

# Screening of Extracellular Hydrolytic Enzymes from Halophilic bacteria and Biodegradation of LDPE

Summera Rafiq<sup>1,2</sup>, R. Ishrath Razia<sup>1</sup>, SK. Jasmine Shahina<sup>1</sup> and K. Vijaya Ramesh<sup>2\*</sup>

<sup>1</sup> Department of Microbiology, Justice Basheer Ahmed Sayeed College for Women, Chennai, Tamilnadu, India

<sup>2</sup> \* Department of Plant Biology and Plant Biotechnology, Quaid-E-Millath Government College for Women, Chennai, Tamilnadu, India

## Abstract

Halophilic and halotolerant microorganisms are present in solar salt pans which are existing all over the world. Halophiles are salt loving bacteria which require salt for their growth. Extreme environments lead to natural attenuation of such microorganisms helps them in adapting to these environments. These microorganisms could produce biotechnologically essential molecules with applications ranging from producing industrially significant enzymes, to bioremediation and other useful products. Extremozymes produced by these halophiles have unique properties to sustain the metabolic and physiological process under high saline conditions. In the present study, a total of three overlaying saltpan water samples were collected from different sites of Chennai, of which 11 distinct halophilic bacterial isolates were obtained. Of the 11 isolates 6/11(54%) produced amylase enzyme and all the 11 (100%) isolates were positive for lipase and protease activity. Polymer degrading enzymes were detected which offers a solution for the biodegradation. Colonization studies, dry weight method, Fourier-transform infrared spectroscopy (FTIR) and Scanning Electron Microscopy (SEM) analysis showed the biodegradation potential of LDPE by halophilic bacteria.

**Keywords:** Halophilic bacteria, Salt pans, Extracellular enzymes, LDPE.

## 1. Introduction

Marine salts are produced from solar salt pans by removal of sea water and represents hypersaline condition which are optimum growth factor for

halophilic bacteria. They have a great potential towards industrial and biotechnological applications (Gilinsky and Tindall, 1992; Margesin and Schinner, 2001). The halophilic bacteria so far isolated and characterized is categorized into four different classes according to NaCl requirement for their growth and includes slight halophiles, moderate halophiles, extreme halophiles, and border line halophiles. The bacteria which are halotolerant on the other hand do not require salts for their growth but can tolerate a high salinity (Rodriguez Valera *et al.*, 1981; Rodriguez-Valera, 1988; Kushner, 1988; Hochstein, and Rodriguez-Valera, 1988).

The optimal activity of industrially important enzymes cannot be met all the times under parameters like pH, ionic strength and temperatures (Arnold, 2001). In such conditions extremozymes, (Eichler, 2001) enzymes produced by extremophiles that are able to thrive in extreme environments, could help in the development innovative biotechnology applications. Halophilic bacteria are found in diverse environments such as hyper saline lakes, saline soils and salted food. The halophilic bacteria are able to produce compounds with great potential in industrial process.

The stability and solubility of the enzymes produced by halophilic bacteria are excellent as these enzymes are active at high temperatures, pH and they are salt tolerant. There are very few reports in the literature about the production and characterization and biotechnological applications of these enzymes (Bhatnagar *et al.*, 2005) (Gomez and Steiner, 2004).

Annually, an estimated 500 billion to 1 trillion plastic bags are consumed worldwide (Roy *et al.*, 2008). Low density polythene (LDPE) has been used widely as packaging material due to its resourceful nature, usefulness, efficient mechanical properties and also able to withstand against water, light weight, low cost and high energy. However, it is resistance to biodegradation and much attention is required for the disposal strategies.

## 2. Materials and Methods:

### Sample collection:

A total of 3 overlaying saltpan water samples were collected in a sterile plastic bottle and was transported to the laboratory. Samples were processed within 24 hours of collection and were stored at 4°C. The samples were collected from three different sites of saltpans located at Theyur, Marakkanam, and Kadambadi of Chennai.

### Isolation of halophilic bacteria:

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

### Screening of extracellular Hydrolytic Enzymes:

#### a) Screening for presence of amylase activity:

The presence of amylolytic activity on plate was determined qualitatively following the method described by Amoozegar *et al.*, using starch agar medium containing 20% (w/v) total salts. The bacterial cultures were inoculated on starch agar and were incubated at 40°C, for 24hrs. After incubation the plates were flooded with iodine solution and observed for zone of clearance. Clearance indicated amylase activity.

#### b) Screening for presence of protease activity:

Proteolytic activity of the isolates was similarly screened on gelatin agar plates containing 10.0 g/l of gelatin. The bacterial cultures were inoculated onto gelatin agar and incubated at 40°C, for 24hrs. After incubation the plates were flooded with mercuric chloride. The isolates showing zones of clearance upon treatment with mercuric chloride were considered as positive protease producing bacteria.

#### c) Screening for presence of lipase activity:

Lipase activity of the cultures was screened on tributyrin nutrient agar plates containing 1% (v/v) of tributyrin. The bacterial cultures were inoculated

onto tributyrin agar and the plates were incubated at 40°C, for 24hrs. Isolates that showed clear zones of hydrolysis were considered as lipase producing bacteria.

### Screening for biodegradation of LDPE by Halophilic bacteria:

#### a) Colonization studies of LDPE:

LDPE sheets were cut into small pieces of 1 cm X 1 cm of similar weight, disinfected with 70% ethanol for 30 min and transferred to sterile distilled water for 20 min. LDPE sheets of same weight were placed in conical flask containing sterile sea water. It was inoculated with bacterial cultures. They were incubated at 40°C and results were observed after 1 week to 10 days.

#### b) Dry weight estimation:

To facilitate accurate measurement of the weight of the residual polyethylene, the polyethylene sheets were taken after the 60 days of incubation for degradation and bacterial biofilm from the polymer surface was washed off with a 2 % (v/v) aqueous sodium dodecyl sulphate solution, a surfactant which denatured the cells and completely washed off from the surface for 4 h (using shaker), followed by distilled water and finally with 70 % ethanol to ensure maximum possible removal of cells and debris. The cleaned polymer pieces were placed on a filter paper and dried overnight at room temperature before weighing (Merina Paul Das & Santosh Kumar, 2014).

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

#### c) FTIR analysis of LDPE sheets :

Fourier transform infrared spectroscopic study was performed for control and bacteria-treated LDPE films. The analysis was done using Perkin-Elmer Spectrum-One FTIR spectroscopy in the horizontal mode with thallium bromide disks.

#### d) SEM analysis of LDPE sheets:

The untreated and treated samples after 60 days of duration were subjected to SEM analysis after washing with 2 % (v/v) aqueous SDS and distilled water repeatedly through mild shaking for few minutes and additionally washed with 70 % ethanol to removal of cells so as to get maximum surface to be exposed for visualization. The samples were pasted onto the SEM Sample Stub using a carbon tape and sample was analyzed under high-resolution scanning electron microscope.

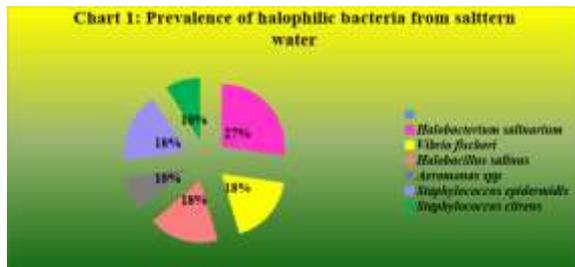
## 3. Results:

A total of 3 overlaying saltpan water samples were collected from three different sites of

salt pans located at Thayur, Marakanam, and Kadambadi of Chennai. Of the 3 samples a total of 11 halophilic bacterial isolates were obtained in this study

### Prevalence of Halophilic bacteria in Slatern water:

Of the 11 halophilic bacteria isolated the prevalence was as follows- 3/11 (27%) *Halobacterium salinarium*, 2/11 (18%) *Halobacillus salinus*, 2/11(18%) *Vibrio fischeri*, 1/11 (10%) *Aeromonas spp*, 1/11(10%) *Staphylococcus citreus* and 2/11(18%) *Staphylococcus epidermidis*. (Chart 1)



### Screening for the presence of Extracellular hydrolytic enzymes:

All the halophilic bacteria were screened for the presence of extracellular enzymes like amylase, lipase and protease. Of the 11 isolates 6/11(54%) were found to produce amylase enzyme and all the 11 (100%) isolates were found to be positive for lipase and protease activity. (Table 1)

Table 1: Prevalence of amylase and protease activity

S.No	Name of the bacteria	Total number	Total positive for amylase	Total positive for lipase	Total positive for protease
		(n=11)	(n=6)	(n=11)	(n=11)
1	<i>Halobacterium salinarium</i>	3	3	3	3
2	<i>Vibrio fischeri</i>	2	-	2	2
3	<i>Halobacillus salinus</i>	2	2	2	2
4	<i>Aeromonas spp</i>	1	1	1	1
5	<i>Staphylococcus epidermidis</i>	2	-	2	2
6	<i>Staphylococcus citreus</i>	1	-	1	1

### Screening for biodegradation of LDPE by Halophilic bacteria:

#### a) Colonization studies of LDPE:

Colonization studies showed an increase in the weight of LDPE sheets due to the attachment of halophilic bacteria on their surface. This is an important step for the occurrence of biodegradation. (Table 2)

Table 2: Colonization studies

S.No	Name of the bacteria	Initial weight of LDPE sheet	Weight of LDPE after 7days	Weight of LDPE sheet after 10days
1	<i>Halobacterium salinarium</i>	10mg	10.8mg	11.2mg
2	<i>Vibrio fischeri</i>	10mg	10.4mg	10.7mg
3	<i>Halobacillus salinus</i>	10mg	10.6mg	10.9mg
4	<i>Aeromonas spp</i>	10mg	10.3mg	10.8mg
5	<i>Staphylococcus epidermidis</i>	10mg	10.5mg	10.9mg
6	<i>Staphylococcus citreus</i>	10mg	10.4mg	10.8mg

#### b) Dry weight estimation (Weight loss) of LDPE

The easiest method to determine the degradation is to measure the weight loss. During the biodegradation period, the set up was maintained as undisturbed with no addition and removal of medium which indicates that the microorganisms used the LDPE film as carbon source. The microbial enzymes catalyzed the depolymerization and thus there was weight reduction of polyethylene. *Halobacterium salinarium* showed maximum weight loss followed by *Halobacillus salinus*, *Vibrio fischeri*, *Aeromonas spp* and *Staphylococcus epidermidis*. The weight loss of the LDPE films is as a result of the breakdown of carbon backbone due to enzymatic degradation by halophilic bacteria. (Table 3)

Table 3: Dry weight estimation

S.No	Name of the bacteria	Initial weight of LDPE sheet	Final weight of LDPE sheet	Weight loss percentage
1	<i>Halobacterium salinarium</i>	10mg	8.1mg	19%
2	<i>Vibrio fischeri</i>	10mg	8.6mg	14%
3	<i>Halobacillus salinus</i>	10mg	8.4mg	16%
4	<i>Aeromonas spp</i>	10mg	9.2mg	8%
5	<i>Staphylococcus epidermidis</i>	10mg	9.5mg	5%
6	<i>Staphylococcus citreus</i>	10mg	9.7mg	3%

#### c) FTIR analysis of LDPE sheets:

In the biodegradation of polyethylene, the initial step involves the oxidation of the polymer

chain leading to the formation of carbonyl groups. These groups eventually form carboxylic groups, which subsequently undergo  $\beta$ -oxidation and are completely degraded via the citric acid cycle resulting in the formation of  $\text{CO}_2$  and  $\text{H}_2\text{O}$ .  $\beta$ -oxidation and the citric acid cycle are catalyzed by microorganisms. Monitoring the formation and disappearance of carbonyl and double bond bands using FTIR helps in detecting biodegradation process. There was formation of alcohols, carboxylic acids, esters, aldehydes, ethers, aromatics, and phenol groups which corresponds to the biodegraded products of LDPE sheet when compared to the control.

#### d) SEM analysis of LDPE sheets

The weight reduction, mineralization level and absorption spectra provide solid evidence of polymer biodegradation, the changes of surface of LDPE films were elucidated by SEM. 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 and pits on the surface which developed after 60 days of treatment. Simultaneously, microbes were also noticed on the film surface indicate its strong adhering capabilities 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.

#### Discussion:

More than 95% of microbes exist in the environment that has not yet been explored (Rappé, and Giovannoni, 2003). Many functionally and taxonomically diversified microbial communities are found in extreme environments like hypersaline habitats (Martins, and Peixoto, 2012). A number of molecules with potential for commercial interest are produced by microorganisms which are adapted to life at high salt concentrations (Waditee-Sirisattha *et al.*, 2016). Salt – loving microorganisms are known as Halo-bacterium which inhabits hypersaline environments. *Halobacterium*, are motile, spore producing aerobic microorganism found distributed all over the world in high salt environments, many in natural hypersaline brines in arid, coastal, even deep sea locations, as well as in artificial salterns used to mine salts from the sea.

Approximately 140 million tons of synthetic polymers are produced each year at international level and the worldwide utility of polyethylene is expanding at an alarming rate of 12% annually (Shimao, 2001). This may lead to huge ecological issue due to the accumulation of polyethylene leading to environmental pollution as its natural

degradation process is very slow. Thus, biodegradation plays a pivotal role for solving this environmental issue among other physical and chemical degradation method.

In the present study, 11 halophilic bacterial isolates were isolated from Theyur, Selayur, and Kadambadi of Chennai. The prevalence was as follows- 3/11 (27%) *Halobacterium salinarium*, 2/11 (18%) *Halobacillus salinus*, 2/11(18%) *Vibrio fischeri*, 1/11 (10%) *Aeromonas spp*, 1/11(10%) *Staphylococcus citreus* and 2/11(18%) *Staphylococcus epidermidis*. These findings were found to be in accord with Kathiresan (2003).

The biodegradation of polyethylene is due to the secretion of extracellular enzymes by the microorganisms which helps in breaking the complex molecular structure of plastics (Kathiresan, 2003). In the present study, all the halophilic bacteria were screened for the presence of extracellular enzymes like amylase, lipase and protease. Of the 11 isolates 6/11(54%) were found to produce amylase enzyme and all the 11 (100%) isolates were found to be positive for lipase and protease activity. Uchida *et al.*, (2000) had reported that during degradation, lipase activity was observed in the culture of a bacterium which causes biodegradation of plastic which was in agreement with our study.

Colonization studies showed an increase in the weight of LDPE sheets due to the colonization of halophilic bacteria on their surface. These findings were found to be in consistent with that of Hadad *et al.*, (2005), Augusta *et al.*, (1993) and Oda *et al.*, (1997). A simple and quick way to measure the biodegradation of polymers is by determining the weight loss. Microorganisms that grow on the surface of the polymer, forming biofilms leads to an increase in weight, whereas a loss of polymer integrity leads to weight loss. Weight loss is proportional to the surface area since biodegradation usually is initiated at the surface of the polymer. In the present study, *Halobacterium salinarium* showed maximum weight loss followed by *Halobacillus salinus*, *Vibrio fischeri*, *Aeromonas spp* and *Staphylococcus epidermidis*. This was found to be in agreement with Kathiresan and Bingham (2001).

In the present study, during the biodegradation of polyethylene, the initial step involves the oxidation of the polymer chain leading to the formation of carbonyl groups. Monitoring the formation and disappearance of carbonyl and double bond bands using FTIR helps in detecting biodegradation process. In the present study, there was disappearance in functional groups upon FTIR analysis of the biodegraded LDPE sheet when compared to the standard. They were similar to the studies done by Arboleda *et al.*, (2004); Drimal *et*

*al.*, (2007). SEM analysis of LDPE film treated with the halophilic bacterial isolate, there were several cracks on the surface which developed after 60 days of treatment. Clear mark of degradation can be seen at places where initially microbes were attached along with the pockets and pits around the LDPE polymer. Similar results were given by Kapri *et al.*, (2010); Shrivastav *et al.*, (2011); Negi *et al.*, (2011); Girdhar *et al.*, (2013).

#### 4. Conclusion:

The problem of plastic waste pollution is rapidly growing. There is no part of the world unharmed from its impact. The present study shows the efficiency of halophilic bacteria to bring about biodegradation of LDPE through the secretion of enzymes like amylase, protease and lipases. *Halobacterium salinarium* showed good biodegrading capacity of LDPE when compared to other bacterial isolates. This biodegradation approach is safe and eco-friendly. The results of the study showed a potential hope to degrade LDPE at an efficient rate than it is degraded naturally.

#### References:

- [1] Galinski, E. A., & Tindall, B. J. (1992). Biotechnological prospects for halophiles and halotolerant micro-organisms. in *Molecular Biology and Biotechnology of Extremophiles*, R. D. Herbert and R. J. Sharp, Eds., Blackie, London, UK, 1992.
- [2] Margesin, R., & Schinner, F. (2001). Potential of halotolerant and halophilic microorganisms for biotechnology. *Extremophiles*, 5(2), 73-83.
- [3] Rodriguez-Valera, F., Ruiz-Berraquero, F., & Ramos-Cormenzana, A. (1981). Characteristics of the heterotrophic bacterial populations in hypersaline environments of different salt concentrations. *Microbial Ecology*, 7(3), 235-243.
- [4] Rodriguez-Valera, F. (1988). Characteristics and microbial ecology of hypersaline environments. *Halophilic bacteria*, 1, 3-30.
- [5] Kushner, D. J. (1988). Physiology of halophilic eubacteria. *Halophilic bacteria*, 109-138.
- [6] Hochstein, L. I., & Rodriguez-Valera, F. (1988). The physiology and metabolism of the extremely halophilic bacteria. *Halophilic bacteria*, 2, 67-83.
- [7] Arnold, F. H. (2001). Combinatorial and computational challenges for biocatalyst design. *Nature*, 409(6817), 253.
- [8] Eichler, J. (2001). Biotechnological uses of archaeal extremozymes. *Biotechnology advances*, 19(4), 261-278.
- [9] Bhatnagar, T., Boutaiba, S., Hacene, H., Cayol, J. L., Fardeau, M. L., Ollivier, B., & Baratti, J. C. (2005). Lipolytic activity from Halobacteria: screening and hydrolase production. *FEMS Microbiology Letters*, 248(2), 133-140.
- [10] Gomes, J., & Steiner, W. (2004). The biocatalytic potential of extremophiles and extremozymes. *Food technology and Biotechnology*, 42(4), 223-235.
- [11] Roy, P. K., Titus, S., Surekha, P., Tulsi, E., Deshmukh, C., & Rajagopal, C. (2008). Degradation of abiotically aged LDPE films containing pro-oxidant by bacterial consortium. *Polymer Degradation and Stability*, 93(10), 1917-1922.
- [12] Amoozegar, M. A., Malekzadeh, F., & Malik, K. A. (2003). Production of amylase by newly isolated moderate halophile, *Halobacillus* sp. strain MA-2. *Journal of microbiological methods*, 52(3), 353-359.
- [13] Das, M. P., & Kumar, S. (2014). Microbial deterioration of low density polyethylene by *Aspergillus* and *Fusarium* sp. *Int J Chem Tech Res*, 6(1), 299-305.
- [14] Rappé, M. S., & Giovannoni, S. J. (2003). The uncultured microbial majority. *Annual Reviews in Microbiology*, 57(1), 369-394.
- [15] Martins, L. F., & Peixoto, R. S. (2012). Biodegradation of petroleum hydrocarbons in hypersaline environments. *Brazilian Journal of Microbiology*, 43(3), 865-872.
- [16] Waditee-Sirisattha, R., Kageyama, H., & Takabe, T. (2016). Halophilic microorganism resources and their applications in industrial and environmental biotechnology. *AIMS Microbiol*, 2, 42-54.
- [17] Shima, M. (2001). Biodegradation of plastics. *Current opinion in Biotechnology*, 12(3), 242-247.
- [18] Kathiresan, K. (2003). Polythene and plastics-degrading microbes from the mangrove soil. *Revista de biologia tropical*, 51(3-4), 629-633.
- [19] Uchida, H., Nakajima-Kambe, T., Shigeno-Akutsu, Y., Nomura, N., Tokiwa, Y., &

- Nakahara, T. (2000). Properties of a bacterium which degrades solid poly (tetramethylene succinate) -co-adipate, a biodegradable plastic. *FEMS microbiology letters*, 189(1), 25-29.
- [20] Hadad, D., Geresh, S., & Sivan, A. (2005). Biodegradation of polyethylene by the thermophilic bacterium *Brevibacillus borstelensis*. *Journal of applied microbiology*, 98(5), 1093-1100.
- [21] Augusta, J., Müller, R. J., & Widdecke, H. (1993). A rapid evaluation plate-test for the biodegradability of plastics. *Applied microbiology and biotechnology*, 39(4-5), 673-678.
- [22] Oda, Y., Oida, N., Urakami, T., & Tonomura, K. (1997). Polycaprolactone depolymerase produced by the bacterium *Alcaligenes faecalis*. *FEMS Microbiology letters*, 152(2), 339-343.
- [23] Kathiresan, K., & Bingham, B. L. (2001). *Biology of Mangroves and Mangrove Ecosystems-1 Introduction*. *Advances in marine biology*, 40, 84-88.
- [24] Arboleda, C. E., Mejía, A. I. G., & López, B. L. O. (2004). Poly (vinylalcohol-co-ethylene) biodegradation on semi solid fermentation by *Phanerochaete chrysosporium*. *Acta Farm. Bonaerense*, 23(2), 123-128.
- [25] Dřimal, P., Hoffmann, J., & Družbík, M. (2007). Evaluating the aerobic biodegradability of plastics in soil environments through GC and IR analysis of gaseous phase. *Polymer testing*, 26(6), 729-741.
- [26] Kapri, A., Zaidi, M. G. H., Satlewal, A., & Goel, R. (2010). SPION-accelerated biodegradation of low-density polyethylene by indigenous microbial consortium. *International Biodeterioration & Biodegradation*, 64(3), 238-244.
- [27] Shrivastav, A., Mishra, S. K., Pancha, I., Jain, D., Bhattacharya, S., Patel, S., & Mishra, S. (2011). Biodegradability studies of polyhydroxyalkanoate (PHA) film produced by a marine bacteria using *Jatropha* biodiesel byproduct as a substrate. *World Journal of Microbiology and Biotechnology*, 27(7), 1531-1541.
- [28] Negi, H., Gupta, S., Zaidi, M. G. H., & Goel, R. (2011). Studies on biodegradation of LDPE film in the presence of potential bacterial consortia enriched soil. *biologija*, 57(4).
- [29] Girdhar, A., Bhatia, M., Nagpal, S., Kanampalliwar, A., & Tiwari, A. (2013). Process parameters for influencing polyhydroxyalkanoate producing bacterial factories: an overview. *J Pet Environ Biotechnol*, 4(155), 2.