UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

CENTRO DE BIOTECNOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

Obtenção e avaliação de linhagens de *Saccharomyces cerevisiae* e de *Wickerhamomyces anomalus* com potencial para aplicação na produção de etanol de segunda geração

TESE DE DOUTORADO

NICOLE TEIXEIRA SEHNEM

PORTO ALEGRE, RS, BRASIL

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Tese apresentada ao Programa de Pós-Graduação em Biologia Celular e Molecular como requisito para a obtenção do Grau de Doutor em Ciências.

PORTO ALEGRE, RS, BRASIL

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ESTRUTURAÇÃO DA TESE

Esta tese encontra-se estruturada da seguinte forma: uma introdução seguida pelo referencial bibliográfico, os objetivos, três capítulos escritos na forma de artigos científicos, os quais foram formatados de acordo com as normas da revista Bioresource Technology, uma discussão geral dos resultados, as conclusões e as perspectivas.

O Capítulo I aborda a seleção de uma linhagem industrial de *Saccharomyces cerevisiae* altamente tolerante a HMF por engenharia evolutiva, a expressão dos genes responsáveis por essa tolerância e suas relações com as respostas fisiológicas para a produção de etanol. Esse capítulo foi publicado na revista Bioresource Technology, e encontra-se no apêndice da tese.

O capítulo II investiga tolerância a compostos furânicos e pressão osmótica da levedura *Wickerhamomyces anomalus*, também submetida à estratégia de engenharia evolutiva. A cinética de produção de etanol dessa levedura foi avaliada quanto à produção de etanol e quanto à produção de enzimas redutoras dos compostos furânicos. Esse capítulo foi submetido à revista Bioresource Technology.

O capítulo III avalia a aplicabilidade dessas leveduras para a produção de etanol de segunda geração simulando condições utilizadas em uma usina de produção de etanol.

Por fim, a discussão geral aborda todos os temas investigados nos três capítulos, fazendo uma relação entre os resultados obtidos, as conclusões finais e perspectivas geradas.

SUMÁRIO

LISTA DE ABREVIATURAS

YPD	Caldo dextrose, peptona, extrato de levedura
YPX	Caldo xilose, peptona, extrato de levedura
HMF	5-hidroximetilfurfural
RID	Detector de índice de refração
UV	Detector de Radiação Ultravioleta
g	Aceleração da gravidade
HPLC	Cromatografia líquida de alta eficiência
CUF	Unidades Formadoras de Colônia
OD	Densidade ótica
ADH	Álcool desidrogenase
RHH	Hidrolisado de casca de arroz
SHH	Hidrolisado de casca de soja

Obtenção e avaliação de linhagens de *Saccharomyces cerevisiae* e de *Wickerhamomyces anomalus* com potencial para aplicação na produção de etanol de segunda geração

Autor: Nicole Teixeira Sehnem

Orientador: Prof. Dr. Marco Antônio Záchia Ayub

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RESUMO

Nos últimos anos, é crescente a busca por tecnologias que permitam que a produção de álcool gerado a partir de fontes renováveis substitua os principais combustíveis utilizados atualmente, que são provenientes do petróleo. A utilização dos resíduos gerados a partir dos processos agrícolas, que são ricos em açúcares, é uma alternativa para a produção de bioetanol pela levedura *Saccharomyces cerevisiae*. Os processos utilizados com esse fim, como a hidrólise ácida diluída, geram uma diversidade de açúcares (pentoses e hexoses), como glicose, manose, xilose e arabinose. Além dos açúcares, ocorre a geração de compostos tóxicos às células, como furfural, 5-hidroximetilfurfural (HMF), compostos fenólicos e ácidos orgânicos, e também alta pressão osmótica. Além disso, *S. cerevisiae* não é capaz de utilizar pentoses como fonte de carbono para fermentação, e a viabilidade econômica desse processo depende da conversão quase total de todos os açúcares a etanol. Com isso é necessário o uso de linhagens fermentadoras de pentoses e hexoses que sejam resistentes às toxinas geradas, para que o hidrolisado seja utilizado sem um processo prévio de destoxificação. Nesse trabalho, foi obtida por engenharia evolutiva uma linhagem de de *S. cerevisiae* resistente ao HMF, nomeada P6H9. Foi avaliada a indução da expressão gênica na presença de HMF e observou-se que os genes *ADH7* E *ARI1* são responsáveis pela resistência a esse composto. Também foi possível observar que as mudanças no metabolismo dessa levedura para destoxificação do meio não possibilitam o aumento na produção de etanol. Também foi obtida uma linhagem da levedura fermentadora de pentoses *Wickerhamomyces anomalus* resistente a furfural e HMF, nomeada WA-HF5,5. Foi possível definir a grande importância das enzimas álcool desidrogenases na destoxificação desses compostos. Finalmente, essas duas leveduras foram avaliadas quanto à capacidade de produção de etanol em hidrolisados lignocelulósicos de casca de soja e casca de arroz, isoladamente, ou em sistemas de co-cultivos em frascos agitados. As melhores produtividades em etanol foram apresentadas em hidrolisado de casca de arroz, e os cultivos foram escalonados para sistemas de biorreatores. Foi observado que o sistema de co-cultivo possui vantagens e grande potencial para a produção de etanol de segunda geração.

1. Tese de Doutorado em Ciências, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil. (137 p.). Maio, 2013.

Obtainment and evaluation of *Saccharomyces cerevisiae* and *Wickerhamomyces anomalus* strains with potential to second-generation ethanol production

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ABSTRACT

In recent years, there is a crescent interest for technologies that enable the production of alcohol originated from renewable sources, in replacement of the fossil fuels. The use of waste generated from agricultural processes, which are rich in sugars, is an alternative for the production of bioethanol by the yeast *Saccharomyces cerevisiae*. The processes used for this purpose, such as dilute acid hydrolysis, releases a diversity of sugars (pentoses and hexoses), such as glucose, mannose, xylose and arabinose. In addition to sugars, toxic compounds to cells are also generated, such as furfural, 5-hydroxymethylfurfural (HMF), phenolic compounds, organic acids, and also high osmotic pressure. The economic feasibility of the process depends of the complete conversion of major sugars present ih the medium to ethanol. As *S. cerevisiae* is not able to metabolize pentoses, it is necessary to use strains that ferment pentoses and hexoses to ethanol, which are resistant to the toxins formed, in order to the hydrolyzate be fermented without previous detoxification processes.In this work, a strain of *S. cerevisiae* resistant to HMF, named P6H9, was obtained by evolutionary engineering. It was observed that the *ARI1* and *ADH7* genes are responsible for HMF resistance. It was also observed that the detoxification process causes changes in the metabolism of this yeast. Because of that, it is not possible increase the ethanol production. A strain of the fermenting pentoses yeast *Wickerhamomyces anomalus* was also obtained by evolutionary engineering, that is resistant to furfural and HMF. This strain is named WA-HF5, 5. On physiological evaluations, it was clear that alcohol dehydrogenase activity possesses importance in furaldehydes detoxification. Finally, the ability of these two strains produce ethanol in lignocellulosic hydrolysates was investigated. Best yields of ethanol were presented in rice hull hydrolysate. It was observed that the co-culture system shows advantages and have potential for the production of second generation ethanol.

1. Doctor thesis of Science Thesis in Cellular and Molecular Biology, Biotechnology Center, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. (137 p.). May, 2013.

1. INTRODUÇÃO

Atualmente, vem ocorrendo um aumento da demanda na produção global de energia, que é consequência das mudanças ocorridas no comportamento de consumo em todo o mundo. No Brasil, a produção de energia vem crescendo ao longo dos anos e mais da metade dessa energia é proveniente de combustíveis fósseis. Devido a isso, nos últimos anos, é crescente a busca por tecnologias que permitam que a produção de álcool gerado a partir de fontes renováveis seja o substituto dos principais combustíveis utilizados, que são provenientes do petróleo. Atualmente, o etanol é gerado a partir da cultura da cana-de-açúcar e da cultura de milho. Além dessas culturas, existe o potencial de produção de etanol a partir de resíduos.

A utilização dos resíduos gerados a partir dos processos agrícolas, que são ricos em açúcares, é uma alternativa para a produção de bioetanol por leveduras. Esses resíduos, formados pelas partes estruturais da planta são compostos por açúcares complexos e de difícil acesso. Por não competir com a indústria de alimentos, o etanol proveniente desses materiais é denominado etanol de segunda geração, que gera grande interesse e tem sido amplamente estudado.

Entretanto, para que os açúcares sejam liberados para a fermentação por leveduras, processos físicos e químicos devem ser empregados sobre esse tipo de fonte. Um dos métodos mais difundidos atualmente é a hidrólise ácida diluída. Esse processo libera uma grande variedade de açúcares, e consequentemente compostos tóxicos às leveduras.

O microrganismo que possui maior capacidade de conversão de açúcares a etanol é a levedura *Saccharomyces cerevisiae*. Porém, essa levedura não possui capacidade de metabolizar açúcares como pentoses, que são liberados a partir dos resíduos lignocelulósicos em grandes quantidades. Entretanto, diversas leveduras possuem capacidade de converter pentoses a etanol, tais como *Pichia stipitis*, *Candida guilliermondii*, *Spathaspora arborariae*, *Pachysolen tannnophilus* e *Wickerhamomyces anomalus*. Essas leveduras possuem características fisiológicas específicas e distintas de *S. cerevisiae*, como a demanda por oxigênio. Essa diferença fisiológica representa um gargalo na produção de etanol de segunda geração.

Além disso, o processo para liberação dos açúcares presentes nos resíduos lignocelulósicos acarreta na desidratação dos açúcares, gerando compostos tóxicos às células, como furfural e 5-hidroximetilfurfural (HMF). Esses compostos atuam sobre o metabolismo celular, interferindo na produtividade do etanol. Atualmente, são necessárias etapas de destoxificação dos hidrolisados para que *S. cerevisiae* seja capaz de converter esses açúcares. Uma alternativa para utilizar os hidrolisados lignocelulósicos na produção de etanol eliminando o passo de destoxificação é o estudo de cepas resistentes aos compostos tóxicos para a direta utilização desse produto sem etapas adicionais. Nesse contexto, o presente trabalho visa contribuir para o desenvolvimento de estratégias para viabilizar a produção de etanol de segunda geração. Buscou-se obter por engenharia evolutiva uma linhagem de *S. cerevisiae* resistente a HMF, identificar os genes responsáveis pela resistência a esse composto e evidenciar as respostas fisiológicas que são resultado das modificações em expressões gênicas. Adicionalmente buscou-se obter, também por engenharia evolutiva, uma linhagem da levedura fermentadora de pentoses *W.*

anomalus resistente a furfural e HMF, e avaliar a capacidade fermentativa dessa levedura na presença de ambos compostos furânicos, além de avaliar sua capacidade natural de resistir a altas pressões osmóticas. Finalmente, essas duas leveduras foram avaliadas quanto à produção de etanol em dois diferentes hidrolisados lignocelulósicos, separadamente ou em sistemas de co-cultivos, simulando uma fermentação de etanol em usinas industriais.

2. REVISÃO BIBLIOGRÁFICA

2.1. Produção e consumo de energia no Brasil

Atualmente, a economia mundial vem sofrendo importantes modificações no comportamento da aquisição de bens e de consumíveis. Devido ao avanço tecnológico, acesso globalizado aos bens de consumo e, no caso do Brasil, aumento do poder aquisitivo da população em geral, ocorre como consequência o aumento na demanda de produção de energia para suprir todas as necessidades atreladas a essas mudanças.

No Brasil, a produção de energia vem crescendo ao longo dos anos, a qual é proveniente de fontes renováveis e fósseis. No ano de 2011, a energia a partir de fontes renováveis na matriz energética brasileira atingiu 44 % da produção total. Fontes de energia renováveis são provenientes de biomassa de cana, hidrelétrica, vegetal, eólica entre outras menos expressivas. O restante da energia produzida no país (56 %) é proveniente de combustíveis fósseis e dentre estes destacam-se os derivados de petróleo, gás natural e carvão mineral (Ministério de Minas e Energia, 2012).

Um setor que exige grande demanda de energia é o setor de transportes, ficando atrás apenas do setor industrial, consumindo 30 % de toda a energia utilizada no Brasil (Ministério de Minas e Energia, 2012). Os combustíveis derivados de petróleo somam 38 % do consumo total de combustíveis do país e um aumento no consumo de gasolina tem sido observado ano após ano. Embora esses combustíveis sejam os mais viáveis economicamente e mais difundidos, o gargalo no seu uso é o fato de serem parte de

uma reserva natural finita. Além disso, desde a extração até a queima desses combustíveis, ocorre a geração de danos ambientais, entre os quais se destaca o efeito estufa (Archer *et al.*, 2009).

A alternativa de combustível disponível atualmente para a gasolina e que é proveniente de uma fonte renovável é o etanol. Entretanto, o uso do etanol hidratado no setor automotivo, antes estimulado pelo baixo preço, nos últimos anos vem se inviabilizando pela falta de oferta devido ao aumento da produção de açúcar. De todo o consumo de combustíveis no país, apenas uma pequena parcela (4,7 %) vem do etanol. A cultura de cana-de-açúcar, que serve de matéria-prima para a produção desse combustível, continua em expansão no Brasil e as áreas em produção seguem com progressivo aumento nos estados da região Centro-Oeste e Sudeste. A área ocupada por esta cultura na safra 2012/2013 está estimada em 8,5 milhões de hectares e do total de cana-de-açúcar destinada para beneficiamento, 50,5 % gera açúcar e 49,5 % gera etanol (CONAB, 2012). A produção de etanol apresenta como efeitos negativos, além da competição com o setor alimentício diretamente, como por exemplo o açúcar, a disputa pela disponibilidade de terras cultiváveis e também de água que é utilizada para a irrigação (Chen, 2011). Nesse contexto, diversas pesquisas têm se empenhado em desenvolver tecnologias alternativas de geração de energia que reduzam a dependência aos combustíveis fósseis e que sejam disponíveis, renováveis e menos poluentes (Canakci e Sanli, 2008).

Uma alternativa energética é a utilização de biomassa vegetal para a produção de etanol. Mas, para que a substituição das fontes fósseis utilizadas hoje por fontes renováveis seja viável, seria necessária a conversão de toda a planta em fonte de energia, especialmente as partes não utilizadas para alimentação (Chiaramonti *et al.*, 2012). Com

esse objetivo, nos últimos anos as fontes renováveis têm sido amplamente estudadas. A biomassa vegetal é composta por duas classes: uma formada por açúcares de reserva, como o amido e a sacarose, os quais são amplamente utilizados pelas indústrias alimentícias e de bioconversão a etanol. O etanol produzido por esse tipo de fonte é chamado *etanol de primeira geração*. Já as partes estruturais da planta, formadas pela lignocelulose, constituem a segunda classe de biomassa vegetal, que é composta por açúcares mais complexos e de difícil acesso produzindo o *etanol de segunda geração* de grande interesse na atualidade.

2.2. Materiais lignocelulósicos como fonte de matéria-prima para produção de etanol

Os materiais lignocelulósicos compreendem resíduos provenientes de agroindústrias, florestas, tratamento de resíduos sólidos municipais, gramas perenes, entre outros (Balat, 2011). Os maiores componentes da parede celular de plantas são a celulose, a hemicelulose e a lignina, além de extrativos e compostos inorgânicos (Kumar *et al.*, 2008) e a proporção destes componentes varia na composição dependendo da espécie da planta, idade, tempo de colheita e condição ou estágio de crescimento.

A celulose é um homopolímero linear de glicose, que possui em sua extensão cerca de 10.000 unidades glicosídicas formando uma cadeia rígida em forma de fibras. A hemicelulose é um heteropolímero ramificado, com grau de polimerização entre 100 e 200, contendo diferentes carboidratos como xilose, arabinose, manose e glicose, assim como

ácidos urônicos, sendo esta a conexão entre a lignina e as fibras de celulose (Stambuk *et al.*, 2008). A lignina por sua vez é uma macromolécula tridimensional composta basicamente por unidades de fenilpropano. Na parede celular, a lignina está associada às frações celulósicas e hemicelulósicas através de interações físicas e ligações covalentes. Essas interações protegem a planta contra o ataque de microrganismos e conferem coesão à estrutura interna, além de resistência ao esforço mecânico (Hofrichter, 2002). A lignocelulose é o elemento chave da estrutura da planta para conferir resistência aos estresses bióticos e abióticos. Porém, essa característica acaba sendo um gargalo na indústria processadora dessa biomassa (Octave e Thomas, 2009).

O grande acúmulo de resíduos lignocelulósicos provém em grande parte do processamento de culturas agrícolas. No Brasil, a cada ano são produzidas toneladas de resíduos oriundos da cultura de cana-de-açúcar, o chamado bagaço de cana, o qual é massivamente estudado para bioconversão a etanol por grupos de pesquisa do sudeste brasileiro (Rabelo *et al.*, 2009; Cardona *et al.*, 2010).

Estados como o Rio Grande do Sul, que não participam do mercado sucroalcooleiro, não produzem bagaço de cana com grande expressão. Em compensação este estado é o maior produtor de arroz e o terceiro maior produtor de soja do país e consequentemente de resíduos provenientes do processamento desses grãos (CONAB, 2012). Na safra 2012/2013 a produção de soja no Brasil atingiu aproximadamente 82,6 milhões de toneladas em 27,3 milhões de hectares de área plantada, sendo que 15 % da produção ocorreram no Rio Grande do Sul (CONAB, 2012). No cenário mundial, os Estados Unidos são os maiores produtores de soja, seguidos pelo Brasil, Argentina China e Índia (FAO, 2008). A casca do grão de soja representa o maior subproduto das indústrias

processadoras dessa semente e constitui por volta de 8% de todo o grão (Gnanasambandam e Proctor, 1999).

A cultura de arroz é produzida no mundo inteiro, sendo a maior concentração dessa cultura no continente asiático. Neste, 80% da produção total do arroz está concentrada em 7 países, totalizando uma produção de mais de 550 milhões de toneladas (FAO, 2008). No continente asiático, o manejo de resíduos da cultura de arroz mais utilizado é a queimada, processo que contribui para a diminuição da qualidade do ar e o surgimento de doenças respiratórias nas regiões próximas às plantações (Thuy *et al.*, 2008). A produção brasileira, que corresponde a aproximadamente 2 % da produção total mundial, atingiu aproximadamente 12 milhões de toneladas em 2,4 milhões de hectares de área plantada na safra 2012/2013. O Rio Grande do Sul é o maior produtor de arroz no Brasil, sendo responsável por 66,5 % da produção total no país (CONAB, 2012). A casca do grão de arroz compõe em torno de 20% do grão e é o maior subproduto das indústrias processadoras dessa semente, sendo que somente 30% desse resíduo é aproveitado para uso energético nas indústrias e o restante é devolvido de maneira indevida ao ambiente (Walter e Rossato, 2010).

Considerando que a produção nacional de resíduos lignocelulósicos na última safra foi de 6,6 milhões de toneladas para soja e 2,4 milhões de toneladas para arroz, o uso desses resíduos para a produção de bioetanol torna-se atrativo devido ao grande volume gerado anualmente, agregando valor a esses compostos lignocelulósicos e diminuindo o descarte indevido no ambiente.

2.3. Pré-tratamentos de materiais lignocelulósicos

Para disponibilizar os açúcares para posterior fermentação ou aumentar sua susceptibilidade à hidrólise enzimática por celulases, os materiais lignocelulósicos devem ser tratados por processos físicos, químicos ou biológicos (Chiaramonti *et al.*, 2012). Muitas tecnologias para o tratamento da lignocelulose têm sido propostas, geralmente combinando esses processos (Galbe *et al.*, 2007).

Os processos físicos possuem objetivo de aumentar a área de superfície e o tamanho dos poros, além de diminuir a cristalinidade e grau de polimerização da celulose (Taherzadeh e Karimi, 2008). Alguns exemplos desse tipo de processo são os diversos tipos de moagem, radiação por raios gama ou ultrassom.

Os processos físico-químicos são capazes de promover a deslignificação parcial ou total do material e de causar a hidrólise total ou parcial da fração hemicelulósica. Alguns exemplos são explosão com vapor, explosão com vapor adicionada de SO_2, explosão de fibra com amônia, explosão com CO_2, hidrólise alcalina, extração por solventes orgânicos, hidrólise ácida diluída e extração por líquidos iônicos (Galbe *et al.*, 2007; Chiaramonti *et al.*, 2012).

Os processos biológicos podem reduzir o grau de polimerização da celulose ou provocar hidrólise parcial da hemicelulose. Esses processos são realizados por fungos basidiomicetos como *Phanerochaete chrysosporium, Trametes versicolor, Ceriporiopsis subvermispora* e *Pleurotus ostreatus* (Taherzadeh e Karimi, 2008).

Dentre os diversos processos de hidrólise empregados atualmente, a hidrólise ácida diluída é o método mais comumente utilizado e pode ser aplicada tanto como pré-tratamento para hidrólise enzimática, quanto como método de liberação de açúcares para fermentação (Taherzadeh e Karimi, 2008; Chiaramonti *et al.*, 2012). As técnicas de explosão a vapor e hidrólise ácida diluída estão sendo testadas atualmente em escala piloto, entretanto, além da liberação de açúcares, ocorre a liberação de uma grande variedade de compostos, os quais são inibitórios para células de leveduras (Margeot *et al.*, 2009). Embora existam métodos capazes de retirar esses compostos inibitórios dos hidrolisados lignocelulósicos, a utilização deles se torna inviável por se tratarem de métodos de alto custo ou de execução lenta. Os métodos mais conhecidos são troca iônica, tratamento alcalino e uso de carbono ativado (Sánchez e Cardona, 2008). Os principais compostos tóxicos serão abordados na próxima seção.

2.4. Compostos secundários gerados durante a hidrólise ácida

Um importante desafio para transformar a produção de etanol a partir de hidrolisados lignocelulósicos em um processo viável é minimizar a degradação de açúcares e a consequente formação de compostos furânicos e fenólicos (Almeida *et al.*, 2007; Almeida *et al.*, 2008; Margeot *et al.*, 2009; Da Cunha-Pereira *et al.*, 2011). A hidrólise ácida consiste na adição de ácido concentrado ou diluído à biomassa e aplicação de altas temperaturas. Dependendo da condição aplicada, o processo pode demorar alguns minutos ou horas (Brodeur *et al.*, 2011).

Durante a hidrólise ácida diluída, dependendo das condições empregadas, são gerados produtos de degradação dos açúcares e da lignina que inibem o metabolismo dos microrganismos, os quais serão empregados como agentes na fermentação. Os açúcares podem ser degradados em compostos furânicos e estes em ácidos fracos, como acético, fórmico e levulínico. Nessas condições, ocorre também a desacetilação da hemicelulose, que gera ácido acético. A partir da quebra da lignina, são gerados ácidos siríngico, vanílico e palmítico e compostos fenólicos (Almeida *et al.*, 2007).

2.4.1. Furaldeídos

Quimicamente, os furaldeídos são compostos por um anel furânico e um grupo aldeído funcional. Os principais inibidores de metabolismo são furfural, que é formado pela desidratação das pentoses e o 5-hidroximetilfurfural (HMF), que é formado pela desidratação das hexoses (Mansilla *et al.*, 1998; Liu *et al.*, 2009; Lenihan *et al.*, 2010).

A reação de desidratação dos açúcares e formação dos compostos furânicos é mostrada da Figura 1.

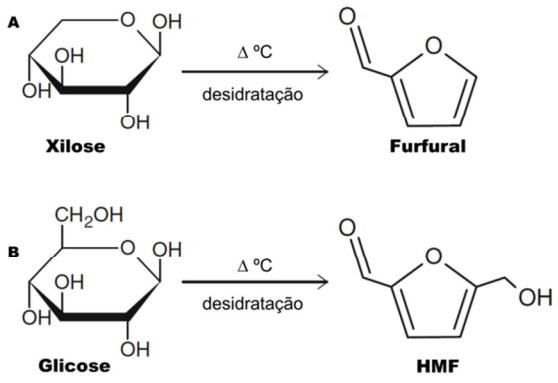

Figura 1. Representação esquemática da reação de desidratação de uma pentose para formação de furfural (A) e de uma hexose para formação de HMF (B). Figura adaptada de Almeida *et al*., (2009).

Compostos químicos que possuem o grupo aldeído podem causar algumas consequências em células eucarióticas. Esses aldeídos reativos em células causam danos oxidativos que podem levar a eventos apoptóticos, assim como causar doenças cardiovasculares e doença de Alzheimer (Almeida *et al.*, 2009).

Em leveduras, a toxicidade dos furaldeídos é detectada sobre o metabolismo, aumentando a fase lag de crescimento, e também sobre a produção de metabólitos. Esses efeitos aumentam de acordo com o aumento da concentração do furaldeído (Liu *et al.*, 2004; Nilsson *et al.*, 2005). Durante o processo de produção de hidrolisados lignocelulósicos, a concentração desses compostos varia de acordo com a composição do material lignocelulósico. Altas concentrações de furaldeídos são geradas, sendo que a concentração de furfural pode alcançar até 5 g L^{-1} na hidrólise de bagaço de cana-de-açúcar (Aguilar *et al.*, 2002), enquanto que HMF pode ser acumulado em até 6 g L^{-1} em hidrolisados de madeira lascada de *Picea abies* (Larsson *et al.*, 1999).

Em geral, os efeitos tóxicos dos furaldeídos podem ser atribuídos à mudança no metabolismo celular, que é direcionado para a reparação dos danos causados por eles, acarretando na redução dos níveis intracelulares de ATP e NAD(P)H. Esses danos podem ser causados pela inibição enzimática ou pelo desequilíbrio no consumo e regeneração dos cofatores (Almeida *et al.*, 2007). Os furaldeídos interferem na produção e atividade de enzimas glicolíticas como hexoquinase, gliceraldeído-3-fosfato desidrogenase, álcool desidrogenase, aldeído desidrogenase e piruvato desidrogenase, assim como na síntese de

proteínas e de RNA (Banerjee *et al.*, 1981; Sanchez e Bautista, 1988; Taherzadeh *et al.*, 1997; Luo *et al.*, 2002; Modig *et al.*, 2002; Liu, 2006)

Devido à sua abundância em diversos hidrolisados lignocelulósicos, furfural tem sido mais amplamente estudado em comparação com HMF. Entretanto, há diferenças importantes entre os efeitos de furfural e de HMF sobre as células de leveduras.

Avaliações dos efeitos tóxicos de furfural sobre o metabolismo de leveduras já foram relatadas em diversas espécies tais como *S. cerevisiae, Kluyveromyces marxianus, P. stipitis, Trichosporon fermentans*, *C. guilliermondii* e *Pachysolen tannophilus* (Tran e Chambers, 1986; Sanchez e Bautista, 1988; Horvath *et al.*, 2003; Zhao *et al.*, 2005; Oliva *et al.*, 2006; Huang *et al.*, 2012). O furfural causa efeitos imediatos sobre o crescimento de microrganismos. Sua capacidade de causar mutações ao DNA é bem estabelecida (Almeida *et al.*, 2009). A adição desse composto a um cultivo de leveduras tem um efeito imediato e drástico sobre o comportamento fisiológico das mesmas, pois na tentativa de diminuir os efeitos tóxicos do furfural ocorre a diminuição da produção de biomassa, consumo de glicose, produção de etanol e glicerol (Lin *et al.*, 2009). Análises de fluxo metabólico mostraram que o furfural afeta a via glicolítica e o ciclo do ácido cítrico (Horvath *et al.*, 2003). Também foi observado que este composto gera espécies reativas de oxigênio, cujos efeitos são danos às membranas mitocondrial e vacuolar, danos à actina e também à cromatina (Allen *et al.*, 2010). A principal estratégia utilizada pelos microrganismos para diminuir a toxicidade do furfural é transformá-lo no seu álcool correspondente, por ser menos tóxico. Os compostos mais comumente encontrados na degradação do furfural são o álcool furfurílico, em condições anaeróbicas e de microaerofilia e o ácido furóico, em condições aeróbicas (Horvath *et al.*, 2003).

Especificamente sobre o HMF, algumas espécies de leveduras já foram avaliadas quanto aos efeitos deste sobre o crescimento e produção de etanol, tais como: *S. cerevisiae, C. guilliermondii* e *P. stipitis* (Taherzadeh *et al.*, 2000; Liu *et al.*, 2004; Keating *et al.*, 2006). A toxicidade do HMF está associada a danos em células de leveduras, já que a presença do grupo hidroximetil confere ao composto a característica de baixa hidrofobicidade e de permeabilidade à membrana, que acarreta em baixas taxas de conversão do composto (Taherzadeh *et al.*, 2000). Entretanto, seus efeitos tóxicos sobre o DNA não estão comprovados (Almeida *et al.*, 2009). A redução deste furaldeído para o álcool correspondente gera como principal produto 2,5-bis-hidroximetilfurano (álcool hidroximetilfurfurílico) em condições anaeróbicas (Liu *et al.*, 2004; Almeida *et al.*, 2007). O composto ácido carboxílico-5-hidroximetilfurano também já foi identificado (Taherzadeh *et al.*, 2000).

Quando uma combinação de furfural e HMF estão presentes em um meio de cultivo, sua toxicidade fica potencializada se comparada com os efeitos que os compostos apresentam separadamente. Esse fenômeno é chamado de efeito sinergístico (Mussatto e Roberto, 2004). Esse comportamento foi observado em duas linhagens de *S. cerevisiae* e uma linhagem de *P. stipitis*. Em todas as linhagens avaliadas, houve produção de biomassa em 30 mM de cada composto, entretanto, quando combinados, produção de biomassa foi observada apenas na concentração de 10 mM (Liu *et al.*, 2004; Mussatto e Roberto, 2004). Em outro estudo, no qual os efeitos de furfural e ácido acético foram avaliados, a interação desses dois compostos diminuiu a taxa de crescimento específica, rendimento de etanol e biomassa em *S. cerevisiae* (Palmqvist *et al.*, 1999).

2.4.2. Pressão osmótica

Após o processo de hidrólise, etapas adicionais de processamento são aplicadas aos hidrolisados lignocelulósicos. Após a retirada da fração sólida, o hidrolisado recuperado é concentrado por evaporação, com o objetivo de aumentar a concentração dos açúcares presentes, e posterior correção do pH, para que o hidrolisado possua condições próprias para fermentação. Esse processo acumula todos os compostos formados, além de gerar grandes quantidades de sais a partir da correção do pH. A consequência desses processos é o aumento da pressão osmótica do meio (Zhao e Bai, 2009). Em *S. cerevisiae*, o metabolismo em meio hiperosmótico é afetado pela diminuição da taxa de multiplicação celular, bem como mudanças na permeabilidade da membrana plasmática (Claro *et al.*, 2007).Uma medida utilizada pela célula para se adaptar a essa condição é a síntese de osmólitos. Osmólitos são grupos de solutos de baixo peso molecular envolvidos na estabilização protéica, em resposta a condições ambientais de estresse (Viana *et al.*, 2005), sendo que *S. cerevisiae* sintetiza principalmente o glicerol (Garay-Arroyo e Covarrubias, 1999).

2.5. O processo de produção de etanol

A produção de etanol pode ser realizada por duas vias. A via química, que consiste na síntese a partir do etileno (Pereira e De Andrade, 1998), ou por via biológica, que ocorre pela fermentação de açúcares, que é realizada por microrganismos. A última via é a principal utilizada atualmente.

A obtenção de etanol por processos fermentativos pode ser realizada por bactérias ou por leveduras e utilizando diversos açúcares que podem ser metabolizados e convertidos a etanol. Açúcares como glicose, frutose, manose, xilose e arabinose podem ser convertidos a etanol. A espécie bacteriana mais conhecida por possuir capacidade de produzir etanol é *Zymomonas mobilis* (Fu *et al.*, 2009). Além disso, o potencial da espécie *Klebsiella pneumoniae* para produção de etanol também tem sido estudado (Rossi *et al.*, 2012). Entretanto, melhores produtividades são obtidas por leveduras.

A levedura *Saccharomyces cerevisiae* é o microrganismo que apresenta maior eficiência na produção de etanol. Além desse emprego, essa levedura possui uma ampla diversidade de aplicações, como por exemplo, a produção de bebidas alcoólicas. (Hirasawa *et al.*, 2010). Apesar de todas as aplicações biotecnológicas de *S. cerevisiae*, essa levedura é capaz de metabolizar apenas hexoses e alguns dissacarídeos, como sacarose. Devido a essa característica, a utilização dessa levedura em alguns processos fica limitada.

Outras leveduras com potencial para a produção de etanol têm sido estudadas, devido a suas características fisiológicas específicas. A capacidade de metabolizar pentoses, tais como xilose, tem sido muito estudada. As espécies *Candida tropicalis*, *C. guilliermondii*, *Candida shehatae*, *Pichia stipitis* e *Pachysolen tannophylus* têm sido amplamente estudadas devido a essa característica (Schirmer-Michel *et al.*, 2008; Zhao *et al.*, 2008; Oberoi *et al.*, 2010; Yadav *et al.*, 2011). Recentemente, a levedura *Spathaspora arborariae* foi identificada e também está sendo avaliada para a produção de etanol a partir de pentoses (Cadete *et al.*, 2009; Da Cunha-Pereira *et al.*, 2011). *Wickerhamomyces anomalus*, antes denominada *Pichia anomala*, também possui características interessantes para a produção de etanol. Entre elas estão resistência a etanol, resistência à pressão

osmótica e capacidade de assimilar diversos açúcares (Passoth *et al.*, 2006; Tao *et al.*, 2011)

Em leveduras, dependendo do açúcar disponível, a via de produção de etanol acontece por duas diferentes rotas bioquímicas. A partir de glicose, frutose, manose, galactose ou sacarose ocorre a formação de piruvato pela glicólise. O piruvato, por sua vez, é convertido a acetaldeído, que gera etanol. Esse processo é anaeróbico (Figura 2).

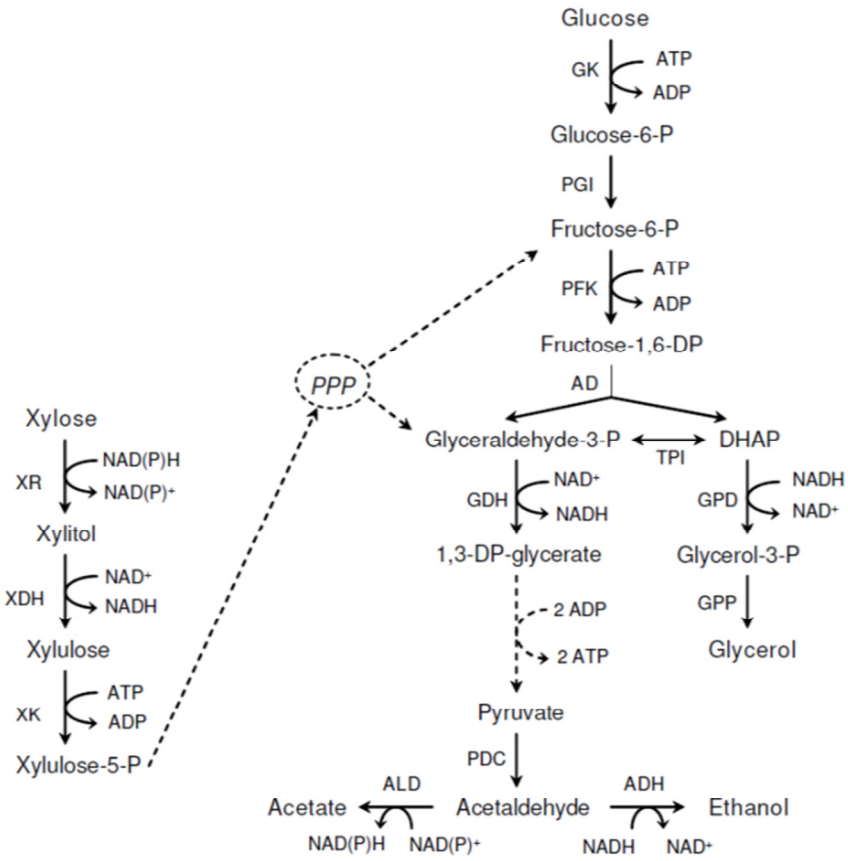

Figura 2: Representação esquemática da conversão de açúcares a etanol em leveduras (Olofsson *et al.*, 2008).

A xilose, por sua vez, é metabolizada na via das pentoses fosfato em duas etapas de oxidação-redução, com formação sequencial de xilitol e xilulose (Figura 2). A

xilulose é convertida a xilulose-5-fosfato e depois a gliceraldeído-3-fosfato, quando participa da via glicolítica (Kuhad *et al.*, 2011). As leveduras fermentadoras de pentoses normalmente são menos tolerantes a baixos valores de pH, altas concentrações de etanol e de inibidores, quando comparadas com *S. cerevisiae* (Kuhad *et al.*, 2011). Além disso, esta via necessita de pequenas quantidades de oxigênio, devido à dependência dos cofatores NAD^+ e NAD(P)H. Ocorre um desequilíbrio nas reações de oxidação-redução das enzimas que convertem xilitol e xilulose. Em condições anaeróbicas, a via das pentoses não é capaz de metabolizar xilose (Johansson e Hahn-Hagerdal, 2002). Além disso, as leveduras tendem a produzir xilitol a partir de xilose por ser uma via mais rápida de obtenção de energia. Como consequência, a produção de etanol é desviada para a produção de xilitol (Schirmer-Michel *et al.*, 2008). O xilitol, por sua vez, também possui diversas aplicações biotecnológicas e pode ser produzido a partir de leveduras assimiladoras de pentoses.

2.6. Estratégias para otimização da produção de etanol a partir de pentoses

Para que o processo de produção de etanol de segunda geração seja viável economicamente, o microrganismo ideal deveria utilizar uma grande diversidade de açúcares, como as hexoses glicose, manose e galactose, além das pentoses xilose e arabinose, convertê-los em altas taxas de rendimento e produtividade de etanol, possuir a habilidade de resistir a altas concentrações de etanol e altas temperaturas, além de ser tolerante aos inibidores presentes nos hidrolisados (ácidos fracos, furaldeídos e fenóis) e possuir atividade celulolítica (Sarkar *et al.*, 2012). Entretanto, até o momento, um único

microrganismo possuidor de todas essas características ainda não foi isolado do meio ambiente, tampouco caracterizado ou obtido por técnicas de melhoramento genético.

Com esse objetivo, grupos de pesquisas em diversos países têm avançado em muitos estudos, os quais envolvem, por exemplo, construção de linhagens *de S. cerevisiae* com capacidade de converter pentoses a etanol (Bettiga *et al.*, 2008), avaliação dos efeitos fisiológicos de compostos tóxicos, bem como mecanismos de resistência utilizados por leveduras (Taherzadeh *et al.*, 2000; Keating *et al.*, 2006; Liu *et al.*, 2008; Da Cunha-Pereira *et al.*, 2011) e estudos de co-culturas de leveduras, ou de leveduras e bactérias, livres e imobilizadas (Fu e Peiris, 2008; Fu *et al.*, 2009).

2.6.1. Melhoramento genético para fermentação de xilose

A levedura *S. cerevisiae* é uma excelente produtora de etanol, entretanto, não possui a capacidade de metabolizar vários açúcares. Devido a esse fator limitante, diversas estratégias têm sido utilizadas para contornar esse problema.

A modificação genética de *S. cerevisiae* que possibilita a metabolização de xilose é uma estratégia muito utilizada. Entre as possíveis vias de metabolização da xilose, há a expressão do gene da xilose isomerase de bactérias, que é capaz de converter xilose diretamente em xilulose. Dentre diversas estratégias utilizadas com esse fim, foi possível expressar apenas o gene da xilose isomerase de *Clostridium phytofermentans* em *S. cerevisiae* (Brat *et al.*, 2009). Embora a expressão heteróloga do gene de xilose isomerase do fungo *Piromyces* sp. tenha apresentado atividade enzimática em *S. cerevisiae*, foi

observado pouco crescimento na presença de xilose (Kuyper *et al.*, 2003). Estudos têm sido realizados no entendimento da estrutura da enzima para avançar nesse campo (Jeffries e Jin, 2004; Brat *et al.*, 2009). As enzimas xilose redutase e xilulose desidrogenase são importantes na via de metabolização de xilose, além da xiluloquinase. A expressão heteróloga dessas enzimas provenientes da levedura *P. stipitis* em *S. cerevisiae* possibilita a fermentação de xilose. Entretanto, a diferença na preferência de cofatores das enzimas altera e tranca o fluxo metabólico de xilose a xilulose (Karhumaa *et al.*, 2007).

2.6.2. Co-cultivo para a produção de etanol a partir de glicose e xilose

A utilização de microrganismos com diferentes capacidades fermentativas no mesmo cultivo está sendo utilizada como uma estratégia para a conversão total de açúcares a etanol. Resultados promissores têm sido observados em sistemas em batelada utilizando co-cultivo de células livres das leveduras *P. stipitis* e *K. marxianus* (Qian *et al.*, 2006; Rouhollah *et al.*, 2007); *S. cerevisiae* e *P. tannophilus* (Qian *et al.*, 2006); cultura contínua com co-cultivo de células livres de *Saccharomyces diastaticus* e *P. stipitis* (Delgenes *et al.*, 1996).

Outra estratégia que está sendo explorada para a produção de etanol é a técnica de imobilização de células. Essa possui algumas vantagens em bioprocessos, já que o uso de suportes inorgânicos está relacionados com o aumento da produtividade de etanol em leveduras e bactérias, além de ser uma técnica relativamente simples e barata (Kuhad *et al.*, 2011). Resultados demonstram que células imobilizadas possuem, em comparação com células livres, maior tolerância a pressão osmótica, temperatura, ampla faixa de pH e maior produção de etanol (Banat *et al.*, 1998). Estudos já foram realizados em células de *S.*

cerevisiae e *P. stipitis* imobilizadas no mesmo suporte inorgânico, demonstrando aumento da produção de etanol e altas taxas de conversão dos açúcares para o álcool (De Bari *et al.*, 2004). Um outro estudo envolveu o co-cultivo de células imobilizadas da bactéria *Zymomonas mobilis* e de células livres da levedura *P. stipitis*. Os resultados mostraram que o co-cultivo apresentou uma eficiência de conversão de glicose e xilose a etanol de quase 100 %, indicando um avanço na pesquisa em co-cultivos (Fu *et al.*, 2009).

Assim, as fermentações em sistemas de co-cultivos otimizados possibilitariam a conversão simultânea de glicose e xilose, a maximização da taxa de utilização de substrato, aumento do rendimento de etanol, além de reduzir custos do processo. Entretanto, segundo Chen (2011) muitos estudos para otimizar este bioprocesso devem ser realizados para que essa alternativa se torne viável.

2.7. Mecanismos de tolerância de *Saccharomyces cerevisiae* ao HMF

Em uma planta de produção de etanol industrial, a situação desejável seria de que as linhagens caracterizadas como resistentes a furfural ou HMF apresentassem as mesmas produtividades de etanol, tanto na presença quanto na ausência dos furaldeídos. Entretanto, até o momento, após extensiva busca bibliográfica, não foram encontradas publicações citando tais linhagens, embora alguns grupos de pesquisa tenham se empenhado em elucidar os mecanismos de toxicidade desses compostos, e as respostas de leveduras sobre eles. Em um estudo utilizando análise de transcrição do genoma na linhagem TMB3000 de *S. cerevisiae*, que possui tolerância a tóxicos de hidrolisados lignocelulósicos, foi

observado que os genes *ADH2, ADH6* e *SFA* apresentaram aumento nos níveis de expressão na presença de HMF. O gene *ADH6* foi superexpresso em uma linhagem laboratorial e os resultados mostraram que o HMF foi reduzido pela proteína Adh6p, utilizando NADPH como cofator (Petersson *et al.*, 2006). Também foi demonstrado que clones de leveduras superexpressando os genes *ADH6* e *ADH7* foram capazes de crescer na presença de HMF (Liu *et al.*, 2008). Recentemente, uma enzima aldeído redutase dependente do cofator NADPH, que é codificada pelo gene *ARI1*, apresentou atividades sobre pelo menos 14 substratos, incluindo HMF (Liu e Moon, 2009). Essa dependência de NADPH induz uma mudança na programação do metabolismo central de leveduras pelo aumento do fluxo em direção à via das pentoses-fosfato na presença de furfural e HMF (Liu *et al.*, 2009; Liu, 2011)

2.8. Engenharia evolutiva como estratégia para o aumento de resistência a furfural e HMF em *Saccharomyces cerevisiae*

A engenharia evolutiva é uma estratégia eficaz, que tem sido utilizada para aumentar ou modificar características de interesse em bioprocessos, incluindo a resistência a furaldeídos em leveduras. A estratégia baseia-se em ciclos repetidos de mutagênese ou recombinação seguidos pela seleção do fenótipo desejado (Petri e Schmidt-Dannert, 2004). A tolerância ao furfural e a hidrolisados lignocelulósicos em geral já foi descrita na linhagem TMB3400 de *S. cerevisiae*, a qual apresentou aumento da resistência ao furfural após 300 gerações de adaptação na presença desse composto (Heer e Sauer, 2008). A tolerância ao HMF e furfural foi aumentada em *P. stipitis* NRRL Y-7124 e em *S.*

cerevisiae NRRL Y-12632, usando um método de adaptação direcionada que consistiu em aumentar a concentração de compostos tóxicos e uma posterior seleção após 100 re-inoculações para se obter uma população estável. As linhagens adaptadas mostraram um aumento na habilidade de reduzir HMF e furfural, além de atingirem a fase estacionária de crescimento antes da linhagem referência (Liu *et al.*, 2005).

3. OBJETIVOS

3.1. Objetivo geral

O presente trabalho teve como objetivos selecionar e estudar a resistência de duas leveduras etanologênicas a compostos furânicos, investigar a fisiologia das mesmas na presença desses compostos e avaliar a capacidade de produção de etanol de segunda geração em hidrolisados lignocelulósicos.

3.2. Objetivos específicos

Avaliar linhagens industriais de *Saccharomyces cerevisiae* isoladas de melaço ou bagaço de cana quanto à sua resistência ao HMF;

Selecionar linhagens de *S. cerevisiae* resistentes a altas concentrações de HMF por meio da estratégia de engenharia evolutiva;

Avaliar a expressão de genes envolvidos na resistência ao HMF e relacionar essa expressão com respostas fisiológicas de *S. cerevisiae* na presença desse composto tóxico;

Selecionar uma linhagem da levedura metabolizadora de pentoses *Wickerhamomyces anomalus* resistente a altas concentrações de furfural e de HMF por meio de engenharia evolutiva;

Realizar cinética de crescimento e de produção de etanol de *W. anomalus* na presença desses compostos tóxicos e relacionar a resistência com a produção de enzimas álcool desidrogenases;

Avaliar a capacidade fermentadora dessas duas leveduras em hidrolisados lignocelulósicos de casca de soja e casca de arroz, comparando a produção de etanol nas culturas puras e em sistemas de co-cultivo em cultivo submerso.

4. CAPÍTULOS

4.1. CAPÍTULO I – 5-hydroxymethylfurfural induces *ADH7* and *ARI1* expression in tolerant industrial *Saccharomyces cerevisiae* strain P6H9 during bioethanol production

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ABSTRACT

The aims of this work were to obtain, by evolutionary engineering, an industrial strain of *Saccharomyces cerevisiae* tolerant to high concentrations of HMF and to determine the expression levels of genes previously described as responsible for this tolerance. Cells were grown under anaerobic and oxygen limited conditions, in the presence of glucose or sucrose as carbon sources. P6H9 strain presented high expression levels for genes *ADH7* and *ARI1* in presence of HMF. This tolerant strain also showed higher ethanol productivity, biomass formation and alcohol dehydrogenase activity comparing to sensitive strains. Results suggest that *S. cerevisiae* P6H9 strain presents potential to be used for second-generation ethanol production.

Keywords: Bioethanol; *Saccharomyces cerevisiae*; 5-hydroxymethylfurfural tolerance; gene expression.

1. INTRODUCTION

The development of second-generation bioethanol from lignocellulosic biomass has become widely important and is attracting global attention, since it is a renewable energy source and does not compete for areas designated for food production (Menon and Rao, 2012). The production of bioethanol from lignocellulosic feedstock requires pretreatments and hydrolyses steps to release sugars for yeast fermentation. Steam explosion and dilute-acid hydrolysis are already in use at pilot scale, but these technologies release, in addition

to sugars, a wide range of compounds, which are inhibitory of yeasts metabolism. Therefore, the main challenge to turn this into an economically feasible process, is to minimize sugar degradation and the formation of furanic and phenolic compounds (Almeida et al., 2008; Margeot et al., 2009).

The main yeast metabolism inhibitors are the 2-furaldehyde (furfural), formed by pentoses dehydration, and 5-hydroxymethyl-2-furaldehyde (5-hydroxymethylfurfural - HMF), which is formed by hexoses dehydration (Liu et al., 2009). The generation of furfural during sugarcane bagasse acid hydrolysis can reach up to 5 g L^{-1}, (Aguilar et al., 2002), while HMF can accumulate to 6 g L^{-1} in hydrolysates from chipped pine wood (Larsson et al., 1999). Furans interfere with microbial growth and subsequent fermentation, interfering with glucolytic enzymes such as triose phosphate dehydrogenase, alcohol dehydrogenase, aldehyde dehydrogenase, and pyruvate dehydrogenase, as well as with protein and RNA synthesis (Modig et al., 2002; Sanchez and Bautista, 1988). In general, the effects of furans can be explained by a re-direction of cell flux energy to damage repairing caused by these compounds and by reduced intracellular ATP and NAD(P)H levels, caused by enzymatic inhibition or consumption/regeneration of cofactors (Almeida et al., 2007).

On the other hand, HMF toxicity is associated with yeast cell damages, since the presence of its hydroxymethyl group leads to a reduced hydrophobicity and membrane permeability, which causes a low conversion rate of this compound (Taherzadeh et al., 2000). In *S. cerevisiae*, HMF toxicity was reported to be dose-dependent (Liu et al., 2004). The reduction of the aldehyde into a less toxic corresponding alcohol is a common detoxification strategy, which is mainly catalyzed by alcohol dehydrogenases. The main product described from HMF conversion in *S. cerevisiae* is 2,5-bis-hydroxymethylfuran

(HMF alcohol) under aerobic and anaerobic conditions (Almeida et al., 2007; Liu et al., 2004). The compound 5-hydroxymethyl furan carboxylic acid (HMFCA) has also been identified (Taherzadeh et al., 2000).

Evolutionary engineering is an efficient strategy, which has been used to increase or modify characteristics of interest in bioprocesses, including resistance to furaldehydes by yeasts. Tolerance towards furfural, and to lignocellulosic hydrolysates in general, was described for *S. cerevisiae* strain TMB3400, which showed increased resistance to furfural after 300 generations of adaptation in the presence of this toxic (Heer and Sauer, 2008). Tolerance towards HMF and furfural was also improved for *Pichia. stipitis* strain NRRL Y-7124 and for *S. cerevisiae* strain NRRL Y-12632, using a directed adaptation method that consisted of increasing the toxics concentration and posterior selection after 100 re-inoculations to obtain a stable population. Adapted strains presented enhanced biotransformation ability to reduce HMF and furfural, and reach growth stationary phase earlier than the control (Liu et al., 2005).

It would be desirable that, in an industrial ethanol production, strains characterized as HMF resistant should have same ethanol productivities, either in the presence or absence of this aldehyde; so far, however, there has been no reports as such strains. Petersson et al. (2006) used genome-wide transcription analysis in the TMB3000 *S. cerevisiae* strain, which is known to tolerate inhibiting lignocellulose hydrolysates, and observed that *ADH2, ADH6* and *SFA1* genes presented increased expressions levels. *ADH6* gene was overexpressed in a laboratory strain, and results showed that HMF was reduced by Adh6p using NADPH as co-factor (Petersson et al., 2006). It has also been demonstrated that yeast clones overexpressing *ADH6* and *ADH7* genes were able to grow in the presence of HMF (Liu et al., 2008). Recently, the *ARI1* gene, coding for an NADPH-

dependent aldehyde reductase from *S. cerevisiae*, was identified as presenting reduction activities towards at least 14 aldehyde substrates, including HMF (Liu and Moon, 2009). This dependence of NADPH induces a reprogramming of the yeast central metabolism by increasing the metabolic flux towards the pentose phosphate pathway in the presence HMF (Liu, 2011; Liu et al., 2009).

In the present study, four industrial strains of *S. cerevisiae* were evaluated regarding HMF tolerance. Evolutionary engineering was then applied to one strain aiming at increasing its tolerance towards HMF, Expression induction in the presence of HMF of four genes reported to be involved in its tolerance (*ADH6*, *SFA1*, *ADH7*, *ARI1*) were studied in anaerobic and oxygen limitation conditions, using either glucose or sucrose as carbon sources. Kinetics of yeasts cultures were conducted to compare the metabolisms of resistant and non-resistant strains in the presence of HMF.

2. Materials and Methods

2.1. Microorganisms and cell maintenance

The strains used in this study were *S. cerevisiae* P6, P18, and JP1 isolated from an ethanol production plant in Brazil (da Silva-Filho et al., 2005). HMF tolerant derivative strains P18R and JP1R were previously isolated by successive platting of their parental on YPD supplemented with 5 g L^{-1} HMF. The laboratory haploid strain BY4741 was used as reference. Yeasts were kept frozen at -20 ^{o}C in stock cultures of 20 % glycerol and 80 % of yeast culture (volume fraction).

2.2 Media and cultivation conditions

Oxygen limitation cultivations were performed in YPD medium (containing, in g L^{-1}: glucose, 20; yeast extract, 10; and peptone, 20) supplemented with HMF as indicated. Cultures were carried out in 250 mL flasks containing 60 mL of medium YPD (for item 2.3) or in 125 mL Erlenmeyer flasks containing 30 mL of medium (for item 2.4) at 30 ºC and 150 rpm on a rotatory shaker. Initial inoculum of 0.1 OD (600 nm) was standardized. Fermentation assays used YPS (sucrose at 120 g L^{-1} instead of glucose) supplemented with HMF as indicated. Yeast cells were collected by centrifugation in graduated conical tubes until the equivalent of 5 mL corresponding to 10 % of inoculum (mass fraction, initial wet cell weight) and then the tubes were filled to 50 mL with YPS. Fermentations were carried out at 30 °C without agitation and samples were collected at defined time periods for determination of glucose, fructose, sucrose, ethanol, glycerol, acetate, and HMF concentration, and for biomass production.

2.3. Selection of strains and evolutionary engineering for HMF resistance

Yeast strains JP1, P6, and P18R were cultivated in YPD medium in the presence (4 g L^{-1}) or absence of HMF for 30 h and samples were taken at defined intervals for growth determination. Evolutionary engineering was used for further increment of the HMF resistance. Cells of JP1, P6, and P18R strains were submitted to an adaptation process by inoculating (OD of 0.1, 600 nm, 10 % volume fraction) them in YPD medium with increments of HMF concentration of 0.5 g L^{-1} each batch, for 48 h at 30 ºC. This procedure

was repeated successive times until final cell biomass reached high and constant value. Samples of each culture cycle were stored on glycerol 20 % at -18 ºC.

2.4. Gene expression analysis

2.4.1. Cell treatments for gene expression analysis

The expression of genes previously described as responsible for HMF resistance were compared between treated and untreated cells grown under oxygen limitation condition and anaerobically to test the effects of physiology (see item 2.2). Cells were harvested at suitable intervals for analysis. They were centrifuged at 13 000 *g* for 5 min, 4 °C and frozen at -18 °C for RNA extraction.

2.4.2. RNA extraction, cDNA synthesis and primer design

Yeast total RNA was isolated using NucleoSpin® RNA II kit following manufacturer's instructions (Macherey-Nagel, USA). RNA was quantified using spectrophotometric method (Nanovue, GE HealthCare). For cDNA synthesis, 1 µg of total RNA was used using ImProm-II™ Reverse Transcription System Promega II kit (Promega, USA). Different *S. cerevisiae* genes were used as reference according to validation experiments (Teste et al., 2009): for oxygen limitation cultivations (*TAF10* and *UBC6* for P6H9 strain; *TFC1* and *UBC6* for JP1 strain; *ALG9* and *TFC1* for BY4741 strain); and for anaerobic

fermentation (*TAF10* and *UBC6* for all strains). Nucleotide sequences of *ADH6*, *ADH7*, *SFA1*, and *ARI1* genes are available at *Saccharomyces cerevisiae* Genome Database (http://www.yeastgenome.org/). Primers were designed using the software Primer Express (Applied Biosystems, Foster City, USA) and are described in Table 1.

Table 1. Primers used for gene expression analyses.

Primer	Sequence (5'-3')
ADH6F	AGTGGGTTGAAAGTCGGTCAA
ADH6R	CAACGGTCACATTCCAAGCAT
ADH5F	GCTGCGGGACTGTAGGACTCT
ADH5R	TGTCAATGGCAATGATCTTGG
ADH7F	GCAAAGGATTGGAAGCATCC
ADH7R	CGCAGATACCACAGGCTTCA
ARI1F	GCCCATTTATTGACGTGCGT
ARI1R	TTGGCCGGTACATTCTGGTT

2.4.3. Real Time RT-PCR Assays

Real-time PCR assays were performed using SYBR Green PCR Master Mix (Applied Biosystems, USA) and the parameters were previously reported (Elsztein et al., 2011). Temperature-time profile (95 °C for 10 min and 40 cycles of 95 °C for 15 sec and 60 °C for 1 min) was optimized for ABI Prism 7300 (Applied Biosystems). Amplification curves were analyzed with software SDS v.2.0 (Applied Biosystems). Negative PCR control (no template) and negative RT control (RNA not reverse-transcribed to cDNA) were run as

internal controls. All samples were run in technical triplicates for each biological duplicate of cell cultivation. Normalization was performed for all genes at once using geNorm applet (Vandesompele et al., 2002), thus minimizing errors due to shifts in a single reference gene (Elsztein et al., 2011); the optimal number of reference genes to be used was also determined after submitting the raw data to the geNorm tool (http://medgen.ugent.be/genorm).

2.5. Analytical methods and kinetic parameters calculation

Growth rate of yeast cultures were measured as optical density (OD) at 600 nm. Cultivations were run for 48 h and samples were taken every 6 h. Cultures were centrifuged at 3 500 *g*, and cells were kept on -18 °C for further enzymatic activity assays. Yeast biomass was determined using a standard curve correlating the OD and cell dry weight (g L^{-1}). Glucose, glycerol, ethanol, and acetate concentrations were determined by HPLC (Shimadzu, Japan) equipped with a refractive index detector and Bio-Rad HPX-87H column (300 × 7.8 mm) using 5 mM sulfuric acid as eluent at 45 °C, flow rate of 0.6 mL min^{-1} and sample volumes of 20 µL. HMF concentration was determined by HPLC with a UV detector (at 276 nm) using a Nucleosil C18 column (250 × 4.6 mm) at room temperature, using acetonitrile–water (2:8) containing 10 g L^{-1} acetic acid as eluent, flow rate of 1.1 mL min^{-1} and sample volumes of 20 µL. The ethanol conversion yield ($Y_{P/S}$, g g^{-1}) was defined as the ratio of the concentration of ethanol produced and glucose consumed. The volumetric productivity (Q_p, g L^{-1} h^{-1}) was calculated using the ethanol production versus time.

2.6. Enzyme activity assay

Crude protein extracts were prepared using the glass beads method. Cells were resuspended in 400 μL potassium phosphate buffer 100 mM, pH 7.0, and 2 μL of a solution of 100 mM PMSF with an equal volume of glass beads (450 × 500 μm). Cells were disrupted by vortexing six times for 60 s, and the samples were cooled on ice for 60 s in between the vortex steps. Protein extracts were collected by centrifugation at 13 000 *g* for 5 min at 4 °C and the concentration was determined using Lowry assay method. Alcohol dehydrogenase activity was monitored by recording decreased absorbance at 340 nm using NADPH as cofactor. The reaction mixture consisted of a final concentration of 10 mM HMF substrate and 100 μM of NADPH in 100 mM potassium phosphate buffer, pH 7.0. All reagents were maintained at 25 °C prior to use. Assays were carried out in a volume of 1 ml at 25 °C for 1 min. The protein samples were kept on ice. To start the reaction, 25 μl of crude extract protein was added to the reaction mix. The NADPH molar absorption coefficient was 6.22 mM^{-1} cm^{-1}.

3. RESULTS AND DISCUSSION

3.1. Selection of industrial strains with HMF tolerance

Four *S. cerevisiae* strains were tested for their tolerance to HMF under oxygen limitation metabolism. Figure 1 A shows the extended lag growth phase for all strains when in the presence of HMF, with P18R strain showing the highest biomass production at the end of

cultivation. Although all strains showed the ability to reduce HMF concentration of media, P18R and P6 were able to completely deplete the toxic at the end of run (Figure 1B).

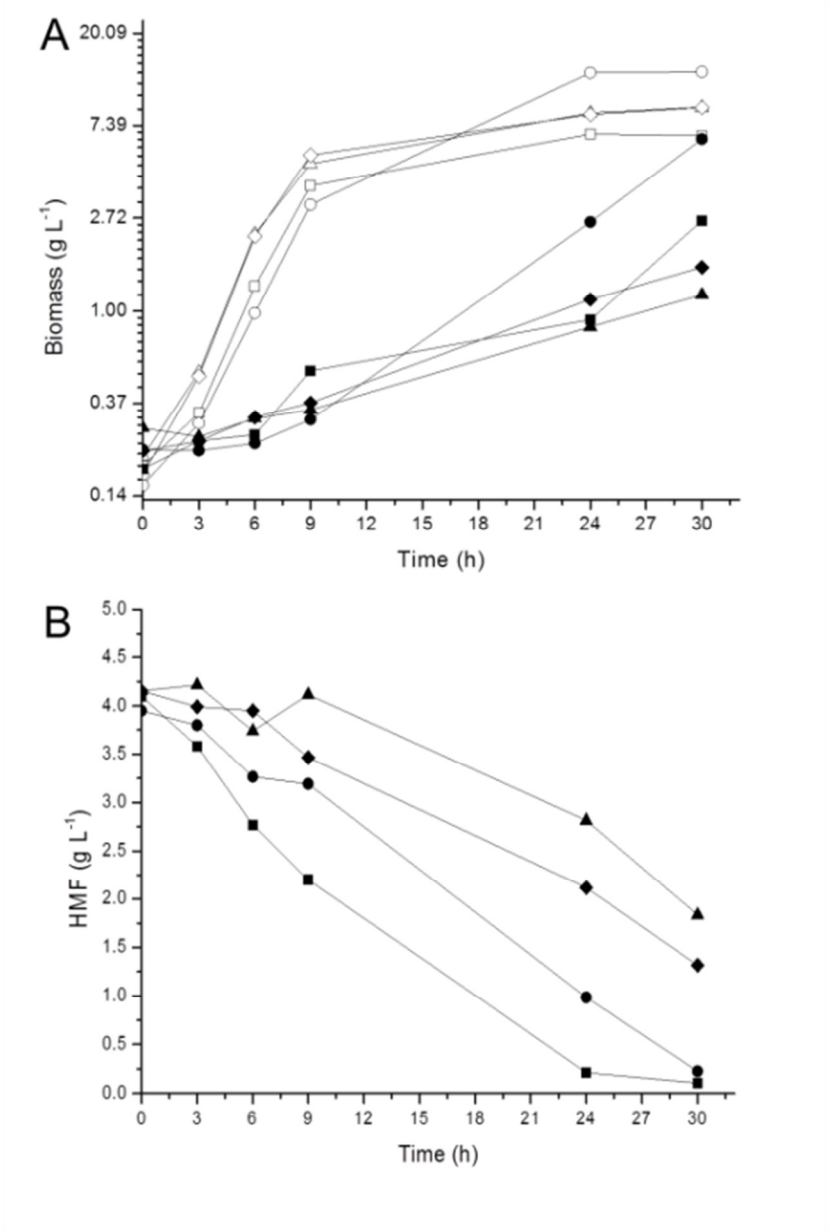

Fig. 1: Kinetics of biomass production (A) and HMF assimilation (B) of *S. cerevisiae* strains JP1 (▲), JP1R (◆), P6 (■), and P18R (●). Open symbols, condition control; filled symbols, presence of 5 g L^{-1} HMF. Results represent the mean of triplicate.

Glucose consumption was not affected, independent of strains (data not shown). In the presence of HMF, three out of four strains presented only about 10 % of the ethanol yield ($Y_{P/S}$) and volumetric productivity (Q_p) compared to controls without HMF (Table 2).

Table 2. Comparison between yield and productivity of ethanol of four industrial strains of *S. cerevisiae* growing in presence or absence of 5 g L^{-1} HMF.

Strain	JP1		JP1R		P6		P18R	
HMF	-	+	-	+	-	+	-	+
$Y_{P/S}$ (g g^{-1})	0.45	0.04	0.46	0.06	0.37	0.06	0.13	0.06
Q_p (g L^{-1} h^{-1})	0.64	0.08	0.55	0.05	0.40	0.05	0.10	0.04

P18R strain, already presenting lower fermentative capacity in the reference medium, showed 50 % yield and productivity reductions. The dose-dependent toxicity of HMF has been reported for *S. cerevisiae* ATCC 211239 and NRRL Y-12632 strains and even at the lowest concentration evaluated (1.2 g L^{-1}) the yeast final biomass did not reach that in the reference condition without HMF (Liu et al., 2004). Ethanol production was also affected in *S. cerevisiae* T1 and Y-1528 strains upon exposure to 3 and 4 g L^{-1} HMF, respectively (Keating et al., 2006). Finally, when furfural and HMF were added to cultures of *Spathaspora arborariae* strain UFMG-HM19.1A, their presence caused significant reductions on biomass formation and ethanol productivity (da Cunha-Pereira et al., 2011). The biochemical explanation seems to be the inhibition of essential enzymes, such as triose phosphate dehydrogenase and alcohol dehydrogenase (Sanchez and Bautista, 1988). HMF

and furans were reported to affect CO2 evolution by yeast cells (Banerjee et al., 1981). Due to their ability to form biomass in the presence of HMF, and to show HMF assimilation, P18R and P6 strains were selected for new rounds of evolutionary engineering for HMF resistance. JP1 was also selected, but in this case for its ability to produce high concentrations of ethanol in absence of HMF.

3.2. Evolutionary engineering for HMF resistance

Evolutionary engineering was performed aiming at selecting strains hyper-resistant to HMF. After 24 days of successive cultivations with increasing concentrations of HMF, it was possible to increase the tolerance of JP1 strain to 3.5 g L^{-1} HMF, while P18R strain was improved to 6 g L^{-1} HMF (results not shown). Moreover, a tolerant mutant of P6 strain was able to grow up to 9 g L^{-1} HMF after 36 days of cultivations. This mutant was named P6H9 and it was selected for further experiments. In the present work it was, therefore, possible to further increase HMF tolerance of JP1R strain by 20 % and to select a hyper-tolerant strain by increasing the HMF tolerance of P6 by 360 %. Evolutionary engineering has been efficiently applied to obtain several interesting mutants of *S. cerevisiae* showing improved xylose-glucose co-assimilation by recombinant xylose-fermenting *S. cerevisiae* strain (Kuyper et al., 2005), and for the selection of sulfuric acid-tolerant JP1 strain (de Melo et al., 2010), which was also used in this work.

3.3. Gene expression analyses

Gene expression analyses were performed comparing the three *S. cerevisiae* strains BY4741, JP1, and P6H9. In the reference medium without HMF, these strains showed similar growth rates, reaching 0.5 DO in 3 h of oxygen limitation cultivation. However, in the presence of HMF there was a marked difference among strains to reach that cell concentration: 12.5 h for BY4741; 5 h for JP1; and 3 h for the mutant P6H9. In JP1, high expression level was observed for *ADH6* gene (>11-fold), while a moderate induction (>2-fold) was observed for *ADH7*, *ARI1*, and *SFA1* genes. On the other hand, there were surprisingly high inductions of *ADH7* (>550-fold) and *ARI1* (>20-fold) genes detected for the P6H9 mutant. Comparatively, the sensitive BY4741 strain showed discreet (>2-fold) inductions of *ADH7* and *ARI1* genes.

When simulating industrial conditions (high-sugar, high-biomass inoculum, anaerobic fermentation), *ADH6* gene was repressed and *ARI1* gene was moderately induced in JP1 strain (>2-fold). Similar to observations for the oxygen limitation condition, under anaerobiosis, *ADH7* was also over-expressed in P6H9 strain, followed by *ARI1* gene. *ADH6* was the first gene reported to be involved with HMF tolerance in yeast (Petersson et al., 2006). Genome-wide DNA Microarray showed the over-expression of ADH6 gene in *S. cerevisiae* TMB3000 strain exposed to HMF and its importance to cell tolerance was demonstrated by overexpressing ADH6 in the recombinant strain CEN.PK 113-5D, also resulting in an increase of NAPDH-dependent alcohol dehydrogenase activity by the cells (Petersson et al., 2006). Adh6p enzyme is active towards a wide spectrum of linear, branched-chain and aromatic primary alcohols and aldehydes such as cinnamaldehyde, benzaldehyde, veratraldehyde, and panisaldehyde using NADPH as the coenzyme (Larroy

et al., 2002a). SFA1 gene overexpression from *S. cerevisiae* TMB3000 was also evaluated, and the NADH-dependent HMF-reducing activity was observed. However, this activity did not result in increased HMF uptake rate (Petersson et al., 2006). Sfa1p presents molecular structure and biological function similar to alcohol dehydrogenases (Wehner et al., 1993), and has been proposed to be a bifunctional enzyme with glutathione-dependent formaldehyde dehydrogenase and long-chain alcohol dehydrogenase activities (Dickinson et al., 2003).

The product of *ARI1* gene was first described as a NADPH-dependent reductase accepting ethyl acetoacetate as substrate (Katz et al., 2003). This gene was also used to produce the 2-phenylethanol from phenylpyruvate, with carbonyl reductase function (Hwang et al., 2009). Recently, Liu and Moon (2009) showed that the partially purified Ari1p displayed NADPH-dependent reduction activities toward at least 14 aldehyde substrates, including furfural, HMF, acetaldehyde, butyraldehyde, cinnamic aldehyde, and phenylacetaldehyde. The expression of ARI1 gene showed a 10-fold increase in *S. cerevisiae* Y-12632 strain upon exposure to HMF (Liu and Moon, 2009), similar to the results for P6H9 strain in this work.

The product of ADH7 gene, a reductase from the NADPH-dependent cinnamyl alcohol dehydrogenase family, has been pointed as an important enzyme for detoxification of cinnamaldehyde, furfural, phenylacetaldehyde, vanillin, and 3-methylbutanal (Larroy et al., 2002b), and the ADH7 overexpression confers yeast tolerance to HMF (Liu et al., 2008). The HMF-tolerant *S. cerevisiae* Y-50049 strain showed a 35-fold increased ADH7 transcription when compared to sensitive strain Y-12632 (Liu et al., 2009).

3.4. Culture kinetics in presence and absence of HMF

Physiological analysis under oxygen limitation cultivation was performed for P6H9 strain and compared with the industrial JP1 and laboratory BY4741 strains (Fig. 2). The three strains showed similar growth rate (Fig. 2A), ethanol (Fig. 2B) and glycerol (Fig. 2C) production in the reference medium, showing that the genetic mutation that conferred HMF hyper-tolerance did not affect yeast fermentative capacity. Yet, the production of acetate in the reference medium, which was already low for industrial strain JP1, was practically abolished for P6H9 tolerant strain compared to BY4741 (Fig. 2D). In the presence of HMF, the yeast cells experienced a change from reductive to oxidative metabolism. The highest growth rate in HMF medium observed for P6H9 strain (Fig. 2A) was also correlated with the highest kinetics of HMF assimilation by this strain (Fig. 2E) and the highest enzymatic activity of its cell-free extract to reduce this compound using NADPH as cofactor (Fig. 2F). This intense metabolic capacity can be explained by the greatly enhanced up-regulation of *ADH7* gene in P6H9 strain. The industrial strain JP1 showed a moderate tolerance towards HMF (Fig. 2A), also showing some limited assimilation of this toxic compound (Fig. 2E), possibly caused by the up-regulation of *ADH6* gene . However, no increased NADPH-dependent enzymatic activity was observed for this strain (Fig. 2F).

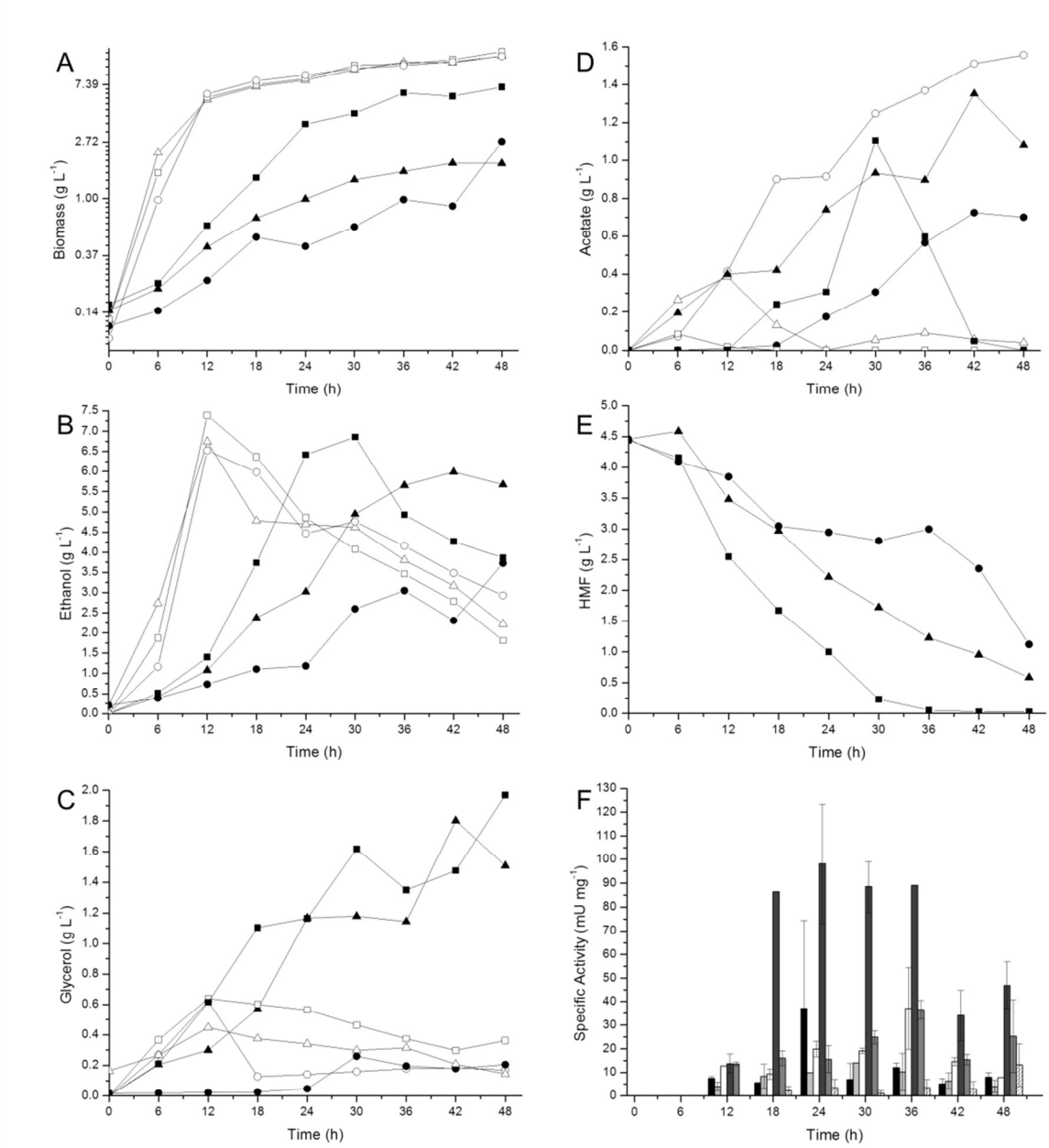

Fig. 2: Physiological analysis of of *S. cerevisiae* strains JP1 (▲), P6H9 (■), and BY4741 (●) cultivated in YPD (open symbols) or YPD containing HMF at 5 g L^{-1} (filled symbols). Production of biomass (A), ethanol (B), glycerol (C), acetate (D), HMF assimilation (E), and NADPH-dependent HMF reduction activity (F) are presented. Black bars, JP1; light gray bars, JP1 with HMF; white bars, P6H9; dark gray bars, P6H9 with HMF; gray bars, BY4741; hatched bars, BY4741 with HMF. Results represent the mean of duplicate.

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Ethanol production was delayed in the presence of HMF for all tested strains, but P6H9 strain was less affected and the final maximal concentration of ethanol achieved was almost the same in the presence or absence of HMF. As expected, ethanol volumetric productivity fell from 0.62 g L^{-1} h^{-1} to 0.23 g L^{-1} h^{-1} in the presence of HMF (Table 3).

Table 3. Comparison between yield and productivity of ethanol for *S. cerevisiae* industrial strains P6H9, JP1, and BY4741, in presence or absence of HMF.

Strain	BY4741		JP1		P6H9	
HMF	-	+	-	+	-	+
$Y_{P/S}$ (g g^{-1})	0.32	0.26	0.32	0.32	0.35	0.33
Q_p (g L^{-1} h^{-1})	0.54	0.07	0.54	0.16	0.62	0.27

Another important observation is the fact that glycerol production was greatly induced by HMF in the industrial strain JP1 and in the HMF-tolerant P6H9 strain (Fig. 2C), despite the reduced growth rate (Fig. 2A), linking its formation to a stress-induced phenomenon caused directly by the assimilation of HMF or indirectly, by some sort of metabolic imbalance. Studies on glycerol production reported that lowered activity of different ADH isoenzymes, especially Adh1p, results in an increase of glycerol production in *S. cerevisiae* (Johansson and Sjostrom, 1984). The protein coded by ADH1 is an enzyme responsible for the anaerobic cytosolic reduction of acetaldehyde to ethanol (Taherzadeh et al., 2002). Analysis of the glucose metabolism of adh0 cells shows that the lack of all known ADH isozymes results in the formation of glycerol as a major fermentation product,

followed by a significant production of acetaldehyde and acetate (DREWKE et al., 1990). In an earlier study, ADH1, ADH2, ADH3, and ADH4 genes expression did not change in tolerant aldehyde *S. cerevisiae* strain. However, glycerol production was not reported for the same strain in that study (Liu et al., 2009).

Except for strain BY4741, which showed a poor growth in the presence of HMF, all other tested strains showed an increased production of acetate in the presence of this toxic (Fig. 2D). Increased acetate production was reported for ADH6- overexpressing strain in batch fermentation (Almeida et al., 2008). In *S. cerevisiae*, there are two main metabolic routes responsible for NADPH formation, namely the pentose-phosphate pathway, and the acetaldehyde dehydrogenase reaction. The first route drains carbon from glycolysis and together with the stress-associated glycerol-3P dehydrogenase reduces the supply of NADH; the second pathway drains acetaldehyde for the production of acetate. Thus, the metabolic requirement for NADPH could lead to increased production of acetate, and decreased production of ethanol as consequence.

In this study, the sensitive strain BY4741 presented low expression levels for all four genes previously described as being involved with HMF tolerance; JP1 strain, however, showed increased expression levels of *ADH6* gene, but this was not reflected in HMF reduction during cultivation, or ethanol and biomass production. These results contrast with results of a previous study, in which tolerance towards HMF in yeast strains overexpressing *ADH6* and *ADH7* genes led to improved growth rate in the presence of this toxic (Liu et al., 2008). However, in the present study, the mutant strain P6H9 showed the highest HMF tolerance so far described for a yeast strain, at the same time capable of producing ethanol. In the presence of this toxic compound, high expression levels for *ADH7* and *ARI1* genes were observed in the two different metabolic conditions tested. In

particular, *ADH7* gene showed a significant increase in its expression levels. This was reflected in the biomass and ethanol production and in the enzymatic activity.

4. CONCLUSION

A high-tolerant *S. cerevisiae* mutant strain P6H9 was obtained by evolutionary engineering from the industrial strain P6, which was isolated from molasses-using ethanol producing plant, and this phenotype was correlated to the high expression levels of *ADH7* and *ARI1* genes and increased enzymatic activity for NADPH-dependent HMF reduction. In the presence of HMF, this strain showed a better physiological performance than JP1 industrial strain and BY4741. Overall, the results obtained in this work shows the importance of several genes on the phenotype for HMF tolerance and strain P6H9 can be further exploited in research for second generation ethanol production.

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4.2. CAPÍTULO II Second-generation ethanol production by *Wickerhamomyces anomalus* strain tolerant to furfural, 5-hydroxymethylfurfural (HMF) and osmotic pressure

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ABSTRACT

The aims of this work were to improve the tolerance to high concentrations of furfural and 5-hydroxymethylfurfural (HMF) of an osmotolerant strain of *Wickerhamomyces anomalus* by means of evolutionary engineering, and to determine its ethanol production capabilities under stress conditions. Cells were grown in the presence of furfural, HMF, and both compounds under high osmotic pressure conditions, and the most toxic condition for the parental strain was the combination of both furans. While the parental strain did not produce biomass or ethanol, the tolerant mutated strain achieved a yield of sugar conversion to ethanol of 0.43 g g^{-1} in the presence of furfural and HMF, with alcohol dehydrogenase activity of 0.68 mU mg $protein^{-1}$. For this strain, osmotic pressure, did not affect growth rate. Results suggest that *W. anomalus* WA-HF5.5 strain presents high potential to be used for second-generation ethanol production.

Keywords: Bioethanol; *Wickerhamomyces anomalus*; furaldehydes tolerance; osmotic pressure, lignocellulosic hydrolysates.

1. INTRODUCTION

There is a worldwide effort to ensure the production of energy from various sources of renewable raw materials. Second-generation ethanol, also called *bioethanol*, is obtained from lignocellulosic biomass, especially from agriculture residues, which is abundant,

inexpensive, and a desirable feedstock for the sustainable production of biofuels it because does not compete with food production (Hasunuma et al., 2012). The production of bioethanol from lignocellulosic feedstock requires pretreatments of biomass and hydrolyses steps to release sugars for yeast fermentation, producing inhibitory compounds of yeast metabolism. This fact turns the use of biomass hydrolysates challenging to bioprocess, since the operational costs of medium detoxification are, at present, prohibitive if bioethanol is to compete with first-generation sugarcane or starch based ethanol.

Another important aspect in second-generation ethanol production is the presence of pentoses and hexoses in the hydrolysates, requiring specific cell metabolisms for the total conversion of sugars. *S. cerevisiae*, the most efficient yeast for conventional ethanol production, cannot assimilate pentoses, including xylose, usually present in great amounts in lignocellulosic hydrolysates (da Cunha-Pereira et al., 2011). As a consequence, several works were carried out in the search for pentose-fermenting yeasts. Literature reports on several strains of *Candida guilliermondii*, *Pichia stipitis*, *Pachysolen tannophylus*, *Spathaspora arborariae,* and *Wickerhamomyces anomalus* (da Cunha-Pereira et al., 2011; Schirmer-Michel et al., 2008; Tao et al., 2011; Yadav et al., 2011; Zhao et al., 2008) used for pentose bioco nversions.

The ascomycetous yeast *W. anomalus* (formerly *Pichia anomala* and *Hansenula anomala*) has been isolated from many different habitats and shows a remarkable physiological robustness towards environmental stresses, such as extreme pH or low water activity, is highly competitive and can inhibit growth of other microorganisms (Passoth et al., 2011). This specie has been tested for several biotechnological applications, including food and beverage applications (as probiotics, sourdough fermentations, volatile aromas in wine), environmental bioremediation (sophorolipis as biosurfactants), biopharmaceuticals

(production of aminobutiric acid), and biofuels (ethanol and isobutanol productions) (Walker, 2011).

During ethanol fermentation, substrate and product inhibition, and osmotic pressure are the most important adverse conditions (Zhao & Bai, 2009). In second-generation ethanol production, there is a further important problem, which is the degradation of sugars during hydrolyses and the consequent formation of toxic compounds (Almeida et al., 2008; da Cunha-Pereira et al., 2011; Margeot et al., 2009). The main yeast metabolism inhibitors are the 2-furaldehyde (furfural), formed by pentoses dehydration, and 5-hydroxymethyl-2-furaldehyde (5-hydroxymethylfurfural - HMF), which is formed by hexoses dehydration (Lenihan et al., 2010; Liu et al., 2009; Mansilla et al., 1998). Furaldehydes interfere with microbial growth and any subsequent fermentation, interfering with glycolytic enzymes, as well as with protein and RNA synthesis (Almeida et al., 2007; Luo et al., 2002; Modig et al., 2002).

Synergistic effects of HMF and furfural have been demonstrated for *S. cerevisiae* and *Spathaspora arborariae* (da Cunha-Pereira et al., 2011; Taherzadeh et al., 2000). A common furaldehyde detoxification metabolism in yeasts is the reduction of the aldehydes into the less toxic corresponding alcohols. This reaction is mainly catalyzed by alcohol dehydrogenases. These enzymes are NAD(P) H-dependent oxidoreductases that catalyze the reversible oxidation of alcohols to aldehydes or ketones (de Smidt et al., 2008; Almeida et al., 2007; Liu et al., 2004).

In recent years, several yeast strains have been extensively studied for bioethanol production, in which yeast cells are exposed to diverse environmental stresses such as high temperature, ethanol inhibition, osmotic pressure, as well as to the presence of inhibitory substances released from pretreatments of lignocellulosic biomass (Zhao & Bai, 2009).

Some biochemical basis of yeast stress tolerance has been elucidated. For example, osmotic pressure has been shown to induce intracellular glycerol levels in *S. cerevisiae* and *W. anomalus* strains (Djelal et al., 2012a), and the accumulation of trehalose and heat shock proteins in response to oxidative stress, heat and ethanol have been reported (Zhao & Bai, 2009).

In the present study, a strain of *W. anomalus* was submitted to evolutionary engineering in order to increase its resistance to furfural and HMF present in the medium. Both the parental and its tolerant-derived strain had their physiological characteristics compared, their abilities to produce biomass and ethanol, correlated to alcohol dehydrogenase enzymatic activity, and the reduction of the toxic furan concentrations in the medium. Their ability to resist to high osmotic pressure was also evaluated and compared.

2. Materials and Methods

2.1. Microorganisms, cell maintenance and chemicals.

The strain used in this work is part of the yeast collection of Bioteclab, named *W. anomalus* WA-001. This strain was isolated by this research group from piles of decomposing rice hulls deposited in the environment and it was identified comparing ITS1 and ITS4 amplicon sequences with GenBank database (www.ncbi.nlm.nih.gov/BLASTn). Stock cultures were kept frozen at -20 ºC in medium containing 20 % glycerol and 80 % of mid-exponential cell suspentions. All chemicals used in this research were of analytical grade and purchased from Sigma-Aldrich (St. Louis, USA).

2.2. Evolutionary engineering for HMF and furfural resistance

Evolutionary engineering was used aiming at increasing furaldehydes resistance of *W. anomalus* WA-001. Cultivations were performed in YPD medium (containing, in g L^{-1}: glucose, 20; yeast extract, 10; and peptone, 20), carried out in 125 mL Erlenmeyer flasks containing 30 mL of medium, at 28 ºC and 150 rpm on a rotatory shaker. Cells were inoculated (OD 0.1, 600 nm, 10 % volume fraction) into YPD medium added of HMF and furfural (0.25 g L^{-1} each chemical), for 48 h. From this mother culture, successive batches were run by transferring 10 % (volume fraction) of cells (OD 0.1) to re-inoculate YPD medium containing increasing concentrations of furaldehydes, up to 1 g L^{-1}.

2.3. Kinetics of ethanol production in presence of furaldehydes

Cultivations were performed in YPD medium and supplemented with furaldehydes as indicated. Cultures were carried out in 250 mL flasks containing 60 mL of medium at 28 ºC and 150 rpm on a rotatory shaker. Initial inoculum of 0.1 OD (600 nm) was standardized. Strains were cultivated in absence (control) and presence of furfural (3 g.L^{-1}), HMF (3 g.L^{-1}), or combination of both (1.5 g.L^{-1} of each). Samples were taken at determined times during 48 h of cultivation for determination of glucose, ethanol, glycerol, furfural, and HMF concentration, and for biomass formation.

2.4. Enzyme activity assay

In order to determine the furfural and HMF reducing metabolic activities, samples were taken at 24 h of cultivation under conditions mentioned in item 2.3. Crude protein extracts were prepared by lysing cells with glass beads attrition. Cells were resuspended in 400 µL of 100 mM K_2HPO_4 buffer, pH 7.0, and 2 µL of a solution of 100 mM phenylmethylsulfonyl fluoride (PMSF) in a 2 mL Eppendorf tube, and added of an equal volume of glass beads (diameter of 500 µm). Cells were disrupted by six cycles of vortexing for 60 s, with samples cooled on ice for 60 s in between each cycle. Protein extracts were collected by centrifugation at 13 000 *g* for 5 min at 4 °C and the concentration was determined using Lowry assay method. Alcohol dehydrogenase activity was monitored by recording decreased absorbance at 340 nm using NADH as cofactor. NADPH was also used as cofactor when only HMF was used as substrate. The reaction mixture consisted of a final concentration of 10 mM HMF substrate and 100 µM of NADPH in 100 mM potassium phosphate buffer, pH 7.0. All reagents were maintained at 25 °C prior to use. Assays were carried out in a volume of 1 ml at 25 °C for 1 min. The protein samples were kept on ice. To start the reaction, 25 µl of crude extract protein was added to the reaction mix. The NADPH molar absorption coefficient was 6.22 mM^{-1} cm^{-1}.

2.5. Osmotic pressure tolerance assay

Cultivations were performed in YPD medium and supplemented with varying concentrations of NaCl. Cultures were carried out in 250 mL flasks containing 60 mL of

medium, incubated at 28 ºC and 150 rpm on a rotatory shaker. Initial inoculum of 0.1 OD (600 nm) was standardized. When cells reached exponential phase (OD = 1), a pulse of NaCl was added into the medium to final concentrations of 0.25, 0.5, 0.75, 1, 1.25, and 1.5 M. Samples were taken at determined times during 12 h for determination of specific growth rate.

2.6. Analytical methods and calculation of kinetic parameters

Growth rate of yeast cultures were estimated as cell optical density (OD) at 600 nm. Cultivations were run for 48 h and samples were taken every 6 h. Cultures were centrifuged at 3 500 *g*, and cells were kept on -18 °C for further enzymatic activity assays. Yeast biomass was determined using a standard curve correlating the OD and cell dry weight (g L^{-1}). Glucose, glycerol, ethanol, and acetate concentrations were determined by HPLC (Shimadzu, Japan) equipped with a refractive index detector and Bio-Rad HPX-87H column (300 × 7.8 mm) using 5 mM sulfuric acid as eluent at 45 °C, flow rate of 0.6 mL min^{-1} and sample volumes of 20 µL. Furfural and HMF concentrations were determined by HPLC with a UV detector (at 276 nm) using a Nucleosil C18 column (250 × 4.6 mm) at room temperature, using acetonitrile–water (2:8) containing 10 g L^{-1} acetic acid as eluent, flow rate of 1.1 mL min^{-1} and sample volumes of 20 µL. Osmotic pressure (mOsm Kg^{-1}) was determined by vapor pressure measurement, in an Osmometer (Wescor, USA). The ethanol conversion yield ($Y_{P/S}$, g g^{-1}) was defined as the ratio of the concentration of ethanol produced and glucose consumed. The volumetric productivity (Q_p, g L^{-1} h^{-1}) was calculated using the maximal ethanol production in time interval to reach it. The specific

growth rate of cells (μ, h^{-1}) was calculated using biomass formation in the time interval to reach it within the exponential growth phase.

3. RESULTS AND DISCUSSION

3.1. Evolutionary engineering for furaldehyde tolerance

Evolutionary engineering was performed in order to select a strain resistant to furfural and HMF. After 22 days of successive cultivations with increasing concentrations of furfural and HMF, it was possible to increase the tolerance of WA-001 strain to 5.5 g L^{-1} of furaldehydes (2.25 g of furfural and HMF, each) (results not shown). In the present work it was, therefore, possible to further increase furaldehydes tolerance of WA-001 strain by 80 %. This mutant was named WA-HF5.5 and it was used in the following experiments. Evolutionary engineering has been used as a powerful tool for the optimization and the introduction of new cellular processes. For instance, this strategy has been efficiently applied to obtain several interesting mutants of *S. cerevisiae,* as well as showing improved gluconate assimilation for wine fermentation (Cadiere et al., 2011), improved xylose-glucose co-assimilation by recombinant xylose-fermenting strain, (Kuyper et al., 2005), improved arabinose consumption by recombinant arabinose-fermenting strain (Wisselink et al., 2007), and recently, improved HMF tolerance in an industrial strain of *S. cerevisiae* (Sehnem et al., 2013). During the processes of hydrolysis of lignocellulosic biomass, the production of furfural and HMF is unavoidable; the toxic synergistic effects of these compounds have been investigated and demonstrated in previous studies for *S. cerevisiae*

and *S. arborariae*. (da Cunha-Pereira et al., 2011; Taherzadeh et al., 2000). Thus, improved tolerance towards both furfural and HMF is an important strategy for second generation ethanol production.

3.2. Culture kinetics behavior in response to furfural and HMF concentration

Physiological analysis under oxygen limitation cultivation was performed for the parental and mutant tolerant strains. High sensibility to furfural and to the synergic effects of both compounds of WA-001 strain resulted in practically no metabolic activity, with results depicted in Figure 1 showing no formation of biomass, ethanol, or glycerol, and with only small alcohol dehydrogenase activity (Fig 2). This blocked metabolic activity is a direct consequence of the inability to reduce furfural and HMF under synergic condition, since the control culture (without furaldehydes) grew normally, producing 7.79 g L^{-1} of biomass (Table 1). For the parental strain, results show that HMF is completely reduced in 24 h of cultivation, while furfural remained even after 48 h of cultivation (Fig. 2A), and when both toxics were added, even the metabolism of HMF was repressed. This result is an evidence of the negative synergic effects of furaldehydes on WA-001 strain. Similar effects were observed in *S. cerevisiae* strain CBS 8066, where the specific ethanol productivity rate was highly affected under synergic conditions (Taherzadeh et al., 2000). *S. arborariae* NRRL Y-48658 also showed high sensitivity to toxics in synergic condition (da Cunha-Pereira et al., 2011). Synergic effects can be related to the immediate furfural effects on glycolytic and TCA pathways, which are involved in the energy metabolism of yeasts. This furaldehyde decreases activity of ADH and the glycolytic enzymes hexokinase and

glyceraldehyde-3-phosphate dehydrogenase, also inducing reactive oxygen species to accumulate, vacuole, mitochondrial membranes, chromatin, and actin damage (Almeida et al., 2007). When HMF is also present, the low conversion rate of this compound also contributes to increase furfural toxicity of yeast cells (Taherzadeh et al., 2000).

Culture kinetics of WA-HF5.5 strain showed the formation of biomass under all conditions of furaldehydes concentration. Under synergic conditions, lag phase lasted 12 h, and biomass production was more affected in presence of 3 g L^{-1} furfural (Fig. 1A). Interesting results were obtained concerning ethanol formation relative to the addition of furaldehydess to culture medium. Although final ethanol concentrations achieved for both strains in control and HMF conditions were similar, productivity was higher for the adapted strain under both conditions. Moreover, when growth was carried out in the presence of furfural, the parental strain almost completely lacked the ability to form ethanol, while strain WA-HF5.5 was able to produce ethanol to reasonable amounts (Table 1). These results show that evolutionary engineering was able to improve the ethanol production in strain WA-HF5.5, even when compared to the control cultivation (Fig. 1B). Strain WA-HF5.5 showed a better metabolism when both furaldehydes were present in the medium than when only furfural was present (Fig. 2B). For strain WA-HF5.5, furfural was reduced in an early stage than HMF, as shown in Fig. 2B. HMF contains hydroxymethyl groups, being less hydrophobic, reducing cell membrane permeability thus causing a low conversion rate of this compound (Taherzadeh et al., 2000).

In this study, the NADH-dependent reduction activity of furaldehydes was strongly related to the alcohol dehydrogenase activity, as can be seen in Fig. 2C. The furfural reducing activity showed higher levels only for the control condition in the parental strain, indicating that it was sensitive to furaldehydes. The enzymatic activity on WA-HF5.5

strain showed different profiles. Higher levels of enzymatic activity were induced in YPD medium with HMF, followed by YPD added of both toxic compounds. These results show that the reducing enzymes are produced in higher levels in the presence of furaldehydes. Alcohol dehydrogenases (ADHs) are enzymes able to catalyze this reaction. This class of enzymes is well characterized for *S. cerevisiae*. ADHs constitute a large family of enzymes responsible for the reversible oxidation of alcohols to aldehydes, and with the concomitant reduction of NAD^+ or $NADP^+$. Physiologically, the ADH reaction in *S. cerevisiae* and in related species plays an important role in sugar metabolism, because specific ADH isozyme serves to regenerate the glycolytic NAD^+, thereby restoring the redox balance through the reduction of acetaldehyde to ethanol (de Smidt et al., 2008).

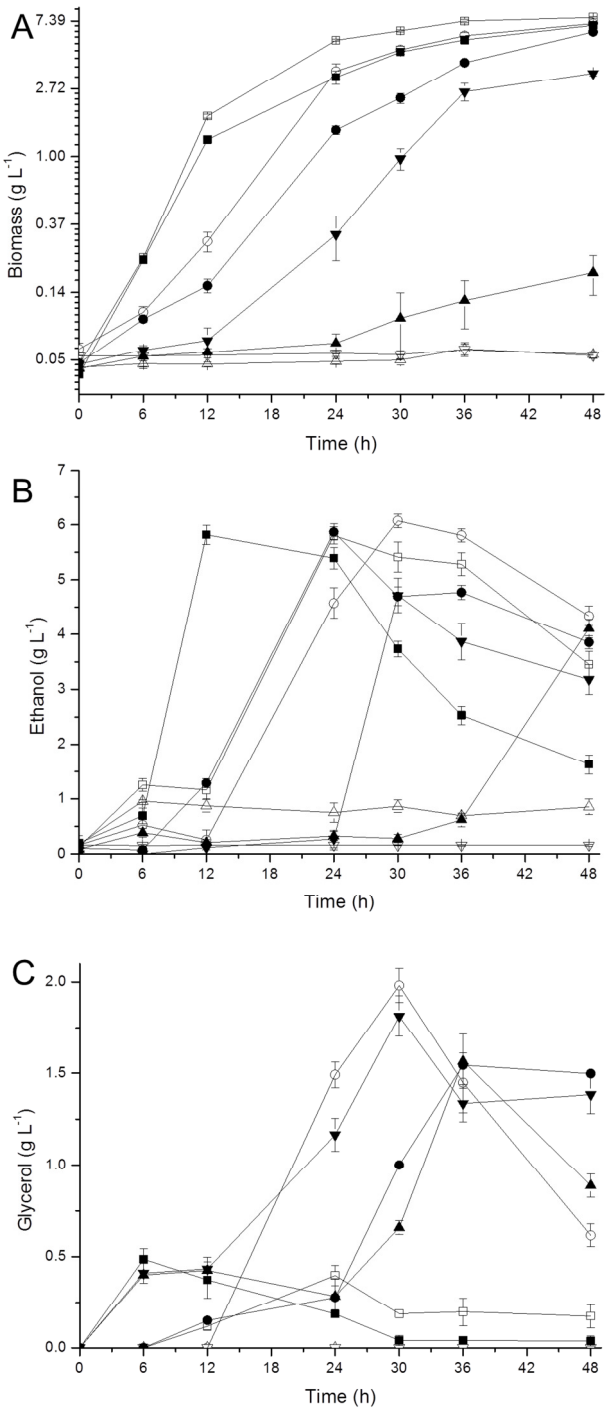

Fig. 1: Physiological analysis of *W. anomalus* strains WA-001 (open symbols) and WA-HF5.5 (filled symbols) cultivated in YPD (■), YPD containing 3 g L^{-1} HMF (●),YPD containing 3 g L^{-1} furfural (▲), or YPD containing 1.5 g L^{-1} of both furaldehydes (▼). Biomass formation (A), ethanol (B) and glycerol concentrations (C). Results represent the mean of triplicate.

On *S. cerevisiae*, enzymes responsible for furaldehydes reducing activities have preference for NADH and NADPH cofactors (Liu, 2011), However, some enzymes have preference only for NADPH, such as Adh6p (Petersson et al., 2006), or NADH for Adh1p (Laadan et al., 2008). In the present work, it can be observed that HMF, when is used as substrate, presents low substrate affinity using NADH or NADPH as cofactors (Fig. 2C). Unlike HMF, furfural is the enzymatic substrate with higher affinity to reducing enzymes present in the crude extract, and similar results were obtained using the mixture of both furaldehydes as substrate. The results in this work suggest that for WA-HF5.5 enzymes catalyzing furaldehydes reduction appear to have more affinity for NADH. These results are consistent to *in vitro* and in *vivo* assays using several *S. cerevisiae* strains, suggesting that HMF and furfural reductions are coupled with NADPH and NADH oxidation, respectively (Almeida et al., 2007). HMF is mainly reduced by NADPH-dependent Adh6p and Adh7p (Petersson et al., 2006; Sehnem et al., 2013), while furfural reduction is catalyzed by NADH-dependent Ald4p, and Gre3p in *S. cerevisiae* (Liu et al., 2008).

Glycerol production was also increased in the presence of furaldehydes for WA-HF5.5, while for the parental strain glycerol production was increased only when HMF was added to cultures (Fig. 1C). Under anaerobic conditions, glycerol is normally produced to regenerate excess NADH formed during biosynthetic processes (Palmqvist & Hahn-Hagerdal, 2000). Production of glycerol is also associated with responses to several stress conditions. In *S. cerevisiae*, changes in internal metabolic fluxes that lead to the production of glycerol helps to counter cell stress or adapt to stressful conditions such as osmotic pressure, high ethanol and CO_2, among others (Pandey et al., 2007). In *W. anomalus*, glycerol production in response to environmental stress was also reported. This yeast survives in media at low water activity resulting from increasing NaCl concentrations in

the culture medium by producing compatible solutes, like glycerol, arabitol, and trehalose (Djelal et al., 2012b).

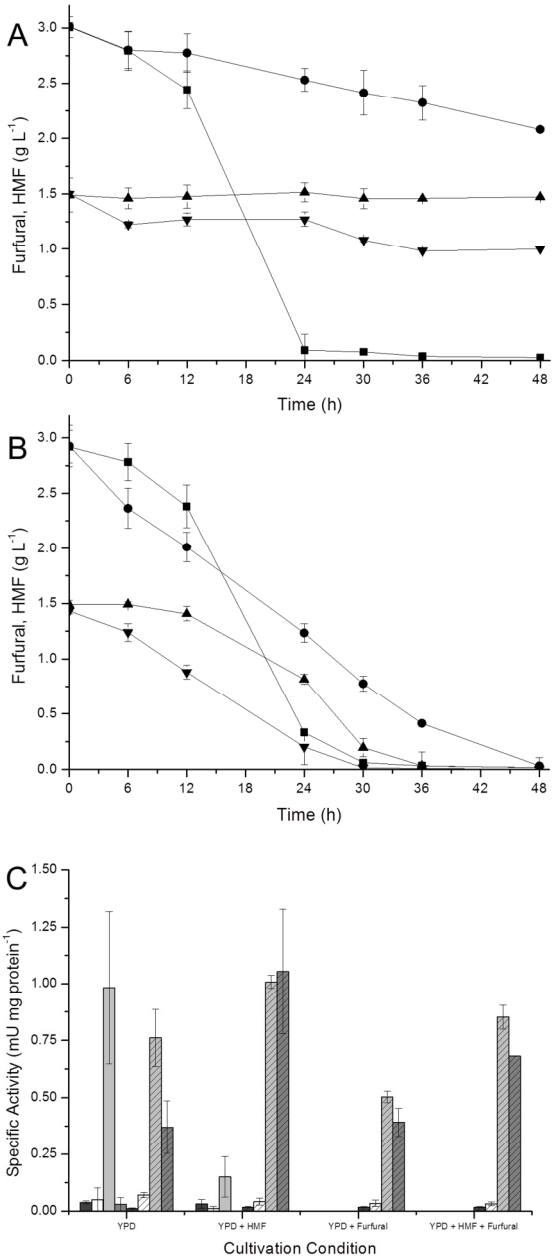

Fig. 2: Furfural and HMF reduction during growth of *W. anomalus* strains WA-001 (A) and WA-HF5.5 (B). YPD containing 3 g L^{-1} HMF (■), YPD containing 3 g L^{-1} furfural (●), or YPD containing 1.5 g L^{-1} furfural (▼) plus HMF at 1.5 g L^{-1} (▲). Comparative analysis of NAD(P)H-dependent reduction activity (C) using as substrates: HMF and NADPH (dark gray bars); HMF and NADH (white bars), furfural and NADH (light gray bars), or HMF and furfural and NADH (dark gray bars) in 24 h of cultivation, solid bars, strain WA-001; hatched bars, strain WA-HF5.5. Results represent the mean of duplicate.

Table 1. Comparison of biomass formation, maximum specific growth rate (μ_{max}), glycerol concentration, ethanol concentration and yields ($Y_{P/S}$), and ethanol productivity (Q_p) for *W. anomalus* strains WA-001 and WA-HF5.5. Cultures in YPD medium containing 3 g L^{-1} HMF, 3 g L^{-1} furfural, or both furans (1.5 g $L^{-1}$of each). Results are the mean of triplicate.

	Strains											
Condition	WA-001	WA-HF5.5	WA-001	WA-HF5.5	WA-001	WA-HF5.5	WA-001	WA-HF5.5	WA-001	WA-HF5.5	WA-001	WA-HF5.5
	Biomass (g L^{-1})		μ_{max} (h^{-1})		Ethanol (g L^{-1})		$Y_{P/S}$ (g g^{-1})		Q_p (g L^{-1} h^{-1})		Glycerol (g L^{-1})	
Control	7.79 ± 0.10	6.93 ± 0.22	0.35	0.30	5.81 ± 0.15	5.82 ± 0.17	0.30	0.35	0.24	0.47	0.40 ± 0.05	0.48 ± 0.05
HMF 3 g L^{-1}	7.12 ± 0.26	6.29 ± 0.08	0.21	0.19	6.07 ± 0.12	5.87 ± 0.14	0.27	0.30	0.18	0.24	1.98 ± 0.09	1.54 ± 0.02
Furfural 3 g L^{-1}	0.05 ± 0.00	0.18 ± 0.05	0.00	0.06	0.51 ± 0.03	4.1 ± 0.17	0.00	0.22	0.00	0.08	0.00 ± 0.00	1.56 ± 0.14
Furfural and HMF	0.00 ± 0.00	3.41 ± 0.12	0.00	0.19	0.00 ± 0.00	4.7 ± 0.31	0.00	0.43	0.00	0.16	0.00 ± 0.00	1.81 ± 0.11

3.3. Analysis for osmotic pressure tolerance

In order to access the effects of medium osmotic pressure on the parental and mutant strain, exposure to varying concentrations of NaCl was conducted. This is important because second-generation ethanol is based on high osmotic pressure media such as hydrolyzed lignocellulosic materials. Several yeast species have been studied in culture conditions of low water activity and high osmotic pressure media in our group, such as *S. cerevisiae*, *C. guilliermondii*, *S. arborariae*, and *C. shehatae* (da Cunha-Pereira et al., 2011; Hickert et al., 2013; Schirmer-Michel et al., 2008) and it would be interesting to know the response of *W. anomalus* to these conditions. Results of NaCl growing concentration exposition for both strains are shown in Table 2. The parental strain showed sensibility to growing osmotic pressure, with growth suppression for 1.5 M NaCl, whereas for WA-HF5.5 strain the specific growth rate was weakly affected. Previous studies with *S. cerevisiae* have shown that the increase in medium osmotic pressure up to 20 % (volumetric fraction) of sorbitol decreased cell viability and growth, and fermentation performance (Pratt et al., 2003).

In this work, the evolutionary engineering for furans tolerance was also positive towards improving osmotic pressure resistance of *W. anomalus*. One possible explanation for this acquired trait is that the analyses of gene expression in response to furfural and HMF in S. *cerevisiae* showed that the responses are distributed across a wide range of functional categories and pathways, which includes the stress-related high-osmolarity glycerol (HOG) pathway and heat shock protein genes (Liu, 2012).

Table 2. Comparison between specific growth rate of *W. anomalus* strains WA-001, and WA-HF5.5, in hyperosmotic stress cultivation. Results represent the mean of duplicate.

Condition	Osmolarity (mM kg^{-1})	Specific Growth Rate μ (h^{-1})	
		WA-001	WA-HF5.5
YPD	209	0.31	0.28
YPD + 0.25 M NaCl	661	0.29	0.29
YPD + 0.50 M NaCl	1 024	0.27	0.27
YPD + 0.75 M NaCl	1 350	0.24	0.28
YPD + 1.00 M NaCl	1 653	0.21	0.25
YPD + 1.25 M NaCl	1 969	0.18	0.23
YPD + 1.50 M NaCl	2 060	0.08	0.21

4. CONCLUSION

An osmotolerant *W. anomalus* mutant strain WA-HF5.5 was obtained by evolutionary engineering, possessing high HMF and furfural tolerance. The mutated strain showed better physiological performances than its parental strain in media containing furfural, HMF or both to concentrations simulating lignocellulosic biomass hydrolysates. Results presented show that the evolutionary engineering that conferred HMF and furfural tolerance improved yeast fermentative capacity, and ethanol productivity, suggesting that this strain can be further exploited in the research for second generation ethanol production.

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4.3. CAPÍTULO III – Bioconversion of soybean and rice hulls hydrolysates into ethanol and xylitol by furaldehyde-tolerant strains of *Saccharomyces cerevisiae*, *Wickerhamomyces anomalus* and their co-fermentations

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ABSTRACT

The aims of this work were to evaluate the ability of furaldehyde-tolerant yeast strains *Saccharomyces cerevisiae* P6H9 and *Wickerhamomyces anomalus* WA-HF5.5, and their co-cultures, to convert soybean and rice hulls hydrolysates into ethanol and xylitol. Batch agitated flasks cultures showed the ability of strains to completely deplete furaldehydes at 12 h cultivations, to tolerate high concentrations of osmotic pressure (1 918 mOsm Kg^{-1}), and to convert the hydrolysate sugars into ethanol, to the best values of conversion yields of 0.37 g. g^{-1} and productivity of 0.31 g L^{-1} h^{-1} for the co-fermentation in the rice hull hydrolysate. Cultivations in soybean hull hydrolysate showed low ethanol productivity (0.08 g L^{-1} h^{-1}). Scaled-up bioreactor cultivations of *S. cerevisiae* using rice hull hydrolysate under anaerobiosis showed high ethanol productivity 6.7 g L^{-1} h^{-1}, while the bioreactor co-culture produced xylitol to yields of 0.86 g g^{-1} under oxygen limited condition.

Keywords: Bioethanol; xylitol, *Saccharomyces cerevisiae, Wickerhamomyces anomalus*; lignocellulosic hydrolysates.

1. INTRODUCTION

There is a global effort to increase the production of biofuels, in special ethanol, to replace fossil fuels. The first generation biofuel is mainly produced from food crops such as sugarcane and maize, therefore, competing for agricultural areas used for food production (Chen, 2011). In contrast, ethanol derived from lignocellulosic feedstocks (second-generation ethanol) represent a renewable source of energy, and offer an environmentally important alternative because these materials do not compete with food production (Keating et al., 2004). Biomass from woods, straws, grasses, and hulls of a variety of vegetables are composed of cellulose, hemicellulose, oils, lignin, and proteins must be treated in order to breakdown their polymeric sugars that make up their complex structure. This is necessary to liberate monomeric fermentable sugars. Diluted acid hydrolysis is one of the commonest pretreatment used to release sugars and is already in place at pilot scale (Almeida et al., 2008; da Cunha-Pereira et al., 2011; Margeot et al., 2009). This pre-treatment depolymerises the hemicellulose fraction of biomasses at low temperatures, releasing hexoses and pentoses (Yadav et al., 2011).

The economical feasibility of second-generation ethanol depends on the complete conversion of sugars during fermentation, and cell tolerance to toxic compounds, which are produced during hydrolysis. *S. cerevisiae* is the most efficient yeast for ethanol production; however, it cannot assimilate pentoses, including xylose that is present in high amounts in lignocellulosic hydrolysates (da Cunha-Pereira et al., 2011). Therefore, the search for efficient pentose-fermenting yeasts has been widely reported on the literature as, for instance, *Candida guilliermondii*, *Pichia stipitis*, *Pachysolen tannophylus*, *Candida utilis, Spathaspora arborariae,* and *Wickerhamomyces anomalus* (da Cunha-Pereira et al., 2011;

Ko et al., 2008; Schirmer-Michel et al., 2008; Tao et al., 2011; Yadav et al., 2011; Zhao et al., 2008). Since xylose is abundant in biomasses, another product of commercial interest with potential for production using lignocellulosic hydrolysates is xylitol, a five-carbon polyalcohol largely used in the food and pharmaceutical industries (Ko et al., 2008; Vandeska et al., 1995).

Concerning the toxic compounds liberated – or produced – during biomasses hydrolyses, are furaldehydes such as furfural and 5-hydroxymethylfurfural (HMF), phenolic compounds, and weak acids such as acetic acid. These toxics are inhibitory of yeasts metabolism, causing a delay in microbial growth and loss of fermentation performances (Almeida et al., 2008; da Cunha-Pereira et al., 2011; Margeot et al., 2009). The high osmotic pressure of hydrolysates, usually caused by the excessive salt formation during the chemical neutralization of acids, is another important adverse condition in these cultivations (Zhao & Bai, 2009). Therefore, detoxification of lignocellulosic hydrolysates is usually required prior to fermentation, using expensive unit operations. Alternatively, the use of inhibitor-tolerant yeasts would make detoxification unnecessary, reducing production costs, and some studies have reported on the improvement of furaldehydes-tolerance in *S. cerevisiae* (Allen et al., 2010; Almeida et al., 2008; Sehnem et al., 2013a).

Due to the complexities of biomass hydrolysates fermentations, different approaches are being tried by researchers, including the use of genetically modified yeasts (Brat et al., 2009; Kuyper et al., 2003; Petersson et al., 2006) fermentations using co-cultures of yeasts (Delgenes et al., 1996; Qian et al., 2006; Rouhollah et al., 2007); and evolutionary engineering (Liu et al., 2009; Sehnem et al., 2013a; Sehnem et al., 2013b), among others.

It was demonstrated in a recent work the ability of *S. cerevisiae* P6H9, an industrial strain modified by evolutionary engineering to resist high HMF concentrations, to produce

ethanol in presence of this toxic (Sehnem et al., 2013a). In another work was reported that an osmotolerant pentose-fermenting *W. anomalus* strain was able to grow in medium containing furfural and HMF and to produce ethanol (Sehnem et al., 2013b). In this context, this work was set up to investigate the use of these strains in fermentations to convert soybean and rice hulls hydrolysates into ethanol and/or xylitol. Yeasts were grown as pure cultures and in co-cultures, under anaerobiosis and oxygen limitation conditions. The lignocellulosic hydrolysate that allowed the best conversion rates was used to scale-up cultures simulating industrial ethanol production in bioreactors.

2. Materials and Methods

2.1. Microorganisms, cell maintenance, and materials

The strains used in this work were the HMF tolerant *S. cerevisiae* P6H9 (Sehnem et al., 2013a), and the furfural and HMF tolerant *W. anomalus* WA-HF5.5 (Sehnem et al., 2013b). Yeasts were kept frozen at -20 ºC in stock cultures of 20 % (volume fraction) glycerol. Soybean hull was supplied by The Solae Company (Esteio, Brazil); rice hull was obtained from a local rice mill. Both were kept as dried material and processed without any further treatments before hydrolysis (see below). Sugarcane worth was obtained from a local sugarcane-producing property (Viamão, Brazil).

2.2. Media composition and inocula preparations

For shaker flasks experiments, inocula were prepared by cultivating the yeasts in YPD_{20} medium containing (in g L^{-1}): glucose, 20; yeast extract, 10; and peptone, 20. A loopful of yeasts was inoculated in 500 mL Erlenmeyer flasks filled with 120 mL of medium. Cultivations were carried out in an orbital shaker (Marconi MA 830, Brazil) at 180 rpm, 30° C for 14 h. Late exponential-phase cells were collected by centrifugation at 3 500 *g* for 10 min, and the pellet formed was washed with sterile distilled water and resuspended directly into the medium to be used in the fermentation to obtain an initial cell concentration of 1.0 OD (optical density) at 600 nm. Volume fractions of 10 % of these cell suspensions were then used as the inocula. For co-culture experiments, volume fractions of 5 % of each yeast were used.

For bioreactor cultivations, inocula were prepared by cultivating the yeasts in YPD_{120} medium containing (in g L^{-1}): glucose, 120; yeast extract, 10; and peptone, 20. A loopful of yeasts was inoculated in YPD_{120} medium in 2 000 mL Erlenmeyer flask filled with 500 mL of medium. Since the aim of this work was to simulate industrial ethanol production, a typical and cheap source was used to produce biomass for fermentation (sugarcane worth). Cell cultures were used to inoculate 5 L volume Biostat B bioreactors (B. Braun Biotech International, Germany) containing 4.5 L of sugarcane wort containing 180 g L^{-1} of sucrose, at 30 ºC, 400 rpm and 2 vvm air flow. Cells were cultivated during 24 h, collected by centrifugation at 3 500 *g* for 10 min, and the pellet formed was washed with sterile distilled water and resuspended directly into the medium to be used in the fermentation to obtain a biomass concentration of 10 % wet weight. For co-culture experiments, volume fractions of 5 % of each yeast were used.

Rice hull hydrolysate (RHH) was obtained by the diluted acid hydrolysis of rice hull in autoclave, on conditions: 121° C, 60 min, solid-liquid ratio of 1:10, 1 % volume fraction of sulfuric acid (da Cunha-Pereira et al., 2011). Soybean hull hydrolysate (SHH) was obtained by acid diluted hydrolysis of soybean hull in an autoclave, on conditions: 121° C, 40 min, solid-liquid ratio of 1:8, 3 % volume fraction of sulfuric acid (Cassales et al., 2011). The liquid fraction was recovered by filtration and the pH was adjusted to 5.5 with solid drops of sodium hydroxide. The acid fraction hydrolysates were vacuum-concentrated at 70 ^{o}C in order to increase their final sugar concentrations. RHH final concentration was (in g L^{-1}): glucose, 20; xylose, 25; arabinose, 6; and osmotic pressure of 1 918 mOsm Kg^{-1} for shaker experiments, and 1 402 mOsm Kg^{-1} for bioreactor experiments. SHH final concentration was (in g L^{-1}): glucose, 4; xylose, 6; arabinose, 2; mannose, 7; and 1493 mOsm Kg^{-1} osmotic pressure. The amount of toxic compounds formed during hydrolysis, in the final RHH was (in g L^{-1}): HMF, 0.07; furfural, 0.02; and for SHH was (in g L^{-1}): HMF, 0.04; furfural, 0.01. Except for glucose supplementation on RHH in bioreactor experiments, neither detoxification nor supplementations were made to the RHH or SHH.

2.3. Comparative kinetics of cultures in SHH and RHH

Yeasts were cultivated in 500 mL Erlenmeyer flask filled with 120 mL of RHH or SHH in an orbital shaker (Marconi MA 830, Brazil) at 180 rpm, 30° C for 72 h of cultivation. Biomasses of *S. cerevisiae* and *W. anomalus* were estimated as viable cells, using colony forming units per mL (CFU mL^{-1}) plated on YPD agar and YPX agar (composed of, in g L^{-1}, xylose, 20; yeast extract, 10; and peptone, 20). Experiments were made in duplicate.

2.4. Bioreactor experiments on RHH

Bioreactor experiments were carried out to simulate industrial conditions in ethanol plants. Initial inoculum size was composed of 10 % wet weight of yeasts, as described on item 2.2.. Experiments were carried out in fully equipped 2 L bioreactors (Biostat B, Braun Biotech International, Germany). The operational volume was 1 500 mL. For the isolated yeasts cultivations, 150 g of wet biomass was added into the medium. For co-cultures, 75 g of each yeast was added. The oxygen limitation concentration experiments were run using an aeration rate of 0.33 vvm, controlled by a needle valve and a rotameter. Temperature and agitation speed were maintained at 30 °C and 180 rpm, respectively. For anaerobic experiments, cells were cultivated at 30 ºC, 200 rpm and no aeration. Samples were collected at stipulated times for determination of biomass and quantification of sugars and alcohols. Experiments were made in duplicate.

2.5. Analytical methods and kinetic parameters calculation

Glucose, mannose, xylose, ethanol, xylitol, and glycerol concentrations were determined by HPLC (Shimadzu, Japan) equipped with a refractive index detector and Bio-Rad HPX-87H column (300 × 7.8 mm) using 5 mM sulfuric acid as eluent at 45 °C, flow rate of 0.6 mL min^{-1} and sample volumes of 20 μL. Furfural and HMF concentrations were determined by HPLC with a UV detector (at 276 nm) using a Nucleosil C18 column (250 × 4.6 mm) at room temperature, using acetonitrile–water (2:8) containing 10 g L^{-1} acetic acid as eluent, flow rate of 1.1 mL min^{-1} and sample volumes of 20 μL. Osmotic pressure

(mOsm Kg^{-1}) was determined by vapor pressure measurement in an Osmometer (Wescor, USA). The ethanol conversion yield ($Y_{P/S}$, g g^{-1}) was defined as the ratio of the concentration of ethanol produced and glucose consumed. The xylitol conversion yield ($Y_{P/S}$, g g^{-1}) was defined as the ratio of the concentration of xylitol produced and xylose consumed. The volumetric productivity (Q_p, g L^{-1} h^{-1}) was calculated using the ethanol or xylitol production versus time.

3. RESULTS AND DISCUSSION

3.1. Comparative kinetics of cultivations in SHH and RHH

The kinetics of *S. cerevisiae* P6H9 and *W. anomalus* WA-HF5.5 cultivated in SHH and RHH are shown Figure 1. Both yeasts were able to produce ethanol in these different hydrolysates. None of the strains were able to metabolize arabinose. For all tests and for both strains, furfural and HMF were depleted from the media in the first 12 hours of cultivation (Fig.1). It was observed that RHH affected the metabolism of *S. cerevisiae*. In this medium, the biomass concentration did not change; and residual glucose (3.6 g L^{-1}) still remained in the medium until 72 h cultivation (Fig. 1D). In SHH, however, this yeast depleted glucose and mannose until 36 h (Fig. 1A). Ethanol yield and productivity were the same for both media (Table 1). Martín et al., (2007), observed that the biomass production and ethanol yield and productivity were severely affected on recombinant xylose-utilizing *S. cerevisiae* strain TMB 3001, which was adapted and exposed to three different formulations of sugarcane bagasse hydrolysates. Glycerol was produced in high

concentrations in both conditions (Fig. 1A, 1D). This is an important metabolite produced in response to stresses, such as high osmotic pressure, high ethanol concentration, high partial pressure of CO_2, among others (Dhar et al., 2011; Pandey et al., 2007). Although P6H9 shows high tolerance to HMF (Sehnem et al., 2013a), there are other components present in lignocellulosic hydrolysates that can be toxic to cells, and hyperosmotic stress is an example. In a previous study, when eight *S. cerevisiae* industrial and laboratory strains were submitted to hyperosmotic medium using NaCl 1M, all strains showed less than 80 % survival (Garay-Arroyo et al., 2004). In this work, RHH osmotic pressure was determined to be 1 918 mOsm Kg^{-1} (equivalent to1.25 M NaCl). Although cell viability was high (CFU values in Fig 1A, 1D), this condition could present some level of toxicity for *S. cerevisiae* P6H9. Furan derivatives, weak acids, and phenolic compounds could also be present in toxic concentrations. These compounds inhibit the biomass formation or produce longer lag phases, as well as decrease the ethanol yield and volumetric productivity. Besides, synergic effects in these compounds also have been reported (Almeida et al., 2007).

W. anomalus physiological response contrasted with that of *S. cerevisiae* (Fig 1B, 1E). This yeast produced 10^9 CFU mL^{-1} cells, and consumed glucose faster than P6H9. No glycerol or xylitol were produced in RHH. Glucose, mannose and xylose were almost completely consumed in 72 h on SHH, but this consumption did not increase the production of ethanol. Xylitol was produced in low concentrations (1.3 g L^{-1}). Low yields and productivities in SHH can be explained by the metabolic preference to biomass production. On RHH, glucose was almost completely consumed in 24 h cultivation, and ethanol showed the highest production (7.7 g L^{-1}). However, xylose still remained in the medium until 72 h cultivation. Results indicate that this strain is less sensitive to the toxics

compounds present in lignocellulosic hydrolysates than P6H9. This trait might be due the physiological robustness of *W. anomalus* towards environmental stresses, such as extreme pH or low water activity (Passoth et al., 2011).

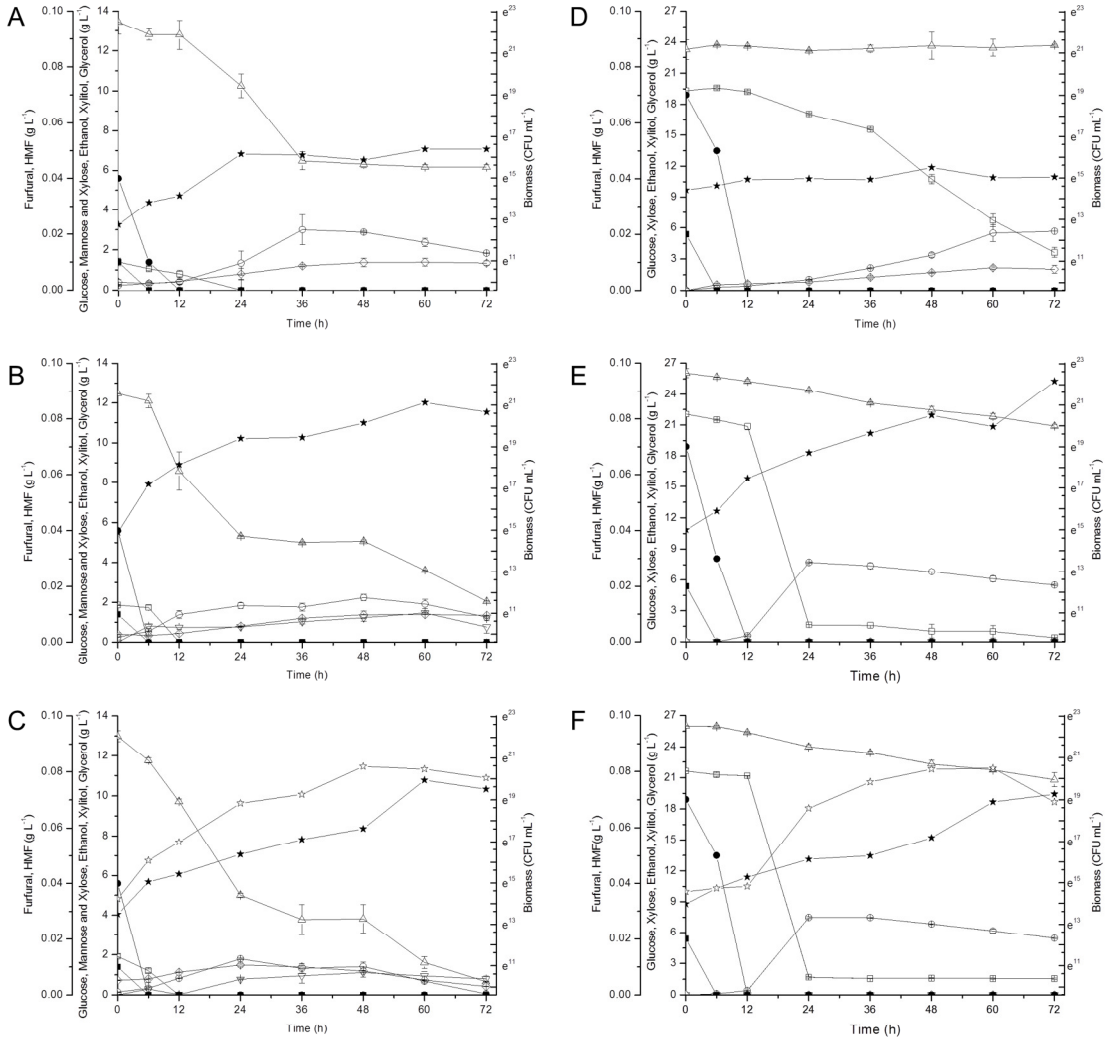

Fig. 1: Kinetics of *S. cerevisiae* P6H9 (A, D), *W. anomalus* WA-HF5.5 (B, E), and their co-cultures (C, F) in SHH (A, B, C), and RHH (D, E, F). Cultivations were run in orbital shaker at 150 rpm, 30 °C. Glucose, (□); Xylose and Mannose, (Δ) for SHH or Xylose for RHH; Ethanol, (○); Glycerol, (◇); Xylitol, (▽). Biomass production; (★). For panels C and F, *S. cerevisiae* biomass (★), and *W. anomalus* biomass (☆).

When *S. cerevisiae* and *W. anomalus* were cultivated together, ethanol yield and productivity did not change in relation to *W. anomalus* alone in SHH. However, using RHH, the ethanol productivity of the co-culture was the sum of the productivities of strains when they were grown alone (Table 1). *W. anomalus* showed stronger biomass formation than *S. cerevisiae* (Fig. 1C, 1F), but this yeast produce more biomass in relation to its sole cultivation, suggesting a synergic effect of these strains for sugar metabolism and ethanol production. A co-culture of *S. cerevisiae* OVB 11 and *P. stipitis* NCIM 3498 in batch fermentation showed improvement in ethanol yield and productivity when compared to the sole cultivation of *S. cerevisiae* (Yadav et al., 2011). The ethanol production of a co-culture of *S. cerevisiae* and *C. shehatae* was increased 22 % in relation to the *C. shehatae* culture in shaker flasks using RHH as medium (Hickert et al., 2013). In the present work, better results for biomass and ethanol productivity were obtained for RHH and this medium was selected for bioreactor tests. This behavior can be related to the difference in sugars composition of the hydrolysates. While RHH is composed by 20 g L^{-1} of glucose, SHH is composed by only 2 g L^{-1} this sugar. Since glucose is the main substrate used for ethanol production, its production is probably affected. Low ethanol production can be related to the presence of mannose. Despite this sugar is present in SHH and can be converted to ethanol, negative effects were reported concerning this hexose. A large variety of signals generated from the environment to the cellular machinery helps controlling metabolism, growth, and development. In *S. cerevisiae,* this signalization can be initiated with the transmembrane protein Gpr1, a member of the superfamily G protein-coupled receptors, and posterior activation of cAMP pathway. Mannose is coupled to Gpr1, and as consequence inhibits the signaling to cAMP (Lemaire et al., 2004).

Table 1. . Values of yields and productivities of ethanol for *W. anomalus* WA-HF5.5, *S. cerevisiae* P6H9, and their co-culture in the lignocellulosic hydrolysates. Results represent the mean of duplicate.

Strain	Soybean hull hydrolysate		Rice hull hydrolysate	
	$Y_{P/S}$	Qp	$Y_{P/S}$	Qp
S. cerevisiae P6H9	0.34±0.03	0.08±0.02	0.35±0.00	0.08±0.00
W. anomalus HF5.5	0.17±0.02	0.05±0.01	0.38±0.01	0.23±0.00
Co-culture	0.17±0.00	0.07±0.00	0.37±0.00	0.31±0.00

3.2. Bioreactor experiments using RHH

Bioreactor cultivations were carried out using RHH for the cultivation of all yeast combinations and results are shown in Figure 2. The pure culture of *S. cerevisiae* strain under anaerobic condition (Fig. 2A) showed an ethanol productivity of 6.7 g L^{-1} h^{-1}, the highest obtained in this work, with a peak of production in 1.5 h. Under oxygen limited condition (Fig. 2D), ethanol productivity was 2.69 g L^{-1} h^{-1} (Table 2). High ethanol productivities were obtained probably due to high initial biomass. A study realized to improve ethanol production by *S. cerevisiae* varying initial inoculum, sugar and temperature showed that increasing ethanol yields can be obtained using increasing inoculum sizes and that a high level of inoculum leads to rapid fermentation (Laluce et al., 2009).

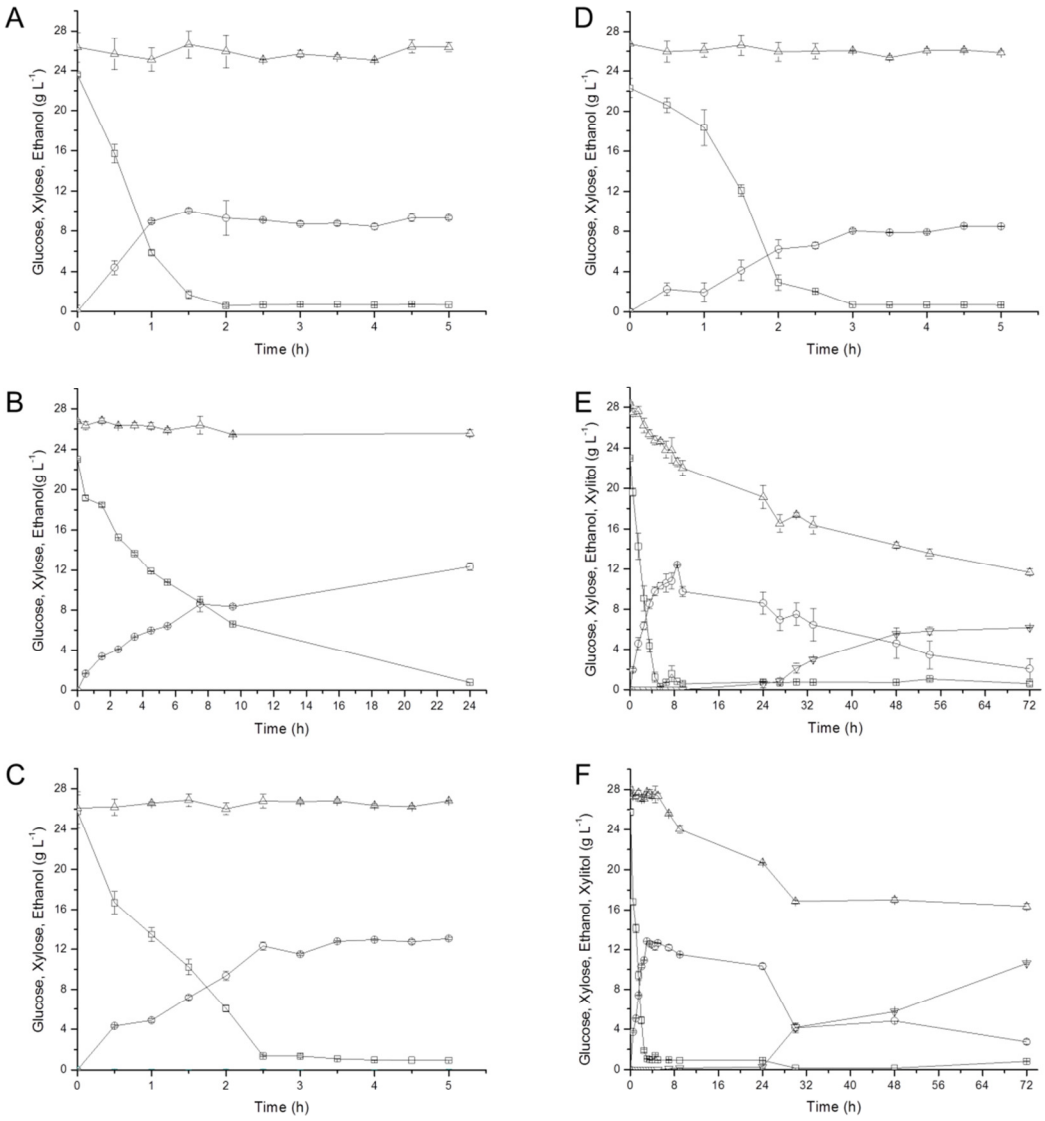

Fig. 2: Kinetics of bioreactor cultivations of *S. cerevisiae* P6H9 (A, D), *W. anomalus* WA-HF5.5 (B, E), and their co-cultures (C, F) growing in RHH under anaerobic conditions (A, B, C), and oxygen limitation conditions (D, E, F). Glucose, (□); Xylose, (Δ); Ethanol, (○); Xylitol, (▽). Results represent the mean of duplicate.

W. anomalus showed low ethanol productivity, even though ethanol yields were close to the maximum theoretical (Table 2), because under anaerobic condition, glucose was completely consumed in the first 24 h cultivation. As expected, xylose was not

consumed in this condition (Fig. 2B). Anaerobic growth of yeasts on xylose has not been reported, probably because there is insufficient ATP formation during anaerobic metabolism to allow xylose consumption (Wahlbom & Hahn-Hägerdal, 2002). However, under oxygen limitation condition, xylose was consumed and xylitol was produced to yields of 0.37 g g^{-1}. Similar metabolisms were reported for *Candida maltosa* Xu316 cultured in xylose-rich medium (50 g L^{-1}) (Guo et al., 2006), and for *C. guilliermondii* cultivated in soybean hull hydrolysate (Schirmer-Michel et al., 2008), both strains showing metabolism of glucose, ethanol and xylose under limited oxygen conditions. When sugars are metabolized under anaerobiosis, NADH formed in the glycolytic pathway is regenerated through the reduction of acetaldehyde to ethanol. Under aerobic conditions, respiration of the accumulated ethanol occurs after depletion of the fermentable sugar, allowing the optimal use of the sugar carbon (de Smidt et al., 2008).

The anaerobic co-cultivation (Fig. 2C) produced ethanol yields of 0.51 g g^{-1}, and productivity reached 3.84 g L^{-1} h^{-1}, which did not improve on the cultivation of *S. cerevisiae* under the same conditions. This might be a consequence of the reduced inoculum size used in the co-cultivation. However, under oxygen limitation conditions, co-culture ethanol productivity was higher, up to 4.28 g L^{-1} h^{-1} when compared to *S. cerevisiae* (2.69 g L^{-1} h^{-1}) and the *W. anomalus* (1.46 g L^{-1} h^{-1}) (Table 2). Although ethanol was been consumed in this condition, xylitol was produced in higher amounts (0.86 g g^{-1}) when compared to *W. anomalus* (0.37 g g^{-1}) (Fig. 2F). Co-cultures of *S. cerevisiae* ICV D254 and *S. arborariae* NRRL Y-48658 showed xylitol yields of 0.45 g g^{-1} (da Cunha-Pereira et al., 2011), while co-culture of *S. cerevisiae* ICV D254 and *C. shehatae* HM 52.2 produced only xylitol yields of 0.11 g g^{-1} (Hickert et al., 2013). Comparatively, the results in this work indicate that *W. anomalus* WA-HF5.5 strain has the potential for xylitol

production on lignocellulosic hydrolysates. Studies of co-cultures of yeasts, or bacteria and yeasts, have frequently shown that there is an advantage for ethanol production, but the reasons of these improvements are not clear (Keating et al., 2004).

Table 2. Values of yields and productivities of ethanol and xylitol for *W. anomalus* WA-HF5.5, *S. cerevisiae* P6H9, and their co-culture in rice-hull hydrolysate, in the bioreactor. Results represent the mean of duplicate.

Strain	Condition	$Y_{P/S\ \text{ethanol}}$	Qp_{ethanol}	$Y_{P/S\ \text{xylitol}}$	Qp_{xylitol}
S. cerevisiae P6H9	Anaerobic	0.46±0.05	6.70±0.14	-	-
S. cerevisiae P6H9	Oxygen limited	0.37±0.02	2.69±0.10	-	-
W. anomalus HF5.5	Anaerobic	0.49±0.04	0.52±0.01	-	-
W. anomalus HF5.5	Oxygen limited	0.50±0.05	1.46±0.09	0.37±0.03	0.09±0.03
Co-culture	Anaerobic	0.51±0.01	3.84±0.06	-	-
Co-culture	Oxygen limited	0.51±0.02	4.28±0.03	0.86±0.14	0.15±0.01

4. CONCLUSION

In this work, a comparative study using the osmotolerant and furaldehyde-tolerant *W. anomalus* strain WA-HF5.5 and the HMF-tolerant *S. cerevisiae* strain P6H9, their co-cultures in SHH and RHH showed that both strains were capable to produce ethanol in both lignocellulosic hydrolysates, and the co-culture presented a synergistic effect on biomass and ethanol production. In the bioreactor experiments, best condition for ethanol

production was anaerobiosis in pure cultures of *S. cerevisiae* strain. However, *W. anomalus* showed high xylitol-producing capability when cultivated under oxygen limitation conditions in co-culture with *S. cerevisiae*. Results indicated that these strains are capable to produce second-generation ethanol and xylitol in RHH and SHH, with potential to be used in biorefinary plants.

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5. DISCUSSÃO GERAL

Este trabalho visou elucidar os mecanismos de resistência de leveduras etanologênicas a furaldeídos, estudar as respostas fisiológicas das mesmas na presença de altas concentrações desses compostos tóxicos, assim como investigar a possibilidade da aplicação industrial dessas leveduras na produção de etanol de segunda geração. Na primeira etapa do trabalho, uma linhagem industrial de *S. cerevisiae* altamente tolerante a HMF foi obtida por engenharia evolutiva, denominada P6H9. A expressão dos genes responsáveis por essa tolerância foi avaliada, assim como a relação dessa expressão com as respostas fisiológicas dessa levedura para a produção de etanol. Na segunda etapa, a levedura fermentadora de pentoses e naturalmente resistente à pressão osmótica *W. anomalus* também foi submetida à estratégia de engenharia evolutiva, resultando em uma linhagem resistente a furfural e HMF e com aumento da resistência à pressão osmótica, nomeada WA-HF5.5. A cinética de produção de etanol dessa levedura foi avaliada quanto à produção de etanol e quanto à produção de enzimas redutoras dos compostos furânicos. Finalmente, para avaliar a aplicabilidade dessas leveduras em um sistema industrial, as mesmas foram cultivadas em cultivo submerso nas condições semelhantes a plantas industriais de produção de etanol, utilizando como substratos hidrolisados de casca de arroz e de casca de soja, em cultivos isolados ou em sistemas de co-cultivos.

5.1. Estudos de tolerância ao HMF em *Saccharomyces cerevisiae*

Das quatro linhagens de *S. cerevisiae* cultivadas na presença de 5 g L^{-1} de HMF, duas apresentaram maior sensibilidade ao composto, ao passo que apenas as linhagens P6 e P18R foram capazes de reduzir o HMF a níveis próximos de zero. Essa diversificação era esperada, já que a capacidade de tolerância a compostos furânicos difere entre linhagens de *S. cerevisiae* (Petersson *et al.*, 2006). Além disso, a toxicidade do HMF é diretamente proporcional à sua concentração. Estudos apontaram que, nas linhagens de *S. cerevisiae* ATCC 211239 e NRRL Y-12632, a produção de biomassa na presença desse composto tóxico, mesmo em baixas concentrações, não alcançou a produção da condição controle (Liu *et al.*, 2004). Esse padrão também foi observado no perfil da produção de etanol das linhagens de *S. cerevisiae* T1 e Y-1528 (Keating *et al.*, 2006). Essa diminuição do metabolismo pode ser ocasionada pela inibição que os furaldeídos causam sobre enzimas da via glicolítica, como triose fosfato desidrogenase e álcool desidrogenase e, por consequência, afetam o metabolismo oxidativo das leveduras (Banerjee *et al.*, 1981; Sanchez e Bautista, 1988).

Após ser submetida a altas concentrações de HMF, a linhagem P6H9 apresentou alta tolerância ao composto (9 g L^{-1}). Essa característica aumentou durante o processo de engenharia evolutiva pois essa linhagem foi isolada de melaço de cana, o qual é submetido a altas temperaturas e possivelmente possua furaldeídos em sua composição (Da Silva-Filho *et al.*, 2005). A engenharia evolutiva é uma estratégia que tem sido aplicada para se obter diversos mutantes de *S. cerevisiae* para inúmeras aplicações como o aumento da capacidade de assimilação de gluconato com aplicação em vinhos (Cadiere *et al.*, 2011) ou a co-assimilação de glicose e xilose em uma linhagem recombinante (Kuyper *et al.*, 2005),

e também para a seleção de uma linhagem mutante que seja tolerante ao ácido sulfúrico (De Melo *et al.*, 2010).

A linhagem P6H9 foi cultivada na ausência e presença de HMF usando glicose como fonte de carbono em microaerofilia, ou utilizando sacarose como fonte de carbono em anaerobiose. Os mecanismos de tolerância ao HMF têm sido investigados e estudos apontam para os genes *ADH6*, *ADH7*, *SFA1* e *ARI1* como envolvidos na tolerância a esse composto, os quais foram investigados neste trabalho (Petersson *et al.*, 2006; Liu *et al.*, 2009; Liu e Moon, 2009). Foi possível observar que a expressão dos genes *ADH7* e *ARI1* foram altamente induzidas na linhagem P6H9 nas duas condições testadas.

O gene *ADH6* foi o primeiro a ser relacionado à tolerância ao HMF na levedura *S. cerevisiae* por análise de expressão gênica global, e a sua importância na redução do HMF foi demonstrada pela superexpressão deste gene na linhagem CEN.PK 113-5D. Esse estudo demonstrou um aumento da atividade das enzimas álcool desidrogenases produzidas pela levedura (Petersson *et al.*, 2006). O produto codificado pelo gene *ADH6* é álcool desidrogenase 6 (Adh6p). Essa enzima possui atividade redutora sobre uma grande variedade de compostos que variam entre álcoois aromáticos e aldeídos, os quais utilizam NADPH como cofator (Larroy *et al.*, 2002).

A superexpressão do gene *SFA1* também foi detectada na linhagem TMB3000, no entanto, não foi observado aumento da velocidade de redução do HMF (Petersson *et al.*, 2006). A proteína Sfa1p possui uma estrutura molecular de função biológica similar às enzimas álcool desidrogenases (Wehner *et al.*, 1993) e foi evidenciado que essa enzima possui uma característica bifuncional, tanto com atividade de formaldeído desidrogenase dependente de glutationa, quanto na atividade de álcool desidrogenase de cadeia longa (Dickinson *et al.*, 2003).

A enzima que é codificada pelo gene *ARI1* foi descrita primeiramente como uma redutase dependente de NADPH que utiliza acetoacetato de etila como substrato (Katz *et al.*, 2003). Também, possui capacidade de converter fenilpiruvato a feniletanol, na função de carbonil redutase (Hwang *et al.*, 2009). Recentemente, Liu e Moon (2009) demonstraram que a proteína codificada por esse gene, aldeído redutase (Ari1p) possui atividades redutoras dependentes de NADPH sobre pelo menos 14 substratos de função aldeído, entre estes furfural e HMF.

O produto codificado pelo gene *ADH7* é uma enzima da família das cinamil álcool desidrogenases dependentes de NADPH. Essa enzima tem sido relatada pela sua importância na destoxificação de vários compostos, entre esses furfural e vanilina (Larroy *et al.*, 2002). A superexpressão desse gene também foi relacionada à capacidade de redução do HMF (Liu *et al.*, 2008).

Quando as leveduras foram avaliadas em cultivo submerso na presença e ausência de HMF, foi possível estabelecer algumas relações entre as respostas fisiológicas das mesmas com a indução da expressão dos genes estudados. A linhagem tolerante P6H9, na presença de HMF, apresentou maior capacidade de produção de biomassa, maior velocidade na redução de HMF e maior atividade enzimática. Além disso, a produção de etanol nessa linhagem foi a menos prejudicada. Todas essas características estão relacionadas com o aumento da expressão do gene *ADH7*. A produção aumentada de glicerol pode ter sido induzida pelo estresse ou por algum tipo de desequilíbrio metabólico, causado direta ou indiretamente pela redução do HMF. Estudos na produção de glicerol demonstraram que a baixa atividade de diferentes isoenzimas de álcool desidrogenases, especialmente Adh1p, resulta no aumento da produção de glicerol em *S. cerevisiae* (Johansson e Sjostrom, 1984). Essa enzima é responsável pela redução anaeróbica de

acetaldeído a etanol (Taherzadeh *et al.*, 2002). Também, análises relacionadas ao metabolismo de glicose em células com genes *ADH* deletados mostram que a ausência de todas as suas isoenzimas conhecidas gera glicerol em maior concentração, seguido pela formação de acetaldeído e acetato (Drewke *et al.*, 1990).

No presente trabalho também foi observado aumento na produção de acetato na presença de HMF. Esse mesmo fenômeno foi relatado para uma linhagem de *S. cerevisiae* recombinante que superexpressava o gene *ADH6* em sistema de batelada (Almeida *et al.*, 2008). Na levedura *S. cerevisiae*, existem duas principais rotas metabólicas responsáveis pela formação de NADPH, a via das pentoses fosfato e a reação da enzima acetaldeído desidrogenase. A primeira rota direciona o carbono da glicólise e junto com a enzima relacionada ao estresse glicerol 3-fosfato desidrogenase, reduz o estoque de NADH, enquanto a segunda via direciona o acetaldeído para a produção de acetato. Então a demanda metabólica por NADPH poderia levar ao aumento da produção de acetato, e como consequência, diminuir a produção de etanol.

A linhagem P6H9 apresentou altos níveis de tolerância ao HMF. De acordo com as buscas bibliográficas, até o momento, essa levedura é a que apresenta a maior tolerância ao HMF. Além disso, essa levedura é capaz de produzir alta produtividade de etanol na presença de elevadas concentrações desse composto tóxico.

5.2. Estudos de tolerância a furaldeídos e pressão osmótica em *Wickerhamomyces anomalus*

Embora *S. cerevisiae* seja o microrganismo conhecido com maior capacidade de conversão de hexoses a etanol, essa levedura não possui capacidade de metabolizar pentoses. Porém, para que a produção de etanol de segunda geração torne-se viável, existe a necessidade de utilização de todos os açúcares disponíveis. Além disso, durante o processo de hidrólise de materiais lignocelulósicos, por consequência, ocorre a formação de furfural juntamente com HMF.

Por essas razões, a levedura *W. anomalus*, que possui capacidade fermentadora de pentoses e é naturalmente resistente à pressão osmótica, foi submetida a altas concentrações de furfural e HMF. Após o período de seleção, foi obtida uma linhagem tolerante a furfural e HMF. Essa linhagem foi nomeada WA-HF5.5. A importância de se obter uma linhagem de levedura tolerante aos dois compostos é para a diminuição à inibição devida aos efeitos tóxicos sinérgicos dos mesmos sobre microrganismos, os quais já foram investigados nas leveduras *S. cerevisiae* e *S. arborariae* em trabalhos anteriores. A adição de HMF e furfural no cultivo da linhagem CBS8066 de *S. cerevisiae* causou diminuição na taxa específica de crescimento (Taherzadeh *et al.*, 2000), enquanto que na levedura *S. arborariae*, o crescimento celular não foi observado na presença furfural, HMF e ácido acético (Da Cunha-Pereira *et al.*, 2011).

As análises de cinética de crescimento e produção de etanol realizadas nessa levedura mostraram que a linhagem WA-HF5.5 possui maior tolerância a furfural e HMF com relação à linhagem parental. Essa linhagem possuiu capacidade de produzir biomassa,

reduzir os furaldeídos e produzir etanol em todas as condições testadas. Um resultado muito interessante é o fato dessa linhagem ter reduzido os compostos a níveis perto de zero quando associados, indicando um possível um mecanismo de resistência aos dois compostos. Na levedura *S. cerevisiae* NRRL Y-50049, a qual possui um certo nível de tolerância a furfural e HMF, foi demonstrado que a tolerância a esses compostos é dependente da regulação de diversos genes presentes na via glicolítica e na via das pentoses fosfato (Liu *et al.*, 2008). Entretanto, na levedura *W. anomalus*, não existem trabalhos que abordem a sensibilidade ou tolerância a furaldeídos. Nessa condição, o furfural foi reduzido anteriormente ao HMF. Resultados semelhantes já haviam sido relatados em um estudo utilizando a levedura *S. cerevisiae*. A diferença nas velocidades de assimilação podem ser causadas pelas diferenças na estrutura química dos dois compostos. O HMF possui o grupo hidroximetil, que confere ao composto características de baixas hidrofobicidade e permeabilidade à membrana, acarretando em baixas taxas de conversão do HMF (Taherzadeh *et al.*, 2000).

Também foi possível observar que nessa levedura a capacidade redutora dos furaldeídos está fortemente ligada à atividade enzimática específica. Na presença dos furaldeídos, tanto furfural quanto HMF, a combinação de ambos, há indução da produção de enzimas com capacidade redutora desses compostos. As enzimas conhecidas por catalisar essa reação são as álcool desidrogenases, as quais são bem caracterizadas em *S. cerevisiae*. As álcool desidrogenases fazem parte de uma grande família de enzimas responsáveis pela oxidação reversível de álcoois a aldeídos, com a redução concomitante de NAD^+ ou $NADP^+$ (De Smidt *et al.*, 2008). As enzimas produzidas pela linhagem WA-HF5.5 demonstraram alta afinidade pelo NADH. Em *S. cerevisiae*, as enzimas identificadas como responsáveis pela atividade redutora dos furaldeídos podem apresentar

preferência tanto por NADH quanto por NADPH (Liu, 2011), Entretanto, algumas enzimas possuem preferência pelo NADPH, como por exemplo as Adh6p (Petersson *et al.*, 2006), ou por NADH, como a Adh1p (Laadan *et al.*, 2008).

Assim como observado na linhagem P6H9 de *S. cerevisiae*, a produção de glicerol foi aumentada na presença dos compostos tóxicos na linhagem WA-HF5.5. Como discutido anteriomente, a produção desse metabólito pode ser devida a respostas ao estresse e também por desequilíbrio metabólico. Na levedura *S. cerevisiae*, mudanças no fluxo metabólico acarretam na produção de diversos compostos, como glicerol ou trealose, os quais auxiliam na proteção da célula a condições de estresse, como por exemplo pressão osmótica, altas concentrações de etanol ou altas concentrações de CO_2 (Pandey *et al.*, 2007). Na levedura *W.anomalus*, a produção de glicerol em resposta a condições de estresse ou de mudança no meio já foi relatada (Djelal *et al.*, 2012).

Com o objetivo de avaliar se o processo de adaptação a compostos furânicos havia afetado a capacidade dessa levedura resistir a altas pressões osmóticas, a mesma foi submetida a altas concentrações de NaCl. Foi possível observar que a taxa específica de crescimento só foi diminuída a partir da concentração de 1M de NaCl e não foi observada morte celular em nenhuma condição. A tolerância da levedura *W. anomalus* a condições de baixa atividade de água é bem conhecida. Nessas condições, o principal composto produzido para realizar o ajuste osmótico intracelular é o glicerol (Djelal *et al.*, 2012). Estudos anteriores demonstraram que o aumento da pressão osmótica causa uma diminuição na produção de etanol e na viabilidade celular (Pratt *et al.*, 2003). Entretanto, no presente trabalho, os resultados demonstraram que as alterações genéticas que ocorreram em várias gerações possibilitaram o aumento da resistência a furaldeídos, e parecem ter influenciado positivamente na resistência ao aumento da pressão osmótica.

Uma possível explicação para esse aumento pode estar relacionada com a via da produção de glicerol em resposta à alta osmolaridade (via HOG). Estudos já apontaram essa via como relacionada à resposta a estresse osmótico e também à tolerância a furaldeídos (Liu, 2012).

A linhagem parental WA-001 de *W. anomalus*, a qual possui capacidade de fermentar pentoses e resistir a altas pressões osmóticas, também possui capacidade de tolerar a presença de furfural e HMF, e ao mesmo tempo, produzir etanol. De acordo com as buscas bibliográficas realizadas, essa é a primeira levedura fermentadora de pentoses que possui tais características. Assim, essa levedura possui grande potencial para a produção de etanol de segunda geração.

5.3. Avaliação da capacidade de produção de etanol de *Saccharomyces cerevisiae* e *Wickerhamomyces anomalus* simulando condições industriais

Com o objetivo de investigar a capacidade de produção de etanol de segunda geração pelas leveduras *S. cerevisiae* P6H9 e *W. anomalus* WA-HF5.5 em hidrolisados lignocelulósicos, testes foram realizados em hidrolisados de casca de arroz (RHH) e casca de soja (SHH).

Com relação ao experimento comparativo em agitador orbital, as duas linhagens apresentaram capacidade de produzir etanol nos dois diferentes meios. Entretanto, a linhagem P6H9 demostrou maior sensibilidade no meio RHH, apresentando concentração de biomassa estável e glicose residual até o final do cultivo. Embora essa linhagem seja altamente tolerante ao HMF, outros componentes presentes no meio de cultivo podem ser

tóxicos para leveduras, como por exemplo a pressão osmótica. A sensibilidade de *S. cerevisiae* a meio hiperosmótico foi relatada por Garay-Arroyo et al. (2004) e todas as linhagens estudadas, dentre elas quatro industriais e quatro laboratoriais, quando submetidas a meio com NaCl 1M, apresentaram taxa de sobrevivência abaixo de 80 %. No presente trabalho, a pressão osmótica do RHH foi de 1918 mOsm Kg^{-1} e essa linhagem poderia ser sensível a essa condição. Além da pressão osmótica, os hidrolisados lignocelulósicos são formados por uma grande variedade de compostos liberados durante o processo de hidrólise, como os compostos furânicos, ácidos fracos e compostos fenólicos, que também poderiam interferir no crescimento dessa levedura. Como mencionado anteriormente, também podem ocorrer efeitos sinérgicos tóxicos sobre leveduras (Almeida *et al.*, 2007).

Com relação à linhagem WA-HF5.5 de *W. anomalus*, os resultados demonstraram que essa levedura apresentou baixa sensibilidade aos hidrolisados lignocelulósicos, sendo capaz de produzir biomassa, etanol e consumir todos os açúcares disponíveis no meio. Esse perfil metabólico pode ser devido às respostas fisiológicas a condições de estresse características dessa espécie, tais como resistência a pHs extremos ou resistência à alta pressão osmótica (Passoth *et al.*, 2011).

No experimento de co-cultivo em SHH, os resultados de rendimento e produtividade foram semelhantes aos obtidos por WA-HF5.5 cultivada isoladamente. Esse comportamento ocorreu provavelmente devido à baixa concentração de açúcares nesse tipo de hidrolisado. Já no meio RHH, o valor de produtividade obtido na co-cultura foi a soma das produtividades dos cultivos isolados das duas leveduras. Além disso, a produção de biomassa em *S. cerevisiae* foi favorecida pela presença da levedura *W. anomalus*. Esses resultados indicam que uma possível interação positiva influenciou na produção de

biomassa e etanol. Outros trabalhos já relataram a presença de interações positivas em co-cultivos para a produção de etanol. Uma co-cultura de *S. cerevisiae* OVB 11 e *P. stipitis* NCIM 3498 em sistema de batelada resultou em um aumento no rendimento e produtividade de etanol quando comparado com o cultivo de *S. cerevisiae* isolada (Yadav *et al.*, 2011). E mais recentemente, um estudo comparativo da produção de etanol entre co-cultivo de *S. cerevisiae* e *C shehatae* e *C. shehatae* cultivada isoladamente em RHH demonstrou aumento do rendimento de etanol no co-cultivo (Hickert *et al.*, 2013). Estudos realizados em co-culturas de bactérias e leveduras, ou entre leveduras, têm demonstrado que existe uma vantagem na produção de etanol quando as características metabólicas de cada linhagem são exploradas, entretanto, as razões dessa vantagem ainda não foram elucidadas (Keating *et al.*, 2004).

Com relação aos cultivos em biorreatores e em RHH, nos quais as leveduras foram cultivadas simulando-se um sistema de produção de etanol industrial, com alta quantidade de biomassa, foi possível observar que o melhor resultado de produtividade de etanol foi obtido no cultivo de *S. cerevisiae* em anaerobiose.

Embora o rendimento de etanol tenha ficado em valores próximos ao teórico, *W. anomalus* apresentou baixa produtividade de etanol em anaerobiose em 72 horas de cultivo. Como esperado, a xilose não foi metabolizada nessa condição. O crescimento anaeróbico a partir de xilose em leveduras ainda não foi evidenciado, provavelmente devido à geração insuficiente de ATP que é necessário durante o metabolismo desse açúcar (Wahlbom e Hahn-Hägerdal, 2002). Já em microaerofilia, após o consumo da glicose, foi observado consumo de xilose e produção de xilitol. Porém, o etanol também foi consumido ao mesmo tempo que a xilose. A preferência de consumo de glicose já foi demonstrada por Guo et al. (2006) na levedura recombinante *Candida maltosa* (Xu316) cultivada em meio

sintético, e também por Schirmer-Michel et al. (2008) em *C. guilliermondii* cultivada em SHH. Nesses trabalhos, em condições de microaerofilia, após o consumo total de glicose, etanol e xilose foram metabolizados. Já com relação ao etanol, esse produto é consumido por causa de um mecanismo que as leveduras possuem para utilizar completamente as fontes de carbono disponíveis. Quando a glicose é metabolizada por via fermentativa, o NADH formado na via glicolítica é regenerado a NAD^+ através da redução de acetaldeído a etanol. Sob condições aeróbicas, o consumo do etanol acumulado ocorre após a glicose ser completamente consumida (De Smidt *et al.*, 2008). O consumo do etanol ser favorecido com relação ao consumo de xilose ocorre porque ele é mais facilmente metabolizado que a xilose.

Nos experimentos de co-cultivo, a condição de microaerofilia apresentou altos valores de rendimentos e produtividade de etanol. Nesse caso, o sistema de co-cultivo demonstra vantagens em relação ao sistema de cultivo de culturas isoladas. Além disso, foi observado alto rendimento na produção de xilitol (0.86 g g^{-1}), indicando um grande potencial para produção de xilitol em hidrolisados lignocelulósicos. Estudos realizados anteriormente por outros colaboradores do nosso grupo de pesquisa, também com RHH, demonstraram que outras leveduras também possuem capacidade de produzir xilitol nesse meio, porém em menores quantidades. A co-cultura de *S. cerevisiae* ICV D254 e *S. arborariae* NRRL Y-48658 apresentou rendimento de etanol de 0.45 g g^{-1} (Da Cunha-Pereira *et al.*, 2011), enquanto que a co-cultura da mesma linhagem de *S. cerevisiae* e *C. shehatae* HM 52.2 mostrou baixos rendimentos de xilitol (0.11 g g^{-1}) (Hickert *et al.*, 2013). Altos rendimentos de xilitol também foram observados para *C. guilliermondii* (0,81 g g^{-1}), entretanto o regime de cultivo foi realizado em células imobilizadas (Carvalho *et al.*, 2005).

O xilitol é um produto de grande interesse econômico e também possui potencial de produção a partir de hidrolisados lignocelulósicos. Esse composto é um poliálcool de cinco carbonos e, em humanos, não é metabolizado (Ko *et al.*, 2008) Além disso, possui capacidade adoçante equivalente à sacarose, o que faz dele um adoçante com baixo valor calórico. Também, devido às suas propriedades anticariogênicas, o xilitol se torna um açúcar de interesse nas áreas de processamento de alimentos, nutrição e farmacêutica (Vandeska *et al.*, 1995; Ko *et al.*, 2008) .

Concluindo, os cultivos em hidrolisado de casca de soja apresentaram baixos valores de produtividade de etanol, por essa razão não foram testados em biorreatores. Em hidrolisado de casca de arroz em biorreatores, a maior produção de etanol foi em cultivo de *S. cerevisiae*, entretanto, o sistema de co-cultivo em microaerofilia apresentou altas produções de etanol e xilitol. Finalizando, as duas linhagens avaliadas possuem grande potencial para a produção de etanol, e também de xilitol, a partir de hidrolisados lignocelulósicos.

6. CONCLUSÕES

No presente trabalho, a linhagem P6H9 de *S. cerevisiae*, mutante proveniente de uma linhagem industrial isolada de melaço de cana, foi obtida pela estratégia de engenharia evolutiva para tolerância a altas concentrações de HMF. Essa característica foi relacionada com os altos níveis de expressão dos genes *ADH7* e *ARI1*. Na presença de HMF, essa linhagem apresentou aumento na atividade enzimática redutora de HMF, além de demonstrar melhor perfil cinético de produção de biomassa e etanol com relação às linhagens sensíveis JP1 e BY4741.

A linhagem osmotolerante WA-HF5.5 de *W. anomalus* foi obtida por engenharia evolutiva para tolerância a altas concentrações de furfural e HMF. Seu perfil cinético demonstra que a presença de furaldeídos aumenta os níveis de atividade de álcool desidrogenases, ao mesmo tempo que produz etanol com maior eficiência que a linhagem parental. Também, a mutação que confere tolerância aos furaldeídos aumentou a resistência à pressão osmótica nessa linhagem.

As duas linhagens investigadas nesse trabalho possuem capacidade de produção de etanol em hidrolisados lignocelulósicos de casca de soja e de casca de arroz. Em sistemas de biorreatores, a condição de co-cultivo em microaerofilia apresentou grande potencial para produção de etanol de segunda geração, já que foram obtidos altos valores de rendimento de etanol e de xilitol.

Os resultados obtidos nesse trabalho indicam que as linhagens P6H9 de *S. cerevisiae* e WA-HF5.5 de *W. anomalus* possuem grande potencial para produção de etanol e xilitol de segunda geração.

7. PERSPECTIVAS

Este trabalho apresentou a possibilidade de produção de etanol de segunda geração com o emprego de duas linhagens tolerantes a furaldeídos, tanto em cultivos isolados quanto em sistemas de co-cultivos. Portanto, é fundamental a continuidade na realização de trabalhos visando aumento das produtividades de álcoois e escalonamento dos processos.

Uma possibilidade de melhoramento da produtividade de etanol é a manipulação genética da levedura *S. cerevisiae*, com o objetivo de aumentar a disponibilidade de cofatores enzimáticos, aumentando assim a produção de etanol na presença de HMF.

Com relação à levedura *W. anomalus*, é importante aprofundar o conhecimento a respeito do mecanismo de tolerância aos compostos furânicos e também de resistência à pressão osmótica, visto que essa é a primeira levedura fermentadora de pentoses descrita com tal característica.

Também é importante investigar as razões pelas quais o sistema de co-cultivo apresenta vantagens na produção de etanol com relação aos cultivos isolados em hidrolisados lignocelulósicos.

A otimização das condições de cultivo com o objetivo de aumentar a produção de etanol, ou de xilitol, além de evitar consumo de produto, é um fator importante para viabilizar esse processo. Além disso, estudos de imobilização celular devem ser realizados.

Por fim, realizar estudos de escalonamento em biorreatores são de suma importância para avaliar a viabilidade econômica da produção de etanol de segunda geração.

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5-Hydroxymethylfurfural induces *ADH7* and *ARI1* expression in tolerant industrial *Saccharomyces cerevisiae* strain P6H9 during bioethanol production

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HIGHLIGHTS

- Evolutionary engineering was used to select industrial *S. cerevisiae* tolerant to HMF.
- Expression levels of genes involved with HMF tolerance were studied.
- *ARI1* and *ADH7* genes were overexpressed in the presence of HMF.
- P6H9 showed higher ethanol productivities in HMF than sensitive strains.
- *S. cerevisiae* P6H9 has potential to be used for second-generation ethanol production.

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ABSTRACT

The aims of this work were to obtain, by evolutionary engineering, an industrial strain of *Saccharomyces cerevisiae* tolerant to high concentrations of HMF and to determine the expression levels of genes previously described as responsible for this tolerance. Cells were grown under anaerobic and oxygen limited conditions, in the presence of glucose or sucrose as carbon sources. P6H9 strain presented high expression levels for genes *ADH7* and *ARI1* in presence of HMF. This tolerant strain also showed higher ethanol productivity, biomass formation and alcohol dehydrogenase activity comparing to sensitive strains. Results suggest that *S. cerevisiae* P6H9 strain presents potential to be used for second-generation ethanol production.

1. Introduction

The development of second-generation bioethanol from lignocellulosic biomass has become widely important and is attracting global attention, since it is a renewable energy source and does not compete for areas designated for food production (Menon and Rao, 2012). The production of bioethanol from lignocellulosic feedstock requires pretreatments and hydrolyses steps to release sugars for yeast fermentation. Steam explosion and dilute-acid hydrolysis are already in use at pilot scale, but these technologies release, in addition to sugars, a wide range of compounds, which are inhibitory of yeasts metabolism. Therefore, the main challenge to turn this into an economically feasible process, is to minimize sugar degradation and the formation of furanic and phenolic compounds (Almeida et al., 2008; Margeot et al., 2009).

The main yeast metabolism inhibitors are the 2-furaldehyde (furfural), formed by pentoses dehydration, and 5-hydroxymethyl-2-furaldehyde (5-hydroxymethylfurfural–HMF), which is formed by hexoses dehydration (Liu et al., 2009). The generation of furfural during sugarcane bagasse acid hydrolysis can reach up to 5 g L^{-1}, (Aguilar et al., 2002), while HMF can accumulate to 6 g L^{-1} in hydrolysates from chipped pine wood (Larsson et al., 1999). Furans interfere with microbial growth and subsequent fermentation, interfering with glucolytic enzymes such as triose

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phosphate dehydrogenase, alcohol dehydrogenase, aldehyde dehydrogenase, and pyruvate dehydrogenase, as well as with protein and RNA synthesis (Modig et al., 2002; Sanchez and Bautista, 1988). In general, the effects of furans can be explained by a redirection of cell flux energy to damage repairing caused by these compounds and by reduced intracellular ATP and NAD(P)H levels, caused by enzymatic inhibition or consumption/regeneration of cofactors (Almeida et al., 2007).

On the other hand, HMF toxicity is associated with yeast cell damages, since the presence of its hydroxymethyl group leads to a reduced hydrophobicity and membrane permeability, which causes a low conversion rate of this compound (Taherzadeh et al., 2000). In *S. cerevisiae*, HMF toxicity was reported to be dose-dependent (Liu et al., 2004). The reduction of the aldehyde into a less toxic corresponding alcohol is a common detoxification strategy, which is mainly catalyzed by alcohol dehydrogenases. The main product described from HMF conversion in *S. cerevisiae* is 2,5-bis-hydroxymethylfuran (HMF alcohol) under aerobic and anaerobic conditions (Almeida et al., 2007; Liu et al., 2004). The compound 5-hydroxymethyl furan carboxylic acid (HMFCA) has also been identified (Taherzadeh et al., 2000).

Evolutionary engineering is an efficient strategy, which has been used to increase or modify characteristics of interest in bioprocesses, including resistance to furaldehydes by yeasts. Tolerance towards furfural, and to lignocellulosic hydrolysates in general, was described for *S. cerevisiae* strain TMB3400, which showed increased resistance to furfural after 300 generations of adaptation in the presence of this toxic (Heer and Sauer, 2008). Tolerance towards HMF and furfural was also improved for *Pichia. stipitis* strain NRRL Y-7124 and for *S. cerevisiae* strain NRRL Y-12632, using a directed adaptation method that consisted of increasing the toxics concentration and posterior selection after 100 re-inoculations to obtain a stable population. Adapted strains presented enhanced biotransformation ability to reduce HMF and furfural, and reach growth stationary phase earlier than the control (Liu et al., 2005).

It would be desirable that, in an industrial ethanol production, strains characterized as HMF resistant should have same ethanol productivities, either in the presence or absence of this aldehyde; so far, however, there has been no reports as such strains. Petersson et al. (2006) used genome-wide transcription analysis in the TMB3000 *S. cerevisiae* strain, which is known to tolerate inhibiting lignocellulose hydrolysates, and observed that *ADH2, ADH6* and *SFA1* genes presented increased expressions levels. *ADH6* gene was overexpressed in a laboratory strain, and results showed that HMF was reduced by Adh6p using NADPH as co-factor (Petersson et al., 2006). It has also been demonstrated that yeast clones overexpressing *ADH6* and *ADH7* genes were able to grow in the presence of HMF (Liu et al., 2008). Recently, the *ARI1* gene, coding for an NADPH-dependent aldehyde reductase from *S. cerevisiae*, was identified as presenting reduction activities towards at least 14 aldehyde substrates, including HMF (Liu and Moon, 2009). This dependence of NADPH induces a reprogramming of the yeast central metabolism by increasing the metabolic flux towards the pentose phosphate pathway in the presence HMF (Liu, 2011; Liu et al., 2009).

In the present study, four industrial strains of *S. cerevisiae* were evaluated regarding HMF tolerance. Evolutionary engineering was then applied to one strain aiming at increasing its tolerance towards HMF, Expression induction in the presence of HMF of four genes reported to be involved in its tolerance (*ADH6*, *SFA1*, *ADH7*, *ARI1*) were studied in anaerobic and oxygen limitation conditions, using either glucose or sucrose as carbon sources. Kinetics of yeasts cultures were conducted to compare the metabolisms of resistant and non-resistant strains in the presence of HMF.

2. Methods

2.1. Microorganisms and cell maintenance

The strains used in this study were *S. cerevisiae* P6, P18, and JP1 isolated from an ethanol production plant in Brazil (da Silva-Filho et al., 2005). HMF tolerant derivative strains P18R and JP1R were previously isolated by successive platting of their parental on YPD supplemented with 5 g L^{-1} HMF. The laboratory haploid strain BY4741 was used as reference. Yeasts were kept frozen at −20 °C in stock cultures of 20% glycerol and 80% of yeast culture (volume fraction).

2.2. Media and cultivation conditions

Oxygen limitation cultivations were performed in YPD medium (containing, in g L^{-1}: glucose, 20; yeast extract, 10; and peptone, 20) supplemented with HMF as indicated. Cultures were carried out in 250 mL flasks containing 60 mL of medium YPD (for item 2.3) or in 125 mL Erlenmeyer flasks containing 30 mL of medium (for item 2.4) at 30 °C and 150 rpm on a rotatory shaker. Initial inoculum of 0.1 OD (600 nm) was standardized. Fermentation assays used YPS (sucrose at 120 g L^{-1} instead of glucose) supplemented with HMF as indicated. Yeast cells were collected by centrifugation in graduated conical tubes until the equivalent of 5 mL corresponding to 10 % of inoculum (mass fraction, initial wet cell weight) and then the tubes were filled to 50 mL with YPS. Fermentations were carried out at 30 °C without agitation and samples were collected at defined time periods for determination of glucose, fructose, sucrose, ethanol, glycerol, acetate, and HMF concentration, and for biomass production.

2.3. Selection of strains and evolutionary engineering for HMF resistance

Yeast strains JP1, P6, and P18R were cultivated in YPD medium in the presence (4 g L^{-1}) or absence of HMF for 30 h and samples were taken at defined intervals for growth determination. Evolutionary engineering was used for further increment of the HMF resistance. Cells of JP1, P6, and P18R strains were submitted to an adaptation process by inoculating (OD of 0.1, 600 nm, 10% volume fraction) them in YPD medium with increments of HMF concentration of 0.5 g L^{-1} each batch, for 48 h at 30 °C. This procedure was repeated successive times until final cell biomass reached high and constant value. Samples of each culture cycle were stored on glycerol 20% at −18 °C.

2.4. Gene expression analysis

2.4.1. Cell treatments for gene expression analysis

The expression of genes previously described as responsible for HMF resistance were compared between treated and untreated cells grown under oxygen limitation condition and anaerobically to test the effects of physiology (see item 2.2). Cells were harvested at suitable intervals for analysis. They were centrifuged at 13 000*g* for 5 min, 4 °C and frozen at −18 °C for RNA extraction.

2.4.2. RNA extraction, cDNA synthesis and primer design

Yeast total RNA was isolated using NucleoSpin® RNA II kit following manufacturer's instructions (Macherey-Nagel, USA). RNA was quantified using spectrophotometric method (Nanovue, GE HealthCare). For cDNA synthesis, 1 μg of total RNA was used using ImProm-II™ Reverse Transcription System Promega II kit (Promega, USA). Different *S. cerevisiae* genes were used as reference according to validation experiments (Teste et al., 2009): for oxygen

Table 1
Primers used for gene expression analyses.

Primer	Sequence (5′–3′)
ADH6F	AGTGGGTTGAAAGTCGGTCAA
ADH6R	CAACGGTCACATTCCAAGCAT
ADH5F	GCTGCGGGACTGTAGGACTCT
ADH5R	TGTCAATGGCAATGATCTTGG
ADH7F	GCAAAGGATTGGAAGCATCC
ADH7R	CGCAGATACCACAGGCTTCA
ARI1F	GCCCATTTATTGACGTGCGT
ARI1R	TTGGCCGGTACATTCTGGTT

limitation cultivations (*TAF10* and *UBC6* for P6H9 strain; *TFC1* and *UBC6* for JP1 strain; *ALG9* and *TFC1* for BY4741 strain); and for anaerobic fermentation (*TAF10* and *UBC6* for all strains). Nucleotide sequences of *ADH6*, *ADH7*, *SFA1*, and *ARI1* genes are available at *Saccharomyces cerevisiae* Genome Database (http://www.yeastgenome.org/). Primers were designed using the software Primer Express (Applied Biosystems, Foster City, USA) and are described in Table 1.

2.4.3. Real time RT-PCR assays

Real-time PCR assays were performed using SYBR Green PCR Master Mix (Applied Biosystems, USA) and the parameters were previously reported (Elsztein et al., 2011). Temperature–time profile (95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min) was optimized for ABI Prism 7300 (Applied Biosystems). Amplification curves were analyzed with software SDS v.2.0 (Applied Biosystems). Negative PCR control (no template) and negative RT control (RNA not reverse-transcribed to cDNA) were run as internal controls. All samples were run in technical triplicates for each biological duplicate of cell cultivation. Normalization was performed for all genes at once using geNorm applet (Vandesompele et al., 2002), thus minimizing errors due to shifts in a single reference gene (Elsztein et al., 2011); the optimal number of reference genes to be used was also determined after submitting the raw data to the geNorm tool (http://medgen.ugent.be/genorm).

2.5. Analytical methods and kinetic parameters calculation

Growth rate of yeast cultures were measured as optical density (OD) at 600 nm. Cultivations were run for 48 h and samples were taken every 6 h. Cultures were centrifuged at 3500*g*, and cells were kept on −18 °C for further enzymatic activity assays. Yeast biomass was determined using a standard curve correlating the OD and cell dry weight (g L^{-1}). Glucose, glycerol, ethanol, and acetate concentrations were determined by HPLC (Shimadzu, Japan) equipped with a refractive index detector and Bio-Rad HPX-87H column (300 × 7.8 mm) using 5 mM sulfuric acid as eluent at 45 °C, flow rate of 0.6 mL min^{-1} and sample volumes of 20 μL. HMF concentration was determined by HPLC with a UV detector (at 276 nm) using a Nucleosil C18 column (250 × 4.6 mm) at room temperature, using acetonitrile–water (2:8) containing 10 g L^{-1} acetic acid as eluent, flow rate of 1.1 mL min^{-1} and sample volumes of 20 μL. The ethanol conversion yield ($Y_{P/S}$, g g^{-1}) was defined as the ratio of the concentration of ethanol produced and glucose consumed. The volumetric productivity (Q_p, g L^{-1} h^{-1}) was calculated using the ethanol production versus time.

2.6. Enzyme activity assay

Crude protein extracts were prepared using the glass beads method. Cells were resuspended in 400 μL potassium phosphate buffer 100 mM, pH 7.0, and 2 μL of a solution of 100 mM PMSF with an equal volume of glass beads (450 × 500 μm). Cells were disrupted by vortexing six times for 60 s, and the samples were cooled on ice for 60 s in between the vortex steps. Protein extracts were collected by centrifugation at 13000*g* for 5 min at 4 °C and the concentration was determined using Lowry assay method. Alcohol dehydrogenase activity was monitored by recording decreased absorbance at 340 nm using NADPH as cofactor. The reaction mixture consisted of a final concentration of 10 mM HMF substrate and 100 μM of NADPH in 100 mM potassium phosphate buffer, pH 7.0. All reagents were maintained at 25 °C prior to use. Assays were carried out in a volume of 1 ml at 25 °C for 1 min. The protein samples were kept on ice. To start the reaction, 25 μl of crude extract protein was added to the reaction mix. The NADPH molar absorption coefficient was 6.22 mM^{-1} cm^{-1}.

3. Results and discussion

3.1. Selection of industrial strains with HMF tolerance

Four *S. cerevisiae* strains were tested for their tolerance to HMF under oxygen limitation metabolism. Fig. 1 A shows the extended lag growth phase for all strains when in the presence of HMF, with

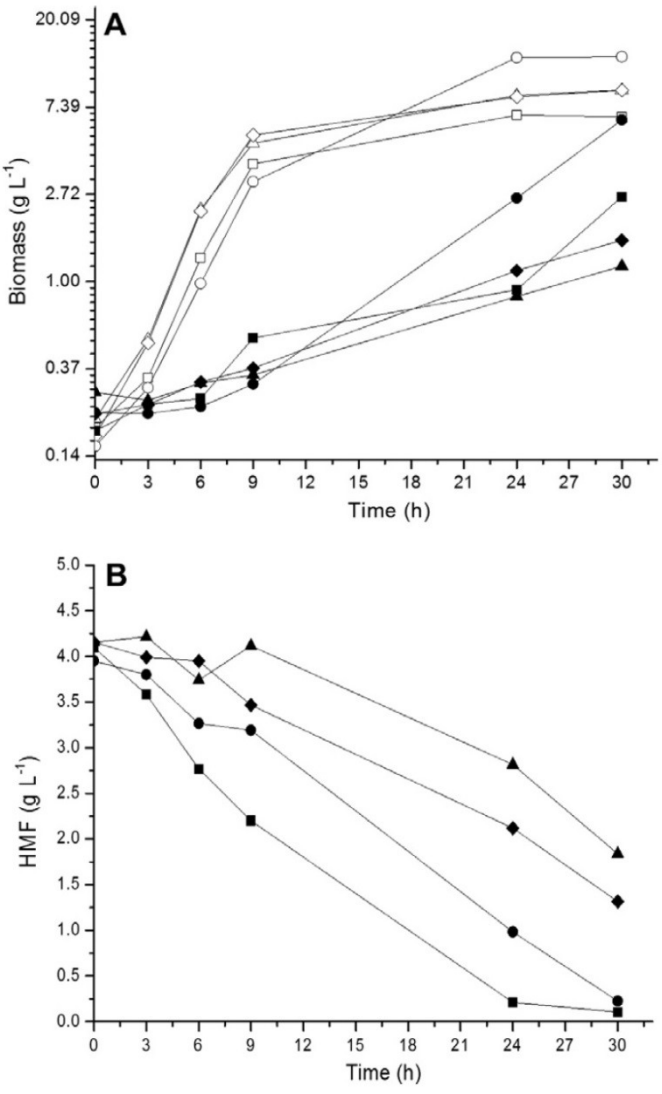

Fig. 1. Kinetics of biomass production (A) and HMF assimilation (B) of *S. cerevisiae* strains JP1 (▲), JP1R (♦), P6 (■), and P18R (●). Open symbols, condition control; filled symbols, presence of 5 g L^{-1} HMF. Results represent the mean of triplicate.

Table 2
Comparison between yield and productivity of ethanol of four industrial strains of *S. cerevisiae* growing in presence or absence of 5 g L^{-1} HMF.

Strain	JP1		JP1R		P6		P18R	
HMF	–	+	–	+	–	+	–	+
$Y_{P/S}$ (g g^{-1})	0.45	0.04	0.46	0.06	0.37	0.06	0.13	0.06
Q_p (g L^{-1} h^{-1})	0.64	0.08	0.55	0.05	0.40	0.05	0.10	0.04

P18R strain showing the highest biomass production at the end of cultivation. Although all strains showed the ability to reduce HMF concentration of media, P18R and P6 were able to completely deplete the toxic at the end of run (Fig. 1B). Glucose consumption was not affected, independent of strains (data not shown). In the presence of HMF, three out of four strains presented only about 10 % of the ethanol yield ($Y_{P/S}$) and volumetric productivity (Q_p) compared to controls without HMF (Table 2). P18R strain, already presenting lower fermentative capacity in the reference medium, showed 50 % yield and productivity reductions. The dose-dependent toxicity of HMF has been reported for *S. cerevisiae* ATCC 211239 and NRRL Y-12632 strains and even at the lowest concentration evaluated (1.2 g L^{-1}) the yeast final biomass did not reach that in the reference condition without HMF (Liu et al., 2004). Ethanol production was also affected in *S. cerevisiae* T1 and Y-1528 strains upon exposure to 3 and 4 g L^{-1} HMF, respectively (Keating et al., 2006). Finally, when furfural and HMF were added to cultures of *Spathaspora arborariae* strain UFMG-HM19.1A, their presence caused significant reductions on biomass formation and ethanol productivity (da Cunha-Pereira et al., 2011). The biochemical explanation seems to be the inhibition of essential enzymes, such as triose phosphate dehydrogenase and alcohol dehydrogenase (Sanchez and Bautista, 1988). HMF and furans were reported to affect CO_2 evolution by yeast cells (Banerjee et al., 1981). Due to their ability to form biomass in the presence of HMF, and to show HMF assimilation, P18R and P6 strains were selected for new rounds of evolutionary engineering for HMF resistance. JP1 was also selected, but in this case for its ability to produce high concentrations of ethanol in absence of HMF.

3.2. Evolutionary engineering for HMF resistance

Evolutionary engineering was performed aiming at selecting strains hyper-resistant to HMF. After 24 days of successive cultivations with increasing concentrations of HMF, it was possible to increase the tolerance of JP1 strain to 3.5 g L^{-1} HMF, while P18R strain was improved to 6 g L^{-1} HMF (results not shown). Moreover, a tolerant mutant of P6 strain was able to grow up to 9 g L^{-1} HMF after 36 days of cultivations. This mutant was named P6H9 and it was selected for further experiments. In the present work it was, therefore, possible to further increase HMF tolerance of JP1R strain by 20 % and to select a hyper-tolerant strain by increasing the HMF tolerance of P6 by 360%. Evolutionary engineering has been efficiently applied to obtain several interesting mutants of *S. cerevisiae* showing improved xylose-glucose co-assimilation by recombinant xylose-fermenting *S. cerevisiae* strain (Kuyper et al., 2005), and for the selection of sulfuric acid-tolerant JP1 strain (de Melo et al., 2010), which was also used in this work.

3.3. Gene expression analyses

Gene expression analyses were performed comparing the three *S. cerevisiae* strains BY4741, JP1, and P6H9. In the reference medium without HMF, these strains showed similar growth rates, reaching 0.5 DO in 3 h of oxygen limitation cultivation. However, in the presence of HMF there was a marked difference among strains to reach that cell concentration: 12.5 h for BY4741; 5 h for JP1; and 3 h for the mutant P6H9. In JP1, high expression level was observed for *ADH6* gene (>11-fold), while a moderate induction (>2-fold) was observed for *ADH7*, *ARI1*, and *SFA1* genes. On the other hand, there were surprisingly high inductions of *ADH7* (>550-fold) and *ARI1* (>20-fold) genes detected for the P6H9 mutant. Comparatively, the sensitive BY4741 strain showed discreet (>2-fold) inductions of *ADH7* and *ARI1* genes.

When simulating industrial conditions (high-sugar, high-biomass inoculum, anaerobic fermentation), *ADH6* gene was repressed and *ARI1* gene was moderately induced in JP1 strain (>2-fold). Similar to observations for the oxygen limitation condition, under anaerobiosis, *ADH7* was also over-expressed in P6H9 strain, followed by *ARI1* gene. *ADH6* was the first gene reported to be involved with HMF tolerance in yeast (Petersson et al., 2006). Genome-wide DNA Microarray showed the over-expression of *ADH6* gene in *S. cerevisiae* TMB3000 strain exposed to HMF and its importance to cell tolerance was demonstrated by overexpressing *ADH6* in the recombinant strain CEN.PK 113-5D, also resulting in an increase of NAPDH-dependent alcohol dehydrogenase activity by the cells (Petersson et al., 2006). Adh6p enzyme is active towards a wide spectrum of linear, branched-chain and aromatic primary alcohols and aldehydes such as cinnamaldehyde, benzaldehyde, veratraldehyde, and panisaldehyde using NADPH as the coenzyme (Larroy et al., 2002a). *SFA1* gene overexpression from *S. cerevisiae* TMB3000 was also evaluated, and the NADH-dependent HMF-reducing activity was observed. However, this activity did not result in increased HMF uptake rate (Petersson et al., 2006). Sfa1p presents molecular structure and biological function similar to alcohol dehydrogenases (Wehner et al., 1993), and has been proposed to be a bifunctional enzyme with glutathione-dependent formaldehyde dehydrogenase and long-chain alcohol dehydrogenase activities (Dickinson et al., 2003).

The product of *ARI1* gene was first described as a NADPH-dependent reductase accepting ethyl acetoacetate as substrate (Katz et al., 2003). This gene was also used to produce the 2-phenylethanol from phenylpyruvate, with carbonyl reductase function (Hwang et al., 2009). Recently, Liu and Moon (2009) showed that the partially purified Ari1p displayed NADPH-dependent reduction activities toward at least 14 aldehyde substrates, including furfural, HMF, acetaldehyde, butyraldehyde, cinnamic aldehyde, and phenylacetaldehyde. The expression of *ARI1* gene showed a 10-fold increase in *S. cerevisiae* Y-12632 strain upon exposure to HMF (Liu and Moon, 2009), similar to the results for P6H9 strain in this work.

The product of *ADH7* gene, a reductase from the NADPH-dependent cinnamyl alcohol dehydrogenase family, has been pointed as an important enzyme for detoxification of cinnamaldehyde, furfural, phenylacetaldehyde, vanillin, and 3-methylbutanal (Larroy et al., 2002b), and the *ADH7* overexpression confers yeast tolerance to HMF (Liu et al., 2008). The HMF-tolerant *S. cerevisiae* Y-50049 strain showed a 35-fold increased *ADH7* transcription when compared to sensitive strain Y-12632 (Liu et al., 2009).

3.4. Culture kinetics in presence and absence of HMF

Physiological analysis under oxygen limitation cultivation was performed for P6H9 strain and compared with the industrial JP1 and laboratory BY4741 strains (Fig. 2). The three strains showed similar growth rate (Fig. 2A), ethanol (Fig. 2B) and glycerol (Fig. 2C) production in the reference medium, showing that the genetic mutation that conferred HMF hyper-tolerance did not affect yeast fermentative capacity. Yet, the production of acetate in the reference medium, which was already low for industrial strain JP1, was practically abolished for P6H9 tolerant strain compared to BY4741 (Fig. 2D). In the presence of HMF, the yeast cells

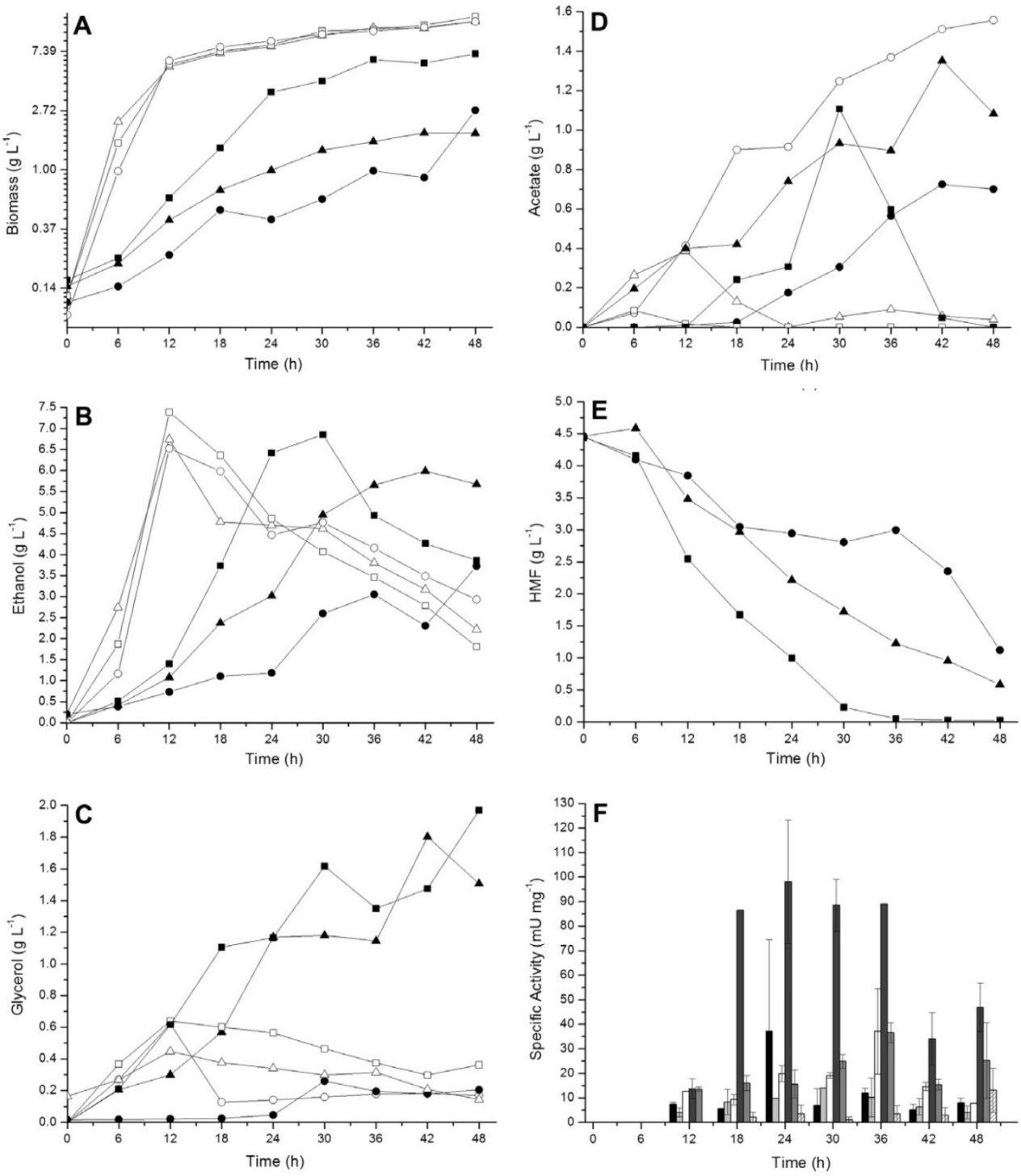

Fig. 2. Physiological analysis of of *S. cerevisiae* strains JP1 (▲), P6H9 (■), and BY4741 (●) cultivated in YPD (open symbols) or YPD containing HMF at 5 g L^{-1} (filled symbols). Production of biomass (A), ethanol (B), glycerol (C), acetate (D), HMF assimilation (E), and NADPH-dependent HMF reduction activity (F) are presented. Results represent the mean of duplicate.

experienced a change from reductive to oxidative metabolism. The highest growth rate in HMF medium observed for P6H9 strain (Fig. 2A) was also correlated with the highest kinetics of HMF assimilation by this strain (Fig. 2E) and the highest enzymatic activity of its cell-free extract to reduce this compound using NADPH as cofactor (Fig. 2F). This intense metabolic capacity can be explained by the greatly enhanced up-regulation of *ADH7* gene in P6H9 strain. The industrial strain JP1 showed a moderate tolerance towards HMF (Fig. 2A), also showing some limited assimilation of this toxic compound (Fig. 2E), possibly caused by the up-regulation of *ADH6* gene. However, no increased NADPH-dependent enzymatic activity was observed for this strain (Fig. 2F).

Ethanol production was delayed in the presence of HMF for all tested strains, but P6H9 strain was less affected and the final maximal concentration of ethanol achieved was almost the same in the presence or absence of HMF. As expected, ethanol volumetric productivity fell from 0.62 g L^{-1} h^{-1} to 0.23 g L^{-1} h^{-1} in the presence of HMF (Table 3).

Table 3
Comparison between yield and productivity of ethanol for *S. cerevisiae* industrial strains P6H9, JP1, and BY4741, in presence or absence of HMF.

Strain	BY4741		JP1		P6H9	
HMF	–	+	–	+	–	+
$Y_{P/S}$ (g g^{-1})	0.32	0.26	0.32	0.32	0.35	0.33
Q_p (g L^{-1} h^{-1})	0.54	0.07	0.54	0.16	0.62	0.27

Another important observation is the fact that glycerol production was greatly induced by HMF in the industrial strain JP1 and in the HMF-tolerant P6H9 strain (Fig. 2C), despite the reduced growth rate (Fig. 2A), linking its formation to a stress-induced phenomenon caused directly by the assimilation of HMF or indirectly, by some sort of metabolic imbalance. Studies on glycerol production reported that lowered activity of different ADH isoenzymes, especially Adh1p, results in an increase of glycerol production in *S. cerevisiae* (Johansson and Sjostrom, 1984). The protein coded by *ADH1* is an enzyme responsible for the anaerobic cytosolic reduction of acetaldehyde to ethanol (Taherzadeh et al., 2002). Analysis of the glucose metabolism of adh^0 cells shows that the lack of all known ADH isozymes results in the formation of glycerol as a major fermentation product, followed by a significant production of acetaldehyde and acetate (DREWKE et al., 1990). In an earlier study, *ADH1*, *ADH2*, *ADH3*, and *ADH4* genes expression did not change in tolerant aldehyde *S. cerevisiae* strain. However, glycerol production was not reported for the same strain in that study (Liu et al., 2009).

Except for strain BY4741, which showed a poor growth in the presence of HMF, all other tested strains showed an increased production of acetate in the presence of this toxic (Fig. 2D). Increased acetate production was reported for *ADH6*-overexpressing strain in batch fermentation (Almeida et al., 2008). In *S. cerevisiae*, there are two main metabolic routes responsible for NADPH formation, namely the pentose-phosphate pathway, and the acetaldehyde dehydrogenase reaction. The first route drains carbon from glycolysis and together with the stress-associated glycerol-3P dehydrogenase reduces the supply of NADH; the second pathway drains acetaldehyde for the production of acetate. Thus, the metabolic requirement for NADPH could lead to increased production of acetate, and decreased production of ethanol as consequence.

In this study, the sensitive strain BY4741 presented low expression levels for all four genes previously described as being involved with HMF tolerance; JP1 strain, however, showed increased expression levels of *ADH6* gene, but this was not reflected in HMF reduction during cultivation, or ethanol and biomass production. These results contrast with results of a previous study, in which tolerance towards HMF in yeast strains overexpressing *ADH6* and *ADH7* genes led to improved growth rate in the presence of this toxic (Liu et al., 2008). However, in the present study, the mutant strain P6H9 showed the highest HMF tolerance so far described for a yeast strain, at the same time capable of producing ethanol. In the presence of this toxic compound, high expression levels for *ADH7* and *ARI1* genes were observed in the two different metabolic conditions tested. In particular, *ADH7* gene showed a significant increase in its expression levels. This was reflected in the biomass and ethanol production and in the enzymatic activity.

4. Conclusion

A high-tolerant *S. cerevisiae* mutant strain P6H9 was obtained by evolutionary engineering from the industrial strain P6, which was isolated from molasses-using ethanol producing plant, and this phenotype was correlated to the high expression levels of *ADH7* and *ARI1* genes and increased enzymatic activity for NADPH-dependent HMF reduction. In the presence of HMF, this strain showed a better physiological performance than JP1 industrial strain and BY4741. Overall, the results obtained in this work shows the importance of several genes on the phenotype for HMF tolerance and strain P6H9 can be further exploited in research for second generation ethanol production.

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ESTÁGIO

Desenvolvimento de pesquisa (iniciação científica) objetivando a caracterização e o aumento da produção das enzimas celulases do fungo *Penicillium echinulatum* em cultivo líquido e semi-sólido. Laboratório de Biotecnologia – Instituto de Biotecnologia – **Universidade de Caxias do Sul**. MARÇO/1999 A DEZEMBRO/2003

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