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Bio-degradation of Polyethylene Waste by Simultaneous Use of Two Bacteria: *Bacillus licheniformis* for production of Bio-surfactant and *Lysinibacillus fusiformis* for Bio-degradation.

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A unique method of biodegradation of commercial polyethylene by using simultaneously a bio-surfactant produced by *Bacillus licheniformis* and *Lysinibacillus* bacterium in various combinations was investigated in this study. Bio-surfactant produced by *B.licheniformis* did not have any anti-adhesive property for bio-film formation unlike other types of bio-surfactant produced by other strains of the same bacterium. It was also observed that the lower the surface tension, the higher is the level of oxidation of polyethylene. *Lysinibacillus* was able to form a bio-film on the control polyethylene without any pre-oxidation step and simultaneously oxidizing that polyethylene. Polyethylene samples which were treated with *Lysinibacillus* along with bio-surfactant showed weight loss. Maximum weight loss of 2.97 ± 0.05 % was achieved in the case of polyethylene treated with *Lysinibacillus* for 1 month, then treated with bio-surfactant for 1 month, followed by treatment with *Lysinibacillus* for another 1 month. Polyethylene is biodegraded via conversion of carbonyl groups into unsaturated hydrocarbon by both bio-surfactant and *Lysinibacillus* bacterium. In GC-MS analysis, partial oxidation of anti-oxidant used in commercial polyethylene was also observed. So, our present method of biodegradation by simultaneous use of two bacteria is very environmental friendly as well as very efficient.

1.Introduction:

Need for an environmental friendly and efficient degradation process rises with the drastic increase in the usage of polyethylene and its subsequent accumulation in the environment in the last three decades. Additional inertness of commercial polyethylene towards oxidation and bio-degradation can be due to the presence of anti-oxidants. Anti-oxidants are mixed with commercial polyethylene (less than 1%) as polyethylene can undergo oxidations during high temperature processing; this results in the loss of physical properties of polyethylene, leading to its premature failure [1].

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Supplementary Figure S1-S8 are added as supplementary information section. . See DOI: 10.1039/x0xx00000x

Several studies have been reported that the microbial degradation or bio-degradation can be used as an environmental friendly method for degradation of polyethylene. Bio-degradation of polyethylene mainly consists of two steps. The primary step is the oxidation of polyethylene and the secondary step is the bacterial incubation of oxidised polyethylene for bio-degradation. Microbes cannot utilise polyethylene due to its highly hydrophobic nature and large molecular weight. It is reported that the pre-oxidation step is used to increase hydrophilic nature of polyethylene by introducing polar groups like carbonyl groups into the carbon-hydrogen backbone of polyethylene. Increased hydrophilic nature and carbonyl groups introduced into the polymer backbone can enhance utilisation of polyethylene by microbes and thereby increasing the bio-degradation[2]. Photooxidition by irradiation with U.V. light at temperature above 50˚C, thermal and chemical oxidation are the examples of abiotic oxidation generally used in studies for oxidation of polyethylene [3]. Polyethylene mixed with U.V. photosensitizer (pro-oxidant) has been photooxidised by irradiation with U.V. at 70˚C for 60 hour before subjecting it to the biodegradation by *Rhodococcus ruber* and *Brevibacillus borstelensis*[4,5]. Pre-oxidation of polyethylene, prior to biodegradation by fungi, has been done by two methods which are by accelerated aging at 70˚C under U.V. light for 29 days and by thermal treatment at 105˚C and 150˚C for 120 hour[6]. Prior to biodegradation, polyethylene has also been photooxidised by natural weathering for 93 days, followed by thermal treatment at temperature ranging from 45˚C to 65˚C for 200 days. In this case, polyethylene mixed with pro-oxidant has been used [1]. Although, oxidation level of polyethylene mixed with pro-oxidant is high, when oxidised by abiotic method; but, these methods have inherent disadvantages. Use of high temperature, U.V. light and chemicals is not cost effective. A more economical way can be the biotic method i.e using microbial sp. In our previous study, commercial polyethylene is oxidised by the bio-surfactant produced by *Bacillus sp*(ATCC- 39307) [7]. Oxidation of polyethylene by bio-surfactant cause similar effect in the chemical structure of polyethylene as reported in case of abiotically oxidised polyethylene like an increase in the carbonyl index. Surfactant is an amphiphilic molecule. Hydrophobic part of the bio-surfactant remains attached with the hydrophobic surface of the polyethylene while hydrophilic part remains protruding towards the aqueous solution. This method increases polyethylene's availability to dissolved oxygen which leads to oxidation of polyethylene. So, even after proper washing and drying, surfactant can remain attached with the surface of the polyethylene. On other hand, bio-surfactant isolated from other variants of *Bacillus licheniformis* bacterium like *B.licheniformis* strain 603, *B.licheniformis* BAS50, *B.licheniformis* V9714 have shown anti-microbial and anti-adhesive property for bio-film formation against different microbial sp[8,9,10,11]. So, further investigation is required to confirm whether microbes can form bio-film on the biotically oxidised polyethylene. Due to higher metabolic activity of bio-film forming microbial population than that of the suspended bacteria, formation of bio-film on polyethylene surface is important for bio-degradation. Another advantage is that the carbon availability is much greater where solid surface serves as the support and substrate for the bio-film formation[4]. Several bacterial sp have been reported for their ability to form bio-film on the polyethylene surface. *Rhodococcus ruber*, *Brevibacillus borstelensis* are such bacteria which have the ability to form bio-film on the polyethylene surface[4,5]. One bacterial sp,

identified as *Lysinibacillus fusiformis* has been isolated from Kolkata municipal wastewater and it is previously reported that this bacterium is able to form bio-film on di (2-ethylhexyl) phthalate which has short $–CH_2$ chain [12]. Two bacterial sp, one for oxidation of polyethylene (*Bacillus licheniformis*) and another for degradation (*Lysinibacillus fusiformis*) of polyethylene has never been used simultaneously for biodegradation of commercial polyethylene waste bags.

In this study, polyethylene was treated with *Bacillus licheniformis* and *Lysinibacillus fusiformis* in different combinations for 3 months when the time allowed for each treatment was 1 month to obtain maximum weight loss due to bio-degradation. *B.licheniformis* was used for biotic oxidation of polyethylene by the formation of bio-surfactant. *Lysinibacillus* sp. was used for its ability to form bio-film on the oxidized and un-oxidized polyethylene samples.

2. Methods and materials:

2.1. Test materials:

Daily used 0.01 mm thick, transparent colourless polyethylene bags were collected from the waste bins of Kolkata Municipal Corporation. Rectangular pieces (5mm × 5mm) of the polyethylene bags were vigorously washed with soap water and distilled water. Rectangular pieces were dried at 60°C for overnight and were used as control polyethylene in this study.

2.2. Microbial culture:

Bacillus licheniformis JF2 (ATCC No. 39307, MTCC No. 2454) was used for bio-treatment study. This microbial culture was obtained from Institute of Microbial Technology, Chandigarh, India. Microbial culture was maintained in nutrient broth (Himedia). One bacterial strain was isolated from the waste water collected from the Bangur area of Kolkata Municipal Corporation through serial dilution method. After purification, isolated strain was maintained in nutrient media (Himedia) at 37°C. The isolated strain was identified as *Lysinibacillus fusiformis* and partial nucleotide sequence of 16s rDNA was submitted in NCBI database with accession number: HE648060.

Mediums used for treatment of polyethylene by *B.licheniformis* were YPD media containing yeast extract 10 g, glucose 20 g, peptone 20 g and sodium chloride (NaCl) 10 g in 1 litre of double distilled water and mineral media containing glucose 10 g, sodium chloride 10 g, NH_4NO_3 4 g, Na_2HPO_4 1.5 g, $MgSO_4$ 0.4 g, $CaCl_2$ 0.1 g in 1 litre of double distilled water. Medium used for treatment of polyethylene by *Lysinibacillus* sp. contained glucose 10 g, NH_4NO_3 3 g, KH_2PO_4 0.4 g, K_2HPO_4 0.5 g, $MgSO_4$ 0.2 g per litre of double distilled water. Liquid culture mediums were sterilised at 120°C for 15 minutes. Control polyethylene samples were treated as described in Table 1 with *B.licheniformis* and *Lysinibacillus* sp. For environmental aging, polyethylene samples were kept under sun continuously for 1 month during day and night in an enclosed glass beaker with a white paper to prevent polyethylene samples from dust and other air polluting agent. Each bacterial treatment was carried out in triplicate at 37°C for 1 month. Control polyethylene (commercial unoxidized) was incubated with *Bacillus licheniformis* in YPD medium (PE 1.1), in mineral media (PE 6.1) and with *Lysinibacillus* bacterium in mineral media (PE 4.1) for 1 month. Then PE 1.1 was aged under Sun light for 1 month (PE 1.2) and PE 1.2 was incubated with *B.licheniformis* in YPD medium for 1 month (PE 1.3). Control polyethylene was incubated with *B.licheniformis* in YPD medium for 2

months (PE 2.2) and PE 2.2 was further incubated with *Lysinibacillus* for 1 month (PE 2.3). PE 1.1 was incubated with *Lysinibacillus*. Samples were collected after 1 month (PE 3.2) and 2 months (PE 3.3). PE 4.1 was subjected to natural aging under Sun light for 1 month (PE 4.2) and PE

4.2 was further incubated with *Lysinibacillus* for 1 month (PE 4.3). PE 4.1 was incubated with *B.licheniformis*in YPD medium for 1 month (PE 5.2) and PE 5.2 was further incubated with *B.licheniformis*for 1 month (PE 5.3). PE 6.1 was treated similarly as PE 3.2 (PE 6.2) and PE 6.2 was treated similarly as PE 3.3 (PE 6.3). PE 6.1 was treated similarly as PE 1.2 (PE 7.2) and PE 7.2 was treated similarly as PE 1.3, but using mineral medium instead of YPD medium (PE 7.3). Control polyethylene was incubated with *B.licheniformis* for 2 months in mineral media (PE 8.2) and PE 8.2 was incubated with *Lysinibacillus* for 1 month (PE 8.3) (Table 1). Polyethylene kept in three different media without any bacterial sp was kept as negative control. After each treatment, polyethylene samples were carefully washed and dried at 60˚C. These treated polyethylene samples were then characterized by FTIR, SEM, and XRD. Initial weight and final weight of each treated polyethylene samples was noted.

2.3. Characterization:

Surface tension (σ) of culture media incubated with *B.licheniformis* was measured by stalagmometer at 25^{o}C at day zero and at different intervals of time (days) [13]. Reduction of surface tension is an indirect measure of bio-surfactant production by *B.licheniformis*. Surface tension was calculated by following formula.

Sample name	Starting material	Types of treatment	Name of the bacteria	Types of Medium	Duration
PE 1.1	Commercial polyethylene	Bio-surfactant treatment	*Bacillus licheniformis*	YPD medium	1 month
PE 1.2	PE 1.1	Natural aging under Sunlight	----------	---------	1 month
PE 1.3	PE 1.2	Bio-surfactant treatment	*Bacillus licheniformis*	YPD medium	1 month
PE 2.2	PE 1.1	Bio-surfactant treatment	*Bacillus licheniformis*	YPD medium	1 month
PE 2.3	PE 2.2	Bacterial treatment	*Lysinibacillus fusiformis*	Mineral medium	1 month
PE 3.2	PE 1.1	Bacterial treatment	*Lysinibacillus fusiformis*	Mineral medium	1 month
PE 3.3	PE 3.2	Bacterial treatment	*Lysinibacillus fusiformis*	Mineral medium	1 month
PE 4.1	Commercial polyethylene	Bacterial treatment	*Lysinibacillus fusiformis*	Mineral medium	1 month
PE 4.2	PE 4.1	Natural aging under Sunlight	----------	----------	1 month
PE 4.3	PE 4.2	Bacterial treatment	*Lysinibacillus fusiformis*	Mineral Medium	1 month
PE 5.2	PE 4.1	Bio-surfactant treatment	*Bacillus licheniformis*	YPD Medium	1 month
PE 5.3	PE 5.2	Bacterial treatment	*Lysinibacillus fusiformis*	Mineral Medium	1 month
PE 6.1	Commercial polytehylene	Bio-surfactant treatment	*Bacillus licheniformis*	Mineral Medium	1 month
PE 6.2	PE 6.1	Bacterial treatment	*Lysinibacillus fusiformis*	Mineral medium	1 month
PE 6.3	PE 6.2	Bacterial treatment	*Lysinibacillus fusiformis*	Mineral medium	1 month
PE 7.2	PE 6.1	Natural aging under Sunlight	----------	----------	1 month
PE 7.3	PE 7.2	Bio-surfactant treatment	Bacillus licheniformis	Mineral Medium	1 month
PE 8.2	PE 6.1	Bio-surfactant treatment	*Bacillus licheniformis*	Mineral medium	1 month
PE 8.3	PE 8.2	Bio-surfactant treatment	*Bacillus licheniformis*	Mineral medium	1 month

Table 1: Information of samples and their respective treatment procedure during 3months of study

$$\sigma_{water} \times \frac{weight\ of\ N\ drops\ of\ solvent}{weight\ of\ N\ drops\ of\ water} = \sigma_{solvent}$$

Fourier transform infrared spectra (FTIR) analysis was carried out with ATR-FTIR (model alpha, Bruker, Germany) spectrometer, scanning from 4000 cm^{-1} to 500 cm^{-1} at room temperature. The resolution was set at 4 cm^{-1} with 42 scans per spectrum. Carbonyl index (C.I.) and double bond index (D.B.I.) were calculated using the ratio of absorbance frequency of the carbonyl peak (1740 cm^{-1}) and double bond peak (1650 cm^{-1}) to that of the CH_2 group bending frequency (1465 cm^{-1}) respectively.

All polyethylene samples were sputter coated with gold layer by a Hitachi sputter coater (model-E1010 Ion Sputter), Japan. Photomicrographs were observed under scanning electron microscope (EVO 18, Carl Zeiss, Germany). X-ray diffraction study of all types of polyethylene samples were recorded with an X-ray diffractometer (PANalytical, Netherlands) at an angle of 2θ from 3° to 50° and fixed scan rate of 1° min^{-1}. Percentage (%) of crystallinity was calculated by using following formula.

$$\%Crystallinity = \frac{Area\ under\ crystalline\ peaks}{Total\ Area\ under\ all\ peaks} \times 100\%$$

Difference of crystallinity in percentage (%) was calculated by using following formula.

BATH' (Bacterial adhesion to hydrocarbon) is a test to measure bacterial hydrophobicity[4]. Affinity of bacterial cells towards hydrocarbon increases with bacterial hydrophobicity. Due to this property, bacterial cells with higher affinity for hydrocarbon [hexadecane ($C_{16}H_{34}$) in the present case], transfer from aqueous suspension to organic phase, leading to the reduction in the turbidity of the culture. BATH' method was carried out to check hydrophobicity of *Lysinibacillus* sp. as described by Gilan et al[4]. *E.coli* was used as negative control.

Extraction of the degraded part of polyethylene samples in chloroform was performed as per procedure reported by Roy et al [14]. Unoxidized commercial polyethylene was used as control. The presence of different compounds in commercial polyethylene and in the oxidation products and degradation products of commercial polyethylene was identified by GC-MS (Thermo Scientific TSQ 8000) analysis. The oven temperature was programmed at 40°C for 3 min, then rose to 280°C at a rate of 10°C/min, and then held for 4 min at 280°C. Helium was used as carrier gas. The identification of degradation products was established by comparison of their mass spectra with NST database.

3. Result and Discussion:

3.1. Bacterial Hydrophobicity:

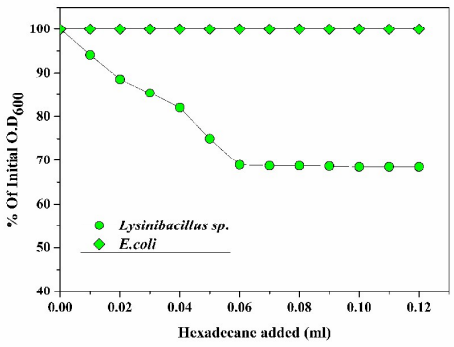

Figure 1: Hydrophobicity of bacterium *Lysinibacillus* sp represent in BATH assay

BATH assay is the measure of bacterial hydrophobicity. The graph depicted from BATH assay of *Lysinibacillus*sp and *E.Coli* is represented in Figure 1. From the BATH assay, affinity of *Lysinibacillus* towards hydrocarbon is evident. More than 30% reduction in cell turbidity is observed after addition of 0.6 ml of hexadecane. After this concentration, reduction of cell turbidity becomes stabilized. Reduction of turbidity occurs as hydrophobic bacterial cells get attached with the hydrocarbon, due to which the transfer of bacterial cells from aqueous phase to organic phase occurs. Similar result has been observed in case of another bacterium, *Rhodococcus rubber*. These bacteria have been able to form bio-film on the surface of polyethylene, leading to the biodegradation of polyethylene [4]. So, *Lysinibacillus* sp can be used for bio-film formation on the surface of the polyethylene.

E.coli used as the negative control, does not show any change of cell turbidity during addition of different concentration of hexadecane.

3.2. Surface Tension:

Surface tension reduction by *B.licheniformis* incubated in two different mediums is represented in Figure 2. From this observation, it is clear that the maximum surface tension

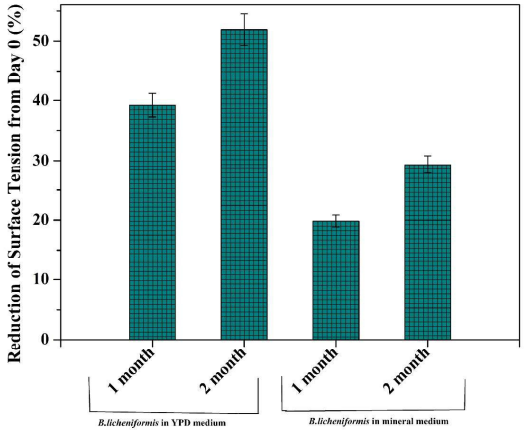

Figure 2: surface tension of biosurfactant produced by *B.licheniformis* grown in different medium

reduction i.e 51.9% is achieved in case of the *B.licheniformis* grown in YPD medium for 2 months. Surface tension reduction in case of *B.licheniformis* grown in mineral media for 2 months is 29% which is much less than the earlier case. Higher amount of bio-surfactant produced by *B.licheniformis* in YPD medium is due to the abundant presence of carbon and nitrogen sources in the YPD medium. In the case of *B.licheniformis* grown in the mineral medium, surfactant production is much less due to the presence of limited amount of nitrogen and carbon sources. As *B.licheniformis* is able to produce bio-surfactant in both YPD and mineral media, both media has been used for the treatment of polyethylene to study the effect of surface tension on polyethylene oxidation.

3.3. Bio-surfactant induced oxidation followed by natural aging under sunlight and biodegradation treatment:

Polyethylene sample oxidised by bio-surfactant produced by *B.licheniformis* grown in YPD medium for 1 month was subjected to natural aging under sunlight for 1 month to improve oxidation level. In the next step of treatment, this oxidized polyethylene incubated with *Lysinibacillus* for 1 and

2 months. It was observed that *Lysinibacillus* was able to form a bio-film on the oxidised polyethylene sample.

3.3.1. FTIR analysis:

In case of PE 1.1, a strong absorbance peak at 1500-1800 cm^{-1} is observed in the FTIR spectra (Supplementary Figure S1). From the broad absorbance peak at 1700-1785 cm^{-1} in the FTIR spectra of PE 1.1, it is apparent that more than one type of C=O containing oxidation product is formed. Mainly ketones (1740 cm^{-1}), aldehydes (1733 cm^{-1}) and unsaturated hydrocarbons (1650 cm^{-1}) are formed as oxidation product [15,16]. After further oxidation by natural aging under sunlight (PE 1.2), another absorbance peak appears at 1715 cm^{-1}. This peak is due to formation of acids as the oxidation product of polyethylene [17]. Polyethylene sample oxidised in the presence of bio-surfactant and natural aging, again treated with bio-surfactant for 1 month (PE 1.3). In this case of PE 1.3, drastic change in the absorbance can be observed in the 1500-1800 cm^{-1} region in the FTIR spectra. Absorbance peak at 1650 cm^{-1} increases rapidly, indicating formation of higher amount of unsaturated hydrocarbons. In our previous study, similar observation of conversion of carbonyl groups into unsaturated hydrocarbons during oxidation by bio-surfactant has been observed[7].

Absorbance peak at 1650 cm^{-1} in the FTIR spectra of PE 2.2 increases and this is due to the formation of unsaturated hydrocarbons as oxidation product (Supplementary Figure S2). This oxidised Polyethylene was incubated with *Lysinibacillus* sp. for 1 month for bio-film formation (PE 2.3). After 1 month, absorbance peak at 1740 cm^{-1} almost disappears and absorbance peak at 1650 cm^{-1} increases. This may be due to the utilization of oxidation product by *Lysinibacillus* sp and also conversion of carbonyl groups into unsaturated hydrocarbon by the same bacterium. Similar phenomenon exhibited by other polyethylene degrading bacteria is also reported [18].

After oxidation of polyethylene in the presence of bio-surfactant for 1 month, it was incubated with *Lysinibacillus* for 2 months (PE 3.3). After 2 months incubation with *Lysinibacillus*, absorbance peak at 1500-1800 cm^{-1} region increases further than that of the PE 1.1; this can be due to formation of ketones, aldehydes and unsaturated hydrocarbons (Supplementary Figure S3).

Carbonyl index (C.I.) and double bond index (D.B.I.) of all samples is represented in Figure 3. C.I. of P.E 1.2 is found to be higher than PE 1.1. It is reported that the presence of carbonyl groups in polyethylene can act as the initiator of

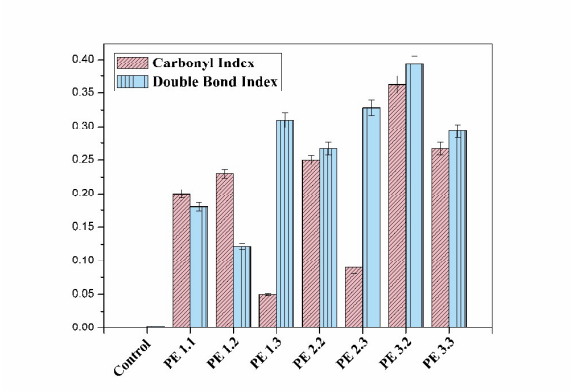

Figure 3: Carbonyl Index and Double Bond Index of control polyethylene and PE 1.1-PE 3.3

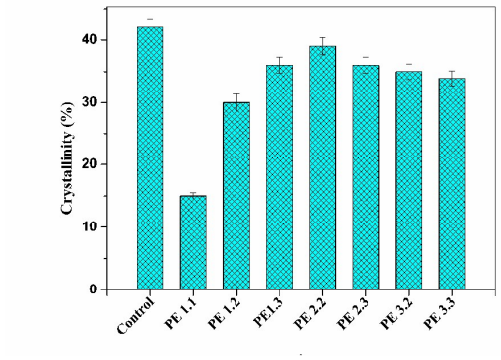

Figure 4: Crystallinity graph of control polyethylene and PE 1.1 to PE 3.3

photo-oxidation during natural aging under sunlight [15] due to which, oxidation level of PE 1.2 is much higher than that of the PE 1.1. But in case of PE1.3, C.I. decreases and D.B.I. increases drastically. In this case, oxidation product of polyethylene gets solubilised into the aqueous media. During oxidation by bio-surfactant, initially, C=O containing oxidation product i.e ketones, aldehydes along with unsaturated hydrocarbons are formed. During later stage of this oxidation, solubilisation of oxidation products and conversion of carbonyl groups into unsaturated hydrocarbon is reported in the previous study [7]. This phenomenon can be resulted due to hydrocarbon solubilisation ability of bio-surfactant which is also reported by other studies [19]. In case of PE 2.3, its C.I. decreases, whereas its D.B.I. increases in comparison to that of the PE 2.2. In this case, *Lysinibacillus* is able to utilize the oxidation product for bio-film formation and is also able to convert the carbonyl groups into unsaturated hydrocarbons. In case of PE 3.2 and PE 3.3, both C.I. and D.B.I. increases. *Lysinibacillus* bacterium may be able oxidize polyethylene further, due to which, more carbonyl groups and unsaturated hydrocarbons are formed. But in case of PE 2.3, such oxidation is not observed. *Lysinibacillus* only utilizes that oxidized part of polyethylene for forming the bio-film due to the presence of higher amount of the oxidation product in PE 2.2. But in case of PE 1.1, the amount of oxidation product is comparatively less, enabling *Lysinibacillus* to oxidise the polyethylene sample further as well as to utilize oxidation product.

3.3.2. XRD analysis:

The level of crystallinity of control polyethylene is compared for the samples PE 1.1 to 3.3 in Figure 4. After oxidation in the presence of bio-surfactant, reduction of crystallinity of polyethylene is reported in our previous study (PE 1.1) [7]. This reduction is mainly due to the formation of oxidation products which act as the impurities and may be due to the alteration of crystalline structure by attached surfactant to the polyethylene surface [20]. After natural aging, crystallinity of oxidised polyethylene again increases (PE 1.2). Increase of crystallinity during natural aging under sunlight is a common phenomenon and reported in several studies [21,22]. During photooxidation, small molecular weight chains resulted due to chain scission, can initiate or promote secondary crystallization. Formation of new polar bonds between oxidised polyethylene molecules can also initiate secondary crystallization. During natural aging of polyethylene at a relatively low temperature during night time can also lead to this effect. Crystallinity increases after oxidation in the presence of bio-surfactant (PE 1.3). Crystallinity of PE 1.3 increases due to the erosion of oxidised amorphous region of polyethylene into aqueous media in the presence of bio-surfactant [15]. In case of PE 2.2, crystallinity increases with the increase in the oxidation level (Figure 3). But after incubation with *Lysinibacillus* (PE 2.3), crystallinity slightly decreases. During bio-film formation, *Lysinibacillus* first utilises oxidised amorphous part and simultaneously further oxidises the crystalline region of polyethylene. In case of PE 3.3, crystallinity also decreases with the increase in the oxidation level. During initial phase, amorphous region is readily oxidised followed by biodegradation by *Lysinibacillus* due to easier availability of amorphous region to the dissolved oxygen. Due to further oxidation and biodegradation by *Lysinibacillus*, some part of crystalline region may also further be oxidised, resulting in a decrease in the crystallinity.

3.4. Bio-film formation and oxidation of control polyethylene by *Lysinibacillus* bacterium:

Additional oxidation ability of *Lysinibacillus* along with its ability to form bio-film on polyethylene is observed during the first part of this study. In this part, control (unoxidized) polyethylene was incubated with *Lysinibacillus*. Natural aging under sunlight and bio-surfactant induced oxidation were also used to enhance oxidation level (Table 1). Bio-film formation was observed on the control polyethylene within 7 days of incubation with *Lysinibacillus*.

3.4.1. FTIR analysis:

In the FTIR spectra of polyethylene incubated with *Lysinibacillus* for 1 month (PE 4.1), certain changes are observed in the absorbance peak region at 1500-1800 cm $^{-1}$ (Supplementary Figure S4). This change corresponds to the formation of ketones, aldehydes, acids and unsaturated hydrocarbons as oxidation product. Polyethylene is oxidized by extracellular enzymes of *Lysinibacillus* and by dissolved oxygen. Then, this oxidation product is further utilized by *Lysinibacillus* for the formation of bio-film on the polyethylene surface. Abiotically oxidised polyethylene has been biodegraded by another strain of *Lysinibacillus* in the identical manner i.e conversion of carbonyl group into unsaturated hydrocarbons in another study [23]. This oxidized polyethylene was then subjected to natural aging under sunlight for 1 month to improve the level of oxidation (PE 4.2), followed by the incubation of polyethylene with *Lysinibacillus* for another 1 month (PE 4.3). After each treatment, appearance or disappearance of no new peak is observed in the 1500-1800 cm $^{-1}$ region (Supplementary Figure S4). This indicates that polyethylene is further oxidized by each treatment.

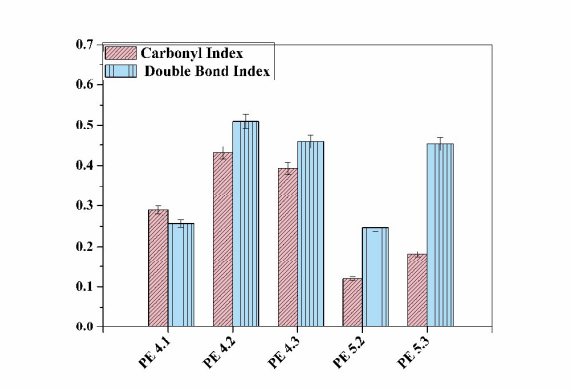

Figure 5: Carbonyl Index and Double Bond Index of control polyethylene and PE 4.1-PE 5.3

After incubating polyethylene with *Lysinibacillus*, polyethylene was incubated in the presence of bio-surfactant for 1 month (PE 5.2). In the FTIR spectra of PE 5.2, absorbance peak at 1740 cm^{-1} decreases, while the absorbance peak at 1650 cm $^{-1}$ increases (Supplementary Figure S5). This is due to the solubilisation of oxidation products into aqueous media and subsequent conversion of carbonyl groups into unsaturated hydrocarbons in the presence of bio-surfactant. This bio-surfactant treatment of polyethylene is followed by 1 month incubation with *Lysinibacillus* for biodegradation (PE 5.3). In the FTIR spectra of PE 5.3, a drastic increase in the absorbance peak at 1650 cm^{-1} is observed; this is due to the utilization of oxidized part by *Lysinibacillus* and subsequent conversion of carbonyl groups into unsaturated hydrocarbons.

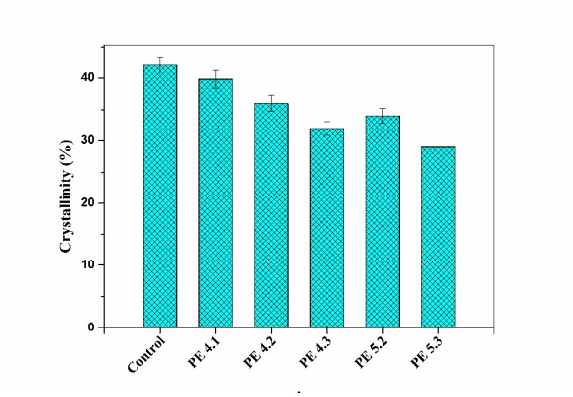

Figure 6: Crystallinity graph of control polyethylene and PE 4.1 to PE 5.3

After 1 month incubation of control polyethylene with *Lysinibacillus*, significant rise in the values of both C.I. and D.B.I can be observed (Figure 5). Carbonyl groups present in the polyethylene act as the initiator of photo-oxidation, due to which higher oxidation level is resulted in PE 4.2. Slight decrease in both of C.I. and D.B.I. of PE 4.3 is due to the utilization of oxidation products by *Lysinibacillus*. In case of PE 5.2, the decrease in C.I. and D.B.I. is possibly due to the solubilisation of oxidation product into the aqueous media in the presence of bio-surfactant. In case of PE 5.3, drastic increase in D.B.I. and comparatively less increase in C.I. are apparent. So, *Lysinibacillus* bacterium utilizes carbonyl groups and converts it into unsaturated hydrocarbon. Slight increase in the C.I. in case of PE 5.3 is due to the oxidation of polyethylene by extracellular enzymes of *Lysinibacillus* and by the dissolved oxygen present in the bacterial media.

3.4.2. XRD analysis:

The level of crystallinity of control polyethylene is compared to that of the treated polyethylene i.e PE 4.1-5.3 (Figure 6). In the case of PE 4.1 to PE 4.3, the crystallinity is found to decrease from that of the control polyethylene. During the oxidation of polyethylene by dissolved oxygen and extracellular enzymes of *Lysinibacillus*, oxidation products can act as the impurities. This phenomenon can cause the reduction in the crystallinity [20]. Crystallinity of PE 4.2 decreases after the oxidation by natural aging under sunlight, though the crystallinity of PE 1.2 increases due to the secondary crystallization initiated by oxidation product. From FTIR studies, C.I. of PE 4.2 is comparatively higher than that of the PE 1.2 as both were oxidised by natural aging under sunlight, indicating higher oxidation level of PE 4.2 (Figure 5). Oxidation of amorphous region as well as crystalline region of polyethylene can be resulted due to higher oxidation level of PE 4.2 which consequently causes the decrease in the crystallinity of PE 4.2. Further decrease in the crystallinity level in case of PE 4.3 is due to the utilization of oxidised product and further oxidation by *Lysinibacillus* bacterium. Crystallinity of PE 5.2 and PE 5.3 decreases from that of the control polyethylene. This decrease is due to the solubilisation of oxidized part and further oxidation during incubation with bio-surfactant. The solubilisation of oxidation product can be observed in FTIR studies where C.I. decreases in case of PE 5.2 from that of the PE 4.1 (Figure 5). Crystallinityof PE 5.3 decreases though oxidation level is not high. In case of PE 4.1, polyethylene is oxidised and that part is eventually utilised simultaneously by *Lysinibacillus* bacterium. Then, this polyethylene is again oxidised, followed by its solubilisation into the aqueous media simultaneously by bio-surfactant (PE 5.2). Then PE 5.2 is incubated with *Lysinibacillus* (PE 5.3). In this stage, oxidised part of polyethylene already present in the sample is utilised by *Lysinibacillus* and also this polyethylene sample is further oxidised simultaneously by the same bacterium. In this way, treated polyethylene gets bio-degraded and bio-deteriorated simultaneously. Total disruption of crystalline region of the

polyethylene may be resulted due to this phenomenon which is indicated from the decrease in the crystallinity of PE 5.3 to a comparatively lower value.

3.5. Oxidation of polyethylene by bio-surfactant produced by *B.licheniformis* in mineral media and subsequent biodegradation:

Similar type of treatment of polyethylene as first part of this study was done in this section (Table 1). Use of mineral media as growth media for *B.licheniformis* instead of YPD media was the only difference in this case. Higher surface tension was observed in case of *B.licheniformis* grown in mineral media than *B.licheniformis* grown in YPD media. Treatment used for PE 6.1 and PE 1.1 were the same except the difference in the growth media used for bio-surfactant production. Similarly, treatment used for PE 6.2-6.3, PE 7.2-7.3, and PE 8.2-8.3 were the same for PE 3.2-3.3, PE 1.2-1.3, PE 2.2-2.3, respectively (Table 1).

3.5.1. FTIR analysis:

New peak appears in the 1500-1800 cm $^{-1}$ region of the FTIR spectra of polyethylene incubated with the bio-surfactant produced by *B.licheniformis* using mineral media as growth media (Supplementary Figure S6). Peaks at 1740 cm^{-1} and 1650 cm^{-1} are due to the formation of ketones, aldehydes and unsaturated hydrocarbons as oxidation product. In case of PE 6.3, absorbance peak at 1740 cm^{-1} almost disappears due to the utilization of carbonyl groups by *Lysinibacillus* bacterium (Supplementary Figure S6). Changes in the FTIR spectra of PE 7.2 and PE 7.3 after treatment are identical to that of the PE 1.2 and PE 1.3 (Supplementary Figure S7, S1). Similarly, changes in the FTIR spectra of PE 8.2 and PE 8.3 after treatment are identical to that of the PE 2.2 and PE 2.3 (Supplementary Figure S8, S2). Only difference in case of PE 8.3 is that the increase in the absorbance peak at 1650 cm^{-1} is comparatively less than that of the PE 2.3.

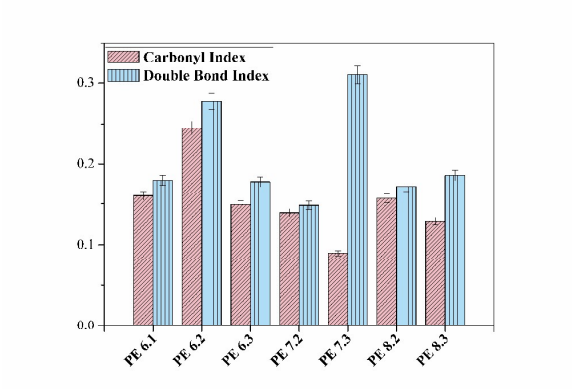

Figure 7: Carbonyl Index and Double Bond Index of control polyethylene and PE 6.1- PE 8.3

C.I. of PE 6.1 is comparatively less than the C.I. of PE 1.1 though both of the polyethylene samples are oxidised by bio-surfactant (Figure 7). Higher surface tension is recorded in case of *B.licheniformis* grown in mineral media; this can be a reason for lower level of oxidation in case of PE 6.1. Characteristic higher D.B.I and lower C.I. of PE 3.2 and 3.3 is also observed in case of PE 6.2 and 6.3. But, the value of both C.I. and D.B.I. of PE 6.2 and 6.3 is comparatively less than that of the PE 3.2 and 3.3. This is also due to the lower oxidation level of polyethylene. In case of PE 7.2-7.3 and PE 8.2-8.3, changing pattern of C.I. and D.B.I. value is identical to that of the PE 1.2-1.3 and PE 2.2-2.3 respectively. But, the value of C.I. and D.B.I. of PE 7.2-7.3 and PE 8.2-8.3 are comparatively low. From this observation, this is apparent that the lower the surface tension of the culture media, the higher is the oxidation level of polyethylene by bio-surfactant and vice-versa.

3.5.2. XRD analysis:

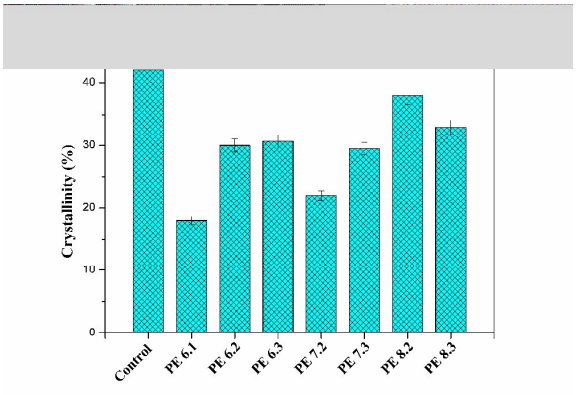

Figure 8: Crystallinity graph of control polyethylene and PE 6.1 to PE 8.3

Crystallinity level of control polyethylene is compared to that of the treated polyethylene i.e. PE 6.1 to PE 8.3 (Figure 8). Changing pattern of crystallinity of PE 6.1, PE 6.2-6.3, PE 7.2-7.3 and PE 8.2-8.3 is almost identical to that of the PE 1.1, PE 3.2-3.3, PE 1.2-1.3 and PE 2.2-2.3 respectively. But the corresponding changes are comparatively less in amount due to the lower level of oxidation.

3.6. Morphological analysis:

SEM images of the control polyethylene and other treated polyethylene samples are represented in Figure 9. Rough surface is observed in all eight treated polyethylene samples by different methods. Solubilisation of oxidation product and release of volatile oxidation products may give rise to such cracks on the polyethylene surface. In case of the polyethylene samples incubated with *Lysinibacillus* at the last stage of treatment, cracks and rough surface are formed due to bio-degradation and bio-deterioration of oxidised polyethylene surface by this bacterium. Such cracks and rough surface has also been observed in other studies after oxidation and biodegradation of polyethylene [18,14]. These cracks are the weak points for the bacteria for bio-film formation on the polyethylene surface. Such bio-film formation around the cracks on the bio-degraded polyethylene sample can be observed in case of PE 6.3 and 8.3 [24]. Comparatively smoothed surface is resulted from this kind of bio-film formation around cracks due to the bio-degradation of the oxidized polyethylene. Surface of PE 2.3 and 3.3 are comparatively smoother than the surface of PE 7.3, 8.3 and 6.3 respectively. Comparatively smoothed surface is resulted due to higher rate of bio-degradation by *Lysinibacillus* as the oxidation level in both the samples PE 2.3 and PE 3.3 is high.

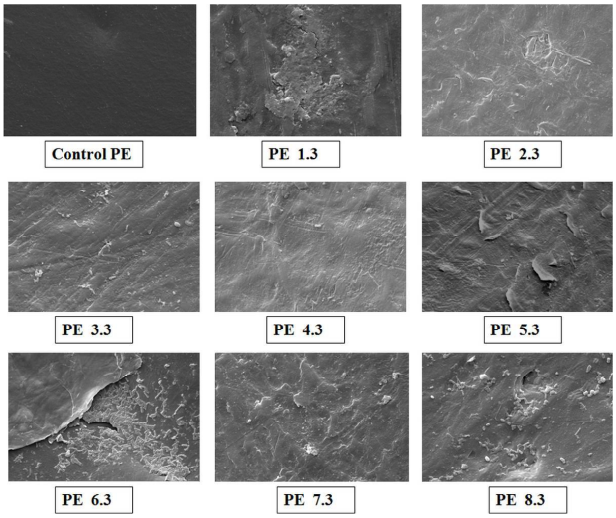

Figure 9: Scanning Electron Microscope image of PE 1.3-PE 8.3 and control polyethylene

3.7. Gravimetric Analysis:

Out of eight treated polyethylene samples, six shows weight-loss after treatment for 3 months (Figure 10).

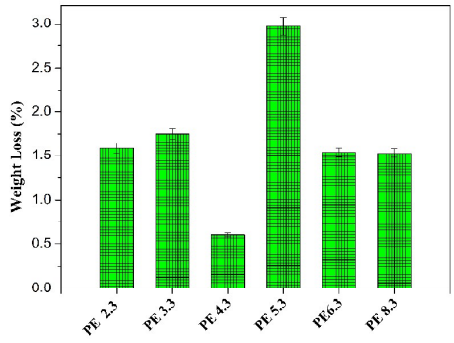

Figure 10: The bio-degradation of polyethylene. Dry weight loss of polyethylene after 90 days or 3 months

No weight loss is observed after 3 months of treatment in case of PE 1.3 and PE 7.3 which were also not treated with *Lysinibacillus* bacterium. Weight-loss is observed in other six treated polyethylene samples i.e PE 2.3, PE 3.3, PE 4.3, PE 5.3, PE 6.3 and PE 8.3 which were treated with *Lysinibacillus* during last stage of the treatment (Table 1). So, it is apparent that biodegradation of polyethylene is only caused by *Lysinibacillus* bacterium. Maximum weight loss is achieved in case of PE 5.3 which is 2.97 ± 0.5%. In case of PE 5.3, during first month of treatment, control polyethylene is simultaneously oxidised and that oxidised part is utilised by *Lysinibacillus* bacterium (PE 4.1). Then during second month, that polyethylene is oxidised and the oxidised part of polyethylene is solubilised by bio-surfactant produced by *B.licheniformis* (PE 5.2). During third month of treatment, that polyethylene is again oxidised and biodegraded by *Lysinibacillus* simultaneously (PE 5.3) (Table 1). In case of PE 4.1, 5.2 and 5.3, these changes can be observed in FTIR analysis by the respective increase and decrease in the value of C.I. and D.B.I. (Figure 5). This way, polyethylene is biodegraded. In a recent study, less than 1% weight loss is achieved during the biodegradation of thermally treated polyethylene by *Bacillus cereus* and *Bacillus sphericus* after 3 months [16]. In another study, rate of polyethylene degradation ranging from 3.5% to 8.4% in 10 years has been reported [25]. After 15 years of treatment in soil, 16% weight-loss of polyethylene has been achieved in another study[26]. The achieved rate of degradation in this study is higher than the rate of degradation of polyethylene as reported in the previous studies.

3.8. GC-MS analysis:

Summarized list of all products identified in GCMS analysis of treated polyethylene is represented in Table 2. Con_PE is the control polyethylene. Con_lyn is the control polyethylene

Antioxidants:

	Compound name	Con_PE	Con_lyn	Con_lich	PE3.3	PE5.3
1	Phenol, 2,4-bis(1,1-dimethylethyl)-	Y	Y	Y	Y	Y
	Pentanoic acid, 5-hydroxy-, 2,4-di-t-butylphenyl esters	Y	Y	Y	Y	Y
	Phenol, 2,6-bis(1,1-dimethylethyl)-	Y	Y	Y	Y	Y

CH_2 group:

2	Tetracosane, 11-decyl- (24C)	Y	N	N	N	N
3	Heneicosane (21C)	N	Y	Y	N	N
4	Eicosane, 7-hexyl- (20C)	Y	N	N	N	N
	Eicosane	Y	Y	Y	N	N
	Eicosane, 2-methyl-	N	N	N	N	N
	Cycloeicosane	N	N	N	N	Y
	Eicosane, 10-methyl-	N	N	Y	N	N

5	Nonadecane	N	N	Y	N	N
	Nonadecane, 9-methyl- (19C)	N	Y	N	N	N
6	Octadecane, 3-ethyl-5-(2-ethylbutyl)- (18C)	N	Y	N	N	N
	Octadecane	N	Y	N	N	N
7	Heptadecane, 9-hexyl- (17C)	Y	Y	N	N	N
	Heptadecane, 7-methyl	N	N	N	N	N
	Heptadecane	Y	N	Y	N	N
8	Hexadecane, 2,6,11,15-tetramethyl- (16C)	N	Y	N	N	N
	Hexadecane, 2,6,10,14-tetramethyl-	N	N	N	N	N
	Cyclohexadecane		N	Y	N	N
	Hexadecane	Y	Y	Y	N	N
9	Pentadecane (15C)	N	Y	N	N	N
	Pentadecane, 7-methyl	N	Y	N	N	N
	Pentadecane, 3-methyl-	N	N	N	N	N
10	Tetradecane, 2,6,10-trimethyl- (14C)	N	N	Y	N	N
	Tetradecane, 4-methyl-	N	N	N	N	N
	Tetradecane, 2-methyl-	N	N	N	N	N
	Cyclotetradecane	N	N	N	Y	Y
	Tetradecane	Y	Y	N	Y	N
	Tridecane	N	N	Y	N	N
11	Dodecane, 5,8-diethyl- (12C)	N	Y	N	N	N
	Dodecane, 2,5-dimethyl-	N	N	N	N	N
	Dodecane, 2,6,10-trimethyl	Y	N	N	Y	Y
	Dodecane	Y	Y	Y	Y	Y
12	Undecane (11C)	N	Y	N	N	N
	Undecane, 2,6-dimethyl-	N	N	N	Y	N
13	Decane, 3,6-dimethyl- (10C)	N	Y	Y	N	N
	Decane	N	N	N	Y	Y
	Decane, 2-methyl-	N	N	N	N	
14	Octane, 3,5-dimethyl-	N	N	N	Y	N
15	Benzene, 1,3-bis(1,1-dimethylethyl)- (6C)	Y	Y	N	N	N
	Benzene, 1,4-bis(1,1-dimethylethyl)-	Y	Y	N	N	N
16	m-Cymene, 5-tert-butyl-	Y	Y	N	N	N

603

604 Oxidation product:

605 Acids:

17	9-Hexadecenoic acid	N	Y	Y	N	N

	Pentadecanoic acid	N	N	Y	N	N
	Oleic acid, 3-(octadecyloxy)propyl ester	N	Y	Y	N	N
	Octadecanoic acid	N	N	Y	N	N
	Docosahexaenoic acid, 1,2,3-propanetriyl ester	N	Y	N	N	N
	Oleic acid, eicosyl ester	N	N	Y	N	N
	9-Octadecenoic acid (Z)-, tetradecyl ester	N	N	Y	N	N
	Erucic acid	N	N	Y	N	N
	22-Tricosenoic acid	N	N	Y	N	N
	cis-13-Octadecenoic acid	N	N	Y	N	N
	cis-Vaccenic acid	N	N	Y	N	N
	Nonahexacontanoic acid	N	N	Y	N	N
	Cyclopropaneoctanoic acid, 2-[(2-pentylcyclopropyl)methyl]-, methyl ester, trans,trans-	N	N	Y	N	N

606

607 Ketones:

18	2-Pentanone, 4-hydroxy-4-methyl-	N	Y	Y	N	N

608

609 Alcohol:

N19	1-Heptatriacotanol	N	N	Y	N	N
	2-Hexanol, 2-methyl-	N	Y	Y	N	N
	2-Pentanol, 2,4-dimethyl-	N	N	Y	N	N
	1-Octanol, 2-butyl-	N	N	Y	N	N
	1-Undecanol	N	N	N	N	Y
	Ethanol, 2-(9-octadecenyloxy)-, (Z)-/ 2-(tetradecyloxy)-	N	N	Y	N	N
	E,E,Z-1,3,12-Nonadecatriene-5,14-diol	N	N	Y	N	N

610

611 Esters:

20	E-8-Methyl-9-tetradecen-1-ol acetate	N	N	Y	N	N

612

613 Other oxidation product:

21	3,8,12-Tri-O-acetoxy-7-desoxyingol-7-one	N	Y	N	N	N
	9-Octadecene, 1-[2-(octadecyloxy)ethoxy]-	N	Y	N	N	N
	Dodecane, 1-methoxy-	N	N	Y	N	N
	1-Tetradecanol, methyl ether	N	N	Y	N	N
	1-Docosanol, methyl ether	N	N	N	Y	N

	Citral	N	N	N	Y	N
	2,6-Octadienal, 3,7-dimethyl-, (Z)-	N	N	N	Y	N
	Benzaldehyde, 2,5-dimethyl-	N	N	N	Y	Y
	Benzaldehyde, 2,4-dimethyl-	N	N	N	Y	Y
	Benzaldehyde, 2-ethyl-	N	N	N	Y	Y
	Benzaldehyde, 2-hydroxy-/ 3-hydroxy/ 4-hydroxy	N	N	Y	N	N

Unsaturated Hydrocarbon:

22	3-Tetradecene, (Z)-	N	N	N	Y	N
	trans-3-Decene	N	N	N	Y	N
	1-Dodecene	N	N	N	Y	N
	10-Heneicosene (c,t)	N	N	N	Y	Y
	6-Dodecene, (Z)-/ (E)	N	N	N	Y	Y
	9-Nonadecene	N	N	N	Y	Y
	1-Octadecene	N	N	N	N	Y

Table 2: List of identified chemicals in GC-MS analysis of treated polyethylene samples.

Con_PE: control polyethylene**. Con_lyn**: control polyethylene incubated with *Lysinibacillus* for 1 month.**Con_lich**: control polyethylene incubated with *B.licheniformis* for 1month

incubated with *Lysinibacillus* for 2 months. Con_lich is the control polyethylene incubated with bio-surfactant produced by *B.licheniformis* for 1 month using YPD medium. Acids, alcohols and ether are formed during oxidation of polyethylene by *Lysinibacillus* and bio-surfactant [22,14]. But only one type of ketones and esters formed after oxidation. As observed in Figure 3 and Figure 5, the value of C.I. and D.B.I. of PE 3.3 is comparatively higher than that of the PE 5.3. Presence of comparatively higher variety of alcohols, acids and unsaturated hydrocarbons in PE 3.3 compared to PE 5.3 is in correspondence with the observed value of C.I. and D.B.I (Figure 3, 5). In case of PE 5.3, biodegradation of treated polyethylene is apparent from the presence of small molecule of hydrocarbons i.e. decane, dodecane. But less variety of oxidation products is identified in case of PE 5.3 which is due to utilization of oxidation product by *Lysinibacillus*. From GC-MS analysis, it is also apparent that polyethylene is bio-degraded via conversion of carbonyl group into unsaturated hydrocarbons by the two bacteria.

Different varieties of nitrogenous compounds are identified in the case of control polyethylene incubated with *Lysinibacillus* and PE 3.3, PE5.3, which are parts of bio-film of bacterium formed on the polyethylene surface. Benzaldehyde present in oxidized polyethylene is formed due to partial oxidation of anti-oxidant i.e. Phenol, 2,4-bis(1,1-dimethylethyl) present in the commercial polyethylene [27].

Two negative control polyethylenes kept in mineral medium and YPD medium without any bacterium do not exhibit any chemical, structural and morphological changes during 3 months of incubation.

4. Conclusion:

From the above observation, it is apparent that *Lysinibacillus* is a unique bacterium with the ability of oxidation and bio-degradation of commercial polyethylene waste bags. Mainly oxidation of polyethylene by *Lysinibacillus* is observed when used polyethylene is either unoxidized or oxidation level is very low. But, when the oxidation level of polyethylene is very high, then the conversion of the carbonyl groups into unsaturated hydrocarbons is observed rather than formation of more carbonyl groups. So, polyethylene is oxidised by *Lysinibacillus* for the formation of oxidation product from polyethylene which can later be utilized by the same bacterium for bio-film formation. Bio-surfactant produced by *B.licheniformis* JF2 (ATCC- 39307) does not have any anti-adhesive property. *Lysinibacillus* has been able to form bio-film on the surface of the polyethylene, oxidised by bio-surfactant as well as on the surface of the control polyethylene. Out of the eight treated polyethylene samples, six samples have been incubated with *Lysinibacillus*. Weight-loss is observed in these six polyethylene samples after treatment of 3 months. Oxidation level of other two polyethylene samples i.e. PE 1.3 and 7.3, treated with bio-surfactant and aged under Sun-light is comparatively higher, though no weight-loss is observed for these two polyethylene samples. So, *Lysinibacillus* is mainly responsible for bio-degradation process of polyethylene and this biodegradation process of polyethylene mainly advanced through the conversion of carbonyl group into unsaturated hydrocarbons. In this study, biodegradation is carried out using two bacteria. One bacterium *B.licheniformis* is used for the production of bio-surfactant and oxidation of polyethylene followed by solubilisation of oxidation product by that bio-surfactant. Another bacterium *Lysinibacillus* is used for biodegradation as well as oxidation of commercial polyethylene. Using these two bacteria simultaneously, one can lead to the biodegradation and bio-deterioration of commercial polyethylene. This method is also very environmental friendly.

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