

Full Length Research Paper

# Isolation and characterization of a thermostable $\alpha$ -amylase from *Bacillus subtilis*

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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  $236 \text{ Uml}^{-1}$ . *B. subtilis* showed optimum growth at pH 8 and optimum temperature for the growth of bacterial isolate was found to be  $45^\circ\text{C}$ . The optimal pH and temperature of the amylase activity were 7.0 and  $37^\circ\text{C}$ , respectively. The enzyme was found to be stable in the pH range of 5 to 10. Maximum  $\alpha$ -amylase activity was determined in 1% starch. The enzyme was found to be in two forms with relative molecular mass of 43 and 18 kDa in SDS-polyacrylamide gel electrophoresis. *B. subtilis* can be exploited for starch conversion biotechnologies.

**Key words:** Starch,  $\alpha$ -amylase, *Bacillus subtilis*.

## 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,  $\alpha$ -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  $\alpha$ -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).

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 recently (Becker et al., 1997; Beg et al., 2000). 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). They are usually *stearothermophilus* (Boyer and Ingle, 1972; Sajedi et al., 2005). The properties of each  $\alpha$ -amylase such as

industrial applications such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens* and *Bacillus* thermostability, pH profile, and Ca-independency must be matched to its application. For example,  $\alpha$ -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  $\alpha$ -amylase from *B. subtilis*. Some properties of the amylase are also determined.

## MATERIALS AND METHODS

### Sample collection and screening of $\alpha$ -amylase producing bacteria

Bacteria for the production of  $\alpha$ -amylase were isolated from 20 soil samples collected from different industries, gardens and fruit markets (Pakistan). One gram of soil sample was added to a glass tube containing 10 ml sterilized distilled water, shaken and placed in a water bath at  $80^\circ\text{C}$  for 15 min. After cooling, 50  $\mu\text{l}$  of the sample was spread on nutrient agar plates (prepared by dissolving 0.6 g peptone, 0.4 g casein hydrolyzate, 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  $121^\circ\text{C}$  and 15 lb pressure for 15 min) and incubated at  $37^\circ\text{C}$  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.

Further screening of  $\alpha$ -amylase producing bacteria was carried out in 1% starch medium containing ( $\text{g l}^{-1}$ ) of distilled water: NaCl

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(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 121°C and 15 lb pressure for 15 min.

#### Determination of optimum growth conditions

For optimum growth of the bacterial isolate, two parameters that are temperature and pH were considered. For determination of optimum temperature, 5 ml LB broth was added in 4 sets, each of three test tubes, autoclaved and inoculated with 20 µl of freshly prepared culture of bacterial isolate by overnight growth at 37°C in LB broth. The four sets of tubes were incubated at 20°C, 30°C, 37°C and 45°C. After an incubation period of 12 h, their absorbance was taken at 600 nm using a LAMBDA 650 UV/Vis Spectrophotometer (PerkinElmer, USA). For determination of optimum pH, test tubes having 5 ml LB broth were prepared in 6 sets, each containing 3 test tubes and their pH was adjusted at 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 then autoclaved. These tubes were inoculated with 20 µl freshly prepared culture of bacterial isolate. After an incubation period of 12 h, their absorbance was taken at 600 nm.

#### Effect of starch on bacterial growth

Growth curves of *B. subtilis* were determined in 1% starch and in LB broth medium. For bacterial isolate 100 ml medium was taken in one set consisting of 3 flasks, autoclaved and then inoculated with 100 µl of the freshly prepared inoculums. The cultures were incubated at 45°C in an incubator shaker at 150 rpm. An aliquot of culture was taken at regular intervals (0, 4, 8, 12, 16, 20, 24 and 28 h) to measure absorbance at 600 nm.

#### Physical, biochemical and molecular characterization

The isolate was Gram stained. For biochemical characterization the isolate was tested for Voges-Proskauer test, sporulation test and utilization of different sugars. The procedures adopted for all the above physical and biochemical tests were taken from Cappuccino and Sherman (2001). 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 (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 0.5 kb 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. The 16S rRNA gene sequences were compared with known sequences in the GenBank database to identify the most similar sequence alignment.

#### 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 25°C 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 1 µmol glucose per minute under the assay condition. All analytical measurements were performed at least in triplicates.

#### Effect of pH, temperature and various substrates 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 5 to 10, containing 1% soluble starch for 5 min at 100°C. 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 30 to 100°C. After treatment the residual enzyme activity was assayed.

The amylase activity was also assayed by measuring the reducing sugar released during the reaction, using complex polysaccharide substrates (starch, carboxymethylcellulose, molasses and sugarcane bagasse). The reaction mixture contained 50 µl of 1% solution of the substrates separately prepared in 2 mM imidazole-HCl buffer of pH (7.0) and 250 µl of enzyme solution. The reaction was stopped by adding 100 µl dinitrosalicylic acid solution after incubation at 37°C for 1 h. The reaction mixture was then heated in boiling water for 5 min and the absorbance was taken at 540 nm after cooling at room temperature.

#### SDS- polyacrylamide gel electrophoresis

Protein profile of bacterial cells, grown with 1% starch and without starch, was done to compare the inducibility of amylase. The crude enzyme was precipitated with ammonium sulphate (60%) and allowed to stand over night at 4°C with constant stirring. The mixture was centrifuged at 10,000 g for 20 min at 4°C. The precipitate was collected, re-dissolved in distilled water. The proteins of *B. subtilis* were isolated according to Rehman et al. (2009), estimated according to Lowry et al. (1951), and electrophoresed according to Laemmli (1970). Aliquots of 15 to 20 µl were loaded onto a 12% SDS polyacrylamide linear resolving gel overlaid with a 6% stacking gel. Gels were stained with Coomassie Brilliant blue R-250.

#### Statistical analysis

Observations were made and all the experiments run in triplicate. At least three separate flasks were usually maintained for one treatment. Each time three readings were taken, their mean, and standard error of the mean were calculated.

## RESULTS AND DISCUSSION

#### Isolation and identification of the bacterial isolate

A total of 20 soil samples collected from different ecological environments were analyzed for bacteria producing amylase. About 28 cultures of *Bacillus* species were isolated, purified and screened for the production of α-amylase in 1% starch. Of all the cultures tested, bacterial isolate SA-J3 gave the maximum hollow zone around the agar plates containing 1% starch and was

**Table 1.** Morphological and biochemical characteristics of bacterial isolate.

Characters	<i>B. subtilis</i>
Shape	Irregular
Size	Large
Colour	White
Consistency	Dry
Margin	Filamentous
Elevation	Flat
Gram's staining	+ rods
Spore staining	+
Voges Proskauer test	+
Sugar fermentation	
Glucose	+
Manitol	+
Lactose	-

(+) positive; (-) negative.

selected for further studies. Table 1 shows some physical and biochemical characteristics of the bacterial isolate. On the basis of this characterization the bacterial isolate has been identified as *Bacillus* sp.

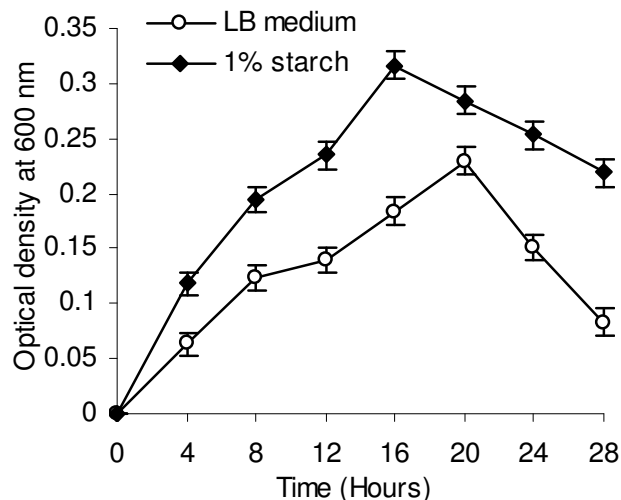
The partially amplified (500 bp) and sequenced 16S rRNA gene from local isolates (SA-J3) was uploaded to the NCBI (National Center for Biotechnology Information) website to search for similarity to known DNA sequences and to confirm the species of the local isolate. The nucleotide sequences coding for the 16S rRNA gene after BLAST query revealed that this gene is 94% homologous to *Bacillus subtilis* (SA-J3). The nucleotide sequences coding for the 16S rRNA gene of *B. subtilis* have been submitted to the GenBank database under accession numbers JF804982.

### Optimum growth conditions

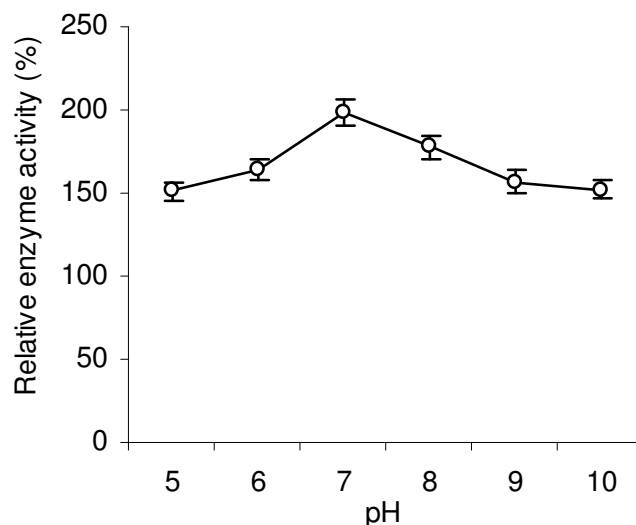
*B. subtilis* showed optimum growth at pH 8 and the most suitable temperature for the growth of bacterium was found to be 45°C. The growth curve pattern was studied by growing the bacterium in 1% starch and comparing with the control culture in which no starch was added. The growth pattern of *B. subtilis* was significantly different from those of control and the growth rate of bacterial isolate was higher in the presence of 1% starch as compared to growth in the presence of LB medium. The maximum growth (O.D) was determined after 16 and 20 h of growth in 1% starch and LB medium, respectively (Figure 1). This shows that the bacterium has efficient ability to convert starch into its sub-products and eventually into glucose.

### Effect of pH, temperature and various substrates on amylase activity

The effect of pH on  $\alpha$ -amylase activity as a function of pH

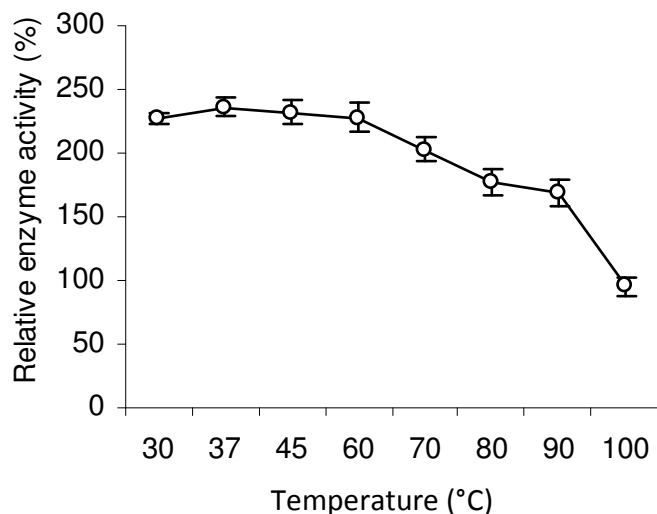


**Figure 1.** Effect of 1% starch on the cell growth of *B. subtilis* in LB broth medium after incubation at 37°C.

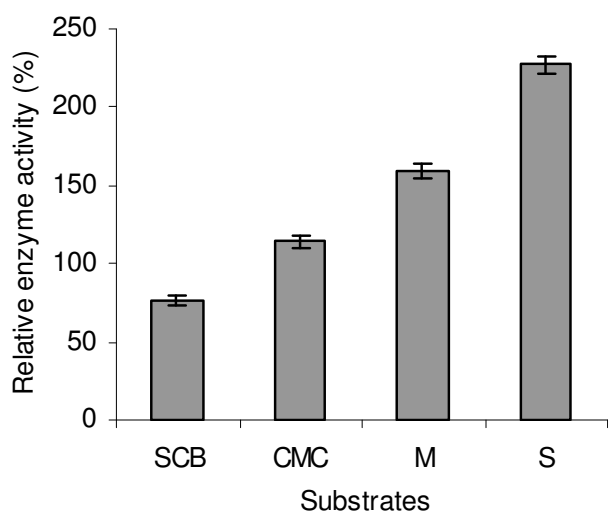


**Figure 2.** Effect of pH on the  $\alpha$ -amylase activity.

is shown in Figure 2. Optimum pH was found to be 7 where the enzyme activity was 199 Uml<sup>-1</sup>. The enzyme activity at pH 5.0 and 10.0 were 151 and 152% of that at pH 7, respectively. The crude enzyme was found to be stable in the pH range of 5.0 to 10.0. These results suggest that the activity of enzyme is fairly higher in alkaline pH, making this enzyme attractive for the detergent industry. The optimum temperature, pH and incubation period for amylase production in *B. subtilis* were 50 to 70°C, 5.0 to 9.0 and 36 h, respectively (Swain et al., 2006). 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).



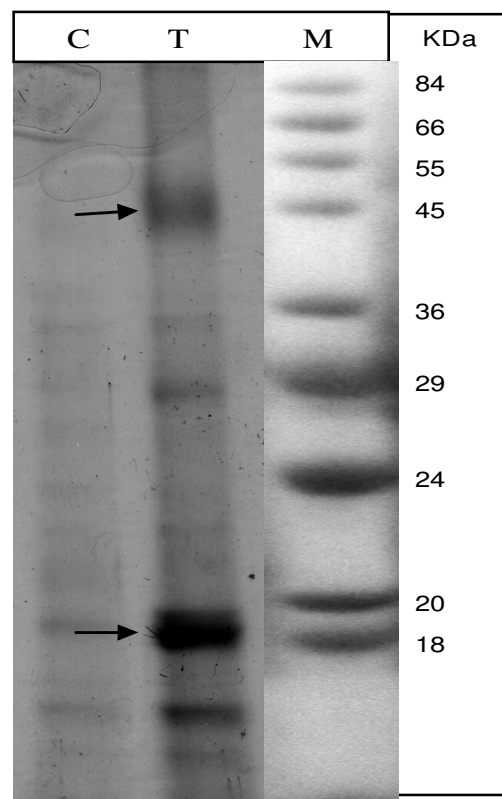
**Figure 3.** Effect of temperature on the  $\alpha$ -amylase activity.



**Figure 4.** Effect of sugarcane bagasse (SCB), carboxymethylcellulose (CMC), molasses (M) and starch (S) on  $\alpha$ -amylase activity.

Figure 3 shows the activity of the enzyme assayed at temperatures ranging from 30 to 100°C at pH 7 and starch concentration of 1%. A slow decrease in enzyme activity was observed at values above 70°C. The optimum temperature of this  $\alpha$ -amylase was 37°C (236%), which was higher or similar to that described for other *Bacillus*  $\alpha$ -amylases (Sidhu et al., 1997; Burhan et al., 2003; Konsula and Liakopoulou-Kyriakides, 2004). The residual activities of crude amylase at 90°C was 169%, maximum enzyme activity lost as compared to optimum temperature (37°C) was 67%.

Figure 4 shows the substrate specificity of  $\alpha$ -amylase. Different polysaccharides at concentration of 1% were used and it was found that maximum  $\alpha$ -amylase activity



**Figure 5.** SDS-PAGE pattern of extracellular ammonium sulfate precipitated proteins of *B. subtilis*. M, protein marker; T, treated (with 1% starch) and C, control (without starch). The gel is 12% stained with Coomassie blue.

was observed in 1% starch.

### SDS-PAGE analysis

In the present study two alkaline  $\alpha$ -amylases with molecular weights of 45 and 18 kDa were observed in SDS-PAGE (Figure 5). Swain et al. (2006) reported the purified  $\alpha$ -amylases from *B. subtilis* were in two forms with molecular mass of  $18.0 \pm 1$  and  $43.0 \pm 1$  kDa in native SDS-PAGE. Shaw et al. (1995) reported the protein of 59 kDa 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  $\alpha$ -amylases having molecular masses of 105 and 75 kDa, respectively. A protein with molecular weight of 53 kDa was found in *B. subtilis* WB600 (Liu et al., 2008). Another protein with molecular weight of 51.4 kDa was reported by Wang et al. (2008).

### Conclusion

In the present study we have isolated  $\alpha$ -amylase, from

locally isolated *B. subtilis*, with maximum enzyme activity of 236 Uml<sup>-1</sup>. The optimal pH and temperature of the enzyme were 7.0 and 37°C. Two forms with molecular masses of 45 and 18 kDa were determined by SDS-PAGE and the enzyme was stable at 30 to 100°C and at pH 5.0 to 10.0. The highest  $\alpha$ -amylase activity was determined in 1% starch. *B. subtilis* can be exploited for industrial biotechnologies.

## REFERENCES

- Adams MWW, Kelly RM (1998). Finding and using thermophilic enzymes. Trends Biotechnol., 16: 329-332.
- Aquino ACMM, Jorge JA, Terenzi HF, Polizeli MLTM (2003). Studies on a thermostable  $\alpha$ -amylase from the thermophilic fungus *Scytalidium thermophilum*. Appl. Microbiol. Biotechnol., 61: 323-328.
- Becker P, Abu-Reesh I, Markossian S (1997). Determination of the kinetic parameters during continuous cultivation of the lipase-producing thermophile *Bacillus* sp. IHI-91 on olive oil. Appl. Microbiol. Biotechnol., 48: 184-190.
- Beg QK, Bhushan B, Kapoor M, Hoondal GS (2000). Production and characterization of thermostable xylanase and pectinase from *Streptomyces* sp. QG-11-3. J. Ind. Microbiol. Biotechnol., 24: 396-402.
- Bon EPS (1995). A tecnologia enzimatica no Brasil. ENZITEC 95: 9-14.
- Boyer EW, Ingle MB (1972). Extracellular alkaline amylase from a *Bacillus* species. J. Bacteriol., 110: 992-1000.
- Burhan A, Nisa U, Gokhan C, Omer C, Ashabil A, 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.
- Cappuccino JG, Sherman N (2001). Microbiology: A Laboratory Manual. 6<sup>th</sup> ed., Pearson education. Benjamin Cummings, San Francisco.
- Carozzi NB, Kramer VC, Warren GW, Evola S, Koziel MG (1991). Prediction of insecticidal activity of *Bacillus thuringiensis* strains by polymerase chain reaction product profiles. Appl. Environ. Microbiol., 57: 3057-306.
- Collins BS, Kelly CT, Fogarty WM, Doyle EM (1993a). The high maltose-producing  $\alpha$ -amylase of the thermophilic actinomycetes, *Thermomonospora curvata*. Appl. Microbiol. Biotechnol., 39: 31-35.
- Fitter J, Heberle J (2000). Structural equilibrium fluctuations in mesophilic and thermophilic  $\alpha$ -amylase. Biophys. J., 79: 1629-1636.
- Konsula Z, Liakopoulou-Kyriakides M (2004). Hydrolysis of starches by the action of an  $\alpha$ -amylase from *Bacillus subtilis*. Process Biochem., 39: 1745-1749.
- Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.
- Leveque E, Janecek S, Haye B, 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, Gao C (2008). Characterization of mutagenised acid-resistant alpha-amylase expressed in *Bacillus subtilis* WB600. Appl. Microbiol. Biotechnol., 78: 85-94.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193: 265-275.
- Murakami S, Nishimoto H, Toyama Y, Shimamoto E, Takenaka S, Kaulpiboon J, Prousoontorn M, Limpaseni T, Pongsawasdi P, Aoki K (2007). Purification and characterization of two alkaline, thermotolerant  $\alpha$ -amylases from *Bacillus halodurans* 38C-2-1 and expression of the cloned gene in *Escherichia coli*. Biosci. Biotechnol. Biochem., 71: 2393-2401.
- Nielsen JE, Borchert TV (2000). Protein engineering of bacterial  $\alpha$ -amylase. Biochim. Biophys. Acta, 1542: 253-274.
- Rehman A, Ali A, Muneer B, Shakoori AR (2007). Resistance and biosorption of mercury by bacteria isolated from industrial effluents. Pakistan J. Zool., 39(3): 137-146.
- Rehman A, Shakoori FR, Shakoori AR (2009). Heavy metal induced total proteins profile of ciliates isolated from industrial wastewater. Pakistan J. Zool., 41(4): 245-251.
- Sajedi RH, Naderi-Manesh H, Khajeh K, Ahmadvand R, Ranjbar B, Asoodeh A, Moradian F (2005). A Ca-independent  $\alpha$ -amylase that is active and stable at low pH from the *Bacillus* sp. KR-8104. Enzyme Microb. Technol., 36: 666-671.
- Sidhu GS, Sharma P, Chakrabarti T, Gupta JK (1997). Strain improvement for the production of a thermostable  $\alpha$ -amylase. Enzyme Microb. Technol., 21: 525-530.
- Shaw J-F, Lin F-P, Chen S-C, Chen H-C (1995). Purification and properties of an extracellular  $\alpha$ -amylase from *Thermus* sp.. Bot. Bull. Acad. Sin., 36: 195-200.
- Swain MR, Kar S, Padmaja G, Ray RC (2006). Partial characterization and optimization of production of extracellular alpha-amylase from *Bacillus subtilis* isolated from culturable cow dung microflora. Pol. J. Microbiol., 55(4): 289-296.
- Uehara H, Yamane K, Maruo B (1979). Thermosensitive, extracellular neutral protease in *Bacillus subtilis*: isolation, characterization and genetics. J. Bacteriol., 139: 583-90.
- Vander Maarel MJ, Van der Veen B, Uitdehaag JC, Leemhuis H, Dijkhuizen L (2002). Properties and applications of starch-converting enzymes of the  $\alpha$ -amylase family. J. Biotechnol., 94: 137-155.
- Wang S, Lu Z, Lu M, Qin S, Lie H, Deng X, Lin Q, Chen J (2008). Identification of archaeon-producing hyperthermophilic  $\alpha$ -amylase and characterization of the  $\alpha$ -amylase. Appl. Microbiol. Biotechnol., 80: 605-614.
- Yang CH, Cheng KC, Liu WH (2003). Optimization of medium composition for production of extracellular amylase by *Thermobifida fusca* using a response surface methodology. Food Sci. Agric. Chem., 5: 35-40.
- Yang C-H, Liu W-H (2007). Cloning and characterization of a maltotriose-producing  $\alpha$ -amylase gene from *Thermobifida fusca*. J. Ind. Microbiol. Biotechnol., 34: 325-330.