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Optimization of the Production of Thermostable endo-β-1,4 Mannanases from a Newly Isolated *Aspergillus niger gr* and *Aspergillus flavus gr*

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Abstract The aim of this work was to establish optimal conditions for the maximum production of endo-β-1,4 mannanases using cheaper sources. Eight thermotolerant fungal strains were isolated from garden soil and compost samples collected in and around the Gulbarga University campus, India. Two strains were selected based on their ability to produce considerable endo-β-1,4 mannanases activity while growing in liquid medium at 37 °C with locust bean gum (LBG) as the only carbon source. They were identified as *Aspergillus niger gr* and *Aspergillus flavus gr*. The experiment to evaluate the effect of different carbon sources, nitrogen sources, temperatures and initial pH of the medium on maximal enzyme production was studied. Enzyme productivity was influenced by the type of polysaccharide used as the carbon source. Copra meal defatted with *n*-hexane showed to be a better substrate than LBG and guar gum for endo-β-1,4 mannanases production by *A. niger gr* (40.011 U/ml), but for *A. flavus gr* (33.532 U/ml), the difference was not significant. Endo-β-1,4 mannanases produced from *A. niger gr* and *A. flavus gr* have high optimum temperature (65 and 60 °C) and good thermostability in the absence of any stabilizers (maintaining 50% of residual activity for 8 and 6 h, respectively, at 60 °C) and are stable over in a wide pH range. These new strains offer an attractive alternative source of enzymes for the food and feed processing industries.

Keywords Endo-β-1 · 4 Mannanase production · *Aspergillus niger gr* · *Aspergillus flavus gr* · Optimization · Screening · Copra meal · Food processing

Introduction

Man (mannose or D-mannopyranose)-containing polysaccharides are widely distributed in the cell walls of higher plants [1]. The chemical structures of these polysaccharides are based on a backbone of (1,4)-linked β-D-mannosyl residues, although other glycosyl residues

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are sometimes present in the main chain. Structurally, Man-containing polysaccharides fall into four main classes: (1) unsubstituted (1,4)-linked β-D-mannans, (2) galactomannans, (3) glucomannans, and (4) galactoglucomannans. Unsubstituted (1,4)-linked β-D-mannans are found in both leguminous and nonleguminous seeds, where they function as a carbohydrate reserve [1].

Endo-β-1,4 mannanases (E.C. 3.2.1.78) are a kind of hemicellulases, widely distributed in microorganisms and plants. They can randomly hydrolyze the (1,4)-β-D-mannosidic linkages within the main chain of mannans and heteropolysaccharides consisting mainly of mannose, such as galactomannans or glucomannans, producing mannooligosaccharides. These oligosaccharides can be further cleaved by β-D- mannosidase, β-D-glucosidase, and α-D-galactosidase to produce mannose, glucose, and galactose [2]. The extent of hydrolysis depends on the degree of substitution and the distribution of the substituents. Hydrolysis of glucomannans is affected by the proportion of glucose and mannose [3].

Many microorganisms are capable of decomposing mannans; however, enzymes from fungi such as *Aspergillus* sp. [4–6], *Trichoderma reesei* [7], and *Sclerotium rolfsii* [8] deserve the most attention. Endo-β-1,4 mannanases have found several industrial applications. They were employed for preparation of monooligosaccharides used as non-nutritional food additives for selective growth of human-beneficial intestinal microflora (*Bifido* bacteria and *Lactobacilli*; [9–11]). They were useful in many fields including biobleaching of pulp in the paper industry [12], bioconversion of biomass wastes to fermentable sugars [13], upgrading of animal feed stuff [14], and reducing the viscosity of coffee extracts [15].

Many mannan-based carbon sources have been used to cultivate filamentous fungi. These included locust bean gum (LBG) [5], guar gum [16], Konjac flour [17], and copra meal [4, 18]. Although LBG represents the most common carbon source, there are only a few reports in literature for the best carbon source to cultivate microorganisms [4].

Copra, a well-dried coconut kernel, is one of the high mannan content [19]. It is usually regarded as a waste after coconut water is consumed, and it is a byproduct of coconut oil extraction, which has been reported to be qualitatively poor due to low concentrations of several limiting amino acids [20, 21]. From the literature survey, it is clear that there are only a few reports on the utilization of copra waste as a carbon source for the growth of filamentous fungi in submerged fermentation and in solid-state fermentation for the production of mannanase [4, 22]. The locally available copra waste needs to be exploited into industrially important endo-β-1,4 mannanase and so produce an enzyme in combination with α-galactosidase and β-D-glucosidase that can be effectively used in the food-processing industry. Owing to the increasing biotechnological importance of thermostable mannanases, the present study was undertaken. The objectives of the present study were (1) the isolation and identification of mannanase-producing fungi and (2) mannanase production using cheaper sources and partial characterization of enzymes from potential strains.

Materials and Methods

Materials

LBG and guar gum were procured from Sigma Chemicals, USA. Copra was brought from the local market (Gulbarga, India). All other chemicals used were of analytical grade.

Preliminary Screening and Identification of Fungi

Different soil samples collected locally from garden and composts were used in the present study to isolate mannanase-producing fungi. The mannanase-producing fungal strains from soil samples were isolated using the dilution-plating technique. One gram of garden or compost sample was mixed in 9 ml distilled water. This suspension was serially diluted to 10^{-4}. one milliliter of the diluted samples from 10^{-3} to 10^{-4} dilutions was plated on a sterile copra meal agar (copra meal—1 g, agar—15 g, $NaNO_3$—1 g, K_2HPO_4—1 g, $MgSO_47H_2O$—0.5 g, yeast extract—1 g, in 1,000 ml distilled water and to eliminate the bacterial contamination, 0.080 g or 0.008% streptomycin was added to one of the media) surface and incubated at 37±2 °C for 3–4 days. Microscopic observation was performed to determine the morphological characteristics of the fungal isolates. The isolates were sent to the Mycology and Plant Pathology Group, Agharkar Research Institute (Pune, India) for further identification, and the isolates were preserved on potato dextrose agar slants for further study (potato infusion—200 g, dextrose—20 g, agar—15 g, in 1,000 ml distilled water) at 4±1 °C.

Secondary Screening

Those isolated organisms from the preliminary screening were cultured in liquid media containing 2% LBG, 0.1% K_2HPO_4, and 0.05% $MgSO_47H_2O$ at pH 5.5 in Erlenmeyer flasks. After incubation on a rotary shaker (37 °C, 180 rpm) for 7 days, the culture broth was centrifuged (12,085.2×*g* for 20 min), and the supernatant was collected for enzyme assay. Among the six isolates tested, two isolates were found to be a potent mannanase producer and were designated as *Aspergillus niger gr* and *A. flavus gr*. The strains were maintained on potato dextrose agar and used for further study on mannanase production.

Assay for Mannanase Activity

Endo-β-1,4 mannanase was assayed using 0.5% (*w*/*v*) LBG as substrate. The substrate was suspended in 0.2 mM acetate buffer, pH 5.0, by heating at 121 °C for 20 min, and insolubles (less than 5% of the substrate) were removed by centrifugation. The enzyme sample (0.1 ml) was incubated with 0.9 ml of the substrate solution in 25-ml test tubes at 50 °C for 20 min. The amount of reducing sugars produced in the enzyme reaction was measured as D-mannose-reducing equivalents by the Somogyi–Nelson method [23, 24]. Substrate and enzyme controls were used with the addition of distilled water instead of the enzyme or substrate, respectively. One unit of mannanase activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar as a D-mannose standard per minute under the conditions described above.

Defatting Methods of Copra

To remove the oil content from the copra, the treatment of copra was carried out according to the methods of Lin and Chen [4]. The copra was finely ground with a grinder for 5 min and sieved (30 mesh), and the powder was designated as CO. CO was boiled for 2 h with 2 vol. of distilled water. The cooled CO suspension was then placed at 4 °C overnight to allow the oil to solidify and finally be removed. The dried and sieved residues were designated as FCO. FCO was then defatted by the solvent extraction using *n*-hexane for 24 h. One liter of solvent *n*-hexane was mixed with 100 g of ground copra in a beaker and

left overnight. The copra suspension was then filtered through Whatman no. 1 filter paper. The product was designated as dFCO, after the residues were oven dried and sieved. All samples were kept in a desiccator until used.

Effect of Physico-chemical Parameters

The optimization of the composition of the medium and cultural conditions was carried out based on stepwise modification of the governing parameters for mannanase production. The effect of commercial mannans as a carbon source (LBG, guar gum) and simple sugars (glucose, sucrose, galactose, and xylose) as a sole carbon source for the production of enzyme was studied. The effect of supplementation of additional nitrogen sources to copra meal (CO) was examined. Nitrogen sources, such as peptone, urea, yeast extract, sodium nitrate, ammonium sulfate, ammonium nitrate, meat extract, and beef extract, were also tried. the effects of incubation temperature between 30 and 80 °C and of initial pH on mannanases production were studied.

Effect of pH and Temperature on Enzyme Activities

The enzyme extract was preincubated at different temperatures ranging from 20 to 80 °C for different time intervals and then assayed for enzyme activity. The effects of pH on mannanase activities were studied between pH 3.0 and 8.0 using citrate/phosphate (pH 3.0–7.2) and Tris–HCl (pH 7.2–8.0) buffers (50 mM). All the experiments were conducted in triplicate, and the results show the mean values of the activities.

Time Course of Mannanase Production

The time course of mannanase production was carried out before and after media formulation. After inoculation, mannanase activity in the supernatant was followed from the first to seventh day.

Effect of pH and Thermal Stability on Enzyme Activities

To determine the pH stability, the enzyme was incubated at the desired pH for 16 h at 4 °C. Residual activity was calculated in each sample by mannanase assay against the enzyme control sample at optimum pH and temperature as explained above.

For the determination of thermal stability, the enzyme was preincubated without any stabilizers at 65 (*A. niger gr*) and 60 °C (*A. flavus gr*) for different time intervals of 2, 4, 6, 8, 10, and 12 h. Residual activity in each sample was calculated by doing the assay against enzyme control sample at optimum pH and temperature as explained above.

Results and Discussion

Screening, Identification, and Enzyme Induction

Active endo-β-1,4 mannanase-producing strains that formed colonies on the mannan–agar plate were selected for secondary screening. In the secondary screening, the liquid medium with LBG as the sole carbon source was used for the isolation of potent endo-β-1,4

mannanase-producing strains. In the preliminary screening, a total of six isolates were obtained from the soil sample. In the secondary screening, out of six isolates, two isolates (*A. niger* and *A. flavus*) showed good mannanase activity, and these strains were selected for detailed taxonomical and optimization studies. The isolates were identified as *A. niger gr* and *A. flavus gr*.

Commercial mannans, simple sugars, copra meal, and defatted copra meal were used as potential inducers of the enzyme activities in both *A. niger gr* and *A. flavus gr* strains. Results of these mannanase activities are given in Table 1. From the table, it is evident that copra meal defatted (dFCO) was the best inducer of mannanase synthesis in both strains. The maximum production of extracellular mannanase from both the *A. niger gr* and *A. flavus gr* was 26.029 and 24.126 U/ml, respectively. The use of commercial mannans such as LBG, guar gum, and konjac flour as the substrate is uneconomical for large-scale production of mannanases. To use locally available copra waste into value added products like enzymes, in this study, defatted copra meal was used as a potent inducer of mannanase production. Among commercial mannans used for the induction of mannanase, 2% LBG induced the maximum production of mannanase (5.994 and 5.173 U/ml) as well as 2% guar gum (4.324 and 3.820 U/ml), respectively. These results are well comparable with the results reported in literature by various authors for the production of mannanase using commercial mannans [5, 16, 17]. Copra meal without defatting will not support the fungal growth because of a high oil content, which inhibited the mannanase production. Coconut oil separated the culture broth from contacting with air, and the amount of dissolved oxygen in the cultural broth decreased, which in turn affected the growth of the fungi [25]. The simple sugars like glucose, sucrose, galactose, and xylose will not induce the enzyme production. The monosugars could cause a catabolite repression, as reported for *Aspergillus* sp. [26–28]. Hence, the induction of enzyme activity was not observed. The fourfold enhanced production of the enzyme was observed in both the strains using 2% defatted copra meal as the carbon source compared to LBG. Similarly, Lin and Chen [4] have observed a fourfold enhanced production of mannanase by growing *A. niger* in a medium containing 2% defatted copra meal. The enhanced production of mannanase by the defatted

Table 1 Effect of different carbon sources on production of endo-β-1,4 mannanases.

Carbon source (2% *w*/*v*)	Maximum mannanase activity (U/ml)	
	Aspergillus niger gr	*Aspergillus flavus gr*
Commercial mannans		
LBG	5.994±0.29	5.173±0.25
Guar gum	4.324±0.21	3.820±0.19
Copra mannans		
Copra meal (CO; unprocessed)	1.966±0.09	1.325±0.06
Copra meal (dFCO; defatted)	26.029±1.30	24.126±1.20
Simple sugars		
Glucose	0.001±0.00	0.001±0.00
Sucrose	0.004±0.00	0.002±0.00
Lactose	0.021±0.00	0.010±0.00
Galactose	0.001±0.00	0.001±0.00
Mannose	0.001±0.00	0.001±0.00
Xylose	0.001±0.05	0.001±0.00

Results are the representative of average of three experiments in duplicate. SE at 5% level

Table 2 Effect of various nitrogen sources on production of endo-β-1,4 mannanases (using 2% defatted copra meal, dFCO).

Nitrogen source (0.5% in the medium)	Maximum mannanase activity (U/ml)	
	Aspergillus niger gr	*Aspergillus flavus gr*
Inorganic nitrogen source		
Ammonium sulfate	27.896±1.39	24.539±1.22
Ammonium nitrate	31.023±1.55	26.731±1.33
Sodium nitrate	25.006±1.25	21.925±1.09
Potassium nitrate	26.108±1.30	24.293±1.21
Ammonium acetate	22.862±1.14	21.251±1.06
Urea	26.199±1.30	23.721±1.18
Organic nitrogen source		
Yeast extract	32.596±1.62	25.871±1.29
Beef extract	26.095±1.30	23.958±1.19
Peptone	29.523±1.47	25.321±1.26
Casein	26.001±1.30	24.021±1.20
Soybean meal (defatted)	19.923±0.99	18.101±0.90
Combined form[a]		
Yeast extract + ammonium nitrate	40.011±2.00	33.532±1.67
Peptone + ammonium nitrate	36.156±1.80	27.896±1.39
Casein + ammonium nitrate	28.775±1.43	24.259±1.21
Soybean meal + ammonium nitrate	26.021±1.30	22.007±1.10

Results are the representative of average of three experiments in duplicate. SE at 5% level

[a] 0.25% each in the medium

copra meal was possibly due to the mannan content of the carbon sources [4]. In LBG, the ratio of mannose to galactose was 4:1 [29], while this was 2:1 for guar gum [29]. The ratio of glucose to mannose was 1.6:1 in konjac glucomannan [30]. In copra mannan, the ratio of mannose and galactose reached 14:1 [6]. Therefore, based upon the same amount of carbon sources, the defatted copra carried the higher mannan content, which would induce more enzyme production by the microorganisms. Defatted copra also contained more protein and minor elements [31], which were more beneficial to fungal growth than other carbon sources.

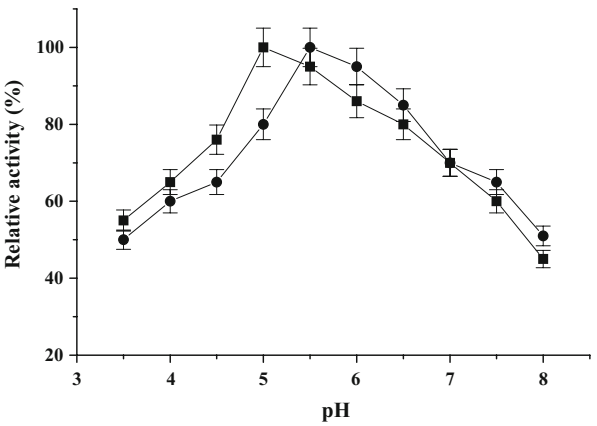

Fig. 1 Mannanase production by *A. niger gr* (*circles*) and *A. flavus gr* (*squares*) at different initial pH

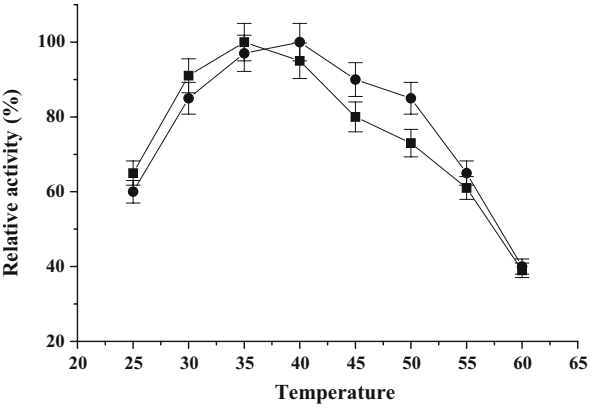

Fig. 2 Effect of temperature on the production of endo-β-1,4 mannanases in *A. niger gr* (*circles*) and *A. flavus gr* (*squares*)

Effect of Nitrogen Sources

The mechanisms that govern the formation of extracellular enzymes are influenced by the availability of precursors for protein synthesis. Furthermore, the nitrogen source can significantly affect the pH of the medium during the course of fermentation [4]. The effect of various inorganic and organic nitrogen sources on mannanase synthesis was also studied, and the results are given in Table 2. From the above findings, it is clear that both the *Aspergillus* strains showed significant enzyme activity even in the absence of any organic and inorganic nitrogen sources. These results are in concordance with the data of many investigators, who have reported that fungi produce more enzymes on the addition of complex organic nitrogen sources [4, 32]. Concerning the inorganic nitrogen sources used as shown in Table 2, a maximum activity was observed with ammonium nitrate in both the *Aspergillus* species (31.023 and 26.735 U/ml, respectively). In case of organic nitrogen sources, a maximum increase was observed with yeast extract rather than with peptone, beef extract, casein, and soybean meal (Table 2). In addition, yeast extract showed a maximum production comparable with different inorganic nitrogen sources. The highest product yield of mannanase (40.011 U/ml) in Table 2 by *Aspergillus* species are several folds higher than the values reported by other workers on *Aspergillus* sp. [4, 6]. The possibility of using the locally available substrate copra meal with an additional

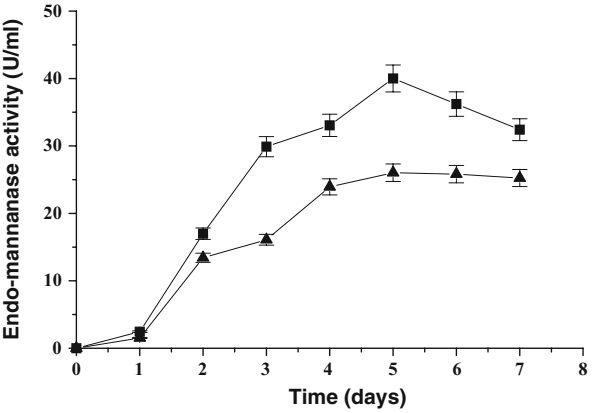

Fig. 3 Time course of endo-β-1,4 mannanase production by *A. niger gr* in submerged fermentation on defatted copra meal before and after optimization (*squares*, optimized and *triangles*, nonoptimized)

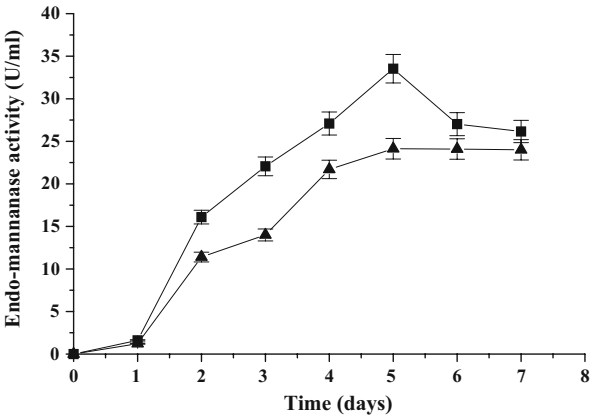

Fig. 4 Time course of endo-β-1,4 mannanase production by *A. flavus gr* in submerged fermentation on defatted copra meal before and after optimization (*squares*, optimized and *triangles*, nonoptimized)

supplemented nitrogen source in the form of ammonium nitrate and yeast extract in a fermentation medium yielded mannanase to a level greater than 1.537-fold in case of *A. niger gr* and 1.389-fold in case of *A. flavus gr* compared to that induced by the defatted copra meal.

Effect of Initial pH and Temperature

The influence of initial pH of the culture media on mannanase production was studied in the range of pH 3.5–8.0 in the absence of pH control for both the strains. Maximum mannanase production occurred at pH 5.5 for *A. niger gr*, where as in the case of *A. flavus gr* the maximum enzyme production occurs at pH 5.0 (Fig. 1). After pH 5.5, mannanase production slowly declined in both the strains. Similarly, Rajoka et al. [32] have reported the maximum production of inulinase from *A. niger* grown in an a fermentation medium having an initial pH at the range of 5.5–6.5. The effect of growth temperature on mannanase production was examined at the optimum medium composition described above. Eight fermentation temperatures in the range of 25–60 °C were tested. Optimum enzyme activities were obtained at 40 °C for *A. niger gr* and 35 °C for *A. flavus gr* (Fig. 2). However, it was found that a decrease in enzyme production above 40 °C for both the *Aspergillus* strains occurs. The studies indicated that in the absence of pH control, an initial pH of 5.5 for *A. niger gr* and an initial pH of 5.0 for *A. flavus gr* are regarded as optimal for mannanase production at 40 and 35 °C, respectively.

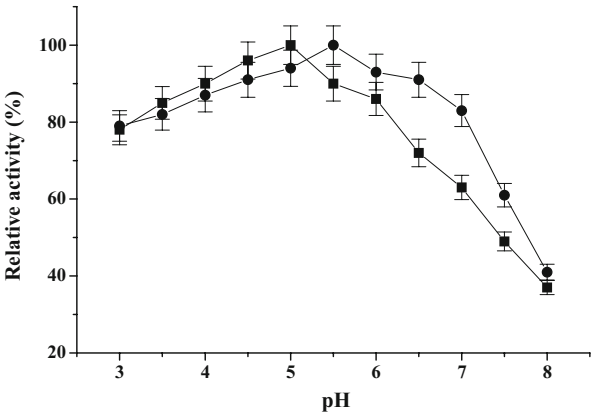

Fig. 5 Effect of pH on endo-β-1,4 mannanases activity in *A. niger gr* (*circles*) and *A. flavus gr* (*squares*)

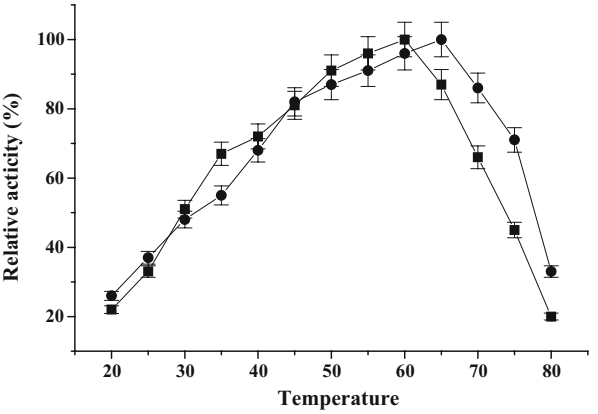

Fig. 6 Effect of temperature on endo-β-1,4 mannanases activity in *A. niger gr* (*circles*) and *A. flavus gr* (*squares*)

Time Course of Mannanase Production

The time course of mannanase production was studied on both *Aspergillus* species with nonoptimized and optimized media. The results presented in Figs. 3 and 4 showed that mannanase was maximally produced at the fifth day of fermentation, in the optimized and nonoptimized media, in both the *Aspergillus* species. When the fungal cultures were cultivated on optimized media with defatted copra meal as the carbon source, the peak in mannanase production on optimized media was on day 5 for the both *Aspergillus* species (Figs. 3 and 4). The measured enzyme activities were 40.01 and 33.126 U/ml, respectively.

Enzyme Characterization

Effects of pH and Temperature on Activity and Stability

The optima of pH and temperature for extracellular mannanase activities were 5.5 and 65 °C for *A. niger gr* and 5.0 and 60 °C for *A. flavus gr* (Figs. 5 and 6). The observed temperature optima of 65 °C for *A. niger gr* and 60 °C for *A. flavus gr* are higher by the results reported for β-mannanases from most microbial sources. Both enzymes are stable at wide pH ranges of 4–8 (Fig. 7). Slightly acidic pH optima of the enzymes (4.5–5) match the

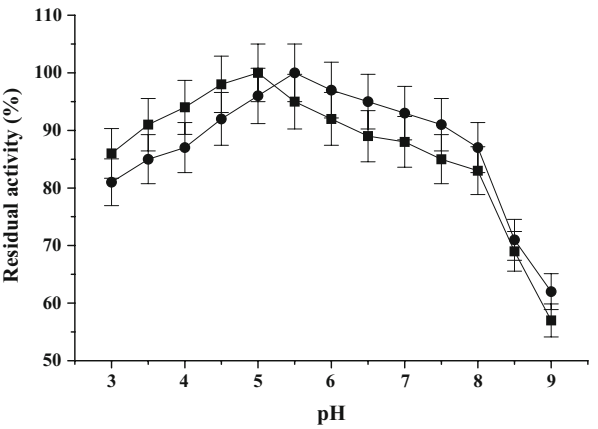

Fig. 7 pH stability of endo-β-1,4 mannanases from *A. niger gr* (*circles*) and *A. flavus gr* (*squares*)

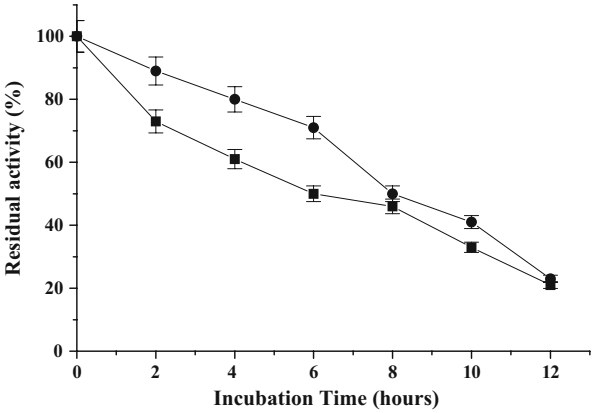

Fig. 8 Thermostability of endo-β-1,4 mannanases from *A. niger gr* (*circles*) and *A. flavus gr* (*squares*)

values characteristic for the fungal glycoside hydrolases [33, 34]. From Fig. 8, it is evident that endo-β-1,4 mannanases produced from *A. niger gr* and *A. flavus gr* have showed good thermostability in the absence of any stabilizer (maintaining 50% of residual activity for 8 and 6 h, respectively, at 60 °C). Similarly, Puchart et al. [34] have observed high temperature optima and stability for mannanase from the fungus *A. fumigatus IMI* 385708.

Conclusions

In conclusion, the present study focuses on the optimization of culture parameters for the maximal production of extracellular mannanase from newly isolated *Aspergillus* species by using cheaper sources. From the study, it is evident that by using locally available copra waste into the production of industrially important enzyme is possible. Defatted copra was one of the best carbon sources for the cultivation of *Aspergillus* species, and after media optimization, the production of enhanced enzyme activity increased up to eight times in comparison to the other carbon sources tested. Mannanase produced by *A. niger gr* seems to be more efficient as compared with that of *A. flavus gr* mannanase, and it exhibited its optimal activity at 65 °C compared to *A. flavus*, which showed its optimal activity at 60 °C, and it exhibited stability at a broad pH range (4–8) and temperature. Moreover, *A. niger* does not produce toxins; their metabolic products enjoy Generally Recognized as Safe status, and this can be used in the food industry [35]. Finally, it is concluded that the properties exhibited by the new strains are promising for their use in food- and feed-processing industries.

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