UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE CIÊNCIAS BIOLÓGICAS
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Identificação molecular da levedura *Dekkera bruxellensis* como principal contaminante do processo de fermentação alcoólica industrial

ANNA THERESA DE SOUZA LIBERAL

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Dissertação apresentada ao curso de Pós-graduação em Genética da Universidade Federal de Pernambuco como parte dos requisitos para obtenção do grau de Mestre em Genética.

Orientador: Marcos Antônio de Morais Júnior

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PARECER DA COMISSÃO EXAMINADORA

Parecer da Comissão Examinadora de defesa de **Dissertação de Mestrado**, com área de concentração em Genética Molecular de Anna Theresa de Souza Liberal, sob o título: "Identificação molecular da levedura *Dekkera bruxellensis* como principal contaminante do processo de fermentação alcoólica industrial". Tendo como orientador Profº Drº Marcos Antônio de Morais Júnior, Departamento de Genética – UFPE

A comissão Examinadora composta pelos professores abaixo, sob a presidência do primeiro, considera a candidata **Anna Theresa de Souza Liberal, aprovada.**

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Recife, 20 de fevereiro de 2006

DEDICATÓRIA

A Deus por iluminar meu caminho.
À minha mãe Maria Aparecida Liberal e minha tia Evany Liberal por serem meu apoio em todas as escaladas da minha vida.

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RESUMO

As indústrias de álcool combustível realizam a fermentação a partir de um processo aberto e não asséptico permitindo que leveduras de baixo rendimento fermentativo se instalem no processo. Identificar e caracterizar a principal espécie de levedura contaminante detectada em usinas de álcool combustível na região Nordeste do Brasil utilizando métodos moleculares foi, portanto o objetivo deste trabalho. O isolado não-*Saccharomyces cerevisiae* mais freqüente foi identificado por provas bioquímicas e por seqüênciamento de DNA como pertencente à espécie *Dekkera bruxellensis*, e diversas linhagens genéticas puderam ser discriminadas entre os isolados. O estudo da dinâmica da população de leveduras mostrou a alta adaptabilidade de *D. bruxellensis* às condições industriais, causando episódios de contaminação recorrentes e severos. Contaminações severas do processo de fermentação industrial por leveduras *Dekkera* têm um impacto negativo no rendimento global das destilarias, explicado neste trabalho pela menor produtividade volumétrica desta levedura. A partir do exposto, a rápida detecção de *D. bruxellensis* em mosto industrial poderá evitar problemas operacionais nas usinas de álcool combustível.

1.0 – INTRODUÇÃO

A produção industrial de álcool combustível é um processo aberto que se baseia na fermentação dos açúcares do caldo da cana e/ou melaço por células da levedura *Saccharomyces cerevisiae*. Sendo um processo não asséptico, são poucas as possibilidades de eliminação das leveduras selvagens provenientes dos substratos, ambiente e equipamentos industriais. Desta forma, tem sido verificada freqüentemente a instalação de leveduras selvagens não-*Saccharomyces cerevisiae* que prejudicam o rendimento da fermentação, pois estas não possuem a capacidade fermentativa das linhagens inicialmente inoculadas. Estes episódios de contaminação só são percebidos pela queda do rendimento fermentativo e, *a posteriori*, após a inspeção das placas de meio de cultura semeadas com amostras do mosto de fermentação. Entretanto, os resultados obtidos por essas inspeções só servem para quantificar o nível de contaminação, ocorrido há cerca de cinco ou seis dias. Desta forma, a utilização de técnicas moleculares já existentes pode indicar episódios de contaminação mais precocemente.

A identificação destas leveduras pode ser feita de maneira precisa a partir do uso de técnicas microbiológicas convencionais. Entretanto, estas são muito demoradas e não podem ser utilizadas para tomadas de decisões imediatas no processo. Por isso, técnicas moleculares têm sido cada vez mais usadas para caracterizar e identificar microorganismos isolados de diferentes processos industriais de forma rápida, específica e sensível. Dentre as técnicas moleculares, aquelas baseadas na reação em cadeia da DNA polimerase (PCR) permitem não apenas a identificação molecular a partir do uso de iniciadores espécie-específicos, como também discriminar diferenças genômicas que correspondem a separação das diferentes linhagens genéticas.

Episódios de contaminação microbiana no processo só são percebidos pela queda do rendimento. Este problema na maioria das vezes só pode ser

revertido com a total remoção da população invasora e a re-inoculação da linhagem original no processo fermentativo, resultando em aumentos no custo de produção. A partir disso, buscamos identificar e caracterizar a principal levedura contaminante detectada na produção de álcool combustível, aqui mostrado como sendo a levedura da espécie *Dekkera bruxellensis*. Desta forma, o presente trabalho apresenta como objetivo geral e específicos:

- Identificar e caracterizar a principal levedura contaminante detectada na produção de álcool combustível utilizando marcadores moleculares.

1. Identificar o principal padrão não-*Saccharomyces cerevisiae* amplificado com o iniciador $(GTG)_5$ em amostras de destilarias de álcool combustível.
2. Identificar por métodos moleculares de sequenciamento, análises bioquímicas e de características morfológicas a espécie não-*Saccharomyces cerevisiae* predominante em episódios de contaminação de destilarias de álcool combustível.
3. Identificar as diferentes linhagens genéticas de *Dekkera bruxellensis* provenientes do processo de fermentação alcoólica aplicando diferentes iniciadores de PCR.
4. Levantar, de forma inicial, alguns dados sobre a capacidade fermentativa de diferentes isolados industriais de *D. bruxellensis*.

2.0 – REVISÃO DA LITERATURA

2.1 – Fermentação Alcoólica

Leveduras são fungos eucarióticos unicelulares, de fácil manipulação microbiológica, sendo bastante utilizados como modelos para estudos biológicos. Estes microorganismos são ideais para estudos genéticos da biologia celular eucariótica porque se reproduzem rapidamente e têm um genoma reduzido em comparação com grupos como os de mamíferos. As células de leveduras se reproduzem assexuadamente por fissão, a partir da formação de um septo central no bastonete em crescimento, ou por brotamento, pela formação de um broto que se separa da célula-mãe após a mitose (Alberts *et al.* 1997). A reprodução sexuada ocorre pela formação de esporos no interior de estruturas especializadas, tais como os ascos nos ascomicetos. As leveduras têm sido utilizadas em processos industriais importantes, tais como a produção de pães, bebidas, álcool combustível, ácidos orgânicos, glicerol, dentre outros (Walker, 1998). Nestes processos, as células de levedura transformam os açúcares dos diferentes substratos nos produtos de interesse comercial através do processo de fermentação (Champe and Harvey, 1996). As indústrias de bebidas a base de fermentação alcoólica dependem da habilidade de leveduras como *Saccharomyces cerevisiae* para converter os açúcares dos diferentes substratos (maltose, sacarose, amido) em glicose, e este em dióxido de carbono e etanol. No processo de produção de álcool combustível no Brasil, o caldo de cana cru ou o melaço são fermentados pelas células de *S. cerevisiae* ao longo de toda a safra. Após cada batelada de fermentação, bem como durante o processo de fermentação contínua, as células de levedura são separadas do meio fermentado por centrifugação e reutilizadas no processo, após serem tratadas com ácido sulfúrico para redução da contaminação bacteriana. A alta concentração de açúcar no meio de fermentação,

temperaturas de 33ºC a 35ºC e o ambiente anaeróbico contribuem para a maior eficiência na produção de etanol. O curto tempo de fermentação (6 a 10 h) permite a reutilização das leveduras até três vezes por dia, ao longo de aproximadamente 200 dias de atividade fermentativa (Wheals *et al.* 1999).

Leveduras *S. cerevisiae* dominam o processo de fermentação alcoólica e já existem até linhagens comerciais específicas para esta fermentação (Lallemand Inc., Canadá; AEB Group, Brasil). Entretanto, episódios de contaminação ao longo da safra podem fazer com que espécies não-*Saccharomyces cerevisiae* provenientes do caldo de cana substituam o inóculo inicial. Este problema, embora em menor escala, também ocorre em indústrias que possuem algum tratamento prévio do caldo. As causas deste grande crescimento das leveduras contaminantes na dorna de fermentação ainda não são conhecidas, mas suspeita-se que fatores abióticos como variações de pH, temperatura, osmolaridade, presença de ácidos orgânicos e a concentração de etanol devem contribuir para este processo. Portanto, informações sobre a dinâmica das leveduras do processo são fundamentais para o apropriado controle microbiológico (Granchi *et al.* 1999; Silva-Filho *et al.* 2005a), bem como para a identificação e seleção de novas linhagens com alta capacidade fermentativa (Silva-Filho *et al.* 2005b).

2.2 – Técnicas Moleculares para Tipagem Genética

A identificação convencional de leveduras através das características morfológicas e fisiológicas é trabalhosa e algumas vezes leva a classificação e identificação incorreta, além de consumirem bastante tempo (Chen *et al.* 2000; Granchi *et al.* 1999). A partir disto, tem-se buscado cada vez mais técnicas mais precisas para a identificação de leveduras através da análise do DNA.

A cariotipagem molecular por meio da eletroforese em campo pulsado - Pulsed Field Gel Electrophoresis (PFGE) - é usada para seleção de linhagens

e controle microbiológico devido a sua capacidade de identificar a diversidade cromossômica entre linhagens de modo superior a outras técnicas. Entretanto este é um método laborioso, demorado e caro para ser utilizado como rotina nas destilarias. Além disso, trabalhos do nosso laboratório mostraram que o alto polimorfismo cromossômico evidenciado por esta técnica é devido a rearranjos cromossômicos. Os resultados encontrados por Lucena (2004) mostraram que uma linhagem pode apresentar polimorfismo cromossômico tanto no número quanto em relação ao peso molecular dos cromossomos observados ao longo de várias gerações celulares no processo de fermentação contínua, fato este decorrente de processos de recombinação mitótica que ocorre entre cromossomos não-homólogos.

A análise do Polimorfismo de Tamanho de Fragmentos de Restrição do DNA Mitocondrial (RFLP-mtDNA) de leveduras, consiste na extração e digestão com enzimas de restrição do DNA, e permite o acompanhamento da população devido ao polimorfismo de tamanho gerado. Recentemente, Martorell *et al.* (2005) relataram a identificação de três diferentes padrões de restrição do DNA mitocondrial utilizando a endonuclease *Hinf*I em isolados da levedura *Dekkera bruxellensis* provenientes de amostras de vinho de diferentes regiões da Europa.

A técnica da Reação em Cadeia da Polimerase - *Polymerase Chain Reaction* (PCR) - apresenta-se como uma ferramenta muito útil tanto na identificação de espécies de leveduras como na discriminação de diferentes linhagens de uma espécie. Tem sido utilizada em áreas de segurança biológica como microbiologia médica, controle higiênico em hospitais e controle de qualidade microbiológica em indústrias produtoras de alimentos (Scheu *et al.* 1998) ou indústrias transformadoras, como as destilarias de álcool combustível (Silva-Filho *et al.* 2005a; Silva-Filho *et al.* 2005b). Esta técnica apresenta variações baseadas na possibilidade de uso de diferentes iniciadores da replicação *in vitro* e que dependem também da região do genoma do organismo alvo a ser amplificada.

A técnica de RAPD é uma variação da PCR que consiste em amplificar seqüências do genoma através de iniciadores curtos com seqüências arbitrárias que, em baixa temperatura de pareamento, hibridizam em regiões distribuídas aleatoriamente no genoma. Esta técnica tem sido utilizada com algum sucesso para discriminar linhagens industriais de leveduras (Gomes *et al*. 2002). Entretanto, problemas de reprodutibilidade podem comprometer seu uso rotineiro. A utilização de iniciadores de seqüências mais longas e de reações com temperaturas de pareamento mais elevadas podem minimizar este problema. Algumas técnicas como o RFLP/mtDNA, apesar de identificarem polimorfismos genéticos, não são capazes de uma grande distinção. A associação da técnica de RAPD e RFLP/mtDNA permitiu descriminar subgrupos dentro dos grupos de perfil gerado somente com o DNA mitocondrial em linhagens industriais de *D. bruxellensis* (Martorell *et al.* 2005).

A técnica da PCR pode ser utilizada para identificação do polimorfismo de comprimento das regiões conservadas dos agrupamentos dos genes de RNA ribossomal (rDNA) para tipagem das leveduras. A mais recente re-classificação de leveduras da classe Ascomycetes foi baseada na análise da seqüência de nucleotídeos do gene 26S rDNA (Kurtzman and Robnett, 1998). Entretanto, estas análises apresentaram algumas dificuldades em identificar as diferentes espécies desta classe. As regiões do DNA que codificam para RNA ribossômico apresentam-se como um agrupamento gênico que contém o gene 18S, o gene 5.8S e o gene 28S. Entre estes genes encontram-se as regiões ITS1 e ITS2, seqüências internas transcritas mais não-traduzidas, que apresentam variação genética tanto de comprimento quanto de seqüências de nucleotídeos. O fato das regiões ITS serem flanqueadas por segmentos conservados (genes de rDNA 18S e 28S) e de aparecerem em grande número de cópias no genoma permite que sejam amplificadas com facilidade (Fungaro, 2000). A análise do comprimento aparente do produto da PCR das regiões ITS1 e ITS2 tem permitido identificar diferentes tipos de

leveduras. O tamanho do produto de amplificação que corresponde ao *locus* ITS1-5.8S-ITS2 pode ser utilizado para identificação rápida de leveduras. Entretanto, várias espécies apresentam produtos de amplificação com o mesmo tamanho. Este problema pode ser resolvido a partir da digestão desses fragmentos com diferentes enzimas de restrição, principalmente com as enzimas *CfoI, DdeI, HaeIII* e *HinfI*. A soma dos padrões de restrição parece ser espécie-específico e pode ser utilizado com segurança na identificação das leveduras (Esteve-Zarzoso *et al.* 1999). A análise do comprimento das regiões ITS1 e ITS2, isoladamente ou em combinação, permitiu discriminar 30 de 40 isolados clínicos da levedura *Saccharomyces cerevisiae* (Chen *et al.* 2001). Recentemente, existe a disposição dos pesquisadores que trabalham em taxonomia e sistemática de leveduras um banco público de dados da coleção espanhola de culturas tipo (CECT) que permite identificação rápida de seus isolados a partir dos resultados de amplificação e digestão do *locus* ITS1-5.8S-ITS2 (www.yeast-id.com).

Vinte espécies do gênero *Saccharomyces* são divididas em três grupos: *Saccharomyces* sensu stricto, *Saccharomyces sensu* lato e *S. Kluyveri.* A região ITS das 13 espécies que compõem o gênero *Saccharomyces* foi amplificada e seqüenciada. Nas duas árvores filogenéticas derivadas da região ITS1 e ITS2 todas as espécies foram distinguidas entre si (Oda *et al.* 1997). O grupo *Saccharomyces sensu stricto* inclui *Saccharomyces cerevisiae, S. paradoxus, S. bayanus*, *S. cariocanus, S. mikatae* e *S. kudriavizevii*. A amplificação da região ITS por PCR produziu uma banda de aproximadamente 850pb para as seis espécies. Entretanto, outras espécies de *Saccharomyces* e espécies não-*Saccharomyces* apresentam bandas de amplificação com diferentes tamanhos as quais podem ser utilizadas como identificadores moleculares (McCullough *et al.* 1998).

O método de tipagem molecular baseado na reação em cadeia de DNA polimerase (PCR) – PCR fingerprinting - utilizando um iniciador que amplifica regiões simples entre duas seqüências de microssatélites tem se mostrado

uma técnica capaz de rotineiramente acompanhar o processo fermentativo e sua população de leveduras. Através do uso do marcador molecular $(GTG)_5$ (Lieckfeldt *et al*. 1993), pelo nosso laboratório, foi possível a discriminação de linhagens de *S. cerevisiae* em processos industriais de produção de álcool combustível. Nos trabalhos de Silva-Filho *et al*. (2005a) e Santos *et al.* (2005) foi demonstrada a sucessão de leveduras ao longo do processo de fermentação que possibilitou a identificação de linhagens de *S. cerevisiae* dominantes no processo. Iniciou-se, assim, a caracterização da dinâmica da população de leveduras ao longo do período de fermentação em diferentes destilarias dos Estados de Pernambuco e Paraíba. Adicionalmente leveduras contaminantes, assim nomeadas por não apresentarem o padrão de amplificação típico da espécie *S. cerevisiae*, foram identificadas e quantificadas por este método.

O trabalho de Basílio *et al*. (2005) mostrou que a técnica de PCR fingerprinting com o primer $(GTG)_5$ foi capaz de diferenciar 29 padrões de bandas distintos para leveduras contaminantes. Estes isolados não-*S. cerevisiae* foram identificados pelo sequenciamento dos domínios D1/D2 da região 26S do rDNA. A levedura contaminante identificada como sendo da espécie *Dekkera bruxellensis* foi o padrão de amplificação não-*S. cerevisiae* mais freqüentemente encontrado no monitoramento, estando presente em 52% de todas as amostras com contaminação. A levedura *D. bruxellensis* foi responsável também pela maior parte dos episódios de contaminação severa nas diferentes destilarias estudadas (Basílio *et al.* 2005), bem como em destilarias da América do Norte (Abbott *et al*. 2005). Esta espécie, cujo anamorfo corresponde à espécie *Brettanomyces bruxellensis*, apresenta posição de destaque nos problemas de qualidade do vinho e de contaminações dos processos fermentativos em geral (Mitrakul *et al.* 1999). Análises fisiológicas de isolados desta espécie mostraram que esta apresenta uma reduzida capacidade fermentativa em relação à levedura do processo *S. cerevisiae*. Estas características fazem com que destilarias contaminadas

com esta levedura apresentem tanto queda no rendimento em etanol quanto aumento no tempo de fermentação (Araújo *et al.* 2005).

Vários métodos moleculares de detecção de leveduras contaminantes têm sido desenvolvidos, se valendo principalmente das características de facilidade de execução e da rapidez e confiabilidade dos resultados. Reações de PCR utilizando os primers específicos DB90F DB394R foram desenvolvidas para a identificação de *Brettanomyces/Dekkera bruxellensis* e *Brettanomyces/Dekkera anomalus* em diversas amostras (Cocolin *et al.* 2003) ao mesmo tempo que o uso da PCR em tempo real foi proposto para detectar esta levedura em vinho (Phister and Mills, 2003). Detecções inespecíficas, mas indicadoras da presença de leveduras contaminantes em amostras industriais, podem se valer da diversidade de comprimento do *locus* ITS1-5.8S-ITS2 do DNA ribossomal das leveduras (Esteve-Zarzoso *et al*. 1999). Neste sentido, um sistema de detecção de leveduras contaminantes em amostras de mosto de fermentação alcoólica foi desenvolvido pelo nosso grupo de pesquisa (de Souza-Liberal *et al.* 2005), o qual pode ser utilizado em conjunto com os primers DB90F/DB394R para detectar e quantificar a presença de *D. bruxelensis* nestas amostras de forma rápida, sensível e precisa.

2.3 – *Dekkera bruxellensis*

Em 1960, van der Walt e van Kerken relataram a formação de esporos em linhagens de leveduras previamente classificadas como leveduras *Brettanomyces*, tidas como não esporulantes, e propuseram um novo gênero para acomodar esta forma teleomorfa. Van der Walt propôs o nome *Dekkera* para o novo gênero. Barnett *et al*. (1990) reconheceu quatro espécies neste gênero: *D. anomala, D. bruxellensis, D. custersiana* e *D. naardenensis*.

A validação do gênero *Dekkera* foi feita a partir das análises de restrição do DNA ribossomal, a qual estabeleceu inequivocamente a

equivalência entre os gêneros *Brettanomyces* (anamorfo) e *Dekkera* (teleomorfo) e suas espécies (Molina *et al*. 1993). Posteriormente a separação das duas espécies *D. bruxellensis* e *D. anomala* foi validada a partir das análises filogenéticas que se utilizaram do seqüenciamento do gene rDNA-18S (Cai *et al.* 1996). Estudos posteriores se valeram destas informações para estabelecer padrões específicos de amplificação baseados nas divergências das seqüências do gene rDNA-18S para identificar as espécies *D. bruxellensis* e *D. anomala* (Cocolin *et al.* 2003).

A classificação atual desta espécie corresponde a: reino Fungi, divisão Ascomycota (Saccaromycota), sub-divisão Ascomyccotina (Saccharomycotina), classe Saccharomycetes, ordem Saccharomycetales, família Saccharomycetacea, gênero *Dekkera*, espécie *D. bruxellensis* (Barnett *et al.* 1990).

Existe na literatura um problema de uniformização da taxonomia relacionada a esta levedura e em muitos artigos pode-se encontrar a denominação composta *Dekkera*/*Brettanomyces* para designar linhagens deste táxon. Por questões de uniformização, utilizaremos neste texto apenas o gênero *Dekkera*, mesmo para referir os trabalhos que citam o gênero *Brettanomyces*.

Atualmente a levedura *Dekkera* está em posição de destaque nos problemas de qualidade do vinho. A detecção precoce e específica destas leveduras é, portanto, necessária (Mitrakul *et al.* 1999). Os produtos metabólicos responsáveis pela contaminação do vinho por *Dekkera* são principalmente os voláteis fenólicos, ácido acético e tetrahidropiridinas, que estão associados a grandes prejuízos econômicos em indústrias de bebidas fermentadas. A descoberta do papel desta levedura no desenvolvimento dos compostos fenólicos prejudiciais ao vinho renovou o interesse por este gênero por parte dos micologistas (Rodrigues *et al.* 2001).

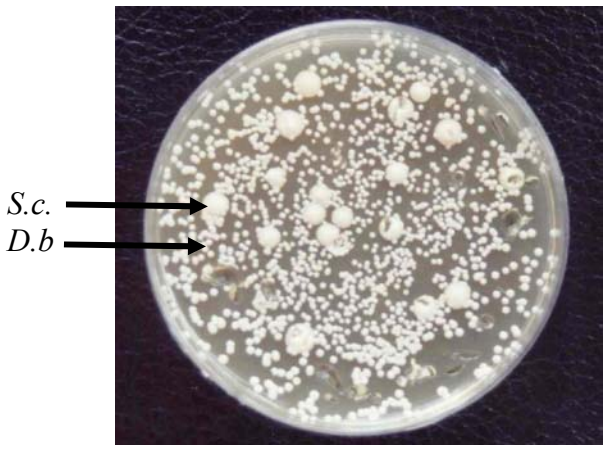

Figura 1. Placa com colônias grandes de *Saccharomyces cerevisiae* (*S.c.*) e colônias pequenas de *Dekkera bruxellensis* (*D.b.*).

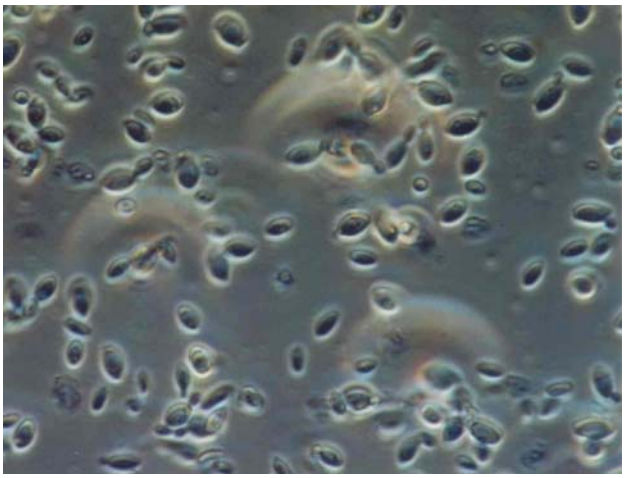

Figura 2. Células da levedura *Dekkera bruxellensis*. 400x.

As leveduras do gênero *Dekkera* foram isoladas e descritas durante a produção de cerveja, no mosto de uva e em vinhos de vários países (Rodrigues *et al.* 2001). Entretanto, pouco se sabe sobre a presença desta levedura em outros nichos ecológicos, que pode ser explicado pela dificuldade de sua recuperação devido ao seu crescimento lento. Isto faz com que, em materiais altamente contaminados com outras leveduras ou fungos filamentosos possam facilmente evitar a detecção de *Dekkera*. Para superar esta dificuldade alguns meios de cultura seletivos têm sido desenvolvidos para manipular o tipo e a concentração do agente antimicrobiano escolhido e

fontes de carbono para suprimir o crescimento de outras espécies de leveduras e bactérias. Em adição, meios bacteriológicos têm sido descritos como essenciais na diferenciação de *Dekkera* de outras leveduras (Rodrigues *et al.* 2001).

Experimentos de determinação de taxas específicas de crescimento mostram que as células de *S. cerevisiae* crescem mais rápido que as de *Dekkera* em todos os meios testados até o momento. Células de *Dekkera* crescem mais rápido que as de *S. cerevisiae* apenas quando as concentrações de ácido acético estão em níveis elevados (>0.45%, w/v), condições não encontradas em processos industriais que não estejam relacionados com a produção deste composto. Recentemente estas leveduras que causam prejuízos devido a sua habilidade em produzir ácido acético e capacidade de estragar vinhos engarrafados, tornaram-se um assunto importante na produção de álcool combustível, particularmente em fermentação contínua. As indústrias de álcool combustível observaram uma queda na produção de etanol diminuindo ou estacionando o processo de fermentação devido ao que se acredita pela presença de leveduras selvagens. Apesar da percepção notória de *Dekkera* na fermentação de álcool combustível pesquisas mostram que estas leveduras não apresentam condições de afetar a fermentação, pois apresentam condições fisiológicas menos adequadas que as leveduras *S. cerevisiae* para suportar o processo fermentativo (Abbott *et al.* 2005).

Problemas operacionais causados por leveduras podem conduzir a enormes perdas econômicas durante a produção, processamento, conservação e armazenagem de produtos que utilizam a fermentação em alguma etapa de seu processo. Por essa razão as indústrias precisam rapidamente de métodos simples e confiáveis para detectar se leveduras estão causando estragos e rapidamente identificar possíveis rotas de contaminação (Hierro *et al.* 2004). Identificações de leveduras *Dekkera* utilizando testes fisiológicos são obtidas em 4 semanas e 3 semanas para

testes de assimilação. E mesmo utilizando linhagens tipo de coleção para avaliação os resultados foram ambíguos (Mitrakul *et al.* 1999). Apesar de linhagens de leveduras selecionadas do vinho terem sido estudadas por décadas, sua classificação inequívoca tem sido possível somente recentemente com o advento de técnicas moleculares (Pramateftaki *et al.* 2000).

A levedura do gênero *Dekkera* tem sido apontada como principal contaminante nos processos de fermentação industrial, tanto para vinhos (Cocolin *et al.* 2003) como para álcool combustível (Guerra, 1998; Abbott *et al*. 2005). A utilização de técnicas moleculares para a identificação de leveduras permite respostas rápidas necessárias ao processo fermentativo. Tendo em vista a problemática que a *D. bruxellensis* pode causar nos procesos fermentativos foi desenvolvido um iniciador específico para a identificação de *Brettanomyces/Dekkera bruxellensis* e *Brettanomyces/ Dekkera anomalus* (Cocolin *et al.* 2003).

No trabalho de Mitrakul *et al.* (1999) a cariotipagem foi escolhida inicialmente por causa do sucesso dessa técnica em diferenciar linhagens de leveduras *Saccharomyces cerevisiae* no alto polimorfismo cromossômico das linhagens industriais. Entretanto, os resultados para a levedura *Dekkera bruxellensis* não permitiram estabelecer padrões para diferenciar linhagens entre si devido ao alto grau de conservação genética entre todas as linhagens. Melhores resultados foram alcançados recentemente por Martorell *et al.,* (2005) com a combinação das técnicas de RFLP-mtDNA e RAPD para discriminação de isolados de *D. bruxellensis*, com a distinção de 12 agrupamentos genéticos dos 63 isolados provenientes de vinícolas de diferentes partes da Europa.

2.4 – Considerações

Vários artigos podem ser encontrados na literatura sobre a caracterização fisiológica de isolados de *Dekkera* por conta de sua participação em processos de contaminação de vinhos. Mesmo estes trabalhos mais fisiológicos apresentam questões mal resolvidas em termos da taxonomia destes isolados. Alguns artigos tratam de *Dekkera/Brettanomyces bruxellensis*, enquanto que outros trazem problemas taxonômicos ainda maiores como a citação de isolados de *Dekkera* sp. ou de *Brettanomyces* sp. Esta falta de uniformidade prejudica uma análise comparativa entre os trabalhos.

Por outro lado, pouco se tem encontrado sobre informações genéticas acerca desta levedura. Pose-se dizer que o melhor trabalho publicado até o momento corresponde ao de Martorell *et al.* (2005) no uso de marcadores RFLP-mtDNA e RAPD para discriminar isolados de vinho. Os outros trabalhos mais genéticos tratam apenas de métodos de detecção desta levedura por PCR específico ou PCR em tempo real. Neste aspecto, o presente trabalho deve contribuir para a caracterização sistemática desta levedura proveniente do processo de produção de álcool combustível, nicho do qual tem sido isolada mas com pouca confirmação da real identidade deste importante contaminante. Os resultados mostrados no corpo deste trabalho deverão iniciar uma trajetória de estudos que visarão comparar a estrutura genética de isolados de vinho, cerveja e álcool combustível para elucidar questões filogenéticas e identificar os fatores que levam esta espécie a apresentar uma adaptabilidade tão relevante para estes contextos industriais das destilarias de álcool combustível.

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4.0 – ARTIGO

Identificação de *Dekkera bruxellensis* como a principal levedura contaminante do processo de fermentação de álcool combustível

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Identification of the yeast *Dekkera bruxellensis* as major contaminant in continuous fuel ethanol fermentation.

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ABSTRACT

Aims: To identify and characterize the main contaminant yeast species detected in fuel-ethanol production plants in Northeast region of Brazil by using molecular methods. **Methods and Results:** Total DNA from yeast colonies isolated from the fermentation must of industrial alcohol plants was submitted to PCR fingerprinting, D1/D2 28S rDNA sequencing and species-specific PCR analysis. The most frequent non-*S. cerevisiae* isolates were identified as belonging to the species *Dekkera bruxellensis*, and several genetic strains could be discriminated among the isolates. The yeast population dynamics was followed on a daily basis during a whole crop harvesting period in a particular industry, showing the potential of *D. bruxellensis* to grow faster than *S. cerevisiae* in industrial conditions, causing recurrent and severe contamination episodes. **Conclusions:** The results showed that *D. bruxellensis* is one of the most important contaminant yeasts in distilleries producing fuel-ethanol from crude sugar cane juice, specially in continuous fermentation systems. **Significance and Impact of Study:** Severe contamination of the industrial fermentation process by *Dekkera* yeasts has a negative impact on ethanol yield and productivity. Therefore, early detection of *D. bruxellensis* in industrial musts may avoid operational problems in alcohol producing plants.

Key words: bioethanol, contaminant yeast, *Dekkera*, molecular identification, PCR fingerprinting, sugar cane fermentation.

IINTRODUCTION

The most recent yeast classification compendium presents 761 species based on conventional taxonomy and new DNA analysis (Kurtzman and Fell 1998), many of them of human interest by their clinical importance or application in biotechnology. Among them, *Saccharomyces cerevisiae* is the most used yeast species worldwide in terms of industrial fermentative processes, mainly by its application in baking, brewing, winemaking and fuel ethanol manufacture. Its application for the production of fermented and distilled beverages is well known. However, other yeast species have been identified in most of these fermentative processes, either as active contributors for the final product properties or as contaminant yeasts. In food microbiology, contaminant yeasts can be regarded as adventitious yeasts, if they can survive or even grow in foods without affecting their properties, or spoilage yeasts, such as those that spoil foods after processing and packing under good manufacturing practices (Loureiro and Malfeito-Ferreira 2003).

In fermented alcoholic beverages that distinction is even more complex since the fermentation generally occurs in the presence of a mixed yeast population, and probably each one might contribute to a certain point to the final characteristics of the product (Loureiro and Malfeito-Ferreira 2003). Nevertheless, the same yeast can act as a contributor or a spoiler. For example, *Dekkera bruxellensis* (teleomorph of *Brettanomyces bruxellensis*) is important yeast in wine fermentation because it produces 4-ethyl-phenol, an ester that contributes to wine taste and aroma. However, at concentrations above 620 $\mu g.l^{-1}$ this metabolite is considered detrimental, and the species may then be seen as spoilage yeast (Chatonnet et al. 1995). Also in wine production the presence of yeast cells after bottling may be detrimental, and in this case even *S. cerevisiae*

may be considered as spoilage yeast (Loureiro and Malfeito-Ferreira 2003). Therefore, the production stage in which a yeast species is found must be taken into account to define its spoilage or contributing status. During traditional Irish cider fermentation, the indigenous yeasts succession includes phases where *Kloekera/Hanseniaspora*, *Saccharomyces* or *Dekkera/Brettanomyces* normally predominate (Morrissey et al. 2004). During the fermentation of crude sugar cane juice for the production of the Brazilian distilled beverage cachaça, a complex microbiology has been reported (Guerra et al. 2001; Schwan et al. 2001; Gomes et al. 2002). In this process, the production of some metabolites such as aldehydes, esters and higher alcohols by some species may be either beneficial or detrimental for the product.

In fuel-ethanol fermentation, the definition of a microbial contaminant may take into account mainly its effect on ethanol yield and productivity. Industrial ethanol fermentation plants in Brazil use sugar cane juice or molasses as substrate, and in some molasses-using distilleries the intermittent addition of sugar cane juice is a common practice. Sugar cane juice is a complex substrate that enables growth of different yeasts and lactic acid bacteria. Despite the economical importance of bioethanol, only a few reports describe the yeast community associated to sugar cane, which may contain species that could act as industrial contaminants. Sherata (1960) first reported the identification of 14 yeast species in sugar cane and in its juice, belonging to the genera *Candida, Endomyces, Hansenula, Kloeckera, Pichia, Saccharomycodes, Schizosaccharomyces* and *Torulopsis*. Later on, species of *Debaromyces, Rhodotorula* and *Cryptococcus* were also reported for this substrate (de Azeredo et al. 1998). In the last few years, other yeast species belonging to many genera such as *Candida, Hanseniaspora, Kloekera, Kluyveromyces, Schizosaccharomyces* and *Pichia* have been isolated in these substrates (Guerra et al. 2001; Schwan et al. 2001; Gomes et al. 2002; Olasupo et al. 2003).

In a previous report, we showed that non-*S. cerevisiae* isolates can be readily detected by DNA fingerprinting of yeast colonies obtained from highly diluted samples taken in different fuel alcohol distilleries in Northeastern Brazil (Silva-Filho et al. 2005a,b), which indicates that some of these isolates appear at high frequency in the yeast population. In the present work, the most frequent of those non-*S. cerevisiae* yeast isolates was identified and characterized as the major industrial contaminant based on its detrimental effects on the process yield and productivity, which are observed when the contamination exceeds a threshold level. The contaminant species was identified as *Dekkera bruxellensis* and different genetic strains responsible for severe contamination episodes could be discriminated. However, the specific factors favoring the outbreak of this species under the prevailing industrial conditions remain unknown.

MATERIAL AND METHODS

Sampling, cell maintenance and cultivations

Must samples were collected at different crop harvesting periods from six bioethanol producing distilleries of Northeastern Brazil, henceforth designated A to F. After appropriate dilutions, yeast cells were plated on WLN medium containing 0.1% bromocresol green. The plates were incubated at 30^{o}C until the development of yeast colonies, which typically required between two (for *S. cerevisiae*) and six days (for contaminant yeasts). Yeast isolates were maintained in slant tubes containing WLN medium, and cultivated in YPD medium for DNA extraction, or in growth medium (described below) for fermentation assays. The strain MA64 was isolated from one of the distilleries of study and identified as *S. cerevisiae* according to Silva-Filho et al. (2005b).

Biochemical taxonomy tests

Conventional taxonomy tests based on assimilation and fermentation of different carbon sources and on assimilation of nitrogen sources were performed at the Culture Collection of the Department of Mycology, Federal University of Pernambuco, Recife, Brazil, according to Kurtzman and Fell (1998). Yeast sporulation tests were performed by inoculation of YPD-growing cells in SPA medium (1% potassium acetate, 0.25% yeast extract, 0.1% glucose), followed by incubation for at least seven days. Ascospore formation was visualized in optical microscope at 400x.

DNA extraction, PCR analysis and molecular identification

Cells from overnight cultures were withdrawn and submitted to DNA extraction according to Silva-Filho et al. (2005a) as follows. Yeast cells were cultivated in YPD medium for 16 h at 30^{o}C and one ml of cell culture was centrifuged. The cells were suspended in 600 µl lysis buffer (0.2 mol.l^{-1} Tris-HCl; 25 mmol.l^{-1} EDTA; 1% SDS; 25 mmol.l^{-1} NaCl, pH8) and incubated at 65^{o}C for 30 minutes with occasional agitation. The lysate were extracted once with phenol:chloroform (1:1), centrifuged at 13000 rpm for 15 minutes and the aqueous phase was extracted once with chloroform:isoamyl alcohol (24:1), and centrifuged as above. DNA from the aqueous phase was precipitated for 2h at –20^{o}C with two volumes of cold ethanol, washed with 70% ethanol, air-dried and suspended in 100 µl TE buffer (10 mmol.l^{-1} tris-HCl, 1 mmol.l^{-1} EDTA, pH8). DNA concentration was determined by spectrophometry at 260 nm before use as a template for molecular analysis.

PCR-Fingerprinting with the microsatellite primer $(GTG)_5$ and the E1 intron splicing site primer were performed as described by Silva-Filho et al. (2005b) and de Barros-Lopes et al. (1998), respectively. PCR for the amplification of ITS1-5.8S-ITS2 rDNA used primers Its4 (5`-TCCTCCGCTTATTGATATGC-3') and Its5 (5`-GCAAGTAAAAGTCGTAACAA-3`), as

described by de Souza-Liberal et al. (2005). For RFLP-PCR, rDNA amplification products were digested with *Hae*III. The primers DB90F (5'-GAYACTAGAGAGAGRRG GARGGC-3') and DB394R (5'-ACGAGGAACGGGCCGCT-3') were used to perform species-specific PCR to identify *D. anomala/D. bruxellensis* according to Cocolin et al. (2004). The amplification products were digested with *DdeI* (Cocolin et al. 2004). DNA fragments were separated in 1.3% agarose gel at 7.5 $V.cm^{-1}$ for 150 minutes in 0.5x TBE, stained with ethidium bromide and the gel was recorded under UV light in a digital photo documenting apparatus (Vilber-Loumart, France). The lengths of the amplicons were estimated by comparison with standard molecular markers using PhotoCapture software (Vilber-Loumart, France).

Molecular identification was performed according to Kurtzman and Robnett (2003). The 650-bp fragments of the D1/D2 region of 26S rDNA amplified with the primers NL-1 and NL-4 were purified (Wizard PCR purification kit, Promega Co.) and submitted to automatic DNA sequencing in a MegaBACE 1000 device (Amersham Biosciences). Nucleotide sequences of D1/D2 26S rDNA loci of C1-type and C4-type *D. bruxellensis* were deposited at NCBI under the accession number DQ409181 and DQ409182, respectively.

Cluster analysis

The fingerprinting amplification patterns of yeast isolates were submitted to pair-wise comparison by the Unweighted Pair Group Method with Arithmetical Average (UPGMA) using Jaccard's coefficient with the NTSYS software pc2.1. Sequencing data were analyzed in BioEdit software and submitted to BLASTn analysis using NCBI database (www.ncbi.nlm.nih.gov). Further, multiple alignment of yeast sequences recovered from BLASTn was performed by CLUSTALW (www.ibi.co.uk) and used for phylogenetic analysis in MEGA 3.0 software.

Fermentation assays

Yeast cells were cultivated in 15x150 mm culture tubes for 16 hours at 30ºC without agitation in 10 mL growth medium containing ($g.l^{-1}$): sucrose, 20; $(NH_4)_2SO_4$, 7.5; KH_2PO_4, 14.4; $MgSO_4.7H_2O$, 0.5. Two milliliters of sterile trace elements solution and one milliliter of sterile vitamin solution were added per liter of medium at pH 6.5 (Verduyn et al. 1992). The cultures were transferred for 500 mL flasks containing 90 mL growth medium and cultivated for additional 24 h and 30ºC at 125 rpm. At the end of growth, cell density in the cultures were determined at 600 nm and the cells were collected by centrifugation, washed in sterile 0.85% saline and suspended in defined fermentation medium for initial optical density of 120. Defined fermentation medium contained ($g.l^{-1}$) sucrose (100), $(NH_4)_2SO_4$ (5.0), KH_2PO_4 (3.0), $MgSO_4.7H_2O$ (0.5) supplemented with trace elements and vitamins as above. Cell suspensions were transferred to 20 x 180 mm closed tubes with a small off gas tubing, and incubated at 33ºC with low agitation. Fermentation was considered finished as the cultures stopped producing CO_2. Cell growth was evaluated by measuring the optical density at the start and the end of fermentation. Sugar consumption, ethanol and glycerol production were evaluated from the concentrations found in the supernatant after centrifugation at the fermentation end.

Glycerol concentration was measured by using the enzymatic GPO-PAP kit (Laborlab, Brazil). Ethanol concentration was measured in a Varian 3600 gas chromatography device at distillery A. Sucrose was measured by the dinitrosalisilic acid method with the following modification: one milliliter of the fermentation must was mixed with 100 μl of 1 M NaOH and incubated at 100^{o}C for five minutes. The calibration curve was prepared with pure sucrose.

RESULTS

Yeast contamination in alcohol production plants fermenting crude sugar cane juice

In must samples from several distilleries of Northeastern Brazil fermenting crude sugar cane juice, it is common to observe the presence, in high counts, of yeast cells that are morphologically different from the *S. cerevisiae* cells used as the starter culture. In WLN plates, those cells form small, dark-green, smooth-bordered colonies with 1-2 mm diameter which appear only after 5 days of incubation. It contrasts to *S. cerevisiae* colonies appearing after 2 days of incubation, which are pale-green, smooth-bordered, large colonies with 3-4 mm diameter with a dark-green region in the center. At the microscope, different cell morphologies are also promptly recognizable, with *S. cerevisiae* cells being three times larger than the non-*S. cerevisiae* cells. Based on biochemical tests, these isolates were consistently identified as belonging to the species *Dekkera (Brettanomyces) bruxellensis* (data not shown). Molecular identification of such small cells were done (see below). Additionally, sporulation experiments performed with several of such isolates invariably showed ascospore formation, indicating that they belong to the teleomorph *Dekkera* and not to the anamorph *Brettanomyces* species.

Using the readily recognizable morphology of *D. bruxellensis* plate colonies, one of the distilleries surveyed in this study (distillery A) recorded on a daily basis the dynamics of the yeast cell population during the six-month period of fermentation of the 2004-2005 sugar cane harvesting season. It can be observed in Fig. 1a that during the first 15 days only *S. cerevisiae* cells were detected in the process. Thereafter, the sub-population of *D. bruxellensis* started rising to achieve almost 50% of the yeast population. At that point, the entire yeast biomass in the industrial plant was replaced with a pure batch of newly propagated *S. cerevisiae* cells, bringing down *D. bruxellensis* cells count to less than 2% (Fig. 1a). Despite this intervention, the *D. bruxellensis* sub-population increased again and was able to overcome *S. cerevisiae* in about 30 days. That oscillation in the yeast population lasted for the whole crop-harvesting season, with periodic interventions by injecting new batches of pure *S. cerevisiae* to the process. A small but

economically relevant decrease of the industrial ethanol yield was observed when *D. bruxellensis* counts increase (Fig. 1a).

The recurrent rise of *D. bruxellensis* subpopulation could not be related to any particular operational condition. The average values of pH and total acidity concentration were 3.68 (± 0.2) and 2.70 $g.l^{-1}$ (± 0.39), respectively, without noticeable changes in the periods where *D. bruxelensis* counts were high (Fig. 1b). The ethanol content in the fermented must varied from 5.62% to 8.37% (v/v), mainly due to fluctuations in the sugar content of the feeding flow, but no correlation to the yeast population dynamics could be established for lower or higher values (Fig. 1b). The same can be said regarding the temperature or the bacterial cell count in the must (data not shown).

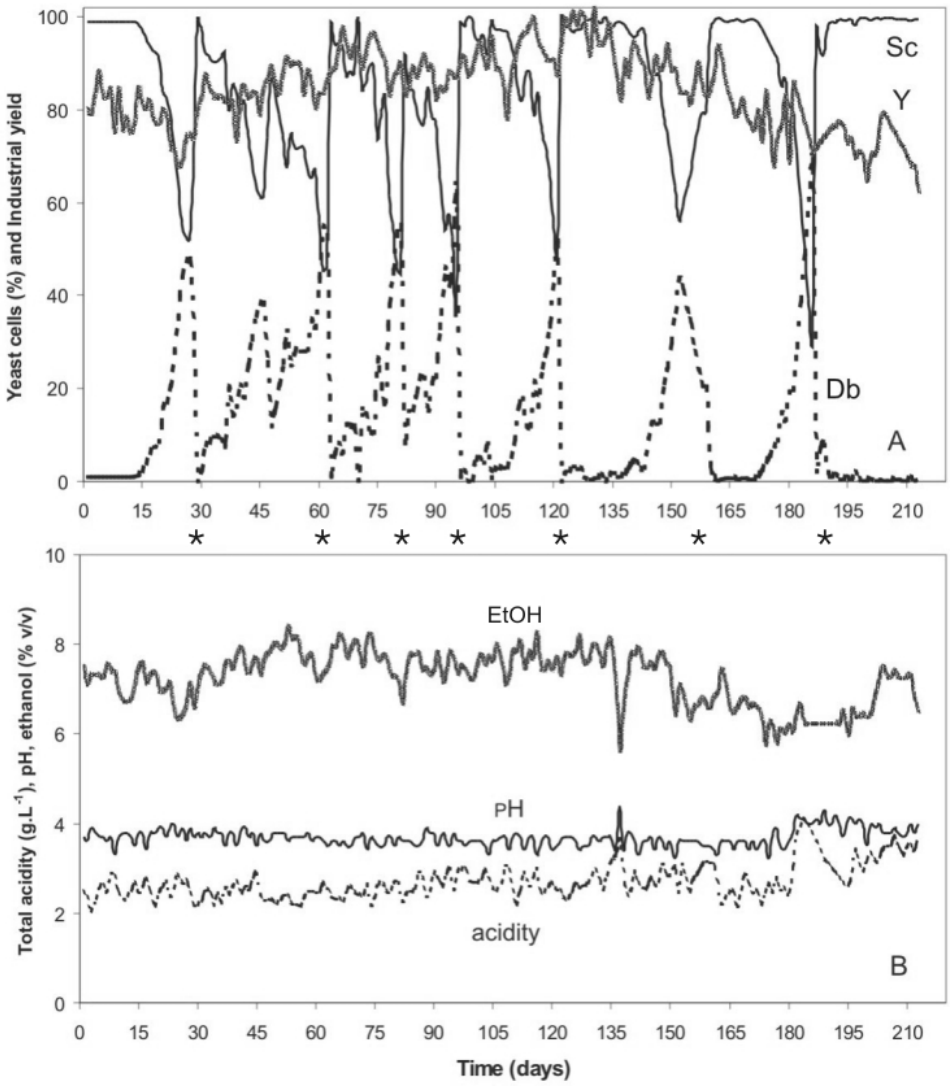

Figure 1. Industrial data of the fermentation process at distillery A in the harvesting period 2004-2005. **A.** The dynamics of the yeast population was followed and represented by *S. cerevisiae* cells (Sc line) and *D. bruxellensis* cells (Db line). Industrial yield is plotted (Y line). **B.** Ethanol concentration (EtOH line), pH variation (pH line) and total acidity concentration (acidity line) in the course of fermentation. The asterisks indicate the period when contaminated population was replaced by pure *S. cerevisiae* batches.

To become predominant in the yeast population, *D. bruxellensis* cells must be grow at a higher rate than *S. cerevisiae* cells in the same conditions. From the population data presented in Fig. 1, it is not possible to quantify the growth rate of each subpopulation alone, but one can estimate the difference between the growth rates of the two subpopulations that is needed to

explain the observed dynamics (see details in the Appendix). As shown in Fig. 2, calculations made over two different time periods of the harvesting season indicate that *D. bruxellensis* specific growth rate was, in average, 0.014 h^{-1} higher than that of *S. cerevisiae* cells in the fermentation conditions prevailing in distillery A.

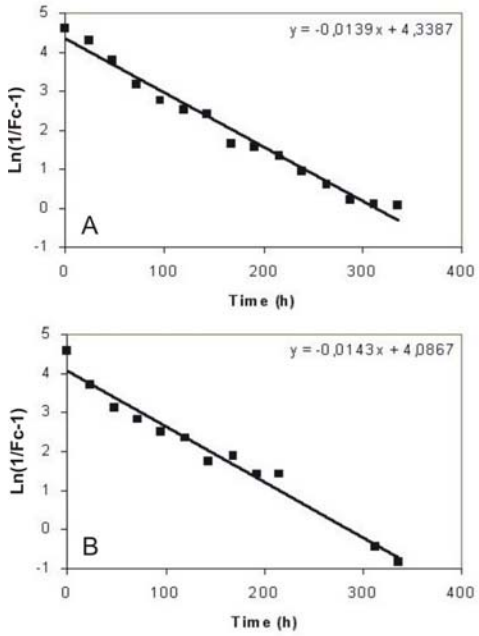

Figure 2. Estimate of the difference between the growth rate of *S. cerevisiae* and *D. bruxellensis* at the fermentative process in distillery A, during the harvesting season 2004-2005. The growth rate gap (μSc – μDb) is given by the slope of the straight lines following the prodedure described in the Appendix. The calculations were made from the estimations using dynamic data from day 15 to 30 (panel A) and from day 175 to 190 (panel B) calculated with the population dynamic data on Figure 1. Those periods represent August 2004 (panel A) and January 2005 (panel B). See details in the appendix for the calculations.

Molecular characterization of industrial *D. bruxellensis* isolates

Different yeast isolates from distillery A were typed by using a microsatellite-based primer (GTG_5), and only two main fingerprinting patterns, named Sc and C1, were obtained (Fig. 3). The pattern Sc (shown in Fig. 3a, lanes 4-5 and 10-11) corresponds to the typical *S. cerevisiae* $(GTG)_5$ fingerprinting pattern described in a previous report (Silva-Filho et al. 2005a). In that

work it was shown that a subset of much conserved bands of the amplification pattern could be used for *S. cerevisiae* species recognition, whereas polymorphic bands can be used for intra-specific discrimination of *S. cerevisiae* strains. The second fingerprinting pattern found in distillery A (pattern C1) corresponds to different *D. bruxellensis* isolates (Fig. 3a, lanes 1-3 and 6-9), which indicates that these two yeast species are readily distinguished by $(GTG)_5$ typing. This C1 pattern has been previously observed (Silva-Filho et al. 2005b). Specific PCR analysis confirmed these results, as described below. First, DNA extracts from the small dark-green colonies produced the same fragment of 510 bp after amplification of the ITS1-5.8S-ITS2 rDNA region, in contrast with *S. cerevisiae* isolates, which yielded an 850 bp fragment for the same DNA *locus* (Fig. 3b). After digestion with *Hae*III, the *S. cerevisiae*-derivative rDNA fragment produced four typical bands of 325, 230, 175 and 125 bp, while the digestion of *D. bruxellensis* rDNA fragments yielded two bands of 380 and 100 bp (data not shown). Second, the use of *Dekkera*-specific PCR primers (Cocolin et al. 2004) produced a 310 bp fragment for the same DNA samples (Fig. 3c), but no amplification was obtained from *S. cerevisiae* isolates (Fig. 1c, lanes 4-5 and 10-12). The specificity of DB primers for *D. bruxellensis* was confirmed since no amplification was observed for other yeast species previously isolated in our laboratory from fuel-ethanol distilleries (Fig. 4). This species-specific PCR results also support the biochemical identification of the C1-type isolates as *D. bruxellensis*.

We further investigated the presence of this contaminant yeast in three other distilleries, two of them fermenting crude sugar cane juice (distilleries B and C) and the other using a blend of cane juice and molasses (distillery D) (Fig. 5). In those analysis, DNA fingerprinting with the microsatellite primer $(GTG)_5$ yielded two fingerprinting patterns for colonies presenting *D. bruxellensis*-like morphology. These patterns were classified as C1-type (Fig. 3 and Fig. 5a) and C4-type (Fig. 5a, lanes 6, 10 and 11), both presenting variations due to polymorphic bands.

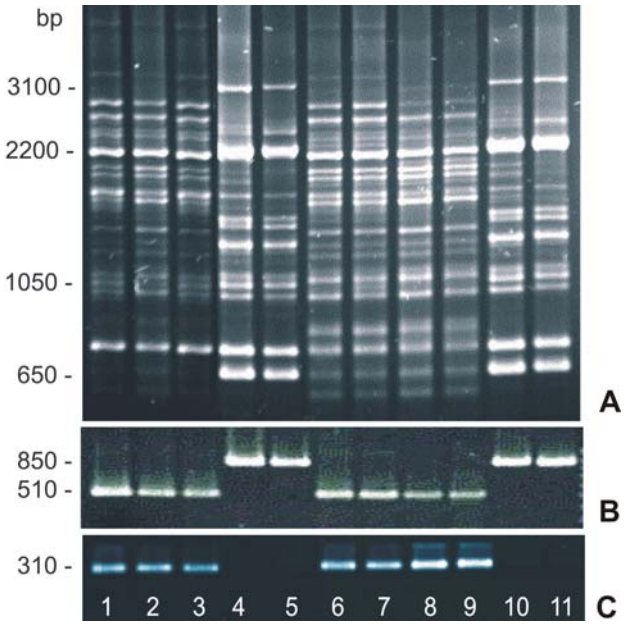

Figure 3. Amplification profile of yeast isolated from the fermentation process at Distillery A. Industrial samples were plated onto WLN plates and the DNA from individual yeast colonies were submitted to amplification with primers $(GTG)_5$ (A), Its4-Its5 (B) and DB90F-DB394R (C).

Figure 4. DNA amplification of yeast isolates belonging to *D. bruxellensis* (lanes 2-6) and a representative of yeast species isolates from the fermentation process and identified as *S. cerevisiae* (lanes 7-8), *Candida tropicalis* (lane 9), *Pichia ohmeri* (lane 10), *Hanseniaspora guilliermondii* (lane 11) and *Zygoascus hellenicus* (lane 12).

A second PCR marker based on the intron splicing site sequence (primer E1) was used as an additional tool for yeast discrimination. Again, two distinct amplification patterns were generated for C1-type isolates and for C4-type isolates (Fig. 5b), both presenting polymorphic bands. However, despite the differences between their fingerprinting patterns, isolates of both C1 and C4-types yielded the same 510 bp ITS1-5.8S-ITS2 fragment (Fig. 5c), as well as the 310 bp *Dekkera*-specific fragment (Fig. 5d). Restriction analysis of the rDNA fragment produced by C1

and C4 isolates also yielded identical profiles (data not shown). In view of these results, C4-type isolates were also temptatively identified as *D. bruxellensis*.

The presence of several polymorphic bands in the fingerprinting patterns of *D. bruxellensis* isolates from different distilleries indicates the prevalence of a high intra-specific genetic variability among these industrial isolates. By clustering those amplification patterns it was possible to limit the C4-type pattern to distillery D (Fig. 6), and is worth noting that no representative of the C4-type was ever isolated in other distilleries we have studied so far (data not shown). However, in the same distillery D, C1-type *D. bruxellensis* isolates were found to coexist with C4-type isolates (Fig. 6). It was also observed that the same C1-type pattern was shared by isolates from different distilleries, while isolates having two different variations of the C1 pattern could be found in the same distillery (Fig. 6). It indicates that, in contrast to the geographical delimitation observed so far for C4-type isolates, there was no specific location to C1-type *D. bruxellensis*. Finally, it is worth noting that we never found C1 or C4 isolates in distilleries E and F that ferment molasses (data not shown).

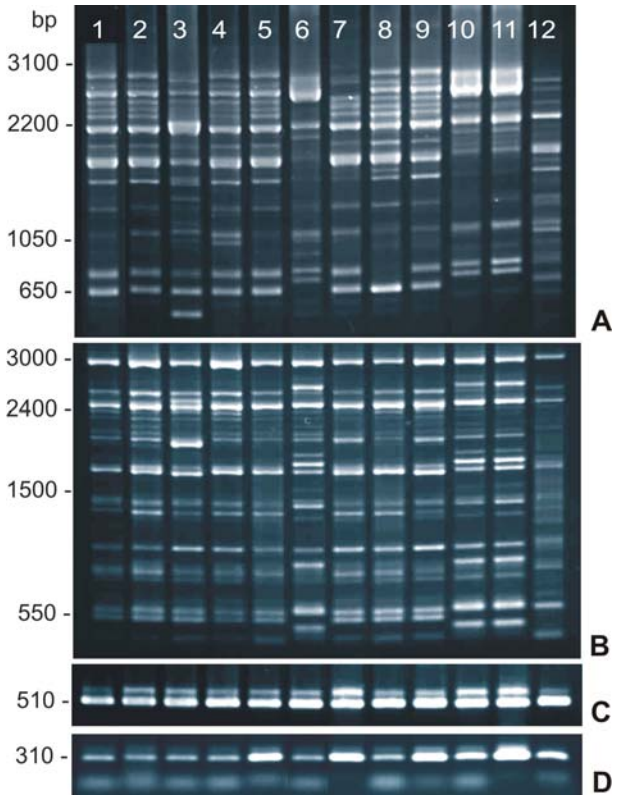

Figure 5. Fingerprinting analysis of different *D. bruxellensis* isolates from different distilleries. DNA samples were amplified with primers $(GTG)_5$ (A), Intron E1 (B), Its4-Its5 (C) and DB90F-DB394R (D).

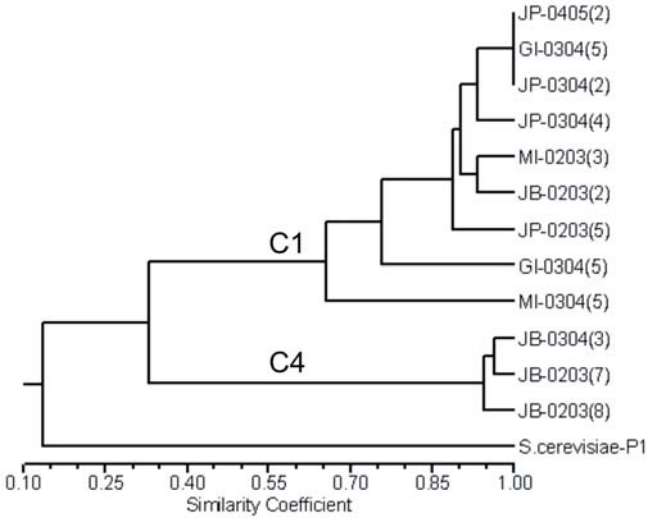

Figure 6. Dendrogram for genetic similarity by using the DNA fingerprinting data of different industrial isolates of *D. bruxellensis*. Distance matrix was done by Jaccard's method and the yeast isolates were clustered by UPGMA tree-making method (MEGA software 2.1). The *S. cerevisiae* P1 strain (Silva-Filho et al 2005a) was used as out-group.

One isolate of each C1 and C4 types were submitted to DNA sequencing analysis of the D1/D2 domain of the 26S rDNA gene. BLAST analysis revealed that both sequences were 99.54% homologous to *D. bruxellensis* sequences at Gene Bank. Significant sequence similarities to other yeast species were also observed, especially to *D. anomala* (96.29% homology, with ten mismatches and six gaps). A phylogenetic analysis of these sequences confirmed the position of C1 and C4 isolates at the *D. bruxellensis* cluster (Fig. 7).

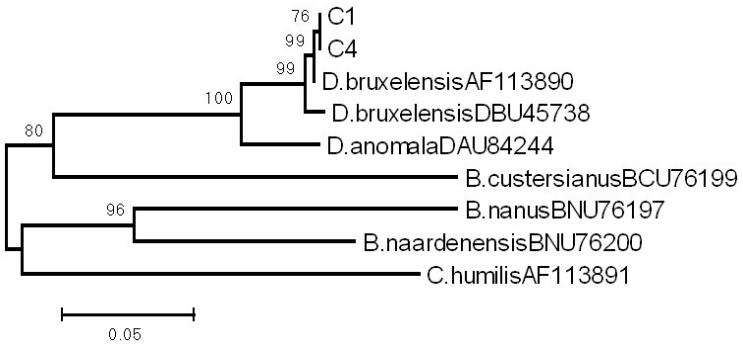

Figure 7. Phylogenetic tree by using the 26S rDNA gene sequences of *Dekkera/Brettanomyces* clade. Nucleotide sequences of C1 and C4 industrial isolates were used for BLAST analysis and the most similar yeast sequences were collected for distance matrix construction using Tamura-Nei (with Gamma distribution) method and clustered in a cladogram by Neighbor-Joining tree-making method (MEGA software 2.1). The consistence of each branch was tested by 1000 bootstrap replicates and the statistics are shown. The scale bar accounts the substitution rate differences between nucleotides.

Physiological characterization of industrial *D. bruxellensis* isolates

Physiological analysis of several *D. bruxellensis* isolates was performed in non-aerated, high cell density batch cultures using a defined fermentation medium. It must be noted that this medium has higher nitrogen content than the natural media actually used in industrial distilleries. These batch experiments also differ from industrial conditions because during the assay the C-source concentration is in average much higher than that prevailing in industrial continuous fermentation processes, where residual sugar concentration is quite low. Table 1 shows that in

laboratory experiments different C1 isolates showed different physiological patterns. Glycerol production varied from 0.22 to 9.31 $g.l^{-1}$ and a huge difference was observed in total sugar consumption during the experiment. However, the ethanol yield of C1 isolates was in average similar to that observed for a *S. cerevisiae* strain isolated from the fermentation process, which is in contrast with the lowering of the ethanol yield perceived in severe *D. bruxellensis* contamination episodes in industrial plants (Fig. 1). In comparison to the *S. cerevisiae* strain, the most significant difference was observed in fermentation time. At similar cell densities, *S. cerevisiae* finished the fermentation in two hours, while C1 isolates took at least four hours to convert sugar to ethanol. Therefore, ethanol productivity of the *S. cerevisiae* industrial strain was at least twice as high as all *D. bruxellensis* isolates tested (Table 1).

DISCUSSION

In Brazil, the fuel alcohol fermentation process is carried out in continuous fermentors on non-sterilized cane juice and/or molasses, and yeast cells are recycled after centrifugation. The bacterial population, composed mainly by lactic acid bacteria, is normally maintained at relatively low counts with the addition of sulfuric acid (and, in some cases, of industrial antibiotics). However, the same procedure is not effective to control the population of non-*S. cerevisiae* yeasts. Occasionally, operational problems of decreasing ethanol yield and slow fermentation are associated to high counts of these contaminant yeasts. In this report, we show that because of its ability to displace *S. cerevisie* cells in the yeast population, *Dekkera bruxellensis* represents one of the most important contaminant yeasts in industrial distilleries fermenting crude sugar cane juice in

continuous process systems in Brazil. *Dekkera/Bretanomyces* has been reported as important contaminant yeast of fermentation processes in USA and Canada (Abbott el et. 2005) and in Europe (de Miniac 1989; Ciani et al. 2003).

Table 1. Physiological characterization of different *D. bruxelensis* strains isolated from the fuel-ethanol process.

Yeast	Glycerol ($g.l^{-1}$)	Residual sugar ($g.l^{-1}$)	Ethanol ($g.L^{-1}$)	$Y_{E/S}$ g ethanol (g.sucrose)$^{-1}$	Fermentation time (h)	Ethanol ($g.l^{-1}.h^{-1}$)
C1-11	0.27 ± 0.09	0.13 ± 0.05	39.15 ± 6.23	0.33	5	7.83
C1-12	0.28 ± 0.09	0.11 ± 0.15	42.26 ± 1.54	0.35	5	8.45
C1-20	0.22 ± 0.04	13.53 ± 3.11	28.07 ± 6.19	0.26	5	5.64
C1-31	7.94 ± 0.79	1.31 ± 1.67	41.56 ± 1.02	0.35	5	8.31
C1-32	9.31 ± 0.84	0.68 ± 0.55	37.80 ± 4.13	0.32	4	9.45
C1-41	0.67 ± 0.22	8.8 ± 8.64	39.45 ± 2.59	0.35	8	4.93
C1-42	0.47 ± 0.03	20.47 ± 20.33	35.25 ± 8.33	0.35	8	4.41
C1-51	0.42 ± 0.11	20.76 ± 8.16	31.64 ± 9.49	0.32	6	5.27
C1-52	0.42 ± 0.04	21.12 ± 6.99	30.39 ± 0.89	0.31	5	6.08
MA64	7.50 ± 0.18	1.66 ± 1.36	38.48 ± 4.71	0.32	2	19.24

The growth rate of *Dekkera*/*Brettanomyces* is usually much lower than that of *S. cerevisiae* when tested in fully supplemented, freshly inoculated natural media in laboratory batch cultures, either in aerobic or anaerobic conditions (Ciani et al. 2003; Abbot et al. 2005). However, the population dynamics data presented above indicates that *D. bruxellensis* can grow faster than *S. cerevisiae* cells in an industrial continuous system, with an average gap between specific growth rates of about 0.014 h^{-1}. The higher fitness shown by *D. bruxellensis* in these conditions could be related either to a nutritional factor (e.g. a higher affinity for a limiting substrate), or to an enhanced tolerance to an environmental stress, such as the accumulation of inhibitory compounds in the industrial must.

It has been suggested that a greater tolerance of *Dekkera/Brettanomyces* to acetic acid in comparison to *S. cerevisiae* could explain outbreaks of the former species in alcohol producing

plants (e. g. de Miniac 1989). Acetic acid is a normal by-product of the alcoholic fermentation, and can also be produced by contaminant microrganisms, including *Dekkera/Brettanomyces* yeasts (Free et al. 2003). In whole corn mash medium, the presence of acetic acid at concentration above 0.45% (w/v) in combination to 0.25% (w/v) lactic acid did inhibit the growth of *S. cerevisiae* and only retarded *Brettanomyces* growth (Abbott et al. 2005). This last compound may be produced by lactic acid bacteria, which are present in counts in the range of 10^6 to 10^7 cell.ml^{-1} (Skinner and Leathers 2004). But detailed inhibition studies carried out in batch cultures on different natural media have shown that the concentration of acetic acid required to favor the growth of *Dekkera/Brettanomyces* yeasts over *S. cerevisiae* is well above those usually found in industrial musts (Phowchinda et al. 1995; Abbott et al. 2005). Moreover, the industrial outbreaks of *Dekkera* yeasts reported in this work were not correlated to an increase of the medium acidity (Fig. 1b), suggesting that the accumulation of acetic acid was not the primary cause for the slower growth of *S. cerevisiae*. In a continuous fermentation system with cell recycle, very high cell densities are reached and the supply of nutrients for cell growth becomes limited. In such a nutrient-limited environment, competing populations are growing at sub-maximal rates, and competition is determined mainly by the ability to scavenge the growth-limiting factor. If *Dekkera* cells metabolize an available nutrient more efficiently, or if *Dekkera* cells are able to uptake a limiting nutrient with greater affinity than *S. cerevisiae*, its growth rate may well be greater than that of *S. cereviae* in that particular condition. A better understanding of the factors involved in the enhanced fitness shown by *Dekkera* yeasts in continuous fermentation plants is needed for the development of a more effective strategy to prevent outbreak events.

It is worth noting that during this study no *D. bruxellensis* isolates were recovered in samples from distilleries fermenting sugar cane molasses. Molasses is a by-product of sugar production and contains a number of potentially growth inhibiting compounds such as furfural

and SO_2, the later being added during the fabrication process for sugar bleaching. Sulfite is a well-known conservative used to preserve wine and other beverages from spoilage by yeast. As many *Dekkera/Brettanomyces* strains show sensitivity to sulfite (e.g. du Toit et al. 2005), this could explain the absence of *D. bruxellensis* in distilleries fermenting molasses. This observation may prove useful in preventing contamination by this yeast in distilleries fermenting crude sugar cane juice.

An inspection in the literature discussed above indicates that different strains of *D. bruxellensis* were studied, and even not completely identified B*rettanomyces* spp. isolates were used. Strain variation may explain the differences in the physiological response of acetic acid production and sulfite resistance presented in the literature. We have used PCR-fingerprinting analysis that resulted in 10 amplification patterns for *D. bruxellensis* isolates from different distilleries, or from the same distillery at different times. Two main clusters were observed, C1 and C4-type patterns. Despite their molecular identification as *D. bruxellensis*, both types displayed a considerable difference between them. Nevertheless, a number of conserved bands were observed in each type, and some polymorphic bands can be used for discriminating isolates of each C1 and C4 (Figs. 3 and 4). Similarly, wine isolates of *D. bruxellensis* from Californian wineries could be discriminated and separated into two groups by karyotyping and both groups were discriminated for different vintages by extending the analyses of those isolates by RAPD-PCR (Mitrakul et al. 1999). In the present work, not only different isolates were found in the same distillery but a given strain was isolated in different distilleries at the same harvesting period, i.e. the patterns GI-0304(5) and JP-0304(2). Therefore, different *D. bruxellensis* strains can share the same population and might have different contribution to fermentation problems.

In laboratory fermentations *D. bruxellensis* cells presented ethanol yield similar to one industrial *S. cerevisiae* strain, although they showed lower ethanol yield when compared to

commercial strains (Silva-Filho et al. 2005b). Moreover, they presented longer fermentation times and higher residual sugar comparing to *S. cerevisiae*. This is in accordance to previous report, where fermentation time for *Dekkera* spp. was twice higher than that of *S. cerevisiae* (Ciani et al. 2003). Those problems were related by the Distillery A during episodes of severe contamination by *D. bruxellensis* showed in Fig. 1, which implies in operational problems and decrease the overall industrial yield. Therefore, rapid detection of *D. bruxellensis* cells in the process is desirable at the industry. Several reports have proposed the use of real-time PCR approach to detect and quantify this yeast in wine (Phister and Mills 2003). Despite its sensitivity and accuracy, this approach is quite expensive for industrial routine use. We recently presented a method based on rDNA-PCR amplification of industrial samples to detect the presence of non-*S. cerevisiae* (de Souza-Liberal et al. 2005), which still requiring improvements to be considered quantitative method. Nevertheless, this method is now being industrially used in the harvesting period 2005-2006 by using both ITS (Fig. 4c) and the species-specific DB primers (Fig. 4d) for direct amplification of fermentation samples, so that we can rapidly detect the presence of this contaminant yeast. This approach could help the distilleries to shortly avoid severe contaminations and eliminate economical lost as consequence.

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APPENDIX

Estimation of the growth rate gap between *S. cerevisiae* and the contaminant species

Selected sets of the industrial population dynamics data depicted in Fig. 1 were used to estimate the growth rate gap between the two subpopulations. The two cell types were considered to be growing at constant but different growth rates in a continuous fermentation system with cell recycle by centrifugation. In this system, the dynamics of each subpopulation can be written as follows:

$$\frac{d(VX_i)}{dt} = \mu_i(VX_i) - QX_{Vi} \qquad \text{Eq. I}$$

where μ_i is the specific growth rate of the subpopulation i, X_i and X_{Vi} respectively are the cell counts in the fermentation system and in the centrifugation efflux stream, V is the system volume and Q is the volumetric flow rate leaving the system from the centrifuge. For each set of dynamic data, V and Q can be considered to be constant during the period of observation. Considering, in addition, that for each subpopulation the cell count in the efflux stream is proportional to the cell count in the system (i.e. the centrifuge operates with a constant concentration factor $\alpha_i = X_{Vi}/X_i$), Eq. I may be rewritten as follows:

$$\frac{dX_i}{dt} = (\mu_i - \alpha_i q)X_i \qquad \text{Eq. II}$$

where $q = Q/V$. For each subpopulation, the integration of Eq. II over time gives

$$X_i = X_{i0} e^{(\mu_i - \alpha_i q)t} \qquad \text{Eq. IIII}$$

The fraction of contaminant cells in the population (F_C) is then given by the following equation, where the subscripts *S* and *C* refer to the subpopulations of *S. cerevisiae* and the contaminant species:

$$F_C = \frac{X_C}{X_S + X_C} = \frac{X_{C0} e^{(\mu_C - \alpha_C q)t}}{X_{S0} e^{(\mu_S - \alpha_S q)t} + X_{C0} e^{(\mu_C - \alpha_C q)t}} \qquad \text{Eq. IV}$$

Assuming that the concentration factors α_S and α_C of both cell types are equal (i.e. that is no preferential separation during centrifugation), after some rearrangement Eq. IV can be simplified to

$$\ln\left(\frac{1}{F_C} - 1\right) = \ln\frac{X_{S0}}{X_{C0}} + (\mu_S - \mu_C)t \qquad \text{Eq. V}$$

The difference between the growth rates of the two subpopulations can then be obtained from the slope of a straight line by plotting $\ln(1/F_C - 1)$ against time.

ABSTRACT

The aim of thois work was to identify and characterize the main contaminant yeast species detected in fuel-ethanol production plants in Northeast region of Brazil by using molecular methods. Total DNA from yeast colonies isolated from the fermentation must of industrial alcohol plants were submitted to PCR fingerprinting, D1/D2 28S rDNA sequencing and species-specific PCR analysis. The most frequent non-*S. cerevisiae* isolates were identified as belonging to the species *Dekkera bruxellensis*, and several genetic strains could be discriminated among the isolates. The yeast population dynamics was followed on a daily basis during a whole crop harvesting period in a particular industry, showing the potential of *D. bruxellensis* to grow faster than *S. cerevisiae* in industrial conditions, causing recurrent and severe contamination episodes. The results showed that *D. bruxellensis* is one of the most important contaminant yeasts in distilleries producing fuel-ethanol from crude sugar cane juice, specially in continuous fermentation systems. Severe contamination of the industrial fermentation process by *Dekkera* yeasts has a negative impact on ethanol yield and productivity. Therefore, early detection of *D. bruxellensis* in industrial musts may avoid operational problems in alcohol producing plants.

5.0 – CONCLUSÕES

- O principal isolado não-*S. cerevisiae* proveniente da fermentação de álcool combustível em destilarias de Pernambuco e da Paraíba foi indentificado a partir de provas bioquímicas e seqüênciamento de DNA como pertencente a espécie *Dekkera bruxellensis*;
- Os dados industriais mostraram a alta adaptabilidade desta levedura ao processo industrial, contrariamente aos resultados da literatura que mostram que essas leveduras não apresentariam condições de afetar a fermetação por apresentar menor taxa de crescimento específico em relação à *S. cerevisiae*;
- Os padrões de amplificação por DNA-fingerprinting proporcionaram a identificação desta levedura nas várias destilarias estudadas;
- Dois padrões distintos de amplificação foram evidenciados para *Dekkera bruxellensis*, mostrando a grande variabilidade genética nesta espécie;
- Os resultados fisiológicos distintos encontrados para os diferentes isolados concordam com a variabilidade genética mostrada;
- Os marcadores genéticos utilizados servirão para o estabelecimento de procedimentos de análises da população de leveduras visando o controle microbiológico do processo.

6.0 – ANEXOS

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A, Absorbance

approx. or *ca,* approximately

at. wt., atomic weight

bp, base pairs

by vol, by volume (for greater than two component liquids)

cm2, per square centimeter

cpDNA, chloroplast DNA

D, attenuance

(see http://www.chem.qmul.ac.uk/iubmb/newsletter/1996/news3.html

Da (kDa), daltons (kilodaltons)

edn, edition

ed., editor(s)

ergs. sq. mm-1, ergs per square millimeter

IU, International unit

kbp, kilobase pair

Mabs, monoclonal antibodies

MIC, minimal inhibitory concentration

mol 1-1, moles per litre

Mr,molecular mass

nm, nanometre

OD, optical density

OFAGE, orthogonal field alteration gel electrophoresis

ORF or orf, open reading frame

P, probability

PFG, pulsed field gradient

ppm, parts per million

recDNA, recombinant DNA

rev min-1, revolution per minute

SD, standard deviation

SE, standard error

subsp., subspecies

U, enzyme unit

UV, ultraviolet

vs, versus

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w/v, weight per volume

w/w, weight per weight

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