Original article

Use of agroindustrial wastes for the production of cellulases by *Penicillium* sp. FSDE15

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A B S T R A C T

Cellulase refers to a group of enzymes like endoglucanases (CMCase), exoglucanases and β-glucosidase that have great industrial importance for the production of second-generation ethanol, e.g. The present study aimed to evaluate the CMCase and β-glucosidase activities of the fungal strain *Penicillium* sp. FSDE15 using different agroindustrial wastes (wheat bran, sugarcane bagasse and sugarcane straw) as carbon sources to the basal medium during Submerged Fermentation (SFm). When the medium presented wheat bran, highest CMCase and β-glucosidase yields were 1.03 U/mL (96 h) and 1.68 U/mL (168 h), respectively. For sugarcane bagasse medium, maximum enzymatic activity values were observed in 120 h culture (1.38 U/mL for CMCase and 0.55 U/mL for β-glucosidase). Sugarcane straw medium presented highest enzymatic yields after 120 h culture (1.02 U/mL of CMCase and 0.39 U/mL of β-glucosidase). The response surface methodology showed highest CMCase (1.3 U/mL) and β-glucosidase (6.5 U/mL) activities in the presence of lower levels of wheat bran (1%) (w/w), inoculum concentration of 10^5 spores/mL, temperatures of 41 °C and 33 °C, respectively. *Penicillium* sp. FSDE15 presented two isoforms of CMCase (150 kDa and around 30 kDa) and one isoform of β-glucosidase (70 kDa). Our study demonstrated that the production of cellulases with high activity by *Penicillium* sp. FSE15 in a culture medium with agroindustrial wastes, such as wheat bran, might be a promising alternative to reduce the cost of these molecules and facilitate their use in various industrial processes.

1. Introduction

Lignocellulosic materials are mainly composed of lignin, cellulose and hemicellulose. They have low cost and potential use in the production of biomolecules and biofuels that offer benefits, both environmental and strategic. Among these materials, sugar cane bagasse, sugar cane straw, corn cob, wheat bran, among others (Zoghlami and Paës, 2019).

Cellulose is a fibrous, stringent, crystalline and water insoluble organic carbon source that can be completely hydrolyzed into cellobiose and glucose monomers by cellulases. These enzymes systems consisting of three main components: endoglucanases (CMCases), exoglucanases and β-glucosidases (Thapa et al., 2020). Cellulases are essential enzymes for the conversion of lignocellu-

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losic biomass into fermentable sugars for the production of second-generation ethanol (Vaishnav et al., 2018).

Fungi of the genus *Penicillium* are also profitable options for the production of cellulases on an industrial scale, in addition to producing β-glucosidases with high activity (Vaishnav et al., 2018). β-Glucosidases are often present at lower levels than the other two enzymatic components – endo and exoglucanases - in microbial preparations. Maintaining optimal amounts of this enzyme is essential for achieving high yields of glucose during biomass degradation (Gao et al., 2012). CMCase and β-glucosidase are key enzymes in the process of cellulose degradation to glucose. Recently, our research group reported the *Penicillium* FSDE15 isolate as an outstanding producer cellulase (Santos et al., 2021). However, these results prompted the question, is it possible to improve cellulase production in *Penicillium* sp. FSDE15? It is evident that there are several variables that can be manipulated for this purpose, such as carbon source with an experimental design by statistical models. Therefore, the aim of this study was to evaluate the CMCase and β-glucosidase production by the fungal strain *Penicillium* sp. FSDE15 using different agroindustrial wastes such as wheat bran, sugarcane bagasse and sugarcane straw as well as to optimize the enzymatic activity.

2. Materials and methods

2.1. Microorganism

This study was carried out with a new fungal strain *Penicillium* sp. FSDE15 obtained from the collection of cultures of the Laboratory of Applied Microbiology (UFPB, Brazil). The strain was isolated by screening soil samples from sugarcane plantation located at Santa Rita, Paraiba, Brazil (6°99′20.67″S 35°02′55.61″) and stored at 4 °C on potato dextrose agar plates (Sigma Aldrich, St. Louis, USA). The microorganism was maintained in Petri dishes containing PDA medium under sterile conditions at 37 °C for seven days. Spore suspension was counted at 10^6 spores/mL according to Santos et al. (2021).

2.2. Cellulases production by submerged state fermentation (SFm)

The basal medium was adapted from Mandels and Weber medium (MW) (1969). Three different lignocellulosic residues, wheat bran (WB), sugarcane bagasse (SB) and sugarcane straw (SS), were used as carbon source. Before their use, each residue underwent milling processes in a knife mill (Wiley Mill, Solab, SL-31) and sieving (mesh 32). Subsequently, residues were submitted to thermal pretreatment in autoclave (121 °C, 1 atm for 15 min). A volume of 50 mL of MW medium were added to 125 mL Erlenmeyer flasks, as well as 5% (w/v) of pretreated WB, SB and SS, separately, final pH was 5.7. The medium was autoclaved for 15 min at 121 °C and, after cooling, received an inoculum of 10^6 spore/mL. Enzymatic production under SmF occurred in duplicates in rotary shaker (Solab, SL-223) at 150 rpm, 37 °C for 8 days. Every 24 h, two flasks were removed and the sample were filtrated using Whatman n° 1 filter paper and centrifuged at 5000 rpm, 10 °C for 5 min (Quimis, Q222RT). Afterwards, the pH of all centrifuged samples was measured. The crude enzymatic extract was used for determination of enzymatic activities.

2.3. Enzymes activities measurement

CMCase activity was assayed in triplicate according to Santos et al. (2021). Calibration curve for CMCase was performed using different glucose concentrations, each level in three replicates

(Miller, 1959). One unit (U) of CMCase activity corresponded to 1 μmol of glucose released per minute.

β-glucosidase activity was determined using 2 mM p-nitrophenyl-β-D-glucopyranoside (PNPG, Sigma-Aldrich) in 50 mM sodium acetate buffer (pH 5.0) and 0.125 mL of the crude extract. The trials were in triplicate according to Gao et al. (2008). Calibration curve for β-glucosidase was made similarly as CMCase, although using p-nitrophenol as substrate (Shi et al., 2018). One unit of β-glucosidase was defined as the releasing of 1 μmol of p-nitrophenol per minute.

2.4. Experimental design and optimization of cellulase production by response surface methodology (RSM)

In order to optimize the cultivation conditions and enhance CMCase and β-glucosidase production by *Penicillium* sp. FSDE15, experimental design was performed using RSM by the software Statistica (Statsoft 7.0, EUA). Then a 2^3 experimental design was used with 3 repetitions in the central point and 8 unique combinations, totaling 11 assays. The factors were inoculum concentration (X_1) (10^5–10^7 spores/mL), temperature (X_2) (30–40 °C) and wheat bran concentration (X_3) (1–5%). Each factor in the design matrix was studied at three different levels (-1, 0, +1) and their level values are shown in Table 1. The variables or responses were activity of CMCase (U/mL) and β-glucosidase (U/mL) are depicted in Table 2.

The fungal strain was cultivated in 125 mL Erlenmeyer flasks containing 50 mL of modified MW medium with 5% (w/v) wheat bran for 4 days, under constant agitation in orbital shaker at 150 rpm. The enzymatic extract was centrifuged at 12.000 rpm at 10 °C for 5 min and enzymatic activity was assayed.

2.5. Enzymatic profile analysis with SDS-PAGE electrophoresis

For CMCase profile analysis, the cell-free supernatant was assayed using polyacrylamide denaturing gel (12% w/v; 0.75 mm) (Padilha et al., 2015). The β-glucosidase gel was washed with distilled water and incubated at 55 °C for 15 min in 0.5 mM 4-methyl-lumbelliferyl β-D-glucopyranoside (MUG) diluted in citrate buffer 50 mM (pH 4.8). The molecular mass markers used were those for low molecular weight ranges (GE, USA) including phosphorylaseb (97 kDa), serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-Lactalbumin (14.4 kDa). Finally, for enzymatic activity visualization and photography, the CMCase gel was submerged in Congo Red (0.1 %) for 15 min and decolored in NaCl 1 M, while β-glucosidase gel was visualized under UV 365 nm (UltraBright transilluminator) (Ng et al., 2010).

2.6. Statistical analysis

The statistical analysis was performed by variance analysis (ANOVA) aiming to obtain the regressions' significance at a 95 % level of confidence. Softwares such as Graph Prism 5.0 (GraphPad software, CA, EUA) and Statistica (Statsoft 7.0, EUA) were used to analyze the data obtained.

3. Results

3.1. CMCase and β-glucosidase production by SmF

All three carbon sources (WB, SB and SS) used in our study induced growth and production of CMCase and β-glucosidase by the *Penicillium* sp. FSDE15. The initial activity of CMCase and β-glucosidase before 24 h, in all culture media, was zero. When the

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Table 1
Independent variables and coded levels for statistical experimental design.

Independent variables	Symbol	Codified levels		
		−1	0	1
Inoculum concentration (spore/mL)	X_1	10^5	10^6	10^7
Incubation temperature (°C)	X_2	33	37	41
Wheat bran concentration (%)	X_3	1	3	5

Table 2
Experimental conditions and results of the statistical experimental design for CMCase and β-glucosidase production by *Penicillium* sp. FSDE15.

Test	X1	X2	X3	CMCase		β-glucosidase	
				Observed	Predicted	Observed	Predicted
1	10^5	33	1	1.05	1.09	6.50	6.74
2	10^7	33	1	0.74	0.78	5.99	6.22
3	10^5	41	1	1.30	1.31	0.52	0.75
4	10^7	41	1	0.90	0.94	1.29	1.52
5	10^5	33	5	0.12	0.16	0.69	0.92
6	10^7	33	5	0	0.04	3.82	4.05
7	10^5	41	5	0.62	0.66	2.49	2.69
8	10^7	41	5	0.40	0.44	0.80	1.13
9	10^6	37	3	0.80	0.68	3.53	3
10	10^6	37	3	0.78	0.68	4.18	3
11	10^6	37	3	0.81	0.68	3.13	3
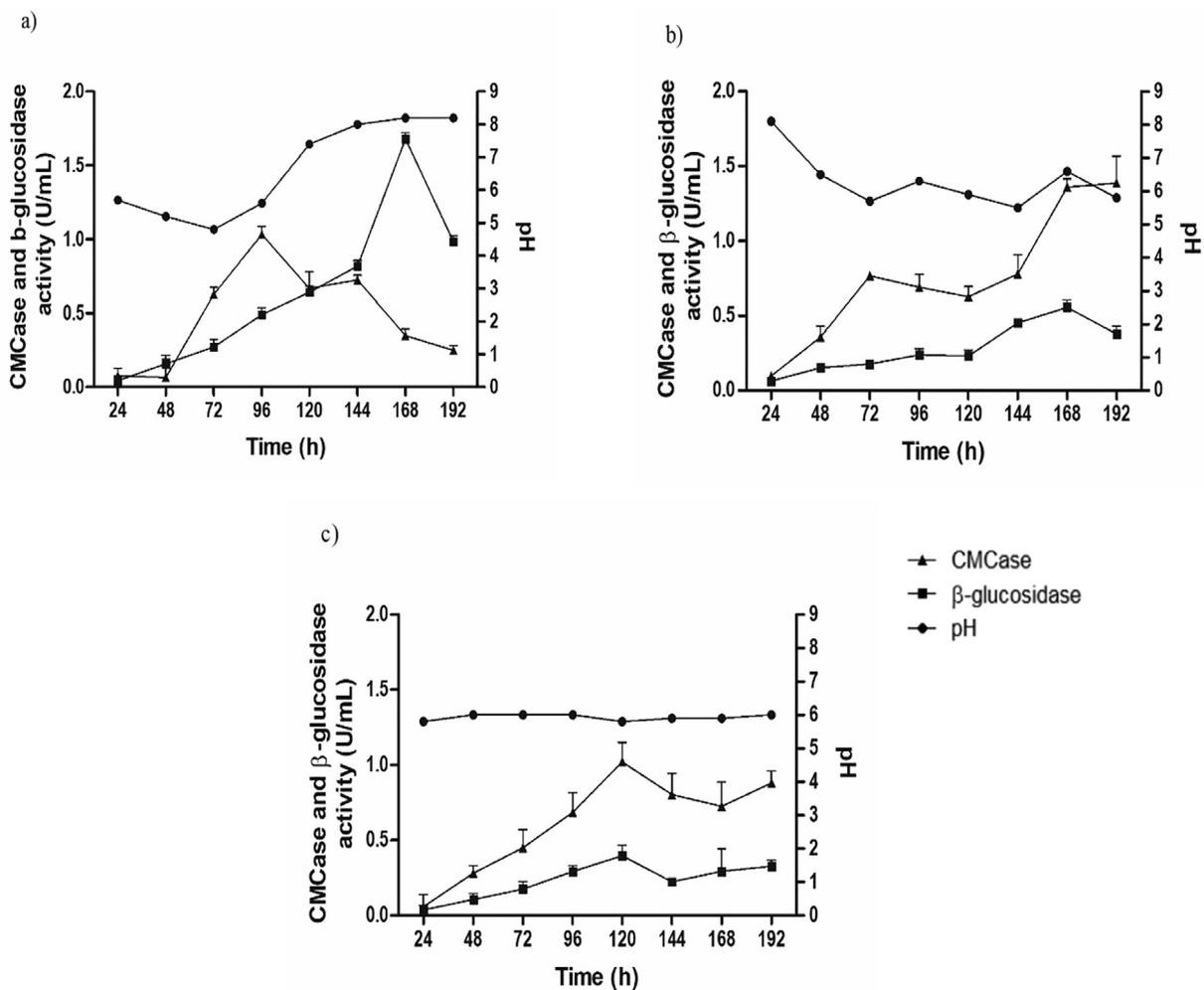
Fig. 1. Enzymatic activity (EA) of Penicillium sp. FSDE15 in basal media containing wheat bran (a), sugarcane bagasse (b) and sugarcane straw (c).

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medium was composed of WB, maximum CMCase and β-glucosidase yields were 1.03 (96 h) and 1.68 (168 h), respectively (Fig. 1A). With the SB medium, maximum values of enzymatic activity observed were after 120 and 168 h culture to CMCase (1.38 U/mL) and β-glucosidase (0.55 U/mL), respectively (Fig. 1B). Although, when the SS medium was used, the highest enzymatic activity was 1.02 U/mL of CMCase and 0.39 U/mL of β-glucosidase after 120 h growth (Fig. 1C).

Regarding the pH variation, alkalinization of the WB medium was directly related to the reduction in CMCase activity, where the pH increased from 5.7 (96 h) to 7.4 (120 h) and reduced about 35% of the enzyme activity (Fig. 1). On the order hand, highest β-glucosidase activity were observed at pH 8.2, 5.7 and 6.6, when the medium WB, SB and SS were used, respectively.

3.2. Cellulase production by RSM

The results presented in Table 2 showed a wide variation of CMCase activity (0 to 1.3 U/mL) and β-glucosidase activity (0.52 to 6.5 U/mL), which reflects the importance to optimize the process parameters. Both CMCase and β-glucosidase showed highest activity in the presence of lower levels of WB (1%) (w/w), inoculum concentration of 10^5 spores/mL and temperatures of 41 and 33 °C, respectively (Table 2).

In this study, R^2 for CMCase and β-glucosidase were 0.96 and 0.95, respectively. Analysis of variance (ANOVA) was used to assess the fit of the polynomial model to CMCase and β-glucosidase activity. The results tabulated in Table 3 showed that calculated F value was 1.28 for CMCase and 0.92 for β-glucosidase (P < 0.05 at 95% confidence level), indicating that the model was significant only to CMCase.

Statistical analysis showed that the two independent variables (WB concentration and temperature) were significant for CMCase. For β-glucosidase, independent variable temperature and interaction between temperature-WB concentration was significant at 5% significance level, as observed in X_3 and X_2 bars (Fig. 2A) and X_2 and X_2*X_3 bars (Fig. 2B).

The final response function to predict CMCase activity was:

$$y = 0.684 - 0.131X_1 + 0.164X_2 - 0.356X_3 - 0.024X_1X_2 + 0.046X_1X_3 + 0.061X_2X_3 - 0.001X_1X_2X_3$$

For β-glucosidase, the final response function for activity prediction was:

$$y = 2.995 + 0.213X_1 - 1.488X_2 - 0.813X_3 - 0.443X_1X_2 + 0.148X_1X_3 + 1.183X_2X_3 - 0.763X_1X_2X_3$$

Fig. 3 shows the optimized response surface, built based on the model for the CMCase activity. It is possible to verify that with the increase in temperature and decrease in the concentration of WB,

A)

B)
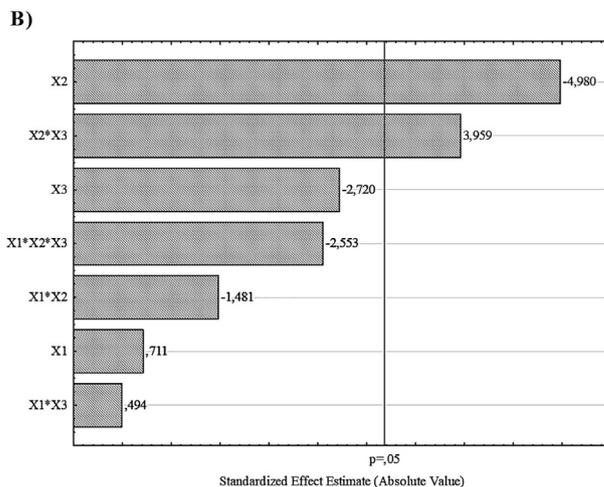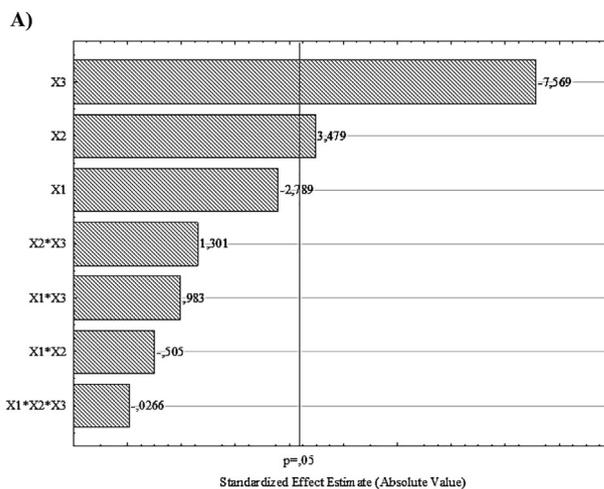
Fig. 2. Pareto chart showing the significance of variables X1 (inoculum concentration), X2 (temperatura) and X3 (wheat bran concentration) on the CMCase (a) and β-glucosidase (b) activity.

CMCase activity increases, for any spore concentration used, obtaining a maximum value of 1.3 U/mL under the conditions studied in this work.

3.3. Enzymatic profiling by SDS-PAGE electrophoresis

The enzymatic profile of *Penicilium* sp. FSDE15 in the crude enzymatic extract by zymogram presented two isoforms of

Table 3
Analysis of variance for the third order polynomial model for CMCase and β-glucosidase activity.

Source of Variation	Sum of Squares		Degrees of Freedom		Mean Square		F-value	
	CMCase	β-glucosidase	CMCase	β-glucosidase	CMCase	β-glucosidase	CMCase	β-glucosidase
Regression	1.4193	40.922	7	7	0.2028	5.846	11.39	8.19
Residual	0.0532	2.141	3	3	0.0178	0.714		
Lack of Fit	0.0527	1.580	1	1				
Pure Error	0.0005	0.562	2	2				
Total	1.4725	43.063	10	10				
R^2	0.96	0.95						

F0.05; 7; 3 = 8.89.
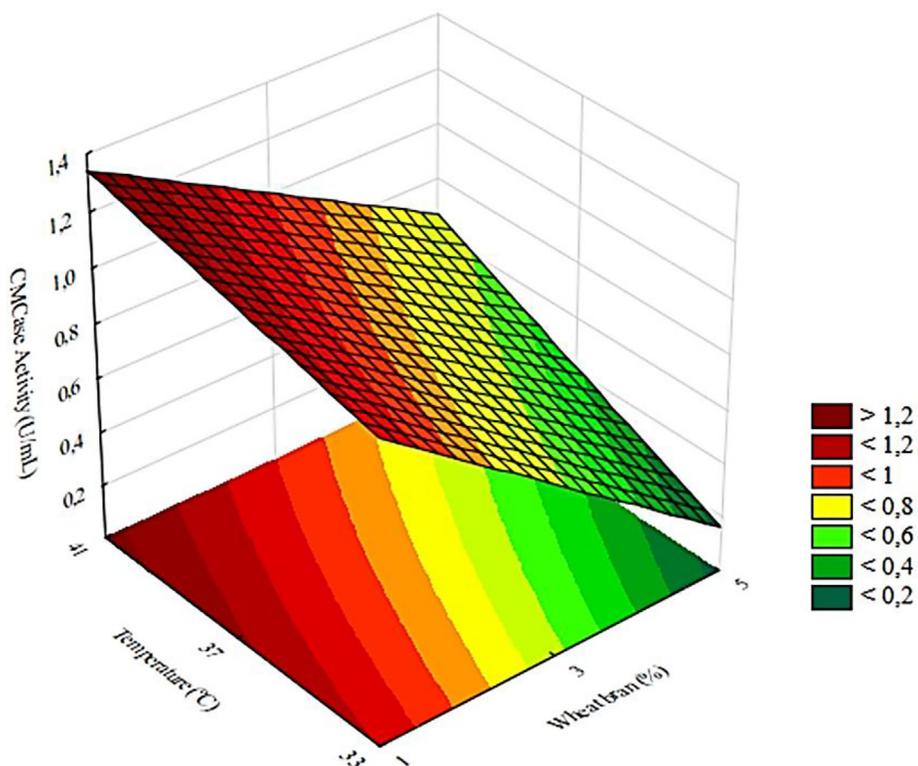
Fig. 3. Effect of temperature and wheat bran concentration on CMCase activity for the fixed spore concentration of 10^5.
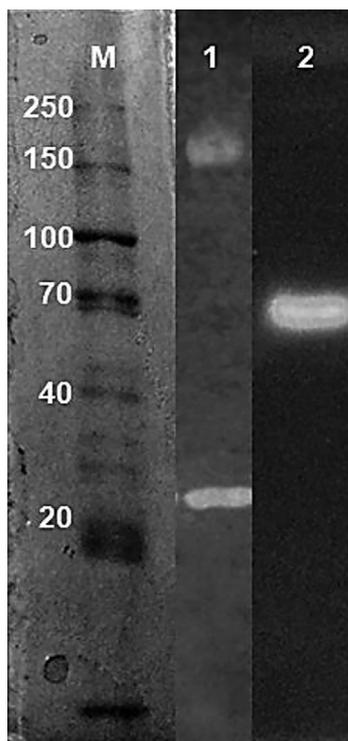
Fig. 4. Enzymatic profile of cellulases from *Penicilium* sp. FDSE15. CMCase and β-glucosidase isoforms are shown in lanes 1 and 2, respectively. M: molecular marker (bp).

CMCase and one isoform of β-glucosidase. The CMCase isoforms were highly different in molecular weight with 150 kDa and between 20 and 30 kDa. β-glucosidase isoform weighed closely to 70 kDa (Fig. 4).

4. Discussion

4.1. Cellulase production using different agroindustrial wastes

In this study, the agroindustrial wastes (WB, SB and SS) induced the production of CMCase and β-glucosidase by *Penicillium* sp. FSDE15. While the SB medium was considered exceptional for CMCase activity, β-glucosidase yield was low for this carbon source. Thus, the WB medium was selected for the experimental design.

Before the experimental design by RSM, the highest CMCase and β-glucosidase activity were 1.03 U/mL and 1.68 U/mL in the WB medium, respectively. Zimbardi et al. (2013) investigated the action of agroindustrial wastes such as corn straw, wheat bran, corn cob, cassava meal and sugarcane bagasse in the production of CMCase and β-glucosidase. They observed that wheat bran was the best induction source for the production of these enzymes. Studies presented by Kumar et al. (2011) demonstrated that the highest production of CMCase occurred in 126 h, pH 4.6, and in a carbon source (a combination of wheat bran, corn bran and kinnow peel at the proportion of 2:1:2, respectively) concentration of 65 g/L in SmF. Prasanna et al. (2016) reported that the highest β-glucosidase activity presented by *Penicillium* sp. was 1.04 U/mL, when the culture medium presented a mixture of cellulose at 0.5%, lactose at 0.5% and sawdust at 0.5% and that at the morphological changes, enzyme secretion and product stability in the medium can be affected by pH change during the growth of microorganisms.

The present study showed that the highest CMCase production by the microorganism evaluated in WB, SB and SS medium was observed at pH 5.7, showing that these enzymes are more active at acidic pH, independent of the carbon source. Sohail et al. (2009) showed that the maximum CMCase yield by *A. niger* MS82 was obtained at pH 4.0, while at pH 5 enzyme production

decreased drastically and remained constant up to 7. Unlike CMCase, the highest levels of β-glucosidase were obtained at pH 4.0, followed by a reduction in its activity as pH increases to 5, and a continuous activity decrease while pH reaches 7.

Sulyman et al. (2020) demonstrated that the optimum pH for maximum CMCase and β-glucosidase production by *Aspergillus niger* in the submerged fermentation was 4.0. Prasanna et al. (2016) observed that the highest CMCase and β-glucosidase production by *Penicillium* sp. was at pH 5.0. The highest β-glucosidase activity by *Penicillium roqueforti* ATCC 10,110 occurs in pH 5.5 (Neves et al., 2020).

The results obtained after experimental design by RSM demonstrate a significant effect on the optimization of CMCase and β-glucosidase activity. After carrying out the experimental design by RSM, using WB as carbon source, the largest CMCase activity was observed at 41 °C (1.3 U/mL), with an increase of 26% in relation to the initial values of 1.03 U/mL for this same carbon source.

Matkar et al. (2013) optimized SmF conditions for *Aspergillus sydowii* using solely lactose as carbon source and reported an optimal CMCase activity (0.39 U/mL) at 40 °C. Gomathi et al. (2012), on the other hand, studied CMCase production by *Aspergillus flavus* using wheat bran as carbon source and registered the highest production of cellulases (0.8 U/mL) at 30 °C.

For β-glucosidase, optimal activity was observed at 33 °C (6.5 U/mL), with an increase of 287% in relation to the initial values of 1.68 U/mL for WB medium. Saini et al. (2015) obtained similar results in optimization studies with *Penicillium oxalicum*, where temperature of cultivation was held at 28 °C. Our results are also comparable to those obtained by Singhvi et al. (2011) in SmF experiments with mutant strains (EMS-UV-8, 5.1 U/mL and EU-2D-21, 3.1 U/mL) and the parent strain (6.8 U/mL) of *Penicillium janthinellum*.

Few studies have been made to compare the β-glucosidase production between the widely used *T. reesei* and other fungal strains, like *Penicillium* and *Aspergillus*. Singhania et al. (2013) performed comparative studies of SmF between a reference strain of *T. reesei* and four strains of *Penicillium*. The maximum yield of 0.03 U/mL of β-glucosidase was obtained from the *T. reesei* strain, while the *Penicillium* strains produced between 0.97 and 2.45 U/mL. Results like these demonstrate the importance of screening studies for finding novel biological sources of industrially applicable enzymes.

The β-glucosidase is as a "rate-limiting" enzyme due to its key role in determining efficiency of biomass hydrolysis. If its amount during hydrolysis is low, cellobiose concentration rises and provokes feedback inhibition of the other components of the cellulolytic system, reducing the rate of glucose release (Monteiro et al., 2020).

4.2. Enzymatic profiling

Various fungi are known for producing multiple cellulases. The presence of different isoforms of CMCase can be explained by different genes that encode enzymes of the same type and this can happen when the fungus grow in different culture media and culture conditions (Bonfá et al., 2018). The existence of multiple isoforms may be important for the microorganism's ability to respond well and rapidly to environmental changes (Oberoi et al., 2014).

5. Conclusion

The present study aimed to evaluate the CMCase and β-glucosidase activities of the fungal strain *Penicillium* sp. FSDE15. We have demonstrated how different variables, such as spore and carbon source concentration and temperature, can drastically affect CMCase and β-glucosidase production by fungal microorganisms, and that the agroindustrial wastes such as wheat bran are potential inducers in the production of these enzymes. Our findings revealed the *Penicillium* sp. FSDE15, obtained from the soil of sugarcane plantation, as a promising fungal strain to be submitted to scale-up studies for industrial cellulase production, especially for enhancement of CMCase and β-glucosidase yields.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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