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Biodegradation of Low Density Polyethylene (LDPE) by Halophilic Bacteria Isolated from Solar Saltpans, Kovalam, Chennai

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ABSTRACT

Saltpan is an extreme environment, which inhabit organisms that survive at very high salinities, high temperatures and withstand severe solar radiations. Halophilic bacteria have been isolated from different hypersaline conditions like salt lakes, natural and artificial solar saltpans. In the present study, a total of two overlaying saltpan water samples were collected from different sites of saltpans from Kovalam in Chennai, of which 8 distinct halophilic bacterial isolates were obtained. Optimization of growth parameters of the isolated halophilic bacteria was done in order to determine the optimum NaCl, temperature, pH and LDPE source required for their growth. The optimum NaCl, temperature, pH, and LDPE source required for their growth were as follows: 20%, 40°C, pH 9-10 and 0.5% LDPE source. Out of the 8 isolates which were tested, only two of them showed some measure of hydrophobicity. Clear zone assay was done to detect the biodegradation of LDPE (Low Density Polyethylene) by halophilic bacteria. Of the 8 isolates 2/8 (28%) isolates showed clearance around the colony showing their potential to degrade LDPE. SEM analysis of LDPE film treated with the halophilic bacterial isolate showed that there were several cracks and pits on the surface which developed after 60 days of treatment in comparison to the control film. The halophilic bacteria *Nesiotobacter exalbescens* and *Bacillus vietnamensis* were perhaps for the first time reported from the hypersaline lakes of Chennai in this study.

INTRODUCTION

Hypersaline environments are widely distributed on the earth's continent where they exist either as natural water bodies such as permanent saline lakes or artificially created solar salterns (Litchfield 2002). Bacteria thriving in these environments are known as halophilic bacteria. The halophilic Archaea are characterized as organisms capable of growing from around 8% (1.5 M) sodium chloride (NaCl) to approximately 36% (5 M) NaCl, which is at saturation for NaCl. They are chemoorganotrophic, utilizing amino acids or carbohydrates as carbon source and occur ubiquitously in nature where the salt concentration is high (Grant 1989, Thongthai 1992)

Plastics are one of the synthetic polymers or man-made polymers (Scott 1999). Commonly used plastics are polyethylene (LDPE, MDPE, HDPE and LLDPE), polyethylene terephthalate (PET), polybutylene terephthalate (PBT), nylons, polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), and polyurethane (PUR) (Rivard 1995). These are the synthetic polymers which accumulate in the environment due to the absence of efficient methods for safe disposal and posing an ever increasing ecological threat to flora and fauna. One of the major strategies to facilitate disintegration and subsequent degradation is by direct degradation of LDPE by microorganisms using only the polymer as sole carbon source (Roy 2008). Several previous studies have reported on biodegradation of polythene by bacterial and fungal species (Kathiresan 2003, Kim 2003). The present study was, therefore, taken up to isolate halophilic bacteria from saltpans and to detect their ability to bring about biodegradation of LDPE (low density polyethylene).

MATERIALS AND METHODS

Sample Collection

Water from saltpans was collected in a sterile plastic bottle and was transported to the laboratory. Samples were processed within 24 hours of collection and stored at 4°C. The samples were collected from two different sites of saltpans from Kovalam of Chennai.

Optimization of Growth Parameters

Optimization of growth parameters of the isolated halophilic bacteria was done in order to determine the optimum tem-

perature, pH, NaCl and LDPE source required for their growth.

Sodium chloride (NaCl) concentration: The bacterial isolates were inoculated on to the nutrient agar plates supplemented with different concentration of salt ranging from 10%, 15%, 20%, 25% and 30%. The plates were incubated at 40°C for 24 hours and the growth was observed.

Temperature: 100 mL of saltpan water was taken in a sterile conical flask with the salt concentration of 20% and the flasks were incubated at different temperatures of 25°C, 37°C and 40°C for 24 hours to determine the optimum temperature for the growth of halophilic bacteria. Growth curve was determined using UV-VIS spectrophotometer.

pH: 100 mL of saltpan water was taken in a sterile conical flask and four different pH conditions were provided - 7, 8, 9 and 10 for the growth of halophilic bacteria. Salt concentration was maintained at 20% and the flasks were incubated at 40°C for 24 hours. Growth curve was determined using UV-VIS spectrophotometer.

LDPE as a carbon source: 100 mL of saltpan water was taken in a sterile conical flask with the salt concentration of 20% and the flasks were incubated at 40°C for 24 hours with different concentration of LDPE - 0.5%, 1% and 2% to determine the optimum carbon source for the growth of halophilic bacteria. Growth curve was determined using UV-VIS spectrophotometer.

Isolation of Halophilic Bacteria

A loopful of the incubated water sample was inoculated onto Seawater Agar (SA) and the plates were incubated at 40°C for 24 to 48 hrs. Next day the colonies were picked up and Gram staining and motility was done. The colonies were observed for pigmentation and the isolates were identified by the standard biochemical methods.

Biodegradation of LDPE Sheets by Halophilic Bacteria

Initial screening for the biodegradation of LDPE sheets by halophilic bacteria was done using clear zone assay and by BATH test.

Preparation of LDPE Powder

LDPE sheets were cut into small pieces and immersed in xylene. It was boiled for 15 min and then crushed while the solution was still warm. The LDPE powder so obtained was washed with ethanol to remove residual xylene, allowed to evaporate for 2-3 hrs to remove ethanol and dried at 60°C. The LDPE powder was stored in closed container for further analysis.

Determination of hydrophobicity (BATH) test (Rosenberg et al. 1980): Bacterial cell surface hydrophobicity was determined by the BATH (bacterial adhesion to hydrocarbon) test. 24 hour culture (5 mL) in the nutrient broth was centrifuged at 10,000 rpm for 15 min and washed twice with phosphate urea magnesium (PUM) buffer. Supernatant was discarded and the pellet re-suspended in PUM buffer. Absorbance of the suspension was measured at 400 nm by UV-VIS spectrophotometer. 0.2 mL of hexadecane was added to the suspension and shaken for 20 min. Test tubes were kept undisturbed for 5 min, which formed two phases viz., organic and aqueous phase. Absorbance of aqueous layer was measured at 400 nm by UV-VIS spectrophotometer. Culture free buffer was used as a blank.

The percent hydrophobicity was calculated using the relation:

$$\text{Hydrophobicity (\%)} = \frac{\text{OD of initial bacterial suspension} - \text{OD of aqueous phase} \times 100}{\text{OD of initial bacterial suspension}}$$

Clear zone assay (Augusta et al. 1993): LDPE powders were added to the mineral salt medium at a concentration of 0.5% respectively. Mixture was sonicated for 1 hour. The medium was sterilized at 121°C and a pressure of 15 lbs/inch^2 for 20 minutes. The medium was poured in the Petri plates and allowed to solidify. It was inoculated with halophilic bacterial culture and incubated at 40°C for 5 to 7 days. The plates were flooded with coomasie blue solution and allowed to stand for 30 min. It was then decolourised with 10% acetone. The plates were observed for zone of clearance around the colonies. Zone of clearance indicated the biodegradable capacity of the halophilic bacteria.

SEM analysis: The pure LDPE sheet and treated LDPE sheet (test sample) after 60 days of duration were subjected to SEM analysis in order to detect surface changes such as cracks, pits and fissures. The pure LDPE sheet served as a control over the test.

Table 1: BATH test.

S.No	Name of the bacteria	OD value before adding hexadecane	OD value after adding hexadecane
1.	HA1	0.16	0.06
2.	HA2	0.38	0.13

RESULTS

A total of 2 overlaying saltpan water samples were collected from different sites of saltpans from Kovalam of Chennai. Of the 2 samples a total of 8 distinct halophilic bacterial isolates were obtained in this study. After hydrophobicity test only two of the bacterial isolates showed biodegradation activity which were taken for further analysis.

Colony Morphology

Most of the isolates produced pigments of different colours like red, orange, yellow and cream, and off-white colonies which are one of the characteristic features of halophilic bacteria.

(a) HA 1 - *Nesiotobacter exalbescens* (Gene Bank accession number BankIt1890612 Contig_HA1 KU661973): Smooth, circular, off-white colonies were formed on seawater agar. They were found to be Gram negative motile rods. Catalase and oxidase were produced. They were indole negative, VP negative, nitrate positive. Acid was produced from ribose, D-mannose, D-fructose and D-glucose,

(b) HA 2 - *Bacillus vietnamensis* (Gene Bank accession number BankIt1890616 Contig_HA2 KU661974): Smooth, circular and raised orange colonies were formed on seawater agar. They were found to be Gram positive motile rods. They produced catalase and oxidase. They were indole positive, nitrate positive and urease negative. Casein, starch and gelatin were hydrolysed. Acid was produced from D-ribose, D-glucose, D-fructose and mannitol.

Optimization of Growth Parameters

Optimization of growth parameters of the isolated halophilic bacteria was done in order to determine the optimum NaCl, temperature, pH and LDPE source required for their growth. The optimum NaCl, temperature, pH, and LDPE source required for their growth were 20%, 40°C, pH 9-10 and 0.5% LDPE source.

Biodegradation of LDPE Sheets by Halophilic Bacteria

Determination of hydrophobicity (BATH) test: Rosenberg et al. (1980) had described BATH test to estimate the bacterial cell surface hydrophobicity that can be directly related to the ability to form an effective biofilm over any hydrophobic surfaces. BATH test revealed increase in the hydrophobicity of the strains. Out of the 8 isolates tested, only two showed some degree of hydrophobicity (Table 1).

Clear zone assay: Clear zone assay was done to detect the biodegradation of LDPE by halophilic bacteria. Of the 8 isolates, 2/8 (28%) showed clearance around the colony showing their potential to degrade LDPE (Fig. 1).

SEM analysis: Two halophilic bacteria which produced positive results for BATH test and clear zone assay were selected to study their ability to bring about biodegradation of LDPE sheet. Control sample had an appearance of smooth surface having no pits, cracks or any particles attached on the surface. In case of LDPE film treated with the halophilic bacterial isolate, there were several cracks on the surface which developed after 60 days of treatment. Simultaneously, microbes were also noticed on the film surface which indicate their strong adhering as well as LDPE utilization capacities. Clear mark of degradation can be seen at places where initially microbes were attached along with the pockets and pits around the LDPE polymer (Fig. 2).

DISCUSSION

The presence of vast areas of saline water around the earth's surface has provided favourable conditions for the evolution and emergence of salt loving organisms called halophiles. Halophilic microorganisms are primarily found in hypersaline environments and have been reported throughout the world (Oren 2002, Surve et al. 2012). Hyper saline environments are predominantly inhabited by both extremely halophilic and halotolerant microorganisms such as *Halobacterium* sp., *Haloferax* sp., *Haloarcula* sp., *Halobacillus* sp., *Salinibacter ruber*, *Virgibacillus salarius*, *Bacillus* spp. and *Micrococcus luteus* (Paterekt & Smith 1985, Arahal et al. 1996, Anton et al. 2002, Solanki & Kothari 2012, Solomon & Viswalingam 2013). Halophilic microorganisms require at least 0.2 M salt for their growth and cannot grow in the absence of it. On the other hand, halotolerant bacteria grow in absence of salt and can also grow in presence of relatively higher salt concentrations (e.g., *Staphylococcus aureus* and *Vibrio* sp.) (Ara et al. 2013).

In the present study, a total of 2 overlaying saltpan water samples were collected from different sites of saltpans from Kovalam of Chennai of which 8 distinct halophilic bacterial isolates were obtained. This was found to be in

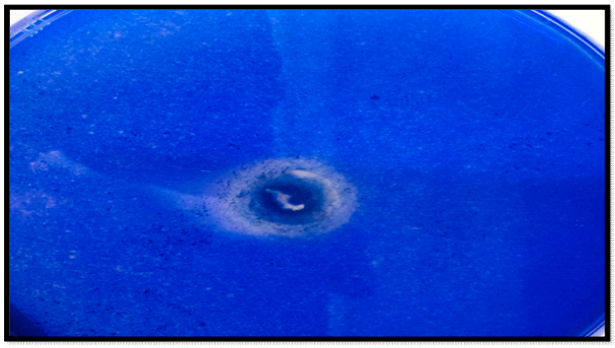

Fig. 1: Clear zone assay.

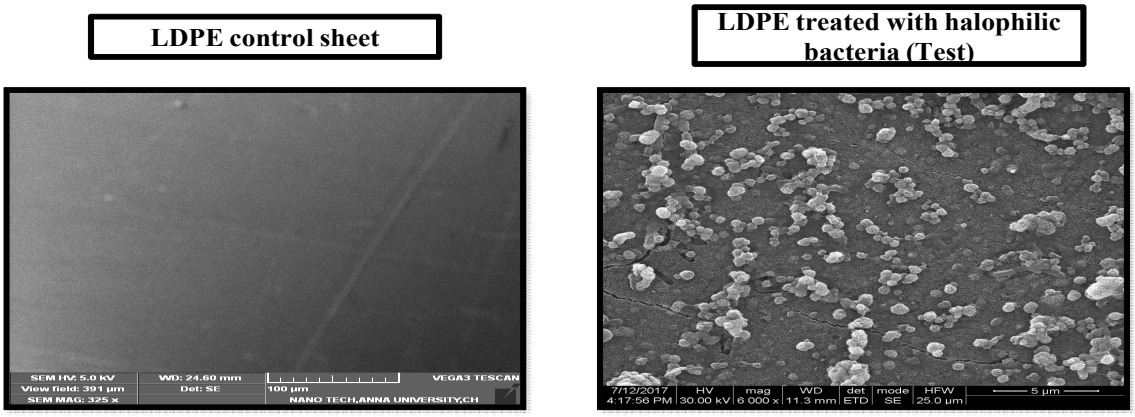

Fig. 2: SEM analysis of LDPE sheet.

agreement with Ganesan et al. (2010) and Saju et al. (2011) who isolated halophilic bacteria from Pichavaram and Kovalam (Kanyakumari). *Nesiotobacter exalbescens* and *Bacillus vietnamensis* were isolated in our study and to the best of our knowledge this is the first study to be done in Chennai, South India which shows the isolation of these two halophilic bacteria from hypersaline lakes.

The microorganisms adhere to the growth substrate to consume it and exponentially grow and multiply in the log phase of their growth curve. This adherence of the bacteria to the substrate can be hydrophilic or hydrophobic depending on many external factors like the surface of the substrate and the property of the bacteria to adhere. Bacterial adhesion in case of hydrophilic bacteria is done by its ability to attach to any surface, whereas hydrophobic bacteria require a hydrophobic surface. Thus, as the polyethylene exhibits a hydrophobic surface, the bacterial adhesion has higher interaction with it because of its hydrophobic nature (Orr 2004).

Bacterial adherence to hydrocarbon assay (BATH) was done to determine the bacterial adhesion in which the rate of interaction of bacterial cell with the polyethylene in both log and stationary phases demonstrated the hybrophobicity of the isolates. The cells in the log phase displayed hydrophobic ability more than those in the exponential phase. The greater affinity to hydrocarbon resulted in an increased colonisation interaction of the positive isolate with the polyethylene surface. In our study, out of the 8 isolates tested, only two showed some degree of hydrophobicity.

Augusta et al. (1993) reported that under shaking condition, the target organisms have enhanced production of hydrolysing enzymes over a period of 2, 4 and 6 months, which cause the extracellular degradation of the plastic and polyethylene. Clear zone assay was performed to demonstrate the ability of the bacteria to degrade polyethylene. Of the 8 isolates, 2/8 (28%) showed clearance around the colony showing their potential to degrade LDPE.

SEM analysis of LDPE film treated with the halophilic bacterial isolate showed that there were several cracks and pits on the surface which developed after 60 days of treatment in comparison to the control. These findings were found to be in agreement with Kapri et al. (2010), Shrivastav et al. (2011), Negi et al. (2011) and Girdhar et al. (2013) who demonstrated the cracks and disruption on surface of degraded LDPE film.

CONCLUSION

Eight halophilic bacteria were isolated from saltpans of Kovalam, of which two isolates demonstrated their potential to bring about biodegradation of LDPE. This indicates that the biodegradation of LDPE could be brought about by halophilic bacteria in hypersaline conditions. The halophilic bacteria *Nesiotobacter exalbescens* and *Bacillus vietnamensis* were perhaps for the first time reported from the hypersaline lakes of Chennai in this study.

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