Screening of Efficient Polymer Degrading Bacteria and Influence of Various Pre-treatment on Degradation

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Submitted By

Surbhi Bhingradiya (15MMB004)

Prity Kumari (15MMB018)

Rohit Shastri (15MMB021)

Under the guidance of

Dr. Nasreen Munshi

INSTITUTE OF SCIENCE

NIRMA UNIVERSITY

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ABBREVIATIONS

- PET: Polyethylene Terephthalate
- PP: Polypropylene
- PS: Polystyrene
- HDPE: High Density Polyethylene
- LDPE: Low Density Polyethylene
- PVC: Polyvinyl Chloride
- PVA: Polyvinyl Acetate
- CLPP: Community Level Physiological Profiling
- PCA: Principle Component Analysis
- AWCD: Well Colour Development Data
- R: Richness
- C: Substrate
- S: No of carbon sources utilized by microbial community after incubtion
- H: Shannon-Weaver Index Value
- MSM: Minimum Salt Medium
- PCR: Polymerase Chain Reaction
- CV: Crystal Violet

- PDS: Plastic Dump Site Soil
- TAE: Tris acetate EDTA
- EtBr: Ethidium Bromide
- ANOVA: Analysis of Varience
- PC: Principal Component
- SEM: Scanning Electron Microscopy
- UTS: Universal Tensile Strength
- FT-IR: Fourier Transform Infrared

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INTRODUCTION

The word 'polymer' is Greek words: **poly** – "many" and **mer** – "unit or part". Many of the polymers are organic in nature and made from the hydrocarbon molecules. They are chemical compounds made up of repeating units of small molecules called monomers and this whole process is known as polymerization. Due to the chemical and the physical nature, polymers are resistant to the natural forces of degradation and has ensured longitivity and long-lived properties (Raghwan *et al.*, 2006).

Types of polymers

Synthetic or man-made polymers are also known as plastics. Plastics show a wide range of applications (Hopewell *et al.,* 2009) as they are lightweight, inexpensive, and durable materials, which can be molded into various products. Different types of polymers are different from one another on the basis of types of molecules used in their preparation and in the way they are joined together as described in table 1 (Billmeyer et al., 2007).

Table 1: classification of plastic materials based on molecular weight, monomeric structure, and their uses.

Name of compounds	Molecular weight (g/mol)	Chemical structure	Monomer structure	Uses
Polyethylene Terepthalate (PET)	192.2	$(C_{10}H_8O_4)_n$		Manufacturing of Beer bottles, Mineral water, some shampoo and fibre for clothes and carpets, etc.
Polypropylene (PP)	42	$(C_3H_6)_n$		Biscuit wrappers, yougert containers, drinking straw, bottle tops, ketchup and syrup bottles, etc.

Polystyrene (PS)	108	$(C_8H_8)_n$		Egg boxes, video cases, brittle toys,fast food trays, etc.
High Density Polyethylene (HDPE)	104	$(CH_2{=}CH_2)_n$		Plant pots, rigid pipes, snack food boxes, cereal boxes, fabric conditioner bottles, detergent, etc.
Low Density Polyethylene (LDPE)	104	$(CH_2{=}CH_2)_n$		Fertilizer bag, shopping bag, some bottle tops, bubble wrap flexible bottles, irrigation pipes, etc.
Polyvinyl chlorides (PVC)	83.5	$CH_2{=}CHCL$		carpet and other floor covering window and door frames, Credit cards, guttering pipes
Polytetrafluoro ethylene (Teflon)	100	$CF_2{=}CF_2$		Nonstick surfaces, chemically resistant films
Polyvinyl acetate (PVA)	44	$[CH_2CH(OH)]_n$		latex paints, Adhesives, textile coatings, chewing gum, etc.

Environmental problems due to polymers

Because of advance technology and the increase in the global population, plastic materials have found wide range of applications in every aspect of life and industries (Tokiwa *et al*., 2009). A major portion of plastic produced each year is used to make disposable items for packaging or other short-lived products that are discarded within a year of manufacture. These two observations alone indicate that our current use of plastics is not sustainable. However usage and disposable of plastics has leads to major environmental issues (Hopewell *et al.*, 2009). In addition, because of the durability of the polymers involved, substantial quantities of discarded end-of-life plastics are accumulating as debris in landfills and in natural habitats worldwide.

Effect of polymers on Ecosystem

Plastic accumulation has caused nuisance in ecosystem (Yabhannavar *et al.*, 1994). When plastic debris is exposed to UV radiation, it undergoes photo oxidation which results in plastic deterioration by loosening its tensile strength, becomes brittle and crumbles to small fragments and particles called micro plastics. These micro plastics can be ingested by various marine animals that are mistakenly identified as planktons. Thus, the plastic debris enter into the environment and accumulate in the food chain, leading to multiple hazards (Sivan *et al.*, 2011). Disposal of HDPE type of plastics causes threat to biological ecosystem. It accumulates in water bodies it the form of garbage waste which sometime causes blockage in intestine of fish, birds and marine mammals (Spear *et al*., 1995). It is impossible to prevent, even in part, the release of these materials into the environment (Caccari *et al*., 1993).

Alternatives for polymer disposal

Today, about 69% of the plastic solid waste in the United States ends up in landfills. In most developed regions of the world, waste is collected, transferred to landfills and is typically covered with soil daily (Rayne *et al*., 2008). But space available for landfills is becoming scarce in some countries (Hopewell *et al.,* 2009). A well-managed landfill site results in limited environmental harm but it has caused damage of the building which were constructed upon those landfills, although there are long-term risks of contamination of soils and groundwater by some additives and breakdown by products in plastics, which can become persistent organic pollutants. There is

no such alternative which can solely solve the problem, so multiple approaches must be taken. The primary methods are (i) Source reduction, (ii) Incineration, (iii) Composting, and (iv) Recycling (Stein *et al.*, 1992).

These trends are likely to continue, but some significant challenges still exist from both technological factors and economic or social behavior issues relating to the collection of recyclable wastes, and substitution for virgin material. Consequently, it is important to discover ways to biodegrade these compounds, including the optimal environmental conditions and the possible biological mechanisms involved.

Polymer degradation

Increased levels of urbanization and industrialization and multipurpose applications have led various types of polymers to accumulate in the environment causing pollution. Hence it has become priority to address these issues and identify novel ecofriendly strategies for removal of such contaminants from the environment.

Biologically initiated degradation also is strongly related to chemical degradation as far as microbial attack is concerned. Microorganisms produce variety of enzymes which are capable of reaction with natural and synthetic polymers (Dindar and Icli *et al.,* 2001).

"Biodegradation" is defined as reduction in the molecular weight of a substance by naturally occurring microorganisms such as bacteria, fungi, and Actinomycetes (Arkatkar *et al.*, 2009) which are involved in the degradation of both natural and synthetic plastics. Optimal environmental condition and possible biological mechanisms to degrade plastic material from ecosystem have been now area of interest by researchers all over the globe.

Different steps of plastic degradation by microorganisms

Several steps occur in the plastic biodegradation process (Figure 1) and could be identified by specific terminology (Lucas *et al.,* 2008):

-Bio-deterioration is defined as the action of microbial communities and other decomposers responsible for the physical and chemical deterioration that results in a superficial degradation and modification of the mechanical, physical and chemical properties of the plastic.

-Bio-fragmentation refers to the catalytic action that cleave polymeric plastics into oligomeric dimeric or monomeric forms by ecto-enzymes or free-radicals secreted by microorganisms.

-**Assimilation** characterizes to the integration of molecules transported in the cytoplasm in the microbial metabolism.

-**Mineralisation** refers to the complete degradation of molecules that resulted in the excretion of completely oxidized metabolites (CO_2, N_2, CH_4, H_2O).

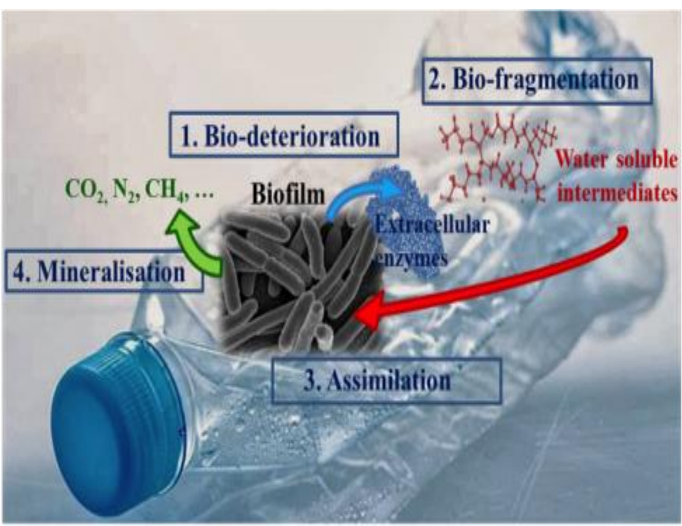


Figure 1: Steps involved in plastic degradation by microorganism

Parameters Affecting Polymer Biodegradation

Polymeric materials released into the environment can undergo changes induced by physical, chemical, and biological forces (Arutchelvi *et al.*, 2008) or combination of all these due to the presence of air, moisture, temperature, light, high energy radiation or microorganisms. Various parameters like chemical and physical pre-treatments have been used for loosening of carbon backbone of various polymers which may influence the rate of biodegradation positively. It is also being observed that addition of required nutrient supplement may help in promotion of growth, adhesion of microbes to polymer and in some cases co-metabolism for polymer degradation.

Degradation of polymer using microbial community

Due to immense use and continuous disposal of plastics to environment, microbial community present in the system gets exposed to the polymers in nature. It becomes important to explore and identify the population of polymer degrading microorganism in ecosystems for development of new degradation approaches. Adherence of microorganism on the surface of plastics followed by the colonization of the exposed surface is major mechanism involved in the microbial degradation of plastics (Tokiwa *et al.*, 2009). Till now there has been only one report of complete degradation of plastic under *in vitro* conditions. Apart from that *Pseudozyma japonica*-Y7-09, Consortia of *Enterobacter* sp. bengaluru-btdsce01, btdsce02 and *Pantoea* sp., and *Engyodontium album* are found to degrade polymer of polyester (PCL), LDPE, polypropylene kind within 15 days, 120 days and 1 year of incubation respectively (Skariyachan *et al.*, 2016; Abdel-Motaal *et al.*, 2013). Efficiency of degradation of different types of plastics by a range of microorganism is listed in table 2:

Table 2: Polymer degradation by different microorganism and its parameters.

Sr. no	Types of polymer	Name Of Organism	% Degradation	Incubation Required	Pre-Treatment	Reference
1	Polyethylene	*Tricoderma harzianum*	23% 40% 13%	3 months	Autoclaved, Uv – treated, Surface- sterilized	16
2	Polyethylene	Consortia formulated by *Pseudomonas spp.*	40%	90 days	-	13
3	Polyethlene	UV and EMS induced *Pseuodomonas putida*	20.54%	1 month	Alkali treated	1
4	pro-oxidant blended (MI-PP)	*Engyodontium album*	79%	1 year	Uv- treated	1
5	Low density polyethylene (LDPE)	*Staphylococcus* spp.	52%	_	_	3
6	Low density polyethylene (LDPE)	*Pseudomonas* Spp.	11%	_	_	3
7	high-density polyethylene (HDPE)	*Achromobacter xylosoxidans*	9%	150 days	_	4
8	Polyethylene	*Bacillus cereus*	14% 7.2% 2.4%	3 months	Uv –treated, Autoclaved, Surface –sterilized	5
9	Aliphatic polyesters poly (€caprolactone) (PCL)	*Pseudozyma japonica-Y7-09*	93.33%	15 days	_	7
10	foam plastic	*Pseudozyma japonica-Y7-09*	43.2 %	30 days	_	7

11	Polyethylene	*S. badius* 252 and *S. setonii* 75Vi2	31% & 36%	4 weeks	UV-treated films	8
12	Polyethylene	*S. viridosporus*	68%	8 days	heat-treated films	8
13	Polyethylene	*Pseudomonas fluorescence*	8.06%	1 month	_	14
14	Polyvinylchloride (Pvc)	*Bacillus cereus* strain	22.22%	1 month	_	12
15	Polyethylene	*Bacillus cereus* strain	17.39%	1 month	_	12
16	low-density polyethylene (LDPE) strips	Consortia of *Enterobacter* sp. bengaluru-btdsce01, *Enterobacter* sp. bengaluru-btdsce02, and Pantoea sp.	81 ± 4%	120 days	_	14
17	low-density polyethylene (LDPE) pellets	Consortia of *Enterobacter* sp. bengaluru-btdsce01, *Enterobacter* sp. bengaluru-btdsce02, and *Pantoea* sp.	38 ± 3 %	120 days	_	14
18	low-density polyethylene (LDPE)	*Pseudomonas* sp. AKS2	5±1 %	45 days	_	15

Community analysis of soil in dumpsite areas

Monitoring the soil microbial community and its activity can be a powerful tool for understanding basic and applied ecological contexts. Rapid growth of microorganisms in the soil allows for comprehensive study of community interactions. Enzymes released by microorganisms during the degradation of solid waste play a key role in the biological and biochemical transformations that take place in the soil environment. Due to the environmental factors and ecological stress, biological parameters of soil are affected. Microbial enzymes are also responsible for the decomposition of complex organic compounds. It seems necessary to consideration community-level cultural approach, called CLPP, developed by Garland and Millis (1991). It was indicated that less than the 95 substrates were sufficient to analyse changes in functional microbial community. The CLPP method provides alternative for time consuming culture-based analyses. The study includes determination of dehydrogenase and respiratory activity, and the response of soil microbial activity and functional diversity to range of environmentally relevant sole carbon sources using the Biolog EcoPlates™ system.(Franc *et al*., 2012).

The Biolog EcoPlate contains 31 of the most useful carbon sources for soil community analysis. These 31 carbon sources are repeated 3 times to provide more replicates of the data (table 3). The community reaction patterns are typically analyzed at defined time intervals over 2 to 5 days. The changes in the pattern are compared and analyzed using statistical analysis software. The most popular method of analysis of the data is Principle Components Analysis (PCA) of average well color development (AWCD) data, but alternative methods may also offer advantages.(Rev *et al*., 2007) The changes observed in the fingerprint pattern provide useful data about the microbial population changes over time.(Oszust *et al*., 2012

Table 3: Carbon source in Ecoplate

A1 Water	A2 β-Methyl-DGlucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine	A1 Water	A2 β-Methyl-DGlucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine	A1 Water	A2 β-Methyl-DGlucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine
B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine	B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine	B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine
C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine	C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine	C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine
D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine	D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine	D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine
E1 α-Cyclodextrin	E2 N-Acetyl-DGlucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine	E1 α-Cyclodextrin	E2 N-Acetyl-DGlucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine	E1 α-Cyclodextrin	E2 N-Acetyl-DGlucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine
F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-LGlutamic Acid	F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid	F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid
G1 D-Cellobiose	G2 Glucose-1Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethylamine	G1 D-Cellobiose	G2 Glucose-1Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethylamine	G1 D-Cellobiose	G2 Glucose-1Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethylamine
H1 α-D-Lactose	H2 D,L-αGlycerol Phosphate	H3 D-Malic Acid	H4 Putrescine	H1 α-D-Lactose	H2 D,L-αGlycerol Phosphate	H3 D-Malic Acid	H4 Putrescine	H1 α-D-Lactose	H2 D,L-αGlycerol Phosphate	H3 D-Malic Acid	H4 Putrescine

AIM OF STUDY

According to the current scenario of global environmental problems due to polymers, it has become a priority to search for efficient microbial strains from various sources. All the microorganisms present in various environmental niche may not be capable for utilization of polymer as source of carbon for their growth. But the organisms present in soil of solid waste dumpsite or waste landfill might be capable for primary adhesion and degradation of polymer due to their prolonged exposure to various concentrated pollutants and polymers. Pre-treatment of polymer may also help in weakening of plastic backbone and growth of selected strains on polymer. Hence the aim of these present study was to isolate bacteria from waste dumpsite and other environments and to explore these along with a known hydrocarbon degrading bacteria for polymer degradation with or without physical/chemical pre-treatment of plastic polymer.

OBJECTIVES

1. Isolation and screening of polymer degrading microorganisms:

    a) To determine the functional microbial diversity using community level physiological profiling by Ecoplate® method.
    b) To screen various landfill sites for isolation and characterization of polymer degrading organisms.

2. To study the effect of various physical and chemical pretreatment on polymer degradation by wastewater bacteria *pseudomonas citronellolis*

3. To study in degradation efficiency of organism isolated from the dumpsite.

MATERILS

AND

METHODS

1. Sample collection

Plastic waste and soil samples were collected from five different waste disposal site dumped with polyethylene bags and plastic waste located at Pirana dumpsite in Ahmedabad, Gujarat, India. The samples were collected randomly from the surface layer of soil (up to10 cm) in depth, and were transferred into sterile plastic bags and stored at 4°C till use. All the sample were dumpsite samples which accumulated soils from respective sities. The sample collected were

1. Dry soil from the surface layer
2. Wet soil from the depth (upto 10 cm)
3. Waste dumped plastic

Figure 2: A) Pirana dumpsite located in Ahmedabad B) Soil sample collected from dumpsite C) Solid waste collected from the dumpsite

2. Community level physiological profiling (CLPP) analysis of four different soil sample

Following different soil sample were used for the CLPP analysis:

a) Scrapped soil from plastic waste
b) Soil sample collected from dumpsite
c) Bulk soil collected from plastic waste site
d) virgin soil sample (highway soil)

One g soil was shaken in 10 mL of distilled sterile water and prepared serial dilution were prepared (1:10, 1:100, 1:1000) using N. Saline. Next 150 µL of each sample were inoculated into each well of Biolog Ecoplates asceptically and incubated at 30 °C. The rate of utilization was indicated by the reduction of the tetrazolium, a redox indicator dye that changes from colorless into purple. Data were recorded with a plate reader at 590 nm at regular interval until 120 h. Microbial response in each microplate that expressed total activity and average well-color development (AWCD) was determined as follows:

$$\text{Total activity} = \text{SUM}(\Sigma OD_i)$$

$$AWCD = \Sigma OD_i/31$$

where OD_i is optical density value from each well, corrected by subtracting the blank well (inoculated, but without a carbon source) values from each plate well.

Richness (R) values were calculated as the number of oxidized C substrates, and the Shannon–Weaver index values (H) (*i.e.*, the richness and evenness of response) were calculated as follows:

$$H = -\Sigma p_i(\ln p_i)$$

where p_i is the ratio of the activity on each substrate (OD_i) to the sum of activities on all substrates ΣOD_i.

$R_{margelf}$ value was found by following equation:

$$R_{margelf} = S\text{-}1/LN(\text{total activity})$$

$R_{menhinick}$ value were found by following equation:

$$R_{menhinick} = S/SQRT(\text{total activity})$$

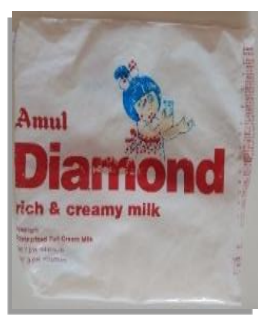
Where S is total carbon source Out of 31 utilized by microbial community present in each well.

Plate readings at all incubation hours were used to calculate AWCD, R and H, and finally the communities were compared on the basis of data at all incubation hours as substrate. Since it was the shortest incubation time that allowed the best resolution (Franc *et al*., 2012).

3. Screening of polymer/plastic

Different types of plastic for use as substrate were collected from the local market which are listed as follows:

Sr. no.	Types of plastic sample	Resin code	Category
1	Milk pouch	4	Low Density Polyethylene
2	Plastic cup	5	Polypropylene
3	Polyethylene bag	2	High Density Polyethylene
4	Snacks packet	7	Composite



4. Isolation of polymer degrading bacteria

Isolation of polymer degrading bacteria were explored in two different way:

1) using plastic powder and 2) using waste plastic strips as sole source of carbon into the MSM media.

4.1) Isolation of plastic degrading microorganisms using plastic powder

i) powder preparation (Singh *et al.*, 2015)

Various types of plastics such as plastic bag, plastic cup, snacks packet, milk pouch were cut into equal size of pieces and weighed (~8 gram). All the weighed materials were mixed and immersed into 200 ml of xylene and boiled for 20 minutes with constant stiring. Then allowed this mixture to cool down. One hundred twenty ml ethyl alcohol was added into the slury of this plastic mixture to remove the xylene and mixed it properly. The xylene-ethyl alcohol was evaporated keeping under sunlight and thus obtained plastic powder was allowed to evaporate the tinge of xylene by keeping under sunlight for 1 hour and air dried overnight.

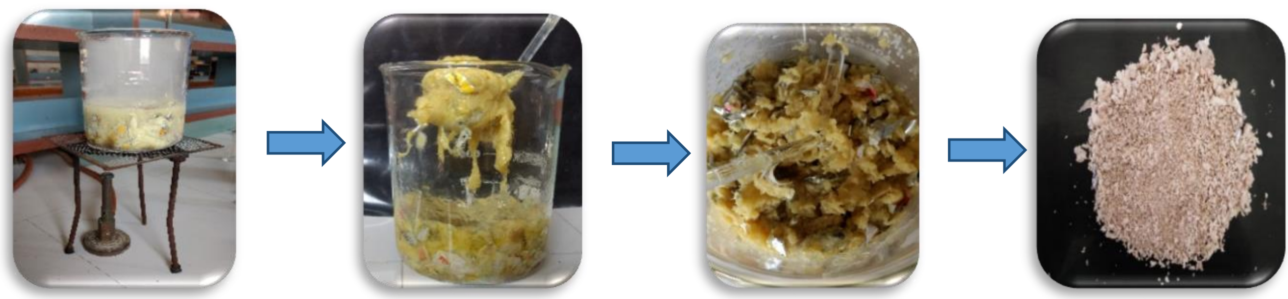


Figure 3: Steps followed for the plastic powder preparation

ii) Isolation method

To isolate the polymer degrading bacteria enrichment culture method was performed. One gram of soil sample which was collected from the different location of the pirana dumpsite was suspended into 10 ml of sterile distilled water, vortexed and the particles were allowed to settle down. Serial dilutions of this Soil sample were prepared and 5 ml soil suspension from these dilution were inoculated into separate 100 ml of autoclaved MSM(minimal salt media) media containing 0.1% plastic powder (plastic powder was added after autoclaving). All the flasks were incubated at 30°C and at 80 rpm for one week. After one week of incubation 5 ml of enrichment culture was transferred into another freshly prepared autoclaved 100 ml MSM media containing 0.1% plastic powder. Similarly second and third transfer were performed. After third incubation

0.1 ml culture from the both flask were plated onto MSM agar plate containing 0.1% plastic powder and incubated for the 14 days at 30°C. After nearly 14 days of incubation when growth was observed, individual colonies were picked up and streaked on sterile nutrient agar plates for culture purification (Singh *et al.*, 2015**)**

4.2 Isolation of plastic degrading microorganisms using waste plastic strips

i) preparation of strip from plastic waste sample

Different types of plastic samples were cut into 6 cm×2 cm strips for the isolation of polymer degrading bacteria using MSM as basal media.

ii) Isolation Method

Previously prepared five different types of plastic strips to which some attached soil were inoculated into 100 ml MSM media and incubated at 30°C and at 80 rpm for 1 week. After one week of incubation 5 ml of this culture was transferred into another freshly prepared autoclaved 100 ml MSM media containing 5 different types of plastic attached soil strips. Plastic strips were added after autoclaving MSM media. Similarly second and third transfers were performed. Before each transfer 0.1ml of culture from flasks were also transferred onto MSM agar plate containing 0.1% plastic powder and incubated for 14 days at 30ºC. Figure 4 displays and medium containing powder/strips [experimental setup]. After 14 days of incubation when growth was observed, individual colonies were picked up and streaked on sterile nutrient agar plates for purification.

Figure 4: Experimental set up for isolation of organism from plastic powder and plastic strips

5. Identification of isolates

The identification were carried out on the basis of microscopic examination, morphological features and biochemical tests which are listed in table as follow:

Sr. no	**Biochemical test**
1	MR-VP test
2	Oxidative Fermentative test
3	Citrate utilization test
4	Deaminase test
5	Gelatin hydrolysis test
6	Triple sugar iron agar slant test
7	Indol production test
8	Sugar utilization test
9	EMB agar plate

Moreover genomic DNA of the selected isolates were extracted and PCR was performed based for 16S rDNA gene amplification and sequencing for molecular identification of bacteria.

6. Pretreatment

6.1 Heat treatment

Plastic chips and strips were cut into 2 cm×2 cm and 15 cm×2 cm. All the chips and strips were put into the hot air oven for 8 hours for 3 days at the following temperature for different plastic categories.

Sr. no.	Sample name	Temperaure
1	LDPE	50 °C
2	HDPE	70 °C
3	PP	70 °C
4	COMPOSITE	70 °C

This heat treated chips and strips were further used for experiment (Lee *et al.*, 1991).

6.2 UV treatment

Plastic chips and strips were cut into 2 cm×2 cm and 15cm×2 cm. All the chips and strips were put under the UV light for 1 hour for 7 days. This UV treated chips and strips were further used for experiment (Lee *et al*, 1991).

6.3 Alkali treatment

Plastic chips and strips were cut into 2 cm×2 cm and 15 cm×2 cm. There strips and chips were added into the mixture of 450 ml of distilled water, 36 ml of tween 20, and 20 ml of bleach (bleach was prepared by mixing 10 g of NAOH, 10 g of NaCl and 20 ml of glacial acetic acid) and stirred it for 1 hour. After 1 hour strips and chips were transferred into 450 ml distilled water and again stirred it for half hour. They were transferred to ethanol solution (70% v/v) for 30 min. for disinfection. Then they removed from the ethyl alcohol and allowed for drying into hot air oven. These alkali treated strips and chips were packed into sterile zip bag and used further for experiment (Muralidhar *et al.*, 2014).

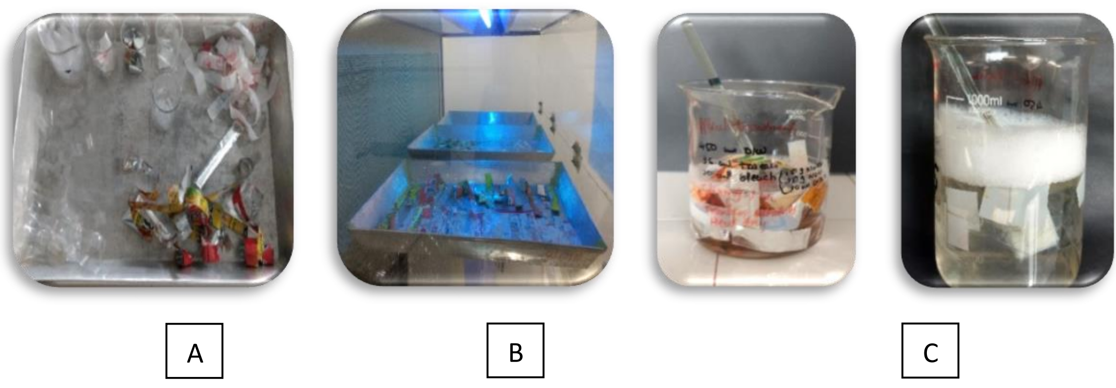


Figure 5: A) Heat treatment B) UV treatment C) Alkali treatment

7. Effect of various pre-treatment on polymer degradation by *Pseudomonas citronellolis*

Effect of Heat pre-treatment:

Three or one heat pre-treated plastic strip of (15cm×2 cm) and five heat pre-treated plastic chips (2 cm×2 cm) of every individual plastic samples (HDPE, PP, Composite, LDPE) were inoculated into 40 ml autoclaved MSM media tube with 5% inoculum of *Pseudomonas citronellolis.*

Similarly three untreated plastic strip of (15cm×2 cm) and five untreated plastic chips (2 cm×2 cm) of every individual plastic samples were inoculated into 40 ml autoclaved MSM media tube with 5% inoculum of *Pseudomonas citronellolis.*

Along with these control was also kept for both heat pre-treated plastic and untreated plastic samples without inoculum of *Pseudomonas citronellolis.*

All the tubes were incubated for 30 days at 30°C at 80 rpm and growth was observed at every 10 days interval by measuring absorbance was measured at 560 nm with spectrophotometer (Agilent technologies cary60(UV-vis)).
Similarly effect of UV pre-treatment and alkali pre-treatment on polymer degradation by *Pseudomonas citronellolis* were analyzed**.**

8. Efficiency of isolates for polymer degradation

Twelve different kinds of isolates (5 % inoculum) were inoculated into 100 ml of autoclaved MSM media with the untreated LDPE and composite plastic sample (three strip of 15cm×2 cm and five chips of 2 cm×2 cm). All the flask were incubated for 30 days into shaker at 80 rpm at 30°C.

9. Assesment of *invitro* biodegradation assay

Changes in the polymer properties were investigated by methods of (Jeyakumar *et al.*, 2014; Sujith *et al.*, 2012 and Sowmya *et al.*, 2014).

9.1 Visual Observation and Weight Determination

i. Visual observation of polymer chips

Visual observation was carried out by looking for change in the surface properties of polymers chips and strips after incubation of 30 days with *P. citronellolis* in MSM.

ii. Weight determination of polymer chips

Weight loss of polymer chips and strips after incubation of 30 days were determined on analytical balance (Nanda and Sahu *et al*., 2010).

9.2 Crystal Violet (CV) Assay for Assessment of cell adhesion on polymers

Materials:

1. 0.1% Crystal Violet (CV)
2. 30% acetic acid
3. Methanol
4. Sterile distilled water

Method:

- The plastic chips from inoculated medium were removed at regular interval and were washed three times with 5 ml of sterile distilled water.
- The remaining adhered bacterial cells were fixed with 2.5 ml of methanol.
- Plastic chips were stained with 0.1% crystal violet (CV) solution and incubated at room temperature for 10-15 min.
- Then the excess stain was washed off with the running tap water and chips were air dried.
- The dye bound to the adhered cells were resolubilized with 2.5 ml of 33% acetic acid and this liquid was poured into a cuvette.
- Glacial acetic acid was taken as a blank and O.D. was taken at 585 nm (Adetunji *et al*., 2011).

9.3 Scanning Electron Microscopy

Scanning Electron Microscopy was done for the heat pre-treated, UV pre-treated, Alkali pre-treated, untreated plastic inoculated with *P. citronellolis (*biologically treated*)* and un-inoculated untreated composite sample (control) at FOOD TESTING LABORATORY, Junagadh Agricultural University, Gujarat to observe if there was colonization of bacterial cell or any deformity on the surface of plastic and to observe colonization of bacterial cells on surface (Dey *et al.*, 2012; Bhatia *et al.,* 2014).

9.4 Tensile strength analysis

The force per unit area (MPa or psi) required to break a material in such manner is the ultimate tensile strength. The inoculated heat pre-treated, UV pretreated, Alkali pretreated, untreated inoculated with *P. citronellolis (*biologically treated*)* and uninoculated untreated composite sample (control) were analyzed for tensile strength after incubation of 30 days by HERTZ TRAINING AND TESTING CENTRE, at Vatva, Ahmedabad, Gujarat.(Esmaeili *et al.,* 2013).

9.5 FTIR (Fourier Transform Infrared Spectrophotometry)

Fourier Transform Infrared Spectroscopy (FTIR) is a tool for identifying various functional groups present in a compound. During the process of degradation, formation or disappearance of functional groups in the polymer can be monitored by FTIR The inoculated Heat pretreated UV pre-treated, Alkali pretreated, untreated inoculated with *P. citronellolis* (biologically treated) and uninoculated untreated composite sample (control) were treated with NAOH to remove aluminium foil from the surface after incubation of 30 days and then analyzed for structural deformation or any change in chemical properties by FTIR analysis by SICART, Vidhyanagar, Gujarat (Jeyakumar et al., 2013).

RESULTS

AND

DISCUSSION

1. Characterization and identification of polymer degrading bacteria

The identification of bacteria was carried out on the basis of macroscopic examination, morphological features and biochemical test. Total 39 bacteria were isolated from the dump site soil sample and solid waste material. Table 4 describe all the isolates as observed on media plate.

Table 4: Colony morphology and Gram's reaction of 39 organism isolated from the dump site soil sample and plastic waste material.

Organism designation	Size	Shape	Margin	Elevation	Surface	Consistency	Opacity	Pigmentation	Gram's Reaction
PDS1	0.5 mm	Round	entire	convex	Smooth	Moist	Opaque	Pink	Positive
PDS2	1 mm	Round	entire	Convex	Smooth	Viscous	Opaque	Yellow	Positive
PDS3	1 mm	Round	entire	convex	Smooth	Viscous	Opaque	Yellow	Negative
PDS4	2 mm	Round	Entire	Convex	Smooth	Moist	Opaque	No	Positive
PDS5	1mm	Round	Entire	Flat	Smooth	Moist	Opaque	Light Yellow	Positive
PDS6	2mm	Round	Entire	Convex	Smooth	Moist	Translucent	No	Positive
PDS7	1 mm	Round	Entire	Flat	Smooth	Viscous	Translucent	No	Positive
PDS8	1mm	Round	Entire	Flat	Smooth	Viscous	Opaque	No	Positive
PDS9	1 mm	Round	Entire	Convex	Smooth	Moist	Translucent	No	Positive
PDS10	2 mm	Round	Entire	Flat	Smooth	Viscous	Opaque	No	Positive
PDS11	1 mm	Round	Entire	Flat	Smooth	Viscous	Translucent	No	Positive
PDS12	1 mm	Round	Entire	Flat	Smooth	Viscous	Translucent	No	Positive
PDS13	2 mm	Round	Entire	Convex	Smooth	Moist	Translucent	No	Positive
PDS14	1mm	Round	Entire	Flat	Smooth	Viscous	Opaque	No	Positive

PDS15	1 mm	Round	Entire	Convex	Smooth	Viscous	Translucent	No	Positive
PDS16	2mm	Round	Entire	Flat	Smooth	Viscous	Opaque	No	Positive
PDS17	2 mm	Round	Entire	Convex	Smooth	Moist	Translucent	No	Positive
PDS18	1 mm	Round	Entire	Flat	Smooth	Viscous	Translucent	No	Positive
PDS19	2 mm	Round	Entire	Flat	Smooth	Viscous	Opaque	No	Positive
PDS20	0.5 mm	Irregular	Entire	Flat	Rough	Viscous	Opaque	No	Positive
PDS21	1 mm	Round	Entire	Flat	Smooth	Viscous	Opaque	No	Positive
PDS22	2 mm	Round	Entire	Flat	Smooth	Moist	Translucent	No	Positive
PDS23	1 mm	Round	Entire	Convex	Smooth	Moist	Opaque	No	Positive
PDS24	2 mm	Round	Entire	Flat	Smooth	Moist	Opaque	Orange	Positive
PDS25	2 mm	Round	Entire	Flat	Smooth	Moist	Opaque	No	Positive
PDS26	1 mm	Round	Entire	Convex	Smooth	Viscous	Opaque	Yellow	Positive
PDS27	1 mm	Round	Entire	Flat	Smooth	Viscous	Translucent	pink	Positive
PDS28	1 mm	Round	Entire	Convex	Smooth	Viscous	Opaque	Yellow	Positive
PDS29	2mm	Round	Entire	Convex	Smooth	Viscous	Opaque	Yellow	Positive
PDS30	1mm	Round	Entire	Convex	Smooth	Viscous	Opaque	Yellow	Positive

PDS31	2 mm	Round	Entire	Convex	Smooth	Viscous	Opaque	Slight Yellow	Positive
PDS32	0.5 mm	Round	Entire	Convex	Smooth	Moist	Translucent	Yellow	Positive
PDS33	1mm	Round	Entire	Flat	Smooth	Moist	Translucent	No	Positive
PDS34	2 mm	Round	Entire	Flat	Smooth	Viscous	Opaque	translucent	Positive
PDS35	1 mm	Round	Entire	Convex	Smooth	Viscous	Opaque	Yellow	Negative
PDS36	1 mm	Round	Entire	Convex	Smooth	Viscous	Opaque	Yellow	Negative
PDS37	1mm	Round	Entire	Convex	Smooth	Viscous	Opaque	Lemon Yellow	Negative
PDS38	1 mm	Round	Entire	Flat	Smooth	Viscous	Opaque	Slight Yellow	Negative
PDS39	1 mm	Round	Entire	Convex	Smooth	Viscous	Opaque	Yellow	Negative

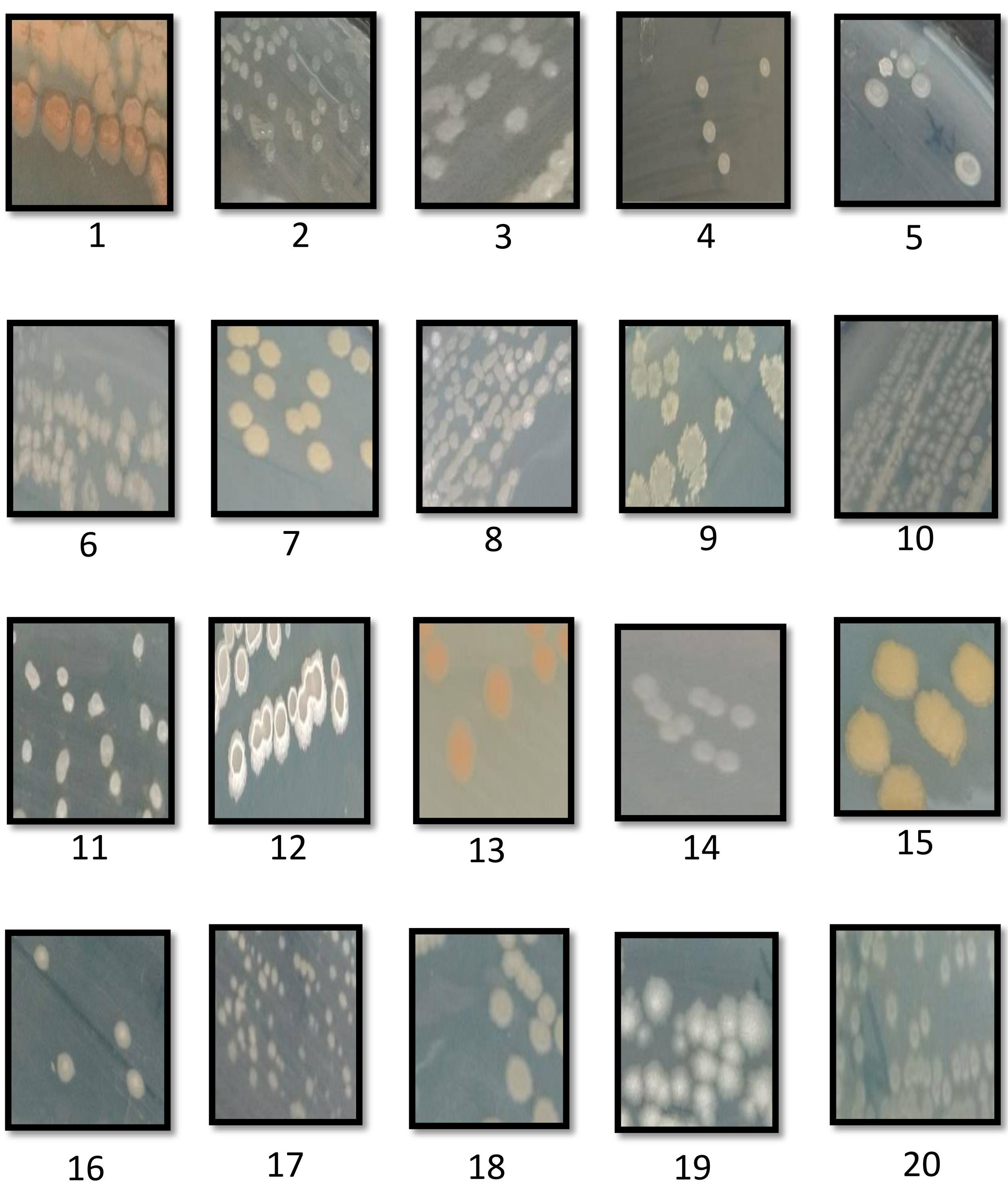


Figure 6: Visual observation of 39 organism isolated from the dump site soil sample and waste plastic materials on nutrient medium

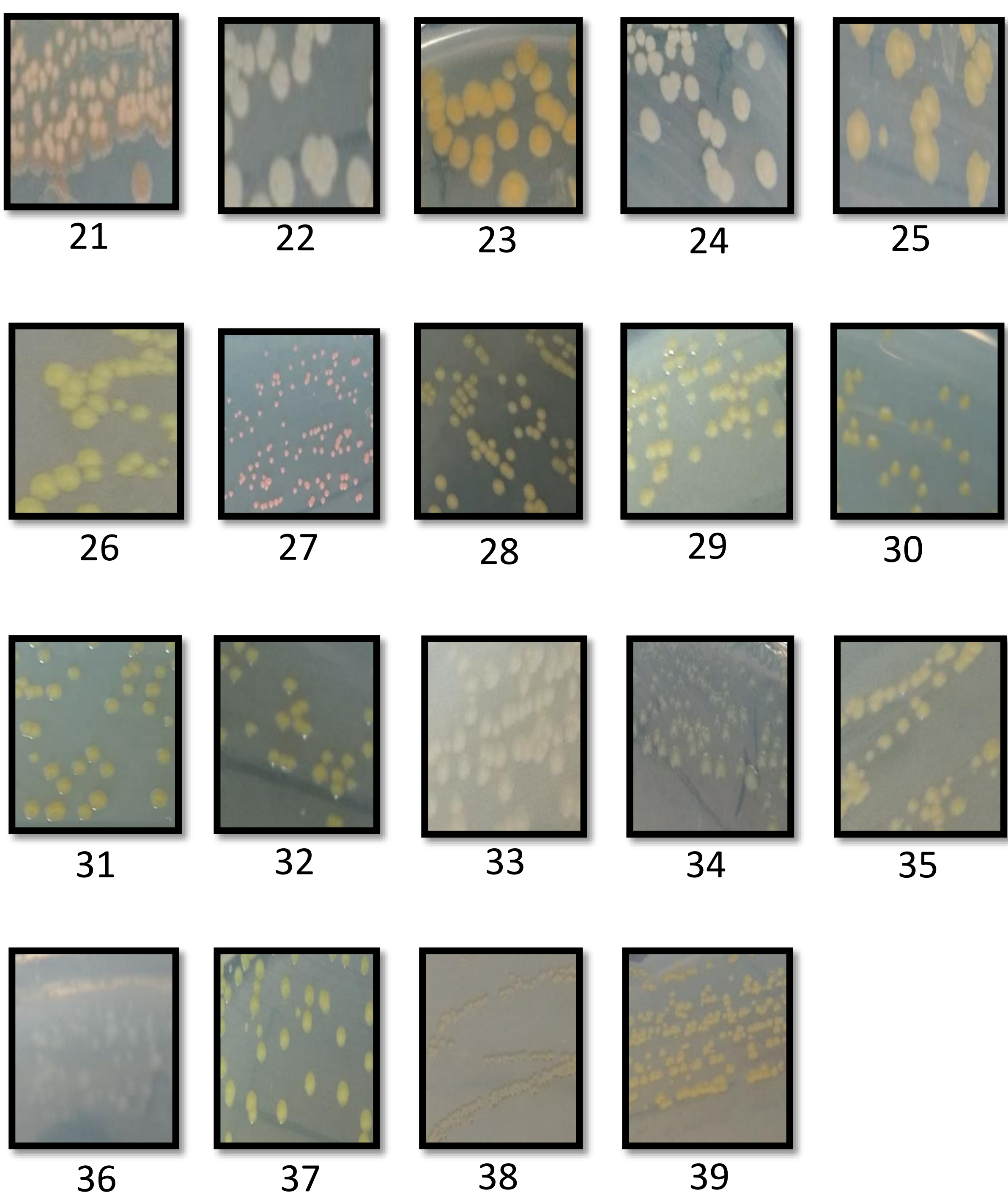


Figure 6: Visual observation of 39 organism isolated from the dump site soil sample and waste plastic materials on nutrient medium.

Out of 39 isolates, 12 organisms which were unique in their colony characteristics were selected further for biochemical test. Table 5 displays the biochemical characteristics of all those 12 isolates which were used for further experiments.

Table 5: Results of biochemical test of organisms isolated from dump site.

Organism	**MR test**	**VP test**	**OF test**	**Deaminase test**	**Gelatin hydrolysis test**	**Triple sugar iron agar slant**	**Indol production**	**Sugar utilization test**	**Citrate utilization test**
PDS1	_	+	+	Only growth	+	_	_	+	+
PDS2	+	+	+	Only growth	_	Only growth	_	+	-
PDS3	+	+	+	Only growth	_	Only growth	_	+	+
PDS4	_	_	+	Only growth	_	Only growth	_	+	+
PDS7	_	_	+	Only growth	_	Only growth	_	+	-
PDS12	+	_	+	Only growth	_	Only growth	_	+	-
PDS15	+	_	+	Only growth	_	Only growth	_	+	-
PDS26	-	_	+	Only growth	_	_	_	+	-
PDS27	+	+	+	Only growth	+	_	_		+
PDS31	_	+	+	Only growth	+	_	_	+	+
PDS34	_	_	+	Only growth	_	_	_	+	+
PDS36	+	_	+	Only growth	+	_	_	+	+

1.1 Molecular identification of isolated polymer degrading bacteria

For molecular identification, genomic DNA was isolated from selected organisms. Electrophoresis was performed using 1X TAE buffer at 100 V at room temperature. DNA bands were resolved with EtBr (Samberbrook and Ruseal, 2001). Figure 7 represents Agarose gel electrophoresis of genomic DNA isolated from selected strains.

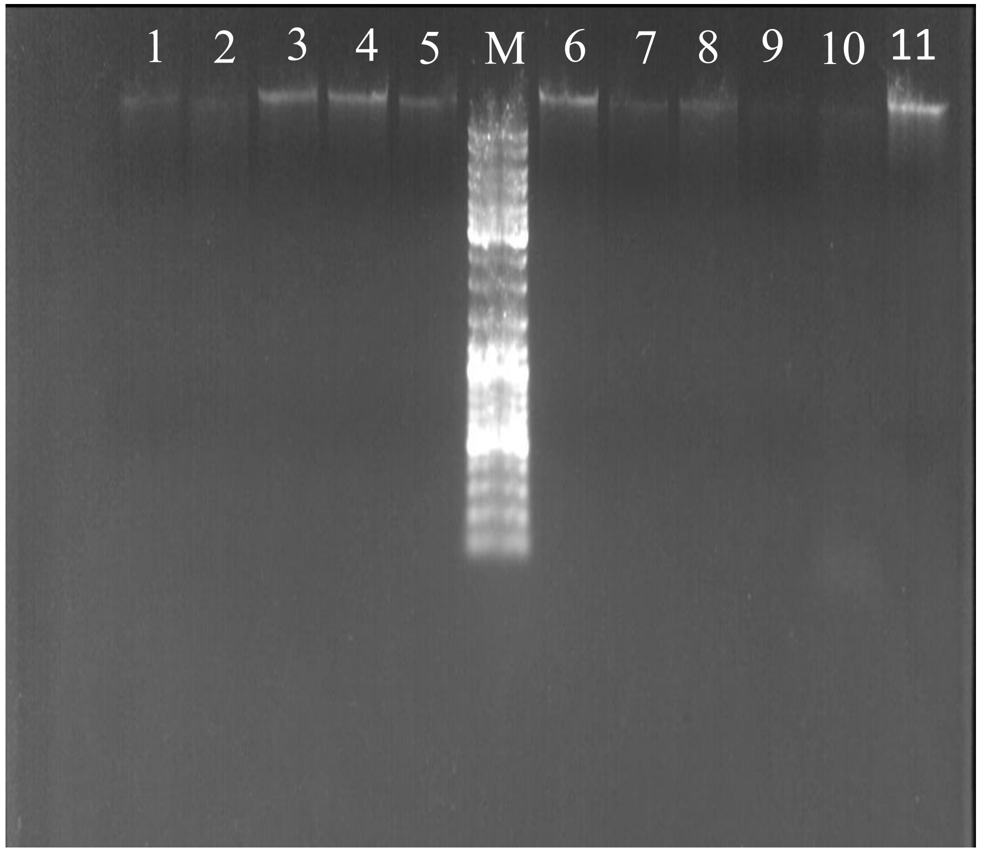


Figure 7: Gel image of isolated genomic DNA

(M: marker, Bacterial isolates; 1: PDS2, 2: PDS7, 3: PDS3, 4: PDS31, 5: PDS36, 6: PDS37, 7: PDS4, 8: PDS15, 9: PDS27, 10: PDS1, 11: PDS26)

For Amplifying 16S rRNA gene of isolated organism, PCR was performed using universal primers (table 6) and run on the 0.8% agarose gel. After running the DNA the gel was observed under gel quant software (Applied Bio system India) and for molecular identification, amplicons were sent for 16 rDNA sequencing.

Table 6: Universal primer used for 16S rDNA Amplification

Primer	Sequence 5' – 3'
F27	AGAGTTTGATCMTGGCTCAG
R1492	TACGGYTACCTTGTTACGACTT

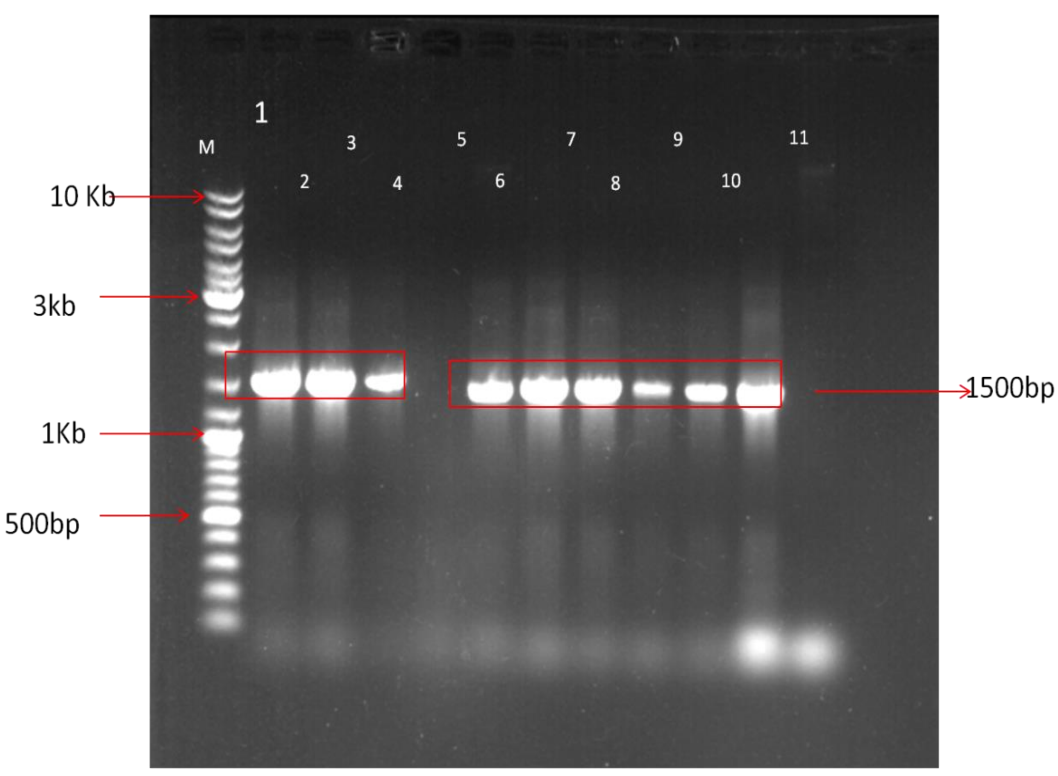


Figure 8: Amplified product of bacterial isolated DNA

(M: marker (mix gene ruler), 1: PDS2, 2: PDS7, 3: PDS3, 4: PDS31, 5: PDS36, 6: PDS37, 7: PDS4, 8: PDS15, 9: PDS27, 10: PDS1, 11: PDS26)

2. Functional diversity analysis

The functional diversity (catabolic potential) was assessed using CLPP approach and by calculating the diversity indices, Richness (R), Evenness (E) and Shannon–Weaver index (H). These indices were calculated from data obtain from the community level physiological profiling (CLPP) using Biolog Eco Plates. The result are presented in Table 7.

Table 7: Diversity indices obtained from the substrate utilization pattern of different soil samples at 60 h of incubation time.

Sample	AWCD	SUM	S	Evenness	$R_{_margelf}$	$R_{_menhinick}$
Attached soil	0.895	3.415	27	1.036	26.699	5.123
Dumpsite Soil	0.502	2.718	14	1.029	13.635	3.546
Highway Soil	0.985	3.270	28	0.981	27.707	5.066
Bulk Soil	1.335	3.335	23	1.063	22.731	3.574

AWCD represent the average well color development which display the overall extent of carbon source utilization, S is substrate richness, $R_{margalef}$ and $R_{menhinick}$ are two different richness indices. In our study carbon substrate utilization appeared quite similar in bulk and highway soil while lower carbon source utilization pattern of dumpsite soil. There was significant difference in carbon source utilization patterns of dumpsite and attached soil. Microbial community of dumpsite soil had utilized only 14 carbon sources out of 31 carbon sources. Similarly community of bulk soil had utilized 23 carbon sources. Attached soil and highway soil have utilized maximum carbon source (27 and 28). The community present in highway soil is not able to utilize only 3 carbon sources - D-Galactonic Acid γ-Lactone, D-Xylose, and 2-Hydroxy Benzoic Acid. Similarly community of attached soil is not able to utilize only 4 carbon sources which are D-Xylose, 2-Hydroxy Benzoic Acid, γ-Hydroxybutyric Acid, and D-Glucosaminic Acid. So it can be concluded that highway soil and attached soil sample are very rich in microbial community which utilizes maximum carbon sources. Evenness is very high in all four types of soil sample which shows that microbial population is evenly distributed and are able to equally utilize the maximum carbon

sources. All richness values are comparable with substrate richness which indicates the presence of resilient community.

Analysis of variance (ANOVA) and principal component analysis (PCA) were used to depict the differences of the soil bacterial functional diversity of microbial communities of different habitats, ranging from natural native soil environments to organic compost soil. Figure 9 represent the principal component analysis of CLPP of all form of soil microbial community.

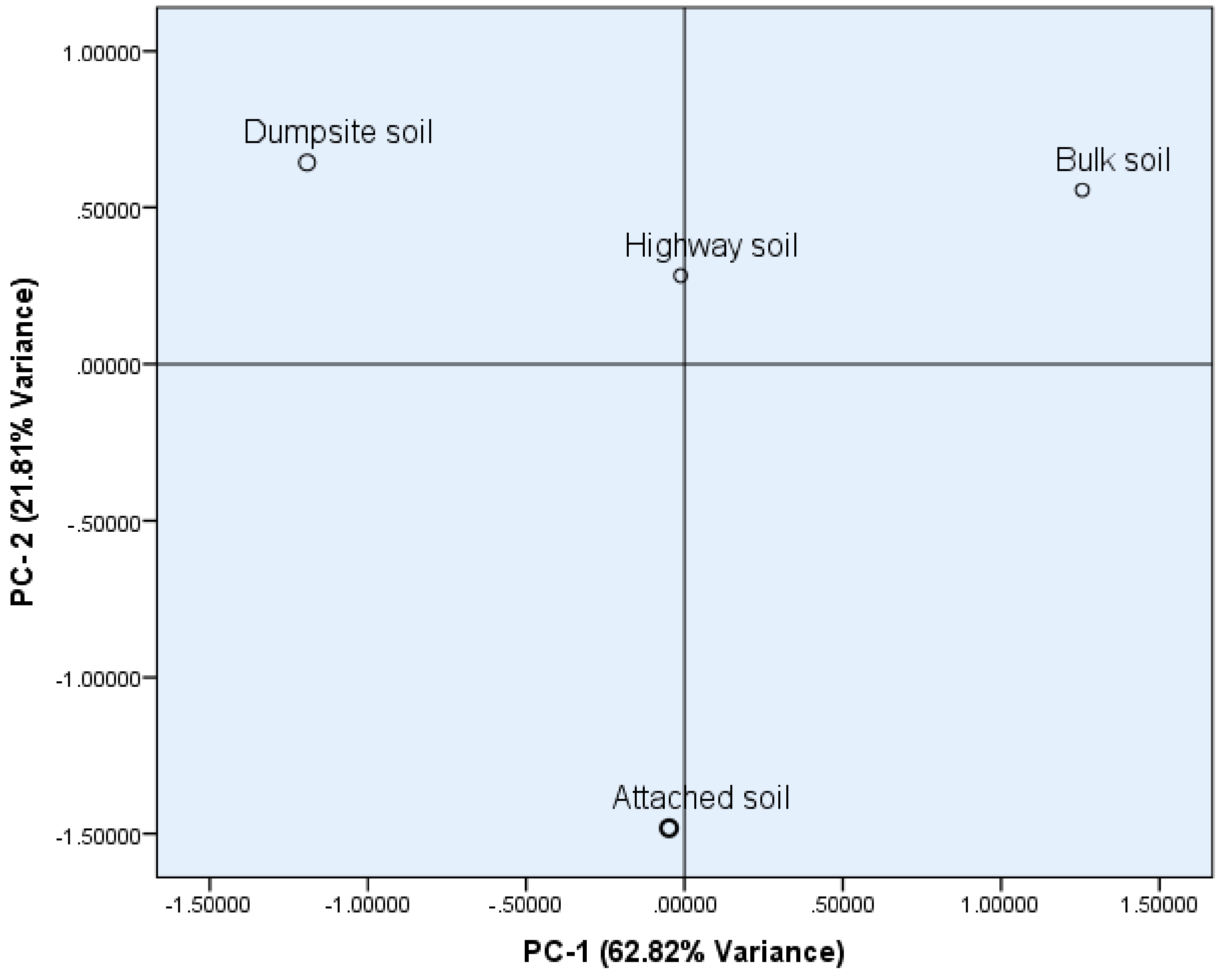


Figure 9: CLPP of Ecoplate data obtained from 60 h incubation from contaminated soil samples

Figure 9 interprets the normalized data from incubation time of 60 h subjected to principle component analysis (PCA), which was performed with correlation matrix. PCA generated three factor above eign value of 1 and scores were designated as PC1, PC2, PC3 etc. scatter plot of PC1

and PC2 at 60 h are presented in figure , where just two principle components could explain as high as 62.82% and 21.81% of the variance present in data at 60 h of incubation. According to above graph, all four soil sample showed completely different microbial community when compared with each other.

3. *In vitro* biodegradation of polymer

3.1 Growth of *P. citronellolis* on heat, UV, and alkali pre-treated various polymer samples

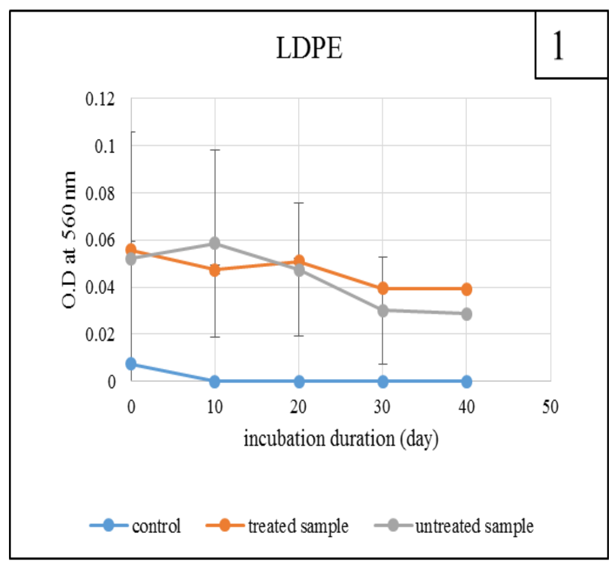
Growth of organism in MSM with polymer (plastic) as sole carbon source was measured by withdrawing 1 ml of inoculated medium at regular time intervals and measuring its absorbance at 560 nm.

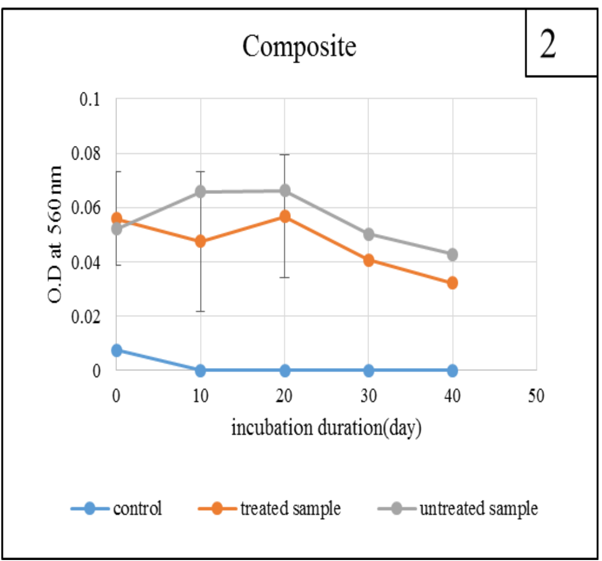
3.1.1 Growth of *P. citronellolis* on heat pre-treated polymer chips

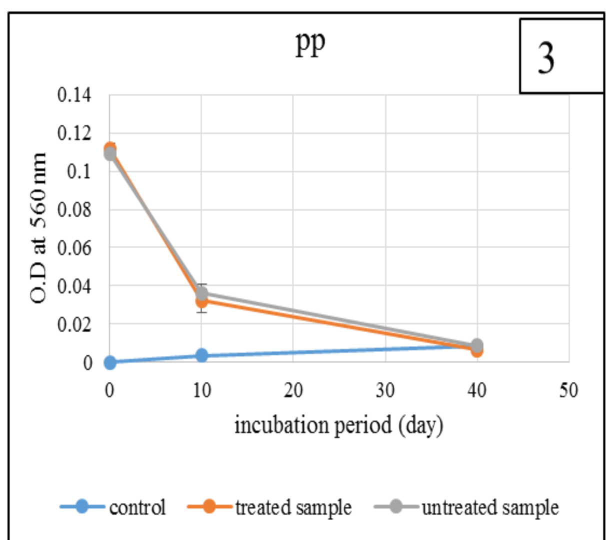
Lee *et al*, (1991) reported that heat pre-treatment has effect on degradation of HDPE polymer upto 68% within 8 days of incubation with *S. viridospores.*

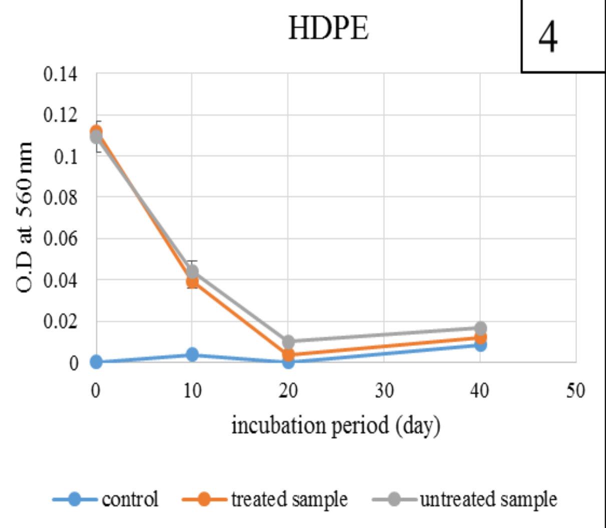
In our study heat pre-treated polymers did not show effect on growth of *P. citronellolis* and also on polymer degradation. The cell population continuously decreased in all type of pre-treated and untreated polymers when taken as individual carbon source . But mixture of all heat pre-treated polymers inoculated with *P. citronellolis* showd positive effect on growth of after 30 days of incubation. No considerable changes was observed during 30 days of incubation and organism reached to decline phase.

Figure 10 indicates growth of *P. citronellolis* in MSM containing heat pre-treated polymer chips as a sole source of carbon at regular intervals of 10 days for 40 days.


LDPE
1
O.D at 560 nm
0.12
0.1
0.08
0.06
0.04
0.02
0
0
10
20
30
40
50
incubation duration (day)
control
treated sample
untreated sample


Composite
2
O.D at 560 nm
0.1
0.08
0.06
0.04
0.02
0
0
10
20
30
40
50
incubation duration(day)
control
treated sample
untreated sample


pp
3
O.D at 560 nm
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
10
20
30
40
50
incubation period (day)
control
treated sample
untreated sample


HDPE
4
O.D at 560 nm
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
10
20
30
40
50
incubation period (day)
control
treated sample
untreated sample

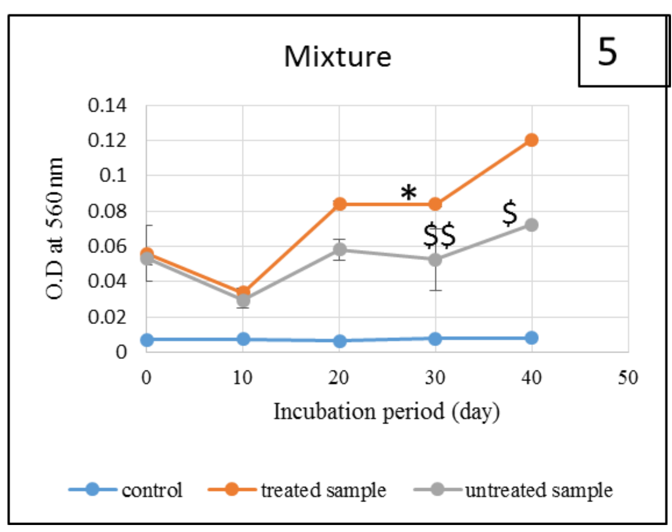
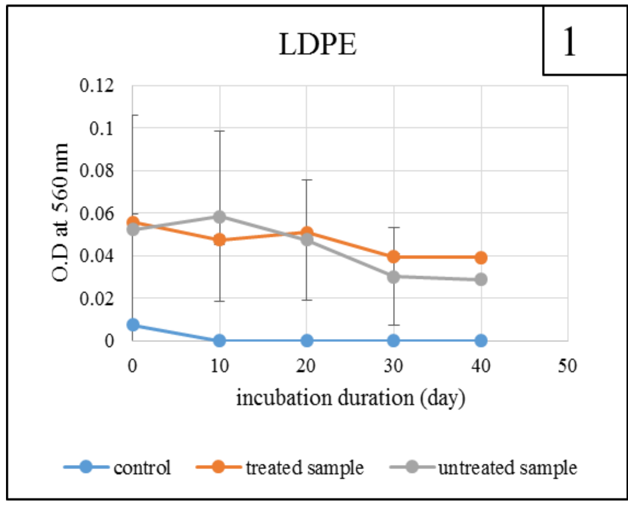

Mixture
5
O.D at 560 nm
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
*
$$
$
0
10
20
30
40
50
Incubation period (day)
control
treated sample
untreated sample

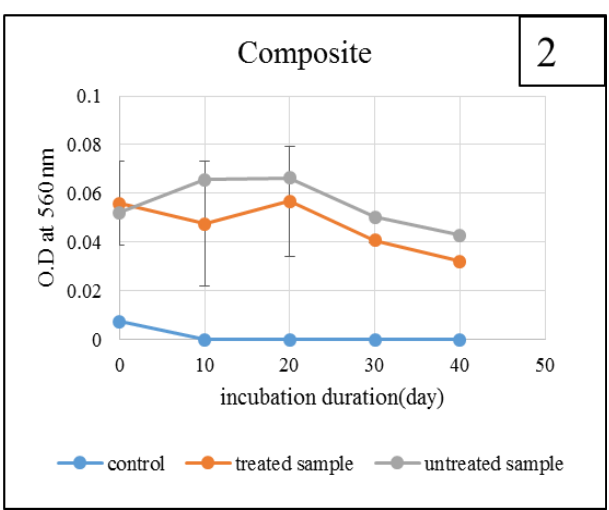
Figure10: Analysis of growth of *P. citronellolis* in MSM media containing heat pre-treated (1) LDPE, (2) composite, (3) PP, (4) HDPE and (5) Mixture of all polymers as sole carbon source.

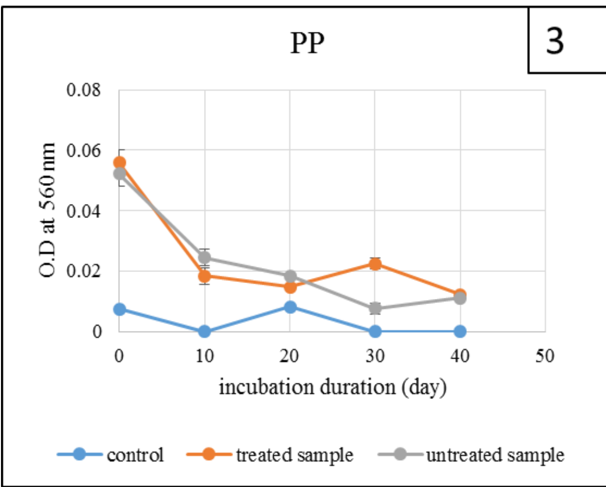
(Note:'*'= P value < 0.05 for heat pre-treated mixture;'$'= P value < 0.05 for heat pre-treated mixture; '$$'= P value < 0.01 for heat pre-treated mixture: when compared to control; n = 2)

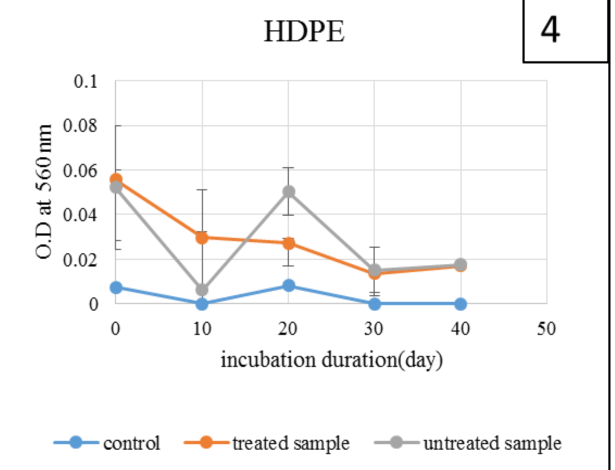
3.1.2 Growth of *P. citronellolis* on UV pre-treated polymer chips

Figure 11 indicates growth of *P. citronellolis* in MSM containing UV pretreated polymer chips as a sole source of carbon and observed at regular intervals of 10 days for 30 days.









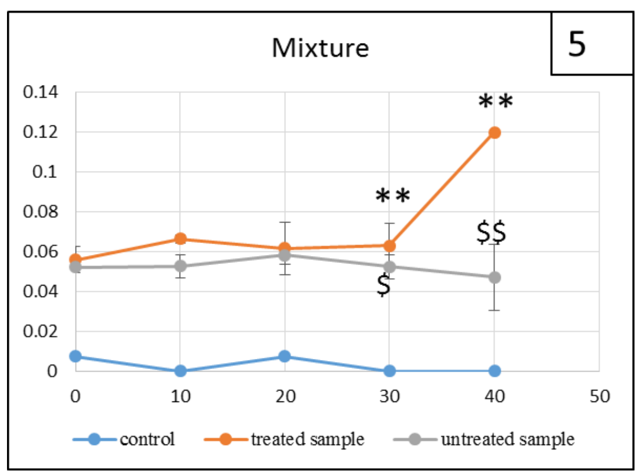


Figure11: Analysis of growth of *P. citronellolis* in MSM media containing UV pre-treated (1) LDPE, (2) composite, (3) PP, (4) HDPE and (5) Mixture of all polymers as sole carbon source

(Note:'**'= P value < 0.01 for UV pre-treated mixture; '$'= P value < 0.05 for UV pre-treated mixture; '$$'= P value < 0.01 for UV pre-treated mixture: when compared to control; n = 2)

Figure11 shows that there was no significant growth was observed except composite polymer with the incubation time of 20 days. Turbidity was not observed as well as clumps of cell were also absent. UV pretreatment was not found to be effective for growth and degradation with incubation time of 40 days in LDPE, PP and HDPE sample. No growth was observed in untreated polymer sample as well.

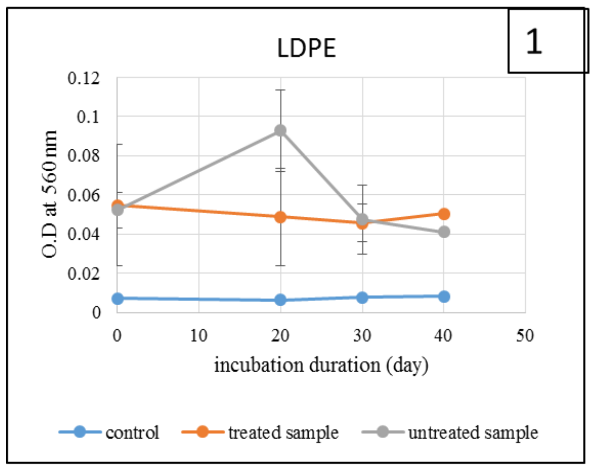
Exponential growth of *P. citronellolis* was found in only UV pretreated composite sample in compare to untreated composite polymer which shows that the UV exposure would have weakend the composite polymer and make them easily available carbon source for the organism and facilitated colonization of *P. citronellolis*.

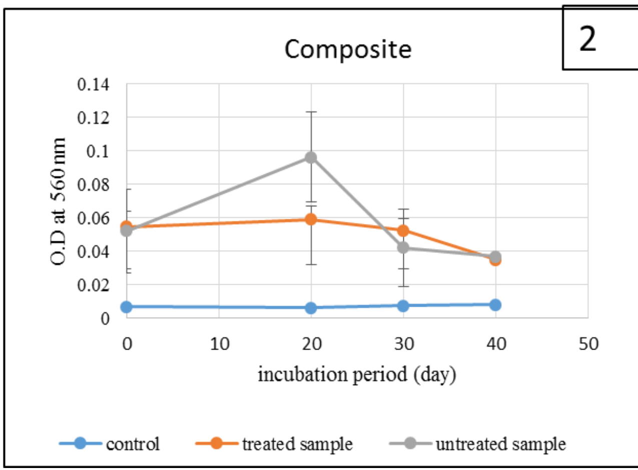
Significant growth of *P. citronellolis* was found in mixture of UV pretreated polymer sample after 30 days of incubation. It is assumed that addition of growth supplement and further incubation may enhance the growth condition. It was also reported (Sowmya *et al*, 2014) that *Bacillus cereus* had shown 14 % of degradation of HDPE by the influence of UV pretreatment within 3 month of incubation whereas only 3.4 % degradation of surface sterilized HDPE was found within 3 months of incubation.

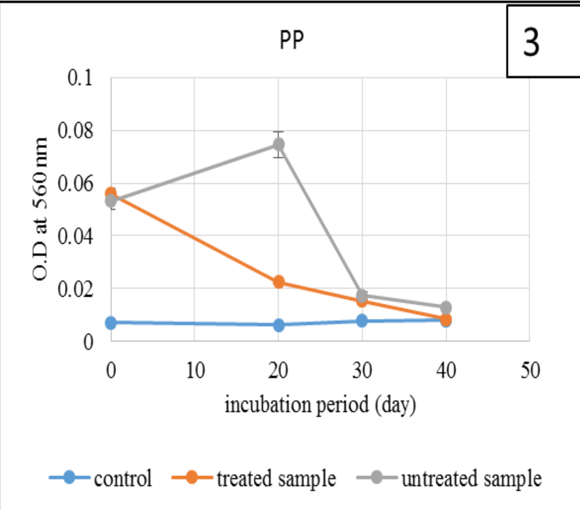
3.1.3 Growth of *P. citronellolis* on alkali pre-treated polymer chips

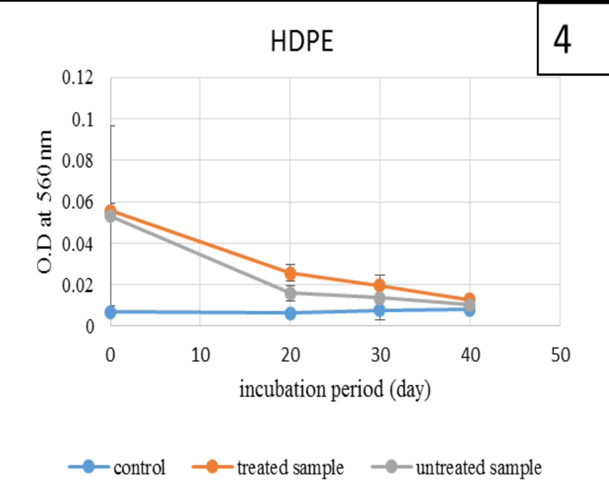
Figure 12 showed the increase in growth of *P. citronellolis* in the untreated LDPE, PP and composite sample as compared to alkali pretreated polymer samples after incubation of 10 days. Continuous decrease in growth of *P. citronellolis* was observed in all type of alkali pretreated polymer sample. No considerable increase in O.D. was observed over 40 days of incubation. Further incubation may enhance the growth condition.

So it can be concluded that heat, UV and alkali pretreatment is not much effective for the growth of *P. citronellolis*. Since adhesion of cell to surface of polymers can be considered to induce the polymer degradation following colonization (ref), hence the biofilm formation of bacterial cells on surface was examined using crystal violet assay.









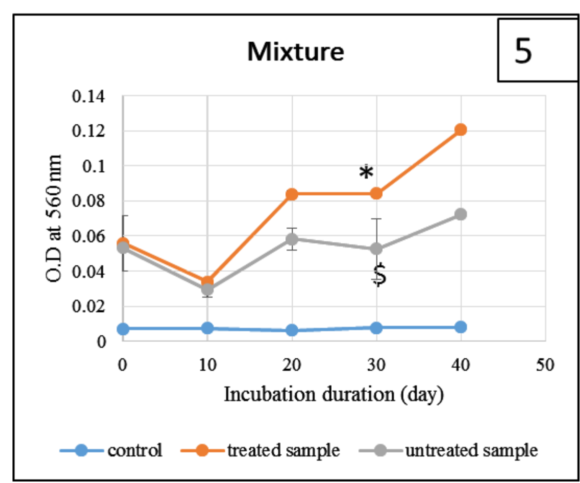


Figure 12: Analysis of growth of *P. citronellolis* in MSM media containing alkali pre-treated (1) LDPE, (2) composite, (3) PP, (4) HDPE, and (5) Mixture of all polymers as sole carbon source.

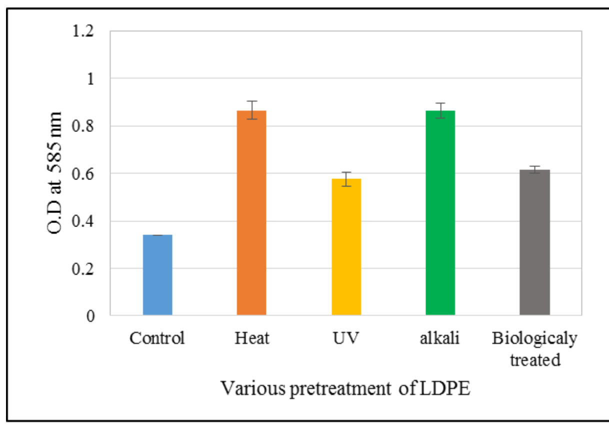
(Note:'*'= P value < 0.05 for alkali pre-treated mixture; '$'= P value < 0.05 for alkali pre-treated mixture when compared to control; n = 2)

3.2 Biofilm formation determination by CV assay

In one of the mechanisms of adhesion to surface, organisms are found to produce polysaccharide or related compound to establish first association with the surface. Such strains are proved to be primary invaders and useful in generation of biofilm on tough surfaces. In order to qualitatively assess the ability of *P. citronellolis* for developing such film formation, crystal violet assay was performed.

3.2.1 CV assay of heat, UV and alkali pre-treated various polymer samples

Because of adherence of *P. citronellolis* on Heat, UV, alkali pre-treated and biological treated four different polymer- LDPE, COMPOSITE, PP, HDPE, highest significant differences in absorbance (OD_{585}) were observed in case of heat and alkali pre-treated LDPE plastic. Whereas low difference in absorbance values were observed in case of UV pretreated LDPE plastics compare to heat and alkali pre-treated LDPE plastic whereas similar to biological treated plastic after 40 days of incubation (Figure:13-1).



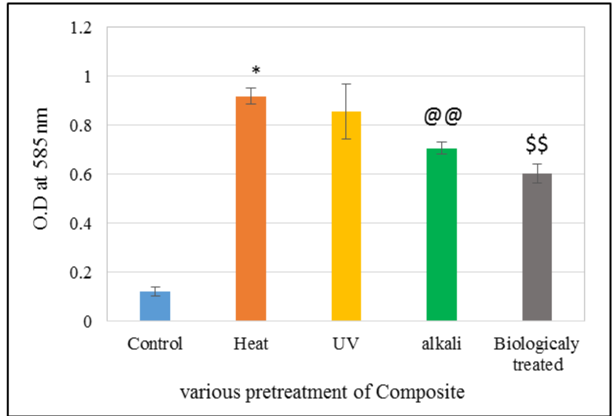
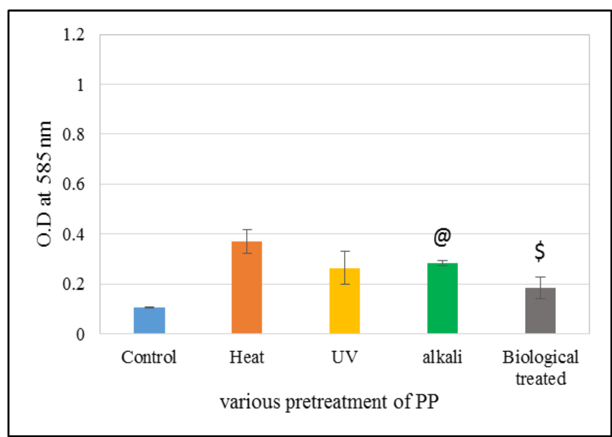


Figure 13: 1) LDPE Pre-treated samples 2) composite pre-treated samples

(Note: "***" = P value < 0.001; '**' = P value < 0.05; '*'= P value < 0.01; n = 2 when compared to Control)

In case of composite pretreated samples (figure:13-2), compare to biological treated composite sample heat pre-treated shows the maximum biofilm formation while alkali pre-treated shows the minimum biofilm formation on the surface of the composite polymer.



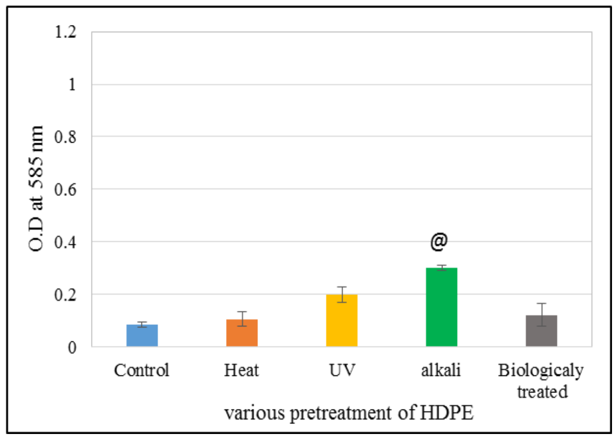


Figure13: 3) PP Pretreated samples 4) HDPE pretreated samples

(Note: "***" = P value < 0.001; '**' = P value < 0.01; '*'= P value < 0.05; n = 2 when compared to Control)

In case of PP pretreated samples (figure:13-3), Biofilm formation is quite similar to the LDPE pretreated samples. In case of HDPE pretreated samples (figure:13-4), bacterial adherence was higher in alkali pretreated sample compare to heat and UV pretreated sample. Minimum biofilm formation was found in heat pretreated sample compare to biological treated sample.

3.4 Determination of biofilm formation by SEM analysis

Details of surface changes and microbial attachments at the microscopic scale was observed using SEM. The comparative analysis of scanning electron micrograph was done with the pretreated, biologically treated and untreated composite sample at different magnification of 1000 X to 10,000 X. Sepperumal *et al.*, (2013) were reported that SEM micrograph of UV, heat and HNO_3 treated PET reveals cracks, pinhole and degradation in the form of crystals and cavities. PET treated with heat, HNO3, UV and inoculated with bacteria has underwent obvious changes. Adhesion of bacteria and formation of cracks on the surface was observed Thus UV, heat, HNO_3 and *Pseudomonas sp.* induce changes in the morphology of PET surface.

Micrograph of heat pretreated composite sample inoculated with *P. citronellolis* into MSM medium (Figure:14-1 and 14-2) clearly indicates the bacterial colonization after 40 days of incubation due to the utilization of polymer as a carbon source. No structural deformation was found on the surface of the heat pre-treated composite polymer.

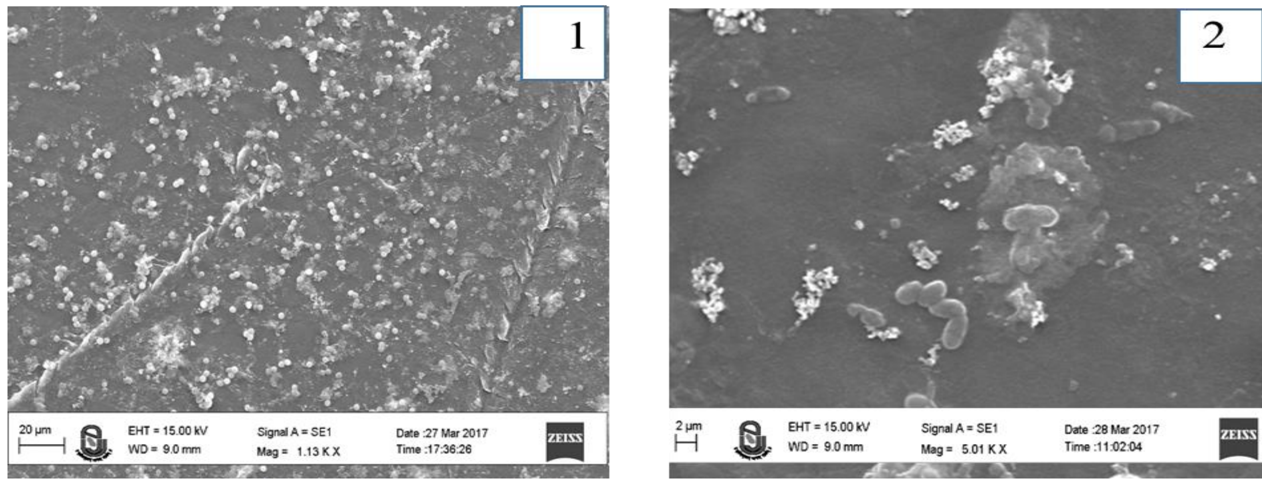


Figure 14: 1) Micrograph of heat pretreated composite sample at 1130 X magnification;
2) Micrograph of heat pretreated composite sample at 5010 X magnification

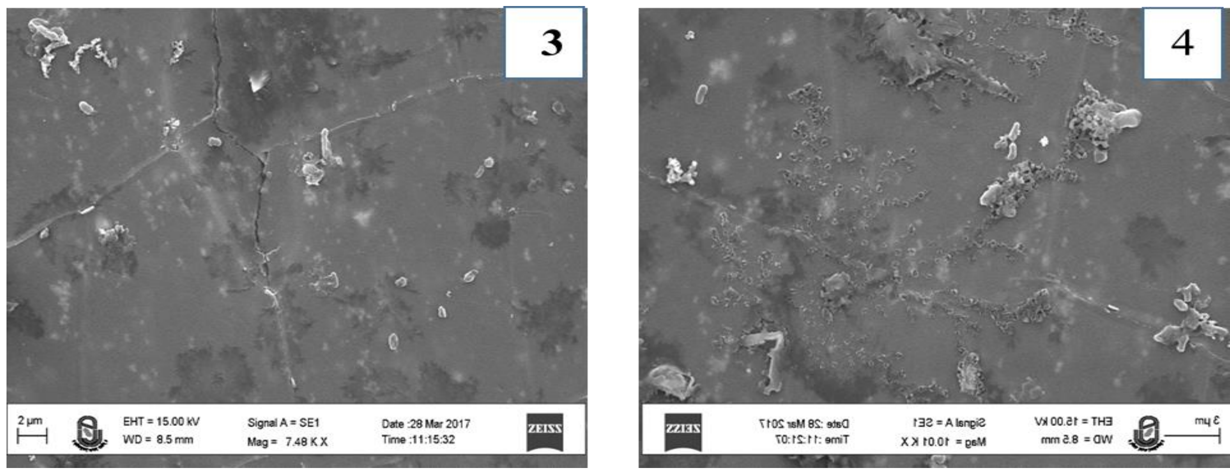


Figure 14: 3) and 4) Micrograph of UV pretreated composite sample at 7480 X and 10010 X magnification respectively.

Micrograph of UV pretreated composite sample inoculated with *P. citronellolis* into MSM medium (Figure 14-3 and 14-4) shows the dense microbial colonization on the surface of polymer compare to micrograph of heat pretreated composite sample after 40 days of incubation. Partial structural deformation like crack, physical damage (Figure:14-3) and patches (figure:14-4) was also found which may be due to colonization of *P. citronellolis.*

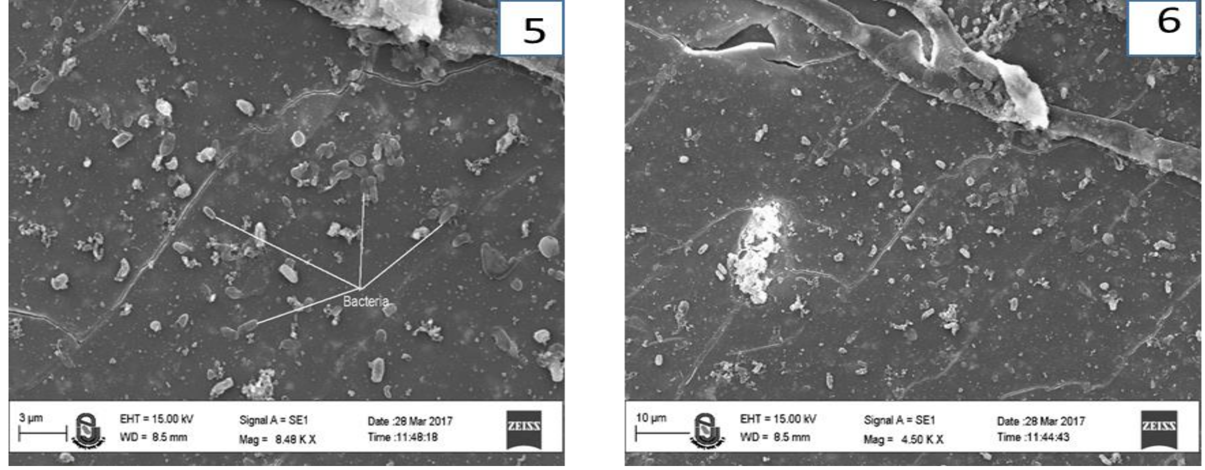
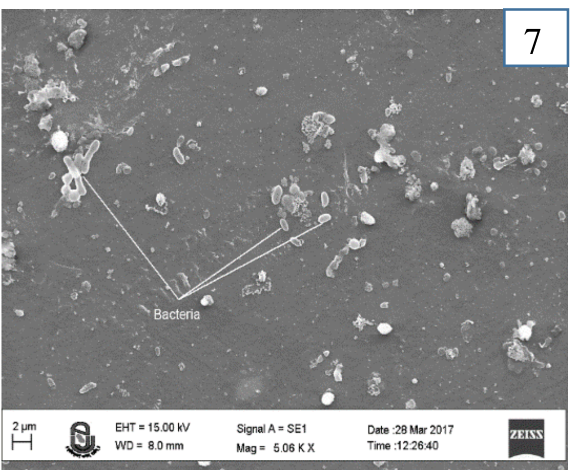


Figure 14: 5) and 6) Micrograph of alkali pretreated composite sample at 8480 X and 4500 X magnification respectively.

Micrograph of alkali pretreated composite sample (Figure:14-5and 14-6) clearly shows single type of bacterial colonization upon incubation with *P. citronellolis* due to the utilization of polymer as a carbon source and crack appears after 40 days of incubation indicating polymer degradation which may be due to chemical damage or combination of both physical and chemical damage. Similarly physical damage clearly seen into the figure 14-6.



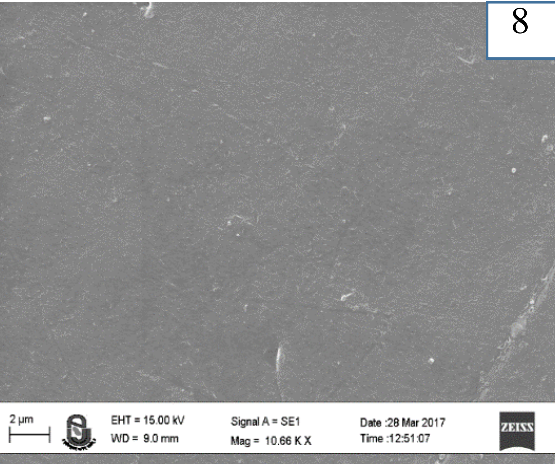


Figure 14: 7) Micrograph of biologically treated composite sample (with inoculum) at 5060 X magnification. 8) Micrograph of untreated composite sample (without inoculum) at 10,660 X magnification.

Micrograph of biologically treated composite sample (figure14-7) showed very low bacterial colonization as compared to heat, UV and alkali pretreated composite samples when inoculated with *P. citronellolis*. Structural deformation was also not observed after 40 days of incubation. Micrograph of untreated composite sample (figure14-8) clearly indicates that polymer was free from defects having smooth surface without any crack or patches and bacterial colonization after 40 days of incubation.

3.5 Weight loss determination

The plastic film can act as a substratum for adhesion by microorganisms. However, no significant decrease in weight and area of any polymeric material was observed. But *Pseudomonas citronellolis* was found to form visible film like layer on various pretreated and biologically treated polymer samples. There was no significant increase in the optical density of medium due to growth of organism in suspended form. Prolonged incubation may result in statistically singificant

increase O.D and reduction of weight. Nanda and Sahu (2010) reported that degradation of polythene 40.5%, 37.5% and 33% by *Pseudomonas, Brevibacillus, Rhodococcus* spp. respectively with incubation time of 3 weeks. In our study we found that *P. citronellolis* did not substantial reduction in weight of polymer with the incubation time of 36 days.

4. Tensile strength analysis of various pretreated composite polymer strips

The force per unit area (MPa or psi) required to break a material in such a manner is the ultimate tensile strength (UTS) or tensile strength at break.

Figure 15 displays tensile strength of heat, UV, alkali and biologically treated composite sample were used in this study. Tensile analysis was done on 15 cm×2 cm strips of various pretreated composite polymer strips after 40 days of incubation.

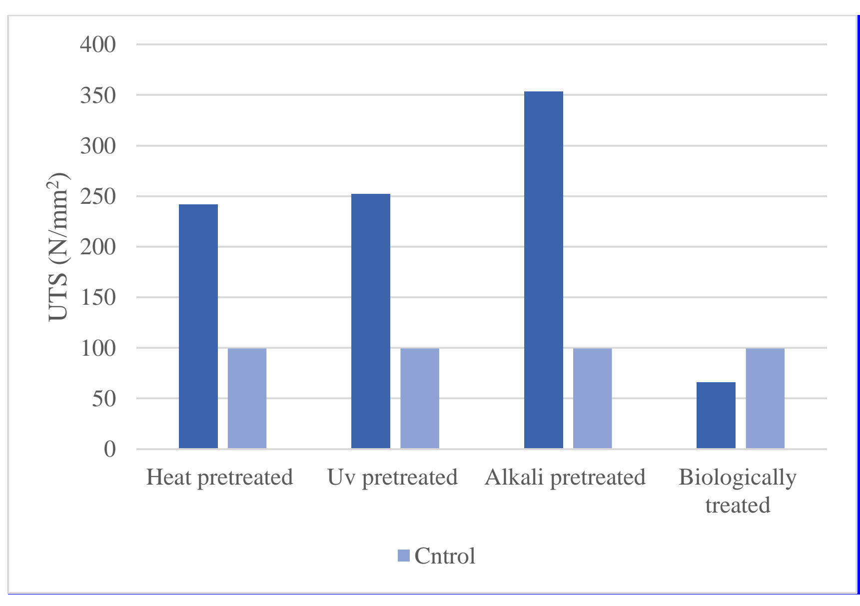


Figure15: UTS of various pretreated composite sample

Mathur *et al* (2011) reported 61% reduction in tensile strength of polyethylene film after 1 month of incubation with fungal isolates. In present study reduction in tensile property was only affected by the only biologically treated composite sample without pre treatment. In our study, signifciant Increase in tensile strength in heat, UV and highly significant increase in alkali pretreated composite sample was found.Nearly 30 % reduction in tentsile strength was observed in biologically treated composite strips after 40 days of incubation. According to UTS analysis, heat, UV and alkali pretreatment has no significant effect on the loss of mechanical property of the

composite polymer. Whereas UTS of untreated polymers was reduced by biological treatment. Reduction in tensile strength is indicator of weakening of polymer structure at base level and prolonged incubation with further detailed analysis may help in optimization of polymer degradation.

5. Fourier Transform Infrared Spectrophotometry analysis

FT-IR analysis widely applied to identify the interactions between the macromolecules during degradation process. It was performed to measure the IR of the films in the frequency range of 4000–400 cm^{-1}. FT-IR analysis was carried out for heat, UV and alkali pre-treated composite polymer, biologically treated composite polymer and control. Control spectra of composite polymer film (not treated with microbes) displayed a number of peaks reflecting the complex nature of the composite polymer (Figure 20).

When various pre-treated and biologically treated composite polymer samples (after incubation with *P. citronellolis*) were analyzed, variation in intensity of bands were observed in different regions (Fig.20). For control spectrum, the characteristic absorption bands were assigned at 3333.41 cm^{-1} (O-H bond), 2904.33 cm^{-1} (C-H bond), 2663.93 cm^{-1} (O-H bond), 1600.07 cm^{-1} (C=O bond), 1174.59 cm^{-1} (C-O bond), 506.33 cm^{-1} (C-Br bond), 613.86 cm^{-1} (C-Cl bond). The structural variations observed in the heat pre-treated composite polymer are shown in Figure 16. The intensity of the major peak value was increased compare to control which indicates the presence of high concentration of similar groups. The peak at 1174.59 cm^{-1} corresponds to C-O tertiary bond having strong stretching vibration and similarly the peak at 1043.73 cm^{-1} corresponds to C-O primary bond that has been disappeared in heat pre-treated composite after incubation with *P. citronellolis*. The significant increase was found at the peak value 632.68 correspond to C-Cl stretching bond.

Similarly, the FTIR spectra of UV pre-treated composite polymer illustrated major structural variation (peaks between 2500-3000 and 1500-1900 cm^{-1}) in comparison with the control (figure 17). new band has been observed at 1828.35 cm^{-1} (C=O bond) and 552.35 cm^{-1} (C-Br bond) which supports the depolymerization activity of the *P. citronellolis*. The strong absorption peaks at 1523.32 cm^{-1} became weaker after microbial treatment to UV pre-treated composite polymer. Deformation at the peak value 3333.41 cm^{-1} (strong and broad O-H bond), 2663.93 cm^{-1} (strong and very broad O-H bond), 1043.73 cm^{-1} (N-O strong stretching bond) were also observed which

shows the significant effect of UV pre-treatment on composite polymer degradation. FTIR spectra of Alkali pre-treated composite polymer also showed microbial activity pattern little similar to the UV pre-treated composite polymer (figure 18). In addition, the intensity of those peaks reduced more compare to heat pre-treated composite polymer. While in only biologically treated composite polymer, no major structural changes was observed campare to various pre-treated composite polymer and control (figure 19).The reduction in intensity at two peaks were observed only. This demonstrated that various pre-treated composite polymer underwent major structural changes which are a direct indication of biodegradation (Corti et al. 2010; Esmaeili et al. 2013) by the *P. citronellolis*. The changes in the peak values of almost all functional groups support the conformational change on composite sample. Das and Kumar (2014) noticed the formation of new and disappearance of functional group in their LDPE degradation studies by *Bacillus amyloliquefaciens* strain.

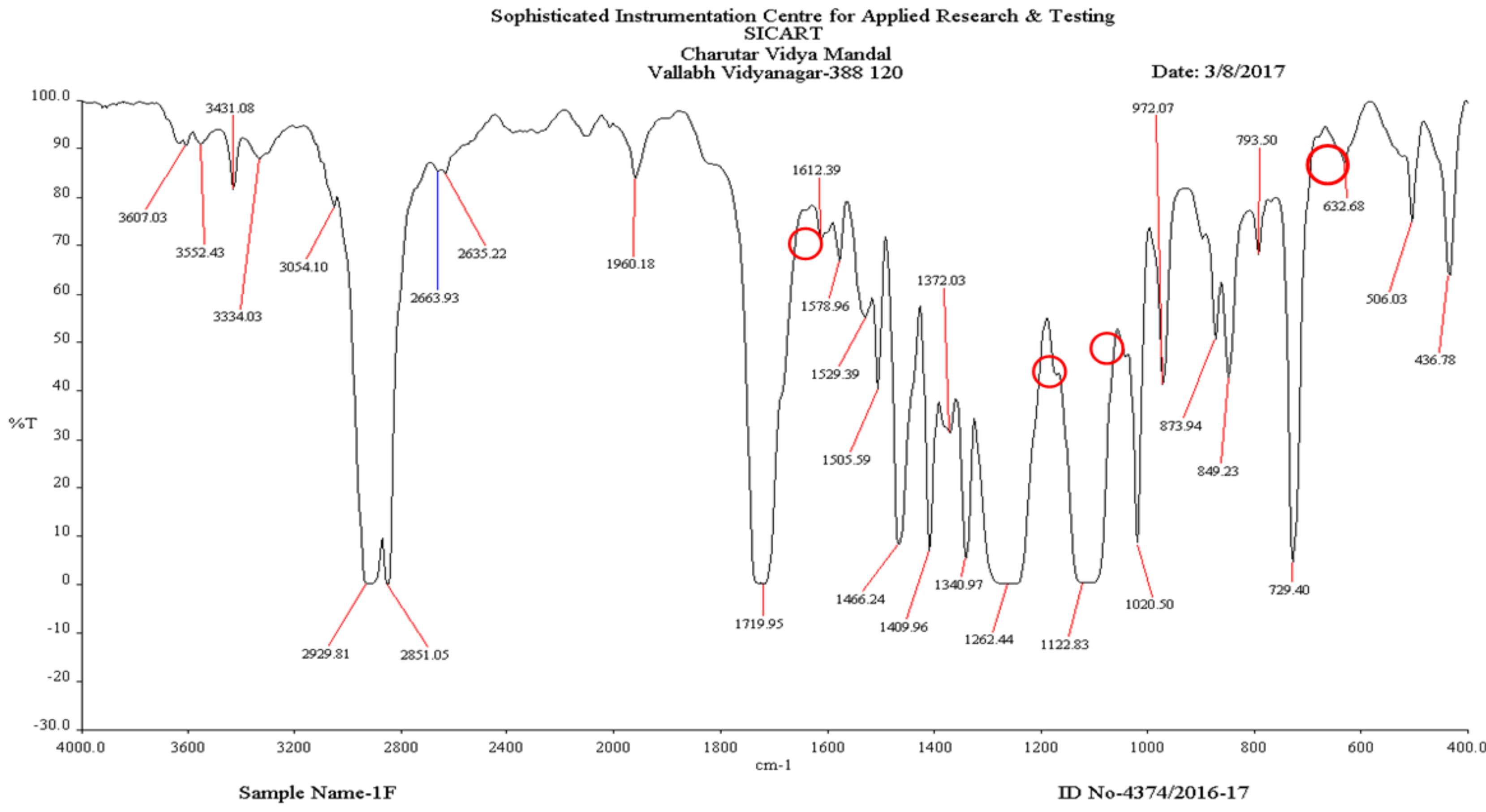


Figure 15: Spectra of heat pre-treated composite polymer

(Sample name: 1F-Heat pre-treated composite polymer; 2F-UV pre-treated composite polymer; 3F-Alkali pre-treated composite polymer; 4F- biologically treated composite polymer; 5F- control)

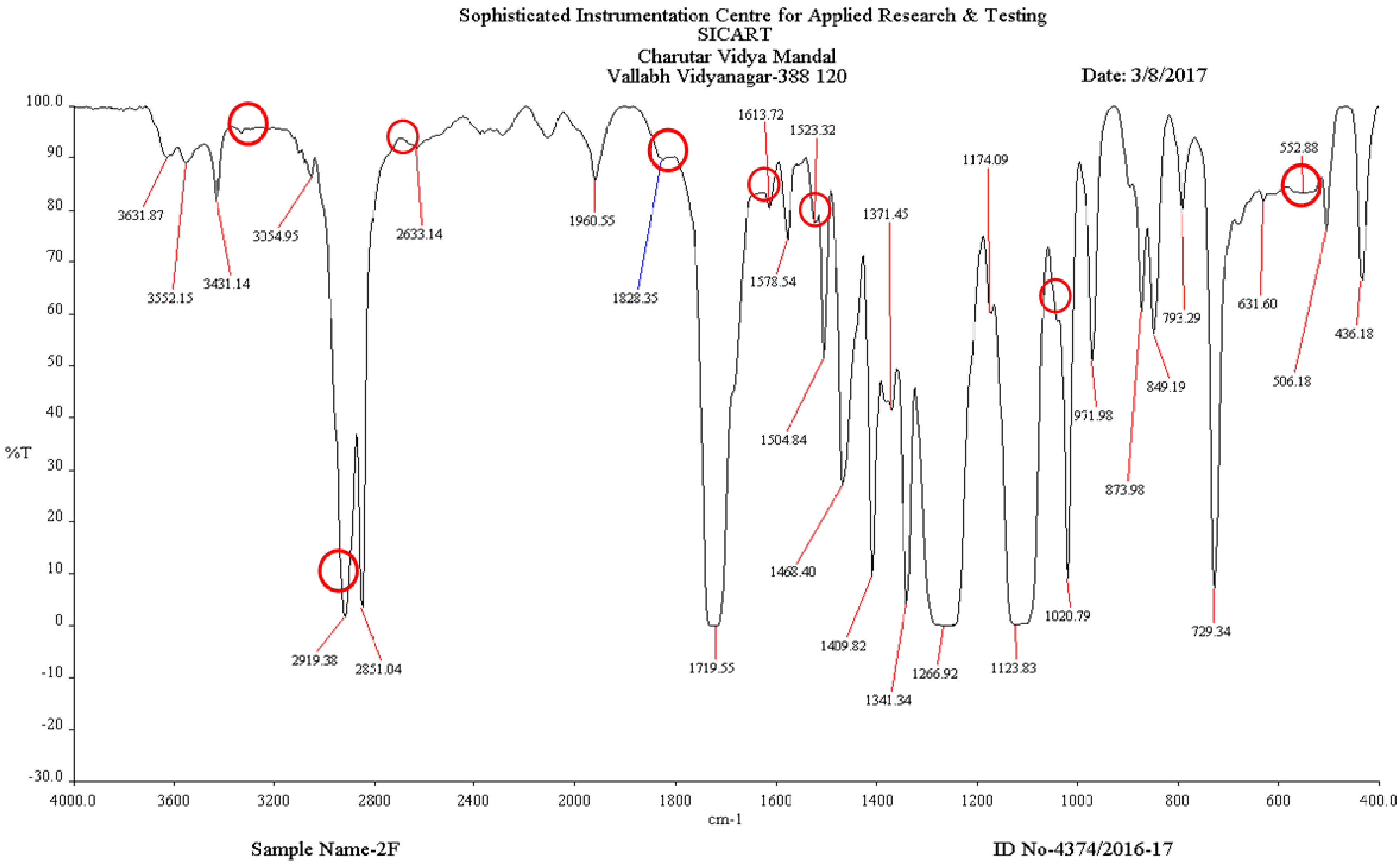


Figure 16: Spectra of UV pre-treated composite polymer

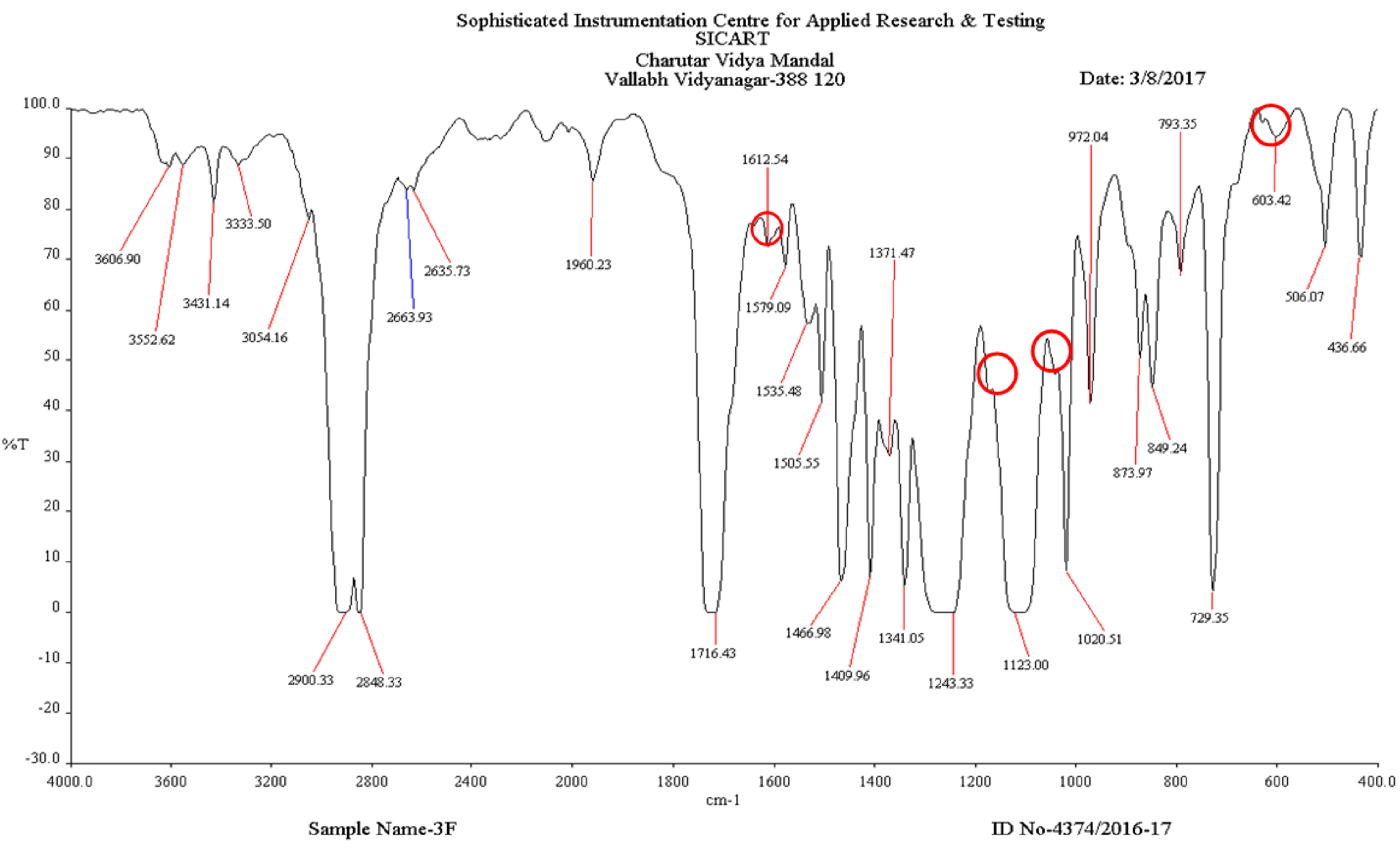


Figure 17: Spectra of alkali pre-treated composite polymer

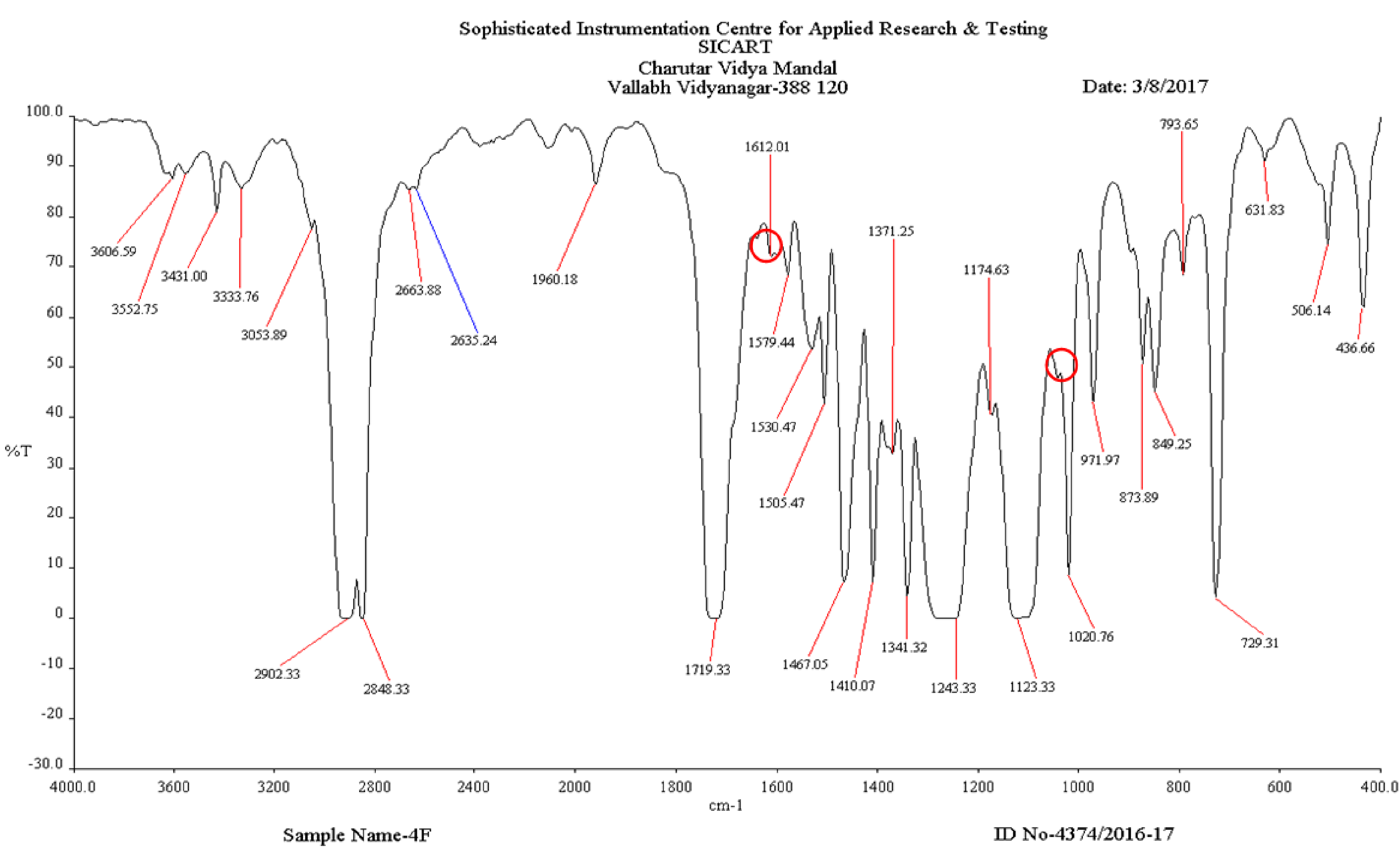


Figure 18: Spectra of biologically treated composite polymer

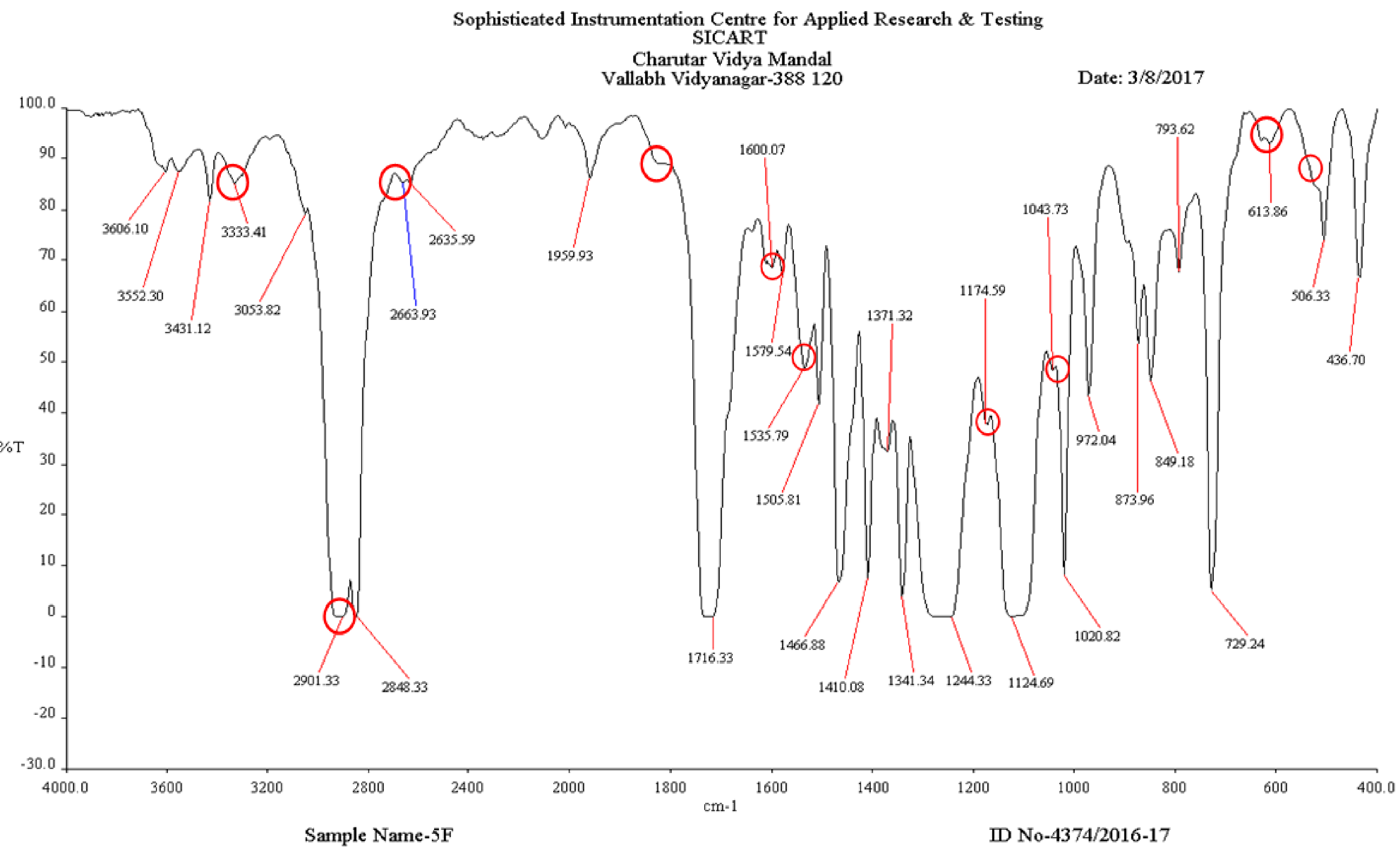


Figure 19: Spectra of untreated composite polymer

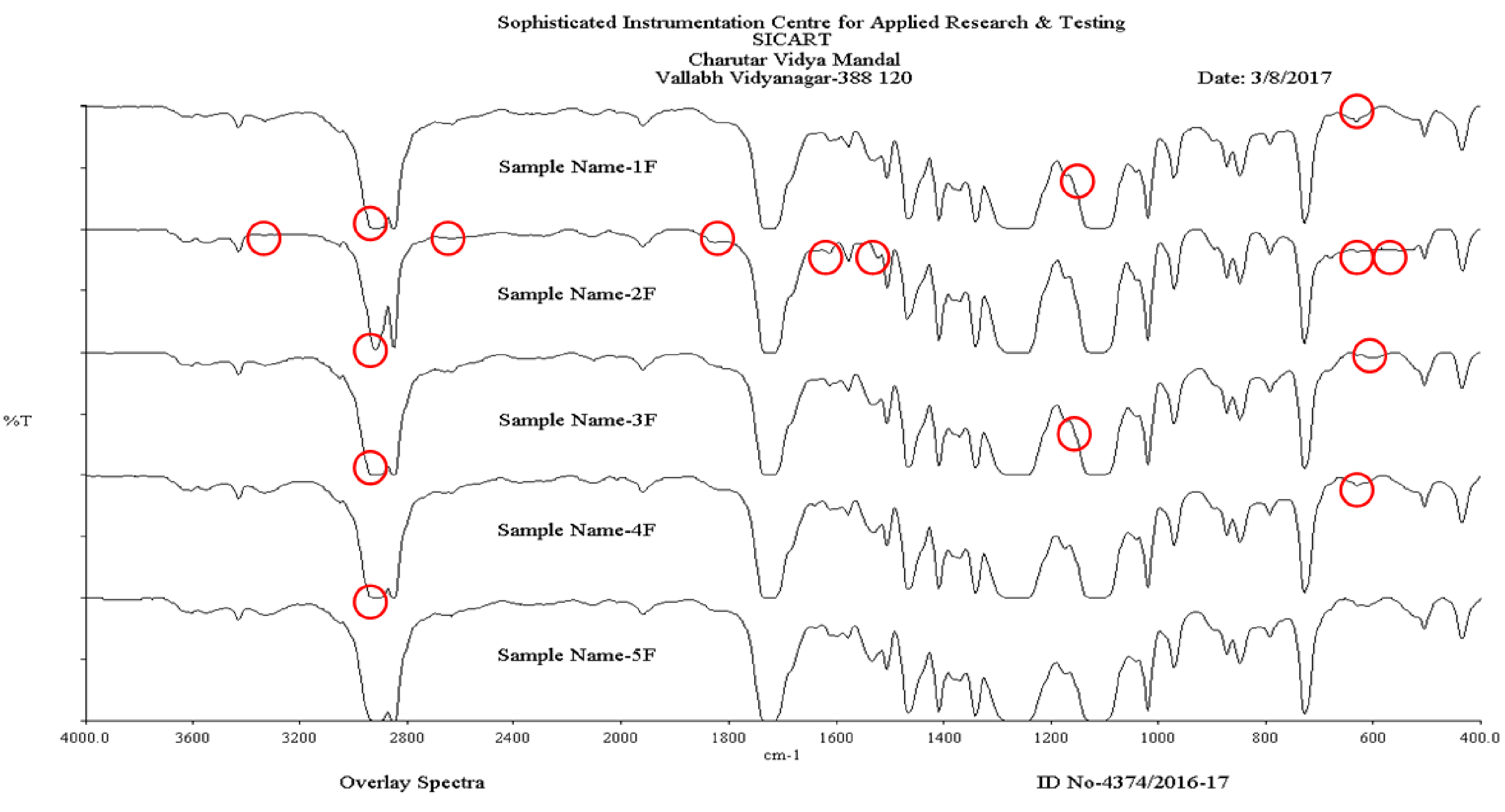


Figure 20: Overlay spectra of various pre-treated and biologically treated composite polymer with control

6. Degradation efficiency of organisms isolated from the dump site

6.1 Growth and CV assay of isolated organism on untreated LDPE chips and strips used for biodegradation

Growth analysis of organisms in MSM with polymer (plastic) as sole carbon source was measured by withdrawing 1 ml of inoculated medium at regular time intervals of 10 days and measuring its absorbance at 560 nm.

Figure 11 shows highest suspended growth in case of isolate PDS1 organism followed by PDS27 and PDS31 isolate as compare to control. The isolates PDS34 and PDS36 are similar in suspended growth while low difference was observed in case of isolates PDS3, PDS4, and PDS26 after 30 days of incubation. Prolonged incubation more than 30 days, may result in increased O.D. under laboratory conditions. Moreover, it can also be concluded that LDPE polymer is not suitable for growth of PDS7 organism.

Previous studies by Adetunji and Isola (2011) have reported biofilm formation by bacterial strain *Listeria monocytogenes* (SLM) and *Listeria spp.* on three different surfaces (wood, glass and steel).

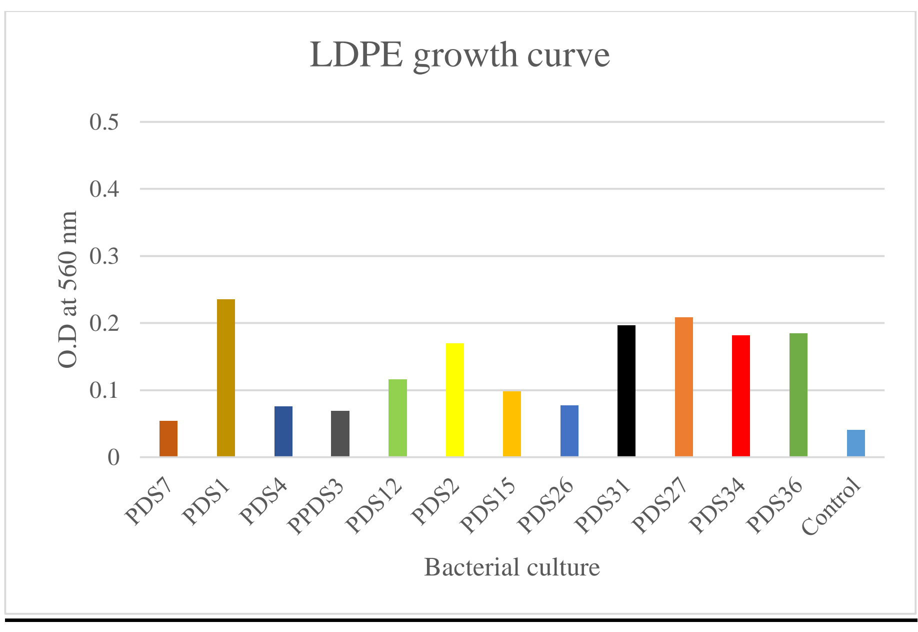


Figure 11: Suspended growth on untreated LDPE sample inoculated with 12 different isolated organism after 20 days of incubation time

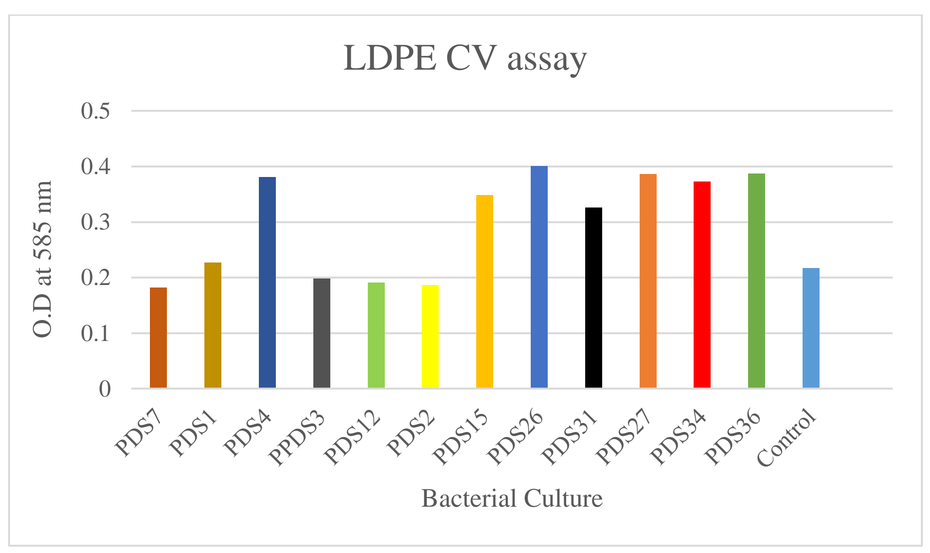


Figure12: CV assay of LDPE sample (without any pre-treatment) inoculated with 12 different isolated organism.

Figure 12 displays ability of 12 different organism for developing biofilm formation. The organism such as PDS27, PDS31, PDS34, and PDS36 having the higher significance difference in their suspended growth compare to control, also showed the higher capacity for biofilm formation. Whereas isolates PDS1 showing the highest suspended growth has lowest biofilm formation capacity. Isolate PDS26, showing the lowest suspended growth has the highest capacity for adherence to the LDPE polymer surface. Slow increase of the turbidity could be due to the formation of biofilms and the residues occurred during the biodegradation process.

6.2 Growth and CV assay of isolated organism on untreated composite chips and strips used for biodegradation

Figure: 13 depicts highest significant differences in suspended growth of PDS1 organism followed by PDS34 and PDS27 isolates compared to control. Isolate PDS3 and PDS36 are similar in their suspended growth while lower difference was observed in case of PDS7, PDS4, and PDS2 isolates

after 30 days of incubation. Prolonged incubation more than 30 days, may result in increased O.D. under laboratory conditions. It can also be concluded that composite polymer is not suitable for growth of PDS15 and PDS26 isolates.

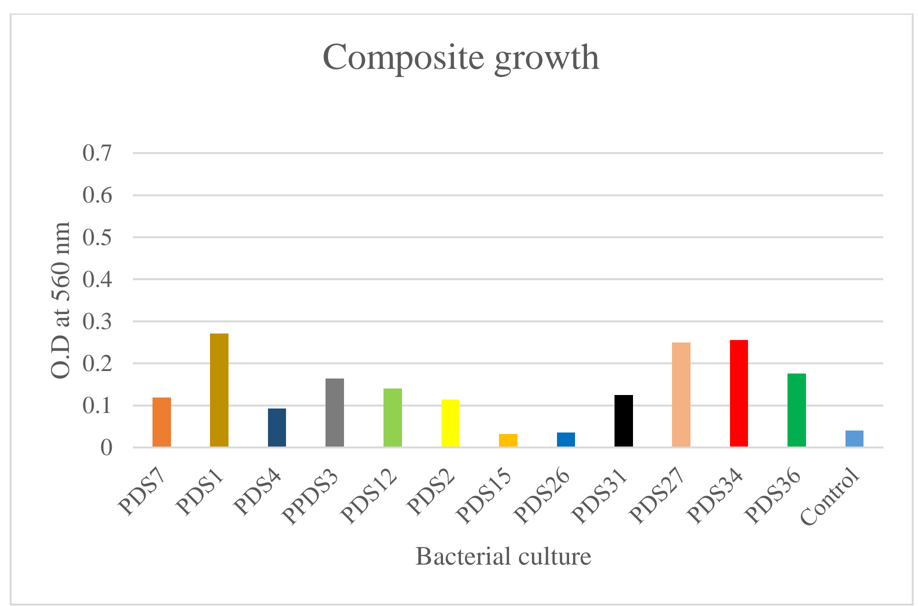


Figure:13 Suspended growth on untreated composite sample inoculated with 12 different isolated organism with control

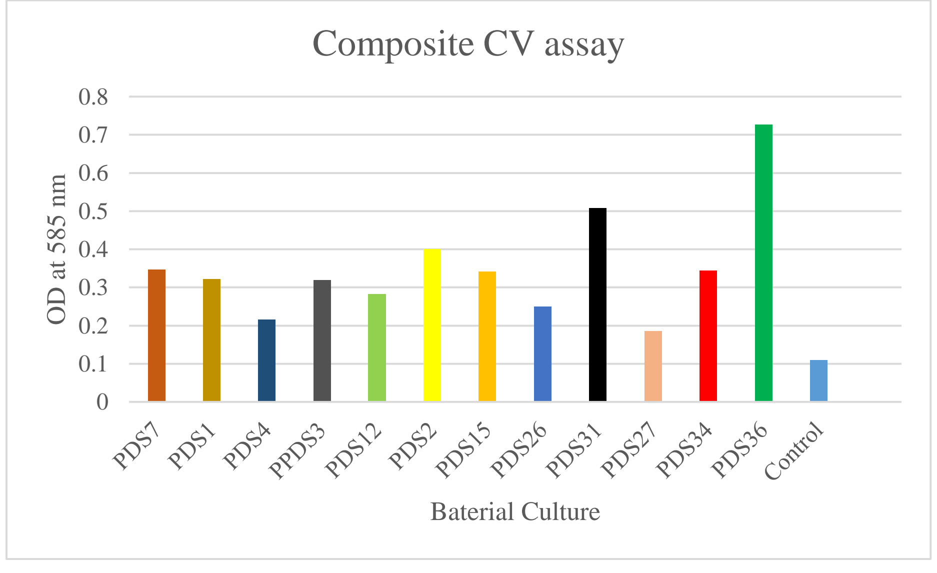


Figure:14 CV assay of untreated composite sample inoculated with 12 different isolated organism with control.

Figure 14 depicts that isolate PDS36 have the highest capacity for biofilm formation. PDS 15 and PDS26 organism showing the lowest suspended growth has the highest capacity for adherence to the composite polymer surface. Overall all the organism have the lower capacity for their suspended growth on composite sample but have higher capacity for the biofilm formation.

By comparing the degradation efficiency of isolates with the degradation efficiency of *P. citronellolis*, it can be concluded that *P. citronellolis* have higher capacity for their suspended growth and biofilm formation on the LDPE and composite polymer. Moreover it can be also said that heat-pre-treatment, UV pre-treatment and alkali pre-treatment have significant effect on influence of biodegradation pocess.

SUMMARY

Community level physiological profiling (CLPP) by Ecoplate® method was performed for characterization of whole microbial community. All the soil sample were differ in their substrate utilization pattern. Microbial community is evenly distributed in all the soil environment and utilize maximum carbon sources as a substrate except dumpsite soil sample.

Total 39 bacteria were isolated from soil samples and plastic attached soil collected from the Pirana dumpsite, Ahmedabad. From this 12 isolates having different colony characteristics were used for the study of degradation efficiency. This study showed that isolates PDS31, PDS34 and PDS36 have higher efficiency for biofilm formation compare to other isolates. While isolates PDS15 and PDS2 showed the invert proportion in suspended growth and biofilm formation. Biofilm formation efficiency and suspended growth of *P. citronellolis* was found higher when inoculated into MSM medium as compare to isolated organism from the dump site. Heat, UV, and Alkali pretreatment was given to the four types of plastics such as HDPE, LDPE, PP, Composite.

The minimum growth of *P. citronellolis* was confirmed by spectrophotometer analysis on all types of pre-treated polymer. When it was analyzed for its ability to survive in presence of such complex substrate, it was found to grow slowly on the different types of polymer. It was further confirmed by SEM analysis. Bacterial colonization, cracks, holes, and patches were observed on the surface of UV and alkali pre-treated composite polymer. So it can be concluded that heat pre-treatment is not much effective as other pre-treatment for the colonization. The change in physical and chemical properties was observed by UTM and FT-IR analysis. UTM analysis shows the 30 % reduction in tensile strength of only biologically treated composite (without any pre-treatment) compare to control. Whereas it showed higher significant increase in tensile strength of all three pre-treated sample which may be due to effect of pre-treatment on the annealing process of polymer. Prolong incubation may enhance the suspended growth of *P. Citronellolis* and shows the significant effect on polymer degradation.

Appendix

1. Media composition

Table 8: Media Composition For Liquid Media (Minimal Salt Media)

Components	Amount(g/L)
K_2HPO_4	1
KH_2PO_4	0.2
NaCl	1
$Cacl_2.2H_2O$	0.002
$(NH_4)_2SO$	1
$MgSO_4.7H_2O$	0.5
$CuSO_4.5H_2O$	0.001
$ZnSO_4.7H_2O$	0.001
$MnSO_4.H_2O$	0.001
$FeSO_4.7H_2O$	0.01

2. Reagent preparation

2.1 0.1% Crystal violate:

Take 0.1 g crystal violate, add in to distilled water and make up the final volume with 100 ml and covered with aluminum foil.

2.2 33% glacial acetic acid:

Add 33 ml acetic acid and make up the final volume 100 ml with distilled water.

2.3 TAE buffer

Buffer	TAE buffer
Stock solution/liter	242 g of Tris base 57.1 ml of glacial acetic acid 100 ml of 0.5 M EDTA (pH 8.0)
Working solution	40 mM Tris-acetate 1 mM EDTA

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