Comparative Characterization and Amylase Activity Assessment of Certain Garden Bacterial Isolates

Munia Islam[a] *and Tamanna Zerin*[a*]

Abstract

Background: Microorganisms are the most important sources of enzymes because of their stability and reduced price in production. The enzyme, amylase has a wide spectrum of application in biotechnology including food, fermentation, textile and laundry, paper and pulp industries. As the use of amylase is increased, it is necessary to search for a new source to produce amylase with better productivity in continuous practice. **Method:** In our present investigation, amylase producing bacteria were screened by starch hydrolysis test as bacteria are more potent in amylase production than other microorganisms. The bacteria were identified by Bergey's manual of systematic bacteriology. Enzyme assay and optimization of enzyme activity were performed by the 3,5-dinitrosalicylic acid method. **Results:** A total of eight bacterial isolates were identified with starch degrading capabilities and they were presumptively placed in the genus, *Bacillus* due to their characteristic features. All the isolates have potential for amylase activity. At any temperature (25˚C, 35˚C, 45˚C, 50˚C and 55˚C), pH (6, 7, 8) and starch concentrations (0.5%, 1% and 1.5%), the highest amylase activity was observed by isolate 1A followed by isolate 2C, 1B, 3A, 4B, 3B, 4A, and 4C. Optimum conditions for the highest amylase activity in our laboratory for isolate 1A was 35˚C (4.105 U/ml), pH 6 (3.343 U/ml) and 1.5% starch concentration (4.381 U/ml). **Conclusion:** Our study reveals that the isolates, collected from garden soil, are good amylase producers and they could be exploited in different industries in optimized conditions.

Keywords: Amylase, Enzyme activity, DNS, Soil, Optimization.

Abbreviations: DNS, 3,5 dinitrosalicylic acid; MR, methyl red; VP, voges-proskauer; GPB, glucose phosphate broth; MIU, motility indole urea.

***Significance* | Screening of soil *Bacillus* spp. for amylolytic activity**

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Introduction

Starch, a glucose polymer, is the main constituent of human diet that is physically and chemically treated industrially to generate starch hydrolysates, glucose syrups, fructose, maltodextrin derivatives or cyclodextrins for various food industries. For various industrial applications, starch is hydrolyzed to smaller oligosaccharide by the enzyme, amylase (de Souza and de Oliveira Magalhães, 2010). Amylases occupy nearly 25% of the world enzyme market has a huge application in a range of industries as food, detergent, paper, textile, and fermentation (Rajagopalan and Krishnan, 2008; Reddy *et al*., 2003). The extent of biotechnology has widened the potential use of amylase in clinical, medicinal, analytical chemistry, brewing and distillation industries as well (Gupta *et al*., 2003; Kandra, 2003; Pandey *et al*., 2000). Amylase can be produced by different sources like animals, plants, and microbes (Kathiresan and Manivannan, 2006). Out of approximately, 100 industrially important enzymes, around one half is acquired from yeast and fungi, and around one third from bacteria, and the rest are collected from plants (4%) and animals (8%) (Patel, 2015). However, microbial sources of amylase are considered beneficial due to cost-effectiveness, consistency as well as, less space and time (Gopinath SC *et al*., 2017; Rao *et al*., 1998). Thermo-stability is a desirable property of most of the industrial enzy-

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mes and in that case, thermostable bacterial α-amlylase enzyme from *Bacillus subtilis*, *B. stearothermophilus*, *B. licheniformis* and *B. amyloliquefaciens* are produced industrially for various applications (Prakash and Jaiswal, 2010). Furthermore, in some harsh industrial processes where high salt concentration could resist enzymatic conversion in most cases, halotolerant amylases from halophilic bacteria can be a choice that is also considered as thermotolerant. Thermotolerant amylase producing bacteria includes *Chromohalobacter* sp. (Prakash *et al.*, 2009), *Halobacillus* sp. (Amoozegar *et al.,* 2003), *Haloarcula hispanica* (Hutcheon *et al., 2005*), *Halomonas meridiana* (Coronado *et al.*, 2000), and *Bacillus dipsosauri* (Deutch, 2002).

Due to the increased usage of amylase enzyme, it is fundamental to search for new indigenous amylase producing microbes with better productivity in a continuous process and optimize the process to improve their activity. Researchers are using different sources to isolate efficient amylase producing microbes (Aiba *et al.*, 1983; Tonkova *et al.*, 1993; Kathiresan and Manivannan, 2006, Mishra and Behera, 2008). Soil is a habitat where a diverse array of microflora and fauna reside and improve soil fertility by the biochemical transformation of complex organic materials to easily accessible nutrients for plants (Patel, 2015). In our study, we chose soil from the garden as it is very rich in nutrients. Following the primary screening, we isolated and characterized amylase producing bacteria and compared their amylolytic activity by optimizing temperature, pH and substrate concentrations.

Methods

Sample collection and processing

Soil samples were collected in a sterile beaker using sterile spatula at a depth of 2-3 cm from rich garden soil in Siddeshwari campus of Stamford University Bangladesh, Dhaka, Bangladesh. One gram of soil sample was dispensed into 99 ml of sterile normal saline and homogenized as described previously (Cappuccino and Sherman, 1996). One ml of homogenized soil sample was transferred into 9 ml sterile normal saline and serial dilution was carried out up to 10^{-6} dilution.

Isolation and screening of amylase producing bacteria

Bacterial isolates were isolated and screened for amylase production by starch hydrolysis test on starch agar media as described previously (Bala *et al.*, 2013; Kaur *et al.*, 2012). Serially diluted bacterial cultures (100 µl) were spread on nutrient agar media and incubated at 37˚C for 24 h. Subsequently, isolated colonies were streaked on starch agar media containing starch as the only carbon source for starch hydrolysis test to detect their amylolytic activity. The plates were incubated at 37˚C for 24-48 h. Following incubation, plates were flooded with Gram's iodine (Gram's iodine- 250 mg iodine crystals added to 2.5 g potassium iodide solution, and 125 ml of water, kept at room temperature) to identify the zone of clearance around the colony. Deep blue color around the growth indicates a negative result that is no amylolytic activity where the zone of clearance produced by amylase producers. The pure cultures showing clear zones were subcultured at regular interval and maintained on to nutrient agar slants at 4˚C.

Identification of amylase producing bacteria

Isolated amylase producing bacteria were presumptively identified by Gram staining, spore staining, motility test, cultural and biochemical characteristics using the taxonomic scheme of Bergey's manual of determinative bacteriology. The biochemical tests included methyl red, voges-proskauer, citrate utilization, indole production, H_2S production, motility, gelatine hydrolysis, sugar hydrolysis, oxidase, catalase and carbohydrate fermentation.

Preparation of cell free enzyme

To extract amylase enzyme, a loopful of pure culture was inoculated in production media containing starch (10 g/l), peptone (5 g/l), $(NH_4)_2SO_4$ (2 g/l), KH_2PO_4 (1 g/l), K_2HPO_4 (2 g/l), $MgCL_2$ (0.01 g/l) at pH 7 and incubated at 37˚C in a shaking water bath at 120 rpm for 24 h (Vaidya and Rathore, 2015). Following incubation, 10 ml of 24 h old culture was centrifuged at 5000 rpm for 15 min. Cells were discarded and the supernatant was decanted for the crude enzyme that was used for the optimization of assay condition for amylase activity.

Amylase assay

Amylase activity was assayed by employing 3,5 dinitrosalicylic acid (DNS) method as described (Bernfeld, 1955) with few modifications. In brief, 1% starch solution was prepared freshly by dissolving 1 g of soluble starch in 100 ml of 0.02 M sodium phosphate buffer (pH, 7). To prepare the assay condition, 1 ml of 1% starch solution and 0.5 ml of crude enzyme extract were incubated at 50˚C for 30 min. The assay was stopped by adding 3 ml of DNS reagent and heated the solution in a boiling water bath for 10 min. Then, with running tap water the solution was cooled. The solution volume was brought up to 10 ml by distilled H_2O and the absorbance was recorded using a spectrophotometer. A blank was always prepared without the enzyme. Enzyme activity was measured by preparing a standard graph with known concentrations of the standard (glucose), and plotted. Here, one unit (U/ml) of amylase activity is defined as the amount of amylase required to catalyze 1 µmol of reducing sugar (glucose) from starch per minute under the assay condition.

Characterization of the crude amylase enzyme

To determine the effect of different parameters enzyme assay was performed at different temperature, pH and starch concentrations.

Effect of temperature

A range of temperature as 25˚C, 35˚C, 45˚C, 50˚C, and 55˚C was used to measure the optimal temperature for enzyme assay, otherwise, all the procedure are the same for enzyme assay as previously mentioned.

Table 1| Microscopic observation of the amylase producing bacterial isolates

Bacterial isolates	Microscopic observation			
	Shape	Arrangement	Gram Reaction	Spore Staining
1A	short rod	single	+ve	-ve
1B	long rod	single, chain	+ve	+ve
2C	long rod	single, chain	+ve	+ve
3A	long rod	single, chain	+ve	+ve
3B	short rod	single, chain	+ve	-ve
4A	short rod	single	+ve	+ve
4B	long rod	single, chain	+ve	+ve
4C	short rod	single	+ve	-ve

Effect of pH

Optimum pH for enzyme activity was determined by performing the enzyme assay in three different pH conditions as 6, 7 and 8 at their optimum temperature determined before. All the experiments for enzyme assay were performed as previously mentioned.

Effect of starch concentration

Three different starch concentrations as 0.5%, 1%, and 1.5% were used as a substrate to obtain optimum substrate concentration for enzyme assay. Optimum temperature and pH condition as determined previously were maintained to determine the effect of different starch concentrations on enzyme activity. The enzyme activity was performed as previously mentioned.

Results

Isolation and characterization of amylase producing bacteria

Amylase producing bacteria were screened by starch hydrolysis test. A total of 8 bacterial isolates as 1A, 1B, 2C, 3A, 3B, 4A, 4B and 4C were found to exhibit a zone of clearance with iodine solution.

All those isolates were subjected to characterization by morphological (Table 1), cultural (Table 2) and biochemical tests (Table 3) and found to belong to the genus, *Bacillus*. A representative picture for endospore former (a) and clear zone for starch hydrolysis test (b) by isolate 1B is showed in Figure 1.

Effect of different parameter on enzyme activity

The amylolytic activity was performed by DNS method with glucose standard curve that was presented in Supplementary Figure 1. The effect of temperature on amylase activity of eight bacterial isolates was observed and showed in Figure 2. Among them, five isolates (1B, 2C, 3A, 3B and 4A) showed the highest activity at 50˚C, whereas, the rest three isolates (1A, 4B and 4C) at 35˚C. The highest amylase activity was observed by the isolate 1A (4.105 U/ml), followed by isolates 2C, 1B, 3A, 4B, 3B, 4A and 4C.

The consequence of pH condition also affects amylase activity that was presented in Figure 3. Amylase activity was observed under three different pH conditions with their optimum temperature as determined previously where 6 isolates (1A, 1B, 2C, 3A, 4A, and 4B) showed the highest activity at pH 6. Others (3B and 4C) showed the highest activity at pH 7. As previously observed, the highest amylase activity was observed by the isolate 1A (3.875 U/ml) followed by isolates 2C, 1B, 3A, 4B, 3B, 4A and 4C.

Amylase activity was performed using 3 different starch concentrations as 0.5%, 1% and 1.5% by maintaining optimum temperature and pH conditions obtained from the previous result. All the isolates showed the highest activity at the highest starch concentration (Figure 4). The highest activity for the enzyme, amylase by the isolates followed the similar pattern as mentioned in temperature and pH optimization experiments.

With the use of optimal temperature, pH and starch concentrations, the activity was greatly improved for all the isolates. All the isolates showed a temperature-, pH- and substrate concentration-dependent increase in activity, while it reached its optimal condition, the activity decreased afterward.

Discussion

Starch degrading bacteria collected from rich in starchy material may have a greater potential to produce amylase enzyme (Mishra and behera, 2008). In our study, we collected soil from garden containing various types of flowering and fruit plants. We believed that the soil would be a good choice to isolate amylase producing bacteria as they constantly exposed to soil containing enormous amount of starchy material from those plants. We were able to isolate eight starch degrading bacteria from primary screening by starch hydrolysis test using iodine solution. Following morphological, cultural and biochemical analysis, they were presumptively found to belong to the genus, *Bacillus*. This finding is in agreement with others' work who found that *Bacillus* is the predominant amylase producing bacteria from different sources as soil from bakery waste (Bala *et al*., 2013), soil from the potato field (Kaur *et al*., 2012), soil from the garden (Patel, 2015), soil receiving kitchen wastes (Mishra and Behera, 2008). We found the highest amylolytic activity that was 4.381 U/ml by the isolate, 1A at 35˚C, pH 6 and 1.5% starch concentration which is higher than other *Bacillus* spp. isolated from different sources (Islam *et al*., 2016; Kaur *et al*., 2012).

We observed the highest enzyme activity at 35˚C by the isolates 1A (4.105 U/ml), 4B (0.887 U/ml), 4C (0.639 U/ml), and at 50˚C by 1B (2.428 U/ml), 2C (2.956 U/ml), 3A (1.625 U/ml), 3B (0.734 U/ml), and 4A (0.685 U/ml). It was reported that the thermal stability of some wheat α-amylases was stable up to 50˚C and some upto 40˚C following incubation (Mohamed *et al*., 2009). Whereas, α-amylases isolated from microorganisms had a wider range of stability that suits

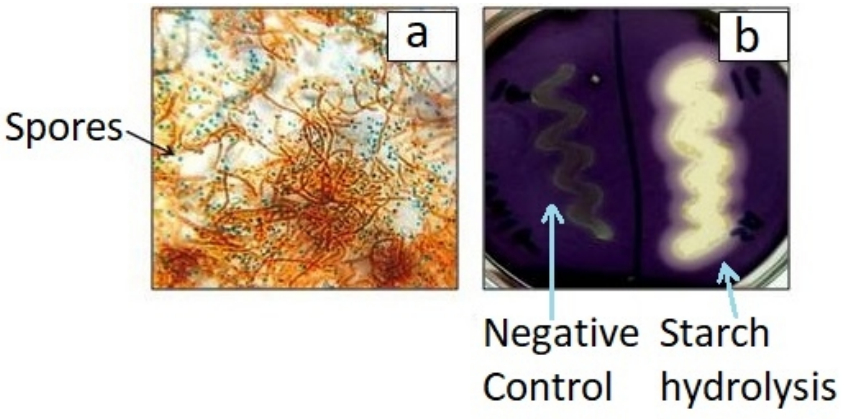

Figure 1| A representative picture of endospore former by isolate 1B with malachite green staining, and the spores were marked as green 1(a). Clear zone by starch hydrolysis test was observed by the isolate, 1A at the right side of the plate, and a negative control is presented at the left side of the plate that showed no hydrolysis 1(b).

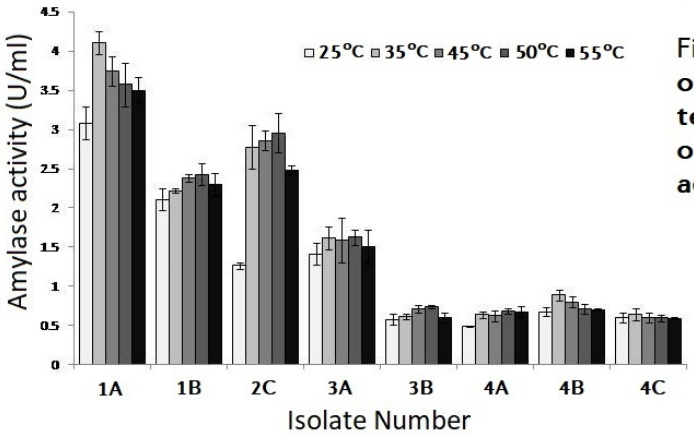

Figure 2| Effect of different temperature on amylase activity.

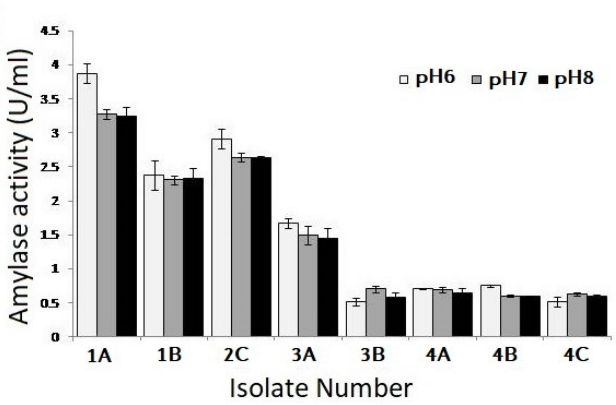

Figure 3| Effect of different pH on amylase activity by maintaining their optimal temperature for amylase activity.

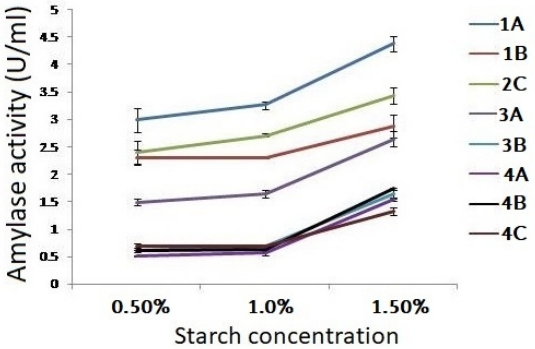

Figure 4| Effect of different starch concentrations on amylase activity at optimal temperature and pH conditions.

Table 2| **Colony characteristics of the amylase producing bacterial isolates on nutrient agar media.**

Characteristics	1A	1B	2C	3A	3B	4A	4B	4C
Size	Medium	Large	Large	Medium	Medium	Small	Large	Small
Shape	Irregular	Irregular	Round	Round	Irregular	Round	Round	Round
Margin	Serrate	Lobate	Entire	Entire	Undulate	Entire	Undulate	Entire
Elevation	Flat	Flat	Umbonate	Umbonate	Flat	Flat	Flat	Flat
Consistency	Moist	Butyrous	Butyrous	Butyrous	Butyrous	Moist	Butyrous	Moist
Texture	Rough	Smooth	Smooth	Smooth	Smooth	Rough	Smooth	Smooth
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
Odor	Earthy	Earthy	Earthy	Earthy	Earthy	Earthy	Earthy	Earthy
Pigmentation	White	Creamy white	Creamy white	Creamy white	Yellow	Creamy white	White	White

Table 3| **Biochemical characteristics of the amylase producing isolates.**

Tests	Media	Observation							
		1A	1B	2C	3A	3B	4A	4B	4C
Starch hydrolysis	Starch agar plate	+++	+++	+++	++	+	+	+	+
MR test	GPB broth	-	-	+	-	-	-	+	-
VP test	GPB broth	-	-	-	-	-	+	-	+
Citrate utilization	Simmons citrate agar slant	+	-	-	+	-	+	-	-
Indole production	1% peptone	-	-	-	-	-	-	-	-
H_2S production	2% peptone	-	-	-	-	-	-	-	-
Motility test	MIU media	-	+	+	+	-	+	+	+
Gelatin hydrolysis	Gelatin media	-	-	-	-	-	-	-	-
Sugar hydrolysis	Triple sugar iron agar slant	K/A	A/A	A/A	A/A	K/A	A/A	K/A	K/A
Oxidase	Nutrient agar	-	+	+	+	+	+	+	+
Catalase	Nutrient agar	+	+	+	+	+	+	+	+
Carbohydrate fermentation	Glucose	+	+	+	+	+	+	+	+
Carbohydrate fermentation	Fructose	-	-	+	+	-	-	+	-
Carbohydrate fermentation	Maltose	-	+	+	+	-	-	+	+
Carbohydrate fermentation	Lactose	-	+	-	+	+	-	+	+
Carbohydrate fermentation	Dextrose	-	-	+	+	-	-	+	+
Carbohydrate fermentation	Sucrose	+	-	+	+	+	+	+	+

them in various industrial applications comparative to those isolated from plants and animals (Tanyildizi *et al.*, 2005). We maintained growth temperature for all those isolates at a fixed temperature that is 37˚C.

The maximum amylase activity in our study was found at pH 6-7. None of the enzymes isolated from bacterial isolates showed the highest activity at pH 8. This data correlates with another study where it was observed that most of the starch degrading bacteria had a pH range 6-7 for both growth and enzyme activity (Gupta *et al.*, 2003) although, amylase enzyme from thermostable bacteria revealed the highest activity at pH 8 (Behal *et al.*, 2006).

The activity of the enzyme was influenced by reaction conditions as temperature, pH, substrate concentration, etc. Among several parameters, we only chose those three parameters to optimize amylase activity as they are considered very crucial for the activity of the enzyme, and they are mostly selected for optimizing enzyme activity by other resear chers (Megahati et al., 2017; Dutta et al., 2016). Our data showed the greatest activity with the highest starch concentration that was 1.5% although it was necessary to do experiment to determine enzyme activity using the concentration of

starch more than 1.5%. The previous study also showed that increased starch concentration from 0.15% to 2% enhanced amylase activity (Mishra and Behera, 2008).

Conclusion

Amylase is one of the most widely used enzymes that are required in various industries. We isolated starch degrading bacteria from rich garden soil and tried to characterize enzyme assay conditions as temperature, pH and starch concentration. With optimal assay condition, the activity was greatly improved. Our data can be useful for further improvement of assay conditions and thereby, increasing enzyme activity.

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Author Contribution

TZ designed the study, Both TZ and MI analyzed the data, prepared the manuscript and revised the data. All the authors approved the manuscript.

Competing financial interests

There is no competing financial interest.

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