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## Isolation and identification of a new *Bacillus* strain for amylase production

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The objective of the study was to isolate amylase producing bacteria from Syrian soil samples. Amylolytic *Bacillus* sp were isolated on starch agar medium. The *Bacillus* SY134D was the best amylase producing isolate among two hundred and ten isolates showed the amylolytic activity. The closest phylogenetic neighbours according to 16S gene sequence, the *Bacillus* SY134D isolate was identified as *Bacillus subtilis*. The optimum temperature for amylase production was found to be 37°C. Amylase production occurred at pH 3.0-9.0 with a maximum at pH 5.0. The best enzyme activity was observed at pH 6.0 and temperature 70°C.

**Key Words:** *Bacillus* sp; solid culture;  $\alpha$ -amylase.

Alpha amylase (EC3.2.1.1, 1,4-alpha-D-glucan-glucanohydrolase) is an extracellular enzyme, which is involved in the starch processing industries where it breaks starch down into simple sugar constituents (Reddy *et al.*, 2003; Asgher *et al.*, 2007). Alpha amylases have extensive applications in starch processing, brewing and sugar production (Leveque *et al.*, 2000), in textile industries and in detergent manufacturing processes (Asgher *et al.*, 2007). Interestingly, the first enzyme produced industrially was an amylase from a fungal source in 1894, which was used as a pharmaceutical aid for the treatment of digestive disorders (Pandey *et al.*, 2000). Amylases are among the most important enzymes and account for about 30% of the world's enzyme production (Kandra, 2003).

These enzymes are found in animals, plants, bacteria and fungi. Sources of amylases in yeast, bacteria and fungi have been reported and their properties described by (Chi *et al.*, 2007; Liu and Xu, 2008; Gupta *et al.*, 2008). Although there are many microbial sources available for producing amylases, the capacity of *Bacillus* strains to produce large quantities of enzymes has placed them among the most important industrial enzyme producers. Indeed, they produce about 60% of commercially available enzymes (Burhan *et al.*, 2003).

Improvement of the yield of  $\alpha$ -amylase and consequent cost reductions depend on the selection of strains, the optimization of the factors affecting biosynthesis, genetic improvements, kinetic studies and the biochemical

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characterization of the enzyme. Each application of  $\alpha$ -amylase requires unique properties with respect to specificity and stability (Konsula and Liakopoulou-Kyriakides, 2004). Considering the industrial importance of amylase, the purpose of the current investigation was to screen *Bacillus* species isolated from soil in order to study their suitability with regard to  $\alpha$ -amylase production.

## Materials and Methods

### Bacterial strains and growth conditions

*Bacillus* isolates analyzed in this study were obtained from a national screening programme carried out in 2006-2007 from soil samples that covered areas throughout the interior of Syria (Ammounh *et al.*, 2011). Isolates were routinely cultured on nutrient agar plates (NA). The plates were incubated at 30 °C until bacterial colonies developed, kept at 4°C and subcultured every fifteenth day. Subsequently, the selected isolates were maintained in 20% glycerol at -80 °C.

### Inoculum preparation

5 mL of medium containing nutrient broth was transferred to a 50 mL tube and sterilized in an autoclave at 121°C for 20 min. After cooling, a loopfull of bacterial culture was aseptically transferred and rotated at 200 rpm (30°C) in a shaking incubator overnight. 1% of this culture was used to inoculate 20 mL of the same medium in 100 ml flask and incubated in orbital shaker at 30°C until the optical density at 600nm (OD<sub>600</sub>) reached 0.15 (cell density about 2x10<sup>8</sup> colony-forming unit (CFU)/mL).

### Screening of amylase producing bacteria

25  $\mu$ L of the prepared inoculum from each of *Bacillus* isolates was aliquoted onto starch agar plates contained (0.1% Remazol Starch), incubated at 30°C for 72 h and screened for amylase detection. Isolates having a higher ratio of clearing zone to colony size were chosen for further investigation.

### Solid state fermentation

Enzyme production was checked for selected *Bacillus* isolates in solid state fermentation using wheat bran procured from local market. Enzyme production was carried out in 100 mL Erlenmeyer flasks containing 5 g of wheat bran and nutrients (based on 100 mL of Liquid medium) plus distilled water to adjust the moisture to 60%. The fermentation medium consisted of: (g/L) K<sub>2</sub>HPO<sub>4</sub> 1; NaCl 3; MgSO<sub>4</sub>.7H<sub>2</sub>O 0.3; and yeast extract 3 and peptone 5, as nitrogen source. 1 mL of the prepared inoculum from each of the selected *Bacillus* isolates was transferred into the solid medium and placed in the incubator. Flasks were removed after 3 days of cultivation and the enzyme was extracted by adding 25mL distilled water containing 0.1% TritonX100. The flasks contents were stirred for 1.5 h on a magnetic stirrer. The clear supernatant was obtained by centrifugation (9800g for 15 min) used as enzyme source.

### Amylase assay

Amylase activity was determined as described by Okolo *et al.* (1995). The reaction mixture consisted of 1.25 mL of 1% soluble starch, 0.25 mL of 0.1 M acetate buffer (pH 5.0), 0.25 mL of distilled water, and 0.25 mL of crude enzyme extract. After 10 min of incubation at 50°C, the liberated reducing sugars (glucose equivalents) were estimated by the dinitrosalicylic acid (DNS) method of Miller (Miller *et al.*, 1959). The blank contained 0.5 mL of 0.1 M acetate buffer (pH 5.0), 1.25 mL of 1% starch solution and 0.25 ml of distilled water. One unit (IU) of amylase is defined as the amount of enzyme releasing 1  $\mu$ mol glucose equivalent per minute under the assay conditions.

### Effect of medium pH and temperature incubation

The influence of initial medium pH on amylase production was assessed by cultivating the strain in the basal media of pH ranging from 3.0 to 11.0. The effect of temperature was studied by performing the

fermentation at different temperatures, 30, 37, 40, 45 and 50 °C.

#### Effect of pH on amylase activity

The optimal pH for enzyme activity was determined by changing the assay reaction mixture pH using the following buffers (0.1 M): sodium acetate (pH 5.0), sodium phosphate (pH 6.0-7.0), Tris-HCl (pH 8), glycine-NaOH buffer (pH 9-10) and 1% soluble starch as substrate.

#### Effect of temperature on amylase activity and stability

The optimum temperature for the enzyme activity was evaluated by measuring the amylase activity at different temperatures (40-80°C) in 0.1 M sodium phosphate buffer (pH 7.0) and 1% soluble starch. The temperature stability was determined by measuring the residual activity at 55 °C, after incubation of the enzyme in different temperature ranging from 30 to 80 °C for 30 and 60 min at optimum pH.

#### Polymerase Chain Reaction (PCR) Amplification and 16S rDNA Sequencing

The primers flanked a highly variable sequence region of 545 bp towards the 5' end of the 16S rDNA region were used BcaF (5'- CTT TAC GCC CAA TAA TTC C -3') and BacR (5' -GTG CCT AAT ACA TGC AAG TC -3') (Nair *et al.*, 2002). Genomic DNA was extracted and purified using DNA extraction kit (BIOTOOLS, Cat. NO. 21.002). PCR mixtures were prepared using 10-20 ng of template DNA, 0.4 µM of each primer, 1U of *Taq* DNA polymerase (Promega), 0.2 mM each of dATP, dCTP, dGTP and dTTP (Promega), 2 mM MgSO<sub>4</sub>, and 3% dimethyl sulfoxide (DMSO). Amplification was done in a Bio-Rad T gradient thermocycler under the following conditions: a 5 min denaturation step at 95°C, followed by 30 amplification cycles (1 min at 95 °C, 1 min at 54 °C and 1 min at 72 °C) and an extra extension step of 10 min at 72 °C. PCR products were separated on a 1 % agarose gel to which ethidium bromide

was added and photographed under UV light. The amplification products were purified using QIAquick Gel Extraction kit (QIAGEN, Cat. No. 28704) and DNA sequencing on both strands was directly performed on an ABI 310 sequencer machine (Department of Molecular Biology and Biotechnology, AECS) and subjected to a BLAST search against the full EMBL / GenBank database available at NCBI public database (<http://www.ncbi.nlm.nih.gov>).

#### Results and Discussion

Five hundred and twenty five *Bacillus* isolates obtained from various types of soil samples collected from different Syrian regions were evaluated for amylase production. Of 525 isolates, 210 isolates were able to produce amylase. From this group, 21 isolates showed a highest ratio of clearing zone to colony size  $\geq 3$  cm on starch agar plates were selected, and tested for their amylase production in solid culture (Fig.1). Figure 1 shows the isolate SY134D was the highest amylase producer and therefore was selected for further studies.

DNA sequence analysis methods are an objective, reproducible, and rapid means of identification, therefore, they have been widely used (Li *et al.*, 2007). Identification of the isolate SY134D was done using 16S DNA gene sequences. The nucleotide BLAST similarity search analysis, based on the 16S DNA gene sequence revealed that this isolate belongs to the genus *Bacillus*. The closest phylogenetic neighbour according to the 16S DNA gene sequence data for SY134D isolate was *Bacillus subtilis* with 100 % of homology.

The pH of the growth medium plays an important role in terms of inducing enzyme production and morphological changes in the microbes (Pederson and Nielson, 2000; Kathiresan and Manivannan, 2006). The production of  $\alpha$ - amylase was investigated at different pH values ranging

from 3.0 to 9.0. The results presented in Fig. (2) shows that enzyme synthesis of *Bacillus* sp. SY134D was observed between pH 3.0 to 9.0. The pH 5 and 6 were the optimum pH value for  $\alpha$ -amylase production. When pH is altered below or above the optimum the activity is decreased or becomes denatured. Different organisms have different pH optima and decrease or increase in pH on either side of the

optimum value results in poor microbial growth and reduction in enzyme production (Gangadharan *et al.*, 2006; Ul-Haq *et al.*, 2002). Gangadharan *et al.* (2006) reported that pH=4 to be the best for the production of amylase by *Bacillus amyloliquefaciens*. Ul-Haq *et al.* (2002) reported pH=7.5-8.0 to be the best for the production of alpha amylase by *Bacillus subtilis*.

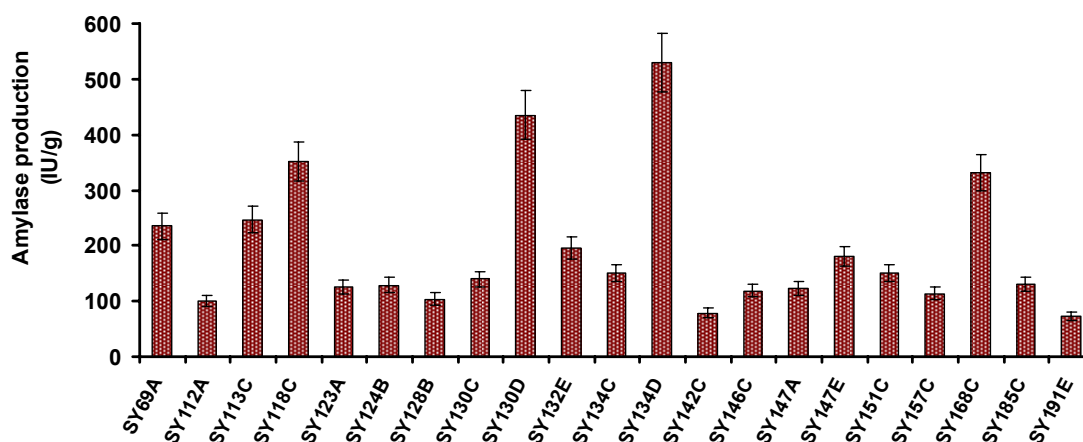


Fig. 1. Extracellular amylase production in solid state fermentation from selected *Bacillus* isolates obtained from Syrian soils.

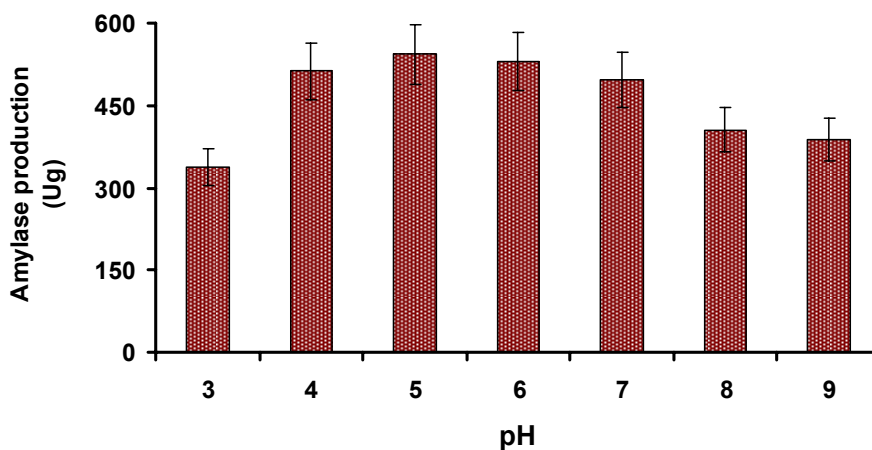


Fig. 2. Effect of media pH on amylase Production from *Bacillus* SY134D.

The effect of temperature on amylase production from SY134D strain was studied. The production of enzyme was determined at different temperatures ranging from 25°C to 45°C and optimum enzyme production was observed at 37°C

(Fig. 3). Other investigators also reported that maximum amylase production occurred at 37°C (Gangadharan *et al.*, 2006; Ul-Haq *et al.*, 2009). It might be due to the fact that 37°C is the optimal temperature of growth of bacterial culture and

subsequently for enzyme production. At temperature higher than 37°C amylase production was decreased. It might be due to that at high temperature, the growth of

the bacteria was greatly inhibited and enzyme formation was also inhibited (Pandey et al., 2000; Vidyalakshmi et al., 2009).

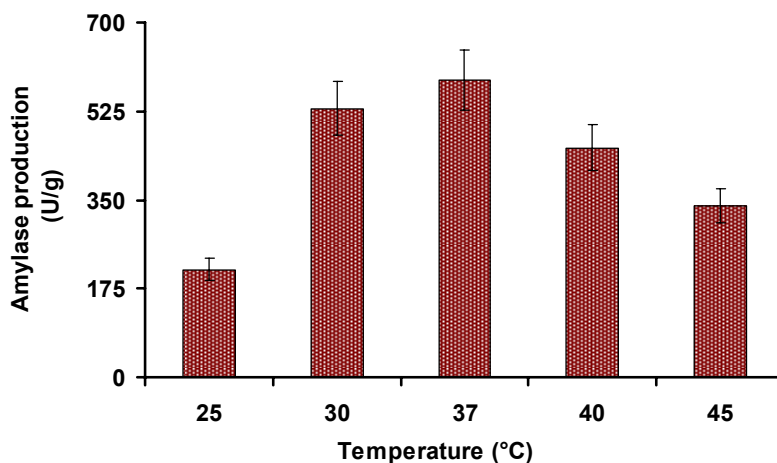


Fig. 3. Effect of incubation temperature on  $\alpha$ -amylase production from *Bacillus* SY134D.

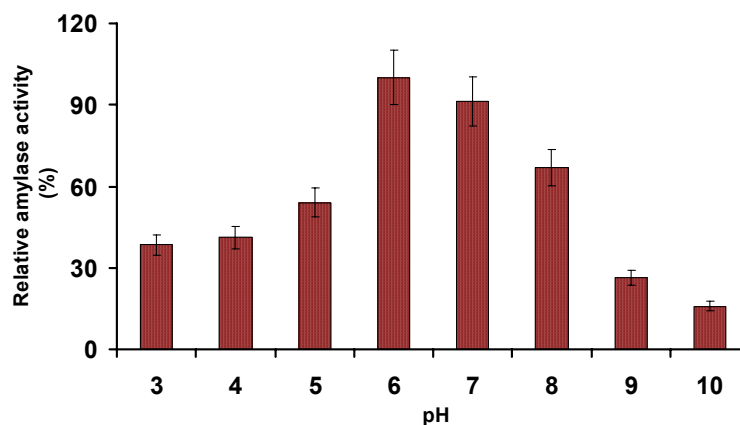


Fig.4. Influence of pH on the activity of amylase produced from *Bacillus* SY134D. Relative activity was determined at pH 6.

Enzyme activity is markedly affected by pH. This is because substrate binding and catalysis are often dependent on charge distribution on both, substrate and, in particular enzyme molecules (Shah and Madamwar, 2005). A pH range from 3 to 10 was used to study the effect of pH on amylase activity and the result given in Fig.4. A relatively low level of activity was observed at pH values 3.0 and 5.0. Amylase activity of *Bacillus* SY134D reached its

maximum at pH 6. At pH 7 and 8, amylase retained 91.26 and 66.95 from its activity, respectively. This indicates that amylase activity from *Bacillus* SY134D has high activity at neutral pH. Similar results were observed for *Bacillus* sp (Teodore and Martins, 2000; Devi et al., 2010; Mahdavi et al., 2010 ). It has been reported that optima of amylase activity vary from 3 - 11 (Vihinen and Mantsala, 1989; Silvaramakrishnan et al., 2006).

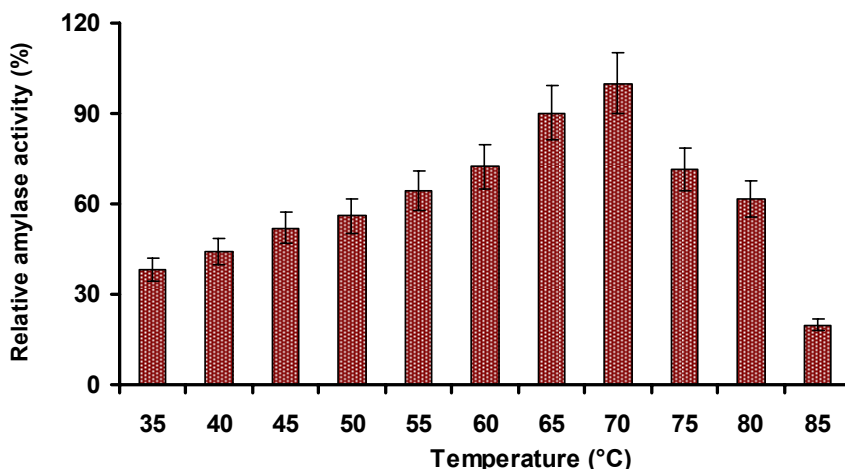


Fig.5. Influence of temperature on the activity of amylose produced from *Bacillus SY134D*. Relative activity was determined at 70 °C.

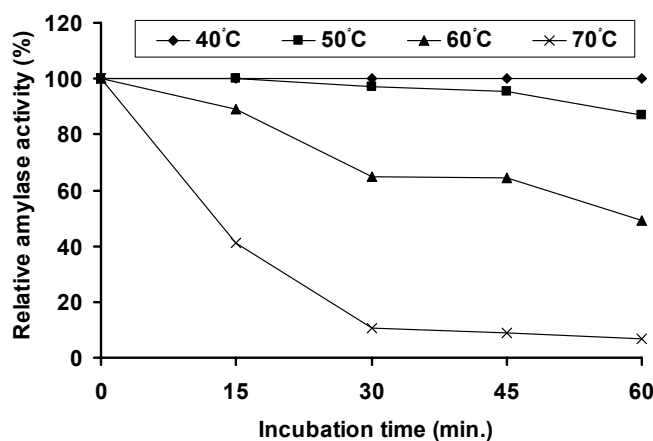


Fig.6. Thermostability of amylose from *Bacillus subtilis SY134D*.

The results illustrated in Fig. (5) indicate that the optimum temperature for  $\alpha$ -amylase activity was 70°C, maintained about 61.64 % of the maximal enzyme activity at 80°C. The optimum temperature for amylase activity from *Bacillus subtilis SY134D* was similar or quite higher to that of optimum temperature of amylase from some *Bacillus sp* strain sources reported in a previous study. Maximum  $\alpha$ -amylase activity produced by *Bacillus sp.* TS-23 and *Bacillus subtilis JS-2004* was achieved at 70°C (Asgher et al., 2007; Lin et al., 1998). On the other hand, *Bacillus sp.* DM-15 (Ozcan et al., 2010), *Bacillus subtilis KIBGEHAR* (Riaz et al., 2003) and *Bacillus subtilis MA9* (Devi et al., 2010) presented optimal

temperature for a maximum  $\alpha$ -amylase activity at 60°C. Thermal stability studies were carried out by preincubation the enzyme up to 1 h in the range of temperatures from 40 - 70 °C (Fig.6). The amylase remained stable after 1 h of incubation at 40 °C, while at 50 °C the residual amylase activity was 87%. At 60 °C, the enzyme retains 49.3 % activity after 1 h. These results indicated that the suitable temperature range for industrial application for amylase from *Bacillus subtilis SY134D* was 40-60°C.

The results of this investigation demonstrate that *Bacillus subtilis SY134D* is a potential source of thermophilic  $\alpha$ -

amylase production. The temperature properties for enzyme activity and stability make the enzyme quite suitable for biotechnological applications, especially in starch processing, laundry and textile industry. The process of amylase production from *Bacillus subtilis* SY134D can be commercialized after further optimization for enhanced enzyme production.

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