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Research Article

# INFLUENCE OF CELL SURFACE HYDROPHOBICITY IN COLONIZATION AND BIOFILM FORMATION ON LDPE BIODEGRADATION

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**ABSTRACT**

Objective: Solid surfaces that are in contact with water in environment tend to form microbial colonization. The purpose of this research is to determine the role of biofilm formation and colonization of bacteria during the biodegradation of low density polyethylene (LDPE) films by *Bacillus amyloliquefaciens* strains BSM-1 and BSM-2.

Methods: Here two active strains were isolated and identified as *Bacillus amyloliquefaciens* BSM-1 and BSM-2. The cell surface hydrophobicity behavior of those strains was determined. The bacteria were tending to colonize on polymer which was evaluated and the formation of biofilm was quantified. The bacterial biofilm was visualized by scanning electron microscope (SEM).

Results: During the study it was found that BSM-2 strain have the maximum capability to colonize, form biofilm on the inert LDPE films and facilitated maximum polymer degradation compared to prototype BSM-1. BSM-2 also exhibited higher cell surface hydrophobicity of 45.23% compared to strain BSM-1.

Conclusion: The present finding implies that determination of cell surface hydrophobicity is important to decide the potentiality of strain for LDPE biodegradation.

**Keywords:** LDPE biodegradation, Hydrophobicity, Biofilm, Surface active compounds.

## INTRODUCTION

Plastics are composed of small monomer units arranged continuously to form long chain polymer in which polyethylene is the most problematic one. Polyethylene is an inert synthetic polymer. Several physical and chemical methods were employed to degrade this polyethylene but it did not showed satisfactory results. Using microorganisms, the desired level of polymer degradation might be obtained. Formation of biofilm plays a major role to increase the level of biodegradation. Bacteria tend to adhere to different kinds of surfaces, ranging from surfaces in the human body, and plants and clays, to plastics and metals. Once bacteria are attached to a surface, a multi-step process starts, resulting in a complex adhering microbial community called a 'biofilm'[1]. Surface adhesion of bacteria is an essential step and is required for the bacteria to arrange themselves favorably in their environment [2].

Costerton et al. [3] defined a biofilm as "a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface." The molecular nature of the bacterial cell surface is crucial in the interaction between the microorganisms and the host [4]. These surface-associated microorganisms contribute substantially to degrade the xenobiotic compounds present on the attached surfaces. Generally microbes have already been observed to form the biofilm under certain circumstances like nutrition cues, inhibitory agents like antibiotics or toxins [5, 6] i.e. under threat conditions thus may be termed as outcome of phenomenon to form oriented structure for protection and survival. This ability to form the protective structure provides several advantages like increased access to nutrient, protection against toxins and antibiotics, maintenance of extracellular activities and shelter from predation [7]. Furthermore, estimation of carbon dioxide evolved in the degradation of LDPE does not represent the potential polymer biodegraders. Thus the methods for determination of biofilm [8, 9] formed on the surface of substrate seems to be the most relevant to find microorganisms capable for its degradation [10].

In the present study, cell surface hydrophobicity of plastic degrading strains was characterized and the effect of hydrophobic nature of these bacteria on biofilm formation, colonization, and thus biodegradation of polyethylene was evaluated.

## MATERIALS AND METHODS

### Microbial strains source

The microbes were isolated from LDPE films collected from the solid waste dump region Pallikaranai (12.9377 N / 80.2153 E, 7 meters above sea level), Chennai, India and kept in sterile container for further use.

### Preparation of LDPE powder

Low density polyethylene (LDPE) films were obtained from B.N. Polymers, Bangalore, India. LDPE films were cut into small pieces, immersed into xylene and boiled for 15 min, crushed with blender at 3000 rpm. The obtained LDPE powder was later washed with ethanol, dried overnight in hot air oven at 60 °C to obtain dry powder, stored at room temperature for further use.

### Culture Preparation

Cultures were grown using the synthetic medium [$NH_4NO_3$ 1.0, $K_2HPO_4$ 0.7, $KH_2PO_4$ 0.7, $MgSO_4.7H_2O$ 0.7, NaCl 0.005, $ZnSO_4.H_2O$ 0.002, $MnSO_4.4H_2O$ 0.001, $FeSO_4.H_2O$ 0.002 g/l] supplemented with 0.3% LPDE powder for the screening of active biodegrading strain, study of cell surface hydrophobicity and other parameters.

### Phylogenetic identification of bacterial strain

To identify the LDPE degraders, a polymerase chain reaction (PCR) was performed to amplify the 16S rRNA gene from the genomic DNA of the strain using universal primers fP1 (5'-GAGTTTGATCCTGGCTCA-3') and rP2 (5'-ACGGCTACCTTGTTACGACTT-3') as described by Weisberg et al [11]. The amplified sequence similarity search was done for the 16S rDNA sequence using online search tool called National Center for Biotechnology Information (NCBI) BLAST database (http://www.nih.nov.ncbi). The unknown organism was identified using the maximum aligned sequence through BLAST search. The sequences were aligned together with those of the representative members of the selected genera with the CLUSTAL W program [12]. The nucleotide sequence of the 16S rRNA gene was determined and compared with published 16S rRNA sequences at GenBank data base of NCBI. The phylogenetic tree was inferred from the neighbor-joining method with MEGA version 3.1 [13].

**Study methodology**

The study was designed to detect the capability of the microbial strain to show the hydrophobic characteristics while forming the biofilm so that possible correlation could be made for the concerned strain behavior to degrade LDPE with the help of cell surface hydrophobicity.

**Cell surface hydrophobicity evaluation**

Identified isolates were incubated with synthetic media supplemented with LDPE and hydrophobicity was determined after 2nd, 4th and 60th d of incubation. To evaluate the percentage of hydrophobicity, 5 ml of 24 h culture was taken, centrifuged and pallets were re-suspended in Phosphate-magnesium buffer, centrifuged supernatant pooled into one, OD was taken at 400 nm using UV-vis spectrophotometer [Shimadzu UV-vis spectrophotometer-8500 II] called Initial Bacterial Suspension. Again 5 ml of culture was taken and mixed 0.2 ml of Hexadecane. Mixed to get two phases, OD was taken at 400 nm for aqueous phase called as Final concentration in aqueous phase [14].

The Percentage hydrophobicity was calculated using the relation

$$\text{Hydrophobicity}[\%] = \frac{\text{OD of Initial bacterial suspension} - \text{OD of aquous phase}}{\text{OD of Initial bacterial suspension}} \text{x}100$$

**Biofilm quantification**

The LDPE films of 3cm X 3cm dimension were taken and disinfected with 70% ethanol for 30 min, washed with distilled water for 10 min, taken into 100 ml synthetic media inoculated with 24 h culture, kept in magnetic stirrer. After every 10 d, 1 LDPE film was taken washed in 10% ethanol by vigorous shaking. OD was taken at 540 nm using UV-vis spectrophotometer [Shimadzu UV-vis spectrophotometer-8500 II], 95% ethanol served as blank [15].

**Determination of biomass colonizing capability**

The LDPE films of 3cm X 3cm dimension were taken and disinfected with 70% ethanol for 30 min, washed with distilled water for 10 min, taken into 100 ml synthetic media inoculated with 24 h old culture, kept in magnetic stirrer. After every 10 d, 1 LDPE film was taken, boiled with 0.5 N NaOH and centrifuged for 15 min at 10,000 rpm. The filtrate is subjected for estimation of protein concentration using Lowry's method. Concentration of protein was calculated using BSA standard plot [16]. LDPE films colonized by bacterial strains for period of 60 d were analyzed by scanning electron microscope (JEOL, Model JSM-6390LV) after gold coating.

**RESULTS AND DISCUSSION**

In the present investigation hydrophobicity of cell and variation in biofilm formation has been studied and correlated with the supporting evidences such as colonization after biodegradation.

**Screening and identification of LDPE degrading bacteria**

Two bacterial strains were isolated from municipal solid waste landfill area, which were able to degrade the LDPE. The bacterial isolates were identified by 16S rRNA method. The nucleotide sequences obtained here has been submitted to GenBank database. The strains were identified as *Bacillus amyloliquefaciens* BSM-1 and *Bacillus amyloliquefaciens* BSM-2 and assigned accession numbers, KC924446 for BSM-1 and KC924447 for BSM-2. Figure 1 and 2 shows the phylogenetic tree for the isolated strains by using the BLAST search tool of NCBI web server, based on the analysis of the 16S rRNA sequence, and on the homology between the 16S rRNA sequences of the registered strains.

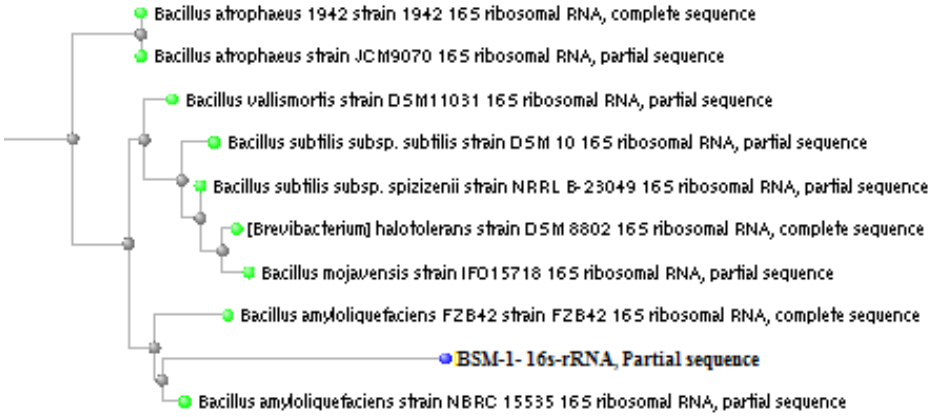


**Fig. 1: Phylogenetic tree of *Bacillus amyloliquefaciens* BSM-1**

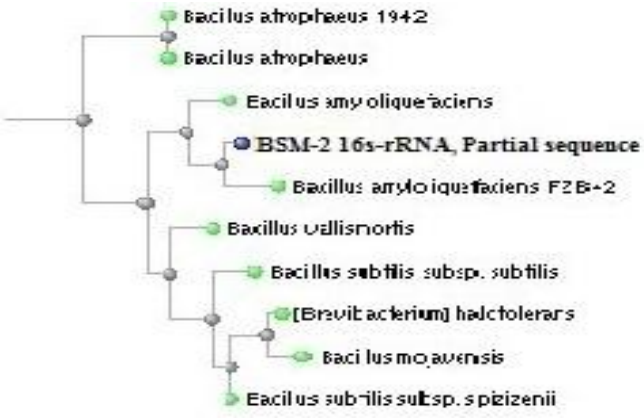


**Fig. 2: Phylogenetic tree of *Bacillus amyloliquefaciens* BSM-2**

Both the species were compared from each other in respect of variation in genetic makeup through Hit matrix plot using BLAST facility (Figure 3). In hit matrix, the sequences are compared with each other for the homology and as a result a straight line is observed in case of similar strains while little difference is denoted by a break in the straight line or gaps. The hit matrix of strains BSM-1 and BSM-2 was showed slight differences around 300-400 bp and 1300-1370 bp range.

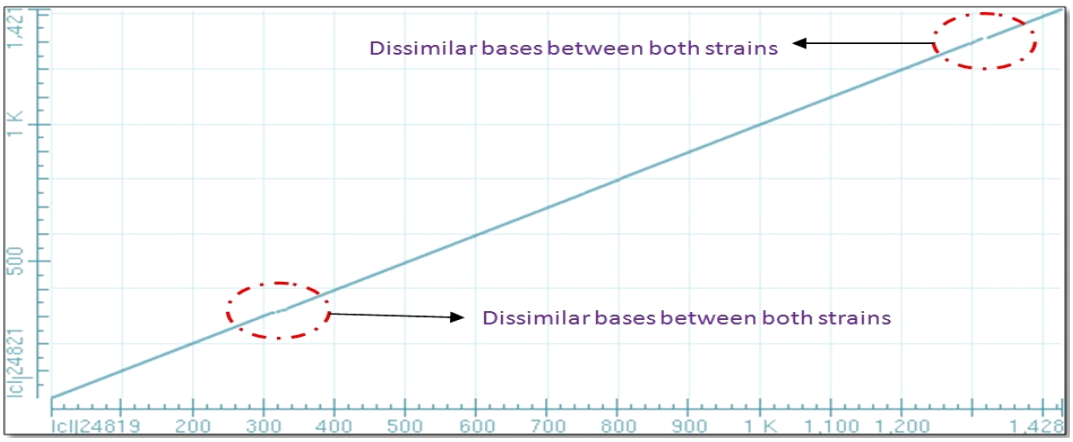


**Fig. 3: Hit Matrix comparison Plot**

As the two strains were identified as *Bacillus amyloliquefaciens* and were isolated from the same site and on the same collected sample thus it may be conferred that these changes and variation in the base pairs has been arrived in the strains due to the environmental adaptation. In the scarcity of the simple nutrients for the cells, has been forced to adopt in the harsh condition and were compelled to utilize the complex medium of carbon source i.e. LDPE films. This led to the survival of the strain which allowed the changes in themselves and developed the enzyme system to utilize the available substrate. It also led to the outcome of the strain capability i.e. higher in BSM-2 to utilize and degradation of the LDPE films as compared to the prototype strain BSM-1.

**Bacterial Hydrophobicity**

It is also well known that microbes tend to change their cell surface hydrophobicity in different growth phases, under different growth conditions, morphogenesis and differentiation [17, 18]. In the present study, BSM-2 strain showed more hydrophobicity i.e. 45.23% than BSM-1 which is less hydrophobic in nature i.e. 36.04% (Figure 4), thus it will not be unfair to state that in present study that environmental adaptation forced the strain BSM-2 to be more hydrophobic. Similarly, *Bacillus amyloliquefaciens* BSM-2 exhibited 16% LDPE degradation whereas BSM-1 11%. The result suggested that the maintenance of higher cell surface hydrophobicity of microbes produce high degree of LDPE biodegradation.

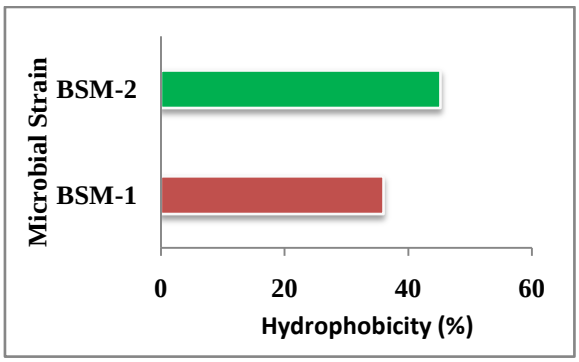


**Fig. 4: Hydrophobicity percentage of the bacterial isolates**

**Colonization of Biomass**

Biodegradation of the polyethylene becomes more efficient if the degrading microorganism forms a biofilm on the surface, but generally hydrophobic nature of the polyethylene hinders the formation of microbial biofilm [16]. Few factors such as motility [19, 20], Zeta potential [21], release of extracellular substances like polysaccharides [22, 23], proteins [24], biosurfactants [25] has influence on bacterial adhesion capability [26], however cell surface hydrophobicity has been attributed the most important in biofilm formation [27].

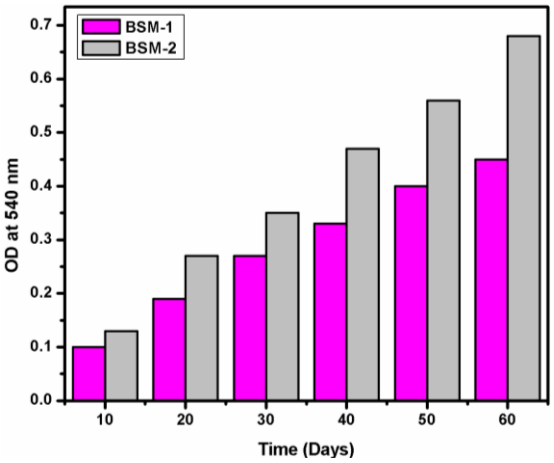


**Fig. 5: Quantification of biofilm by bacterial isolates**

The high hydrophobic nature of BSM-2 helped it for maximum biofilm formation on the LDPE (Figure 5) and thus showed highest capability of colonization. The colonization on the polyethylene samples were observed through SEM photomicrographs (Figure 6). It is evident that the bacterial isolate *Bacillus amyloliquefaciens* BSM-2 (Figure 6a) have significant colonization than BSM-1 (Figure 6b) after 60 d of incubation, resulting formation of dense biofilm. As large numbers of bacterial cell of BSM-2 were attached on the LDPE, the level of extracted protein concentration from the film was high (101µg/ml) (Figure 7) and facilitated efficient biodegradation of as compared to strain BSM-1 prototype.

The evidences from published research, taken together with suggested approach could give a satisfactory explanation for this behavior of cells. The hindrance offered by hydrophobic film is nothing but the formation of the interfaces of water and hydrophobic surface due to repulsion of the duo facilitated due to opposite nature of surface charge. In this respect surface active compounds (SACs) have found to play a vital role to help the microbes to interact through interfaces [28] by forming conditional

film which is mainly composed of lipids, proteins, complex polysaccharides and humic substances [29]. It changes the wet ability and surface charge of the substrate which may be found out through contact angle and free surface energy measurement. Significance of SACs were firstly described in 1987 [30] supported by Gerson [31] which suggests its role in growth of microbes on water insoluble substrates and possibly valid in this case. Additionally it is also well known that gram negative bacteria with a reduced O-specific chain in their lipopolysaccharides appear to be less hydrophilic than strains having the full O-specific chain [28], unlikely here as *Bacillus amyloliquefaciens* strains are gram positive in nature but definitely its hydrophobicity helped the cells to form cumulated biofilm as compared to rival prototype and enabled cells to utilize more nutrients from LDPE films as sole carbon source resulted in more biodegradation by BSM-2 because all other parameters were kept similar during in vitro study.

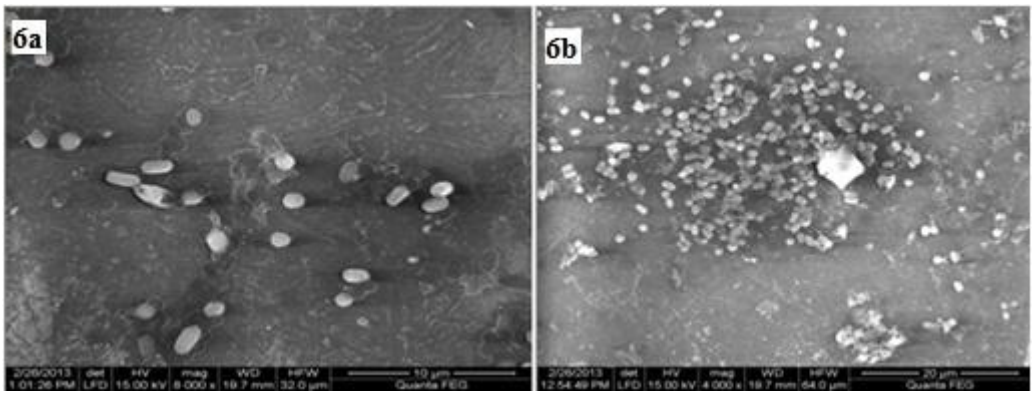


**Fig. 6: Scanning electron micrograph of microbial colonization by BSM-1(6a) and BSM-2 (6b)**

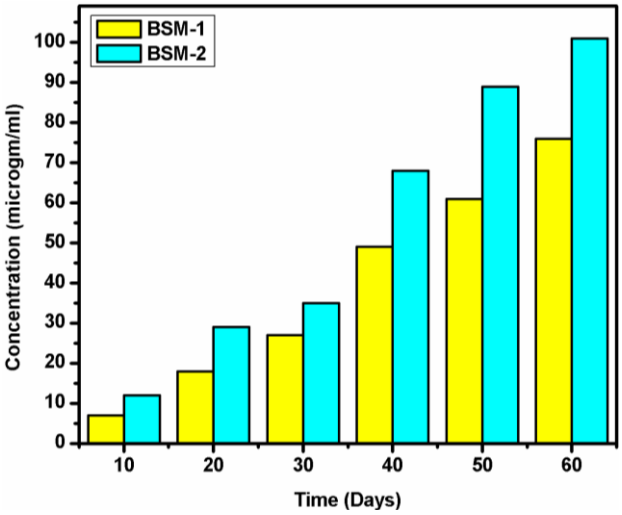


**Fig. 7: Determination of protein concentration from colonized film**

## CONCLUSION

From the above study it was concluded that cell surface hydrophobicity may be regarded as most relevant parameter for the assay of microbes for their biodegradation capability and suggested for the screening of such microbial strains from the mixed population. The cells having more hydrophobic nature has shown maximum capability of biofilm formation and biodegradation compared to its prototype strain as suggested by the study while intense work in this regard is needed at molecular level which will open new possibilities & better understanding of the behavior and its importance in biodegradation.

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