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Purification and Characterization of α-Amylase from *Bacillus subtilis* Isolated from Local Environment

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Abstract.- The present study is aimed at assessing the ability of *Bacillus subtilis* to convert starch into reducing sugars. Maximum enzyme activity showed by *B. subtilis* was 228 U/ml. *B. subtilis* showed optimum growth at pH 7 and optimum temperature for the growth of the bacterial isolate was found to be 37ºC. The optimal pH and temperature of the purified amylase were 7.0 and 37ºC. The purified enzyme was found to be stable in the pH range of 5.0 to 9.0. The enzyme was stable for 1 h at temperatures ranging from 25-80ºC, while at 80ºC, 38% of its maximum activity was lost. Maximum α-amylase activity was determined in 1% starch concentration. The purified amylase could be detected as a single band of 59 kDa by SDS polyacrylamide gel electrophoresis. Alpha-amylase activity was improved by treating it with an alkylating agent, N-methyl-N nitro-N-nitroso guanidine. Out of 39 mutants of *B. subtilis*, m-39 showed 3.16 fold higher activities (721.5 U/ml) than the parent strain. *B. subtilis* can be exploited for starch conversion biotechnologies.

Keywords: Starch; α-amylase; *Bacillus subtilis*; purification.

INTRODUCTION

For years microorganisms have been the principal source of many different enzymes, which were identified after extensive research and currently find their main uses in industrial applications (Bon, 1995). The majority of industrial enzymes used belong to the hydrolase group, which are active on many natural substrates. Amylase (EC 3.2.1.1, α-1,4-D-glucanohydrolyase) is capable of catalyzing the production of high yields of specific maltooligosaccharides on degrading starch and is of considerable commercial interest (Collins *et al*., 1993a). Thermostable α-amylases have extensive commercial applications in starch processing, brewing and sugar production, desizing and textile industries and in detergent manufacturing process (Leveque *et al*., 2000; De Souza and Magalhães, 2010).

Amylases can be obtained from several sources (Van der Maarel *et al*., 2002; Aquino *et al*., 2003). Thermophilic microorganisms have gained a great deal of attention (Beg *et al*., 2000; Shafaat *et al*., 2011; Raul *et al*., 2014). Enzymes from these microorganisms are of special interest since they are not usually denatured by high temperatures and are even active at elevated temperatures (Adams and Kelly, 1998; Fitter and Heberle, 2000; De Souza and Magalhães, 2010). They are usually produced by bacteria belonging to the genus *Bacillus* for industrial applications such as *B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens* and *B. stearothermophilus* (Sajedi *et al*., 2005; De Souza and Magalhães, 2010; Shafaat *et al*., 2011). The properties of each α-amylase such as thermostability, pH profile, and Ca-independence must be matched to its application. For example, α-amylases used in starch industry must be active and stable at low pH but in detergent industry at high pH values (Nielsen and Borchert, 2000).

The present study is concerned with the isolation and characterization of the α-amylase from *B. subtilis*. Some properties of the purified amylase are also determined.

MATERIALS AND METHODS

Isolation of α-amylase producing bacteria

Bacteria for the production of α-amylase were isolated from 50 soil samples collected from different industries, gardens and cultivated areas (Pakistan). One gram of soil sample was added to a

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glass tube containing 10 ml sterilized distilled water, shaked and placed in a water bath at 80ºC for 15 min. After cooling, 50 µl of the sample was spread on nutrient agar plates (prepared by dissolving 0.6 g peptone, 0.4 g casein hydrolysate, 0.15 g beef extract, 0.3 g yeast extract and 0.2 g starch in 100 ml distilled water, pH adjusted at 7 and then 1.5 g agar was added in the 250 ml Erlenmeyer flasks, autoclaved at 121ºC and 15 lb pressure for 15 min) and incubated at 37ºC for 48 h. The colonies forming clear zones around them were picked up and streaked on nutrient agar plates to get pure culture and to confirm zone formation.

Production of α-amylase was carried out in a medium containing (g/l of distilled water): NaCl-0.4, peptone-2.0, yeast extract-1.0 and starch-10.0 (Uehara *et al.*, 1979). The pH was adjusted to 7 with NaOH and medium was sterilized by autoclaving at 121ºC and 15 lb pressure for 15 min.

Amylase activity assay

Amylase activity was determined by measuring the release of reducing sugar from soluble starch. The reaction mixture contained 0.5 ml of crude enzyme and 1 ml of sodium phosphate buffer (pH 7.0) containing 1% soluble starch and incubated at 25ºC for 10 min, the amount of reducing sugar released in the mixture was determined by the addition of 2 ml of 3, 5 dinitrosalicylic acid method (Yang *et al*., 2003) followed by boiling for 10 min and to develop colour. The absorbance of the mixture was measured at 540 nm, and D-glucose was used to create a standard curve. One unit of enzyme activity was defined as the amount of enzyme releasing reducing sugar equivalent to 1µmol glucose per minute from starch under the assay condition. All analytical measurements were performed at least in triplicates.

Physical, biochemical and molecular characterization

The isolate was Gram positive. For biochemical characterization the isolate was tested for catalase activity, motility, oxidase acivity, nitrate reduction and Voges-Proskauer test. Some specific tests were performed for further characterization of isolates such as sporulation test, acid formation test, utilization of different sugars, hydrolysis of casein, hydrolysis of starch, growth at 20ºC, 30ºC, 37ºC, 45ºC, 50ºC, growth in medium containing 2%, 5%, 7% and 10% NaCl and growth at different pH. The procedures adopted for all the above physical and biochemical tests were taken from Benson (1994). For molecular characterization genomic DNA was extracted as described by Carozzi *et al*. (1991) and the 16S rRNA gene was amplified by PCR using 16S rRNA primers covering variable region V6 to variable region V9
(RS-1; 5′-AAACTC-AAATGAATTGACGG-3′,
and RS-3; 5′-ACGGGCGGTGTGTAC-3′)
(Rehman *et al*., 2007). PCR was performed by initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 5 min. The PCR product of 0.5kb was removed from the gel and cloned in pTZ57R/T vector. The amplified 16S rRNA gene was purified with a Fermentas purification kit (#K0513) and the amplified products were electrophoresed on 1% agarose gel. Sequencing was carried out by Genetic analysis system model CEQ-800 (Beckman) Coulter Inc. Fullerton, CA, USA. Nucleotide sequence similarities were determined using BLAST (NCBI database; http://www.ncbi.nlm.nih.gov/BLAST). The sequence was aligned with close matches using ExPasy-Tools (Clustal W) multiple sequence alignment program (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and a dendrogram based on the homologous sequences was created using the same package.

Effect of pH, temperature and starch concentration on amylase activity

The effect of pH on the activity of α-amylase was measured by incubating 0.5 ml of the diluted enzyme and 0.5 ml of phosphate buffer ranging pH from 6 to 12, containing 1% soluble starch for 10 min at 90ºC. The stability of the enzyme at different pH values was also studied by incubating the enzyme at various pH values ranging from 3 to 10 for 2 h and then estimating the residual activity.

The effect of temperature on the enzyme activity was determined by performing the standard assay procedure for 10 min at pH 8.0 with in a

temperature range of 25-90ºC. Thermostability was determined by incubation of the enzyme at temperatures ranging from 25-90ºC for 1 h in a constant temperature water bath. After treatment the residual enzyme activity was assayed.

The effect of starch concentration on the activity of α-amylase was measured by incubating 0.5 ml of the diluted enzyme and 0.5 ml of phosphate buffer, containing 0.5, 1, 1.5, 2, 2.5 and 3% soluble starch for 10 min at 90ºC.

Protein purification, gel electrophoresis and enzyme assay

The crude enzyme was precipitated with ammonium sulphate (80%) and allowed to stand over night at 4ºC with constant stirring. The mixture was centrifuged at 10,000*g* for 20 min at 4ºC. The precipitate was collected, re-dissolved in 10 mM phosphate buffer (pH 6.9) and dialyzed against same buffer for 24 h at 4ºC. The dialyzed mixture after centrifugation (10,000*g*) for 20 min at 4ºC was further purified through membrane filtration by using centricons (Amicon Company). The partially purified enzyme solution was passed through centricon of 100 kDa membranes and then followed by 50 kDa membranes and used for subsequent studies.

The molecular mass of the purified enzyme was estimated by 12% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (Laemmli, 1970). Non denaturing polyacrylamide gel electrophoresis (Native PAGE) was also performed in a large (HoeferModel Se 600) gel apparatus to confirm the molecular weight of purified α-amylase. Thirty microlitre samples were loaded in duplicate. After electrophoresis, the gel was divided longitudinally into two halves. One half of the gel along with marker was silver stained (Bloom *et al*., 1987) and other half was used for electrophoretic α-amylase assay. The gel size was 13cm in length and 26 slice of each lane were made and the number of lanes was seven. Each slice was immediately placed into 1 ml of 1% soluble starch and incubated at 25ºC for 12 h. The reaction was stopped by the addition of 2 ml of DNS. Optical density was taken at 540 nm and a 0.5cm slice of the gel without sample was used as blank.

Chemical mutagenesis

The actively growing cells (1 x 108 c.f.u/ml) of *B. subtilis* were treated with 377.35 µg/ml of MNNG (N-methyl-N nitro-N-Nitroso guanidine, Sigma Chemicals) at 37ºC for 1, 2 and 3 h. After centrifugation at 8000 rpm for 190 min, supernatant was discarded and pellet was washed three times for the complete removal of MNNG. Cells were resuspended in nutrient agar broth. After 24 h of incubation at 37ºC the cell suspension was diluted (up to 10-5, 10-6) and were spread on nutrient agar containing starch medium Petri plates and incubated at 37ºC for 24 h. Colonies appeared were counted for cell survival and mutants forming clear zones were picked up and transferred to the nutrient agar medium slants.

RESULTS AND DISCUSSION

Isolation and identification of the bacterial isolate

A total of 50 soil samples collected from different ecological environment were analyzed for bacteria producing amylase. About 72 cultures of *Bacillus* species were isolated, purified and screened for the production of α-amylase in the shake flask containing 50 ml of M-9 medium. Of all the cultures tested, bacterial isolate 10-R gave the maximum enzyme production *i.e.*, 228 U/ml and was selected for further studies. Table I shows physical and biochemical characteristics of the bacterial isolate. The partially amplified (500bp) and sequenced 16S rRNA gene from local isolate (10-R) was uploaded to the NCBI (National Center for Biotechnology Information) website to search for similarity to known DNA sequences and to confirm the species of this local isolate. The nucleotide sequences coding for the 16S rRNA gene after BLAST query revealed that this gene is 100% homologous to *Bacillus subtilis*. The nucleotide sequences coding for the 16S rRNA gene of *B. subtilis* have been submitted to the GenBank database under accession number JN546607. Other close matches included *B. subtilis* strain BPR7 (JN208240.1; 100% similarity), *B. amyloliquefaciens* strain NSP6 (JF802178.1; 100% similarity), and *B. tequilensis* strain YC5-2 (HM770882.1; 100% similarity). Dendrogram indicates the identity of the isolate as a species of *Bacillus*.

Table I.- Morphological and physiological characteristics of the isolated strain.

Characteristics	*B. subtilis*
Gram stain	Positive
Motility	Positive
Sporulation	Positive
Behavior of oxygen	Aerobic
Casein hydrolysis	Positive
Starch hydrolysis	Positive
Reduction of nitrate to nitrite	Negative
Catalase test	+
Voges proskauer test	+
Production of acid from	
Glucose	+
Mannitol	+
Xylose	+
Arabinose	-
Growh in NaCl	
2%	+
5%	+
7%	+
10%	-
Temperature range for growth	20-50
pH range for growth	7-11

(+) positive; (-) negative

Effect of pH, temperature and starch concentration on amylase activity

The effect of pH on α-amylase activity as a function of pH is shown in Figure 1A. Optimum pH was found to be 7 where the enzyme activity was 245 U/ml. The enzyme activity at pH 4.0 and 10.0 were 28% and 18.5% of that at pH 7, respectively. After incubation of the enzyme solution for 2 h at pH 3.0-10.0, the original activity at pH 8.0 decreased by 12% and at pH 9.0 the decreased in enzyme activity was 51%. The partial purified enzyme was found to be stable in the pH range of 5.0 to 9.0. These results suggest that the activity of the enzyme is fairly higher in alkaline pH, making this enzyme attractive for the detergent industry. Alpha-amylases produced by other *Bacillus* sp. have shown optimum activities at pH values as low as 3.5 or as high as 12 (Burhan *et al*., 2003; Konsula and Liakopoulou-Kyriakides, 2004; De Souza and Magalhães, 2010).

Figure 1B shows the activity of the enzyme assayed at temperatures ranging from 25-90ºC at pH 7 and a substrate concentration of 1%. Enzyme activity increased with temperature with in the range of 25 to 80ºC. A sharp decrease in enzyme activity was observed at values above 80ºC. The optimum temperature of this α-amylase was 37ºC, which was higher or similar to that described for other *Bacillus* α-amylases (Sidhu *et al*., 1997; Burhan *et al*., 2003; Konsula and Liakopoulou-

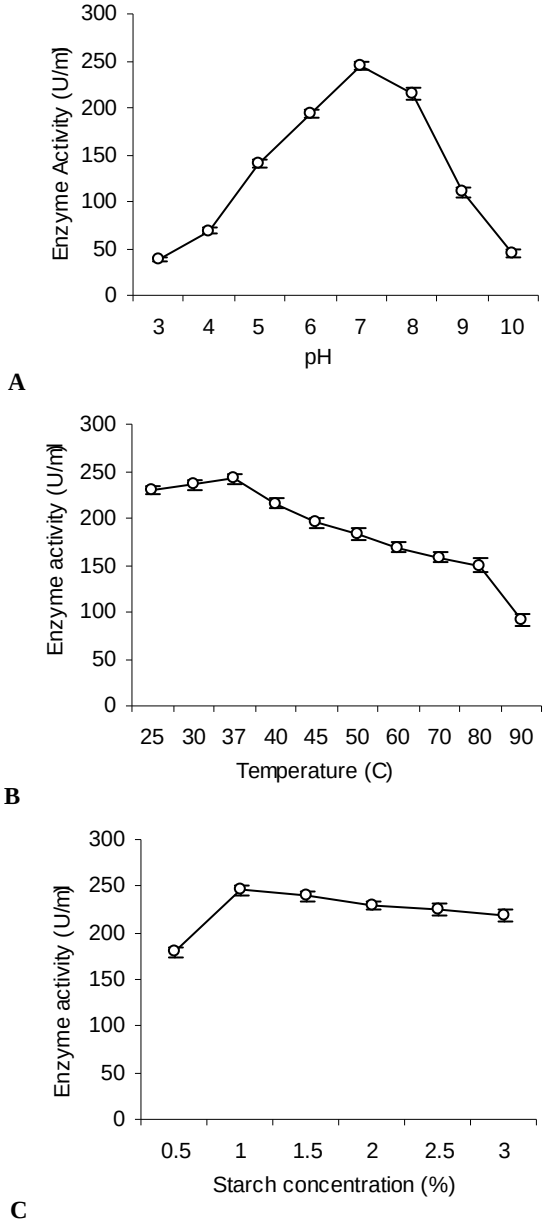

Fig. 1. Effect of pH (A), temperature (B) and starch (C) on the α-amylase activity. *Error bars* show standard deviation among three observations. Some values are too small to be visible.

Kyriakides, 2004; Raul *et al*., 2014). The residual activities of crude amylase incubated at different temperature for a period of 1 h were estimated at optimum temperature. The enzyme was stable for 1 h at temperatures ranging from 25-80ºC while at 80ºC, 38% of its maximum activity was lost. At 90ºC maximum enzyme activity lost was 62%.

Figure 1C shows the effect of starch concentration on the activity of α-amylase. Different starch concentrations were used and it was found that maximum α-amylase activity was observed in 1% starch. It was also found that starch concentration greater than 1% did not show any increase in amylase activity.

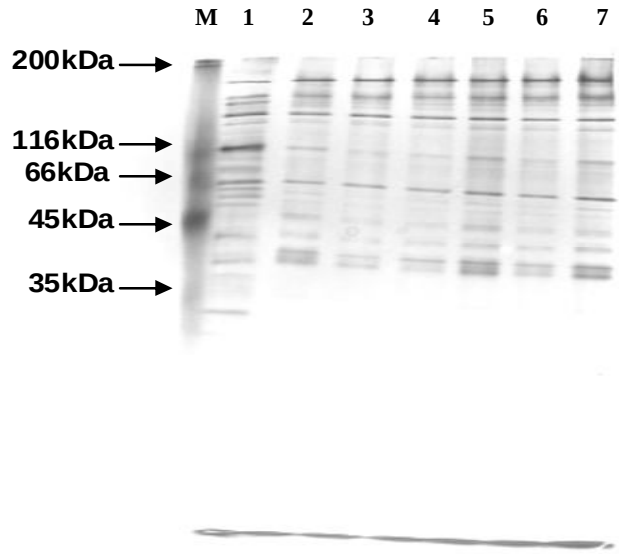

Fig. 2. Native PAGE of extracellular total protein from *B. subtilis Lane M*, molecular weight; *Lane 1*, wild type; *Lane 2*, mutant-9; *Lane 3*, mutant-11; *Lane 4*, mutant-17; *Lane 5*, mutant-25; *Lane 6*, mutant-30; *Lane 7*, mutant-39. The gel is 12% silver stained.

SDS-PAGE and enzyme assay

A protein band (approx.59 kDa) was found in native PAGE (Fig. 2) and α-amylase band was confirmed by dinitrosalicylic acid method. The molecular weight of the purified enzyme which appeared as a single band on SDS-PAGE was found to be 59 kDa (Fig. 3). The banding pattern is not same in two protein gels because the native PAGE was performed from the extracellular total proteins (contains many protein bands) while SDS-PAGE was performed from the purified sample (contains one major protein band). The specific activity of the partially purified enzyme was 280 U/mg (1.2 fold increased as compared to crude enzyme). Shaw *et al*. (1995) reported the similar weight of protein in *Thermus* sp. Yang and Liu (2007) reported amylase as a single band of about 65 kDa by SDS-polyacrylamide gel electrophoresis. Murakami *et al*. (2007) reported two alkaline thermotolerant α-amylases having molecular masses of 105 and 75 kDa, respectively. A protein with molecular weight of 53 kDa was found in *Bacillus subtilis* WB600 (Liu *et al*., 2008). Another protein with molecular weight of 51.4 kDa was reported by Wang *et al*. (2008).

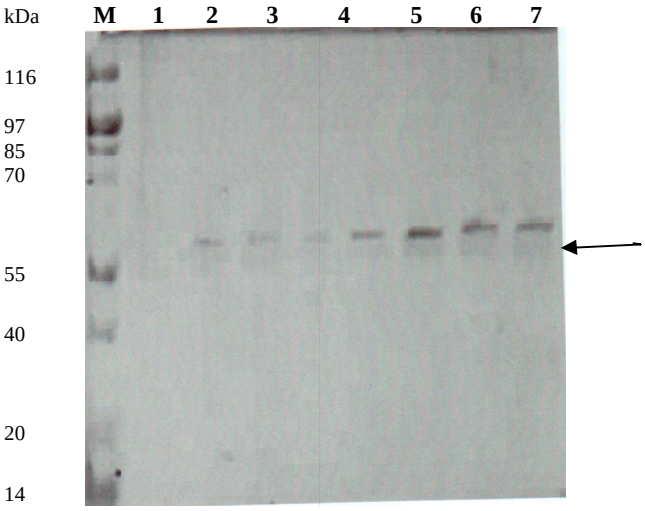

Fig. 3. SDS-PAGE of the purified amylase from *B. subtilis Lane M*, molecular weight; *Lane 1*, wild type; *Lane 2*, mutant-9; *Lane 3*, mutant-11; *Lane 4*, mutant-17; *Lane 5*, mutant-25; *Lane 6*, mutant-30; *Lane 7*, mutant-39. The gel is 12% silver stained.

Mutagenic effect on the amylase activity

From 3 h treatment of MNNG, 39 mutants were selected on the basis of morphology and the production of α-amylase (zone diameter). On the basis of enzyme activity 8 mutants were found positive and remaining were found negative. After repeated cultivation of the positive mutants only three mutants (m-25, m-30, m-39) appeared stable. The proteolytic activity of m-25, m-30 and m-39 was 375.00, 503.50 and 721.50 U/ml, respectively.

The m-39 gave the greater yield of α-amylase in all mutants and therefore was selected for further studies. Bokhari *et al*. (2009) reported that mutant *Thermomyces lanuginosus* showed 5.3 fold improvement as compared to the parental strain to produce xylanases.

In the current investigation, *B. subtilis* amylase showed promising properties and might find applications in starch conversion biotechnologies.

CONCLUSION

In the present study we have isolated α-amylase, from locally isolated *Bacillus subtilis*, with maximum enzyme activity of 228 U/ml. The optimal pH and temperature of the purified amylase were 7.0 and 37ºC. The highest α-amylase activity was determined in 1% starch. A molecular mass of 59 kDa was determined by SDS-PAGE and the enzyme was stable at 25-80ºC and at pH 5.0 to 9.0. Alpha-amylase activity was improved by treating it with an alkylating agent, N-methyl-N nitro-N-Nitroso guanidine. Out of 39 mutants of *B. subtilis*, m-39 showed 3.16 fold higher activities (721.5 U/ml) than the parent strain. *B. subtilis* can be exploited for industrial biotechnologies.

REFERENCES

ADAMS, M.W.W. AND KELLY, R.M., 1998. Finding and using thermophilic enzymes. *Trends Biotechnol*., **16:**329-332.

AQUINO, A.C.M.M., JORGE, J.A., TERENZI, H.F. AND POLIZELI, M.L.T.M., 2003. Studies on a thermostable α-amylase from the thermophilic fungus *Scytalidium thermophilum*. *Appl. Microbiol. Biotechnol*., **61:** 323-328.

BEG, Q.K., BHUSHAN, B., KAPOOR, M. AND HOONDAL, G.S., 2000. Production and characterization of thermostable xylanase and pectinase from *Streptomyces* sp. QG-11-3. *J. Indust. Microbiol. Biotechnol*., **24:** 396-402.

BENSON, H.J., 1994. *Microbiological applications. laboratory manual in general microbiology*. Wan C. Brown Publishers, Dubuque. ISBN 0-03-018394-4.

BLOOM, H., BEIER, H. AND GROSS, H.S., 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis*, **8:** 93-99.

BOKHARI, S.A.I., LATIF, F. AND RAJOKA, M.I., 2009. Purification and characterization of xylanases from *Thermomyces lanuginosus* and its mutant derivative possessing novel kinetic and thermodynamic properties. *World J. Microbiol. Biotechnol*., **25:** 493-502.

BON, E.P.S., 1995. A teenologia enzimatica no Brasil. *Enz. Technol. (ENZITEC)*, **95:** 9-14.

BURHAN, A., NISA, U., GOKHAN, C., OMER, C., ASHABIL, A. AND OSMAN, G., 2003. Enzymatic properties of a novel thermostable, thermophilic, alkaline and chelator resistant amylase from an alkaliphilic *Bacillus* sp. isolate ANT-6. *Process Biochem*., **38:** 1397-403.

CAROZZI, N.B., KRAMER, V.C., WARREN, G.W., EVOLA, S. AND KOZIEL, M.G., 1991. Prediction of insecticidal activity of *Bacillus thuringiensis* strains by polymerase chain reaction product profiles. *Appl. environ. Microbiol*., **57:** 3057-306.

COLLINS, B.S., KELLY, C.T., FOGARTY, W.M. AND DOYLE, E.M., 1993a. The high maltose-producing α-amylase of the thermophilic actinomycetes, *Thermomonospora curvata*. *Appl. Microbiol. Biotechnol*., **39:** 31-35.

DE SOUZA, P.M. AND MAGALHÃES, P. O., 2010. Application of microbial amylase in industry-a review. *Braz. J. Microbiol.*, **41:** 850-861.

FITTER, J. AND HEBERLE, J., 2000. Structural equilibrium fluctuations in mesophilic and thermophilic α-amylase. *Biophys. J*., **79:** 1629-1636.

KONSULA, Z. AND LIAKOPOULOU-KYRIAKIDES, M., 2004. Hydrolysis of starches by the action of an α-amylase from *Bacillus subtilis*. *Process Biochem*., **39:** 1745-1749.

LAEMMLI, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227:** 680-685.

LEVEQUE, E., JANECEK, S., HAYE, B. AND BELARBI, A., 2000. Thermophilic archaeal amylolytic enzymes. *Enzyme Microb. Technol*., **26:** 3-14.

LIU, Y.-H., LU, F.-P., LI, Y., YIN, X.-B., WANG, Y. AND GAO, C., 2008. Characterization of mutagenised acid-resistant alpha-amylase expressed in *Bacillus subtilis* WB600. *Appl. Microbiol. Biotechnol*., **78:** 85-94.

MURAKAMI, S., NISHIMOTO, H., TOYAMA, Y., SHIMAMOTO, E., TAKENAKA, S., KAULPIBOON, J., PROUSOONTORN, M., LIMPASENI, T., PONGSAWASDI, P. AND AOKI, K., 2007. Purification and characterization of two alkaline, thermotolerant α-amylases from *Bacillus halodurans* 38C-2-1 and expression of the cloned gene in *Escherichia coli*. *Biosci. Biotechnol. Biochem*., **71:** 2393-2401.

NIELSEN, J.E. AND BORCHERT, T.V., 2000. Protein engineering of bacterial α-amylase. *Biochim. Biophys.*

Acta, **1542:** 253-274.

RAUL, D., BISWAS, T., MUKHOPADHYAY, S., DAS, S.K. AND GUPTA, S., 2014. Production and partial purification of alpha amylase from *Bacillus subtilis* (MTCC 121) using solid state fermentation. *Biochem. Res. J*., **2014:** 1-5.

REHMAN, A., ALI, A., MUNEER, B. AND SHAKOORI, A.R., 2007. Resistance and biosorption of mercury by bacteria isolated from industrial effluents. *Pakistan J. Zool.*, **39:** 137-146.

SAJEDI, R.H., NADERI-MANESH, H., KHAJEH, K., AHMADVAND, R., RANJBAR, B., ASOODEH, A. AND MORADIAN, F., 2005. A Ca-independent α-amylase that is active and stable at low pH from the *Bacillus* sp. KR-8104. *Enzyme Microb. Technol*., **36:** 666-671.

SHAFAAT, S., AKRAM, M. AND REHMAN, A., 2011. Isolation and characterization of a thermostable α-amylase from *Bacillus subtilis*. *Afr. J. Microbiol. Res.*, **5:** 3334-3338.

SIDHU, G.S., SHARMA, P., CHAKRABARTI, T. AND GUPTA, J.K., 1997. Strain improvement for the production of a thermostable α-amylase. *Enzyme Microb. Technol.*, **21:** 525-530.

SHAW, J.-F., LIN, F.-P., CHEN, S.-C. AND CHEN, H.-C., 1995. Purification and properties of an extracellular α-amylase from *Thermus* sp. *Bot. Bull. Acad. Sin.*, **36:** 195-200.

UEHARA, H., YAMANE, K. AND MARUO, B., 1979. Thermosensitive, extracellular neutral protease in *Bacillus subtilis*: isolation, characterization and genetics. *J. Bact*., **139:** 583-590.

VANDER MAAREL, M.J., VAN DER VEEN, B., UITDEHAAG, J.C., LEEMHUIS, H. AND DIJKHUIZEN, L., 2002. Properties and applications of starch-converting enzymes of the α-amylase family. *J. Biotechnol*., **94:** 137-155.

WANG, S., LU, Z., LU, M., QIN, S., LIE, H., DENG, X., LIN, Q. AND CHEN, J., 2008. Identification of archaeon-producing hyperthermophilic α-amylase and characterization of the α-amylase. *Appl. Microbiol. Biotechnol.*, **80:** 605-614.

YANG, C.H., CHENG, K.C. AND LIU, W.H., 2003. Optimization of medium composition for production of extracellular amylase by *Thermobifida fusca* using a response surface methodiology. *Fd. Sci. Agric. Chem*., **5:** 35-40.

YANG, C.-H. AND LIU, W.-H., 2007. Cloning and characterization of a maltotriose-producing α-amylase gene from *Thermobifida fusca*. *J. indust. Microbiol. Biotechnol*., **34:** 325-330.

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