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Cellulase Production by *Aspergillus japonicus* URM5620 Using Waste from Castor Bean (*Ricinus communis* L.) Under Solid-State Fermentation

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Abstract The activity of β-glucosidase (βG), total cellulase (FPase) and endoglucanase (CMCase), produced by *Aspergillus japonicus* URM5620, was studied on solid-state fermentation using castor bean meal as substrate. The effect of the substrate amount, initial moisture, pH, and temperature on cellulase production was studied using a full factorial design (2^4). The maximum βG, FPase, and CMCase activity was 88.3, 953.4, and 191.6 U/g dry substrate, respectively. The best enzyme activities for all three enzymes were obtained at the same conditions with 5.0 g of substrate, initial moisture 15% at 25 °C and pH 6.0 with 120 h of fermentation. The optimum activity for FPase and CMCase was found at pH 3.0 at an optimum temperature of 50 °C for FPase and of 55 °C for CMCase. The cellulases were stable in the range of pH 3.0–10.0 at 50 °C temperature. The enzyme production optimization demonstrated clearly the impact of the process parameters on the yield of the cellulolytic enzymes.

Keywords *Aspergillus japonicus* · Cellulolytic activities · Castor bean meal · Solid-state fermentation · Optimum activity · Stability

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Introduction

The castor bean is a promising candidate for biodiesel production. The Petrobras Research Center has been developing a biodiesel production process from castor bean seeds. After a transesterification reaction, an unwanted by-product named castor bean meal is produced [1]. This extremely alkaline waste presents no utility after the biodiesel production process, and it cannot be used as animal feed. Furthermore, castor bean seeds contain a potent toxin (ricin) and an allergenic protein fraction (CB-1A or 2S albumin isoforms), which severely limit the utility of castor bean meal waste after the oil extraction [2, 3].

The allergenic compounds of castor bean meal are stable proteins that exhibit an extraordinary capacity to sensitize individuals exposed to low concentrations of castor bean powder or castor bean waste. Once industrial-scale production of biodiesel from castor bean is established, a great amount of the waste will inevitably be produced. The total elimination or inactivation of toxic compounds from castor bean waste is extremely important before it can be considered useful as animal feed, fertilizer, or used in wastewater pretreatment (as solid enzymatic preparation) [1].

Biological detoxification, using solid-state fermentation (SSF) with filamentous fungi, has been used to detoxify other residues, showing good results [4]. In addition to promoting residue detoxification, the SSF of castor bean waste represents an interesting and low-cost alternative for generating useful enzymes, such as cellulases [5, 6].

Cellulose, which constitutes the highest proportion of municipal and plant wastes, represents a major source of renewable energy and raw materials. Therefore, the utilization of cellulosic wastes to produce energy is potentially of great importance. Cellulases bring about the hydrolysis of cellulose, a homopolymer of β-1,4 linked glucose units that comprises amorphous and crystalline regions, by synergistic action of its constituent enzymes. These enzymes include: (a) β-1,4-endoglucanase (1,4-β-D-glucan 4-glucanohydrolase; EC 3.2.1.4, cellulase), which cleaves internal β-1,4-glycosidic bonds; (b) cellobiohydrolase (1,4-β-D-glucan cellobiohydrolase; EC 3.2.1.91, cellulase 1,4-β-cellobiosidase), an exo-acting enzyme which releases cellobiose from reducing and nonreducing ends of cellulose; and (c) β-glucosidase (β-D-glucoside glucohydrolase; EC 3.2.1.21, cellulase 1,4-β-glucosidase) that hydrolyzes cellobiose to glucose [7]. β-glucosidase is generally responsible for the regulation of the whole cellulolytic process and is a rate-limiting factor during enzymatic hydrolysis of cellulose, as both endoglucanase and exoglucanase activities are often inhibited by cellobiose [8].

In the intervening time, commercial cellulases and hemicellulases are used in different ways through a wide range of applications, including, as detergents and in the textile pulp and paper industry, for animal feeding, fruit extraction, and vegetable juices as well as for starch processing [9, 10].

The goal of this study was to evaluate the production of βG, total cellulase (FPase), and endoglucanase (CMCase) by *Aspergillus japonicus* URM5620 in SSF, using castor bean meal (*Ricinus communis* L.) as substrate and also to characterize the enzymes.

Materials and Methods

Castor Bean Meal

The castor bean meal used in this study was supplied by the Brazilian Agricultural Research Corporation, EMBRAPA/Agribusiness Tropical, located in Fortaleza, Ceará, Brazil, which is currently working on a local Castor Development Program.

Microorganism

The culture *A. japonicus* URM5620 utilized in this study was obtained from the Mycology Department's Mycoteca—URM, at Federal University of Pernambuco, Brazil. The strain was maintained on malt extract agar [11] and kept at 28 °C for 7 days.

Cellulase Production by Solid-State Fermentation

For cellulase production, the castor cake was used as a substrate with a particle size between 3 and 8 mm to provide improved absorption and porosity to facilitate transport of oxygen as well as nutrients during SSF [12]. The substrate was autoclaved at 120 °C for 15 min in Erlenmeyer flasks of 250 mL capacity. The inoculum was prepared by suspending the spores present on the malt extract agar plates in 0.05 M citrate buffer. The number of spores was determined in a Neubauer counting chamber, and the inoculum of 10^7 spores per gram was inoculated in the substrate used for SSF. The initial moisture of the substrate was determined in accordance with the standards of the Institute Adolfo Lutz [13].

Experimental Design and Statistical Analysis

The influence of the substrate amount, initial moisture, pH, and temperature on the cellulase production was evaluated from the results of the experiments performed according to a 2^4 factorial design [14], plus four central points (Table 1). The choice of variables and their levels was made according to Gao et al. [6]. All statistical analyses were carried out using Statistica 8.0 software [15].

Enzyme Extraction

The cellulase production was followed for 120 h. The contents of the flasks were harvested at regular intervals (24 h). A mass of 5 g of the fermented mixture was mixed with 30 mL of 0.05 M citrate buffer (each 1 g of substrate/2.5 mL of buffer). After maceration, extraction was performed with filter paper (Whatman no. 1) under vacuum. The extract was clarified by filtration and centrifugation at 4,500 rpm for 15 min [12]. The supernatant was used as crude enzymatic extract, and it was subjected to enzymatic analysis.

Analytical Techniques

Protein content was determined according to Bradford [16] at 595 nm. Bovine serum albumin was used as standard. The determinations were done in triplicate on each sample.

Table 1 Variable levels of the 2^4 experimental design for the production of cellulases by *A. japonicus* URM5620

Variables	Levels		
	Low (−1)	Central (0)	High (+1)
Sa (g)	5.0	7.5	10.0
Im (%)	15	25	35
pH	4.0	5.0	6.0
T (°C)	25	30	35

Sa substrate amount, *Im* initial moisture, *T* temperature

The activities of FPase (filter paperase activity) and CMCase (carboxymethyl cellulase activity) were determined as reported by Ghose [17], βG activity was determined according to Deshpande and Eriksson [18]. FPase activity was assayed by incubating 1 mL of the suitably diluted enzyme solution with citrate buffer (50 mM, pH 5.0) containing Whatman no. 1 filter paper (50 mg, 1×6 cm). The reaction mixture was incubated at 50 °C for 60 min. CMCase activity was carried out in the total reaction mixture of 1 mL containing 0.5 mL of suitably diluted enzyme and 0.5 mL of 1% (*w*/*v*) CMC solution in citrate buffer (50 mM, pH 5.0). This mixture was incubated at 50 °C for 30 min. βG activity was estimated using p-nitrophenyl-β-D-glucopyranoside (pNPG) as substrate. The total of assay mixture (1 mL) consisting of 0.9 mL of pNPG (1 mM) and 0.1 mL of suitably diluted enzyme was incubated at 60 °C for 30 min. The p-nitrophenol liberated was measured at 420 nm after developing the color with 2 mL of sodium carbonate (2 M). One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of glucose or p-nitrophenol from the appropriate substrates per minute under the assay conditions, and it was expressed as units per gram dry substrate. The release of reducing sugars was determined by the 3,5-dinitrosalicylic acid (DNS) method [19].

Enzyme Characterization

FPase and CMCase activities from the crude enzymatic extract were measured at different pH and temperature values.

Optimum pH and Temperature for Enzyme Activity The effect of pH on enzymes activity was measured using the following buffers: sodium acetate buffer (pH 3.0–5.0), citrate–phosphate buffer (pH 5.0–7.0), and glycine–NaOH buffer (pH 8.0–10.0). The optimum temperature within the 30–90 °C range was determined by incubation of the reaction mixture at optimum pH.

pH and Temperature Stability Crude enzymatic extract was diluted (1:2) in different buffers (pH 3.0–10.0, 0.05 M for buffers as the ones cited above) and maintained at 25 °C for 24 h. After incubation, the FPase and CMCase activities were measured for their values of pH and temperature optimum. For determination of the thermal stability, the crude enzymatic extract was incubated at temperatures ranging from 30 to 90 °C for 90 min. After that, the FPase and CMCase activities were determined.

Results and Discussion

Production of Cellulolytic Enzymes

Considering the 186 fungi isolated from the castor bean meal, ten were acknowledged as cellulase producers [20]. Of these, the *A. japonicus* URM5620 used in this work had not been previously described as a producer of cellulases. We evaluated the enzyme production at different substrate amount, temperature, pH, and initial moisture by solid-state fermentation using castor bean meal. High activities were obtained using this residue, denoting that this agro-industrial residue is a good substrate for cellulase production by *A. japonicus*. This substrate contains considerable amounts of valuable substances like sugars, oils, fibers, polyphenols, and cellulose [21], which contribute to the production of cellulases by filamentous fungi.

The *A. japonicus* URM5620, when cultured in the castor cake without any addition of nutrient solution, produced βG, FPase, and CMCase, implying that cellulase expression is solely regulated by carbon sources. The enzymes produced in SSF were analyzed during 120 h, and the maximum βG, FPase, and CMCase activity was 88.3, 953.4, and 191.6 U/g dry substrate, respectively at 120 h of fermentation, using 5.0 g of substrate with an initial moisture of 15% at 25 °C and pH 6.0 (Table 2).

In an attempt to clarify the relationship between the production of lipids and the secretion of cellulases using wheat bran as substrate, Hui et al. [22] worked with *Aspergillus oryzae* A-4. They analyzed the use of the following different culture conditions: the inoculum size of 0.5 mL/g dry substrate, the proportion of straw meal 2:8, the fermentation volume 6 g, the substrate's proportion to the solution 4:7 and 25 °C, and as a result, they obtained 2.31 U/g dry substrate with 148 h of SSF. This value was lower than the one obtained in this present work, which found values more satisfactory without the addition of any carbon source to increase the cellulases production.

Using rice straw as substrate, Nazir et al. [23] found CMCase activity (28 U/g dry substrate) in SSF at 45 °C for 120 h from *Aspergillus terreus* AN_1 with 5 g of substrate. Using horticultural waste, Xin and Geng [24] obtained the following activity values:

Table 2 Results of the 2^4 design for the βG, FPase, and CMCase production in SSF by *A. japonicus* URM5620

Run	Sa (g)	Im (%)	pH	T (°C)	βG_{120h}		$FPase_{120h}$		$CMCase_{120h}$	
					(U/g dry substrate)	(U/mg)	(U/g dry substrate)	(U/mg)	(U/g dry substrate)	(U/mg)
1	5.0	15	4.0	25	59.3	213.4	319.9	1150.6	138.8	499.2
2	5.0	15	4.0	35	2.6	11.8	22.6	102.1	9.8	44.4
3	5.0	35	4.0	25	31.4	84.4	12.7	34.1	4.7	12.8
4	5.0	35	4.0	35	47.9	131.1	19.7	54.0	6.8	18.7
5	10.0	15	4.0	25	9.0	40.5	43.7	197.1	14.3	64.5
6	10.0	15	4.0	35	16.0	61.2	82.4	315.7	26.9	103.2
7	10.0	35	4.0	25	16.8	46.5	9.3	25.8	2.8	7.8
8	10.0	35	4.0	35	20.9	62.0	12.7	37.9	2.8	8.3
9	5.0	15	6.0	25	88.3	188.7	953.4	2037.0	191.6	409.5
10	5.0	15	6.0	35	8.5	19.2	108.2	244.8	25.9	58.7
11	5.0	35	6.0	25	39.6	87.9	25.4	56.4	5.2	11.7
12	5.0	35	6.0	35	23.7	58.7	17.4	43.2	4.4	10.9
13	10.0	15	6.0	25	8.9	23.8	34.9	93.8	13.9	37.4
14	10.0	15	6.0	35	16.4	45.2	64.7	178.6	26.1	72.0
15	10.0	35	6.0	25	18.7	43.1	6.9	16.0	2.9	6.9
16	10.0	35	6.0	35	22.4	57.5	11.2	28.8	5.2	13.4
17 (C)	7.5	25	5.0	30	25.2	71.9	10.5	30.0	5.4	15.5
18 (C)	7.5	25	5.0	30	29.3	89.4	16.5	50.4	9.8	30.0
19 (C)	7.5	25	5.0	30	33.3	87.1	14.8	38.9	6.9	18.1
20 (C)	7.5	25	5.0	30	30.8	83.9	15.9	43.2	6.3	17.2

For βG, FPase, and CMCase, the subscript gives the cultivation time in hours

Sa substrate amount, *Im* initial moisture, *T* temperature, *C* central points

15.0 U/g dry substrate (FPase), 90.5 U/g dry substrate (CMCase), and 61.6 U/g dry substrate β-glucosidase at 172 h of SSF. The values of all activities obtained with castor bean meal were higher when compared to the results obtained by Nazir et al. [23] and Xin and Geng [24].

Gao et al. [6] tested various lignocellulose carbon sources and their effect on cellulase production of *A. terreus* M11 in SSF. The results showed 581 U/g of dry substrate CMCase, 243 U/g of dry substrate FPase, and 128 U/g of dry substrate β-glucosidase per gram of carbon source produced at 45 °C pH 3 and moisture of 80% with corn stover and 0.8% yeast extract as sources of carbon and nitrogen. The production levels of FPase, CMCase, βG were observed by Gottschalk et al. [25] when they worked with *Trichoderma reesei* ATCC 56765 and *Aspergillus awamori* 2B.361 U2/1 and had it cultured in medium containing untreated and pretreated sugarcane bagasse as substrates and incubated in an orbital shaker for up to 72 h at 30 °C and 200 rpm. The activities were detected in *T. reesei* FPase (1.7 U/mL), CMCase (20.0 U/mL), βG (0.34 U/mL) and *A. awamori* FPase (0.42 U/mL), CMCase (4.9 U/mL), βG (45.6 U/mL). The preparations presented the same FPase load (10 U/g) and different loads of endoglucanases (from 111 to 170 U/g), βG (from 2 to 1,086 U/g). The fungi mixture resulted in increased enzyme activity, which demonstrated the bagasse synergistic effect on hydrolysis. These results showed that SSF was suitable for cellulases production by *A. japonicus* URM5620 using castor cake as substrate.

Our results showed that βG, FPase, and CMCase activity had maximum production under the same conditions and time of cultivation. This condition contributes to the cellulolytic production in large scale, facilitating and minimizing costs and production time for the industry.

The substrate condition of 5.0 g, initial moisture of 15% at 25 °C and pH 6.0 with 120 h of fermentation, was chosen using statistical analysis, which is a common condition for the production of βG, FPase, and CMCase activity. The results of the statistical analysis with the effects of each variable studied in the experimental design in order to produce βG, FPase, and CMCase activity, at 120 h of SSF are shown in Table 3 (Fig. 1).

The initial moisture and pH were not a significant variable at a 95% confidence level for the βG activity production. Substrate amount and temperature showed significant and negative effects on this enzyme during the 120 h of fermentation, which indicated that an increase in βG activity was obtained by reducing the substrate amount and temperature (Table 3). Thus, the use of 5.0 g of the substrate, an initial moisture of 15%, and a temperature of 25 °C provided a maximum activity to this enzyme (run 1, 59.3 U/g of dry substrate and run 9, 88.3 U/g of dry substrate) (Table 2). This could be explained by the fact that in such fermentation conditions the fungus had good growth, and it was capable of producing cellulases to hydrolyze cellulose residues in carbon sources.

The pH variable had a positive effect on FPase and CMCase activity, indicating that the higher the pH the better the activity of enzymes, while for temperature, initial moisture, and substrate amount showed significant and negative effects during the 120 h of fermentation (Table 3). This means that the production of CMCase and FPase was enhanced when the fermentation conditions were as follows: a pH level of 6.0, 5 g of substrate, 15% of initial moisture, and a temperature of 25 °C. Most of the interaction effects were significant, both for a second order interaction (between two variables) and third order (three variables) (Table 3), demonstrating a dependent relationship between them.

However, our results were different from those obtained by Dogaris et al. [26]. These authors studied the production of cellulolytic enzymes by *Neurospora crassa* DSM1129, and their highest enzyme activities were obtained when a higher initial moisture was used (70.5%). Higher moisture levels (80%) were necessary for maximal cellulase production

Table 3 Effects calculated from the responses of the 2^4 design for the production of the βG, FPase, and CMCase with 120 h of SSF by *A. japonicus* URM5620

Variables/interactions	βG	FPase	CMCase
(1) Temperature	−8.34[a]	−98.52[a]	−34.95[a]
(2) Initial moisture	0.89	−139.84[a]	−54.0[a]
(3) Substrate amount	−12.6[a]	−112.04[a]	−38.36[a]
(4) pH	1.65	64.55[a]	8.98[a]
1×2	9.59[a]	99.79[a]	35.85[a]
1×3	11.64[a]	112.61[a]	42.04[a]
1×4	−4.07[a]	−52.71[a]	−4.97[a]
2×3	3.29[a]	105.58[a]	36.40[a]
2×4	−3.51[a]	−63.35[a]	−8.79[a]
3×4	−1.12	−70.16[a]	−8.62[a]
1×2×3	−10.57[a]	−111.00[a]	−41.77[a]
1×2×4	−0.75	50.10[a]	4.79[a]
1×3×4	4.07[a]	51.21[a]	5.43[a]
2×3×4	3.96[a]	67.52[a]	9.78[a]

[a] Statistically significant values (at the 95% confidence level, $p<0.05$)

when the same strain was grown in industrial by-products of the citrus processing industry under SSF [27].

The cellulase production was favored by the high carbon/nitrogen ratio present in the castor bean meal, associated with static cultivation and low initial moisture. Cultivation of microbial cells in excess of water can lead to sticking particles, limited gas exchange, and higher vulnerability to bacterial contamination, while low moisture levels are correlated with reduced microbial growth, enzyme stability, substrate swelling, and diffusion of nutrients [28].

Substrate amount and temperature have played an important role in influencing the process parameters on the response of enzymes production under solid-state fermentation [29]. In contrast, the results obtained by Alam et al. [30] with *Trichoderma harzianum* showed that a variation of the substrate concentration (0.5–1.0%) did not affect the FPase activity much (>6 U/ml). Although the substrate concentration has demonstrated positive interaction effect on some other variables, it might not be favorable for the temperature effect. Nevertheless, linear temperature effect on the response can be described as highly significant [30].

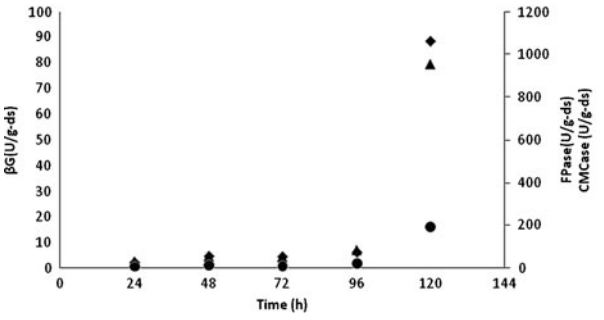

Fig. 1 Activity of β-glucosidase (*diamond*), total cellulase (*circle*) and endoglucanase (*triangle*) in the best condition of production of three enzymes (5.0 g of substrate, initial moisture of 15% at 25 °C, pH 6.0 with 120 h of fermentation—run 9)

Enzyme Characterization

For characterization of FPase and CMCase from *A. japonicus* URM5620, the crude enzymatic extract was used and was chosen as the best one for the production of two enzymes (Table 2, Run 09).

The effect of pH on FPase and CMCase activities is shown in Fig. 2. The optimum activity for FPase and CMCase was pH 3.0. It was remarkable that the enzymes were more stable at acidic pHs than at neutral pHs. These cellulases from *A. japonicus* URM5620 showed a second peak of activity at pH 5.5 and pH 4.0, 96%, and 90%, respectively. The optimum pH for cellulases was much lower than those reported by other literature [6, 31]. Gao et al. [6] obtained maximum CMCase activity at pH 2.0 and suggested this property could be used to degrade cellulosic residue and to produce cellulases under acidic condition. CMCase activities from *Penicillium citrinum* MTCC6489 showed two different peaks at pH 5.5 and 8.0, but FPase showed only one peak at pH 6.5 [32]. Xin and Geng [24], working with horticultural waste as the substrate for cellulase and hemicellulase production by *T. reesei* RUT-C30 under SSF, obtained the maximum activity for CMCase, xylanase, and β-xylosidase with the optimal initial pH value of 5.0. Generally speaking, a lower pH level (4.5–5.5) favored the production of all the five enzymes investigated. Each microorganism possesses a pH range for its growth and activity with an optimum value within the range. Filamentous fungi have reasonably good growth over a broad range of pH 2–9, with an optimal range of 3.8 to 6.0 [33].

The optimum temperature was 50 °C for FPase and 55 °C for CMCase (Fig. 2). Probably, the most important factor among all the physical variables affecting the production of enzymes and metabolites is usually the incubation temperature because the enzymatic activities are sensitive to temperature [34]. The results are in accordance with those obtained by Dutta et al. [32], studying cellulases from *P. citrinum* MTCC6489, who observed that the maximum activity of FPase and CMCase was set at 65 and 60 °C, respectively. Gao et al. [6], working with CMCase from *A. terreus* M11, obtained maximum enzyme activity at 70 °C. CMCase of *Penicillium* sp. CR-316 exhibited maximal activity at 65 °C [35]. The results obtained by Xin and Geng [24] showed an optimum activity at

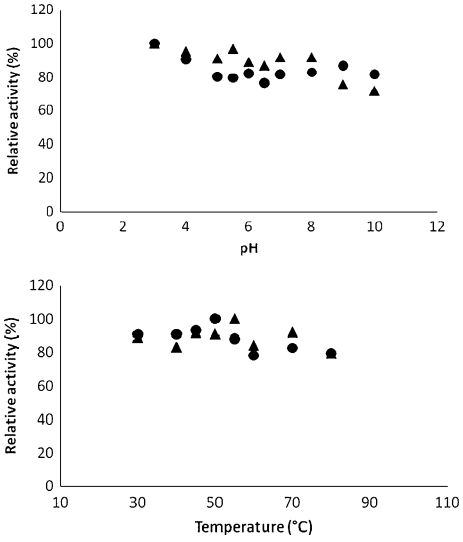

Fig. 2 Optimum pH and temperature on total cellulase (*circle*) and endoglucanase (*triangle*) from *A. japonicus* URM5620

30 °C for FPase, CMCase of *T. reesei* RUT-C30. This fact confirms that the optimum temperature for cell growth could be different from that for enzymatic metabolites formation [34].

FPase and CMCase were stable at 50 °C after 90 min, but the CMCase decreased as the temperature increased, and the FPase was stable at 90 °C at pH 8, keeping 97% of its activity after 90 min in this temperature (Fig. 3). CMCase kept 89% of its activity after 90 min of incubation at 50 °C. The thermal stability achieved by Picart et al. [35] showed that the CMCase remained stable at 60 °C and at pH 4.5 for at least 3 h, while at 65 °C, the enzyme lost 75% of its activity after 1 h of incubation. Gao et al. [6] showed 65% stability after incubation at pH 2 at 70 °C, for 6 h, for CMCase from *A. terreus* M11. The CMCase was stable in the range of pH 2–5, but its activities markedly decreased at above pH 5 (Fig. 3). CMCase thermal stability studies of *P. citrinum* MTCC6489 showed that the enzyme was fairly stable within 50–70 °C temperature ranges, for 2 h of incubation. However, the FPase was less thermostable than the CMCase [32]. For the majority of the microorganisms such as *T. reesei*, *Aspergillus niger*, etc., the optimum temperature for the cellulase was within the range of 40–50 °C [36]. The high stability at a certain temperature and the pH suggested that the cellulases (FPase and CMCase) are sufficiently acceptable for commercial application.

Conclusions

The ability of the newly isolated *A. japonicus* URM5620 to produce cellulases in the SSF with castor bean meal as carbon source was investigated. The results suggested the potential use of the *A. japonicus* URM5620 for cellulase production in a shorter period with cheap and available raw material. The condition of 5.0 g of substrate, initial moisture 15% at

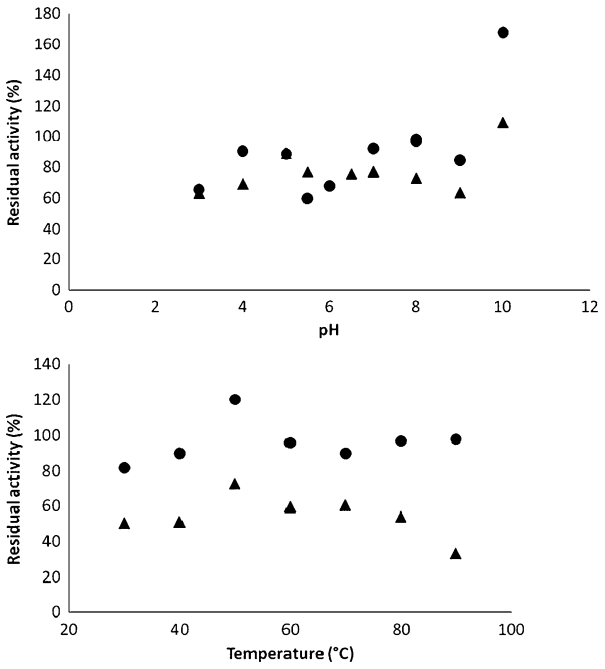

Fig. 3 pH and temperature stability on total cellulase (*circle*) and endoglucanase (*triangle*) from *A. japonicus* URM5620

25 °C and pH 6.0 with 120 h of fermentation was defined as the best for production of βG, FPase, and CMCase at the same time. The maximum βG, FPase, and CMCase activity was 88.3, 953.4, and 191.6 U/g of dry substrate, respectively. The variables analyzed (substrate amount, initial moisture, pH, and temperature) showed significant effects on βG, FPase, and CMCase production. The optimum activity for FPase and CMCase was at pH 3.0, and the optimum temperature for FPase was 50 °C and for CMCase, 55 °C. The cellulases were stable in the range of pH 3.0–10.0 and temperature 30–50 °C. The optimization of the enzyme production clearly demonstrated the impact of the process parameters on the yield of the cellulolytic enzymes.

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References

1. Godoy, M. G., Gutarra, M. L. E., Maciel, F. M., Felix, S. P., Bevilaqua, J. V., Machado, O. L. T., et al. (2009). Use of a low-cost methodology for biodetoxification of castor bean waste and lipase production. *Enzyme and microbial technology, 44*, 317–322.
2. Audi, J., Belson, M., Patel, M., Shier, J., & Osterloh, J. (2005). Ricin poisoning – A comprehensive review. *J. Am. Medical. Assoc., 294*, 2342–2351.
3. Thorpe, S. C., Kemeny, D. M., Panzani, R. C., Mcgurl, B., & Lord, M. (1988). Allergy to castor bean. II. Identification of the major allergens in castor bean-seeds. *The Journal of Allergy and Clinical Immunology, 21*, 67–72.
4. Brand, D., Pandey, A., Roussos, S., & Soccol, C. R. (2000). Biological detoxification of coffee husk by filamentous fungi using a solid state fermentation system. *Enzyme and Microbial Technology, 26*, 127–133.
5. Hölker, U., Höfer, M., & Lenz, J. (2004). Biotechnological advantages of laboratory-scale solid state fermentation with fungi. *Applied Microbiology and Biotechnology, 64*, 175–186.
6. Gao, J., Weng, H., Zhu, D., Yuan, M., Guan, F., & Xi, Y. (2008). Production and characterization of cellulolytic enzymes from the thermoacidophilic fungal *Aspergillus terreus* M11 under solid-state cultivation of corn stover. *Bioresource Technol., 99*, 7623–7629.
7. Bhat, M. K., & Bhat, S. (1997). Cellulose degrading enzymes and their potential industrial application. *Biotechnology Advances, 15*(3), 583–620.
8. Harhangi, H. R., Steenbakkers, P. J. M., Akhmanova, A., Jetten, M. S. M., Drift, C. V. D., Camp, O. D., et al. (2002). A highly expressed family 1 β-glucosidase with transglycosylation capacity from the anaerobic fungus *Piromyces* sp. E2. *Biochimica et Biophy. Acta., 1574*(3), 293–303.
9. Bhat, M. K. (2000). Cellulases and related enzymes in biotechnology. *Biotechnology Advances, 18*, 355–383.
10. Polizeli, M. L. T. M., Rizzatti, A. C. S., Monti, R., Terenzi, H. F., Jorge, J. A., & Amorim, D. S. (2005). Xylanases from fungi: properties and industrial applications. *Applied Microbiology and Biotechnology, 67*, 577–591.
11. Klich, M. A. (2002). *Identification of common Aspergillus species* (1st ed.). Utrecht: Centraalbureau voor Schimmelcultures.
12. Spier, M. R., Greiner, R., Rodriguez-León, J. A., Woiciechowski, A. L., Pandey, A., Soccol, V. T., et al. (2008). Phytase production using citric pulp in SSF. *Food Technol. Biotechnol., 46*, 178–182.
13. Zenebon, O., & Pascuet, N. S. (2005). *Métodos físico-químicos para análise de alimentos* (4th ed.). São Paulo: Instituto Adolfo Lutz.
14. Bruns, R. E., Scarminio, I. S., & Neto, B. B. (2006). *Statistical Design-Chemometrics* (1st ed.). Elsevier: Amsterdam.
15. Statsoft (2008), Inc. *STATISTICA* (data analysis software systems) version 8.0.

16. Bradford, M. M. (1976). A rapid and sensitive method for the quantification of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry, 72*, 248–254.
17. Ghose, T. K. (1987). Measurement of cellulase activities. *Pure and Applied Chemistry, 59*, 257–268.
18. Deshpande, V., and Eriksson, K.E. 1,4-ß-glucosidases of *Sporotrichum pulverulentum*. In: Wood, W.A.; Kellogg, S.T. (Ed.). (1988), *Methods in Enzymology.* San Diego: Academic Press, 160: 415–424.
19. Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Analytical Chemistry, 31*, 426–428.
20. Herculano, P. N., Lima, D. M. M., Fernandes, M. J. S., Neves, R. P., Souza-Motta, C. M., & Porto, A. L. F. (2011). Cellulases from two *Penicillium* sp. strains isolated from subtropical forest soil: production and characterization. *Current Microbiology, 62*, 1416–1422.
21. Laufenberg, G., Rosato, P., & Kunz, B. (2004). Adding value to vege table waste: oil press cakes as substrates for microbial decalactone production. *Eur. J. Lipid Sci. Technol., 106*, 207–217.
22. Hui, L., Wan, C., Hai-Tao, D., Xue-Jiao, C., Qi-Fa, Z., & Yu-Hua, Z. (2010). Direct microbial conversion of wheat straw into lipid by a cellulolytic fungus of *Aspergillus oryzae* A-4 in solid-state fermentation. *Bioresource Technol., 101*, 7556–7562.
23. Nazir, A. S., Saini, R. H. S., Kaur, A., & Chadha, B. S. (2010). Profiling Differential Expression of Cellulases and Metabolite Footprints in *Aspergillus terreus*. *Applied Biochemistry and Biotechnology, 162*, 538–547.
24. Xin, F., & Geng, A. (2010). Horticultural Waste as the Substrate for Cellulase and Hemicellulase Production by *Trichoderma reesei* Under Solid-State Fermentation. *Applied Biochemistry and Biotechnology, 162*, 295–306.
25. Gottschalk, L. M. F., Oliveira, R. A., & Bon, E. P. S. (2010). Cellulases, xylanases, β-glucosidase and ferulic acid esterase produced by *Trichoderma* and *Aspergillus* act synergistically in the hydrolysis of sugarcane bagasse. *Biochemical Engineering Journal, 51*, 72–78.
26. Dogaris, I., Vakontios, G., Kalogeris, E., Mamma, D., & Kekos, D. (2009). Induction of cellulases and hemicellulases from *Neurospora crassa* under solid-state cultivation for bioconversion of sorghum bagasse into ethanol. *Industrial Crops and Products, 29*, 404–411.
27. Mamma, D., Kourtoglou, E., & Christakopoulos, P. (2008). Fungal multienzyme production on industrial by-products of the citrus-processing industry. *Bioresource Technology, 99*, 2373–2383.
28. Botella, C., Diaz, A., Ory, I., Webb, C., & Blandino, A. (2007). Xylanase and pectinase production by *Aspergillus awamori* on grape pomace in solid state fermentation. *Process Biochemistry, 42*, 98–101.
29. Christen, P., Bramorski, A., Revaha, S., & Soccol, C. R. (2000). Characterization of volatile compounds produced by *Rhizopus* strains grown on agro-industrial solid wastes. *Bioresource Technology, 71*, 211–215.
30. Alam, M. Z., Muyibi, S. A., & Wahid, R. (2008). Statistical optimization of process conditions for cellulase production by liquid state bioconversion of domestic wastewater sludge. *Bioresource Technology, 99*, 4709–4716.
31. Prasetyo, J., Sumita, S., Okuda, N., Park, E. Y., & Park, E. Y. (2010). Response of Cellulase Activity in pH-Controlled Cultures of the Filamentous Fungus *Acremonium cellulolyticus*. *Applied Biochemistry and Biotechnology, 162*, 52–61.
32. Dutta, T., Sahoo, R., Sengupta, R., Ray, S. S., Bhattacharjee, A., & Ghosh, S. (2008). Novel cellulases from an extremophilic filamentous fungi *Penicillium citrinum*: production and characterization. *J. Ind. Microb. Biotechnol., 35*, 275–282.
33. Gowthaman, M. K., Krishna, C., & Moo-Young, M. (2001). Fungal solid state fermentation – an revision. *Appl. Mycol. Biotechnol., 1*, 305–352.
34. Krishna, C. (2005). Solid-State Fermentation Systems – An Overview. *Critical Rev. Biotechnol., 25*, 1–30.
35. Picart, P., Diaz, P., & Pastor, F. I. J. (2007). Cellulases from two *Penicillium* sp. strains isolated from subtropical forest soil: production and characterization. *Letters in Applied Microbiology, 45*, 108–113.
36. Sreenath, H. K., Yang, V. W., Burdsall, H. H., Jeffries, T. W. (1996). Toner removal by alkaline-active cellulases from desert Basidiomycetes. In: Jeffries T.W., Viikari, L. (Eds.), The enzymes for pulp and paper processing. Washington: American Chemical Society, 267–279.