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Immobilization of *Spathaspora passalidarum* cells in sugarcane bagasse for second-generation ethanol production

Florianópolis, setembro de 2023.

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Tese submetida ao Programa de Pós-Graduação em Engenharia Química da Universidade Federal de Santa Catarina como requisito para obtenção do título Doutora em Engenharia Química.

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RESUMO EXPANDIDO

Introdução

Com o intuito de reduzir os impactos ambientais causados pela larga utilização de combustíveis fósseis, a pesquisa e o desenvolvimento no setor de biocombustíveis vêm ganhando força. O conceito de biorrefinaria, que promove a integração de instalações e processos utilizando matérias-primas renováveis e os transformando em produtos de maior valor agregado é considerado atualmente promissor e desejável (Bonan et al., 2021; Cherubini et al., 2009). O etanol é o mais próspero biocombustível produzido atualmente no mundo e a sua produção, baseada na utilização de biomassas lignocelulósicas, reforça seu viés sustentável (Abud & Silva, 2019).

Os principais componentes da biomassa lignocelulósica são hemicelulose, celulose e lignina. A celulose é um homopolímero de moléculas de glicose ligadas entre si por ligações glicosídicas; a hemicelulose é constituída, em sua maioria, por pentoses (xilose e arabinose), ramificações diversas e hexoses (glicose, galactose e manose); enquanto a lignina é uma macromolécula de estrutura química a, com a predominância de anéis aromáticos.

Etapas de pré-tratamento e hidrólise são necessárias para liberar os açúcares presentes nessas frações, visando a posterior produção de compostos de valor agregado (Robak & Balcerek, 2020). O pré-tratamento da biomassa pode ser realizado por meio de processos físicos, químicos e biológicos. Quando o foco é a liberação dos carboidratos, o objetivo dessa etapa é degradar parcialmente a lignina, diminuindo a compactação das fibras de hemicelulose e celulose, a fim de tornar essas moléculas mais acessíveis para as etapas posteriores do fracionamento da biomassa em açúcares fermentescíveis (Chiaramonti et al., 2012). Dependendo do tipo de pré-tratamento empregado é possível se obter açúcares monoméricos já nesta etapa, correspondendo principalmente à fração de pentoses advinda da hemicelulose (Aditiya et al., 2016), denominado hidrolisado hemicelulósico. A etapa posterior ao pré-tratamento é a hidrólise enzimática, que utiliza especialmente enzimas que hidrolisam a celulose em monômeros de glicose. A partir daí, os açúcares podem ser utilizados em bioprocessos para a produção de diversos produtos (Aslanzadeh et al., 2014).

Um dos maiores desafios na utilização de hidrolisados hemicelulósicos é a presença de inibidores formados durante o pré-tratamento, principalmente os que utilizam ácidos, devido à formação e/ou liberação de compostos provenientes da lignina e da hemicelulose (Aditiya et al., 2016; Taherzadeh & Karimi, 2011). A presença desses compostos (ácidos orgânicos, compostos furânicos e fenólicos) no hidrolisado hemicelulósico atrapalha o metabolismo dos microrganismos produtores de etanol, diminuindo a produtividade do processo ou até mesmo impedindo a fermentação. Diversas técnicas já foram utilizadas com o objetivo de minimizar a ação dos inibidores para as células, como a retirada desses compostos por meio de processos de detoxificação antes da fermentação, desenvolvimento de cepas mais resistentes, utilização de alta densidade celular, imobilização das células, entre outras (Karagoz et al., 2019; Taherzadeh & Karimi, 2011).

A imobilização celular é a técnica na qual as células são fixadas em um suporte sólido que restringe sua movimentação. Dessa maneira, é possível protegê-las dos efeitos dos inibidores presentes nos hidrolisados hemicelulósicos, além de favorecer a reutilização das células em processos sequenciais e contínuos (Rodríguez-Restrepo & Orrego, 2020). A imobilização de células pode ser realizada em suportes diversos e para a produção de etanol de segunda geração o material mais utilizado é alginato de cálcio, com a formação de esferas com células imobilizadas em seu interior (Navarrete et al., 2021). Alguns autores utilizaram a biomassa lignocelulósica do próprio processo de produção de etanol de segunda geração (E2G) como suportes para as células. A biomassa, ao ser utilizada como suporte de imobilização de células apresenta vantagens como alta disponibilidade, biocompatibilidade e robustez (Rodríguez-Restrepo & Orrego, 2020), e resultados promissores foram descritos na literatura na produção de etanol de E2G utilizando bagaço de cana-de-açúcar como suporte (Balderas et al., 2016; Chandel et al., 2009; Gajula et al., 2011; Singh et al., 2013).

O bagaço de cana-de-açúcar é atualmente o principal material lignocelulósico disponível no Brasil para a produção de etanol de segunda geração. A cada tonelada de cana-de-açúcar são produzidos cerca de 140 kg de bagaço seco que podem ser utilizados para produção de etanol de segunda geração (E2G) (Santos, et al, 2019).

A cepa de levedura *Spathaspora passalidarum* é considerada atualmente como microrganismo promissor na utilização de pentoses, devido a sua robustez quando comparada a outros microrganismos consumidores desse açúcar (Bonan et al., 2021; Martinez-Jimenez et al., 2021). Apesar do potencial de utilização da levedura *S. passalidarum* no consumo da fração de pentoses, há poucos relatos na literatura da imobilização desse microrganismo para a produção de E2G utilizando hidrolisados (Dal Cortivo et al, 2022; Silveira et al, 2021) e não há trabalhos que utilizem bagaço de cana-de-açúcar como suporte para a imobilização deste microrganismo.

Portanto, considerando os bons resultados descritos na literatura para a utilização de bagaço de cana-de-açúcar como suporte para outras cepas, o objetivo deste estudo é o desenvolvimento de uma tecnologia de fermentação utilizando a imobilização de *S. passalidarum* em bagaço de cana-de-açúcar como estratégia para proteção das células contra a ação nociva dos inibidores em hidrolisado hemicelulósico de bagaço de cana-de-açúcar, visando melhorias no processo de produção de etanol de segunda geração.

Objetivos

O objetivo principal deste estudo foi analisar a produção de etanol de segunda geração utilizando a imobilização de *S. passalidarum* em bagaço de cana-de-açúcar como estratégia para proteger as células contra a ação nociva de inibidores presentes no hidrolisado hemicelulósico de bagaço de cana-de-açúcar.

Metodologia

A imobilização de *S. passalidarum* em bagaço de cana-de-açúcar foi desenvolvida adicionando as células em alta densidade celular em contato com o suporte sólido em três versões: *in natura*, após pré-tratamento alcalino e após pré-tratamento ácido. O processo de imobilização foi realizado em agitador orbital a 30ºC e agitação de 100 rpm. A migração das células do líquido para o sólido foi medida indiretamente através de gravimetria do líquido. O sólido que proporcionou a melhor adesão das células foi escolhido como suporte para seguir com os experimentos.

Após, foi realizada fermentação com as células imobilizadas no bagaço de cana-de-açúcar utilizando hidrolisado hemicelulósico resultante de pré-tratamento ácido. O meio de fermentação, que continha inibidores, foi suplementado com nutrientes como extrato de levedura, $MgSO_4$ e ureia e um ensaio controle com células livres foi realizado a critério de comparação de desempenho fermentativo. Foram coletadas amostras do líquido ao longo do processo para quantificação de açúcares, metabólitos e inibidores por cromatografia líquida de alta eficiência. Amostras do sólido foram avaliadas através de microscópio eletrônico de varredura a fim de visualizar as células no interior do bagaço de cana-de-açúcar no inicio e fim do processo de fermentação.

Foram realizadas fermentações em modo contínuo com as células de *S. passalidraum* imobilizadas utilizando, separadamente, dois tipos de suportes: bagaço de cana-de-açúcar e esferas de alginato de cálcio. Para a imobilização em alginato, as células de levedura em alta concentração celular foram misturadas a uma solução de alginato de sódio. Essa solução foi gotejada em cloreto de cálcio gelado através de uma bomba peristáltica e mangueira para a formação das esferas com as células imobilizadas em seu interior. As esferas permaneceram na solução de cloreto de cálcio por aproximadamente 24h para a cura. Após esse tempo, as esferas foram utilizadas na fermentação contínua. A imobilização em bagaço de cana-de-açúcar foi realizada de acordo com a metodologia descrita anteriormente. A fermentação contínua foi realizada em um reator cilíndrico de vidro boro-silicato encamisado, com diâmetro de 2,5 cm (diâmetro interno de 2,0 cm) e 15,0 cm de comprimento. Os leitos ocuparam o correspondente a 70% do volume do reator. A coluna foi alimentada a partir do fundo com auxílio de uma bomba peristáltica, primeiramente com meio sintético e depois com meio contendo hidrolisado hemicelulósico, ambos saturados em ar atmosférico estéril. Foi aplicada vazão de alimentação de $0.5h^{-1}$, resultando em 2h de tempo de residência. O efluente e as amostras do fermentado foram recolhidas no topo da coluna, na mesma vazão de alimentação. A temperatura do reator foi mantida a 30º C pela circulação de água na camisa com auxílio de um banho aquecido. Amostras para análise de metabólitos e produtos foram retiradas em intervalos de 3h nas primeiras 36h e após com espaçamentos de 6h e 12h.

Resultados e Discussão

Este estudo demonstrou que é possível utilizar o bagaço de cana-de-açúcar como suporte para a imobilização de células de *Spathaspora passalidarum*. O pré-tratamento ácido do suporte foi o que proporcionou melhor adesão da levedura ao sólido (60,03 mg/g) em comparação com o bagaço de cana-de-açúcar *in natura* (37,56 mg/g) ou após pré-tratamento alcalino (43,66 mg/g). Dessa forma, o pré-tratamento ácido foi escolhido para, além de gerar os açúcares para fermentação, tratar a superfície do suporte de imobilização.

A fermentação em batelada por *S. passalidarum* imobilizada em bagaço de cana-de-açúcar obteve fator de crescimento e rendimento de produto de $Y_{P/S}$ (0,35 g/g) e $Y_{X/S}$ (0,43 g/g), respectivamente, contra $Y_{P/S}$ (0,27 g/g) e $Y_{X/S}$ (0,086 g/g) para o ensaio para células livres. Após 24 h de fermentação foi possível atingir uma produtividade de 0,153 g/(L.h) e um rendimento de 68,37% com células imobilizadas, que também foram superiores aos parâmetros da fermentação com células livres (0,148 g/(L.h) e 54%). A comparação com o desempenho das células livres mostrou que a proteção dada às células no interior do sólido é eficaz, resultando em melhores parâmetros de fermentação.

Foi possível utilizar um biorreator contínuo com células de *S. passalidarum* imobilizadas separadamente, em bagaço de cana-de-açúcarou e em esferas de alginato de cálcio para fermentar. Com meio sintético e sem a presença de inibidores, tanto a glicose quanto a xilose são consumidas e uma fase estacionária de produção de etanol foi alcançada Para células imobilizadas em esferas de alginato de cálcio, a produtividade atingiu 2.35 g/(L.h) e para as imobilizadas em bagaço de cana-de-açúcar a produtividade foi de 2,0 g/(L.h). Porém, quando foi alimentado meio sintético contendo ácido acético ou hidrolisado hemicelulósico a fermentação foi inibida, independente do suporte usado. A inibição da fermentação pode estar relacionada a alta concentração dos compostos inibitórios do hidrolisado (2,7 g/L de ácido acético, 0,11 g/L ácido fórmico, 0,04 g/L de ácido levulinico, 0.03 g/L de 5-hidroximetilfurfural e 0,10 g/L de furfural) e também ao alto tempo de residência (2h) utilizado no processo Contudo, mesmo em condições de inibição da fermentação, as células permanecem imobilizadas e viáveis. Dessa maneira, mais estudos são necessários para implementar um processo contínuo de produção de etanol de segunda geração, uma vez que não foi possível fermentar eficientemente o hidrolisado hemicelulósico com células imobilizadas em bagaço de cana-de-açúcar ou em esferas de alginato de cálcio nas condições testadas neste trabalho. A diminuição da vazão de alimentação, resultando um tempo de residência maior pode ser uma estratégia válida para fermentação contínua de hidrolisados hemicelulósicos.

Considerações Finais

Este estudo avaliou a aplicação da estratégia de imobilização celular para melhorar o processo de fermentação etanólica de hidrolisado hemicelulósico contendo inibidores de *Spathaspora passalidarum.*

Em relação ao bagaço de cana-de-açúcar in natura e após pré-tratamento alcalino, o pré-tratamento ácido deste material resultou a maior a adesão das células de *S. passalidarum* ao suporte, o que tornou vantajoso o processo de imobilização deste microrganismo - já que é um consumidor natural de xilose e este tipo de pré-tratamento é o que melhor recupera a fração de pentose da biomassa. Nesse sentido, é possível realizar um processo integrado de obtenção de suporte para imobilização celular e obtenção de açúcares de segunda geração em uma única etapa.

Células imobilizadas em bagaço de cana-de-açúcar tiveram melhor desempenho que células livres em modo batelada na fermentação de hidrolisado hemicelulósico contendo inibidores, tornando a

imobilização celular de *S. passalidarum* uma estratégia eficaz para melhorar parâmetros fermentativos na produção de etanol de segunda geração por esse microrganismo.

Foi possível realizar fermentação contínua de meio sintético com células de *S. passalidarum* imobilizadas em bagaço de cana-de-açúcar e também em esferas de alginato de cálcio. Sem a presença de inibidores, foram consumidas glicose e xilose e estabelecida uma produção contínua de etanol. Com a introdução de inibidores na alimentação, a fermentação foi inibida, embora as células permanecessem imobilizadas e viáveis para crescer.

Palavras-chave: imobilização de células; etanol de segunda geração; hidrolisado hemicelulósico; bagaço de cana-de-açúcar.

RESUMO

Um dos principais materiais lignocelulósicos brasileiro, bagaço de cana-de-açúcar, tem um grande potencial energético. O bagaço é composto de três macromoléculas principais: celulose, hemicelulose e lignina. O desenvolvimento de tecnologias para utilizar os carboidratos obtidos a partir da hidrólise de celulose (glicose) e hemicelulose (xilose, arabinose e glicose) para a produção de etanol de segunda geração (E2G) é promissor e muitos grupos de pesquisa estudam o desenvolvimento deste processo. O pré-tratamento utilizando ácido sulfúrico diluído, por exemplo, gera o hidrolisado hemicelulósico, fração rica em xilose, porém, gera também compostos inibitórios ao metabolismo dos microrganismos. Dentre os principais inibidores presentes no hidrolisado hemicelulósico estão os ácidos orgânicos e os compostos furânicos e fenólicos. A imobilização das células é uma estratégia consolidada para a proteção dos microrganismos frente aos inibidores, além de facilitar a reutilização dos biocatalisadores em fermentações sequenciais ou em processos contínuos. Este trabalho estudou a imobilização de *Spathaspora passalidarum* em bagaço de cana-de-açúcar como suporte. Diferentes tratamentos foram testados para a escolha do suporte: pré-tratamento alcalino, pré-tratamento com ácido diluído e bagaço de cana-de-açúcar sem nenhum tratamento. Após, foram realizados testes para o melhoramento do processo de imobilização e as variáveis concentração de células e tempo foram estudadas. Bagaço de cana-de-açúcar após pré-tratamento ácido foi o que proprocionou maior adesão de células ao suporte e aumentos no tempo de processo e na concentração celular no inóculo melhoraram a razão de imobilização no suporte de 60,0 para 77,6 mg/g. Além disso, foram realizadas fermentações com as células imobilizadas em bagaço de cana-de-açúcar utilizando hidrolisado hemicelulósico contendo inibidores como fonte de carbono, a fim de verificar o desempenho das células protegidas pelo suporte em comparação as células livres. As células imobilizadas em bagaço de cana-de-açúcar resultaram melhor desempenho que células livres, melhorando os parâmetros cinéticos de fermentação, a exemplo do fator de conversão de substrato em produto ($Y_{P/S}$) de 0,349 g/g para imobilização em bagaço de cana-de-açúcar e 0,274 g/g para células livres. Também foram realizadas fermentações contínuas com as células imobilizadas em bagaço de cana-de-açúcar e em esferas de alginato de cálcio, porém mais estudos são necessários para desenvolvimento de um processo contínuo de produção de etanol de segunda geração com *S.passalidarum* imobilizada, uma vez que não foi possível fermentar de forma eficiente o hidrolisado hemicelulósico em modo contínuo com as células imobilizadas nos suportes testados.

Palavras-chave: imobilização de células; etanol de segunda geração; hidrolisado hemicelulósico; bagaço de cana-de-açúcar.

ABSTRACT

One of the main Brazilian lignocellulosic materials, sugarcane bagasse, has a great energy potential. Bagasse is composed of three main macromolecules: cellulose, hemicellulose and lignin. The development of technologies to use carbohydrates obtained from the hydrolysis of cellulose (glucose) and hemicellulose (xylose, arabinose and glucose) for the production of second generation (2G) ethanol is promising and many research groups are studying the development of this process. Pre-treatment using diluted sulfuric acid, for example, in addition to generating the hemicellulose hydrolysate, a fraction rich in xylose, generates compounds that inhibit the metabolism of microorganisms. Among the main inhibitors present in the hemicellulosic hydrolysate are organic acids and furan and phenolic compounds. Cell immobilization is a consolidated strategy for the protection of microorganisms against inhibitors, in addition to facilitating the reuse of biocatalysts in sequential fermentations or in continuous processes. This work studied the immobilization *Spathaspora passalidarum* on sugarcane bagasse as a support. Different treatments were tested for the choice of support: alkaline pre-treatment, pre-treatment with diluted acid and sugarcane bagasse without any treatment. Afterwards, tests were carried out to improve the immobilization process and the variables cell concentration and time were studied. Sugarcane bagasse after acid pretreatment was what provided greater cell adhesion to the support and increases in process time and cell concentration in the inoculum improved the immobilization rate on the support from 60.0 to 77.6 mg/g. In addition, fermentations were carried out with cells immobilized on sugarcane bagasse using hemicellulosic hydrolysate containing inhibitors as a carbon source, in order to verify the performance of cells protected by the support compared to free cells. Cells immobilized in sugarcane bagasse resulted in better performance than free cells, improving the kinetic parameters of fermentation, such as the substrate-to-product conversion factor ($Y_{P/S}$) of 0.349 g/g for immobilization in sugarcane bagasse and 0.274 g/g for free cells. Continuous fermentations were also carried out with cells immobilized in sugarcane bagasse and in calcium alginate spheres, but more studies are needed to develop a continuous process for the production of second-generation ethanol with immobilized *S.passalidarum*, since it was not possible to efficiently ferment the hemicellulosic hydrolysate in continuous mode with the cells immobilized on the tested supports.

Keywords: cells imobillization; second generation etanol; hemicellulosic hydrolysate; sugarcane bagasse.

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Immobilization of *Spathaspora passalidarum* cells for second-generation ethanol production

Why? For what?

- The search for sustainable alternatives in fuel production raises interest in second-generation ethanol, which has the potential to increase the production of this input by 50% and replace the use of fossil fuels;
- Acid pretreatment has been the most used in industrial scale because recover high carbohydrate content to second-generation ethanol production;
- The intrinsic presence of inhibitory compounds in hemicellulosic hydrolysates from acid pretreatment is one of the biggest challenges for second generation ethanol become economically viable;
- The inhibithors like organic acids decrease intracellular pH interfering on metabolic pathways; furan and phenolic compounds damage the cell wall and can cause mutations;
- The immobilization of cells helps the fermentation of hydrolysates containing inhibitors due to the protection provided by the solid, in addition to facilitating the reuse of supports in sequential fermentations and in continuous processes;
- Sugarcane bagasse has advantages when used as a support for cell immobilization, since it is a low-cost material already available in the second-generation ethanol process itself.

Who already did?

- Chandel et al. 2009 used sugarcane bagasse as a support for the immobilization of *Saccharomyces cerevisiae* VS3 and fermented cellulosic hydrolysate of sugarcane bagasse pretreated followed by enzymatic hydrolysis. The results showed that the kinetic parameters related to the process with immobilized cells were higher when compared to those with free cells such as volumetric productivity (0.405 for free and 0.601 g/L.h for immobilized cells).
- Balderas et al. 2016 used sugarcane bagasse to immobilize *Scheffersomyces stipitis*. Comparison between free and immobilized cells was performed with hemicellulose hydrolysate of sugar cane containing 2.4 g/L of acetic acid and 0.8 g/L of furfural. After 60 h of fermentation, the assay with immobilized cells resulted in a yield 1.85 times greater than the process with free cells.
- Mishra et al. 2016 immobilized *S. cerevisiae* in calcium alginate spheres and performed continuous fermentations using non-detoxified rice straw hydrolysate. The authors also applied the strategy of first feeding with synthetic medium until a steady state was established and then feeding the already stable reactor with hemicellulose hydrolysate. Upon reaching steady state, the immobilized cells responded with almost no variation in the process parameters, maintaining ethanol production around 40 g/L for 216 h.
- Silveira et al. 2021 immobilized *Spathaspora passalidarum* in calcium alginate spheres and fermented in a synthetic medium simulating the glucose and xylose sugar concentrations of an acidic hemicellulosic hydrolysate of sugarcane bagasse. The authors, when using synthetic medium without inhibitors, did not notice a significant difference between fermentations of immobilized cells when compared to free cells.

Hypothesis

- It is possible to use sugarcane bagasse as a support for the immobilization of *S. passalidarum;*
- It is possible to improve the consumption of sugars and the production of ethanol by *S. passalidarum* in hemicellulosic hydrolysates containing inhibitors through cell immobilization.

Scientific methodology

- Immobilization of *S. passalidarum* in sugarcane bagasse under different treatments with the aim to improve cell adhesion to the support;
- Fermentation of the hemicellulosic hydrolysate with the cells immobilized on the sugarcane bagasse and comparison with free cells in batch mode;
- Coninuous fermentation with cells immobilized on sugarcane bagasse and calcium alginate spheres;
- 2G fermentation kinetic parameters analysis to verify inhibitors resistance by immobilization.

Answers

- Acid pretreatment of sugarcane bagasse enabled greater adhesion of cells to the support;
- Cells immobilized in sugarcane bagasse performed better than free cells in batch mode in the fermentation of hemicellulosic hydrolysate containing inhibitors;
- Continuos fermentation of hemicellulosic hydrolysate by *S. passalidarum* immobilized on sugarcane bagasse and calcium alginate spheres should be better investigated.

Chapter 1: Introduction and Justificative

In order to reduce the environmental impacts caused by the wide use of fossil fuels, research and development in the biofuel sector has been gaining strength. The integration of facilities and processes aimed at using renewable raw materials and transforming them into products with greater added value constitutes the concept of biorefinery and is considered the main way to develop a sustainable future [1, 2]. Ethanol is the most successful biofuel currently produced in the world and its production, based on the use of lignocellulosic biomass, increases its importance as the major substitute for fossil fuels [3].

The main components of lignocellulosic biomass are hemicellulose, cellulose and lignin. Cellulose is a homopolymer of glucose molecules linked together by glycosidic bonds; hemicellulose consists mostly of pentoses (xylose and arabinose), diversified branches, such as those of the acetyl group and hexoses (glucose, galactose and mannose); while lignin is a macromolecule with a diverse chemical structure, with a predominance of aromatic rings [4].

Sugarcane bagasse is currently the main lignocellulosic material available in Brazil for the production of second-generation ethanol. Each ton of sugarcane produces about 140 kg of dry bagasse that can be used to produce second-generation (2G) ethanol [5]. Pretreatment and hydrolysis steps are necessary to release the sugars present in these fractions, aiming at the subsequent production of value-added compounds [6].

The pretreatment of biomass can be carried out through physical, chemical and biological processes. For the production of second generation ethanol, the objective of this step is to partially degrade the lignin, reducing the compaction of the hemicellulose and cellulose fibers, in order to make these molecules more accessible for the subsequent steps of biomass fractionation into fermentable sugars [7]. Depending on the

type of pretreatment used, in special the acid pretreatments, it is possible to obtain monomeric sugars already at this stage, corresponding mainly to the fraction of pentoses derived from hemicellulose [8]. The step after pretreatment is enzymatic hydrolysis, which uses enzymes that specifically hydrolyze cellulose into glucose monomers. The sugars obtained in these steps can be used by fermentation for the production of different platforms of products [9].

The yeast *Saccharomyces cerevisiae* is the microorganism widely used for the production of E2G from hexoses (also for first generation ethanol), but it is not able to efficiently ferment pentoses. In this sense, there are microorganisms that naturally ferment pentoses, such as *Scheffersomyces stipitis* and *Spathaspora passalidarum* [10, 11]. The yeast *S. passalidarum* is currently considered a promising microorganism in the use of pentoses, due to its robustness when compared to other microorganisms that consume this sugar [1, 11].

Intrinsically to the availability of sugars from the lignocellulosic material, the biomass deconstruction process originates compounds that inhibit the metabolism of microorganisms, harming the next stage of ethanol production. Among the main inhibitors present in the hemicellulose hydrolysate are weak organic acids, such as acetic and formic acid; degradation products of pentose and hexoses such as furfural and 5-hydroxymethylfurfural; and products from the degradation of lignin, such as phenolic and furan compounds [12]. Organic acids are small molecules capable of permeating the cell membrane. Inside the cell, they lower the cytosolic pH and cause the cell to divert energy in the form of reestablishing the internal pH, which disrupts growth and ethanol production metabolisms. On the other hand, furan and phenolic compounds are capable of damaging the cell membrane, leaving the intracellular content exposed. They can also interact with the DNA of cells, causing mutations. [13].

Much research aim to overcome the action of the inhibitors in the microorganisms as a way to enable the production of 2G ethanol. Strategies such as the removal of these compounds through detoxification processes before fermentation, development of more resistant strains, use of high cell density, cell immobilization, among others [12, 14].

Cell immobilization is a strategy that aims to protect cells from external factors, confining them in supports that restrict their movement. When compared to processes with free cells, this technique has advantages such as the application of high cell density, reuse of cells in sequential processes with relative ease of product separation, ease of application in continuous processes, among others [15, 16]. For 2G ethanol production in particular, it is reported that immobilization increases xylose conversion, reducing the catabolic repression of glucose on xylose, due to diffusion limitation - which restricts glucose concentration within the support [17] . Likewise, it reduces ethanol inhibition and concentrates cells in a small volume, thus increasing ethanol yield and productivity [17]. Immobilization techniques can be divided into: flocculation, mechanical containment, involvement in porous matrices and immobilization on solid supports [14].

Due to the ease of the process, the most used method for cell immobilization is involvement in porous matrices. This methodology consists of adding the concentrated cell solution to a gelatinous solution, which by dripping will form spheres with the cells immobilized inside. Natural polymers such as calcium alginate, agar-agar, k-carrageenan and chitosan are used in these processes because they are compatible with cells and because they form spheres quickly [18, 19]. Also easy to perform, immobilization by adsorption on solid supports is also used for cell immobilization in processes and 2G ethanol production. The technique consists of interactions or

connections between the cells and the solid, which can be of the *Van der walls* type, electrostatic, ionic, or covalent. The cells and the solid are placed in contact and there is migration of microorganisms from the liquid to the support [20]. Supports such as polymers, glass, sponge loofa, charcoal and lignocellulosic materials are used as supports for cell immobilization [18]. Among all the supports described in the literature, lignocellulosic materials, when used as cell immobilization matrices, have advantages over other materials, such as being non-toxic, mechanically resistant and having high porosity [21–23], in addition to being already abundantly available in the context of 2G ethanol production. In the context of ethanol production in Brazil, sugarcane bagasse is seen with great potential to be used as a support for cells and the literature shows some studies using this biomass with immobilized cells inside for the 2G ethanol process.

S. cerevisiae VS3 was immobilized on sugarcane bagasse to ferment cellulosic hydrolysate of sugarcane bagasse pretreated with NH_3 followed by enzymatic hydrolysis [21]. Free and immobilized cells were compared in sequential fermentations and the results showed that the kinetic parameters related to the process with immobilized cells were higher when compared to free cells, such as ethanol yield in 6% for the immobilized cells and volumetric productivity in 48%. It was possible to carry out eight sequential batches with the same immobilized biocatalyst without significant loss in the values of the kinetic parameters, demonstrating that sugarcane bagasse resulted in a robust matrix for cell immobilization. Singh et al. (2013) [24] pre-treated sugarcane bagasse in microwave in the presence of alkali (NaOH) and used this material both to immobilize *S. cerevisiae* and to perform enzymatic hydrolysis and obtain the cellulosic hydrolysate used in fermentations. The results showed that it was possible to use the support for up to eight times without substantial loss in the kinetic parameters of the process, mainly the productivity, which remained around 0.43 g/(L.h). In addition,

the work compared the performance in repeated fermentations of the hemicellulosic matrix with gelatinous matrices, such as spheres of calcium alginate and agar-agar, and it was observed that after four repetitions, the gelatinous matrices fragmented, which indicates that the lignocellulosic material used was more robust by supporting twice as many recycles. Balderas et al. 2016 [25] used sugarcane bagasse to immobilize *Scheffersomyces stipitis*. Comparison between free and immobilized cells was performed with hemicellulose hydrolysate of sugar cane containing 2.4 g/L of acetic acid and 0.8 g/L of furfural. After 60 h of fermentation, the assay with immobilized cells resulted in a yield 1.85 times greater than the process with free cells. Gajula et al, 2011 [26] immobilized *S. stipitis* in sorghum bagasse and used the support with the immobilized cells to ferment peanut shell cellulosic hydrolyzate. The results showed that immobilization favored ethanol production for the process with immobilized cells, which reached a maximum ethanol concentration of 20.45 g/L, yield of 0.47 g/g and productivity of 0.243 g/(L.h)1, values 14, 7 and 14% higher, respectively, than with free cells. The authors also carried out repeated fermentations with immobilized cells and obtained good results, managing to reuse the supports up to 10 times without significant loss in the ethanol values produced (up to the 5th with the same ethanol production)

Continuous fermentation has also been studied with immobilized cells, but the most common support used is gelatinous materials. *S. cerevisiae* was immobilized on calcium alginate spheres for continuous fermentation using non-detoxified rice straw hydrolysate as a carbon source [27]. First, the synthetic medium was fed into the column until a steady state was established, and then the already stable reactor was fed with hemicellulosic hydrolysate (1.2 g/L of acetic acid, 0.46 g/L of 5-HMF and 0 .52 g/L of furfural). After reaching the steady state with the addition of medium without

inhibitor, the authors reported that the immobilized cells maintained the process parameters almost without variation, with ethanol production around 40 g/L for 216 h, after the addition of the hemicellulosic hydrolysate. Cortivo et al, 2022 [28] evaluated the performance of fluidized bed and packed bed bioreactors using *S. passalidarum* UFMGCM-469 cells immobilized in LentiKats® in the fermentation of oat and soybean hull hydrolysate. This support is an improved polyvinyl alcohol material commercially available from GeniaLab (Braunschweig, Germany) which forms lens-shaped solids, with the cells immobilized by entrapment. The process began with first applying a batch fermentation to acclimatize the microorganisms in the hydrolysate, and after consuming the sugars in the medium, they initiate to feed continuously on the same medium. For the tests, the hemicellulosic hydrolysate medium contained 4.8 g/L of glucose, 26.7 g/L of xylose, 1.1 g/L of acetic acid, 0.08 g/L of furfural and 0.01 g/L of 5-hydroxymethylfurfural and it took 48 hours for the sugars to be consumed by the cells immobilized in the fluidized bed reactor. Afterwards, the authors started continuous feeding, using a dilution rate of 0.05 h^{-1} and demonstrated that the continuous cultures in the fluidized bioreactor reached steady state after 96 h of feeding, reaching production values similar to the batch phase, but highlighting the productivity, which increased by 28%.

Considering the promising results described in the literature for the use of the cell immobilization strategy in the production of second-generation ethanol, and mainly, the use of sugarcane bagasse as a support, the objective of this study is the development of a fermentation technology using the immobilization of S. passalidarum in sugarcane bagasse as a strategy to protect cells against the harmful action of inhibitors in hemicellulosic hydrolysate, aiming to inprove the process of E2G production of sugarcane bagasse biomass.

1.2 Objectives

The main objective of this study was to analyze the production of second-generation ethanol by using the immobilization of *S. passalidarum* in sugarcane bagasse as a strategy to protect cells against the harmful action of inhibitors in hemicellulosic hydrolysate of sugarcane bagasse.

1.2.1 Specific objectives

● Evaluate the feasibility of immobilizing *S. passalidarum* in sugarcane bagasse;

● Improvement of cells adhesion to the sugarcane bagasse avaliating the support pretreatment, cell concentration and time of immobilization parameters;

● Evaluate the fermentative performance of cells immobilized in sugarcane bagasse and free cells of *S. passalidarum* in batch mode fermentations of hemicellulosic hydrolysate of sugarcane bagasse;

● Produce second-generation ethanol from hemicellulosic hydrolysate of sugarcane bagasse containing inhibitors in continuous mode fermentations, using a fixed-bed bioreactor with *S. passalidarum* cells immobilized, separately, in sugarcane bagasse and in calcium alginate spheres,

2. Chapter 1 references

1. Bonan CIDG, Tramontina R, dos Santos MW, et al (2021) Biorefinery Platform for Spathaspora passalidarum NRRL Y-27907 in the Production of Ethanol, Xylitol, and Single Cell Protein from Sugarcane Bagasse. Bioenergy Res. https://doi.org/10.1007/s12155-021-10255-7

2. Cherubini F, Jungmeier G, Wellisch M, et al (2009) Toward a common classification approach for biorefinery systems. Biofuels, Bioprod Biorefining 3:534–546. https://doi.org/10.1002/bbb.172

3. Abud AK de S, Silva CE de F (2019) Bioethanol in Brazil: Status, Challenges and Perspectives to Improve the Production. Elsevier Inc.

4. Fengel D, Wegener G (1989) Wood. Chemistry, Ultrastucture, Reactions. Walter de Gruyter

5. Santos F, Eichler P, Queiroz JH de, Gomes F (2019) Production of second-generation ethanol from sugarcane. Elsevier Inc.

6. Robak K, Balcerek M (2020) Current state-of-the-art in ethanol production from lignocellulosic feedstocks. Microbiol Res 240:126534. https://doi.org/10.1016/j.micres.2020.126534

7. Chiaramonti D, Prussi M, Ferrero S, et al (2012) Review of pretreatment processes for lignocellulosic ethanol production, and development of an innovative method. Biomass and Bioenergy 46:25–35. https://doi.org/10.1016/j.biombioe.2012.04.020

8. Aditiya HB, Mahlia TMI, Chong WT, et al (2016) Second generation bioethanol production: A critical review. Renew Sustain Energy Rev 66:631–653. https://doi.org/10.1016/j.rser.2016.07.015

9. Aslanzadeh S, Ishola MM, Richards T, Taherzadeh MJ (2014) An Overview of Existing Individual Unit Operations. Elsevier B.V.

10. Azhar SHM, Abdulla R, Jambo SA, et al (2017) Yeasts in sustainable bioethanol production: A review. Biochem Biophys Reports 10:52–61. https://doi.org/10.1016/j.bbrep.2017.03.003

11. Martinez-Jimenez FD, Neitzel T, Biazi LE, et al (2021) Exploiting the Non-conventional Yeast Spathaspora passalidarum as a Platform for Hemicellulosic Hydrolysate Conversion into Bioproducts: a Mini Review. Bioenergy Res. https://doi.org/10.1007/s12155-021-10257-5

12. Taherzadeh MJ, Karimi K (2011) Fermentation inhibitors in ethanol processes and different strategies to reduce their effects, 1st ed. Elsevier Inc.

13. Palmqvist E, Hahn-Hägerdal B (2000) Fermentation of lignocellulosic hydrolysates. I: Inhibition and detoxification. Bioresour Technol 74:17–24. https://doi.org/10.1016/S0960-8524(99)00160-1

14. Karagoz P, Bill RM, Ozkan M (2019) Lignocellulosic ethanol production: Evaluation of new approaches, cell immobilization and reactor configurations. Renew Energy 143:741–752. https://doi.org/10.1016/j.renene.2019.05.045

15. Ghorbani F, Younesi H, Esmaeili A, Najafpour G (2011) Cane molasses fermentation for continuous ethanol production in an immobilized cells reactor by Saccharomyces cerevisiae. Renew Energy 36:503–509. https://doi.org/10.1016/j.renene.2010.07.016

16. Milessi TS, Silva CR, Moraes GS, et al (2020) Continuous 2G ethanol production from xylose in a fixed- bed reactor by native Saccharomyces cerevisiae strain through simultaneous isomerization and fermentation. 7:4429–4442. https://doi.org/10.1007/s10570-020-03108-7

17. Abbi M, Kuhad RC, Singh A (1996) Bioconversion of pentose sugars to ethanol by free and immobilized cells of Candida shehatae (NCL-3501): Fermentation behaviour. Process Biochem 31:555–560. https://doi.org/10.1016/s0032-9592(95)00104-2

18. Navarrete HC, Matín C, García JM (2021) Yeast immobilization systems for second-generation ethanol production: actual trends and future perspectives. Biofuels, Bioprod Biorefining 1–17. https://doi.org/10.1002/bbb.2250

19. Willaert RG (2007) Cell immobilization and its applications in biotechnology: Current trends and future prospects. Ferment Microbiol Biotechnol Third Ed 313–368. https://doi.org/10.1201/b11490-18

20. Rodríguez-Restrepo YA, Orrego CE (2020) Immobilization of enzymes and cells on lignocellulosic materials. Environ Chem Lett 18:787–806. https://doi.org/10.1007/s10311-020-00988-w

21. Chandel AK, Lakshmi Narasu M, Chandrasekhar G, et al (2009) Use of Saccharum spontaneum (wild sugarcane) as biomaterial for cell immobilization and modulated ethanol production by thermotolerant Saccharomyces cerevisiae VS3. Bioresour Technol 100:2404–2410. https://doi.org/10.1016/j.biortech.2008.11.014

22. Escobar LMA, Álvarez US, Peñuela M (2012) Yeast immobilization in lignocellulosic wastes for ethanol production in packed bed bioreactor. Rev Fac Ing 66–76

23. Yu J, Zhang X, Tan T (2007) An novel immobilization method of Saccharomyces cerevisiae to sorghum bagasse for ethanol production. J Biotechnol 129:415–420. https://doi.org/10.1016/j.jbiotec.2007.01.039

24. Singh A, Sharma P, Saran AK, et al (2013) Comparative study on ethanol production from pretreated sugarcane bagasse using immobilized Saccharomyces cerevisiae on various matrices. Renew Energy 50:488–493. https://doi.org/10.1016/j.renene.2012.07.003

25. Balderas BSY, Muñiz BO, Rodríguez JG, et al (2016) Ethanol production by Pichia stipitis immobilized on sugarcane bagasse. Bioethanol 2:44–50. https://doi.org/10.1515/bioeth-2016-0001

26. Gajula C, Chandel AK, Konakalla R, et al (2011) Fermentation of groundnut shell enzymatic hydrolysate for fuel ethanol production by free and sorghum stalks immobilized cells of Pichia stipitis NCIM 3498. Int J Chem React Eng 9:. https://doi.org/10.1515/1542-6580.2514

27. Mishra A, Sharma AK, Sharma S, et al (2016) Lignocellulosic ethanol production employing immobilized Saccharomyces cerevisiae in packed bed reactor. Renew Energy 98:57–63. https://doi.org/10.1016/j.renene.2016.02.010

28. Cortivo PRD, Raquel L, Carlos H, et al (2022) Continuous bioreactor bioprocess using immobilized Spathaspora passalidarum to convert hydrolysates of oat and soybean hulls into ethanol. Biomass Convers Biorefinery. https://doi.org/10.1007/s13399-022-02531-z

Chapter 2: Literature review – "An overview on fermentation strategies to overcome lignocellulosic inhibitors in second-generation ethanol production using cell immobilization"

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An overview on fermentation strategies to overcome lignocellulosic inhibitors in second-generation ethanol production using cell immobilization

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Abstract

The development of technologies to ferment carbohydrates (mainly glucose and xylose) obtained from the hydrolysis of lignocellulosic biomass for the production of second-generation ethanol (2G ethanol) has many economic and environmental advantages. The pretreatment step of this biomass is industrialy performed mainly by steam explosion with diluted sulfuric acid and generates hydrolysates that contains inhibitory compounds for the metabolism of microorganisms, harming the next step of ethanol production. The main inhibitors are organic acids, furan, and phenolics. Several strategies can be applied to decrease the action of these compounds in microorganisms, such as cell immobilization. Based on data published in the literature, this overview will address the relevant aspects of cell immobilization for the production of 2G ethanol, aiming to evaluate this method as a strategy for protecting microorganisms against inhibitors in different modes of operation for fermentation. This is the first overview until now that show the relation between inhibitors, cells immobilization and fermentation operation modes for 2G ethanol. In this sense, the state of the art regarding the main inhibitors in 2G ethanol and the most apllied techniques for cell immobilization, besides batch, repeated batch and continuous fermentation using immobilized cells, in addition to co-culture immobilization and co-immobilization of enzymes are presented in this work.

Keywords: supports, sequential batches; continuous process, co-immobilization, co-culture immobilization

1. Introduction

Research and development in the biofuels sector have been gaining attention to reduce the environmental impacts caused by the widespread use of fossil fuels. The biorefinery concept, which promotes the integration of facilities and processes using renewable raw materials and transforming them into higher value-added products, is currently considered promising and desirable [1, 2]. Ethanol is the most promising biofuel produced globally, and its products based on the use of lignocellulosic biomass reinforces its sustainable bias [3].

The main components of lignocellulosic biomass are cellulose, hemicellulose, and lignin, and their percentages depend mainly on the type of biomass and plant growth mode. Cellulose is a homopolymer of glucose molecules linked by glycosidic bonds; hemicellulose consists mostly of pentoses (xylose and arabinose), some hexoses (glucose, galactose, and mannose) and acetyl group branches; while lignin is a macromolecule of diverse chemical structure, with predominance of aromatic rings with the alcohol function. Pretreatment and hydrolysis steps are necessary to release the sugars present in cellulose and hemicellulose, aiming at the subsequent production of value-added compounds based on carbohydrates [4].

Biomass pretreatment can be carried out through physical, chemical, and/or biological processes. The objective of this step is to partially degrade lignin, decreasing the compaction of cellulose and hemicellulose fibers, making these molecules more accessible for the later step of biomass fractionation into fermentable sugars [5]. Depending on the type of pretreatment used, it is possible to obtain monomeric sugars at this stage, corresponding mainly to the fraction of pentoses from hemicellulose [6], designated hemicellulosic hydrolysate (steam explosion with dilute sulfuric acid

pretreatment is the current process used industrially). The step after this kind of pretreatment is the enzymatic hydrolysis, in which enzymes are applied to hydrolyze cellulose into glucose monomers. From there, sugars can be used in bioprocesses to produce various products [7].

One of the biggest challenges in using hemicellulosic hydrolysate is the presence of inhibitors, which are obtained during certain types of pretreatment by the formation or release of compounds from lignin and hemicellulose [6,8]. The presence of these compounds (organic acids, furanic and phenolic compounds) in the hemicellulosic hydrolysate hinders the metabolism of ethanol-producing microorganisms, reducing the productivity of the process or even preventing fermentation. Several techniques have been used to minimize the action of inhibitors on cells, such as the removal of these compounds through detoxification processes before fermentation, development of more resistant strains, use of high cell density, cell immobilization, among others [8,9].

Cell immobilization is a general term that describes the physical confinement of viable cells in a defined region in space - usually called support - to limit the environment where the microorganisms will remain. As a result, different hydrodynamic characteristics than the surrounding environment are promoted [10]. This technique brings multiple advantages compared to the process with free cells, including relative ease of product separation, biocatalyst reuse, high cell density application, and high volumetric yield. Additionally, it protects the cells against external factors, such as pH, temperature, and toxic compounds [11,12], including the effects of inhibitors present in hemicellulosic hydrolysates. It also favors the reuse of cells in sequential and continuous processes [13].

Immobilization can be performed using different supports. The most used material for second-generation ethanol production is calcium alginate, forming spheres with cells

immobilized inside [14]. Some authors also used lignocellulosic biomass from the 2G ethanol production process as cell supports. The improvement of fermentative parameters with cell immobilization may be related to the low diffusion of inhibitors in the supports and improvements in the stress response because the cells are confined in the solid [15,16].

Therefore, this review heavily focuses on different strategies to overcome inhibitors for 2G ethanol production based on cell immobilization. In this sense, the state of the art characterizing the main inhibitors in 2G process and the most apllied techniques for cell immobilization, besides batch, repeated batch and continuous fermentation using immobilized cells, in addition to co-culture immobilization and co-immobilization of enzymes are presented in this work.

2. Scientometric analysis on immobilization for 2G ethanol

Considering that research on immobilized cells for 2G ethanol is very limited, the analysis presented herein was based on empirical research of published articles in various indexed journals. The relevant literature was shortlisted and categorized following the terms such as "cell immobiliz*" OR "immobiliz* cells" AND "ethanol" OR "bioethanol" AND lignocellulos* OR "second generation" OR "2nd generation" OR "2G" by search in Scopus database (). It was possible to observe the evolution of publications in the area over the years: from 1987 to 2010, one to three research manuscripts were published per year; however, in 2012, there were nine publications regarding this topic; and from 2013 to 2018, an average of four annual publications in this field were noted. According to the consulted "database", it was found that in 2019, 2020 and 2021 around eight, nine and five articles were published on this topic, respectively. Figure 1 shows the bibliometric analysis of publications on

2G ethanol and cell immobilization that appeared in the last ten years by international scientific journal, i.e., from January 2012 to January 2022. The journal Bioresource Technology leads the chart, followed by the Applied Energy and the Biochemical Engineering Journal. This analysis suggests that the subject has been addressed in relevant scientific journals in the area of 2G ethanol production, showing the relevance of the topic for the production of this biofuel.

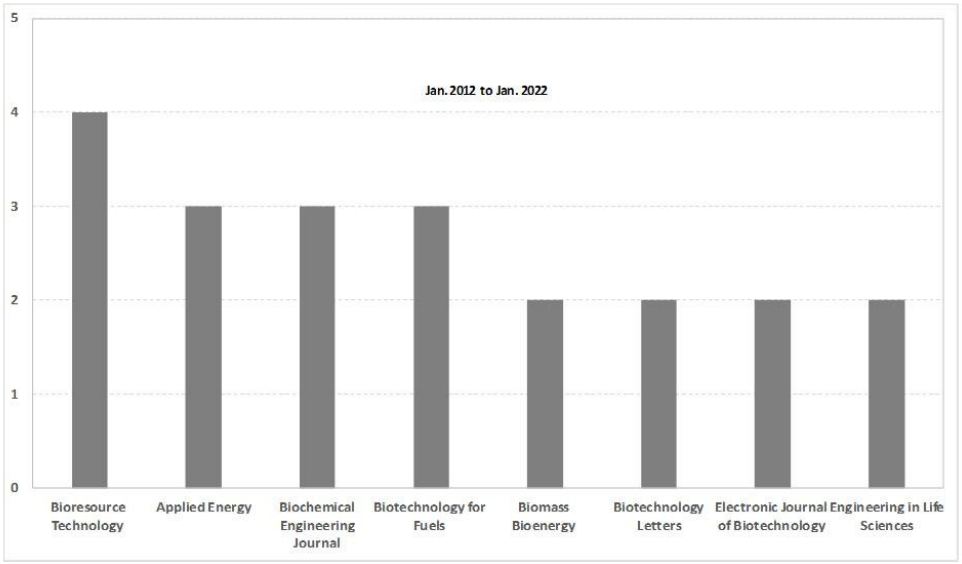

Figure 1. Bibliometric analysis by international scientific journal about second-generation ethanol production and cell immobilization (Jan. 2012–Jan. 2022).

3. Second-generation ethanol: production and current challenges

The development of economically viable biorefineries depends on the efficient fractionation of lignocellulosic biomass. [17]. The lignocellulosic biomass is essentially composed of cellulose (38-50%), hemicellulose (23-32%), and lignin (15-25%) [18]. Cellulose is a linear polymer of D-glucose units linked by β-1→4 glycosidic bonds. Hemicellulose is a heteropolymer composed predominantly of pentoses and hexoses

with short ramifications, such as D-xylose, D-glucose, L-arabinose, D-galactose, and acetyl groups. Lignin is a polyphenolic macromolecule consisting of basic units of 3-5-dimethoxy-4-hydroxy-phenylpropane, 3-methoxy-4-hydroxy-phenylpropane, and 4 hydroxy-phenylpropane [18,19].

The 2G ethanol is a biofuel obtained through lignocellulosic biomasses. For the production of 2G ethanol, the hemicellulosic and cellulosic polymeric chains must be transformed into fermentable sugars through sequential pretreatment and hydrolysis. The sugars released are then converted into ethanol through microbial fermentation [4]. Sugarcane bagasse, residues from the processing of corn and rice; forest residues such as soft and hardwood and wood chips, as well as agricultural and non-food residues, such as grass and alfalfa [9], are examples of biomasses.

Pretreatment is the first step for the development and industrialization of efficient 2G ethanol processes, promoting the separation of the biomass components into easily accessible fractions that are then subjected to hydrolysis and fermentation. Depending on the type of physical-chemical pretreatment applied to the biomass, it removes part of the structural lignin as phenolic compounds, reduces the crystallinity of the cellulose, and increases the porosity of this material, partially releasing monomeric/oligomeric sugars from the hemicelluloses for microbial conversion to ethanol. This fraction is called hemicellulosic hydrolysate and contains the fermentable sugars xylose (mainly), arabinose, glucose, galactose, and mannose [4,5]. Several pretreatment methods have been studied and improved over the years, such as steam explosion [20], acid [21,22], and alkaline [23] pretreatments.

After physical-chemical pretreatment, an additional step should be carried out by using enzymes to hydrolyze the recalcitrant structure and to release monomeric sugars from the cellulosic fraction [6,7]. Enzymes such as endoglucanases, exoglucanases, β-

glucosidases, and oxidoreductases are used in this step, breaking cellulose into glucose. The liquor obtained in this step is called cellulosic hydrolysate and contains mostly glucose.

After obtaining the monomeric sugars from biomass, they are converted into ethanol through the metabolism of microorganisms in the fermentation process. For hemicellulosic hydrolysates, microorganisms capable of fermenting pentose sugars are used, such as *Scheffersomyces stipitis*, *Scheffersomyces shehatae,* and *Spathaspora passalidarum* [1, 24, 25] or genetically modified *Saccharomyces cerevisiae* [26–29]. For fermentation of cellulosic hydrolysates, composed mainly of hexoses, the most used microorganisms are *Zymomonas mobilis* and *Saccharomyces cerevisiae* [4].

The fermentation step can be carried out using separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF). In SHF, the process conditions are specific for each step, whereas in SSF the two processes occur in the same tank under the same conditions, which may be interesting from an economic point of view [25] but requires process conditions to be the same for microorganism and enzyme. There is also the possibility of mixing pentose and hexose fractions and carrying out a co-culture (simultaneous saccharification co-culture fermentation, SSCF) with different microorganisms to favor the consumption of different sugars [30]. Figure 2 shows the steps of SHF, SSF, and SSCF for second-generation ethanol processes.

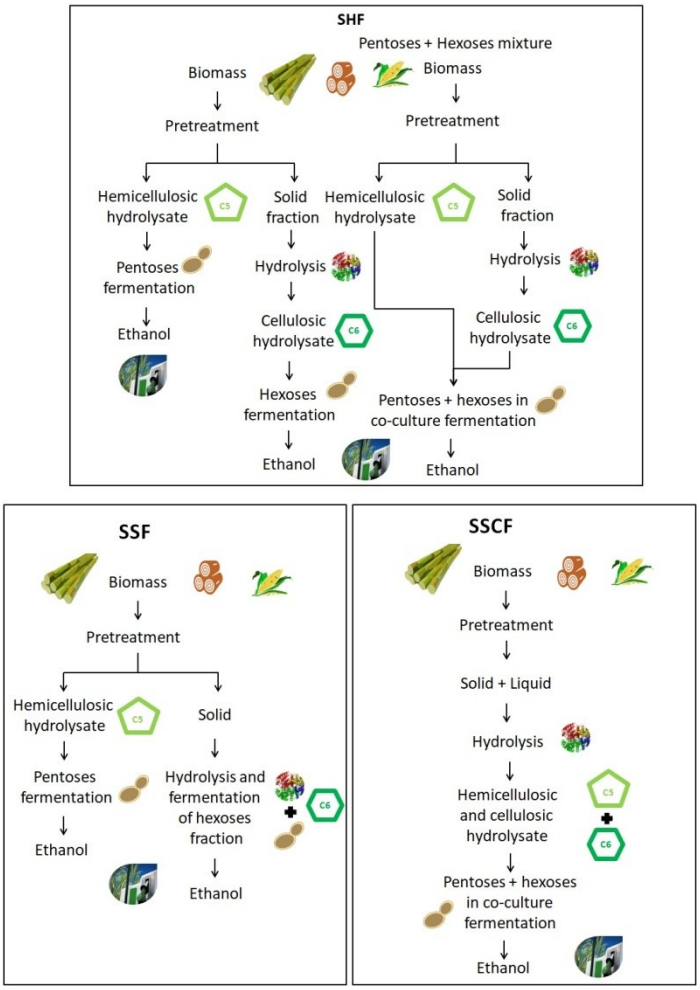

Figure 2. Steps of separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), and simultaneous saccharification co-culture fermentation (SSCF) for second-generation ethanol processes.

The fermentation process can be carried out in batch mode, the simplest and most easy process. The substrate is supplied initially without adding or removing the broth until the total conversion of sugars to ethanol. The disadvantages of this operation mode are, among others, the inhibition by the high initial substrate concentration and the low productivity. Another method used for ethanol production is the fed-batch mode, in which the substrate is fed at rates close to the sugar consumption rate of the microorganism employed. Thus, substrate inhibition is overcome, and ethanol productivity is increased. Continuous fermentation consists of the constant addition of substrate and constant removal of the fermented medium, decreasing substrate and

ethanol inhibition [25]. Some variations of the processes presented above can be used, such as cell recycling and operation in single or multiple stages.

3.1 Inhibitors of 2G fermentation process

Regardless of the fermentation process used for the 2G ethanol, there is an intrinsic bottleneck related to the deconstruction of the biomass in the physical-chemical pretreatment step, which is the formation of inhibitors. These compounds reduce process yield and productivity and specifically act on cells causing internal energy expenditure, membrane rupture, mutations, and even cell death [8, 31].

Inhibitors are classified according to their main organic function. Organic acids are generated when the acetyl structure of the hemicellulose is degraded. Furanic compounds are produced from the dehydration of pentoses and hexoses. Phenolic compounds result from the degradation of lignin [8, 19, 32, 33]. The main inhibitory compounds found in hemicellulosic hydrolysates from sugarcane bagasse are (in varying concentrations): acetic acid (from 2.0 to 6.0 $g.L^{-1}$), furfural (from 0.05 to 5.6 $g.L^{-1}$), 5-hydroxymethylfurfural (from 0.1 to 1.0 $g.L^{-1}$), and phenolics (~0.03 $g.L^{-1}$) [34–38].

The inhibition mechanisms of organic acids are related to the acidification of the cytoplasm, causing the cell to expend energy in an attempt to reestablish the internal pH [39]. On the other hand, furanic compounds disrupt the cell membrane and expose the cytoplasm. They also interact with segments of DNA, causing mutations [40]. Likewise, phenolic compounds interact and disrupt the cell membrane; and the smaller the molecular structure, the more toxic they are to cells [8, 31].

Figure 3 illustrates, in general, the obtainment of microorganisms (yeast pentoses) and sugars for the use of lignocellulosic fractions and shows the interaction of

inhibitor compounds with cells. Native pentose-consuming microorganisms are generally associated with wood-degrading insects, such as beetles and termites. These microorganisms are present in the guts of these insects, helping them to obtain energy from biomass [25].

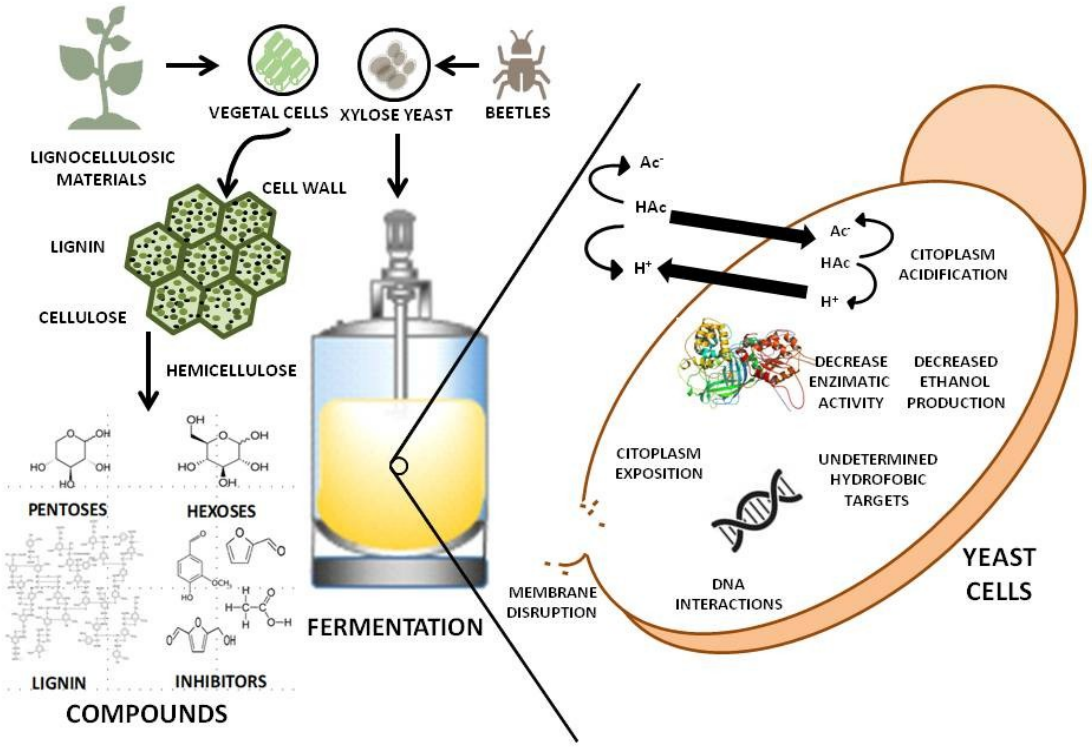

Figure 3. Obtainment of microorganisms (pentose yeasts) and sugars to utilize lignocellulosic fraction and shows the interaction of inhibitory compounds with cells.

Many strategies have already been used to reduce the action of inhibitors in 2G ethanol fermentation processes [8]. Previous detoxification of the hydrolysates can be performed through chemical, physical and biological processes. Detoxification *in situ* can also be applied to remove compounds during fermentation by microorganisms that can metabolize the inhibitors [41]. Process strategies are also studied, such as the use of adapted strains [42, 43], genetically modified microorganisms with increased tolerance to inhibitors [44], use of high cell density [45, 46], application of continuous or fed-

batch operation modes to dose the addition of inhibitors [8], dilution of inhibitors without decreasing the concentration of sugars by adding another source of carbon (such as molasses) [29, 47], and immobilization of cells to protect the direct exposure of microorganisms to the toxic environment [4, 9, 13].

In the last decades, immobilization has overcome the interference of inhibitors in the production of 2G ethanol and many research groups have focused on this strategy. In the following topics, we summarize the state of the art of the main fermentation strategies proposed to improve the production of 2G ethanol by fermentation using immobilization, focusing mainly on its use as a protection strategy against the action of inhibitors on microorganism cells.

4. Cells immobilization for 2G ethanol

Some authors have already described the benefits of cells immobilization as a strategy for fermenting hemicellulosic hydrolysate with inhibitors (Table 1). The immobilization techniques can be divided into flocculation, mechanical containment, entrapment in porous matrices and immobilization on solid supports [9], as shown in Figure 4.

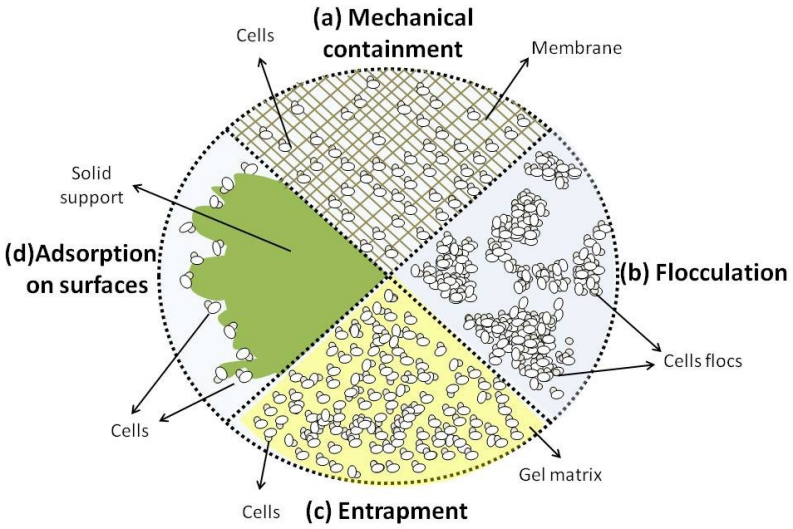

Figure 4. Common immobilization techniques for microbial cells: (a) mechanical containment; (b) flocculation; (c) entrapment in a porous matrix; and (d) adsorption on surfaces.

From Table 1, the most used techniques for cell immobilization in the production of 2G ethanol are encapsulation and surface adsorption due to ease of operation and low input cost.

Table 1 – Information about cells immobilization for 2G ethanol production reported in the literature.

Microorganism	Technique / Support	Substrate	Inhibitors ($g.L^{-1}$)	Fermentation conditions	Increase in parameters after cell immobilization (compared to free cells) Yield (%)	Q_P (%)	Number of cycles / Why stop	Reference
S.cerevisiae CEN.PK113-7D strong flocculant mutant	Flocculation Self-aggregation	Spruce tree hydrolysate	AA:2.4 FURF:3.28 HMF: 0.69	Batch Erlenmeyer No agitation information 15 h	8.6	18.9	No cycles	[48]
S. cerevisiae KF-7	Flocculation Self-aggregation	Wood biomass hydrolysate	AA: 4.5	Continous 0.45L fermenter 0.3 h^{-1} 1920 h	No comparison with free cells		No cycles	[49]
S. cerevisiae CBS 8066	Mechanical containment Polyethersulfone and polyvinyl-pyrrolidone membrane	Spruce sawdust hydrolysate	AA:1.18 FURF: 0.47 HMF: 2.88	Continous 600mL membrane fermenter 0.2 - 0.8 h^{-1} 50 h	No comparison with free cells		No cycles	[50]
Industrial strain *S. cerevisiae*	Mechanical containment Ceramic membrane	Oak wood hydrolysate	Not mentioned	Continous 1.5L membrane fermenter 0.75 $L.min^{-1}$ 55 h	No comparison with free cells		No cycles	[51]
S.cerevisiae T0936	Mechanical containment Polysulfone and polyvinyl-pyrrolidone membrane	Wheat straw hydrolysate	AA:8.9 FURF:9.2 HMF: 1.1	Continous 3L membrane fermenter 0.6 $L.min^{-1}$ 150 h	No comparison with free cells		No cycles	[52]
Adapted *Sc. stipitis* PSA30	Encapsulation Ca-alginate	Corn cob hydrolysate	AA: 4.5	Repeated batch 1L fermenter 100 rpm / 48h	4.9	64.5	8 no explicit reason for the stop	[53]

Recombinant *S. cerevisiae* T18	Encapsulation Ca-alginate	Sugarcane bagasse hydrolysate	AA:6.5 FURF: 0.37 HMF: 0.02	Repeated batch 10 mL mini fermenter 150 rpm / 4 h	No comparision with free cells		3 successive loss in parameters	[12]
				Repeated batch 100 mL fixed bed reactor 150 rpm / 4 h	No comparison with free cells		3 successive loss in parameters	
Recombinant *S. cerevisiae* T18	Encapsulation Ca-alginate	Sugarcane bagasse hydrolysate	AA:8.31 FURF: 0.27 HMF: 0.23	Repeated batch 10 mL mini fermenter 150 rpm / 40 h	No comparison with free cells		3 successive loss in parameters	[54]
S.cerevisiae MTCC 3089 + *Sc. stipitis* NCIM 3498	Encapsulation Ca-alginate	Apple pomace hydrolysate	Not mentioned	Batch Erlenmeyer No agitation information 36 h	28.8	29.3	No cycles	[55]
S. cerevisiae CBS 8066	Encapsulation Ca-alginate + carboxymethylcellulose	Spruce tree hydrolysate	AA:2.2 FURF: 0.19 HMF: 0.79 VAN: 0.08	Batch Erlenmeyer No agitation 80h	No comparison with free cells		No cycles	[15]
S. cerevisiae	Encapsulation Ca-alginate + chitosan	Corn straw hydrolysate	Not mentioned	Batch Erlemmeyer 150 rpm / 24 h	Not mentioned	148.0	No cycles	[56]
Pachysolen thanophilus						268.8		
S.cerevisiae+ *P. thanophilus*						224.9		
S.cerevisiae CTCRI	Encapsulation Ca-alginate	Mahula flower hydrolysate	Not mentioned	Repeated batch Erlenmeyer 120 rpm / 96h	8.5	3.9	3 successive loss in parameters	[57]
	Encapsulation Ágar-ágar cubes				8.8	0.5	3 successive loss in parameters	

Thermotolerant *S cerevisiae* VS3	Adsorption on surface Sugarcane bagasse	Sugarcane bagasse hydrolysate	Not mentioned	Batch Erlenmeyer 50 rpm / 72h	5.9	48.4	8 successive loss in parameters	[58]
Sc. stipitis NCIM 3498	Adsorption on surface Sorghum bagasse	Soybean hull hydrolysate	Not mentioned	Repeated batch Erlenmeyer 150 rpm / 84h	6.8	14.6	5 no explicit reason for stop	[59]
Sc. stipitis ACL 2.1	Adsorption on surface Sugarcane bagasse	Sugarcane bagasse hydrolysate	AA:2.4 FURF: 0.8	Repeated batch Erlenmeyer 250 rpm/ 36h	85.7	100.0	25 no explicit reason for the stop	[60]
	Adsorption on surface/ Sugarcane bagasse						7 successive lost in parameters	
S.cerevisiae MTCC 174	Encapsulation Ágar-ágar cubes	Sugarcane bagasse hydrolysate	Not mentioned	Repeated batch Erlenmeyer no agitation information 36 h	No comparison with free cells		4 successive loss in parameters, cubes rupture	[61]
	Encapsulation Ca-alginate						4 successive loss in parameters, beads rupture	
	Adsorption on surface Terracota beads						5 no explicit reason for stop	
Sc.shehatae ATCC 22984	Adsorption on surface Coconut bract	Rice straw hydrolysate	Not mentioned	Repeated batch Erlenmeyer 50 rpm / 168 h	No comparison with free cells		5 no explicit reason for stop	[62]
	Adsorption on surface Corn cob						4 successive loss in parameters,	
Sc.. shehatae NCL-3501	Encapsulation Ca-alginate	Rice straw hydrolysate	Not mentioned	Batch Erlenmeyer 150 rpm / 24h	27.0	46.7	No cycles	[63]

				Continuous Packed bed reactor 37,5 mL.h^{-1} 216 h	No comparison with free cells		
S. cerevisiae CBS 8066	Encapsulation Ca-alginate+ carboxymethylcellulose	Spruce tree hydrolysate	AA: 4.95 FURF: 0.39 HMF: 0.74	Batch Erlenmeyer 130 rpm / 24 h	The free cells did not fermented in 24h. The immobilized cells fermented with good parameters (see on text)	No cycles	[64]
Recombinant *S. cerevisiae* ZU-10	Encapsulation Ca-alginate	Corn stover hydrolysate	AA: 1.16	Batch Erlenmeyer 120 rpm / 72 h	44.0	145.3	5 no explicit reason for stop	[65]
S.cerevisiae NRRL 2034	Encapsulation Ca-alginate	Rice straw hydrolysate	AA:1.92 FURF: 0.52 HMF: 0.46	Repeated batch Erlenmeyer 150 rpm / 24 h	No comparison with free cells		17 no explicit reason for the stop	[66]
Recombinant *S.cerevisiae* GSE1618				Continuous Packed bed reactor 0.37.h^{-1} 232 h			No cycles	
S. cerevisiae CBS 8066	Encapsulation Ca-alginate+ carboxymethylcellulose	Spruce tree hydrolysate	AA:2.5 FURF: 1.35 HMF: 0.37	Continuous Packed bed reactor 0.1- 0.5 h^{-1} 50 h	29.4	33.7	No cycles	[67]
Sc.shehatae UFMG - HM 52.2	Encapsulation Ca-alginate	Sugarcane bagasse hydrolysate	AA: 0.88	Repeated batches Erlenmeyer 200 rpm / 72 h	No comparison with free cells		no explicit reason for the stop	[68]

Note: abbreviations refer to AA: acetic acid; FURF: furfural; HMF: hydroxymethylfurfural; VAN: vanline.

The immobilization method by encapsulation is performed by trapping the cells in porous matrices. The cell solution is added to the gelatinous solution, which, through the process of extrusion or dripping, forms spheres with the cells immobilized [14,69]. The most used materials are natural polymers such as calcium alginate, agar-agar, k-carrageenan, and chitosan [69,70]. Alginate has been widely applied for cell immobilization as calcium alginate spheres in the fermentation of different hydrolysates [16,53,57,63,65] and it has shown to be a good support choice for 2G ethanol. As a natural polymer, alginate is non-toxic and has biocompatibility with microorganisms. The application in cell immobilization consists of a mixture of alginate in concentrations between 1 to 4% with cells solution (in variated inoculums concentrations) that are dripped into a gelling solution, usually of a divalent cation, such as calcium, which promotes the formation of spheres. The resulting spheres have sizes between 2.0 and 5.0 mm and undergo a curing time, varying from minutes to days. After the curing process, the supports can be used in the fermentation [12,53,54,61,65,66,68]. However, it is known that mass transfer may be a problem in the diffusion of gases, substrates, and products through the supports, especially if the immobilized microorganism is aerobic or depends on microaerophilia for the consumption of sugars and cell growth, such as some pentose consuming strains [1]. This problem can be minimized by applying sufficient agitation in the fermentation process to improve the mass transfer between liquid and solid. However, since the spheres are usually made of gelatinous material, a prior assessment should be made for an adjustment that provides improved diffusion and, at the same time, does not harm the integrity of the supports.

Immobilization by adsorption on the surface is based on the formation of interactions or bonds between cells and the solid, which can occur naturally or induced by using binding agents (metal oxides or covalent binding agents, such as glutaraldehyde or aminosilane) [69]. Van der Walls type, electrostatic, ionic, or covalent bonds can be formed. There are no

barriers between the liquid and solid phases; thus, cells can be displaced throughout the process. The immobilization method is done by the simple contact of the support with the cells solution, which migrates from the liquid to the solid [13]. Many authors who used this immobilization technique to produce 2G ethanol bet on low-cost materials as supports. In this context, lignocellulosic materials are a cheaper alternative and a more abundant cell immobilization support [13]. Some advantages of using lignocellulosic materials as support are the physical and chemical properties (such as porosity and rigidity); they are also ecologically correct, reneweable, biodegradable and non-toxic for cells [13]. The porosity of these materials is especially interesting, as they positively affect the diffusion of nutrients and products compared to other solid supports, such as calcium alginate spheres. However, as the cells will be adsorbed on the surface generally by weak bonds, there is a high chance of displacement or leakage to occur depending on process conditions, such as pH and temperature, reducing the efficiency of the protection of the cells conferred by the immobilization.

Regardless of the technique, in addition to the physical protection that the supports provide to cells, the improvement in the kinetic parameters of fermentation of hydrolysates with inhibitors achieved by applying cell immobilization may be related to the low diffusion of inhibitory compounds through the supports and the ability to transform these inhibitors into less toxic species. There is also evidence that the external stress response is strengthened when performing immobilization. It was also observed that *S. cerevisiae* cells on the surface of the alginate spheres were able to convert the toxic compounds present in forest residue hydrolysate, thus leaving the medium less inhibitory for the cells in the innermost layers of the support [15]. In another study [16], *S. cerevisiae* cells were immobilized in calcium alginate spheres and used to ferment a cellulosic hydrolysate from forest residues with addition of inhibitors. The authors observed that the immobilized cells could metabolize the

inhibitors with higher consumption rates than in the process with free cells. With real-time PCR analysis, the authors identified genes related to the stress response (*YAP1* genes: phenolic resistance and apoptosis suppressor and genes: *ATR1* and *FLR1*: membrane transport proteins). These same genes were investigated in immobilized cells before being placed in contact with the inhibitors. The results showed that encapsulated cells also increased the expression of these genes, indicating that the metabolism responds to stress related to the cell being confined on the stand. This initial activation of the response to the stress situation may be related to better results about the subsequent increase in stress imposed by the addition of supports in the medium with inhibitors.

Together with the existing immobilization techniques, it is possible to use different operation modes of the fermentation process, such as batch, repeated batch, continuous process, co-cultures, or processes with cells and enzymes immobilized together. Depending on the operation mode, the cell immobilization confers advantages such as easy separation of the cells from the medium and working at high cell density in the process. These advantages will be discussed in the following topics, based on the methods already described in the literature for the production of 2G ethanol.

5. Fermentation strategies using immobilized cells for 2G ethanol

5.1 Batch fermentations

Batch fermentation is commonly used in 2G ethanol production [58,63,65,71]. This process consists on supplying all the substrate at the beginning of the fermentation, inoculating the microorganism, and removing fermented broth and biocatalysts at the end of the process, which means both nutrients and inhibitors are present at the beginning of the process. This process can be operated in different bioreactors, such as Erlenmeyer flasks [57]

and stirred tanks [12]. Sterilization is extremely important to operate since it prevents contamination, but it demands a considerate amount of preparation time. Figure 5 presents a comparison of the performance of free and immobilized cells in various supports for 2G ethanol in fermentations under batch mode.

The results reported in the literature (Figure 5, Table 1) show that the cell immobilization strategy in batch processes substantially improves the performance of the microorganism against inhibitors of the hydrolysates in comparison to the performance of free cells. All of the authors cited in this overview (in this topic and on the others) used real hemicellulosic hydrolysates containing inhibitors (such as acetic acid, furfural, hydroxymethylfurfural, valine), and the presence of these components is one of the most challenging aspects of hydrolysates fermentation. The fact that fermentation parameters are improved when immobilized cells are applied proves that this is a viable alternative to bypass the difficulties caused by those compounds. Certainly, *S. cerevisiae* strains are the microorganisms mostly used in immobilization for 2G ethanol processes and are more capable of reaching better parameters, because of their metabolic characteristics, either genetically modified or not.

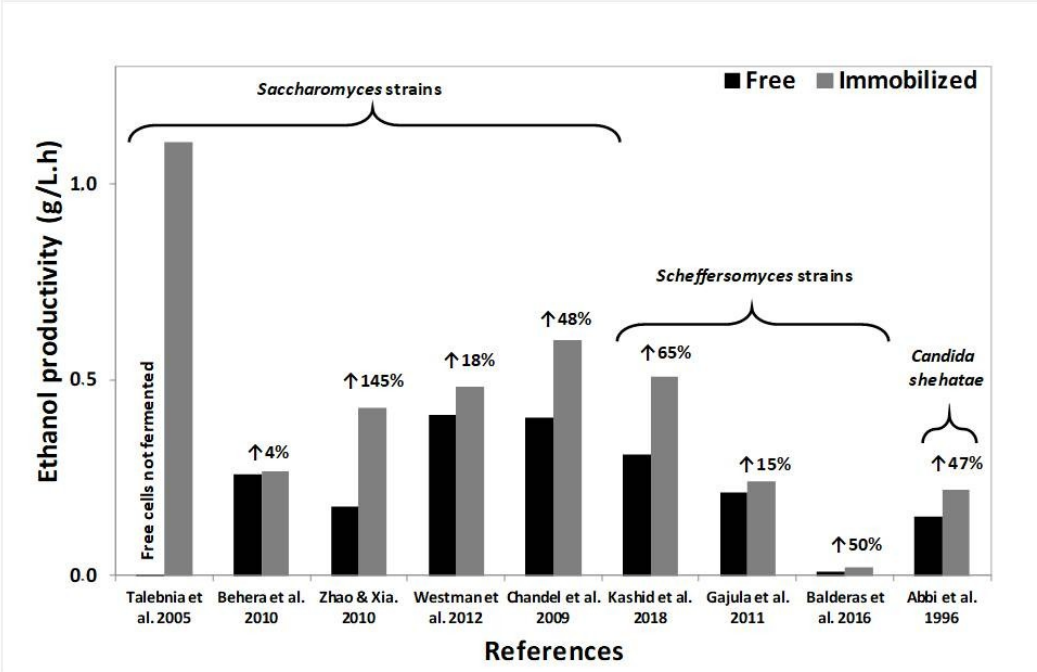

Figure 5. Comparison between the productivity of free and immobilized cells in batch processes for the production of 2G ethanol.

For example, the immobilization of *S. cerevisiae* in calcium alginate spheres improved fermentation parameters of mahula flower hydrolysate [57], in which the technique favored ethanol yield, reaching 97% (0.483 $g.g^{-1}$) for immobilized cells, against 89% (0.445 $g.g^{-1}$) for free cells. The productivity for immobilized cells was also higher (0.268 $g.L^{-1}.h^{-1}$) when compared to free cells (0.258 $g.L^{-1}.h^{-1}$). The authors also tested entrapment immobilization in agar-agar spheres. Results were not as promising as those obtained using calcium alginate, since a small increase of 0.5% in productivity was obtained comparing to free cells. The tests were carried out in Erlenmeyer flasks containing the hydrolysate and 10% inoculum, which were incubated for 96 h statically at room temperature. Calcium alginate entrapment was a great immobilization technique choice, as it improved *S. cerevisiae* performance in the fermentation of mahula flower hydrolysate. However, productivity values were still low compared to studies that applied different operation modes, such as continuous fermentation or even using a shaker, which would improve mass transfer and ethanol

production since the authors did not shake the fermentation flasks [57]. Although the authors do not mention the concentration of inhibitors in the hydrolysate, mahula flowers are rich in fermentable sugar (40–47%; on a fresh weight basis), which makes this biomass interesting in a biorefinery concept.

Others authors [65] immobilized recombinant *S. cerevisiae* ZU10 in calcium alginate and fermented corn straw hemicellulosic hydrolysate (1.16 $g.L^{-1}$ of acetic acid) in 250-mL Erlenmeyer flasks (30 mL of cells and 150 mL of hydrolysate) with an agitation of 120 rpm at 30 °C. The results showed an increase in ethanol production when immobilization was applied. After 96 h, the free cells consumed 78% of the available xylose and produced 21.6 $g.L^{-1}$ of ethanol, with a yield of 0.282 $g.g^{-1}$. In contrast, after 72 h, the immobilized cells consumed 97% of the xylose and produced 31.1 $g.L^{-1}$ of ethanol, resulting in a yield of 0.406 $g.g^{-1}$. Besides, productivity increased from 0.176 to 0.431 $g.L^{-1}.h^{-1}$. Sugar consumption is an important fermentation parameter, and the residual sugar content at the end of batches is not ideal for an industrial process. Hence, the improvement observed by those authors after immobilization is a great advantage for fermentation of hydrolysates.

Another study [64] initially compared the performance for ethanol production of free and immobilized cells of *S. cerevisiae* CBS 8066 in synthetic medium, containing 20 $g.L^{-1}$ of glucose, with and without the presence of 5 $g.L^{-1}$ of furfural, as a preliminary investigation (before hydrolysates fermentation) of the benefits of cell encapsulation. The effect of furfural in the synthetic medium fermentation with free cells was monitored along with 24 h; and no cell growth, sugar consumption nor ethanol production were observed during this period. In contrast, after 20 h of the process with immobilized cells, all the sugar available in the medium was consumed. Some concentration of inhibitor was also metabolized, transforming furfural into furfuryl alcohol and 2-furic acid, a molecule less toxic for the cells than the original compound. Other authors have already observed this ability to transform inhibitors

into less toxic species for free and immobilized cells, but immobilized cells can metabolize inhibitors at a higher rate [15]. In the mentionated study [64], besides synthetic medium, a hemicellulosic hydrolysate of forest residues (inhibitors, in $g.L^{-1}$: acetic acid: 4.95; furfural: 0.39; HMF: 0.74) was also used by the authors. In this case, the authors did not observe fermentative activity during 24 h in the experiment with free cell. However, immobilized cells consumed all the sugar in the hemicellulosic hydrolysate and produced ethanol at a rate of 1.108 $g.L^{-1}.h^{-1}$ in 18 h of process, and a yield of 0.43 $g.g^{-1}$ was reached. All batch fermentations were carried out in anaerobic conditions in 300-mL conical flasks placed in a shaker bath at 30 °C; for the hydrolysate fermentation, the pH was adjusted to 5.0.

Calcium alginate and carboxymethylcellulose spheres were also used to immobilize recombinant yeast *S. cerevisiae* CBS8066 [15]. Batch experiments were performed in 250-mL conical flasks at 30 °C for, approximately, 80 h. When fermenting a hemicellulosic hydrolysate of forest residues (inhibitors, in $g.L^{-1}$: acetic acid: 2.2; furfural: 0.19; HMF: 0.79; vanillin: 0.08 $g.L^{-1}$), yields of 0.411 $g.g^{-1}$ and 0.484 $g.g^{-1}$ were achieved for free and immobilized cells, respectively, therefore increasing the yield by 18%. It is possible to notice that cell immobilization applied in batch fermentations promoted, once again, the increase in parameters such as yield and productivity even in the presence of inhibitors that are known for harming cell performance in sugar consumption for ethanol production. In another study [63], *Sc. shehatae* was immobilized in calcium alginate, and fermentations of synthetic medium containing xylose or rice straw hydrolysate were carried out in 250-mL Erlenmeyer flasks containing 150-mL of the medium, at 30 ºC and agitation of 150 rpm. Calcium alginate immobilization improved yield and productivity by 27 and 47%, respectively, compared to free cells (with the same cell density in both processes) (Table 1). A yield of 0.47 $g.g^{-1}$ and a productivity of 0.22 $g.L^{-1}.h^{-1}$ were reached when fermenting rice straw hydrolysates. Batch mode made it possible to notice the improvement in parameters caused by cell

immobilization. However, productivity did not reach a value as high as the one obtained in continuous mode operation (0.33 $g.L^{-1}.h^{-1}$), which will be addressed in topic 5.3.

Sc. stipitis was immobilized in calcium alginate spheres and applied in batch fermentations of a hemicellulosic hydrolysate from corn residues (xylose: 55 $g.L^{-1}$ and acetic acid: 4.5 $g.L^{-1}$)[53]. The fermentation batch was carried out in a 1-L Brunswick BioFlow fermenter with 0.7 L of working volume, at a aeration rate of 0.2 vvm, and 30 °C. When comparing to free cells, the results for immobilization led to a faster xylose consumption (48 h compared to 96 h). As a result, productivity increased from 0.31 to 0.51 $g.L^{-1}.h^{-1}$ and the yield from 0.41 to 0.43 $g.g^{-1}$. The productivity and yield improvement obtained by cell immobilization is a great advantage in the industrial process, as it promotes higher ethanol production in less time which has a positive economic impact. However, the authors did not make it clear if they used the same initial cell concentration in both processes.

As mentioned before, given all the costs, difficulties, and weaknesses of alginate spheres in cell immobilization, some authors have chosen lignocellulosic materials as cell supports due to their availability and robustness to perform fermentations. The study carried out by Chandel et al. (2009) [58] used delignified sugarcane bagasse treated with ammonia as a support for the immobilization of the thermotolerant yeast *S. cerevisiae* VS3. The authors compared fermentations of free and immobilized cells on batch fermentations carried out in 500-mL Erlenmeyer flasks, at 42 °C and 50 rpm, for 72 h. The results showed that the fermentative parameters with immobilized cells were higher, such as the ethanol yield (0.410 $g.g^{-1}$ for free cells and 0.434 $g.g^{-1}$ for immobilized cells) and volumetric productivity (0.405 $g.L^{-1}.h^{-1}$ for free cells and 0.601 $g.L^{-1}.h^{-1}$ for immobilized cells). The cell immobilization process also favored the absorption of sugars, expressed in the sugar consumption rate (0.658 $g.L^{-1}.h^{-1}$ for free cells and 1.386 $g.L^{-1}.h^{-1}$ for immobilized cells). The maximum production of ethanol (21.66 $g.L^{-1}$) occurred in 36 h for immobilized cells, while

for free cells, the highest ethanol titer (19.45 $g.L^{-1}$) was detected in 48 h.. In another study [60], *Sc. stipitis* was immobilized in sugarcane bagasse pretreated with H_2SO_4. The authors performed batch experiments in Erlenmeyer flasks at an agitation of 250 rpm for 36 h. The comparison between free and immobilized cells was performed by fermenting a hemicellulosic sugarcane hydrolysate (inhibitors, in $g.L^{-1}$: acetic acid: 2.4; furfural: 0.8). The same initial cell concentration was used in both processes. After 60 h of fermentation, the process with immobilized cells resulted in a 1.85-fold higher yield (from 0.14 to 0.26 $g.g^{-1}$) and, despite the productivity being low for both assays, it was 2-fold higher when immobilization was applied (0.01 for free cells and 0.02 $g.L^{-1}.h^{-1}$ for immobilized cells). *Sc. stipitis* was also immobilized in sorghum bagasse and applied to ferment soybean hull hydrolysate [59]. Batch experiments were carried out in 250-mL Erlenmeyer flasks at an agitation of 150 rpm, 30 °C and pH of 5.5, for 84 h. The results showed that the immobilization favored the production of ethanol, reaching the maximum ethanol concentration (20.45 $g.L^{-1}$), yield (0.4 $g.g^{-1}$), and productivity (0.243 $g\ L^{-1}\ h^{-1}$), values 14.0, 6.8, and 14.6% higher, respectively, than the fermentation with free cells. Although the authors did not report the concentration of lignocellulosic hydrolysate inhibitors, the analysis of the peanut shell biomass indicated a content of 16% lignin, which is an indication that it is possible to have concentrations of inhibitors in this substrate.

When lignocellulosic materials were used as support, fermentations were performed at higher agitation than those with calcium alginate in the previously cited works, which indicates that the lignocellulosic material is much more robust than the gelatinous matrix, withstanding greater agitation. Efficient agitation provides better homogenization of nutrients, and in the case of processes with immobilized cells, it improves the diffusion of compounds through the supports.

In general, it is possible to notice that the cell immobilization improves fermentation of hemicellulosic hydrolysate for fermentations under batch mode. Overall, hemicellulosic hydrolysate presents low fermentability by non-adapted free cells, however an increased yield and productivity are observed when strategies of immobilization are applied in 2G process. Fermentations under batch mode seems to be a good choice of operation, since it allowed the authors to have a clearer idea of the immobilization effects on ethanol production, as it provides a fair comparison with free cells processes. Besides, the cells immobilization allows the improvement of fermentation kinetic parameters by repeating batches, whose thematic will be addressed in the next topic.

5.2 Repeated batch fermentations

In addition to protection against inhibitors, another advantage of immobilized cells is the possibility of reusing them in sequential fermentations due to easier separation from the fermentation medium. In addition, it saves time and inputs for inoculum preparation and allows a better recovery of the final product [9, 13]. The repeated batch process consists of sequential batches that reuse the cells to ferment fresh medium at each batch. Some authors tested the reuse of immobilized cells until there was a significant decrease of fermentation parameters [12] or any physical damage on the support was noticed [61]. Repeated batches are a well-established strategy in the industry, mainly for first-generation ethanol production. Cell recycling is also known for exposing the cells to various stress conditions that select the strains better adapted to industrial processes (Amorim et al., 2011).

In a work previously mentioned [65] (Table 1), using a corn straw hemicellulosic hydrolysate (acetic acid: 1.16 $g.L^{-1}$), the authors carried out five batches reusing the alginate calcium beads with recombinant *S. cerevisiae* ZU-10. The batch experiments were performed

in Erlenmeyer flasks at an agitation of 120 rpm for 72 h. The beads were collected and washed with sterile water three times between each batch and then added to the new batch. The authors observed that the ethanol production and the yield remained stable throughout the five batches (30 $g.L^{-1}$ and 0.4 $g.g^{-1}$, respectively). The possibility of reusing cells throughout the cycles achieving great ethanol concentration, and yield is one of the benefits of cell protection by immobilization, exemplified in this work. *S. cerevisiae* T18 cells were also used for others authors [54], which were immobilized on calcium alginate to perform repeated fermentations using sugarcane bagasse hemicellulosic hydrolysate as substrate (inhibitors, in $g.L^{-1}$: acetic acid: 8.31; furfural, 0.27; HMF: 0.23; total phenolics: 3.2). In this work, repeated-batches were carried out in a 10-mL mini reactor with a stirring rate of 150 rpm for 40 h each, and consisted in the removal of the fermented medium at the end of each cycle and addition of fresh medium to start a new fermentation cycle. The authors reported that it was possible to reuse immobilized cells up to four times without decreasing ethanol titer. In the fourth batch, cell viability was still around 70%. Altough the productivity decreased over the sequential fermentations (from 8.33 to 1.33 $g.L^{-1}.h^{-1}$ from the first to the fourth cycle), it must be remarked that the microorganisms metabolized a hydrolysate with a high concentration of inhibitors. Compared to other works, the number of tested cycles was low, but the fact that cell viability was kept around 70% shows that immobilization protected the cells during batches.

Different natural xylose-fermenting yeasts have been immobilized and applied for 2G ethanol production in repeated fermentations. Authors [68] studied the immobilization of *Sc. shehatae* in calcium alginate spheres and used them in repeated-batches fermentations of a detoxified sugarcane bagasse hemicellulosic hydrolysate (Table 1). After optimizing the immobilization conditions, the authors performed five sequential batches reusing the spheres with immobilized cells. Repeated-batch fermentations were conducted in 150-mL Erlenmeyer

flasks at 30 °C and 200 rpm for 72 h each. After the end of each batch cycle, the fermented medium was discharged, and the immobilized cells were washed with distilled water to remove fractions of fermented broth and used as inoculum for the next batch with fresh medium. The ethanol productivity and the conversion factor of sugars into ethanol were 0.18 $g.L^{-1}.h^{-1}$ and 0.32 $g.g^{-1}$, respectively, using a fermentation medium with 0.88 $g.L^{-1}$ of acetic acid. In this study, the fermentative parameters did not change over the cycles, demonstrating the stability of the immobilization, which is a great achievement.

Although the majority of authors report the discontinuity of the process mostly due to the decrease in the fermentative parameters rather than to the stability of the supports, some parameters can help the reuse of calcium alginate spheres, such as the curing time of the spheres in the gelling solution, the concentration of alginate and the stirring of the fermentation process. The study that presents the highest number of sequential batches fermentations with immobilized yeasts was reported by Mishra et al. [66]. The authors immobilized *S. cerevisiae* in calcium alginate spheres and performed fermentations using synthetic medium and non-detoxified rice straw hydrolysate (inhibitors, in $g.L^{-1}$: acetic acid: 1.92; furfural: 0.52; HMF: 0.46) (Table 1). Repeated batches were performed in Erlenmeyer flasks at an agitation of 150 rpm for 24 h each. After each fermentation, the beads were filtered, rinsed with sterile distilled water, and added to the fresh medium. The results of repeated fermentations indicated that, with the optimized conditions (4 mm of diameter, 2% alginate, 30 °C, and 120h of curing time), it was possible to recycle immobilized cells without losses in kinetic parameters for 36 times using synthetic medium containing glucose, reaching an average of 97% ethanol yield on most fermentation cycles. The same experiment was carried out with hemicellulosic rice straw hydrolysate and, between the 3^{rd} and 17^{th} cell recycle, the fermentation parameters remained constant (approximately 30 $g.L^{-1}$ of ethanol titer and a yield of 90%). The higher number of fermentation cycles reached by this study

may be related to the rigidity of the support caused by the long curing time (120 h). Although the authors did not mention the reason for the process discontinuity after 17 fermentation cycles in hemicellulosic hydrolysate, the microscopy analysis revealed that beads remained unbroken for the assays with synthetic glucose medium until the 24th cycle, and the yeast adhered to the surface of the support increased. After the 30th cycle, cracks began to appear in the structures, and in the 40th cycle, the supports lost 1/3 of their initial volume due to mechanical wear caused by agitation in the fermentation [66], which demonstrates that even with long curing times, the supports have a deadline of use. Interestingly, among the related works, the lowest value applied for curing time (1 h) also resulted in a moderate value of cycles: a total of eight cycles were performed by these authors [53], who used low agitation (100 rpm), which may be related to the lesser wear of the supports during the process. In general, the alginate concentration used in the methodologies was between 1 and 2%. This study [53] was the one that used an alginate concentration of 3%. The authors performed sequential batches using corn residues hemicellulosic hydrolysate as substrate (acetic acid: 4.5 g.L^{-1}) with *Sc. stipitis* cells immobilized in calcium alginate. Repeated-batches experiments were conducted in 1-L fermenters with a stirring rate of 100 rpm for 48 h each. Eight batches were performed without losses in the ethanol production parameters (Table 1).

Curing time promotes more rigidity to the beads due to the gelling solution and its interaction with the support. Based on the studies cited [53,66], the curing time seems extremely important to the immobilization and beads utilization. Longer curing time enables a higher number of fermentation cycles, but shorter times do not mean they will not be rigid enough - it depends on the conditions used in the fermentation process, such as agitation. The agitation conditions interfere in the robustness of the supports for their reuse and the nutrients homogenization. Additionally, it impacts the establishment of the aerobic or anaerobic condition since, within the supports, the diffusion of gases is hampered. Thus, studies that

focus on optimizing the aeration conditions of the medium through agitation but without harming the integrity of the supports are necessary.

The lignocellulosic materials, due to their robustness, seem to be ideal to be used in repeated batch fermentations. In the previously cited study [61], the authors compared sugarcane bagasse, calcium alginate, and agar-agar cubes with immobilize *S. cerevisiae* cells in repeated batch fermentations of sugarcane bagasse cellulosic hydrolysate. The fermentations cycles were performed for 36 h each. At the end of each cycle, the support was harvested from the fermentation broth, washed with sterile water, and inoculated to a fresh medium. This procedure was performed from four to ten times. The sugarcane bagasse matrix was used up to seven times without occurring a substantial loss in the fermentative parameters, mainly productivity, which remained around 0.42 $g.L^{-1}.h^{-1}$ until the end of the seventh cycle. The other matrices presented worse parameters in the second batch and could not be recycled more than three times, when they lost their physical rigidity and wore out (Table 1). The tests carried out by these authors were important to compare lignocellulosic material as a support with the most used material for this purpose, the calcium alginate spheres. Comparing to the standard support (alginate spheres), the sugarcane bagasse proved to be more robust for use in sequential batches.

Other authors already previously mentioned also used sugarcane bagasse as support to cells immobilizations in repeated batches. They showed good reproducibility in the assays, as the authors [58] were able to carry out eight sequential batches without having significant loss in the the kinetic parameters. The repeated-batch experiments were performed in Erlenmeyer flasks, at 50 rpm, for 72 h each with *S. cerevisiae* immobilized on sugarcane bagasse. The immobilized *Saccharum* stalks were retained at the end of each batch, washed with sterile water, and then transferred to a similar volume of fresh medium for the next cultivation cycle, until the parameters dropped to considerably lower levels. In another study

[60], the authors reached 25 repetitions with the same support with immobilized *Sc*. *stipitis*. The authors used Erlenmeyer flasks at an agitation of 250 rpm for 36 h each batch.

In addition to sugarcane bagasse, other materials were also used for cell immobilization in repeated fermentations. Peanut shell cellulosic hydrolysate was used as substrate and the authors carried out repeated fermentations in Erlenmeyer flasks (at 150 rpm, for 84 h) with the *Sc. stipitis* immobilized in sorghum stalks [59]. At the end of each fermentation cycle, the immobilized sorghum stalks were retained, washed with sterile water, and then transferred to a similar volume of fresh medium for the next cultivation cycle. The authors obtained good results, managing to reuse the supports up to ten times without significant loss in the ethanol values produced (up to the 5^{th} with the same ethanol production). *Sc. shehatae* was tested for immobilization in different lignocellulosic materials (terracotta beads, coconut bracts, corn cobs) [62]. Repeated-batch experiments were performed in Erlenmeyer flasks at an agitation of 150 rpm for 168 h. Although the results of repeated fermentations with free and immobilized cells were very similar (for corn cob, for example, an average of 16.7 $g.L^{-1}$ and 17.2 $g.L^{-1}$ of ethanol for free and immobilized cells, respectively), the authors reached five repetitions of 168 h for the process with immobilized cells, which demonstrates the reuse ability of lignocellulosic material in repeated batch fermentations.

Repeated batch fermentations are especially interesting for the production of 2G ethanol, given the already known capacity of yeasts to adapt and improve their performance throughout the cycles. This capacity is widely exploited and brings many advantages in the industrial production of first-generation ethanol and, associated with the immobilization of cells for fermentation of lignocellulosic hydrolysates, it seems promising to improve the process in the presence of inhibitors, as previously discussed. In the support criterion, more rigid materials, such as lignocellulosic materials, were the ones that presented better

performances in terms of process repetition; however, more fragile materials, such as gelatinous matrices, also delivered good improvements results, despite fewer repetitions.

5.3 Continuous fermentation

The continuous process consists of the substrate and fermented flow out of the bioreactor. In this type of process, the culture medium containing inhibitors can be dosed so that the maximum concentration of these compounds in the feed does not impair the performance of the microorganisms, thus facilitating the conversion of sugars into ethanol. In addition, the immobilization facilitates the retention of cells in the reactor when prisoning them in a section, decreasing the possibility of cell washout, which allows a long operating time.

In the work mentioned before [66], *S. cerevisiae* was immobilized in calcium alginate and applied in continuous fermentations of a non-detoxified rice straw hydrolysate in a packed bed reactor. The authors initially fed synthetic medium until a steady state was established; then proceeded to feed a hemicellulosic hydrolysate (inhibitors, in $g.L^{-1}$: acetic acid: 1.92; furfural: 0.52; HMF: 0.46) in different dilution rates. Upon reaching the steady-state, the results showed that the immobilized cells responded with almost no variation in the process parameters, maintaining ethanol production at around 40 $g.L^{-1}$ for 216 h, even with the addition of the inhibitors to the incoming flow. In another work [67], the authors compared the performance of free and immobilized cells of *S. cerevisiae* in continuous fermentations of cellulosic hydrolysate obtained from forest residues (inhibitors, in $g.L^{-1}$: acetic acid: 2.5; furfural: 1.35; HMF: 0.37). The experiments were carried out anaerobically in a bioreactor with 1.0-L working volume at 30 °C and pH 5. Different dilution rates were applied, and the yeasts were trapped in alginate and carboxymethylcellulose supports. In the

cultivation of free cells, the steady-state was reached after 50 h of cellulosic hydrolysate feeding at a dilution rate of 0.1 h^{-1}. The sugar conversion reached 90%, with a productivity of 0.857 $g.L^{-1}.h^{-1}$ and a conversion factor of sugars into ethanol of 0.34 $g.g^{-1}$. However, by increasing the dilution rate by 2-fold, they observed the washing of the reactor cells, and this fact was attributed to the presence of inhibitors in the fermentation medium. The same process was carried out with immobilized cells in a fluidized bed bioreactor, resulting in increased parameters. 95% of the available glucose was consumed, with ethanol productivity and yield of 1.146 $g.L^{-1}.h^{-1}$ and 0.44 $g.g^{-1}$, respectively, both values approximately 1.3-fold higher than those achieved in the process with free cells. In addition, with immobilized cells, the authors were able to apply higher dilution rates, reaching up to 0.5 h^{-1}. It was observed that the higher the dilution rate, the lower the percentage of sugar consumed and the higher the productivity, reaching 4.206 $g.L^{-1}.h^{-1}$ in the process with the highest dilution rate (0.5 h^{-1}). The authors also reported that the experiments lasted about 20 days without damaging the immobilized cells. The fact that immobilization minimized cell wash in continuous process is an important contribution to the 2G process as it allows the process to run longer while keeping the kinetic parameters high.

The influence of alginate entrapment on the tolerance of recombinant *S. cerevisiae* in a fixed bed reactor using non-detoxified sugarcane bagasse hemicellulosic hydrolysate (inhibitors, in $g.L^{-1}$: acetic acid: 6.5; furfural: 0.368; HMF: 0.016 $g.L^{-1}$) as substrate was studied [73]. The authors obtained ethanol yield and productivity of 0.38 $g.g^{-1}$ and 5.7 $g.L^{-1}.h^{-1}$, respectively.

The majority of studies proved that cell confinement improves the residence time of cells in continuous reactors, which prevents the washout that usually happens to free cells. Moreover, as observed in other process operations, such as batch and repeated batch, cell immobilization improves fermentation parameters in continuous systems. However, difficult

substrate diffusion occasioned by support choice may be a challenge when operating in continuous mode, especially if working with a Crabtree negative yeast (main representatives of xylose native consumers) to whom microaerophilia is extremely important for the fermenting metabolism. These microorganisms present the increase in cell production with increased aeration, and the choice to produce ethanol or cells depends on the concentration of O_2 available to the cells (Agbogbo & Coward-Kelly, 2008; Bonan et al., 2020)]. In this case, the selected system can be favorable or unfavorable to cell metabolism. Compacted (Mishra et al., 2016)] and fluidized reactors are commonly studied, and the distribution of supports with the cells immobilized during the process is the main differential. Figure 6 shows how immobilized cells are established within different mode operation bioreactors. In fixed bed reactors (Fig. 6-A), the supports immobilized with the cells are distributed throughout the reactor, not moving significantly in its extension. There are high flow rates of liquid passing through, which facilitates mass transfer between the medium and cells; however, air cannot be introduced directly to avoid bed clogging. The cells are in constant movement in fluidized reactors (Fig. 6-B). As a result, the possibility of clogging is reduced, allowing aeration directly into the reactor, avoiding the supports movement through the extension of the reactor. On the other hand, a stirred tank reactor (Fig. 6-C) can operate with an air inlet, and the supports will be randomly distributed throughout the reactor. However, supports need to be resistant to the shear imposed by the stirring of the impeller, as mentioned before.

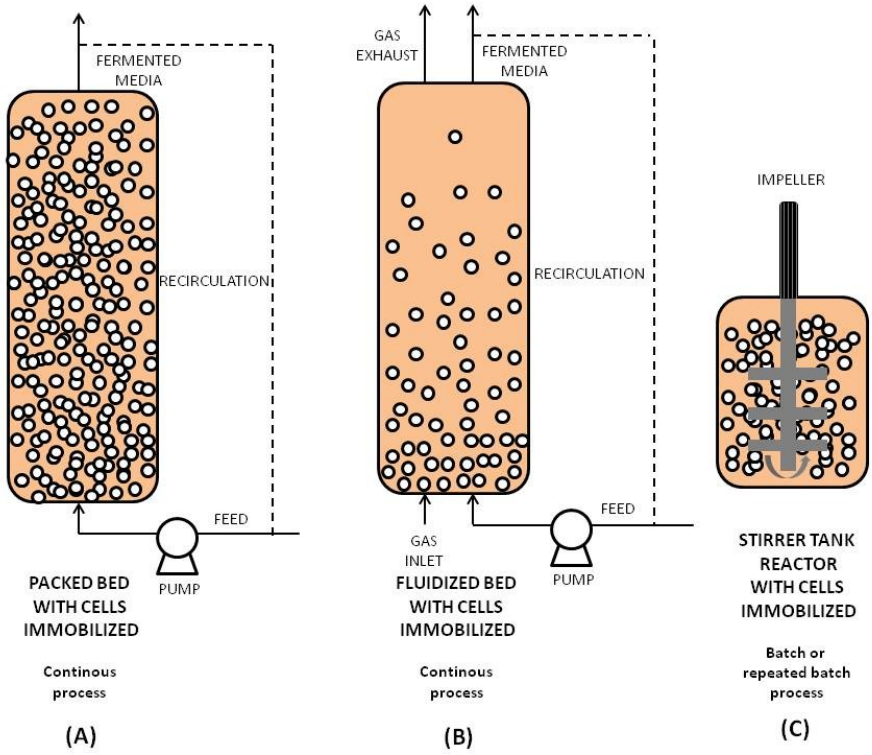

Figure 6. Different operation mode bioreactors with immobilized cells and the distribution of the supports in those systems.

5.4 Co-culture fermentations

It is known that hemicellulosic hydrolysates are composed of hexoses and pentoses. Not all microorganisms have the required metabolic pathways or transporters for the efficient consumption of pentoses, such as *S. cerevisiae*, which is not able to metabolize xylose at convenient rates, unless it is genetically modified to improve this metabolic route [26, 27]. On the other hand, naturally pentose-consuming microorganisms, such as those of the genus *Scheffersomyces, Spathaspora, Schizosaccharomyces,* and *Pachysolen,* suffer from catabolic repression in the presence of glucose [1,25]. Therefore, some authors apply the co-immobilization of two strains to favor the consumption of both sugars in the hemicellulosic hydrolysate or its mixture with cellulosic hydrolysate in a single fermentation stage (please see Figure 2). Table 2 shows the comparative performance of volumetric productivity between free and immobilized for single culture or co-cultures in 2G ethanol.

In this context, authors immobilized *S. cerevisiae* (hexose fermenter) and *P. tannophilus* (pentoses fermenter) in calcium-alginate-chitosan support [56]. They used them separately or in co-culture to ferment corn straw hydrolysate. When individually applied, the immobilized *S. cerevisiae* reached a productivity of 0.621 $g.L^{-1}.h^{-1}$ against 0.252 $g.L^{-1}.h^{-1}$ with free cells. The improvement in productivity with immobilized cells was also achieved with *P. tannophilus* (0.592 $g.L^{-1}.h^{-1}$ against 0.159 $g.L^{-1}.h^{-1}$). These results already demonstrate that the immobilization of these microorganisms provides better performance in the hydrolysate fermentation since this strategy resulted in a productivity increase of 59% and 73% for S. *cerevisiae* and *P. tannphilus*, respectively. When a co-culture of both yeasts was used for fermentation, the immobilized cells reached a productivity of 0.868 $g.L^{-1}.h^{-1}$. On the other hand, for free cells, this value was only 0.257 $g.L^{-1}.h^{-1}$. Comparing the co-culture with single cultures in the immobilized system, productivity increased 43% when the cells were used together inside the support. Although the authors did not describe the amounts of sugars consumed in each process or the concentration of inhibitors on the corn straw hydrolysate, there was a substantial increase in the productivity of the processes with the immobilized cells plus co-culture systems. It suggests that both strategies used together protected the cells inside the sodium-alginate-chitosan matrix against inhibitors favored cellular metabolism for higher ethanol production rates and utilized the sugars efficiently.

In another research, the authors immobilized *Sc. stipitis* (pentose fermenter) and *S. cerevisiae* (hexose fermenter) on Ca-alginate in different proportions of pentose/hexose [76]. The authors used co-culture to ferment wheat straw hydrolysate in continuous fermentation in a fixed bed reactor. At a hydraulic retention time of 0.75 h, the presence of *Sc. stipitis* in co-culture increased the productivity of the process by 10% when compared to the performance of *S. cerevisiae* single culture. Using co-culture in the same proportions of *S. cerevisiae* and *Sc. stipitis*, 92.59% of total glucose and 40.46% of total xylose present in wheat straw

hydrolysate were consumed. It must be remarked that xylose, even in co-culture, was underutilized. It is known that the metabolism of microorganisms that consume xylose is slower than those that consume hexoses. Therefore, the lower consumption of xylose in this study probably occurred due to the low residence time of the substrate in the reactor, which was not enough for consumption of xylose by *Sc. stipitis*.

Table 2 – Performance comparison of volumetric productivity between free and immobilized for single culture and co-cultures in 2G ethanol production systems.

Microorganism	**Support**	**Substrate**	**Volumetric productivity Q_P ($g.L^{-1}.h^{-1}$)**		**Co-culture improvement on Q_P** (for immobilized cells)	**Immobilization improvement on Qp** (compare to free cells)	**Reference**
			Free	Immobilized			
S. cerevisiae	Ca-alginate + chitosan	Corn straw hydrolysate	0.252	0.621	40%	59%	[56]
P. thanophilus			0.159	0.593	46%	73%	
S. cerevisiae + *P. thanophilus*			0.257	0.868	-	70%	
S. cerevisiae ATCC 26602	Ca-alginate	Wheat straw hydrolysate	Not evaluated	8.9	-	-	[76]
S. cerevisiae ATCC 26602 + *Sc. stipitis* DSM 3651				9.8	10%	-	
S. cerevisiae MTCC 3089 + *Sc. stipitis* NCIM 3498	Ca-alginate	Apple pomace hydrolysate	0.957	1.238	-	29%	(Pathania et al., 2017)]

Note: Co-culture improvement on Q_P values was calculated by vertically comparing the values from the volumetric productivity column for the immobilized cells. Immobilization improvement on Q_P values was calculated by comparing the volumetric productivity column values between free and immobilized cells.

S. cerevisiae and *Sc. stipitis* were applied in co-culture (in the same proportion) and immobilized on sodium alginate to ferment apple pomace hemicellulosic hydrolysate [55]. The authors compared the fermentations of immobilized cells with free cells. They observed an increase of almost 30% in the final concentration of ethanol when the process was carried out with the immobilized cells. The fermentation yield was also higher (58% against only 45% for free cells). Unfortunately, the authors did not evaluate the performance of the cultures separately; thus, it was not possible to verify the increase in ethanol production by

applying co-culture. Despite this, the comparison of co-culture of free and immobilized cells suggests that the improvement in fermentation parameters by immobilization was due to the protection of cells in the spheres. However, the authors did not mention the concentrations of sugars or inhibitors present in the hydrolysate so that the improvement with immobilization was justified. The authors reported that the biochemical composition of apple pomace was 36.6% cellulose, 11% hemicellulose, 16.6% pectin, 8% starch, and 19% lignin, which may be able to generate more glucose than xylose.

S. cerevisiae (hexose fermenter), *Sc. shehatae* and *Spathaspora arborariae* (pentose fermenters) were immobilized individually and in co-cultures to ferment peanut shell hemicellulosic hydrolysate (inhibitors, in $g.L^{-1}$: HMF, 0.58; furfural, 0.08; acetic acid, 2.1) [77]. When the cells were applied in the fermentation individually and in free form, viability decreased after 24 h of process. Only 50% of the available glucose was consumed, with no xylose consumption. In contrast, the immobilized cells consumed the sugars and produced ethanol with good yields individually and in co-culture: the single cultures obtained yields of 0.43 $g.g^{-1}$ for *S. cerevisiae*; 0.47 $g.g^{-1}$ for *Sc. shehatae*; 0.38 $g.g^{-1}$ for *Sp. arborariae* and the co-culture attained yields of 0.48 $g.g^{-1}$ for *S. cerevisiae* + *Sc. shehatae* and 0.40 $g.g^{-1}$ for *S. cerevisiae* + *Sp. arborariae*. The association of *S. cerevisiae* + *Sc. shehatae* was the one that reached the highest ethanol yield, in addition to being the one that resulted in the lowest residual xylose, demonstrating that the immobilized co-culture of these microorganisms worked well for the consumption of two sugars. In addition, the immobilization provided the possibility of fermentation for hydrolysate that, with free cells, it was not possible to efficiently ferment.

5.5 Enzymes and cells co- immobilization for 2G ethanol

As previously mentioned, the yeast *S. cerevisiae* cannot efficiently consume xylose. Thus, one of the alternatives for consumption of the pentose sugars present in hemicellulosic hydrolysates by this yeast is to carry out the isomerization of xylose into xylulose and use this carbohydrate in fermentations with *S. cerevisiae* [78]. The enzyme used is xylose isomerase, catalyzing xylose into xylulose in a reversible isomerization. The application of this enzyme co-immobilized with yeast is an alternative to bypass substrate inhibition in enzymatic reaction since the consumption of xylulose by yeast displace the isomerization balance towards producing more carbohydrates. This process of immobilizing cells and enzymes together is called simultaneous isomerization and fermentation (SIF) and can be applied in the different process modes.

In a previously mentioned study, the authors immobilized the enzyme xylose isomerase on chitosan microparticles crosslinking with glutaraldehyde and co-immobilized these particles together with *S. cerevisiae* cells on calcium alginate beads [78]. The authors carried out fermentations in a synthetic xylose medium, and the proposed combined immobilization resulted in the transformation of xylose into xylulose by the enzyme. The released xylulose was continuously transformed into ethanol by the action of *S. cerevisiae*. Later, other researchers [73] used the biocatalysts developed by the previous work [78] to ferment hemicellulosic hydrolysate from sugarcane bagasse (inhibitors, in $g.L^{-1}$: acetic acid: 3.0; furfural: 0.4; HMF: 0.05) in a continuous process. The authors compared the results to a fermentation using synthetic xylose. When the hemicellulosic hydrolysate was used in the reactor with enzymes and immobilized cells, the xylose into xylulose conversion reached 92% and the ethanol productivity reached 1.8 $g.L^{-1}.h^{-1}$. Compared to the synthetic xylose assay, productivity and xylose to xylulose conversion values were very similar, 2.1 $g.L^{-1}.h^{-1}$ and 99%, respectively. The results demonstrate that the strategy of immobilizing both enzyme and cells on the same support is interesting, since practically all the xylose available

from the hemicellulose hydrolysate was isomerized and the yeast *S. cerevisiae* was able to efficiently convert xylulose into ethanol, although the medium contains inhibitors. Despite being an interesting process, scaling up is challenging, since it is necessary to follow the enzymatic activity of the catalysts, and once inside the supports, a reactivation or replacement of the enzymes becomes a challenge, due to the presence of cells which need to be viable to ferment.

6. Production of 2G ethanol employing immobilized cells: conclusions and future perspectives

In general, the benefits obtained through the application of cell immobilization are numerous. The advantages of applying this strategy for fermentations of hemicellulosic hydrolysates seem to be even more relevant. In the presence of inhibitors, immobilized cells of several microorganisms showed a better performance when compared to the free forms, resulting in superior kinetic parameters for ethanol production, regardless of the operating mode. Therefore, when there is the presence of inhibitors in the culture medium, cell immobilization has proven to be a fast and easy application strategy that protects the cells and helps to produce second-generation ethanol.

In addition to helping to overcome the toxic action of acids, furans, and phenolics in the fermentation medium, the immobilization of cells in the production of 2G ethanol provided the possibility and facility of reusing the cells in sequential batches, with some processes reaching a large amount of recycles. It is an interesting approach, as it saves time and inputs for inoculum preparation and allows for better separation and purity of the final product.

The immobilization of microorganisms also allows high cell concentration in the fermentation systems. Besides, it also provides the possibility of using process configurations

that favor the production of 2G ethanol, either by positioning the reactors with the immobilized cells, that is, by the possibility of dosing the quantity of different cells in a co-culture to favor the consumption of the predominant sugar in the hydrolysate composition. In addition, confinement improves the residence time of cells in continuous reactors, as the cells in free form are more likely to be washed out when fermenting hemicellulosic hydrolysates.

The spheres of calcium alginate are the most studied matrix for cell immobilization and improved the production of 2G ethanol, despite presenting challenges inherent to its structure, such as making mass transfer difficult and presenting less resistance compared to other materials. Lignocellulosic materials were shown to be a robust support for cells in the production of 2G ethanol, mainly in terms of reuse, providing a larger amount of fermentation recycles. In addition, lignocellulosic materials have additional advantages such as ease of obtaining, low cost, and, due to their less dense structure, mass transfer between liquid and the adhered cells are facilitated. However, as the interaction between the support and the cells is achieved through a weak bond, it is possible to observe the escape of microorganisms into the medium. In this regard, it is also worth mentioning that the cell growth inside the supports is not an easy parameter to analyze, and probably this is the reason there are no works in the literature that quantify the growth of cells immobilized entrapped in supports. According to the consulted literature, only the superficial loss of cells from the support are discussed, for example, of cells immobilized on alginate beads [78].

According to the consulted literature, there are no reports regarding economic analysis on the implementation of cell immobilization at industrial scale for 2G ethanol. It is important to emphasize that cell immobilization can add costs and inputs to the process, as it requires additional encapsulation or adsorption (or even others) techniques. In this sense, the use of lignocellulosic biomass as a support presents itself an advantage, by reusing materials that are already available in the process [13]. Another important challenge of this technique

that deserves attention, but is rarely described in literature, is the two-phases fermentation process (solids of immobilized cells and liquid media) that requires additional cost to the process due to agitation. Although cell immobilization has several advantages for processes with inhibitors, as widely discussed in this review, little emphasis has been given to the implementation of these processes on a large scale for the production of ethanol. Since there are few biorefineries in the world producing 2G ethanol at commercial scale [80, 81], different operation modes, among which cell immobilization, must be taken into account, since new facilities in this field can be designed for the near future.

Although there are still many obstacles to implementing large-scale 2G ethanol production, cell immobilization has great potential for improving the fermentation process. Process stability, high productivity rates, improved control of contaminants, and improved performance against inhibitors are some of the advantages that cell immobilization adds to the 2G ethanol production, enabling fermentation of lignocellulosic hydrolysates. Notwithstanding, studies with other microorganisms, including the application and cost of scale-up, are needed to consolidate the strategy of cell immobilization for the production of second-generation ethanol. Moreover, it is interesting that the study of different supports may increase cell reuses and increase fermentation kinetic parameters.

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8. Conflict of Interest

The authors declare that they have no conflict of interest.

9. Authors' contributions

LBS, JMS, LEB, and LL search for articles construction of tables, writing the manuscript, structuring the manuscript; DO, AFJ and JLI: writing the manuscript, structuring the manuscript, revision of the manuscript. All the authors read and approved the final manuscript.

10. Chapter 2 references

1. Bonan CIDG, Tramontina R, dos Santos MW, Biazi LE, et al. Biorefinery Platform for *Spathaspora passalidarum* NRRL Y-27907 in the Production of Ethanol, Xylitol, and Single Cell Protein from Sugarcane Bagasse. Bioenergy Res. 2021;
2. Cherubini F, Jungmeier G, Wellisch M, et al. Toward a common classification approach for biorefinery systems. Biofuels, Bioprod Biorefining. 2009 Sep;3(5):534–46.
3. Abud AK de S, Silva CE de F. Bioethanol in Brazil: Status, Challenges and Perspectives to Improve the Production. Bioethanol Production from Food Crops. Elsevier Inc.; 2019. 417–443 p.
4. Robak K, Balcerek M. Current state-of-the-art in ethanol production from lignocellulosic feedstocks. Microbiol Res. 2020;240(June):126534.
5. Chiaramonti D, Prussi M, Ferrero S, et al. Review of pretreatment processes for lignocellulosic ethanol production, and development of an innovative method. Biomass and Bioenergy. 2012;46:25–35.

6. Aditiya HB, Mahlia TMI, Chong WT, et al. Second generation bioethanol production: A critical review. Renew Sustain Energy Rev. 2016;66:631–53.
7. Aslanzadeh S, Ishola MM, Richards T, et al. An Overview of Existing Individual Unit Operations. Biorefineries: Integrated Biochemical Processes for Liquid Biofuels. Elsevier B.V.; 2014. 3–36 p.
8. Taherzadeh MJ, Karimi K. Fermentation inhibitors in ethanol processes and different strategies to reduce their effects. 1st ed. Biofuels. Elsevier Inc.; 2011. 287–311 p.
9. Karagoz P, Bill RM, Ozkan M. Lignocellulosic ethanol production: Evaluation of new approaches, cell immobilization and reactor configurations. Renew Energy. 2019;143:741–52.
10. Zur J, Wojcieszyńska D, Guzik U. Metabolic responses of bacterial cells to immobilization. Molecules. 2016;21(7).
11. Ghorbani F, Younesi H, Esmaeili A, et al. Cane molasses fermentation for continuous ethanol production in an immobilized cells reactor by Saccharomyces cerevisiae. Renew Energy. 2011;36(2):503–9.
12. Milessi TS, Perez CL, Zangirolami TC, Corradini FAS, et al. Repeated batches as a strategy for high 2G ethanol production from undetoxified hemicellulose hydrolysate using immobilized cells of recombinant Saccharomyces cerevisiae in a fixed-bed reactor. Biotechnol Biofuels. 2020;13(1):1–12.
13. Rodríguez-Restrepo YA, Orrego CE. Immobilization of enzymes and cells on lignocellulosic materials. Environ Chem Lett. 2020;18(3):787–806.
14. Navarrete HC, Matín C, García JM. Yeast immobilization systems for second-generation ethanol production: actual trends and future perspectives. Biofuels, Bioprod Biorefining. 2021;1–17.
15. Westman JO, Manikondu RB, Franzén CJ, et al. Encapsulation-induced stress helps

Saccharomyces cerevisiae resist convertible lignocellulose derived inhibitors. Int J Mol Sci. 2012;13(9):11881–94.

16. Westman JO, Ylitervo P, Franzén CJ, et al. Effects of encapsulation of microorganisms on product formation during microbial fermentations. Appl Microbiol Biotechnol. 2012;96(6):1441–54.
17. Ienczak JL, Poletto P, Robl D, et al. Transforming the Lignocellulosic Biomass into High Value-Added Bioproducts. 2021. 21–51 p.
18. Chandel AK, Garlapati VK, Jeevan Kumar SP, et al. The role of renewable chemicals and biofuels in building a bioeconomy. Biofuels, Bioprod Biorefining. 2020;14(4):830–44.
19. Fengel D, Wegener G. Wood. Chemistry, Ultrastucture, Reactions. Walter de Gruyter; 1989. 613 p.
20. Kumar P, Barrett DM, Delwiche MJ, et al. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Ind Eng Chem Res. 2009;48(8):3713–29.
21. McIntosh S, Zhang Z, Palmer J, et al. Pilot-scale cellulosic ethanol production using eucalyptus biomass pre-treated by dilute acid and steam explosion. Biofuels, Bioprod Biorefining. 2016 Jul;10(4):346–58.
22. Roque LR, Morgado GP, Nascimento VM, et al. Liquid-liquid extraction: A promising alternative for inhibitors removing of pentoses fermentation. Fuel 2019;242:775–87.
23. Nakanishi SC, Nascimento VM, et al. Comparative material balances and preliminary technical analysis of the pilot scale sugarcane bagasse alkaline pretreatment to 2G ethanol production. Ind Crops Prod. 2018;120:187–97.
24. Biazi LE, Martínez-Jimenez FD, Bonan CIDG, et al. A differential evolution approach to estimate parameters in a temperature-dependent kinetic model for second generation

ethanol production under high cell density with *Spathaspora passalidarum*. Biochem Eng J.. 2020;161:107586.

25. Azhar SHM, Abdulla R, Jambo SA, et al. Yeasts in sustainable bioethanol production: A review. Biochem Biophys Reports. 2017;10:52–61.

26. Cadete RM, De Las Heras AM, Sandström AG, et al. Exploring xylose metabolism in *Spathaspora* species: XYL1.2 from Spathaspora passalidarum as the key for efficient anaerobic xylose fermentation in metabolic engineered *Saccharomyces cerevisiae*. Biotechnol Biofuels. 2016;9(1):1–14.

27. Jansen MLA, Bracher JM, Papapetridis I, et al. *Saccharomyces cerevisiae* strains for second-generation ethanol production: from academic exploration to industrial implementation. FEMS Yeast Res. 2017;17(5):1–20.

28. Nogué VS, Karhumaa K. Xylose fermentation as a challenge for commercialization of lignocellulosic fuels and chemicals. Biotechnol Lett. 2015;37(4):761–72.

29. Pereira I de O, dos Santos ÂA, Gonçalves DL, et al. Comparison of *Spathaspora passalidarum* and recombinant *Saccharomyces cerevisiae* for integration of first- and second-generation ethanol production . FEMS Yeast Res. 2021;21(6):1–14.

30. Canilha L, Chandel AK, Milessi TSS, et al. Bioconversion of sugarcane biomass into ethanol: An overview about composition, pretreatment methods, detoxification of hydrolysates, enzymatic saccharification, and ethanol fermentation. J Biomed Biotechnol. 2012;2012.

31. Palmqvist E, Hahn-Hägerdal B. Fermentation of lignocellulosic hydrolysates. I: Inhibition and detoxification. Bioresour Technol. 2000;74(1):17–24.

32. Almeida JR, Modig T, Petersson A, et al. Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. J Chem Technol Biotechnol. 2008;8:340–9.

33. Jönsson LJ, Alriksson B, Nilvebrant N-O. Bioconversion of lignocellulose: inhibitors and detoxification. Biotechnol Biofuels. 2013;6(16):1–10.

34. Dionísio SR, Santoro DCJ, Soares LB, et al. Second-generation ethanol process for integral use of hemicellulosic and cellulosic hydrolysates from diluted sulfuric acid pretreatment of sugarcane bagasse. Fuel. 2021;304:121290.

35. Martins LH da S, Rabelo SC, da Costa AC. Effects of the pretreatment method on high solids enzymatic hydrolysis and ethanol fermentation of the cellulosic fraction of sugarcane bagasse. Bioresour Technol. 2015;191:312–21.

36. Nakasu PYS, Ienczak LJ, Costa AC, et al. Acid post-hydrolysis of xylooligosaccharides from hydrothermal pretreatment for pentose ethanol production. Fuel. 2016;185:73–84.

37. Rocha GJM, Martin C, Soares IB, et al. Dilute mixed-acid pretreatment of sugarcane bagasse for ethanol production. Biomass and Bioenergy. 2011;35(1):663–70.

38. Zhang Z, O'Hara IM, Doherty WOS. Pretreatment of sugarcane bagasse by acid-catalysed process in aqueous ionic liquid solutions. Bioresour Technol. 2012;120:149–56.

39. Pereira RS, Mussatto SI, Roberto IC. Inhibitory action of toxic compounds present in lignocellulosic hydrolysates on xylose to xylitol bioconversion by *Candida guilliermondii*. J Ind Microbiol Biotechnol. 2011;38(1):71–8.

40. Mills TY, Sandoval NR, Gill RT. Cellulosic hydrolysate toxicity and tolerance mechanisms in *Escherichia coli*. Biotechnol Biofuels. 2009;2(1):1–11.

41. Zhang Q, Huang H, Han H, et al. Stimulatory effect of in-situ detoxification on bioethanol production by rice straw. Energy. 2017;135:32–9.

42. Biazi LE, Santos SC, Kaupert Neto AA, et al. Adaptation Strategy to Increase the Tolerance of *Scheffersomyces stipitis* NRRL Y-7124 to Inhibitors of Sugarcane

Bagasse Hemicellulosic Hydrolysate Through Comparative Studies of Proteomics and Fermentation. Bioenergy Res. 2021;

43. Morales P, Gentina JC, Aroca G, et al. Development of an acetic acid tolerant *Spathaspora passalidarum* strain through evolutionary engineering with resistance to inhibitors compounds of autohydrolysate of Eucalyptus globulus. Ind Crops Prod. 2017;106:5–11.

44. Chen Y, Stabryla L, Wei N. Improved acetic acid resistance in *Saccharomyces cerevisiae* by overexpression of the WHI2 gene identified through inverse metabolic engineering. Appl Environ Microbiol. 2016;82(7):2156–66.

45. Soares LB, Bonan CIDG, Biazi LE, et al. Investigation of hemicellulosic hydrolysate inhibitor resistance and fermentation strategies to overcome inhibition in non-*saccharomyces* species. Biomass and Bioenergy. 2020;137:105549.

46. Cassells B, Karhumaa K, Nogué VS, et al. Hybrid SSF/SHF Processing of SO2 Pretreated Wheat Straw—Tuning Co-fermentation by Yeast Inoculum Size and Hydrolysis Time. Appl Biochem Biotechnol. 2017;181(2):536–47.

47. Farias D, Maugeri Filho F. Co-culture strategy for improved 2G bioethanol production using a mixture of sugarcane molasses and bagasse hydrolysate as substrate. Biochem Eng J. 2019;147:29–38.

48. Westman JO, Mapelli V, Taherzadeh MJ. Flocculation Causes Inhibitor Tolerance in *Saccharomyces cerevisiae* for Second-Generation Bioethanol Production. Appl Environ Microbiol. 2014;80(22):6908–18.

49. Tang Y, An M, Liu K, et al. Ethanol production from acid hydrolysate of wood biomass using the flocculating yeast *Saccharomyces cerevisiae* strain KF-7. Process Biochem. 2006;41(4):909–14.

50. Ylitervo P, Doyen W, Taherzadeh MJ. Fermentation of lignocellulosic hydrolyzate

using a submerged membrane bioreactor at high dilution rates. Bioresour Technol. 2014;164:64–9.

51. Lee WG, Park BG, Chang YK, et al. Continuous ethanol production from concentrated wood hydrolysates in an internal membrane-filtration bioreactor. Biotechnol Prog. 2000;16(2):302–4.

52. Ishola MM, Ylitervo P, Taherzadeh MJ. Co-Utilization of glucose and xylose for enhanced lignocellulosic ethanol production with reverse membrane bioreactors. Membranes (Basel). 2015;5(4):844–56.

53. Kashid M, Ghosalkar A. Critical factors affecting ethanol production by immobilized *Pichia stipitis* using corn cob hemicellulosic hydrolysate. Prep Biochem Biotechnol. 2018;48(3):288–95.

54. Perez CL, Milessi TS, Sandri JP, et al. Unraveling continuous 2G ethanol production from xylose using hemicellulose hydrolysate and immobilized superior recombinant yeast in fixed-bed bioreactor. Biochem Eng J. 2021;169.

55. Pathania S, Sharma N, Handa S. Immobilization of co-culture of *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* in sodium alginate for bioethanol production using hydrolysate of apple pomace under separate hydrolysis and fermentation. Biocatal Biotransformation. 2017;35(6):450–9.

56. Zhang W, Bai A, Chen X, Wei G. Ethanol production from lignocelluloses hydrolyzates with immobilized multi-microorganisms. Energy Sources, Part A Recover Util Environ Eff. 2012;34(13):1206–12.

57. Behera S, Kar S, Mohanty RC, Ray RC. Comparative study of bio-ethanol production from mahula (Madhuca latifolia L.) flowers by *Saccharomyces cerevisiae* cells immobilized in agar agar and Ca-alginate matrices. Appl Energy 2010;87(1):96–100.

58. Chandel AK, Lakshmi Narasu M, Chandrasekhar G, et al. Use of *Saccharum*

spontaneum (wild sugarcane) as biomaterial for cell immobilization and modulated ethanol production by thermotolerant *Saccharomyces cerevisiae* VS3. Bioresour Technol. 2009;100(8):2404–10.

59. Gajula C, Chandel AK, Konakalla R,et al. Fermentation of groundnut shell enzymatic hydrolysate for fuel ethanol production by free and sorghum stalks immobilized cells of *Pichia stipitis* NCIM 3498. Int J Chem React Eng. 2011;9.

60. Balderas BSY, Muñiz BO, Rodríguez JG, et al. Ethanol production by *Pichia stipitis* immobilized on sugarcane bagasse. Bioethanol. 2016;2(1):44–50.

61. Singh A, Sharma P, Saran AK, et al. Comparative study on ethanol production from pretreated sugarcane bagasse using immobilized *Saccharomyces cerevisiae* on various matrices. Renew Energy. 2013;50:488–93.

62. Yuvadetkun P, Reungsang A, Boonmee M. Comparison between free cells and immobilized cells of *Candida shehatae* in ethanol production from rice straw hydrolysate using repeated batch cultivation. Renew Energy. 2018;115:634–40.

63. Abbi M, Kuhad RC, Singh A. Bioconversion of pentose sugars to ethanol by free and immobilized cells of *Candida shehatae* (NCL-3501): Fermentation behaviour. Process Biochem. 1996;31(6):555–60.

64. Talebnia F, Niklasson C, Taherzadeh MJ. Ethanol production from glucose and dilute-acid hydrolyzates by encapsulated *S. cerevisiae*. Biotechnol Bioeng. 2005;90(3):345–53.

65. Zhao J, Xia L. Ethanol production from corn stover hemicellulosic hydrolysate using immobilized recombinant yeast cells. Biochem Eng J. 2010;49(1):28–32.

66. Mishra A, Sharma AK, Sharma S, et al. Lignocellulosic ethanol production employing immobilized *Saccharomyces cerevisiae* in packed bed reactor. Renew Energy. 2016;98:57–63.

67. Talebnia F, Taherzadeh MJ. In situ detoxification and continuous cultivation of dilute-acid hydrolyzate to ethanol by encapsulated *S. cerevisiae*. J Biotechnol. 2006;125(3):377–84.

68. Antunes FAF, Santos JC, Chandel AK, et al. Repeated batches as a feasible industrial process for hemicellulosic ethanol production from sugarcane bagasse by using immobilized yeast cells. Cellulose. 2019;26(6):3787–800.

69. Willaert RG. Cell immobilization and its applications in biotechnology: Current trends and future prospects. Ferment Microbiol Biotechnol Third Ed. 2007:313–68.

70. Verbelen PJ, De Schutter DP, Delvaux F, et al. Immobilized yeast cell systems for continuous fermentation applications. Biotechnol Lett. 2006;28(19):1515–25.

71. Westman JO, Engineering B. Ethanol production from lignocellulose using high local cell density yeast cultures. 2014.

72. Amorim HV, Lopes ML, Oliveira JVC, et al. Scientific challenges of bioethanol production in Brazil. Appl Microbiol Biotechnol. 2011;91(5):1267–75.

73. Milessi TS, Silva CR, Moraes GS, et al. Continuous 2G ethanol production from xylose in a fixed- bed reactor by native *Saccharomyces cerevisiae* strain through simultaneous isomerization and fermentation. 2020;7:4429–42.

74. Bonan CIDG, Biazi LE, Dionísio SR, et al. Redox potential as a key parameter for monitoring and optimization of xylose fermentation with yeast *Spathaspora passalidarum* under limited-oxygen conditions. Bioprocess Biosyst Eng. 2020;43(8):1509–19.

75. Agbogbo FK, Coward-Kelly G. Cellulosic ethanol production using the naturally occurring xylose-fermenting yeast, *Pichia stipitis*. Biotechnol Lett. 2008;30(9):1515–24.

76. Karagöz P, Özkan M. Bioresource Technology Ethanol production from wheat straw

by *Saccharomyces cerevisiae* and Scheffersomyces stipitis co-culture in batch and continuous system. 2014;158:286–93.

77. Hickert LR, Cruz MM, Dillon AJP, et al. Fermentation kinetics of acid-enzymatic soybean hull hydrolysate in immobilized-cell bioreactors of *Saccharomyces cerevisiae, Candida shehatae, Spathaspora arborariae*, and their co-cultivations. Biochem Eng J. 2014;88:61–7.

78. Silva CR, Zangirolami TC, Rodrigues JP, et al. Enzyme and Microbial Technology An innovative biocatalyst for production of ethanol from xylose in a continuous bioreactor. Enzyme Microb Technol. 2012;50(1):35–42.

79. Silveira JM da, Soares LB, Karina C, et al. Immobilization of *Spathaspora passalidarum* NRRL Y - 27907 in Calcium Alginate Aiming the Production of Second - Generation Ethanol. 2021;(0123456789).

80. Patel A, Shah AR. Integrated lignocellulosic biorefinery: Gateway for production of second generation ethanol and value added products. J Bioresour Bioprod 2021;6(2):108–28.

81. Santos LV, Grassi MCB, Gallardo JCM, et al. Second-Generation Ethanol: The Need is Becoming a Reality. Ind Biotechnol. 2016;12(1):40–57.

Chapter 3: “Evaluating the production of second-generation ethanol by S*pathaspora passalidarum* immobilized on sugarcane bagasse”

This chapter presents tests with different treatments on sugarcane bagasse to identify which modification on the surface would promote the adhesion of *S. passalidarum* cells to the solid support. These results were presented at the XXIII National Symposium of Bioprocesses - SINAFERM in August 2022.

Then, batch fermentation of the cells immobilized on the solid was carried out using hemicellulosic hydrolysate carbon obtained after acid pretreatment of sugarcane bagasse as a carboun source. These results were added to the initial immobilization tests and published in the international journal *Bioenergy Research*. The study was published in July 24, 2023. https://doi.org/10.1007/s12155-023-10634-2

EVALUATING THE PRODUCTION OF SECOND-GENERATION ETHANOL by *Spathaspora passalidarum* IMMOBILIZED ON SUGARCANE BAGASSE

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ABSTRACT

Second-generation (2G) ethanol is obtained from the processing of lignocellulosic biomasses, such as sugarcane bagasse. However, several obstacles need to be overcome to make the industrial fermentation of the sugarcane bagasse hydrolysates viable, such as the time and capital expense of the process when compared with first-generation (1G) ethanol (produced by sugary and starchy raw materials), the inhibitors generated and the process scaling-up. The intrinsic release of inhibitors compounds during the deconstruction of lignocellulosic material into sugars is one of the biggest challenges in the fermentation step. Cell immobilization can be used as a strategy to protect microorganisms from these inhibitory compounds. Immobilization can add costs to the process; therefore, the use of materials already available in the ethanol production is interesting from an economic point of view. In this sense, the objective of this study was to evaluate the immobilization of *Spathaspora passalidarum* in raw, alkaline and acid pretreated sugarcane bagasse. In addition, the fermentation of hemicellulosic hydrolysate (HH) from acid pretreatment of sugarcane bagasse was evaluated by immobilized and the free cells. Fermentation by *S. passalidarum* immobilized on sugarcane bagasse obtained growth and product yield factor of $Y_{P/S}$ (0.35 g/g) and $Y_{X/S}$ (0.43 g/g), respectively, against $Y_{P/S}$ (0.27 g/g) and $Y_{X/S}$ (0.086 g/g) for the cell-free assay. After 24 h of fermentation it was possible to reach a productivity of 0.153 g/(L.h) and a yield of 68.37% with immobilized cells, which were also superior to the fermentation with free cells (0.148 g/(L.h) and 54%). Based on the results, it was possible to verify that sugarcane bagasse can be used not only as an effective source of carbon for the production of 2G ethanol, but also as a support for cell immobilization, increasing the productivity of the process

Keywords: alkaline pretreatment; sulfuric acid pretreatment; non-conventional yeast; volumetric productivity; xylose uptake rate

1. Introduction

The concern about the energetic and environmental crisis, arising from dependence on fossil fuels, brings the importance of developing sustainable energy production such as liquids biofuels (bioethanol and biodiesel) and gaseous biofuels (biohydrogen and methane) [1]. The first-generation ethanol (1G) is produced by raw materials such as sugarcane, beet, and maize. The production of 1G ethanol, the most used liquid biofuel in the world [2], raised around 15 million gallons in 2022 according to U.S. Energy Information Administration. The second generation (2G) ethanol produced from lignocellulosic raw materials, such as sugarcane bagasse, is an interesting alternative to replace the use of fossil fuels and it is a way of sustainable development within the context of biorefineries. The lignocellulosic biomass is composed mainly by cellulose, hemicelluloses and lignin, while its processing, for the production of 2G ethanol, occurs through four main stages: i) biomass fractionation (pretreatment); ii) enzymatic hydrolysis; iii) fermentation; and iv) distillation [2].

Pretreatment is the first stage of the 2G ethanol production process and aims to modify the crystalline structure of plant biomass, separating it into fractions of interest for the biorefinery platform. For the production of 2G ethanol, pretreatment increases the accessibility and biodegradability of cellulose and hemicelluloses for later steps, enzymatic hydrolysis and fermentation [3], in addition some compounds that negatively affect these subsequent steps can be formed, for example, the inhibitors furfural and hydroxymethylfurfural. Several types of physical-chemical pretreatment are reported in the literature, and one of the most used is the pretreatment with dilute sulfuric acid, which consists in the solubilization of hemicelluloses, ensuring high recovery of pentoses in the liquid fraction (hemicellulosic hydrolysate, HH) and obtaining a pulp rich in cellulose and lignin content (cellulignin) [1, 4]. The solid fraction (cellulignin) can be subjected to a hydrolysis step to depolymerize the cellulose chains into glucose monomers, which can be

fermented by conventional yeasts, like *Saccharomyces cerevisiae,* while the hemicellulosic fraction, rich in xylose [5], needs microorganisms able to consume pentoses, such as the yeast *Spathaspora passalidarum*. Results in literature pointed that this pretreatment achieved around 89.5% of hemicellulosic solubilization, in which 82% was recovered as monomeric sugars (xylose and arabinose) [6].

S. passalidarum was isolated from the gut of wood beetles in 2006, and this yeast reproduces asexually by budding [4, 7]. *S. passalidarum* has shown great potential in the production of 2G ethanol, mainly due to its natural ability to consume xylose at higher rates and to present better performance in HH when compared to other xylose fermenters [8, 9]. However, the fermentation of HH for 2G ethanol production presents challenges, like the time and capital expense of the process when compared with 1G ethanol, the inhibitors generated in pretreatments, the scale-up process, among others. In the fermentation step, the main one is the presence of inhibitor compounds, such as furfural (from cellulose), 5-hydroxymethylfurfural and organic acids (from hemicelluloses) and phenolic compounds (from lignin), which affect directly cell metabolism during fermentation, decreasing the efficiency and productivity of ethanol [8, 10]. Challenges like these must be overcome to enable the production of 2G ethanol, and cell immobilization is a promising alternative for this purpose.

Cell immobilization is a biotechnological technique in which biocatalysts are fixed in a matrix that limits their movement, increasing their stability, facilitating the reuse of these microorganisms, protecting them against pH variations and contaminations, in addition to promote increased functionality in continuous systems of production [11]. Flocculation, adsorption on surfaces and encapsulation are the most used techniques to immobilize microorganisms [12]. Flocculation is a natural process of immobilization that occurs when cells adhere to each other in a non-sexual and reversible way through cell wall proteins called

adhesins or floculins [13, 14]. Immobilization by flocculation is strongly impacted by the temperature and pH of the process [14]. Surface adsorption occurs through van de Waals forces, ionic interactions and/or hydrogen bonds between the cell wall and the support surface. The effectiveness of microorganisms adhesion to the support is also influenced by the broth characteristics when this immobilization technique is used [11]. The encapsulation technique consists of applying a porous matrix synthesized in the form of spheres around the cells. Hydrogels such as Ca-alginate, k-carrageenan and agar are used. Encapsulation shows disadvantages such as limiting the diffusion of nutrients, metabolites and oxygen. [15, 16].

Furthermore, due to diffusion and concentration gradients within the support materials, immobilized yeast cells are more tolerant to ethanol and exhibit a lower degree of substrate inhibition compared to free cells [17, 18]. In 2G ethanol, the improvement in the kinetic parameters of the hydrolysate fermentation with inhibitors by using immobilized cells may be related to the low diffusion of inhibitory compounds through the beads and the ability to convert them into less toxic substances. *S. cerevisiae* cells on the surface of the beads were able to transform assembled compounds, leaving a less inhibitory medium for the inner cells [16]. In another study, the same authors also observed that sphere confinement induced responses to environmental stress increased overall stress tolerance by proteomic analysis, which may be related to better performance in environments containing inhibitory compounds [19]. It is important to emphasize that in 2G ethanol the costs of all steps (pretreatment, enzymatic hydrolysis, fermentation, and distillation) to obtain the final product are very important factors to make possible the implementation of the process on an industrial scale. In view of this, the addition of an immobilization step could increase both the costs with inputs, as well as with processes and energy, and this could be a disadvantage about this technique.

Cell immobilization can be done mainly by adsorption or entrapment, with immobilization by passive adhesion to surfaces being preferred due to problems related to diffusion [20]. The most used matrix as support for cells is calcium alginate, due to its biocompatibility and speed in the gelling process [21]. However, several authors pointed to the use of lignocellulosic materials, such as sugarcane bagasse, as a support for cell immobilization, claiming the success of these systems in the biotechnological, pharmaceutical and environmental fields [11]. Furthermore, lignocellulosic supports have desirable physical and mechanical properties, for example, they can resist to several fermentation cycles and allow cell growth due its porous matrix [22]. In addition, they are ecologically correct, renewable, biodegradable and non-toxic [23], which facilitates their use and implementation on an industrial scale [11, 24, 25].

Sugarcane bagasse has great potential as a support for cell immobilization in the production of 2G ethanol, because it is a material capable of overcoming challenges present in use of others types of supports; keeping the protection of microorganisms against the inhibitors present in the HH, a factor that leads to a higher yield in the production of ethanol [11]. In this study, the immobilization of the yeast *S. passalidarum* in raw sugarcane bagasse and pretreated with sulfuric acid and alkaline was compared with the objective of evaluating which kind of support retains a greater amount of cells and which results in a higher rate of cell immobilization. Furthermore, fermentations of the HH obtained from sugarcane bagasse were carried out comparing free and immobilized cells in sugarcane bagasse. To the best of our knowledge this is the first time that *S. passalidarum* was immobilized in sugarcane bagasse and tested in fermentations of sugarcane bagasse hemicellulosic hydrolysate.

2. Material and methods

2.1 Microorganism, seed culture and cell propagation

The yeast used in the study was *Spathaspora passalidarum* NRRL Y-27907 [7]. The microorganism was stored in a cryotube containing YPDX medium, containing (g/L) yeast extract (10.0), peptone (20.0) dextrose (10.0) and xylose (10.0), and glycerol (volume proportion 1:1) in a freezer at -80 ºC. In order to obtain high cell density, *S. passalidarum* was submitted to pre-inoculum, inoculum and cell propagation stages.

The microorganism from the stock culture was transfer to a 250 mL Erlenmeyer flask containing 100 mL of the pre-inoculum medium YPD containing (in g/L) yeast extract (10.0), peptone (20.0) and dextrose (20.0). The flasks were incubated in an orbital shaker (Tecnal TE-424), at constant temperature of 30 °C and shaking at 150 rpm for 24 h. After the pre-inoculum time, the entire volume was centrifuged (1200 xg for 10 min, Kasvi, K14-0815C) and resuspended in sterile water in an amount corresponding to 10% (25 mL) of the total inoculum volume and was aseptically transferred to 500 mL Erlenmeyer flasks containing 250 mL of medium. Medium was the same described by Silva et al.[26] and Santos et al. [27] composed of (g/L): xylose, (12.0); glucose, (1.32); urea, (2.3); yeast extract, (3.0) and $MgSO_4.7H_2O$, (1.0), and incubated under the same conditions of pre-inoculum for 24 h.

Propagation step was carried out in a benchtop bioreactor (New Brunswick BioFlo 115), with a total capacity of 7 L and an initial working volume of 2.8 L of the medium described by Santos et al. [27] composed of (g/L): dilute sugarcane molasses (30.0); urea (5.0); and KH_2PO_4 (2.0). The cells obtained in the inoculum step were centrifuged (1200 xg for 10 min, Kasvi, K14-0815C) and the pellet cells was dispersed in sterile water, in a volume corresponding to 10% (280 mL) of the initial bioreactor volume. Propagation took place at 30 °C and pH 6.0; with initial agitation of 200 rpm and initial aeration of 0.1 vvm. The control of agitation and aeration was established to keep the concentration of dissolved oxygen above 50% in relation to the saturation of air at atmospheric pressure [27].

Propagation was divided into two stages: the first step was carried out in batch mode, until the yeast reached the exponential growth phase (around 12 h of process). Next, a pulse of nutrients KH_2PO_4 (2.0 g/L) and urea (5.0 g/L) was performed and a fed batch operation mode was started with a supply in the feed flow of 3 g/(L.h) of pure sugarcane molasses (467.21 g/L), in order to establish linear growth (for approximately 12 h). At the end of the propagation, the entire volume was centrifuged at 3000 xg for 20 min (Avanti J-30I Beckman Coulter) and the pellet was resuspended in sterile water. The yeast cream obtained was quantified in terms of cell concentration and stored under refrigeration (4 °C) in a sterile flask for later use [27].

2.2 Sugarcane bagasse support preparation

Sugarcane bagasse (*Saccharum officinarum*) from a local market (São Miguel do Oeste, Santa Catarina, Brazil) was used in this study. This material has some differences in relation to a bagasse from a mill, mainly for juice extraction. The biomass was washed in running water to remove dirties and manually cut into pieces of approximately 2.0 cm in length with a scissor. Bagasse moisture was measured with a moisture analyzer (CEM-Smart Turbo). The material was subjected to different pretreatments. The first one was the alkaline pretreatment that was carried out in an autoclave at 121 °C with a NaOH solution of 1.5 %, w/v, in a solid/liquid ratio of 1:15 (dry basis) for 30 min, adapted from Nakanishi et al. [9]. The last one was an acid pretreatment carried out in a 316 L stainless steel reactor (Metalquim, 5.0 L) at 130 °C in a H_2SO_4 solution of 0.5%, v/v, in a solid/liquid ratio of 1:10 (dry basis) for 15 min, adapted from Roque et al. [28]. Solid-liquid separation of both pretreatments was performed by filtration and the solid fraction was washed separately with water until neutral pH and oven dried at 105 °C for 24 h.

2.3 *S. passalidarum* immobilization on sugarcane bagasse

After sugarcane bagasse support preparation, pretreated and raw materials were added separately in 500 mL Erlenmeyer flasks containing 250 mL of 1.0 g/L peptone solution, in a solid:liquid ratio 1:20 (dry basis) for *S. passalidarum* immobilization. *S. passalidarum* cells (8.0 g/L) obtained from propagation were added into the flasks, which were incubated in an orbital shaker, at 30 °C and 100 rpm for 24 h, following methodology adapted from Singh et al. [22]. As a standard assay, a flask under the same conditions but without pretreated or raw sugarcane bagasse was incubated with free cells. Samples were collected for quantification of free cell dry weight, in order to establish the migration of yeasts from the media to the solid support. All assays, in triplicate, were subjected to immobilization kinetic analysis, and the support with best performance for *S. passalidarum* immobilization was used for subsequent optimization of immobilization and HH fermentations.

2.4 Optimization of *S. passalidarum* immobilization on sugarcane bagasse

Two parameters were optimized for *S. passalidarum* immobilization: time and cell concentration. For time, the 1st. order kinetic Equation 1 was used to describe the adsorption behavior of cells on the support over 48 h and predict the equilibrium cell concentration.

$$q_X = q_E * [1 - e^{(-kt)}] \qquad (1)$$

Where q_x is the concentration of cells on the support (mg.g^{-1}), q_E is the concentration of cells on the support at equilibrium (mg/g), t is the time (h), k is the model constant (1/h).

With the aim to improve cell adhesion into the bagasse, an upgrade of cell concentration from 8 to 15 g/L (based on the higher cell concentration according the consulted literature [29]) was carried out.

2.5 Fermentation

The aforementioned acid pretreatment (please see **Sugarcane bagasse support preparation)** was performed to obtain HH used in fermentations. After solid-liquid separation, the HH (liquid fraction of H_2SO_4 pretreatment) had its pH corrected to 5.0 with 1.0 M NaOH; it was centrifuged at 3000 xg for 20 min (Avanti J-30I Beckman Coulter) to remove suspended material, followed by sterilization at 115 °C, 10 min, and was stored under freezing until use.

After characterization, HH was diluted around 5 times to result in approximately 1.5 g/L of acetic acid, based on Soares et al. [8] that previously described acetic acid tolerance of *S. passalidarum*, and it was added to the fermentation flask that contained immobilized cells. The media was supplemented with (in g/L): urea (2.3) (main nitrogen source), yeast extract (nitrogen and micronutrients source) (10.0) and $MgSO_4.7H_2O$ (nutrient essential) (1.0)[10]. The same initial concentration of cells (~3.8 g/L) was applied in the fermentation with immobilized cells and with free cells, allowing a comparison between the fermentation processes. The batch fermentations were carried out in Erlenmeyer flasks, incubated at 30 ºC for 24 h, under agitation at 150 rpm in an orbital shaker.

2.6 Analytical methods

The chemical composition (cellulose, hemicellulose, lignin) of raw sugarcane bagasse and the solid fraction after acid and alkaline pretreatments was determined according to the Laboratory Analytical Protocol of the National Renewables Energy Laboratory (NREL) [30].

The percentage of cellulose and hemicellulose were calculated considering the extractives content of the sugarcane bagasse (Equations 2 and 3).

$$\boldsymbol{C}\ (\%) = \left\{\frac{[(0.9\times C_{Glu})+(0.95\times C_{Cel})+]\times V}{M_{BE}}\right\} \times \left\{\left[1-\left(\frac{E}{100}\right)\right]\times 100\right\} \quad (2)$$

$$\boldsymbol{H}\ (\%) = \left\{\frac{[(0.88\times C_{X})+(0.88\times C_{A})+(0.72\times C_{AA})]\times V}{M_{BE}}\right\} \times \left\{\left[1-\left(\frac{E}{100}\right)\right]\times 100\right\} \quad (3)$$

where C (%) is the cellulose content of biomass; C_{Glu} is the glucose concentration (g/L); C_{Cel} is the cellobiose concentration (g/L); H (%) is the hemicellulose content of the biomass; C_X is the xylose concentration (g/L); C_A is the arabinose concentration ($g.L^{-1}$); C_{AA} is the acetic acid concentration (g/L); V is the final filtration volume (L); M_{BE} is the mass of biomass free of extractives (g); E is the total extractive content, expressed in mass per mass percentage (% m/m).

The concentration of free cells was measured by dry cell weight. A volume of 1.0 mL samples was collected in triplicate and centrifuged at 3.000 xg (Eppendorf HsiangTai CN2160) for 5 min in previously weighed 2.0 mL microtubes. For the samples of immobilization process, the supernatant was discarded, while the cell pellet was resuspended with distilled water and centrifuged again under the same conditions. The precipitate of washed cells was subjected to drying in an oven at 105 °C for 24 h. After the drying time, the microtubes were weighed on an analytical balance (Shimadzu ATY224) and the cell concentration was calculated by mass difference.

Samples of the fermentation process were collected every 6 h and centrifuged to separate possible solids in suspension, while the supernatant was frozen for further analysis of metabolites. The supernatant was thawed, filtered (PVDF Millex 0.22 μm) and injected (10 μL) into the High Performance Liquid Chromatography (HPLC) system (Shimadzu LC-

20A) to analyze the concentrations of glucose, xylose, acetic acid, ethanol and xylitol. Compounds were separated at 50 °C with Aminex HPX 87H (300 μm 7.8 mm, BIO-RAD, Hercules, CA) using 5 mM H_2SO_4 as mobile phase at a rate of 0.6 mL/min. For furfural and hydroxymethylfurfural analysis, the samples are injected into the HPLC system, using diode array detector (DAD). Compounds were separated at 30 °C with Nova-Pak C18 (4 um, Waters) using acetonitrile/water (1:8 with 1% acetic acid) as mobile phase at a rate of 0.8 mL/min.

The morphologies of sugarcane bagasse with *S. passalidarum* cells immobilized in the initial and final times of fermentation were analyzed using scanning electron microscopy (SEM). Sample preparation consisted of submersion in glutaraldehyde 25 g/L for 2 h for cells fixation. Afterwards, the samples were dipped in ethanol solution with increasing concentrations (10, 20, 30, 50, 70, 80, 90 and 100% - 3 times) for 20 min each [31]. Subsequently, the samples were stored in desiccators for drying for 24 hours and after, covered with a thin layer of gold. The micrographs of the immobilized cells were analyzed with magnification between 25 and 1500X and electron beam with energy of 8kV (Jeol, model JSM – 6390 LV).

2.7 Kinetic parameters

Fermentation parameters were calculated to evaluate the performance of *S. passalidarum* in ethanol production. The yield (η, %) of each fermentation is given by Equation 4. The volumetric productivity of ethanol (Q_P, g/(L.h)) was calculated according to Equation 5. Yield factors (observable) of reducing sugars in ethanol ($Y_{P/S}$, g/g) and in cell biomass ($Y_{X/S}$, g/g) were calculated according to Equations 6 and 7, respectively.

$$\eta = \frac{P_f - P_0}{S_0 - S_f} \times \frac{100}{0.511} \quad (4)$$

$$Q_P = \frac{P_f - P_0}{t_f} \quad (5)$$

$$Y_{\frac{P}{S}} = \frac{P_f - P_0}{S_0 - S_f} \quad (6)$$

$$Y_{\frac{X}{S}} = \frac{X_f - X_0}{S_0 - S_f} \quad (7)$$

Where P_f and P_0 represent the final and initial concentrations of ethanol, respectively, S_f and S_0 represent the final and initial concentrations of reducing sugars, respectively, X_f and X_0 represent the final and initial concentrations of cells, respectively, and t_f the fermentation time. The value 0.511 represents the stoichiometric yield of carbon sources (glucose and xylose) in ethanol (g/g).

To determine the kinetic parameters of fermentation, profile of cell growth (X), glucose (S_g), xylose (S_x) and ethanol (P) were plotted *versus* time. Specific growth rate (μ_X, Equation 8), specific uptake rate (μ_S, Equation 9) and specific production rate (μ_P, Equation 10) were also evaluated.

$$\mu_X = \frac{1}{X} . \frac{dX}{dt} \quad (8)$$

$$\mu_S = \frac{1}{X} . \left(-\frac{dS}{dt}\right) \quad (9)$$

$$\mu_P = \frac{1}{X} . \frac{dP}{dt} \quad (10)$$

3. Results and discussion

3.1 *S. passalidarum* immobilization profile on sugarcane bagasse

Figure 1 shows the results of immobilization of *S. passalidarum* in raw sugarcane bagasse (a) pretreated with NaOH (b) and pretreated with H_2SO_4 (c) (compositions are presented in Table 1). First, when analyzing the values of the standard assay (control, without sugarcane bagasse), it is possible to notice that the concentration of free cells in the medium showed some oscillation over time, but remained around 8.0 g/L over the 24 h of process (Fig. 1 a, b and c, black bars). This data demonstrates that the immobilization medium and the agitation conditions did not favor neither the growth nor the cell disruption of the yeast, since the cell concentration neither decreased nor increased substantially. The lack of cell growth may be related to the low concentration of the carbon source in the medium and/or low agitation applied (100 rpm), which provides low aeration of the medium, important nutrient for *S. passalidarum* growth, since it is a negative *Crabtree* microorganism [32].

It was also possible to observe that the concentration of free cells in the medium decreased over the time for the assays with sugarcane bagasse (Fig. 1 a, b and c, gray bars). Indeed, it is possible to note the decrease of free cells concentration in the medium containing sugarcane bagasse, mainly by the migration of these cells from the liquid to the solid support. Immobilization ratio is presented in Figure 1d and it is observed that the sugarcane bagasse after the acid pretreatment was able to retain more cells (60.03 mg/g) than the raw sugarcane bagasse (37.56 mg/g) or pretreated with NaOH (43.66 mg/g).

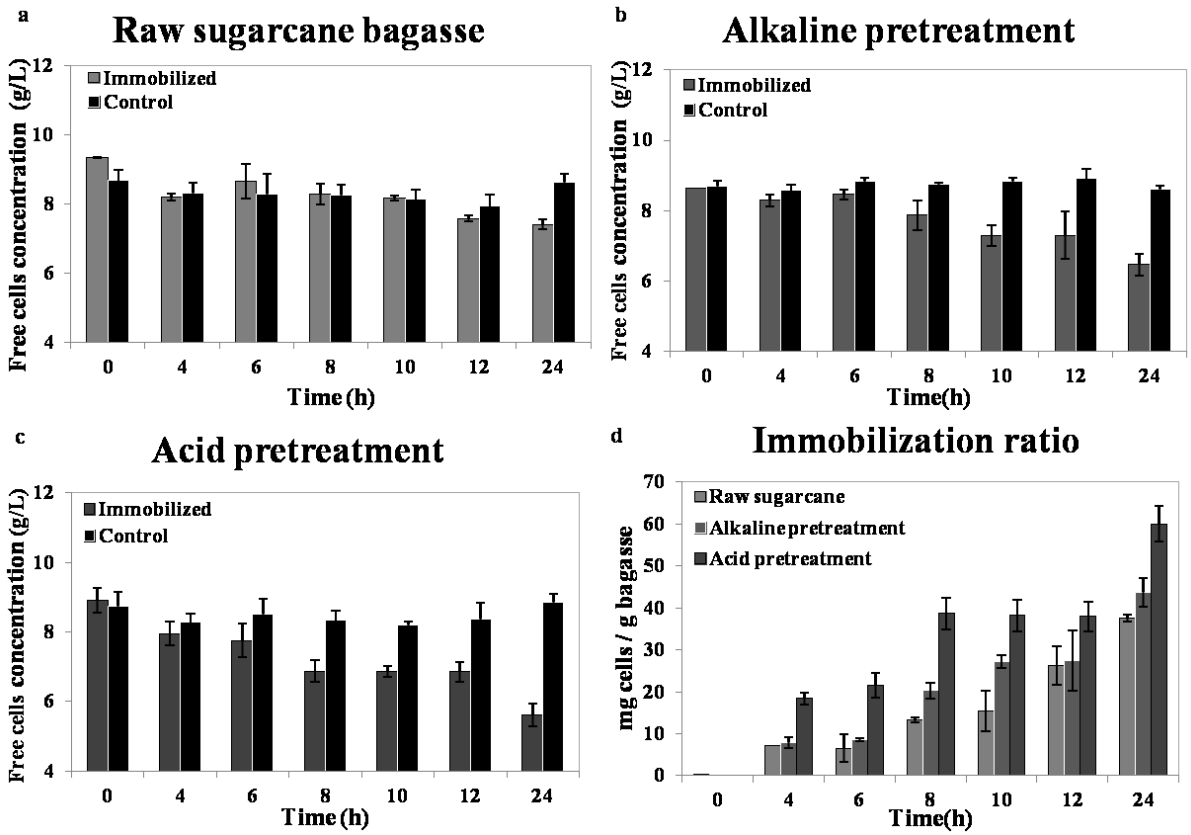

Fig. 1: Results for the immobilization of *S. passalidarum* on raw sugarcane bagasse (a), pretreated with NaOH (b), pretreated with H_2SO_4 (c), and immobilization ratio (d). The grays bars are related to immobilized assays and the black bars are the control assays for each condition.

Table 1 - Chemical composition of the raw sugarcane bagasse and solid fraction after pretreatment with acid and alkaline (dry mass).

	Compounds					
Biomass type	**Cellulose (%)**	**Hemicellulose (%)**	**Lignin (%)**	**Extractives (%)**	**Ashes (%)**	**Others (%)**
Raw	44.79 ± 1.15	29.75 ± 0.70	17.71 ± 0.96	3.75 ± 0.39	0.98 ± 0.07	3.01
Pretreated with acid	53.43 ± 0.14	8.88 ± 0.10	27.70 ± 0.04	n.d.	n.d.	9.99
Pretreated with alkaline	51.98 ± 0.77	21.05 ± 0.33	12.45 ± 0.41	n.d.	n.d.	14.52

n.d = not detected

Figure 2 shows the micrographs of the materials used for the immobilization step. In Fig. 2a, 2b and 2c, refer to raw sugarcane bagasse, it is possible to observe under different magnifications, the chains referring to the crystalline structure of the untreated material. Fig. 2c, 2d and 2e show the structure of sugarcane bagasse after the alkaline pretreatment, which smoothes it out, resulting in a material that is flatter than that seen in Fig. 2g, 2h and 2i, which refer to the material after acid pretreatment. It is possible to observe that the surface of the material after this treatment is more porous, which probably facilitates the adhesion of cells to the solid, forming shelters where the cells can accommodate. These results also agree with those previously reported by Godoy-Salinas [33], which evaluated different pretreatments in sugarcane bagasse to immobilize *S. cerevisiae* and observed grea er adhesion of the microorganism to the material after the acid pretreatment. Associated with these observations, the increase of the lignin content in the pretreated material with H_2SO_4 in relation to raw and alkaline pretreated material (Table 1) seems to favor cell adhesion in this support, probably by the chemical interaction between cell-support. However additional studies should be performed to confirm this hypothesis.

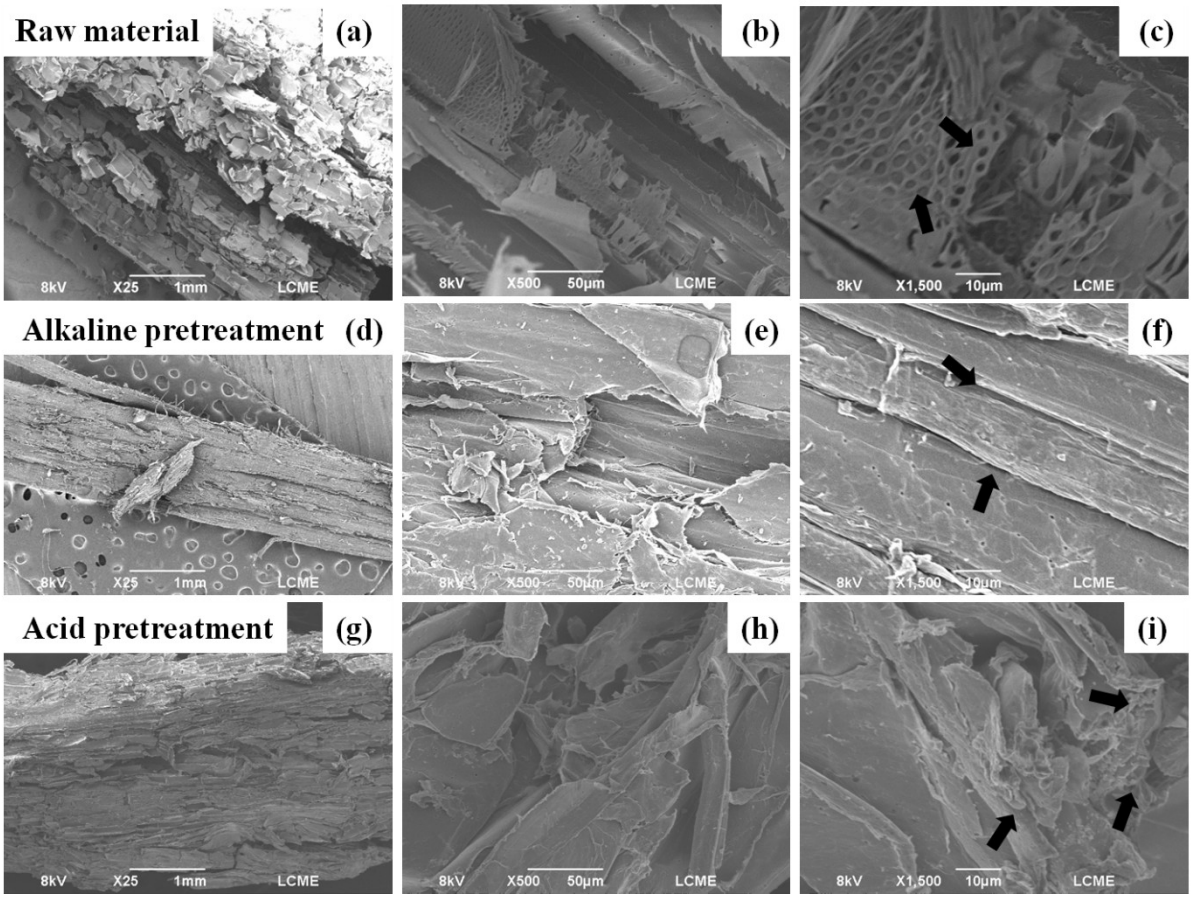

Fig. 2: Micrographs of the materials used for the immobilization step (a) raw material 25X, (b) raw material 500X, (c) raw material 1500X - the crystalline structure evidenced by the black arrows, (d) alkaline pretreatment 25X, (e) alkaline pretreatment 500X, (f) alkaline pretreatment 1500X - the flatter surface evidenced by the black arrows, (g) acid pretreatment 25X, (h) acid pretreatment 500X and (i) acid pretreatment 1500X - the shelters structures evidenced by the black arrows.

Heris Anita and coworkers [34] immobilized *S. cerevisiae* (2.0 $g.L^{-1}$) in sugarcane bagasse pretreated in water at 121 °C for 30 min and the results showed 5.0 mg of immobilized cells per gram of bagasse at the end of 24 h of immobilization. The pretreatment of sugarcane bagasse in water reported by the same authors [34] resulted in less cell adhesion to the support compared to all assays of the present work. In another work [29], different inoculum size (0.27; 0.55; 1.10; 2.75; 6.00; 8.25 and 15.00 g/L) were evaluated in the process

of immobilization of *Scheffersomyces stipitis* in sugarcane bagasse (without pretreatment, only sterilized) [29]. The authors performed immobilization kinetics and the results showed that both contact time and inoculum size influenced the immobilization process. For the highest cell concentrations, maximum cell adhesion to the support was obtained after 18 h of the process. When the authors tested similar cell concentration value (8.25 g/L) to that used herein (8.0 g/L), around 100.0 mg of cells per gram of sugarcane bagasse were immobilized, a value higher than that observed in the present study.

3.2 Optimization of *S. passalidarum* immobilization on sugarcane bagasse

Immobilization by surface adsorption takes place through two successive steps. The first is characterized by the prevalence of physical-chemical interactions in the system; the cells are transported and bonded to a solid support, by contact with the surface. Subsequently there may be attachment of the adjacent suspended cells to the cells of the first layer and/or the desorption of cells and buds to the medium or to a new place on the support. Changes in environmental ionic strength, pH, temperature, along with physical stresses such as agitation and abrasion can induce cellular desorption. Another limitation of adsorption cell carriers is the possibility of non-specific binding of materials within the fermentation medium. The thickness of the biofilm attached to the surface of these carriers varies from a monolayer of cells to a layer of cells one millimeter thick [35, 36].

With the results obtained from acid pretreated sugarcane bagasse immobilization, Equation 1 was used to predict the amount of immobilized cells in 48 h of immobilization process. Figure 3 the linearization of the immobilized cells/sugarcane bagasse ratio (mg cell/g bagasse) versus time and the 1st order kinetic model obtained (Figures 3a and 3b, respectively). By 1st order kinetic model it was possible to estimate the amount of cell immobilized per sugarcane bagasse pretreated with H_2SO_4 (mg cell/g bagasse) in 48 h. The

result obtained by this equation (q_E value was estimated at 80.0 mg/g, k= 0.0597 1/h and R^2 = 0.9603) showed that the immobilization process for 48 h could improve immobilization ratio in the support from 60.0 (Figure 1d) to 75 mg/g (Figure 3).

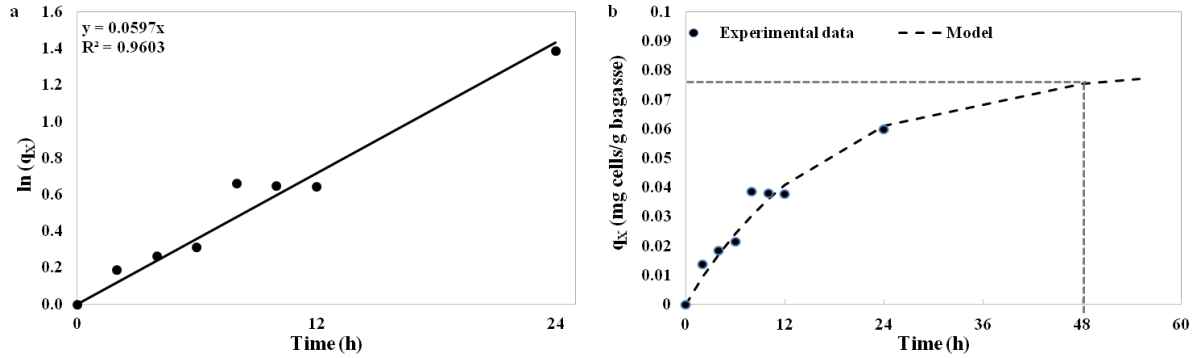

Fig. 3: (a) Linearization of the ratio (mg cell/g bagasse) time (ln (q_X) *versus* time) to obtain 1st order kinetic equation and (b) the amount of cell immobilized per sugarcane bagasse pretreated with H_2SO_4 (mg cell.g/bagasse) by the obtained 1st order kinetic equation model. The dotted lines represent the data estimated by the model.

The results of ~16 g/L inoculum assay are shown in Figure 4. The concentration of free cells in the medium decreased over the time for the assay with sugarcane bagasse (Fig. 4, black bars) indicating that it is possible to increase the concentration of cells in the inoculum to provide greater cell adhesion to the solid.

In this sense, considering the increase in the cell concentration value predicted by the adsorption model for 48 h, and the results with an increase of inoculum size, the cellular immobilization procedure of *S. passalidarum* in sugarcane bagasse pretreated with H_2SO_4 was performed once again, following the same protocol previously described in Material and methods, adapted from Singh et al.[22] during 48 h and 15 g/L from inoculum was used. Experimental result of this immobilization process was 77.6 mg/g of immobilized cells.

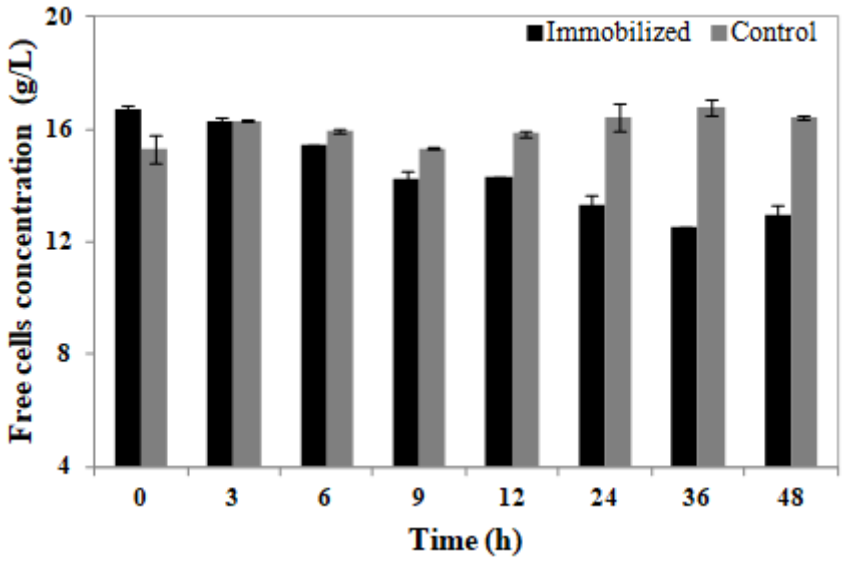

Fig. 4: Results for the immobilization of *S. passalidarum* on acid pretreated sugarcane bagasse with 16 g/L inoculum

3.3 Comparison of free and immobilized *S. passalidarum* for 2G ethanol production

The composition of acid hemicellulosic hydrolysate (HH) obtained after acid pretreatment was (in g/L): cellobiose (2.51), glucose (17.79), xylose (46.44), arabinose (4.23), acetic acid (5.38), furfural (0.015) and hydroxymethylfurfural (0.04). These values are similar to those obtained in study of Dionísio et al. [6] that used similar pretreatment conditions.

Figure 5 illustrates the profile of glucose, xylose, acetic acid, ethanol, xylitol, free and immobilized cells for 2G ethanol production from HH and the specific rates of cell growth (μ_x), sugars consumption (μ_{Sg} for glucose and μ_{Sx} for xylose) and ethanol production (μ_P) for free and immobilized cells fermentations.

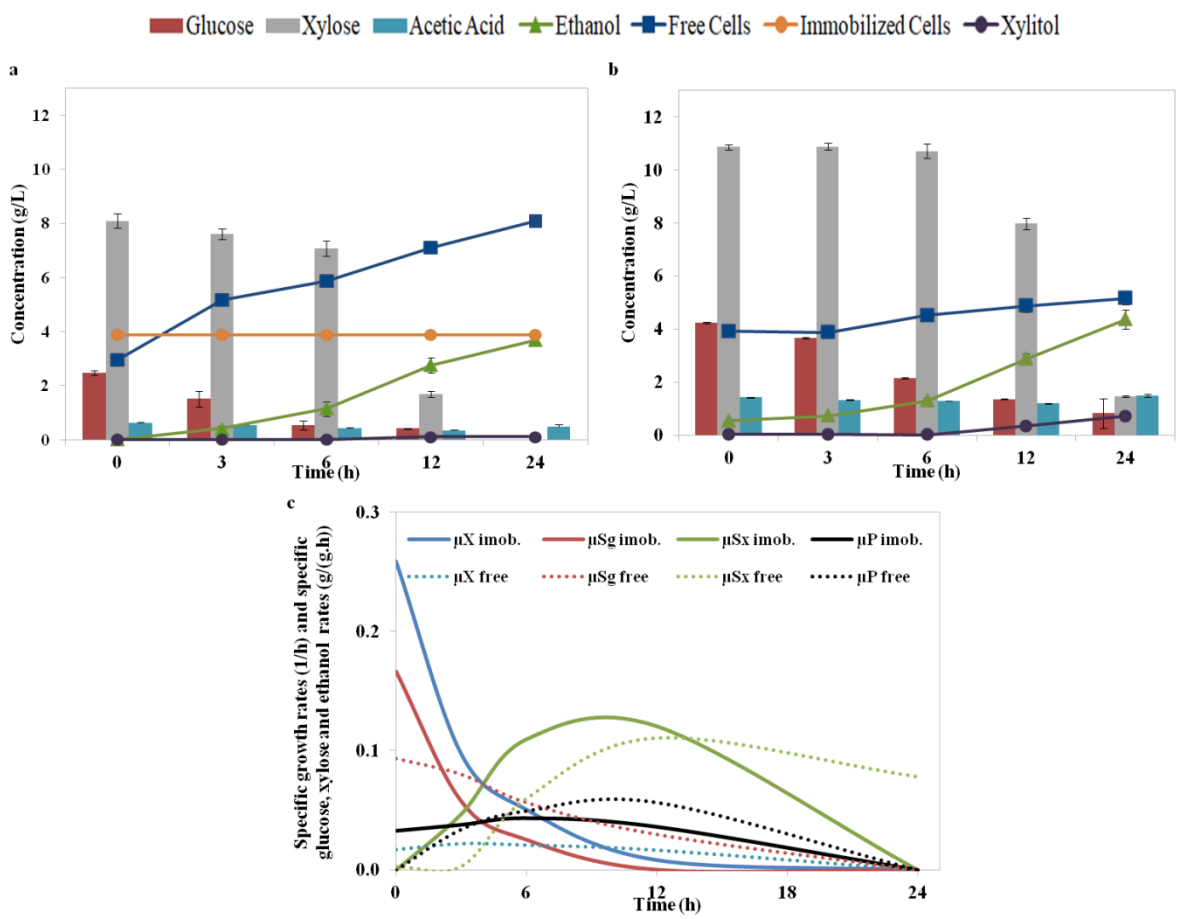

Fig. 5: Fermentation kinetics for (a) immobilized cells on sugarcane bagasse pretreated with H_2SO_4, (b) free cells to produce 2G ethanol from HH and (c) xylose (μ_{Sx}) and glucose (μ_{Sg}) specific uptake rates and cells (μ_x) and product (μ_P) specific production rates for immobilized cells (full lines) and free cells (dotted lines) fermentations to produce 2G ethanol from HH.

Is possible to observe that the initial concentration of sugars was diluted with the addition of the immobilized *S. passalidarum* in the fermentation medium (Figure 5a). This phenomenon was also observed by other authors [37, 38]. The solid addition (support with cells) not only diluted the sugars, but also diluted the inhibitors, resulting concentrations of 0.6 g/L acetic acid, 0.006 g/L furfural and no concentration of hydroxymethylfurfural was detected in HPLC. On the other hand, free cells fermentation presented initial concentrations of 1.43 g/L acetic acid, 0.003 $g.L^{-1}$ furfural and 0.008 g/L of hydroxymethylfurfural.

The specific rates of cell growth (μ_x), sugars consumption (μ_{Sg} for glucose and μ_{Sx} for xylose) and ethanol production (μ_P) for free and immobilized cells fermentations had different behaviors, as shown in Figure 5c. It is possible to notice that the specific uptake rate of xylose was slightly higher for the immobilized cells fermentation. Through the profile of μ_{Sg} and μ_{Sx} of both the fermentations it is possible to note concomitant consumption of glucose and xylose This behavior is observed from 3 h for the free cells' fermentation and from the beginning of the fermentation for immobilized cells (Fig. 5c), and mainly when the glucose concentration was below 4 g/L (please see Figure 5a and 5b). Similar behavior was observed by Martinez-Jimenez et al., (2021) [4] in fermentations with *S. passalidarum.*

In the fermentation with immobilized cells there was an expressive cell growth, as it is possible to observe in Fig. 5b, blue line. This cell growth suggests that the addition of the solid may help with the solubilization of oxygen since contributes with agitation in Erlenmeyer flasks, making this nutrient more available, favoring cell growth. This same behavior was already observed in other studies with solid supports in the fermentation medium [21]. The ethanol production profile was similar for both fermentations, reaching around 4 g/L at the end of 24 h of process.

Table 2 shows the kinetic parameters obtained from the present study in comparison with that reported in the literature for the strategy of immobilization in HH fermentation. According to data from Table 2*, S. cerevisiae* cells were immobilized on sugarcane bagasse pretreated with NaOH (2.75%) and fermented with a concentrated cellulosic hydrolysate to produce ethanol. Fermentation of 50 g/L of sugars took place for 72 h, with maximum ethanol production in 36 h, when 0.42 g/g of ethanol conversion, 0.42 g/(L.h) productivity and 84% yield were attained [22]. The work by Balderas et al. [29] used sugarcane bagasse pretreated with sulfuric acid as a support for the immobilization of *S. stipitis*. Comparison between free and immobilized cells was performed with sugarcane HH containing 2.4 g/L of

acetic acid and 0.8 g/L of furfural. After 60 h of fermentation, the test with immobilized cells resulted in 1.85 times higher yield than the process with free cells. Productivity doubled when immobilized cell were applied. In another study, *S. stipitis* was immobilized in calcium alginate spheres and used to ferment HH from corn waste [39]. The HH contained 4.5 g/L of acetic acid. The authors compared the performance of fermentations with immobilized cells with free cells and the results showed that immobilization resulted in higher xylose consumption rate than in the process with free cells. Productivity increased from 0.31 to 0.51 g/(L.h) [39]. In another study the sorghum bagasse was also used to immobilize *S. stipitis* in and used to ferment cellulosic hydrolysate from peanut shells [40]. The authors compared the fermentation performance with that of free cells. The results showed that the immobilization favored the production of ethanol for the process with the immobilized cells, which reached the maximum ethanol concentration of 20.45 g/L, yield of 0.47 g/g and productivity of 0.243 g/(L.h).

Table 2 - Comparison of kinetic parameters for HH fermentations performed in the present study and in the literature for immobilized cells.

Microorganism	Support	Substrate	Free Cells				Immobilized Cells				Increaseo on yield (%)	Increase on Q_P (%)	Reference
			$Y_{X/S}$ (g/g)	$Y_{P/S}$ (g/g)	Yield (%)	Q_P (g/(L.h))	$Y_{X/S}$ (g/g)	$Y_{P/S}$ (g/g)	Yield (%)	Q_P (g/(L.h))			
S. passalidarum NRRL Y-7124	Sugarcane bagasse	Sugarcane bagasse hydrolysate	0.086	0.274	54%	0.148	0.431	0.349	68%	0.153	27.4	3.4	This work
S. cerevisiae	Sugarcane bagasse	Sugarcane bagasse hydrolysate	-	-	-	-	-	0.42	84%	0.420	-	-	[22]
S. stipitis ACL 2.1	Sugarcane bagasse	Sugarcane bagasse hydrolysate	-	0.14	27%	0.01	-	0.26	51%	0.02	85.7	100.0	[29]
Adapted *S. stipitis* PSA30	Ca- alginate beads	Corn cob hydrolysate	-	0.41	80%	0.31	-	0.43	84%	0.51	4.9	64.5	[39]
S. stipitis NCIM 3498	Sorghum bagasse	Soybean hull hydrolysate	-	0.44	86%	0.212	-	0.47	92%	0.243	6.8	14.6	[40]
Thermotolerant *S. cerevisiae* VS3	Sugarcane bagasse	Sugarcane bagasse hydrolysate	-	0.41	80%	0.405	-	0.434	85%	0.601	5.9	48.4	[41]

Although the immobilization process is widely used in ethanol production, there is a lack of literature investigating this strategy for *S. passalidarum* using sugarcane bagasse as a support. Silveira et al.[21] immobilized *S. passalidarum* in alginate spheres, using a synthetic medium that simulated sugarcane bagasse hydrolysate pretreated with dilute sulfuric acid, in terms of sugar composition (56.87 g/L of xylose; 17.49 g/L of glucose), but without addition of inhibitors. As a result, in 24 h of fermentation, 82.7% of sugars were consumed; 0.22 g/g $Y_{P/S}$; 0.55 g./(L.h) productivity and 42.31% yield.

Figure 6 shows micrographs of cells immobilized on sugarcane bagasse at the initial and final times of fermentation. In Fig. 6a it is possible to visualize a general aspect of the sample and the place that was enlarged in the subsequent images. Fig. 6b shows the sample with 500X magnification, where the pores of the sugarcane bagasse structure richly filled with *S. passalidarum* cells can be seen. The magnifications of 1000 and 1500X in Fig. 6c and 6d show in cells adhered to the surface of the pores. In Fig. 6e is show the general aspect of the final fermentation time sample micrograph. The enlargements show that after 24 h of fermentation the cells are still adhered to the surface of the sugarcane bagasse, using pores of different sizes (Fig 6f (500x); g (1000x) and h (1500x)).

It should be noted that samples taken during fermentation do not return to the process, since they need to be treated to obtain micrographs (as described in item Analytical methods). Therefore, the sample observed in Figure 6 a-d is not the same sample observed in Figure 6 e-h. The difference between the number of cells observed between the samples is not only a result of the fermentation time, but also of the heterogeneous structure of the support itself and randomness in the sampling.

In this sense, the results of this study shows that was possible to immobilize *S. passalidarum* strain using sugarcane bagasse as a support, and the acid pretreatment allowed high cell content in the support. This material can also used in the sequential fermentations, since the micrographs showed the cells still adhered to the support at the end of the fermentation performed. The high yield and productivity in fermentations of HH, in relation to the free cells fermentation also demonstrate the improvement of process with the use of cells immobilization strategy.

Despite the advantages mentioned above, the heterogeneity of the material can become one of the challenges of the immobilization of microorganisms in lignocellulosic biomasses, being possible to occur differences in cell adhesion between batches of immobilization, which can make this process difficult to control on an industrial scale, in addition to making it difficult to clean the tanks of a 2G ethanol industrial facilities.

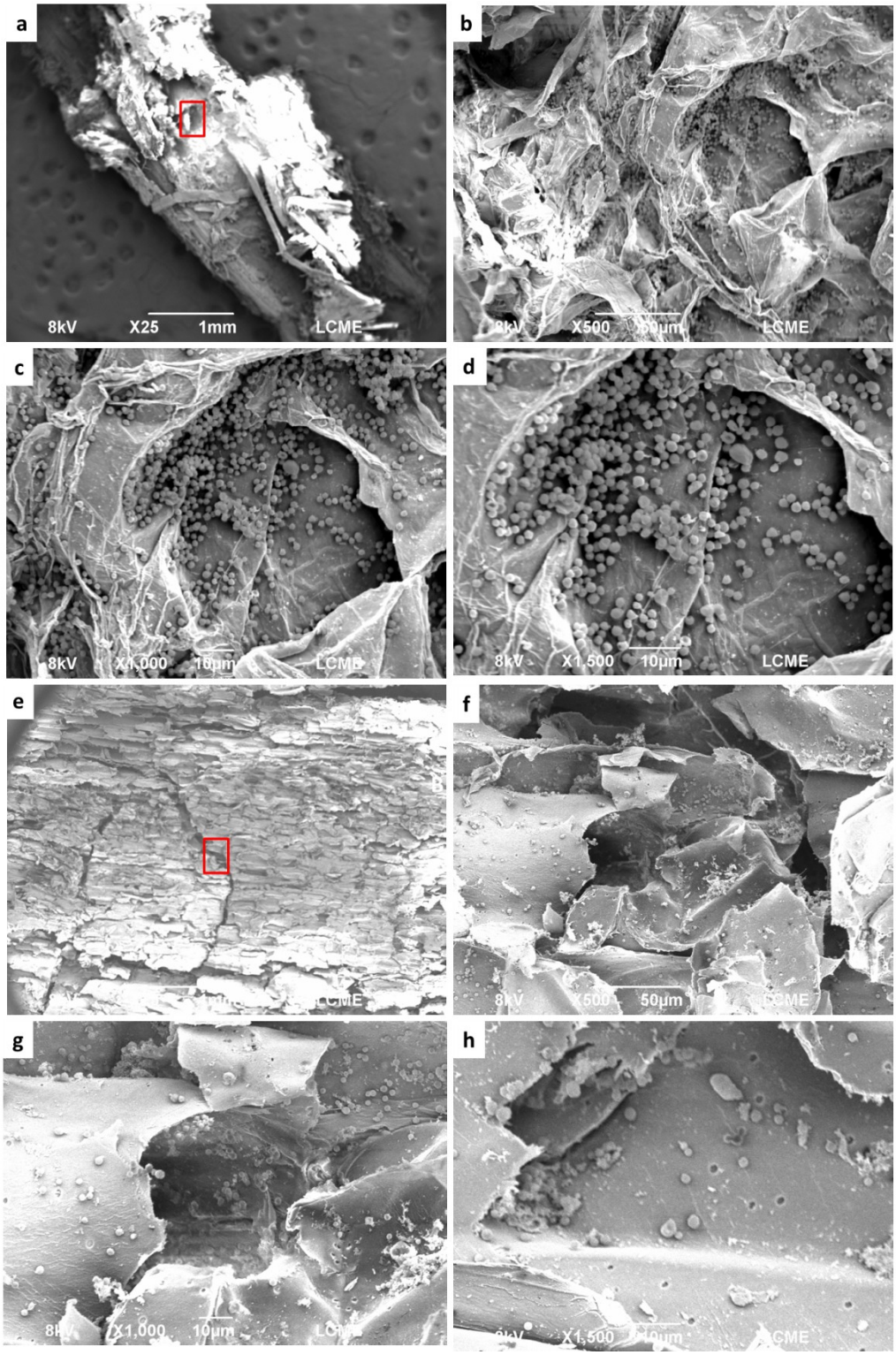

Fig. 6: Morphology of acid pretreated sugarcane bagasse with *S. passalidarum* cells immobilized in the initial fermentation time. (a) 25X; (b) 500; (c) 1000; and (d) 1500X; and at the final fermentation time (e) 25X; (f) 500X; (g) 1000X; and (h) 1500X.

4. Conclusions

This study demonstrated that it is possible to use sugarcane bagasse as a support for the immobilization of *Spathaspora passalidarum* cells. The acid pretreatment of the support was the one that shows best adhesion of yeast to the solid. Comparison with the performance of free cells showed that the protection given to cells inside the solid is effective, resulting in better fermentation parameters. Sugarcane bagasse proved to be efficient not only to obtain the carbon source used for ethanol production, but also as a strategic support in improving the productivity of the process itself.

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6. Authors contributions

LBS, MBSJ; JMS; LLN; MYM: Investigation, conceptualization and experimental execution; data accuracy; writing – original draft. ROH, EZ, MFA, BUS and ACC: experimental execution; data accuracy, writing – review & editing. JLI and AFJ: Writing – review & editing, supervision, project administration, funding acquisition, conceptualization.

7. Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that influenced the work reported in this paper.

8. Chapter 3 references

1. Robak K, Balcerek M (2020) Current state-of-the-art in ethanol production from lignocellulosic feedstocks. Microbiol Res 240:126534. https://doi.org/10.1016/j.micres.2020.126534

2. Naik SN, Goud V V., Rout PK, Dalai AK (2010) Production of first and second generation biofuels: A comprehensive review. Renew Sustain Energy Rev 14:578–597. https://doi.org/10.1016/j.rser.2009.10.003

3. Soltanian S, Aghbashlo M, Almasi F, et al (2020) A critical review of the effects of pretreatment methods on the exergetic aspects of lignocellulosic biofuels. Energy Convers Manag 212:112792. https://doi.org/10.1016/j.enconman.2020.112792

4. Martinez-Jimenez FD, Neitzel T, Biazi LE, et al (2021) Exploiting the Non-conventional Yeast *Spathaspora passalidarum* as a Platform for Hemicellulosic

Hydrolysate Conversion into Bioproducts: a Mini Review. Bioenergy Res. https://doi.org/10.1007/s12155-021-10257-5

5. Rocha GJ de M, Martin C, Soares IB, et al (2011) Dilute mixed-acid pretreatment of sugarcane bagasse for ethanol production. Biomass and Bioenergy 35:663–670. https://doi.org/10.1016/j.biombioe.2010.10.018

6. Dionísio SR, Santoro DCJ, Soares LB, et al (2021) Second-generation ethanol process for integral use of hemicellulosic and cellulosic hydrolysates from diluted sulfuric acid pretreatment of sugarcane bagasse. Fuel 304:121290. https://doi.org/10.1016/j.fuel.2021.121290

7. Nguyen NH, Suh S-O, Marshall CJ, Blackwell M (2006) Morphological and ecological similarities: wood-boring beetles associated with novel xylose-fermenting yeasts, *Spathaspora passalidarum* gen. sp. nov. and *Candida jeffriesii* sp. nov. Mycol Res 110:1232–1241. https://doi.org/10.1016/J.MYCRES.2006.07.002

8. Soares LB, Bonan CIDG, Biazi LE, et al (2020) Investigation of hemicellulosic hydrolysate inhibitor resistance and fermentation strategies to overcome inhibition in non-*saccharomyces* species. Biomass and Bioenergy 137:105549. https://doi.org/10.1016/j.biombioe.2020.105549

9. Nakanishi SC, Soares LB, Biazi LE, et al (2017) Fermentation strategy for second generation ethanol production from sugarcane bagasse hydrolyzate by *Spathaspora passalidarum* and *Scheffersomyces stipitis*. Biotechnol Bioeng 114:2211–2221. https://doi.org/10.1002/bit.26357

10. Lima CS, Neitzel T, de Oliveira Pereira I, et al (2021) Effect of the Sugarcane Bagasse Deacetylation in the Pentoses Fermentation Process. Bioenergy Res. https://doi.org/10.1007/s12155-020-10243-3

11. Rodríguez-Restrepo YA, Orrego CE (2020) Immobilization of enzymes and cells on lignocellulosic materials. Environ Chem Lett 18:787–806. https://doi.org/10.1007/s10311-020-00988-w

12. Zur J, Wojcieszyńska D, Guzik U (2016) Metabolic responses of bacterial cells to immobilization. Molecules 21:. https://doi.org/10.3390/molecules21070958

13. Westman JO, Franzén CJ (2015) Current progress in high cell density yeast bioprocesses for bioethanol production. Biotechnol J 10:1185–1195. https://doi.org/10.1002/biot.201400581

14. Zhao XQ, Bai FW (2009) Yeast flocculation: New story in fuel ethanol production. Biotechnol Adv 27:849–856. https://doi.org/10.1016/j.biotechadv.2009.06.006

15. Verbelen PJ, De Schutter DP, Delvaux F, et al (2006) Immobilized yeast cell systems for continuous fermentation applications. Biotechnol Lett 28:1515–1525. https://doi.org/10.1007/s10529-006-9132-5

16. Westman JO, Manikondu RB, Franzén CJ, Taherzadeh MJ (2012) Encapsulation-induced stress helps *Saccharomyces cerevisiae* resist convertible lignocellulose derived inhibitors. Int J Mol Sci 13:11881–11894. https://doi.org/10.3390/ijms130911881

17. Karagoz P, Bill RM, Ozkan M (2019) Lignocellulosic ethanol production: Evaluation of new approaches, cell immobilization and reactor configurations. Renew Energy 143:741–752. https://doi.org/10.1016/j.renene.2019.05.045

18. Qun J, Shanjing Y, Lehe M (2002) Tolerance of immobilized baker's yeast in organic solvents. Enzyme Microb Technol 30:721–725. https://doi.org/10.1016/S0141-0229(02)00048-0

19. Westman JO, Ylitervo P, Franzén CJ, Taherzadeh MJ (2012) Effects of encapsulation of microorganisms on product formation during microbial fermentations. Appl Microbiol Biotechnol 96:1441–1454. https://doi.org/10.1007/s00253-012-4517-y

20. Survase SA, Van Heiningen A, Granström T (2012) Continuous bio-catalytic conversion of sugar mixture to acetone-butanol-ethanol by immobilized *Clostridium acetobutylicum* DSM 792. Appl Microbiol Biotechnol 93:2309–2316. https://doi.org/10.1007/s00253-011-3761-x

21. Silveira JM da, Soares LB, Karina C, et al (2021) Immobilization of *Spathaspora passalidarum* NRRL Y - 27907 in Calcium Alginate Aiming the Production of Second - Generation Ethanol. https://doi.org/10.1007/s12155-021-10309-w

22. Singh A, Sharma P, Saran AK, et al (2013) Comparative study on ethanol production from pretreated sugarcane bagasse using immobilized *Saccharomyces cerevisiae* on various matrices. Renew Energy 50:488–493. https://doi.org/10.1016/j.renene.2012.07.003

23. Elgharbawy AA, Alam MZ, Moniruzzaman M, Goto M (2016) Ionic liquid pretreatment as emerging approaches for enhanced enzymatic hydrolysis of lignocellulosic biomass. Elsevier B.V.

24. Thangaraj B, Solomon PR (2019) Immobilization of Lipases – A Review. Part II: Carrier Materials. ChemBioEng Rev 6:167–194. https://doi.org/10.1002/cben.201900017

25. Velasco-Lozano S, López-Gallego F, Mateos-Díaz JC, Favela-Torres E (2016) Cross-linked enzyme aggregates (CLEA) in enzyme improvement – a review. Biocatalysis 1:166–177. https://doi.org/10.1515/boca-2015-0012

26. Silva JPA, Mussatto SI, Roberto IC, Teixeira JA (2012) Fermentation medium and oxygen transfer conditions that maximize the xylose conversion to ethanol by *Pichia stipitis*. Renew Energy 37:259–265. https://doi.org/10.1016/j.renene.2011.06.032

27. Santos SC, de Sousa AS, Dionísio SR, et al (2016) Bioethanol production by recycled *Scheffersomyces stipitis* in sequential batch fermentations with high cell density using xylose and glucose mixture. Bioresour Technol 219:319–329. https://doi.org/10.1016/j.biortech.2016.07.102

28. Roque LR, Morgado GP, Nascimento VM, et al (2019) Liquid-liquid extraction: A promising alternative for inhibitors removing of pentoses fermentation. Fuel 242:775–787. https://doi.org/10.1016/j.fuel.2018.12.130

29. Balderas BSY, Muñiz BO, Rodríguez JG, et al (2016) Ethanol production by *Pichia stipitis* immobilized on sugarcane bagasse. Bioethanol 2:44–50. https://doi.org/10.1515/bioeth-2016-0001

30. Sluiter A, Hames B, Ruiz R, et al (2012) Determination of Structural Carbohydrates and Lignin in Biomass Determination of Structural Carbohydrates and Lignin in Biomass

31. Puligundla P, Poludasu RM, Rai JK, Reddy Obulam VS (2011) Repeated batch ethanolic fermentation of very high gravity medium by immobilized *Saccharomyces cerevisiae.* Ann Microbiol 61:863–869. https://doi.org/10.1007/s13213-011-0207-8

32. Bonan CIDG, Tramontina R, dos Santos MW, et al (2021) Biorefinery Platform for *Spathaspora passalidarum* NRRL Y-27907 in the Production of Ethanol, Xylitol, and Single Cell Protein from Sugarcane Bagasse. Bioenergy Res. https://doi.org/10.1007/s12155-021-10255-7

33. Godoy-Salinas AJ, Ortiz-Muñiz B, Rodríguez JG, et al (2021) Bioethanol production by *S. cerevisiae* ITV-01 RD immobilized on pre-treated sugarcane bagasse. Biomass Convers Biorefinery. https://doi.org/10.1007/s13399-021-01602-x

34. Heris Anita S, Mangunwardoyo W, Yopi Y (2016) Sugarcane Bagasse as a Carrier for the Immobilization of *Saccharomyces cerevisiae* in Bioethanol Production. Makara J Technol 20:73. https://doi.org/10.7454/mst.v20i2.3059

35. Veliky IA, McLean RJC (1994) Immobilized Biosystems. Theory and Practical Applications

36. Santos DT, Sarrouh BF, Rivaldi JD, et al (2008) Use of sugarcane bagasse as biomaterial for cell immobilization for xylitol production. J Food Eng 86:542–548. https://doi.org/10.1016/j.jfoodeng.2007.11.004

37. Perez CL, Milessi TS, Sandri JP, et al (2021) Unraveling continuous 2G ethanol production from xylose using hemicellulose hydrolysate and immobilized superior recombinant yeast in fixed-bed bioreactor. Biochem Eng J 169:. https://doi.org/10.1016/j.bej.2021.107963

38. Milessi TS, Perez CL, Zangirolami TC, et al (2020) Repeated batches as a strategy for high 2G ethanol production from undetoxified hemicellulose hydrolysate using immobilized cells of recombinant *Saccharomyces cerevisiae* in a fixed-bed reactor. Biotechnol Biofuels 13:1–12. https://doi.org/10.1186/s13068-020-01722-y

39. Kashid M, Ghosalkar A (2018) Critical factors affecting ethanol production by immobilized *Pichia stipitis* using corn cob hemicellulosic hydrolysate. Prep Biochem Biotechnol 48:288–295. https://doi.org/10.1080/10826068.2018.1425715

40. Gajula C, Chandel AK, Konakalla R, et al (2011) Fermentation of groundnut shell enzymatic hydrolysate for fuel ethanol production by free and sorghum stalks

immobilized cells of *Pichia stipitis* NCIM 3498. Int J Chem React Eng 9:. https://doi.org/10.1515/1542-6580.2514

41. Chandel AK, Lakshmi Narasu M, Chandrasekhar G, et al (2009) Use of *Saccharum spontaneum* (wild sugarcane) as biomaterial for cell immobilization and modulated ethanol production by thermotolerant *Saccharomyces cerevisiae* VS3. Bioresour Technol 100:2404–2410. https://doi.org/10.1016/j.biortech.2008.11.014

Chapter 4: Continuous fermentation of *S. passalidarum* immobilized in calcium alginate and sugarcane bagasse as supports

1. Introduction

In a scenario of great demand for clean energy, biofuels such as ethanol and biodiesel have been gaining prominence. Ethanol, when produced from lignocellulosic materials, optimizes the production of this biofuel without increasing the cultivation area of commodities such as sugarcane and corn [1]. Lignocellulosic biomass is composed of three main polymers: cellulose, hemicellulose, and lignin. The development of technologies to use carbohydrates obtained from the hydrolysis of cellulose (glucose) and hemicellulose (xylose) to produce second-generation ethanol is promising [2]. The first stage of this process is the pre-treatment, which, when done with acids, generates the hemicellulose hydrolysate, a fraction rich in xylose [3, 4]. In this step, other compounds are also generated, such as organic acids, furan, and phenolic compounds, which inhibit the metabolism of microorganisms that will convert sugars into ethanol [5]. Many strategies aim to reduce the action of these compounds in cells, such as detoxifying [6]; using adapted or genetically modified strains that are more robust against inhibitors [7, 8]; using high cell density [9]; application of dilution of inhibitors with other less toxic carbon sources [10, 11]; immobilizing cells [2, 12, 13]; between others. Cell immobilization is a consolidated strategy for protecting microorganisms against inhibitors, in addition to facilitating the reuse of biocatalysts in sequential fermentations or in continuous processes [14, 15].

The continuous fermentation process is characterized by a continuous input of culture medium and a constant output of fermented product. The second generation (2G) ethanol production in this reactor operation mode show advantages like the ease

retention of cells in the reactor, allowing the application of high cell density, which is also known as an interesting strategy to overcome inhibitors action.

Some studies in literature have shown the benefit of cells immobilization for continuous 2G ethanol production. Rice straw hydrolysate was used in continuous fermentation of *Saccharomyces cerevisiae* immobilized in calcium alginate [16]. First, synthetic medium was fed until a steady state was established; then, a hemicellulosic hydrolysate (inhibitors, in g/L: acetic acid: 1.92; furfural: 0.52; 5-HMF: 0.46) was fed at different dilution rates. After establishing the steady state, the addition of the hemicellulosic hydrolysate with inhibitors did not significantly alter the process parameters, maintaining ethanol production at around 40 g/L for 216 h. Others authors [17] tried a different strategy to ferment second generation sugars, evaluating the performance of fluidized bed bioreactor using *S. passalidarum* UFMGCM-469 cells immobilized in LentiKats®. The study used oat and soybean hull hydrolysate (inhibitors, in g/L: acetic acid: 1.1; furfural: 0.08; 5-HMF: 0.01). First, the authors applied batch fermentation to familiarize the microorganisms in the inhibitors on hydrolysate. After 48h of process, there were no sugars reminiscent in the medium, so then the same fresh media was continuous feed, using a dilution rate of 0.05 h^{-1}. After 96 h of feeding, the cells reached a steady state, with production ethanol concentration similar to the batch phase, but with an increasing of 28% on productivity. Co-culture is also used in continuous fermentation: *Scheffersomyces stipitis* and *S. cerevisiae* are immobilized on calcium alginate spheres and continuous ethanol production was performed in packed bed immobilized cell reactor [18]. Using 4 mL/min of flow rate, the authors obtained an increase of 10% on ethanol production from wheat straw hydrolysate when co-culture was used, in compare with the assay with only *S. cerevisiae.*

In this study, continuous fermentation was performed with *S. passalidarum* NRRL Y-7124 cells immobilized in different supports. Two strategies of feed were tested. The first assay was carried out with the introduction of synthetic medium followed by hemicellulosic hydrolysate, using cells immobilized in calcium alginate spheres, the most commum support use for 2G ethanol process. A second test was performed with cells immobilized in sugarcane bagasse, with the feeding of three different media: synthetic, synthetic + acetic acid and, finally, hemicellulosic hydrolysate.

2. Materials and methods

2.1 Hemicelullosic hydrolysate

Due to the large volumes required for continuous fermentation, the hemicellulosic hydrolysate (HH) used in this step was produced in the Pilot Plant for Process Development of the National Biorenewables Laboratory – Campinas, São Paulo, Brazil. The applied methodology was that described by Dionísio et al., 2021 [3], which uses diluted sulfuric acid and a temperature of 140 °C in a 350 L steel alloy reactor (Pope Scientific Inc., Saukville, USA), using about 15 kg of raw bagasse (50% (w/w) of humidity), with a solid loading of 9% (w/w) and 0.5% (v/v) of sulfuric acid solution, with stirring at 150 rpm for 15 min. At the end of the process, the reactor was cooled, depressurized and the resulting material was divided into liquid and solid fractions through a Nutsche filter (Pope Scientific Inc., Saukville, USA) with a capacity of 140 L. The hemicellulosic hydrolysate was concentrated 7 times in an evaporator for conservation and storage. At the moment of use, the pH of the hydrolysate was

corrected for 5.5 with NaOH powder, centrifuged at 3000 xg for 20 min (Avanti J-30I Beckman Coulter) to remove suspended material, followed by sterilization at 115 °C, 10 min, and used to compose the fermentation media.

2.2 Microorganism, seed culture and cell propagation

Spathaspora passalidarum NRRL Y-27907 was used in the study [19]. The microorganism was stored in a cryotube containing, containing in (g/L): yeast extract (10.0), peptone (20.0) dextrose (10.0) and xylose (10.0) and glycerol (volume proportion 1:1) to configure the YPDX medium, in a freezer at -80 ºC. In order to obtain high cell density, *S. passalidarum* was submitted to pre-inoculum, inoculum and cell propagation stages.

The stock culture of the microorganism was transfer to a 250 mL Erlenmeyer flask containing 100 mL of the pre-inoculum medium YPD containing (in g/L) yeast extract (10.0), peptone (20.0) and dextrose (20.0). The flasks were incubated in an orbital shaker (Tecnal TE-424), at constant temperature of 30 °C and 150 rpm for 24 h. After the pre-inoculum time, the entire volume was centrifuged (1200 xg for 10 min, Kasvi, K14 0815C) and resuspended in sterile water in an amount corresponding to 10% (25 mL) of the total inoculum volume and was aseptically transferred to 500 mL Erlenmeyer flasks containing 250 mL of medium. Medium was the same described by Silva et al. [20] and Santos et al. [21] composed of (g/L): xylose, (12.0); glucose, (1.32); urea, (2.3); yeast extract, (3.0) and $MgSO_4.7H_2O$, (1.0), and incubated under the same conditions of pre-inoculum for 24 h.

Propagation step was carried out in a benchtop bioreactor (New Brunswick BioFlo 115), with a total capacity of 7.0 L and an initial working volume of 2.8 L of the medium described by Santos et al. [21] composed of (g/L): dilute sugarcane molasses

(30.0); urea (5.0); and KH_2PO_4 (2.0). The cells obtained in the inoculum step were centrifuged (1200 xg for 10 min, Kasvi, K14-0815C) and the pellet cells was dispersed in sterile water, in a volume corresponding to 10% (280 mL) of the initial bioreactor volume. Propagation took place at 30 °C and pH 6.0; with initial agitation of 200 rpm and initial aeration of 0.1 vvm. The control of agitation and aeration was established to keep the concentration of dissolved oxygen above 50% in relation to the saturation of air at atmospheric pressure [21].

Propagation was divided into two stages: the first step was carried out in batch mode, until the yeast reached the exponential growth phase (around 12 h of process). Next, a pulse of nutrients KH_2PO_4 (2.0 g/L) and urea (5.0 g/L) was performed and a fed batch operation mode was started with a supply in the feed flow of 3 g/(L.h) of pure sugarcane molasses (467.21 g/L), in order to establish linear growth (for approximately 12 h). At the end of the propagation, the entire volume was centrifuged at 3000 xg for 20 min (Avanti J-30I Beckman Coulter) and the pellet was resuspended in sterile water. The yeast cream obtained was quantified in terms of cell concentration and stored under refrigeration (4 °C) in a sterile flask for later use [21].

2.3 Sugarcane bagasse support preparation and cells imobilization

Sugarcane bagasse (*Saccharum officinarum*) from a local market (São Miguel do Oeste, Santa Catarina, Brazil) was used in this study. The solid was washed in running water to remove dirt and manually cut into pieces of approximately 2.0 cm in length with scissors and the moisture content of the biomass was measured using a moisture analyzer (CEM-Smart Turbo). The sugarcane bagasse was pretreated in a 316 L stainless steel reactor (Metalquim, 5.0 L) at 130 °C in a H_2SO_4 solution of

0.5% v/v, in a solid/liquid ratio of 1:10 (dry basis) for 15 min, adapted from Roque et al. [4]. The solid fraction was separated by filtration; the retained material was washed with water until neutral pH and dried in an oven at 105 °C for 24 h. Before immobilization, the *S. passalidarum* cells concentrated in the previous step were reactivated in YPD medium containing (in g/L): yeast extract (10.0), peptone (20.0) and dextrose (20.0) in a volume corresponding to 20g/L of cells. The flasks were incubated in an orbital shaker (Tecnal TE-424), at a constant temperature of 30 °C and 150 rpm for 12 h. Then the cells were centrifuged (1200 xg for 10 min, Kasvi, K14 0815C) and resuspended in sterile water. Reactivated cells were added to immobilization medium containing peptone (1 g/L) and sugarcane bagasse pretreated with acid in a solid:liquid ratio 1:20 (dry basis). The flasks were incubated in an orbital shaker at 30 °C and 100 rpm for 48 h [22]. After the immobilization time, the solid support with the immobilized cells was drained from the medium and used in continuous fermentation.

2.4 Immobilization in calcium alginate spheres

Sodium alginate was dissolved in a mixture of sterile distilled water and concentrated reactivated *S. passalidarum* cells solution to result in a final alginate concentration of 2% m/m and that of cells corresponding to 20 g/L in the continuous reactor [7]. This mixture was dripped (flow rate: 2 mL/s, 3mm internal diameter silicone hose), with a peristaltic pump (Watson-Marlow 120S), in 2% $CaCl_2$ to form the spheres. The spheres resulted present approximate 3.0 mm of diameter and were kept in this solution for approximated 16 hours at 4°C for curing time [16]. Afterwards, the calcium alginate spheres were drained from the gelling solution and used in continuous fermentation.

2.5 Continous fermentation

Continuous fermentation was carried out in a cylindrical jacketed borosilicate glass reactor, with a diameter of 2.5 cm (internal diameter of 2.0 cm) and length of 15.0 cm (30 mL of util volume). The bioreactor was equipped with a polypropylene mesh at the upper end to retain solids in the system. Two continuous fermentations were performed with cells immobilized on different supports. First, a calcium alginate support was used with *S. passalidarum* cells. The spheres filled 70% of the reactor volume and the column was fed from the bottom with a peristaltic pump, with a dilution rate of 0.5 h^{-1} (residence time 2h) in the first 24 h with synthetic medium (Table 1) at 30ºC. The medium (Table 1) was constantly saturated in atmospheric air through the injection of this gas in a sterile flow rate of 0.5 L/min. After 24h, the synthetic medium was replaced by hemicellulosic hydrolysate media (Table 1), fed under the same conditions for another 48 h (adapted from [16]) at the same dilution rate. The feeding flasks were kept warm at 30°C and stirred using a stirrer/heating plate. The effluent was collected at the top of the column reactor, at the same feed rate. The reactor temperature was maintained at 30°C by circulating water through the jacket using a heated circulation bath. The reactor setup with cells immobilized in calcium alginate spheres is shown in Fig. 1 (a) and highlighting the reactor bed in (b).

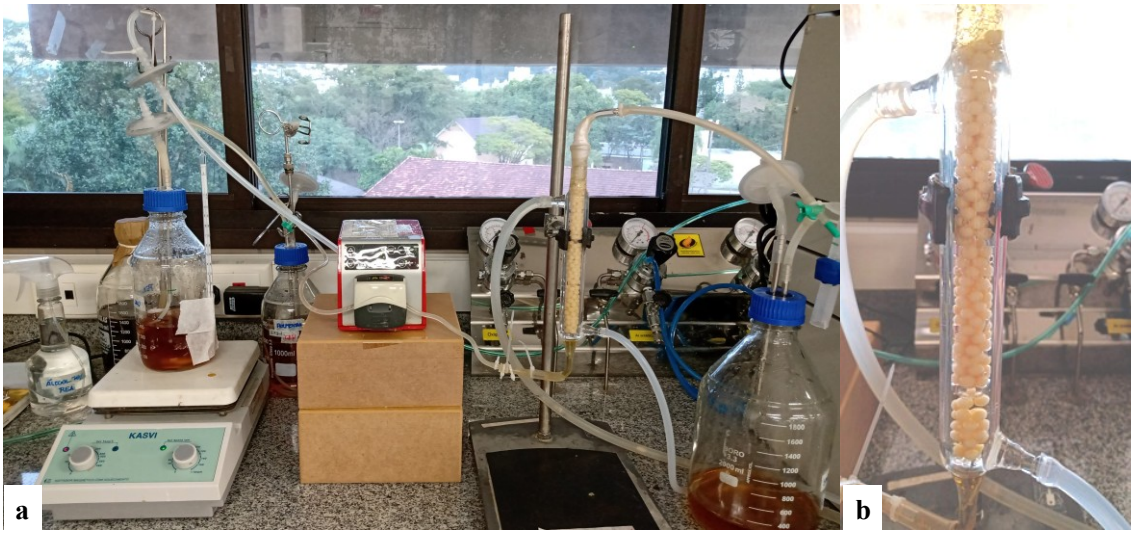

Figure 1. Reactor configuration with cells immobilized in calcium alginate spheres (a) and highlighting the reactor bed (b).

Table 1 Composition of continuous fermentation media with *S. passalidarum* cells immobilized in calcium alginate spheres.

Nutrient	Concentration (g/L)	
	Sintetic media	Hemicelullosic hydrolysate media
Yeast extract	10.0	10.0
Glucose	7.0	-
Xylose	70.0	-
TRS* in HH	-	90.0
$MgSO_4$	1.0	1.0
Urea	2.4	2.4
KH_2PO_4	3.0	3.0

TRS* corresponds a total reducing sugars.

Cells immobilized on sugarcane bagasse were also evaluated in continuous fermentation. In this test, the support as well filled 70% of the reactor volume (1.5 g of bagasse, 4.0 g/L of cells inside) and the column was fed from the bottom with a peristaltic pump, with a dilution rate of 0.5 h^{-1} in the first 48 h with synthetic medium saturated in atmospheric air through the constant injection of this gas in a sterile flow rate of 0.5 L/min (Table 2) at 30 ºC. After 48 h, the synthetic medium was replaced by buffered synthetic medium, pH 5.75, containing acetic acid (acid 2.46 g/L + buffer

12.81 g/L; Table 2), fed under the same conditions for another 48 h. Afterwards, hemicellulosic hydrolysate was fed for 96 h, under the same conditions. The feeding flasks were kept warm at 30°C and stirred using a stirrer/heating plate. The effluent was collected at the top of the column, at the same feed rate. The reactor temperature was maintained at 30°C by circulating water through the jacket using a heated circulation bath. The configuration of the reactor with the cells immobilized in sugarcane bagasse is shown in Figure 2 (a), with the reactor bed highlighted in (b).

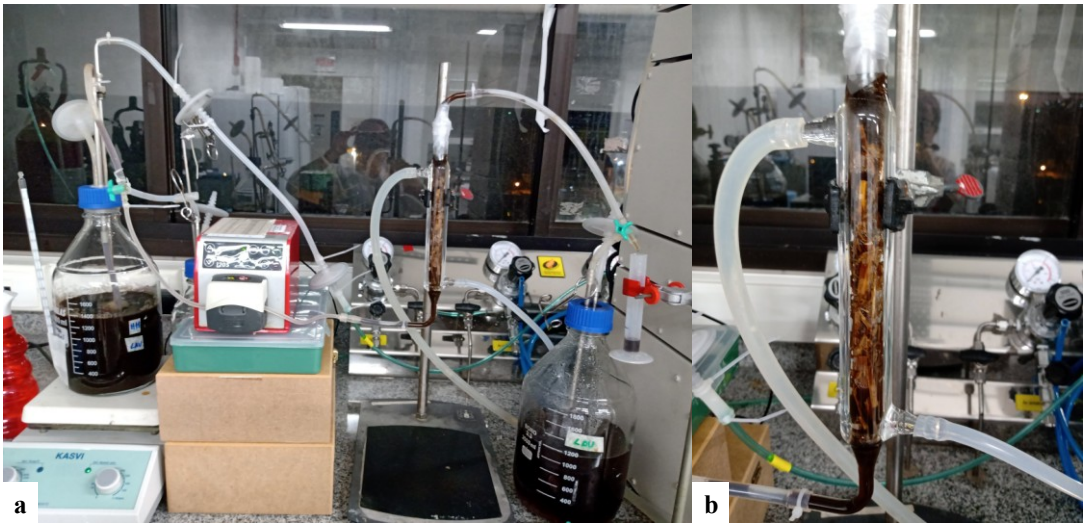

Figure 2. Reactor configuration with cells immobilized in sugarcane bagasse (a) and highlighting the reactor bed (b).

Table 2: Compositions of continuous fermentation media with *S. passalidarum* cells immobilized in sugarcane bagasse.

Nutrient	Concentration (g/L)		
	Sintetic media	Sintetic media + acetic acid	Hemicelullosic hydrolysate media
Yeast extract	10.0	10.0	10.0
Glucose	7.0	7.0	-
Xylose	70.0	70.0	-
TRS* in HH	-	-	90.0
$MgSO_4$	1.0	1.0	1.0
Urea	2.4	2.4	2.4
KH_2PO_4	3.0	3.0	3.0
Acetic acid	-	2.7	2.7
Sodium acetate buffer 0.1 M	-	12.81	-

TRS* corresponds to a total reducing sugars.

2.6 Analytical methods

Samples were collected at 3 h intervals at the first 36 h of each feeding bottle and then spaced between periods of 4, 6 or 12 h. The concentration of free cells in the outlet of reactor was measured by dry cell weight. A volume of 1.0 mL samples was collected in triplicate and centrifuged at 3.000 xg (Eppendorf HsiangTai CN2160) for 5 min in previously weighed 2.0 mL microtubes. The cell pellet was resuspended with distilled water and centrifuged again under the same conditions. The precipitate of washed cells was subjected to drying in an oven at 105 °C for 24 h. After the drying time, the microtubes were weighed on an analytical balance (Sartorius) and the cell concentration was calculated by mass difference.

The liquid supernatant was thawed, filtered (PVDF Millex 0.22 μm) and injected (10 μL) into the High-Performance Liquid Chromatography (HPLC) system (Shimadzu LC-20A) to analyse the concentrations of glucose, xylose, acetic acid, ethanol, and xylitol. Compounds were separated at 50 °C with Aminex HPX 87H (300

μm 7.8 mm, BIO-RAD, Hercules, CA) using 5 mM H_2SO_4 as mobile phase at a rate of 0.6 mL/min.

The morphologies of sugarcane bagasse with *S. passalidarum* cells immobilized in the initial and final times of fermentation were analyzed using scanning electron microscopy (SEM). Sample preparation consisted of submersion in glutaraldehyde 25 g/L for 2 h for cells fixation. Afterwards, the samples were dipped in ethanol solution with increasing concentrations (10, 20, 30, 50, 70, 80, 90 and 100% - 3 times) for 20 min each [24]. Subsequently, the samples dried with CO_2 supercritic and covered with a thin layer of gold. The micrographs of the immobilized cells were analyzed with magnification between 25 and 1500X and electron beam with energy of 8kV (Jeol, model JSM – 6390 LV).

2.7 Kinetic parameters

Volumetric ethanol productivity and the yield factor of reducing sugars in ethanol were used to evaluate the performance of *S. passalidarum* in steady-state ethanol production. The volumetric productivity of ethanol (Q_P, g/(L.h)) was calculated according to Equation 1. Yield factor of reducing sugars in ethanol ($Y_{P/S}$, g/g) was calculated calculated according to Equation 2 [17]:

$$Q_P = D * P_{out} \quad (1)$$

$$Y_{\frac{P}{S}} = \frac{P_{out}}{S_{in} - S_{out}} \quad (2)$$

Where D is the dilution rate, 0.5 h^{-1}, P_{out} is the ethanol concentration [g/L] in the outlet of reactor, S_{in} represents the substrate concentration [g/L] at inlet of reactor, S_{out} [g/L] that of outlet of reactor.

3. Results and discussion

The results of continuous fermentation in hemicellulosic hydrolysate and *S. passalidarum* cells immobilized in calcium alginate spheres are shown in Figure 3.

For the initial 24 hours of synthetic medium feeding the glucose concentration at the reactor outlet decreased over time, being no longer detected at the end of this period, indicating a complete consumption of this substrate. Xylose is also consumed when continuous synthetic medium was fed, but, partially approximately 16% of the inlet concentration (inlet 75.71 g/L and outlet 63.6 g/L) of what was fed in the same period. The production of ethanol for feeding the synthetic medium showed a plateau: between 12 and 24 h of the process, approximately 4.7 g/L of ethanol was achieved, characterizing a stationary production phase. For this ethanol stationary phase, the substrate conversion factor in ethanol ($Y_{P/S}$) was 0.29 ± 0.04 g/g and the productivity in ethanol was 2.35 g/(L.h). During the synthetic medium feeding period there was a small production of xylitol (average concencentration of 0.46 ± 0.18 g/L) and a constant escape of cells from the spheres (average 0.14 ± 0.07 g/L) detected in the reactor effluent. Then, the average production of xylitol was 6.9 mg/h and the average cell escape rate was 2.1 mg/h.

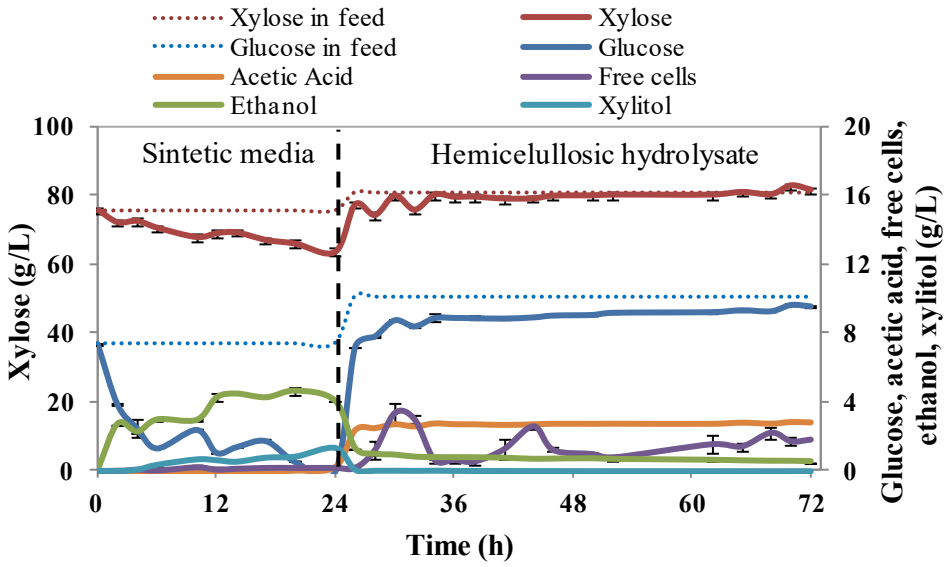

Figure 3. Results of fermentation in a continuous bioreactor (D=0.5 h^{-1}, V=30 mL) with *S. passalidarum* cells immobilized in calcium alginate spheres. The dotted lines on the X-axis indicate the concentration of xylose and glucose in the feed. The solid lines indicate concentrations of xylose, glucose, acetic acid, free cells, ethanol, and xylitol in the reactor effluent. The horizontal dotted line indicates the moment of switching from the synthetic medium to the medium containing hemicellulosic hydrolysate.

After 24 h of feeding the synthetic medium, the feeding of hemicellulosic hydrolysate (Table 1) was started. It is possible to notice that at the beginning of the feeding, between 24 and 30 h, there was a certain consumption of sugars and low ethanol production, in lower levels than those reached in the synthetic substrate. After 30 h (6 h of HH feeding) an inhibition of fermentation is noticed, with low glucose consumption. There´s no detection of xylitol concentration in this phase and an increase of free cells in the effluent reactor is noticed after 40 h of process, which could characterize the beginning of the wash-out of cells. The medium containing hemicellulosic hydrolysate had 2.7 g/L of acetic acid, in addition to other inhibitors such as formic acid (0.11 g/L); levilinic acid (0.04 g/L); 5-hydroxymethylfurfural (0.03 g/L) and furfural (0.10 g/L), which have already been reported in the literature as

toxic to *S. passalidarum*. It was reported that with 1.5 g/L of acetic acid in a batch medium, this microorganism already descrease the consumption of sugars [9]. As the level of this inhibitor was much higher in the continuous fermentation (2.7 g/L) than reported in literature, this could be one of the reasons for the fermentation not proceeding after feeding the hydrolysate.

Since the test with synthetic medium followed by medium with hemicellulosic hydrolysate did not favor the consumption of second-generation sugars, a different strategy was used in the continuous fermentation with cells immobilized in sugarcane bagasse: the feeding time of synthetic medium was increased to 48 h and a synthetic medium with a concentration of acetic acid similar to that of the medium containing hydrolysate was introduced after for another 48 h of feeding. Thus, it was intended to adapt the immobilized microorganism to the most severe inhibitor for *S. passalidarum* [9] so that the hydrolysate could then be fed.

The results for cells immobilized in sugarcane bagasse in Figure 4 show glucose consumption similar to that observed for fermentation with cells immobilized in alginate, with the concentration of this sugar no longer detected in the reactor effluent at the end of the 48 h of feeding. It is also possible to note the consumption of xylose between 8 and 48h for this type of support fermentation. The ethanol production reached two plateaus, one between 4 and 36 h, reaching a concentration of around 2.3 g/L of ethanol and another between 36 and 48 h, producing around 3.3 g/L of ethanol in the reactor output. A small escape of the immobilized cells was observed after 36h of the process. For this period, the average of substrate (glucose + xylose) conversion factor in ethanol ($Y_{P/S}$) was 0.26 ± 0.08 g/g, value similar to the obtained in calcium alginate spheres fermentation.The productivity in ethanol was lower that the first assay, 2.0 g/(L.h). This difference may be correlated with the difference between

the concentrations of cells immobilized on two supports (alginate ~ 20.0 g/L and sugarcane bagasse ~ 4.0 g/L).

For the buffered acetic acid sintetic media phase, it is observed in Figure 4 that the glucose concentration increased on reactor output in the first moments of feeding and remained stable, showing that there was a small consumption of this sugar. The same occurred for xylose, which maintained a level very similar to that contained in the fresh medium. No expressive amount of ethanol or xylitol was detected in this feeding phase and the escape of cells that had started in the previous feeding phase lasted until approximately 72 h of process. This behavior may be related to the high concentration of acetic acid in the medium, showing that the strategy of trying to adapt the immobilized microorganism to ferment in media with the most severe inhibitor with this feeding phase was not effective for *S. passalidarum*. Despite the inhibition observed, there is a small consumption of sugars. As there is no apparent production of ethanol and no cell output after 72 h, it is the immobilized cells that are consuming, which indicating that they are still viable.

After 48 h of buffered acetic acid sintetic media phase (96 h of process), the feeding of the medium with hemicellulosic hydrolysate was initiated. There was no consumption of glucose in this phase and the small amount of xylose consumed was transformed into xylitol (average 0.81 ± 0.34 g/L or 12.15 mg/h). In this feeding phase, there was also a possible detachment of cells of surface of bagasse into the liquid or even growth, as evidenced by the increase in free cells at the output of the continuous reactor (average 1.99 ± 0.76 g/L or 29.85 mg/h).

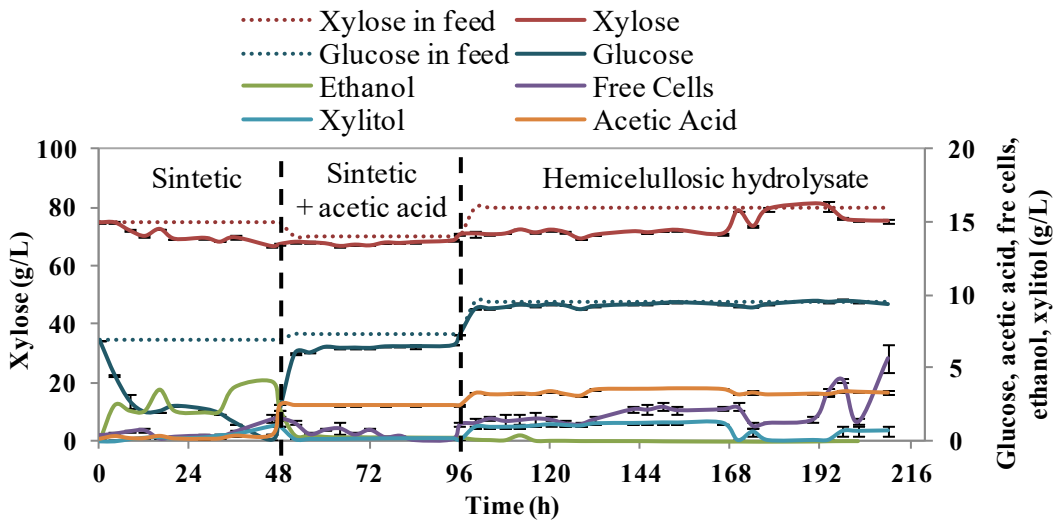

Figure 4. Results of fermentation in a continuous reactor (D=0.5 h^{-1}, V=30 mL) with *S. passalidarum* cells immobilized in sugarcane bagasse. The dotted lines on the X-axis indicate the concentration of xylose and glucose in the feed. The solid lines indicate concentrations of xylose, glucose, acetic acid, free cells, ethanol and xylitol in the reactor effluent. The horizontal dotted lines indicate the time of change from synthetic medium to synthetic medium with acetic acid and then to medium containing hemicellulosic hydrolysate.

The morphological analysis of sugarcane bagasse was also carried out with cells immobilized by scanning electron microscopy, at the initial and final times of continuous fermentation. Two different samples at initial fermentation time are shown in Fig. 5 (a) and (b), where it is possible to observe the interior of the sugarcane bagasse containing the *S. passalidarum* cells immobilized on its surface, including the presence of cells budding (highlights). For the fermentation time of 210 h (Fig. 5 c, d), the solid samples still contained yeast cells inside, making it clear that the sugarcane bagasse was able to retain the cells until the end of the continuous process.

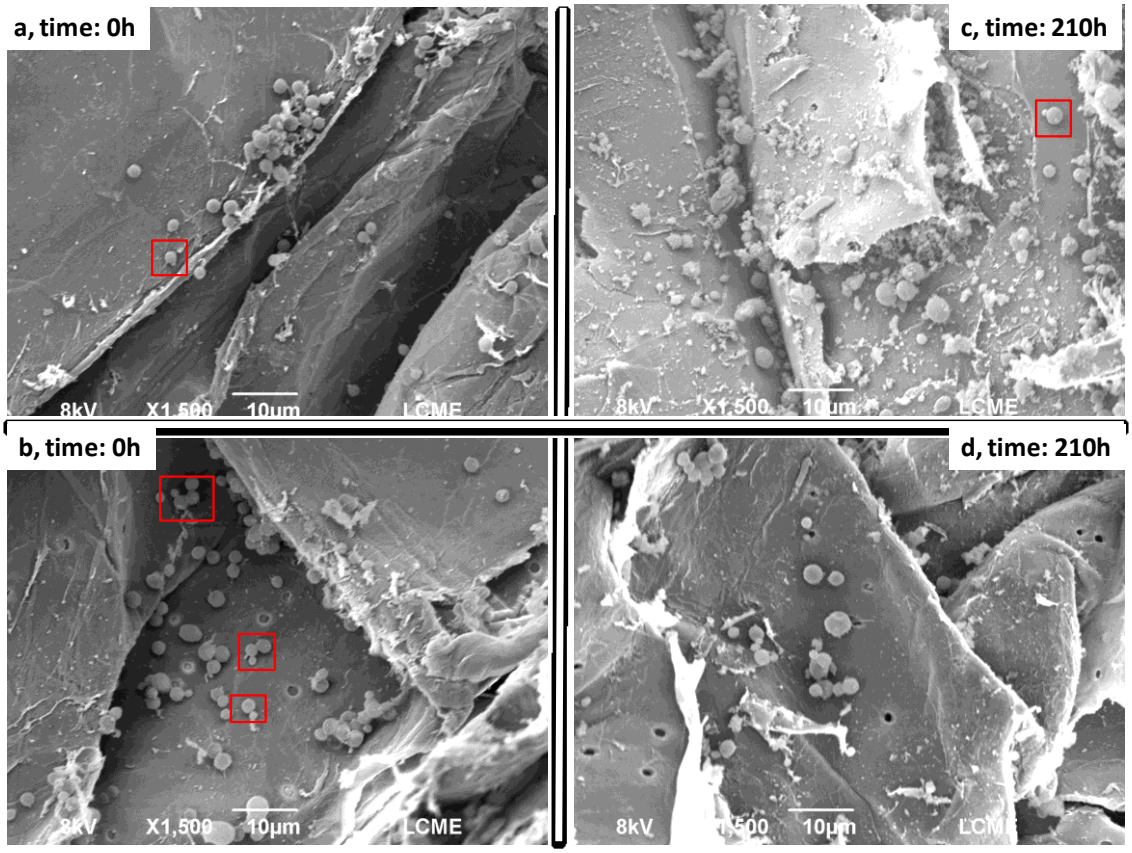

Figure 5. Morphology sugarcane bagasse with *S. passalidarum* cells immobilized in the initial continous fermentation time (a), (b); and in the final continous fermentation time (c), (d).

After 210 h of continuous fermentation, a sample of sugarcane bagasse was seen on solid media. Interestingly, after 24 hours of incubation, it was possible to notice the growth of colonies, as shown in Figure 5, demonstrating that the microorganism was, even not producing ethanol in the bioreactor, still viable to growth.

The feed flow used in this study was limited by the physical structure (peristaltic pump, hose caliber and reactor column length) existing in our laboratory, which may also have caused the limitation of fermentation in both supports used. Since using sugarcane bagasse as support in batch fermentations (Chapter 3), immobilized *S. passalidarum* cells take almost 24 hours to consume the xylose from the medium. In

this way, the strategy of increasing the residence time in the reactor could improve sugar consumption in continous process.

Figure 6. Colonies of cells grown after 24 hours of plating sugarcane bagasse used in continuous fermentation, showing the viability of *S. passalidarum* cells immobilized in the solid after 210 hours of process.

Table 3 shows literature studies that use continuous fermentation with cells immobilized for 2G ethanol production. Cortivo et al, 2022 [17] evaluated the performance of fluidized bed and packed bed bioreactors using *Spathaspora passalidarum* UFMGCM-469 cells immobilized in LentiKats® in the fermentation of oat and soybean hull hydrolysates. The authors used the strategy of first applying a batch fermentation to acclimatize the microorganisms in the hydrolysate, and after consuming the sugars in the medium, they began to feed continuously on the same medium. After batch phase (48 h), using a dilution rate of 0.05 h^{-1} (6 h of residence time) the continuous process reached production values similar to the batch phase, but with an emphasis on productivity, which increased by 28%. For other microorganisms, continuous fermentation into hemicellulosic hydrolysates with immobilized cells also

showed promise. Mishra et al, 2016 [16] immobilized *S. cerevisiae* in calcium alginate spheres and performed continuous fermentations using non-detoxified rice straw hydrolysate. The authors also applied the strategy of first feeding with synthetic medium until a steady state was established (both in sugar consumption and in ethanol production), and then feeding the already stable reactor with hemicellulosic hydrolysate (1.2 g/L of acetic acid, 0.46 g/L of 5-HMF and 0.52 g/L of furfural). The results showed that, upon reaching steady state, the immobilized cells responded with almost no variation in the process parameters, maintaining ethanol production around 40 g/L for 216 h, even with the introduction of inhibitors to the inflow.

Table 3: Literature studies that use continuous fermentation with cells immobilized for 2G ethanol production.

			Parameters on stationary phase				
Support	**Media**	**Microorganism**	**Diluition rate (h^{-1})**	**$Y_{P/S}$ (g/g)**	**Productivity g/(L.h)**	**Maximum ethanol (g/L)**	**Reference**
Calcium alginate	Sintetic	*S. passalidarum* NRRL Y-27907	0.5	0.29 ± 0.04	2.35	4.7	This work
Sugarcane bagasse	Sintetic	*S. passalidarum* NRRL Y-27907	0.5	0.26 ± 0.08	2.0	4.0	This work
Lentikats®	Oat and soybean hull hydrolysate	*S. passalidarum* UFMGCM-469	0.05	0.36	0.58	11.5	[17]
Calcium alginate	Rice straw enzymatic hydrolysate	Recombinant *S. cerevisiae* GSE1618	0.61	0.509	26.06	42.72	[16]

4. Conclusion

In summary, the results obtained at this stage show it is possible to use a continuous bioreactor with *S. passalidarum* cells immobilized in sugarcane bagasse and in calcium alginate spheres to ferment. With synthetic medium and no inhibitors present

both glucose and xylose are consumed and a stationary ethanol production phase was achieved. The addition of acetic acid caused fermentation inhibition, since there was no expressive consumption and production when the continuous reactor was fed with synthetic medium containing acetic acid or hemicellulosic hydrolysate. However, even under conditions of fermentation inhibition, the cells remain immobilized and viable.

Thus, further studies are needed to implement a continuous ethanol production process, since it was not possible to efficiently ferment the hemicellulosic hydrolysate with cells immobilized in sugarcane bagasse or in calcium alginate spheres under the conditions tested in this work.

5. Chapter 4 references

1. Dias MOS, Junqueira TL, Cavalett O, et al (2013) Biorefineries for the production of first and second generation ethanol and electricity from sugarcane. Appl Energy 109:72–78. https://doi.org/10.1016/j.apenergy.2013.03.081

2. Robak K, Balcerek M (2020) Current state-of-the-art in ethanol production from lignocellulosic feedstocks. Microbiol Res 240:126534. https://doi.org/10.1016/j.micres.2020.126534

3. Dionísio SR, Santoro DCJ, Soares LB, et al (2021) Second-generation ethanol process for integral use of hemicellulosic and cellulosic hydrolysates from diluted sulfuric acid pretreatment of sugarcane bagasse. Fuel 304:121290. https://doi.org/10.1016/j.fuel.2021.121290

4. Roque LR, Morgado GP, Nascimento VM, et al (2019) Liquid-liquid extraction: A promising alternative for inhibitors removing of pentoses fermentation. Fuel 242:775–787. https://doi.org/10.1016/j.fuel.2018.12.130

5. Taherzadeh MJ, Karimi K (2011) Fermentation inhibitors in ethanol processes and different strategies to reduce their effects, 1st ed. Elsevier Inc.

6. Zhang Q, Huang H, Han H, et al (2017) Stimulatory effect of in-situ detoxification on bioethanol production by rice straw. Energy 135:32–39. https://doi.org/10.1016/j.energy.2017.06.099

7. Biazi LE, Santos SC, Kaupert Neto AA, et al (2021) Adaptation Strategy to Increase the Tolerance of *Scheffersomyces stipitis* NRRL Y-7124 to Inhibitors of Sugarcane Bagasse Hemicellulosic Hydrolysate Through Comparative Studies of Proteomics and Fermentation. Bioenergy Res. https://doi.org/10.1007/s12155-021-10267-3

8. Morales P, Gentina JC, Aroca G, Mussatto SI (2017) Development of an acetic acid tolerant *Spathaspora passalidarum* strain through evolutionary engineering with resistance to inhibitors compounds of autohydrolysate of Eucalyptus globulus. Ind Crops Prod 106:5–11. https://doi.org/10.1016/j.indcrop.2016.12.023

9. Soares LB, Bonan CIDG, Biazi LE, et al (2020) Investigation of hemicellulosic hydrolysate inhibitor resistance and fermentation strategies to overcome inhibition in non-*saccharomyces* species. Biomass and Bioenergy 137:105549. https://doi.org/10.1016/j.biombioe.2020.105549

10. Farias D, Maugeri Filho F (2019) Co-culture strategy for improved 2G bioethanol production using a mixture of sugarcane molasses and bagasse hydrolysate as substrate. Biochem Eng J 147:29–38. https://doi.org/10.1016/j.bej.2019.03.020

11. Pereira I de O, dos Santos ÂA, Gonçalves DL, et al (2021) Comparison of *Spathaspora passalidarum* and recombinant *Saccharomyces cerevisiae* for integration of first- and second-generation ethanol production . FEMS Yeast Res 21:1–14. https://doi.org/10.1093/femsyr/foab048

12. Karagoz P, Bill RM, Ozkan M (2019) Lignocellulosic ethanol production: Evaluation of new approaches, cell immobilization and reactor configurations. Renew Energy 143:741–752. https://doi.org/10.1016/j.renene.2019.05.045

13. Rodríguez-Restrepo YA, Orrego CE (2020) Immobilization of enzymes and cells on lignocellulosic materials. Environ Chem Lett 18:787–806. https://doi.org/10.1007/s10311-020-00988-w

14. Navarrete HC, Matín C, García JM (2021) Yeast immobilization systems for second-generation ethanol production: actual trends and future perspectives. Biofuels, Bioprod Biorefining 1–17. https://doi.org/10.1002/bbb.2250

15. Soares LB, Machado J, Biazi LE, et al (2022) An overview on fermentation strategies to overcome lignocellulosic inhibitors in second- generation ethanol production using cell immobilization. Crit Rev Biotechnol 0:1–22. https://doi.org/10.1080/07388551.2022.2109452

16. Mishra A, Sharma AK, Sharma S, et al (2016) Lignocellulosic ethanol production employing immobilized Saccharomyces cerevisiae in packed bed reactor. Renew Energy 98:57–63. https://doi.org/10.1016/j.renene.2016.02.010

17. Cortivo PRD, Raquel L, Carlos H, et al (2022) Continuous bioreactor bioprocess using immobilized *Spathaspora passalidarum* to convert hydrolysates of oat and soybean hulls into ethanol. Biomass Convers Biorefinery. https://doi.org/10.1007/s13399-022-02531-z

18. Karagöz P, Özkan M (2014) Bioresource Technology Ethanol production from wheat straw by *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* co-culture in batch and continuous system. 158:286–293. https://doi.org/10.1016/j.biortech.2014.02.022

19. Nguyen NH, Suh S-O, Marshall CJ, Blackwell M (2006) Morphological and ecological similarities: wood-boring beetles associated with novel xylose-fermenting yeasts, *Spathaspora passalidarum* gen. sp. nov. and *Candida jeffriesii* sp. nov. Mycol Res 110:1232–1241. https://doi.org/10.1016/J.MYCRES.2006.07.002

20. Silva JPA, Mussatto SI, Roberto IC, Teixeira JA (2012) Fermentation medium and oxygen transfer conditions that maximize the xylose conversion to ethanol by *Pichia stipitis*. Renew Energy 37:259–265. https://doi.org/10.1016/j.renene.2011.06.032

21. Santos SC, de Sousa AS, Dionísio SR, et al (2016) Bioethanol production by recycled *Scheffersomyces stipitis* in sequential batch fermentations with high cell density using xylose and glucose mixture. Bioresour Technol 219:319–329. https://doi.org/10.1016/j.biortech.2016.07.102

22. Singh A, Sharma P, Saran AK, et al (2013) Comparative study on ethanol production from pretreated sugarcane bagasse using immobilized *Saccharomyces cerevisiae* on various matrices. Renew Energy 50:488–493. https://doi.org/10.1016/j.renene.2012.07.003

23. Silveira JM da, Soares LB, Karina C, et al (2021) Immobilization of *Spathaspora passalidarum* NRRL Y - 27907 in Calcium Alginate Aiming the Production of Second - Generation Ethanol. https://doi.org/10.1007/s12155-021-10309-w

24. Puligundla P, Poludasu RM, Rai JK, Reddy Obulam VS (2011) Repeated batch ethanolic fermentation of very high gravity medium by immobilized *Saccharomyces cerevisiae.* Ann Microbiol 61:863–869. https://doi.org/10.1007/s13213-011-0207-8

Chapter 5. Final considerations and suggestions for futures studies

This study proved the feasibility of *S. passalidarum* immobilization in sugarcane bagasse to improve the second-generation ethanol production in hemicellulosic hydrolysate from diluted surfuric acid pretreatment.

The validation of *S. passalidarum* immobilization in sugarcane bagasse shown that the solid material obtained after diluted sulfuric acid pretreatment was the best support for the adhesion of cells in comparison to sugarcane raw material and the material obtained after alkaline pretreatment. The selected support allowed a greater number of cells and a higher rate of cell immobilization and the process to obtain it is the one that best recovers the pentose fraction of the biomass. In this sense, it was possible to perform an integrated process for obtaining support for cell immobilization and for obtaining second-generation sugars in a single step.

Optimization of the time and *S. passalidarum* concentration in the process of immobilization was possible, and allowed higher cell adhesion to the support, improving characteristics of process intensification. The strategy of intensification is described in literature to overcome inhibitors present in hemicellulosic hydrolysates.

Fermentation of the hemicellulosic hydrolysate by immobilized *S. passalidarum* in sugarcane bagasse performed better than free cells in batch mode, making this process an effective strategy to improve fermentative parameters in the production of 2G ethanol by this microorganism.

It was possible to perform continuous fermentation of synthetic medium with immobilized *S. passalidarum* cells in sugarcane bagasse and calcium alginate spheres. Without the presence of inhibitors, glucose and xylose were consumed and a continuous production of ethanol was established. With the introduction of inhibitors in the feed,

fermentation was inhibited, although the cells remained immobilized and viable to grow.

The author suggests for future studies:

- Study of a methodology for direct quantification of cells immobilized on sugarcane bagasse;
- Study of the stability of the support and the improvement of hemicellulosic hydrolysate fermentation through sequential fermentations with cells immobilized in sugarcane bagasse;
- Study of different concentrations of sugar and inhibitors in the continuous reactor feed;
- Study of different concentrations of cells immobilized in sugarcane bagasse in continuous fermentation;
- Study of different dilution rates in continuous fermentation with cells immobilized in sugarcane bagasse;
- Study of the application in a batch mode in the continuous reactor until the exhaustion of the sugars in the hemicellulosic hydrolysate so that a continuous feed of these sugars can then be established;
- Study of the application of fluidized bed in continuous fermentation to improve xylose consumption by *S. passalidarum* immobilized in sugarcane bagasse.