

Research Article

Characterization of plastic degrading bacteria isolated from landfill sites

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Abstract

The plastic pollution is threatening the environment because it has very slow degradation rate and high usage in regular activities. The present study aims at the isolation of novel microorganisms that would assist faster degradation process of polyethylene. The waste samples were collected from different landfills and dumpsites. Out of forty samples, eight samples were found to degrade polythene strips in liquid medium. Further screening of these samples showed that two strains of microbes had high potential for polythene degradation. Biochemical tests and ribotyping were performed for characterization of isolated bacteria. Resultantly, two novel bacterial strains were identified named; *Bacillus wudalianchiensis_UMT* (2A) and *Pseudomonas aeruginosa_UMT* (6). Analysis of these microbes further revealed that *Bacillus wudalianchiensis_UMT* and *Pseudomonas aeruginosa_UMT* have capability to degrade 6.6% and 4.8% polyethylene respectively. So, the results disclosed that these bacteria have great potential to degrade polythene in less time as compare to natural degradation process and can contribute to reduce pollution from our environment.

Introduction

The use of plastic is growing day by day. Its durability and stability has made it an indispensable part of our life. Despite its superiority over other materials, it is posing a major threat to the environment because these long chain polymeric molecules take thousand years to decompose naturally. According to UNDP, more than 3.3 million tons of plastic are wasted in Pakistan each year. So, it is a much needed step to reduce its lifespan in order to protect the environment. Biodegradation is an environmental friendly method to degrade polyethylene as it does not generate any harmful byproducts. The speed of degradation depends upon the chemical nature of polymer, on the environmental conditions in which it is placed and on the amount of polymer. Statistically stated, 38% plastic is used for packaging and this rate of utilization is increasing 12% every year. 12% plastic is used for construction, 21% for indoors and the outdoors, 7% for automotive, 6% for electronics and 28% plastic is used in other sectors. When these plastics are introduced into the environment, they cause potential hazards [1]. Though, plastic is useful in many applications but it poses serious effect to the environment. Because plastic is durable that's why it is highly persistent to decay. There is chemical bonding in its molecules which make is resilient to degrade. Plastic takes hundreds of years to degrade naturally [2].

Statistical data of worldwide consumption of plastic shows that North America utilizes 26%, Europe 23%, India 5%, Japan 6% and Asia contributes 40% of total plastic consumption in the world. Plastic can be disposed by different methods like incineration, landfills and through process of recycling. There are benefits and limitations in each case. When plastic waste is disposed in landfills, it apparently reduces wastes from upper surface of land but in true terms it reduces the land for agriculture [3]. As it takes so many years to degrade it naturally, so the area of landfill cannot be used for other purpose [4]. Incineration is a better option as compared to the landfills as it does not require much space and it is also better in terms of energy recovery [5]. However, it is not beyond the limitations because it causes greenhouse gases and it is also responsible for PCBs and free radical exposure [6]. Regardless of this, the limitations of landfills and incineration can be overcome through recycling though this process is expensive and the quality of end products is also low [7].

Biodegradation is the best methods as compare to the others. It is cheap and does not generate harmful pollutants into the environment. Plastic can be degraded through microorganisms. Thus, microbes play an important role in degradation process [8].



Materials and methods

Sample collection

Forty soil samples were collected from different dumpsites and landfills from Lahore and Bhera (Pakistan) with the help of sterilized spatula and were stored in polythene bags. Each sample was given a specific ID.

Screening of microbes having ability to degrade plastic

Flasks of 5ml liquid broth were prepared. 10mg soil of each sample was put in every flask. All flasks were kept in shaker overnight to get maximum growth of microbes [9]. Took 1ml from primary inoculums and was put in 150ml enriched media. This enriched media was composed of yeast extract 0.1g, MgSO₄·H₂O, 0.25g; KH₂PO₄, 5.8g; K₂HPO₄, 3.7g; KNO₃, 2.0g in 1000ml of distilled water. 100mg LMWPE strips were put in each enriched media flask. These flasks were then autoclaved. After that, flasks were kept in shaker for seven days. During seven days all other microbes died except of those who had capacity to degrade polymer [10]. Agar plates were prepared from enriched media by using three different substrates to examine the efficiency of microbes in each substrate. The used substrates were consisted of 1g Polyethylene glycol (PEG), mixture of 200 µl hexadecane and 20µl Tween80 and 100mg plastic powder [11].

Methods for screening of microbes

Clear zone method: Emulsified agar plates having substrate were streaked by dipping the L shaped loop in enriched median flasks which were previously inoculated in shaker for seven days. These plates were kept in incubator at 37°C for seven days. After seven days, the diameter of clear zone around the colonies was observed and measured [12].

Determination of weight loss: The pre weighed plastic strips were removed from the flasks. These strips were rinsed thoroughly once with distilled water and then with absolute ethanol in order to remove the impurities. Strips were over dried at 90 °C. The weight was determined by using the following formula [13]:

$$\text{Weight loss (\%)} = \frac{\text{Initial Weight} - \text{Final Weight}}{\text{Initial Weight}} \times 100$$

Sequencing

After isolation of genomic DNA, PCR was run for those eight samples. Universal primers 16S rRNA (5'-AAACTC/TAAAG/TGAATTGACGG-3') and (5'-ACGGCGGGTGTGTA/GC-3') were used. The PCR product was analysed on 1% agarose gel and purified using gel extraction kit (Fermentas, USA). M13 forward and reverse primers were used to sequence the rRNA amplified products in a Beckman Coulter CEQTM 8000 Genetic Analyser. National Centre for Biotechnology Information BLAST database was used to amplify nucleotide sequence. These sequences were aligned with selected genera

representative members by using CLUSTAL W program [14]. After sequencing, two samples (2A, 6) were preceded further which had high potential to degrade plastic more and which had new strains.

Quantification of DNA

After isolation, the genomic DNA was quantified at 260nm using UV-1280 Spectrophotometer using water as blank. Liquid media was inoculated with bacterial colonies and kept in shaker at 37°C for overnight. After 16 hours, 1ml culture from overnight media was taken and put in 50ml media flask. The flasks were then kept in shaker and readings were taken after every two hours [15].

Effect of temperature on growth

The isolated bacterial strains were grown at temperature 37°C, 45°C, 50°C and 60°C to determine the temperature for optimal growth. After seven hours, the absorbance was assessed at 260nm by using spectrophotometer [16].

Phylogenetic tree

The sequenced 16S rRNAs amplified from both bacterial strains were compared with sequences available in nucleotide databases. *Pseudomonas aeruginosa_UMT* sequence was used as out group for *Bacillus* strains and *Bacillus wudalenchinesis_UMT* sequence was used as an out group for *Pseudomonas aeruginosa*. Best fit model was deduced and was implemented to draw phylogenetic tree.

Identification tests for microbes

Preliminary tests: Gram staining method: This is a widely used method in bacteriology. In this method, a slide was used which was grease free and clean. A smear from bacterial culture was spread in this slide by using a sterilized loop. This slide is then fixed with the heat and was passed by staining reagents [17].

Hanging drop method: This is a motility test which is used to observe live microbes and their mobility. A drop was placed on the cover slip to observe microbes in this drop and was covered by the cavity slide. Microbes in droplet were observed under light microscope [18].

Biochemical tests

Catalyze test: Culture sample was placed on the slide and then hydrogen peroxide was added on the slide drop by drop. Samples which showed bubble formation indicated positive results [19].

Motility test: The mobility test of both isolated strains was performed in semi-solid agar medium containing 0.4 g agar, 2 mg peptone and 1 g NaCl in 200ml of distilled water. The sterilized straight wire was used to inoculate the culture in a single stab centrally from top to bottom of the medium. The incubation of tube was carried out at 30°C for overnight. A diffused hazy growth represented motile bacterium, spread throughout the medium.

Caesin hydrolysis

In this method a loop was used to transfer culture on the agar plate. Plates were inverted and kept in incubator for one to two days [20].

Gelatin hydrolysis

Suspension was prepared from overnight culture media. This suspension is then pipette in two tubes that contained gelatin agar base. Both of these tubes were kept in incubator at 37°C. One tube was tested after 4 hours and one tube was tested after 24 hours. Acid was added and reading was recording after 30 minutes against blank gelatin agar tube.

Urea broth

In this method equal volume of urea buffer and suspension were mixed. Capillary tubes were incubated at 37 °C and alkali production was recorded after 4 and 24 hours [21].

Oxidase test and mannitol test

The smear of a colony of isolated bacterium was introduced on a filter paper containing few drops of oxidase reagent (0.1 g of *tetramethyl-p-phenylenediamine dihydrochloride* in 100 ml of distilled water). The colony having oxidase activity, gave a characteristic color. In order to check the ability of isolated bacteria for fermenting mannitol, phenol red mannitol broth with 0.5% - 1.0% mannitol was used. Single colony of each isolated bacterium was incubated at 37°C for 24 hours. The change in color from red to yellow indicated that bacteria have ability to ferment mannitol.

Results and discussion

Total forty samples were collected from different dumpsites and landfill sites of Bhera and Lahore respectively. Out of which only nine samples showed positive results i.e. reduction in weight loss (Table 1).

Plate assay showing plastic degradation by microbes

Culture flasks in which weight of strips were reduced was streaked on nutrient agar plates in which PEG as substrate was added previously. After seven days of incubation, clear zones were observed around single colony having PEG substrate (Figure 1).

Isolation of genomic DNA from microbes

After isolation, the quality and quantity of genomic DNA was analyzed on 1% agarose gel (Figures 2,3).

Gel of gene clean

After PCR, gene clean was done. Six samples were run for gene clean on agarose gel. After running these samples on agarose gel, positive results were seen in all samples (Figure 4).

Ribotyping

After gene clean, ribotyping was done in which nucleotides sequences of isolated bacteria were used to analyze through NCBI BLAST database. The homology was determined by

aligning the multiple sequences through CLUSTAL W program. Sequencing was done for all samples, out of which sample 2A and sample 6 had new strains.

Table 1: Loss of weight of plastic by isolated microbes.

Serial No	Sample ID	Final weight	Initial weight	Weight loss (%)
1	1A	96.6	100 mg	3.4
2	2A	93.6	100 mg	6.4
3	8A	96.3	100 mg	3.7
4	8B	97.7	100 mg	2.3
5	6	95.7	100 mg	4.3
6	N6	99	100 mg	1
7	N7(i)	97	100 mg	3
8	N7(ii)	98.5	100 mg	1.5
9	N9	99	100 mg	1



Figure 1: Formation of enriched zones by microbes.

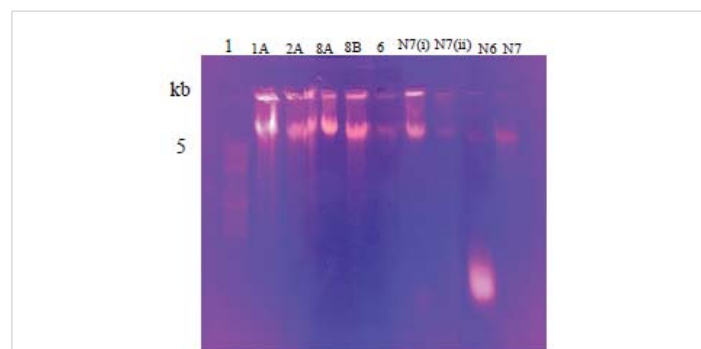


Figure 2: Ethidium Bromide stained Agarose gel showing genomic DNA isolate from different strains. Lanes 1A, 2A, 8A, 8B, 6, N7. (i) showing bands of polyethylene degrading strains but very weak bands in lane N7. (ii), N6 and N9. Lane 1 is showing DNA marker.

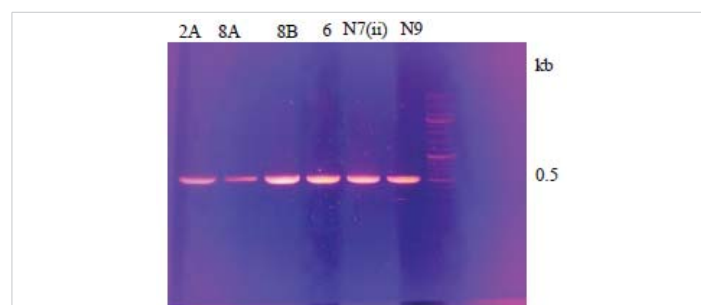


Figure 3: Ethidium Bromide stained Agarose gel showing PCR product from different strains. Lane 1 is showing ladder and lanes 2A, 8A, 8B, 6, N7(ii) and N9 showing amplified PCR product of 0.5 kb.

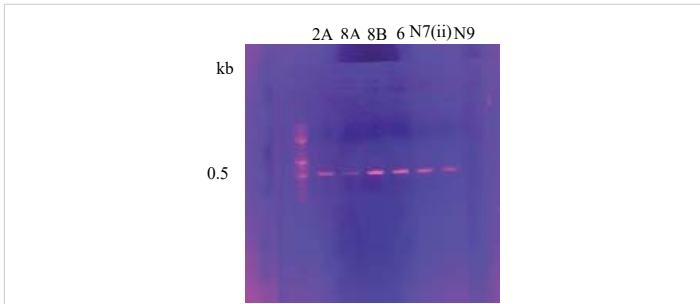


Figure 4: Ethidium Bromide stained Agarose gel showing DNA purification gel from different samples. Lane 1 is showing ladder and lanes 2A, 8A, 8B, 6, N7(ii) and N9 showing bands of gene clean of 0.5 kb.

Bacillus Sp. (Sample 2A):

CATGTGGTTTATTTCGAAGCAACGCGAAGAACCTTACCAGGT
 CTTGACATCCCGCTGACCGGTCTGGAGACARATCTTCCCTTCG
 GGGACAGCGGTGACAGGTGGTYCATGGTTGTCGTCAGCTCGTG
 TCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC
 TTGATCTTAGTTGCCAGCATTAGTTGGGCACTCTAAGGT
 GACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAA
 TCATCATGCCCTTATGACCTGGGCTACACACGTGCTACAATG
 GATGGTACAAAGGGCTGCAAGACCGCAAGGTTTAGCCAATCCC
 ATAAAACCATTTCTCAGTTCCGATTGCAGGCTGCAACTCGCCTG
 CATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCATGCCGG
 GTGAATACGTTCCCGGGCCTTGWACACACCGCCCAA.

Pseudomonas Sp. (Sample N9):

CATGTGGTTTATTTCGAGCAACGCGAAGAACCTTACCTGG
 CCTT\GACATGCTGAGAACTTTCAGAGATGGATTGGKGCCTT
 CGGGAACCTCAGACACAGGTGCTGCWTTGGCTGTCGTCAGCTCG
 TGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCC
 TTGTCCTTAGTTACCAGCACCTCGGGTGGGCACTCTAAGGAGA
 CTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAA
 GTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATG
 GTCGGTACAAAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCC
 CATAAAACCGATCGTAGTCCGATCGCAGTCTGCAACTCGA
 CTGCGTG AAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCA
 CGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGT.

In vitro degradation of plastic by *Pseudomonas aeruginosa_UMT* and *Bacillus wudalianchiensis UMT*:

After sequencing new bacterial strains were identified that were *Bacillus wudalianchiensis_UMT* (2A) and *Pseudomonas aeruginosa_UMT* (6). The seven days experiment was repeated again that was explained previously for the confirmation of their weight reduction potential (Figure 5) (Table 2).

Effect of temperature on growth

The growth temperature of the *Bacillus wudalianchiensis_UMT* and *Pseudomonas aeruginosa_UMT* ranged from 37°C to 60°C. Growth stopped above 60°C [22] (Figure 6) (Tables 3,4).

Biochemical characterization

Bacillus wudalianchiensis_UMT exhibited catalase, caesin

Hydrolysis, Gelatin Hydrolysis and Urea broth activities. It indicated negative results for the tests of oxidase, mannitol and hanging drop method. *Pseudomonas aeruginosa_UMT* showed positive results for oxidase, mannitol, catalase, caesin hydrolysis, gelatin hydrolysis, urea broth activities and hanging drop method [23].

Phylogenetic tree of *Bacillus wudalianchiensis_UMT*:

The phylogenetic tree was constructed using amplified 16S rRNA from both bacteria, sequenced and comparing with those in nucleotide databases. *Pseudomonas aeruginosa_UMT* was used as an out group for *Bacillus wudalianchiensis_UMT*. Phylogenetic tree gives a clear indication that there is homology between different strains of *Pseudomonas Sp.* It is clear from phylogenetic tree that sample 2A is close in cluster with *Bacillus wudalianchiensis_UMT*. Each name at the *wudalianchiensis_UMT* (Figure 7).

Phylogenetic tree of *Pseudomonas aeruginosa_UMT*:

Bacillus wudalianchiensis_UMT was used as an out-group for *Pseudomonas aeruginosa*. The phylogenetic tree shows that there is 90% homology between different strains of *Pseudomonas* (Figure 8).

Conclusion

The increase in use of plastic is growing concern and major contributor to pollution across the globe. Scientists are working to develop environmental friendly methods to degrade the plastic causing pollution. Different microbes has been identified having the potential to degrade polythene

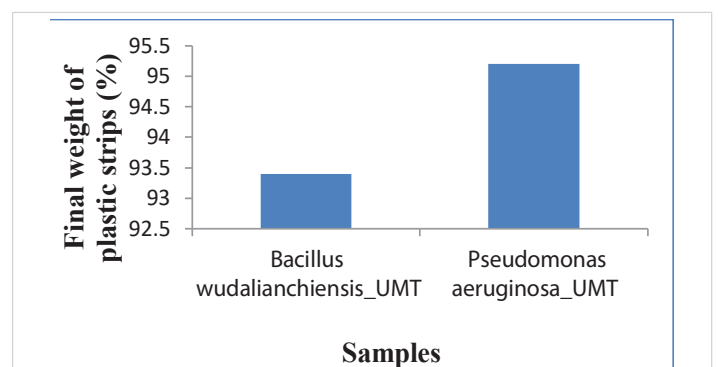


Figure 5: Graph showing comparison of final weight of both samples.

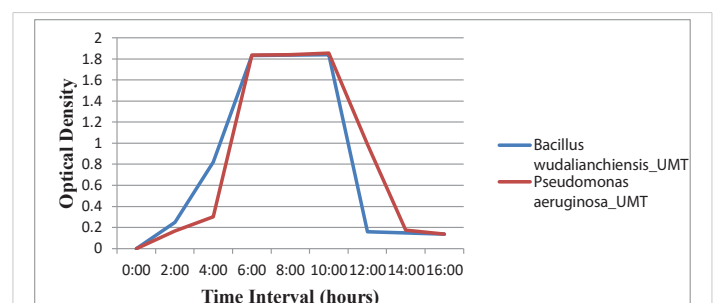


Figure 6: Growth comparison of *Bacillus wudalianchiensis_UMT* and *Pseudomonas aeruginosa_UMT* on different time intervals at 37°C. The growth curve indicates maximum growth at 10 hours after which it starts decreasing.

Table 2: Percentage of weight loss by *Bacillus wudalianchinesis_UMT* (2A) and *Pseudomonas aeruginosa_UMT* (6):

Serial No	Sample ID	Final weight (mg)	Initial weight (mg)	Percentage of weight lose (%)
1	2A	93.4	100 mg	6.6
2	6	95.2	100 mg	4.8

Table 3: Determination of optimum growth of *Bacillus wudalianchinesis_UMT*(2A) at different temperatures.

Serial No	Temperature	Maximum OD at 260 nm
1	37°C	1.839
2	45°C	0.239
3	50°C	0.342
4	60°C	0.34

Table 4: Determination of optimum growth of *Pseudomonas aeruginosa_UMT*(6) at different temperatures

Serial No	Temperature	Maximum OD at 260 nm
1	37°C	1.99
2	45°C	0.38
3	50°C	0.224
4	60°C	0.038

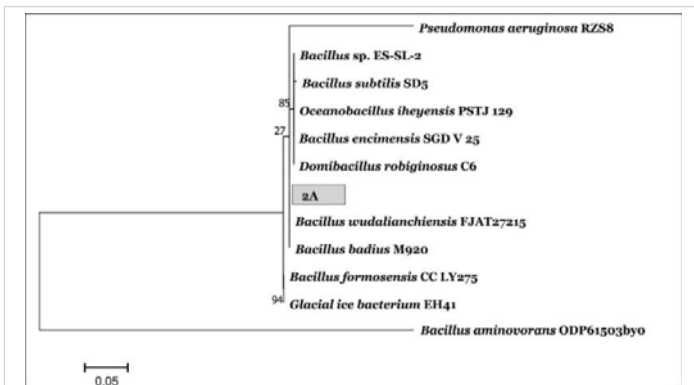


Figure 7: A phylogenetic tree of *Bacillus_UMT* (2A) showing relationships between 16S rRNA sequences.

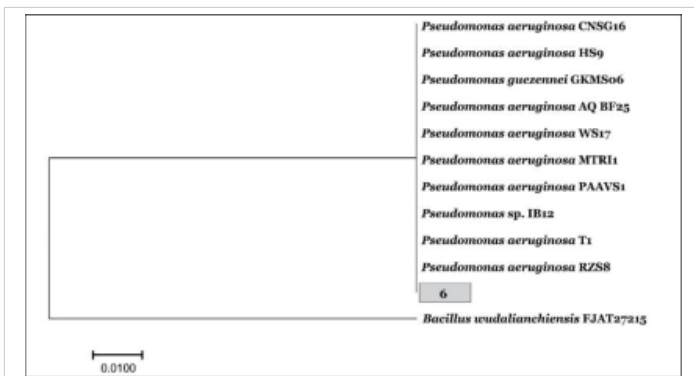


Figure 8: A phylogenetic tree of sample *Pseudomonas aeruginosa_UMT* (6) showing relationships between 16S rRNA sequences.

and other plastics. This study is aimed at isolation and characterization of plastic degrading bacterial from landfill sites. Samples from landfill sites were used to isolate different bacteria having ability to degrade plastic. Two bacterial strains have shown great potential to degrade plastic with substantial growth in culture media. On the basis of biochemical tests and phylogenetic tree it was revealed that these two bacteria are

Bacillus wudalianchensis_UMT and *Pseudomonas aeruginosa_UMT*. *Bacillus wudalianchensis_UMT* degraded 6.6% plastic and *Pseudomonas aeruginosa_UMT* degraded 4.8% in culture media. We concluded that these two bacteria are suitable to be used at large scale for degrading plastic at landfill sites and in industries to degrade plastics. In future studies, the cloning and expression of plastic degrading genes can be done.

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