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Research Article

α-Amylase Production by Thermophilic Isolates of Bacillus licheniformis

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Abstract

Screening for amylolytic properties of isolates from four local soil samples was were carried out on starch agar plates. The isolated organisms were identified as four sub-strains of *Bacillus licheniformis*. Physiological studies of the isolates showed that temperature of 50°C, starch (1% w/v), and supplementation of growth medium by 5 mM Mg^{2+} supported optimum amylase production. Crude characterization of α-amylase revealed optimum amylolytic activity at pH 9. The four *Bacillus licheniformis* isolates produced thermostable α-amylase with characteristics suitable for use in starch processing or other industries.

Keywords: *α-Amylase; Thermostable; Bacillus; Starch*

Introduction

Microbes in soil are the key to carbon and nitrogen recycling in nature. Aerobic endospore-formers are important in the soil nitrogen and carbon cycles [1]. *Bacillus* species are very common soil micro-organisms. They are Gram positive, mesophilic, aerobic or facultative anaerobic spore-forming heterotrophs that produce heat-resistant endospores [2].

Bacillus licheniformis, a close relative of *Bacillus subtilis*, is widely distributed in the environment. As a facultative anaerobe, this may allow it to grow in a wide range of ecological niches. *B. licheniformis* is used in the biotechnology industry to produce enzymes, antibiotics, biochemicals such as glutamic acid and consumer products. Certain *B. licheniformis* isolates are capable of denitrification. The relevance of this characteristic to environmental denitrification may be small, as the species generally persists in soil as endospores [3]. As an endospore-forming bacterium, the ability of the organism to survive under unfavorable environmental conditions may enhance its potential as a natural biocontrol agent. *B. licheniformis* can be differentiated from other bacilli on the basis of metabolic and physiological tests [4,5]. However, biochemical and phenotypic characteristics may be ambiguous among closely related species. Molecular taxonomic studies indicate that *B. licheniformis* is closely related to *B. subtilis* and *B. amyloliquefaciens* on the basis of comparisons of 16S rDNA and 16S-23S internal transcribed spacer (ITS) nucleotide sequences [6].

B. licheniformis produces extracellular enzymes that may contribute to nutrient cycling and work as catalysts of biological processes in nature. Amylases from *B. licheniformis* are deployed for the hydrolysis of starch [7]. The α-amylases (1,4-α- D-glucan-glucan hydrolase, EC. 3.2.1.1) comprise a group of enzymes that catalyse the hydrolysis of the α-1,4 glycoside linkages in starch and related carbohydrates with retention of the α-anomeric configuration in the products. They are found in family 13 of the classification of glycosyl-hydrolases [8]. The production of bacterial α-amylase depends on the strain, composition of medium, method of cultivation, cell growth-phase, nutrient requirements, metal ions, pH, temperature, time of incubation and thermostability [9]. α-Amylases can be found in microorganisms, plants

and animals; where they play a dominant role in carbohydrate metabolism. These enzymes and related amylolytic enzymes are widely used in biotechnology for starch degradation [10], and in synthetic chemistry for the formation of oligosaccharides by transglycosylation [11,12]. Furthermore, these enzymes are used as targets for drug design in attempts to treat diabetes, obesity, hyperlipemia and dental caries [13].

Some researchers studied the effect of medium composition on alpha amylase production by *B. licheniformis*. For instance, in Pakistan, wheat bran (complex substrate) was used as substrate [14], while in Iraq, potato peels (carbon source) and wastes of fish (nitrogen source) have been added to medium for optimum production of alpha amylase [15]. In China, starch was the best carbon source for enzyme production [16]. In India, the oilseed cakes made of groundnut or mustard could completely replace the conventional peptone-beef extract medium as the fermentation base for the production of α-amylase by *B. licheniformis* [17].

There was a variation in the optimum pH and temperature for α-amylase activity in the above reported works. Hamad., *et al*. [14] reported a pH value of 7.5 and temperature of 40˚C, while Amal [15] reported pH 7.0 and temperature of 45˚C as optima. Tan and Tsay [16] reported pH 9.0 and temperature 90˚C as an optimal value for maximum production of α-amylase. In other studies such as in Indonesia, acidic pH of 5.0 was shown to be the best at temperature of 50˚C for α-amylase production [18].

In this research, we studied the effects of some environmental conditions (pH and temperature) and medium composition (carbon source and metal ions) on production of the enzyme α-amylase to determine the optimum conditions for the production and activity of alpha-amylase produced by each of 4 isolates of *Bacillus licheniformis* isolated from soil samples obtained from 4 different sites in Al-Bayda, AL- Jabal AL-Akhdar, Libya.

Here we report on the optimization of environmental conditions (medium composition, pH and temperature) for production of the enzyme alpha amylase by four local isolates of the bacterium *Bacillus licheniformis* isolated from soils in Al-Jabal Al-Akhdar, Libya.

Materials and Method

Isolation and identification of bacteria

Four soil samples were collected from four different sites in plastic bags, and then transferred to sterile bottles. For each soil sample 10 grams of soil were suspended in 90 of sterile saline phosphate buffer (pH 7.0). The suspensions were heated at 80˚C for 10 minutes, to kill vegetative cells. Ten-fold serial dilutions of samples were prepared, where dilution of 10^{-3} and 10^{-4} of each sample were plated on prepared sterile nutrient agar plates. Plates were incubated at 37˚C for 24 hours, and then examined for pure isolated colonies. Pure colonies (from each sample) were screened for amylase production by starch hydrolysis test. Plates of starch agar (1% soluble starch, 0.2% yeast extract, 0.5% peptone, 0.1% $MgSO_4$, 0.1% NaCl, 0.02% CaCl2 and 2% agar at pH 7.0) were inoculated, incubated at 37˚C and tested by flooding with 1% of iodine solution. Amylolytic isolates (from each sample) that produced a clear zone around colonies were selected to perform standard identification techniques such as culture characteristics, morphology, Gram reaction, spore formation, and biochemical tests.

Enrichment of bacterial growth

Broth medium (10g peptone, 3g K_2HPO_4, 1g $MgSO_4.7H_2O$, 0.5g starch/L) was prepared, and 100 mL of which were added to four 500-mL Erlenmeyer flasks separately, which were then autoclaved (121˚C, 15 minutes). Each flask was inoculated with a loopful of a different culture isolate, flasks were put on a rotary shaker (200 rpm) and incubated at 37˚C for 24 hours.

Effect of temperature on amylase production

Amylase production was tested as follows: For each isolate 7 flasks each contained 100 mL of prepared broth medium, were inoculated with 5 ml of primary inoculum. Flasks for each isolate were incubated at different temperature 25˚C, 30˚C, 35˚C, 40˚C, 45°C, 50˚C and 55˚C for 24 hours. Twenty ml of solution from each flask were centrifuged and enzyme activity was measured at 40˚C, pH 7.5.

Effect of pH on amylase activity

Effect of different levels of pH (5.0, 6.0, 7.0, 8.0, 9.0, 10.0) on amylase activity derived from each isolate was tested by varying the pH of assay reaction at enzyme incubation by the addition of acidic buffers (sodium acetate) and alkaline buffers (sodium phosphate), followed by measuring enzyme activity.

Effect of different carbon sources on amylase production

Effect of different carbon sources on amylase production by each isolate was tested by replacement of carbon source in broth culture medium. Different carbon sources included starch, sucrose, maltose, lactose, fructose and glucose at a concentration of 1% (w/v) each were tested. Amylase production was measured by determining amylase activity after centrifugation at 40°C, pH 7.5.

Effect of supplementation by different metal ions on amylase production

Preparations of broth culture medium were supplemented separately by different metal ions which were Mg2+, Ca2+, Ba2+, Mn2+, and Fe2+ in a concentration of 5 mM each. Production of the enzyme was measured by measuring amylase activity at 40°C, pH 7.5.

Amylase activity measurement

Enzyme was first extracted by taking 20 ml of broth culture for each treatment, followed by centrifugation at 10,000 rpm for 10 minutes. The supernatant contained the crude enzyme needed for activity assay. The activity of amylase was assayed according to the procedure of Miller [19] by incubating 0.5 ml enzyme with 0.5 ml soluble starch (1% w/v) prepared in 0.1 M sodium phosphate buffer (pH 7.05). After incubating the mixture at 40°C for 60 minutes the reaction was stopped by the addition of 2 ml of 3-5-dinitrosalicylic acid reagent (DNS) followed by incubation in a boiling water bath for 10 minutes followed by cooling. The absorbance was recorded using spectrophotometer at 560 nm. The released reducing sugars were calculated from absorbance versus concentration curve using maltose as standard. One unit of enzyme activity is defined as the amount of enzyme that released one micromole of reducing sugar as maltose per ml of supernatant per min under the conditions described above

$$Enzyme\,activity\,(unit\ /\ ml) = \frac{micromole\ maltose\ released}{ml\,enzyme\ in\ reaction\ mixture \times minutes}$$

Protein content of enzyme solution was determined by the method of Bradford [20] using bovine serum albumin as standard. A 100 µl of enzyme solution following centrifugation at 10,000 rpm/20 minute was added to 5.0 ml of Coomassie Brilliant Blue reagent then mixed by vortexing. The absorbance was read at 595 nm wavelength. The concentrations of the enzyme was calculated from bovine albumin concentrations versus absorbance curve.

Specific activity is calculated by dividing the enzyme activity by its protein content

$$Specific\ activity\ (U\ /\ mg) = \frac{unit\ /\ ml\ enzyme}{mg\,protein\ /\ ml\,enzyme}$$

Results and Discussion

The four isolates were characterized based on cultural, morphological and biochemical characteristics and were all identified as different isolates of *B. licheniformis* (Figures 1, 2, 3, 4 and Table 1). The isolates were numbered *B. licheniformis 1, B. licheniformis 2, B. licheniformis 4,* and *B. licheniformis 0.*

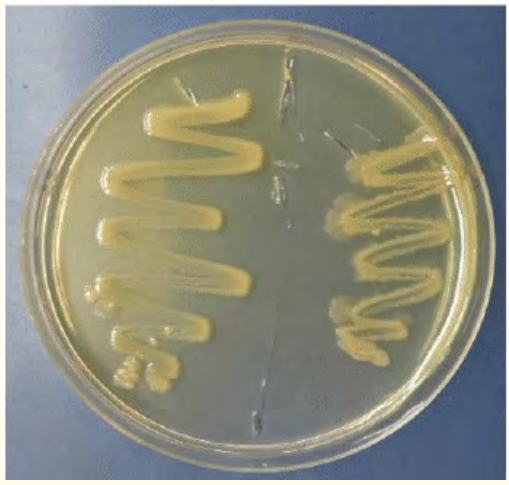

Figure 1: *Two of the isolates of Bacillus licheniformis grown at 37°C: colonies on nutrient agar plates with irregular (undulate, fimbriate) margins, with a rough and wrinkled surface showing a "licheniform", or hair-like growths. Color ranges from opaque to white.*

Figure 2: *A starch agar plate showing amylase activity with clear white zone at the center surrounding the single colony of one of the isolates of B. licheniformis isolated from soil.*

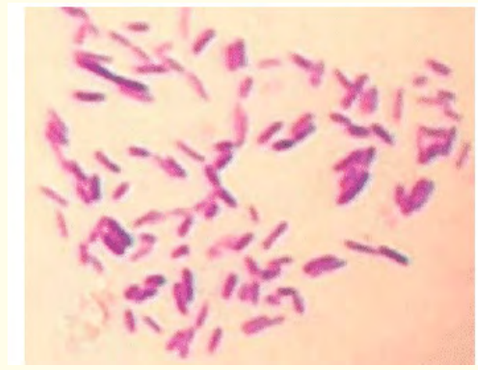

Figure 3: *Gram stain of Bacillus licheniformis: Gram positive rods.*

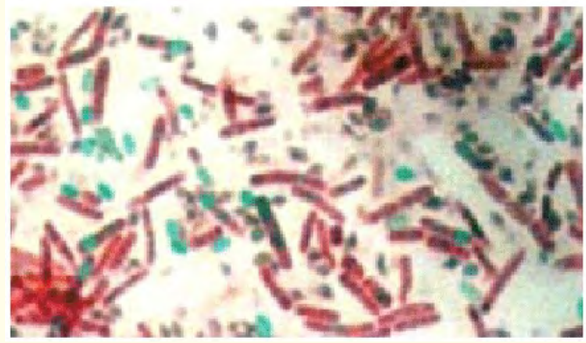

Figure 4: *Tpore stain of Bacillus licheniformis (ellipsoidal and cylindrical spore that do not exceed the diameter of the sporangium).*

Characterization test	Bacterial strain
Catalase	Positive
Nitrate reduction	Positive
Gelatin liquefaction	Positive
Voges-Proskauer (acetoin)	Positive
Citrate utilization	Positive
Oxidative-Fermentative test	Oxidative
Oxygen relationship	Facultative anaerobe
Indole production	Negative
Anaerobic growth	Positive
Acid from sugar fermentation	
Glucose	Positive
Sucrose	Positive
Mannitol	Positive
Xylose	Positive
Maltose	Positive
Growth at 45°C	Positive
Probable identity	*Bacillus licheniformis*

Table 1: *Biochemical characteristics of the 4 isolated amylolytic bacterial strain.*

Amylase production increased as the temperature of the reaction mixture was increased. *Bacillus licheniformis* was capable of maximum amylase production at 50°C (Figure 5), further increase to 55°C led to decrease in amylolytic production. The increase in amylase activity with temperature can be attributed to increase in the collision between substrate and enzyme and further increase in temperature beyond 70°C destabilized the 3-dimensional structure of the enzyme resulting in its denaturation.

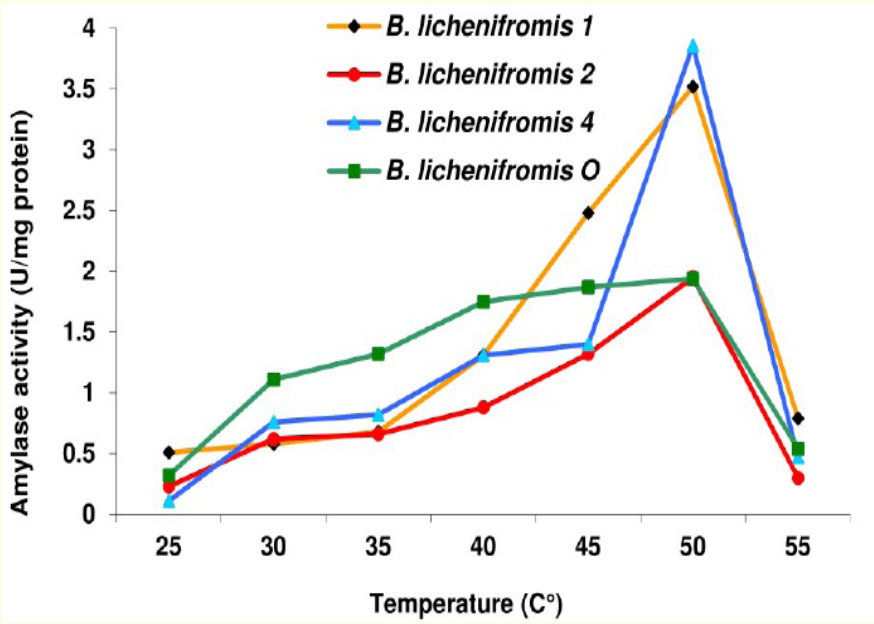

Figure 5: *Effect of temperature on amylase production produced by different isolates of Bacillus licheniformis.*

The effect of pH on the activity of α-amylase is shown in figure 6. The activity of amylase was gradually increasing as the pH of the substrate increased from pH 5 to pH 9. Maximum amylase activity was observed at pH 9. This is in line with Vihinen and Mantsala [21] who reported that the pH optima of α-amylases vary from pH 2 - pH 12, although Iraj., *et al*. [22] reported that *B. licheniformis* Shahed-07 had an optimum activity at pH 7.0.

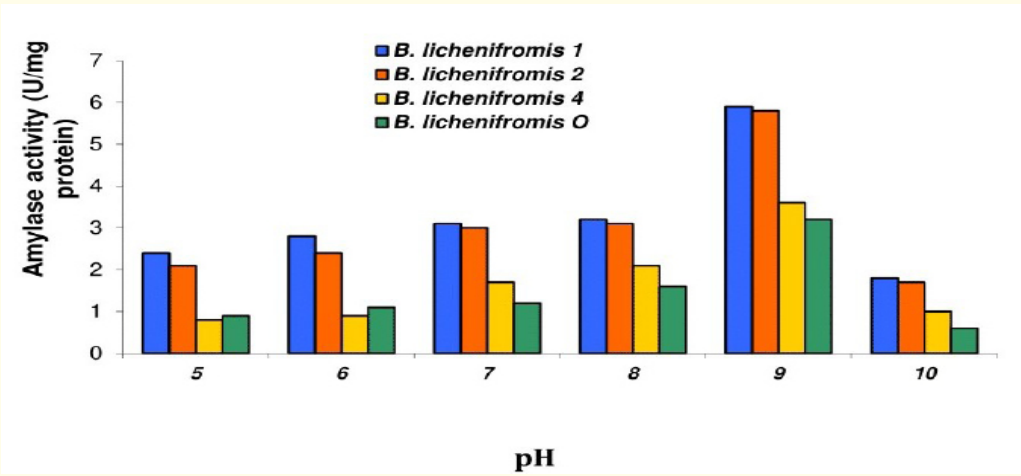

Figure 6: *Effect of pH on amylase activity produced by different isolates of Bacillus licheniformis.*

The nature of carbon source in culture media is an important factor for the production of extracellular amylase [23]. Figure 7 shows the effect of different carbon sources on the production of α-amylase. Among the different carbon sources, maximum enzyme production was exhibited by starch (1% w/v). This is in accordance with the reported maximum α-amylase production when starch was used as the carbon source [24-29]. Amylolytic activity was the least when the medium was supplemented with fructose or glucose. This suggested that fructose and glucose were repressors of α-amylase enzyme in this study which is similar to the observed catabolic repression by glucose and fructose in *Bacillus coagulans* reported by Babu and Satyanarayana [30].

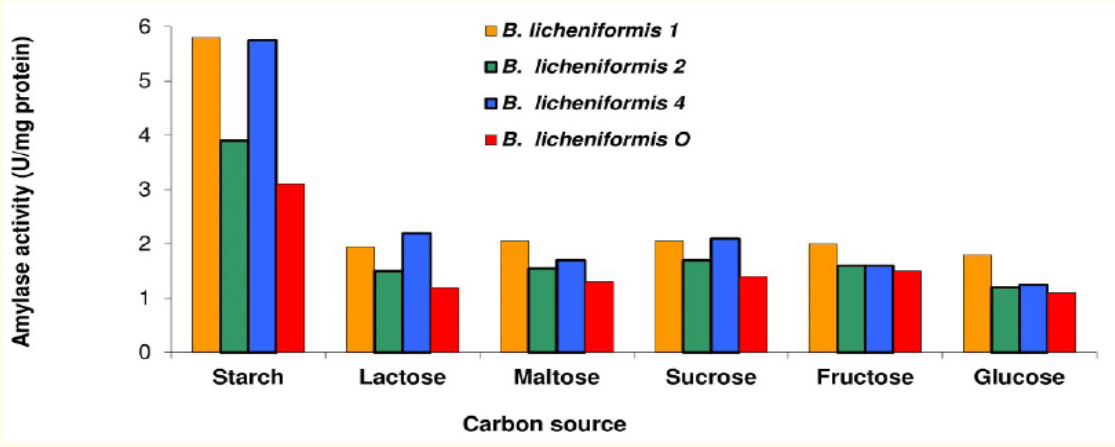

Figure 7: *Effect of different carbon sources on amylase production by different isolates of Bacillus licheniformis.*

The effects of various metal ions at concentrations of 5 mM on the α-amylase enzyme production were assessed (Figure 8). Optimum enzyme production was shown when Mg^{+2} was used. However a decrease in the enzyme production was shown when using Ca^{+2}, Ba^{+2}, Mn^{+2}, and Fe^{+2} ions. This is in contrast with [31,32] who reported that Ca^{+2} increased the amylase production and stability.

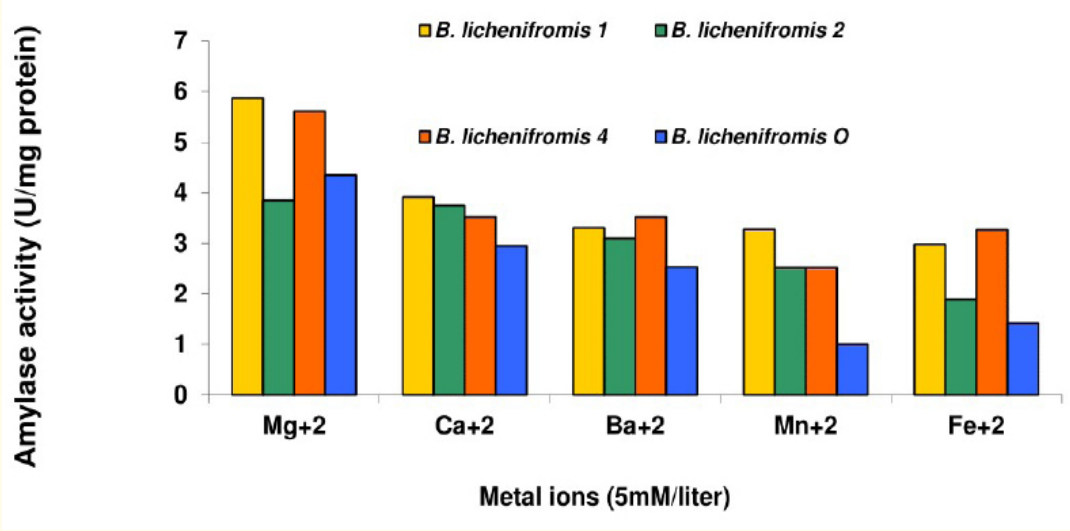

Figure 8: *Effect of different metal ions on amylase production by different isolates of Bacillus licheniformis.*

Conclusion

In conclusion, the findings of this work indicate that *B. licheniformis* produced a thermostable and high pH tolerant α-amylase at 1% (w/v) soluble starch, and medium supplementation with 5 mM magnesium.

The enzyme has characteristics that are suitable for starch processing which can be used to replace non-renewable energy sources by renewable starch-containing agricultural waste in an attempt to provide a tenable solution to the energy crisis.

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