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ISOLATION AND IDENTIFICATION OF AMYLOLYTIC BACTERIA FROM GARBAGE AND GARDEN SOIL

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Abstract

An analysis for the abundance and diversity of amylolytic bacteria of two different soil types *viz.* garbage and garden soil was carried out. pH of the garbage and garden soil samples ranged between 7.73 and 9.84, 6.88 and 7.93, respectively. Average bacterial load on both NA and PYG agar media was found to be higher in garbage than garden soils. Bacterial load of garbage soil samples ranged from 2.08×10^8 to 3.79×10^8 cfu/g and 1.45×10^8 to 2.74×10^8 cfu/g on NA and PYG agar, respectively. On the other hand, bacterial load of the garden soil samples ranged from 3.3×10^6 to 9.7×10^6 cfu/g on NA and 2.9×10^6 to 9.35×10^6 cfu/g on PYG agar. A total of 200 bacterial isolates (100 from each soil type) were primarily selected for their amylolytic potential. Among them, the percentage of amylolytic bacteria was higher in garbage soil (46) than garden soil (38). Finally, a total of 8 (4 from each soil type) amylolytic potential isolates were selected for detailed study and identification. All 4 isolates from garbage soil and 3 from garden soil were found to be Gram positive and by conventional identification belonged to the genus *Bacillus* with six different species *viz. Bacillus azotoformans* (2), *B. stearothermophilus* (1), *B. acidocaldarius* (1), *B. subtilis* (2) and *B. megaterium* (1) and the only Gram negative isolate was identified as *Acetobacter liquefaciens.* The conventional identification was further confirmed by molecular technique and isolates were identified as *Bacillus* sp. T5-12, *B. cereus* MSW, *Bacillus* sp. FJAT-14266, *B. toyonensis* KK25A, *B. cereus* T10, *Stenotrophomonas* sp. ZJZG10, *B. subtilis* XF-1 and *Pseudomonas* sp. NCCP-1179. As significance of amylase enzyme in various industries and biotechnological processes are on the rise, it is important to find better and cheaper source for it. This piece of work focuses on finding out which can be a better source for amylolytic bacteria between two different soil types.

Introduction

Soil a vibrant habitat for diversified life-forms shelters many animals from invertebrates such as worms and insects to mammals like rabbits, rodents and badgers. It is also an ideal habitat of complex groups of microorganisms including bacteria, archaea, fungi and protozoa (Bhattarai *et al*. 2015). Bacteria, involved in decomposition of organic substances, nutrient cycling, soil aggregation and humus formation can reach up to 10 billion cells/g in soil (Morris and Blackwood 2015). Bacteria constitute the principal group of soil microbes mostly because of their capacity to produce diverse extracellular enzymes such as amylase, protease, lipase, pectinase, cellulase and chitinase.

Bacterial population can be manipulated to produce enzymes which are commercially important in organic compound synthesis, clinical analysis, pharmaceuticals, detergents, food production and fermentation (Logeswaran *et al*. 2014). Microbial, mainly bacterial production of amylase is more fruitful than that of other sources like plants or animals, because of their short growth period, biochemical diversity and the ease with which enzyme production capability might be increased by environmental and genetic manipulation (Mishra and Behera 2008).

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Soil is one of the richest sources of starch degrading microorganisms as it contains abundant starchy substances. Different types of soils are comprised of varying amounts of minerals and nutrients. As a result, there are several reports on starch degrading microorganisms from different soil sources with varying amylase activity (Serin *et al.* 2012). Considering the importance of various soil physico-chemical factors on the presence, abundance and types of amylolytic bacterial population, the present investigation was undertaken to compare two different soil types *viz.* garbage soil and garden soil as a better source of such bacteria. From the findings of the present study, the better source could be searched further to find potential bacterial isolates capable of producing the industrially significant and widely used enzyme, amylase.

Materials and Methods

Two types of soil samples were collected aseptically in sterile plastic bags; one from garbage dump sites of five local markets of Dhaka city *viz.* Ananda Bazar, Hatirpool Bazar, New Market Bazar, Palashi Bazar and Shantinagar Bazar and the other from an experimental plot (from four corners and middle of the plot) of Dhaka University Botanical garden. Collected samples were immediately brought to laboratory for analysis. The samples were sieved to discard debris and pH of the samples was measured by a pH meter (TOA-DKK, HM-31P, Japan).

Serial dilution technique (Greenberg *et al.* 1998) was employed using nutrient agar (NA) (Eklund and Lankford 1967) and peptone yeast extract glucose (PYG) agar (Atlas 1997) media for enumeration of aerobic heterotrophic bacteria. The pH of the media was adjusted to 7.0 ± 0.2. Inoculated plates were inverted and incubated at 37°C for 24 hrs in an incubator (Memmert GmbH + Co Kg 8540 Schwabach, Germany). After 24 hrs, plates having well discrete colonies were counted.

Isolation of amylolytic bacterial colonies was made in starch nutrient agar (SNA) medium (Claus 1995). Primary selection of the bacterial isolates was made on the basis of their distinctive colony morphology. Selected isolates were purified by repeated streaking and stored in NA slants at 4°C for further analysis. Starch hydrolysis test (Claus 1995) was then used to evaluate the amylolytic potentiality of the isolates. For this test, SNA plates were point inoculated with the isolated organisms and the plates were incubated at 37°C for 24 hrs. After incubation, the surface of the plates was flooded with iodine solution. The isolates showing clear halo zones around their colonies were able to degrade starch and supposed to possess amylolytic activity. Diameter of the colonies and clear zones around the colonies were measured by mm scale. The following formula was used to determine the starch hydrolysis ratio (SHR).

$$\text{Starch hydrolysis ratio (SHR)} = \text{Zone diameter (mm)} \div \text{Colony diameter (mm)}$$

Bacterial isolates from both soil types showing better SHR were selected for detailed analysis. Simple and Gram staining of the selected isolates were done according to the methods described by SAB (1957). Important physiological and biochemical tests *viz.* KOH solubility test, VP test, MR test, utilization of propionate and citrate, tyrosine degradation etc. (Sneath *et al.* 1986, Schand 1988, Atlas 1997) were also carried out by the isolates**.** Conventional identification of the isolates was done according to Bergey's Manual of Systematic Bacteriology Vol. I (Krieg and Holt 1984) and Vol. II (Sneath *et al.* 1986).

Molecular identification of the bacterial isolates was conducted by amplifying ~600 bp fragments of 16S rDNA using CC [F] 5'-CCAGACTCCTACGGGAGGCAGC and CD [R] 3'-CTTGTGCGGGCCCCCGTCAATTC primer pairs. Supernatant of heat lysed cell suspension was used as the source of template DNA for PCR amplification following protocol mentioned in Khan *et al.* (2017). The amplified products were separated electrophoretically on 1% agarose gel. DNA bands were observed on UV-transilluminator and photographed by a gel documentation system

(Microdoc DI□HD, MUV21□254/365, Cleaver Scientific, UK). The amplified bands were gel purified using Gel purification kit (Invitrogen) and sequenced from Macrogen, South Korea. Sequences were analyzed through NCBI-BLAST (http://blast.ncbi.nlm.nih.gov/) and rRNA BLAST program (http://bioinformatics.psb.ugent.be/beg/) to find out possible similar organisms in the databases. The data were analyzed to determine the descriptive statistics *viz.* statistical mean and standard deviation (SD) with SPSS v.16.0 for Windows (SPSS, SAS Institute Inc. Cary, USA).

Results and Discussion

The pH of the collected samples presented in the Table 1 shows that garbage soil samples were somewhat alkaline, but garden soil was mostly neutral. Though it has been observed in most studies that, maximum amylase activity of the bacteria is recorded at an alkaline pH, the enzyme is generally stable over a wide range of pH from 4 to 11 (Khoo *et al.* 1994). In a study, Krishma and Radhathirumalaiarasu (2017) reported maximum amylase activity at high alkaline pH (10.0) whereas, at neutral pH, there was moderate activity. So, it can also be speculated that bacteria producing this enzyme would also be available in environment with broad pH spectrum.

Table 1. Sampling sites and pH of collected soil samples.

Garbage soil		Garden soil	
Sampling site	pH	Sampling site	pH
Ananda Bazar	7.90	Northeast corner	7.23
Hatirpool Bazar	8.03	Southeast corner	7.10
New Market Bazar	9.84	Centre position	7.34
Palashi Bazar	8.60	Northwest corner	6.88
Shantinagar Bazar	7.73	Southwest corner	7.93

Both NA and PYG agar media were found to be suitable for enumeration of bacteria from the soil samples. Bacterial load of garbage soil was found to be higher than that of garden soil on both NA and PYG agar (Table 2). Higher bacterial count in the garbage soil samples may be due to availability of diversified nutrients as waste substances which also was evident in the experiment conducted by Ogunmwonyi *et al.* (2008). He reported mean total bacterial count from park and garbage soil sample to be 9.5×10^7 cfu/g. In another study, Krishna *et al.* (2012) reported bacterial load ranged between 1.41×10^7 and 2.71×10^7 cfu/g soil of Mahatma Gandhi University campus of India.

The difference in bacterial counts for two different types of soil may be due to various biochemical factors which influence microbial growth and survival in soils. One of the most influential factors affecting the microbial community in soil is pH as reported by Baath and Arnebrant (1995). They reported that treatment of forest soils with lime and ash resulted in pH change from acidic to alkaline which also increased bacterial growth about five-folds. Similarly, a study including 19 different soils from areas with various land uses, spanning a pH range from 4 to 8, showed an increase in bacterial growth with higher pH (Baath 1998).

Primarily, a total of 200 bacterial isolates (100 from each soil type) were isolated from SNA plates based on their different colony morphology. All the isolates were then checked for their amylolytic activity by starch hydrolysis test on SNA medium. Among them, 46 and 38 isolates from garbage and garden soil samples, respectively demonstrated positive amylolytic activity. The

occurrence of amylolytic activity showing organisms from the soil agrees with the report by Madhav *et al.* (2011) showing that the soil is an enriched repository of amylolytic bacteria. Percentage of amylolytic activity showing bacteria was higher in garbage soils than garden soils (Fig. 1). The diversified starchy waste substances available in the garbage soil samples may again be responsible for this result.

Table 2. Bacterial load of the collected soil samples.

Bacterial load (cfu/g) on garbage soil			Bacterial load (cfu/g) on garden soil		
Sampling site	Media		Sampling site	Media	
	NA	PYG		NA	PYG
Ananda Bazar	2.35×10^8	1.91×10^8	Northeast corner	9.50×10^6	8.40×10^6
Hatirpool Bazar	3.67×10^8	2.86×10^8	Southeast corner	3.40×10^6	2.90×10^6
New Market Bazar	3.79×10^8	2.74×10^8	Centre position	4.70×10^6	4.10×10^6
Palashi Bazar	2.08×10^8	1.45×10^8	Northwest corner	9.70×10^6	9.35×10^6
Shantinagar Bazar	2.31×10^8	1.62×10^8	Southwest corner	3.30×10^6	3.60×10^6
Mean ± SD	2.84×10^8 ± 0.82	2.12×10^8 ± 0.65	Mean ± SD	6.12×10^6 ± 3.23	5.67×10^6 ± 2.98

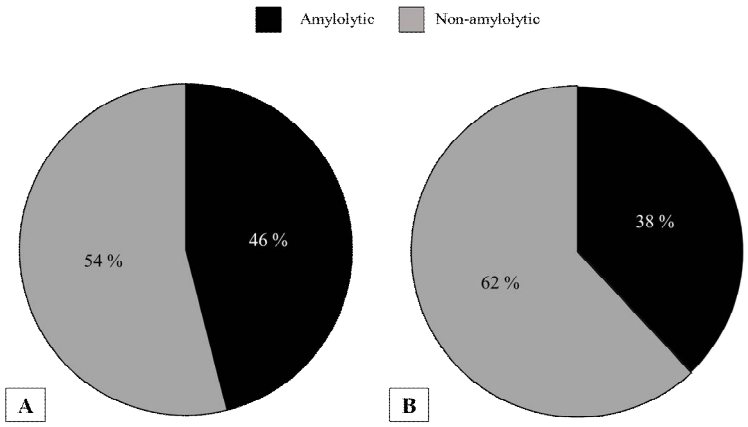

Fig. 1. Percentage of amylolytic activity showing isolates. (A) Garbage soil and (B) garden soil.

Starch hydrolysis ratio (SHR) of top 10 amylolytic isolates from both soil types were shown in Table 3. SHR of the isolates from garbage soil ranged between 1.80 ± 0.18 and 3.10 ± 0.10 whereas, SHR of the isolates from garden soil ranged between 2.40 ± 0.41 and 2.68 ± 0.58. In a research, Padhiar and Kommu (2016) reported the highest SHR as 1.90. In another study, Oyeleke and Odiwole (2009) reported, the highest SHR (3.10) showing organism to be *Bacillus subtilis* isolated from a cassava waste dumpsite in Minna, Nigeria.

A total of 8 better (4 from each soil) SHR showing isolates were selected for detailed study and identification. All four isolates from garbage soil were found to be Gram positive while garden soil, 3 were Gram positive and one was Gram negative (Table 4). In this study, majority of the amylase producing bacteria were Gram positive. Similar conclusion was drawn in the

investigation by Kumar and Shree (2016) where they worked with 6 Gram positive amylolytic isolates. This finding is further supported by the work of Parmar and Pandya (2012) where out of 18 amylolytic bacterial isolates, 13 were found to be Gram positive. The isolates of the present study showed variable characteristics in terms of their vegetative cells (Table 4).

Table 3. SHR of top 10 isolates of two different soil types.

	Isolate No.	Colony diameter (mm) (Mean ± SD)	Zone diameter (mm) (Mean ± SD)	SHR (Mean ± SD)
Garbage soil	S1//11	4.57 ± 1.64	16.80 ± 2.17	3.10 ± 0.10
	S5/11	6.60 ± 0.65	16.70 ± 1.09	2.57 ± 0.39
	S11/17	7.60 ± 1.39	12.00 ± 1.73	2.50 ± 0.44
	S8/1	7.00 ± 0.87	14.50 ± 2.15	2.10 ± 0.25
	S7/2	6.55 ± 0.42	13.35 ± 1.78	2.10 ± 0.34
	S1/3	9.06 ± 2.52	19.50 ± 2.18	2.10 ± 0.45
	S1/7	6.20 ± 2.05	11.48 ± 1.58	2.10 ± 1.36
	S9/1	6.57 ± 0.82	12.40 ± 0.82	2.09 ± 0.41
	S11/12	6.90 ± 0.74	11.70 ± 0.27	2.07 ± 0.58
	S10/3	5.80 ± 0.91	10.30 ± 1.86	1.80 ± 0.18
Garden soil	S/N/2/7	4.75 ± 0.96	10.75 ± 2.75	2.68 ± 0.58
	S/N/2/2	6.38 ± 0.85	12.25 ± 2.21	2.67 ± 0.77
	S/P/1/1	4.00 ± 0.71	10.6 ± 0.75	2.60 ± 0.19
	S/N/1/1	6.75 ± 1.04	15.25 ± 1.89	2.58 ± 0.50
	S/P/2/17	4.87 ± 1.18	10.50 ± 3.00	2.53 ± 0.38
	S/N/2/9	3.88 ± 0.85	10.50 ± 0.58	2.49 ± 0.47
	S/N/5/2	4.62 ± 0.75	10.75 ± 0.96	2.43 ± 0.43
	S/P/1/5	4.13 ± 0.86	8.75 ± 0.96	2.42 ± 0.50
	S/P/1/10	6.63 ± 0.75	11.25 ± 1.50	2.42 ± 0.67
	S/N/4/1	7.00 ± 0.00	10.75 ± 4.92	2.40 ± 0.41

Table 4. Gram reaction and vegetative cell characteristics of the selected isolates.

	Isolate No.	Gram reaction	Vegetative cell characteristics
Garbage soil	S1/11	+	Long rods, occur in long chains
	S5/11	+	Long rods, occur in short chains
	S8/1	+	Very short rods, occur as single cells
	S11/17	+	Rod-shaped, occur in short chains
Garden soil	S/N/1/1	+	Short rods, occur singly or in cluster
	S/P/1/1	+	Short rods, occur as single cells
	S/N/2/7	+	Very short rods, occur singly or in cluster
	S/N/2/2	-	Short rods, occur as single cells

Some of the major physiological and biochemical tests of the selected isolates are presented in Table 5. Considering all available morphological, physiological and biochemical characteristics and following Bergey's Manual of Systematic Bacteriology Vol. I and Vol. II, the bacterial isolates were provisionally identified (Table 5) and all 7 Gram positive isolates belonged to the genus *Bacillus* with five different species while the only Gram negative isolate was *Acetobacter liquefaciens.* These identifications agree with Pandey *et al.* (2000) who reported *Bacillus* spp. to be the most prominent among various amylase producing bacteria. According to Prakash and Jaiswal (2009), *B. subtilis, B. stearothermophilus, B. lecheniformis* and *B. amyloliquefaciens* are known to be the good producers of thermostable α-amylase. Basma *et al.* (2015) also reported amylolytic activity from *B. amyloliquefaciens* under submerged fermentation using some agro-industrial by-products. Similar result was reported by Prasad (2014) who reported amylase producing *Bacillus* species from soil samples from various apartment garbage dumping sites.

Table 5. Major physiological and biochemical characteristics of the isolates and their conventional identification.

	Isolate No.	KOH solubility test	VP test	MR test	Utilization of		Tyrosine degradation	Conventional identification
					Propionate	Citrate		
Garbage soil	S1/11	-	+	+	+	-	-	*Bacillus azotoformans*
	S5/11	-	+	+	-	-	-	"
	S8/1	-	+	+	+	+	-	*B. stearothermophilus*
	S11/17	-	+	+	-	-	-	*B. acidocaldarius*
Garden soil	S/N/1/1	-	+	+	+	-	-	*B. subtilis*
	S/P/1/1	-	+	+	+	-	-	"
	S/N/2/7	+	+	+	-	+	+	*B. megaterium*
	S/N/2/2	-	+	+	+	+	+	*Acetobacter liquefaciens*

+ = Positive result, - = Negative result

Table 6. Comparison between provisional and molecular identification of the selected isolates.

Isolate No.	Conventional identification	Molecular identification			
		Scientific name	Strain	Max. coverage score	Identity match (%)
S1/11	*B. azotoformans*	*Bacillus* sp.	T5-12	893	96
S5/11	*B. azotoformans*	*B. cereus*	MSW	1013	99
S8/1	*B. stearothermophilus*	*Bacillus* sp.	FJAT-14266	1027	99
S11/17	*B. acidocaldarius*	*B. toyonensis*	KK25A	520	91
S/N/1/1	*B. subtilis*	*B. cereus*	T10	588	98
S/P/1/1	*B. subtilis*	*Stenotrophomonas* sp.	ZJZG10	920	96
S/N/2/2	*B. megaterium*	*B. subtilis*	XF-1	1085	100
S/N/2/7	*Acetobacter liquefaciens*	*Pseudomonas* sp.	NCCP-1179	941	97

For authentication, the selected isolates were confirmed through molecular identification based on 16S rDNA sequencing. Amplified DNA bands were found to be approximately 600 bp (Fig. 2). The isolates were identified as *Bacillus* sp. T5-12, *B. cereus* MSW, *Bacillus* sp. FJAT-14266, *B. toyonensis* KK25A, *B. cereus* T10, *Stenotrophomonas* sp. ZJZG10, *B. subtilis* XF-1 and *Pseudomonas* sp. NCCP-1179 based on sequence analysis. Similarly, Ghazala *et al.* (2016) identified an amylolytic bacterial strain by 16S rDNA gene sequencing, belonging to the genus *Bacillus* with the closest relation to *B. mojavensis*. In another study, Krishma and Radhathirumalaiarasu (2017) also identified a potential amylolytic bacterial isolate to be *Bacillus cereus* KR9.

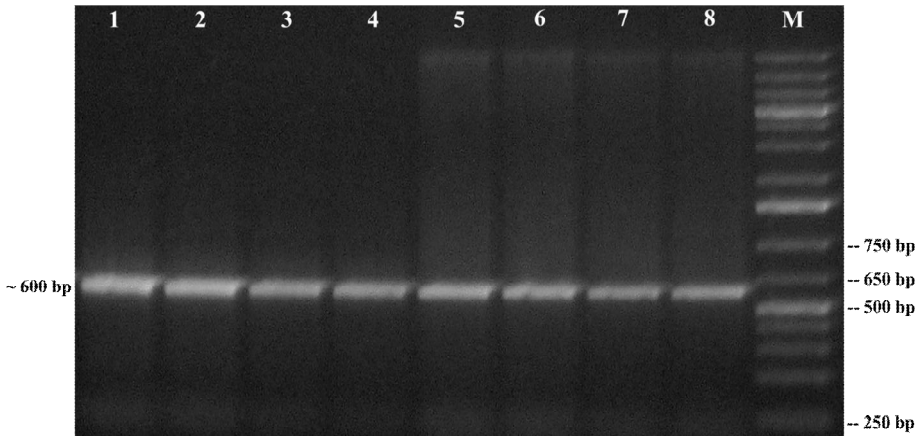

Fig. 2. PCR amplification of part of the 16S rRNA. Lane M is the 1.0 kb ladder and lanes 1-8 are representing 8 different bacterial isolates.

In this study, molecular identification of 6 Gram-positive isolates correlated with their provisional identification up to generic level and 2 isolates were found to be different. So, conventional identification of bacteria based on their morphology, physiological and biochemical profile was found to be valid to some extent. In this experiment, pH of samples, mean bacterial count and SHR of bacteria isolated from garbage soil were found to be higher than garden soil suggesting the former as a better source for amylolytic bacteria. In addition, garbage soil can be a potentially cheap source of amylase enzyme producing bacteria and when utilized properly this source can contribute to the growing needs of amylase enzyme in various industries. Further research is needed for production, optimization, purification and characterization of amylase enzyme by these bacterial isolates and possible biotechnological application of the enzyme.

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