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Optimization of Cultural Conditions for β-mannanase Production by a Local *Aspergillus niger* Isolate

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ABSTRACT

A survey of eight locally isolated fungal strains was carried out to select the most potent fungus producing an extracellular β-mannanase activity. In both static and shaking cultures, *Aspergillus niger* recorded the highest β-mannanase activity (3.00 & 2.69 Um L^{-1}, respectively) among the eight fungal isolates. Optimization of the fermentation medium for maximization of β-mannanase activity in *A. niger* batch culture was performed. The highest β-mannanase activity (3.00 U mL^{-1}) was achieved after 6 days of static incubation. Coconut at a concentration of 5 g 50 mL^{-1} proved an optimum natural substrate for reaching the highest β-mannanase activity (8.56 U mL^{-1}), and protein content (1.689 mg mL^{-1}). Ammonium chloride (as the best nitrogen source) and di-potassium hydrogen phosphate at concentrations of 2.5 and 1 g L^{-1} culture medium, respectively yielded the highest β-mannanase activity (20.91 U mL^{-1}). On the other hand, the use of magnesium sulphate at 1.25 g L^{-1} resulted in maximum enzyme activity (25.64 U mL^{-1}) as well as maximum protein content and growth. Maximum enzyme activity (26.86 U mL^{-1}) protein content and growth were all obtained when using potassium chloride at concentration of 0.75 g L^{-1}. β-mannanase activity increased by 9.6 -fold yielding 28.98 U mL^{-1} when the optimized culture medium was initially adjusted to pH 6.0, inoculated with 2 x 10^6 spore mL^{-1} and incubated at 30°C.

Key Words: *A. niger*; β-mannanase activity; Optimization; Protein content

INTRODUCTION

There is a considerable interest in the biological degradation of lignocelluloses as the most abundant renewable resource in nature and its potential for industrial application (El-Naggar *et al*., 2006). The main carbohydrate constituents of lignocellulosic material (i.e. cellulose, mannan & xylan) consist of chains of β- 1, 4 -linked pyranosyl units, which can be variously substituted. These β- 1, 4 -glycosidic bonds within the polysaccharide backbones are hydrolyzed by cellulases, mannanases, and xylanases, respectively (Sachslehner *et al*., 1998). Efforts have been directed to microbial mining for production of β-mannanase including bacteria (Emi *et al*., 1972; Tamaru *et al*., 1995; Hossain *et al*., 1996), fungi (Gübitz *et al*., 1996b) and actinomycetes (Takahashi *et al*., 1984). Fungi like *A. tamarii* (Civas *et al*., 1984), *A. niger* (Ademark *et al*., 1998), *Sporotrichum cellulophilum* (Araujo & Ward, 1991), *Thielavia terrestris* (Araujo & Ward, 1990) and *Trichoderma reesei* (Arisan-Atac *et al*., 1993) have been targeted for β-mannanases isolation. β-endomannanases and β-endomannosidases, which are commonly produced by Aspergilli are responsible for degradation of galactoglucomannan backbone. β-endomannanases, generally referred to as β-mannanases, hydrolyze the backbone of galactoglucomannans, producing mannooligosaccharides.

Mannanases recently attracted the attentions of industrial sector due to their positive role in the pulp and paper industry to remove the hemicelluloses from the dissolving pulps (Gübitz *et al*., 1997). Their effective role in pulp bleaching processes minimized the use of environmentally harmful bleaching chemicals in pulp and paper industry (Lahtinen *et al*., 1995; Cuevas *et al*., 1996). Mannanases have been also used in the food industry for the extraction of vegetable oils from leguminous seeds and the clarification of fruit-juices (Christgau *et al*., 1994). They are also used in the reduction of the viscosity of extracts during manufacturing of instant coffee, chocolate and cacao liquor (Belitz & Grosch, 1987; Francoise *et al*., 1996) to lower the cost for subsequent evaporation and drying (Wong & Saddler, 1993). In addition, mannanases are potentially applied in the pharmaceutcal industry for the production of physiologically interesting oligosaccharides (Christgau *et al*., 1994).

The objective of this study was to screen 8 fungal strains for the production of β-mannanase. The desired combination of physiological conditions that would yield the highest enzyme production by the most potent fungal strain was studied.

MATERIALS AND METHODS

Microorganisms. In this study, 8 fungal strains were isolated on Dox-medium. The fungal isolates were identified in the Mycological Center, Assiut University,

Assiut, Egypt. The isolates were: *A. ochraceous*, *Alternaria alternata, A. veriscolor* (isolated from Siwa soil, Egypt); *A. niger, Stachybotrys chartarum, Rhinocladiella atrovirens* (isolated from coconut fibers) and *Neosartorya fischeri, Penicilium* sp. (isolated from garden soil, Alexandria, Egypt).

Culture medium and growth conditions. Dox-medium was used for maintenance of the isolated fungi throughout the work. This medium contained the following ingredients (g L^{-1}): locust bean gum 10, $NaNO_3$ 2, K_2HPO_4 1, $MgSO_4.7H_2O$ 0.5, KCl 0.5, $FeSO_4.7H_2O$ traces, agar-agar 20. Final pH was adjusted to 5. The inoculated slants were incubated for 7 days at 30°C then stored at 4°C until used.

Cultivation of the fungi for enzyme production. Fifty mL aliquots of the Dox medium were dispensed in 250 mL Erlenmeyer flasks. The medium was adjusted to pH 5 and then sterilized at 121°C for 15 min. After sterilization, each flask was inoculated with 1 mL fungal spores suspension, obtained from 7 day old slant culture; the flasks were then incubated statically at 30°C for 7 days. Thereafter, the content of each flask were taken for analysis. Each treatment was carried out in triplicates and the results obtained throughout the work were the arithmetic mean of at least 2 experiments.

Preparation of the crude enzyme. At the end of the incubation period, the fungal cells were separated from the culture by centrifugation at 6000 rpm, 4°C for 15 min in a cooling centrifuge (Chilspin, MSE Fisons, UK). The clear supernatant was considered as the crude enzyme source (El-Naggar *et al.,* 2006).

Estimation of protein content. The protein content of the enzyme preparation was determined by the method of Lowry *et al.* (1951) and the developed color was measured at 750 nm (Pharmacia Biotech/Novaspec® II spectrophotometer). The standard curve was constructed using crystalline BSA (Sigma Chemical Company, St. Louis, USA).

Assay for β-mannanase activity. The reaction mixture containing 1 mL of 1% locust bean gum dissolved in 0.05 M acetate buffer at pH 5 and 1 mL enzyme solution was incubated in water-bath at 40°C for 5 min, 1 mL of this mixture was removed and added to 1 mL dinitrosalycilic acid (DNS). This mixture was boiled for 10 min and then the absorbance was measured at 550 nm. The released mannose due to mannanase activity was determined by DNS method (Miller, 1959). One unit of mannanase activity is defined as the amount of enzyme, which produced 1 μmol mL^{-1} of mannose under the assay conditions.

Determination of dry weight. The cells were separated from the media by centrifugation at 5000 rpm, washed twice with distilled water and dried at 100°C to a constant weight.

RESULTS

Survey of the fungal isolates for the production of extracellular β-mannanase. The results indicated the potential differences of fungal isolates to produce an extracellular mannanase enzyme to degrade mannan. In both static (Fig. 1a) and shaken (Fig. 1b) conditions *A. niger* displayed the highest extracellular mannanase activity followed by *A. alternata* and *S. chartarum*, whilst it was the lowest in *R. atrovirens* cultures. The extracellular mannanase activity in shaken culture was generally lower than that of static one. It was interesting to note that the final pH value of *A. niger* was at the acidic range, while those of most cultures of the other tested organisms was alkaline (7.2 - 8.8). The highest protein content was recorded for *A. niger*, although, there was no direct relationship between the protein content of the tested cultures and the production of mannanase. It was also observed that there was no relation between the growth of the fungus and the mannanase activity.

Physiological factors influencing β-mannanase production by *A. niger*: Effect of incubation period. β-mannanase activity present in the culture increased regularly during the first 6 days of incubation and the highest mannanase activity was obtained at the day 6 recording 2.48 -fold increase compared to the day 1 value (Fig. 2). At longer incubation periods the activity decreased gradually and after 14 days the β-mannanase activity was 72% lower than the activity obtained at 6th day of incubation. It was also noticed that the growth estimated as a dry weight increased gradually with increasing the incubation period until it reached its maximum value after 8 days of incubation followed by a gradual decrease. The pH of the culture filtrate decreased with the prolonged incubation up to day 6 (pH 4.0) then increased gradually at longer incubation periods. However it was still in the acidic range (data not shown).

Effect of natural substrates and some sugars on enzyme activity. The effect of carbon source on the production of β-mannanase was studied using the basal culture medium supplemented with 2% mannan as a control. In other trials mannan was replaced by equal amounts of different carbon sources, which included glucose, fructose, sucrose and mannose as a sole carbon source or milled date seeds, coconut, soybean meal, rabbit feed and milled carob as natural substrates that might be useful for production of the enzyme in a commercial scale.

Final pH value and the protein content of the culture filtrates were greatly affected by the nature of the carbon source used (Table I). Coconut was the most preferable carbon source yielding a maximum β-mannanase activity, and protein content. This activity was 2.85, 7.1 folds of that obtained when mannan or milled carob were used, respectively. On the other hand, the lowest β-mannanase activity was obtained in cultures containing sugars, where glucose showed an activity lower than that of coconut by about 98%. Cultures containing fructose, mannose or sucrose showed nearly no activity.

Effect of different concentrations of coconut pulp. The different concentrations (1 - 10 g 50 mL^{-1} culture medium)

Fig. 1. Production of extracellular β-mannanase by some local fungi in (a) static and (b) shaken cultures

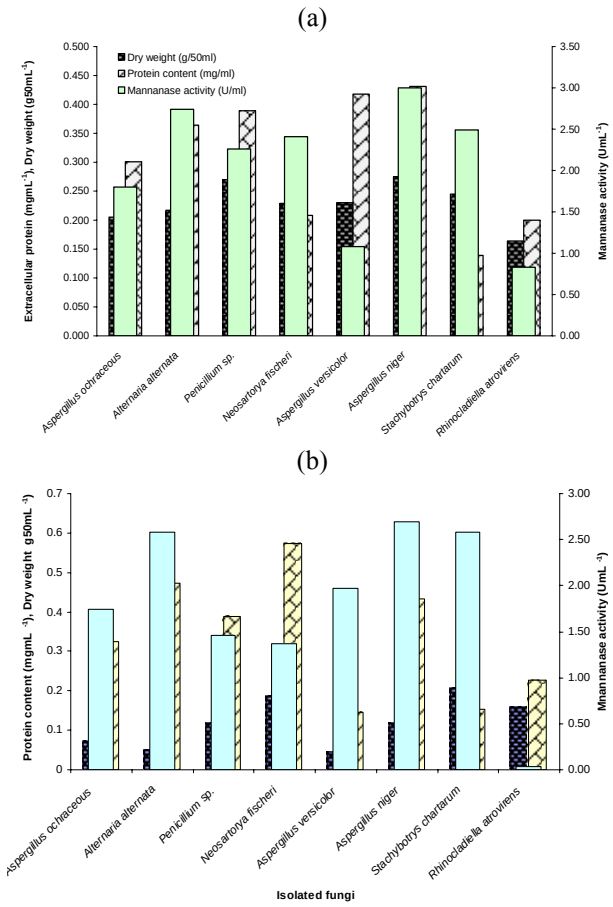


Fig. 2. Production of β-mannanase by *A. niger* at different incubation times

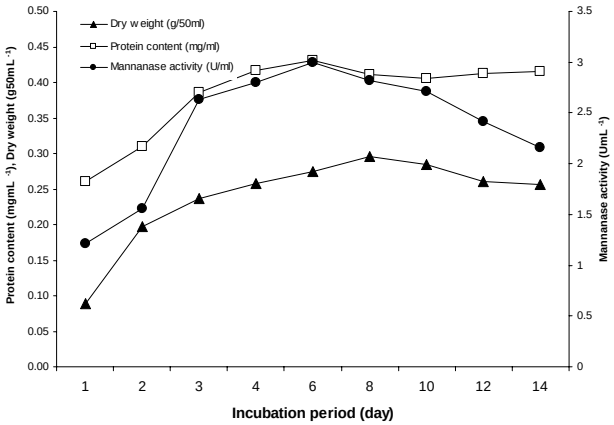
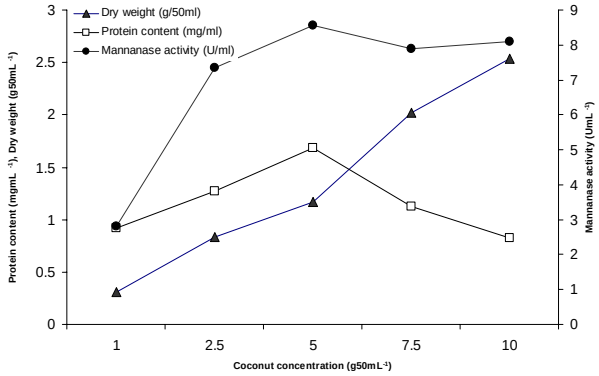


Fig. 3. Effect of different concentrations of coconut on the production of β-mannanase by *A. niger*



of coconut pulp were individually supplemented to the basal medium. The optimum coconut concentration was 5 g 50 mL^{-1}, yielding a highest mannanase activity and protein content (Fig. 3). The final pH value increased with increasing the coconut concentration till it became constant (pH 4.0) when the coconut concentration of 5 g 50 mL^{-1} medium or any higher coconut concentration was used (data not shown).

Effect of nitrogen source. The effect of nitrogen source on the production of β-mannanase was studied using the culture medium supplemented with coconut (5 g 50 mL^{-1} medium). Sodium nitrate (0.2%) used as a nitrogen source in the basal medium was replaced on equal nitrogen basis, by ammonium sulphate, ammonium nitrate, ammonium chloride, peptone, whey and yeast extract plus peptone, each at a time. Growth, final pH value and protein content of the culture filtrate were differently affected by the nature of the nitrogen source (Table II). All the tested nitrogen sources showed a β-mannanase activity higher than that obtained with $NaNO_3$ used in the original medium. Ammonium chloride was the most preferable nitrogen source yielding a maximal β-mannanase activity, which was 1.65 -fold the value of sodium nitrate.

The results in Fig. 4 indicate that a parallel increase in biomass, protein content and enzyme activity occurred with raising the NH_4Cl concentration until a concentration of 2.5 g L^{-1} NH_4Cl and then it decreased gradually by using higher concentrations. The pH value of the culture medium decreased gradually with increased NH_4Cl concentration to reach 3.8 at 2.9 g L^{-1}. At higher concentrations the pH value increased and the activity decreased showing 41.5% decrease of the maximum value at an ammonium chloride concentration of 5 g L^{-1} (data not shown).

Effect of di-potassium hydrogen phosphate level. The basal medium was supplemented with various concentrations of K_2HPO_4 ranging from 0.1 to 2.5 g L^{-1}. The β-mannanase activity increased gradually by increasing the K_2HPO_4 level in the medium and reached the maximum activity at a concentration of 1 g L^{-1}, 3.4 -fold that obtained in the presence of the low K_2HPO_4 concentration of 0.1 g L^{-1} (Table III). Similarly, the highest growth and maximum protein content were obtained at K_2HPO_4 level of 1 g L^{-1}. A gradual decrease in the pH value of the culture filtrate was also noticed with an increase in K_2HPO_4 levels until the

Table I. Effect of some natural substrates and sugars as carbon sources on the production of β-mannanase in *A. niger* cultures

Natural substrate	Dry weight (g 50 mL^{-1})	Final pH value	Protein content (mg mL^{-1})	Mannanase activity (U mL^{-1})
Date seeds	0.341±0.00	4.6±0.3	0.245±0.03	3.21±0.04
Coconut pulp	1.171±0.08	4.0±0.2	1.689±0.15	8.56±1.26
Soy bean meal	1.421±0.07	4.0±0.3	0.536±0.02	3.78±0.07
Rabbit feed	1.506±0.03	5.2±0.1	0.376±0.04	3.82±0.13
Carob pods	0.318±0.00	4.0±0.2	0.292±0.01	1.21±0.00
Mannan	0.275±0.04	4.0±0.0	0.431±0.05	3.00±0.13
Glucose	0.336±0.02	2.6±0.1	0.721±0.09	0.189±0.00
Fructose	0.338±0.00	3.1±0.1	0.914±0.06	0.002±0.00
Sucrose	0.321±0.01	2.7±0.2	0.800±0.13	0.005±0.00
Mannose	0.341±0.05	2.8±0.0	0.770±0.08	0.010±0.00

Mean±SE

Table II. Effect of nitrogen sources on the production of β-mannanase by *A. niger*

Nitrogen source	Dry weight (g 50 mL^{-1})	Final pH value	Protein content (mg mL^{-1})	Mannanase activity (U mL^{-1})
$NaNO_3$	1.171±0.04	4.0±0.2	1.689±0.10	8.56±0.08
$(NH_4)_2SO_4$	1.495±0.06	3.8±0.1	2.321±0.07	13.51±1.04
NH_4NO_3	1.656±0.05	3.7±0.0	2.631±0.05	13.56±2.07
NH_4Cl	1.554±0.07	4.0±0.3	1.820±0.06	14.18±1.06
Peptone	1.669±0.05	4.2±0.0	2.310±0.13	13.67±0.90
Yeast extract + peptone	1.691±0.05	3.0±0.2	2.230±0.14	12.92±1.00
Whey	1.581±0.03	3.0±0.0	1.860±0.08	12.37±1.05

Mean±SE

concentration value of 1 g L^{-1}, while at higher concentrations the pH increased gradually. Higher or lower K_2HPO_4 levels showed an adverse effect on the activity, protein content and growth, where much lower values were obtained.

Effect of magnesium sulphate level. The effect of $MgSO_4.7H_2O$ concentration on the production of mannanase by *A. niger* was investigated using the medium containing coconut (10%), NH_4Cl (0.25%), K_2HPO_4 (0.1%) and different concentration of $MgSO_4.7H_2O$ ranging from 0.1 to 1.5 g L^{-1}. A maximum β-mannanase activity was recorded on using a concentration of 1.25 g L^{-1} $MgSO_4.7H_2O$ (Table IV). Likewise, the growth reached a maximum value as well as the protein content achieved its highest value at the same level. It was also observed that concentrations higher or lower than 1.25 g L^{-1} showed a variable effect on the enzyme activity, protein content and the growth of *A. niger*.

Effect of potassium chloride. The results indicated that the β-mannanase activity increased gradually by increasing the KCl level in the medium and reached a maximum value of 26.86 U mL^{-1} at a concentration of 0.75 g L^{-1} and then the activity decreased gradually (Table V). At this concentration, the highest growth and protein content were achieved. It was also noticed that there was a little variation in pH value ranging from 3.8 to 4.0.

Effect of incubation temperature. The inoculated flasks were incubated for 6 days at different temperatures covering a range from 20 to 40 °C (Fig. 5). The maximum mannanase activity, protein content and growth were achieved at an incubation temperature of 30°C. At higher or lower temperatures, the enzyme activity in the culture showed a lower value.

Effect of initial pH value of the culture medium. The medium used was of the same composition as that used in the previous experiment. The medium was initially adjusted using NaOH or HCl to cover a pH range from 3.0 to 8.0 (All adjustments were made before sterilization). After inoculation, the flasks were incubated under static condition for 6 days at 30°C. Initial pH 6.0 gave the highest mannanase activity, protein content and dry weight (Fig. 6). The initial pH values lower or higher than 6.0 had an adverse effect on the growth, protein content and mannanase activity. At initial pH 8.0 the enzymatic activity dropped to about 34.98% of that obtained at initial pH 6.0.

Effect of inoculum size. In this experiment, the fermentation flasks (each one containing 50 mL basal medium) received different inocula ranging from 0.1-$4.0x10^6$ spores $flask^{-1}$ from a spore suspension obtained from a 6 -day old culture of *A. niger*. The flasks were incubated at 30°C in a static incubator for 6 days. Fig. 7 exhibits the effect of the inoculum's size of *A. niger* on the enzyme activity, protein content and growth. An increase of 2.11 -fold and 3.3 -fold in protein content and enzyme activity, respectively was noticed on raising the inoculum's size from 0.1 to 2×10^6 spores 50 mL^{-1}medium.

DISCUSSION

In the present work, the optimization of the culture medium components and some environmental parameters could improve the mannanase activity in *A. niger* batch

Table III. Effect of different concentrations of K_2HPO_4 on the production of β-mannanse by *A. niger*

K_2HPO_4 concentration (g L^{-1})	Dry weight (g 50 mL^{-1})	Final pH value	Protein content (mg mL^{-1})	Mannanase activity (U mL^{-1})
0.1	0.834±0.05	4.8±0.2	1.104±0.2	6.11±0.26
0.25	1.286±0.11	4.2±0.3	1.770±0.3	14.25±1.20
0.5	1.582±0.13	4.0±0.1	2.100±0.0	18.23±2.00
1.0	1.607±0.09	3.8±0.0	1.970±0.1	20.91±1.97
1.5	1.466±0.08	4.0±0.3	2.261±0.4	19.61±1.54
2.0	1.445±0.04	4.2±0.4	2.242±0.2	18.24±1.34
2.5	1.326±0.07	4.4±0.1	2.200±0.0	16.89±2.00

Mean±SE

Table IV. Effect of different concentrations of $MgSO_4.7H_2O$ on the production of β-mannanase by *A. niger*

$MgSO_4.7H_2O$ concentration (g L^{-1})	Dry weight (g 50 mL^{-1})	Final pH value	Protein content (mg mL^{-1})	Mannanase activity (U mL^{-1})
0.1	0.978±0.04	4.0±0.1	0.982±0.07	10.12±1.02
0.25	1.225±0.02	4.0±0.0	1.548±0.16	18.73±2.00
0.5	1.607±0.05	3.8±0.2	1.970±0.18	20.91±1.90
0.75	1.786±0.20	3.8±0.1	1.986±0.04	22.44±1.83
1.0	1.792±0.10	3.8±0.0	2.102±0.18	24.71±1.90
1.25	1.862±0.10	3.8±0.1	2.314±0.07	25.64±1.70
1.50	1.842±0.06	4.0±0.1	2.204±0.10	20.66±1.63

Mean±SE

cultures. β-mannanase enzyme is very important for the digestion of hemicelluloses, one of the most abundant groups of polymers in nature. This enzyme hydrolyzes mannan yielding mannotriose and mannobiose (Stålbrand *et al*., 1993). In our screening program, *A. niger* cultures showed the highest mannanase activity, protein content and growth among the isolated fungi in static and shaken conditions. The highest β-mannanase activity in the static culture compared to shaken one could be attributed to the oxygen limitation that could be a serious problem in the shaken cultivation due to the highly non-Newtonian medium caused by the filamentous growth of the fungus (Groβwindhager *et al.*, 1999).

The substitution of mannan in the culture medium by coconut resulted in a maximum β-mannanase activity. Although, many investigators used galactomannan (mannan) as a sole carbon source for the cultivation of some fungi (Zakaria *et al*., 1998). However, still few investigators used glucose as a sole carbon source (Großwindhager *et al*., 1999; Sachslehner & Haltrich, 1999).

Generally, the synthesis of mannanase enzyme necessary for the degradation of energy-yielding polymeric materials as lignocelluloses is inducible in microbial cells and appears to be controlled by carbon repression when more easily metabolizable carbon sources e.g. glucose are present in the culture medium together with a substrate suitable for inducing endoglycanase synthesis (Ronne, 1995; Ruijter & Visser, 1997). So, enzyme formation in fungi starts only when the repressing glucose is completely metabolized, although very few levels of endoglycanases were produced when glucose was used as the only carbon source (Beguin & Aubert, 1994; Groβwindhager *et al.*, 1999). A generally accepted mechanism for induction of endoglycanases is that the respective polysaccharide is first partly hydrolyzed by hydrolases, which are constitutively produced in very low amounts. The soluble low molecular weight catabolites can easily enter the cell, signaling the presence of an extracellular substrate and providing the stimulus for the accelerated synthesis of the respective

Fig. 4. Effect of different concentrations of NH4Cl on the production of β-mannanse by A. niger

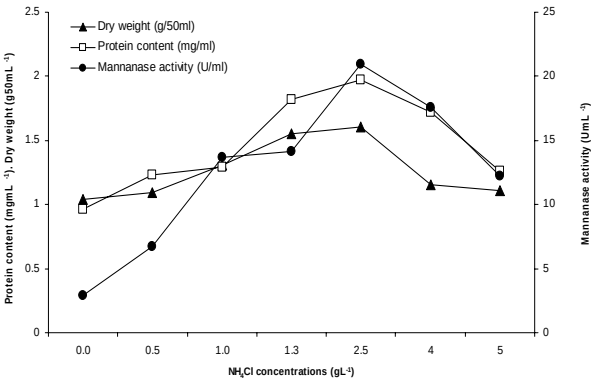


Fig. 5. Effect of incubation temperature on the production of β-mannanase by A. niger

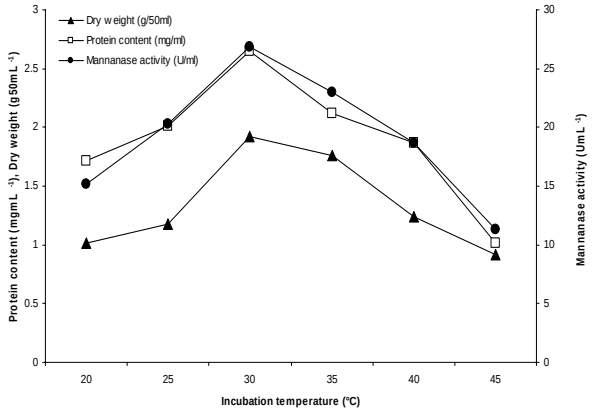


Table V. Effect of different concentrations of KCl on the production of β- mannanase by *A. niger*

KCl concentration (g L^{-1})	Dry weight (g 50 mL^{-1})	Final pH value	Protein content (mg mL^{-1})	Mannanase activity (U mL^{-1})
0.1	1.231±0.00	4.0±0.2	1.694±0.17	19.58±1.00
0.25	1.468±0.05	4.0±0.0	1.933±0.06	21.72±1.30
0.5	1.862±0.07	3.8±0.1	2.314±0.14	25.64±1.15
0.75	1.925±0.13	3.8±0.3	2.365±0.12	26.86±1.55
1.0	1.624±0.04	4.0±0.0	2.353±0.19	24.47±1.48
1.25	1.550±0.09	4.0±0.1	2.217±0.21	20.63±1.37

Mean±SE

Fig. 6. Effect of initial pH value on the production of β-mannanase by A. niger

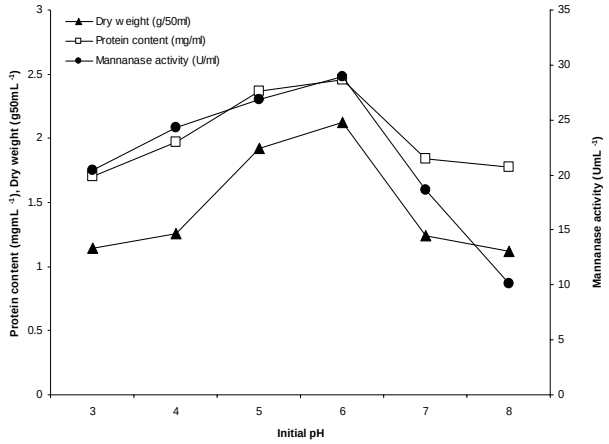
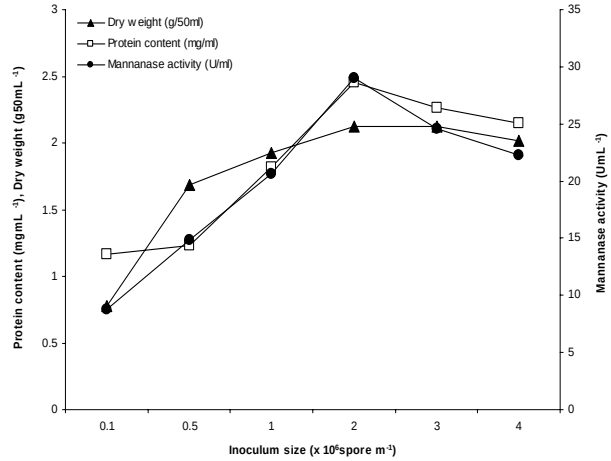


Fig. 7. Effect of inoculum size on the production of β-mannanase by A. niger



enzymes (Biely, 1993). A maximum β-mannanase activity produced when *A. niger* grown on glucose was 6.3% of the corresponding activity in response to mannan as an inducing substrate.

Regarding the effect of different nitrogen sources on the mannanase activity by *A. niger* it was found that the use of ammonium chloride (2.5 g L^{-1}) was best followed by peptone. Contrarily, peptone and ammonium nitrate were the best nitrogen sources for enzyme activity of *Sclerotium rolfsii* (Gübitz *et al*., 1996a, b; Großwindhager *et al*., 1999; Sachslehner & Haltrich, 1999).

Use of 0.1% dipotassium hydrogen phosphate also resulted in the maximal mannanase activity by *A. niger*. The $MgSO_4.7H_2O$ level (0.13%) was responsible for the maximum enzyme activity. This was comparable to the other fungal strains for the production of mannanase as *Sclerotium rolfsii* (Gübitz *et al*., 1996a, b; Feng *et al*., 2003). The use of 0.08% KCl resulted in the production of a maximum β-mannanase activity, which was very close to the finding of Sachslehner *et al*. (1998), who used 0.06% KCl to induce mannanase activity in *Sclerotium rolfsii* cultures.

Regarding the environmental factors, the optimum temperature for growth and for β-mannanase production by *A. niger* was found to be 30°C. The activity of β-mannanase by *A. niger* was also considerably affected by the initial pH values, where the initial pH 6.0 was the most favorable for the β-mannanase activity. In general, the organism preferred the acidic range especially the low acidic range, which was similar to *Sclerotium rolfsii* for producing mannanase at pH 5.0 (Großwindhager *et al*. (1999). Finally, the inoculum size affected the ability of *A. niger* to produce mannanase in culture. The increase of the inoculum size from 0.1 - 2 x 10^6 spore 50 mL^{-1} medium enhanced the production of the enzyme by about 3.3 fold.

This work collectively suggests a possible role of the locally isolated *A. niger* or its extracellular enzyme to play a significant role towards the enhancement of the industrial solubilization of lignocellulose under the optimized environmental and nutritional conditions.

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