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STRATEGIES FOR CONSOLIDATED BIOPROCESSING OF EUCALYPTUS CHIPS WITH HIGH SOLID LOADS

Paulo S. Santos Júnior[1], Suzana M.P. Costa[1], Márcio D.N. Ramos[1], Isadora A.C. Pupin[2], Johan M. Thevelein[3], Jesus J. Ascencio[4], Anuj K. Chandel[4], & Thais S. Milessi[1,2]*

[1] *Graduate Program of Chemical Engineering (PPGEQ)/ Federal University od São Carlos, São Carlos, Sp, Brazil.*
[2] *Department of Chemical Engineering (DEQ)/ Federal University od São Carlos, São Carlos, Sp, Brazil.*
[3] *NovelYeastbv /Leuven, Belgium.*
[4] *Department of Industrial Biotechnology/ University of São Paulo, São Paulo, Brazil.*
* *Corresponding author's email address: thais.milessi@ufscar.br*

ABSTRACT

Consolidated bioprocessing (CBP) is a technology for the valorization of biomass where enzyme production, hydrolysis and fermentation are conducted in a single reactor simultaneously. This strategy is promising once it does not require the addition of high-cost commercial enzymes. The use of high solid loads during the biomass hydrolysis stage is an important strategy to increases the final concentration of sugars released and, consequently, the final product titer. In this context, the present study investigated different strategies to improve the efficiency of the CBP of eucalyptus chips using 10% of solid load using a recombinant yeast that secretes seven hydrolytic enzymes. For this purpose, three different systems were evaluated: eucalyptus pulp without additives, eucalyptus pulp with the addition of soy protein as lignin blocker, and eucalyptus pulp with the addition of Cellic CTec2 enzyme cocktail to increase enzyme concentration in CBP. The results showed that the addition of soy protein prevented enzyme adsorption in lignin and enhanced CBP performance. However, the addition of Cellic CTec2 into the system was the most effective approach, resulting in increase in ethanol concentration of 92% and 67% when comparing the conditions with eucalyptus without additives and eucalyptus plus soy protein, respectively. This underscores the critical importance of developing microorganisms with high enzyme production to enhance the efficiency of CBP.

Keywords: Consolidated bioprocessing. Eucalyptus pulp. High solid load. Cellulases.

1 INTRODUCTION

Eucalyptus chips are a forest residue that can be used in the production of value-added products and bioethanol through a biorefinery approach. The structure of this lignocellulosic biomass is composed of approximately 45% cellulose and 30% hemicellulose[1] which represents an important carbon source for biotechnological applications. One of the main challenges associated with the utilization of biomass in a biorefinery context is the effective release of monosaccharides from both cellulose and hemicellulose, which requires a pretreatment and an enzymatic hydrolysis steps[2]. Furthermore, the high costs of the enzyme cocktails required for the enzymatic hydrolysis represents a significant obstacle to the application of second generation (2G) processes at industrial scale[3].

A notable advance in the production of 2G bioproducts, particularly bioethanol, is the development of the Consolidated Bioprocessing (CBP) of biomass. This technology integrates the production of enzymes, enzymatic hydrolysis, and the fermentation of hexoses and pentoses within a single bioreactor[2]. This implies a reduction in operational costs as it does not require the addition of exogenous enzymes. However, a microorganism that is capable of producing hydrolytic enzymes, as well as being resistant to toxic environments and capable of consuming both fractions of sugars generated after the hydrolysis of lignocellulose (hexose and pentose) it is necessary. In this context, Claes et al.[4] developed the recombinant yeast *Saccharomyces cerevisiae* AC14 strain, which stands out from works in literature as it is capable of secreting seven enzymes involved in biomass degradation: endoglucanase, β-glucosidase, cellobiohydrolase I and II, xylanase, β-xylosidase, and acetyl-xylan esterase. Previous studies have yielded promising results with this yeast. Perez et al.[5] investigated the CBP of sugarcane bagasse using the AC14 yeast and achieved 89% of theoretical yield in 7 hours. However, the authors used low solid loads (1% w/v), making important to evaluate the AC14 yeast's performance at higher solid loads of biomass.

The use of high solid loading during the biomass hydrolysis stage is an important strategy because it increases the final concentration of released sugars and, consequently, the final ethanol titer produced[6]. However, at high solids loads, hydrolysis conversion can be negatively affected by factors such as mass transfer limitations, unproductive lignin adsorption, and inhibition of the final product by sugars released during enzymatic hydrolysis[7]. Seeking strategies to address these problems, Florencio et al.[7] indicated that soybean protein is a lignin-blocking additive that can enhance ethanol production from enzymatic hydrolysates of pretreated sugarcane bagasse once it provides efficient blockage, doubles the amount of sugars, and is low cost. The authors tested three different solids loads (10.15 and 20% w/v) and found that the highest glucose concentrations were obtained when using the highest solid loading. However, the increase in the yield of glucose released with the addition of soy protein was more pronounced with a solid load of 10% (w/v).

Another possible solution to increase the hydrolysis efficiency would be to increase the production of enzymes by the AC14 yeast. In this context, the aim of this work was to evaluate different strategies to improve the performance of the CBP of eucalyptus chips

at 10% solid loading by the AC14 yeast by using soy protein as a lignin blocker and by increasing the enzyme content in the reactor.

2 MATERIAL & METHODS

Eucalyptus pulping: Kraft pulping of eucalyptus was carried out in a Regmed AU/E–20 rotary reactor equipped with a 20 L digester vessel with 25% sulfide and 13% active alkali content. Eucalyptus chips were cooked at 170ºC for 3h in a wood-to-liquor ratio of 4:1 (w/v), following the methodology described by Gomes et al.[8]. The resulting cellulosic pulp was washed and filtered before being stored at 4°C.

Microorganism and Inoculum: The yeast *S. cerevisiae* AC14 was used in all experiments. This yeast was obtained by sequential genomic integration of seven heterologous genes encoding lignocellulolytic enzymes[4]. The inoculum was prepared according to an adaptation of the protocol of Ramos et al.[3] where a loop of the stock culture (stored at − 80 ºC) was spread on YP-CBP solid agar medium (20 g/L de peptone, 10 g/L yeast extract, 15 g/L agar, 20 g/L glucose, 20 g/L de xylose, 10 g/L corncob xylan,10 g/L cellobiose and 5 g/L carboxymethylcellulose) and incubated at 30 ºC for 48 h. A single colony of the plate was resuspended in 75 µL of sterile distilled water and spread with a Drigalski loop onto a new YPDX-agar (YP-CBP without polymers) solid medium plate and incubated at 30ºC for 24 h. The "cell mat" formed in the Petri dish was completely resuspended and inoculated into 300 mL of YPDX medium (YPDX - 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 used in CBP experiments.

CBP experiments: CBP experiments were performed in mini-reactors with high cell load (OD_{600}=100), temperature of 35ºC, pH 5.5 and magnetic stirrer[3]. The CBP medium consisted of eucalyptus pulp (10 % supplemented with yeast extract 10 g/L, peptone 20 g/L and xylan 5 g/L). Soy protein (5% w/v) or commercial enzyme Cellic CTec2 (0.978 U/mL) was added to the experiments to evaluate the influence of lignin adsorption and enzyme concentration on CBP. The operating systems were identified as without additive using only eucalyptus (EUC), with additive using soy protein (Euc+Soy) and with Cellic CTec2 (EUC+Celic CTec2). All experiments were performed in replicates under the same experimental conditions.

Analytical methods: Quantification of the enzymatic activity of total cellulases was evaluated according to Ghose[9] based on the release of glucose from 15 mm Whatman No.1 filter paper discs. The reducing sugars released were quantified using the DNS method[10]. One unit of activity (U) was defined as the amount of enzyme required to release 1 µmol of glucose in 1 minute under the assay conditions. A control was performed by replacing the enzyme extract with distilled water. The concentrations of products and residual substrate were quantified by high performance liquid chromatography (HPLC) on a Waters e2695 chromatograph with a RezexTM ROA-Organic acid H+ ion exclusion column[11]. Fermentative parameters (ethanol productivity - Q_P; and ethanol yield – Y, %) were calculated according to Ramos et al. [3].

3 RESULTS & DISCUSSION

The different systems of eucalyptus CBP with 10% (w/v) of solids loading were evaluated and the ethanol production and cellulase activity are shown in Figure 1. The pretreated eucalyptus pulp was composed of 59% cellulose, 12% hemicellulose, and 19% of lignin, with a maximum ethanol production of 42 g/L, considering the stoichiometric factors. The fermentative parameters of the three conditions are shown in Table 1. The system using only eucalyptus (EUC) resulted in the lower ethanol production, yield and productivity. Probably, part of the enzymes produced by AC14 yeast were adsorbed on lignin or the amount of enzymes was insufficient in this condition. As can be seen in Figure 1B, this condition presented the lower cellulase activity.

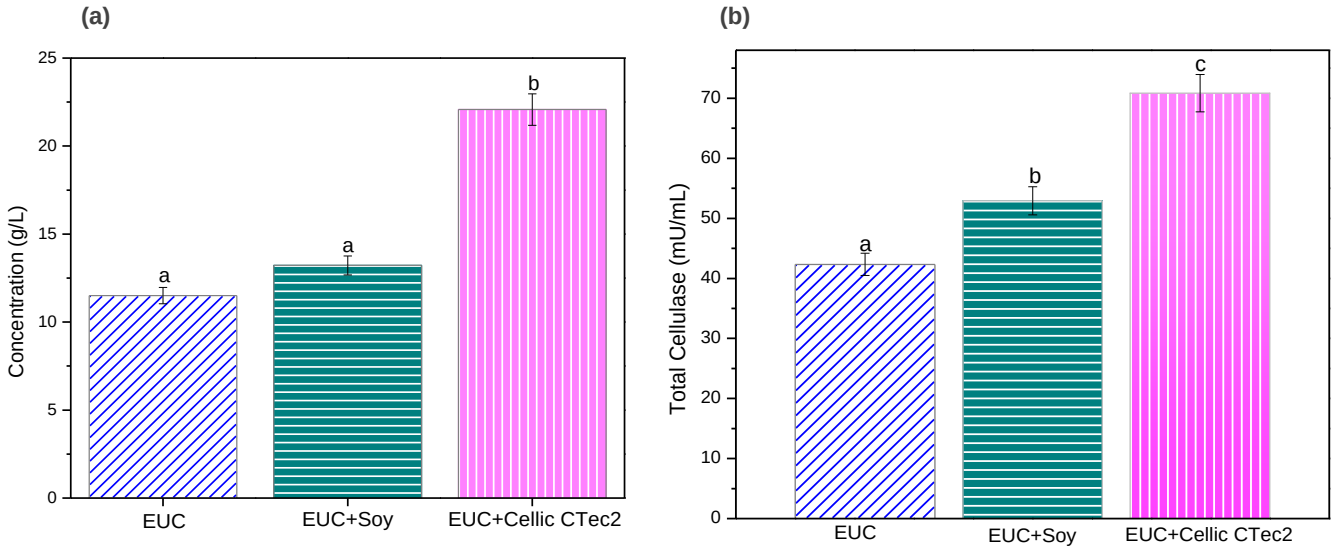

Figure 1 – (a) Ethanol concentration and (b) Final enzymatic activity of cellulase obtained from the CPB of eucalyptus chips (10% w/v), at 35ºC and pH 5.6 after 12 hours of process with eucalyptus (EUC), EUC plus soy protein and EUC plus the commercial enzyme Cellic CTec2. Different letter indices mean that the values are significantly different according to Tukey's test (95% confidence)

To overcome this limitation, soy protein was tested as an additive, and a 15% increase in ethanol production was observed due to the doping effect on lignin. This result is expected, since the addition of lignin blockers improves the saccharification efficiency and reduces the number of enzymes lost in the enzymatic hydrolysis[12]. The Cellic CTec2 system, on the other hand, showed the highest ethanol concentration, about 22 g/L, and the best fermentation parameters (Table 1), with emphasis on the productivity, which was almost doubled compared to EUC. This can be attributed to the enzyme increase in the system, which increased the hydrolysis of the material and the production of ethanol, respectively. As can be seen in Figure 1b, the production of total cellulases in EUC was equivalent to 42 mU/mL, in this condition the amount of enzyme produced by AC14 was lower when compared to the other conditions (70.83 mU/mL for EUC+CelicCTec2), which reduced the hydrolysis yield by decreasing the availability of free cellulase enzymes. With the use of soy protein, there was a significant increase in the enzymatic activity of total cellulases due to the decrease in the adsorption of residual lignin from eucalyptus[7], however it was not sufficient to achieve the same enzymatic activity when using exogenous cellulase.

Table 1 Productivity (Qp) and yield (Y%) of the different system for the production of ethanol by CBP of from eucalyptus chips: EUC, EUC plus soy protein (5% w/v) and EUC plus Cellic CTec2 (0.978 U/mL)

	Qp (g/L/h)	Y%
EUC	0.96	27%
EUC + Soy	1.10	31%
EUC + Cellic CTec2	1.84	51%

The addition of Cellic CTec2 to the system provided an increase in cellulose degradation efficiency, suggesting that the cellulase enzyme present in the enzyme cocktail played an important role in the breakdown of cellulose and its simpler components. It is also important to emphasize that enzymatic hydrolysis of biomass requires a wide range of different enzymes acting synergistically; besides the remarkable capacity of AC14 yeast to produce 7 hydrolytic enzymes, the commercial cocktail probably contains an important accessory enzyme that the AC14 does not produce, which can also significantly increase the hydrolysis efficiency. However, besides the lower yields, the results obtained in EUC and EUC+Soy are superior compared to other works in the literature using other microorganisms, where yields and productivities of 0.15 and 0.05 are reported for CBP[13].

4 CONCLUSION

The use of soy protein as a lignin blocker during CBP of eucalyptus resulted in a slight improvement in biomass hydrolysis, fermentation parameters and final cellulase activity. However, the system with the addition of Cellic CTec2 showed the best results in terms of productivity (1.84 g/L/h) and ethanol yield (51%). In this sense, the development of microorganisms with high enzyme production is of paramount importance to increase the efficiency of CBP leading to higher rates of substrate conversion into desired products, reducing costs and increasing the commercial viability of biotechnological processes.

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