REPEATED BATCHES OF SUGARCANE BAGASSE CONSOLIDATED BIOPROCESSING IN A VORTEX BIOREACTOR USING MAGNECTIC IMMOBILIZED RECOMBINANT YEAST

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ABSTRACT

Consolidated bioprocessing (CBP) of biomass is an emerging technology where enzyme production, hydrolysis and fermentation of biomass occur in a single vessel. Cell recycling, on the other hand, is an important approach to achieve process feasibility, but the difficulty of separating and recovering cells from the remaining solid biomass after CBP makes it challenging. Therefore, the present work aimed to evaluate the operation of the CBP of hydrothermally pretreated sugarcane bagasse in repeated batches using a recombinant yeast strain immobilized in magnetic alginate beads. The CBP was carried out at 35ºC in a vortex flow reactor with a reaction volume of 220 mL using initial optical density (OD) of 10, pH 5.5, and solid content of 1% w/v. Following each experimental batch, the magnetic beads were retrieved using a magnet and a new batch was started. The conversion rate increased 2-fold during the batches due to yeast adaptation to the industrial medium, reaching an ethanol productivity of 0.47 g/L/h in the third batch. Notably, it was possible to perform repeated batches in the presence of residual solids with 100% of cell recovery and reuse in the CBP at industrial conditions

Keywords: Consolidated bioprocessing 1. Cell recycles 2. Cell immobilization 3. Vortex bioreactor 4.

1 INTRODUCTION

The development of biorefineries that utilize the entire biomass through a diverse array of technologies for the sustainable production of biofuels, bioenergy, and chemicals represents the most promising strategy for the transition of the global matrix from fossil processes to sustainable ones[1]. However, technological advancements are still necessary for the feasibility and implementation of 2G technologies on a large scale, particularly in the light of the lack of cost-effective technologies to overcome the recalcitrance of biomass structure. Ideed, the high cost of the enzyme cocktails required for the release of fermentable sugars from cellulose and hemicellulose represents a significant impact on the overall cost-effectiveness of the biomass processing[2].

The consolidated bioprocessing (CBP) of biomass is a promising technology where the production of hydrolytic enzymes, hydrolysis of biomass and fermentation occur simultaneously in the same bioreactor. This is achieved by using a microorganism or a consortium that produces hydrolytic enzymes, eliminating the need for the addition of commercial enzymes[3]. In order for a biorefinery to be feasible, it is necessary to utilize all components of biomass. Therefore, a robust CBP process requires a microorganism that is not only capable of producing enzymes but is also capable of fermenting both sugar fractions produced after the hydrolysis of lignocellulosic biomass (hexoses and pentoses). This has stimulated the development a microorganism suitable for application in CBP[2,4]. In this context, the yeast *Saccharomyces cerevisiae* AC14 developed by Claes et al.[5] stands out due to its ability to consume xylose and secrete seven hydrolytic enzymes, achieving promising yields and productivities in previous works[3,6]. However, the use of free cells during CBP may impair cell recycling due to the difficulty in separating and recovering cells from the remaining solid biomass. This issue is not exclusive to CBP; it also pertains to all technologies that use microorganisms along with solid biomasses, such as simultaneous saccharification and fermentation (SSF)[6].

One potential solution to this operational issue is the immobilization of microorganisms. This approach allows for the easy recovery of cells and increases biocatalyst stability due to the protective effect of the immobilization matrix[6]. Among the available technologies for yeast immobilization, the entrapment in calcium alginate gel is a widely used method due to its biocompatibility and biodegradability [7]. Furthermore, the use of magnetic particles as immobilization support makes biocatalyst recovery easier and more efficient[8].

The use of heterogeneous catalysts with solid biomass presents diffusional limitations that can impair the enzyme diffusion and its access to the biomass polymers. Therefore, the bioreactor choice is a crucial factor in ensuring bioprocess homogenization and good mass transfer rates. Additionally, the shear stress must be controlled to avoid disruption of immobilized biocatalyst. In this context, the use of a vortex flow reactor, based on the Taylor-Couette flow generated between two concentric cylinders, is seen as an interesting alternative due to its low shear stress while the vortices ensure a gentle but efficient agitation[9].

The objective of the present study was to demonstrate the feasibility of recycling cells during sugarcane bagasse CBP, performed in repeated batches using magnetic immobilized cells and a low-shear stress vortex bioreactor. This approach represents a successful proof-of-concept for cell recovery from the residual solid after biomass hydrolysis.

2 MATERIAL & METHODS

Microorganism and inoculum: The *S. cerevisiae* AC14 strain is a xylose-fermenting inhibitor-tolerant industrial yeast that can secrete endoglucanase, cellobiohydrolase I and II, β-glucosidase, xylanase, β-xylosidase and acetyl-xylan esterase[5]. The inoculum preparation was conducted in accordance with the methodology described by Ramos et al.[6], where a loop of the stock culture was spread in YP-CBP solid agar medium (20 g/L peptone, 10 g/L yeast extract, 15 g/L agar, 20 g/L glucose, 20 g/L xylose, 10 g/L corncob xylan, 10 g/L cellobiose and 5 g/L carboxymethylcellulose) and incubated at 30 ◦C for 48 h. A single colony from the plate was resuspended in 75 µL sterile distilled water and spread with a Drigalski loop onto a new YPDX-agar solid medium plate (20 g/L peptone, 10 g/L yeast extract, 15 g/L agar, 20 g/L glucose and 20 g/L xylose) and incubated at 30ºC for 24 h. The resulting "cell carpet" formed in the Petri dish was completely resuspended and inoculated into 300 mL of YPDX medium (YPDX-agar without agar) in 1 L baffled Erlenmeyer flasks and incubated for 12 h at 30 ºC and 150 rpm. Yeast cells in the exponential growth phase were recovered by centrifugation (2500 rpm for 10 min at 4ºC) and immediately immobilized.

Magnetic biocatalyst preparation: The immobilization of yeast by entrapment in Ca-alginate gel was performed in accordance with the procedure previously described by Ramos et al. [6]. A cell suspension (10% w/w) containing 1% w/w of sodium alginate and 25% w/w of magnetic microparticles was dropped into a coagulation solution (0.25 M of $CaCl_2$) to form microspheres (beads) which were then kept at 5 ºC for 12 h in a hardening solution (20 g/L peptone and 10 g/L yeast extract, pH 5.5).

CBP repeated batches: The assays were conducted at 35ºC in a vortex flow reactor with a reaction volume of 220 mL. The magnetic biocatalysts (OD=10) were added together with the liquid (pH 5.5) and solid (1% w/v) fractions of the hydrothermally pretreated sugarcane bagasse obtained in conditions previously defined[3]. After the end of each batch, the beads were retrieved using a magnet and new liquid and solid fractions were added. Samples were collected at regular intervals to quantify the substrates and products.

Analytical methods and calculations: The enzyme activities of cellulases and hemicellulases were performed as described by Ramos et al.[6], whereby the hydrolysis of Whatman No. 1 filter paper, and birchwood xylan, respectively, at pH 5.5 and 50ºC. The concentration of free cells (Cx) was determined by turbidimetry, while yeast viability was quantified by the methylene blue methodology. The concentrations of ethanol, glycerol, xylitol, xylose and glucose were quantified by high performance liquid chromatography (HPLC). The fermentative parameters (ethanol productivity - QP; substrate conversion - X and ethanol yield – Y,%) were calculated as described in Ramos et al.[6] The Tukey test (95% confidence level) was performed in Origin®Pro 8.5 software to evaluate the significant differences between the results.

3 RESULTS & DISCUSSION

The conversion during the repeated batches and the production of enzymes after each batch are shown in Figure 1, while process parameters of each batch are presented in Table 1. It can be observed that the conversion rate and productivity increased from the first to the subsequent batches, achieving similar ethanol yields in half the time (12h). This may be attributed to yeast adaptation to the industrial medium, with ethanol yields (Y%) remaining at approximately 50% of the maximum theoretical ethanol produced.

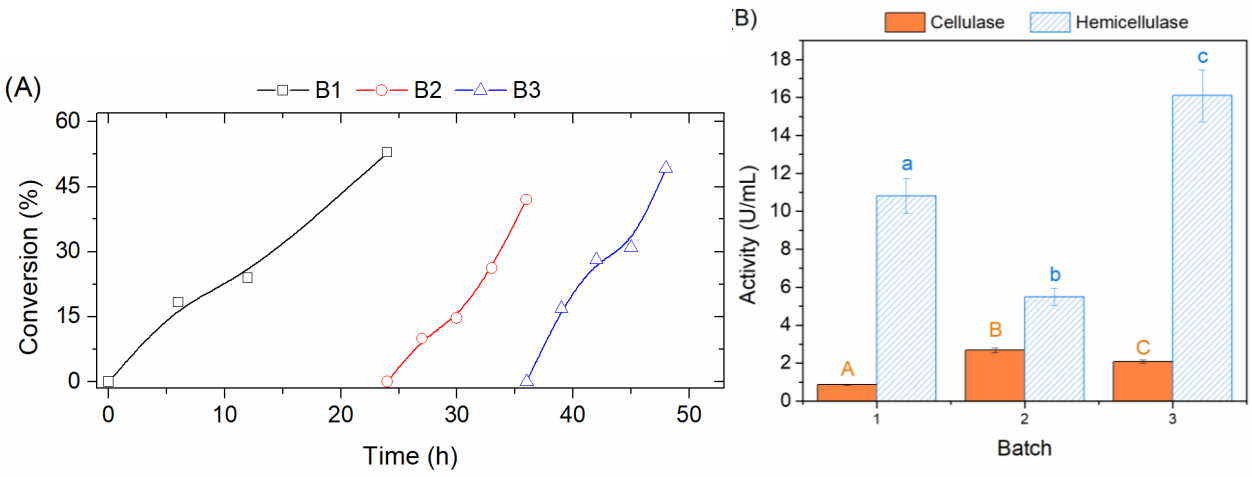

Figure 1 Repeated batch of the CBP of sugarcane bagasse in a vortex bioreactor using AC14 yeast immobilized in Ca-alginate magnetic particles (35ºC, pH 5.5, OD 10, 1% w/v solid). (A) Process conversion profile where B1, B2 and B3 means each repeated batch performed and (B) Cellulase and hemicellulase activities in each batch. Tukey's test was performed separately for each parameter and different letters mean that values are significantly different (95% of confidence).

Table 1 Fermentation parameters in the repeated bacthes of the CBP of sugarcane bagasse in a vortex bioreactor using AC14 yeast immobilized in magnetic particles (35ºC, pH 5.5, OD 10, 1% w/v of pretreated bagasse).

Batch	Ethanol (g/L)	Qp (g/L/h)	Y (%)	Viability (%)
B1	6.1	0.25	52.8	94
B2	4.8	0.40	41.9	95
B3	5.7	0.47	49.2	95

*Qp- ethanol productivity, Y – percentage of the maximum ethanol that can be produced

In a study conducted by Ramos et al.[10], 10 repeated batches of 12h in duration were performed using the AC14 strain in its free form, resulting in conversions above 80%. However, it is important to note that the authors used free cells and synthetic medium (without the presence of solids). In the present work, the lower process rate can be attributed to the complexity of the substrate and diffusional limitations due to biocatalyst immobilization, which lead to lower conversion (approximately 50%), that could be overcome by applying longer processes and higher cell loads. Indeed, the higher productivities achieved by Perez et al[3] (1.9 g/L/h) and by Ramos et al.[6] (1 g/L/h) in previous works with the AC14 yeast in batch experiments can be attributed to the use of synthetic medium and free cells.

It is also noteworthy that the enzyme produced and secreted into the medium exhibited considerable variation across the three batches, which could attributed to diffusional effects within the support gel matrix, whereby enzymes must pass through the gel diameter to reach the medium, as previously observed by Ramos et al. [6]. However, a notable achievement is that the agitation provided by the vortex bioreactor had no adverse impact on yeast viability, maintaining it at approximately 95% and preserving the beads integrity.

There is a paucity of literature on repeated batches of CBP. The few existing works generally report a lengthy duration for each cycle and utilize synthetic media. Liu et al. [4] studied a yeast that secretes cellulases (β-glucosidase, endoglucanase and cellobiohydrolase I). Using 10 g/L of phosphoric acid-swollen cellulose as the raw material they conducted three cycles of 96 h each. Matano et al. [2] performed five cycles of 72 h with a strain of *S. cerevisiae* that produces only cellulolytic enzymes (β-glucosidase, endoglucanase and cellobiohydrolase II). In this case, pretreated rice straw (200 g/L) was used as substrate. However, the authors had previously performed a liquefaction treatment on the solid biomass using commercial enzymes. Furthermore, the authors recovered the cells through a two-step centrifugation process, with the first step conducted at low gravity and the second at high gravity. This approach inevitably resulted in the transfer of the residual solids along with the cells to the subsequent batch, leading to a cell recovery of only 50%.

In this context, the effective reuse of AC14 cells in the present work using a real industrial medium comprising complex polysaccharides, without the use of commercial enzymes, demonstrates that this yeast possesses significant characteristics that make it a valuable candidate for application in CBP at industrial conditions. Most notably, it was possible to perform repeated batches in the presence of residual solids with 100% of cell recovery from one batch to another, overcoming the challenge of cell recovery in the presence of solid substrates derived from agro-industrial processing.

4 CONCLUSION

The immobilization of yeast in magnetic beads allowed the complete recovery of cells in the presence of solids after a CBP and its operation in repeated batches. However, mass transfer limitations due to the presence of immobilization support reduced the process rate, which can be overcome by longer batches and higher cell loads. The use of a vortex bioreactor can provide good homogenization without compromising cell viability and beads integrity. Overall, the technology reported here has demonstrated considerable potential and may be extended to other process (such as SSF) and bioproducts of biorefineries other than bioethanol.

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ACKNOWLEDGEMENTS

The authors would like to thank the São Paulo Research Foundation (FAPESP) - grants #2016/10.636-8, # 2022/11189-6 and # 2023/15508-1, and the Brazilian National Council for Scientific and Technological Development (CNPq) – process 407716/2021-1. This work was carried out with the support of CAPES, a Brazilian Government entity focused on the training of human resources (Finance code 001).