the present study, we used disulfiram (DSF) to inhibit acetaldehyde dehydrogenase

(ACDH) activity to evaluate the influence of cytosolic acetate on cell metabolism. *D. bruxellensis* was more tolerant to DSF than *Saccharomyces cerevisiae* and the use of different carbon sources revealed that the former yeast might be able to export acetate (or acetyl-CoA) from mitochondria to cytoplasm. Fermentation assays showed that ACDH inhibition re-oriented yeast central metabolism to increase ethanol production and decrease biomass formation. However, glucose uptake was reduced, which ultimately represents economical loss to the fermentation process. This might be the major challenge for future metabolic engineering enterprises on this yeast.

Keywords: *Dekkera bruxellensis*; acetaldehyde dehydrogenase; acetyl-CoA; carbon distribution; disulfiram; ethanol fermentation.

Introduction

In the past decade, several studies have been performed regarding the biology of the yeast *Dekkera bruxellensis*, the teleomorph of *Brettanomyces bruxellensis.* These reports ranged from identification as contaminant in fermentation processes to the perception of its potential to produce bio-compounds (Blomqvist and Passoth 2015; Radecka et al. 2015; Steensels et al. 2015). In the case of fuel-ethanol fermentation, it has been revealed that *D. bruxellensis* has high adaptability and capacity to replace the initial population of *Saccharomyces cerevisiae*, as well as potential to produce ethanol at high yields (De Souza Liberal et al*.* 2007; Passoth et al*.* 2007). This profile places *D. bruxellensis* within a particular group of fermenting yeasts of the Saccharomycetacea family. Evolutionary analyses estimated that *D. bruxellensis* and *S. cerevisiae* diverged from a common ancestor around 200 million years ago (Rozpedowska et al. 2011). However, they have converged to unique characteristics that enables the production of ethanol: (i) both are *Crabtree* positive, that is, at high concentrations of glucose they can perform the fermentative metabolism even in aerobic condition; (ii) they exhibit tolerance to high ethanol content in the medium and (iii) both can survive without mitochondrial DNA (petite positive phenotype), meaning that it can grow anaerobically (Piskur et al. 2006). However, *D. bruxellensis* is also considered as acetogenic yeast with the production of acetic acid and ethanol at similar yields in aerobic condition (Leite et al. 2013). This physiological trait is related to the so-called Custer effect, which negatively impacts glycolysis due to the depletion of NAD^+ cofactor that is used to oxidize acetaldehyde to acetate (Van Diijken et al. 1986).

Metabolically, acetate is the product of acetaldehyde oxidation by $NAD(P)^+$-dependent acetaldehyde dehydrogenase (ACDH). In *S. cerevisiae*, five genes were assigned to encode ACDHs: *ALD1*/*ALD6* (YPL061w), *ALD2* (YMR170c) and *ALD3* (YMR169c), that encode cytosolic isoforms, as well as *ALD4* (YOR374w) and *ALD5* (YER073w), that encode mitochondrial isoforms (Navarro-Avino et al. 1999). Acetate is mainly produced in the cytoplasm as the product of PDH (pyruvate dehydrogenase) bypass, which works by supplying cytosolic acetyl-CoA used for anabolic reactions towards biomass production, as well as for regulatory acetylation of proteins and chromatin (reviewed by Van Rossum et al. 2016). This PDH bypass is

the same involved in the production of ethanol, which starts with the action of pyruvate decarboxylase (PDC) to convert pyruvate from glucose to acetaldehyde. Thus, this molecule is the branch point to produce ethanol by NADH-dependent alcohol dehydrogenase (ADH) and/or acetate by ACDH. Therefore, the balance between ADH and ACDH activities and the redox state of the cell metabolism directs the fate of acetaldehyde and might define the fermentative capacity of the yeast. Besides the PDH bypass, acetate can also be provided by the mitochondria through the action of acetyl-CoA hydrolase, encoded by the gene *ACH1* (Van Rossum et al. 2016). Physiologically, Ach1 enzyme of *S. cerevisiae* works mainly in the direction of transferring CoA from succinyl-CoA to the acetate, which enters the mitochondria from the cytosol. Moreover, Ach1 would also display acetyl-CoA transferase activity, to ensure the functioning of TCA cycle even in cells lacking PDH activity (Chen et al. 2015). In *S. cerevisiae*, this reverse activity (acetyl-CoA + succinate > acetate + succinyl-CoA) can be detected only when cells are released from Glucose Catabolite Repression (GCR) (Lafuente et al. 2000; Schulte *et al.* 2000), and, in this case, mitochondrial acetate could flow to cytosol (Chen et al. 2015). This explains the fact that cells lacking PDC activity are unable to grow on glucose unless external acetate is provided in the medium (Flikweert et al. 1999) or when these mutant cells are released from GCR (Chen et al. 2015; Zhang et al. 2015).

Since the diversion of acetaldehyde to acetate reduce ethanol production, we hypothesized whether the low ethanol yield reported for *D. bruxellensis* (Pereira et al. 2012, 2014) is somehow linked to its acetogenic profile, as well as to the *Custer* effect itself. To address this question, we used the compound disulfiram (DSF), a drug used to treat chronic alcoholism in humans by blocking the PDH bypass. This compound inhibits ACDH and then impairs the conversion of acetaldehyde to acetate, causing accumulation of acetaldehyde and depletion of cytosolic acetate (Kwolek-mirek et al. 2012). Kinetic analyses of the fermentative metabolism were performed using the industrial strain GDB 248 as model due to the significant amount of knowledge already available in the literature. Despite the variability among the strains related to date, the present study aims the identification of important genetic/physiological factors related to the potential use of this strain in ethanol fermentation processes. In overall terms, we observed that the addition of external acetate did not recover its growth in the presence of the ACDH inhibitor in different carbon sources. Carbon balance estimated from fermentation data pointed out to the

action of ACDH in controlling the distribution of carbon in the pyruvate metabolic branch point in *D. bruxellensis.* Herein, we propose that *D. bruxellensis*, like *Kluyveromyces lactis,* is able to export acetate from mitochondria, in order to fulfil C2 requirements in the cytosol. So far, studies have addressed issues regarding to acetate production and tolerance in *D. bruxellensis* (Capusoni et al*.* 2016). Therefore, the present work is a pioneer study on the acetate metabolism in *D. bruxellensis* and might contribute to the understanding of regulatory mechanism behind the control of ethanol production in this yeast.

Materials and methods

Strain and chemicals

The strain *D. bruxellensis* GDB 248, isolated from industrial fuel-ethanol fermentation process, was used as it has been a model for physiological studies of this species in our laboratory (Leite et al*.* 2013). The industrial strain *S. cerevisiae* JP1 was used as reference (Pereira et al*.* 2012). Disulfiram (DSF) or tetraethylthiuram disulphide, was kindly provided by the Department of Tropical Medicine of the Federal University of Pernambuco. The antioxidants glutathione (GSH) and N-acetylcysteine (NAC) were purchased from Sigma Aldrich (St Louis, USA). Solutions were prepared by dissolving the compound in deionised water and filter sterilising with 0.22 μm filter. Acetic acid and sulphuric acid were purchased from Vetec Co. (Rio de Janeiro, Brazil).

Culture conditions

Cells were maintained in solid YPD medium (yeast extract: 10 g L^{-1}; peptone: 20 g L^{-1}; glucose: 20 g L^{-1}; agar: 20 g L^{-1}). Cells were cultivated in flasks containing liquid synthetic defined (SD) medium (yeast nitrogen base: 1.7 g L^{-1}; ammonium sulphate: 5 g L^{-1}; glucose or galactose or glycerol: 20 g L^{-1}; pH 5.5) at 30 ^{o}C and 180 rpm for 48 h. Afterwards, yeast cells were transferred to fresh SD media to initial concentration of 0.1-0.2 OD units at 600nm. Whenever necessary, medium pH was adjusted with pure sulphuric acid. DSF, antioxidants or acetic acid were added to the concentrations indicated in each figure. Cultivations were performed in 150 μL volume in sterile microtitre plates in multireader Synergy HT (Biotek, Switzerland)

with automatic recording of OD at 600 nm. Yeast growth rates were calculated from the slope of exponential growth phase (Leite et al. 2013). All experiments were performed by two independent biological duplicates with technical triplicates each. The results represent the average of six measurements for each point in the curves (±SD).

Fermentation assays

Cells were cultivated in flaks as above, collected by centrifugation (4.000 g for 5 min), washed with distilled water and re-suspended to 50 mL final volume with SD medium containing 120 g glucose L^{-1} to initial biomass concentration of 50 g L^{-1} (5% w/v) in 125 mL flasks. Incubations were performed at 30 ^{o}C in rotatory orbital shaker at 120 rpm to ensure minimal oxygenation to induce acetate production. Samples were taken at defined intervals up to 24 h and centrifuged (10.000 g for 6 min). Supernatants were frozen at -20 ^{o}C for extracellular metabolite measurements and yeast cells were frozen in liquid nitrogen and stored at -80 ^{o}C for RNA extraction. All experiments were performed by two independent biological duplicates and the results represent the average value (±SD).

Metabolites quantification

Samples were thawed, filtered with 0.22 µm filters and used for metabolite analysis by HPLC (Waters device) using Aminex HPX-87H colunm (BioRad) heated at 60 ^{o}C, with mobile phase of 8 mmol L^{-1} H_2SO_4 at 0.6 mL min^{-1} flow rate. Metabolites (glucose, ethanol, glycerol and acetate) were detected by refraction index and their concentrations calculated by integrating the peak area with the calibration factor determined for each metabolite as performed by Pereira et al. (2012) and Leite et al. (2013). Physiological, fermentative and kinetic parameters were calculated according to Leite et al. (2013). Mass and carbon balance were calculated from the final concentration of the products and the consumed glucose, and stoichiometric CO_2 was calculated from the sum of ethanol, acetate and biomass according to elemental composition of the biomass provided by Leite et al. (2013).

Relative gene expression analysis

Primer design, synthesis and quality and efficiency evaluations followed the procedures described by De Barros Pita *et al.* (2012). Cells were thawed, centrifuged at 4 ºC and re-suspended in 250 µL of AE buffer (50 mM sodium acetate and 10 mM EDTA, pH5.3) plus 60 µL 10% SDS. Cells were lysed by vortexing followed by incubation at 65 ºC for 10 minutes. The lysates were centrifuged at 10,000 rpm for five minutes at 4 ºC and the supernatants were transferred to new tubes. Total RNA was purified with NucleoSpin® RNA II kit (Macherey-Nagel, Germany) according to manufacturer instructions. Quantification was performed in Nanodrop device (GE Health Care, EUA), with purity evaluated by 260nm/280nm ratio, and integrity evaluated in 1% agarose gel in DEPC-treated TAE buffer and dying with ethidium bromide. cDNA was produced with the aid of ImProm-IITM Reverse Transcription System Promega II kit (Promega, USA), following manufacturer instructions.
Amplifications were performed in ABI Prism 7300 device (Applied Biosystems, Foster City, USA) using SYBR Green PCR master Mix kit (Applied Biosystems). Reference genes for data normalisation and analysis by geNorm algorithm as well as relative quantity determination followed the MIQE Guidelines as recommended for *D. bruxellensis* by de Barros Pita *et al.* (2012).

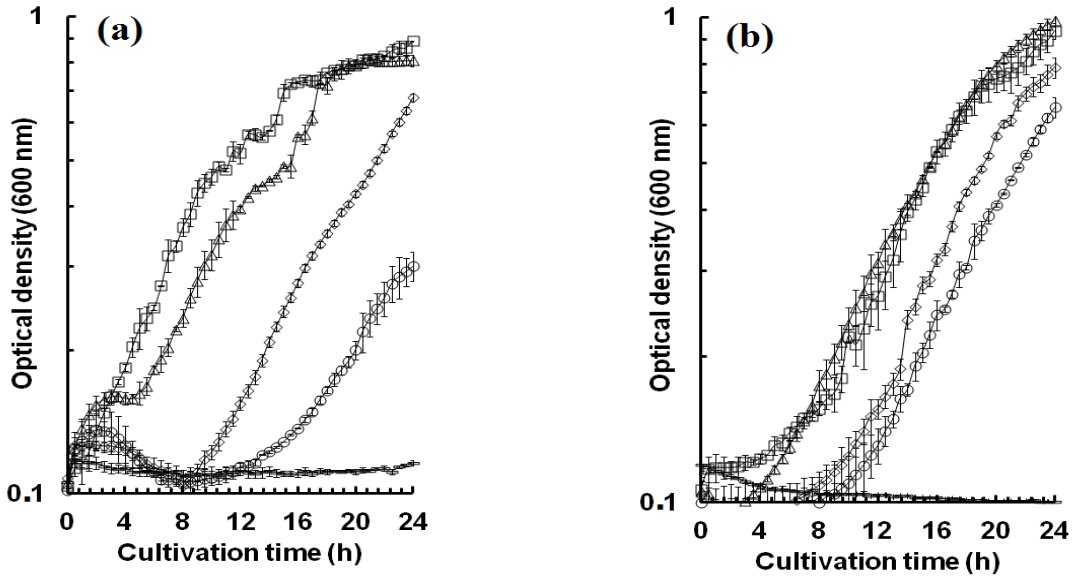
Results and discussion

Our previous results showed the tendency of *D. bruxellensis* to produce acetate and ethanol at the same levels in aerobic shake flask cultivations in low-glucose medium as well as after pulsing in C-limited chemostat with glucose (Leite et al*.* 2013). This is a profile not shared with *S. cerevisiae*, despite various similarities between both yeasts. Thus, the acetogenic characteristic of this yeast might be linked to its natural tendency for respiration, probably a consequence of the P/O ratio = 3, which means three active proton pumping sites in its respiratory chain (Leite et al*.* 2013).

In the present study, we used the compound DSF in order to inhibit ACDH activity and compare its effects between *D. bruxellensis* GDB 248 and *S. cerevisiae* JP1 industrial strains (Fig. 1). Our results revealed that *D. bruxellensis* GDB 248 is more tolerant to this compound than *S. cerevisiae* JP1. DSF at 15 µM reduced growth and extended lag phase in the latter (Fig. 1a). In *D. bruxellensis*, the duration

of lag phase was extended only when cells were grown in concentrations above 15 μM (Fig. 1b). Therefore, it seems that DSF is, indeed, inhibiting its ACDH, however, this fact seems not to be affecting the cytosolic supply of acetate in *D. bruxellensis* as it does in *S. cerevisiae*. The minimal inhibitory concentration (MIC) for both yeasts was defined as 100 μM of DSF (Fig. 1). The cytotoxic effect of DSF at MIC value for both yeasts was completely reverted when the thiol compounds glutathione or N-acetylcysteine (NAC) were added to the medium (data not shown). This is in accordance to previous work reporting that NAC chemically interacts with DSF and inhibits its toxic effect in *S. cerevisiae* (Kwolek-Mirek et al. 2012).

Figure 1. Effect of disulfiram (DSF) on the growth of *Saccharomyces cerevisiae* (panel a) and *Dekkera bruxellensis* (panel b) after 24 hours culture in YNB medium containing 15 μM (open triangles), 60 μM (open diamond), 75 μM (open circles) or 100 μM (traits) of the compound. DSF-untreated culture was represented (open squares). Bars represent standard deviation from the mean value of two independent experiments, with technical triplicate for each one.



It is well known that decarboxylase-negative *pdc1Δpdc5Δpdc6Δ* triple mutant strain of *S. cerevisiae* is not capable of growing in glucose as sole carbon source without acetate supplementation (Flikweert et al. 1999; Van Maris et al. 2004). The explanation for these results is that no mitochondrial acetate is exported to cytosol in the presence of glucose to overcome the negative effect of impairment of PDH bypass (Van Maris et al. 2003). A mutant strain lacking of Pdc activity has partially

restored the cytosolic supply of acetate in presence of a mutation in the *MTH1* gene, which codes for a repressor of genes subjected to GCR (Oud et al. 2012). This deletion reduces the uptake of glucose from the medium and releases GCR mechanism (Lafuente et al. 2000; Schulte et al. 2000). Moreover, double mutant *mth1Δhxt3Δ* fully restored the growth of *pdc* mutants in glucose by releasing the expression of *ACH1* gene, which encodes acetyl-CoA hydrolase. In this case, part of the mitochondrial acetyl-CoA is hydrolysed or its CoA group is transferred to succinate to release acetate that, in turn, flows to the cytosol to fulfil the C2 requirement of *pdc* mutant (Chen et al. 2015; Zhang et al. 2015). Altogether, those results indicate that acetate flows from mitochondria to cytosol whenever GCR is relieved in *S. cerevisiae*. On the other hand, *pdc* mutant of *Kluyveromyces lactis* grows normally in glucose, but not *pda* mutant (Bianchi et al. 1996). It indicates that in this yeast there is an export of acetate to cytosol even when glucose is present, which might also be the case for *D. bruxellensis*.

In order to test this hypothesis, we supplemented the cultivation medium with acetate containing DSF and measured the effect of this supplementation on the yeast growth (Fig. 2). The results showed that acetate partially restored growth in *S. cerevisiae* (Fig. 2a), indicating that, indeed, the presence of DSF at a sub-MIC dose leads to cytosolic acetate shortage. This was in accordance to previous report in which external acetate overcome the effect of PDH bypass inactivation (Flikweert et al. 1999). However, the external acetate did not reduce the duration of the lag phase in this yeast caused by DSF (Fig. 2b). On the other hand, this external acetate supply exacerbated the negative effect of DSF on *D. bruxellensis* cells (Fig. 2c) and increased even more the extension of lag phase in presence of this inhibitor (Fig. 2d). It suggested that acetate might be supplied from mitochondria to cytoplasm in this yeast in presence of DSF. A recent study involving 29 strains of *D. bruxellensis* showed acetate tolerance up to 120 mM. Even the most tolerant strain, CBS 4482, was negatively affected at this acetate concentration (Moktaduzzaman et al. 2015a; Capusoni et al. 2016).

The hypothesis that *D. bruxellensis* is capable of exporting acetate or acetyl-CoA from mitochondria to cytoplasm was ultimately checked by cultivating the cells in two other C sources: galactose and glycerol (Fig. 3). The presence of DSF reduced significantly the growth of *S. cerevisiae* in galactose, which was completely reverted with the external supply of acetate (Fig. 3a). On the other hand, no effect of DSF on

cell growth was observed when *D. bruxellensis* was cultivated in galactose even at MIC dose (Fig. 3b). Galactose metabolism in ammonium-based medium, as in the case of the present work, is slow and might avoid the overflow to the upper part of the glycolysis, leading to a fully respiratory metabolism and releasing cells from GCR (Moktaduzzaman et al. 2015b). In addition, glycerol was used as a carbon source that is exclusively metabolised by the mitochondria in a respiratory metabolism. Also in this case, no GCR mechanism should be active, allowing mitochondrial acetyl-CoA to be constitutively exported to the cytosol. Indeed, the inhibitory effect of DSF at MIC dose was not observed in *S. cerevisiae* cultivated in glycerol or galactose (GCR-free cells) (Fig. 3a,c) as it was in glucose (Fig. 1). No effect of DSF at MIC dose was observed whatsoever for *D. bruxellensis* in glycerol, even in presence of external acetate (Fig. 3d). As galactose and glycerol might relieve GCR mechanism, cells of both yeasts could export the mitochondrial acetate in order to fulfil the cytosolic requirement for C2 in the absence of PDH bypass.

Figure 2. Effect of acetate on relative growth rate (panels a and c) and time of lag phase (panels b and d) in cultures of *Saccharomyces cerevisiae* JP1 (panels a and b) and *Dekkera bruxellensis* GDB 248 (panels c and d) in mineral medium containing different concentrations of disulfiram (DSF). Open symbols represent data from cultures not supplemented with acetate. Closed symbols represent data from cultures supplemented with acetate to 8.3 mM initial concentration. Relative growth rate represents the percentage of growth rate in DSF-treated (with or without acetate) relative to DSF-untreated reference condition (100%). Bars represent standard deviation from the mean value of two independent experiments, with technical triplicate for each one.

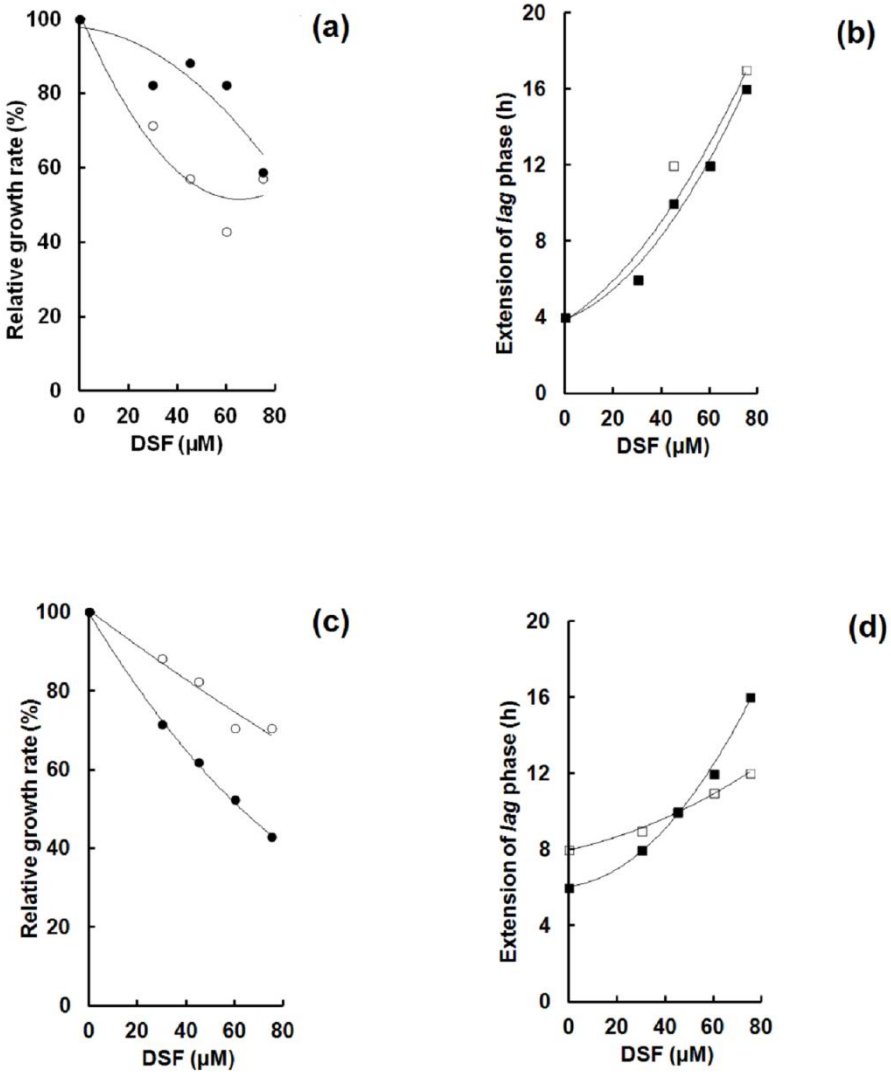
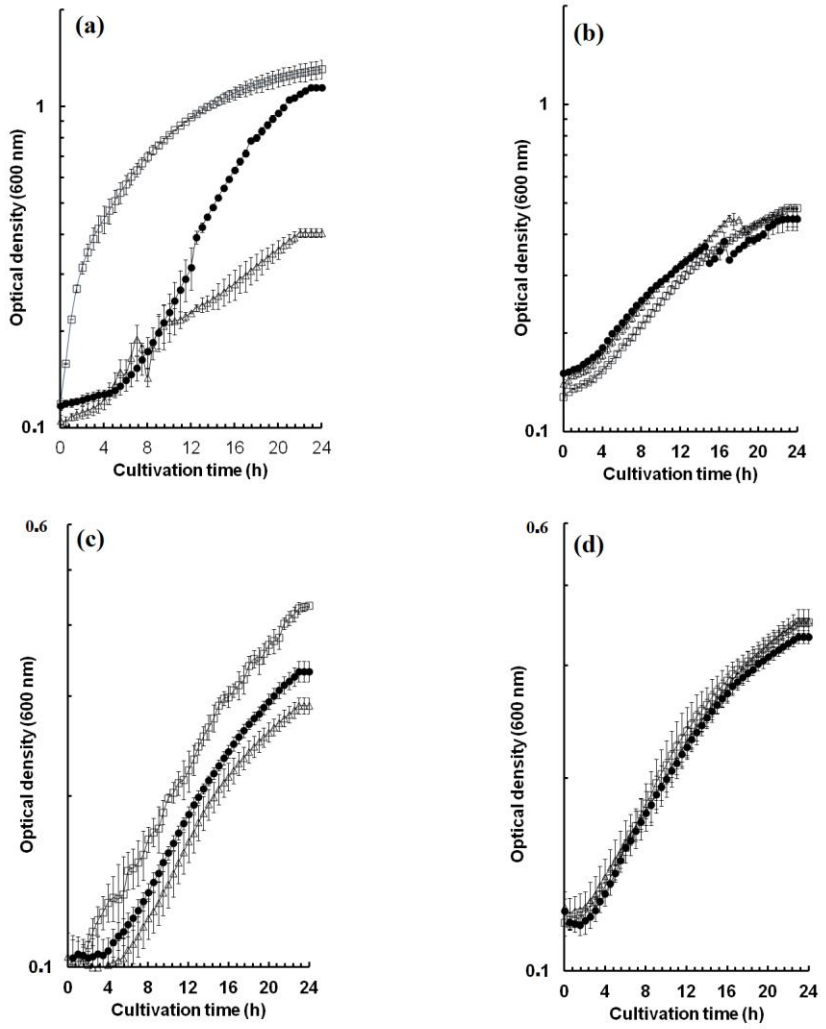


Figure 3. Growth of *Saccharomyces cerevisiae* JP1 (panels a and c) and *Dekkera bruxellensis* GDB 248 (panels b and d) in mineral medium containing galactose (panels a and b) or glycerol (panels c and d) as carbon source in the presence of disulfiram (DSF). Open squares represent data from untreated reference condition. Open triangles represent data from growth in the presence of DSF (100 μM). Closed circles represent data from growth in the presence of DSF (100 μM) and acetate (8.3 mM). Bars represent standard deviation from the mean value of two independent experiments, with technical triplicate for each one.



Afterwards, we performed fermentation tests of *D. bruxellensis* GDB 248 in medium containing high glucose concentration (130 g L) and slight agitation to induce acetate production in presence of 60 µM DSF and compared to reference condition without the inhibitor. It was observed that the presence of DSF reduced overall glucose consumption by 37%. In reference condition, the specific glucose consumption rate was calculated as 4.32 mmol gDW^{-1} h^{-1}. This kinetic parameter was reduced to 2.96 mmol gDW^{-1} h^{-1} when DSF was added to fermentation, which represented a reduction of 43% in glucose uptake. Oxygen supply was imposed to the process by gently agitating the flasks with high initial biomass and sugar content with the aim to induce the production of acetate. However, the oxygen-limited condition reduced biomass yield to 20% of that achieved when fully aerobic (oxidative) metabolism was operating in C-limited chemostat with glucose (Leite et al. 2013). Moreover, biomass yield dropped even more when ACDH activity was

inhibited by DSF in this oxygen-limited condition, followed by 70-80% reduction in glycerol production and yield (Table 1).

Table 1. Mass and carbon balance of oxygen-limited 24 h fermentation assays by *Dekkera bruxellensis* GDB 248 in mineral media containing disulfiram (DSF) at 60 μM.

Mass balance	-DSF	+DSF	Carbon balance	-DSF	+DSF
Glucose consumed (g)	114.58	72.61	Cmol glucose	3.819	2.420
Y_{etoh} ($g.g^{-1}$)	0.328	0.494	Cmol ethanol	1.629	1.561
Y_{acet} ($g.g^{-1}$)	0.029	0.033	Cmol acetate	0.109	0.081
Y_{glyc} ($g.g^{-1}$)	0.015	0.004	Cmol glycerol	0.053	0.010
Y_{biom} ($g.g^{-1}$)	0.059	0.018	Cmol biomass	0.266	0.051
Y_{CO2} ($g.g^{-1}$)	0.519	0.470	Cmol CO_2	1.784	0.775
Mass recovery (%)	98	102	Carbon recovery (%)	100	102

Ethanol yield in the reference fermentation (Table 1) was 27% higher that calculated for sugar cane juice (Pereira et al. 2012). When DSF was added, ethanol yield increased even more (Table 1) to the levels of *D. bruxellensis* fermenting sugar cane molasses (Pereira et al. 2014) and *S. cerevisiae* fermenting sugar cane juice and molasses (Pereira et al. 2012, 2014). Ethanol yield of 0.16 $g.g^{-1}$ was obtained when respiratory-growing cells of *D. bruxellensis* were pulsed with glucose (Leite et al. 2013). Different fermentation conditions regarding the oxygen supply can be compared. As it can be seen in Table 2, there is an opposite relation between oxygen availability and ethanol yield, while acetate yield was dependent on oxygen supply. In terms of ethanol, the presence of DSF increased fermentation yield in synthetic medium to the level of the industrial substrate molasses, which was twice higher than observed for the industrial substrate cane juice (Table 2). Molasses, as a waste residue of sugar production, contains oxidised molecules and high content of minerals that interfere in the redox state of the yeast cells, in a similar way to DSF acting on ACDH activity. It causes changes in the ratio ethanol/acetate yields (from 11 to 17) in the direction of the first metabolite (Table 2). Actually, there was no significant difference in the final concentration of ethanol (35 g L^{-1}) in the presence or absence of DSF. Hence, the higher efficiency of carbon conversion from glucose to

ethanol was the consequence of reduced glucose uptake, which ultimately resulted in higher residual glucose in DSF-containing medium (Table 1), as well as in molasses (Pereira et al. 2014).

Table 2. Ethanol and acetate yields of *Dekkera bruxellensis* GDB 248 in different fermentation conditions**.**

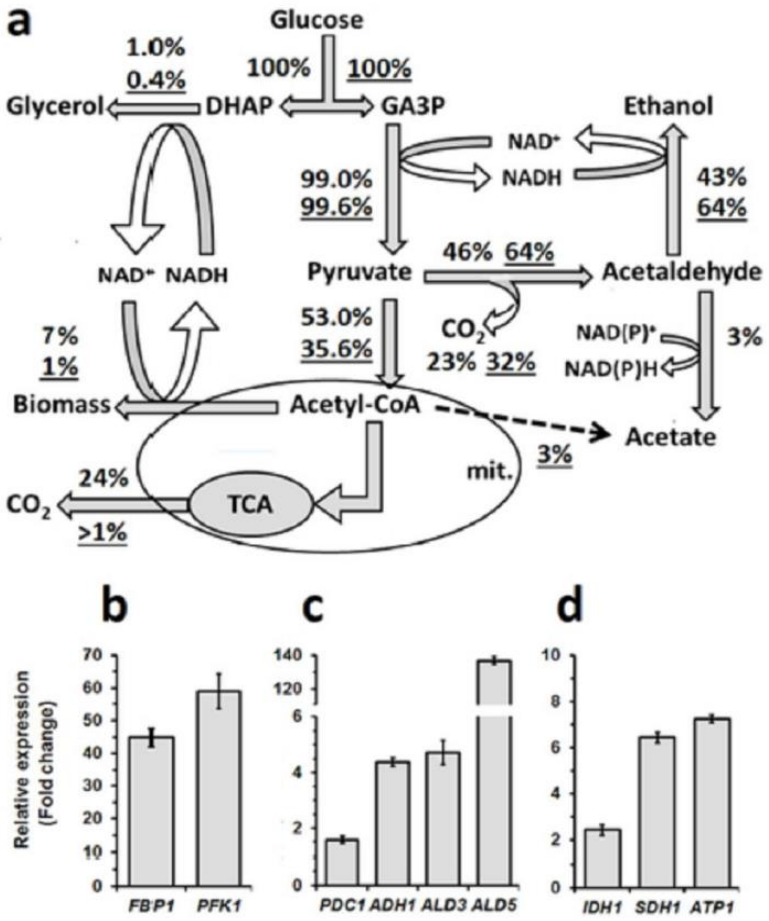
Condition	Y_{etoh} ($g.g^{-1}$)	Y_{acet} ($g.g^{-1}$)	Reference
Aerobic C-limited chemostat pulsed with glucose	0.16	0.20	Leite et al (2013)
Oxygen-limited fermentation of sugar cane juice	0.22	0.00	Pereira et al (2012)
Oxygen-limited fermentation of sugar cane molasses	0.45	0.02	Pereira et al (2014)
Oxygen-limited fermentation of reference medium	0.33	0.03	This work
Oxygen-limited fermentation of reference medium with DSF	0.49	0.03	This work

Overall carbon distribution in the central metabolism of DSF-treated and untreated cells of *D. bruxellensis* was calculated (Fig. 4a) on the basis of carbon balance from the end points of fermentation experiments (Table 1). These data were co-related with the expression of genes involved in glycolysis, fermentation, TCA and respiration (Fig. 4b,c,d). The uptake of glucose was reduced in the presence of DSF as showed above. However, glycolytic gene *PFK1* showed 60-fold upregulation in the presence of DSF when compared to reference condition (Fig. 4b). Unexpectedly, an increase was observed in the transcript level of the gluconeogenic gene *FBP1,* by 50-fold (Fig. 4b). The products of these genes work in opposite directions and the second is subjected to GCR also in *D. bruxellensis* (Leite et al. 2016). As hypothesised above, this result is the confirmation that the inactivation of ACDH relieves (or partially relieves) the repressive activity of GCR mechanism. Furthermore, the inhibition of PDH bypass by DSF at 60 μM increased by 30% the carbon distribution from pyruvate towards acetaldehyde through PDC activity (Fig. 4a), which was not accompanied by upregulation of *PDC1* gene (Fig. 4c). On the other hand, the consequent overproduction of ethanol co-related with 4.3-fold upregulation of *ADH1* gene (Fig. 4c). Interestingly, a slight induction was observed for *ALD3* (3.7-fold) and a significant upregulation of *ALD5* (140-folds) in presence of DSF (Fig. 4c). *ALD5* encodes a mitochondrial acetaldehyde dehydrogenase (mtACDH) while *ALD3* encodes the cytosolic counterpart (ctACDH). This should

represent a compensatory mechanism intending to ensure acetate production due to inactivation of ACDH activity by DSF. Deletion of the *ALD5* gene in *S. cerevisiae* and consequent loss of mtACDH activity led to a significant reduction in acetate production (24%), indicating the importance of the mitochondrial isoform in acetate biosynthesis (Saint-Prix et al. 2004). In the same work, authors analysed the acetate production in a series of single and multiple mutations in *ALD* genes and concluded that Ald5p is the main isoform for the production of acetate during fermentation (Saint-Prix et al. 2004). Thus, it is also possible that the overexpression of Ald5p reported in the present work could provide minimal ACDH activity in the mitochondria, being capable of converting the excess of acetaldehyde to acetate, which flows to the cytosol.

This model also proposes that DSF causes shortage of mitochondrial acetyl-CoA by reducing by 30% carbon delivery from pyruvate to TCA cycle through pyruvate dehydrogenase (Pdh) activity. This led to a proportional increase of metabolism through PDH bypass as proposed above. Then, the oxidative metabolisation of pyruvate through TCA pathway is so reduced that in a condition of oxygen limitation it cannot support biomass formation (Fig. 4a). This scenario triggered the upregulation of *IDH1, SDH1* and *ATP1* genes (Fig. 4d) from the oxidative metabolism to compensate the shortage of TCA intermediates.

Figure 4. Relative carbon distribution (%) for product formation from glucose in cells of *Dekkera bruxelensis* in oxygen-limited fermentative condition in the absence or presence of disulfiram (DSF) at 60 □M (panel a) and relative expression of genes of glycolysis/gluconeogenesis (panel b), fermentative pathway (panel c) and TCA/respiration (panel d). Underlined values refer to DSF-treated fermentations. Fold change indicates the amount of gene transcripts in DSF-treated fermentations relative to DSF-untreated reference fermentations. Bars represent standard deviation from the mean value of two independent experiments, with technical triplicate for each one.



It is interesting that under ACDH inhibition the proportion of carbon directed to acetate is similar to the reference condition (Fig. 4a). So, where does it come from? We propose that when the conversion from acetaldehyde to acetate is blocked, pyruvate is decarboxylated to mitochondrial acetyl-CoA by PDA activity, which serves as substrate for CoA transferase, resulting in mitochondrial acetate that flows to cytosol. On the other hand, most of the pyruvate is decarboxylated to acetaldehyde by PDC activity that, in turn, is reduced to ethanol. However, in spite of increasing ethanol production, DSF causes accumulation of glucose in the medium. Therefore, the control of cytosolic acetate seems to be the key for transforming this rather industrially adapted yeast to high-fermenting yeast. The metabolic challenge to be addressed is the combination between reduction in ACDH activity with the maintenance of high glucose assimilation rate.

Acknowledgements

We appreciate the support of all colleagues in the metabolic engineering group, the Federal University of Pernambuco (UFPE) and the University of Pernambuco (UPE) for technical support. This work was supported by the Bioethanol Research Network of the State of Pernambuco (CNPq-FACEPE/PRONEM program, grant nº APQ-1452-2.01/10) and by grants of the National Council of Science and Technology (CNPq nº 472533/2013-4, 474847/2013-6 and 446927/2014-7).

Conflict of interest

The authors declare no conflict of interest.

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6 ARTIGO III - METABOLIC AND BIOTECHNOLOGICAL INSIGHTS ON THE ANALYSIS OF PDH BYPASS AND ACETATE PRODUCTION IN THE YEAST *DEKKERA BRUXELLENSIS*

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Abstract

Dekkera bruxellensis is a microorganism that emerges in distilleries of diverse industrial processes, being placed in a generic group of species, often called non-conventional yeasts (NCY). These microorganisms usually present industrial relevance and, therefore, studies aiming to understand their major metabolic aspects are important, in order to explore their biotechnological potential. In this sense, we analyzed respiro-fermentative parameters of *D. bruxellensis* under the effect of glucose or sucrose as carbon (C) source, ammonium or nitrate as nitrogen (N) source and in the presence or absence of the biochemical inhibitor of the enzyme acetaldehyde dehydrogenase (Acdh) disulfiram (DSF). Physiological and genetic data were connected by mean of qPCR assays to evaluate the expression levels of genes from respiro-fermentative metabolism in *D. bruxellensis*. Our results showed a 27% (glucose) and 14% (sucrose) increase in fermentative efficiency when Acdh was blocked by DSF. Therefore, it is possible to assume that any acetate produced by cells in this condition is being generated in the mitochondria, which is later exported to the cytoplasm in order to support the cell biosynthetic needs. In the presence of nitrate, we observed that *D. bruxellensis* cells increased carbon targeting for acetate production. The use of DSF and nitrate hampered the assimilation of carbon. Cultures with sucrose showed a similar profile of ethanol and acetate production regardless the N source. It was hypothesized that fructose released from sucrose might have some regulatory action on the carbon fluxes in the cell, improving yeast growth in the presence of nitrate, as well as allowing the production of ethanol. Finally, *PDC1, ADH1, ALD3, ALD5, PFK1* and *ATP1* gene expression data and clustering analyses provided an important basis for the understanding of several aspects of the respiro-fermentative metabolism of the industrial yeast *D. bruxellensis* when in the presence of different carbon and nitrogen sources, as well as with the impediment of the production of cytosolic acetate.

Keywords: Carbon distribution; Crabtree effect; Custer effect; Fermentation; Nitrate assimilation; Pyruvate branchpoint

1. Introduction

The yeast *Dekkera bruxellensis*, teleomorph of *Brettanomyces bruxellensis,* was identified as a contaminant in bioethanol production process, yet several studies identified important characteristics in this species, namely its wide ability to assimilate carbon sources and its high fermentative capacity (De Souza Liberal et al., 2007; Blomqvist et al., 2012; De Barros Pita et al., 2013; Pereira et al., 2014; Teles et al., 2018; Da Silva et al., 2019; Peña-Moreno et al., 2019). In this regard, *D. bruxellensis* can obtain ethanol yields similar to *Saccharomyces cerevisiae*, the main yeast in industrial processes, such as in synthetic media (Blomqvist et al., 2012), or even in fermentation of sugarcane juice and sugar cane molasses (Pereira et al., 2012,2014). Additionally, *D. bruxellensis* is also capable of producing ethanol from cellobiose, being defined as a potential yeast in the production of second-generation ethanol (Reis et al., 2014). In addition to the industrial process in bioethanol distilleries (De Souza Liberal et al., 2007), *D. bruxellensis* is also essential for the composition of the sensory panel of Belgian Lambic beer (Spitaels et al., 2014), as well as in the production of kombucha and cider (Teoh et al., 2004; Gamero et al., 2014). The release of sensory compounds can also introduce *D. bruxellensis* as a potential microorganism in the production of the Brazilian spirit cachaça (Parente et al., 2015).

Another important metabolic trace in *D. bruxellensis* is its ability to metabolize nitrate as a nitrogen source, which might be fundamental for its maintenance in sugarcane-derived bioethanol production, since it provides means for sustainable growth after the depletion of ammonium (De Barros Pita et al., 2011). However, nitrate assimilation demands a huge number of reduced equivalents: four mols of NADPH for the reduction of nitrate to nitrite by nitrate reductase (Ntr) and one mol of NADPH for the reduction of nitrite to ammonium by nitrite reductase (Nir). The intracellular ammonium is further assimilated in the carbon skeleton of 2-oxoglutarate to produced glutamate at expenses of one mol of NAPDH by glutamate dehydrogenase (Gdh) (Siverio, 2002). This reducing power can be mainly provided by two metabolic pathways: the pentose phosphate pathway and/or the Pdh bypass (Fig. 1). Alternatively, these Ntr and Nir reductases can also use the glycolysis-derived NADH as reducing power to convert nitrate to ammonium (Galafassi et al., 2013), draining the reduced cofactors required for ethanol production. In nitrate,

several oxygen-limited fermentation assays were performed and showed the ability of *D. bruxellensis* to produce ethanol (Blomqvist et al., 2012; De Barros Pita et al., 2013a; Peña-Moreno et al., 2019). However, in aerobiosis, the presence of nitrate promotes higher yields of acetate compared to ethanol (Galafassi et al., 2013).

The ability of *D. bruxellensis* to produce ethanol even under aerobic conditions is called the crabtree effect, and in this case, a high concentration of sugar is required. (Prochazka et al., 2010; De Barros Pita et al 2013). In *S. cerevisiae,* the saturation of the respiratory capacity forces the cells to re-oxidise the glycolytic NADH by reducing acetaldehyde to produce ethanol by the alcohol dehydrogenase (Adh) (Pronk et al., 1996). In this yeast, the carbon flux of glycolytic enzymes can be 11 times higher in the absence than in the presence of oxygen to compensate the lower ATP production at substrate level, in the oxidative part of glycolysis (Daran-Lapujade et al., 2007). The adequate regulation of central metabolism from glucose metabolization is, therefore, essential for several metabolic processes not only for the cell homeostasis but also for metabolic engineering and industrial production strategies (Daran-Lapujade et al., 2004, 2007).

In the end of the glycolytic pathway, the pyruvate formed face a branchpoint in which they can be decarboxylated by pyruvate dehydrogenase (Pdh) to produce mitochondrial acetyl-CoA or decarboxylated by pyruvate decarboxylase (Pdc) to acetaldehyde (Fig. 1). Acetaldehyde can be further converted to ethanol by alcohol dehydrogenase (Adh) in the fermentative pathway or to cytosolic acetate by acetaldehyde dehydrogenase (Acdh) in the so-called Pdh bypass (Fig. 1). In a previous report, we proposed that the carbon flux through the Pdh bypass has some regulatory effect on the carbon flux in the central metabolism when in fermentation conditions, and the inhibition of Acdh promoted greater carbon targeting for ethanol production (Teles et al., 2018). It is also expected that the decrease in the Pdh activity reduces the production of both ethanol and cytosolic acetate in *S. cerevisiae* (Remize et al., 2000). In the case of acetate production, *D. bruxellensis* exhibits the "Custers effect", a phenomenon characterized by a halt in fermentation metabolism (Wijsman et al., 1984) brought about by the depletion of NAD^+ that was used to oxidize acetaldehyde to acetate (Scheffers, 1966). Other yeasts achieve redox reorganization through the production of glycerol (Pronk et al., 1996). However, no glycerol production was observed in assays with *D. bruxellensis* (Teles et al., 2018;

Penã-Moreno et al., 2019). The "Custer's effect" can be abolished under anaerobic conditions in the presence of nitrate as a nitrogen source (Galafassi et al., 2013).

In the present paper, we evaluate physiogenomic profiles of the respiro-fermentative metabolism of *D. bruxellensis* with an emphasis on crossroads aspects at the level of pyruvate. The first strategy was to force the functioning of the Phd bypass by cultivating the cells in nitrate (De Barros Pita et al, 2011, 2013), while in the second strategy the Pdh bypass was impaired by the Acdh inhibition by disulfiram (DSF) (Mirek et al., 2012). Regarding this second strategy, experiments were carried out in aerobic growth conditions, different from the oxygen-limited fermentation essays carried out previously (Teles et al., 2018). Furthermore, it was quantified the expression of the three genes involved in the PDH bypass (*PDC1* coding the Pdc enzyme, *ADH1* coding the Adh enzyme and *ALD3* coding the cytosolic Acdh), the gene *ALD5* coding the mitochondrial Acdh ($_{mt}$Acdh) (Teles et al., 2018), the gene *ATP1* coding the alpha subunit F1 of mitochondrial F1F0 ATP synthase that is directly involved in energy production in the respiratory chain (Francis et al., 2007) and the gene *PFK1* coding the phosphofructokinase (Pfk) that controls the flow of the glycolytic pathway (Uyeda, 1979; Nishino et al., 2015). Furthermore, a cluster analysis was performed from the results of the tests in the presence of glucose and sucrose as carbon sources and ammonium sulfate and nitrate as nitrogen input to evallate the connection of the physiological parameters.

2. Materials and methods

2.1. Yeast strain and culture media

Dekkera bruxellensis GDB248 (strain URM 8346) was used in the present work (Peña-Moreno et al., 2019). Experiments were also performed using the strain *S. cerevisiae* JP1 as a reference (Pereira et al., 2012). Cell maintenance was performed in YPD medium (10 g L^{-1} yeast extract; 20 g L^{-1} peptone; 20 g L^{-1} dextrose and 20 g L^{-1} agar). Prior to the assays, cells were washed in 0.9% saline to eliminate waste from YPD medium and seed cultures were prepared by inoculating synthetic medium YNB (Yeast Nitrogen Base w/o amino acids and ammonium sulphate, 1.7 g L^{-1}) supplemented with glucose (20 g L^{-1}) and ammonium sulphate (5 g L^{-1}). The initial cell density was of 0.1 OD units (600 nm) and cultivations lasted for 24h at 30 ^{o}C and 150 rpm.

2.2. Fermentation and aerobic cultivation assays

Aerobic cultivations were carried out in synthetic YNB media containing glucose or sucrose as carbon source at initial equimolar carbon concentration of 660 mM and ammonium sulphate or sodium nitrate at initial equimolar nitrogen concentration of 75 mM. Disulfiram (DSF), inhibitor of the enzyme acetaldehyde dehydrogenase, was used at 60 µM (Mirek et al., 2012; Teles et al., 2018). The following cultivation conditions were used: Glucose+ammonium (C1), Glucose+ammonium+DSF (C2), Glucose+nitrate (C3), Glucose+nitrate+DSF (C4), Sucrose+ammonium (C5), Sucrose+ammonium+DSF (C6), Sucrose+nitrate (C7) and Sucrose+nitrate+DSF (C8). A volume of 30 ml of synthetic media in 125 ml flasks were inoculated with seed cultures to intital cell concentration of 0.5 OD units (at 600 nm). The cultures were incubated in orbital shaker at 30 ^{o}C and 150 rpm for 24h. All experiments were performed in biological triplicates. Samples were withdrawn during cultivations and analysed for cell density at 600 nm. Growth rate (µ h^{-1}) was calculated from the slope of the logarithmic growth phase as previously described (Leite et al., 2013). At defined times, samples were centrifuged at 4 ^{o}C to separate the supernatants and the cell sediments were immediately frozen in liquid nitrogen and stored at -80 °C until RNA extraction.

2.3. Quantification of extracellular metabolites

Supernatants were collected, filtered with 0.22 µm membranes (Millipore) and used for HPLC analyses for the following metabolites: glucose, sucrose, acetate, ethanol and glycerol. These compounds were separated by an Aminex HPX-87H BioRad column at 60 °C, using 5 mM H_2SO_4 as the mobile phase at 0.6 mL min^{-1} flow rate and detected by the refraction index. Standard calibration curves were performed and metabolite parameters were calculated as described by Leite et al. (2013). CO_2 yield was estimated from the stoichiometry of gas production from biomass (Leite et al., 2013) along with the stoichiometric estimated production from fermentation products (acetate+ethanol).

2.4. Relative gene expression analysis

Cells were submitted to cell lysis by suspending in 200 µL of lyticase solution. Afterwards, total RNA was extracted using Maxwell® 16 LEV simplyRNA Blood Kit in Maxwell device (Promega Co., USA), according to the manufacturer instructions. RNA was quantified in Nanodrop device (Thermo Fischer Scientific, USA) and its integrity assessed by 1% agarose gel electrophoresis in DEPC-treated TAE buffer

and dyed with ethidium bromide. cDNA was then synthesized using the GoScript™ Reverse Transcription Mix, Oligo(dT) (Promega, USA) using 2,5 µg total RNA for each 20 µl reaction tube, thus standardizing the RNA input concentration for the quantification of gene expression. qPCR assays were performed on the ABI Prism 7300 detection system (Applied Biosystems, USA) using the GoTaq® qPCR Master Mix kit. The choice for reference genes, primer validation procedures and data analysis were performed as described by De Barros Pita et al. (2012) for *D. bruxellensis*. All experiments were performed in biological duplicate with technical triplicates for each condition.

2.5. Principal component analysis

The data from the eight conditions analyzed were loaded into the ClustVis tool for principal component analysis as described by Metsalu and Vilo (2015). After loading, the tool automatically detects the number of default rows and columns from that analysis. Principal component analysis was performed using the pcaMethods R package (Stacklies et al., 2007), using the standard SVD (Singular Value Decomposition) method. Subsequently, the heatmap was generated using pheatmap R package (version 0.7.7).

2.6. Equations, stoichiometry and calculations

- Glycolysis: 1 mol glucose + 2 mols ADP + 2 mols Pi + 2 mols NAD^+ = 2 mols pyruvate + 2 mols ATP + 2 mols NADH
- Ethanol fermentation: 2 mols pyruvate + 2 mols NADH = 2 mols CO_2 + 2 mols ethanol + 2 mols NAD^+
- Acetate production: 2 mols pyruvate + 2 mols $NAD(P)^+$ = 2 mols CO_2 + 2 mols acetate + 2 mols NAD(P)H
- Pentose Phosphate Pathway: 3 mols glucose-6P + 6 mols $NADP^+$ = 1 mol fructose-6P + 3 mols glyceraldehyde-3P + 3 mols CO_2 + 6 mols NADPH
- Nitrate assimilation: 1 mol nitrate + 1 mol 2-oxoglutarate + 5 mols NADPH = 1 mol glutamate + 5 mols $NADP^+$
- *D. bruxellensis* biomass formation (Leite et al., 2013): $C_6H_{12}0_6$ + 0.68 NH_3 + 1.27 O_2 = 4.57 $CH_{1.754}O_{0.583}N_{0.149}$ + 1.43 CO_2 + 3.01 H_2O
- Yield: grams of products/grams of sugar consumed
- Sugar uptake rate: sugar consumed (mM)/time of consumption (h)

- Mol of carbon in the yeast biomass: [[gDW/L]·0.48]/12 (the biomass of *D. bruxellensis* is composed by 48% of carbon. Carbon molecular mass = 12)
- Carbon efficiency assimilation: [gDW/L]/[mmol consumed sugar · nr. of pyruvate equivalent] (glucose = 2 pyruvate equivalent; sucrose = 4 pyruvate equivalent)

3. Results and discussion

Growth experiments of *D. bruxellensis* were performed with the combination of the following variables: (i) two fermentable C sources (glucose and sucrose), (ii) two different N sources (ammonium or nitrate) and (iii) in the absence or presence of disulfiram (DSF), known for the inhibition of the enzyme acetaldehyde dehydrogenase (Acdh, EC 1.2.1.10) (Mirek et al., 2012; Teles et al., 2018). Physiological parameters were calculated based on the quantifications, except with reference to CO_2 that was stoichiometrically calculated (Leite et al., 2013; Teles et al., 2018). Mass balance and carbon balance were then calculated to evaluate data consistence. The reference condition consisted of YNB medium containing glucose as C source and ammonium as N source. The physiological data in this reference condition, including mass and carbon balance calculations, indicated that all measurements were done with reliability (Table 1). All results were compared with those very accurated quantitative physiology data reported by Leite et al. (2013) that included both shake flask and chemostat cultivations.

3.1. The Pdh bypass regulates the ethanol production

Aerated growth in glucose is known to induce the respiro-fermentative metabolism, a common trait between *S. cerevisiae* and *D. bruxellensis* from which the yeasts use the sugar via respiratory/oxidative metabolism but also deviate carbon to fermentation. Hence, both yeasts are classified as Crabtree positive (Leite et al., 2013). Analysis of the data in reference condition aimed to setup the basic quantitative physiological profile of this yeast in order to estimate the carbon distribution and its basic metabolic behavior.

Table 1. Respiro-fermentative parameters at the end of 24 h of aerated cultivations of *Dekkera bruxellensis* GDB 248 in the presence of glucose as carbon source, ammonium (NH_4) or nitrate (NO_3) as nitrogen source and biochemical inhibitor of the enzyme acetaldehyde dehydrogenase disulfiram (DSF).

	NH_4	NH_4+DSF	NO_3	NO_3+DSF
Mass balance				
Sugar consumption (g L^{-1})	20.2	17.5	16.3	5.22
Growth rate (μ, h^{-1})	0.14	0.13	0.08	0.09
Biomass yield (g g^{-1})	0.07	0.06	0.03	0.08
Ethanol yield (g g^{-1})	0.35	0.48	0.00	0.00
Acetate yield (g g^{-1})	0.16	0.21	0.46	0.00
CO_2^* yield (g g^{-1})	0.47	0.63	0.34	0.02
Mass recovery (%)	105	138	83	10
Carbon balance				
Substrate (mmol C)	673.3	583.3	543.3	174.0
Products (mmol C)	685.3	783.4	394.3	18.9
Biomass	57.1	43	19	16.1
Ethanol	308.3	366.5	0.0	0.0
Acetate	104.7	123.0	248.3	0.0
CO_2^*	215.3	250.9	127	2.9
Carbon recovery (%)	102	134	73	11

The typical eight hours of lag phase period for GDB248 strain was observed (Fig. 2a) followed by an exponential phase in which the cell population grew at 0.14 h^{-1} (Table 1), as previously reported (Leite et al., 2013). All sugar was consumed at the end of 24h of cultivation (Table 1), with estimated final rate about 7 mmol of glucose consumed per hour of cultivation (Fig. 2b). In this condition, ethanol was produced by the cells to 7.1 g L^{-1} (Fig. 2c) as the result of the respiro-fermentative metabolism (Fig. 2a), with calculated yield of 0.35 g g^{-1} (Table 1). It represented almost 46% of the consumed carbon being converted to ethanol (Table 1). This in the range of what

was previously reported for aerobic cultivations (De Barros Pita et al., 2013b; Teles et al., 2018) and lower than found in anaerobiosis (Peña-Moreno et al., 2019) and oxygen-limited fermentation (Galafassi et al., 2011). Acetate was also produced at 3.4 g L^{-1} (Fig. 2c) with calculated yield of 0.16 g g^{-1} (Table 1), representing 15% of the consumed carbon deviated via Acdh to acetate (Table 1). This is a typical trait of this acetogenic yeast (De Barros Pita et al., 2013b; Leite et al., 2013; Teles et al., 2018). In molar terms, the final ethanol concentration was tree times higher than acetate (154 mM vs. 52 mM, respectively). Hence, it can be concluded that in this reference condition the metabolic flux towards ethanol via Adh was tree times higher than the flux through acetate via Acdh (see Fig. 1 for metabolic routes). A previous study reported a lower ethanol:acetate proportion both for shake flask (1.8:1) and after pulsing C-limited chemostat with glucose (1.5:1) (Leite et al., 2013). It should be noted that those experiments were performed with much higher agitation, which stimulates the oxidative metabolism and acetate production. In *S. cerevisiae*, these proportions are higher (5:1), leading to the preference for ethanol production in that yeast (Van Maris et al., 2003). Our results confirm the acetogenic character of *D. bruxellensis* and indicates an intense metabolic dispute at the branchpoint of acetaldehyde between the fermentative pathway (Adh) and the Pdh bypass (Acdh) (Fig. 1).

Figure 1. Schematic representation of the central metabolism of *Dekkera bruxellensis* connecting the carbon and the nitrogen assimilatory routes. Dissimilatory reactions were highlighted as the carbon is lost to the medium in the form of CO_2, glycerol, ethanol and acetate. Abbreviations: ATP, adenosine triphosphate; CoA, coenzyme A; CO_2, carbonic dioxide; DHA-3P, dihydroxyacetone 3-phosphate; (GA-3P) glyceraldehyde 3-phosphate; NADH, nicotinamide adenine dinucleotide reduced; NADPH, nicotinamide adenine dinucleotide phosphate reduced; NH_4^+, ammonium; NO_2^-, nitrite; NO_3^-, nitrate; PPP, Pentose Phospate Pathway; Pi, phosphate; TCA, TriCarboxilic Acid cycle.

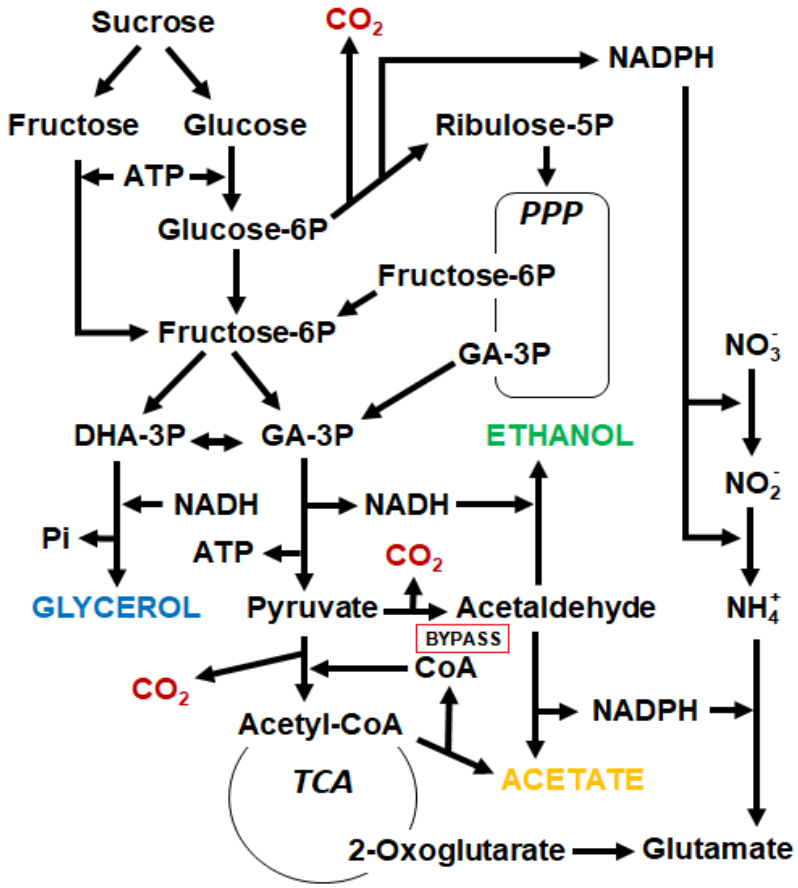
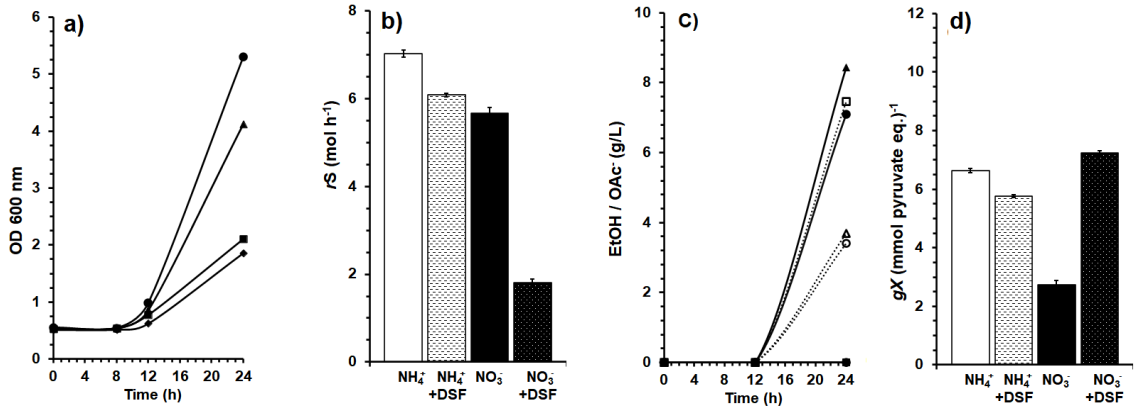


Figure 2. Growth profile (panel a), sugar consumption rate (panel b), production of metabolites (panel c) and biomass formation per mol of equivalent pyruvate consumed (panel d) of *Dekkera bruxellensis* GDB 248 cells cultivated under respiro-fermentative condition in synthetic defined medium containing GLUCOSE. Symbols are as follow. Growth condition 1 in the presence of ammonium: full circle for growth (panel a), white column for sugar uptake (panel b), full circle straight line for ethanol (EtOH) and open circle dotted line for acetate (OAC^-) (panel c), and white column for biomass formation (panel d). Growth condition 2 in the presence of ammonium+disulfiram: full triangle for growth (panel a), white dotted column for sugar uptake (panel b), full triangle straight line for ethanol (EtOH) and open triangle dotted line for acetate (OAC^-) (panel c), and white dotted column for biomass formation (panel d). Growth condition 3 in the presence of nitrate: full square for growth (panel a), black column for sugar uptake (panel b), full square straight line for ethanol (EtOH) and open square dotted line for acetate (OAC^-) (panel c), and black column for biomass formation (panel d). Growth condition 4 in the presence of nitrate+disulfiram: diamond square for growth (panel a), black dotted column for sugar uptake (panel b), full diamond straight line for ethanol (EtOH) and open diamond dotted line for acetate (OAC^-) (panel c), and black dotted column for biomass formation (panel d). Standard deviations of curves and metabolites were less than 5%.



Together, the results on ethanol and acetate production indicated that 90.4% of the carbon in the consumed glucose was pushed towards the fermentative pathway at the pyruvate crossroad in the end of glycolytic pathway. First, the pyruvate was decarboxylated to acetaldehyde by the Pdc, with initial lost of 30.1% of the carbon in the form of CO_2. Then, 45% of carbon as acetaldehyde was converted to ethanol by the Adh while 15.3% was converted to acetate by the Acdh. This is indicative of the potential of *D. bruxellensis* in producing ethanol, despite the tendency to produce acetate and presence of three phosphorilation sites (P/O) in the respiratory chain of the yeast (Leite et al., 2013). Therefore, the challenge to increase the fermentative capacity of *D. bruxellensis* to the level of *S. cerevisiae* is to increase the metabolic conversion at the branchpoint of acetaldehyde by Adh at the expense of the Acdh activity. The remaining 9.6% of the carbon went to the TCA cycle and to the oxidative metabolism by its conversion to acetyl-CoA by the Pdh. In this reaction, 1.3% of the carbon was dissimilated as CO_2 while the remaining 8.3% of the carbon in the form of acetyl-CoA was used in the anabolic reaction for biomass production (Table 1). Based on this carbon distribution, it was possible to estimate that each mol of pyruvate that entered the TCA cycle was enough to produce 6.63 g of yeast biomass (Fig. 2d).

From the energetic point of view, the 112 mmols of glucose metabolised through the glycolytic pathway (calculated from the glucose uptake – Table 1) led to the production 224 mmols of NADH. However, 154 mmols of NADH were directed to reduce acetaldehyde to ethanol via Adh (calculated from the ethanol produced – Table 1), with a surplus of 70 mmoles of NADH. Moreover, 52 mmols of NADH were produced by the Pdh bypass in the oxidation of acetaldehyde to acetate via the Acdh (calculated from the acetate produced – Table 1). In addition to the cytosolic NADH,

the glycolytic pathway might be produced 224 mmols of ATP at substrate level. Then, the 18 mmols of pyruvate (8% of the carbon entering the TCA cycle) would produce 72 mmols of NADH and 18 mmols of $FADH_2$. These reducing equivalent would produce a total of 252 mmols of ATP in the respiratory chain in the theoretical terms (see Material and Methods for calculations). In conclusion, during 24h of cultivation it might be estimated that 60 mmols of carbon plus 122 mmols of cytosolic reduced equivalents NAD(P)H and 476 mmols of ATP were avalible to generate 1.49 g of yeast biomass.

These calculations setup the physiological parameters for *D. bruxellensis* GDB248 shake flask cultivated in YNB medium with glucose and ammonium. Despite of the tendency for biomass and acetate production, it is clear that this yeast has a significant potential for ethanol production. This characteristic is directly connected with the activity of the cytosolic acetyl-CoA production pathway, also known as Pdh bypass, given that the activity of Acdh drains carbon from the acetaldehyde. To test this hypothesis, we used two strategies: chemical inhibition of Acdh using DSF and physiological induction of Acdh using nitrate as a nitrogen source.

3.2. The blockage of Acdh signals for lower biomass, which reduces glucose uptake rate yet increases the ethanol yield

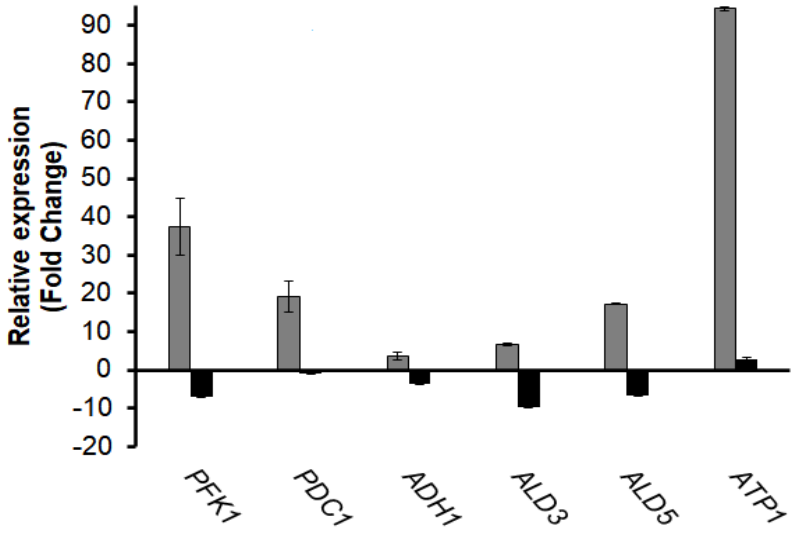
At the end of glycolytic pathway, pyruvate can be decarboxylated by the Pdc to acetaldehyde and this acetaldehyde can take two metabolic pathways: reduced to ethanol by Adh or oxidised to acetate by Acdh (Fig. 1). Therefore, we investigated how Acdh inhibition by DSF would influence the aerobic cell metabolism relative to the reference condition. The presence of this inhibitor reduced the final yeast biomass by 25% (Fig. 2a) and the estimated glucose consumption rate by 14% (Fig. 2b). It resulted in the detection of residual glucose at the end of 24h of cultivation (Table 1), in the same way as when Dsf was used in fermentation assays (Teles et al., 2018). In *S. cerevisiae*, DSF also interfered in several cellular parameters, such as growth decrease, glutathione depletion, mitochondrial disintegration and decrease in cell metabolic activity (Mirek et al., 2012). On the other hand, ethanol production was increased by 19% (Fig. 2c). This result corroborated the previous work reporting the effect of DSF in stimulating ethanol production by *D. bruxellensis* (Teles et al., 2018). The combination of reduced glucose consumption with increased ethanol production led to a significant increase of 37% in ethanol yield in aerobiosis (Table

1). In this case, the calculated ethanol yield in aerobiosis approached that calculated for fully anaerobic cultivation condition (Peña-Moreno et al., 2019). Paradoxically, acetate yield was also raised by 36% in a condition of Pdh bypass impairement (Table 1).

In a previous work, we showed that under fermentative condition acetate yield was unchanged by the presence of DSF. In that case, it was proposed that acetate was produced in the mitochondria and exported to the cytosol to fulfil the metabolic acetyl-CoA requirement for anabolic reactions, and that the excess leaked to medium (Teles et al., 2018). It might explain why less carbon was assimilated into the biomass in the present work (Fig. 2d), lost as exported acetate. These physiological data were corroborated by the relative expression data of genes involved in the fermentative pathway. The general expression profile was similar to that presented by Teles et al (2018), with the differences in values being a consequence of differences in the cultivation conditions (aerobic growth in the present work and fermentation assay in the previous work). In general, the increased expression of *PFK1*, *PDC1* and *ADH1* genes (Fig. 3) showed that the metabolic flux towards the fermentative pathway was increased by the presence of DSF. The upregulation of *ALD3* also shows that the Pdh bypass pathway is stimulated, although it is not functional by the presence of DSF. On the other hand, upregulation of the *ATP1* gene also showed high mitochondrial activity in this condition (Fig. 3). Previously, DSF was shown to inhibit ATP hydrolysis and was an important chemical in combating cellular resistance to anti-cancer drugs (Sauna et al., 2004). Finally, the higher upregulation of *ALD5* induced by DSF (Fig. 3) supported the hypothesis that the acetate detected comes from the mitochondria by the $_{mt}$Acdh, as previously reported (Teles et al., 2018). The *ALD5* expression profile also establishes the idea that the presence of DSF together with ammonium as the N source lightens the catabolic repression imposed by glucose, since low transcript levels should be detected in glucose, as previously observed in *S. cerevisiae* (Jacobson and Bernofsky, 1974; Aranda and Olmo, 2003) and *D. bruxellensis* (Teles et al., 2018).

Figure 3. Relative expression of *Dekkera bruxellensis* GDB 248 genes involved in the glycolysis (*PFK1*), the fermentative metabolism and Pdh bypass (*PDC1, ADH1, ALD3*), mitochondrial acetate production (*ALD5*) and mitochondrial ATP production (*ATP1*). Gray bars: gene expressions in synthetic defined medium containing glucose+ammonium+disulfiram relative to synthetic defined

media containing glucose+ammonium reference condition. Black bars: gene expressions in glucose+nitrate+disulfiram relative to glucose+nitrate reference condition. The values represent the average of two biological replicates with technical triplicates for each condition (standard error bars are shown).



In the scenario described above, the calculated flux of pyruvate towards Pdc activity was reduced to 70.2% in comparison to 90.4% in the reference condition. However, in this case all carbon might be converted to ethanol (46.8%) and to CO_2 (23.4%). On the other hand, the carbon flux to TCA (as acetyl-CoA) was increased from 9.6% to 29.8% in the presence of DSF. However, less biomass was produced (Fig. 2a), most likely due to the production of mitochondrial acetate, which accounts for 17% of the carbon that went to the TCA. This acetate was further dissimilated to the cultivation medium. In the passage through the Pdh, 8.6% of the carbon was dissimilated as CO_2 while only 5.5% was assimilated to biomass formation. At this point, it can be concluded that the capacity of *D. bruxellensis* in producing ethanol is indeed controlled by the functioning of the Phd bypass, defined by the efficiency of the cytosolic Acdh activity. However, blocking this pathway causes a decrease in glucose assimilation by signalling for lower biomass formation, which leaves residual sugar in the medium. This would harm industrial efficiency and does not prove to be a good metabolic strategy. Therefore, the challenge would be to increase the carbon flux to the fermentative pathway without altering the cells' ability to capture sugar from the substrate.

3.3. Nitrate assimilation impairs the fermentation pathway in aerobiosis

Nitrate assimilation has been considered a paramount trait of *D. bruxellensis* since it represents a cheaper source of nitrogen for industrial applications. Its cytosolic assimilation to organic nitrogen is very costly to cells in terms of reducing equivalents and is reported to be dependent on the Pdh bypass pathway (see Material and Metods for stoichiometry). Nitrate represents a selective advantage for this yeast in substrates like sugarcane (De Barros Pita et al., 2011) and can increment ethanol production under strict anaerobiosis (Peña-Moreno et al., 2019), despite its negative effect in aerobiosis (De Barros Pita et al., 2013; Peña-Moreno et al., 2021). Indeed, the use of nitrate in the present work reduced biomass formation to one third of that observed in ammonium (Fig. 2a) and the growth rate by almost half of the reference condition (Table 1), as previously reported (De Barros Pita et al., 2013b). Residual sugar accounted for 20% of its initial concentration (Table 1), as the result of the reduction in its consumption rate (Fig. 2b). In this case, no ethanol was detected while acetate was twice higher than in the reference condition with ammonium (Fig. 2c). It contrasted with those reporting high ethanol yields in oxygen-limited cultivation conditions (Blomqvist et al., 2012; Peña-Moreno et al., 2019).

The bulk damand for NADPH deviated 94.5% of the consumed carbon in the form of pyruvate to the Pdh bypass to be converted to acetate (63%) and CO_2 (31.5%). Therefore, only 5.5% of the carbon were available for the TCA cycle and to the oxidative metabolism. As consequence, most of carbon was dissimilated as acetate with a huge reduction in biomass formation (Fig. 2d). Nitrate also induced the production of acetate in agitated cultivations (De Barros Pita et al., 2013a), and fermentative tests with glucose (Peña-Moreno et al., 2019). The increased demand for NADPH makes Acdh highly active in the convertion of acetaldehyde to acetate. It negatively affected Adh and, consequently, the ethanol production (Table 1). It corroborates with the above-mentioned assumption that the ethanol fermentation capacity of *D. bruxellensis* is dependent of the Pdh bypass functioning. In addition to the fact that nitrate stimulates the Pdh bypass, gene expression and proteomic data indicated that the PPP pathway is highly induced in *D. bruxellensis* cells when nitrate is the N source (De Barros Pita et al., 2013; Barbosa Neto et al., 2014). It has also been pointed out that the aerobic assimilation of nitrate imposes severe oxidative stress to the yeast cells, as revealed by the proteomic data (Penã-Moreno et al., 2021).

In the context reported above, two highly reducing power demanding processes were activated: the nitrate assimilation pathway and the oxidative stress tolerance mechanism. Both processes require the activity of NADPH-producing pathways (the PPP pathway and the Pdh bypass). The theoretical amount of NADPH produced by Pdh bypass was calculated in 124 mmols, far less than the 375 mmols needed to convert the initial 75 mmoles of nitrate in the medium to intracellular glutamate. If all consumed glucose was flowing through the PPP pathway it could produce additional 182 mmols of NADPH, totaling 306 mmols of NADPH. Still, this amount would be enough to assimilate only 82% of the nitrate. However, we had only 33% of the biomass from the reference condition (Table 1), which indicates that most of the NADPH was in fact used to protect cells from oxidative stress. Finally, the high demand for reducing power and low NADPH production may make glycolytic NADH to be used also for biomass production and/or cell protection. This means that there is no reducing equivalent to be used by Adh to reduce acetaldehyde to ethanol, preventing alcoholic fermentation as it was observed (Table 1). These data led to the conclusion that over-stimulation of the Pdh bypass by nitrate in fact hampered the fermentation capactity of *D. bruxellensis.* However, the excess of acetate produced and lost in the substrate would have great biotechnological importance if it was completely converted to acetyl-CoA, which would play an important role as a building block of molecules and polymers of industrial interest. Its disposal seems to be associated with a great decrease in biomass formation. Therefore, this loss could be avoided with a metabolic strategy that involved an acetyl-CoA synthetase that was activated independently of molecular signaling for biomass generation.

3.4. Medium aeration negatively interacts with nitrate assimilation

Afterwards, we blocked the Pdh bypass in nitrate medium using DSF in order to understand the role of each of the NADPH-producing pathways in nitrate assimilation. There was no additive effect of DSF on the decrease in biomass formation and on the growth rate caused by nitrate (Fig. 2a; Table 1), although the consumption of glucose was severely decreased (Fig. 2b). Neither ethanol nor acetate was produced in this condition (Fig. 2c). It indicated that the flux of carbon through the Pdc was blocked and that there was no excess of mitochondrial acetate to be dissimilated to the medium, as observed in ammonium in the presence of DSF. Almost all genes showed downregulation in relation to the nitrate-based medium (Fig.

3). Only the *ATP1* gene was upregulated, which once again shows the interference of DSF in mitochondrial metabolism, as previously reported. This analysis of the relative expression of genes showed that, in fact, cell metabolism was negatively affected by the concomitant presence of nitrate and DSF. On the other hand, it resulted in the highest level of carbon assimilation to the yeast biomass among all conditions tested in glucose, with the calculated value of 7.23 g of biomass produced per mol of pyruvate equivalent generated at the end of the glycolytic pathway (Fig. 2d). Glucose consumption was only 5.2 g, which would produce only 58 mmoles of NADPH if all this glucose were metabolized via PPP pathway to compensate the lack of the Pdh bypass. In this condition, the demand for glycolytic NADH is even greater than described for nitrate-based medium. Therefore, without available NADH, ethanol production does not occur and the fermentation pathway is impeded. Thus, all pyruvate produced would be metabolized by Pdh for oxidative metabolism and biomass formation. At this point, it can be concluded that the lack of the Pdh bypass pathway, allied to the high reducing power demand, slowdown the glycolytic flux to a level in which 100% of the carbon would have to be directed towards biomass formation (84.9%) and for the biomass-associated CO_2 (15.1%), and not wasted as fermentation products. Even so, the formation of biomass is very limited by the great oxidative stress imposed by the aerobic assimilation of nitrate. In other words, the industrial use of nitrate as a nitrogen source would be limited by the supply of oxygen to the cells, so that the smaller the aeration, the greater the formation of biotechnological products (biomass and/or building blocks).

3.5. The sucrose-derived Fructose would positively regulate the carbon flux via the Pdh bypass

Sucrose is a disaccharide present in one of the most abundant industrial substrates used for fuel-ethanol fermentation, sugarcane juice and molasse, and *D. bruxellensis* has been identified in several distilleries using this substrate (De Souza Liberal et al., 2007). Its capacity to convert sucrose to ethanol had been tested ever since (Pereira et al., 2012, 2014). Different from *S. cerevisiae, D. bruxellensis* uptakes sucrose and hydrolyzes it by an internal invertase (Leite et al., 2013). In addition, growth rate in this sugar was reported to be higher than in its constituint monosaccharides, glucose and fructose (Leite et al., 2013). Similarly, *S. cerevisiae* cells engineered for internal hydrolysis of sucrose also presents higher growth rate

than the wild-types (Badotti et al., 2008). That recombinant yeast expends more ATP for the active transport of sucrose, which speeds up the glycolytic flux for the substrate-level ATP production. As consequence, ethanol production can be also incremented (Basso et al., 2011). The same metabolic phenomenon does occur naturally in *D. bruxellensis* with the transport and the intracellular breakdown of sucrose (Leite et al., 2013). Shake flask cultivation showed an exponential growth rate of 0.16 h^{-1} (Table 2) after the typical eight hours of lag phase period of GDB248 strain (Fig. 4a), higher than the observed for glucose (Table 1). *D. bruxellensis* isolates from wine also showed higher growth rates in fructose compared to glucose (Da Silva et al., 2019) and isolates from fuel ethanol reached final biomass in aerobic cultures with fructose higher than in glucose (Da Silva et al., 2019). This is similar to our findings in the present work, however, in this case with sucrose (Fig. 2a and fig. 4a).

Figure 4. Growth profile (panel a), sugar consumption rate (panel b), production of metabolites (panel c) and biomass formation per mol of equivalent pyruvate consumed (panel d) of *Dekkera bruxellensis* GDB 248 cells cultivated under respiro-fermentative condition in synthetic defined medium containing SUCROSE. Symbols are as described in the legend of figure 2. Standard deviations of curves and metabolites were less than 5%.

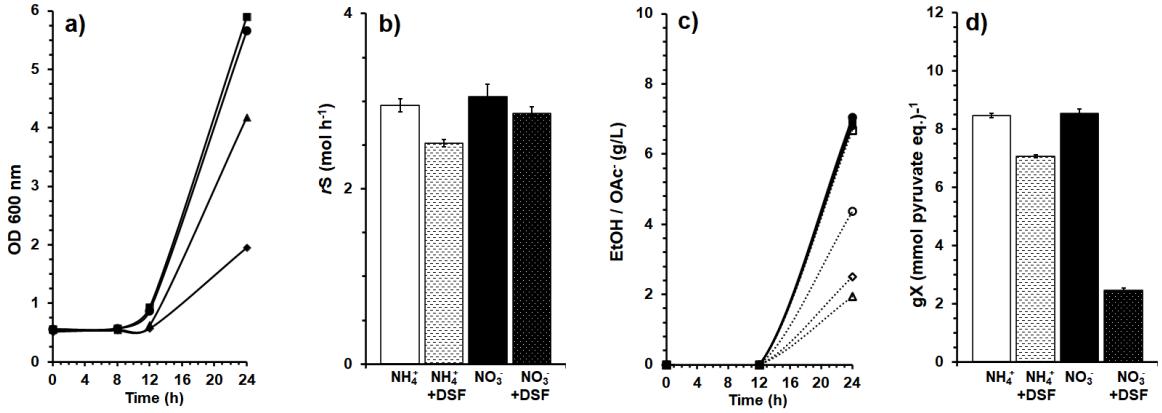


Table 2. Respiro-fermentative parameters at the end of 24h of aerated assays of *D. bruxellensis* GDB248 in the presence of sucrose as carbon source, ammonium (NH_4) or nitrate (NO_3) as nitrogen source and biochemical inhibitor of the enzyme acetaldehyde dehydrogenase disulfiram (DSF).

	NH_4	NH_4+DSF	NO_3	NO_3+DSF

Mass balance				
Sugar consumption (g L^{-1})	17.0	14.5	17.6	16.5
Growth rate (μ, h^{-1})	0.16	0.16	0.15	0.10
Biomass yield (g g^{-1})	0.09	0.08	0.09	0.03
Ethanol yield (g g^{-1})	0.41	0.48	0.39	0.41
Acetate yield (g g^{-1})	0.26	0.13	0.32	0.15
CO_2^* yield (g g^{-1})	0.61	0.58	0.64	0.64
Mass recovery (%)	137	126	145	123
Carbon balance				
Substrate (mmol C)	567.0	483.3	586.7	549.3
Products (mmol C)	749.3	604.5	810.5	588.5
Biomass	61.3	43.6	64.1	17.3
Ethanol	306.6	306.6	301.0	295.2
Acetate	145.7	64.3	189.0	83.3
CO_2^*	235.7	189.9	256.5	192.7
Carbon recovery (%)	132	125	138	107

The rate of sucrose uptake was almost half of the calculated for glucose (Fig. 4b), which represented 16% less carbon equivalent uptake as sucrose (567 mmol, Table 2) than as glucose (673 mmol, Table 1). Only 85% of the initial sugar was consumed and the final consumption rate was calculated as 2.95 mmol per hour of cultivation (Fig. 4b). Slightly less ethanol was produced from sucrose than glucose (Fig. 4c). Nevertheless, the ethanol yield was 20% higher with the disaccharide, indicating that 40.9% of the consumed carbon as sucrose was converted to ethanol (Table 2). Acetate was more produced in sucrose medium (4.7 g L^{-1}; Fig. 4c) than in glucose, which is in accordance with our previous work (Leite et al., 2013). Therefore, during the assimilation of sucrose, 30.2% of the consumed carbon were deviated via Acdh to acetate (Table 2), reducing to 2:1 the ethanol to acetate molar proportion. Similar to glucose, about 90.5% of the pyruvate from sucrose was pushed towards the fermentative pathway by the Pdc. However, there was a metabolic reorientation in sucrose at the acetaldehyde crossroads in which more carbon flowed through the Acdh. In fact, the genetic data showed that the *PDC1*, *ADH1* and *ALD3* genes are more expressed in sucrose than in glucose (Fig. 5a), supporting the

concomitant higher ethanol and acetate yields observed (Table 2). The *PFK1, ALD5* and *ATP1* genes were also up-regulated in the presence of the disaccharide. The same percentage of carbon was lost as CO_2 in both sugars for all metabolic reaction (31.5%). More carbon was directed to etanol in glucose while more carbon was deviated to acetate in sucrose. The remaining 9.5% of the carbon consumed went to the oxidative metabolism for biomass formation by the Pdh, the same percentage observed for glucose, representing 8.5 g of biomass being produced from each mmol of pyruvate that entered the TCA cycle (Fig. 4d).

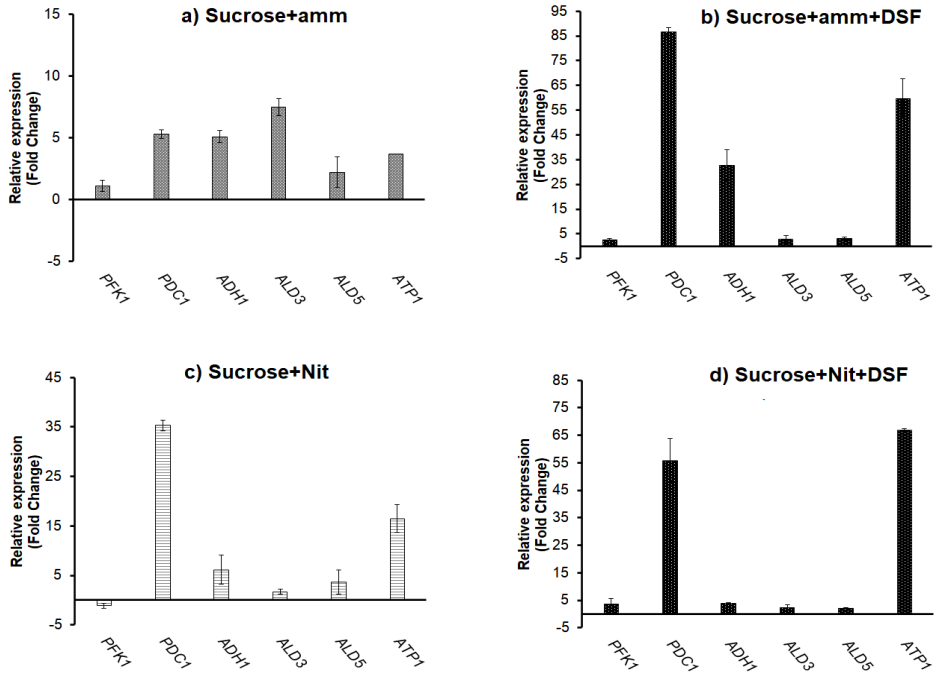
Therefore, even though less carbon was consumed, the efficiency of its conversion to the metabolic products biomass, ethanol and acetate was higher in sucrose than in glucose. One difference in the metabolization of these sugars is the production of fructose after sucrose breakdown, representing half of the sucrose molecule. Whether this monossacharide has any regulatory influence on the crossroad of acetaldehyde remains a matter of future studies. One possible explanation is that fructose is phosphorylated to fructose-6P that enters the glycolytic pathway after the branchpoint for the PPP pathway (Fig. 1). So, as less carbon enters the PPP pathway, decreasing the net production of NADPH, more carbon would be required by the Pdh bypass pathway to meet the anabolic requirement for this reduced cofactor.

3.6. The fructose might signal for carbon assimilation in the biomass when the Pdh bypass is not operational

The metabolic reorientation caused by sucrose in the flow through the Pdh bypass pathway was tested with the inhibition of Acdh by DSF. Final biomass (Fig. 4a) and sugar consumption rate (Fig. 4b) were reduced, leaving almost 30% of the initial sugar in the medium (Table 2). The production of ethanol was similar to the one observed when DSF was absent (Fig. 4c), but lower than in glucose. On the other hand, ethanol yield raised to the level observed for glucose in the presence of DSF (Table 2). Once again, inhibition of Acdh by DSF stimulated the production of ethanol by *D. bruxellensis* (Teles et al., 2018) to reach the yield calculated for fully anaerobic cultivations (Peña-Moreno et al., 2019), or with oxygen limitation in the presence of industrial substrates (Pereira et al., 2012, 2014). Unlike the observed in glucose, acetate production and yield were reduced by DSF in sucrose (Fig. 4c; Table 2). Then, about 76.1% of the carbon assimilated in the form of sucrose flowed from the

end of glycolysis to the fermentative pathway, with the first dissimilation of 25.4% in the form of CO_2 by the Pdc and the remaining 50.7% being conveted to ethanol by Adh. The genetic data showed that in fact *PDC1* and *ADH1* genes were significantly upregulated, while the *ALD3* gene was only mildly upregulated in presence of DSF (Fig. 5b). In this case, the acetate production detected in the culture medium seems to have been completely carried out in the mitochondria despite the mildly induction of *ALD5* gene (Fig. 5b).

Figure 5. Relative expression of *Dekkera bruxellensis* GDB 248 genes involved in the glycolysis (*PFK1*), the fermentative metabolism and Pdh bypass (*PDC1, ADH1, ALD3*), mitochondrial acetate production (*ALD5*) and mitochondrial ATP production (*ATP1*). Relative gene expressions were evaluated in cells cultivated in synthetic defined media containing sucrose+ammonium (panel a), sucrose+ammonium+disulfiram (panel b), sucrose+nitrate (panel c) and sucrose+nitrate+disulfiram (panel d). The condition of glucose+ammonium was used as reference for all four test conditions. The values represent the average of two biological replicates with technical triplicates for each condition (standard error bars are shown).



Similar to the observed in glucose (Fig. 3), *ATP1* gene was highly upregulated, which proves once again the interference of DSF in the energy metabolism of the *D.*

bruxellensis, clearly in response to all its effect on yeast growth pathways. The remaining 23.9% of the carbon from sucrose flowing through the glycolytic pathway was metabolized in the mitochondria via Pdh, with 6.1% dissimilated as CO_2 by this reaction to produce acetyl-CoA to feed the TCA cycle. Then, 7.2% was assimilated to the biomass and 10.6% converted to acetate by *Ald5p*, which was transported to the cytoplasm and dissimilated to the medium.

In glucose and ammonium, the presence of DSF did not significantly alter the carbon flux to ethanol. However, in the presence of sucrose this flux decreases in relation to glucose but increases in the presence of DSF. On the other hand, the carbon flux to biomass was reduced in glucose by the presence of DSF, while it remained practically stable between sucrose and sucrose plus DSF. Therefore, the presence of fructose derived from sucrose hydrolysis, in a situation of inactivation of the Pdh bypass pathway, in fact seems to induce a signaling system for greater carbon fixation in the biomass, with less carbon loss in the form of mitochondrial acetate. This would occur without loss of ethanol production, which is an industrial advantage, as little cell formation could keep the yeast population more active during biomass recycling. Nevertheless, once again the major industrial problem was the significant residual sugar left at the end of the process, which compromises the global industrial yield.

3.7. The sucrose-derived fructose seems to release the cells from the fungistatic effect of nitrate

The presence of nitrate in sucrose-rich substrate such as sugarcane juice increased the fermentative capacity of *D. bruxellensis* (De Barros Pita et al., 2011). Therefore, it makes relevant the study of this substrate combination. Unexpectedly, the presence of nitrate in the medium with sucrose provided the same final biomass (Fig. 4a), growth rate (Table 2) and sugar consumption rate (Fig. 4b) as those observed in ammonium. Also surprisingly, ethanol was produced at the same level as in ammonium (Fig. 4c), with a slight decrease in ethanol yield (Table 2). Even more acetate was produced (Fig. 4c), which increased the acetate yield (Table 2). The carbon distribution resembles that calculated for ammonium, with 90.7% being converted to acetaldehyde via Pdc. This validates the hypothesis that fructose from sucrose hydrolysis increases carbon flux via the Pdh bypass. However, unlike what was observed in glucose, in sucrose the fermentative pathway remained active in the

presence of nitrate. The confirmation of this metabolic signaling was made by detecting the high expression of the *PDC1* gene (Fig. 5c). In this context, the molar ratio of ethanol to acetate dropped further to 1.5:1. Clearly, the explanation for this is based on the more balanced partitioning between the fermentative pathway and the Pdh bypass, as it is evident from the fact that both *ADH1* and *ALD3* were only mildly upregulated in this medium (Fig. 5c) compared to glucose. This allowed us to calculate that 23.3% of the carbon metabolized by Pdc resulted in acetate and 37.1% was converted to ethanol (Table 2). The amount of consumed sucrose would produce 196 mmoles of NADH in the glycolytic pathway plus 94 mmoles of NADPH generated in the Pdh bypass during acetate production by Acdh, totaling 290 mmols of reduced cofactors. Considering that the observed production of ethanol required 150 mmoles of NADH, only 140 mmols of NAD(P)H was left for nitrate assimilation (and biomass formation) and for cell protection against oxidative damage. If all glucose was first metabolized via PPP pathway, it could produce 98 mmols of extra NADPH. Still, these 238 mmols of NADPH appear to be insufficient for cellular needs. So far, we have no plausible explanation for this massive production of biomass under these conditions. However, this scenario of large production of biomass and ethanol in sucrose was practically the same as observed by Peña-Moreno et al. (2021), even if it was in anaerobiosis. In that work, we show that there is a substitution of hexokinase for glucokinase. Added to other factors, the results indicated that the glucose catabolic repression mechanism was reliefed, allowing the induction of proteins from ribosome biogenesis and nucleotide production. Thus, protein and DNA synthesis could be induced, promoting superior growth even in this condition of oxidative stress. Therefore, although the hypothesis of oxidative stress is still upheld, these oxidizing agents seem to signal more for the induction of cell cycle arrest than to promote cell damage. As in the case above of the presence of DSF in a medium with ammonium, this scenario of biomass production and maintenance of high levels of ethanol seems to be very relevant from an industrial point of view. But again, the issue of residual sugar must be considered.

At this point in the study, it was evident that the type of metabolic regulation in *D. bruxellensis* when nitrate is the nitrogen source is different in glucose and sucrose. The hypothesis put forward is that the co-metabolization of fructose interferes with the functioning of the fermentative pathway and the Pdh bypass pathway. Then, we

block the Pdh bypass in the culture with sucrose and nitrate to try to unravel how much this pathway is responsible for this metabolic balance.

Biomass formation and growth rate were similar to values observed in glucose plus nitrate and DSF (Fig. 4a; Table 2), while sucrose uptake rate was similar to ammonium medium (Fig. 4b). Residual sucrose was also high, as observed for three other conditions using sucrose (Table 2). Once again, the inhibition of Acdh in nitrate did not affect ethanol production, which was 6.8 g L^{-1} (Fig. 4c), with ethanol yield of 0.41 g g^{-1} (Table 2). Acetate production and yield dropped when compared to the nitrate without DSF (Fig. 4c; Table 2). However, these parameters remained above those observed in ammonium plus DSF. The gene expression data showed that the type of sugar, either glucose or sucrose, promoted important differences in cell metabolism when in nitrate plus DSF: genes of ethanol fermentation and the Pdh bypass were all downregulated in glucose (Fig. 3) while upregulated in sucrose (Fig. 5d). It was calculated that 75.2% of the carbon from sucrose was converted to acetaldehyde by Pdc, a result that was supported by the genetic data of upregulation of *PDC1* gene (Fig. 5d). Hence, 50.2% of the carbon metabolised via Pdc were converted to ethanol and 25.1% was dissimilated as CO_2 (Table 2). Of the 24.8% of carbon that entered the TCA cycle via Pdh, it was estimated that 14.2% was converted to mitochondrial acetate and only 2.9% was assimilated into biomass, with 7.7% dissimilated as CO_2. In this condition, the reducing equivalents for nitrate assimilation were provided by the PPP pathway. Furthermore, it should be considered that the production of NADPH can occur due to the action of $_{mt}$Acdh, encoded by the *ALD5* gene, in the conversion of acetaldehyde to acetate in the mitochondria. Different from that observed in glucose, the efficiency of carbon assimilation in biomass was significantly reduced to only 2.5 g of biomass per mol of equivalent pyruvate (Fig. 4d). Therefore, there would be the potential to produce 92 mmols of NADPH through PPP and 42 mmols for through $_{mt}$Acdh calculated from the acetate detected. The sum of this reducing potential would be enough to fix only 27 mmols of nitrate to glutamate, or about one third of the total nitrate in the medium. Perhaps this is the explanation for the fact that the formation of biomass in this condition was only a third of that observed for media with sucrose and ammonium and for sucrose and nitrate. On the other hand, the amount of sugar flowing through the glycolytic pathway, considering that all the glucose from sucrose made an alternative route through the PPP pathway, was able to produce 183 mmols of

NADH, more than the 148 mmols required to produce the ethanol detected in the medium of culture.

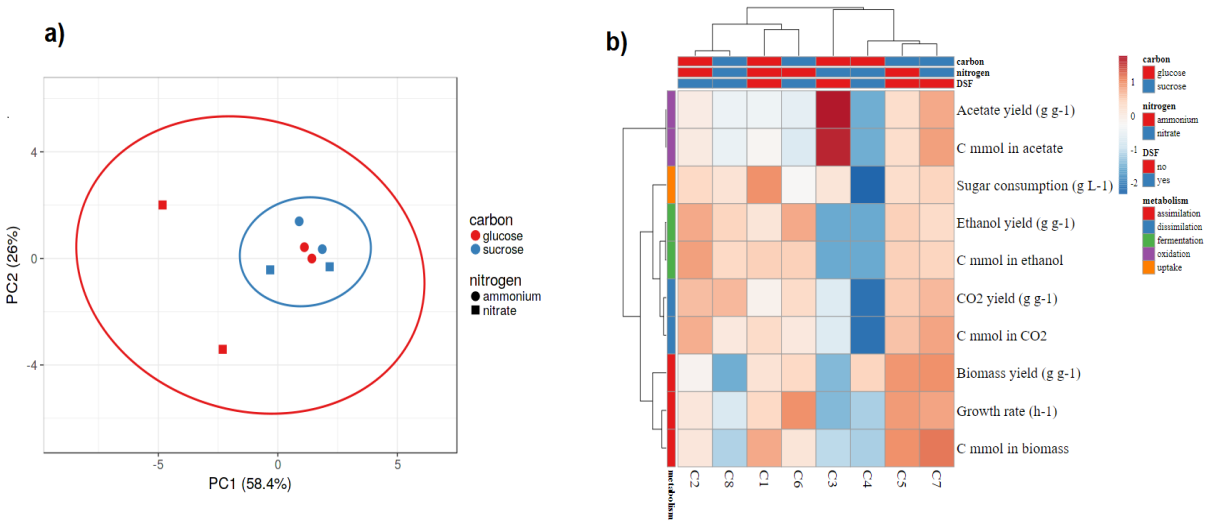
3.8. Clustering data confirm that sucrose induces positive effects on fermentative metabolism in *D. bruxellensis*

Eight cultivation conditions were tested by combining two carbon and two nitrogen sources, in the absence or presence of DSF. Overall, we calculated that 68% of the carbon consumed by the cells during the 24 hours of cultivations were converted to products (biomass, ethanol and acetate) while 32% was dissimilated as CO_2. The exception was the condition of glucose with nitrate and DSF which presented values of 85% and 15%, respectively. The difference between these conditions was the form of carbon distribution in the central metabolism. In ammonium, regardless of sugar and DSF, between 40% and 50% of the carbon was recovered in the form of ethanol, even though the cultures were aerated. This shows that, in fact, *D. bruxellensis* has a very relevant fermentative capacity and that it can be better exploited. In nitrate, this fermentative capacity is only maintained in sucrose, while abolished in glucose. This shows that fructose, generated from sucrose hydrolysis, must play a very important regulatory role in the distribution of carbon flux in central metabolism.

Principal component analysis was performed using all physiological parameters presented in Tables 1 and 2. The results showed that data from media containing glucose and ammonium formed a homogeneous group that included all conditions with sucrose (Fig. 6a). This does not mean that the data were similar between conditions, but that they varied evenly in each of them. On the other hand, the data from the conditions with glucose and nitrate were quite spread and with large internal variation (Fig. 6a). This shows that the presence of fructose from sucrose hydrolysis relieves the metabolic pressure exerted by nitrate, converting it from a secondary or non-preferred nitrogen source to a preferred one, such as ammonium. This has been reported when cells were cultured on glucose and nitrate in the absence of oxygen (Parente et al., 2018; Peña-Moreno et al., 2019).

Figure 6. Integrative analysis of physiological data of *Dekkera bruxellensis* GDB 248 obtained from eight cultivation conditions in synthetic defined media: glucose+ammonium (C1),

glucose+ammonium+disulfiram (C2), glucose+nitrate (C3), glucose+nitrate+disulfiram (C4), sucrose+ammonium (C5), sucrose+ammonium+ disulfiram (C6), sucrose+nitrate (C7) and sucrose+nitrate+disulfiram. Principal component (panel a) and data clusterisation (panel b) were shown.



When these parameters were clustered, those that had the same database were well-defined groups, such as ethanol yield and the conversion of consumed carbon to ethanol (Fig. 6b). This shows that the fermentation pathway is the biggest carbon sink in the metabolism of this yeast, as it happens in *S. cerevisiae*. Furthermore, the clustering of data on ethanol with sugar consumption shows that the flow through the glycolytic pathway is essential for the control of fermentative metabolism. Ethanol production would also be expected to be linked with acetate production, as both metabolites share a common input via Pdc activity. The fact that they do not form a group shows that the presence of DSF causes acetate to have a different origin, as postulated for its mitochondrial production by Ald5.

The metabolic disturbance that the assimilation of nitrate causes in *D. bruxellensis* cells in the presence of glucose, regardless of the functioning of the Pdh bypass, is reflected in the positioning of conditions 3 and 4 as single groups in the clustering analysis (Fig. 6b). Therefore, we propose that nitrate should not be used as a preferential N source when the substrate sugar is glucose, especially if the industrial process has some type of aeration. On the other hand, the metabolic homogeneity presented by conditions 5 and 7 was evident. This indicates that if the metabolic pathways are functioning properly, especially the Pdh bypass pathway, the use of sucrose makes the assimilation of ammonium and nitrate metabolically similar

from the energy-cost point of view. Regarding the genetic profile, condition 7 has a 35x increase in *PDC1* expression (Fig. 5c) while in condition 5 this increase was only 5x (Fig. 5a). This is the result of induction of the Pdh bypass by nitrate assimilation, while induced oxidative metabolism with the 15x overexpression of *ATP1* gene (Fig. 5c). This metabolic fit allows cells to behave very similarly in both culture conditions. An important factor that can connect these two scenarios is the theoretically lower production of NADPH via PPP, which would avoid an energy imbalance, keeping constant the production of biomass, ethanol and acetate. The relationship between these conditions was observed in strict fermentation experiments, that is, in the absence of oxygen, in which the physiological parameters of cells grown on sucrose with ammonium or nitrate were practically the same (Peña-Moreno et al., 2019).

Unexpectedly, cultivation conditions 2 and 8 formed a physiological cluster (Fig. 6b), despite the differences found in the production of biomass and acetate that were higher in condition 2 (Tables 1 and 2). Inhibition of the Pdh bypass by DSF caused cellular metabolism in nitrate to be generally lowered in sucrose as in the case of condition 8. This contrasts with the discussed above for conditions 5 and 7. However, this same inhibition of the Pdh bypass pathway promoted an overall increase in fermentation parameters in the presence of glucose and ammonium. A relevant fact here is the impressive level of induction of the *ATP1* gene of 95x in condition 2 (Fig. 3) and 70x in condition 8 (Fig. 5d), which indicates a high activity of oxidative phosphorylation in both culture conditions. Therefore, the maintenance of cellular balance in the assimilation of nitrate when sucrose is the available sugar only takes place when the Pdh bypass pathway is functioning properly.

Finally, conditions 1 and 6 were grouped by the fact that physiological variables present some similarity (Fig. 6b). The relative expression of the genes tested was higher in the medium containing sucrose, ammonium and DSF (condition 6) than in the one containing glucose and ammonium (condition 1). Another important aspect is the fact that the ethanol:acetate ratio increased from 3:1 in condition 1 to 5:1 in condition 6. This is further evidence that sucrose increases the carbon flux in the form of acetaldehyde through the fermentation pathway to the detriment of its entry in the TCA cycle. As the Pdh bypass is blocked by DSF, this carbon ends up being completely converted to ethanol.

4. Conclusion

In conclusion, the data reported in the present work show the ability of the yeast *D. bruxellensis* to shift carbon towards the oxidative metabolism of acetate production, establishing a lower proportion of ethanol:acetate ratio compared to the main industrial yeast*, S. cerevisiae.* Nevertheless, it is important to emphasize the efficient capacity of *D. bruxellensis* to produce ethanol with relevant yields. In this regard, when the enzyme Acdh is inhibited by DSF, the increase in the flow for ethanol production promoted yields close to the theoretical maximum and compared to anaerobic or oxygen limited assays. Furthermore, we identified that the acetate produced must come from the mitochondria when in presence of cytosolic impediment. Gene expression data relate to these results. Therefore, the capacity for ethanol production by *D. bruxellensis* is widely influenced by an adequate flux control of the pdh bypass, and the activity of the Acdh plays a fundamental role in this process. In the presence of nitrate as a nitrogen source, the data show the importance of the reducing power generated from the pdh bypass due to acetate production, which prevents the production of ethanol, but may represent a relevant strategy for the production and use of acetyl-CoA as building blocks for the production of molecules of industrial interest. The presence of sucrose in cultures with *D. bruxellensis* promoted an increase in ethanol and acetate yields, compared to glucose. Ethanol yield increased even more when Acdh was inhibited, which shows that high yields can also be achieved with another carbon source when the inhibitor is added. In this case, the significant increase in the expression of fermentation-related genes was a preponderant factor. Additionally, the combination of sucrose with nitrate allowed *D. bruxellensis* cells to produce the ethanol that was not produced with glucose as a carbon source. We propose that the fructose present in sucrose must be exerting a differential factor in this metabolic reorientation, as shown in the clustering analyses. This study provides valuable contributions to the understanding of the crossroads of ethanol and acetate production by the yeast *D. bruxellensis* and represents an advance for the characterization of this industrial microorganism.

Acknowledgements

We appreciate the technical support of all colleagues in the metabolic engineering group, the Federal University of Pernambuco (UFPE) and the University of Pernambuco (UPE).

Funding information

This work was supported by the Bioethanol Research Network of the State of Pernambuco (CNPq- FACEPE/PRONEM program, grant nº APQ-1452-2.01/10) and by grants of the National Council of Science and Technology (CNPq nº 472533/2013-4, 474847/2013-6 and 446927/2014-7).

Conflict of Interest

The authors declare that they have no conflict of interest.

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7 ARTIGO IV - THE METABOLISM OF RESPIRING CARBON SOURCES BY *DEKKERA BRUXELLENSIS* AND ITS RELATION WITH THE PRODUCTION OF ACETATE

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Abstract

Dekkera bruxellensis presents relevant industrial characteristics and the understanding of several aspects of its metabolism over the past years has expanded the comprehension of its predominant role in the alcoholic fermentation environment. Acetate is a metabolite often found in *D. bruxellensis* aerobic cultivations, whereas its production is linked to decreased ethanol yields. In a previous publication, we intended to know how its metabolism affected the yeast fermentation capacity. In the present work, we evaluated the role of its metabolism in respiring cells using ammonium or nitrate as nitrogen sources. The results showed that galactose is a strictly respiratory sugar and that a relevant part of its carbon is lost and the remaining is metabolised thought the Pdh bypass pathway before being assimilated into biomass. When this pathway was blocked, the yeast growth was reduced while more carbon was assimilated to the biomass. In nitrate, more acetate was produced as expected, which increased carbon assimilation, although less galactose was uptake from the medium. This picture was not affected by the Pdh bypass inhibition. The confirmation that acetate production was paramount for carbon

assimilation was brought by cultivations in pyruvate. All physiological data fitted to the expression patterns of *PFK1, PDC1, ADH1, ALD3, ALD5* and *ATP1* genes. Other respiring carbon sources could only be properly used by the cells when some external acetate was supplied. Therefore, the results reported in this paper aided in providing valuable contributions to the understanding of the oxidative metabolism in this potential industrial yeast.

Keywords: *Dekkera bruxellensis*; oxidative sources; disulfiram; acetate production; real-time PCR

Introduction

The yeast *Dekkera bruxellensis (*Van der Walt 1964*)* is well-known for its participation in industrial processes, being fairly linked to the production of alcoholic beverages, such as wine and beer (Renouf et al. 2006). Previous studies have identified *D. bruxellensis* as a main contaminant in the bioethanol production process in northeast Brazil, with differential factors in the maintenance and adaptability to this environment, such as the advantageous assimilation of nitrate (Basílio et al. 2008; De Barros Pita et al. 2011; De Souza Liberal et al. 2007). However, despite being identified as a contaminant, other studies have also pointed that *D. bruxellensis* has potential for industrial application, since it is also capable of producing ethanol with yields similar to *Saccaromyces cerevisiae* (De Barros Pita et al. 2013; Galafassi et al. 2010; Leite et al. 2013; Pereira et al. 2014). This fermentative capacity observed in *D. bruxellensis* is related to the Crabtree effect, i.e., the ability to ferment under aerobic conditions and high sugar concentration, which is shared with *S. cerevisiae* (Piskur et al. 2006; Rozpędowska et al. 2011). On the other hand, the fermentative performance of *D. bruxellensis* has some relationship with the metabolic deviations for either biomass or acetate production from the oxidation of acetaldehyde (Pereira et al. 2014; Teles et al. 2018), featuring *D. bruxellensis* as a yeast that exhibits a preferentially oxidative metabolism (Leite et al. 2013). In this respect, the fermentation efficiency of *D. bruxellensis* increased considerably when the yeast decreased its biomass production in oxygen-limited synthetic cultures (Teles et al. 2018).

The ability to assimilate different carbon sources is one of the characteristics of the yeast *D. bruxellensis* (Conterno et al. 2006; Da Silva et al. 2019a; De Barros Pita et al. 2013; Galafassi et al. 2010; Leite et al. 2016; Reis et al. 2014). Nevertheless, few studies have evaluated the growth physiology of *D. bruxellensis* in so-called “poor” carbon sources such as ethanol and glycerol (Rodrigues et al. 2001; Teles et al. 2018), or with galactose (Moktaduzzaman et al. 2015; Da Silva et al. 2019a). In *S. cerevisiae*, ethanol is oxidized to acetaldehyde by the activity of the enzyme alcohol dehydrogenase isoform 2 (Adh2) (Kusano et al. 1998) and glycerol catabolism produces dihydroxyacetone phosphate by the enzymes glycerol kinase (Gut1) and dihydroxyacetone kinase (Dak), from glycerol-3-phosphate and dihydroxyacetone, respectively (Semkiv et al. 2017). Galactose, on the other hand, is metabolized to glucose-6-phosphate through the Leloir pathway (Frey 1996; Ostergaard et al. 2000; Bhat and Murthy 2001). Successive reactions after the production of glucose-6-phosphate (glycolytic pathway) lead to the production of pyruvate, which can be oxidized to acetyl-CoA by the enzyme pyruvate dehydrogenase (Pdh) or decarboxylated to acetaldehyde by the enzyme pyruvate decarboxylase (Pdc) (Flikweert et al. 1996; Pronk et al. 1996).

Acetaldehyde is a central compound and an important branch point in the fermentative metabolism, in which its oxidation or reduction determines the production of acetate or ethanol, respectively (Medina et al. 2016). In fermentative yeasts, the major branch is the production of ethanol from glucose via *ADH1* gene, which encodes the main enzyme isoform 1 of alcohol dehydrogenase (Ganzhorn et al. 1987; Stahlberg et al. 2008). When it comes to acetate, the second branch, the positive regulation of the *ALD3* gene coding for acetaldehyde dehydrogenase plays a major role in the production of cytosolic acetic acid (Heit et al. 2018). This step provides the necessary reducing power NADPH for the maintenance of the redox balance in *S. cerevisiae*. In addition, the expression of genes *ALD4* and *ALD5* (encodes mitochondrial isoforms) and *ALD6* (encodes cytosolic isoform) also has some contribution to the formation of acetic acid in yeast (Heit et al. 2018). The tendency to produce acetate in *D. bruxellensis* is related to the Custer effect, in which there is inhibition of alcoholic fermentation due to a redox imbalance (depletion of NAD^+ that was used to oxidize acetaldehyde) (Pronk et al. 1996; Van Diijken; Scheffers, 1986). Therefore, it is important to state that acetate and biomass production are considered energy deviations which lead to a reduction in the

availability of carbon for fermentation, directly affecting ethanol yields (De Barros Pita et al. 2013; Leite et al. 2013). The production of cytosolic acetate in yeasts is primarily performed through the so-called alternative PDH reactions, called the Pdh-bypass, which has as key components the enzymes pyruvate decarboxylase (Pdc), acetaldehyde dehydrogenase (Ald) and acetyl-CoA synthetase (Acs) (Pronk et al. 1996; Remize et al. 2000).

Recently, our research group sought to block acetate production by using disulfiram (DSF), a biochemical Ald inhibitor, and showed that *D. bruxellensis* increases its ethanol yield when the cytosolic enzyme Ald3 is inhibited, preferentially redirecting carbon to the fermentation pathway (Teles et al. 2018). The present work is a follow up study, expanding the characterization of oxidative metabolism in *D. bruxellensis*, evaluating the effects of oxidative sources on the physiology of the yeast. Gene expression analyses are discussed, and the results revolve around the influence of the availability of acetate in the respiro-fermentative metabolism of *D. bruxellensis.*

Materials and methods

Yeast strain and culture media

Dekkera bruxellensis GDB 248 (strain URM 8346) was used in the present work (Peña-Moreno et al. 2019). This strain is deposited at the Department of Mycology Culture Collection (URM-Recife), Federal University of Pernambuco, which is part of the World Directory of Collections of Culture of Microorganisms (WFCC) under the registration number 604 and can be released for research purpose upon request. Experiments were also performed using the strain *S. cerevisiae* JP1 as a reference (Pereira et al. 2012). Cell maintenance was performed in YPD medium (10 g L^{-1} yeast extract; 20 g L^{-1} peptone; 20 g L^{-1} dextrose and 20 g L^{-1} agar). Subsequently, cells were washed in 0.9% saline to eliminate waste from YPD medium. The reference medium for precultures and specific assays was synthetic YNB (Yeast Nitrogen Base w/o amino acids and ammonium sulfate, 1.7 g L^{-1}) and ammonium sulphate. Seven carbon sources were used for growth evaluation with initial equimolar concentration defined as 660 mM of carbon: galactose, pyruvate, citrate, alpha-ketoglutarate, acetate, glycerol and ethanol. Moreover, nitrogen was added either as ammonium sulphate (5 g L^{-1}) or sodium nitrate (6.5 g L^{-1}) in a concentration of 75 mM of total nitrogen. Disulfiram (DSF) was used in the

concentration of 60 μM (Teles et al. 2018) and the antioxidant N-acetylcysteine (NAC) at concentrations of 5 mM (Kwolek et al. 2012).

Growth assays in microtiter plate and flasks

Aerobic growth cultures were performed on a multi-detection microplate reader (Biotek Synerg HT), using 96-well microplate or shaker incubator flasks. For growth in microplate reader and flasks, cells were primed at 0.1 OD 600nm in a volume of 150 μL and 0.5 OD 600nm in 30 ml of medium in 125 ml flasks, respectively. Cultivations were performed under 30 ^{o}C and continuous agitation for 24 or 48 hours. All experiments were performed in biological duplicates with technical triplicates and negative control containing culture medium only. Growth rate (μ, h^{-1}) was calculated from the slope of the logarithmic growth phase as previously described (Leite et al. 2013). From flask cultivations, supernatants were collected and submitted to analysis by high performance liquid chromatography (HPLC) and cells were immediately frozen in liquid nitrogen and stored at -80 °C until RNA extraction (Teles et al. 2018).

Quantification of extracellular metabolites

Samples collected during growth assays were filtered in a 0.22 μm filter (Millipore) and used for HPLC measurement of the following metabolites: glucose, galactose, pyruvate, citrate, ethanol, glycerol and acetate. These compounds were separated by an Aminex HPX-87H BioRad column at 60 °C, using 5 mM H_2SO_4 as the mobile phase at 0.6 mL/min flow rate and detected by the refractive index of the samples. In order to determine the concentrations, a standard calibration curve was used. Metabolite parameters were calculated as described by Leite et al (2013).

Relative gene expression analysis

Total RNA was extracted using Maxwell® 16 LEV simplyRNA Blood Kit, quantified on Nanodrop device (Thermo Fischer Scientific, USA), and its integrity assessed by 1% agarose gel electrophoresis in DEPC-treated TAE buffer and dyed with ethidium bromide. cDNA was then synthesized using the GoScript^{TM} Reverse Transcription Mix, Oligo(dT) (Promega, USA) using 2.5 μg total RNA for each 20 μl reaction tube, thus standardizing the RNA input concentration for the quantification of gene expression.

Real-time PCR assays were performed on the ABI Prism 7300 detection system (Applied Biosystems, USA) using the GoTaq® qPCR Master Mix kit. The choice for reference genes, primer validation procedures and data analysis were

performed as described by De Barros Pita et al (2012). All experiments were performed in biological duplicate with technical triplicates for each condition.

Results

Galactose metabolization wastes most of the carbon in a pointless dissimilation

Cells of *D. bruxellensis* GDB 248 (URM 8346) were cultivated in mineral medium containing galactose as C source and ammonium or nitrate as N source, in the absence or presence of disulfiram (DSF) (Fig. 1). Growth curves in galactose and ammonium showed the typical eight hours of lag phase for GDB 248 followed by an exponential phase in which the cell grew at 0.11 h^{-1} (Table 1). Yeast growth rates were progressively reduced in ammonium+DSF, nitrate and nitrate+DSF, respectively (Fig. 1a; Table 1). The rate of galactose assimilation (Fig. 1b) was 17% lower than those calculated for glucose and sucrose (considering the mol of hexose monomer equivalent) (manuscript submitted).

Furthermore, the results indicated that the aerobic assimilation of nitrate reduces the flux of galactose metabolization (Fig. 1; Table 1) and no further negative effect on the yeast physiology was observed in the presence of DSF (Fig. 1a). On the other hand, biomass yield doubled in the presence of DSF or nitrate, or both (Table 1). This effect was evident from the calculation of biomass formation per mol of substrate consumed, thereof denominated carbon assimilation efficiency (Fig. 1c). For this calculation, we considered the amount of pyruvate equivalent, as this metabolite is the final product of hexose metabolism through the glycolysis and represents the metabolic branch point between respiration and fermentation.

Figure 1 Physiological analysis of cell growth (panel a), substrate uptake rate (panel b) and biomass production by consumed carbon, denominated as carbon assimilation efficiency, (panel c) of the *Dekkera bruxellensis* GDB 248 cultivated in mineral medium containing galactose as carbon source and ammonium (NH_4) (circle symbols) or nitrate (NO_3) (tringle symbols) as nitrogen source. Media were also supplemented with the acetaldehyde dehydrogenase inhibitor disulfiram (DSF) (square symbols for ammonium or diamond symbols for nitrate). Bars represent standard deviation from the mean value of two independent experiments, with technical triplicate for each one.

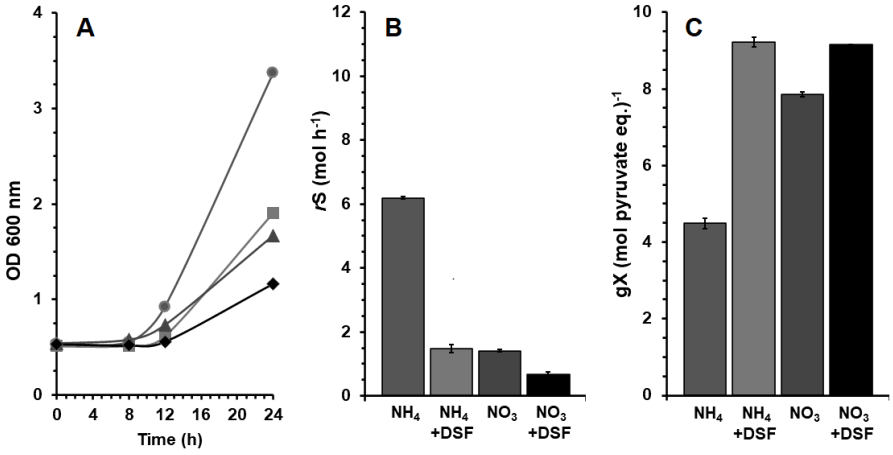


Table 1 Physiological data calculated at the end of 24h cultivation of *Dekkera bruxellensis* GDB 248 in mineral medium containing galactose as carbon source and ammonium (NH_4) or nitrate (NO_3) as nitrogen source. Media was also supplemented with the acetaldehyde dehydrogenase inhibitor disulfiram (DSF).

	Mass distribution					Carbon distribution			
	NH_4	NH_4+DSF	NO_3	NO_3+DSF		NH_4	NH_4+DSF	NO_3	NO_3+DSF
Growth rate (μ, h^{-1})	0.11	0.09	0.07	0.06	Consumption (mmol C)	600.7	148.0	141.3	71.0
Sugar consumption (g L^{-1})	18.02	4.45	4.22	2.13	Production (mmol C)	41.9	121.3	108.0	9.3
Biomass yield (g g^{-1})	0.05	0.10	0.08	0.09	Biomass	35.6	17.4	14.1	7.9
Ethanol yield (g g^{-1})	0.00	0.00	0.00	0.00	Ethanol	0.0	0.0	0.0	0.0
Acetate yield (g g^{-1})	0.00	0.45	0.43	0.00	Acetate	0.0	67.3	61.0	0.0
CO_2* yield (g g^{-1})	0.02	0.36	0.34	0.03	CO_2*	6.3	36.6	32.9	1.4
Mass balance (%)	7	92	86	12	Carbon recovery (%)	7	82.0	76.4	13.1
Mass loss (%)	93	8	14	88	Carbon loss (%)	93	18.0	23.6	86.9

* Stoichiometricaly calculated (Teles et al. 2018).

In ammonium medium, the calculated rate of galactose uptake by *D. bruxellensis* was 17% lower than glucose (manuscript submitted), resulting in 23% reduction in growth rate and 40% reduction in final biomass (Fig. 1b; Table 1). It means that the carbon flux through the glycolytic pathway was slower in galactose than in glucose. Hence, the glycolytic NADH might be fully re-oxidised by respiration, instead of by fermentation as in *S. cerevisiae*. In this culture condition, acetate was also undetected (Table 1).

The relevant problem in the measuremnts was the extremely low mass balance and carbon recovery calculated for this condition, showing that only 7% of the consumed sugar mass was recovered as biomass and biomass-associated CO_2 (Table 1). All chromatograms from HPLC analyses were very clear and showed no

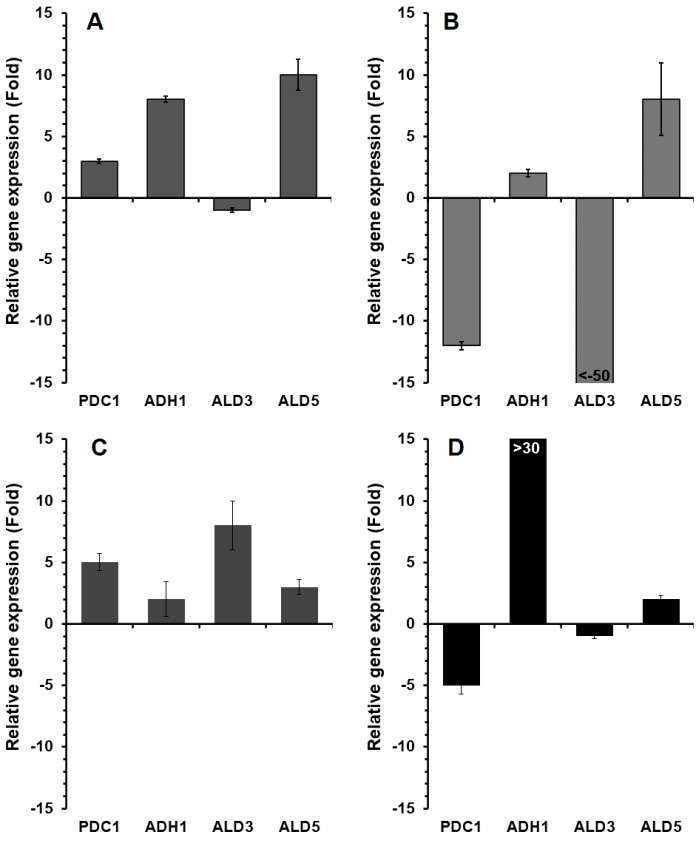
other growth product in the range of 23 minutes of retention time (data not shown). Besides, the results of cultivation on sucrose and glucose showed closed both mass and carbon balances (data not shown), indicating the reliability of the measurements. This metabolic scenario was partially reverted when DSF was added to the ammonium-based medium. In this condition, biomass yield doubled and acetate was detected in the medium (Table 1). In addition, the carbon assimilation efficiency was also higher (Fig. 1c). The carbon distribution calculation showed that 10% was fixed as yeast biomass, 45% was wasted as excreted acetate and 36% was dissipated as biomass-associated CO_2 (Table 1). Both biomass balance and carbon recovery were far higher than in the absence of DSF (Table 1), attesting the reliability of the measurements.

Afterwards, cells were cultivated in medium containing galactose and nitrate as N source (Table 1; Fig. 1). As expected, nitrate promoted lower growth (Fig. 1A) and much lower galactose uptake (Fig. 1B) than ammonium. Acetate was produced at high yield while ethanol was absent (Table 1). The presence of acetate allowed the recovery of the majority of the carbon assimilated by the cells, with much higher mass balance than in ammonium and similar to what was calculated for ammonium+DSF medium (Table 1). Despite of the negative effects on cell growth, the carbon assimilation efficiency was higher in nitrate than in ammonium (Fig. 1C). The calculated carbon distribution in nitrate medium was practically the same as in ammonium+DSF, with 8%, 43% and 34% recovered as biomass, acetate and biomass-associated CO_2, respectively (Table 1). When DSF was added to nitrate medium, biomass formation, carbon consumption and assimilation rate were reduced while acetate production was blocked (Fig. 1A and B; Table 1). On the other hand, growth rate, biomass yield and carbon assimilation efficiency remained unaltered (Fig. 1C; Table 1). The lack of acetate production coincided with the decreasing of biomass balance and carbon recovery to 12% and 13%, respectively, similar to what was observed for ammonium medium (Table 1).

The physiological results indicated that the carbon from galactose entered to the glycolytic pathway and was distributed in different ways in the four culture conditions. A relevant point was the observation that this distribution is related to acetate production. To help understand this distribution, we evaluated the expression of four genes whose proteins are involved in acetate biosynthesis (Fig. 2). Medium with glucose+ammonium was used as reference for all tested conditions. Despite the

lack of ethanol fermentation, cultivation in galactose+ammonium promoted the overexpression *PDC1*, *ADH1* and *ALD5* and the down-expression of *ALD3* (Fig. 2A). In the presence of DSF, there was a significant overexpression of *ADH1* and *ALD5* and the down-expression of *PDC1* and *ALD3* (Fig. 2B). In nitrate, all four genes of the Pdh bypass pathway were overexpressed in galactose compared to glucose, with *ALD3* showing three times more readouts than *ALD5* (Fig. 2C). When DSF was added to nitrate medium, there was the overexpression of *ADH1* and *ALD5* and the down-expression of *PDC1* and *ALD3* (Fig. 2D), as observed for ammonium+DSF. The physiological and genetic results revealed that the fate of the pyruvate pool, whether directed to the TCA cycle or the Pdh bypass, is related to acetate production as well as to carbon assimilation in the biomass. In view of this, the experiments were repeated using pyruvate directly as a carbon source.

Figure 2 Expression of genes coding for pyruvate decarboxylase (*PDC1*), alcohol dehydrogenase (*ADH1*), cytosolic (*ALD3*) and mitochondrial (*ALD5*) acetaldehyde dehydrogenase in *Dekkera bruxellensis* GDB 248 cultivated in mineral medium containing galactose as carbon source and ammonium (panel a), ammonium plus disulfiram (panel b), nitrate (panel c) or nitrate plus disulfiram (panel d). Relative expression in all four cultivation conditions was normalised by the reference condition containing glucose and ammonium. Bars represent standard deviation from the mean value of two independent experiments, with technical triplicate for each one.



Pyruvate metabolism and the relationship with acetate production

The analyses presented above indicated that the metabolic direction taken by the pyruvate formed at the end of glycolytic pathway defined the fate of carbon in the central metabolism, and might be decided by the production of acetate. Therefore, we decided to investigate this distribution using pyruvate directly. Yeast growth was equally low under all four growing conditions (Fig. 3A), although the pyruvate uptake rate was different (Fig. 3B). An inversely proportional relationship was observed between the pyruvate consumption rate and the carbon assimilation efficiency for biomass formation (Fig. 3C). As expected, no ethanol was detected in the media (Table 2). Acetate was not produced when ammonium was used as a source of nitrogen, regardless of the presence of DSF. In this case, the mass balance and carbon recovery were extremely low, even less than was calculated when galactose was used as a carbon source. (Table 2).

When nitrate was used as a nitrogen source, the biomass yield reached a value higher than that observed in the medium with ammonium (Table 2), despite the lower consumption rate of pyruvate (Fig. 3B). Consequently, this represented an increase in the efficiency of carbon assimilation (Fig. 3C). In this medium, the yield of acetate production was similar to that observed in medium with galactose (Table 2). The quantification of acetate allowed the calculation of much higher values for mass balance and carbon recovery. This result indicated that the very low massa balance in ammonium the consequence of carbon deviation to products not predicted in our analysis, like more CO_2 than expected from stoichiometric calculations, rather than by erros in measurements.

When DSF was added to nitrate medium, it was observed an increase in pyruvate uptake rate (Fig. 3b) that was not associated to biomass formation (Table 2), i.e., the carbon assimilation efficiency decreased (Fig. 3c). Acetate was produced in this condition, accounting for 64% of the consumed carbon (Table 2). This allowed the increment in mass balance and carbon recovery relative to ammonium medium (Table 2). These results corroborated the assumption that pyruvate decarboxylation followed by the excretion of CO_2 and acetaldehyde could indeed be the major cause of carbon loss in both galactose and pyruvate cultivations.

Figure 3 Physiological analysis of cell growth (panel a), substrate uptake rate (panel b) and biomass production by consumed carbon (panel c) of the *Dekkera bruxellensis* GDB 248 cultivated in mineral medium containing pyruvate as carbon source and ammonium (NH_4) (circle symbols) or nitrate (NO_3) (triangle symbols) as nitrogen source. Media were also supplemented with the acetaldehyde dehydrogenase inhibitor disulfiram (DSF) (square symbols for ammonium or diamond symbols for nitrate). Bars represent standard deviation from the mean value of two independent experiments, with technical triplicate for each one.

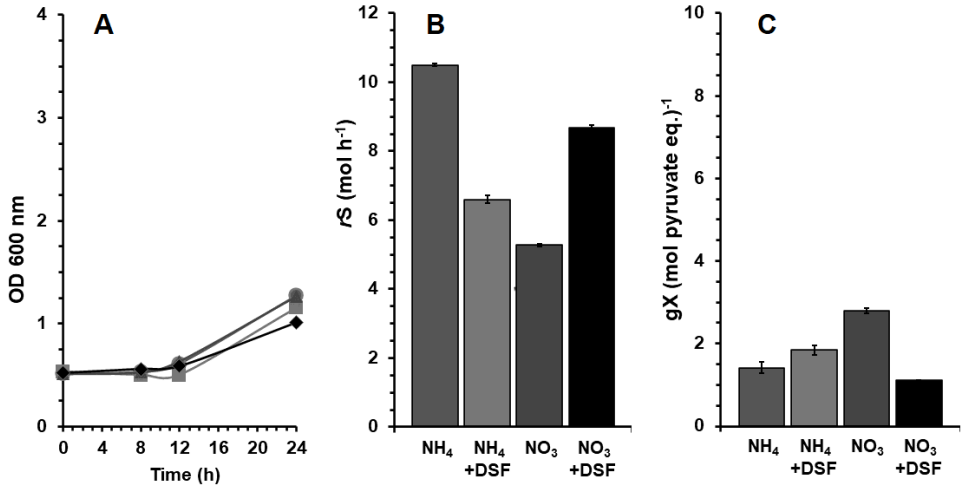


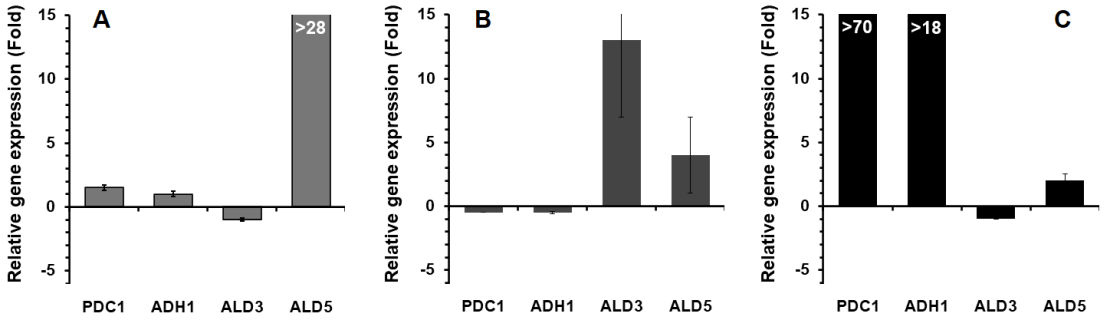
Table 2 Physiological data calculated at the end of 24h cultivation of *Dekkera bruxellensis* GDB248 in mineral medium containing pyruvate as carbon source and ammonium (NH_4) or nitrate (NO_3) as nitrogen source. Media was also supplemented with the acetaldehyde dehydrogenase inhibitor disulfiram (DSF).

	Mass distribution					Carbon distribution			
	NH_4	NH_4+DSF	NO_3	NO_3+DSF		NH_4	NH_4+DSF	NO_3	NO_3+DSF
Growth rate (μ, h^{-1})	0.06	0.07	0.06	0.05	Consumption (mmol C)	504.0	316.7	253.0	416.7
Sugar consumption (g L^{-1})	15.12	9.50	7.59	12.50	Production (mmol C)	11.2	9.1	167.8	192.9
Biomass yield (g g^{-1})	0.02	0.02	0.03	0.01	Biomass	9.5	7.8	9.4	6.1
Ethanol yield (g g^{-1})	0.00	0.00	0.00	0.00	Ethanol	0.0	0.0	0.0	0.0
Acetate yield (g g^{-1})	0.00	0.00	0.41	0.30	Acetate	0.0	0.0	104.7	124.0
CO2 yield* (g g^{-1})	0.00	0.01	0.31	0.22	CO_2^*	1.7	1.4	53.8	62.8
Mass balance (%)	2	3	76	53	Carbon recovery (%)	2	3	66	46
Mass loss (%)	98	97	24	47	Carbon loss (%)	98	97	34	54

*Stoichiometricaly calculated (Teles et al. 2018).

Regarding gene expression, Pdh bypass genes were less affected in ammonium+DSF when pyruvate was the C source than in galactose, while the expression of *ALD5* was much higher in the first condition (Fig. 4a). This aids to explain while acetate was detected in galactose but not in pyruvate. When nitrate was the N source, the production of acetate was be explained by the increased metabolic flux through Pdh bypass both by the overexpression of *ALD3* in the absence of DSF (Fig. 4b) and overexpression of *PDC1* in the presence of DSF. Besides, *ALD5* remained overexpressed in both condictions (Fig. 4c).

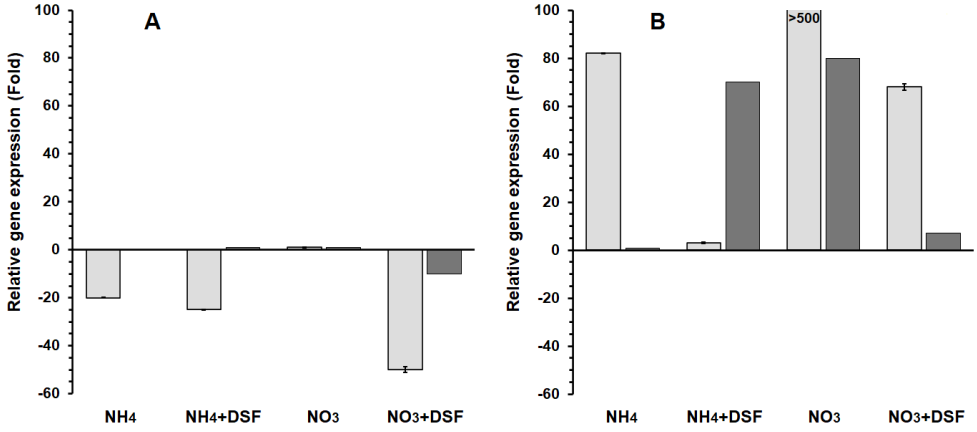
Figure 4 Expression of genes encoding for pyruvate decarboxylase (*PDC1*), alcohol dehydrogenase (*ADH1*), cytosolic acetaldehyde dehydrogenase (*ALD3*) and mitochondrial acetaldehyde dehydrogenase (*ALD5*) in *Dekkera bruxellensis* GDB 248 cultivated in mineral medium containing pyruvate as carbon source and ammonium plus disulfiram (panel a), nitrate (panel b) or nitrate plus disulfiram (panel c). Relative expression in all four cultivation conditions was normalised by the reference condition containing glucose and ammonium. Bars represent standard deviation from the mean value of two independent experiments, with technical triplicate for each one.



The metabolic differences promoted by the use of galactose or pyruvate were tested by the analysis of *PFK1* and *ATP1* genes. The first gene encodes the phosphofructokinase (Pfk), enzyme that catalyses the second reaction of the glycolytic pathway in the phosphorylation of fru-6P to fructose-1,6 bisphosphate (fru-1,6BP). This is one of the key enzymes in regulating the flow of the glycolytic pathway (Nishino et al. 2015). The level of *PFK1* readouts was either unchanged or decresed in galactose or pyruvate than in glucose in almost all conditions tested (Fig. 5a). This shows that, in fact, the flow through the glycolytic pathway must be decreased, as indicated by the physiological data.

On the other hand, the expression of the *ATP1* gene is greatly increased in these two carbon sources in relation to glucose (Fig. 5b). This gene codes the alpha subunit of the F1 subunit of mitochondrial F1F0 ATP synthase that is directly involved in energy production in the respiratory chain (Francis et al. 2007).

Figure 5 Expression of genes coding for the phosphofructokinase – *PFK1* (panel a) and the F1 subunit of mitochondrial F1F0 ATP synthase – *ATP1* (panel b) in *Dekkera bruxellensis* GDB 248 cultivated in mineral medium containing galactose (light grey columns) or pyruvate (dark grey columns) as carbon source and ammonium (NH_4), ammonium plus disulfiram (NH_4+DSF), nitrate (NO_3) or nitrate plus disulfiram (NO_3+DSF). Relative expression in all four cultivation conditions was normalised by the reference condition containing glucose and ammonium. Bars represent standard deviation from the mean value of two independent experiments, with technical triplicate for each one.



External supply of acetate enables the growth of *D. bruxellensis* in other alternative respiratory C sources

We complemented the analysis on the *D. bruxellensis* respiratory metabolism by using four other respiratory C sources: glycerol that is converted to pyruvate with the production of NADH, ethanol that is converted to acetate with the production of both NADH and NADPH, and mitochondrial intermediates of the TCA cycle citrate and 2-oxoglutarate (Fig. 6a). In this context, 2-oxoglutarate is the only that cannot produce acetate backwards (Fig. 6a). Very poor cell growth was observed for ethanol and glycerol in mineral medium with ammonium, independent of the presence of DSF (Fig. 6b). In addition, no growth was observed when nitrate was used as N source with these substrates (data not shown). The addition of the antioxidant agent N-

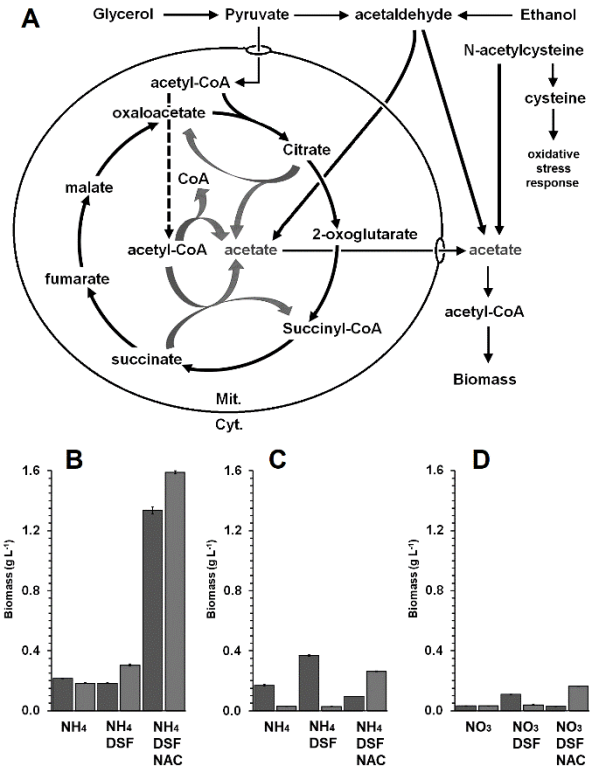
acetylcysteine (NAC) to 5 mM in ammonium+DSF stimulated biomass formation by 6 to 7 times in ethanol and glycerol (Fig. 6b). On the other hand, it was not observed cell growth in glycerol or ethanol when nitrate was the N source, even in the presence of NAC (data not shown). In this case, the oxidative burden caused by respiratory activity together with nitrate assimilation might be far beyond the NAC detoxification capacity. Citrate produced low biomass in ammonium similar to glycerol and ethanol (Fig. 6c). This metabolite can be hydrolysed back to oxaloacetate and acetate (or acetyl-CoA) and part of the produced acetate could be secreted from the mitochondria to produce cytosolic acetyl-CoA (Fig. 6a). Increment in biomass formation was observed in the presence of DSF, while NAC reduced biomass production (Fig. 6c). So far, we have no clues on any possible explanation for these phenomena. At least, only a residual growth was observed when 2-oxoglutarate was tested as C source in ammonium or ammonium+DSF media (Fig. 6c). This molecule is formed by the decarboxylation of citrate and cannot produce the acetate and acetyl-CoA required for growth (Fig. 6a). However, the addition of NAC increased yeast biomass formation by ten times (Fig. 6c). This phenomenon was also observed in nitrate+DSF (Fig. 6d).

Discussion

Galactose is a hexose that, different from glucose and fructose, does not directly enter in the glycolytic pathway. Instead, this sugar must be first converted to glucose 6-phosphate (Glu-6P) through the Leloir pathway, which then enter the glycolytic pathway (Bhat and Murthy 2001). The reduced flux in the Leloir pathway is associated to lower carbon flow in the central metabolism when comparing to glucose and fructose, reducing the fermentative metabolism in *S. cerevisiae* (Ostergaard et al. 2000). In C-limited steady-state *S. cerevisiae* cultivations, the intracellular concentration of Glu-6P and fructose 6-phosphate (Fru-6P) were 3.6 and 2.5 times higher in glucose than in galactose, respectively (Ostergaard et al. 2001), which attests the slower metabolic flux in galactose. So far, there are few studies addressing the physiological features of *Dekkera/Brettanomyces bruxellensis* grown in galactose (Da Silva et al. 2019a; Moktaduzzaman et al. 2015).

Figure 6 Biomass formation of *Dekkera bruxellensis* GDB 248 cultivated in mineral medium containing ethanol, glycerol, citrate or 2-oxoglutarate as carbon source. (panel a) Metabolic map was drawn to

show the fate of each carbon substrate used. (panel b) Final biomass was calculated for cultivations in ethanol (dark-grey columns) or glycerol (light-grey columns) in the presence of ammonium (NH_4), ammonium plus disulfiram (NH_4+DSF) or ammonium plus disulfiram and N-acetylcysteine (NH_4+DSF+NAC). (panel c) Final biomass was calculated for cultivations in citrate (dark-grey columns) or 2-oxoglutarate (light-grey columns) in the presence of ammonium (NH_4), ammonium plus disulfiram (NH_4+DSF) or ammonium plus disulfiram and N-acetylcysteine (NH_4+DSF+NAC). (panel d) Final biomass was calculated for cultivations in citrate (dark-grey columns) or 2-oxoglutarate (light-grey columns) in the presence of nitrate (NO_3) or nitrate plus disulfiram (NO_3+DSF) or nitrate plus disulfiram and N-acetylcysteine (NO_3+DSF+NAC). Bars represent standard deviation from the mean value of two independent experiments, with technical triplicate for each one.

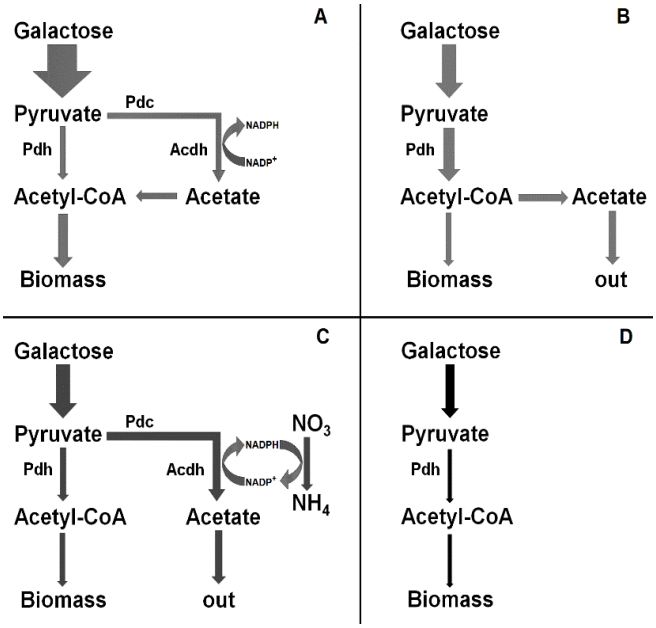


Both physiological and genetic results taken from the aerobic cultivations helped to build up a metabolic model to explain the ways used by *D. bruxellensis* GDB 248 to metabolise galactose (Fig. 7). The low growth rate calculated is below those reported for glucose and sucrose (Da Silva et al. 2019a; Leite et al. 2013), indicating the lower efficiency of the galactose assimilatory pathway. It can be explained by the low rate of galactose assimilation relative to glucose and sucrose, considering the mol of hexose monomer equivalent (manuscript submitted). Interestingly, the calculated rate of galactose uptake by *D. bruxellensis* of 7 mmol gDW^{-1} h^{-1} was more than two times higher than the calculated for *S. cerevisiae* 3

mmol gDW^{-1} h^{-1} in batch cultivations (Ostergaard et al. 2000) and after galactose pulse of C-limited galactose chemostat cultivation (Ostergaard et al. 2001). The lack of ethanol production indicated that galactose is exclusively metabolised via respiration by the strain GDB 248. A lower growth rate and consumption of galactose in relation to glucose was also observed in European wine strain *D. bruxellensis* CBS2499 (Moktaduzzaman et al. 2015). The strain-dependence galactose metabolism was reported, since Chilean wine isolates grew more in galactose than Brazilian bioethanol strains (Da Silva et al. 2019a). Therefore, it is important to considered this strain-associated differences. Nevertheless, the strain GDB 248 seems an interesting platform to study the effects of this metabolic reorientation in *D. bruxellensis.* In this condition, the large amount of carbon from galactose would be converted to pyruvate. And from there, they would go to the Pdh and Pdh bypass routes (Fig. 7a). The small amount of carbon that was assimilated as acetyl-CoA from both pathways should allow the formation of biomass as the only metabolic product (Fig. 7a).

The parameters of growth rates and sugar uptake were progressively reduced in the conditions of ammonium+DSF, nitrate and nitrate+DSF. Dissulfiram (DSF) was reported to inhibit the activitity of acetaldehyde dehydrogenase (Acdh), resulting in depletion of cytosolic acetate (Kwolek-mirek et al. 2012). This drug reduced the metabolism of glucose in *D. bruxellensis* while increased ethanol production (Teles et al. 2018). DSF also reduced galactose uptake (Fig. 1b) but did not lead to ethanol production (Table 1) as observed for glucose (Teles et al. 2018). Furthermore, the results indicated that the aerobic assimilation of nitrate reduces the flux of galactose metabolization, as previously reported for glucose (Cajueiro et al. 2017; De Barros Pita et al. 2013; Peña-Moreno et al. 2021). There was an additive negative effect on the yeast physiology by the presence of DSF in nitrate medium. However, biomass yield doubled in the presence of DSF or nitrate, or both (Table 1). It indicated that cellular anabolism was more efficient when the carbon distribution in the central metabolism was modified by the inhibition of Acdh and/or by the induction of the energy-demanding nitrate assimilatory pathway, changing the carbon assimilation efficiency profile.

Figure 7 Proposed models for carbon distribution in *Dekkera bruxellensis* GDB 248 cultivated in mineral medium containing galactose as carbon source and ammonium (NH_4) (panel a), ammonium plus disulfiram (NH_4+DSF) (panel b), nitrate (NO_3) (panel c) or nitrate plus disulfiram (NO_3+DSF) (panel d). Data were compilated from physiological (Fig. 1 and Table 1) and genetic (Fig. 2). results.



Ethanol was not detected in any cultivation conditions tested (Table 1), showing that galactose is an exclusive respiratory hexose for *D. bruxellensis* GDB 248. This result was also observed for *D. bruxellensis* CBS 2499 strain (Moktaduzzaman et al. 2015). On the other hand, galactose is assimilated by the respiro-fermentive metabolism in *S. cerevisiae*, although the ethanol yield from galactose is lower than glucose (Ostergaard et al. 2000). This yield is increased the more galactose is consumed, at the same time that the oxidative metabolism is reduced (Ostergaard et al. 2000). It indicates that the rate of galactose uptake is the constraint of the fermentation capacity of the yeast cells (Ostergaard et al. 2000). In the strain GDB 248, the meatbolisation of galactose is made exclusively by a respiratory metabolism, meaning that galactose is useless in fermentation processes.

In the respiratory metabolism, the oxidative decarboxylation of pyruvate by the pyruvate dehydrogenase (Pdh) generates acetyl-CoA for the mitochondrial TCA cycle, while in the fermentation the redox-neutral decarboxylation by pyruvate decarboxylase (Pdc) lead to cytosolic acetaldehyde and carbon dioxide (Pronk et al. 1996). In *S. cerevisiae*, three genes encode enzymes necessary for Pdc activity

(PDC1, PDC5 and *PDC6)* and its efficient function is a key mechanism for ethanol production (Nosaka et al. 2012). The isoforms Pdc1 is responsible for most of the enzyme activity (Schaaff et al. 1989). From that point, acetaldehyde can be reduced by the NADH-dependent alcohol dehydrogenase (Adh) to ethanol and/or oxidised by the NADPH-dependent acetaldehyde dehydrogenase (Acdh) to acetate. This acetate is converted to cytosolic acetyl-CoA at expenses of ATP by the acetyl-CoA syntase (Acs), which is further used for anabolic and regulatory processes. This alternative acetyl-CoA producing pathway (Pdc → Acdh → Acs) is refered as the Pdh bypass (Pronk et al. 1996; Remize et al. 2000). Different to what was observed in glucose, galactose did not stimulate the production of acetate (Table 1), meaning that the flux through the Pdh by is slow and all cytosolic acetate is converted to acetyl-CoA. This metabolic scenario was partially reverted when DSF was added to the mediumin which 45% of the consumed carbon was wasted as excreted acetate. This large acetate production seemed a paradox, since DSF is expected to block the activity of cytosolic Acdh (Ald3). In glucose fermentation, acetate production in the presence of DSF is maintained by the activation of mitochondrial Acdh (Ald5) as revealed by the high expression of *ALD5* orthologous gene (Teles et al. 2018), and the overflow caused by the metabolic re-orientation produced the excess of mitochondrial acetate that was expelled to the medium. This seems to be the case in galactose, in which the blockade of cytosolic acetate production might cause an excess of acetaldehyde, which entered to the mitochondria and is then oxidized to acetate. Therefore, carbon loss in cultivations without DSF must occur at the level of highly volatile acetaldehyde.

Nitrate stimulates the pathway for acetate production, the Phd bypass, as the major NADPH provider for the functioning of the nitrate assimilatory enzymes. In the CBS 2499 strain, nitrate in cultures with galactose promotes decrease biomass yield, increase consumption rate and induces the targeting of part of the pyruvate to produce acetate, when compared to ammonium (Moktaduzzaman et al. 2015). The assimilation of one mol of nitrate requires four mols of NADPH to convert it intracellular ammonium, plus one mol of NADPH to assimilate this ammonium as glutamate (Siverio, 2002). In this context, the Pdh bypass seems paramount as the most important NADPH provider in aerobiosis. On the other hand, the cells cultivated in anaerobiosis seems to re-direct its metabolism in a way that the pentose phosphate pathway (PPP) and the tetrahydrofolate pathway (THF) take the lead as

major NAPDH producers (Peña-Moreno et al. 2021). Carbon distribution analysis indicated that the acetate detected in culture medium was originated from the mitochondria in the case of ammonium+DSF medium, and from the cytosol in the case of nitrate. When the cytosolic Acdh was blocked by DSF, the metabolic flux in nitrate medium as N source was directed to the mitochondria and no acetate was produced whatsoever (Table 1). In this case, the reducing power for nitrate metabolization should be provided solely by the PPP, similar to what was proposed for anaerobic cultivation (Peña-Moreno et al. 2021). Despite the high efficiency of carbon assimilation, carbon recovery was very low in this condition.

The profile of gene expression was quantified to help to understand the regulatory mechanisms involved in this metabolic re-orientation. Despite the high expression of *ADH1* (Fig. 2a), we propose that the shortage of cytosolic NADH caused by the slow glycolytic flux would be the best explanation for the lack of ethanol production in galactose. Furthermore, the down-expression of *ALD3* (Fig. 2a) indicated that the flux through cytosolic Acdh was limited. In this context, there should be a massive decarboxylation of pyruvate to acetaldehyde, which might be poorly metabolised to acetyl-CoA citosolic. Our hypothesis is that both volatile compounds (CO_2 and acetaldehyde) were lost, jeopardising both mass and carbon balance as discussed above (Table 1). On the other hand, the overexpression of *ALD5* indicated that part of cytosolic acetaldehyde entered in the mitochondria to be converted to acetate. Afterwards, the mitochondrial acetyl-CoA syntase (Acs) could convert acetate to acetyl-CoA that is further used as building block for biomass formation. The significant down-expression of *PDC1* and *ALD3* in the presence of DSF, together with the overexpression of *ALD5* (Fig. 2b), is an indicative that the flux towards Pdh bypass was blocked and the whole pyruvate was metabolised via Pdh. It might result in an excess of acetate inside the mitochondria, as suggested by the physiological data above. In *S. cerevisiae*, mitochondrial acetate is produced from the function of the *ACH1* gene product (Acetyl-CoA transferase) in cells that do not have Pdc activity (Chen et al. 2015). Ach1 hydrolase activity (conversion of acetyl-CoA to acetate and CoA) has also been previously proposed (Buu et al. 2003). It means that cells might overcome the defect in the Pdh bypass by secreting mitochondrial acetate and/or acetyl-CoA to the cytosol for the required anabolic and regulatory processes. In the yeasts *Kluyveromyces lactis* and *K. marxianus*, the deletion of single *PDC* gene did not affect growth compared to wild-type strains,

physiology that is associated with mitochondrial acetyl-CoA shuttle (Bianchi et al. 1996; Choo et al. 2018; Porro et al. 1999). In the present case, the huge down-expression of *PDC1* could partially simulate gene deletion and showed that *D. bruxellensis* display this metabolic similarity with those *Kluyveromyces* species as previously proposed (Teles et al 2018).

In nitrate, the over-expression of all four Pdh bypass genes explains the acetate production by the metabolic re-orientation of the cell metabolism at the pyruvate crossroad to the Pdh bypass. On the other hand, the presence of DSF impaired the expression of *PDC1* and *ALD3*, but not *ALD5*. These results aided to explain why the mitochondrial acetate was detected in the supernatant ammonium+DSF medium, but not in the nitrate+DSF medium (Table 1). All these gene expression profiles fitted quite well with the metabolite profile detected.

It is proposed that there is an equilibrium between the flux through Pdh and the Pdh bypass to produce mitochondrial and cytosolic acetyl-CoA, respectively, which allows anabolic reactions andFig. 3 biomass formation (Fig. 3a). Whenever this equilibrium is disrupted by inhibiting Acdh (in ammonium+DSF) or over-activating this enzyme (in nitrate), an excess of acetate is formed in both mitochondria (Fig. 3b) or cytosol (Fig. 3c), respectively, which is excreted to the medium. These results showed that Pdh bypass is the major source of reduced equivalent required for nitrate assimilation in aerobiosis. The interesting aspect was the fact that the more acetate was detected, the more carbon was recovered in the calculations. The common aspect of both conditions was the fact that more carbon was assimilated when the production of NADPH was limited either by the inhibition of Acdh (Fig. 3b) or by its draining for nitrate assimilation (Fig. 3c). When the induction of Pdh bypass by nitrate was counteracted by Acdh inhibition (in nitrate+DSF), there seems to have a slowdown of the whole central metabolism in a way that biomass production was drastically affected (Fig. 3d). However, it is notewort the high carbon assimilation efficiency in this condition (Fig. 1c).

We could use the carbon distribution from galactose reported for *S. cerevisiae* (Ostergaard et al. 2001) to try to figure out the possible metabolic ways taken by the galactose-based carbon in *D. bruxellensis*. In the stady state of the C-limited galactose chemostat, 58% of the carbon was assimilated in the biomass while 42% was wasted as CO_2. When the cultures were pulsed with galatose, the cells left steady-state and enter in dynamic (batch) state. In that case, 38% of the carbon was

assimilated in the biomass, 20% was wasted as CO_2 and 35% and 7% was excreted as ethanol and glycerol, repectively (Ostergaard et al. 2001). More carbon from galactose is pushed to the pentose phosphate pathway in the stady-state (40%) than after pulse (7%) (Ostergaard et al. 2001). In the present work, we showed that carbon assimilation was higher when the NADPH from Pdh-bypass was not available by the presence of DSF and/or nitrate (Fig. 1c; Fig. 3b,c,d), just when the growth and galactose uptake rates were reduced (Fig. 1d). Therefore, we might suppose that *D. bruxellensis* in galactose+ammonium was metabolic similar to *S. cerevisiae* after pulsing condition, using less NADPH from PPP and more Pdh-bypass, but facing the shortage of glycolytic NADH. On the other hand, in nitrate or in the presence of DSF it was closer to *S. cerevisiae* steady-state like condition by using more PPP-derived NADPH for cell anabolism.

Taken in consideration the results above, it was made clear that the fate of pyruvate is the key factor for the regulation of the central metabolism in *D. bruxellensis.* In *S. cerevisiae,* pyruvate is exclusively assimilated by a respiratory metabolism because the lack of glycolytic NADH that is used to reduce acetaldehyde to ethanol (Pronk et al. 1996). Hence, pyruvate is almost exclusively metabolised by the Pdh complex to produce mitochondrial acetyl-CoA (Kresze and Ronft 1981; Remize et al. 2000). The results in this work showed that pyruvate is a poor C source for *D. bruxellensis* GDB 248 regardless the functioning of the Phd bypass. Despite the relative high carbon uptake rate, its carbon assimilation efficiency very low and no product was observed. It means that more carbon has been dissimilated as CO_2 than that calculated from biomass formation. Therefore, 98% of the consumed carbon from pyruvate was lost in that hypothetic above-mentioned futile reaction of decarboxylation that might occurs either by Pdh or by Pdc activities. The presence of DSF increase in the carbon assimilation efficiency without acetate production, but still only a small amount of carbon was recoreved. The situation was clearer in nitrate, with less carbon uptake and more carbon assimilated in the biomassion efficiency was twice higher than in ammonium (Fig. 4c). In this case, the production of acetate to fulfil the nitrate assimilatory pathway made that less carbon was dissimilated. On the opther hand, the addition of DSF increase pyruvate uptake rate and reduced the carbon assimilation efficiency decreased, while maintained acetate production. These results corroborated the assumption that pyruvate decarboxylation followed by

the excretion of CO_2 and acetaldehyde would indeed be the major cause of carbon loss in both galactose and pyruvate cultivations.

Regarding gene expression, *PDC1, ADH1* and *ALD3* were less affected compared to galactose (Fig. 4a and 2b), however, the *ALD5* gene remained highly expressed. In the presence of nitrate, the acetate detected can be attributed to the increased expression of the *ALD3* gene (Fig. 4b) or the overexpression of the *PDC1* gene (Fig. 4c), that is, the bypass on actived. In the latter case, the amount of acetaldehyde would be converted into acetate by Ald5, with the gene also overexpressed (Fig. 4c). The growth of a *S. cerevisiae* strain in ethanol was associated with an overexpression of the cytosolic *ALD6* gene, which produced more acetate compared to a reference strain that was unable to grow in this substrate (Boubekeur et al. 2001). A previous study proposed the existence of a mitochondrial acetaldehyde bypass, where the cytosolic acetaldehyde can be oxidized by the mitochondrial Ald to produce acetate, which returns to the cytosol (Boubekeur et al. 1999).

Subsequently, the analysis of the *PFK1* gene, important for glycolytic flow, was performed (Fig. 5a). Under most conditions, *PFK1* gene expression was decreased (Fig. 5a), regardless of the carbon source, which indicates decreased glycolytic flux, according to the calibrator condition in glucose. Ostergaard et al. (2001) reported three times less Fru-6P in the biomass of *S. cerevisiae* in galactose than in glucose. Hence, the possible lower level of Fru-6P would result in the lack of *PFK1* expression, which could reduce the level of Fru-6P kinase. In *S. cerevisiae, PFK1* mutants show reduced glucose metabolization rates compared to the wild-type (Heinisch 1986). It produces an approximately 10-fold increase in the concentration of Fru-6P, with a consequent significant reduction in Fru-1,6BP and dihydroxyketone phosphate (DHAP) (Nishino et al. 2015). Therefore, it very much reduces the supply of NADH in the oxidative part of the glycolysis, impairing ethanol production as observed for galactose (Table 1). We could presume that the lack of *PFK1* induction in *D. bruxellensis* would result in the low flux through the oxidative part of glycolysis, reducing the NADH production and impairing ethanol fermentation to take place. On the other hand, the data show that the *ATP1* gene was highly expressed under the conditions of the study (Fig. 5b). These indicate that both carbon sources are metabolised by an exclusive oxidative/respiratory pathway, as showed by the physiological data. The energetics of *S. cerevisiae* cells in galactose is lower than in

glucose, both regading the amount of intracellular ATP and energy charge (Ostergaard et al. 2001). It probably might be the same in *D. bruxellensis,* given that the growth rate in galactose is lower than in glucose (Da Silva et al. 2018). Therefore, this over-expression of *ATP1* gene (Fig. 5b) seems to be the attempt of the yeast cells to increase energy production. This scenario did not depend on the nitrogen source and the Phd bypass inhibition. In addition, the expression of *ATP1* was significantly higher in galactose+DSF compared to the fold change found in fermentation tests with glucose+DSF (Teles et al. 2018). The expression of other genes involved in the respiratory metabolism of *D. bruxellensis* was also increased in the presence of galactose, such as cytochrome-c oxidase gene *COX5A* and ubiquinol cytochrome-c reductase *QCR2* (Moktaduzzaman et al. 2015).

Furthermore, we extended the spectrum of the analysis of the respiratory metabolism with the use of others carbon sources not related to the central metabolism. Very poor growth was observed in the presence of glycerol and ethanol for *D. bruxellensis* (Fig 6b). Minimal growth in glycerol has been previously observed for this *D. bruxellensis* strain (Teles et al. 2018). Another explanation was that the excessive respiratory activity induced a massive oxidative stress for the yeast cells, which halts cell metabolism and growth. We have reported that *D. bruxellensis* GDB 248 is more sensitive to oxidative stress than *S. cerevisiae* (Leite et al. 2013). *D. bruxellensis* cells also did not grow with these carbon sources in the presence of nitrate (data not shown). We recently showed that oxidative damages were causes of lower growth in aerobic cultivations with nitrate (Peña-Moreno et al. 2021). Therefore, the complete absence of growth under these conditions might be the result of the action of these two effects (low carbon metabolism and massive oxidative stress) that considerably affect the *D. bruxellensis* metabolism. In view of this, we used NAC as protector against oxidative stress caused, for exemplo, by increased repiratory activity. It restored the growths under the ethanol and glycerol culture conditions in ammonium (Fig. 6b), but did not recover the growth on nitrate (data not shown). Furthermore, growth in citrate followed the same line, poor growth, however, improvement in the presence of Dsf and worsening with NAC was somewhat surprising (Fig 6c). The result was opposite with 2-oxoglutarate, that is, growth improvement when NAC was added, what could be occurring? (Fig. 6c). The explanation of oxidative stress protection was fited, but it did not explain how the cells could grow without acetyl-CoA. The reasonable expalanation came from a

report showing that NAC is split down in the yeast cytosol to produce acetate and cysteine (Deffieu et al. 2009). Hence, NAC might have a duo function as an antioxidant by its sulphur amino acids cysteine as well as an acetate supplier (Fig. 6a). Therefore, the observed growth could be credited to the 5 mmol of acetate proposed to be released from 5 mmol NAC. In this case, the carbon from 2-oxoglutarate feeds the TCA cycle while the acetate fulfils the requirement for cytosolic acetyl-CoA. This phenomenon was also observed in nitrate+DSF (Fig. 6d). We had recently provided evidences of the importance of acetate consumption in the lactic acid bacteria *Lactobacillus vini* by providing extra ATP for biomass formation (Da Silva et al. 2019b). In that work, it was shown that less external acetate was required when citrate was present, while more external acetate was required when pyruvate was the used (Da Silva et al. 2019b). This last condition could be metabolic similar to the cultivation of *D. bruxellensis* in 2-oxoglutarate (Fig. 6).

The present work provides important pieces to the respiratory metabolism puzzle of *D. bruxellensis* in which the production of acetate seems of paramount importance. From the results above, it was possible to calculate the stoichiometry of 240 mg of biomass produced by mmol of external acetate consumed in medium containing a carbon source that can produce internal acetate, like ethanol and glycerol. In 2-oxoglutarate, that cannot produce acetate, only 62 mg and 34 mg of biomass can be produced by mmol of external acetate consumed in ammonium or nitrate, respectively. Therefore, we conclude by proposing that the intracellular concentration of acetate is the key point for that regulation of *D. bruxellensis* central metabolism functioning.

Acknowledgements

The authors express their gratitude to the administration of the Federal Univeristy of Pernambuco and to the goverment of the state of Pernambuco that kindly supported this work with all basic needs in this period of absolute denaying of the federal government of our countrie to science, education, art and social rights.

Funding information

This work was sponsored by grants of the National Council of Science and Technology (CNPq/process 409767/2018-2 and CNPq/process 303551/2017-8) and

by the Bioethanol Research Network of the State of Pernambuco (CNPq-FACEPE/PRONEM APQ-1452-2.01/10).

Conflict of Interest

The authors declare that they have no conflict of interest.

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8 CONCLUSÕES

- A disponibilidade de acetato é um fator chave para o direcionamento do carbono para etanol em *D. bruxellensis* GDB248. A utilização do inibidor bioquímico de acetato citosólico, dissulfiram, aumenta a produção e rendimentos em etanol por *D. bruxellensis* em ensaios fermentativos.

- A utilização do Dsf diminui consumo de açúcar, taxas de crescimentos e biomassa final em *D. bruxellensis*, o que indica a sua interferência no metabolismo respiratório da levedura.

- *D. bruxellensis* mostrou-se mais tolerante aos efeitos metabólicos provocados pelo Dsf, em comparação a levedura *S. cerevisiae*, no entanto, a adição de ácido acético diminui os crescimentos de *D. bruxellensis* na presença do inibidor, o que indica uma sobrecarga intracelular.

- Dados fisiológicos e de expressão gênica sugerem que a produção de acetato pode ser mitocondrial em *D. bruxellensis*, com o composto fluindo para o citosol e restabelecendo as necessidades biossintéticas de *D. bruxellensis* na presença do Dsf.

- *A* inibição da Acdh alivia a repressão catabólica exercida pela glicose em *D. bruxellensis* em cultivos respiro-fermentativos.

- *D. bruxellensis* direciona mais carbono para etanol mesmo em condições aeróbicas na presença de glicose e sacarose, sendo que o dissacarídeo melhora os parâmetros respiro-fermentativos da levedura.

- A enzima Acdh pode controlar o fluxo de carbono no metabolismo central;

- O bypass PDH é a principal fonte de poder redutor para assimilação de nitrato em *D. bruxellensis.*

- *D. bruxellensis GDB248* utiliza a galactose exclusivamente pela via respiratória.

- O antioxidante N-acetilcisteína restaura os crescimentos de *D. bruxellensis* na presença de Dsf.

- *D. bruxellensis* é capaz de produzir etanol com altos rendimentos, mas o acetato ainda é produzido em grandes quantidades.

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APÊNDICE A - THE BIOTECHNOLOGICAL POTENTIAL OF THE YEAST *DEKKERA BRUXELLENSIS*

World Journal of Microbiology and Biotechnology (2019) 35:103
https://doi.org/10.1007/s11274-019-2678-x

REVIEW

The biotechnological potential of the yeast *Dekkera bruxellensis*

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Received: 17 March 2019 / Accepted: 15 June 2019

Abstract

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

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

APÊNDICE B - FIRST ASPECTS ON ACETATE METABOLISM IN THE YEAST *DEKKERA BRUXELLENSIS*: A FEW KEYS FOR IMPROVING ETHANOL FERMENTATION

Received: 25 February 2018 | Revised: 25 April 2018 | Accepted: 23 May 2018

DOI: 10.1002/yea.3348

RESEARCH ARTICLE

WILEY Yeast

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

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Funding information
Conselho Nacional de Desenvolvimento Científico e Tecnológico, Grant/Award Numbers: 446927/2014-7, 472533/2013-4 and 474847/2013-6; Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco, Grant/Award Number: APQ-1452-2.01/10

Abstract

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

KEYWORDS

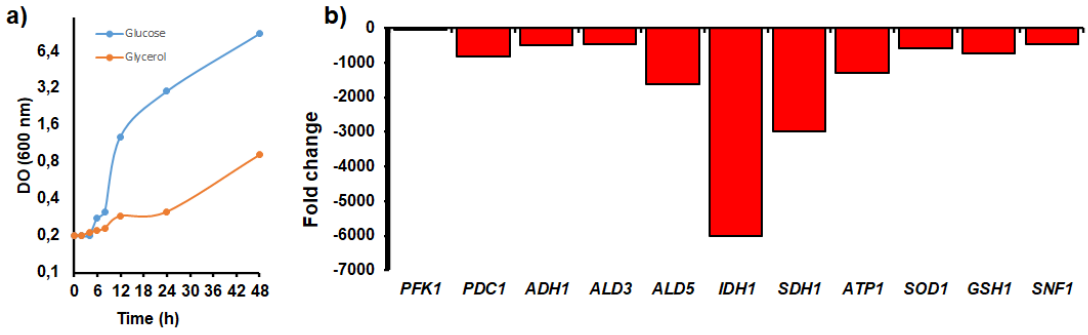
acetaldehyde dehydrogenase, acetyl-CoA, carbon distribution, *Dekkera bruxellensis*, disulfiram, ethanol fermentation

APÊNDICE C - RELAÇÃO ENTRE A VIA *PDH BYPASS* E A REGULAÇÃO DO METABOLISMO RESPIRATÓRIO EM *DEKKERA BRUXELLENSIS*

a. Glicerol é uma fonte pobre de carbono e energia para *Dekkera bruxellensis*

As células de *D. bruxellensis* GDB248 foram cultivadas em dois tipos de fontes de carbono: 1) glicose, uma fonte que é respirável e fermentável, portanto, menos dependente da cadeia fosforilativa para geração de energia; 2) glicerol, uma fonte que é estritamente respirável. O perfil de crescimento mostrou que o glicerol não é eficientemente utilizado pela levedura, gerando pouca biomassa (Figura 1a). O perfil de expressão gênica relativa mostrou que todos os genes testados apresentaram menor expressão em glicerol do que em glicose (Figura 1b). Isto indica que o metabolismo da levedura está diminuído. Paradoxalmente ao que se esperava com glicerol como fonte estritamente respirável, a expressão dos genes de resposta a estresse oxidativo tiveram suas expressões bastante diminuídas. Com isso, pode-se especular que pouco carbono e elétrons cheguem à cadeia respiratória e, por isso, ocorre pouca ou nenhuma produção de espécies reativas de oxigênio (EROs).

Figura 1. Relação entre o metabolismo respiro-fermentativo e oxidativo em *D. bruxellensis* GDB 248. (Painel a) Curvas de crescimento aeróbio levedura em meio sintético definido contendo glicose ou glicerol. (Painel b) Expressão gênica em meio com glicerol relativa ao meio contendo glicose.



Em *S. cerevisiae*, glicerol é uma fonte alternativa de carbono produzindo metade da energia e metade rendimento em biomassa em relação a glicose (Nevoigt and Stahl, 1997). Em *D. bruxellensis,* a produção de biomassa foi apenas 1/10 daquela observada em glicose (Figura 1a). Além disso, os genes da via de assimilação de glicerol em *S. cerevisiae* são submetidos a repressão catabólica pela glucose (GCR) e, portanto, o consumo desta fonte de carbono só se faz na exaustão da glicose no

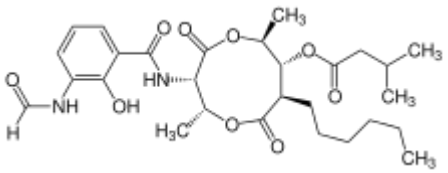
meio ou na sua ausência (Nevoigt and Stahl, 1997). Embora nossos dados indiquem que o mecanismo GCR em *D. bruxellensis* é menos restritivo do que em *S. cerevisiae* (Leite et al., 2016), não temos ainda informações sobre seu efeito na utilização de glicerol.

b. O direcionamento do carbono para a via fermentativa é maior quando a cadeia fosforilativa e a via *Pdh bypass* estão inibidos

Na quase totalidade dos eucariotos aeróbios, a cadeia fosforilativa no mecanismo respiratório é formado por três sítios (sítios I, III e IV) de óxido-redução que transferem próton H^+ da matriz mitocondrial para o periplasma na medida em que o elétron proveniente do NADH seja transferido de um sítio para o outro. Ao final, esse elétron é finalmente usado para reduzir o oxigênio molecular O_2 na formação de uma molécula de H_2O (Malina et al. 2018). Os três prótons retornam para a matriz mitocondrial pela bomba de próton F0F1, cada um formando uma molécula de ATP. Com isso, a chamada razão P/O é de 3:1, com três moles de ATP produzido para cada mol de NADH oxidado. Essa da cadeia respiratória é encontrada em *D. bruxellensis* (Leite et al 2013). No entanto, o sítio I de *S. cerevisiae* é formado por uma NADH desidrogenase que não tem função de bomba de próton e, por isso, a razão P/O é de 2:1 nessa levedura (Bakker et al. 2001). Isso é um dos motivos que fazem com que *S. cerevisiae* seja um organismo fermentador por excelência. Nossos resultados têm mostrado que a levedura *D. bruxellensis* tem também grande capacidade fermentativa, apesar da sua condição P/O=3 e de sua tendência ao metabolismo oxidativo. Portanto, o objetivo desses experimentos foi de avaliar a alteração causada pela inibição da via de produção citosólica de acetato quando a cadeia fosforilativa também está bloqueada, com inibição do metabolismo respiratório.

A antimicina é um inibidor da respiração que possui a formamida e o salicilato ligado a um anel dilactona como componentes estruturais (Labs et al. 2016). Esse anel dilactona liga-se aos grupos acil e alquil (Figura 2), o que deriva a nomenclatura AA da antimicina A (Labs et al. 2016). Esse composto inibe o complexo III, ao nível dos citocromos b e c (Potter; Reif, 1952; Trumpower; Katki, 1975), interrompendo o bombeamento de prótons durante a cadeia respiratória (Labs et al. 2016).

Figura 2. Estrutura química da antimicina A (retirado de https://en.wikipedia.org/wiki/Antimycin_A em 14/03/2022)

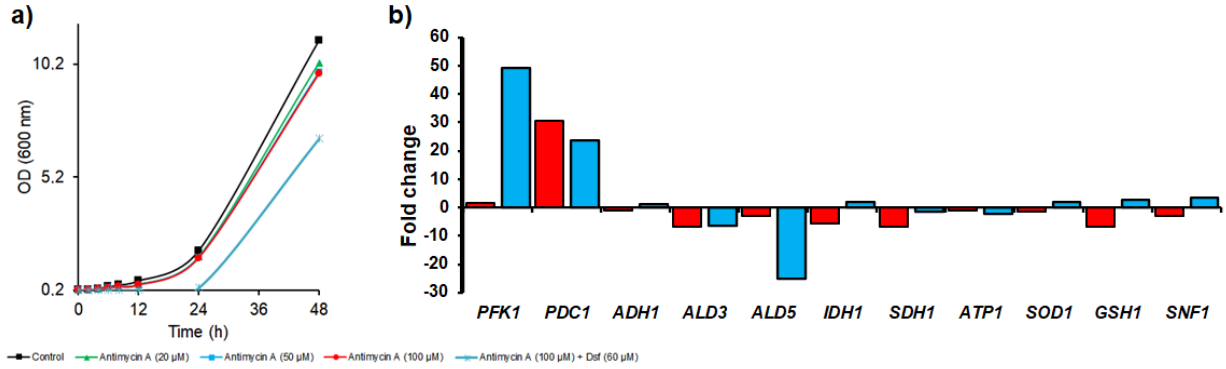


Os resultados dos cultivos de *D. bruxellensis* utilizando antimicina A mostraram ausência de interferência do inibidor no crescimento celular em concentração até 100 µM no crescimento na presença de glicose como fonte de carbono (Figura 3a). Em estudos anteriores mostramos que o crescimento de *D. bruxellensis* é melhor em anaerobiose do que em aerobiose (Peña-Moreno et al 2018). Portanto, a inibição da cadeia fosforilativa poderia mimetizar bioquimicamente uma condição de anaerobiose, fazendo com que as células priorizassem o metabolismo fermentativo. Adicionalmente, a associação do inibidor da respiração com o dissulfiram (Dsf) estendeu a fase lag por até 24h, a partir da qual as células voltaram a crescer em velocidade semelhante na ausência de DSF. Portanto, a inibição da via citosólica de produção de acetil-CoA (chamada de *Pdh bypass*) promoveu retardo no crescimento celular quando o metabolismo respiratório foi inibido. Em relação a expressão gênica, a super-expressão de *PFK1* indica que a via glicolítica foi estimulada quando os dois processos, respiração e *Pdh by-pass*, foram inibidos (Figura 3b). A sub-expressão de *ALD5* foi observada pela primeira vez e indica que de fato o carbono é prioritariamente desviado para a via fermentativa, possivelmente produzindo muito mais etanol do que tem sido observada apenas na presença de DSF (Teles et al., 2018). Os dados de análise metabólica por HPLC deverão confirmar essa hipótese.

Em glicose, a presença de antimicina A não produziu grande efeitos fisiológicos (Figura 3a). Isso se refletiu no padrão de expressão gênica (Figura 3b). O gene *PFK1* da via glicolítica não apresentou alteração na expressão na presença de antimicina A em relação ao meio de referência apenas com glicose (Figura 3a). Isso mostra que não deve ter ocorrido alteração no fluxo glicolítico. A maior expressão do gene *PDC1* indica que a inibição da respiração induz o desvio do piruvato para a via fermentativa. A inibição da respiração pela antimicina A fez com que os genes de resposta a estresse oxidativo fossem sub-expressos (Figura 3b), o que pode ser explicado pela maior atividade fermentativa e menor produção de espécies reativas

de oxigênio. A inibição concomitante da cadeia fosforilativa e do *Pdh by-pass* não teve efeito sobre os genes da via fermentativa (Figura 3a), mas levou a indução dos genes de resposta a estresse oxidativo. Neste caso, o aumento do fluxo do piruvato para a produção de acetil-CoA mitocondrial parece esbarrar na diminuição do funcionamento do ciclo de Krebs como indicado pela sub-expressão do gene SDH (succinato desidrogenase) e a baixa expressão do gene *IDH* (isocitrato desidrogenase).

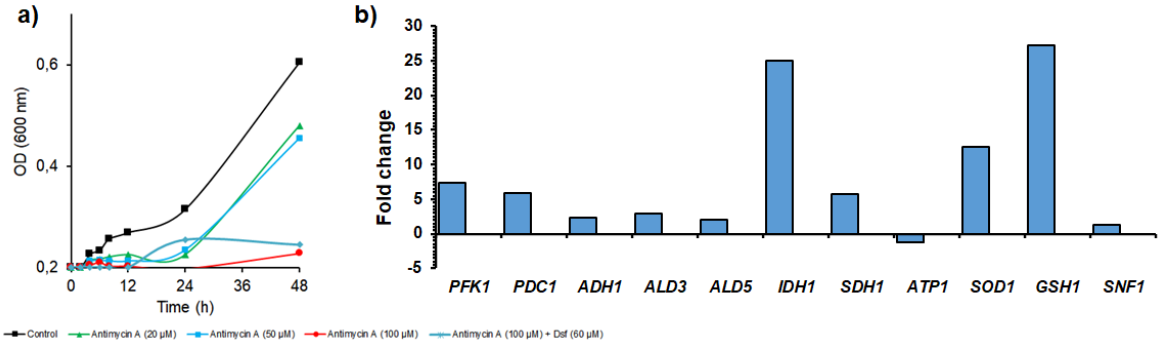
Figura 3. Metabolismo respiro-fermentativo em *D. bruxellensis* GDB 248. (Painel a) Curvas de crescimento aeróbio levedura em meio sintético definido contendo glicose. (Painel b) Expressão gênica em meio com glicose e antimicina A 100 µM (colunas vermelhas) e glicose com antimicina A 100 µM e DSF 50 µM (colunas azuis) relativa ao meio contendo glicose.



Em glicerol, a presença de antimicina A diminuiu o crescimento celular já em 20 µM e inibiu completamente o crescimento de *D. bruxellensis* em 100 µM (Figura 4a). É interessante notar que apenas 1 µM de antimicina A é capaz de inibir o crescimento de *S. cerevisiae* JP1 utilizando etanol ou glicerol como fontes de carbono (dados não mostrados) durante o mesmo período de cultivo. Foi necessário, portanto, 100 vezes mais do inibidor para produzir o mesmo efeito no crescimento de *D. bruxellensis* no cultivo com glicerol. Isso mostra que a estrutura do sítio III da cadeia fosforilativa de *D. bruxellensis* deve apresentar alguma variação em relação a de *S. cerevisiae*, que o torna muito menos sensível a antimicina A. Além disso, a presença do DSF inibiu o crescimento já na presença de antimicina A a 50 µM. Ou seja, a inibição da via *Pdh bypass* interfere no funcionamento correto do metabolismo respiratório, e torna as células mais sensíveis a inibição da cadeia fosforilativa. Diferente do observado em glicose, praticamente todos os genes testados apresentaram maior expressão na presença de antimicina A (Figura 4b).

Neste caso, os genes da resposta a estresse oxidativo indicam que ocorreu a produção de EROs. Uma hipótese para esse fenômeno é que o pouco NADH produzido no ciclo de Krebs pelo restrito uso de glicerol (vide Figura 1) pode ter sido re-oxidado no sítio I da cadeia fosforilativa. No entanto, o bloqueio do sítio III pela antimicina A deve ter causado o vazamento do elétron para a produção mitocondrial de EROs. Nesse caso, a eficiência energética do glicerol é ainda menor, com apenas um próton sendo bombeado para o espaço periplasmático e, com isso, a provável razão P/O igual a 1, com um ATP por mol de NADH, representando um terço da eficiência energética do glicerol. Esses resultados serão avaliados juntamente com análises por HPLC para o melhor estabelecimento de respostas sobre o metabolismo de *D. bruxellensis.*

Figura 4. Metabolismo oxidativo em *D. bruxellensis* GDB 248. (Painel a) Curvas de crescimento aeróbio levedura em meio sintético definido contendo glicerol. (Painel b) Expressão gênica em meio com glicerol e antimicina A 50 µM relativa ao meio contendo glicerol.

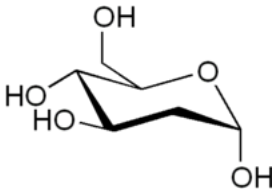


c. A repressão catabólica em *D. bruxellensis* GDB 248 é aliviada pela inibição da via *Pdh bypass*

A 2-desoxiglicose (2-DG) é um análogo da glicose no qual ocorre a substituição de um grupo hidroxila por um hidrogênio no segundo átomo de carbono da molécula. O composto tem sido utilizado sob vários aspectos, entre eles em estudos sobre captação de glicose, bloqueador metabólico, inibição enzimática e agente depletor de ATP (Laussel; Léon, 2020). Esta molécula é normalmente fosforilada a 2-deoxiglicose-6P (2DG-6P), mas a ausência na hidroxila no carbono 2 impede sua posterior isomerização ao que seria uma 2-deoxifrutose-6P. (kuo; Lampen, 1972). Portanto, 2DG6P se acumula no interior das células e induz os mecanismos biológicos de repressão catabólica, que vai reprimir os genes de utilização de fontes

alternativas de carbono e da respiração celular. Portanto, 2-DG tem sido usado para identificação de genes relacionados a repressão catabólica pela glicose (Witt et al. 1966; Randez-Gil et al. 1995; Hedbacker; carlson, 2006). Além disso, tem sido utilizado para selecionar linhagens mutantes resistentes à repressão catabólica, como no caso de leveduras que foram capazes de crescer em rafinose e sacarose mesmo na presença de 2-DG (Entian; fröhlich, 1984). Diferentes efeitos são observados com a utilização da 2-DG, dependendo se as células estão crescendo em abundância ou limitação de glicose, ou mesmo em fontes alternativas de carbono (Mccartney et al. 2014). Devido aos efeitos da 2-DG no metabolismo celular, vários estudos com o composto também foram realizados para o entendimento de características de diversos tumores (Kurtoglu et al. 2007; Stein et al. 2010; Xi et al. 2013).

Figura 5. Estrutura química da 2-deoxiglicose (retirado de https://en.wikipedia.org/wiki/Antimycin_A em 14/03/2022)

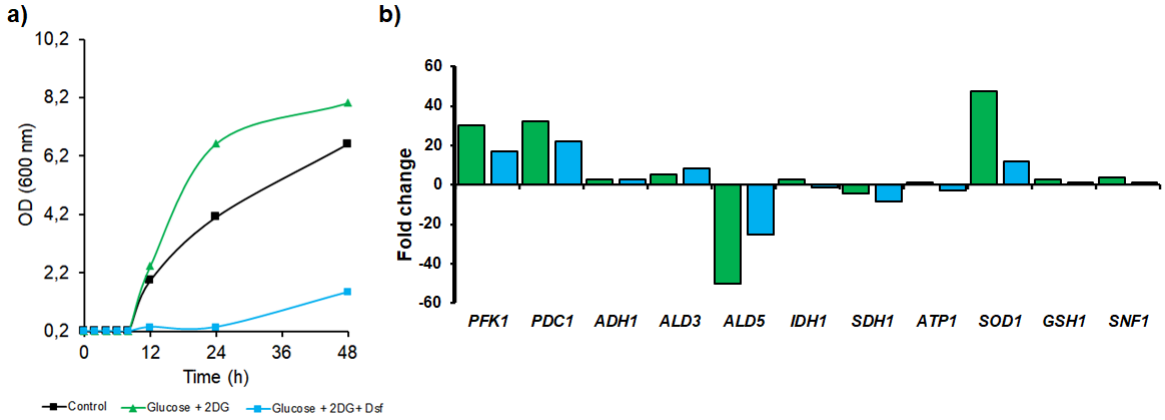


Os resultados mostraram que a presença de 2-DG induz maior crescimento celular (Figura 6a). Esse resultado de alguma forma vai ao encontro de nossos dados anteriores que mostram que as células de *D. bruxellensis* crescem mais em anaerobiose do que em aerobiose (Penã-Moreno et al 2018). Nesse caso, a presença de 2-DG estaria inibindo o metabolismo respiratório pela indução da GCR e estimulando o metabolismo fermentativo. Trabalhos anteriores mostram que *D. bruxellensis* é mais sensível a estresse oxidativo do que *S. cerevisiae* (Leite et al 2016). Então, a baixa produção de estresse oxidativo resultante da baixa atividade respiratória poderia promover maior crescimento celular como observado. Essa hipótese encontrou respaldo pela super-expressão principalmente dos genes *PFK1* e *PDC1* (Figura 6b), o que indicam alto fluxo pela glicólise e maior distribuição do carbono para a via fermentativa.

Por outro lado, foi observada uma queda considerável no crescimento celular quando DSF foi adicionado ao meio contendo 2-DG (Figura 6a). A associação 2-

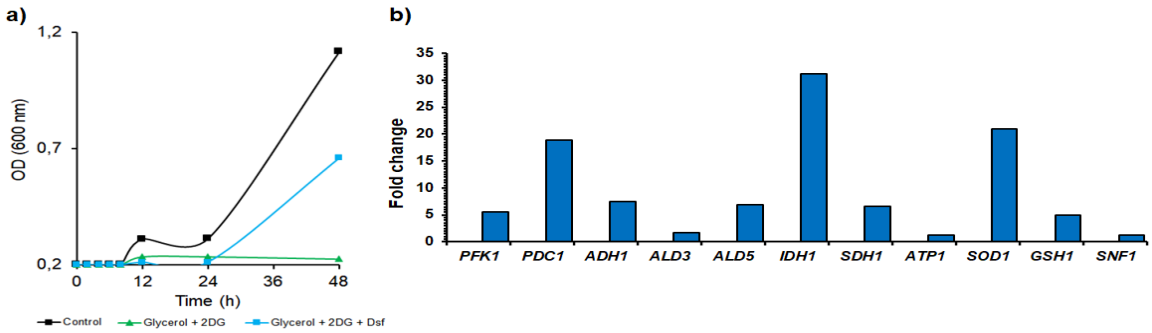
DG+Dsf inibiu o crescimento por até 24h, seguido de baixo crescimento celular depois da saída desta fase lag. Neste caso, não está ainda claro como a inibição do *Pdh by-pass* poderia produzir esse feito deletério para as células de *D. bruxellensis*.

Figura 6. Metabolismo respiro-fermentativo em *D. bruxellensis* GDB 248. (Painel a) Curvas de crescimento aeróbio levedura em meio sintético definido contendo glicose na presença de 2-deoxiglicose a 1 mM e disulfiram a 60 µM. (Painel b) Expressão gênica em meio com glicose na presença de 2-deoxiglicose a 1 mM (clunas verdes) e em glicose na presença de 2-deoxiglicose a 1 mM e disulfiram a 60 µM (colunas azuis) relativa ao meio contendo glicose.



Em glicerol, a ausência de crescimento de *D. bruxellensis* na condição glicerol+2-DG mostra o efeito da repressão catabólica (Figura 7a), inibindo a utilização de uma fonte alternativa de carbono como esperado. No entanto, diferente do que ocorreu em glicose, a presença de DSF induziu o crescimento em glicerol mesmo que na presença de repressão catabólica. O alívio metabólico causado pela inibição do *Pdh by-pass* também não está claro até o momento. Nessa condição, todos os genes testados foram super-expressos (Figura 7b). Com esse alívio, o glicerol pode ser metabolizado, gerando crescimento celular.

Figura 7. Metabolismo oxidativo em *D. bruxellensis* GDB 248. (Painel a) Curvas de crescimento aeróbio levedura em meio sintético definido contendo glicerol presença de 2-deoxiglicose a 1 mM e disulfiram a 60 µM. (Painel b) Expressão gênica em meio com glicerol e 2-deoxiglicose a 1 mM e disulfiram a 60 µM relativa ao meio contendo glicerol.


a)
1,2
0,7
0,2
OD (600 nm)
0
12
24
36
48
Time (h)
Control
Glycerol + 2DG
Glycerol + 2DG + Dsf
b)
35
30
25
20
15
10
5
0
Fold change
PFK1
PDC1
ADH1
ALD3
ALD5
IDH1
SDH1
ATP1
SOD1
GSH1
SNF1

APÊNDICE D – ARTIGO PUBLICADO EM COAUTORIA

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

Jackeline Maria da Silva, Gilberto Henrique Teles Gomes da Silva, Denise Castro Parente, Fernanda Cristina Bezerra Leite, Carolina Santos Silva, Patrícia Valente, Angélica Maria Ganga, Diogo Ardaillon Simões, Marcos Antonio de Morais Jr

FEMS Yeast Research, Volume 19, Issue 3, May 2019, foz022, https://doi.org/10.1093/femsyr/foz022
Published: 08 March 2019 **Article history**

ABSTRACT

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

Keywords: anaerobic cultivation, carbon assimilation, Crabtree effect, disaccharides, glucose catabolite repression, selective adaptation
Issue Section: Research article

APÊNDICE E – ARTIGO PUBLICADO EM COAUTORIA

Received: 19 March 2020 | Revised: 26 July 2020 | Accepted: 1 September 2020
DOI: 10.1002/yea.3519

RESEARCH ARTICLE

Yeast WILEY

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

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Funding information
Conselho Nacional de Desenvolvimento Científico e Tecnológico, Grant/Award Number: 409767/2016-7

Abstract

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

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

APÊNDICE F – ARTIGO PUBLICADO EM COAUTORIA

Isolamento e caracterização morfológica de *Acanthamoeba* spp em caixas de água de edifícios residenciais

Isolation and morphological characterization of *Acanthamoeba* spp in water tanks of residential buildings

Aislamiento y caracterización morfológica de *Acanthamoeba* spp en tanques de agua de edificios residenciales

Recebido: 05/02/2021 | Revisado: 07/02/2021 | Aceito: 12/02/2021 | Publicado: 19/02/2021

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Resumo

Acanthamoeba spp um dos gêneros que conforma o grupo das amebas de vida livre é o agente causal da ceratite amebiana e encefalite granulomatosa, doenças associadas ao uso das lentes de contato, deficiência na higiene pessoal e exposição a água contaminada. O objetivo desse trabalho foi isolar formas evolutivas de *Acanthamoeba* spp na água de consumo em um condomínio residencial no bairro as Nieves da cidade de Bogotá, Colômbia. Foram coletadas dez amostras no total, a técnica empregada foi a filtração por membrana e o cultivo no meio agar não nutritivo com uma suspenção de *Escherichia coli* para garantir o desenvolvimento da ameba. Como controle positivo foi usada uma cepa de *Acanthamoeba* spp e controle negativo água destilada estéril. As características morfológicas e tamanho das formas evolutivas foram determinadas por microscopia convencional facilitando a classificação nos grupos descritos por Pussard e Pons. De uma amostra foram identificados 5 trofozoítos e 6 cistos correspondentes aos grupos II e III respetivamente, indicando a presencia de várias espécies de *Acanthamoeba*. A amostra positiva corresponde a uma caixa que nunca foi feita a limpeza, o que foi confirmado pelo administrador do condomínio, infringindo com as normativas de saneamento das caixas de água para consumo. Concluindo que as más condições sanitárias favorecem o desenvolvimento dessa ameba e faz-se necessária a identificação das espécies junto com o genótipo para correlacionar a capacidade infecciosa.

Palavras-chave: Ameba de vida livre; Caixa de água; Ceratite amebiana; Encefalite granulomatos.