ON-SITE PRODUCED ENZYME COCKTAILS FOR SACCHARIFICATION OF HYDROTHERMAL PRETREATED SOYBEAN HULLS

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ABSTRACT

Bioprospecting of microorganisms that produce lignocellulolytic enzymes, *on-site* enzyme production, and the combination of enzymatic extracts produced by different microorganisms (blends) are some alternatives to reduce the cost of obtaining of biocatalysts and increase the biomass saccharification velocity and efficiency. In the present study two enzyme cocktails were produced by blending crude enzyme extracts from different fungi. These fungi were selected from eight distinct fungi isolated from pretreat soybean hulls and cultivated on soybean hulls in natura in two ways: solid-sated and submerged fermentation. The enzyme activities were determined and evaluated for synergism aiming to biomass lignocellulosic conversion. The blend of fungal extracts provided a release of glucose about 40-55% and the xylose about 13-119% more than individual extracts, highlighting the synergistic effect of the enzymes during the hydrolysis of soybean hulls.

Keywords: Lignocellulolytic enzymes. Fungi. Enzymatic hydrolysis. Biomass conversion.

1 INTRODUCTION

Soybean hulls is an agricultural residue of soybean whose yield is up to 80 kg per kg of soybean. The existing literature shows the vast potential of soybean hulls as an environmentally friendly carbon source to produce value-added chemicals, materials, fuels, and energy in a similar way to oil refineries. However, the use of this biomass on a commercial scale in biorefineries still faces some challenges like as the high cost of the cellulolytic enzymatic cocktails used in the saccharification step that significantly affects the economics of industrial large-scale conversion processes.

The on-site production of enzymes, integrated to the biorefinery plant, is being considered as a potential strategy that could be used to reduce costs. In such approach, the microbial production of enzymes can be carried out using the same lignocellulosic biomass as feedstock for fungal development and biofuels production. Most of the microbial cultivation processes for the production of industrial enzymes have been developed using the conventional submerged fermentation. However, recently, a sequential solid-state followed by submerged fermentation has been described as a potential alternative cultivation method for cellulolytic enzymes production. [1] Since the composition of lignocellulosic biomass is quite diverse, there is an increasing demand for the development of optimized enzymatic cocktails. [2] Bioprospecting of microorganisms that produce lignocellulolytic enzymes, *on-site* enzyme production, and the combination of enzymatic extracts produced by different microorganisms (blends) are some alternatives to reduce the cost of obtaining of biocatalysts and increase the saccharification velocity and efficiency. [3]

In the present study two enzyme cocktails were produced by blending crude enzyme extracts from different fungi isolated from pretreated soybean hulls. These fungi were also cultivated on soybean hulls in natura in two ways: solid-sated and submerged fermentation and the enzyme activities were determined and evaluated for synergism aiming to biomass lignocellulosic conversion.

2 MATERIAL & METHODS

Microrganisms and cultivation. The fungi were isolated from hydrothermally pretreated soybean hulls. Eight morphologically distinct fungi were placed on PDA plates at 28 °C and subcultured periodically. The microorganisms were grown by Submerged fermentation (Sbm) and Solid state fermentation (SSF) according to the method described by Visser and coauthors. [4] After the obtainment of enzymes produced by both fungi, a nylon cloth was used to separate solids by filtration, followed by centrifugation at 15,000g for 10 minutes. The clarified enzyme extracts of each fungi were frozen and stored for further analysis.

Enzyme activities and determination of synergy. All the enzymatic assays were carried out in sodium acetate buffer, 100 mM, pH 5, at 50° C. They were performed in triplicate and the mean values were calculated. Relative standard deviations of the measurements were below 5%. FPase and endoglucanase activities were determined using Whatman No. 1 filter paper (1 x 6 cm, 50 mg) and 1.25% (w/v) CMC as substrates, respectively, according to previously described standard conditions. [5] The total reducing sugars released during the enzymatic assays were quantified by the dinitrosalicylic acid (DNS) method using glucose as the standard. [6] Xylanase, β-Glucosidase, β-xylosidase and cellobiohydrolase activities were measured using xylan from birchwood (1% w/v), ρPNGlc, ρNPXyl and ρNPCel (1 mM) as substrates, respectively. The enzymatic assays wre perfomed as described by Maitan-Alfenas et al., (2015). One unit of enzymatic activity (U) was defined as the amount of enzyme that released 1 µmol of corresponding product per minute, under the assay conditions used. The level of synergy presents in the

enzyme cocktails was calculated by comparison between the measured enzymatic activities of this extract and the theoretical activity expected of the same enzyme according to the equations below:

$$Theoretical\ enzyme\ activity = \frac{Enzyme\ activity\ 1 + Enzyme\ activity\ 2}{2} \quad (1)$$

$$Synergy\ (\%) = \frac{Enzyme\ activity\ 1 \times 100}{Theoretical\ enzyme\ activity} \quad (2)$$

Production of enzyme cocktails. The best enzyme extract produced by submerged fermentation (Sbm) was used to extract the enzymes secreted during SSF by other fungi also selected as the best, under agitation of 150 rpm for 60 min at room temperature. Solids were separated by filtration through nylon cloth followed by centrifugation at 15,000*g* for 10 min; and the clarified supernatants were frozen and stored for subsequent analysis.

Enzymatic hydrolysis. Enzymatic hydrolysis was performed in 125 mL Erlenmeyer flasks containing 2%(w/v) of hydrothermally pretreated soybean hulls at 160ºC, 30 min in 100mM sodium citrate buffer at pH 4.8 in a final volume of 25 mL. Enzyme loading was specified as 8 units per gram of biomass. The reaction was carried out in an orbital shaker at 200 rpm and 50°C for 72 hr. This was followed by the samples being immediately heated to 100°C to denature the enzymes, centrifuged for 5 min at 15,000 g, and then cooled until use. Glucose, xylose and arabinose concentrations were analyzed by HPLC equipped with Aminex ion exclusion HPX-87H (Bio-Rad Labs, Hercules, CA). The column was eluted with a mobile phase (5 mmol/L H_2SO_4) at a flow rate of 0.6 mL/min and it operated at 65°C. All analytical values were calculated from triplicates.

3 RESULTS & DISCUSSION

The use of on-site enzyme cocktails produced by submerged (Sbm) and Solid-state fermentation (SSF) using agroindustrial wastes, such as soybean hulls in natura, can be a viable option to improve the efficiency of bioconversion and reduce its cost.

Table 1. Analyses of crude extracts from fungi cultivated by submerged and Solid State fermentation.

Fungo		Enzyme activity (U/mL)					
		FPase	CMCase	β-glucosidase	β-celobiohydrolase	Xilanase	β-xylosidase
A	Sbm	0.03 ± 0.01	0.64 ± 0.02	0.05 ± 0.001	0.01 ± 0.00	0.69 ± 0.03	0.02 ± 0.001
	SSF	0.06 ± 0.001	1.50 ± 0.03	0.25 ± 0.03	0.39 ± 0.02	5.34 ± 0.03	0.14 ± 0.03
B	Sbm	0.04 ± 0.01	1.87 ± 0.05	0.02 ± 0.00	0.04 ± 0.001	4.7 ± 0.08	0.04 ± 0.02
	SSF	0.05 ± 0.00	1.68 ± 0.02	0.007 ± 0.00	0.03 ± 0.00	2.17 ± 0.09	0.04 ± 0.01
C	Sbm	0.05 ± 0.01	1.02 ± 0.02	0.26 ± 0.02	0.03 ± 0.00	1.41 ± 0.03	0.003 ± 0.00
	SSF	0.004 ± 0.00	5.53 ± 0.07	0.20 ± 0.001	0.21 ± 0.02	9.65 ± 0.06	0.16 ± 0.02
D	Sbm	0.03 ± 0.01	5.05 ± 0.09	0.01 ± 0.00	0.08 ± 0.001	11.8 ± 0.6	0.05 ± 0.01
	SSF	0.06 ± 0.001	1.92 ± 0.03	0	0.02 ± 0.00	2.17 ± 0.03	0
E	Sbm	0.03 ± 0.00	0.61 ± 0.03	0.03 ± 0.00	0.03 ± 0.002	0.9 ± 0.07	0.004 ± 0.00
	SSF	0.05 ± 0.00	2.82 ± 0.03	0.18 ± 0.001	0.35 ± 0.04	4.69 ± 0.4	0.02 ± 0.00
F	Sbm	0.02 ± 0.00	0.59 ± 0.01	0.001 ± 0.00	0.02 ± 0.00	4.65 ± 0.5	0.002 ± 0.00
	SSF	0.07 ± 0.002	1.89 ± 0.02	0.16 ± 0.02	0.19 ± 0.02	1.90 ± 0.05	0.03 ± 0.001
G	Sbm	0.003 ± 0.00	0.54 ± 0.03	0.25 ± 0.04	0.03 ± 0.001	0.45 ± 0.08	0.02 ± 0.00
	SSF	0.05 ± 0.002	3.18 ± 0.04	0.43 ± 0.06	0.92 ± 0.07	7.40 ± 0.04	0.01 ± 0.00
H	Sbm	0.02 ± 0.00	0.45 ± 0.05	0.01 ± 0.00	0.004 ± 0.00	2.3 ± 0.3	0.02 ± 0.001
	SSF	0.04 ± 0.00	3.36 ± 0.08	0.01 ± 0.00	0.04 ± 0.01	2.97 ± 0.08	0.01 ± 0.00

All eight fungi were able to secrete the lignocelluloytic enzymes by submerged and solid state fermentation when cultivated on soybean hulls. It shows that soybean hulls is an interesting inducing source, mainly for fungus D cultivated by Sbm fermentation and for fungus C and G cultivated by SSF fermentation. These fungi were selected as the best due to the variety of enzymes secreted as well as the highest values of the main cellulases and hemicellulases presented.

As can it observed in Table 2, the blend of crude enzymatic extracts of fungi C, D and G led to the synergy of the most of enzymes evaluated.

Table 2. Comparative analysis of cellulases and hemicellulases activities present in the cocktails applied in the saccharification experiment.

Enzyme	Crude enzymatic extracts			Enzyme cocktails			
	Fungus D (Sbm)	Fungus C (SSF)	Fungus G (SSF)	C + D		C + G	
				U/mL	Synergy level (%)	U/mL	Synergy level (%)
FPase	0.1 ± 0.01	0.71	0.54	0.27	n.d.	0.14	n.d.
CMCase	6.42 ± 0.4	5.61	4.2	12.9	114	8.00	63
β-glucosidase	1.54 ± 0.2	0.53	1.51	2.99	189	2.13	109
Cellobiohydrolase	1.15 ± 0.2	0.25	0.99	1.15	64	0.62	n.d.
Xylanase	10.98 ± 0.5	7.01	4.6	25.1	179	17.5	200
β-xylosidase	0.26 ± 0.01	0.26	0.03	0.09	n.d.	0.17	183

n.d., not detected

Synergy is defined as the interactions that occur between two or more hydrolytic components, producing an effect total greater than the sum of the effects of the individual components. [4] Previous studies have demonstrated that fungal species cultivated in monocultures and consortia by SSF can be employed for the production of lignocellulose-degrading enzymes with synergistic actions, which exhibited satisfactory sugar yields for biomass hydrolysis. [7]

Overall the cellulose and hemicellulose conversion yields on soybean hulls saccharification were low using all enzyme sources tested. However, the positive effect from mixtures of fungal enzyme extracts was observed. For instance, the enzyme cocktail containing the enzymes secreted by the fungi C and D was able to release 44.1% and 13.5% more glucose and xylose, respectively, than the enzymes from fungus C applied alone. If compared to crude enzyme extract from D, the release of sugars was even greated (55% of glucose and 119% of xylose). The same behaviour was observed for mixture of enzyme from G and D fungi. The blend of fungal extracts provided a greater release of glucose and xylose, highlighting the synergistic effect of the enzymes during the hydrolysis of soybean hulls.

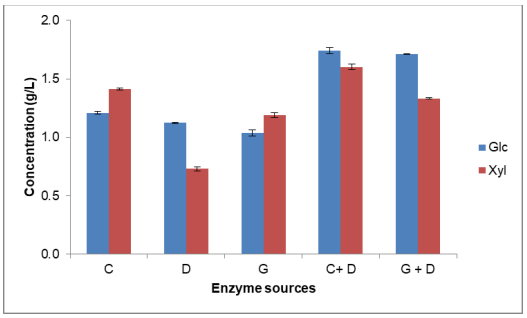

Figure 1 Enzymatic hydrolysis of hydrothermal soybean hulls pretreated (2% w/v) with different *on-site* produced ezymes cocktails (8 FPU/ g biomass) at 50° C, 200 rpm for 72h.

4 CONCLUSION

Soybean hulls is an environmentally friendly carbon source to produce enzymes from different fungi either by submerged or solid state fermentation. In this study, enzyme blend from mixture of different *on-site* crude enzymatic extract was able to improve the release of glucose and xylose during the hydrothermal pretreated soybean hulls when compared the individual use of them. The results provide the importance of the joint and synergistic effect of lignocellulolytic enzymes in order to achieve better yields in the biomass saccharification.

REFERENCES

[1] FARINAS, C.S., FLORENCIO, C., BADINO, A.C. 2018. On-Site Production of Cellulolytic Enzymes by the Sequential Cultivation Method. In: Lübeck, M. (eds) Cellulases. Methods in Molecular Biology, vol 1796. Humana Press, New York, NY.
[2] DE ALBUQUERQUE, M.F.G., LEAL, T.F., LADEIRA AZAR, R.I.S., MILAGRES, A.M.F., GUIMARÃES, V.M., DE REZENDE, S.T. 2021.
Hydrothermal pretreatment as strategy for the improvement of sugarcane bagasse saccharification by fungal enzyme blend. Braz. arch. biol. technol. 64:1-13.
[3] MAITAN-ALFENAS, G.P., VISSER, E.M., GUIMARÃES, V.M. 2015. Enzymatic hydrolysis of lignocellulosic biomass: Converting food waste in valuable products. Curr Opin Food Sci. 1:44–49.
[4] VISSER, E.M., FALKOSKI, D.L., DE ALMEIDA, M.N., MAITAN-ALFENAS, G.P, GUIMARÃES, V.M. 2013. Production and application of an enzyme blend from *Chrysporthe cubensis* and *Penicillium pinophilum* with potential for hydrolyses of sugarcane bagasse. Bioresour Technol. 144:587–594.
[5] GHOSE, T.K. Measurement of cellulase activities. 1987. Pure Appl Chem. 59 (2) 257–68.
[6] MILLER, G.L. Use of dinitrosalicyclic reagent for determination of reducing sugar. 1959. Anal Chem. 31:426–428.
[7] RODRIGUES, P. O., BARRETO, E.S., BRANDÃO, R.L., GURGEL, L.V.A., PASQUINI, D., BAFFI, M.A. 2022. Waste and Biomass Valorization. 13:95-106.

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