



Increased *Malbranchea pulchella* β -glucosidase production and its application in agroindustrial residue hydrolysis: A research based on experimental designs

Lummy Maria Oliveira Monteiro^a, Ana Claudia Vici^b, Josana Maria Messias^b, Paulo Ricardo Heinen^a, Vanessa Elisa Pinheiro^a, Carem Gledes Vargas Rechia^c, Marcos S. Buckeridge^d, Maria de Lourdes Teixeira de Moraes Polizeli^{a,b,*}

^a Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Bandeirantes Av., 3.900, 14049-900, Ribeirão Preto, SP, Brazil

^b Faculdade de Filosofia Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Bandeirantes Av., 3.900, 14040-900, Ribeirão Preto, SP, Brazil

^c Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Bandeirantes Av., 3.900, 14040-903, Ribeirão Preto, SP, Brazil

^d Instituto de Biociências, Universidade de São Paulo, Matão Street, 277, 05508-090, São Paulo, SP, Brazil

ARTICLE INFO

Article history:

Received 2 January 2019

Received in revised form 7 September 2020

Accepted 8 April 2021

Keywords:

Thermophilic fungus

Second-generation ethanol

Composite central design

Mixture design

ABSTRACT

β -Glucosidases are a limiting factor in the conversion of cellulose to glucose for the subsequent ethanol production. Here, β -glucosidase production by *Malbranchea pulchella* was optimized using Composite Central Designs and Response Surface Methodologies from a medium designed. The coefficient of determination (R^2) was 0.9960, F -value was very high, and the lack of fit was found to be non-significant. This indicates a statistic valid and predictive result. *M. pulchella* enzymatic extract was successfully tested as an enzymatic cocktail in a mixture design using sugarcane bagasse, soybean hull and barley bagasse. We proved that the optimization of the β -glucosidase production and the application in hydrolysis using unexpansive biomass and agricultural wastes can be accomplished by means of statistical methodologies. The strategy presented here can be useful for the improvement of enzyme production and the hydrolysis process, arising as an alternative for bioeconomy.

© 2021 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

The use of lignocellulosic biomass for the production of second-generation ethanol can be considered advantageous in relation to the use of sugar and starch for the production of first-generation ethanol, due to the lack of competition with food production, besides causing less change in the use of the soil and less deforestation [1–3]. The use of lignocellulosic materials as a raw material to produce second-generation ethanol is not a global industrial reality, mainly due to the lack of efficient and low-cost technologies and low investment in the production [4,5]. Both, the structure of the plant cell wall and the difficulties in hydrolyzing its main components (cellulose, hemicellulose and lignin), limit the application of plant biomass [6,7].

Brazil is one of the main agricultural producers in the world and, therefore, accumulating a large number of lignocellulosic

residues. Approximately 260 kg of sugarcane bagasse are produced from one ton of sugarcane (considering 50 % moisture), and as its composition is mainly by sugar polymers, this residue can be recycled for the production of high-value compounds such as second-generation ethanol, food additives, organic acids and enzymes [8,9].

The lignocellulosic biomass can be hydrolyzed by enzymes with catalytic power to cellulose, hemicellulose and lignin, which are the major components of plant biomass. This enzymatic system includes cellulases such as endo-1-4- β -glucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91) and β -glucosidases (EC 3.2.1.21); hemicellulases such as endo-1,4- β -xylanases (EC 3.2.1.8), 1,4- β -xylosidases (EC 3.2.1.37); ligninases such as laccase (EC 1.10.3.2). Therefore, these enzymes have a high added value, since they are responsible for the hydrolysis of the most diverse lignocellulolytic residues. Lignocellulosic biomass residue-mediated biofuels could provide environmental benefits, would be economically feasible and could be produced in large quantities, without competing with food supplies, for biofuel production [10,11].

β -Glucosidases (BGLs) under physiological conditions, catalyze the hydrolysis of β -1,4-glycosidic bonds from the non-reducing end presented in alkyl- and aryl- β -D-glycosides, as well as different

* Corresponding author at: Faculdade de Filosofia Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Bandeirantes Av., 3.900, 14040-900, Ribeirão Preto, São Paulo, Brazil.

E-mail address: polizeli@ffclrp.usp.br (M.d.L. Teixeira de Moraes Polizeli).

oligosaccharides (containing 2–6 monosaccharides) [11–14]. The deficiency in BGL activity causes the accumulation of cellobiose, leading to the repression of enzyme biosynthesis and end-product inhibition of the upstream enzymes, which results in a limited hydrolysis yield [14–16]. BGLs are the most important rate-limiting factor in the conversion of cellulose to glucose for the subsequent ethanol production. Due to this important role, there is an increasing demand for the identification and production of new BGLs, especially with good catalytic power [11,16,17].

The cost of an enzyme is an economically limiting factor for the industrial process. Reducing the enzyme costs by optimizing the culture medium and the fermentation process is the goal of basic research for industrial applications [18–20]. In general, optimization by the traditional ‘one-factor-at-a-time’ technique is used. However, this method, although simple, often requires a considerable amount of work and time. A good alternative is the use of statistical designs, a method that has proved to be powerful and useful [21–23]. The goal of this methodology based on statistical validations is that it can be applied in the most diverse areas, such as bioengineering [24], pharmaceuticals [25], fine chemistry [26], microbiology [27–29] and chemical engineering [30,31].

In order to produce enzymes from fungal species with the aim of optimizing and cheapening the fermentation, carbon source is an important variable that must be analyzed. The use of agroindustrial residues, such as sugarcane bagasse, may contribute to the cost reduction of enzyme production, generating an inexpensive final product. Enzymes of some fungal species are widely used in industries due to their great potential for biotechnological applications [11,14,32].

Previous studies have shown that *M. pulchella* is a good producer of trehalases [33] and xylanases [34,35], and may be considered as promising for the production of other enzymes of biotechnological interest, yet there are no data on BGL production by *M. pulchella*. Thus, the aim of this work was to optimize the production of BGLs by *M. pulchella* in submerged fermentation (SbmF) according to central composite design (CCD) and surface response methodology analysis, and evaluate the potential of the enzymatic extract produced as an enzymatic cocktail for the hydrolysis of lignocellulosic residues by a mixture design.

2. Material and methods

2.1. Microorganism conditions

M. pulchella strain is deposited in a filamentous fungi collection located in the Laboratory of Microbiology and Cell Biology at Department of Biology from Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, University of São Paulo, Brazil. The fungus was maintained at 40 °C in solid Emerson medium [36] for 7 days for mycelial growth.

2.2. *Malbranchea pulchella* cultivation

All experiments were performed in 100-mL Erlenmeyer flasks containing 25 mL of the culture medium. 1 mL of the suspension was inoculated containing 10^6 spores per mL of *M. pulchella*, which were cultured for 72 h at 40 °C and 180 rpm. Lummy medium (g/100 mL distilled water) (0.4 yeast extract, 0.9 Na₂HPO₄, 0.05 MgSO₄, and 0.35 citric acid), supplemented with 1 % cellobiose (w/v) (Sigma-Aldrich) and buffered at pH 6.0 ensuring optimal buffering for BGLs stability.

2.3. Screening of different carbon sources

Screening with different carbon sources was performed as mentioned: cellobiose, wheat bran, ground soybean, sugarcane

bagasse, carboxymethylcellulose (CMC) and Avicel. In all experiments, 1% (w/v) of the carbon source was used (Fig. 1).

2.4. Optimization by central composite design and response surface analysis

A 2² central composite design (CCD) and response surface analysis were performed to evaluate the concentration effect of cellobiose (C) and sugarcane bagasse (SB) *in natura*, in culture media composition (%), (mg/mL), which were the most significant carbon sources for the BGL production (U/mL). Independent variable levels were defined according to a complete experimental design and a set of 12 experiments was carried out, four of which were central point repetitions (Table 1).

The answer to the variables, where Y is the BGL activity (U/mL), can be approximated by the quadratic polynomial (Eq. (1)):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \quad (1)$$

where: β_0 is the intercept, β_1 and β_2 are the first order model coefficients, β_{12} is the ratio between products, X_1 is the variable cellobiose and X_2 is the variable sugar cane bagasse. Regression coefficient (R^2) was used to express quality of the fit of the polynomial model, and its statistical significance was determined by the F test (Fisher's test) (Table S1).

2.5. Protein assays

Proteins were measured with Bradford method [37], using the standard curve 0–200 µg of bovine serum albumin with intervals of 0.05 µg between points. The values of protein were expressed as mg of protein per mL of solution. Specific activity was expressed as units per mg of protein (U/mg) present in the enzymatic extract.

2.6. Measurement of reducing sugars

The reducing sugars (mg/mL) were quantified through the Miller method, using dinitrosalicylic acid (DNS) [38]. After assay, the mixture was boiled at 100 °C for 5 min, the tubes were cooled, and 1 mL of distilled water was added to each mixture. Aliquots of 100 µL were withdrawn for the reading in a Molecular Devices Gemini XS microplate reader at 540 nm.

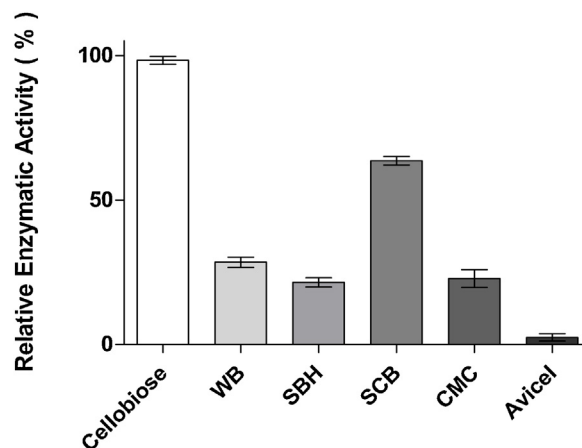


Fig. 1. BGL relative activity when induced by different carbon sources. Cellobiose = Cellobiose, WB = Wheat bran, SBH = Soybean hulls, SCB = Sugarcane Bagasse, CMC = Carboxymethylcellulose, Avicel = Avicel, all at 1% (w/v).

Table 1

CCD to evaluate the effect of cellobiose and sugarcane bagasse on BGL production.

	Variables		BGL activity (U/mL)		
	Cellobiose	Sugarcane bagasse	Experimental value	Predicted value	RD ^d
1 ^a	0.18 (-1)	2.0 (-1)	0.25	0.28	0.11
2 ^a	1.02 (+1)	2.0 (-1)	0.27	0.28	0.04
3 ^a	0.18 (-1)	6.0 (+1)	0.24	0.28	0.14
4 ^a	1.02 (+1)	6.0 (+1)	0.28	0.28	0.00
5 ^b	0.008 (-1.41)	4.0 (0)	0.27	0.27	0.01
6 ^b	1.192 (+1.41)	4.0 (0)	0.29	0.27	0.06
7 ^b	0.6 (0)	1.18 (-1.41)	0.31	0.29	0.06
8 ^b	0.6 (0)	6.82 (+1.41)	0.32	0.29	0.09
9 ^c	0.6 (0)	4.0 (0)	0.92	0.91	0.01
10 ^c	0.6 (0)	4.0 (0)	0.92	0.91	0.01
11 ^c	0.6 (0)	4.0 (0)	0.89	0.91	0.02
12 ^c	0.6 (0)	4.0 (0)	0.90	0.91	0.01

The encoded values of the variables are in parentheses.

^a Assays from the factorial design.^b Axial points.^c Central points.^d RD - Relative Deviation.

2.7. Measurement of lignocellulolytic enzymes

Enzymes present in the crude extract optimized for CCD were assessed: CMCase, avicelases, FPases, xylanases, pectinases, amylases, arabinofuranosidases, cellobiohydrolases, β -xylosidases, arabinanases, laccases, β -glucosidase, β -glucanases, mannanases and lipases. All enzyme assays were performed at 50 °C and pH 6.0 McIlvaine buffer, as Table S2.

2.7.1. CMCase, avicelases, FPases, xylanases, pectinases, amylases, arabinanases, β -glucanases and mannanases

These enzymes were measured from its natural substrates (Table S2). The enzyme activity unit was defined as the amount of enzyme capable of releasing 1 μ mol of reducing sugar per minute. The reducing sugars released were quantified with the Miller method [38]. The mixtures were boiled at 100 °C for 5 min, then 1 mL of distilled water was added to each mixture and an aliquot of 100 μ L was quantified at 540 nm.

2.7.2. Laccases

Laccases were measured from the syringaldazine substrate. The assay mixture was composed of 0.6 mL of crude extract, 0.1 mL of pH 6.0 McIlvaine buffer, 0.1 mL of distilled water and 0.1 mL syringaldazine (0.1 % in ethanol). The oxidation of syringaldazine ($\epsilon_{525} = 65,000 / \text{Mcm}$) to the quinone was monitored for 10 min at 525 nm, and the activity expressed in U/L (unit/Liter - which means $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{unit}^{-1}$) [39 modified].

2.7.3. β -Glucosidases, β -xylosidases, arabinofuranosidases, lipases and cellobiohydrolases

β -Glucosidase activity was determined by *p*-nitrophenyl- β -D-glucopyranoside (pNPG) hydrolysis, measured at 405 nm. The assay was initiated with 15 μ L of the crude extract added to 10 μ L of McIlvaine buffer pH 6.0 and 25 μ L of pNPG (4 mM in H₂O), and incubated for 5 min at 50 °C. The assay was stopped by adding 50 μ L of 0.2 M Na₂CO₃ solution. The measurement was performed in a spectrophotometer at 405 nm. The enzymatic unit (U) was defined

Table 2Mixture design: sugarcane bagasse (A), soybean hulls (B) and barley bagasse (C) hydrolysis by *M. pulchella* enzymatic extract during 48 h. It was evaluated the yields of reducing sugars (mg/mL), glucose (%) e xylose (%).

Assay ^a	Mixture components	Mixture proportions		Answer evaluated		
		Encoded values	Real values	Reducing sugars (mg/mL) ^g	Glucose ^h (%) ^j	Xylose ⁱ (%) ^j
		100 % = 1 ^b	(mg)			
1	A ^d	(1;0;0)	(50;0;0)	0.21	19.1	0
2	B ^e	(0;1;0)	(0;50;0)	0.57	19.5	17.5
3	C ^f	(0;0;1)	(0;0;50)	2.05	72.4	6.1
4	A + B	(¹ / ₂ ; ¹ / ₂ ; 0)	(25;25;0)	0.35	28.2	11.9
5	A + C	(¹ / ₂ ; 0; ¹ / ₂)	(25;0;25)	1.13	64.2	0.7
6	B + C	(0; ¹ / ₂ ; ¹ / ₂)	(0;25;25)	1.44	53.2	9.1
7	A + B + C	(¹ / ₃ ; ¹ / ₃ ; ¹ / ₃)	(16.66;16.66;16.66)	0.84	68.5	10.2
8 ^c	A + B + C	(¹ / ₃ ; ¹ / ₃ ; ¹ / ₃)	(16.66;16.66;16.66)	0.89	66.9	10.6
9 ^c	A + B + C	(¹ / ₃ ; ¹ / ₃ ; ¹ / ₃)	(16.66;16.66;16.66)	0.87	70.1	12.0

^a Experimental points in the mixture design.^b The sum of all components in the assays was always 100 %.^c Replicates from the assay number 7 or central point.^d Sugarcane bagasse (mg).^e Soybean hulls (mg).^f Barley bagasse (mg). 100 % or 1 = 50 mg of wastes.^g Analysis performed by Miller method.^h Analysis performed in HPLC.ⁱ Analysis performed in HPLC.^j The percentage of glucose and xylose was obtained in relation to the amount of reducing sugar (mg/mL).

as the amount of enzyme required to hydrolyze one μmol of the substrate per minute. β -xylosidases, arabinofuranosidases, lipases and cellobiohydrolases were assayed from artificial substrates, as previously described to β -glucosidase activity (Table S2). The unit (U) was defined as the amount of enzyme required to hydrolyze one μmol of the substrate in one minute.

2.8. Residue composition

2.8.1. Sugarcane bagasse in natura and Barley bagasse

The pectin, hemicellulose and cellulose compounds of sugarcane bagasse in natura and barley bagasse were isolated by modified protocol developed by Redgwell and Selvendran [40]. The powdered samples of sugarcane bagasse in natura and barley bagasse (25 mg) were suspended in 50 mL of the following: (1) 0.1 M of cyclohexanediamino-N,N,N',N'-tetraacetic acid (CDTA) pH 6.5, at room temperature (RT), 2 h, three times; (2) NaOH, 4 M, with 20 mM NaBH_4 at RT, 2 h; (3) cadmium ethylenediamine solvent (cadoxen), 2 h at RT. After each step of the extraction, the residues were separated by centrifugation at $11,200 \times g$, 5 min. NaOH extract was neutralized with concentrated acetic acid. All extracts were analyzed with the phenol sulfuric acid method [41].

2.8.2. Soybean hulls

The composition of Soybean hulls used in this work was the one published and described by Marco et al. [42].

2.9. Mixture design

A simplex centroid experimental design was carried out to evaluate the interaction effects of the mixtures of in natura sugarcane bagasse (A), soybean hulls (B) and barley bagasse (C) in obtaining reducing sugars, glucose and xylose, through the action of the previously optimized enzyme extract. Assays were composed by 50 mg of the residue (Table 2), 5 mL of the crude extract buffered in McIlvaine, pH 6.0 and 15 mM sodium azide to prevent bacterial contamination. The hydrolysis was carried out at 50°C at 700 rpm in a dry bath with stirring. Aliquots of 600 μL were withdrawn up to 48 h and immediately boiled for 5 min to inactivate the enzymes. The reducing sugars were quantified by the DNS method (mg/mL) [38]. Glucose and xylose were calculated by HPLC and the results were shown in percentages related to the total quantity of reducing sugars.

Models for mixture design consisting of three components include: linear, quadratic and special cubic models. The models for the case of three variables are:

$$Y = \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 \quad (2)$$

Linear model

$$Y = \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 \quad (3)$$

Quadratic model

$$Y = \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{123} x_1 x_2 x_3 \quad (4)$$

Special cubic model where Y is the dependent variable, β_i is the regression coefficients (calculated from experimental data) and x_i is the independent variables. Each parameter was presented by a model that helps to explain the variability [43–45].

Models of mixture considered adequate were chosen based on the results of variance analysis (ANOVA) (Table S3) because they did not present lack of fit, had a high percentage of explained variation (R^2) and the calculated value of the F test was greater than the tabulated value (in the degrees of freedom considered). Since

the models were predictive, it was possible to generate the respective response surfaces (Fig. 5) [45].

2.10. Thin-layer chromatography analysis

The sugars formed by the residue hydrolysis were analyzed by means of thin-layer chromatography (TLC – Merck, TLC aluminum sheets 20×25 cm silica gel 60). A volume of 10 μL of the assay product was applied showing a higher amount of reducing sugar at times 0 h, 3 h, 6 h, 24 h and 48 h; also, 1 μL standard containing glucose, maltose, maltotriose, maltotetraose and maltopentaose (0.1 % w/v) and 1 μL standard containing xylose, xylobiose, xylotriose, xylotetraose (0.1 % w/v). The plate was then placed in a glass vessel containing a solution of *n*-butanol solution, ethanol and distilled water (5:3:2). The run was held once. For developing the plate, a solution containing 18 mL methanol, 2 mL of sulfuric acid (PA) and 0.04 g of orcinol was sprayed; the plate was then placed in an oven at 100°C for about 2 min – until the appearance of bands.

2.11. High-performance liquid chromatography analysis of the mixture design products

The hydrolysis product of the twelve points at time zero and 48 h were analyzed through high-performance liquid chromatography (HPLC). Aliquots of the mixture (1 mL) were filtered on a cellulose nitrate membrane (0.45 mm). Twenty microliters of each filtered sample or standard were injected into a Waters HPLC system using a Supelcogel C611 column (7.8×300 mm; 60°C) eluted with NaOH 0.1 mM as the mobile phase at a flow rate of $0.5 \text{ mL} \cdot \text{min}^{-1}$, controlled by a peristaltic pump. Eluted products were monitored with a differential refractometer (model R401, Waters). The monosaccharides (glucose and xylose) present in the samples were identified by their retention time in comparison with those of commercial standards.

2.12. Reproducibility of experiments and statistical analysis

The experiments performed by the "one-at-a-time" procedure were conducted in triplicate and the mean and standard deviation values were reported. The pure error of the CCD experiments and the mixture design were calculated based on the values of the center point response variable (minimum of three replicates). The software Statistica v.13.0 (STATSOFT, USA) was used to analyze the experimental regression and for the graphical analysis data. Analysis of variance (ANOVA) was performed at the significance levels of 5%.

3. Results and discussion

3.1. Screening of different carbon sources

A screening of promising carbon sources for the BGL production was performed and it could be concluded that cellobiose was the best carbon source to induce BGL production. Although cellobiose is a good carbon source for inducing the BGL production, it can be regarded as expensive and unpromising for industrial applications. However, in search for carbon sources economically viable, sugarcane bagasse showed a promising result. In Fig. 1, it can be observed that when induced by 1% cellobiose (w/v), *M. pulchella* produced a relative activity of 100 % (0.129 mg/mL and 1.55 U/mg), achieving the best production. The other residues produced the following results: wheat bran 28.4 % (0.037 mg/mL and 0.44 U/mg), soybean hulls 21.4 % (0.021 mg/mL and 0.33 U/mg), sugarcane bagasse 63.4 % (0.081 mg/mL and 0.98 U/mg), CMC 22.8 % (0.029 mg/mL and 0.35 U/mg) and Avicel 2.4 %

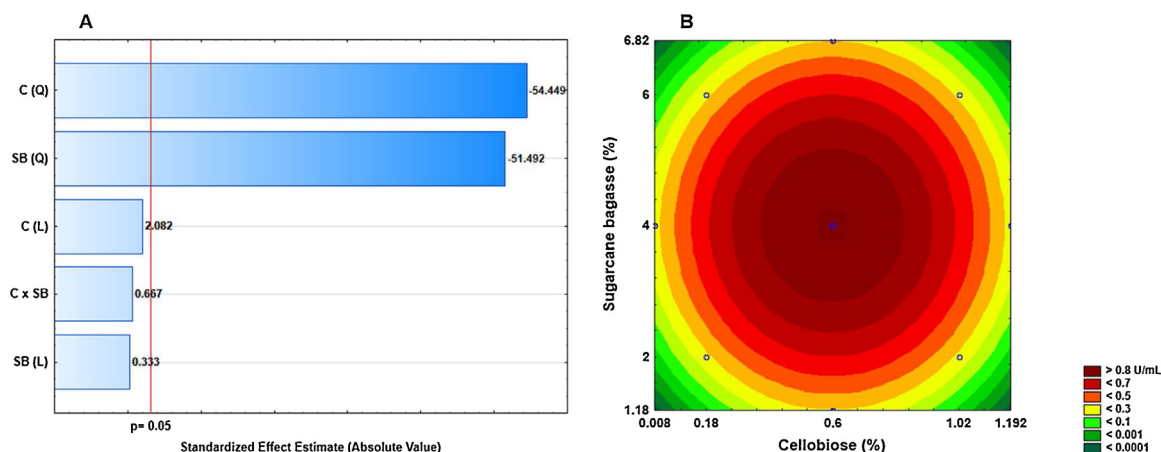


Fig. 2. Central composite design (CCD) 2^2 for optimization of *M. pulchella* BGL (U/mL) production. (A) Pareto chart showing the effect of cellobiose and sugarcane bagasse variables on BGL (U/mL) production. (B) Response surface contours obtained by the reduced model calculated from a CCD 2^2 .

(0.003 mg/mL and 0.03 U/mg) of the relative enzyme activity. Thus, it can be seen that sugarcane bagasse, as well as cellobiose, are good carbon sources for the BGL production. It is worth mentioning that sugarcane bagasse is a cheap carbon source when compared to cellobiose, allowing the cheapening of enzyme production. This way, we sought for the association of cellobiose and sugarcane bagasse in order to reduce costs and increase production. Cellobiose and sugarcane bagasse have separately been used as inducers of BGL production for several fungi such as *Trichoderma reesei*, *Aspergillus awamori*, *Ganoderma lucidum* and *Humicola grisea* [46–48].

3.2. Optimization by central composite design (CCD) and response surface analysis

Cellobiose and sugarcane bagasse were jointly investigated by a CCD since they had a strong influence on BGL activity. Previous experiments have shown that the combination of cellobiose and sugarcane bagasse increased BGL production by *M. pulchella* more than each compound separately (data not shown). These same experiments showed that increasing the amount of sugarcane bagasse is beneficial for the enzyme production; therefore, the range of sugarcane bagasse and cellobiose concentration tested was 1.18–6.82 % and 0.008–1.192 %, respectively. The best CCD results can be observed in the central points (Table 1), with concentrations of 4.0 % sugarcane bagasse and 0.6 % cellobiose. The Pareto chart (Fig. 2A) showed variable effects where cellobiose and sugarcane bagasse present only the quadratic effect on the enzymatic production. Thus, the reduced model obtained for the BGL activity (U/mL) is expressed in Eq. (5):

$$\text{BGL activity (U/mL)} = 0.91 - 0.32(C)^2 - 0.31(SB)^2 \quad (5)$$

where, C and SB correspond to the encoded values for cellobiose and sugarcane bagasse, respectively.

The predicted activities using this equation show that the equation is predictive, since the relative deviation (RD) is very low (Table 1). Table S1 shows the ANOVA results for the second-order reduced model. In this way, Fig. 2B shows the contour curves for the BGL production (U/mL) obtained by the reduced model of second order. The results show that the highest activity occurs in the central point (which also reveals the robustness of the generated model), corresponding to the midpoints and 4.0 % sugarcane bagasse and 0.6 % cellobiose. This was also confirmed by the desirability profile [49], which detailed the precise proportions

of the independent variables. Fig. 3 showed the maximum BGL production that could be achieved by the model, that is, 0.91 U / mL value obtained with 98.2 % desirability.

Factorial design has increasingly been used in order to optimize the production and products in addition to reducing production costs, for example, to evaluate the compound effects on the growth and metabolism of microorganisms, or to optimize the biodiesel production, or also, to optimize the fermentation factors on bioethanol production in batch bioreactor [27–29]. Interestingly, with the aid of this technique, it was possible to considerably increase BGL production and make it cheaper, since it began to use sugarcane bagasse with cellobiose as carbon source, instead of using only cellobiose. The use of agroindustrial waste such as sugarcane bagasse reduces enzyme production costs, decreases environmental damage caused by agribusiness and adds value to a byproduct of the sugar industry.

3.3. Residue composition

The composition of sugarcane bagasse and barley bagasse were determined for future hydrolysis experiments. The amount sugarcane bagasse components were, 27.1 % of cellulose, 41.1 % of hemicellulose, 22.9 % of lignin and 8.9 % of pectin. On the other hand, barley bagasse presented 33.7 % of cellulose, 39.7 % of hemicellulose, 14.3 % of lignin, 11.7 % of pectin and 0.6 % of starch. As it was possible to observe, most residues are composed of cellulose, hemicellulose and lignin as expected. Amounts of cellulose present in sugarcane bagasse and in the soybean hulls are very close, with 27.1 % and 24.4 % respectively. The amounts of hemicellulose are quite similar, 41.1 % in sugarcane bagasse, 33.8 % in ground soybean husk and 39.7 % in barley bagasse. On the other hand, it is noteworthy that lignin, an important factor that may hinder the enzymatic action during hydrolysis, has a varied composition in the different residues, with 22.9 % in sugarcane bagasse, 36.6 % in ground soybean husk and 14.3 % in barley bagasse. Moreover, a small portion of 0.6 % starch was found only in the barley bagasse composition.

3.4. Enzyme activity of lignocellulolytic complex

Because sugarcane bagasse is a complex carbon source, several enzymes could be found in the enzymatic extract. In order to study them, *M. pulchella* was cultured in optimal condition found by the result of CCD for BGL production. Then, this enzymatic extract was

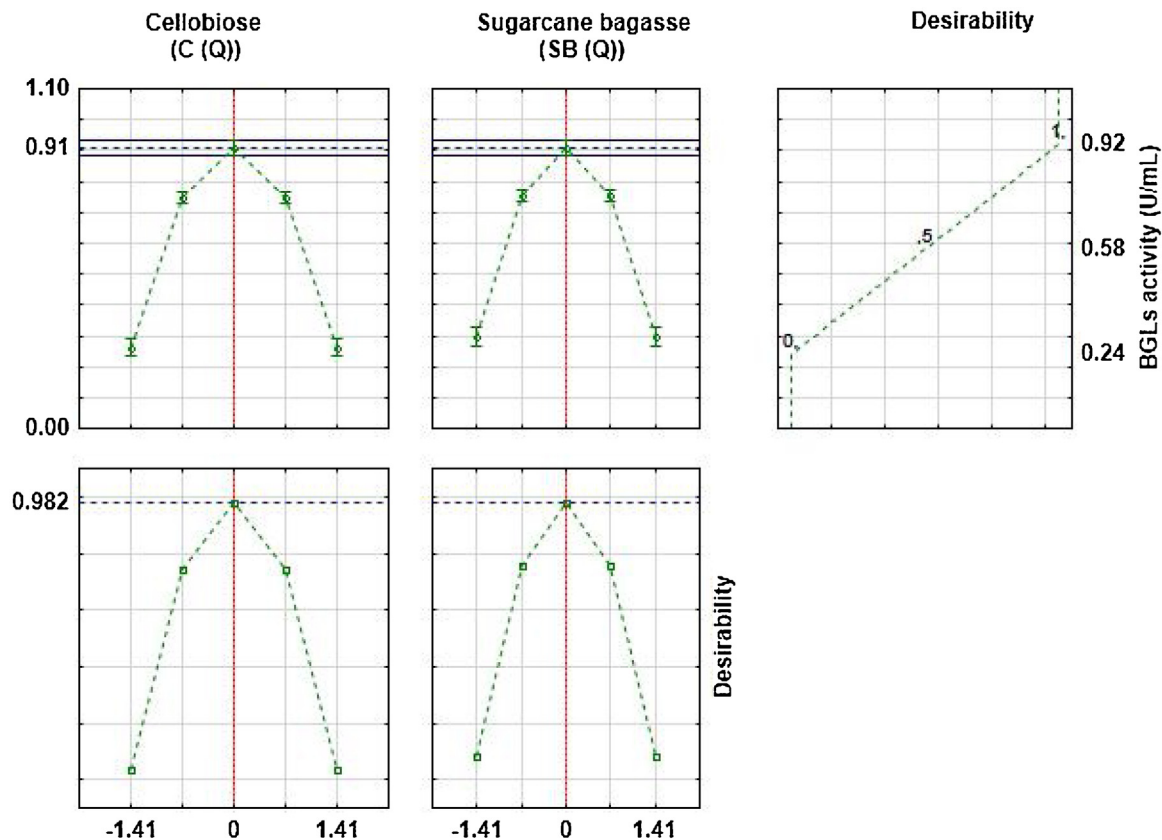


Fig. 3. Desirability profile for BGL activity (U/mL). Cellobiose and sugarcane bagasse were the independent variables of CCD 2².

Table 3

Enzymatic activities (U/mL) in *M. pulchella* crude extract.

Enzyme	Activity (U/mL)	Specific activity (mg/mL)
Xylanase	2.38 ± 0.09	11.90
Amylase	2.05 ± 0.07	10.25
CMCase	0.09 ± 0.05	0.45
FPase	0.07 ± 0.01	0.35
Avicelase	0.03 ± 0.01	0.15
Pectinase	0.15 ± 0.01	0.75
β-glucanase	0.71 ± 0.01	3.55
Arabinanase	0.10 ± 0.01	0.50
Mannanase	0.00	0.00
β-glucosidase	2.90 ± 0.08	14.50
Arabinofuranosidase	0.04 ± 0.00	0.20
Lipase	1.20 ± 0.03	6.00
Cellobiohydrolase	0.40 ± 0.00	2.00
β-xylosidase	0.40 ± 0.00	2.00
Laccase	0.00	0.00

concentrated 3-fold and the volume recovered was used in the measurement of lignocellulolytic enzymes. Results (U/mL) are shown in Table 3.

It is interesting to note the presence of several enzymes that can hydrolyze agroindustrial residues. It can be concluded that the enzymes β-glucosidase, xylanase and amylase are present in larger amounts (2–2.9 U/mL); β-glucanase, lipase, cellobiohydrolase and β-xylosidase in average amounts (0.4–1.2 U/mL) and, finally, the enzymes arabinofuranosidase, arabinanase, pectinase, avicelase, FPase, laccase and CMCase were not present at significant levels Table 3.

3.5. Mixture design

Due to the presence of several enzymes capable of degrading lignocellulosic biomass, the following question was made: “Which residues would be more hydrolysable by the enzyme cocktail?” In order to answer this question, a mixture design was carried out with 3 residues rich in lignocellulosic biomass: sugarcane bagasse, soybean hulls and barley bagasse.

The hydrolysis was possible due to the presence of several enzymes capable of degrading lignocellulosic biomass. The reducing sugars (mg/mL) and amounts of glucose and xylose (%) produced in this experiment can be observed in Table 2. For reducing sugars (mg/mL), the analysis within 48 h hydrolysis, the ANOVA values and the model used are shown in Table S3. The Pareto chart (Fig. 4A) showed that sugarcane bagasse, soybean hulls and barley bagasse have an effect separately ($p \leq 0.05$). Thus, the reduced linear model obtained for the reducing sugar (mg/mL) is expressed in Eq. (6):

$$\text{Reducing sugar (mg/mL)} = 0.15A + 0.57B + 2.06C \quad (6)$$

where A, B and C correspond to encoded values for sugarcane bagasse, soybean hulls and barley bagasse, respectively. The predicted values are very close to the values observed (Fig. 4B), indicating that the relative deviation is very low.

The linear model showed that $C > B > A$, where barley bagasse hydrolysis (assay number 3) promoted a greater amount of reducing sugars in 48 h hydrolysis (Table 2), it can be seen in the response surface (Fig. 5A). There was no lack of adjustment in the degrees of freedom used, the correlation coefficient value (R^2) was 0.9874 and the $F_{\text{calculated}}$ (237.7) for the model was greater than the F_{listed} (5.14), which means that the model is statistically

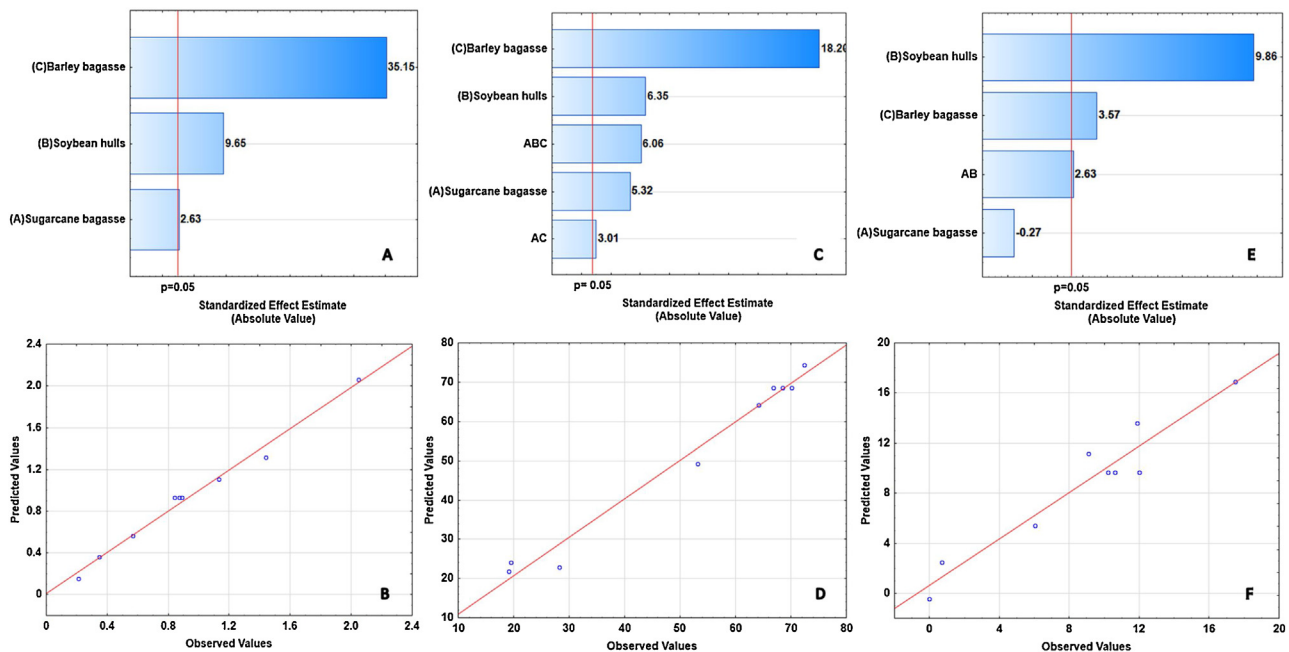


Fig. 4. Pareto charts showing the significant effects (A, C, E) and comparison between the expected and observed responses from the mixture design (B, D, F) which used sugarcane bagasse, soybean hulls and barley bagasse as substrates for *M. pulchella* cocktail action. Dependent variables: reducing sugar (mg/mL) (A, B), glucose (%) (C, D) and xylose (%) (E, F).

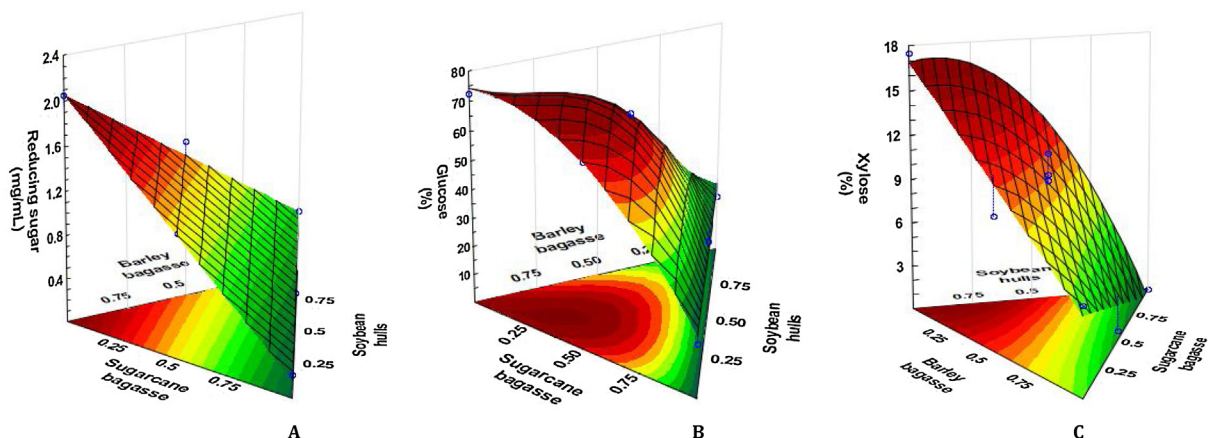


Fig. 5. Response surfaces from the mixture design which used sugarcane bagasse, soybean hulls and barley bagasse as substrates for *M. pulchella* cocktail action. Dependent variables: reducing sugars (mg/mL) (A), glucose (%) (B) and xylose (%) (C).

significant and predictive ($p \leq 0.05$) (Table S3). When the optimized mixing condition for the response (Fig. S1) was estimated, 2.062 mg/mL of reducing sugar was obtained by Eq. (6) and this result was very close to the experimental value (2.05 mg/mL), which supports the good predictive capacity of this model.

However, what will be the exact amount of small sugars (glucose and xylose) at each point of the experimental design? Also, will the amount of oligosaccharides be decreasing, i.e., is the hydrolysis happening as expected? In order to quantify the percentage of monosaccharide formed after the hydrolysis by the enzymatic extract during the mixture design, an HPLC analysis was performed.

For the glucose formation (%), the Pareto chart (Fig. 4C) showed that sugarcane bagasse, soybean hulls and barley bagasse presented synergic effect (Table 2) when they are together (ABC) ($p \leq 0.05$ and $R^2 = 0.9803$). It also happened in the binary mixture between sugarcane bagasse and barley bagasse (AC),

however, the most influential component in the mixture was barley bagasse ($A > B > C$). For the glucose (%), the special cubic model was given by Eq. (7) and the response was obtained as a function of proportions of each component in the mixture (Table S3).

$$\text{Glucose (\%)} = 21.74A + 24.11B + 74.38C + 64.57AC + 573.73ABC \quad (7)$$

where A, B and C correspond to encoded values for sugarcane bagasse, soybean hulls and barley bagasse, respectively. The variation in the quality of the adjustments is shown by the graph of expected responses against the observed responses [45] and according to Fig. 4D, the yield of glucose (%) was well modeled.

According to Table S3 (ANOVA), the R^2 value and the F ($F_{\text{calculated}} > F_{\text{listed}}$) value obtained for the regression showed that the model is reliable and significant and since the lack of fit was not significant ($F_{\text{calculated}} < F_{\text{listed}}$), the model is also predictive ($p \leq 0.05$), which means that it is suitable to describe the results by

response surface (Fig. 5B). From the glucose results generated by the HPLC, it can be concluded that the most hydrolysable substrate is barley bagasse, and it can be seen in Table 2, which indicates that within 48 h of hydrolysis using 100 % of barley bagasse as substrate (assay number 3), there is the formation of 72.4 % of glucose. The formation of this monosaccharide was also good with the binary mixture (assay number 5) between sugarcane and barley bagasse, since 64.2 % and similar values were obtained for the ternary mixture (68.48 ± 1.61), represented by the central point (assays 7, 8 and 9, Table 2).

The idea that barley bagasse was the most hydrolysable residue is correct, but this mixture design also valued sugarcane bagasse and soybean hulls (widely found in Brazil) as substrates for hydrolytic action of enzymatic cocktail from *Malbranchea pulchella* (Table 2). It was possible to validate the predictive capacity of the model for the formation of glucose (%) when the mixing condition evaluated was that of the central point (Fig. S2), because the value found (68.5 %) was very close to the average value obtained experimentally.

On the other hand, by analyzing the xylose results generated by HPLC, it could be concluded that the enzyme cocktail was able to act more efficiently in the production of xylose on the soybean hull residue. It can be seen in Table 2 that within 48 h of hydrolysis using 100 % of soybean hulls as substrate (assay 2), there is the formation of 17.5 % of xylose, the best result achieved for xylose production. It may also be noted that the points that have xylose intermediate percentages formed are those that contained soybean hulls in its initial composition: assays 4, 6, 7, 8, 9 with the xylose formation of 11.9 %, 9.1 %, 10.2 %, 10.6 % and 12.0 %, respectively. Thus, this result supports the idea that the soybean hulls probably have a more available xylan to the action of xylanolytic enzymes present in the cocktail.

The Pareto chart (Fig. 4E) showed that sugarcane bagasse itself did not have significant effect on the cocktail hydrolysis, but soybean hulls, barley bagasse and the binary interaction between sugarcane bagasse and soybean hulls did ($p \leq 0.05$). So, the reduced quadratic model obtained for the xylose production (%) is expressed in Eq. (8) and the terms in bold are predictive:

$$\text{Xylose (\%)} = -0.46A + 16.90\mathbf{B} + 5.41\mathbf{C} + 21.49\mathbf{AB} \quad (8)$$

where A, B and C correspond to encoded values for sugarcane bagasse, soybean hulls and barley bagasse, respectively, and $B > C > A$. The predicted values were very close to the observed values (Fig. 4F) indicating that the modeling is satisfactory. The ANOVA results are available at Table S3. Fig. 5C shows the response surface for the xylose formation (in percentage) obtained by the quadratic model. It can be concluded that the soybean hulls were the most hydrolysable residue and that the binary mixture between sugarcane bagasse (25 mg) and soybean hulls (25 mg) was a synergistic substrate for the action of enzymes from *M. pulchella*.

Knowing the composition of sugarcane bagasse, soybean hulls and barley bagasse used in the mixture design, and the fact that barley bagasse presented greater capacity for glucose formation can be explained as follows: (i) by the lignin composition, since barley bagasse has approximately 14.3 % of lignin, while the sugarcane bagasse and soybean hulls have 22.9 % and 36.6 %, respectively. From this composition, it could be concluded that the lower amount of lignin facilitated the action of the enzyme consortium. Lignin is normally present in the plant cell wall conferring stiffening, waterproofness and resistance to microbial attack and oxidative stress [50,51] therefore, hindering the action of enzymes that degrade cellulose and hemicellulose. The enzyme extract had no laccase activity; so that, the less lignin on the residue composition, the better the action of other enzymes. A

possible way to improve hydrolysis of these residues would be the use of alkaline pretreatment, oxidation, biological and AFEX (Ammonia fiber explosion) mainly removing the fraction of lignin. Thus, the biomass would mainly be composed of hemicellulose and cellulose at the end of the process; (ii) by the presence of a small fraction of starch in the barley bagasse composition. The large amount of amylase in the enzyme extract may have contributed to a better hydrolysis and consequently to an increase in fermentable sugars such as glucose. The cellulose and hemicellulose composition do not seem to have any direct influence on the three different residues, since their compositions are very similar.

3.6. Thin-layer chromatography analysis of the mixture design products

Considering the measurements of reducing sugars with the Miller method, it can be stated that the enzyme cocktail acts more efficiently on barley bagasse, producing a larger amount of sugars. In order to analyze the evolution of sugars formed during the barley bagasse hydrolysis, a TLC of the point 3 (100 % barley bagasse) on 0, 3, 6, 24 and 48 h was performed. As it can be seen from Fig. S3, at 0 h there is no sugars present, but with 3 h of hydrolysis, it is already possible to observe the presence of oligosaccharides, disaccharides and monosaccharides. The same result could be observed after 6 h of hydrolysis, but the bands appear with greater intensity suggesting that there was a rise in the amount of sugars formed. By 24 h of hydrolysis, the carbohydrate profile formed changed and there was a larger amount of disaccharides and monosaccharides, resulting in the reduction of higher sugars as trisaccharide and oligosaccharides, confirming the efficiency of the present enzymes. Finally, after 48 h, a similar profile as seen for 24 h could be observed, but with more intensity in these bands showing the increased sugars in comparison with 24 h, like glucose and xylose.

4. Conclusion

In this work, we show that the search for a cheaper and novel culture media can be an alternative for increasing the production of enzymes of biotechnological interest. The strategy used here demonstrates that statistical analysis and experimental designs can be helpful to optimizing enzyme production with a limited number of experiments allowing the prediction for the best condition of hydrolysis. Using this methodology, the production of BGL and several lignocellulolytic enzymes were optimized, demonstrating the importance of experimental designs for the enzyme cocktail production.

Although nowadays there is a steady application of enzymes in the industries, the addition of hydrolytic enzymes to increase hydrolyses of wastes is still not widely employed, mainly because of the overprice in the process of production [52–56]. However, the bioenergy production process developed with an enzymatic pre-treatment of wastes, such as barley bagasse, is a great possibility that leads to a greater degradation of the complex compounds present in the plant biomass in order to obtain a greater, easier and faster fermentation. This pre-treatment can lead to a higher productivity of bioethanol, for example. The use of enzymatic cocktail pre-treatment represents a shorter retention time of the raw material in the biodigester, increasing its productivity and making the process more viable.

In this way, the search for novel strategies based on experimental designs and statistical analysis using unexpensive resources is important for the field. CCD has been used for the optimization for several proposes, demonstrating its importance

for the improvement based on statistical analysis and composite design [52–55]. This strategy brings a lot of possibilities for those trying to improve the hydrolysis, due to the prediction potential based on a small number of tests facilitating the validation. CCD helped increase the production of β -glucosidases, the mixture design carried out in this work showed that barley bagasse is an excellent substrate for the *M. pulchella* optimized cocktail, and revealed the remarkable synergism of the ternary mixture of vegetable residues for glucose yield (%).

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgments

This work was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP 2010/52322-3, 2014/50884-5 and 2018/07522-6), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Finance Code 001) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, process 563260/2010-6 and 465319/2014-9). M. L. T. M. P. is a Research Fellow of CNPq (process 301963/2017-7). L.M.O.M. was a recipient of a CAPES Master's scholarship. We thank Mariana Cereia, Ricardo Alarcon and Maurício de Oliveira for technical assistance. We also thank teacher Abilio Borghi and Mariana Cereia for the grammar review of the manuscript.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.btre.2021.e00618>.

References

- [1] Z. Fu, M.T. Holtzapfel, Consolidated bioprocessing of sugarcane bagasse and chicken manure to ammonium carboxylates by a mixed culture of marine microorganisms, *Bioresour. Technol.* 101 (2010) 2825–2836.
- [2] P.S. Nigam, A. Singh, Production of liquid biofuels from renewable resources, *Prog. Energy Combust. Sci.* 37 (2011) 52–68.
- [3] D. Maga, N. Thonemann, M. Hiebel, D. Sebastião, T.F. Lopes, C. Fonseca, F. Gírio, Comparative life cycle assessment of first- and second-generation ethanol from sugarcane in Brazil, *Int. J. Life Cycle Assess.* (2018) 1–15.
- [4] C.A. Cardona, J.A. Quintero, I.C. Paz, Production of bioethanol from sugarcane bagasse: status and perspectives, *Bioresour. Technol.* 101 (2010) 4754–4766.
- [5] K. Pandiyan, A. Singh, S. Singh, A.K. Saxena, L. Nain, Technological interventions for utilization of crop residues a 606 nd weedy biomass for second generation bio-ethanol production, *Renew. Energy* 132 (2018) 723–741.
- [6] S.Y. Ding, Y.S. Liu, Y. Zeng, M.E. Himmel, J.O. Baker, E.A. Bayer, How does plant cell wall nanoscale architecture correlate with enzymatic digestibility? *Science* 338 (2012) 1055–1060.
- [7] D. Loqué, H.V. Scheller, M. Pauly, Engineering of plant cell walls for enhanced biofuel production, *Curr. Opin. Plant Biol.* 25 (2015) 151–161.
- [8] G.P. Maitan-Alfenas, E.M. Visser, V.M. Guimarães, Enzymatic hydrolysis of lignocellulosic biomass: converting food waste in valuable products, *Curr. Opin. Food Sci.* 1 (2015) 44–49.
- [9] J.E. Seabra, L. Tao, H.L. Chum, I.C. Macedo, A techno-economic evaluation of the effects of centralized cellulose ethanol and co-products refinery options with sugarcane mill clustering, *Biomass Bioenergy* 34 (2010) 1065–1078.
- [10] J. Portugal-Pereira, R. Soria, R. Rathmann, R. Schaeffer, A. Szklo, Agricultural and agro-industrial residues-to-energy: techno-economic and environmental assessment in Brazil, *Biomass Bioenergy* 81 (2015) 521–533.
- [11] R.R. Singhania, A.K. Patel, A. Pandey, E. Ganansounou, Genetic modification: a tool for enhancing beta-glucosidase production for biofuel application, *Bioresour. Technol.* 245 (2017) 1352–1361.
- [12] A. Sørensen, M. Lübeck, P.S. Lübeck, B.K. Ahring, Fungal beta-glucosidases: a bottleneck in industrial use of lignocellulosic materials, *Biomolecules* 3 (2013) 612–631.
- [13] L.M.O. Monteiro, M.G. Pereira, A.C. Vici, P.R. Heinen, M.S. Buckeridge, M.L.T.M. Polizeli, Efficient hydrolysis of wine and grape juice anthocyanins by *Malbranchea pulchella* β -glucosidase immobilized on MANAE-agarose and ConA-Sepharose supports, *Int. J. Biol. Macromol.* 136 (2019) 1133–1141.
- [14] J. Mallerma, L. Papinutti, L. Levin, Characterization of β -glucosidase produced by the white rot fungus *Flammulina velutipes*, *J. Microbiol. Biotechnol.* 25 (2015) 57–65.
- [15] F.F. Zanoelo, M.L.T.M. Polizeli, H.F. Terenzi, J.A. Jorge, β -Glucosidase activity from the thermophilic fungus *Scytalidium thermophilum* is stimulated by glucose and xylose, *FEMS Microbiol. Lett.* 240 (2004) 137–143.
- [16] J.C.S. Salgado, L.P. Meleiro, S. Carli, R.J. Ward, Glucose tolerant and glucose stimulated β -glucosidases—a review, *Bioresour. Technol.* 267 (2018) 704–713.
- [17] J.H. Noh, V.A. Hoang, Y.J. Kim, J.P. Kang, D.C. Yang, *Chryseobacterium ginsengiterrae* sp. nov., with Beta-glucosidase activity isolated from soil of a ginseng field, *Curr. Microbiol.* 74 (2017) 1417–1424.
- [18] D. Klein-Marcuschamer, P. Oleskowicz-Popiel, B.A. Simmons, H.W. Blanch, The challenge of enzyme cost in the production of lignocellulosic biofuels, *Biotechnol. Bioeng.* 109 (2012) 1083–1087.
- [19] A. Ranganathan, O.P. Smith, N.H. Youssef, C.G. Struchtemeyer, H.K. Atiyeh, M.S. Elshahed, Utilizing anaerobic fungi for two-stage sugar extraction and biofuel production from lignocellulosic biomass, *Front. Microbiol.* 8 (635) (2017) 1–10.
- [20] H. Guo, X.D. Wang, D.J. Lee, Proteomic researches for lignocellulose degrading 554 enzymes: a mini-review, *Bioresour. Technol.* 265 (2018) 532–541.
- [21] N.B. Ghanem, H.H. Yusef, H.K. Mahrouse, Production of *Aspergillus terreus* xylanase in solid-state cultures: application of the Plackett–Burman experimental design to evaluate nutritional requirements, *Bioresour. Technol.* 73 (2000) 113–121.
- [22] C.M. Judd, J. Westfall, D.A. Kenny, Experiments with more than one random factor: designs, analytic models, and statistical power, *Annu. Rev. Psychol.* 68 (2017) 601–625.
- [23] Y. Park, S. Kang, J. Lee, S.L. Hong, S. Kim, Xylanase production in solid state fermentation by *Aspergillus niger* mutant using statistical experimental designs, *Appl. Microbiol. Biotechnol.* 58 (2002) 761–766.
- [24] T. Keskin Gündoğdu, İ. Deniz, G. Çalıskan, E.S. Şahin, N. Azbar, Experimental design methods for bioengineering applications, *Crit. Rev. Biotechnol.* 36 (2016) 368–388.
- [25] M.J. Curtis, R.A. Bond, D. Spina, A. Ahluwalia, S. Alexander, M.A. Giembycz, A. Gilchrist, D. Hoyer, P.A. Insel, A.A. Izzo, A.J. Lawrence, D.J. MacEwan, L.D.F. Moon, S. Wonnacott, A.H. Weston, J.C. McGrath, Experimental design and analysis and their reporting: new guidance for publication in BJP, *Br. J. Pharmacol.* 172 (2015) 3461–3471.
- [26] B.S. Kaith, R. Sharma, S. Kalia, M.S. Bhatti, Response surface methodology and optimized synthesis of guar gum-based hydrogels with enhanced swelling capacity, *RSC Adv.* 4 (2014) 40339–40344.
- [27] B. Speranza, A. Racioppo, M. Sinigaglia, M.R. Corbo, A. Bevilacqua, Use of central composite design in food microbiology: a case study on the effects of secondary phenols on lactic acid bacteria from olives, *Int. J. Food Sci. Nutr.* 66 (2015) 520–525.
- [28] R. Rajendran, B. Animozhi, P. Prabhavathi, D. Kumar, P. Santhanam, M. Abirami, K. Sundaram, A. Manikandan, A Method of Central Composite Design (CCD) for optimization of biodiesel production from *Chlorella vulgaris*, *J. Pet. Environ. Biotechnol.* 6 (2015) 1000219.
- [29] D.S.N. Benarji, C. Ayyanna, Statistical optimizations of fermentation factors on bioethanol production from Mahua flower (*Madhuca indica*) with *Saccharomyces cerevisiae* by Response Surface Methodology in batch bioreactor, *Microbiol. J.* 2 (2016) 01–07.
- [30] T.M. Alsaiibi, I. Abustan, M.A. Ahmad, A.A. Foul, Application of response surface methodology (RSM) for optimization of Cu^{2+} , Cd^{2+} , Ni^{2+} , Pb^{2+} , Fe^{2+} , and Zn^{2+} removal from aqueous solution using microwaved olive stone activated carbon, *J. Chem. Technol. Biotechnol.* 88 (2013) 2141–2151.
- [31] R.H. Hesas, A. Arami-Niya, W.M.A.W. Daud, J.N. Sahu, Preparation of granular activated carbon from oil palm shell by microwave-induced chemical activation: optimisation using surface response methodology, *Chem. Eng. Res. Des.* 91 (2013) 2447–2456.
- [32] A. Maller, A.C. Vici, F.D.A. Facchini, T.M. da Silva, E.S. Kamimura, M.I. Rodrigues, J.A. Jorge, H.F. Terenzi, M.L.T.M. Polizeli, Increase of the phytase production by *Aspergillus japonicus* and its biocatalyst potential on chicken feed treatment, *J. Basic Microbiol.* 54 (2014) S152–S160.
- [33] M.G. Pereira, L.H.S. Guimarães, R.P.M. Furriel, M.L.T.M. Polizeli, H.F. Terenzi, J.A. Jorge, Biochemical properties of an extracellular trehalase from *Malbranchea pulchella* var. sulfurea, *J. Microbiol.* 49 (2011) 809–815.
- [34] M. Matsuo, T. Yasui, Properties of xylanase of *Malbranchea pulchella* var. sulfurea No. 48, *Agric. Biol. Chem.* 49 (1985) 839–841.
- [35] L.F. Ribeiro, R.C. De Lucas, G.L. Vitcosque, L.F. Ribeiro, R.J. Ward, M.V. Rubio, A.R. L. Damasio, F.M. Squina, R.C. Gregory, P.H. Walton, J.A. Jorge, R.A. Prade, M.S. Buckeridge, M.L.T.M. Polizeli, A novel thermostable xylanase GH10 from *Malbranchea pulchella* expressed in *Aspergillus nidulans* with potential applications in biotechnology, *Biotechnol. Biofuels* 7 (2014) 115.
- [36] D.G. Cooney, R. Emerson, Thermophilic Fungi. An Account of Their Biology, Activities, and Classification, W.H. Freeman, San Francisco, 1964.
- [37] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.* 72 (1976) 248–254.
- [38] G.L. Miller, Use of dinitrosalicylic acid reagent for determination of reducing sugar, *Anal. Chem.* 31 (1959) 426–428.
- [39] G.D. Szklarz, R.K. Antibus, R.L. Sinsabaugh, A.E. Linkins, Production of phenol oxidases and peroxidases by wood-rotting fungi, *Mycologia* 81 (1989) 234–240.
- [40] R.J. Redgwell, R.R. Selvendran, Structural features of cell-wall polysaccharides on onion *Allium cepa*, *Carbohydr. Res.* 157 (1986) 183–199.

- [41] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for determinations of sugars and related compounds, *Anal. Chem.* 28 (1956) 350–356.
- [42] J.C.I. Marco, G.P. Souza Neto, C.F.S. Castro, M. Michelin, M.L.T.M. Polizeli, E.X. Ferreira Filho, Partial purification and characterization of a thermostable β -mannanase from *Aspergillus foetidus*, *Appl. Sci.* 5 (2015) 881–893.
- [43] E.C. Giese, R.F.H. Dekker, I.S. Scarminio, A.M. Barbosa, R. Silva, Comparison of β -1,3-glucanase production by *Botryosphaeria rhodina* MAMB-05 and *Trichoderma harzianum* Rifai and its optimization using a statistical mixture-design, *Biochem. Eng. J.* 53 (2011) 239–243.
- [44] J.A. Cornell, Experiments with Mixtures – Designs, Models and the Analysis of Mixture Data, 3rd ed., John Wiley and Sons Inc, New York, USA, 2002.
- [45] B. Barros Neto, I.S. Scarminio, R.E. Bruns, Como fazer experimentos: pesquisa e desenvolvimento na ciência e na indústria, 4. ed., Ed. da Unicamp, Campinas, 2007, pp. 315–339.
- [46] L.M.F. Gottschalk, R.A. Oliveira, E.P. da Silva Bon, Cellulases, xylanases, β -glucosidase and ferulic acid esterase produced by *Trichoderma* and *Aspergillus* act synergistically in the hydrolysis of sugarcane bagasse, *Biochem. Eng. J.* 51 (2010) 72–78.
- [47] C.V. Nascimento, F.H.M. Souza, D.C. Masui, F.A. Leone, R.M. Peralta, J.A. Jorge, R. P.M. Furriel, Purification and biochemical properties of a glucose-stimulated β -D-glucosidase produced by *Humicola grisea* var. *thermoidea* grown on sugarcane bagasse, *J. Microbiol.* 48 (2010) 53–62.
- [48] T. Manavalan, A. Manavalan, K.P. Thangavelu, K. Heese, Secretome analysis of *Ganoderma lucidum* cultivated in sugarcane bagasse, *J. Proteomics* 77 (2012) 298–309.
- [49] W. Ouedrhiri, M. Balouiri, S. Bouhdid, S. Moja, F.O. Chahdi, M. Taleb, H. Greche, Mixture design of *Origanum compactum*, *Origanum majorana* and *Thymus serpyllum* essential oils: optimization of their antibacterial effect, *Ind. Crops Prod.* 89 (2016) 1–9.
- [50] J. Pérez, J. Munoz-Dorado, T. de la Rubia, J. Martínez, Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview, *Int. Microbiol.* 5 (2) (2002) 53–63.
- [51] K. Ratanakhanokchai, R. Waenukul, P. Pason, C. Aachaapaikoon, K.L. Kyu, K. Sakka, Y. Mori, *Paenibacillus curdlanolyticus* Strain B-6 multi-enzyme complex: a novel system for biomass utilization, in: M.D. Matovic (Ed.), *Biomass Now – Cultivation and Utilization*, IntechOpen, London, 2013, pp. 369–394.
- [52] M. Dastkhoo, M. Ghaedi, A. Asfaram, R. Jannesar, F. Sadeghfhar, Magnetic based nanocomposite sorbent combination with ultrasound assisted for solid-phase microextraction of Azure II in water samples prior to its determination spectrophotometric, *J. Colloid Interface Sci.* 513 (2018) 240–250.
- [53] A. Asfaram, M. Ghaedi, M.K. Purkait, Novel synthesis of nanocomposite for the extraction of Sildenafil Citrate (Viagra) from water and urine samples: process screening and optimization, *Ultrason. Sonochem.* 38 (2017) 463–472.
- [54] A. Asfaram, E.A. Dil, P. Arabkhani, F. Sadeghfhar, M. Ghaedi, Magnetic Cu: CuO-GO nanocomposite for efficient dispersive micro-solid phase extraction of polycyclic aromatic hydrocarbons from vegetable, fruit, and environmental water samples by liquid chromatographic determination, *Talanta* (2020) 121131.
- [55] A. Asfaram, M. Ghaedi, H. Javadian, A. Goudarzi, Cu-and S-@ SnO₂ nanoparticles loaded on activated carbon for efficient ultrasound assisted dispersive μ SPE-spectrophotometric detection of quercetin in Nasturtium officinale extract and fruit juice samples: CCD-RSM design, *Ultrason. Sonochem.* 47 (2018) 1–9.
- [56] L.M.O. Monteiro, A.C. Vici, M.P. Pinheiro, P.R. Heinen, A.H.C. de Oliveira, R.J. Ward, R.A. Prade, M.S. Buckeridge, M.L.T.M. Polizeli, A highly glucose tolerant β -glucosidase from *Malbranchea pulchella* (mp bg3) enables cellulose saccharification, *Sci. Rep.* 10 (1) (2020) 1–12, doi:<http://dx.doi.org/10.1038/s41598-020-63972-y>.