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ALK –B GENE EXPRESSION IN BACTERIA ISOLATED FROM PLASTIC ACCUMULATED MUNICIPAL WASTES OF THANJAVUR

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ABSTRACT: The efficacy of biodegradation of plastic material was analyzed using indigenous flora present in the municipal dump solid waste. The microbial species associated with the polythene materials were identified as *Bacillus amylolyticus, Bacillus firmus, Pseudomonas putida, Pseudomonas fluorescence, Bacillus subtilis* and *Streptomyces sps.* The efficiency of microbes utilizing LDPE as carbon sources were analyzed. Among the isolated strains, *Pseudomonas putida*, *Pseudomonas fluorescence* and *Streptomyces sps.* could able to utilize LDPE as carbon source. Further to the preliminary screening, gene specific primers for alkane monooxygenase was synthesized and gradient PCR was performed to determine the presence of alkane monooxygenase gene in the bacterial isolates. Among the five bacteria selected, three bacteria such as *Pseudomonas putida, Pseudomonas fluorescence* and *Streptomyces sps* could able to express ALKB gene. Hence those three bacteria may produce the key enzyme alkane monoxygenase, which is a crucial enzyme for the biotransformation of many xenobiotic compounds including LDPE.

KEYWORDS: Low density poly ethylene, PCR, ALKB gene.

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1. INTRODUCTION

The recalcitrance of several synthetic chemicals for biodegradation is mostly due to absence of enzymes that can carry out crucial steps in a catabolic pathway. This concept can be particularly adapted for low-molecular weight halogenated complexes. These xenobiotic chemicals are usually water soluble and bioavailable, and hypothetically could be transformed by short metabolic paths to

intermediates that would serve as a nutrient source for cellular growth under aerobic conditions. But the potential candidate microbes which could utilize chloroform, trichloroethylene, 1,1,1-trichloroethane, 1,2-dichloropropane and 1,2,3-trichloropropane are yet be explored that oxidatively transform and use as a carbon source. Efforts to find improvements or pure cultures that aerobically grow on these chemicals have encountered no accomplishment. Though, some other halogenated chemicals are simply biodegradable, and cultures that use chloroacetate, 2-chloropropionate and 1-chlorobutane can be freely augmented from nearly any soil sample [1, 2]. For still other compounds, degradative organisms can be isolated, but only after prolonged adaptation or if a suitable inoculum is used in which the catabolic activity that is searched for apparently is already enriched due to pre-exposure to halogenated chemicals in the environment. Compounds of this class of intermediate degradability include dichloromethane, 1,2- dichloroethane and the nematocides 1,2-dibromoethane and 1,3-dichloropropene. Plastic is the most useful synthetic 'manmade' substance, made up of elements extracted from the fossil fuel resources. It has made possible most of the industrial and technological revolutions of the 19th and 20th centuries. During the past 30 years plastic materials such as polyethylene (LDPE, MDPE, HDPE, LLDPE), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), polyurethane (PUR), Polybutylene terephthalate (PBT), nylons have been used widely in food, clothing, shelter, transportation, construction, medical and leisure industries because they are lightweight, low cost, extremely durable and relatively unbreakable [3]. Environmental pollution by synthetic polymers, such as waste plastics and water-soluble synthetic polymers in waste-water has been recognized as a major problem. In view of this, energetic, chemical and biological polymer-degrading techniques have been studied extensively during the last three decades. The energetic agencies can be either thermal or radiant. The radiant energy may be high-energy radiation like gamma rays, ion beams, and electrons or even low energy radiation like ultra-violet (UV) rays. Chemical degradation is caused using certain chemicals like acids and alkalis, etc. Usage of certain microorganisms and enzymes to degrade polymers are classified as the biodegradation method of polymers [4]. The microbial species are associated with the degrading materials. Microbial degradation of plastics is caused by certain enzymatic activities that lead to a chain cleavage of the polymer into oligomers and monomers. These water soluble enzymatically cleaved products are further absorbed by the microbial cells and then are metabolized. Aerobic metabolism results in carbon dioxide and water [5] and anaerobic metabolism results in the production of carbon dioxide, water and methane. Plastic that has been enzymatically broken down further absorbed by the microbial cells to be metabolized [6]. The variety of alkB-related alkane hydroxylase sequences and the association between alkB gene expression and the hydrocarbon pollution level had been studied in the chronically polluted Etang-de-Berre sediments [7]. For this determination, these deposits were preserved in microcosms and introduced in to a precise oil input miming an oil spill. New degenerated PCR primers targeting alkB-related alkane hydroxylase

sequences were designed to explore the diversity and the expression of these genes using terminal restriction fragment length polymorphism fingerprinting and gene library investigations. Induction of alkB genes was detected immediately after oil addition and their expression detected only during 2 days, although the n-alkane degradation was observed throughout the 14 days of incubation. The alkB gene expression within triplicate microcosms was heterogeneous probably due to the low level of alkB transcripts. Moreover, the alkB gene expression of dominant OTUs has been observed in unoiled microcosms indicating that the expression of this gene cannot be directly related to the oil contamination. Although the dominant alkB genes and transcripts detected were closely related to the alkB of Marino bacteraquaeolei isolated from an oil-producing well, and to alkB genes related to the obligate alkanotroph *Alcanivorax borkumensis*. No clear relationship between the oil contamination and the expression of the alkB genes could be established. This finding suggests that in such coastal environments, alkB gene expression is not a function relevant enough to monitor bacterial response to oil contamination.

2. MATERIALS AND METHODS

Sources and Collection of Municipal Solid Waste

The municipal solid wastes(MSW) sample used in this study were aseptically collected from five different dumpsites located within the Thanjavur municipality; and these dumpsites were designated as dump sites Sample-A (Srivivasapuram South), Sample-B (Srivivasapuram North) Sample-C (Srivivasapuram East) Sample-D (Srivivasapuram West) and Sample-E (Near Tamil Universty campus). During the collection of samples from the respective dumpsites, the surface debris at each sampling points were carefully removed with a sterilized trowel and the subsurface scooped at a depth of 10 cm with another sterilized sampling scooper. The waste samples were transferred into sterile conical flasks, labelled and transported to the Microbiology Laboratory for analysis.

Estimation of densities of heterotrophic bacteria in the MSW

The isolation of bacteria from the MSW was carried out by pour plate method. 10 g of MSW sample was placed into a sterile saline in a conical flask, and serial dilution was carried out by pipetting out 10ml into sterile 100 ml of the diluents and the procedure was continued up to 10^6. One ml of diluted samples were taken from different dilutions and inoculated into a sterile Petri dish labeled appropriately, and 20 ml of sterile nutrient agar was then poured and the plates were swirled back and forth and allowed to solidify. It was then incubated at 30^{o}C for 2 days.

Identification of Bacteria isolated from MSW

The isolated bacteria were identified based on their cultural and morphological characteristics and biochemical methods. The unknown bacteria isolates were identified based on standard microbiological identification techniques as per Bergy'smanual.

Determination of LDPE utilization potential of the microbial isolates

The ability of the microorganisms isolated from MSW to effectively utilize the LDPE as their source of carbon source was determined on mineral salt medium (MSM) using the method of Okpokwasili and Okorie [8]. Mineral salt agar media was prepared and sterile disintegrated LDPE were mixed in the molten state of the media. The bacterial isolates to be screened for plastic utilizing ability was spot inoculated and incubated at 30^{o}C for 7 days.

Isolation of Chromosomal DNA

The bacterial culture was grown at 37^{o}C for 24 hours in LB broth and 10ml of culture was taken and centrifuged at 5000 rpm for 10 minutes. 1mL extraction buffer was added. 1ml of lysozyme solution was added to the above suspension and incubated at 37^{o}C for 30 minutes with intermittent stirring. After the incubation, the lysis was completed by adding 2mL of 25% SDS solution. The preparation was heated for 10 minutes at 60^{o}C in a water bath and finally cooled down to room temperature. 5M perchloride was sufficiently added to the lysed preparation to the final concentration of 1M.Equal volume of chloroform: isoamyl alcohol was added to the lysed preparation suspended in 1M percholride and shaken slowly (30-60 oscillations/min) in a highly stoppered flask for 30 minutes at room temperature. The resulting emulsion was separated by centrifuging for 5 min at 10000 rpm at room temperature. After centrifugation, the top clear aqueous phase was carefully pipetted out from the coagulated protein emulsion, at the inner phase. The aqueous phase containing the nucleic acid was placed in the beaker. The nucleic acid solution was gently stirred with a sterilized glass rod and 2 volumes of 95% ethanol was added slowly down the side of the beaker. So that the ethanol was layered over the viscous aqueous phase. The stirring was continued up to the preparation to mix throughout the phase and spool all of the gelatinous thread like DNA rich precipitate on the glass rod. The excess fluid was drained off from the spooled crude DNA by pressing the rod against the wall of the beaker until no further fluid can be squeezed from the spooled preparation if the squeezing was not done sufficiently, the alcohol adhering the crude DNA will make it difficult to dissolve the DNA. The crude DNA was dissolved in 9ml of diluted saline citrate. To the even suspension,1mL of 3M acetate and 1M EDTA were added and transferred the preparation to 100ml beaker containing 5.4ml of isopropanol. For the storage of DNA, the crude DNA was dissolved in 9ml of saline citrate and stored at 2^{o}C with few drops of chloroform. The isolated DNA was further analyzed by Nanovue- spectrophotometry for purity and gel electrophoresis for integrity.

PCR

Gene expression study to determine alkB gene was carried out. 38 µl of sterile triple distilled water was added to a sterile microfuge tube. Then 5 µl of 10X Taq polymerase assay buffer with $MgCl_2$ were added. .3 µl of 2.5 mm dNTP mixed solution and 1 µl of control template DNA was added. Followed by that, 1 µl of each of forward and reverse primers were added (Table No.1). The reaction

mixture was layered with 50 µl of mineral oil to avoid evaporation. The amplification was carried out using the following reaction conditions.

1. Initial denaturation at 94^0C for 1 min
2. Denaturation at 94^0C for 30 seconds
3. Annealing at 48^0C for 30 seconds.
4. Extension at 72^0C for 1 minute
5. Final extension at 72^0C for 2 minutes.

3. RESULTS AND DISCUSSION

In our present study isolation of effective polythene waste resistant bacteria from municipal solid waste were carried out. Morphologically distinct isolates were further used to determine its ability to use low grade plastic as carbon source. Among the different isolates, only three isolates could able to grow in a media containing powdered LDPE. Those bacteria were identified as *Pseudomonas putida*, *Pseudomonas fluorescence* and *Streptomyces sps.* Exhausted investigation on the degradation of polyethylene has been conducted in order to astounded environmental complications connected with synthetic plastic waste. Current work has comprises of the isolation of most competent microorganism using diverse soil samples taken from five waste disposal sites Fig No.1). In the previous study, numerous microorganisms were isolated from the soil samples, grown in an inorganic media (M9 media). There are some microbes that have the ability to degrade plastic waste up to 51.5%. This result was accomplished due to addition of starch as additive in M9 media. This study reports that Pseudomonas sp. are having greater potential to degrade polyethylene.

A. South B. North

C. East D. West

Figure No 1: CRC Municipal Dumping Area Srinivasanpuram (A, B, C &D)

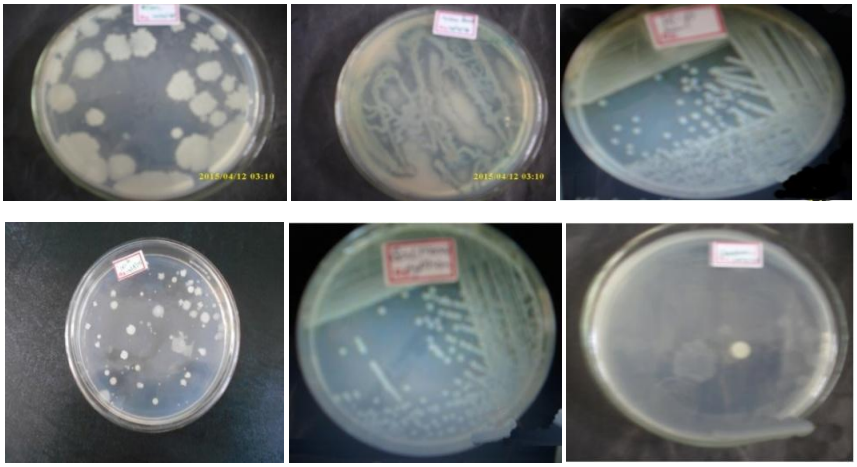

Figure No 2: Plate culture

Figure No: 2. Depicts the pure cultures of isolated colonies from the municipal solid waste. Which was further subjected to staining methods, cultural characterization and biochemical examinations and the unknown bacteria were identified as *Bacillus amylolyticus, Bacillus firmus, Pseudomonas putida*, *Pseudomonas fluorescence*, *Bacillus subtilis* and *Streptomyces sps.*

PCR

ALKB gene specific primer synthesized by Biosource Ltd, Bangalore was used to determine the alkane monooxygenase gene and the genomic DNA from the selected isolates were used as a template and amplified by PCR and result is depicted in figure No: 3. The product size of the alkB gene is 330bp and which was determined by gel electrophoresis.

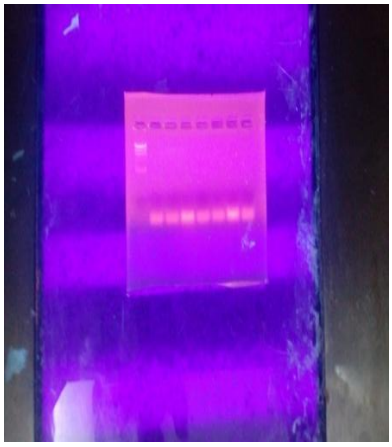

Figure No 3: PCR Amplified product of ALKB gene

Lane 1 - 500bp ladder

Lane 2 & 3 – Genomic DNA of *Pseudomonas putida,*

Lane 4 & 5 – *Pseudomonas fluorescence,*

Lane 6, 7 & 8 - *Streptomyces sps.*

Kloos *et al.* [10] implemented the PCR technique for the determination of total DNA of the soil sample at Bavaria, Germany. They acquired 58 PCR products whose bands seemed at 550 ± 50 bp. The hybridization of the PCR products produced 42 alkB clones attached with the alkB complementary probe. Examination of the alkB homology by means of the cDNA library cloning exposed that the genetic consistency between the 42 alkB clones was in the range of 57~90 %. On the other hand, the PCR for the total DNA of the agricultural soil samples were identified 21 alkB genes among which the 2 alkB genes displayed genetic change less than 1% and the 19 alkB genes showed the genetic homogeny in the variance of 50~90%. Heiss-Blanquet *et al.* [11] determined the amount of the gene in the soil testers to measure the ecological significance of the bacteria for the biodegradation of alkanes of short-chain size by means of the alkB. They observed that the alkB be influenced by the environmental and ecological conditions and that the amount of the alkB was higher in the soil polluted with hydrocarbons. Very nearly all the genes identified as alkB existed in *P. aeruginosa, P. putida* and *P. mendocina.* Saadoun *et al*. [12] identified microbes from an oil polluted soil and investigated their growth using diesel oil as a sole carbon source. They determined the presence of the alkB through the PCR analyses. Two groups of the bands appeared at 316~334 bp and 460~550 bp, correspondingly, on the agarose gel, and the alkB genes showed bands between 320 and 550 bp. Smits *et al.* [13] observed that a single alkB precise primer cannot notice all the alkane degrading microbes due to the microbial species-specificity in the discovery of alkane degraders through the PCR method. The alkB was found to be completely different dependent on the microbes due to the great dissimilarity in the alkB sequence. Because of these reasons, Kohno *et al.* [14] equipped 3 different PCR primers and amplified them to put on for the discovery of the alkane hydroxylase gene in the examination of the alkane degrading microbes. They categorized the

discovered microorganisms into 3 groups: Group I with 185 bp was encompassed of *Acinetobacter, Pseudomonas* and *Corynebacterium* spp. Categorized to Group II with 271 bp and Group III with 330 bp belonged to *Pseudomonas* and *Rhodococcus* spp. Our present investigation has evidenced the same. Polyethylene is the maximum exploited preparation of synthetic polymer and extremely hydrophobic in nature [15]. In the case of polyethylene, the biodegradability is opposite to its molecular weight. Less than 620 molecular weight hydrocarbon oligomers good turn the development of microbes; high molecular weight polyethylene is resilient to biodegradation. Its hydrophobic nature hampers its bioavailability. Certain physical and chemical management strategies used beforehand biodegradation intensify its effectiveness. Such treatments include UV irradiation, photo-oxidation, thermal treatment and oxidation with nitric acid. Oxidation of polyethylene increases the superficial hydrophilicity, which eventually upsurges the biodegradation [16]. Chemical treatments (0.5 M HNO3 and 0.5 M NaOH) of polyethylene speed up the biodegradation by *Pseudomonas* sp. [17]. Polyethylenes and polystyrenes with ether bond are vulnerable to monooxygenases dose [18]. Biodegradation of polyethylene was improved by the physical dealings reasons pre-ageing via light or heat acquaintance. A hot air oven was used for physical oxidation and formed a molecular weight delivery at 60 °C monitored by nurtured with polymer degrading microbes. Genera *Gordonia* and *Nocardia* are accompanying with the biodegradation procedure [19]. Certain categories are also described for polyethene degradation (*Bacillus, Lysinibacillus, Pseudomonas, Staphylococcus, Streptococcus, Micrococcus, Streptomyces, Rhodococcus, Proteus, Listeria, Vibrio, Bravibacillus, Serratia, Nocardia, Diplococcus, Moraxella, Penicillium, Arthrobacter, Aspergillus, Phanerochaete, Chaetomium and Gliocladium*) [20, 21, 22, 23, 24]. Polyethylene is employed as a carbon source by microbes, and biofilm development on it indications their efficiency. Biofilm establishment enhanced with the accumulation of mineral oil (0.05%) to the medium. Nonionic surfactants can encourage the polymer biodegradation by aggregate the hydrophilicity of the polymer, which supports in the linkage of microbes on the polymer. Polyethylene can be ruined by hydro- or oxo-biodegradation. The biodegradation technique is determined by upon the constituents used in the development. Fungi (*Mucorrouxii* NRRL 1835, *A. flavus*) and strains of *Streptomyces* are also intricate in starch-based polyethylene degradation. Degraded polymers are explicated by superficial breakages, link rubbing and additional fluctuations like color, etc., and such modifications observed by scanning electron microscopy and FT-IR. Degraded polymer demonstrations conformational changes in its texture could support the extent of the microbial inhabitants [25]. Microorganisms take part in degradation via modification of their metabolic functional pathways according to environmental conditions to utilize xenobiotic compounds. A bioremediation process is more affordable by discovering novel catabolic mechanisms [25]. The biodegradation process is slow but this does not indicate that ingredients in plastic material and polymers are not bioactive.

Polycarbonate plastics undergo leaching of bioactive bisphenol-A monomer when undergoing salt exposure in seawater. Commercial use of several synthetic polymers made with bioactive additives monomers, which are non-stick compounds; softeners and UV stabilizers are found in nature. Their degradation rate depends on the environmental circumstances. Symphony is a type of polymeric material that is used in polyethylene formation and degradable in nature [26, 27]. Low molecular weight polyethylene degrading bacteria was explored and identified *Pseudomonas* sp. E4. They found 28.6, 14.9, 10.3 and 4.9% carbon mineralized from different molecular weight (1700, 9700, 16,900, 23,700) polyethylene samples within 80 days at 37 °C that evolved CO2 [28]. The alkB gene encoded the enzyme alkane hydroxylase, and Yoon *et al.* [28] cloned the alkB from *Pseudomonas* sp. E4 to E. coli strain BL21 and found their carbon mineralization potential as 19.3% in 80 days at 37 °C. [29] concluded that alkB gene played a key role in polyethylene degrading. Bacterial originated copper-binding laccase from R. ruber for enzymatic degradation of polythene was identified [29]. Nowadays different groups of microorganisms are reported for biofilm formation. Similarly, [30] studied *Pseudomonas* sp. AKS2 for biofilm formation and reported LDPE degradation. They found enhanced microbial growth with 26% surface hydrophobicity and 31% hydrolytic activity. The surface malformations of plastic was detected when preserved with *S. marcescens*; and an additional deprivation was assessed by the decrease in glass change temperature (Tg) and decrease in crystallinity via DSC examination. Likewise a marine bacterial strain was identified as *Achromobacter denitrificans* S1 for LDPE degradation that was determined by NMR, XRD, TGA and GCMS examination [31]. They also examined a model of metropolitan solid soil for delivery of polymer degrading microbes and secluded *Bacillus amyloliquefaciens* BSM-1 and *B. amyloliquefaciens* BSM-2. [31] found improved biodegradation with strain *B. amyloliquefaciens* BSM-2 in appraisal to *B. amyloliquefaciens* BSM-1. The rate and competence of polymer degradation were unwavering by pH modification in media, CO2 evolution, weight loss, SEM and FT-IR examination. Likewise, [32] inaccessible fungus from landfill soil was recognized as *A. clavatus* (strain JASK1). These findings were paving a way and strengthening biological means of solving the problems associated with polyethylene.

Table No: 1 Primer Used for this Present study

AlK primer	Sequence	GC content (%)	PCR product (bp)	Reference [9]
Forward	5`-TCGAGCACATCC-GCGGCCACCA-3`	68	330	
Reverse	5`-CCGTAGTGCTC GACGTAGTT-3`	55		

4. CONCLUSION

Mesophilic bacteria for the LDPE biodegradation was isolated from a municipal dump solid wastes soil sample and were used for their ability to utilize LDPE as carbon source. They were identified as *Pseudomonas putida, Pseudomonas fluroscence, & Streptomyces sps.* through phenotypic methods. The isolated strains mineralized LDPE whose molecular weight was in the range of 1,700~23,700 with considerable action at 37˚C in the dung under the organized circumstances. Though, the biodegradation action reduced with upsurge in the molecular weight of LDPE. Presence of alkB gene in the selected strains were determined to rule out its efficacy to be explored as potential microbe to be employed for biodegradation of LDPE and *Pseudomonas putida, Pseudomonas fluroscence, & Streptomyces sps.* showed the presence of alkB gene.

CONFLICT OF INTEREST

Authors have no any conflict of interest.

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