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Bioethanol production by immobilized co-culture of *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* in a novel continuous 3D printing microbioreactor

PEDRO HENRIQUE F. RODRIGUES, ELIZABETH G. DA SILVA, ALEX S. BORGES, GABRIEL LUIS CASTIGLIONI, CARLOS ALBERTO G. SUAREZ & INTI DORACI C. MONTANO

Abstract: Biorefineries require low-cost production processes, low waste generation and equipment that can be used not only for a single process, but for the manufacture of several products. In this context, in this research a continuous 3D printing microbioreactor coupled to an Arduino-controlled automatic feeding system was developed for the intensification of the ethanol production process from xylose/xylulose (3:1), using a new biocatalyst containing the co-culture of *Scheffersomyces stipitis* and *Saccharomyces cerevisiae* (50/50). Initially, batch fermentations of monocultures of *S. cerevisiae* and *S. stipitis* and co-culture were carried out. Subsequently, the immobilized co-culture was used as a biocatalyst in continuous fermentations using the developed microreactor. Fermentations carried out in the microbioreactor presented a 2-fold increase in the ethanol concentration and a 3-fold increase in productivity when compared to monocultures. The microbioreactor developed proved to be efficient and can be extended for other bioproducts production. This approach proved to be a promising alternative for the use of the hemicellulose fraction of biomasses without the need to use modified strains.

Key words: hemicellulose, xylose, xylulose, biocatalyst, ethanol, continuous fermentation.

INTRODUCTION

The main raw material for the production of second generation ethanol (2G ethanol) is lignocellulosic waste. Lignocellulosic residue is called the by-product of processes that consume agro-industrial inputs, such as corn, wheat, malt, sugar cane, among others, and is mainly composed of cellulose, hemicellulose and lignin (Pellera & Gidarakos 2018, Silva et al. 2019, Johnston et al. 2020). In the context of biorefinery, these lignocellulosic materials have advantages for their use as raw materials due, among other things, to the large amount available and, in most cases, to their low cost, making their use interesting in the production of fuels and chemicals with higher added value (Santos et al. 2011, Delivand & Gnansounou 2013, Agostinho & Ortega 2013, Yu et al. 2018). To increase the economic viability of the 2G ethanol production process, it is important that the main sugars present in cellulose (glucose) and hemicellulose (xylose) are fermented (Menon et al. 2010, Rosales-Calderon & Arantes 2019). In this sense, this study aims at the use of xylose, the main sugar in the hemicellulose fraction.

The main microorganism used in biorefineries for ethanol production is the yeast *Saccharomyces cerevisiae*, due to its tolerance to high concentrations of ethanol, tolerance to temperature changes,

and high efficiency to ferment monosaccharides for ethanol production. However, this microorganism cannot metabolize xylose (Yu et al. 1995, Zhang et al. 2008, Robak & Balcerek 2018). The inability of *S. cerevisiae* to ferment xylose is believed to be because xylose uptake is non-specifically mediated by hexose transporters, with xylose uptake through these transporters being significantly less efficient compared to glucose (Hamacher et al. 2002, Jiang et al. 2020).

Additionally, once xylose is inside the cell, it must be converted to xylulose to continue the metabolic route to ethanol, but although *S. cerevisiae* contains all the necessary genes for metabolizing this pentose, the intracellular enzymes xylose reductase (responsible for reduce xylose to xylitol) and xylitol dehydrogenase (responsible for oxidizing xylitol to xylulose) have low activities (Toivari et al. 2004, Jiang et al. 2020).

However, *S. cerevisiae* is able to ferment externally obtained xylulose by the isomerization of xylose by the enzyme glucose isomerase. Xylose isomerization generates a xylose/xylulose mixture in a ratio (3:1). Since xylose is the component that is present in greater proportion in the mixture, in this study it was proposed to use a co-culture of *S. cerevisiae* with the yeast of *Scheffersomyces stipitis*, since this yeast is naturally capable of fermenting xylose into ethanol with good rates of conversion (Su et al. 2015, Farias et al. 2017). Until the present study, there was no information about the ability of *S. stipitis* to metabolize xylulose.

An important advantage of co-cultures is the possibility that one of the microorganisms is able to consume substances from the environment that the other cannot, this work in synergy leads to an increase in the efficiency of the process (Kim et al. 2012, Singh et al. 2014, Kim et al. 2016, Zhang et al. 2017). The co-culture of *S. stipitis*/*S. cerevisiae* has some advantages: the two microorganisms have compatible pH and temperatures and both need a low level of oxygen for the formation of ethanol (Chen 2011).

Experiments using co-culture of *S. stipitis*/*S. cerevisiae* using glucose/xylose mixtures show that the efficiency of the bioprocess of conversion of total sugars into ethanol increased from 55% to 95% with the use of co-culture (Yadav et al. 2011). Studies on the co-culture of microorganisms using xylose and xylulose as carbon sources simultaneously have not been reported in the literature, nor have studies on the consumption of xylulose by *S. stipitis* been reported, which is an important point in this study.

Furthermore, in this research, a mini-scale bioreactor was used for continuous operation, allowing a slightly more detailed study of the metabolites produced using a smaller amount of raw material. Small-scale devices can be built in different types of materials that present high sensitivity, large mass and thermal exchange area, low volumes of necessary reagents, low production cost and reduced amount of generated effluents, being a very promising alternative both in research involving reagents and enzymes of high cost, as well as in the generation of medical and food supplies of small quantity and high commercial value (Meldrum & Holl 2012, Roper et al. 2018, Sarafraz et al. 2019).

Thus, the present study aimed to analyze the viability of using a co-culture of *S. cerevisiae* and *S. stipitis* for the production of 2G ethanol, using a D-xylose/D-xylulose mixture as substrate, obtained after the isomerization of xylose by the enzyme xylose isomerase, this procedure allows the use of *S. cerevisiae*, yeast with good tolerance to ethanol, widely used in industry, but inefficient in the fermentation of D-xylose. Experiments with the monoculture of *S. stipitis* showed that in the presence of the mixture, the yeast consumed both substrates, indicating that the microorganism is able to

metabolize xylulose, although it preferentially consumed xylose. On the other hand, experiments carried out with *S. cerevisiae* showed that only D-xylulose was consumed. It was found that the use of co-culture led to increased productivity and further higher production of 2G ethanol. Subsequently, experiments using the immobilized co-culture were carried out, in batch and continuous, using a microbioreactor. The approach used in this work allowed doubling the production of 2G ethanol, using a process that is easy to operate industrially, without the need to use genetically modified microorganisms or to use processes where enzymes and microorganisms operate simultaneously under suboptimal conditions. Therefore, this study contributes to the consolidation of the biofuel sector, presenting alternatives for the use of the hemicellulose fraction and contributing to the improvement of the economic viability of the 2G ethanol production process.

MATERIALS AND METHODS

Microorganisms and Enzyme

Scheffersomyces stipitis acquired from Fundação André Tosello –Campinas/SP, *Saccharomyces cerevisiae* PE-2 (Usina Pedra). Enzyme Xylose Isomerase Sweetzyme It Extra Novozymes.

Isomerization process

1,0 g of Xylose isomerase enzyme was added to 400 mL of pH 7.0 solution containing 10 g/L of KH_2PO_4, 4 g/L of $MgSO_4$, 0.2 g/L of $CoCl_2$ and 120 g/L of d -xylose. Isomerization was carried out at 60°C and 200 rpm for 24 hours in a shaker incubat (Silva et al. 2012, Stahlberg et al. 2012, Yu et al. 2012).

Inoculum preparation

The yeast strains stored in agar medium plates were cultivated in YPX/X medium containing 10g/L of yeast extract, 20 g/L of peptone and around 30 and 10 g/L of xylose/xylulose respectively, at 30ºC, 200 rpm and pH 5.5. After incubation for 24h, cells were recovered by centrifugation at 10,000 rpm for 10 min and used as inoculum to ferment the medium.

Fermentation of pure microorganisms

The YPX/X medium was inoculated with a sufficient amount of concentrated cell suspension to reach an optical density of 2 at 600nm and subsequently incubated in a shaker with heating at 150 rpm and 30ºC. Samples were taken every 4 hours to measure cell concentration and analyze sugars, alcohols and organic acids.

Co-culture of *S. cerevisiae* and *S. stipitis* in shaker

The YPX/X medium was inoculated with a sufficient amount of concentrated *S. cerevisiae*/*S. stipitis* to reach an optical density of 2 at 600nm. For this, inoculums of pure microorganisms prepared separately as explained in Inoculum preparation, were mixed in the necessary proportion to obtain the desired ratio. Subsequently, it was incubated in a heated shaker at 150 rpm and 30°C.

Immobilization of yeast co-culture

The strain inoculums were prepared separately and subsequently centrifuged to separate the cells from the culture medium. In order to obtain pellets with 10% (w/w) yeast on a dry basis, the previously

centrifuged yeasts were weighed on a Bel Engineering moisture scale, model: i-Thermo 163 L, to calculate dry mass content by subtracting the sample moisture content value. Thus, the yeasts were weighed in the corresponding values so that they maintained the same proportion (50% of each yeast), and added to a solution containing sodium alginate (1% w/w), calcium carbonate (0.50% w/w) and buffer solution pH 8 .0 disodium phosphate/monobasic potassium phosphate (88.50%).

This solution was homogenized on the vortex and dropped through a syringe into a calcium chloride solution (30%). After coagulation of the pellets, they were stored in a refrigerator in a curing solution containing magnesium sulfate heptahydrate (4.0 g/L), monobasic potassium phosphate (10.0 g/L), urea (3.0 g /L), cobalt II chloride hexahydrate (0.2 g/L), calcium chloride (4.0 g/L), xylose (30.0 g/L) and xylulose (10.0 g/L) (Trovati et al. 2009, Silva et al. 2012).

Batch fermentation of YPX/X medium with immobilized *S. stipitis*/*S. cerevisiae* co-culture

In order to estimate the appropriate residence time to be used in continuous experiments in a 3D printing microbioreactor, batch experiments were carried out to evaluate the time required for substrate consumption and thus estimate the value of the feed flow for continuous experiments. For this purpose, 2.0 mL eppendorfs were filled with 900 microliters of YPX/X medium and 0.7g of *S. cerevisiae*/*S. stipitis*. The eppendorfs were then placed in a shaker-type incubator heated at 30ºC and 50 rpm. Every 4 hours an eppendorf was removed, the pellets were separated from the liquid, and the latter was analyzed via HPLC (High performance liquid chromatography). The calculation of the feed flow value for continuous experiments is presented in section Batch fermentation with immobilized *S. stipitis*/*S.cerevisiae* yeasts.

3D printing microbioreactor

The 3D printing microbioreactor was designed using AutoCAD 3D software and printed on translucent ABS (Acrylonitrile Butadiene Styrene) material using the Sethi 3D AiP A3 printer. The dimensions and specifications of the microbioreactor are presented in section Results and discussion. Inside the bioreactor, a stainless steel screen was installed in order to retain the immobilized yeast inside the microbioreactor.

Continuous fermentation of immobilized *S. stipitis*/*S.cerevisiae* co-culture in YPX/X medium using a 3D printing microbioreactor

The 3D printing microbioreactor was filled with 0.7g of *S. cerevisiae*/*S. stipitis* and placed in a shaker-type incubator without agitation to maintain the temperature inside the bioreactor at 30°C. A gas outlet was attached to the top of the microbioreactor in order to relieve the pressure inside it due to the accumulation of CO_2 resulting from fermentation. The microbioreactor has a useful volume of 900 microliters and inside the pellets are confined between two stainless steel screens (Figure 1).

YPX/X medium at a flow rate of 16 microliters per hour (see the feed flow calculation in section Batch fermentation with immobilized *S. stipitis*/*S.cerevisiae* yeasts) was injected into the microbioreactor with the aid of a syringe coupled to an arduino-controlled continuous injection system. For the beginning of the experiment, the microbioreactor was filled with the YPX/X medium and the initial sample was collected. The following samples were taken every 12 hours and analyzed via HPLC (High performance liquid chromatography).

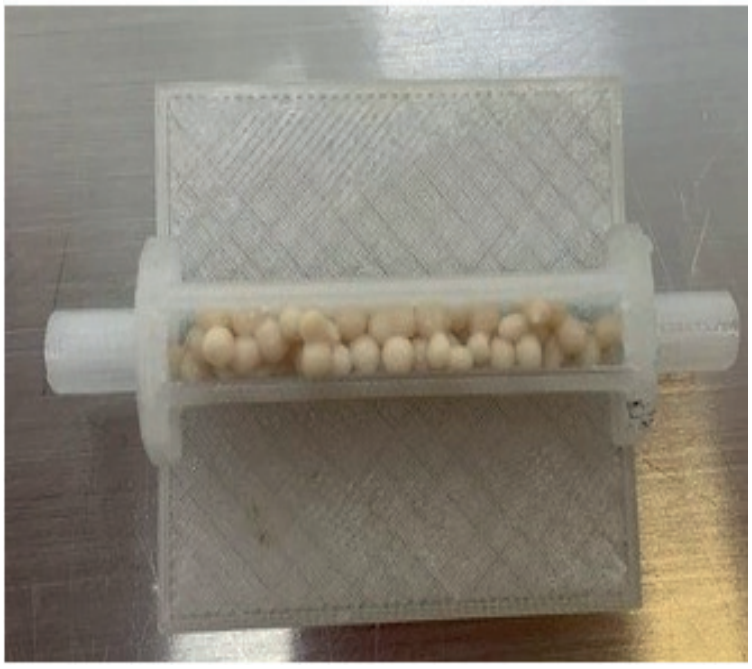

Figure 1. 3D printing microbioreactor filled with 0.7g of *S. cerevisiae*/*S. stipitis* immobilizeds, containing 50% yeast *S. stipitis* and 50% yeast *S. cerevisiae*.

Quantification of sugars, ethanol and xylitol

The amounts of xylose, xylulose, ethanol and xylitol present in the samples were analyzed using ion exchange HPLC (High performance liquid chromatography) (Shimadzu Prominenc chromatograph with Ultraviolet (UV) and Infrared (IR) detectors), Shimpack SCR-102 column (H) with an aqueous solution of 5 mM perchloric acid, eluting at a flow rate of 0.6 mL/min, as the mobile phase The temperature for separating the components was 50 ºC.

Cell viability

The viability of the cells immobilized in calcium alginate was monitored by counting the cells using a Neubauer Chamber with the aid of a microscope. About 25 mg of pellets were solubilized in 1 mL of 8% sodium citrate solution (w/v) and stirred until the pellets were completely dissolved.

The resulting solution was diluted 20 times using sodium citrate solution, and then diluted 40 times in methylene blue dye. After 5 minutes, 4.0 microliters of the resulting solution were placed over the Neubauer Chamber and viable and non-viable cells were counted. The percentage was calculated as follows: number of viable cells (not stained) / number of total cells (stained and not stained).

Fermentation performance indexes

The results of the experiments were evaluated and compared by determining performance indices, including ethanol yield ($Y_{ethanol/s}$) ($g_{ethanol}/g_{substrate}$), xylitol yield ($Y_{xylitol/s}$) ($g_{xylitol}/g_{substrate}$), volumetric productivity ($g_{ethanol}$/L.h) and substrate conversion (%), according to Eqs. (1) – (4) (Silva et al. 2019, Shuler & Kargi 2002).

- Ethanol yield ($Y_{ethanol/s}$, $g_{ethanol}/g_{substrate}$)

$$Y_{ethanol/S} = \left(P_{ethanol} - P_{ethanoli}\right) / \left(S_i - S\right) \quad (1)$$

Where: $P_{ethanol}$ = final concentration of ethanol (g/L); S = final substrate concentration (g/L); $P_{ethanoli}$ = initial ethanol concentration (g/L); S_i = initial substrate concentration (g/L).

- Xylitol yield ($Y_{xylitol/s}$, $g_{xylitol}/g_{substrate}$)

$$Y_{Xylitol/S} = (P_{Xylitol} - P_{Xylitoli}) / (S_i - S) \quad (2)$$

Where: $P_{xylitol}$ = final concentration of xylitol (g/L); $P_{xylitoli}$ = initial concentration of xylitol (g/L).

- Ethanol volumetric productivity ($Pr_{ethanol}$, g/L.h).

$$Pr_{ethanol} = P_{ethanol} - P_{ethanoli} / t \quad (3)$$

Where: t = final process time (h).

- Substrate conversion (X, %).

$$X = \frac{(S_i - S)}{S_i} \times 100 \quad (4)$$

RESULTS AND DISCUSSION

Fermentation of the D-xylose/D-xylulose by monocultures of *S. cerevisiae* and *S. stipitis* yeasts.

Figures 2 and 3 shows cell concentrations and metabolites during fermentations by *S. cerevisiae* and *S. stipitis* yeasts. As observed in other works (Ochoa-Chacón et al. 2022, Senac & Hahn-Hagerdal 1990), the yeast *S. cerevisiae* did not present significant consumption of xylose, consuming the xylulose present in the mixture for the production of xylitol and ethanol.

During the first 24 hours of fermentation, the yeast *S. cerevisiae* (Figure 2) showed a lower xylulose consumption rate, which increased after that time. This behavior was also observed by Lee et al. in 2003, where during the fermentation of the xylose/xylulose mixture in fed batch, the consumption

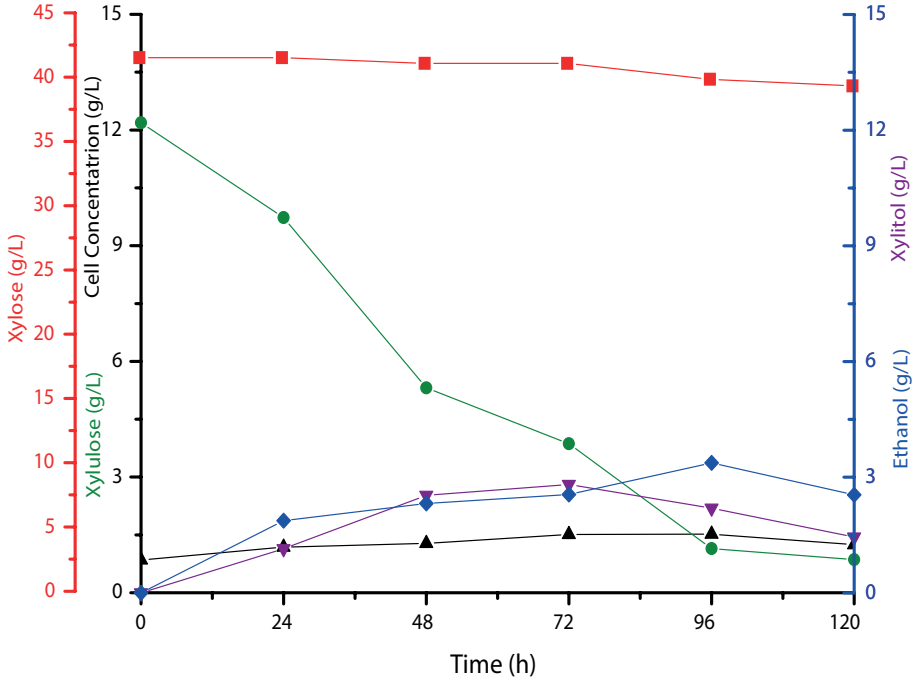

Figure 2. Growth in the YPX/X medium and metabolic profile of *S. cerevisiae*. (■) xylose, (●) xylulose, (♦) xylitol, (▼) ethanol) and (▲) cellular concentration. Operating conditions are 30ºC, pH 5.5 and 150 rpm.

rate of xylulose increased after 20 hours of fermentation, considered as the time of the lag phase of this yeast when metabolizing this substrate. Additionally, the limitation of consumption of this substrate occurs because *S. cerevisiae* lacks specific pentose transporters, and these sugars enter the cell with low affinity through hexose transporters of the Hxt family (Luyten et al. 2002, Saloheimo et al. 2007, Nijland et al. 2018, Nijland & Driessen 2020).

The highest concentration of ethanol was observed at 96 hours of fermentation (Figure 2), reaching 3.373g/L of ethanol, whereas xylitol reached a concentration of 2.891 g/L. The presence of xylitol is basically due to two factors: the metabolic reaction that converts D-xylulose to xylulose-5-phosphate, catalyzed by the enzyme xylulokinase (XK), in *S. cerevisiae* has low activity and is limiting in fermentation, causing it to happen an accumulation of xylitol inside the cell and thus leading to the need to excrete xylitol into the culture medium. Another factor has to do with oxygen limitation, causing the need to regenerate the cofactors present in the metabolic chain (NAD+) (Yu et al. 1995, Lee et al. 2003, Silva et al. 2019, Mouro et al. 2020, Nwinyi & Kalu 2021).

In the case of the yeast *S.stipitis*, the metabolic profile allows identifying that this yeast is able to metabolize D-xylulose (Figure 3), however, it preferentially consumed the D-xylose contained in the mixture. This preferential consumption of xylose may be related to a greater affinity of transporters for this pentose, since the capacity of yeasts to assimilate pentoses is strongly dependent on the transport proteins of the plasma membrane (Donzella et al. 2021). Also in Figure 3, it can be seen that the yeast *S. stipitis* produced 4.509 g/L of ethanol, a higher amount when compared to the fermentation using the *S. cerevisiae* monoculture (Figure 2). For the yeast *S. stipitis*, after 96h of cultivation, xylose reached a residual value of around 6 g/L and, after this period, ethanol began to be consumed. This also happened in experiments carried out by Silva et al. 2019, where fermentations with oxygen limitation showed that, before the total depletion of xylose (residual xylose concentrations less than 8 g/L) ethanol begins to be consumed. On the other hand, in the

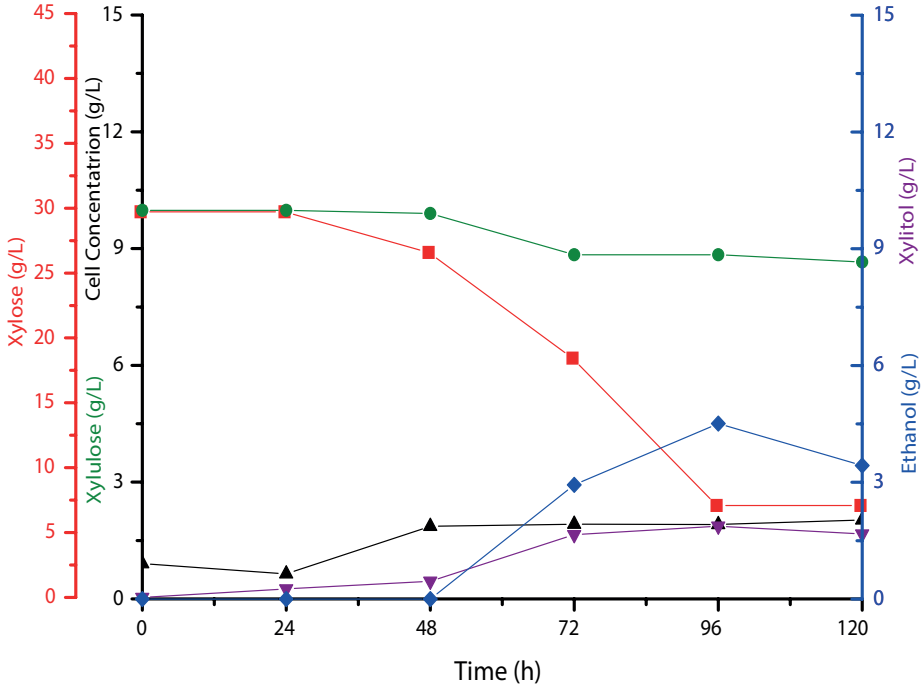

Figure 3. Growth in the YPX/X medium and metabolic profile of *S. stipitis*. (■) xylose, (●) xylulose, (♦) xylitol, (▼) ethanol) and (▲) cellular concentration. Operating conditions are 30ºC, pH 5.5 and 150 rpm.

cultivation of *S. stipitis*, the production of xylitol was also observed, reaching a concentration of 1.644g/L of xylitol, a lower amount when compared to *S. cerevisiae*. In the case of xylose metabolism in *S. stipitis*, there is also a need to regenerate the cofactors present in the metabolic chain (NAD+), leading to the accumulation of xylitol, but in comparison with *S. cerevisiae*, in *S. stipitis* the enzymes of this metabolic pathway present greater activity, leading to a lower accumulation of xylitol and a greater production of ethanol. This behavior is reflected in the xylitol yield ($Y_{xylitol}$/s) shown in Table I for the two yeasts. With regard to the yield of ethanol ($Y_{ethanol}$/s) in *S. cerevisiae*, it is higher even with lower ethanol production, due to being related only to the consumed xylulose, in the case of *S. stipitis*, even producing a greater amount of ethanol, it metabolized so much xylose how much xylulose, making the amount of substrate taken into account for the calculation of the factor greater.

Fermentation of the D-xylose/D-xylulose mixture using *S. cerevisiae*/*S. stipitis*.

Experiments using 50% of each of the yeasts were carried out. In Figure 4, the consumption and production profiles of metabolites can be observed. Note that the highest amounts of ethanol and xylitol were reached in 72 hours of fermentation. At this time, ethanol and xylitol concentrations reached 10.010g/L and 8.456 g/L respectively. After 72 hours of experiment, due to the low concentration of xylose and xylulose in the culture medium, a decrease in cell mass and consumption of metabolites produced for cell maintenance is observed. Table II presents a comparison of the concentrations

Table I. Yield parameters for monoculture of *S. cerevisiae* and *S. stipitis*.

S. cerevisiae		***S. stipitis***	
Yxilitol/s	Yetanol/s	Yxilitol/s	Yetanol/s
0,169	0,259	0,081	0,195

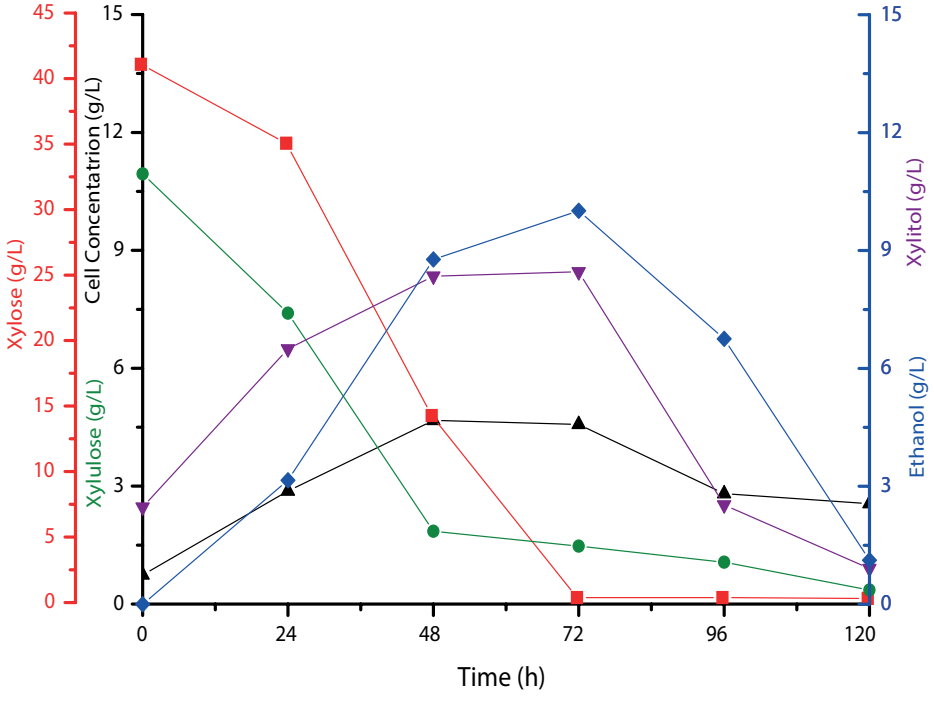

Figure 4. Growth in the YPX/X medium and metabolic profile of 50% *S. stipitis* - 50% *S. cerevisiae* co-culture. (■) xylose, (●) xylulose, (♦) xylitol, (▼) ethanol) and (▲) cellular concentration. Operating conditions are 30ºC, pH 5.5 and 150 rpm.

achieved for both fermentation products. The use of co-culture allowed an increase of 196.8% in the production of ethanol and 284.9% in the production of xylitol in relation to experiments using only the yeast *S. cerevisiae*. On the other hand, using only the yeast *S. stipitis*, the use of co-culture allowed an increase of 122% and 351.46% in the production of ethanol and xylitol, respectively.

From the productivity results of ethanol and xylitol compared in Figure 5 it is possible to verify that the use of co-culture significantly increased the concentration and productivity of ethanol compared to the results obtained in fermentations with yeast monocultures.

The use of co-culture allowed a 4-fold increase in ethanol productivity in relation to monoculture experiments with the yeast *S. cerevisiae* and of 3-fold in relation to monoculture experiments using *S. stipitis*. The synergy between the two yeasts is evident, since *S. cerevisiae* exclusively uses xylulose to produce ethanol and *S. stipitis* mainly uses xylose to produce ethanol, thus allowing both yeasts to produce simultaneously. It can also be seen in Figure 3 that the use of a yeast proportion of 50% led to the depletion of the two substrates present in the culture medium at approximately the same fermentation time, thus leading to an increase in productivity, since there is no period of fermentation where the two yeasts need to compete for the same substrate and there is no need for additional fermentation time for one of the yeasts to continue consuming the substrate until it is exhausted. This proportion of 50% was the condition that presented the best results when compared to the other yeast proportions tested (data not shown).

This type of results, where there is an improvement in the process when using co-culture of microorganisms, was also observed by other researchers. Sadoudi et al. 2012, tested the co-culture of *M. pulcherrima* and *S. cerevisiae* yeasts. In the research, the authors also observed a synergistic

Table II. Ethanol and Xylitol concentrations produced in YPX/X medium fermentations under conditions of 0% (*S. cerevisiae*), 50% (*S. stipitis*/*S. cerevisiae co-culture*) and 100% (*S. stipitis*).

Proportion of *S. stipitis* in the co-culture	0%	50%	100%
Ethanol concentration (g/L)	3,373	10,010	4,509
Xylitol concetration (g/L)	2,197	8,456	1,873

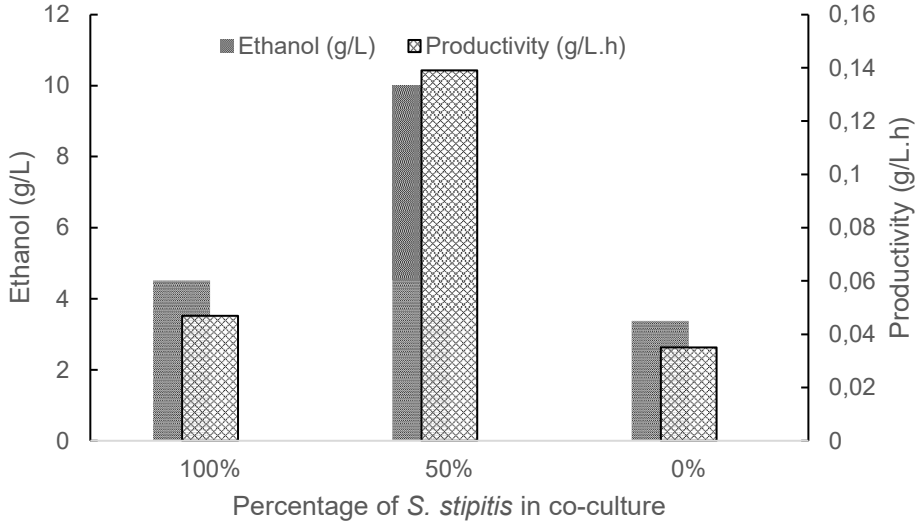

Figure 5. Comparison of productivity and ethanol concentration for monoculture (100% S. stipitis), co-culture (50% *S. stipitis* - 50% *S. cerevisiae*) and monoculture (100% S. cerevisiae). Operating conditions are 30ºC, pH 5.5 and 150 rpm.

effect on the production of aromatic compounds during alcoholic fermentation for wine production, because through the evaluation of the profiles of volatile compounds, they demonstrated that the production of aromatic compounds can be altered, in view that the entire metabolic pathway is affected by interactions between yeasts, which are complex and largely unknown. Rijswijck et al. 2017, tested the co-culture of *Saccharomyces cerevisiae* with yeasts such as *Cyberlindnera fabianii* and *Pichia kudriavzevii*. In the experiments, the authors showed the feasibility of using these yeast species in co-cultivation with *S. cerevisiae* and how their interactions in different proportions allowed adjusting the aroma profiles, as well as the final alcohol content of the beer.

Another type of interactions such as bacteria-yeast has been studied with promising results. Tusher et al. 2022, developed a system to produce bioethanol from phytomass, in this study the researchers using a recombinant yeast of *Kluyveromyces marxianus* and a recombinant strain of *Bacillus subtilis* showed that the bacteria-yeast co-culture gave a higher yield of ethanol from phytomass when compared to monoculture. Ferreira et al. 2022 also evaluated the performance of co-cultures composed of a strain of the bacterium *Lactiplantibacillus plantarum* with strains of the yeasts *Pichia kluyveri, Pichia guilliermondii* and *Debaryomyces hansenii* separately as probiotics in the production of plant-based fermented beverages. In the study, it was possible to observe that the sensory profile of the drink varied with the type of co-cultivation and that the co-culture of *D. hansenii* and *L. plantarum* showed greater antioxidant activity. Thus, as in this present study, these results show that co-culture approaches, even of the yeast-bacteria type, have shown potential and an alternative to improve the production process.

Other approaches for using the xylose present in the hemicellulose fraction of lignocellulosic materials are found in the literature, among which the use of genetically modified *S. cerevisiae* to be able to metabolize this pentose can be highlighted. Coimbra et al. 2023, studied recombinant strains derived from the industrial strain *S. cerevisiae* CAT-1 were developed by performing several genetic changes: insertion of genes involved in the assimilation and transport of D-xylose, genes for the metabolism of D-xylose via oxido -reducing, genes for the enzyme D-xylose isomerase, as well as extra copies of homologous genes for xylulose kinase and transaldolase, among other changes. Additionally, the researchers carried out an evolution process, in which after 10 subcultures, the modified CAT-1 strain consumed 74% D-xylose and managed to obtain an ethanol productivity of 0.095 g/L/h.

In another study, carried out by Dzanaeva et al. 2021, researchers evaluated the influence of transcription factors on xylose catabolism. *S. cerevisiae* strains with genetic alterations were evaluated in the regulation of growth and xylose fermentation. In the research it was possible to verify that the microorganism achieved a 1.8-fold increase in ethanol production from xylose when compared to the wild strain, reaching an ethanol productivity value equal to 0.16 g/L/h.

On the other hand, the study carried out by Rahmadhani et al. 2022, used mutants of *P. kudriavzevii* R-T3 and the industrial yeast *S. cerevisiae* BY4741 to ferment a mixture of glucose and xylose. In this study, the researchers tested monocultures and co-cultures in various proportions of inoculum of the two yeasts. In the research, it was possible to verify that the *P. kudriavzevii* R-T3 strain showed a low use of mixed substrate, but presented higher ethanol production than the *S. cerevisiae*. Additionally, they observed that in co-culture there was around a 30% reduction in ethanol production. In this study they managed to obtain productivity between 0.21 – 0.16 g/L/h, but using glucose in the mixture.

From the results obtained in the present work, it can be observed that the productivity obtained by the co-culture of wild microorganisms *S. stipitis*/*S. cerevisiae* (0.16 g/L/h) is competitive with those obtained in studies using genetically modified microorganisms. This approach is interesting, since working industrially with genetically modified microorganisms can bring some disadvantages such as: high cost and complex process, especially for small companies or countries with limited resources; unknown environmental and human impact, which may lead to facing resistance due to concerns about food safety, environmental impacts and ethical issues; need for regulatory policies for the use of this type of microorganisms (Wesseler et al. 2022).

Batch fermentation with immobilized *S. stipitis*/*S.cerevisiae* yeasts.

In order to define the necessary residence time for the carbon sources in the YPX/X medium to be consumed and ethanol to be produced inside the microbioreactor, a batch experiment containing immobilized cells was carried out.

For this, the co-culture *S. stipitis*/*S. cerevisiae* (50%/50%) was immobilized in pellets (according to section Immobilization of yeast co-culture), at the beginning of the experiment the cell viability was 94%, after fermentation the cell viability of the pellets reached 82%. From the batch using the co-culture with the immobilized cells, it was possible to determine the consumption time of most of the carbon sources present in the culture medium. This time allowed defining the residence time with which the microbioreactor should be operated in order to maximize the production of the metabolites of interest. The evolution of the concentration of the metabolites produced during the fermentation by the co-culture are shown in Figure 6. After 60 h the concentrations of the xylose and xylulose substrates reached a residual value of 1.042g/L and 0.279g/L respectively, and remained constant up to 80 hours of fermentation. In this fermentation, the main metabolites produced were ethanol and xylitol, which also reached their maximum concentrations after 60 h of fermentation, reaching 9.907g/L and 6.950g/L respectively. Comparing ethanol production using co-culture with free cells and co-culture with immobilized cells, it is observed that there is a small drop in ethanol production with a value of approximately 1.0%, caused by diffusional resistance, since the substrate

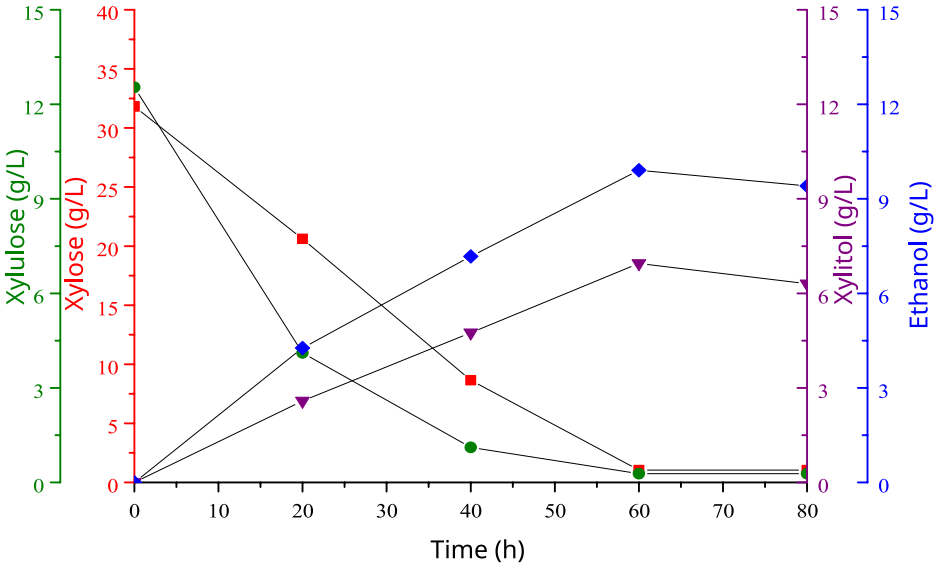

Figure 6. Co-culture of *S. stipitis*/*S.cerevisiae* (50/50) immobilized on calcium alginate pellets. Concentration of metabolites ((■) xylose, (●) xylulose, (♦) xylitol, (▼) ethanol) in fermentation of YPX/X medium in batch. Operating conditions are 30ºC, pH 5.5 and 150 rpm.

must diffuse through the pellet to reach the yeast. This value can be considered small, since in the research carried out by Oyaas et al. 1995 the researchers reported that the diffusivities in 2% Ca alginate granules were only 15% lower than those measured in water. Generally, diffusivity decreases with increasing molecular weight of the solute and with increasing polymer content, and in some carbohydrates it depends on the position of the carbonyl group (Venâncio & Teixeira 1997, Riley et al. 1999).

Thus, based on the experimental data of the batch fermentations with the immobilized co-culture, a residence time of 60 h was defined for the operation of the continuous microbioreactor.

Continuous fermentation with immobilized *S. stipitis*/*S.cerevisiae* co-culture using continuous 3D printing microbioreactor

In order to study the behavior of the immobilized co-culture system in a continuous regime, and thus perform a preliminary analysis of the possible implementation for ethanol production on a larger scale, a small-scale bioreactor was developed. The use of small-scale equipment makes it possible to carry out system studies using reduced amounts of substrates and cells, which leads to a reduction in costs and waste generated. The microbioreactor was built through 3D printing, this type of technology is currently used to manufacture miniaturized microfluidic devices and a wide variety of objects for use in the field of biotechnology, ranging from miniaturized culture chambers to lab-on-lab microfluidic devices a-chip for diagnostics, which are already being implanted in laboratories all over the world. Consequently, this type of recent developments shows that the use of 3D printing has potential for applications in biotechnology in the coming years (Shah et al. 2019, Heuer et al. 2021).

In Figure 7 shows the dimensions and geometry of the microbioreactor developed in the present study, the measurements are in the millimeter scale.

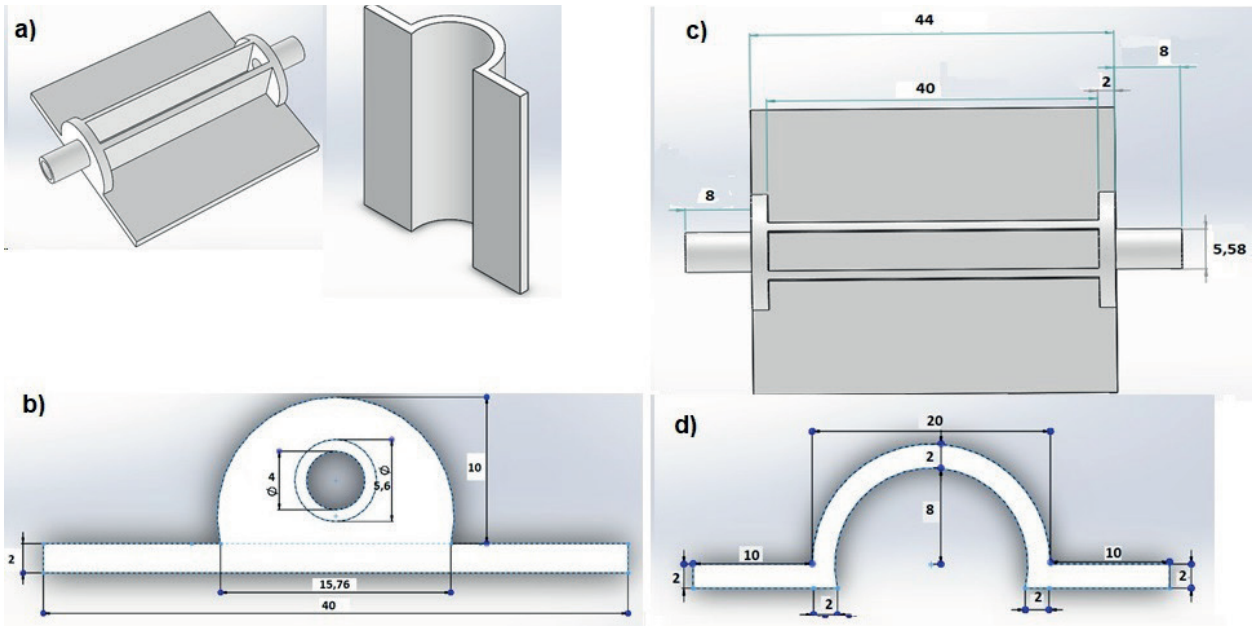

Figure 7. Perspective view of the continuous 3D printing microbioreactor: (a) bottom and top of the microbioreactor; (b) front view and bottom dimensions; (c) Top view and bottom dimensions. (d) Front and top view with top dimensions.

The microbioreactor was operated with a residence time of 60 h, adjusting the flow rate of the culture medium to 17 microliters per hour, since the useful volume of the microbioreactor is 900 microliters. Inside the microbioreactor, the immobilized yeasts are kept in a fixed bed, in this sense, part of the available volume inside the microbioreactor is occupied by the biocatalyst, thus leaving a useful volume of around 1 mL available for the liquid flow. Fermentations were carried out under the same conditions as in batch experiments. Figure 8 shows the concentration of metabolites for continuous fermentation using YPX/X medium and co-culture (50% *S. stipitis* – 50% *S. cerevisiae*) immobilized on pellets, performed in the 3D printing microbioreactor. The substrate concentrations in the culture medium and supplementation were 30.204 g/L and 12.190g/L for xylose and xylulose respectively, the continuous fermentation process using the microbioreactor was carried out during 168 hours of operation.

At the end of 60 hours of experiment, it was possible to reach a maximum production of 9.540g/L and 6.841g/L of ethanol and xylitol respectively, a concentration very close to the concentration observed in batch fermentation with immobilized cells. After 72 hours, substrate and product concentrations remained stable until the end of fermentation, thus characterizing this interval as the steady state of the experiment. Under these conditions, the system showed substrate conversion above 95% for 7 days (168h). In this experiment, the ethanol yield ($Y_{ethanol}/s$) was 0.227 g/g and the xylitol yield ($Y_{xylitol}/s$) was 0.163 g/g, close to the values obtained for monocultures.

There are few reports in the literature addressing continuous fermentation of xylose or the xylose/xylulose mixture as a substrate, however, Milessi et al. 2020 carried out a continuous fermentation using only xylose with a concentration of 65 g/L using yeast immobilized with the enzyme xylose isomerase using a 100 mL reactor, obtaining xylose conversion above 85% in 168h and 0.31$g_{ethanol}/L.g_{xylose}$. In the experiment carried out by the researchers, the system was able to operate for 168 h, with xylose conversion above 85%, but after this time the conversion showed a continuous drop until it

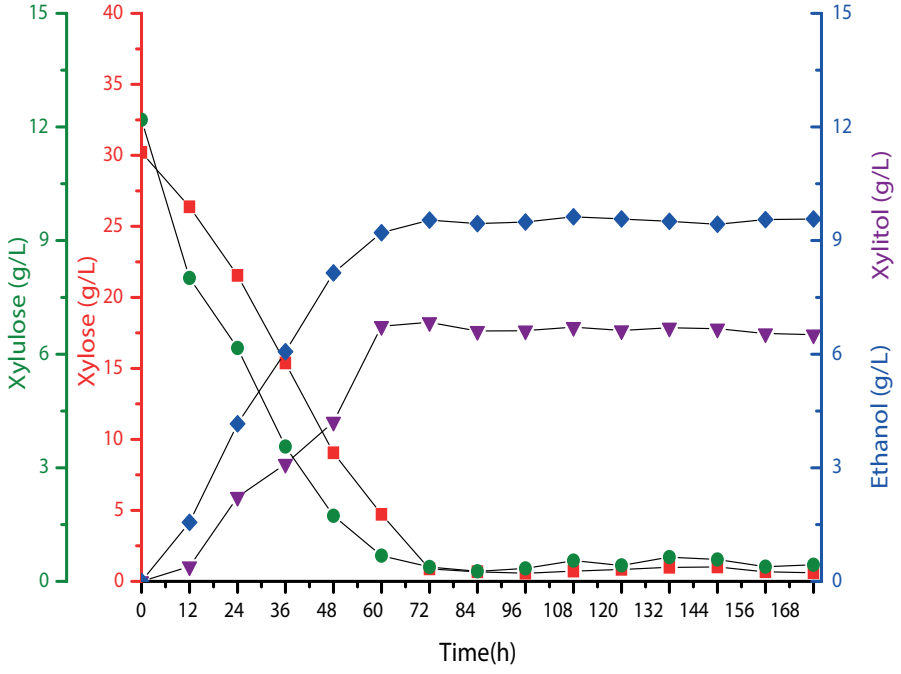

Figure 8. Concentration of metabolites in the continuous 3D printing microbioreactor in fermentation of YPX/X medium by immobilized co-culture (50% *S. stipitis* - 50% *S. cerevisiae*) (■) xylose, (●) xylulose, (♦) ethanol, (▼) xylitol. Operating conditions are 30ºC, pH 5.5, flow rate of 16 microliters per hour. pH 5.5, flow rate of 16 microliters per hour.

reached 66% after 264 hours of operation. Among the main products obtained, ethanol and xylitol were reported, with concentrations of 20 g/L and 12 g/L respectively. The results in the present study are similar to those obtained by Milessi et al. 2020, since the sugar concentration used in the present study was around half and, therefore, the concentration of the products obtained also with values of 9.5 g/L for ethanol and a little above 6 g/L for xylitol, however, in the approach of the present work, the process is easier to operate industrially. Additionally, the results obtained in the present study show that the use of the microbioreactor is an alternative for the study of biotechnological processes in general, using less amount of reagents and generating less amount of waste.

The process presented in this work is applicable to hemicellulosic liquors arising from pre-treatment processes of lignocellulosic raw materials and also to liquors originating from the hydrolysis stage of the cellulose fraction of these materials. Some authors used the yeast *S. stipitis* to carry out the fermentation of hemicellulose liquor resulting from the hydrolysis of lignocellulosic raw materials for the production of ethanol, showing that the yeast present in the biocatalyst of this work can ferment this type of raw material (Silva et al. 2019, Deshavath et al. 2018, Brito et al. 2018). Operationally, the liquors obtained from hydrolyzed lignocellulosic raw materials must first go through a filtration process to avoid clogging of the stainless steel screen that is responsible for confining the biocatalyst inside the microbioreactor.

CONCLUSION

Monocultures using *S. stipitis* showed that this yeast can metabolize D-xylulose, however it presents preferential consumption by D-xylose. The use of *S. stipitis*/*S. cerevisiae* allowed a synergistic action for the fermentation of the two substrates (xylose - xylulose) showing to be a process with potential for the production of 2G ethanol using the hemicellulose fraction of lignocellulosic materials. The microbioreactor developed using additive manufacturing (3D printing) showed good performance, allowing the use of small amounts of substrates, generating little waste and keeping the cells confined within the equipment. Finally, the continuous fermentation system using the microbioreactor developed in this work can be adapted for the production of other products of interest, and specific modifications can be made to the equipment thanks to the flexibility offered by additive manufacturing technology.

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PEDRO HENRIQUE F. RODRIGUES[1]
https://orcid.org/0009-0005-5545-6878

ELIZABETH G. DA SILVA[1]
https://orcid.org/0000-0002-2658-3756

ALEX S. BORGES[1]
https://orcid.org/0000-0002-2746-6906

GABRIEL LUIS CASTIGLIONI[2]
https://orcid.org/0000-0001-6941-148X

CARLOS ALBERTO G. SUAREZ[1]
https://orcid.org/0000-0002-6307-2031

INTI DORACI C. MONTANO[1]
https://orcid.org/0000-0001-8509-0911

[1]Universidade Federal de Goiás, Instituto de Química, Av. Esperança, s/n, Chácaras de Recreio Samambaia, 74690-900 Goiânia, GO, Brazil

[2]Universidade Federal de Goiás, Departamento de Engenharia de Alimentos, Av. Esperança, s/n, Chácaras de Recreio Samambaia, 74690-900 Goiânia, GO, Brazil

Correspondence to: **Inti Doraci Cavalcanti Montano**
E-mail: inti@ufg.br

Author contributions

Pedro Henrique Rodrigues: Data curation, Writing - original draft, Investigation, Software. Elizabeth Gonçalves da Silva: Data curation, Investigation. Alex Souza Borges: Software and Arduino-controlled automatic feeding system development. Gabriel Luis Castiglioni: analysis and quantification of samples. Carlos Alberto Galeano Suarez: Resources, Visualization, Formal analysis, Writing, Acquisition of the financial support. Inti Doraci Cavalcanti Montano: Conceptualization, Methodology, Supervision, Acquisition of the financial support for the project leading to this publication, Writing - review & editing, Project administration.