UNIVERSIDADE FEDERAL DE PERNAMBUCO

CENTRO DE CIÊNCIAS BIOLÓGICAS

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

FISIOLOGIA DA LEVEDURA *Dekkera bruxellensis* DURANTE A FERMENTAÇÃO COM SUBSTRATOS INDUSTRIAIS

LUCIANA FILGUEIRA PEREIRA

Recife
2013

LUCIANA FILGUEIRA PEREIRA

FISIOLOGIA DA LEVEDURA *Dekkera bruxellensis* DURANTE A FERMENTAÇÃO COM SUBSTRATOS INDUSTRIAIS

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

ORIENTADOR:
Prof. Dr. Marcos Antonio de Morais Júnior. Departamento de Genética – UFPE
Co orientadora: Dra. Bereneuza Tavares Ramos Valente Brasileiro.

Recife
2013

Catalogação na Fonte:
Bibliotecário Bruno Márcio Gouveia, CRB-4/1788

P436f Pereira, Luciana Filgueira
Fisiologia da levedura *Dekkera bruxellensis* durante a fermentação com substratos industriais / Luciana Filgueira Pereira. – Recife: O Autor, 2013.

102. f. : fig., tab.
Orientador: Marcos Antonio de Morais Júnior
Coorientador: Bereneuza Tavares Ramos Valente Brasileiro
Tese (doutorado) – Universidade Federal de Pernambuco. Centro de Ciências Biológicas. Pós-graduação em Ciências Biológicas, 2013.
Inclui bibliografia e anexos

1. Fungos 2. Fermentação 3. Microbiologia industrial I. Morais Júnior, Marcos Antonio (orientador) II. Brasileiro, Bereneuza Tavares Ramos Valente (coorientadora) III. Título.

579.5 CDD (22.ed.) UFPE/CCB-2013-196

LUCIANA FILGUEIRA PEREIRA

FISIOLOGIA DA LEVEDURA *Dekkera bruxellensis* DURANTE A FERMENTAÇÃO COM SUBSTRATOS INDUSTRIAIS

Orientador: Prof. Dr. Marcos Antonio de Morais Júnior

Co orientadora: Dra. Bereneuza Tavares Ramos Valente Brasileiro

Banca Examinadora:

_____

Prof. Dr. Marcos Antonio de Morais Junior (Depto. de Genética / UFPE).

_____

Profa. Dra. Maria Teresa dos Santos Correia (Depto. de Bioquímica / UFPE)

_____

Profa. Dra. Cristina Maria de Souza Motta (Depto. de Micologia / UFPE).

_____

Prof. Dra. Márcia Vanusa da Silva (Depto. de Bioquímica / UFPE)

_____

Prof. Dr. Luiz Carlos Basso (Depto. de Ciências Biológicas / ESALQ-USP)

A minha Mãe Maria Cleide pela confiança em mim depositada.

Dedico

AGRADECIMENTOS

Á Universidade Federal de Pernambuco, através do Programa de Pós Graduação do Centro de Ciências Biológicas (CCB) pela oportunidade de realização desse Doutorado;

A Fundação de Amparo Ciência e Tecnologia do Estado de Pernambuco (FACEPE) pelo suporte financeiro;

Ao meu orientador, Prof. Dr. Marcos Antonio de Morais Júnior, pela oportunidade e pelos valiosos ensinamentos acadêmicos;

A Profa. Dra. Bereneuza Tavares Ramos Valente Brasileiro pela amizade construída e colaboração nas etapas iniciais deste trabalho;

A Professora Dra. Sandra Regina Ceccato-Antonini da Universidade Federal de São Carlos (UFSCAR) pela parceria desenvolvida e pelos aprendizados;

Ao Dr. Luiz Carlos Basso da Escola Superior de Agricultura (ESALq-USP) pelos conhecimentos passados e pela colaboração neste trabalho;

Aos meus colaboradores Ana Paula Guarnieri Bassi (UFSCAR), Elisa Lucatti (UNIMEP) e Luiz Lucatti pela oportunidade de trabalhar com vocês;

A todos os colegas do Laboratório de Engenharia Metabólica do Departamento de Bioquímica- UFPE- Theresa Liberal, Carol Elsztein, Denise Castro, Raquel Fátima, Emanuella Maria, Fernanda Bezerra, Rodrigo Mendonça, Carol Almeida e Daiana Rocha;

Aos amigos César Costa Júnior e Rosângela Arruda pelo apoio em todos os momentos de minha estada aqui em Recife;

Ao meu namorado James Silva Moura Buchmeier por todo carinho e paciência;

A minha família, por todo carinho e paciência nos momentos difíceis.

RESUMO

A levedura *Dekkera bruxellensis* tem sido conhecida por sua adaptação aos processos fermentativos industriais, tendo chegado a situações que suplanta a levedura do processo *Saccharomyces cerevisiae* possuindo rendimentos equivalentes. Apesar de possuir grande adaptabilidade ao ambiente industrial, ainda são escassos trabalhos que se dediquem ao estudo de suas características fisiológicas e bioquímicas e que expliquem seu sucesso adaptativo. Este trabalho teve por objetivo descrever o metabolismo fermentativo da levedura GDB 248 em situações que simulem os processos fermentativos industriais na produção do álcool combustível bem como, sua tolerância aos diferentes fatores de estresse presentes no ambiente industrial. Foram realizadas fermentações com reciclo celular em meios sintético e caldo de cana. Os resultados mostraram que as células de *D. bruxellensis* possuem baixa eficiência no consumo da sacarose (consumo máximo 30%), maior tendência de conversão de açúcar em biomassa, mas exibem rendimentos em etanol próximos ao de *S. cerevisiae*. Os experimentos de tolerância aos fatores de estresse adicionados nas fermentações mostraram que *D. bruxellensis* possui, quando comparada a *S. cerevisiae,* tanto tolerância similar ao etanol quanto sensibilidade a ácidos orgânicos (acético e lático). Não foi detectada a produção de acido acético nas culturas de *D. bruxellensis,* tendo em ácido lático produção similar a *S. cerevisiae*. Para avaliar a tolerância de *D. bruxellensis* ao estresse, foram realizadas curvas de sobrevivência celular na presença dos agentes de estresse: H_2O_2, etanol, KCl, e após um choque térmico, relacionando o envolvimento de três proteínas de choque térmico no padrão transcricional desta resposta. Em conjunto, os resultados mostraram que as células de *D. bruxellensis* foram mais sensíveis aos fatores de estresse testados, quando comparados a *S. cerevisaie,* apesar de exibir tolerância similar ao etanol. Os genes HSP22, HSP24 e HSP82 são apenas responsivos ao choque térmico, mas suas proteínas não conferem tolerância às células. Por fim, para avaliar o comportamento desta levedura em outro substrato, frequentemente utilizado em destilarias no Brasil, e assim, compararmos o melhor desempenho desta espécie realizamos fermentações com reciclo em melaço e, desta vez, determinamos o conteúdo intracelular de trealose e glicogênio. No melaço, *D. bruxellensis* mostrou comportamento similar quanto à assimilação de sacarose, tendo tendência ao desvio do açúcar para biomassa e rendimento final em etanol equivalente ao produzido pelas células de *S. cerevisiae*. Mas, neste substrato, foi detectado a produção de ácido acético por esta levedura em quantidades superiores ao detectado nas culturas com *S. cerevisiae*. Quanto ao conteúdo intracelular dos carboidratos de reserva, só foi possível a detecção de glicogênio em quantidades inferiores a *S. cerevisiae,* pois nenhuma produção de trealose foi detectada em nossos ensaios.

Pavaras chaves: *Dekkera bruxellensis,* Fermentação de etanol, Adaptação industrial, resistência a estresse.

ABSTRACT

The yeast *Dekkera bruxellensis* has been known for its adaptation to industrial fermentations processes with situations which overcome the process of the yeast *Saccharomyces cerevisiae*. Despite it having great adaptability to the industrial environment, there are still few researches which deal to the study of its physiological and biochemical characteristics that explain its success adaptation. This study aimed at describing the fermentative metabolism of the GDB 248 yeast in situations that simulate the industrial fermentations processes for the production of fuel alcohol as well as their tolerance to different stresses factors present in the industrial environment. Fermentations were performed with cell-recycle in synthetic area and sugarcane juice. The results showed that *D. bruxellensis* cells have low efficiency in the consumption of sucrose (maximal consumption of 30%) and a higher tendency of converting sugar into biomass, but showing the yields of ethanol similar to the *S. cerevisiae* yeast. The experiments of tolerance to stress factors added in fermentations showed that *D. bruxellensis* has both similar tolerance to ethanol as sensitivity to organic acids (acetic and lactic). Acetic acid production was not detected in *D. bruxellensis* cultures, the production in lactic acid was similar to *S. cerevisiae*. To evaluate the tolerance of *D. bruxellensis* to stress, cell survival curves were performed in the presence of stress agents: H_2O_2, ethanol, KCl, and after heat shock linking the involvement of three heat-shock proteins in transcriptional standard on this response. Together, the results showed that *D. bruxellensis* cells are more sensitive to stress factors tested compared to *S. cerevisiae*, even displaying similar tolerance to ethanol. The genes HSP22, HSP24 and HSP82 are only responsive to thermal shock, but their proteins do not seem to confer tolerance to the cells. Finally, to evaluate the behavior of the yeast on another substrate, often used in distilleries in Brazil, and so, we compared the best performance of that kind of specie, we made fermentations of recycle molasses and, this time, we determined the intracellular content of trehalose and glycogen. In molasses, *D. bruxellensis* showed a similar behavior regarding assimilation of sucrose, with a tendency to the diversion of sugar to biomass and final yield in ethanol equivalent to that produced by *S. cerevisiae* cells. However, in this substrate, was detected a superior amounts of production of acetic acid by this yeast in relation to the amounts detected in cultures with *S. cerevisiae*. Regarding the intracellular content of carbohydrate of reserve, it was only possible to detect lower amounts of glycogen in *S. cerevisiae*, because no production of trehalose was detected in our tests.

Keywords: *Dekkera bruxellensis*, Ethanol fermentation, Industrial adaptation, and Stress resistance.

LISTA DE FIGURAS

REVISÃO BIBLIOGRÁFICA Página

ARTIGO 1

ARTIGO 2

ARTIGO 3

LISTA DE TABELAS

ARTIGO 1

ARTIGO 2

ARTIGO 3

LISTA DE ABREVIAÇÕES

cDNA	DNA complementar
CO_2	Dióxido de carbono
H_2O_2	Peróxido de hidrogênio
Cq	Ciclo quantitativo
KCl	Cloreto de Potássio
Mb	Megabase
NADH	Nicotinamida adenina dinucleotídeo reduzida
OD	Densidade óptica
pb	Pares de base
PFGE	Eletroforese em gel de campo pulsátil
pH	Potencial hidrogeniônico
qPCR	PCR quantitativa
RNA	Ácido ribonucleico
mRNA	Ácido ribonucleico
RT-qPCR	PCR quantitativa com transcrição reversa
sHsps	Pequenas proteínas de choque térmico
YNB	Nitrogênio levedura base
YPD	Extrato de levedura peptona Dextrose
YPG	Extrato de levedura peptona Glicerol

SUMÁRIO

INTRODUÇÃO

A levedura *Dekkera bruxellensis* tem sido reportada como bem adaptada a ambientes de fermentação alcoólica industrial, tanto na produção de álcool combustível como de vinhos. Apesar disso, ainda são restritos trabalhos acerca do metabolismo fermentativo desta levedura em substratos frequentemente utilizados nas indústrias brasileiras. No artigo intitulado: **"The physiological characteristics of the yeast *Dekkera bruxellensis* in fully fermentative conditions with cell recycling and in mixed cultures with *Saccharomyces cerevisiae*"** foram avaliados e comparados aspectos fisiológicos desta levedura com *Saccharomyces cerevisiae* pela primeira vez em caldo de cana, focando em fatores como capacidade fermentativa e tolerância a diferentes agentes inibitórios do processo industrial tais como, etanol e ácidos orgânicos.

Em 2010, foram publicados os primeiros dados, através da técnica de PCR em tempo real, sobre o padrão de expressão de genes ao estresse ambiental nas fermentações do vinho, sendo identificado, o gene codificante da proteína de choque térmico Hsp82p em *D. bruxellensis*. Numa tentativa de melhor compreender a resposta molecular de células de *D. bruxellensis* aos agentes de estresse dos processos industriais, avaliamos o envolvimento nestes processos de três proteínas de choque térmico no artigo intitulado: "**Response of *Dekkera buxellensis* to stress conditions and involvement of heat shock proteins".**

E por fim, com o intuito de avaliar o melhor desempenho desta levedura no ambiente de fermentação alcoólica, propusemos um trabalho a fim de, avaliar sua capacidade fermentativa quando se utiliza o melaço como substrato. O artigo intitulado: **"Fermentation of sugarcane molasses by *Dekkera bruxellensis* and mobilization of reserve carbohydrates"** aborda aspectos fisiológicos desta levedura bem como, a determinação e mobilização do conteúdo intracelular dos carboidratos de reserva glicogênio e trealose.

Diante do exposto, o objetivo geral deste trabalho foi caracterizar a fisiologia da linhagem industrial GDB 248 da levedura *D. bruxellensis* em condições fermentativas com substratos industriais. Estes resultados são bastante promissores na tentativa de se conhecer quais os reais fatores que contribuem para o sucesso adaptativo desta espécie no ambiente de fermentação alcoólica industrial.

1 REVISÃO BIBLIOGRÁFICA

1.1 A levedura *Dekkera bruxellensis*

A levedura *D. bruxellensis* forma teleomórfica (produtora de ascosporos) de *Brettanomyces bruxellensis*, é um microrganismo pertencente ao grupo dos hemiascomicetos da família Saccharomycetacea (MOLINA; SHEN; JONG, 1993). A morfologia das células da levedura *D. bruxellensis* é bastante diversificada, podendo apresentar-se na forma elipsoidal a esférica, frequentemente ogival, ou ainda cilíndrica a alongada (Figura 1). Frequentemente, é observada a formação de pseudomicélio (VAN DER WALT, 1964).

Os primeiros isolados de leveduras do gênero *Brettanomyces* foram feitos a partir de fermentados de cerveja inglesa. O estabelecimento do gênero foi feito em 1940 (CUSTERS et al., 1940) e mais tarde, em 1964, foi criado o gênero *Dekkera* após a observação de formação de ascosporos em algumas linhagens de *Brettanomyces* (VAN DER WALT, 1964). A validação do gênero *Dekkera* foi feita a partir das análises de restrição do DNA ribossomal, a qual estabeleceu de forma errônea à equivalência entre os gêneros *Brettanomyces* (anamorfo) e *Dekkera* (teleomorfo) e suas espécies (MOLINA et al., 1993). Atualmente, apenas cinco espécies são reconhecidas como pertencentes a estes gêneros: *D. bruxellensis*, *D. anomala*, *B. custersianus*, *B. nanus e B. naardenensis* (MITRAKUL; HENICK-KLING; EGLI, 1999; RÖDER; KÖNIG; FRÖHLICH, 2007).

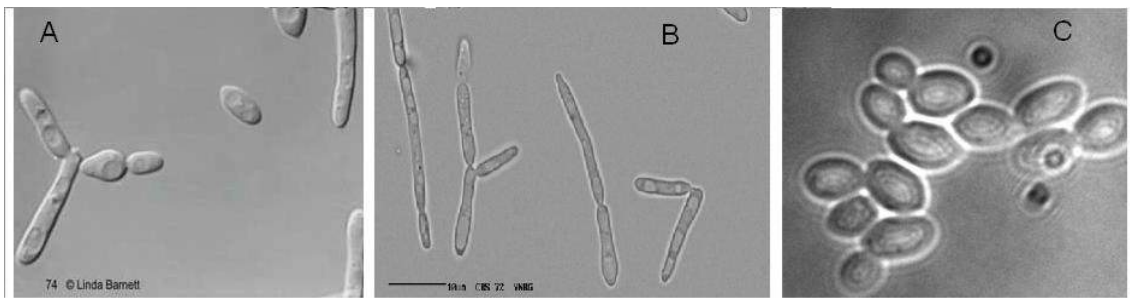

Figura 1. Células da espécie *Dekkera bruxellensis*. (A) Linhagem CBS 74 (http://www.cbs.knaw.nl/); (B) Linhagem CBS 2499 (http://www.cbs.knaw.nl/); (C) Linhagem industrial GDB 248 em meio YPD (Microscopia de contraste de fase).

1.2 Genoma e diversidade genética da levedura *D. bruxellensis*

Apesar da grande importância da levedura *D. bruxellensis* como contaminante de vários processos fermentativos, pouco se sabe sobre suas características genéticas, e poucos trabalhos relatam a diversidade genética desta levedura. Entre os isolados de *D. bruxellensis* do processo de fermentação alcoólica foram identificados dois fenótipos muito distintos a partir do uso de marcadores de PCR do tipo ISSR (Inter Single Sequences Repeats), mostrando a existência de duas variantes dentro desta espécie (DE SOUZA LIBERAL et al., 2007). Resultados similares mostraram a presença de três padrões distintos de restrição do DNA mitocondrial entre isolados da fermentação de vinho na Espanha (MARTORELL et al., 2006) e dois padrões distintos de cariótipo molecular entre isolados da fermentação de vinho na Califórnia (MITRAKUL; HENICK-KLING; EGLI, 1999).

Análises de cariotipagem por eletroforese em gel de campo pulsado (PFGE) mostrou que o genoma de *Dekkera* possui um tamanho bastante variável sendo estimado um genoma compreendido na faixa de 20 a 30 Mb (SIURKUS, 2004). Nas linhagens isoladas da fermentação industrial em destilarias da região Nordeste do Brasil este número cromossômico parece variar entre 4 a 6 cromossomos possuindo tamanhos entre 0,67 Mb e 5,7 Mb (LIBERAL, 2010).

Em 2007, foi realizado o sequenciamento do genoma de um isolado de vinícolas da levedura *D. bruxellensis* mostrando que o genoma desta espécie deve conter cerca de 7.340 genes (WOOLFIT et al., 2007). Foram identificadas 2.606 sequências completas ou parciais de genes codificadores de proteínas ortólogas as de *S. cerevisiae* e outros 277 não ortólogos *S. cerevisiae*, mas ortólogos a outras espécies Saccharomycetales, por exemplo, *Candida albicans*, *Hansenula polymorpha* e *Pichia pastoris* (WOOLFIT et al., 2007). As proteínas ortólogas entre *D. bruxellensis* e *S. cerevisiae* apresentam cerca de 48,8% de identidade em aminoácidos enquanto *S. cerevisiae* e *C. albicans* compartilham cerca de 52,9%.

Estudos recentes de agrupamento filogenético utilizando análise multigênica mostraram que, *D. bruxellensis* ocupa um dos grupos dos hemiascomycetos, juntamente com *Saccharomyces cerevisiae*, *Kluyveromyces lactis* e *Candida glabrata* (WOOLFIT et al., 2007). Graças a essas informações genéticas vários estudos já foram desenvolvidos em busca de genes relacionados ao metabolismo fermentativo (LIBERAL, 2010; NARDI; REMIZE; ALEXANDRE, 2010), via de assimilação do nitrato (DE BARROS PITA et al., 2011), resposta ao estresse ambiental e choque térmico (NARDI; REMIZE; ALEXANDRE, 2010).

Recentemente, Piskur et al. (2012) realizaram o sequenciamento total do genoma da linhagem Y879 (CBS2499) de *D. bruxellensis* isolada do vinho mostrando possuir tamanho de 13,4 Mb. Neste estudo foi possível também posicionar evolutivamente as espécies do gênero *Dekkera* como estando mais perto do grupo de leveduras consideradas "pobres" produtoras de etanol como, por exemplo, *Pichia pastoris* (Komagataella). Além disso, o resultado deste sequenciamento gerou um banco público de dados favorecendo ainda mais os estudos sobre os aspectos genéticos e filogenéticos dessa espécie (http://genome.jgi.doe.gov/Dekbr2/Dekbr2.home.html).

1.3 FISIOLOGIA DA LEVEDURA *D. BRUXELLENSIS*

1.3.1 O feito *Crabtree* em células de *Dekkera bruxellensis*

Apesar de estarem separada evolutivamente a cerca de 200 milhões de anos (WOOLFIT et al., 2007), as leveduras do gênero *Dekkera* compartilham algumas características com *S. cerevisiae* e uma dessas é a capacidade de produzir etanol, mesmo em presença de oxigênio caracterizando, assim o chamado efeito *Crabtree* positivo (PROCHÁZKA et al., 2010). Em *S. cerevisiae* e em outras leveduras, este comportamento fisiológico tem sido explicado pela saturação da capacidade respiratória em reoxidar o NADH resultante da glicólise. Este "gargalo" formado no nível do metabolismo do piruvato faz com que este seja direcionado ao metabolismo fermentativo, possibilitando que o NADH seja reoxidado via fermentação alcóolica (PRONK et al., 1996).

1.3.2 Assimilação de açúcares

A levedura *D. bruxellensis* é capaz de assimilar uma grande variedade de fontes de carbono tais como, glicose, frutose, galactose, sacarose, maltose, celobiose e trealose (CONTERNO et al., 2006). Dentre estas fontes de carbono, glicose, frutose e sacarose apresentam relevância industrial por fazer parte da composição de meios industriais como o caldo de cana e o melaço (PITA, 2009).

Ao longo dos anos o estudo de espécies do gênero *Dekkera* tem sido focado na detecção e identificação por serem tratadas como contaminante dos processos industriais. Apesar dessa ideia, ainda são escassos dados sobre a fisiologia desta levedura principalmente quando se fala em metabolismo de dissacarídeos, que já é bem descrito para *S. cerevisiae* (DIJKEN; WEUSTHUIS; PRONK, 1993; BASSO; BASSO; ROCHA, 2011). O conhecimento acerca das características fermentativas de *D. bruxellensis* em caldo de cana ainda é limitado, o que torna difícil avaliar os impactos dessa espécie neste substrato. A dinâmica de crescimento desta espécie em caldo de caldo de cana foi avaliada em diferentes trabalhos mostrando sua capacidade de adaptação a este substrato, fato este que normalmente não ocorre em leveduras desta espécie em meio rico e/ou suplementado quando comparada a *S. cerevisiae* (ABBOTT; HYNES; INGLEDEW, 2005; CIANI; MACCARELLI; FATICHENTI, 2003).

Meneghin (2007) realizou ensaios fermentativos em condições de limitações de oxigênio com uma linhagem industrial de *D. bruxellensis* (CCA 59) e, mostrou que esta linhagem, tanto em culturas pura ou mista com *S. cerevisiae* (PE-02) foi capaz de crescer em meios com caldo de cana independente do inóculo inicial (10^1-10^3 células/mL). Da mesma forma, um estudo realizado em diversas destilarias do nordeste do Brasil observou que em sistemas contínuos de fermentação, que utilizam caldo de cana, a subpopulação de *D. bruxellensis* foi capaz de substituir a de *S. cerevisiae*, mesmo com repetidas ocasiões de troca da biomassa celular total (DE SOUZA LIBERAL et al., 2007). Apesar de exibir capacidade de crescimento no caldo de cana, análises fisiológicas de espécies do gênero *Dekkera* mostraram que essas leveduras possuem, nestes substratos, baixa capacidade fermentativa quando comparadas a *S. cerevisiae.* Isto implica, em maior tempo de fermentação e menor rendimento industrial resultando em acúmulo de açúcar nas dornas industriais (ARAÚJO, 2005). A baixa capacidade fermentativa de leveduras do gênero *Dekkera* pode estar relacionada a limitações na taxa de assimilação de sacarose. É conhecido que a enzima invertase das células de *S. cerevisiae* hidrolisa a maior parte da sacarose no meio extracelular originando glicose e frutose. Por outro lado, estudos recentes mostraram que culturas de

células de uma linhagem industrial de *D. bruxellensis* exibiram apenas 10% da atividade dessa enzima como sendo extracelular. Isto implica na necessidade de transporte da sacarose para o interior das células, com provável gasto de energia (LEITE et al., 2012).

A produção de etanol de segunda geração a partir de hidrolisados ligno celulósicos tem crescido bastante ao longo dos anos. Galafassi et al. (2011) analisaram cerca de 50 linhagens pertencentes ao gênero *Dekkera/Brettanomyces* quanto ao seu potencial fermentativo relativo a estas fontes de carbono. Os resultados mostraram linhagens de *D. bruxellensis* capazes de assimilar celobiose e amido, mas não xilose e arabinose. Apesar de na descrição inicial da espécie ter sido relatado que *D. bruxellensis* não era capaz de assimilar e fermentar celobiose (VAN DER WALT, 1964), Blomqvist et al. (2010) mostraram que as células da linhagem CBS 11269 da levedura *D. bruxellensis* também foi capaz de assimilar e fermentar este dissacarídeo, porém, mais lentamente e menos eficiente que a assimilação e fermentação de glicose. Além disso, recentemente foi avaliada a capacidade de uma linhagem industrial (GDB 248) em assimilar e fermentar celobiose e os resultados mostraram que a linhagem foi capaz de assimilar e fermentar a celobiose, mas com consumo mais lento quando comparado ao de sacarose (LEITE, 2012).

As leveduras que pertencentes ao gênero *Saccharomyces* possuem a capacidade de assimilar e degradar açúcares a compostos formados por dois carbonos, em especial etanol, mesmo em condições de excesso de oxigênio. Esta característica é chamada de efeito *Crabtree* positivo o qual é a “chave” para conferir as estas espécies uma estratégia de sobrevivência chamada “Make-Accumulate-Consume”. Esta é caracterizada por um rápido consumo de açúcares, alta capacidade em produzir e acumular etanol e excelente tolerância a este composto conferindo as espécies um melhor desempenho competitivo em seu ambiente natural. Rozpedowska et al. (2011) estudaram a aquisição desta estratégia no grupo *Dekkera/Brettanomyces* e observaram que apesar de indivíduos destes gêneros terem divergindo antes do desenvolvimento desta estratégia, os dados apontam para uma teoria de que houve uma evolução paralela entre esses grupos, uma vez que, foram submetidos a pressões seletivas semelhantes no mesmo nicho.

1.3.3. Efeito *Custers* em células de *Dekkera/Brettanomyces*

As leveduras do gênero *Brettanomyces e Dekkera* possuem como característica bioquímica a inibição da fermentação alcoólica em condições anaeróbicas evento este, denominado de efeito *Custers* (WIJSMAN et al., 1984). A este efeito é atribuído a tendência das células de produzir ácido acético a partir da glicose com concomitante redução de NAD^+ (CARRASCOSA et al., 1981).

Segundo Scheffers (1966), o efeito *Custers* poderia ser explicado pela escassez de NAD^+ causada pelo seu uso para a oxidação do acetaldeído a acetato. Em condições aeróbicas, a coenzima é reoxidado pela via respiratória. No entanto, sob condições anaeróbicas e na ausência de um aceptor de próton, a produção de ácido acético ocorrerá em menor quantidade resultando numa diminuição da proporção NAD^+/NADH e consequentemente estagnação do fluxo glicolítico a nível da gliceraldeído 3-fosfato desidrogenase (SCHEFFERS; NANNINGA, 1977). Em condições limitantes de oxigênio as leveduras produzem glicerol a fim de, reoxidação do NADH produzido durante os processos de oxidação (VAM DIJKEN; SCHEFFERS, 1986). Entretanto, parece que leveduras que exibem o efeito *Custers* são de alguma forma, incapaz de fechar o balanço redox através da produção de glicerol ou outros compostos altamente reduzidos. De Souza Liberal et al. (2007) mostraram que *D. bruxellensis* pode produzir glicerol em condições anaeróbicas, mas, em pequenas quantidades. Em células de *D. bruxellensis* com essa característica o efeito *Custers* pode ser abolido pela adição de oxigênio ou um aceptor orgânico de elétron, por exemplo, acetoína ao meio, possibilitando que o NADH formado via glicólise e formação de acetato seja reoxidado via cadeia respiratória (CARRASCOSA et al., 1981). Estudos recentes mediram a atividade enzimática da glicerol 3-fosfato desidrogenase durante o crescimento celular e observaram que sua atividade pode ser observada apenas em condições limitantes de oxigênio (GALAFASSI et al., 2011).

1.3.4 Produção de ácido acético por *D. bruxellensis*

As linhagens de *D. bruxellensis* tem uma importante característica de produzir ácido acético como subproduto da assimilação de glicose em condições aeróbicas. Em leveduras, o ácido acético é produzido a partir da oxidação do acetaldeído, que pode ser formado como um produto da reação da piruvato descarboxilase (PDC) durante o crescimento em glicose ou como produto da reação da enzima álcool desidrogenase (ADH) durante o crescimento em etanol (POSTMA et al., 1989). Já é conhecido que as linhagens de *D. bruxellensis* podem

produzir ácido acético a partir glicose e etanol (AGUILAR USCANGA; DELIA; STREHAIANO, 2003; BLOMQVIST et al., 2010; FREER, 2002; FREER; DIEN; MATSUDA, 2003). Por muito tempo foi proposto que o ácido acético produzido pelas células *D. bruxellensis* pudesse conferir uma vantagem competitiva sobre as células *S. cerevisiae* (DE MINIAC, 1989). Entretanto, em condições industriais o nível de oxigênio é limitado, o que não proporcionaria uma elevação da concentração de ácido acético que fosse capaz de inibir ou mesmo, interferir no crescimento das *S. cerevisiae* (ABBOTT; HYNES; INGLEDEW, 2005; BLOMQVIST et al., 2010; PHOWCHINDA; DÉLIA-DUPU; STREHAIANO, 1995). Além disso, a inibição do crescimento e interferência da capacidade fermentativa das células de *D. bruxellensis* só ocorre, quando a concentração de ácido acético chega a níveis superiores a 2 g/L (YAHARA e et al., 2007). Blomqvist (2011), utilizando cultivos contínuos e com limitação de oxigênio (5% de oxigênio dissolvido), observou que as células de *D. bruxellensis* produziram quantidades de ácido acético inferiores 1g/L, concentração esta que não foi suficiente para afetar o crescimento e desenvolvimento das leveduras *D. bruxellensis* e *S. cerevisiae* nos processos de fermentação industrial.

1.3.5 Assimilação de fontes de nitrogênio

As leveduras encontram em seu ambiente natural, uma ampla variedade de fontes de nitrogênio que podem ser utilizadas tais como, amônia, prolina, arginina e nitrato (CONTERNO et al., 2006; GODARD et al., 2007). Apesar disso, a capacidade de assimilação do nitrato e/ou nitrito é restrita a poucas espécies (SIVERIO, 2002). Dentre as diferenças metabólicas entre *S. cerevisiae* e *D. bruxellensis* destaca-se a capacidade desta última de assimilar nitrato e nitrito como fontes de nitrogênio, característica que é utilizada na taxonomia bioquímica do gênero (BARNETT et al., 2002). A via de assimilação do nitrato *em D. bruxellensis* possuem muitos genes ortólogos aos da levedura *Hansenula polymorpha* diferindo apenas na posição relativa e orientação (WOOLFIT et al., 2007). Essa via é composta por transportadores de alta afinidade (DbYNT1) e duas enzimas redutases, a nitrato (DbYNR1) e nitrito redutase (DbYNI1). Esta dupla redução da molécula de nitrato resulta em amônia e reoxidação de 4 moléculas de NAD(P)H (SIVERIO, 2002).

O caldo de cana pode apresentar concentrações significativas de nitrato oriundo do processo de adubação do campo com fertilizantes a base de nitrato (nitrato de cálcio, nitrato

de magnésio ou mesmo nitrato de amônia), muito utilizado nos canaviais. Desta forma, o nitrato poderia constituir uma fonte de nitrogênio que seria assimilada pelas células de *D. bruxellensis* para seu crescimento (PITA, 2009). De Barros Pita et al. (2011) a partir de estudos de competição entre *D. bruxellensis* e *S. cerevisiae* mostraram que *D. bruxellensis* é capaz de superar a população de *S. cerevisiae* em meios contendo uma maior concentração de nitrato.

1.4 *DEKKERA BRUXELLENSIS* NO CONTEXTO INDUSTRIAL

A levedura *Dekkera bruxellensis* tem sido por muito tempo considerada como a responsável por contaminações na produção de vinhos visto que, produz metabólitos, sendo os mais significantes o ácido acético e etilfenóis, como 4-etilfenol e 4-etilguaiacol. A presença destes compostos está relacionada especificamente a atividade de *D. bruxellensis* e aos aromas desagradáveis encontrados em vinhos contaminados (CHATONNET et al., 1995; 1997).

Um trabalho de monitoramento em destilarias da região nordeste, que utilizam caldo de cana como substrato, sugeriu a levedura *D. bruxellensis* como principal contaminante da produção do álcool combustível. Outros relatos desta levedura já foram observados em destilarias de álcool de álcool dos EUA e Canadá, onde a matéria-prima é o milho (ABBOTT; HYNES; INGLEDEW, 2005), e ainda na Europa, onde o substrato é beterraba (DE MINIAC, 1989; CIANI; MACCARELLI; FATICHENTI, 2003). O melhor desempenho competitivo de *D. bruxellensis* em relação ao de *S. cerevisiae* no ambiente industrial indica um elevado *fitness* da espécie ao ambiente da fermentação alcoólica. Em princípio, a vantagem adaptativa de *D. bruxellensis* nestas condições pode estar relacionada a um fator nutricional ou a uma maior tolerância a um estresse ambiental. Já que, estudos recentes mostram *D. bruxellensis* exibindo maior adaptação ao ambiente industrial quando comparada a *S. cerevisiae* e isto pode estar relacionado à maior tolerância a baixos pH e elevada resistência a etanol (ROZPEDOWSKA et al., 2011). Apesar de trabalhos anteriores sugerirem esta espécie como contaminante industrial de processos de produção de álcool combustível, dados atuais tem mostrado que *D. bruxellensis* é capaz de produzir etanol a rendimentos próximos ao de *S. cerevisiae* (DE SOUZA LIBERAL et al., 2007; DE BARROS PITA et al., 2011). Além disso,

Passoth et al. (2007) mostraram que essa espécie quando utilizada em consórcio com *Lactobacillus vini* mostra boa contribuição para a produção do etanol.

1.5 FATORES QUE AFETAM O METABOLISMO DE *DEKKERA BRUXELLENSIS*

Os estudos de *D. bruxellensis* no que ser relaciona a sua capacidade fermentativa e metabolismo tem sido focado em alguns fatores tais como, influência de oxigênio (AGUILAR USCANGA; DELIA; STREHAIANO, 2003; CIANI; FERRARO, 1997), agitação e temperatura (CASTRO MARTINEZ et al., 2005), fonte de carbono (AGUILAR USCANGA et al., 2007), pH (FREER; DIEN; MATSUDA, 2003; ROZPEDOWSKA et al., 2011; BLOMQUIST et al., 2010) e temperatura (BRANDAM et al., 2008). Aguilar Uscanga et al. (2003) observaram que a concentração de oxigênio disponível afeta a drenagem do substrato para a produção de etanol ou ácido acético. Assim, quanto mais aerado for o sistema de cultivo, as células de *D. bruxellensis* tendem a drenar mais carbono para a produção de ácido acético do que para etanol.

Em relação à preferência por fontes de carbono para produção de ácido acético, estudos mostram que as células de *D. bruxellensis* tendem a produzir mais ácido acético a partir de glicose do que etanol (FREER, S.; DIEN, B.; MATSUDA, 2003). Aguilar Uscanga et al. (2007) avaliaram o efeito de diferentes fontes de carbono sobre o crescimento de *Brettanomyces bruxellensis* e a influência das mesmas sobre a produção de ácido acético e etanol e observaram que, de todas as fontes de carbono utilizadas o rendimento máximo de ácido acético (0,24 g/g) e produtividade (0,14 g/L/ h) foram obtidos das culturas que continham melaço com 60g/L de sacarose como fonte de carbono.

Estudos mostram que, para a levedura *D. bruxellensis* a temperatura é também um fator de suma importância nos processos fermentativos. Já foi observado que quando esta espécie é submetida à temperatura de 35ºC ocorre uma parada no seu crescimento, antes mesmo, que todo o substrato seja consumido (BRANDAM et at al., 2008; BISSON; BUTZKE, 2000). Brandam et al. (2008) avaliaram a influência da temperatura sobre a fermentação, formação de metabólitos e taxa de crescimento da linhagem IHEM 6037 de *D. bruxellensis* e foi observado que, na faixa de temperatura entre 15 e 32ºC, a formação de biomassa foi muito semelhante e que a temperatura de 35ºC causou uma queda de 65% na

formação de biomassa e de 40% na viabilidade celular para a linhagem por eles testada. Consequentemente, nesta mesma condição de 35º C, houve uma queda nas taxas de produção de biomassa e metabólitos como etanol e ácido acético.

A capacidade de crescimento e tolerância a ambientes com baixo pH também tem sido investigada para linhagens de *D. bruxellensis*. Em um estudo recente, Blomqvist (2011) investigou a influência do pH e temperatura sobre a taxa de crescimento e o rendimento em etanol para uma linhagem industrial de *D. bruxellensis*, e observou que a faixa de pH (3 a 5) e temperatura (25º a 37º C) testados não afetaram os parâmetros analisados levando a conclusão de que *D. bruxellensis* é bastante resistente a mudanças nas condições ambientais. Por fim, *D. bruxellensis* tem mostrado ser mais bem adaptada ao ambiente industrial do que *S. cerevisiae* e isto, pode está relacionado a uma maior tendência que estas células possuem em tolerar baixos pH além de possuir elevada resistência ao etanol (ROZPEDOWSKA et al., 2011).

1.6 INFLUÊNCIA DE FATORES DE ESTRESSE SOBRE OS PROCESSOS FERMENTATIVOS

Durante os processos fermentativos as leveduras estão sujeitas a vários fatores de estresses que afetam seu crescimento e metabolismo fermentativo. Estes podem ser ambientais ou mesmo, decorrentes do próprio metabolismo da levedura (BAI et al., 2008; BASSO et al., 2008). Os agentes de estresse industriais os quais as leveduras são expostas estão exibidos na figura 2. Alguns destes, podem agir sinergisticamente (DORTA et al., 2006; BAI et al., 2008), particularmente quando há reciclo de células (BASSO et al., 2008) afetando mais ainda a viabilidade da levedura e consequentemente produção de etanol.

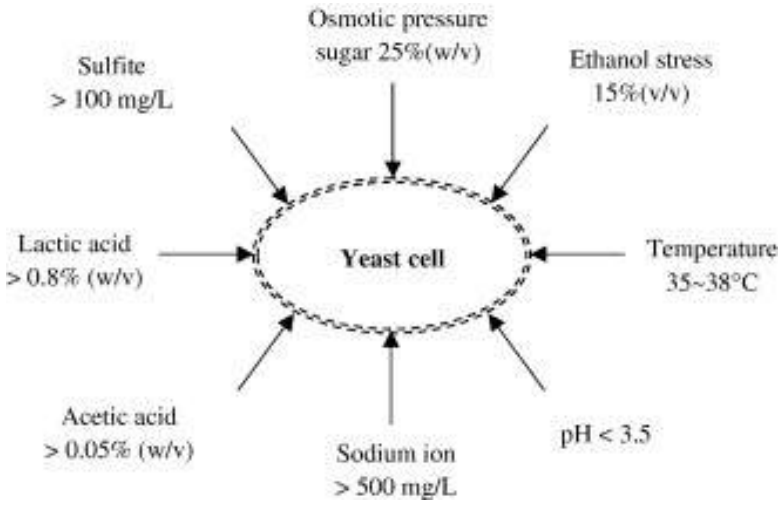

Figura 2. Fatores físico-químicos que induzem estresse em células de *Saccharomyces cerevisiae* durante a fermentação alcoólica (INGLEDEW, 1999; BAI et al., 2008).

É conhecido que a levedura *S. cerevisiae* exibe capacidade de tolerância ao etanol nos processos fermentativos (JENSEN et al., 2009). Porém, altas concentrações de etanol quando em sinergismo com elevadas temperaturas ou baixos pH afetam até mesmo, linhagens industriais de *S. cerevisiae* consideradas resistentes a altas temperaturas (MONACO, 2007).

Muitas das modificações que ocorrem nas leveduras após choque térmico são idênticas às causadas pela exposição ao estresse etanólico. Existe, um sinergismo entre esses dois fatores fazendo com que o etanol seja mais severo sob altas temperaturas (PIPER, 1995; ALDIGUIER et al., 2004). Outro fator que age em sinergismo com o etanol é o aumento da pressão osmótica. Este em células de *S. cerevisiae* quando associado ao etanol tende a potencializar o efeito tóxico do mesmo levando a uma redução da viabilidade celular (JOHN et al., 2012). O aumento da pressão osmótica também causa perda rápida de água intracelular (HOHMANN, 1997), danos intracelulares e parada do crescimento na fase G1 (BELLÍ et al., 2001) e G2 (ALEXANDER et al., 2001), contração ou enrugamento da célula (MORRIS at al., 1983) e numa fase posterior, acúmulo de glicerol no citoplasma, como forma de neutralizar a desidratação (MAGER; SIDERIUS, 2002).

O estresse osmótico torna-se preocupante em fermentações com melaço de cana de açúcar visto que, este substrato mostra grandes quantidades de sais. Elevados níveis de cálcio, potássio e magnésio encontrados neste substrato excedem os requisitos nutricionais da levedura. Os níveis médios de potássio (4.000 mg/L), presentes nos processos fermentativos, são suficientes para induzir uma resposta ao estresse com aumento na formação de glicerol,

diminuição do conteúdo intracelular dos carboidratos de reserva e, consequentemente diminuição do rendimento em etanol (ALVES, 2000). Nos processos de fermentação de produção de álcool combustível as leveduras são separadas do mosto por centrifugação, seguida por uma lavagem ácida de ácido sulfúrico antes de uma nova fermentação (SILVA-FILHO et al., 2005). Este procedimento, embora seja utilizado para tentar reduzir a contaminação bacteriana, provoca perturbações na fisiologia da levedura levando a uma lixiviação de minerais (N, P, K, Mg). Essa situação na levedura *S. cerevisiae* provoca uma diminuição do conteúdo intracelular de trealose seguido de queda de viabilidade (FERREIRA; AMORIM; BASSO, 1999).

A presença de ácidos orgânicos pode ser um dos fatores para a redução do pH intracelular da levedura e prejudicar sua homeostase. Ácidos orgânicos quando em baixos pH adquirem uma forma protonada, que facilmente atravessa a membrana plasmática, e dentro do citoplasma, onde o pH está próximo à neutralidade, a maioria dos ácidos fracos se dissocia, liberando prótons e ânions, acidificando assim, o citoplasma. Prótons e ânions, podem ser expelidos para o exterior celular a custo de ATP, estes últimos se associam novamente com os prótons, devido ao baixo pH do meio e então, podem difundir-se novamente através da membrana, formando um ciclo que ocasiona um gasto dispendioso de energia pela célula no intuito de manter sua homeostase (ORIJ; BRUL; SMITS , 2011). Os ácidos orgânicos que são frequentemente produzidos em ambientes de fermentação são o lático e acético. O ácido lático é produzido por bactérias láticas contaminantes dos processos industriais, e o ácido acético é sintetizado em pequenas quantidades por leveduras. Narendranath, Thomas e Ingledew (2001) observaram que a concentração inibitória mínima do ácido acético sobre o crescimento de linhagens de *S. cerevisiae* foi de 0,6% (p/v) ou 100 mM enquanto que, para o ácido lático foi de 2,5% (p/v) ou 278 mM. O efeito inibitório tanto do ácido acético como lático torna-se maior quanto menor for o pH do meio, devido a uma maior concentração da forma não dissociada dos mesmos (GRAVES et al., 2006).

Por fim, embora os processos fermentativos sejam em condições de anaerobiose, o estresse oxidativo pode ser causado pelo crescimento aeróbico durante a propagação de biomassa podendo gerar, espécies reativas de oxigênio (ROS), levando a vários tipos de danos celulares (GÓMEZ-PASTOR; PÉREZ-TORRADO; MATALLANA, 2010). Para lidar com os danos causados pelo estresse oxidativo e assim, manter o estado “redox”, os organismos tendem a se adaptar por meio de defesas antioxidantes formados por sistemas enzimáticos e não enzimáticos (FOLCH-MALLOL et al., 2004).

1.7 RESPOSTA MOLECULAR AO ESTRESSE

Células de *S. cerevisiae* desenvolveram uma rápida resposta molecular para reparar danos e proteger as estruturas celulares dos efeitos causados pelo estresse (ESTRUCH, 2000). Entretanto, parece que o efeito STR deve estar mais relacionado com a adaptação das células, induzindo genes e ativando mecanismos que irão proteger as células de mudanças ambientais posteriores (BERRY; GASH, 2008).

Os elementos de transcrição de resposta a estresse, ou STRE (Elementos de transcrição de choque térmico) são motivos presentes nas sequências promotoras dos genes que respondem aos estímulos induzidos pelos agentes causadores de estresse celular. Estes medeiam à expressão de um número de genes dentro do mecanismo de resposta geral ao estresse (MARTINEZ-PASTOR et al., 1996). Os STREs correspondem a cópias simples ou múltiplas da sequência de nucleotídeos CCCCT/AGGGG que servem como local de ligação dos fatores de transcrição Msn2p ou Msn4p, ativados por estresses diversos tais como, choque térmico, osmótico, oxidativo ou mesmo, em situações de carência de nutrientes (RUIS; SCHÜLLER, 1995). Outro elemento de transcrição ativado pelo estresse é o elemento de choque térmico, ou HSE (Elemento de choque térmico). Este contém pelo menos três repetições de sequência de nucleotídeos de nGAAn, e serve como local de ligação para um outro fator de transcrição de choque térmico o chamado Hsf1p (BAUER; PRETORIUS, 2000; ESTRUCH, 2000).

1.7.1 Proteínas de choque térmico

As chaperonas moleculares são definidas como um grupo de proteínas interativas que modulam, tanto a dobra e desdobramento de outras proteínas ou mesmo, a montagem e desmontagem de complexos proteína-proteína, proteína-DNA e proteína-RNA (HARTL; HAYER-HARTL, 2002; DEUERLING, BUKAU, 2004; SAIBIL, 2008). Em eucariotos, o fator de transcrição de choque térmico (HSF) é o principal modulador de resposta ao choque térmico, levando a indução de expressão de proteínas de choque térmico (HSP) que

geralmente servem como chaperonas moleculares que ajudam no dobramento de proteínas. Muitas destas estão presentes na ausência de estresse, no entanto, na presença dos mesmos, os genes que a codificam tem sua expressão aumentada e em muitos casos, contribuem para a tolerância celular em associação com o mecanismo de resposta geral ao estresse (BAUER, PRETORIUS, 2000; ESTRUCH, 2000).

As proteínas de choque térmico são classificadas de acordo com suas massas moleculares, gerando as famílias Hsp100, Hsp90, Hsp70, Hsp60, e as sHSPs que são pequenas chaperonas ubíquas presentes nas células eucarióticas (MORANO; GRANT; MOYE-ROWLEY, 2011). As sHSPs são proteínas de baixo peso molecular que formam grandes estruturas homo oligoméricas. A característica marcante destas proteínas é a o aumento na expressão dos genes que a codificam durante condições de estresse, fazendo com que as mesmas participem dos processos de proteção celular (HASLBECK et al., 2005; WELKER et al., 2010). Devido ainda a poucos dados sobre as estruturas e propriedades das sHSPs, muitas estão anotadas como pertencentes a família HSP20 (POULAIN; GELLY; FLATTERS, 2010). As sHSPs têm massas moleculares variando entre 10 e 30 kDa e possuem em sua região “C”- terminal, uma sequência conservada entre os membros de sua família chamada de domínio alfa cristalino (Figura 3). Este domínio possui cerca de 90 aminoácidos sendo flanqueado por um braço “N” terminal de sequência divergente e variável em comprimento (média de 55 aminoácidos) e por uma extensão “C”- terminal com aproximadamente menos de 20 resíduos (DE JONG; LEUNISSEN; VOORTER, 1993; POULAIN; GELLY; FLATTERS, 2010). Dentre as várias funções das sHSPs pode-se citar o envolvimento da Hsp26p e Hsp42p na manutenção do proteoma da levedura em estado solúvel durante o choque térmico. (HASLBECK et al., 2004). Em *S. cerevisiae* a Hsp12p atua em diversas condições tais como, choque térmico, osmótico, oxidativo, elevadas concentrações de etanol, bem como, no início da fase estacionária (WELKER et al., 2010; VARELA et al., 1995). Outra importante sHSPs é a Hsp104p que além de, ser induzida por uma variedade de estresses ambientais, possui um papel essencial na aquisição de termotolerância celular e quando associada ao carboidrato trealose atua no combate a desnaturação proteica (HOHMANN; MAGER, 1997**;** SANCHEZ; LINDQUIST, 1990).

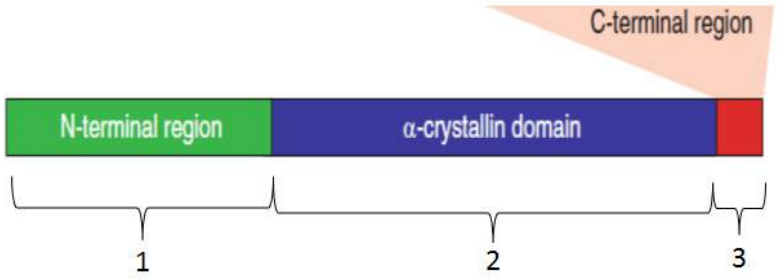

Figura 3. Arquitetura do domínio Alfa Cristalino das Proteínas de choque térmico adaptado de Hilton et al. (2013). 1- Região "N"- terminal com cerca de 55 aminoácidos. 2. Domínio alfa cristalino com cerca de 90 aminoácidos e 3. Região "C"- terminal contendo resíduos menores que 20 aminoácidos.

1.7.2 Proteção cruzada em células de leveduras

Um importante aspecto de resposta ao estresse em células de levedura é o fenômeno da resistência adquirida. Células de diversos organismos podem resistir a estresses severos quando são previamente expostas a uma forma branda deste estresse (SIDERIUS; MAGER, 1997). A existência do mecanismo de proteção cruzada levantou a hipótese de que as condições de estresse requerem um maior esforço do mecanismo de resposta geral e, este, envolve vários tipos de funções celular tais como, proteção celular, metabolismo de energia, produção de proteínas protetoras e síntese de carboidratos de reserva como, por exemplo, trealose. Estas funções celulares e as proteínas de choque térmico são as respostas mais notáveis associadas ao mecanismo de proteção cruzada (SOTO et al., 1999; TROTT; MORANO, 2003).

Os processos de adaptação celular, os efeitos de proteção cruzada e o agente de estresse a qual as células são expostas parecem sugerir respostas celulares distintas. Jamieson (1992) avaliou o efeito de proteção cruzada em células de *S. cerevisaie* quando expostas aos agentes oxidantes menadiona (1mM) e H_2O_2 (0.7mM) durante um período de 1h. Os resultados mostraram que, apesar de ser obter uma resposta cruzada contra o H_2O_2 por tratamento de células com menadiona, o pré-tratamento das mesmas com H_2O_2 sensibilizou as células a menadiona.

1.8 METABÓLITOS INDICATIVOS DE FATORES ESTRESSANTES

Nos processos de fermentação, ocorrem mudanças no perfil dos metabólitos intracelulares tais como, trealose, glicogênio, esteróis e glicerol, o que pode estar relacionado com mecanismos adaptativos que permitem que as células de levedura possam lidar contra os fatores de estresse presente nesses processos (PEREIRA et al., 2011). A trealose e o glicogênio chegam a constituir 25% do peso seco da levedura, possuindo funções de reserva de carbono e energia cujos níveis intracelulares podem variar de acordo com diferentes mudanças ambientais (BASSO et al., 2011; SCHULZE; LARSEN; VILLADSEN; FRANCOIS; PARROU, 2001). Na levedura *S. cerevisiae*, glicogênio e trealose estão envolvidos na tolerância a diferentes estresses (ATTFIELD, 1997; PARROU; TESTE; FRANÇOIS, 1997; SINGER; LINDQUIST, 1998). Entretanto, os carboidratos de reserva trealose e glicogênio podem ser mobilizados para a formação de álcool quando são utilizados como fonte alternativa de carbono em processos conhecidos como fermentação endógena (FERREIRA; AMORIM; BASSO, 1999).

1.8.1 Trealose

A trealose é um dissacarídeo não redutor formado por duas unidades de glicose unidas por uma ligação α–1,1 glicosídica. Este açúcar esta presente em vários organismos tais como, bactérias, fungos, invertebrados e plantas servindo como fonte de energia e carbono (ELBEIN et al., 2003). Na fase exponencial de crescimento de leveduras, são observados baixos níveis intracelulares de trealose resultante da repressão exercida pela glicose sobre a expressão dos genes envolvidos na biossíntese desse metabólito (BOULTON; QUAIN, 2001). Por outro lado, em condições normais de crescimento, ocorre um acúmulo deste carboidrato logo após, as células entrarem em fase estacionária. Outro tipo de situação que pode favorecer o acúmulo de trealose é quando as células estão expostas a condições de estresse, donde a trealose pode atuar como um protetor contribuindo para a sobrevivência celular (ALCARDE; BASSO, 1997; THEVELEIN, 1984; VAN LAERE, 1989; WIEMKEN, 1990). Essa situação de mobilização do “pool” de trealose intracelular é acompanhada de altos níveis de mRNA de genes que codificam para as subunidades do complexo trealose sintase (*TPS1, TPS2, TPS3* e *TSL1*) e também, acúmulo da proteína Tps1p (PARROU; TESTE; FRANÇOIS, 1997). Esta

mobilização, embora pareça um ciclo fútil de síntese/degradação, está também relacionada com a renovação da reserva de fosfato dentro das células (Figura 4) (PARROU; TESTE; FRANÇOIS, 1997).

Na levedura *S. cerevisiae* os estresses térmicos e salinos favorecem o acúmulo de trealose. Para a recuperação da viabilidade celular nessas condições é necessário que a trealose acumulada seja mobilizada, permitindo que as células retornem às condições normais (WERA et al., 1999; GARRE; MATALLANA, 2009). O acúmulo de trealose é também observado em situações de privação de nitrogênio e fosfato podendo sugerir, uma reposta comum a vários fatores nutricionais. Aranda, Salgado e Taillandier (2004) mostraram que em situações de limitações de carbono e nitrogênio o acúmulo de trealose nas células de *S. cerevisaie* pode chegar até 13% do peso em biomassa seca.

Os processos fermentativos estão sujeitos a diferentes fatores de estresse (PATARO et al., 2000; 2002) e o acúmulo de trealose parece estar envolvido na capacidade de sobrevivência das leveduras a esses ambientes. Pataro et al. (2002) observaram que as linhagens de *Sacharomyces* de processos de produção de cachaça que mais mostraram acúmulo de trealose, foram as submetidas a um estresse térmico numa temperatura subletal de 40ºC por um período de 60 min. Por outro lado, todas as linhagens testadas apresentaram queda de viabilidade e de trealose quando transferidas diretamente da temperatura de 28ºC para 50ºC por um período de 8 min. Situação semelhante foi observada por Hottiger, Schmutz e Wiemken (1987) quando ao transferirem cultivos de *S. cerevisiae* previamente incubados a 27ºC para uma temperatura de 40ºC, observaram que houve um aumento rápido do conteúdo intracelular de trealose enquanto que, quando o cultivo foi retornado para a temperatura de 27ºC a trealose tinha sido degradada.

O acúmulo de trealose em linhagens de *S. cerevisiae* está relacionado com a manutenção da viabilidade celular e tolerância ao etanol durante a fermentação alcoólica (MANSURE; SOUZA; PANEK, 1997). Sharma (1997), observou que células de *S. cerevisiae*, quando crescidas previamente sob concentrações crescentes de NaCl e, em seguida, expostas a uma concentração de 16% de etanol (v/v), mostraram maior tolerância ao etanol, maior viabilidade e maior acúmulo de trealose do que as células controle. Segundo Benaroudj, Lee e Goldberg (2001) a trealose protege as células contra os danos induzidos pelos radicais de oxigênio, bem como, da toxicidade do etanol. O pré-tratamento de linhagens de *S. cerevisiae* a 38ºC proporcionou um aumento exponencial no conteúdo de trealose além de, aumentar a viabilidade das células quando expostas ao estresse oxidativo (BENAROUDJ; LEE; GOLDBERG; 2001). Outro papel exercido pela trealose é o de proteção das células de

levedura contras os danos causados à parede celular por agentes biocidas (ELSZTEIN et al., 2008).

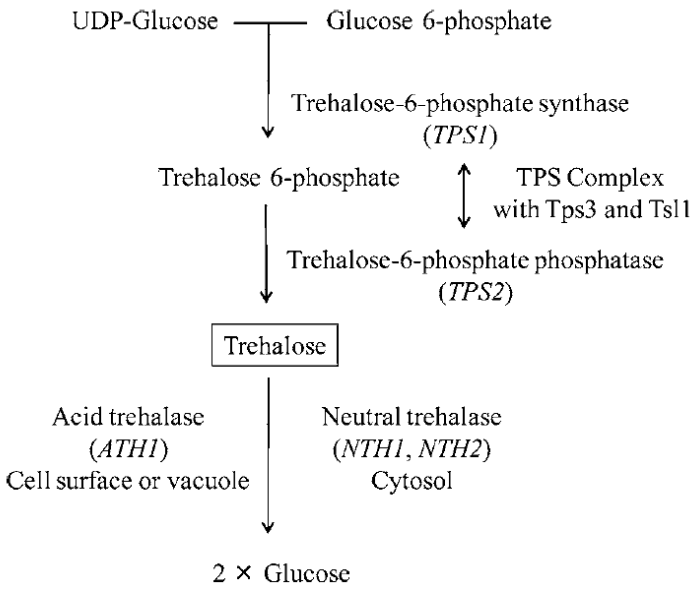

Figura 4. Via de síntese de trealose na levedura *Saccharomyces cerevisiae* (SHIMA; TAKAGI, 2009). A via de síntese de trealose inicia-se com a formação da trealose-6-fosfato a partir da UPD-glicose e glicose-6-fosfato. As enzimas que catalisam a síntese, trealose sintase 6-P (TPS1) e trealose 6-P fosfatase (TPS2), fazem parte de um complexo em que, as proteínas Tsl1 e TPS3, participam. Por fim, a degradação da trealose é feita por duas trealases distintas, uma ácida confinada nos vacúolos celulares e outra neutra, localizada no citossol.

1.8.2 Glicogênio

O glicogênio é uma grande reserva de polímero intracelular constituído por subunidades α-1,4-glicose com ligações α-1,6-glicose nos pontos de ramificação (WILSON et al., 2010). O conteúdo deste metabólito pode variar entre 1% a 23% do peso total de matéria seca da levedura e por isso, é considerado o principal carboidrato de reserva, de grande importância para sobrevivência celular durante situações de privação de nutrientes (LILLIE; PRINGLE, 1980; SILLJEET et al., 1999). Entretanto, quando as células estão expostas a um estado quiescente prolongado, o glicogênio é degradado para induzir a síntese de trealose a fim de, proteger as células contra o estresse nutricional (SHI et al., 2010). A síntese do glicogênio (Figura 5) é aumentada em situações de limitações de fontes de carbono, nitrogênio, fósforo e enxofre (LILLIE; PRINGLE, 1980). O glicogênio está envolvido na tolerância e adaptação celular da levedura ao estresse etanólico. Segundo Dake, Khetmalas e Amarapurkar (2011) o

crescimento de células de *S. cerevisiae* na presença de 2-8% (v/v) de etanol resulta no aumento do glicogênio insolúvel, bem como, do conteúdo total de carboidratos como forma de proteção contra o estresse osmótico. Por outro lado, concentrações de etanol superiores a 8% v/v promovem a redução do teor de glicogénio insolúvel. Resultados recentes mostraram que linhagens industriais da levedura *S. cerevisiae* apresentam aumento de seu conteúdo de glicogênio após 12 h de fermentação sendo esse aumento, acentuado ao final da fermentação. As cepas industriais PE e CA1185 chegaram a apresentar acúmulo de glicogênio na faixa de 2,6 a 4,2 vezes maiores do que a linhagem de laboratório CEN. PK113-7D (PEREIRA et al. 2011).

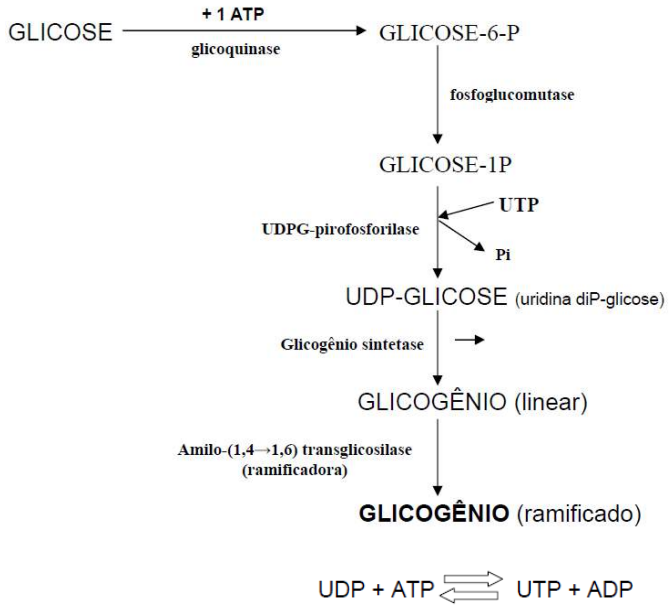

Figura 5. Via de síntese de glicogênio. Fonte: Gutierrez, 1997. p. 238

1.8. 3 Glicerol

As células de leveduras quando expostas ao etanol sintetizam trealose e glicerol para serem utilizados com função de proteção (GUYOT, S.; FERRET, E.; GERVAIS, P, 2005). Durante a fermentação alcoólica, a formação do glicerol ocorre em função de vários fatores tais como, tipo de linhagem utilizada, aumento de temperatura, pH e da concentração de sacarose do meio (GUTIERREZ, 1991). Nos processos fermentativos o glicerol é o principal subproduto das células de *S. cerevisiae*, formados principalmente como o resultado de reações re-oxidação para consumir o excesso de NADH, formado durante a biossíntese sob condições anaeróbicas (VAN DIJKEN; SCHEFFERS, 1986; BAI; ANDERSON; MOO-YOUNG,

2008). A formação do glicerol em condições anaeróbicas está relacionada com a manutenção do balanço de redox intracelular. Nessa condição, o NADH é reoxidado a NAD^+ através da formação do glicerol. A síntese de 1 mol de glicerol a partir da glicose leva a oxidação de um 1 mol de NADH (ANSELL et al., 1997; WANG et al., 2001). Como a síntese do glicerol utiliza o poder redutor (NADH), a produção do mesmo é aumentada quando há excesso de NADH na célula, que ocorre quando processos oxidativos se desenvolvem, sejam decorrentes da produção de biomassa ou formação de ácidos orgânicos (BASSO; ALVES; AMORIM, 1996).

2 CONSIDERAÇÕES FINAIS

A adaptabilidade das células de leveduras aos processos de fermentação alcoólica bem como, sua capacidade fermentativa é influenciada por uma série de fatores relacionados com a disponibilidade de nutrientes ou mesmo a presença de compostos produzidos pela microbiota do processo. A levedura *D. bruxellensis* mostra-se bem adaptada aos processos industriais para produção de etanol, fato este que a torna um microrganismo que vem despertando o interesse científico e tecnológico pela capacidade de produção de etanol e de competição com a levedura *S. cerevisiae.* No Brasil, o caldo de cana e melaço ou misturas destes, são os substratos mais frequentemente utilizados nos processos fermentativos para produção de álcool combustível. Estes substratos parecem bem adequados para o crescimento das células de *D. bruxellensis*, pois trabalhos de monitoramento em destilarias da região Nordeste mostraram que a levedura *D. bruxellensis* pode suplantar a população de células da levedura do processo. Na tentativa de uma melhor compreensão acerca da fisiologia desta espécie nestes substratos, o presente estudo buscou em situações fermentativas miméticas a dos processos industriais avaliar as características fisiológicas desta espécie no intuito de compreender seus mecanismos de adaptação a estes ambientes. Os resultados alcançados podem contribuir para o emprego biotecnológico desta levedura nos processos fermentativos de produção de álcool combustível.

REFERÊNCIAS

ABBOTT, D.; HYNES, S.; INGLEDEW, W. Growth rates of Dekkera/Brettanomyces yeasts hinder their ability to compete with Saccharomyces cerevisiae in batch corn mash fermentations. **Applied microbiology and biotechnology**, v. 66, n. 6, p. 641-647, 2005.

AGUILAR USCANGA, M.; DELIA, M. L.; STREHAIANO, P. *Brettanomyces bruxellensis*: effect of oxygen on growth and acetic acid production. **Applied microbiology and biotechnology,** v. 61, n. 2, p. 157-162, 2003.

AGUILAR USCANGA, M.G.; ABARCA, B. I. E; RODRIGUEZ, J.G.; GARCIA, R. C. Carbon Sources And Their Effect On Growth, Acetic Acid And Ethanol Production By *Brettanomyces Bruxellensis* In Batch Culture. **Journal of Food Process Engineering,** v. 30, n.1 p.13-23, 2007.

ALCARDE, A.R.; BASSO, L.C. Efeito da trealose na manutenção da viabilidade de células de leveduras desidratadas por liofilização. **Scientia Agricola**, Piracicaba, v. 54, n. 3, p. 189-194, 1997.

ALDIGUIER, A.S; ALFENORE, S.; CAMELEYRE, X.; GOMA, G.; URIBELARREA, J.L.; GUILLOUET, S.E.; MOLINA-JOUVE, C. Synergistic temperature and ethanol effect on Saccharomyces cerevisiae dynamic behaviour in ethanol bio-fuel production. **Bioprocess and Biosystems Engineering**, New York, v. 26, p. 217-222, 2004.

ALEXANDER, M.R.; TYERS, M.; PERRET, M.; CRAIG, B.M.; FANG, K.S.; GUSTIN, M.C. Regulation of cell cycle progression by Swe1p and Hog1p following hypertonic stress. **Molecular Biology of the Cell,** v. 12, n. 1, p. 53-62, 2001.

ALVES, D.M.G. **Respostas fisiológicas de duas linhagens de *Saccharomyces cerevisiae* frente ao potássio durante a fermentação alcoólica**. Rio Claro: Instituto de Biociências/UNESP, 2000. 118p.

ANSELL, R.; GRANATH, K.; HOHMANN, S.;THEVELEIN, J.M.; ADLER, L. The two isoenzymes for yeast NAD+ -dependent glycerol 3-phosphate dehydrogenase encoded by GPD1 and GPD2 have distinct roles in osmoadaptation and redox regulation. **The EMBO Journal**,v. 16, n. 9, p. 2179-2187, 1997.

ARANDA, J.S.; SALGADO, E.; TAILLANDIER, P. Trehalose accumulation in *Saccharomyces cerevisiae* cells: experimental data and structured modeling. **Biochemical Engineering Journal**, v. 17, n.2, p. 129-140, 2004.

ARAÚJO, P.R.L.; BASÍLIO, A.C.M.; SIMÕES, D.A.; MORAIS JR, M.A.; MORAIS, J.O.F. Informação sobre algumas leveduras contaminantes da fermentação alcoólica industrial isoladas no nordeste de Brasil. In: XV Simpósio de Nacional de Bioprocessos,15., 2005, Recife, **Anais...,**Recife,2005.

ATTFIELD, P.V. Stress tolerance: the key to effective strains of industrial baker´s yeast. **Nature Biotechnology**, v. 15, n.13, p. 1351-1357, 1997.

BAI, F.W.; ANDERSON, W.A.; MOO-YOUNG, M. Ethanol fermentation technologies from sugar and starch feedstocks. **Biotechnology Advances**, New York, v. 26, n. 1, p. 89-105, 2008.

BARNETT, J.A.; PAYNE, R.W.; YARROW, D. **Yeasts**: Characteristics and Identification. Cambridge: Cambridge University Press, 2002. 1002p.

BASSO, L.C.; ALVES, D.M.G.; AMORIM, H.V. Fermentação alcoólica e alguns fatores que afetam o desempenho fermentativo. In: AMORIM, H.V. (Ed.). **Processo de produção de álcool**: controle e monitoramento. 2 ed. Piracicaba: FERMENTEC/FEALQ/ESALq-USP, 1996. cap. 4, p. 38-85.

BASSO, L.C.; AMORIM, H.V.; OLIVEIRA, A.J.; LOPES, M.L. Yeast selection for fuel ethanol in Brazil. **FEMS Yeast Research**, Amsterdam, v. 8, n. 7, p. 1155-1163, 2008.

BASSO, L.C.; BASSO, T.O.; ROCHA, S.N. Ethanol production in Brazil: the industrial process and its impact on yeast fermentation. In: DOS SANTOS BERNARDES, M.A. (Ed.).

Biofuel production - recent developments and prospects. Croatia: INTECH, 2011. cap. 5, p. 85-100.

BAUER, F.F.; PRETORIUS, I.S. Yeast stress response and fermentation efficiency: how to survive the making of wine. **A review. South African Journal of Enology and Viticulture**, vol. 21, pp. 27-51, 2000.

BELLÍ, G.; GARÍ, E.; ALDEA, M.; HERRERO, E. Osmotic stress causes a G1 cell cycle delay and downregulation of Cln3/Cdc28 activity in Saccharomyces cerevisiae. **Molecular Microbiology**, Salem, v. 39, n. 4, p. 1022-1035, 2001.

BENAROUDJ, N.; LEE, D.H.; GOLDBERG, A.L. Trehalose accumulation during cellular stress protects cells and cellular proteins from damage by oxygen radicals. The **Journal of Biological Chemistry**, v. 276, n. 26, p. 24261-24267, 2001.

BERRY, D. B; GASCH, A. P. Stress-activated genomic expression changes serve a preparative role for impending stress in yeast.**Molecular Biology of the Cell**, v.19, p. 4580-4587, 2008.

BISSON, L.F.; BUTZKE, C. E. Diagnosis and rectification of stuck andsluggish fermentations. **American Journal of Enology and Viticulture**, v. 51, n.2, p. 168-177, 2000.

BLOMQVIST, J. ***Dekkera bruxellensis* - a competitive yeast for ethanol production from conventional and non-conventional substrate**s. Uppsala: Swedish University of Agricultural Sciences, 2011. 59p.

BLOMQVIST, J.; EBERHARD, T.; SCHNÜRER, J.; PASSOTH, V. Fermentation characteristics of *Dekkera bruxellensis* strains. **Applied microbiology and biotechnology,** v. 87, n. 4, p. 1487-1497, 2010.

BOULTON, C.; QUAIN, D. **Brewing Yeast and Fermentation**. Oxford: Blackwell Science Ltd, 2001.

BRANDAM, C. B. C.; CASTRO-MARTÍNEZ, C. C. M. C.; MARIE-LINE DÉLIA, M. L. D.; RAMÓN-PORTUGAL, F. R. P. F.; STREHAIANO, P. S. P. Effect of temperature on

Brettanomyces bruxellensis: metabolic and kinetic aspects. **Canadian journal of microbiology,** v. 54, n. 1, p. 11-18, 2008.

CARRASCOSA, J.; VIGUERA, M. D.; NÚÑEZ DE CASTRO, I.; SCHEFFERS, W. Metabolism of acetaldehyde and custers effect in the yeast. **Antonie van Leeuwenhoek**, v. 47, n. 3, p. 209-215, 1981.

CASTRO-MARTINEZ, C.; ESCUDERO-ABARCA, B.I., GOMEZ RODRIGUEZ, J., HAYWARD-JONES, P.M., AGUILAR-USCANGA, M.G. Effect of physical factors on acetic acd production in *Brettanomyces* strains. **Journal of food process engineering**, v. 28, n.2, 2005.

CHATONNET, P.; DUBOURDIEU, D.; BOIDRON, J.N. The influence of *Brettanomyces/Dekkera* sp. yeasts and lactic acid bacteria on the ethylphenol content of red wines.**American Journal of Enology and Viticulture**, v. 46, p. 463-468, 1995.

CHATONNET, P.; VIALA, C.; DUBOURDIEU, D. Influence of polyphenol components of red wines on the microbial synthesis of volatile phenols. **American Journal of Enology and Viticulture**, v.48, p. 463-468, 1997.

CIANI, M.; FERRARO, L. Role of oxygen on acetic acid production by *Brettanomyces/Dekkera* in winemaking. **Journal of the Science of Food and Agriculture,** v.75, p. 489-495, 1997.

CIANI, M.; MACCARELLI, F.; FATICHENTI, F. Growth and fermentation behaviour of *Brettanomyces/Dekkera* yeasts under different conditions of aerobiosis. World **Journal of Microbiology and Biotechnology**, v. 19, n. 4, p. 419-422, 2003.

CONTERNO, L.; JOSEPH, C.; ARVIK, T. J.; HENICK-KLING, T.; BISSON, L. F. Genetic and physiological characterization of *Brettanomyces bruxellensis* strains isolated from wines. **American journal of enology and viticulture**, v. 57, n. 2, p. 139-147, 2006.

CUSTERS, M. T. **Onderzoekingen over het gistgeslacht Brettanomyces**. Deltf: Deltf University, 1940.178p.

DAKE, M. S.; KHETMALAS, M. B.; AMARAPURKAR. S. V. Role of insoluble glycogen in ethanol adaptation mechanism of *Saccharomyces italicus*. **Indian Journal of Science and Technology**, v. 4, n.1, p.52, 2011.

DE BARROS PITA, W.; LEITE, F. C. B.; DE SOUZA LIBERAL, A. T.; SIMÕES, D. A.; DE MORAIS, M. A. The ability to use nitrate confers advantage to *Dekkera bruxellensis* over *S. cerevisiae* and can explain its adaptation to industrial fermentation processes. **Antonie van Leeuwenhoek**, v.100, n. 1, p. 99-107, 2011.

DE JONG, W.W.; LEUNISSEN, J.A.; VOORTER, C. E. Evolution of the alphacrystallin/small heat-shock protein family. **Molecular Biology and Evolution**, v.10,n.1, p. 103-126, 1993.

DE MINIAC, M. Contamination des fermentations alcooliques industrielles par les levures du genre: *Brettanomyces*. **Industries alimentaires et agricoles**, v. 106, n. 7-8, p. 559-563, 1989.

DE SOUZA LIBERAL, A.; BASILIO, A.; DO MONTE RESENDE, A.; BRASILEIRO, B.; DA SILVA FILHO, E.; DE MORAIS, J.; SIMOES, D.; DE MORAIS JR, M. Identification of *Dekkera bruxellensis* as a major contaminant yeast in continuous fuel ethanol fermentation. **Journal of applied microbiology**, v. 102, n. 2, p. 538-547, 2007.

DEUERLING, E.; BUKAU, B.Chaperone-assisted folding of newly synthesized proteins in the cytosol.**Critical Reviews in Biochemistry and Molecular Biology**, v.39,n. 5-6, p.261–277, 2004.

DIJKEN, J. P.; WEUSTHUIS, R. A.; PRONK, J. T. Kinetics of growth and sugar consumption in yeasts. **Antonie van Leeuwenhoek**, v. 63, n. 3, p. 343-352, 1993.

DORTA, C.; OLIVA-NETO, P.; DE-ABREU-NETO, M.S.; NICOLAU-JUNIOR, N.; NAGASHIMA, A.I. Synergism among lactic acid, sulfite, pH and ethanol in alcoholic fermentation of *Saccharomyces cerevisiae* (PE-2 and M-26).**World Journal of Microbiology and Biotechnology,** Oxford, v. 22, p. 177-182, 2006.

ELBEIN, A.D.; PAN, Y.T.; PASTUSZAK, I.; CARROLL. New insights on trehalose: a multifunctional molecule. **Glycobiology**, v.13, n. 4, p. 17–27, 2003.

ESTRUCH, F. Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. **FEMS Microbiology Reviews**, v. 24, n. 4, p. 469-486, 2000.

FERREIRA, L.V.; AMORIM, H.V.; BASSO, L.C. Fermentação de trealose e glicogênio endógenos em *Saccharomyces cerevisiae*. **Ciência e Tecnologia de Alimentos**, v. 19, n. 1, p. 29-32, 1999.

FOLCH-MALLOL, J. L.; GARAY-ARROYO, A.; LLEDÍAS, F.; ALEJANDRA, A.; ROBLES, C. La respuesta a estrés em la levadura *Saccharomyces cerevisiae*. **Revista Latino americana de Microbiología,** v. 46, n.1-2, p.24-26, 2004.

FRANCOIS, J.; PARROU, J. L. Reserve carbohydrates metabolism in the yeast *Saccharomyces cerevisiae*. **FEMS Microbiology Reviews**, v. 25, n.1, p. 125-145, 2001.

FREER, S. Acetic acid production by *Dekkera/Brettanomyces* yeasts. **World Journal of Microbiology and Biotechnology,** v. 18, n. 3, p. 271-275, 2002.

FREER, S.; DIEN, B.; MATSUDA, S. Production of acetic acid by *Dekkera/Brettanomyces* yeasts under conditions of constant pH. **World Journal of Microbiology and Biotechnology,** v. 19, n. 1, p. 101-105, 2003.

GALAFASSI, S.; MERICO, A.; PIZZA, F.; HELLBORG, L.; MOLINARI, F.; PIŠKUR, J.; COMPAGNO, C. *Dekkera/Brettanomyces* yeasts for ethanol production from renewable sources under oxygen-limited and low-pH conditions. **Journal of industrial microbiology and biotechnology**,v.38, n.8, p. 1079-1088, 2011.

GARRE, E.; MATALLANA, E. The three trehalases Nth1p, Nth2p and Ath1p participate in the mobilization of intracellular trehalose required for recovery from saline stress in *Saccharomyces cerevisiae*. **Microbiology**,v. 155, n.9, p. 3092-3099, 2009.

GODARD, P.; URRESTARAZU, A.; VISSERS, S.; KONTOS, K.; BONTEMPI, G.; VAN HELDEN, J.; ANDRÉ, B. Effect of 21 different nitrogen sources on global gene expression in the yeast *Saccharomyces cerevisiae*. **Molecular and Cellular Biology**, v. 27, n. 8, p. 3065, 2007.

GÓMEZ-PASTOR, R.; PÉREZ-TORRADO, R.; MATALLANA, E. Improving yield of industrial biomass propagation by increasing the Trx2p dosage. **Bioeng Bugs**, v. 1, n. 5, p. 352-353, 2010.

GRAVES, T.; NARENDRANATH, N.V.; DAWSON, K.; POWER, R. Effect of pH and lactic or acetic acid on ethanol productivity by *Saccharomyces cerevisiae* in corn mash. **Journal of Industrial Microbiology and Biotechnology**, v. 33, n. 6, p. 469-474, 2006.

GUTIERREZ, L. E. Produção de glicerol por linhagens de *Sacharomyces cerevisiae* durante fermentação alcoólica. In; Anais da escola superior de Agricultura “Luiz de queiroz”,1991, n. 48 p.55-69, Piracicaba, **Anais**..., Piracicaba,1991.

GUTIERREZ, L.E. **Bioquímica de Leveduras**. Piracicaba: ESALq. Departamento de Ciência de Tecnologia Industrial, 1997, 270 p.

GUYOT, S.; FERRET, E.; GERVAIS, P. Responses of *Saccharomyces cerevisiae* to thermal stress. **Biotechnology and Bioengineering**, v.92, n.4, p. 403-409, 2005.

HARTL, F.U.; HAYER-HARTL, M. Molecular chaperones in the cytosol: from nascent chain to folded protein. **Science**,v. 295,n. 5561, p. 1852–1858, 2002.

HASLBECK, M.; BRAUN, N.; STROMER,T.; RICHTER,B.; MODEL, N.; WEINKAUF, S.; BUCHNER J. Hsp42 is the general small heat shock protein in the cytosol of *Saccharomyces cerevisiae.* **EMBO Journal**, v. 23,n. 3, p. 638-649, 2004.

HASLBECK, M.A.; MIESS, T.; STROMER, S.; WALTER; J. BUCHNER, J. Disassembling protein aggregates in the yeast cytosol. The cooperation of Hsp26 with Ssa1 and Hsp104. **The Journal of Biological Chemistry**, v. 280, n. 25, p. 23861-23868, 2005.

HILTON, G. R; LIOE, H. H.; STENGEL, F.; BALDWIN, A. J.; BENESCH, J. L. P. Small Heat-Shock Proteins: Paramedics of the Cell. **Topics in Current Chemistry**, v. 328, p. 69-98, 2013.

HOHMANN, S.; MAGER, W.H. **Yeast stress responses**. New York: Springer, 1997. 252p.

HOTTIGER, T.; SCHMUTZ, P.; WIEMKEN, A.Heat-induced accumulation and futile cycling of trehalose in *Saccharomyces cerevisiae*. **Journal of Bacteriology**,v.169, n. 12, 5518–5522, 1987.

INGLEDEW, W.M. **Alcohol production by Saccharomyces cerevisiae: a yeast primer, in the alcohol textbook.** 3rd ed. UK: Nottingham University Press, 1999. p. 49-87.

JAMIESON, D. J. *Saccharomyces cerevisiae* has distinct adaptive responses to both hydrogen peroxide and menadione. **Journal of Bacteriology**, v.174, n. 20, p. 6678-6681, 1992.

JENSEN, S. L.; UMIKER, N. L.; ARNEBORG, N.; EDWARDS, C. G. Identification and characterization of *Dekkera bruxellensis, Candida pararugosa*, and *Pichia guilliermondii* isolated from commercial red wines. **Food microbiology**, v. 26, n. 8, p. 915-921, 2009.

JOHN, G.S.M.; GAYATHIRI, M.; ROSE, C.; MANDAL, A.B. Osmotic shock augments ethanol stress in *Saccharomyces cerevisiae* MTCC 2918. **Current Microbiology,** v. 64, n. 2, p. 100-105, 2012.

LEITE, F. C. B. **Fisiologia molecular da levedura *Dekkera bruxellensis***. Recife: Universidade Federal de Pernambuco, 2012. 80p.

LEITE, F. C. B.; GOMBERT, A. K.; MORAIS JR, M. A.; SIMOES, D. A. Quantitative aerobic physiology of the yeast *Dekkera bruxellensis*, a major contaminant in bioethanol production plants. **FEMS Yeast Research**, 2012.

LIBERAL, A. T. D. S. **Análise da constituição genética de linhagens industriais da levedura *Dekkera bruxellensis***. Recife: Universidade Federal de Pernambuco, 2010. 88p.

LILLIE, S. H.; PRINGLE, J.R. Reserve Carbohydrate metabolism in *Saccharomyces cerevisiae*: responses to nutrient limitation. **Journal of Bacteriology**, v.143, n.3, p. 1384-1394, 1980.

MAGER, W.H.; SIDERIUS, M. Novel insights into the osmotic stress response of yeast. **FEMS Yeast Research**, v. 2, n. 3, p. 251-257, 2002.

MANSURE, J.J.; SOUZA, R.C.; PANEK, A. D. Trehalose metabolism in *Saccharomyces cerevisiae* during alcoholic fermentation. **Biotechnology Letters**, v. 19, n. 12, p. 1201-1203, 1997.

MARTINEZ-PASTOR, M. T., G. MARCHLER, C. SCHULLER, A. MARCHLER-BAUER, H. RUIS,; ESTRUCH, F. The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). **Embo Journal**, v. 15, n.9, p.2227-2235,1996.

MARTORELL, P.; BARATA, A.; MALFEITO-FERREIRA, M.; FERNÁNDEZ-ESPINAR, M.T.; LOUREIRO, V.; QUEROL, A. Molecular typing of the yeast species *Dekkera bruxellensis* and *Pichiaguilliermondii* recovered from wine related sources. **International Journal of Food Microbiology**, v.106, p. 79–84, 2006.

MENEGHIN, M. C. **Caracterização e comportamento fermentativo de linhagens de Dekkera contaminantes da fermentação alcoólica**. Piracicaba: Ciência e Tecnologia de alimentos, Escola Superior de Agricultura Luiz de Queiroz., 2007. 86p.

MITRAKUL, C.; HENICK-KLING, T.; EGLI, C. Discrimination of *Dekkera/Brettanomyces* yeast isolates from wine by using various DNA fingerprinting methods. **Food Microbiology**, v. 16, p. 3-14, 1999.

MOLINA, F. I.; SHEN, P.; JONG, S. C. Validation of the species concept in the genus *Dekkera* by restriction analysis of genes coding for rRNA. **International journal of systematic bacteriology,** v. 43, n. 1, p. 32, 1993.

MONACO, M.A.S.L. **Efeito protetor do magnésio no choque térmico e estresse pelo etanol em leveduras *Saccharomyces cerevisiae***. Piracicaba: Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo, 2007. 67p.

MORANO, K. A.; GRANT, C. M.; MOYE-ROWLEY,W. S. The Response to Heat Shock and Oxidative Stress in *Saccharomyces cerevisiae*. **Genetics**, v. 190, n.4, p. 1157-1195, 2012.

MORRIS, G. J; WINTERS, L.; COULSON, G.E.; CLARKE, K.J. Effect of osmotic stress on the ultrastructure and viability of the yeast *Saccharomyces cerevisiae*. **Journal of General Microbiology**, v. 132, n. 7, p. 2023-2034, 1986.

NARDI, T.; REMIZE, F.; ALEXANDRE, H. Adaptation of yeasts *Saccharomyces cerevisiae* and *Brettanomyces bruxellensis* to winemaking conditions: a comparative study of stress genes expression. Applied microbiology and biotechnology, p. 1-13, 2010.

NARENDRANATH, N.V.; THOMAS, K.C.; INGLEDEW, W.M. Effects of acetic acid and lactic acid on the growth of *Saccharomyces cerevisiae* in a minimal medium. **Journal of Industrial Microbiology and Biotechnology**, v. 26, p. 171-177, 2001.

ORIJ, R.; BRUL, S.; SMITS, G.J. Intracellular pH is a tightly controlled signal in yeast. **Biochimica et Biophysica Acta**, v. 1810, n. 10, p. 933-944, 2011.

PARROU, J.L.; TESTE, M. A.; FRANÇOIS, J. Effects of various types of stress on the metabolism of reserve carbohydrates in *Saccharomyces cerevisiae*: genetic evidence for a stress-induced recycling of glycogen and trehalose. **Microbiology**, 143, n.6, p. 1891-1900, 1997.

PASSOTH, V.; BLOMQVIST, J.; SCHNURER, J. *Dekkera bruxellensis* and *Lactobacillus vini* form a stable ethanol-producing consortium in a commercial alcohol process. **Applied and environmental microbiology**, v.73, n. 13, p. 4354-6, 2007.

PATARO C, GUERRA, J.B.; GOMES, F.C.O.; NEVES, M.J.; PIMENTEL, P. F.; ROSA, C.A. Trehalose accumulation, invertase activity and physiological characteristics of yeasts

isolated from 24 h fermentative cycles during the production of artisanal Brazilian cachaça. **Brazilian Journal of Microbiology**, São Paulo, v. 33, n.3, p. 33:202–208, 2002.

PATARO, C.; GUERRA, J.B.; PETRILLO-PEIXOTO, M.L.; MENDONCA-HAGLER, L.C.; LINARDI, V.R.; ROSA, C. A. Yeast communities and genetic polymorphism of *Saccharomyces cerevisiae* strains associated with artisanal fermentation in Brazil. **Journal of Applied Microbiology,** v.89, n. 1, p. 24-31, 2000.

PEREIRA, F. B; GUIMARÃES, P.M.R.; TEIXEIRA, J. A.; DOMINGUES, L. Robust industrial *Saccharomyces cerevisiae* strains for very high gravity bio-ethanol fermentations. **Journal of Bioscience and Bioengineering**, v. 112, n.2, p.130–136, 2011.

PHOWCHINDA, O.; DÉLIA-DUPUY, M.; STREHAIANO, P. Effects of acetic acid on growth and fermentative activity of *Saccharomyces cerevisiae*. **Biotechnology letters**, v. 17, n. 2, p. 237-242, 1995.

PIPER, P.W. The heat shock and ethanol stress responses of yeast exhibit extensive similarity and functional overlap. **FEMS Microbiology Letters**, Amsterdam, v. 134, n. 2-3, p. 121-127, 1995.

PISKUR, J.; LING, Z.; MARCET-HOUBEN, M.; ISHCHUK, O.P.; AERTS, A.; LABUTTI, K.; COPELAND, A.; LINDQUIST, E.; BARRY, K.; COMPAGNO, C.; BISSON, L.; GRIGORIEV, I.V.; GABALDÓN, T.; PHISTER, T. The genome of wine yeast *Dekkera bruxellensis* provides a tool to explore its food-related properties. **Intrernational Journal Food Microbiology**, p. 202-9, 2012.

PITA, W. D. B. **Análise da expressão dos genes relacionados à assimilação do nitrato na levedura *Dekkera Bruxellensis*.** Recife: Univerdidade Federal de Pernambuco, 2009.

POSTMA, E.; VERDUYN, C.; SCHEFFERS, W. A.; VAN DIJKEN, J. P. Enzymic analysis of the crabtree effect in glucose-limited chemostat cultures of *Saccharomyces cerevisiae.* **Applied and environmental microbiology**, v. 55, n. 2, p. 468, 1989.

POULAIN, P.; GELLY, J.C.; FLATTERS, D. Detection and architecture of small heat shock protein monomers. **PLoS ONE**, v.5, n,4, p.e9990, 2010.

PROCHÁZKA, E.; POLÁKOVÁ, S.; PIŠKUR, J.; SULO, P. Mitochondrial genome from the facultative anaerobe and petite positive yeast *Dekkera bruxellensis* contains the NADH dehydrogenase subunit genes. **FEMS yeast research**, v. 10, n. 5, p. 545-557, 2010.

PRONK, J. T.; STEENSMA, H. Y.; VAN DIJKEN, J. P. Pyruvate metabolism in *Saccharomyces cerevisiae*. **Yeast**, v. 12, n. 16, p. 1607-1633, 1996.

RÖDER, C.; KÖNIG, H.; FRÖHLICH, J. Species specific identification of *Dekkera/Brettanomyces* yeasts by fluorescently labeled DNA probes targeting the 26S rRNA. FEMS yeast research, v. 7, n. 6, p. 1013-1026, 2007.

ROZPEDOWSKA, E.; HELLBORG, L.; ISHCHUK, O. P.; ORHAN, F.; GALAFASSI, S.; MERICO, A.; WOOLFIT, M.; COMPAGNO, C.; PIŠKUR, J. Parallel evolution of the make–accumulate–consume strategy in *Saccharomyces* and *Dekkera* yeasts. **Nature Communications**, v. 2, p. 302, 2011.

RUIS, H.; SCHULLER, C. Stress signaling in yeast. **Bioessays**, v.17,n.11, p. 959-965,1995.

SAIBIL, H.R. Chaperone machines in action.**Current Opinion in Structural Biology**, v.18, n. 1, p. 35-42, 2008.

SANCHEZ, Y.S. L.; LINDQUIST. HSP104 required for induced thermotolerance. **Science**, v. 248, n. 4959, p. 1112-1115, 1990.

SCHEFFERS, W. A.; NANNINGA, G. L. The anaerobic inhibition of fermentation (Custers effect) in *Brettanomyces*. -- EUCHEM Conference on Metabolic Reactions in the Yeast Cell in Aerobic and Anaerobic Conditions, 1977. **Proceedings…** Helsinki, Finland, p. 53-55,1977.

SCHEFFERS, W. Stimulation of fermentation in yeasts by acetoin and oxygen. **Nature**, v. 210, p. 533-534, 1966.

SCHULZE, U.; LARSEN, M. E.; VILLADSEN, J. Determination of intracellular trehalose and glycogen in *Saccharomyces cerevisiae*. **Analytical Biochemistry**, v. 228, n. 1, p.143-149, 1995.

SHARMA, S. C. A. Possible role of trehalose in osmotoleranceand ethanol tolerance in *Saccharomyces cerevisiae*. **FEMS Microbiology Letters**, v. 152, n.1, 11–15, 1997.

SHI, L.; SUTTER, B.M.; YE, X.; TU, B. P. Trehalose Is a Key Determinant of the Quiescent Metabolic State That Fuels Cell Cycle Progression upon Return to Growth. **Molecular Biology of the Cell**, v.21, n.12, p. 1982–1990, 2010

SHIMA, J.; TAKAGI, H. Stress-tolerance of baker's-yeast (*Saccharomyces cerevisiae*) cells: stress-protective molecules and genes involved in stress tolerance. **Biotechnology and Applied Biochemistry**, v.53, n. Pt 3, p.155-164, 2009.

SIDERIUS, M; MAGER, W.H. General Stress response: In search of a common denominator. In: HOHMANN, S.; MAGER, W.H. **Yeast stress responses**. New York: Springer, 1997.

SILLJE, H. H., PAALMAN, J. W., TER SCHURE, E. G., OLSTHOORN, S. Q., VERKLEIJ, A. J., BOONSTRA, J.; VERRIPS, C. T. Function of trehalose and glycogen in cell cycle progression and cell viability in *Saccharomyces cerevisiae.* **Journal of Bacteriology**, v. 181, n.2, p. 396–400, 1999.

SILVA-FILHO, E. A.; SANTOS, S. K. B.; RESENDE, A. M.; MORAIS, J. O. F.;MORAIS JR, M. A.; SIMÕES, D.A. Yeast population dynamics of industrial fuelethanol fermentation process assessed by PCR-fingerprinting. **Antonie Van Leeuwenhoek**, v. 88, n. 2, p. 13-23, 2005.

SINGER, M.A.; LINDQUIST, S. Multiple effects of trehalose on protein folding in vitro and in vivo. **Molecular Cell**, Cambridge, v. 1, n.5, p. 639-648, 1998.

SIURKUS, J. **Preliminary molecular biology studies of *Dekkera bruxellensis* yeast**. Lyngby: Technical University of Denmark Lyngby, 2004.

SIVERIO, J. M. Assimilation of nitrate by yeasts. **FEMS microbiology reviews**, v. 26, n. 3, p. 277-284, 2002.

SOTO, T., FERNANDEZ, J., VICENTE-SOLER, J., CANSADO, J. AND GACTO, M. Accumulation of trehalose by overexpression of tps1, coding for trehalose-6-phosphate synthase, causes increased resistance to multiple stresses in the fission yeast *Schizosaccharomyces pombe*. **Applied and Environmental Microbiology**, v. 65, n.5, p. 2020-2024, 1999.

THEVELEIN, J. M. Regulation of trehalose mobilization in fungi. **Microbiological Review**, v.48, n.1, p. 42–59, 1984.

TROTT, A.; MORANO, K. A. The Yeast response to heat shock. In: HOHMANN, S.; MAGER, P. W. H.(Ed) **Yeast Stress Responses**. p. 172–200. Berlin: Heidelberg Springer., 2003.

VAN DER WALT, J. *Dekkera*, a new genus of the Saccharomycetaceae. **Antonie van Leeuwenhoek**, v. 30, n. 1, p. 273-280, 1964.

VAN DIJKEN, J.P.; SCHEFFERS,W. A. Redoxbalancesinthe metabolism of sugars by yeasts. **FEMS Microbiology Letters**, v.32, p. 199-224, 1986.

VAN LAERE A. Trehalose, reserve and/or stress metabolite? **FEMS Microbiology Review**, v. 63, n. 3, p. 201-209, 1989.

VARELA, J. C.; PRAEKELT, U. M.; MEACOCK, P. A.; PLANTA, R. J.; MAGER, W. H. The *Saccharomyces cerevisiae* HSP12 gene is activated by the high-osmolarity glycerol pathway and negatively regulated by protein kinase A. **Molecular and Cellular Biology,** v.15, n.11, p. 6232-6245, 1995.

WANG, Z.X.; ZHUGE, J.; FANG, H.; PRIOR, B.A. Glycerol production by microbial fermentation: a review. **Biotechnology Advances**, v. 19, n. 3, p. 201-223, 2001.

WELKER, S.; RUDOLPH, B.; FRENZEL, E.; HAGN, F.; LIEBISCH, G.; SCHMITZ, G.; SCHEURING, J.; KERTH, A.; BLUME, A.; WEINKAUF, S.; HASLBECK, M.; KESSLER, H.; BUCHNER, J. Hsp12 is an intrinsically unstructured stress protein that folds

upon membrane association and modulates membrane function. **Molecular Cell**, v. 39, n. 4, p.507-20, 2010.

WERA, S.; DE SCHRIJVER, E.; GEYSKENS, I.; NWAKA, S.; THEVELEIN, J. M. Opposite roles of trehalase activity in heat-shock recovery and heat-shock survival in *Saccharomyces cerevisiae*. **Biochemical Journal**, v.343, n.3, p. 621–626, 1999.

WIEMKEN A. Trehalose in yeast, stress protectant rather than reserve carbohydrate. **Antonie Van Leeuwenhoek,**v. 58,n. 3, p. 209–217, 1990.

WIJSMAN, M. R.; DIJKEN, J. P.; KLEEFF, B. H. A.; SCHEFFERS, W. A. Inhibition of fermentation and growth in batch cultures of the yeast *Brettanomyces intermedius* upon a shift from aerobic to anaerobic conditions (Custers effect). **Antonie van Leeuwenhoek**, v. 50, n. 2, p. 183-192, 1984

WILSON, A.; ROACH, P.J.; MONTERO,M.; BAROJA-FERNÁNDEZ, E.; MUÑOZ, F. J.; EYDALLIN, G.; VIALE, A. M.; POZUETA-ROMERO, J. Regulation of glycogen metabolism in yeast and bacteria. **FEMS Microbiology Letters**, v. 34, n. 6, p. 952-985, 2010.

WOOLFIT, M.; ROZPEDOWSKA, E.; PISKUR, J.; WOLFE, K. H. Genome survey sequencing of the wine spoilage yeast *Dekkera (Brettanomyces) bruxellensis*. **Eukaryotic cell,** v. 6, n. 4, p. 721-33, 2007.

YAHARA, G. A.; JAVIER, M. A.; TULIO, M. J. M.; JAVIER, G. R.; GUADALUPE, A. U. M. Modeling of yeast *Brettanomyces bruxellensis* growth at different acetic acid concentrations under aerobic and anaerobic conditions. **Bioprocess and biosystems engineering**, v. 30, n. 6, p. 389-395, 2007.

ARTIGO 1- The physiological characteristics of the yeast *Dekkera bruxellensis* in fully fermentative conditions with cell recycling and in mixed cultures with *Saccharomyces cerevisiae***.**

Publicado em Antonie van Leeuwenhoek

http://www.springer.com/life+sciences/microbiology/journal/10482

doi: 10.1007/s10482-011-9662-2. Epub 2011 Oct 22

Antonie van Leeuwenhoek
DOI 10.1007/s10482-011-9662-2

ORIGINAL PAPER

The physiological characteristics of the yeast *Dekkera bruxellensis* in fully fermentative conditions with cell recycling and in mixed cultures with *Saccharomyces cerevisiae*

Luciana Filgueira Pereira · Ana Paula Guarnieri Bassi · Simoni Helena Avansini · Adauto Gomes Barbosa Neto · Bereneuza Tavares Ramos Valente Brasileiro · Sandra Regina Ceccato-Antonini · Marcos Antonio de Morais Jr.

Received: 9 September 2011 / Accepted: 14 October 2011

Abstract The yeast *Dekkera bruxellensis* plays an important role in industrial fermentation processes, either as a contaminant or as a fermenting yeast. In this study, an analysis has been conducted of the fermentation characteristics of several industrial *D. bruxellensis* strains collected from distilleries from the Southeast and Northeast of Brazil, compared with *Saccharomyces cerevisiae*. It was found that all the strains of *D. bruxellensis* showed a lower fermentative capacity as a result of inefficient sugar assimilation, especially sucrose, under anaerobiosis, which is called the Custer effect. In addition, most of the sugar consumed by *D. bruxellensis* seemed to be used for biomass production, as was observed by the increase of its cell population during the fermentation recycles. In mixed populations, the surplus of *D. bruxellensis* over *S. cerevisiae* population could not be attributed to organic acid production by the first yeast, as previously suggested. Moreover, both yeast species showed similar sensitivity to lactic and acetic acids and were equally resistant to ethanol, when added exogenously to the fermentation medium. Thus, the effects that lead to the employment of *D. bruxellensis* in an industrial process and its effects on the production of ethanol are multivariate. The difficulty of using this yeast for ethanol production is that it requires the elimination of the Custer effect to allow an increase in the assimilation of sugar under anaerobic conditions.

Keywords Ethanol fermentation · Industrial adaptation · Organic acid · Stress resistance · Sugarcane

L. F. Pereira · M. A. de Morais Jr.
Interdepartmental Research Group in Metabolic Engineering, Federal University of Pernambuco, Recife, PE 50760-901, Brazil

A. P. G. Bassi · S. H. Avansini · S. R. Ceccato-Antonini
Department of Agroindustrial Technology and Social-Rural Economy, Federal University of São Carlos, Araras, SP 13600-970, Brazil

A. G. B. Neto · B. T. R. V. Brasileiro · M. A. de Morais Jr.
Genetech Bioproductivity Ltd, Recife, PE 50670-901, Brazil

B. T. R. V. Brasileiro
Center for Biological and Health Sciences, Catholic University of Pernambuco, Recife, PE 50050-900, Brazil

M. A. de Morais Jr. (✉)
Departament of Genetics, Federal University of Pernambuco, Recife, PE 50760-901, Brazil
e-mail: marcos.morais@pesquisador.cnpq.br
URL: www.ufpe.br/nem

Published online: 22 October 2011

Introduction

The yeast *Dekkera bruxellensis* (telomorph of *Brettanomyces bruxellensis*) has attracted attention in recent years because of its involvement in wine spoilage, and

2007) during the harvesting season (2010–2011). After appropriate dilution, the yeast cells were spread onto a Wallerstein Lab Nutrient (WLN) agar (Himedia, Mumbai, India) or WLN + actidione (WLD) medium containing bromocresol green dye. As well as colony morphology discrimination, the identity of the yeast was confirmed by DNA fingerprinting analysis with $(GTG)_5$ primer. Data for fermentation efficiency was provided by the distilleries at the time of sampling.

Fermentative assays

Cell pre-inoculum was prepared by inoculating 125 ml flasks containing 50 ml sugar cane at 40 g sucrose l^{-1} or synthetic medium containing 20 g glucose l^{-1} with fresh yeast colonies and incubated for 24 h at 30°C under orbital agitation at 160 rpm. Yeast cells were recovered by centrifugation and suspended in different fermentation media depending on the type of fermentations described below. Cell density was determined by microscopic count in Neubauer chamber. Cell viability was evaluated after dyeing with methylene blue before the microscopy analysis (Lee et al. 1981). Fermentation conditions (FC) were performed as below, all in biological triplicates:

(a) *FC1* single-batch single-cell complete fermentations performed in 500-ml flasks containing 200 ml of sugar cane juice with sucrose at 120 g l^{-1} inoculated with yeast cells from pre-inoculum to 10^8 cells ml^{-1}. The cultures were incubated for 24 h at 33°C without agitation.
(b) *FC2* similar to FC1 by changing fermentation medium to semi-synthetic medium.
(c) *FC3* similar to FC1 by changing fermentation medium to synthetic medium with glucose at 120 g l^{-1}, with agitation at 150 rpm in an orbital shaker.
(d) *FC4* single-batch single-cell half-time fermentations performed similar to FC1 by decreasing incubation time to eight to 12 h at 33°C without agitation.
(e) *FC5* single-batch mixed-cell half-time fermentations similar to FC4 by mixing *S. cerevisiae* and *D. bruxellensis* cells to the indicated proportions, keeping the initial cell concentration.
(f) *FC6* successive-batch single-cell half-time cell recycling fermentations performed in 500-ml flasks containing 200 ml sugar cane juice with sucrose at 140 g l^{-1} inoculated with yeast cells from pre-inoculum to 10^8 cells ml^{-1}. The cultures were incubated for 12 h at 33°C without agitation. At the end of each batch, yeast cells were recovered by centrifugation (1200 g for 5 min) and suspended in the same medium for batch fermentation. This procedure was performed for eight consecutive cycles.
(g) *FC7* successive-batch mixed-cell half-time cell recycling fermentations similar to FC6 by mixing *S. cerevisiae* and *D. bruxellensis* cells to 10^8 cells ml^{-1} and 10^3 cells ml^{-1}, respectively.
(h) *FC8* similar to FC7 by adding acetic acid and lactic acid to initial concentrations of 0.1 and 0.2 g l^{-1}, respectively, at the beginning of cycles no. 3 to no. 6.
(i) *FC9* similar to FC7 by adding ethanol to 8% at the beginning of cycles no. 3 to no. 6.

At the end of each batch (single and recycled fermentations), samples were taken for cell concentration and viability by cell count in a Neubauer chamber after methylene blue dyeing, and differentiated by spreading onto WLN and WLD medium supplemented with actidione (Basílio et al. 2008). The remaining volume was centrifuged and the fermented worts were used for residual sugar analysis by the DNSA method (Basílio et al. 2008), suspended solids (°Brix) with manual refratometer and by HPLC (Waters Co., USA) using column SHODEX Sugar SP0810 (BioRad, USA) for carbohydrates. Measurements of organic acids, glycerol and ethanol were carried out by HLPC, using Aminex HPX-87H column (BioRad, USA). Total acidity was measured by titration with 50 mM NaOH solution until pH 7.0 was reached using digital pHmeter and the values were expressed as mEq l^{-1}. The production of CO_2 was measured by the weight loss approach, as described by Basílio et al. (2008).

Results

Effect of the presence of *D. bruxellensis* cells on the industrial process

To extend our knowledge on the behavior of *D. bruxellensis* in industrial processes, we conducted

2007) during the harvesting season (2010–2011). After appropriate dilution, the yeast cells were spread onto a Wallerstein Lab Nutrient (WLN) agar (Himedia, Mumbai, India) or WLN + actidione (WLD) medium containing bromocresol green dye. As well as colony morphology discrimination, the identity of the yeast was confirmed by DNA fingerprinting analysis with $(GTG)_5$ primer. Data for fermentation efficiency was provided by the distilleries at the time of sampling.

Fermentative assays

Cell pre-inoculum was prepared by inoculating 125 ml flasks containing 50 ml sugar cane at 40 g sucrose l^{-1} or synthetic medium containing 20 g glucose l^{-1} with fresh yeast colonies and incubated for 24 h at 30°C under orbital agitation at 160 rpm. Yeast cells were recovered by centrifugation and suspended in different fermentation media depending on the type of fermentations described below. Cell density was determined by microscopic count in Neubauer chamber. Cell viability was evaluated after dyeing with methylene blue before the microscopy analysis (Lee et al. 1981). Fermentation conditions (FC) were performed as below, all in biological triplicates:

(a) *FC1* single-batch single-cell complete fermentations performed in 500-ml flasks containing 200 ml of sugar cane juice with sucrose at 120 g l^{-1} inoculated with yeast cells from pre-inoculum to 10^8 cells ml^{-1}. The cultures were incubated for 24 h at 33°C without agitation.
(b) *FC2* similar to FC1 by changing fermentation medium to semi-synthetic medium.
(c) *FC3* similar to FC1 by changing fermentation medium to synthetic medium with glucose at 120 g l^{-1}, with agitation at 150 rpm in an orbital shaker.
(d) *FC4* single-batch single-cell half-time fermentations performed similar to FC1 by decreasing incubation time to eight to 12 h at 33°C without agitation.
(e) *FC5* single-batch mixed-cell half-time fermentations similar to FC4 by mixing *S. cerevisiae* and *D. bruxellensis* cells to the indicated proportions, keeping the initial cell concentration.
(f) *FC6* successive-batch single-cell half-time cell recycling fermentations performed in 500-ml flasks containing 200 ml sugar cane juice with sucrose at 140 g l^{-1} inoculated with yeast cells from pre-inoculum to 10^8 cells ml^{-1}. The cultures were incubated for 12 h at 33°C without agitation. At the end of each batch, yeast cells were recovered by centrifugation (1200 g for 5 min) and suspended in the same medium for batch fermentation. This procedure was performed for eight consecutive cycles.
(g) *FC7* successive-batch mixed-cell half-time cell recycling fermentations similar to FC6 by mixing *S. cerevisiae* and *D. bruxellensis* cells to 10^8 cells ml^{-1} and 10^3 cells ml^{-1}, respectively.
(h) *FC8* similar to FC7 by adding acetic acid and lactic acid to initial concentrations of 0.1 and 0.2 g l^{-1}, respectively, at the beginning of cycles no. 3 to no. 6.
(i) *FC9* similar to FC7 by adding ethanol to 8% at the beginning of cycles no. 3 to no. 6.

At the end of each batch (single and recycled fermentations), samples were taken for cell concentration and viability by cell count in a Neubauer chamber after methylene blue dyeing, and differentiated by spreading onto WLN and WLD medium supplemented with actidione (Basílio et al. 2008). The remaining volume was centrifuged and the fermented worts were used for residual sugar analysis by the DNSA method (Basílio et al. 2008), suspended solids (°Brix) with manual refratometer and by HPLC (Waters Co., USA) using column SHODEX Sugar SP0810 (BioRad, USA) for carbohydrates. Measurements of organic acids, glycerol and ethanol were carried out by HLPC, using Aminex HPX-87H column (BioRad, USA). Total acidity was measured by titration with 50 mM NaOH solution until pH 7.0 was reached using digital pHmeter and the values were expressed as mEq l^{-1}. The production of CO_2 was measured by the weight loss approach, as described by Basílio et al. (2008).

Results

Effect of the presence of *D. bruxellensis* cells on the industrial process

To extend our knowledge on the behavior of *D. bruxellensis* in industrial processes, we conducted

a survey of four distilleries in Northeast Brazil during the harvest season (2010–2011). The results showed that *D. bruxellensis* cells still constitute up to 70% of the yeast population in the process (Fig. 1), despite the injection of fresh batches of pure *S. cerevisiae* cells (industrial data not shown). The survey corroborated the exceptional ability of this yeast to adapt to industrial processes, as previously reported (Liberal et al. 2007). Different distilleries showed their own profile of yeast dynamics and the way that they might be dependent on particular operational practices. Moreover, despite the huge increase in the *D. bruxellensis* population, no direct effect of this yeast was observed when the overall efficiency of industrial fermentation was taken into account (Fig. 1). Four different pictures were observed: non-contaminated periods showing high or low industrial fermentation efficiencies and periods with high counts of *D. bruxellensis* showing high and low industrial efficiencies (Fig. 1). The total titrable acidity in industrial wort was in the range of 20 to 40 mEq l^{-1} and no direct relation to the presence of *D. bruxellensis* cells was observed (data not shown).

In order to replicate this industrial profile, half-time mixed single fermentations (FC5) were performed by varying the proportion of *S. cerevisiae* and *D. bruxellensis* in the medium. No effect on the cell viability of *S. cerevisiae* JP1 was observed as a result of the increasing proportion of *D. bruxellensis* GDB 248 cells (data not shown). However, it was observed that the higher the *D. bruxellensis* population, the lower was the fermentation efficiency (Fig. 2). The fall in fermentation efficiency was followed by a fall in ethanol and CO_2 production (Fig. 2) when there was an increase of *D. bruxellensis* cells in the yeast population. On the other hand, the sugar consumption was higher when there was an increase in the *D. bruxellensis* population (Fig. 2).

D. bruxellensis fermentation efficiency in a single batch assay using synthetic medium

These experiments were performed to compare the fermentation efficiency of the two yeast species (*D. bruxellensis* and *S. cerevisiae*), and the results were shown as the average of all the strains within a species, with a triplicate for each isolate. The mean ethanol yield ($Y_{p/s}$) of 0.38 g ethanol (g sucrose)$^{-1}$ was obtained for *D. bruxellensis* while the mean ethanol yield ($Y_{p/s}$) of 0.47 g ethanol (g sucrose)$^{-1}$ was obtained for *S. cerevisiae* JP1 when the fermentations were left to reach completion (FC1). A significant difference was observed between the isolates, with GDB 248 remarkably showing a 10% higher fermentation efficiency ($Y_{p/s} = 0.42$ g g^{-1}) within the yeast group, while TPC2-1 showed a 15% lower fermentation capacity ($Y_{p/s} = 0.32$ g g^{-1}). Sugar tended to accumulate in a way that corresponded with the lower fermentation capacity, being 20% higher in low fermentative isolates. The average of final pH in the fermented wort was 4.1 (±0.04) and 3.5 (±0.02) for *D. bruxellensis* and *S. cerevisiae* cultures, respectively, suggesting a slight tendency of *D. bruxellensis* cells for lower medium acidification. In contrast, the ethanol yield dropped to 0.25 g g^{-1} when *D. bruxellensis* cultures were left to ferment for half the time (FC4). Similarly, yeast isolates from Southeast distilleries were also analyzed for half-time fermentations and showed mean ethanol yields of 0.23 g/g (±0.04) and 0.22 (±0.09) g g^{-1} with glucose (FC3) and sucrose (FC2), respectively, which is quite close to their Northeast isolate counterparts.

D. bruxellensis fermentation efficiency with cell recycling

Three isolates of *D. bruxellensis* from Southeast Brazil were evaluated by recycling fermentation assays and compared to three industrial *S. cerevisiae* strains. Again, the results were shown as the average of all the strains within a species, with triplicate for each isolate, and comprised 12 and 9 experiments for *D. bruxellensis* and *S. cerevisiae,* respectively, for each fermentative cycle (FC6). During the recycling procedure with sugar cane, the cell viability remained at 93 to 97% for both yeasts. There was also an increase in the number of cells for both yeasts, although *D. bruxellensis* showed a higher increase of the cell population (Fig. 3a). The final pH of the fermented wort was higher for *D. bruxellensis* (3.9 ± 0.09) than for the *S. cerevisiae* (3.3 ± 0.26) fermentations. This result was corroborated by the analysis of total titratable acidity in the fermented wort, which was higher for *S. cerevisiae* than for the *D. bruxellensis* fermentations (Fig. 3b). The total acidity for *D. bruxellensis* varied from 20 to 30 mEq l^{-1}, which was close to the values for industrial samples observed above.

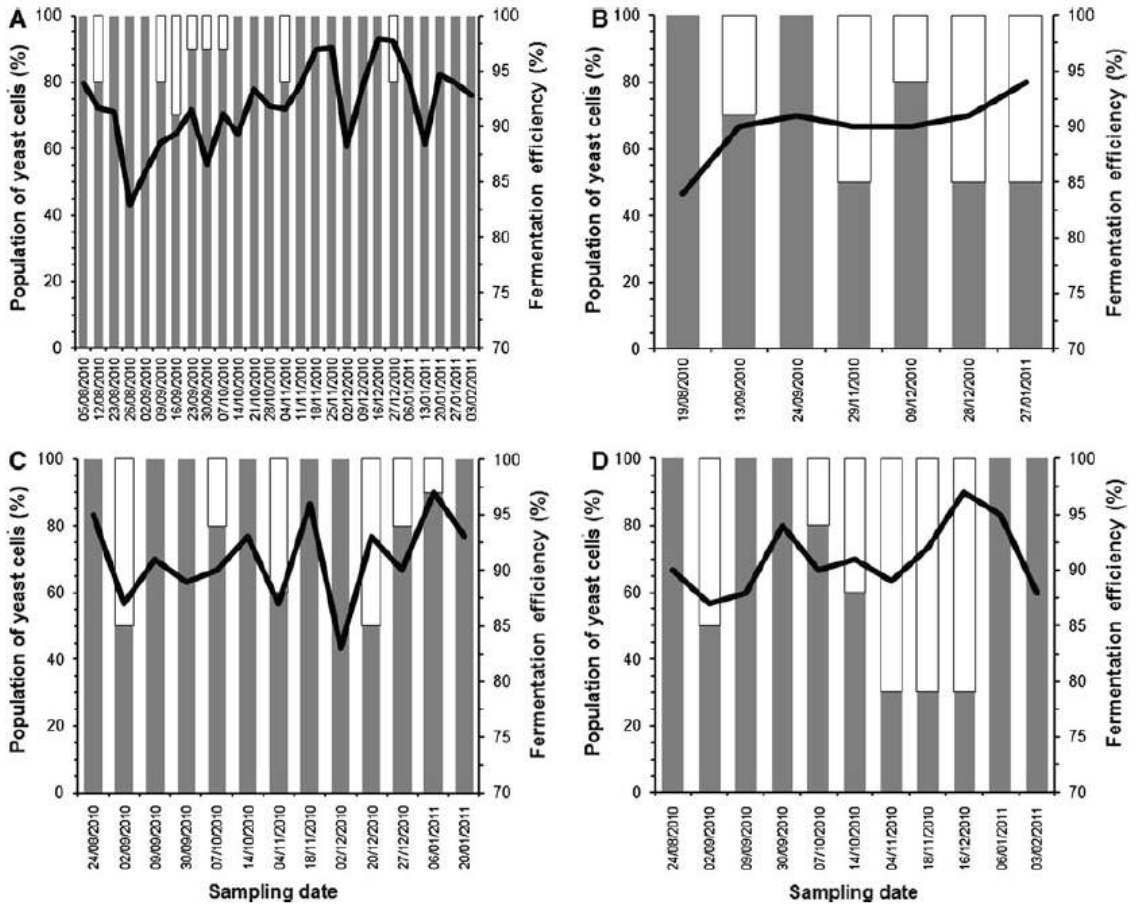

Fig. 1 Dynamics of yeast population in four ethanol distilleries in Northern Brazil during the harvest season 2010–2011. The percentage of *S. cerevisiae* cells (*gray columns*) and *D. bruxellensis* cells (*white columns*) in different sampling periods was related to the industrial fermentation efficiency (*black lines*) provided by the distilleries on the day of the sampling

Sucrose consumption increased after each fermentation cycle, which suggests that the yeast cells were adapting to the higher sugar content of 140 g l^{-1} at the beginning of each cycle (Fig. 3c). After the third cycle, the *S. cerevisiae* cells consumed over 80% of the sugar present in the medium at the end of 12 h fermentation, while *D. bruxellensis* maximally consumed only 30% of the total sugar available in the sugar cane wort (Fig. 3c). Similarly, ethanol production was three times higher in the *S. cerevisiae* fermentations than in the *D. bruxellensis* fermentations (Fig. 3d). The average ethanol yield of 0.47 (±0.02) g g^{-1} for *S. cerevisiae* from Southern Brazil was similar to the JP1 strain from Northeast Brazil in single batch experiments. A low ethanol yield of 0.22 (± 0.09) g g^{-1} was calculated for *D. bruxellensis*, which is in the same range as in the above single cycle experiments.

The isolates CCA 077 and CCA 155 from southern Brazil were further used in mixed cultures to test any sort of antagonistic activity against the *S. cerevisiae* cells during the recycled fermentations (FC7). It was observed that the sugar assimilation, ethanol production and medium acidity were similar whatever *D. bruxellensis* isolate was present in the fermentation (Fig. 4). The proportion of *S. cerevisiae* cells was affected by the presence of *D. bruxellensis* cells, particularly by the CCA 077 isolate (Fig. 4a). This effect was attributed to the increase of the *D. bruxellensis* cell count in the cultures (Fig. 3a)

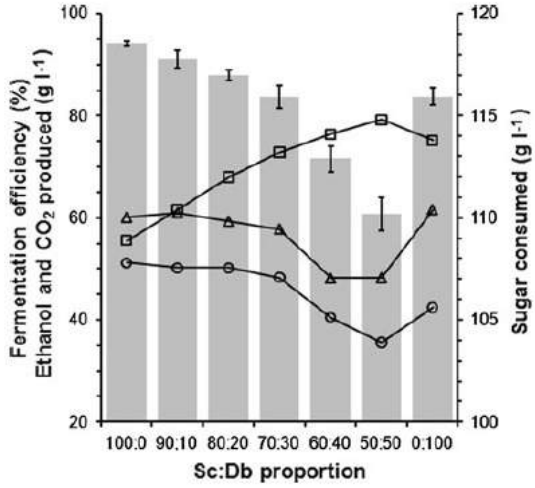

Fig. 2 The half-time single fermentation profile of a mixed population of *S. cerevisiae* (Sc) JP1 and *D. bruxellensis* (Db) GDB 248 cells and in sugar cane medium at recommended proportions. Culture supernatants were taken to determine the rate of sugar consumption (*squares*) and ethanol production (*circles*) by analytical methods, while the production of CO_2 was measured by the weight loss of the system (*triangles*).These data were used to calculate the efficiency of ethanol fermentation (*gray columns*) referring to percentage of the maximal theoretical yield

rather than to the production of inhibitory organic acids, since no further acidification was detected (Figs. 3b and 4a) when there was an increase in the *D. bruxellensis* population.

Metabolite in the fermentation wort with recycling

A detailed analysis of the metabolites in the fermented wort was performed in the fermentations of *S. cerevisiae* JP1 and *D. bruxellensis* GDB 248 at different stages of the recycling experiments (FC6) (Table 1). The sucrose tended to accumulate in the fermentations with *D. bruxellensis* cells, while *S. cerevisiae* was able to hydrolyze almost all the initial sucrose. The glucose and fructose concentration at the end of the fermentation was decreasing during the cycles for both yeasts, although they accumulated more in the *D. bruxellensis* fermentations (Table 1). Less fructose was consumed than glucose, particularly by the *D. bruxellensis* cells (Table 1). Despite the higher cell growth (Fig. 3a), the *D. bruxellensis* cells produced less glycerol than the *S. cerevisiae* cells, while lactic acid accumulated to the same level at the end of each fermentation cycle for both yeasts (Table 1). No acetic or succinic acid was detected in the fermented wort of either of the yeasts (data not shown).

Yeast tolerance to inhibitory compounds

Given the fact that *D. bruxellensis* cannot produce enough organic acids under full fermentative conditions to inhibit *S. cerevisiae* cells (Table 1), experiments were performed by adding exogenous organic acids to the medium (FC8). Lactic and acetic acids were added at the beginning of cycle no. 3 and the population of both *S. cerevisiae* and *D. bruxellensis* cells began to decline to 0.1% of the initial population at the end of cycle no. 6. The remaining cells were suspended in sugar cane juice without the stress agents at the beginning of cycle no. 7 and the yeast population began to recover at an equal rate in the fermentations that followed (Fig. 5). There was a sharp fall in ethanol production after acid treatment, which was recovered after removal of the acid (data not shown). A similar experiment was performed by adding ethanol (FC9). A slight decrease was observed in yeast population in the presence of the stress agent, which was subsequently recovered even in the presence of the ethanol stress agent (Fig. 5). This suggested that both yeasts were equally resistant to ethanol.

Discussion

Despite being regarded as a contaminant for the ethanol fermentation process (Liberal et al. 2007; Basílio et al. 2008), *D. bruxellensis* has been reported as a potential yeast for ethanol production from glucose (Passoth et al. 2007) and cellobiose (Blomqvist et al. 2010). However, there is still little information on the physiology of this yeast with regard to its fermentative profile. Apart from its capacity for ethanol production, this yeast shares several characteristics with *S. cerevisiae,* such as the ability to propagate in the absence of oxygen (anaerobic growth) and to produce offspring without mitochondrial DNA (petite positivity) (Rozpedowska et al. 2011). We have previously described the astonishing capacity of this yeast to play a dominant role in industrial fermentation processes in Brazil (Liberal et al. 2007).

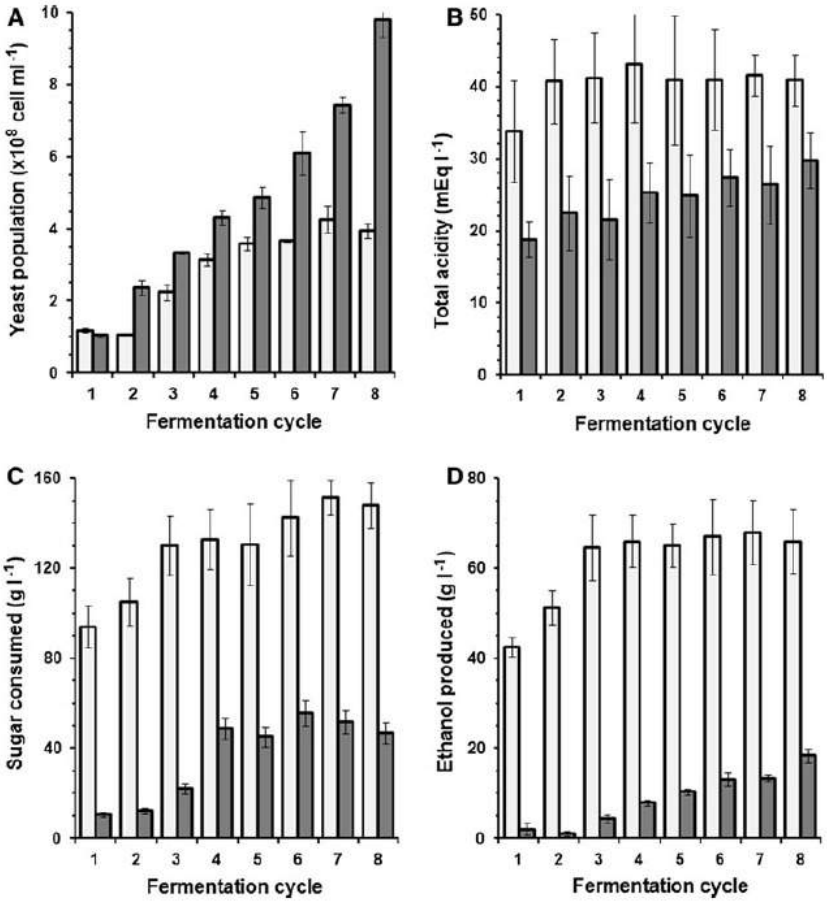

Fig. 3 Fermentation assays with cell recycling using sugar cane wort as substrate. The results are the average of three commercial strains of *S. cerevisiae* (*light gray columns*) and three industrial isolates of *D. bruxellensis* from southeast distilleries (*dark gray columns*). The following parameters were evaluated at the end of each 12-h cycle: cell density (**a**), total acidity of the wort (**b**), sucrose consumed (**c**) and ethanol produced (**d**)

In the industrial fermentation of sugar cane, yeast cells are submitted to an anaerobic environment, sucrose as the main sugar and repeated pitching. During this process a succession of yeast species and strains occurs (Silva-Filho et al. 2005; Basso et al. 2008) and *D. bruxellensis* cells can bypass the process (Liberal et al. 2007; Basílio et al. 2008), even as an operational maneuver, by the continuous injection of *S. cerevisiae* (Fig. 1). In this study, it is shown that *D. bruxellensis* may be very efficient in converting the available sugar to biomass during fermentation assays (Fig. 3a). Under anaerobiosis, this physiological trait might be aided by the capacity of this yeast to use nitrate present in sugar cane to bypass this oxygen requirement for growth and fermentation (De Barros Pita et al. 2011). It is noteworthy that many reports describe *D. bruxellensis* as a slow-growing yeast in a laboratory medium (Abbott et al. 2005; Liberal et al. 2007; Blomqvist et al. 2010; Galafassi et al. 2010; De Barros Pita et al. 2011), whatever the strains or culture conditions employed. However, the higher energy efficiency of *D. bruxellensis* compared to *S. cerevisiae* can be calculated on the basis of its high biomass production, while glycerol production was hardly detected even in anaerobiosis (Uscanga et al. 2003; Blomqvist et al. 2010; Galafassi et al. 2010). In this study, the production of glycerol was not detected in fermentative conditions with high sugar concentration

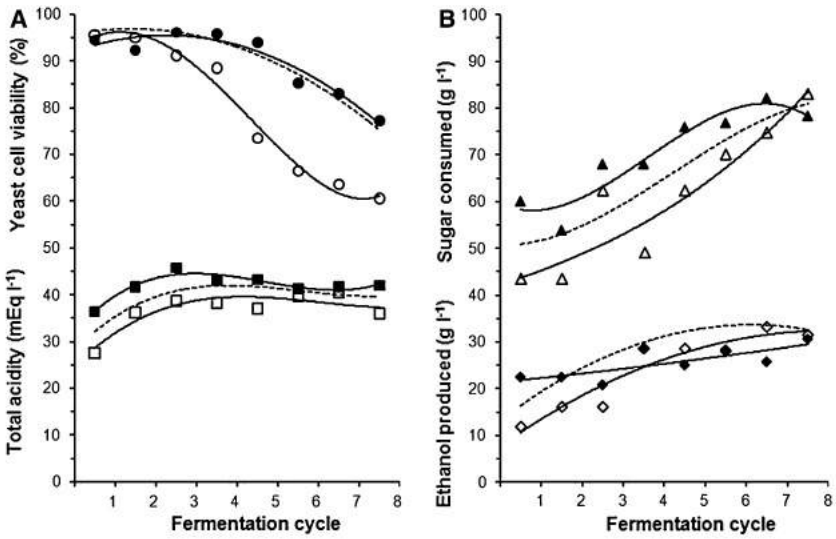

Fig. 4 Physiological characteristics of the half-time mixed recycled fermentations in the sugar cane medium. Cells of *Saccharomyces cerevisiae* (Itaiquara™) strain were mixed with cells of *Dekkera bruxellensis* CCA077 isolate (*open symbols*) or CCA155 isolate (*closed symbols*) and samples were withdrawn after each 12 h cycle of fermentation for determination of cell viability (*circles*) and medium acidity (*squares*) and for sugar consumed (*triangles*) and ethanol produced (*diamonds*). Fermentations with pure *S. cerevisiae* cells were plotted as reference-points (*dotted lines*)

Table 1 Metabolites at the end of three recycling stages of fermentation (C1, C5 and C10) of pure cultures of *S. cerevisiae* and *D. bruxellensis* in sugar cane wort (FC6)

Metabolite	*S. cerevisiae* JP1			*D. bruxellensis* GDB 248		
	C1	C5	C10	C1	C5	C10
Sucrose consumed (%)	97.4	97.2	97.2	40.3	44.6	52.2
Residual glucose (g l^{-1})	35.9	4.3	4.6	31.8	14.8	13.9
Residual fructose (g l^{-1})	53.9	19.1	17.7	33.1	24.8	23.7
Glycerol produced (g l^{-1})	3.0	4.8	4.8	0.0	0.6	0.3
Lactic acid produced (g l^{-1})	1.1	1.2	1.1	1.0	1.1	1.0

(Table 1). The lack of glycerol production might be an advantageous trait that is worth exploring for ethanol fermentation, since less carbon and energy is deviated for this redox sink. In fact, it is acetate and not glycerol that is produced during the aerobic growth of *D. bruxellensis* (Van Dijken and Scheffers 1984; Uscanga et al. 2003) and little or no acetic acid is produced in anaerobiosis (Uscanga et al. 2003; Blomqvist et al. 2010). In this study, we did not observe any acetate formation during the fermentation of the sugar cane wort (Table 1).

Given the efficient way that sugar is converted to biomass, it is expected that the presence of *D. bruxellensis* cells at high counts may decrease the efficiency of ethanol production. The evidence of industrial data is not conclusive in this respect (Fig. 1), since other parameters unrelated to the composition of the yeast population may affect the process. Despite its potential for ethanol production, it has long been known that *D. bruxellensis* requires at least a low oxygen supply for sugar consumption (Wijsman et al. 1984) and to stimulate the fermentation process, which is called the Custer effect (Van Dijken and Scheffers 1984). This supply of oxygen is hard to obtain in industrial conditions, even at low levels, due to problems of gas transference and aeration costs. Thus,

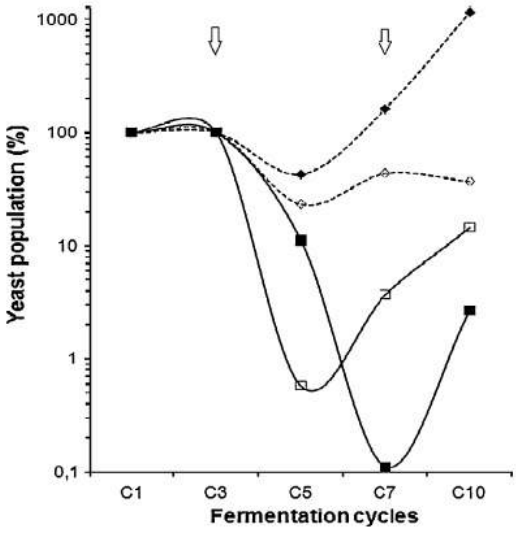

Fig. 5 Variation in the yeast population of *S. cerevisiae* (*open symbols*) and *D. bruxellensis* (*closed symbols*) cells during half-time recycled fermentation in sugar cane juice in response to the addition of acetic acid and lactic acid (*solid lines*) or ethanol (*dashed lines*). Stress agents were added at the beginning of cycles no. 3 (*arrow*) to no. 6. At cycle no. 7 (*arrow*) the yeast cells were suspended in medium without the stress agents

the real effectiveness of *D. bruxellensis* for ethanol production must be tested under controlled conditions, even though they are close to those that apply in industry. Thus, the fermentation experiments with mixed populations were performed in a way that differed from that described by De Barros Pita et al. (2011) which was carried out in a shorter (industrial) fermentation time. It was observed that when the *D. bruxellensis* cell number increased, the fermentation capacity of the yeast population decreased correspondingly despite of the increasing on sugar consumption (Fig. 2). One explanation could be that the presence of *S. cerevisiae* rapidly depletes oxygen in the medium. Thus, when *D. bruxellensis* population is high the sugar consumed could be converted to biomass, the Custer effect. In the situation that *D. bruxellensis* is pure, the depletion of oxygen could be slower, keeping ethanol fermentation for longer time. In fact the reported high yields of ethanol were always achieved only after a long period of fermentation (Blomqvist et al. 2010; Galafassi et al. 2010), and took account of cell growth. Overall, in this study we observed similar low ethanol yields (c.a. 0.23 g g^{-1}) for isolates from Northeast and Southeast Brazilian distilleries when pure cultures were submitted to high-sucrose high-biomass conditions, either in single or successive batches. When submitting pure cultures of *D. bruxellensis* to short-term recycling experiments, it was observed that the sugar consumption was slow (Fig. 3c; Table 1). However, the sugar consumption increased when both yeasts were present in the medium (Figs. 2, 4). An increased rate of sugar consumption was also observed in wine fermentation experiments by co-cultivation of *S. cerevisiae* and *D. (Brettanomyces) bruxellensis* compared to that obtained through pure *S. cerevisiae* fermentation (Renouf et al. 2006). One plausible explanation for this is the inefficient hydrolysis of sucrose by *D. bruxellensis* which was detected during the fermentation cycles (Table 1). Thus, this sugar could be efficiently broken down by *S. cerevisiae* invertases and then glucose and fructose could be uptake by both cells. However, glucose and fructose were not efficiently assimilated by *D. bruxellensis* cells in sugar cane wort (Table 1), which is similar to what has been reported for grape wort fermentation by the pure culture of *D. (Brettanomyces) bruxellensis* (Renouf et al. 2006).

It was widely believed that acetic acid production by *D. bruxellensis* could affect cell viability and the fermentation capacity of *S. cerevisiae* (De Miniac 1989; Ciani and Ferraro 1997; Abbott et al. 2005). However, for a long time, it has been stated that this compound cannot be produced in inhibitory concentrations (Phowchinda et al. 1995). Under fully fermentative conditions, we did not observe any correlation between acidity, which represents the total concentration of weak organic acids in the medium, and the proportion of *D. bruxellensis* in the population (Liberal et al. 2007; Fig. 1). In fact, the production of weak acids seemed to be higher in pure cultures of *S. cerevisiae* (Fig. 3b) and showed no correlation with the decrease of *S. cerevisiae* cells in a mixed population (Fig. 4). Our data showed that no acetic acid was produced by *D. bruxellensis* in fully fermentative conditions, while lactic acid is produced in equal amounts by both yeasts in this condition (Table 1). In view of this, we decided to add acetic and lactic acids to the fermentation medium and the results showed that both yeasts were equally sensitive to this increased acidity in anaerobic conditions (Fig. 5). Thus, it can be postulated that the presence of acetic acid in the medium is not the primary cause for the

excessive growth of *D. bruxellensis* in industrial processes. Moreover, it was also observed that both yeasts were equally sensitive (or resistant) to the increase of ethanol in the medium, which invalidates the belief by the distilleries that if fermentation is kept with a high ethanol content, it should eliminate *D. bruxellensis* from the process. In a similar way, Renouf et al. (2006) showed that wine isolates of *D. (Brettanomyces) bruxellensis* were more resistant to ethanol than wine *S. cerevisiae* strains.

In conclusion, the effects that lead to the establishment of *D. bruxellensis* in an industrial process and its effect regarding ethanol yield, are multivariate, but largely dependent on the capacity of this yeast to assimilate nutrients differentially such as nitrate. In addition, the tendency of this yeast for biomass production (Fig. 3a) may lead to lower ethanol yield, although ethanol production seems to be closely associated with biomass formation. We have recent evidences from chemostat cultivation on the tendency of its cells for respiration over fermentation (manuscript in preparation). Thus, the challenges that must be overcome before this industrially robust yeast can be employed for ethanol production are to find a way of eliminating the Custer effect and to increase sugar assimilation under fully anaerobic conditions.

Acknowledgments The authors express their gratitude to Prof. Andreas Gombert, laboratory of Biochemistry Engineering at University of São Paulo, for the HPLC analysis, and the Brazilian funding agencies CNPq, FACEPE and FAPESP for sponsoring this study and providing financial support. This study is part of the activities being undertaken by the Bioethanol Research Network of the State of Pernambuco (CNPq-FACEPE/PRONEM).

References

Abbott DA, Hynes SH, Ingledew WM (2005) Growth rates of *Dekkera/Brettanomyces* yeasts hinder their ability to compete with *Saccharomyces cerevisiae* in batch corn mash fermentations. Appl Microbiol Biotechnol 66:641–647

Basílio ACM, Araújo PRL, Morais JOF, Silva-Filho EA, Morais MA Jr, Simões DA (2008) Detection and identification of wild yeast contaminants of the industrial fuel ethanol fermentation process. Curr Microbiol 56:322–326

Basso LC, de Amorim HV, de Oliveira AJ, Lopes ML (2008) Yeast selection for fuel ethanol production in Brazil. FEMS Yeast Res 8:1155–1163

Blomqvist J, Eberhard T, Schnürer J, Passoth V (2010) Fermentation characteristics of *Dekkera bruxellensis* strains. Appl Microbiol Biotechnol 87:1487–1497

Ciani M, Ferraro L (1997) Role of oxygen on acetic acid production by *Brettanomyces/Dekkera* in winemaking. J Sci Food Agric 75:489–495

Ciani M, Maccarelli F, Fatichenti F (2003) Growth and fermentation behaviour of *Brettanomyces/Dekkera* yeasts under different conditions of aerobiosis. World J Microbiol Biotechnol 19:419–422

De Miniac M (1989) Contamination des fermentations alcooliques industrielles par les levures du genre *Brettanomyces*. Ind Aliment Agric 106:559–563

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

Galafassi S, Merico A, Pizza F, Hellborg L, Molinari F, Piskur J, Compagno C (2010) *Dekkera/Brettanomyces* yeasts for ethanol prodution from renewable sources under oxygen-limited and low-pH conditions. J Ind Microbiol Biotechnol 38:1079–1088

Gerós H, Azevedo MM, Cássio F (2000) Biochemical studies on the production of acetic acid by the yeast *Dekkera anomala*. Food Technol Biotechnol 38:59–62

Lee SS, Robinson FM, Wang HY (1981) Rapid determination of yeast viability. Biotechnol Bioeng 11:641–649

Lema C, Garcia-Jares C, Orriols I, Angulo L (1996) Contribution of *Saccharomyces* and non-*Saccharomyces* populations to the production of some components of Albariño wine aroma. Am J Enol Viticult 47:206–216

Liberal ATS, Basílio ACM, Resende AM, Brasileiro BTRV, Silva-Filho EA, Morais JOF, Simões DA, Morais MA Jr (2007) Identification of *Dekkera bruxellensis* as a major contaminant yeast in continuous fuel ethanol fermentation. J Appl Microbiol 102:538–547

Oelofse A, Pretorius IS, du Toit M (2008) Significance of *Brettanomyces* and *Dekkera* during winemaking: a synoptic review. S Afr J Enol Vitic 29:128–144

Passoth V, Blomqvist J, Schnürer J (2007) *Dekkera bruxellensis* and *Lactobacillus vini* form a stable ethanol producing consortium in a commercial alcohol production process. Appl Environ Microbiol 73:4354–4356

Phowchinda O, Deliadupuy ML, Strehaiano P (1995) Effects of acetic acid on growth and fermentative activity of *Saccharomyces cerevisiae*. Biotechnol Lett 17:237–242

Renouf V, Falcou M, Miot-Sertier C, Perello MC, De Revel G, Lonvaud-Funel A (2006) Interactions between *Brettanomyces bruxellensis* and other yeast species during the initial stages of winemaking. J Appl Microbiol 100:1208–1219

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

Silva-Filho EA, Melo HF, Antunes DF, Dos Santos SKB, Resende MA, Simões DA, Morais MA Jr (2005) Isolation by genetics and physiological characteristics of a fuel ethanol fermentative *Saccharomyces cerevisiae* strain with potential for genetic manipulation. J Ind Microbiol Biotechnol 32:481–486

Uscanga MGA, Delia ML, Strehaiano P (2003) *Brettanomyces bruxellensis*: effect of oxygen on growth and acetic acid production. Appl Microbiol Biotechnol 61:157–162

Van Dijken JP, Scheffers WA (1984) Studies on alcoholic fermentation in yeasts. In: Houwink EH, Van der Meer RR (eds) Innovations in biotechnology. Elsevier, Amsterdam, pp 497–506

Wijsman MR, van Dijken JP, van Kleeff BH, Scheffers WA (1984) Inhibition of fermentation and growth in batch cultures of the yeast *Brettanomyces intermedius* upon a shift from aerobic to anaerobic conditions (Custers effect). Antonie Van Leeuwenhoek 50:183–192

ARTIGO 2- Response of the yeast *Dekkera bruxellensis* to stress conditions and involvement of heat shock proteins

Submetido a Current Microbioloy

http://www.springer.com/life+sciences/microbiology/journal/284

Current Microbiology

Current Microbiology

Response of the yeast Dekkera bruxellensis to stress conditions and involvement of heat shock proteins

Journal:	*Current Microbiology*
Manuscript ID:	Draft
Manuscript Type:	Original Manuscripts
Date Submitted by the Author:	n/a
Complete List of Authors:	Pereira, Luciana; Federal University of Pernambuco, Interdepartmental Research Group on Metabolic Engineering Liberal, Anna Theresa; Federal University of Pernambuco, Interdepartmental Research Group on Metabolic Engineering de Morais, Marcos; Universidade Federal de Pernambuco, Genetica
Keywords:	cell tolerance, ethanol fermentation, industrial adaptation, heat shock, oxidative stress

Response of the yeast *Dekkera bruxellensis* to stress conditions and involvement of heat shock proteins

Running head: Stress tolerance in *Dekkera bruxellensis*

Luciana Filgueira Pereira[1], Anna Theresa de Sousa Liberal[1] and Marcos Antonio de Morais Júnior[1,2]

[1]Interdepartmental Research Group on Metabolic Engineering and [2]Department of Genetics, Federal University of Pernambuco. Av. Moraes Rego 1235, 50670-901, Recife, PE, Brazil.

Correspondence:

Marcos A. Morais Jr

Departamento de Genética - Universidade Federal de Pernambuco

Av. Moraes Rego, 1235 Cidade Universitária 50.670-901 Recife PE Brasil

Phone/Fax: 00-55-81-21268522

E-mail: marcos.morais@pesquisador.cnpq.br

Web site: www.ufpe.br/nem

Abstract

The yeast *Dekkera bruxellensis* has been recognized as well suited to industrial fermentation processes due to its reported vigor and adaptation. Thus, this work seeks to contribute for the understanding of the tolerance of *D. bruxellensis* to stressing conditions of the industrial environment, drawing attention to the possible involvement of three heat shock proteins in the cell protection against those agents. Our results showed that *D. bruxellensis* is more sensitive to thermal and oxidative stresses than *Saccharomyces cerevisiae*, and that an adaptive mechanism does exist only for oxidative stress in this yeast. Transcriptional response of three heat-shock genes indicated that their corresponding proteins may not be essential for survival. However, the transcriptional profile indicated that each of the three heat-shock proteins do specifically participate in the molecular mechanism for cell tolerance to different forms of stress.

Keywords: cell tolerance, ethanol fermentation, industrial adaptation, heat shock, oxidative stress

Introduction

The processes of industrial fermentation present a series of stresses to yeasts cells that includes high sugar concentration at the beginning and high concentration of ethanol at the end, production of weak organic acids and other inhibitory compounds, and fluctuation of temperatures and external pH [9]. These factors impose constraints on growth and metabolism of the yeast and, when acting in synergy, further affect cell

viability leading to reduced fermentation yield.

In *S. cerevisiae* the General Stress Response (GSR) mechanism consist of a number of genetic responses that transcribe a set of genes responsive to cell injuries [5]. This acquired stress resistance can be trigged by a different agent upon induction of genes whose proteins act on the cross-stress protection phenomenon, which constitutes the Environmental Stress Response (ESR) [5]. One important part of the ESR mechanism is the set of so-called heat shock proteins (HSPs) that allow cell survival after irreversible aggregation of misfolded proteins produced by stressing conditions [15]. HSPs are groups of proteins with alpha crystalline domain at C-terminus that in view their heterogeneous composition are annotated as belonging to HSP families [15].

The yeast *Dekkera bruxellensis* has been reported as very adapted to industrial ethanol fermentation process and its adaptive success may be related to some physiological characteristics such as tolerance to ethanol [10] and robustness to variations in pH and temperature [3]. However, whether this adaptation is regarded to tolerance to industrial stress remains to be elucidated. The first announcement of *D. bruxellensis* genome was done in 2007 [17] and so far only one gene encoding HSP was described for this yeast [13]. According to the expression profile during wine fermentation, it seems that Hsp82 protein could be associated with increased tolerance to ethanol in *D. bruxellensis* [13]. Nevertheless, there are still few data to elucidate the protective mechanisms used by this yeast cell that allow survival in environments subject to various stress conditions as present in industrial fermentation. In the present study, we evaluated the tolerance of an industrial strain of *D. bruxellensis* to different stressing agents and the putative involvement of three heat shock proteins in this response: Hsp82p and two newly described Hsp22p and Hsp24p. The results showed that, despite they do not seem to be essential for cell survival, these proteins may play

roles in response to different stresses.

Material and Methods

Yeast strains and growth conditions

Dekkera bruxellensis industrial strain GDB 248 and *Saccharomyces cerevisiae* industrial strain JP1, used as reference, were used in the present work [14]. Cells were maintained in YPD medium (1% yeast extract, 2% peptone, 2% glucose and 2% agar). For liquid cultivation, both YPD and YPG (2% glycerol instead of glucose) without agar were used.

Cytotoxicity assays

Yeast cultivation was performed in 125 ml flasks containing YPD medium for 24 h at 30°C and 150 rpm in rotatory shaker. Yeast cells were used to inoculate the same medium to 0.1 DO_{600nm} initial concentration and cultivated to around 1 DO_{600nm} that represents the exponential growth phase. Cell suspensions were diluted to $2x10^7$ cells/ml in 50 ml YPD and submitted to different treatments. For heat shock treatment cell suspensions were incubated for 60 min in different temperatures. By using chemical agents, cell suspensions were incubated for 30 min at 30°C in the presence of different concentrations of hydrogen peroxide (oxidative stress), KCl (osmotic stress) and ethanol (ethanolic stress). After the treatments, cell suspensions were diluted to $1:10^4$ in 0.85% sterile saline and aliquots of 100 µL were plated onto YPD medium. The number of CFUs were scored after two (for *S. cerevisiae*) or four (for *D. bruxellensis*) days of incubation at 30°C. Cell viability was calculated as the number of viable cells after each treatment by the number of viable cells before treatments. The LD_{50}, lethal dose that inactivate 50% of the cells, was calculated from the slope of the dose-response curves.

In order to evaluate the capacity of *D. bruxellensis* to adapt to stress agents, yeast cells were submitted to sub-lethal doses (<LD_{20}) prior to cytotoxic treatments (Table 1).

Identification of HSP genes in D. bruxellensis genome and primer design

Search for chaperonines as keyword was performed in the *Dekkera* Genome Project database (www.lge.ibi.unicamp.br/dekkera/) and the nucleotide sequences of the contigs were downloaded for BLASTX analysis (http://www.ncbi.nlm.nih.gov/projects/gorf/). Determination of the ORFs for each gene was performed by the ORF FINDER tool (http://www.ncbi.nlm.nih.gov/projects/gorf/).

Gene expression analysis

Cells were pre-grown for 24 hours with shaking at 30°C in YPD (for respiro-fermentative metabolism) or YPG (for respiratory metabolism) and cultivated to exponential growth phase as above. After washing with sterile saline, yeast cells were re-suspended in YPD medium and treated for 15 min with sub-lethal doses of different stressing agents. In order to establish the reference condition, the cells were incubated in temperatures ranging from 30°C to °34C, which did not affect cell viability.

Primer design, total yeast RNA extraction, cDNA synthesis and RT-qPCR assays were performed according to De Barros Pita et al [7]. All parameters of quality and validations were applied and the statistical analysis and data normalization was performed by GeNorm applet (http://medgen.ugent.be/genorm). The samples were run in technical triplicates for each biological duplicates of cell cultivation.

Results and Discussion

Sensitivity of yeast cells to stressing agents

In the course of the fermentative processes the yeast cells are submitted to several stressing conditions produced by exposure to physical and chemical agents [9]. It has been recently shown that *D. bruxellensis* maintain the same fermentative capacity in medium pH and temperature ranging from 3 to 5 and 25°C and 37°C, respectively [3]. However, a decrease of c.a. 30% in its growth rate can be observed in that work when the temperature increased from 25° to 37°C. This is in accordance to the high sensitivity reported for this yeast to 35°C during wine fermentation, with 40% loss of cell viability [4]. Overall, the results of the present study confirmed that *D. bruxellensis* cells are less tolerant to thermal treatments than *S. cerevisiae*, with loss of cell viability at 42°C after 30 minutes of exposure (Fig. 1). After 30 minutes of incubation at 42°C and 44°C the percentage of viable cells of *D. bruxellensis* fall to 50% and 10%, respectively (Fig. 1A), while practically no killing effect was observed for *S. cerevisiae* cells in those conditions (Fig. 1B). Cultivation in glycerol promoted tolerance of *D. bruxellensis* cells to treatment at 44°C, indicating that induction of respiratory metabolism does protect the cells against the killing effect of high temperature.

Regarding to other stress treatments, *D. bruxellensis* cells was only slightly sensitive to osmotic stress by KCl (1 M) and to ethanol (10%) similar to *S. cerevisiae* (Fig. 2A, B). Previous results showed that *D. bruxellensis* display tolerance to ethanol up to 13% [2], which is higher than the average ethanol concentration (7 to 10%) in industrial fermentation processes. On the other hand, this yeast was more sensitive than *S. cerevisiae* to oxidative stress caused by hydrogen peroxide (Fig. 2C). However, this

sensitivity was suppressed when the cells were pre-grown in glycerol, so under respiratory metabolism. The cell viability of *D. bruxellensis* grown in glycerol was similar that found for *S. cerevisiae* grown in glucose (Fig. 2C). This result indicated that respiratory metabolism induced resistance to oxidative stress in *D. bruxellensis*.

Afterwards, the adaptive response was tested by exposing the cells to sub-lethal doses of different stress agents (Table 1). The results showed that pre-treating the cells with H_2O_2 or ethanol resulted in almost 3-times increase of cell survival to killing dose of H_2O_2. This indicates the adaptive mechanism to oxidative stress in *D. bruxellensis*. On the other hand, no pre-treatment recovered cell survival from heat shock treatment. Thus, it is suggestive of lack of cellular adaptation to heat shock by the induction of the oxidative stress response, different to what is observed for *S. cerevisiae* [6]. Thus, the results indicate that from the industrial point of view the increasing of the temperature of fermentation seems very adequate operational management if the decision is to prevent the settlement of *D. bruxellensis* in the process.

Identification and analysis of two novel chaperonines in D. bruxellensis

Short-Term Response (STR) enables the cells to protect against the damages caused by a variety of environmental stressing conditions and to repair the damages caused by such conditions [5,8]. It is an evolutionary conserved mechanism that involves the production of, for example, small heat shock proteins (sHsp). BLASTx analysis in the present work recovered two contigs from the *D. bruxellensis* genome that corresponded to Hsp22 (Genebank access EIF 49321.1) and Hsp24 (Genebank access EIF 45496.1), both presenting the alpha-crystallin domain (ACD) typical of sHsp proteins called chaperonines that belong to Hsp23 super-family. The biological involvement of these two sHsp proteins and the previously reported large heat shock

protein Hsp82p of *D. bruxellensis* [13] were evaluated by quantifying the expression of their corresponding genes under stress condition.

All three *HSP* genes were up-regulated after 15 minutes of exposure to 44°C, especially *HSP22* and *HSP24* that showed 100-fold increase in transcripts (Figure 3A). However, that high level of induction did not seem to protect the yeast cells for longer exposure to that temperature as observed from the cytotoxicity assay (Fig. 1A), neither induced any adaptive response to heat shock (Table 1). Considering that these genes were induced during the pre-treatment with heat shock (Table 1), the sHsp did not seem to protect the cells against the killing effect of H_2O_2 either. On the other hand, all three genes were down-regulated after cell treatment with H_2O_2 (Fig. 3A). This decrease in genes transcripts could account for the higher sensitivity of *D. bruxellensis* cells to H_2O_2 (Fig. 2A). The *HSP24* gene was down-regulated under ethanolic stress and up-regulated under osmotic stress (Fig. 3A). In contrast, its orthologous gene in *S. cerevisiae* was induced after exposure to 7% ethanol [1]. Over-expression of *HSP26* gene by strong gene promoter seems to increase *S. cerevisiae* cell viability after ethanolic (10% ethanol) and osmotic (25% glucose) stresses [11]. The results suggest that Hsp24p may be involved in response and tolerance to osmotic stress in *D. bruxellensis*. On the other hand, *HSP82* gene was slightly up-regulated under ethanolic stress and very much down-regulated under osmotic stress (Fig. 3A). This gene was also induced at ethanol concentration of 11% during wine fermentation [13]. Its orthologous *HSP82* in *S. cerevisiae* showed induction by ethanol at 6% [13] or 7% [1] and under osmotic stress [16]. In *S. cerevisiae* large HS proteins such as Hsp82, Hsp90, Hsp104 and Hsp150 were shown to be connected to cell resistance to ethanol [12]. Thus, it suggests that Hsp82p could take part of the mechanism of tolerance to ethanolic stress in both yeasts. The *HSP22* gene was very much down-regulated under ethanolic stress

and slightly down-regulated under osmotic stress compared to *HSP24* gene (Fig. 3A). Together, those results pointed that *HSP22* gene may take part in the mechanism of cell resistance to osmotic stress (Fig. 2A) in *D. bruxellensis*.

When the cells were grown aerobically in YPG medium, glycerol imposed strict respiratory metabolism that lead to induction of adaptive response to oxidative stress (Fig. 2C). In such condition, *HSP24* gene showed up-regulation under all stressing conditions tested (Fig. 3B), while *HSP22* and *HSP82* displayed similar regulation profile of that observed for glucose medium. It indicates that Hsp24p may be more produced when the yeast cells are grown under respiratory conditions, which leads to increased tolerance to thermal and oxidative stresses (Figs. 1A and 2C).

Taking together the results on cell survival and genes expression, it is clear that *HSP22, HSP24* and *HSP82* are only responsive to heat shock, and that their proteins are not involved in cell tolerance mechanism. Moreover, Hsp24p should participate somehow in cell tolerance to oxidative stress, but it is not essential, while Hsp82p may be involved in cell response and tolerance to ethanol linked to maintenance of the integrity of yeast cell wall in *D. bruxellensis*.

References

1. Alexandre H, Ansanay-Galeote V, Dequin S, Blondin B (2001) Global gene expression during short-term ethanol stress in Saccharomyces cerevisiae. FEBS Letters 498:98-103
2. Barata A, Pagliara D, Piccininno T, Tarantino F, Ciardulli W, Malfeito-Ferreira M, Loureiro V (2008) The effect of sugar concentration and temperature on growth and volatile phenol productions by *Dekkera bruxellensis* in wine. FEMS Yeast Res

8:1097-1102

3. Blomqvist J, Eberhard T, Schnürer J, Passoth V (2010) Fermentation characteristics of Dekkera bruxellensis strains. Appl Microbiol Biotechnol 87(4):1487-1497

4. Brandam CBC, Castro-martínez CCMC, Marie-line délia MLD, Ramón-Portugal FRP F, Strehaiano PSP (2008) Effect of temperature on Brettanomyces bruxellensis: metabolic and kinetic aspects. Can J Microbiol 54 (1):11-18

5. Causton HC, Ren B, Koh SS, Harbison CT, Kanin E, Jennings EG, Lee TI, True HL, Lander ES, Young RA (2001) Remodeling of yeast genome expression in response to environmental changes. Mol Bio Cell 12:323-337

6. Coote PJ, Cole MB, Jones MV (1991) Induction of increased thermotolerance in *Saccharomyces cerevisiae* may be triggered by a mechanism involving intracellular pH. J Gen Microbiol 137:1701-1708

7. De Barros Pita W, Leite FCB, De Souza Liberal AT, Simões, DA, De Morais, M A (2011) The ability to use nitrate confers advantage to *Dekkera bruxellensis* over *S. cerevisiae* and can explain its adaptation to industrial fermentation processes. Antonie Leeuwenhoek 100(1):99-107

8. Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz G, Botstein D, Brown PO (2000) Genomic expression programs in the response of yeast cells to environmental changes. Mol Biol Cell 11: 4241-4257

9. Graves T, Narendranath N, Power R (2007) Development of a "Stress Model" Fermentation System for Fuel Ethanol Yeast Strains. J Inst Brew 113(3):263-271

10. Jensen SL, Umiker NL, Arneborg N, Edwards CG (2009) Identification and characterization of *Dekkera bruxellensis*, *Candida pararugosa* and *Pichia guilliermondii* isolated from commercial red wines. Food Microbiol 26: 915-921

11. Jiménez-Martí E, Zuzuarregui A, Ridaura I, Lozano N, Del Olmo M (2009) Genetic

manipulation of HSP26 and YHR087W stress genes may improve fermentative behaviour in wine yeasts under vinification conditions. Int J Food Microbiol 130:122-130

12. Ma M, Liu LZ (2010) Quantitative transcription dynamic analysis reveals candidate genes and key regulators for ethanol tolerance in *Saccharomyces cerevisiae*. BMC Microbiol 10:169

13. Nardi T, Remize F, Alexandre H (2010) Adaptation of yeasts Saccharomyces cerevisiae and Brettanomyces bruxellensis to winemaking conditions: a comparative study of stress genes expression. Appl Microbiol Biotechnol 88 (4): 925-937

14. Pereira LF, Bassi APG, Avansini SH, Neto AGB, Brasileiro, B T R V, Ceccato-antonini S R, De Morais, M A (2012) The physiological characteristics of the yeast Dekkera bruxellensis in fully fermentative conditions with cell recycling and in mixed cultures with Saccharomyces cerevisiae. Antonie Leeuwenhoek 101(3):529-539

15. Poulain P, Gelly JC, Flatters D (2010) Detection and architecture of small heat shock protein monomers. Doi: 10.1371/journal.pone.0009990

16. Trott A, KA Morano (2003) The yeast response to heat shock. In: Hohmann S P W H Mager (ed) Yeast stress responses, Springer-Verlag, Heidelberg, Germany, pp 71–119

17. Woolfit M, Rozpedowska E, Piskur J, wolfe kH (2007) Genome survey sequencing of the wine spoilage yeast Dekkera (Brettanomyces) bruxellensis. Eukaryotic cell 6 (4):721-733

Legend to figures

Figure 1. Cell survival of *Dekkera bruxellensis* (panel A) and *Saccharomyces cerevisiae* (panel B) after 30 min exposure to 42°C (○), 44°C (□)and 46°C (◇). Cells were grown in YP medium containing glucose (straight lines) or glycerol (dashed lines).

Figure 2. Cell survival of *Dekkera bruxellensis* (●) and *Saccharomyces cerevisiae* (○) to 30 minutes treatments with different doses of KCl (panel A), ethanol (panel B) or hydrogen peroxide (panel C). Cells were pre-grown in YP medium containing glucose (straight lines) or glycerol (dashed lines).

Figure 3. Relative quantification of *Dekkera bruxellensis* genes *HSP24* (open columns), *HSP22* (closed columns) and *HSP82* (dotted columns) after 15 minutes exposure to heat shock (44°C), H_2O_2 (0.05 mM), ethanol (8%) or KCl (0.4 M). Cells were pre-grown in YP medium containing glucose (panel A) or glycerol (panel B).

Table 1. Cell survivor to thermal and oxidative stresses after pre-treatments with sub-lethal doses (LD_{25}) of heat, hydrogen peroxide or ethanol.

Pre-treatment			Treatment	
Agent	Dose	Time	46°C for 15min	0.5 mM H_2O_2 for 30 min
None			2.7% (±0.7)	29% (±3.3)
Heat	44°C	15 min	<0.1%	35.8% (±0.25)
H_2O_2	0.1 mM	30 min	2.1% (±0.17)	94% (±3.6)
Ethanol	8%	30 min	3.9% (±0.25)	86% (±0.7)

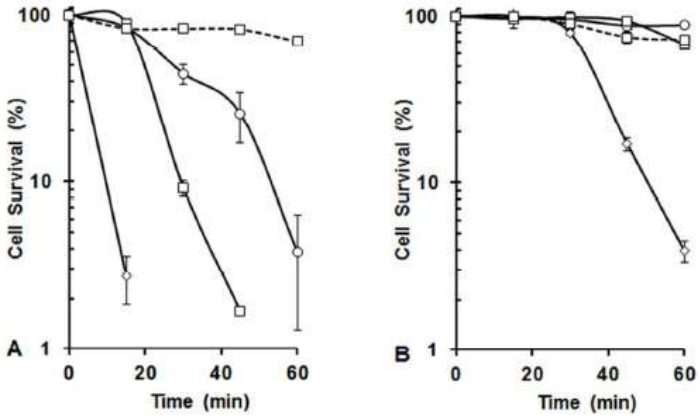

Figure 1. Cell survival of Dekkera bruxellensis (panel A) and Saccharomyces cerevisiae (panel B) after 30 min exposure to 42ºC (circle), 44ºC (square)and 46ºC (losang). Cells were grown in YP medium containing glucose (straight lines) or glycerol (dashed lines).
75x42mm (300 x 300 DPI)

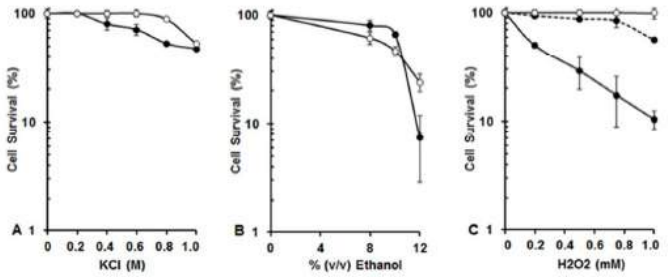

Figure 2. Cell survival of Dekkera bruxellensis (closed circles) and Saccharomyces cerevisiae (open circles) to 30 minutes treatments with different doses of KCl (panel A), ethanol (panel B) or hydrogen peroxide (panel C). Cells were pre-grown in YP medium containing glucose (straight lines) or glycerol (dashed lines). 62x26mm (300 x 300 DPI)

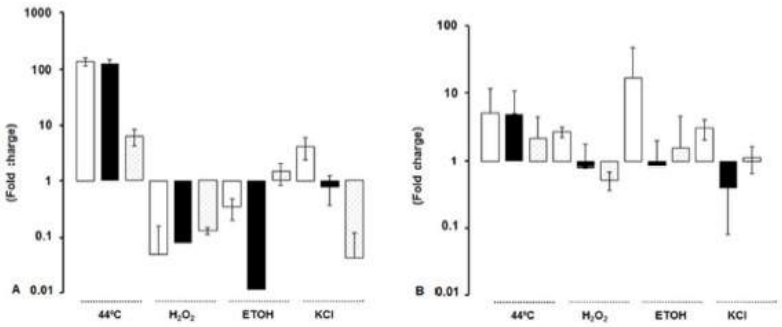

Figure 3. Relative quantification of Dekkera bruxellensis genes HSP24 (open columns), HSP22 (closed columns) and HSP82 (dotted colunms) after 15 minutes exposure to heat shock (44ºC), H2O2 (0.05 mM), ethanol (8%) or KCl (0.4 M). Cells were pre-grown in YP medium containing glucose (panel A) or glycerol (panel B).
62x25mm (300 x 300 DPI)

Supplementary data

Pereira et al. [Response of the yeast *Dekkera bruxellensis* to stress conditions and involvement of heat shock proteins]

Table S1. Primers used for RT-qPCR of *Dekkera bruxellensis* genes.

Gene	Primers	(5'-3')	Reference
HSP24	HSP24-F	GGCGCTGCCAAGGATAACT	This work
	HSP24-R	CGTGCTTGGAATTTCACCTTC	
HSP22	HSP22-F	AAATGGTCCTTTGCAGCACCT	This work
	HSP22-R	CCAGCATTAGATGTGCACGAA	
HSP82	HSP82-BB-F	GAGGTTTTGTTCCTTGTTGATCCTA	Nardi et al (2010)
	HSP82-BB-R	CCTTGGTCAATGGCTCAAACTC	
YNA1	YNA1-Db-F	TTTATATGGCGGGCATTGTA	De Barros Pita et al (2011)
	YNA1-Db-R	CCGTGAGCATTCAAGACATC	
EFA	EFA-Db-F	GGATGGTCAGACCAGACAGCA	De Barros Pita et al (2011)
	EFA-Db-R	TGACGGCAACAATAAGCTGC	
EFB	EFB-Db-F	CATGGGATGACGAGACCGAT	De Barros Pita et al (2011)
	EFB-Db-R	CACCAAACCGTCCATCTTGAT	
ACT1	ACT1-BB-F	GGTTATTGATAACGGTTCTGGTATGTG	Nardi et al (2010)
	ACT1-BB-R	TGACCCATACCGACCATGATAC	

Table S2. Optimization of the reference temperature for the analysis of the expression of heat shock genes *HSP24* and *HSP22* of *Dekkera bruxellensis*.

Temperature (°C)	*Cq*	
	HSP 24	***HSP22***
30	37.9	28.7
32	33.3	29.7
33	31.4	28.75
34	29.6	27.15

ARTIGO 3 - Fermentation of sugarcane molasses by *Dekkera bruxellensis* and mobilization of reserve carbohydrates

Artigo a ser submetido a Antonie van Leeuwenhoek
http://www.springer.com/life+sciences/microbiology/journal/10482

Fermentation of sugarcane molasses by *Dekkera bruxellensis* and mobilization of reserve carbohydrates.

Luciana Filgueira Pereira[1], Elisa Lucatti[3], Luiz Carlos Basso[3] and Marcos Antonio de Morais Júnior[1,2]

[1]Interdepartmental Research Group on Metabolic Engineering and [2]Department of Genetics, Federal University of Pernambuco. Av. Moraes Rego 1235, 50670-901, Recife, PE, Brazil.

Correspondence:

Marcos A. Morais Jr

Departamento de Genética - Universidade Federal de Pernambuco

Av. Moraes Rego, 1235 Cidade Universitária 50.670-901 Recife PE Brasil

Phone/Fax: 00-55-81-21268522

E-mail: marcos.morais@pesquisador.cnpq.br

Web site: www.ufpe.br/nem

Abstract

The yeast *D. bruxellensis* is considered very well adapted to the environment in industrial alcoholic fermentation using different substrates in Brazil, USA, Canada and Europe. Our previous study described its fermentative profile in sugarcane juice substrate. In the present study, we extended its physiological evaluation in situations fermentation using sugarcane molasses as substrate. It confirmed the inefficiency of *D. bruxellensis* cells in assimilating sucrose, which seems to be the main bottleneck for its use as fermentative yeast. Furthermore, cells of *D. bruxellensis* showed greatest deviation of sugar for biomass and organic acids compared to *Saccharomyces cerevisiae.* This is a particular concern in molasses by the induction of acetate production, which was not observed when sugarcane juice was fermented. It is noteworthy that this yeast do not accumulate trehalose while glycogen intracellular content was smaller than *S. cerevisiae*. Therefore, the adaptive success of *D. bruxellensis* under industrial fermentation conditions seems unrelated to the production of those reserve carbohydrates, which suggests that this species has another type of mechanism that favors this success adaptive to the industrial environment.

Keywords: molasses, adaptation industrial, organic acids, carbohydrate reserves, ethanol fermentation

Introduction

The alcoholic fermentation environment is endowed with stressors, sometimes environmental or resulting from the yeast metabolism that when they act synergistically, particularly when there are cell recycle, cause greater effect on cell viability and, consequently, on fermentation yield (Dorta et al. 2006; Bai et al. 2008; Basso et al. 2008). The yeast *D. bruxellensis* has proven to be well suited to industrial fermentation processes coming to ethanol yield similar to that of *S. cerevisiae* in laboratory media and sugarcane juice (De Souza Liberal et al. 2007; Pereira et al. 2012) and to higher biomass yield than *S. cerevisiae* in these media (Pereira et al. 2012; Leite et al. 2012). The ability of this yeast to adapt to industrial processes has been attributed to its resistance to weak organic acids (Ciani et al. 2003), the advantage in nutritional substrate containing nitrate as nitrogen source (De Barros Pita et al. 2011), tolerance to ethanol (Jensen et al. 2009) and robustness to variation of pH and temperature (Blomqvist et al. 2010). The physiological and metabolic causes of such adaptation remain unclear. Despite its robustness in industrial condition, *D. bruxellensis* cells are very sensitive to long periods of storage in laboratory medium (unpublished data) and its growth rate is lower in laboratory medium than in industrial medium such as sugarcane juice, for example (De Barros Pita et al. 2011; Leite et al. 2012; Pereira et al 2012). In a recent study we observed that cells of *D. bruxellensis* GDB 248 industrial strain are less tolerant to thermal and oxidative stresses than *S. cerevisiaie* JP1 industrial strain (manuscript submitted). Thus, deeper metabolic analyses of this yeast are required in order to figure out the mechanisms beyond such adaptation.

Yeast cells are subjected to stressful conditions of fermentation showed low levels of glycogen reserve carbohydrates and trehalose. This may affect their survival in the environment as elevated levels of these carbohydrates are important for yeast withstand the

stress present in the industrial processes (Basso et al. 2011). It is well established that the intracellular content of glycogen and trehalose in yeast cells varies significantly depending on the growth conditions, the availability of nutrients in the media and with the presence of stress-inducing factors (Francois and Parrou 2001). Moreover, studies show the relationship between the ethanolic stress and the mobilization of these carbohydrates, since situations may occur during fermentation that decrease trehalose content up to 60% (Pataro et al. 2002). Likewise, increasing the intracellular content of glycogen during fermentation is related to the adaptation and tolerance to ethanol (Mansure et al. 1997; Dake et al. 2010). We recently showed that in fermentation of sugarcane juice with recycle cells *D. bruxellensis* low efficiency have lower consumption of sucrose, large deviation biomass, same tolerance to ethanol and same sensitivity to organic acids as compared to *S. cerevisiae* (Pereira et al. 2012). In the present study, we evaluated the fermentation profile of *D. bruxellensis* in fermentation assays with cell recycle using sugarcane molasses as substrate, with an emphasis on the analysis of intracellular content and mobilization of trehalose and glycogen, weaving a parallel with this biochemical trait with yeast cell adaptability to the industrial environment.

Material and Methods

Strains and growth conditions

Dekkera bruxellensis industrial strain GDB 248 and *Saccharomyces cerevisiae* industrial strain JP1, used as reference, were used in the present work (Pereira et al 2012). Cells were maintained in YPD medium (1% yeast extract, 2% peptone and 2% glucose). Solid medium contained 2% agar.

Fermentative assays

Yeast cells were pre-grown in 5 mL YPD medium at 30ºC for 48 h in rotatory shaker at 140 rpm, recovered by centrifugation (800 g for 20 min at room temperature) and suspended in propagation molasses medium containing initial sugar at 10 g l^{-1} (sucrose, glucose and fructose expressed as hexose content) for propagation of the biomass at 30ºC. Cells were collected by centrifugation, the supernatant was removed at most and the wet sediment was weighed. Cell suspensions were prepared in fermentation molasses medium containing initial sugar at 86.7 g l^{-1} to initial biomass concentration of 10% (wet weight/vol) in a total volume of 40 ml. The cultures were transferred to 50 ml flasks for incubation for 24 h at 32ºC without agitation (first cycle). Afterwards, cells were collected by centrifugation, washed with sulfuric acid solution at 1.56 mM (pH 2.5) for one hour and re-suspended as above in fermentation molasses medium for a new batch of fermentation (second cycle). This procedure was repeated for three times for a total of five cycles. At the end of each cycle, yeast cells were collected for viability determination after dying with eosine by microscope inspection and the supernantant was used for measurement of metabolites consumption and production. Single batches experiments for 24 h were performed as above, with the modification of initial volume of 100 ml in order to allow sampling at every two hours.

Metabolite measurements

Residual sugars, lactate, acetate and glycerol were determined by ion Exchange chromatography in Dionex DX-300 device equipped with CarboPac PA-1 (4 x 250 mm) column and pulse amperometric detector. Mobile phase was 100 mM NaOH at a constant flux of 0.9 ml min^{-1} (Basso et al. 2008). Fermentation yields were calculated as the metabolite

produced by consumed sugar. Production of CO_2 was measured by the weight loss procedure (Basílio et al. 2008). The intracellular content of glycogen and trehalose were determined at the end of each fermentative cycle, as well along the kinetics experiments. After being collected, yeast cells were submitted to 0.5M trichloroacetic acid treatment at 0ºC for 20 min for extraction of reserve carbohydrates. Trehalose was quantified in Dionex DX-300 device (Basso et al. 2008) while glycogen was determined according to Rocha-Leão et al. (1984). All experiments were performed in biological triplicates with two technical replicate. The results of each were submitted to analysis of variance (ANOVA) and the averages were compared by Tukey test ($p \leq 0.05$). Only averages were plotted in graphics and average ± standad deviation were presented in Tables.

Results

Sugarcane molasses fermentation with cell recycling

Cell viability over the recycles fermentation was calculated to be 96% and 98% for *D. bruxellensis* and *S. cerevisiae*, respectively (Fig. 1a) and the cell population was kept constant at the end of each fermentation cycle for both yeasts (Fig. 1b). The final pH of the fermentation was higher in cultures of *S. cerevisiae* (5.06 ± <0.01) than *D. bruxellensis* (4.8 ± 0.02). The mass balance over the recycles was calculated as 99% (± 0.03) and 94% (± <0.01) for *D. bruxellensis* and *S. cerevisiae*, respectively, distributed as follows according to the consumption of sugars and production of metabolites and biomass. Biomass yield ($Y_{x/s}$) of *D. bruxellensis* cultures (0.05 ± <0.01) was greater than *S. cerevisiae* cultures (0.02 ± <0.01) (Fig. 2a), whereas the yield of organic acids (lactate+acetate) was similar for the two yeasts (0.04 ± <0.01) for *D. bruxellensis* and 0.05 ±> 0.01 for *S. cerevisiae*). For the production of

those acids, no difference in the final concentration of lactic acid between the two yeasts, while the cultures of *D. bruxellensis* showed almost three times higher acetate production than *S. cerevisiae* (Table 1). The residual concentration of glucose and fructose were low for both yeasts (Table 1). In contrast, only 36% of the initial sucrose was consumed on average over the recycles fermentation by *D. bruxellensis* cells, whereas the cells of *S. cerevisiae* consumed almost all of sucrose at the end of each fermentation cycle (Table 1). As consequence, both production of ethanol and CO_2 were lower in cultures of *D. bruxellensis* than in cultures of *S. cerevisiae* (Table 1). Nevertheless, ethanol yield (Fig. 2b) it was similar for both yeasts (0.45 g g^{-1}).

Regarding reserve carbohydrates, trehalose could not be detected in cells of *D. bruxellensis* after each recycle fermentation, while cells of *S. cerevisiae* showed values up to 17% of this metabolite corresponding to cell dry weight, with average 13.34% (± 0.26) over the cycles. The average intracellular glycogen content was lower in *D. bruxellensis* cells (0.24% ± 3.6) than in *S. cerevisise* (5.11% ± 0.48) over the recycles (Table 1). These results show the difference in the intracellular content of the carbohydrate reserves between both yeasts. Under aerobic cultivation in YPD medium no measureable trehalose was observed in *D. bruxellensis* cells, while lower glycogen content was measured in this yeast compared to *S. cerevisiae* (unpublished data). Therefore, this seems to be a metabolic trait of this yeast.

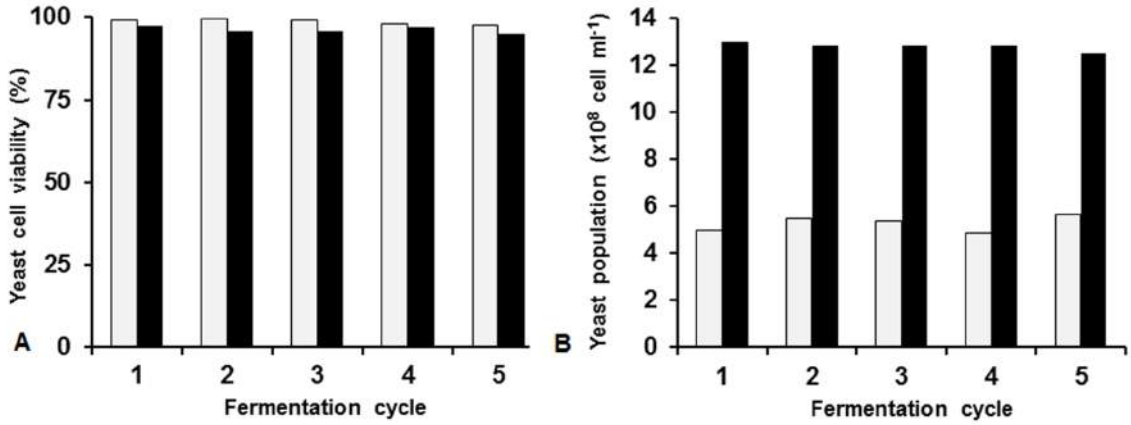

Figure 1. Cell viability (A) and cell count (B) of *Saccharomyces cerevisiae* JP1 strain (gray columns) and *Dekkera bruxellensis* GDB248 (black columns) at the end of fermentation cycles in molasses medium.

Table 1. Metabolites at the end of three recycling stages of fermentation (C1, C3 and C5) of pure cultures of *S. cerevisiae* and *D. bruxellensis* in molasses

Metabolite	***S. cerevisiae* JP1**			***D. bruxellensis***		
	`C1	**C3**	**C5**	**C1**	**C3**	**C5**
(Sucrose consumed (g l^{-1})	86,3	86,6	86,1	43,82	31,43	32,35
Residual glucose (g $l^{-1)}$	0.19	0,45	0.43	0.29	0.1	0,29
Residual fructose (g $l^{-1)}$	0,1	0.15	0.14	0.3	0.49	0.31
Glycerol (g $l^{-1)}$	3,3	3,5	3,9	1.4	1.2	1.2
Acetate (g $l^{-1)}$	0.42	0.58	0.5	1.4	1.4	1.5
Lactic acid (g $l^{-1)}$	0.61	0.64	0.68	0.97	0.83	0.97
Glycogen (%)	3.9	6.3	6.6	3.2	4.5	4.2
Trehalose (%)	13.2	16.8	13.3	0.0	0.0	0.0
Ethanol (%v/v)	7.45	8.45	8.28	4.71	4.73	4.8
CO2 (g $l^{-1)}$	1.97	2.23	2.18	1.15	1.10	1.19

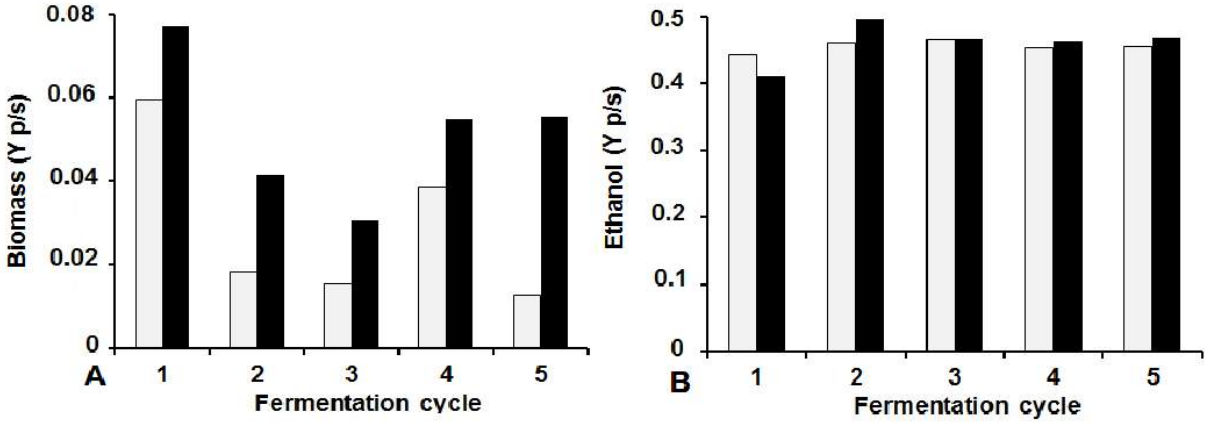

Figure 2. Yields for biomass (A) and ethanol (B) of *Saccharomyces cerevisiae* JP1 strain (gray columns) and *Dekkera bruxellensis* GDB248 (black columns) at the end of fermentation cycles in molasses medium.

Fermentation kinetics of sugarcane molasses fermentation

The dynamic behavior of the yeast physiology was analyzed in single batch experiments. Slower sucrose consumption was observed for *D. bruxellensis* along the 24 h of fermentation compared to *S. cerevisiae* (Fig. 3a), while glucose and fructose have been completely consumed within 6 and 4 hours of fermentation, respectively for both yeasts (Fig. 3bc). The initial accumulation of glucose and fructose in the medium in cultures of *S. cerevisiae* (Fig. 3bc) might be explained by the action of extracellular invertase, while this enzyme is almost exclusively intracellular in *D. bruxellensis* (Leite et al. 2012). Glycerol production by cells of *D. bruxellensis* was very low when compared to *S. cerevisiae* (Fig. 4a). The production of organic acids was detected in both yeasts, with acetate production being more relevant in *D. bruxellensis* cultures (Fig. 4b), while the lactic acid production being similar in both yeasts (Fig. 4c).

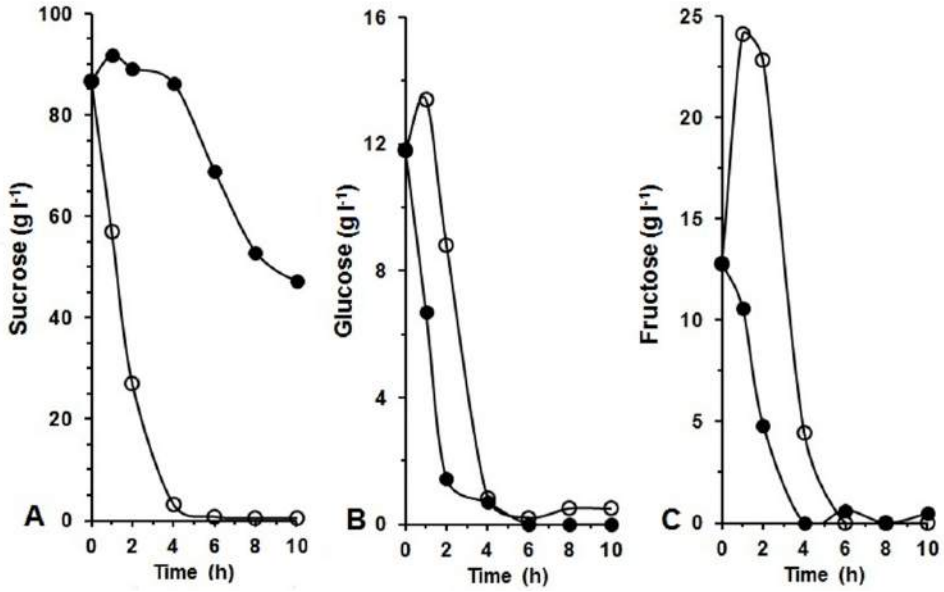

Figure 3. Kinetics of sugar consumption by cells of *Saccharomyces cerevisiae* JP1 strain (open circles) and *Dekkera bruxellensis* GDB248 (closed circles) within a fermentation cycle in molasses medium.

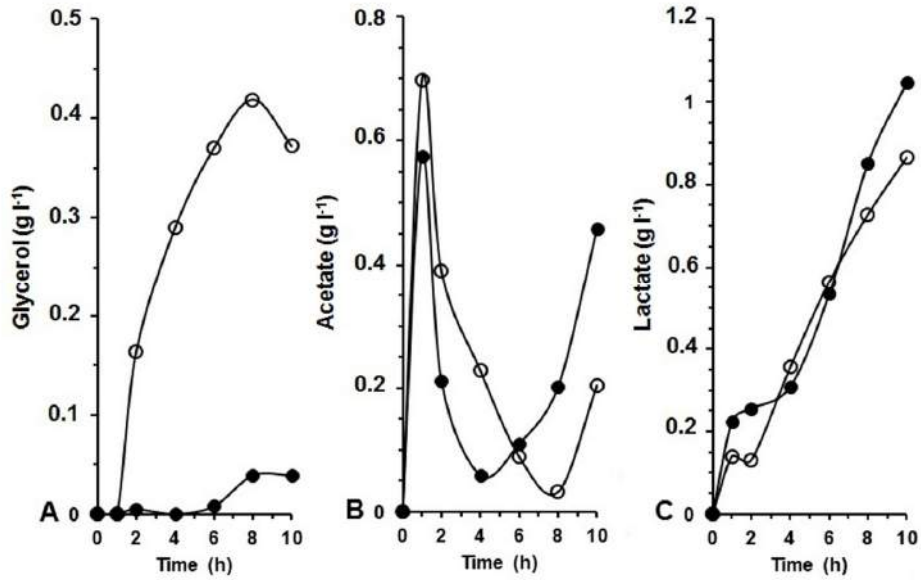

Figure 4. Kinetics of extracellular metabolites production by cells of *Saccharomyces cerevisiae* JP1 strain (open circles) and *Dekkera bruxellensis* GDB248 (closed circles) within a fermentation cycle in molasses medium.

It was not detected the presence of trehalose in the cells of *D. bruxellensis* during fermentation, whereas this metabolite has accounted for about 11% of the cell mass of *S. cerevisiae* at the end of fermentation (Fig. 5) On the other hand, glycogen accumulation was observed for *D. bruxellensis* cells throughout the fermentation, although smaller than *S. cerevisiae* (Fig. 5). The intracellular content of glycogen reached 10% in *S. cerevisiae* and only 4% in *D. bruxellensis* (Fig. 5).

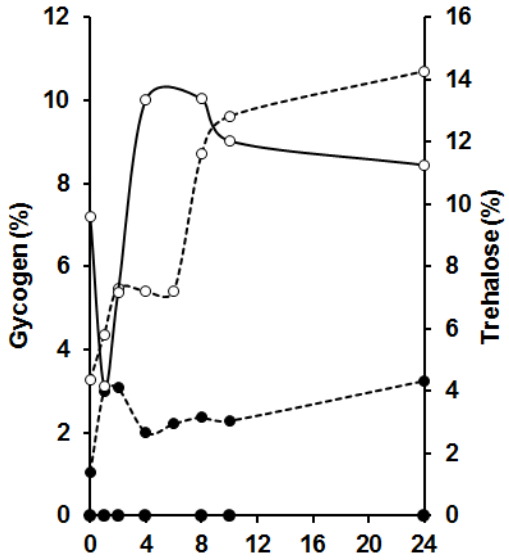

Figure 5. Kinetics of intracellular reserve carbohydrate trehalose (straight lines) and glycogen (dotted lines) accumulation in the cells of *Saccharomyces cerevisiae* JP1 strain (open circles) and *Dekkera bruxellensis* GDB248 (closed circles) within a fermentation cycle in molasses medium.

Discussion

The yeast *D. bruxellensis* has been regarded as well suited to industrial fermentation processes due to its persistence in the fermentation tanks even when it is not used as inoculant (De Souza Liberal et al. 2007; Passoth et al. 2007; Basílio et al. 2008; Pereira et al. 2012; De

Souza Barros et al. 2012). Although there are few data on the characteristics that confer this adaptability, it is accepted that this trait may be related to greater tolerance for various forms of stress process (Rozpedowska et al. 2011). The processes for producing ethanol from sugarcane substrates are characterized by a succession of non-*Saccharomyces* yeasts (Silva-Filho et al. 2005; Basílio et al. 2008; Basso et al. 2008) and the yeast *D. bruxellensis* presented greater growth than *S. cerevisiae* and thus greater efficiency in sugar conversion to biomass in sugarcane juice medium (Pereira et al. 2012). Similarly, it was observed in the present work greater tendency for biomass production by this yeast also in molasses medium (Fig. 2a). Current data has shown that *D. bruxellensis* is capable of achieving ethanol yields close to *S. cerevisiae* (De Souza Liberal et al. 2007; Blomqvist et al. 2010; De Barros Pita et al. 2011), and it may be in situations with consortium of lactic acid bacteria considered a good ethanol producer (Passoth et al. 2007; De Souza Barros et al. 2012). However, it is currently reported that high ethanol yields were observed only after long periods of fermentation (De Souza Liberal et al. 2007; Blomqvist et al. 2010). Differently to the short-term fermentation experiments performed in sugarcane juice (Pereira et al. 2012), in the present work it was observed ethanol yield similar to *S. cerevisiae* after long-term 24 h molasses fermentation (Fig. 2c; Table 1). Nevertheless, the most prominent problem still regarding to the lower productivity of this yeast with high residual sugar left in the process.

This low productivity is due to the inefficiency of sucrose assimilation by *D. bruxellensis* when compared to *S. cerevisiae* both during fermentation of grape must (Renouf et al. 2006), laboratory medium (Basilio et al 2008) and sugarcane juice (Pereira et al. 2012) as well as sugarcane molasses (Fig. 3a). One possible explanation for this phenomenon is the intracellular metabolism of sucrose that requires a very efficient system of transporting sugar (Leite et al. 2012). *Dekkera bruxellensis* showed inefficiency in the assimilation of glucose and fructose during fermentation of grape juice using pure cultures (Renouf et al. 2006) or

sugarcane juice using cultures mixed with *S. cerevisiae* cells (Pereira et al. 2012). On the other hand, glucose and fructose assimilation was in the range of that observed for *S. cerevisiae* (Fig. 3bc). It led to the conclusion that physical-chemical characteristics of the molasses are very stimulatory of the fermentative metabolism from hexoses. Therefore, the metabolic problem of low productivity seems restricted to the inefficiency of sucrose assimilation and breakdown.

Under anaerobic conditions little or no production of glycerol is accomplished by cells of *D. bruxellensis* (Blomqvist et al. 2010; Pereira et al. 2012) compared to already well described glycerol production by *S. cerevisiae* during fermentation. This effect is regarded to the low capability of *D. bruxellensis* to restore the redox balance through the production of reduced metabolites such as glycerol (Wijsman et al. 1984). Furthermore, and unlike what happens in *S. cerevisiae*, the yeast *D. bruxellensis* produces much acetic acid under aerobic conditions (Leite et al. 2012) and very little or no amount under anaerobic environments or oxygen-limited condition (Uscanha et al. 2003; Pereira et al. 2011). In the present work we found glycerol in the cultures of *D. bruxellensis*, although about three times lower than in *S. cerevisiae* (Table 1). It is known for long date that *Dekkera* yeasts require a supply of oxygen to stimulate fermentation, a phenomenon known as Custer effect that could be abolished by the presence of oxygen or an electron acceptor like acetoin (Van Dijken and Scheffers 1984). De Barros Pita (2011) suggested that the nitrate in the sugarcane juice can be an ultimate electron acceptor, removing this effect and increasing production of ethanol by *D. bruxellensis*. Similarly, we suggest that some component of molasses could induce the production of acetic acid by the cells of *D. bruxellensis* even in anaerobiosis (Table 1; Fig. 4b), which does not occur in the sugarcane juice medium (Pereira et al. 2012).

Trehalose and glycogen are important intracellular carbohydrate reserves that maintain carbon and energy sources as well as play an important role in cell protection under stress

(François and Parrou 2001). The lack of trehalose in the interior of *D. bruxellensis* cells, together with the low glycogen content, point to the fact that increase in biomass yield should be related to high metabolic flux towards amino acids biosynthesis in this yeast. If it comes to be true, the production of *D. bruxellensis* cell biomass as a source of single cell protein may be economically advantageous as compared to *S. cerevisiae* (Leite et al. 2012).

Another important biological function of those carbohydrates is their participation in cell viability and longevity. During the alcoholic fermentation processes various stresses can affect the viability of yeast cells (Graves et al. 2007). Elevated levels of carbohydrate make the cells supporting the acid wash imposed by industrial processes and become them more tolerant industrial conditions (Basso et al. 2011). Trehalose is a metabolite of paramount importance to the tolerance of yeast to face different situations of stress associated with the plasma membrane while maintaining its integrity (Martini et al. 2006; Elsztein et al. 2008). This metabolite seems to be also involved in ethanol stress tolerance (Mansure et al. 1997). There was no detectable accumulation of trehalose by *D. bruxellensis* cells in the course of fermentation (Fig. 5) or along the fermentation cycles (Table 1). Additionally, the absence of this metabolite does not appear to affect cell viability during fermentation in which ethanol accumulates in the medium (Fig. 1a), neither to organic acids (Pereira et al. 2012). Besides, accumulation of trehalose is involved in acquisition of thermotolerance in *S. cerevisiae* (Virgilio et al. 1994). Our recent results show that *D. bruxellensis* is more sensitive to heat stress than *S. cerevisiae* (manuscript submitted). Together, these findings show that in *D. bruxellensis* tolerance to heat and ethanolic stresses are not connected to the accumulation in intracellular trehalose.

The intracellular content of glycogen in the cells of *D. bruxellensis* was one third of that observed for *S. cerevisiae* at the end of fermentation cycle in molasses (Table 1; Fig. 5). This metabolite is considered the major reserve of energy in *Saccharomcyes cerevisiae* (Dake

et al. 2010) and seems to be also involved in the tolerance to ethanol (François and Parrou 2001). Thus, similar to trehalose, glycogen might not be involved in *D. bruxellensis* tolerance to ethanol or to organic acid (Pereira et al. 2012), nor to thermal or oxidative stresses (manuscript submitted).

In conclusion it seems that fermentation in sugarcane molasses presents some particularities when comparing to sugarcane juice, the most relevant being the stimulation of acetate production even in anaerobiosis. It might be regarded to the differences in mineral/chemical composition differences between these substrates. In both cases, sucrose assimilation seems the major problem for completion of fermentation in reasonable period of industrial processes, without leaving high residual sugar. And lastly, the lack of trehalose and the low content of glycogen inside the yeast cells may be not related neither to the success of *D. bruxellensis* adaptation nor to the eventual failure in its settlement to industrial production plants. But may support the suggestion that *D. bruxellensis* is very effective for single cell protein production.

References

Bai FW, Anderson WA, Moo-Young, M (2008) Ethanol fermentation technologies from sugar and starch feedstocks. Biotech Adv 26: 89-105

Basílio ACM, Araújo PRL, Morais JOF, Silva-Filho EA, Morais MA Jr, Simões DA (2008) Detection and identification of wild yeast contaminants of the industrial fuel ethanol fermentation process. Curr Microbiol 56: 322-326

Basso LC, Amorim HV, Oliveira AJ, Lopes, ML (2008) Yeast selection for fuel ethanol in Brazil. FEMS Yeast Res 8: 1155-1163

Basso LC, Basso TO, Rocha SN (2011) Ethanol production in Brazil: the industrial process and its impact on yeast fermentation. In: Dos Santos Bernardes MA (Ed.) Biofuel production - recent developments and prospects. Intech, Croatia, pp 85-100

Blomqvist J, Eberhard T, Schnürer J, Passoth V (2010) Fermentation characteristics of Dekkera bruxellensis strains. Appl Microbiol Biotechnol 87:1487-1497

Ciani M, Maccarelli F, Fatichenti F (2003) Growth and fermentation behaviour of Brettanomyces/Dekkera yeasts under different conditions of aerobiosis. World J Microbiol Biotechnol 19: 419–422

Dake MS, Jadhv JP, Patil N B (2010) Variations of two pools of glycogen and carbohydrate in *Saccharomyces cerevisiae* grown with various ethanol concentrations. J Ind Microbiol Biotechnol 37: 701–706.

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

De Souza Barros R, Dos Santos BM, De Souza RFR, Da silva PQN, Lucena BL Marcos Morais M A Jr. (2012) The consequences of Lactobacillus vini and *Dekkera bruxellensis* as contaminants of the sugarcane-based ethanol fermentation. J Ind Microbiol Biotechnol. Doi DOI 10.1007/s10295-012-1167-0

De Souza Liberal AT, Basílio ACM, Resende AM, Brasileiro BTRV, Silva-Filho EA, Morais JOF, Simões DA, Morais MA Jr (2007) Identification of *Dekkera bruxellensis* as a major contaminant yeast in continuous fuel ethanol fermentation. J Appl Microbiol 102: 538–547

De Virgilio C, Hottiger T, Dominguez J Boller T, Wiemken A (1994) The role of trehalose synthesis for the acquisition of thermotolerance in yeast I. Genetic evidence that trehalose is a thermoprotectant. Eur J Biochem 219: 179-186

Dorta C, Oliva-Neto P, De-Abreu-Neto MS, Nicolau-Junior N, Nagashima AI (2006) Synergism among lactic acid, sulfite, pH and ethanol in alcoholic fermentation of *Saccharomyces cerevisiae* (PE-2 and M-26). World J Microbiol Biotechnol 22:177-182

Elsztein C, Menezes JAS, Morais Junior MA (2008) Polyhexamethyl biguanide can eliminate contaminant yeasts from fuel-ethanol fermentation process. J Ind Microbiol Biotechnol 35: 967-973

Francois J, Parrou, JL (2001) Reserve carbohydrates metabolism in the yeast Graves T, Narendranath N, Power R (2007) Development of a "Stress Model" Fermentation System for Fuel Ethanol Yeast Strains. J Inst Brew 113: 263-271

Graves T, Narendranath N, Power R (2007) Development of a "Stress Model" Fermentation System for Fuel Ethanol Yeast Strains. J Inst Brew 113: 263-271

Jensen SL, Umiker NL, Arneborg N, Edwards CG (2009) Identification and characterization of *Dekkera bruxellensis*, *Candida pararugosa* and *Pichia guilliermondii* isolated from commercial red wines. Food Microbiol 26: 915-921

Leite FCB, Basso TO, Pita WB, Gombert A, Simões D, Morais Junior MA (2012) Quantitative aerobic physiology of the yeast Dekkera bruxellensis, a major contaminant in bioethanol production plants. FEMS Yeast Research. doi:10.1111/j.1567-1364.2012.12007.x

Mansure JJ, Souza RC, Panek AD (1997) Trehalose metabolism in *Saccharomyces cerevisiae* during alcoholic fermentation. Biotechnol Lett 19: 1201-1203

Martini S, Ricci M, Bartolini F, Bonechi C, Braconi D, Millucci L, Santucci A, Rossi C (2006) Metabolic response to exogenous ethanol in yeast: an in vivo NMR and mathematical modeling approach. Biophys Chem 120: 135–142

Parrou JL, Teste MA, Francois J (1997) Effects of various types of stress on the metabolism of reserve carbohydrates in Saccharomyces cerevisiae: genetic evidence for a stress-induced recycling of glycogen and trehalose. Microbiol 143: 1891–1900

Pataro C, Guerra JB, Gomes FCO, Neves MJ, Pimentel PF, Rosa CA (2002) Trehalose accumulation, invertase activity and physiological characteristics of yeasts isolated from 24 h fermentative cycles during the production of artisanal Brazilian cachaça. Braz J Microbiol 33: 202–208

Pereira LF, Bassi APG, Avansini SH, Neto AGB, Brasileiro BTRV, Ceccato-antonini SR, De Morais M A (2012) The physiological characteristics of the yeast *Dekkera bruxellensis* in fully fermentative conditions with cell recycling and in mixed cultures with *Saccharomyces cerevisiae.* Antonie Leeuwenhoek 101: 529-539

Renouf V, Falcou M, Miot-Sertier C, Perello MC, De Revel G, Lonvaud-Funel, A (2006) Interactions between Brettanomyces bruxellensis and other yeast species during the initial stages of winemaking. J Appl Microbiol 100: 1208–1219

Rocha-Leão MHM, Panek AD, Costa-Carvalho, VLA (1984) Glycogen accumulation during growth of *Saccharomyces cerevisiae*: catabolite repression effects. IRCS Med Sci 12: 411–412.

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

Silva-Filho EA, Melo HF, Antunes DF, Dos Santos SKB, Resende MA, Simões DA, Morais MA Jr (2005) Isolation by genetics and physiological characteristics of a fuel ethanol

fermentative *Saccharomyces cerevisiae* strain with potential for genetic manipulation. J Ind Microbiol Biotechnol 32:481–486

Uscanga Aguilar MG, Delia ML, Strehaiano P (2003) *Brettanomyces bruxellensis*: Effect of oxygen on growth and acetic acid production. Appl Microbiol Biotechnol 61: 157-10 162

Van Dijken JP, Scheffers WA (1984) Studies on alcoholic fermentation in yeasts. In: Houwink EH, Van der Meer RR (eds) Innovations in biotechnology. Elsevier, Amsterdam, pp 497–506

Wijsman MR, van Dijken JP, van Kleeff BH, Scheffers WA (1984) Inhibition of fermentation and growth in batch cultures of the yeast Brettanomyces intermedius upon a shift from aerobic to anaerobic conditions (Custers effect). Antonie Van Leeuwenhoek 50: 183–192

3 CONCLUSÃO

- Em fermentações com reciclo celular as células de *D. bruxellensis* mostram-se menos eficiente na hidrólise da sacarose do que as células de *S. cerevisiae*.

- As células de *D. bruxellensis* produzem menor quantidade de ácidos orgânicos fracos. Sendo assim, em condições fermentativas não há qualquer relação entre produção de ácidos orgânicos fracos e queda de viabilidade celular de *S. cerevisiae*.

- As células de *D. bruxellensis* exibem perfil similar de tolerância ao etanol quando comparado a *S. cerevisaie* o que invalida a possibilidade de eliminação de *Dekkera* dos processos industriais por fermentações com altos teores alcoólicos.

- As sequências dos contigs 959 e 1728 do banco de dados genômico da levedura *D. bruxellenis* referem-se aos genes HSP22 e HSP24 codificantes de proteínas de baixo peso molecular (sHsP) pertencentes a super família HSP23.

- Os genes HSP22, HSP24 e HSP82 são apenas responsivos ao choque térmico e que suas proteínas não estão envolvidas no mecanismo de tolerância celular. Embora, Hsp24p deva participar na tolerância da célula de alguma forma contra o estresse oxidativo.

- Os ensaios de sensibilidade das células de *D. bruxellensis* (glicose) a diferentes agentes de estresse mostram que as mesmas são mais sensíveis aos estresses oxidativo e térmico do que *S. cerevisiae* mais exibem perfil similar de tolerância ao etanol e estresse osmótico.

- Os cultivos em glicerol promovem a tolerância das células *D. bruxellensis* ao tratamento térmico (44°C) e oxidativo (H_2O_2) indicando que a indução do metabolismo respiratório protege as células contra a queda de viabilidade por estes agentes.

- A células de *D. bruxellensis* apresenta um mecanismo de adaptação celular ao estresse oxidativo quando pré induzida por dosagens subletais de etanol (8%) e (H_2O_2) resultando em aumento de viabilidade celular;

- Nas fermentações com reciclo celular utilizando como substrato o melaço as células de *D. bruxellensis* mostram rendimentos em etanol similares ao de *S. cerevisiae.*

- As células de *D. bruxellensis* não sintetizam trealose em condições fermentativas e seu conteúdo de glicogênio (4%) é inferior (4%) ao detectado nas células de *S. cerevisiae* (10%).

- Os resultados obtidos neste trabalho contribuíram para uma melhor compreensão acerca do sucesso adaptativo das células de *D. bruxellensis* aos ambientes de fermentação alcoólica industrial além de, favorecer os estudos na busca de utilização desta espécie como potencial produtora de etanol.

ANEXOS COMPLEMENTARES

1 Artigos completos publicados em periódicos

De Barros Pita W, Leite FC, de Souza Liberal AT, **Pereira LF**, Carazzolle MF, Pereira GA, de Morais MA Jr. A new set of reference genes for RT-qPCR assays in the yeast *Dekkera bruxellensis*. Can J Microbiol. 2012 Dec;58(12):1362-7. doi: 10.1139/cjm-2012-0457. Epub 2012 Nov 25.

2 Resumos publicados em anais de congressos

Barros Pita, Will; Leite, Fernanda Cristina Bezerra; Souza Liberal, Anna Theresa; **Pereira, Luciana Filgueira**; Morais Junior, Marcos Antonio. Validação de genes de referência para análises de expressão gênica por PCR em Tempo Real em *Dekkera bruxellensis.* In: 27ª Reunião de Genética de Microrganismos, 2010, Guarujá-SP.

Pereira, L.F.; De Souza Liberal,A.T.; Costa Júnior, C.R.L.; Morais JR, M.A. Análise da expressão gênica por PCR em tempo real dos genes HSP13 e HSP26 codificantes de chaperoninas em linhagem industrial da levedura Dekkera bruxellensis. In: XIX Encontro de Genética do Nordeste, 2012, Petrolina-PE.