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Effective application of immobilized second generation industrial *Saccharomyces cerevisiae* strain on consolidated bioprocessing

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ABSTRACT

Integrated bioprocessing strategies can facilitate ethanol production from both cellulose and hemicellulose fractions of lignocellulosic biomass. Consolidated bioprocessing (CBP) is an approach that combines enzyme production, biomass hydrolysis and sugar fermentation in a single step. However, technologies that propose the use of microorganisms together with solid biomass present the difficulty of the recovery and reuse of the biocatalyst, which can be overcome by cell immobilization. In this regard, this work applied immobilized cells of AC14 yeast, a recombinant yeast that secretes 7 hydrolytic enzymes, in the CBP process in a successful proof-of-concept for the enzyme access to the substrate polymers. The most appropriate cell load for CBP under the conditions studied with immobilized cells was selected among three optical densities (OD) 10, 55 and 100. These experiments were performed with free cells to ensure that the results were not biased by mass limitations effects. OD 10 achieved 100% of the sugar consumption and the higher specific production of enzymes, being selected for further studies. Diffusional effects were observed with immobilized cells under static conditions. However, mass transfer limitations were mitigated under agitation, with an 18.5% increase in substrate consumption rate (from 2.7 to 3.5 g/L/h), reaching the same substrate uptake rates as free cells. In addition, immobilized cells achieved 100% hydrolysis and consumption of all substrates offered within only 12 h. Overall, this is the first report of a successful application of immobilized yeast cells in CBP processes for bioethanol production, a promising technology that can be extended to other biorefinery bioproducts.

Introduction

Biomasses are considered as the most foreseeable raw materials for the transition of the global matrix from fossil processes to sustainable ones [1]. Their energy potential is closely related to the lignocellulosic structure of biomass, which is mainly composed of polymers (cellulose and hemicellulose) that can be made available as carbon sources for the production of biofuels and valuable bioproducts through second generation (2 G) biochemical processes in biorefineries [2]. Brazil produces a large amount of biomass annually, in particular derivatives from sugarcane processing (sugarcane bagasse and straw), having a considerable potential to contribute to the world's primary energy needs and to the development of biorefineries [3]. Currently, most of the Brazilian biorefineries co-produce first generation (1 G) bioethanol, sugar and energy [4], which makes 2 G bioethanol a natural biofuel to be incorporated in these plants once an integrated 1 G/2 G bioethanol

Abbreviations: 1 G, first generation; 2 G, second generation; CBP, Consolidated bioprocessing; DNS, 3,5-Dinitrosalicylic acid; FPU, filter paper unit; GOD-PAP, enzymatic colorimetric glucose determination after enzymatic oxidation by glucose oxidase; HPLC, high performance liquid chromatography; OD, optical densities; p-NPX, 4-nitrophenyl β-D-xylopyranoside; SSF, simultaneous saccharification and fermentation; U, unit of activity; YP-CBP, medium composed of peptone, yeast extract, glucose, xylose, corncob xylan, cellobiose and carboxymethylcellulose; YPDX, medium composed of peptone, yeast extract, glucose and xylose.

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production process can potentially improve bioprocess economics [5]. However, technological developments are still required for the feasibility and implementation of 2 G technologies on a large scale in these plants [3].

One of the main technological challenges for the widespread use of biomass in 2 G plants is the lack of sustainable and cost-effective technologies to overcome the recalcitrant biomass structure [1]. The most commonly used approach to overcome biomass recalcitrance and to depolymerize cellulose and hemicellulose into predominantly glucose and xylose, respectively, involves a biomass pretreatment step that is required to alter the biomass structure. These changes in biomass structure are critical to facilitate enzyme access to fermentable sugars during the next step, which comprises enzymatic hydrolysis of the remaining pretreated solid fraction and requires the use of expensive commercial hydrolytic enzymes cocktails [6,7].

In this context, the consolidated bioprocessing (CBP) of biomass emerges as a promising technology where the production of hydrolytic enzymes, hydrolysis of biomass and fermentation of sugars take place sequentially and simultaneously in the same bioreactor once a microorganism or a consortium that produces hydrolytic enzymes is used [8,9]. This system has the advantage of not requiring the addition of commercial enzymes in the bioreactor, which significantly contributes to the feasibility of the bioprocess due to the high cost of enzyme cocktails [9,10]. In addition, osmotic stress and feedback inhibition of hydrolyzing enzymes due to high sugar concentrations in biomass hydrolysates are avoided once fermentable sugars are immediately converted to ethanol upon release [11].

Considering that the feasibility of industrial 2 G plants and biorefineries requires the complete utilization of all biomass components [12,13], CBP requires a microorganism that is not only capable of producing enzymes and fermenting both sugar fractions produced after lignocellulosic biomass hydrolysis (hexoses and pentoses), but is also tolerant to ethanol and hydrolysate inhibitors. However, the efficient consumption of xylose by wild strains of *S. cerevisiae* is still a challenge, which has stimulated synthetic biology and genetic engineering tools to develop a microorganism suitable for application in CBP [14–16]. However, low ethanol titers and long fermentation times with low ethanol productivity have been reported [17–19].

In this scenario, the yeast *Saccharomyces cerevisiae* AC14 developed by Claes et al. [20] stands out due to its ability to consume xylose and secrete seven hydrolytic enzymes: β-glucosidase, endoglucanase, cellobiohydrolase I and II, β-xylosidase, xylanase and acetylxylan esterase. In a first study with this strain, promising results were obtained [21], achieving ethanol productivities of 4.46 g/L/h and 1.86 g/L/h in synthetic and real sugarcane bagasse industrial media, respectively. In this report, the authors used free cells of the AC14 yeast, which may impair cell recycling between batches due to the difficulty in separating and recovering cells from the remaining solid biomass. This issue relates not only to CBP but to all technologies that propose the use of microorganisms together with solid biomasses, such as simultaneous saccharification and fermentation (SSF) processes [22].

To improve this operational issue, the immobilization of microorganisms is an interesting alternative, which allows the easy recovery of cells and can also increase the stability of the biocatalyst due to the protective effect of the immobilization matrix, thus increasing the efficiency of the process [23]. Among the available technologies for yeast immobilization, the entrapment in calcium alginate gel is widely used due to its biocompatibility and biodegradability [23,24]. The use of Ca-alginate immobilized cells has been well studied in the literature in a variety of bioprocesses, including the production of 2 G ethanol using recombinant yeasts [25–27]. However, to the best of our knowledge, no reports in the literature have applied immobilized cells in the CBP of biomass. In fact, there are some concerns for the use of heterogeneous catalysts with solid biomass, once diffusional limitations could impair the enzyme diffusion and its access to the biomass polymers.

Taking advantage of AC14 yeast high enzyme production and potential as a 2 G ethanol producer, the present work reports, for the first time, the application of immobilized cells in CBP process, in a successful proof-of-concept for the enzyme access to polymers substrate, efficient hydrolysis and ethanol production using a heterogeneous system as shown in Fig. 1.

Materials and methods

Materials

Yeast extract was purchased from Fisher Bioreagents (Mexico City, Mexico), peptone was kindly donated by Kerry (Campinas, Brazil). Sodium alginate, cellobiose and 4-nitrophenyl β-D-xylopyranoside (p-NPX) were purchased from Sigma-Aldrich (Burlington, USA; Gillingham, UK; Jerusalem, Israel, respectively). Corn cob xylan and beechwood xylan were purchased from Carl Roth (Karlsruhe, Germany). Filter paper was purchased from Cytiva (Shanghai, China). Carboxymethylcellulose was provided by Synth (Diadema, Brazil) and GOD-PAP reagent by Bioclim (Belo Horizonte, Brazil). All other reagents were of analytical grade.

Microorganism and inoculum

The *S. cerevisiae* AC14 strain developed in [20] is a xylose-fermenting inhibitor-tolerant industrial yeast obtained by sequential genomic integration of seven heterologous genes encoding seven lignocellulolytic enzymes by using the CRISPR-Cas9 method. All the genetic modifications made in yeast chromosomes can be accessed in details in [20]. The recombinant yeast can express and secrete endoglucanase, cellobiohydrolase I and II, β-glucosidase, xylanase, β-xylosidase and acetyl-xylan esterase, as mentioned above. In all experiments, inoculum preparation followed a procedure adapted from [25], where a loop of the stock culture (stored at −80 °C) 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 30°C for 24 h. The “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 °C and 150 rpm. Yeast cells in exponential growth phase were recovered by centrifugation (2500 rpm for 10 min at 4°C) and immediately immobilized.

Cell immobilization

Immobilization by entrapment in Ca-alginate gel was performed according to the procedure described previously [25]. A cell suspension (10% w/w) containing 1% w/w of sodium alginate was dropped into a coagulation solution (0.25 M of $CaCl_2$) to form microspheres (beads) of ~3.5 mm. At the end of immobilization, the microspheres were kept at 5 °C for 12 h in a hardening solution (20 g/L peptone and 10 g/L yeast extract, pH 5.5).

CBP experiments

CBP experiments were performed in 5 mL mini-reactors equipped with CO_2 outlet, as described in [28] (Fig. 2). The process was monitored by measuring the mass of CO_2 produced during the fermentation. The experiments were terminated when mass stabilization was reached, indicating the end of fermentation. The CBP medium was composed of yeast extract (10 g/L), peptone (20 g/L), glucose (5 g/L), xylose (5 g/L), cellobiose (5 g/L), corncob xylan (5 g/L), and carboxymethylcellulose (2.5 g/L).

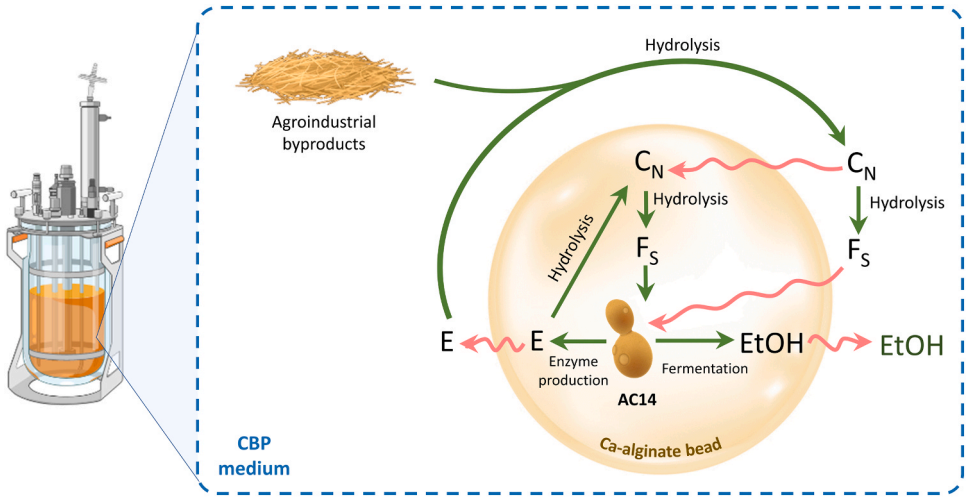

Fig. 1. – Scheme of consolidated bioprocessing (CBP) of biomass using Ca-alginate immobilized *S. cerevisiae* AC14 yeast, where red lines represent steps possibly limited by diffusion; EtOH (ethanol); E (hydrolytic enzymes); C_N (small oligomers capable of crossing gel pores); and F_S (fermentable C5 and C6 sugars).

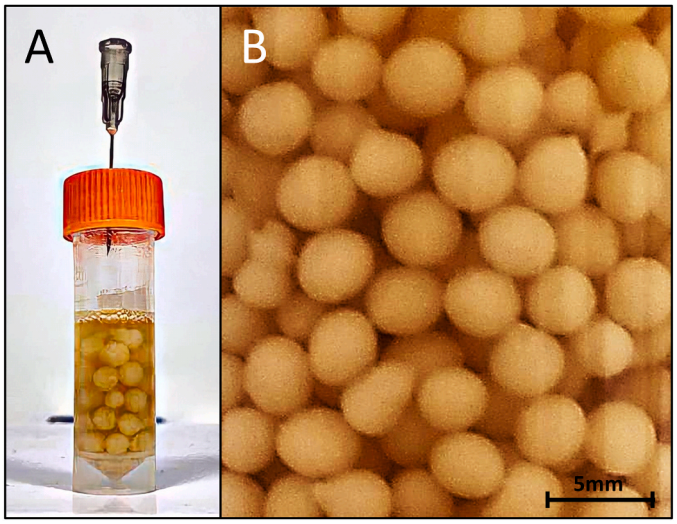

Fig. 2. Mini-reactor used for CBP studies with immobilized cells (A) and beads of Ca-alginate immobilized cells (B).

The first set of experiments was carried out with three different cell loads (Optical Density – OD 10, 55 and 100) in order to select the most appropriate cell load for CBP studies. These experiments were performed with free cells to ensure that mass transfer limitations did not affect the results. Once the cell load was selected, a second set of CBP experiments were performed with immobilized cells at the same cell load (g_{dcw}/mL) in the mini-reactors incubated statically and under agitation (250 rpm) to access the influence of cell immobilization on substrate consumption and product formation and consequently on process efficiency. All experiments were performed at 35 °C, 4 mL of reaction volume and pH 5.5. At the end of the experiments, the medium was recovered (centrifuged or filtered for free and immobilized cells, respectively) and the supernatant obtained was characterized as enzymatic extract as well as regarding its composition in substrate and products. All experiments were performed under aseptic conditions.

Analytical methods

Cell concentration and viability

The concentration of free cells (Cx) was determined by turbidimetry in a spectrophotometer (Bioespectro SP-22/China), at 600 nm, and correlated with the dry weight of the cells (g_{dcw}/L) using a corresponding previously established calibration curve. Yeast viability was quantified by the methylene blue method followed by counting in a Neubauer chamber [27]. To assess the viability of immobilized cells, beads were dissolved in sodium citrate 8% (w/v) at a proportion of 100 mg_{beads}/mL under vortex agitation at room temperature, according to [25]. Non-viable cells were those that stained blue and viable cells remained clear. Cell viability was defined as the ratio of viable cells to total cells (viable and non-viable) counted in a defined space of the counting chamber.

Enzymatic activities

The enzymatic extracts obtained at the end of the CBP experiments were characterized in respect to their activities in cellulase, xylanase, β-glucosidase and β-xylosidase.

Total cellulase activity was measured by a method adapted from [29], based on the release of glucose from Whatmam No. 1 filter paper (15 mm diameter) by the action of the enzymatic extract at pH 5.0 (50 mM citrate buffer), 50 °C for 1 h. The reducing sugars released were quantified by the DNS method [30]. One unit of activity (U) was the amount of enzyme required to release 1 μmol of glucose in 1 min under the assay conditions. A control was performed by replacing the enzyme with distilled water.

The enzymatic activity of β-glucosidase was measured by a method adapted from [31], which quantifies the release of glucose from cellobiose at 50°C, pH 5.5 (50 mM citrate buffer) for 15 min. The samples were heated to 100 °C for 10 min in order to stop the reaction. The released glucose was then quantified using the commercial enzyme kit GOD-PAP method. One unit of activity (U) was the amount of enzyme required to release 1 μmol of glucose in 1 min under the assay conditions. A control was performed by replacing the enzyme with distilled water.

Xylanase activity was measured according to [32], based on the release of xylose from birchwood xylan at 50°C and pH 5.5 (50 mM citrate buffer). Aliquots were taken at intervals of 2 min to determine the rate of product formation (dP/dt) and the reducing sugars were quantified by the DNS method [30]. One unit of activity (U) was the amount of enzyme necessary to release 1 μmol of xylose in 1 min under the experimental conditions. The control was the aliquot taken at time zero.

The enzymatic activity of β-xylosidase was measured by the method adapted from [33], based on the increase in absorbance at 405 nm caused by the release of 4-nitrophenol ($\varepsilon = 1950$ mol/L/cm) during the hydrolysis of p-NPX at 50°C and pH 6.0 (50 mM phosphate buffer). One unit of activity (U) was the amount of enzyme required to release 1 μmol

of 4-nitrophenol in 1 min under the assay conditions. A control was performed with a sample prior to before incubation.

Eq. (1) was used to calculate all enzymatic activities, where (dC_P/dt) is the rate of product formation (mg/mL/min); V_R is the reaction volume (mL); D is the dilution of the enzymatic extract; MM is the molar mass of the product (mg/μmol); and V_E is the volume of enzyme extract used. Enzyme volumetric activities were expressed in U/mL, while enzyme specific activities were expressed in U/g_{dcw}, indicating how efficient cells are in secreting enzymes, once it relates the amount of enzyme secreted per mass of cells used.

$$Activity(U/mL) = \frac{\left(\frac{dC_P}{dt}\right) \times V_R \times D}{MM \times V_E} \quad (1)$$

Quantification of substrates and products concentrations

The concentrations of ethanol, glycerol, xylitol, acetic acid, xylose and glucose were quantified by high performance liquid chromatography (HPLC) on a Waters e2695 chromatograph equipped with refractive index and UV–VIS detectors (λ = 210 nm) with a RezexTM ROA-Organic acid H+ ion exclusion column at 65ºC with 5 mM H_2SO_4 as eluent at a flow rate of 0.6 mL/min [34]. Prior to injections, the samples were frozen for at least 24 h, centrifuged twice at and filtered through a 0.22 μm filter to remove insoluble solids as described in [34].

Quantification of total oligomers

The total concentration of oligomers remaining from CMC and xylan after CBP, representing the unhydrolyzed polymer fraction, was quantified by total hydrolysis of 5 mL samples with 174 μL of sulfuric acid 72% at 121 °C for 1 h [35]. After the acid hydrolysis, the samples were cooled, neutralized with $CaCO_3$ and analyzed by HPLC. The total concentration of oligomers was estimated by the difference between the concentration of sugars obtained after the hydrolysis and its initial concentration before the acid hydrolysis.

Calculations and statistical analysis

Fermentative parameters (ethanol productivity - Q_P; substrate conversion - X and substrate consumption rate - r_S) were calculated according to [36]. The xylose consumption rate (r_S, g/L/h) was calculated according to the Eq. (2), by fitting a polynomial to the xylose concentration (C_S) over time curve and obtaining the tangent of the curve (dC_S/dt) through derivation of the polynomial, as also described by [23] and [34].

$$r_S = -dC_S/dt \quad (2)$$

Ethanol yields (Y, %) were calculated according to Eq. (3), where the theoretical maximum ethanol was estimated according to the stoichiometric factors for mono-, di- and polysaccharides (0.511, 0.538 and 0.57 g/g, respectively) [37]. The specific enzyme activity (U/g_{dcw}) was calculated by dividing the measured enzyme activity by the dry weight of the cell load used (g_{dcw}/L). The statistical significance of the differences between the results was verified using the Tukey test in the Origin®Pro 8.5 software at a 95% confidence level.

$$Y(\%) = \frac{ethanol\ \ produced(g/L)}{theoretical\ \ maximum\ \ ethanol(g/L)} x100 \quad (3)$$

Results and discussion

Selection of cell loads in CBP experiments

The use of high cell loads in the fermentation process is required to achieve high productivities, which is already applied in the Brazilian 1 G ethanol mills that generally run fermentations with high ODs in the tanks, resulting in a robust process that lasts around 10 h [38]. In a previously reported work [21] with the yeast AC14, the authors achieved promising ethanol productivities using high cell loads (OD 100). However, achieving such high cell mass loading can be challenging and time consuming on industrial scale. In addition, high cell loads can lead to limitations of mass transfer in the reactor and to idle cell layers in the system [39]. For this reason, in order to select the most suitable cell load for CBP studies with immobilized cells, experiments were carried out at different cell loads (OD 10, 55 and 100). These experiments were performed with free cells to ensure that all conditions were free from mass limiting effects caused by the presence of the immobilization support, allowing the observation of intrinsic CBP rates.

The profiles of sugar conversion and fermentative parameters for all cell loads studied are shown in Fig. 3 and Table 1, respectively. In all experimental conditions, the potential sugars of all substrates were completely consumed (100%). No differences were observed between the profiles of sugar conversion and substrate consumption rate (r_S) for the different cell loads studied (Tukey test with 95% of confidence), nor in the production of xylitol, glycerol and acetic acid, which reached about 0.81, 0.38 and 1.22, respectively.

Considering the enzyme volumetric activities observed for each cell load studied (Fig. 4A-D), only cellulase differs between the experiments, being higher at OD 10. It is important to note that the cells have limited resources (coming from substrates and nutrients) to supply building blocks for metabolic protein synthesis as well as to produce the seven recombinant hydrolytic enzymes. Thus, although the OD changed, the resources remained at the same level (same medium composition), as did the sugar uptake rate (Table 1). Consequently, it is likely that similar enzyme production occurs regardless the OD value, resulting in similar volumetric activities for each enzyme as observed for xylanase, β-xylosidase and β-glucosidase in Fig. 4 B, C and D. However, for cellulase, a decrease in volumetric activity was observed with increasing OD values. This different pattern may also be related to substrate limitation, which is more severe at higher ODs. The cellulase activity measured by the FPU is based on the synergistic action of 3 enzymes, endoglucanase, cellobiohydrolase I and II [10], thus, if the supply of a building block for the synthesis of only one of these 3 enzymes is reduced, its production will be impaired and overall cellulase activity will decrease, even if the production of other enzymes is unaffected. In addition, quorum sensing may also play a role in the enzyme production at different ODs [40]. Fig. 4 E–H show the enzyme specific activity and the cell response to lower substrate fluxes by reducing the fluxes of enzyme production can be clearly observed.

Similar substrate conversion profiles (Fig. 3) as well as similar results

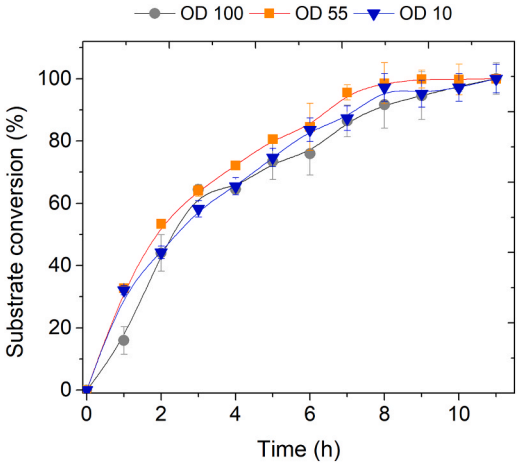

Fig. 3. Profiles of substrate conversion during CBP experiments using different free cell loads of AC14 yeast (optical density – OD = 10; 55 and 100). CBP conditions: 35 ºC, pH 5.50, static.

Table 1
Fermentation indexes of CBP processes using free cells at different loads and Ca-alginate immobilized cells. All experiments were carried out with AC14 yeast at 35 ºC and pH 5.5. Free cells CBP were conducted statically while immobilized CBP were static or stirred (250 rpm). Tukey's test was performed separately for each parameter (data from the same column) and different letters indexes mean that values are significantly different (95% of confidence).

Experiment	Ethanol (g/L)	Y (%)	Q_P (g/L/h)	Viability (%)	r_S (g/L/h)
FC OD 10	9.15 ± 0.64^{a}	74.8 ± 7.7^{c,d}	0.83 ± 0.06^{e}	97.5 ± 0.4^{g}	3.5 ± 0.2^{h,i}
FC OD 55	9.16 ± 0.64^{a}	74.9 ± 7.7^{c,d}	0.83 ± 0.06^{e}	96.2 ± 2.0^{g}	4.3 ± 0.2^{h}
FC OD 100	8.75 ± 0.61^{a}	71.5 ± 5.8^{d}	0.80 ± 0.06^{e}	98.1 ± 1.3^{g}	3.7 ± 0.2^{h}
Immob static	11.76 ± 0.43^{b}	96.1 ± 3.8^{c}	1.09 ± 0.04^{f}	98.9 ± 1.9^{g}	2.7 ± 0.2^{j}
Immob stirred	10.61 ± 0.39^{a,b}	86.7 ± 3.9^{c}	1.08 ± 0.04^{f}	97.6 ± 1.9^{g}	3.2 ± 0.3^{i}

FC: free cells; Immob: immobilized cells; OD: optical density; Y: ethanol yield compared to theoretical; Q_P: ethanol productivity; r_S: substrate consumption rate.

regarding ethanol production, yield and productivity (Table 1) are expected, since the volumetric enzyme activities of 3 key enzymes (Fig. 4 B – D) did not change at different ODs and the hydrolysis rate is the limiting step of the process (once the sugars are released, they are readily fermented). Regarding the influence of cellulase on the sugar conversion (Fig. 3) or performance indexes (Table 1), CMC is present at a low concentration (2.5 g/L) in the medium and its complete hydrolysis occurs even at the reduced cellulase activity (Fig. 4A) observed at higher ODs.

The results observed in the present work reinforce the sensitivity of the enzyme production to cultivation conditions [41,42], suggesting that enzyme production is favored under less limiting nutrient and substrate conditions, and with higher carbon uptake fluxes ($g_{substrate}/g_{dcw}$) [43], which are achieved at the lower cell load. Thus, under the conditions studied, the lower OD 10 achieved the higher specific enzyme productivities for all enzymes quantified (Fig. 4E-F), producing significantly more enzymes per gram of cells than OD 55 and OD 100. The specific enzyme activity values obtained at OD 10 are higher than the total cellulase activity (0.234 U/g_{dcw}) reported in [44], using a *S. cerevisiae* strain expressing endoglucanase and celobiohydrolase from *Trichoderma reesei* and β-glucosidase from *Aspergillus aculeatus*. Claes et al. [20] quantified 19.1 U/g_{dcw} for β-glucosidase and 18.1 U/g_{dcw} for cellulase using AC14 yeast at an initial OD 1.0, which are also lower than the values shown in Fig. 4G and E, respectively. However, the authors used different methods based on specific enzymatic assay kits to determine each enzyme activity (4-nitrophenyl-β-D-glucopyranoside -pNPG, and CellG5 kit, respectively), which may make the comparison to the results of the present study difficult. Overall, the results obtained here with AC14 stand out achieving higher specific enzymatic activities.

There are few studies in the literature that address the influence of cell load on CBP processes. In [45] the influence of yeast load on bioethanol production from sugarcane bagasse by native *S. cerevisiae* was evaluated and a cocktail of hydrolytic enzymes in the SSF using design of experiments and found that the process was favored in lower yeast loads. In another report [46], the SSF of steam exploded corn stover was studied also using a hydrolytic enzyme preparation and *S. cerevisiae,* observing that too low or high inoculation ODs impaired the process, selecting OD 4 as optimal. According to both reports, this behavior is due to the higher sugar consumption rate necessary at high cell loads and its deviation for cell maintenance. Thus, considering that CBP with OD 10 achieved 100% of sugar consumption and a higher specific enzymatic activity, requiring less previous effort for cell propagation, this cell load was select for further experiments with immobilized cells.

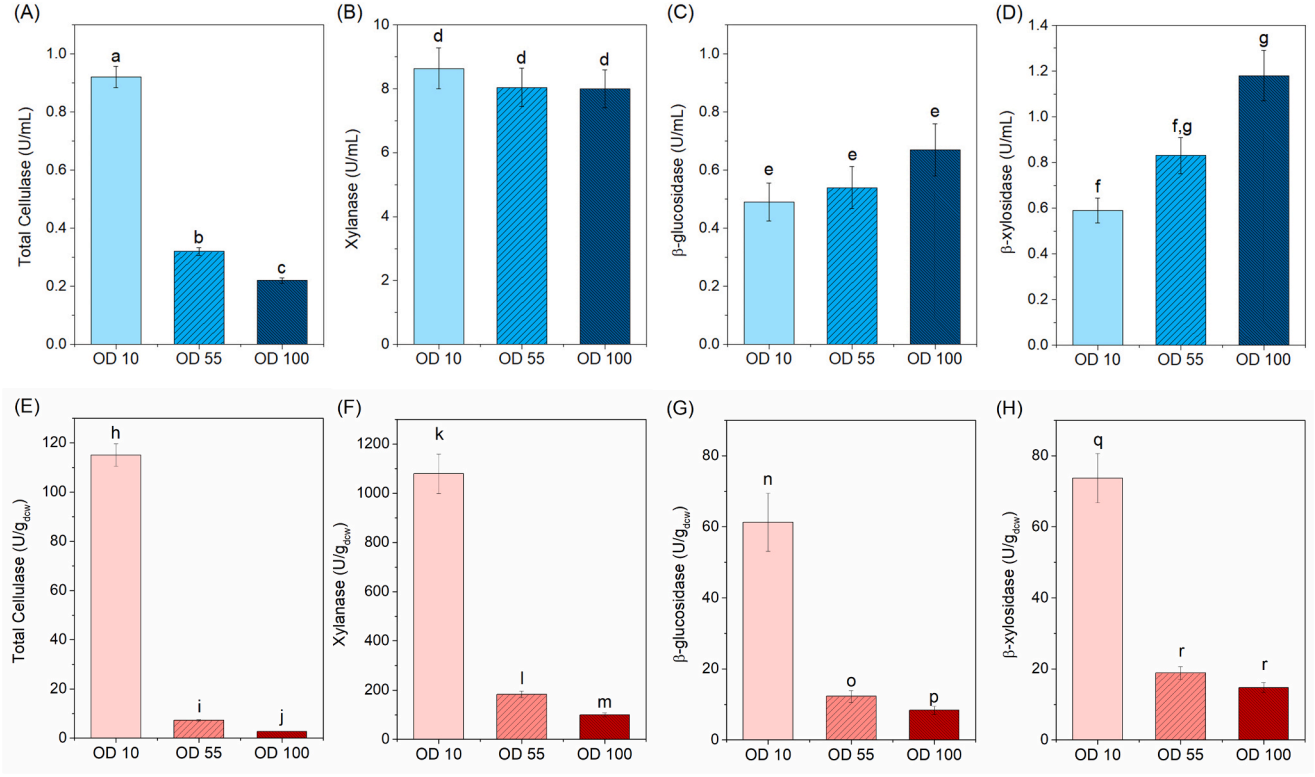

Fig. 4. Final volumetric activities of secreted enzymes (U/mL) (A-D) and final specific enzyme activities (U/g_{dcw}) (E-H). CBP experiments using AC14 yeast at different free cell loads (optical density – OD = 10; 55 and 100) at 35 ºC, pH 5.5 and static. Tukey's test was performed separately for each parameter (data from Fig. 4A-D and E-H) and different letters indexes mean that values are significantly different (95% of confidence).

CBP using immobilized cells

The performance of immobilized cells of AC14 yeast in the CBP process was evaluated to determine if the secreted enzymes would be able to diffuse though the support matrix and access the substrate, providing efficient hydrolysis and release of fermentable sugars for ethanol production. Considering the diffusional effects that would be associated with this heterogeneous system, the CBP was performed under static and stirred conditions. According to Table 1 and Fig. 5A, only immobilized CBP incubated statically presented substrate consumption rate and conversion profile slightly lower than free cells (OD 10), which was expected due to the presence of the gel matrix and mass transfer issues (Fig. 5B). Interestingly, in [47] the same behavior was not observed when comparing the fermentation of xylose by immobilized and free cells of *S. cerevisiae* T18 yeast in fermentations, suggesting that the limitations of mass transfer affecting substrate consumption in static CBP are most related to the diffusion of enzymes and small oligomers through the gel matrix, once glucose and xylose monomers are not affected by diffusional effects.

It is also worth mentioning that despite the lower substrate uptake rate in static immobilized experiment, both CBPs with immobilized cells achieved higher ethanol titers, yields and productivities compared to CBPs with free cells at the same OD. This behavior is one of the advantages of cell immobilization when the microenvironment inside the beads can influence cell metabolism and, in this case, redirect it for ethanol production. According to [48], immobilization can affect cellular regulation and control functions within the glycolytic pathway, changing the preferred metabolic pathway.

The diffusional effect was attenuated when CBP with immobilized AC14 was carried out under agitation, with an increase of 18.5% in the substrate consumption rate (from 2.7 to 3.5 g/L/h, Table 1) and in most of the enzyme activities (Fig. 6), which represents the produced enzymes that diffuse through the gel beads and reach the bulk medium once the activities were measured in the supernatant. The external mass resistance is related to the dimensionless Dankoler factor. This type of limitation can then be overcome by increasing the temperature, which is not always possible in bioprocesses, or by reducing the stagnant film around the solid immobilization support (Fig. 5B) [49,50] by stirring, as observed here.

Besides the external mass resistance, the intraparticle resistance to diffusion can also influence the apparent process rate, since only part of the support matrix volume might be available for diffusion due to support porosity and internal cavities as well as individual enzyme characteristics. Among the enzymes quantified, only cellulases were unaffected by the reduction of the stagnant film around the gel beads

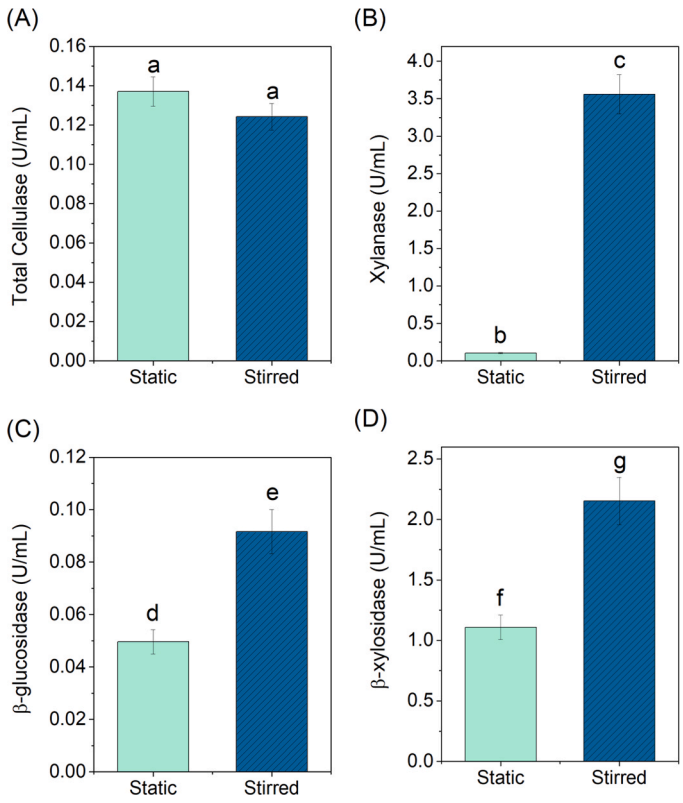

Fig. 6. Final volumetric (U/mL) (A-D) activities using immobilized AC14 yeas at 35 °C, pH 5.50 and static or stirred. Different letters indexes means that values are significant different according to Tukey's test (95% of confidence).

(Fig. 6A) once its activity did not increase in the stirred CBP. This indicates that the diffusion of cellulases is mainly influenced by internal mass transfer limitations. This behavior can be explained by the fact that the interaction between each enzyme and the gel matrix is directly related to the enzyme properties, once it is based on its different surface forces, including van der Waals forces, hydrogen bonds, and, in particular, electrostatic and hydrophobic interactions [51]. As can be seen in Table 2, the 7 hydrolytic enzymes secreted by the AC14 yeast present a wide range of properties, differing significatively in the size (from 24 to 87 kDa), number of amino acids (224−804), isoelectric point (PI) and residues charges, especially Cellobiohydrolase I, which has a much lower PI and number of negatively charged residues compared to the

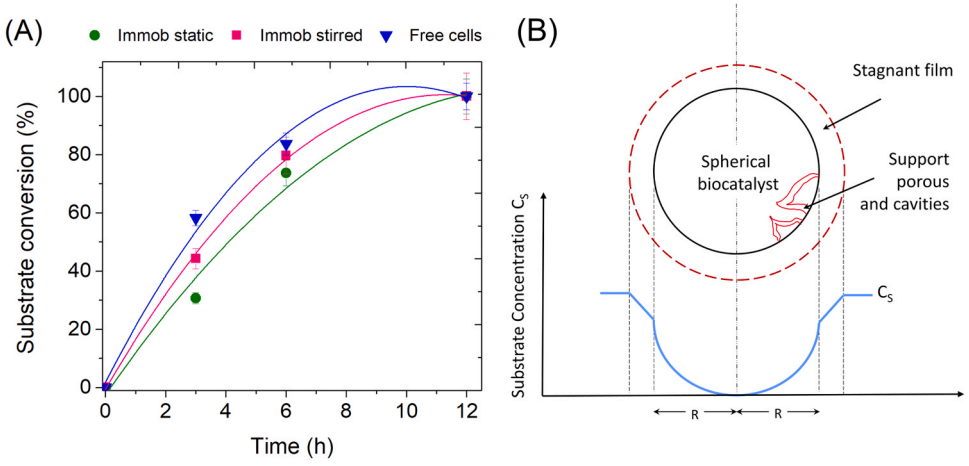

Fig. 5. Profiles of substrate conversion of CBP using immobilized AC14 yeast at 35 °C, pH 5.50 and static or stirred (A) and illustration of substrate concentration variation pattern due to diffusional resistances on immobilized cells solid beads (B).

Table 2
Properties of the hydrolytic enzymes produced and secreted by the recombinant yeast *S. cerevisiae* AC14.

Enzyme	Access number	Number of aminoacids	Molecular weight (kDa)	Isoelectric point (PI)[a]	Number of residues positively charged	Number of residues negatively charged
Cellobiohydrolase I	Uniprot: Q8TFL9	455	48.7	4.19	23	53
Cellobiohydrolase II	GenBank: HH793136.1	482	52.4	5.53	28	35
β-glucosidase	Uniprot: Q12715	744	78.44	6.38	54	57
β-xylosidase	Uniprot: O00089	804	87.2	4.75	52	87
Xylanase	Uniprot: P55330	225	24.1	5.20	11	16
Endoglucanase	Uniprot: Q2TX26	416	44.5	4.96	27	40
Acetylxylan esterase	Uniprot: Q8GH59	520	57.0	8.01	46	44

[a] Estimated from enzyme aminoacid sequence using https://web.expasy.org/

other enzymes. As already mentioned, the measured cellulase activity is based on the synergistic action of endoglucanase, cellobiohydrolase I and cellobiohydrolase II, which implies that if only one of these three enzymes faces a stronger interaction with gel matrix and a higher diffusional limitation, it will directly impair on cellulase activity, as is observed in Fig. 6.

In the case of intraparticle diffusional limitations, the effective diffusivity is also strongly influenced by solid porosity and tortuosity [50]. The dimensionless Thiele modulus, which correlates the reaction and diffusion rates, describes this phenomenon, indicating that the reduction of the biocatalyst particle diameter can overcome the limitations related to intraparticle effects [49]. In addition, the variation of substrate concentration with the particle radius (Fig. 5B) indicates that negligible substrate levels could be reached in the particle center, creating a "biological inactive region" of idle cells [39]. According to this, future experiments to optimize the diameter of immobilized cells beads and the cell load inside the beads would be crucial to achieve an immobilized CBP free from diffusional effects, especially at higher polymers concentrations. It is also important to note that immobilization conditions may need to be validated for different lignocellulosic substrates used due to changes in media properties.

The use of immobilized cells in CBP with polymeric substrates is rarely reported in the literature. Bioethanol production from bagasse pith was studied by immobilized *Pichia stipitis* [52] through SSF process and 10.62 g/L of ethanol was obtained using 50 FPU/g of a commercial cellulase after 5 days of process (0.09 g/L/h of ethanol). On the other hand, [53] achieved 1 G ethanol productivities of 2.13 g/L/h during SSF of corn meal with immobilized cells of *Saccharomyces cerevisiae* var. *ellipsoideus* in synergism with α-amylase enzyme. However, these reports used commercial high-cost hydrolytic enzymes, which compromises the feasibility of the process. As mentioned above, the use of immobilized microorganisms that produce enzyme on CBP is not reported in the literature. Regarding immobilized enzyme-producing microorganisms, only the work of [54] describes the use of immobilized *Thrichoderma viride* cellulase producer for simultaneous delignification and saccharification of rice straw, which released 8.52 g/L of glucose after 10 days. Comparing this value with the substrate consumption rates of Table 1, AC14 yeast stands out as a great enzyme producer, with high hydrolysis rate. However, it is important to remember that the lignocellulose structure of bagasse [52], corn meal [53] or rice straw [54] is more complex than cellobiose, CMC and xylan-based medium used in the present study and certainly it contributes to the better results reported here.

Finally, despite diffusion limitations, in both immobilized cells experiments it was possible to achieve 100% of hydrolysis and consumption of all substrates offered in only 12 h, without significant production of xylitol, glycerol and acetic acid (0.16, 0.27 and 1.91, respectively), being superior in ethanol titer and productivity of recent CBP reports in literature using free cells [14–17]. In a nutshell, this is the first report of a successful proof-of-concept application of immobilized yeast cells in CBP processes for bioethanol production, an innovative process with high potential for industrial application that can overcome the difficult of cell recovery in the presence of solid substrates derived from agro-industrial processing. Overall, the technology reported herein proved to be promising and expandable to other bioproducts of biorefineries than bioethanol.

Conclusion

The application of immobilized cells of AC14 yeast in CBP process was assessed and it was shown that it is possible to achieve 100% of hydrolysis and consumption of the substrate offered in a heterogeneous system. These results demonstrate the functionality of this concept. This work also shows the potential of AC14 as a whole cell biocatalyst suitable for replacing commercial enzymatic cocktails in industrial processes of 2 G ethanol production. Good substrate consumption and conversion rates were achieved, and the efficiency of CBP using immobilized cells depends on the presence of stirring to improve mass transfer. Further studies with industrial media and higher solid loads would provide important additional information for the scale-up of this technology. Overall, the application of AC14 yeast immobilization in Consolidate Bioprocessing for bioethanol production has potential for industrial use once it can overcome the difficulty of cell recovery in a heterogeneous system.

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CRediT authorship contribution statement

Thais S. Milessi, Teresa C. Zangirolami: Conceptualization, Supervision. **Thais S. Milessi, Teresa C. Zangirolami, Marcio D.N. Ramos**: Methodology. **Marcio D.N. Ramos, Juliana P. Sandri**: Investigation, Formal analysis. **Johan M. Thevelein, Arne Claes, Bruna T. Carvalho**: Resources. **Marcio D.N. Ramos, Thais S. Milessi**: Writing – original draft preparation. **Teresa C. Zangirolami, Bruna T. Carvalho, Johan M. Thevelein, Arne Claes**: Writing – review & editing. **Thais S. Milessi**: Funding acquisition. All authors have read and approved the final manuscript and agree with its publication.

Declaration of Competing Interest

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

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