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- Short communication

ISOLATION AND CHARACTERIZATION OF BACTERIA GROWN ON POLYETHYLENE TEREPHTHALATE PLASTIC BOTTLES

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Abstract

Polyethylene terephthalate (PET) is a polymer used in the production of water and beverage bottles as well as textiles, and packaging materials. The aim of this study is to isolate and characterize PET-associated bacteria. The samples were collected from different sites in Dhaka and inoculated into enriched culture media. The predominant bacteria were *Bacillus* (31%), *Pseudomonas* (31%), and *Enterobacter* (10%), while others were *Listeria, Enterobacter, Citrobacter, Klebsiella, Kluyerea, Staphylococcus* and *Morganella*. Most bacteria can grow at 30 - 40°C temperature and 6.5 - 7.5 pH.

Polyethylene terephthalate (PET) bottles are commonly used for water and soft drinks (Sinha *et al*. 2010). PET is created via chain polymerization of ethylene glycol and terephthalic acid (Kim and Lee 2012). The production of single-use plastic bottles and another disposable packaging from PET has increased significantly during the past few decades (Geyer *et al.* 2017). Few bacteria and fungi have been described for the partial degradation of PET to oligomers or monomers (Wei and Zimmermann 2017). Some of the most well-studied PET-degrading bacteria belong to the genera *Thermobifida, Ideonella, Pseudomonas,* and *Bacillus* (Roberts *et al.* 2020). The present study was aimed to determine the bacterial communities associated with PET in natural environments and their physiological characteristics.

Polyethylene terephthalate plastic bottles were collected from natural habitats where it was dumped for longer period (Table 1). After the collection of PET bottles, it was transported to the laboratory as soon as possible and immediately analysis was started. The bottles were cut into small pieces (around 10 × 9 mm) and washed with sterile distilled water three times. The pieces of PET films were inoculated in different enrichment media such as nutrient agar, Luria-Bertani agar (LB) and Peptone Yeast extract Glucose (PYG) agar using pour plate and spread plate techniques and incubated at 37°C for 24 hrs.

Table 1. The physical factors and location of sampling sites.

Sampling site	GPS reading		Sample collection	Temperature (°C)	
	North	East		Air	Water
Shiv bari temple pond	23°43´45.5´´	90°23´47.32´´	18/10/21	24.0	22.5
Shiv bari temple pond	23°43´45.47´´	90°23´47.51´´	8/12/21	23.0	22.0
Shiv bari temple pond	23°43´44.31´´	90°23´46.76´´	28/12/21	22.0	23.0
Shiv bari temple pond	23°43´44.28´´	90°23´46.78´´	28/12/21	25.0	24.0
Nazira bazar pond	23°43´11.51´´	90°24´27.12´´	23/01/22	23.0	21.0
Ramana lake	23°43´4.28´´	90°23´30.78´´	02/03/22	22.0	21.0

The streak-plate technique was used for quick qualitative isolation. A loopful of culture was spread around the surface of a nutrient agar plate using streak-plate technique to assure individual

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colony growth. Different bacterial colonies from the media were chosen based on their morphological and microscopic characterization of simple, gram and spore staining. The isolates were subjected to physiological characterization following Bergey's Manual of Systematic Bacteriology, Vol. II and III (Garrity *et al.* 2005, De Vos *et al.* 2009), Bergey's Manual of Determinative Bacteriology (Holt *et al.* 1994) and the Society of American Bacteriology (1957).

To amplify 16S rRNA gene of the isolates, their genomic DNA was extracted using heat lysis technique (Karim and Happy 2020). The extracted bacterial DNA was used as a template for PCR based partial amplification of 16S rRNA gene using universal primers as CC [F] 5'-CAGACTCCTACGGGAGGCAGCC-3′ And CD [R] 5´-CTTCTGCGGGCCCCCGTCAATTC 3´. The amplifications were repeated 30 times, for initial denaturation 5.0 min at 95°C, denaturation 1.0 min at 94°C, primer annealing 30.0 sec at 55°C, polymerization 1.0 min at 72°C, final extension 5.0 min at 72°C and the final product was stored at 4°C for further processing. The PCR product was run in 1.1% agarose gel electrophoresis under UV light after ethidium bromide staining (Ameresco). DNA bands were observed on UV-transilluminator and photographed by a Gel Documentation system (Microdot DI-HD, MUV2I-254/365, Cleaver Scientific, UK). The amplified bands were purified by using the FAVORGEN PCR purification kit (Biotech. Corp) following the manufacturer's instructions. Concentrations of the purified DNA were measured using BioDrop µLITE spectrophotometer and purified products were sent to Centre for Advanced Research in Science, University of Dhaka (CARS) for DNA sequencing. The partial sequence of 16S rRNA gene was compared with the existing sequences available in GenBank database of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/GenBank) by means of the basic local alignment search tool (BLAST) to identify close phylogenetic relatives. The sequence data of the isolates were deposited in the GenBank nucleotide sequence database for accession number.

The nucleotide 16S rRNA gene sequences along with reference sequences from the GenBank were aligned and a phylogenetic tree was generated using neighbor joining (NJ) distance-based algorithm to understand intergeneric relationship of the bacterial isolates (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985). MUSCLE was used to perform nucleotide multiple sequence alignments. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.* 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated (complete deletion option). Evolutionary analyses were conducted in MEGA 11(Tamura *et al.* 2021).

Microbial populations naturally coexist with a variety of other cell types rather than dividing into distinct species (Fig. 1). A total of 260 isolates were initially isolated based on their cultural and morphological characteristics and finally after microscopic characterization, 30 isolates were selected for detailed study. Among them, 12 were gram-positive, and they were *Bacillus* (9), *Listeria* (2) and *Sporolactobacillus* and 18 were gram-negative as *Pseudomonas* (8), *Enterobacter* (3), *Citrobacter*, *Klebsiella*, *Kluyvera*, *Staphylococcus*, and *Morganella*. The *Bacillus*, *Enterobacte*r and *Pseudomonas* were found significantly higher variation in association with PET of different habitats (Fig. 2).

The sequences of P1/2, P2/4, SP/3/6, and PY15/4 were used to find its close relatives using phylogenetic analysis, clustered together in the bootstrap test (1,000 replicates) and they were shown next to the branches. The isolates P1/2, P2/4, SP/3/6, and PY15/4 were identified as *Pseudomonas aeruginosa* P1/2, *Bacillus cereus* P2/4, *Pseudomonas aeruginosa* SP3/6 and *Staphylococcus* sp. PY15/4, respectively (Table 2). The level of precision of the sequence

similarity of the 16S rRNA of the identified organisms with that of the GenBank reference organisms was shown (Table 2).

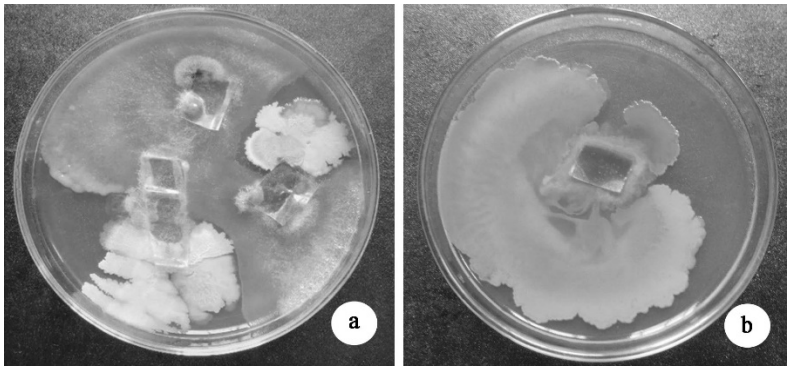

Fig. 1. Bacterial colonies developed at different stages. PET associated bacteria grow on PYG agar.

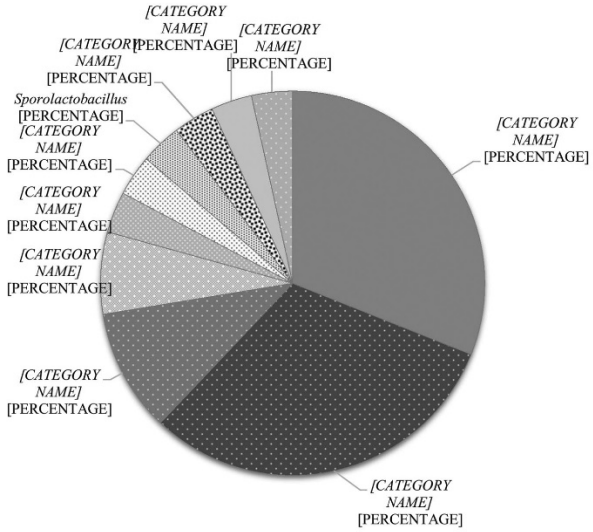

Fig. 2. Proportions of bacteria in association with PET.

The phylogenetic relationship was constructed based on neighbor-joining (NJ) distance-based algorithm using the 16S rRNA gene sequences (Fig. 3). The phylogenetic tree was shown with two main branches, one contains *Pseudomonas aeruginosa* P1/2 and *Pseudomonas aeruginosa* SP3/6 and another contains two subgroups *Bacillus cereus* P2/4 and *Staphylococcus* sp., where *Staphylococcus* sp. PY15/4 is closely related with reference *Staphylococcus haemolyticus* BM27 (Fig. 3).

The bacterium *Pseudomonas putida* SP3/1 was found to be alkalophilic to neutral, while *Klebsiella oxytoca* Na5/1 grew well at pH 9.0 (Fig. 4). The bacteria *Staphylococcus* sp. PY15/4 OR272116, *Citrobacter amalonaticus* P2/10/2, *Sporolactobacillus inulinus* P2/18/3, *Pseudomonas aeruginosa* P1/2 OR272113, *Enterobacter aerogenes* NB7/2 and *Bacillus subtilis* P4/3/3 were

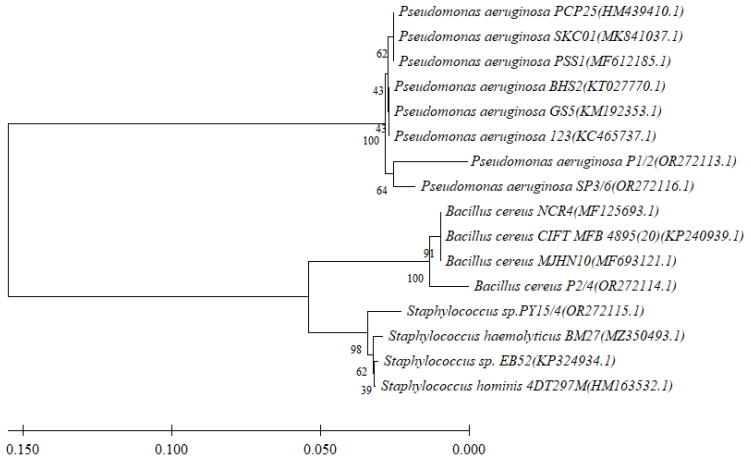

Fig. 3. Phylogenetic tree is constructed based on partial sequences of 16S rRNA genes of isolates and corresponding references using MEGA 11. Bootstrap values are represented by numbers at the nodes based on 1000 replications. The scale is the evolutionary distance value.

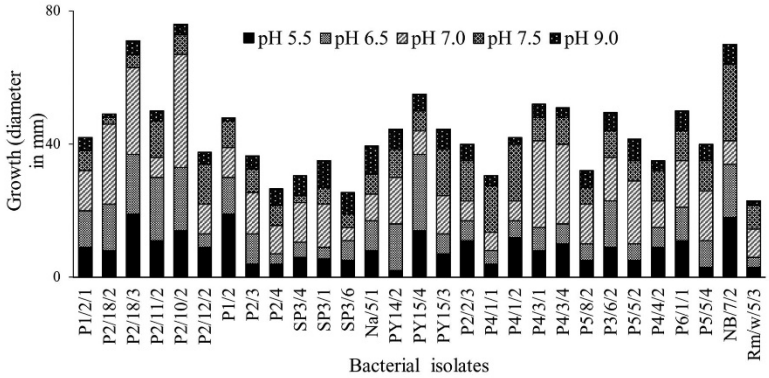

Fig. 4. Bacterial growth response at different pH.

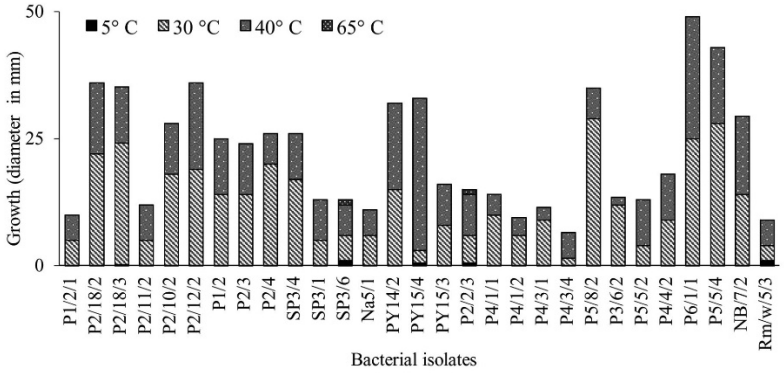

Fig. 5. Bacterial growth response at different temperatures.

Table 2. Sequence-based identity profile of the isolates.

Isolates	Bacterial species from the GenBank database showing the closest comparison	Similarity with GenBank data		Bacteria	
	Species name and accession No.	Max. coverage	Identities match %	Name	Accession No.
P1/2	*Pseudomonas aeruginosa* SKC01 MK841037.1	99%	96.28%	*Pseudomonas aeruginosa* P1/2	OR272113
	Pseudomonas aeruginosa PSS1 MF612185.1	98%	96.45%		
	Pseudomonas aeruginosa PCP25 HM439410.1	98%	96.45%		
P2/4	*Bacillus cereus* CIFT MFB 4895 (20) KP240939.1	99%	97.86%	*Bacillus cereus* P2/4	OR272114
	Bacillus cereus MJHN10 MF693121.1	99%	97.86%		
	Bacillus cereus strain NCR4 MF125693.1	99%	97.86%		
SP3/6	*Pseudomonas aeruginosa* PSS1 MF612185.1	99%	98.03%	*Pseudomonas aeruginosa* SP3/6	OR272115
	Pseudomonas aeruginosa BHS2 KT027770.1	99%	97.85%		
	Pseudomonas aeruginosa GS5 KM192353.1	99%	97.85%		
PY15/4	*Staphylococcus* sp. EB52 KP324934.1	98%	97.31%	*Staphylococcus* sp. PY15/4	OR272116
	Staphylococcus haemolyticus BM27 MZ350493.1	98%	97.13%		
	Staphylococcus hominis 4DT297M HM163532.1	98%	97.13%		

slightly acidophilic (Fig. 4) Most bacteria were shown growth between 30 and 40°C, while no growth was observed at 5 and 65°C reveals mesophilic (Fig. 5). However, *Pseudomonas aeruginosa* SP3/6 OR272115 and *Pseudomonas aeruginosa* P2/2/3 growing between 5 and 65°C reveals psychrotolerant (Fig. 5).

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