Isolation and identification of microbial consortium from corporation dump yard towards biological degradation of polymer

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Abstract

Polymer is made up of group of monomers which interact with terrestrial as well as maritime environment and cause severe pollution on earth. Low Density PolyEthylene (LDPE) is a type of light-weight polymer responsible for major pollution causing substance in the environment. Therefore, the present investigation was carried to isolate and identification of LDPE degrading bacterial consortium from a chronically dumped site. Subsequently, the isolated bacterial strains were identified as *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* through VITEK 2 COMPACT system along with biochemical analysis. Next, the percentage weight loss was calculated to explore the degradation efficacy of isolated bacterial consortium. Furthermore, FT-IR and SEM analysis was done in detail. FT-IR results indicated the obvious changes in functional groups occurred between treated and untreated LDPE. The Scanning Electron Microscopy results indicated that the erosions were observed on LDPE. Over all, this study hypothesized the isolated bacterial species from a chronically dumped site displayed high potential towards degrading the LDPE within a short span of time.

Keywords: Low Density PolyEthylene, Microbial degradation, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa.*

Introduction

Recent reports revealed that various human activities ultimately lead to the dumping of non – biodegradable wastes in the terrestrial and the aquatic environment (Ejaz et al., 2010). Among all, wastes such as plastic wraps, plastic containers, melt blown filter masks, straws, carrying bags, car covers, food packing covers, fishing nets etc., forms the major parts in the solid waste pollution (Isangedighi et al., 2020). It is well known that plastics are divided into four groups based on their biodegradability and raw materials and off them, Polyethylene (PE), polypropylene (PP), and poly (ethylene terephthalate) (PET) are the plastics which cannot have the ability to degrade by far (Tokiwa and Pranamuda, 2002).

In accordance with the scientific reports, plastic is one of the biggest risks to the world's well-being today since it takes more than 500 years to decompose (Warscheid et al., 2009). This automatically leads to the accumulation of plastic bed on our planet. The plastic bed accumulation affects both terrestrial and marine habitat. The factors that responsible for transferring plastic wastes from terrestrial to marine habitat includes rivers, streams and the wind. Various Non – Governmental Organizations voluntarily have taken steps to evacuate plastics from maritime environment. Increasing of plastic waste in aquatic environment shows that their attempt ends in failure.

Interestingly, Low Density PolyEthylene (LDPE) is used as the major packaging material for food items (Ncube et al., 2020). When the food items are packed and stored in LDPE covers, the toxic chemicals such as Bisphenol A, Dioxin and phthalates are released from these packs and all these hazardous chemicals from LDPE cause Cancer, Cardiovascular and Hepatic diseases in human being and other animals (Sung, 2010). Scientists have found that when animals eat plastics, the toxic chemicals transferred to their bodies (Liboiron et al., 2016). Approximately, one million birds and ten thousand marine animals die each year due to ingestion of plastics. Several researches were carried out by scientists to develop degradable plastics (Gajendiran et al., 2016).

The sole solution to deteriorate the dumped plastics in terrestrial and marine ecosystem can be achieved by the microbial consortium. The secretion of exoenzymes and endoenzymes by microbes attracts to the polymer surface and convert them into monomers (Huang et al., 1990).The main bacterial and fungal species that degrades plastics are *Pseudomonas aeruginosa, Pseudomonas stutzeri, Streptomyces badius, Streptomyces setonii, Rhodococcus ruber, Comamonas acidovorans, Clostridium thermocellum and Butyrivibrio fibrisolvens, Aspergillus niger, Aspergillus flavus, Fusarium lini, Pycnoporus cinnabarinus and Mucor rouxii* (Pathak et al., 2017). This will increase the degradation rate without any harm to the environment (Bhardwaj et al., 2012). Photodegradation, thermo – oxidative degradation, hydrolytic degradation and biodegradation by microbes are the four main mechanisms responsible for the polymer degradation (Andrady, 2011).

Therefore, an attempt was made in the laboratory to explore the degradation of Low Density PolyEthylene using bacterial

consortium isolated from the soil sample at Corporation Dump Yard. Interestingly, two different bacterial strains have been isolated and were chosen by screening from opacity method and they were identified based on their morphological and biochemical characteristics. Further, the isolated bacterial strains were used for the biodegradation of Low Density PolyEthylene.

Material and Methods

Sample collection

The soil sample was collected from a chronically dumped site located in Tamil Nadu's Corporation Dump Yard, Kodungaiyur, Chennai at latitude 13.0802″ N and longitude 80.1609″E. Approximately 10 grams of soil sample from 20 cms deep was collected and packed in LDPE packing cover and sealed. The sealed cover with the soil sample was preserved in thermocol chiller for transportation.

Collection of LDPE films

Twenty micron thick and translucent LDPE packs were procured from Integra Poly Packs, Coimbatore. To remove the debris, the LDPE packs were washed with 70% ethanol and then distilled water. The washed LDPE packs were air dried in Laminar Air Flow Chamber.

Isolation & Culturing of LDPE degrading bacterial consortium

To culture the LDPE degrading bacterial consortium, the Mineral Salt Medium (MSM) was used (Munir et al., 2018). The following constituents were dissolved in 100 ml of distilled water and sterilized. 1. K2HPO4 - 0.18 g, 2. NH4Cl - 0.4 g, 3. MgSO4.7H2O - 0.02 g, 4. NaCl - 0.01 g, FeSO4.7H2O - 0.001 g (Zajic et al., 1972). LDPE sheets (0.5 g) and 2 g of soil sample were added to the sterilized medium and incubated. Following, the setup was maintained in orbital shaker for two weeks at 36ºC and 100 RPM.

Isolation of LDPE degrading microbes

To isolate the LDPE degrading bacteria, the spread plate technique was followed from the incubated MSM. To obtain a pure culture, the microbial colony were picked from a selective spread plate and streaked over a newly prepared sterilized Nutrient agar Medium with the aid of inoculation loop. The plates were kept in incubator for 24 hours at 37ºC.

Screening of the efficacy of isolated bacteria for LDPE degradation

Opacity method

This method is employed to confirm the efficacy of isolated microorganisms for LDPE degradation. The Nutrient Agar Medium was prepared along with 1 g of starch and the surface of the agar was inoculated with isolated microorganisms (Orhan et al., 2000). The plate was left in incubator for 24 hours at 37ºC. The emergence of erosion in the surface of agar medium indirectly assures that the isolated bacterial species have the ability to degrade LDPE films.

Identification tests for isolated bacteria

Morphological characterization

Colony morphology

Bacteria develop quite quickly when provided with an abundance of nutrients. Diverse bacteria generate colonies with different appearances (Bergey et al., 1939). Bacterial colonies have some characteristics such as size, color, texture, form, margin and elevation. Nutrient agar with microbial samples is viewed by naked eye under laminar air flow chamber and then the characteristics of the isolated bacteria were recorded.

Gram's staining

The LDPE degrading bacterial isolates were identified using Gram's staining method by following Prescott's microbiology (Willey et al., 2011). The Gram's staining method is simple staining technique and this classifies the microbes into two groups namely Gram positive and Gram negative based on the form of cell walls. The smear was stained with a primary stain called crystal violet. Then the mordant, Iodine was added which helps to attach the dye to the cell wall. Then, it was decolorized by using alcohol. This step retains the Gram positive bacteria in crystal violet whereas, Gram negative bacteria become colorless. Finally, the smear was counterstained using safranin stain which gives pink color to Gram negative bacteria and leaves Gram positive bacteria in dark purple color.

Biochemical characterization

Motility test

To observe the motility of the isolated bacterial strains, hanging drop method was used. The cavity slide was cleaned and sterilized with ethanol. A microscopic cover glass was taken where a small drop of bacterial suspension in the Nutrient broth was added in the middle and the Vaseline was placed in the four corners. The cover glass with the bacterial suspension was placed in inverted position on the cavity slide and viewed under microscope (Mondal et al., 2008).

VITEK 2 COMPACT

The selected organisms were identified by using VITEK 2 COMPACT system. This is a computational technique which continually monitors the growth and the action of the bacterial isolates inside the well of the test cards called ID – GN card (Gram Negative). The reagent card contains 64 wells with different substrates. This technique works based on fluorogenic method and turbidimetric method (Pincus, 2006). This gives a high accuracy result with genus and species name which is based on the measurement of various activities mainly, acidification, enzyme hydrolysis and alkanization.

Microbial growth studies

To monitor the growth of bacterial isolates, HB and L UROQUATTRO was used. The number of colonies formed was identified by light scattering technology and it provides real time growth curves and the quantitative bacterial counts in CFU/ml.

Microbial degradation assay of LDPE

Weight loss calculation

To determine the quantity of plastic degraded by the selected bacterial isolates, the percentage weight loss method was followed. The Pre-weighed LDPE (0.5g) was transferred to the conical flask containing 100 ml of Mineral Salt broth and it was inoculated with isolated bacterial species. Control was maintained with 0.50 g of LDPE in the microbe-free medium. Different flasks were maintained for both control and treatment. After one month of incubation, the LDPE was collected, washed thoroughly using distilled water and ethanol, shade dried and then weighed for final weight.

Weight loss of LDPE was calculated by the following formula,

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

Characterization studies

Fourier Transform Infrared Analysis (FT-IR)

To determine the formation of new or disappearance of functional groups was carried out on treated and untreated LDPE using FT-IR in the frequency range 3600 – 600 cm-1.

Scanning Electron Microscopy of Polyethylene

The change in the morphology after the incubation period, the treated LDPE was washed with ethanol, distilled water, dried and analyzed using SEM.

Results and Discussion

Culturing of LDPE degrading bacterial consortium

The Mineral Salt Medium is a Carbon free medium, where microbes in the soil sample take the LDPE as a carbon source. Hence forth, we observed the growth of bacterial strains responsible for LDPE degradation were grown utilizing carbon source from LDPE.

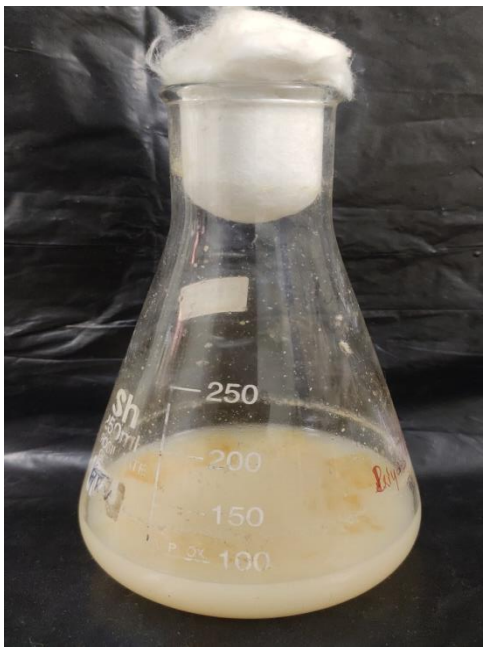

Fig 1. Culturing of LDPE degrading bacterial consortium

Isolation of LDPE degrading microbes

The LDPE degrading bacteria were isolated using spread plate and streak plate technique. In spread plate technique, the growth of bacteria was seen based on the dilution factor. High numbers of colonies were seen in the dilution factor 10-1 and least number of colonies was seen in 10-10. In streak plate technique, two different and separated colonies (i.e pure culture) were obtained.

Screening of LDPE bacteria

Opacity method

Plastic degrading ability of the isolated bacterial strains was confirmed with the opacity method. The isolated bacterial strains were translucent in Mineral Salt Agar medium and the opacity was not observed. However, in Nutrient Agar Medium along with starch, clear opacity has been noticed. It is known that starch contains *n* number of carbon atoms. Besides, miniature pit like (opacity) observations were seen on the surface of the Nutrient Agar Medium. This may be due to the consumption of starch by the isolated bacterial strains and forms opacity (Vignesh et al., 2016).

Identification assays for isolated bacteria morphological characterization

Colony morphology

The colony morphology of the isolated microorganisms was summarized in table 1.

Table 1. Colony morphology for isolated bacteria

ISOLATES	SIZE	COLOUR	TEXTURE	FORM	ELEVATION	MARGIN
ISOLATE 1	Medium	Off white	Shiny	circular	Flat	Smooth
ISOLATE 2	Medium	Creamy white	shiny	circular	convex	Smooth

Morphological Characteristics

The morphological observation of the bacterial isolates namely, isolate 1 and isolate 2 were identified as Gram negative bacilli. The isolate 1 was identified as highly motile organism whereas isolate 2 as non-motile.

VITEK 2 COMPACT technique

The isolated microorganisms were identified through Biomeriux VITEK 2 COMPACT system and results were given in table 2. Among two isolates, the isolate 1 was identified as Pseudomonas aeruginosa and the test substrates present in the isolates are Glutamyl Arylamidase, D-Glucose, Gamma-Glutamyl Transferase, Beta-Alanine arylamidase, L-Proline Arylamidase, Citrate, Malonate, L-Lactate alkanisation, Succinate alkanisation, L- Malate and L- Lactate. The substrates present in the isolate, Pseudomonas aeruginosa are similar to the study Satapute and Kaliwal, 2016. The isolate 2 was identified as Klebsiella pneumoniae and the test substrates present in the isolates are Adonitol, D- Cellubiose, Beta-Galactosidase, D-Glucose, Gamma-Glutamyl-Transferase, Glucose, Beta-Glucosidase, D-Maltose, D-Mannitol, D- Mannose, Beta-Xylosidase, Palatinose, Urease, D-Sorbitol, Sucrose, D-Trehalose, Citrate, Malonate, L-Lactate, Alpha-Galactosidase, Phophotase and Lysine decarboxylase. The substrates such as Adonitol, Cellobiose, Beta-Galactosidase, D-Glucose, Lysine decarboxylase and Phophotase are positive for the same species in the research findings Rosenblueth et al., 2004; Wu et al., 2012; Wang et al., 2014; Feng et al., 2017; Kim et al., 2016. From the report, the probability was scrutinized as 97% for Pseudomonas aeruginosa and 99% for Klebsiella pneumoniae. All these inferred that both the isolates were the confidence of excellent identification.

TABLE 2. Biochemical results for isolated bacteria using VITEK 2 COMPACT

Well No	Name of the substrate	Presence/ Absence of the substrate	
		Isolate 1	Isolate 2
2	Ala – Phe – Pro – ARYLAMIDASE	-	-
3	ADONITOL	-	+
4	L – Pyrrolydonyl - ARYLAMIDASE	-	-
5	L – ARABITOL	-	-
7	D – CELLOBIOSE	-	+
9	BETA – GALACTOSIDASE	-	+
10	v PRODUCTION	-	-

11	BETA- N– ACETYL - GLUCOSAMINIDASE	-	-
12	GLUTAMYL ARYLAMIDASE pNA	+	-
13	D-GLUCOSE	+	+
14	GAMMA-GLUTAMYL-TRANSFERASE	+	+
15	FERMATATION GLUCOSE	-	+
17	BETA-GLUCOSIDASE	-	+
18	D-MALTOSE	-	+
19	D-MANNITOL	-	+
20	D-MANNOSE	-	+
21	BETA-XYLOSIDASE	-	+
22	BETA-ALANINE ARYLAMIDASE	+	-
23	L-PROLINE ARYLAMIDASE	+	-
26	LIPASE	-	-
27	PALATINOSE	-	+
29	TYROSINE ARYLAMIDASE	-	-
31	UREASE	-	+
32	D-SORBITOL	-	+
33	SACCHAROSE/SUCROSE	-	+
34	D-TAGATOSE	-	-
35	D-TREHALOSE	-	+
36	CITRATE(SODIUM)	+	+
37	MALONATE	+	+
39	5-KETO-D-GLUCONATE	-	-
40	L-LACTATE ALKANISATION	+	+
41	ALPHA-GLUCOSIDASE	-	-
42	SUCCINATE ALKALINISATION	+	-
43	BETA-N-ACETYL-GALACTOSAMINIDASE	-	-
44	ALPHA-GALACTOSIDASE	-	+
45	PHOSPHATASE	-	+
46	GLYCINE ARYLAMIDASE	-	-
47	OENITHINE DECARBOXYLASE	-	-
48	LYSINE DECARBOXYLASE	-	+
53	L-HISTIDINE ASSIMILATION	-	-
56	COUMARATE	-	-
57	BETA-GLUCORONIDASE	-	-
58	O/129 RESISTANCE	-	+
59	Glu-Gly-Arg-ARYLAMIDASE	-	-
61	L-MALATE	+	-
62	ELLMAN	-	-
64	L-LACTATE ASSIMILATION	+	-

Microbial growth studies

The HB and L UROQUATTRO monitored the growth phase of bacterial isolates and the quantitative bacterial count in the form of Colony Forming Unit/ml. The figure 2 and 3 shows the growth curve of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae.* The quantitative bacterial count of 10,000,000 CFU/ml has been observed in both the isolates.

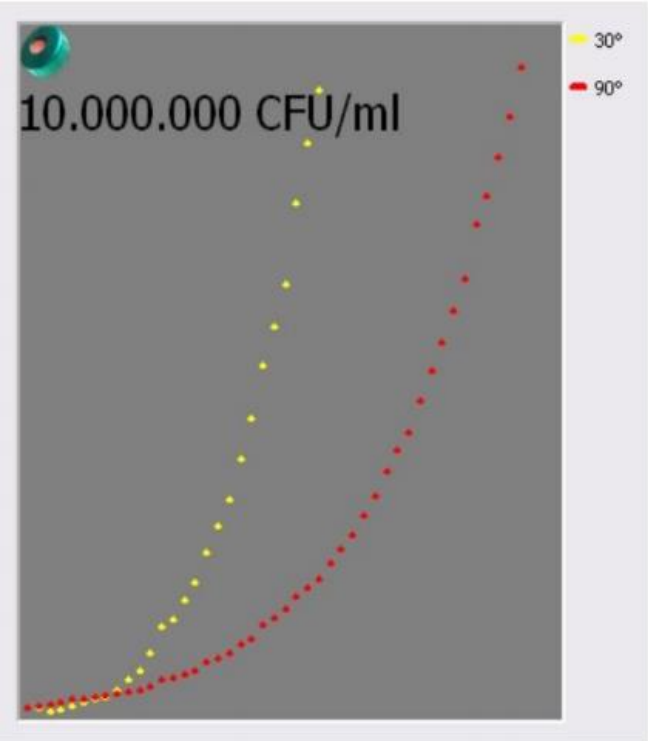

Fig 2. Growth phase of *Pseudomonas aeruginosa*

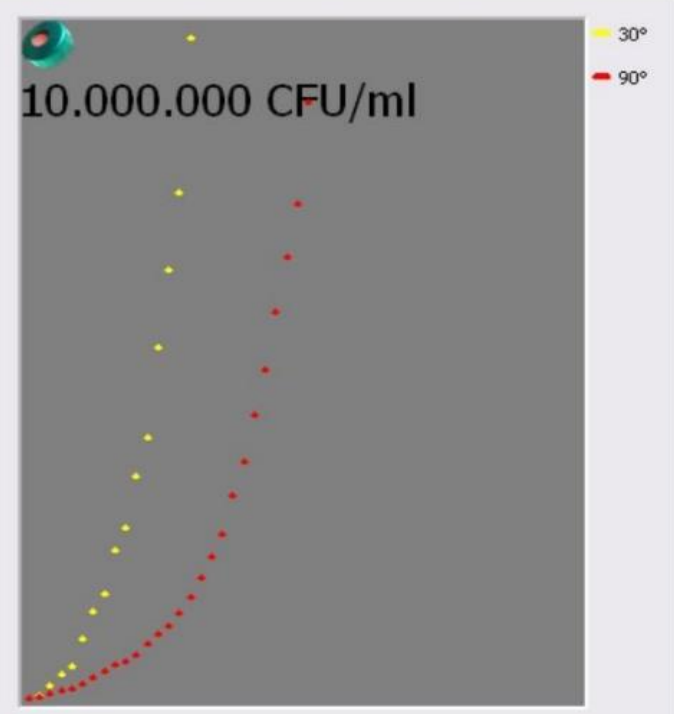

Fig 3. Growth phase of *Klebsiella pneumoniae*

Microbial degradation assay of LDPE using weight loss method

The percentage weight loss method has been followed to estimate the quantity of LDPE degradation by the two isolated bacterial strains, *P. aeruginosa and K. pneumoniae.*

Determination of weight loss

The initial weight of LDPE film was measured as 0.50 g. After microbial degradation assay using isolated bacterial strains, the weight was reduced to 0.32 g. This shows the efficacy of the isolated bacterial strains in degrading LDPE film within a month. The percentage weight loss was calculated from the initial and final weight as 36%. This invention gives a hope to cent percent degradation using the isolated bacterial strains.

Characterization studies

Fourier Transform Infrared Spectroscopy

The untreated LDPE film showed peaks at 3849 cm^{-1}, 1651 cm^{-1} and 1388 cm^{-1}. These peaks indicated a shift and decrease in the peak intensity at 3834 cm^{-1}, 1642 cm^{-1} and 1373 cm^{-1}. The peaks observed in the LDPE control films were 2553 cm^{-1}, 1203 cm^{-1}, 856 cm^{-1} and 547 cm^{-1} indicated shifts and increase in the peal intensity at 2360 cm^{-1}, 1913 cm^{-1}, 1273 cm^{-1}, 864 cm^{-1}, 555 cm^{-1}. However, the new peaks were observed from the treated LDPE film at 3394 cm^{-1}, 1427 cm^{-1}, 1033 cm^{-1}, 478 cm^{-1} and 439 cm^{-1}. The disappearance of functional groups in the treated LDPE in the peak intensity at 2846 cm^{-1}, 2021 cm^{-1}, 1686 cm^{-1}, 925 cm^{-1} and 516 cm^{-1}. All these changes conforms that the degradation occurred in LDPE. The increase and decrease of peak intensity clearly indicates that the degradation occurred in LDPE. The figures 4 and 5 show the FTIR peak absorbance in treated and untreated LDPE film.

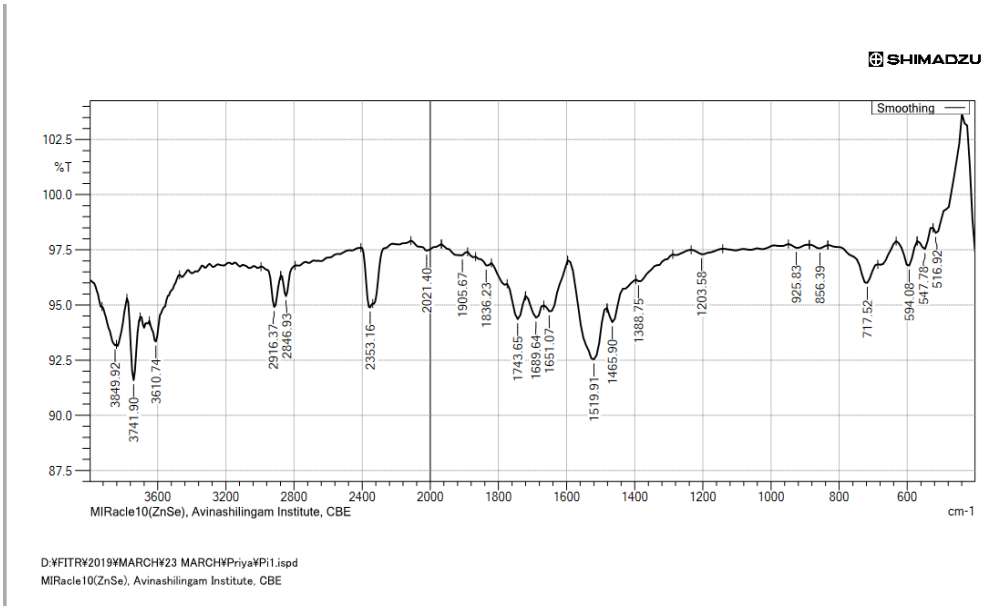

Fig 4. FTIR spectra for untreated LDPE

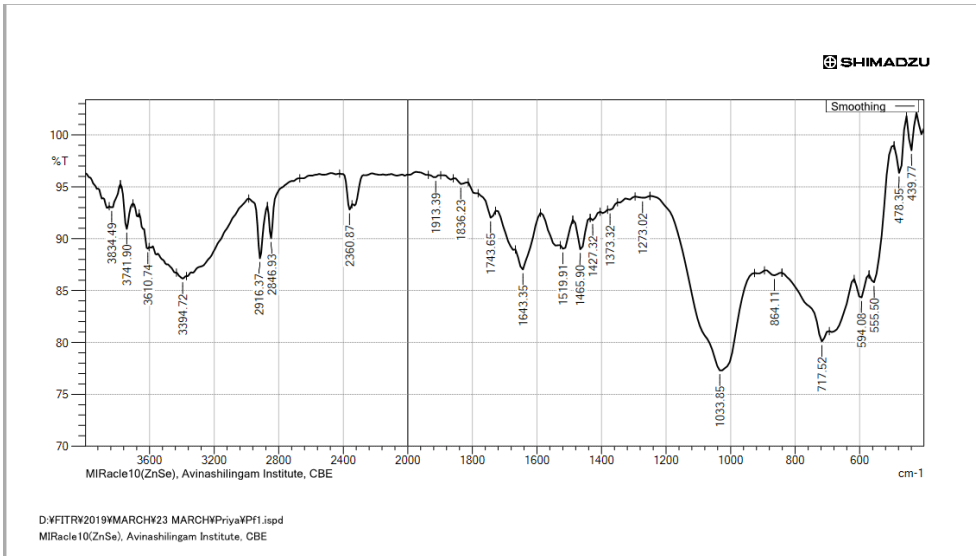

Fig 5. FTIR spectra for treated LDPE

Scanning Electron Microscopy

The surface morphology of the treated LDPE was analyzed through Scanning Electron Microscopy to observe the structural changes 1 month after degradation. Figure 6 shows the micrographs of the Scanning Electron Microscopy of LDPE film after the 30 days of incubation with bacterial isolates. The surface topography of LDPE film revealed that there are two areas such as dark and light. The light color area denotes non decomposition while the darker area with cavities and erosions indicates the decomposition by the bacterial consortium.

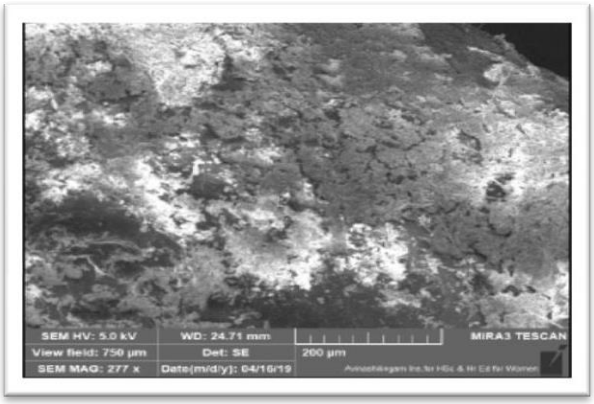

Fig 6. SEM image for treated LDPE

Conclusion

The overall investigation concludes that two bacterial strains, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* as potential, polymer degrading microbial consortia which can be exploited as efficient decomposing strains to Corporation Dump Yards. It clearly shows that there are more possibility of finding plastic degrading microbial consortia in dump yards and contaminated areas. In future study, the virulence of isolated bacterium could be identified and must be reduced to avoid pathogenic effect on living entity. Therefore the modified and reengineered bacterium promises to degrade the complete plastic bed on the earth.

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