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Isolation and Identification of a Bacterial Strain Producing Thermostable α- Amylase

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ABSTRACT. *This study was aimed at isolating and identifying thermostable α-amylase producing bacteria, and characterization of α- amylase produced by the selected strains. A total of 72 bacterial strains were isolated from different sources such as soil containing decaying materials (42), gruel of rice (09), soil receiving kitchen waste (06), bakery waste (08), flour mill waste (04) and tea waste (03) by incubating at 37°C for 24 h in a medium containing nutrient agar (25.0 g/L) and starch (3.0 g/L). Bacterial colonies (72) capable of hydrolyzing starch were purified and transferred to starch - nutrient agar slants for activation and then to fermentation medium. The highest α-amylase activity producing [7.0 ±0.21 Um/L at 24 h] strain (BS_1) was isolated from soil receiving bakery waste. The strain BS_1 was identified as Bacillus licheniformis based on genus, species analysis, morphological and biochemical characterization. Crude α-amylase showed zero order kinetics for 5 min and gave the highest activity at 90°C and pH 7.0. Michaelis constant of the crude enzyme to soluble starch was 2.85 g/L at 90°C and pH 7.0. In the absence of additives α-amylase retained 37.6% of its initial activity at 90°C at 30 min and 10.4% of its activity at 1 h, whereas at 80°C and pH 7.0 it retained 68.8% of its initial activity at 30 min and 59.1% of its initial activity at 1 h. Half life of the enzyme was 21 min at pH 7.0 and 90°C.*

Key words*: Activity, α-Amylase, Bacillus licheniformis, Half life, Thermostable.*

INTRODUCTION

Enzymes from fungal and bacterial sources have been increasingly applied in industrial sectors (Pandey *et al*., 2000). Amylases contribute as a class of industrial enzymes constituting approximately 25% of the enzyme market (Sindhu *et al*., 1997; Rao *et al*., 1998). It is desirable that α-amylases should be active at the high temperatures of gelatinization (100-110°C) and liquefaction (80-90°C) to economize the processes. Therefore, there has been a need for more thermophilic and thermostable α-amylases (Sindhu *et al*., 1997). The most widely used thermostable enzymes in the starch industry are the amylases (Poonam and Dalel, 1995; Crab and Mitchinson, 1997; Sarikaya *et al*., 2000). An extremely thermostable α-amylase is produced by *B. licheniformis* (Morgan and Priest, 1981). The objective of this work was to isolate and identify a bacterial strain, which can produce thremostable α-amylase with desired characters that can be used in industrial sectors.

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MATERIALS AND METHODS

Sample collection

Samples were collected in containers under sterile conditions from soil contaminated with decaying materials i.e. soil receiving kitchen waste, bakery waste, flour mill waste and tea waste and hot white rice gruel and compost.

Media and culture conditions for enzyme production

The starch-nutrient agar plates and slants containing 25 g/L of nutrient agar and 3 g/L of starch at pH 7 were used for the storage of strains. The activation medium contained 3.0 g/L of starch and 25 g/L of nutrient broth at pH 7. Two loops of bacterial strains grown on starch-nutrient agar slants were transferred to activation medium (10 mL) and incubated in a shaker water bath at 42°C at 120 rpm for 12 h. Fermentation medium contained soluble starch (2 g/L) peptone (2 g/L), $(NH_4)_2 SO_4$ (2 g/L), NaCl (2 g/L), KH_2PO_4, (1 g/L), K_2HPO_4, (2.5 g/L), $FeCl_3$, (1 g/L), $MgCl_2$, (0.01 g/L), and $CaCl_2$, (0.01 g/L) at pH 7.The fermentation medium was inoculated with the activated culture (20%, v/v) and incubated at appropriate temperatures. Volume ratio of 1:10 (media: flask) was maintained in experiments performed in shaker flasks. All the experiments were carried out in triplicate.

Determination of α-Amylase activity

The assay mixture consisted of 0.5 mL of diluted enzyme solution and 0.5 mL of 20g/L starch in 0.01M phosphate buffer (pH 7.0), incubated at 90°C for 5 min and the increase in the reducing sugar was determined by dinitrosalicylic acid method (Miller, 1959). One unit of α-amylase activity was defined as the amount of enzyme that releases one μmol reducing sugar equivalent to glucose per min. at 90°C and at pH 7.0 with 20 g/L starch.

Isolation and characterization of starch utilizing strains

From each sample, 1 g was drown and mixed with 9 mL of sterile saline (9 g/L NaCl). The samples were then serially diluted from 10^{-4} to 10^{-6} with saline and spread plated on starch-nutrient agar plate. After 24 h of incubation at 37°C, single colonies of different sizes were selected and the diameters of colonies were measured. Single colonies showed different morphological characteristics such as size, shape, colour, elevation and margin were identified from different plates streaked with diluted samples. Single colonies which formed clear halos with Gram's iodine were identified as starch utilizing strains. The halo diameters of selected single colonies were measured after 24 h of incubation to determine the halo diameter to colony diameter ratio. Selected single colonies were purified by repeated streaking and transferred to starch-nutrient agar slant.

Screening for α-amylase producing bacteria

Purified bacterial strains were activated, transferred into the fermentation medium and incubated in a shaker water bath at 42°C at 120 rpm for 24 h. The spent medium was centrifuged at 3000 rpm for 20 min. Cell-free filtrate was used as enzyme source for the assay of α-amylase activity.

Selection of the best thermostable α-amylase producer

The selected bacterial strains were activated at different temperatures (42, 45, 50 and 55ºC), transferred to the fermentation medium and incubated at respective temperatures at 120 rpm.

Effect of fermentation period

The selected strains were activated, transferred to the fermentation medium and incubated in a shaker water bath at 42ºC and 120 rpm. Effect of fermentation period on enzyme activity was assessed by monitoring the α-amylase activity produced at pH 7.0 and 90ºC.

Determination of the genus of the selected strain

Colony morphology such as form, elevation, margin, diameter (mm) after 24 h, colour and surface of the best α-amylase producer, when grown on starch-nutrient agar plate were observed. Biochemical tests such as catalase and oxidase tests and Kligler iron agar pattern, motility and oxygen requirement were studied and finally the genus of the selected strain was identified **(**Cheesbrough, 1984).

Determination of the species of selected strain

Biochemical tests such as citrate utilization test, indole test, Voges-Proskauer (VP) test, production of urease, nitrate reduction test, decomposition of tyrosine and haemolysis studies on blood agar were carried out. Growth temperature and salt tolerance test were also carried out **(**Cheesbrough, 1984).

Kinetic properties of the crude enzyme

Starch solution (20 g/L, 0.25 mL, pH 7.0) was mixed with 0.25 mL of diluted crude enzyme at 90ºC and the amount of glucose produced was monitored. The effect of temperature, pH and substrate concentration on α-amylase activity was studied and K_m and Vmax values of the enzyme were calculated from Lineweaver-Burk (double-reciprocal) plot.

Temperature stability of the enzyme

The thermal stability of the α-amylase from *Bacillus licheniformis* was studied at pH 7.0 and at 80 and 90ºC without additives and the half life of the enzyme was calculated.

RESULTS AND DISCUSSION

Isolation and selection of thermostable α-amylase producing bacteria

Bacteria isolated from starch rich materials may have better potential to produce enzyme under adverse conditions (Mishra *et al*., 2008). Microorganisms that produce amylases could be isolated from places such as soil around mills, cassava farms and processing factories as well as flour markets (Fossi, *et al*., 2005). During the study, α-amylase producing bacterial strains were isolated from soil contaminated with decaying materials including kitchen waste, bakery waste, flour mill waste, soil receiving tea waste, hot white

rice gruel of and compost heap. On starch-nutrient agar plate, 72 single bacterial colonies, which produced clear halos with iodine solution were selected and purified. When α-amylase production by these selected 72 strains was measured, five strains did not produce α-amylase at pH 7.0 and at 42ºC at 24 h, while 62 strains produced α-amylase activities less than 1.0 Um/L. Among the rest, three strains which produced α-amylase at between 1.0 and 7.0 Um/L were labelled as strain BS_1 (from soil receiving bakery waste), strain FS_2 (from soil receiving flour mill waste) and strain GS_3 (from hot gruel of white rice). The halo diameters of the strains BS_1, FS_1 and GS_1 were 20, 18 and 17 mm and their halo diameter to colony diameter ratios were 4.0, 3.6 and 3.4, respectively (Table 1).

Table 1. Activities of α-amylases (at 90ºC and at pH 7.0), halo diameter and colony diameter (after 24h) produced by the strains BS_1, FS_1 and GS_1

Strain	Diameter of clear halo (mm)	Diameter of colony (mm)	Halo diameter to colony diameter ratio	Activity (at 24 h) (UmL^{-1})
BS_1	20	05	4.0	7.01
FS_1	18	05	3.6	5.52
GS_1	17	05	3.4	4.74

Among the three strains, BS_1, which was isolated from soil receiving bakery waste produced the highest α-amylase activity [7.0 ±0.21 Um/L] at pH 7.0 and at 90˚C and hence it was selected for further studies. The strain BS_1 also showed the highest value for halo diameter to colony diameter ratio (4.0) from among the strains, which showed highest α-amylase activities.

Selection of highest titre thermostable α-amylase producing bacteria

In order to select the best thermostable α-amylase producing bacteria, effect of fermentation temperature on α-amylase production was studied in the temperature range of 42-55ºC (Fig. 1). All three strains produced the highest α-amylase activities at 42ºC and with increase in temperature the production of α-amylase decreased. However, among the three strains, BS_1 showed the highest activity at all the temperatures.

Effect of fermentation period

The suitable fermentation period at which highest α-amylase activity production takes place was studied. All three strains produced high α-amylase activities [strain BS_1 7.01±0.21, strain FS_1 5.52± 0.34 and strain GS_1 4.73± 0.51 Um/L] at 24 h (Fig. 2), while growth of all three strains reached maximum at 12 h (Fig. 3). Based on the highest α-amylase producing ability at high temperature (42ºC), the strain BS_1, which was selected as the best strain was further characterized.

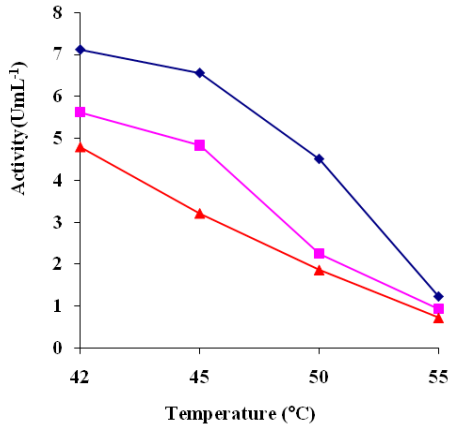

Fig. 1. Production of α-amylases by the Strains BS_1 (◆), FS_1 (■) and GS_1 (▲) at different temperatures and pH 7.0 at 24 h in fermentation medium.

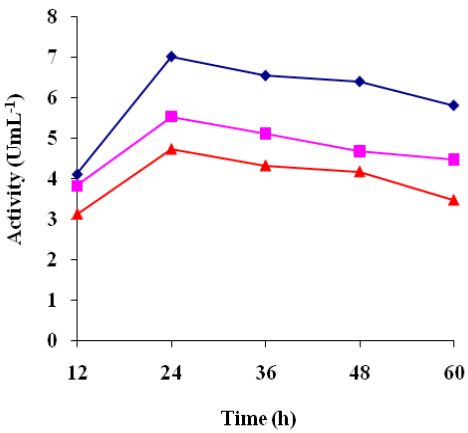

Fig. 2. Production of α-amylases by the strains BS_1 (◆), FS_1 (■) and GS_1 (▲) in fermentation medium at 42ºC and at pH 7.0.

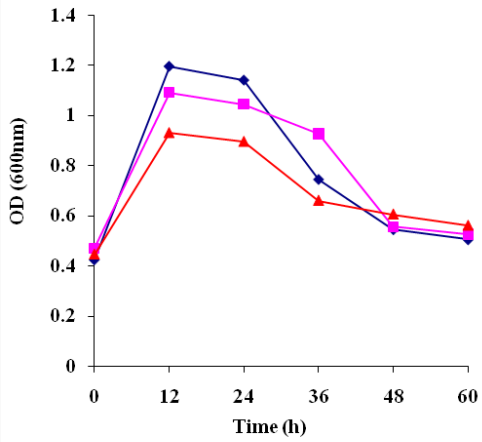

Fig. 3. Cell growth of strains BS_1 (◆), FS_1 (■) and GS_1 (▲) in fermentation medium at 42ºC and at pH 7.0. Identification of the selected strain

Identification of the genus

Morphological and biochemical (Table 3) characteristics were used to identify the Genus of the strain (Table one and two). The strain BS_1 was gram positive, rod shaped, motile, and gave positive results for catalase test and negative results for oxidase test. It produced acid slant, acid butt, no gas and no H_2S in the KIA reaction. Based on the results, the strain BS_1 was identified as belonging to Genus *Bacillus*.

Identification of species

Biochemical characteristics were used to identify the species of the strain. The strain BS_1 gave positive results for VP-test, citrate and nitrate reduction tests and gave negative results for indole, urease and tyrosine utilisation tests. It gave β-haemolysis on blood agar and growth in the medium containing 7% NaCl (Table 3). Based on the above results the strain BS_1 belongs to species *licheniformis*. The strain BS_1 was identified as *Bacillus licheniformis* based on the Genus and Species identification and comparing with the characters of *Bacillus licheniformis* (Table 4).

Table 2. Microscopic characteristics of the strain BS_1

Characteristic	Results
Form	Irregular
Elevation	Flat
Margin	Irregular
Opacity	Opaque
Colour	Pale
Surface	Moist, shiny
Diameter of colony after 24 h (mm)	05
Diameter of clear halo (mm)	20
Gram staining	(+) ve
Shape of vegetative cell	Rod
Spore formation	(+) ve
Motility	Actively motile

Table 3. Biochemical and cultural characteristics of the strain BS_1

Biochemical test	Results
Growth in air	(+) ve
Anaerobic growth	(+) ve
Indole production	(-) ve
Voges-Proskaeur test	(-) ve
Catalase production	(+) ve
Citrate production	(+) ve
Oxidase production	(-) ve
Urease test	(-) ve
KIA pattern	Acid slant, Acid butt, H_2S (-) ve , Gas (-) ve Lactose fermentation(+) ve
Nitrate reduction test	(+) ve
Hyrolysis of tyrosine	(-) ve
Haemolysis on blood agar	β- haemolysis
Growth in 7% NaCl	(+) ve
Growth at 55ºC	(+)ve

Table 4. Comparision of biochemical and cultural characteristics of known strains of *Bacillus licheniformis* with the selected strain BS_1

Characteristic	*Bacillus licheniformis*	BS_1
Gram reaction	Positive	Positive
Motility	Positive	Positive
position of spore	Central	Central
Shape of spore	Ellipsoidal	Ellipsoidal
Growth at 45°C	Positive	Positive
Growth at 50°C	Positive	Positive
Growth at 55°C	Positive	Positive
Growth in 7% NaCl	Positive	Positive
Anaerobic growth	Positive	Positive
Acid from glucose	Positive	Positive
Acid from xylose	Positive	Positive
Acid from mannose	Positive	Positive
Utilization of citrate	Positive	Positive
Production of urease	Negative	Negative
Production of indole	Negative	Negative
Results for VP test	Positive	Negative
Nitrate reduction	Positive	Positive
Starch hydrolysis	Positive	Positive
Production of oxidase	Negative	Negative
Production of catalase	Positive	Positive
Chains of cells	Positive	Negative
Hydrolysis of Tyrosin	Negative	Negative
Hemolysis	Variable	(β- hemolysis)
Swelling of cells	Negative	Negative
Score		81%

(Fisher, 1895; Barrow and Feltham, 1993)

Kinetic properties of crude α-amylase

The α-amylase obtained from *Bacillus licheniformis* showed zero order kinetics for 5 min. Therefore, the reaction time of the enzyme was fixed as 5 min (Fig. 4) in the following experiments.

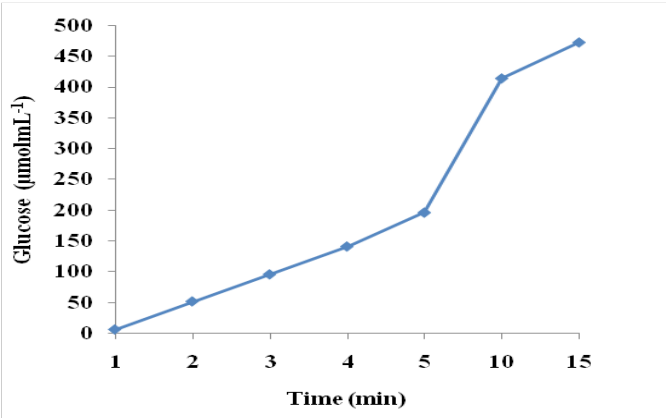

Fig. 4. Activity of α-amylase produced by *B. licheniformis* with soluble starch as a function of time at pH 7.0 and at 90ºC.

The activity of the α-amylase obtained from *Bacillus licheniformis* was assayed at different temperatures ranging from 30-95ºC at pH 7.0. The optimum temperature for activity of α-amylase was 90ºC (Fig. 5). The purified α-amylase of *Bacillus licheniformis* CUMC305 showed maximal activity at 90°C and pH 9.0 (Krishnan and Chandra, 1983). The enzyme showed 86.1, 93.6 and 95.1% of its maximum activity at 95, 80 and 85ºC, respectively, at pH 7.0.

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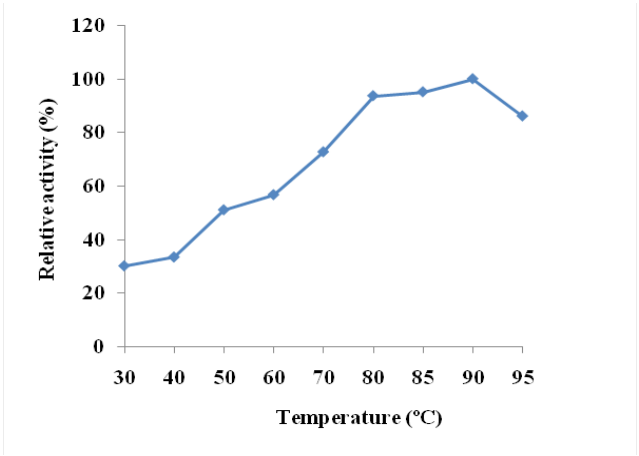

Fig. 5. Effect of temperature on the activity of α-amylase from *B. licheniformis* at pH 7.0.

In the pH range between 6.0 -10.0, the activity of α-amylase produced by *B.licheniformis* was studied at 90°C and the optimum was 7.0 (Fig. 6). α-Amylase from *Bacillus licheniformis* showed 55.8 and 83.9% of its maximum activity at pH 6.0 and 8.0, respectively. Neutral pH was found to be optimal for amylase activity by *B. thermooleovorans* NP54 and also reported in *B.coagulans* (Medda and Candra, 1980), *B.licheniformis* (Krishnan and Candra, 1983) and *B. Brevis* (Tsvetkov and Emanyilova, 1989).

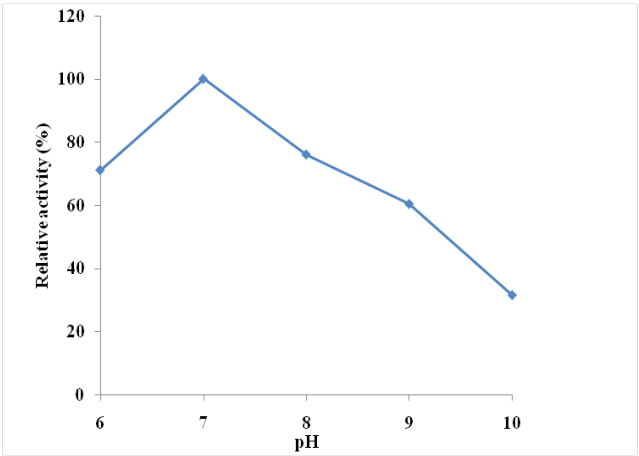

Fig. 6. Effect of pH on the activity of α-amylase from *B. licheniformis* at 90ºC.

When the substrate concentration was increased from 5 to 30 g/L the activity of the enzyme increased up to 20 g/L (Fig. 7) at pH 7.0 and at 90ºC. Michaelis constant (K_m) of the crude enzyme to soluble starch was 2.85 g/L and Vmax value was 238 Um/L at 90ºC and pH 7.0 (Fig. 8).

Effect of temperature on stability

Bacillus subtilis AX20 α-amylase showed 60% and 35% of maximum activity at 40 and 70ºC, respectively and the amylase showed stability at 50ºC for 45 min. (Mohsen *et al*., 2005). Thermal stability of α-amylase was studied without additives. α-Amylase produced by *Bacillus licheniformis* retained 37.6% of its initial activity at 90ºC and at pH 7.0 at 30 min of incubation and 10.4% of its activity at 1 h, whereas the enzyme retained 68.75% of its initial activity at 80ºC and at pH 7.0 at 30 min of incubation and 59.1% of its initial activity at 1 h. Half life of enzyme was 21 min at 90°C and pH 7.0.

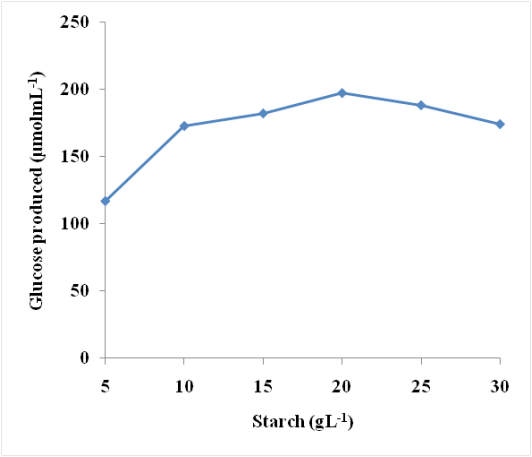

Fig. 7. Effect of substrate concentration on the activity of the α-amylase from *B.licheniformis* at pH 7.0 and 90ºC.

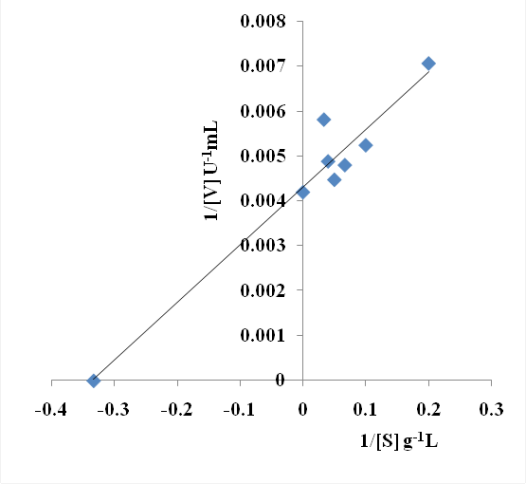

Fig. 8. Lineweaver-Burk (double-reciprocal) plot for α-amylase obtained from *B. licheniformis.*

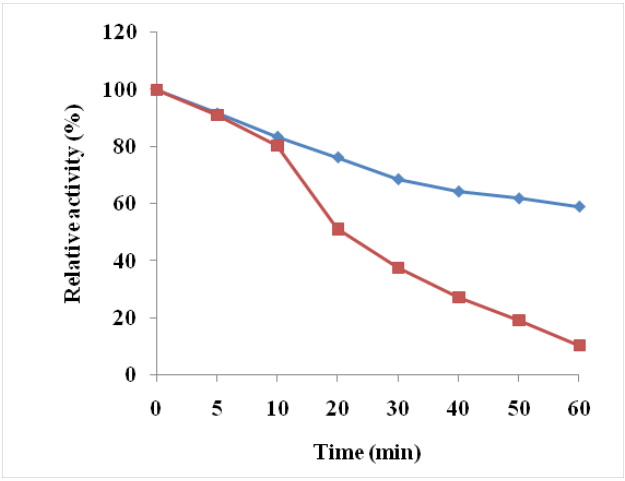

Fig. 9. Thermal stability of α-amylase (◆) at 80°C, and (■) at 90°C without additives and at pH 7.0.

CONCLUSION

A total of 72 bacterial strains which produced clear halos in the starch- nutrient agar medium were isolated and purified. Among the 72 bacterial strains, one strain was selected as best α-amylase producer and identified as *Bacillus licheniformis*. The optimum temperature and pH for the activity of the α-amylase obtained from this strain were 90°C and 7.0, respectively. Michaelis constant (K_m) of the crude enzyme to soluble starch was 2.85 g/L and Vmax value was 238 Um/L. As the *Bacillus licheniformis* was able to produce maximum α-amylase activity at 24 h and the enzyme showed neutral pH optimum and temperature stabilities at 90°C without additives, this strain can be recommended for industrial applications.

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