Isolation and Characterization of Plastic Degrading Bacteria from Soil Collected from the Dumping Grounds of an Industrial Area

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Abstract: Microorganisms play a very important role in the biological decomposition of various materials in the natural environment, this is called biodegradation. Synthetic materials including plastic and polyethylene waste accumulates in the environment and poses an ever increasing ecological threat. Biodegradation of these plastic wastes using potent microbial strains could provide a solution to the problem. In the present study, the in vitro biodegradation of polyethylene and PVC strips were analyzed using the microorganisms isolated from the soil over a period of 1 month of incubation. The microbial species associated with the degrading capibilities were identified as one Gram (+)ve and one Gram (-)ve bacteria. The efficiencies of these two bacteria in the degradation of plastics were compared and one with the higher degrading capacity is identified to be ***Bacillus cereus strain NBAII B7*** (GenBank Accession Number: **KF322125.1**)

Keywords: Biodegradation, Plastics, PVC, *Bacillus cereus*

Introduction: Plastics are the polymer which becomes mobile upon heating and can be casted into moulds. They are non-mettalic compounds and plastic derived materials can be pushed into any desirable shapes. Plastics are used primarily in the packaging industry such as food, pharmaceuticals, cosmetics etc. The most commonly used plastics in the industries are polyethylene (LDPE, MDPE, HDPE, LLDPE), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), polybutyrene tetraphthalate (PBT), nylon.

Low density polyethylene (LDPE) is one of the major sources of environmental pollution. Polyethylene is a polymer made up of repeating units of ethylene monomers. The use of this synthetic polymer is growing at a rate of 12% per year and about 140 million tons of synthetic polymers are produced worldwide each year. It causes serious threats to the environment both during its production and after its disposal.

Microorganisms can degrade plastics over 90 genera from bacteria, fungi, among them *Bacillus megaterium, Pseudomonas sp, Azotobacter sp, Ralstonia eutropha , Halomonas* sp etc (Chee et al 2010). Microbes cause cleavage of the polymer chain using certain enzymes and convert them into monomers and oligomers. The diverse metabolic capability of microbes can be exploited for bioremediation of plastic wastes that uses microbial strain developed through selection, strain improvement and genetic modifications. The aim of this study is to isolate the bacteria from the soil having a potential to degrade polyethylene plastics.

Materials and Methods

1) Sample Collection

Soil sample was collected from the dumped area of a plastic industry in Kolkata and its physical and chemical standards were studied.

2) Isolation:

After the sample collection the pre-weighed 1gm of plastics were cut into 9 pieces and added to 9ml of sterile water to make 1:10 dilution. Using the standard serial dilution technique 1:1000 dilution factor was made. Now 1 ml from each of the dilution factor was poured on 3 sterile Petri-plates. Freshly made nutrient agar was added in each of the plates. The plates were stirred slowly and was allowed to solidify after which they were kept in the incubator at 37° for 24 hours.

Total heterotrophic count:
C.F.U. /g= Number of colonies/ inoculums size (ml) X dilution factor.

3) Identification:
Identification of the isolates were performed according to their morphological, cultural and biochemical characteristics.

[A] Morphological

Gram Staining Method A clean grease free slide was taken and a smear of the bacterial culture was made on it with a sterile loop. The smear was air dried and then heat fixed. Then it was subjected to the following staining reagents:

(a)Flood with crystal violet for 1min followed by washing with distill water
(b) Again flooded with Gram's iodine for 1 min and followed by washing with alcohol.
(c)The slide was counterstained with saffranin for 30 seconds followed by washing with distill water.

Colony Morphology: The above stained slide was then observed under microscope to determine the morphology of selected strain on the basis of shape, size and colour.

[B] Biochemical: Biochemical identification of the isolated strains was done by using manual biochemical methods.

Catalase Test
The catalase test was performed to detect the presence of catalase enzyme by inoculating a loopful of culture into tubes containing 3% of hydrogen peroxide solution. Positive result was indicated by formation of effervescence or appearance of bubbles due to the breaking down of hydrogen peroxide to O_2 and H_2O.

Mannitol Test
This experiment is generally performed to determine whether the bacteria is capable of fermenting mannitol sugar or not. Whenever organism ferment mannitol sugar, the pH of the media becomes acidic due to the production of acids. The fermentation of the media from red to yellow show positive result.

Motility Test
The motility test was done to determine the motility of the organism. Approximately 5ml of the nutrient broth was inoculated with the isolated microorganism and was incubated for an overnight growth. Then a drop of the NB media was taken on a grooved slide (hanging drop method) and was observed under phase contrast microscope.

Citrate Test
This test determines the ability of the bacteria to convert citrate into oxaloacetate. Citrate is the only carbon source available to the bacteria in this method. Positive results are seen if the bacteria grows and the media turns into bright blue colour.

Luminescence Production
One plate each of the two isolated strain were kept under U.V meter and checked for the production of any bioluminescence.

Chitinase and Glucanase Test.
The isolates were analysed for the presence of chitinase and glucanase enzyme and the measurement of these enzyme activity were examined.

4) Interaction between the isolated sample with other organisms:
Nutrient agar plates were made and was spreaded with the sample suspension. The petri plates were then incubated at 37° for 24hrs.Mixed colonies were observed on the nutrient agar plates. There were 2 types of colonies. Pure culture was obtained on fresh NA plates. Then 4 fresh agar plates were made. Two of them were spread with Pseudomonas suspension and the other two were spread with yeast suspension. Three agar cups were bored out of the NA plate using sterile cork-borer.One of the three cups made on all the 4 plates were filled with distill water and the other two were filled with the sample solution from pure culure plate in nutrient broth grown overnight. The two isolated sample solution was filled in separate plates containing yeast and Pseudomonas culture. All the 4 plates were then incubated at 37° for 24 hrs and was observed the next day.

5) Detection of the Toxin Producing Ability Of The Isolated Strain: Nutrient agar plates were made and were spreaded with the sample suspension. The petriplates were incubated at 37° for 24 hours. Next day mixed colony were observed on the plates. Pure culture was obtained on fresh NA plate. Then four fresh nutrient agar plates were made, two of which was spreaded with the yeast suspension and the remaining two were spreaded with bacterial suspension(*Pseudomonas* sp). Then three agar cups were bored out using sterile cork-borer. One of these three cups, made on all NA plates, was filled with distilled water (control) and the other two wells contained the sample suspension. Now for sample suspension the pure culture grown in the overnight NA plates were re-suspended in nutrient broth medium in a falcon tube and was centrifuged at 5000 rpm for two minutes. The pellets were discarded and the soup of the sample was used as bacterial suspension which contains the endogenous enzymes of the bacteria under assay. These plates were then incubated at 37° and were observed the next day.

6) Microbial Degradation of Plastics in Laboratory Condition:
The analysis of plastic degrading ability of the microorganism were done in two ways:-
Determination of weight-loss: Preweighed strips of polythene bags and PVC were aseptically transferred to the conical flask containing 50ml culture broth medium inoculated with different bacterial species in flasks along with a control set. This set-up was maintained for one month and periodic replacement of the broth with fresh bacterial suspension was done. This eliminated the chance of saturation of the microbial growth in the medium. After one month of shaking the plastic strips were washed thoroughly using distill water, shade-dried and then weighed for final weight.
Testing of PVC film degradation:
PVC strips were made in the laboratory. Pour-plate technique was used for inoculation of the cell-suspension in the agar medium. Cell suspension of the culture was added to sterile petriplate followed by addition of warm NA media. The plate was swirled and the culture was homogenously mixed. The PVC strips were then aseptically placed on each plate containing the two bacterial samples. The plates were then sealed with polythene bags to avoid desiccation and were kept at 30° for 45 days .The plates were periodically removed and the film was observed for any sign of microbial growth.

RESULTS

1: Physical Table no.and Chemical Standards

Location	Dumping Ground
Quantity	10-12 gms
Texture	Rough, Coarse
Nature	Soil with polyethylene
Colour	Blackish brown
Odour	Odourless
pH	5.85
ElectricalConductivity	38.75
Temperature	25
Disssolved Oxygen	0.4 ppm

Table no. 2: Colony morphology of the bacterial strain on the basis of serial dilution.

Serial no.	Dilution factor	No. of colonies	Colony Characteristics
1	10^{-1}	110 (2types)	White,Round,Opaque[B]
			White, Branched Cottony Opaque [A]
2	10^{-2}	52 (2types)	White, Round, Big ,Opaque [B]
			White, Branched, Opaque [A]
3	10^{-3}	17 (1 type)	White, Branched, Irregular Opaque [A]

Table no.3) Total Heterotrophic Count (CFU)

Dilution	No. of colonies	Inoculum Size	CFU/g
10^{-1}	110	0.1	110
10^{-2}	52	0.1	5.2
10^{-3}	17	0.1	0.17

For our convenience the colony with White, Branched and opaque characteristics are designated as **A** and the colony with White, Round characteristics are designated as **B**

Morphological Identification

Table no. 4: Result of GRAM STAINING:

The bacterial strains are identified as:

Strain	Shape	Colour	Characteristics
A	Bacilli	Violet	Gram +
B	Bacilli	Red	Gram -

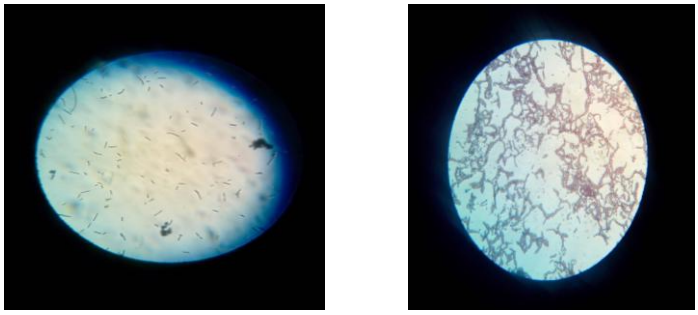


Fig.1 Gram Staining of two selected strains (A,B) based on colony morphology

Table no. 5:
RESULT OF BIOCHEMICAL TEST

Biochemical Tests	Strain A	Strain B
CATALASE	-	+
MANNITOL	-	-
CITRATE	+	-
MOTILITY	-	-
Bio-luminescence	-	-
CHITINASE	0.59u/ml	0.83u/ml
GLUCANASE	0.34u/ml	0.41u/ml

Table no. 6) Result of interactive studies of the isolated microbes:

Plate Composition	Observed Interaction
Yeast + Sample Solution A	Ample amount of yeast colonies seen around the cup containing bacterial suspension. No yeast colony in the control set.
Yeast + Sample solution B	Colonies were widely dispersed throughout the plate. Yeast was profusely grown around the bacterial suspension.
Pseudomonas + Sample Solution A	No colonies
Pseudomonas + Sample Solution B	No colonies

Both the strains isolated from the soil sample showed characteristic interaction with other groups of bacteria and fungi. In the first two cases were the yeast colonies grew around the bacterial culture but not around the well containing water explains that the bacterial strain produces certain compounds or metabolites which promotes the growth of yeast which was absent in case of the well containing water (control) since the medium was selective for bacteria. Hence here the sample and yeast shows **Agonistic relationship** with each other.
On the other hand, the plates containing Pseudomonas culture with the two sample solutions show no colonies, which suggest that the Pseudomonas might secrete certain compounds or anti-metabolites which inhibits the growth of other competent bacterial strains. Hence here the sample bacteria and Pseudomonas shows **Antagonistic relationship** with each other.

8. Result of the toxin producing ability of the isolated strains.

Clear zones were observed on the NA plates spreaded with yeast suspension which indicates that the bacterial suspension did not allow the growth of the fungus in it's vicinity (i.e. antifungal in nature). Clear zones were not observed on the plates spreaded with *Pseudomonas* sp. From the results it was concluded that both the samples isolated from the soil exhibits antifungal activity [Fig2 (a)] and hence does not allow the growth of other fungus in their vicinity. The bacterium produces a metabolite (probably toxin) which does not allow the growth of yeast in their vicinity. But neither of the two samples isolated showed antibacterial activity [Fig2 (b)] because no clear zones were observed on the plates containing *Pseudomonas* sp.

Fig.2 (a) Plate showing antifungal nature of the bacterial isolates.

Fig2 (b) Plates showing absence of antibacterial activity.

Serial	Strain	POLYTHENE				PVC			
		Initial Wt.(mg)	Final Wt.(mg)	Diff	Weight Loss/month	Initial Wt.(mg)	Final Wt.(mg)	Diff	Weight Loss/month
1	A	23	19	4	17.39%	63	49	14	22.22%
2	B	23	21	2	8.69%	63	53	7	15.87%
3	-	23	23	0	0	63	63	0	0

Table no. 7) Table showing the observed difference in the weight of the plastic samples with different microbial strains after an incubation period of 1 month.

9. Result of degradation of plastic sample

1) Weight-loss method :

The results observed after one month of incubation of the experiment set-up are tabulated in Table no 7 above. During the incubation period, the bacterial sample along with the plastic strip were kept at constant shaking condition. Also the culture media were periodically replenished in order to eliminate any chances of saturation. At the end of the incubation period it was inferred that the bacterial isolates possessed the capability to degrade the plastic strips which was shown by the weight difference of the treated strips compared to the control ones. Among the two isolates it was seen that degradation ability of Sample A was greater in comparison to sample B. The % weight difference of Sample A is 22.22% (in case of PVC) and 17.39% (in case of polyethene) while that of Sample B is 15.87% (in case of PVC) and 8.69% (in case of polyethene).Also it was noted that the isolates degraded PVC more than the polythene strips.

2)PVC film degradation:

The plates were observed after 1.5 month of incubation. During the test period, microbial colony started appearing after two weeks of incubation from the edges of the film and covered the entire film within sixth week of incubation. However due to dehydration of the culture media, it was not possible to do the test after that. The pictures of the biofilms were taken after 6th week of incubation.

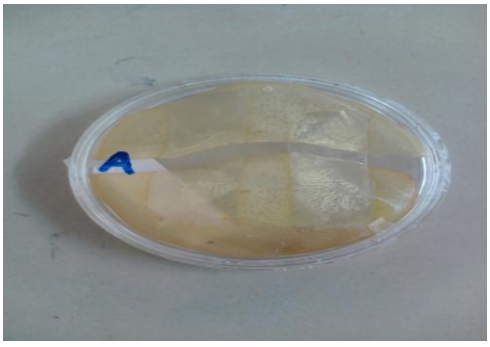


Fig 3: Biofilm formed on PVC strip by strain A

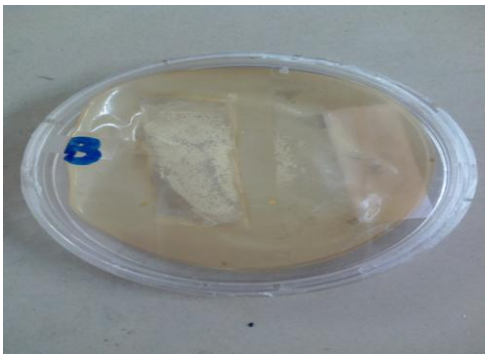
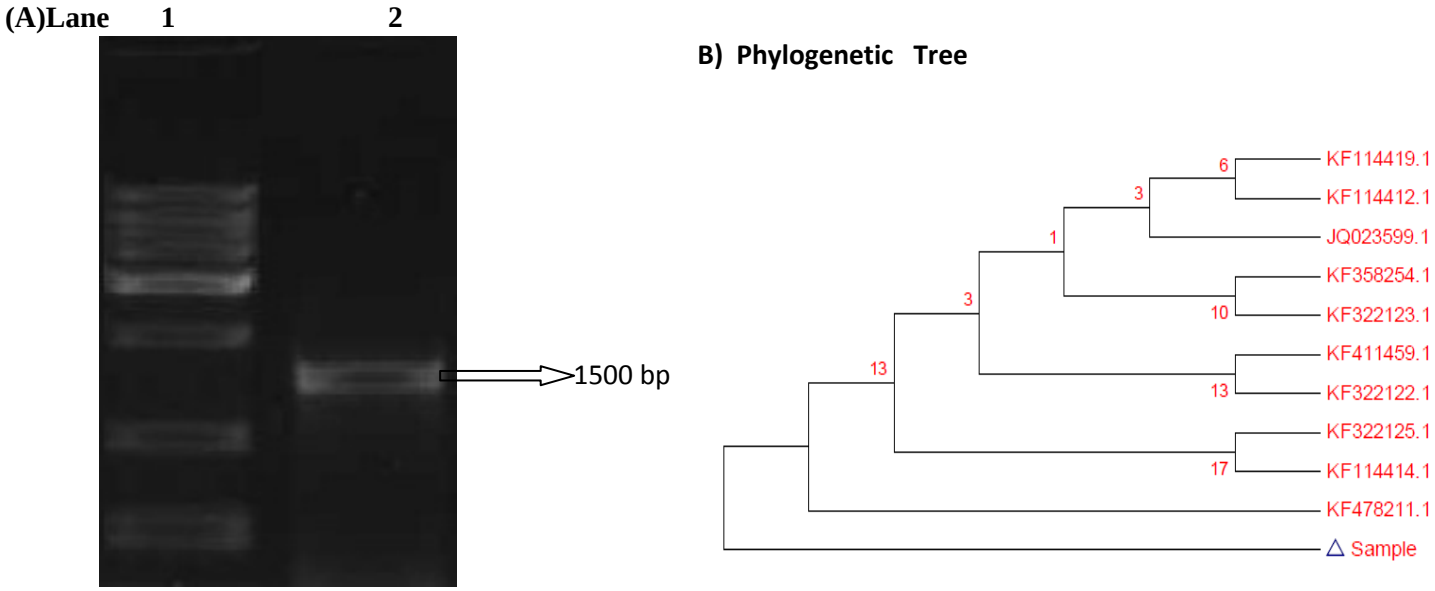


Fig4: Biofilm formed on PVC strip by strain B



Lane 1:DNA marker
Lane 2: 16S rDNA amplicon band

Fig. 5 (A) Gel Image of 16s RDNA of the unknown organism (B) Phylogenetic Tree of the identified organism *Bacillus cereus strain NBAII B7*

Discussion:

The present study deals with the isolation , identification and degradative ability of plastic degrading microorganisms from soil. Different types of changes are observed by the isolates during morphological and chemical analysis. Another area examined has been the biodegradation of plastic by the liquid culture method. It is clear that the polymers to some extent can be degraded in the appropriate environment in right concentration.Here,it was concluded that Sample A has high degradation activity both on PVC and Polyethylene. It is also observed that the degradation of Polyvinyl chloride was faster than Polyethylene as both the Sample A and B showed greater % difference in weight for PVC than for Polyethylene.As the PVC was prepared in the laboratory so there might be some error during the preparation. Microbial degradation of a solid polymer like polyethylene or PVC requires the formation of biofilm which has also been tested in this study. The microbes utilize these insoluble substrates by enzymatic activities (chitinase and glucanase assay). Development of such biofilms on the surface of synthetic waste can prove to be a very efficient method for degradation of these polymers in vitro. The results obtained in the study showed that the isolated strain survived on plastic film surface. Biofilms were observed on the surface of the PVC strips, it can be concluded that the bacterial strains degraded the plastic and also gives a positive intimation that the isolate can be used as a potent biodegrading agent for plastic waste material. The large scale application of this method may prove as an environmentally profitable research. Since organism A, degraded the plastic more, it was sent for sequencing at the **Xcelris Labs Ltd. Ahmadabad** and was identified to be *Bacillus cereus strain NBAII B7*

Conclusion

In conclusion, the microbial strains from the collected sample were successfully isolated with potential to degrade the synthetic polymers like polyvinyl chloride and polyethylene. *Bacilus cereus* strain NBA II B7 was found to possess plastic degrading potential and its efficiency was maximum compared to other bacteria. These findings have important application in solving plastic waste problem through bioremediation where modern approach developed for remediation can be combined and applied with these organisms.

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