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LAUREN BERGMANN SOARES

O efeito de inibidores do hidrolisado hemicelulósico e seu impacto no desempenho da fermentação etanólica de *Scheffersomyces stipitis* NRRL Y-7124 e *Spathaspora passalidarum* NRRL Y-27907

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Orientadora: Aline Carvalho da Costa
Co-orientadora: Jaciane Lutz Ienczak

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_____

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Albert Einstein

RESUMO

SOARES, L. B. O efeito de inibidores do hidrolisado hemicelulósico e seu impacto no desempenho da fermentação etanólica de *Scheffersomyces stipitis* NRRL Y-7124 e *Spathaspora passalidarum* NRRL Y-27907. Campinas, UNICAMP. Setembro. 2018. 116 fls. Departamento de Desenvolvimento de Processos e Produtos da Faculdade de Engenharia Química da Universidade Estadual de Campinas.

O principal material lignocelulósico brasileiro, bagaço de cana-de-açúcar, é composto de três polímeros principais: celulose (60-50%), hemicelulose (25-30%) e lignina (25-30%). O desenvolvimento de tecnologias para utilizar carboidratos obtidos a partir da hidrólise de celulose e hemicelulose para produzir bioetanol de segunda geração é promissor e grupos de pesquisa fazem esforços para desenvolver este processo, visando elevar a produção de bioetanol por área de cultivo. Porém, a etapa de processamento do bagaço para disponibilização de açúcares gera compostos inibitórios ao metabolismo dos microrganismos, prejudicando a etapa seguinte de produção de bioetanol. Dentre os principais inibidores presentes no hidrolisado hemicelulósico estão os ácidos orgânicos, como ácido acético e fórmico; produtos da degradação de pentose e hexoses, como furfural e 5-hidroximetilfurfural; e produtos provenientes da degradação da lignina, como os compostos fenólicos. Este estudo teve como objetivo comparar o impacto desses compostos inibitórios no metabolismo de crescimento celular, consumo açúcares e produção de bioetanol entre as leveduras *Scheffersomyces stipitis* NRRL Y-7124 e *Spathaspora passalidarum* NRRL Y-27907, visto que a formação de inibidores é intrínseca à obtenção do hidrolisado hemicelulósico e tratá-lo para retirada desses compostos adiciona etapas ao processo, tornando-o mais dispendioso e demorado. O trabalho objetivou ainda estabelecer estratégias de aprimoramento de parâmetros de processo, como a utilização de cepa adaptada e diferentes concentrações de células iniciais como alternativas de viabilizar a fermentação de hidrolisados. Baseando-se nos testes de resistência individual dos inibidores foram estabelecidas que concentrações de 2,5 $g.L^{-1}$ de ácido acético e 2,0 $g.L^{-1}$ de furfural são os valores limites que os hidrolisados hemicelulósicos podem conter para que se obtenha um desempenho satisfatório na fermentação com *S. stipitis* NRRL Y-7124. Para *S. passalidarum* NRRL Y-27907, concentrações de 0,5 $g.L^{-1}$ de ácido acético, 1,0 $g.L^{-1}$ de furfural, 3,0 $g.L^{-1}$ de 5-HMF são os valores limites que as frações hemicelulósicas podem conter. O trabalho também demonstrou que uso de leveduras adaptadas durante o processo com açúcares

de segunda geração resultou em melhor desempenho quando comparado com a linhagem não adaptada, observada no aumento da taxa de consumo de xilose em 32% e de 57% de aumento na taxa de produção de etanol em relação à levedura selvagem. Também foi observado que o aumento na concentração inicial de células no processo influencia positivamente o uso de hidrolisados contendo inibidor, uma vez que os meios fermentativos considerados não fermentescíveis (devido à baixa concentração de células aplicada) tornaram-se viáveis para fermentações quando foram utilizadas densidades celulares elevadas. Quando se triplicou a concentração celular inicial, houve aumento de 5% em rendimento, resultando em aproximadamente 25 $g.L^{-1}$ de etanol a mais no final do processo. Com base nos resultados, é possível estabelecer uma relação entre os inibidores e sua toxicidade para essas leveduras e compará-las entre si em relação ao seu desempenho fermentativo quando sujeitas à ação dos inibidores. O conhecimento detalhado do comportamento na presença destes compostos permite estabelecer estratégias de detoxificação do meio de fermentação e direcionamento dos microrganismos de acordo com seu grau de tolerância, objetivando processos fermentativos de maior desempenho.

Palavras-chave: inibidores; fermentação de pentoses; produção de bioetanol de segunda geração; levedura adaptada, tamanho de inóculo.

ABSTRACT

SOARES, L. B. The effect of inhibitors of the hemicellulosic hydrolysate and the impact on the performance of the ethanolic fermentation of *Scheffersomyces stipitis* NRRL Y-7124 and *Spathaspora passalidarum* NRRL Y-27907. Campinas, UNICAMP. September. 2018. 116 fls. Department of Process and Product Development, School of Chemical Engineering, State University of Campinas.

The main Brazilian lignocellulosic material, sugarcane bagasse, is composed of three main polymers: cellulose (60-50%), hemicellulose (25-30%) and lignin (25-30%).The development of technologies to use carbohydrates obtained from the hydrolysis of cellulose and hemicellulose to produce second generation ethanol is promising and many research groups make efforts to develop this process, aiming at increasing the production of bioethanol by area of cultivation. However, the stage of bagasse processing to produce sugar yields inhibitory compounds to the metabolism of the microorganisms, impairing the next stage of ethanol production. Among the main inhibitors present in the hemicellulosic hydrolysate are organic acids, such as acetic and formic acid; degradation products of pentose and hexoses, such as furfural and 5-hydroxymethylfurfural; and products from lignin degradation, such as phenolic compounds. The study aims to compare the impact of inhibitory compounds present in the hemicellulosic hydrolysate on the metabolism of cell growth, sugar consumption and ethanol production among the yeasts *Scheffersomyces stipitis* NRRL Y-7124 and *Spathaspora passalidarum* NRRL Y-27907, since inhibitors formation is intrinsic to the hemicellulosic hydrolysate production and treating it for withdrawal of such compounds adds steps to the process, making it more expensive and time consuming. It also aims at establishing strategies to improve process parameters, such as the use of adapted strains and different concentrations of initial biomass as alternatives to enable the fermentation. Based on the individual inhibitors resistance tests it was established that concentrations of 2.5 $g.L^{-1}$ of acetic acid and 2.0 $g.L^{-1}$ of furfural are the limit values that the hemicellulosic hydrolysates may contain for performance satisfactory in fermentation with *S. stipitis* NRRL Y-7124. For *S. passalidarum* NRRL Y-27907, concentrations of 0.5 $g.L^{-1}$ of acetic acid, 1.0 $g.L^{-1}$ of furfural, 3.0 $g.L^{-1}$ of 5-HMF are the limit values that the hemicellulosic fractions can contain. The work also demonstrated that the use of evolved yeasts during the process with second generation sugars resulted in better performance when compared to the unadapted strains, observed in the increase of the of

consumption xylose rate by 32% and of a 57% increase in the ethanol production rate in relation to wild yeast. It has also been observed that the increase in the initial concentration of cells in the process positively influences the use of inhibitor-containing hydrolysates, since fermentative media considered non-fermentable (due to the low concentration of cells apllied) have become viable for fermentations when high cell densities were used. When the initial cell concentration tripled, there was a 5% increase in yield, resulting in approximately 25 $g.L^{-1}$ of more ethanol. Based on the results, it is possible to establish a relationship between the inhibitors and their toxicity to these yeasts and to compare them with each other in relation to their fermentative performance when subjected to the action of the inhibitors. The detailed knowledge of the behavior in the presence of these compounds allows the establishment of detoxification strategies of the fermentation medium and the targeting of the microorganisms according to their degree of tolerance, aiming at higher performance fermentative processes.

Keywords: inhibitors, pentose fermentation, second generation ethanol production, evolved yeast, inoculum size.

SUMÁRIO

CAPÍTULO 1 – INTRODUÇÃO

1.1 Diagrama conceitual do trabalho

O efeito de inibidores do hidrolisado hemicelulósico e seu impacto no desempenho da fermentação etanólica de *Scheffersomyces stipitis* NRRL Y-7124 e *Spathaspora passalidarum* NRRL Y-27907.

Por quê? Para quê?

- Os inconvenientes gerados na utilização de combustíveis fósseis resultam no crescimento da demanda de bioetanol a níveis nacional e mundial, e estimula a busca por alternativas que visem maximizar sua produção.

- A presença de compostos inibitórios nos hidrolisados hemicelulósicos é visto como uma barreira para que o etanol celulósico seja viável tecnicamente;

- Considerando o potencial de *Spathaspora passalidarum* na conversão de xilose em bioetanol, utilizando hidrolisados hemicelulósicos, um estudo mais detalhado envolvendo inibidores é necessário.

Quem já fez?

- Liu *et al* (2004) testou a influência de furfural e 5-hidroximetilfurfural em *S. stipitis* Y-NRRL 7124 e observou efeito sinérgico de inibição de crescimento celular.

- Oliveira (2010) verificou a influência da adição de ácido acético, furfural e 5- hidroximetilfurfural para *S. stipitis* CBS 5774 e sugere uma maior dificuldade de assimilar a xilose na presença dos compostos inibitórios.

- Morales *et al* (2016) verificaram que a tolerância para ácido acético da cepa *S. passalidarum* é pequena, uma vez que a produtividade volumétrica em etanol decresceu em quase 90% ao adicionar 4,5 $g.L^{-1}$ de ácido acético no meio contendo glicose.

- Agbogbo *et al* (2007) demonstraram aumento na taxa de consumo de xilose quando altas concentrações de células foram aplicadas nas fermentações.

- Santos *et al* (2016) demonstrou melhora nas condições fermentativas adaptando a levedura *S. Stipitis* ao longo de reciclos de células.

Hipóteses

- É possível determinar, entre os compostos estudados, qual inibidor presente em hidrolisado hemicelulósico é o mais severo;

- É possível determinar, entre as cepas estudadas, qual a mais resistente em relação aos inibidores do hidrolisado hemicelulósico;

- É possível determinar concentrações máximas de inibidores em hidrolisados hemicelulósicos para torná-lo fermentescível utilizando as cepas *Scheffersomyces stipitis* NRRL Y-7124 e *Spathaspora passalidarum* NRRL Y-27907;

- É possível, mediante estratégias de processo, viabilizar a fermentação de frações hemicelulósicas que contenham inibidores.

Metodologia Científica

- Análise dos efeitos dos inibidores ácido acético, furfural, 5-hidroximetilfurfural e vanilina, isoladamente e em conjunto, no crescimento celular, consumo de açúcares e produção de etanol para as leveduras *Scheffersomyces stipitis* NRRL Y-7124 e *Spathaspora passalidarum* NRRL Y-27907 em meio padrão de fermentação;

- Fermentações em batelada com cepa selvagem e adaptada utilizando o inibidor mais severo como modelo de inibição;

- Fermentações em batelada em meio contendo inibidores utilizando diferentes concentrações de células iniciais.

Respostas

- Classificação de severidade dos inibidores presentes no hidrolisado hemicelulósico, dentre os estudados;

- Definição do metabolismo mais tolerante, dentre as cepas de leveduras estudadas,

- Definição da utilização de leveduras adaptadas durante o próprio processo como estratégia para melhorar a fermentação de pentoses;

- Definição do tamanho de inóculo como fator determinante para viabilizar a fermentação de hidrolisados hemicelulósicos contendo inibidores.

A demanda crescente do uso de petróleo e carvão, fontes de energia não renováveis, tem levantado questões sobre seu impacto no meio ambiente e na economia. Com o intuito de reduzir a dependência aos combustíveis fósseis e reduzir a susceptibilidade econômica dos países perante flutuações de preço dessas *commodities*, muito esforço tem sido dedicado na pesquisa de combustíveis renováveis (Gupta e Verma, 2015; UNCTAD, 2016).

Dentre os combustíveis renováveis em destaque estão biodiesel, biogás e bioetanol, sendo que o bioetanol é o principal biocombustível produzido e utilizado no Brasil; maior produtor mundial a partir de cana-de-açúcar (Basso *et al.*, 2008). A utilização de cana-de-açúcar como matéria-prima para a produção de bioetanol é vantajosa do ponto de vista ambiental, apresentando balanço energético superior ao processo de produção de bioetanol a partir de milho (que ocorre nos Estados Unidos) (Basso *et al.*, 2008; Leite; Cortez, 2008).

O crescimento da demanda de bioetanol a níveis nacional e mundial estimula a busca por alternativas que visem maximizar sua produção. A utilização de novas matérias-primas para produção, como o aproveitamento da biomassa gerada (bagaço e palha de cana-de-açúcar) no próprio processo produtivo de geração de bioetanol, pode resultar em um aumento de até 40 % na produção, sem a necessidade de aumento da área plantada (Dias *et al.*, 2013).

Dados da safra de 2017/2018 mostram que o Brasil processou 596.313 milhões de toneladas de cana-de-açúcar, produzindo 25.092 bilhões de litros de bioetanol (UNICA, 2018). Estima-se que foram gerados, aproximadamente, 239 milhões de toneladas de bagaço, uma vez que uma tonelada de cana-de-açúcar processada gera aproximadamente 250 kg de bagaço úmido (50% de umidade) (Vasconcelos *et al.*, 2013). Atualmente, o bagaço e a palha (em algumas usinas brasileiras) gerados são

queimados em caldeiras para produção de vapor e eletricidade, com o objetivo de suprir a demanda energética do próprio processo de produção de açúcar e bioetanol (Dias *et al*., 2009).

A integração entre o processo convencional de produção de bioetanol a partir de caldo e melaço de cana-de-açúcar (primeira geração) e o processo com aproveitamento da biomassa (segunda geração) é uma aposta para aumento da produtividade, pois com a integração desses processos pode haver redução nos custos fixos de produção, justamente pelas operações unitárias serem comuns a ambos os processos. Com isso, o desenvolvimento da tecnologia de produção de bioetanol de segunda geração torna-se econômica e estrategicamente atrativa (Dias *et al*., 2013).

O bagaço da cana-de-açúcar é constituído por três componentes principais: celulose, hemicelulose e lignina (Fengel & Wegener, 1989). A produção de bioetanol a partir da biomassa depende de etapas de pré-tratamento, que resulta na exposição das cadeias de celulose e solubilização das cadeias de hemiceluloses; seguida de hidrólise para liberação dos açúcares monoméricos; e, por fim, da fermentação e recuperação do bioetanol (Canilha *et al.*, 2012).

O pré-tratamento consiste em uma das etapas operacionais mais relevantes em termos de custo para conversão da biomassa em açúcares fermentescíveis. Nela estão acoplados os requisitos especiais para equipamentos, o grande consumo de água e a tecnologia complexa de solubilização da lignina, além de influenciar consideravelmente nas etapas posteriores do processo de produção de bioetanol (Su e Cheng, 2002; Mosier, 2003a). Porém, dentre os principais desafios relacionados ao desenvolvimento da tecnologia de produção de bioetanol celulósico, destaca-se a presença de compostos inibidores ao metabolismo microbiano presentes nos hidrolisados lignocelulósicos,

advindos da degradação dos açúcares (Almeida *et al.*, 2007) ou da própria estrutura da biomassa hidrolisada.

Quando as hemiceluloses são degradadas xilose, ácido acético e glicose são liberados. A celulose é hidrolisada a glicose. À alta temperatura e pressão, as pentoses são ainda degradadas a furfural. Da mesma forma, 5-hidroximetilfurfural (HMF) é formado por degradação de hexoses. Ácido fórmico é formado quando furfural e HMF são degradados e ácido levulínico é formado por degradação do HMF. Compostos fenólicos como ácido 4-hidroxibenzóico, vanilina e ácido siríngico são gerados a partir da degradação parcial da lignina (Palmqvist *et al.*, 2000; Taherzadeh e Karimi, 2011).

Os ácidos fracos, como ácido acético, são lipossolúveis e difundem pela membrana celular, diminuindo o pH citosólico. Com isso, a célula gasta energia procurando restabelecer seu pH interno, desviando o metabolismo do crescimento celular e produção de bioetanol (Palmqvist *et al.*, 2000; Taherzadeh e Karimi, 2011).

Os compostos furânicos, como furfural e 5-HMF são observados interagindo com enzimas importantes na multiplicação celular e na produção de bioetanol, como a hexoquinase, gliceraldeído 3-fosfato desidrogenase, álcool desidrogenase e piruvato desidrogenase (Modig *et al.*, 2002; Mussatto *et al.*, 2004; Almeida *et al.*, 2007).

Existe uma ampla variedade de compostos fenólicos gerados no processamento de materiais lignocelulósicos, e por esse motivo seu mecanismo de ação não foi totalmente elucidado. O que se sabe é que interagem com os compostos da parede celular, causando problemas de integridade da membrana e, por isso, afetam as atividades metabólicas, diminuindo a produção de bioetanol e o crescimento celular (Taherzadeh e Karimi, 2011).

A levedura *Saccharomyces cerevisiae* é o microrganismo amplamente utilizado para produção de bioetanol, seja para o processo de primeira ou de segunda geração.

Porém, ela não é capaz de metabolizar pentoses (principalmente xilose), a não ser que seja geneticamente modificada para expressar as rotas de assimilação desse açúcar (Fujii *et al.*, 2011; Kim *et al.*, 2013).

É nesse cenário, portanto, que leveduras naturalmente capazes de fermentar pentoses ganham destaque, como *Scheffersomyces stipitis*, tanto na sua forma selvagem (Nakanishi *et al.*, 2017; Santos *et al.*, 2016; Scordia *et al.*, 2012), quanto geneticamente transformada (Jeffries e Jin, 2004). Recentemente isolada, *Spathaspora passalidarum* também é capaz de metabolizar xilose e pode co-utilizar glicose, xilose e celobiose como fontes de carbono (Nakanishi *et al.*, 2017; Hou, 2012; Long *et al.*, 2012; Nguyen *et al.*, 2006).

As leveduras *S. passalidarum* e *S. stipitis* são próximas no que diz respeito a perspectivas taxonômicas. Além disso, também foram isoladas de locais semelhantes, sendo que ambas são encontradas no intestino de besouros (larvas e adultos) (Wohlbach *et al*., 2011). Provavelmente vem do fato de encontrarem-se associadas aos besouros, que por sua vez vivem e alimentam-se de madeira podre, as suas capacidades de metabolizarem uma ampla faixa de açúcares presentes em materiais lignocelulósicos e de crescerem em ambientes com pouca concentração de oxigênio (Garcia-Ochoa e Gomez, 2009; Nguyen *et al.*, 2006).

Associado ao uso de linhagens nativas para utilizar a fração de pentoses está o desenvolvimento estratégias de fermentação para superar os problemas causados pelos inibidores. Quando altas concentrações de células são aplicadas no início do processo fermentativo, há maior chance de sobrevivência das linhagens frente ao ambiente tóxico formado por compostos inibitórios provenientes do hidrolisado hemicelulósico (Taherzadeh e Karimi, 2011). Também é relatado que o aumento da concentração inicial de células aumenta a taxa de consumo de xilose para *S. stipitis* (Agbobo *et al.*, 2007).

Esta prática é comum na produção de bioetanol de primeira geração (Basso *et al.,* 2011) e tem sido utilizada como uma alternativa para obtenção de elevado desempenho do processo de produção de bioetanol a partir de matéria prima lignocelulósica (Nakanishi *et al.*, 2017; Santos *et al.*, 2016).

No caso de *S. stipitis*, a discussão sobre os efeitos dos inibidores do hidrolisado hemicelulósico em seu metabolismo já foi bastante debatida (Delgenes *et al.*, 1996, Liu *et al.*, 2004, Díaz *et al.*, 2009, Oliveira, 2010, Bellido *et al.*, 2011, Ortiz-Muñiz *et al.*, 2014). Por outro lado, por ter sido isolada recentemente, existem poucos trabalhos elucidando a ação de inibidores para o metabolismo de *S. passalidarum* (Hou, 2011; Costa, 2016; Morales *et al.*, 2016).

Considerando seu potencial de conversão de xilose em bioetanol na utilização de hidrolisados hemicelulósicos, um estudo mais detalhado envolvendo o impacto da presença dos inibidores no metabolismo de *S. passalidarum* se faz necessário, objetivando a comparação com o microrganismo modelo para fermentação de pentoses, *S. stipitis*.

1.3 Objetivo

Estudar a influência dos compostos inibitórios presentes no hidrolisado hemicelulósico de bagaço de cana–de-açúcar para as leveduras *Scheffersomyces stipitis* NRRL Y-7124 e *Spathaspora passalidarum* NRRL Y-27907, por meio de parâmetros de fermentação, além de avaliar alternativas de processo a fim de viabilizar a produção de bioetanol a partir da fração de pentoses.

1.3.1 Objetivos específicos

1) Determinar os efeitos de diferentes concentrações de ácido acético, furfural, 5-hidroximetilfurfural e vanilina no crescimento celular, consumo de açúcares e produção de bioetanol para as leveduras *S. stipitis* NRRL Y-7124 e *S. passalidarum* NRRL Y-27907 em meio de fermentação;

2) Definir concentrações-limite para o crescimento celular, consumo de açúcares e produção de bioetanol para os inibidores ácido acético, furfural, 5-hidroximetilfurfural e vanilina no metabolismo das leveduras *S. stipitis* NRRL Y-7124 e *S. passalidarum* NRRL Y-27907;

3) Definir o inibidor mais severo em termos de crescimento, consumo e produção para ambas as leveduras e realizar ensaios contendo o inibidor mais severo como modelo de inibição para cepa adaptada *S. stipitis* evoluída CTBE S_A-100 (Santos *et al*, 2016) em comparação com a cepa *S. stipitis* NRRL Y-7124;

4) Verificar a eficácia da estratégia da utilização de alta densidade celular aplicando concentrações iniciais de células (5, 15 e 30 $g.L^{-1}$) em fermentação contendo concentrações de inibidores baseadas em hidrolisados hemicelulósicos provenientes de pré-tratamento com ácido diluído;

5) Definir parâmetros de processo de acordo com a análise de consumo de carboidratos, produção de bioetanol e subprodutos e crescimento celular ao longo das fermentações, utilizando velocidade específica de consumo de carboidratos, velocidade específica de

produção de bioetanol, produtividade volumétrica, fator de conversão de carboidratos em bioetanol, e rendimento.

CAPÍTULO 2 - REVISÃO BIBLIOGRÁFICA

2.1 Produção de bioetanol

O álcool etílico, ou etanol, de fórmula molecular CH_3CH_2OH, pode ser obtido por via química, através da hidratação direta ou indireta do etileno; ou por via biológica, a partir do processamento e fermentação de materiais vegetais como cana-de-açúcar, milho, beterraba açucareira, batata, entre outras. A cana-de-açúcar é a matéria-prima largamente utilizada na indústria sucroalcooleira brasileira, e pode ter dois destinos: produção de açúcar ou de bioetanol. Atualmente, o Brasil produz bioetanol de 1ª geração a partir de xarope e/ou melaço de cana-de-açúcar e, recentemente, com o intuito de aumentar a oferta de álcool combustível (e ao mesmo tempo tentar resolver o problema de excedente de resíduos na produção tradicional de bioetanol), pesquisas têm sido desenvolvidas para obtenção do bioetanol de 2ª geração através do aproveitamento da biomassa residual.

Os automóveis que circulam no Brasil usam duas categorias de bioetanol: o hidratado e o anidro. O hidratado (96%) é usado diretamente em motores desenvolvidos para este fim ou em motores com tecnologia flex. O bioetanol anidro é misturado à gasolina, sem prejuízo para os motores, em proporções variáveis, de acordo com a vigência legal (Agência Nacional de Petróleo, Gás Natural e Biocombustíveis (ANP), 2017).

Segundo a ANP, existem 349 plantas produtoras de bioetanol em operação no país, correspondendo a uma capacidade total autorizada de 186.176 m^3/dia de produção de bioetanol hidratado e 99.848 m^3/dia de produção de bioetanol anidro. A cana-de-

açúcar é a matéria-prima utilizada em 97,1% das plantas de bioetanol autorizadas. A Figura 1 mostra o histórico de produção de bioetanol no Brasil no período compreendido entre 2012 e 2016.

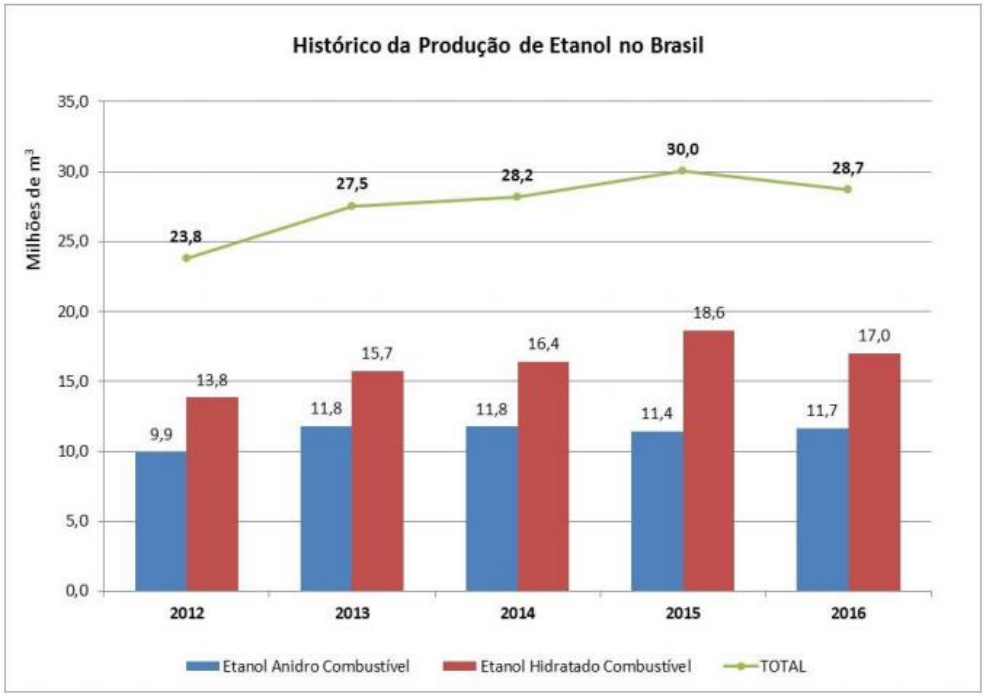

Figura 1 - Produção de bioetanol no Brasil entre 2012 e 2016. Fonte: Boletim do Etanol nº 9 – ANP, Fevereiro, 2017.

O processo de produção de bioetanol de 1ª geração no Brasil compreende as etapas de recebimento e limpeza da cana, extração do caldo nas moendas ou difusor, clarificação e concentração do caldo para posterior fermentação a bioetanol através do metabolismo de microrganismos. As destilarias brasileiras utilizam processo de fermentação melhorado, patenteado em 1941 por F. Boinot. O processo consiste em fermentação em batelada alimentada do mosto de cana-de-açúcar por leveduras *Saccharomyces cerevisiae*. Após o fim de cada fermentação, emprega-se o processo de centrifugação para separar as células de levedura (creme) do vinho, este último é submetido à destilação para separação do produto final. O creme de leveduras é destinado a um tratamento com ácido sulfúrico diluído em água (pH 2,0-2,5 por

30 min), e então um novo ciclo de fermentação é iniciado com o retorno da biomassa celular às dornas, que recebem novamente a alimentação de mosto de cana de açúcar (*United States Patent Office*, PI 2, 1941).

2.2 Biomassa lignocelulósica

O bagaço e a palha gerados no processamento de bioetanol de 1ª geração são queimados em caldeiras para produção de vapor e eletricidade, com o objetivo de suprir a demanda energética do processo de produção de açúcar e bioetanol (Dias *et al*., 2009). Porém, como em todo material lignocelulósico, a estrutura vegetal contém polímeros, que, com adequado tratamento, podem aumentar a produção de bioetanol.

O bagaço da cana-de-açúcar é constituído por três componentes principais: celulose, hemicelulose e lignina (Fengel e Wegener, 1989). Enquanto a celulose é um polímero linear constituído por unidades de D-glicose unidas por ligações glicosídicas β-1→4, as hemiceluloses são heteropolímeros compostos predominantemente por pentoses e hexoses com curtas ramificações, tais como D-xilose, D-glicose, L-arabinose e D-galactose. Já a lignina é uma macromolécula polifenólica, constituída por unidades básicas de 3-5-dimetoxi-4-hidroxi-fenilpropano, 3-metoxi-4-hidroxi-fenilpropano e 4-hidroxi-fenilpropano (Fengel e Wegener, 1989).

Para que a transformação desses componentes em açúcares fermentescíveis seja possível, o bagaço de cana-de-açúcar deve ser submetido a um pré-tratamento, cujo objetivo é o aumento da área superficial para atuação de enzimas através de mudança na composição química da biomassa. Este pré-tratamento pode ser realizado por processos físicos, químicos, biológicos e/ou pela combinação destes (Sun e Cheng, 2002). Dentre as técnicas, podem-se destacar a explosão a vapor, os tratamentos hidrotérmicos e

AFEX (*Ammonia Fiber Expansion*) e o pré-tratamento com ácido diluído. Esses são os métodos mais estudados e promissores no processo de obtenção de produtos de interesse industrial a partir da biomassa vegetal (Mosier *et al.*, 2005; Sánchez e Cardona, 2008). Um dos esquemas possíveis para a desconstrução da biomassa vegetal é mostrado na Figura 2.

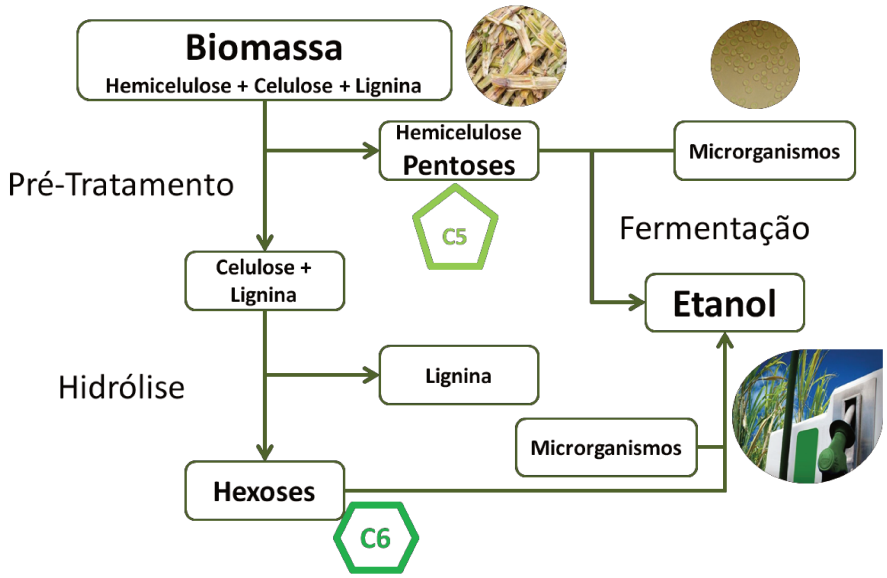

Figura 2 - Esquema da desconstrução da biomassa para a geração dos açúcares de segunda geração e posterior fermentação a etanol.

2.3 Inibidores de fermentação presentes em hidrolisados hemicelulósicos

De acordo com Palmqvist e Hahn-Haagerdal (2000), durante o pré-tratamento, além dos carboidratos, outros compostos são liberados, podendo ser divididos em vários grupos de acordo com a sua origem. A Figura 3 mostra os compostos gerados durante a desconstrução da biomassa.

Além da liberação dos carboidratos presentes na biomassa, como glicose, xilose, manose e galactose, o pré-tratamento libera compostos que são separados em grupos. Dentre esses grupos, incluem-se os ácidos orgânicos, como ácido acético, gerado quando a estrutura acetil das hemiceluloses é degradada; compostos furânicos como

furfural, produzido a partir da desidratação de pentoses, e o 5-hidroximetilfurfural (5-HMF), produzido a partir da desidratação de hexoses; e compostos fenólicos, oriundos da degradação de lignina (Fengel e Wegener, 1989; Almeida *et al.*, 2007; Taherzadeh e Karimi, 2011; Jönsson *et al.* 2013).

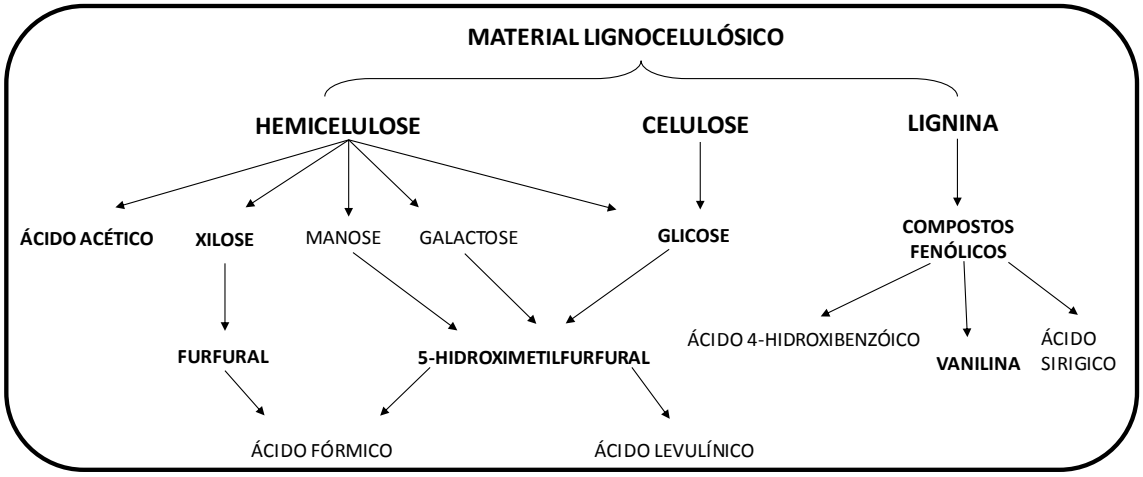

Figura 3 - Compostos gerados durante a desconstrução da biomassa lignocelulósica. Adaptado de Taherzadeh e Karimi (2011).

A Figura 4 mostra o mecanismo de interação dos ácidos fracos com a célula microbiana. A inibição relacionada à ação de ácidos orgânicos geralmente é causada pela dissociação e acumulação de ânions intracelularmente. A toxicidade dos ácidos é dependente do pH, pois a forma não dissociada é predominante em meios com pH abaixo de sua constante de dissociação (para ácido acético: 4,75). Dessa forma, o ácido permeia a membrana celular e se dissocia no interior da célula, promovendo a acidificação do citoplasma (Pereira *et al.*, 2011).

Alguns autores (Verduyn *et al.*, 1992; Russel, 1992; Imai e Ohono, 1995; Modig *et al.*, 2002) propuseram mecanismos para a ação do ácido dentro da célula. O mecanismo de desacoplamento, em que a queda no pH intracelular é resultante do fluxo de ácido para dentro do citoplasma, neutralizada pela ação da ATPase da membrana plasmática, resultando no bombeamento prótons para fora da célula. É necessário gerar

ATP´s adicionais a fim de manter o pH intracelular. Quando a concentração intracelular de ácido é muito elevada, a capacidade de bombeamento de prótons da célula é esgotada, o que resulta no empobrecimento do teor de ATP, dissipação da força motriz de retirada de prótons e ocorre a acidificação do citoplasma. Dessa forma, esse processo interfere no consumo de ATP, pois a célula se ocupa em reestabelecer o pH interno, desviando parte do ATP que seria utilizado para crescimento celular e produção de bioetanol.

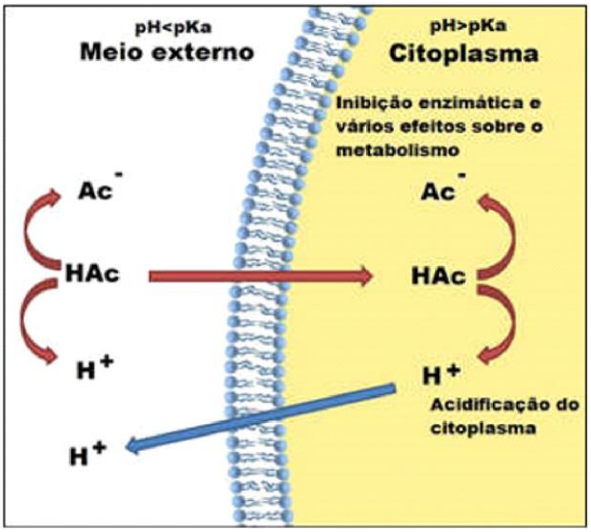

Figura 4 - Mecanismo de interação dos ácidos fracos com a célula microbiana. Lawfordet al. (1993), Mills et al. (2009), adaptado por Zeferino (2013).

A Figura 5 mostra o mecanismo de interação dos compostos furânicos com a célula microbiana. Por ter caráter hidrofóbico, são capazes de interagir e romper a membrana celular. Uma vez dentro da célula, interagem também com os segmentos de DNA, principalmente nas regiões de adenina e timina, causando mutações (Mills *et al.*, 2009; Shahabuddin *et al.*, 1991). Os efeitos dos compostos furânicos também se relacionam à inibição do crescimento e ao aumento da fase *lag* dos microrganismos. Modig e colaboradores (2002) estudaram a cinética da inibição do furfural nas enzimas álcool desidrogenase, aldeído desidrogenase e do complexo piruvato desidrogenase em

fermentações com *S. stipitis* e observaram que a presença desse inibidor diminuiu a atividade dessas enzimas, indicando que o furfural interfere nos mecanismos importantes na glicólise, síntese de biomassa celular e produção de bioetanol. Este mesmo trabalho reporta o efeito inibidor do HMF nas enzimas aldeído desidrogenase, álcool desidrogenase e piruvato desidrogenase. A enzima mais prejudicada foi a álcool desidrogenase, que apresentou atividade muito baixa na presença de 1,26 $g.L^{-1}$ de HMF.

Segundo Palmqvist *et al (*1999a), o crescimento celular é mais sensível à presença de furfural do que a produção de bioetanol. Em seu trabalho foi proposto um modelo que descreve a velocidade específica de crescimento, consumo de glicose, formação de bioetanol e glicerol na presença de furfural. O modelo foi baseado assumindo duas hipóteses: redução de furfural para álcool furfurílico e furfural causando inativação da replicação celular. O modelo descrito foi ajustado com precisão aos dados experimentais de *S. cerevisiae* na ausência e na presença de furfural, sugerindo que as suposições estavam corretas.

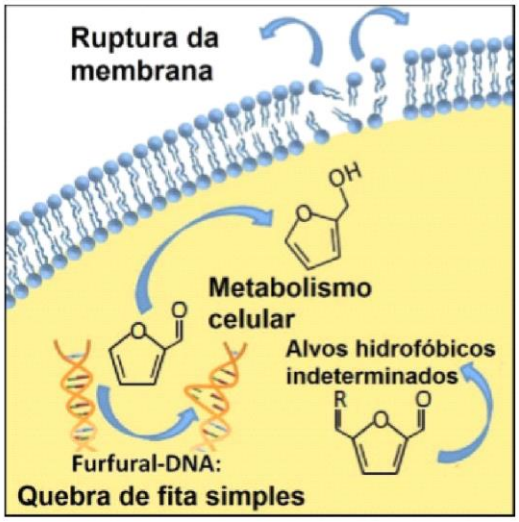

Figura 5 - Mecanismo de interação dos compostos furânicos com a célula microbiana. Mills et al. (2009), adaptado por Zeferino (2013).

Devido ao elevado número de compostos fenólicos presentes em hidrolisados lignocelulósicos, as análises qualitativas e quantitativas são de difícil realização e, por isso, o efeito deste grupo de moléculas ainda não foi completamente elucidado. O que se tem conhecimento é que seus efeitos estão relacionados ao caráter hidrofóbico da molécula. Por essa propriedade, são capazes de romper a membrana celular, como mostra a Figura 6, causando maior fluidez entre componentes intra e extracelulares. Com isso, a célula se torna menos seletiva e permite a passagem de prótons para o citoplasma. Além disso, quanto menor a massa molar do composto fenólico, maior é sua toxicidade para a célula microbiana (Palmqvist *et al.*, 1999; Palmqvist e Hahn-Hagerdal, 2000; Taherzadeh e Karimi, 2011).

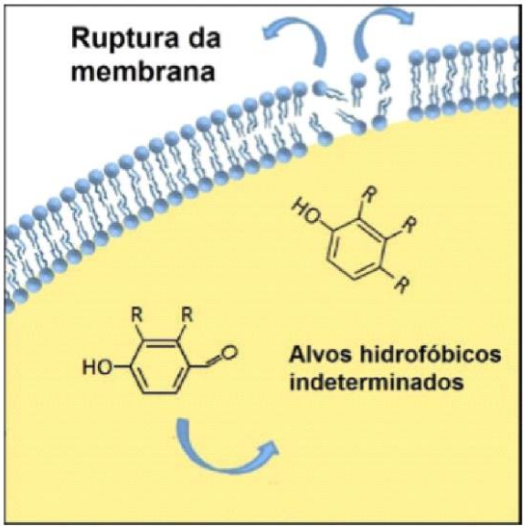

Figura 6 - Mecanismo de interação dos compostos fenólicos com a célula microbiana. Mills et al. (2009), adaptado por Zeferino (2013).

A Tabela 1 lista a concentração dos inibidores obtidos empregando tipos diferentes de pré-tratamento em bagaço de cana de açúcar. Convém salientar que é comum a etapa de concentração desses hidrolisados a fim de se obter uma alta concentração de açúcar para posterior alta conversão a etanol. Com isso, a tendência é

que os inibidores aumentem de concentração também, tornando-se uma barreira para processo de produção de bioetanol.

Tabela 1 - Concentração de inibidores em hidrolisados hemicelulósicos de bagaço de cana de açúcar.

Autor	**Rocha *et al*, 2011**		**Zhang *et al*, 2012**	**Martins *et al*, 2015**			**Nakasu *et al*, 2016**
Biomassa (sólido: líquido)	Bagaço de Cana de Açúcar (1,5: 10)	Bagaço de Cana de Açúcar (1:10)	Bagaço de Cana de Açúcar (1:10)	Bagaço de Cana de Açúcar (2:10)			Bagaço de Cana de Açúcar (2:10)
Pré-Tratamento	Ácido	Ácido	Ácido	Ácido	Peróxido de hidrogênio alcalino	Hipoclorito-peróxido de hidrogênio	Hidrotérmico com pós-hidrólise ácida
Glicose, g.L^{-1}	3,67	3,09	1,00	n.d	n.d	n.d	n.d
Xilose, g.L^{-1}	9,04	9,33	5,00	41,54	22.93	26,99	47,00
Ácido Acético, g.L^{-1}	2,21	2,89	4,60	2,95	3,56	6,15	6,14
Furfural, g.L^{-1}	0,05	0,10	5,60	0,26	0,23	1,10	0,80
5-HMF, g.L^{-1}	0,01	0,02	0,30	0,12	0,03	0,95	0,21
Fenólicos, g.L^{-1}	n.d	n.d	n.d	n.d	n.d	n.d	7,0*

n.d. - não determinado * sendo vanilina 0,104.

2.4 O efeito dos inibidores do hidrolisado hemicelulósico em microrganismos

Os efeitos de inibição por ácido acético (5,0; 10,0 e 15,0 g.L^{-1}) nos microrganismos consumidores de xilose *S. stipitis* e *Candida shehatae* e consumidores de glicose *Saccharomyces cerevisiae* e *Zymomonas mobilis* foram estudados por Delgenes e colaboradores (1996). Os resultados do trabalho indicaram que *C. shehatae* foi mais resistente à presença de ácido acético que *S. stipis* no que se refere à crescimento celular e produção de bioetanol, sendo esse último o metabolismo mais

afetado pela ação do inibidor. A presença de ácido acético em 15,0 $g.L^{-1}$ fez com que a concentração de bioetanol final diminuísse para *S. cerevisiae* (62 % do controle) e *Z. mobilis* (83 % do controle).

Díaz e equipe (2009) observaram o decaimento da velocidade específica de crescimento celular da levedura *S. stipitis*, de 0,09 para 0,06 h^{-1} com 3,0 $g.L^{-1}$ de ácido acético no meio de fermentação. A produtividade volumétrica em bioetanol também caiu, de 0,69 $g.L^{-1}.h^{-1}$ no controle para 0,17 $g.L^{-1}.h^{-1}$ na maior concentração de inibidor (6 $g.L^{1}$).

Morales *et al* (2016) verificaram que a tolerância para ácido acético da cepa *S. passalidarum* é baixa, uma vez que a produtividade volumétrica em bioetanol decaiu em quase 90% ao adicionar 4,5 $g.L^{-1}$ de ácido acético no meio contendo glicose.

Díaz e colaboradores (2009) estudaram o efeito do furfural para a levedura *S. stipitis* em concentrações de 2,0 e 4,0 $g.L^{-1}$. Observaram que a produção de bioetanol e o crescimento celular foram completamente bloqueados na presença da maior concentração de inibidor.

Foi testada a adição de concentrações entre 1,0 e 11,0 $g.L^{-1}$ de furfural e entre 1,3 e 15 $g.L^{-1}$ de HMF no trabalho de Liu e colaboradores (2004) para as cepas *S. stipitis* e *S. cerevisiae.* Os resultados mostraram que as leveduras foram mais sensíveis ao furfural que ao HMF, com a fase *lag* de crescimento pronunciada para todas as concentrações de inibidores e verificou-se que a mistura dos dois componentes inibiu totalmente o crescimento celular. Liu *et al* (2004) reporta a transformação de 5-HMF em 2,5 hidroximetilfurano após a fase *lag* de crescimento de *S. cerevisiae*, o que demonstra que a célula conseguiu se adaptar e transformar o inibidor em um composto menos tóxico.

Hou, em sua tese de doutorado (2011), avaliou o efeito da adição de 5-HMF e furfural em fermentações anaeróbicas com a cepa *S. passalidarum* em concentrações de até 3,0 $g.L^{-1}$. Os resultados mostraram que o crescimento celular foi mais inibido por furfural do que HMF. Com apenas 0,5 $g.L^{-1}$ de furfural a cepa teve metade do seu crescimento inibido, enquanto precisou de 2,0 $g.L^{-1}$ de HMF para que o mesmo efeito fosse observado. Hou (2011) observou a conversão de furfural a ácido furfurílico pela ação de *S. passalidarum* após 48h de processo. Estudos indicando o efeito do ácido furfurílico para esta cepa mostraram que o ácido inibe de forma muito sutil o crescimento celular em concentrações de até 1,0 $g.L^{-1}$ (Hou, 2011).

Delgenes *et al* (1996) testou a influência dos compostos fenólicos vanilina, siringaldeído e hidroxibenzaldeído no desempenho de *C. shehatae* e *S. stipitis*. Para a vanilina, os testes foram realizados com 0,5; 1,0 e 2,0 $g.L^{-1}$ desse composto no meio de cultura, e na menor quantidade testada já foi capaz de influenciar o crescimento e produção dos microrganismos, sendo esse efeito mais pronunciado em *S. stipitis*, que obteve 11 % de crescimento e 12 % de produção quando comparado ao seu ensaio controle. Na maior concentração, a vanilina inibiu totalmente os dois parâmetros estudados. Para seringaldeído, esse efeito foi observado para os dois microrganismos com 1,5 $g.L^{-1}$ de inibidor adicionado. Esta mesma concentração (1,5 $g.L^{-1}$) também foi a concentração de hidroxibenzaldeído que fez com que as cepas não crescessem e tampouco produzissem bioetanol.

2.5 Detoxificação do hidrolisado hemicelulósico

Os processos de detoxificação do hidrolisado hemicelulósico são uma alternativa para tornar possível a produção de bioetanol de 2ª geração e visam à retirada dos compostos inibitórios ao metabolismo celular dos microrganismos necessários para a

conversão dos açúcares à bioetanol. As estratégias de detoxificação envolvem técnicas utilizando diversos fatores como aditivos químicos, agentes redutores, enzimas, extração líquido-líquido, carvão ativado, troca iônica além de processos simples como o de evaporação.

Alriksson e colaboradores (2006) estudaram a detoxificação de hidrolisados hemicelulósico de resíduos de pinheiro. O hidrolisado continha 3,1 $g.L^{-1}$de 5-hidroximetilfurfural, 0,8 $g.L^{-1}$de furfural, 1,7 $g.L^{-1}$de ácido acético e a concentração total de fenóis foi estimada pela concentração de vanilina, 3,0 $g.L^{-1}$. Foram utilizados álcalis NaOH e NH_4OH imprimindo altas temperaturas e diferentes valores de pH (; 10; 11 e 12), sendo quantificada a remoção de HMF, furfural e fenóis desse material. Alguns dos resultados da otimização indicaram que o tratamento foi capaz de retirar em torno 90 % da concentração de furfural e 89 % da concentração de 5-HMF, porém não removeu os fenólicos, houve perda dos açúcares e baixo desempenho fermentativo quando comparado à fermentação padrão Os resultados de Alriksson e colaboradores (2006) indicam que é necessário encontrar um ponto de equilíbrio entre a remoção de inibidores e perda dos açúcares.

Cavka e Jonsson (2013) testaram a remoção de furfural e compostos aromáticos em hidrolisados de bagaço de cana e de resíduos de pinheiro com a adição do agente oxidante boro-hidreto de sódio ($NaBH_4$) aplicado diretamente na etapa de hidrólise. Seus resultados mostraram que houve 86 % de remoção de furfural e a detoxificação *in situ* resultou em pequena concentração residual de compostos aromáticos. A fermentação de *S. cerevisiae* também não foi afetada pela adição do composto na etapa anterior, alcançando produtividades volumétricas maiores do que no ensaio com os hidrolisados não tratados.

Cannella e colaboradores (2014) realizaram processo para a remoção de 45 % de ácidos alifáticos (acético e fórmico), 32 % de 5-HMF e 50 % de furfural, utilizando polímero catiônico de polietilenoimina (PEI) em hidrolisado de pinheiro.

Chandel et al. (2007) utilizaram resina de troca iônica em hidrolisado de cana-de-açúcar advindo de tratamento ácido e alcançaram redução máxima de furanos (63,4%) e fenólicos totais (75,8%). Nesse mesmo trabalho, estratégias de utilização de carvão ativado no hidrolisado causaram redução de 38,7 % e 57,5 % em furanos e fenólicos totais, respectivamente. Também foi utilizada a enzima lacase como estratégia de detoxificação para reduzir fenóis totais (77,5%), sem afetar os furanos e o teor de ácido acético no hidrolisado. A fermentação destes hidrolisados com *Candida shehatae* NCIM 3501 mostrou rendimento máximo de bioetanol (0,48 $g.g^{-1}$) a partir de hidrolisado tratado com troca iônica, seguido de carvão ativado (0,42 $g.g^{-1}$) e lacase (0,37 $g.g^{-1}$).

O estudo de Carvalho e colaboradores (2004) utilizou de quatro resinas de troca iônica diferentes, em sequência, para detoxificar o hidrolisado hemicelulósico do bagaço de cana-de-açúcar. O tratamento resultou em remoção de 82,1 % de furfural, 66,5 % de hidroximetilfurfural, 61,9 % de compostos fenólicos, contudo, a remoção de ácido acético não foi significativa.

O trabalho de Chaud e colaboradores (2010) utilizaram duas metodologias de detoxicação para o hidrolisado hemicelulósico do bagaço de cana-de-açúcar: aumento de pH para 7,0 com óxido de cálcio, seguido da diminuição para 2,5 com ácido fosfórico (*liming-overliming*), combinado com adsorção com carvão ativo e posterior floculação com polímero vegetal. A eficiência desses procedimentos foi avaliada pela análise de remoção de fenólicos e atingiu 80% do objetivo. Ainda foi reportado que o

polímero vegetal proporcionou perda de íons cromo, ferro e zinco (acima de 90%) no hidrolisado.

Apesar de existirem variados métodos de detoxificação, existem também pontos negativos para serem levados em conta na implementação desses processos. Sem dúvida, eles elevam os custos da cadeia produtiva de bioetanol, pois aumentam a demanda de energia, de capital de investimento em equipamentos e operação, além de requererem etapa de recuperação dos agentes detoxificantes, que encarecem o custo do produto final, tornando-o ainda menos competitivo com os biocombustíveis de primeira geração e/ou combustíveis fósseis (Jonsson et al., 2016). Outro fator é que praticamente todas as metodologias citadas acima resultam na perda de açúcares durante o processo de detoxificação.

2.6 Microrganismos consumidores de pentoses

A Figura 7 mostra o esquema da degradação de xilose devida à ação do metabolismo de leveduras. Inicialmente a D-xilose sofre redução a xilitol pela enzima xilose redutase (XR) e, em seguida, sofre oxidação à D-xilulose pela enzima xilitol desidrogenase (XDH). A D-xilulose, sofre fosforilaçao à D-xilulose-5-fosfato, segue para a via pentose fosfato, produzindo gliceraldeído-3-fosfato e frutose-6-fosfato, as quais são posteriormente convertidas a etanol pela via glicolítica (Agbogbo *et al.*, 2007; Kuhad *et al*., 2011).

Microrganismos capazes de produzir bioetanol a partir de pentoses podem ser naturalmente fermentadores ou microrganismos geneticamente modificados. Entre os naturais estão as linhagens de *Candida, Scheffersomyces, Schizosaccharomyces,*

Spathaspora, Kluyveromyces e Pachysolen, os fungos filamentosos *Fusarium, Mucor, Monilia e Paecilomyces*, e bactérias *Clostridium, Bacillus* . Dentre estes os que se destacam por apresentarem bons rendimentos são *Candida shehatae*, *Scheffersomyces stipitis* (anteriormente denominada *Pichia stipitis*) e *Fusarium oxysporum* (Millati; Edebo; Taherzadeh, 2004).

A levedura de *Scheffersomyces stipitis* tem sido amplamente estudada por sua capacidade de fermentação de pentose. Alguns dos estudos disponíveis utilizaram a xilose como fonte exclusiva de carbono (Shi *et al.*, 2014) ou numa combinação com a glicose (Santos *et al.*, 2015; Sliniger *et al*, 2011; Agbobo *et a.l*, 2008). Porém, esta espécie tem uma baixa tolerância ao bioetanol e ao açúcar, o que restringiu a sua utilização como uma estirpe industrial para produção de bioetanol em grande escala a partir da xilose. No entanto, continua sendo a levedura mais utilizada para fornecer os genes para a expressão das enzimas XR e XDH em *S. cerevisiae* geneticamente modificada.

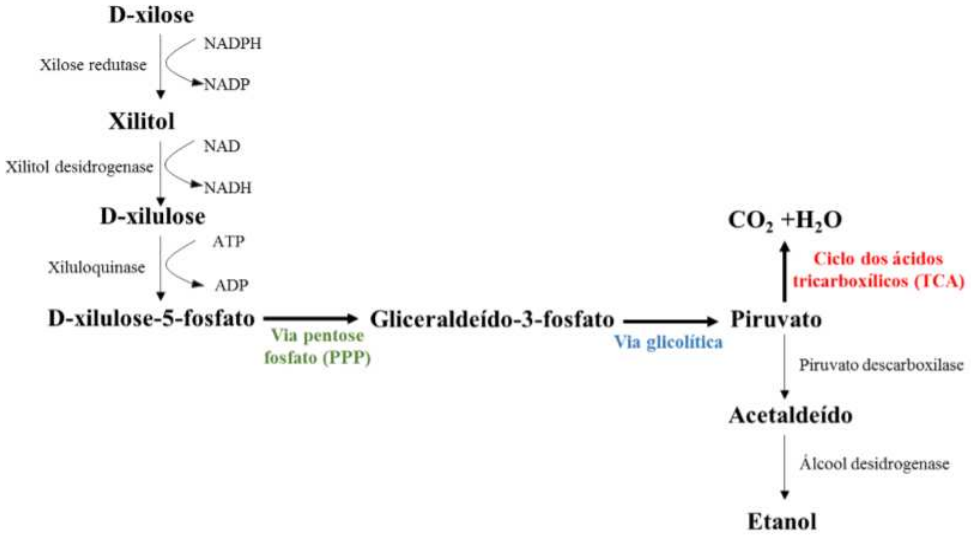

Figura 2 - Esquema da degradação da xilose devido ao metabolismo de leveduras. Kuhad et al.(2013), adaptado por Costa (2016).

A espécie *Spathaspora passalidarum*, isolada por Nguyen *et al* (2006) e estudada por Hou (2012), apresentou resultados mais promissores que os previamente encontrados para *S. stipitis*. Em anaerobiose, a levedura apresentou produção de xilitol de 0,09 $g.g^{-1}$ por xilose consumida, valor inferior a linhagens notadamente consumidoras de xilose, e rendimento em bioetanol superior a 0,45$g.g^{-1}$. Já em aerobiose, os açúcares glicose e xilose foram consumidos concomitantemente (Hou, 2012). A vantagem da utilização de *S. passalidarum* estaria na especificidade de seus cofatores, que seriam naturalmente oxidados e reduzidos durante o metabolismo, reduzindo o acúmulo de xilitol no meio de cultivo (Hou, 2012). No Brasil, leveduras do gênero *Spathaspora* foram inicialmente isoladas de madeira em decomposição e de insetos relacionados à decomposição em reservas da Mata Atlântica e do Cerrado brasileiro, em 2009 (Cadete *et al*., 2009).

Long e colaboradores (2012) estudaram *S. passalidarum* em co-fermentações de glicose, xilose e celobiose e verificaram que a assimilação dos outros açúcares não sofreu repressão pela presença de glicose no meio fermentativo. A análise metabólica dessas fermentações mostrou que o fluxo de intermediários glicolíticos é significativamente maior para xilose do que para glicose. A alta afinidade de suas atividades de xilose-redutase para NADH e xilose; combinada com a ativação alostérica da glicólise, provavelmente explica em parte essa capacidade de não sofrer diauxia que não é comum nas leveduras reportadas na literatura. No trabalho de Long *et al.,* (2012) *S. passalidarum* NN245 alcançou rendimento em bioetanol de 0,42 $g.g^{-1}$.

Nakanishi e colaboradores (2017) compararam o desempenho de *S. passalidarum* com *S. stipitis* em hidrolisado de bagaço de cana de açúcar. Utilizando a estratégia de alta densidade celular, quatro reciclos de células foram realizados e os resultados indicaram que a produção de bioetanol atingiu valores mais altos para *S.*

passalidarum (21 $g.L^{-1}$) que para *S. stipitis* (15 $g.L^{-1}$) em ensaios nas mesmas condições. A produtividade volumétrica de *S. passalidarum* atingiu 0,81 $g.L^{-1}.h^{-1}$, enquanto que para *S. stipitis* esse valor não ultrapassou 0,38 $g.L^{1}.h^{1}$ para a mesma condição experimental.

Yu *et al (*2017) utilizou *S. passalidarum* U1 – 58 em fermentações empregando hidrolisado hemicelulósico de sabugo de milho e obteve resultados satisfatórios, atingindo 35 $g.L^{-1}$ de bioetanol no final do processo.

Analisando sua potencialidade na conversão de xilose para produção de bioetanol de segunda geração utilizando hidrolisados hemicelulósicos, um estudo mais particularizado envolvendo a presença dos inibidores e seu impacto no metabolismo de *S. passalidarum* é imprescindível, objetivando a comparação com o microrganismo modelo para fermentação de pentoses, *S. stipitis*.

CAPÍTULO 3 - COMPARATIVE BEHAVIOUR OF NON CONVENTIONAL YEASTS *Scheffersomyces stipitis* AND *Spathaspora passalidarum* IN THE PRESENCE OF DIFFERENT HEMICELLULOSIC HYDROLYSATE INHIBITORS

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Soares, L. B. [a, b]; Bonan, C. I. D. G. [a, b], Dionísio S. R. [a], Bonatelli, M. L. [a], Costa, A. C.[b]; Ienczak, J. L. [c]

[a] Brazilian Bioethanol Science and Technology Laboratory - CTBE/CNPEM, 10000 Giuseppe Maximo Scolfaro St, Zip Code 13083-852 Campinas, SP, Brazil

[b] School of Chemical Engineering, State University of Campinas - UNICAMP, 500 Albert Einstein Av, Zip Code 13083-852 Campinas, SP, Brazil

[c] Chemical Engineering and Food Engineering Department - Santa Catarina Federal University. CP 476, Zip Code 88040-900, Florianópolis - SC, Brazil.

3.1 Abstract

The present study aims to compare the impact of inhibitory compounds (acetic acid, furfural, 5-hydroxymethylfurfural and vanillin) on cell growth, sugar consumption and ethanol production of the yeasts *Scheffersomyces stipitis* and *Spathaspora passalidarum*. The results have shown that acetic acid (2.5 $g_{AA}.L^{-1}$ for *S. stipitis* and 0.5 $g_{AA}.L^{-1}$ for *S. passalidarum*) and furfural (2.5 $g_{FURF}.L^{-1}$ for *S. stipitis* and 1.5 $g_{FURF}.L^{-1}$ for *S. passalidarum*) are the strongest inhibitors for both yeasts. From 0.5 $g_{5\text{-}HMF}.L^{-1}$ of 5-hydroxymethylfurfural, ethanol production by *S. passalidarum* was negatively influenced. Vanillin only influenced the cellular growth of *S. passalidarum*, which is not interesting for processes with cellular recycle. Considering sugar consumption, xylose uptake is more inhibited than glucose uptake. Regarding the synergic effect with the other inhibitors studied; acetic acid seems to increase the inhibition effect of all the inhibitors. *S. stipitis* was more resistant than *S. passalidarum* in the presence of the inhibitors studied; however, even more inhibited, *S. passalidarum* was more efficient in ethanol production. By the results obtained it was possible to determine the maximum concentrations of each inhibitor that can be present in hemicellulosic hydrolysates without a decrease of more than 20 % in fermentation performance compared to fermentation in the absence of the inhibitor.

3.2 Introduction

Sugarcane bagasse consists of three main components: cellulose, hemicellulose and lignin (1). The industrial production of bioethanol from biomass presents a pretreatment stage, which results in the exposure of the cellulose chains and sometimes in the solubilization of hemicellulose and/or lignin; followed by enzymatic hydrolysis to release monomeric sugars and, finally, fermentation and recovery of bioethanol (2). Pretreatment is a process bottleneck and it causes the generation of inhibitors for the enzymatic hydrolysis and fermentation steps. The study of the inhibitors impact on fermentation is of great interest to produce ethanol from biomass, since the effect on growth, carbohydrate consumption, ethanol production and the limits of tolerance must be known. It is possible to highlight acetic acid (AA), furfural (FURF), 5-hydroxymethylfurfural (5-HMF) and phenolic compounds as the main inhibitors (3).FURF and 5-HMF are compounds formed by the degradation of xylose and glucose, respectively. Acetic acid is generated by the deacetylation of hemicelluloses and phenolic compounds are formed by lignin degradation (4). These inhibitors are mainly present on the liquid fraction generated after pre-treatment, the hemicellulosic hydrolysate, which is rich in xylose (around 70 - 80 % of the concentrations of total sugars) and glucose (20 – 30%).

Xylose is the main sugar in hemicellulosic hydrolysate and yeasts naturally capable of fermenting pentoses such as *Scheffersomyces stipitis* (5, 6, 7) and *Spathaspora passalidarum* (5, 8, 9) can be used to metabolize this fraction of biomass for ethanol production. The effects of hemicellulosic hydrolysate inhibitors on *S. stipitis* metabolism has been widely discussed (10, 11, 12, 13). On the other hand, only one paper has been published on this subject for *S. passalidarum* (14). In this context, this

work aimed to compare the effect of acetic acid, FURF, 5-HMF and vanillin on *S. passalidarum* and *S. stipitis* yeasts growth, ethanol production and sugars consumption.

3.3 Material and Methods

3.3.1 Yeasts, Inoculum and Propagation

The xylose-fermenting yeasts *Scheffersomyces stipitis* NRRL Y-7124 and *Spathaspora passalidarum* NRRL Y-27907 were used in this study. Yeasts were stored at -80 °C in YPX media containing glycerol (0.8% w/v). For cellular reactivation, cells were transferred to pre-inoculum that was performed in 250 mL Erlenmeyer flasks containing 100 mL of YPX media ($g.L^{-1}$): yeast extract (10.0); peptone (20.0) and xylose (20.0) and then incubated for 24 h at 30 ° C and 200 rpm (Orbital Innova® 44 Shaker, New Brunswick). After this period, 10% (v/v) of the pre-inoculum was transferred to the inoculum media composed of ($g.L^{-1}$): xylose (10.0), glucose (12.0), urea (2.3), yeast extract (3.0) and $MgSO_4$.$7H_2O$ (1.0) (15). Inoculum was carried out in 1000 mL Erlenmeyer flasks with 250 mL of working volume and were kept at 30 °C and 200 rpm for a period of 24 h in orbital shaker. Posteriorly, the entire volume was centrifuged at 8000 rpm, 4 °C for 20 min (Thermo Scientific, Sorvall RC-6 Plus). After discarding the supernatant, the cells were resuspended in sterile water (the total volume represented 10 % of the initial volume of the propagation bioreactor).

Cells from inoculum were transferred to the propagation bioreactor (Bioflo®, New Brunswick Scientific Co., Inc., Edison, NJ, 7.0 L), which was maintained at 50 % saturation relative to atmospheric air, at 30 °C and contained a media composed of ($g.L^{-1}$): sugarcane syrup (30.0), urea (2.3) and KH_2PO_4 (2.0) (6). After the exhaustion of the total reducing sugars (TRS) (detected with medium infrared MIR, FT-IR), a

continuous feed of sterile sugarcane syrup at a feed rate of 3 $g_{TRS}.L^{-1}.h^{-1}$ and a pulse of 3.0 $g.L^{-1}$ of urea and 1.0 $g.L^{-1}$ of KH_2PO_4 were established (6). After 24 h of propagation, the feed was stopped and the media was centrifuged in a sterile vessel at 8000 rpm for 20 min and 4 °C. Pellet cells were resuspended in sterile water and the concentration was determined by dry cell weight. The yeast creams of *S. stipitis* and *S. passalidarum* were stored separately at 4 °C and used as the stock cells solution for the later stages.

3.3.2 Individual and synergic inhibitor resistance test

For the individual inhibitor resistance test, cells from yeast creams of *S. stipitis* and *S. passalidarum* were reactivated in YPX media for 24 h, 30ºC and 200 rpm in a rotatory orbital. After the reactivation time, the cells were centrifuged separately at 8000 rpm for 20 min and resuspended in sufficient sterile water to reach an active initial cell concentration of 5 $g.L^{-1}$.

A 96-deepweel plate was prepared containing 2 mL of the fermentation control media, with the composition in $g.L^{-1}$: yeast extract (3.0), glucose (27.0), xylose (63.0), urea (2.3), $MgSO_4.7H_2O$ (1.0), ampicillin (1 $\mu L.mL^{-1}$) for *S. stipitis* and the same media with addition of NH_4Cl (3.0) for *S. passalidarum* (16). In the wells were added the inhibitors acetic acid (AA): 0.5; 1.5; 2.5; 3.5; 4,5; 5.5; 6.5 and 7.5 $g.L^{-1}$ (in citrate buffer pH 5.0 media), furfural (FURF): 1.0; 1.5; 2.0; 2.5; 3.0; 3.5 and 4.0 $g.L^{-1}$, 5-hydroxymethylfurfural (5-HMF): 0.5; 1.0; 1.5; 2.0; 2.5; 3.0; 3.5 and 4.0 $g.L^{-1}$ and vanillin (VAN): 0.1; 0.2; 0.3; 0.4; 0.5; 0.6; 0.7; 0.8 and 1.0 $g.L^{-1}$. Each assay was performed in triplicate. After inoculation, the plate was sealed with gaseous exchange

lace (Greiner, Bi-One Brazil), and maintained in a rotary incubator for 24 h at 30° C and 200 rpm.

Fermentations with combined concentrations of acetic acid, furfural, 5-HMF and vanillin were also performed to evaluate the synergistic effect of the inhibitors on the strains studied. These tests were done on deep-well plates as described for the individual inhibitors test and the concentrations of the compounds used are listed in Table 1.

Table 1. Inhibitors concentration tested in fermentations by *S. stitpis* and *S. passalidarum* to verify the synergic impact on growth, ethanol production and sugars consumption.

Inhibitor	Concentration ($g.L^{-1}$)				
	I	II	III	IV	V
Acetic acid	0.0	0.0	3.0	1.5	4.5
Furfural	0.0	1.0	1.0	0.5	0.5
5-HMF	0.0	1.0	1.0	0.5	0.5
Vaniline	0.0	0.2	0.0	0.3	0.3

Samples were taken at the initial and final fermentation time (72 h for *S. stipitis* and 48 h for *S. passalidarum*) for optical density analysis at 600 nm in a spectrophotometer (Infinite 200 PRO, Tecan, Austria). Thereafter, the samples were centrifuged and the supernatants were used for metabolites analysis. The cellular concentration was determined using a correlation curve between optical density and dry cell weight previously established. The analysis of carbohydrates, alcohols and organic acids were carried out using high performance liquid chromatography (HPLC). The samples were filtered using Millex 0.20 μm filters (PVDF), diluted and analyzed in an Agilent Infinit 1260 (Santa Clara, CA, USA) HPLC system coupled to the Refractive Index (IR) detector equipped with Aminex HPX-87H column (300 x 7.8 mm, Bio-Rad) at 35 °C. The mobile phase employed was a solution of 5mM sulfuric acid in the

isocratic mode with flow of 0.6 mL.min^{-1}, injection volume of 30 µL and analysis time of 30 min. The phenolic compounds (vanillin, HMF and furfural) were analyzed using an Agilent Infinit 1260 HPLC system (Santa Clara, CA, USA) coupled to the Variable Wavelength Detector (VWD) equipped with a C-18 reverse phase C-18 Acclaim PA2 3µm 120Å (150 x 4.6 mm, Thermo Scientific) at 25 °C. A water: acetonitrile solution in a ratio of 8: 1 (v / v) containing 1% acetic acid with isocratic elution, flow of 0.8 mL.min^{-1}, 30 µL injection volume , analysis time of 40 minutes and detection at wavelength λmax = 274 nm. The product quantifications were made from the construction of calibration curves performed with external standards. All standards and solvents used were of chromatographic and analytical grade (purity greater than 99.0%, Sigma Aldrich, Merck). The mobile phase is filtered through a 0.45 µm membrane and degassed by ultrasound for 30 min prior to use.

The concentrations of cell, substrates and ethanol were evaluated in relation to the control assay for each yeast, in which no inhibitor was added. Cell growth and sugar consumption were calculated using the difference between the final and initial concentrations. In equation 1, S_0 is the initial glucose and xylose concentration, E_0 and E are the initial and final ethanol concentration, respectively. The value of 0.511 $g_{EtOH} \cdot g_{substrate}^{-1}$ corresponds to the stoichiometric conversion of xylose and glucose into ethanol for the yeasts.

$$yield = \frac{E - E_0}{S_0} \times \frac{100}{0.511} \quad (1)$$

The average results of cell growth, sugar consumption and ethanol production were compared using the Kruskall-Wallis test for non-parametric data considering a 95 % confidence level, using the statistical Software R (17).

3.4 Results and Discussion

Experiments were conducted to compare the fermentative performance of *S. stipitis* and *S. passalidarum* regarding the presence of hemicellulosic hydrolysate inhibitors. The performance of yeasts was compared with the control assay (without inhibitors) for cell growth, sugar consumption and ethanol production.

All acetic acid concentrations tested were considered to be significantly different from the control assay and influenced negatively cell multiplication of both yeasts (Figure 1 (A) - I). Cell growth of *S. stipitis* did not exceed 45 % of the control assay (without acetic acid addition) for concentrations from 2.5 $g_{AA}.L^{-1}$. For *S. passalidarum*, at the same inhibitor concentration, growth was of about 70 % of the control assay. Acetic acid also influenced the sugars assimilation (glucose and xylose), highlighting the most pronounced influence for *S. passalidarum* (Figure 1 (B) - I), for which a concentration of 1.5 $g_{AA}.L^{-1}$ inhibited the consumption in about 40 % in relation to the control. For *S. stipitis*, the concentration that reaches this inhibition level was higher than for *S. passalidarum* and equal to 3.5 $g_{AA}.L^{-1}$. Ethanol production was more influenced than cell growth and sugars consumption (Figure 1(C) - I). Again, for ethanol production, *S. passalidarum* was the most inhibited and in the presence of 1.5 $g_{AA}.L^{-1}$, ethanol production dropped to 20 % of the control assay production. *S. stipitis* also showed ethanol production inhibited from the presence of acetic acid, but its degree of tolerance was higher, an abrupt decline in production was only observed from 3.5 $g_{AA}.L^{-1}$.

Others authors (12) have tested the addition of 0.5; 1.5 and 2.5 $g_{AA}.L^{-1}$ in glucose and xylose fermentations with *S. stipitis* DSM 3651. The results showed that, in fermentations with 1.5 and 2.5 $g_{AA}.L^{-1}$, cell grow was about one-third of that achieved in

the control assay, and ethanol production was of 60 % and 40 % of the control assay production, respectively. For *S. passalidarum*, the tolerance to acetic acid is low, since the volumetric productivity in ethanol declined by almost 90 % when $4.5 g_{AA}.L^{-1}$ was added to the media containing glucose as substrate(14).

Cell growth was not severely influenced by the presence of FURF. Growth for both yeasts was not lower than 80 % of the control growth (Figure 1 (A) - II) for all FURF concentrations tested. As observed for sugar consumption (Figure 1 (B) - II), *S. passalidarum* was more impaired in comparison with *S. stipitis*. For this yeast, the presence of 1.5 $g_{FURF}.L^{-1}$ drastically decreased sugar consumption to 60 % of the control, while for *S. stipitis* the percentage of consumption was not lower than 80 % of the control, even at the highest concentration of inhibitor tested. For ethanol production (Figure 1 (C) - II), once again the most significantly affected yeast was *S. passalidarum*. From 1.5 $g_{FURF}.L^{-1}$, the production dropped sharply to less than half of the production of the assay without inhibitor. In the highest concentration of FURF *S. stipitis* reached around 60% of the production of the control assay.

Figure 1 (A) - III shows cell growth for the yeasts in the presence of increasing concentrations of 5-HMF. For *S. stipitis*, the highest concentration of inhibitor tested (4.0 $g_{5\text{-}HMF}.L^{-1}$) had low influence on cell multiplication, and growth was not lower than 80 % of the control growth. *S. passalidarum* showed cellular growth corresponding to 40 % of the control from 3.0 $g_{5\text{-}HMF}.L^{-1}$. The presence of 5-HMF had little influence on sugars consumption for *S. stipitis*; however, in all concentrations of this inhibitor, sugars consumption was inhibited for *S. passalidarum*, and its performance was around of 60% of the control (Figure 1 (B) - III). The addition of 5-HMF also did not influence *S. stipitis* ethanol production, but the assays for *S. passalidarum* showed about 60 % of production relative to the control assay (Figure 1 (C) - III).

When other studies in literature are observed for 5-HMF inhibitor effect it is possible to notice that its influence on *Saccharomyces cerevisiae* has shown that 5-HMF is consumed by this yeast at a slow rate, causing a pronounced lag phase of cell growth. Production of 5-HMF alcohol is also reported from the consumption of 5-HMF (20). It was verified the inhibitory action of 5-HMF in fermentations of *S. stipitis* DSM 3651 at concentrations of 0.1 and 0.5 $g_{5\text{-}HMF}.L^{-1}$ using glucose and xylose as carbon sources. The results showed that the yeast had growth behavior similar to the control assay, with all assays displaying the same final concentration of cells (12). That compound was also tested in anaerobic glucose fermentations by the *S. passalidarum* strain ATCC MYA-4345 and the results showed from 40 to 50 % inhibition in cell growth when 1.0 to 2.0 $g_{5\text{-}HMF}.L^{-1}$ were added in the fermentation media (21).

The addition of 0.4 $g_{VAN}.L^{-1}$ weakly influenced the cell growth of *S. stipitis*, which reached about 80% of the control growth. The growth of *S. passalidarum* has been influenced even by the lowest concentration added, with the sharpest decrease at 0.4 $g_{VAN}.L^{-1}$(Figure 2 (A) - IV), what does not favor processes that uses cell recycling. Despite the pronounced inhibition for cell growth of *S. passalidarum*, both yeasts could consume the sugars and produce ethanol in a manner similar to their control assays, as shown in Figures 1 (B) - IV and (C) - IV.

The behavior of *S. stipitis* NRRL Y-7124 at concentrations of 0.5; 1.0 and 2.0 $g_{VAN}.L^{-1}$ in fermentation media containing xylose as the carbon source was studied by Delgenes et al. (10). For the lowest vanillin concentration, *S. stipitis* growth was 12 % of the growth in the fermentation without inhibitor. Ethanol production was also affected by the presence of vanillin, reaching only 11, 9 and 6 % of the control assay production at concentrations of 0.5; 1.0 and 2.0 $g_{VAN}.L^{-1}$, respectively. These results differ from the obtained in the present work, where growth and production were higher

than 80% of the obtained in the control assay in the same range of inhibitor concentrations. A possible explanation for the different behavior is the use of different fermentation media, since in the present work the media contained glucose and xylose mixture and the study performed by Delgenes et al (10) used only xylose as a carbon source. The cell concentration used in the tests was not reported, which can also influence the results. In the present work the cell concentration was of 5 $g.L^{-1}$ at the beginning of the fermentation.

From Figure 1 (C), it is possible to observe that, although ethanol production by *S. passalidarum* is strongly inhibited, the final concentrations of ethanol reached by this yeast are similar to those obtained by *S. stipitis*. For example, considering the concentration of 3.5 $g_{AA}.L^{-1}$, ethanol production by *S. passalidarum* was 10% of the production in the control assay, whereas in that same condition, *S. stipitis* production was 40% of the control assay. However, in actual values of ethanol concentration, both yeasts produced approximately 7 $g_{EtOH}.L^{-1}$. The same behavior was observed at 1.5 $g_{FURF}.L^{-1}$, with a 50% inhibition of ethanol production by *S. passalidarum*, while *S. stipitis* showed only 25% inhibition. In this condition, *S. passalidarum* produced 7 $g_{EtOH}.L^{-1}$, while *S. stipitis* produced 10 $g_{EtOH}.L^{-1}$. In the presence of 5-HMF and vanillin, ethanol production is also similar for both yeasts, despite some fluctuations in the data.

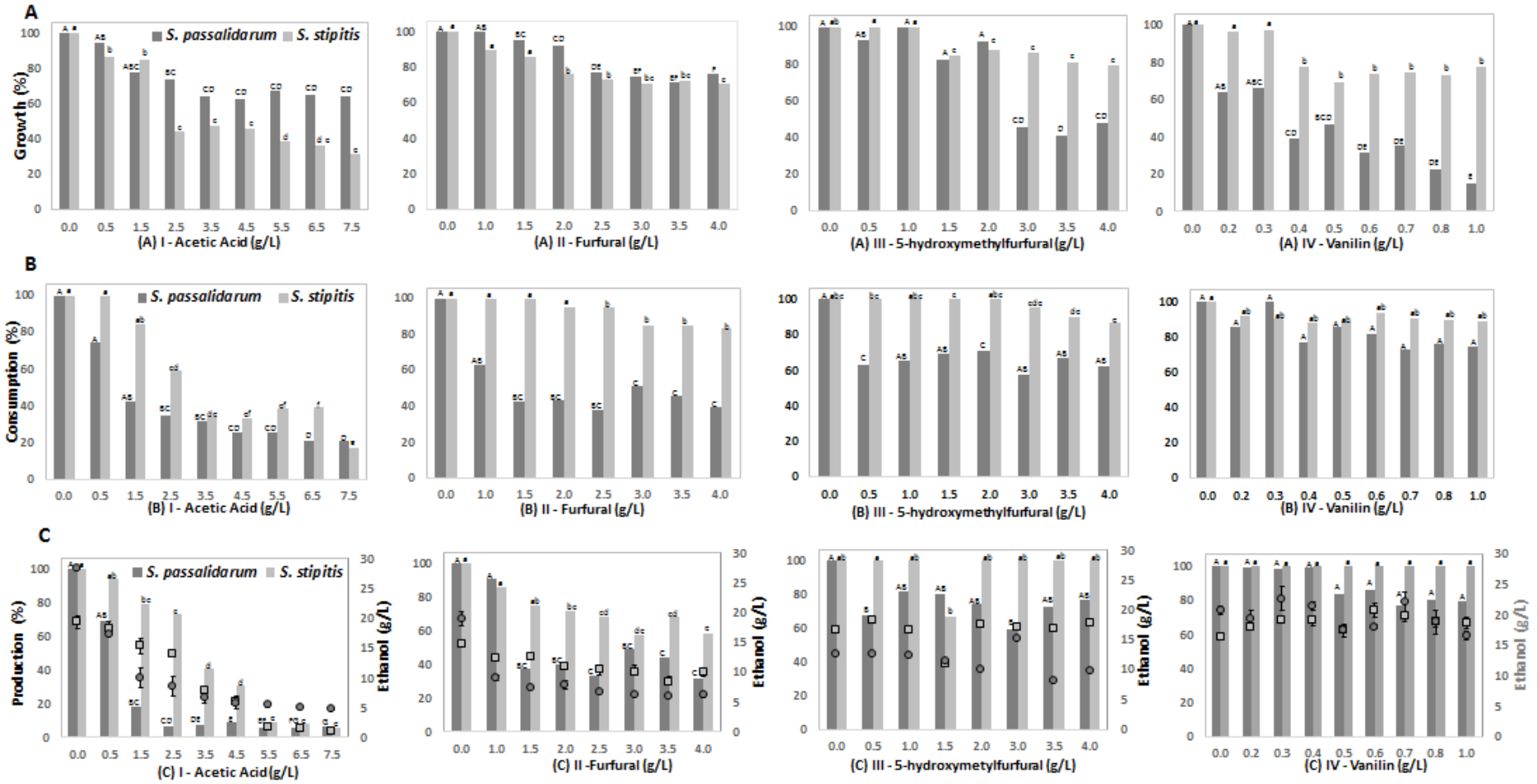

Figure 1 Comparison of *S. stipitis* (light gray) and *S. passalidarum* (dark gray) performance in the presence of individual acetic acid (I), furfural (II), 5-hydroxymethylfurfural (III) and vanillin (IV), considering cell growth (A), sugar consumption (B) and ethanol production (C) in relation to the control assay. The bars represent the percentage values and the circles and squares represent the final concentration of ethanol obtained in each assay. The assays with the same letter are not significantly different in Kruskall-Wallis test at the 95% confidence level.

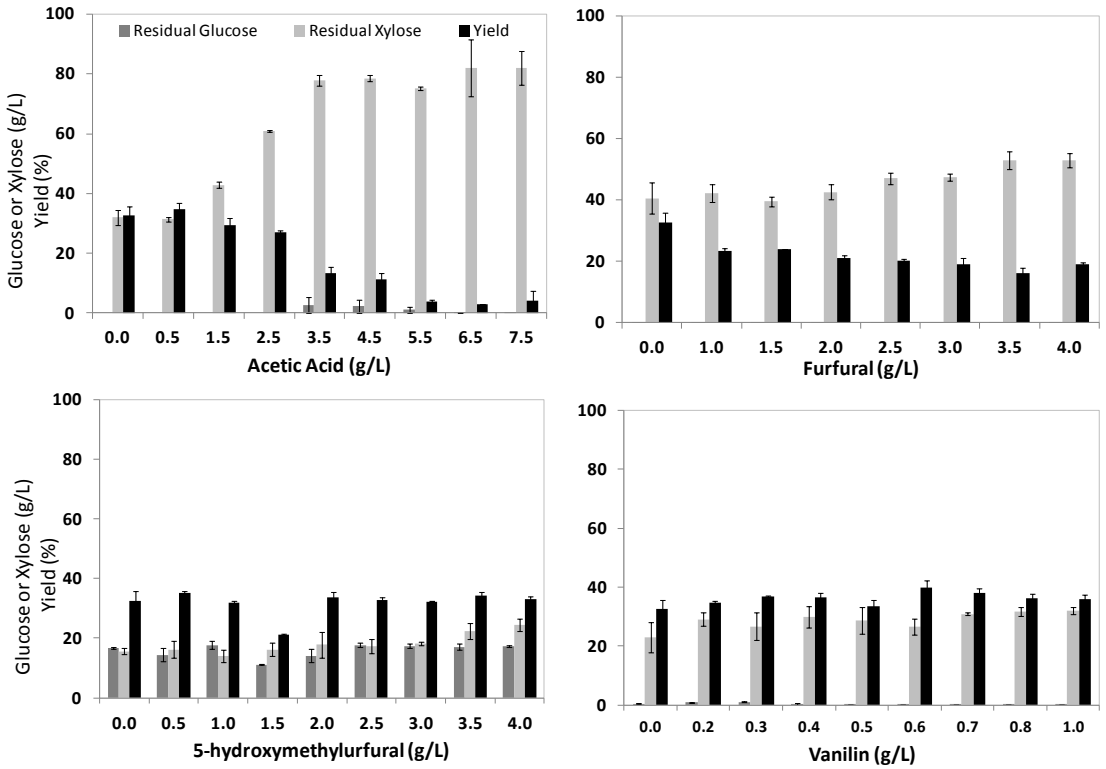

Figure 2. Ethanolic fermentation for *S. stipitis* in the presence of inhibitors: (A) acetic acid; (B) furfural, (C) 5-HMF and (D) vanillin. The bars represent the values of residual glucose (black gray), residual xylose (light grey) and yield (black).

Literature describes *S. passalidarum* as the best ethanol producer when compared to *S. stipitis*. The comparison on fermentation kinetics of *S. passalidarum* NN45 and *S. stipitis* NRRL Y-7124 in a media containing glucose or xylose and mixtures of glucose and xylose at various dissolved oxygen conditions was studied and, in most cases, it was found that *S. passalidarum* produces more ethanol than *S. stipitis,* especially when xylose is the sole carbon source (22). Other comparison made for the two yeasts in fed-batch fermentations of a mixture of both sugars (xylose and glucose) in a sugarcane bagasse hydrolysates found that ethanol production was higher for *S. passalidarum* (23 $g.L^{-1}$) than for *S. stipitis* (17 $g.L^{-1}$) in assays under the same conditions. In addition to the higher production, *S. passalidarum* also presents higher

production rates, with productivity reaching 0.81 $g.L^{-1}.h^{-1}$, while for *S. stipitis* the highest value was no more than 0.40 $g.L^{-1}.h^{-1}$. (5)

It is possible to observe in Figure 2 for the fermentation with *S. stipitis* that xylose consumption was the most inhibited by the presence of the different inhibitors. In some fermentations performed, there was no xylose consumption, mainly for the fermentations with acetic acid (up to 3.5 $g_{AA}.L^{-1}$) and with all furfural concentrations tested. In turn, glucose consumption was only inhibited by the presence of 5-HMF. Yield showed a decrease with increasing concentrations in the acetic acid tests, and it was not much influenced by the presence of furfural, 5-HMF and vanillin.

For *S. passalidarum*, the same analysis of final glucose and xylose concentrations and yield (Figure 3) was performed and it has shown that glucose consumption was less inhibited by the presence of acetic acid and furfural in comparison to xylose again. Xylose consumption was affected by all the inhibitors considered, and acetic acid and furfural resulted in drastic influence for consumption. It is known that the net yields of ATP are substantially lower when the yeast is growing in xylose in comparison to glucose [22]. Consequently, cells need to consume more carbon to produce the same amount of energy when xylose is present in greater amounts, which can be worse with the presence of inhibitors, so the cell will not expend energy with that sugar. The presence of acetic acid and furfural dropped ethanol production (measured by yield) sharply, but 5-HMF and vanillin had little influence on the production.

It is important to emphasize that the yield of the control assay for both the yeasts was lower than values reported in literature (around 60% for *S. stipitis* and 85% for *S. passalidarum*(5)), probably due to the conditions offered by 96-deepweel plate (difficult in transfer mass).

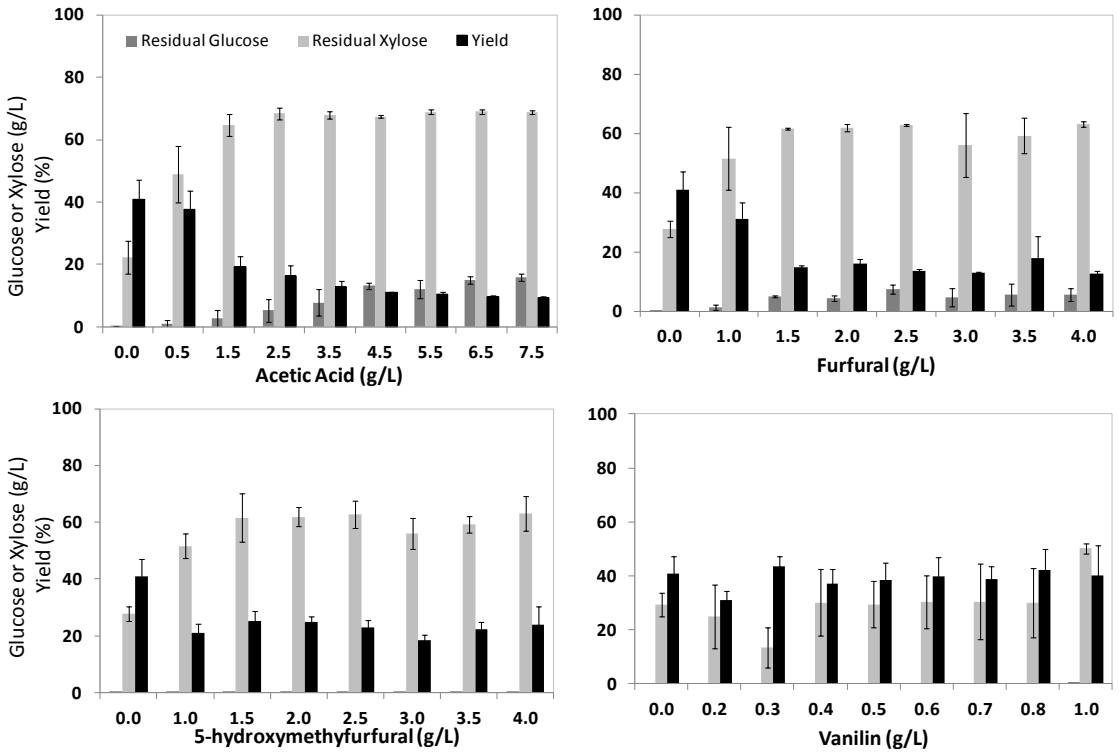

Figure3. Ethanolic fermentation for *S. passalidarum* in the presence of inhibitors: (A) acetic acid; (B) furfural, (C) 5-HMF and (D) vanillin. The bars represent the values of residual glucose (black gray), residual xylose (light grey), and yield (black).

According to the results obtained until this point of the work, among the inhibitors present in second generation liquors, acetic acid and furfural have the strongest influence on the yeasts metabolism. However, it is important to consider the synergism between the inhibitors. In this way, experiments were conducted to compare the fermentative performance of *S. stipitis* and *S. passalidarum* regarding the presence of hemicellulosic hydrolysate inhibitors in aleatory mixtures (see Table 1). The performance of yeasts was compared with the control assay (without inhibitors) for cell growth, sugar consumption and ethanol production

Analyzing the effect of combined inhibitors tested in the *S. stipitis* fermentation, it was possible to notice that the presence of furfural, 5-HMF (both at 1.0 $g.L^{-1}$) and vanillin (0.2 $g.L^{-1}$) only reduced glucose consumption by the yeast when compared with

control (Figure 4 A I and II). Keeping the furanic concentration, removing the vanillin and increasing the acetic acid concentration to 3.0 $g.L^{-1}$ (Fig 4 A III) it was observed the drastic drop in the sugars consumption, ethanol and cell production when compared to the assay with vanillin but no acetic acid (Fig 4 A II).

Even at lower concentrations, the mixture of acetic acid (1.5 $g\ L^{-1}$) with furfural, 5-HMF (both at 0.5 $g\ L^{-1}$) and vanillin (0.3 $g\ L^{-1}$), affected significantly the metabolism of *S. stipitis*, reducing xylose consumption, ethanol production and cell growth (Fig 4 A IV). These results suggest that the presence of acid acetic is more harmful for yeast than the other inhibitors. In the presence of higher concentration of acid acetic (4.5 $g\ L^{-1}$) in addition to the other inhibitors in the same concentration, lower values of sugar consumption, ethanol production and cell growth were reported, just confirming the significant role of acid acetic as inhibitor for *S. stipitis* (Figure 4 A IV and V)

Considering S. *passalidarum*, the effect of inhibitors was similar to those observed with *S. stipitis*, but it was even more severe. The presence of furfural, 5-HMF (both at 1.0 $g.L^{-1}$) and vanillin (0.2 $g.L^{-1}$) reduced sugar consumption, when compared with the control (Figure 4 B I and II). The same effect observed for *S. stipitis* occurred when the concentration of acetic acid was increased and the level of furfural and HMF was not modified. In this condition there was neither sugar consumption nor cell production.

The presence of acid acetic (1.5 $g\ L^{-1}$), furfural, 5-HMF (both at 0.5 $g\ L^{-1}$) and vanillin (0.3 $g\ L^{-1}$) (Figure 4 B IV) nearly prevented yeast sugar consumption and ethanol production. The effect of this mixture was even more severe when the acid acetic concentration increased to 4.5 $g\ L^{-1}$ (Figure 4 B V).

Studies (18) have shown the synergic action between acid acetic (3.0 $g\ L^{-1}$) and furfural (2.0 $g\ L^{-1}$) in the same yeast and noticed that *S. stipitis* did not produce ethanol

or consume sugars, but this effect was not noticed when the inhibitors were tested separately. In another study (12) using a mix of acid acetic (1.52 $g.L^{-1}$), furfural (0.14 $g.L^{-1}$) and 5-HMF (0.05 $g.L^{-1}$) the authors observed the reduction of fermentation performance in *S. stipitis* DSM 3651 and lowering sugar consumption, as we have observed in our experiments.

Weak acids, such as acetic acid, are liposoluble and diffuse through the cell membrane, decreasing the cytosolic pH. Thus, the cell spends energy seeking to restore its internal pH, diverting the metabolism from cell growth and ethanol production (23; 4). Furan compounds such as furfural and 5-HMF interact with important enzymes in cell multiplication and ethanol production, such as hexokinase, glyceraldehyde 3-phosphate dehydrogenase, alcohol dehydrogenase and pyruvate dehydrogenase (24, 25, 3). There is a wide variety of phenolic compounds from different lignocellulosic materials described in the literature, and for this reason their mechanisms of action have not yet been fully elucidated. What is known is that they interact with cell wall compounds, causing damage to membrane integrity and therefore affect metabolic activities, decreasing ethanol production and cell growth (4). The origin of sugars, inhibitors, microorganisms and their interactions in second generation ethanol production is schematized in Figure 5.

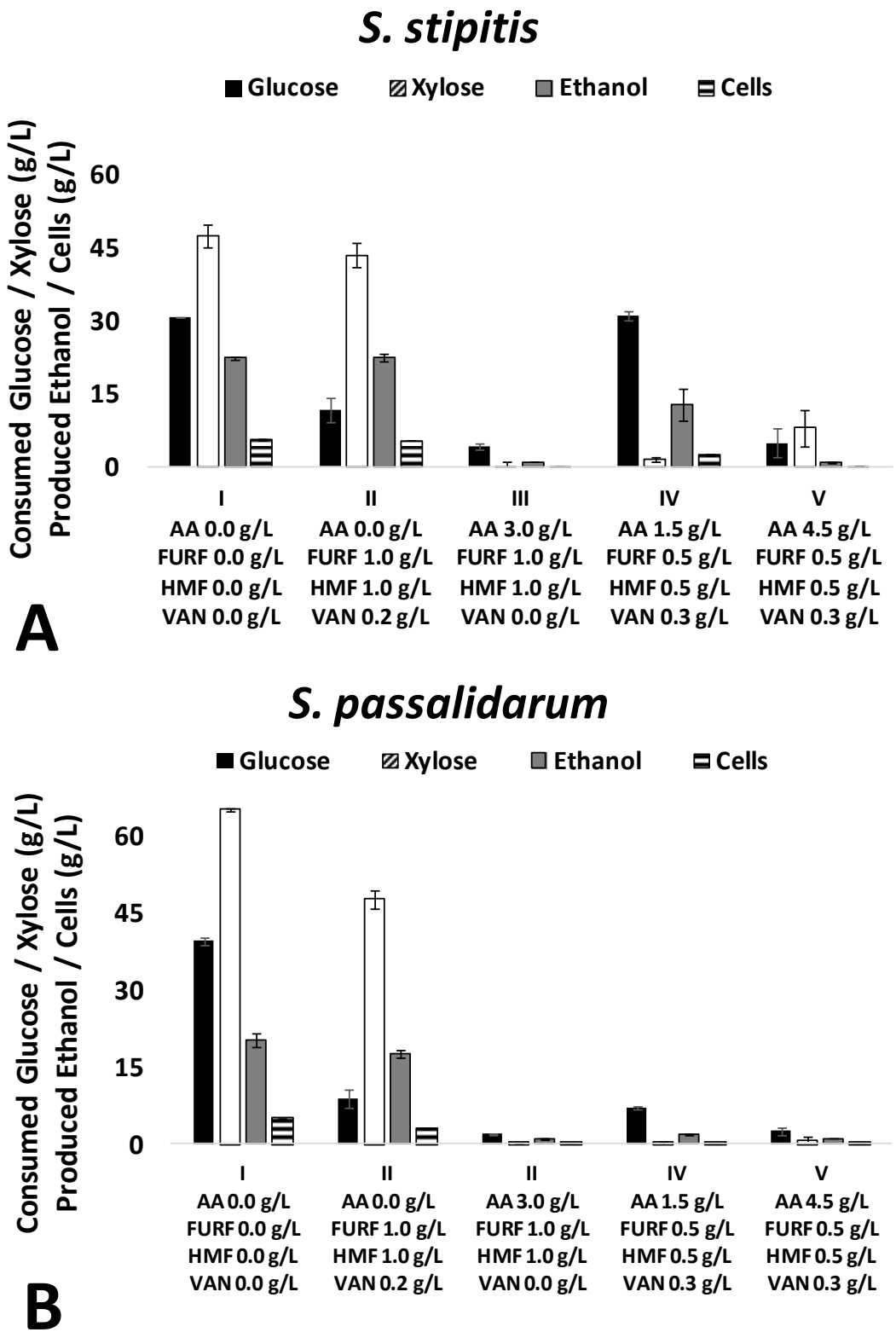

Figure 4. Fermentation results of *S. stipitis* (A) and *S. passalidarum* (B) in the presence of combined inhibitors: acetic acid, furfural, 5-HMF and vanilin. The bars represent the values of consumed glucose (black), consumed xylose (inclined bars), produced ethanol (grey) and cells growth (parallel bars).

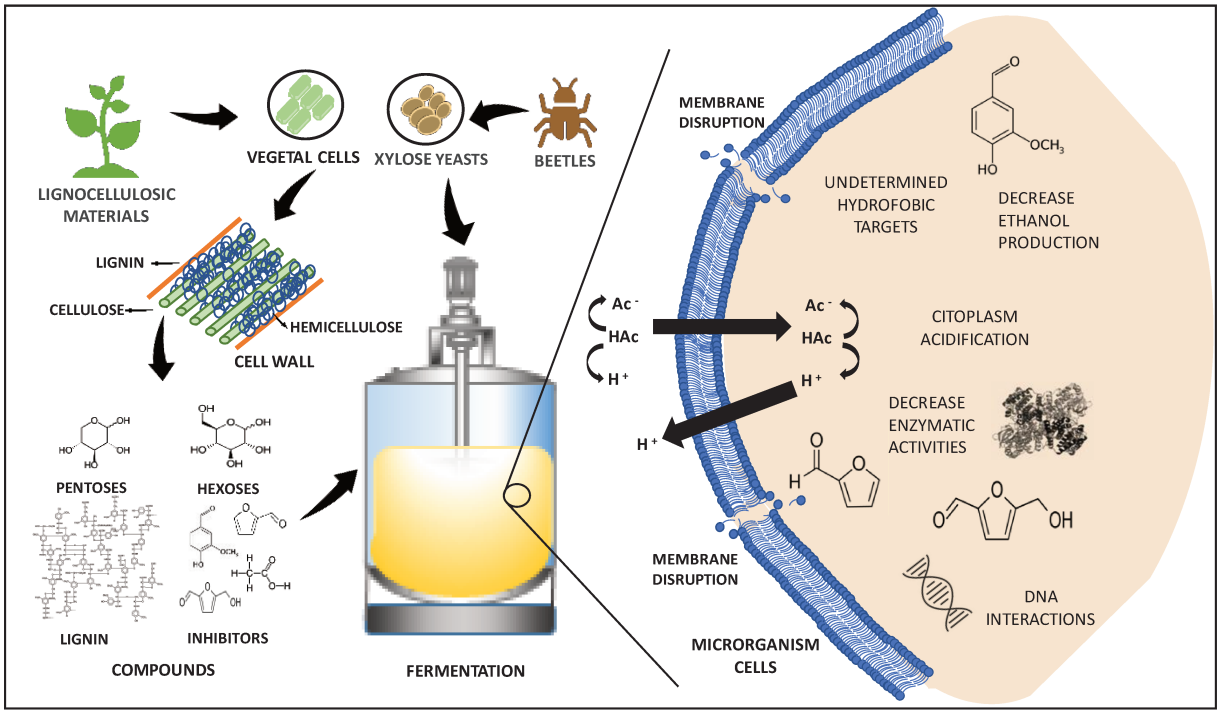

Figure 5. Graphical representation of the action mechanisms for the main inhibitors present in lignocellulosic in yeasts cells. Based on Mills et al. (2009), adapted.

Acetic acid and furfural were the most severe inhibitors of ethanol production for both yeasts, and since ethanol is the final product of interest, they are the inhibitors that require the most attention. Based on the individual inhibitors tests, and considering 80% of the control assay performance as the minimum acceptable for ethanol production, the concentrations of 2.5 $g.L^{-1}$ for acetic acid and furfural could be indicated as the limit values acceptable in hemicellulosic hydrolysates in order to guarantee satisfactory performance in the fermentation with *S. stipitis* NRRL Y-7124. These concentration limits are even lower for *S. passalidarum* NRRL Y-20272, 0.5 $g.L^{-1}$ of acetic acid and 1.5 $g.L^{-1}$ of furfural. This yeast also presented performance lower than 80% of the control assay for concentrations above 0.5 $g.L^{-1}$ of 5-HMF (Table 2). These values were selected based on the effect of each inhibitor tested in the present work in their isolate form without their synergistic effects. For comparison, Table 2 also lists the concentration of inhibitors AA, FURF, 5-HMF and VAN obtained in different

sugarcane bagasse pre-treatments. The inhibitors limiting concentrations found in this work were much lower than those obtained in real hemicellulosic hydrolysates (please see Table 2), except for very specific biomass conversion processes, which generate a small amount of inhibitors [5]. Alternatives to overcome these problems are the use of detoxification processes and the adaptation of yeasts to tolerate the most severe inhibitors, such as acetic acid and furfural.

It is difficult to attribute the effect of each inhibitor when we are studying them synergistically, but it seems that, in the concentrations that we used in this study, acid acetic played a major role in inhibiting yeasts fermentation. Concentrations above 1.5 $g.L^{-1}$ of this acid (along with other toxic compounds) were detrimental to the fermentation of *S. stipitis*. The synergism of other inhibitors in the presence of the lowest concentration of acetic acid (tested in this work) inhibited completely the fermentation by *S. passalidarum*.

It is known that the transformation of lignocellulosic biomass into fermentable sugars is advantageous and strategic from the economic and environmental point of view. However, such a conversion aggregates known and unknown amounts of inhibitors to the fermentation process and the treatment for withdrawal of these compounds adds costs to the final product. Thus, many alternatives to make viable ethanol production have been studied, such as the development of genetically modified microorganisms (16; 26), or strategies for adaptation of strains (27).The knowledge about how the microorganisms are influenced by the compounds present in the hemicellulosic hydrolysate can direct the studies in this way.

Table 2. Inhibitors limit concentrations of for *S. stipitis* and *S. passalidarum* considering 80 % of performance in individual inhibitors test for ethanol production when compared with the control assay (results obtained in this work and results obtained in hemicellulosic hydrolysate in literature for comparison).

Compound	Concentration indicated by results obtained in this work ($g.L^{-1}$)	
	S. stipitis	*S. passalidarum*
Acetic acid	2.5	0.5
Furfural	2.5	1.5
5-HMF	not inhibit	0.5
Vanilin	not inhibit	not inhibit
Acid pretreatment (Santoro et al. 2015)		
Xylose	21.54	
Acetic acid	4.84	
Furfural	0.77	
5-HMF	0.49	
Vaniline	n.a.	
Hydrothermal pretreatment (Nakasu et al. 2016)		
Xylose	47.00	
Acetic acid	6.0	
Furfural	0.8	
5-HMF	0.21	
Vaniline	0.104	
Alkaline pretreatment (C5 + C6 fraction) (Nakanishi et al. 2017)		
Xylose	14.9	
Acetic acid	0.0	
Furfural	0.0	
5-HMF	0.0	
Vaniline	n.a.	

3.5 Conclusions

S. stipitis showed to be more resistant in the presence of the studied inhibitors, considering cell growth, sugars consumption and ethanol production. Even more

inhibited by the compounds studied, the yeast *S. passalidarum* showed an ethanol production capacity very close to *S. stipitis*.

Detailed knowledge of the behavior of the yeasts in the presence of inhibitors allows the development of specific detoxification strategies for hemicellulosic hydrolysates aiming to reach inhibitor concentrations below the limit values in order to maximize the performance of the fermentation process.

3.6 Acknowledgments

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CAPÍTULO 4 - COMPARISON OF TWO STRATEGIES TO OVERCOME THE INHIBITORS OF HEMICELLULOSIC HYDROLYSATES: EVOLVED YEASTS AND HIGH CELL DENSITY

Artigo submetido à revista internacional *Biomass & Bioenergy*.

Soares, L. B.[1,2]; Andrade, A. L. D.[1]; Renzano, E. C.[1]; Bonan C. I. D. G.[2]; Biazi, L. E.[2]; Dionísio, S. R.[1]; Costa, A. C. [2]; Ienczak, J. L.[1,3]

[1] Brazilian Bioethanol Science and Technology Laboratory - CTBE/CNPEM, 10000 Giuseppe Maximo Scolfaro St, Zip Code 13083-852 Campinas, SP, Brazil

[2] School of Chemical Engineering, State University of Campinas - UNICAMP, 500 Albert Einstein Av, Zip Code 13083-852 Campinas, SP, Brazil

[3]Chemical Engineering and Food Engineering Department - Santa Catarina Federal University. CP 476, Zip Code 88040-900, Florianópolis - SC, Brazil.

4.1 Abstract

Economic, environmental and social benefits of using hemicellulosic wastes to increase energy production and biofuels have been largely discussed. However, the presence of toxic compounds, generated together with sugars released from biomass hydrolysis, constitutes a technological barrier for its use in industrial processes that aim at the production of chemical blocks, such as ethanol. Treatment processes to minimize the concentrations of the inhibitors in hemicelullosic fractions impact economically on the ethanol final value and make the use of such raw material economically unfeasible.

Several strategies have been proposed considering the scenario of operation with non-detoxified hemicellulosic hydrolysates. In this work, the use of adapted strains during the fermentation process with hemicellulosic hydrolysate containing inhibitors as an adaptive factor and the increase of the initial concentration of cells applied in the fermentation were used as strategies to overcome the intrinsic presence of toxic compounds in hemicellulosic hydrolysates. The adapted strain presented better performance in the presence of inhibitors when compared to the wild-type strain, especially for xylose consumption. It was also observed that increasing the inoculum size makes hemicellulosic hydrolysate previously defined as non-fermentable (due to the presence of inhibitor concentrations) viable for fermentations, obtaining significant concentrations of ethanol.

4.2 Introduction

In the current scenario, more than 80 % of the total global energy has its origin on fossil fuels. The transport sector is responsible for 58 % of this consumption [1]. Considering that reserves of fossil fuels are expected to be exhausted in 40-50 years [2] and they highly contribute to the emission of greenhouse gases and, consequently, to global warming, the use of renewable, sustainable and cost–effective energy sources is stimulated [3]. Among renewable fuels, biodiesel, biogas and bioethanol are some of the examples, of which bioethanol is the main biofuel produced and used in Brazil (the biggest world sugarcane producer). The use of sugarcane as feedstock for bioethanol has some environmental advantages, with an energetic balance superior to the bioethanol from corn, for example [4], [5]. The crescent demand for bioethanol stimulates the development of alternatives to maximize the production. In this context, the exploitation of the lignocellulosic biomass (sugarcane bagasse and straw) generated in the bioethanol production process can increase the production in 40% [6], which is so denominated second-generation bioethanol (E2G).

Sugarcane bagasse is composed by three major components: cellulose, hemicellulose and lignin [7]. While cellulose is a linear polymer composed by units of D-glucose linked by glycosidic bonds β-1→4, hemicelluloses are heteropolymers composed mainly by pentose and hexose sugars with short branches, as D-xylose, D-glucose, L-arabinose and L-galactose. Lignin is a polyphenolic macromolecule composed by basic units of 3,5-dimethoxy-4-hydroxyphenylpropane, 3 methoxy-4-hydroxyphenylpropane e 4-hydroxyphenylpropane [7]. The sugars that constitute the lignocellulosic biomass are not promptly available to be fermented by microorganisms. Before fermentation, sugarcane bagasse must be subjected to a

pretreatment with the objective of increasing the superficial area of cellulose and enabling a more efficient biomass hydrolysis by enzymes (subsequent step for E2G production). This increases the availability of fermentable sugars, which is desirable for a more-efficient ethanol production process.

Saccharomyces cerevisiae yeast is still the most used microorganism for ethanol production. However, it is not capable of fermenting pentose sugars at a considerable rate, unless it is genetically modified to express genes for pentose assimilation [9], [10]. Considering the scenario of ethanol production from lignocellulosic biomass, the use of natural xylose-fermenting microorganisms is desired, of which yeasts *Scheffersomyces stipitis* and *Spathaspora passalidarum* are promising candidates for industrial application [11], [12], [13] [14].

Although the use of pretreatments is a way to increase ethanol production from lignocellulosic biomass, other compounds are released together with the fermentable sugars and they can be grouped according to their origin [15]. When the acetyl structure of hemicelluloses is degraded, organic acids are released, as acetic acid. Furanic compounds are generated through dehydration of pentose and hexose sugars, such as furfural and 5-hydroxymethylfurfural (5-HMF), respectively. Degradation of lignin is responsible for the release of phenolic compounds [7], [16], [17], [18]. The presence of these components in the fermentative medium is detrimental to the process, as they act as inhibitory compounds in the cell metabolism. Considering acetic acid, some authors observed a decrease in the specific cell growth rate for the yeast *S. stipitis*, from 0.09 to 0.06 h^{-1}, by adding 3 $g.L^{-1}$ of acetic acid in the culture medium compared to the medium with no acid. Ethanol volumetric productivity also decreased from 0.69 to 0.17 $g.L^{-1}.h^{-1}$ comparing the experiment with no acid concentration to an experiment in which 6 $g.L^{-1}$of acetic acid were added [19]. For the yeast *S. passalidarum*, a low tolerance for

acetic acid was verified, once the ethanol volumetric productivity decreased almost 90 % by adding 4.5 $g.L^{-1}$, in a medium rich in glucose [20].

Considering furanic compounds, studies have been done to evaluate the effect of furfural on the yeast *S. stipitis* in concentrations of 2.0 and 4.0 $g.L^{-1}$. The work [19] showed that ethanol production and cell growth were completely blocked in the highest concentration of the inhibitor [19]. In another study, the effect of furfural and HMF in concentrations from 1.0 to 11.0 $g.L^{-1}$ and 1.3 to 15.0 $g.L^{-1}$, respectively, for the yeasts *S. stipitis* and *S. cerevisiae*, was evaluated [21]. Results showed that the strains were more sensitive to furfural than to HMF and that the mixture of the two compounds completely inhibited cell growth [21]. For the yeast *S. passalidarum*, the effect of furfural and HMF in concentrations up to 3 $g.L^{-1}$ was tested in anaerobic fermentations and it was also found that the microorganism was more affected by furfural [13].

Due to the several types of phenolic compounds in lignocellulosic hydrolysates, qualitative and quantitative analyses are difficult and, for that reason, the negative effect of this inhibitor group is not completely elucidated. In this context, researches to verify the influence of vanillin, syringaldehyde and hydroxybelzaldehyde in the performance of *Candida shehatae* and *S. stipitis* were made. The authors found that the strains were highly affected by vanillin, which, in the highest concentration tested (2.0 $g.L^{-1}$), completely inhibited cell growth and ethanol production. For syringaldehyde and hydroxybenzaldehyde, 1.5 $g.L^{-1}$ of each substance caused the same effect. [22].

Thus, it is clear that the release of inhibitory compounds is detrimental to the process of ethanol production from lignocellulosic biomass. Once pretreatment is a key step to increase the amount of available sugar for posterior fermentation, feasible alternatives must be adopted to contour this problem. Among the possibilities, three strategies have been reported as alternatives: (i) detoxification of hemicellulosic

hydrolysates; (ii) fermentations with high initial cell concentration; or (iii) fermentations with evolved microorganisms with higher tolerance to the inhibitors. Considering the first alternative, the application of detoxification methods aims to remove or decrease the concentrations of inhibitory compounds in the fermentation medium by applying techniques with chemical reagents, reducing agents, enzymes, liquid-liquid extractions, activated charcoal, ionic changes and simple processes as evaporation. Although there are several possible methods for detoxification of hydrolysates, the implementation of this step elevates the cost of the ethanol production process, increasing the demand for energy, investment in equipment and operations and, additionally, a step for recovery of detoxifying agents used in the process. Thus, the use of this alternative increases the cost of the final product, making it less competitive than other first-generation biofuels and/or fossil fuels [23].

Therefore, the viability of other strategies is an important step to overcome the intrinsic problem of inhibitors in the hemicellulosic hydrolysate. In this context, working with high cell concentration has been reported as a method to improve fermentative parameters. When high cell density is applied, there is a possibility of survival when strains are facing a toxic environment created by the presence of inhibitory compounds [17], as well as an increase in xylose consumption rate [24]. This condition is common on first-generation ethanol plants [25] and has been used as a great alternative to attain an improved second-generation ethanol production process [11], [12].

In another try to decrease the detrimental effect of inhibitory compounds, many research groups have been working to construct an improved and more tolerant microorganism. In a study, the authors were capable to develop a D-xylose fermenting and inhibitor tolerant industrial *S. cerevisiae* strain using metabolic and evolutionary

engineering, demonstrating an efficient fermentation rate of glucose and D-xylose in inhibitor-rich lignocellulosic hydrolysates [25]. Similarly, the ability of producing ethanol from xylose was improved in the yeast *Scheffersomyces shehatae* using UV-mutagenesis. The strain exhibited high resistance to lignocellulosic inhibitors along with high ethanol yield from dilute-acid lignocellulosic hydrolysate with no need for detoxification, proving that a non-recombinant microorganism can be efficient for ethanol production from pentose sugars facing a toxic environment [26]. Considering that working with non-recombinant strains is cheaper and simpler than working with genetically modified microorganisms, the development of inhibitor tolerant strains by adaptive evolution is desired.

Therefore, considering the scenario of operating with non-detoxified lignocellulosic hydrolysates and the potential of xylose-fermenting yeast *S. passalidarum* for second-generation ethanol production, this work aims to evaluate the effect of different initial cell concentrations on fermentative parameters when a toxic environment is faced. In addition, the performance of an evolved *S. stipitis* is compared to a wild strain over different concentrations of inhibitors. The inhibitors considered were acetic acid, furfural, 5-hydroxymethylfurfural and vanillin.

4.3 Materials and Methods:

4.3.1 Yeasts, Inoculum and Propagation

The xylose-fermenting yeasts *Scheffersomyces stipitis* NRRL Y-7124, *Scheffersomyces stipitis* CTBE S_A-100 and *Spathaspora passalidarum* NRRL Y-27907 were used in this study. The yeasts were stored at -80 °C in YPX media containing glycerol (0.8% m/v).

For cellular reactivation, cells were transferred to pre-inoculum that was performed in 250 mL Erlenmeyer flasks containing 100 mL of YPX media ($g.L^{-1}$): yeast extract (10.0); peptone (20.0) and xylose (20.0) and then incubated for 24 h at 30 °C and 200 rpm (Orbital Innova® 44 Shaker, New Brunswick). After this period, 10 % (v/v) of the pre-inoculum was transferred to the inoculum media composed of ($g.L^{-1}$): xylose (10.0), glucose (12.0), urea (2.3), yeast extract (3.0) and $MgSO_4$.$7H_2O$ (1.0) [27]. Inoculums was carried out in 1000 mL Erlenmeyer flasks with 250 mL of working volume and were kept at 30 °C and 200 rpm for a period of 24 h on a rotary orbital. After, the entire volume was centrifuged at 8000 rpm, 4 °C for 20 min (Thermo Scientific, Sorvall RC-6 Plus). After discarding the supernatant, the cells were resuspended in sterile water (the total volume represented 10 % of the initial volume of the propagation bioreactor).

Cells were transferred to the propagation bioreactor (Bioflo®, New Brunswick Scientific Co., Inc., Edison, NJ, 7.0 L), which was maintained at 50 % saturation relative to atmospheric air, at 30 °C and contained a media composed of ($g.L^{-1}$)sugarcane syrup (30.0), urea (2.3) and KH_2PO_4 (2.0). After the exhaustion of the total reducing sugars (TRS) (detected with medium infrared MIR, FT-IR), a continuous feed of sterile sugarcane syrup, at a feed rate of 3 $g_{TRS}.L^{-1}.h^{-1}$ and a pulse of 3.0 $g.L^{-1}$ of urea and 1.0 $g.L^{-1}$ of KH_2PO_4,were established [12]. After 24 h of propagation, the feed was stopped and the media was centrifuged in a sterile vessel at 8000 rpm for 20 min and 4 °C. The pellet cells were resuspended in sterile water and the concentration was determined by cell dry weight. The yeast creams of *S. stipitis* and *S. passalidarum* were stored separately at 4 °C and used as the stock cells cream for the later stages.

4.3.2 First strategy: The use of adapted strains

4.3.2.1 Wild and adapted *S. stipitis* inhibitor fermentation

Scheffersomyces stipitis CTBE SA-100 was obtained submitting the wild strain to the sequential batch fermentation protocol previously described [12]. The fermentation medium used was hemicellulosic hydrolysate from acid pretreatment (data not published).

Acetic acid was used as an inhibition model to compare the performance of the adapted strain *S. stipitis* CTBE SA-100 with the wild strain *S. stipitis* NRRL Y-7124. Erlenmeyer flasks (250 mL) were prepared containing 100 mL (in $g.L^{-1}$): xylose (63.0), glucose (27.0), urea (2.3), yeast extract (3.0) and $MgSO_4.7H_2O$ (1.0). Aliquots of acetic acid were added so that they resulted in concentrations of 0.2; 0.7; 1.1; 1.7; 2.2; 2.6; 3.2 and 3.6 $g.L^{-1}$. Cells of *S. stipitis* NRRL Y-7124 and *S. stipitis* CTBE SA-100 from the propagation step were inoculated separately in order to compose 5 $g.L^{-1}$ of cells at the beginning of each assay. After inoculation, the flasks were held on a rotary shaker for 72 h, 30 °C and 200 rpm. Samples were taken for the evaluation of cell growth, sugar consumption and ethanol production kinetics.

4.3.3 Second strategy: The use high cell density

4.3.3.1 Effect of initial cells concentration

Tests were carried out containing synthetic medium adding inhibitors in the typical concentration [28] of hemicellulosic hydrolysates from acid pretreatment at theoretical concentrations of 5, 15 and 30 $g.L^{-1}$ of initial cells to investigate the

influence of the initial cellular concentration variation on the performance of *S. stipitis* (wild and its adapted variation) and *S. passalidarum* for second generation ethanol production.

The assays were performed in duplicate in 500 mL Erlenmeyer flasks containing 250 mL of fermentation medium, in $g.L^{-1}$: yeast extract (3.0); glucose (27.0); xylose (63.0); $MgSO_4$ $.7H_2O$ (1.0); urea (2.3); and the inhibitors: acetic acid (2.0) furfural (0.15) and 5-hydroxymethylfurfural (0.06). These concentrations were based on the dilution of xylose contained in the concentrated hemicellulosic hydrolysate resulting from acid pretreatment from literature to obtain about 90 $g.L^{-1}$ of sugars in the fermentation medium [28]. Vanillin was added to the fermentations at the concentration of 0.42 $g.L^{-1}$.

After inoculation, the vials were held in a rotary incubator for 72h, at 30 °C and 200 rpm. Samples were taken at determined times for the evaluation of cell growth, sugar consumption and ethanol production kinetics.

4.4 Analytical Methods

The fermentations samples were centrifuged and yeasts concentrations were measured by gravimetrical analysis in duplicate, whereas 2 mL of each sample were submitted to centrifugation (14000 rpm for 3.5 min), re-suspended in distilled water and directed to a biomass drying oven at 80 °C. The analysis for the determination of the concentration of the compounds present in the supernatant of the fermentation samples (carbohydrates, alcohols and inhibitors) were carried out using high performance liquid chromatography (HPLC).

The samples were filtered using Millex 0.20 μm filters (PVDF), diluted and analyzed in an Agilent Infinit 1260 (Santa Clara, CA, USA) CLAE system coupled to the Refractive Index (IR) detector equipped with Aminex HPX-87H column (300 x 7.8 mm, Bio-Rad) at 35 ° C. The mobile phase employed was a solution of 5mM sulfuric acid in the isocratic mode with flow of 0.6 $mL.min^{-1}$, injection volume of 30 μL and analysis time of 30 minutes. The phenolic compounds (vanillin, HMF and furfural) were analyzed using an Agilent Infinit 1260 CLAE system (Santa Clara, CA, USA) coupled to the Variable Wavelength Detector (VWD) equipped with a C-18 reverse phase C-18 Acclaim PA2 3μm 120Å (150 x 4.6 mm, Thermo Scientific) at 25 °C. A water: acetonitrile solution in a ratio of 8: 1 (v / v) containing 1% acetic acid with isocratic elution, flow of 0.8 $mL.min^{-1}$, 30 μL injection volume , analysis time of 40 minutes and detection at wavelength λmax = 274 nm. The product quantifications were made from the construction of calibration curves performed with external standards. All standards and solvents used were of chromatographic and analytical grade (purity greater than 99.0%, Sigma Aldrich, Merck). The mobile phase is filtered through a 0.45 μm membrane and degassed by ultrasound for 30 min prior to use.

4.5 Kinetic parameters

Specific ethanol production rate, q_P ($g_{EtOH}.g_{cel}^{-1}.h^{-1}$):

$$q_P = \frac{1}{X} \cdot \frac{dP}{dt} \qquad (1)$$

where:

X = biomass concentration (on dry basis) at the final fermentation time, $g.L^{-1}$

P = Ethanol concentration, $g.L^{-1}$

t = time, h

Volumetric productivity, Q_P ($g_{EtOH}.L^{-1}.h^{-1}$):

$$Q_P = \frac{P - P_0}{t} \quad (2)$$

where:

P = final ethanol concentration, $g.L^{-1}$

P_0 = initial ethanol concentration, $g.L^{-1}$

t = final fermentation time, h

Conversion factor carbohydrates in to ethanol, $Y_{P/S}$ ($g_{EtOH}.g_S^{-1}$):

$$Y_{P/S} = \frac{P - P_0}{S_0 - S} \quad (3)$$

where:

P = final ethanol concentration, $g.L^{-1}$

P_o = initial ethanol concentration, $g.L^{-1}$

S_0 = initial substrate concentration, $g.L^{-1}$

S = final substrate concentration, $g.L^{-1}$

Sugars consumption rate, r_S ($g.L^{-1}.h^{-1}$):

$$r_S = -\frac{dS}{dt} \quad (4)$$

Yield (%):

$$Yield = \frac{Y_{P/S}}{0.511} \times 100 \quad (5)$$

Where the value 0.511 $g.g^{-1}$ corresponds to the stoichiometric conversion of xylose and glucose to ethanol.

4.6 Results and Discussion

4.6.1 First strategy: The use of adapted strains

Acetic acid is a compound commonly found in lignocellulosic fractions, resulting from the degradation of the acetyl groups of hemicellulose. This acid affects the fermentative performance of microorganisms and is classified as a toxic and inhibitory compound [17].

In this sense, acetic acid in increasing concentrations was used as an inhibitor compound model, aiming to evaluate and compare the performance of the previous adapted *S. stipitis* CTBE SA 100 and wild *S. stipitis* NRRL Y-7124. Concentrations of 0.2; 0.7; 1.1; 1.7; 2.2; 2.6; 3.2 and 3.6 $g.L^{-1}$ of acetic acid were added in the fermentation medium containing glucose and xylose as carbon sources.

Figure 1 shows the kinetic results of the control processes and with addition of acetic acid concentrations. It is possible to observe that, for both strains and regardless of the inhibitor concentration, there was catabolic xylose repression, but it was more pronounced for higher inhibitor concentrations due to the decrease of glucose consumption by the yeasts. In the control experiment (Fig. 1-A), both strains reached similar concentration of ethanol at the end of the process, however, the adapted strain produced it faster than the wild-type. In this experiment, after the exhaustion of sugars (48h), the adapted strain consumed the ethanol present in the medium as a carbon source, probably for cell maintenance. The adapted cell was also able to grow more than the wild-type strain (growth of 15 and 11 $g.L^{-1}$, respectively).

When 1.1 $g.L^{-1}$ acetic acid was used (Fig. 1-D), the glucose took about 24 h to be consumed, double the time of the control experiment (12h), evidencing that this

inhibitor delays the consumption of this sugar. In this test, the effect of catabolic repression on xylose consumption is most pronounced mainly due to the glucose consumption delay. For the wild strain, however, the drop in sugar concentrations occurred at a small rate (please see Table 1), reflecting the accumulation of this carbohydrate in a residual form. In this assay, the ethanol concentration was higher for the adapted strain, which reached about 10 $g.L^{-1}$ more ethanol titer than did the wild strain. Adaptation of the strain was also favorable for cell growth, which reached about 14 $g.L^{-1}$, against only 4 $g.L^{-1}$ of growth for the wild strain in the fermentation with 1.1 $g.L^{-1}$ of acetic acid.

The results for the highest acetic acid concentration (3.6 gL^{-1}, Fig. 1-I) tested in the present work showed that the increase in inhibitor concentration delayed glucose uptake for the adapted strain, because the sugar was fully consumed only after 48 h process. In turn, it was with 48 h of process that the wild strain began to consume glucose. The consumption of xylose presented again catabolic repression, being initiated only after 48 h of fermentation in the assay with the adapted strain. The wild-type strain was not able to consume xylose in this condition, because glucose had not yet been exhausted when the process was stopped (72 h). Although its metabolism was inhibited, the adapted strain produced ethanol, reaching a concentration of 20 $g.L^{-1}$ at the end of the process. Due to the slow consumption of sugars, the wild-type strain grew and produced little ethanol, about 3 and 5 $g.L^{-1}$, respectively. The adapted strain showed cell growth of about 10 $g.L^{-1}$. Acetic acid caused cell lysis in this condition once dry cell weight was reduced compared to the beginning of the experiment, but after 48 h of fermentation it was possible to observe a slight growth associated to ethanol production for both yeasts (Fig. 1-I).

Table 1 shows the results of glucose and xylose consumption rates in fermentations with wild and adapted *S. stipitis* strains. The increase in acetic acid concentration in the fermentative medium caused the rates of consumption of both sugars to drop, which was most pronounced in xylose. In the control experiment, both strains had the same rate of glucose consumption (2.33 $g.L^{-1}.h^{-1}$). However, the adapted strain showed higher consumption rate for xylose (1.09 to 1.44 to $g.L^{-1}$) in relation to the wild for control fermentation. The rate of glucose consumption (1.25 $g.L^{-1}.h^{-1}$) and xylose (0.56 $g.L^{-1}.h^{-1}$) reaches about half of its control value in the 0.7 $g.L^{-1}$acetic acid test for the wild strain. For the adapted strain, the same observation occurs with higher values of inhibitor: 50% of the consumption rate of glucose was demonstrated in the experiment with 2.2 $g.L^{-1}$ acetic acid. From the control experiment to this acetic acid concentration (2.2 $g.L^{-1}$), xylose consumption rates were higher (between 1.44 and 1.15 $g.L^{-1}.h^{-1}$) than for the wild-type strain in the control experiment (1.09 $g.L^{-1}.h^{-1}$). For the highest concentrations of inhibitor (2.6, 3.2 and 3.6 $g.L^{-1}$) xylose consumption rates were very close to zero when the wild type strain was used, which did not happen for the adapted strain. Thus, it was possible to verify that the consumption of sugars was influenced by the concentration of the acetic acid and the consumption of xylose was more affected by the presence of the toxic compound. In this sense, adaptation of the yeast during the process itself resulted in the improvement of that response in relation to the wild strain.

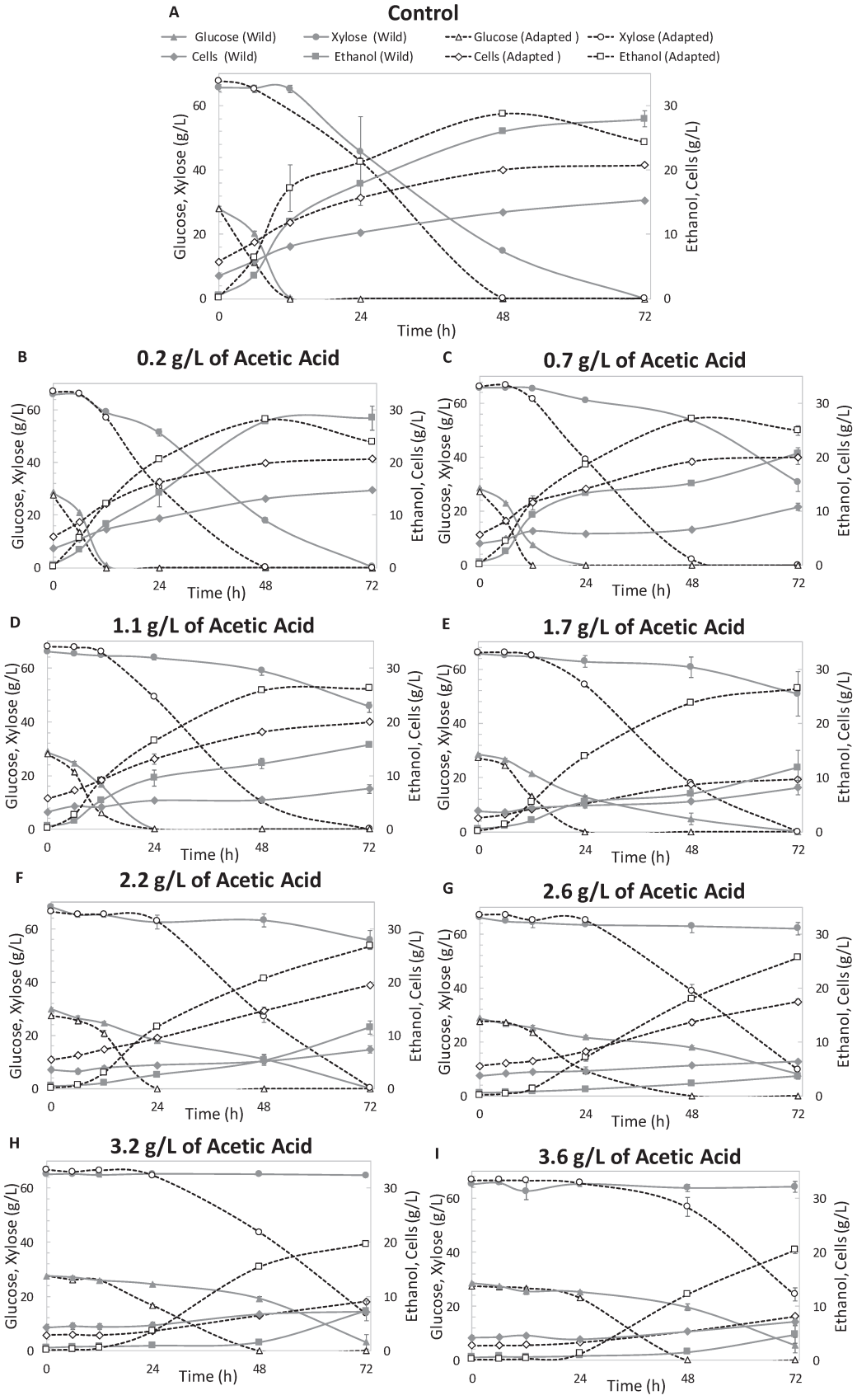

Figure 1: Fermentation data without inhibitor (A) and in the presence of 0.2 (B); 0.7 (C); 1.1 (D); 1.7 (E); 2.2 (F); 2.6 (G); 3.2 (H); e 3.6 (I) $g.L^{-1}$ of acetic acid for wild (full line, full marker) and adapted (dotted line, marker) *Scheffersomyces stipitis* strains. Concentrations of glucose (▲or △), xylose (● or ○), cells (◆ or ◇) and ethanol (■ or □) are shown over 72 hours of processing.

Table 1: Sugars consumption rate for fermentations with wild and adapted *S. stipitis* at different acetic acid concentrations.

Sugars consumption rate r_S ($g.L^{-1}.h^{-1}$)				
Acetic acid	**Wild**		**Adapted**	
($g.L^{-1}$)	**Glucose**	**Xylose**	**Glucose**	**Xylose**
0.0	2.33	1.09	2.33	1.44
0.2	2.30	1.03	2.29	1.59
0.7	1.25	0.56	2.28	1.57
1.1	1.23	0.26	1.21	1.13
1.7	0.51	0.19	1.19	1.08
2.2	0.40	0.13	1.17	1.15
2.6	0.27	0.00	0.63	0.88
3.2	0.32	0.00	0.60	0.78
3.6	0.29	0.00	0.58	0.54

Figure 2 shows residual concentrations of glucose, xylose, ethanol and volumetric productivity for the wild (A) and adapted (B) strains for all concentrations of acetic acid tested. It is noteworthy the decrease in performance of the wild strain compared to the adapted one.

Concentrations from 2.6 $g.L^{-1}$ of acetic acid impaired glucose uptake for the wild-type strain. In the assay with the adapted strain, the presence of this sugar was not observed at the end of fermentations, even in the highest concentration of inhibitor tested. For xylose, concentrations higher than 0.7 $g.L^{-1}$ of acetic acid affected the performance of the wild-type strain: from this value accumulation of this carbon source was registered, and at higher concentrations of inhibitor (2.6, 3.2 and 3.6 $g.L^{-1}$) none of the initially available xylose was metabolized by the microorganism (Figure 2B). Also for the concentration of 2.6 $g.L^{-1}$of acetic acid, xylose was not consumed and its accumulation as residual sugar was observed for the adapted strain, however, with residual values of this sugar being much lower.

The decline of the ethanol production for the wild strain occurred from 0.7 $g.L^{-1}$ acetic acid, which was not observed for the adapted strain, which maintained the ethanol

concentration practically constant along the increase of the presence of inhibitor. The rate of ethanol production in the control experiment was significantly higher for the adapted strain (0.60 $g.L^{-1}.h^{-1}$) than for the wild type strain (0.38 $g.L^{-1}.h^{-1}$). As a result of the increasing concentrations of acetic acid, the volumetric productivity decreased to the wild-type strain. There was also a decrease in productivity for the adapted strain (from 0.7 $g.L^{-1}$ of acetic acid), but the value was kept constant until the highest concentration of inhibitor tested.

In general terms, the interference in the performance of the wild yeast occurred from 0.7 $g.L^{-1}$ of acid in the fermentation medium, with a decrease in yield. Higher inhibitor content also affected glucose uptake (from 2.6 $g.L^{-1}$). On the other hand, the data for the adapted strain showed lower inhibition than the wild-type, and a marked inhibition for acetic acid content higher than 2.6 $g.L^{-1}$, when it showed inhibition for xylose consumption.

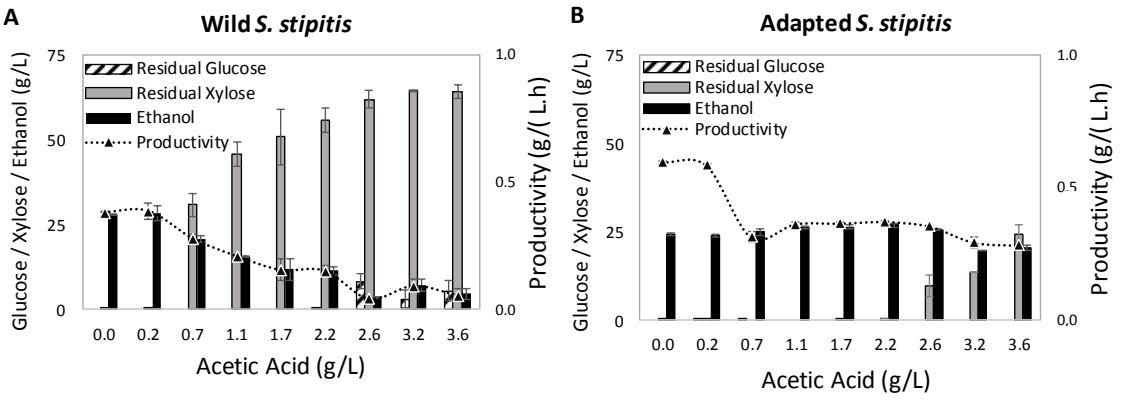

Figure 2: Residual concentrations of glucose (striped bars), xylose (grey bars), ethanol (black bars) and productivity (▲) for wild (A) and adapted (B) *Scheffersomyces stipitis* strains in increasing concentrations of acetic acid.

Other studies have shown the power of the adaptation of microorganisms throughout the fermentation process itself. In corn hydrolysates, adapted strains showed better

performance in the consumption of sugars, mainly xylose [29,30]. Improvements were also reported in relation to ethanol production rate due to adaptation. The sequence transfer (by selection pressure) of *S. stipitis* made it resistant to up to 15 $g.L^{-1}$ of acetic acid and 2.4 $g.L^{-1}$ of furfural [29]. Adapted in wood hydrolysates containing approximately 4 $g.L^{-1}$ acetic acid, *S. stipitis* could increase the consumption of sugars by 16%, resulting in a 22% increase in ethanol yield [30].

4.6.2 Second strategy: The use of high cell density

Three initial concentrations of cells from three different strains were inoculated into a media containing inhibitor blends in order to simulate the use of a real hemicellulosic hydrolysate. Concentrations of 5; 15 and 30 $g.L^{-1}$ cells for wild-type *S. stipitis*, adapted *S. stipitis* and *S. passalidarum* were used. The fermentation media contained 90 $g.L^{-1}$ of the sugars glucose and xylose (in proportions 30% and 70%, respectively), and a mixture of inhibitors was added with the contents: ($g.L^{-1}$) acetic acid (2.07 ± 0.10), furfural (0.15 ± 0.02), 5-hydroxymethylfurfural (0.06 ± 0.00) and vanillin (0.42 ± 0.02).

Regardless of the initial concentration of cells used, the wild *S. stipitis* strain was strongly inhibited by the inhibitor mixture. Even at the highest cell concentration (30 $g.L^{-1}$), the cell was not able to consume the sugars, grow and produce ethanol. For simplification, these data were not shown.

Figure 3 shows the profile of glucose, xylose and ethanol and cell production for adapted *S. stipitis* and *S. passalidarum* strains at different initial cell concentrations.

At the lowest cell concentration applied, 6.22 $g.L^{-1}$, the adapted *S. stipitis* was able to metabolize 70% of available glucose (17 $g.L^{-1}$), but xylose was not consumed. Because of this, there was low ethanol production (about 4 $g.L^{-1}$), (Fig 3-A). By raising

the cell concentration to 17.8 $g.L^{-1}$, the yeast could metabolize 100% of the glucose, and in 72 h there was a discrete consumption of xylose (about 6 $g.L^{-1}$) yielding about 10 $g.L^{-1}$of ethanol (Fig 3-C). When the highest cell concentration (33.6 $g.L^{-1}$) was applied, the adapted strain also consumed all of the glucose present and part of the xylose (19 $g.L^{-1}$), yielding about 13 $g.L^{-1}$ of ethanol (Fig. 3-E). In this assay we can observe a decay of the cell concentration during fermentation, from 33 to 22 $g.L^{-1}$, evidencing that there was cellular disruption throughout the process.

The results for the *S. passalidarum* were shown to be similar to the adapted *S. stipitis* to 5.9 $g.L^{-1}$cells. In this assay, *S. passalidarum* consumed part of the glucose present at the beginning of the fermentation (about 21 $g.L^{-1}$) and showed low xylose consumption (about 4 $g.L^{-1}$) resulting in a low ethanol production (7 $g.L^{-1}$) (Fig 3-B). When the concentration of 16.4 $g.L^{-1}$of cells was used in the fermentation, it can be observed that, in addition to the total glucose consumption, there was little residual xylose, that is, the metabolization of sugars was more efficient. As a consequence of this increase in the carbohydrates consumption, a significant ethanol production was noted, reaching the range of 32 $g.L^{-1}$ (Fig 3-D). For the 31.1 $g.L^{-1}$ cell assay, there was no residual sugar and ethanol production reached its highest value among the experiments performed for the different yeasts, 38 $g.L^{-1}$ of ethanol (Fig. 3-F).

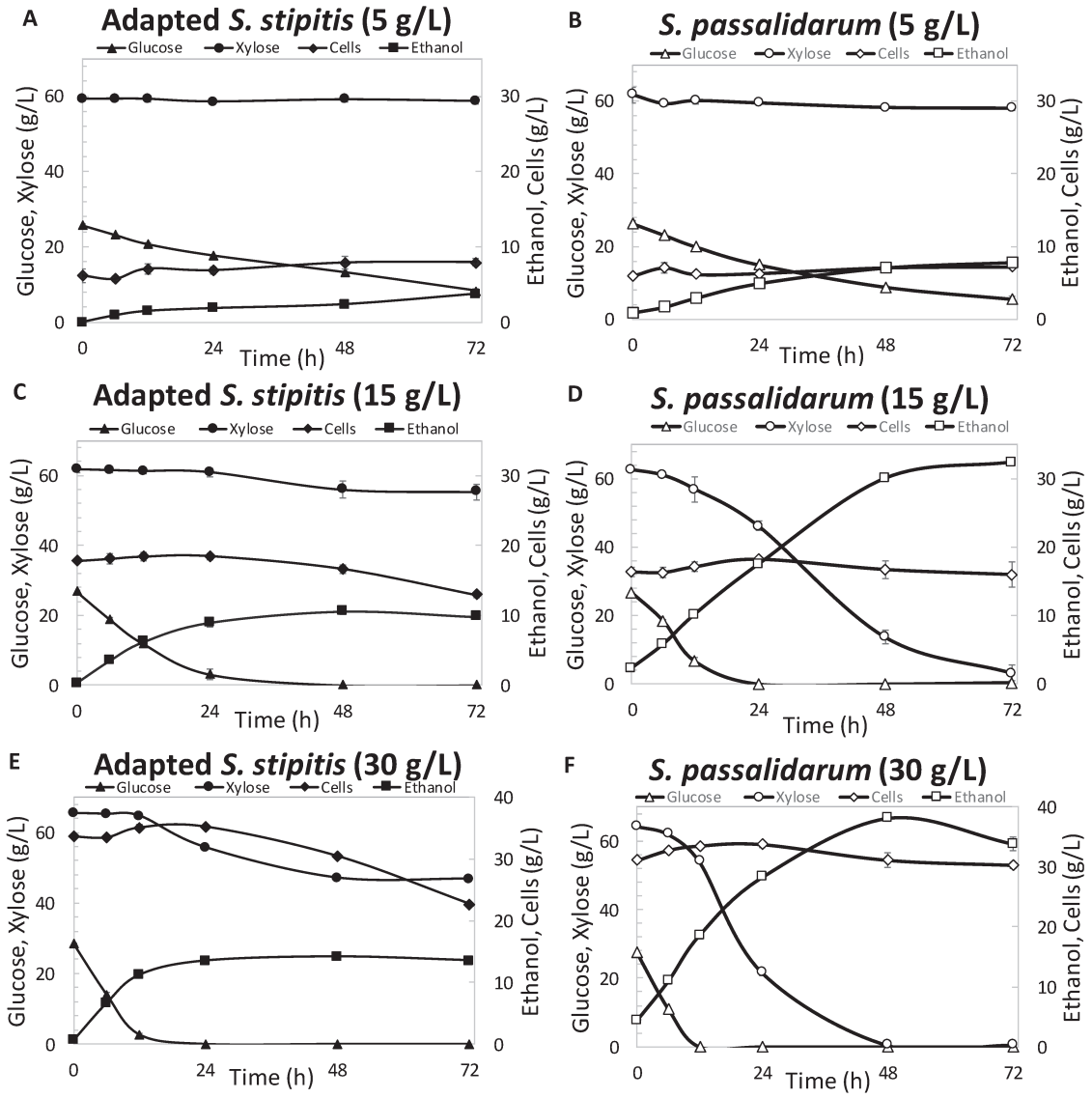

Figure 3: Kinetic data of fermentation in the presence of combined inhibitors for adapted *Scheffersomyces stipitis* with 5 (A), 15 (C) and 30 (E) $g.L^{-1}$ of initial cells and for *Spathaspora passalidarum* with 5 (B), 15 (D) and 30 (E) $g.L^{-1}$ of initial cells. Concentrations of glucose (▲ or △), xylose (● or ○), cells (◆ or ◇) and ethanol (■ or □) are shown over 72 hours of processing.

Table 2 shows the kinetic parameters for fermentations with different initial cells concentrations. As mentioned, no fermentation occurred at any concentration of wild *S. stipitis* tested and for this reason the kinetic parameters were not calculated.

For adapted *S. stipitis* it is possible to notice a difference in the yield, specific ethanol production rate and volumetric productivity between 6.22 and 17.8 $g.L^{-1}$of initial cell, since the lowest concentration was not able to transform all available sugars into ethanol, which in fact happened at 17.8 g L^{-1} of cells. At the highest cell concentration evaluated, 33.6 $g.L^{-1}$, the ethanol yield and productivity parameters were not much improved compared to the initial cell concentration of 17.8 g L^{-1}. The performance of the *S passalidarum* yeast is superior to that for the adapted *S. stipitis*, presenting higher yields in all cellular concentrations.

It is clear, observing the specific rates of ethanol production, that both yeasts maintain their performance with different inoculum concentrations (between 0.02 and 0.03 $g.g^{-1}.h^{-1}$). However, a significant change in volumetric productivity values was observed, reaching an increase of 5 times in the case of *S. passalidarum* (from 0.13 to 0.70 $g.g^{-1}.h^{-1}$ when the initial cell concentration increased from 5.9 g L^{-1} to 31.1 gL^{-1}), that is, a larger cell mass decreases the fermentation time, which is an economic gain for the fermentation process.

It is interesting to note that *S. passalidarum* with 5.9 $g.L^{-1}$ of initial cells reaches values of yield and specific production rate similar to the assays using the highest concentration of adapted *S. stipitis* (33.6 $g.L^{-1}$).

Table 2: Kinetic parameters of fermentation for the processes with 5, 15 and 30 $g.L^{-1}$ of initial cells for adapted *S. stipitis* and *S. passalidarum*.

Initial cell concentration ($g.L^{-1}$)	Adapted *S. stipitis*			Wild *S. passalidarum*		
	5	**15**	**30**	**5**	**15**	**30**
Yield (%)	40.72±3.6	56.46±2.5	56.40±3.0	55.48±3.2	68.97±0.6	72.42±1.80
q_P ($g_{EtOH}.g_{cell}^{-1}.h^{-1}$)	0.01±0.00	0.02±0.00	0.02±0.00	0.02±0.00	0.03±0.00	0.03±0.00
Q_P ($g_{EtOH}.L^{-1}.h^{-1}$)	0.05±0.00	0.13±0.01	0.18±0.00	0.13±0.01	0.58±0.02	0.70±0.01

The improvement of the fermentative parameters with the increase of the inoculum size was also observed by other authors. *S. stipitis* was tested at 1.8; 4.0 and 6.5 $g.L^{-1}$ of initial cells and after 192h of process, using only xylose as a carbon source, the assays obtained 20.9; 34.3 and 41.09 $g.L^{-1}$ of ethanol, respectively. Although yields were similar for all three conditions tested, trials using 1.8 and 4.0 $g.L^{-1}$ of starting cells did not consume all available carbon source for conversion to ethanol [24]. These results are in agreement with what happened in this work, when an inoculum of 6.22 $g.L^{-1}$ was applied. These authors also observed that the initial cell uptake also increased consumption and production speeds, but decreased selectivity in the formation of ethanol and xylitol. This fact was not observed in present work, xylitol concentrations remained low even with the increase of ethanol concentrations (data not shown). When recombinant *S. cerevisiae* MA-R4 was used in fermentations with up to 13 $g.L^{-1}$ of initial cells, high concentrations of ethanol were obtained, but there was also a loss in selectivity to xylitol and acetic acid [31]. Another authors also observed an increase in xylitol production with increased cell concentration for the recombinant yeast *S. cerevisiae* C5LT 1202. The authors applied up to 8 $g.L^{-1}$ of cells in fermentations with corn-straw hydrolysate, and in the higher concentrations used, the cells not only produced more ethanol but were able to consume the furfural present in the liquor, detoxifying the medium and favoring the ethanol fermentation [32].

4.7 Conclusions

The use of adapted strains during the fermentation process itself and the use of high cell densities in the fermentation have been proposed as a process modification to deal with the toxic compounds present in an intrinsic form in the second-generation sugars. The two strategies presented in this study proved to be satisfactory to enable the use of lignocellulosic fractions to obtain products by fermentative route.

The use of adapted yeast during the process with second generation sugars resulted in better performance when compared to the unadapted strain. It has also been observed that the increase in the initial concentration of cells in the process positively influences the use of inhibitor-containing hydrolysates, since fermentative media considered non-fermentable (due to the low cell concentration used in the process) have become viable for fermentations when high cell densities were used.

As additional results of this work, it was possible to verify that, against the presence of inhibitors, the consumption of xylose was markedly affected. It was also possible to verify that *S. passalidarum* presented the best performance among the strains studied in a non-favorable environment, indicating that this is an interesting microorganism for adaptation processes and genetic engineering, observing its better performance in relation to the *S. stipitis* strain.

As an alternative to the feasibility of the use of hemicellulosic fractions, the joint application of the two strategies presented in this study - high cell density process of a strain already adapted to inhibitory compounds - in the usual processes of fermentation as fed batch and/or recycle of cells can add even more robustness to the second-generation ethanol production process.

4.8 Acknowledgements

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CAPÍTULO 5 – CONSIDERAÇÕES FINAIS E SUGESTÕES PARA TRABALHOS FUTUROS

Scheffersomyces stipitis mostrou-se mais resistente na presença dos inibidores estudados, considerando o crescimento celular, o consumo de açúcares e a produção de etanol. Ainda mais inibida pelos compostos estudados, a levedura *Spathaspora passalidarum* mostrou uma capacidade de produção de etanol muito próxima de *S. stipitis*. Se o critério de escolha do microrganismo produtor de etanol for somente a resistência aos inibidores, a cepa que se destaca é *S. stipitis*. Porém, se o critério for a quantidade final de etanol produzido, mesmo com inibição, a cepa *S. passalidarum* se sobressai. Essa dualidade - entre não ser tolerante aos inibidores e mesmo assim produzir maior quantidade de etanol que a cepa mais resistente - é uma contradição para a utilização de *S. passalidarum* em processos que empregam frações de pentose. Dessa forma, os resultados obtidos pelo presente trabalho mostram o potencial no desenvolvimento de cepas adaptadas ou geneticamente modificadas de *S. passalidarum*, a fim de torná-la mais resistente aos compostos inibidores do hidrolisado hemicelulósico.

Ácido acético foi o inibidor mais severo, dentre os estudados, para ambas as leveduras. Furfural também apresentou acentuado efeito inibidor. O composto 5-hidroximetilfurfural influenciou no metabolismo de *S. passalidarum*. Baseando-se nos testes de resistência individual dos inibidores, foram estabelecidas que concentrações de 2,5 $g.L^{-1}$ de ácido acético e 2,0 $g.L^{-1}$ de furfural são os valores limites que os hidrolisados hemicelulósicos podem conter para que se obtenha um desempenho satisfatório na fermentação com *S. stipitis* NRRL Y-7124. Para *S. passalidarum* NRRL Y-27907, concentrações de 0,5 $g.L^{-1}$ de ácido acético, 1,0 $g.L^{-1}$ de furfural, 3,0 $g.L^{-1}$ de 5-HMF são os valores limites que as frações hemicelulósicas podem conter para que se obtenha um desempenho satisfatório na fermentação. Esses valores não levam em consideração a ampla variedade de outros compostos inibitórios contidos em hidrolisados reais (que não foram alvos do presente estudo) e seus efeitos sinérgicos, que podem diminuir ainda mais a faixa de operação listada acima. Além disso, foi

demonstrado que o ácido acético interferiu da maneira significativa na ação dos outros inibidores, quando foram realizadas fermentações com mistura desses compostos.

Dessa forma, o conhecimento detalhado do comportamento das leveduras na presença de inibidores permite o desenvolvimento de estratégias específicas de detoxicação para hidrolisados hemicelulósicos visando alcançar concentrações de inibidores abaixo dos valores limites para a utilização de cada cepa, a fim de maximizar o desempenho do processo de fermentação.

O uso de leveduras adaptadas durante o processo com açúcares de segunda geração resultou em melhor desempenho quando comparado com a linhagem não adaptada. Também foi observado que o aumento na concentração inicial de células no processo influencia positivamente o uso de hidrolisados contendo inibidor, uma vez que os meios fermentativos considerados não fermentescíveis (devido à baixa concentração de células usada no processo) tornaram-se viáveis para fermentações quando foram utilizadas densidades celulares elevadas.

Como resultados adicionais deste trabalho, foi possível verificar que, contra a presença de inibidores, a assimilação de xilose foi afetada de maneira mais severa quando comparada à assimilação de glicose. Também foi possível verificar que *S. passalidarum* apresentou o melhor desempenho entre as linhagens estudadas em um ambiente não favorável quando as modificações de processo foram empregadas.

Como alternativa à viabilidade do uso de frações hemicelulósicas, a união das duas estratégias apresentadas neste estudo - processo de alta densidade celular de uma cepa já adaptada a compostos inibitórios - nos processos usuais de fermentação como batelada alimentada e/ou reciclagem de células podem acrescentar ainda mais robustez ao processo de produção de etanol de segunda geração.

Para trabalhos futuros, sugere-se uma investigação com outros inibidores não listados neste estudo ou em referências na literatura, visando à análise do efeito desses compostos em microrganismos-chave para a produção de etanol de segunda geração, pois, mesmo que em concentrações muito pequenas, sua presença em hidrolisados hemicelulósicos pode interferir de forma expressiva nos metabolismos celulares. Sugere-se também estudo detalhado da membrana celular das leveduras em questão, a fim de direcionar as modificações genéticas no próprio envoltório celular, com o

objetivo torná-lo mais resistente à permeabilidade dos ácidos e às interações com compostos furânicos e fenólicos. Além disso, sugere-se estudo da utilização da levedura *Spathaspora passalidarum* em hidrolisados hemicelulósicos reais, a fim de adaptá-la a presença de inibidores e assim obter melhores rendimentos no processo de obtenção e etanol celulósico.

CAPÍTULO 6 – REFERÊNCIAS

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APÊNDICE A – MATERIAIS E MÉTODOS

Materiais & Métodos

Microrganismos

Linhagens

- Meio sólido de ágar YPD / 4 °C
- Meio líquido YPD + glicerol / - 80°C

Pré-Inóculo → **Inóculo** → **Propagação** → **Armazenamento**

- Meio YPD
- 24 h, 30 °C e 200 rpm

- Xilose, glicose, ureia, extrato de levedura e $MgSO_4.7H_2O$
- 24 h, 30 °C e 200 rpm

- Biorreator a 50% de oxigênio dissolvido
- Xarope de cana, ureia e KH_2PO_4
- Centrifugação

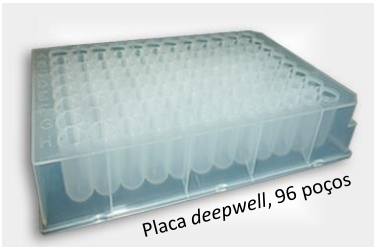
Santos *et al*, 2016

- 8°C

Fonte: autora

Materiais & Métodos

Teste de Resistência a inibidores individual



- Extrato de levedura (3,0 g.L $^{-1}$)
- Glicose comercial (27,0 g.L $^{-1}$)
- Xilose comercial (63,0 g.L $^{-1}$)
- Ureia (2,3 g.L $^{-1}$)
- $MgSO_4.7H_2O$ (1,0 g.L $^{-1}$)
- Ampicilina (1 µL.mL^{-1})
- Biomassa (5 g.L $^{-1}$)

Santos *et al*, 2016; modificado

Absorbância (600 nm)

(crescimento)

Cromatografia

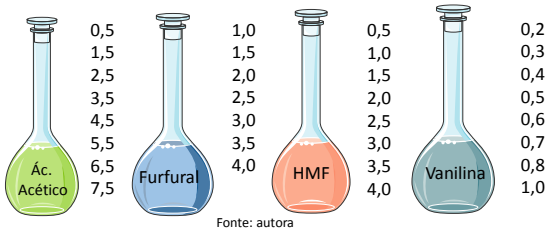
(açúcares e etanol)

% crescimento

% consumo

% produção

Controle

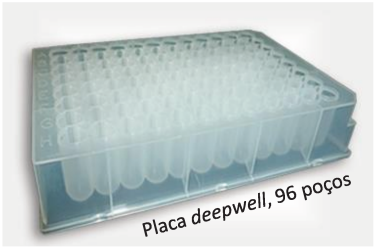


Fonte: autora

21

Materiais & Métodos

Teste de Resistência a inibidores combinados



Extrato de levedura (3,0 g.L $^{-1}$)

Glicose comercial (27,0 g.L $^{-1}$)

Xilose comercial (63,0 g.L $^{-1}$)

Ureia (2,3 g.L $^{-1}$)

$MgSO_4.7H_2O$ (1,0 g.L $^{-1}$)

Ampicilina (1 µL.mL^{-1})

Biomassa (5 g.L $^{-1}$)

Santos *et al*, 2016; modificado

Absorbância (600 nm)

(crescimento)

Cromatografia

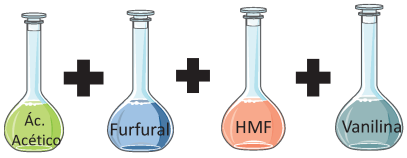
(açúcares e etanol)

% crescimento

% consumo

% produção

Controle



Inibidor	Concentração (g.L^{-1})				
	I	II	III	IV	V
Ácido Acético	0.0	0.0	3.0	1.5	4.5
Furfural	0.0	1.0	1.0	0.5	0.5
5-HMF	0.0	1.0	1.0	0.5	0.5
Vanilina	0.0	0.2	0.0	0.3	0.3

Fonte: autora

Materiais & Métodos

Teste de resistência para levedura adaptada

Concentrações do inibidor mais severo

Batelada

30° C; 200 rpm

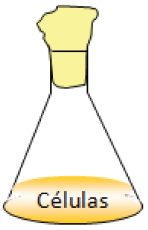
72h

Cinética de Fermentação

Crescimento

Consumo de açúcares

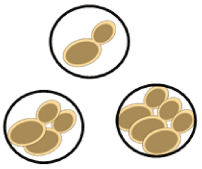
Produção de etanol e subprodutos



Fonte: autora

Materiais & Métodos

Teste de Resistência a inibidores combinados aplicando alta densidade celular



Mistura de inibidores

Células:
5 g.L^{-1}
15 g.L^{-1}
30 g.L^{-1}

2,0 g.L^{-1} AA
0,04 g.L^{-1} FURF
0,3 g.L^{-1} HMF
0,5 g.L^{-1} VAN
Santoro *et al.*, 2015

Batelada
30° C; 200 rpm
72h

Cinética de Fermentação
Crescimento Celular
Consumo de açúcares
Produção de etanol e subprodutos

Fonte: autora

Materiais & Métodos

Metodologia Analítica

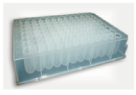
Concentração celular:

Análises gravimétricas

Concentrações de açúcares, metabólitos e inibidores:

Cromatografia Líquida de Alta Eficiência

Testes em *deepwell:*
Amostragem inicial e final



Testes em Erlenmeyer:
Amostras de 0, 6, 12, 24, 48, e 72h

Parâmetros cinéticos:

$$\mu_X = \frac{1}{X}\left(\frac{dx}{dt}\right)$$

Vel. Específica Crescimento Celular

$$Q_P = \frac{P - P_0}{t}$$

Produtividade Volumétrica

$$q_s = \frac{1}{X}\left(-\frac{dS}{dt}\right)$$

Vel. Específica Consumo de Açúcares

$$Y_{P/S} = \frac{P - P_0}{S_0 - S}$$

Fator de conversão de substrato em etanol

$$q_P = \frac{1}{X}\left(\frac{dP}{dt}\right)$$

Vel. Específica Produção de Etanol

$$Y_{X/S} = \frac{X - X_0}{S_0 - S}$$

Fator de conversão de substrato em células

$$\text{rendimento} = Y_{P/S} \cdot \frac{100}{0{,}511}$$

APÊNDICE B – DADOS COMPLEMENTARES

Tabela complementar 1: Concentrações de ácido acético, furfural, 5-hidroximetilfurfural e vanilina de ensaios com mistura de inibidores.

	Concentação do ensaio			
Ensaio	Ác. Acético	Furfural	5-HMF	Vanilina
1	1,5	0,5	0,5	0,1
2	4,5	0,5	0,5	0,1
3	1,5	1,5	0,5	0,1
4	4,5	1,5	0,5	0,1
5	1,5	0,5	1,5	0,1
6	4,5	0,5	1,5	0,1
7	1,5	1,5	1,5	0,1
8	4,5	1,5	1,5	0,1
9	1,5	0,5	0,5	0,3
10	4,5	0,5	0,5	0,3
11	1,5	1,5	0,5	0,3
12	4,5	1,5	0,5	0,3
13	1,5	0,5	1,5	0,3
14	4,5	0,5	1,5	0,3
15	1,5	1,5	1,5	0,3
16	4,5	1,5	1,5	0,3
17	0,0	1,0	1,0	0,2
18	6,0	1,0	1,0	0,2
19	3,0	0,0	1,0	0,2
20	3,0	2,0	1,0	0,2
21	3,0	1,0	0,0	0,2
22	3,0	1,0	2,0	0,2
23	3,0	1,0	1,0	0
24	3,0	1,0	1,0	0,4
25	3,0	1,0	1,0	0,2
26	3,0	1,0	1,0	0,2
27	3,0	1,0	1,0	0,2
28	3,0	1,0	1,0	0,2

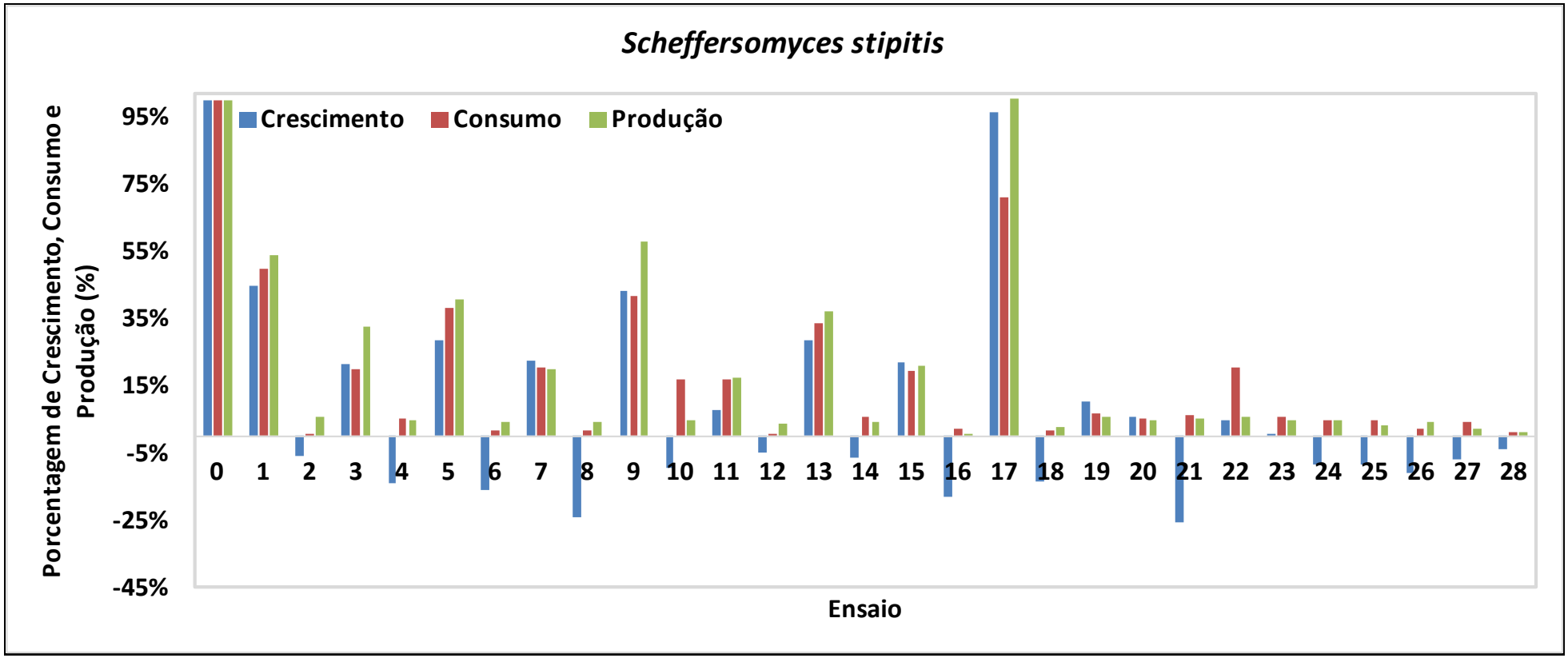


Figura Complementar 1: Resultados de porcentagens de crescimento celular, consumo de açúcares e produção de etanol para *S. stipitis* para os ensaios com as concentrações de inibidores listadas na Tabela Complementar 1, em relação ao ensaio controle.

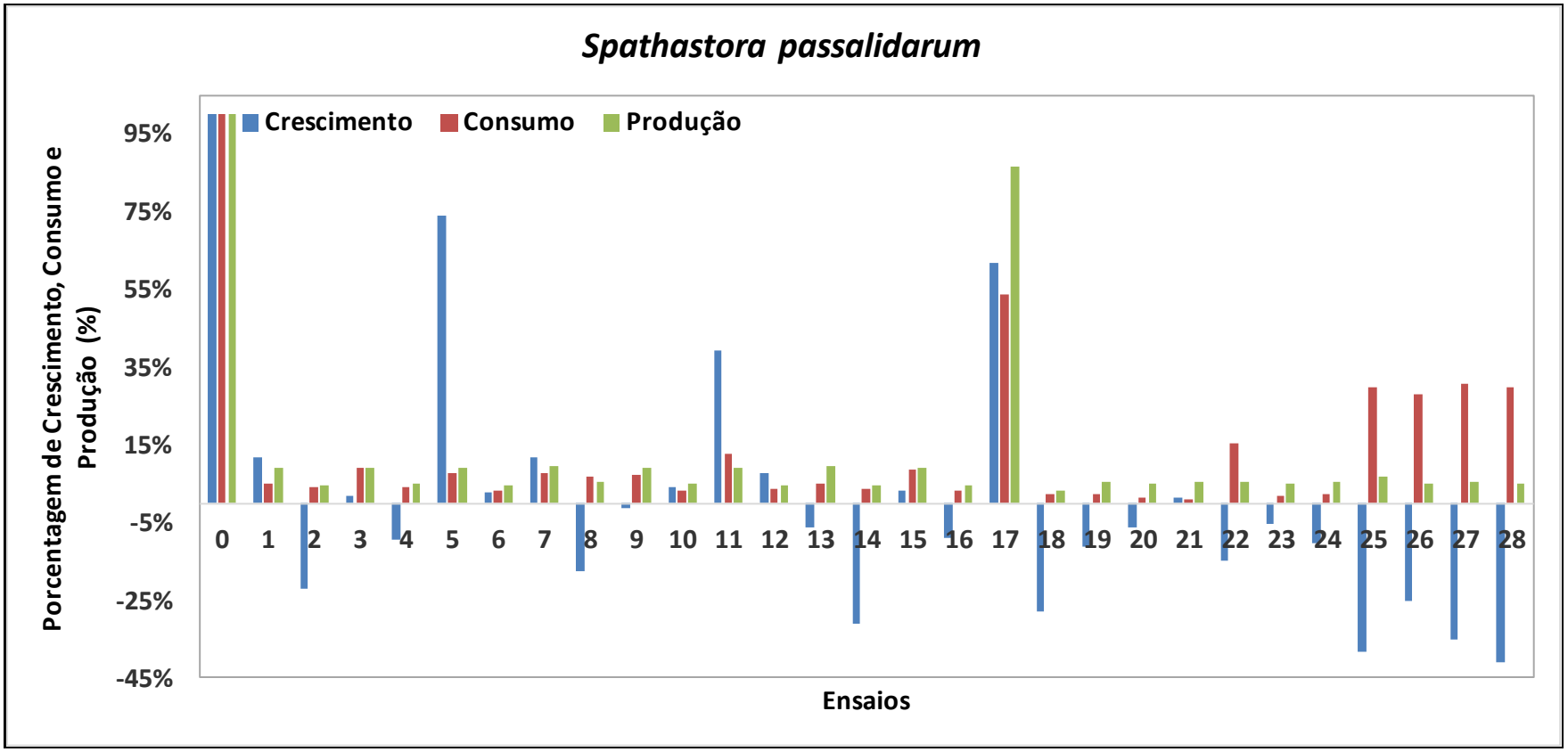


Figura Complementar 2: Resultados de porcentagens de crescimento celular, consumo de açúcares e produção de etanol para *S. passalidarum* para os ensaios com as concentrações de inibidores listadas na Tabela Complementar 1, em relação ao ensaio controle .