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Lignocellulosic bioconversion by an optimized enzymatic cocktail from *Irpex lacteus*

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Abstract

Lignocellulosic residues generated by the agro-forestry industry are low-cost renewable biomass, rich in cellulose, and can be valorized by converting it into second-generation bioethanol. This work aimed to convert the rice straw, pine, and eucalyptus sawdust into fermentable sugars by applying an enzymatic cocktail from *Irpex lacteus* LBM 034 optimized for enhancing cellulase levels. The most influential medium compounds in the production of the fungal cellulolytic enzymes were identified by a Plackett Burman design. The most significant compounds were xylan, carboxymethyl-cellulose, and peptone. The physical conditions for fungal incubation were optimized using response surface methodology, with 32 °C and pH 5.5 without agitation identified as the optimal conditions. Under these conditions, the level of endo-β-1,4-glucanases in the cocktail was 2.6 times higher than the original. The optimized fungal cocktail was then applied to hydrolyze the polysaccharides of NaOH-pre-treated rice straw, pine, and eucalyptus sawdust into fermentable sugars. The use of this enzymatic cocktail enabled efficient hydrolysis of rice straw, yielding in 27.19% and 38.18% of hemicellulose and cellulose conversion, respectively. This bioconversion of cellulose and hemicellulose of renewable biomass into fermentable sugars represents a cost-effective bioprocess for applications such as the production of lignocellulosic bioethanol.

Keywords – cellulose – cellulases – endo-β-1,4-glucanase – fermentable sugars – hemicellulose – Plackett-Burman design – response surface methodology – saccharification

Introduction

Lignocellulosic wastes generated by agroforestry industries such as wheat straw, corn stover, and sawdust are abundant, renewable, and low-cost sources. These wastes can be valorized as feedstock for biotechnological processes such as the second-generation (2G) bioethanol. The enzymatic hydrolysis of cellulose to fermentable sugars is a critical step to maximize the efficiency of the 2G bioethanol production (Soccol et al. 2010, Díaz et al. 2019, Sharma et al. 2020).

Cellulose is a linear glucose polymer composed of β-1,4-glucose units linked by β-1,4-D-glycosidic bonds (Sohail et al. 2016). The bioconversion of cellulose to simple sugars requires the use of cellulases. A representative cellulase system includes at least three hydrolytic enzyme groups: endo-β-1,4-glucanases (EG) (EC 3.2.1.4), cellobiohydrolases (CBH) (EC 3.2.1.91), and β-1,4-

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glucosidases (BGL) (EC 3.2.1.21) (Barbosa et al. 2020). Copper-dependent enzymes such as the lytic polysaccharide monooxygenases (LPMOs) can also degrade the cellulase through an oxidative mechanism (Johansen 2016). Endo-β-1,4-glucanases are one of the major component enzymes of the cellulase complex and have an affinity for amorphous regions of the cellulose (Barbosa et al. 2020). They catalyze the hydrolysis of cellulose by randomly cleaving internal β-glycosidic bonds, releasing oligomers of different lengths, such as cellobiose and cello-oligosaccharides (Barbosa et al. 2020). Exoglucanases, or cellobiohydrolases are processive enzymes that act on the reducing ends of cellulose chains (cellobiohydrolases I) or on the non-reducing ends (CBH II), releasing β-cellobiose, that are then hydrolyzed to glucose by cellobioases or β-1,4-glucosidases (Barbosa et al. 2020).

The use of enzymes represents a high operational cost for 2G bioethanol production, and only a limited number of multinational companies produce enzymatic cocktails (Zhang et al. 2006; Barbosa et al. 2020). Therefore, optimizing enzyme production is essential (Singhal et al. 2022). In this context, the Plackett-Burman design (PBD) is initially employed as an initial screening to identify the most influential factors affecting the process. PBD is a statistical screening method that allows for the evaluation of a large number of variables using a minimal number of experimental trials (Plackett & Burman 1946). To further refine and optimize these most significant factors, Response Surface Methodology (RSM) is widely applied. RSM is a multivariate statistical technique based on ANOVA that enables the modeling and optimization of experimental conditions to enhance process efficiency (Singhal et al., 2022). Furthermore, screening, characterizing, and optimizing novel sources of enzymes from organisms has been extensively studied (Jurado et al. 2011, Coniglio et al. 2017, Giorgio et al. 2018, Díaz et al. 2021). Several fungi and bacteria are capable of producing multiple enzymes that can degrade the cellulose (Barbosa et al. 2020). A promising alternative is to search for novel sources from biodiversity-rich environments such as the Paranaense rainforest (Díaz et al. 2021, Vignale et al. 2022). Exploration of new fungal strains can reveal abilities for bioprocesses such as bioconversion (Giorgio et al. 2012). One such fungus, *Irpex lacteus* LBM 034, was isolated from this rainforest and selected due to its high cellulase production in previous study (Giorgio et al. 2018). This work aimed to optimize the cellulolytic enzyme production by the fungus *I. lacteus* LBM 034, a strain isolated from the subtropical rainforest of Misiones province, Argentina, and transform lignocellulosic residues by applying the enzymes produced by this fungus.

Materials & Methods

Organism and cultivation conditions

The organism used for this study was *Irpex lacteus* LBM 034. The fungus was selected due to its high levels of cellulase filter paper and EG activities as reported in previous work (Giorgio et al. 2018). The fungus was deposited in the culture collection of the Laboratorio de Biotecnología Molecular of the Instituto de Biotecnología Misiones, Universidad Nacional de Misiones, Argentina, and maintained in stock by periodic sub-culturing on malt extract agar (MEA; 12.7 g L^{-1} malt extract and 20 g L^{-1} agar) plates at 4 °C (Saparrat et al. 2002).

Screening of nutritional sources for cellulase production by I. lacteus LBM 034

To identify the significant nutritional factors affecting the production of cellulases by *I. lacteus* LBM 034, the effects of thirteen factors were evaluated by applying a Plackett Burman Design (PBD) (Plackett & Burman 1946). Among factors, six carbon sources, three nitrogen sources, a surfactant, and three dummy variables were each screened at two levels (-1 and +1 for low and high levels, respectively) (Table 1) across 20 experimental runs. Erlenmeyer flasks containing 30 mL of Czapek minimum medium supplemented with the combination of factors of each experimental run according to the PBD matrix (Table 2) were sterilized at 121 °C for 20 min previous pH adjustment to 4.8. Each medium was inoculated with agar plugs (5 mm diameter) covered by young mycelium of *I. lacteus* LBM 034 and incubated at 28 ± 2 °C for 14 days. Samples of the culture broths were aseptically collected every 48 hours to monitor axenic fermentation. After the incubation, culture broths were

centrifuged at 10000 g and 4 °C for 20 min to obtain the cell-free enzymatic cocktails, which were used for determining cellulase activities.

Table 1 Nutritional sources of the culture medium, including their real and coded values (-/+) evaluated in the PBD for the production of cellulases by *I. lacteus* LBM 034.

Factors		Symbol	Coded factor level	
			Low -1 (gL^{-1})	High +1 (gL^{-1})
Carbon sources	Lactose	X_1	0	10
	Glucose	X_2	0	10
	Xylose	X_3	0	10
	Xylan	X_4	0	10
	CMC	X_5	0	10
	CC	X_6	0	10
	Dummy variable I	X_7	--	--
Nitrogen sources	Urea	X_8	0	1
	Peptone	X_9	0	1
	Ammonium Sulphate	X_{10}	0	1
	Dummy variable II	X_{11}	--	--
Surfactant	Tween 20*	X_{12}	0	1
	Dummy variable III	X_{13}	--	--

*Tween 20 is expressed in ml L^{-1}.

The effect of each factor on the cellulase production of *I. lacteus* LMB 034 was calculated according to the following equation

$$E_i = \Sigma R_{i+} - \Sigma R_{i-} /N \qquad (1)$$

where E_i is the effect of each factor i under study, R_{i+} and R_{i-} are the responses of the cellulolytic production of the experimental runs, in which the nutritional source was at its upper and lower levels, respectively, and N is the total number of experimental runs.

The experimental matrix was designed, and the results were analyzed using the software Statgraphics Centurion XVI.I (StatPoint, Inc. version 15.2.05). The variables with confidence levels above 95% were considered to be significant.

Optimization of nutritional sources for EG production by I. lacteus LBM 034

To enhance EG production, the selected nutritional factors were optimized using Response Surface Methodology. Among these methodologies, the central composite design is one of the most widely used experimental designs to model response surfaces (Marget & Morris 2019). In this study, an orthogonal central composite design (RCCD) with 17 runs at five levels (-α, -1, 0, +1, +α) and four replicates at its central point was employed (Table 3). Erlenmeyer flasks containing 30 mL of Czapek minimum medium, supplemented with the combination of factors for each experimental run according to the RCCD matrix (Table 3), were sterilized, inoculated, and incubated as described for PBD experimental runs. Samples of the culture broths were taken in sterile conditions at every 48 h for monitoring an axenic fermentation and determining EG activity of the resulting enzymatic cocktails.

The behavior of the design is explained by the following quadratic equation:

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ij} X_i X_j + \Sigma\beta_i X_i^2 \qquad (2)$$

where Y is the predicted response; X_i and X are the input variables that influence the response variable Y; β_0 is the intercept; β_i is the linear coefficient of X_i; β_{ij} is the interaction coefficient between X_i and X_j; β_{ii} is the quadratic coefficient of X_i.

The RCCD matrix and the analysis of experimental results were performed using the software Statgraphics Centurion XVI.I (StatPoint, Inc. version 15.2.05).

Optimization of physical variables for EG production by I. lacteus LBM 034

Three physical variables, temperature, pH, and agitation speed (AS), were optimized for enhancing EG production by *I. lacteus* LBM 034 using an RCCD of 17 runs at five different levels (-α, -1, 0, +1, +α) and four replicates at its central points (Table 4). Erlenmeyer flasks containing 30 mL of MMC supplemented with the optimized nutritional sources were sterilized and inoculated as described earlier. Flasks were incubated at different conditions according to the experimental runs of the RCCD matrix (Table 4). Samples of the culture broths were taken in sterile conditions at every 48 h for monitoring an axenic fermentation and determining EG activity of enzymatic cocktails.

The behavior of the design is explained by the quadratic equation (3). RCCD matrix and the analysis of experimental results were performed using the software Statgraphics Centurion XVI.I (StatPoint, Inc. version 15.2.05).

Biological validation of the optimization of physical variables for EG production

To validate the theoretical optimization of the model, three experiments were carried out under the predicted conditions decoded by the following equation:

$$X_i = (X_i - X_0)/\Delta X_i \qquad (3)$$

where, x_i is the dimensionless value of an independent variable; X_i is the real value of an independent variable; X_0 is the value of X_i at the center point, and ΔX_i is the step change.

Erlenmeyer flasks containing media were sterilized, inoculated, and incubated under the predicted physical variables by the model. Enzymatic cocktails were used for determining EG activity.

Determination of enzyme activities

EG activity was assayed according to Ghose & Bisaria (1987) using 2% (w/v) carboxymethyl-cellulose (CMC, Sigma Aldrich, USA) in 0.05 sodium acetate buffer (pH 4.8) as the substrate; reducing sugar released was measured by the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). EG activity was expressed in U, defined as the amount of enzyme required to release 1 μmol of glucose per minute under the assay conditions.

CBH and BGL activities were determined through the quantification of the ρ-nitrophenol method according to Wu et al. (2006) and Ghose & Bisaria (1987), respectively, using 0.007% (w/v) 4-nitrophenyl-β-D-cellobioside (p-NPC, Sigma-Aldrich, USA) and 4-nitrophenyl-β-glucopyranoside (p-NPG, Sigma-Aldrich, USA) in 0.05 M sodium acetate buffer pH 4.8 as substrates, respectively. CBH and BGL activities were expressed in U, defined as the amount of enzyme required to release 1 μmol of p-nitrophenol per minute under the assay conditions.

Lignocellulosic residues preparation

Eucalyptus (*Eucalyptus grandis*) sawdust was collected at the Ligna sawmill, Corrientes, Argentina. Pine (*Pinus taeda*) sawdust was collected at the Valerio Oliva S. A. sawmill, Misiones, Argentina. Rice (*Oryza sativa*) straw was collected at San Salvador, Entre Ríos, Argentina. All residues were dried at 60 ºC for 24 h and milled by the Brabender-Duisburg (242-WI267) grinder to obtain a material retained through a 40-mesh.

Bioconversion of lignocellulosic residues

Eucalyptus, pine sawdust, and rice straw were pre-treated with a thermo-alkaline technique. For that, 2.5 g (dry weight) of each residue was incubated in 20 mL NaOH 1.5 or 2.0 N at 120 °C, 1 atm for 60 min (López-Miranda et al. 2009). Liquid fractions were sampled from each experiment to further determination of reducing sugars (Miller, 1959), and total phenolic content (TPC) (Singleton & Rossi 1965). Then, the residues were washed with water, 0.5 M sodium acetate buffer pH 4.0; filtered; and dried at 45 °C for 24 h.

Pre-treated lignocellulosic residues were hydrolyzed using the optimized enzymatic cocktail of *I. lacteus* LBM 034 according to the protocol by the National Renewable Energy Laboratory (NREL) (Selig et al. 2008) with modifications. Also, the hydrolysis was carried out by the commercial cellulase from *Aspergillus niger* (C1184-5KU, Sigma-Aldrich, USA) to compare their performance. Each pre-treated lignocellulosic biomass was incubated in bioreactors at 1% (m/v) consistency, containing the optimized fungal cocktail (10 FPU U g^{-1} of biomass) or the commercial cellulase, Tween 80 (2.5 g L^{-1}) and sodium acetate buffer 50 mM pH 4.0. Hydrolysis was carried out at 50 °C, 120 rpm for 72 h. Samples were taken at 0, 6, 12, 24, 36, 48, and 72 h, heated at 100 °C for 10 min, and centrifugated at 10000 g for 5 min. Then, released reducing sugars and glucose were determined according to Miller (1959) and the enzymatic commercial kit Glicemia (Wiener Laboratory SAIC), respectively. Values were used to build the progress curves for the bioconversion of lignocellulosic residues.

The percentage of cellulose conversion was calculated according to the following equation (Selig et al. 2008):

$$\textit{Cellulose conversion}\ (\%) = \left(\frac{\text{released reducing sugars } (g/L) \times 0.9}{\text{cellulose in lignocellulosic residue } (g/L)}\right) \times 100 \quad (4)$$

The percentage of holocellulose conversion was calculated using the following equation:

$$\textit{Holocellulose conversion}\ (\%) = \left(\frac{\text{released reducing sugars } (g/L) \times 0.9}{\text{holocellulose in lignocellulosic residue } (g/L)}\right) \times 100 \quad (5)$$

Results

Screening of the most significant nutritional sources for cellulase production by I. lacteus LBM 034

The experimental values of production of cellulases in the different runs of the PBD were varied, from not detected (ND) to 499.95 U L^{-1} for EGs; from ND to 109.74 U L^{-1} for CBH; and from ND to 110.44 U L^{-1} for BGL (Table 2).

Multiple regression analysis for the production of the three cellulases showed R^2 of almost 0.9, indicating that the model adequately represented the observed production of EG, CBH, and BGL. Moreover, the lack of fit was not significant, indicating that the models adequately represented the observed at a 95% confidence level.

Factors that presented statistically significant effects ($p \leq 0.05$) on cellulase production are shown in Fig. 1. CMC, xylan, peptone, urea, and ammonium sulfate were the most significant nutritional sources at their +1 level for EG production while xylose was significant at its -1 level (Fig. 1a). CMC, xylan, peptone, and urea were the most significant nutritional sources for CBH and BGL production at their +1 level (Fig. 1b, c).

Excluding the nonsignificant variables, the equations of the model for the production of EGs, CBHs, and BGLs are Eq. 6, 7, and 8, respectively:

$$y_{\text{EG production}} = 202.6 - 50.0{*}\text{Xylose} + 72.2{*}\text{Xylan} + 91.8{*}\text{CMC} + 41.1{*}\text{Urea} + 91.1{*}\text{Peptone} + 39.8{*}\text{Ammonium sulfate} - 43.4{*}\text{Dummy variable II} \quad (6)$$

$$y_{\text{CBH production}} = 32.4245 + 7.0115*\text{Xylan} + 10.4225*\text{CMC} - 9.0915*\text{Dummy variable I} + 12.2545*\text{Urea} + 26.9615*\text{Peptone} \quad (7)$$

$$y_{\text{BGL production}} = 36.403 + 8.704*\text{Xylan} + 8.682*\text{CMC} - 9.548*\text{Dummy variable I} + 9.632*\text{Urea} + 30.315*\text{Peptone} \quad (8)$$

As the fungus *I. lacteus* LBM 034 reached the highest levels of EGs ($p < 0.05$), the optimization of the nutritional sources and the physical variables were focused on this enzyme production. In addition, the medium used for carrying out the following optimizations was the medium of the experimental run No. 17 because it yielded the highest EG production.

Table 2 PBD matrix for screening nutritional sources and cellulolytic activities of *I. lacteus* LBM 034.

Run no.	*Coded variables*													*Activity (U L^{-1})*		
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	EG	CBH	BGL
1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	ND	4.13	ND
2	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	ND	ND	ND
3	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	ND	ND	ND
4	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	269.11	20.33	20.01
5	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	ND	ND	ND
6	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	325.23	ND	19.94
7	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	ND	5.18	6.01
8	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	364.76	8.45	17.17
9	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	325.22	33.28	49.40
10	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	358.39	86.72	105.58
11	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	350.73	67.39	79.66
12	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	ND	8.21	11.18
13	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	ND	4.17	ND
14	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	457.86	78.43	78.91
15	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	ND	61.19	71.78
16	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	ND	29.60	40.00
17	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	499.95	109.74	110.44
18	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	350.73	109.18	91.46
19	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	306.09	9.56	13.14
20	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	443.84	14.93	13.39

EG: endo-β-1,4-glucanase; CBH: cellobiohydrolase activity; BGL: β-glucosidase activity; ND: Not detected.

Optimization of nutritional sources for EG production by I. lacteus LBM 034

The three most significant nutritional sources for EG production were optimized by an RCCD. Levels of EGs of days 5, 7, 9, 11, and 13 are shown in Table 3. Despite the lack of fit indicating that the model was not adequate for predicting the influence of nutritional sources on EG production, there was a 155% improvement in EG production regarding the PBD values, from 499.95 U L^{-1} (PBD run No. 17, Table 2) to 777.98 U L^{-1} (RDCC run No. 10 at day 5, Table 3).

Optimization of physical variables for EG production by I. lacteus LBM 034

Irpex lacteus LBM 034 produced different levels of EGs according to the different combinations of temperature, pH, and AS (Table 4). The quadratic expressions for temperature ($p < 0.0315$) and pH ($p < 0.0032$) were significant on EG production indicating that their optimal levels were around their central points, 30 °C and pH 5, respectively. The coefficient of determination R^2 was 80.70 and the lack of fit was not significant ($p > 0.5405$) indicating that the model could appropriately explain the variability in the response.

Table 3 RCCD matrix for optimizing nutritional sources to enhance EG production by *I. lacteus* LBM 034.

Run no.	*Coded value (and real values, gL^{-1})*			*EG Activity ($U\ L^{-1}$)*				
	CMC	**Peptone**	**Xylan**	**Day 5**	**Day 7**	**Day 9**	**Day 11**	**Day 13**
1	-1 (4)	-1 (3)	-1 (3)	584.12	474.44	283.13	ND	ND
2	1 (8)	-1 (3)	-1 (3)	568.82	512.70	499.95	459.14	474.44
3	-1 (4)	1 (6)	-1 (3)	443.83	528.01	464.24	290.79	ND
4	1 (8)	1 (6)	-1 (3)	ND	ND	ND	ND	ND
5	-1 (4)	-1 (3)	1 (10)	556.07	438.73	581.57	507.60	408.12
6	1 (8)	-1 (3)	1 (10)	586.67	617.28	619.83	586.67	298.44
7	-1 (4)	1 (6)	1 (10)	443.83	528.01	464.24	290.79	ND
8	1 (8)	1 ()	1 (10)	681.05	686.15	714.21	686.15	464.24
9	-1.68 (1.27)	0 (4.5)	0 (6.5)	471.89	469.34	364.76	209.16	ND
10	1.68 (14.73)	0 (4.5)	0 (6.5)	777.98	734.62	688.71	711.66	405.57
11	0 (12)	-1.68 (1.98)	0 (6.5)	586.67	668.30	543.31	364.76	209.16
12	0 (12)	1.68 (7.02)	0 (6.5)	619.83	594.33	556.07	403.02	ND
13	0 (12)	0 (4.5)	-1.68 (0.61)	604.53	476.99	428.53	326.50	255.08
14	0 (12)	0 (4.5)	1.68 (12.39)	612.18	614.73	637.69	645.34	456.59
15	0 (12)	0 (4.5)	0 (6.5)	484.64	497.40	512.70	387.72	255.08
16	0 (12)	0 (4.5)	0 (6.5)	448.93	522.91	548.41	323.95	244.87
17	0 (12)	0 (4.5)	0 (6.5)	456.59	545.86	548.41	410.67	275.48

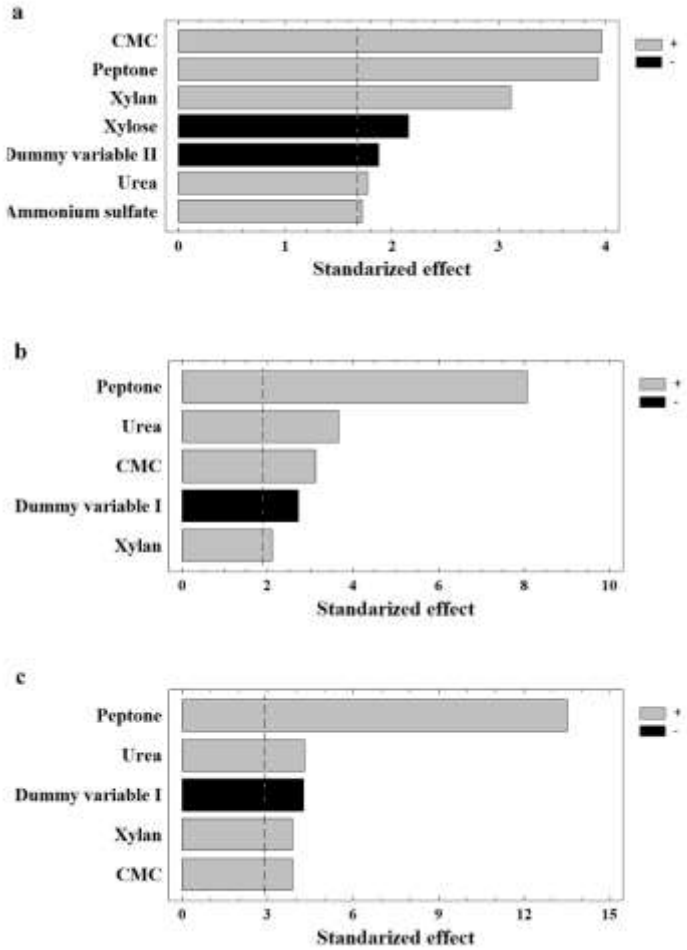

Fig. 1 – Pareto charts for cellulases production by *I. lacteus* LBM 034. The length of the bars is proportional to the standardized effect of nutritional sources on a EG. b CBH. and c BGL production. Bars extending beyond the vertical line correspond to statistically significant at a 95% confidence level. EG: endo-β-1,4-glucanase; CBH: cellobiohydrolase; BGL: β-glucosidase.

Table 4 RCCD matrix for optimizing physical variables to enhance EG production by *I. lacteus* LBM 034.

Run no.	*Coded value (and real values)*			*Response*
	T (°C)	**pH**	**AS (rpm)**	**EG Activity (U L^{-1})**
1	-1 (23)	-1 (3.5)	-1 (30.36)	ND
2	+1 (35)	-1 (3.5)	-1 (30.36)	ND
3	-1 (23)	+1 (6.5)	-1 (30.36)	207.53
4	+1 (35)	+1 (6.5)	-1 (30.36)	487.46
5	-1 (23)	-1 (3.5)	+1 (119.64)	ND
6	+1 (35)	-1 (3.5)	+1 (119.64)	ND
7	-1 (23)	+1 (6.5)	+1 (119.64)	220.98
8	+1 (35)	+1 (6.5)	+1 (119.64)	ND
9	-1.68 (18.91)	0 (5)	0 (75)	140.23
10	+1.68 (39.09)	0 (5)	0 (75)	400.01
11	0 (29)	-1.68 (2,48)	0 (75)	ND
12	0 (29)	+1.68 (7.52)	0 (75)	ND
13	0 (29)	0 (5)	-1.68 (0)	800.54
14	0 (29)	0 (5)	+1.68 (150)	385.17
15	0 (29)	0 (5)	0 (75)	800.14
16	0 (29)	0 (5)	0 (75)	539.76
17	0 (29)	0 (5)	0 (75)	450.87

EG: endo-β-1,4-glucanase activity; T: temperature; AS: agitation speed; ND: Not detected.

RSM plots showing interactive effects were drawn from experimental data to show the optimum value of each physical variable for maximal EG production (Fig. 2). EG production increased when the temperature was around 30 °C, with pH fixed at its middle level (Fig. 2a). The maximal EG production occurred when the AS was at its lowest level, 0 rpm, with the temperature fixed at its middle level (Fig. 2b). The highest EG production was achieved when both pH and temperature were at their center levels, pH 5 and 30 °C, respectively, with agitation speed (AS) fixed at its middle level (Fig. 2c).

The equation of the adjusted model for EG production (y) without the nonsignificant factors is the following:

$$y_{\text{EG production}} = 606.875 + 36.3075*\text{Temperature} + 67.0704*\text{pH} - 85.8599*\text{AS} - 149.821*\text{Temperature}^2 + 7.36875*\text{Temperature}*\text{pH} - 62.6138*\text{Temperature}*\text{AS} - 245.323*\text{pH}^2 - 59.2513*\text{pH}*\text{AS} - 35.7164*\text{AS}^2 \quad (9)$$

The optimal physical conditions for EG production predicted by the model equation were 32 °C, and pH 5.5 without agitation. Under these conditions, the model estimated 713.24 U L^{-1} of EG activity. Three experimental runs were carried out under the predicted conditions. The average EG production obtained was 692.54 ± 20.91 U L^{-1}, showing concordance with the predicted value (Table 5).

Table 5 Model validation of RCCD for physical variables to enhance EG production by *I. lacteus* LBM 034.

Physical variables	*Optimal*		*EG activity (U L^{-1})*		$VC_{Predicted\ \&Real}$
	Coded	**Real**	**Predicted**	**Real**	
T (°C)	0.48118	32			
pH	0.346947	5.5	713.24	692.54 ± 20.91	2.1%
AS	-1.68179	0			

EG: endo-β-1,4-glucanase activity; T: temperature; AS: agitation speed; VC: variation coefficient

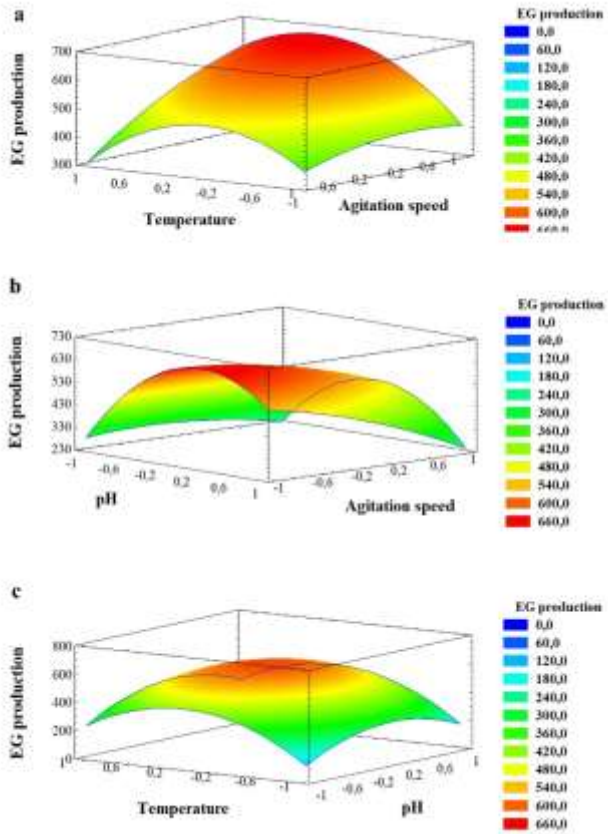

Fig. 2 – Response surface plots showing the interactive effects of physical variables on EG production by *I. lacteus* LBM 034. a Three-dimensional surface plot for AS and T when pH is fixed at its middle level. b Three-dimensional surface plot for pH and AS when T is fixed at its middle level. c Three-dimensional surface plot for T and pH when AS is fixed at its middle level. The maximum EG production is shown in U mL^{-1}. EG activity. AS, agitation speed; T, temperature; EG, endo-β-1,4-glucanase.

Table 6 NaOH concentrations employed in the pre-treatment of the lignocellulosic residues. Release of reducing sugars and soluble phenols in the liquid fraction after the alkaline pre-treatment of the lignocellulosic residues.

Lignocellulosic material	***NaOH [N]***	***Reducing sugars*** **mg g^{-1} (dry biomass)**	***Total phenols*** **mg g^{-1} (dry biomass)**
Eucalyptus sawdust	1.5	9.07 ± 1.44[a]	1.95 ± 0.24[a]
	2	10.16 ± 0.82[a]	2.01 ± 0.08[a]
Pine sawdust	1.5	3.37 ± 0.65[b]	1.07 ± 0.18[b]
	2	3.53 ± 0.08[b]	1.15 ± 0.04[b]
Rice straw	1.5	3.56 ± 0.58[b]	2.13 ± 0.02[a]
	2	3.95 ± 1.35[b]	2.23 ± 0.08[a]

[a,b] Values with the same letters in the same column indicate no significant differences (p>0.05) among them, according to the Tukey test.

Bioconversion of lignocellulosic residues by enzymatic hydrolysis

Eucalyptus sawdust, pine sawdust, and rice straw were physically and chemically pre-treated before the enzymatic hydrolysis. The alkaline pre-treatment of the lignocellulosic residues enhanced the accessibility of cellulose in these materials. There was no significant difference ($p > 0.05$) between the pre-treatments carried out with two NaOH concentrations regarding the release of

reducing sugars and total phenols. Hence, we chose the alkaline pre-treatment at 1.5 N because it reduced the operative costs. On the other hand, the pine sawdust showed the highest release of total phenols ($p < 0.05$), while the eucalyptus sawdust exhibited the most loss of reducing sugars ($p < 0.05$) (Table 6).

The bioconversion of the pre-treated lignocellulosic residues was carried out using the optimized enzymatic cocktail from *I. lacteus* LBM 034 and the commercial enzyme from *A. niger* C1184-5KU (Sigma, Aldrich) (Fig. 3). The bioconversion of the holocellulose and cellulose of the eucalyptus sawdust and pine sawdust showed a similar profile. During the initial hours, the conversion of the holocellulose of the eucalyptus sawdust (Fig. 3a) and the pine sawdust (Fig. 3b) was similar by using both cocktails, that came from *I. lacteus* LBM 034 and the commercial one.

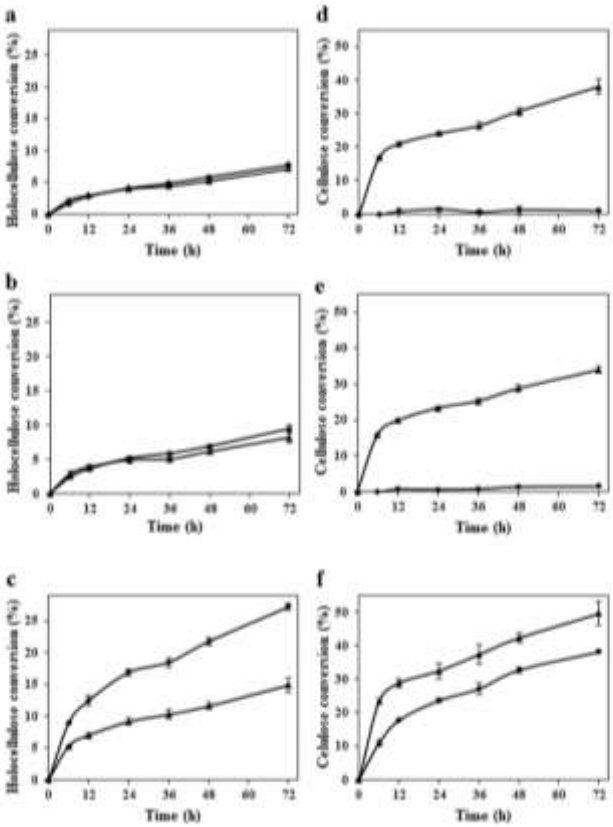

Fig. 3 – Bioconversion of the holocellulose and cellulose of the lignocellulosic residues pre-treated with 1.5 NaOH using the enzymatic cocktail from *I. lacteus* LBM 034 and the commercial enzyme from *A. niger* C1184-5KU. a Conversion of the eucalyptus sawdust holocellulose. b Conversion of the pine sawdust holocellulose. c Conversion of the rice straw holocellulose. d Conversion of the eucalyptus sawdust cellulose. e Conversion of the pine sawdust cellulose. f Conversion of the rice straw cellulose. (◆) represents the enzymatic cocktail from *I. lacteus* LBM 034 and (▲), the commercial enzyme from *A. niger* C1184-5KU.

However, at 72 h, the percentage of the holocellulose was higher ($p < 0.05$) using the enzymatic cocktail from *I. lacteus* LBM 034 (7.74 ± 0.06% and 9.44 ± 0.54% for eucalyptus sawdust and pine sawdust, respectively) than the commercial enzyme (7.19 ± 0.35% and 8.21 ± 0.75% for eucalyptus sawdust and pine sawdust, respectively). On the other hand, the conversion of the cellulose of the eucalyptus sawdust (Fig. 3d) and pine sawdust (Fig. 3e) was significantly higher ($p < 0.01$) during the process when the commercial enzyme was used instead of the cocktail produced by *I. lacteus* LBM 034. The conversion of the holocellulose of the rice straw (Fig. 3c) was significantly higher ($p < 0.01$) by using the enzymatic cocktail from *I. lacteus* LBM 034 than the commercial enzyme being 27.19 ± 0.57% and 14.91 ± 1.14%, respectively. Regarding the cellulose conversion of the rice straw

(Fig. 3f), the commercial enzyme reached a higher ($p < 0.05$) conversion percentage during the process (49.59 ± 3.53%) than the yielded by the enzymatic cocktail produced by *I. lacteus* LBM 034 (38.18 ± 0.37%).

Discussion

Lignocellulosic biomass conversion into fermentable sugars such as glucose is a common strategy for producing 2G bioethanol utilizing microbial cellulases, including those of fungal origin (Broda et al. 2022). However, the actual cost of the commercial enzyme cocktails limits their application in several small and medium-sized enterprises (Singhvi et al. 2011, Díaz et al. 2019). Hence, the optimization through the variation of the culture media composition and incubation conditions is mandatory. In this work, we performed a PBD followed by two RCCDs whose widely experimental values reflect the importance of screening nutritional sources and optimizing the physical factors to maximize fungal cellulase production. Santos et al. (2022) also highlighted the importance of studying the factors that affect the extracellular enzymes by microorganisms and stated that the response surface methodology (RSM) is considered effective for analyzing the effect of these factors and optimizing parameters for the desired response.

We optimized the EG production of *I. lacteus* LBM 034 by identifying the most influential nutrient sources by a PBD which were xylan, CMC, and peptone. CMC and xylan induced EG, CBH, and BGL activities of *I. lacteus* LBM 034, while xylose caused a decrease only in EG activity levels. The nature of the carbon source plays an important role in the differential induction of the fungal enzyme system (Nazir et al. 2010, Singhania et al. 2013, Wang et al. 2020). While the production of cellulases is repressed in the presence of simple carbon sources such as glucose, it is induced via soluble compounds such as cellobiose and lactose (Suto & Tomita, 2001, Sehnem et al. 2006, Mattam et al. 2022). Several studies report a strong induction of cellulolytic system by CMC in filamentous fungi such as *Phlebia gigantea* (Niranjane et al. 2007), *Trametes hirsuta* (Jeya et al., 2009), and *Coriolus versicolor (*Phuengjayaem et al. 2014). Xylan was also described as an inducer of cellulase production in *Sclerotinia sclerotiorum* (Issam et al., 2003). Suto & Tomita (2001) and Smaali et al. (2004) identified xylose as one of the main inducers of the cellulolytic system in *Penicillium purpurogenum* and *Trichoderma reesei.* In contrast, we found xylose as a cellulolytic repressor in *I. lacteus* LBM 034, reflecting the species-specific and concentration-dependent nature of the differential response to an inducer (Biely et al. 1980, Wang et al. 2020). In this context, the response level to an inducer varies among the organisms, potentially acting as an effector that enhances enzymatic activity in one fungal species while inhibiting it in another. Moreover, it is well established that induction is effective at low inducer concentrations, whereas at high concentrations, the inductive effect is reduced due to catabolic repression mediated by degradation products (Biely et al. 1980). Similar to the results obtained in this study, Niranjane et al. (2007) did not detect cellulolytic activity in *Phlebia gigantea* grown in a medium supplemented with glucose or xylose at 20 g L^{-1}. However, they observed strong induction of the cellulolytic enzyme system when the fungus was cultivated on polymeric substrates such as CMC and Avicel (also at 20 g L^{-1}).

Nitrogen is another key modulator of the cellulolytic enzymes in wood-degrading fungi. The effects are variable and depend on the species and their interaction with the source of nitrogen and its concentration (Zhang et al. 2012, Coniglio et al. 2019). Among the nitrogen sources analyzed in our PBD, peptone significantly increased the production of the three cellulases, EG, CBH, and BGL, followed by urea and ammonium sulfate. Han et al. (2009) also reported higher levels of EG activity in *Penicillum waksmanii* F10-2 cultures supplemented with peptone (1170 U L^{-1}) compared to the levels detected with inorganic sources such as NH_4NO_3 (450 U L^{-1}). Jeya et al. (2009) reported a higher level of BGL in cultures of *T. hirsuta* supplemented with organic sources of nitrogen compared to the levels detected using inorganic nitrogen compounds. Ammonium sulfate has been reported as the most effective source of nitrogen for cellulase production by *A. niger* HQ-1 and *C. versicolor* TD17 (Zhang et al. 2012, Phuengjayaem et al. 2014).

In our work, the EG production by *I. lacteus* LBM 034 was increased to 663.97 U L^{-1} in the RCCD by supplementing the medium with CMC, peptone, and xylan. This EG level is higher than

those reported by Giorgio et al. (2017) in *C. versicolor* BAFC 266 (32.35 U L^{-1}), *Pycnoporus sanguineus* BAFC 2126 (57.17 U L^{-1}), and *P.* BAFC 633 (116.7 U L^{-1}), cultivated in liquid medium containing malt extract and corn syrup as carbon source.

We also adjusted the best physical conditions for incubating the fungal enzyme production using an RSM. The optimal conditions for achieving enzymatic levels were 2.6-fold higher than the original conditions, demonstrating the importance of physical conditions over extracellular enzyme production in liquid cultures. Although several studies have focused on physical parameters that affect the production of cellulolytic enzymes, such as pH and temperature, most of them described the evaluation of one parameter at a time (Farinas et al. 2010, Saqib et al, 2010). We analyzed the effect and the interaction of three physical parameters on EG production of *I. laceteus* LBM 034 by an RCCD. We obtained a maximal enzyme production of 692.54 U L^{-1} when pH and temperature were around their center points (32 °C and pH 5.5) while the agitation speed was at its lowest level, 0 rpm. Most filamentous fungi grow at acidic pH between 5 to 6, but there is still a discussion about the optimum level that maximizes cellulose activity; moreover, it has been observed that the pH of the culture below 5 or above 7 drastically affects cellulase production (Niranjane et al. 2007, Santos et al. 2022).

The optimization of EG production by *I. lacteus* LBM 034 allows the enzymatic hydrolysis of the lignocellulosic wastes studied in this work, pine sawdust, eucalyptus sawdust, and rice straw. The mechanical and alkaline pre-treatment of these wastes made the cellulose accessible for further enzymatic hydrolysis. The reduction of the particle size before the alkaline pre-treatment is an energy-consuming process; however, it is necessary for cellulose bioconversion because it enhances the surface area of the material (Mood et al. 2013). Following the mechanical treatment, we applied a soft alkaline pre-treatment to the residues using NaOH in its lowest concentration since it successfully extracted the lignin and preserved the cellulose by modifying the acetyl groups of lignocellulose and substituting uronic acids (Carvalheiro et al. 2008, Díaz et al. 2022). Concerning reducing sugars, a significant loss was observed in eucalyptus sawdust after the pre-treatment likely due to the hydrolysis of hemicellulose. Alkalis treatments produce cleavage of hydrolyzable linkages in lignin and glycosidic bonds in polysaccharides. This process reduces the degree of polymerization and crystallinity, swells the fibers, and disrupts the lignin structure. The dissolution of hemicellulose and lignin significantly varied concerning pre-treatment conditions. High alkali loading, elevated temperature, and prolongated pre-treatments can lead to undesired sugar loss due to hemicellulose dissolution and degradation (Chen et al. 2013). The differences in lignocellulosic residues observed in our study compared to other studies could be attributed to various causes, such as lignin content in the material and the pre-treatment methods employed. These methods, which involved physical, physicochemical, or chemical processes, differently impact the substrate, depending on the specific pre-treatment applied (Van Dyk & Pletschke 2012). Size reduction, such as chipping, grinding, and milling, are examples of physical methodologies focused on the increase of the surface area of the material and the reduction of the crystalline nature of cellulose (Pedersen & Meyer 2009). Other physical methodologies employ irradiation, such as microwave, gamma-ray, electron beam, or ultrasound frequencies. Applying a high hydrostatic pressure and a high-pressure homogenization are other physical pre-treatments that proportionally distribute the pressure in all parts of the biomass. Regarding physicochemical methodologies, the auto-hydrolysis of hemicellulosic biomass employs high temperatures from 150 to 250 °C to leave cellulosic content in a solid mass. Steam explosion pre-treatment uses physical methods (high temperature and pressure) and chemicals, such as sulfur dioxide (SO_2-added steam explosion), carbon dioxide (CO_2 explosion), liquid hot water, or ammonia (Ammonia Fiber Explosion, AFEX). Chemical pre-treatments aim to disrupt the biomass structure (Beig et al. 2021). Alkaline pre-treatments use lime, sodium, potassium, calcium, or ammonium hydroxides, as well as wet oxidation, while acidic pre-treatments apply sulfuric, hydrochloric, phosphoric or nitric acid (Beig et al. 2021).

The hydrolysis of the holocellulose of the pre-treated residues showed a similar profile by the enzymatic cocktail of *I. lacteus* LBM 034 and the commercial cellulase. Moreover, the conversion of the holocelloluse of the rice was 1.8 times higher using the cocktail of *I. lacteus* LBM 034 than

the commercial enzyme. Higher percentages of bioconversion have been reported for NaOH-pre-treated corn stubble (79.4%, Varga et al. 2002) and sorghum stubble (42.5%, Rodhe et al. 2011); however, in these works, the enzyme dose was 2.5 times more than in our work. Regarding the cellulose conversion, the commercial enzyme yielded higher levels of glucose than the cocktail from *I. lacteus* LBM 034, which can be explained by the specific characteristics of each enzymatic extract (Falkoski et al. 2012). A cellobiose accumulation in the hydrolysis carried out by the cocktail from *I. lacteus* LBM 034 can cause an inhibition of EG and CBH enzymes which reduces the cellulose bioconversion percentage (Gottschalk et al. 2010). In addition, the commercial enzyme can have higher levels of BGL than the cocktail from *I. lacteus.* Most cellulolytic fungal cocktails including those from *Trichoderma* spp. show low BGL/cellulases ratio, which is solved by adding extra BGLs from *Aspergillus* and *Penicillium* spp. (Falkoski et al. 2012, Singhania et al. 2014, Díaz et al. 2022). Moreover, we found differences in the holocellulose and cellulose bioconversion of the rice straw by the *I. lacteus* cocktail. This could be attributed to hemicellulolytic enzymes such as polygalacturonases and hemicellulases present in the fungal cocktail (Laothanachareon et al. 2015). Further studies are necessary to elucidate the behavior of the cellulases of the *I. lacteus* LBM 034 cocktail, particularly on the hydrolysis of rice straw, since its holocellulose and cellulose bioconversion highlighted from the other wastes making it a promising bioprocess for lignocellulose bioethanol energy.

In summary, we identified the most influential medium compounds (xylan, carboxymethyl-cellulose, and peptone) on cellulase production of the fungus *Irpex lacteus* LBM 034 using a PBD. Then, we enhanced fungal cellulase production 2.6 times-fold using an RSM. The optimal physical conditions were 32 °C, pH 5.5 without agitation. We applied this improved cocktail to efficiently hydrolyze NaOH-pre-treated lignocellulosic residues, rice straw, pine, and eucalyptus sawdust, into fermentable sugars. The highest hemicellulose (27.19%) and cellulose (38.18%) bioconversion were achieved in rice straw during enzymatic hydrolysis. These fermentable sugars are now available for cost-effective bioethanol production.

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Accessibility of data

Not applicable

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