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ISOLATION OF BIODEGRADABLE POLYMER PRODUCING BACTERIA FROM WASTE MATERIALS OF DHAKA METROPOLITAN CITY

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Abstract

Bacterial polyhydroxyalkanoates (PHAs) are the most feasible substitute for fossil fuel based-plastics owing to their biocompatibility and biodegradability. The study was aimed to isolate and identify polyhydroxyalkanoates producing bacteria from various readily available and unusable waste materials. 75 bacterial isolates were identified as PHAs producers from the collected samples. Among them, 33 isolates were selected for their in-depth analysis and identification. All of the selected bacterial isolates were Gram positive rods except one. Considering morphological, biochemical, and physiological characteristics, isolated bacteria were provisionally identified. Interestingly, 32 isolates corresponded to the single genus *Bacillus*, whereas the remaining isolate was *Enterococcus* sp. PHAs production was confirmed by the presence of key functional groups notably C=O, C-H, C-O, and –OH, respectively.

Introduction

The diverse qualities of fossil fuel-based plastics, including hardness, resistance to deterioration, lightness, flexibility, and resilience, have made them highly popular. It has become a crucial commodity in modern society due to its usefulness, versatility, and broad applicability in the domestic, medicinal, and commercial sectors (Yadav *et al*. 2018). The demand for plastic and plastic materials has been steadily rising in response to increased population growth, industrialization, globalization, and urbanization, resulting in major environmental issues known as plastic pollution (Rajvanshi *et al.* 2023). Biopolymers can be an excellent remedy to overcome this problem as well as an appealing substitute to fossil fuel-based synthetic plastics due to their biodegradability and biocompatibility. It can be entirely decomposed by microbial enzymes. It can be categorized into three groups *viz.*, natural biopolymers, synthetic biopolymers, and microbial biopolymers (Narayanan *et al*. 2020a).

Polyhydroxyalkanoates (PHAs) are the most environmentally friendly biopolymer which accumulates as granular form within the cellular structure under the conditions of ample carbon source and exhaustion of a single nutrients particularly N, S, P, K or Oxygen (Novelli *et al*. 2021). PHA has a variety of potential applications due to its biodegradability, biocompatibility, durability, flexibility, elasticity, thermoplasticity, and reduced lethality to cells. For this, PHAs are gaining ubiquity in a variety of fields, including agriculture, medical sector, clothing, packaging, cosmetics, and coating materials as a substitute for synthetic plastics (Samrot *et al.* 2021). A multitude of Gram positive and Gram negative bacteria including *Bacillus*, *Pseudomonas*, *Methylotrophs*, *Alcaligenes*, *Azotobacter*, *Acetobacter*, *Enterococcus*, *Enterobacter*, *Archaebacteria*, recombinant *Escherichia coli*, etc. play important role in the production of PHAs (Giraldo-Montoya *et al.* 2020).

Considering environmental hazards, this research was framed to isolate polyhydroxy-alkanoates producing bacteria from various types of waste in and around Dhaka Metropolitan City, extract PHAs, and characterize PHAs using FTIR-ATR.

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Material and Methods

Eight distinct samples including soil, rotten vegetables and dairy products were gathered carefully in sterile plastic boxes and polybags from different locations of Dhaka Metropolitan City. The pH of the samples was checked by using a pH meter (HANNA HI 8424). Serial dilution plate technique was followed for the enumeration and isolation of bacteria from the collected samples using NA (Nutrient Agar) and LBA **(**Luria-Bertani Agar) media containing 1% dextrose. The CFU/g (solid samples) and CFU/mL (liquid samples) were done according to Tripathi *et al.* (2025).

PHAs selective media (Narayanan *et al.* 2020b) and 0.3 % (w/v) alcoholic Sudan Black B solution (Babu *et al.* 2014) were used to screen PHAs producing bacteria. Bacterial colonies that were capable to produce PHAs could retain the pigment of the Sudan Black B dye and appeared as bluish black. Intercellular lipid staining was carried out using 0.3% (w/v) alcoholic Sudan Black B solution and examined under oil immersion fluorescent microscope (BioBlue.Lab, Netherland) to observe PHA granules.

PHAs were extracted from the bacterial isolates following the sodium hypochlorite-chloroform method (Ali *et al.* 2017). The dry weight of PHAs was measured using Electronic Precision Balance (model: EK 600i-600) and data were recorded. FTIR-ATR (Fourier Transform Infrared-Attenuated Total Reflectance) was carried out at room temperature using a spectrophotometer (IRPrestige-21, Fourier Transform Infrared Spectrophotometer SHIMADZU) to illustrate the functional units present in the extracted PHA sample, within the limits of 4000-700 cm^{-1} (Samrot *et al.* 2021). The obtained FTIR-ATR results were compared with the available literatures related to PHAs production.

In addition, Gram staining and major biochemical tests were also performed for bacterial identification as per Bergey's Manual of Systematic Bacteriology (Sneath *et al.* 1986).

Results and Discussion

The number of aerobic heterotrophic bacterial colonies obtained from the collected samples varied from 3×10^5 to 5.2×10^7 cfu/g or cfu/mL on NA and 3.4×10^5 to 15.1×10^8 cfu/g or cfu/mL on LBA media (Table 1). Sample collected from Lalbagh sweet shop yielded the highest bacterial count of 15.1×10^8 cfu/ml in LBA medium, while a soil sample from Shivbari dumpsite showed the lowest bacterial count of 3×10^5 cfu/g in NA medium. The highest amount of PHA producers was observed in dumpsite soil sample of Samsun Nahar Hall, Dhaka University (Table 1). The findings demonstrated that PHA-producing bacteria are widely dispersed throughout various waste habitats and dairy products due to presence of various nutritional substances.

Seventy five bacterial isolates were screened out as PHAs producers from the collected samples. This was done using PHAs selective media (Fig. 1A), 0.3% alcoholic Sudan Black B (Fig. 1B), and microscopic observation (Fig. 2). Of these, 33 bacterial isolates were selected for thorough analysis and identification. Out of 33 isolates, only one was Gram-positive coccus and the rest 32 were Gram-positive rods. In a different study, Rathore (2011) found 9 Gram negative rods, 12 Gram positive rods and 2 Gram positive cocci as PHAs producers from various sources of waste materials. Whereas, another study reported higher number of Gram positive rods as polyhydroxybutyrate (PHB) producers from various waste material samples (Khan *et al.* 2019) which is similar to this present findings. This finding indicated that the majority of PHA generating bacteria were Gram positive bacilli and can be obtained from various waste habitats.

The results of major biochemical tests and traditional identification of bacterial isolates as per Bergey's Manual of Systematic Bacteriology (Sneath *et al.* 1986), 32 isolates were identified as the genus *Bacillus* which includes 9 individual species namely *Bacillus subtilis, B. cereus, B.*

pumilus, B. schlegelii, B. licheniformis, B. alcalophilus, B. macquariensis, B. polymyxa, and *B. firmus*. The remaining isolate was identified as *Enterococcus* sp. (Table 2). The identified *Bacillus* spp. of this research work was reported earlier in the study of Kumar *et al.* (2016), Getachew and Woldesenbet (2016), and Khan *et al.* (2019). Different species of *Bacillus* including *B. cereus, B. megaterium, B. licheniformis*, and *B. thuringiensis* were also found as PHA producers isolated from various soil samples in accordance with Alia *et al.* (2016).

Table 1. Bacterial load and number of PHAs bacteria of the collected samples.

Sample No.	Sampling sites	Samples	pH	Bacterial load (cfu/g or cfu/mL*) of sample		No. of PHAs positive bacteria
				NA	LBA	
1	Dumpsite, Shivbari	Soil	5.51	3×10^5	3.4×10^5	7
2	Ananda Bazar, Dhaka	Rotten vegetable	7.56	7×10^5	1×10^6	6
3	Dumpsite, Azimpur	Ash and soil	7.27	3.7×10^5	5.9×10^5	9
4	Dumpsite, JN Hall, DU	Soil	7.51	5×10^7	4.3×10^7	7
5	Lalbagh, Dhaka	Curd	6.46	7×10^5	6.7×10^5	12
6	Lalbagh, Dhaka	Whey	4.19	**5.2×10^7**	**15.1×10^8**	10
7	Dumpsite, Motijheel	Soil	8.33	1.95×10^6	1.8×10^6	9
8	Dumpsite, SN Hall, DU	Soil	7.22	5×10^5	7×10^5	**15**

Table 2. Major biochemical tests and provisional identification of the bacterial isolates.

Bacterial Isolates	Catalase	Starch	Casein	Oxidase	VP	MR	Citrate	Propionate	Indole	Glucose Fermentation		Provisional identification
										Gas	Acid	
A5, E5, E6, E9, D10, B2, and B5	+	+	+	+	+	+	+	-	-	-	+	*Bacillus subtilis*
C1	+	+	+	-	+	-	+	+	-	-	+	*B. cereus*
B1, LS2, LS10, F18, F21, and S8	+	-	+	+	+	-	+	-	-	-	+	*B. pumilus*
B3, B6, D11, F17, S3, and S10	+	-	+	-	-	-	-	-	-	-	-	*B. schlegelii*
F20	+	+	+	+	+	-	+	+	-	-	-	*B. licheniformis*
A6, B4, and S7	+	+	+	-	-	-	-	-	-	-	+	*B. alcalophilus*
S6	+	+	-	-	-	-	-	-	-	-	+	*B. macquariensis*
D9	+	+	+	-	+	-	-	-	-	+	+	*B. polymyxa*
C5, D6, E1, E3, S1, and S14	+	+	+	-	-	-	-	-	-	-	+	*B. firmus*
F15	-	+	-	-	+	-	-	-	-	+	+	*Enterococcus* sp.

+, - represent positive and negative result, respectively.

Sodium hypochlorite-chloroform technique was followed for extraction and quantification of PHAs from the selected bacterial isolates. The interpretation showed that dry weight of the extracted PHAs was ranged between 0.14 mg/mL and 31.96 mg/ml (Fig. 3). The highest accumulation of PHA (31.96 mg/mL) was found in *B. schlegelii* (D11) and the lowest (0.14 mg/mL) in *B. polymyxa* (D 9). PHA accumulations are widely variable among different species of

Bacillus depending on the type of bacterial strains or food sources used in the process of PHA production (Mascarenhas and Aruna 2017).

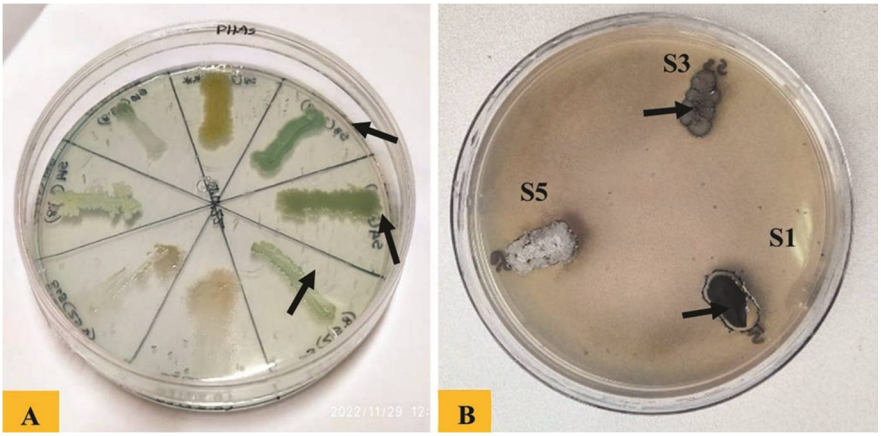

Fig. 1. Screening and detection of PHAs producing bacteria. A: Growth on PHAs selective media and B: Sudan Black B plate staining. Arrows indicate PHA producers.

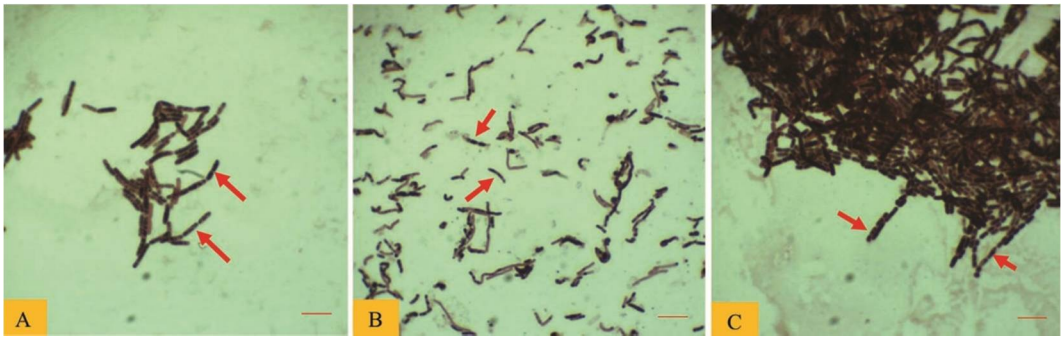

Fig. 2. Photomicrographs of bacterial isolates showing PHAs granules within the cell. A: *Bacillus firmus* (S1), B: *B. subtilis* (S3) and C: *B. pumilus* (S8). Arrows indicate PHA granules. Bar = 10 µm.

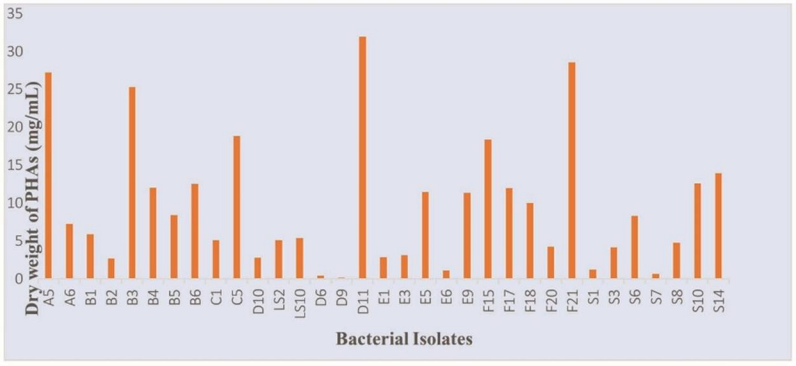

Fig. 3. PHAs production by selected bacterial isolates after 3 days of incubation.

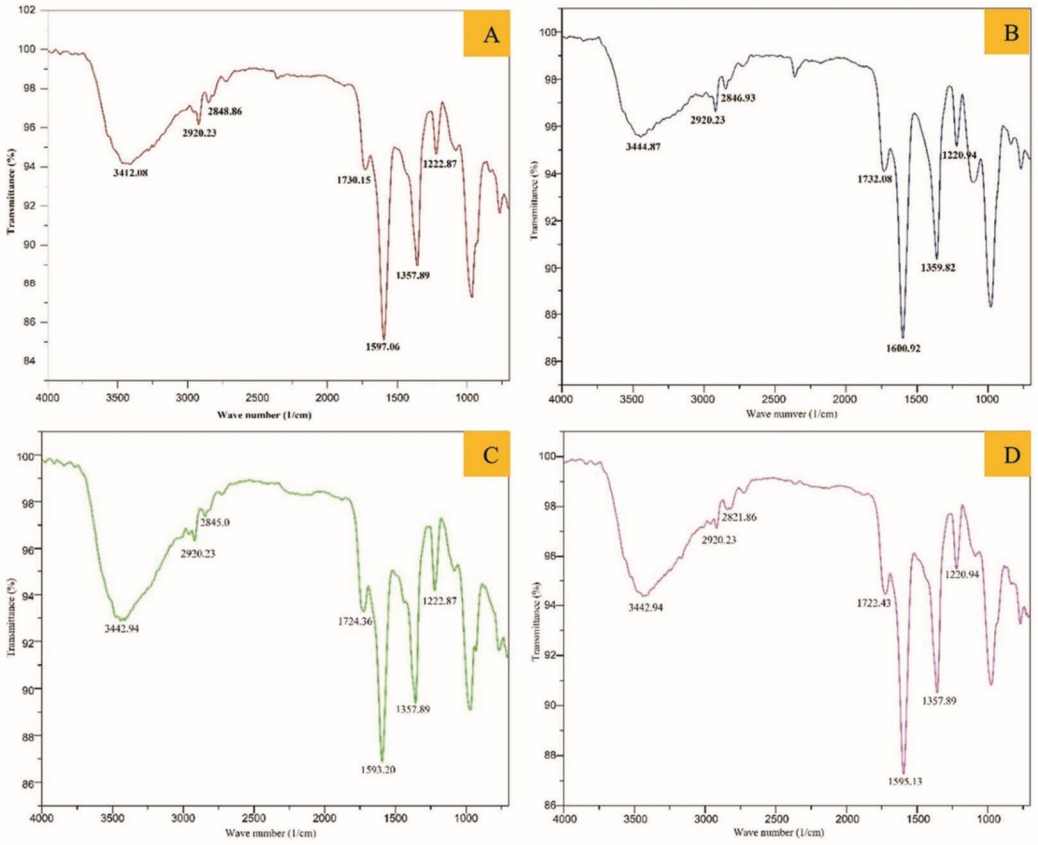

Fig. 4. FTIR-ATR spectra of polyhydroxyalkanoate polymer extracted from bacterial isolates A: *Bacillus pumilus* (LS2), B: *B. firmus* (E3), C: *B. subtilis* (B5) and D: *B. macquariensis* (S6).

Ten extracted PHA samples were analyzed to figure out of their functional units using FTIR-ATR spectroscopy but only four of them (LS2, E3, B5 and S6) showed potential PHA characteristic bands (Fig 4). The spectra at 3412.08 cm^{-1} (Fig 4A), 3444.87 cm^{-1} (Fig 4B) 3442.94 cm^{-1} (Fig 4C) and 3442.94 cm^{-1} (Fig 4D) indicated as -OH group. Devi *et al.* (2015) also observed strong peak at 3436 cm^{-1} which stated -OH stretching extracted from *B. cereus*. The bands at 2920.23/2848.86 cm^{-1} (Fig 4A), 2920.23/2846.93 cm^{-1}, (Fig 4B), 2920.23/2845 cm^{-1} (Fig 4C), and 2920.23/2821.86 cm^{-1} (Fig 4D) corresponded to C-H stretching of methylene and methyl groups. These results were consistent with Chen *et al.* (2016) and Baria *et al.* (2023). Furthermore, the spectra at 1730.15 cm^{-1} (Fig 4A) 1732.08 cm^{-1} (Fig 4B) 1724.36 cm^{-1} (Fig 4C) and 1722.43 cm^{-1} (Fig 4C) indicated the presence of carbonyl (C=O) stretching of ester group. The presence of C=O had been labeled as a PHA marker according to the study of Morya *et al.* (2018). Mascarenhas and Aruna (2017) also documented similar findings when analyzing the PHA extracted from *Bacillus* spp. The spectra at 1600.92 cm^{-1} indicated a weak C=O bond and a series of bands between the range of 1300-1200 cm^{-1} showed the C-O polymeric group. The presence of these functional groups, specifically the C=O group, confirmed that the polymers extracted from the bacterial isolates were polyhydroxyalkanoates (PHAs).

Based on the scientific information gathered in this work, it can be concluded that isolated *Bacillus* spp. seems to be a potential source and can be implemented in the production of PHAs.

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