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Extracellular β-Mannanase Production by the Immobilization of the Locally Isolated *Aspergillus niger*

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ABSTRACT

The production of β-mannanase by the immobilization of the local *Aspergillus niger* strain isolated from the coconut fibres was studied. The fungal spores were entrapped in different gel materials. Alginate (1%) was the best gel matrix for β-mannanase production, although alginate entrapment showed a relatively low β-mannanase activity compared to free culture system. The entrapped cells in alginate were reused for 8 successive times. The activity increased up-to the third run and then showed a gradual decrease up to 8th run. The production of β-mannanase by adsorption of *A. niger* onto sponge cubes, luffa pulp, pumice particles, clay particles and ceramic pieces was also investigated. The results showed that adsorption on pumice particles resulted in a relatively high β-mannanase activity when compared with free cultures. Factorial design was applied for production of β-mannanase by *A. niger* adsorbed on pumice particles. The optimized medium raised the activity of β-mannanase to 90.87 U/ mL which is approximately 2.3 times the value recorded for the basal medium yield.

Key Words: Adsorption; *A. niger;* Entrapment; Immobilization; Mannanase; Plackett-Burman design

INTRODUCTION

Immobilization of microbial cells is a useful technique to investigate the potential of new industrial production processes and academic research (El-Naggar *et al*., 2003). The wide ranges of applications for immobilized biocatalysts include generally the production of chemicals, bioactive materials, pharmaceuticals and food materials. Immobilization techniques can be grouped into four major categories: adsorption on porous inert supports or ion exchange materials, entrapment in cross linked gel, cross-linking by some reagents and covalent binding to solid polymeric materials (El-Naggar *et al*., 1998).

Adsorption of microbial cells to solid supports proved to be a mild, non-specific processes involving binding of the cells to support materials by ionic or less powerful bonds. Cell adsorption may be carried out on several porous materials, such as ceramics, sintered glass, wood ships, sponge cubes, polyvinyl chloride fibers, resins and others (Moo-Young *et al*., 1980; Bandyopadhyay & Ghose, 1982; El-Naggar *et al*., 1998; El-Naggar *et al*., 2003).

The nutritional and environmental conditions have a great influence on the extracellular mannanase activity, which is responsible for the reductive cleavage of the β-mannan polymer. Optimization of these fermentation conditions is traditionally done by varying one factor, while keeping the other factors at a constant level. The one-factor-at-a-time approach used frequently to study the effect of several factors is inefficient and time consuming and ignores the interactions between variables (Teruel *et al*., 1997), but statistical experimental design would allow a reliable short-listing of a few significant variables for further optimization studies and considers the statistical interaction between variables that are ignored by traditional techniques (Ooijkaas *et al*., 1998; Ooijkaas *et al*., 2000).

In order to develop an efficient mannan degradation process, knowledge regarding the environmental factors affecting the rate of degradation by the locally isolated fungus has to be identified. In this study, the mannanase activity and its protein content as parameters were considered for the degradation process of mannan polymer by the immobilized *A. niger* using pumice as a solid support. Factors affecting the objectives were evaluated by application of the Plackett-Burman experimental design (Plackett & Burman, 1946).

MATERIALS AND METHODS

Microorganisms. In the present work, *Aspergillus niger* was isolated from Coconut fibers on Dox-medium and was identified in the Mycological Center, Assiut University, Egypt.

Culture medium. Dox-medium was used for maintenance of *A. niger* throughout the work. This medium contained the following ingredients (g/ l) Locust bean gum 10 gm, NaNO3, 2 gm, K_2HPO_4, 1 gm, $MgSO_4.7H_2O$, 0.5 gm, KCl, 0.5 gm, $FeSO_4.7H_2O$, traces, agar-agar, 20 gm, and final pH was adjusted to 5. The inoculated slants were incubated for

7 days at 30°C then stored at 4°C until used.

Preparation of the crude enzyme. At the end of the incubation period, the fungal cells were separated from the culture by centrifugation at 6.000 rpm, 4°C for 15 min in a cooling centrifuge (Chilspin, MSE Fisons, UK). The clear supernatant was considered as the crude enzyme source.

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 previously constructed using crystalline bovine serum albumin (Sigma Chemical Company, St. Louis, USA).

Assay for β-mannanase activity. The reaction mixture (1 mL) containing 1 mL of 1% locust bean gum dissolved in 0.05 M acetate buffer at pH 5 and 1 mL enzyme solution were 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 μ mole of mannose per mL 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.

Immobilization of *A. niger* by Gel Entrapment

Entrapment in Ca-alginate. Fungal cells were entrapped in 2% calcium alginate gel beads according to the procedure described by El-Naggar *et al.* (1998). 2% sodium alginate solution (Sigma Chemical Company, St. Louis, USA with a viscosity of 3500 centipoises at 25°C) was prepared by dissolving 2 g in 90 mL distilled water and then autoclaving at 108°C for 10 min. Ten mL fungal spore suspension obtained from 6 days old slant culture was added to the sterile alginate solution to obtain 2% final concentration. 10 mL of the alginate- spore mixture were drawn with the aid of a sterile syringe and allowed to drop into a cross linking solution (100 mL of 2% $CaCl_2$ solution) in 250 mL Erlenmeyer flask to obtain spherical beads (3 - 4 mm diameter) of calcium alginate gel entrapping the fungal spores. The beads were left in the calcium chloride solution for 1 h for complete hardening and then washed several times with sterile distilled water. The resulted beads from 10 mL alginate were added to 50 mL sterile medium in 250 mL Erlenmeyer flask. The flasks were incubated statically at 30°C for 6 days.

Immobilization of fungal cells by adsorption. One mL spore suspension was added to the Erlenmeyer flasks (250 mL capacity) containing sterilized 50 mL culture medium and sponge, luffa, pumice or ceramic (purchased from the Egyptian local market). They were cut to small cubes (sponge) or pieces (ceramic, luffa pulp, clay, pumice), washed several times with water before use. These flasks were then placed in an incubator at 30°C for 6 days Adsorbed mycelia were estimated by washing the solid support several times with water and then drying the adsorbed mycelia at 60°C over night to determine their dry weight. Dry weight of adsorbed mycelia was determined by subtraction of an average pre-determined dry weight of the used support from the weight of support plus mycelia (Vassilev *et al*., 1993).

Plackett-Burman experimental design. The Plakett-Burman experiment design, a fractional factorial design, (Plackett & Burman, 1946) was used in this research to reflect the relative importance of various environmental factors on the activity of mannanase enzyme for degradation of mannan. In this experiment, for example, seven independent variables were screened in eight combinations organized according to the Plackett-Burman design matrix. For each variable, a high (+) and low (-) level was tested. All trails were performed in duplicates and the averages results were treated as the responses.

The main effect of each variable was determined with the following equation:

$$E_{xi} = (\Sigma M_{i+} - \Sigma M_{i-}) / N$$

Where E_{xi} is the variable main effect, M_{i+} and M_{i-} are the activity percentage in trails where the independent variable (xi) was present in high and low concentrations, respectively, and N is the number of trials divided by 2. A main effect figure with a positive sign indicates that the high concentration of this variable is near to the optimum and a negative sign indicates that the low concentration of this variable is near to the optimum. Using Microsoft Excel, statistical t-values for equal un-paired samples were calculated for determination of variable significance.

Electron microscopy. Fungal growth within different porous supporting materials were harvested as small pieces, washed with phosphate buffer and fixed with 2% glutaraldehyde followed by 1% osmium tetraoxide treatment. Samples were then washed in a buffer solution, and dehydrated in ethanol. The samples were dried completely in a critical point dryer, and finally coated with gold in JEOL-JFG 1100 E ion-sputter-coater. The specimens were viewed in JEOL-JSM 5300 scanning electron microscope operated at 20 kV with a beam angle of 45°.

RESULTS

Production of extracellular β-mannanase by immobilized *A. niger*. The aim of the present part of the work was to study the influence of immobilization of *A. niger* on the production of extracellular mannanase. Cell adsorption on solid supports and gel entrapment in different polymers were examined in this study.

Effect of different gel materials on the production of extracellular β-mannanase by *A. niger* cells. This experiment deals with the study of extracellular mannanase

production by *A. niger* mycelia entrapped in different gel materials. The cells were entrapped in alginate, carrageenan, agarose, agar-alginate mixture and a free culture was used as a control.

An optimized culture medium in 250 mL Erlenmeyer flasks was introduced to porous beads of gel material including fungal cells. After 6 days in static culture at 30°C, the mannanase activity and protein content of the culture filtrate were determined.

The results (Fig. 1) showed that mannanase activity with free cells was higher than that of entrapped cells by approximately 3.7 fold. On other hand, the data illustrate that the alginate gel recorded the highest mannanase activity (7.89 U/ mL) for the entrapped cells, while the agar-alginate exhibited the highest protein content (1.573 mg/ mL) with a slightly lower mannanase activity than alginate gel.

Effect of alginate gel concentrations on the production of extracellular β-mannanase by *A. niger*. Effect of different concentrations of alginate on the mannanase production was tested using a concentration range of 1 - 4%. The experiment was carried under static condition and at 30°C.

The result given in Table I showed that, the best concentration for producing a maximum mannanase activity (9.91 U/ mL) and highest protein content (1.731 mg/ mL) was obtained at 1% level. It was noticed that the activity at 1% was 1.6-fold of that obtained at level 4% and the protein content at this level was higher by about 71%. It was observed that there was a gradual decrease in the growth behavior of *A. niger* as a high growth at 1% while the lowest one at level 4%.

Effect of reusing entrapped *A. niger* mycelia on the production of extracellular β-mannanase. The reusing of entrapped *A. niger* mycelia on the production of mannanase using alginate polymer was investigated. For this purpose, 50 mL fractions of the production media were used. The experiment was carried out by batch wise reuse of the entrapped mycelia.

At the end of each reuse the culture media were decanted and fresh media were added under aseptic conditions to the entrapped fungus. The time interval for the reuse was 6 days. Incubation was carried out at 30°C.

The results in Fig. 2 showed that reusing the entrapped mycelia resulted in an increase in the activity up-to the third run as a maximum mannanase activity was observed (34.17 U/ mL) with a high protein content (2.367 mg/ mL). After the third run the reused culture showed a gradual decrease up to the 8th run where, the entrapped mycelia showed the lowest mannanase activity which loosed 33.6% of its activity at the last run.

Effect of *A. niger* adsorption on the production of extracellular β-mannanase. The results recorded in Fig. 3, showed that a high cell adsorption occurred on all the used supports. However, the protein content of adsorbed cultures was slightly lower than that of free cultures.

Cultures containing adsorbed mycelia on pumice particle, luffa pulp and ceramic particles showed a relatively high mannanase activity while culture containing sponge cubs and clay pieces showed lower mannanase activity. On other hand, cultures containing pumice particles as a solid support exhibited the highest mannanase activity 35.67 U/ mL.

Many trials were done for existing a continuous production of extracellular β-mannanase from a continuous culture. But all of them were failed due to a high viscosity of mannan, which ceased the mechanical process of peristaltic pump.

Scanning electron microscopy. Scanning electron micrographs showed that there are highly condensed fungal mycelia adsorbed onto the pumice particles followed by the

Fig. 1. Production of β-mannanase by *A. niger* entrapped in different gel matrices.

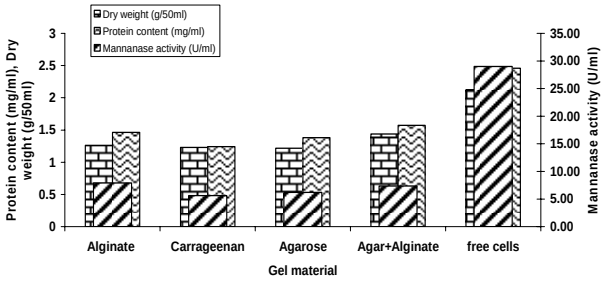


Fig. 2. Effect of reusing the entrapped *A. niger* mycelia on the production of β-mannanase.

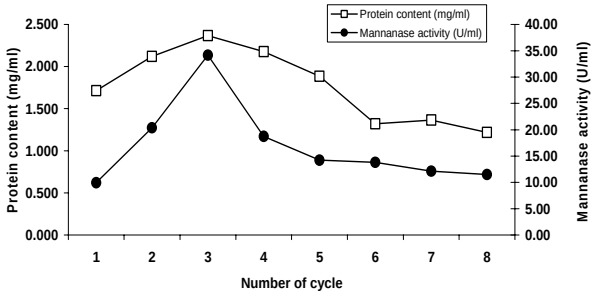


Fig. 3. Production of β-mannanase by *A. niger* mycelia adsorbed on different solid porous supports.

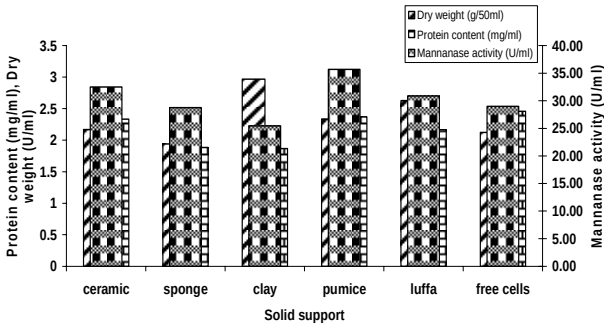


Table I. Effect of different alginate concentrations on the production of β-mannanase by entrapped *A. niger*

Alginate concentration (%)	Dry weight (g/ 50 ml)	Final pH value	Protein content (mg/ ml)	Mannanase activity (U/ml)
1	1.477	3.8	1.713	9.91
2	1.285	3.8	1.582	8.56
3	1.264	3.8	1.463	7.89
4	1.017	4.0	1.211	6.05

Table II. Semi-continuous production of β-mannanase by entrapped *A. niger*.

Run Number	Protein content (mg/ml)	Mannanase activity (U/ml)
1	1.713	9.91
2	2.121	20.35
3	2.367	34.17
4	2.177	18.73
5	1.883	14.21
6	1.321	13.82
7	1.365	12.11
8	1.218	11.47

Table III. Independent variables and their levels in the Plackett-Burman design.

Factor (g/L)	Level		
	-1	0	1
Mannan	15	20	25
NH_4Cl	2	3.9	6
K_2HPO_4	0.25	0.5	0.75
$MgSO_4.7H_2O$	0.65	1.25	1.75
KCl	0.35	0.75	1.5
$FeSO_4$	0.0005	0.001	0.002
Inoculum size	$1x10^6$	$2x10^6$	$3x10^6$

Table IV. The Plackett-Burman experimental design for 7 factors.

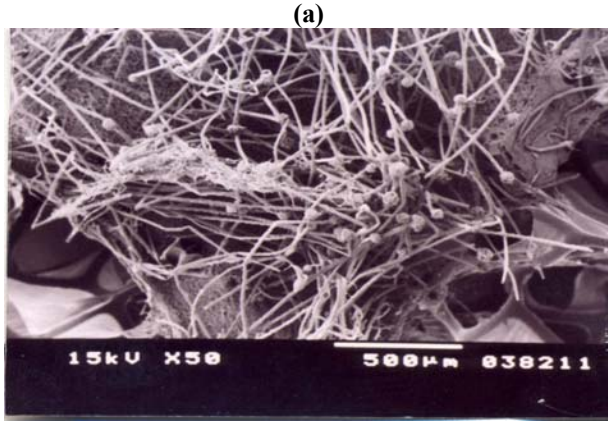
Trials	Independent Variable						
	Mannan	NH_4Cl	K_2HPO_4	$MgSO_4.7H_2O$	KCl	$FeSO_4$	Inoculum size
1	-1	-1	-1	1	1	1	-1
2	1	-1	-1	-1	-1	1	1
3	-1	1	-1	-1	-1	-1	1
4	1	1	-1	-1	1	-1	-1
5	-1	-1	1	1	-1	-1	1
6	1	-1	1	1	1	-1	-1
7	-1	1	1	-1	-1	1	-1
8	1	1	1	1	1	1	1
9	0	0	0	0	0	0	0

sponge cubes, whereas, the least adsorption occurred in the case of luffa pulp particles (Fig. 4a-c).

Determination of fermentation factors affecting the production of β-mannanase by immobilized *A. niger*. Seven culture variables were examined and presented in Table III. The design was applied with 9 different fermentation conditions as shown in Table IV. The culture filtrate of each filtrate was taken where the protein content and mannanase activity of each trial were determined. The reducing sugars produced as a result of enzyme activity were determined as mannose reducing equivalents, using the dinitrosalycilic acid (DNS) method as mentioned before. All experiments were performed in duplicates and the averages of results of mannanase activity (U/ mL) and protein content (mg/ mL) are presented in Table V. The principal statistical analysis of this experiment is shown in Table VI. The main effect of each variable upon enzyme production as well as protein content was studied. The data indicated that the presence of high levels of Mannan, K_2HPO_4, NH_4Cl and KCl in the growth medium affects mannanases production by *Aspergillus niger* positively. On the other hand, the same figure suggests that the presence of MgSO4.7H2O, FeSO4 and inoculum size at their lowest levels would result in high mannanase activity. The main effect results pointed out those high levels of mannan, inoculum size and NH_4Cl in the medium with low levels of K_2HPO_4, $FeSO_4$, $MgSO_4.7H2O$ and KCl induced the

Fig. 4. Scanning electron micrographs of *A. niger* adsorbed on Pumice particles (a); Sponge cubes (b) and Luffa pulp (c). Bar = 500 μm.

(a)



(b)

(c)

existence of high protein content in the culture filtrate of the immobilized fungal cells.

According to the results it can be predicted that the optimum medium for producing an extracellular β-mannanase from the culture of *A. niger* with a relatively high mannanase activity is (g/ l): Mannan, 25; NH_4Cl, 6; K_2HPO_4, 0.25; $MgSO_4.7H_2O$, 0.65; KCl, 1.5; $FeSO_4$, 0.0005; Inoculum size, 1 X 10^6 spore/ 50 mL.

In order to evaluate the accuracy of the applied Plackett-Burman screening test, a verification experiment was carried out in triplicate. The predicted optimum levels of independent variables were examined and compared to the basal condition setting and the average of protein content and mannanase activity are shown in Table VII. Where the mannanase activity reached about 90.87 U/ mL, which is approximately 2.3 times higher than that obtained from the basal medium (38.69 U/ mL). Moreover, the protein content of the culture filtrates of the new medium increased by 2 times than the basal medium.

DISCUSSION

Aspergillus niger, a filamentous fungus, is one of the most used organisms in the industrial production of fermented food, organic acids, and enzymes. In the present work, the production of mannanase by *A. niger* entrapped in different gel materials demonstrated that alginate gel polymer was the best for production of mannanase. Mannanase activity was higher than those obtained from other gel materials. But the activity of free fungal cells was higher than that obtained from the entrapped fungal cells in alginate gel material by 3.7 times. This could be explained on the ground that the constraints created by the polysaccharide gel that can modify the physiological behavior and cause the low deficiency due to the transport limitation of a substrate-controlling substrate or co-substrate (oxygen) resulting from the boundary layer diffusion or intraparticle pore diffusion compared to the free cell cultures (El-Naggar *et al*., 2000; El-Naggar *et al*., 2003). However, the production of mannanase by entrapped *A. niger* was studied in semi-continuous cultures. The entrapped cells were reused for 8 successive cycles covering a period of 48 days. The reuse of the entrapped mycelia enhanced the production of the enzyme until third successive cycle where gradual increase of mannanase activity values was recorded. At third cycle the mannanase activity reached about 34.17 U/ mL with a high protein content 2.367 mg/ mL. However, a gradual decrease was observed in the next reuses.

The production of mannanase by *A. niger* immobilized by adsorption on different porous solid supports in a batch mode was studied. The highest mannanase activity was observed in culture containing pumice particles as a solid support followed by ceramic particles, the activity was 1.2 and 1.1 fold of that obtained from free cells. Also luffa pulp showed a higher activity than free cells. Only clay pieces and sponge cubes showed slightly lower activities. Other investigators reported the enhancement of enzyme production by adsorbed fungal cells on porous support. Aleksieva and Tchorbanov (1990) demonstrated the use of *Humicola Lutea* 120 - 5 spores that were entrapped in polyurethane sponge cubes for the production of acid proteinases in batch mode cultures. Bliyeva (1982) also demonstrated the immobilization of *Aspergillus oryzae* and *Aspergillus awamori* on an immobile solid carrier of porolon for the production of alpha-amylase and pectinase enzymes in batch cultures, respectively.

Scanning electron microscopy revealed that there was a relatively condensed mass of fungal mycelia on a pumice particles than other porous solid materials but varying degrees of mycelial growth were shown within sponge cubes and luffa pulp. The successful adsorption obtained on many of these solid support with the simplicity of the immobilization procedure and low price of the used supports, present some advantages for the possibility of using adsorbed *A. niger* for mannanase production.

Statistically based experimental designs for optimizing nutrients, which involve simultaneously varying factors in a deliberate manner, are useful in identifying the important

Table V. Protein content (mg/ ml) and β-mannanase activity (U/ ml) responses in the Plackett-Burman experimental design of the immobilized *A. niger*.

Trial No.	Response	
	Protein content (mg/ ml)	Mannanase activity (U/ ml)
1	1.965	54.96
2	5.077	13.32
3	3.385	57.35
4	4.859	46.05
5	1.965	23.26
6	4.750	86.12
7	2.402	47.02
8	5.008	78.22
9	2.427	35.67

Table VI. Statistical analysis of the Plackett-Burman experiment

Variable	Protein content		Mannanase activity (U/ml)	
	Main effect	t-value	Main effect	t-value
Mannan	3.1	7.28	10.28	0.56
NH_4Cl	0.5	0.45	12.75	0.704
K_2HPO_4	-0.3	-0.27	15.74	0.98
$MgSO_4.7H_2O$	-0.5	-0.43	-0.33	-0.017
KCl	-1.1	-1.13	21.15	1.37
$FeSO_4$	-0.1	-0.12	-4.82	-0.26
Inoculum size	0.4	0.34	-15.5	-0.69

Table VII. A verification experiment for mannan degradation by immobilized cells of *A. niger* prepared on basal versus optimized medium.

Response	Basal medium	Optimized medium
Protein content (mg/ml)	2.537	4.993
Mannanase activity (U/ml)	35.67	90.87

nutrients and interactions between two or more factors (Zhang *et al*., 1996; Ooijkaas *et al*., 1998; El-Helow & El-Ahawany, 1999; Ooijkaas *et al*., 2000). Furthermore, applying multifactorial experiments consider the interaction between the non-linear natures of the response in short experiments (Gresham & Inamine, 1986). Plackett-Burman experimental design was effective in the determination of the medium components which have a significant effect on the production of mannanases enzyme and protein content of the immobilized fungus. Yu *et al*. (1997) applied the Plackett-Burman experimental design to determine the relative importance of constituents within a complex medium on the growth and sporulation of *Colletotrichum coccodes*. The conclusion obtained from the present work was evaluated by a verification experiment in which a predicted optimum culture medium showed a 2.3 fold increase in mannanase activity when compared to the basal medium settings.

The results of the present investigation collectively indicate the possibility of using free or immobilized cells of *A. niger* for the production of a relatively highly active mannanase preparation, which can be applied in many fields such as food industry, paper and pulp industry.

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