INFLUENCE OF THE SOLID LOAD ON THE PERFORMANCE OF CONSOLIDATED BIOPROCESSING OF EUCALYPTUS CHIPS

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

[1] *Postgraduate Program of Chemical Engineering (PPGEQ)/Federal University of São Carlos, São Carlos-SP, Brazil.*
[2] *Chemical Engineering/Department of Chemical Engineering (DEQ)/Federal University of São Carlos, São Carlos-SP, Brazil.*
[3] *NovelYeast bv, Open Bio-Incubator, Leuven, Belgium.*
[4] *Department of Industrial Biotechnology/University of São Paulo, São Paulo, Brazil.*
* *thais.milessi@ufscar.br*

ABSTRACT

Consolidated bioprocessing (CBP) is a technology for the valorization of biomass where enzyme production, hydrolysis and fermentation occur simultaneously in a single reactor, dismissing the addition of high-cost commercial enzymes. Considering that the use of high solid loads is an important strategy to increases process efficiency, this study investigated the influence of biomass solid load in the CBP of eucalyptus chips using the recombinant yeast *Saccharomyces cerevisiae* AC14, which secrets seven hydrolytic enzymes. CBP processes were conducted at varying solid loads, ranging from 0.4% to 10% (w/v). An increase in ethanol production of 55% and productivity of 57% were achieved using higher solid loads of biomass. However, a significant decrease in ethanol yield was observed with 10% of eucalyptus chips. Strategies to mitigate these challenges, including bioreactor design and lignin-blocking additives, can be explored in the future to enhance CBP efficiency. This study underscores the importance of optimizing operational conditions for maximizing CBP performance and highlights the effectiveness of the AC14 yeast strain in facilitating ethanol production.

Keywords: Consolidated Bioprocessing. Eucalyptus chips. Ethanol. Recombinant yeast.

1 INTRODUCTION

Biomass is considered as the most foreseeable raw materials for the transition of the global matrix from fossil to sustainable processes[1]. However, one of the main technological challenges for the widespread use of biomass in 2G plants is the lack of sustainable and cost-effective technologies to overcome the recalcitrant biomass structure. The most commonly used approach to overcome biomass recalcitrance and depolymerize cellulose and hemicellulose into monomeric fermentable sugars involves the enzymatic hydrolysis of a pretreated biomass using hydrolytic enzyme cocktails. However, the high cost of these enzymes is a major drawback for biomass utilization[2].

Consolidated Bioprocessing (CBP) is an emerging technology where the production of hydrolytic enzymes, the hydrolysis of biomass and the fermentation of released sugars occur simultaneously in a single bioreactor. In this sense, CBP requires the use of native or recombinant microorganisms, or a consortium of microorganisms, that secrete hydrolytic enzymes, eliminating the need for costly enzymatic preparations to carry out the hydrolysis/saccharification step of lignocellulosic biomass[3]. In this sense, the yeast *Saccharomyces cerevisiae* AC14 stands once it is able to secrete seven hydrolytic enzymes (endoglucanase, β-glucosidase, cellobiohydrolase I and II, xylanase, β-xylosidase, and acetyl-xylan esterase) and consume xylose, reaching interesting process yields and productivities compared to other microorganisms developed for CBP[4].

In addition to an efficient enzyme-producing microorganism, it is important to achieve an industrially relevant ethanol titer in CBP to make its distillation and integration into a large-scale plant viable. In this sense, the use of high solid loads of biomass in CBP can increase the sugar concentration available for the subsequent fermentation stage and thus contribute to the economic recovery of ethanol by distillation. The use of high solid loads improves the economics of lignocellulosic biomass conversion to fuels and chemicals by reducing both capital and operational costs, as the increase in the final product concentration reduces equipment volumes in addition to the costs of purification steps[5]. Nevertheless, the high solids concentration also brings challenges such as the slurry's high viscosity, ineffective mixing, and heat/mass transfer limitations[6]. In addition, the residual lignin present in the pretreated solid biomass can promote the unproductive adsorption of the hydrolytic enzymes, reducing the enzymatic hydrolysis yield by reducing the availability of free cellulases[7].

The influence of high solid loads in CBP is not explored in the literature, which must be accessed to contribute with crucial information for its development and operational scalability[1]. Considering that eucalyptus chips are a forest byproduct, largely obtained in the pulp and paper industry and still not explored in CBP, the present work evaluated the influence of the solid load on the CBP of eucalyptus wood chips pretreated hydrothermally using the recombinant yeast *S. cerevisiae* AC14.

2 MATERIAL & METHODS

Eucalyptus pulping: Eucalyptus kraft pulping was performed in a Regmed AU/E-20 model rotary reactor equipped with a 20 L digester vessel with 25% sulfide and 13% active alkali content. Eucalyptus chips were cooked at 170 °C for 3 hours in a wood-to-liquor ratio of 4:1 (w/v)[8]. The resulting cellulose pulp was washed and filtered before being stored at 4 °C.

Microorganism and inoculum: The *Saccharomyces cerevisiae* AC14 yeast containing seven heterologous genes encoding lignocellulolytic enzymes was used in all experiments. Pre-inoculum and inoculum were prepared by spreading a loop of the stock culture 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 30ºC for 48 hours. A single colony was selected from the plate and resuspended in 75 µL of sterile distilled water and spread with a Drigalski loop onto a YPDX-agar solid medium (YP-CBP without polymers) and incubated at 30 ºC for 24 h to generate the "cell carpet", which was completely resuspended and inoculated into 300 mL of YPDX liquid medium in 1 L baffled Erlenmeyer flasks and incubated for 12 h at 30 ºC and 150 rpm. Yeast cells in exponential growth phase were recovered by centrifugation (2500 rpm for 10 min at 4 ºC) [1].

CBP experiments: The experiments were carried out in mini reactors of 10 mL containing a CO_2 output, filled with 4 mL of medium and high cell load (OD_{600} = 100) at 35 °C, pH 5.5 and magnetic stirring. The CBP medium was composed of yeast extract (10 g/L), peptone (20 g/L) and xylan (5 g/L)[9]. The experiments were carried out using pretreated eucalyptus at different solid loads: 0.4, 2, 4, and 10%. Samples were periodically collected, centrifuged (4 ºC, 10000 rpm) and the supernatant were quantified by high performance liquid chromatography (HPLC) and the enzyme activities.

Analytical methods: The concentrations of ethanol, glycerol, xylitol, xylose and glucose were quantified by HPLC on a Waters e2695 chromatograph with a RezexTM ROA-Organic acid H+ ion exclusion column[10]. Enzyme activities of cellulases, and hemicellulases were performed based on the release of glucose and xylose from 15 mm Whatman No.1 filter paper discs and birchwood xylan, respectively[1]. Reducing sugars released were quantified by the DNS method[11]. The concentration of free cells (Cx) was determined by turbidimetry, and yeast viability was quantified by the methylene blue method[10]. Fermentative parameters (ethanol productivity - QP; substrate conversion - X and ethanol yield – Y, %) were calculated[1].

3 RESULTS & DISCUSSION

The fermentative parameters of eucalyptus CBP at different solid loads are presented in Table 1. The pretreated eucalyptus pulp was composed of 59% cellulose and 12% hemicellulose, with a maximum ethanol production varying between 7.4 and 42.9 g/L from 0.4% to 10% of solid loading, considering the stoichiometric factors. The use of 10% solid load led to an increase in ethanol production of 55% (from 7.4 to 11.5 g/L) and 57% in productivity (from 0.61 to 0.96 g/L/h), as expected. Higher productivities were also achieved using sugarcane bagasse at high solid loads in simultaneous saccharification and fermentation (SSF) processes[12-13], however this is the first report of the solid load effect on CBP performance.

Table 1 Productivity (Qp) and yield (Y%) of the eucalyptus CBP at different solid loads.

Solid Load (%)	Ethanol (g/L)	Q_P (g/L/h)	$EtOH_{max}$ (g/L)	Y (%) *
0.4	7.4	0.61	7.4	100
2.0	6.5	0.54	13.7	47
4.0	6.3	0.53	21.8	29
10.0	11.5	0.96	42.9	27

*$EtOH_{max}$: maximum theoretical ethanol production; Y (%): ethanol yield compared to theoretical; Q_P: ethanol productivity.

The ethanol yield (Y) decreased with the increase in eucalyptus solid load (Figure 1), achieving a maximum yield of 27% for 10% of solid loading. The use of high solid loads of biomass can negatively affect the enzymatic hydrolysis step due to lignin acting as a steric barrier to enzymes and the unproductive enzyme adsorption on residual lignin present in the pretreated solid[7]. Also, with high solids loads the amount of free water is reduced and the fibrous material slurry reach high viscosity, resulting in poor mixing and mass and heat transfer limitations, reducing the efficiency of the process in the first steps of the enzymatic hydrolysis, known as the liquefaction stage[5].

In this regard, it is evident that the solid load influences the CBP of biomass, making it imperative to develop strategies to enable high solid load operation, for example, by using specially design bioreactor or adding compounds that act as a lignin-blocking additive, to adsorb in residual lignin in the place of the enzyme. Previous studies observed that the addition of soybean protein in the hydrolysis of sugarcane bagasse potentiated the production of ethanol due to efficient blockade of lignin adsorption, doubling glucose released with a low operational cost[7].

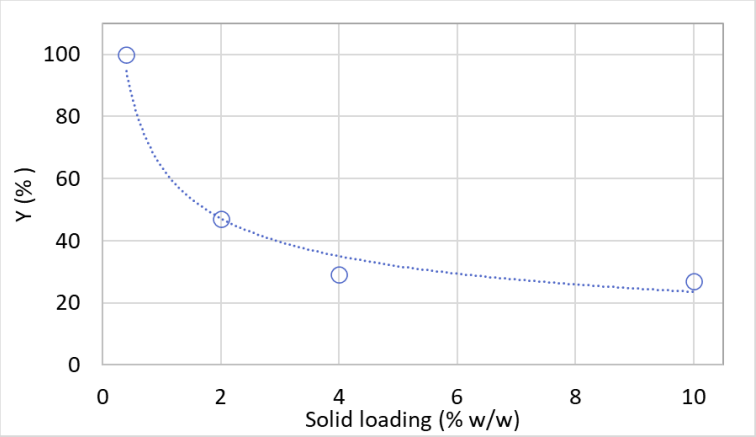

Figure 1. Ethanol yield during CBP of eucalyptus at different solid loads using AC14 yeast.

It is also worth to emphasize that the type of biomass influences in CBP performance due to similarities of its composition. CBP was performed with pretreated sugarcane bagasse (1% solid load) using the AC14 yeast and achieved a productivity of ethanol of 1.9 g/L/h[14]. This can be explained by the fact of sugarcane being a grass and eucalyptus being a hardwood, the latter being harder to hydrolyze. Still, when compared to other works from literature using other microorganisms, the yield and productivities obtained in the present study stands out, once productivities of 0.15 and 0.05 are reported for CBP[15].

CONCLUSION

The results obtained are promising in terms of increasing ethanol production with CBP in a higher solid load, although, when compared to the theoretical maximum, production remains at a lower value. In this sense, the development of strategies to solve the operational problems already discussed, such as proper mixing and adjustment of mass and heat transfer, are necessary, such as different configurations of bioreactors and the addition of lignin-blocking compounds to mitigate enzymatic adsorption on residual lignin. The results of this study demonstrate the effectiveness of the AC14 strain of *Saccharomyces cerevisiae* in CBP and highlight the importance of optimizing operating conditions to achieve desirable yields and productivity. These findings provide valuable insights for the continued development and scalability of CBP, thereby contributing to the global transition to sustainable energy matrix processes.

REFERENCES

[1] Ramos, M. D. N., Sandri, J. P., Claes, A., Carvalho, B. T., Thevelein, J. M., Zangirolami, T. C., & Milessi, T. S. New Biotechnology, 78, 153–161.

[2] Maitan-Alfenas, G. P., Visser, E. M., & Guimarães, V. M. Current Opinion in Food Science, 1, 44–49.

[3] Olguin-Maciel, E., Singh, A., Chable-Villacis, R., Tapia-Tussell, R., & Ruiz, H.A. Agronomy, 10, 1834.

[4] Claes, A., Deparis, Q., Foulquié-Moreno, M. R., & Thevelein, J. M. Metabolic Engineering, 59, 131–141.

[5] da Silva, A.S., Espinheira, R.P., Teixeira, R.S.S., De Souza, M. F., Ferreira-Leitão, V., & Bon, E. P. S. *Biotechnol Biofuels* 13, 58

[6] Shiva, N., Barba, F. C., Rodríguez-Jasso, R. M., Sukumaran, R. K., & Ruiz, H. A. *Bioresource Technology*, *351*, 127044.

[7] Florencio, C., Badino, A. C., & Farinas, C. S. BioEnergy Research/BioEnergy Research, 12(1), 81–93.

[8] Gomes, R. M., & Silva Júnior, F. G. da. *BioResources*, 15, 2, 3945-3961.

[9] Ramos, M.D.N., Zangirolami, T.C., Milessi, T.S. Development of consolidated bioprocessing using yeast with hydrolytic enzymes. Masters dissertation, Federal University of São Carlos/UFSCar.

[10] Sandri, J. P., Ramos, M. D., Perez, C.L., Mesquita, T.J., Zangirolami, T. C., Milessi, T. S. 2023. Biochemical Engineering Journal, 191,108777.

[11] Miller, G.L. Journal of Analytical Chemistry, 31, 426-428.

[12] Gao, Y., Xu, J., Yuan, Z., Jiang, J., Zhang, Z., Li, C. Energy Sci. Eng. 6, 810–818.

[13] da Conceição Gomes, A., Moysés, D.N., Santa Anna, L.M.M., de Castro, A.M. Ind. Crops Prod. 119, 283–289.

[14] Perez, C. L., Pereira, L. P. R. da C., Milessi, T. S., Sandri, J. P., Demeke, M., Foulquié-Moreno, M. R., Thevelein, J. M., & Zangirolami, T. C. Renewable Energy, 185, 363–375.

[15] Cunha, J.T., Romaní, A., Inokuma, K., Johansson, B., Hasunuma, T., Kondo, A., Domingues, L. Biotechnol Biofuels 13, 138.

ACKNOWLEDGEMENTS

Authors are thankful to grants #2016/10636-8, #2022/11189-6 and # 2023/15508-1 from São Paulo Research Foundation (FAPESP), and #407716/2021-1 from Brazilian National Council for Scientific and Technological Development (CNPq). This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brasil (CAPES, Finance Code 001).