FEDERAL UNIVERSITY OF SÃO CARLOS
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“Development of integrated strategies using non-recombinant yeast for 2G ethanol production from lignocellulosic materials”

Juliana Passamani Sandri

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"Desenvolvimento de estratégias integradas utilizando leveduras não recombinantes para produção de etanol 2G a partir de materiais lignocelulósicos"

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"Sometimes we feel that what we do is just a drop in the sea. But the sea would be less if it lacked a drop."

Mother Teresa of Calcutta

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"Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar. Mas o mar seria menor se lhe faltasse uma gota."

Madre Teresa de Calcutá

Abstract

The viability of second-generation (2G) ethanol production still requires overcoming some bottlenecks, such as the underutilization of the hemicellulosic fraction of biomass and process improvements to reach higher productivity and economic viability. In this sense, the present project aimed to study and develop strategies for efficient 2G ethanol production, taking full advantage of biomass sugars and using non-recombinant yeasts and commercial enzymes, besides evaluating processes integration for first (1G) and 2G ethanol production. Simultaneous Isomerization and Fermentation (SIF) studies were carried out with commercial yeasts (*S. cerevisiae* Itaiquara and FT858L) and enzymes (xylose isomerases IGI-HF and Sweetzyme IT extra). Both enzymes were adequate to operate under SIF conditions (pH 5.2, 35 °C), whilst the baker's yeast Itaiquara had better performance in fermenting xylulose. Mass transfer limitations in the SIF were circumvented by designing and operating the denominated "enzyme-multiple-packed bed reactor" (EMPBR), consisting of several tubes for enzyme load (53 IU/mL), which enabled its recycling in a process that resulted in ethanol productivities of about 0.87 g_{etOH}/L/h in xylose synthetic medium. The EMPBR was also applied for Simultaneous (Saccharification) Isomerization and Co-Fermentation (SICF/SSICF). The best strategy for hexoses and pentoses co-fermentation was evaluated in a defined medium mimicking industrial hydrolysate, with 12% of the total ethanol produced coming from xylose. Then, EMPBR performance was evaluated in industrial media, consisting of hemicellulosic hydrolysates mixed with pretreated solids (2G) or molasses (1G), supplemented with the commercial enzyme preparation Cellic®CTec2, under the concepts of 1G/2G process integration and whole sugarcane bagasse use. Hydrothermal hydrolysate fermented in a sequential strategy with molasses resulted in 76% of xylose conversion and 46 g/L of ethanol titer. Co-cultures with the yeasts *K. marxianus* and *S. cerevisiae*, aiming at improvements in the co-fermentation of hexoses and pentoses, were carried out in 24 deep-well plates, which allowed the screening of different culture conditions. A promising xylose conversion (83%) was achieved compared to monoculture (52%, *K. marxianus*), in hemicellulosic hydrolysate medium supplemented with nutrients (35 °C, 150 rpm, V_{medium}: 2/5 of deep-well volume, C_{Xi}: 5 g/L). Further studies of the enzymatic hydrolysis process of the entire fraction of pretreated sugarcane bagasse were conducted on a small scale, reaching 37.5% cellulose conversion with 20% (w/v) of solids after 72 h at 35 °C. Scale-up of the process was carried out in a bioreactor designed for high solid load (HSL) processes. Solid feeding strategies were evaluated, achieving 64 and 75% cellulose conversion with 22.5% solids (w/v) after 72 h at 35 and 50 °C, respectively. Simultaneous Saccharification and Co-Fermentation (SSCF) with 15% solids were conducted with co-culture under the optimal conditions found in the 24-well plate study, resulting in 98% glucose and 52% xylose conversions, respectively, in 72 h, ethanol yield of 0.23 g_{etOH}/g_S and productivity of 0.28 g_{etOH}/L/h. However, cell viability loss was observed during the HSL bioreactor operation, requiring modifications in the culture strategy. Overall, a great advance in the 2G processes using non-recombinant yeasts and commercial enzymes was achieved, as well as promising results for integrating the 1G/2G ethanol production processes.

Resumo

A viabilização da produção de etanol de segunda geração (2G) ainda requer a superação de alguns gargalos, como a subutilização da fração hemicelulósica da biomassa e melhorias no processo para aumentar sua produtividade e alcançar viabilidade econômica. Nesse sentido, o presente projeto visa estudar e desenvolver estratégias para produção eficiente de etanol 2G, aproveitando integralmente os açúcares das frações celulósicas e hemicelulósicas da biomassa e utilizando leveduras não recombinantes e enzimas comerciais, além de avaliar a integração dos processos de produção de primeira e segunda geração (1G/2G). Estudos de Isomerização e Fermentação Simultâneas (SIF) foram realizados com leveduras (*Saccharomyces cerevisiae* Itaiquara e FT858L) e enzimas (xilose isomerases IGI-HF e Sweetzyme IT extra) comerciais. Ambas as enzimas foram adequadas para operar em condições SIF (pH 5,2; 35 °C), enquanto a levedura de panificação (Itaiquara) teve melhor desempenho na assimilação de xilulose. Limitações de transferência de massa observadas durante a SIF foram contornadas através do projeto e operação do denominado "reator de múltiplos leitos empacotados de enzima" (EMPBR), o qual é composto por vários tubos para carga das enzimas (53 UI/mL), permitindo seu reciclo. Produtividades em etanol de aproximadamente 0,87 g_{etOH}/L/h foram alcançadas em SIF conduzidas em meio sintético com xilose. O EMPBR também foi utilizado para estudos de Isomerização (Sacarificação) e Co-Fermentação Simultâneas (SICF/SSICF). A melhor estratégia para co-fermentação de hexoses e pentoses foi avaliada através de cultivos em meio definido, mimetizando hidrolisados industriais, resultando em 12% do etanol produzido vindo apenas de xilose. Posteriormente, avaliou-se o desempenho do EMPBR em meio industrial, composto por misturas de hidrolisados hemicelulósicos, sólidos pré-tratados (2G) e melaço (1G), suplementado com o coquetel enzimático comercial Cellic®CTec2, contemplando a integração 1G/2G. Fermentação conduzida com hidrolisado hidrotérmico e melaço em estratégia sequencial resultou em 76% de conversão de xilose e 46 g/L de etanol. Co-culturas com as leveduras *K. marxianus* e *S. cerevisiae*, visando melhorias na co-fermentação de hexoses e pentoses, foram realizadas em placas de 24 poços, o que permitiu a triagem de diferentes condições de cultivo. Conversão promissora de xilose (83%) foi alcançada em comparação com a monocultura (52%, *K. marxianus*), em meio contendo hidrolisado hemicelulósico suplementado com nutrientes (35 °C, 150 rpm, V_{meio}: 2/5 do volume do poço, C_{Xi}: 5 g/L). Estudos do processo de hidrólise enzimática de ambas as frações pré-tratadas do bagaço de cana-de-açúcar foram conduzidos em pequena escala, alcançando 37,5% de conversão de celulose com 20% (p/v) de sólidos após 72 h a 35 °C. O escalonamento do processo foi realizado em um biorreator projetado para processos com alta carga de sólidos (ACS). Estratégias para alimentação de sólidos foram avaliadas, sendo alcançados 64 e 75% de conversão de celulose com 22,5% de sólidos (p/v) após 72 h a 35 e 50 °C, respectivamente. Sacarificação e Co-Fermentação Simultâneas (SSCF) foram conduzidas em co-cultura com 15% (p/v) de sólidos, nas condições ótimas encontradas no estudo em placa de 24 poços, resultando em 98 e 52% de conversões de glicose e xilose, respectivamente, em 72 h, com 0,23 g_{etOH}/g_S e 0,28 g_{etOH}/L/h de rendimento e produtividade em etanol. No entanto, perda de viabilidade celular foi observada durante a operação do biorreator com ACS, exigindo modificações na estratégia de cultivo. Conclui-se que o presente projeto permitiu alcançar grande avanço nos processos 2G usando leveduras não recombinantes e enzimas comerciais, bem como resultados promissores para a produção integrada de etanol 1G/2G.

Summary

Acknowledgments 5
Agradecimentos 6
Abstract 7
Resumo 8
Figure captions 10
Table captions 13
Acronyms and abbreviations 15
Chapter 1. Introduction 16
Purpose 21
Specific purposes 21
Chapter 2. Bioreactor and process design for 2G ethanol production from xylose using industrial *S. cerevisiae* and commercial xylose isomerase 23
Chapter 3. Integrated first and second-generation ethanol production through a sequential process in an enzyme-multiple-packed bed reactor 52
Chapter 4. High throughput screening of yeast co-culture using crude hydrolysate for co-fermentation of pentose and hexose 75
Chapter 5. Solid feeding and co-culture strategies as tools for efficient enzymatic hydrolysis and ethanol production in a helical stirred bioreactor at high solid loads of sugarcane bagasse 97
Chapter 6. General conclusions and future perspectives 120
References 123
Supplementary material (Chapter 2) 135

Figure captions

Figure 1.1. Illustrative scheme of the steps performed in the SIF, SICF, SSCF, and SSICF processes. 19

Figure 2.1. Illustrative scheme **(a)** and real picture **(b)** of the NSR; Enzyme bag **(c)**; illustrative scheme **(d)** and real picture **(e)** of EBagR; Enzyme-multiple-packed bed module **(f)** and running process in the EMPBR **(g)**. All reactors were specially designed and set up for running the proposed SIF strategy. (NSR: Nitrogen sparging reactor; EBagR: Enzyme bag reactor; EMPBR: Enzyme-multiple-packed bed reactor). 30

Figure 2.2. Influence of pH on IGI-HF and Sweetzyme activities, measured at 35 °C. Relative activity was calculated having pH 7.0 as a reference (100%). [a, b, c, d] the Tukey test indexes with 95% confidence. Different letters mean statistically different results. 33

Figure 2.3. Residual activities of the xylose isomerase´s Sweetzyme and IGI-HF, measured at 60 °C after incubation at typical SIF temperature (35 ºC) at different pH **(a, b)**, ethanol **(c, d)**, and acetic acid **(e, f)** concentrations. Incubation in ethanol and acetic acid solutions carried out at pH 5.2 (SIF condition). Error bars are standard deviations of the duplicates. [a-h] Tukey test indexes with 95% confidence. Different letters mean statistically significant differences between the last experimental point of each condition. 36

Figure 2.4. Xylose conversion and isomerization rate profiles during SIF; **(a)** and **(b) -** different S. cerevisiae strains FT 858L and Itaiquara ($[Xylose]_{Initial}$: 105 g/L, static); **(c)** and **(d) -** Fermentations with and without magnetic stirring ($[Xylose]_{Initial}$: 65 g/L). Cell load: 50 g_{DCW}/L, IGI-HF: 120 IU/mL, 35 °C, pH_i: 5.20. Standard deviations are less than 10% 38

Figure 2.5. Profiles of xylose conversion and ethanol production for the SIF in **(a)** N_2 sparging reactor (NSR) (4 vvm), IGI-HF: 120 IU/mL; **(b)** Enzyme bag reactor (EBagR), IGI-HF: 53 IU/mL; **(c)** the three batches (B1, B2, and B3) performed in the Enzyme-multiple packed bed reactor (EMPBR), IGI-HF: 53 IU/mL. $[Xylose]_{initial}$: 65 g/L, 35 °C, pH_i: 5.20. Itaiquara: 50 g_{DCW}/L. 44

Figure 3.1. Simplified flowchart of the integrated 1G and 2G ethanol production process proposed. 60

Figure 3.2. Fermentation indexes **(a)** Xylose isomerization rate (r_{xylose}), **(b)** Xylose and total sugars conversion (X_{xylose} and $X_{totalsugars}$), **(c)** Overall ethanol productivities, and **(d)** Ethanol titers reached at the end of Strategies 1 to 5, within 11.5 h of fermentation (total time), except S4* (1.5 h).

Different letters on top of the bars mean statistically significant differences between the strategies, pair-compared (Tukey's test, 95% of confidence), for each index. 64

Figure 3.3. Hexoses (sucrose, glucose, fructose, and cellobiose), pentoses (xylose and XOs*) concentration profiles and ethanol production in the batch fermentations conducted with **(a)** AH+M, **(b-d)** HH+M (B1, B2, and B3), and **(e)** HH+ HPS+M. Molasses feeding was done at 15 h. The banners in the upper part of the figure indicate the duration of each step. IGI-HF: 53 IU/mL, CC2: 315 IU/mL (for HH), C_X: 50 g_{DCW}/L, 35 °C, pH_0: 5.20. *XOs were only present in the media with HH. AH: Acid hydrolysate, HH: Hydrothermal hydrolysate, HPS: Hydrothermally pretreated solids, M: Molasses. 67

Figure 4.1. Sugar conversion (glucose, Gluc HHm, and xylose, Xyl HHm) and biomass concentration (Cx HHm) obtained during the culture of evolved K. marxianus strains **(a)** 1 A, **(b)** 2 A, **(c)** 3 A, and **(d)** 4 A in hemicellulosic hydrolysate medium (HHm), 35 °C, 250 rpm, C_{X0}: 1 g/L, pH_0: 5. Different letters at the top of the graph symbols represent statistically significant differences for conditions at the last point, pair-compared, according to Tukey's test. 84

Figure 4.2. Sugar conversion (glucose and xylose) and biomass concentration obtained during the co-culture of K. marxianus 3 A and S. cerevisiae in synthetic (S) and hemicellulosic hydrolysate (HHm) media with aeration conditions **(a)** 1:5 (2 mL) and **(b)** 1:2.5 (4 mL), 35 °C, 150 rpm, C_{X0}:1 g/L, pH_0:5. 85

Figure 4.3. Sugar conversion (glucose and xylose) and biomass concentration obtained during the co-culture in S, HHm and HHs media with S. cerevisiae: K. marxianus 3 A cell ratio of **(a)** 1:5 (C_{X0}: 3 g/L); **(b)** 1:9 (C_{X0}: 5 g/L), and **(c)** 1:1 (C_{X0}: 5 g/L) (plus HHs), 150 rpm, and **(d)** 1:5 (C_{X0}: 3 g/L) and **(e)** 1:1 (C_{X0}: 5 g/L), with 100 rpm of agitation. Aeration ratio: 1:2.5, $V_{deep\text{-}well}$: 4 mL, 35 °C, pH_0:5.. 91

Figure 4.4. Sugar conversion (glucose and xylose) and biomass concentration in S and HHs media during the sequential fermentation with S. cerevisiae: K. marxianus 3 A (2.5:2.5 g/L), **(a)** 100 rpm, and **(b)** 100 (24 h) and 150 rpm (72 h) (K. marxianus 3 A inoculation was at 24 h), and 72 h mono-culture (C_{X0}: 5 g/L) of K. marxianus 3 A at **(c)** 150 rpm, and S. cerevisiae at **(d)** 100 rpm, 35 °C, aeration ratio: 1:2.5, $V_{deep\text{-}well}$: 4 mL, pH_0: 5. 94

Figure 5.1. Cellulose conversion (CC) **(a)**, xylose yield (XY) **(b)**, and total sugars* concentration **(c)** after enzymatic hydrolysis (72 h) of PSB and HH using Cellic®Ctec2 at 35 and 50 ºC, 50-mL flasks with 20 mL HH and 15 or 20% of the solid load (w/v), 45 FPU/$g_{cellulose}$, PEG (0.05

g/$g_{drybiomass}$), pH_0: 5, 150 rpm. Error bars are standard deviations of the duplicates. Different letters at the top of the bars mean statistically significant differences between conditions, pair-compared (Tukey's test). *Glucose, xylose, arabinose, and cellobiose.. 106

Figure 5.2. Concentrations of glucose, xylose, and ethanol during SSCF co-culture of S. cerevisiae and K. marxianus 3A **(a, b)**; mono-culture of K. marxianus 3A **(e);** and PSSSCF co-culture **(c, d)** in 50-mL flasks and 24 deep-well plates (10 mL). Error bars are standard deviations of the duplicates. Dashed lines represent the control experiment; solid lines represent the cultures (C_{X0}: 5 g/L, 35 ºC, pH_0: 5, 150 rpm, Cellic®Ctec2: 45 FPU/$g_{cellulose}$, PEG: 0.05 g/$g_{drybiomass}$). 109

Figure 5.3. Cellulose conversion **(a)** and xylose yield **(b)** correlated to accumulated solid load (black diamonds connected by a black line) during fed-batch enzymatic hydrolysis in BTB at 35 and 50 °C. The labels B1 to B6 represent each sequential batch at the corresponding time in which each solid feeding was performed. Error bars are standard deviations of the duplicates. Cellic®Ctec2: 45 FPU/$g_{cellulose}$, PEG (0.05 g/$g_{drybiomass}$), pH_0: 5, 150 rpm, HH+ PSB (700 mL). 112

Figure 5.4. Concentrations of glucose, xylose, and ethanol, and solid load (%) during SSCF with solid fed-batch in HSB-SF1 **(a)** Dashed lines represent the control experiment (EH) (no cells) while solid lines represent the co-culture (C_{X0}: 5 g/L, 35 ºC, 150 rpm). Error bars are standard deviations of the duplicates. **(b)** Torque, pO_2, and pH during the 72 h fermentation, red dashed lines indicate solids feed times. 114

Figure 5.5. Compounds concentrations and solid load (SL) during SSCF with SF2 strategy for solid fed-batch in HSB **(a)** Dashed lines represent the control experiment (EH) (no cells) while solid lines represent the co-culture (C_{X0}: 5 g/L, 35 °C, 150 rpm). **(b)** Torque, pO_2, and pH during the 72 h SSCF. Red dashed lines indicate solids feed times. **(c)** Cell viability followed by plating at three sampling times (SSCF). Compounds and biomass concentrations during SqSCF **(d)** (C_{X0}: 5 g/L, 35 ºC, 150 rpm). **(e)** Torque, pO_2, and pH during the 72 h SqSCF. Error bars are standard deviations of the duplicates. 116

Table captions

Table 2.1. Xylulose, pH, and calculated fermentation indexes obtained in SIF performed with **(a)** *S. cerevisiae* FT858L and Itaiquara ($[Xylose]_{Initial}$: ~105 g/L, static); And in static and magnetic stirred configuration **(b)** ($[Xylose]_{Initial}$: 65 g/L). Cell load: 50 g_{DCW}/L, IGI-HF: 120 IU/mL, pH_i: 5.20, 35 °C. Standard deviations were lower than 10%. Different letters in front of the performance index values mean significant differences between them, pair-compared (Tukey's test, 95% of confidence). 40

Table 2.2. Culture time, pH, cell viability, and performance indexes (real and estimated*- NSR*) obtained at the batches performed in the N_2 sparging reactor (NSR) (4 vvm), IGI-HF: 120 IU/mL (**a**), Enzyme bag reactor (EBagR) **(b)**, and Enzyme-multiple-packed bed reactor (EMPBR) **(c)**, IGI-HF: 53 IU/mL. $[Xylose]_{initial}$: 65 g/L, Itaiquara: 50 g_{DCW}/L, static, 35 °C, pH: 5.20. Standard deviations were less than 10%. Different letters in front of the performance index values mean statistically significant differences between them, pair-compared (Tukey's test, 95% of confidence). 45

Table 2.3. Main fermentation performance indexes for the present study and reported SIF processes. 49

Table 3.1. Sugarcane bagasse liquors (Acid- AH, and hydrothermal hydrolysates- HH), hydrothermally pretreated solid fraction (HPS), and molasses (M) composition. Standard deviations were lower than 10% 58

Table 3.2. Fermentation strategies evaluated to improve xylulose uptake rate. 59

Table 3.3. Fermentation indexes for 2 steps fermentations conducted in **(a)** Defined medium (S1), and industrial ones, **(b)** Acid (AH+M), **(c)** Hydrothermal hydrolysates (HH+M), and (d) Whole fraction (HH+HPS+M) media. IGI-HF: 53 IU/mL, CC2: 315 IU/mL (for HH), C_{X0}: 50 g_{DCW}/L, 35 °C, pH_0: 5.2. Standard deviations were less than 10%. Different letters in front of the performance index values mean statistically significant differences between them, pair-compared (Tukey's test, 95% of confidence)..................... 70

Table 4.1. Chemical composition of sugarcane bagasse hemicellulosic hydrolysate (HH) obtained by hydrothermal pretreatment (10% (w/v) of solids, dry basis, 195 °C for 45 min). 79

Table 4.2. Final ethanol concentration (C_{etOH}), cell ($Y_{X/S}$), ethanol ($Y_{etOH/S}$), xylitol ($Y_{xylOH/S}$) yields, and xylose conversion (X_{xylose}) in different media (HHm, HHs, and S) and culture conditions

evaluated in co-culture studies. Values are replicates of averages, with less than 10% standard error. Glucose was depleted at a maximum of 48 h in all studies. Different letters in front of the values represent statistically significant differences between them, pair-compared for each condition in each study, according to Tukey's test (95% of confidence). 89

Table 5.1. Characterization of the solid (PSB) and liquid (HH) fractions of pretreated sugarcane bagasse obtained by hydrothermal pretreatment (10% w/v of solids, 195 °C for 45 min). 101

Table 5.2. Fermentation performance parameters for SSCF and PS SSCF co-culture (*S.cerevisiae* + *K.marxianus* 3A)/ mono-culture (*K.m.* 3A) in Flasks/ deep-well plates (C_{X0} of 5 g/L, 35 ºC, pH_0: 5, 150 rpm, Cellic®Ctec2: 45 FPU/$g_{cellulose}$, PEG: 0.05 g/$g_{drybiomass}$). Standard deviations were lower than 10%. Different letters mean statistically significant differences between the values pair-compared for each parameter (Tukey's test), 95% of confidence. 111

Acronyms and abbreviations

- **1G:** First-generation ethanol production;
- **2G:** Second-generation ethanol production;
- **GMOs:** Genetically modified organisms;
- **SIF:** Simultaneous Isomerization and Fermentation;
- **SICF:** Simultaneous Isomerization and Co-Fermentation;
- **SSCF:** Simultaneous Saccharification and Co-Fermentation;
- **SSICF:** Simultaneous Saccharification Isomerization and Co-Fermentation;
- **SqSCF:** Sequential Saccharification and Co-Fermentation;
- **PS:** Pre-saccharification;
- ***S. cerevisiae*:** *Saccharomyces cerevisiae;*
- ***K. marxianus*:** *Kluyveromyces marxianus;*
- **XI:** Xylose isomerase;
- **HSL:** High solid load;
- **CC2:** CellicCtec2;
- **IGI-HF:** Gensweet IGI-HF;
- **Sweetzyme:** Sweetzyme IT extra;
- **Y:** Product yield (ethanol or xylitol);
- **Q_P:** Overall ethanol productivity;
- **q_P:** Specific ethanol productivity;
- **X:** Sugar conversion;
- **S:** Selectivity ethanol/xylitol;
- **r_S:** Sugar uptake rate;
- **C_P:** Product titer (ethanol or xylitol);
- **g_{DCW}:** Grams of dry cell weight;
- **g_{DER}:** Grams of enzyme derivative.

Chapter 1. Introduction

Environmental concerns, as well as the high fluctuation in the prices of fossil-derived products, have motivated researchers in the development of renewable alternatives (CUNHA et al., 2019a). Among them, biofuels stand out due to their success in reducing the market energy dependence on fossil fuels and, consequently, decreasing greenhouse gas emissions. In the Brazilian scenario, bioethanol, produced from sugarcane sugars in a process denominated as first generation (1G), is a strong competitor in the transport sector, given that the country has a large fleet of *flex-fuel* vehicles (CHANDEL et al., 2021; MAITAH et al., 2019). However, to increase its production, an extension in the arable lands intended for sugarcane crops would be necessary, generating competition with those used for food crops. To deal with this impasse, second-generation (2G) processes, which address the use of carbon sources derived from lignocellulosic materials, have been widely studied (CHANDEL et al., 2021).

Lignocellulosic materials, also called biomasses, are the world's most available residues/coproducts. They are mainly composed of cellulosic and hemicellulosic fractions, rich in hexose and pentose sugars, respectively, in proportions that vary for each material (MUSSATTO; DRAGONE, 2016). Generally, these biomasses can be obtained directly at the industries, favoring their further use *in loco*. In the Brazilian sugar-ethanol industry, bagasse is the main coproduct from the sugarcane milling process, with a generic composition of approximately 50% cellulose, 25% hemicellulose, and 25% lignin (CANILHA et al., 2012). Currently, most of this material is burned in the industrial plants themselves to produce energy for the process (steam and electricity), but the remaining fraction still has important content in sugars that can be converted into value-added products (CHANDEL et al., 2021).

Many improvements have already been done to the 2G ethanol production processes, such as defining conditions and strategies for biomass pretreatment, enzymatic hydrolysis, and fermentation (CHANDEL et al., 2021; DOS SANTOS et al., 2016; HANS et al., 2021; MILESSI et al., 2020c; MODENBACH; NOKES, 2013; PRATTO et al., 2020). However, 2G processes still did not reach market competitive prices. Process viability is reported as dependent on the whole use of biomass sugars in a biorefinery scenario, taking maximum advantage of these materials, besides integrating the 1G/2G production processes, for energy and bioproducts, contributing to a

circular economy (CHANDEL et al., 2021; CHERUBINI, 2010). For that, many bottlenecks still need to be overcome.

Biomass pretreatment and enzymatic hydrolysis are steps required for the 2G process and are the main differences between the 1G and 2G production routes. These steps are responsible for providing fermentable sugars for the later ones since the sugars are not directly available in the lignocellulosic biomass (CHANDEL et al., 2021). The fermentation of hexoses like glucose by microorganisms is a well-established process that results in high product yields, but pentoses, such as xylose, the second most available sugar in sugarcane bagasse, require specific microorganisms/strategies for its assimilation. The yeast *S. cerevisiae*, a robust microorganism widely used for the 1G ethanol process, cannot assimilate xylose, which impairs ethanol production from pentoses.

There are two main routes for xylose conversion into ethanol: *ex-vivo* and *in-vivo*. Currently, the most popular is the *in vivo* route, which mainly applies genetically modified organisms (GMOs) capable of directly assimilating xylose, such as recombinant *S. cerevisiae* (JO et al., 2016; MILESSI et al., 2020a, 2020c; PEREZ et al., 2021). However, the use of GMOs in industrial environments is still limited, mainly due to possible competition with wild/contaminating strains and difficulties in large-scale implementation in countries with strict biosafety regulations like Brazil (MILESSI et al., 2020b). *Kluyveromyces marxianus, Scheffersomyces shehatae,* and *Scheffersomyces stipitis*, among other natural pentose fermenting strains (*in-vivo*), are an alternative to the recombinants (ROBAK; BALCEREK, 2018). In addition, *K. marxianus* is thermotolerant, favoring its use in the industrial ethanol production process, which commonly faces problems of loss of cell viability, related to temperature increase in the fermentation vessels (HUA et al., 2019). However, natural pentose fermenting strains tend to have low ethanol productivity and yield, as well as low tolerance to hydrolysate inhibitors. In addition, they require specific culture conditions (ROBAK; BALCEREK, 2018), which brings the need of developing strategies for process improvement.

In the *ex-vivo* route, enzymes such as xylose isomerases (XI, E.C. 5.3.1.5) are added to the process. They catalyze the isomerization of xylose into xylulose, an isomer that can be assimilated by native *S. cerevisiae*. The isomerization reaction is reversible with equilibrium around 1 xylulose:5 xylose (SILVA et al., 2012). Simultaneous Isomerization and Fermentation (SIF) processes, in which isomerization and fermentation are carried out simultaneously in the same

reactor, favor shifting the equilibrium towards xylulose production, increasing xylose conversion. However, differences between the optimal conditions for both processes are the main bottleneck for the good performance of SIF (RAO et al., 2008), requiring optimization studies. An intensification of the SIF process can be reached through the whole use of biomass sugars (hexoses and pentoses) in high-performance reactors, through Simultaneous Isomerization and Co-Fermentation (SICF), and represent the main motivation of the present doctoral project.

The intensification of 2G ethanol production can also be achieved through Simultaneous Saccharification and Co-Fermentation (SSCF). In this strategy, while the cellulolytic and hemicellulolytic enzymes hydrolyze the biomass polymer fractions, the microorganism assimilates the released sugars gradually converting them into ethanol and other by-products. Simultaneous Saccharification, Isomerization, and Co-Fermentation (SSICF) is another alternative that could be performed with the introduction of XI enzymes, enabling the use of native/industrial *S. cerevisiae*. SSCF is conducted with commercial enzyme preparations using recombinant *S. cerevisiae* to assimilate xylose or natural xylose-fermenting yeasts such as *K. marxianus* (HUA et al., 2019; LI et al., 2019). Co-culture strategies, which combine strains with different abilities, have also been studied for application in SSCF (NOSRATI-GHODS et al., 2018). In the saccharification process, to obtain the necessary ethanol concentrations (8 to 11% v/v) to enable the distillation step (PEREIRA et al., 2018), it is important to operate the reactors with high solid loads (HSL) to reach the desired sugar concentration (ROBERTO et al., 2020). However, this leads to additional challenges in reactor operation, related to the low density of some biomasses, such as sugarcane bagasse, and the high viscosity of suspensions containing HSL (AFEDZI; RATTANAPORN; PARAKULSUKSATID, 2022). In addition to the need of selecting appropriate conditions to conduct the process, such as pretreatment, enzyme, microorganism, and the type of bioreactor (ROBERTO et al., 2020). An illustrative scheme of the mentioned processes (SIF, SICF, SSCF, SSICF) is presented in **Fig. 1.1**.

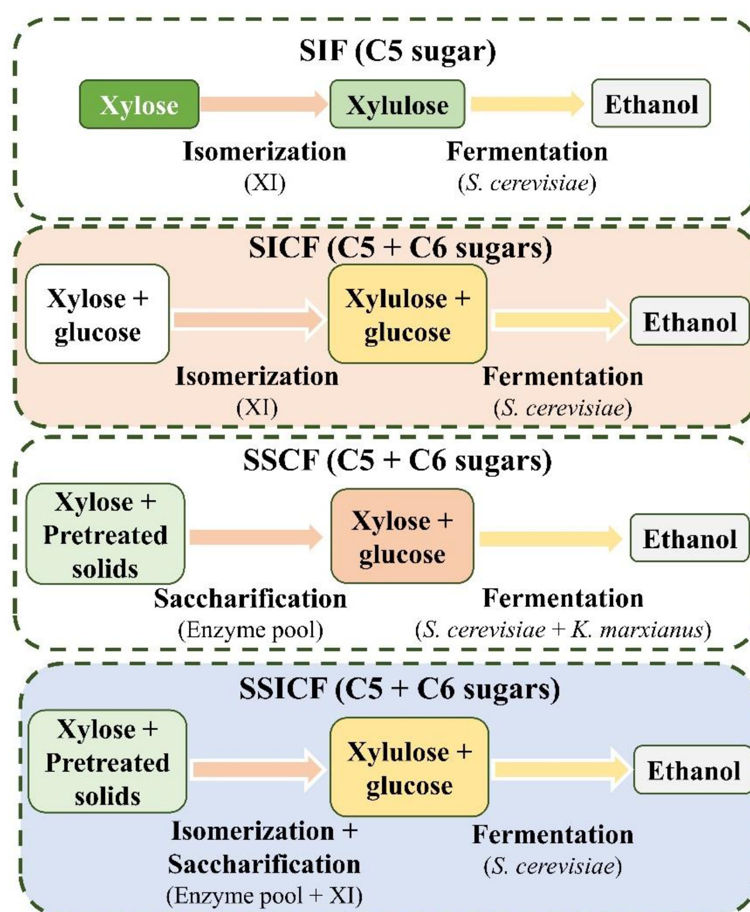

Figure 1.1. Illustrative scheme of the steps performed in the SIF, SICF, SSCF, and SSICF processes.

The importance of 1G/2G integration is evident for the technical and economic feasibility of producing 2G ethanol with greater productivity, as well as the exploration of this technology in the use of the hemicellulosic fraction in the context of the biorefinery, which is a valuable source of raw material and currently underused. The proposal for an integrated process for the production of 1G/2G ethanol deposited with the INPI as a patent privilege of the invention in December 2019 (MILESSI-ESTEVES et al., 2019b), describes this technology, which will be developed in this doctoral project. So, this doctoral thesis intends to present and discuss the achieved advances related to the previously reported challenges, through the study and development of technologies for SIF, SICF, SSICF, and SSCF processes, embracing the use of hemicellulosic and cellulosic fractions, in operational conditions compatible with those used in the current industrial plants. As a basis for the development of this work, the progress in the development of 2G ethanol production processes, by researchers from the Laboratory of Enzymatic Technologies (LabEnz) and the

Laboratory of Development and Automation of Bioprocesses (LaDABio) at DEQ/UFSCar (GIORDANO et al., 2014; MILESSI-ESTEVES et al., 2019a, 2019b; MILESSI et al., 2020b, 2020c; PEREZ et al., 2021; SILVA et al., 2012) was considered. This doctoral project is part of the thematic project “From the cell factory to the integrated biodiesel-bioethanol biorefinery: a systemic approach applied to complex problems at micro and macroscales” (FAPESP process 2016/10.636-8).

Purpose

The main purpose of this doctoral project is the study of integrated fermentation strategies for efficient 2G, with the potential for integration with the 1G production, taking full advantage of the sugar fractions from lignocellulosic biomass, and using non-GMO yeast strains and commercial enzymes.

Specific purposes

i) Evaluate commercial xylose isomerases' performance by characterizing their activities and stabilities in SIF conditions and in the presence of inhibitors commonly found in industrial media;

ii) Study and improve the SIF using commercial enzyme and yeast, evaluating different culture strategies;

iii) Study Simultaneous (Saccharification) Isomerization and Co-Fermentation (SICF/SSICF) technology for 1G/2G integration in a medium prepared with sugarcane derivatives (molasses, hemicellulosic hydrolysate, and pretreated bagasse), in single and repeated batches;

iv) Evaluate and define the best co-culture (*K. marxianus* and *S. cerevisiae*) conditions for hexoses and pentoses co-fermentation in hemicellulosic hydrolysate (HH);

v) Study the enzymatic hydrolysis process using the whole pretreated fraction of sugarcane bagasse, and develop an efficient strategy for bioreactor operation with high solid load;

vi) Evaluate yeast co-culture in a medium containing hemicellulosic hydrolysate and pretreated bagasse with high solid load in different culture strategies (SSCF and pre-saccharification + SSCF).

This doctoral thesis presents the studies carried out to achieve the proposed purposes, and from now on will be divided into 5 main chapters. Chapter 2, entitled "Bioreactor and process design for 2G ethanol production from xylose using industrial *S. cerevisiae* and commercial xylose isomerase" comprises the progress made in SIF studies. Chapter 3 is entitled "Integrated first and second-generation ethanol production through a sequential process in an enzyme-multiple-packed bed reactor" and addresses SICF and SSICF studies using industrial media. Both were conducted at the Federal University of São Carlos (UFSCar). Chapters 4 e 5, on the other hand, refer to the results obtained during the one-year split-site doctorate held at Denmark Technical University

(DTU), in the BCBT (Biomass Conversion and Bioprocess Technology) group. Chapter 4 is entitled “High throughput screening of yeast co-culture for pentose and hexose co-fermentation of crude hydrolysate”, and provides valuable information on the improvements of using a yeast-yeast co-culture for co-assimilation of pentoses and hexoses. Chapter 5 is entitled “Helical stirred bioreactor as a tool for efficient enzymatic hydrolysis and 2G ethanol production from the whole fraction of pretreated sugarcane bagasse using non-GMO yeast co-culture”, and its main focus is to present hydrolysis and SSCF co-culture studies performed using the whole pretreated biomass with high solid loads (HSL). The scale-up of the process was carried out in a bioreactor for processes with HSL. Finally, the general conclusions and future perspectives for the present project are presented in Chapter 6.

Chapter 2. Bioreactor and process design for 2G ethanol production from xylose using industrial *S. cerevisiae* and commercial xylose isomerase

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Abstract

Second-generation ethanol has gained attention since the increase in demand for renewable fuels, but some issues still impair its feasibility, such as the conversion of xylose from biomass. Many routes can be explored for this purpose, but Simultaneous Isomerization and Fermentation (SIF) stand out due to the possibility of working with robust, non-genetically modified *Saccharomyces cerevisiae* strains. This work addresses bioreactor and process designs for SIF, using high enzyme and yeast load in their commercial forms, that can be introduced in the current ethanol-sugar industry. Characterization of two xylose isomerases (IGI-HF and Sweetzyme) in SIF conditions showed that both are suitable for the process. Of the two industrial yeast strains evaluated, *S. cerevisiae* "Itaiquara" (baker's yeast) was selected due to its superior xylulose assimilation. Problems with mass transfer and cell damage due to the presence of enzyme particles at high loads were faced, and different strategies were evaluated. Promising ethanol productivity (0.87 g_{EtOH}/L/h, for 50 g_{DCW}/L and 53,000 IU/L) was achieved using an enzyme-multiple-packed bed reactor. In addition, it allowed easy enzyme recovery and reuse, enabling its application in repeated batch operations integrated into the 1G ethanol process.

Keywords: Bioreactor design; Simultaneous Isomerization and Fermentation (SIF); 2G ethanol; Xylose isomerase; Mass transfer; Hemicellulose.

2.1. Introduction

Renewable biofuels attract much attention due to their benefits in environmental and economic aspects. Worldwide, the bioethanol market has made remarkable progress recently, showing a growth perspective from US$ 33.7 billion (2020) to US$ 64.8 billion by 2025 (MARKETS AND MARKET, 2020). North America takes the first position regarding its production, followed by Brazil. In the Brazilian scenario, ethanol fuel has a high demand mainly due to the large existing fleet of flex-fuel vehicles (more than 85%), proving good engine efficiency (MAITAH et al., 2019). In addition, the use of ethanol as a fuel blend is mandatory in the country, and this biofuel has competitive prices with non-renewable ones.

To increase ethanol production, second-generation (2G) processes, which encompass the use of lignocellulosic biomass sugars, have been widely studied. The use of naturally abundant and easily accessible raw materials can lead to boosting biofuel production, without needing to extend arable land and compete with food crops (RASTOGI; SHRIVASTAVA, 2017). In the biorefinery context, 2G ethanol production highly contributes to sustainable and carbon-neutral processes (ÖZDENKÇI et al., 2017), but its economic feasibility requires the use of whole sugar fractions (cellulose and hemicellulose) from biomass (MILESSI et al., 2020c).

After glucose, released during the hydrolysis of cellulose, xylose is the second-largest carbon source available in lignocellulosic materials, coming from their hemicellulosic fraction. However, pentose sugars such as xylose are more challenging to process compared to hexoses due to the difficulty of converting them into valuable products (LI; CHEN; NIELSEN, 2019). In the context of ethanol production, the robust yeast *Saccharomyces cerevisiae*, which is the most used in the sugar-energy industry, does not metabolize xylose, which is a major hindrance to biofuel production based on this sugar. Xylose assimilation for ethanol production requires either the use of in-vivo routes present in natural pentose-fermenting strains and genetically modified organisms (GMOs) or ex-vivo, in which native/industrial strains are associated with extracellular enzymes that allow xylose conversion. In general, xylose fermentation by any route is not as efficient regarding ethanol yield and productivity as the fermentation found in the 1G ethanol mills (JO et

al., 2016). Thus, developing the proper technology for xylose fermentation is crucial to achieving titers, yields, and productivities compatible with industrial standards.

The most studied in-vivo route addresses the use of recombinant *S. cerevisiae* strains, capable of directly assimilating xylose (JO et al., 2016). However, the use of GMOs in industrial environments is still limited, due to difficulties of large-scale implementation in countries with strict biosafety regulations such as Brazil (DELLA-BIANCA et al., 2013; MILESSI et al., 2020b). Yeast strains like *Kluyveromyces marxianus*, *Candida shehatae*, and *Scheffersomyces stipitis,* among other natural pentose-fermenting microorganisms, are an alternative to recombinant strains (ROBAK; BALCEREK, 2018), but generally, they reach low ethanol productivities and yield, in addition to presenting low tolerance to hydrolysate inhibitors and requiring specific process conditions (ROBAK; BALCEREK, 2018).

On the other hand, in the ex-vivo route, the robust *S. cerevisiae* strain can be used in its native form. Xylose is converted into xylulose through a reversible isomerization reaction catalyzed by the enzyme xylose isomerase (XI, E.C. 5.3.1.5), and this isomer can be assimilated by the yeast. The assimilation comprises xylulose uptake into the cell through the plasma membrane, followed by its phosphorylation into xylulose-5-phosphate by the action of the xylulokinase enzyme (XK, E.C. 2.7.1.17) and subsequently metabolization in the native pentose phosphate pathway (PPP) (CUNHA et al., 2019b). XI is also known as glucose isomerase as it can isomerize glucose into fructose, and is widely used for the industrial production of high fructose corn syrup (GIORDANO; GIORDANO; COONEY, 2000). Its optimal temperature and pH range between 60 - 80 °C and 7 - 9 (BHOSALE; RAO; DESHPANDE, 1996), respectively, with few exceptions.

The process conducted in the presence of XI and microorganisms is denominated Simultaneous Isomerization and Fermentation (SIF), consisting of the isomerization of xylose by the enzyme XI, and the simultaneous assimilation of xylulose by the yeast. This strategy favors the shift of the reversible isomerization reaction (1 xylulose:5 xylose) toward xylulose formation (MILESSI et al., 2018). Thus, in an adequate balance of enzyme and yeast concentration, xylulose can be assimilated as produced without accumulating in the culture medium (SILVA et al., 2012). However, the conduction of SIF presents some bottlenecks, such as the differences between optimal pH and temperature for the enzyme and the microorganism (MILESSI et al., 2018). Thus, studies to identify conditions that favor the performance of both are required.

The SIF technology began to be studied and implemented in the 1980s and the main issues are generally related to the low rate of xylulose assimilation and the high production of xylitol during fermentation, a compound that can inhibit XI activity (CHIANG et al., 1981; GONG et al., 1981; HAHN-HÄGERDAL; BERNER; SKOOG, 1986; LASTICK et al., 1990). Additionally, the enzyme is responsible for a significant part of the process cost, therefore its feasibility, catalyst recovery, and its recycling in the process are also important (SILVA et al., 2012).

Many strategies have been reported to improve the SIF process, such as the use of high cell concentrations and enzyme load, strain selection, the use of a higher temperature for fermentation, the use of compounds that favors the isomerization reaction towards xylulose production (sodium tetraborate), the co-immobilization of enzymes and yeasts, enzyme immobilization in different supports and/or enclosed in a compartment (CHIANG et al., 1981; HAHN-HÄGERDAL; BERNER; SKOOG, 1986; RAO et al., 2008; SILVA et al., 2012). However, considering further integration into the 1G industry, the development of a scalable technology is also required, aiming to facilitate the process implementation in the current industrial facilities. In this context, there is a lack of studies in the literature regarding processes that work in conditions that could be easily introduced into the industry, such as high cell load, use of enzymes in their commercial form, as well as operating under the repeated batch mode, coupled with good ethanol productivity and enzyme recycling.

Relevant advances have been achieved in our research group in the last 10 years regarding SIF operating conditions, such as pH, temperature, culture medium composition, use of different yeasts, and strategies for process conduction (MILESSI et al., 2018, 2020b; RAMOS et al., 2021; SILVA et al., 2012). Silva et al (2012) proposed a catalyst with XI and *S. cerevisiae* co-immobilized for use in SIF of xylose, reporting success in initial studies with about 75% conversion in 48 h (35 °C), but leaving room for improvements and process optimization. Milessi et al. (2020b) evaluated the continuous production of ethanol by SIF using the biocatalyst proposed by Silva et al. (2012) with optimized cell:enzyme proportion. The authors evaluated five different industrial yeasts, and the fresh baker's yeast Itaiquara had the best performance in the cultures (Y: 0.34 g_{EtOH}/g_S and Q_P: 2.1 g_{EtOH}/L/h). Continuous SIF were carried out in a fixed bed reactor filled with XI immobilized in chitosan and co-immobilized with *S. cerevisiae* as a biocatalyst. The continuous culture was run for 7 days and high values of ethanol yield and productivity were obtained (0.37 g_{EtOH}/g_S and 1.9 g_{EtOH}/L/h). Although immobilization in alginate beads showed to be effective to

operate at a high cell and enzyme load, confining the cells inside the beads left room for the growth of direct xylose uptake bacteria outside the beads, and contamination spread after day 8, causing the operation to halt. Furthermore, the proposed technology (immobilized biocatalyst), is not easy to implement at an industrial scale.

The present paper proposes an innovative reactor prototype and process design that combines a commercial immobilized enzyme and a suitable industrial yeast, kept as a free cell suspension culture, for the performance of SIF in repeated batches, which is compatible with the current industrial scenario of 1G ethanol production.

2.2. Material and methods

2.2.1. Enzymes, microorganisms, culture medium, and inoculum preparation

The experiments were carried out with the commercial enzymes Gensweet® IGI-HF, a xylose isomerase enzyme from *Streptomyces rubiginosus*, kindly provided by DuPont - Genencor (Palo Alto, USA), and Sweetzyme® IT Extra (G4166), from *Streptomyces murinus*, purchased from Novozymes Corporation, marketed by Sigma Aldrich. From now on they will be addressed as IGI-HF and Sweetzyme, respectively. Both products are commercialized as bulk particulate material and are not soluble in the medium.

Industrial yeasts *S. cerevisiae* FT 858L, donated by the Fermentec Ltda (Piracicaba, SP, Brazil) and *S. cerevisiae* Itaiquara® (Baker's yeast, Tapiratiba, SP, Brazil), both in lyophilized form, were used. For inoculum and SIF experiments, the culture medium (non-sterile) was prepared either with commercial glucose or xylose, in concentrations that will be described in each section. The medium was supplemented with KH_2PO_4 (5 g/L), $MgSO_4.7H_2O$ (2 g/L), $CoCl_2.6H_2O$ (0.1 g/L), and urea (1.5 g/L), similar to the defined medium used by Milessi et al. (2018). The initial pH was adjusted to 5.2 with an HCl solution (1 mol/L). The antibiotic Kamoran (4 ppm), kindly supplied by Química Real, MG, Brazil, was also added.

For inoculum preparation, the lyophilized yeasts were hydrated and activated according to Mesquita et al. (2021). The medium used for activation was prepared with glucose (65 g/L). Optical density (OD) at 600 nm and cell viability (Section 2.2.5.2) were measured before the inoculation of the SIF runs.

2.2.2. Commercial enzymes performance in SIF and industrial conditions

The commercial enzymes were characterized regarding pH influence on the enzyme activity, and stability when exposed to different pH as well as ethanol and acetic acid concentrations. The main purpose was to verify the ability of both catalysts to fulfill the requirements to work under SIF conditions (pH around 5.0 and temperature of 35 °C), which are usually applied in industrial fermentations with *S. cerevisiae* for 1G ethanol production (DELLA-BIANCA et al., 2013). Similarly, an industrial medium usually contains ethanol and acetic acid at moderate to high concentrations (DELLA-BIANCA et al., 2013; VANMARCKE et al., 2021) and it is important to assess enzyme stability under these conditions.

2.2.2.1. pH influence on enzyme activity: Enzyme activity was measured at pH values of 5, 6, and 7 at 35 °C (temperature used at SIF cultures), according to the methodology described in Section 2.2.5.1. Results were presented as relative activity related to the value measured at pH 7, considered 100% (control), which is the standard value used for optimal XI activity (MILESSI et al., 2018; SILVA et al., 2012). The relative activities at each pH were compared between both enzymes by the Tukey test (Section 2.2.6).

2.2.2.2. Enzymes stability: The stability of the enzymes incubated in different conditions was assessed by activity measurements (60 °C, pH 7), every 24 h for at least 120 h. For the periodic monitoring, samples of the enzyme were incubated in 1 mL of solutions corresponding to each condition studied in a water bath at 35 °C. Samples were taken periodically, in replicate, to measure enzymatic activity. The results were presented as a percentage of residual activity related to the initial value (100% at time 0 h) and statistically compared by Tukey's test applied to the final value of each study.

The influence of different pH (5, 6, and 7), ethanol (0, 30, and 70 g/L), and acetic acid concentrations (0, 10, 20 g/L) on the stability of each commercial enzyme were evaluated. All ranges of evaluated conditions were defined based on values that can be found in industrial processes (DELLA-BIANCA et al., 2013), and already aiming for further studies using real hydrolysates (VANMARCKE et al., 2021). The different pH buffers consisted of sodium citrate (50 mmol/L) for pH 5, and tris-maleate (50 mmol/L) for pH 6 and 7, both buffers were supplemented with $MgSO_4.7H_2O$ (50 mmol/L) and $CoCl_2.6H_2O$ (2.5 mmol/L). The different ethanol and acetic acid concentrations were prepared in a solution containing the salts described for SIF cultures (Section 2.2.1), with pH adjusted to 5.2.

2.2.3. Influence of yeast strain on SIF performance

Screening studies addressed the influence of *S. cerevisiae* strain on SIF performance. The yeasts *S. cerevisiae* FT 858L, with a great performance in hexose medium and high ethanol tolerance (MESQUITA et al., 2021), and Itaiquara, baker's yeast, with a known wide spectrum of sugars assimilation (MILESSI et al., 2020b), were inoculated in SIF medium containing about 105 g/L of initial xylose concentration. Loads of cell and enzyme (IGI-HF) were fixed at 50.0 (g_{DCW}/L) and 120 IU/mL, respectively. Fermentation batches were conducted in 15 mL mini-reactors (V_{medium}: 6 mL, V_{final}: 9 mL after enzyme addition) equipped with CO_2 exit and followed periodically by weighting to quantify the CO_2 mass released, as described by Mesquita et al. (2021). A control experiment (non-inoculated mini-reactor) was also conducted to quantify the mass lost due to evaporation. The mini-reactors were statically incubated at 35 °C. At the end of the batches, sample suspensions were centrifuged (10000 rpm, 10 min, 5 °C), the supernatants had their pH measured, and were stored (-20 °C) for further HPLC analysis.

2.2.4. SIF under improved mass transfer conditions

Due to mass transfer limitation related to the presence of the high enzyme load observed in the screening studies, several strategies were evaluated to improve the process as detailed in the sections below. The yeast *S. cerevisiae* Itaiquara (50 g_{DCW}/L) and the enzyme IGI-HF were used. All cultures were conducted at 35 °C with the defined SIF media described in Section 2.2.1. The initial xylose concentration was 65 g/L and the initial pH was 5.2.

2.2.4.1. Mini-rectors with magnetic stirring

The effect of direct agitation over fermentation performance was verified by conducting SIF experiments with and without the magnet presence (~250 rpm). IGI-HF volumetric activity in the medium was 120 IU/mL. The fermentations were performed in mini-reactors (V_{medium}: 6 mL), followed by CO_2 mass released, as described in Section 2.2.3. Samples collected at the end of the fermentations were analyzed regarding cell viability, pH, and medium composition (HPLC).

2.2.4.2. Nitrogen sparging reactor (NSR)

This reactor design was inspired by bubble column reactors and adapted to a 250-mL Schott flask reactor, containing about 130 mL of suspension (**Fig. 2.1 a-b**). The bubbling was promoted

by a continuous supply of N_2 (4 vvm) through a sintered metal (porous) sparger, and the reactor ($V_{working}$: 250 mL) was equipped with a condenser. The SIF medium (100 mL) was prepared with 65 g/L of xylose and the antibiotic ampicillin (20 ppm), as well as Kamoran, were added. IGI-HF volumetric activity was 120 IU/mL. Periodic sampling was performed, followed by centrifugation (10000 rpm, 10 min, and 5 °C) and storage for further compound analyses (HPLC). Cell viability and enzyme activity were assessed at the end of the fermentation. Ethanol drag was observed due to the N_2 flow, and therefore ethanol concentrations were estimated using the ethanol yield found in SIF without magnetic agitation (Section 2.2.4.1). The estimated values were used in the calculations of ethanol productivity and selectivity.

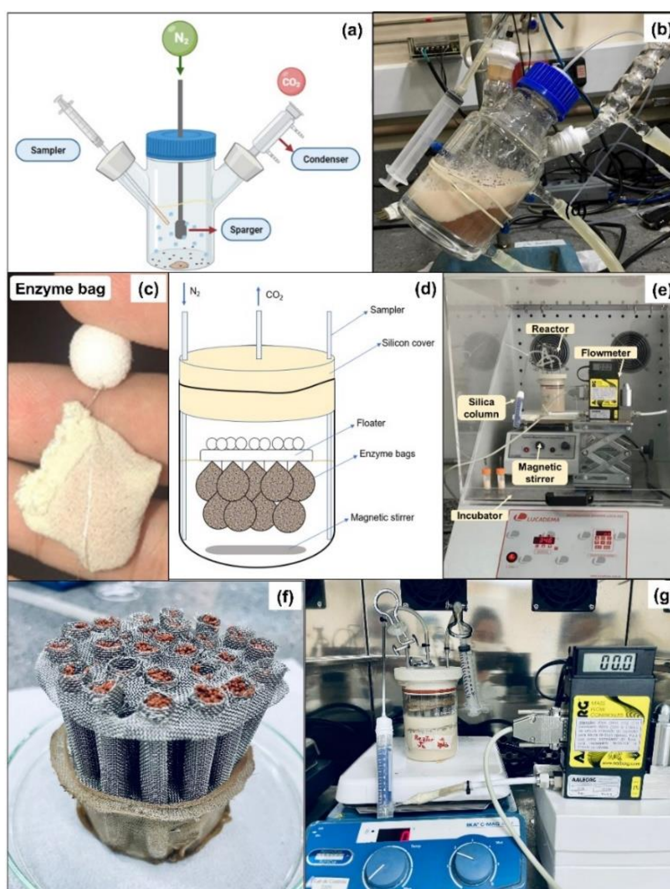

Figure 2.1. Illustrative scheme **(a)** and real picture **(b)** of the NSR; Enzyme bag **(c)**; illustrative scheme **(d)** and real picture **(e)** of EBagR; Enzyme-multiple-packed bed module **(f)** and running process in the EMPBR **(g)**. All reactors were specially designed and set up for running the proposed SIF strategy. (NSR: Nitrogen sparging reactor; EBagR: Enzyme bag reactor; EMPBR: Enzyme-multiple-packed bed reactor).

2.2.4.3. Enzyme bag reactor (EBagR)

A new reactor design (**Fig. 2.1 c-e**) was proposed aiming to improve the SIF performance. The reactor consisted of a glass vessel with a silicon rubber lid, equipped with a tube for N_2 supply and two exits, one for collecting samples and the other for a gas outlet. The gas leaving the reactor passed through a silica column before entering a flowmeter (GFC, Aalborg) connected to a notebook for online acquisition of released CO_2 flow rate and data treatment. The data acquisition interval was 60 s. The volume of CO_2 produced was estimated by the numerical integration of the flow rate data and converted to mass using the ideal gas law. A plot of total accumulated CO_2 volume as a function of time was automatically displayed on the notebook screen (**Fig. SM1**, Supplementary Material). This assembly (flowmeter, computer, and software developed for data acquisition and treatment) was set up to provide an online monitoring system, allowing easy follow-up of the fermentation progress.

The enzyme (IGI-HF) to be loaded was distributed in nylon net bags containing 1 g of the dry enzyme each, and the bags were connected to floaters (**Fig. 2.1c**). Before inoculation, 10 enzyme bags were placed inside the reactor (~5300 IU), 100 mL of medium was added and the reactor was closed. The air remaining in the headspace was stripped by passing nitrogen for a few minutes and the activated cell suspension was transferred. The assembly was maintained inside an incubator **(Fig. 2.1e)**. Samples were taken periodically and further analyzed regarding cell viability, pH, and compound concentration (HPLC). Enzyme activity was also measured at the end of the fermentation.

2.2.4.4. Enzyme-multiple-packed bed reactor (EMPBR)

In the same reactor structure used for the EBagR, a new internal module was designed, denominated as the enzyme-multiple-packed bed (**Fig. 2.1 f-g**). The in-house made module consisted of 26 tubes (inner diameter of ~7 mm and height of 70 mm) made of stainless steel screen mesh 40 to pack the enzyme and allow a good percolation of the fermentation media through it. The tubes (enzyme-multiple-packed bed) were assembled closely together (**Fig. 2.1f**) and also fixed to the silicon rubber lid, allowing easy enzyme module removal from the fermented medium at the end of each batch. Fifteen grams (dry weight) of IGI-HF was previously hydrated in SIF medium for 15 min and added to the enzyme module. The enzyme-containing module was then placed in the reactor vase (**Fig. 2.1g**) together with 150 mL of SIF medium, resulting in a volumetric activity

of 53 IU/mL. The mixing was provided through a magnet in the bottom of the reactor, kept on a magnetic stirrer, as for the EBagR. The procedure to start and follow the fermentation was the same as that described for the EBagR, also using the online monitoring system. In this reactor, 3 repeated batches were conducted. To perform them, the rubber lid with the multiple-packed bed was removed, the suspension with the cells was centrifuged, and the recovered cells were resuspended in a fresh medium, to reproduce the cell recycle operation (Melle-Boinot process). Then, the empty reactor was loaded with the suspension in fresh medium and the enzyme module plus the lid was placed again in the reactor. Cell viability was correct to 100% before beginning each new batch. Samples were taken periodically and further analyzed regarding their compounds (HPLC). Enzyme activity, cell viability, and pH were measured at the end of each batch.

2.2.5. Analytical methods

2.2.5.1. Xylose isomerase activity: It was determined by the initial reaction rate of fructose conversion into glucose, as described by Silva et al. (2012). The substrate consisted of fructose (2 mol/L) in tris-maleate buffer (50 mmol/L) (pH 7), supplemented with $MgSO_4.7H_2O$ (50 mmol/L) and $CoCl_2.6H_2O$ (2.5 mmol/L). For the activities measured at pH 6, the tris-maleate buffer was also used. At pH 5, fructose was diluted in sodium citrate buffer (50 mmol/L). Glucose concentration was determined by the commercial enzymatic kit GOD-PAP. One international unit (IU) was defined as the amount of enzyme that produces 1 μmol of glucose per minute (LADISCH; EMERY; RODWELL, 1977). Activity measurements were performed at least in duplicate.

2.2.5.2. Cell viability: It was determined by the methylene blue technique and defined as the ratio between viable cells and total cells counted in a defined space of the counting chamber (SMART et al., 1999b).

2.5.3. Substrate and product quantification: Previous to HPLC analyses, fermentation samples were prepared as described by Perez et al. (2022). The equipment Waters e2695 chromatograph, equipped with refractive index and UV-VIS detectors (210 nm) was used for compound analyses. Ethanol, glycerol, xylitol, acetic acid, xylose, and xylulose were identified using an ionic exclusion column (Rezex ROA-Organic acid H+ (8%), H_2SO_4 (5 mmol/L) as the mobile phase (0.6 mL/min) at 65 °C (PEREZ et al., 2022).

2.2.6. Calculation procedures and statistical analysis

For the experiments carried out in mini-reactors (Sections 2.2.3 and 2.2.4.1), ethanol and xylitol yields, overall ethanol productivity, ethanol/xylitol selectivity, xylose isomerization rate, and xylose conversion were calculated according to that described by Milessi et al. (2020c) and Mesquita et al. (2021) using both CO_2 released data as well as HPLC analysis. For the experiments conducted in the reactors NSR, EBagR, and EMPBR (Sections 2.2.4.2 to 2.2.4.4), all calculations were performed using HPLC data only (MILESSI et al., 2020c). The specific ethanol productivity was calculated by dividing the overall productivity by the enzyme load (in IU/L) used. The statistical significance of the differences between the results evaluated was verified by conducting the Tukey test in the Origin®Pro 8.5 software, with a confidence level of 95%.

2.3. Results and Discussion

2.3.1. Commercial enzymes performance in SIF and industrial conditions

The commercial xylose isomerases IGI-HF and Sweetzyme were characterized regarding activity and stability under common conditions found at 2G ethanol production processes.

2.3.1.1. pH influence on enzyme activity

Considering the influence of acidic pH on XI catalytic activity (SILVA et al., 2012), and the need for its application in SIF processes (pH around 5), IGI-HF and Sweetzyme were characterized regarding their activities measured at 35 °C in different pH (5, 6, and 7). The results are given as relative activity (to pH 7) and are presented in **Fig. 2.2**.

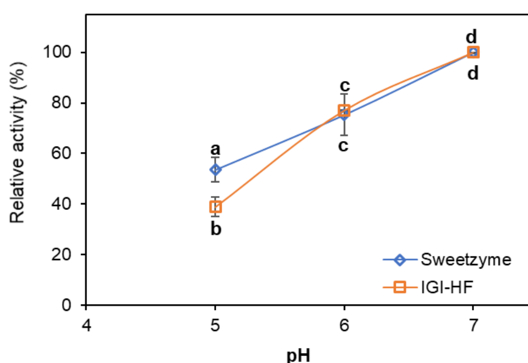

Figure 2.2. Influence of pH on IGI-HF and Sweetzyme activities, measured at 35 °C. Relative activity was calculated having pH 7.0 as a reference (100%). [a, b, c, d] the Tukey test indexes with 95% confidence. Different letters mean statistically different results.

Both enzymes showed a similar decrease in relative activity from pH 7 to 6, however, from pH 6 to 5, IGI-HF had a greater loss (38.2%) compared to Sweetzyme (21.7%). Despite the drop in relative activity, the enzymes still presented 38.1 IU/g_{der} (IGI-HF) and 65.7 IU/g_{der} (Sweetzyme) at pH 5, corresponding to relative activities of 40% and 55%, respectively. It is important to emphasize that these activities were measured at 35 °C, a temperature that will be further used at SIF, providing important information about the enzyme in similar conditions to the real process.

According to Bhosale et al. (1996), the optimal pH and temperature ranges for XI are generally between 7- 9, and 60- 80 °C, respectively. An exception reported in the literature is a XI produced by *Candida boidinii*, with maximum activity between pH 4.5- 5, however, its activity under these conditions (0.77 IU/g_{der}) is very low compared to other XI (CHANDRAKANT; BISARIA, 2000a). Miyamoto et al. (2020) evaluated a soluble XI (XylA2F1) similar to the homolog from *S. murinus* (SweetZyme®, sequence identity 95%). The authors reported a drastic drop in relative activity (60 °C) from pH 7 to 6 (about 58%), but this value remained at pH 5. This drop was higher than that observed in the present study with the Sweetzyme (46.5%, 35 °C), which could be due to the immobilization support that may protect the biocatalysts (MILESSI et al., 2020c).

Silva et al. (2012) show important results of relative activity for comparison with the present work as they also evaluated the XI enzymes aiming for further use in SIF and conducted experiments in similar conditions to those applied in the present work (35 °C, pH 5.3, and high enzyme load). The authors evaluated Genencor XI in its soluble (Gensweet® SGI) and in-house immobilized (IGI-Ch: SGI immobilized in chitosan-glutaraldehyde) forms. At pH 5.3 and 35 °C, IGI-Ch produced by Silva et al. (2012) presented similar enzymatic activity (69 IU/g_{der}) to that found in the present work for the immobilized Sweetzyme, but 1.8-fold higher than that for IGI-HF. Nevertheless, all immobilized enzymes presented lower activities at the pH required for the SIF, with relative activities decreasing to 55% and 40% for Sweetzyme and IGI-HF (**Fig. 2.2**), respectively, as well as for IGI-Ch (about 48%), compared to the values observed in pH 7.0. Thus, the use of high enzyme loads in the reactor to reach adequate reaction rates in SIF conditions are recommended. It is worth mentioning that soluble enzyme SGI had its activity reduced to about zero, highlighting once again the benefits of enzyme immobilization for special application conditions such as the SIF.

2.3.1.2. Enzyme stability in different pH

Commercial enzymes were evaluated for stability under prolonged exposure to pH 5, 6, and 7 (35 °C). The results are presented as residual activities in **Fig. 2.3 a-b**.

IGI-HF (**Fig. 2.3b**) presented a bigger drop in residual activity over time, maintaining 57.2% of its initial activity after 7 days (168 h) of incubation in a pH 5 buffer. Sweetzyme kept a higher value (77.8%) under the same conditions (**Fig. 2.3a**). Having high stability under SIF conditions is an important characteristic because it enables the reuse of the enzyme in the process for several cycles, as required for repeated batches operation commonly performed in the 1G ethanol mills. It is worth mentioning that both enzymes showed greater stability under exposure to pH 5 than at higher pH (6 and 7). Furthermore, IGI-HF did not show significant differences between pH 6 and 7, while Sweetzyme had similar results at pH 5 and 6 (Tukey's test).

Several studies present pH stability results for XI, but most of them only evaluate a short exposure time. Gaikwad and Deshpande (1992) evaluated a XI of *Streptomyces sp.* NCIM 2730 (immobilized on Indion 48-R anion exchange resin), kept at 60 °C for 20 min in different pH buffers (4.5- 8.5). The enzyme showed greater stability between pH 5- 8, with lower activities measured above and below these values, showing evidence of enzymatic inactivation in too acidic/ basic media. Hahn-Hägerdal; Berner and Skoog (1986) reported stability results of the commercial Optisweet-P (immobilized in whole cells) exposed for about 50 h to different pH buffers (4- 6) at 30 °C. The enzyme kept about 75% of its initial activity at pH 4, 5, and 5.5, while at pH 6 it was 95%.

As described, most of the reported works in the literature have evaluated XI stabilities for a very short time of exposure, making a proper comparison difficult with the results obtained in the present work. Dokuzparmak et al. (2020) was the only work found that evaluated stability for more than 5 days. The authors studied four different XI incubated at pH 6 and obtained residual activities between 30 and 80%. According to them, the differences between the results are mainly due to immobilization characteristics. Factors such as flexibility in the structure, hydrophobicity index, and amount of salt bridges influence XI's stability, and therefore the introduction of hydrogen bonds in the enzymatic structure can significantly increase it. Thus, this would be a possible explanation for the greater stability observed by the enzymes IGI-HF and Sweetzyme at pH 5 than at higher pH (6 and 7) in the present study. They are multimeric enzymes and, in the condition assessed (pH 5), there is greater availability of H^+ in the medium, and therefore the greater

possibility of hydrogen bond formation and stabilization of the quaternary structure. However, as can be seen, stability results also vary for each enzyme and immobilization type, which justifies the difference between IGI-HF and Sweetzyme.

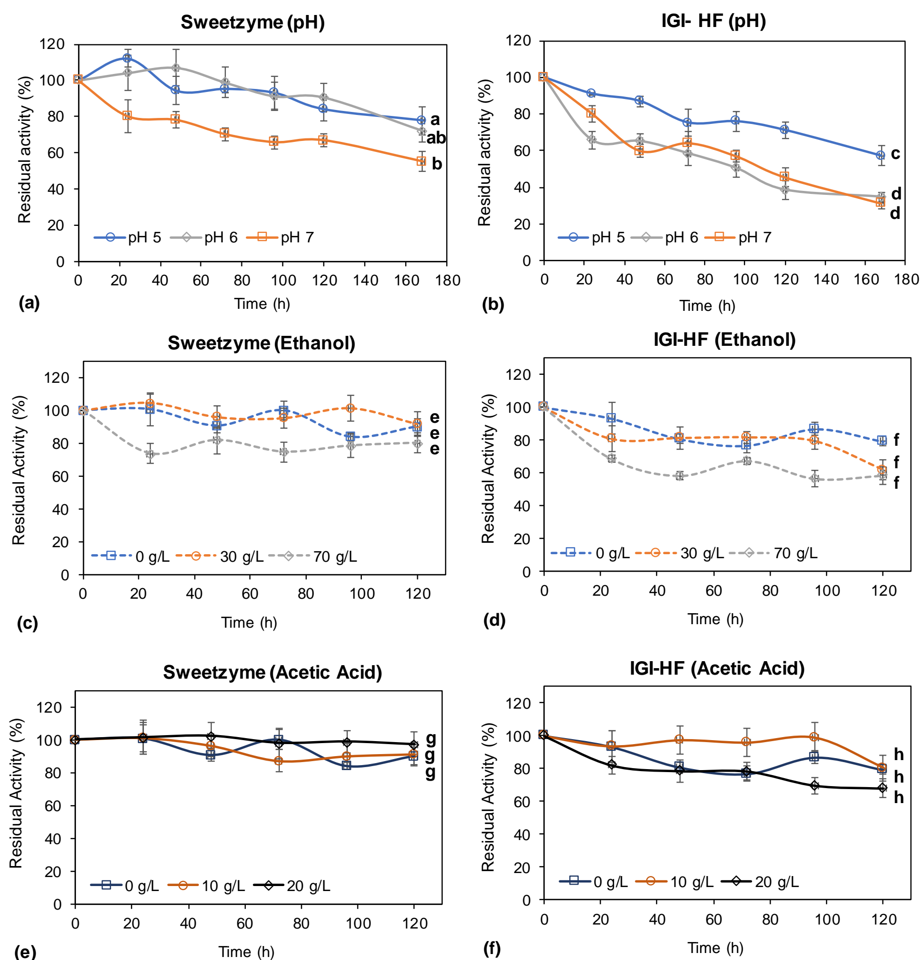

Figure 2.3. Residual activities of the xylose isomerase´s Sweetzyme and IGI-HF, measured at 60 °C after incubation at typical SIF temperature (35 ºC) at different pH **(a, b)**, ethanol **(c, d)**, and acetic acid **(e, f)** concentrations. Incubation in ethanol and acetic acid solutions carried out at pH 5.2 (SIF condition). Error bars are standard deviations of the duplicates. $^{a\text{-}h}$ Tukey test indexes with 95% confidence. Different letters mean statistically significant differences between the last experimental point of each condition.

2.3.1.3. Enzymes stability in different ethanol concentrations

Enzyme stability was also evaluated under exposure to ethanol, the main fermentation product, which can reach concentrations of 60- 95 g/L in the broth (DELLA-BIANCA et al., 2013). Incubation was at 35 °C and pH 5.2 (SIF conditions). **Fig. 2.3 c-d** shows promising results for both

enzymes. Sweetzyme kept 80.2% of its initial activity after 5 days of exposure to 70 g/L of ethanol (**Fig. 2.3c**), while IGI-HF maintained 58.5% (**Fig. 2.3d**). According to the Tukey test applied to the last point of all conditions, no significant difference was observed between them, thus concluding that 120 h exposure of ethanol (up to 70 g/L) does not influence the enzymes' stability. These findings also allow us to infer that the main reason for enzyme loss of activity is the pH (5.2) to which they were exposed, as previously discussed, because even in the absence of ethanol a noticeable decrease in residual activity was observed, especially for the IGI-HF.

Silva et al. (2012) evaluated soluble and immobilized XI incubated at 35 °C in a medium similar to the one used in the present study, with 30 and 70 g/L of ethanol (pH 5). Soluble XI lost more than 50% of its initial activity after 120 h, while the immobilized one (IGI-Ch) kept about 90%, showing a low influence of ethanol up to 70 g/L on the activity of immobilized enzymes. According to the authors, one of the main causes of the loss of activity of multimeric enzymes is the dissociation of their subunits, which can be avoided or reduced through immobilization. In comparison with these results, the present study confirms that the stability of commercially immobilized enzymes is not affected by exposure to ethanol.

Other studies also evaluated the ethanol influence over the stability of different XI (HAHN-HÄGERDAL; BERNER; SKOOG, 1986; MIYAMOTO et al., 2020; VILONEN et al., 2004), however, in very different conditions to that assessed in the present paper. However, the main conclusions were that only super-high concentrations of ethanol are harmful to the enzyme's stability.

2.3.1.4. Enzymes stability in different acetic acid concentrations

Considering that acetic acid is the inhibitor present in higher concentrations in hemicellulosic hydrolysates, which are potential feedstocks for 2G ethanol production (VANMARCKE et al., 2021), the enzyme stability at different concentrations of this compound was also verified, and the results are shown in **Fig. 2.3 e-f**. Similar to ethanol, Sweetzyme, and IGI-HF showed no difference between the conditions evaluated (Tukey's test). The former kept about 90% of its initial activity after 5 days, while IGI-HF (**Fig. 2.3f**), showed a bigger decrease, keeping about 75% of residual activity after 120 h.

It is reported that the XI's produced by *Streptomyces*, such as IGI-HF and Sweetzyme, are generally more than 90% similar in their sequencing (RASMUSSEN et al., 1994). Thus, it is likely

that the differences observed in the stability of both enzymes are more related to their immobilization process than to their source. According to Yuan (2010) and Yu et al. (2011), Gensweet® IGI-HF is produced from a genetically modified *S. rubiginosus* and immobilized on an ion exchange resin cross-linked with polyethyleneimine and glutaraldehyde. Sweetzyme IT extra is produced from a selected *S. murinus* and immobilized on silica. Although Sweetzyme had a better overall performance in the studies, no big differences were observed between both enzymes, and since a generous donation of IGI-HF was provided to our research group, this enzyme was used in the following studies.

2.3.2. Influence of yeast strain on SIF performance

The main objective of the studies presented in this topic was to evaluate the influence of the yeast strain on the SIF process performance. The xylose conversion and xylose isomerization rate profiles for the two *S. cerevisiae* strains evaluated (FT 858L and Itaiquara) are presented in **Figs. 2.4a-b**.

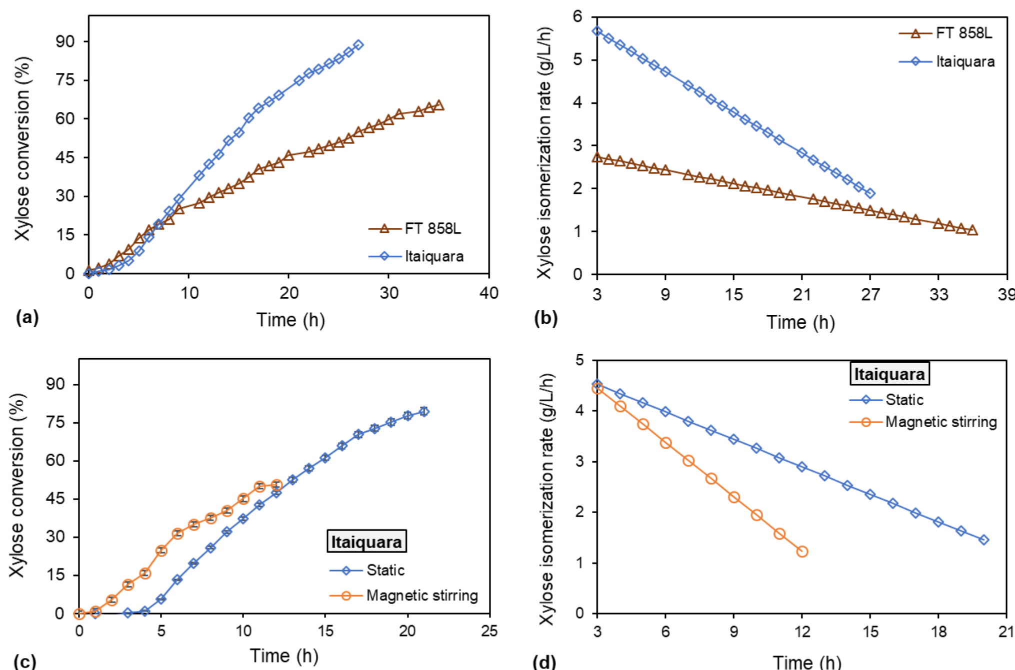

Figure 2.4. Xylose conversion and isomerization rate profiles during SIF; **(a)** and **(b) -** different *S. cerevisiae strains* FT 858L and Itaiquara ($[Xylose]_{Initial}$: 105 g/L, static); **(c)** and **(d) -** Fermentations with and without magnetic stirring ($[Xylose]_{Initial}$: 65 g/L). Cell load: 50 g_{DCW}/L, IGI-HF: 120 IU/mL, 35 °C, pH_i: 5.20. Standard deviations are less than 10%.

Xylose conversion reached 89% after 27 h of SIF for Itaiquara, whereas only 67% of conversion was attained for FT 858L after 36 h (**Fig. 2.4a**). For both yeasts, a linear decrease in xylose isomerization rate can be observed during the culture (**Fig. 2.4b**), which can be either related to a slower isomerization rate due to a decrease in xylose concentration along with fermentation progress (SHULER; KARGI, 2002), or to the increase in the concentration of bioproducts in the medium, such as ethanol and acetic acid, which can inhibit cell activity (CHANDRAKANT; BISARIA, 2000a; KOVALEVSKY et al., 2012). In fact, besides ethanol and xylitol (**Table 2.1a**), glycerol and acetic acid were also produced in the cultures. For FT 858L and Itaiquara, glycerol production was 4.5 and 3.2 g/L and acetic acid was 3.5 g/L and 1.1 g/L, respectively.

The ethanol yield was about 0.28 g_{EtOH}/g_S (55% of the theoretical) (**Table 2.1a**) for both strains, a low value compared to other SIF studies, which report average yields of 71% of the theoretical (0.51 g_{EtOH}/g_S) (SILVA et al., 2012). The low ethanol production is related to the co-production of xylitol (0.16 g_{xylOH}/g_S), the second major bioproduct. High production of xylitol by native/industrial strains is commonly reported in the literature and is related to the catabolism of xylulose by the metabolic pathways of native *S. cerevisiae*, but can also be accentuated according to culture conditions such as high pH, low temperatures, and high xylose concentrations (CHIANG et al., 1981; MILESSI et al., 2018; PATIÑO et al., 2019).

Unlike FT 858L, Itaiquara achieved superior results regarding ethanol productivity, xylose conversion, and isomerization rates, as observed in **Table 2.1a** and **Figs. 2.4a-b**. These results show the greater affinity of the baker's yeast for the pentose xylulose compared to the industrial FT 858L. Baker yeasts, such as Itaiquara, are commonly used in a less specific environment than industrial yeast and are exposed to different types of sugars, which probably contributed to providing them with a better ability to ferment xylulose. Furthermore, it is well known that industrial strains of *S. cerevisiae* such as FT 858L, selected in the industrial environment, are experts in the assimilation of hexoses present in molasses/sugarcane juice (MESQUITA et al., 2021; MILESSI et al., 2020b). Thus, the longer fermentation time observed in the present study (about 36 h) may be related to the lower affinity of FT 858L for xylulose. A similar behavior was observed in studies conducted by Milessi et al. (2020c), who evaluated five yeast strains and reported the best results for xylulose assimilation with the fresh baker's yeast Itaiquara, compared to industrially isolated or laboratory strains. From these observations, it can be settled that the Itaiquara strain is the best choice to perform the following SIF experiments.

Table 2.1. Xylulose, pH, and calculated fermentation indexes obtained in SIF performed with **(a)** *S. cerevisiae* FT858L and Itaiquara ($[Xylose]_{Initial}$: ~105 g/L, static); And in static and magnetic stirred configuration **(b)** ($[Xylose]_{Initial}$: 65 g/L). Cell load: 50 g_{DCW}/L, IGI-HF: 120 IU/mL, pH_i: 5.20, 35 °C. Standard deviations were lower than 10%. Different letters in front of the performance index values mean significant differences between them, pair-compared (Tukey's test, 95% of confidence).

(a) Influence of yeast strain on SIF performance									
Strain	**t h**	**Final xylulose g/L**	**Final pH**	**Y_{EtOH} g_{EtOH}/g_S**	**Y_{XylOH} g_{XylOH}/g_S**	**Q_P g_{EtOH}/L/h**	**q_p g_{EtOH}/IU/h**	**S g_{EtOH}/g_{XylOH}**	**X %**
FT 858L	36	2.38	5.10	0.26^a	0.16^a	0.60^a	$0.50x10^{-5a}$	1.61^a	65.6^a
Itaiquara	27	0.59	6.15	0.29^b	0.16^a	0.79^b	$0.66x10^{-5b}$	1.85^b	88.7^b
(b) Mini-reactor with magnetic stirring									
Agitation	**t h**	**Final xylulose g/L**	**Final pH**	**Y_{EtOH} g_{EtOH}/g_S**	**Y_{XylOH} g_{XylOH}/g_S**	**Q_P g_{EtOH}/L/h**	**q_p g_{EtOH}/IU/h**	**S g_{EtOH}/g_{XylOH}**	**X %**
No	20	0.51	5.52	0.34^c	0.22^b	0.82^c	$0.68x10^{-5b}$	1.51^c	79.5^c
Yes	12	1.45	5.56	0.26^d	0.13^c	0.72^c	$0.60x10^{-5b}$	2.08^d	50.6^d

* Q_P/q_P: Overall and specific productivity; S: Selectivity; X: Xylose conversion.

As expected, the Itaiquara strain also showed superiority in the fermentation process (**Table 2.1a**). Its faster xylulose assimilation led to a smaller content of this sugar remaining at the end of the batch (0.59 g/L), compared to FT 858L (2.38 g/L). Itaiquara also presented higher ethanol yield (0.29 g_{EtOH}/g_S) and selectivity (1.85 g_{EtOH}/g_{XylOH}) than FT 858L, once again highlighting that working with 50 g_{DCW}/L of Itaiquara strain in consortium with IGI-HF favors ethanol yield and selectivity, besides substrate conversion.

However, ethanol productivity (0.79 g_{EtOH}/L/h) is still low due to the long fermentation time (27 h) compared with the SIF reported in similar conditions (MILESSI et al., 2018; SILVA et al., 2012), requiring process improvements to achieve better performance. Using baker's yeast co-immobilized on alginate beads with XI immobilized in the chitosan support, Milessi et al. (2018) reported ethanol productivity of 2.07 g/L/h (50 g_{DCW}/L of yeast and 120 IU/mL of enzyme load), which is a consequence of the short SIF time (about 12 h) required for the total conversion of 65 g/L of xylose.

It is important to note that, during the SIF with Itaiquara or FT 858L, enzyme accumulation in the bottom of the mini-reactors was observed (**Fig. SM2**, Supplementary Material). As the SIF were carried out in static condition, mass transfer problems can be the main reason impairing the

rate of xylose isomerization and xylulose fermentation. The specific ethanol productivities enrolled in **Table 2.1a** were equal to or less than 0.66×10^{-5} g_{EtOH}/IU/h, whereas 1.72×10^{-5} g_{EtOH}/IU/h was reported for the SIF using the alginate immobilized biocatalyst (MILESSI et al., 2018), suggesting that the enzyme load supplied to the mini-reactors was underused. Therefore, strategies to improve mixing and substrate access to the enzyme will be studied from now on.

It is worth mentioning that both yeasts presented final cell viability higher than 95% and the final pH did not change much in the studies and neither decreased as expected in alcoholic fermentation, due to the production of organic acids and ethanol. The culture with Itaiquara even presented an increase in pH, which was not observed with FT 858L, likely due to metabolic differences between both strains, such as the production of some unquantified acid that contributed to the pH decrease. This possibility was reported by Aquino (2013), who observed a greater pH drop in the culture medium using the industrial strains CAT-1 and BG-1 compared to fresh baker's yeast Itaiquara. On the other hand, the pH increase observed for Itaiquara could be related to a buffered environment created by the solubilization of compounds present in the enzyme's immobilization support, which was more evident due to lower acids production by this baker yeast compared to FT 858L. It is important to remember that this enzyme is originally produced to be used in the high fructose corn syrup industry, with an optimal pH of around 7 (CRABB; SHETTY, 1999). This event was positive in the sense of minimizing enzymatic deactivation due to the pH decrease, a problem commonly reported in SIF studies (MILESSI et al., 2018; SILVA et al., 2012).

2.3.3. SIF under improved mass transfer conditions

Mixing is crucial when the reaction system contains solid particles to avoid mass transfer limitations. Depending on the particle characteristics, only the CO_2 released during fermentation can be enough to promote the homogeneity of the suspension, even when the studies are carried out statically (MILESSI et al., 2018; SILVA et al., 2012). However, IGI-HF particles have high density and two phases were observed throughout the cultures performed statically in mini-reactors (**Fig. SM2**, Supplementary Material). Thus, strategies to improve mixing and xylose access to the enzyme and, consequently, xylulose production and assimilation by the yeast, were evaluated. The strategies were developed considering the observations and problems faced in each proposal.

2.3.3.1. Mini-reactor with magnetic stirring

The first approach to maintaining a homogeneous suspension was the use of magnetic stirring. Therefore, SIF cultures were performed in mini reactors in the presence and absence of a magnet. Results regarding xylose conversion and isomerization rate during the fermentation are presented in **Figs. 2.4c-d**.

The xylose conversion profile with magnetic stirring shows a slight improvement in the first 8 h of fermentation, which can be related to a better mass transfer. However, from this time onwards, it decreased, approaching the static culture conversion profile, and within 12 h of the process, the CO_2 production ceased at only 50% of xylose conversion (**Fig. 2.4c**). On the other hand, in the static culture, xylose conversion continued for an additional 8 h. Analyzing the xylose isomerization rate profiles (**Fig. 2.4d**), the stirred fermentation showed a steeper decreasing slope compared to the static culture.

For a complete picture of the stirring influence on the fermentation performance, the calculated indexes are presented in **Table 2.1b**. The initial xylose concentration (65 g/L) used in this study allowed a higher ethanol yield (0.34 g_{EtOH}/g_s) in the static culture than that reached in the previous experiment evaluating the best yeast strain (0.29 g_{EtOH}/g_s), which was carried out with initial xylose concentration of 105 g/L. In terms of specific ethanol productivity, the static culture showed 0.68 x10^{-5} g_{EtOH}/IU/h (**Table 2.1b**), which is similar to the value obtained with magnetic stirring, 0.60 x10^{-5} g_{EtOH}/IU/h (Tukey's test); and also close to the value previously found for the static culture with higher initial xylose concentration, 0.66 x10^{-5} g_{EtOH}/IU/h (**Table 2.1a**).

The ethanol yield obtained in this study was close to that reported by Milessi et al. (2018) with Itaiquara yeast co-immobilized with XI (0.35 g_{EtOH}/g_s, 50 g_{DCW}/L of yeast, 120 IU/mL of enzyme, 35 °C). It is important to mention that mass transfer, even in a static condition, is not such a critical issue for the SIF technology studied by Milessi et al. (2018) and related works (MILESSI et al., 2020a; PEREZ et al., 2021; RAMOS et al., 2021), as the enzyme and yeast cells are confined in the same environment, inside the alginate beads. The authors achieved a total conversion of xylose (65 g/L) in 11 h. The results obtained by Milessi et al. (2018) are superior to the ones achieved so far for the SIF based on commercial enzymes and free cells (79.5% of conversion in 20 h) carried out under the same conditions, highlighting the need of overcoming reaction limitations to achieve better results in the present work.

In the presence of magnetic stirring, the performance indexes (yields, selectivity, and conversion) obtained were lower than in static SIF, which is directly related to the observed stop

in CO_2 release within 12 h of the process. The early interruption of CO_2 release and, consequently, of the sugar conversion, raised doubts about cell viability. When this analysis was performed at the end of the cultures, only 40% of viable cells were observed in the magnet presence, while no loss of viability was observed in the static culture. Thus, the magnetic stirring coupled with the presence of a high enzyme load in its immobilized form seems to be the cause of the gradual loss of viability of the cells during the SIF, probably due to a combined shear effect caused by the presence of the magnet and the solids over the cells for 10 h. Problems related to the solid's presence and different types of mixing in contact with cells in suspension are often reported in the literature, especially when performing simultaneous saccharification and fermentation with high biomass solid load, which commonly leads to a loss of cell viability and lysis (KOPPRAM; OLSSON, 2014; SASSNER; GALBE; ZACCHI, 2006; UNREAN; KHAJEERAM; LAOTENG, 2016).

Therefore, based on the previously discussed results and the literature reports, stirring in the presence of high solids load was not suitable for SIF operation. Having said that, a suitable mixing in the presence of solids is fundamental to ensure an efficient isomerization reaction. Some alternatives are suggested to deal with the damage caused to the cells by the shear effect such as the co-immobilization of cells and enzymes (SILVA et al., 2012), but this option has low viability for implementation in the ethanol industry.

In this study, different reactor designs based on alternative mixing strategies, suitable for repeated batches operation, were proposed and evaluated. Instead of using a stirrer, a different pattern of mixing can also be promoted by a gas flowing through the reactor. The results obtained using the N_2 sparging reactor are described in the next section. Alternatively, the damage caused by the solid particles can be avoided if they are confined separately inside the reactor, instead of moving freely in the suspension, together with the cells. The results of these strategies are discussed in Sections 2.3.3.3 and 2.3.3.4.

2.3.3.2. Nitrogen sparging reactor (NSR)

Airlift bioreactors are well known for their flexibility, in which the shear stress can be modulated by choosing the proper bioreactor type and gas flow rate (SHULER; KARGI, 2002). Therefore, a new mixing system, resembling the bubble column bioreactor was evaluated. Nitrogen gas at 4 vvm was continuously sparged in the suspension (**Fig. 2.1a-b**) to promote the mixture

whilst maintaining anaerobic conditions. Xylose conversion and ethanol production profiles are shown in **Fig. 2.5a**.

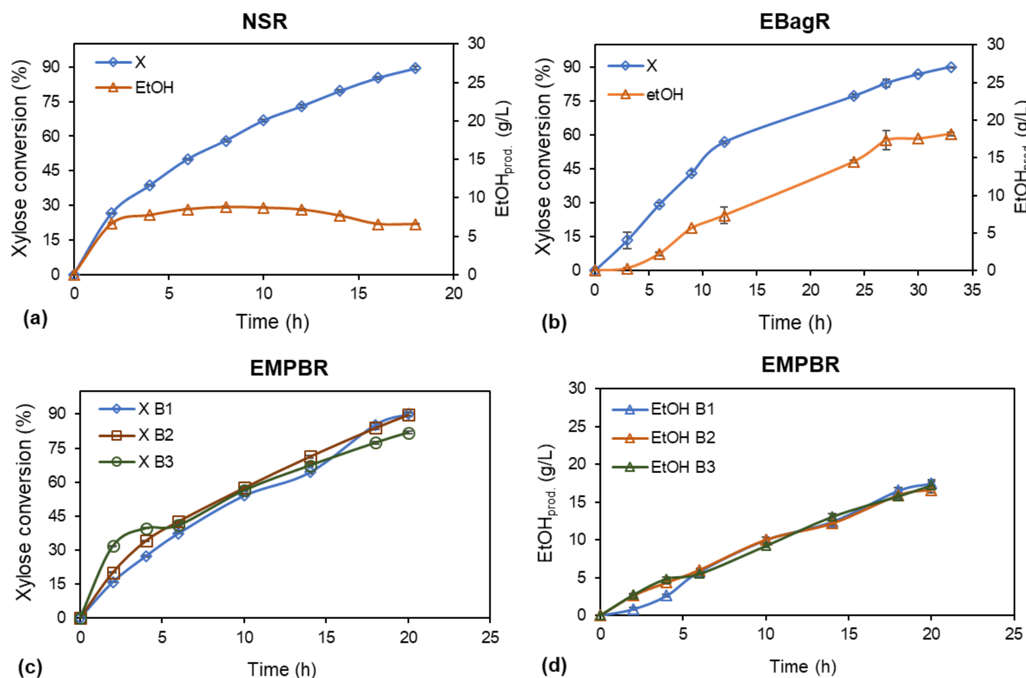

Figure 2.5. Profiles of xylose conversion and ethanol production for the SIF in **(a)** N_2 sparging reactor (NSR) (4 vvm), IGI-HF: 120 IU/mL; **(b)** Enzyme bag reactor (EBagR), IGI-HF: 53 IU/mL; **(c)** the three batches (B1, B2, and B3) performed in the Enzyme-multiple packed bed reactor (EMPBR), IGI-HF: 53 IU/mL. $[\text{Xylose}]_{\text{initial}}$: 65 g/L, 35 °C, pH_i: 5.20. Itaiquara: 50 g_{DCW}/L.

The importance of the mixture promoted by N_2 sparging was confirmed. Xylose conversion of 89.4% (**Fig. 2.5a, Table 2.2a**) was obtained in 18 h, showing a superior performance compared to the SIF performed statically in the mini-reactor, in which 79.5% of conversion was achieved after 20 h (**Table 2.1b**). The residual xylulose ranged from 1.9 to 0.3 g/L from the beginning to the end of the culture. This gradual decrease in the xylulose concentration may be related either to a progressive decrease in the xylose available to be isomerized or to the presence of xylitol. According to Yuan et al. (2012), xylitol concentrations as low as 0.5 g/L are enough to reduce the rate of xylulose production by half.

Unfortunately, as a consequence of the best mixture by using a high N_2 flow rate, ethanol was dragged by the gas phase. Even with the presence of a condenser at the reactor gas exit, the recovery of the ethanol present in the gas phase was not effective. Thus, ethanol production

increased for a few hours and then tended to stabilize or decrease (**Fig. 2.5a**), as a result of dragging. As an alternative to infer ethanol production data and better evaluate the performance of this SIF configuration, total ethanol concentration values (ethanol in the medium + dragged) were estimated by using the ethanol yield obtained in the previous experiment without magnetic stirring (0.34 g_{EtOH}/g_S). From the estimated ethanol concentrations, ethanol selectivity and productivity were also calculated. The real fermentation indexes along with the estimated ones are described in **Table 2.2a**.

Table 2.2. Culture time, pH, cell viability, and performance indexes (real and estimated*- NSR*) obtained at the batches performed in the N_2 sparging reactor (NSR) (4 vvm), IGI-HF: 120 IU/mL **(a)**, Enzyme bag reactor (EBagR) **(b)**, and Enzyme-multiple-packed bed reactor (EMPBR) **(c)**, IGI-HF: 53 IU/mL. $[Xylose]_{initial}$: 65 g/L, Itaiquara: 50 g_{DCW}/L, static, 35 °C, pH: 5.20. Standard deviations were less than 10%. Different letters in front of the performance index values mean statistically significant differences between them, pair-compared (Tukey's test, 95% of confidence).

		pH and viability		**SIF performance indexes**						
Batch	**t h**	Final pH	Cell viability loss %	X %	Residual enzyme activity %	Y_{XylOH} g_{XylOH}/g_s	Y_{EtOH} g_{EtOH}/g_s	Q_P g_{EtOH}/L/h	q_p g_{EtOH}/IU/h	S g_{EtOH}/g_{XylOH}
(a) Nitrogen sparging reactor- NSR										
							Estimated*			
1	**18**	5.49	2.00	89.4^a	87.9 ^{a}	0.19^a	**0.34^a**	**1.08^a**	**0.90x10^{-5a}**	**1.68^a**
(b) Enzyme bag reactor (EBagR)										
1	**33**	5.06	0.00	90.7^a	100.0^b	0.09^b	0.39^a	0.55^b	1.04x10^{-5b}	4.26^b
(c) Enzyme-multiple-packed bed reactor (EMPBR)										
1	**20**	5.69	2.00	90.1^a	100.0^b	0.13^b	0.38^a	0.87^c	1.64x10^{-5c}	3.05^c
2		5.53	5.00	89.7^a	100.0^b	0.10^b	0.38^a	0.83^c	1.57x10^{-5c}	3.66^c
3		5.18	5.00	82.5^b	89.3^a	0.10^b	0.35^a	0.83^c	1.57x10^{-5c}	3.47^c

*Q_P/q_P: Overall volumetric and specific productivities; S: Selectivity; X: Xylose conversion.

The best mixing and, consequently, the shorter SIF time improved the estimated values of overall volumetric and specific ethanol productivities, which were higher (1.08 g_{EtOH}/L/h and 0.90x10^{-5} g_{EtOH}/IU/h) (**Table 2.2a**) than those obtained in the static mini-reactor experiment (0.82 g_{EtOH}/L/h and 0.68x10^{-5} g_{EtOH}/IU/h) (**Table 2.1b**). However, these values are still about 2-fold lower than those reported by Milessi et al. (2018) (2.07 g_{EtOH}/L/h and 1.72x10^{-5} g_{EtOH}/IU/h),

suggesting that the mass transfer problems persisted. The enzyme particles have a high density and even the intense flow of N_2 (24 L/h) continuously injected into the reactor was not enough to efficiently move them in the medium and promote a homogeneous mixture. During the culture, most of the enzyme load remained mainly in the bottom of the flask (**Fig. 2.1b,** brownish sedimented particles). However, it is important to highlight that the loss in enzymatic activity (12%) and cell viability (2%) was very low.

2.3.3.3. Enzyme bag reactor (EBagR)

Based on the previous issues faced by the NSR, regarding ethanol dragging and enzyme accumulation in the bottom of the reactor, a new reactor design was proposed. The idea behind it was to remove enzyme particles from suspension by confining them separately in the reactor. In addition, this configuration enables the operation with a high load of cells and enzymes, enzyme reuse, as well as cell recycling. Two alternative reactor designs based on this idea were set up. In both designs, magnetic stirring was used to improve the mixture. Previous experiments of the group proved that the yeast cells could bear the magnetic stirring for 18 h without any loss of viability in the absence of enzyme particles (data not shown).

In the enzyme bag reactor (EBagR), the enzyme load was confined into nylon net bags (**Fig. 2.1c**), avoiding the damage caused to the cells by particles in suspension. The results regarding xylose conversion profile and fermentation indexes are presented in **Fig. 2.5b** and **Table 2.2b**, respectively.

The SIF batch in this reactor lasted 33 h for about 90% of xylose conversion, almost double the time necessary with NSR. However, higher values of ethanol yield (0.39 g_{EtOH}/g_S), specific productivity (1.04×10^{-5} g_{EtOH}/IU/h), and selectivity (4.26 g_{EtOH}/g_{XylOH}) were achieved. In addition, xylitol yield (0.09 g_{XylOH}/g_S) was significantly reduced when compared to NSR (0.19 g_{XylOH}/g_S). These positive fermentation indexes show that there was a more effective use of the enzyme load present in the process, probably due to the mixing improvements.

The main reason for the longer SIF in EBagR was the limited amount of enzyme that could be added to this reactor. IGI-HF (as well as Sweetzyme) particles showed a huge absorption capacity when immersed in the medium, causing particle swelling and, consequently, an increase in the overall volume of about 2-fold. Therefore, after swelling, only 10 enzyme bags could be accommodated inside the reactor, leading to a load of 53 IU/mL used in the SIF, which was less

than half the load used in the NSR or mini-reactors. The specific ethanol productivity parameter was introduced to enable a fair comparison to the previous results and shows remarkable process improvements with this reactor. The specific productivity value became closer to that achieved by Milessi et al. (2018) using 120 IU/mL as the load.

This reactor design also showed the effectiveness of the magnetic stirring in maintaining a homogeneous cell suspension, without hampering the viability of the cells as previously observed in the mini-reactor (Section 2.3.3.1). In addition, no loss of enzymatic activity was observed, compared to a 12% reduction in the NSR (**Table 2.2 a-b**), which may be related to the fact that in this reactor the enzymes were freely percolating through the medium and sheared each other.

Nevertheless, there is still room for improvement. The geometry of the bags changed from flat cushions to balloons after enzyme swelling and it was not simple to remove them from the reactor or place them back. The nylon net was also susceptible to tearing apart, mainly after the swelling. Furthermore, the swelled enzyme bag is likely to have internal mass transfer limitations. Thus, aiming to facilitate the process conduction and further implementation in industrial facilities, a new reactor design, which changed the modules' geometry from flat cushions to long tubes was proposed and is presented in the following sections.

2.3.3.4. Enzyme-multiple-packed bed reactor (EMPBR)

Packed-bed reactors are widely used with chemical and enzymatic catalysts. They can consist of single or multiple beds (ZHU et al., 2020). An in-house prototype of a multi-tubular fixed bed reactor was constructed and packed with commercial IGI-HF particles (**Fig. 2.1 f-g; Section 2.2.4.4**). It was a natural choice to overcome the limitations observed in the EBagR, enabling easy module removal, enzyme reuse, and cell recycling. Three sequential SIF batches were conducted in this reactor with the same enzyme load used in the EBagR (53 IU/mL). The profiles of xylose conversion and ethanol production, along with the main performance indexes are presented in **Fig. 2.5c-d** and **Table 2.2c**, respectively.

This reactor allowed a significant reduction in fermentation time (20 h) to reach 90% of xylose conversion in batches 1 and 2, which proves the achievement of an even better mixing during the fermentations than that obtained with the EBagR. Moreover, an ethanol yield of 0.38 g_{EtOH}/g_S (75% of the theoretical) was reached in both batches, coupled with xylitol yields of 0.13 g_{XylOH}/g_S (B1) and 0.10 g_{XylOH}/g_S (B2 and B3). These results are similar to those achieved in the

EBagR. Overall volumetric and specific ethanol productivities of 0.87 g_{EtOH}/L/h and $1.64x10^{-5}$ g_{EtOH}/IU/h (B1), as well as 0.83 g_{EtOH}/L/h and $1.57x10^{-5}$ g_{EtOH}/IU/h (B2 and B3), were the indexes that had the best improvements in comparison to the previous studies. The specific ethanol productivity was indeed very similar to the value reached by Milessi et al. (2018, 2020b), and more than 2-fold higher than that obtained by Silva et al. (2012) (**Table 3**) using a biocatalyst containing $35.5x10^3$ IU/L of XI and 20 g/L of yeast. The main issue faced by the authors was related to limitations in the isomerization step due to a high pH decrease in the process, which impaired enzyme activity.

Coupled with these results, it is also worth mentioning that the ethanol/xylitol selectivity obtained in this reactor (about 3.05 g_{EtOH}/g_{XylOH}) and in the EBagR (4.26 g_{EtOH}/g_{XylOH}) was more than 2-fold higher than that achieved in the static mini-reactor SIF (1.5 g_{EtOH}/g_{XylOH}) **(Table 2.1b)** and higher than most of the SIF studies reported at **Table 2.3**. These results suggest the direct relation of xylulose production and assimilation rate with the system mixture, which drive the metabolic substrate flux preferentially towards ethanol formation rather than for xylitol synthesis. Milessi et al. (2018) reported high ethanol/xylitol selectivity (**Table 2.3**) in a SIF using the alginate immobilized biocatalyst by increasing the enzyme/yeast ratio, which favored xylulose availability and consequently, ethanol production, reducing xylitol concentration. As previously mentioned, mass transfer problems in this study were less relevant, as both enzyme and yeast were co-immobilized, and therefore they could observe this influence directly by varying the enzyme/yeast ratio. The relation between xylulose availability and selectivity was also mentioned by Yuan et al. (2012), who studied SIF in a fixed bed reactor with yeast and enzyme co-immobilized with urease. The authors highlight the importance of a good xylulose production rate for the process, which besides improving ethanol production, also prevents enzyme inhibition due to exposition to high xylitol concentrations.

Regarding batch 3, a slightly lower xylose conversion (83%) was obtained than in the other batches within 20 h of the process, which is coupled with a small decrease in the enzyme activity (11%) as well as in the cell viability (5%). The other fermentation indexes (ethanol, xylitol yield, and productivity) had no significant differences between all batches (**Table 2.2c**, Tukey's test). The observed small viability loss can be attributed to the stress caused to cells by continuous exposure to the compounds produced in the medium, such as ethanol, xylitol, glycerol, and acetic acid as the cells were not washed between batches, but only centrifuged and resuspended in fresh

medium. Glycerol and acetic acid were produced in all batches, but in concentrations lower than 1 g/L (data not shown). It is also important to emphasize that no contamination was observed during repeated batches, even after 60 h of operation. Contamination was the main problem for long SIF operations using immobilized biocatalysts, probably because the contaminating bacteria present in the medium could assimilate xylose/xylulose without any direct competition, once yeast cells were confined into the beads (MILESSI et al., 2020b). Thus, our results show that the SIF operation with cells in suspension has the advantage of preventing contamination.

Table 2.3. Main fermentation performance indexes for the present study and reported SIF processes.

$[Xylose]_i$ g/L	X_{xylose} %	C_{EtOH} g/L	Y_{EtOH} g_{EtOH}/g_S	Q_P g_{EtOH}/L/h	q_P g_{EtOH}/IU/h	S g_{EtOH}/g_{XylOH}	Reference
65	77	12.0	0.24	0.25	0.70×10^{-5}	1.26	(SILVA et al., 2012)
65	98	22.5	0.35	2.07	1.72×10^{-5}	2.42	(MILESSI et al., 2018)
65	99	22.5	0.34	2.10	1.75×10^{-5}	2.45	(MILESSI et al., 2020b)
65	90.1	17.5	0.38	0.87	1.64×10^{-5}	3.05	**Present study**

*Q_P/q_P: Overall volumetric and specific productivities; S: Selectivity; X: Xylose conversion.

Overall, the results previously discussed support the feasibility of the multiple-packed bed reactor for ethanol production from xylose, which led to improved SIF efficiency and more effective use of the enzyme loaded. In addition to the good fermentation indexes achieved, the proposed configuration has the potential to accomplish the aimed 1G/2G process integration, as the enzyme module can be incorporated into the existing 1G ethanol mill facilities, requiring minor modifications in the process. The multi-tubular module can be placed in and removed from any cylindrical tank fermenter, allowing enzyme recycling in industrial conditions, in association with the Melle-Boinot process.

Certainly, the performance of the SIF using the enzyme-multiple-packed bed reactor can be further improved. Despite the suitable characteristics of the commercial enzymes for the SIF process, such as good activity and stability in SIF conditions, availability on the market, and ease of reuse, they are a product developed for high fructose corn syrup industrial production, not for SIF. The commercial enzymes intensely absorb the liquid phase where they are immersed, causing

a significant increase in the particulate material volume. Consequently, the volumetric GI activity is reduced because less enzyme mass can be loaded in the tubes when the particles are swollen. Their high density is also an undesirable feature for the SIF process because they quickly settle at the bottom of the reactor vessel and the smooth agitation caused by CO_2 bubbles is not enough to even move the enzyme particles.

On the other hand, the development of tailor-made biocatalysts for SIF, using low-density support, with high specific activity per gram of derivative and good stability at fermentation conditions could significantly improve the performance of the multiple packed bed reactor, reduce SIF time extent and increase productivity. However, it is not an easy task. The biocatalyst IGI-Ch developed by Silva et al. (2012) using chitosan as support, has a density similar to water and does not absorb liquid. In addition, this biocatalyst presented a specific activity of 1,700 IU/g, which is more than 3-fold superior to the one measured for IGI-HF (~500 IU/g, in dry weight). However, at SIF pH, IGI-Ch exhibits only 10% of its maximum activity, while IGI-HF keeps 38.9%, and both biocatalysts would present similar performances.

Finally, the choice of the most suitable biocatalyst must consider its impact on process economics. Thus, this decision should be based on proper technical-economic analysis. Similarly, the enzyme-multiple-packed reactor studied in the present work is a prototype and can be improved by applying formal engineering approaches. Nevertheless, important advances were achieved in this work, contributing to the development of a promising SIF process.

2.4. Conclusions

The two commercially available xylose isomerases characterized fulfilled the stability and activity requirements to conduct isomerization under SIF conditions. Similarly, both industrial yeast strains evaluated in the SIF process assimilated xylulose and produced ethanol. Despite this, the Itaiquara strain (Baker's yeast) presented a faster xylulose uptake rate, which is a key feature to increase process productivity. Overall, the success of the proposed technology to produce 2G ethanol from hemicellulosic hydrolysates using commercial xylose isomerase and industrial *S. cerevisiae* strains relies on a high-performance biocatalyst as well as a proper reactor design, which allows good mixing, enzyme reuse and embedding into the existing 1G ethanol mill facilities, requiring minor modifications in the process to accomplish the aimed 1G/2G integration. Thus, the evaluated prototype of the multiple-packed bed reactor evaluated has the potential to fulfill the

desired characteristics. The enzyme module can be improved for further scale up, and be placed in, and removed from any cylindrical tank fermenter, allowing the enzyme to recycle in industrial conditions, in easy association with the Melle-Boinot process.

Chapter 3. Integrated first and second-generation ethanol production through a sequential process in an enzyme-multiple-packed bed reactor

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Abstract

Integration into a biorefinery scenario is the main goal of current research that seeks bioprocess viability. In this framework, second-generation (2G) ethanol production has attracted great attention since its feasible consolidation into the current first-generation (1G) industry is still a big challenge. The present work explored alternatives for efficiently integrating 1G/2G ethanol production. Simultaneous (Saccharification) Isomerization and Co-Fermentation (SICF and SSICF) were performed with non-recombinant yeast, mediated by a commercial xylose isomerase (XI) entrapped in a specially designed enzyme-multiple-packed bed reactor (EMPBR), allowing the performance of repeated batches. To evaluate the best strategy to improve hexose/pentose co-fermentation in a 1G/2G process, studies were conducted in defined media, mimicking the composition of industrial ones. Sequential batches (1st step containing mainly pentoses followed by hexoses feeding in a 2nd step) were the foremost option for a faster xylose isomerization rate (r_{xylose}: 2.18 g/L/h). This strategy was further evaluated in industrial media, formulated with sugarcane bagasse acid (AH) or hydrothermal (HH) hydrolysates (2G) sequentially fermented with molasses (1G). Promising xylose conversion (89.6 and 76.3%) and ethanol titers (44 and 46 g_{etOH}/L) were achieved, respectively, for AH and HH based media. However, as expected, overall and specific productivities were lower than those achieved in the defined medium. Three repeated batches were performed with HH (total of 54 h) and a decrease in the SIF performance was observed under enzyme and cells recycle operation. Full integration using both bagasse pretreated fractions and molasses (SSICF) faced a negative influence of the hexose presence, which was also

coming from the gradual hydrolysis of the pretreated solids, on the assimilation of pentose. Overall, great progress was made for 1G/2G integration through SICF.

Keywords: Simultaneous (Saccharification) Isomerization and co-Fermentation (SICF/ SSICF), Process integration, Hydrolysates, Molasses, Pretreated biomass.

3.1. Introduction

Increasing biofuel production, such as ethanol, is hugely important for a more sustainable and less fossil fuel-dependent world. Biorefineries are the main ally to reaching this purpose (MUSSATTO; DRAGONE, 2016). They are characterized as industrial units that integrate equipment and processes to extract the maximum yield from the raw materials used in different bioproducts (AGUIAR et al., 2021). The integration of processes, proposed in the biorefineries, is the key to the processes' economic viability.

Nowadays ethanol production is mainly conducted through the enzymatic hydrolysis and/or fermentation of hexose sugars (sucrose, glucose, and fructose) directly extracted from sugar-rich plants such as sugarcane, beetroot, and corn, followed by a distillation step (DELLA-BIANCA et al., 2013). This process is denominated as first-generation (1G) ethanol production. The main residues/coproducts from the 1G industry are the processed biomass after the removal of the fermentable juice, such as sugarcane bagasse, straw, brewer's spent grain, and corn stover, which are fibrous materials mainly composed of cellulose, hemicellulose, and lignin, in different percentages depending on the material source (AGUIAR et al., 2021).

The main coproduced biomass in the Brazilian 1G industry is the sugarcane bagasse. Currently, this material is burned in the sugar-energy plants themselves to provide energy and steam for the process (CHANDEL et al., 2021). However, a significant amount remains non-used with great potential in sugars, coming either from cellulosic or hemicellulosic fractions (BEZERRA; RAGAUSKAS, 2016). The utilization of these biomass-derived sugars for additional bioproducts has been widely studied and is denominated as a second-generation process (2G) (BEZERRA; RAGAUSKAS, 2016; CHANDEL et al., 2021). The main difference between 2G and 1G processes is the availability of the sugars for further use, which for the former is found in oligomeric form, trapped in a tangle composed of the cellulose, hemicellulose, and lignin structures of each biomass (RASTOGI; SHRIVASTAVA, 2017). This condition requires additional and

challenging steps for the sugars' release and conversion into monomers before fermentation, such as pretreatment and enzymatic hydrolysis, an extensive work that is widely discussed in the literature, with great advances having already been made (DOS SANTOS ROCHA et al., 2017; PINTO et al., 2021; PRATTO et al., 2016; RASTOGI; SHRIVASTAVA, 2017; YAMAKAWA; QIN; MUSSATTO, 2018).

In addition to sugar access, 2G processes also involve a diversified range of sugars that comes from biomass fractionation, such as xylose, the second most available sugar from biomass, that comes from the hemicellulosic fraction (NARISETTY et al., 2022). This pentose is not directly assimilated by the most conventional and robust yeast applied in the 1G ethanol industry, *Saccharomyces cerevisiae*, being commonly put aside since it requires specific solutions for its conversion, such as the use of naturally pentose fermenting strains, genetically modified organisms (GMOs), or the introduction of specific enzymes to the process (MILESSI et al., 2018, 2020b; PEREZ et al., 2021; SANDRI et al., 2023a). Either route does not provide such an efficient and good product yield/titer process as that found in the 1G ethanol production, through hexoses' fermentation. However, the sugar amount represented by xylose is important for 2G process feasibility, in which the use of whole sugar fraction from biomass matters. In this sense, integrating 1G/2G processes is a way of fitting into the biorefinery context (CHANDEL et al., 2021). For this purpose, and considering the current industrial scenario in developing countries like Brazil, the use of non-recombinant yeasts is easiest to be introduced in conventional plants since it does not face the severe biosecurity laws required for GMO application (MILESSI et al., 2020b).

Therefore, the options for xylose conversion are either the use of naturally pentose fermenting strains or the addition of enzymes to the process. Our research group has wide experience in enzymes application (MILESSI-ESTEVES et al., 2019a; MILESSI et al., 2018, 2020b; RAMOS et al., 2021; SANDRI et al., 2023b; SILVA et al., 2012), such as xylose isomerase (XI), which performs the isomerization of xylose into xylulose, an isomer that can be assimilated by *S. cerevisiae*, through a reversible reaction (1xylulose: 5xylose). A strategy commonly used to dislocate the equilibrium of this reaction into xylulose production is Simultaneous Isomerization and Fermentation (SIF), which consists of adding enzyme and yeast simultaneously to the process, so the isomer can be assimilated as it is produced (RAMOS et al., 2021; SILVA et al., 2012). Several optimizations have already been done for SIF processes, from which appropriate yeast and enzyme loads have been defined, besides the design of a special reactor that proved to be efficient

for conducting the conversion of xylose/xylulose into ethanol (SANDRI et al., 2023b). However, Simultaneous Isomerization and Co-Fermentation (SICF), the process that is characterized by the co-fermentation of hexoses and pentoses, presents some additional issues, such as the repression of pentose assimilation by the yeast due to hexose's presence (RASTOGI; SHRIVASTAVA, 2017), requiring further investigation.

Regarding the SICF studies reported in the literature (CHANDRAKANT; BISARIA, 2000a, 2000b; DE BARI et al., 2014), great progress is presented in the development of in-house made biocatalysts for improved isomerization reaction, but most of the processes still face slow xylulose fermentation rate and consequently low xylose conversion, a strategy to improve these limitations is the use of high enzyme and cell loads, as reported by Milessi et al. (2018, 2020b) and Silva et al. (2012). However, working with enzymes, especially in high loads, is expensive, so taking maximum advantage of the biocatalyst during the process is fundamental for its feasibility, such as by conducting recycling (HANS et al., 2021; VIJAYALAXMI et al., 2013). Despite that, it is important to keep good enzyme activity and cell viability during repeated batches (SILVA et al., 2012). Guilherme et al. (2022) is the first reported SSICF process (including saccharification of pretreated biomass solid fraction) to perform recycling batches. However, the authors also did not evaluate strategies to improve the xylulose uptake rate, which was very limited in the presence of the hexoses hydrolyzed from the solid fraction, the result was pointed out as dependent on the low efficiency of the wild-type *S. cerevisiae* in the xylulose uptake, with consequent side-production of xylitol.

Therefore, based on the current know-how found in the literature, the present work proposes the study and selection of an adequate strategy to perform sugar co-fermentation allowing good xylose conversion to fulfill this gap. Additionally, processing integration for 1G/2G ethanol production is proposed, providing hexoses and pentoses coming either from 2G or from 1G sources, derived from sugarcane, which is not still mentioned in the literature. SICF mediated by the enzyme xylose isomerase was conducted in an enzyme multiple-packed-bed reactor (EMPBR), previously developed by the research group (SANDRI et al., 2023b), with the performance of repeated batches. The SSICF process was also evaluated by adding pretreated solids and a hemicellulolytic cocktail to the reactor. To the best of our knowledge, this is a new proposal for 1G/2G integration since it involves either a special bioreactor configuration, as well as the evaluation of different strategies for improving xylulose uptake, besides using non-recombinant yeast and enzyme in its

commercial form, with process recycling in a strategy that can be easily introduced in the current 1G ethanol production process.

3.2. Material and Methods

3.2.1. Microorganisms, enzymes, and inoculum

3.2.1.1. Microorganism and inoculum

Fermentations were performed with a baker's yeast *Saccharomyces cerevisiae* (Itaiquara®, Brazil), commercialized in lyophilized form. The inoculum was prepared as detailed by Mesquita et al. (2021) using the defined media described in Section 3.2.2.1 with 65 g/L of glucose. Optical density measurement (OD) at 600 nm and cell viability (Section 3.2.5) were performed before inoculating the reactor.

3.2.1.2. Enzymes

The enzyme used for xylose isomerization was the immobilized xylose isomerase (XI) Gensweet® IGI-HF, produced from *Streptomyces rubiginosus*, and commercialized as a bulk particulate material, with activity of 532 IU/mL (SILVA et al., 2012). It was kindly provided by DuPont- Genencor (Palo Alto, USA). For xylooligomers (XOs) and pretreated solids hydrolysis, the enzymatic cocktail Cellic®CTec2 (CC2) (Novozymes, Araucária, Brazil) was used, with cellulase (106.3±6.6 FPU/mL), xylanase (10938.0±184.4 IU/mL), and β-xylosidase (139.1±4.6 IU/mL) activities previously measured according to Ghose (1987), Bailey; Biely; Poutanen (1992), and Lachke; Deshpande (1988), respectively.

3.2.2. Culture media

Defined and industrial media, non-sterilized, were used for the fermentation.

3.2.2.1. Defined media

It was prepared according to Sandri et al. (2023b), containing KH_2PO_4 (5.0 g/L), $MgSO_4.7H_2O$ (2.0 g/L), $CoCl_2.6H_2O$ (0.1 g/L), urea (1.5 g/L), and sugars (glucose, xylose and/or sucrose) in concentrations that will be described in each section. The initial medium pH was adjusted to 5.2 with HCl (1 mol/L). After pH adjustment, the antifoam polypropylene glycol (PPG 3%: 1 mL/L), and the antibiotics Kamoran (4 ppm), kindly provided by Química Real (MG, Brazil), and ampicillin (20 ppm) were added to the media.

3.2.2.2. Industrial media: Hydrolysates, pretreated biomass, and molasses

Industrial media consisted of combinations of sugarcane bagasse acid/hydrothermal hydrolysates, pretreated solids, and molasses, as will be described in the following sections regarding each study. Nutrient supplementation, antibiotics, and antifoam addition were also performed, as described for the defined media. The initial pH was adjusted to 5.2.

Acid hydrolysate (AH) was kindly supplied by Prof. Silvio S. Silva and Prof. Julio C. Santos (USP, Brazil). It was produced by sugarcane bagasse acid hydrolysis with H_2SO_4 (100 $mg/g_{dry\ bagasse}$, solid:liquid ratio of 1:10 m/v at 121 ºC for 20 min) (ALVES et al., 1998). The Hydrothermal hydrolysate (HH) was produced in-house as described by Milessi et al. (2021). Sugarcane bagasse, provided by Ipiranga Agroindustrial (SP, Brazil), was the biomass used. It was pretreated in a 2-L Parr reactor (model 4848) (195 ºC for 10 min, solid:liquid ratio of 1:10 m/v) (MILESSI et al., 2021). After pretreatment, liquid (HH) and solid (HPS) fractions were separated through filtration and stored for further characterization (SLUITER et al., 2008a, 2008b, 2008c) and use.

The molasses (M), a coproduct from the sugar production in the 1G sugar ethanol industry, was also provided by Ipiranga Agroindustrial (SP, Brazil). The compositions of the hydrolysates, pretreated solids (HPS), and molasses (M) are presented in **Table 3.1**.

Table 3.1. Sugarcane bagasse liquors (Acid- AH, and hydrothermal hydrolysates- HH), hydrothermally pretreated solid fraction (HPS), and molasses (M) composition. Standard deviations were lower than 10%.

Hydrolysates and Molasses			
Compounds (g/L)	**AH**	**HH**	**M**
Sucrose	-	-	737.37
Fructose	-	-	59.25
Cellobiose	0.96	1.15	-
Glucose	1.85	0.38	59.90
Xylose	19.29	4.22	-
Arabinose	2.00	1.41	-
Xylooligomers (XOs)	-	14.68	-
Acetic acid	2.70	1.67	0.03
HMF	0.03	0.26	-
Furfural	0.33	1.97	-
Hydrothermally pretreated sugarcane bagasse (HPS)			
Components (%)			
Cellulose	59.29		
Hemicellulose	9.95		
Acetyl group	2.11		
Total lignin	25.71		
Ash	1.96		

3.2.3. Fermentation strategies to improve xylulose uptake rate in an integrated 1G/2G process

The influence of hexoses' presence over pentoses assimilation by native yeasts is well-known. The lack of specific xylulose transporters in those microorganisms requires that the hexose ones are shared between both substrates, generating competition and hindering the co-fermentation of these sugars (GONÇALVES et al., 2014). The enzyme invertase is present at the periplasmatic membrane of *S. cerevisiae* cells, and it readily converts sucrose (from molasses) into glucose and fructose. Therefore, the purpose of the present section is to evaluate different alternatives to improve xylulose, glucose, and fructose co-fermentation, a process denominated as simultaneous isomerization and co-fermentation (SICF). Xylose, glucose, fructose, and sucrose are the main sugars present or coming from hemicellulosic hydrolysates (2G process), and cellulosic

hydrolysates (2G) or molasses/sugarcane juice (1G process) and, therefore, the strategy consisted of combining different concentrations of xylose and sucrose (defined media) in 5 different experiments, either as single batches or sequential ones, as described in **Table 3.2**.

Table 3.2. Fermentation strategies evaluated to improve xylulose uptake rate.

Strategy	1st step		2nd step (Sequential batch)
	Xylose (g/L)	Sucrose (g/L)	Sucrose feed (g/L)
S1	20	0	80
S2	20	10	70
S3	20	40	40
S4	0	80	0
S5	20	80	0

Xylose concentration was fixed at 20 g/L, based on a value commonly found in sugarcane bagasse hemicellulosic hydrolysates. Regarding sucrose, it was supplemented from 10 to 80 g/L, aiming to evaluate its influence over xylulose uptake from low to high concentration, and also offer about 100 g/L of total sugars to strategies S1, S2, S3, and S5, which allows a significant ethanol titer and makes easier the comparison between experiments. Strategy 4 was conducted with 80 g/L of sucrose to allow further analysis of the contribution of the ethanol coming from additional pentose sugars to the medium. The media used for the 1st steps were prepared as described in Section 3.2.2.1. Sequential batches, conducted at Strategies 1, 2, and 3, were performed through the addition of concentrated sucrose solution (700 g/L) directly to the fermented broth in the volume necessary to reach the desired concentration. The sucrose feed was performed as a single pulse when a high consumption (about 80%) of the sugars offered in the 1st step was observed by an online CO_2 monitoring system used to follow the experiments, which consisted of a CO_2 flowmeter connected to the reactor and to a computer, giving in-situ information of CO_2 production rate and accumulated volume during the fermentation.

The fermentations were conducted in an Enzyme-Multiple-Packed Bed Reactor (EMPBR), which was designed by our research group to perform SIF and had its proof of concept published by Sandri et al. (2023b). The reactor consisted of a packed bed for XI enzyme retention that allows a good percolation of the fermentation medium through it and easy enzyme recycling at the end of

each batch. The volume of fermentation medium added to the reactor, referring to each strategy, was 130 mL. The packed enzyme load was 53 IU/mL (IGI-HF). Cells were inoculated for an initial concentration of 50 g_{DCW}/L. The reactor mixing was provided by a magnet in its bottom, kept on a magnetic stirrer, and inside an incubator (35 °C). In addition to the online monitoring system, the fermentations were followed through periodic sampling for HPLC analysis (Section 3.2.5).

3.2.4. 1G/2G fermentations with industrial media

To accomplish a 1G/2G integration process closer to industrial conditions, new fermentations were performed using the best strategy found in Section 3.2.3, with industrial media, as described in **Fig. 3.1**. In these studies, it was possible to evaluate the fermentation performance when exposed to the inhibitors and specific compounds present in the sugarcane bagasse hydrolysates, pretreated biomass, and molasses.

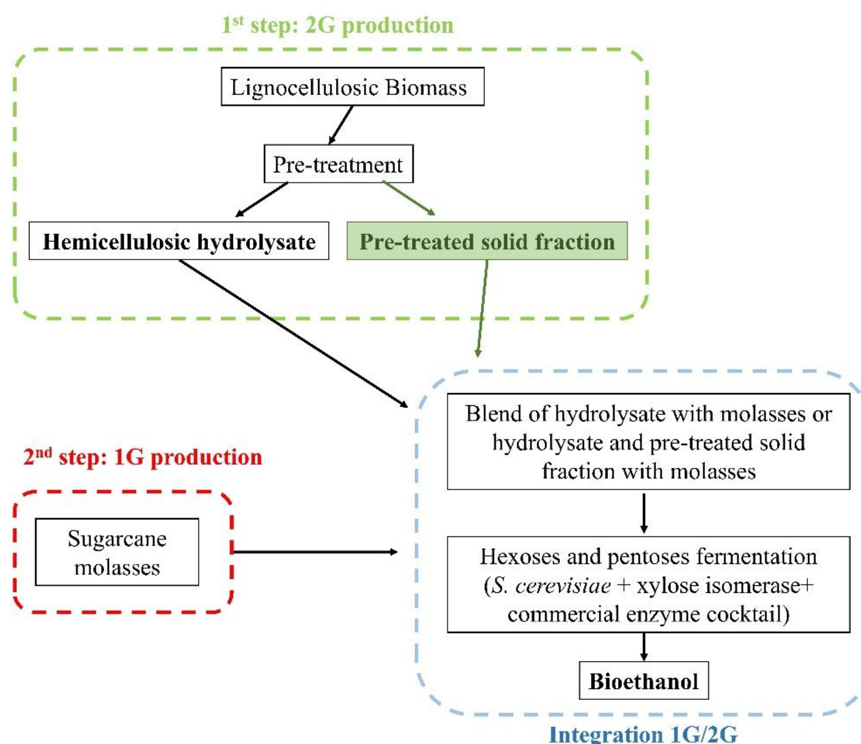

Figure 3.1. Simplified flowchart of the integrated 1G and 2G ethanol production process proposed.

3.2.4.1. Acid hydrolysate (AH) plus molasses (M) medium

The presence of sucrose in the fermentation media proved to be interfering with the xylulose uptake rate when in concentrations higher than 10 g/L. In this way, sequential fermentations as described in Strategies 1 and 2 were performed (**Table 3.2**). As shown in **Table 3.1**, glucose concentration in the acid hydrolysate is less than 2 g/L, so that should not interfere with the xylulose uptake. The 1st step was conducted with AH medium only, and the sequential batch consisted of the addition of molasses (M) to the fermented broth, providing an additional 80 g/L of hexose sugars. The fermentation conditions were the same as used in the previous studies (C_{X0}: 50 g_{DCW}/L, enzyme load: 53 IU/mL, pH_0: 5.2). The online CO_2 monitoring system was also used to follow the fermentation, besides periodic sampling for further HPLC analysis. At the end of the batch, cell viability and enzyme activity were assessed (Section 3.2.5). The final pH of the fermented broth was measured.

3.2.4.2. Repeated batches in hydrothermal hydrolysate (HH) plus molasses (M) media

Different from the AH, hydrothermal hydrolysates are reported as more environmentally friendly since chemicals are not used in their production, leading to less damage to the pretreatment reactor. Besides, they usually present smaller concentrations of inhibitors than the acid hydrolysates, which was confirmed by their lower acetic acid concentration (**Table 3.1**). However, the main difference between both is the form in which sugars are available. While the AH presents mainly xylose monomers directly, in the hydrothermal pretreatment carried out under mild conditions, the hemicellulose is partially hydrolyzed, yielding mainly xylooligomers (XOs), as **Table 3.1** shows. Thus, an additional hydrolysis step is required to convert XOs into monomers, for further simultaneous isomerization to xylulose and its fermentation by the yeast. Regarding the hexose's presence in the HH, it contains less than 3 g/L considering cellobiose and glucose.

For hydrolyzing the XOs, the enzymatic cocktail CC2 was used, which is composed of cellulases, xylanases, and β-xylosidases, capable of breaking down these compounds into xylose and glucose. The enzyme load used was 315 IU/mL of medium, based on its xylanase activity. Besides the addition of the enzymatic cocktail, the other applied conditions and fermentation procedures were the same as described in Section 3.2.4.1. However, with the HH medium, three repeated batches (2 steps each) were conducted to assess the feasibility of running a long process with XI enzyme and cell recycling, reproducing the cell recycle operation performed in most of the Brazilian ethanol industries (Melle-Boinot process). The repeated batches were conducted by

removing the reactor rubber lid with the multiple-packed bed from the reactor vessel after the 2nd step of each batch was finished. The fermented broth was centrifuged and the recovered cells were resuspended in a fresh medium. The new addition of the enzymatic cocktail CC2 was performed, and the enzyme module plus the lid was placed again in the reactor. Cell viability was correct to 100% before beginning each new batch. Cell viability, enzyme activity, and pH were also measured at the end of each batch.

3.2.4.3. Integrated process using HH and hydrothermally pretreated solids (HPS) plus molasses (M) medium

Aiming for the whole use of the biomass after the hydrothermal pretreatment, a new fermentation was performed with the addition of the pretreated solids (HPS) remaining from this process. The HPS (**Table 3.1**) was added into 5 of the 26 packed-bed tubes that compose the EMPBR, which allowed this biomass to be percolated by the medium with CellicCtec2 and cells, and consequently be hydrolyzed and fermented without being mixed in direct contact with the cells, avoiding problems of shear effect that causes loss of cell viability, as previously reported by Sandri et al. (2023b) in the presence of solids and magnetic stirring. The enzyme XI mass that should be in these 5 cylinders was redistributed in the other ones, keeping the immersed enzyme load of 53 IU/mL in the reaction.

The solid load added was 10 g/L (dry basis), in the 1st step, corresponding to 1.3% (w/v) of solids in 130 mL of HH medium, initial pH 5.2, and incubated at 35 °C. After 1st step, 2nd step was performed with the addition of about 80 g/L of molasses. The fermentation was performed and followed in the same way as described in Section 3.2.4.2. At the end of the process, the remaining HPS was washed and dried to estimate the residual mass and consequently the biomass conversion due to enzymatic hydrolysis.

3.2.5. Analytical methods

3.2.5.1. Activity of xylose isomerase

It was determined by the initial reaction rate of fructose conversion into glucose, as described by Silva et al. (2012). Fructose (2 mol/L) in tris-maleate buffer (50 mmol/L) (pH 7) was used as a substrate, supplemented with $MgSO_4.7H_2O$ (50 mmol/L) and $CoCl_2.6H_2O$ (2.5 mmol/L). The enzymatic kit GOD-PAP was used to quantify glucose concentration. One international unit

(IU) was defined as the amount of enzyme that produces 1 $\mu mol_{glucose}$/min (LADISCH; EMERY; RODWELL, 1977). The measurements were performed in duplicate.

3.2.5.2. Cell viability

It was determined by the methylene blue technique and defined as the ratio between viable cells and total cells counted in a defined space of the counting chamber (SMART et al., 1999a).

3.2.5.3. Compounds analysis

Compounds quantification was performed through HPLC analysis. Samples were previously prepared as described by Perez et al. (2021). Waters e2695 chromatograph was used for the analysis, equipped with RID and UV-VIS detectors (210 nm). Ethanol, glycerol, xylitol, acetic acid, xylose, xylulose, HMF, and furfural were identified using an ionic exclusion column (Rezex ROA$^-$Organic acid H^+ (8%), H_2SO_4 (5 mmol/L) as the mobile phase (0.6 mL/min) at 65 °C (PEREZ et al., 2021). Sucrose was quantified using a Sugar Pak I column 6.5×300 mm (Waters, USA) coupled to a RID detector. Ultrapure water with 50 mg/g Ca-EDTA was used as eluent at a flow rate of 0.6 mL/min. The temperature of the column was 80 ºC (MILESSI-ESTEVES et al., 2019a).

3.2.6. Calculation procedures and statistical analysis

Ethanol and xylitol yields, overall ethanol productivity, ethanol/xylitol selectivity, xylose isomerization rate, and xylose conversion were calculated according to Milessi et al. (2018) using HPLC data. The specific ethanol productivity was calculated by dividing the overall productivity by the enzyme load (IU/L) used. Tukey test (Origin®Pro 8.5) was performed to analyze the statistically significant differences between some results evaluated, with a confidence level of 95%.

3.3. Results and Discussion

3.3.1. Fermentation strategies to improve xylulose uptake rate in an integrated 1G/2G process

Xylulose uptake rate is the main limiting factor in integrated processes containing pentose and hexose. Considering SICF processes, different fermentation strategies were evaluated to assess the influence of hexose concentration on xylulose assimilation, as presented in **Table 3.2**. Fermentation indexes were calculated at the end of each experiment and are presented in **Figs.**

3.2a-d. The xylose isomerization rate was obtained considering sugar consumption data in the linear range.

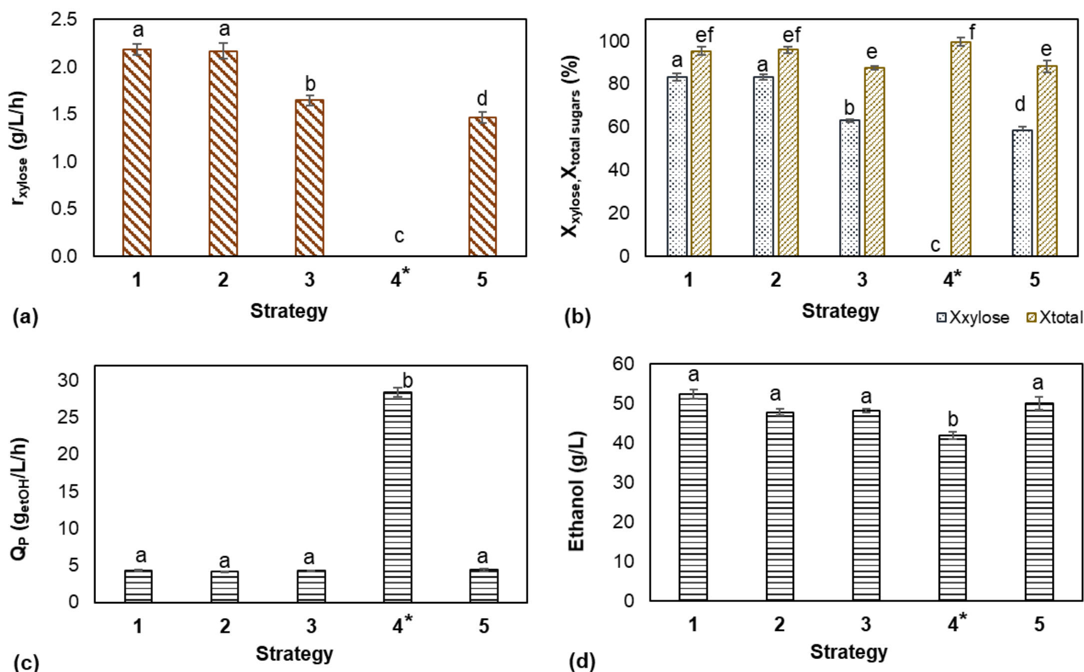

Figure 3.2. Fermentation indexes **(a)** Xylose isomerization rate (r_{xylose}), **(b)** Xylose and total sugars conversion (X_{xylose} and $X_{totalsugars}$), **(c)** Overall ethanol productivities, and **(d)** Ethanol titers reached at the end of Strategies 1 to 5, within 11.5 h of fermentation (total time), except S4* (1.5 h). Different letters on top of the bars mean statistically significant differences between the strategies, pair-compared (Tukey's test, 95% of confidence), for each index.

According to Tukey's test, no significant difference was observed between the xylose isomerization rates (r_{xylose}) for Strategies 1 and 2 (**Fig. 3.2a**), which means that sucrose presence up to 10 g/L did not influence the xylulose assimilation rate, around 2.18±0.07 g_{xylose}/L/h. That is an important observation since it is very unlikely to find an industrial media containing only xylose, generally, the hemicellulosic hydrolysates contain some hexoses coming either from hemicellulosic or cellulosic fraction, as shown by the produced hydrolysate's composition presented in **Table 3.1**. However, increasing sucrose concentration 4-fold, to 40 g/L (S3), a decrease of about 25% in the isomerization rate (1.65±0.06 g_{xylose}/L/h) was observed, followed by a 33% decrease when hexose concentration was raised to 80 g/L (S5), co-fermented in one batch with 20 g/L of xylose. Strategy 4 consisted of a hexose-only fermentation, which required 1.5 h to deplete 80 g/L of sucrose.

The decrease in xylose isomerization rate with hexoses presence above 10 g/L consequently influenced other fermentation indexes such as sugars conversion. It is important to highlight that in the studies performed in 2 steps (**Table 3.2**), the 1st step was conducted for 10 h and the 2nd one for an additional 1h30min. Within 10 h of fermentation, xylose conversion (**Fig. 3.2b**) decreased from about 83% (S1 and S2) to 63 and 58% at S3 and S5, respectively, due to the repression caused by the hexose's presence over xylulose assimilation. No additional xylose conversion was observed in the 1.5 h following the sequential batch (2nd step) when sucrose was added and rapidly assimilated by the yeast cells. The total sugar conversion (**Fig. 3.2b**), which considers sucrose, glucose, fructose, xylose, and xylulose uptake, reached in the co-fermentations S1 (95.4), S2 (95.0), S3 (86.7), and S5 (88.3%) were significantly similar, as well as S1 and S2 conversions were similar to S4 (99.6%) process, reaffirming the benefits of fermenting xylulose and hexoses in sequential steps, in the presence of on top 10 g/L of hexoses, over the final total sugar conversion.

Overall ethanol productivities (**Fig. 3.2c**) were also similar in the SICF S1, S2, S3, and S5, around 4.28 g_{etOH}/L/h, a 6-fold lower value than that reached in S4 with only sucrose. Even so, SICF values were about 5.5-fold higher than that reported by Sandri et al. (2023b) when conducting a SIF process in the same reactor used in the present work (0.87 g_{etOH}/L/h), with about 65 g_{xylose}/L, and the same yeast and enzyme loads, within 20h. Specific ethanol productivity obtained after the 1st step of S1 (1.18x10^{-5} g_{etOH}/IU/h) had the same value order as that found in the SIF (1.64x10^{-5} g_{etOH}/IU/h) reported by Sandri et al. (2023b), proving the reproducibility of fermentations in this reactor and efficiency in the XI reaction, even having reduced the concentration of xylose supplied in the present work (20 g/L). It is important to mention that no significant loss of cell viability was observed after the 11.5 h (maximum time) of fermentation in the strategies.

SICF studies have been explored in the literature, however, without including the hemicellulosic fraction in 1G/2G integration proposals (CHANDRAKANT; BISARIA, 2000a, 2000b; DE BARI et al., 2014; HANS et al., 2021). Chandrakant and Bisaria (2000a), for instance, performed simultaneous isomerization and co-fermentation in a defined medium, also offering 100 g/L of total sugars (30 g/L glucose and 70 g/L xylose). However, with this proportion between hexoses and pentoses, present together in the reactor, xylose conversion was only 42.8%, and 22.3 g/L of ethanol was produced. The authors later evaluated a different proportion between xylose and glucose (20:80 g/L), which consequently benefited ethanol titer (44.3 g/L). However, the process required 56 h to reach that value and 94.3% of total sugar conversion, still lacking

improvements in xylose consumption (CHANDRAKANT; BISARIA, 2000b). Based on the literature reports, it can be affirmed that an efficient fermentation of hexoses and pentoses requires specific strategies, such as the proposed in the present work, of performing sequential steps, each one focused on one sugar, considering that up to 10 g/L of hexose does not impair the xylulose uptake rate. Allied with this, working on strategies to intensify the SIF step is the key to increasing the productivity of the process and the conversion of xylose, as has been explored by our research group, by the conduction of fermentations with high enzyme and cell load, development of biocatalysts and enzymatic immobilization techniques, and design of reactors (MILESSI et al., 2018, 2020b; RAMOS et al., 2021; SANDRI et al., 2023b; SILVA et al., 2012). Another good strategy to intensify the 2G process would be to perform adaptive evolution to improve the assimilation of xylulose by the yeast *S. cerevisiae*, an alternative to resorting to genetic engineering.

Besides all the fermentation indexes analyzed in the present study, what matters the most for showing the importance of pentoses' utilization is their contribution to the ethanol titer at the end of the process. At S1, 6.26 $g_{ethanol}$/L was produced only from xylose within the 1st step, which refers to 12% of the total ethanol produced in this process. In addition, supplying 20 g/L of xylose to 80 g/L of sucrose significantly increased (Tukey test) the ethanol titer (**Fig. 3.2d**) at all strategies, compared to S4. Thus, founding a proper way of integrating 1G/2G processes including hemicelluloses is important, and sequential fermentation, as proposed in Strategies 1 and 2 is a good way of performing it.

3.3.2. Industrial media for integrated 1G/2G fermentations

As a proof of concept for the strategies evaluated and selected (S1 and S2) in Section 3.3.1, fermentations using industrial media, referring to combinations of sugarcane bagasse hydrolysates produced through different pretreatments, pretreated solid fraction (2G), and sugarcane molasses (1G), were performed. The integration of 1G/2G ethanol production is a promising advance for the intensification and introduction of 2G processes in conventional sugarcane processing industries. The strategies explored in the present work were designed for this integration by the conduction of SICF with commercial yeast and enzyme in high loads in a special reactor that allows the easy performance of repeated batches.

3.3.2.1. Acid (AH) or hydrothermal (HH) hydrolysates plus molasses (M) media

As previously described, the main differences between the acid (AH) and hydrothermal (HH) hydrolysates are the forms of potential sugars available and the inhibitors' concentration (**Table 3.1**). Based on these differences, both were evaluated regarding their performance in fermentations mediated by the enzymes IGI-HF and CC2 (for the HH). The 1st step of fermentation lasted 15 h, after that, molasses, from the 1G sugar-ethanol industry, was added to the fermented broth aiming to offer about 80 g/L of additional sugars and contemplate the 1G/2G integration. Hexoses and pentoses assimilation profiles and ethanol production are shown in **Figs. 3.3a-d** for both hydrolysates and the fermentation indexes are in **Tables 3.3a-d**.

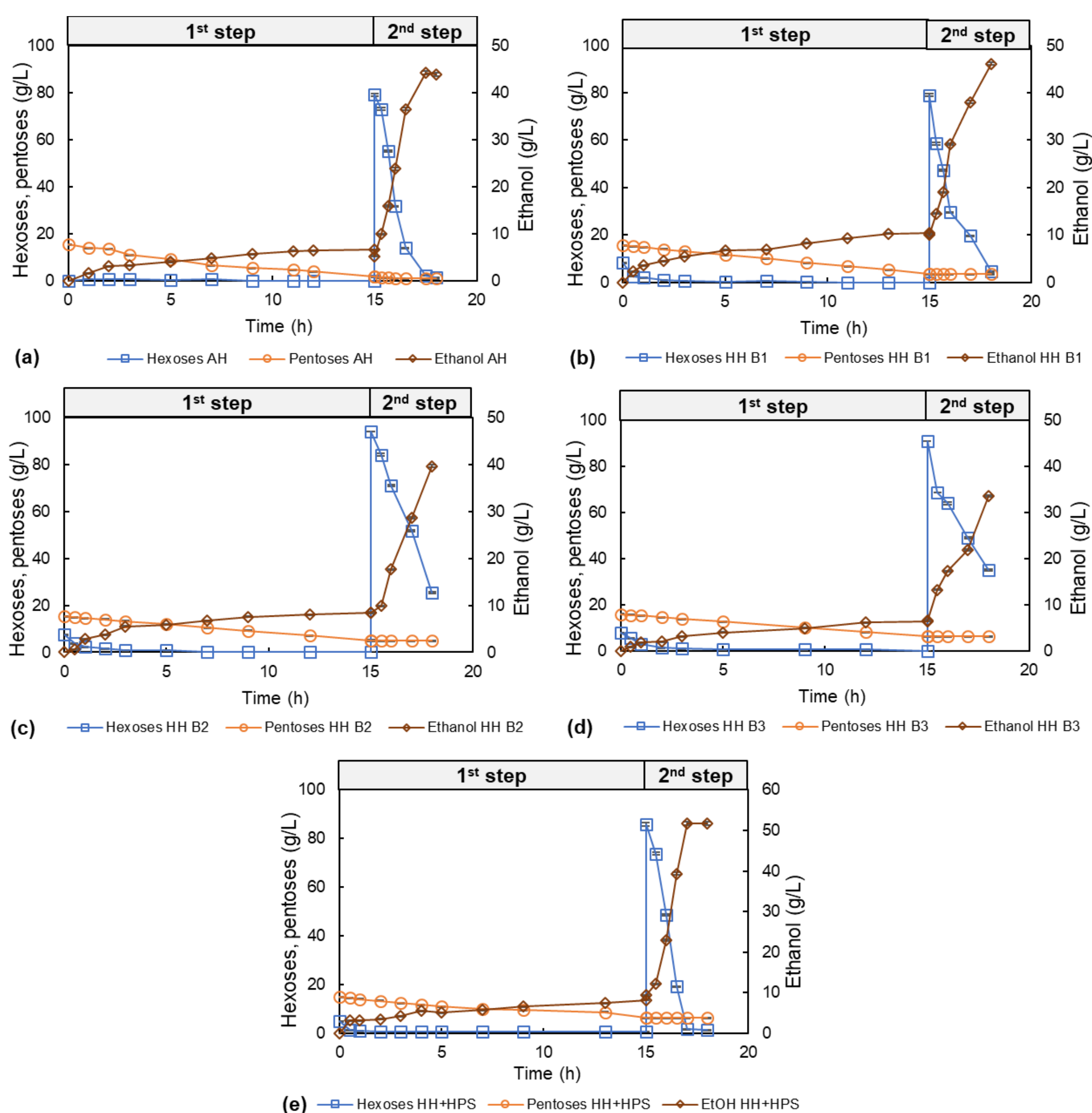

Figure 3.3. Hexoses (sucrose, glucose, fructose, and cellobiose), pentoses (xylose and XOs*) concentration profiles and ethanol production in the batch fermentations conducted with **(a)** AH+M, **(b-d)**

HH+M (B1, B2, and B3), and **(e)** HH+ HPS+M. Molasses feeding was done at 15 h. The banners in the upper part of the figure indicate the duration of each step. IGI-HF: 53 IU/mL, CC2: 315 IU/mL (for HH), C_X: 50 g_{DCW}/L, 35 °C, pH_0: 5.20. *XOs were only present in the media with HH. AH: Acid hydrolysate, HH: Hydrothermal hydrolysate, HPS: Hydrothermally pretreated solids, M: Molasses.

Comparing the pentose assimilation profiles (1st step, **Figs. 3.3 a-b**) between AH and HH, a slower consumption can be observed in the latter, which is probably due to the dependence of this medium on two enzymatic reactions before xylulose is available for fermentation. First, CC2 hydrolyses the XOs into xylose, which then is isomerized into xylulose. Additionally, lower availability of xylose (1.5-8.4 g/L) was observed during the 1st step with HH medium since it was coming from XOs hydrolysis, compared to 1.6-15.0 g/L in the AH medium, and as known by Michaelis-Menten equation (SHULER; KARGI, 2002), substrate concentration directly influences the enzymatic rate, which is lower at low substrate concentrations. So, synergic limitations between both enzymatic processes can be limiting the process. It is worth highlighting that both enzymes were working in SICF conditions, which are suboptimal for them. Regarding the HH medium, a higher initial hexose concentration (~8.2 g/L, **Fig. 3.3b**) than the AH one can be observed, which came from the commercial CC2 enzymatic pool added. Considering these sugars, a higher final ethanol titer was achieved (**Table 3.3b**). However, the slower pentose assimilation rate, previously mentioned, resulted in a 15% lower xylose conversion than that achieved with the AH (89.63%) within 15 h of 1st step of fermentation.

Comparing the fermentation indexes (**Tables 3.3 a, b**), great differences can be observed between both studies. The ethanol/xylitol selectivity is about 4-fold higher in the 1st step with HH than in the AH medium, 7.28 and 1.97 g_{etOH}/g_{xylOH} (Tukey test, 95% of confidence), respectively. According to Yuan et al. (2012) and Milessi et al (2018), the higher ethanol selectivity comes from a higher availability of xylulose in SIF processes. Milessi et al (2018) also mention that the need for cofactor regeneration can vary depending on broth conditions, such as pH variations, which can shift cell metabolism either to ethanol or xylitol production, a process related to the activity of the enzyme xylitol dehydrogenase (XDH), responsible for the reduction of xylulose to xylitol by oxidizing NADH. Based on the previous observations regarding pentose assimilation profiles, it cannot be said that the xylulose availability was higher in the HH medium, however, the xylulose concentration quantified was more constant for this medium during the 1st step (data non-shown), while it started decreasing in the AH when xylose available got lower than 9 g/L, with a consequent increase in xylitol concentration that had a 3-fold higher yield (0.25 g_{xylOH}/g_S) than in HH.

Additionally, the different compositions of the two media may also be interfering with the selectivity, shifting the metabolic pathway between the two products.

The high ethanol selectivity was a great advantage of the HH over the AH, allowing higher overall/specific ethanol productivities and yield in this medium than in the AH. In this way, despite the limitations in the enzymatic processes, comprising XOs conversion into xylose by the CC2 (84% in Batch 1), and isomerization, the HH is a promising hydrolysate to be combined with molasses in the proposed integration 1G/2G process, in addition to its well-known positive operational and environmental characteristics of production. It is worth mentioning that the CC2 used for XOs hydrolysis in the present medium can also be useful for hydrolyzing pretreated solids, as will be further evaluated.

For comparison with both industrial media, **Table 3.3I** presents the calculated indexes for the defined medium used in Section 3.3.1 at Strategy 1, which is considered a control experiment. According to Tukey's test, the only significant similarity between the processes is regarding total sugar conversion reached with S1 and HH-B1 (about 82%) in the 1st step. Ethanol yield, selectivity, and specific productivity were lower in the S1 than in AH and HH, due to the absence of hexoses in the 1st step of the process, which were rapidly converted into ethanol in the industrial media. Fermentation time was the main difference between defined and industrial media, while the 1st step of S1 lasted 10h, in AH and HH it had to be extended to 15 h to reach xylose conversion greater than 75%. The 2nd step was also longer, requiring double the time in the industrial media (3h). Besides these differences, considering the presence of inhibitory compounds and sugars in oligomeric form (HH), the results obtained in industrial media were very satisfactory. Ethanol titers of around 45 g/L were reached after both steps were performed, referring to about 92% of the stoichiometric yield.

Table 3.3. Fermentation indexes for 2 steps fermentations conducted in **(I)** Defined medium (S1), and industrial ones, **(a)** Acid (AH+M), **(b-d)** Hydrothermal hydrolysates (HH+M), and **(e)** Whole fraction (HH+HPS+M) media. IGI-HF: 53 IU/mL, CC2: 315 IU/mL (for HH), C_{X0}: 50 g_{DCW}/L, 35 °C, pH_0: 5.2. Standard deviations were less than 10%. Different letters in front of the performance index values mean statistically significant differences between them, pair-compared (Tukey's test, 95% of confidence).

Batch	Time h	C_{EtOH} g/L	X_{xyl} %	X_{tot} %	Y_{EtOH} g/g	Y_{xylOH} g/g	Q_P g_{EtOH}/L/h	q_P g_{EtOH}/IU/h	$S_{EtOH/XylOH}$ g/g
(I) Defined medium (Strategy 1)									
B1+seq (I)	10	8.43^{a}	83.35^{a}	83.35^{a}	0.35^{a}	0.32^{a}	0.63^{a}	$1.18x10^{-5a}$	1.01^{a}
	+1.5	52.03^{b}	83.35^{a}	95.38^{b}	0.50^{b}	0.05^{b}	4.34^{b}	-	8.37^{b}
(a) Acid hydrolysate (AH) + molasses (M)									
B1+seq (a)	15	6.57^{c}	89.63^{b}	90.28^{c}	0.45^{c}	0.25^{c}	0.44^{c}	$8.26x10^{-6b}$	1.97^{c}
	+3	43.97^{d}	89.63^{b}	97.66^{d}	0.47^{d}	0.04^{b}	2.44^{d}	-	11.46^{d}
(b-d) Hydrothermal hydrolysate (HH) + molasses (M)									
B1+seq (b)	15	10.49^{e}	76.25^{c}	81.97^{a}	0.49^{b}	0.07^{d}	0.70^{e}	$1.32x10^{-5c}$	7.28^{e}
	+3	46.18^{d}	76.25^{c}	91.36^{c}	0.47^{d}	0.02^{e}	2.57^{d}	-	25.16^{f}
B2+seq (c)	15	8.35^{a}	68.96^{d}	77.19^{e}	0.42^{e}	0.09^{f}	0.56^{f}	$1.05x10^{-5d}$	4.71^{g}
	+3	39.64^{f}	68.96^{d}	75.78^{e}	0.40^{e}	0.02^{e}	2.20^{d}	-	20.18^{hj}
B3+seq (d)	15	6.48^{c}	60.71^{e}	68.73^{f}	0.41^{e}	0.09^{f}	0.43^{c}	$8.14x10^{-6b}$	4.70^{g}
	+3	33.54^{f}	60.71^{e}	61.94^{g}	0.48^{b}	0.02^{e}	1.86^{g}	-	21.30^{h}
(e) HH + Hydrothermally pretreated solids (HPS) + molasses (M)									
B1+seq (e)	15	8.19^{a}	45.62^{f}	77.95^{e}	0.43^{e}	0.06^{d}	0.55^{f}	$1.03x10^{-5d}$	3.99^{i}
	+3	51.77^{b}	45.62^{f}	91.96^{c}	0.51^{b}	0.01^{e}	1.92^{g}	-	19.00^{j}

Considering the good performance achieved in the ‘first’ batch (B1) with HH+M, two additional batches were carried out with this medium to assess the feasibility of cell and enzyme recycling. The fermentation indexes are also presented in **Table 3.3c-d** and the hexose and pentose concentration, and ethanol production profiles are in **Figs. 3.3c-d**. A drop in the process efficiency can be seen at B2 and B3, which had a significant decrease in xylose conversion, 20% from B1 to B3, and consequently in other parameters such as ethanol/xylitol selectivity, which decreased 35% from B1 to B3 (1st step) (**Table 3.3b-d**), due to an increase in xylitol production. Related to that, total sugar conversion (91 - 62%) and ethanol titer (46 - 34 g/L) also decreased within the batches (B1 - B3), consequently increasing the amount of residual sugars at the end of the processes (**Figs. 3.3b-d**), since the same fermentation time was kept in each step of all batches for better comparison. The loss in the process performance raised many doubts, as three reactions were happening

simultaneously, hydrolysis, isomerization, and fermentation. Regarding the XOs hydrolysis by the enzymatic cocktail CC2, a reduction happened through the batches, from 84.1% (B1) to 66.2% (B3), but significant concentrations of xylose were quantified during the fermentation, showing that this hydrolysis step was not limiting the isomerization reaction.

A possible reduction of the isomerization rates was verified by the measurements of IGI-HF enzymatic activity, performed after each batch. A decrease of 10% was observed from B1 to B3, but xylulose concentrations similar to that found in the first batch were quantified during the whole process. Even though, more xylose was deviated to xylitol production at B2 and B3, increasing the xylitol yield and raising the possibility of inhibition over the XI performance. Xylose isomerase inhibition by fermentation byproducts such as xylitol is often reported in the literature (DE BARI et al., 2014; LASTICK et al., 1990). Coupled with that, the xylulose assimilation rate by the yeast could also have been affected due to the cell's long exposure to the inhibitory compounds present in the hydrolysate, impairing the SIF efficiency, synergistically with the limitations in the IGI-HF performance. Additionally, the baker's yeast used in the process is not evolved to handle the conditions found in industrial media. This strain has already been evaluated in repeated batches previously, but in defined media, showing great stability in the fermentation indexes through the 3 batches performed (SANDRI et al., 2023b).

There are few reports in the literature regarding SICF processes in hydrolysates. The first of them was described by Chandrakant and Bisaria (2000b). The authors evaluated co-fermentation in three different compositions of peanut-shell hydrolysates, achieving the highest ethanol concentration in that with a higher concentration of glucose, but most of the xylose remained in the medium. When performing the SICF in a condition favorable for pentose assimilation (medium containing 50 g/L of xylose and 3.5 g/L of glucose), xylose conversion was only 36% within 56 h, providing 6 g/L of ethanol, probably due to limitations in the isomerization rate for providing xylulose to the yeast or in its uptake rate by the strain used. De Bari et al. (2014) evaluated SICF similarly to the present work, using commercial enzymes and a high yeast load. The authors used an acid-catalyzed hemicellulosic hydrolysate produced from corn stover and found out that increasing cell load from 50 to 75 g/L allowed an increase in ethanol titer, but xylose conversion was similar for both processes (about 87%) within 72 h. These results show the superiority of the ones obtained in the present work, which although still requires improvements for process stability

in the repeated batches, provided promising fermentation indexes in industrial media (46 g_{etOH}/L in 18 h with HH+M) with shorter fermentation time.

3.3.2.2. Integrated 1G/2G process using HH and hydrothermally pretreated solids (HPS) plus molasses (M) media

The full 1G/2G integration was also evaluated. An experiment with 10 g/L (dry basis) of hydrothermally pretreated sugarcane bagasse (HPS) supplemented with the fermentation media (HH+M) was performed, characterizing a Simultaneous Saccharification, Isomerization, and Co-Fermentation (SSICF) process. In the SSICF, the CellicCtec2 enzymatic cocktail, already present in the HH+M fermentation for XOs hydrolysis, had the additional hole of hydrolyzing the cellulose. The media consisted of HH+HPS in the 1st step, followed by the addition of molasses (2nd step). The hexoses and pentoses assimilation profile and ethanol production are presented in **Fig. 3.3e**, and fermentation indexes are in **Table 3.3e**.

From **Fig. 3.3e**, a slower pentose assimilation can be observed compared to the other fermentations in absence of solids (HH+M and AH+M, **Figs. 3.2a-b**), which can be attributed to hexose's preference over pentose's assimilation, since during the 1st step, glucose was slowly but constantly being provided to the media through the solid's hydrolysis by the CC2, competing with the xylulose uptake. Xylose conversion after 15 h was 45.62%, a value 40% lower than that reached in HH+M (B1). Despite the lower xylose conversion, the hydrolysis of the solids present in the process into hexose sugars was very efficient, about 71% of the 10 g/L solids provided were hydrolyzed, which theoretically provided 6.92 g/L of extra sugar to the process (potential considering 71% conversion of its sugar fractions), coming from the hemicellulosic and mostly cellulosic fractions of the pretreated biomass. This sugar concentration contributed to the ethanol titer, which was about 52 g/L at 18 h of process, a value similar to that achieved in the defined medium. Coupled with that, the xylitol yield was low (0.06 g_{xylOH}/g_S, 1st step) and significantly similar to that found in HH+M (B1) (**Table 3.3b**).

An SSICF for ethanol production was previously reported by Guilherme et al. (2022), who used a commercial enzymatic cocktail, supplemented with an in-house made multi-enzymatic system for the co-fermentation of pentoses and hexoses. The authors performed 4 repeated batches in a complex medium with 30 g/L of cellobiose and 20 g/L of xylose, 48 h each, keeping an ethanol yield of 0.37 g_{etOH}/g_S and overall ethanol productivity of about 0.46 g_{etOH}/L/h. However, about 50% of the xylose offered remained in the medium in the 3rd and 4th batches, even though, the xylose

conversion increased through the batches, due to a decrease in the offer of free glucose from cellobiose, caused by a reduction in the cellobiose hydrolysis efficiency during enzyme recycling. As a proof of concept for the process in industrial media, the authors ran a batch of SSICF with pretreated sugarcane bagasse as substrate. It was accomplished in 2 stages, first a pre-saccharification, and then the fermentation, but xylose consumption was not observed, on the contrary, this sugar concentration increased with time due to the hydrolysis of some hemicellulose present in the biomass. The low xylulose uptake was pointed out as dependent on the low enzyme (XI) load used and on the low efficiency of the wild-type *S. cerevisiae*, which led to the consequent side-production of xylitol. The yeast used by the authors was an industrial strain (PE-2) selected in the Brazilian ethanol mills, which according to Sandri et al. (2023b) is not so efficient in the uptake of the pentose xylulose, due to its specificity of being selected in hexose rich environments.

Considering the negative impact caused by the continuous hydrolyzed hexoses over pentoses assimilation, but the positive contribution of the ethanol produced by these hexoses in the final titer, an improvement of this strategy is necessary. The proposal would be maintaining the same fermentation strategy used previously, i.e., using only HH in the 1st step and adding HPS together with M in the 2nd step, the two stages of fermentation proposed showed to be a valuable approach to effectively reach high xylose consumption and extra ethanol production.

3.4. Conclusions

The potential of using the generally neglected pentose sugars is widely addressed in the literature, but its implementation in the industrial scenario is still a big challenge. The present work proposed a simple and feasible strategy for 1G/2G ethanol process integration based on a specially designed bioreactor configuration, commercially available enzymes and non-GMO yeast, and a sequential fermentation procedure to maintain high pentose conversion in the 1st step as well as achieve high ethanol titers after molasses supplementation in the 2nd step. The fermentation strategy was evaluated with hemicellulosic hydrolysates, either acid or hydrothermal pretreated, in single or repeated batches operation, and it was further validated in the SSICF process using hydrothermally pretreated sugarcane bagasse. Impaired overall productivity due to slow xylulose uptake rate and decreased performance in repeated batches due to the inhibitors present in the hydrolysates remain the main issues to be faced for further process improvement. The isomerization reaction plays a relevant role in the overall productivity, and solutions to run SIF

with higher enzyme volumetric activity, such as developing biocatalysts with higher specific XI activity (IU/mg) and improving EMPBR design to increase its enzyme load capacity, must be pursued. Concerning fermentation, adaptive evolution poses a promising approach to select *S. cerevisiae* cells with a better affinity for xylulose and more tolerance to inhibitors, leading to faster fermentation rates.

Chapter 4. High throughput screening of yeast co-culture using crude hydrolysate for co-fermentation of pentose and hexose

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Abstract

The use of all sugars from biomass fractionation, *i.e.,* hexoses and pentoses, is essential to develop feasible processes in a biorefinery context. Hexoses are converted into different products by most microorganisms, the main issue being the conversion of pentoses, especially when mixed with hexoses. Improving sugar conversion in co-fermentation is a major challenge that was explored in this work. Co-culture using *S. cerevisiae* and evolved *K. marxianus* strains was evaluated by a high throughput screening using 24 deep-well plates. Glucose depletion and 83% of xylose conversion were achieved from supplemented hemicellulosic hydrolysate, using 2/5 of deep-well volume filled with medium (at 150 rpm) and initial cell concentrations of 2.5 g/L for each yeast. The screening strategy provided reliable data that confirmed the co-culture as a promising approach for the co-assimilation of biomass-derived sugars. The importance of aeration control for effective xylose assimilation was also pointed out.

Key-words: Lignocellulosic biomass; Yeast co-culture; Xylose conversion; Co-fermentation; Hydrolysate; Ethanol

4.1. Introduction

Lignocellulosic material is the most available kind of biomass in the world. It is mainly composed of cellulose, hemicellulose, and lignin, the first two being rich in monomeric sugars. To

make these sugars accessible for further use, the biomass needs to go through some processes such as pretreatment and enzymatic hydrolysis (MUSSATTO; DRAGONE, 2016). In a biorefinery context, the utilization of all biomass sugars is of great importance, but this goal is limited by the lack of industrially suitable organisms able to efficiently convert both glucose and xylose (NOSRATI-GHODS et al., 2018). These sugars are sustainable sources for the production of many valuable bioproducts, such as xylitol, carotenoids (BONFIGLIO et al., 2021; LIU et al., 2020), ethanol (PEREZ et al., 2021), butanol (MAGALHÃES et al., 2018), and single-cell protein (FENG et al., 2021).

Most microorganisms widely and naturally assimilate hexoses. In the sugar-ethanol industry, sucrose from sugarcane molasses is broken by periplasmic invertase into glucose and fructose, which are readily consumed by *Saccharomyces cerevisiae,* producing high ethanol titers (PEREIRA et al., 2018). However, this robust yeast does not assimilate pentoses like xylose (MILESSI et al., 2018). Finding a yeast that can co-assimilate glucose and xylose efficiently is a big challenge, which brings many studies working toward a good solution. There are several options to enable xylose assimilation, such as the use of genetically modified organisms (GMOs), natural pentose fermenting strains, or yet supplementation with enzyme preparations. Similarly, different fermentation strategies have been investigated to enhance the co-fermentation of pentoses and hexoses, such as co-cultures and sequential cultures (NOSRATI-GHODS et al., 2018).

Currently, GMOs are one of the most studied and developed options (HOANG NGUYEN TRAN et al., 2020; LI et al., 2019). However, genetic modifications often imply the loss of other important traits of the strains, such as product and substrate tolerance (DEPARIS et al., 2017). In addition, the application of GMOs to industrial processes is conditioned on several regulations, depending on the country, which can make their use difficult and even increase the fermentation cost (MILESSI et al., 2018). In this sense, using generally regarded as safe (GRAS) microorganisms makes their application in industrial conditions easier (LEONEL et al., 2021).

Another alternative for xylose conversion is the addition of enzymes to the culture media, such as xylose isomerase (XI), which performs the isomerization of xylose into xylulose that can be assimilated by many microorganisms, including *S. cerevisiae*, in a process denominated Simultaneous Isomerization and Fermentation- SIF (MILESSI et al., 2018; MIYAMOTO et al., 2021). Even though, the use of XI presents several bottlenecks, such as the difference between the optimal conditions for the enzyme reaction and the microorganism to be used for fermentation,

besides the loss of enzymatic activity (MIYAMOTO et al., 2021). In addition, enzymes are expensive and can contribute to increasing the costs of the process (DRAGONE et al., 2020).

The use of natural pentose-fermenting strains is an alternative for the co-consumption of hexoses and pentoses since it does not require additional enzymes supplemented to the media (ARORA et al., 2019; NOSRATI-GHODS et al., 2018). However, these strains usually present specific demands for their cultivation, like media supplementation and controlled aeration. In addition, they usually exhibit slow growth and sugar uptake rate, low productivity, and reduced tolerance to ethanol and hydrolysate inhibitors, which make it fundamental to study and optimize their culture conditions (BONAN et al., 2020; NOSRATI-GHODS et al., 2018). Among the many pentose-fermenting strains, *Spathaspora passalidarum*, *Scheffersomyces stipitis*, and *Kluyveromyces marxianus* stand out for glucose and xylose fermentation, but as mentioned, their culture conditions have to be evaluated to deal with the many existing bottlenecks (ARORA et al., 2019; BONAN et al., 2020; NADAL; SHARMA; ARORA, 2020).

Some culture strategies can be performed to improve sugar co-assimilation, like sequential and co-cultures. Co-culture is a promising technology that consists in inoculating a mix of microorganisms in the media, generally each one with its particular benefit to the process (MUSSATTO; DRAGONE, 2016), which may show advantages to the process due to synergistic interactions of their metabolic pathways, like improving pentose assimilation, avoiding higher expenses related to using enzymes for xylose conversion or GMOs, and reducing fermentation time (CHEN, 2011). Otherwise, the main challenges are to find a condition that suits all microorganisms, such as temperature, inoculum size, and aeration, and deal with possible catabolic repression (NOSRATI-GHODS et al., 2018). For this purpose, detailed screening is necessary. Apart from co-culture, sequential culture is a strategy that allows optimizing fermentation conditions for each microorganism separately, facilitating the process conduction. Nevertheless, it requires fermentation to be carried out in two stages, impairing the conduction of repeated batches and reducing the synergistic interactions between the microorganisms.

Small-scale screening is a reliable and efficient way of conducting experiments that have been widely used for several purposes, such as defining optimal culture conditions or selecting the best microorganism from a range (BONFIGLIO et al., 2021; MORATO et al., 2021). This high throughput strategy allows the evaluation of different cultivation conditions at once with several repetitions, besides providing a replicable culture environment and saving experimental time and

material. The scalability of small-scale processes in bioreactors has also shown reproducible results (GARRIGÓS-MARTÍNEZ et al., 2021; HEMMERICH et al., 2014; MESQUITA et al., 2021).

To achieve a promising co-culture process, many details must be evaluated, but before starting, the main decision is which microorganisms to use. As mentioned, *S. cerevisiae* is a robust yeast widely applied for ethanol production at sugarcane mills, with a rapid hexose assimilation rate, making it a great candidate for the consumption of hexoses in co-culture. Regarding pentoses, the yeast *K. marxianus* stands out for its characteristics of assimilating a wide variety of substrates, thermotolerance, and robustness, besides being a GRAS microorganism (LEONEL et al., 2021). This yeast also has a very flexible metabolism that can be redirected to the desired pathway through changes in the culture condition. Ethanol production from xylose, for instance, occurs at an optimum oxygen level, being poor in conditions close to aerobiosis or anaerobiosis (ZHANG et al., 2011). Yeast cell mass, a relevant co-product with commercial value, also has its production influenced by the aeration condition (FENG et al., 2021; SHULER; KARGI, 2002).

Based on the above, a high throughput screening of a co-culture process with an industrial *S. cerevisiae* strain and an evolved *K. marxianus* strain is presented in this work, targeting high conversions of glucose and xylose into products like ethanol and yeast cell mass. To the best of our knowledge, this is the first work addressing high throughput screening of a co-culture using *S. cerevisiae* and *K. marxianus* strains aiming at glucose and xylose co-fermentation using crude sugarcane bagasse hydrolysate based medium. Our results show that the co-culture significantly improves xylose consumption in the crude hydrolysate, and support the great potential of this approach in a biorefinery context.

4.2. Material and methods

4.2.1. Preparation of hemicellulosic hydrolysate

Sugarcane bagasse (SB) was kindly provided by Ipiranga Agroindustrial (Descalvado, São Paulo, Brazil). The raw material was dried at 45 °C until reaching approximately 10% of moisture; milled in a Wiley type knife mill (TE-650/1 Tecnal, Brazil) to a particle size of 10 mesh, and then characterized according to Sluiter et al. (2012). Its composition was (w/w%) cellulose (44.15 ± 2.01), hemicellulose (24.35 ± 2.48), total lignin (19.16 ± 0.22), acetyl group (3.52 ± 0.20), ashes (1.86 ± 0.16), and extractives (3.81 ± 0.36).

The SB was pretreated hydrothermally in 600-mL Parr Series 4760 pressure vessels (Parr Instrument Company, USA) using 300 mL volume for the reaction (YAMAKAWA et al., 2020). The pretreatment was conducted with 10% (w/v) of solid load (dry basis) at 195 °C for 45 min. The reactors were heated by immersion in a silicone oil bath and then immediately cooled in an ice bath for 30 min. After pretreatment, the liquid (crude hemicellulosic hydrolysate, HH) was separated from the solid fraction by vacuum filtration and stored at 5 °C for further use and analysis to determine its content of monomeric sugars, xylooligomers, carboxylic acids, furans, and phenolic compounds. The composition of the HH is presented in **Table 4.1**.

Table 4.1. Chemical composition of sugarcane bagasse hemicellulosic hydrolysate (HH) obtained by hydrothermal pretreatment (10% (w/v) of solids, dry basis, 195 °C for 45 min).

Compounds	Concentration (g/L)
Glucose	0.04 ± 0.00
Xylose	1.92 ± 0.15
Arabinose	0.52 ± 0.02
5-Hydroxymethylfurfural (HMF)	0.05 ± 0.01
Furfural	0.74 ± 0.04
Formic acid	0.07 ± 0.00
Acetic acid	1.50 ± 0.01
Xylooligomers	13.85 ± 0.13
Total phenolics	0.48 ± 0.02

4.2.2. Microorganisms

The commercial baker´s yeast *Saccharomyces cerevisiae* Itaiquara® in pure form (purchased from Itaiquara Alimentos S.A., Tapiratiba/ Brazil) and four evolved strains of *Kluyveromyces marxianus* NRRL (Y-2265 1 A, Y-8287 2 A, Y-6860 3 A, and Y-6373 4 A) of the research group "Biomass Conversion and Bioprocess Technology" (Technical University of Denmark), with increased tolerance to biomass-derived inhibitors (unpublished data), were evaluated.

For the inoculum preparation of all yeast strains, one slope of the agar plate containing the cells was transferred to a 250-mL baffled shaker flask containing 50 mL of synthetic medium (22 g/L glucose, 8 g/L xylose, 3 g/L yeast extract, 5 g/L $(NH_4)_2HPO_4$, 3 g/L KH_2PO_4, 0.5 g/L

$MgSO_4.7H_2O$, 0.025 g/L of trace microminerals solution, and 100 μg/mL of ampicillin) with initial pH adjusted to 5 with H_2SO_4 (2 mol/L) solution. The flasks were incubated at 35 °C, 250 rpm for 24 h. Afterward, the cells were recovered by centrifugation (10000 rpm, 5 min, 5 °C), washed with saline solution and the pellet was inoculated in the medium at the initial concentration defined for each strain, which changed according to the experimental condition studied.

4.2.3. Experimental strategy

First, a screening of evolved *K. marxianus* strains, candidates for co-culture studies, was carried out. The selected strain was further studied to improve xylose conversion as well as product yield in co-cultures with *S. cerevisiae*. Finally, experiments to investigate the performance of the strains in sequential cultures as well as in mono-cultures were performed for comparison.

All fermentations described from now on were performed in 24 deep-well plates (Enzyscreen, Netherlands) with 10 mL of working volume (BONFIGLIO et al., 2021). The plates were incubated in an orbital shaker (New Brunswick, USA) at 35 °C, and samples were taken for further analysis at 0, 6, 12, 24, 48, and 72 h (Section 4.2.4). The volume of media added per well and the agitation applied varied according to each study. All studies were performed in duplicate.

4.2.3.1. Yeast screening

The purpose of the screening was to define the best xylose-fermenting yeast (among the 4 *K. marxianus* mentioned in Section 4.2.2) for combining with *S. cerevisiae* in co-culture studies. The strains were cultivated in crude hemicellulosic hydrolysate (HH) supplemented with 55 g/L glucose and 20 g/L xylose (without nutrient addition), denominated as HH medium (HHm). The concentrations of glucose and xylose supplemented were defined based on concentrations that could be achieved through the enzymatic hydrolysis of the whole pretreated biomass, considering 15% (w/v) of solid load, and approximately 60% of cellulose and 90% of hemicellulose conversion (PINTO et al., 2021). The initial pH was adjusted to 5 with NaOH (2 mol/L) solution. The volume of media added per well was 2 mL with an initial cell concentration (C_{X0}) of 1 g/L. The deep-well plates were incubated at 250 rpm (same agitation used in the evolution of these strains) for 72 h.

4.2.3.2. Co-culture assays: influence of process conditions on xylose conversion and product yield

All studies described in this section were conducted as co-cultures with the strain previously selected in the yeast screening: *K. marxianus* NRRL Y-6860 3 A, which from now on will be called *K. marxianus* 3 A, in association with *S. cerevisiae* (Itaiquara®).

Initially, for a better understanding of co-culture performance and responses, several conditions of medium volume, agitation, and initial cell concentration ratios were evaluated in different media: hemicellulosic hydrolysate medium (HHm) and HHm enriched with nutrients (3 g/L yeast extract; 2 g/L $(NH_4)_2HPO_4$; 2 g/L KH_2PO_4; 0.25 g/L $MgSO_4.7H_2O$) (HHs). For control experiments, a synthetic medium (S) containing 55 g/L glucose and 20 g/L xylose (same sugar composition of HHm or HHs) was used, and it was supplemented with salts and nitrogen sources (same composition described in Section 4.2.2). All media had the initial pH adjusted to 5 with NaOH (2 mol/L) or H_2SO_4 (2 mol/L) solutions and were supplemented with 100 μg/mL ampicillin.

To assess the influence of aeration on co-culture performance, two aeration conditions, defined as the ratio between the volume of medium and the volume of the deep-well (V_{medium}:$V_{deep-well}$), were evaluated, 1:5 (2 mL of inoculated medium in 10 mL total volume) and 1:2.5 (4 mL of inoculated medium). The strains were inoculated in the same initial cell concentration (C_{X0}) of 0.5 g/L for each yeast (1 g/L of total concentration) and the fermentations were conducted at 150 rpm for 72 h. These experiments had the main purpose of observing the influence of the combination of medium volume and agitation on fermentation performance since these parameters are related to the availability of dissolved O_2 in the medium.

The ratio between *S. cerevisiae* and *K. marxianus* 3 A initial concentrations inoculated to run the co-cultures was also investigated. Considering the condition that led to the best results in the previous studies regarding xylose conversion and product yield, the co-cultures were conducted at 150 rpm, with V_{medium}:$V_{deep-well}$ of 1:2.5, 4 mL of medium (S and HHm) in each deep-well. The initial cell concentration ratios (*S. cerevisiae: K. marxianus* 3 A) evaluated were 0.5:2.5 g/L and 0.5:4.5 g/L. A study with an equal cell ratio of both strains (2.5:2.5 g/L), C_{X0} of 5 g/L, was also conducted in S, HHm, and HHs media. The HHs was a plus condition for verifying if the presence of nutrients could improve the fermentation process.

Finally, a lower agitation rate was evaluated to reduce the dissolved O_2 level of the culture and verify its influence on the fermentation performance. Co-culture was conducted at 100 rpm

with 4 mL (V_{medium}:$V_{deep-well}$ of 1:2.5) of S and HHs medium per deep-well. The initial cell concentration ratios assessed were 0.5:2.5 g/L and 2.5:2.5 g/L (*S. cerevisiae*: *K. marxianus* 3 A).

4.2.3.3. Sequential fermentation

Sequential fermentations were performed to assess whether there is hexose repression on pentose assimilation. In this strategy, fermentations were started only with *S. cerevisiae*, at an initial cell concentration of 2.5 g/L. After 24 h, the average time previously observed for glucose depletion, *K. marxianus* 3 A was inoculated (C_{X0}: 2.5 g/L) in the medium. Fermentations were carried out in S and HHs media, with 4 mL of media per deep-well (V_{medium}:$V_{deep-well}$ of 1:2.5). The agitation rates evaluated were 100 rpm, and also a combination of 100 rpm in the first 24 h (*S. cerevisiae* only) followed by an increase to 150 rpm when adding *K. marxianus* 3 A.

4.2.3.4. Mono-cultures

To compare and validate the benefits of the co-culture strategy, the performance of the yeasts *S. cerevisiae* and *K. marxianus* 3 A as mono-cultures was evaluated in S and HHs media. The fermentations were carried out in the best conditions obtained after the previous studies: initial cell concentration of 5 g/L, 4 mL of medium (V_{medium}:$V_{deep-well}$ of 1:2.5), and agitation rates of 100 and 150 rpm for *S. cerevisiae* and *K. marxianus* 3 A, respectively.

4.2.4. Analytical methods

During the cultures, samples taken were analyzed regarding pH and optical density (OD), measured at 600 nm using a V-1200 spectrophotometer (VWR, Denmark), which was used to determine cell concentration, by a dry weight calibration curve obtained for each yeast. Then, samples were centrifuged (10000 rpm, 5 min, 5 °C) and the supernatants were filtered in a 0.22 μm filter and stored in a freezer at -20 °C for further analysis of the compounds.

The concentrations of cellobiose, glucose, xylose, arabinose, acetic acid, formic acid, furfural, and 5-HMF were determined by high-performance liquid chromatography (HPLC) using a Dionex Ultimate 3000 $HPLC^+$ Focused system (Thermo Scientific) with a Bio-Rad Aminex® column HPX-87H at 60 °C, with H_2SO_4 (5.0 mM) as mobile phase, at a flow rate of 0.6 mL/min. Sugars and acids were detected using a refractive index detector (RefractoMax 520 series), whereas 5-HMF and furfural were obtained using a UV detector at 254 nm.

For determining the content of total xylooligomers in the hemicellulosic hydrolysate, this medium was acid-hydrolyzed with sulfuric acid (72%) in an autoclave (121 $^{\circ}$C for 60 min), according to Nascimento et al. (2016). Using the hydrolysis stoichiometry, total xylooligomers concentration was calculated from the difference between the concentration of xylose in the acid-hydrolyzed and the original hydrolysate samples after HPLC analysis (MILESSI et al., 2021). Total phenolic compounds were quantified by colorimetric method (BALLESTEROS; TEIXEIRA; MUSSATTO, 2014).

4.2.5. Calculation procedures and statistical analysis

The cultures were evaluated and compared regarding some performance indicators including substrate conversion X (%), product yield $Y_{P/S}$ ($g_{coproduct}/g_{substrate}$), and sugar uptake rate Q_S (g_{sugar}/L/h), which were calculated as described in the literature (MILESSI et al., 2018; SHULER; KARGI, 2002). The results were compared statistically regarding their significant differences through the Tukey test, using the Origin®Pro 8.5 software at a confidence level of 95%.

4.3. Results and discussion

4.3.1. Yeast screening

The purpose of the screening was to find the best xylose-fermenting yeast for joining *S. cerevisiae* in the co-culture experiments. The fermentation profile for each strain evaluated in hemicellulosic hydrolysate medium (HHm), is presented in **Fig. 4.1**. The agitation applied in these studies was the same used for the evolution of these strains (unpublished data), which favors cell growth. Therefore, the formation of fermentative products (ethanol and xylitol) was not detected. In contrast to *S. cerevisiae,* according to Bellaver et al. (2004), when the yeast *K. marxianus* is cultivated under aerobic conditions with high glucose concentration (metabolic overflow conditions), it results in very low to no ethanol production but favors the production of cells. It is worth noticing that in this harsh environment, HHm without nutrients supplementation and with a high initial glucose concentration, the strains needed a long time for complete glucose consumption. Consequently, xylose assimilation was even slower. Its assimilation improved after about 70% of glucose was consumed, which means that less than 20 g/L of glucose remained in the medium, for strains 2 A and 3 A (**Figs. 4.1 b-c**). Similar final biomass concentrations (about

3.8 g/L) and glucose conversion (about 90%) were reached for all strains (**Figs. 4.1 a-d**) (Tukey's test).

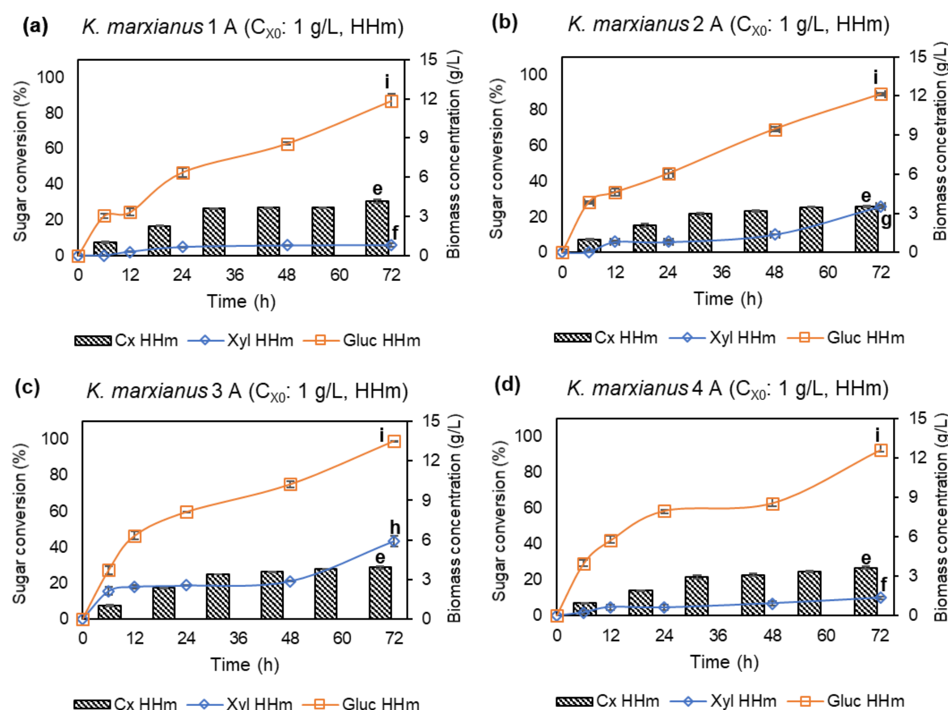

Figure 4.1. Sugar conversion (glucose, Gluc HHm, and xylose, Xyl HHm) and biomass concentration (Cx HHm) obtained during the culture of evolved *K. marxianus* strains **(a)** 1 A, **(b)** 2 A, **(c)** 3 A, and **(d)** 4 A in hemicellulosic hydrolysate medium (HHm), 35 °C, 250 rpm, C_{X0}: 1 g/L, pH_0: 5. Different letters at the top of the graph symbols represent statistically significant differences for conditions at the last point, pair-compared, according to Tukey's test.

From the sugar conversion profiles, *K. marxianus* 3A (**Fig. 4.1c**) showed the best performance for glucose (100%) and xylose assimilation (43%) in 72 h. The volumetric xylose consumption rate (Q_{xyl}) was calculated for each strain considering data in a linear range after more than 60% of glucose was converted, reflecting the increase in xylose assimilation. The results obtained were 1.9±0.5, 156.6±1.3, 188.4±1.7, and 27.9±1.1 mg_{xylose}/L/h for *K. marxianus* 1 A, 2 A, 3 A, and 4 A, respectively. Thus, *K. marxianus* 3 A was selected for further studies.

4.3.2. Co-culture assays: influence of process conditions on xylose conversion and product yield

Having selected the most promising pentose-fermenting yeast, the challenge became optimizing fermentation conditions to obtain high xylose conversion combined with favorable

product yields in co-culture experiments. Aeration is an important factor when working with *K. marxianus* as this yeast deviates its metabolism according to the conditions to which it is exposed, such as oxygenation and medium composition (BONFIGLIO et al., 2021; LEONEL et al., 2021). For instance, although *K. marxianus* can assimilate xylose well, producing ethanol from this sugar requires adjustments in aeration for redirecting its metabolic route (ZHANG et al., 2011). For the cultivation of *S. cerevisiae*, on the other hand, it is well known that high ethanol yield is achieved in an anaerobic environment, as found in the conventional "Brazil fuel-ethanol plant". The presence of oxygen over a threshold limit can deviate *S. cerevisiae* metabolism into biomass production (MESQUITA et al., 2019). Therefore, a condition that benefits both strains in co-culture must be found.

4.3.2.1. Influence of agitation rate and headspace volume on co-culture performance

Initially, co-culture fermentations were performed under two aeration conditions by using different ratios between medium and deep-well volumes of 1:5 and 1:2.5, which means 2 and 4 mL of inoculated medium added per deep-well (10 mL), respectively. The agitation applied in these experiments was lower than that used in the screening step, from 250 to 150 rpm, aiming to approach a microaerobic environment and obtain the desired metabolic responses that trigger product formation. The profiles of xylose and glucose conversion, as well as biomass production during the fermentations at each aeration condition evaluated, are presented in **Fig. 4.2**. Performance indicators such as product concentrations and yields are presented in **Table 4.2**.

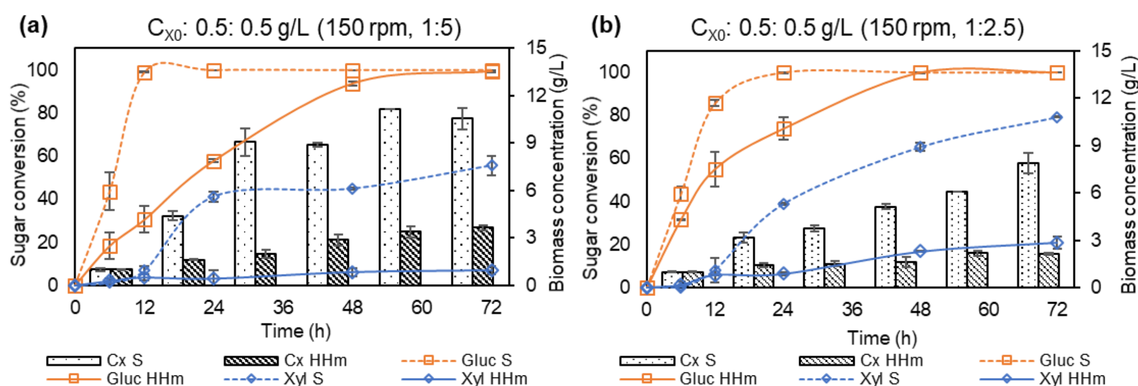

Figure 4.2. Sugar conversion (glucose and xylose) and biomass concentration obtained during the co-culture of *K. marxianus* 3 A and *S. cerevisiae* in synthetic (S) and hemicellulosic hydrolysate (HHm) media with aeration conditions **(a)** 1:5 (2 mL) and **(b)** 1:2.5 (4 mL), 35 °C, 150 rpm, C_{X0}:1 g/L, pH_0:5.

In both aeration conditions evaluated, glucose was depleted in less than 24 h in the S medium. Glucose exhaustion was also observed in HHm, but it took about 48 h (**Figs. 4.2 a-b**). Even so, glucose assimilation was faster in HHm with a lower headspace volume (V_{medium}:$V_{deep\text{-}well}$ of 1:2.5) than with the higher one (V_{medium}:$V_{deep\text{-}well}$ of 1:5). The glucose uptake rates (Q_{gluc}) were 1.40±0.11 $g_{glucose}$/L/h and 2.56±0.22 $g_{glucose}$/L/h for the aeration conditions 1:5 and 1:2.5 in HHm, respectively, while in S medium it was 4.84±0.42 $g_{glucose}$/L/h (1:5) and 3.98±0.04 $g_{glucose}$/L/h (1:2.5). Comparing the uptake rates between the two headspaces volumes (V_{medium}:$V_{deep\text{-}well}$ of 1:5 and 1:2.5) for each medium (HHm and S), and between the two media with the same headspace, it can be verified the coupled influence of HHm inhibitors and aeration condition on yeasts' performance, even if *K. marxianus* is evolved for a better inhibitors tolerance. The higher glucose uptake rate was obtained with an aeration condition of 1:5 in the S medium, despite no big differences in uptake rate values being observed between the two headspaces in this medium.

Regarding xylose conversion, as a consequence of glucose assimilation rate, it was higher in both media at smaller headspace (V_{medium}:$V_{deep\text{-}well}$ of 1:2.5), with significant differences for all conditions (Tukey's test). In HHm, the improvement was 3-fold compared to 1:5 experiments (7%), reaching 21% of xylose conversion after 72 h (**Fig. 4.2 b**, **Table 4.2**), which proves the importance of providing a suitable microaeration level over xylose conversion. The influence of aeration in *K. marxianus* CICC 1727−5 cultures was investigated by Feng et al. (2021), growing this yeast in a mimicked hydrolysate medium. By keeping constant aeration of 0.2 vvm, the authors could reduce the residual xylose in the medium significantly compared to the condition without an air supply. The results were further improved at 0.5 vvm, allowing the depletion of xylose and higher xylitol production, but the worst responses in xylitol yield and productivity were obtained with 1.0 vvm, highlighting the importance of suitable aeration for xylose conversion through the fermentative route. In the present study, a low amount of xylitol was obtained in all conditions with both media, with a similar yield of about 0.02 g_{xylOH}/g_S, showing a redirecting of the metabolic pathway into other products formation, such as ethanol and biomass.

From **Fig. 4.2**, it is also possible to observe that the patterns of xylose and glucose conversion are associated. As previously mentioned, the co-utilization of glucose and xylose only started after about 70% of glucose conversion was reached, which means about 20 g/L of glucose remaining in the medium, suggesting that at glucose concentrations higher than this threshold value xylose uptake might be repressed. This ability to co-ferment both sugars is a positive trait of the

evolved *K. marxianus* used in this work since several studies report pentose-fermenting strains that present a long lag phase after glucose depletion for xylose assimilation to get started (DOS SANTOS et al., 2013; RODRUSSAMEE et al., 2011; SLININGER et al., 2011). According to Hua et al. (2019), the ability to co-assimilate xylose and glucose is mainly related to the regulation of xylitol dehydrogenase (KmXYL2) gene expression. Rodrussamen et al. (2011) and dos Santos et al. (2013) reported that xylose consumption by *K. marxianus* DMKU3-1042 and UFV-3, respectively, is competitively inhibited by hexoses during diauxic growth in the presence of 20 g/L glucose. In the presence of glucose, xylose-specific enzymes, such as xylose reductase (XR) and xylitol dehydrogenase (XHD), and sugar transporters are repressed in xylose-fermenting microorganisms (SLININGER et al., 2011). To promote the complete conversion of glucose and xylose into ethanol, the conduction of separate/sequential cultivation is commonly proposed, which allows the cultivation of either hexose and pentose assimilating microorganisms under their best culture conditions (NOSRATI-GHODS et al., 2018; SLININGER et al., 2011).

Contrary to xylose conversion, biomass concentration reached in the co-cultures in S medium was higher in the more aerated condition (V_{medium}:$V_{deep\text{-}well}$ 1:5), showing that this change was able to shift the yeast metabolism towards the growth pathway. Cell yields were about 3-fold (0.13 g_X/g_S) and 5-fold (0.09 g_X/g_S) higher in S medium than in HHm, with ratios V_{medium}:$V_{deep\text{-}well}$ of 1:5 and 1:2.5, respectively. A 3-fold increase in biomass production after 72 h was reported in a co-culture of *S. cerevisiae* and *S. stipitis* for ethanol production in detoxified hydrolysate (ROJAS-CHAMORRO et al., 2019). According to the authors, the high biomass production is directly related to the difficulty of maintaining a microaerobic environment during fermentation, which would favor ethanol production, as shown in the present work. No significant differences were observed between cell and ethanol yields in HHm (Tukey's test, **Table 4.2**). Even though, in the S medium, the ethanol yield was higher under lower headspace (0.20 g_{etOH}/g_S).

Overall, it can be observed that besides ethanol production and yield, the xylose conversion and cell yield (**Table 4.2**) were higher in S media than in HHm at both aeration conditions. As previously mentioned, glucose assimilation was also faster in this medium, especially with a V_{medium}:$V_{deep\text{-}well}$ of 1:2.5, allowing a greater xylose conversion and less biomass formation compared to the 1:5 experiment. The better performance in the S medium can be related to the inhibitor's presence in the HHm, which may be impairing cells' metabolism, especially of *S. cerevisiae*, which is not evolved to handle this stress. Another reason may be the lack of nutrients

in HHm, which were present in the S medium. However, considering the xylose conversions reached in HHm, medium of greatest interest, the aeration ratio that provided the highest value was 1:2.5. So, this condition was defined for use in the subsequent studies.

Table 4.2. Final ethanol concentration (C_{etOH}), cell ($Y_{X/S}$), ethanol ($Y_{etOH/S}$), xylitol ($Y_{xylOH/S}$) yields, and xylose conversion (X_{xylose}) in different media (HHm, HHs, and S) and culture conditions evaluated in co-culture studies. Values are replicates of averages, with less than 10% standard error. Glucose was depleted at a maximum of 48 h in all studies. Different letters in front of the values represent statistically significant differences between them, pair-compared for each condition in each study, according to Tukey's test (95% of confidence).

Agitation (rpm)	**V_{medium}:$V_{deep\text{-}well}$**	***S. cerevisiae: K. marxianus* 3 A**	**Time (h)**	**C_{etOH} (g/L)**		**$Y_{X/S}$ (g_X/g_S)**		**$Y_{etOH/S}$ (g_{etOH}/g_S)**		**$Y_{xylOH/S}$ (g_{xylOH}/g_S)**		**X_{xylose} (%)**	
4.3.2.1. Influence of agitation rate and headspace volume on co-culture performance													
Media:				**HHm**	**S**	**HHm**	**S**	**HHm**	**S**	**HHm**	**S**	**HHm**	**S**
150	1:5	0.5:0.5 g/L	72	12.54^{a}	9.13^{b}	0.04^{a}	0.13^{b}	0.21^{a}	0.14^{b}	0.01^{a}	0.02^{ab}	7.10^{a}	55.57^{c}
	1:2.5			12.94^{a}	17.86^{a}	0.02^{a}	0.09^{c}	0.24^{a}	0.20^{a}	0.02^{ab}	0.02^{b}	21.08^{b}	72.23^{d}
4.3.2.2. Influence of initial yeast concentrations and medium supplementation													
150	1:2.5	0.5:2.5 g/L	72	11.18^{d}	13.66^{d}	0.05^{df}	0.09^{ef}	0.21^{d}	0.23^{d}	0.03^{c}	0.07^{cd}	16.19^{f}	66.82^{e}
		0.5:4.5 g/L		10.30^{d}	14.87^{de}	0.04^{df}	0.11^{e}	0.18^{d}	0.26^{de}	0.03^{c}	0.08^{de}	30.36^{g}	69.79^{e}
		2.5:2.5 g/L		13.46^{d}	17.64^{del}	0.02^{d}	0.10^{ef}	0.21^{d}	0.25^{del}	0.02^{c}	0.04^{e}	45.83^{h}	66.59^{e}
Media:				**HHs**	**S**	**HHs**	**S**	**HHs**	**S**	**HHs**	**S**	**HHs**	**S**
150	**1:2.5**	**2.5:2.5 g/L**	**72**	**22.40^{el}**	**-**	**0.07^{f}**	**-**	**0.29^{elm}**	**-**	**0.07^{d}**	**-**	**82.88^{itu}**	**-**
4.3.2.3. Influence of reduced agitation													
100	1:2.5	0.5:2.5 g/L	72	19.09^{f}	15.01^{f}	0.06^{g}	0.07^{g}	0.28^{f}	0.23^{f}	0.08^{f}	0.08^{f}	49.54^{j}	64.79^{l}
		2.5:2.5 g/L		20.68^{fk}	20.21^{fk}	0.06^{g}	0.07^{g}	0.31^{fk}	0.28^{fk}	0.07^{f}	0.08^{f}	34.53^{k}	68.49^{lrv}
4.3.2.4. Sequential fermentations													
100	1:2.5	2.5:2.5 g/L	96	21.78^{gk}	20.68^{ghk}	0.05^{h}	0.07^{h}	0.33^{gk}	0.30^{ghk}	0.06^{gi}	0.08^{gi}	60.36^{mv}	79.03^{or}
100/150				20.13^{ghl}	17.11^{hl}	0.10^{i}	0.10^{i}	0.26^{ghl}	0.23^{hl}	0.10^{h}	0.07^{i}	88.59^{nou}	89.78^{ot}
4.3.3. Mono-cultures													
100	1:2.5	*S. cerevisiae* 5 g/L	48/24	27.47^{i}	26.82^{i}	0.07^{j}	0.12^{k}	0.46^{i}	0.49^{i}	0.03^{j}	0.03^{jk}	-	-
150	1:2.5	*K. marxianus* 3A 5 g/L	72	20.09^{gj}	22.00^{j}	0.05^{j}	0.05^{j}	0.29^{jm}	0.33^{j}	0.08^{k}	0.06^{jk}	51.93^{p}	46.01^{q}

4.3.2.2. Influence of initial yeast concentrations and medium supplementation

Since the pentose assimilation depends strictly on *K. marxianus* 3 A, a new set of co-cultures was conducted increasing the initial concentration of this yeast and keeping *S. cerevisiae's* initial concentration fixed at 0.5 g/L. As defined in the previous experiments, the aeration condition used was V_{medium}: $V_{deep\text{-}well}$ of 1:2.5. The results can be seen in **Figs. 4.3 a-b**, and **Table 4.2**. Comparing the results of **Fig. 4.3 a-b** with **Fig. 4.2 b**, it is noticeable that the increase in total C_{X0} from 1 to 3 g/L did not improve xylose conversion in HHm. A small improvement was obtained with C_{X0} of 5 g/L (0.5:4.5 g/L), from 21 to 30% of xylose conversion in 72 h, which can be related to a shorter diauxic lag period due to faster glucose depletion (SLININGER et al., 2011). Regarding xylitol, ethanol yield, and production, no significant differences were observed between the values obtained for C_{X0} of 3 (0.21 g_{etOH}/g_S,11.18 g_{etOH}/L) and 5 g/L (0.18 g_{etOH}/g_S, 10.30 g_{etOH}/L) (**Table 4.2**). The xylitol yield was on average 0.025 g_{xylOH}/g_S for all conditions. In S medium, the xylose conversion achieved was similar for all cell concentrations evaluated, about 67% with 3 and 5 g/L, a slight decrease compared to the conversion reached with an equal cell ratio of *S. cerevisiae* and *K. marxianus* 3 A, C_{X0} of 1 g/L (aeration ratio 1:2.5), which was 72.23%, probably due to less limitation related to the O_2 supply for this lower cell concentration.

In summary, the increase in *K. marxianus* initial concentration (**Fig. 4.3 a-b**) did not improve co-culture performance. Thus, new experiments in S and HHm media were conducted with an increased initial concentration of *S. cerevisiae* (C_{X0} of 2.5 g/L), keeping both strains at the same initial concentration (total initial C_{X0} of 5 g/L). In addition to the increase in *S. cerevisiae* concentration, a co-culture experiment was also performed in supplemented hemicellulosic hydrolysate (HHs), besides HHm and S media, to assess nutrients' influence over xylose assimilation and product formation in HH. Sugar conversion profiles and biomass production are presented in **Fig. 4.3 c** and the fermentation performance indicators are in **Table 4.2**.

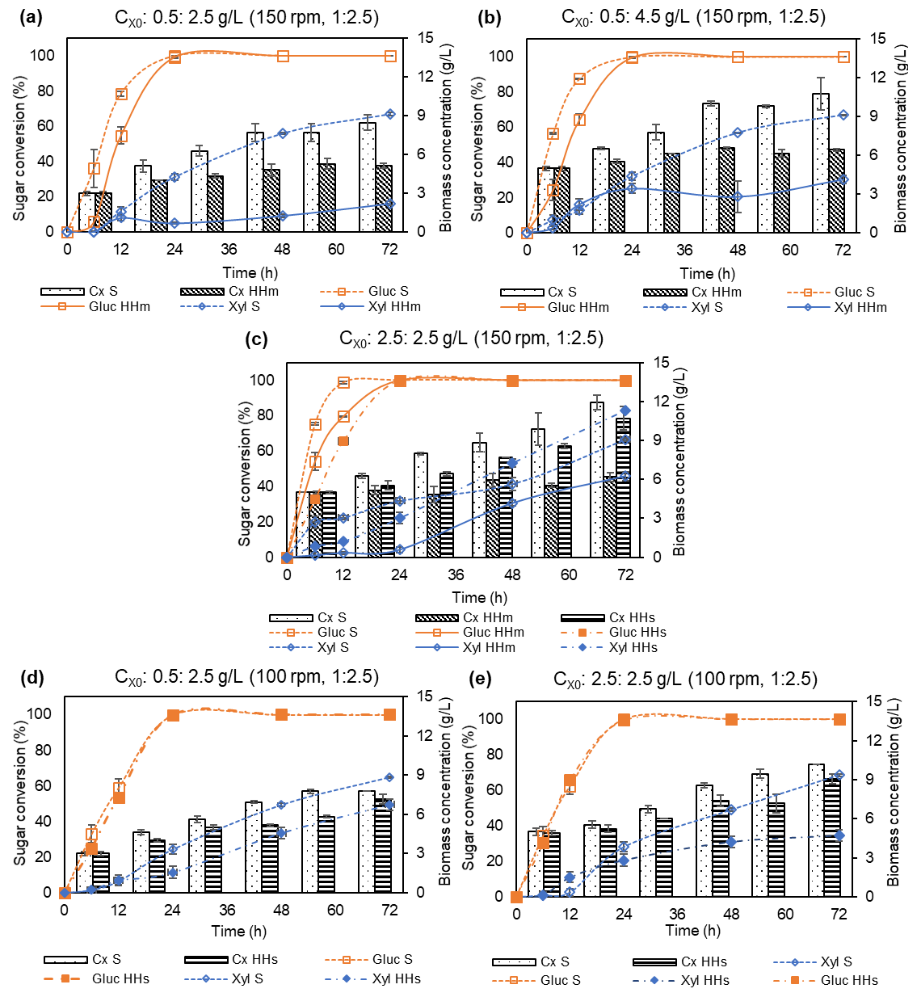

Figure 4.3. Sugar conversion (glucose and xylose) and biomass concentration obtained during the co-culture in S, HHm and HHs media with *S. cerevisiae*: *K. marxianus* 3 A cell ratio of **(a)** 1:5 (C_{X0}: 3 g/L); **(b)** 1:9 (C_{X0}: 5 g/L), and **(c)** 1:1 (C_{X0}: 5 g/L) (plus HHs), 150 rpm, and **(d)** 1:5 (C_{X0}: 3 g/L) and **(e)** 1:1 (C_{X0}: 5 g/L), with 100 rpm of agitation. Aeration ratio: 1:2.5, $V_{deep\text{-}well}$: 4 mL, 35 °C, pH_0:5.

Xylose conversion was significantly improved in HHs (83%) compared to HHm (46%) and even to S medium (67%) **(Fig. 4.3 c)**, as well as ethanol yield, reaching 0.29 g_{etOH}/g_S, a value similar to that achieved in S medium (**Table 4.2**). Xylitol yield reached 0.07 g_{xylOH}/g_S (HHs), a higher and significantly different value than that obtained in HHm. Media enrichment was important to accelerate cell metabolism, and even improve inhibitors tolerance, benefiting the co-culture. The nutrients' influence on the culture performance was mentioned by Bonfiglio et al. (2021), who worked with one of the evolved strains evaluated in the present work (*K. marxianus* 4 A), and observed an increase in cell mass besides xylitol production in the supplemented medium. The increase in biomass concentration was also observed in the present work, obtaining 2-fold the

C_{X0} (final C_X of 10.70 g/L, 0.07 g_X/g_S) in HHs, a statistically similar value to that reached in S medium (final C_X of 12.51 g/L, 0.10 g_X/g_S) and 1.7-fold higher than reached in HHm. These results clearly show the importance of medium supplementation with nutrients for this co-culture strategy, aiming at improved xylose conversion and product yield.

It can also be inferred that increasing *S. cerevisiae* concentration in the co-culture improved xylose conversion (**Fig. 4.3 a-c**), as it allowed faster glucose depletion. The presence of a higher amount of *S. cerevisiae* (2.5:2.5 g/L) seemed to play a good synergism with *K. marxianus*, improving the xylose volumetric consumption rate about 5-fold in HHm (150.0±10.0 mg_{xylose}/L/h) compared to the condition 0.5:4.5 g/L (30.0±10.0 mg_{xylose}/L/h). In HHs with 2.5 g/L of each strain, the Q_{xylose} calculated was 270.0±0.0 mg_{xylose}/L/h. Due to the promising results achieved with the HHs medium, from now on only HHs and S media were evaluated in the cultures.

4.3.2.3. Influence of reduced agitation

The assays performed so far pointed out that the proposed co-culture is a promising tool for the fermentation of glucose-xylose using crude biomass hydrolysates. However, relevant process conditions, such as aeration deserve better attention, mainly when the cell concentration is changed. Reducing the agitation rate from 150 to 100 rpm will provide a more microaerobic environment that can improve ethanol yield. For this purpose, two new conditions with different C_{X0} ratios of *S. cerevisiae*: *K. marxianus* 3 A were investigated at 100 rpm: one with a cell ratio of 1:5 (C_{X0}: 3 g/L) and another with 1:1 (C_{X0}: 5 g/L). The aeration ratio (V_{medium}:$V_{deep-well}$) was kept at 1:2.5 (4 mL of S and HHs medium per deep-well). Results regarding sugar conversion, biomass production, and fermentation indicators for this study are presented in **Figs. 4.3 d-e**, and **Table 4.2**.

For both cell ratios, glucose was depleted at a similar uptake rate within 24 h in S and HHs media, showing improvements in *S. cerevisiae* performance under less aerated condition (100 rpm), coupled with the presence of *K. marxianus* 3 A. However, reducing agitation rate hampered final xylose conversion in HHs, dependent on *K. marxianus* 3 A, which decreased to 50 and 35% for 3 and 5 g/L of C_{X0}, respectively (**Table 4.2**). Yet, in S medium, final xylose conversions were similar to the values achieved in previous studies in the same cell concentration at 150 rpm, 65 and 68% for 3 and 5 g/L of C_{X0} (**Table 4.2**), respectively. Besides the highest xylose conversion in S medium than in HHs, biomass production, xylitol, and ethanol yields were statistically similar for all conditions (**Table 4.2**). Highlighted ethanol production and yield were obtained in HHs,

corresponding to 7.17 g_{etOH}/L and 0.28 g_{etOH}/g_S for C_{X0}: 3 g/L, and 9.06 g_{etOH}/L and 0.31 g_{etOH}/g_S for C_{X0}: 5 g/L; while biomass yield was 0.06 g_X/g_S at both conditions.

These results confirm the importance of defining and controlling the aeration conditions for co-cultures. The effect of aeration was stronger than that observed for inoculum size if comparing the results between the two cell concentration ratios evaluated (**Table 4.2**). The co-culture carried out at 150 rpm and C_{X0} of 5 g/L reached 83% of xylose conversion, whereas at 100 rpm the conversion dropped to 35%. On the other hand, when C_{X0} was reduced to 3 g/L (100 rpm), the conversion increased to 50% since the O_2 supply was less limiting for a lower cell concentration. So, integrated biomass conversion to valuable products can be accomplished in a co-culture strategy, but it requires precise control of the O_2 supply, which can be done with a properly equipped bioreactor and a control algorithm developed for running microaerated fermentations (MESQUITA et al., 2019, 2021).

4.3.2.4. Sequential fermentation

The hexose repression over pentose assimilation is a well-known issue (Leonel et al., 2021). So, to evaluate if the concentration of glucose present at the beginning of the co-culture could be impairing better results, sequential fermentations were conducted. The strategy consisted in conducting a 24 h fermentation with only *S. cerevisiae* (C_{X0} 2.5 g/L), and then, *K. marxianus* 3 A (C_{X0} 2.5 g/L) was inoculated to the fermented media. These studies were carried out either at 100 rpm throughout the sequential batches or with an increase in agitation to 150 rpm when *K. marxianus* was added. In the sequential culture at 100 rpm (**Fig. 4.4 a**), a significant increase in xylose conversion was achieved in HHs, compared to the co-culture at this same agitation rate (**Fig. 4.3 b**), from 35 to 60% at 72 and 96 h of fermentation, respectively.

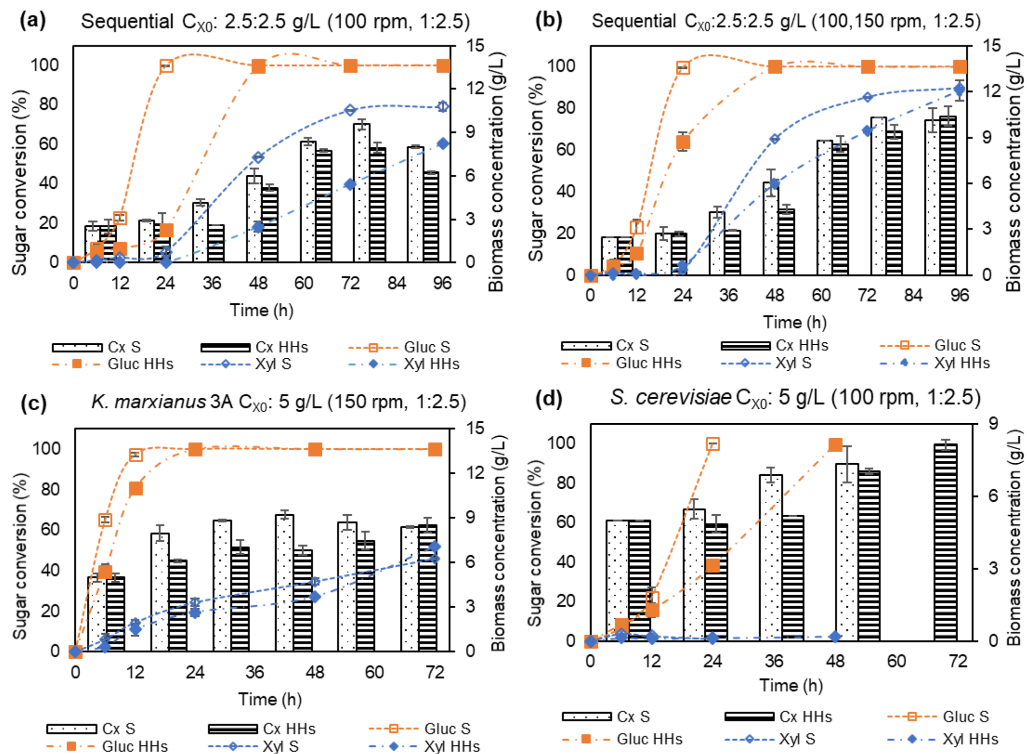

Figure 4.4. Sugar conversion (glucose and xylose) and biomass concentration in S and HHs media during the sequential fermentation with *S. cerevisiae*: *K. marxianus* 3 A (2.5:2.5 g/L), **(a)** 100 rpm, and **(b)** 100 (24 h) and 150 rpm (72 h) (*K. marxianus* 3 A inoculation was at 24 h), and 72 h mono-culture (C_{X0}: 5 g/L) of *K. marxianus* 3 A at **(c)** 150 rpm, and *S. cerevisiae* at **(d)** 100 rpm, 35 °C, aeration ratio: 1:2.5, $V_{deep\text{-}well}$: 4 mL, pH_0: 5.

Regarding the sequential fermentation with the change in agitation rate (**Fig. 4.4 b**), which provided more aeration when *K. marxianus* was inoculated, xylose conversion remained at the same level (83% in the co-culture at 150 rpm, C_{X0} of 5 g/L, and 89% in sequential culture) for HHs medium, and increased from 67 to 90% for S medium. Thus, although the sequential strategy with agitation rate changes presented good results in terms of xylose conversion, biomass formation, and ethanol production (**Fig. 4.4 b**) after 96 h, they were similar to those achieved in co-culture carried out at the best condition (150 rpm, 4 mL of medium, and C_{X0} of 5 g/L, **Fig. 4.3 c**) in a shorter cultivation time (72 h) (**Table 4.2**). Glucose conversion profiles displayed in **Figs. 4.3 c** and **4.4 b** were very similar for S and HHs media in both co-culture and sequential culture strategies. However, at 100 rpm, the improvement of xylose conversion in the sequential fermentations shows that the performance of co-culture carried out at 100 rpm was probably affected by glucose repression **(Fig. 4.3 e**), even though if glucose was not depleted in 24 h, its

concentration was reduced at the moment *K. marxianus* was inoculated. For the same reason, in the S medium, xylose conversion was higher in the sequential fermentations than in co-cultures for both agitation conditions. However, the extra xylose assimilated did not contribute to a change in ethanol production or biomass formation, which were of 0.30 g_{etOh}/g_S and 0.08 g_X/g_S, respectively, for the S medium (**Table 4.2**).

Based on the results achieved for the conditions studied so far, it can be concluded that the co-culture at 150 rpm with equal initial concentrations of *S. cerevisiae*: *K. marxianus* 3 A (C_{X0} of 5 g/L) is the best condition for yeast-yeast fermentation of glucose and xylose.

According to Nosrati-Ghods et al. (2018), sequential fermentation is a way to overcome the slower xylose fermentation often observed in co-culture, which presents issues with catabolite repression, and varying oxygen requirement of the combined microorganisms. However, in the present study, this approach did not bring relevant improvements compared to the best co-culture conditions found, especially considering that in the sequential strategy, about 24 h of additional fermentation time was necessary to decrease glucose concentration in the beginning. This discrepancy in the findings can be attributed to the differences in the strategies, cultivation conditions, used microorganisms, and the synergistic relationship between them.

4.3.3. Mono-cultures

To better evaluate the co-culture performance, it is important the comparison with mono-cultures of *S. cerevisiae* or *K. marxianus* 3 A. Thus, these strains were cultured in HHs and S media in the best conditions for each one. Comparing the mono-culture of *K. marxianus* 3 A (**Fig. 4.4 c**) with the co-culture (**Fig. 4.2 c**) it is clear that the presence of *S. cerevisiae* improved xylose conversion (from 52 to 83% in HHs and from 46 to 67% in S medium), making it faster and reaching similar ethanol yield after 72 h (0.29 g_{etOH}/g_S, HHs) (**Table 4.2**). Rojas-Chamorro et al. (2019) also reported improvements in sugar conversion in a co-culture of *S. stipitis* with *S. cerevisiae* compared to an *E. coli* mono-culture in brewer's spent grains (BSG) hydrolysates. On the contrary, Farias and Maugeri Filho (2019) observed slower xylose assimilation when using co-culture fermentation of *S. passalidarum* and *S. cerevisiae* compared to mono-cultures, probably due to oxygen limitation and faster ethanol accumulation by hexose fermenting species.

Regarding *S. cerevisiae* mono-culture (**Fig. 4.4 d**), xylose was not assimilated, as expected. A point to highlight is the difference between the glucose volumetric consumption rate ($Q_{glucose}$) in

HHs (1.31±0.10 $g_{glucose}$/L/h) and S media (2.93±0.53 $g_{glucose}$/L/h). Since this strain is not evolved like *K. marxianus* 3 A, the fermentation performance in the S medium was better than in the hydrolysate (containing inhibitors), whereas *K. marxianus* 3 A showed a more similar sugar conversion profile in both media, for xylose and glucose.

The ethanol yield presented for *S. cerevisiae* at 24 h of fermentation was 0.46 in HHs and 0.49 g_{etOH}/g_S in S medium, values expected and widely reported for hexoses assimilation by this yeast (MESQUITA et al., 2021; PEREIRA et al., 2018). However, using only this strain does not allow the integral conversion of biomass sugars in a biorefinery context. For this purpose, it would be necessary to use commercial enzyme preparations or genetically modified organisms (GMOs), which could increase the costs of the process and lead to issues regarding the use of GMO strains in an industrial environment (DRAGONE et al., 2020; MILESSI et al., 2018). It is worth mentioning that the pentose fermenting strain used in this work is non-GMO, being only subject to an adaptation process for selecting the best phenotype.

4.4. Conclusions

High throughput screening allowed defining a promising co-culture strategy with *S. cerevisiae* and *K. marxianus* to assimilate biomass sugars (glucose depletion and 83% of xylose conversion), which was efficient to overcome glucose repression and inhibition caused by the hydrolysate compounds. Although sequential fermentation and co-cultures achieved similar conversions, the former was not considered a good option as it increased the fermentation time by 24 h, besides being a more complex cultivation procedure to carry out. After all, the combination of ideal inoculum size and adequate aeration showed to be the key to a promising yeast-yeast co-culture in biomass hydrolysate.

Chapter 5. Solid feeding and co-culture strategies as tools for efficient enzymatic hydrolysis and ethanol production in a helical stirred bioreactor at high solid loads of sugarcane bagasse

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Abstract:

The feasibility of second-generation (2G) processes still highly depends on improvements in the hydrolysis and fermentation steps, ensuring the whole use of biomass sugars in an integrated approach. The main bottlenecks are the differences between the optimal conditions for both steps, high solid load (HSL) hydrolysis limitations, and hexoses and pentose co-fermentation. The present work explored a feeding strategy to improve HSL enzymatic hydrolysis of hydrothermally pretreated sugarcane bagasse (PSB), at optimal and suboptimal temperatures, further integrated with a non-recombinant yeast co-culture (*Saccharomyces cerevisiae* and *Kluyveromyces marxianus*) for sugar co-fermentation. The entire PSB fraction was used without conditioning. Efficient enzymatic hydrolysis was achieved using a feeding strategy in a helical stirred bioreactor (64 and 75% cellulose conversion with 22.5% of solids, 72 h at 35 and 50 °C, respectively), obtaining hydrolysates with high glucose and xylose concentrations (100 g/L). Simultaneous saccharification and co-fermentation (SSCF) provided high glucose (98%) and xylose (52%) conversion in 24 deep-well plates, with ethanol yield of 0.23 g_{etOH}/g_S, but faced problems of cell viability loss in the bioreactor. Sequential SCF (SqSCF) co-culture stood out as a promising strategy for the use of biomass sugars. The results provided significant contributions to the intensification and feasibility of 2G production, in addition to highlighting the difficulties faced at

process integration aiming at the full conversion of sugars from pretreated biomass coming from high solid load enzymatic hydrolysis.

Keywords: Helical stirred bioreactor; Enzymatic hydrolysis; Solid feeding strategy; Integrated process; Co-culture; Ethanol.

5.1. Introduction

The global energy matrix is transitioning to less dependence on non-renewable sources due to environmental issues or the high fossil fuel prices oscillating, increasing the development of clean processes (MUSSATTO et al., 2021). Lignocellulosic materials have great potential for this purpose, being renewable feedstocks widely available in nature, mainly composed of cellulose, hemicellulose, and lignin, which can be converted into various valuable chemicals (VU et al., 2020). In Brazil, the bagasse, a co-product of the sugarcane industry, is a rich source of fermentable sugars (SILVA et al., 2015). Currently, most of this biomass is burned in sugarcane mills to produce energy, but a significant amount remains, representing a great potential for sugar conversion into bio-based products, and defining second-generation (2G) processes (CHANDEL et al., 2021).

Using renewable feedstocks in the biorefinery context encompasses the synergic production of fuels and chemicals by biological and/or chemical routes. However, its success depends on many techno-economic issues, such as the biomass pretreatment required, the variety of feedstocks, the high costs of additional processes required for 2G biomass conditioning, and the materials needed, such as enzymes and chemicals (BACHOSZ et al., 2022; ÖZDENKÇI et al., 2017; PREETHI et al., 2021). Process integration is a way of reaching 2G production feasibility and intensification, by reducing expenses with new equipment acquisition, and saving time (MUSSATTO et al., 2021), but is very challenging.

Simultaneous saccharification and co-fermentation (SSCF) is an integrated approach to carry out enzymatic hydrolysis and fermentation in the same reactor. Allows fermentation of all biomass sugars by microorganisms as soon as they are released by enzymes, which helps to avoid substrate repression in cell metabolism, reduce the risks of contamination, and enzyme inhibition (MILESSI et al., 2020; MUSSATTO et al., 2008). However, an efficient sugar uptake requires specific microorganisms or combinations (co-cultures) capable of co-fermenting pentoses and hexoses (SANDRI et al. 2023a). Co-cultures are a valuable strategy for this, often reported as

superior to mono-cultures, it allows the best of each strain through positive synergism between them (NOSRATI-GHODS et al., 2018; SANDRI et al., 2023a). It is also a way to convert pentoses without using recombinants, which faces difficulties in large-scale implementation due to the biosafety laws, and possible loss in competition with wild/contaminating organisms (DELLA-BIANCA et al., 2013; MILESSI et al., 2020b). A yeast co-culture of *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* was recently screened by Sandri et al. (2023a), with great potential for hydrolysate sugars co-fermentation.

Despite the many advantages, SSCF present several bottlenecks regarding combining the optimal conditions for hydrolysis and fermentation, bringing a range of possibilities to be studied and optimized. Great progress has been achieved (SILVA et al., 2015; BRONDI et al., 2021; PINTO et al., 2021; ZHAO et al., 2020; PRATTO et al., 2020), but there is a lack of studies performed under more realistic conditions for process integration, such as lower temperatures for the hydrolysis and the use of non-conditioned biomass, besides few reports using non-recombinant strains for efficient pentose and hexose co-fermentation.

Several characteristics are required for successful enzymatic hydrolyses such as proper enzyme load and solids amount, feeding strategies, and proper mixing (DA SILVA et al., 2020). A suitable solids amount is related to the desired concentration of sugars, which is generally high, requiring a high solid load (HSL), considered from 15% (w/v) of solids, based on the available free water. However, working with HSL is not useful if not with a proper reactor configuration or feeding strategy (UNREAN et al., 2016). A detailed review regarding impeller selection for HSL processes is presented by Afedzi et al. (2022). De Oliveira et al. (2018) got a significant improvement in cellulose conversion with a helical kind (39 to 60%, 48 h, 15% w/v of washed solids in buffer solution) compared to a Rushton one.

A feeding strategy has great potential to improve enzymatic hydrolysis since it provides time for the solids to liquefy before new additions and endures a suitable amount of free water for the reaction (PRATTO et al., 2022). But the feedings and enzyme addition must be well planned, to obtain high sugar release and also save energy to reach a feasible process (DA SILVA et al., 2020). Zhang et al. (2010) reported that the energy for mixing HSL pretreated corn stover using a helical impeller significantly increased from 15 to 30% (w/w), highlighting the importance of balancing the benefits of having an HSL and the energy needed for efficient stirring.

Besides suitable mixing and solid feeding, the water available is also important in hydrolysis, especially at HSL, when it is low at the beginning and possibly interferes with the process efficiency (DA SILVA et al., 2020). Biomass conditioning before hydrolysis, such as washing, is another issue. This step is commonly performed to remove possible inhibitors from pretreatment, but it increases costs, discards a significant portion of sugars, and is not environmentally friendly (VU et al., 2020). So, using unconditioned biomass is the best option, even if requiring robust enzymes to handle these conditions.

Based on the above, the present study applied both fractions of pretreated sugarcane bagasse (PSB), hemicellulose hydrolysate, and cellulignin, as coming from the pretreatment, for enzymatic hydrolysis and SSCF studies. The main purpose was the development of a solid feeding strategy to improve the whole biomass hydrolysis with HSL, at optimal and suboptimal temperatures, by using a helical stirred bioreactor, for further evaluation of process integration with a yeast co-culture (SSCF) for hexoses and pentoses co-fermentation. To the best of our knowledge, exploring HSL enzymatic hydrolysis at different temperatures using the entire biomass without conditioning and evaluating process integration with a non-recombinant yeast co-culture for co-fermentation is new in the literature. The study provided great contributions to 2G process intensification in a biorefinery context, as well as highlighted the challenges and improvements required for process feasibility.

5.2. Material and methods

5.2.1. Biomass and pretreatment

Sugarcane bagasse (SB) was kindly provided by Ipiranga Agroindustrial (SP, Brazil). The raw material was characterized according to Sluiter et al. (2008), presenting (%) cellulose (44.15 ± 2.01), hemicellulose (24.35 ± 2.48), total lignin (19.16 ± 0.22), acetyl group (3.52 ± 0.20), ash (1.86 ± 0.16), and extractives (3.81± 0.36).

The SB was hydrothermally pretreated as described by Sandri et al. (2023a), with 10% (w/v) of solid load (dry basis) at 195 °C for 45 min. The solid and liquid fractions obtained after the pretreatment were called pretreated sugarcane bagasse (PSB) and hemicellulosic hydrolysate (HH), respectively. Both were separated by vacuum filtration. The wet PSB was dried for 3 h at 50 °C, reaching ~60% of moisture. PSB and HH were stored at 5 °C for further use and characterization (**Table 5.1**).

Table 5.1. Characterization of the solid (PSB) and liquid (HH) fractions of pretreated sugarcane bagasse obtained by hydrothermal pretreatment (10% w/v of solids, 195 °C for 45 min).

Solid (PSB)		Liquid (HH)	
Components (%)		**Compounds (g/L)**	
Cellulose	59.29 ± 1.58	**Glucose**	0.04 ± 0.00
Hemicellulose	9.95 ± 0.21	**Xylose**	1.92 ± 0.15
Acetyl group	2.11 ± 0.03	**Arabinose**	0.52 ± 0.02
Total lignin	25.71 ± 0.20	**5- Hydroxymethylfurfural (HMF)**	0.05 ± 0.01
Ash	1.96 ± 0.27	**Furfural**	0.74 ± 0.04
		Acetic acid	1.50 ± 0.01
		Xylooligomers	13.85 ± 0.13
		Total phenolics	0.48 0.02

5.2.2. Enzyme, microorganisms, and inoculum

The enzymatic cocktail Cellic®CTec 2 (Novozymes, Denmark) was used for hydrolysis, with cellulase (167 FPU/mL) and xylanase (13107 IU/mL) activities measured according to Adney and Baler (2008), and Bailey et al. (1992), respectively. The evolved strain of *Kluyveromyces marxianus* NRRL Y-6860 (3A) (BCBT group, DTU, Denmark), with increased tolerance to biomass-derived inhibitors (unpublished data), and the yeast *Saccharomyces cerevisiae* Itaiquara® (Baker's yeast, Brazil) were used in SSCF (simultaneous saccharification and co-fermentation), PS SSCF (pre-saccharification followed by SSCF) and SqSCF (Sequential saccharification and co-fermentation) cultures. The inoculum was prepared as described by Sandri et al. (2023a). Afterward, cells were recovered by centrifugation (10000 rpm, 5 min, 5 °C), washed with saline solution, and the pellet was inoculated in each culture medium.

5.2.3. Small-scale studies

Different enzymatic hydrolysis conditions and fermentation strategies were assessed in a small-scale to define the best conditions for further process scale-up in the bioreactor.

5.2.3.1. Enzymatic hydrolysis

Enzymatic hydrolysis (EH) experiments were performed to evaluate the conditions of solid load (15 and 20%) (w/v, dry basis) and temperature (35 and 50 °C), resulting in four different conditions performed in duplicate. The experiments were carried out in 50-mL flasks, to which PSB and HH were added to obtain the required solid load for a final volume of 20 mL (V_{medium}:V_{flask} of 1:2.5). Enzyme load was 45 FPU/$g_{cellulose}$. Ampicillin (100 µg/mL) and polyethylene glycol (PEG: 0.05 g/$g_{drybiomass}$) (CEBREIROS; FERRARI; LAREO, 2019) were added. The initial pH of HH was adjusted to 5 previous to the mix with PSB, using NaOH (3 mol/L). For each condition, six equal flasks were incubated at 150 rpm and removed one by one at each sampling time (0, 6, 12, 24, 48, and 72 h). Samples taken were heated (100 °C, 10 min) in a dry block (ThermoMixer^{TM} C) for enzyme deactivation, followed by centrifugation (10000 rpm, 10 min, 5 °C) to obtain the supernatant, which was further analyzed by HPLC.

5.2.3.2. Simultaneous saccharification and co-fermentation (SSCF) and Pre-saccharification followed by SSCF (PS SSCF)

The simultaneous saccharification and co-fermentation (SSCF) experiment consisted of the simultaneous addition and incubation of media (HH and PSB), enzymes, and microorganisms to the reactor. This process was followed for 72 h. The PS SSCF had as its main difference a pre-saccharification step (PS), characterized as short enzymatic hydrolysis conducted for 12 h at 50 °C (optimum temperature for the enzymatic cocktail). Afterward, the pre-saccharified suspension was cooled down (35 °C), had its pH adjusted to 5, and was inoculated with the yeasts, starting the SSCF, which was followed for additional 72 h.

SSCF was conducted with co-culture (*S. cerevisiae* and *K. marxianus 3A*) and mono-culture (*K. marxianus 3A*) of yeasts, while PS SSCF was performed only with co-culture. The initial cell load applied for all cases was 5 g/L (2.5:2.5 g/L for the co-culture), a condition previously optimized by Sandri et al. (2023a). The solid load used was 15% (w/v) of PSB in HH, defined as appropriate in the enzymatic hydrolysis small-scale studies. The enzyme load was 45 FPU/$g_{cellulose}$. For fermentation, the HH was supplemented (HHs) with salts and nitrogen sources (3 g/L yeast extract; 2 g/L $(NH_4)_2HPO_4$; 2 g/L KH_2PO_4; 0.25 g/L $MgSO_4.7H_2O$), besides ampicillin (100 µg/mL) and PEG (0.05 g/$g_{drybiomass}$). The initial pH of HHs was also adjusted to start at 5. SSCF and PF SSCF were performed either in 50-mL flasks or 24 deep-well plates ($V_{working}$ of 10 mL), to

verify the reproducibility of the data on different scales. Control experiments, consisting of enzymatic hydrolysis in the same medium composition without cells, were used as a reference for the final sugar concentration reached during hydrolysis and used for the calculation of fermentation performance indexes. For 50-mL flasks, the final volume of medium was 20 mL, while for 24 deep-well plates, it was 4 mL (V_{medium}:V_{flask} of 1:2.5). The cultures were incubated at 35 °C and 150 rpm for 72 h, with samples taken periodically for HPLC analysis. All conditions were performed in duplicate.

5.2.4. High solid load hydrolysis and co-cultures in bioreactor

From the small-scale experiments, the conditions selected either for enzymatic hydrolysis or integrated process were tested in a bioreactor with the main purpose of evaluating the cultivation and solid feeding strategies on a bioreactor scale.

A BioBench Biobased 3 L Benchtop Bioreactor (Biostream International BV, Netherlands) designed to work with high solid loads was used. Its base unit contains flow controllers, hardware, and off-gas analyzers. The bioreactor jacketed glass vessel, 3-L total volume, was connected to a water bath. HSL hydrolyses, SSCF, and PS SSCF co-cultures were carried out with a helical impeller, which allows improved mass transference in the reactions (AFEDZI; RATTANAPORN; PARAKULSUKSATID, 2022). The process parameters were set and controlled on the control board through a touchscreen display. Lucullus® software was used for data acquisition. The sensors used will be mentioned in the description of each study. The bioreactor was also used for Sequential saccharification and co-fermentation (SqSCF) experiments using a Rushton impeller, recommended for submerged cultures. No aeration was provided in either study.

5.2.4.1. Enzymatic hydrolysis

To reach a high final solid load and conversion, enzymatic hydrolysis was conducted using a solid fed-batch strategy. The bioreactor was filled with PSB and HH at an initial solid load of 3.5% (w/v) in a final volume of 700 mL. The enzyme load added was 45 FPU/$g_{cellulose}$. Ampicillin (100 µg/mL) and PEG (0.05 g/$g_{drybiomass}$) were also added. The reaction was conducted for 72 h at both 35 and 50 °C, 150 rpm, and with an initial pH of 5. The bioreactor was equipped with a pH meter and the pH control was conducted manually. During the process, the torque was monitored (Biobased motor with torque measurement) and 6 additional feeds of about 3.0% (w/v) of solids

were performed when a decrease in the torque value was observed, allowing to reach a final load of about 22.5%. At each feeding, enzyme, ampicillin, and PEG were also added proportionally and the pH was again adjusted to 5. During the hydrolysis, samples were taken at the beginning and end of each sequential batch.

5.2.4.2. Simultaneous saccharification and co-fermentation (SSCF) strategy

SSCF was also carried out at a high final solid load. For the conduction of the SSCF co-culture, two strategies of PSB supply were studied. In the first strategy (SF-1), the procedure already described for enzymatic hydrolysis was reproduced (initial solid load of 3.5% and sequential feeds of about 3.0%), with the addition of cells (C_x: 5 g/L) and supplementation to the HH (HHs), as mentioned in Section 5.2.3.2. In the second strategy (SF-2), the co-culture SSCF was also started with 3.5% (w/v) of solid load. However, additional 14 solid feedings of about 1.25% (w/v) were made during the 72 h, reaching around 22.6% of solids at the end. This strategy of several feedings with very low solid load aimed to investigate the influence of solids addition in the co-culture performance and cell viability. Fermentations were performed at 35 °C and the bioreactor was additionally equipped with a dissolved oxygen sensor. During the fermentation, samples were taken periodically for HPLC analysis. For some samples, one slope of the co-culture suspension was transferred to a YPD-agar plate and incubated for 36 h at 35 °C to observe colony growth and cell viability.

5.2.4.3. Sequential saccharification and co-fermentation (SqSCF)

A new cultivation strategy combining hydrolysis and co-fermentation in the sequence was developed after obtaining the results of the previous SSCF, which suggested the harmful effects of solids' presence on yeast cell viability. In the SqSCF, a submerged co-culture with whole biomass hydrolysate (WBH), free of solids, was performed. The enzymatic hydrolysate obtained after the 35 °C hydrolysis of PSB and HH with Cellic®CTec 2 (Section 5.2.4.1) was centrifuged (10 min, 10000 rpm, 5 °C) to separate the residual solids. The liquid fraction was sterilized in a 0.22 μm membrane and supplemented with salts, nitrogen sources, and ampicillin, (as described in Section 5.2.3.2). The supplemented WBH was used as fermentation medium in a process conducted in the bioreactor using a Rushton impeller. The bioreactor was also equipped with a pH meter and dissolved oxygen sensor. The co-culture cells were inoculated in the bioreactor as previously

described (C_{X0}: 5 g/L of *S. cerevisiae* and *K. marxianus* 3A), and kept at 35 °C, 150 rpm for 72 h. During the process, samples were collected (0, 6, 12, 24, 48, and 72 h) for optical density (OD_{600}) measurements, and HPLC analysis.

5.2.5. Analytical methods

The optical density (OD_{600}) of the inoculum was measured at 600 nm (V-1200 spectrophotometer, VWR, Denmark) and used to determine the cell concentration through a dry weight calibration curve obtained for each yeast. The samples collected were centrifuged at 10000 rpm, 5 min, and 5 °C (Micro Star 17 R centrifuge, VWR, Denmark). The supernatants were filtered in 0.22 µm membranes and stored at -20 °C for further analysis.

The quantification of sugars, alcohols, organic acids, and inhibitors was carried out using a Dionex UltiMate 3000 UHPLC^{+} Focused system (Thermo Scientific) with a Bio-Rad Aminex® column HPX-87H (300 mm×7.8 mm) (SANDRI et al., 2023a). The concentration of xylooligomers in the HH was determined by post-hydrolysis conducted in an autoclave (121 °C for 60 min, H_2SO_4 72%) (NASCIMENTO et al., 2016). After acid hydrolysis, the samples were analyzed by HPLC. Using the hydrolysis stoichiometry, the difference between the post-hydrolyzed monomeric sugars and the original hydrolysate resulted in the total xylooligomers concentration. The concentration of total phenolic compounds was quantified by a colorimetric method according to Ballesteros et al. (2014).

5.2.6. Calculation procedures

Results of enzymatic hydrolysis were expressed as cellulose conversion (CC) and xylose yields (XY), calculated by the mass ratio of glucose and cellobiose per cellulose (MUSSATTO et al., 2008), and by the xylose from the liquid (xylooligomers) and from the remained hemicellulose in solid fraction, respectively. The hydrolysis, SSCF, PS SSCF, and SqSCF performance indexes including substrate conversion X (%), products yield $Y_{P/S}$ ($g_{coproduct}/g_{substrate}$), and/or productivity Q_P ($g_{coproduct}$/L/h) were calculated as described elsewhere (MILESSI et al., 2018; SHULER; KARGI, 2002). For the cultures, these indexes were calculated regarding the component concentration present in the control experiment (Section 5.2.3.2), for each corresponding time.

5.2.7. Statistical Analysis

The statistical significance of the differences between the results was verified by the Tukey test using the Origin®Pro 8.5 software at a confidence level of 95%.

5.3. Results and Discussion

5.3.1. Small-scale studies

5.3.1.1. Enzymatic hydrolysis

Enzymatic hydrolysis of the whole fraction obtained after hydrothermal pretreatment of sugarcane bagasse was performed at two high solid loads (HSL) and two temperatures. PEG polymer was added to all conditions based on previous results (data not shown) that showed a 10.5% improvement in cellulose conversion (CC) when using the compound in 20% of solids hydrolysis at 35 °C. The potential of using PEG to reduce the nonproductive adsorption of enzymes and increase CC is already mentioned in the literature, together with other media additives (BRONDI; PINTO; FARINAS, 2021; CEBREIROS; FERRARI; LAREO, 2019). Cellulose conversion, xylose yield, and total sugar concentration after 72 h are presented in **Fig. 5.1**.

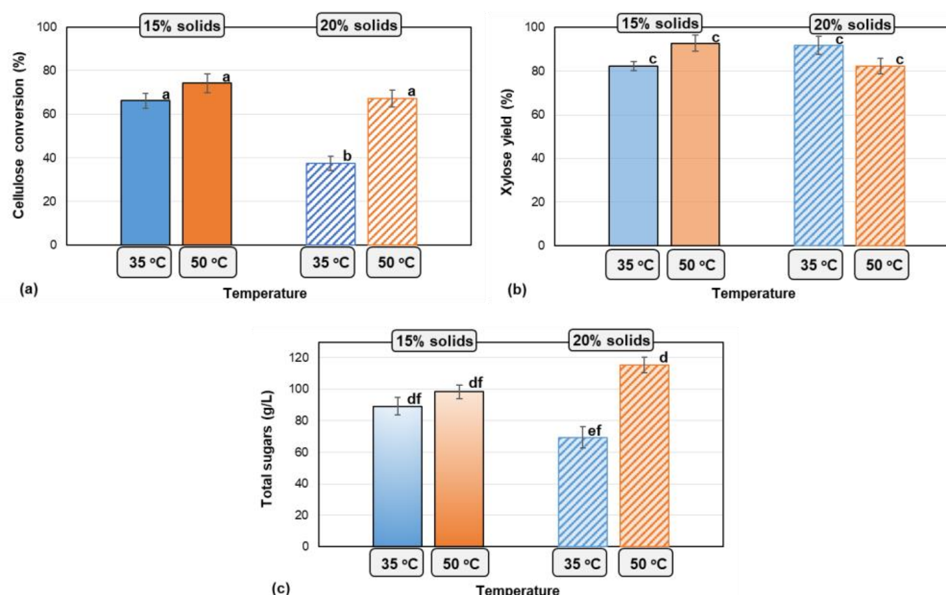

Figure 5.1. Cellulose conversion (CC) **(a)**, xylose yield (XY) **(b)**, and total sugars* concentration **(c)** after enzymatic hydrolysis (72 h) of PSB and HH using Cellic®Ctec2 at 35 and 50 ºC, 50-mL flasks with 20 mL HH and 15 or 20% of the solid load (w/v), 45 $FPU/g_{cellulose}$, PEG (0.05 $g/g_{drybiomass}$), pH_0: 5, 150 rpm. Error bars are standard deviations of the duplicates. Different letters at the top of the bars mean statistically significant differences between conditions, pair-compared (Tukey's test). *Glucose, xylose, arabinose, and cellobiose.

From **Fig. 5.1a** it is clear the decrease in CC (35 °C|) along with the solid load increase, from 66.25 (15% w/v) to 37.51% (20% w/v) at 35 °C, which is related to mass transfer problems. The high amount of solid coupled with the lack of proper mixing in the shaker flasks impaired the effectivity of the enzyme in hydrolysis. Partial deactivation and adsorption of the enzymes caused by the lignin present in the PSB could also have influenced the performance. Besides the previously reported effects, according to Modenbach and Nokes (2013), the presence of hemicellulose-derived products, like xylose and xylooligomers, can impair cellulose conversion and reduce glucose yields due to enzyme inhibition. This effect was also observed by Pinto et al. (2021), who obtained lower conversion values in the enzymatic hydrolysis when using hydrothermal hydrolysate of sugarcane bagasse (liquor), which is rich in xylose and xylooligomers instead of buffer solution.

Regarding the temperatures evaluated, no significant differences were observed for CC with 15% of solids at 35 °C (66.25%) and 50 °C (74.19%) according to Tukey's test, showing that the enzyme load used was enough to compensate for its lower activity at 35 °C compared to 50 °C, which is the optimal temperature for the CellicCtec2. On the other hand, CC with 20% of solids at 35 °C was significantly lower than at 50 °C, showing that the limitations related to the high solid load and low temperature could not be circumvented in this condition. These results are extremely valuable for further SSCF process that requires a lower temperature than the optimal for enzymatic hydrolysis. At 20% of solids and 50 °C, the CC reached 67.21%. The results at 50 °C were similar to those obtained by da Silva et al. (2016), who reported 68% (15%) and 69% (20%) of glucose yield in shake flasks also using Cellic®Ctec2 (20 FPU/g_{glucan}). However, with the benefits of performing the hydrolysis in buffered media. The authors also mentioned the superior performance of the Cellic®Ctec2 enzymatic cocktail, after comparison with other commercial and in-house-made ones, which may be associated with its improved resistance to end-product inhibition.

In **Fig. 5.1b**, it can be seen that the XY obtained are over 80% in all conditions, with no statistically significant differences (Tukey's test), highlighting the efficiency of Cellic®Ctec2 to break the xylooligomers and xylan into xylose, due to adequate activities of xylanases, β-xylosidases, and accessory enzymes in the cocktail (NASCIMENTO et al., 2016; QIN et al., 2018), and the greater ease of converting the hemicellulosic fraction compared to the cellulosic one (MUSSATTO and DRAGONE, 2016). It is important to highlight that according to the sampling performed at different times (data not shown), both CC and XY increased gradually during the hydrolysis in both temperatures evaluated, probably due to the limitations in the access of the

enzymes to the substrate, related to the slow liquefaction of the suspension. Visually, the liquefaction started only after 12 h at 50 °C, and after 24 h at 35 °C, with more noticeable free water in the lowest solid load runs.

As for the sugar concentrations reached after hydrolysis (Fig. 1c), they are proportional to the amount of the solid provided and its cellulose conversion and xylose yield. Thus, although cellulose conversion with 20% of solids at 35 °C was not efficient, this high solid load provided a similar total sugar concentration to that obtained with 15% of solids, a condition that stood out for further process integration in small-scale, reaching about 90 g/L of fermentable sugars at 35 °C. In summary, the enzyme load provided was satisfactory for conducting shake-flask small-scale enzymatic hydrolysis with on top 15% of solids at 35 °C, above that, improvements are necessary, such as the solid feedings exploited in the helical stirred bioreactor (DA SILVA et al., 2020).

Based on these results and considering the need to work at mild temperatures for the SSCF process, it can be settled that the condition with 15% of solids in HH at 35 °C is more suitable for further studies with cultures in the same configuration (small-scale). Even though there is room for improvement of the hydrolysis yield, as it will be further presented.

From the previous discussions, it is worth highlighting the importance of the enzymatic cocktail chosen and its load for enzymatic hydrolysis. Pant et al. (2021) used an in-house produced mixture of cellulases for the hydrolysis of delignified Brassica juncea biomass (15% of solids, 50 °C), also performed in shake flasks, obtaining about 62 g/L of fermentable sugars in 48 h, and showing the potential of developing novel and efficient enzymatic cocktails as alternatives to the commercially available ones. Biocatalyst development was also assessed by Bachosz et al. (2022), who developed and assessed a co-immobilized multi-enzymatic biocatalytic system with cofactor regeneration, allowing its recycling in repeated batches, and dispensing the addition of cofactors to the medium, important features for the techno-economic feasibility of 2G processes (Vu et al., 2021).

5.3.1.2. Simultaneous saccharification and co-fermentation (SSCF) and Pre-saccharification followed by SSCF (PS SSCF)

After getting relevant results in the enzymatic hydrolysis at 35 °C, co-culture and mono-culture fermentations were evaluated at two strategies, SSCF and PS SSCF, under the same hydrolysis conditions to verify the performance of the integrated process. Results regarding sugar

consumption and ethanol production are presented in **Figs. 5.2 a-e** and the fermentation indexes are shown in **Table 5.2**.

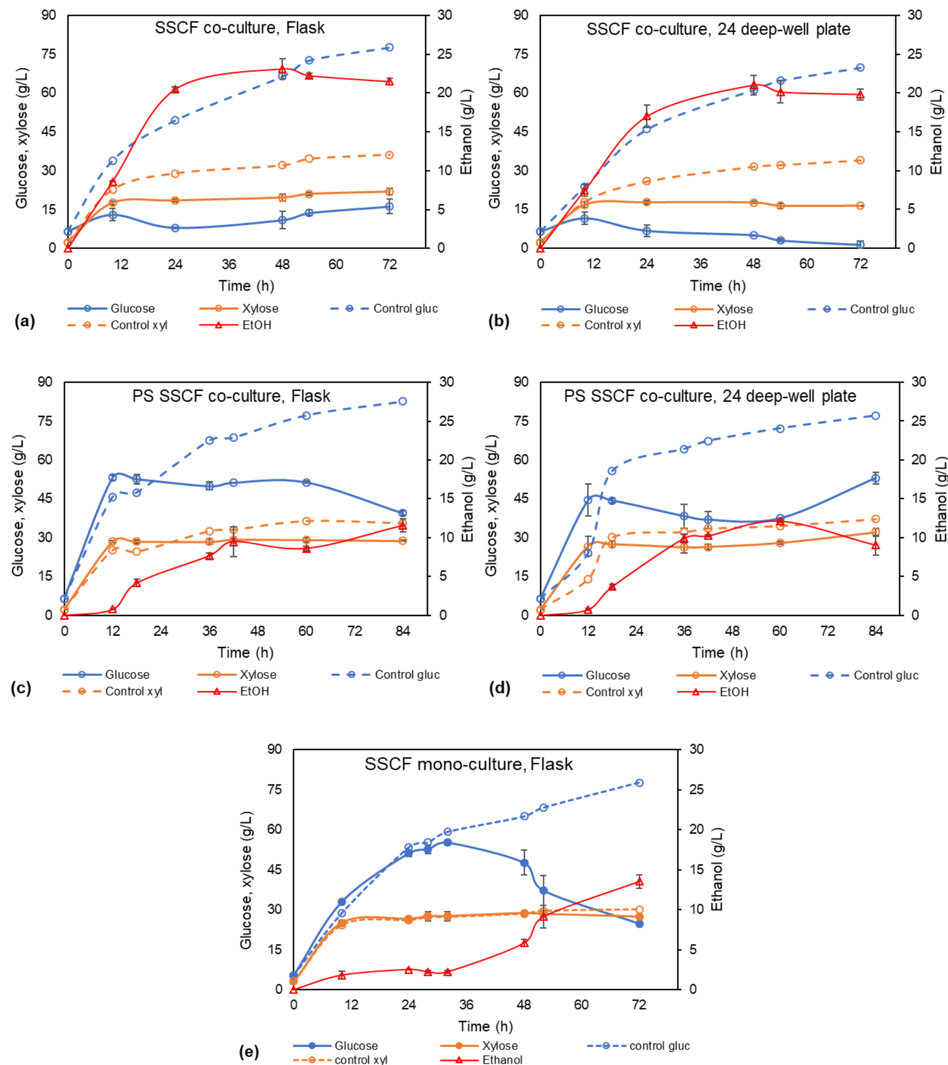

Figure 5.2. Concentrations of glucose, xylose, and ethanol during SSCF co-culture of *S. cerevisiae* and *K. marxianus* 3A **(a, b)**; mono-culture of *K. marxianus* 3A **(e);** and PSSSCF co-culture **(c, d)** in 50-mL flasks and 24 deep-well plates (10 mL). Error bars are standard deviations of the duplicates. Dashed lines represent the control experiment; solid lines represent the cultures (C_{X0}: 5 g/L, 35 ºC, pH_0: 5, 150 rpm, Cellic®Ctec2: 45 FPU/$g_{cellulose}$, PEG: 0.05 g/$g_{drybiomass}$).

Regarding SSCF co-culture (**Figs. 5.2 a-b**) either in flasks or in 24 deep-well plates, it can be seen that the xylose assimilation required at least 12 h to begin, from the moment glucose concentration in the media became lower than 15 g/L. This agrees with Rodrussamee et al. (2011), who observed repression in the xylose assimilation by a *K. marxianus* strain (DMKU3-1042) in

the presence of 20 g/L of glucose. It can also be observed that both glucose and xylose concentrations are mostly stable during the fermentation, which is mainly due to a similar assimilation and production rate of these sugars since the saccharification is continuously occurring within the fermentation, giving the impression that no sugar is being assimilated. The ethanol production increased until 48 h, then a slight decrease was observed, probably caused by a re-assimilation of the bioproduct, together with the xylose uptake, which is intensified after glucose depletion in the media. This behavior can be either observed for *S. cerevisiae* or *K. marxianus*, due to a lack of substrate (FARIAS AND MAUGERI FILHO, 2019) or flexibility in the metabolic routes to replace needed cofactors (FENG et al., 2021), respectively.

To verify a possible improvement in the fermentation performance, a co-culture PS SSCF was conducted. However, as previously observed by Sandri et al. (2023a), by culturing the same co-culture in liquid HH media, glucose repression over xylose assimilation occurred when its concentration is greater than 20 g/L. As shown in **Figs. 5.2 c-d**, an initial glucose concentration of about 50 g/L was present at the beginning of the fermentation due to the pre-hydrolysis step, which impaired xylose assimilation. The glucose conversion by the yeast was slow and its concentration was not lower than 40 g/L during the whole fermentation. As shown in Table 1, xylose conversions either in flask or in the 24 deep-well plates were only 19.26 and 13.71% after 84 h, compared to final xylose conversions of 39.50 and 51.69% in SSCF performed in flask and 24 deep-well (72 h), respectively, coupled with high glucose conversions. These experiments allowed concluding that SSCF co-culture is a promising strategy for 2G production process integration, especially considering that it was conducted in media containing HH and PSB without any conditioning.

It is also noteworthy that the best results of sugar conversion were obtained in the 24 deep-well plate SSCF compared to the flask, with significant differences (Tukey's test), highlighting the influence of agitation, and consequently aeration, on the performance of this co-culture proposal, which is related to the configuration of the deep-well plate system used. Glucose depletion was faster in the 24 deep-well plates than in the flask, which improved xylose conversion. However, the sugar assimilation profiles (**Fig. 5.1a**) show that higher conversions could be reached in flasks by increasing fermentation time. In this sense, the 24 deep-well plates could represent the flasks very well, besides allowing conducting a higher amount of experiments using less material and time (SANDRI et al., 2023a). It is important to highlight that Sandri et al. (2023a) reported glucose depletion and 83% of xylose conversion when culturing the same co-culture used in the present

study in 24 deep-well plates. However, the results were obtained in liquid hydrolysate, allowing to infer a possible inhibition caused by the compounds present in the cellulignin on the cells, in addition to the influence of aeration, related mass transfer limitations in the non-heterogeneous system, which is valid for both strategies evaluated here (SSCF and PS SSCF).

Fig. 5.2e presents a mono-culture (*K. marxianus* 3A) conducted in the SSCF strategy. This fermentation shows the superiority of the co-culture strategy for the co-assimilation of glucose and xylose. From the previous co-culture results, it is clear that the presence of *S. cerevisiae* was fundamental to increase the glucose assimilation rate, and consequently, xylose conversion, reaffirming the results presented by Sandri et al. (2023a). Regarding the fermentation parameters (**Table 5.2**), glucose conversion after 72 h was 68%, referring to less than 30 g/L remaining in the medium. Only from 48 h, it was possible to see the start of xylose assimilation. Accordingly, ethanol productivity (0.19 g/L/h) was lower than in the co-culture (0.30 g/L/h) performed in the same condition, with significant differences. Regarding ethanol yield, significant differences were observed only between the two configurations of PS SSCF performed. Xylitol yields were similar and presented low values.

Table 5.2. Fermentation performance parameters for SSCF and PS SSCF co-culture (*S.c*erevisiae + *K.marxianus* 3A)/ mono-culture (*K.m.* 3A) in Flasks/ deep-well plates (C_{X0}: 5 g/L, 35 ºC, pH_0: 5, 150 rpm, Cellic®Ctec2: 45 FPU/$g_{cellulose}$, PEG: 0.05 g/$g_{drybiomass}$). Standard deviations were lower than 10%. Different letters mean statistically significant differences between the values pair-compared for each parameter (Tukey's test), 95% of confidence.

Strategy:	**SSCF co-culture**					
	Flask					
Time (h)	**X_{gluc} (%)**	**X_{xyl} (%)**	**C_{etOH} (g/L)**	**Y_{etOH} (g/g)**	**Y_{xylOH} (g/g)**	**Q_{etOH} (g/L/h)**
72	79.07^{a}	39.50^{a}	21.49^{a}	0.28^{ab}	0.01^{a}	0.30^{a}
	24 deep-well plate					
	97.99^{b}	51.69^{b}	19.83^{a}	0.23^{a}	0.04^{a}	0.28^{a}
	PS SSCF co-culture					
	Flask					
84	52.23^{c}	19.26^{cd}	11.54^{bc}	0.23^{a}	0.01^{a}	0.14^{b}
	24 deep-well plate					
	31.23^{d}	13.71^{de}	8.54^{b}	0.31^{b}	0.00^{a}	0.10^{b}
	SSCF mono-culture, Flask					
72	68.07^{e}	9.19^{d}	13.55^{c}	0.24^{ab}	0.02^{a}	0.19^{c}

5.3.2. Bioreactor for high solid load hydrolysis and co-cultures

5.3.2.1. Solid fed-batch enzymatic hydrolysis

As a strategy to reach high solid load and cellulose conversion in the enzymatic hydrolysis, fed-batch experiments gradually supplying PSB to the bioreactor equipped with a helical impeller were conducted either at 35 or 50 °C. The results of CC and XY are shown in **Fig. 5.3**. These values were calculated at each sampling time, and are related to the previous initial solid load present in the bioreactor.

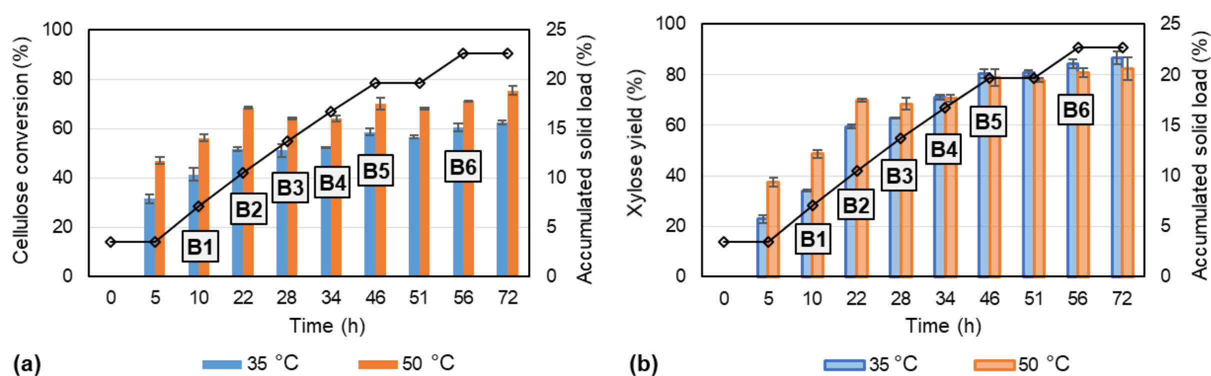

Figure 5.3. Cellulose conversion **(a)** and xylose yield **(b)** correlated to accumulated solid load (black diamonds connected by a black line) during fed-batch enzymatic hydrolysis in BTB at 35 and 50 °C. The labels B1 to B6 represent each sequential batch at the corresponding time in which each solid feeding was performed. Error bars are standard deviations of the duplicates. Cellic®Ctec2: 45 FPU/$g_{cellulose}$, PEG (0.05 g/$g_{drybiomass}$), pH_0: 5, 150 rpm, HH+ PSB (700 mL).

As shown in **Fig. 5.3**, the feeding strategy consisted of several additions of a low solid load during the whole process. The load increment of 3.5% (w/v) was defined based on the concentration of total sugars that this amount of solids would provide, which was less than 20 g/L in the first 10 h. This concern was based on the further process integration with the co-culture (SSCF), as previously presented in the small-scale studies, since it is known the repression that hexoses concentration over this value causes in xylose assimilation (SANDRI et al., 2023a). Another key condition in the strategy applied was the use of biomass with high moisture (~60%), which besides saving energy in the drying step, can facilitate the hydrolysis, by saving time for the increase in free-water content in the bioreactor. The water constraint is an important operation condition for hydrolysis, and according to da Silva et al. (2020) surfaces capable of constraining more water are the most efficiently hydrolyzed.

After 10 h with 3.5% of solids, about 40 and 56% of cellulose conversion (CC) were reached at 35 and 50 °C, respectively (**Fig. 5.3a**). At this time, the first solid feeding (B1) was performed, providing about 3.4% of additional solids to the suspension. From this moment to the next batch, about 50 and 68% of CC were achieved. Similar CC values were observed from B2 to B6 at both temperatures, showing a stable process that reached homogeneity due to proper mixing during the 72 h, as well as a sign that the proportional addition of fresh enzyme in each batch was working well on keeping high CC and XY. After 72 h of hydrolysis, CC of 62.52% (35 °C), and 75.45% (50 °C) were achieved with about 22.5% of solids provided to the reaction. At this time, the total sugar concentration released was 97.38 g/L (35 °C) and 112.56 g/L (50 °C) (glucose, xylose, arabinose, and cellobiose), values reported as enough to provide a feasible distillation step after fermentation of these fermentable sugars into ethanol (da Silva et al., 2020). Comparing these results with the previous hydrolysis performed in shake flasks at 20% of solids (the closest solid load) (**Fig. 5.1a**), it is clear the improvement provided by the bioreactor configuration, using a proper impeller for HSL hydrolysis, especially at 35 °C (37.51% in shake flask), which presented a 67% increase in CC, against 12% at 50 °C. Consequently, the use of the bioreactor also increased the sugar concentration in the media, which was only 69.40 g/L in the shake flask at 35 °C (**Fig. 5.1c**).

Zhang et al. (2012) also studied a fed-batch approach for the conversion of NaOH-pretreated sugarcane bagasse and wheat straw. The solid feedings performed were 9%, 8%, 7%, and 6% throughout 48 h, achieving a final solid load of 30% (w/v). Different from the present work, the enzymes were all added at the beginning of the reaction, resulting in a gradual decrease in glucan conversion, from an initial 60% to 39%, after 72 h. With sugarcane bagasse biomass, the conversion profile increased throughout the reaction, except for the last feeding time (6% solids at 48 h), resulting in a sharp conversion decrease. These results reinforce the importance of a gradual feed of fresh enzymes with the solids, which, as in the present work, allowed a more constant CC during the whole process.

Regarding the xylose yield (**Fig. 5.3b**), the results were similar to the ones previously reached in flasks at both temperatures (**Fig. 5.1b**), since very high yields, over 80%, have already been achieved. It is also important to point out the gradual increase in xylose yield during the time, which shows continuous hydrolysis of the xylan still attached to the solids and/or the xylooligomers. Both xylose sources were supplied in each solid feeding since the biomass had

moisture of about 60% (HH). After B5 (46 h), a stabilization close to a maximum value of xylan and xylooligomers hydrolysis was observed.

5.3.2.2. Simultaneous saccharification and co-fermentation co-culture feeding strategy 1- SF1

Based on the encouraging results obtained during the hydrolysis with the fed-batch strategy (Section 5.3.2.1), the same approach was then studied in co-fermentation coupled with simultaneous saccharification.

A co-culture (*S. cerevisiae* and *K. marxianus* 3A) was performed in the same conditions of the enzymatic hydrolysis previously described (35 °C), considered the control experiment (no cells). This co-culture will be denominated as feeding strategy 1 (SF1). The results of sugars and ethanol concentration, torque, pO2 (percentage of oxygen related to the saturation), and pH are presented in **Fig. 5.4**.

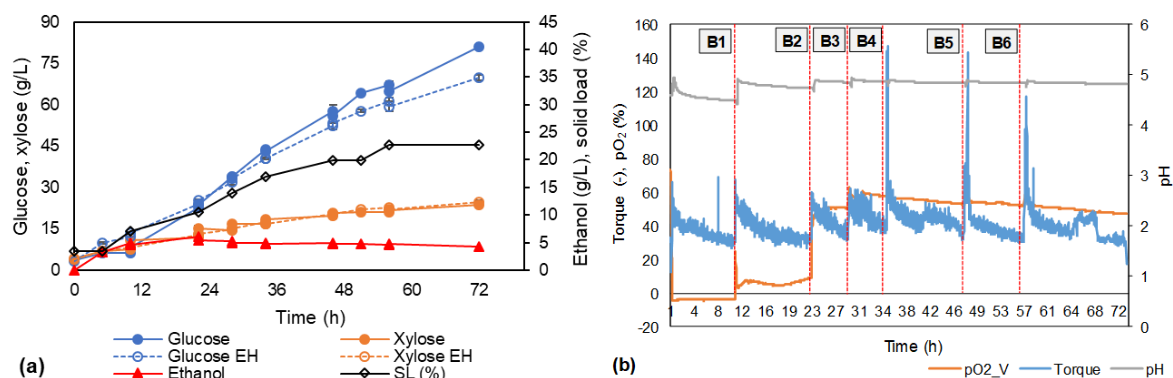

Figure 5.4. Concentrations of glucose, xylose, and ethanol, and solid load (%) during SSCF with solid fed-batch in HSB-SF1 **(a)** Dashed lines represent the control experiment (EH) (no cells) while solid lines represent the co-culture (C_{X0}: 5 g/L, 35 ºC, 150 rpm). Error bars are standard deviations of the duplicates. **(b)** Torque, pO_2, and pH during the 72 h fermentation, red dashed lines indicate solids feed times.

As shown in **Fig. 5.4a**, the concentrations of glucose and xylose during the co-culture did not decrease in the course of the fermentation. Up to 24 h of co-culture, some glucose assimilation could be observed, associated with low ethanol production, around 6 g/L, but later its production stabilized. Combined with the results provided in **Fig. 5.4b**, which shows the profile of pO_2 followed during the fermentation, it can be inferred a loss of viability of the cells during the co-culture. After each solid batch was made, an increase in the oxygen available was observed, which

did not decrease due to O_2 uptake by the cells of *K. marxianus* or *S. cerevisiae*, as expected. From B3 (34 h) the oxygen profile remained stable, and the sugar concentration increased. Regarding torque values, the profile shows a big increase at the moment of the feedings, followed by a plateau a few hours after it, due to the action of the enzymes added at each batch and the resulting liquefaction of the solid suspension. The new feedings were performed when the torque value reached about half of its initial value.

After 72 h, one slope of the co-culture suspension was transferred to a YPD-agar plate and incubated to verify cell viability. No colonies were observed, confirming cell death. This finding exposes the cell damage during the HSL operation, probably due to the shear effect caused by solids addition, coupled with the type of impeller and stirring speed used.

5.3.2.3. Simultaneous saccharification and co-fermentation co-culture feeding strategy 2- SF2

After facing the problems regarding cell viability in the previous SSCF experiment carried out under the SF1 strategy, a new feeding strategy (SF2) was evaluated. It consisted of increasing the number of feedings but decreasing the solid load in each one (about 1.25%), aiming to reach the same final load after 72 h of the process (about 22.5%). The main idea behind the SF2 strategy was to reach the liquefaction of the solid suspension faster and reduce the exposure of the cells to the shear effect. The results of sugars and ethanol concentrations, solid load, torque, pO_2, pH profiles, and viability are presented in **Figs. 5.5 a-c**.

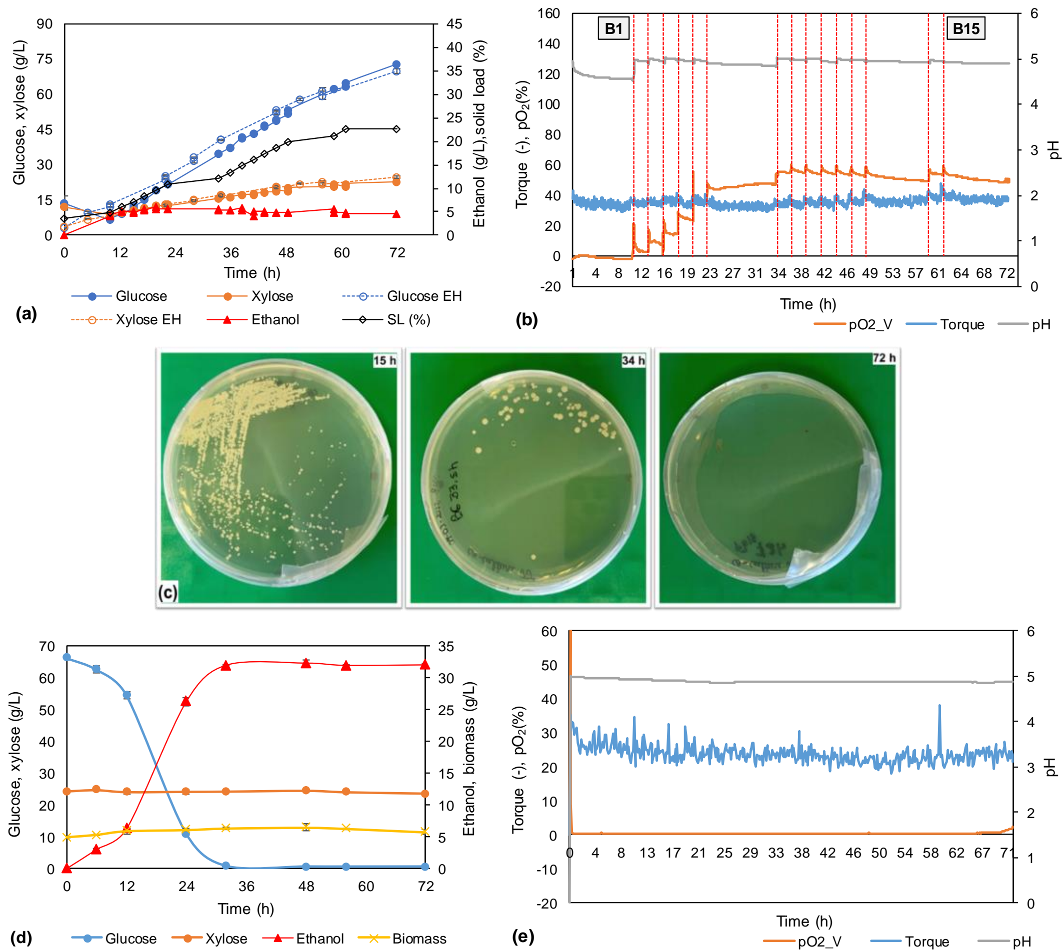

Figure 5.5. Compounds concentrations and solid load (SL) during SSCF with SF2 strategy for solid fed-batch in HSB **(a)** Dashed lines represent the control experiment (EH) (no cells) while solid lines represent the co-culture (C_{X0}: 5 g/L, 35 °C, 150 rpm). **(b)** Torque, pO_2, and pH during the 72 h SSCF. Red dashed lines indicate solids feed times. **(c)** Cell viability followed by plating at three sampling times (SSCF). Compounds and biomass concentrations during SqSCF **(d)** (C_{X0}: 5 g/L, 35 ºC, 150 rpm). **(e)** Torque, pO_2, and pH during the 72 h SqSCF. Error bars are standard deviations of the duplicates.

As in the SF1 strategy, glucose and xylose concentrations accumulated during the fermentation. Regarding ethanol production, once again the highest value was around 6 g/L within 20 h of process, from this moment its production stopped (**Fig. 5.5a**). The pO_2 profile in **Fig. 5.5b** shows a more gradual increase in its value compared to the previous feeding strategy. However, the increase was also coupled with the solids feeding and reached a maximum value at 34 h. Nevertheless, it is important to observe that the torque values were kept continuously low in this strategy, probably because the suspension remained at the intended liquefaction condition, a positive characteristic for saving energy in the process (MODENBACH; NOKES, 2013). To verify

cell damage, the cell viability was followed by plating one slope of the medium suspension three times in the process (**Fig. 5.5c**). From the pictures, it is clear the decrease in the number of colonies during the fermentation, reaffirming the damage caused to the cells even when the suspension was kept at the liquefied condition and lower torque.

Some authors have reported a loss of cell viability when performing SSF processes. According to Unrean et al. (2016) and Koppram and Olsson (2014), the observed cell death may be caused by a combined effect of ethanol inhibition and the inhibitory effect of inhibitors such as acetic acid present in pretreated bagasse. These authors have presented the performance of cell feedings with the solid ones as the main solution to decrease the viability drop. As seen in the present work, the loss of viability was not relevant in the small-scale studies since sugars conversion and ethanol production were observed throughout 72 h of culture (**Fig. 5.2**). Thus, cell damage seems to be rather caused by the stirring and solids addition than the presence of the inhibitors, highlighting the fact that an evolved yeast (*K. marxianus* 3A) with improved inhibitors tolerance was used in the co-culture.

Overall, these results show that further research in bioreactor design and operation is required to scale-up SSCF. A decrease of the stirring speed combined with gas supply at a suitable flow rate, or the enclosure of the PSB would probably be effective in reducing the shear effect and enabling the SSCF. However, at the expense of a significant drop in the enzymatic hydrolysis efficiency.

5.3.2.4. Sequential saccharification and co-fermentation (SqSCF)

Considering the high cellulose conversion and sugar yields achieved in the PSB+HH enzymatic hydrolysis (**Fig. 5.1**), the direct co-fermentation of the WBH obtained after removal of the remaining solids posed as a natural choice to bioreactor operation and process scale-up without cell damage. The medium consisted of the enzymatic hydrolysate obtained in the solid fed-batch enzymatic hydrolysis at 35 °C, previously discussed in Section 3.2.1. The results of sugars consumption, ethanol and biomass production, torque, pO_2, and pH profiles are presented in **Figs. 5.5 d-e**.

As shown in **Fig. 5.5d**, this hydrolysate initially contained about 65 g/L of glucose and 24 g/L of xylose. Glucose was rapidly assimilated and converted into ethanol, leading to values of ethanol yield and productivity of 0.48 g_{etOH}/g_S and 0.44 g_{etOH}/L/h, respectively. Biomass

concentration (**Fig. 5.5d**) remained fairly constant throughout the fermentation, proving that no stressful condition affecting cell viability was established in the WBH fermentation. **Fig. 5.5e** shows the torque profile during the cultivation in the hydrolysate, which remained around 25, and was slightly lower than the torque imposed by the helical impeller at the SF2 strategy (**Fig. 5.5b**). Thus, it suggests that the torque itself was not the main cause of cell damage, but it seems that the presence of PSB fragments, even after suspension liquefaction, affected the hydrodynamics and intensified the shear effect on the cells.

Our results are in accordance with the findings of Sassner et al. (2006) who found the best results of ethanol production from glucose when fermenting the liquid fraction of the hydrolysis, and avoiding mass transfer problems or stress due to high viscosity at high solid concentration. On the other hand, **Fig. 5.5d** and **Table 5.2** show that xylose remained almost at the same concentration after 72 h of fermentation (~3.4% of conversion). In **Fig. 5.5e**, it is interesting to see the pO_2 profile, which remained close to 0 throughout the cultivation, indicating that the cell metabolism was strongly oxygen-limited. This is probably the main reason for the lack of xylose consumption since, as reported by Sandri et al. (2023a), a suitable micro aerated environment is very important to improve xylose conversion. The authors reached 83% of xylose conversion when conducting the co-culture on small-scale under the optimal conditions established after a high throughput screening in 24 deep-well plates. Feng et al. (2021) also reported that xylose was minimally consumed after glucose depletion under non-aeration conditions by the yeast *K. marxianus* CICC 1727−5. The authors observed improved xylose conversion at an aeration rate of 0.5 vvm.

Assuming that xylose uptake could be considerably improved by setting-up a suitable air supply, the SqSCF poses a promising approach for the consumption of whole biomass fractions, which could be scaled-up and integrated with already available 1G ethanol production facilities. The early removal of the solids remaining after enzymatic hydrolysis is an important action that allows repeated batch operation with the recycling of *K. marxianus* and *S. cerevisiae* cells, reducing the inoculum preparation effort. In addition, in the biorefinery context, the removed solid residue can be easily mixed with sugarcane bagasse and directly used for steam and bioenergy generation.

5.4. Conclusion

This study showed that the utilization of an appropriate solid feeding strategy coupled with a suitable bioreactor configuration is essential to achieve efficient enzymatic hydrolysis of

lignocellulosic biomass. The hydrolyses of the whole fraction of hydrothermally pretreated sugarcane bagasse, conducted in a bioreactor with high solid loads, resulted in cellulose conversions of 63% (35 °C) and 75% (50 °C), as well as xylose yields higher than 80%. In addition, different *S. cerevisiae* and *K. marxianus* co-culture strategies, aiming at hexoses and pentoses co-fermentation, were addressed, both in small-scale and in bioreactor experiments, and revealed that the non-recombinant yeasts were able to directly ferment the unconditioned enzymatic hydrolysate. It was concluded that the strategies of simultaneous saccharification and co-fermentation (SSCF), as well as sequential SCF (SqSCF), have great potential for large-scale implementation. These findings contribute to the intensification of 2G production processes since by using the whole biomass for fermentation and integrating operational steps, costs can potentially be minimized, positively impacting the feasibility of the industrial operation. However, as highlighted in the paper, some challenges still have to be overcome for process improvements, requiring additional research in bioreactor designs and operation to scale up the SSCF approach, whilst the SqSCF requires optimization studies to define the best aeration rate to allow glucose and xylose co-fermentation.

Chapter 6. General conclusions and future perspectives

Integrated strategies aiming for the whole use of biomass sugars have been widely addressed in the literature as the key to feasible second-generation (2G) ethanol production processes. However, there are still some gaps to be fulfilled, such as the use of the generally neglected hemicellulosic sugars, as well as the development of processes that can be easily introduced in the current industrial/biorefinery context. In the Brazilian scenery, for instance, the use of GMOs faces some specific biosecurity requirements that make difficult their application. In this sense, the present doctoral thesis explored several fermentation strategies, using non-recombinant yeasts and commercial enzymes, for ethanol production by 2G process and integrated first generation (1G) and 2G. The performance of Simultaneous Isomerization and Fermentation (SIF), Simultaneous Isomerization and Co-Fermentation (SICF), and Simultaneous Saccharification (Isomerization) and Co-Fermentation (SSCF/SSICF) were evaluated and greatly improved, using commercial *S. cerevisiae* either combined with xylose isomerase (XI) or in co-culture with natural pentose fermenting yeast (*K. marxianus*). Sugarcane bagasse, a coproduct from the Brazilian 1G industry, was the biomass applied in this work. Its whole use was investigated by fermentations of its liquor (hydrothermal/acid pretreated hydrolysates) and solid pretreated fractions. Molasses (hexose-rich syrup from the industrial sugar production) addition was also evaluated, accomplishing integrated 1G/2G processes.

A special bioreactor ("Enzyme-multiple-packed bed reactor", EMPBR), originally designed for SIF, had a fundamental contribution to the advances achieved. The EMPBR can be easily scaled-up, since it consists of a module of several tubes for enzyme load that can fit in any cylindric tank, having great potential for large-scale implementation. In SIF studies, good ethanol yield and productivity (0.38 g_{etOH}/g_S, 0.85 g_{etOH}/L/h) were attained from 90% of xylose converted in synthetic medium, besides keeping good stability in the repeated batches performed, which allowed cell and enzyme recycling. The performance of the developed reactor was also assessed for SICF/SSICF (integration 1G/2G) processes. Our results clearly showed that the rate of the SIF process is severely reduced in the presence of hexoses. Thus, a sequential fermentation strategy was set-up and validated. The hemicellulosic hydrolysate (containing pentoses) was fermented first, followed by a fast fermentation of molasses. Together with EMPBR, the sequential fermentation strategy coupled with the supplementation with molasses was a smart approach to

introduce the crude hemicellulosic hydrolysates into the 1G production process (as the dilution stream of molasses), preserving xylose high conversion in the 1st fermentation step and using the sugar-rich molasses to reach high ethanol concentration in the 2nd step. The same sequential strategy seems promising for SSICF, inasmuch as the pretreated solid fraction, rich in cellulose, if added to the reactor in the 2nd fermentation step, along with the molasses.

Despite the process intermediated by the XI, a yeast co-culture combining *S. cerevisiae* and *K. marxianus* (pentose fermenting strain evolved for improved hydrolysate inhibitors tolerance) showed to be another feasible alternative for hexoses and pentoses co-fermentation. The co-culture performance was evaluated in high throughput studies (24 deep-well plates) that allowed finding the best conditions for combining both strains and having high xylose conversion (83%), a 30% improvement compared to that reached in mono-culture with *K. marxianus*. The optimized co-culture was further conducted in an industrial medium with a high solid load (HSL), but before, enzymatic hydrolysis studies were performed, aiming at evaluating the process, improving its conditions, and increasing the solid amount. The lack of proper agitation limited the solid load in shake flasks to 15% (w/v) as cellulose conversion decreased by 43% when increasing that to 20%. Solid feeding strategies applied in a helical stirred bioreactor were the key to increasing solid load (up to 22.5%) and reaching good cellulose conversion (63 and 73%, at 35 and 50 °C, respectively) even at optimal fermentation temperature (35 °C). Performing the co-cultures in shake-flasks with 15% of solids, 52% xylose conversion, and 0.23 g_{etOH}/g_S of ethanol yield were achieved. However, when scaling-up to the bioreactor with the solid feeding strategy, sugar assimilation was impaired by the cells' viability loss, related to the shear effect caused by the combined presence of the helicoidal impeller and the solids. The harm of solids freely agitated over cells viability was previously observed in the SIF studies. Sequential hydrolysis and liquid co-fermentation (SqSCF) were proposed for the co-culture to reach the 24 deep-well performance when scaled-up.

Based on all the results gathered from the fermentation and enzymatic hydrolysis strategies explored in this Ph.D. research, the potential of non-recombinant yeasts for 2G processes is evident, making co-culture or the addition of commercial enzymes a good alternative for process integration in the biorefinery context, achieving the goals proposed in the present doctoral thesis, which consisted of exploiting processes with non-GMO yeast and commercial enzymes for the production of 1G/2G ethanol using the entire biomass fraction and molasses. Even though, the SICF, using the enzyme xylose isomerase in industrial medium, stood out for second generation ethanol production,

due to its higher yield and productivity, as well as the facilities for conducting the process compared to the co-culture strategy, which requires optimal aeration conditions. Regardless of the costs that accompany the enzyme requirement, repeated batches, a well-established process in the 1G industry, can help make the process viable. However, some issues still need to be addressed in both processes, as highlighted in this thesis, bringing possibilities for improvements to achieve better processes performance.

Regarding Chapter 2, it was clear the importance of an adequate bioreactor design for good SIF performance, and studies for optimizing the EMPBR design still have a spot in this research. Aligned with that, the integrated 1G/2G ethanol production discussed in Chapter 3, an improvement of the SSICF strategy is necessary. The initial proposal would be using only the hemicellulosic hydrolysate in the 1st step and adding the pretreated bagasse solid fraction together with molasses in the 2nd step. The stability in repeated batches operation also deserves attention. It depends on improving the performance of *S. cerevisiae* in industrial media and adaptative evolution poses a promising approach to achieve this goal. Reducing the load of the CelliCtec2 enzymatic cocktail or finding good options for its immobilization are also possible points to be studied since this commercial enzyme preparation is liquid and cannot be recovered after each fermentation batch, adding costs to the process. In Chapters 4 and 5, the use of a yeast co-culture provided great insights for the possibility of taking advantage of the whole biomass sugars without requiring enzyme addition, but specific aeration requirements are demanded for better results of xylose uptake. Setting-up a suitable air supply could significantly improve that and poses a further step in these studies. Similarly, the Simultaneous Saccharification and Co-Fermentation (SSCF) seems to be a promising approach for the consumption of whole biomass fractions, but it faced problems regarding loss of cell viability due to the combined shear effect of the bioreactor impeller and solids over the yeast's cells. A liquid co-culture of the pre-enzymatic hydrolyzed fraction of sugarcane bagasse, in a high solid load, with the hemicellulosic hydrolysate coming from the biomass pretreatment should be evaluated. The early removal of the solids remaining after enzymatic hydrolysis is an important action that allows repeated batch operation with the recycling of *K. marxianus* and *S. cerevisiae* cells, reducing the inoculum preparation effort. In addition, in the biorefinery context, the removed solid residue can be easily mixed with sugarcane bagasse and directly used for steam and bioenergy production.

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Supplementary material (Chapter 2)

'Bioreactor and process design for 2G ethanol production from xylose using industrial *S. cerevisiae* and commercial xylose isomerase'

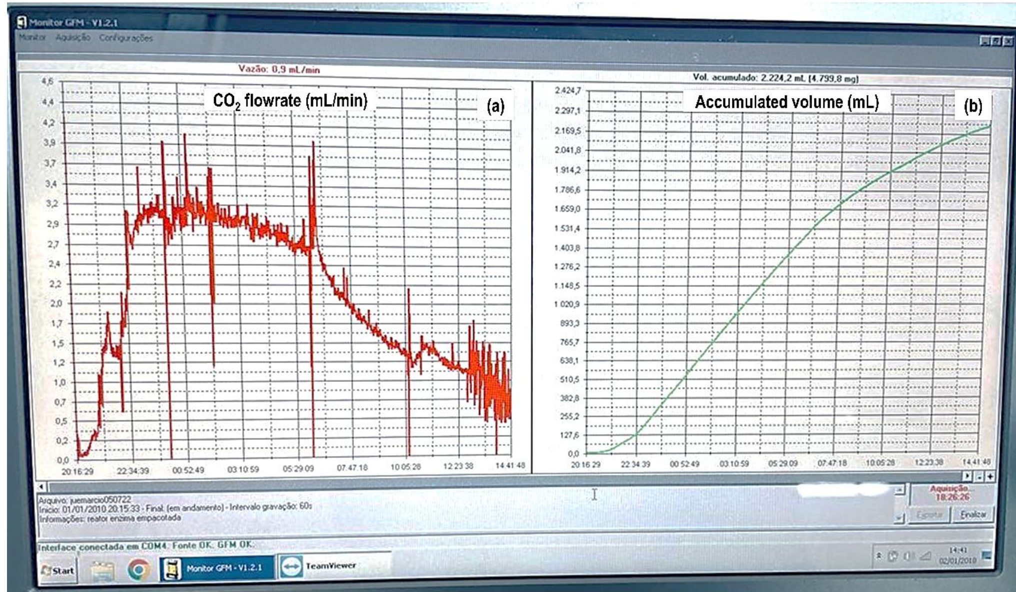

Figure SM1. Picture of the notebook screen showing the CO_2 acquisition data through to the flowmeter connected to the reactor: flowrate in mL/min (a) and accumulated volume in mL (b).

Figure SM2. Mini-reactors containing cell suspension and commercial xylose isomerase IGI-HF.