ISOLATION AND BIOCHEMICAL CHARACTERIZATION OF AN AMYLASE PRODUCING THERMOPHILIC BACTERIUM FROM GARDEN SOIL

Isha Kohli[1], Rakesh Tuli[2] and Ved Pal Singh[1]*

[1]Applied Microbiology and Biotechnology laboratory, Department of Botany, University of Delhi, Delhi-110 007, India.
[2] National Agri-Food Biotechnology Institute, Mohali, Punjab-160 071, India.
** E-mail: vpsingh_biology@rediffmail.com*

Abstract: A thermophilic bacterium (strain Th-3), which was able to degrade starch maximally, was isolated from the soil of Delhi University Botanical Garden. The temperature and pH optima and incubation time for the maximum growth of isolated bacterium were found to be 45°C, pH 6.0 and 24 h, respectively. In addition to amylase production, the bacterium had also shown positive results for production of protease, lipase and catalase as well as for nitrate reduction. Th-3 exhibited maximum amylolytic activity, when assayed at 45°C at pH 6.5 in the culture harvested at 24 hours of growth. The bacterium was non-pathogenic, as tested on Himedia sheep blood agar plates. The strain was sensitive to most of the antibiotics tested, except ampicillin and kanamycin to which it had shown resistance. The biochemical, microscopic and morphological features of the isolated strain indicated that it was Gram-positive, rod-shaped and closely resembled *Bacillus* species.

Keywords: Amylase, Amylolytic activity, Starch degrading enzyme, Thermophilic amylase, Thermophilic bacterium

INTRODUCTION

The starch degrading enzyme (amylase) is among the most important enzymes widely used in industries and commercial sectors. Microbial amylases are more stable, economical and easily available. Amylases are the enzymes that catalyse the breakdown of starch into sugars by breaking polysaccharides bonds (Gupta *et al*., 2003). Isolation of amylases can be done from a number of sources, such as plants, animals and microbes, though microbial amylases are most preferred and used in industry (Dey and Banerjee, 2012). The major advantages of using microorganisms for the production of amylases are their ability to produce them in bulk and ease at which they can be manipulated for desired products (Roses and Guerra, 2009). Amylases can be divided in to three groups, i.e., α-amylases, β-amylases and glucoamylases. α-Amylases [endo-1,4-α-D-glucan glucohydrolase, EC 3.2.1.1] are extracellular amylases. These are endoacting enzymes that cleave 1, 4-α-D-glucosidic linkages between adjacent glucose units in the linear amylase chain. β-amylases [α-1, 4-glucan maltohydrolase, EC 3.2.1.2] are exoacting enzymes that cleave non-reducing chain ends of amylose, amylopectin and glycogen molecules. Glucoamylases [exo-1, 4-α-D-glucan glucohydrolase, EC 3.2.1.3] are able to hydrolyze 1, 6- α-linkages at the branching points of amylopectin (Pandey *et al.*, 2000). The history of industrially produced amylase from fungal source began in 1894, which was used for the treatment of digestive disorder (Swargiari and Baruah, 2013). Today, amylase has great significance in present day biotechnology with its applications, ranging from textile, paper, food, fermentation and pharmaceutical industry (Vijayaraghavan *et al*., 2011). The role of amylases has also been acknowledged in clinical, medical and analytical chemistry. The aim of the present study was to isolate and characterize a starch degrading bacterium from soil sample collected from Botanical Garden, University of Delhi, India. Present study is focussed on the standardization of production and assay optima of amylase with respect to temperature, pH and incubation period of growth.

MATERIAL AND METHOD

Collection of Sample and Isolation of Bacterial Strain

One gram of garden soil sample collected from Botanical Garden, Department of Botany, University of Delhi, Delhi, India was dried and suspended in Erlenmeyer flasks containing 50 ml of enrichment medium for selective growth of the starch degrading (amylase producing) bacteria.

Preparation of Enrichment Media

In brief, 100 ml enrichment media was prepared by 2 g of starch (sole source of carbon), 0.5 g of ammonium sulphate, 0.5 g of di-potassium-hydrogen phosphate, 0.1 g magnesium sulphate, and 0.5 g calcium chloride in 100 ml of distilled water. The media was sterilized in an autoclave and then 1 g of dried soil sample was suspended in it for selective growth of starch utilizing bacteria. The flask was kept at 45°C at 250 rpm for 24 h in rotary shaker.

Isolation of Strains and Maintenance of Pure Culture

The enriched culture was serially diluted from 10^{-1} to 10^{-6} and was spread plated on Nutrient Agar media that contained (g/L): 1 g beef extract, 5 g peptone, 5 g sodium chloride, 2 g yeast extract, 15 g agar. Five strains that grew optimally at 45°C were selected (Th-1, Th-2, Th-3, Th-4 and Th-5) and their pure cultures were prepared and maintained.

Tests for Amylolytic Activity
Plates with bacterial colonies were flooded with Lugol's Iodine solution (2 g potassium iodine and 1 g iodine crystal dissolved in 300 ml distilled water, filtered and stored in brown bottle) and observed for clear and transparent zone of degradation of starch under and around colonies. Of the various colonies, the one that exhibited highest degradation was selected for biochemical and other physico-chemical characterization.

Identification of the Selected Microbial Strain
Isolated strain was identified by morphological, biochemical and physiological analysis. Colonial characteristics and microscopic observations were also done.

Pathogenicity Test
Pathogenicity test was performed, using Himedia sheep blood agar plates. Sterile sheep blood agar plate was streaked with 24 hours old bacterial culture. It was incubated at 45°C for 24 hours in an incubator for the detection of fastidious organism. The blood culture pattern of the selected bacterial strain was checked for gamma-hemolysis.

Biochemical Characterization
The biochemical tests were performed for carbohydrate utilization (using Hi-Carbohydrate Utilization Test Kit), motility, Gram staining, urea hydrolysis, nitrate reduction and hydrogen sulphide production.

Production of Hydrolytic Enzymes
Screening of the selected bacterial strain was done to check the production of other hydrolytic enzymes such as protease, lipase, xylanase, catalase and oxidase.

Antibiotic Susceptible Test
Antibiotic susceptibility test was done to determine the sensitivity or resistance of the bacterial strain as per procedure adopted for aerobic and facultative anaerobic bacteria to various antimicrobial compounds (Bauer *et al.*, 1966), using Himedia antibiotic discs. Bacterial culture was grown overnight in nutrient broth at 45ºC and 250 rpm in a rotary shaker. The freshly grown culture was spread on nutrient agar plates and the antibiotic discs were mounted on the surface of the plates carefully. Plates were then incubated overnight at 45ºC and the inhibition zones were measured using Hi antibiotic zone scale^{TM}-c (Table 3). On the basis of inhibition zone, bacteria have been characterized as antibiotic resistant (less than 15 mm of zone), antibiotic intermediate (16-20 mm) and susceptible (21 mm or greater) (Ammor *et al.*, 2007).

Optimization of Growth Conditions With Respect to Temperature, pH and Incubation Period
Optimizations of various parameters, such as temperature, pH and incubation period, are necessary for the maximum production of amylase. The growth of the organism was observed at different ranges of temperature (30°C- 55°C), pH (5-9) and incubation period (8-72 h).

Amylase Production
The nutrient medium containing starch was inoculated with bacterial colony and was cultured for 24 h at 45ºC at 250 rpm in rotary shaker. From this, inoculum (1% v/v) was transferred to amylase production medium (sucrose- 15g/l, peptone- 15g/l, NH_4Cl- 25g/l, $MgSO_4$- 0.7g/l, K_2HPO_4- 2g/l, starch- 2g/l) and was incubated again for 24 h at 45ºC at 250 rpm.

Isolation of Enzyme
In order to obtain crude enzyme, 24 h old culture grown in amylase production medium was transferred to centrifuge tubes and centrifuged at 12,000 rpm for 15 min. The resultant supernatant was used as the crude enzyme extract.

Amylase Assay
The enzyme activity was assayed following the method of Bernfeld (1955) using 3, 5 – dinitrosalicylic acid (DNS). In this reaction 2 ml of 1% (w/v) soluble starch was prepared in 50 mM phosphate buffer and was incubated at 45°C for 15 min with 0.2 ml of diluted enzyme solution. To this, 4 ml of DNS solution was added to stop the reaction, which was further followed by heating in water bath for 5 min. After the contents were cooled at room temperature, absorbance was measured at 540 nm, using UV-visible spectrophotometer. Standard curve of maltose was constructed that helped in estimating concentration of reducing sugars present in our sample. The enzyme activity was expressed as Units/ml.

Effect of Different Incubation Periods
The experiment was carried out individually at varying incubation periods (i.e., 8, 12, 24, 48 and 72 h) at 45°C at 250 rpm in a rotary shaker and was then analysed for amylase activity. The absorbance was measured at 540 nm with UV-visible spectrophotometer.

Effect of Temperature on Production of Amylase
To study the effect of temperature on amylase activity, the assay was carried out at different temperatures in the range of 30, 35, 40, 45, 50, 55 ºC for 24 h at 250 rpm in a rotary shaker. Amylase activity was then calculated using standard procedure.

Effect of pH on Production of Amylase
The effect of pH for amylase production was studied by culturing the bacterium at different pH of the production medium (in the range of pH 5, 5.5, 6, 6.5, 7, 7.5 and pH 8) for 24 h. The spectrophotometric analysis was done at 540 nm and amylase activity was calculated using standard procedure.

Statistical Analysis
Each experiment was conducted in triplicate. Standard Deviation (± SD) was calculated and represented in the form of line bars in the figures.

RESULT

Test for Amylolytic Activity
Initially, the soil sample, when plated on nutrient agar media, revealed the presence of 5 bacterial strains that grew at 45ºC. When tested with lugol's iodine solution, although all the strains exhibited clear zones, strain Th-3 had shown maximum clear zone under and around the colony, exhibiting its amylolytic nature (Fig: 1). Therefore, strain Th-3 was selected for further studies and its pure culture was maintained.

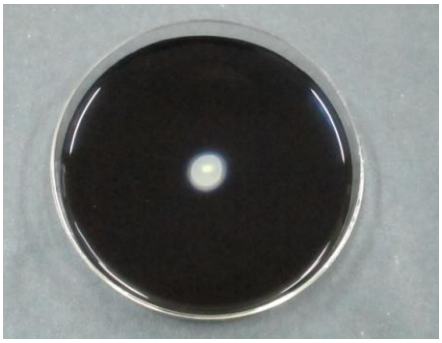


Fig 1. Qualitative screening of culture of Th-3 for the production of extracellular amylase. The zone clearence under and around the colony at the centre of the Petriplate represents amylolytic activity

Morphological and Microscopic Features
Morphologically, Th-3 colonies were translucent and creamy white in color. All the colonies were with entire regular margin. Gram staining and microscopic observation revealed that the bacterium was Gram +ve and rod-shaped.

Pathogenicity Test
While performing pathogenicity test on Himedia sheep blood agar plates, it was observed that the strain Th-3 was non-pathogenic, as agar under and around the colony was unchanged (this is also called non-hemolysis) (Fig: 2).

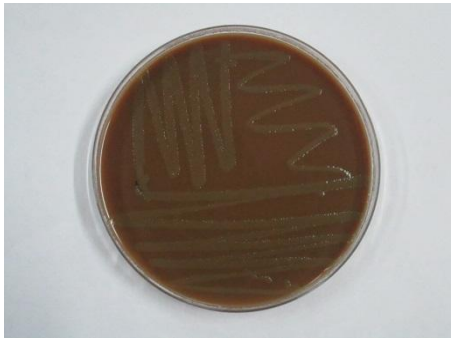


Fig 2. Pathogenicity test of the culture of Th-3 exhibiting non-hemolysis, representing its non-pathogenic nature

Carbohydrate Utilization Test
The carbohydrate utilization test was performed by incubating bacterial isolate Th-3 with different carboohydrates (Table 1). The observations indicated that there was differential utilization of carbohydrate sources by this bacterium.

Table 1: Utilization of carbohydrates by the bacterial strain Th-3

S.No	Carbon Source	Bacterial Strain Th-3
1	Lactose	Positive
2	Xylose	Positive
3	Maltose	Positive
4	Fructose	Positive

5	Dextrose	Positive
6	Galactose	Negative
7	Raffinose	Positive
8	Trehalose	Positive
9	Melibiose	Positive
10	Sucrose	Positive
11	L-Arabinose	Positive
12	Mannose	Negative
13	Inulin	Negative
14	Sodium Gluconate	Negative
15	Glycerol	Negative
16	Salicin	Negative
17	Dulcitol	Positive
18	Incsitol	Negative
19	Sorvitol	Negative
20	Mannitol	Negative
21	Adonitol	Positive
22	Arabitol	Positive
23	Erythritol	Negative
24	α-Methyl-D-glucoside	Positive
25	Rhamnose	Negative
26	Sellobiose	Negative
27	Melezitose	Negative
28	α-Methyl-D-mannoside	Positive
29	Xylitol	Negative
30	ONPG	Negative
31	Esculin Hydrolysis	Positive
32	D-Arabinose	Negative
33	Citrate utilazation	Negative
34	Malonate utilazation	Positive
35	Sorbose	Negative

Biochemical Characterization and Production of Hydrolytic Enzymes

Strain Th-3 was characterized with respect to the biochemical parameters given in Table 2. Among the hydrolytic enzymes, this strain was screened positive for protease, lipase and catalase. The bacterium was motile and was found to be positive for nitrate reduction.

Table 2: Different biochemical tests performed on bacterial isolate Th-3

S.No	**Biochemical Test**	**Result**
1	Protease activity test	Positive
2	Lipase activity test	Positive
3	Xylanase activity test	Negative
4	Catalase activity test	Positive
5	Oxidase activity test	Negative
6	Motility test	Motile
7	Gram staining	Gram +ve
8	Urea hydrolysis	Negative
9	Nitrate reduction test	Positive
10	Hydrogen sulphide production test	Negative

Antibiotic Susceptibility Test

The susceptibility of bacterial strain Th-3 was studied against different antibiotics listed in Table 3. The strain was sensitive to most of the antibiotics tested, except ampicillin and kanamycin to which it had shown resistance.

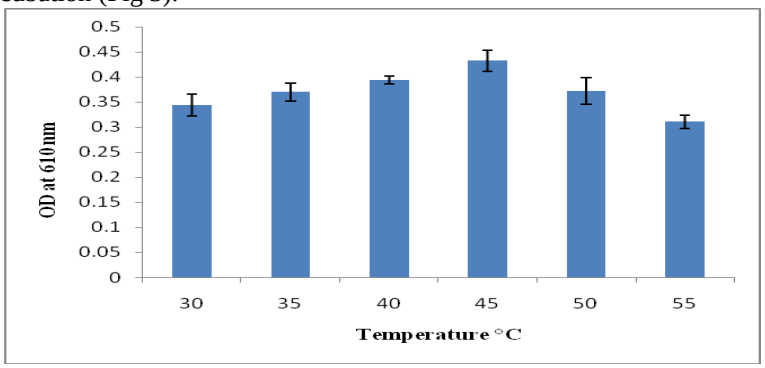
Table 3: Sensitivity/resistance of the bacterial strain Th- 3 to different antibiotics

Antibiotic Discs	**Symbols**	**Concenteration**	**Diameter of zone of inhibiton (mm)**
Amikacin	AK	30mcg/disc	17 (sensitive)
Ampicillin	AMP	10mcg/disc	Resistant

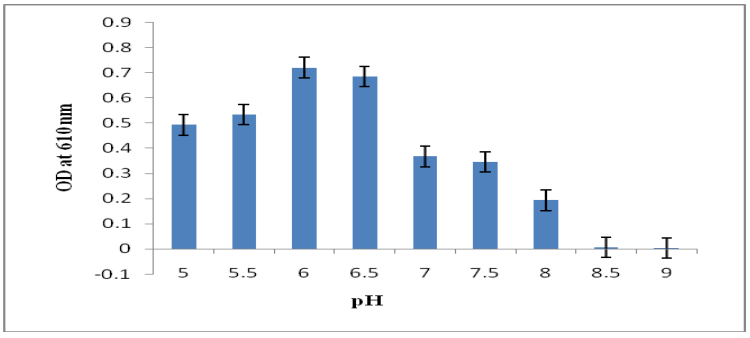
Carbenicillin	CB	100mcg/disc	18 (sensitive)
Cefaclor	CF	30mcg/disc	15 (sensitive)
Cefazolin	CZ	30mcg/disc	23 (sensitive)
Cefotaxime	CTX	30mcg/disc	19 (sensitive)
Ceftazidime	CAZ	30mcg/disc	18 (sensitive)
Ceftriaxone	CTR	30mcg/disc	21 (sensitive)
Chloramphenicol	C	30mcg/disc	16 (sensitive)
Ciprofloxacin	CIP	5mcg/disc	18 (sensitive)
Erythromycin	E	10mcg/disc	15 (sensitive)
Gentamicin	GEN	10mcg/disc	22 (sensitive)
Kanamycin	K	5mcg/disc	Resistant
Norfloxacin	NX	10mcg/disc	25 (sensitive)
Ofloxacin	OF	5mcg/disc	16 (sensitive)
Penicillin-G	P	2 units/disc	19 (sensitive)
Rifampicin	RIF	30mcg/disc	21 (sensitive)
Streptomycin	S	10mcg/disc	17 (sensitive)
Tetracycline	TE	10mcg/disc	18 (sensitive)

Optimization of Growth Conditions

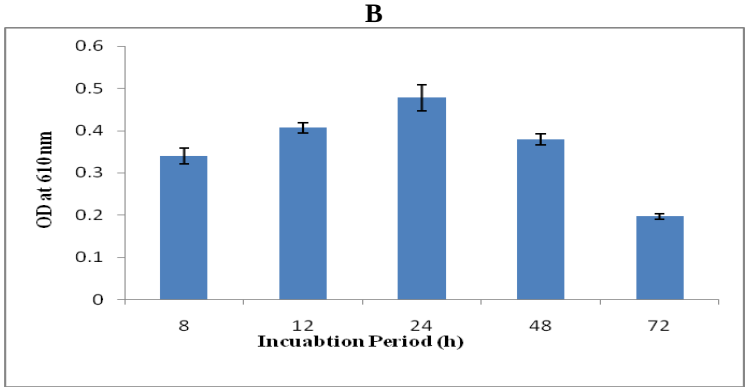
On standardization of growth conditions, it was observed that the best growth of Th-3 occured at 45°C, at pH 6 and at 24 hours of incubation (Fig 3).



A



B

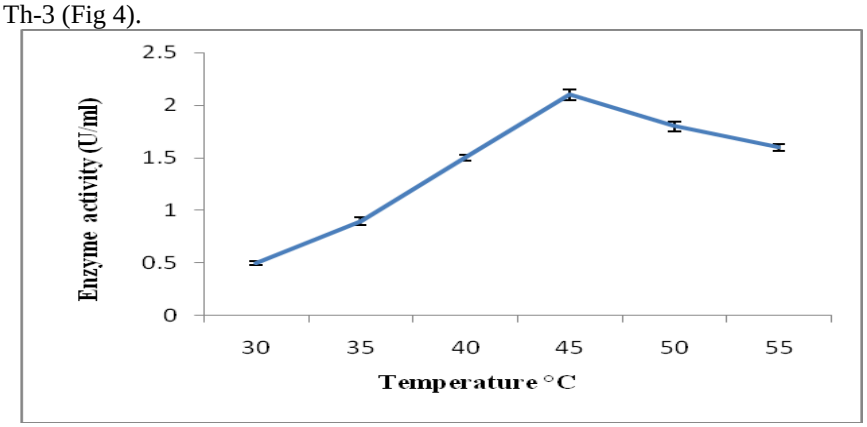


C

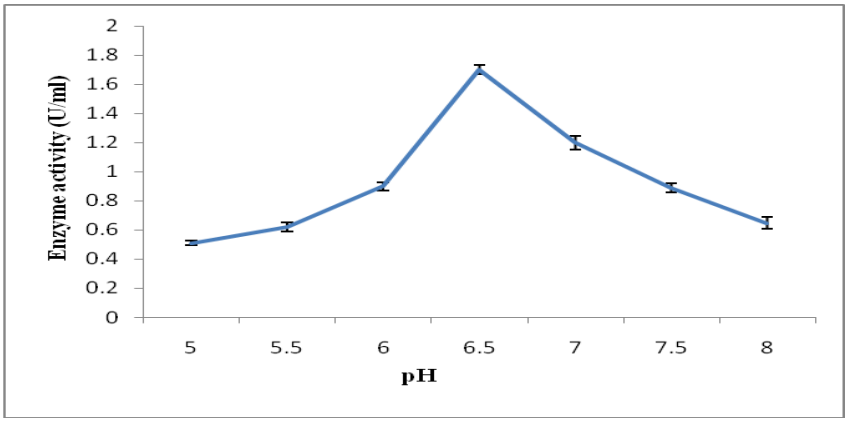
Fig 3. Optimization of growth conditions of the isolated bacterial strain Th-3 at different temperatures (A), pH (B) and incubation period of growth (C)

Effect of Temperature, pH and Incubation Period on Amylase Activity

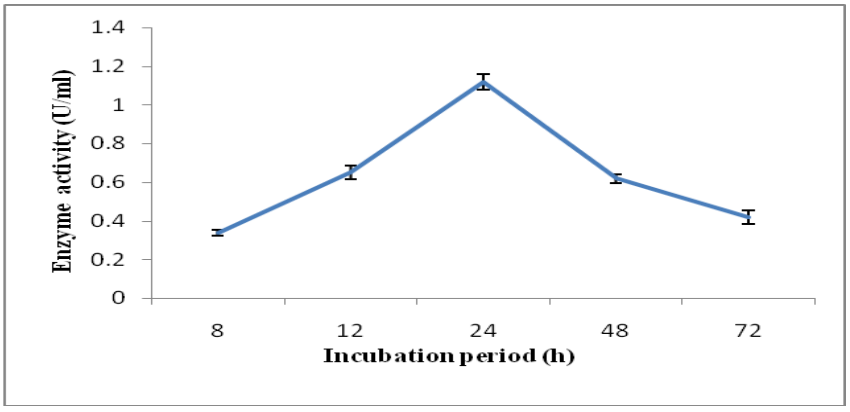
When assays for amylase activities were performed at different temperatures, pH and incubation period, it was found that the activity of this enzyme was maximum at 45°C, at pH 6.5 and at 24 hours of growth of the bacterial strain Th-3 (Fig 4).



A



B



C

Fig 4. Amylase activity, assyed at different temperatures (A), pH (B) and incubation period of growth (C) of the isolated bacterial strain Th-3

DISCUSSION

The genus *Bacillus* produces a large variety of extracellular enzymes, of which amylases are of considerable industrial importance (Swain *et al.*, 2006). In addition to amylase production, the isolated bacterial strain Th-3 had also shown positive results for production of protease, lipase and catalase as well as nitrate reduction, which were in accordance with the observations made by other authors (Deb *et al.*, 2013). Optimization of growth conditions are the prime step in using microorganisms for the production of enzymes (Kathiresan and Manivannan, 2006). In this context, temperature is a vital factor that controls the synthesis of bacterial extracellular enzymes. Bacterial amylases are produced at a much wider range of temperature. *Bacillus subtilis*, *B. licheniformis* and *B. stearothermophilus* are among the most commonly used *Bacillus* spp. reported to produce α-amylase within the temperature range of

37–60°C (Mendu *et al.*, 2005). In the present study, we observed that the optimum temperature for maximum growth of the bacterium and amylase production was 45°C, suggesting its thermophilic nature. The higher temperature (50°C and above) inhibited its growth and amylase activity. Aiba et al. (1983) also reported that the high temperature may inactivate expression of gene(s) responsible for amylase synthesis. pH of the growth medium is also among the important physical parameter that has to be optimized for the enzyme secretion. The pH range observed during the growth of microbes also affects enzyme production in the medium (Banargee and Bhattacharya, 1992). Most of the amylase secreting bacterial strains revealed pH range between 6 and 7 for the best growth of the organism and enzyme specific activity (Bose and Das, 1996; Mishra and Behera, 2008). Similarly, under this catagory the optimum pH for maximal growth of Th-3 was 6, and optimum pH for its maximum amylase activity was 6.5. In another study, the activity of enzyme was also observed at slighly alkaline pH (at around pH 9) (Deb *et al*., 2013).

It has already been reported that the enzyme activity is directly dependent on the period of incubation of bacterial strain in the culture medium (Smitt *et al*., 1996; Mishra and Behera, 2008). Some reports signify that with the increase in incubation time, enzyme activity decreased (Aiyer, 2004). In the present investigation, both growth as well as amylase activity increased with increasing period of incubation up to 24 h, followed by their decrease at further increase in the period of incubation. This suggested that the enzyme production in the isolated bacterial strain Th-3 is a growth associated phenomenon. In some studies, maximum activity of amylase was reported at 12 h in *Bacillus* species (Bozic *et al.*, 2011); nevertheless, more often amylase cannot be detected in the culture broth of *Bacillus* sp. before 12 h of incubation (Asgher *et al.*, 2007). In *B. stearothermophilus* AN 002 maximum activity occurred at 6 h of cultivation. Among all the antibiotics screened for, strain Th-3 was resistant to ampicillin and kanamycin. The results were similar to those reported by Samanta et al. (2012) for bacterial strain *Bacillus* sp. isolated from municipal waste. The isolated bacterium was non-pathogenic in nature, as tested on Himedia sheep blood agar plates. The biochemical, microscopic and morphological features of the isolated strain indicated that it was Gram-positive, rod-shaped and closely resembled *Bacillus* species. Thus, the non-pathogenicity of the starch degrading Th-3 and its sensitivity to most of the antibiotics suggested the possibility of exploiting this thermophilic bacterium for commercial production of amylase for diverse industrial applications.

CONCLUSION

Amylases are important enzymes used for industrial purposes and biotechnological research. Though amylases can be produced from various sources and are prevalent in industries from many decades, the microbial sources have shown significant role in their commercial production. It is also important to note that the commercial production of amylase from microbes is limited to few selected strains of fungi and bacteria. Therefore, it was necessary to isolate efficient microbial strains that can produce high titres of enzyme that can actively work on starch. For industrial applications, high temperature catalysis of enzyme is an extremely important feature; therefore, an attempt was made to look for production of amylase from a thermophilic bacterium. Protein engineering or chemical modification of the existing enzyme is also necessary to make this enzyme efficient in various other industrial sectors apart from food, textile, paper and pharmaceutical industry. The present investigation highlights the importance of a bacterial soil isolate which was non-pathogenic Gram-positive rod and which could grow at high temperature and produce thermophilic extracellular amylase, suggesting its applicability for high temperature catalysis of diverse industrial processes.

ACKNOWLEDGEMENT

The study was supported by the Department of Science and Technology (DST), Govt. of India through the award of Inspire Fellowship to Isha Kohli. The financial support under R & D Programme by the University of Delhi is gratefully acknowledged.

REFERENCES

Aiba, S.; Kitai, K. and Imanaka, T. (1983). Cloning and expression of thermostable α-amylase gene from *Bacillus stearothermophilus* in *Bacillus stearothermophilus* and *Bacillus subtilis. Applied Environmental Microbiology*, **46**: 1059- 1065.

Aiyer, P.V. (2004). Effect of C:N ration on alpha amylase production by *Bacillus licheniformis* SPT 27. *African Journal of Biotechnology.* **3**: 519-522.

Ammor, M. S., Belén Flórez, A. and Mayo, B. (2007). Antibiotic resistance in non- enterococcal lactic acid bacteria and bifidobacteria. *Food Microbiology*, **24**: 559-570.

Asgher, M.; Asad, J.M.; Rahman, S.U. and Legge, R.L. (2007). A thermostable α-amylase from a moderately thermophilic *Bacillus subtilis* strain for starch processing. *Journal of Food Engineering*, **79**: 950-955.

Banargee, R. and Bhattacharya, B.C. (1992). Extracellular alkaline protease of newly isolated *Rhizopus oryzae*. *Biotechnology letter*, **14**: 301-304.

Bauer, A.W.; Kirby W.M.; Sherris, J.C. and Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology*, **45**: 493-496.

Bernfeld, P. (1955). Enzymes of starch degradation and synthesis. *Advances in Enzymology*, **12**: 379-428.

Bose, K. and Das, D. (1996). Thermostable α-amylase production using *Bacillus licheniformis* NRRL B1438. *Indian Journal of Experimental Biology*, **34**: 1279-1282.

Bozic, N.; Ruiz, J.; Santin, J.L. and Vujcic, Z. (2011). Optimization of the growth and α-amylase production of *Bacillus subtilis* IP 5832 in shake flask and laboratory fermenter batch cultures. *Journal of the Serbian Chemical Society*, **76**: 965-972.

Deb, P.; Talukdar, S.A.; Mohsina, K.; Sarker, P.K. and Sayem, A. (2013). Production and partial characterization of extracellular amylase enzyme from *Bacillus amyloliquefaciens* P-001. *Springerplus*, doi:10.1186/2193-1801-2-154.

Dey, T.B. and Banerjee, R. (2012). Hyperactive α-amylase production by *Aspergillus oryzae* IFO 30103 in a new bioreactor. *Letter in Applied Microbiology*, **54**: 102-107.

Gupta, R.; Gigras, P.; Mohapatra, H.; Goswami, V.K. and Chauhan, B. (2003). Microbial α-amylases: a biotechnological perspective. *Process Biochemistry*, **38**: 1599-1616.

Kathiresan, K. and Manivannan, S. (2006). α-amylase production by *Penicillium fellutanum* isolated from mangrove rhizospheric soil. *African Journal of Biotechnology*, **5**: 829-832.

Mendu, D.R.; Ratnam, B.V.V.; Purnima, A. and Ayyanna, C. (2005). Affinity chromatography of α-amylase from *Bacillus licheniformis. Enzyme and Microbial Technology*, **37**:712–717.

Mishra, S. and Behera, N. (2008). Amylase activity of starch degrading bacteria isolated from soil receiving kitchen wastes. *African Journal of Biotechnology*, **7**: 3326-3331.

Pandey, A.; Nigam, P.; Soccol, C.R.; Soccol, V.T.; Singh, D. and Mohan, R. (2000). Review: Advances in microbial amylases. *Biotechnology and Applied Microbiology*, **31**: 135-152.

Roses, R.P. and Guerra N.P. (2009). Optimization of amylase production by *Aspergillus niger* in solid-state fermentation using sugarcane bagasse as solid support material. *World Journal of Microbiology and Biotechnology*, **25**: 1929-1939.

Samanta, A.; Bera, P.; Khatun, M.; Sinha, C.; Pal, P.; Lalee, A. and Mandal, A. (2012). An investigation on heavy metal tolerance and antibiotic resistance properties of bacterial strain *Bacillus* sp. isolated from municipal waste. *Journal of Microbiology and Biotechnology Research*, **2**: 178-189.

Smitt, J.P.; Rinzema, J.; Tramper, H.; Van, M. and Knol, W. (1996). Solid state fermentation of wheat bran by *Trichoderma reesei* QMQ414. *Applied Environmental Microbiology*, **52**: 179-184.

Swain, M.R.; Kar, S.; Padmaja, G. and Ray, R.C. (2006). Partial characterization and optimization of production of extracellular α-amylase from *Bacillus subtilis* isolated from culturable cow dung microflora. *Polish Journal of Microbiology* **55**: 289-296.

Swargiari, BN. and Baruah, PK. (2013). Isolation and screening of amylolytic *penicillium* species from soil. *International Journal of Pharma and Bio Sciences*, **4**: 575-581.

Vijayaraghavan, P.; Remya, C.S. and Prakash, S.G. (2011). Production of α-Amylase by *Rhizopus microsporus* using agricultural by-products in solid state fermentation. *Research Journal of Microbiology*, **6**: 366-375.