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Biodegradation of Low Density Polyethylene (LDPE) using marine bacteria isolated from tropical beaches of megacity Mumbai

A B Fulke*, K Khade & A K Sasidharan

CSIR-National Institute of Oceanography (CSIR-NIO), Regional Centre, Mumbai – 400 053, India

*[E-mail: afulke@nio.org; abhay_fulke@yahoo.co.in]

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Plastics, although as useful and versatile as they are, have led to major environmental pollution. Its resistance to degradation has caused a major global threat to the environment. Plastic waste is disposed of in landfills causing soil pollution, incinerated releasing toxic fumes and causing air pollution, and dumped in the ocean causing a wide range of problems like entanglement and ingestion by marine creatures. Since the introduction of plastic, microorganism, though incapable of degrading it, is known to associate with its surface. Marine ecosystem is one such environment where the disposal of a large amount of plastic waste is found, and microorganisms are known to be associated with it. Hence, the marine environment can serve as a potential source for indigenous plastic-degrading microorganisms. In the following study, plastic pieces rooted deep in sediments from seven recreational beaches in Mumbai were collected for the isolation of possible plastic degrading bacteria associated with the surface of the collected plastic pieces. Altogether, nine isolates were isolated after three months of incubation of collected samples in a medium containing no carbon source. On identification by 16S rRNA sequencing, isolates were confirmed to be *Pseudomonas*, *Bacillus* and *Lysinibacillus.* The NCBI accession numbers were obtained for all the isolates. For further experimentation, only four *Pseudomonas* isolates (Strains ABFPD01, ABFPD02, ABFPD03 and ABFPD05) and one each isolate of *Bacillus* and *Lysinibacillus* were used. In the weight loss experiment, the ABFPD02 & ABFPD03 strains of *Pseudomonas* degraded 11 % of pre-weighed polyethylene strips in 2 months incubation period. The marine environment needs to be studied extensively for more potential microorganisms for plastic degradation.

[**Keywords**: *Bacillus*, *Lysinibacillus*, Marine microbes, Plastic degradation, *Pseudomonas*]

Introduction

The properties of plastic, like moulding easiness, resistance to corrosion, insulation, low production cost and waterproofing, have led to its wide applications and use in every aspect of human life. Plastic is used in the making of all sorts of products, from packing materials to medical devices. On an average, 8.7 per cent of growth in plastic production was observed from 1950 to 2012(ref. 1). In 2015, 6300 Mt of plastic waste had been generated worldwide[2]. Depending upon the type of plastic, it takes anywhere between 10 to 1000 years for its decomposition[3]. About 79 % of plastic produced is disposed of in landfills, and it may take up to a billion years for its degradation naturally[2,4]. The non-biodegradable nature of plastic has resulted in a major threat to the environment. For example, 4.8 to 12.7 million tons of plastic entered the ocean in 2010(ref. 5). Plastic waste was known first to enter the ocean in the 1940s(ref. 6). Around 580,000 plastic pieces per km^2 are found in ocean[7]. Large amount of plastic waste is thus known to enter the ocean, causing the deleterious effects. A huge number of marine species as well as birds, has been known to be affected by ingestion and entanglement due to plastic[8,9]. Large plastic pieces are broken down into smaller pieces due to UV radiation and weathering into microplastics[10,11]. The pieces of microplastics are known to enter the food chain by ingestion by marine organisms[12]. Most of the one-time-use plastic objects are made of polyethylene and are discarded immediately[13]. It is highly resistant to acids, alcohols, bases and esters. It is also biologically inactive and considered a recalcitrant material. The inertness of plastic makes its degradation by the microbes difficult[14]. Hence, a suitable eco-friendly method for their disposal must be found.

Microorganisms are ubiquitous and are known to degrade almost all kinds of organic matter over a course of time. Biodegradation of plastic using microbes has always been an ambitious area of study within the scientific community. Reports of degradation of plastic by marine microbes are well published and are known to degrade plastic by enzymatic degradation[15-17].

In this study, the potential Low Density Polyethylene (LDPE) degrading bacteria were screened and isolated from the coastal area and recreational beaches contaminated with plastic litter. The *Pseudomonas* spp. was found to be dominating plastic-degrading microbes among the nine potential isolates.

Materials and Methods

Sample collection and processing

Samples were collected from anthropologically contaminated beaches. Plastic pieces from coastal areas contaminated with plastic waste were collected in zip-lock bags. The sampling locations are mentioned in Figure 1 and Table 1.

Isolation of plastic degrading microbes

The plastic rags collected from the beaches were incubated in Bushnell Hass medium (Himedia, India) for a period of 3 months at 30 °C. After 3 months, the broth was used as inoculums and plated on marine agar plates. All the isolates obtained were characterized using morphological and molecular methods. DNA extraction was performed using a DNA Exgene Cell SV extraction kit (GeneAll, South Korea). The DNA was subjected to 16S rRNA amplification using a Thermo cycler machine (Himedia, India).

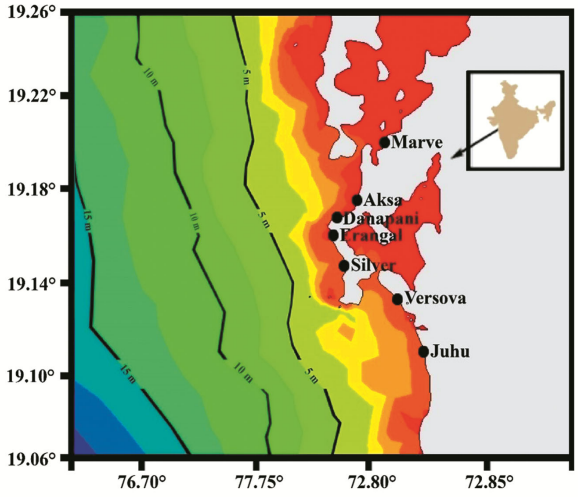

Fig. 1 — Geographical locations of studied beaches

Table 1 — Details of sampling locations

Sr. No.	Sampling site (Beaches)	Coordinates
1	Marve	19°12'1.548" N; 72°47'56.796" E
2	Aksa	19°10'21" N; 72°47'38.4" E
3	Danapani	19°10'0.534" N; 72°47'13.4232" E
4	Erangal	19°09'41.472" N; 72°47'5.064" E
5	Silver	19°08'57.552" N; 72°47'22.848" E
6	Versova	19°07'27.12" N; 72°48'59.76" E
7	Juhu	19°06' 3.3408" N; 72°49'28.092" E

16S rRNA gene sequencing

The extracted DNA was amplified using 16S universal primers (forward) 5'CCAGCAGCC-GCGGTAAC3' and (reverse) 5'ATCGGYTACG-TTGTTACGAT3'. The amplicons were sent for sequencing to the Berylls Pharma Pvt Ltd, India and were sequenced using the Sanger sequencing method.

Microcosm experiment

Based on the initial screening, potential bacterial strains were selected for the microcosm experiment. Wherein, the individual isolate was incubated in a sterile 500 ml Erlenmeyer flask containing carbon-deficient Bushnell Hass broth along with a sterilized (using 70 % ethanol) pre-weighed (2×10 cm, 51 microns thickness) LDPE strip. The strips were removed at equal intervals of 15 days for weight loss determination.

Weight loss determination

The plastic strips (2×10 cm) were washed with 2 % sodium dodecyl sulphate (Fisher) for 2 h. The detergent was washed off by cleaning the strip in a sterile distilled water. It was then immersed in ethanol for 2 h and dried in a hot air oven overnight at 40 °C. The weight of the strip was then taken. The strip was then re-inoculated in the respective flask after thorough washing. The procedure was performed four times, and four weight loss readings were obtained using a weighing balance (CB-9 series, Contech). The final weight loss percentage was calculated using the following formula:

$$\%\ weight\ loss = \frac{W1 - W2}{W1}\ x\ 100$$

Where, $W1$ is the initial weight, and $W2$ is the weight after incubation.

Viability test

The stock of 5 mg/ml of fluoresce in diacetate (FDA) (Himedia, India) was prepared in dimethyl sulfoxide (DMSO)[18]. A 40 µL of the diluted (1:100) FDA working solution was mixed with 1 ml of inoculated broth and incubated for 15 min at room temperature. A small

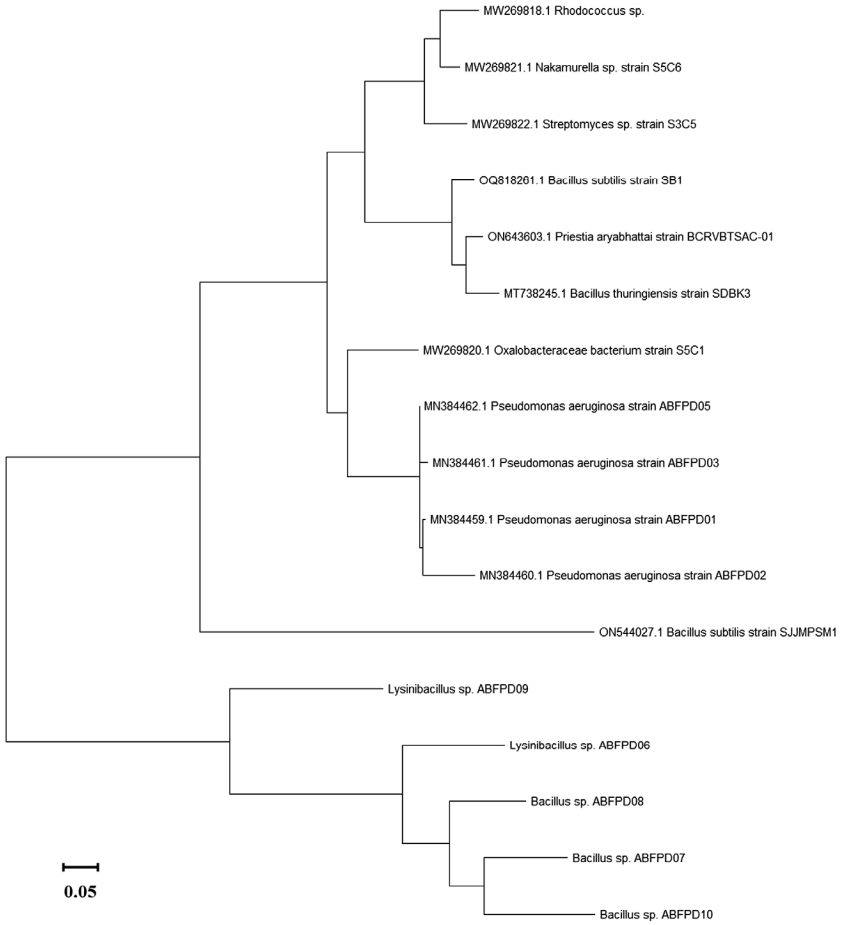

Fig. 2 — Phylogenetic tree of the bacterial isolates having potential to degrade plastics

amount of the solution containing FDA-stained cells (1 or 2 drops) was placed onto a microscope slide, covered with a cover slip and observed under the FITC filter in a fluorescent microscope (BX53 Olympus, Japan). Similarly, plastic strips inoculated with respected culture were checked for biofilm formation by FDA staining.

Scanning Electron Microscopy (SEM)

The most efficient plastic-degrading bacteria from this study, *Pseudomonas* spp. was further inoculated with LDPE film in Bushnell Hass media to check plastic-bacterial interaction and also to confirm plastic degradation by SEM. After 30 days of incubation, LDPE film was removed aseptically and was fixed with 2.5 % glutaraldehyde for 2 h followed by dehydration by 50, 75 and 100 % ethanol for 15 m each[19]. Further, LDPE film was mounted on a screw and imaged using SEM (Quanta 200, FEI).

Results and Discussion

A sterile flask containing Bushnell Hass broth was inoculated with plastic pieces rooted deep in sand collected from the anthropogenically contaminated beaches. Out of these, the isolates from Versova Beach were used in further study, because polluted Malad creek meets to Versova beach making it polluted further and hence a high probability of getting potential plastic degrading microbes. Further, these isolates were identified using 16S rRNA gene sequencing. About nine isolates in total were obtained, three of which belonged to *Bacillus* spp., two to *Lysinibacillus* spp. and 5 to *Pseudomonas* spp. The five isolates identified as *Bacillus* and *Lysinibacillus* species were found not much efficient in the degradation of plastic. The four *Pseudomonas* species were identified as *Pseudomonas aeruginosa* and the respective strains were named ABFPD01, ABFPD02, ABFPD03 and ABFPD05. The phylogenetic tree was constructed using MEGA 6 and is illustrated in Figure 2. Further, only 4 *Pseudomonas*

aeruginosa strains and one each unidentified *Bacillus* and *Lysinibacillus* species strains were inoculated with pre-weighed plastic strips in Bushnell Hass broth. The polyethylene strip was checked for weight loss at equal intervals of time. As discussed by Nanda & Sahu, 2010, who isolated three *Pseudomonas* species from different sites, out of which the most efficient strain degraded 29.1 % of polyethylene[20]. Also, Kathiresan[21] has reported *Pseudomonas* spp. degrading 20.54 % of Polythene. *Pseudomonas* spp. has been isolated in various studies on plastic degradation and it is also a predominant species that has potential degrading activity[22]. Reports of *Bacillus* spp. exhibiting plastic degrading properties are well published. *Bacillus pumilus* M27, *Bacillus subtilis* H1584, *Bacillus* sp. AF8, are certain *Bacillus* species isolated showing polythene, polyurethane,etc. degradation properties[23,24]. Other organisms known to have the potential for degrading polyethylene include *Streptomyces*, *Micrococcus*, *Rhodococcus*, etc.[24]. Microorganisms degrade plastics slowly, mainly by the production of various extracellular enzymes like lipases, depolymerase, protease, cutinase, esterase, proteinase K, pronase, hydrogenase, etc.[25,26] .

The weight loss performed to determine the degradation of polyethylene strips showed a slight decrease in weight after 60 days of incubation. Strains ABFPD01, ABFPD02, ABFPD03 and ABFPD05 caused 8, 11, 11 and 9 % of weight loss (Fig. 3). The unidentified *Bacillus* species showed less than 5 % weight loss, whereas the weight loss by the *Lysinibacillus* spp. was negligible. The weight loss may be due to the metabolic activity of bacteria on the strips. The presence of bacteria on the strips was determined by performing a viability test on LDPE strips using FDA staining (Fig. 4). Live bacteria were present on the film by the end of the two months incubation. The negative controls (no culture + LDPE strip and culture + no LDPE strip) did not show the presence of live bacteria as shown in Figure 3(g, h). The number of cells of the isolates ABFPD02 and ABFPD03 were comparatively more than ABFPD01, ABFPD05, and unidentified *Bacillus* and *Lysinibacillus* isolates. The presence of live cells on the LDPE strips indicates the formation of biofilm and may favour the degrading ability of the microorganisms[12]. The adherence of bacterial cells on the plastic surface can be promoted by decreasing the hydrophobicity. It is well-known that the metabolic activity in biofilm is higher than in freely suspended cells. This is also stated by Gilan *et al.*[27] that the carbon availability is greater in a

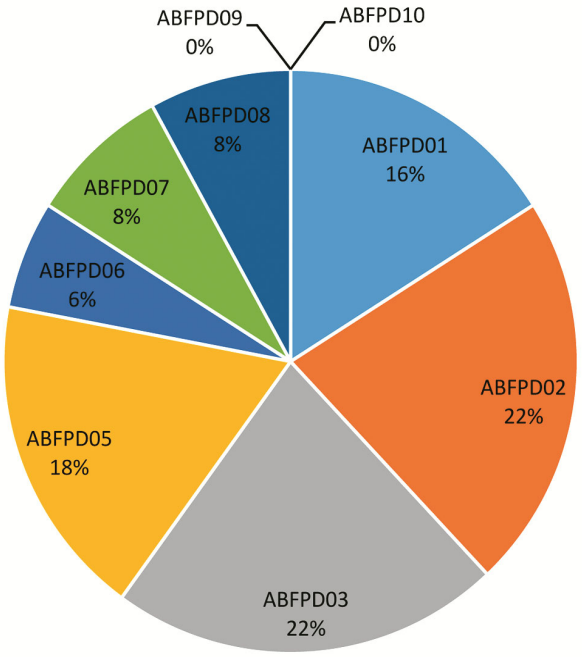

Fig. 3 — Percentage weight loss of LDPE using different bacterial strains

biofilm since more bacteria in the biofilm degrades more plastic. Thus, the availability of nutrients, especially hydrocarbon source from polyethylene has available to the bacteria in large amount due to biofilm formation (since, biofilm formation enhances the process). An approach of plastic pre-treatment prior to bacterial degradation can be helpful. Further, addition of surfactant can reduce the hydrophobicity. For example, tween 80, mineral oil, etc., are used to reduce hydrophobicity. Thus, by facilitating the attachment and formation of biofilm to the polyethylene, degradation can be promoted. The four *Pseudomonas aeruginosa* strains isolated had potential plastic degrading activity. The strains ABFPD02 and ABFPD03 were promising isolates and need to be characterized further.

In addition to the microcosm experiment and LDPE weight loss studies, the LDPE films were also analyzed using SEM (Fig. 5) to check plastic-bacterial interaction and to confirm plastic degradation by bacteria. The only hydrocarbon source (macronutrient) available to bacteria is LDPE, as Bushnell Hass broth was used for inoculation, which is a hydrocarbon depleting media. Due to this, bacteria (plastic degrading in this case) attached to the LDPE film enzymatically breaks it into small soluble molecules, which is taken up by bacterial cells and metabolized further.

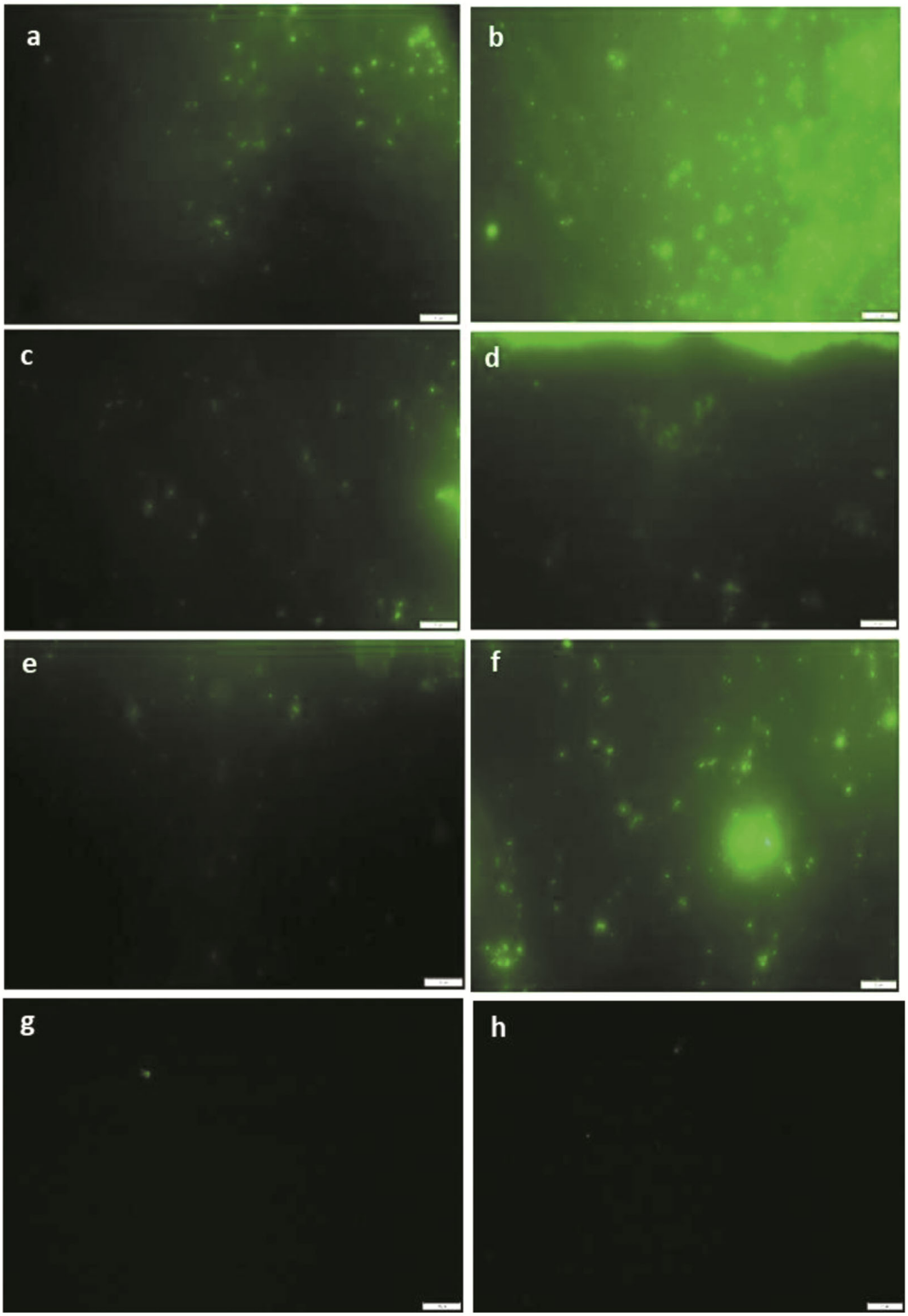

Fig. 4 — Biofilm viability determination on LDPE strips using FDA staining: a) *Pseudomonas aeruginosa* strain ABFPD01, b) *Pseudomonas aeruginosa* ABFPD02, c) unidentified *Bacillus* Strain, d) *Pseudomonas aeruginosa* ABFPD05, e) *Lysinibacillus* spp., f) *Pseudomonas aeruginosa* ABFPD03, and g & h) Negative control (without addition of bacteria). Scale for images (a – h) is 20 µm

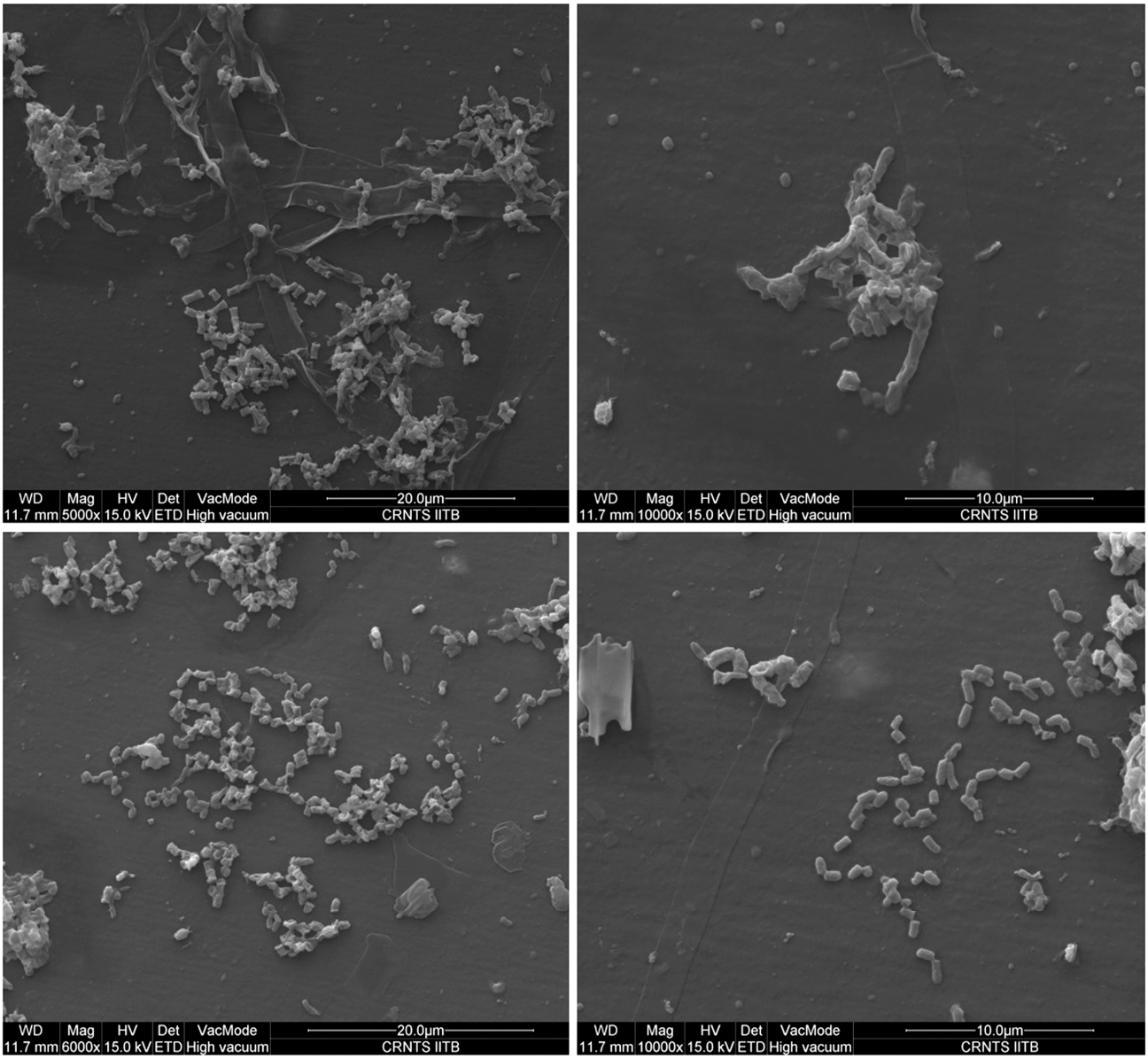

Fig. 5 — Scanning Electron Microscope (SEM) images showing interaction of plastic degrading bacteria with LDPE film as a result of biofilm formation

Conclusion

The sizable portion of the plastics or plastic end products subsequently finds its way to the marine environment causing deleterious effects. The current study highlights that the further use of plastic can be justified only if a proper, safe and environment-friendly way of disposing of the plastic, is found. Although the retention time of the plastics once discarded is quite high, the marine environment can still assist in their degradation through indigenous plastic-degrading microorganisms. Degradation of plastic by microbes is one of the most efficient ways for disposal of plastic. The present study showed that the *Pseudomonas* species isolated from the marine environment has the potential to deplete LDPE. Though the degradation action was gentle than reported for various other isolated *Pseudomonas* species, the study helps in knowing that the marine environment has potential plastic-degrading microbes that need to be explored.

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Conflict of Interest

The author's state there is no conflict of interest.

Author Contributions

ABF: Designing of the study and guidance; KK: Sampling, pre-treatment and experimentation under the supervision of ABF; KK & AKS: Maps and logistics; KK & ABF: Revision of the manuscript.

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