Pak. J. Bot., 37(1): 155-161, 2005.

A SURVEY OF AMYLOLYTIC BACTERIA AND FUNGI FROM NATIVE ENVIRONMENTAL SAMPLES

MUHAMMAD SOHAIL[1], AQEEL AHMAD[1], SALEEM SHAHZAD[2] AND SHAKEEL AHMED KHAN[1*]

[1]Department of Microbiology, University of Karachi, Karachi-75270, Pakistan
[2]Department of Botany, University of Karachi, Karachi-75270, Pakistan

Abstract

Starches are one of the most important naturally occurring glucose polymers that are present in plants. They are used as the starting material for the production of a variety of different types of compounds of commercial importance. The usual process is the treatment of starch at an elevated temperature to gelatinize followed by liquefaction by chemicals or by enzymes. Enzymatic liquefaction has an edge over chemical treatment process. Microbes are, in general, the source of commercial enzymes. Present study was initiated to screen the microbial population for their ability to possess amylolytic potential where 117 strains of bacteria and 130 fungal strains were isolated and screened. A considerable number of both were found to produce amylase. *Bacillus* sp., and *Aspergillus* sp., were the most active amylase producers. Bacterial enzymes showed maximum activity at slightly elevated temperature and at alkaline pH while one of the fungal enzymes retained most of its activity even at a temperature of 80ºC, however, level of activity was reduced when reaction was carried out at alkaline pH. Genetic studies revealed that starch is a suitable inducer in case of fungi, while maltose seems to be a better inducer than that of starch in case of bacteria.

Introduction

Starch, a homopolysaccharide is an important and an abundant food reserve and energy source in plants. It is found in seeds, fruits, leaves, bulbs and tubers (Baum, 1995; Fogarty & Kelly, 1990). Structurally it is a composite polymer of amylose and amylopectin which vary in proportion depending on its source (Baum, 1995). In amylose around 10^3 glucose residues are linked through α-1, 4 bonds (Mayes, 1996). Amylopectin, a highly branched polymer contains 10^4–10^5 glucose residues which comprises of α-1, 4-linked glucose chains (20–25 residues long) with α-1, 6 linkages present at branch points (Baum, 1995; Morrison, 1990). Natural Starches are processed to yield different end products which find many industrial applications. Traditionally, processing is carried out by acid treatment and heating. However, enzymes ("starch-hydrolases" or simply "Amylases") have replaced chemicals due to an edge over chemicals (Fogarty & Kelly, 1990).

Amylases are widely used in various fields, from analysis (glycogen determination), industries (Brewing, baking, laundry, textiles etc) to clinical and pharmaceutical applications (diagnostic aids). Soil and other natural microorganisms are a good source of amylases. In the present study, soil microbial population was surveyed for their starch degrading ability. An attempt was made to screen indigenous microbial flora for their amylolytic potential. Since, industrial processes are carried out at high temperature and alkaline pH, so efforts were also made to isolate thermophiles and/or thermotolerant microbes along side the mesophiles, which produce amylolytic enzymes that work under alkaline conditions.

[*]All correspondences.

Materials and Methods

Isolation of starch hydrolase producers

Samples were collected from soil, infected barks and leaves. Isolation of mesophilic microbes was carried out by inoculating the samples on to Sabouraud's Dextrose Agar (Merck) supplemented with soluble starch (Sigma), Soil extract agar (prepared by autoclaving 10% suspension of garden soil for 30 minutes, left at room temperature for 24 hours followed by filtration; the filtrate was considered as soil extract medium), Peptone-Starch agar (containing 1 % peptone and 0.5% starch) and nutrient agar (Merck); and incubating plates either at 37°C (for bacteria) and at room temperature (for fungi) for a varying time period.

Thermodurics were isolated by heating the suspensions at 60°C for 15 minutes followed by the procedure mentioned above. Thermophilic-amylolytic microbes were isolated by incubating the samples in peptone-starch medium for 14 days at 60°C followed by sub-culturing at regular interval.

Screening and enzyme assay

Screening of isolates for amylolytic activity was carried out by growing the organisms on a nutrient medium plate containing 0.5% starch and subsequently staining it with iodine vapor. Presence of a halo around the colony was indicative of amylolytic enzyme producing strain.

Enzyme assay was carried out by growing the cultures in a broth medium and obtaining the cell-free culture supernatant after centrifugation for 5 min., at 3000 rpm. Amylase activity was determined by a modified method of Fuwa (1954). Briefly, 60 µL of the crude enzyme preparation was diluted in a 200 µL reaction mixture containing 40 µL of 0.5M Sodium acetate buffer (pH 5.9) and 0.5% starch solution. The reaction was allowed to take place for 30 min., at 40°C. After incubation, reaction was stopped by adding 200 µL of 1M acetic acid. The volume was made up to 10 ml by adding 9.4 ml of distilled water. Readings were recorded at $O.D_{660}$ after adding an aliquot of 200 µL of iodine solution (1:1:3 ratio of 1% (w/v) mixture of ethanolic solution of iodine, 10% aqueous potassium iodide and distilled water). One unit of enzyme activity was defined as the quantity of enzyme able to hydrolyze 0.1 mg starch under the standard assay conditions.

Induction and/or repression of amylase production

The isolates were grown in the presence of either 1% glucose, 0.5% maltose or 0.5 % starch, as a sole source of carbon in an Erlenmeyer flask containing minimal medium (0.7% K_2HPO_4, 0.3% KH_2PO_4, 0.1% Na_2SO_4, 0.1% $(NH_4)_2SO_4$, 01% $MgSO_4$ and 0.05% Na citrate) and incubated for 4 days at 30ºC (for fungi) and for 24 hours at 37ºC (for bacteria). Cells were harvested by centrifugation and washed twice with normal saline and finally with distilled water. Washed cells from glucose containing medium were transferred to the minimal medium containing 0.5% starch or 0.5% maltose and the ones grown in starch or maltose were transferred to minimal medium containing 1% glucose as a sole carbon source. The cell-free culture supernatant collected from each of the medium was used to determine the amount of enzyme liberated by the organisms.

Result

Isolation, identification and screening

A total of 40 out of 117 bacterial species/strains (34.2%) possessed amylolytic activity (Table 1) while 21 out of 130 fungal isolates (16.15%) were found to be amylolytic. All the good bacterial amylase producers belonged to the genus *Bacillus* (as identified by standard microbiological procedures). Fungal isolates were identified on the basis of cultural as well as morphological characteristics (Table 2). Good over-producers of amylase were selected for further study.

Table 1. Frequency of bacterial isolates screened for their ability to possess amylolytic activity

Bacterial genera	No. of isolates	No. of α amylase producers	% of α amylase producers
Bacillus sp.	89	34	38.20
Klebsiella sp.	21	6	28.57
Micrococcus sp.	5	0	0
Lactobacillus sp.	2	0	0
Total	**117**	**40**	**34.18**

Table 2. Genera of fungi isolated for screening to have possessed amylolytic activity.

Genus	No. of Isolates	No. of α Amylase producers	% of α Amylase producers
Aspergillus niger group	54	8	14.80
Aspergillus flavus group	22	3	13.64
Aspergillus sp.	18	7	38.88
Fusarium sp.	5	0	0
Alternaria sp.	13	1	7.69
Trichoderma sp.	5	0	0
Paecilomyces sp.	1	0	0
Penicillium sp.	3	2	66.67
Monilia sp.	2	0	0
Curvularia sp.	3	0	0
Cladiosporium sp.	4	0	0
Total	**130**	**21**	**16.15**

Effect of reaction temperature and enzyme activity

Cell-free culture supernatants, both from bacterial as well as fungal origin, showed maximum enzyme activity at 60°C and only one of the bacterial isolates produced amylase which remained highly active even at 80°C (Table 3). All the amylases produced by fungi were however heat labile and their activity decreased drastically when enzyme activity was carried out at 80°C (Fig. 1).

Effect of pH on enzyme activity

A variable pattern of enzyme activity for enzymes from different microbial origin was observed (Fig. 2). Maximum activity was noted at pH 8.0 for the enzymes obtained from bacterial origin, while in case of fungi maximum enzyme activity was noted at pH 4.0.

Table 3. Cell-free culture supernatants assayed for α-amylase activity by incubating the reaction mixture at various temperatures.

Bacterial isolates	Enzyme activity (U/ml) incubation temperature (ºC)		
	40 ºC	60 ºC	80 ºC
B6	3.341	3.831	1.93
B14	3.483	3.859	3.596
B17	3.276	3.84	2.315
B25	2.909	3.003	1.783
B27	3.567	3.906	2.127
B36	3.69	3.897	2.541

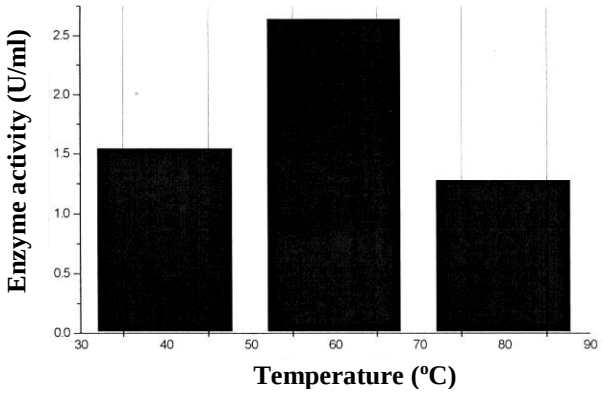

Fig 1. Cell-free culture supernatants assayed for α-amylase activity by incubating the reaction mixture at various temperatures.

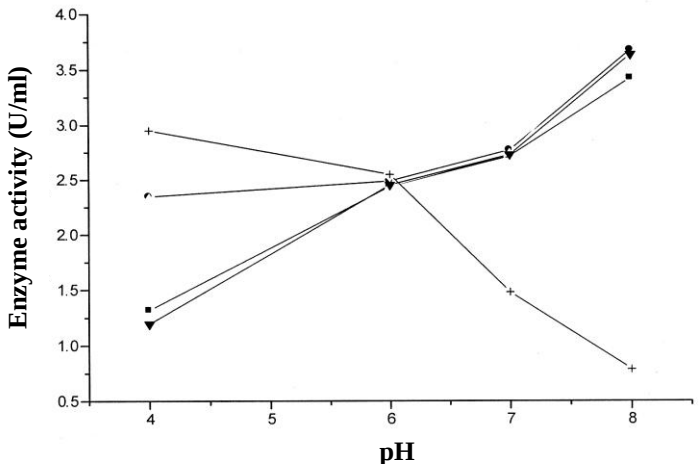

Fig 2. Enzyme preparations from bacterial (■B6, ●B14, ▲B17 & ▼B25, ○B36) and fungal (+F1) isolates assayed after equilibrating the enzyme preparation in 0.5M sodium acetate buffer (pH 4.0 and 6.0), 0.5M phosphate buffer (pH 7.0) and 0.5M Tris buffer (pH 8.0) and enzyme activity in terms of units per ml calculated.

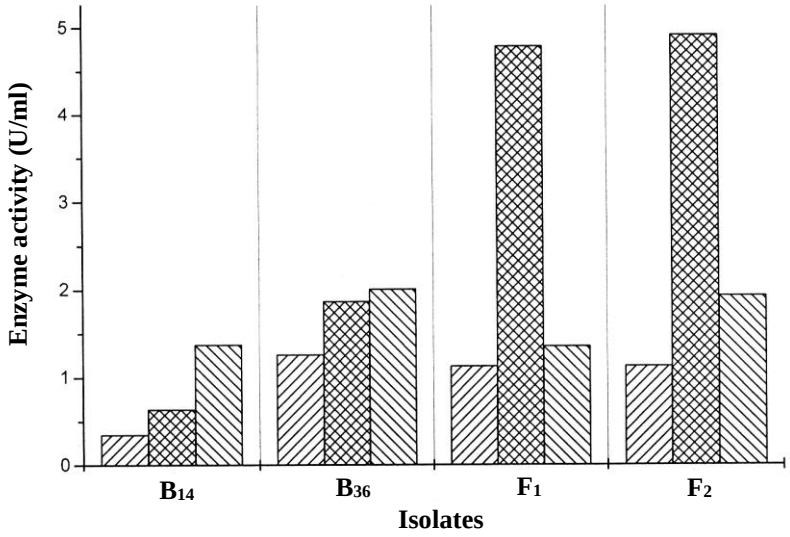

Fig. 3. Induction of α-amylase. Cells were grown in the presence of 1% glucose ( ) and then shifted to minimal medium containing 0.5% starch ( ) or maltose ( ) as sole carbon source. Supernatants were collected and used as enzyme preparations to assay for α-amylase activity. Isolates B_{14} and B_{36} are bacteria and F_1 and F_2 are fungi.

Induction of α–amylase synthesis

A significant enzyme activity was observed when the amylase producing strains were grown in a glucose containing minimal medium. The activity however increased by two-folds (for bacterial) and four-folds (for fungi), after a period of 24 hrs incubation when the cells were shifted from glucose containing minimal medium to starch containing minimal medium. It was also noted that the enzyme activity continued to increase with time in case of bacterial isolates, i.e. minimum after an hour to maximum after 24 hours (data not shown). However, after shifting the cells from starch-containing minimal medium to glucose containing medium, a 2- 4-fold repression in enzyme activity was noted in both bacterial and fungal isolates respectively. Maltose was not a better inducer than starch, in most cases (Fig. 3).

Discussion

In the present study it was observed that the garden soil harbors a dominant amylolytic bacterial population than fungi. *Bacillus* and *Aspergillus* were the most common and abundant amongst amylolytic soil microbial flora. This finding is in agreement with the studies reported earlier (Godfrey *et al*., 1996). Despite repeated attempts to isolate thermophilic starch-degrading microorganisms no attempt was found successful, thermodurics were, however, found to be present which play a potentially important role in the degradation of starches in soils.

A considerable amount of available data suggests that most of the bacterial α-amylases optimally catalyzes a reaction at alkaline pH values (Bajpai, 1989; Highara *et al.*, 2001; Lin *et al.*, 1998). When we analyzed the enzyme preparations from bacterial origin our data has reaffirmed the earlier findings. Contrary to bacterial enzymes, fungal

α-amylases were less active under alkaline conditions but show their optimum activity at pH 4.0 as reported by Duran-Paranco *et al*., (2000).

Temperature optima for enzyme activity is one of the most important parameters in starch-processing industry, as the starch granules are kept at high temperatures during the initial process of starch hydrolysis, 'gelatinization', followed by the process of enzymatic liquefaction where the use of thermostable amylolytic enzymes is indispensable. One of the fungal strains isolated during this study was found to produce amylase that remained stable by retaining its catalytic activity at a temperature of 80°C. Maximum rate of activity from all the other isolate (both, bacteria and fungi) was, however, observed at 60°C. Narang & Satyanarayama (2001); Fitter *et al*., 2001 and Lin *et al*., (1999) have also reported that some of the α-amylases works better at high temperature

Studies on enzyme production and induction kinetics reveal that both, bacteria and fungi can produce significant levels of α-amylase even in the absence of starch. Abate *et al.* (1999) and RajDevi & Yogeesvaran (1999) reported that the maximum enzyme production takes place after 24 hrs in case of *Bacillus amyloliquefaciens* and 48 hrs from *Micrococcus halobius*. Present study suggests that the enzyme production decreases as the concentrations of starch increases in the medium. It is probably due to the presence of glucose in a complex medium which provides an alternate and simpler source of carbon or it could be due to the formation of oligomers formed during the degradation of starches, which was not determined during this study.

Carleson & Neilson (2001) reported that the glucose acts as an inducer during α-amylase production. Duran-Paranco *et al.,* (2000) demonstrated a direct correlation between the glucose and the production of α-amylase from *B. subtilis* which is also in agreement with the data generated during the present study. Although Busch *et al*., (1996) found that maltose acts as a good inducer for amylase production in maltogenic bacteria. In case of fungi, maltose was however, not proved to act as a good inducer as was starch rather it suppressed the production of amylase.

References

Abate, M., R. Carlos, C. Guillermo, F.S. Danley and A.S. Callieri. 1999. Production of amylolytic enzyme by *Bacillus amyloliquefaciens* in pure culture and co-culture with *Zymomonas mobilis*. *Biotechnol. Lett*., 21(3): 249–252.

Bajpai, P. 1989. High temperature alkaline α - Amylase from *Bacillus licheniformis*. *Biotech. Bioeng*., 33: 72–76.

Baum J.S. and W. John-Hill. 1995. Polysaccharides. In: *Introduction to Organic and biological chemistry*. MacMillan Publishing Company. New York.

Busch, J.E., E.G. Porter and F.J. Stutzberger. 1996. Induction of α-amylase by maltooligosaccharide in *Thermomonospora curvata*. *J. Appl. Microbiol*., 82: 669–676.

Carlsen, M. and J. Nielsen. 2001. Influence of Carbon source on α-amylase production by *Aspergillus oryzae*. *Appl. Microbiol. Biotechnol*., 57(3): 346–349.

Duran-Paranco, E., O. Garcia-Kirchner, J.F. Hervagault, D. Thomas and J.N. Barbotin. 2000. α-amylase production by free and immobilized *Bacillus subtilis*. *Appl. Biochem. Biotechnol*., 84**(**86): 479–485.

Fitter, J., R., Herrmann, N.A., Dencher, A., Blume and T. Hauss. 2001. Activity and stability of a thermostable α-amylase compared to its mesophilic homologue : mechanism of thermal adaptation. *Biochem*., 40(35): 10723–10731.

Fogarty, W. and C.T. Kelly. 1990. Microbial Amylases. In: *Industrial Enzymes.* (Ed.) W. M. Fogarty. Academic Press Inc. New York

Fuwa, H. 1954. A new method of microdetermination of amylase activity by the use of amylose as a substrate. *J. Biochem*., 41: 583–603.

Godfrey, T.S.W. 1996. Starch Conversion. In: *Industrial Enzymology*. Second Ed. The MacMillan Press Ltd., New York.

Highara, H.., K. Igarashi, Y. Hayashi, K. Endo, K. Ikawa-Kitayama, K. Ozaki, S. Kawai and S. Ito. 2001. Novel α-amylase that is highly resistant to chelating agents and chemical oxidants from the alkalophilic *Bacillus* isolate KSM – K38. *Appl. Environ. Microbiol*., 67(4): 1744–1750.

Lin, L.L., C.C., Chyan and W.H. Hsu. 1998. Production and properties of a raw starch-degrading amylase from the thermophilic and alkaliphilic *Bacillus* Sp. TS – 23. *Biotechnol. Appl. Biochem*., 28(1): 61–68.

Mayes, A.P. 1996. Carbohydrate of physiological significance. In: *Harper's Biochemistry*. (Ed.): K. Murray, D.K. Robert, P.A. Granner, Mayes, Victor W. Rodwell. Appleton and Lange, USA

Morrison, R.W. and J. Karkalas. 1990. Starch. In: *Methods in plant biochemistry*. Vol. 2. Academic Press Limited.

Narang S. and T. Satyanarayama. 2001. Thermostable α-amylase by extreme thermophile *Bacillus thermooleovorans*. *Lett. Appl. Microbiol*., 32(1): 31–5.

Raj Devi, P.K. and G. Yogeesvaran. 1999. Co-expression of saccharifying alkaline amylase and pullulanase in *Micrococcus halobius* OR-1 isolated from tapioca cultivar soil. 1999. *World J. Microbiol. Biotechnol*., 15(2): 223 – 229.

(Received for publication 6 October 2004)