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Isolation and Identification of Indigenous Poly-β-Hydroxy-butyrate (PHB) Producing Bacteria from Different Waste Materials

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Key words: Poly-β-hydroxybutyrate (PHB), Indigenous, PHB-producing bacteria

Abstract

Attempt was made to isolate and identify poly-β-hydroxybutyrate **(**PHB) producing bacteria from different domestic and industrial waste materials from in and around Dhaka Metropolitan city. The bacterial load of the collected samples ranged in between 5.50 ×10^3 and 2.52 × 10^7, 3.10 × 10^4 and 2.23 × 10^7, 7.50 × 10^3 and 1.01 × 10^7 cfu/g or cfu/ml in NAG (nutrient agar glucose), PYG and LB media, respectively. The maximum bacterial count (2.52 × 10^7 cfu/g) was observed in NAG medium from soil sample of BDR market and the lowest bacterial load (5.5 × 10^3 cfu/ml) was observed in NAG medium from the garments wastewater. The highest number (33) of PHB producing bacteria was found in the kitchen waste soil and the lowest number (9) was in water sample of the Turag river. All the Sudan Black B positives isolates were tested for positive PHB production. Primarily, 160 PHB positive bacteria were isolated and finally 30 isolates were selected for identification. Provisionally identified potential 10 PHB positive bacterial isolates were identified on molecular basis. Nine of them were matched with their conventional identification but conventionally identified *Rhizobium leguminosarum* was found to be as *Sinorhizobium* sp. The isolated PHB producing bacteria could be used for biotechnological application.

Introduction

At present non-biodegradable plastic is one of the major environmental hazards. Plastic causes serious damage to environment both during its production and disposal. So the scientific community is very much worried about the inevitable use of synthetic plastics. Environmentally the friendly polymers could be the substitute of synthetic plastics. Different biodegradable plastics have been developed either by incorporating natural polymers into conventional plastics formulations, or by microbial fermentations.

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However, physical limitations of these materials still exist (Shaaban et al. 2012). Biopolymers have attracted great interest due to their excellent biodegradability, biocompatibility and varied applications. Polyhydroxyalkanoates (PHAs) are biodegradable polyesters produced by bacteria which are gaining importance as alternative biopolymers to petroleum-based plastics due to their eco-friendly nature. Bacteria of different genera accumulate PHAs as intracellular carbon and energy storage granules in response to growth media containing excess carbon substrate and limited quantities of nitrogen source (Narayanan and Ramana 2012). They are readily degraded by the depolymerases present in the environmental microflora resulting in the formation of water and CO_2 (Suriyamongkol et al. 2007).

Among the entire bio-based and bio-degradable polymers, polyhydroxyalkanoates (PHAs) are well-known. The endocellular PHAs are biosynthesized hydroxy-fatty-acids stored as lipid inclusions when carbon source is in abundance and nutrients like nitrogen, phosphorus, oxygen or sulfur are limited. These are secondary metabolites produced by various microbes in response to environmental stress. More than 90 hydroxyalkanoic acids have been reported as constituents of PHAs and among these, polyhydroxybutyrate (PHB) represents a common type synthesized by different microbes (Mohapatra et al. 2017). Poly-hydroxybutyrate (PHB) belongs to the class of biodegradable plastics PHAs. PHB was first among the family of PHAs to be detected by Lemoigne in 1926 as a constituent of bacterium *Bacillus megaterium* (Lemoigne 1926). The physical properties of the PHB are similar to those of some conventional plastic. Because of their good biodegradability and biocompatibility PHB have attracted interest in their use as an alternative to petroleum based plastic including fine chemicals, plastics, printing materials bio fuel, agriculture, marine, medical and other fields (Singh et al. 2011).

PHB production is increased by excess of carbon source and limiting the nutrients such as nitrogen, phosphorus, sulfur, magnesium, iron, oxygen, and potassium (Madison and Huisman 1999). It is an intracellular polymer accumulated under stress conditions but with excess carbon source. It is produced by fermentation process of microorganisms (Mikkili 2014). Considering environmental hazards the present study was aimed to isolate and identify indigenously PHB producing bacteria from different waste samples for biotechnological application.

Materials and Methods

Ten samples were collected from ten different industrial waste dumping sites. Water and soil samples were collected aseptically in sterile bottles and plastic bags. Collected samples were immediately brought to laboratory for analysis and pH of the samples was measured by a pH meter (ToA-DKK, HM-31P, Japan). Serial dilution technique (Greenberg et al. 1998) was followed using nutrient agar with 1% glucose (NAG)

(Eklund and Lankford 1967) and peptone yeast extract glucose (PYG) agar (Atlas 1997) and Luria-Bertani (LB) agar (Atlas 1997) media for enumeration of aerobic heterotrophic bacteria associated with collected samples. 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 incubation, plates having well discrete colonies were counted. For the rapid detection and isolation of PHB producing bacteria, 0.02% alcoholic solution of Sudan black B was applied to stain bacterial colonies and the plates were kept undisturbed for 30 min. The excess dye was then decanted and plates were rinsed gently by adding ethanol. Colonies unable to incorporate the Sudan black B appeared white, while PHB producers appeared bluish black (Fig. 1) (Juan et al. 1998). Simple and Gram staining of the selected isolates were done following standard methods (SAB 1957). For conventional identification major 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 and Atlas 1997) were also carried out and identification was done following Bergey's Manual of Systematic Bacteriology (Krieg and Holt 1984, Sneath et al. 1986).

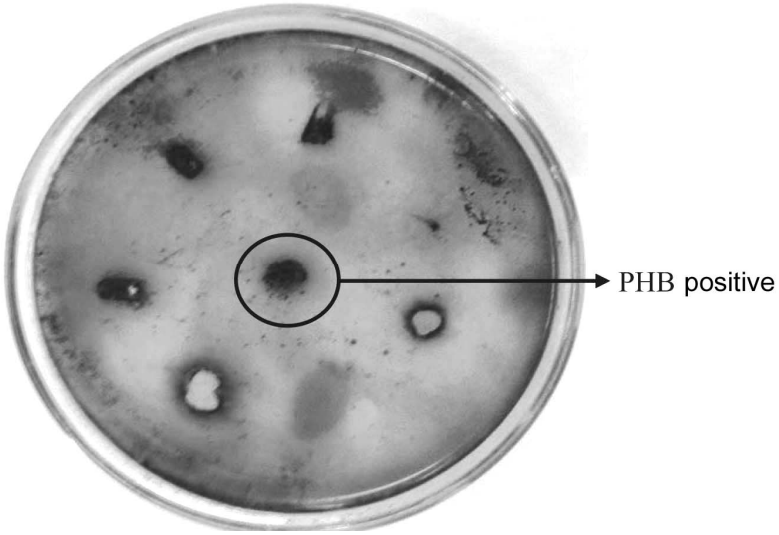

Fig. 1. Screening of PHB positive bacteria after purification.

Molecular identification of potential ten isolates was conducted by amplifying ~600bp fragments of 16S rDNA followed by sequence analysis using NCBI-BLAST search (Fig. 2). Amplification of DNA was done the primer pair designed by Rudi et al. (1997) using CC [F] 5'-CCAGACTCCTACGGGAGGCAGC-3 ′ and CD [R] 5'-CTTGTGCGGGCC CCCGTCAATTC- 3′. Supernatant of heat lysed cell suspension was used as the source of template DNA for PCR amplification. 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). DNA sequencing was performed in an automated gene sequencer and sequences were analyzed through NCBI-BLAST database (http://blast.ncbi.nlm.nih.gov/) and r RNA BLAST (http://bioinformatics.psb.ugent.be/cgi-bin/rRNA/blastform.cgi) programs.

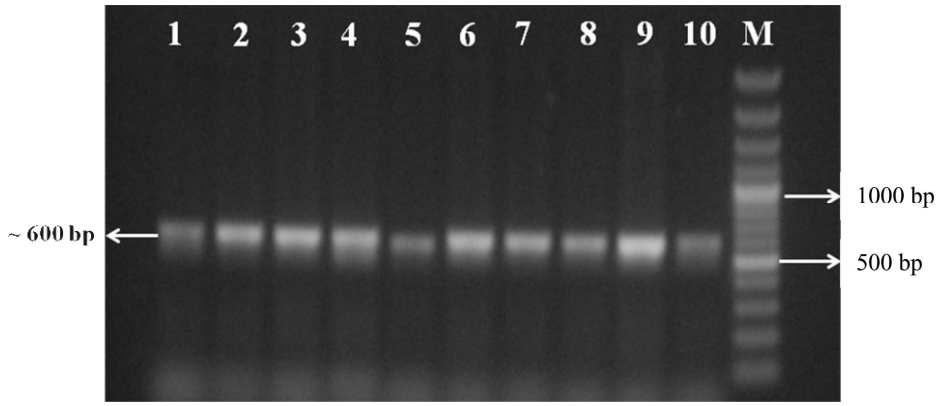

Fig. 2. PCR amplification of part of the 16S rRNA. Lane M is the 100 bp ladder and lanes 1- 10 are representing 10 different bacterial isolates.

Results and Discussion

A total ten samples were collected from different domestic and industrial areas in and around Dhaka Metropolitan city. The bacterial load of different samples is shown in Table 1. The heterotrophic bacterial load of the collected samples ranged in between 5.55 × 10^3 and 2.52 × 10^7, 3.10 × 10^4 and 2.23 × 10^7, 7.50 × 10^3 and 1.01 × 10^7 cfu/g or cfu/ml in NAG, PYG and LB media, respectively. The maximum bacterial count (2.52 × 10^7 cfu/g) was observed in NAG medium from the soil sample of BDR market, Uttara, Dhaka. On the other hand, the lowest bacterial load (5.5 × 10^3 cfu/ml) was observed in NAG medium from the garments wastewater. The difference in bacterial count may be due to different biochemical or organic factors which influence bacterial growth and survival. The highest number (33) of polyhydroxyalkanoates producing bacteria was found to be in the kitchen waste soil and the lowest number (9) was found in the Turag river water sample. Higher bacterial count in the garbage soil samples might be due to availability of diversified nutrients as waste substances. The results revealed that PHB producing bacteria are widely distributed in different waste habitats.

All the Sudan Black B positives isolates were subjected to qualitative screening for PHB producing strains. Primarily, 160 PHB positive bacteria were isolated from the different samples and finally 30 isolates were selected for conventional identification. Out

of 30 isolates, 21 were Gram positive rods and 9 were Gram negative rods. The result showed that most of the PHB producing bacteria were Gram positive. This was in conformity with the findings of Shah (2014) where they reported 10 Gram positive bacilli and 5 Gram negative bacilli out of 15 isolates cultured from soil sample. Similarly, Raju et al. (2012) also found that among 23 isolates 12 were Gram positive bacilli, 2 were Gram positive cocci and 9 were Gram negative bacilli cultured from soil and waste water sample.

Table 1. Bacterial load of collected samples.

Sample No.	Sampling sites	Samples	Bacterial load (cfu/ml of water and cfu/g of soil)			No. of PHB positive bacteria
			NAG	PYG	LB	
1	Dairy farm, Savar, Dhaka	Soil	4.35×10^6	5.30×10^6	ND	18
2	The Bangshi river, Savar, Dhaka	Water	2.33×10^7	5.10×10^6	ND	10
3	The Turag river, Dhaka	Water	2.76×10^4	1.15×10^5	4.40×10^4	9
4	The Buriganga river, Dhaka	Water	1.90×10^4	3.40×10^4	1.99×10^4	10
5	Dumping waste, Gabtoli, Dhaka	Soil	2.23×10^5	2.92×10^5	1.23×10^6	14
6	Navy dockyard, Narayanganj	Water	3.55×10^5	3.10×10^4	7.50×10^3	12
7	Kitchen waste dumpsite, Uttara, Dhaka	**Soil**	1.35×10^6	8.75×10^6	3.80×10^5	**33**
8	BDR market, Uttara, Dhaka	Soil	$\mathbf{2.52 \times 10^7}$	$\mathbf{2.23 \times 10^7}$	$\mathbf{1.01 \times 10^7}$	19
9	Garments waste, Fotulla, Dhaka	Water	5.55×10^3	5.60×10^5	2.40×10^5	13
10	Balur Math, Kazla, Dhaka	Soil	4.10×10^5	1.58×10^5	8.45×10^4	22

ND = Not done.

Results of some of major biochemical tests for provisional identification are shown in Tables 2 and 3. The isolated Gram positive rods were provisionally identified as the different species of the genus *Bacillus*. The result of provisional identification of Gram positive PHB producing bacteria agrees with Ghate et al. (2011). Narayanan and Ramana (2011) also reported similar type of observation. On the other hand, Gram negative members were identified as the genera of *Acetobacter, Enterobacter, Klebsiella, Neisseria, Pseudomonas* and *Tatumella*. Mercan et al. (2002) and Tombolini and Nuti (1989) also reported *Pseudomonas* and *Rhizobium* having the ability of PHB production.

Bacterial abundance of the isolates is shown in Table 4. Among the indigenous isolates *Bacillus anthracis*, *B. cereus*, *B. mycoides* and *B. megaterium* were the highest abundance (10.0%). The lowest abundance represented by *Bacillus lentus*, *Acetobacter liquefaciens*, *A. aceti*, *Enterobacter cloaceae*, *Klebsiella oxytoca*, *Neisseria elongate*, *Pseudomonas aeruginosa* and *Rhizobium leguminosarum* (3.33%).

Identification 10 potential PHB positive isolates were further confirmed through molecular analysis based on 16S rDNA sequencing. Following BLAST search analysis the isolates were identified as *B. anthracis* S2CB42, *Bacillus cereus* AK9, *B. cereus* HB45, *B. cereus* WCF2, *B. cereus* PU, *B. megaterium* LY6, *B. mycoides* TCCC11292, *B. subtilis* NXUSASNFB008, *B. thuringiensis* B43 and *Sinorhizobium* sp. R25067 (Table 5).

Table 2. Major biochemical tests and provisional identification of Gram positive bacteria.

Isolates	Biochemical tests							Provisional identification
	Catalase	VP	Starch	Casein	Citrate	Propionate	Nitrate	
S_1P-7	+	-	+	+	-	-	+	*Bacillus anthracis*
S_2N-4	+	+	+	+	-	-	+	*B. cereus*
S_2P-2	+	-	+	+	-	-	-	*B. mycoides*
S_3P-1	-	+	+	-	+	-	+	*B. pumilus*
S_4P-2	+	+	+	+	-	-	+	*B. subtilis*
S_4L-3	+	-	+	+	-	-	+	*B. thuringiensis*
S_6P-4	+	+	+	+	-	-	-	*B. megaterium*
S_6P-7	+	+	+	+	-	-	-	*B. megaterium*
S_6N-8	+	+	+	+	-	-	-	*B. lentus*
S_7N-5	+	-	+	+	-	-	+	*B. cereus*
S_7P-27	**+**	**+**	**+**	**+**	**-**	**-**	**-**	*B. mycoides*
S_7P-29	+	-	+	+	-	-	+	*B. anthracis*
S_7P-32	+	+	+	+	-	-	+	*B. schlegelii*
S_7L-25	+	+	+	+	-	-	+	*B. pumilus*
S_7L-26	+	+	+	+	-	-	+	*B. subtilis*
S_8N-5	+	-	-	+	-	-	-	*B. anthracis*
S_8N-6	+	+	+	+	-	-	-	*B. mycoides*
S_8P-11	+	-	+	+	-	-	+	*B. thuringiensis*
S_8L-18	+	+	+	+	-	-	-	*B. cereus*
S_9P-2	-	+	+	+	-	-	+	*B. schlegelii*
S_{10}L-16	+	-	+	+	-	-	-	*B. megaterium*

+ = Positive result, - = Negative result.

Molecular identification of nine Gram positive isolates matched with their provisional identification up to generic level and only one Gram negative isolates were found to be different. Therefore, conventional identification of bacteria based on their morphology, physiological and biochemical profile was found to be valid and authentic to some extent in compared to genotyping results obtained during this study.

Table 3. Major biochemical tests and provisional identification of Gram negative bacteria.

Isolates	Biochemical tests							Provisional identification
	Catalase	Oxidase	Arginine	Indole	H_2S	Tyrosine	Levan	
S_2P-3	+	-	-	+	-	+	-	*Klebsiella oxytoca*
S_3N-1	+	+	-	-	-	+	-	*Neisseria elongata*
S_4N-2	+	+	+	-	-	+	-	*Acetobacter liquefaciens*
S_4L-10	+	-	-	-	-	+	-	*Tatumella ptyseos*
S_6P-3	+	-	-	-	-	+	-	*Tatumella ptyseos*
S_6N-1	+	-	-	-	-	+	-	*Acetobacter aceti*
S_7P-19	+	-	+	-	+	+	-	*Pseudomonas aeruginosa*
S_8P-2	+	-	-	-	-	+	-	*Rhizobium leguminosarum*
S_{10}P-3	+	-	+	-	-	-	-	*Enterobacter cloaceae*

+ = Positive result, - = Negative result.

Table 4. Number of isolates and their percentage of abundance.

	Name of isolates	Number of occurrence	Percentage
Gram positive (21)	*Bacillus anthracis*	3	10.00
	B. cereus	3	10.00
	B. mycoides	3	10.00
	B. megaterium	3	10.00
	B. subtilis	2	6.67
	B. schlegelii	2	6.67
	B. pumillus	2	6.67
	B. thuringiensis	2	6.67
	B. lentus	1	3.33
Gram negative (9)	*Acetobacter liquefaciens*	1	3.33
	Acetobacter aceti	1	3.33
	Enterobacter cloaceae	1	3.33
	Klebsiella oxytoca	1	3.33
	Neisseria elongate	1	3.33
	Pseudomonas aeruginosa	1	3.33
	Rhizobium leguminosarum	1	3.33
	Tatumella ptyseos	2	6.67

Table 5. Comparison between provisional and molecular identification of the selected isolates.

Isolates	Provisional identification	Molecular identification			
		Scientific name	Strain	Max. coverage score	Identity match (%)
S_7N-5	*Bacillus cereus*	*Bacillus cereus*	AK9	784	95
S_{10}L-16	*B. megaterium*	*B. megaterium*	LY6	913	97
S_2N-4	*B. cereus*	*B. cereus*	HB45	680	93
S_4P-2	*B. subtilis*	*B. subtilis*	NXUSASNFB008	913	97
S_4L-3	*B. thuringiensis*	*B. thuringiensis*	B43	1007	99
S_7P-29	*B. anthracis*	*B. anthracis*	S2CB42	691	91
S_8N-6	*B. mycoides*	*B. mycoides*	TCCC11292	1007	98
S_8P-11	*B. thuringiensis*	*B. cereus*	WCF2	1013	99
S_8L-18	*B. cereus*	*B. cereus*	PU	1007	99
S_8P-2	*Rhizobium leguminosarum*	*Sinorhizobium* sp.	R25067	961	98

The isolation of the indigenous PHB producing bacteria was found to be successful from different waste habitats of Dhaka Metropolitan city. Nowadays researchers are focusing on biopolymer producing microorganisms for developing biodegradable plastics. The search for promising strains of PHB producers is a continuous process and development of efficient PHB producing bacteria is a prime need to control environmental hazards due to synthetic non-biodegradable polymer compounds. Finally, it could be concluded that the isolated potential *Bacillus* species with PHB producing potentiality can be employed in the production of PHB as well as in the biotechnological application.

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