Sugar Tech (Jan-Feb 2023) 25(1):86–98
https://doi.org/10.1007/s12355-022-01191-7

RESEARCH ARTICLE

Multi-Response Optimization Using the Desirability Function of Exoglucanases, Endoglucanases and β-Glucosidases Production by *Aspergillus Niger* ITV-02 from Delignified Sugarcane Bagasse

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Received: 20 January 2022 / Accepted: 24 June 2022 / Published online: 14 July 2022

Abstract Multi-objective optimization using the desirability function is a useful tool, which allows you to select the better conditions to maximize multiple objectives/responses simultaneously. Enzymatic hydrolysis of lignocellulosic residues requires synergistic action of three cellulase enzymes: exoglucanases (FPase), endoglucanases (CMCase) and β-glucosidase (BGL), to the complete conversion of lignocellulosic residue to fermentable sugars. In this work the production of cellulases (FPase, CMCase and BGL) in *Aspergillus niger* ITV-02 using delignified sugarcane bagasse (DSB), was optimized using a Box–Behnken design, where independent variables were: DSB concentration, Tween 80 concentration and pH. The multi-objective optimization method was applied to maximize the three-enzymatic activities (FPase, CMCase and BGL) simultaneously and find an optimal value between simple and multi-objective optimization. The optimal parameters were 20.69 g/L of DSB, 0.24% v/v of Tween 80 and pH 5.67, obtaining an activity FPase 0.42 U/mL, CMCase 0.35 U/mL and BGL 10.23 U/mL. The activity FPase, CMCase and BGL after optimization increased 47.62, 65 and 47.4%, respectively, with respect to the activity before optimization. Multi-objective or multi-response optimization using the desirability function (d) allowed to obtain an enzymatic extract rich in exoglucanase, endoglucanase and *β*-glucosidase activity by *A. niger* ITV-02 using DSB, in order to improve the enzymatic hydrolysis process of lignocellulosic residues and thus the production of 2G biofuels.

Keywords Sugarcane bagasse · Aspergillus niger ITV-02 · Box–Behnken design · Desirability function · Cellulases

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Introduction

The application of biotechnological processes to produce biofuels and value-added chemicals from lignocellulosic residues has received a lot of attention in recent decades (Jampala et al. 2017). The enzymatic hydrolysis of lignocellulosic residue is a limiting stage in biofuels production. The multi-component enzyme "cellulases" hydrolyze cellulose through the synergistic action of three enzymes; endoglucanases are also known as CMCases (EC 3.2.1.4), exoglucanases or cellobiohydrolase (EC 3.2.1.91) and *β*-glucosidase (EC 3.2.1.21) (Matkar et al. 2013; Srivastava et al. 2018). These enzymes can be present in variable

proportions in a certain cellulolytic microorganism; in the case of cellulase enzymes, they vary depending on the substrate used to growth (Matkar et al. 2013). Cellulase use in the biofuels industry is projected to have an annual growth rate (CAGR) of approximately 7% over the next 5 years. The cellulase market was approximately US$ 1.5 billion in 2019, expected to reach US$ 2320 million in 2024 (Thapa et al. 2020). Therefore, the production and efficiency of cellulase has become one of the main points of attention on an industrial scale.

Fungi are equipped with machinery for the secretion of a large number of enzymes and therefore play an important role in cellulase production. Fungal strains belonging to different genera such as *Trichoderma*, *Aspergillus*, *Penicillium*, *Talaromyces* produce cellulases and xylanases (Baskaran et al. 2020). Fungi such as *T. reesei* and *T. viride* secrete a large amount of endoglucanases and cellobiohydrolases; however, the amount of β-glucosidase secreted by *Trichoderma* species is very low, which leads to an accumulation of cellobiose and therefore to an incomplete hydrolysis when the enzymatic extracts of cellulases produced by these fungi are used in the hydrolysis process (Strakowska et al. 2014). Species of the genus *Aspergillus* spp. such as *A. niger*, *A. fumigatus*, *A. flavus*, y *A. nidulans* are reported as cellulase producers (Prajapati et al. 2020). *Aspergillus* secretes β-glucosidase and it is frequently used to supplement commercial enzyme cocktails (Chauve et al. 2010). One of the advantages of *Aspergillus* is that it can grow over a wide range of temperature (10–50 °C), pH (2–11), water activity (aw) (0.6–1) and salinity (0–0.34%) (Kaschuk et al. 2020). The main setback in enzyme production is its cost of production. This limitation can be overcome by finding and isolating new microorganisms, using an accessible, low-cost carbon source and optimizing growing conditions in cellulase enzyme production.

Lignocellulosic biomass such as cane bagasse, sorghum bagasse, agave and corn stubble is known as good carbon source for the production of enzymes *by Aspergillus.* In 2021 Mexico reported an annual production of 51.92 million tons of sugarcane (*Saccharum officinarum*) (CONADESUCA 2020–2021), and it has been reported that each ton of ground sugarcane is generated 270 kg of cane bagasse (Castañon-Rodriguez et al. 2015). Cane bagasse is an accessible substrate, economical and due to its composition, 40–50% cellulose, 25–35% hemicellulose and the rest is lignin, is a potential source for the production of cellulases by microorganisms.

Cellulase production depends on various factors such as pH, fermentation time, surfactants and composition of the culture medium and profoundly affects the production of enzymes (Baskaran et al. 2020; Nanjundaswamy et al. 2020). pH is an important factor in cellulase production, and Prasetyo et al. (2010) reported optimal pH for endoglucanases (pH 4), exoglucanases and *β*-glucosidase (pH 5.5–6.0) for *Acremonium cellulolyticus.* Li et al. (2013) assessed the effect of pH on the production of cellulase by *T. reesei* 30 s-3–13 observing that cellulase production is related to mycelial morphological changes at different pH. In order to increase cellulase activity various authors have evaluated the addition of non-ionic surfactants in cellulase production, favoring cell membrane permeability and promoting the release of enzymes bound to cells (Suwannarangsee et al. 2014; Soni et al. 2010). One way to increase cellulase production (exoglucanases, endoglucanases and *β*-glucosidase) is through the optimization of the composition of the culture medium and the physicochemical conditions of production, resulting in cost-effective production. A design frequently used for enzyme production optimization is the Box–Behnken design, using the response surface methodology, which determines the optimal concentration of each component, the interaction between independent components and their effect on response (Kalantzi et al. 2019). However, when the optimization procedure involves more than one response, it is not possible to optimize each one in a separate way, because what is optimal for a response may not be for other responses. Simultaneous consideration of multiple responses requires first building an appropriate response surface model for each response and then trying to find a set of operating conditions that optimizes all responses in some sense or at least keeps them in the desired ranges. The multi-objective optimization method, using Derringer's desirability feature, is the best-known methodology, which seeks a combination of factor levels that simultaneously meets the requirements for each response in the design (Derringer et al. 1980). Jampala et al. (2017) evaluated cellulase and xylanase production by *T. reesei* NCIM1186 through multi-objective optimization by desirability function using a central composite experimental design. The optimal values reported 3.03 and 0.439 U/mL for cellulase and xylanase, respectively, achieving a trade-off between the two responses optimized by the central composite design. In a previous study we demonstrated that the effect of nitrogen source (urea, ammonium sulfate and yeast extract) has an effect on cellulase production in *A. niger* ITV-02, favoring the production of endoglucanases (Infanzón-Rodríguez et al. 2020). In this study a Box–Behnken design was used to evaluate the synergistic effect of three factors, concentration of DSB, Tween 80 and pH on enzymatic activity FPase, CMCase and BGL, with the focus of the desirability function to simultaneously optimize these three responses, in order to obtain an enzyme extract from *A. niger* ITV-02 efficient in the process of enzymatic hydrolysis of lignocellulosic residues.

Materials and Methods

Microorganism

A. niger ITV-02 belonging to the Bioengineering Laboratory of the Veracruz Institute of Technology cell culture collection was isolated from sugarcane bagasse (Infanzón-Rodríguez et al. 2021). *A. niger* ITV-02 was stored at 4 °C in the culture medium containing 10 g/L yeast extract, 20 g/L casein peptone, 20 g/L glucose and 25 g/L agar. Spores were recovered by adding a solution of 0.1% (v/v) Tween 80 in the Petri dishes and then used to inoculate the fermentation medium with an inoculum concentration of 6×10^6 spores/mL.

Sugarcane Bagasse Delignificated (DSB)

Sugarcane was provided by Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP) after grinding and juice extraction, and it was dried at room temperature until 10% humidity. To improve the digestibility and access of *A. niger* ITV-02 to the substrate, it was necessary to partially remove the lignin from the lignocellulosic residue. This bagasse was pretreated by alkaline hydrolysis with H_2O_2 (6% v/v) in a 12:1 v/w liquid: solid ratio, pH 11.5 with NaOH 10 M, for 37 h (Moran-Aguilar, 2018). Cellulase, hemicellulose and lignin were measured using the methodology of National Renewable Energy Laboratory (NREL) (Sluiter et al. 2008). Bagasse morphology was analyzed before and after pretreatment, as well as after submerged fermentation with *A. niger* ITV-02, by a scanning electron microscope (Tescan, Mira3).

Enzyme Production

Fermentations were carried out in 250-mL Erlenmeyer flasks with 100 mL culture medium consisting of delignified sugarcane bagasse (DSB), 0.3 g/L KH_2PO_4, 0.3 g/L $CaCl_2$, 0.3 g/L $MgSO_4.7H_2O$, 1 g/L peptone, 0.9 g/L urea, 1.5 g/L yeast extract, 2.4 g/L ammonium sulfate, 5 mg/L $FeSO_4.7H_2O$, 1.6 mg/L $MnSO_4$. H_2O, 1.4 mg/L $ZnSO_4.7H_2O$ and 2 mg/L $CoCl_2$, sterilized for 15 minutes at 121 °C. The fermentation was carried out at 31 °C, 200 rpm for 168 hours (Miranda-Sosa, 2019). The resulting fermentation samples were centrifuged for 20 minutes at 4500 x*g*, the supernatant collected and subsequently microfiltered with 0.20 μm nylon membranes (Merck Millipore, 0.20 μm GNWP04700). The determination of biomass was not determined because the substrate is sugarcane bagasse. The determination of proteases was carried out, and the results indicated that there was not presence of these.

Experimental Design

The delignified sugarcane bagasse concentration effect, DSB (X_1), surfactant concentration, Tween 80 (X_2) and pH (X_3), were optimized by response surface methodology using Stat graphics Centurion XVI software. For the response surface methodology, the Box–Behnken design was selected with three variables and three levels, medium (0), high (+ 1) and low (−1) (Table 1), which was designed with 12 different combinations of the evaluated parameters and 3 center points, for three responses (Y_j) FPase, BGL and CMCase activity (Table 2). The experiments were performed tripled. The ANOVA was performed by Statgraphics Centurion XVI software. Based on the quadratic model, the polynomial quadratic equation (Eq. 1) was generated, followed by the 3D graphs of response surface, in order to analyze the individual and interactive effect of each of the variables on the responses.

$$Y_j = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 \quad (1)$$

where Y_j is the predicted answer; b_0 is the intercept; b_1, b_2, b_3 are linear coefficients; b_{11}, b_{22}, b_{33} are the quadratic coefficients; b_{12}, b_{13}, b_{23} are the coefficients interaction with the independent variables, X_1 (substrate concentration, DSB), X_2 (surfactant concentration Tween 80) and X_3 (pH).

Analysis of Multi-Response Optimization

Multi-response optimization is used when there is more than one objective function to optimize simultaneously. In this study, to maximize the three response variables (FPase activity, CMCase and BGL) simultaneously, an optimization was performed using the desirability function (d), which consists of converting each response into a single desirability function (di), 0–1 ($0 \leq di \leq 1$) where di-0, indicating that optimization is not acceptable and di-1, suggesting that optimization is adequate (Jampala et al. 2017; Hossain et al. 2018; Maeda et al. 2010). Numerical and graphical analysis was performed using Statgraphics Centurion XVI version 16.1.03 statistical software.

Validation of Models

The optimal conditions of each variable for the FPase, BGL, CMCase activity and the desirability function, predicted by the models, were experimentally validated by triplicate.

Table 1 Independent variables and their levels employed in the Box–Behnken design

Independent Variables	Symbols	Coded and Actual Levels		
		Low (-1)	Middle (0)	High (+ 1)
DSB (g/L)	X_1	10	20	30
Tween 80 (% v/v)	X_2	0.1	0.35	0.6
pH	X_3	4	5	6

Table 2 Box–Behnken design matrix with actual values of the independent variables

Runs	X_1 DSB (g/L)	X_2 Tween 80 (%v/v)	X_3 pH
1	10	0.1	5
2	30	0.1	5
3	10	0.6	5
4	30	0.6	5
5	10	0.35	4
6	30	0.35	4
7	10	0.35	6
8	30	0.35	6
9	20	0.1	4
10	20	0.6	4
11	20	0.1	6
12	20	0.6	6
13	20	0.35	5
14	20	0.35	5
15	20	0.35	5

Determination of Enzymatic Activity

Endoglucanase activity (CMCase) was measured by the release of reducing sugars in 1 mL reaction volume containing 1% CMC (w/v) using the enzyme extract in a sodium acetate buffer pH 5, 0.05 mM for 30 min at 50 °C. Reducing sugars formed were quantified by the DNS (dinitrosalicylic acid) method (Miller, 1959). One unit of enzyme activity was defined as the amount of enzyme that produces 1 µmol reducing sugars per minute under the evaluated conditions. β-Glucosidase activity (BGL) was determined using 10 mM 4-nitrophenyl β-D-glucopyranoside (β-pNPG, Sigma) as the substrate in 0.05 M sodium acetate buffer pH 5. The reaction mixture involved incubating the substrate with the enzymatic extract at 50 °C for 10 min; the reaction was stopped by adding 2 mL 0.2 M Na_2CO_3 to one mL of the reaction mixture and absorbance was measured at 400 nm. One unit of β-glucosidase activity was defined as the micromol of p-nitrophenol released per milliliter of enzyme per minute under the conditions evaluated in the assay (Singhania et al. 2011). Filter paper activity (FPase) was quantified according to Ghose (1987). Reducing sugars formed were quantified by the DNS method (Miller, 1959). One FPase unit (FPU) was defined as the quantity of enzyme required to release 1 µmol glucose per min under the assay conditions (Prajapati et al. 2020). All experiments were conducted in triplicate.

Results and Discussion

Optimization of Activity Exoglucanases, Endoglucanases and β-Glucosidase

Sugarcane bagasse showed a composition of 33.4% glucans, 24.25% xylan and 27.95% lignin. Figure 1a shows the compact structure of sugarcane bagasse in its native form, a compact structure can be observed due to the interconnection between the biopolymers of cellulose and hemicellulose embedded in lignin, they can be observed in two different magnifications. The delignified sugarcane bagasse used as a carbon source in cellulase production *by A. niger* ITV-02 was characterized by NREL, whose composition obtained was 54% cellulose, 27.88% hemicellulose and 18.2% lignin. The effect of alkaline pretreatment is shown in Fig. 1b. The compact structure of cellulose and hemicellulose fibers showed pores in the fibers in addition to the breakdown of the fibers in an irregular shape. Alkaline pretreatments are effective in reducing lignin, altering the fiber structure, reducing cellulose crystallinity and promoting porosity, allowing microorganisms or enzymes to more easily access the cellulosic substrate (Maeda et al. 2010; Rodrigues et al. 2017). In Fig. 1c the DSB is shown after cellulases production by *A. niger* ITV-02 under optimal conditions, and it can be seen how the structure of the linear cellulose chain of the DSB was destroyed and the formation of holes in the residue lignocellulosic. The composition of the culture medium is one of the most important parameters that has an effect on the growth and production of enzymes in microorganisms; the combined effect of the concentration of delignified sugarcane bagasse substrate (DSB), concentration of Tween 80 and pH on cellulase production by *A. niger* ITV-02 was studied in this work. The Box–Behnken design matrix included 15 experiments with different combinations of DSB concentration, Tween 80 and pH (Table 2). Figure 2 shows the FPase, CMCase and BGL activity obtained in each treatment at 168 h. High activity can be observed FPase (0.47 U/mL), BGL (12.46 U/mL) and CMCase (0.50 U/mL) in three different treatments; 11 (DSB 20 g/L, Tween 80 0.1% v/v, pH 6), 12 (DSB 20 g/L, Tween 80 0.6% v/v, pH 6) and 6 (DSB 30 g/L, Tween 80 0.35% v/v, pH 4), respectively, at 168 h of submerged fermentation. Treatments with pH greater than 5 increased

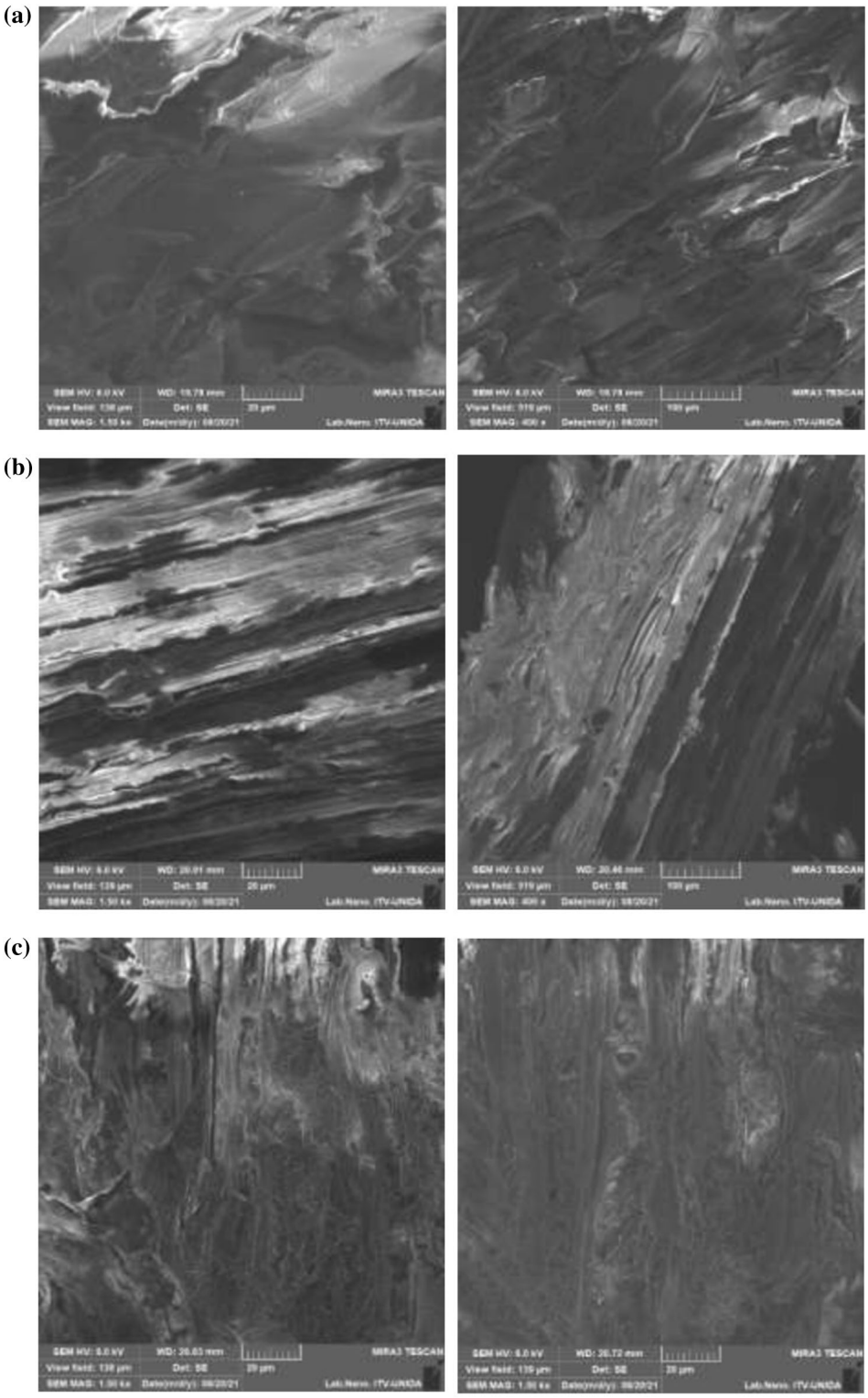

Fig. 1 Scanning electron micrograph (SEM) of (a) native sugarcane bagasse, (b) of pretreated sugarcane bagasse (DSB), (c) of DSB after fermentation with *A. niger* ITV-02

FPase and BGL activity; however, CMCase activity decreased. This can be attributed to the production of cellulase in filamentous fungi being influenced by pH (Virgilio et al. 2018). Li et al. (2013) evaluated the pH effect of the culture medium on the production of three cellulolytic enzymes, endoglucanases, exoglucanases and β-glucosidase, reporting that pH changes in the fermentation medium have an effect on the micelial morphology of *T. reesei,* which is correlated with cellulase activity. These authors report that CMCase activity was high at pH 4

values, when mycelium had fewer branches (primary mycelium), while the production of exoglucanases and β-glucosidase showed maximum activity at pH 5.5 and 6.0, when the mycelium showed greater branching (secondary mycelium). Long, branched hyphae increase the surface area of the fungus, possibly improving interaction with the substrate and thus improving enzyme productivity (Nicolas-Santiago et al. 2006).

On the other hand, the Tween 80 favored the CMCase and BGL activity. Surfactants generally play a positive role in improving enzymatic activities, creating a dispersion effect on fermentation media resulting in better aeration and subsequent increase in enzyme secretion (Nanjundaswamy et al. 2020), due to the stimulating effect and increased permeability in the cell membrane (Pardo, 1996; Kumar, 2020). These treatments showed that substrate concentration, the addition of surfactant (Tween 80) and pH are variables that can increase or decrease cellulase activity (FPase, CMCase and BGL), clearly indicating that it is necessary to optimize the production conditions of cellulase enzymes where all three activities are favored.

With the experimental results of the Box–Behnken design (Fig. 2) the multiple regression analysis was performed to determine the relationship between the three factors evaluated concentration of DSB, concentration of Tween 80 and pH (independent variables), for the optimization of the activity FPase, CMCase and BGL (dependent variables) by *A. niger* ITV-02.

Experimental data were adjusted to second-order polynomial equations for a respective response, and regression coefficients were calculated. The following regression equations demonstrate an empirical relationship between selected variables and enzymatic activities.

$$FP_{ase} = 0.531244 + 0.0127808x_1 + 0.418607x_2 - 0.164567x_3 - 0.000756333x_1^2 + 0.125867x_2^2 + 0.0196167x_3^2 + 0.0156x_1x_2 + 0.00225x_1x_3 + 0.011x_2x_3 \quad (2)$$

$$CMC_{ase} = 0.067032 + 0.00577x_1 + 0.92366x_2 + 0.096715x_3 + 0.000143x_1^2 - 0.3448x_2^2 - 0.00805x_3^2 + 0.0001x_1x_2 - 0.001975x_1x_3 - 0.1304x_2x_3 \quad (3)$$

$$BGL = -36.0461 + 1.00307x_1 + 13.1686x_2 + 12.0623x_3 - 0.0146596x_1^2 - 11.4473x_2^2 - 0.998208x_3^2 + 0.1233x_1x_2 - 0.0506x_1x_3 - 0.6x_2x_3 \quad (4)$$

where FPase is the activity in filter paper, CMCase activity endoglucanase and BGL activity β-glucosidase (U/mL), while X_1, X_2 and X_3 represent substrate concentration, DSB (g/L), concentration of Tween 80 (%v/v) and pH, respectively. The regression coefficients (R^2) of each equation were 95.83%, 85.74% and 93.29% for FPase, CMCase and BGL, indicating that only 4.17% (FPase), 14.26% (CMCase) and 6.71% (BGL) of the variability in the response cannot be explained by the model.

Variance analysis (ANOVA) (Table 3) shows that Tween 80, DSB concentration and pH have a significant effect ($p < 0.05$) on BGL activity. pH had a significant effect ($p < 0.05$) on FPase and CMCase activity. It has been reported by Lee et al. (2017) that Tween 80 influences the morphology of fungi, which probably improves the fungus's accessibility to nutrients to improve enzyme production level and activity. The stimulating effect of surfactant may be a consequence of its action on the cell

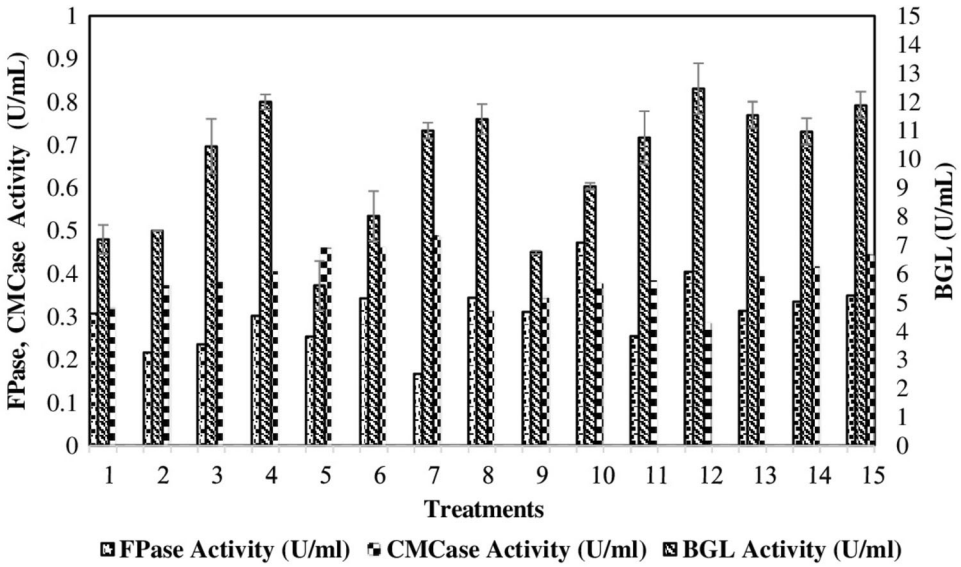

Fig. 2 Effect of DSB, Tween 80 and pH concentration on FPase, CMCase and BGL activity on each Box–Behnken design treatment

Table 3 ANOVA for the second-order polynomial models and coefficient values for FPase, CMCase and BGL activity obtained from the growth culture supernatant

Source	Sum of squares	Mean square	F value	P value
FPase activity				
X_1: DSB	0.000630	0.000630	1.89	0.3029
X_2:Tween 80	0.002701	0.002701	8.11	0.1044
X_3: pH	0.041760	0.041760	125.33	0.0079*
X_1^2	0.021121	0.021121	63.39	0.0154*
X_1X_2	0.006084	0.006084	18.26	0.0506
X_1X_3	0.001980	0.001980	5.94	0.1350
X_2^2	0.000228	0.000228	0.69	0.4947
X_2X_3	0.000030	0.000030	0.09	0.7916
X_3^2	0.001421	0.001421	4.26	0.1749
Lack of Fit	0.002692	0.000897	2.69	0.2823
Pure Error	0.000666	0.000333		
Cor Total	0.080595			
R^2 = 95.83%				
CMCase activity				
X_1: DSB	0.002178	0.002178	7.46	0.1120
X_2:Tween 80	0.000522	0.000522	1.79	0.3131
X_3:pH	0.038005	0.038005	130.15	0.0076*
X_1^2	0.000755	0.000755	2.59	0.2491
X_1X_2	0.000002	0.000002	0.00	0.9793
X_1X_3	0.001560	0.001560	5.34	0.1470
X_2^2	0.001715	0.001715	5.87	0.1363
X_2X_3	0.004251	0.004251	14.56	0.0623
X_3^2	0.000239	0.000239	0.82	0.4609
Lack of Fit	0.007630	0.002543	8.71	0.1047
Pure Error	0.000584	0.000292		
Cor Total	0.057603			
R^2 = 85.74%				
BGL Activity				
X_1: DSB	34.2254	34.2254	160.08	0.0062*
X_2:Tween 80	10.6791	10.6791	49.95	0.0194*
X_3:pH	5.89274	5.89274	27.56	0.0344*
X_1^2	7.93489	7.93489	37.11	0.0259*
X_1X_2	0.38007	0.38007	1.78	0.3140
X_1X_3	1.02414	1.02414	4.79	0.1601
X_2^2	1.89002	1.89002	8.84	0.0969
X_2X_3	0.09	0.09	0.42	0.5830
X_3^2	3.67909	3.67909	17.21	0.0535
Lack of Fit	4.1922	1.3974	6.54	0.1356
Pure Error	0.42759	0.213797		
Cor Total	68.8446			
R^2 = 93.29%				

*Indicates that the effect is significant at $P < 0.05$. No asterisk indicates that the effect is not significant

membrane causing an increase in permeability by promoting the release of enzymes bound to cells (Goukanapalle et al. 2020). The addition of surfactant has a positive effect on cellulase production. Nanjundaswamy and Okeke (2020) evaluated the use of surfactant in cellulase production by *Trichoderma* SG2 using powdered wastepaper,

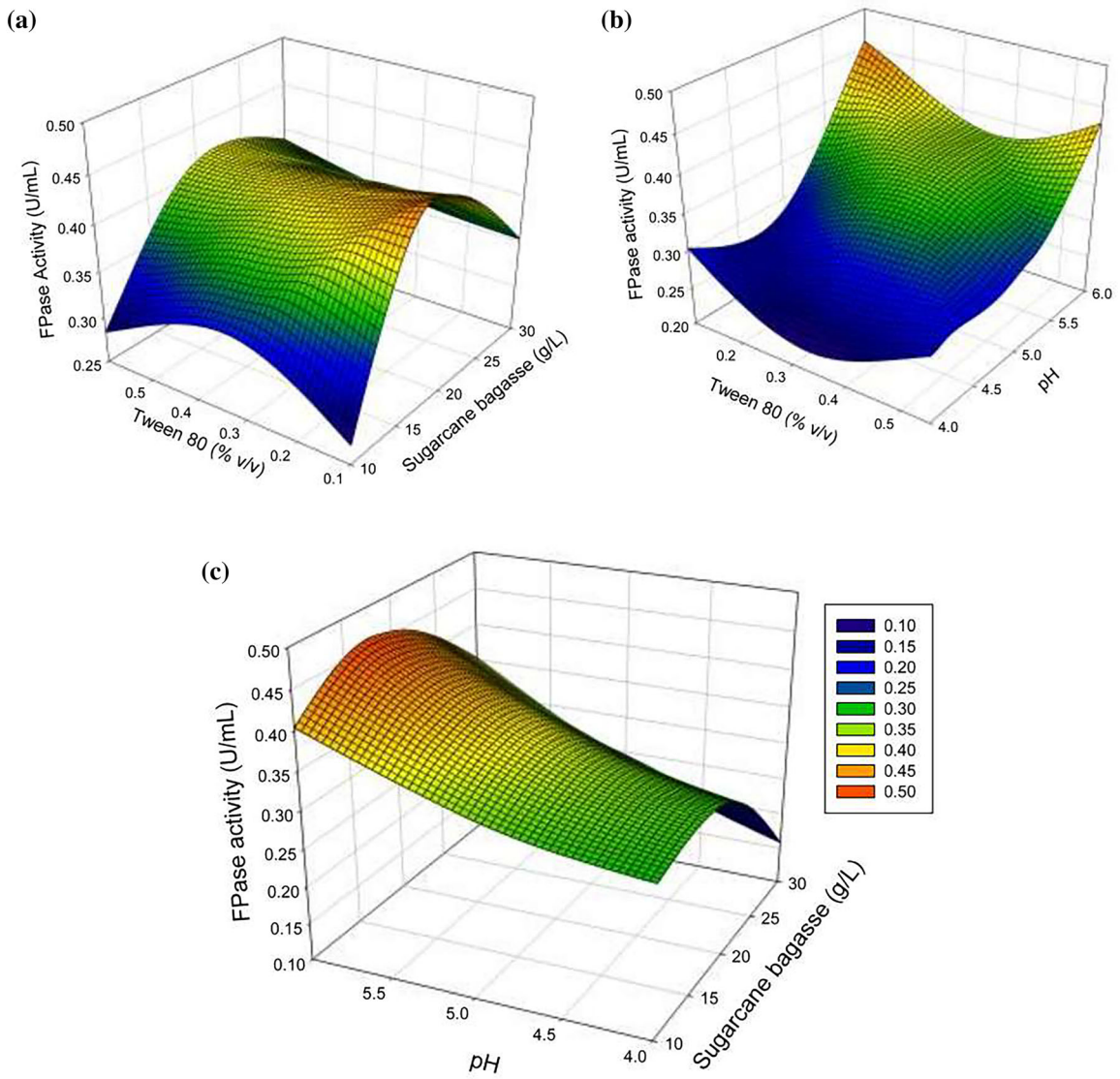

Fig. 3 Response surface graphs for the FPase activity. a) Concentration effect of DSB (g/L) versus Tween 80 (% v/v), b) effect of Tween 80 (% v/v) versus pH and c) effect of pH versus DSB concentration (g/L)

reporting that Tween 60 and 80 (0.5 g/L) favored BGL activity (9.56 U/mL). Kalsoom et al. (2019) reported that concentrations of 0.3% of Tween 80 favored cellulase activity, due to a reduction in pellet formation.

Figures 3a, 4a and 5a show the response surface plot for the activity FPase, CMCase and BGL, depending on the concentration of substrate and Tween 80, can be observed that intermediate concentrations (20 g/L) of DSB and low concentrations of Tween 80 (0.1% v/v) favor FPase activity, while BGL and CMCase activity increases by increasing the concentration of DSB (30 g/L) and Tween 80 (0.6% w/v). These results show that by increasing the concentration of substrate and the addition of Tween 80 favors cellulase activity. Li et al. (2019) evaluated the effect of substrate concentration (Kraft hardwood pulp) on cellulase production by *T. reesei* rut C-30 reporting that 40 g/L favored cellulase activity; however, by increasing the concentration of kraft hardwood pulp cellulase activity decreased due to the limitation of mass transfer to a higher substrate load. Almeida et al. (2019) reported that the best concentration of substrate (gamba grass) was between 25 and 55 g/L for the production of endoglucanases for *Fusarium verticillioides.* pH had a significant effect ($p < 0.05$) on the three activities analyzed, FPase, CMCase and BGL (Table 3). In response surface plot (Fig. 3b, 3c, and 5b, c) it can be observed that by increasing the pH to 5 the FPase and BGL activities are favored; however, CMCase activity decreases and increases to pH 4 values (Fig. 4b and c). Several authors have reported a correlation between the initial pH of the medium and the production of

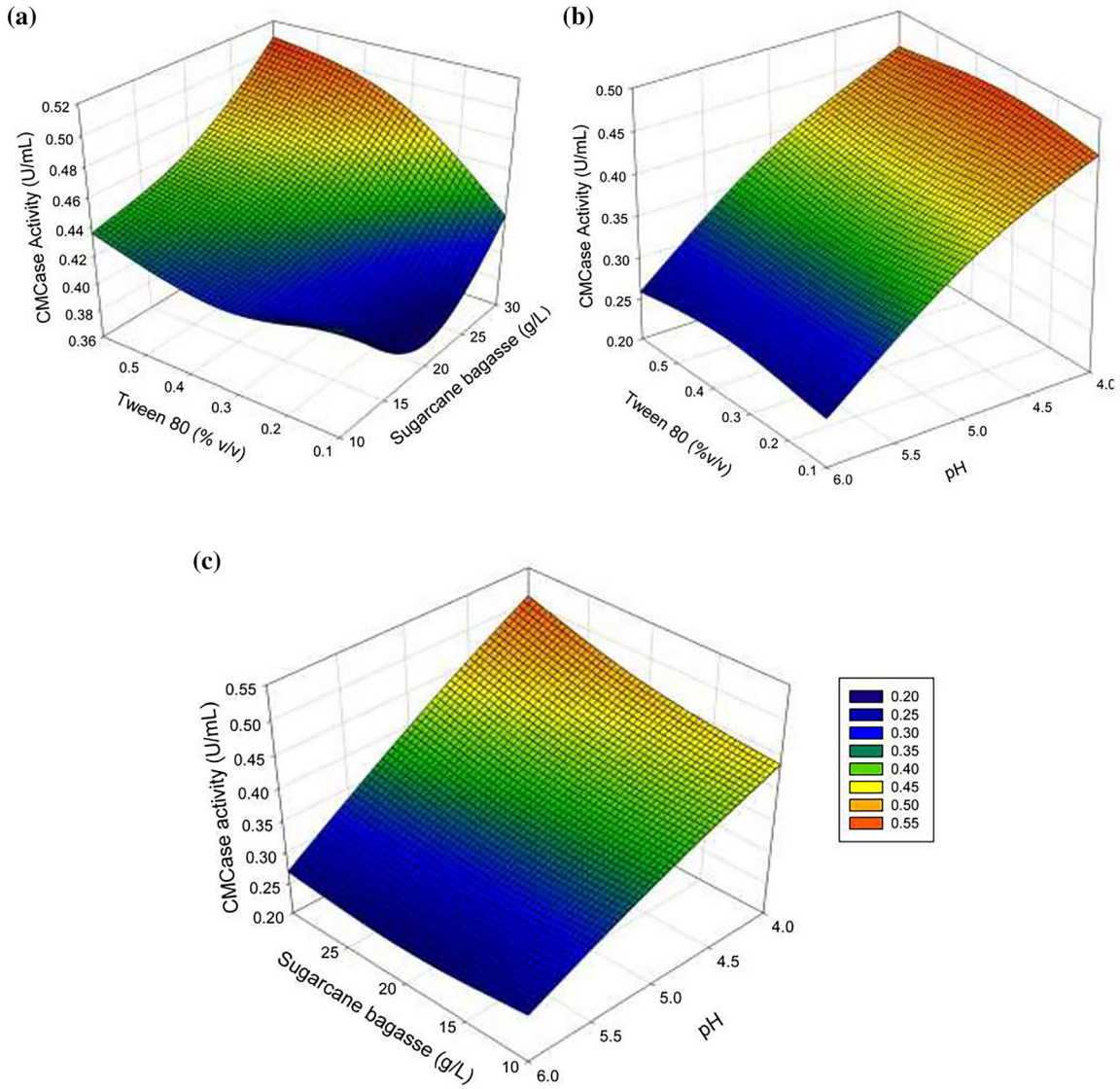

Fig. 4 Response surface graphs for CMCase activity. a) Concentration effect of DSB (g/L) versus Tween 80 (% v/v), b) effect of Tween 80 (% v/v) versus pH and c) effect of pH versus DSB concentration (g/L)

cellulase (Sohail et al. 2009; Toor et al. 2014; Teixeira et al. 2016). Sohail et al. (2009) reported that acidic pH (4.0) of the medium induces cellulase production (CMCase and BGL). Kalsoon et al. (2019) reported a maximum enzyme production of 1,165 U/mL at pH 6, while the increase in pH to 8 decreased cellulase activity in *T. reesei*. Liu and Yang (2007) also reported that acidic conditions (pH 5) are the best conditions for cellulase production. The optimum level of each independent variable can be seen in the response surface plots (Fig. 3, 4 and 5) and in Table 4; concentration of DSB (X_1), concentration of Tween 80 (X_2) and pH (X_3) in optimization for each response (FPase, CMCase and BGL).

Validation Model

To validate the mathematical model and the optimal conditions of the variables (X_1, X_2 and X_3), the experiments were performed with the values predicted by the model. Table 4 shows the optimal conditions of each model for maximum activity FPase (Eq. 2), CMCase (Eq. 3) and BGL (Eq. 4) obtaining the predicted and experimental values of the other two activities, respectively. Optimization models showed an excellent correlation between the experimentally obtained response and the predicted response (Table 4).

In optimization to maximize a single response, the maximum FPase activity was 0.41 U/mL where CMCase and BGL activity were 0.302 U/mL and 8.55 U/mL,

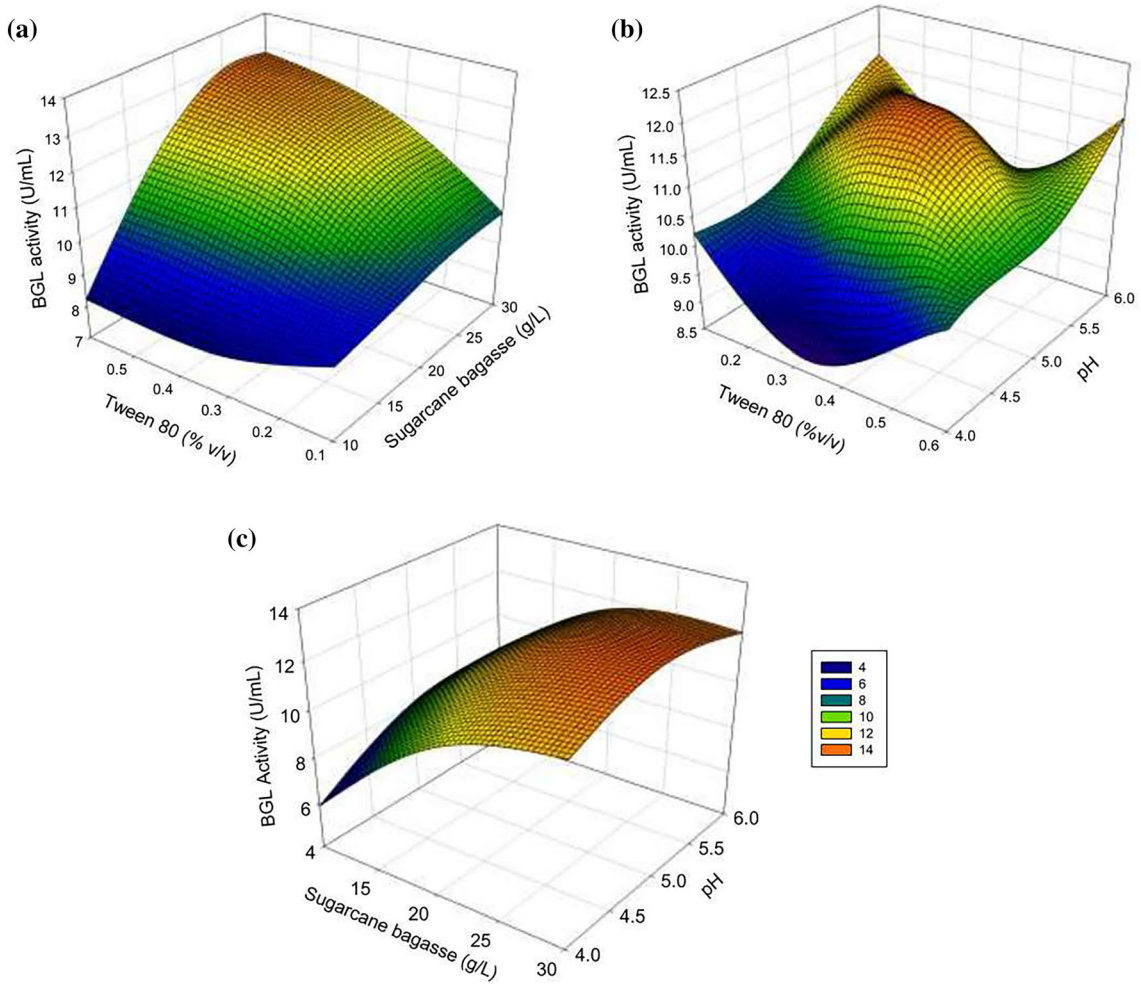

Fig. 5 Response surface graphs for the activity of β-glucosidase. a) Concentration effect of DSB (g/L) versus Tween 80 (% v/v), b) effect of Tween 80 (% v/v) versus pH and c) effect of pH versus DSB concentration (g/L)

Table 4 Predicted and experimental values of the responses at optimum conditions

	Optimal levels of variables			(U/mL) Optimized values (predicted values)[a]			(U/mL) Experimental values [b]			Correlation between experimental and theoretical value		
Model	DSB (g/L)	Tween 80 (% v/v)	pH	FPase	CMCase	BGL	FPase	CMCase	BGL	FPase	CMCase	BGL
Not optimized	10	0	5.3	–	–	–	0.2 ± 0.01	0.23 ± 0.03	4.85 ± 0.32	–	–	–
FPase (Eq. 2)	18.3	0.1	6	0.4563	0.3050	9.35	0.41 ± 0.05	0.302 ± 0.035	8.55 ± 0.20	0.90	0.99	0.91
CMCase (Eq. 3)	29.993	0.58	4	0.2021	0.508	11.59	0.18 ± 0.015	0.42 ± 0.03	10.3 ± 0.25	0.89	0.83	0.89
BGL (Eq. 4)	27.78	0.583	5.16	0.312	0.3699	12.89	0.315 ± 0.014	0.354 ± 0.03	11.81 ± 0.38	1.00	0.96	0.92
Desirability function	20.69	0.2408	5.67	0.4	0.3303	11.1	0.42 ± 0.04	0.354 ± 0.015	10.23 ± 0.22	1.05	1.07	0.92

[a]Predicted using response surface quadratic model

[b]Mean ± standard deviation of triplicate determinations from experiments

respectively. The model to optimize CMCase obtained 0.42 U/mL, where the FPase and BGL activity were 0.18 and 10.3 U/mL. Finally, in the model to optimize BGL activity 11.81 U/mL, 0.31 U/mL FPase and 0.35 U/mL CMCase were reached. Because the optimal conditions of each model for maximum FPase, CMCase and BGL

activity are in a substrate concentration range of 18.3–29.99 g/L of DSB, 0.1 to 0.58% w/v of Tween 80 and pH from 4 to 6, it was necessary to resort to multi-objective optimization using the desirability function in order to find the optimal parameters to simultaneously meet the requirements for each response in the design, without adversely affecting the activity of one type of enzyme in favor of another. Therefore, optimal compensation was achieved between the production of FPase, CMCase and BGL. The results were compared with those of optimizing a single response variable. The predicted maximum response obtained by multi-objective optimization was as follows: activity FPase 0.4 U/mL, CMCase 0.33 U/mL and BGL 11.1 U/mL at the optimal level of variables (DSB, 20.69 g/L; Tween 80, 0.24% v/v; pH, 5.67) in a desirability of 0.91. The FPase, CMCase and BGL activity obtained from multi-objective optimization was found slightly less than the optimization values for a single response. However, it can be seen in Table 4 that using the single response model to promote the desired response sacrifices the activity of the other two enzymes. Such is the case of the CMCase model (Eq. 3) where the predicted value was 0.5 U/mL, the predicted FPase activity was 0.2 U/mL, decreasing 50% compared to predicted values using multi-objective optimization (FPase 0.4 U/mL).

The three experimentally obtained FPase, CMCase and BGL activities were favored using the conditions obtained in multi-objective optimization. Experimentally obtained FPase, CMCase and BGL activity increased 2, 1.54 and 2.11 times more in multi-objective optimization than under in optimized conditions in the cellulase enzyme production process (Table 4), unestimated. Similar results of BGL activities (9.24 U/mL) for *A. sydowii* on the sixth day of submerged fermentation were reported by Matkar et al. (2013). Sirohi et al. (2019) assessed the production conditions of pre-treated Pea Hulls using *T. reesei* QM9414 in submerged fermentation reaching an FPase activity of 0.372 U/mL at 91 h of fermentation time. A study conducted by De Castro et al. (2010) reported the production of an enzyme extract from sugarcane bagasse by *T. harzianum* IOC-4038 for the production of cellulases in submerged fermentation, reaching 0.09, 0.559 and 0.745 U/mL of FPase, CMCase and BGL at 166 h of fermentation. Infanzón-Rodríguez et al. (2020) reported the production of cellulase by *A. niger* ITV-02, using the delignified sweet sorghum bagasse and optimizing through the Box–Behnken design three nitrogen sources (urea, ammonium sulfate and yeast extract) and a single response (CMCase activity), reporting a CMCase activity of 0.61 U/mL and BGL of 0.41 U/mL. The previously mentioned works report the optimization of a single responses; in this work, through multi-objective optimization it was possible to find an optimal compensation between the three activities FPase (0.42 U/mL), CMCase (0.354 U/mL) and BGL (10.23) in order to obtain an efficient enzymatic extract in the hydrolysis of lignocellulosic residues. Maeda et al. (2010) optimize nitrogen sources in cellulases production by *P. funiculosum* ATCC 11,797 in acid-alkaline pretreated sugarcane bagasse using a composite central design and the desirability function (d = 0.872) achieved an FPase activity of 0.227, CMCase 6.917 and BGL 1.37 U/mL. Santos et al. (2022) evaluated the cellulases production: FPase and CMCase by *A. niger* using urban lignocellulosic residues, through the central composite design, they evaluated the interactive effect of pH and moisture content and optimized through the desirability function, reporting an FPase activity of 0.413 U/mL and CMCase of 0.23 U/mL, respectively.

In this work under the optimal conditions of DSB concentration, Tween 80 and pH of the multi-objective model (d = 0.91) FPase 0.42 U/mL CMCase 0.354 U/mL and BGL 10.23 U/mL activity was achieved, optimizing the three enzymatic activities simultaneously. The BGL (10.23 U/mL), FPase (0.42 U/mL) activity of the enzymatic extract of *A. niger* ITV-02 was higher than that reported by Maeda et al. (2010) and Santos et al. (2022). It has been reported that an FPase: BGL ratio greater than 1: 4 prevents cellobiose accumulation during hydrolysis, which reduces enzyme inhibition (Saine et al. 2015). In this study the FPase: BGL activity ratio (1: 24.3) is high enough for efficient saccharification compared to the reported values of 1: 5 (Gottschalk et al. 2010) and 1: 7.1 (Matkar et al. 2013). Obtaining an enzyme cocktail with cellulase activity can be applied in enzymatic hydrolysis of lignocellulosic residues (sugarcane bagasse, sorghum bagasse, corn stubble, wheat straw, among others) for fermentable sugars productions and its application in 2G bioethanol production.

Conclusions

Multi-objective optimization using the desirability function allowed to find the optimal parameters of DSB concentration (20.69 g/L), Tween 80 (0.24% v/v) and pH (5.67) to simultaneously improve and maximize all three activities with respect to the optimization of a single response, achieving an FPase activity of 0.42 U/mL, CMCase of 0.35 U/mL and BGL of 10.23 U/mL. FPase, CMCase and BGL activity increased by 47.62, 65 and 47.4%, respectively, with respect to optimized treatment. This allows to obtain an extract with cellulase activity and a FPase: BGL with a relation 1: 24.3 that can be applied in the enzymatic hydrolysis of lignocellulosic residues efficiently for the production of fermentable sugars, which can be used to produce second-generation ethanol. The enzymatic activity

of the enzymatic extract of *A. niger* ITV-02 can be increased by means of a concentration stage prior to the enzymatic hydrolysis of lignocellulosic residues in order to increase percentage conversion and sugar concentration in the hydrolysis. However, additional experiments should be carried out during the optimization stage of the enzymatic hydrolysis of lignocellulosic residues using the enzymatic extract of *A. niger* ITV-02 produced directly under the optimal parameters established in this work.

Acknowledgements The authors acknowledge the economic support from CONACyT by the postdoctoral granted to M.I Infanzon-Rodríguez.

Declarations

Conflict of Interest The authors declare that they have no conflict of interest.

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