Universidade Federal de Minas Gerais
Instituto de Ciências Biológicas
Departamento de Microbiologia

TESE DE DOUTORADO

Potencial biotecnológico de leveduras fermentadoras de D-xilose isoladas de regiões de Floresta Amazônica Brasileira

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Belo Horizonte

2013

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Tese de Doutorado apresentada ao programa de Pós-Graduação em Microbiologia do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais como requisito parcial para obtenção do grau de Doutor em Microbiologia.

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“Eu acredito muito na sorte. E tenho constatado que, quanto mais duro trabalho, mais sorte eu tenho.”

Thomas Jefferson

Agradecimentos

Durante a realização deste trabalho, fui contemplada com oportunidades incríveis que contribuíram não só para a minha formação como cientista e pesquisadora, mas também para que eu me tornasse um ser humano melhor. Esta trajetória proporcionou o conhecimento e a convivência com pessoas fantásticas, as quais eu não poderia agradecer tão somente por meio de palavras. A todos vocês, o meu muito obrigada! E gostaria, humildemente, de expressar meus agradecimentos àqueles que são muito especiais para mim.

Ao meu orientador (de toda uma vida), Carlos Rosa, com quem venho caminhando há 10 anos pelo mundo da ciência. Carlos, você é o mentor da minha carreira como pesquisadora e eu serei eternamente grata a você por isso! Eu te agradeço pela confiança, ensinamentos, oportunidades, lições de vida e puxões de orelha, por ter estado sempre ao meu lado quando precisei e por ter me transformado numa pessoa apaixonada por leveduras!

Ao meu co-orientador, César Fonseca: obrigada por todo o apoio durante a realização do meu Doutorado Sanduíche e após o mesmo. Agradeço por ter aprendido tanto com você (e continuar a aprender), pela ajuda, confiança, oportunidades e conquistas, e por sempre ter estado ao meu lado. Muito obrigada, pá!

Ao meu co-orientador, Sílvio Silvério da Silva, agradeço pela ajuda, apoio e pela confiança depositada em mim em todas as colaborações que estabelecemos.

À Marie-Françoise, colaboradora desta tese, agradeço pela oportunidade e acolhida em um grupo de pesquisa que sempre foi a principal referência do meu trabalho.

A todos os professores, alunos e funcionários do Departamento de Microbiologia da UFMG, agradeço pela convivência, ajuda, apoio, pelo conhecimento proporcionado e pelas amizades conquistadas!

Aos queridos amigos do Laboratório de Taxonomia, Biodiversidade e Biotecnologia de Fungos da UFMG, eu agradeço pelos anos de boa convivência, lições de vida no âmbito profissional e pessoal e pelas amizades construídas! Gostaria principalmente de agradecer às amigas mais do que especiais que este laboratório colocou em minha vida: Carla Lara, Cris Guamán, Elsiene, Iara, Mari Vieira, Mona, Piló, Polly e Rê. Meninas, sou muito abençoada pelo amor de vocês!

Aos amigos da Escola de Engenharia de Lorena (EEL-USP), muito obrigada pela convivência tão prazerosa. Um agradecimento especial às minhas queridas Kelly e Adriana, por toda ajuda, carinho e alegria!

Aos amigos “tugas” do Laboratório Nacional de Energia e Geologia (é pá, como não vos agradecer?), obrigada pelo maravilhoso convívio! Gostaria de agradecer especialmente à Carla Ferreira e Nuno, pela amizade, companheirismo, ajuda e ensinamentos.

Aos amigos da Universidade de Lund, agradeço por todo o apoio e pela experiência de uma convivência extraordinária! Um agradecimento especial aos meus companheiros de trabalho Anders e Alejandro, pelos conhecimentos adquiridos e resultados conquistados.

Aos amigos de toda uma vida, principalmente às minhas biólogas Lu, Nany, Paty, Rosa e Zuppi, e às amigas de infância Ana, Bia e Nath. Vocês são a prova de que uma pessoa é feliz por poder contar com verdadeiros amigos!

À minha maravilhosa família, meus pais Eduardo e Matilde, meu irmão Dé, minhas vovós Otília e Tereza, meu vovô Tãozinho, e minha tia Nena. Obrigada pelas orações, amor incondicional, por serem meus exemplos de vida, acreditarem em mim e estarem sempre ao meu lado!

Agradeço ao Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq, pela bolsa de estudos do Doutorado no Brasil e auxílio financeiro; à Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES, pela bolsa de estudos do Doutorado Sanduíche modalidade PDEE; e à Fundação de Amparo à Pesquia do Estado de Minas Gerais – FAPEMIG, pelo auxílio financeiro.

E finalmente, o meu agradecimento mais importante de todos: a Deus, eu agradeço por ter me proporcionado uma vida iluminada e contemplada de bênçãos, e principalmente pela força nos momentos de desânimo, solidão e tristeza. Obrigada, Senhor, por tudo!

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Resumo

A atual busca pela produção de combustíveis sustentáveis tem sido conduzida por propósitos ambientais, econômicos, políticos, geográficos e sociais. Neste cenário, o etanol de segunda geração (2G) é considerado o principal combustível renovável, e a conversão eficiente dos açúcares advindos de materiais lignocelulósicos em etanol tornou-se uma prioridade mundial para a produção ambientalmente favorável dessa fonte de energia a custos viáveis. Os objetivos deste trabalho foram isolar, identificar e caracterizar leveduras capazes de fermentar D-xilose, segundo monossacarídeo mais abundante da biomassa vegetal, tendo em vista o emprego desses micro-organismos em processos fermentativos de obtenção do etanol 2G. Duzentas e vinte e quatro leveduras foram isoladas a partir de 40 amostras de madeira em decomposição coletadas em áreas de Floresta Amazônica Brasileira. Trinta e três espécies, 26 das quais já descritas pela literatura e sete novas espécies foram identificadas. Dentre as novas espécies, cinco são atribuídas aos dois principais clados detentores de leveduras fermentadoras de D-xilose: clado *Scheffersomyces*, representado pela espécie *Sc. amazonensis*, e clado *Spathaspora*, representado pelas espécies *Sp. brasiliensis, Sp. roraimanensis, Sp. suhii* e *Sp. xylofermentans*. Além dessas novas espécies, seis linhagens de *Sp. passalidarum* também foram encontradas. A partir de ensaios de fermentação de D-xilose conduzidos em meio complexo e hidrolisado de bagaço de cana-de-açúcar, e da determinação das atividades enzimáticas e utilização de co-fatores das enzimas xilose redutase (XR) e xilitol desidrogenase (XDH), foi possível a caracterização das leveduras estudadas de acordo com o principal metabólito obtido, etanol ou xilitol. As espécies produtoras de xilitol (*Sc. amazonensis*, *Sp. brasiliensis, Sp. roraimanensis, Sp. suhii* e *Sp. xylofermentans*) apresentaram atividades de XR estritamente dependentes de NADPH. *Spathaspora arborariae* e *Sp. passalidarum*, espécies produtoras de etanol, exibiram XR dependente de NAD(P)H, e adicionalmente, em *Sp. passalidarum*, esta enzima exibiu preferência por NADH. A identificação dos genes codificadores de XR (*XYL1*) nas espécies de *Spathaspora* analisadas resultou na constatação de que *Sp. passalidarum* é a única que alberga dois genes, *XYL1.1* e *XYL1.2*, dotados de tal função. Nas demais espécies, somente um gene, *XYL1*, foi encontrado, responsável pela codificação de uma XR (XYL1p) que exibe maior homologia à XR codificada por *XYL1.1* (XYL1.1p) que *XYL1.2* (XYL1.2p). XYL1.2p dispõe de uma atividade enzimática com preferência por NADH, comportamento este capaz de manter o balanço de co-fatores nas etapas iniciais do metabolismo de D-xilose, conferindo à *Sp. passalidarum* uma notável capacidade de produzir etanol durante este processo. Esta enzima apresenta exclusivamente na posição 271 da região promovedora da ligação ao co-fator um resíduo de ácido aspártico em substituição ao resíduo de asparagina, este último encontrado na posição correpondente na maioria das demais sequencias de XR identificadas. Esse atributo está diretamente relacionado à

preferência por NADH mostrada por XYL1.2p. A expressão heteróloga dos genes *XYL1.1* e *XYL1.2* da linhagem tipo e de uma linhagem brasileira de *Sp. passalidarum* permitiu a averiguação do comportamento de cada gene no metabolismo de D-xilose em *S. cerevisiae*. O transformante gerado com o gene *XYL1.2* da linhagem brasileira exibiu uma rápida e elevada conversão de D-xilose a etanol em anaerobiose juntamente com uma baixa produção de xilitol. Os resultados deste trabalho contribuem para a demonstração do potencial de estudos de novas espécies e linhagens de leveduras fermentadoras de D-xilose isoladas de biomas brasileiros no que diz respeito à evolução, taxonomia e aplicações biotecnológicas desses micro-organismos. A construção de uma linhagem de *S. cerevisiae* a partir de um gene inédito e capaz de fermentar D-xilose em anaerobiose com elevado rendimento e produtividade em etanol destaca-se como uma importante ferramenta no aprimoramento da obtenção do etanol de segunda geração via fermentação eficiente de D-xilose.

Abstract

Environmental, economical, political, geographical and social purposes have recently driven the search for the production of sustainable fuels. In this scenario, second generation (2G) ethanol is considered the main renewable fuel, and an efficient conversion of sugars from lignocellulosic materials into ethanol has become a world priority for an environmentally friendly production of this energy source, at feasible costs. The aims of this study were to isolate, identify and characterize yeasts able to ferment D-xylose, the second main monosaccharide in plant biomass, regarding to the employment of these microorganisms in the production of 2G ethanol. A total of 224 yeast strains were isolated from 40 rotting wood samples collected in areas of Brazilian Amazonian Forest. Of the 33 species identified, 26 were previously known and seven were new. Within these new species, five were ascribed to the main xylose-fermenting clades, *Scheffersomyces* clade, represented by *Sc. amazonensis*; and *Spathaspora* clade, represented by *Sp. brasiliensis, Sp. roraimanensis, Sp. suhii* e *Sp. xylofermentans*. Besides these new species, six strains of *Sp. passalidarum* were also found. From D-xylose fermentation assays performed with complex medium and sugarcane bagasse hydrolysate, and the determination of enzymatic activities and co-factors usage of xylose reductase (XR) and xylitol dehydrogenase (XDH), it was possible to characterize the studied yeasts according to achievement of ethanol or xylitol as main metabolic product. Xylitol-producing species (*Sc. amazonensis*, *Sp. brasiliensis, Sp. roraimanensis, Sp. suhii* and *Sp. xylofermentans*) showed XR activities absolutely dependent of NADPH. *Spathaspora arborariae* and *Sp. passalidarum*, ethanol-producing species, presented XR activity using both NADH and NADPH as cofactors, and in *Sp. passalidarum* this is accomplished by a XR with preference for NADH. The identification of XR encoding genes (*XYL1*) in the evaluated *Spathaspora* species revealed that *Sp. passalidarum* is the sole harboring two *XYL1* genes, *XYL1.1* and *XYL1.2*. In the remaining species, a unique gene, *XYL1*, was found, encoding a XR (XYL1.p) showing a higher homology to the XR encoded by *XYL1.1* (XYL1.1p) than by *XYL1.2* (XYL1.2p). XYL1.2p is a NADH-preferred XR, feature able to sustain a co-factor balance in the first steps of the D-xylose metabolism, giving the yeast a remarkably ability to produce ethanol during this process. This enzyme exclusively has an aspartic acid residue in the position 271 of the co-factor binding site instead of an asparagin residue, the latter found in the corresponding position in the majority of the remaining analyzed XRs. This feature is directly related to the preference for NADH showed by XYL1.2p. Heterologous expression of *XYL1.1* and *XYL1.2* from *Sp. passalidarum* type strain and a Brazilian strain allowed the investigation of each gene behavior during the metabolism of D-xylose in *S. cerevisiae*. The transformant generated with *XYL1.2* from the Brazilian strain displayed a fast and high conversion of D-xylose to ethanol under anaerobic conditions together with a low production of xylitol. The results of this work contributes to demonstrate the potential of studying new D-xylose-fermenting

yeast species and strains isolated in Brazilian biomes with regard to evolutionary, taxonomic and biotechnological applications of these micro-organisms. The construction of a *S. cerevisiae* strain with an unprecedented gene and capable of fermenting D-xylose under anaerobic conditions, displaying high ethanol yield and productivity, stands as a relevant contribution in the improvement of second generation ethanol production through efficient D-xylose fermentation.

1. Relevância e Justificativa

A crescente preocupação com o impacto ambiental negativo relacionado ao uso e esgotamento de fontes não renováveis de combustível, tais como os combustíveis fósseis, particularmente a poluição e a emissão de gases do efeito estufa, além da instabilidade do mercado do petróleo têm influenciado a procura por fontes de energia renováveis menos poluentes, como o etanol de segunda geração, e estimulado o interesse em otimizar o processo de fermentação para a obtenção deste produto em larga escala.

O etanol lignocelulósico ou de segunda geração pode ser produzido a partir de produtos ou resíduos agrícolas, além de resíduos agroindustriais e florestais, como palha, bagaço, madeira e capim. A utilização de tecnologias que empregam esses materiais lignocelulósicos apresenta como vantagens o baixo custo e a abundância de matéria-prima, impactos positivos na conservação do ambiente e da biodiversidade, além de diminuir a dependência dos combustíveis fósseis.

A biomassa lignocelulósica é composta de lignina (25%) e polímeros de carboidratos (75% de celulose e hemicelulose) e consiste na maior fonte conhecida renovável de carbono. O processo industrial de produção do etanol lignocelulósico envolve o pré-tratamento da biomassa, seguida da hidrólise, fermentação e destilação. Durante os processos de pré-tratamento e hidrólise, as porções de hemicelulose e celulose são despolimerizadas em açúcares, dentre os quais destacam-se, respectivamente, pentoses (principalmente D-xilose) e hexoses (principalmente glicose). A produção convencional de etanol implica no uso de hexoses ou seus dissacarídeos por *Saccharomyces cerevisiae*. No entanto, essa levedura não é capaz de fermentar pentoses. Sabendo-se que a conversão altamente produtiva da hemicelulose em etanol é um fator decisivo para a economia total do processo, a obtenção e identificação de micro-organismos capazes de converter seletivamente D-xilose a etanol em altas taxas tem sido o foco de extensivas pesquisas nos últimos anos. O estudo desses micro-organismos pode contribuir para um melhor entendimento da aquisição e evolução desta característica entre seus representantes, das etapas do metabolismo de pentoses, e servir como ferramenta para indução de mutação em linhagens naturalmente fermentadoras, ou ainda, para o desenvolvimento de linhagens recombinantes de *S. cerevisiae*.

Nesse contexto, a realização do presente trabalho é baseada no fato de que são necessárias pesquisas para a descoberta, descrição e caracterização de leveduras capazes de fermentar D-xilose, principal pentose encontrada em matérias-primas lignocelulósicas, dotadas de boa produtividade e rendimento na produção do etanol, visando o emprego desses micro-organismos na obtenção economicamente viável do etanol de segunda geração.

2. Introdução

2.1 Fontes de energia: fundamentação e caracterização

No que diz respeito ao desenvolvimento dos setores de comércio e indústria, e, de modo geral, ao bem-estar da população mundial, a energia pode ser reconhecida como um fator chave necessário ao estabelecimento dessas considerações. A demanda por energia e combustíveis para transporte tem aumentado constantemente em nível mundial durante o último século. Como resultado, a energia é considerada a força motriz capaz de impulsionar a competição e o desenvolvimento econômico de nações. Com o intuito de se alcançar o desenvolvimento contínuo e sustentável da economia, torna-se essencial que o suprimento de energia seja adequado e seguro, a preços razoáveis a longo prazo (Pasha e Rao, 2009).

Somente no século passado, o consumo mundial de energia aumentou 17 vezes em relação ao período anterior (Ture et al., 1997). Futuramente, a procura energética é projetada para crescer mais de 50% no ano de 2025, com grande parte deste aumento devido à demanda emergente de várias nações em rápido desenvolvimento, ao crescimento da população mundial e à industrialização dos países. De acordo com Stocker (2008), uma maneira de se garantir o futuro abastecimento energético reside no aumento do uso da energia advinda de fontes renováveis em relação aos combustíveis fósseis. Do ponto de vista do desenvolvimento da energia sustentável, as fontes renováveis estão amplamente disponíveis, o que garante uma maior segurança do fornecimento de energia e reduz a dependência das importações de petróleo. As energias renováveis são menos poluentes, em termos de emissões locais (tais como particulados, enxofre e chumbo) e de gases do efeito estufa (dióxido de carbono e metano) que causam o aquecimento global (Goldemberg, 2004). Dessa maneira, as energias renováveis são mais favoráveis ambientalmente quando comparadas aos combustíveis fósseis. Adicionalmente, esse tipo de energia é visto como uma solução a longo prazo para uma fonte segura e infinita de abastecimento energético. Existem vários tipos de energias renováveis, nomeadamente a energia solar, eólica, geotérmica, hidrelétrica e os bicombustíveis (Tan et al., 2008). Particularmente, dentre os biocombustíveis, o bioetanol é considerado um dos mais importantes e consumidos recursos energético no mundo (Hahn-Hägerdal e al., 2006; Basso et al., 2011).

2.1.1 Bioetanol

O etanol combustível é frequentemente associado ao conceito de energia verde, ou seja, a fontes eficientes de energia que contribuem para a redução das emissões de gases causadores do efeito estufa e outros impactos ambientais (Oliveira et al., 2005). Aproximadamente 9% do etanol são produzidos sinteticamente, e consequentemente os processos de fermentação são responsáveis por 91% da produção global de etanol (Wheeler et al., 1991).

As variadas matérias-primas que podem ser utilizadas na fabricação do etanol por meio da fermentação por micro-organismos são convencionalmente classificadas em três tipos principais: açúcares, amidos e materiais lignocelulósicos. Açúcares (cana-de-açúcar, beterraba, melaço e frutas) podem ser convertidos em etanol diretamente; amidos (milho, mandioca, batata e raízes) e a lignocelulose (madeira, resíduos agrícolas, resíduos da indústria de papel) devem primeiramente ser hidrolisados a açúcares fermentáveis, geralmente pela ação de enzimas e ácidos. Depois que os açúcares simples são formados, os micro-organismos podem fermentá-los a etanol (Lin e Tanaka, 2006). O processo de obtenção de etanol a partir da biomassa vegetal é sustentado em grande parte pelo amido de milho nos Estados Unidos e pela sacarose da cana-de-açúcar no Brasil (Parikka, 2004). Esses países, utilizando a tecnologia de primeira geração (1G) para a produção de bioetanol – culturas agrícolas cujo uso pode ser voltado para a produção de alimentos – são responsáveis por cerca de 90% da produção mundial (Thomsen e Haugaard-Nielsen, 2008). No Brasil, atualmente mais de 325 usinas sucroalcooleiras processam aproximadamente 649 milhões de toneladas de cana-de-açúcar por ano, e quantidades equivalentes de caldo de cana são utilizadas para a produção de açúcar e etanol. Esta indústria vem crescendo rapidamente: são estimadas 86 novas usinas em operação no país até 2015 (Goldemberg, 2008).

Apesar da atual produção de bioetanol por meio do uso de culturas como as de cana-de-açúcar e milho encontrar-se bem estabelecida (Karhumaa et al., 2006), o esperado é que haverá limites para o suprimento dessas matérias-primas num futuro próximo. Uma das barreiras principais no papel do etanol em relação ao consumo de combustíveis é o preço diferencial em relação à gasolina. Na produção de etanol a partir de milho ou cana-de-açúcar, a matéria-prima constitui cerca de 40 a 70% do custo total de produção (Quintero et al., 2008), e uma redução nos valores da aquisição desses materiais poderia levar à diminuição do custo final desse produto (Wyman, 1996; Andersson et al., 2006). Para uma produção em larga escala de etanol combustível, é desejável o uso de substratos mais baratos e mais abundantes (Cardona et al., 2010). Dessa forma, o caminho para se reduzir o custo das matérias-primas é alterar a utilização das culturas tradicionais baseadas em sacarídeos e amidos para o uso de substratos não alimentícios de lignocelulose, matéria-prima necessária à produção do etanol de segunda geração (2G). A lignocelulose é considerada um substrato atrativo para se expandir a produção renovável e sustentável do bioetanol devido à sua disponibilidade em grandes quantidades a baixo custo (Cardona e Sánchez, 2007) e por reduzir a concorrência com áreas destinadas à produção de alimentos. No entanto, a fim de se obter um maior desempenho nas projeções econômicas de produção do etanol 2G a partir da biomassa lignocelulósica, uma série de avanços tecnológicos é ainda necessária (Hamelinck, 2005).

2.2 Biomassa lignocelulósica

Existe uma ampla variedade de potenciais matérias-primas lignocelulósicas que possam ser utilizadas para a produção de etanol. A natureza e a disponibilidade dessas matérias-primas em diferentes regiões do planeta dependem do clima e de outros fatores ambientais, práticas agrícolas e desenvolvimentos tecnológicos (Claassen et al., 1999). De modo geral, os materiais lignocelulósicos podem ser divididos em cinco grupos principais: resíduos de culturas (bagaço de cana e sorgo, palha de milho, trigo, arroz e cevada, casca de arroz, polpas, caules e folhas em geral), resíduos florestais (madeiras), culturas herbáceas (gramíneas), subprodutos industriais e resíduos sólidos municipais (Saha, 2003; Cardona et al., 2010). No Brasil, a agroindústria do milho, cana-de-açúcar, arroz, mandioca, trigo, cítricos, côco e capim ocupa coletivamente uma área de 28 milhões de hectares e gera 597 milhões de toneladas de resíduos por ano. Essa escala de operação agroindustrial clama indiscutivelmente por novas soluções visando à utilização adequada desses recursos valiosos. Como cada tipo de matéria-prima exige o desenvolvimento de uma tecnologia apropriada, a diversidade dos materiais supramencionados pode permitir novas soluções para a produção de químicos, combustíveis e energia uma vez aplicados em processos de sacarificação e fermentação (Ferreira-Leitão et al., 2010).

2.2.1 Composição da biomassa lignocelulósica

Os custos da produção do etanol 2G encontram-se diretamente relacionados à matéria-prima lignocelulósica utilizada. A composição bioquímica da biomassa desempenha um importante papel na rentabilidade desse processo, uma vez que influencia no rendimento do etanol via composição da hemicelulose e dos açúcares da matéria-prima em questão. A maioria dos sistemas de conversão de biomassa em etanol encontrados na literatura é baseada em uma única matéria-prima ou cultura agrícola. No entanto, considerando-se os processos de hidrólise e fermentação, é possível utilizar múltiplos tipos de matérias-primas lignocelulósicas. Essa perspectiva é necessária para que se possa alcançar a desejável produção do etanol 2G em larga escala no futuro (Hamelinck, 2005).

A lignocelulose consiste em um complexo de dois polímeros, celulose (40-55%) e hemicelulose (10-40%) e uma macromolécula tridimensional amorfa, lignina (20-30%) interligados quimicamente via ligações covalentes e não covalentes (Hahn-Hagerdal et al., 1991; Ingram et al., 1999; Zaldivar et al., 2001; Perez et al., 2002; Sun e Cheng, 2002). A celulose, considerada o principal componente da ligocelulose, é um polímero estrutural linear composto exclusivamente por monômeros de D-glicose ligados por ligações glicosídicas β,1-4 (Lynd, 1996), o que a torna o polímero de carboidratos mais abundante do planeta (Guo et al., 2008), e, consequentemente, o material orgânico disponível em maior quantidade no mundo (Chi et al., 2009). Devido às ligações β-1,4, a celulose é altamente cristalina e compacta, e tal conformação estrutural, assim como sua estreita associação à lignina, hemicelulose, amido, proteínas e minerais tornam a celulose

altamente resistente à hidrólise e ao ataque biológico (Aristidou e Penttila, 2000; Zaldivar et al., 2001; Perez et al., 2002; Gray et al., 2006).

A hemicelulose, o segundo grande componente da lignocelulose, e o segundo polissacarídeo natural mais abundante no mundo, é um heteropolímero complexo e altamente ramificado que está presente na parede celular e na lamela média das células vegetais (Collins et al., 2005). O termo hemicelulose abrange uma gama de polissacarídeos não celulósicos, compostos, em variadas proporções, por unidades monossacarídicas, como D-xilose, D-manose, D-glicose, L-arabinose, D-galactose, ácido D-glucurônico e ácido D-galacturônico. Existem classes de hemiceluloses, que são nomeadas de acordo com a unidade do açúcar principal constituinte. Desse modo, as hemiceluloses podem ser formadas por xilana (homopolímero de D-xilose), xiloglucana (heteropolímero de D-xilose e D-glicose), glucomanana (heteropolímero de D-glicose e D-manose), galactoglucomanana (heteropolímero de D-galactose, D-glicose e D-manose) e arabinogalactana (heteropolímero de D-galactose e L-arabinose). Devido à natureza amorfa e ramificada desse polímero, ao contrário da celulose, a hemicelulose é facilmente hidrolisada aos seus monossacarídeos constituintes (Aristidou e Penttila, 2000; Ebringerova, 2000; Zaldivar et al., 2001; Perez et al., 2002; Shallom e Shoham, 2003; Hamelinck, 2005). Vários resíduos agrícolas, como palha de milho, trigo e arroz e bagaço de cana-de-açúcar contêm cerca de 20 a 40% de hemicelulose em sua composição. Nos últimos anos, a bioconversão da hemicelulose tem recebido uma grande atenção por causa de suas aplicações em vários processos agroindustriais, como uma eficiente conversão da fração hemicelulósica a combustíveis e químicos (Saha, 2003).

A lignina constitui 20-30% do peso seco da biomassa; é uma macromolécula aromática que contém resíduos fenólicos tais como álcool trans-p-cumaril, álcool trans-p-coniferil e álcool trans-p-sinapil (Hahn-Hagerdal et al., 1991; Ingram et al., 1999; Zaldivar et al., 2001; Perez et al., 2002). Apesar da fração de lignina não contribuir como fonte de carbono fermentável, ela é relevante como fonte potencial de inibidores microbianos. Além disso, a lignina pode estar covalentemente ligada à hemicelulose via ligações éster, o que confere à estrutura da parede celular vegetal uma maior solidez e resistência (Gray et al., 2006).

2.3 Etapas de produção do etanol de segunda geração

Numerosos estudos para o desenvolvimento da produção em larga escala do etanol lignocelulósico têm sido realizados no mundo. Entretanto, o principal fator limitante é o elevado grau de complexidade inerente ao processamento da matéria-prima, que está de acordo com a natureza e composição da biomassa lignocelulósica. Os materiais lignocelulósicos não contêm monossacarídeos prontamente disponíveis para bioconversão. Alternativamente, contêm polissacarídeos, como a celulose e as hemiceluloses, que uma vez hidrolisados por meio de ácidos ou enzimas a açúcares fermentáveis podem ser convertidos em etanol ou outros produtos de valor

agregado, como por exemplo, o xilitol. No entanto, esse processo de degradação é complicado, não completamente desenvolvido e demanda gastos energéticos (Sánchez e Cardona, 2008).

De uma maneira geral, a produção de etanol a partir de materiais lignocelulósicos inclui cinco etapas principais: o pré-tratamento ou pré-hidrólise da biomassa; a hidrólise da celulose; a destoxificação do hidrolisado; a fermentação de hexoses e/ou pentoses; e a separação, purificação e tratamento de efluentes (Cardona et al., 2010). No entanto, várias configurações de processos de obtenção desse combustível já foram desenvolvidas e têm sido estudadas. No processo designado como hidrólise e fermentação em separado (SHF), a hidrólise da celulose e a fermentação das hexoses liberadas a partir desse polissacarídeo ocorrem separadamente, em unidades diferentes. O organismo mais utilizado na etapa de fermentação desse processo é a levedura *S. cerevisiae*. Neste caso, as pentoses resultantes da hidrólise da hemicelulose não são utilizadas (Sánchez e Cardona, 2008; Gírio et al., 2010). Alternativamente, quando, além da fração de hexoses, a fração de pentoses é também submetida à fermentação após a hidrólise, a configuração é designada hidrólise e co-fermentação em separado (SHCF). Neste processo, fermentações sequenciais são empregadas e ambas realizadas independentemente (co-fermentação). A fermentação de pentoses acoplada a uma unidade independente deve-se à necessidade do emprego de micro-organismos específicos, capazes de fermentar pentoses, comportamento este que não é exibido por linhagens indígenas de *S. cerevisiae*. Visto que a co-fermentação representa uma opção tecnológica para uso de todos os açúcares liberados durante o pré-tratamento da biomassa e da hidrólise da celulose, micro-organismos geneticamente modificados, como linhagens recombinantes de *S. cerevisiae,* capazes de fermentar tanto hexoses quanto pentoses com elevada produção de etanol também podem ser utilizados. Nesta configuração, a fermentação pode ser conduzida em uma única etapa, sem a necessidade de unidades independentes (Cardona et al., 2010; Gírio et al., 2010). O processo denominando de sacarificação e fermentação simultâneas (SSF) implica na realização simultânea da hidrólise da celulose e da fermentação, numa única etapa. Quando essa configuração inclui a co-fermentação das hexoses e pentoses, é chamada de sacarificação e co-fermentação simultâneas (SSCF). Finalmente, quando as enzimas necessárias à quebra das frações oligoméricas e das ligações monoméricas dos polissacarídeos são produzidas também durante o processo, em uma via de produção enzimática sem a necessidade da adição de enzimas exógenas (comerciais ou não), a hidrólise e a fermentação dos açúcares são realizadas em uma única etapa, e o processo é denominado bioprocesso consolidado (CBP) (Gírio et al., 2010).

De acordo com Gírio e colaboradores (2010), a produção de etanol combustível a partir de hidrolisados hemicelulósicos é essencial para o sucesso econômico do etanol lignocelulósico, e deve ser realizada juntamente com a produção a partir da fração celulósica. No entanto, a conversão da hemicelulose é particularmente um desafio devido à variedade dos açúcares usualmente encontrados nessa fração (incluindo D-xilose, L-arabinose, D-galactose, D-glicose e D-

manose) e a necessidade de se utilizar linhagens microbianas aptas a convertê-los a etanol. Geralmente, a D-xilose é o principal açúcar hemicelulósico (Santos et al., 2005; Silva et al., 2007; Rocha et al., 2011), e sua bioconversão é um importante pré-requisito para o uso de materiais lignocelulósicos na obtenção do etanol 2G (Silva et al., 2010).

Durante as etapas de conversão da lignocelulose a etanol, mencionadas a seguir, os micro-organismos estão previstos para contribuir com os principais eventos fundamentais a este processo: produção de enzimas sacarolíticas, hidrólise de polissacarídeos presentes na biomassa, fermentação de hexoses e fermentação de pentoses (Gírio et al., 2010).

2.3.1 Pré-tratamento

O propósito da etapa de pré-tratamento é a remoção da lignina e da hemicelulose, a redução da cristalinidade da celulose e o aumento da porosidade deste material, o que leva à melhora da susceptibilidade enzimática deste polímero na etapa de hidrólise (Sun e Cheng, 2002; Keller et al., 2003). Os resultados do pré-tratamento são o aumento da área da superfície interna das partículas dos substratos constituintes acoplada à solubilização parcial e/ou degradação da hemicelulose, o fracionamento dos três componentes da biomassa e a abertura da estrutura da celulose (Pandey et al., 2000). Como consequências destes resultados, o pré-tratamento é útil no melhoramento da digestibilidade desses materiais, facilitando o acesso da ação de micro-organismos (Doran et al., 1994).

O processo de pré-hidrólise pode ser realizado por métodos físicos, físico-químicos, químicos ou biológicos (Olsson e Hahn-Hagerdal, 1996; Sun e Cheng, 2002), e tem sido investigado para diferentes materiais lignocelulósicos (Cardona e Sánchez, 2007; Sánchez e Cardona, 2008). Esses métodos incluem tratamentos termais, explosão a vapor, explosão de fibras com amônia (AFEX), explosão de CO_2, radiação gama, tratamento com ácidos concentrados ou diluídos (ácido sulfúrico, clorídrico, fosfórico) e álcalis (hidróxido de sódio, amônia), peróxido de hidrogênio, extração por solventes orgânicos (organosolv) e tratamentos biológicos (empregando-se, por exemplo, micro-organismos produtores de enzimas lignocelulolíticas, como fungos causadores da podridão branca) (Olsson e Hahn-Hagerdal, 1996; Pandey et al., 2000; Saha, 2003; Cardona et al., 2010). Cada tecnologia de pré-tratamento apresenta vantagens e desvantagens, e o pré-tratamento ideal possivelmente não existe. Os pré-tratamentos mais apropriados dependem de vários fatores, como o tipo de matéria-prima e sua recalcitrância. O desafio de qualquer estratégia de pré-tratamento da lignocelulose é o fracionamento adequado da hemicelulose, celulose e lignina, juntamente com uma baixa degradação dos açúcares que constituem esses polímeros a fim de se obter elevados rendimentos e taxas de fermentação no processo empregado (Gírio et al., 2010).

2.3.2 Hidrólise

A fração residual de celulose obtida a partir da etapa de pré-tratamento pode ser hidrolisada a glicose, processo também conhecido como sacarificação (Saha, 2003; Cardona et al., 2010). Esta etapa é geralmente conduzida por meio do uso de ácidos ou enzimas. No primeiro caso, ácidos concentrados ou diluídos podem ser utilizados (Olsson e Hahn-Hagerdal, 1996). Em relação aos ácidos concentrados, no estudo de Wright e colaboradores (1985), ao se empregar o ácido clorídrico os processos de pré-hidrólise e hidrólise puderam ser conduzidos em uma única etapa. Em contrapartida, um processo de hidrólise com ácido fraco ou diluído é frequentemente combinado com uma pré-hidrólise com ácidos fracos (Keim e Venkatasubramanian, 1989). Ainda que a utilização de ácidos possa ser um método de escolha para o processo de hidrólise da celulose, este procedimento, no entanto, pode dar origem a uma ampla gama de compostos no hidrolisado resultante o que pode vir a influenciar negativamente as etapas subsequentes da produção de etanol (Olsson e Hahn-Hagerdal, 1996).

Uma alternativa à hidrólise ácida, a hidrólise enzimática é realizada por celulases microbianas e tem mostrado melhores resultados para a etapa de fermentação, uma vez que não são formados componentes derivados da degradação da glicose. Embora a hidrólise enzimática apresente tal vantagem, o processo de conversão da celulose a glicose por este método é lento (Cardona et al., 2010) e, ainda, de alto custo, visto que são utilizadas enzimas comerciais. Entretanto, de acordo com Lynd (1996), a hidrólise enzimática é a abordagem mais promissora para se obter elevados rendimentos em produtos críticos ao sucesso econômico do processo de obtenção do etanol lignocelulósico.

2.3.3 Destoxificação

Dependendo do processo e das condições utilizadas durante o pré-tratamento da matéria-prima lignocelulósica, os açúcares hemicelulósicos podem ser degradados a derivados do furano, como o furfural e o hidroximetilfurfural. Além disso, ácidos alifáticos, como o ácido acético, fórmico e levulínico e compostos fenólicos derivados da lignina são também formados (Palmqvist e Hahn-Hagerdal, 2000b; Cardona et al., 2010; Gírio et al., 2010). A formação dessas substâncias é mais provável quando ácidos e/ou altas temperaturas são utilizados (Cardona et al., 2010). Particularmente, o ácido acético, o furfural e o hidroximetilfurfural são liberados durante a hidrólise ácida (Silva et al., 2005). Esses compostos agem inibindo o metabolismo microbiano, razão pela qual são chamados de inibidores, dificultando a bioconversão dos açúcares nos produtos desejados (Canilha et al., 2010), e consequentemente afetando o desempenho da produção de etanol pelos micro-organismos fermentadores (Cardona et al., 2010).

A presença de substâncias inibidoras muitas vezes impõe a necessidade de purificação dos hidrolisados antes da utilização e/ou adaptação dos micro-organismos ao açúcar a ser utilizado (Winkelhausen e Kuzmanova, 1998). No processo de destoxificação, uma variedade de tratamentos físico-químicos e também tratamentos biológicos pode ser utilizada (Palmqvist e Hahn-Hagerdal, 2000a). Já foram propostas como estratégias a esta etapa, conduzidos tanto de forma individual quanto combinados, os métodos de neutralização, adsorção por resinas de troca iônica, adsorção por carvão ativado e *overliming* com hidróxido de cálcio (Carvalheiro et al., 2005; Canilha et al., 2010).

2.3.4 Fermentação

Existem vários obstáculos tecnológicos para o processo de obtenção do bioetanol a partir da lignocelulose, um dos quais sendo a eficiente fermentação dos açúcares derivados da biomassa a etanol (Slininger et al., 2006). Uma conversão eficiente de todos esses açúcares é um pré-requisito para maximizar a rentabilidade do processo industrial e para melhorar a competitividade da produção deste combustível (Fu et al., 2009), e mesmo pequenos aumentos na utilização da matéria-prima podem melhorar significativamente o processo geral (von Sivers e Zacchi et al., 1996).

Os açúcares provenientes de hidrolisados lignocelulósicos compõem uma mistura de hexoses (principalmente glicose) e pentoses (principalmente D-xilose), de acordo com o substrato analisado (Gray et al., 2006). Dependendo da matéria-prima e das condições de reação, o pré-tratamento dos resíduos de lignocelulose pode recuperar de 80 a 95% das pentoses presentes (Kim et al., 2001; Aguilar et al., 2002). Particularmente, a conversão microbiana de pentoses, que representam 25-40% dos açúcares totais dos materiais lignocelulósicos, tem sido identificada como o principal desafio para a tecnologia do etanol 2G (Fromanger et al., 2010). A levedura *S. cerevisiae* é o micro-organismo mais comumente utilizado em fermentações etanólicas industriais, e pode ser empregada na etapa de fermentação do hidrolisado celulósico, rico em monômeros de glicose, durante a produção de etanol. Em contrapartida, esta levedura não pode ser utilizada para a fermentação de hidrolisados hemicelulósicos devido à presença, nesses hidrolisados, de pentoses, açúcares que não são fermentados por *S. cerevisiae* (Kumar et al., 2009). No entanto, a conversão custo-efetiva, sustentável e economicamente eficiente da biomassa a etanol implica na utilização de linhagens microbianas capazes de fermentar todos os açúcares presentes em hidrolisados lignocelulósicos, com alto rendimento e produtividade (van Maris et al., 2006; Hahn-Hagerdal et al., 2007; Bettiga et al., 2008; Fukuda et al., 2009). Particularmente, visto que a fermentação de D-xilose representa um desafio único a esta etapa, durante as últimas décadas pesquisas tem sido focadas na descoberta e no estudo de micro-organismos fermentadores de D-xilose (Hahn-Hagerdal et al., 2007; Agbogbo et al., 2008).

Os micro-organismos são o fator chave na conversão de açúcares a etanol. Organismos capazes de fermentar pentoses presentes na biomassa lignocelulósica podem ser divididos em dois grupos: micro-organismos naturalmente fermentadores e micro-organismos geneticamente modificados. Os micro-organismos naturalmente fermentadores incluem linhagens das leveduras *Scheffersomyces stipitis*, *Sc. shehatae* e *Pachysolen tannophilus*, dentre outras espécies (Agbogbo et al., 2008). Organismos geneticamente modificados são representados pela levedura *S. cerevisiae*, e as bactérias *Escherichia coli* e *Zymomonas mobilis* (Skoog e Hahn-Hagerdal, 1988). Para esses micro-organismos, são desejadas propriedades necessárias à fermentação de hidrolisados de lignocelulose como: uma eficiente utilização de hexoses e pentoses; taxas de fermentação rápidas; alta produção de etanol; alta tolerância ao etanol e aos inibidores presentes no hidrolisado; fermentação em valores baixos de pH e a altas temperaturas; alta viabilidade e rentabilidade; e utilização de uma ampla gama de substratos (Pasha et al., 2007). No entanto, nenhum micro-organismo capaz de satisfazer a todas essas características foi encontrado ou desenvolvido até o momento (Pasha e Rao, 2009). Dessa forma, o futuro desempenho global da etapa de fermentação do etanol 2G depende da descoberta e obtenção de micro-organismos mais eficientes para emprego no processo fermentativo. Novos micro-organismos podem também permitir a combinação de etapas, como a fermentação de diferentes açúcares, em um único passo (Lynd, 1996).

2.3.4.1 Fermentação de D-xilose

Em hidrolisados de biomassa vegetal, a D-xilose pode estar presente em concentrações que variam de 1 a 22% da composição total dos principais açúcares encontrados em matérias-primas agrícolas. Micro-organismos como leveduras e bactérias são essenciais para a fermentação de xilose (Dien et al., 2003; Jeffries e Jin, 2004). O maior tamanho, parede celular mais espessa, melhor crescimento a baixos pH, requerimento nutricional menos rigoroso, e maior resistência a contaminações fornece às leveduras vantagens em relação às bactérias em processos de fermentação comerciais (Jeffries, 2006). Leveduras produtoras de etanol a partir de D-xilose têm sido isoladas de vários habitats, tais como exudatos de árvores (Nigam et al., 1985), insetos habitantes de madeira (Toivola et al., 1984; Suh et al., 2003; Nguyen et al., 2006; Urbina et al., 2012a), madeira em decomposição (Toivola et al., 1984; Cadete et al., 2009), frutas em decomposição e cascas de árvores (Rao et al., 2008). Essas leveduras naturalmente fermentadoras de xilose incluem principalmente as espécies *Sc. stipitis, Sc. shehatae, Sc. lignosa, Sc. insectosa, P. tannophilus, Candida tenuis* (Agbogbo et al., 2008; Ferreira et al., 2011; Wohlbach et al., 2011), *Spathaspora passalidarum* (Nguyen et al., 2006; Wohlbach et al., 2011; Hou et al., 2012) e *Sp. arborariae* (Cadete et al., 2009; Cunha-Pereira et al., 2011).

A produção anaeróbica de etanol a partir de D-xilose foi primeiramente mostrada em 1951 por Karczewska (Karczewska, 1959), e as primeiras observações acerca da fermentação de D-xilose por leveduras foram realizadas no início de 1980. No tratado taxonômico de leveduras editado por Kreger-van Rij (1984), 64% das espécies listadas eram citadas como capazes de assimilar D-xilose aerobicamente, mas nenhuma foi citada como capaz de fermentar esse açúcar. Em outra sinopse taxonômica (Barnett et al., 1983), entretanto, algumas espécies de leveduras – *Sc. stipitis, Sc. shehatae, P. tannophilus* e *Brettanomyces naardenendis* – são relatadas como capazes de fermentar D-xilose a etanol em diferentes taxas. Existe a necessidade de um maior número de pesquisas na área de leveduras capazes de atuarem no processo de transformação da lignocelulose em bioetanol (Pasha et al., 2007). Melhorias nas taxas de fermentação específicas de D-xilose e concentração final de etanol foram obtidas principalmente pelo isolamento de linhagens fermentadoras de substratos naturais e pela seleção e indução de mutação de linhagens em laboratório (Jeffries, 1985a).

Dentre as leveduras que assimilam D-xilose, um pequeno número é capaz de fermentar esse açúcar a etanol (van Maris et al., 2006). Essa aparente discrepância é intrínseca à via metabólica da D-xilose. Essa via, descrita pela primeira vez em 1955 (Gunsalus et al., 1955), liga o metabolismo da D-xilose à via pentose fosfato por meio da conversão da D-xilose em xilulose 5-fosfato (Figura 1). Este processo pode ser conduzido por meio de duas reações: a oxido-redução, comumente realizada por fungos e leveduras (Bruinenberg et al., 1984), e a isomerização, utilizada por certos fungos e pela maioria das bactérias capazes de assimilar D-xilose (Bhosale et al., 1996; Harhangi et al., 2003). A primeira etapa no metabolismo da D-xilose é o transporte dessa pentose através da membrana celular mediado por transportadores de glicose na ausência de um transportador específico de D-xilose (Jeffries e Jin, 2004). Sistemas de transporte próton-simporte de baixa e alta afinidade por D-xilose operam simultaneamente na levedura *Sc. stipitis*. A glicose compete com a D-xilose pelo transporte mediado pelo sistema de baixa afinidade e inibe o transporte de xilose pelo sistema de alta atividade (Kilian e van Uden, 1988). Posteriormente a esta primeira etapa, na via oxido-redutora a D-xilose internalizada é metabolizada por duas enzimas, xilose redutase, (XR - EC 1.1.1.21) e xilitol desidrogenase (XDH - EC 1.1.1.9). XR reduz a D-xilose a xilitol e XDH oxida o xilitol a D-xilulose. Na via da isomerase, somente uma etapa é necessária, uma vez que a D-xilose é diretamente convertida a D-xilulose por meio da enzima xilose isomerase (XI – EC 5.3.1.5). A D-xilulose formada é fosforilada a D-xilulose 5-fosfato pela xilulocinase (XK - EC 2.7.1.17), e metabolizada por meio da via pentose fosfato em etanol (Granstrom e Leisola, 2002). A conversão de pentoses em xilulose 5-fosfato é um pré-requisito para sua utilização por vias catabólicas centrais (Slininger et al., 1987). A via da pentose fosfato consiste de uma fase oxidativa que converte hexoses fosfato em pentoses fosfato, suprindo o NADPH necessário na via biossintética, e uma fase não-oxidativa, na qual as pentoses fosfato são convertidas em hexoses

fosfato e trioses fosfato (Jeffries, 1983). O gliceraldeído 3-fosfato e a frutose 6-fosfato são produzidos na fase não oxidativa. Ambos podem ser convertidos a piruvato na via Embden-Meyerhof-Parnas. O piruvato pode tanto ser descarboxilado e reduzido a etanol quanto entrar no ciclo do ácido cítrico. A via da pentose fosfato também é responsável pela geração de ribose 5-fosfato, utilizada na síntese de ácidos nucléicos, e histidina e eritrose 4-fosfato, necessários na síntese de aminoácidos aromáticos (Winkelhausen e Kuzmanova, 1998).

As principais oxido-redutases do metabolismo de D-xilose, xilose redutase e xilitol desidrogenase, apresentam diferentes especificidades pelos co-fatores necessários a esta reação: XR é dependente de NADPH ou NADH como co-fator, apresentando, em geral, uma maior preferência por NADPH; XDH é dependente de NAD^+ ou $NADP^+$, com majoritária preferência por NAD^+. Dessa forma, na maioria dos micro-organismos fermentadores de D-xilose até então estudados, a conversão da D-xilose em xilulose implica na produção de um $NADP^+$ e um NADH, sendo que NADPH e NAD^+ precisam ser regenerados a fim de se manter o balanço redox. Para o NADPH isso pode ser obtido pelo desvio de parte da frutose 6-fosfato produzida na etapa oxidativa para a via da pentose fosfato. Em condições aeróbicas, o NADH pode ser reoxidado via cadeia respiratória com o oxigênio molecular. Contudo, sob condições anaeróbicas, outro aceptor de elétron é necessário para reoxidar este co-fator (Bruinenberg et al., 1983). Se nenhum aceptor de elétrons estiver disponível, a célula não pode manter o balanço redox e não será capaz de fermentar D-xilose. A ocorrência da conversão direta da D-xilose a xilulose via isomerase independe da utilização de co-fatores. Entretanto, essa via não é naturalmente relatada em leveduras (Bhosale et al., 1996; Harhangi et al., 2003).

Saccharomyces cerevisiae é o micro-organismo produtor de etanol mais comumente utilizado em fermentações industriais (Bengtsson et al., 2009). Apesar de esta levedura não ser capaz de metabolizar D-xilose, propriedades como a elevada produção de etanol a partir de hexoses e a alta tolerância a inibidores presentes em hidrolisados de biomassa tornam-na o micro-organismo de escolha para a produção industrial de bioetanol (Hahn-Hägerdal et al., 2001). Diversas estratégias para a obtenção de linhagens transformantes de *S. cerevisiae* aptas a converter D-xilose a etanol já foram desenvolvidas. Dentre elas, a expressão heteróloga da via oxido-redutora, utilizando-se genes codificadores de XR e XDH (Kötter et al., 1990; Kostrzynska et al., 1998; Watanabe et al., 2007; Bengtsson et al., 2009; Runquist et al., 2010); a expressão heteróloga de xilose isomerase bacteriana (Walfridsson et al., 1996; Karhumaa et al., 2005) ou fúngica (Kuyper et al., 2003); a superexpressão da xilulocinase e de enzimas da via pentose fosfato (Karhumaa et al., 2005) e a expressão heteróloga de transportadores de D-xilose (Kötter et al., 1990). Ainda que estas técnicas tenham alcançado sucesso na construção de linhagens capazes de fermentar D-xilose e produzir etanol, desafios como uma produção associada de xilitol (Jeppsson et al., 2002) aliada a baixas produtividades em etanol (Runquist et al., 2010) devem ser

superados. O estudo de novas espécies e linhagens de leveduras capazes de fermentar D-xilose, proposta motivadora da realização do presente trabalho, pode contribuir para uma maior compreensão da via metabólica desta pentose e servir como fonte de novos genes e novas estratégias na busca pela produção custo-efetiva e bem sucedida do etanol de segunda geração.

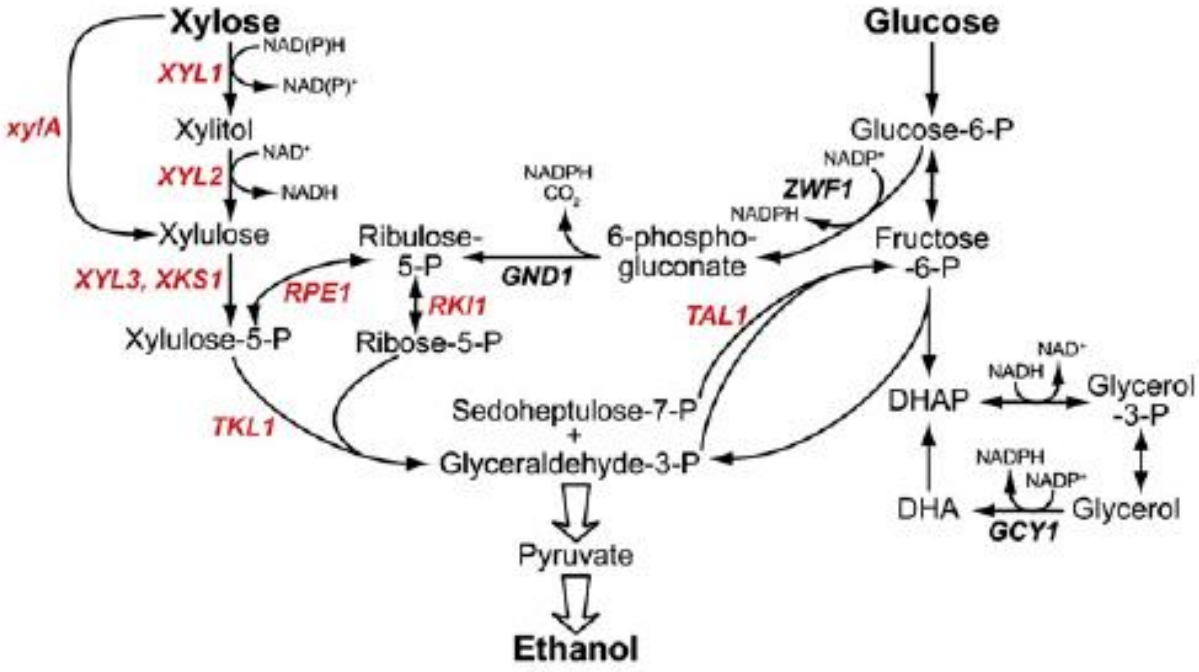

Figura 1. Visão geral do metabolismo de D-xilose e glicose a etanol. A via simplificada inclui genes utilizados para a transformação de *S. cerevisiae* (em destaque). *XYL1* – xilose redutase; *XYL2* – xilitol desidrogenase; *xylA* – xilose isomerase*; XYL3/XKS1* – xilulocinase*; TKL1* – transcetolase; *RPE1* – ribulose 5-fosfato epimerase; *RKI1* – ribose 5-fosfato isomerase; *TAL1* – transaldolase (Wohlbach et al., 2011).

3. Objetivos

3.1 Objetivo Geral

Isolar e identificar leveduras associadas à amostras de madeira em decomposição coletadas na Floresta Amazônica Brasileira, e caracterizar a fermentação de D-xilose pelos isolados encontrados, tendo em vista o emprego desses micro-organismos no processo de fermentação de hidrolisados de biomassa lignocelulósica para a produção do etanol de segunda geração.

3.2 Objetivos Específicos

1. Isolar e identificar leveduras provenientes de amostras de madeira em decomposição coletadas na Floresta Amazônica Brasileira capazes de assimilar D-xilose;

2. Selecionar, dentre os microrganismos isolados, leveduras capazes de fermentar D-xilose;

3. Verificar a fermentação de D-xilose em meio complexo e hidrolisado de bagaço de cana-de-açúcar pelas leveduras fermentadoras e selecionadas;

4. Avaliar a influência de diferentes condições de oxigenação na fermentação de D-xilose em meio complexo pelas leveduras selecionadas;

5. Determinar as atividades enzimáticas de xilose redutase e xilitol desidrogenase e a dependência de co-fatores dessas enzimas pelas leveduras selecionadas submetidas aos ensaios de fermentação de D-xilose em condições limitantes de oxigenação;

6. Identificar os genes responsáveis pela codificação da enzima xilose redutase em *Spathaspora* spp.;

7. Promover a expressão heteróloga dos genes codificadores de xilose redutase identificados em linhagens de *Sp. passalidarum* em uma linhagem laboratorial de *Saccharomyces cerevisiae*;

8. Verificar a fermentação de D-xilose em meio mínimo em condições de anaerobiose pelos transformantes de *S. cerevisiae* produzidos;

9. Determinar as atividades enzimáticas de xilose redutase pelos transformantes de *S. cerevisiae* submetidos aos ensaios de fermentação de D-xilose em condições anaeróbicas.

4. Capítulos

Esta tese foi elaborada com base nos artigos produzidos durante a realização do presente trabalho, e será organizada e referenciada por meio dos seguintes capítulos:

Capítulo I – Isolamento e caracterização de leveduras fermentadoras de D-xilose

Cadete, R.M.; Melo, M.A.; Dussán, K.J.; Rodrigues, R.C.L.B.; Silva, S.S.; Zilli, J.E.; Vital, M.J.S.; Gomes, F.C.O.; Lachance, M.A.; Rosa, C.A. Diversity and physiological characterization of D-xylose-fermenting yeasts isolated from the Brazilian Amazonian Forest. *PLOS One*. v.7, e43135, 2012.

Capítulo II – Descrição de novas espécies de leveduras fermentadoras de D-xilose

Cadete, R.M.; Melo, M.A.; Lopes, M.R.; Pereira, G.M.D.; Zilli, J.E.; Vital, M.J.S.; Gomes, F.C.O.; Lachance, M-A.; Rosa, C.A. *Candida amazonensis* sp. nov., an ascomycetous yeast isolated from rotting wood in the Amazonian forest. *International Journal of Systematic and Evolutionary Microbiology*. v.62, p.1438-1440, 2012.

Cadete, R.M.; Melo, M.A.; Zilli, J.E.; Vital, M.J.S.; Mouro, A.; Prompt, A.H.; Gomes, F.C.O.; Stambuk, B.U.; Lachance, M.A.; Rosa, C.A. *Spathaspora brasiliensis* sp. nov.; *Spathaspora suhii* sp. nov.; *Spathaspora roraimanensis* sp. nov. and *Spathaspora xylofermentans* sp. nov., four novel D-xylose-fermenting yeast species from Brazilian Amazonian forest. *Antonie van Leeuwenhoek*. v.103, p.421-431, 2013.

Capítulo III – Metabolismo de D-xilose em *Spathaspora* spp: fermentação, atividades enzimáticas e identificação dos genes codificadores de xilose redutase

Cadete, R.M.; Sandström, A.; Ferreira, C.; Gorwa-Grauslund, M-F.; Rosa, C.A.; Fonseca, C. Exploring xylose metabolism in *Spathaspora* species: *XYL1.2* from *Spathaspora passalidarum* UFMG-HMD-1.1 as the key for efficient anaerobic xylose fermentation in metabolic engineered *Saccharomyces cerevisiae.* 2013*.* Manuscrito em preparação.

Devido à importância do artigo citado a seguir para a realização deste trabalho, este se encontra disponível para consulta na seção de Anexos desta tese:

Cadete, R.M.; Santos, R.O.; Melo, M.A.; Mouro, A.; Gonçalves, D.L.; Stambuk, B.U.; Gomes, F.C.O.; Lachance, M.A.; Rosa, C.A. *Spathaspora arborariae* sp. nov., a D-xylose-fermenting yeast species isolated from rotting wood in Brazil. *FEMS Yeast Research.* v.9, p.1338-1342, 2009.

No decorrer da realização do presente estudo contribuiu-se também para a produção dos seguintes artigos, apresentados na seção de Anexos:

Barbosa, A.C.; Cadete, R.M.; Gomes, F.C.O.; Lachance, M-A.; Rosa, C.A. *Candida materiae* sp. nov. a yeast species isolated from rotting wood in the Atlantic Rain Forest. *International Journal of Systematic and Evolutionary Microbiology*. v.59, p.2104-2106, 2009.

Santos, R.O.; Cadete, R.M.; Badotti, F.; Mouro, A.; Wallheim, D.; Gomes, F.C.O.; Stambuk, B.; Lachance, M.A.; Rosa, C.A. *Candida queiroziae* sp. nov., a cellobiose-fermenting yeast species isolated from rotting wood in Atlantic Rain Forest. *Antonie van Leeuwenhoek*. v.99, p.635-642, 2011.

Ferreira, A.D.; Mussato, S.I.; Cadete, R.M.; Rosa, C.A.; Silva, S.S. Ethanol production by a new pentose-fermenting yeast strain, *Scheffersomyces stipitis* UFMG-IMH 43.2, isolated from the Brazilian forest. *Yeast*. v.28, p.547-554, 2011.

Morais, C.G.; Cadete, R.M.; Uetanabaro, A.P.P.; Rosa, L.H.; Lachance, M-A.; Rosa, C.A. D-xylose-fermenting and xylanase-producing yeast species from rotting wood of two Atlantic Rainforest habitats in Brazil. *Fungal Genetics and Biology*. 2013. Manuscrito submetido.

4.1 Capítulo I

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PLOS ONE

Diversity and Physiological Characterization of D-Xylose-Fermenting Yeasts Isolated from the Brazilian Amazonian Forest

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Abstract

Background: This study is the first to investigate the Brazilian Amazonian Forest to identify new D-xylose-fermenting yeasts that might potentially be used in the production of ethanol from sugarcane bagasse hemicellulosic hydrolysates.

Methodology/Principal Findings: A total of 224 yeast strains were isolated from rotting wood samples collected in two Amazonian forest reserve sites. These samples were cultured in yeast nitrogen base (YNB)-D-xylose or YNB-xylan media. *Candida tropicalis, Asterotremella humicola, Candida boidinii* and *Debaryomyces hansenii* were the most frequently isolated yeasts. Among D-xylose-fermenting yeasts, six strains of *Spathaspora passalidarum*, two of *Scheffersomyces stipitis*, and representatives of five new species were identified. The new species included *Candida amazonensis* of the *Scheffersomyces* clade and *Spathaspora* sp. 1, *Spathaspora* sp. 2, *Spathaspora* sp. 3, and *Candida* sp. 1 of the *Spathaspora* clade. In fermentation assays using D-xylose (50 g/L) culture medium, *S. passalidarum* strains showed the highest ethanol yields (0.31 g/g to 0.37 g/g) and productivities (0.62 g/L·h to 0.75 g/L·h). *Candida amazonensis* exhibited a virtually complete D-xylose consumption and the highest xylitol yields (0.55 g/g to 0.59 g/g), with concentrations up to 25.2 g/L. The new *Spathaspora* species produced ethanol and/or xylitol in different concentrations as the main fermentation products. In sugarcane bagasse hemicellulosic fermentation assays, *S. stipitis* UFMG-XMD-15.2 generated the highest ethanol yield (0.34 g/g) and productivity (0.2 g/L·h), while the new species *Spathaspora* sp. 1 UFMG-XMD-16.2 and *Spathaspora* sp. 2 UFMG-XMD-23.2 were very good xylitol producers.

Conclusions/Significance: This study demonstrates the promise of using new D-xylose-fermenting yeast strains from the Brazilian Amazonian Forest for ethanol or xylitol production from sugarcane bagasse hemicellulosic hydrolysates.

Citation: Cadete RM, Melo MA, Dussán KJ, Rodrigues RCLB, Silva SS, et al. (2012) Diversity and Physiological Characterization of D-Xylose-Fermenting Yeasts Isolated from the Brazilian Amazonian Forest. PLoS ONE 7(8): e43135. doi:10.1371/journal.pone.0043135

Editor: Eric A. Johnson, University of Wisconsin, Food Research Institute, United States of America

Received March 21, 2012; **Accepted** July 17, 2012; **Published** August 13, 2012

Funding: This work was funded by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq – Brazil, process 560715/2010-2), Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG), Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP, process 2008/57926-4), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Procad-NF, process 2280/2008) and the Natural Science and Engineering Research Council of Canada (M.A.L.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors declare that no competing interests exist.

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Introduction

Growing environmental concerns over the use and depletion of non-renewable fuel sources, together with the rising price of oil and the instability of the oil market, have stimulated interest in optimizing fermentation processes for the large-scale production of alternative fuels such as ethanol [1]. The largest potential feedstock for ethanol is lignocellulosic biomass, which includes materials such as agricultural residues (corn stover, crop straws, sugarcane bagasse), herbaceous crops, short rotation woody crops, forestry residues, waste paper and other plant wastes [2].

Lignocellulosic biomass varies among plant species but generally consists of ~25% lignin and ~75% carbohydrate polymers (cellulose and hemicellulose). It is the largest known renewable carbohydrate source. The cellulosic and hemicellulosic portions of biomass can be separated from the lignin and depolymerized by hydrolysis to obtain their constituent sugars, mainly glucose from cellulose and D-xylose from hemicellulose [3]. As the major sugar in hemicellulose, D-xylose is the second most abundant sugar in lignocellulose [4]. The successful conversion of hemicellulose into fuel ethanol at high yields is the deciding factor for the economic viability of the process [5]. Thus, the efficient use of lignocellulosic biomass as a substrate for ethanol production requires effective utilization of D-xylose [4].

Yeasts that produce ethanol from D-xylose have been isolated from various locations, including tree exudates [6], wood-boring insects [7,8], decaying wood [7,9], rotten fruit and tree bark [10]. Known D-xylose-fermenting yeasts are principally from the

species *Scheffersomyces (Pichia) stipitis, Candida shehatae, C. lignosa, C. insectosa, C. tenuis, Pachysolen tannophilus* [11–13], *Spathaspora passalidarum* [14] and *S. arborariae* [9,15]. Among these naturally D-xylose-fermenting yeasts, *S. stipitis* and *S. passalidarum* are considered the best ethanol producers [11,16]. Despite the existence of these microorganisms, it is still challenging to reach high yields of ethanol from pentose sugars on a large scale [17] because no microorganisms that robustly convert pentose sugars into ethanol at high yields while withstanding fermentation inhibitors have been identified [18].

According to Jeffries and Kurtzman [19], the identification of yeast strains that ferment hemicellulosic sugars will improve prospects for lignocellulosic ethanol production. The strains can be obtained by isolating them from the environment, by mutating and selecting strains in the laboratory [20] or by engineering strains of *Saccharomyces cerevisiae* that can ferment pentoses [21].

The Amazon basin sustains almost 60% of the world's remaining tropical rainforest, with Brazilian Amazonia alone comprising ~30% of the world's current primary tropical rainforests. This environment plays crucial roles in biodiversity conservation, carbon storage, and regional hydrology and climate [22,23]. Even considering all of the research that has been performed on biodiversity in the Amazonia to date, clearly much more research is needed to understand the enormous diversity and complexity of this region. Few studies have characterized the yeast diversity of Brazilian Amazonian environments [24–26]. Works related to yeast diversity in the region have identified a number of potential new species, but only one species, *Candida amapae* (*Saccharomycopsis* clade), from the region was characterized [25]. In this work, we studied yeast diversity in rotting wood collected from two Amazonian sites, focusing on the isolation of new D-xylose-fermenting yeasts that might potentially be used in the production of ethanol from sugarcane bagasse hemicellulosic hydrolysates.

Materials and Methods

Yeast Isolation

Yeasts were isolated from rotting wood samples collected in two sites of Amazonian Forest in the state of Roraima, in northern Brazil. These sites are maintained by Embrapa (Empresa Brasileira de Pesquisa Agropecuária)-Roraima for long-term experiments and are located in the municipalities of Mucajaí (2° 25′ 48′′N and 60° 55′ 11′′W) and São João da Baliza (00° 56′ 58′′N and 59° 54′ 41′′W). The collection sites were the experimental ecological reserve Serra da Prata (Mucajaí), belonging to Embrapa-Roraima, and the ecological reserves belonging to the owners of private landOsvaldo Antônio Sant'ana (Mucajaí) and José Lopes (São João da Baliza). All necessary permits were obtained from Embrapa-Roraima (collection permission obtained by Jerri E. Zilli) and from the owners of the private lands for the described field studies. The predominant vegetation in these sites is characterized as an Amazonian Forest biome. The climate is hot and humid, with an annual precipitation between 1,300 and 2,900 mm, and the average temperature ranges from 25.6 to 27.6°C. The Amazonian Forest comprises a *continuum* of nine main, floristically distinct vegetal formations, and 70% of it is occupied by upland forests that are characterized by their high richness and diversity of tree species [27]. The field collections were made according to the Brazilian diversity rules.

Forty decayed wood samples, 20 samples from each site, were collected in October 2009. Each sample was collected approximately 5 m from the other. The samples were stored in sterile plastic bags and transported under refrigeration to the laboratory within 24 h. One gram of each sample was placed, separately, in 125 mL Erlenmeyer flasks with 20 mL of sterile YNB-D-xylose medium (yeast nitrogen base, 6.7 g/L; D-xylose, 5 g/L; chloramphenicol, 0.2 g/L) or 20 mL sterile YNB-xylan medium (yeast nitrogen base, 6.7 g/L; xylan, 10 g/L; chloramphenicol. 0.2 g/L; pH 5.0±0.2). D-xylose, xylan and YNB solutions were sterilized separately. The flasks were incubated at 25°C on an orbital shaker at 150 rpm for 3–10 days. When growth was detected, 0.5 mL was transferred to a tube containing 5 mL sterile YNB-D-xylose or YNB-xylan media. The tubes were incubated on an orbital shaker as described above. After growth detection, one loopful of each tube was streaked on YNB-D-xylose or YNB-xylan agar media. The plates were incubated at 25°C until yeast colonies developed [9]. The different yeast morphotypes were purified by restreaking on yeast extract–malt extract agar (YMA – glucose, 10 g/L; peptone, 5 g/L; yeast extract, 3 g/L; malt extract, 3 g/L; agar, 20 g/L) and stored at −80°C or in liquid nitrogen for later identification.

Yeast Identification

The yeasts were preliminarily grouped according to various characteristics including their colony morphology and standard tests for growth on different carbon and nitrogen sources [28]. Physiology-based groupings were confirmed by PCR fingerprinting using the Intron Splice Site primer EI-1 (5′CTGGCTTGGTGTATGT) [29]. Yeast strains with identical DNA banding patterns were grouped and putatively considered to belong to the same species [30]. At least one representative strain from each EI-1 PCR group was subjected to sequence analysis of the D1/D2 region and internal transcribed spacer (ITS) domains of the large subunit of the rRNA gene as described below. Physiologically distinct strains with unique EI-1 PCR banding patterns were also selected for direct identification by sequencing of the D1/D2 region and ITS domains.

The D1/D2 and ITS domains were amplified by PCR directly from whole cells as described previously [31]. The amplified DNA was concentrated, cleaned and sequenced in a Mega-BACE™ 1000 automated sequencing system (Amersham Biosciences, USA). Potential new species were also sequenced using an ABI sequencer at the John P. Robarts Research Institute, London, Ontario, in Canada. The sequences were assembled, edited and aligned with the program MEGA5 [32]. Sequences of the new species isolated in this work were deposited in GenBank. The existing sequences for other yeasts were retrieved from GenBank. Phylogenetic placements of new species were based on maximum parsimony analysis of the sequences of the D1/D2 domains of the large subunit of the rRNA gene.

Screening of D-xylose-fermenting Yeasts

The ability to ferment D-xylose was tested in Durham tubes containing a 2% (w/v) solution of the sugar. The tubes were incubated at 25°C on an orbital shaker at 100 rpm for 25 days and observed daily for gas production. *Candida shehatae* CBS 5813, *C. insectosa* CBS 4286, *C. lignosa* CBS 4705 and *S. stipitis* NRRL Y-7124 were used as positive controls for D-xylose fermentation [9].

Yeasts showing the development of gas inside the Durham tubes were tested for fermentation in D-xylose culture medium (YPX: yeast extract-peptone-D-xylose medium) as described below. In addition, the yeast isolates identified by D1/D2 rRNA gene sequencing as belonging to the D-xylose-fermenting clades *Spathaspora* or *Scheffersomyces* were also tested for fermentation in YPX medium. Yeasts with the best D-xylose consumption and highest ethanol yields ($Y_{p/s}^{et}$) were assayed for their ability to ferment sugars in sugarcane bagasse hemicellulosic hydrolysate.

Candida lignosa CBS 4705 and *S. stipitis* NRRL Y-7124 were used as positive controls.

Fermentation Assays

Inoculum preparation. Yeast inocula were prepared on yeast extract–malt extract agar (YMA – glucose, 10 g/L; peptone, 5 g/L; yeast extract, 3 g/L; malt extract, 3 g/L; agar 20 g/L) plates at 30°C for 24–48 h. Cells were cultured in 50 mL YPX liquid medium (yeast extract, 10 g/L; peptone, 20 g/L; D-xylose, 30 g/L) in 125 mL Erlenmeyer flasks at 30°C with continuous shaking (200 rpm) for 24 h at 30°C. D-xylose and yeast extract-peptone solutions were sterilized separately. Cells were recovered by centrifugation at 2,600×*g* for 20 min, washed twice and resuspended in the fermentation media to a final concentration of 0.5 g/L.

Medium composition and cultivation conditions. Batch fermentation experiments were carried out in 50 mL D-xylose culture medium (yeast extract, 10 g/L; peptone, 20 g/L; D-xylose, 50 g/L), pH 6.0, in 125 mL Erlenmeyer flasks incubated as described above for 48 h. Fermentation was monitored by taking samples at 0, 12, 24 and 48 h.

Sugarcane bagasse was supplied by Usina São Francisco (Sertãozinho, SP, Brazil). Hemicellulosic hydrolysate was prepared as described previously [33] in a 250 L stainless steel reactor loaded with sugarcane bagasse and sulfuric acid solution (100 mg acid/g dry matter). The reactor was operated with a solid/liquid ratio of 1:10 at 121°C for 20 min. After hydrolysis, the resulting solid material was removed by filtration. The hemicellulosic hydrolysate was concentrated in a 30 L evaporator at 70±5°C to obtain a xylose concentration of about 70 g/L. To reduce inhibitors, a detoxification assay was performed as described in Alves et al. [34] by first raising the pH to 7.0 with calcium oxide and then decreasing it to pH 5.5 with phosphoric acid, adding active charcoal (2.5% w/v) and incubating at 200 rpm at 30°C for 1 h. The precipitates resulting after each procedure were removed by vacuum filtration. The sugar composition of the hydrolysate before autoclaving was 59 g/L D-xylose, 6.2 g/L glucose and 6.4 g/L L-arabinose. The treated hydrolysate was autoclaved at 111°C for 15 min and supplemented with yeast extract solution (3 g/L). Experiments were carried out in 250 mL flasks with 100 mL supplemented hemicellulosic hydrolysate. The average hydrolysate was at pH 5.1 and composed of 50.2 g/L D-xylose, 5.3 g/L glucose, 5.8 g/L L-arabinose, 3.2 g/L acetic acid, 0.01 g/L furfural, 0.01 g/L hydroxymethylfurfural, and 1.3 g/L total phenols. The flasks were incubated as described above for 96 h. Samples were taken at 0, 12, 24, 48, 72 and 96 h. Samples were stored at −20°C until analysis. All experiments were performed in duplicate.

Analytical Methods

Cell concentrations were determined by correlating optical density (OD) measurements taken with a Beckman DU 640B spectrophotometer at 600 nm with a previously constructed calibration curve (dry weight × optical density). After the cell concentration determined, samples were centrifuged at 2,600×*g* for 15 min, and the supernatant was diluted and filtered using a Sep-Pak C18 (Millipore) filter. Monosaccharides (glucose, D-xylose and L-arabinose), xylitol, glycerol, ethanol, and acetic acid levels were determined by HPLC (Waters 410, Milford, MA, USA) using a Bio-Rad Aminex HPX-87H (300×7.8 mm) column at 45°C with a sample injection volume of 20 µL, a Waters 410 refraction index, a mobile phase of 0.01 N H_2SO_4 and a flow rate of 0.6 mL/min.

Fermentation Parameter Calculation

The fermentation parameters $Y_{p/s}^{et}$ (g/g, ethanol yield), $Y_{p/s}^{xy}$ (g/g, xylitol yield), Qp (g/L·h, ethanol productivity), η (%, fermentation efficiency) and D-xylose and/or glucose consumption (%) were experimentally determined. Ethanol ($Y_{p/s}^{et}$, g/g) and xylitol ($Y_{p/s}^{xy}$, g/g) yields were calculated following the methods of Schmidell et al. [35]), which correlated ΔP produced ($\Delta P_{ethanol}$ or $\Delta P_{xylitol}$) with ΔS consumed (substrate consumed to product obtained, derived by determining the total, initial and consumed substrate). The slope of the line through the origin provided the estimate of $Y_{p/s}^{et}$ and $Y_{p/s}^{xy}$. Ethanol productivity (Qp, g/L·h) was determined by the ratio of ethanol concentration (g/L) to fermentation time (h). Conversion efficiency (η, %) was calculated as a percentage of the maximum theoretical ethanol yield (0.51 g ethanol/g D-xylose and/or glucose). D-xylose and/or glucose consumption (%) was determined as a percentage of the initial sugar concentration.

Results and Discussion

Yeast Isolation and Diversity

In this work, we studied the diversity of rotting-wood-associated yeasts from two Amazonian sites. A total of 224 yeast strains were isolated from rotting wood samples from the forest reserve sites of São João da Baliza (114 yeast strains) and Mucajaí (110 yeast strains). Of these strains, 118 were obtained following growth in YNB-D-xylose medium, and 106 were obtained from growth in YNB-xylan medium.

Table 1 shows the results of yeast species identification, the occurrence of each species by isolation site, the number of samples cultured in both media (YNB-D-xylose and xylan) and the results of the Durham tube fermentation tests. Of the 33 yeast species identified, 26 species were previously known and seven were new (Table S1). Eleven species were isolated from both isolation sites, whereas 22 species were observed in only one site. Sixteen of the 22 species were observed in the São João da Baliza forest reserve and the remaining six were observed in the Mucajaí forest reserve. Species in the genus *Candida* (namely 16 species related to the *Candida glaebosa*, *Kurtzmaniella*, *Lindnera*, *Lodderomyces/Spathaspora*, *Metschnikowia*, *Ogataea*, *Wickerhamomyces* and *Yamadazyma* clades were the most prevalent, followed by the genus *Spathaspora*, with four species. *Candida tropicalis* (*Lodderomyces/Spathaspora* clade) was the most frequently isolated yeast, occurring in 15 samples cultured on YNB-D-xylose medium and 13 samples in YNB-xylan medium, followed by *Asterotremella humicola* (eight samples on YNB-D-xylose medium and 10 in YNB-xylan medium) and *Candida boidinii* (*Ogataea* clade; 10 samples on YNB-D-xylose medium and seven samples on YNB-xylan medium). Strains of *C. tropicalis* have been reported in fruit, flowers, soil, water, and clinical specimens [36], and this species has already been shown to produce ethanol and, mainly, xylitol from D-xylose [37–40]. Strains belonging to the genus *Asterotremella,* including *A. humicola*, have been isolated from soil, plants and mushrooms [41]. *Candida boidinii* has been found with high regularity in the sap of many tree species in geographically distinct regions of the world. Specific substrates associated with this species are largely linked to its ability to assimilate the methanol produced in decaying plant tissues [36].

The species restricted to the São João da Baliza forest reserve included all *Spathaspora passalidarum* isolates and four new species (*Candida* sp. 2, *Candida* sp. 3, *Spathaspora* sp. 1 and *Spathaspora* sp. 3). *Candida amazonensis*, *Candida* sp. 1 and *Spathaspora* sp. 2 were recovered in the Mucajaí forest reserve. Fifteen species were isolated on only one cultivation media, four in YNB-D-xylose medium (*C. blattae*, *C. labiduridarum*, *Naumovazyma castelli* and

Table 1. Identification, occurrence and fermentation in Durham tube test of yeasts isolated in Amazonian forest reserves.

Yeast species	Sampled medium		Fermentation in Durham tube test
	YNB-D-xylose (n = 40)	YNB-xylan (n = 40)	
São João da Baliza Forest Reserve			
Asterotremella humicola	2[1]	4	–
Blastobotrys mokoenaii[3]	1	1	–
Candida amphixiae[3,5]	–	1	–
C. boidinii	5	5	–
C. gorgasii[2,3]	1	1	–
C. intermedia	4	3	–
C. labiduridarum[3,4]	2	–	–
C. palmioleophila[3,5]	–	1	–
C. pseudointermedia[3,5]	–	1	–
C. quercitrusa	1	–	–
C. tropicalis	1	3	–
Candida sp. 2[2,3]	2	1	–
Candida sp. 3[2,3,5]	–	2	–
Cryptococcus diffluens[3,5]	–	1	–
Cr. laurentii[5]	–	1	–
Debaryomyces hansenii	5	2	–
Kodamaea ohmeri[3,5]	–	1	–
Lindnera saturnus	5	1	–
Meyerozyma guilliermondii	–	1	–
Naumovazyma castellii[3,4]	1	–	–
Scheffersomyces stipitis	1	–	+
Schwanniomyces polymorphus[3]	6	2	–
Sc. vanrijiae[3]	2	2	–
Spathaspora passalidarum[3,5]	–	5	+
Spathaspora sp. 1[2,3]	1	1	–
Spathaspora sp. 3[2,3,5]	–	1	–
Trichosporon mycotoxinivorans	1	2	–
Mucajaí Forest Reserve			
A. humicola	6	6	–
Candida amazonensis[2,3]	3	1	–
C. blattae[3,4]	1	–	–
C. boidinii	5	2	–
C. intermedia	–	1	–
C. maltosa[3]	2	2	–
C. natalensis[3,5]	–	1	–
C. quercitrusa	–	1	–
C. tropicalis	14	10	–
Candida sp. 1[2,3,5]	–	2	–
Cr. laurentii[5]	–	1	–
D. hansenii	2	2	–
L. saturnus	1	1	–
M. guilliermondii	4	1	–
S. stipitis	–	1	+
Spathaspora sp. 2[2,3,4]	1	–	–
T. mycotoxinivorans	–	3	–

[1]Number of samples in which the yeast was isolated.
[2]Novel yeast species.
[3]Occurrence restricted to one isolation site.
[4]Occurrence restricted to YNB-D-xylose medium.
[5]Occurrence restricted to YNB-xylan medium.
doi:10.1371/journal.pone.0043135.t001

Spathaspora sp. 2) and 11 species in YNB-xylan medium (*C. amphixiae, C. natalensis, C. palmioleophila, C. pseudointermedia, Candida* sp. 1, *Candida* sp. 3, *Cryptococcus diffluens, Cr. laurentii, Kodamaea ohmeri, S. passalidarum*, and *Spathaspora* sp. 3). Most known yeast species found in YNB-D-xylose or xylan media have previously been linked to terrestrial environments, such as soil, flowers, fruit, rotting wood, beetle guts, and floricolous insects [42]. Suh et al. [8] reported that *C. intermedia, Meyerozyma guilliermondii, Lindnera (Williopsis) saturnus*, and *S. stipitis* were associated with wood-ingesting beetles, and Nguyen et al. [14] observed the same for *S. passalidarum*. Bhadra et al. [43] isolated *Cr. laurentii, D. hansenii* and *K. ohmeri* from tree bark samples. *Candida tropicalis* and *C. maltosa* were isolated from rotten fruit by Rao et al. [10]. *Schwanniomyces polymorphus* and *Sc. vanrijiae* were also isolated from several samples collected in the São João da Baliza forest reserve. These metabolically versatile species have been isolated from soil, tree exudates, and ant hills [44]. Some species, namely *D. hansenii, C. intermedia* and *M. guilliermondii*, were isolated in both culture media and from both collection sites. Two isolates of *B. mokoenaii* were obtained from the São João da Baliza site. This species is rare and known previously from only a single strain obtained from soil in South Africa [45]. Du Preez et al. [46] reported that *B. mokoenaii* is thermotolerant and produces an extracellular endo-β-xylanase comparable to that of *Aureobasidium pullulans*.

Molecular Identification and Phylogenetic Relationships

Some yeast isolates showed three or more non-contiguous nucleotide differences in the D1/D2 domains of the LSU rRNA gene when compared with the most closely related known species, indicating that they might represent novel yeast species. According to Kurtzman et al. [42], isolates of the same species typically have zero to two nucleotide differences in the D1/D2 region of the large subunit of the rRNA gene. Based on this concept, seven new yeast species were found in our studies. Five isolates have been described as a new species named *Candida amazonensis* [47]. The new species belongs to the *Scheffersomyces* clade and differs by nine nucleotide substitutions and six indels in the D1/D2 region of the LSU rRNA gene from *C. coipomoensis*, nine nucleotide substitutions and seven indels from *C. lignicola* and 16 nucleotide substitutions and six indels from *C. queiroziae*, its closest relatives [47]. All yeast species that are phylogenetically related to *C. amazonensis* were also isolated from rotting-wood samples or insects associated with this substrate [36,48,49]. The yeast isolates identified as *Candida* sp. 1, *Spathaspora* sp. 1, *Spathaspora* sp. 2 and *Spathaspora* sp. 3 by sequence analysis belong to the *Spathaspora* clade, which contains D-xylose-fermenting yeasts. *Spathaspora* sp. 3 (UFMG-HMD-19.3) and *Spathaspora* sp. 1 (UFMG-XMD-16.2) belong to the same subclade as do *S. arborariae, Candida jeffriesii*, and *Candida materiae*. *Spathaspora* sp. 3 (UFMG-HMD-19.3) differs in the D1/D2 domains by eight substitutions from its least divergent relative, *C. materiae*. The smallest degree of sequence divergence observed for *Spathaspora* sp. 1 (UFMG-XMD-16.2) was seven substitutions and four indels from *S. arborariae*. The other two new species, *Spathaspora* sp. 2 (UFMG-XMD-23.2) and *Candida* sp. 1 (UFMG-HMD-23.3), are closely related, forming a separate subclade within the *Spathaspora* clade. They differ by 76 changes in the combined D1/D2 domains (6 substitutions and 5 indels) and ITS region (19 substitutions and 46 indels) and are well separated phylogenetically from other clade members (Fig. 1). The D1/D2 sequences of both isolates of *Spathaspora* sp. 1 (UFMG-XMD-16.2 and UFMG-HMD-16.3) were identical. The same was observed for the isolates of *Candida* sp. 1 (UFMG-HMD-23.3 and UFMG-HMD-25.1). Three isolates were identified as *Candida* sp. 2 (GenBank accession number is JQ695901). This new species belongs to the *Wickerhamomyces* clade and it differs by six nucleotide substitutions from *C. myxetangii*. The later species is associated with tree-boring beetles [36]. *Candida* sp. 3 (GenBank accession number is JQ695900) belongs to the *Lindnera* clade and it differs by six nucleotide substitutions from *Lindnera* (*Pichia*) *japonica*, a species associated with tree boring insects [50].

Out of the 33 yeast species identified, strains belonging to only two species, *S. passalidarum* and *S. stipitis*, showed gas formation from D-xylose in the Durham tube test. Both species had previously been shown to ferment D-xylose [11,14]. However, although one new species (*C. amazonensis*) was identified as belonging to the *Scheffersomyces* clade and four as belonging to the *Spathaspora* clade, these isolates exhibited no gas production in the Durham tube test, showing that this test alone is insufficient in screening for D-xylose-fermenting yeasts.

Fermentation Assays

D-xylose culture medium. To evaluate D-xylose fermentation and ethanol production, two strains of *S. stipitis*, six strains of *S. passalidarum*, five strains of *Candida amazonensis* and all strains of the new species belonging to the *Spathaspora* clade were subjected to fermentation assays in D-xylose (50 g/L) culture medium. The results of the fermentation parameters [$Y_{p/s}^{et}$ (g/g), ethanol yield; $Y_{p/s}^{xy}$ (g/g), xylitol yield; Qp (g/L·h), ethanol productivity; η (%), fermentation efficiency; (%), D-xylose consumption (%)] and cells, ethanol and xylitol concentrations (g/L) are summarized in Table 2. These results were calculated according to the fermentation time (time of maximum ethanol production or time of the end of the fermentation experiment) for each yeast strain.

Fermentation results revealed that all yeasts tested were able to consume D-xylose, with the consumption rates ranging from 52.3% to practically 100% in 48 h. Da Cunha-Pereira et al. [15] found an efficiency of 84% for D-xylose utilization in 96 h by *S. arborariae* UFMG-HM 19.1A in synthetic medium containing initial concentrations of 20 g/L xylose, 20 g/L glucose and 10 g/L arabinose. Agbogbo et al. [51] reported 100% D-xylose consumption after 120 h by *S. stipitis* CBS 6054 in synthetic medium formulated with 60 g/L D-xylose. Agbogbo and Wenger [52] demonstrated a D-xylose consumption over 85% in 48 h by *S. stipitis* CBS 6054 in synthetic medium with approximately 21 g/L D-xylose and 5.8 g/L glucose. According to du Preez and Prior [53], *C. shehatae* CBS 2779 consumed 100% xylose in 28 h from medium containing 20 g/L xylose. Among the microorganisms studied in our work, the isolates of *C. amazonensis* showed the highest D-xylose consumption rates, approaching 100% of consumption (values ranged from 99.8% to 99.9%) in 48 h, whereas *S. passalidarum* showed the fastest D-xylose consumption rate, 98%, in 24 h. Among the new species, both *Spathaspora* sp. 1 and *Spathaspora* sp. 2 consumed up to 80% of D-xylose in 48 h, while *Spathaspora* sp. 3 and *Candida* sp. 1 consumed 52.3% to 60.2% of D-xylose, respectively, in 48 h. Strains of *S. stipitis* achieved similar maximum D-xylose consumption values, approximately 98.8%, in 48 h. These results show, in general, high and fast D-xylose consumption by most of the yeasts studied in this work. Furthermore, we also observed that a specifically high and fast D-xylose consumption behavior can likely be associated with *C. amazonensis*, *S. passalidarum* and *S. stipitis* strains isolated in this work, but not with all species from the genus *Spathaspora*.

Ethanol production was observed during the fermentation assay, confirming the ability to ferment D-xylose to ethanol by the microorganisms tested. The highest ethanol concentration values were produced by the *S. passalidarum* strains (a species that exhibited gas production in the Durham tube test), ranging from 15 g/L to 18 g/L in 24 h. *Candida amazonensis*, *Spathaspora* sp. 3 and

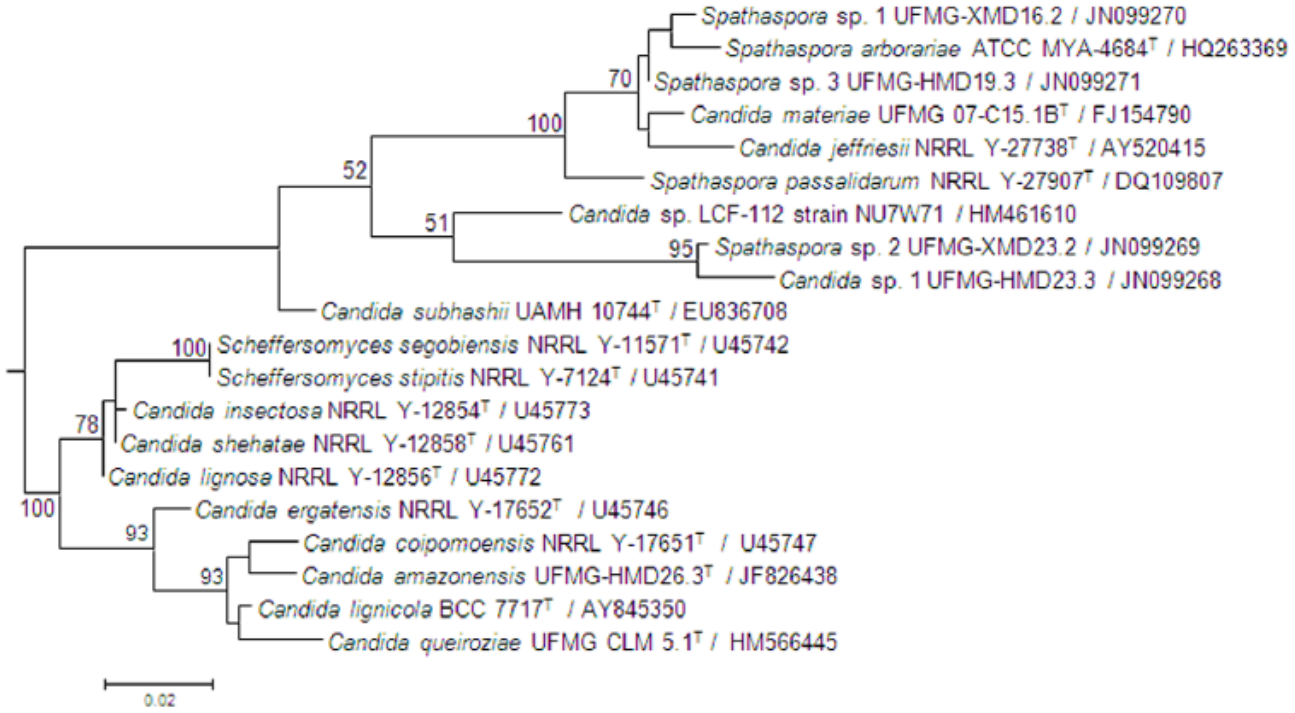

Figure 1. Phylogram of yeast species considered in this study based on the D1/D2 domains of the large subunit ribosomal gene. The maximum likelihood tree was constructed with the Mega5 program following correction of the distances with the Kimura 2-parameter transformation. A total of 499 nucleotide positions were used in the analysis. Bootstrap values of 50% or greater are shown (100 replicates). Bar 0.02 substitutions per nucleotide position.
doi:10.1371/journal.pone.0043135.g001

Candida sp. 1 were at the other end of the spectrum, showing the lowest ethanol concentration values, between 3.1 g/L and 4.9 g/L in 48 h. *Scheffersomyces stipitis* UFMG-XMD-15.2 and UFMG-HMD-32.1, that exhibited gas production in the Durham tube test, produced ethanol with similar concentrations, 12.3 g/L and 11.1 g/L, respectively, but at different fermentation times, 24 h and 48 h, respectively. The variations observed can probably be associated with physiological differences between strains of the same species. Ethanol production of 15 g/L was observed by the yeast *S. arborariae* UFMG-HM-19.1A in approximately 72 h from 20 g/L xylose, 20 g/L glucose and 10 g/L arabinose [15]. *Candida shehatae* CBS 2779 reached 13.1 g/L ethanol in 28 h in synthetic medium containing 20 g/L xylose [53]. *Scheffersomyces stipitis* CBS 6054 was able to produce 24.3 g/L ethanol in 120 h in medium with 60 g/L D-xylose [51]. Agbogbo and Wenger [53] also showed the production of approximately 8.7 g/L ethanol in 48 h by *S. stipitis* CBS 6054 from 20 g/L xylose. In general, *S. passalidarum* strains showed the best potential for ethanol production in the conditions tested because they produced the highest ethanol concentrations in a short period of time.

Candida lignosa CBS 4705 (positive control) presented the highest ethanol yield ($Y_{p/s}^{et}$ = 0.4 g/g) and fermentation efficiency (η = 79.4%). *S. passalidarum* strains showed the highest ethanol productivity (Qp = 0.62 g/L·h to 0.75 g/L·h), with minimum and maximum $Y_{p/s}^{et}$ values between 0.31 g/g and 0.37 g/g and η between 61% and 71.6%. The new species *Spathaspora* sp. 1 UFMG-XMD-16.2, *Spathaspora* sp. 1 UFMG-HMD-16.3 and *Spathaspora* sp. 2 UFMG-XMD-23.2 showed better ethanol fermentation results than *C. amazonensis* strains, *Spathaspora* sp. 3, *Candida* sp. 1 UFMG-HMD-23.3 and *Candida* sp. 1 UFMG-HMD-25.1 (Table 2). Da Cunha-Pereira et al. [15] found a $Y_{p/s}^{et}$ and Qp equivalent to 0.45 g/g and 0.21 g/L·h, respectively, for *S. arborariae* UFMG-HM-19.1A. *Candida shehatae* CBS 2779 and *S. stipitis* CBS 6054 showed $Y_{p/s}^{et}$ values equal to 0.37 g/g and 0.44 g/g, respectively, and Qp equivalent to 0.47 g/L·h and 0.20 g/L·h, respectively [51,53]. Overall, *S. passalidarum* strains showed promising results for ethanol production under the conditions tested, considering their remarkably high ethanol productivity (Qp = 0.62 g/L·h to 0.75 g/L·h) and fermentation efficiency of approximately 70% (η = 61% to 71.6%), in 24 h. However, $Y_{p/s}^{et}$ values found in *S. passalidarum* strains (0.31 g/g and 0.37 g/g) were lower than those found in *S. arborariae* UFMG-HM-19.1A and *S. stipitis* CBS 6054, but it must be taken into consideration the different experimental conditions employed for these yeasts. *Spathaspora arborariae* UFMG-HM-19.1A produced $Y_{p/s}^{et}$ equal to 0.45 g/g in medium containing 20 g/L xylose, but also 20 g/L glucose and 10 g/L arabinose, and its maximum ethanol production was achieved in approximately 72 h [15], while *S. stipitis* CBS 6054 showed $Y_{p/s}^{et}$ equal to 0.44 g/g in medium containing 60 g/L xylose and took 120 h to reach its maximum ethanol concentration [51]. In our study, D-xylose (50 g/L) was used as the sole carbon source, and *S. passalidarum* strains reached maximum ethanol production in 24 h.

A good understanding of ethanol production from xylose requires a knowledge of both the experimental conditions and the enzymes responsible for xylose metabolism. In yeasts, fermentation of xylose to ethanol is strongly dependent on NADH-linked xylose reductase (XR). In *Scheffersomyces stipitis*, XR preferentially uses NADPH, although it can also use NADH [54]. Recently, it was shown that in *S. passalidarum* xylose is converted by means of an NADH-preferring XR [16]. These observations are compatible with the higher ethanol yields achieved by strains of *S. passalidarum*

Table 2. Ethanol yield [$Y_{p/s}^{et}$ (g/g)], xylitol yield [$Y_{p/s}^{xy}$ (g/g)], ethanol productivity [Qp (g/L•h)], fermentation efficiency [η (%)], D-xylose consumption (%), cell concentration (g/L), ethanol concentration (g/L) and xylitol concentration (g/L) in D-xylose culture medium assays.

Yeast species	Yeast strain	D-xylose consumption (%)[1]	Cells (g/L)	$Y_{p/s}^{et}$ (g/g)[2]	Qp (g/L•h)[3]	H (%)[4]	Ethanol concentration (g/L)	$Y_{p/s}^{xy}$ (g/g)[5]	Xylitol concentration (g/L)	Fermentation Time (h)[6]
Candida lignosa	CBS 4705	76.2	7.7	0.40	0.60	79.4	14.5	0.03	1.0	24
Scheffersomyces stipitis	NRRL Y-7124	89.0	10.9	0.35	0.62	69.5	15.0	0.02	1.3	24
S. stipitis	UFMG-XMD-15.2	84.5	12.9	0.28	0.51	55.2	12.3	0.04	1.9	24
S. stipitis	UFMG-HMD-32.1	98.8	14.2	0.22	0.23	44.1	11.1	–	–	48
C. amazonensis sp. nov.	UFMG-XMD-24.1	99.9	10.6	0.08	0.08	15.3	4.0	0.59	25.2	48
C. amazonensis sp. nov.	UFMG-XMD-26.2	99.8	10.7	0.08	0.08	14.6	4.0	0.58	25.2	48
C. amazonensis sp. nov.	UFMG-HMD-26.3	99.9	10.7	0.07	0.08	13.4	3.7	0.57	24.8	48
C. amazonensis sp. nov.	UFMG-XMD-40.2	99.9	11.8	0.07	0.08	14.4	3.8	0.55	23.7	48
C. amazonensis sp. nov.	UFMG-XMD-40.3	99.9	11.1	0.07	0.08	14.0	3.7	0.56	23.9	48
Spathaspora passalidarum	UFMG-HMD-1.1	98.4	8.7	0.36	0.75	70.0	18.0	0.03	1.5	24
S. passalidarum	UFMG-HMD-1.3	98.1	9.8	0.35	0.72	68.4	17.2	0.04	2.2	24
S.passalidarum	UFMG-HMD-2.1	98.4	10.7	0.31	0.62	61.0	15.0	0.02	1.1	24
S. passalidarum	UFMG-HMD-10.2	98.4	10.6	0.33	0.69	65.4	16.6	0.02	1.2	24
S. passalidarum	UFMG-HMD-14.1	98.3	10.2	0.37	0.68	71.6	16.4	0.02	1.1	24
S. passalidarum	UFMG-HMD-16.2	98.3	9.9	0.33	0.64	64.2	15.3	0.02	1.0	24
Spathaspora sp. 1	UFMG-XMD-16.2	86.8	7.4	0.33	0.27	65.4	13.1	0.21	7.8	48
Spathaspora sp. 1	UFMG-HMD-16.3	84.0	6.3	0.27	0.22	53.8	10.7	0.17	6.7	48
Spathaspora sp. 2	UFMG-XMD-23.2	89.8	7.4	0.26	0.21	50.8	10.2	0.19	7.0	48
Spathaspora sp. 3	UFMG-HMD-19.3	55.2	5.6	0.13	0.07	26.2	3.3	0.16	3.9	48
Candida sp 1	UFMG-HMD-23.3	60.2	9.3	0.18	0.10	35.8	4.9	0.13	3.3	48
Candida sp 1	UFMG-HMD-25.1	52.3	9.0	0.14	0.06	27.0	3.1	0.22	4.6	48

[1]D-xylose consumption (%) – percentage of initial D-xylose consumed.
[2]$Y_{p/s}^{et}$ (g/g) – ethanol yield: correlation between ethanol ($\Delta P_{ethanol}$) produced and D-xylose (ΔS_{xylose}) consumed.
[3]Qp (g/L•h) – ethanol productivity: ratio of ethanol concentration (g/L) and fermentation time (h).
[4]η (%) – fermentation efficiency: percentage of the maximum theoretical ethanol yield (0.51 g ethanol/g D-xylose).
[5]$Y_{p/s}^{xy}$ (g/g) – xylitol yield: correlation between xylitol ($\Delta P_{xylitol}$) produced and D-xylose (ΔS_{xylose}) consumed.
[6]Time when the maximum ethanol production (g/L) value was attained or time of the end of the fermentation experiment.
doi:10.1371/journal.pone.0043135.t002

and *S. stipitis* tested in this work. The impact of XR activities and their co-factor preferences in on ethanol production among these new xylose-fermenting yeasts will be the focus of future work.

Yeast strains that achieved their maximum ethanol concentration in 24 h were observed to undergo a decrease in ethanol concentration after this period (data not shown). This decrease probably results from ethanol assimilation by the yeasts as a consequence of the rapid depletion of sugar from the medium while oxygen remains available. This behavior has previously been reported for yeasts in studies utilizing both synthetic medium and/or lignocellulosic hydrolysates [9,12,15,52,55].

Although ethanol was the main product obtained in these fermentation assays, by-products such as xylitol and glycerol were also found. Glycerol was present at low concentrations (on average ≤0.05 g/L, maximum value of 1.4 g/L, data not shown), whereas xylitol was produced at higher concentrations. The lowest xylitol values (0 g/L to 2.2 g/L) were obtained for *S. stipitis*, *C. lignosa* and *S. passalidarum*. Ferreira et al. [12] suggested that the formation of low amounts of by-product is important characteristics of the yeast strain, as they allow elevated formation of the main fermentation product, ethanol, to be obtained. The new *Spathaspora* species that was identified in this study produced xylitol concentrations between 3.3 g/L and 7.8 g/L in 48 h ($Y_{p/s}^{xy}$ between 0.13 g/g and 0.22 g/g) and, together with *C. amazonensis*, produced lower ethanol concentrations than the other species tested. Another species from *Spathaspora* clade, *S. arborariae* UFMG-HMD-19.1A has already been shown to produce xylitol, at 5 g/L in 108 h in synthetic medium [15]. *Candida amazonensis* was responsible for the highest xylitol production values found in this assay (26.1 g/L to 27.8 g/L in 24 h, $Y_{p/s}^{xi}$ between 0.62 g/g and 0.67 g/g, data not shown). These values were much higher than those found for ethanol production by all yeasts tested.

After 24 h, a decrease in the xylitol concentration occurred, indicating that this product was being used as carbon source, presumably following D-xylose depletion and also due the oxygen availability in the medium. Xylitol is one of the most expensive polyol sweeteners in the world market and has been the subject of specific health claims. It is suitable for diabetics and is recommended for oral health and parenteral nutrition [56]. The biotechnological production of xylitol using microorganisms such as yeasts and fungi is of economic interest and presents some advantages when compared with conventional xylitol production by the chemical reduction of D-xylose, as this latter process is characterized by high costs due to difficulties in separation and purification steps [39,57,58]. Thus, *C. amazonensis* isolates obtained in this work can be studied in the future for direct xylitol production from D-xylose in hemicellulosic hydrolysates. Further studies are needed to establish the best cultivation conditions for this process.

Sugarcane bagasse hemicellulosic hydrolysate. After performing D-xylose fermentation assays, we selected a number of strains for fermentation tests in sugarcane bagasse hydrolysate. We picked the strains or species that presented the highest rates of D-xylose consumption and ethanol yields ($Y_{p/s}^{et}$), specifically *S. passalidarum* UFMG-HMD-1.1 and UFMG-HMD-14.1, *S. stipitis* UFMG-XMD-15.2, *Spathaspora* sp. 1 UFMG-XMD-16.2 and *Spathaspora* sp. 2 UFMG-XMD-23.2. *Candida amazonensis*, *Spathaspora* sp. 3 and *Candida* sp. 1 were not selected due to their low ethanol production in D-xylose culture medium.

The fermentation parameter results [$Y_{p/s}^{et}$ (g/g), ethanol yield; $Y_{p/s}^{xy}$ (g/g), xylitol yield; Qp (g/L·h), ethanol productivity; η (%), fermentation efficiency; sugar (D-xylose and glucose) consumption (%)] and cells, ethanol and xylitol concentrations (g/L) in sugarcane bagasse hemicellulosic hydrolysates are summarized in

Table 3. Ethanol yield [$Y_{p/s}^{et}$ (g/g)], xylitol yield [$Y_{p/s}^{xy}$ (g/g)], ethanol productivity [Qp (g/L·h)], fermentation efficiency [η (%)], sugar (D-xylose and glucose) consumption (%), cell concentration (g/L), ethanol concentration (g/L) and xylitol concentration (g/L) in sugarcane bagasse hemicellulosic hydrolysate assays.

Yeast species	Yeast strains	Sugars consumption (%)[1]	Cells (g/L)	$Y_{p/s}^{et}$ (g/g)[2]	Qp (g/L·h)[3]	H (%)[4]	Ethanol concentration (g/L)	$Y_{p/s}^{xy}$ (g/g)[5]	Xylitol concentration (g/L)	Fermentation time (h)[6]
Candida lignosa	CBS 4705	55.3	7.8	0.16	0.05	31.6	4.6	–	–	96
Scheffersomyces stipitis	NRRL Y-7124	34.9	3.4	0.25	0.10	49.3	4.9	–	–	48
S. stipitis	UFMG-XMD-15.2	80.9	12.7	0.34	0.20	65.7	14.1	–	–	72
Spathaspora passalidarum	UFMG-HMD-1.1	84.9	13.4	0.20	0.09	40.0	8.8	–	–	96
S. passalidarum	UFMG-HMD-14.1	91.0	13.1	0.18	0.10	36.0	9.5	–	–	96
Spathaspora sp. 1	UFMG-XMD-16.2	72.9	7.9	0.23	0.10	46.0	9.3	0.57	18.2	96
Spathaspora sp. 2	UFMG-XMD-23.2	68.6	6.4	0.22	0.08	42.4	7.2	0.61	17.1	96

[1]Sugar consumption (%) – percentage of the initial D-xylose and glucose consumed.
[2]$Y_{p/s}^{et}$ (g/g) – ethanol yield: correlation between ethanol ($\Delta P_{ethanol}$) produced and D-xylose and glucose (ΔS_{sugars}) consumed.
[3]$Y_{p/s}^{xy}$ (g/g) – xylitol yield: correlation between xylitol ($\Delta P_{xylitol}$) produced and D-xylose (ΔS_{xylose}) consumed.
[4]Qp (g/L·h) – ethanol productivity: ratio of ethanol concentration (g/L) and fermentation time (h).
[5]η (%) – fermentation efficiency: percentage of the maximum theoretical ethanol yield (0.51 g ethanol/g D-xylose and glucose).
[6]Time when the maximum ethanol production (g/L) value was attained or time of the end of the fermentation experiment.
doi:10.1371/journal.pone.0043135.t003

Table 3. These results were also calculated based on the fermentation time (time of maximum ethanol production or time of the end of the fermentation experiment) for each yeast strain.

Taking only into account the glucose and D-xylose present in the hydrolysates, *S. stipitis* UFMG-XMD-15.2 showed the highest sugar consumption, equal to 93.4% in 96 h (data not shown), followed by *S. passalidarum* strains UFMG-HMD-1.1 (84.9%) and UFMG-HMD-14.1 (91%) in 96 h. *Spathaspora* sp. 2 UFMG-XMD-23.2 and *Spathaspora* sp. 1 UFMG-XMD-16.2 presented values of 68.6% and 72.9%, respectively, whereas *C. lignosa* CBS 4705 (positive control) showed the lowest consumption value, equal to 55.3% in 96 h. *Scheffersomyces stipitis* NRRL Y-7124 (positive control) consumed 69.8% of glucose and D-xylose (data not shown) in 96 h, similar to the amount consumed by *Spathaspora* sp. 2. Da Cunha-Pereira et al. [15] observed that *S. arborariae* UFMG-HM-19.1A consumed glucose and xylose at 100% and 45% in approximately 120 h and 240 h, respectively, in rice hull hydrolysate containing 35 g/L glucose, 13 g/L xylose and 4 g/L arabinose. *Scheffersomyces stipitis* UFMG-IMH-43.2 consumed, on average, 83.6% of glucose and xylose in 192 h, when cultivated in sugarcane bagasse hydrolysate with an initial concentration of 52.5 g/L xylose [12]. Chandel et al. [59] reported a total sugar consumption of 85.9% after 24 h fermentation by *C. shehatae* NCIM 3501 in sugarcane bagasse hydrolysate with 20 g/L total sugars. Among the yeasts tested in our assays, glucose and D-xylose were initially metabolized at the same time, but the glucose consumption rate was clearly faster than that of D-xylose. After glucose exhaustion, D-xylose was consumed much faster, and this behavior was observed after 12 h by *S. passalidarum* strains and *S. stipitis* UFMG-XMD-15.2, after 24 h by *S. stipitis* NRRL-Y 7124 and *C. lignosa* CBS 4705 and after 48 h by *Spathaspora* sp. 1 and *Spathaspora* sp. 2 (data not shown). When compared with the results obtained in other studies on hydrolysate fermentation, *S. stipitis* UFMG-XMD-15.2 and the *S. passalidarum* strains tested in our work showed excellent D-xylose and glucose consumption results, when the initial D-xylose (50.2 g/L) and glucose (5.3 g/L) concentrations and the time of consumption (96 h) of the majority (84.9% and 93.4%) of these sugars are considered. Unlike D-xylose and glucose, L-arabinose consumption was not observed by the yeasts during the fermentation time studied. The absence of L-arabinose consumption was also observed in fermentations carried out with *S. stipitis* UFMG-IMH-43.2 in experiments similar to those performed in this work [12].

Ethanol production was observed within 12 h of cultivation by the yeasts tested. *Scheffersomyces stipitis* NRRL Y-7124 produced a maximum concentration of 4.9 g/L ethanol within 48 h of fermentation. *Candida lignosa* CBS 4705, *S. passalidarum* UFMG-HMD-1.1 and UFMG-HMD-14.1 reached maximum ethanol concentrations of 4.6 g/L, 8.8 g/L and 9.5 g/L, respectively, in 96 h. *S. stipitis* UFMG-XMD-15.2 was responsible for the highest ethanol production, corresponding to 14.1 g/L in 72 h. An ethanol production greater than 15 g/L was observed for *S. arborariae* UFMG-HM-19.1A after 50 h in rice hull hydrolysate [15]. *Candida shehatae* NCIM 3501 produced 5.2 g/L ethanol after 24 h in sugarcane bagasse hydrolysate [59]. *Spathaspora passalidarum* UFMG-HMD-1.1 and UFMG-HMD-14.1 showed ethanol concentration results similar to that reported by *S. stipitis* UFMG-IMH-43.2, which presented an ethanol production equal to 9.1 g/L, but in 192 h [12]. Still, when the strains tested in this study were compared with *S. stipitis* UFMG-IMH-43.2, *S. stipitis* UFMG-XMD-15.2 produced a higher ethanol concentration (14.1 g/L) at a much faster rate (72 h). It must be noted that both studies used sugarcane bagasse hemicellulosic hydrolysate with similar experimental conditions (including the initial concentration of D-xylose, supplementation, agitation and incubation temperature). Considering the initial high D-xylose concentration (50.2 g/L) relative to the glucose concentration (5.3 g/L) and the remarkably high ethanol production by *S. stipitis* UFMG-XMD-15.2 relative to other species tested here, the hydrolysate fermentation study results suggest that this strain is a promising tool for producing ethanol from D-xylose.

Scheffersomyces stipitis UFMG-XMD-15.2 presented the highest ethanol yield ($Y_{p/s}^{et}$ = 0.34 g/g), ethanol productivity (Qp = 0.2 g.L/h) and fermentation efficiency (η = 65.7%). *Spathaspora passalidarum* strains UFMG-HMD-1.1 and UFMG-HMD-14.1 showed $Y_{p/s}^{et}$ values between 0.18 g/g and 0.2 g/g, ethanol productivity between 0.09 and 0.1 g/L·h and η between 36% and 40%, whereas *Spathaspora* sp. 2 and *Spathaspora* sp. 1 presented ethanol yields between 0.22 g/g and 0.23 g/g, Qp between 0.08 g/L·h and 0.1 g/L·h and η between 42.4% and 46%. Da Cunha-Pereira et al. [15] found a $Y_{p/s}^{et}$ and Qp equivalent to 0.45 g/g and 0.16 g/L·h, respectively, for *S. arborariae* UFMG-HM-19.1A in a fermentation assay using rice hull hydrolysate. *Candida shehatae* NCIM 3501 showed $Y_{p/s}^{et}$ equal to 0.3 g/g and Qp equivalent to 0.22 g/L·h in sugarcane bagasse hydrolysate [59], results similar to those found for *S. stipitis* UFMG-XMD-15.2. Ferreira et al. [12] found $Y_{p/s}^{et}$ and Qp equal to 0.17 g/g and 0.05 g/L·h, respectively, for *S. stipitis* UFMG-IMH-43.2, showing that ethanol fermentation parameters presented by the strains isolated in our study were better than those observed for *S. stipitis* UFMG-IMH-43.2. When compared with the ethanol yield of *S. arborariae* [15], the yeast strains isolated in this study showed lower $Y_{p/s}^{et}$ in hemicellulosic hydrolysate. *Spathaspora arborariae* UFMG-HM-19.1A produced $Y_{p/s}^{et}$ equal to 0.45 g/g in rice hull hydrolysate containing glucose as the main sugar (35 g/L), with an initial xylose concentration (13 g/L) that was much lower than that employed in our work (50.2 g/L). Despite the higher $Y_{p/s}^{et}$ presented by *S. arborariae* in rice hull hydrolysates, *S. stipitis* UFMG-XMD-15.2 showed a Qp (0.2 g/L·h) higher than that observed for *S. arborariae* (0.16 g/L·h) in our experiments.

Ethanol consumption was observed by *S. stipitis* NRRL Y-7124 after 48 h and *S. stipitis* UFMG-XMD-15.2 after 72 h (time of maximum ethanol production). Glycerol was found at low concentrations in *S. passalidarum* UFMG-HMD-1.1 (0.05 g/L), *S. passalidarum* UFMG-HMD-14.1 (0.1 g/L), *S. stipitis* UFMG-XMD-15.2 (0.7 g/L), *Spathaspora* sp. 1 UFMG-XMD-16.2 (0.2 g/L) and *Spathaspora* sp. 2 UFMG-XMD-23.2 (0.6 g/L). Except for the new species identified, xylitol production was not observed for the other yeasts studied. Again, as suggested by Ferreira et al. [12], the low production rates of xylitol and glycerol may be important characteristics of the yeast strain that should allow higher production of the main fermentation product, ethanol, by the microorganism, a physiological characteristic desirable in our study.

Spathaspora sp. 1 UFMG-XMD-15.2 and *Spathaspora* sp. 2 UFMG-XMD-23.2 were the only yeasts that were observed to produce xylitol (18.2 g/L and 17.1 g/L, respectively) in this assay (Table 3). These yeasts produced markedly higher xylitol concentrations in hydrolysate than in D-xylose culture medium. Xylitol yield values that were found for these new species were considerably higher than that found for a previously identified strain of *C. tropicalis* from the soil ($Y_{p/s}^{xy}$ = 0.45 g/g), tested in a fermentation assay on sugarcane hydrolysate with an initial xylose concentration of 30 g/L [39]. The xylitol production (g/L) and $Y_{p/s}^{xy}$ results found in our work were also higher than those reported by Silva and Roberto [60] for *C. gulliermondii* FTI 20037. These authors observed a xylitol production of 17 g/L and a $Y_{p/s}^{xy}$ of 0.35 g/g after 120 h for *C. gulliermondii* FTI 20037 in a rice straw hemicellulosic hydrolysate

fermentation assay, with an initial xylose concentration of 90 g/L. The biotechnological production of xylitol from crude hemicellulosic hydrolysates could be a valuable economic alternative to the expensive chemical production of xylitol from D-xylose [39,61]. Our results for xylitol production by *Spathaspora* sp. 1 and *Spathaspora* sp. 2 are promising and warrant future testing of the production of this polyol from xylose fermentation in hemicellulosic hydrolysates.

Our study investigated the ability of yeasts to produce ethanol and/or xylitol while consuming D-xylose in D-xylose culture medium and produce ethanol and/or xylitol while consuming D-xylose and glucose in sugarcane bagasse hemicellulosic hydrolysate. *S. stipitis* UFMG-XMD-15.2 is of particular interest because it efficiently converted D-xylose to ethanol and grew well in sugarcane bagasse hemicellulosic hydrolysate. *Spathaspora passalidarum* strains were also observed to grow in hydrolysates and produce ethanol. *Candida amazonensis* was notable for its potential use in the biotechnological production of xylitol. The newly identified yeast strains *Spathaspora* sp. 1 and *Spathaspora* sp. 2 were similarly found to potentially produce xylitol in hemicellulosic hydrolysate. Future studies are needed to test their production of this polyol. As this work is the first to use these yeasts in fermentation assays, further studies are required to optimize cultivation conditions (nutritional dependence, initial substrate concentration, initial cell concentration, pH, temperature and aeration) for the efficient conversion of D-xylose into ethanol or xylitol.

Supporting Information

Table S1 List of the new yeast species isolated in this work and their respective GenBank deposit numbers. (DOC)

Acknowledgments

We thank the Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA)-Roraima by their technical support. We also thank Dr. Hélio Tonini for his technical support during the field collections, and the owners, Osvaldo Antônio Sant'ana and José Lopes, of the private lands by the permission to collect the samples in the areas of the private ecological reserves of Mucajaí and São João da Baliza, respectively.

Author Contributions

Conceived and designed the experiments: RMC RCLBR SSS JEZ MJSV FCOG CAR. Performed the experiments: RMC MAM KJD MAL. Analyzed the data: RMC KJD RCLBR SSS FCOG MAL CAR. Contributed reagents/materials/analysis tools: RCLBR SSS JEZ MJSV MAL CAR. Wrote the paper: RMC CAR.

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Table S1 - List of the new yeast species isolated in this work and their respective GenBank deposit numbers.

Yeast species/Strain number	GenBank deposit number
Spathaspora sp.3 HMD19.3	JN099271
Candida amazonensis UFMG-HMD26.3	JF826438
Spathaspora sp. 1 UFMG-XMD16.2	JN099270
Candida sp. 1 UFMG-HMD23.3	JN099268
Spathaspora sp. 2 UFMG-XMD23.2	JN099269
Candida sp. 2 UFMG-XMD-16.4	JQ695901
Candida sp. 3 UFMG-HMD-7.2	JQ695900

4.2 Capítulo II

International Journal of Systematic and Evolutionary Microbiology (2012), 62, 1438–1440 DOI 10.1099/ijs.0.036715-0

Candida amazonensis sp. nov., an ascomycetous yeast isolated from rotting wood in the Amazonian forest

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Five strains of a novel yeast species were isolated from rotting wood samples collected in an Amazonian forest site in the state of Roraima, northern Brazil. The sequences of the D1/D2 domains of the large subunit of the rRNA gene showed that this species belongs to the *Scheffersomyces* clade and is related to *Candida coipomoensis*, *Candida lignicola* and *Candida queiroziae*. The novel species *Candida amazonensis* sp. nov. is proposed to accommodate these isolates. The type strain of *C. amazonensis* sp. nov. is UFMG-HMD-26.3^T (=CBS 12363^T=NRRL Y-48762^T).

The genus *Scheffersomyces* was proposed by Kurtzman & Suzuki (2010) to include the species *Scheffersomyces segobiensis*, *S. spartinae* and *S. stipitis*. The asexual species *Candida shehatae*, *C. insectosa*, *C. lignosa*, *C. ergatensis*, *C. coipomoensis*, *C. lignicola* and *C. queiroziae* are also members of the *Scheffersomyces* clade (Jindamorakot *et al.*, 2007; Kurtzman, 2011; Santos *et al.*, 2011). *Scheffersomyces stipitis*, *S. segobiensis*, *C. shehatae*, *C. lignosa* and *C. insectosa* are of industrial interest because they ferment D-xylose from biomass to ethanol (Kurtzman, 2011). *C. queiroziae*, *C. coipomoensis* and *C. lignicola* are able to ferment cellobiose. This is also of interest because this sugar is a competitive inhibitor of cellulases and hinders the saccharification process of cellulosic material during bioethanol production (Bezerra & Dias, 2005; Santos *et al.*, 2011).

During a search for novel D-xylose-fermenting yeasts associated with rotting wood in a region of the Amazonian forest in northern Brazil, we isolated five strains of a novel cellobiose-fermenting species. Sequence analyses of the D1/D2 regions of the large subunit (LSU) of the rRNA gene showed that the novel species belonged to the *Scheffersomyces* clade and was closely related to *C. coipomoensis*, *C. lignicola* and *C. queiroziae*. The name *Candida amazonensis* sp. nov. is proposed for this novel species.

The yeasts were isolated from rotting wood samples collected at a site in the state of Roraima, northern Brazil. This site belongs to a private forest reserve of an estate located in the municipality of Mucajaí (2° 25′ N 60° 54′ W). The predominant vegetation is characterized as Amazonian forest biome. The climate is hot and humid, with annual precipitation between 1500 and 2100 mm and a mean temperature of 25.6–27.6 °C.

Twenty decayed wood samples were collected in October, 2009. The samples were stored in sterile plastic bags and transported under refrigeration to the laboratory over a period of no more than 24 h. One gram of each sample was placed separately in flasks with 20 ml sterile D-xylose medium (yeast nitrogen base 0.67 %, D-xylose 0.5 %, chloramphenicol 0.02 %) and 20 ml sterile xylan medium (yeast nitrogen base 0.67 %, xylan 1 %, chloramphenicol 0.02 %, pH 5.0±0.2), respectively. The flasks were incubated at 25 °C on an incubator shaker (New Brunswick) at 150 r.p.m. for 3–10 days. When yeast growth was detected, 0.5 ml aliquots were transferred to tubes containing 5 ml sterile D-xylose or

Abbreviations: ITS, internal transcribed spacer; LSU, large subunit.

The GenBank/EMBL/DDBJ accession number for the gene sequence of the D1/D2 domain of the 26S rRNA gene of strain UFMG-HMD-26.3^T is JF826438.

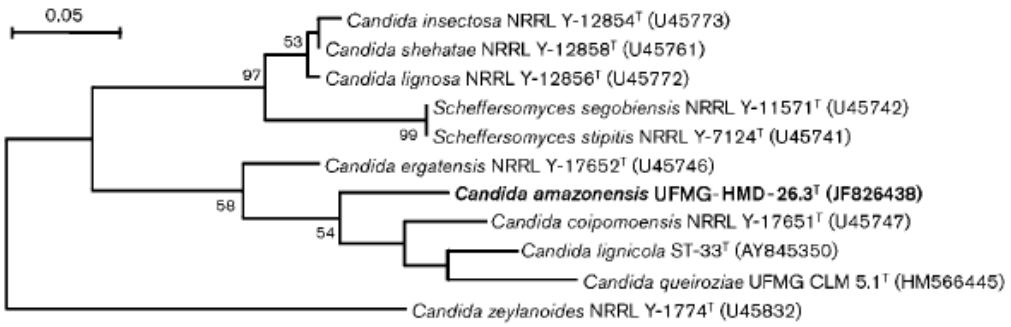

Fig. 1. Phylogenetic placement of *Candida amazonensis* sp. nov. by maximum-parsimony analysis. The bootstrap consensus tree was produced from 1000 iterations using 525 aligned nucleotide positions of the D1/D2 domains of the LSU rRNA gene. The sequence of *Candida zeylanoides* NRRL Y-1774^T was used as the outgroup and all strains analysed were type strains. Bar, 0.05 substitutions per nucleotide position.

xylan medium and the tubes were incubated on an incubator shaker as described above. One loopful of each tube was streaked on D-xylose or xylan agar medium. The plates were incubated at 25 °C until yeast colonies developed (Cadete *et al.*, 2009).

Representatives of the different colony morphotypes were purified by repeated streak inoculation on yeast extract–malt extract agar (YMA) and preserved at −80 °C or in liquid nitrogen for later identification. The yeasts were characterized using standard methods (Kurtzman *et al.*, 2011). Identities were determined by sequencing the D1/D2 domains of the LSU of the rRNA gene. The D1/D2 domains and the internal transcribed spacer (ITS) region of the LSU rRNA gene of the yeast strains were amplified by PCR directly from whole cells as described previously (Lachance *et al.*, 1999). The amplified DNA was concentrated and cleaned on QIAquick PCR columns (Qiagen) and sequenced using an ABI sequencer at the John P. Robarts Research Institute (London, Ontario, Canada). The sequences were assembled, edited and aligned with the program MEGA5 (Tamura *et al.*, 2011). Phylogenetic placement of the strains was based on maximum-parsimony analysis of the sequences of the D1/D2 domains of the LSU rRNA gene. The bootstrap consensus tree was produced from 1000 iterations using 525 aligned nucleotide positions.

The novel species belongs to the *Scheffersomyces* clade and is related to *C. coipomoensis*, *C. lignicola* and *C. queiroziae* (Fig. 1). It differed in the D1/D2 domains by 9 substitutions and 6 gaps from *C. coipomoensis*, 9 substitutions and 7 gaps from *C. lignicola*, and 16 substitutions and 6 gaps from *C. queiroziae*. The D1/D2 and ITS sequences of the five isolates (UFMG-HMD-26.3^T, XMD-24.1, XMD-26.2, XMD-40.2 and XMD-40.3) of the novel species were identical. The isolates, either alone or mixed in pairs, were examined after growth on the most common sporulation media at 17 and 25 °C (cornmeal agar, dilute V8 agar, 5 % malt extract agar and yeast carbon base agar supplemented with 0.01 % ammonium sulfate, among others), but asci or signs of conjugation were not seen.

The isolation of this novel species from rotting wood suggests that this substrate could be its ecological niche. *C. amazonensis* has not been isolated from rotting wood collected in south-eastern Brazil and could be restricted to the Amazonian region. This novel yeast species and its close relatives form a subclade within the *Scheffersomyces* clade in which all species were isolated from rotting wood (Kurtzman, 2011; Santos *et al.*, 2011); this suggests that these yeasts are adapted to this substrate.

The strains of *C. amazonensis* are physiologically similar to strains of the species *C. lignicola*, *C. coipomoensis* and *C. queiroziae*, but can be distinguished from them based on growth at 37 °C, which is positive for the novel species and negative or weak for the other species (Lachance *et al.*, 2011; Santos *et al.*, 2011). In addition, *C. coipomoensis* and *C. amazonensis* do not produce acid from glucose, whereas *C. lignicola* and *C. queiroziae* exhibit weak acid formation.

Latin diagnosis of *Candida amazonensis* Cadete, Melo, Lopes, Zilli, Vital, Gomes, Lachance & Rosa sp. nov.

In medio liquido post dies tres cellulae singulae aut binae; cellulae ovoidae aut ellipsoideae (2–3 × 2–4 μm). *Post unum mensem sedimentum formatur. Cultura in agaro malti post dies* 2 (17 °C) *parva, convexa, rugosa et candida. In agaro farinae Zea mays post dies* 14 *pseudohyphae formantur. Glucosum, galactosum, cellobiosum et trehalosum* (*lente*) *fermentantur. Glucosum, sucrosum, galactosum, trehalosum, maltosum, melezitosum* (*variabile*), *cellobiosum, salicinum,* L-*sorbosum,* D-*xylosum,* D-*arabinosum* (*lente*), *ethanolum* (*lente*), *glycerolum, erythritolum, ribitolum,* D-*mannitolum,* D-*glucitolum, succinatum, citratum* (*lente*), *xylitolum et N-acetyl-*D-*glucosaminum assimilantur, at non inulinum, raffinosum, melibiosum, lactosum, amylum solubile,* L-*rhamnosum,* L-*arabinosum,* D-*ribosum, methanolum, galactitolum, myo-inositolum,* DL-*lactatum,* D-*gluconatum,* D-*glucosaminum nec hexadecanum. Ethylaminum, lysinum et cadaverinum assimilantur at non kalium nitricum et natrium nitrosum. Ad crescentiam vitaminae externae necessaria sunt.*

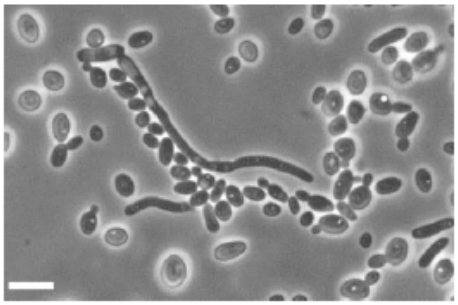

Fig. 2. Phase-contrast micrograph of cells of *Candida amazonensis* sp. nov. grown on yeast nitrogen base with 0.01 % ammonium sulfate after 3 days. Bar, 5 μm.

Augmentum in 37 °C. *Habitat materiam Brazil. Typus* UFMG-HMD-26.3^{T}. *In collectione zymotica* Centraalbureau voor Schimmelcultures, *Trajectum ad Rhenum, sub no.* CBS 12363^{T} *typus stirps deposita est.*

Description of *Candida amazonensis* Cadete, Melo, Lopes, Zilli, Vital, Gomes, Lachance & Rosa sp. nov.

Candida amazonensis (a.ma.zo.nen'sis. N.L. nom. fem. sing. adj., *amazonensis* referring to the region which this yeast was isolated, the Amazonian region).

In yeast extract (0.5 %)/glucose (2 %) broth after 3 days at 25 °C, cells are ovoid to ellipsoidal (2–3 × 2–4 μm). Budding is multilateral (Fig. 2). A sediment is formed after a month, but no pellicle is observed. On YMA after 2 days at 17 °C, colonies are white, convex, rough and opalescent. In Dalmau plates after 2 weeks on cornmeal agar, well-developed pseudohyphae are present. Fermentation of glucose, galactose, cellobiose and trehalose (slow) is positive. Maltose, sucrose and D-xylose are not fermented. Assimilates the following carbon compounds: glucose, sucrose, galactose, trehalose, maltose, melezitose (variable), cellobiose, salicin, L-sorbose, D-xylose, D-arabinose (slow), ethanol (slow), glycerol, erythritol, ribitol, D-mannitol, D-glucitol, succinate, citrate (slow), xylitol and *N*-acetyl-D-glucosamine. No growth occurs on inulin, raffinose, melibiose, lactose, soluble starch, L-rhamnose, L-arabinose, D-ribose, methanol, galactitol, *myo*-inositol, DL-lactate, D-gluconate, D-glucosamine or hexadecane. Positive for assimilation of lysine, ethylamine . HCl and cadaverine, and negative for nitrate and nitrite. Growth in vitamin-free medium is negative. Growth in amino-acid-free medium is positive. Growth at 37 °C is positive. Growth on YM agar with 10 % sodium chloride is positive (slow). Growth in 50 % glucose/yeast extract (0.5 %) is negative. Acid production is negative. Starch-like compounds are not produced. Grows in 100 μg cycloheximide ml^{-1}. Urease activity is negative. Diazonium Blue B reaction is negative.

The type strain is UFMG-HMD-26.3^{T} (=CBS 12363^{T}=NRRL Y-48762^{T}), isolated from rotting wood in the Amazonian forest ecosystem in the state of Roraima, Brazil. Four further reference strains were also isolated from the same source. The Mycobank number is MB 563080.

Acknowledgements

This work was funded by Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq – Brazil), Fundação do Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Procad-NF, process 2280/200) and the Natural Science and Engineering Research Council of Canada (M.-A. L.). We also thank the Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) and Dr Hélio Tonini for their technical support.

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Antonie van Leeuwenhoek (2013) 103:421–431
DOI 10.1007/s10482-012-9822-z

ORIGINAL PAPER

Spathaspora brasiliensis sp. nov., *Spathaspora suhii* sp. nov., *Spathaspora roraimanensis* sp. nov. and *Spathaspora xylofermentans* sp. nov., four novel D-xylose-fermenting yeast species from Brazilian Amazonian forest

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Received: 24 April 2012 / Accepted: 21 September 2012 / Published online: 5 October 2012

Abstract Four new D-xylose fermenting yeast species of the clade *Spathaspora* were recovered from rotting-wood samples in a region of Amazonian forest, Northern Brazil. Three species produced unconjugated asci with a single elongated ascospore with curved ends. These species are described as *Spathaspora brasiliensis*, *Spathaspora suhii* and *Spathaspora roraimanensis*. Two isolates of an asexually reproducing species belonging to the *Spathaspora* clade were also obtained and they are described as *Spathaspora xylofermentans*. All these species are able to ferment D-xylose during aerobic batch growth in rich YP (1 % yeast extract, 2 % peptone and 2 % D-xylose) medium, albeit with differing efficiencies. The type strains are *Spathaspora brasiliensis* sp. nov UFMG-HMD19.3 (=CBMAI 1425=CBS 12679), *Spathaspora suhii* sp. nov. UFMG-XMD16.2 (=CBMAI 1426=CBS 12680), *Spathaspora roraimanensis* sp. nov. UFMG-XMD23.2 (CBMAI 1427=CBS 12681) and *Spathaspora xylofermentans* sp. nov. UFMG-HMD23.3 (=CBMAI 1428=CBS 12682).

Keywords New yeast species · *Spathaspora* · D-xylose-fermenting yeast · Rotting wood · Amazonian forest

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Introduction

Bioethanol production from lignocellulosic material uses substrates, such as agricultural residues and wood waste, that do not compete with food supplies and therefore presents an economic and environmentally sustainable opportunity (Hahn-Hägerdal et al. 2006; Solomon 2010; Cheng and Timilsina 2011). In lignocellulosic feedstocks the second most abundant sugar after glucose is the pentose xylose, and although several yeast species have been shown to be able to

ferment this sugar (including recombinant *Saccharomyces cerevisiae* strains), the efficiency and rates of xylose fermentation are low, challenging the feasibility of industrial production of lignocellulosic bioethanol (Hahn-Hägerdal et al. 2007; Stambuk et al. 2008; Amorim et al. 2011). Therefore, there is still a need for new yeasts capable of efficient xylose fermentation for bioethanol production.

The yeast genus *Spathaspora* contains two sexually-reproducing species, *Spathaspora passalidarum* and *Spathaspora arborariae*, that produce elongate ascospores with curved ends (Nguyen et al. 2006; Cadete et al. 2009). These species are associated with rotting-wood substrates and the insects that occupy this ecological niche. Both species are known for their ability to ferment D-xylose producing ethanol and have some potential for the production of biofuels through fermentation of plant cell wall materials (da Cunha-Pereira et al. 2011; Nguyen et al. 2011). The asexual species *Candida insectamans, Candida jeffriesii*, *Candida lyxosophila, Candida materiae*, and *Candida subhashii* also belong to the *Spathaspora* clade and are also associated with wood substrates or insects (Barbosa et al. 2009; Lachance et al. 2011; Nguyen et al. 2011). Da Cunha-Pereira et al. (2011) reported on the possible use of *S. arborariae* alone or in co-culture with *S. cerevisiae* for ethanol production using rice hull hydrolysates. *S. arborariae* was able to metabolize the xylose and the glucose present in the hydrolysate with an ethanol conversion yield of 0.45 g g^{-1}. *S. passalidarum* simultaneously coferments glucose, cellobiose, and xylose with an ethanol yield of 0.42 g g^{-1}, and it has a specific ethanol production rate on xylose more than 3 times that of the corresponding rate on glucose (Long et al. 2012). Xylose-fermenting species of the *Spathaspora* clade could be used directly for ethanol production or might provide a source of genes, enzymes and/or sugar transporters to engineer industrial strains for the efficient production of bioethanol from renewable biomass (Hahn-Hägerdal et al. 2007; Wohlbach et al. 2011).

During an extensive study of yeasts associated with rotting wood in Brazilian ecosystems, we isolated six D-xylose-fermenting yeasts. Sequence analyses of the ITS region and D1/D2 domains of the large subunit rRNA gene showed that these isolates represented four new species belonging to the clade *Spathaspora*. Three species produced ascospores with curved ends and are here described as *Spathaspora brasiliensis* sp. nov, *Spathaspora suhii* sp. nov. and *Spathaspora roraimanensis* sp. nov. Two isolates representing an asexual *Spathaspora* species closely related to *S. roraimanensis* are described here as *Spathaspora xylofermentans* sp. nov.

Materials and methods

Yeast isolation and identification

The yeasts were isolated from samples of rotting wood collected in two areas of Amazonian forest in the state of Roraima, Northern Brazil. The areas are the site of a long-term experiment maintained by Embrapa-Roraima, located in the municipalities of São João da Baliza (00°56′58″N and 59°54′41″W) and Mucajaí (2°25′48″N and 60°55′11″W). The predominant vegetation is characterized as an Amazonia Forest biome. The climate is hot and humid, with an annual precipitation between 1,300 to 2,900 mm, and an average temperature from 25.6 to 27.6 °C. Forty decayed wood samples were collected, twenty from each area, in October 2009. The samples were stored in sterile plastic bags and transported under refrigeration to the laboratory over a period of no more than 24 h. One gram of each sample was placed separately in 125 mL Erlenmeyer flasks with 20 mL sterile D-xylose medium (yeast nitrogen base 0.67 %, D-xylose 0.5 %, chloramphenicol 0.02 %) or 20 mL sterile xylan medium (yeast nitrogen base 0.67 %, xylan 1 %, chloramphenicol 0.02 %, pH 5.0 ± 0.2), respectively. The flasks were incubated at 25 °C in an incubator shaker (New Brunswick) at 150 rpm for 3–10 days. When growth was detected, 0.5 mL of a culture was transferred to a tube containing 5 mL sterile D-xylose or xylan medium, and the tubes were incubated on an incubator shaker as described above. After growth detection, one loopful of each tube was streaked on D-xylose or xylan agar medium. The plates were incubated at 25 °C until yeast colonies developed (Cadete et al. 2009). The different yeast morphotypes were purified by repeated plating on YM agar (glucose 1 %, peptone 0.5 %, yeast extract 0.3 %, malt extract 0.3 %, agar 2 %) and maintained on GYMP broth (2 % glucose, 0.5 % yeast extract, 0.5 % malt extract, 0.2 % mono sodium phosphate) with 20 % glycerol at −80 °C or in liquid nitrogen for later identification. The yeasts were characterized by

standard methods (Kurtzman et al. 2011a). Preliminary identities were determined using the keys of Kurtzman et al. (2011b). Sporulation was investigated using 1 % glucose–0.01 % yeast extract (GY), Yeast Carbon Base plus 0.01 % ammonium sulfate (YCBAS), dilute (1:9 and 1:19) V8, Fowell's acetate and YM agars at 20 °C for up to four weeks (Cadete et al. 2009).

DNA sequencing and sequence analysis

The ITS1-5.8S-ITS2 region and the D1/D2 variable domains of the large-subunit rRNA gene were amplified by PCR directly from whole cells using primers ITS1 (5′-TAG GTG AAC CTG CGG AAG GAT CAT-3′) and NL4 (5′-GGT CCG TGT TTC AAG ACG G-3′), as described previously (Marinoni and Lachance 2004). The amplified DNA was cleaned on QIAquick PCR columns (Qiagen) and sequenced using an ABI sequencer at the John P. Robarts Research Institute (London, Ontario, Canada). The sequences were assembled, edited and aligned with the program MEGA5 (Tamura et al. 2011). Phylogenetic placement of the new species was based on a neighbor-joining analysis of D1/D2 LSU rRNA gene sequences with Kimura two-parameter distance transforms. The sequence data consisted of 528 aligned positions. The robustness of the tree was assessed by the bootstrap method with 1,000 iterations.

Growth conditions and D-xylose fermentation assays

The ability to ferment D-xylose was tested in Durham tubes containing a 2 % (w/v) solution of the sugar (Kurtzman et al. 2011a). The tubes were incubated at 25 °C on an incubator shaker (New Brunswick) at 100 rpm for up to 25 days. *Candida shehatae* CBS 5813, *C. insectosa* CBS 4286, *C. lignosa* CBS 4705 and *Scheffersomyces stipitis* NRRL Y-7124 were used as positive controls for D-xylose fermentation (Cadete et al. 2009). Ethanol production during aerobic batch growth in YP medium (1 % yeast extract and 2 % peptone) adjusted to pH 5.0 with HCl and supplemented with 2 % glucose or xylose was also performed for all isolates of the new species. For this, the cells were grown in YP medium with shaking at 28 °C (160 rpm) in cotton-plugged Erlenmeyer flasks filled to 1/5 of the volume. The inocula for growth assays were prepared by transferring aseptically a single colony from a plate into 5 mL of the glucose or xylose medium and allowing growth to proceed to stationary phase for 2–3 days. These cells were then used to inoculate fresh media of similar composition at a rate of 1 %. Samples were taken regularly, centrifuged (5,000*g*, 1 min), and their supernatants used for the determination of sugars and ethanol. Glucose was measured by the glucose oxidase and peroxidase method using a commercial kit (BioDiagnostica-Laborclin, Brazil), and xylose was determined with dinitrosalycilic acid as described by Miller (1959). Ethanol was determined with alcohol oxidase (Sigma, USA) and peroxidase (Toyobo do Brasil, Brazil) as described previously (Alves et al. 2007). Growth was followed by turbidity measurements at 570 nm after appropriate dilution of the medium samples in distilled water. The ethanol yields during growth on glucose ($Y_{E/glu}$, g ethanol g^{-1} glucose) or xylose ($Y_{E/xyl}$, g ethanol g^{-1} xylose) were calculated taking into account the amount of sugar consumed at the point of maximum ethanol production. Reported values are averages ± mean deviations obtained from independent duplicate cultures, and were analyzed using the paired *t* test.

Results and discussion

Species delineation, classification, and ecology

Spore morphology of the three sexual species was typical of the genus *Spathaspora* and neighbor-joining analysis of D1/D2 LSU rRNA gene sequences indicated that the four new species belong to the *Spathaspora* clade (Fig. 1). *Spathaspora brasiliensis* and *S. suhii* belong to the same subclade as do *S. arborariae*, *C. jeffriesii*, and *C. materiae*. Only one isolate of *S. brasiliensis* was isolated in the present study. We describe it based on only a single strain because of its ability to ferment D-xylose, a biotechnological trait of industrial interest for production of biofuels through fermentation of plant hydrolysates. *Spathaspora brasiliensis* UFMG-HMD19.3^T differs by eight substitutions in the D1/D2 domains and by 13 differences in the ITS region from its least divergent relative, *C. materiae*. The smallest degree of sequence divergence observed for *Spathaspora suhii* UFMG-XMD16.2^T was seven substitutions and four gaps

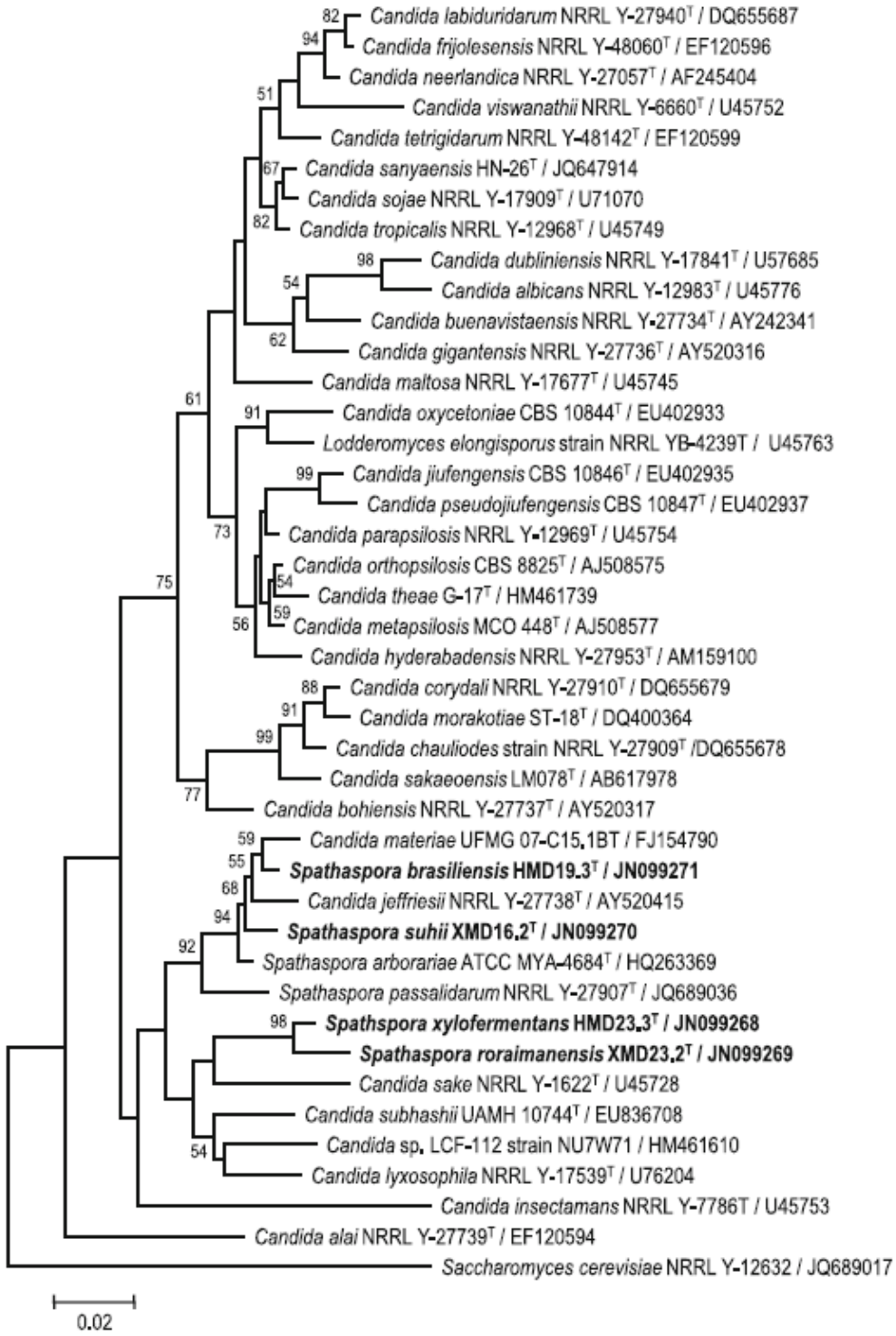

Fig. 1 Phylogenetic placement of *Spathapora brasiliensis, S. roraimanensis, S. suhii*, and *S. xylofermentans* inferred by the neighbor-joining analysis of the D1/D2 LSU rRNA gene regions. Bootstrap values (1,000 replicates) of 50 % and above are shown. Distances were Kimura 2-parameter transformed. A total of 528 positions were used in the analysis, which was conducted with the program MEGA5

from *S. arborariae* in the D1/D2 domains. The ITS and D1/D2 and sequences of the two isolates of *Spathaspora suhii* (UFMG- XMD16.2^{T} and UFMG-HMD16.3) were identical, as were those of the two isolates of *Spathaspora xylofermentans* (UFMG-HMD23.3^{T} and UFMG-HMD 25.1). The two are

sister species but differ by 76 changes in the combined ITS region (19 substitutions and 46 gaps) and D1/D2 domains (6 substitutions and 5 gaps) and are well separated phylogenetically from other members of the genus. In fact, the phylogeny presented in Fig. 1 would suggest that *S. roraimanensis* and *S. xylofermentans* are closer relatives of *Candida sake*, *Candida subhashii*, and *Candida lyxosophila*. However, the low bootstrap values obtained for the relevant subclade betray the weak or conflicting phylogenetic signals present in these sequences. This is further corroborated by the observation that the branching order was greatly dependent on taxon sampling (data not shown), which is why we have included all available species of the *Candida albicans* and *Lodderomyces elongisporus* subclades. Furthermore, analysis of the same data by maximum likelihood or maximum parsimony failed to place all known *Spathaspora* species in the same clade. Given the conserved ascospore morphology observed across the species assigned to this genus, it will be of interest to see eventual multi-locus phylogenies. In particular this is to our knowledge the first suggestion that the enigmatic position of *C. sake* (Lachance et al. 2011) may be on its way to being resolved. Last, given the frail nature of the topology depicted in Fig. 1, the utmost circumspection will be called for in implementing the nomenclatural revisions prompted by the forthcoming Melbourne Code (Norvell 2011), which will require the elimination of dual names and a redefinition of genera such as *Lodderomyces*, *Candida*, and even possibly *Spathaspora*.

All species of the *Spathaspora* clade were isolated from rotting-wood or insects associated with this substrate (Cadete et al. 2009; Nguyen et al. 2011). *Spathaspora brasiliensis* and *S. suhii* were isolated from rotting-wood samples collected in the ecological reserve of São João da Baliza and *S. roraimanensis* and *S. xylofermentans* were obtained from samples collected in the ecological reserve of Mucajaí. All species were minor component of the very diverse yeast communities isolated from rotting wood in these areas (R. M. Cadete and C. A. Rosa, unpublished results).

The new species belonging to the *Spathaspora* clade did not form septate hyphae or pseudohyphae. This morphological characteristic was described as present in *S. passalidarum*, the type species of the genus. Brazilian species belonging to the *Spathaspora* clade (*S. arborariae, S. brasiliensis, S. suhii, S. xylofermentans, S. roraimanensis* and *C. materiae*) be separated from others species based on the assimilation of L-sorbose, which does not occur in the species isolated from other countries (*S. passalidarum, C. insectamans* and *C. jeffriesii*). *Spathaspora roraimanensis* and *S. xylofermentans* can be distinguished from all other species based on the ability to grow in the presence of cycloheximide 0.01 %. The former also assimilated L-arabinose, whereas all other species in the clade do not use this sugar. The new species also differ from *S. passalidarum, S. arborariae*, and *C. jeffriesii* by failing to assimilate D-ribose. Separation of *S. brasiliensis* from *S. suhii* is difficult as these species had identical growth profiles. In this case, sequencing of D1/D2 domains is recommended to differentiate these species.

Growth and fermentation of glucose or xylose by the new species

Although initial results with Durham tubes indicated that the new species were unable to ferment xylose, a detailed analysis of glucose and xylose utilization by these yeasts during aerobic batch growth showed that all can produce ethanol from either sugar, albeit with differing efficiencies (Figs. 2, 3). *Spathaspora xylofermentans* grew well on both carbon sources (Fig. 2a, b) and produced ethanol from both sugars ($Y_{E/glu} = 0.37 \pm 0.02$; $Y_{E/xyl} = 0.34 \pm 0.04$) at yields similar to those reported for other xylose-fermenting *Spathaspora* yeasts (see Cadete et al. 2009). However, the lower levels of ethanol produced during aerobic growth on glucose or xylose by the other three *Spathaspora* species, *S. brasiliensis* ($Y_{E/glu} = 0.08 \pm 0.01$ and $Y_{E/xyl} = 0.12 \pm 0.04$, see Fig. 2c, d), *S. roraimanensis* ($Y_{E/glu} = 0.18 \pm 0.04$ and $Y_{E/xyl} = 0.29 \pm 0.05$, see Fig. 3a, b), and *S. suhii* ($Y_{E/glu} = 0.11 \pm 0.03$ and $Y_{E/xyl} = 0.14 \pm 0.05$, see Fig. 3c, d), indicated that these yeasts (no significant differences for ethanol production were found among these species), like many other yeast species (Suh et al. 2003; Stambuk et al. 2003; Merico et al. 2007; Barbosa et al. 2009; Boonmak et al. 2011; Rozpędowska et al. 2011), preferentially consume these sugars oxidatively. Although the molecular basis for efficient xylose fermentation is complex and not yet fully understood (Hou 2012; Long et al. 2012), the discovery and analysis of these closely related *Spathaspora*

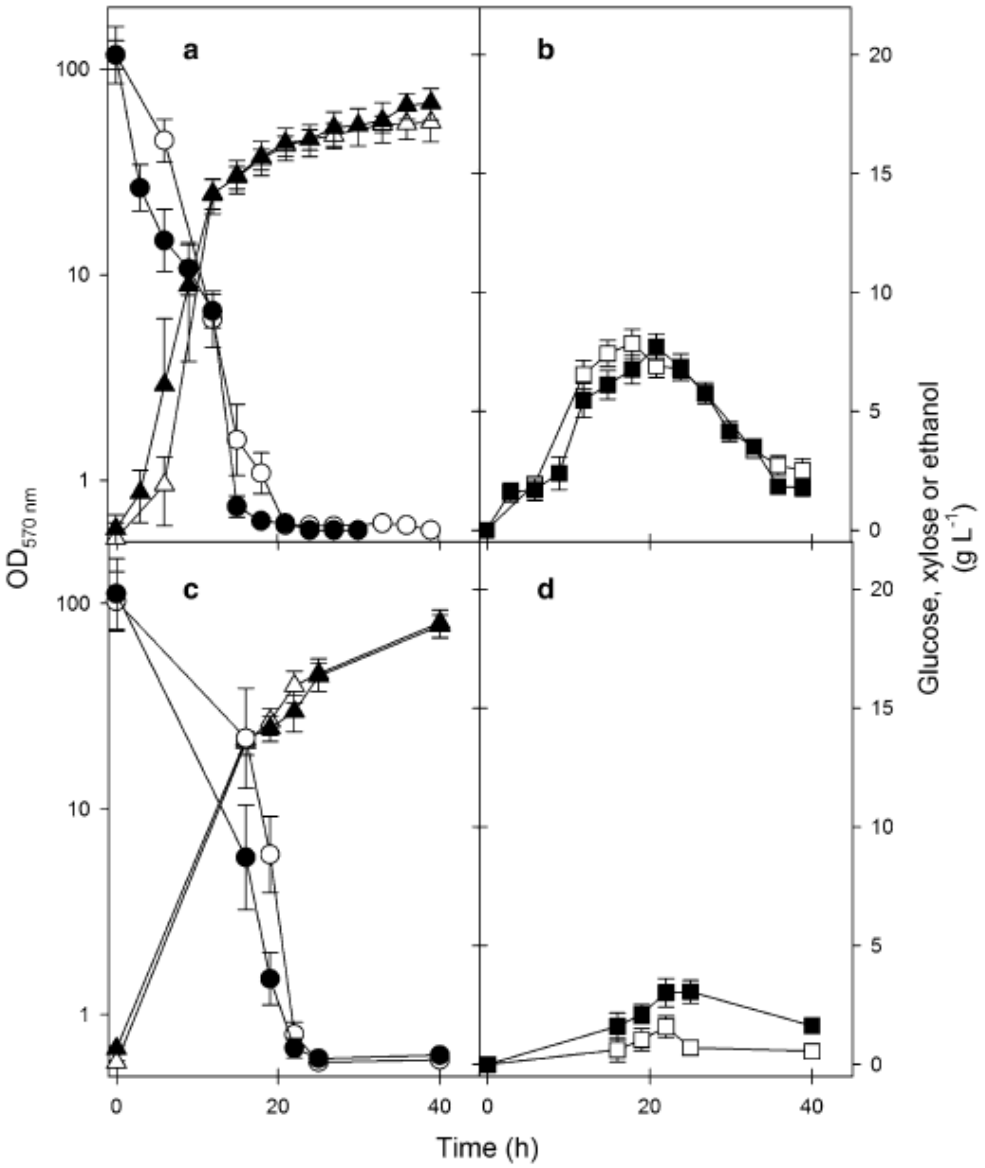

Fig. 2 Typical aerobic batch growth of *Spathaspora xylofermentans* UFMG-HMD25.1 (**a**, **b**) and *Spathaspora brasiliensis* UFMG-HMD19.3 (**c**, **d**) on 20 g L^{-1} of glucose (*open symbols*) or D-xylose (*black symbols*). Cell growth (*triangles*), the consumption of sugars (*circles*), and the production of ethanol (*squares*) by *S. xylofermentans* or *S. brasiliensis* were determined during growth in rich YP medium with shaking (160 rpm) at 28 °C. Reported values are averages ± mean deviations obtained from independent duplicate cultures

species capable of xylose fermentation may contribute important genomic information that could be used to improve the efficiency of pentose assimilation by yeasts (Wohlbach et al. 2011), a significant limitation in cellulosic biofuel production. It will be also important to verify whether the new yeast species can be used directly for bioethanol production from lignocellulosic hydrolysates, as has been recently shown for *S. arborariae* (Da Cunha-Pereira et al. 2011).

Description of *Spathaspora brasiliensis* Cadete, Zilli, Vital, Gomes, Stambuk, Lachance and Rosa sp. nov

In yeast extract (0.5 %), glucose (2 %) broth after 3 days at 25 °C, the cells are ovoid to ellipsoidal (2–3 × 2–4 μm). Budding is multilateral. Sediment is formed after a month, but a pellicle is not observed. On YM agar after 2 days at 17 °C, colonies are white, butyrous, and glistening. In Dalmau plates after

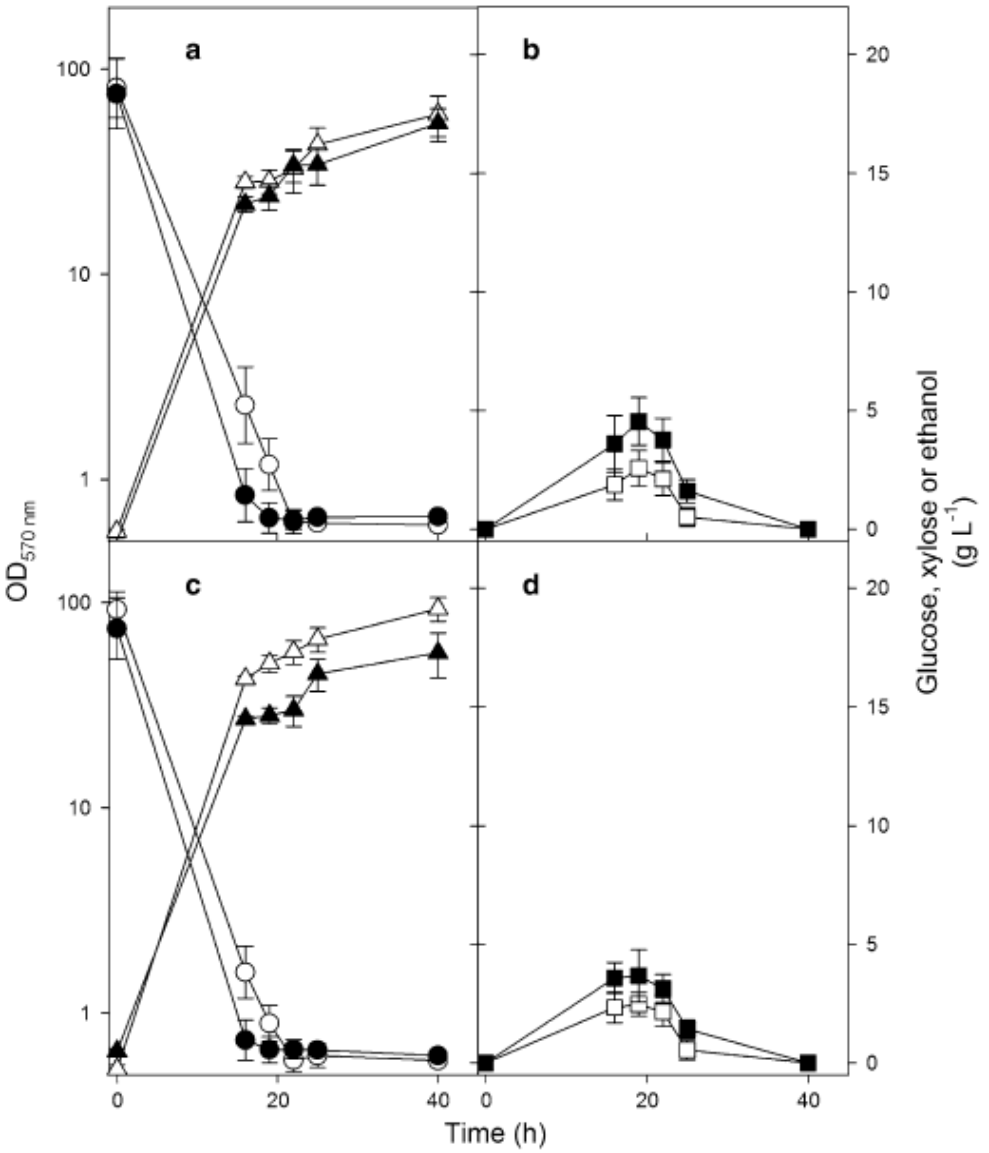

Fig. 3 Typical aerobic batch growth of *Spathaspora roraimanensis* UFMG-XMD23.2 (**a**, **b**) and *Spathaspora suhii* UFMG-XMD16.2 (**c**, **d**) on 20 g L^{-1} of glucose (*open symbols*) or D-xylose (*black symbols*). Cell growth (*triangles*), the consumption of sugars (*circles*), and the production of ethanol (*squares*) by *S. roraimanensis* or *S. suhii* were determined during growth in rich YP medium with shaking (160 rpm) at 28 °C. Reported values are averages ± mean deviations obtained from independent duplicate cultures

2 weeks on cornmeal agar, pseudohyphae are not formed. Sporulation occurs on dilute (1:9 and 1:19) V8 and YCBAS agars at 20 °C after 5 days (Fig. 4). Unconjugated asci are formed from single cells with a single greatly elongated ascospore tapered and curved at the ends. Asci are persistent. Fermentation of glucose, galactose, maltose and trehalose are positive. Xylose fermentation in negative using Durham tubes, but ethanol is produced from xylose when determined with alcohol oxidase and peroxidase tests. Assimilation of carbon compounds: glucose, galactose, L-sorbose, sucrose, maltose, cellobiose, trehalose, melizitose, D-xylose, ethanol (slow or negative), glycerol (slow or negative), erythritol, ribitol, D-mannitol, glucitol, salicin, lactic acid, succinic acid, hexadecane, xylitol, ethyl acetate, and *N*-acetyl-D-glucosamine. No growth occurs on lactose, melibiose, raffinose, inulin, soluble starch, D-arabinose, L-arabinose, D-ribose, L-rhamnose, galactitol, citric acid, myo-inositol, methanol, D-glucosamine, acetone, isopropanol and gluconate. Assimilation of nitrogen compounds: positive for ethylamine–HCl, lysine and

cadaverine, and negative for nitrate and nitrite. Growth in vitamin-free medium is negative. Growth in amino-acid-free medium is positive. Growth at 37 °C is weak. Growth on YM agar with 10 % sodium chloride is negative. Growth in 50 % glucose/yeast extract (0.5 %) is negative. Starch-like compounds are not produced. In 100 μg cycloheximide mL^{-1} the growth is negative. Urease activity is negative. Diazonium Blue B reaction is negative. The habitat is rotting wood in Amazonian forest ecosystem, in the state of Roraima, Brazil.

Type

The type strain accession number of *Spathaspora brasiliensis* is UFMG-HMD19.3^T. It was isolated from rotting wood in Brazil. It has been deposited in the Brazilian Collection of Environmental and Industrial Microorganisms (Coleção Brasileira de Micro-organismos de Ambiente e Industria, CBMAI), Campinas, São Paulo, Brazil, as strain CBMAI 1425^T and in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands, as strain CBS 12679^T. The Mycobank number is MB 801224.

Etymology

The epithet *brasiliensis* (bra.si.li.en′sis) L. nom. f. adj. *brasiliensis*, of or pertaining to the country (Brazil) where this yeast was found.

Description of *Spathaspora roraimanensis* Cadete, Zilli, Vital, Gomes, Stambuk, Lachance and Rosa sp. nov

In yeast extract (0.5 %), glucose (2 %) broth after 3 days at 25 °C, the cells are ovoid to ellipsoidal (2–3 × 2–4 μm). Budding is multilateral. Sediment is formed after a month, but a pellicle is not observed. On YM agar after 2 days at 17 °C, colonies are white, butyrous, and glistening. In Dalmau plates after 2 weeks on cornmeal agar, pseudohyphae are not formed. Sporulation occurs on dilute (1:19) V8 and YCBAS agars at 20 °C after 5 days (Fig. 4). Unconjugated asci are formed from single cells with a single greatly elongated ascospore tapered and curved at the ends. Asci are not dehiscent. Fermentation of glucose, galactose, maltose and trehalose are positive. Xylose fermentation in negative using Durham tubes, but ethanol is produced from xylose when determined with alcohol oxidase and peroxidase tests. Assimilation of carbon compounds: glucose, galactose, L-sorbose, sucrose, maltose, cellobiose, trehalose, D-xylose, L-arabinose (slow), ethanol, erythritol, ribitol, glucitol, salicin, lactic acid, succinic acid, citric acid (variable), hexadecane, xylitol, and *N*-acetyl-D-glucosamine. No growth occurs on lactose, melibiose, raffinose, melizitose, inulin, soluble starch, D-arabinose, D-ribose, L-rhamnose, glycerol, galactitol, D-mannitol, myo-inositol, methanol, D-glucosamine, acetone, isopropanol, ethyl acetate and gluconate. Assimilation of nitrogen compounds: positive for ethylamine–HCl, lysine and cadaverine, and negative for nitrate and nitrite. Growth in vitamin-free medium is negative. Growth in amino-acid-free medium is positive. Growth at 37 °C is weak. Growth on YM agar with 10 % sodium chloride is negative. Growth in 50 % glucose/yeast extract (0.5 %) is negative. Starch-like compounds are not produced. In 100 μg cycloheximide mL^{-1} the growth is positive. Urease activity is negative. Diazonium Blue B reaction is negative. The habitat is rotting wood in Amazonian forest ecosystem, in the state of Roraima, Brazil.

Type

The type strain accession number of *Spathaspora roraimanensis* is UFMG-XMD 23.2^T. It has been deposited in the Brazilian Collection of Environmental and Industrial Microorganisms, Campinas, São Paulo, Brazil, as strain CBMAI 1427^T and in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands, as strain CBS 12681^T. The Mycobank number is MB 801225.

Etymology

The epithet *roraimanensis* (ro.ra.i.ma'ne.sis) L. nom. f. adj. *roraimanenis,* of or pertaining to the state in Brazil where this yeast was found.

Description of *Spathaspora suhii* Cadete, Zilli, Vital, Gomes, Stambuk, Lachance, and Rosa sp. nov

In yeast extract (0.5 %), glucose (2 %) broth after 3 days at 25 °C, the cells are ovoid to ellipsoidal

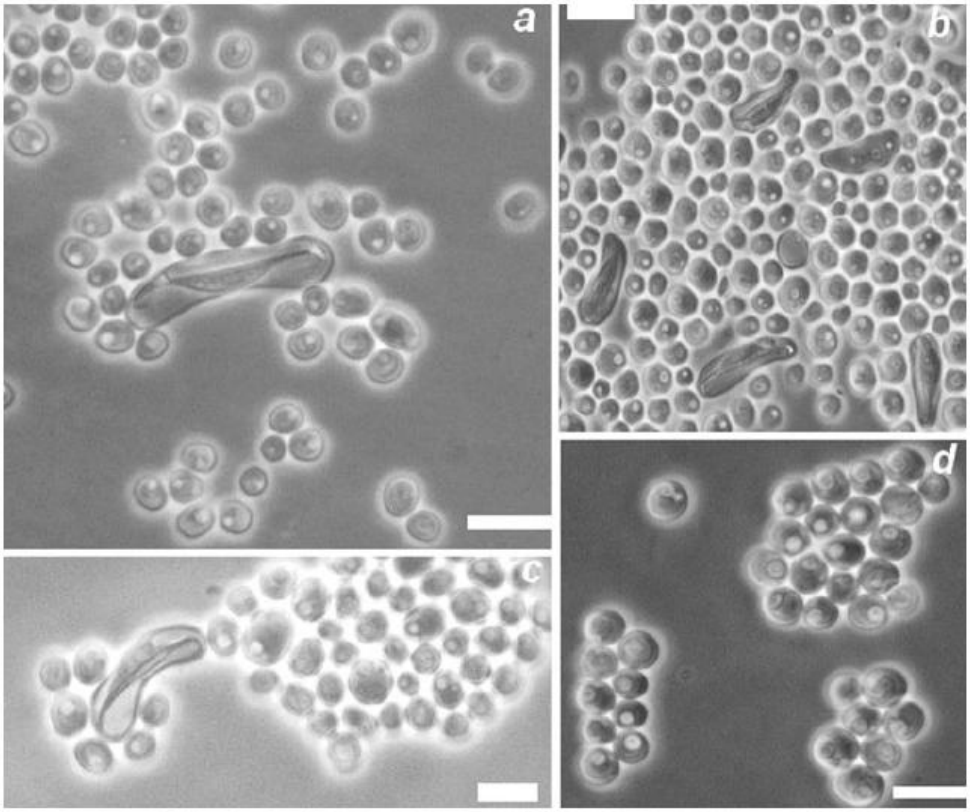

Fig. 4 Budding yeast cells and asci of *Spathaspora brasiliensis* (**a**) *S. roraimanensis* (**b**) *S. suhii* (**c**) and budding cells of *S. xylofermentans* (**d**) on diluted V8 agar after 5 days at 20 °C. *Scale bar* = 5 μm

(2–3 × 2–4 μm). Budding is multilateral. Sediment is formed after a month, but a pellicle is not observed. On YM agar after 2 days at 17 °C, colonies are white, butyrous, and glistening. In Dalmau plates after 2 weeks on cornmeal agar, pseudohyphae are not formed. Sporulation occurs on dilute (1:9) V8 and YCBAS agars at 20 °C after 5 days (Fig. 4). Unconjugated asci are formed from single cells with a single greatly elongated ascospore tapered and curved at the ends. Asci are not dehiscent. Fermentation of glucose, galactose, maltose and trehalose are positive. Xylose fermentation in negative using Durham tubes, but ethanol is produced from xylose when determined with alcohol oxidase and peroxidase tests. Assimilation of carbon compounds: glucose, galactose, L-sorbose, sucrose, maltose, cellobiose, trehalose, melizitose, D-xylose, ethanol (slow), erythritol, ribitol, D-mannitol, glucitol, salicin, lactic acid (variable), succinic acid, hexadecane, xylitol, and *N*-acetyl-D-glucosamine. No growth occurs on lactose, melibiose, raffinose, inulin, soluble starch, D-arabinose, L-arabinose, D-ribose, L-rhamnose, glycerol, galactitol, citric acid, myo-inositol, methanol, D-glucosamine, acetone, isopropanol, ethyl acetate, and gluconate. Assimilation of nitrogen compounds: positive for ethylamine–HCl, lysine and cadaverine, and negative for nitrate and nitrite. Growth in vitamin-free medium is negative. Growth in amino-acid-free medium is positive. Growth at 37 °C is positive. Growth on YM agar with 10 % sodium chloride is negative. Growth in 50 % glucose/yeast extract (0.5 %) is negative. Starch-like compounds are not produced. In 100 μg cycloheximide mL^{-1} the growth is negative. Urease activity is negative. Diazonium Blue B reaction is negative. The habitat is rotting wood in Amazonian forest ecosystem, in the state of Roraima, Brazil.

Type

The type strain accession number of *Spathaspora suhii* is UFMG-XMD16.2^T. It was isolated from rotting wood in Brazil. It has been deposited in the Brazilian Collection of Environmental and Industrial Microorganisms, Campinas, São Paulo, Brazil, as strain

CBMAI 1426^T and in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands, as strain CBS 12680^T. The Mycobank number is MB 801226.

Etymology

The epithet *suhii* (suh'i.i) L. gen. m. n. *suhii,* referring to Dr. Sung-Oui Suh, in recognition of his contributions to yeast systematics and ecology.

Description of *Spathaspora xylofermentans* Cadete, Gomes, Stambuk, Lachance and Rosa sp. nov

In yeast extract (0.5 %), glucose (2 %) broth after 3 days at 25 °C, the cells are ovoid to ellipsoidal (2–3 × 2–4 μm). Budding is multilateral. Sediment is formed after a month, but a pellicle is not observed. On YM agar after 2 days at 17 °C, colonies are white, rough, and glistening. In Dalmau plates after 2 weeks on cornmeal agar, pseudohyphae are not formed. Asci or signs of conjugation were not seen on sporulation media (Fig. 4). Fermentation of glucose, maltose and trehalose are positive. Xylose fermentation in negative using Durham tubes, but ethanol is produced from xylose when determined with alcohol oxidase and peroxidase tests. Assimilation of carbon compounds: glucose, galactose, L-sorbose, sucrose, maltose, cellobiose, trehalose, melizitose, D-xylose, ethanol (slow), glycerol (variable), erythritol, ribitol, glucitol, salicin, lactic acid, succinic acid, citric acid (variable), hexadecane, xylitol, and *N*-acetyl-D-glucosamine. No growth occurs on lactose, melibiose, raffinose, inulin, soluble starch, D-arabinose, L-arabinose, D-ribose, L-rhamnose, galactitol, D-mannitol, myo-inositol, methanol, D-glucosamine, acetone, isopropanol, ethyl acetate, and glucante. Assimilation of nitrogen compounds: positive for ethylamine-HCl, lysine and cadaverine, and negative for nitrate and nitrite. Growth in vitamin-free medium is negative. Growth in amino-acid-free medium is positive. Growth at 37 °C is very weak. Growth on YM agar with 10 % sodium chloride is negative. Growth in 50 % glucose/yeast extract (0.5 %) is negative. Starch-like compounds are not produced. In 100 μg cycloheximide mL^{-1} the growth is positive. Urease activity is negative. Diazonium Blue B reaction is negative. The habitat is rotting wood in Amazonian forest ecosystem, in the state of Roraima, Brazil.

Type

The type strain accession number of *Spathaspora xylofermentans* is UFMG-HMD23.3^T. It was isolated from rotting wood in Brazil. It has been deposited in the Brazilian Collection of Environmental and Industrial Microorganisms, Campinas, São Paulo, Brazil, as strain CBMAI 1428^T and in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands, as strain CBS 12682^T. The Mycobank number is MB 801227.

Etymology

The epithet *xylofermentans* (xy.lo.fer.men'tans) L. nom. f. adj. *xylofermentans,* pertaining to the ability of this yeast to ferment D-xylose.

Acknowledgments This work was supported in part by grants and fellowships from the Brazilian agencies Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq, process no. 552877/2007-7, 551392/2010-0, 551245/2010-7, 560715/2010-2, and 490029/2009-4), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Procad-NF, process no. 2280/2008), Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG), Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP/BIOEN/FAPEMIG, process no. 2008/57926-4), Fundação de Apoio à Pesquisa Científica e Tecnológica do Estado de Santa Catarina (FAPESC, process no. 17293/2009-6), and the Natural Science and Engineering Research Council of Canada (M.-A. L.).

Conflict of interest None.

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4.3 Capítulo III

Exploring xylose metabolism in *Spathaspora* species: *XYL1.2* from *Spathaspora passalidarum* UFMG-HMD-1.1 as the key for efficient anaerobic xylose fermentation in metabolic engineered *Saccharomyces cerevisiae*

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Keywords: *Spathaspora* spp., *Spathaspora passalidarum*, *Saccharomyces cerevisiae*, xylose fermentation, NADH-preferring xylose reductase, bioethanol, *XYL1.2*

Abstract

Background: The deployment of second generation bioethanol is dependent of complete and efficient conversion of xylose usually present in the hemicellulose fraction of lignocellulosic materials. Xylose conversion to ethanol in yeasts is related to a xylose reductase with the capacity to use NADH as co-factor. Among the best xylose-fermenting *Saccharomyces cerevisiae* strains are those expressing a xylose reductase from *Scheffersomyces stipitis*, which is able to use NADH as co-factor but prefers NADPH. Xylose metabolism in *Spathaspora* spp. strains is efficient and the main fermentation products are ethanol and xylitol.

Results: Six strains from different Brazilian *Spathaspora* species were characterized towards xylose fermentation under two different oxygen-limited conditions and the influence on ethanol (and xylitol) production was evaluated. The best xylose-fermenting strains belong to the *Spathaspora passalidarum* species, revealing the highest ethanol titres, yields and productivities under both oxygen-limited conditions. The high ethanol yield from xylose (among 0.47 and 0.48 g/g) observed for *Sp. passalidarum* was associated to xylose reductase activity with preference for NADH as co-factor. Among the different *Spathaspora* species studied, *Sp. passalidarum* is the sole harboring two *XYL1* genes, *XYL1.1* and *XYL1.2*. XYL1.1p is similar to the XYL1p found in other *Spathaspora* and other yeast species, and the coding gene exhibits relatively low expression levels under the conditions tested. Under those conditions, XYL1.2p has relatively high expression levels, the amino acid sequence revealed similarities to engineered XYL1p displaying improved affinity for NADH, notably in one specific amino acid (N2712D). *XYL1.1* and *XYL1.2* from both *Sp. passalidarum* UFMG-HMD1.1 and *Sp. passalidarum* CBS 10155^{T} were expressed in *Sa. cerevisiae* TMB 3044 and their effect compared to the *XYL1* N272D from *Sc. stipitis* in the same isogenic background. XYL1.1p was confirmed to be NADPH-dependent while XYL1.2p to use both NADPH and NADH, with preference for the later. Xylose fermentation by recombinant *Sa. cerevisiae* under anaerobic conditions is successful with the expression of *XYL1.2* genes but not with *XYL1.1*. *XYL1.2* from *Sp. passalidarum* UFMG-HMD1.1 generated the best ethanol yields (78% of theoretical) and volumetric productivities 136% higher (0.26 $g.g^{-1}.h$) than those obtained with *XYL1* N272D from *Sc. stipitis*.

Conclusion: A rational framework for the identification of efficient xylose reductase in *Spathaspora* genus and its heterologous expression in *Sa. cerevisiae* was defined. A newly generated *Sa. cerevisiae* strain harboring *XYL1.2* from *Sp. passalidarum* UFMG-HMD1.1 (TMB3504) represents a

significant advance towards the efficient fermentation of xylose to ethanol, and, consequently, to the effective deployment of lignocellulosic ethanol.

Background

Results

Xylose fermentation by *Spathaspora* spp. under two different oxygen-limited conditions

Six yeasts strains isolated from Brazilian habitats, *Sp. arborariae* UFMG-HM-19.1A^T, *Sp. brasiliensis* UFMG-HMD-19.3^T, *Sp. passalidarum* UFMG-HMD-1.1, *Sp. roraimanensis* UFMG-XMD-23.2^T, *Sp. suhii* UFMG-XMD-16.2^T and *Sp. xylofermentans* UFMG-HMD-23.3^T, plus the reference strain *Sp. passalidarum* CBS 10155^T, were studied under two different oxygen-limited conditions corresponding to an oxygen transfer rate (OTR) of approx. 1-2 mmol/l/min (severe) and 10-15 mmol/l/min (moderate). The results of the fermentation parameters and ethanol and xylitol maximum titres ($g.L^{-1}$) are summarized in Table 1. All yeasts were capable to consume xylose under both oxygen-limited conditions, but the highest xylose consumption rates were attained under moderate conditions (0.58 to 2.78 $g.L^{-1}.h^{-1}$ between 18h and 72h) than in severe conditions (0.05 to 2.08 $g.L^{-1}.h^{-1}$ between 24h and 144h). Overall, regardless the oxygen-limited condition tested, the majority of the yeasts showed xylose consumption over 99%, except *Sp. xylofermentans* under moderate (55.4%) and severe (97.5%) conditions, and *Sp. brasiliensis* (31.5%) and *Sp. suhii* (13.2%) under severe conditions. Both *Sp. passalidarum* strains showed total xylose consumption at remarkably high rateunder both oxygen-limited conditions tested, with a virtually complete consumption between 18h and 24h.

Ethanol and/or xylitol were the major products from xylose fermentation. Accordingly, yeasts were divided into two groups: ethanol-producing yeasts, comprising species showing ethanol as main product, *Sp. passalidarum* strains and *Sp. arborariae*; xylitol-producing yeasts, including the remaining *Spathaspora* species, *Sp. brasiliensis*, *Sp. roraimanensis*, *Sp. suhii* and *Sp. xylofermentans*. Among xylitol-producing yeasts, *Sp. roraimanensis* and *Sp. xylofermentans* showed higher xylitol concentration under severe oxygen-limited conditions (30.7 and 24.4 $g.L^{-1}$, respectively), whereas *Sp. brasiliensis*, *Sp. roraimanensis* and *Sp. suhii* achieved the highest titers under moderate conditions (22.9, 22.7 and 22.4 $g.L^{-1}$, respectively). Lower xylitol production observed for *Sp. brasiliensis* and *Sp. suhii* (11.9 $g.L^{-1}$ and 4.5 $g.L^{-1}$, respectively) under severe oxygen-limited conditions is associated to low xylose consumption rates and uncomplete fermentation (31.5 and 13.2% of xylose consumption, respectively). All xylitol-producing yeasts

revealed low ethanol yields, between 0.05 and 0.15 $g.g^{-1}$. *Spathaspora suhii* was the only species producing no ethanol under severe oxygen-limited conditions. In fact, this yeast hardly grew under this condition of very low oxygen available. *Spathaspora passalidarum*, an ethanol-producing yeast, presented the fastest xylose consumption rate (approx. 2 and 3 $g.L^{-1}.h^{-1}$ under moderate and severe conditions, respectively), acchieved the highest ethanol titers (over 20 $g.L^{-1}$ under both oxygen-limited conditions), yields of (0.47 and 0.48 $g.g^{-1}$) and productivities of 1.13 and 0.85 $g.L^{-1}$.h under moderate and severe oxygen-limited conditions, respectively. Also, *Sp. passalidarum* produced the lowest xylitol concentrations (0.6 $g.L^{-1}$ to 1.4 $g.L^{-1}$) corresponding to yields below 0.05 $g.g^{-1}$. *Spathaspora arborariae* achieved moderate ethanol titers, reaching 10.6 $g.L^{-1}$ and 16.0 $g.L^{-1}$ under moderate and severe oxygen limitation, respectively. However, ethanol productivities were much lower than in *Sp. passalidarum* (< 0.2 $g.L^{-1}$.h). Although this yeast produced ethanol as main fermentation product, a considerable amount of xylitol was obtained, around 6.7 $g.L^{-1}$ and 9.0 $g.L^{-1}$ under moderate and severe oxygen-limited conditions, respectively.

Biochemical characterization of xylose reductase and xylitol dehydrogenase in *Spathaspora* spp.

The enzyme activities associated to the first steps of xylose metabolism, xylose reductase (XR) and xylitol dehydrogenase (XDH) were determined in *Spathaspora* spp. cell extracts after 16 h xylose fermentation under moderate or severe oxygen-limited conditions (Table 2). All the yeasts showed xylitol dehydrogenase activity strictly dependent of NAD^+. On the other hand, xylose reductase activity was NADPH-dependent or accepted both NADH and NADPH depending on the xylose-fermenting groups described above: ethanol- or xylitol-producing yeasts. While xylitol-producing yeasts (*Sp. brasiliensis*, *Sp. roraimanensis*, *Sp. suhii* and *Sp. xylofermentans*) have strictly NADPH-dependent XR activities, ethanol-producing yeasts (*Sp. passalidarum* strains and *Sp. arborariae*) showed XR activities both using NADH and NADPH as co-factor. However, in *Sp. arborariae* xylose metabolism takes place by means of NADPH-preferred XR activity, while in both *Sp. passalidarum* strains this is accomplished by a XR with preference for NADH.

Overall, the XDH activities determined under severe oxygen limitation were higher than in moderate conditions, except for *Sp. suhii*. Instead, higher XR NADPH-dependent activities were detected under moderate oxygen-limited conditions, except for both *Sp. passalidarum* strains and *Sp. xylofermentans*, which showed higher XR NADPH-dependent activities under severe oxygen-limited conditions. XR activities in *Sp. arborariae* UFMG-HM-19.1A are lower under severe oxygen-limited conditions either with NADH or with NADPH as co-factor. These results are correlated to a

higher NADH/NADPH ratio (0.75) under moderate conditions is higher than under severe conditions (0.55). In *Sp. passalidarum*, an increase in XR activities is observed as the limitation of oxygen becomes higher, regardless the co-factor considered. Although it is observed a slight decrease in the NADH/NADPH ratio for both *Sp. passalidarum* strains when the oxygen limitation is shifted from moderate to severe, XRs enzymes in this species have a remarkably preference for NADH, at least over 1.6x than for NADPH. Indeed, in all *Spathaspora* spp. studied in this work, *Sp. passalidarum* was the only species presenting this behavior.

XYL1 gene(s) and encoded proteins in _Spathaspora_ spp.

The amplification, length and identity values of the xylose reductase gene(s) (*XYL1*) for the yeasts studied are summarized in Table 3 and Figure 1, and the amino acids sequences predicted from the amplified genes are characterized in Table 4 and Figure 2. *XYL1 (XYL1.1)* gene was detected in all *Spathaspora* species, with an encoding region of 957 base pairs (bp), resulting in XRs with 318 amino acids (aa). A highly conservative pattern among *XYL1.1* and *XYL1* genes and its encoded proteins (XYL1.1p and XYL1p) was found in this work, with genes identities ranging from 89% to 100% and proteins similarities from 93% to 100%. A phylogram constructed based on the predicted amino acid sequences of the XRs among *Spathaspora* spp is shown in Figure 3. The existence of a second xylose reductase-encoding gene was observed only in *Sp. passalidarum*. *XYL1.2* genes have an encoding region of 954 bp, resulting in XRs with 317 aa. Although the existence of few differences between *XYL1.2* (15 bp) and XYL1.2p (3 aa) among *Sp. passalidarum* CBS 10155 and UFMG-HMD-1.1, the nucleotide and amino acid sequences are highly homologous, with identity values equal to 98% and 99%, respectively, within both strains.

The comparison of *XYL1.1* and *XYL1.2* genes, and XYL1p and XYL1.2p within *Sp. passalidarum* strains showed that, besides the difference of one amino acid in the XRs length, in *Sp. passalidarum* CBS 10155 *XYL1.2* is 77% identical to *XYL1.1*, and XYL1.2p is 74% identical to XYL1.1p. These results are quite similar to those observed for *Sp. passalidarum* UFMG-HMD-1.1, which presented identities of 78% and 75% among its *XYL1* genes and encoded proteins, respectively.

XYL1.1 and _XYL1.2_ transcript analysis in _Spathaspora passalidarum_

The levels of *XYL1.1* and *XYL1.2* transcripts were evaluated by Real-Time RT-PCR using actin and 18S ribosomal RNAs as internal controls (Table 7). *XYL1.2* is more expressed than *XYL1.1* in both *Sp. passalidarum* strains, 5-fold in *Sp. passalidarum* CBS 10155 and 10-fold in *Sp. passalidarum* UFMG-HMD1.1 under both oxygen-limited conditions. While *XYL1.2* expression is slightly lower

under severe oxygen-limited conditions in *Sp. passalidarum* CBS 10155, on contrary, in *Sp. passalidarum* UFMG-HMD1.1, its expression is slightly higher in severe than in moderate oxygen-limited conditions.

The effect of *SpXYL1.1* and *SpXYL1.2* expression in *Saccharomyces cerevisiae*

Within the *Spathaspora* species studied in this work, *Sp. passalidarum* is the sole presenting XR activities with preference for NADH and harboring two *XYL1* genes, *XYL1.1* and *XYL1.2*. In order to study the influence of *XYL1.1 and XYL1.2* from the two *Sp. passalidarum*, the genes were individually expressed in *Sa. cerevisiae* TMB 3044, the most suitable TMB strain for testing novel *XYL1* genes. The expression of the four genes in *Sa. cerevisiae* TMB 3044 generated strains *Sa. cerevisiae* TMB 3501, TMB 3502 (*XYL1.1* from *Sp. passalidarum* CBS 10155 and UFMG-HMD-1.1, respectively), TMB 3503 and TMB 3504 (*XYL1.2* from *Sp. passalidarum* CBS 10155 and UFMG-HMD-1.1, respectively).

Since *Sa. cerevisiae* TMB 3501 and TMB 3502 were unable to grow anaerobically, the recombinant *Sa. cerevisiae* strains were characterized in terms of xylose reductase activity and co-factor specificity under aerobic conditions in YNB-xylose medium (Table 8). Strains *Sa. cerevisiae* TMB 3501 and TMB 3502 revealed strict NADPH-dependent XR activity, while *Sa. cerevisiae* TMB 3503 and TMB 3504 reveal dual co-factor utilization with preference for NADH (NADH/NADPH ratio of 1,34 and 1,21, respectively). However, the XR activity of *Sa. cerevisiae* TMB 3504 is approx. 3-fold the one determined in *Sa. cerevisiae* TMB 3503.

Anaerobic batch fermentation revealed that among the strains expressing *Sp. passalidarum* XRs, only *Sa. cerevisiae* TMB 3504 (harbouring *S. passalidarum* UFMG-HMD-1.1 *XYL1.2*) was able to anaerobically perform a successful conversion of xylose into ethanol. Strains *Sa. cerevisiae* TMB 3501 and TMB 3502, both engineered with *XYL1.1*, failed to grow during the entire assay. Unlike *Sa. cerevisiae* TMB 3504, strain *Sa. cerevisiae* TMB 3503 (CBS 10155 *XYL1.2*) showed a poor performance under the conditions tested, consuming 8.9 $g.L^{-1}$ xylose (16.6% of total xylose) and producing 0.5 $g.L^{-1}$ cells, 2.5 $g.L^{-1}$ ethanol and 0.9 $g.L^{-1}$ xylitol in 95h (data not shown). Thus, *Sa. cerevisiae* TMB 3504 was compared with TMB 3422, which carries the *Sc. stipitis* XR mutation N272D, in anaerobic batch fermentation under controlled conditions with 50 $g.L^{-1}$ xylose (Table 9 and Figure 4). *Saccharomyces cerevisiae* TMB 3504 showed 80% of sugar conversion in approx 50 h (Figure 4, B), with relatively constant xylose consumption rate and ethanol production rate, reaching in that period at approx. 15 $g.L^{-1}$. Than it slightly increase up to 18.4 $g.L^{-1}$ at 72h and 19.7

$g.L^{-1}$ at 142h. Otherwise, ethanol production rate by strain *Sa. cerevisiae* TMB 3422 is constant approx. until 100h (Figure 4, A), when it reaches the maximum concentration (16.2 $g.L^{-1}$). While strain *Sa. cerevisiae* TMB 3422 showed ethanol yield of 0.33 $g.g^{-1}$, *Sa. cerevisiae* TMB 3504 revealed a remarkable yield of 0.40 $g.g^{-1}$. Moreover, ethanol volumetric productivity is 136% higher in *Sa. cerevisiae* TMB 3504 (Qp = 0.26 $g.L^{-1}.h$) than in TMB 3422 (Qp = 0.11 $g.L^{-1}.h$). This results from a remarkable 2-fold higher xylose consumption rate in *Sa. cerevisiae* TMB 3504 (approx. 1.25 $g.L^{-1}.h^{-1}$) compared to *Sa. cerevisiae* TMB 3422 (0.62 $g.L^{-1}.h^{-1}$) and from higher anaerobic growth (3.3 $g.L^{-1}$ and 1.9 $g.L^{-1}$ cell dry wight in *Sa. cerevisiae* TMB 3504 and TMB 3422, respectively). Additionally, *Sa. cerevisiae* TMB 3504 produced almost 2-fold less xylitol (5.4 $g.L^{-1}$) than TMB 3422 (10.4 $g.L^{-1}$).

XR activity was determined in crude cell-free extracts from *Sa. cerevisiae* TMB 3504 and TMB 3422 after approx. 50h of anaerobic batch fermentation (Table 9). Both *Sa. cerevisiae* TMB 3504 and TMB 3422 showed xylose reductase activities NAD(P)H-dependent. However, whereas TMB 3422 has a NADPH-preferred XR, the opposite is attained from XR codified by *Sp. passalidarum* UFMG-HMD-1.1 *XYL1.2*, which preference for NADH is close to 1.2x than for NADPH. This remarkably preference for NADH presented by TMB 3504 is correlated with the great results achivied by this strain in our study relative to its anaerobical conversion of xylose to ethanol.

Discussion

Xylose fermentation by *Sa. cerevisiae*

Conclusions

A rational framework for the identification of efficient xylose reductase in *Spathaspora* genus and its heterologous expression in *Sa. cerevisiae* was defined. The characterization of xylose fermentation, XR/XDH activities and the identification and characterization of the coding genes (*XYL1*) allowed the selection of two genes, one from each *Sp. passalidarum* studied, coding for XR activity with preference for NADH. *Saccharomyces cerevisiae* TMB3504 is a new recombinant xylose-fermenting strain harboring *XYL1.2* from *Sp. passalidarum* UFMG-HMD1.1. This strain revealed an ethanol yield of 0.4 $g.g^{-1}$ with volumetric productivity of 0.26 $g.L^{-1}.h^{-1}$ against 0.33 $g.g^{-1}$ and 0.11 $g.L^{-1}.h^{-1}$ for the reference strain used (*Sa. cerevisiae* TMB3422), previously reported as the best recombinant xylose-fermenting lab strain harbouring an heterologous XR/XDH pathway (from *Sc. stipitis*). These results represents a significant advance towards the efficient fermentation of xylose to ethanol

through the XR/XDH pathway, thus contributing towards the effective deployment of lignocellulosic ethanol.

Methods

Strains and maintenance

Brazilian *Spathaspora* strains *Sp. arborariae* UFMG-HM-19.1A^T, *Sp. brasiliensis* UFMG-HMD-19.3^T, *Sp. passalidarum* UFMG-HMD-1.1, *Sp. roraimanensis* UFMG-XMD-23.2^T, *Sp. suhii* UFMG-XMD-16.2^T and *Sp. xylofermentans* UFMG-HMD-23.3^T were obtained from the Collection of Microorganisms and Cells of Universidade Federal of Minas Gerais (Coleção de Micro-organismos e Células da Universidade Federal de Minas Gerais – UFMG), Brazil, and *Sp. passalidarum* CBS 10155^T was supplied by the Centraalbureau voor Schimmelcultures (CBS) culture collection. *Saccharomyces cerevisiae* strains TMB 3044 and TMB 3422 were provided by the Teknisk Mikrobiologi (TMB) culture collection (Lund, Sweden). All strains were stored in 15% glycerol at – 80°C.

Xylose fermentations under oxygen-limited conditions with *Spathaspora* spp.

Fermentation experiments were carried out on YPX medium (yeast extract, 10 g.L^{-1}; peptone, 20 g.L^{-1}; D-xylose, 40-50 g.L^{-1}) in shake flasks under the pre-established oxygen-limited conditions, being (1) moderate: 100 mL working volume in 250 mL cotton plugged flask and (2) severe: 100 mL working volume in 100 mL rubber plugged flask with needle to allow the release of CO_2. Oxygen transfer rate (OTR) was determined by direct measurements in the culture media (with or without cells), at several time intervals, with an oxygen electrode after sparging with nitrogen. Yeasts were pre-grown on YMA medium (glucose, 10 g.L^{-1}; peptone, 5 g.L^{-1}; yeast extract, 3 g.L^{-1}; malt extract, 3 g.L^{-1}; agar, 20 g.L^{-1}) for 24-48h, and single colonies were transferred to 50 mL YPX in 250 mL shake flasks at 30°C and 200 rpm. Cells were recovered by centrifugation at 2,600 x*g* for 20 min, washed twice with sterile water and resuspended in the fermentation media as inoculum with a final concentration of 0.5 g.L^{-1} (Cadete et al. 2012). The flasks were incubated at 30°C and 200 rpm and the fermentation was monitored by taking samples between 0 and 144h, according to the conditions of oxygen limitation employed. Samples were stored at – 20°C until analysis. All experiments were performed in duplicate. Cell biomass was determined by correlating optical density (OD) measurements taken with a Thermo Spectronic Genesys 20 Model 4001/4 spectrophotometer (Thermo Scientific, Waltham, USA) at 600 nm (OD_{600}) with a previously constructed calibration curve (cell dry weight x optical density). Xylose, ethanol, xylitol, glycerol, and acetate were analyzed

by a high performance liquid chromatography system (Merck Hitachi, Darmstadt, Germany), equipped with an Aminex HPX-87H column (Bio-Rad Hercules, USA) and a refractive index detector (L-7490, Merck Hitachi, Darmstadt, Germany). The column was eluted with 5 mM H_2SO_4 as mobile phase at a flow rate of 0.4 mL.min^{-1}, 50°C. Fermentation parameters [Yp/s^{et} (g.g^{-1}), ethanol yield; Yp/s^{xy} (g.g^{-1}), xylitol yield; Qp (g.g^{-1}.h), ethanol volumetric productivity; $r_{s\ max}$ (g.L^{-1}.h^{-1}), D-xylose (volumetric) consumption rate and D-xylose consumption (%)] were calculated as described previously (Cadete et al. 2012).

Enzyme activities

For the enzymatic activity assay of XR and XDH in *Spathaspora* spp, yeasts were grown in YPX medium as described above under both oxygen-limited conditions tested. After 16h, cells were recovered, washed with sterile deionized water and used to obtain crude cell-free extracts using Y-PER® Yeast Protein Extraction Reagent (Pierce, Rockford, USA). Protein concentrations in the cell-free extract were determined by BCA Protein Assay Kit (Pierce, Rockford, USA). Enzymatic activities were determined spectrophotometrically by following the oxidation or reduction of the coenzymes at 340 nm using UV-2401 PC UV-PIS recording spectophotometer (Shimadzu, Kyoto, Japan) at 25°C, with an interval time of 1 sec for recording and a total measuring time of 90 sec for each reaction. Kinetic parameters of XR for xylose reduction were obtained in a reaction mixture containing 200 mM triethanolamine buffer pH 7.0, 10 mM NAD(P)H, 2 M D-xylose, cell-free extract and deionized water, while kinetic parameters of XDH for xylitol oxidation were obtained in a reaction mixture containing 200 mM glycine buffer pH 9.0, 500 mM $MgCl_2$, 60 mM NAD(P)$^+$, 2 M xylitol, cell-free extract and deionized water. A value of 5.33 mM^{-1}.cm^{-1} was used for the absorption coefficient of NAD(P)H. One unit produced 1 μmol NAD(P)H per min. The specific enzyme activities were given in units (U) per mg protein (Fonseca et al. 2007). This experiment was performed in biological duplicate.

Enzymatic activities of XR in TMB strains (3501, 3502, 3503 and 3504) grown in aerobic conditions, at 30°C and 200 rpm, for 48 h in shake flasks containing 2x YNB medium with 50 g.L^{-1} xylose and 50 mM potassium hydrogen phthalate pH 5.5 were also performed. Reaction mixtures and the calculations of specific enzyme activities were conducted as described above for determination of *Spathaspora* spp. XR activities.

Genetic analysis of *XYL1* gene(s) in *Spathaspora* spp.

Based on genome sequencing of *Spathaspora passalidarum* NRRL Y-27907 (CBS 10155) (Wohlbach et al. 2011) and *Sp. arborariae* UFMG-HM-19.1A (CBS 11463, unpublished), respectively with two and one putative *XYL1* gene(s), the following primers were designed (Table 6): SpspXYL1.1_F and SpspXYL1.1_R to identify/obtain *XYL1.1* (*XYL1*); and SppaXYL1.2_F and SppaXYL1.2_R2 to identify/obtained a putative *XYL1.2*, previously assigned in *Sp. passalidarum* (Wohlbach et al. 2011). The amplified fragments were submitted to DNA sequencing (STAB Vida, Portugal) to confirm *XYL1.1* gene sequence in *Sp. passalidarum* CBS 10155, *XYL1* gene sequence in *Sp. arborariae* UFMG-HM-19.1A, and to identify *XYL1* genes sequences in the remaining strains. The sequences obtained were used to predict the amino acid residues forming the XR proteins and DNA and amino acid sequences were aligned using the free software BioEdit Sequence Alignment Editor (Ibis Biosciences).

Transcript analysis of *XYL1.1* and *XYL1.2* expression in *Spathaspora passalidarum*

Samples from *Sp. passalidarum* cultures (CBS 10155 and UFMG-HMD1.1) were taken for transcript analysis of *XYL1.1* and *XYL1.2* along with samples for enzyme activities experiments (i.e. YPX medium, under moderate and severe oxygen-limited conditions, after 16h). Samples were immediately frozen in liquid nitrogen, and stored at -80ºC prior to extraction. RNA extraction was performed with Direct-zol™ RNA MiniPrep w/ TRI-Reagent®. First-Strand cDNA Synthesis was obtained with High-Capacity cDNA Reverse Transcription Kit for 200 Reactions (Applied Biosystems) with the following cycle: 25ºC 10 min, 37ºC 120 min and 85ºC 5 min, in C1000 Touch™ Thermal Cycler (BIORAD). Real-time primers a are listed in Table 6. This step was performed in duplicate with Real-time Power SYBR® Green PCR (Applied Biosystems) with the following cycle: 94ºC 3 min, 40 cycles of 94ºC, 55ºC and 72ºC, 0.5 min each temperature, 72ºC 4 min. The mRNA expression levels of *XYL1.1* and *XYL1.2* were evaluated using actin and 18S ribosomal RNAs as internal controls, analysed with Pfaffl method (Pfaffl, 2001).

Construction of *Sa. cerevisiae* TMB 3501, TMB 3502, TMB 3503 and TMB 3504

Plasmids and strains used for metabolic engineering of xylose-fermentation in *Sa. cerevisiae* are listed in Table 5. *Escherichia coli* NEB 5-alpha (New England BioLabs, Ipswich, USA) used for cloning was grown at 37°C on LB medium (tryptone, 10 $g.L^{-1}$; yeast extract, 5 $g.L^{-1}$; NaCl, 5 $g.L^{-1}$. *Saccharomyces cerevisiae* strains were grown at 30°C on YPD medium (yeast extract, 10 $g.L^{-1}$; peptone, 20 $g.L^{-1}$; glucose, 20 $g.L^{-1}$) supplemented with 20 $g.L^{-1}$ agar whenever necessary. Plasmid DNA was prepared with GeneJET™ Plasmid Miniprep Kit (Thermo Scientific, Waltham, USA). Agarose gel DNA extraction was performed with QIAquick® Gel Extraction Kit (Qiagen GmbH,

Hilden, Germany). PCR amplification was conducted in C1000™ Thermal Cycler (Bio-Rad, Hercules, USA) using Phusion™ Hot Start High-Fidelity DNA Polymerase (Thermo Scientific, Waltham, USA) and dNTP from Thermo Scientific (Waltham, USA). PCR product purification was carried out with GeneJET™ PCR Purification Kit (Thermo Scientific, Waltham, USA). DNA sequencing was performed by Eurofins MWG Operon (Ebersberg, Germany) or by STAB Vida (Caparica, Portugal). Restriction endonucleases, Thermosensitive Alkaline Phosphatase and T4 DNA Ligase from Thermo Scientific (Waltham, USA) were used for DNA manipulation. *Sp. passalidarum* strains CBS 10155 and UFMG-HMD-1.1 *XYL1.1* and *XYL1.2* genes were respectively amplified with the designed primers SppaXYL1.1_XbaIF, SppaXYL1.1_XbaIR2, SppaXYL1.2_XbaIF and SppaXYL1.2_XbaIR, listed in Table 6. Purified amplicons were digested with *Xba*I and the resulting fragments were inserted into the plasmid YIpOB8 (Bengtsson et al. 2008) previously digested with *Xba*I to excise *Sc. stipitis XYL1* gene, creating YIpRC1, YIpRC2, YIpRC4 and YIpRC5. Correct orientation and sequence of the inserts were verified by restriction fragment analysis and sequencing. Competent *E. coli* NEB 5-alpha cells were transformed as described previously (Inoue et al. 1990), and transformed *E. coli* strains were selected on LB plates containing 100 $mg.L^{-1}$ ampicillin. The constructed plasmids were purified and subsequently cleaved with *EcoRV* within the *URA3* gene and transformed into TMB 3044 (Karhumaa et al. 2005) by the lithium acetate method (Güldener et al. 1996). Transformed yeast strains were selected on YNB plates (Yeast Nitrogen Base w/o amino acids, 6.7 $g.L^{-1}$; agar, 20 $g.L^{-1}$) containing 20 $g.L^{-1}$ D-xylose.

Xylose fermentations under anaerobic conditions with recombinant *Sa. cerevisiae*

Anaerobic batch fermentation was carried out in a flat-bottomed1.4-liter Multifors bioreactor vessel (Infors AG, Bottmingen, Switzerland) with a working volume of 800 mL. Cells were pre-cultivated at 30°C and 180 rpm in 250 mL shake flasks containing 50 mL of 2x YNB medium (Yeast Nitrogen Base w/o amino acids, 13.4 $g.L^{-1}$) with 50 $g.L^{-1}$ D-xylose and 50 mM potassium hydrogen phthalate pH 5.5, recovered by centrifugation at 2,600 x *g* for 20 min, washed twice with sterile water and inoculated into the bioreactor to an optical density at 620 nm (OD_{620}) of ~ 0.2. Fermentation was conducted on 2x YNB medium with 50 $g.L^{-1}$ D-xylose, 0.01 $g.L^{-1}$ ergosterol and 0.5 $mL.L^{-1}$ antifoam RD emulsion (Dow Corning®, Midland, USA). Temperature was maintained at 30°C, pH was controlled at 5.5 through addition of 3 M KOH and stirring was set to 200 rpm. Anaerobic conditions were attained by sparging with nitrogen gas containing less than 5 ppm O_2 (AGA GAS AB, Sundbyberg, Sweden) at a flow rate of 200 mL min^{-1} before inoculation. During fermentation, anaerobic conditions were maintained by the produced CO_2 that diffused through a water lock.

Cultures were sampled aseptically between 0 and 142h and stored at – 20°C until analysis. Strain *Sa. cerevisiae* TMB 3422 (Runquist et al. 2010), carrying *Sc. stipitis XYL1* (N272D) gene was used reference for *Sa. cerevisiae* TMB 3503 and TMB 3504 carrying native *S. passalidarum XYL1.2* genes. The experiments were performed in biological duplicates. Growth was determined by measuring OD_{620} with a Ultrospec 2100 pro spectrophotometer (Amersham Biosciences, Uppsala, Sweden). Xylose, ethanol, xylitol, glycerol, and acetate were analyzed by high performance liquid chromatography system (Waters, Milford, USA) with an Aminex HPX-87H ion exchange column (Bio-Rad Hercules, USA) and a refractive index detector (RID-6A, Shimadzu, Kyoto, Japan). The mobile phase was 5 mM H_2SO_4, at a flow rate of 0.6 ml, 45°C. Cell dry weight was determined in triplicate by filtering a known volume of culture broth through a pre-weighed 0.45 μm Supor® 450 Membrane filters (Pall Corporation, Port Washington, USA), washing with distilled water, drying in a microwave oven and weigh. Fermentation parameters were calculated as described above.

References

Acknowledgements

RMC acknowledge … and CAPES for the PDEE fellowship.

The authors acknowledge to EU-Brazil Project ProEthanol2G (FP7-251151).

CNPq and FAPEMIG

Table 1. D-Xylose consumption and product formation (biomass, ethanol and xylitol) ($g.L^{-1}$) in YPX fermentation assays with *Spathaspora* spp. under moderate (1) and severe (2) oxygen-limited conditions.

Oxygen-limited conditions	Yeast species	Yeast strains	D-xylose consumption (%)[1]	$r_{s\,max}$ ($g.L^{-1}.h^{-1}$)[2]	Cells final biomass ($g.L^{-1}$)	Maximum ethanol concentration (g/L)	$Y_{p/s}^{et}$ ($g.g^{-1}$)[3]	Qp ($g.L^{-1}.h$)[4]	Maximum xylitol concentration (g/L)	$Y_{p/s}^{xy}$ (g/g)[5]
1. Moderate	*Spathaspora arborariae*	UFMG-HM-19.1A ^{T}	99.2	0.69	12.6	10.6	0.21	0.15	6.7	0.14
	Sp. brasiliensis	UFMG-HMD-19.3 ^{T}	99.5	0.69	10.9	3.7	0.08	0.05	22.9	0.49
	Sp. passalidarum	UFMG-HMD-1.1	99.3	2.78	8.9	20.2	0.47	1.12	1.1	0.02
	Sp. roraimanensis	UFMG-XMD-23.2 ^{T}	100	1.04	5.2	5.8	0.13	0.12	22.7	0.47
	Sp. suhii	UFMG-XMD-16.2 ^{T}	100	0.83	7.1	6.1	0.14	0.10	22.4	0.49
	Sp. xylofermentans	UFMG-HMD-23.3 ^{T}	55.4	0.58	8.7	2.6	0.09	0.05	10.4	0.31
	Sp. passalidarum	CBS 10155 ^{T}	99.1	2.78	9.4	20.3	0.48	1.13	0.6	0.01
2. Severe	*Sp. arborariae*	UFMG-HM-19.1A ^{T}	99.3	0.42	6.0	16.0	0.32	0.13	9.0	0.19
	Sp. brasiliensis	UFMG-HMD-19.3 ^{T}	31.5	0.22	3.6	2.6	0.15	0.04	11.9	0.53
	Sp. passalidarum	UFMG-HMD-1.1	99.4	2.08	4.6	20.5	0.47	0.85	1.4	0.03
	Sp. roraimanensis	UFMG-XMD-23.2 ^{T}	100	0.35	5.6	3.0	0.05	0.02	30.7	0.67
	Sp. suhii	UFMG-XMD-16.2 ^{T}	13.2	0.05	0.7	-	-	-	4.5	0.63
	Sp. xylofermentans	UFMG-HMD-23.3 ^{T}	97.5	0.34	7.3	3.7	0.08	0.03	24.4	0.55
	Sp. passalidarum	CBS 10155 ^{T}	99.4	2.08	5.0	20.5	0.48	0.85	1.4	0.03

[1] D-xylose consumption (%) – percentage of initial D-xylose consumed
[2] $r_{s\ max}$ ($g.L^{-1}.h^{-1}$) – D-xylose (volumetric) consumption rate
[3] $Yp_{/s}^{et}$ ($g.g^{-1}$) – ethanol yield: correlation between ethanol ($\Delta P_{ethanol}$) produced and D-xylose (ΔS_{xylose}) consumed
[4] Qp ($g.L^{-1}.h$) – ethanol volumetric productivity: ratio of ethanol concentration ($g.L^{-1}$) and fermentation time (h)
[5] $Y_{p/s}^{xy}$ ($g.g^{-1}$) – xylitol yield: correlation between xylitol ($\Delta P_{xylitol}$) produced and D-xylose (ΔS_{xylose}) consumed

Table 2. *Spathaspora* spp. xylose reductase (XR) and xylitol dehydrogenase (XDH) activities expressed in units (U) per mg protein [U. $(mg\ protein)^{-1}$] after 16 h of fermentation in YPX medium under moderate (1) and severe (2) oxygen-limited conditions.

Oxygen-limited conditions	Yeast species	Yeast strains	XR		XDH	Ratio NADH/NADPH
			NADH	NADPH	NAD^+	
1. Moderate	*Spathaspora arborariae*	UFMG-HM-19.1A [T]	0.54±0.02	0.72±0.06	0.57±0.05	0.75
	Sp. brasiliensis	UFMG-HMD-19.3 [T]	-	0.39±0.02	0.18±0.01	-
	Sp. passalidarum	UFMG-HMD-1.1	0.83±0.12	0.46±0.06	0.60±0.07	1.81
	Sp. roraimanensis	UFMG-XMD-23.2 [T]	-	0.64±0.04	0.74±0.02	-
	Sp. suhii	UFMG-XMD-16.2 [T]	-	0.51±0.03	0.74±0.04	-
	Sp. xylofermentans	UFMG-HMD-23.3 [T]	-	0.09±0.01	0.12±0.01	-
	Sp. passalidarum	CBS 10155 [T]	2.81±0.31	1.62±0.17	1.80±0.14	1.74
2. Severe	*Sp. arborariae*	UFMG-HM-19.1A [T]	0.33±0.04	0.61±0.01	0.84±0.07	0.55
	Sp. brasiliensis	UFMG-HMD-19.3 [T]	-	0.35±0.01	0.60±0.01	-
	Sp. passalidarum	UFMG-HMD-1.1	1.09±0.12	0.63±0.14	1.17±0.12	1.72
	Sp. roraimanensis	UFMG-XMD-23.2 [T]	-	0.26±0.03	0.87±0.01	-
	Sp. suhii	UFMG-XMD-16.2 [T]	-	0.20±0.01	0.63±0.04	-
	Sp. xylofermentans	UFMG-HMD-23.3 [T]	-	0.27±0.01	0.54±0.01	-
	Sp. passalidarum	CBS 10155 [T]	4.07±0.55	2.46±0.27	2.19±0.05	1.66

Table 3. Amplification of *XYL1 (XYL1.1)* and *XYL1.2* genes in *Spathaspora* spp. with the primers SpspXYL1.1_F, SpspXYL1.1_R, SppaXYL1.2_F and SppaXYL1.2_R2.

Yeast species	Yeast strains	*XYL1 (XYL1.1)* [length bp]	Identity (%)[1]	*XYL1.2* [length bp]	Identity (%)[1]	Reference
Sp. passalidarum	CBS 10155^T	+ [957]	100	+ [954]	100	Wohlbach et al., 2011; this work
Sp. arborariae	UFMG-HM-19.1A^T	+ [957]	89	-	-	Unpublished data; this work
Sp. brasiliensis	UFMG-HMD-19.3^T	+ [957]	93	-	-	This work
Sp. passalidarum	UFMG-HMD-1.1	+ [957]	98	+ [954]	98	This work
Sp. roraimanensis	UFMG-XMD-23.2^T	+ [957]	89	-	-	This work
Sp. suhii	UFMG-XMD-16.2^T	+ [957]	99	-	-	This work
Sp. xylofermentans	UFMG-HMD-23.3^T	+ [957]	100	-	-	This work

[1] Relative identity of nucleotide sequence of *XYL1*/*XYL1.1* or *XYL1.2* genes with those reported for *Sp. passalidarum* CBS 10155^T

Table 4. Length and identity of xylose reductases XYL1p, XYL1.1p and XYL1.2p in *Spathaspora* spp.

Yeast species	Yeast strains	XYL1p (XYL1.1p) [size aa]	Identity (%)[1]	XYL1.2p [size aa]	Identity (%)[1]	Reference
Sp. passalidarum	CBS 10155^T	318	100	317	100	Wohlbach et al., 2011; this work
Sp. arborariae	UFMG-HM-19.1A^T	318	93	-	-	Unpublished data; this work
Sp. brasiliensis	UFMG-HMD-19.3^T	318	96	-	-	This work
Sp. passalidarum	UFMG-HMD-1.1	318	99	317	99	This work
Sp. roraimanensis	UFMG-XMD-23.2^T	318	93	-	-	This work
Sp. suhii	UFMG-XMD-16.2^T	318	99	-	-	This work
Sp. xylofermentans	UFMG-HMD-23.3^T	318	100	-	-	This work

[1] Relative identity of amino acid sequence of xylose reductases (XYL1p/XYL1.1p or XYL1.2p) with those reported for *Sp. passalidarum* CBS 10155^T

Table 5. Yeast strains and plasmids constructed and/or used in this study.

Strains and plasmids	Relevant features	References
Plasmids		
YpOB8	*URA3 TDH3p-XYL1-ADH1t, PGK1p-XYL2-PGK1t*	Bengtsson et al. 2008
YpDR7	*URA3 TDH3p-XYL1(N272D)-ADH1t, PGK1p-XYL2-PGK1t*	Runquist et al. 2010
YIpRC1	pOB8 *XYL1.1 S. passalidarum* CBS 10155	This work
YIpRC2	pOB8 *XYL1.1 S. passalidarum* UFMG-HMD-1.1	This work
YIpRC4	pOB8 *XYL1.2 S. passalidarum* CBS 10155	This work
YIpRC5	pOB8 *XYL1.2 S. passalidarum* UFMG-HMD-1.1	This work
Sa. cerevisiae strains		
TMB 3044	CEN.PK 2-1C, *MAT*a, *ura3-52*, Δ*gre3*, *his3::HIS3 PGK1p-XKS1-PGK1t, TAL1::PGK1p-TAL1-PGK1t, TKL1::PGK1p-TKL1-PGK1t, RKI1::PGK1p-RKI1-PGK1t, RPE1::PGK1p-RPE1-PGK1t*	Karhumaa et al. 2005
TMB 3422	TMB 3044, *ura3*::YIpDR7	Runquist et al. 2010
TMB 3501	TMB 3044, *ura3*:: YIpRC1	This work
TMB 3502	TMB 3044, *ura3*:: YIpRC2	This work
TMB 3503	TMB 3044, *ura3*:: YIpRC4	This work
TMB 3504	TMB 3044, *ura3*:: YIpRC5	This work

Table 6. Primers used in this study.

Primer	Sequence (5'-3')
SpspXYL1.1_F	TTTTGTGTAATCTCGGAT
SpspXYL1.1_R	TTTTATTAATGCTAATCGATAAC
SppaXYL1.2_F	ATTACTACTTACAAGTAAAACACA
SppaXYL1.2_R2	ATTATCAGTGATCCAAAGAATGAA
SppaXYL1.1_XbaIF	GACTCTAGAATGGCTACTATTAAATT
SppaXYL1.1_XbaIR2	GACTCTAGATTAAACGAAGATTGGAATG
SppaXYL1.2_XbaIF	GACTCTAGAATGTCTTTTAAATTATCT
SppaXYL1.2_XbaIR	GACTCTAGATTAAACAAAGATTGGAAT
Sppa_xyl1.1_rtFor	CTCAGGTCACTTGATGCCTTTAG
Sppa_xyl1.1_rtRev	TCTTTAAACCGTCACCGACTTCC
Sppa_xyl1.2_rtFor	GCCACCATTCCTCAGCAAATTTAC
Sppa_xyl1.2_rtRev	CTCTTAACAAGACCGTCTTCAATAGC
Sppa_act1_rtFor	AGATACCCAATTGAACACGGTATCG
Sppa_act1_rtRev	GATTTAGGATTCATTGGAGCTTCAG
Sppa_rdn18_rtFor	TCACCAGGTCCAGACACAATAAG
Sppa_rdn18_rtRev	GGTTAAGGTCTCGTTCGTTATCG

Table 7. mRNA expression levels of *XYL1.1* and *XYL1.2* genes from *Sp. passalidarum* CBS 10155 and *Sp. passalidarum* UFMG-HMD-1.1 after 16h fermentation in YPX medium under moderate or severe oxygen-limited conditions. *XYL1.1* at moderate oxygen-limited conditions was used as reference for each *Sp. passalidarum* strain and mRNA levels of *ACT1* or *RDN18* used as internal control. Analysis were performed according to Pfaffl method (Pfaffl, 2001).

Internal control gene	Oxygen-limited condition	*Sp. passalidarum* CBS 10155		*Sp. passalidarum* UFMG-HMD-1.1	
		XYL1.1	*XYL1.2*	*XYL1.1*	*XYL1.2*
ACT1	Moderate	1	7.1 ± 1.4	1	13.8 ± 2.5
	Severe	0.6 ± 0.1	5.0 ± 0.6	2.4 ± 1.2	23.6 ± 9.2
RDN18	Moderate	1	7.1 ± 1.4	1	13.8 ± 2.5
	Severe	0.5 ± 0.1	4.2 ± 0.1	2.0 ± 0.8	20.3 ± 8.6

Table 8. Xylose reductase (XR) activity (200 mM xylose), expressed in units (U) per mg protein [U. (mg protein)$^{-1}$], of *Sa. cerevisiae* TMB 3501, TMB 3502, TMB 3503 and TMB 3504 after 48h of aerobic cultivation in YNB-xylose medium.

Yeast species	Yeast strains	XR		Ratio NADH/NADPH
		NADH	NADPH	
Sa. cerevisiae	TMB 3501	0	2.88±0.12	0
Sa. cerevisiae	TMB 3502	0	2.38±0.08	0
Sa. cerevisiae	TMB 3503	1.77±0.13	1.32±0.01	1.34
Sa. cerevisiae	TMB 3504	4.97±0.30	4.12±0.36	1.21

Table 9. D-xylose consumption, product formation (biomass, ethanol and xylitol) and xylose reductase (XR) activity in anaerobic xylose fermentation (50 g/L) with recombinant *Sa. cerevisiae* strains TMB 3422 and TMB 3504.

Strains	D-xylose consumption (%)[1]	$r_{s\ max}$ ($g.L^{-1}.h^{-1}$)[2]	Final biomass ($g.L^{-1}$)	Ethanol concentration (g/L)	$Y_{p/s}^{et}$ ($g.g^{-1}$)[2]	Qp ($g.L^{-1}.h$)[3]	Xylitol concentration (g/L)	$Y_{p/s}^{xy}$ (g/g)[6]	XR		Ratio NADH/NADPH
									NADH	NADPH	
TMB 3422	93.0	0.33	1.9	16.2	0.33	0.11	10.4	0.22	0.67±0.03	0.94±0.02	0.71
TMB 3504	95.2	0.66	3.3	18.4	0.40	0.26	5.4	0.12	1.13±0.08	0.96±0.01	1.18

[1] D-xylose consumption (%) – percentage of initial D-xylose consumed

[2] $r_{s\ max}$ ($g.L^{-1}.h^{-1}$) – D-xylose (volumetric) consumption rate

[3] $Yp_{/s}^{et}$ ($g.g^{-1}$) – ethanol yield: correlation between ethanol ($\Delta P_{ethanol}$) produced and D-xylose (ΔS_{xylose}) consumed

[4] Qp ($g.L^{-1}.h$) – ethanol volumetric productivity: ratio of ethanol concentration ($g.L^{-1}$) and fermentation time (h)

[5] $Y_{p/s}^{xy}$ ($g.g^{-1}$) – xylitol yield: correlation between xylitol ($\Delta P_{xylitol}$) produced and D-xylose (ΔS_{xylose}) consumed

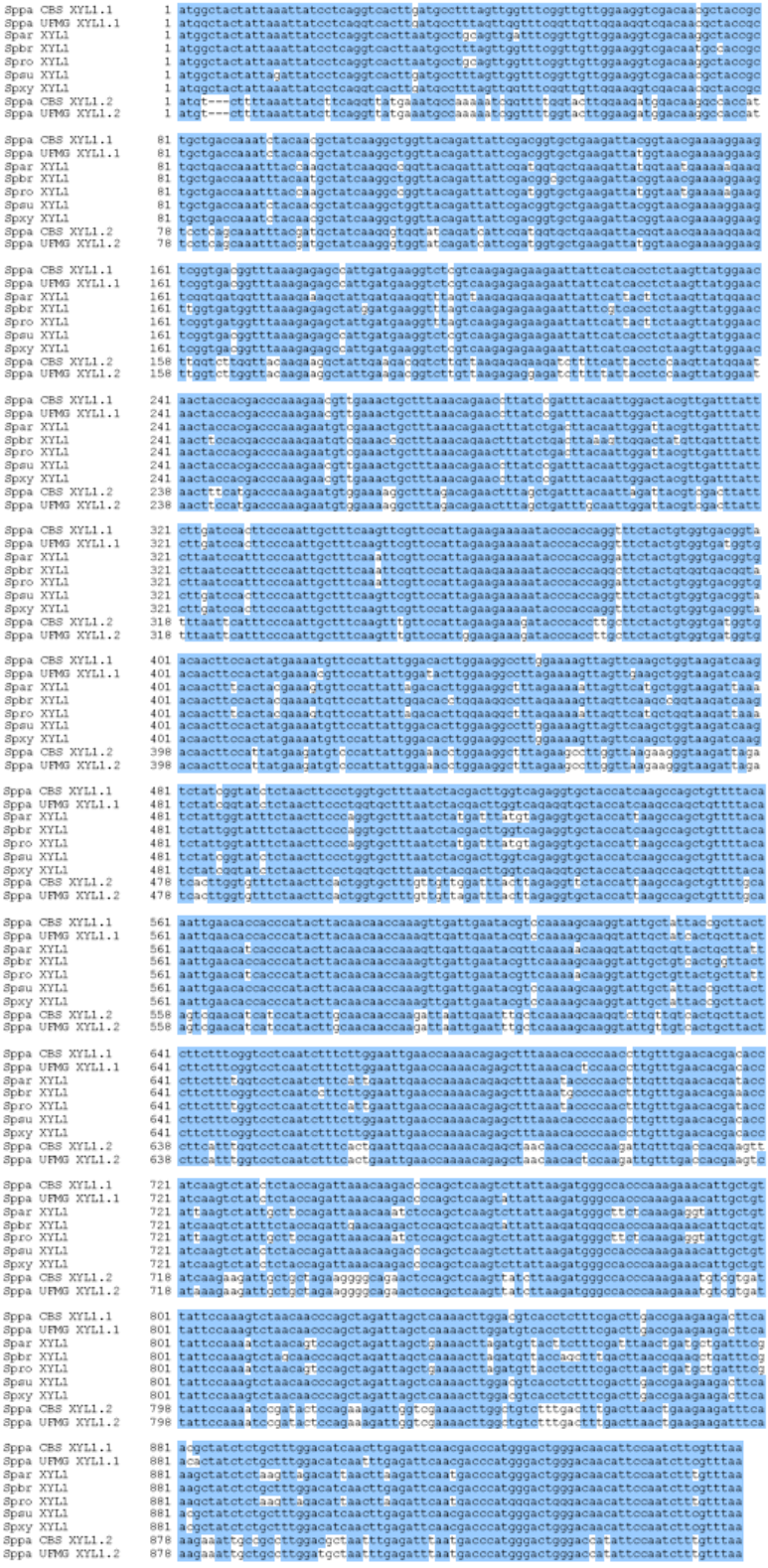

Figure 1. Alignment of *XYL1*, *XYL1.1 and XYL1.2* genes sequences of *Spathaspora* spp. Sppa – *Sp. passalidarum*; Spar – *Sp. arborariae*; Spbr – *Sp. brasiliensis;* Spro – *Sp. roraimanensis;* Spsu – *Sp. suhii*; Spxy – *Sp. xylofermentans*

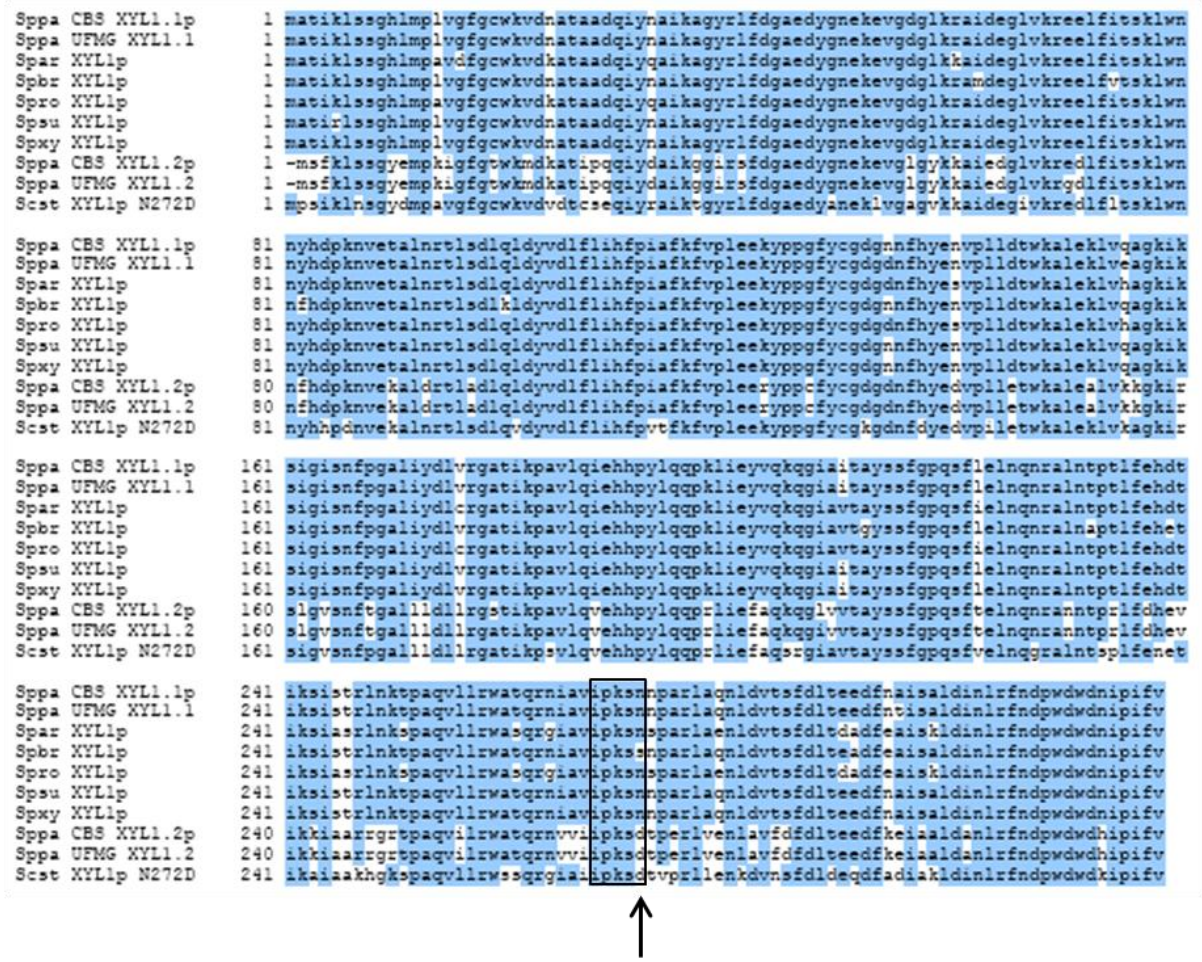

Figure 2. Alignment of XYL1p, XYL1.1p and XYL1.2p amino acid sequences of *Spathaspora* spp and *Scheffersomyces stipitis* (XYL1p N272D). The highly conserved motif Ile-Pro-Lys-Ser referred as the coenzyme binding site is shown enclosed by a rectangle box. The position site 271 (XYL1.2p) or 272 (XYL1p and XYL1.1p) is indicated by an arrow. Sppa – *Sp. passalidarum*; Spar – *Sp. arborariae*; Spbr – *Sp. brasiliensis;* Spro – *Sp. roraimanensis;* Spsu – *Sp. suhii* Spxy – *Sp. xylofermentans*

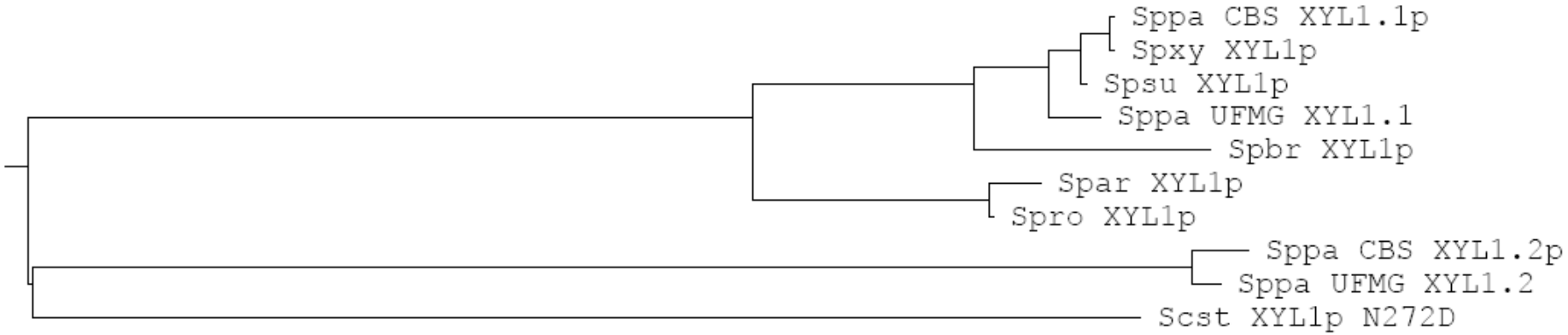

Figure 3. Phylogenetic relationships among *Spathaspora* spp. and *Scheffersomyces stipitis* (XYL1p N272D) based on XYL1p, XYL1.1p and XYL1.2 p amino acid sequences. Sppa – *Sp. passalidarum*; Spar – *Sp. arborariae*; Spbr – *Sp. brasiliensis;* Spro – *Sp. roraimanensis;* Spsu – *Sp. suhii* Spxy – *Sp. xylofermentans*; Scst – *Sc. stipitis*.

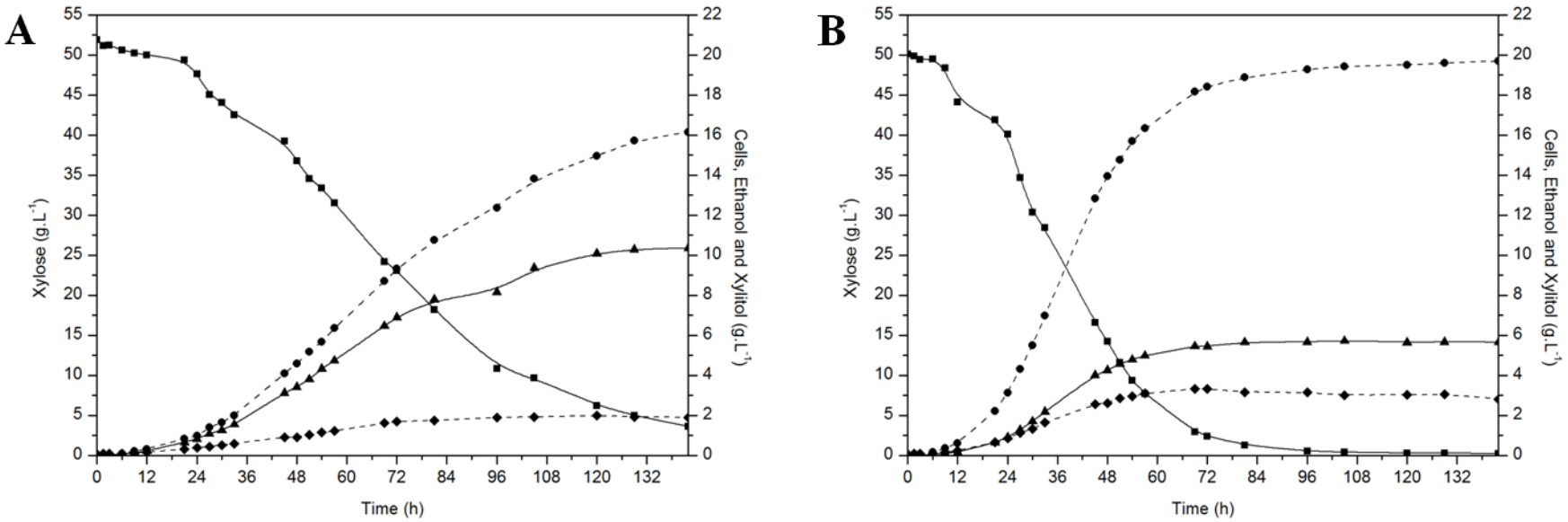

Figure 4. Anaerobic batch fermentation of 50 $g.L^{-1}$ xylose with *Sa. cerevisiae* strains TMB 3422 (A) and TMB 3504 (B). Symbols: square (continuous line) – xylose; circle (dashed line) – ethanol; triangle (continuous line) – xylitol; diamond (dashed line) – cells.

5. Discussão

5.1 Identificação de leveduras fermentadoras de D-xilose

Um total de 224 isolados de leveduras assimiladoras de D-xilose associadas à madeira em decomposição foi obtido neste trabalho. Trinta e três espécies, das quais 26 são espécies já descritas pela literatura e sete representam novas espécies foram identificadas. Apesar do grande número de isolados e da elevada diversidade de espécies, a capacidade de fermentar D-xilose restringiu-se a representantes de apenas oito espécies: *Candida tropicalis, Scheffersomyces stipitis, Sc. amazonensis, Spathaspora passalidarum*, *Sp. brasiliensis, Sp. roraimanensis, Sp. suhii* e *Sp. xylofermentans*. Embora um reduzido número de espécies fermentadoras tenha sido encontrado, é importante salientar que cinco das oito espécies destacadas são novas espécies de leveduras, e representam uma importante contribuição taxonômica à ecologia e biotecnologia desses micro-organismos no que diz respeito à fermentação de D-xilose (Capítulos I e II). A restrição da capacidade de fermentar D-xilose a poucas espécies não é, todavia, surpreendente. De acordo com Kurtzman e colaboradores (2011) cerca de 25 espécies de leveduras dentre as mais de 1500 descritas são aptas a fermentar D-xilose. Este tratado taxonômico, entretanto, utiliza a técnica de tubos de Durham para a constatação da fermentação de fontes de carbono por leveduras, e conforme informado nos Capítulos I e II, não foi observada a formação de gás no interior dos tubos durante o cultivo em D-xilose pelas espécies *Sc. amazonensis, Sp. brasiliensis, Sp. roraimanensis, Sp. suhii* e *Sp. xylofermentans*, o que poderia ser erroneamente interpretado como um resultado negativo no ensaio realizado. Adicionalmente, a capacidade de fermentar D-xilose nem sempre consistiu em um parâmetro rotineiramente utilizado para a descrição de espécies de leveduras, observação consoante à carência de informações a respeito deste teste constatada em tratados taxonômicos. Estudos iniciais de averiguação da fermentação de D-xilose por leveduras apontavam para 41 espécies capazes de desempenhar tal função (Jeffries, 1985b). Dessa forma, o número de leveduras fermentadoras de D-xilose é certamente subestimado, ainda que, em realidade, poucas espécies possam converter esta fonte de carbono a elevadas concentrações de etanol.

Uma vez que a capacidade de fermentar D-xilose, ainda que possivelmente subestimada, não é um comportamento amplamente difundido dentre as leveduras, Kurtzman e colaboradores (2011) sugerem que esta propriedade seja rotineiramente determinada, não somente por funcionar como um marcador filogenético, mas também como um indicador ecológico de decomposição de madeira e outras fontes de biomassa. Essas constatações são sustentadas pela observação de que, salvo algumas exceções, leveduras fermentadoras de D-xilose encontram-se localizadas em dois clados principais, *Scheffersomyces* e *Spathaspora*, e que membros desses clados são descritos como associados aos substratos supramencionados. De fato, as espécies fermentadoras

de D-xilose identificadas neste trabalho pertencem aos clados citados ou encontram-se em íntima associação com os mesmos, caso da espécie *C. tropicalis*, pertencente ao clado *Lodderomyces/Spathaspora.*

Os clados *Scheffersomyces, Spathaspora* e *Lodderomyces* pertencem à mesma família, Debaromycetaceae (Kurtzman et al., 2011), e são filogeneticamente próximos entre si em relação aos demais clados desta família (Figura 2). As espécies ascospóricas atribuídas ao gênero *Scheffersomyces* eram inicialmente identificadas dentro de um gênero polifilético, *Pichia*, cuja classificação baseada em similaridades fenotípicas como a formação de ascosporos com formato de chapéu e ausência de assimilação de nitrato como única fonte de nitrogênio resultava na acomodação de espécies filogeneticamente distantes dentro de um mesmo gênero. A análise combinada das sequências dos domínios D1/D2 da subunidade maior e de genes da subunidade menor do RNA ribossomal mostraram que as espécies descritas como *Pichia stipitis*, *P. segobiensis* e *P. spartinae* encontravam-se distantemente relacionadas à espécie tipo do gênero, *Pichia membranifaciens*, mas filogeneticamente próximas entre si, levando à proposição do gênero *Scheffersomyces* por Kurtzman e Suzuki (2010) para a reclassificação de tais espécies. Atualmente, o gênero *Scheffersomyces* é representado por 16 espécies (Urbina et al., 2012a; Urbina et al., 2012b), incluindo as espécies assexuadas anteriormente identificadas como *Candida* spp. (*C. amazonensis, C. coipomoensis, C. ergatensis, C. gosingica, C. insectosa, C. lignicola, C. lignosa, C. queiroziae, C. shehatae*) e atualmente identificadas como *Scheffersomyces* spp. (*Sc. amazonensis, Sc. coipomoensis* e assim sucessivamente) e pelas espécies *Sc. stipitis* (espécie tipo do gênero), *Sc. segobiensis*, *Sc. spartinae*, *Sc. quercinus, Sc. illinoinensism, Sc. virginianus e Sc. cryptocercus.* Além da atualização do gênero, os autores dividiram o clado *Scheffesomyces* em três subclados, sendo eles: 1) subclado formado pelas espécies *Sc. spartinae e Sc. gosingicus* (*C. gosingica*), esta última capaz de fermentar celobiose; 2) subclado *Sc. ergatensis*, composto pelas espécies fermentadoras de celobiose *Sc. amazonensis*, *Sc. coipomoensis*, *Sc. ergatensis*, *Sc. lignicola* e *Sc. queiroziae*; 3) subclado *Sc. stipitis*, composto pelas demais espécies do gênero, cuja capacidade de fermentar D-xilose é constatada. É interessante observar que apesar de *Sc. amazonensis*, uma das sete novas espécies descritas neste trabalho, ter sido atribuída ao subclado de leveduras fermentadoras de celobiose e não ao subclado de fermentadoras de D-xilose, atribuição esta sustentada pela descrição da espécie (Capítulo II), essa levedura apresenta a capacidade de converter D-xilose a elevadas concentrações de xilitol e baixas concentrações de etanol (Capítulo I). Tal observação destaca, novamente, a importância da realização de testes mais específicos e quantitativos que não a técnica de tubos de Durham para a constatação da fermentação de fontes de carbono por leveduras.

Figura 2. Relações filogenéticas entre espécies dos gêneros atribuídos à família Debaryomycetaceae determinadas por análise neighbor-joining de sequências concatenadas de genes dos domínios D1/D2 da subunidade maior e da subunidade menor do RNA ribossomal. Valores de bootstrap correspondem a 1000 replicatas (Kurtzman et al., 2011).

O clado *Spathaspora* foi primeiramente descrito por Nguyen e colaboradores (2006) para abrigar as espécies *Sp. passalidarum*, primeira espécie teleomórfica do gênero, e *C. jeffriesii*, isoladas a partir de besouros associados a madeira coletados respectivamente em Louisiana nos Estados Unidos e Chiriqui no Panamá. Atualmente, o clado *Spathaspora* é representado pelas espécies teleomórficas *Sp. passalidarum, Sp. arborariae, Sp. brasiliensis, Sp. roraimanensis e Sp. suhii* e pelas espécies anamórficas, previamente conhecidas como pertencentes ao gênero *Candida*, *Sp. jeffriesii, Sp. lyxosophila, Sp. materiae, Sp. insectamans, Sp. subhashii e Sp. xylofermentans* (Capítulos I e II). Dentre tais espécies, seis foram descritas a partir de isolados de madeira em decomposição coletada em biomas brasileiros: *Sp. materiae* (Barbosa et al., 2009), *Sp. arborariae* (Cadete et al., 2009) e as espécies *Sp. brasiliensis, Sp. roraimanensis, Sp. suhii* e *Sp. xylofermentans* encontradas durante a realização do presente trabalho. Além disso, seis novas linhagens de *Sp. passalidarum*, até então uma espécie representada por um único isolado, foram também obtidas em hábitats brasileiros. Espécies desse clado apresentam como características em comum a associação a substratos vegetais em decomposição, tais como madeira e insetos que se alimentam deste material. Apesar de abrigar espécies conhecidas por sua extraordinária capacidade de fermentar D-xilose (*Sp. passalidarum* e *Sp. arborariae* – Capítulos I e III), o clado *Spathaspora* acomoda, contudo, espécies não fermentadoras ou pobremente fermentadoras (Kurtzman et al., 2011). *Spathaspora jeffriesii* é descrita como fermentadora de D-xilose, porém de lento desempenho; *Sp. lyxosophila* e *Sp. materiae* são descritas como não fermentadoras e não há informações disponíveis acerca da fermentação de D-xilose por *Sp. insectamans* e *Sp. subhashii*. Levando-se em consideração a técnica de averiguação da fermentação de carboidratos utilizada por taxonomistas, as espécies de *Spathaspora* descritas como não fermentadoras de D-xilose provavelmente apresentam a capacidade de fermentar esta pentose aliada a uma baixa produção de etanol. As melhores leveduras produtoras de etanol a partir de D-xilose já descritas – *Sp. passalidarum, Sp. arborariae, Sc. stipitis e Sc. shehatae* – produzem gás no interior de tubos de Durham quando cultivadas nesta fonte de carbono.

As espécies pertencentes ao clado *Lodderomyces* (Figura 2) são descritas como capazes de assimilar D-xilose. Entretanto, não existem informações no tratado taxonômico de Kurtzman e colaboradores (2011) acerca da fermentação desta pentose por membros desse clado. Carece-se de tais informações mesmo em relação à *C. tropicalis*, uma espécie amplamente estudada quanto à conversão de D-xilose em xilitol, conforme mencionado no Capítulo I. Visto que o foco inicial deste trabalho baseava-se na busca por leveduras capazes de converter D-xilose a etanol, as linhagens de *C. tropicalis* obtidas não foram utilizadas nos ensaios fermentativos realizados. Contudo, torna-se importante salientar o isolamento desta espécie uma vez que a mesma foi a mais frequentemente isolada de madeira em decomposição nas áreas de Floresta Amazônica estudadas (Capítulo I), além de ser atribuída a um clado filogeneticamente próximo aos clados

Spathaspora e *Scheffersomyces* e pela documentada capacidade desta levedura fermentar D-xilose.

O conhecimento de características e comportamentos inerentes aos membros dos clados *Scheffersomyces* e *Spathaspora* uma vez que estes clados albergam leveduras eficientemente capazes de fermentar ou não D-xilose é de extrema importância para se tentar compreender como esta propriedade surgiu e evoluiu dentre as leveduras. Ainda que leveduras fermentadoras de D-xilose encontrem-se dispersas ao longo do subfilo Saccharomycotina (Kurtzman et al., 2011), o maior número das principais espécies fermentadoras corresponde a representantes dos clados mencionados, ambos classificados dentro de uma mesma família. De acordo com Urbina e colaboradores (2012b) essa particular localização filogenética de leveduras fermentadoras de D-xilose suporta duas alternativas: 1) a capacidade de fermentar D-xilose foi resultado de uma evolução convergente em leveduras ascomicéticas, isto é, esta capacidade evoluiu independentemente entre espécies diferentes, não sendo encontrada no ancestral comum dessas espécies. Este fenômeno ocorre devido à seleção natural, responsável por selecionar positivamente mutações que geram adaptações morfológicas, fisiológicas e até comportamentais mais adequadas para um determinado ambiente (Ridley, 2006); 2) a capacidade de fermentar D-xilose fazia-se presente no mais antigo ancestral comum do subfilo Saccharomycotina e foi retida, principalmente, nas leveduras associadas a insetos lignícolas e substratos relacionados, como madeira em decomposição. Ambas as hipótese estão de acordo com os habitats em comum compartilhados por leveduras fermentadoras de D-xilose. Todavia, Urbina e colaboradores (2012b) apontam para linhas de evidências independentes que favorecem a segunda premissa: i) estudos bioquímicos clássicos têm determinado que a capacidade de assimilar D-xilose e xilitol é comum dentre as leveduras; ii) espécies pertencentes ao subfilo Saccharomycotina, mas não à família Debaryomycetaceae, como *Ogataea polymorpha, Brettanomyces naardenensis* e *Pachysolen tannophilus* fermentam D-xilose; iii) recentemente, a determinação e comparação de sequências dos genomas de leveduras fermentadoras e não fermentadoras de D-xilose confirmaram a presença de genes relacionados ao metabolismo desta pentose em todas as leveduras analisadas (Wohlbach et al., 2011). Dentro desse contexto, a obtenção de novas espécies de leveduras fermentadoras de D-xilose e a utilização de ensaios mais específicos de fermentação com espécies descritas como assimiladoras desta fonte de carbono, como realizados no presente trabalho, tornam-se relevantes contribuições para o entendimento relacionado à aquisição e evolução dessa importante característica taxonômica e biotecnológica.

5.2 Ensaios de fermentação de D-xilose por leveduras *Spathaspora* spp. e determinação das atividades enzimáticas e dependência de co-fatores das enzimas XR e XDH

No presente estudo foram realizados seis ensaios distintos de cultivo em D-xilose em escala de bancada, sendo quatro desses ensaios conduzidos em meio complexo, um em hidrolisado hemicelulósico de bagaço de cana-de-açúcar e um em meio mínimo. Os ensaios realizados em meio complexo apresentaram como característica em comum a utilização de meio rico em extrato de levedura e peptona (YP – extrato de levedura, 10 $g.L^{-1}$ e peptona, 20 $g.L^{-1}$) suplementado com D-xilose como única fonte de carbono, em concentrações que variaram entre 20 e 50 $g.L^{-1}$. Por outro lado, a principal diferença evidenciada entre cada ensaio diz respeito à condição de oxigenação empregada. Sabe-se que a bioconversão de pentoses por leveduras é um processo complexo influenciado por uma variedade de fatores, incluindo a linhagem do micro-organismo utilizado, as condições de cultivo (pH, temperatura, agitação, oxigenação, concentração inicial de D-xilose e de células, suplementação do meio de fermentação com nutrientes) e o tipo de substrato testado no ensaio fermentativo (soluções sintéticas ou hidrolisados lignocelulósicos), este último, ainda, afetado pela presença de outros açúcares e compostos inibitórios (Watson et al., 1984; Parekh et al., 1987; Ferreira et al., 2011). Particularmente, a oxigenação é considerada um fator de grande influência na fermentação de D-xilose, uma vez que determina a divisão do fluxo de carbono entre a multiplicação do micro-organismo e a formação de produtos da via fermentativa (du Preez, 1994). Dessa forma, o emprego de diferentes condições de oxigenação para a avaliação do metabolismo de D-xilose em leveduras *Spathaspora* spp. neste trabalho permitiu uma ampla compreensão deste processo nessas espécies, além da determinação específica da influência exercida pelo oxigênio para cada levedura examinada. Ademais, a caracterização das atividades enzimáticas e preferências de co-fatores das enzimas xilose redutase e xilitol desidrogenase em duas condições de oxigenação também veio a contribuir, conjuntamente, para esta concepção.

A fim de uma melhor compreensão e comparação dos resultados obtidos de acordo com o ensaio de cultivo em meio complexo realizado, as condições de cada experimento serão resumidamente especificadas a seguir, de acordo com o Capítulo correspondente:

1) Capítulo I – condições microaeróbicas: cultivo conduzido em 50 mL de meio YP suplementado com 50 $g.L^{-1}$ de D-xilose em frascos com capacidade de 125 mL tampados com rolha de algodão e gaze, inóculo inicial de 0,5 $g.L^{-1}$ de células, a 30°C e 200 rpm. As amostras foram retiradas assepticamente, por meio de abertura total do frasco.

2) Capítulo II – condições aeróbicas: cultivo conduzido em 50 mL de meio YP suplementado com 20 $g.L^{-1}$ de D-xilose em frascos com capacidade de 250 mL, tampados com rolha de algodão e gaze, inóculo inicial de 1% de células, a 28°C e 160 rpm. As amostras foram retiradas assepticamente, por meio de abertura total do frasco.

3) Capítulo III – condições de limitação moderada de oxigênio (semelhantes às condições microaeróbicas): cultivo conduzido em 100 mL de meio YP suplementado com 50 $g.L^{-1}$ de D-xilose em frascos com capacidade de 250 mL, tampados com rolha de algodão e gaze, inóculo inicial de 0,5 $g.L^{-1}$ de células, a 30°C e 200 rpm. Neste ensaio determinaram-se as atividades enzimáticas de XR e XDH. As amostras foram retiradas assepticamente, por meio de abertura total do frasco.

4) Capítulo III – condições de limitação severa de oxigênio: cultivo conduzido em 100 mL de meio YP suplementado com 50 $g.L^{-1}$ de D-xilose em frascos com capacidade de 100 mL, tampados com rolha de borracha, inóculo inicial de 0,5 $g.L^{-1}$ de células, a 30°C e 200 rpm. Neste ensaio também se determinaram as atividades enzimáticas de XR e XDH. As amostras foram retiradas assepticamente, por meio de agulha, sem abertura do frasco.

As novas espécies *Sp. brasiliensis, Sp. roraimanensis, Sp. suhii* e *Sp. xylofermentans* foram avaliadas em todos os ensaios; linhagens da espécie *Sp. passalidarum* não foram testadas sob condições aeróbicas; *Sp. arborariae* foi analisada sob as condições moderada e severa, entretanto esta levedura já havia sido anteriormente testada sob condições aeróbicas de cultivo em D-xilose (Cadete et al., 2009). *Scheffersomyces amazonensis* foi avaliada nas condições microaeróbicas (Capítulo I) e nas condições moderada e severa (dados não mostrados). Visto que o foco do trabalho referenciado no Capítulo III é direcionado à fermentação de D-xilose por leveduras *Spathaspora* spp, os resultados obtidos por *Sc. amazonensis* não se encontram divulgados neste Capítulo, mas serão mencionados no decorrer da discussão para enriquecimento da mesma. É importante salientar que a condição moderada empregada no Capítulo III foi uma tentativa de reproduzir a condição microaeróbica utilizada no Capítulo I de forma a comparar diretamente os resultados obtidos nesses dois Capítulos. Ambas diferem no tamanho do frasco e na quantidade de meio de cultivo utilizados, mas mantém a razão volume do frasco/volume do meio de cultivo (V_{frasco}:V_{meio}) equivalente a 2,5. Essa razão e a incubação dos frascos a 200 rpm foram estabelecidos por Silva e colaboradores (2010) como características de uma condição microaeróbica, a mais adequada para se atingir uma alta produção de etanol a partir da fermentação de D-xilose. Contudo, dado os diferentes resultados encontrados entre ambas as condições, a oxigenação das mesmas, dentre outras possíveis influências (equipamentos, reagentes e metodologias de análise), provavelmente diferiu, e dessa forma convencionou-se designá-las por nomes distintos. A condição aeróbica apresentou uma razão V_{frasco}:V_{meio} igual a 5, e a condição severa uma razão equivalente a 1. As duas condições de limitação de oxigênio estabelecidas (moderada e severa – Capítulo III) pretenderam definir as melhores condições de arejamento para o processo fermentativo na produção de etanol e/ou xilitol e de caracterizar esse processo com enfoque nas atividades enzimáticas de XR e XDH.

A partir de uma análise geral dos resultados encontrados durante os ensaios fermentativos, não é possível estabelecer um comportamento universal acerca da influência do oxigênio no metabolismo de D-xilose pelas espécies testadas, i.e., não há como salientar a existência de uma condição absoluta de maior produção de etanol ou xilitol para todas as espécies, assim como afirmar que condições relativamente limitantes de oxigênio são as melhores para a ocorrência deste processo fermentativo em todas as espécies. Dentro desse contexto, os resultados observados serão abordados de acordo com cada espécie estudada. Em contrapartida, um padrão relativo aos resultados dos ensaios enzimáticos de XR e XDH pôde ser estabelecido de acordo com o principal produto formado – etanol ou xilitol – por cada espécie, em concordância com os co-fatores enzimáticos utilizados.

A principal constatação a ser feita analisando-se o comportamento da levedura *Sp. brasiliensis* nos cultivos realizados refere-se ao fato de que esta espécie produz baixas concentrações de etanol a partir de D-xilose independentemente da condição de oxigenação estudada. Na condição aeróbica, esta levedura apresentou um rápido consumo de D-xilose (100% de consumo em cerca de 20h) e uma elevada produção de biomassa, verificada por meio do aumento expressivo da leitura de DO a 570 nm, resultados estes esperados quando o consumo de uma fonte de carbono sabiamente assimilada por um organismo heterotrófico é conduzida em condições de elevada oxigenação. A produção de etanol não ultrapassou mais do que 3 $g.L^{-1}$ ($Y_{p/s}^{et}$ = 0,12). A produção de xilitol não foi determinada nesta condição, o que inviabiliza um maior esclarecimento relativo à obtenção deste produto sob condições oxigenadas. Quando cultivada em condições microaeróbicas, *Sp. brasiliensis* (*Spathaspora* sp. 3) apresentou um consumo de D-xilose equivalente a 55% em 48h, e baixa produção de etanol (3,3 $g.L^{-1}$, $Y_{p/s}^{et}$ = 0,13) e xilitol (3,3 $g.L^{-1}$, $Y_{p/s}^{xil}$ = 0,16). A produção de biomassa (5,6 $g.L^{-1}$ de células) foi favorecida em detrimento desses metabólitos. Já a condição moderada revelou-se a melhor condição para a produção de xilitol por esta levedura em comparação às demais condições testadas. A produção de etanol permaneceu baixa (3,7 $g.L^{-1}$, $Y_{p/s}^{et}$ = 0,08) enquanto a concentração de xilitol atingiu 23 $g.L^{-1}$ ($Y_{p/s}^{xil}$ = 0,49) em 72h. Neste período, a D-xilose foi quase 100% consumida, e este elevado consumo refletiu não só na considerável produção de xilitol quanto no aumento da biomassa (cerca de 11 $g.L^{-1}$ de células). Sob condições severas, o consumo de D-xilose decaiu substancialmente para 31,5% em 72h e 50,4% em 144h, e consequentemente as concentrações de células, xilitol e etanol foram menores que na condição moderada, girando em torno de 3,6 $g.L^{-1}$ (72h), 12 $g.L^{-1}$ e $Y_{p/s}^{xil}$ = 0,53 (144h) e 2,6 $g.L^{-1}$ e $Y_{p/s}^{et}$ = 0,15 (72h), respectivamente.

A levedura *Sp. roraimanensis* (*Spathaspora* sp. 2) produziu xilitol ou etanol como principais produtos do metabolismo de D-xilose de acordo com a condição utilizada, destacado-se, entretanto, em relação à uma elevada produção de xilitol. Essa espécie apresentou um comportamento semelhante à *Sp. brasiliensis* no ensaio aeróbico, no que diz respeito ao rápido

consumo de D-xilose e expressiva multiplicação celular, apresentando, todavia, uma maior produção de etanol (cerca de 5 $g.L^{-1}$ e $Y_{p/s}^{et}$ = 0,29) que *Sp. brasiliensis.* Na condição microaeróbica, 90% da D-xilose disponível foi consumida em 48h, e este relevante consumo foi acompanhado da produção de 7,4 $g.L^{-1}$ de células, e uma maior produção de etanol (10 $g.L^{-1}$, $Y_{p/s}^{et}$ = 0,26) que xilitol (7 $g.L^{-1}$, $Y_{p/s}^{xil}$ = 0,19). Os valores do fator de conversão de substrato em etanol ($Y_{p/s}^{et}$) foram próximos entre as condições aeróbicas e microaeróbicas, uma vez que a produção máxima de etanol atingida (5 e 10 $g.L^{-}1$, respectivamente) foi proporcional à concentração inicial de D-xilose utilizada em cada ensaio. Sob moderada oxigenação, a concentração de xilitol foi 2,3 vezes maior (22,7 $g.L^{-1}$, $Y_{p/s}^{xil}$ = 0,47) que o valor observado em microaerobiose, além de uma menor produção de biomassa (5,2 $g.L^{-1}$ de células) e etanol (5,8 $g.L^{-1}$, $Y_{p/s}^{et}$ = 0,13) aliados a um consumo de 100% de D-xilose em 48h. A condição severa de oxigenação foi a condição na qual a maior produção de xilitol por *Sp. roraimanensis* foi encontrada, entretanto, essa elevada produção (30,7 $g.L^{-1}$, $Y_{p/s}^{xil}$ = 0,67) foi atingida somente em 144h. Nesse período, 100% da D-xilose disponível foram consumidos, com a produção de 5,6 $g.L^{-1}$ de células em conjunto com a menor produção de etanol dentre todas as condições testadas (3 $g.L^{-1}$, $Y_{p/s}^{et}$ = 0,05).

Spathaspora suhii (*Spathaspora* sp. 1), espécie representada por duas linhagens, apresentou um comportamento semelhante a *Sp. roraimanensis* nas condições aeróbicas, microaeróbicas e moderadas, com uma maior produção de etanol (10,7 e 13,7 $g.L^{-1}$, e $Y_{p/s}^{et}$ = 0,27 e 0,33, respectivamente) sob microaerobiose, e uma elevada produção de xilitol (22,4 $g.L^{-1}$, $Y_{p/s}^{xil}$ = 0,49) em oxigenação moderada. O grande diferencial encontrado para esta espécie foi o desempenho observado na fermentação em condições severas. A redução da disponibilidade de oxigênio resultou em um drástico consumo de D-xilose, equivalente a 13% em 144h de ensaio. Associada a este resultado, constatou-se uma produção mínima de biomassa (0,7 $g.L^{-1}$ de células), a presença de etanol não foi detectada e uma baixa concentração de xilitol (4,5 $g.L^{-1}$, e $Y_{p/s}^{et}$ = 0,63) foi produzida.

A maior produção de etanol (7,5 $g.L^{-1}$, $Y_{p/s}^{xil}$ = 0,34) determinada para a levedura *Sp. xylofermentans* (*Candida* sp. 1) foi verificada no ensaio conduzido em condições aeróbicas de oxigenação, fato exclusivamente observado para esta espécie. Em microaerobiose, *Sp. xylofermentas* apresentou resultados semelhantes aos alcançados por *Sp. brasiliensis,* como o consumo moderado de D-xilose (52 a 60%) e concentrações equivalentes e reduzidas de etanol (3,1 e 4,9 $g.L^{-1}$, e $Y_{p/s}^{et}$ = 0,14 e 0,18, respectivamente) e xilitol (3,3 e 4,6 $g.L^{-1}$, e $Y_{p/s}^{xil}$ = 0,13 e 0,22, respectivamente). Na condição moderada, essa espécie apresentou o menor índice de consumo de D-xilose quando comparada às demais (55,4% em 48h e 70,1% em 72h) e uma produção de xilitol (10,4 $g.L^{-1}$, e $Y_{p/s}^{xil}$ = 0,31) maior que a de etanol (2,6 $g.L^{-1}$, $Y_{p/s}^{et}$ = 0,09). Sob condições severas de oxigenação, *Sp. xylofermentans* exibiu um comportamento semelhante à *Sp. roraimanensis*, atingindo uma concentração de xilitol de 24,4 $g.L^{-1}$ ($Y_{p/s}^{xil}$ = 0,55), o maior valor detectado para esta

espécie, em 144h, com consumo quase total de D-xilose (97,5%) e baixa produção de etanol (3,7 $g.L^{-1}$, $Y_{p/s}^{et}$ = 0,08).

As linhagens de *Sc. amazonensis* destacaram-se pelas maiores concentrações de xilitol (entre 24 e 25 $g.L^{-1}$, e $Y_{p/s}^{et}$ = 0,55 e 0,59 em 48h) obtidas em relação à todas as espécies testadas na condição microaeróbica de cultivo. Nas condições moderada e severa, o comportamento relativo à uma elevada produção de xilitol (dados não mostrados) persistiu, com uma produção de 26,4 $g.L^{-1}$ em 36h e 34 $g.L^{-1}$ em 72h, respectivamente. Em todas as condições explicitadas, a concentração de etanol detectada foi baixa (entre 3,6 a 5 $g.L^{-1}$).

O metabolismo de D-xilose em leveduras resulta na síntese de uma variedade de produtos que contém carbono, nos quais se incluem dióxido de carbono, etanol, xilitol, ácido acético e polissacarídeos, dentre outros. O rendimento desses produtos é dependente da regulação do fluxo de carbono por meio de vias metabólicas disponíveis (Slininger et al., 1987). De acordo com Prior e colaboradores (1989), o xilitol pode ser secretado extracelularmente como um subproduto metabólico durante a produção de etanol a partir de D-xilose. No entanto, conforme Barbosa e colaboradores (1988), esse poliol pode também consistir no principal produto obtido durante este processo metabólico. Uma vez que todas as novas espécies de *Spathaspora* produziram xilitol como produto principal do metabolismo de D-xilose em pelo menos duas das três condições nas quais esta detecção foi realizada, e para *Sc. amazonensis* este comportamento foi observado em todas as condições avaliadas, essas leveduras podem ser particularmente designadas como espécies detentoras de uma capacidade específica de conversão de D-xilose em xilitol.

A análise dos resultados observados nos ensaios executados deve ainda levar em consideração as principais enzimas responsáveis pela via oxido-redutora da D-xilose e sua dependência de co-fatores, tendo em vista que uma relação direta entre a dependência de co-fatores da enzima xilose redutase e a capacidade de fermentar D-xilose com alta eficiência foi estabelecida por Bruinenberg e colaboradores (1984). Conforme explicado anteriormente (Figura 1), essa via consiste em duas reações sequenciais. Na primeira, a xilose redutase (XR), na presença de NADH e/ou NADPH reduz D-xilose a xilitol (Taylor et al., 1990). Em uma reação subsequente, o xilitol é oxidado a D-xilulose pela xilitol desidrogenase (XDH) ligada majoritariamente a NAD^+ ou minimamente a $NADP^+$ (Skoog e Hahn-Hagerdal, 1988). Estudos de cinética da utilização de D-xilose já foram realizados em culturas de leveduras, e os resultados encontrados mostraram uma íntima relação entre a redução da D-xilose a xilitol, o co-fator ligado a XR e os produtos do metabolismo da D-xilose de acordo com a espécie de levedura estudada e a taxa de oxigenação (Bruinenberg et al., 1984). Em condições anaeróbicas e de limitação de oxigênio, uma atividade de xilose redutase estritamente dependente de NADPH pode acarretar na inativação da enzima xilitol desidrogenase dependente de NAD^+ devido ao aumento da razão $NADH/NAD^+$, uma vez que nessas condições NADH não pode ser oxidado a NAD^+. Esse fenômeno

é resultado da incapacidade das leveduras compensarem o excesso de NADH produzido, já que estes micro-organismos não apresentam atividade de transhidrogenase, capaz de interconverter NADPH e NADH (Dellomonaco et al., 2010). O metabolismo de D-xilose via XR NADH-dependente contorna o desequilíbrio do sistema redox NAD^+/NADH, permitindo a conversão de D-xilose a etanol sob condições anaeróbicas (Bruinenberg et al., 1984). Em contraste, leveduras que consomem D-xilose pela atividade de XR estritamente dependente de NADPH (com completa ausência de XR ligada a NADH) acumulam xilitol (Bruinenberg et al., 1984; Gírio et al., 1994). Os resultados das atividades enzimáticas e dependência de co-fatores de XR e XDH para as leveduras *Sapathaspora* spp. caracterizadas como produtoras de xilitol no presente estudo (Capítulo III) estão de acordo com o contexto apresentado. Estas espécies apresentam atividade de xilose redutase exclusivamente dependente de NADPH e de xilitol desidrogenase associada à NAD^+, comportamento também exibido por *Sc. amazonensis*. O desequilíbrio gerado pela demanda de NADPH e produção de NADH acarreta no bloqueio da reação de oxidação realizada pela XDH, gerando o acúmulo de xilitol e uma baixa produção de etanol. Consequentemente, o xilitol pode ser considerado o principal produto do metabolismo de D-xilose para essas leveduras.

Em relação às espécies de *Spathaspora* remanescentes, *Sp. passalidarum* e *Sp. arborariae*, o etanol é o principal produto do metabolismo de D-xilose independentemente da condição empregada. Por conseguinte, essas leveduras podem ser designadas como detentoras de uma capacidade específica de conversão de D-xilose a etanol. O cultivo aeróbico em D-xilose foi averiguado para a descrição de *Sp. arborariae* (Cadete et al., 2009), e conduzido nas mesmas condições utilizadas para a descrição das novas espécies de *Spathaspora* (Capítulo II). Nesse ensaio, *Sp. arborariae* produziu cerca de 10 $g.L^{-1}$ de etanol. Em ambas as condições moderada e severa, esta levedura atingiu, respectivamente, uma maior concentração de etanol (entre 10,6 e 16 $g.L^{-1}$, e $Y_{p/s}^{et}$ = 0,21 e 0,32 em 72h e 120h) que xilitol (6,7 e 9 $g.L^{-1}$, e $Y_{p/s}^{xil}$ = 0,14 e 0,19) com total consumo da D-xilose disponível. No estudo de Cunha-Pereira e colaboradores (2011), uma eficiência de consumo de 84% de xilose em 96h foi encontrada para esta espécie em meio complexo $G_{20}X_{20}A_{10}$ composto por glicose 20 $g.L^{-1}$, D-xilose 20 $g.L^{-1}$ e L-arabinose 10 $g.L^{-1}$, além de extrato de levedura e peptona, cultivado em condições microaeróbicas. Uma produção acima de 15 $g.L^{-1}$ ($Y_{p/s}^{et}$ = 0,46) de etanol e 2,5 $g.L^{-1}$ ($Y_{p/s}^{xil}$ = 0,13) de xilitol foi observada em aproximadamente 72 e 108h, respectivamente. Esses autores também destacaram um consumo de 37% de L-arabinose no meio $G_{20}X_{20}A_{10}$ e de 45% e 33% de consumo de D-xilose e L-arabinose, respectivamente, em hidrolisado de casca de arroz (RHH) não destoxificado nem suplementado. Neste hidrolisado, *Sp. arborariae* foi capaz de produzir 15 $g.L^{-1}$ ($Y_{p/s}^{et}$ = 0,46) de etanol em 96h e 2,5 $g.L^{-1}$ ($Y_{p/s}^{xil}$ = 0,33) de xilitol em 240h. Quando co-cultivada com uma linhagem vinícola de *S. cerevisiae* em $G_{20}X_{20}A_{10}$ e RHH, os resultados de produção de etanol ultrapassaram 17 $g.L^{-1}$ ($Y_{p/s}^{et}$ = 0,77) e 21 $g.L^{-1}$ ($Y_{p/s}^{et}$ = 0,62), respectivamente. Esses resultados foram considerados

promissores para estudos voltados à otimização da fermentação de D-xilose por *Sp. arborariae* tanto em cultivos individuais quanto em co-cultivos com outros microrganismos etanologênicos.

As seis linhagens brasileiras de *Sp. passalidarum* foram avaliadas quanto à fermentação de D-xilose em condições microaeróbicas. Entretanto, nas condições moderada e severa foram estuadas a linhagem tipo (CBS 10155) da espécie e uma das linhagens brasileiras (UFMG-HMD-1.1). Em todos os ensaios realizados, *Sp. passalidarum* destacou-se por uma eficiente e rápida produção de etanol em elevadas concentrações, um rápido e elevado consumo de D-xilose e pela menor produção de xilitol determinada dentre as demais espécies de *Spathaspora*. Apesar das diferenças intraespecíficas observadas em relação à produção de etanol (entre 15 e 18 $g.L^{-1}$) e xilitol (1 a 2,2 $g.L^{1}$) entre as linhagens brasileiras testadas, todas exibiram o comportamento explicitado. Observaram-se resultados muito semelhantes e algumas vezes idênticos nos ensaios realizados somente com as linhagens CBS 10155 e UFMG-HMD-1.1. Em ambos, uma considerável concentração de etanol acima de 20 $g.L^{1}$ com valores de $Y_{p/s}^{et}$ (0,47 e 0,48) próximos ao máximo teórico (0,51) foram alcançados entre 18 e 24h de fermentação. Recentemente, Hou (2012) demonstrou a capacidade de *Sp. passalidarum* em fermentar D-xilose e glicose em condições anaeróbicas, produzindo praticamente a mesma quantidade de etanol a partir de cada fonte de carbono, com valores próximos a 90% do máximo teórico. Quando cultivada sob condições aeróbicas e anaeróbicas em meio com glicose e D-xilose, esta espécie foi capaz de consumir 100% de ambos os açúcares, e também produzir acima de 90% do máximo teórico de etanol. Em contrapartida, a linhagem de *Sc. stipitis* utilizada neste mesmo ensaio não consumiu mais do que 9% da D-xilose disponível no cultivo anaeróbico, obtendo cerca de 50% do máximo teórico de etanol em ambas as condições (Hou, 2012). No estudo de Long e colaboradores (2012), *Sp. passalidarum* foi capaz de co-assimilar D-xilose e glicose aerobicamente; consumir D-xilose mais rapidamente que glicose quando estes açúcares foram avaliados individualmente; exibir uma taxa de produção específica de etanol três vezes maior em D-xilose que glicose, e co-fermentar glicose, D-xilose e celobiose tanto em meio mineral definido quanto em hidrolisado de madeira e de palha de milho em condições limitantes de oxigenação. Estes autores destacaram essa espécie por sua potencial utilização em processos de sacarificação e fermentação simultâneas (SSF) e para futuros estudos de seus mecanismos enzimáticos e regulatórios que a permitem utilizar simultaneamente hexoses e pentoses.

Bruinenberg e colaboradores (1983) estabeleceram que leveduras produtoras de XR com especificidade dual por co-fatores podem oxidar NADH a NAD^{+}, reduzindo a formação de xilitol e permitindo a fermentação de D-xilose a etanol sob condições limitantes de oxigênio. Dentre as espécies de *Spathaspora* estudadas, aquelas caracterizadas como produtoras de etanol – *Sp. passalidarum* e *Sp. arborariae* – foram as únicas a apresentar atividades de xilose redutase NAD(P)H-dependente. Em *Sp. passalidarum* esta atividade é acompanhada por uma preferência

por NADH em relação a NADPH. Essa descoberta foi recentemente evidenciada por Hou (2012) que reportou uma afinidade de XR 1,8 vezes maior por NADH que NADPH, resultado próximo aos encontrados no presente estudo para ambas as linhagens de *Sp. passalidarum*. Assim como constatado para as demais leveduras avaliadas, *Sp. passalidarum* e *Sp. arborariae* apresentam atividade de xilitol desidrogenase estritamente dependente de NAD^+.

Ainda que um comportamento padrão tenha sido estabelecido em relação à atividade de XR e utilização exclusiva ou dual por NAD(P)H quanto ao principal produto do metabolismo de D-xilose por *Spathaspora* spp. (Capítulo III), não foi possível associar diretamente o valor calculado individualmente para cada atividade enzimática à obtenção e concentração de etanol ou xilitol produzidos em cada condição de oxigenação. Observaram-se valores maiores das atividades de XDH em condições severas em todas as espécies avaliadas, com exceção de *Sp. suhii*, única a apresentar uma maior atividade de XDH na condição moderada. Uma vez que em leveduras XR NADPH-dependente ou com preferência por este co-fator cultivadas em condições limitantes de oxigênio, a indisponibilidade de NAD^+ à atividade da enzima XDH NAD^+-dependente é constatada, esperavam-se menores atividades de xilitol desidrogenase na condição severa, com maior limitação de oxigênio, na qual a regeneração de NADH a NAD^+ é diminuta. Entretanto, as atividades foram estabelecidas decorridas 16h de cultivo para todas as leveduras em ambos os ensaios, e nesse período, com exceção de *Sp. passalidarum*, as demais espécies não apresentavam um elevado consumo de D-xilose ou produção de etanol/xilitol, um possível indicativo de uma expressão não significativa de XDH no intervalo considerado. A determinação das atividades próxima ao ponto máximo de produção de etanol ou xilitol para cada espécie pode, possivelmente, trazer melhores esclarecimentos quanto ao comportamento de XDH em cada levedura e condição de oxigenação, além da própria análise de expressão gênica de *XYL2* para cada espécie.

Uma vez que a atividade de XR correlacionada à NAD(P)H é considerada uma das principais etapas relacionadas ao metabolismo de D-xilose a etanol ou xilitol já que XDH apresenta virtualmente uma atividade exclusivamente dependente de NAD^+, constatação confirmada por este trabalho, procurou-se observar uma correlação entre as atividades de xilose redutase e a quantidade dos metabólitos formados, observação esta que provou ser incerta. Notou-se que para as espécies produtoras de xilitol as maiores atividades de XR encontravam-se em concordância com concentrações de etanol maiores, obtidas na condição moderada para *Sp. brasiliensis*, *Sp. roraimanensis* e *Sp. suhii* e na condição severa para *Sp. xylofermentans*. Adicionalmente, para essas leveduras, com exceção da espécie *Sp. roraimanensis*, as maiores concentrações de xilitol também estavam de acordo com maiores atividades de XR. Em contrapartida, em relação à espécie produtora de etanol *Sp. arborariae*, as maiores atividades de xilose redutase, para ambos os co-fatores, foram encontradas na condição moderada, sendo que uma maior produção de etanol

foi constatada na condição severa. A própria razão advinda das atividades calculadas utilizando-se NADH em relação à NADPH decaiu de 0,75 na oxigenação moderada para 0,55 na condição severa. As atividades de xilose redutase associadas à *Sp. passalidarum* são um tópico à parte nesta discussão, já que elas foram as únicas a apresentar predileção por NADH. Tanto a linhagem tipo quanto a linhagem brasileira exibiram maiores atividades de XR ligada a ambos os co-fatores na condição severa que sob oxigenação moderada. Não obstante, a quantidade de etanol produzida por ambas as leveduras nas duas condições foi praticamente a mesma. É interessante observar que apesar dos valores calculados para as atividades atribuídas à linhagem tipo de *Sp. passalidarum* terem sido consideravelmente maiores que as atividades da linhagem brasileira, as razões NADH/NADPH foram muito próximas entre essas linhagens. Alegando-se a inexistência de uma relação direta entre os valores encontrados para as atividades de XR e XDH ao metabólito principal obtido, assim como a quantidade produzida do mesmo, entende-se que o uso específico e a preferência de co-fatores são as propriedades responsáveis pela determinação de qual produto será predominante no processo de fermentação de D-xilose, e não as atividades enzimáticas *per si*. Evidentemente a influência de outros genes relacionados à via do metabolismo de D-xilose deve ser considerada durante a análise do processo. Contudo, é inegável a relação direta encontrada entre a utilização e predileção de co-fatores à produção predominante de etanol ou xilitol.

O ensaio fermentativo conduzido em hidrolisado hemicelulósico de bagaço de cana-de-açúcar (Capítulo I) foi unicamente realizado em condições microaeróbicas, o que não possibilita a comparação de diferentes condições de cultivo no metabolismo da D-xilose nesse tipo de substrato de acordo com a espécie de levedura. Entretanto, este experimento permitiu o estabelecimento de comparações interespecíficas e entre linhagens selecionadas de uma mesma espécie. Os resultados obtidos devem também ser analisados a partir do conhecimento prévio do comportamento da enzima xilose redutase para cada espécie. Observa-se que as únicas leveduras a produzirem xilitol foram *Sp. suhii* (*Spathaspora* sp. 1) e *Sp. xylofermentans* (*Spathaspora* sp. 2), dotadas de XR NADPH-dependente; nas demais espécies, caracterizadas pela dependência por NAD(P)H, o etanol foi o principal produto advindo da fermentação. Além do papel exercido pelas atividades enzimáticas na interpretação dos resultados de fermentação, a análise do processo deve considerar a natureza heterogênia do substrato utilizado. Os hidrolisados de biomassa compreendem uma complexa mistura de componentes (Olsson e Hahn-Hagerdal, 1996). Durante a etapa de hidrólise, diferentes tipos de açúcares podem ser produzidos pela quebra dos polissacarídeos constituintes da matéria-prima (D-glicose, D-galactose, D-manose, D-xilose, L-arabinose). A eficiente bioconversão de D-xilose é dificultada pela presença de outras pentoses e/ou hexoses. As hexoses (como glicose ou manose) podem inibir o metabolismo da D-xilose pela repressão ou inativação do sistema de transporte ou de enzimas catabólicas (Parajó et al., 1998b). Adicionalmente à composição de açúcares inerente a cada tipo de hidrolisado, observa-se uma

característica crítica em hidrolisados preparados empregando-se a catálise ácida: a presença de inibidores do metabolismo microbiano (Schneider, 1989) o que torna a utilização desse meio fermentativo substancialmente mais difícil para os micro-organismos que misturas sintéticas de açúcares puros correspondentes (Keim e Venkatasubramanian, 1989). Estes compostos inibitórios, comumente representados pelo furfural, 5-hidroximetilfurfural, ácido acético, ácido siríngico, ácido p-hidroxibenzóico e vanilina reduzem a multiplicação e viabilidade celular e o rendimento do produto final de fermentação (Winkelhausen e Kuzmanova, 1998).

As considerações a respeito da composição dos hidrolisados de biomassa são fundamentadas baseando-se no importante papel que esta composição exerce no desempenho da etapa de fermentação. No presente estudo, ainda que a D-xilose tenha sido o principal açúcar constituinte do hidrolisado, glicose, L-arabinose e provavelmente outros açúcares não mensurados pela técnica empregada também estavam presentes. Além da influência exercida por esses açúcares na fermentação de D-xilose, e ainda que utilizadas técnicas de destoxificação do hidrolisado (técnica de *overliming*), inibidores, como furfural, hidroximetilfurfural, fenóis e ácido acético foram detectados. Todas essas observações podem vir a justificar os resultados observados no ensaio de fermentação em hidrolisado, em relação aos resultados encontrados no ensaio em meio complexo: (i) um período de fermentação mais longo (tempo de máxima produção de etanol); (ii) um menor consumo de açúcares; (iii) uma menor produção de etanol, em geral, detectada, e consequentemente, menores valores nos índices de parâmetros fermentativos calculados. Uma vez que a natureza da composição dos hidrolisados apresente uma ampla variação, dificultando o estabelecimento de comparações da eficiência de fermentação (Parajó et al., 1998a), a análise individual de cada levedura testada, comparando-se os resultados em ambos os ensaios fermentativos, permite observar e inferir comportamentos diversos desempenhados por cada espécie ou linhagem. As linhagens da espécie *Sc. stipitis* exibiram comportamentos diferenciados, uma comprovação da influência da linhagem no papel da fermentação dentro de uma mesma espécie. Enquanto a linhagem *Sc. stipitis* NRRL Y-7124 (controle positivo) apresentou as menores taxas de consumo de açúcares, e de produção de biomassa e etanol, a linhagem brasileira *Sc. stipitis* UFMG-XMD-15.2 foi a levedura que obteve o melhor desempenho de produção de etanol em relação às demais. A princípio, este comportamento poderia ter sido o esperado pelas leveduras da espécie *Sc. stipitis*, já que essa espécie é caracterizada por apresentar a enzima XR dependente de NAD(P)H. No entanto, em se tratando da utilização do hidrolisado como meio fermentativo, a composição do mesmo foi determinante no desempenho da fermentação em nível de linhagens. O alto consumo de açúcares, a elevada produção de biomassa e de etanol, este último em uma concentração superior à obtida em meio complexo sugerem que *Sc. stipitis* UFMG-XMD-15.2 é dotada de uma boa “adaptação” às condições encontradas no hidrolisado utilizado. De acordo com Olsson e Hahn-Hagerdal (1996) a sensibilidade do micro-

organismo ao hidrolisado varia tanto em relação às condições de fermentação empregadas quanto à linhagem da espécie de interesse escolhida. Adicionalmente, segundo esses autores, micro-organismos eficientemente produtores de etanol a partir de D-xilose em meios definidos não necessariamente reproduzem tal comportamento em hidrolisados lignocelulósicos.

Ainda que um único ensaio fermentativo tenha sido conduzido em hidrolisado de bagaço de cana-de-açúcar, a utilização de quatro diferentes condições de cultivo em meio complexo para avaliação do consumo de D-xilose e produção de etanol e xilitol pelas leveduras estudadas, além dos ensaios enzimáticos determinados, permitiru um amplo espectro de análise do comportamento de cada espécie frente a esse processo metabólico. Devido ao papel relevante desempenhado pela atividade de XR atrelada à dependência de co-fatores na etapa inicial da fermentação, a identificação dos genes codificadores desta enzima e subsequente predição dos resíduos de aminoácidos que a constituem são de fundamental importância para entendimento do mecanismo inerente à predileção e uso exclusivo de NAD(P)H.

5.3 Identificação dos genes codificadores de XR em *Spathaspora* spp.

O sequenciamento do genoma da linhagem tipo de *Sp. passalidarum* (Wohlbach et al., 2011) resultou na constatação da existência de dois genes responsáveis pela codificação da enzima xilose redutase nesta espécie: *XYL1.1* e *XYL1.2*. Esta observação revelou-se uma potencial peculiaridade a ser investigada, visto que a maioria das leveduras fermentadoras de D-xilose apresenta somente um gene codificador de XR, *XYL1* (Jeffries et al., 2007; Wohlbach et al., 2011). Este fato levantou a hipótese da existência de um gene adicional em *Sp. passalidarum* estar diretamente relacionada à elevada capacidade de conversão de D-xilose a etanol por esta levedura (Cadete et al., 2012; Hou, 2012; Long et al., 2012). No presente estudo, realizou-se a comparação de regiões do genoma de *Sp. passalidarum* com regiões do genoma de *Sp. arborariae* na tentativa de se verificar a existência ou não de um gene codificador de xilose redutase adicional em outra espécie do gênero estudado e, ainda, para que essas comparações funcionassem como ferramentas moleculares na identificação do(s) gene(s) codificador(es) de XR nas demais espécies de *Spathaspora* por meio do desenho de iniciadores específicos.

De acordo com dados advindos do sequenciamento de *Sp. passalidarum* disponibilizados nos portais do NCBI (National Center for Biotechnology Information) e JGI (Joint Genome Institute), *XYL1.1* é constituído por 828 pares de bases (pb), que codificam para uma proteína (XYL1.1p) de 275 aminoácidos (aa) e *XYL1.2* possui um tamanho equivalente a 954 pb, codificando para a proteína XYL1.2p com 317 aa. Quando se efetuou a análise das regiões do genoma de *Sp. passalidarum* atribuídas a esses genes, as informações acerca de *XYL1.2* foram confirmadas; em contrapartida, os resultados encontrados não corresponderam à anotação do *XYL1.1* na base de dados. As análises efetuadas mostraram que *XYL1.1* é na verdade formado por 957 pb

responsáveis pela codificação de uma proteína com 318 aminoácidos. Algumas evidências confirmam como válida a identificação de *XYL1.1* efetuada no presente estudo em relação às informações consultadas: i) a confirmação da existência na linhagem brasileira de *Sp. passalidarum* de uma sequencia gênica de 957 pb codificadora de uma proteína com 318 aa apresentando similaridades de 98% e 99%, respectivamente, com as sequencias equivalentes determinadas na linhagem tipo dessa espécie; ii) a amplificação de uma sequencia gênica de 957 pb nas demais espécies de *Spathaspora* utilizando-se os iniciadores desenhados para a amplificação deste gene nessas leveduras; iii) a posterior confirmação da existência de sequencias e resíduos conservados entre *XYL1.1*/XYL1.1p em relação às sequencias de *XYL1*/XYL1p identificadas em *Spathaspora* spp.

A comparação inicial dos genomas de *Sp. passalidarum* e *Sp. arborariae* indicou que *XYL1*/XYL1p de *Sp. arborariae* apresentavam uma maior identidade com *XYL1.1*/XYL1.1p (89% e 93%, respectivamente) que *XYL1.2*/XYL1.2p (79% e 74%, respectivamente) de *Sp. passalidarum*. Em *Sp. passalidarum*, observou-se que os genes *XYL1.1* e *XYL1.2* estão proximamente localizados, separados por cerca de 1480 pares de bases, e que *XYL1.2* encontra-se à montante de *XYL1.1*. Na tentativa de se encontrar uma sequencia de bases que pudesse corresponder total ou parcialmente a *XYL1.2* no genoma de *Sp.arborarie*, efetuou-se a análise de regiões à jusante e à montante do gene *XYL1* comparando-se essas regiões às regiões adjacentes aos genes *XYL1.1* e *XYL1.2* (dados não mostrados). Um total de 20 mil pares de bases foi individualmente analisado e comparado entre os genomas de cada levedura. Apesar da descoberta de sequencias e genes conservados entre ambas as linhagens em regiões equivalentes do genoma (sintenia gênica), não foram identificados genes ou regiões que pudessem confirmar ou sugerir a existência de um gene codificador de XR adicional em *Sp. arborariae*. Dessa maneira, a partir de sequencias homólogas adjacentes aos genes *XYL1* e *XYL1.1*, foram desenhados iniciadores com o intuito de se amplificar tal região nas demais espécies de *Spathaspora*, e iniciadores capazes de amplificar *XYL1.2* especificamente em linhagens de *Sp. passalidarum* (Capítulo III).

O alinhamento das sequencias dos genes codificadores de xilose redutases em *Spathaspora* spp., assim como das sequencias preditas de aminoácidos dessas enzimas permitiu a identificação de regiões e resíduos conservados e divergentes tanto em nível molecular quanto protéico. Porcentagens de identidade entre 89% e 100% e 93% e 100% foram encontradas comparando-se respectivamente as sequencias de *XYL1* e *XYL1.1* e XYL1p e XYL1.1p entre as linhagens de *Spathaspora* estudadas com a linhagem tipo de *Sp. passalidarum*, mostrando um elevado grau de conservação existente entre esses genes e proteínas nessas espécies. É interessante constatar que o gene *XYL1* de *Sp. xylofermentans* apresenta 100% de identidade com *XYL1.1* da linhagem tipo de *Sp. passalidarum*, enquanto uma menor porcentagem de identidade é identificada quando são comparados esse gene e a proteína por ele codificada entre as linhagens

tipo e brasileira desta espécie. *XYL1.2* e XYL1.2p apresentam uma alta porcentagem de identidade (98% e 99%) entre as duas linhagens de *Sp. passalidarum*; observam-se diferenças de 15 pb e três aminoácidos, respectivamente, entre as sequencias gênicas e proteicas, mesmo padrão de diferenças observado para as sequencias de *XYL1.1* e XYL1.1p. Por outro lado, uma clara divergência é observada comparando-se *XYL1.2/XYL1.1* e XYL1.2p /XYL1.1p em cada linhagem de *Sp. passalidarum*: na linhagem tipo, *XYL1.2* apresenta 77% de identidade com *XYL1.1*, e XYL1.2p é 74% idêntico a XYL1.1p; na linhagem brasileira essas porcentagens são equivalentes a 78% e 75%, respectivamente.

Dentre as várias diferenças de resíduos observadas com relação às sequencias de XYL1.2p e XYL1p/XYL1.1p, uma merece destaque por estar localizada no sítio de ligação da enzima ao co-fator, a tétrade altamente conservada Ile-Pro-Lis-Ser (IPKS) e resíduos adjacentes. Essa região foi determinada mapeando-se sítios homólogos de aldoses redutases de diferentes organismos (Bohren et al., 1991; Kostrzynska et al., 1998; Lee et al., 2003) e por meio de análises de cristalografia de raio-X (Carugo e Argos, 1997). Neste sítio, na posição 271 em XYL1.2p e 272 em XYL1p/XYL1.1p, todas as sequencias de XYL1/pXYL1.1p apresentam um resíduo de asparagina (N) com exceção da linhagem de *Sp. brasiliensis* que possui um resíduo de serina (S). Em contrapartida, XYL1.2p dispõe de um resíduo de ácido aspártico (D). Os demais resíduos do sítio de ligação, correspondentes à tétrade, são iguais em todas as sequencias. Uma vez que a região de ligação da enzima ao co-fator está diretamente relacionada à determinação de qual co-fator poderá ser utilizado, assim como a ordem de magnitude desta reação, pode-se inferir que as diferenças de aminoácidos observadas neste sítio consistem no principal indício de uma atividade NADH-preferencial apresentada por XYL1.2p e uma atividade estrita ou dependente de NADPH por XYL1p/XYL1.1p.

A alteração da preferência de co-fatores em xilose redutases tem sido experimentalmente realizada por meio da indução de mutações randômicas ou sítio-direcionadas nos resíduos de aminoácidos responsáveis pela discriminação da ligação da enzima à NADH ou NADPH (Kostrzynska et al., 1998; Watanabe et al., 2007; Bengtsson et al., 2009; Runquist et al., 2010). Bohren e colaboradores (1991) ao substituírem o resíduo de lisina (K) do motivo Ile-Pro-Lis-Ser por metionina (M) constataram que a lisina encontra-se envolvida na ligação ao NADPH. A mutação K270M na xilose redutase de *Sc. stipitis* aumentou 17 vezes a constante de Michaelis-Menten (K_m) por este co-fator quando D-xilose foi utilizada como substrato (Kostrzynska et a., 1998). A xilose redutase NADH-dependente de *Candida parapsilosis* apresenta naturalmente nesta posição um resíduo de arginina (R) ao invés de lisina, K274R (Lee et al., 2003). A introdução de uma mutação K274R em *Candida tenuis* aparentemente não afetou as afinidades de co-fatores nessa levedura; entretanto observou-se um aumento da K_m por NADPH e uma diminuição dessa constante por NADH (Petschacher et al., 2005). A combinação das duplas mutações K270R e N272D em *Sc.*

stipitis resultou no aumento da preferência por NADH pela xilose redutase desta levedura (Watanabe et al., 2007). Observa-se que a mutação N272D, equivalente à substituição da asparagina (N) por ácido aspártico (D) induzida em *Sc. stipitis* corresponde precisamente à diferença de resíduos observada na região de ligação ao co-fator em XYL1.2p em relação às demais sequencias de XR analisadas. No estudo de Runquist e colaboradores (2010) as mutações N272D e P275Q foram encontradas em linhagens de *S. cerevisiae* capazes de crescer em culturas anaeróbicas de D-xilose, transformadas com genes *XYL1* de *Sc. stipitis* submetidos a mutações randômicas no sítio de ligação ao co-fator. Esses autores avaliaram individualmente a influência de cada mutação ou de ambas as mutações na fermentação anaeróbica de D-xilose por *S. cerevisiae*. Os transformantes TMB 3421 (N272D/P275Q) e TMB 3422 (N272D) foram capazes de crescer em anaerobiose enquanto a linhagem TMB 3423 (P275Q) não cresceu nessas condições. TMB 3421 e TMB 3422 exibiram rendimentos maiores em etanol ($Y_{p/s}^{et}$ = 0,35 e 0,37, respectivamente) que xilitol ($Y_{p/s}^{xil}$ = 0,26 e 0,24, respectivamente), comportamento contrário observado pela linhagem TMB 3423 ($Y_{p/s}^{et}$ = 0,18 e $Y_{p/s}^{xil}$ = 0,57). De fato, segundo esses autores, TMB 3421 e TMB 3422 apresentaram os maiores rendimentos em etanol já relatados para linhagens de *S. cerevisiae* transformadas com a via XR/XDH e capazes de utilizar D-xilose. Essas linhagens também exibiram um significativo aumento na especificidade de XR por NADH, resultado não observado para o transformante TMB 3423. Os autores concluíram, portanto, que a mutação N272D foi a mutação selecionada primariamente nos mutantes obtidos capazes de crescer anaerobicamente em D-xilose. Adicionalmente, a introdução da mutação P275Q em XR, avaliada no transformante TMB 3423, não resultou na obtenção de resultados consideráveis de conversão de D-xilose em etanol, indicando que esta mutação não está relacionada à otimização deste processo.

XYL1.2p, encontrada com exclusividade em *Sp. passalidarum,* apresenta naturalmente um resíduo de ácido aspártico (D) na posição 271, equivalente à mutação N272D do gene XYL1 de *Sc. stipitis* reportada por Runquist e colaboradores (2010) como a responsável direta pelo aumento da atividade de xilose redutase ligada a NADH e decorrente conversão de D-xilose em elevadas concentrações de etanol em anaerobiose. Com base nessas informações e levando-se em consideração que: i) *Sp. passalidarum* é a única dentre as demais espécies de *Spathaspora* analisadas a exibir atividade de XR NAD(P)H-dependente com preferência por NADH; ii) *Spathaspora* spp. possuem um único gene, *XYL1*, que codifica para uma XR estritamente dependente de NADPH ou com preferência por este co-fator, e iii) as sequencias de *XYL1*/XYL1p apresentam uma maior homologia em relação à sequencias de *XYL1.1*/XYL1.1p que *XYL1.2*/XYL1.2p, deduz-se que XYL1.2p é a enzima dotada da capacidade de utilizar exclusivamente ou preferir NADH em *Sp. passalidarum*, enquanto XYL1.1p apresenta uma atividade estritamente dependente ou com preferência por NADPH. A comprovação dessa inferência foi possibilitada após a transformação individual de *S. cerevisiae* com os genes *XYL1.1* e

XYL1.2 (Capítulo III) que veio a demonstrar uma atividade com dependência por ambos os co-fatores, mas com predileção por NADH exibida por XYL1.2p, e uma atividade NADPH-dependente apresentada por XYL1.1p.

A presença de um gene adicional em *Sp. passalidarum* capaz de proporcionar a esta espécie um comportamento específico em relação ao metabolismo de D-xilose pode ser explicada pelo fenômeno da duplicação gênica. Credita-se a este processo a principal fonte de novos genes (Ohno, 1970). Genes duplicados, muitas vezes referidos como parálogos (Zhang, 2003) podem surgir de duplicações de genes individuais, de segmentos do genoma (pequenos segmentos cromossomais e cromossomos inteiros), ou pela duplicação de todo o genoma (Langkjær et al., 2003; Kellis et al., 2004). Uma vez que o surgimento de novos genes pode advir de duplicações gênicas, este evento tem sido proposto como um caminho vantajoso para a inovação evolutiva (Ohto, 1970), posto que genes duplicados podem fornecer "matéria-prima genética" para o surgimento de novas funções por meio de mutação e seleção natural (Kellis et al., 2004). De fato, a "evolução por meio da duplicação gênica" emergiu como um princípio geral da evolução biológica, evidente a partir da prevalência de genes duplicados em todos os genomas sequenciados de Bacteria, Archaea e Eukayota (Zhang, 2003). Apesar da existência universal em virtualmente todos os organismos, esses genes são raramente preservados no genoma. Acredita-se que a manutenção de genes parálogos esteja relacionada à diferenciação e aquisição de uma especialização funcional (Langkjær et al., 2003). Dentre as hipóteses existentes que procuram justificar a permanência de genes duplicados, a hipótese da neofuncionalização (NF) propõe que, após a duplicação, um dos genes retém a função ancestral enquanto o outro adquire novas funções (Ohno, 1970). Um leque de hipóteses NF pode ser considerado, no qual o gene apresentador da nova função pode reter toda, nenhuma ou parte das funções ancestrais (He e Zhang, 2005). A neofuncionalização requer a variação do número de substituições de aminoácidos (Zhang, 2003). Nesse contexto, genes neofuncionais ou dotados de uma especialização funcional estão sujeitos à pressões evolutivas por meio da seleção positiva que acelera a fixação de mutações vantajosas capazes de aumentar a atividade da nova função (Yokoyama e Yokoyama, 1989). A duplicação gênica espécie-específica pode resultar em genes com funções especializadas em nível de espécie, que por sua vez podem vir a facilitar a adaptação da espécie a determinados ambientes. Em outras palavras, a duplicação de genes contribui para a divergência e origem de características específicas em uma espécie (Zhang, 2003). Nesse contexto, a existência de dois genes codificadores de xilose redutase com preferências diferenciadas de co-fatores em *Sp. passalidarum* apontam para a ocorrência de uma duplicação gênica seguida da neofuncionalização espécie-específica atribuída à presença exclusiva de *XYL1.2* nesta levedura.

Uma relevante constatação realizada em complementaridade à presença adicional de *XYL1.2* em *Sp. passalidarum* diz respeito à diferença do nível de expressão desse gene em relação

à expressão de *XYL1.1* (Capítulo III). Na linhagem tipo, *XYL1.2* é cinco vezes mais expresso que *XYL1.1* e na linhagem brasileira este gene é 10 vezes mais expresso. Gu e colaboradores (2005), por meio da determinação de taxas da expressão relativa de genes em leveduras, sugerem que a expressão de genes duplicados tende a evoluir assimetricamente, ou seja, que a expressão de uma das cópias evolui rapidamente enquanto a outra cópia mantém em grande parte o perfil de expressão encontrado no gene ancestral em comum. Os resultados obtidos no presente trabalho vêm a corroborar este comportamento, uma vez que *XYL1.2* é muito mais expresso que *XYL1.1* em ambas as linhagens de *Sp. passalidarum*.

A confirmação de que a existência do gene *XYL1.2* em *Sp. passalidarum* seja efetivamente a principal característica responsável pela extraordinária capacidade desta levedura fermentar D-xilose em anaerobiose, com elevada produtividade e rendimento em etanol, pode ser comprovada por meio da avaliação do comportamento individual deste gene em um hospedeiro incapaz de fermentar D-xilose. Dessa maneira, e para a observação do comportamento específico de *XYL1.1*, os genes codificadores de XR das linhagens tipo e da linhagem brasileira UFMG-HMD-1.1 de *Sp. passalidarum* foram utilizados independentemente na transformação de uma linhagem laboratorial de *S. cerevisiae*.

5.4 Expressão heteróloga dos genes *XYL1.1* e *XYL1.2* de *Spathaspora passalidarum* em *Saccharomyces cerevisiae*

A transformação de *S. cerevisiae* com os genes *XYL1.1* e *XYL1.2* de *Sp. passalidarum* (Capítulo III) resultou na obtenção de quatro linhagens transformantes: TMB 3501 (*XYL1.1* da linhagem tipo, CBS 10155), TMB 3502 (*XYL1.1* da linhagem brasileira, UFMG-HMD-1.1), TMB 3503 (*XYL1.2*, CBS 10155) e TMB 3504 (*XYL1.2*, UFMG-HMD-1.1). Essas linhagens foram submetidas a ensaios de fermentação anaeróbica de D-xilose em meio mínimo. A linhagem TMB 3422 (Runquist et al., 2010) carreando o gene *XYL1* de *Sc. stipitis* com a mutação N272D também foi testada nessas condições. Todos os transformantes apresentam como única diferença o gene codificador de xilose redutase utilizado. A linhagem de *S. cerevisiae* TMB 3044 foi utilizada para a transformação. Essa linhagem apresenta o gene codificador de xilulocinase (*XKS1*) além dos genes codificadores das enzimas da etapa não oxidativa da via pentose fosfato, transaldolase (*TAL1*), transcetolase (*TKL1*), ribose 5-fosfato isomerase (*RKI1*) e ribulose 5-fosfato epimerase (*RPE1*) superexpressados (Figura 1). Adicionalmente, o gene *GRE3*, responsável pela codificação de uma aldose redutase NADPH-dependente, foi deletado de modo a se minimizar eventuais interferências no processo analisado. O vetor YIp (plasmídio integrativo para levedura), capaz de se integrar diretamente no DNA cromossomal por meio de recombinação homóloga, foi empregado para a expressão dos genes de interesse: os genes de xilose redutase e o gene codificador de uma xilitol desidrogenase de *Sc. stipitis* NAD^+-dependente. Dessa maneira, objetivou-se atestar e

comparar individualmente a influência exercida pelas enzimas XYL1p (N272D), XYL1.1p e XYL1.2p no metabolismo de D-xilose em *S. cerevisiae*.

As linhagens transformadas com os genes *XYL1.1* foram incapazes de crescer durante todo o ensaio fermentativo em condições anaeróbicas. Este resultado está diretamente relacionado a um comportamento de XR estritamente dependente de NADPH, uma vez que o metabolismo de D-xilose foi impossibilitado devido à indisponibilidade de co-fatores para a reação em condições anaeróbicas, e utilizando-se D-xilose como única fonte de carbono. A determinação das atividades enzimáticas dos transformantes TMB 3501 e TMB 3502 cultivados em condições aeróbicas confirma estes resultados, visto que XYL1.1p apresentou atividades com dependência exclusiva de NADPH (Capítulo III).

Surpreendentemente, resultados drasticamente diferentes foram obtidos com as linhagens TMB 3503 e TMB 3504. A linhagem TMB 3503 (*XYL1.2*, CBS 10155) apresentou um fraco desempenho fermentativo, consumindo cerca de 16% da D-xilose disponível em 95h de ensaio, aliado a uma baixa produção de células (0,5 $g.L^{-1}$) xilitol (2,5 $g.L^{-1}$) e etanol (0,9 $g.L^{-1}$). Em contrapartida, o transformante TMB 3504 (*XYL1.2*, UFMG-HMD-1.1) foi responsável por uma relevante conversão de D-xilose em etanol, com elevada produtividade e baixo acúmulo de xilitol. Os resultados alcançados por esta linhagem foram consideravelmente melhores que aqueles demonstrados por TMB 3422, transformante considerado por Runquist e colaboradores (2010) como detentor de um dos maiores rendimentos em etanol já relatados para linhagens de *S. cerevisiae* transformadas com a via XR/XDH. Além da conversão de substrato em etanol ter sido maior na fermentação realizada com TMB 3504 ($Y_{p/s}^{et}$ = 0,4) que com TMB 3422 ($Y_{p/s}^{et}$ = 0,33), o grande diferencial encontrado para *S. cerevisiae* transformada com o gene *XYL1.2* da linhagem brasileira diz respeito à produtividade volumétrica em etanol (Qp) e à produção de xilitol. TMB 3504 apresentou uma produtividade volumétrica 136% maior que TMB 3422, correspondente a uma máxima produção de etanol (18,4 $g.L^{-1}$) em 72h, praticamente a metade do tempo de fermentação relativo à outra linhagem, cujo pico de etanol foi alcançado às 142h. A comparação da produção de xilitol entre as duas linhagens merece um destaque à parte: esta produção caiu pela metade com a utilização de TMB 3504 (5,4 $g.L^{-1}$) uma vez que TMB 3422 produziu 10,4 $g.L^{-1}$ de xilitol. Um dos principais gargalos na construção de linhagens transformantes de *S. cerevisiae* via expressão de XR e XDH é fundamentado na considerável produção de xilitol detectada para estas leveduras (Jeppsson et al., 2002) aliada a baixas produtividades em etanol (Runquist et al., 2010). Os resultados do presente trabalho mostram potencial para a resolução dessas questões, uma vez que uma baixa produção de xilitol e uma elevada produtividade em etanol via exploração de um gene codificador de XR inédito foram conquistadas.

Os resultados das atividades enzimáticas determinadas para TMB 3422 e TMB 3504 confirmam os comportamentos observados durante a fermentação de D-xilose em condições

anaeróbicas para essas leveduras. Somente as atividades dessas linhagens foram possíveis de serem determinadas nessas condições, uma vez que a multiplicação do transformante TMB 3503 foi inexpressiva em anaerobiose. Entretanto, a caracterização das atividades deste transformante foi permitida a partir da cultura do mesmo em condições aeradas de cultivo. Confirmou-se que gene *XYL1.2* de *Sp. passalidarum* é responsável por codificar uma XR com utilização dual de co-fatores, mas com predileção por NADH. As razões NADH/NADPH encontradas foram equivalentes a 1,34 e 1,21 para TMB 3503 e TMB 3504, respectivamente. Entretanto, as atividades enzimáticas de XR apresentadas por TMB 3503 foram três vezes maiores que aquelas apresentadas por TMB 3504. O transformante TMB 3422 também apresenta uma atividade NAD(P)H-dependente, mas com preferência por NADPH, confirmando os resultados constatados para esta levedura por Runquist e colaboradores (2010). Os comportamentos encontrados comparando-se TMB 3422 e TMB 3504 cultivados em anaerobiose mostram, ainda, que estas leveduras exibem atividades de XR NADPH-dependentes equivalentes (0.94±0.02 e 0.96±0.01, respectivamente). O grande diferencial em relação à essas linhagens está relacionado às atividades de xilose redutase dependentes de NADH: TMB 3504 apresenta uma atividade 1,7 vezes maior utilizando este co-fator que TMB 3422. Esses resultados são refletidos nas razões NADH/NADPH encontradas, iguais a 1,18 e 0,71, respectivamente, e por repercutirem diretamente no desempenho da fermentação associado a cada transformante avaliado, confirmam o papel determinante da reciclagem de co-fatores no direcionamento da conversão de D-xilose a etanol.

As xilose redutases XYL1p (N272D) de *Sc. stipitis* (TMB 3422) e XYL1.2p de *Sp. passalidarum* apresentaram um resíduo de ácido aspártico em substituição ao resíduo de arginina em posições equivalentes (271/272) da sequencia de aminoácidos da proteína. Entretanto, uma vez que XYL1.2p exibe predileção por NADH e em XYL1p a atividade de XR é decorrente de uma preferência por NADPH, diferenças em outros resíduos entre ambas as enzimas são certamente os responsáveis por determinar comportamentos tão diferenciais e inerentes a cada redutase. No alinhamento das sequencias de aminoácidos dessas enzimas (Capítulo III) observa-se na região que engloba o sítio de ligação ao co-fator resíduos específicos encontrados unicamente em XYL1.2p. Nas posições 264 e 265 de XYL1.2p, equivalente às posições 265 e 266 em XYL1p e XYL1.1p, são identificados resíduos de valina (V) em ambos os sítios ao invés de um resíduo de isoleucina (I) e alanina (A), respectivamente. Nas posições 274 e 277 em XYL1.2p (275 e 276 em XYL1p e XYL1.1p), resíduos de ácido glutâmico (E) e valina (V) estão presentes somente em XYL1.2p, enquanto XYL1p de *Sc. stipitis* apresenta respectivamente os resíduos de prolina (P) e leucina (L), e as demais redutases possuem em ambas as posições resíduos de alanina (A). A atribuição exclusiva desses e de outros resíduos às sequencias de XYL1.2p em *Sp. passalidarum* podem vir a direcionar estratégias futuras de análise da influência de resíduos específicos na predileção de co-fatores em XR, assim como a magnitude dessa reação, e de pesquisas voltadas

para a mutação sítio-dirigida nos genes *XYL1.2* a fim de se aumentar ou até mesmo restringir a atividade de XYL1.2p ao co-fator NADH.

Ao contrário da relevante capacidade converter D-xilose a etanol por *S. cerevisiae* atribuída à *XYL1.2*/XYL1.2p da linhagem brasileira de *Sp. passalidarum*, um fraco desempenho foi alcançado na utilização da levedura transformada com gene *XYL1.2* da linhagem tipo dessa espécie. A transformação foi funcional, visto que em condições aeróbicas de cultivo TMB 3503 foi capaz de crescer em D-xilose e apresentar atividades enzimáticas NAD(P)H-dependentes com predileção por NADH. Entretanto, essas atividades foram baixas, e ficaram muito aquém daquelas encontradas para TMB 3504. Uma explicação para a ocorrência desses resultados pode estar relacionada às diferenças em nível de gene e proteína constatadas para essas leveduras. Apesar da elevada homologia apresentada por XYL1.2 (98%) e XYL1.2p (99%) nas linhagens tipo e brasileira de *Sp. passalidarum*, são observadas diferenças de 15 pb e três aa entre as sequencias gênicas e proteicas, respectivamente. Estas diferenças podem estar diretamente relacionadas à indução de interações em nível gênico e/ou proteico (gene-gene, gene-proteína e proteína-proteína) que venham a ter ocorrido entre *XYL1.2* e XYL1.2p com genes e proteínas encontrados na linhagem de *S. cerevisiae* utilizada, ou mesmo com o vetor de expressão empregado na transformação. Essas interações possivelmente resultaram numa baixa transcrição de *XYL1.2* e/ou baixa atividade funcional de XYL1.2p em *S. cerevisiae*. De acordo com Ito e colaboradores (2000), as interações do tipo proteína-proteína desempenham papéis fundamentais em vários aspectos da organização funcional da célula, e o conhecimento desse mecanismo é indispensável para uma compreensão completa da estrutura celular. Esses pesquisadores afirmam, ainda, que embora os estudos dessas interações sejam relizados com proteínas de interesse à ciência, os genomas abrigam uma série de novas proteínas para as quais, atualmente, não existam informações concretas disponíveis, e, consequentemente, de como essas proteínas estabelecem redes de interação com outras proteínas. Uma possibilidade de interação a nível gênico que possa ter ocorrido especificamente com o gene *XYL1.2* da linhagem CBS 10155 está relacionada à interação gene-ambiente, ou seja, à interação existente entre o gene e o ambiente no qual ele está inserido. Este fenômeno prediz que o efeito de uma variedade genética difere em diferentes ambientes (Smith e Kruglyak, 2008). Levando-se em consideração que no presente trabalho o ambiente pode ser definido como a célula na qual *XYL1.2* está inserido, uma mudança drástica de ambiente foi realizada, visto que este gene foi transferido de uma espécie de levedura para outra. Segundo Smith e Kruglyak (2008), os efeitos das variações genéticas em características fenotípicas frequentemente são dependentes das condições ambientais e fisiológicas, e que, no entanto, tais interações gene-ambiente são mal compreendidas. Uma vez que estudos específicos acerca da influência das diferenças encontradas nos genes *XYL1.2* e nas redutases XYL1.2p entre as linhagens de *Sp. passalidarum* no metabolismo de D-xilose em *S. cerevisiae* não foram realizados,

é imprudente afirmar com exatidão o que pode ter ocorrido para a obtenção de resultados tão adversos. Entretanto, visto que o genoma de *Sp. passalidarum* foi concluído e disponibilizado para análise por Wohlbach e colaboradores em 2011, e que até o presente momento não existem registros na literatura de linhagens de *S. cerevisiae* transformadas com o gene *XYL1.2* da linhagem tipo dessa espécie, os resultados do presente estudo podem vir a confirmar a impossibilidade desta expressão heteróloga ser absolutamente funcional.

A realização deste trabalho proporcionou um conhecimento mais amplo em relação à influência da utilização e preferência de co-fatores pela enzima xilose redutase no metabolismo de D-xilose, principalmente acerca da obtenção dos produtos fermentativos de interesse. *Spathaspora passalidarum* mostrou ser uma levedura dotada de um comportamento único na conversão efetiva de D-xilose a etanol. Constatou-se que o gene *XYL1.2*, encontrado somente nesta espécie, é um dos responsáveis por esta atuação. Entretanto, o estudo de outros genes, tais como genes relacionados ao transporte de D-xilose e codificadores das enzimas da via pentose fosfato apresentam um potencial a ser explorado no que diz respeito à elucidação do metabolismo de D-xilose nessa levedura, e ainda, no emprego em futuras estratégias de transformação de linhagens de *S. cerevisiae*. O isolamento de seis linhagens de *Sp. passalidarum* a partir de regiões de Floresta Amazônica Brasileira abre um leque ainda maior de possibilidades para a investigação de novos genes e proteínas inerentes a esta espécie.

6. Conclusões

- O isolamento de novas espécies e linhagens de leveduras fermentadoras de D-xilose é uma importante contribuição taxonômica, evolutiva e biotecnológica a estudos voltados para a compreensão e aplicações deste processo metabólico, além de salientar o potencial de pesquisas voltadaos para a bioprospecção desses micro-organismos de interesse.

- A disponibilidade de oxigênio e as condições de cultivo influenciam diretamente a fermentação de D-xilose em nível de espécie e linhagem. Contudo, o espectro de utilização de co-fatores da enzima xilose redutase revelou-se a principal característica determinante da obtenção de um produto fermentativo majoritário. As espécies produtoras de xilitol (*Sc. amazonensis, Sp. brasiliensis, Sp. roraimanensis, Sp. suhii* e *Sp. xylofermentans*) apresentaram atividade de XR NADPH-dependente. *Spathaspora arborariae* e *Sp. passalidarum*, espécies produtoras de etanol, exibiram XR dependente de NAD(P)H.

- *Spathaspora passalidarum*, levedura que exibiu os melhores resultados de conversão de D-xilose a etanol, foi a única espécie caracterizada pela expressão de XR com preferência por NADH. Esse comportamento está relacionado à duplicação gênica de *XYL1* identificada nesta levedura. Comprovou-se que o gene adicional *XYL1.2* é responsável pela codificação da xilose redutase com predileção por NADH (XYL1.2p), enquanto *XYL1.1* codifica uma xilose redutase (XYL1.1p) estritamente NADPH-dependente. Além disso, *XYL1.2* é mais expresso que *XYL1.1*, resultando numa maior síntese de XYL1.2p.

- As sequencias de XYL1p identificadas nas demais espécies de *Spathaspora* estudadas são mais similares à sequencia de XYL1.1p que XYL1.2p. A presença exclusiva de resíduos em determinadas posições de XYL1.2p, como um resíduo de ácido aspártico na posição 271 da região promovedora da ligação ao co-fator em substituição ao resíduo de asparagina (N271D) está diretamente relacionada à predileção desta enzima por NADH.

- A expressão heteróloga de *XYL1.2* da linhagem brasileira de *Sp. passalidarum* UFMG-HMD-1.1 em *S. cerevisiae* resultou na obtenção de uma levedura capaz de fermentar D-xilose anaerobicamente, com elevado rendimento e produtividade em etanol, e baixa produção de xilitol. Esta levedura é promissora para futuros ensaios de conversão de D-xilose em etanol utilizando-se outras condições de cultivo e escalas de produção.

- Não foram obtidos resultados satisfatórios com a transformação de *S. cerevisiae* utilizando-se o gene *XYL1.2* da linhagem tipo (CBS 10155) de *Sp. passalidarum*. Interações gene-ambiente e em nível gênico e/ou proteico de *XYL1.2* e XYL1.2p desta linhagem podem ter ocorrido com genes e

proteínas expressas na linhagem de *S. cerevisiae* utilizada, ou mesmo genes e produtos de expressão do vetor carreador de *XYL1.2,* resultando numa baixa transcrição de *XYL1.2* e/ou baixa atividade funcional de XYL1.2p da linhagem CBS 10155 em *S. cerevisiae*.

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Anexos

RESEARCH ARTICLE

Spathaspora arborariae sp. nov., a D-xylose-fermenting yeast species isolated from rotting wood in Brazil

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Received 3 July 2009; revised 27 August 2009; accepted 29 August 2009.
Final version published online 10 October 2009.

DOI:10.1111/j.1567-1364.2009.00582.x

Editor: Cletus Kurtzman

Keywords
new yeast species; *Spathaspora arborariae*; D-xylose-fermenting yeast; rotting wood; tropical ecosystems.

Abstract

Four strains of a new yeast species were isolated from rotting wood from two sites in an Atlantic Rain Forest and a Cerrado ecosystem in Brazil. The analysis of the sequences of the D1/D2 domains of the large-subunit rRNA gene showed that this species belongs to the *Spathaspora* clade. The new species ferments D-xylose efficiently and is related to *Candida jeffriesii* and *Spathaspora passalidarum*, both of which also ferment D-xylose. Similar to *S. passalidarum*, the new species produces unconjugated asci with a single greatly elongated ascospore with curved ends. The type strain of *Spathaspora arborariae* sp. nov. is UFMG-HM19.1A^T (= CBS11463^T = NRRL Y-48658^T).

Introduction

The *Spathaspora* clade contains several D-xylose-fermenting yeast species isolated from rotting wood or wood-boring insects. *Spathaspora passalidarum*, the single teleomorph species of the clade, was isolated from the passalid beetle *Odontotaenius disjunctus* in Louisiana (Nguyen *et al.*, 2006). The D-xylose-fermenting asexual species *Candida jeffriesii* and *Candida lyxosophila* are phylogenetically related to *S. passalidarum*, and were isolated from the surface of a wood-boring beetle and woodland soil, respectively (van der Walt *et al.*, 1987; Nguyen *et al.*, 2006). *Candida insectamans* and *Candida materiae*, two species that do not ferment this pentose, also belong to the *Spathaspora* clade, although with little or no support, and were isolated from the frass of beetle larvae in trees in South Africa and from rotting wood samples in Brazil, respectively (van der Walt *et al.*, 1972; Barbosa *et al.*, 2009).

We have isolated four strains of a new yeast species belonging to the *Spathaspora* clade from rotting wood samples collected in the Atlantic Rain Forest and the Cerrado ecosystem in Brazil. The strains formed asci without conjugation and asci containing a single elongated ascospore resembling that of *S. passalidarum*. Analysis of the sequences of the D1/D2 domains of the large-subunit rRNA gene showed that these strains represented a single species that is closely related to *C. jeffriesii*, *S. passalidarum* and *C. materiae*. The new species is described as *Spathaspora arborariae* sp. nov.

Materials and methods

Yeast isolation and identification

The yeasts were isolated from rotting wood samples collected in the National Park of Serra do Cipó and in the Rio Doce State Park. The National Park of Serra do Cipó is located in Minas Gerais state, Brazil, at the southern part of Serra do Espinhaço, and extends over a total area of

33 800 ha. The local climate is altitudinal tropical with fresh and rainy summers and a well-established dry season, with annual mean temperatures oscillating between 17 and 18.5 °C. The vegetation is varied, although dominated by grasslands (Cerrado) and rupestrian fields. The Rio Doce State Park has an area of approximately 36 000 ha and is the largest preserved semi-deciduous Atlantic Rain Forest found in Minas Gerais state. This state park is characterized by a transition of mesothermic humid tropical climate and mesothermic rainy tropical climate, with annual average temperatures ranging from 21 to 24 °C.

Five decayed wood samples were collected from the National Park of Serra do Cipó in April 2008 and 20 samples from the Rio Doce State Park in August 2008. The samples were stored in sterile plastic bags and transported under refrigeration to the laboratory over a period of no more than 24 h. One gram of each sample was placed separately in flasks with 20 mL sterile D-xylose medium (yeast nitrogen base 0.67%, D-xylose 0.5%, chloramphenicol 0.02%) and 20 mL sterile xylan medium (yeast nitrogen base 0.67%, xylan 1%, chloramphenicol 0.02%, pH 5.0 ± 0.2), respectively. The flasks were incubated at 25 °C on an incubator shaker (New Brunswick) at 150 r.p.m. for 3–10 days. When growth was detected, 0.5 mL of a culture was transferred to a tube containing 5 mL sterile D-xylose or xylan media, and the tubes were incubated on an incubator shaker as described above. One loopful of each tube was streaked on D-xylose or xylan agar media. The plates were incubated at 25 °C until yeast colonies developed. The yeasts isolated were chosen based on colony morphology by repeated streaking on yeast extract–malt extract agar (YMA, glucose 1%, yeast extract 0.3%, yeast malt 0.3%, peptone 0.5%, agar 2% and chloramphenicol 0.02%) and preserved at − 80 °C or in liquid nitrogen for later identification. The yeasts were characterized using standard methods (Yarrow, 1998). The ability to ferment D-xylose was tested in Durham tubes containing a 2% (p/w) solution of the sugar. The tubes were incubated at 25 °C on an incubator shaker (New Brunswick) at 100 r.p.m. for 25 days. *Candida shehatae* var. *shehatae* CBS 5813, *C. shehatae* var. *insectosa* CBS 4286, *C. shehatae* var. *lignosa* CBS 4705 and *Pichia stipitis* CBS 5773 were used as positive controls for D-xylose fermentation (Barbosa *et al.*, 2009). Preliminary identifications followed the taxonomic keys of Kurtzman & Fell (1998).

DNA sequencing and sequence analysis

The D1/D2 variable domains of the large-subunit rRNA gene were amplified by PCR directly from whole cells as described previously (Lachance *et al.*, 1999). The amplified DNA was concentrated, cleaned (Wizard Plus SV Minipreps DNA Purification System, Promega) and sequenced in a MegaBACE™ 1000 automated sequencing system (Amersham Biosciences). The sequence was edited with the program DNAMAN, version 6 (Lynnon BioSoft, Vaudreuil, QC, Canada). Existing sequences for other yeasts were retrieved from GenBank. The CLUSTAL W software (Thompson *et al.*, 1994) was used to align the sequences and to construct a neighbor-joining tree with 1000 bootstrap iterations.

Growth conditions and fermentation assays

Cells were grown on YP medium (1% yeast extract and 2% peptone), adjusted to pH 5.0 with HCl and supplemented with 2% glucose or D-xylose. Cells were grown with shaking at 28 °C (160 r.p.m.) in cotton-plugged Erlenmeyer flasks filled to 1/5 of the volume with medium. The inoculum for growth assays was prepared by transferring a single colony aseptically from a plate into 5 mL of the medium containing glucose or D-xylose, and allowing growth to proceed to the stationary phase for 2–3 days before inoculating cells (by a 100× or a 1000× dilution factor) to new media of a similar composition. Culture samples were harvested regularly, centrifuged (5000 ***g***, 1 min) and their supernatants were used for the determination of sugars and ethanol. For batch fermentations, the yeasts were pregrown on YP-2% sugar to the late exponential phase (~1 g dry yeast L^{-1}), centrifuged (3500 ***g***, 3 min), washed twice with cold water and inoculated at a high cell density (10 g dry yeast L^{-1}) into a YP medium containing the amounts of glucose and/or D-xylose indicated. Batch fermentations were incubated as described above for growth assays, and samples were collected regularly, centrifuged and their supernatants were analyzed as described below.

Analytical methods

Glucose was measured by the glucose oxidase and peroxidase method using a commercial kit (BioDiagnostica-Laborclin, Brazil), and D-xylose was determined as described by Miller (1959). Ethanol was determined with alcohol oxidase (Sigma) and peroxidase (Toyobo do Brasil, Brazil) as described previously (Alves *et al.*, 2007). Growth was followed by turbidity measurements at 570 nm after appropriate dilution, and yeast cell dry weight was determined as described elsewhere (Badotti *et al.*, 2008). Briefly, from 1 to 3 mL of fermentation broth was filtered through preweighed filters (0.45-μm mixed nitrocellulose and cellulose acetate filters), washed with 5 mL of distilled water and, after placing in a small (5 cm diameter) covered Petri dish, the filter membranes were dried for 1 min in a microwave oven at maximum power (900 W) and overnight at 80 °C. The sugar consumption rates were calculated using samples harvested at intervals during which maximal rates were attained. Mean values of dry weight in the specified time intervals were used in the rate calculations. The specific growth rate (μ, h^{-1}) was determined as the slope of a straight

line between ln $OD_{570\,nm}$ and time (h) during the initial (~12 h) exponential phase of growth. Ethanol yield coefficients ($Y_{e/s}$) were obtained at the end of ethanol production.

Results and discussion

Species delineation, classification and ecology

The four strains of the new species were isolated from media with xylan as the sole carbon source. One yeast strain (UFMG-HM19.1A) was isolated from the National Park of Serra do Cipó and the three other strains (UFMG-HM32.1, HM33.2a and HM34.2) were obtained from samples collected in different locations in the Rio Doce State Park. The sequences of the D1/D2 domains of the large-subunit rRNA gene were identical in all four strains and showed that the new species belongs to the *Spathaspora* clade (Fig. 1). The new species (GenBank accession no. GQ149081) differs by 11 substitutions and four gaps from *C. jeffriesii* and 13 substitutions and four gaps from *C. materiae* in the D1/D2 region of the large-subunit rRNA gene. In relation to *S. passalidarum*, the three species differ by 17 substitutions and four gaps. We conclude that the strains represent a new species of the *Spathaspora* clade. Similar to *S. passalidarum*, this new species produced asci with a single elongated ascospore with curved ends (Fig. 2). The name *S. arborariae* sp. nov. is proposed for the new species.

Spathaspora arborariae can be distinguished from *S. passalidarum* based on the assimilation of L-sorbose and growth on 50% glucose, which does not occur in the latter. *Spathaspora arborariae* can grow at 37 °C, but *C. jeffriesii* does not. *Spathaspora arborariae* is easily separated from *C. materiae* based on the ability to ferment D-xylose and the production of ascospores by the former species.

The isolation of *S. arborariae* from rotting wood of two different localities suggests that the yeast is associated with this kind of substrate. *Spathaspora arborariae* assimilates and ferments D-xylose efficiently and is therefore able to utilize this and related compounds from rotting wood. Gas formation from D-xylose fermentation in Durham tube tests was observed after 4 days with the type strain (UFMG-HM19.1A), and after 10 days with the other three strains.

Growth and fermentation of D-xylose by *S. arborariae*

Figure 3 shows the kinetics of growth on glucose and D-xylose by strain UFMG-HM19.1A. The strain exhibited a typical growth curve where the sugar is efficiently fermented, and after the sugar is exhausted from the media, the ethanol produced starts to be consumed and used as a carbon source. The strain grew well on both carbon sources ($\mu = 0.35\,h^{-1}$), producing practically the same amount of biomass and ethanol (Fig. 3) from glucose or D-xylose

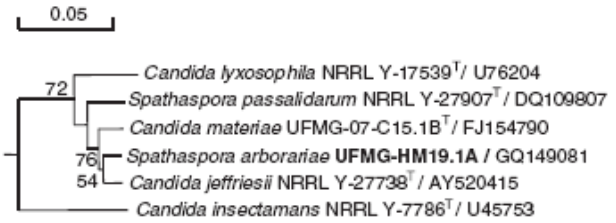

Fig. 1. Neighbor-joining tree showing the placement of *Spathaspora arborariae* among related species in the *Spathaspora* clade. Bootstrap values of 50% or above are shown.

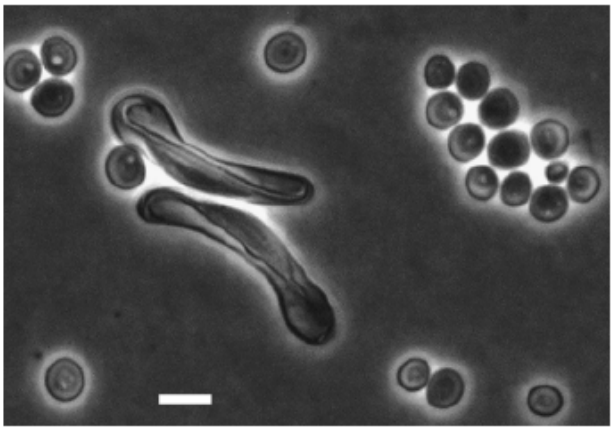

Fig. 2. Budding yeast cells and asci of *Spathaspora arborariae* with a single elongated ascospore with ends curved on dilute V8 agar after 3 days at 20 °C. Scale bar = 5 μm.

($Y_{e/s} = 0.35$–0.37 g ethanol g^{-1} sugar). However, as found typically for other D-xylose-fermenting yeasts (Panchal *et al.*, 1988; Sanchez *et al.*, 2002; Stambuk *et al.*, 2003), this yeast has a clear preference for glucose uptake and fermentation. This characteristic is evident during batch fermentations at high cell densities (see Fig. 4a), where glucose consumption rates by the yeasts ($0.74\,g\,g^{-1}$ cell dry weight h^{-1}) are higher than D-xylose consumption rates ($0.33\,g\,g^{-1}$ cell dry weight h^{-1}), and glucose consumption occurs before D-xylose utilization when both sugars are present at the beginning of the fermentation (Fig. 4b). Nevertheless, given the high ethanol yields ($Y_{e/s}$~0.50 g ethanol g^{-1} sugar) obtained during the batch fermentations described above, *S. arborariae* may provide a source of genes, enzymes and/or sugar transporters to engineer strains for efficient ethanol production from renewable biomass (Hahn-Hagerdal *et al.*, 2007; Stephanopoulos, 2007; Stambuk *et al.*, 2008).

Latin diagnosis of *Spathaspora arborariae* sp. nov. Cadete, Santos, Melo, Gomes, Mouro, Gonçalves, Stambuk, Lachance & Rosa

In medio liquido post dies tres cellulae singulae aut binae; cellulae ovoidae aut ellipsoideae (2–3 × 2–4 μm). Post unum

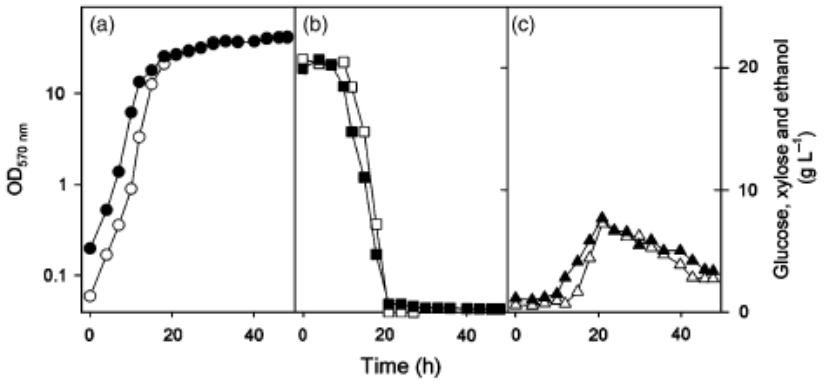

Fig. 3. Aerobic batch growth of *Spathaspora arborariae* on 20 g L^{-1} of glucose (open symbols) or D-xylose (black symbols). Cell growth (a), the consumption of sugars (b), and the production of ethanol (c) by strain UFMG-HM19.1A were determined during growth in rich YP medium.

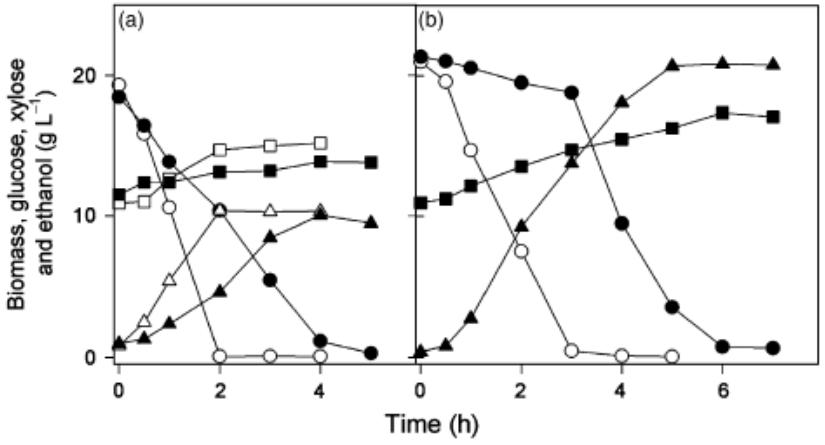

Fig. 4. Sugar batch fermentations by 10 g L^{-1} (dry weight) of *Spathaspora arborariae* yeast cells in rich YP medium. (a) The consumption of sugars (circles), and biomass (squares) and ethanol (triangles) production during batch fermentations of 20 g L^{-1} of glucose (open symbols) or D-xylose (black symbols) were determined with cells of strain UFMG-HM19.1A pregrown on glucose or D-xylose, respectively. In (b) D-xylose pregrown cells were used to ferment a mixture of 20 g L^{-1} of both glucose plus D-xylose [same symbols as in (a)].

mensem sedimentum formatur. Cultura in agaro malti post dies 2 (17 °C) candida, butyrosa et teres. In agaro farinae Zea mays post dies 14 pseudomycelium non formatur. Asci inconjugati et stabiles. Ascosporae alatae constipatae et non liberatio. Glucosum, galactosum, maltosum et D-xylosum fermentantur. Glucosum, galactosum, L-sorbosum (exigue), sucrosum, maltosum, cellobiosum, trehalosum, melezitosum, D-xylosum, D-ribosum (lente), ethanolum, glycerolum (variabile), erythritolum, ribitolum, D-mannitolum, glucitolum, salicinum, acidum succinicum (exigue), D-glucosaminum (exigue), *N*-acetylglucosaminum (exigue) et xylitolum (variabile) assimilantur, et non lactosum, melibiosum, raffinosum, inulinum, amylum solubile, D-arabinosum, L-arabinosum, L-rhamnosum, galactitolum, acidum lacticum, acidum citricum, meso-inositolum, methanolum, hexadecanum, D-glucosaminum, acetonum, isopropanolum nec ethyl acetas. Ethylaminum, lysinum et cadaverinum assimilantur at non kalium nitricum et natrium nitrosum. Ad crescentiam vitamina externa necessaria sunt. Augmentum in 37 °C. Habitat materiam in Brazil. Typus UFMG-HM19.1A. In collectione zymotica Centraalbureau voor Schimmelcultures, Trajectum ad Rhenum, sub no. CBS11463 typus stirps deposita est.

Description of *Spathaspora arborariae* sp. nov. Cadete, Santos, Melo, Gomes, Mouro, Gonçalves, Stambuk, Lachance & Rosa

In yeast extract (0.5%), glucose (2%) broth after 3 days at 25 °C, the cells are ovoid to ellipsoidal (2–3 × 2–4 μm). Budding is multilateral. A sediment is formed after a month, but a pellicle is not observed. On YMA after 2 days at 17 °C, colonies are white, butyrous and glistening. In Dalmau plates, after 2 weeks on cornmeal agar, pseudomycelia are not formed. Sporulation occurs on dilute V8 agar at 20 °C after 3 days (Fig. 2). Unconjugated asci are formed from single cells with a single highly elongated ascospore with curved ends. Asci are not dehiscent. Fermentation of glucose, galactose, maltose and xylose is positive. Assimilation of carbon compounds: glucose, galactose, L-sorbose (slow), sucrose, maltose, cellobiose, trehalose, melizitose, D-xylose, D-ribose (slow), ethanol, glycerol (variable), erythritol, ribitol, D-mannitol, glucitol, salicin, succinic acid (slow), D-glucosamine (slow), xylitol (variable) and *N*-acetyl-glucosamine (slow). No growth occurs on lactose, melibiose, raffinose, inulin, soluble starch, D-arabinose, L-arabinose,

L-rhamnose, galactitol, lactic acid, citric acid, m-inositol, methanol, hexadecane, acetone, isopropanol and ethyl acetate. Assimilation of nitrogen compounds: positive for ethylamine-HCl, lysine and cadaverine, and negative for nitrate and nitrite. Growth in vitamin-free medium is negative. Growth in amino acid-free medium is positive. Growth at 37 °C is positive. Growth on YMA with 10% sodium chloride is negative. Growth in 50% glucose/yeast extract (0.5%) is positive. Starch-like compounds are not produced. In 100 μg cycloheximide mL^{-1} the growth is negative. Urease activity is negative. Diazonium Blue B reaction is negative. The habitat is rotting wood in the Atlantic Rain Forest ecosystem, in the state of Minas Gerais, Brazil. The type strain accession number of *S. arborariae* is UFMG-HM19.1A^T. It was isolated from rotting wood in Brazil. It has been deposited in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands, as strain CBS11463^T (= NRRL Y-48658^T). The epithet *arborariae* (ar.bo.ra′ri.ae′) L. gen. nov. *arborariae*, of or pertaining to trees, and referred to the substrate where this yeast was found.

Acknowledgements

This work was supported in part by grants from the Brazilian agencies Conselho Nacional de Desenvolvimento Cientifico e Tecnologico-CNPq (process no. 552877/2007-7) and Fundação de Amparo a Pesquisa do Estado de São Paulo-FAPESP (process no. 04/10067-6), and the Natural Science and Engineering Research Council of Canada (M.-A.L.). D.L.G., A.M., B.U.S. and C.A.R acknowledge research fellowships from FUNCAMP and CNPq.

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International Journal of Systematic and Evolutionary Microbiology (2009), 59, 2104–2106 DOI 10.1099/ijs.0.009175-0

Candida materiae sp. nov., a yeast species isolated from rotting wood in the Atlantic Rain Forest

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Three strains of a novel yeast species, *Candida materiae* sp. nov., were isolated from rotting wood in an Atlantic rain forest site in Brazil. Analysis of the sequences of the D1/D2 domains of the large-subunit rDNA showed that this species belonged to the *Spathaspora* clade and was related to *Candida jeffriesii* and *Spathaspora passalidarum*. Unlike *C. jeffriesii* and *S. passalidarum*, *C. materiae* sp. nov. did not ferment xylose. The type strain of *C. materiae* sp. nov. is UFMG-07-C15.1B^T (=CBS 10975^T=CBMAI 956^T).

The genus *Spathaspora* was proposed by Nguyen *et al.* (2006) to accommodate the single species *S. passalidarum*, a xylose-fermenting yeast isolated from the beetle *Odontotaenius disjunctus* in Louisiana, USA. The asexual species *Candida jeffriesii*, *Candida lyxosophila* and *Candida insectamans* are phylogenetically related to *S. passalidarum* (Nguyen *et al.*, 2006). *C. jeffriesii* was isolated from an adult of the beetle *Phrenapates bennetti* collected in Panama. van der Walt *et al.* (1972, 1987) isolated *C. insectamans* from a frass sample of beetle larvae in trees and *C. lyxosophila* from surface woodland soil samples in South Africa, respectively. *C. jeffriesii* and *C. lyxosophila* are also able to ferment D-xylose, whereas *C. insectamans* does not ferment this pentose. During a survey of yeasts associated with rotting wood in Atlantic rain forest in Brazil, representatives of a novel yeast species related to the *Spathaspora* clade were isolated. The isolates were not able to ferment D-xylose and analysis of the sequences of the D1/D2 domains of the large-subunit rRNA gene showed that they were closely related to *C. jeffriesii*. The novel species is described as *Candida materiae* sp. nov.

The yeast strains were isolated from rotting wood samples collected in the private Natural Heritage Reserve of the Sanctuary of the Caraça. This is an ecological reserve with 11233 ha of Atlantic rain forest located in the Serra do Espinhaço (20° 05′ S 43° 28′ W), state of Minas Gerais, south-eastern Brazil. The region consists of a mountain complex that constitutes a zone of contact between the 'Cerrado' (savannas) and the Atlantic rain forest ecosystems in the south, and a zone of transition from 'Cerrado' to Atlantic forest to 'Caatinga' ecosystem in the north. The reserve has various floristic formations that include seasonal semideciduous forests, 'high-altitude plateaus' and rupestrian fields (grasslands surrounded by rocky outcrops, as well as shrubs and small trees). Seven decayed wood samples were collected on ecological trails of the Reserve of Caraça in November 2007. The samples were stored in sterile plastic bags and transported under refrigeration to the laboratory over a period of no more than 24 h. One gram of each sample was placed separately in flasks with 20 ml sterile carboxymethylcellulose medium (0.67 % yeast nitrogen base, 1 % carboxymethylcellulose, 0.05 % cellobiose and 0.02 % chloramphenicol), and in 20 ml sterile D-xylose medium (0.67 % yeast nitrogen base, 0.5 % D-xylose, 0.02 % chloramphenicol). The flasks were incubated at 25 °C on an incubator shaker (New Brunswick) at 150 r.p.m. for 3–10 days. When growth was detected, 0.5 ml of the cultures was then transferred separately to tubes containing 5 ml sterile carboxymethylcellulose or D-xylose media and the tubes were incubated as described above. One loopfull of solution from each tube was streaked on yeast extract-malt extract agar (YMA; 1 % glucose, 0.3 % yeast extract, 0.3 % yeast malt, 0.5 % peptone, 2 % agar and 0.02 % chloramphenicol). The plates were incubated at 25 °C until yeast colonies developed. The different yeast morphotypes were purified by repeated streaking on YMA plates and preserved at −80 °C or in liquid nitrogen for later identification. The yeasts were characterized by using standard methods (Yarrow, 1998). Fermentation of D-xylose was tested in Durham tubes containing 2 % (w/v) solution of the sugar. The tubes were incubated at 25 °C on an incubator shaker

The GenBank/EMBL/DDBJ accession number for the gene sequence of the D1/D2 domain of the 26S rDNA of strain UFMG-07-C15.1B^T is FJ154790.

(New Brunswick) at 80 r.p.m. for 3–20 days. *Candida shehatae* var. *shehatae* CBS 5813, *C. shehatae* var. *insectosa* CBS 4286, *C. shehatae* var. *lignosa* CBS 4705 and *Pichia stipitis* CBS 5773 were used as positive controls for D-xylose fermentation. Preliminary identifications followed the taxonomic keys of Kurtzman & Fell (1998).

The D1/D2 variable domains of the large-subunit rDNA were amplified by PCR directly from whole cells as described previously (Lachance *et al.*, 1999). The amplified DNA was concentrated and cleaned on QIAquick PCR columns (Qiagen) and sequenced using an ABI sequencer at the John P. Robarts Research Institute, London, Ontario, Canada. The sequence was edited with the program DNAMAN, version 6 (Lynnon BioSoft). Existing sequences for other yeasts were retrieved from GenBank. The CLUSTAL W software (Thompson *et al.*, 1994) was used to align the sequences and construct a neighbour-joining tree with 1000 bootstrap iterations.

Species delineation, classification and ecology

Analysis of the sequences of the D1/D2 region of the large-subunit rRNA gene showed that *Candida materiae* sp. nov. belonged to the *Spathaspora* clade (Fig. 1). In terms of sequence divergence, *C. materiae* sp. nov. differed by 13 substitutions from *C. jeffriesii*, and by 20 substitutions from *S. passalidarum*. Three strains (UFMG-07-C12.1, UFMG-07-C15.1B^T and UFMG-07-C27.1) of the novel species were isolated from samples of rotting wood collected in the Atlantic rain forest of the Reserve of Caraça. These three strains of *C. materiae* sp. nov. were physiologically very similar to *C. jeffriesii* and *S. passilidarum*. However, the novel yeast was not able to ferment D-xylose.

Two strains of *C. materiae* sp. nov. were isolated from yeast nitrogen base plus D-xylose as sole carbon source, whereas the third was isolated from the carboxymethylcellulose medium. However, the strain isolated using the carboxymethylcellulose medium was not able to produce extracellular cellulase (data not shown). The medium contained cellobiose, which can be utilized by *C. materiae* sp. nov. as a sole carbon source. *S. passalidarum* and *C. jeffriesii* are associated with wood-boring beetles that occur throughout the eastern USA and in Panama (Nguyen *et al.*, 2006). The isolation of *C. materiae* sp. nov. from rotting wood suggests that this yeast was also introduced to this plant material by wood-boring insects that use this substrate.

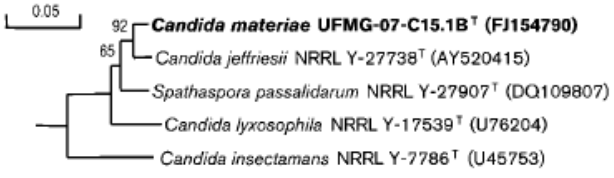

Fig. 1. Neighbour-joining tree showing the placement of *Candida materiae* sp. nov. among related species in the *Spathaspora* clade. Bootstrap values of 50 % or above are shown. Bar, 5 substitutions per 100 nucleotides.

C. materiae sp. nov. could be distinguished from *C. jeffriesii* based on growth at 37 °C and the fermentation of D-xylose. *C. materiae* sp. nov. gave a positive response for the first physiological test and a negative response for the second. *S. passalidarum* could be easily distinguished from *C. materiae* sp. nov. based on the ability to ferment D-xylose and the production of ascospores by the former species (Nguyen *et al.*, 2006). *C. insectamans* was negative for assimilation of galactose and sucrose (Meyer *et al.*, 1998), whereas the other species of the *Spathaspora* clade are able to use these sugar sources. Isolates of *C. materiae* sp. nov. were examined individually or mixed in pairs on cornmeal, V8, dilute V8, 5% malt extract, yeast carbon base supplemented with 0.01% ammonium sulphate and Gorodkowa agars, but asci or signs of conjugation were not observed.

Latin diagnosis of *Candida materiae* Barbosa, Cadete, Gomes, Lachance *et* Rosa sp. nov.

In medio liquido post dies tres cellulae singulae aut binae; cellulae ovoidae aut ellipsoideae (2–3 × 2–4 μm). *Post unum mensem sedimentum formatur. Cultura in agaro malti post dies* 2 (17 °C) *candida, convexa, rugosa et opalescens. In agaro farinae Zea mays post dies* 14 *pseudomycelium formantur. Glucosum at maltosum fermentatur. Glucosum, galactosum,* L-*sorbosum, sucrosum, maltosum, cellobiosum, trehalosum, melezitosum,* D-*xylosum,* D-*ribosum* (*lente*), *glycerolum, erythritolum, ribitolum,* D-*mannitolum, salicinum* (*variabile*), *acidum lacticum* (*variabile*), *acidum succinicum, acidum citricum* (*exigue*), N-*acetylglucosaminum* (*exigue*) *et xylitolum* (*variabile*) *assimilantur, at non-lactosum, melibiosum, raffinosum, inulinum, amylum solubile,* D-*arabinosum,* L-*arabinosum,* L-*rhamnosum, ethanolum, galactitolum, glucitolum, meso-inositolum, methanolum, hexadecanum,* D-*glucosaminum, acetonum,* 2-*propanolum et ethyl acetas. Ethylaminum, lysinum et cadaverinum assimilantur at non-kalium nitricum et natrium nitrosum. Ad crescentiuam vitamina externa necessaria sunt. Augmentum in* 37 °C. *Habitat materiam in* Brazil. *Typus* UFMG-07-C15.1B^T. *In collectione zymotica* Centraalbureau voor Schimmelcultures, *Trajectum ad Rhenum,* sub no. CBS 10975^T *typus stirps deposita est.*

Description of *Candida materiae* Barbosa, Cadete, Gomes, Lachance & Rosa sp. nov.

Candida materiae [ma.te′ri.ae′. L. gen. n. *materiae* of a material, matter, referring to the substrate (wood) where this yeast was found].

In yeast extract (0.5 %), glucose (2 %) broth after 3 days at 25 °C, the cells are ovoid to ellipsoidal (2–3 × 2–4 μm). Budding is multilateral (Fig. 2). A sediment is formed after a month, but a pellicle is not observed. On YMA after 2 days at 17 °C, colonies are white, convex, rugose and opalescent. In Dalmau plates after 2 weeks on cornmeal

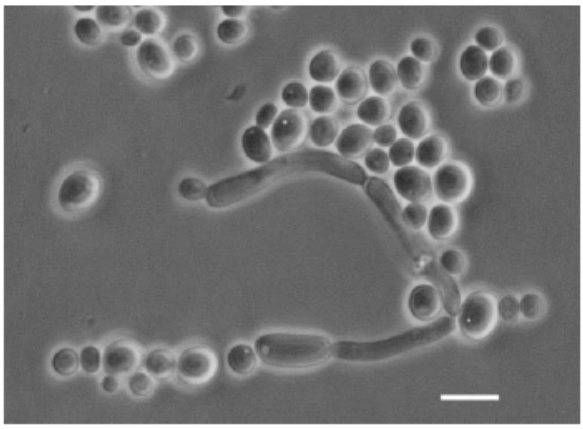

Fig. 2. Phase-contrast micrograph of cells of *Candida materiae* sp. nov. strain UFMG-07-C15.1B^T on yeast extract-malt extract agar after 5 days at 22 °C. Bar, 5 μm.

agar, pseudomycelia are formed. Ascospores are not formed. Fermentation of glucose and maltose is positive. The following carbon compounds are assimilated: glucose, galactose, L-sorbose, sucrose, maltose, cellobiose, trehalose, melezitose, D-xylose, D-ribose (slow), glycerol, erythritol, ribitol, D-mannitol, salicin (variable), lactic acid (variable), succinic acid, citric acid (weak), *N*-acetylglucosamine (weak) and xylitol (variable). No growth occurs on lactose, melibiose, raffinose, inulin, soluble starch, D-arabinose, L-arabinose, L-rhamnose, ethanol, galactitol, glucitol, *myo*-inositol, methanol, hexadecane, D-glucosamine, acetone, 2-propanol and ethyl acetate. Assimilation of nitrogen compounds: positive for lysine, ethylamine-HCl, cadaverine, and negative for nitrate and nitrite. Growth in vitamin-free medium is negative. Growth in amino-acid-free medium is positive. Growth at 37 °C is positive. Growth on YM agar with 10 % sodium chloride is negative. Growth in 50 % glucose/yeast extract (0.5 %) is negative. Starch-like compounds are not produced. In 100 μg cycloheximide ml^{-1} growth is negative. Urease activity is negative. Diazonium Blue B reaction is negative. The habitat is rotting wood in Atlantic rain forest ecosystem, in the state of Minas Gerais, Brazil.

The type strain of *Candida materiae*, UFMG-07-C15.1B^T, was isolated from rotting wood in Brazil. It has been deposited in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, as strain CBS 10975^T (=CBMAI 956^T).

Acknowledgements

This work was funded by Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq, process no. 477528/03-1), Fundação do Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG, process no. CBB–378/04 and CBB APQ-3484-4.01/07), and the Natural Science and Engineering Research Council of Canada (M.-A. L.).

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Antonie van Leeuwenhoek (2011) 99:635–642
DOI 10.1007/s10482-010-9536-z

ORIGINAL PAPER

Candida queiroziae sp. nov., a cellobiose-fermenting yeast species isolated from rotting wood in Atlantic Rain Forest

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Received: 6 September 2010 / Accepted: 23 November 2010 / Published online: 8 December 2010

Abstract Eight strains of a novel yeast species were isolated from rotting wood and wood-boring insects in Atlantic Rain Forest ecosystems in Brazil. Sequences of the D1/D2 domains of the large subunit of the rRNA gene showed that the yeast belongs to the *Scheffersomyces* clade and that it is related to *Candida lignicola* and *Candida coipomoensis*. The new species was isolated from rotting wood of three different localities and a wood-boring insect suggesting that these substrates are its ecological niche. This new yeast species is able to assimilate cellobiose and other compounds related to rotting wood. Strong fermentation of cellobiose in Durham tubes was observed for the strains of this new yeast. The new species produced an intracellular β-glucosidase responsible for cellobiose hydrolysis. The novel species, *Candida queiroziae* sp. nov., is proposed to accommodate these isolates. The type strain of *C. queiroziae* is UFMG-CLM 5.1^T (=CBS 11853^T = NRRL Y-48722^T).

Keywords New yeast species · *Candida queiroziae* · Cellobiose-fermenting yeast · Rotting wood · Atlantic Rain Forest

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Introduction

Discarded cellulosic biomass derived from forestry, agriculture, and municipal sources are potential feed stocks for the production of bio-fuels, in particularly for the synthesis of fuel ethanol (Hahn-Hägerdal et al. 2006). Although cellulose is the most abundant biopolymer in the world, its chemical structure makes it difficult to hydrolyze (Zhu et al. 2010) and current strategies to produce fuel ethanol from cellulose should favour the simultaneous saccharification and fermentation of the substrate (Lynd et al. 2002, 2005). The process requires pre-treatment of the cellulosic feedstock by stem-explosion or acid treatment, followed by addition of exogenously produced cellulolytic enzymes to hydrolyze cellulose chains and release the monomers required for fermentation (Carere et al. 2008).

During the simultaneous saccharification and fermentation process, cellulose is hydrolyzed by the

cellulase enzyme complex (cellobiohydrolases, endoglucanases and β-glucosidases) working in synergy. Endoglucanases (EC 3.2.1.4) randomly cleave the β-1,4 glycosidic linkages of cellulose, cellobiohydrolases (EC 3.2.1.91) attack cellulose chain ends to produce the constitutive unit of cellulose, cellobiose, a disaccharide of glucose linked by a β-1,4 glycosidic bond, and finally β-glucosidases (EC 3.2.1 21) hydrolyze cellobiose into two glucose moieties (Kumar et al. 2008). The use of microorganisms able to ferment cellobiose constitutes a desirable alternative for bio-ethanol production because cellobiose is a competitive inhibitor of cellulases (Bezerra and Dias 2005). These strains also ferment the monomers produced during cellulose hydrolysis, and cellulases are thus not inhibited (Lynd et al. 2005).

In a study on yeast communities associated with rotting wood and wood-boring insects in Atlantic Rain Forest ecosystems in Brazil, we have isolated eight strains of a new asexual cellobiose-fermenting yeast species. Analysis of the sequence of the D1/D2 domains of the large subunit rRNA gene showed that these strains represented a single species closely related to *Candida lignicola* and *C. coipomoensis*. In this work, we describe this new species as *Candida queiroziae* sp.nov.

Materials and methods

Yeast isolation and identification

Five strains were isolated from rotting wood and one from a wood-boring insect collected in the Private Natural Heritage Reserve of the Sanctuary of the Caraça. This is an ecological reserve with 11,233 ha of Atlantic Rain Forest located in the Serra do Espinhaço, state of Minas Gerais, Southeastern Brazil (Cadete et al. 2009). Two other yeast strains were isolated from rotting wood samples collected at the Private Natural Heritage Reserves of Woodstock and Bello & Kerida, respectively, two areas of Atlantic Rain Forest ecosystems located in the city of Nova Friburgo, Rio de Janeiro state, Brazil. The local climate in these ecological reserves is altitudinal tropical, with cold and dry winter and fresh and rainy summers, with annual mean temperatures around 16°C.

Fifteen rotting wood and 16 wood-boring insect samples were collected from the Caraça Ecological Park in April 2008. Ten samples each were collected from the Ecological Reserves of Woodstock and Bello & Kerida in January 2009. Rotting wood samples were stored in sterile plastic bags and transported under refrigeration to the laboratory over a period of no more than 24 h. Insects were stored in sterile flasks and transported under refrigeration to the laboratory as described by Suh et al. (2003). One gram of each wood sample was placed separately in flasks with 20 ml sterile D-xylose (yeast nitrogen base 0.67%, D-xylose 0.5%, chloramphenicol 0.02%) and carboxymethylcellulose (Yeast Nitrogen Base 0.67%, carboxymethylcellulose 1%, cellobiose 0.05% and chloramphenicol 0.02%) minimal media. The flasks were incubated at 25°C on an incubator shaker (New Brunswick, USA) at 150 rpm for 3–10 days. When growth was detected, 0.5 ml of the cultures was then transferred separately to tubes containing 5 ml sterile carboxymethylcellulose or D-xylose media and the tubes were incubated as described above. For yeast isolation from insects 100 µl of a solution of insect gut macerate was inoculated in the same media and conditions mentioned above. One loopful of culture from each tube was streaked on yeast extract-malt extract agar (YM, glucose 1%, yeast extract 0.3%, malt extract 0.3%, peptone 0.5%, agar 2% and chloramphenicol 0.02%). The plates were incubated at 25°C until yeast colonies developed. The different yeast morphotypes were purified by repeated streaking on YM agar plates and preserved at −80°C or in liquid nitrogen for later identification. The yeasts were characterized by standard methods (Yarrow 1998) and identification followed the taxonomic keys of Kurtzman and Fell (1998).

The ability to ferment cellobiose was tested in Durham tubes containing fermentation basal medium with a final sugar concentration of 2% (w/v) (Yarrow 1998). The tubes were incubated at 25°C on an incubator shaker (New Brunswick) at 100 rpm for 25 days.

DNA sequencing and sequence analysis

The D1/D2 variable domains and internal transcribed spacer (ITS) of the large subunit rRNA gene were amplified by PCR directly from whole cells as described previously (Lachance et al. 1999). The amplified DNA was concentrated, cleaned (Wizard Plus SV Minipreps DNA Purification System, Promega) and sequenced in a Mega-BACE™ 1000

automated sequencing system (Amersham Biosciences) in Institute of Biological Sciences of the Federal University of Minas Gerais, Brazil, or sequenced at the Robarts Research Institute, London, Ontario, in Canada. The sequences were edited with the program DNAMAN, version 6 (Lynnon BioSoft, Vaudreuil, QC, Canada). Existing sequences for other yeasts were retrieved from GenBank. The CLUSTAL W software (Thompson et al. 1994) included in DNAMAN was used to align the D1/D2 LSU rRNA gene sequences.The alignment covered 534 positions, of which 63 were polymorphic, 38 were parsimony-informative and 13 were gapped. For tree construction using the built-in Neighbour-Joining algorithm, gapped positions were deleted in pairs and Kimura's two-parameter distance was applied.

Growth conditions and fermentation assays

Cells were grown on YP medium (1% yeast extract and 2% peptone), adjusted to pH 5.0, supplemented with 2% glucose or cellobiose. Cells were grown with shaking at 28°C (160 rpm) in cotton-plugged Erlenmeyer flasks filled to 1/5 of the volume with medium. The inoculum for growth assays was prepared by transferring a single colony aseptically from a plate into 5 ml of the YP medium containing glucose or cellobiose, and allowing growth to proceed to the stationary phase for 2–3 days before inoculating cells (by a 100 or 1,000× dilution factor) to new media of a similar composition. Culture samples were harvested regularly, centrifuged (5000×*g*, 1 min) and their supernatants were used for the determination of sugars and ethanol. For batch fermentations, the yeasts were pre-grown on YP-2% sugar to the late exponential phase (~ 1 g dry yeast l^{-1}), centrifuged (3,500×*g*, 3 min), washed twice with cold water and inoculated at a high cell density (10 g dry yeast l^{-1}) into YP medium containing the amounts of glucose and/or cellobiose indicated. Batch fermentations were incubated as described above for growth assays, and samples were collected regularly, centrifuged and their supernatants were analyzed as described below.

Determination of β-glucosidase activity

Periplasmic or secreted cellobiose hydrolysis by β-glucosidase was determined in vivo with whole cells previously incubated with 50 mM sodium fluoride to block glycolysis (Silveira et al. 1996), using 67 mM cellobiose in either 67 mM succinate–Tris, pH 5.0, or 67 mM HEPES–NaOH, pH 7.0. Intracellular β-glucosidase activity was determined in situ with permeabilized yeast cells (Stambuk 1999) using 100 mM cellobiose in either 100 mM succinate–Tris, pH 5.0, or 100 mM HEPES–NaOH, pH 7.0. Controls using previously boiled cells were used. Enzyme activity is expressed as U/g dry yeast cells, where one unit corresponds to 1 µmol of glucose produced/min at 28°C.

Analytical methods

Glucose was measured by the glucose oxidase and peroxidase method using a commercial kit (BioDiagnostica-Laborclin, Brazil), and cellobiose was determined as described by Miller (1959). Ethanol was determined with alcohol oxidase (Sigma) and peroxidase (Toyobo do Brasil, Brazil) as described previously (Alves et al. 2007). Growth was followed by turbidity measurements at 570 nm after appropriate dilution, and yeast cell dry weight was determined as described elsewhere (Badotti et al. 2008). Ethanol yield coefficients ($Y_{e/s}$) were obtained at the end of ethanol production taking into account the amount of sugar utilized.

Results and discussion

The sequences of the D1/D2 domains and ITS region of the large subunit rRNA gene of the eight strains of the new species were identical. This new species belongs to the *Scheffersomyces* clade and it is closely related to *C. lignicola* and *C. coipomoensis* (Fig. 1). The genus *Scheffersomyces* was proposed by Kurtzman and Suzuki (2010) to include several D-xylose-fermenting yeast species related to *Pichia stipitis*. This clade contains the species *S. stipitis, S. segobiensis, S. spartinae* and the anamorphic yeasts *C. lignosa, C. insectosa, C. shehatae, C. coipomoensis, C. lignicola* and *C. ergastensis*. The new species differs by 11 substitutions and one gap from *C. lignicola,* and 13 substitutions from *C. coipomoensis* in the D1/D2 domains, and by eight indels from *C. lignicola* and six substitutions from *C. coipomoensis* in the ITS region of

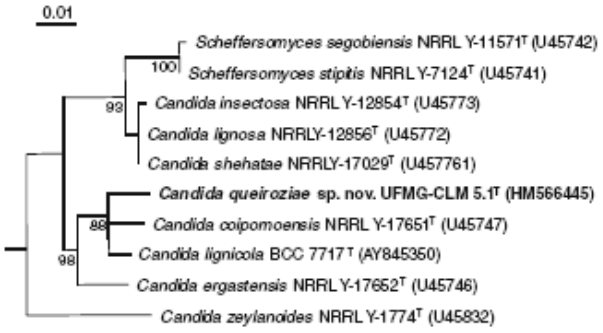

Fig. 1 Neighbour-joining tree of the D1/D2 domains of the large subunit of the rRNA gene showing the placement of *Candida queiroziae* sp. nov. among related species in the *Scheffersomyces* clade. Bootstrap values of 50% and above are shown. *Candida zeylanoides* NRRL Y-1774 is the outgroup species, and all species are analyzed from type strains. *Bar* 0.01 substitutions per nucleotide position

the large subunit rRNA gene. The name *C. queiroziae* sp. nov. is proposed for the new species.

The strains of the new species were isolated from different sources and localities. The new species is not able to ferment D-xylose, but strong fermentation of cellobiose in Durham tubes was observed after 24 h for strain UFMG-IMX 6.1 isolated from a boring wood insect and after 3–6 days for the yeast strains isolated from wooding root samples (Table 1). The strains of *C. queiroziae* are physiologically similar to *C. lignicola* and *C. coipomoensis*. *C. lignicola* is able to assimilate L-arabinose and L-rhamnose, which does not occur with the new species. *C. queiroziae* can be distinguished from *C. coipomoensis* based on the assimilation of D-Gluconate, which is positive for the new species and negative for *C. coipomoensis*. In addition, *C. queiroziae* is able to grow on 10% NaCl plus 5% glucose medium, whereas the other two species did not grow. Isolates of *C. queiroziae* were examined individually or mixed in pairs on cornmeal, V8, dilute V8, 5% malt extract, yeast carbon base supplemented with 0.01% ammonium sulphate, and Gorodkowa agars incubated at 20 or 28°C until 30 days, but asci or signs of conjugation were not seen.

Candida coipomoensis was isolated from wood in advanced stages of degradation in the evergreen rainy Valdivian forest of Southern Chile (Ramirez and González 1984), and *C. lignicola* was isolated from insect frass collected from Khao-Yai National Park of Thailand (Jindamorakot et al. 2007). Similar to their closest relatives, *C. queiroziae* was isolated from rotting wood of three different localities and a wood-boring insect suggesting that these substrates are the ecological niche of this new species.

Growth and fermentation of cellobiose by *Candida queiroziae*

Candida queiroziae is able to assimilate cellobiose and other compounds related to rotting wood, and tests in Durham tubes indicates that this yeast species can ferment cellobiose within variable periods of time (see Table 1). Figure 2 shows the kinetics of growth on cellobiose by the strain UFMG-CLM 5.1^T and UFMG-IMX 6.1. Both strains exhibited a typical growth curve where the sugar is efficiently fermented. After the sugar is exhausted from the medium, the ethanol produced starts to be consumed and used as a carbon source by the yeast. In agreement with the fermentation results in Durham tubes (Table 1), strain UFMG-IMX 6.1 grew and consumed cellobiose faster than strain UFMG-CLM 5.1, although at the end of the fermentation both strains produced practically the same amount of biomass (Fig. 2). An ethanol yield from cellobiose ($Y_{e/s} = 0.32 \pm 0.03$) obtained with these two strains of *C. queiroziae* is in the same range as those obtained during glucose fermentation ($Y_{e/s} = 0.32 \pm 0.01$) by these yeasts (data not shown). Although during growth strain UFMG-IMX 6.1 apparently utilized cellobiose more efficiently than strain UFMG-CLM 5.1, during batch fermentations at high cell densities (Fig. 3) no significant differences in the fermentation of glucose (Fig. 3a) or cellobiose (Fig. 3b) between these two strains was observed, with cellobiose fermentation by both strains occurring at a slightly slower rate than glucose fermentation.

The lack of glucose production during growth on cellobiose (Fig. 2b), or even during batch fermentation of this sugar (Fig. 3b), prompted us to characterize in more detail the subcellular localization (periplasmic or intracellular) of the β-glucosidase responsible for cellobiose hydrolysis by *C. queiroziae*. Our results indicate that both strains UFMG-IMX 6.1 and UFMG-CLM 5.1 lack (or have very low) periplasmic β-glucosidase activity (Fig. 4), with rates of cellobiose hydrolysis of less than $\sim$5 U/g dry yeast cells at either pH 5.0 or 7.0. When the yeast cells were permeabilized, a significant β-glucosidase activity could be seen both at pH 5.0 (29–47 U/g dry yeast cells), and especially at pH 7.0 (167–230 U/g dry

Table 1 Source, origin, culture media for isolation and days for cellobiose fermentation of the strains of *Candida queiroziae*

Strains	Source	Origin (Atlantic Rain Forest ecosystem)	Culture media	Cellobiose fermentation (days to fill the Durham tube)
UFMG-CLM 5.1, UFMG-CLM 8.1,	Rotting wood	Sanctuary of the Caraça.	Carboxymethylcellulose	4
UFMG-CLM 6.1, UFMG-CLM 12.1	Rotting wood	Sanctuary of the Caraça.	Carboxymethylcellulose	3
UFMG-CLM 7.2	Rotting wood	Sanctuary of the Caraça.	Carboxymethylcellulose	6
UFMG-IMX 6.1	Wood-boring insect	Sanctuary of the Caraça.	D-xylose	1
UFMG-CLM 43.1	Rotting wood	Woodstock	Carboxymethylcellulose	3
UFMG-CLM 57.2	Rotting wood	Bello & Kerida	Carboxymethylcellulose	3

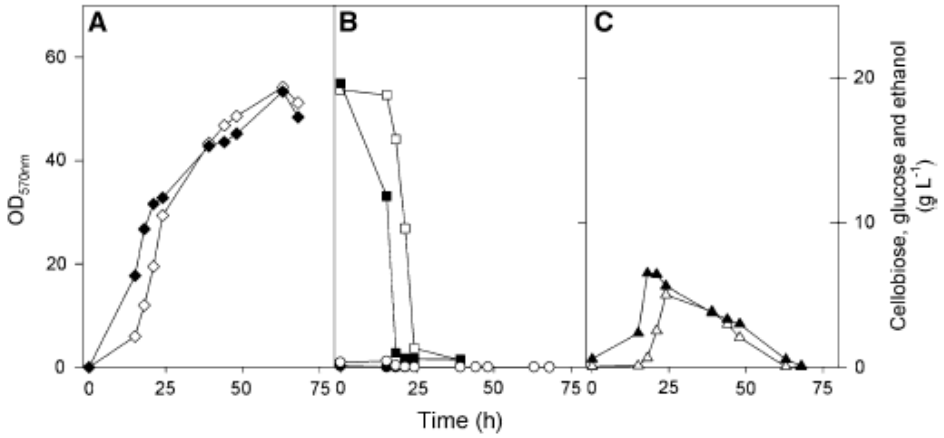

Fig. 2 Typical aerobic batch growth on cellobiose by *Candida queiroziae* UFMG-CLM-5.1^T (*open symbols*), or strain UFMG-IMX-6.1 (*black symbols*). **a** Cell density (*diamonds*), **b** cellobiose (*squares*) consumption, and **c** production of ethanol (*triangles*), were determined during growth in rich YP medium containing 20 g l^{-1} of cellobiose. In **b** are also shown glucose (*circles*) concentrations in the medium during cellobiose utilization

yeast cells) by both strains, which is consistent with an intracellular β-glucosidase as being responsible for cellobiose hydrolysis by *C. queiroziae*. While yeast species (e.g. *Saccharomycopsis fibuligera*, *Wickerhamomyces anomalus* and *C. wickerhamii*) ferment cellobiose by expressing β-glucosidases at the cell surface, very few yeast are reported as capable of fermenting this sugar through an intracellular enzyme, a process which requires the uptake of the disaccharide into the cell. Unfortunately, when compared to our current knowledge of other disaccharide uptake system in several yeast species, cellobiose transport systems have been poorly characterized (Freer and Greene 1990; Spencer-Martins 1994). In view of its efficient cellobiose fermentation *C. queiroziae* may provide a new source of genes, enzymes and/or sugar transporters to engineer industrial strains for efficient ethanol production from renewable biomass (Galazca et al. 2010; Li et al. 2010).

Latin diagnosis of *Candida queiroziae* Santos, Cadete, Badotti, Mouro, Wallheim, Gomes, Stambuk, Lachance & Rosa sp. nov.

In medio liquido post dies tres (25°C) cellulae singulae aut binae; cellulae ovoidae aut ellipsoideae (2–3 × 2–4 μm). Post unum mensem sedimentum formatur. Cultura in agaro malti post dies 2 (17°C) candida, convexa, rugosa et opalescens. In agaro farinae Zea mays post dies 14 pseudomycelium formantur. Glucosum, galactosum et cellobiosum fermentantur, sucrosum variable at non maltosum at D-xylosum. Glucosum, galactosum, L-sorbosum, maltosum, sucrosum, cellobiosum, trehalosum, D-xylosum, D-arabinosum,

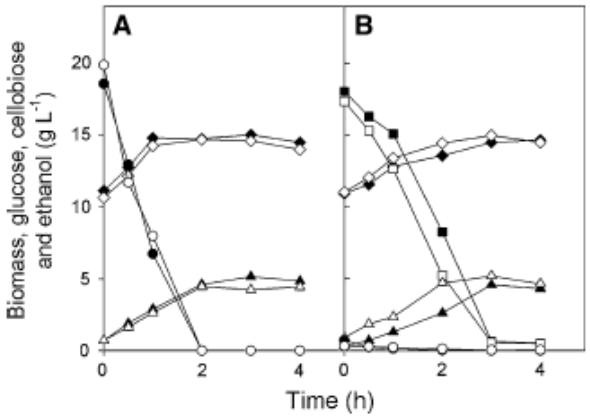

Fig. 3 Sugar batch fermentations by 10 g l^{-1} (dry weight) of *Candida queiroziae* UFMG-CLM-5.1^T (*open symbols*) or strain UFMG-IMX-6.1 (*black symbols*) yeast cells in rich YP medium. Biomass (*diamonds*) and ethanol (*triangles*) production was determined during the consumption of 20 g l^{-1} of **a** glucose (*circles*) or **b** cellobiose (*squares*). In **b** are also shown glucose (*circles*) concentrations in the medium during cellobiose utilization

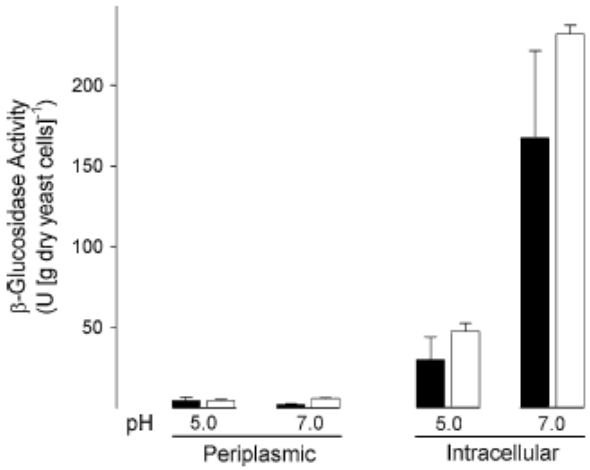

Fig. 4 Cellobiose hydrolysis by a periplasmic or intracellular enzyme was determined in cells of *Candida queiroziae* UFMG-CLM-5.1^T (*open bars*) or strain UFMG-IMX-6.1 (*black bars*) collected during growth on 20 g l^{-1} of cellobiose, and the enzymatic activity assayed at the indicated pH

ethanolum, glycerolum, erythritolum, ribitolum (variable), D-mannitolum, glucitolum, salicinum, acidum succinicum, acidum citricum, D-ribosum (exigue), hexadecanum (exigue), xylitolum, acidum lacticum (exigue), D-gluconate et *N*-acetylglucosaminum assimilantur, at non lactosum, melibiosum, raffinosum, melezitosum, inulinum, amylum solubile, L-arabinosum, L-rhamnosum, galactitolum, meso-inositolum, methanolum, D-glucosaminum, acetonum, ethyl acetatum nec isopropanolum. Ethylaminum, lysinum et cadaverinum assimilantur at non kalium nitricum et natrium nitrosum. Augmentum (exigue) in 37°C. Habitat materiam in Brazil. Typus UFMG–CLM 5.1. In collectione zymotica Centraalbureau voor Schimmelcultures, Trajectum ad Rhenum, sub no. CBS 11853 typus stirps deposita est.

Description of *Candida queiroziae* Santos, Cadete, Badotti, Mouro, Wallheim, Gomes, Stambuk, Lachance & Rosa sp. nov.

In yeast extract (0.5%), glucose (2%) broth after 3 days at 25°C, the cells are ovoid to ellipsoidal (2–3 × 2–4 μm). Budding is multilateral (Fig. 5). A sediment is formed after a month, but a pellicle is not observed. On YM agar after 2 days at 17°C, colonies are white, convex, and opalescent. In Dalmau plates after 2 weeks on cornmeal agar, pseudomycelia are formed. Ascospores are not formed. Fermentation of glucose, galactose and cellobiose is positive. Sucrose fermentation is variable. Maltose and D-xylose are not fermented. Assimilation of carbon compounds: glucose, galactose, L-sorbose, maltose, sucrose, cellobiose, trehalose, D-xylose, D-arabinose, ethanol, glycerol, erythritol, ribitol (variable), D-mannitol, glucitol, salicin, succinic acid, citric acid, D-ribose (very weak), hexadecane (very weak), xylitol, lactic acid (very weak), D-gluconate and *N*-acetyl-glucosamine. No growth occurs on lactose, melibiose, raffinose, melezitose, inulin, soluble starch, L-arabinose,

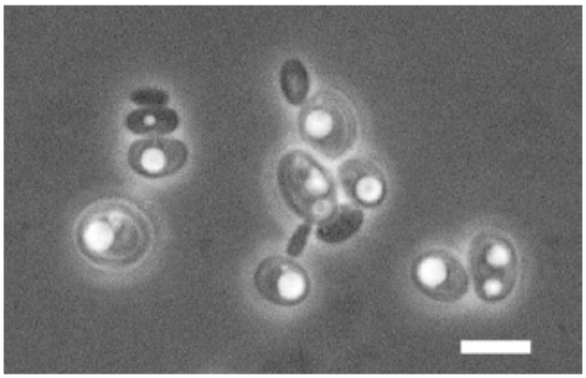

Fig. 5 Phase-contrast micrograph of budding cells of *Candida queiroziae* UFMG-CLM-5.1^T on yeast carbon base agar (Difco) with 0.01% ammonium sulphate after 3 day at 20°C. *Bar* 5 μm

L-rhamnose, galactitol, meso-inositol, methanol, D-glucosamine, acetone, ethyl acetate, isopropanol. Assimilation of nitrogen compounds: positive for lysine, ethylamine–HCl, cadaverine, and negative for nitrate and nitrite. Growth in amino-acid-free medium is positive. Growth at 37°C is weak. Growth on YM agar with 10% sodium chloride is positive. Growth in 50% glucose/yeast extract (0.5%) is negative. Starch-like compounds are not produced. In 100 μg cycloheximide ml^{-1} the growth is positive. Urease activity is negative. Diazonium Blue B reaction is negative. The habitat is rotting wood in Atlantic Rain Forest ecosystem, in the states of Minas Gerais and Rio de Janeiro, Brazil. The type strain accession number of *C. queiroziae* is UFMG-CLM 5.1. It was isolated from rotting wood in Brazil. It has been deposited in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, as strain CBS 11853^T (=NRRL $Y\text{-}48722^T$). The Mycobank number is MB 519120.The epithet *queiroziae* (que′i′ro′zi′ae) L. gen. n. *queiroziae,* of Queiroz, in honor to Dr Luzinete Aciole de Queiroz for her contribution to the mycology in Brazil.

Acknowledgments This work was funded by Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq), Fundação do Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG), and the Natural Science and Engineering Research Council of Canada (M.A.L.). The authors are grateful to Acir Moreno for assistance during the field collection in the Private Natural Heritage Reserves of Woodstock and Bello & Kerida.

Conflict of interest None

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Yeast
Yeast (2011)
Published online in Wiley Online Library
(wileyonlinelibrary.com) **DOI:** 10.1002/yea.1858

Research Article

Ethanol production by a new pentose-fermenting yeast strain, *Scheffersomyces stipitis* UFMG-IMH 43.2, isolated from the Brazilian forest

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Received: 18 February 2011
Accepted: 20 April 2011

Abstract

The ability of a recently isolated *Scheffersomyces stipitis* strain (UFMG-IMH 43.2) to produce ethanol from xylose was evaluated. For the assays, a hemicellulosic hydrolysate produced by dilute acid hydrolysis of sugarcane bagasse was used as the fermentation medium. Initially, the necessity of adding nutrients ($MgSO_4 \cdot 7H_2O$, yeast extract and/or urea) to this medium was verified, and the yeast extract supplementation favoured ethanol production by the yeast. Then, in a second stage, assays under different initial xylose and cell concentrations, supplemented or not with yeast extract, were performed. All these three variables showed significant ($p < 0.05$) influence on ethanol production. The best results (ethanol yield and productivity of 0.19 g/g and 0.13 g/l/h, respectively) were obtained using the hydrolysate containing an initial xylose concentration of 30 g/l, supplemented with 5.0 g/l yeast extract and inoculated with an initial cell concentration of 2.0 g/l. *S. stipitis* UFMG-IMH 43.2 was demonstrated to be a yeast strain with potential for use in xylose conversion to ethanol. The establishment of the best fermentation conditions was also proved to be of great importance to increasing the product formation by this yeast strain. These findings open up new perspectives for the establishment of a feasible technology for ethanol production from hemicellulosic hydrolysates. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: ***Scheffersomyces stipitis*; fermentation; ethanol; sugarcane bagasse; hemicellulosic hydrolysate**

Introduction

In the last decade, ethanol has experienced unseen levels of attention, due to its value as a fuel alternative to gasoline, the increase of oil prices and climatic changes, besides being a renewable and sustainable energy source, efficient and safe to the environment. Currently, worldwide ethanol production is at high levels and corn is the main raw material used for this purpose, but this scenario must change due to technological improvements that are being developed for the production of low-cost lignocellulosic ethanol (Mussatto *et al.*, 2010). Among the variety of lignocellulosic materials, including agricultural and agro-industrial residues that can be used for this purpose, sugarcane bagasse (the solid residue obtained after sugar cane juice extraction) has attracted great interest, since it has a composition rich in polysaccharides convertible to fermentable sugars, which correspond to about 72.5% of the material composition (Rocha *et al.*, 2011).

Large efforts have been made to produce ethanol from lignocellulose. However, this process will only be competitive to the actual technologies from corn or sugarcane if efficient and low cost bioconversion processes are established to convert both cellulose and hemicellulose sugars to ethanol. The hemicellulose conversion is particularly a challenge on ethanol production from lignocellulose, due to the variety of sugars usually found in this fraction (including L-arabinose, D-galactose, D-glucose, D-mannose and D-xylose) and the need to find microbial strains able to convert them to ethanol. Xylose is usually the main hemicellulosic sugar, being the most abundant in sugarcane bagasse (Rocha *et al.*, 2011; Santos *et al.*, 2005a; Silva *et al.*, 2007), and its bioconversion is an important step in the use of lignocellulosic materials for ethanol production (Silva *et al.*, 2010).

Saccharomyces cerevisiae is the most commonly used ethanol producer microorganism, but it may not be used for hemicellulosic hydrolysates fermentation, since this medium contain a mixture of hexose and pentose sugars and *S. cerevisiae* cannot ferment pentoses (Kumar *et al.*, 2009). Therefore, during the last two decades, research has been focused on finding xylose-fermenting microorganisms and understanding their xylose metabolism. Although a large number of yeast species able to metabolize xylose and with fermentative capacity have been found, only approximately 1% of them are capable of fermenting xylose to ethanol (Hahn-Hägerdal *et al.*, 2007). *Scheffersomyces* (*Pichia*) *stipitis* is reported to have the ability to ferment a wide variety of sugars, including xylose, glucose, mannose, galactose and cellobiose along with mannan and xylan oligomers (Jeffries and Van Vleet, 2009), and thus it has attracted great interest for use on ethanol production from hemicellulose. Some recent studies describe the ethanol production by different *S. stipitis* strains, the results being clearly dependent on the microorganism, cultivation medium and conditions employed (Agbogbo and Wenger, 2007; Diaz *et al.*, 2009; Silva *et al.*, 2010).

Isolation of strains from natural or industrial habitats is a useful technique for finding strains with specific properties. Xylose-fermenting yeast strains have been found in black knots (a disease that occurs on numerous cultivated and wild plums, prunes, and cherries), insect frass and tree exudates (Olsson and Hahn-Hägerdal, 1996). *Scheffersomyces stipitis* UFMG-IMH 43.2 is a yeast strain recently isolated from the intestine of insects found in wood samples derived from the Private Natural Heritage Reserve of Woodstock, in the city of Nova Friburgo, Rio de Janeiro state, Brazil (Cadete, 2009), and the present work is the first approach on ethanol production by this microorganism. Fermentation assays were performed using sugarcane bagasse hemicellulosic hydrolysate as the fermentation medium, and the supplementation of this medium with nutrients and the use of different initial xylose and cell concentrations were tested, aiming to understand the yeast metabolism and verify its ability to produce ethanol.

Materials and methods

Hydrolysate preparation

Sugarcane bagasse was submitted to dilute acid hydrolysis in a 100 l stainless steel reactor, using a sulphuric acid solution (100 mg H_2SO_4/g dry matter) in a 1.75 : 10 (g/ml) dry matter : acid solution ratio at 150 °C for 30 min. After hydrolysis, the residual solid material was separated by centrifugation and the liquid phase (hemicellulosic hydrolysate) was vacuum concentrated in a 30 l stainless steel evaporator at 70 °C, aiming to increase the sugars content five-fold. To become a medium suitable for use in fermentation processes, the concentrated hydrolysate was detoxified as previously described (Santos *et al.*, 2005b) and finally autoclaved at 0.5 atm and 112 °C for 15 min. The hydrolysate then obtained contained: xylose 76.1 g/l; glucose 10.1 g/l; arabinose 8.9 g/l; acetic acid 2.9 g/l; furfural 0.02 g/l; and hydroxymethylfurfural 0.01 g/l. This hydrolysate was supplemented or not with nutrients to be used in the first part of the experimental assays. For the other experiments, dilutions with sterile distilled water were performed to obtain the desired initial xylose concentration (30.0, 52.5 or 75 g/l).

Microorganism and inoculum

The recently isolated yeast strain *Scheffersomyces stipitis* UFMG-IMH 43.2 (Cadete, 2009) from the culture collection of the Universidade Federal de

DOI: 10.1002/yea

Table 1. Experimental conditions used to evaluate the effects of sugarcane bagasse hemicellulosic hydrolysate supplementation with nutrients on ethanol production by *Scheffersomyces stipitis* UFMG-IMH 43.2

	Nutritional sources			Responses			
Assays	$MgSO_4 \times 7H_2O$ (g/l)	Yeast extract (g/l)	Urea (g/l)	Sugars consumption* (%)	Ethanol (g/l)	$Y_{P/S}$ (g/g)	Q_P (g/l/h)
1	0.0	0.0	0.0	49.3	4.3	0.10	0.03
2	1.0	0.0	0.0	46.3	4.4	0.11	0.03
3	0.0	5.0	0.0	92.1	10.3	0.13	0.07
4	1.0	5.0	0.0	92.8	8.0	0.10	0.05
5	0.0	0.0	5.0	62.3	4.3	0.08	0.03
6	1.0	0.0	5.0	96.6	8.3	0.10	0.05
7	0.0	5.0	5.0	98.5	10.2	0.12	0.07
8	1.0	5.0	5.0	96.9	10.9	0.13	0.07
9	0.5	2.5	2.5	90.0	8.5	0.11	0.05
10	0.5	2.5	2.5	93.4	9.7	0.12	0.06
11	0.5	2.5	2.5	88.9	9.2	0.12	0.06
12	0.5	2.5	2.5	92.2	9.5	0.12	0.06

* Sugars = xylose + glucose.

Minas Gerais (Brazil) was used in the experiments. Cultures of this yeast were maintained on malt extract agar slants at 4 °C. For inoculum preparation, fresh cells of the yeast cultivated on malt extract agar slants at 30 °C for 24 h were transferred to 125 ml Erlenmeyer flasks containing 50 ml medium composed of: xylose 30.0 g/l; peptone 5.0 g/l; and yeast extract 2.5 g/l. The inoculated flasks were incubated at 30 °C at 200 rpm for 24 h, after which the cells were recovered by centrifugation (3000 × *g*, 15 min) and resuspended in the fermentation medium to obtain the desired initial cell concentration.

Fermentation media and conditions

Fermentation assays for evaluation of the hydrolysate supplementation with nutrients on ethanol production were performed in 125 ml Erlenmeyer flasks containing 50 ml medium (hemicellulosic hydrolysate) supplemented or not with $MgSO_4 \cdot 7H_2O$, yeast extract and/or urea, according to the experimental matrix presented in Table 1. After inoculation with an initial cell concentration of 2 g/l, the flasks were incubated in a rotary shaker at 30 °C and 200 rpm for 156 h.

In a second stage, the experiments were performed in 125 ml Erlenmeyer flasks containing 50 ml medium composed by the hydrolysate diluted with sterile distilled water to obtain different initial xylose concentrations (30.0, 52.5 or 75 g/l). The fermentation media were supplemented or not with yeast extract (up to 5 g/l) and inoculated with initial cell concentrations of 0.5, 1.0 or 2.0 g/l, according to the experimental conditions given in Table 2. Then the flasks were incubated in a rotary shaker at 30 °C and 200 rpm for 192 h.

Analytical methodology

During the experiments, samples were periodically taken for sugars, ethanol and cell growth determinations. Cell concentration was determined by measuring the fermentation broth absorbance at 600 nm, which was correlated to a calibration curve (dry weight × optical density). Glucose, xylose, arabinose, acetic acid and ethanol concentrations were determined by high-performance liquid chromatography (HPLC) in a Waters 410 (Milford, MA, USA) chromatograph, equipped with a refractive index detector and a Bio-Rad Aminex HPX-87H column (300 × 7.8 mm) at 45 °C. 0.005 M sulphuric acid was used as eluent at a flow rate of 0.6 ml/min, and a sample volume of 20 µl was injected. For furfural and hydroxymethylfurfural (HMF) determinations, the HPLC system was equipped with a UV detector (at 276 nm) and a RP 18 (4.6 × 200 mm) column at 25 °C. Acetonitrile : water (1 : 8 with 1% acetic acid) was used as eluent at a flow rate of 0.9 ml/min, and a sample volume of 20 µl was injected.

DOI: 10.1002/yea

Table 2. Experimental conditions used to evaluate the effects of different variables on sugarcane bagasse hemicellulosic hydrolysate fermentation to ethanol by *Scheffersomyces stipitis* UFMG-IMH 43.2

	Variables			Responses				
Assays	Initial xylose concentration (g/l)	Initial cell concentration (g/l)	Yeast extract (g/l)	Sugars consumption* (%)	Ethanol (g/l)	$Y_{P/S}$ (g/g)	Q_P (g/l/h)	Time (h)
1	30.0	0.5	0.0	88.7	2.7	0.09	0.02	168
2	75.0	0.5	0.0	54.2	1.8	0.04	0.01	168
3	30.0	2.0	0.0	75.2	4.0	0.16	0.04	96
4	75.0	2.0	0.0	62.1	5.2	0.10	0.03	192
5	30.0	0.5	5.0	87.5	2.7	0.09	0.05	48
6	75.0	0.5	5.0	71.6	3.0	0.05	0.02	168
7	30.0	2.0	5.0	93.3	6.4	0.19	0.13	48
8	75.0	2.0	5.0	68.1	5.9	0.11	0.03	192
9	52.5	1.0	2.5	84.1	9.0	0.17	0.05	192
10	52.5	1.0	2.5	83.8	9.1	0.17	0.05	192
11	52.5	1.0	2.5	83.7	9.1	0.17	0.05	192
12	52.5	1.0	2.5	83.0	9.1	0.17	0.05	192

* Sugars = xylose + glucose.

Statistical data analysis

Fermentation assays were performed according to 2^3 full-factorial designs with four replicates in the centre point. The ethanol yield ($Y_{P/S}$) and productivity (Q_P) were taken as responses of the experimental designs. Ethanol yield ($Y_{P/S}$, g/g) was calculated by the ratio between ethanol concentration (g/l) and sugars (xylose + glucose) consumed (g/l), while the ethanol productivity (Q_P, g/l) was determined by the ratio between ethanol concentration (g/l) and fermentation time (h). Statistical analysis of the data was carried out using the software Statistica, version 5.0.

Results and discussion

A variety of factors influence xylose fermentation by yeasts, including pH, temperature, agitation, aeration, initial xylose and cell concentrations and supplementation of the fermentation medium with nutrients, among others. In the present study, the influence of three of these factors, viz. the addition of nutrients to the fermentation medium and the initial xylose and cell concentrations, were evaluated, aiming to understand the metabolism of this recently isolated yeast strain. Aeration is considered a factor of great influence on xylose fermentation, since it determines the partitioning of the carbon flow between growth and product formation (du Preez, 1994). As the influence of this variable was not evaluated in the present study, some care was taken to use a condition suitable for ethanol production. Xylose-fermenting yeasts usually do not grow on xylose under anaerobic conditions, and high ethanol yield has been reported under microaerobic conditions. Therefore, fermentations in the present study were performed under microaerobic conditions consisting in the use of 200 rpm agitation and a 2.5 $V_{flask} : V_{medium}$ ratio, which were established in a recent study as being suitable to attain high ethanol production from xylose (Silva *et al.*, 2010).

Hydrolysate supplementation with nutrients

The presence of nutritional sources in a fermentation medium is of fundamental importance for a good performance of the microbial strain, since microorganisms require several substances for synthesis of cell material and energy obtainment. Medium components should primarily satisfy the basic requirements of the microorganism, such as carbon, oxygen, nitrogen, phosphorus and sulphur. Additionally, metals, growth factors and vitamins may also be useful (Olsson and Hahn-Hägerdal, 1996). When hemicellulosic hydrolysates are used as the fermentation medium, supplementation with nutrients may or not be necessary, according to the raw material and conditions employed for

DOI: 10.1002/yea

its hydrolysis, as well as the microorganism utilized and the product that it is desired to obtain. Therefore, the first part of this study consisted in evaluating the effect of the addition of different nutritional sources to sugarcane bagasse hemicellulosic hydrolysate, on ethanol production by *S. stipitis* UFMG-IMH 43.2. Urea, $MgSO_4 \cdot 7H_2O$ and yeast extract were the nutrients chosen to supplement the hydrolysate medium, since they have been proved to be of great importance for a good development of different yeast strains.

Fermentation results revealed that *S. stipitis* UFMG-IMH 43.2 was able to simultaneously consume the sugars xylose and glucose and utilize them for ethanol production in all the evaluated conditions. Such behaviour can be visualized in Figure 1, which shows an example of time evolution of xylose and glucose consumption and biomass and ethanol production by the yeast when cultivated in sugarcane bagasse hemicellulosic hydrolysate. The simultaneous sugars consumption is an important aspect to be considered, since the inhibition of the xylose transport into the cell by the glucose would be a significant disadvantage to the process. Contrary to glucose and xylose, arabinose was not consumed by the yeast in any of the fermentations during the considered fermentation time.

Ethanol was the main product obtained in all the fermentation assays. By-products such as xylitol and glycerol were only found at very low concentration values (<0.5 g/l) and in some cases no xylitol production was observed (assays 1 and 2, Table 1). This low by-products formation can also be pinpointed as a very important characteristic of this yeast strain, since it allows elevated formation of ethanol as the main fermentation product to be obtained.

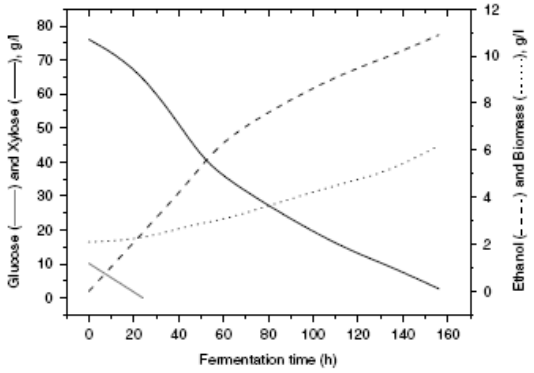

Figure 1. Time evolution of xylose and glucose consumption and biomass and ethanol production by *Scheffersomyces stipitis* UFMG-IMH 43.2 when cultivated in sugarcane bagasse hemicellulosic hydrolysate (conditions of assay 8; Table 1)

As can be seen in Table 1, sugar consumption and ethanol production strongly varied according to the fermentation medium composition. Ethanol production was also observed when no nutrients were added to the medium (assay 1), demonstrating that sugarcane bagasse hemicellulosic hydrolysate contains some nutritional source required by the yeast. However, the fermentation results were improved when the hydrolysate was supplemented with nutrients. Yeast extract or urea addition improved the sugars consumption by the yeast, as can be observed by comparing the assays with and without supplementation of these nutrients. Yeast extract appears also to have had the highest influence on product formation, since the maximum values of ethanol concentration, yield ($Y_{P/S}$) and productivity (Q_P) were obtained in the assays supplemented with the highest amount of this nutrient.

Statistical analysis of these data revealed that, in fact, the addition of yeast extract and urea to the fermentation medium favoured sugars consumption by *S. stipitis*. The individual effect of yeast extract was the most significant ($p < 0.01$), followed by the individual effect of urea ($p < 0.05$) and by the interaction effect between these two variables ($p < 0.1$). Supplementation of the hydrolysate with $MgSO_4 \cdot 7H_2O$ did not influence the sugars consumption by the yeast.

The positive effect of the medium supplementation with yeast extract on ethanol production was also statistically confirmed. Figure 2 clearly shows that yeast extract addition to the medium provided higher improvements in $Y_{P/S}$ and Q_P than $MgSO_4 \cdot 7H_2O$ or urea addition. Yeast extract was the only nutritional source with a highly significant ($p < 0.01$) individual effect on these responses. Interactions between the variables were significant at the 95% confidence level for both $Y_{P/S}$ and Q_P, suggesting that combinations between yeast extract and $MgSO_4 \cdot 7H_2O$ or urea, or between $MgSO_4 \cdot 7H_2O$ and urea, were also favourable for ethanol production by *S. stipitis* UFMG-IMH 43.2. However, when observing the results presented in Table 1, it can be noted that the highest $Y_{P/S}$ value

DOI: 10.1002/yea

was obtained under two different conditions, using the highest level of yeast extract, in combination or not with the highest level of the other two nutrients (assays 8 and 3, respectively). This makes evident that yeast extract was the most important nutrient to improve the ethanol production by *S. stipitis* UFMG-IMH 43.2 from sugarcane bagasse hemicellulosic hydrolysate.

Yeast extract is a rich source of amino acids and vitamins and has been reported to have protective effects on cell growth and viability, which stimulate the fermentation rate and product formation (Bafrncová *et al.*, 1999; Casey *et al.*, 1984). However, since ethanol is a low-value product, the addition of a high-cost nutrient such as yeast extract to the fermentation medium may be economically non-viable. Nevertheless, the knowledge about the yeast requirement to this nutrient made it possible to identify another low-cost alternatives, e.g. vitamins, that can be used to substitute it. This will be the focus of our next studies.

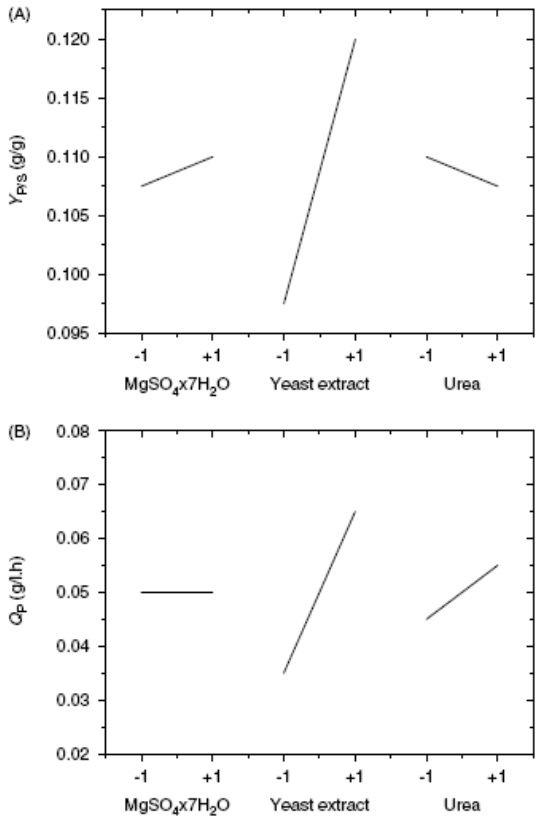

Figure 2. Individual effects of $MgSO_4 \cdot 7H_2O$, yeast extract and urea supplementation to sugarcane bagasse hemicellulosic hydrolysate on ethanol yield ($Y_{P/S}$) and productivity (Q_P) by *Scheffersomyces stipitis* UFMG-IMH 43.2

Effect of initial xylose and cell concentrations and yeast extract supplementation on bioconversion performance

Establishing the most suitable composition of a fermentation medium to each particular strain is of great importance to improve the xylose conversion to ethanol, since the nutritional dependence strongly varies according to the genus and species of the microorganisms utilized, and even by strains within the same genus and species. However, other factors, including the initial substrate concentration and initial cell concentration, also affect xylose conversion to ethanol and need to be studied and defined to each particular case. Thus, in this second stage of the work, the effects of these two variables on ethanol production by *S. stipitis* were evaluated. The addition of yeast extract to the medium, which was previously selected among the three evaluated nutrients, was also evaluated again to verify possible interaction effects among the variables with influence on ethanol production. The fermentation conditions and the ethanol production obtained to each experimental assay are presented in Table 2. As can be seen, sugars consumption and ethanol production varied in each assay, demonstrating that the xylose conversion to ethanol by *S. stipitis* UFMG-IMH 43.2 was affected by the cultivation conditions.

Fermentation assays were maintained for 192 h and for most of the cases, ethanol production was constantly increased during the process, attaining the maximum concentration value at this final time. Nevertheless, different behaviours were observed in some experiments. For assays 1, 2 and 6, for example, the maximum ethanol concentration was achieved at 168 h and remained constant during the last 24 h. On the other hand, assays 5 and 7 achieved the maximum ethanol production at 48 h fermentation, and assay 3 at 96 h (Table 2). After these times the ethanol concentration was notably reduced, probably due to its consumption by the yeast as a consequence of the fast exhaustion of sugars from the media. Similar behaviour has

DOI: 10.1002/yea

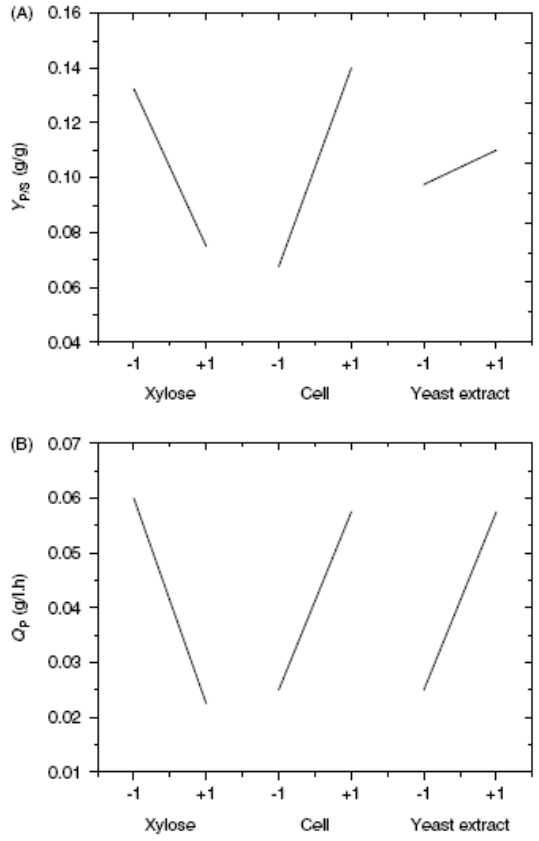

Figure 3. Individual effects of the initial xylose concentration, initial cell concentration and yeast extract supplementation to sugarcane bagasse hemicellulosic hydrolysate on ethanol yield ($Y_{P/S}$) and productivity (Q_P) by *Scheffersomyces stipitis* UFMG-IMH 43.2

already been observed for other yeast strains (Fu *et al*., 2009; Groleau *et al*., 1995).

The differences on ethanol production and sugars consumption observed in the experiments were statistically analysed, aiming to verify the influence of each variable on ethanol formation by *S. stipitis*. Sugars consumption by the yeast was higher, the lower the initial xylose concentration ($p < 0.01$) and the higher the amount of yeast extract ($p < 0.1$) added to the fermentation medium. These results confirm that, even in the presence of different xylose and cell concentrations, yeast extract exerts a positive effect on sugars consumption by the yeast, as previously observed. Regarding ethanol production, all the studied variables presented significant influence ($p < 0.05$) on ethanol yield and productivity, the results being improved when using the lowest xylose (30.0 g/l) and the highest cell (2.0 g/l) and yeast extract (5.0 g/l) concentrations (Figure 3). Besides the individual effect of the variables, the interaction effects between initial xylose concentration and initial cell concentration or yeast extract were also significant for these responses, and responsible for additional improvements in the $Y_{P/S}$ and Q_P values.

In fact, the cell concentration increase is reported to be a possible alternative to increase the ethanol productivity (Olsson and Hahn-Hägerdal, 1996), as was found here. This happens because as more cells will be available for the product formation, more elevated product concentration is expected to be achieved in a shorter fermentation time. The need for medium supplementation with an elevated amount of yeast extract is also justifiable, since with increasing cell mass the requirement of nutrients will also be higher. Finally, low initial xylose concentration (about 50 g/l) was also the most suitable for ethanol production by *P. stipitis* NRRL Y-7124 from rice straw hemicellulosic hydrolysate (Silva *et al*., 2010).

The highest $Y_{P/S}$ (0.19 g/g) and Q_P (0.13 g/l/h) values obtained in the present work (assay 7, Table 2) are not very high values of ethanol yield and productivity, but these results are of interest if considering that this is the first study on ethanol production by this yeast strain. It merits emphasizing that these values are higher than those obtained in the previous assays for evaluation of the hydrolysate supplementation with different nutritional sources (0.13 g/g and 0.07 g/l/h, respectively; Table 1), thus revealing the importance of establishing the best fermentation medium and conditions to achieve elevated ethanol yield and productivity by *S. stipitis* UFMG-IMH 43.2.

In brief, *S. stipitis* UFMG-IMH 43.2 was able to consume sugars and produce ethanol from sugarcane bagasse hemicellulosic hydrolysate, but the bioconversion performance of this yeast was affected according to the experimental conditions used. The initial xylose concentration, initial cell concentration and yeast extract supplementation showed significant influence on ethanol production by this yeast, and the establishment of the best levels of these variables was of great importance to increase the ethanol production results. This study

DOI: 10.1002/yea

allows concluding that *S. stipitis* UFMG-IMH 43.2 is a promising yeast strain for use on ethanol production from xylose, since it was able to quickly consume the sugars present in the hydrolysate medium and convert them to ethanol. This is only the first study on ethanol production by this yeast and the results here achieved may be improved by determining the other conditions to perform the fermentation process. This will be the focus of our next studies.

Acknowledgements

Financial supports provided by the Brazilian agencies FAPESP, CAPES, CNPq, and FAPEMIG are gratefully acknowledged.

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DOI: 10.1002/yea

D-xylose-fermenting and xylanase-producing yeast species from rotting wood of two Atlantic Rainforest habitats in Brazil

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Abstract

This study investigated the yeast species associated with rotting wood in Brazilian Atlantic Rainforest ecosystems focusing on the identification of D-xylose-fermenting and/or xylanase-producing species. A total of 321 yeast strains were isolated from rotting wood samples collected in two Atlantic Rainforest areas. These samples were cultured in yeast nitrogen base (YNB)-D-xylose or YNB-xylan media. *Schwanniomyces polymorphus, Scheffersomyces queiroziae, Barnettozyma californica,* and *Candida (Ogataea) boidinii* were the most frequently isolated yeasts. The rarefaction curves for the yeast communities isolated in YNB-D-xylose and YNB-xylan from both areas continued to rise and did not reach an asymptote, indicating that not all yeast diversity had been recovered. Additionally, the yeast composition was variable among the samples and areas, which was confirmed by the values of the Sorensen and Bray-Curtis similarity indices. Among the 69 species identified, only 12 were found in both areas sampled. Fifteen possible new species were obtained. Among them, two species (*Sugiyamaella* sp. 1 and *Sugiyamaella xylanicola*) showed the

ability to ferment D-xylose into ethanol, and three species (*Spencermartinsiella* sp. 1, *Su. xylanicola* and *Tremella* sp.) were able to produce extracellular xylanases. Indeed, most of the xylanase-producing isolates belong to the new species *Su. xylanicola*, which was also positive for D-xylose fermentation. *Scheffersomyces queiroziae* and *S. stipitis* were the main D-xylose-fermenting yeasts identified. The results of this work showed that rotting wood collected from the Atlantic Rainforests is a huge source of yeasts, including new species, with promising biotechnological properties.

Keywords

Yeasts; Brazilian Atlantic Rainforest; new species; D-xylose fermentation; xylanase production

1. Introduction

Yeasts are unicellular fungi that are ubiquitously distributed throughout all biomes of the world (Rosa and Péter, 2006; Starmer and Lachance, 2011). Currently, although nearly 1500 yeast species are recognised (Kurtzman et al., 2011a), and it is estimated that approximately 90% of the potential diversity of yeasts remain unknown (Lachance, 2006), which supports a need for increasing efforts to study the biodiversity of these microorganisms, especially in mega-diverse countries from the tropical regions of the planet. Particularly, South America is a region that offers great potential for the study of microbial biodiversity (Barriga et al., 2011). Originally considered to be one of the largest rainforests of the Americas, the Atlantic Rainforest once covered approximately 150 million ha of South America, mainly extending along the Brazilian coast (representing 92% of the Brazilian coast) (Ribeiro et al., 2009). This biome is classified as a *hotspot* region, which is an area with high biodiversity, elevated levels of endemism and great anthropogenic pressure. Moreover, this forest is considered to be one of the five leading *hotspots* on the planet (Myers et al., 2000). Today, only 12-16% of the original vegetation remains, mostly as small fragments of land (<100 ha) (Ribeiro et al. ,2009). Studies on yeasts isolated from this ecosystem have concentrated on the search for new species, and several have been described in recent years (Barbosa et al., 2009; Cadete et al., 2009; Morais et al., 2013, 2006; Santos et al., 2011). Some of these new species have showed remarkable biotechnological potential, as they are capable of D-xylose fermentation and xylanase production (Cadete et al., 2009; Morais et al., 2013).

Typically, yeasts are considered to be primarily decomposers and are among the earliest colonisers of nutrient rich substrates, such as land plant tissues, where they are followed by a succession of organisms that degrade dead organic matter (Starmer and Lachance, 2011). Plant cell walls are a source of lignocellulose, a complex and chemically rich material whose structure chiefly consisting of cellulose, a linear glucose polymer, hemicellulose, a highly branched sugar heteropolymer, and lignin, a very high molecular weight and cross-linked aromatic macromolecule, all interacting physico-chemically (Ferreira-Leitão et al., 2010). The bioconversion of renewable lignocellulosic biomass to biofuel (e.g., second generation ethanol) is gaining global importance and involves five major steps: (i) choice of suitable biomass, (ii) effective pretreatment, (iii) production of saccharolytic enzymes such as cellulases and hemicellulases, (iv) fermentation of hexoses and pentoses, and (v) downstream processing (Menon and Hao, 2012). The microbial conversion of the hemicellulose fraction is essential in increasing fuel-ethanol yields. The most relevant hemicelluloses are xylans and glucomannans, with xylans being the most abundant (Gírio et al., 2010). Xylans, composed of β-1,4-linked D-xylosyl subunits, are the richest D-xylose-based plant polymers (Dumon et al., 2012). Their β-1,4 backbone can be randomly cleaved by hydrolytic enzymes called xylanases (Collins et al., 2005), and the full hydrolysis of this polymer leads to the release of D-xylose, a pentose (five carbon) sugar, which is considered the second most abundant monosaccharide in lignocellulose (Nakamura et al., 2008). Thus, a cost-effective conversion of lignocellulosic biomass into bioethanol requires that the D-xylose released from the hemicellulose fraction be fermented (Hou, 2012).

In the lignocellulose to ethanol conversion process, yeasts can act as sources of xylanases and as D-xylose-fermenting agents. Xylanase-producing yeast strains from several species have already been documented (Bhadra et al., 2008; Biely et al., 1980; Laitila et al., 2006; Mokwena et al., 2000; Parachin et al., 2009; Petrescu et al., 2000; Strauss et al., 2001; Scorzetti et al., 2000). To date, the D-xylose-fermenting yeasts that have been described are principally from the *Scheffersomyces* (Kurtzman et al., 2011a; Urbina and Blackwell, 2012) and *Spathaspora* clades (Cadete et al., 2013, 2012a; Kurtzman et al., 2011a; Nguyen et al., 2006). Aside from these studies, few groups have focused on the isolation of D-xylose-fermenting yeasts or the discovery of new species and strains (Bhadra et al., 2008; Cadete et al., 2013, 2012a, 2009; Urbina et al., 2012). Given that previous studies used plant material for the isolation of yeasts, the present study was performed to study yeast richness in rotting wood collected from two Brazilian Atlantic Rainforest sites, focusing on the isolation of D-xylose-fermenting or xylanase-producing yeasts.

2. Materials and methods

2.1 Yeast isolation

The procedures for sample collection, processing and isolation of yeasts were performed as described previously (Barbosa et al., 2009; Cadete et al., 2013, 2012a, 2012b, 2009; Santos et al., 2011). Yeasts were isolated from samples of rotting wood collected in two areas of the Atlantic Rainforest, the Private Natural Heritage Reserve of the Sanctuary of Caraça and the Private Natural Heritage Reserve of Serra Bonita. The Reserve of the Sanctuary of Caraça is an ecological reserve of 11,233 ha located in the Serra do Espinhaço (20° 05' S 43° 28' W), Minas Gerais, in southeastern Brazil (Barbosa et al., 2009). The Reserve of Serra Bonita is an ecological reserve of 2,000 ha located in the Serra Bonita (15° 23' S 39° 33' W), Bahia, in northeastern Brazil (Amorim et al., 2009).

One hundred wood samples (50 from each area) were collected in April (Caraça) and June (Serra Bonita) 2011. The samples were stored in sterile plastic bags and transported under refrigeration to the laboratory within 24 h. One gram of each sample was placed separately into 50 mL sterile shake flasks with 20 mL of sterile YNB-D-xylose (yeast nitrogen base, 6.7 g/L; D-xylose, 5 g/L; chloramphenicol, 0.2 g/L) or 20 mL sterile YNB-xylan (yeast nitrogen base, 6.7 g/L; xylan, 10 g/L; chloramphenicol, 0.2 g/L) media. D-xylose, xylan and YNB solutions were sterilised separately. The flasks were incubated under aerobic conditions at 25˚C on an orbital shaker at 150 rpm for 3-15 days. When growth was detected, 0.5 mL was transferred to a tube containing 5 mL sterile YNB-D-xylose or YNB-xylan media. The tubes were incubated on an orbital shaker as described above. After growth detection, one loopful from each tube was streaked on yeast extract-malt extract agar (YMA – glucose, 10 g/L; peptone, 5 g/L; yeast extract, 3 g/L; malt extract, 3 g/L; agar, 20 g/L). The plates were incubated at 25˚C until yeast colonies developed. The different yeast morphotypes were purified by restreaking on YMA and stored on GYMP broth (glucose, 20 g/L; yeast extract, 5 g/L; malt extract, 5 g/L; Na_2PO_4 2 g/L) with 20% of glycerol at -80˚C for later identification.

2.2 Yeast identification

The yeasts were preliminarily grouped according to their colony morphology and their growth on different carbon and nitrogen sources (Kurtzman et al., 2011b). Physiology-based groupings were confirmed by PCR fingerprinting using the intron splice site primer EI-1 (5´CTGGCTTGGTGTATGT) (de Barros Lopes et al., 1996). Yeast strains with identical DNA banding patterns were grouped and preliminarily considered to belong to the same species (Cadete et al., 2012a; Rosa et al., 2007). At least one representative strain from each EI-1 PCR group, as well as physiologically distinct strains with unique EI-1 PCR banding patterns, were subjected to sequence

analysis of the D1/D2 variable domains and the ITS1-5.8S-ITS2 region of the large-subunit rRNA gene by PCR, directly from whole cells, as described previously (Lachance et al., 1999). The amplified DNA was concentrated, cleaned and sequenced in a Mega-BACE^{TM} 1000 automated sequencing system (Amersham Biosciences, USA). Potential new species were also sequenced using an ABI sequencer. The sequences were assembled, edited and aligned with the program MEGA5 (Tamura et al., 2011). The existing sequences for other yeasts were retrieved from GenBank. Phylogenetic placements of new species belonging to the clades *Spathaspora* and *Sugiyamaella* were based on maximum parsimony analysis of D1/D2 LSU rRNA gene sequences.

2.3 Rarefaction curves and similarities indices

A rarefaction curve was calculated using the Mao Tao index (Colwell et al., 2004). The similarities among yeast species from different areas were estimated using the Sorensen coefficient (QS) and Bray-Curtis (B) measures. All of the results were obtained with 95% confidence, and bootstrap values were calculated from 1,000 iterations using the computer programme PAST, version 1.90 (Hammer et al., 2001). Further information about these indices can be found in Magurran (2011).

2.4 Screening of D-xylose-fermenting yeasts

The ability to ferment D-xylose was tested in cotton plugged tubes containing YPX medium (D-xylose 20 g/L, peptone 20 g/L, yeast extract 10 g/L) inoculated with a cell optical density at 600 nm (OD_{600}) of 0.05. The tubes were incubated at 25˚C on an orbital shaker at 150 rpm for 72 h. Samples were taken at 24 h, 48 h and 72 h and centrifuged at 2,600x *g* for 10 min, and the supernatant was used to determine the ethanol produced. Tubes containing YPD medium (glucose 20 g/L, peptone 20 g/L, yeast extract 10 g/L), as well as the D-xylose-fermenting yeast strains *Spathaspora passalidarum* UFMG-HMD-1.1 and *Scheffersomyces stipitis* NRRL Y-7124, were used as positive controls (Cadete et al., 2012a). In addition, tubes containing YP medium (peptone 20 g/L, yeast extract 10 g/L) without glucose were used as negative control in these experiments to verify the possibility of alcohol production from peptone and yeast extract from the strains. The experiments were performed in biological duplicate. Ethanol was quantified by the alcohol oxidase enzyme (AOD, from *Pichia pastoris*, Sigma, USA) and peroxidase assay (POD, Toyobo do Brasil, Brazil) as described previously (Alves et al., 2007). Fermentation of D-xylose was considered positive when the production of ethanol was detected.

2.5 Screening of xylanase-producing yeasts

The capacity of xylanase production was evaluated in a modified plate assay, according to Bhadra et al. (2008). All isolates were incubated at 25°C for 5 to 15 days in xylan-agar medium (yeast nitrogen base, 6.7 g/L; xylan, 10 g/L, pH 5.0± 0.5). Xylanase-producing strains were identified based on the formation of a clear halo around the colonies. *Aureobasidium pullulans* UFMG-BRO-53 from the Culture Collection of Microorganisms and Cells of the Universidade Federal de Minas Gerais (UFMG) was used as a positive control. These experiments were also performed in biological duplicate.

3. Results and discussion

3.1 Yeast identification and diversity

A total of 321 yeast strains was isolated from 50 rotting wood samples collected in the Sanctuary of Caraça reserve (151 yeast strains) and 50 rotting wood samples from the Serra Bonita reserve (170 yeast strains). Of these strains, 135 were obtained in YNB-D-xylose medium and 186 in YNB-xylan medium. In a similar study, Cadete et al. (2012a) isolated 224 yeast strains from 40 rotting wood samples cultured in YNB-D-xylose and YNB-xylan media and collected from two Brazilian Amazonian Forest sites. The higher number of isolates obtained in our study may be due to the larger number of samples that were collected.

Sixty-nine species were identified. Among them, 30 species were represented by a single isolate, and 39 species were isolated more than once. These later species are characterised in Table 1, which shows the results of yeast species identification, the occurrence of each species by isolation site, the number of isolates obtained from each media (YNB-D-xylose and xylan) and the screening results of D-xylose fermentation and xylanase production assays. Of the 69 species found, 54 were previously known and 15 represented novel yeast species. Cadete et al. (2012a) found 33 yeast species (seven of which were previously uncharacterised) from rotting wood samples collected in Amazonian Forest. The considerably higher number of identified species, including previously undescribed species, presented in this work is likely due to the greater number of wood samples collected and possibly due to the geographically distinct regions used as sampling sites, which is expected to increase the probability of obtaining a higher diversity of species. Thirty-eight species were obtained in the Reserve of the Sanctuary of Caraça, being 26 species isolated only in this region. Forty-three species were isolated in the Serra Bonita reserve, being 31 isolated only in this region. Only 12 species were isolated from both sites, showing different yeast species distribution according to the studied area.

Among the 30 species found as single isolates, which could represent transitory yeast mycota found within the sampling sites, 16 were isolated in the Reserve of the Sanctuary of Caraça, and of them, 10 have been previously described [*Candida (Hyphopichia) homilentoma, C. (Kurtzmaniella) railenensis, C. (Lodderomyces) orthopsilosis, C. (Ogataea) nanaspora, C. (Yamadazyma) michaelii, C. (Yamadazyma) tammaniensis, Geotrichum silvicola, Kodamaea ohmeri, Rhodotorula mucilaginosa* and *Tremella globispora*] and six are new (*Ogataea* sp. 1, *Ogataea* sp. 2, *Saturnispora* sp. 1, *Sugiyamaella* sp. 2, *Sugiyamaella* sp. 3 and *Tremella* sp). The remaining 14 species were found in the Serra Bonita reserve; nine of these species have been previously identified [*Asterotremella musci, Bandoniozyma complexa, C. (Metschnikowia) pseudointermedia, C. (Metschnikowia) tsuchiyae, C. (Nakazawaea) heliconiae, C. albicans, C. solani, Galactomyces candidum, Lindnera fabianii*] and five are new (*Metschnikowia* sp. 1, *Pichia* sp., *Saturnispora* sp. 2, *Spathaspora* sp. 1 and *Sugiyamaella* sp. 1). *Lindnera fabianii* and *Tremella* sp., obtained as single isolates, were capable of producing xylanase, and *Sugiyamaella* sp. 1 of fermenting D-xylose.

The most prevalent genera identified in this work were *Sugiyamaella*, represented by six species (*Su. boreocaroliniensis, Su. lignohabitans, Su. xylanicola, Sugiyamaella* sp. 1, *Sugiyamaella* sp. 2, *Sugiyamaella* sp. 3), followed by *Cryptococcus*, with four species [*Cr. (Bulleromyces) laurentii, Cr. (Asterotremella) humicola, Cr. (Bulleromyces) flavescens* and *Cr. podzolicus*)]. *Schwanniomyces polymorphus* was the most frequently isolated yeast, occurring in both isolation sites and in seven samples cultured on YNB-D-xylose medium and 28 samples on YNB-xylan medium. According to Suzuki and Kurtzman (2011), strains of *Sc. polymorphus* have been isolated from soil, ant-hill and "toadstools", and more recently also from rotting wood (Cadete et al., 2012a). *Scheffersomyces queiroziae* was the second most frequently isolated yeast, found only in the Reserve of the Sanctuary of Caraça, in 18 samples in YNB-D-xylose medium and six in YNB-xylan medium. *Scheffersomyces queiroziae* is a recently discovered species that has been isolated from rotting wood and wood-boring insects in two Atlantic Rainforest ecosystems in Brazil, including the Reserve of the Sanctuary of Caraça (Santos et al., 2011). These results suggest that the Atlantic Rainforest biome is this species' natural habitat. The species with the third highest frequency observed was *Candida boidinii* (*Ogataea* clade), isolated from both reserves, and *Barnettozyma californica*, isolated only from the Reserve of Serra Bonita. Each of these two species was represented by 19 isolates from both sampling media. *Candida boidinii* was also one of the yeasts most frequently isolated from rotting wood collected in the Amazonian Forest by Cadete et al. (2012a), with 10 samples cultured on YNB-D-xylose medium and seven in YNB-xylan medium. *Candida boidinii* has been found frequently in

the sap of many tree species in geographically distinct regions of the world. Specialised substrates associated with this species are largely linked to its ability to assimilate the methanol produced in decaying plant tissues (Lachance et al., 2011). *Barnettozyma californica* appears to have a worldwide distribution, as evident from its isolation from soil, water, tree fluxes and animal dung (Kurtzman, 2011a).

The Mao Tao rarefaction curves for the yeast communities isolated in YNB-D-xylose and YNB-xylan media from both areas continued to rise and did not reach an asymptote (Figure 1), indicating that not all yeast richness had been recovered. Thirty yeast species (43.5%) occurred as singletons, representing rare components of the yeast communities. Additionally, the yeast composition was variable among the samples and areas, which was confirmed by the values of the Sorensen and Bray-Curtis similarity indices (Figure 2) showing that the most similar yeast communities were found in between the YNB-D-xylose and YNB-xylan media of each site collection. Among the 69 species identified, only *C. boidinii*, *C. cellulosicola*, *C. melibiosica*, *C. palmioleophila*, *C. picinguabensis*, *C. sojae*, *C. silvae*, *Cr. humicola*, *G. geotrichum*, *Sc. polymorphus*, *Su. boreocaroliniensis* and *Wickerhamomyces* sp. were found in the two sampled areas. As already observed for the yeast species characterised in this work, these common species are also associated with terrestrial environments and plant materials.

The 57 species, the majority of which were exclusively found in the Reserve of the Sanctuary of Caraça, isolated from only one sampling site included *S. queiroziae* (24 isolates), followed by a new species discovered in this work, *Su. xylanicola* (11 isolates), *Cr. laurentii* (five isolates), *M. guilliermondii* (four isolates) and *Sc. vanrijiae* (four isolates). *Cryptococcus laurentii* has been isolated from various sources, such as soil, seawater, decaying wood, the phyllosphere of grasses, the rhizosphere of plants and clinical sources (Fonseca et al., 2011). Strains from *M. guilliermondii* have been reportedly isolated from insect frass, flowers, fruits, soil, alcohol fermentation processes and clinical specimens (Kurtzman, 2011b), while *Sc. vanrijiae* has been found to be associated with soil, plant exudate and ant hills (Suzuki and Kurtzman, 2011). These last three species were also isolated from rotting wood samples collected in the Brazilian Amazonian Forest (Cadete et al., 2012a). The species exclusively found in the Reserve of Serra Bonita included *B. californica* (19 isolates), *C. (Kurtzmaniella) natalensis* (11 isolates), *T. laibachii* (10 isolates) and *S. stipitis* (seven isolates). The latter species is known for its ability to ferment D-xylose and is associated with insect larvae, the gut of Passalid and other beetles, rotting wood and insect frass (Cadete et al., 2012a; Kurtzman, 2011c, Urbina and Blackwell, 2012). These observations show that despite the great number of different

species found, few of them represent the majority of the isolates identified, predominantly among the remaining yeasts.

Some yeast isolates showed three or more non-contiguous nucleotide differences in the D1/D2 domains of the LSU rRNA gene when compared with the most closely related known species, indicating that they might represent novel yeast species (Kurtzman et al., 2011a). Fifteen possible new yeast species, shown in Table 2, were found in our study. Eight of these new species, namely *Ogataea* sp. 1, *Ogataea* sp. 2, *Saturnispora* sp. 1, *Spencermartinsiella* sp., *Sugiyamaella* sp. 2, *Sugiyamaella* sp. 3, *Sugiyamaella xylanicola* and *Tremella* sp. were isolated exclusively from the Reserve of the Sanctuary of Caraça, whereas six species – *Metschnikowia* sp., *Pichia* sp., *Saturnispora* sp. 2, *Spathaspora* sp. 1, *Spathaspora* sp. 2 and *Sugiyamaella* sp. 1 – were solely isolated from the Reserve of Serra Bonita. Only one species, *Wickerhamomyces* sp., was found at both sites. Eleven isolates have been described as a new species, named *Sugiyamaella xylanicola* (Morais et al., 2013). The new species belongs to the *Sugiyamaella* clade, and it differs by 14 nucleotide substitutions in the D1/D2 domains and six in the ITS region of the LSU rRNA gene from *Su. marionensis*, its closest relative (Figure 3). Among the remaining eight new species found in the Reserve of Sanctuary of Caraça, only *Spencermartinsiella* sp. was represented by more than one isolate. This species presented D1/D2 sequences identical to *Candida* sp. NCAIM Y.01937 (GenBank accession number GQ340914), and both were phylogenetically related to the species *Spencermartinsiella europaea.* These yeasts differed from S. *europaea* by 19 substitutions in D1/D2 sequences. *Sugiyamaella* sp. 2 is related to *Su. novakii*, and *Sugiyamaella* sp. 3 are related to *Su. americana* (differing by 47 substitutions in the D1/D2 domains) (Figure 3). *Saturnispora* sp. 1 differs from *Saturnispora mendocae* by 48 nucleotide substitutions and 10 gaps. *Ogataea* sp. 1 differs from *Ogataea wangdongensis* by 18 substitutions, but this species is most closely related to *Candida* sp. CLIB 1156 (HE574640), a species without formal description isolated from exudates in New Zealand. *Ogatea* sp. 2 differs from *Candida nanaspora* in 29 substitutions and 14 gaps in the D1/D2 domains. However, *Ogataea* sp. 2 is closed related to *Candida* sp. NRRL Y-27166 (EU011603), a species without a formal published description. *Tremella* sp. is related to *Tremella fuciformis*, and these species differ by 11 substitutions in D1/D2 sequences.

All of the new species, except *Spathaspora* sp. 2, found exclusively in the reserve of Serra Bonita were represented by a single isolate each. *Pichia* sp. differs by 19 nucleotide substitutions in the D1/D2 domains from its closest relative, *Candida rugopelliculosa*. *Sugiyamaella* sp. 1 is closed related to *Su. lignohabitans* and *Su. xylanicola* differing by 11 nucleotide substitutions and five gaps

and 13 substitutions and five gaps in the D1/D2 domains, respectively, from these species. Two new species belonging to the genus *Spathaspora* were identified; *Spathaspora* sp. 1 differs from *Sp. brasiliensis* by 12 nucleotide substitutions and one gap, and *Spathaspora* sp. 2 differs by 17 nucleotide substitutions and three gaps from *Candida (Spathaspora) lyxosophila* in the D1/D2 domains (Figure 4). *Metschnikowia* sp. 1 is phylogenetically related to *Candida* sp. GS-2010 (GU592922), differing from this species by 33 substitutions and 16 gaps in D1/D2 sequences, and by 58 differences from *C. blattae*. Lastly, *Saturnispora* sp. 2 is closed related to *C. (Saturnispora) sekii*, differing from the later species by eight substitutions and two gaps. However, *Saturnispora* sp. 2 is most closely related to *Candida* sp. BG02-7-15-015A-2-1 (AY520326), differing from this species by four substitutions in the D1/D2 sequences.

Wickerhamomyces sp. was the only new species common to both collection sites, represented by one isolate in each site. This new species is related to the species *C. quercuum*, differing by 24 substitutions and seven gaps. *Wickerhamomyces* sp. presented identical D1/D2 sequences to *Candida* sp. UWO(PS)00-147.3 (AF530612), a species without formal description, isolated from slime flux of *Hymenaea courbaril* in Costa Rica. The great diversity of species observed in this study, together with the remarkable number of new species obtained, reinforce the characterisation of the Atlantic Rainforest as a biodiversity *hotspot*, which therefore must be correctly studied and preserved.

3.2 D-xylose fermentation assay

Out of the 69 yeast species identified, strains belonging to 14 species showed production of ethanol in the D-xylose fermentation test. These strains are composed of the known species *C. (Ogataea) boidinii, C. (Spencermartinsiella) cellulosicola, C. (Metschnikowia) melibiosica, C. (Lodderomyces/Spathaspora) tropicalis, C. (Metschnikowia) intermedia, M. guilliermondii, S. queiroziae, S. shehatae, S. stipitis, Sc. polymorphus, Su. boreocaroliniensis, W. pijperi* and the new species *Sugiyamaella* sp. 1 and *Su. xylanicola.* Among these species, *S. shehatae* and *S. stipitis* are already described as D-xylose-fermenting yeasts (Kurtzman et al., 2011a), both of which have been extensively studied in several works for their D-xylose-fermenting ability. Also *C. tropicalis* has previously been shown to ferment D-xylose, producing ethanol, but mainly xylitol, from this pentose (Jeffries, 1981; Kim and Oh, 2003; Rao et al., 2006; Sánchez et al., 2008). This is the first study to report that a *S. queiroziae* isolate is capable of producing ethanol from D-xylose. This yeast has been described as a strong cellobiose-fermenting microorganism when assayed in Durham tubes, but no gas formation was observed when D-xylose was the sugar used (Santos et al., 2011). *Candida intermedia* grows well in D-xylose medium and shows a high D-xylose-transport capacity (Gárdonyi

et al., 2003; Leandro et al., 2006). According to Vandeska et al. (1995) and Vongsuvanlert and Tani (1989), *C. boidinii* produces ethanol and xylitol from D-xylose, whereas *C. melibiosica* has the ability to convert xylulose to ethanol (Ueng et al., 1981), but only the xylitol and arabitol production was observed when this species was tested in a D-xylose fermentation assay (Gong et al., 1983). *Candida cellulosicola* and *Su. boreocaroliniensis* are capable of assimilating D-xylose (Guo et al., 2012; Kurtzman, 2007). However, there have been no reports documenting the conversion of D-xylose to ethanol by these species until the present study. The same finding is observed for the yeasts *S. polymorphus* and *W. pijperi*. Species of *Sugiyamaella* are D-xylose-assimilating yeasts (Urbina et al., 2012), but this is the first study to demonstrate the fermentation of D-xylose by a species of this genus, including the new species *Sugiyamaella* sp. 1 and *Su. xylanicola*. The conversion of D-xylose to ethanol has already been reported for *M. guilliermondii* strains (Rao et al., 2008; Toivola et al., 1984). Surprisingly, although two new species (*Spathaspora* sp. 1 and *Spathaspora* sp. 2) were identified as belonging to the *Spathaspora* clade, which is known to contain D-xylose-fermenting yeasts, these isolates exhibited no ethanol production in the screening test for D-xylose fermentation.

3.3 Xylanase production assay

Strains from 20 species produced xylanase on xylan-agar medium. These strains were represented by the known species *C. (Ogataea) boidinii, C. (Spencermartinsiella) cellulosicola, C. (Saturnispora) silvae, Cr. (Bulleromyces) laurentii, Cr. (Bulleromyces) flavescens, Cr. podzolicus, G. geotrichum, L. fabianii, L. saturnus, S. queiroziae, S. shehatae, S. stipitis, Su. boreocaroliniensis, Su. lignohabitans, Trichosporon* spp. and *W. pijperi,* and by the new species *Spencermartinsiella* sp., *Su. xylanicola*, *Tremella* sp. and *Wickerhamomyces* sp. Strains of *C. silvae* have been sporadically isolated from miscellaneous substrates, but this species was associated with horse intestine and necrotic cladode (Lachance et al., 2011). *Candida cellulosicola, Su. boreocaroliniensis* and *Su. lignohabitans* are commonly associated with rotten lignocellulosic materials (Kurtzman, 2007), but no evidence for the production of xylanases by all these yeasts, including *C. boidinii*, has been found. This behaviour was also observed for the yeasts *G. geotrichum* and *L. saturnus*, which are associated with soils (de Hoog and Smith, 2011; Kurtzman, 2011d). Species from the genus *Cryptococcus* have already been shown to produce xylanases (Parachin et al., 2009). Lee et al. (1986) reported that a strain of *Cr. podzolicus* is able to produce xylanase, as well as strains of *S. shehatae* and *S. stipitis*. In addition, the production of xylanase by the species *S. stipitis* has been documented by other authors (Basaran et al., 2001; Ozcan et al., 1991). As verified for D-xylose fermentation, this is also the first study to report that *S. queiroziae* is capable of producing xylanase. The synthesis of xylanases was

demonstrated by species of the genus *Trichosporon* (Stevens and Payne, 1977), and two isolates of this genus were able to produce xylanase in our work. The species *L. fabianii* and *W. pijperi* have been recently shown to produce xylanase when cultivated in xylan-yeast extract medium (Thongekkaew et al., 2012). For the new species, *Su. xylanicola*, the production of an extracellular endo-1,4-ß-xylanase activity is correlated with the use of xylan or D-xylose as inducers but with higher activities under xylan induction (Morais et al., 2013). Interestingly, strains of seven species that have been shown to ferment D-xylose in the present study were also capable of producing xylanase; six of these have already been described (*C. boidinii, C. cellulosicola, S. queiroziae, S. shehatae, S. stipitis* and *W. pijperi*) and one is the new species *Su. xylanicola*. The ability to convert D-xylose to ethanol is shown by fewer than 25 species of the more than 1500 described yeasts (Kurtzman et al., 2011a) and also the production of xylanases is restricted to a few yeast species. D-xylose fermenting yeasts are in general associated to rotten biomass substrates where fungi act as main lignocellulose-degrading organisms, by using a complex system of enzymes responsible to provide the biomass constituents sugars and ensure a rapid colonization of the substrates. On the other hand, the majority of xylanase-producing yeasts are able to consume xylooligosaccharides or D-xylose, but not to ferment these carbon sources since the fermentation of D-xylose is strictly dependent on the oxygenation level, being favoured under microaerophilic conditions, and on the co-factors dependency presented by the enzymes responsible for the first steps of the D-xylose metabolism, xylose reductase and xylitol dehydrogenase. Once this study describes the isolation of new D-xylose-fermenting and/or xylanase-producing strains and species, it constitutes an important contribution to the assessment of the biotechnological potential of yeasts isolated from a biodiversity *hotspot* ecosystem, particularly with regard to the employment of these microorganisms in the production of second generation ethanol.

Acknowledgements

This work was funded by Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq, process n^{o} 551392/2010-0, 551245/2010-7 and 560715/2010-2), Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG), Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP/BIOEN/FAPEMIG), Fundação de Amparo à Pesquisa da Bahia (FAPESB) and the Natural Science and Engineering Research Council of Canada (M.A.L.).

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Figure 1. Species accumulation curves (Mao Tao index) for the yeast communities present in decaying wood in Brazilian Atlantic Rainforest ecosystems. **(a)** YNB-xylose, Reserve of the Sanctuary of Caraça, **(b)** YNB-xylan, Reserve of the Sanctuary of Caraça, **(c)** YNB-D-xylose, Reserve of Serra Bonita, and **(d)** YNB-xylan, Reserve of Serra Bonita.

Figure 2. Dendrograms showing the Sorensen **(a)** and **(b)** Bray-Curtis similarity measures for the yeast communities obtained from decaying wood in Brazilian Atlantic Rainforest ecosystems. SBXylo = YNB-xylose, Reserve of Serra Bonita; SBXyla = YNB-xylan, Reserve of Serra Bonita; SCXylo = YNB-xylose, Reserve of the Sanctuary of Caraça; and SCXyla = YNB-xylan, Reserve of the Sanctuary of Caraça.

Figure 3. Maximum Likelihood phylogram showing the phylogenetic placement of species of the *Sugiyamaella* clade. The large subunit rRNA gene D1/D2 sequence distances were transformed using the General Time Reversible model. Bootstrap values were determined from 100 iterations. The dataset had a total of 516 aligned positions. Alignment and tree construction were performed with the program MEGA5.

Figure 4. Maximum Likelihood phylogram showing the phylogenetic placement of species of the *Spathaspora-Lodderomyces* clade. The large subunit rRNA gene D1/D2 sequence distances were transformed using the General Time Reversible model. Bootstrap values were determined from 100 iterations. The dataset had a total of 511 aligned positions. Alignment and tree construction were performed with the program MEGA5.

Table 1. Identification, occurrence, xylanase production and D-xylose fermentation of yeasts isolated in Atlantic Rainforest forest reserves.

Table 2. Identification of novel yeast species isolated in Atlantic Rainforest reserves.

Table 1. Identification, occurrence, xylanase production and D-xylose fermentation of yeasts isolated in Atlantic Rainforest forest reserves.

Yeast species	Sampled medium		Xylanase production	D-xylose fermentation (alcohol production in g/L)
	YNB-D-xylose (n = 50)	YNB-xylan (n = 50)		
Private Natural Heritage Reserve of the Sanctuary of Caraça				
Candida (Ogataea) boidinii	1	1	-	1.44 ± 0.7 (1)[a]
C.(Spencermartinsiella) cellulosicola	6	0	+ (6)	1.22 ± 0.3 (4)
C.(Metschnikowia) melibiosica	2	8	-	0.98 ± 0.2 (3)
Candida (Kurtzmaniella) oleophila	0	2	-	-
C. palmioleophila	0	1	-	-
C. (Metschnikowia) picinguabensis	1	0	-	-
C. (Lodderomyces/Spathaspora) tropicalis	1	1	-	1.21 ± 0.2 (1)
C. (Saturnispora) silvae	1	0	-	-
C. (Lodderomyces /Spathaspora) sojae	1	0	-	-
Cryptococcus (Bulleromyces) laurentii	1	4	+ (4)	-
Cr. (Asterotremella) humicola	1	3	-	-
Debaryomyces hansenii	0	3	-	-
Galactomyces geotrichum	3	1	+ (2)	-
Meyerozyma guilliermondii	2	2	-	0.84 ± 0.3 (3)
Scheffersomyces queiroziae	18	6	+ (1)	2.21 ± 0.5 (23)

Schwanniomyces polymorphus	6	26	-	1.46 ± 0.3 (7)
Sc. Vanrijiae	0	4	-	-
Spencermartinsiella sp.[b]	2	1	+ (2)	-
Sugiyamaella boreocaroliniensis	7	3	-	1.83 ± 0.3 (7)
Su. lignohabitans	0	2	+ (1)	-
Su. xylanicola sp. nov.[b]	6	5	+ (10)	1.17 ± 0.1 (7)
Wickerhamomyces sp.[b]	0	1	-	-
Private Natural Heritage Reserve of Serra Bonita				
Barnettozyma californica	6	13	-	-
C. (Ogataea) boidinii	10	7	+ (1)	0.72 ± 0.06 (6)
C. (Spencermartinsiella) cellulosicola	2	1	+ (2)	-
C. (Ogataea) cylindracea	1	2	-	-
C. (Metschnikowia) intermedia	1	1	-	1.93 ± 0.9 (1)
C. (Metschnikowia) melibiosica	2	3	-	1.29 ± 0.2 (2)
C. (Kurtzmaniella) natalensis	3	8	-	-
C. palmioleophila	1	4	-	-
C. (Metschnikowia) picinguabensis	3	1	-	-
C. (Saturnispora) silvae	1	3	+ (1)	-
C. (Lodderomyces/Spathaspora) sojae	2	3	-	-
Cr. (Bulleromyces) flavescens	1	3	+ (3)	-
Cr. (Asterotremella) humicola	1	9	-	-
Cr. Podzolicus	0	2	+ (1)	-
G. geotrichum	5	1	+ (1)	-
Geotrichum klebahnii	2	0	-	-

Lindnera saturnus	2	0	+ (1)	-
L. subsufficiens	2	1	-	-
Saturnispora hagleri	0	2	-	-
S. shehatae	2	2	+ (1)	6.53 ± 0.8 (4)
S. stipitis	5	2	+ (2)	6.78 ± 1.4 (7)
Sc. Polymorphus	1	2	-	1.17 ± 0.3 (1)
Spathaspora sp. 2[b]	1	1	-	-
Su. boreocaroliniensis	3	2	+ (3)	-
Trichosporon laibachii	2	8	-	-
T. multisporum	0	3	-	-
Trichosporon spp.	2	1	+ (2)	-
Wickerhamomyces pijperi	3	2	+ (1)	0.98 ± 0.2 (1)
Wickerhamomyces sp. [b]	1	0	+ (1)	-

[a] numbers in parenthesis represent the number of strains positive for xylanase production and/or D-xylose fermentation

[b] novel yeast species

Table 2. Identification of novel yeast species isolated in Atlantic Rainforest reserves based on the sequences of the domains D1/D2 of the large subunit of the rRNA gene.

UFMG code	N° of isolates	Top BLAST search results [GenBank accession number]	Identity (%)	Bp analyzed	Proposed species or taxonomic group [GenBank accession number]
Private Natural Heritage Reserve of the Sanctuary of Caraça					
CA-7.2	1	*Candida* sp. CLIB 1156 [HE574640]	99	358	*Ogataea* sp. 1 [KC832476]
CA-48.1	1	*Candida* sp. NRRL Y-27166 [EU011603]	99	585	*Ogataea* sp. 2 [KC832477]
CA-1.2	1	*Saturnispora mendocae* [EF550214]	89	545	*Saturnispora* sp. 1 [KC832478]
CO-34.2	3	*Candida* sp. NCAIM Y.01937 [GQ340914]	100	581	*Spencermartinsiella* sp. [KC906243]
CO-50.1	1	*Sugiyamaella novakii* [AY618511]	95	608	*Sugiyamaella* sp. 2
CO-31.1	1	*Sugiyamaella. americana* [HM208604]	92	595	*Sugiyamaella* sp. 3
CO-32.1	11	*Sugiyamaella marionensis* [DQ438197]	98	528	*Sugiyamaella xylanicola* [KC493642]

CA-33.2	1	*Tremella fuciformis* [JN043571]	98	635	*Tremella* sp. [KC832479]
CA-5.2	1	*Candida* sp. UWO(PS)00-147.3 [AF530612]	99	716	*Wickerhamomyces* sp.[a] [KC912858]
Private Natural Heritage Reserve of Serra Bonita					
IA-7.2	1	*Candida* sp. GS-2010 [GU592922]	91	529	*Metschnikowia* sp. [KC832481]
IA-11.1	1	*Candida rugopelliculosa* [EF550238]	98	576	*Pichia* sp. [KC832482]
IA-38.2	1	*Candida* sp. BG02-7-15-015A-2-1 [AY520326]	99	564	*Saturnispora* sp. 2 [KC832480]
IA-35.1	1	*Spathaspora brasiliensis* [JN099271]	98	579	*Spathaspora* sp. 1
IO-27.1	2	*Candida (Spathaspora) lyxosophila*	97	582	*Spathaspora* sp. 2
IA-2.1	1	*Sugiyamaella lignohabitans* [DQ438199]	97	573	*Sugiyamaella* sp. 1
IO-22.1	1	*Candida* sp. UWO(PS)00-147.3 [AF530612]	97	838	*Wickerhamomyces* sp.[a]

[a] species found in both Atlantic Rainforest reserves

Figure 1
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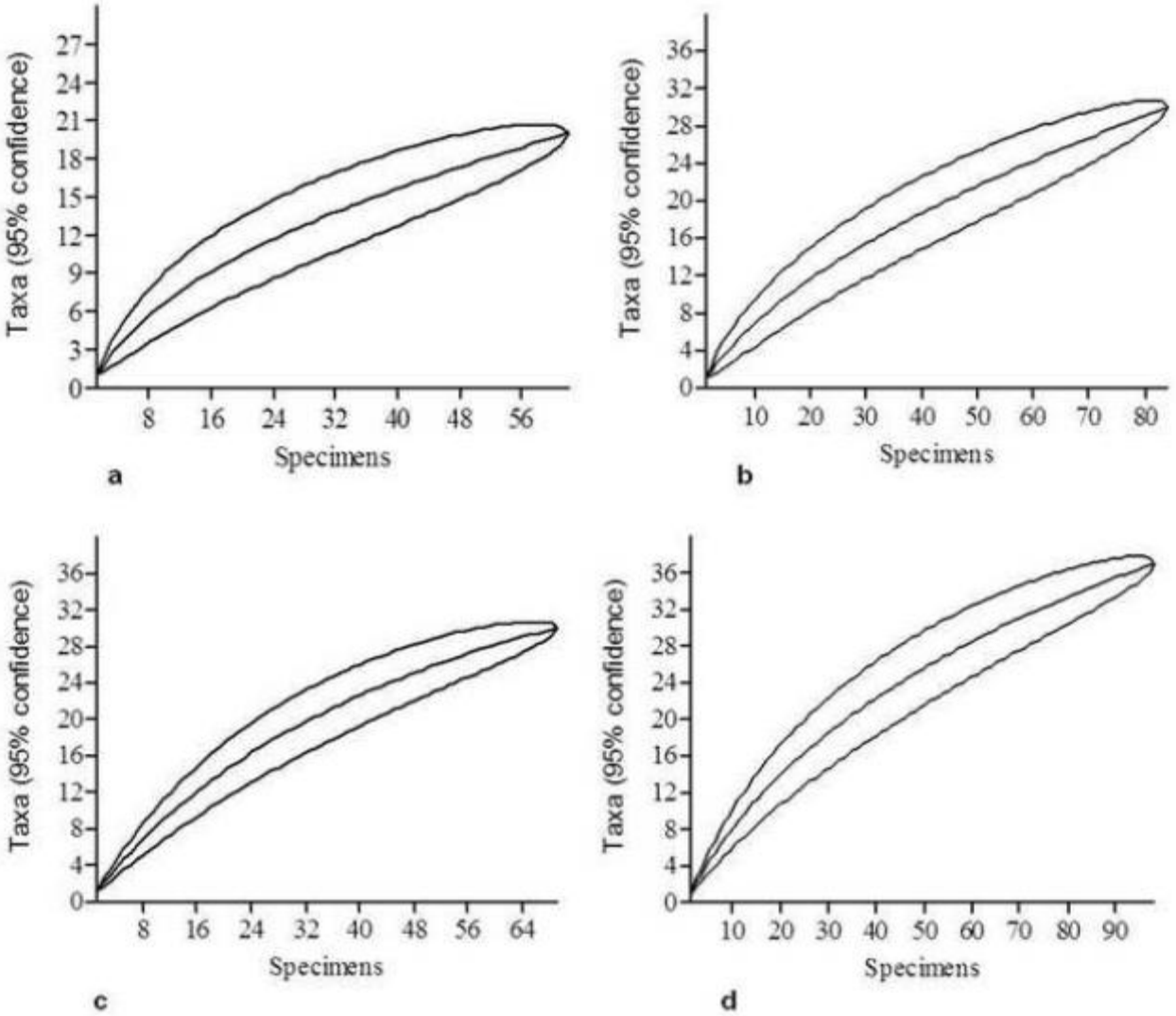

Figure 2
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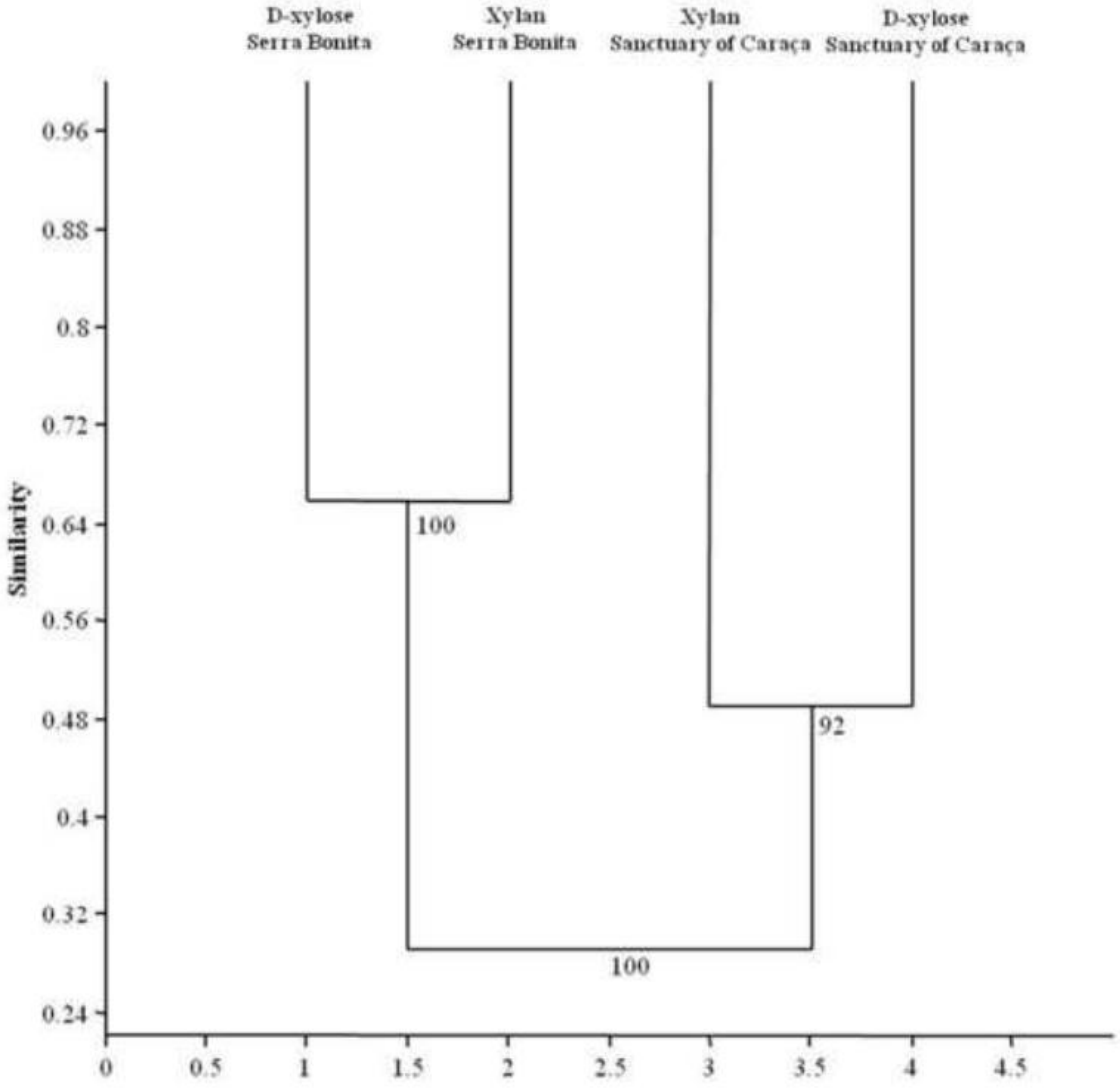


Figure 3

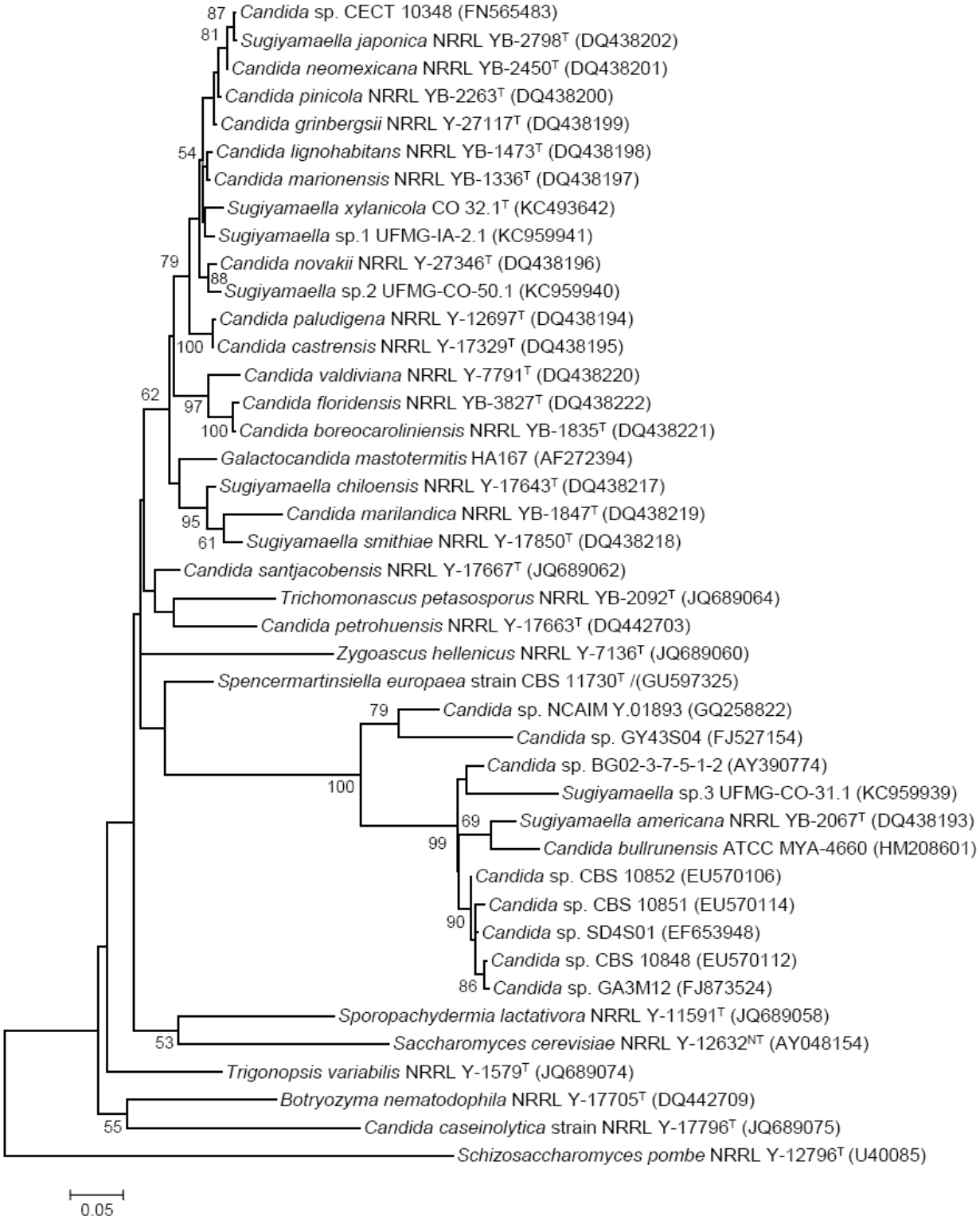
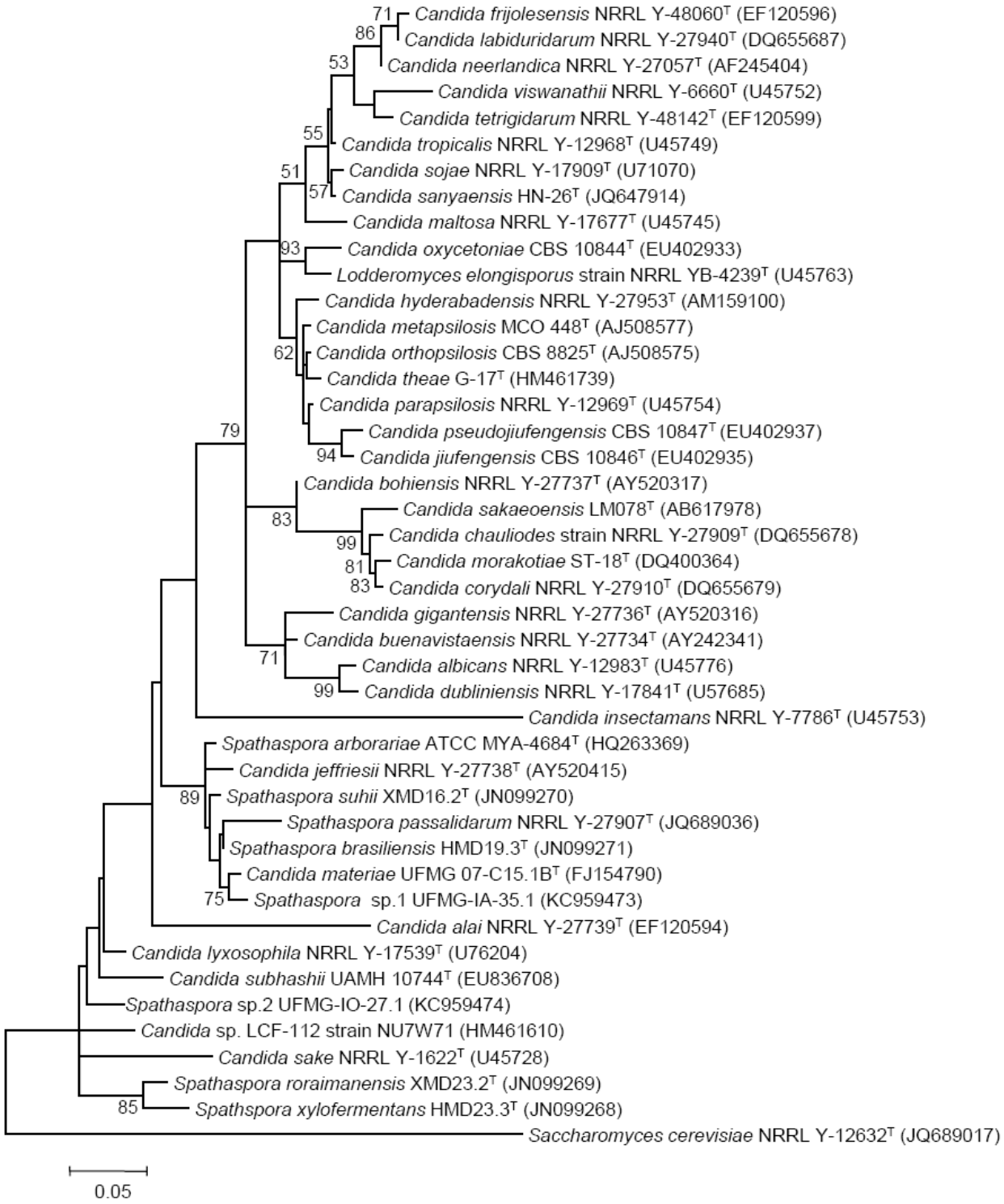

87
81
Candida sp. CECT 10348 (FN565483)
Sugiyamaella japonica NRRL YB-2798T (DQ438202)
Candida neomexicana NRRL YB-2450T (DQ438201)
Candida pinicola NRRL YB-2263T (DQ438200)
Candida grinbergsii NRRL Y-27117T (DQ438199)
54
Candida lignohabitans NRRL YB-1473T (DQ438198)
Candida marionensis NRRL YB-1336T (DQ438197)
Sugiyamaella xylanicola CO 32.1T (KC493642)
Sugiyamaella sp.1 UFMG-IA-2.1 (KC959941)
79
Candida novakii NRRL Y-27346T (DQ438196)
88
Sugiyamaella sp.2 UFMG-CO-50.1 (KC959940)
Candida paludigena NRRL Y-12697T (DQ438194)
100
Candida castrensis NRRL Y-17329T (DQ438195)
Candida valdiviana NRRL Y-7791T (DQ438220)
62
97
Candida floridensis NRRL YB-3827T (DQ438222)
100
Candida boreocaroliniensis NRRL YB-1835T (DQ438221)
Galactocandida mastotermitis HA167 (AF272394)
Sugiyamaella chiloensis NRRL Y-17643T (DQ438217)
Candida marilandica NRRL YB-1847T (DQ438219)
95
61
Sugiyamaella smithiae NRRL Y-17850T (DQ438218)
Candida santjacobensis NRRL Y-17667T (JQ689062)
Trichomonascus petasosporus NRRL YB-2092T (JQ689064)
Candida petrohuensis NRRL Y-17663T (DQ442703)
Zygoascus hellenicus NRRL Y-7136T (JQ689060)
Spencermartinsiella europaea strain CBS 11730T /(GU597325)
79
Candida sp. NCAIM Y.01893 (GQ258822)
Candida sp. GY43S04 (FJ527154)
Candida sp. BG02-3-7-5-1-2 (AY390774)
100
Sugiyamaella sp.3 UFMG-CO-31.1 (KC959939)
69
Sugiyamaella americana NRRL YB-2067T (DQ438193)
99
Candida bullrunensis ATCC MYA-4660 (HM208601)
Candida sp. CBS 10852 (EU570106)
Candida sp. CBS 10851 (EU570114)
90
Candida sp. SD4S01 (EF653948)
Candida sp. CBS 10848 (EU570112)
86
Candida sp. GA3M12 (FJ873524)
Sporopachydermia lactativora NRRL Y-11591T (JQ689058)
53
Saccharomyces cerevisiae NRRL Y-12632NT (AY048154)
Trigonopsis variabilis NRRL Y-1579T (JQ689074)
Botryozyma nematodophila NRRL Y-17705T (DQ442709)
55
Candida caseinolytica strain NRRL Y-17796T (JQ689075)
Schizosaccharomyces pombe NRRL Y-12796T (U40085)
0.05

Figure 4


71 Candida frijolesensis NRRL Y-48060T (EF120596)
86 Candida labiduridarum NRRL Y-27940T (DQ655687)
53 Candida neerlandica NRRL Y-27057T (AF245404)
Candida viswanathii NRRL Y-6660T (U45752)
Candida tetrigidarum NRRL Y-48142T (EF120599)
55 Candida tropicalis NRRL Y-12968T (U45749)
51 Candida sojae NRRL Y-17909T (U71070)
57 Candida sanyaensis HN-26T (JQ647914)
Candida maltosa NRRL Y-17677T (U45745)
93 Candida oxycetoniae CBS 10844T (EU402933)
Lodderomyces elongisporus strain NRRL YB-4239T (U45763)
Candida hyderabadensis NRRL Y-27953T (AM159100)
Candida metapsilosis MCO 448T (AJ508577)
62 Candida orthopsilosis CBS 8825T (AJ508575)
Candida theae G-17T (HM461739)
Candida parapsilosis NRRL Y-12969T (U45754)
79 Candida pseudojiufengensis CBS 10847T (EU402937)
94 Candida jiufengensis CBS 10846T (EU402935)
Candida bohiensis NRRL Y-27737T (AY520317)
Candida sakaeoensis LM078T (AB617978)
83 Candida chauliodes strain NRRL Y-27909T (DQ655678)
99 Candida morakotiae ST-18T (DQ400364)
81 Candida corydali NRRL Y-27910T (DQ655679)
83 Candida gigantensis NRRL Y-27736T (AY520316)
Candida buenavistaensis NRRL Y-27734T (AY242341)
71 Candida albicans NRRL Y-12983T (U45776)
99 Candida dubliniensis NRRL Y-17841T (U57685)
Candida insectamans NRRL Y-7786T (U45753)
Spathaspora arborariae ATCC MYA-4684T (HQ263369)
Candida jeffriesii NRRL Y-27738T (AY520415)
89 Spathaspora suhii XMD16.2T (JN099270)
Spathaspora passalidarum NRRL Y-27907T (JQ689036)
Spathaspora brasiliensis HMD19.3T (JN099271)
Candida materiae UFMG 07-C15.1BT (FJ154790)
75 Spathaspora sp.1 UFMG-IA-35.1 (KC959473)
Candida alai NRRL Y-27739T (EF120594)
Candida lyxosophila NRRL Y-17539T (U76204)
Candida subhashii UAMH 10744T (EU836708)
Spathaspora sp.2 UFMG-IO-27.1 (KC959474)
Candida sp. LCF-112 strain NU7W71 (HM461610)
Candida sake NRRL Y-1622T (U45728)
Spathaspora roraimanensis XMD23.2T (JN099269)
85 Spathspora xylofermentans HMD23.3T (JN099268)
Saccharomyces cerevisiae NRRL Y-12632T (JQ689017)
0.05