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BIODEGRDATION OF PLASTIC (LDPE) BY BACTERIA FROM GARBAGE SOIL

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ABSTRACT

Plastic and polythene waste accumulating in the environment due to their high molecular weight, hydrophobic nature, high C-C crosslink bonding, make it non-biodegradable and harmful to environment and living organisms. In the present study, 19 bacteria were isolated from dumped garbage soil on nutrient agar at 37˚C, after 24h.Among nineteen, eight bacterial isolates were efficiently hydrolyzed hydrocarbons (Starch, gelatin) evaluated by zone of hydrolysis (8 to 29 mm of starch, 14 to 38mm of gelatin). Tween 80, Petroleum ether, Naphthalene powder are proficiently utilized by I8, I10 and I13 bacteria and consortium for their growth and it's confirmed their ability to LDPE biodegradation. Bacterial isolates were identified as *Bacillus subtilis, Bacillus amyloliquefaciens,* and *Bacillus licheniformis* as per Bergey's Mannual, ABIS software and 16s r RNA sequencing. Biodegradation of LDPE 81±3.2% weight loss by *Bacillus licheniformis,* 76.77±1.2% by *Bacillus subtilis* ,76.88±2.6% by consortium and lowest weight loss 16.18±2.9% was by *Bacillus amyloliquefaciens* in without glucose minimal media. LDPE utilization (biodegradation) for growth by bacteria was also analyzed by colorimetric method. Biodegradation of LDPE was confirmed by FTIR analysis and GC-MS. Hence in present work, LDPE sheets was biodegraded by isolates eco-friendly, helps in reducing environment pollution.

Keywords: LDPE, FTIR, Biodegradation, Hydrocarbons

INTRODUCTION

The word plastic comes from the Greek word "plastikos" which means able in molded into varied shapes. Plastic is a linear hydrocarbon polymers consisting of long chain of ethylene monomers **[1]**. Plastics are strong, durable, moisture resistant, light weight polymers of carbon along with hydrogen, nitrogen, sulphur, organic and inorganic elements and are manufactured from fossil fuel which is a non-renewable sources **[2]**. Most common forms of plastics which are used in industries are polyethylene (LDPE, MDPE, HDPE, LLDPE), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), polybutyrenetetraphthalate (PBT) **[3, 4]**. They do not break down in the environment easily because they are resistant to microbial attack, due to their excessive molecular mass, high number of aromatic rings, unusual bonds, or halogen substitutions. As a result they remain in the environment for a very long time without any deterioration and the large-scale accumulation of waste plastics in the biosphere has given rise to the problem of severe environmental pollution **[2]**.

Polythene is resistant to biodegradation due to its high molecular weight, three dimensional structures and its hydrophobicity that interferes with polythene availability to soil microorganisms. Physical pre-treatment such as weathering, UV irradiation and thermal treatment was employed to raise the hydrophilicity of polyethylene by introducing polar groups such as carbonyl groups to the polyethylene backbone chain and cause mechanical damage to the polymer thus facilitates the microbes to metabolize the plastic **[5, 6]**. Microorganisms can degrade plastics over 90 genera from bacteria, fungi, among them *Bacillus megaterium, Pseudomonas* sp*, Azotobacter* sp*, Ralstoniaeutropha, Halomonas* sp etc. *Brevibacillus borstelensis18, Rhodococcus ruber*19, 20*,* as well as fungi (heterotrophic microorganisms) such as *Penicillium simplicissimum* YK21, 22*, Fusarium solani*23 are reported to be used in degradation of both natural and synthetic polyethylene as its potential carbon substrate. Microbes cause cleavage of the polymer chain using certain enzymes and convert them into monomers and oligomers. The diverse metabolic capability of microbes can be exploited for bioremediation of plastic wastes that uses microbial strain developed through selection, strain improvement and genetic modifications **[7, 4]**. One method to overcome environmental issues from plastic wastes is to use biological agent like bacteria, where the bacteria will breaking polymer

bond from plastic into monomer chain and create environmental-friendly plastic **[8]**. Biodegradation of plastics involves excretion of extracellular enzymes by the microorganism, attachment of enzyme to the surface of plastic, hydrolysis to short polymer intermediates, which are ultimately assimilated by microbial cells as carbon source to release CO_2 **[9]**. The attractive part of biodegradation is they do not produce secondary pollutants associated with incineration and landfills **[5]**. The oxidation or hydrolysis by enzyme to create functional groups that improves the hydrophilicity of polymer is the primary mechanism for the biodegradation of high molecular weight polymer. The degradation of most synthetic plastics in nature is a very slow process that involves environmental factors, which follows the action of wild microorganism. Huge amount of polyethylene getting accumulated in the environment and their disposal evokes a big ecological issue. To the marine life polythene waste is recognized as a major threat. Sometimes, it could cause intestinal blockage in the fishes, birds and marine mammals. Due to plastic pollution in the marine environment minimum 267 species are being affected which includes all mammals, sea turtles (86%) and seabirds (44%).The death of terrestrial animals such as cow was reported due to consumption of polythene carry bags **[3]**. Polyethylene cannot be broken down easily in an environment, their accumulation in a land affects the water percolation and fertility of the soil **[5].** The disposal of these used plastic materials by using chemical and physical methods are very expensive and produces persistent organic pollutants (POP's) known as furans and dioxins, which are reported to be toxic irritant products, resulting into infertility of soil, preventing degradation of the other normal substances, depletion of the underground water source and have proved to be dangerous to animal, human and eco-system **[7].** The purpose of our present study is to isolate plastic degrading Bacteria and check its efficiency to degrade plastic eco-friendly.

MATERIAL AND METHODS

Garbage soil samples collection:

The garbage soil samples were collected in zip lock bags from different dumping places of Naregaon, Mukund Nagar of Aurangabad (Maharashtra, India).

Collection of LDPE (plastic bags) and its surface sterilization:

Plastic bags (LDPE) were collected from local shops and household and cut in to small strips then further proceed for surface sterilization by 70% (v/v) ethanol for 30

minutes as method described by **[1]**, after that the plastic strips were dried and weighed (Initial weight).

Isolation and identification of plastic degrading Bacteria:

From five different garbage soil samples, plastic degrading bacteria were isolated by serial dilution method. Aliquots (100µl) of 10^{-7} sample was spread on NA plates and incubated at 37˚C for 24 hours. After incubation bacterial colonies with different color, shape and size are purified on nutrient agar slant and further proceed to evaluate its efficiency to degrade hydrocarbon and then plastic (LDPE) **[7]**. Identification of efficient hydrocarbon and plastic utilizing bacterial isolates was done as per Bergey's manual of systemic bacteriology ,ABIS software and most efficient one was identified by 16s r RNA sequencing .

Efficiency to degrade hydrocarbons

Active culture of bacterial isolates was inoculated on starch (1%) and gelatin (1%) agar plates and incubated at 37°C for 24 h., The bacteria which forms large zone of clearance were chosen for further studies **[10]**. Starch, gelatin hydrocarbon hydrolyzing bacteria was further subjected to various hydrocarbon sources such as 1% tween 80, naphthalene powder, petroleum ether in minimal media at 37°C for 2 to 3 weeks, after incubation growth of these isolates was evaluated at 600nm, the growth of bacterial isolates depends on utilization of these hydrocarbons as source of carbon this indicates that can efficiently degrade (utilize) plastic.

Biodegradation of Plastic by various methods

Weight loss method:

In weight loss method, Dry weight of plastic strips (2×2 cm) (LDPE) were measured (Initial weight) using the weighing balance then added in sterile minimal media and inoculated by 0.5 McFarland concentration of bacterial isolates as *Bacillus subtilis* (I_8) *Bacillus amyloliquefaciens* (I_{10})and *Bacillus licheniformis*(I_{13}) and incubated at 37˚C for three months. After incubation the inoculated plastic strips (2×2 cm) (LDPE) were taken, and washed with sterile distilled water then the plastic strips were dried and weighed for final weight. Experiment carried out in triplicates **[11]**.

Percentage of weight loss of plastic (LDPE) is calculated using below formula.

$$\% \text{ of weight loss} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100$$

Colorimetric method:

Evaluation of plastic utilization by *Bacillus subtilis* (I_8), *Bacillus amyloliquefaciens* (I_{10}), *Bacillus licheniformis (*I_{13})as a carbon source is in the form of growth in indicator minimal

media with and without glucose (Bromothymol Blue) by Colorimetric method. In triplicates sets of sterile indicator minimal media flask plastic strips (2×2 cm) (LDPE) were added and then inoculated by 0.5 McFarland concentration of *Bacillus subtilis* (I_8)*, Bacillus amyloliquefaciens* (I_{10} *), Bacillus licheniformis*(I_{13}), bacterial consortium incubated at 37°C for three months. After incubation check its efficiency is in the form of growth i.e. absorption at 600nm every after 5 days of incubation up to one month then after interval of one month till three months. After incubation observe the change in media color of both test and abiotic control, confirmed color change by absorption **[11]**.

Characterization of biodegraded plastic:

Biodegradation of LDPE strips was Characterized by FTIR (Chemistry Dept., BAMU University) and GC-MS analysis (Wockhardt R & D, Aurangabad) and that is compared with control LDPE. In FTIR, for each LDPE strips, a spectrum was taken from 500 to 4000 wave numbers. LDPE biodegradation is shown by the structural changes in FTIR spectra **[6]**.

RESULT AND DISCUSSION

Collection of Garbage soil sample:

From Naregaon, Mukundnagar dumping areas of Aurangabad (**Figure 1**), the garbage soil samples were collected in zip lock bags and proceed for isolation of plastic degrading bacteria.

Plastic bags (LDPE) surface sterilization:

Before biodegradation, LDPE sheets cut into small strips (2×2cm) **(Figure 2)** and their Surface was sterilized by 70% (v/v) ethanol, initial weight was taken and then proceed for biodegradation by bacterial isolates.

Isolation and identification of plastic degrading Bacteria:

In our present study 19 bacteria were isolated from garbage soil samples from that 3 bacterial isolates **(Figure 3)** showed proficient LDPE degradation and identified as *Bacillus subtilis, Bacillus amyloliquefaciens,* as per Bergey's manual of systemic bacteriology, ABIS software and most efficient I13 was identified as *Bacillus licheniformis* by 16s r RNA sequencing **[12]**. identified *A. niger,A. flavus, Pseudomonas* sp. as a polyethylene degrading microbes in their research work. *E.coli, Staphylococcus* sp., *Pseudomonas* sp.*, Klebsiella* sp. and *Bacillus s*p. will degraded Low Density Polythene was referred by **[5]**. The bacterial isolate 1 is identified as *streptococcus* sp. and isolate 2 is identified as *pseudomonas* sp. and the isolate 3 is identified as *Bacillus* sp. Reported by **[10]**.

Efficiency to degrade hydrocarbons:

Among isolates, *Bacillus subtilis* (I8), *Bacillus amyloliquefaciens (*I10*), Bacillus licheniformis* (I13) were shows highest zone of starch and Gelatin hydrolysis **(Table 1 and Figure 4)** while *Streptococcus sp., Pseudomonas sp.,* and *Bacillus sp,*were reported starch and gelatin hydrolysis **[10]**. These isolates further proceed for secondary screening to evaluate their efficiency for utilization of 1% tween 80, naphthalene powder, and petroleum ether in minimal media after incubation period of 2 to 3 weeks, from three isolates I_{13} shows highest utilization of various hydrocarbons **(Figure 5)** at 600nm. Efficiency of hydrocarbon utilization by these isolates was evaluated is in the form of color change and absorption. Higher the absorption higher the growth, due to utilization of hydrocarbons as source of carbon, this indicates that these isolates have ability to degrade (utilize) LDPE.

Biodegradation of Plastic (LDPE) by various methods:

Weight loss method:

After 3 Months of incubation, LDPE Strips shows morphological changes like color, texture, transparency **(Figure 8)** and its biodegradation was confirmed by weight loss. In absence of microbes i.e. in abiotic control, plastic (LDPE) does not shows weight loss while biodegradation of LDPE 81±3.2%weight loss by *Bacillus licheniformis*76.77±1.2%by *Bacillus subtilis* (I8), consortium 76.88±2.6%and *lowest* weight loss16.18±2.9% was by *Bacillus amyloliquefaciens* in without glucose minimal media .LDPE sheets biodegradation in medium without glucose was found to be more as compared to glucose medium. The percentage of weight loss by *Staphylococcus aureus* and *Bacillus cereus* in minimal medium was 57.3% and 26% respectively reported by **[5]** and **[11]** states that the highest degradation capability is by PDB-1 which is 3.17% at day 5 and by PDB-14 which is 3.47% at day 10 after inoculation. However, the lowest degradation capability is reported by PDB-14 at day 5 which is 0.51% and PDB-11 at day 10 which is 1.62%. Weight loss percentage. Our experimental outputs indicates that the weight loss of plastic (LDPE) during the incubation with respective isolates it is due to utilization of plastic (LDPE) as the sole source of carbon, higher the utilization higher the plastic biodegradation. As compared to cited review our isolates most proficiently showed LDPE biodegradation.

Colorimetric method:

Bromothymol Blue indicator is used to check the pH changes in minimal media, initial color of media is green (alkaline) then changes to yellow (acid),decrease in pH due

to metabolic activity of bacteria , as metabolism is shown by bacterial cells may greatly support the evidence of plastic (LDPE) utilization (biodegradation).After incubation the change in media color in all test flasks was observed and confirmed by absorption at 600 nm as incubation increases (5^{th} day to months) absorption was also found to be increased due to utilization of LDPE by *Bacillus subtilis,Bacillus amyloliquefaciens,Bacillus licheniformis* for their growth, higher the utilization higher the plastic biodegradation. Among all the isolates, the highest pH was shown by PDB-12 in the media containing the highest amount of LDPE powder. All the isolates showed increasing pH except PDB-2 and PDB-11 studied by **[11]**.

Characterization of biodegraded plastic (FTIR and GC-MS):

Biodegradation of LDPE was confirmed by FTIR, analysis of the biodegraded LDPE (*Bacillus licheniformis*) spectral figures.as compare to control (non treated) indicate formation of new peaks at the region 1606.70cm^{-1}. The main bands of the studied LDPE sheets consist of, a band around 1461–1466 cm-1 revealing a bending deformation was observed in biodegraded plastic. The changes in bond scission, chemical transformation and formation and disappearance of new functional groups are the areas of interest that help us to determine whether any changes in the chemical structure of the LDPE has taken place and was determined with the help of FTIR **(Figure 11)** similar result reported by **[6, 7]**. As the rate of degradation increases with the passage of time, the peaks get broader as several monomeric and oxidative forms of the LDPE get produced.In bacterial treated LDPE GC-MS analysis, there was minor changes in peak molecules **(Figure 12)** which should be enhanced after some modification in present protocol, Hence it was confirmed that LDPE strips biodegraded by isolates.

Figure 1: Garbage soil samples

Figure 2: Sterilized plastic strips

Figure 3: Bacterial Isolates

Table 1: Zone of starch and Gelatin hydrolysis:

Bacterial isolates	Starch	Gelatin
I_1	-	27mm
I_2	10mm	34mm
I_3	13mm	21mm
I_4	8mm	28mm
I_5	-	17mm
I_6	7mm	-
I_7	-	14mm
I_8*Bacillus subtilis*	27mm	38mm
I_9	17mm	20mm
I_{10}*Bacillus amyloliquefaciens*	29mm	35mm
I_{11}	15mm	18mm
I_{12}	-	-
I_{13}*Bacillus licheniformis*	22mm	32mm
I_{14}	11mm	34mm
I_{15}	13mm	17mm
I_{16}	10mm	31mm
I_{17}	-	-
I_{18}	12mm	-
I_{19}	10mm	-

Figure 4: Zone of starch and Gelatin hydrolysis by bacteria

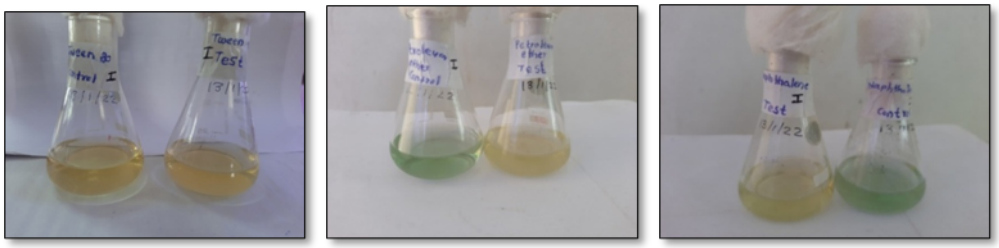


Figure 5: Efficiency of hydrocarbon utilization by *Bacillus licheniformis*

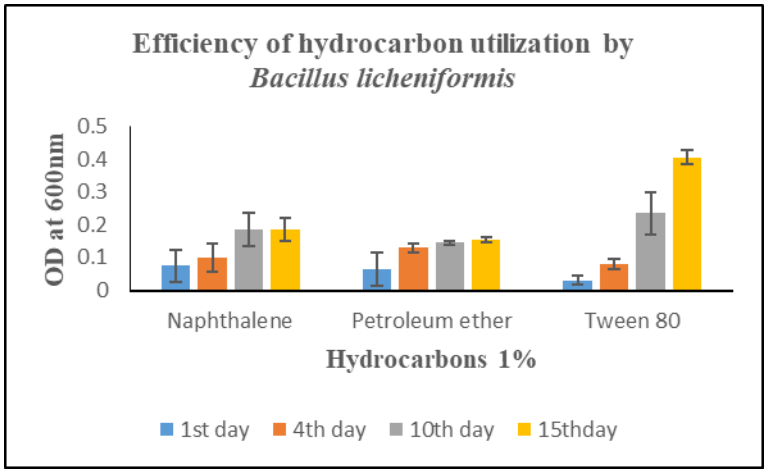


Figure 6: Efficiency of hydrocarbon utilization by *Bacillus licheniformis*

Table 2: Weight loss% of degraded plastic

Plastic (LDPE)	Initial weight				Final weight				% of weight loss			
	I_{10}	I_8	I_{13}	Mixture (Consortium	I_{10}	I_8	I_{13}	mixture	I_{10}	I_8	I_{13}	mixture
WO/G	0.005	0.006	0.005	0.005	0.0042	0.0014	0.0008	0.001	16	76.666	84	80
WO/G	0.0052	0.004	0.0040	0.005	0.0042	0.001	0.0009	0.0012	19.231	75	77.5	78
WO/G	0.015	0.0015	0.0153	0.015	0.013	0.0032	0.0028	0.0038	13.333	78.666	81.699	74.667
W/G	0.0153	0.015	0.0152	0.015	0.0145	0.0082	0.0065	0.0084	5.228	45.33	57.23	44
W/G	0.005	0.005	0.005	0.005	0.0046	0.0024	0.0025	0.0023	8	52	50	54
W/G	0.005	0.005	0.004	0.004	0.0045	0.0023	0.0019	0.002	10	54	52.5	50

Table 3: Weight loss% of degraded plastic by Bacterial Isolates:

Bacterial Isolates	WG	WOG
B.amyloliquefaciens	7.7±2.4	16.18±2.9
B. subtilis	50.44±4.5	76.77±1.8
Bacillus licheniformis	53.2±3.6	81.03±3.2
Consortium	49.33±5	76.88±2.7

W/G = with glucose, WO/G=without glucose

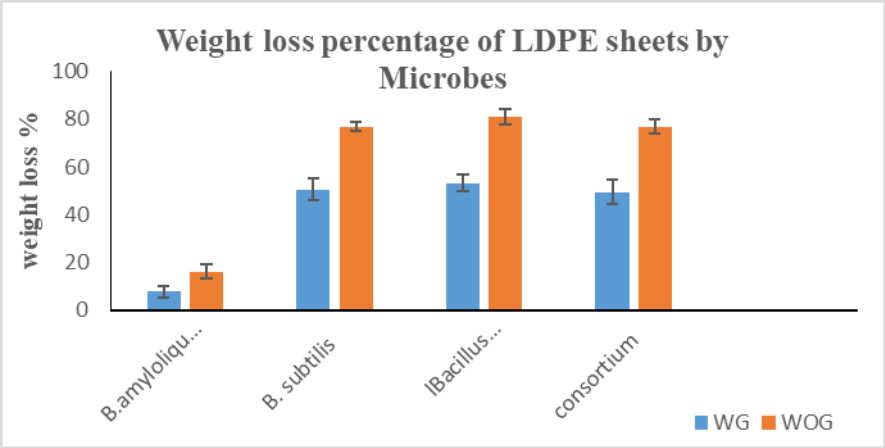
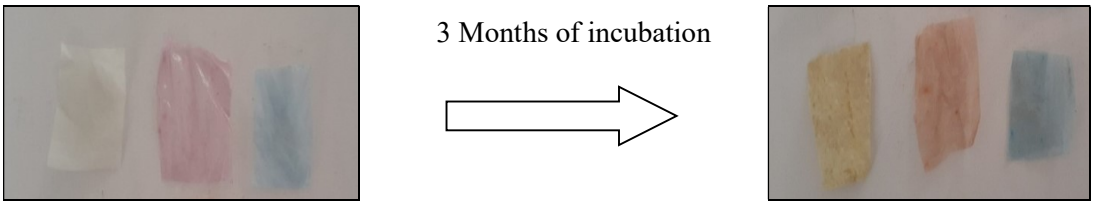
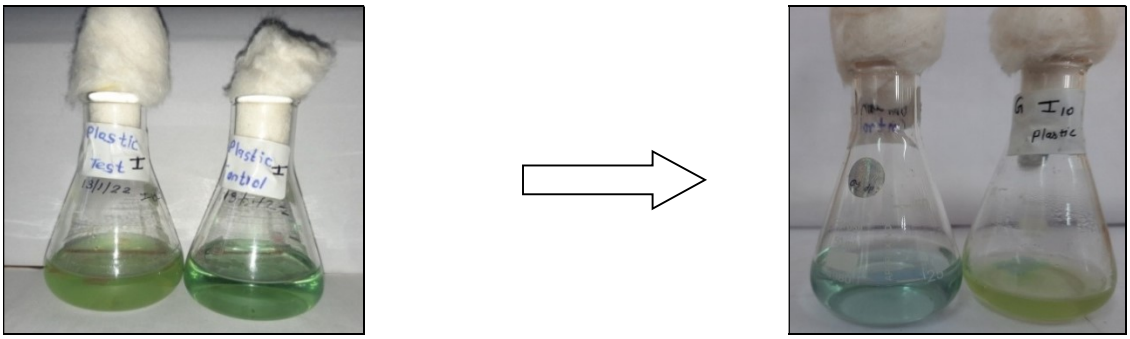


Figure 7: Weight loss% of degraded plastic

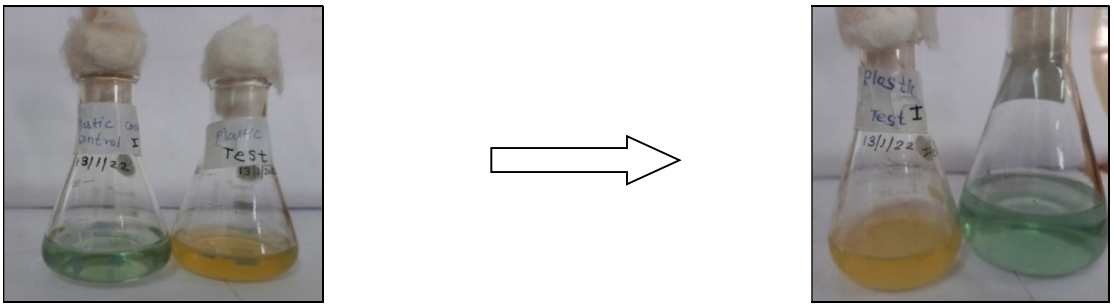


LDPE Strips (Before) **LDPE Strips (After)**

Figure 8: Biodegradation of LDPE strips



5 days of incubation 15 days of incubation



1 Month of incubation 2 Months of incubation

Figure 9: Biodegradation of LDPE by Isolates (Bacteria)

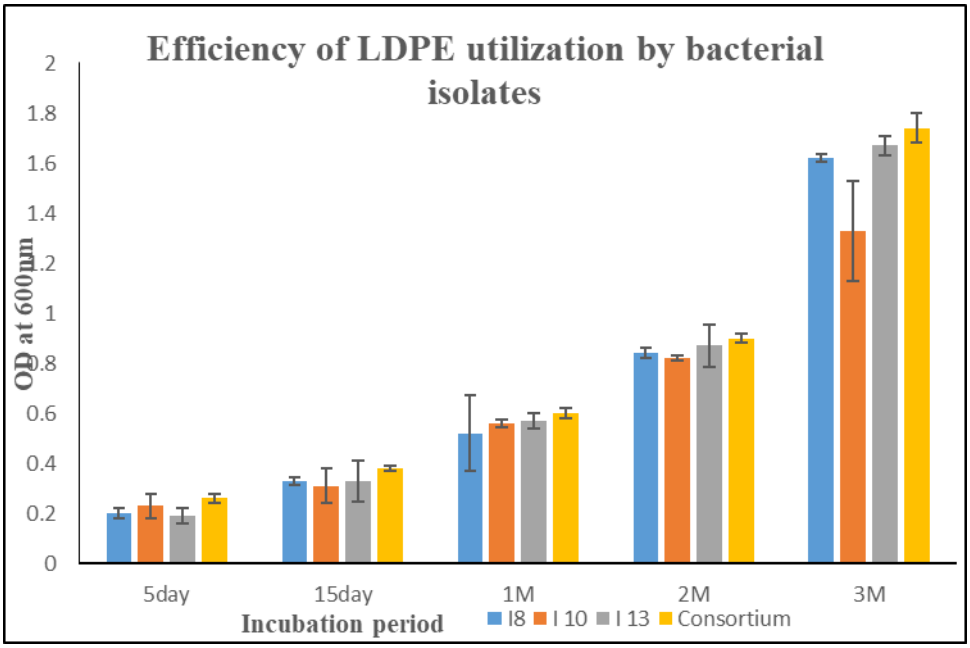


Figure 10: Efficiency of LDPE utilization by bacterial isolates

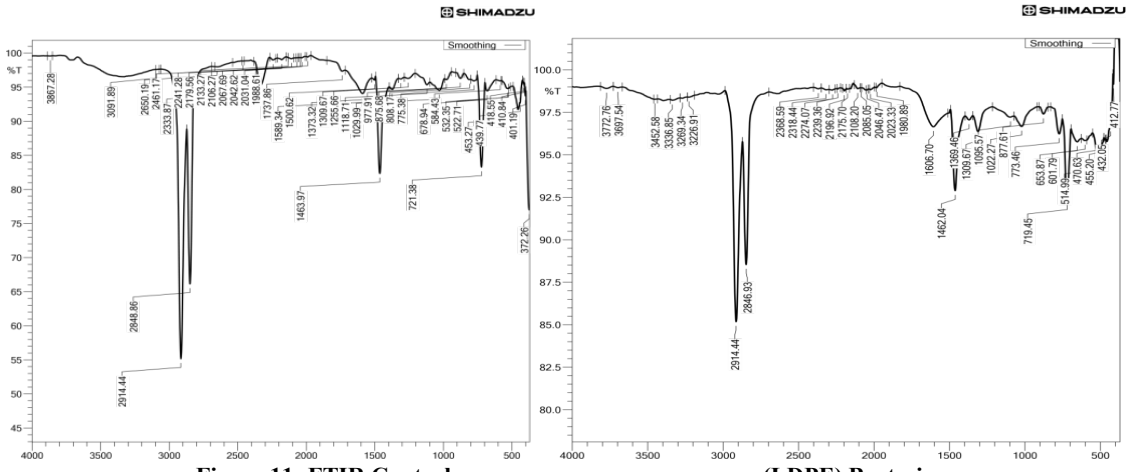
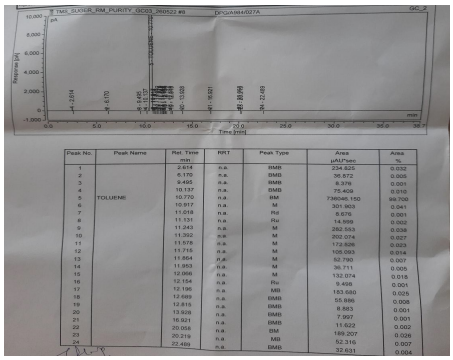


Figure 11: FTIR Control **(LDPE) Bacteria**



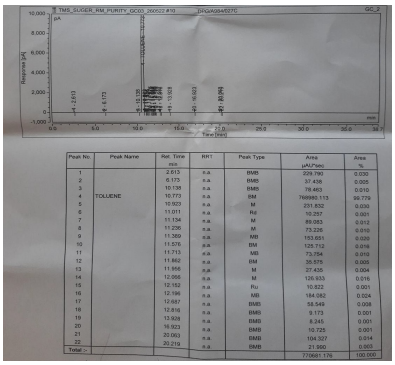


Figure12: GC-MS Control (LDPE) Bacteria

CONCLUSION

Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus licheniformis were found to be most efficient LDPE biodegrading microbes isolated from garbage soil samples. Biodegradation of LDPE 81±3.2%weight loss by *Bacillus licheniformis* 76.77±1.2% by *Bacillus subtilis*(I8), consortium 76.88±2.6%and *lowest* weight loss16.18±2.9% was by *Bacillus amyloliquefaciens* in without glucose minimal media. Approximately 20 % more LDPE sheets biodegradation in medium without glucose was found to be more as compared to glucose medium. LDPE utilization (biodegradation) for growth by microbes was also analyzed by colorimetric method. As incubation increases (5th day to months) absorption was also found to be increased due to utilization of LDPE by *Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus licheniformis* for their growth, higher the utilization higher the plastic biodegradation. Biodegradation of LDPE was confirmed by FTIR and GC-MS analysis. An experimental output indicates that LDPE (plastic) was biodegraded by microbes eco-friendly and help to reduced the environment pollution. Biodegradation of LDPE was by microbes but it is very slow process, so its today's need to enhanced that biodegradation process by composite protocol.

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