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Statistical Optimization and Partial Characterization of Amylases Produced by Halotolerant *Penicillium Sp.*

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Abstract: The production of extracellular amylase by the halotolerant fungus *Penicillium* sp. was statistically optimized in a submerged cultivation. Maximum enzyme production was obtained after two days of incubation in a medium containing 10% (w/v) NaCl, in addition to $MgSO_4.7H_2O$ 1% and 1% (w/v) of starch. The optimum pH for growth and enzyme production was 4. Partial characterization of the crude enzyme revealed the presence of two enzymes with pH-optima at 9 and 11. Further more, the optimum temperature was 30°C at pH 11 and from 30°C to 90°C at pH value of 9. The two enzymes were stable in wide pH range with the maximum stability at pH 9 to 11 after 5 days and at temperature range of 40-60°C. Different starchy materials (barley grains, wheat grains, wheat bran, crushed wheat grains, birds feed grains, maize meal and wheat meal) were tested as substrates for amylase production in solid state fermentation (SSF). The highest enzyme production was obtained in presence of maize meal after 6 days of incubation at 30°C when enzyme assayed at pH 11.

Key words: *Penicillium* · Halotolerant · Amylases · Statistical design

INTRODUCTION

Amylases are a group of enzymes (α-amylase, β-amylase and glucoamylase) that have been found in several microorganisms like bacteria [1-3] and fungi [4-5]. Fungal sources are confined to terrestrial isolates, mostly to *Aspergillus* species and some species of *Penicillium* [6, 7].

Among all industrial enzymes, hydrolytic enzymes account for 85% of them. The market size was approximately US$ 1.6 billions in 2002 and has witnessed about 12% annual growth over the last decade. It is expected that the market will continue to grow fast and reach US$ 3 billions by 2008 [8]. The global market for starch-processing enzymes is around US$ 156 million and the cost of the enzymes used in the liquefaction process represented 24% of the total process cost [9]. Microbial amylases could be potentially useful in the pharmaceutical and fine-chemical industries if enzymes with suitable properties could be prepared. With the advent of new frontiers in biotechnology, the spectrum of amylase application has widened in many other fields, such as clinical, medicinal and analytical chemistry, textile to paper industries, food, brewing and distilling industries, as well as starch saccharification [7,9,10]. A search for highly active amylolytic enzymes with novel properties is necessary to improve biotechnological processes. Thermostable [11], hydrolyzed native starch [12], stable-to-high salt concentrations [13] and also alkaline amylases attract particular attention, because most of the known and widely used industrial fungal amylases are active in the acidic medium. It was believed that microbial communities at high salinities are dominated exclusively by archaea, bacteria and the eukaryotic species *Dunaliella salina*, studies on the microbial diversity in hypersaline environments revealed the presence of melanized fungi, 'considered as a new group of eukaryotic halophiles' [14] and several other fungi including *Penicillium* spp. [15, 16].

In this study, strain of *Penicillium* sp. was isolated from the soil and is capable to grow at high salt concentrations. Optimization of the culture conditions using statistical design was performed. Partial characterization of the crude amylase preparation was also investigated.

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MATERIALS AND METHODS

Microorganism and Culture Conditions: The fungus, *Penicillium* sp. was isolated from the soil (Faculty of Science, Alexandria University) on glucose-peptone medium containing 100 g/l of NaCl, purified on the same medium and stored at 4°C. For amylase production, liquid medium determined after the statistical optimization of component was used and had the following compositions (g L^{-1}) starch; 10, NaCl; 100, $MgSO_4.7H_2O$; 5, casein hydrolysate; 1, $CaCl_2.2H_2O$; KCl; 2, sodium citrate; 2 and the pH was adjusted to 4 before autoclaving. Erlenmeyer flask with 100 ml liquid medium was inoculated with 2 ml spore suspension and incubated at 30±2°C and 120 rpm for 2 to 5 days in the preliminary experiments and only for 2 days in all subsequent experiments.

Optimization of Culture Conditions Using Plackett-burman and D-optimal Statistical Approaches: Medium optimization for maximum amylase production was done in the basal liquid medium. The components were varied according to the statistical designs and the optimum components were incorporated in the basal medium for further experiments.

The preliminary investigations of factors affecting the production of amylolytic enzymes were done using the Plackett-Burman design. Plackett-Burman designs are useful for ruggedness testing where the aim to find little or no effect on the response due to any of the factors assuming no factors interaction is present. This fractional method allows the testing of multiple independent variables within a single experiment. In this study an array for $N = 12$ trials that will test $N-1$ independent variables [11] factors to be tested in the preliminary study as shown in Table 1). Each row represents one trial and each column represents a single variable (medium component). The -1 and +1 element represent the lower and upper levels of each variable present within each trial.

A second round of factor level selection was conducted using the D-optimal design. A D-optimal design minimizes the volume of the confidence ellipsoid for the coefficients and provides the most accurate estimates of the model coefficients. This design allows the study of the factors interactions thus determine the best combinations for better results. In this design four factors were studied in 25 runs as shown in Table 2. After carrying out the different experiments, response, the enzyme activities are measured. The effect of each variable on the measured response is determined by the Stat-Ease Package (Stat-Ease, Minneapolis, USA).

Table 1: Screening for factors affecting the production of amylolytic enzymes by the *Penicillium* sp. using the Plackett-Burman design

Run No.	A	B	C	D	E	F	G	H	I	J	K	U/ml
1	-1	1	1	-1	1	-1	-1	-1	1	1	1	2.1
2	1	1	-1	1	1	-1	1	-1	-1	-1	1	4.8
3	-1	-1	1	1	1	-1	1	1	-1	1	-1	2.0
4	1	1	1	-1	1	1	-1	1	-1	-1	-1	3.5
5	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	3.4
6	1	-1	1	-1	-1	-1	1	1	1	-1	1	4.2
7	1	1	-1	1	-1	-1	-1	1	1	1	-1	1.7
8	1	-1	1	1	-1	1	-1	-1	-1	1	1	3.7
9	-1	1	1	1	-1	1	1	-1	1	-1	-1	4.0
10	1	-1	-1	-1	1	1	1	-1	1	1	-1	5.0
11	-1	1	-1	-1	-1	1	1	1	-1	1	1	2.0
12	-1	-1	-1	1	1	1	-1	1	1	-1	1	2.6

-1 and 1 indicate the lower and upper levels of each factor. Experiments were carried out in duplicate and the mean of activities is shown. Factors are listed in alphabetic order and their levels are given in (g/l), A; Starch (10-20), B; NaCl (75-100), C; $MgSO_4.7H_2O$ (0-10), D; Casein (2-6), E; KCl (2-5), F; Citrate (1-3), G; KNO_3 (0-1), H; $CaCl_2.2H_2O$ (0-0.2), I; Inoculum (2-4, % v/v), J; pH (4-8) and K; Volume (75-100)

Table 2: D-optimal model for the optimization of amylolytic activity of the halotolerant *Penicillium* sp.

Run	NaCl (g/l)	Casein (g/l)	$MgSO_4$ (g/l)	pH	Activity (U/ml)
1	137.5	6.0	10.0	5.5	7.5
2	137.5	3.5	5.0	4.0	7.7
3	100.0	6.0	5.0	5.5	5.4
4	137.5	6.0	5.0	2.5	6.2
5	118.8	4.8	7.5	3.3	5.4
6	137.5	1.0	7.5	4.0	8.7
7	175.0	3.5	7.5	4.0	5.0
8	100.0	1.0	5.0	2.5	6.2
9	175.0	6.0	7.5	5.5	4.3
10	100.0	6.0	10.0	2.5	6.9
11	100.0	1.0	10.0	5.5	7.6
12	175.0	1.0	10.0	5.5	5.4
13	175.0	6.0	5.0	4.0	7.8
14	175.0	1.0	10.0	5.5	5.0
15	100.0	6.0	5.0	5.5	5.2
16	100.0	1.0	10.0	5.5	8.6
17	175.0	3.5	5.0	5.5	8.0
18	175.0	1.0	5.0	2.5	7.1
19	137.5	1.0	10.0	2.5	5.9
20	137.5	3.5	10.0	4.0	5.8
21	175.0	6.0	10.0	2.5	4.8
22	137.5	3.5	7.5	5.5	6.0
23	100.0	1.0	5.0	2.5	6.2
24	137.5	1.0	5.0	5.5	4.2
25	175.0	1.0	5.0	2.5	5.0

Experiments were carried out in duplicate and the mean of activities is shown.

Contrast coefficients allow the determination of the effect of each constituent. A large contrast coefficient either positive or negative indicates that a factor has a large impact on activity, while a coefficient close to zero means that a factor has little or no effect. The *p*-value is the probability that the magnitude of a contrast coefficient is due to random process variability. A low *p*-value indicates a "real" or significant effect [17].

Amylase Production in Solid State Fermentation (SSF): For production of enzyme in SSF, the fungus was grown at 30±2°C in 250 ml Erlenmeyer flasks containing 15 g of the starchy substrates (barley and wheat grains; crushed wheat grains; wheat bran; local bird's feed, maize and wheat meal) enriched with the optimized basal medium without starch. The moisture content was adjusted to 70-80%. At the end of the incubation period, 50 ml of distilled sterile water were added to the cultures; the mixtures were shaken for 1 h at room temperature and centrifuged. The supernatants were assayed for amylolytic activity. Results were expressed as the mean of at least three independent measurements.

Amylase Assay: The amylase enzyme was assayed according to the method described by Miller [18]. The reaction mixture contained 200 µl soluble starch in phosphate buffer [0.5 M, pH 7.5), 200 µl of diluted enzyme and 300 µl phosphate buffer. The reaction was incubated for 15 min. at 30°C, 300 µl Dinitrosalicylic acid (DNS) solution were added and boiled for 15 min. Before cooling 100 µl Rochelle salt (40% sodium potassium tartarate) was added and the color was measured at 575 nm. One unit of amylase activity was defined as the amount of enzyme that releases 1 mg of reducing sugar as glucose per ml per min under the assay conditions.

Effect of pH and Temperature on Activity and Stability of the Crude Enzyme: The effect of pH on the activity of the crude amylases was determined by conducting the reaction at different pH values (0.5 M citrate-phosphate buffer, pH 3-7; Tris-HCl buffer, pH 7-8.5 and Glycin-HCl buffer, pH 9-11). The activity was measured using the standard assay conditions. The optimal temperature for enzyme activity was determined by incubating the reaction mixture at temperature (4-90°C) and pH 9 and 11.

pH Stability and Thermal Stability of the Crude Enzyme: The pH stability was performed by incubating the crude enzyme in different pH (3-11) at 30°C for 5 days and then the residual activity was measured using the standard assay conditions. For thermal stability study, the enzyme was incubated at various temperatures for 30, 60 and 90 min and pH 9 and 11, the residual activity was then assayed at pH 9 and 11.

RESULTS AND DISCUSSION

While there are several reports about the control of extracellular amylase production by fungi [7], optimization of cultivation conditions is expected to improve the enzyme production. In this study, the optimization of amylase production by a halotolerant strain of *Penicillium* sp. using two different statistical designs was investigated.

Major approaches used for screening and media optimization have reported by Parekh *et al*. [19]. However, one of the basic approaches used to design experiments to screen media components based on empirical processes. Statistical techniques for experimental design provide a more accurate and elegant means of designing the best medium [20]. The most widely used statistical experimental designs are Plackett-Burman design and fractional factorial design [21]. Advantages of the designs include simplicity and assessment of a large number of factors on the relative efficiency of the production process.

The Plackett-Burman design does not yield estimates of the extent or type of interaction between variables [22]. As shown in table 1, 11 factors were investigated to determine the optimum medium components suitable for amylolytic enzymes production by the halotolerant *Penicillium* sp. The statistical analysis of the activities obtained from the 12 experiments revealed that starch, KNO_3, $CaCl_2$ and pH are the significant factors influencing the production of amylolytic enzymes (Table 3). Concentrations of starch, $MgSO_4$, KCl, inoculum and KNO_3 were shown to have negative impact

Table 3: Statistical parameters for selected the linear polynomial model using Plackett-Burman design

Source	*p*-value	Coefficient estimates	Sum of squares
Model	0.06	3.4	14.6
A-Starch*	0.03	0.55	3.6
B-NaCl	0.06	-0.25	0.8
C-$MgSO_4$	0.95	0.002	$4.9E^{-5}$
D-Casein	0.15	-0.11	0.2
E-KCl	0.23	0.073	0.1
F-Citrate	0.08	0.22	0.6
G-KNO_3*	0.04	0.42	2.1
H-$CaCl_2$*	0.03	-0.59	4.2
I-Inoculum	0.55	0.024	$6.8E^{-3}$
J-pH*	0.03	-0.51	3.2
K-Volume	0.96	-0.028	$9.2\ E^{-3}$

*Factors with $p<0.05$ are significant.

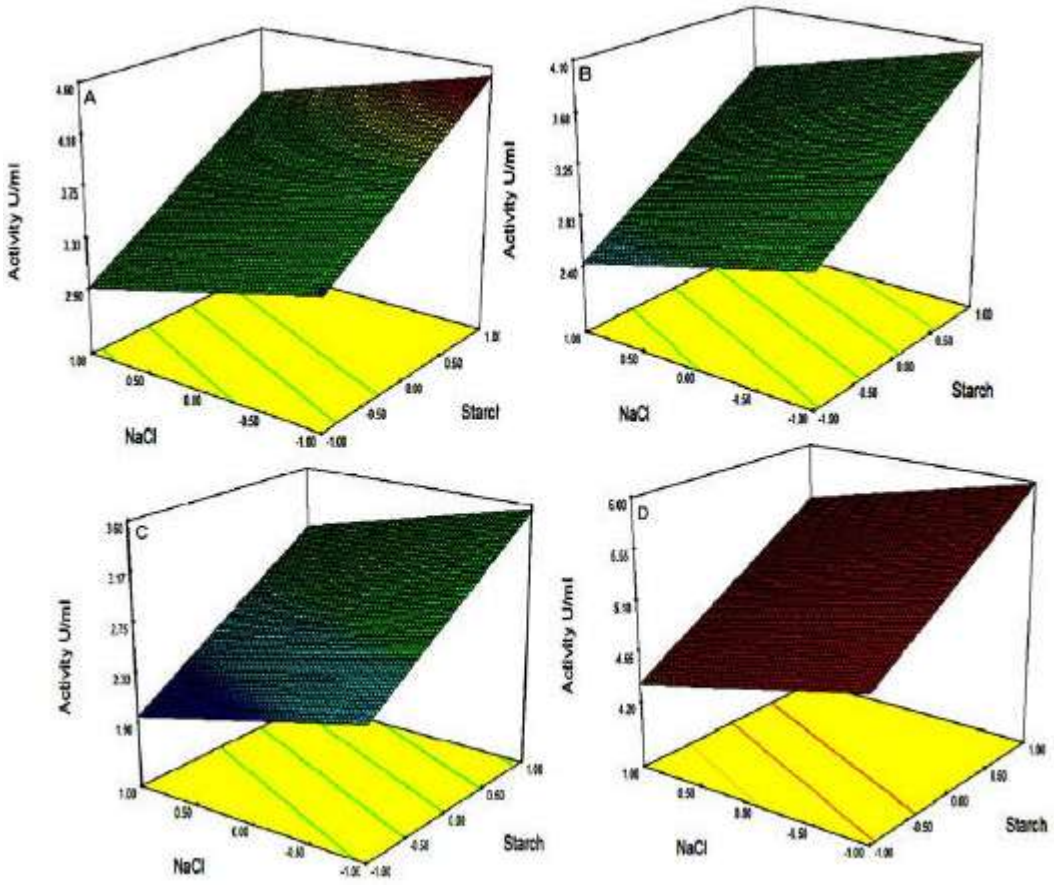

Fig. 1: Screening for factors affecting amylolytic enzymes production by the halotolerant *Penicillium* sp. by the Plackett-Burman design. A; activity determined at the lower level of factors, B; activity determined at the middle level of factors, C; activity determined at the upper level of factors and D; factors were varied to obtain maximum activity ($MgSO_4$; -1, casein; -1, KCl; 1, citrate; 1, KNO_3; 1, $CaCl_2$; -1; inoculum; 1, pH; -1 and volume; 1).

on production of enzymes. The regression analysis of the linear model was 0.999 and was in reasonable agreement with the adjusted R-Squared value of 0.993 indicating the consistence of the predicted activities to the actual activities measured in these experiments.

Figures 1 (a-d) illustrates the effect of different levels of the different factors. At the lower level of all factors, but increasing starch combined with reduced concentration of NaCl resulted in a maximum activity of 4.6 U/ml, middle and higher levels of factors, activities of 4.3 and 3.6 U/ml were obtained, respectively. Mathematical adjustment of different factors (Fig. 1d) resulted in a predicted activity of 6 U/ml. The results obtained from Plackett-Burman design were then used to study the effect of higher concentrations of NaCl, casein and $MgSO_4$ beside pH to reveal the factors interaction using the D-optimal design as shown in Table 2.

The 25 runs were carried out and the amylolytic activities were determined as mentioned in the materials and methods section. The model statistics (data not shown) illustrated that all variables and their interaction are not significant except the interaction between NaCl and $MgSO_4$ was shown to have a significant p value of (0.01) and has negative effect on the production of amylolytic enzymes. In that case, concentration of casein, NaCl and $MgSO_4$ showed negative effect on amylolytic activity of *Penicillium* sp. however, only pH showed a positive effect when shifted into the slightly acidic medium.

The model obtained with the D-optimal design was reduced and showed that interaction of sodium chloride and magnesium sulfate is the only

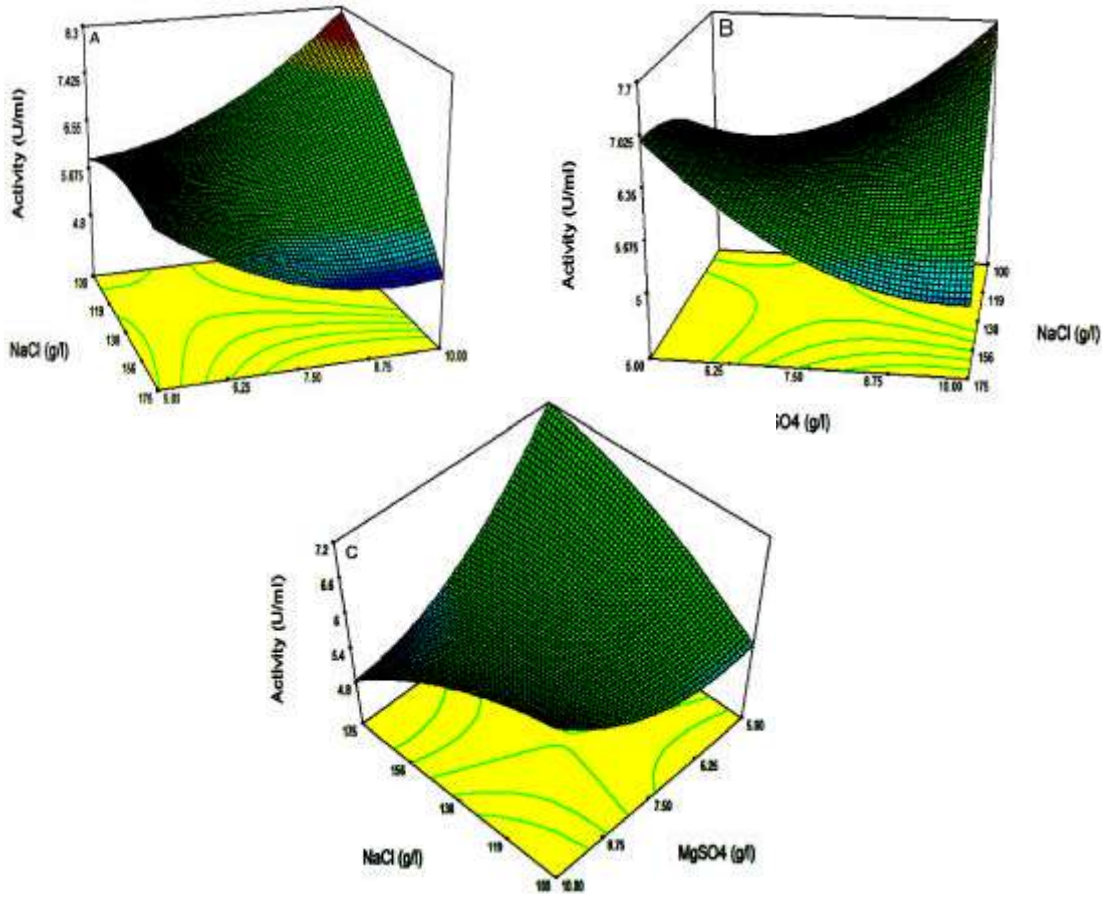

Fig. 2: Optimization of factors affecting amylolytic activity produced by the halotolerant *Penicillium* sp. using the D-optimal design. Activity calculated at varying concentrations of NaCl and $MgSO_4$. A; at pH 2.5 and casein 1 (g/l), B; pH 4 and casein 3.6 (g/l) and C; pH 5.5 and casein 6(g/l)

significant variable which indicated by the curvature in figure 2a-c. The maximum activity obtained in this experiment was 8.7 U/ml which is about twice the activity in the Plackett-Burman experiment.

The necessary modifications of the medium such as reduction of NaCl and $MgSO_4$ concentrations and using pH value of 4 were used in the following experiments. Casein on the other hand was shown to decrease the production of amylolytic enzymes (Figures 2a-c) at variable concentration of NaCl and the maximum theoretical activity was obtained at 1 g/l casein.

Information on the kinetics, pH and thermal stability of an enzyme is mandatory in determining its applicability in biotechnological industries [23]. In this work, the physico-chemical properties of the crude enzyme preparation were investigated.

The results presented in Fig. 3 shows the presence of two pH optima (9.0 and 11.0), this might be due to the presence of two different amylases in the crude extract. The highest activity was recorded at pH 9.0 and then dramatically decreased at pH 9.5. The second peak started at pH 10.0 with the maximum at pH 11.0 and then decreased. Amylases produced by most fungi and bacteria, generally have pH optima within the range 4.0-5.0 [24-26].

However, a few alkalophilic α-amylase-producing strains have also been reported by Fogarty and Kelly [24] and Yamamoto *et al.* [27]. The amylase of Bacillus sp. TS-23 was optimally active at pH 9.0 [28]. The optimum pH for pure α-amylase from the fungus *Thermomyces lanuginosus* was found to be 5.6 and for α-amylase isolated from *Penicillium chrysogenum* was 5.0 [29, 30]. α-amylase produced by *Tricholoma matsutake* was most active at pH 5.0-6.0 [31].

The amylase activity measured at pH 9.0 show better pH stability in the acidic to neutral pH rang than the

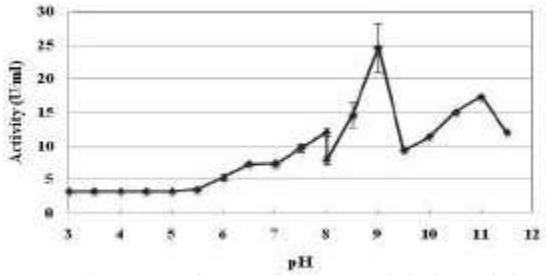

Fig. 3: Optimum pH for maximum amylolytic activity of the crude filtrate of the halotolerant *Penicillium* sp. activity determined at 30°C

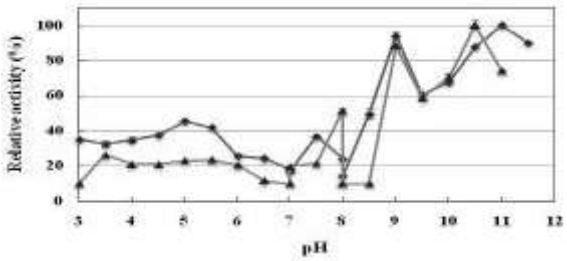

Fig. 4: The pH stability of the amylolytic activity of the crude filtrate of the halotolerant *Penicillium* sp. at different pH values after 5 days incubation at 30°C. Activity was assayed at 30°C and pH values of 9 (•) and 11(▲).

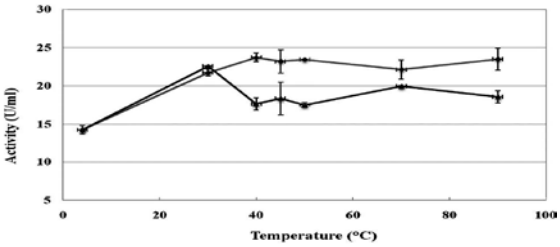

Fig. 5: Optimum temperature for maximum amylolytic activity of the crude filtrate of the halotolerant *Penicillium* sp. activity determined at pH 9 (•) and pH 11(▲)

activity measured at pH 11.0 after 5 days incubation at 30°C (Fig. 4). Both enzymes showed maximum pH stability in the alkaline pH range and retained about 100% of the residual activity at pH 9.0 and 11.0. These results were differ from that obtained with Uma Maheswar Rao and Satyanarayana [32] who reported that approximately 50% of the residual activity was recorded after 7.5 h at pH 7.0. On the other hand, α-amylase from *Tricholoma matsutake* was stable in pH range from 4.0-10.0 [31].

The effect of temperature on the activity of crude amylases was tested at different temperatures and pH 9.0 and 11.0 (Fig. 5). The results revealed that the optimum temperature for the activity measured at pH 11.0 was 30°C;

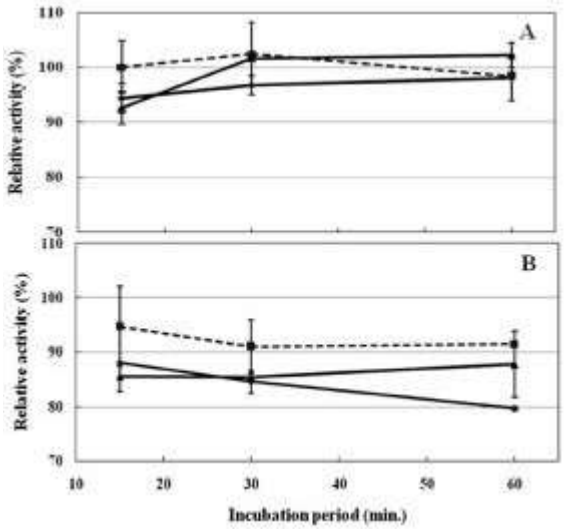

Fig. 6: Heat stability of the amylolytic activity of the crude filtrate of the halotolerant *Penicillium* sp. Stability were tested at different temperatures, 40°C (-♦-), 50°C (-■--) and 60°C (-▲-). Activities were then assayed at 30°C and pH values of 9 (panel A) and 11 (panel B).

increasing the temperature decreased the activity with fluctuations. While the activity measured at pH 9.0 revealed that maximum activity was obtained at 40°C and remains stable over a wide range of temperature up to 90°C. These results may be due to the presence of more than amylase in the crude extract.

Our results are in agreement with that obtained in previous work [33], where *Aspergillus flavus* produced diverse amyloltic complexes and each complex contains one or two amylase with different pH and temperature optima. A raw-starch digesting amylase with optimum temperature at 40°C was also reported by Okolo *et al.* [34], which in partial agreement with our results. The optimum temperature for the α-amylase produced by *P. chrysogenum* was 30-40°C [30]. Moreover, the optimum temperature for amylase enzyme was 35°C was reported by El-Safey and Ammar [35].

Because industrial starch liquefaction is mediated at high temperatures, it was of interest to study the heat stability of the crude enzyme preparation. The stability of the crude extract was determined at pH 9.0 and 11.0. The results demonstrated in Fig. 6 show that there is no loss in the activity measured at pH 9.0 after incubation without substrate at 60°C for 60 min, while the activity at pH 11 lost about 15% of its original activity after the same time and temperature. Generally the activity measured at

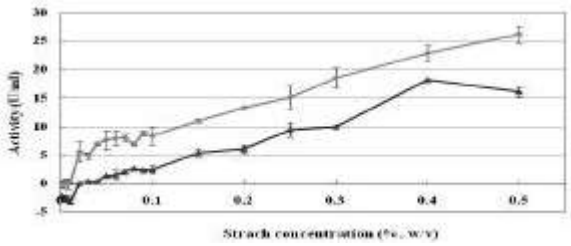

Fig. 7: Effect of starch concentration on the amylolytic activity of the crude filtrate of the halotolerant *Penicillium* sp. Activity were assayed at 30°C and pH values of 9 (▲) and 11(♦).

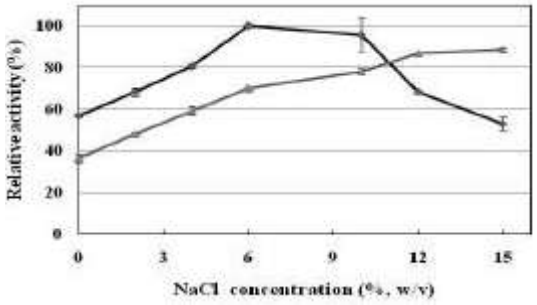

Fig. 8: Effect of NaCl concentration on the relative activity of amylolytic enzymes produced by the halotolerant *Penicillium* sp. activity were assayed at 30°C and pH values of 9 (▲) and 11(♦).

pH 9.0 was more thermostable than that measured at pH 11.0. These results were better than that obtained by other authors [30] whom reported that α-amylase from *P. chrysogenum* was stable at 30°C for 20 min. and lost about 8% at 40°C after the same time and than that obtained [33], which lost about 30% of its original activity after 30 min at 60. The amylase from *A. carbonarius* retained over 85% of initial activity between 30 and 80 for 20 min. [34]. Moreover, a thermostable amylase from the thermophilic fungus *Scytalidium thermophilum* was stable for 1 h at 50°C and it decayed with a half-life of approximately 25 min at 55°C and 12 min at 60°C [36].

The effect of starch concentration on the activity of the crude enzyme preparation was tested at pH 9.0 and 11.0. The results show that increasing the starch concentration increased the activity and the activity at pH 11.0 was better than that obtained with pH 9.0 (Fig. 7). Maximum activity at pH 9 was observed at 0.4% (w/v) starch and decreased after that, while the activity at pH 11 increased up to 0.5% starch [35].

The stability of amylases in presence of high salt concentration (NaCl) may be useful for the processing of starches under high-salt conditions. The effect of different salt concentration (2-15%) in the reaction mixtures was tested at pH 9.0 and 11.0. The results presented in figure 8 revealed that increasing NaCl concentration increased the enzyme activity at the two tested pH. It was observed that the activity measured at pH 9.0 retained 100% of the original activity in presence of 6% (ca 1mol/l) NaCl and remain stable up to 10% NaCl, then lost about 50% at 15% NaCl. On the other hand, at pH 11.0, about 10% of the activity was lost in the presence of 15% NaCl. A salt-tolerant extracellular α-amylase from *Bacillus dipsosauri* retained its full activity in presence of 1mol/l NaCl [37]. Although an α-amylase from *Halomonas meridiana* was active in 15% NaCl, it was inactivated at temperatures above 37°C [38]. Moreover, α--amylase from the salt-tolerant archaeon *Halobacterium halobium* was progressively inactivated by increasing concentrations of NaCl [39].

Solid state cultivation systems (SSF) and submerged liquid cultivation systems have been used for amylase production, although most research has used liquid culture, which allows greater control of culture conditions such as temperature and pH. However, solid state fermentation is gaining interest in recent years due to potential advantages in manufacturing products such as enzymes in high yield, at high concentrations and with high specificity [40]. In this study, the production of amylase in solid state fermentation using the optimized medium from the submerged fermentation was investigated. Different starchy substrates (described under materials and methods) were used. The substrates were hydrated with the optimized mineral medium without starch to give 70-80% moisture content. After 2 and 6 days incubation at 30°C, the enzyme was extracted and measured at pH 9.0 and 11.0. The results obtained demonstrated that the solid state fermentation was better than the submerged fermentation (Fig. 9). In this study, the maximum activity obtained in submerged fermentation at 30°C and pH 11.0 was about 23 U/ml, while 137U/g was obtained in solid state fermentation with maize meal as substrate under the same assay conditions. It was also observed that good activities were obtained with birds' feed and barley grains. The lowest activity was observed in presence of wheat meal as substrate. Different solid substrates were found to affect the production of enzymes [41]. Amylase production by the thermophilic fungus *T. lanuginosus* using solid state was reported by Kunamneni *et al.* [11], who obtained the maximum activity in presence of wheat bran with initial moisture content 90% at pH 6.0. On the other hand, maximum amylase

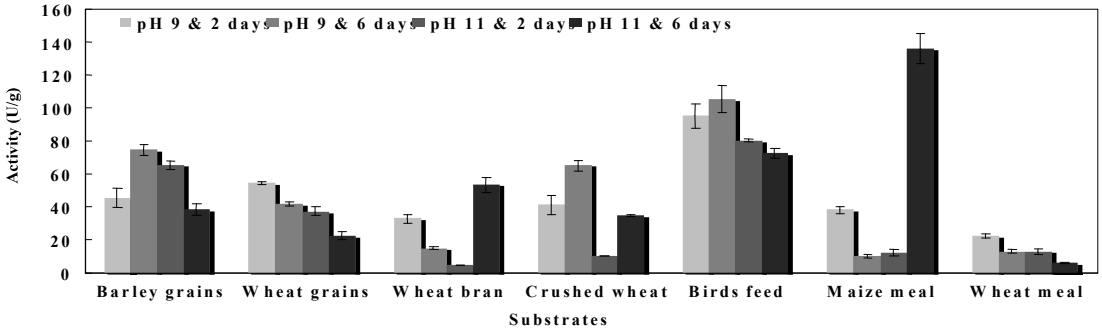

Fig. 9: Amylase production in solid state fermentation (SSF) by the halotolerant *Penicillium* sp. The specified substrates adjusted to 70-80% moisture, incubated at 30°C for 2 or 6 days and the amylolytic activity was determined

activity was obtained with initial moisture content 67% and spent brewing grain as substrate [42]. Using mixture of wheat bran; rice bran and nut oil cake gave the maximum amylase activity [43].

CONCLUSION

In conclusion, this study has shown that the amylolytic enzymes produced by the isolated halotolerant *Penicillium* sp. may have practical applications in the starch industry on account of the stability at alkaline pH, high salt concentration and also high temperature. Further studies to purify and characterize the amylase complexes produced by this strain will be investigated.

REFERENCES

1. Haseltine, C., M. Rolfsmeier and P. Blum, 1996. The glucose effect and regulation of a-amylase synthesis in the hyperthermophilic archaeon *Sulfolobus solfataricus*. J. Bacteriol., 178: 945-950.
2. Leam, F. and B. A. Gashe, 1994. Amylase production by a gram-positive bacterium isolated from fermenting tef (*Eragrostis tef*). J. Appl. Bacteriol., 77: 348-352.
3. Young, M.H., L.D. Gun, Y.J. Hoon, P.Y. Ha and K.Y. Jae, 2001. Rapid and simple purification of a novel extracellular beta-amylase from *Bacillus* sp. Biotechnol. Letters. 23: 1435-1438.
4. Fadel, M., 2000. Production of thermostable amylolytic enzymes by *Aspergillus niger* F-909 under solid state fermentation. Egyptian J. Microbiol., 35: 487-505.
5. Wang, B.D., D.C. Chen and T.T. Kuo, 2001. Characterization of a *Saccharomyces cerevisiae* mutant with oversecretion phenotype. Appl. Microbiol. Biotechnol., 55: 712-720.
6. Haska, R. and Y. Ohta, 1994. Starch/Starke, 46: 480-485.
7. Pandey, A., P. Nigam, C.R. Soccol, V.T. Soccol, D. Singh and R. Mohan, 2000. Advances in microbial amylases. Biotechnol. Appl. Biochem., 31: 135-152.
8. Pandey, A. and S. Ramachandran, 2005. In A. Pandey, C. Webb, C. R. Soccol, & C. Larroche (Eds.), Enzyme Technology, New Delhi: Asiatech Publishers Inc. pp: 1-10.
9. Crabb, W.D. and C. Mitchinson, 1997. Enzymes involved in the processing of starch to sugars. Trends. Biotechnol., 15: 349-352.
10. Lin, L.L., W.H. Hsu and W.S. Chu, 1997. A gene encoding for α -amylase from thermophilic *Bacillus* sp., strain TS-23 and its expression in *Escherichia coli*. J. Appl. Microbiol., 82: 325-334.
11. Kunamneni, A., K. Permaul and S. Singh, 2005. Amylase production in solid state fermentation by the thermophilic fungus *Thermomyces lanuginosus*. J. Bioscience Bioeng., 100: 168-171.
12. Wijbenga, D.J., G. Beldman, A. Veen and D.J. Binnema, 1991. Production of native-starch-degrading enzymes by a *Bacillus firmus/lentus* strain. Appl. Microbiol. Biotechnol., 35: 180-184.
13. Chessa, J.P., G. Feller and C. Gerday, 1999. Purification and characterization of the heat-labile a-amylase secreted by the psychrophilic bacterium TAC 240B.Can. J. Microbiol., 45: 452-457.
14. Butinar, L., S. Sonjak, P. Zalar, A. Plemenitas and N. Gunde-Cimerman, 2005. Melanized halophilic fungi are eukaryotic members of microbial communities in hypersaline waters of solar salterns. Bot. Mar., 48: 73-79.
15. Marbaniang, T. and S. Nazareth, 2007 Isolation of halotolerant Penicillium species from mangroves and salterns and their resistance to heavy metals. Current Science, 92(7): 895-897.

16. Zalarl, P., M.A. Kocuvan, A. Plemenitas and N. Gunde-Cimerman, 2005. Halophilic black yeasts colonize wood immersed in hypersaline water. Bot. Mar., 48: 323-326.
17. Plackett, R.L. and J.P. Burman, 1946. The design of optimum multifactorial experiments. Biometrika, 33: 305-325.
18. Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem., 31: 426-429.
19. Parekh, S., V.A. Vinci and R.J. Strobel, 2000. Improvement of microbial strains and fermentation processes. Appl. Microbiol. Biotechnol., 54: 287-301.
20. Strobel, R.J. and G.R. Sullivan, 1999. Experimental design for improvement of fermentations. In: Demain A.L. and J.E. Davies (Eds.). Manual of industrial microbiology and biotechnology. Am. Soc. Microbiol. Washington, pp: 80-93.
21. Broedel, S.E., S.M. Papciak and W.R. Jones, 2001. The selection of optimum media formulations for improved expression of recombinant proteins in *E. coli*. Technical Bull., 2: 1-6.
22. Motola, S. and S.N. Agharkar, 1992. Preformulation research or parental medications. In: Avis, K.E., H.A. Liberman and M. Lachman (Eds.). Pharmaceutical dosage forms: Parental medications. Marcell Decker, New York, pp: 115-172.
23. Kumar, S. and T. Satyanarayana, 2003. Purification and kinetics of raw starch hydrolysing thermostable and neutral glucoamylase of thermophilic mold *Thermomucor indicae-seudaticae.* Biotechnol. Progress, 19: 936-944.
24. Fogarty, W.M. and C.T. Kelly, 1979. Starch degrading enzymes of microbial origin. In: Bull AH (ed). Progress in Industrial Microbiology, Elsevier, Amsterdam, 15: 87-150.
25. Fogarty, W.M. and C.T. Kelly, 1990. Amylases, amyloglucosidases and related glucanases. In: Microbial Enzymes and Bioconversions (Fogarty, W.M. and Kelly, C.T., (Ed.) Elsevier, London, pp: 71-132.
26. Silva, J.G., H.J. Nascimento, V.F. Soares, E.P.S. Bon and C.E. Wyman, 1997. Glucoamylase iso-enzymes tailoring through medium composition. Appl. Biochem. Biotechnol., 87-96.
27. Yamamoto, M., Y. Tanaka and K. Horikoshi, 1972. Alkaline Amylases of Alkalophilic Bacteria. Agric. Biol. Chem., 36: 1819-1823.
28. Lin, L.L., C.C. Chyau and W.H. Hsu, 1998. Production and properties of a raw-starch-degrading amylase from thermophilic and alkaliphilic Bacillus sp. TS-23. Biotechnol. Appl. Biochem., 28: 61-68.
29. Mishra, R. and R. Maheshwari, 1996. Amylases of the thermophilic fungus *Thermomyces lanuginosus*: Their purification, properties, action on starch and response to heat. J. Biosci., 21(5): 653-672.
30. Balkan, B. and F. Ertan, 2005. Production and properties of α-amylase from *Penicillium chrysogenum* and its application in starch hydrolysis. Prep. Biochem. Biotechnol., 35: 169-178.
31. Kusuda, M., M. Nagai, T.C. Hur, M. Ueda and T. Terashita, 2003. Purification and some properties of α-amylase from an ectomycorrhizal fungus, *Tricholoma matsutake.* Mycoscience, 44: 311-317.
32. Uma Maheswar Rao, J. L. and T. Satyanarayana, 2007. Purification and Characterization of a Hyperthermostable and High Maltogenic α-Amylase of an Extreme Thermophile *Geobacillus thermoleovorans*. Appl. Biochem. Biotechnol., 142: 179-193.
33. Frolova, G.M., A.S. Sil'chenko, M.V. Pivkin and V.V. Mikhailov, 2002. Amylases of the fungus *Aspergillus flavipes* associated with *Fucus evanescens*. Appl. Biochem. Microbiol., 38: 134-138.
34. Okolo, B.N., F.S. Ire, L.I. Ezeogu, C.U. Anyanwu and F.J.C. Odibo, 2000. Purification and some properties of a novel raw starch-digesting amylase from *Aspergillus carbonarius.* J. Sci. Food Agric., 81: 329-336.
35. El-Safey, E.M. and M.S. Ammar, 2004. Purification and characteraization of -A-amylase isolated from *Aspergillus falvus var. columnaris*. Ass. Univ. Bull., 7: 93-100.
36. Aquino, A.C.M.M., J.A. Jorge, H.F. Terenzi and M.L.T.M. Polizeli, 2003. Studies on a thermostable α-amylase from the thermophilic fungus *Scytalidium thermophilum*. Appl. Microbiol. Biotechnol., 61: 323-328.
37. Deutch, C.E., 2002. Characterization of a salt-tolerant extracellular α-amylase from *Bacillus dipsosauri*. Lett. Appl. Microbiol., 35: 78-84.
38. Coronado, M.J., C. Vargas, J. Hofemeister, A. Ventosa and J.J. Nieto, 2000 Production and biochemical characterization of an a-amylase from the moderate halophile Halomonas meridiana. FEMS Microbiol. Lett., 183: 67-71.

39. Good, W.A. and P.A. Hartman, 1970. Properties of the amylase from *Halobacterium halobium*. J. Bacteriol., 104: 601-603.
40. Pandey, A., P. Selvakumar, R.C. Soccol and P. Nigma, 1999. Solid state fermentation for the production of industrial enzymes. Curr. Sci., Bangalore, 77: 149-161.
41. Satyanarayana, T., 1994. Production of bacterial extracellular enzymes by solid state fermentation. In: Pandey, A. (Ed.), Solid state fermentation, Wiley Eastern Limited, New Delhi, pp: 122-129.
42. Bogar, B., G. Szakacs, R.P. Tengerdy, J.C. Linden and A. Pandey, 2002. production of α-Amylase with *Aspergillus oryzae* on spent brewing grain by solid substrate fermentation. Appl. Biochem. Biotechnol., 453: 102-103.
43. Alva, S., J. Anupama, J. Savla, Y.Y. Chiu, P. Vyshali, M. Shruti, B.S. Yogeetha, D. Bhavya, J. Purvi, K. Ruchi, B.S. Kumudini and K.N. Varalakshmi, 2007. Production and characterization of fungal amylase enzyme isolated from *Aspergillus* sp. JGI 12 in solid state culture. African J. Biotechnol., 6: 576-581.