SELECTION OF A RECOMBINANT YEAST PRODUCING HYDROLYTIC ENZYMES FOR BIOMASS CONSOLIDATED BIOPROCESSING

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ABSTRACT

The global energy matrix transition is a global need, driving the development of integrated biorefineries based on vegetal biomass. Considering the high costs associated with the hydrolytic enzymes involved in biomass processing, the Consolidated Bioprocessing (CBP) appears as an emerging technology where enzyme production, biomass hydrolysis and fermentation occur simultaneously in the same bioreactor. A challenge in CBP development is the absence of an efficient microorganisms that secretes hydrolytic enzymes. Therefore, the present work focused on the characterization of the capacity of three different recombinant CBP *Saccharomyces cerevisiae* strains to produce hydrolytic enzymes. The yeasts AC14, Cellusec 1 and Cellusec 3.1 were evaluated aiming the selection of the more suitable yeast for CBP. Cellulases and hemicellulases production were evaluated and the AC14 strain presented the higher production of xylanases (717.07 U/g), while Cellusec 3.1 presented higher production of cellulases (162,75 U/g). In this sense, Cellusec 3.1 is more suitable for hydrolyzing the cellulosic fraction, while AC14 is preferable for hemicellulose. Combining these yeasts in a consortium in CBP could maximize the biomass hydrolysis by degrading both cellulose and hemicellulose fractions.

Keywords: Hydrolytic enzymes. Consolidated Bioprocessing. Recombinant yeast. Biorefinery. Biomass.

1 INTRODUCTION

The replacement of current energy matrices with more sustainable processes is becoming increasingly necessary due to climate changes, which is driving the development of biorefineries. In this context, lignocellulosic biomass is considered to be the most foreseeable feedstock for the production of biofuels and chemicals in biorefineries through second generation (2G) processes[1]. Despite that, there are still significant economic challenges to the implementation of 2G products on an industrial scale, especially for biofuels.

The potential of biomass as a feedstock is related to its complex chemical structure, which is composed mainly of cellulose, hemicellulose, and lignin. Between these components, cellulose and hemicellulose are primarily composed of fermentable sugars, glucose and xylose, respectively[2]. One of the main challenges to the viability of the 2G process is its economic competitiveness with traditional processes, particularly in the context of 2G ethanol, as the processing of lignocellulosic biomass requires complex processes to release the fermentable sugars from cellulose and hemicellulose. These include biomass pretreatment and enzymatic hydrolysis, the latter requiring the use of commercial enzyme cocktails, which are often costly.

In this sense, the Consolidated Bioprocessing (CBP) of biomass represents an emerging technology where the production of hydrolytic enzymes, biomass hydrolysis and fermentation of sugars occur simultaneously in a single bioreactor through the application of microorganisms that secrete enzymes, thereby eliminating the necessity for commercial enzyme cocktails[3]. For CBP to occur, microorganisms or a consortium that secrete the hydrolytic enzymes involved in the biomass degradation process are essential. Cellulases are enzymes involved in the hydrolysis of cellulose. They are conventionally divided into three major groups: endoglucanase, which attacks low crystallinity regions in the cellulose; exoglucanases or cellobiohydrolases which hydrolyze cellobiose; and β-glucosidase which converts cellooligosaccharides and cellobiose into glucose residues. In addition to the three major groups of cellulase enzymes, there are also a number of other enzymes that attack hemicelluloses These include glucoronidase, acetylesterase, xylanase, β-xylosidase, galactomannase and glucomannase. These enzymes work together synergistically to attack cellulose and hemicellulose[4].

However, wild yeasts do not naturally express genes that can secrete all of the aforementioned enzymes together. Therefore, the development of recombinant strains that secrete these enzymes efficiently is an important factor for the success of CPB. In the light of the aforementioned considerations, the yeast *Saccharomyces cerevisiae* AC14 developed by Claes et al.[5] stands once it is able to consume xylose while secreting seven enzymes involved in biomass degradation (endoglucanase, β-glucosidase, cellobiohydrolase I and II, xylanase, β-xylosidase and acetyl-xylan esterase). More recently, the strains Cellusec 1 and Cellusec 3.1 were developed from the AC14 strain and they overexpress the same seven enzymes, in addition to exhibiting resistance to inhibitors formed during the pretreatment of biomass.

In this context, the objective of this work was to characterize the yeasts AC14, Cellusec 1 and Cellusec 3.1 with respect to their capacity to produce hydrolytic enzymes, with the aim of identifying the most suitable yeast for application in CBP.

2 MATERIAL & METHODS

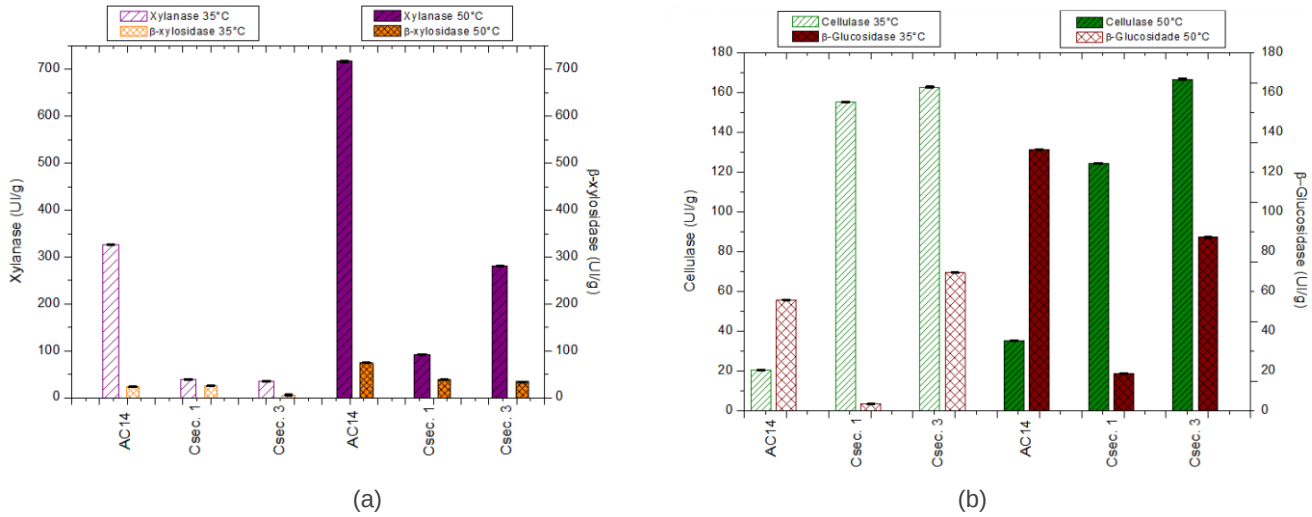
Enzyme extract production: The enzyme extracts were prepared according to the methodology of Ramos et al.[1], which involved spreading a loop of the stock culture on YP-CBP solid agar medium (20 g/L peptone, 10 g/L yeast extract, 15 g/L agar, 20 g/L glucose, 20 g/L xylose , 10 g/L of corncob xylan, 10 g/L of cellobiose and 5 g/L of carboxymethyl cellulose) and incubating it at 30 ºC for 24 h. A single colony from the plate was picked 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 of glucose and 20 g/L of xylose) and incubated at 30ºC for 24 h. The resulting “cell mat” in the Petri dish was completely resuspended and inoculated in 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 separated by centrifugation (2500 rpm for 10 min at 4ºC) and the enzymatic extract was recovered for subsequent characterization.

Enzymatic activities: The enzymatic activities of cellulases and hemicellulases were performed as described by Ramos et al.[1] at 50 and 35ºC, the hydrolysis and fermentation temperatures, respectively. The total cellulases were measured by the release of glucose from Whatman No. 1 filter paper at pH 5.5 and the reducing sugars were quantified by the DNS method[6]. Xylanase was measured by the release of xylose from beechwood xylan at pH 5.5 in a homogeneous enzymatic reactor with mechanical stirring, aliquots were taken every 2 minutes to determine the xylose production rate (dP/dt) and the reducing sugars were quantified by DNS method[6]. Furthermore, the enzymatic activity of β-glucosidases and β-xylosidases were performed by the quantification of glucose released from cellobiose and by the increase in absorbance at 405 nm caused by the release of 4-nitrophenol during the hydrolysis of 4-Nitrophenyl β-D-xylopyranoside (PNPX), respectively. Equation 1 adapted from Ramos et al.[1] was used in all enzyme activities calculations, where (dP/dt) is the product formation rate (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); V_E is the volume of enzyme extract (mL) and C is the cell concentration (g_{dcw}/L).

$$Activity\left(\frac{U}{g_{dcw}}\right) = \frac{\left(\frac{dP}{dt}\right) x V_R x D}{MM x V_E x C} \qquad (1)$$

3 RESULTS & DISCUSSION

The results regarding the enzymatic characterization of the yeast extracts are presented in Figure 1, where it can be seen that the AC14 strains exhibits a higher xylanase enzymatic activity compared to Cellusec 1 and Cellusec 3.1. Conversely, the novel strains demonstrate a higher cellulase enzymatic activity compared to the AC14 strain. This behavior is not observed in the β-xylosidase and β-glucosidase enzymes, however, it is evident that the AC14 yeast has greater activity of hemicellulases, while the Cellusec strains are more efficient at cellulases production.



(a) (b)

Figure 1 Enzymatic activity of xylanases and β-xylosidase (a) and cellulases and β-glucosidase (b) for the three studied yeasts AC14, Cellusec 1 and Cellusec 3.1 at 35ºC (open bars) and 50ºC (filled bars). Results are the means of triplicates.

Table 1 presents the volumetric activity of each enzyme per milliliter of the enzymatic extract of the three studied strains. Once the growth of the three strains were similar (approximately 12 g/L of cells), the same behavior was observed, with AC14 exhibiting

high hemicellulase activity and the Cellusec strains exhibiting high cellulase activity. The enzyme activities were also higher at 50ºC, as expected, as the reaction rate increases with temperature in accordance with the Arrhenius law[7]. However, it is noteworthy that the β-glucosidase activity of the AC14 strain is lower at 50ºC than at 35ºC. This may be related to the enzyme's thermal instability at this temperature, which is an important consideration for future evaluations of the stabilities of these enzymatic extracts at process conditions (50ºC when performing only biomass hydrolysis and 35ºC when performing CBP).

Table 1: Volumetric activity of cellulases, β-glucosidases, xylanases and β-xylosidases for the three studied yeasts AC14, Cellusec 1 and Cellusec 3.1 at 35°C and 50°C.

Yeast	AC14		Cellusec 1		Cellusec 3	
Enzyme (U/mL)	50ºC	35ºC	50ºC	35ºC	50ºC	35ºC
Cellulase	0.3±0.0	0.2±0.0	1.9±0.2	0.9±0.1	1.5±0.2	0.9±02.
Xylanase	10.8±1.1	4.9±0.3	1.2±0.1	0.5±0.1	2.6±0.8	0.3±0.1
β-glucosidase	0.8±0.0	1.2±0.0	0.04±0.01	0.03±0.00	0.64±0.04	0.45±0.02
β-xylosidase	1.1±0.0	0.4±0.0	0.48±0.04	0.31±0.0	0.31±0.01	0.05±0.00

From these results, it is important to highlight that the specific enzymatic activities obtained for all yeasts studied are superior than reported in literature. Yamada et al.[8], using a strain of *S. cerevisiae* expressing endoglucanase, cellobiohydrolase and β-glucosidase obtained a total cellulase activity of 0.234 U/g_{dcw}. Amoah et al[9] with a recombinant strain of *S. cerevisiae* reported cellulase and xylanase activities of 0.2 U/g and 0.4 U/g, respectively. Thus, in a general context, the results obtained here with AC14, Cellusec 1 and Cellusec 3.1 stand out achieving higher activities. Regarding the potential of these yeasts for CBP, Cellusec strains are more suitable for cellulose degradation, while AC14 strain is more suitable when the focus is hemicellulose hydrolysis. Given the importance of all biomass components utilization, a consortium of these yeasts could provide an enzyme cocktail that is efficient in degrading both cellulosic and hemicellulosic fractions of biomass.

4 CONCLUSION

The enzymatic activity of both total cellulases and hemicellulases for the three recombinant strains evaluated can be considered high when compared to the current scenario of recombinant yeasts reported in literature. Therefore, considering the higher cellulase activity of Cellusec strains and the higher hemicellulases activity of AC14 strain, a consortion of these strains could be most suitable for CBP aiming to the hydrolysis of all biomass components.

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