Section A-Research paper

Isolation and Molecular characterization of Low Density Poly Ethylene (LDPE) degrading bacteria

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Abstract:

The usage of Low Density Poly Ethylene has become one of the major sources of environmental pollution. These plastics exist mostly as xenobiotic components which persist in the environment for such a long period. The current study is aimed at isolation of LDPE degrading bacteria from soil sample. Plastic dumped sites are considered to be the habitat for plastic degrading bacteria. So, such a soil sample was used for isolation of these kind of bacteria. Reduction in weight was noted when the serially diluted soil sample was inoculated along with pre weighed LDPE sheets. Further, repeated spread and streak plate techniques helped in the isolation of four distinct bacterial strains JE1, JE2, JE3, JE4. Determination of protein concentration is an indirect estimation of microbial biomass attached to LDPE films. The protein content was estimated by lowry's method and it was found to be 5.22 μg mg^{-1} PE for JE1, 3.72 μg mg^{-1} PE for JE2, 2.15 μg mg^{-1} PE for JE3 and 3.12 μg mg^{-1} PE for JE4 respectively. LDPE films were checked for degradation with individual strains and weight reduction percentage was calculated. When incubated, the strain JE1 showed highest weight loss percentage of about 0.7%. So, the strain JE1 had highest degrading efficiency and used for further studies. FTIR (Fourier transform Infrared Spectroscopy) analysis of treated and untreated LDPE films showed considerable change in the structural confirmation of plastics which is indicative of plastic degradation. Molecular characterization through 16 S rRNA gene sequencing and BLAST (Basic Local Alignment Search Tool) analysis and Phylogenetic revealed that the selected strain is *Bacillus subtilis*. Further, large scale implementation of plastic degradation by the potent strain for longer duration would yield promising results.

Keywords: Plastics, Low density polyethylene, Fourier transform Infrared Spectroscopy, Degradation, Xenobiotic components, gene sequencing.

Introduction:

Polythene is used to meet our desired needs in day to day life. It can be used for various purposes from processing of various products to manufacturing of scientific instruments (Anthoni Agustien *et al.*, 2016). Plastics are organic polymers of high molecular mass. It is non-biodegradable, strong, long chain polymers of carbon and hydrogen.

Plastics can be modified into different shapes or molded using heat and pressure. Most common varieties of plastic are polyethylene, poly-propylene, polystyrene etc. Among the various types of plastics, the most used and popular are Low density polyethylene (LDPE) and High-density polyethylene (HDPE) (Priya Trivedi *et al*., 2010).

One of the major sources of environmental pollution is Low density polyethylene (LDPE). It is the most produced polymer accounting for 21% of global production. Under high pressure and high temperature using oxide initiators LDPE is prepared from gaseous ethylene. These processes produce a polymer structure with long and short branches. Low-density polyethylene shows inert function towards microorganisms. Hence, they are slowly degraded in both stabilized and un-stabilized forms (Sen and Raut., 2015).

An estimation of around 57 million tons of plastic waste has been generating worldwide annually. These plastics do not break down easily due to its strong mechanical and thermal properties. As a result, they remain in the environment for a very long period and cause environmental pollution. To prevent accumulation, it should be properly disposed (Patil R.C 2018). Different methods such as landfilling, incineration and recycling are used to dispose plastics. In each case there are limitations and benefits (Afreen Bakht *et al*., 2020).

Over the past few years there has been a significant interest in the degradation of already existing plastic and development of biodegradable plastic (Nur Haedar *et al*., 2019). Biodegradation is a process in which microorganisms degrade natural or synthetic polymers. Microorganisms use polythene as substrate for the growth when they are being degraded. However, biodegradation plays a major role in degrading plastic by reducing the molecular weight using microorganisms such as bacteria, fungi and actinomycetes in the natural environment (Deepika and Jaya Madhuri., 2014).

Biodegradation is cheap, pollution friendly, less toxicity and does not produce any harmful effect to the environment. Microbial degradation process is an enzymatic activity by cleaving polymers into oligomer and monomer. Enzymes namely endoenzymes and exoenzymes of microorganisms has the ability to degrade a substrate into simpler component (Leja K., 2009).

These enzymatically digested polymers are used as carbon and energy source by the microorganisms. Among various Microorganisms certain species such as *Pseudomonas, Bacillus,*

Streptococcus, Aspergillus niger and *Aspergillus* galcus has been identified as efficient polythene degrading microorganisms (Usha *et al*., 2011)

It is expected that plastic dumped sites contain Plastic Degrading Bacteria. So such soil would be ideal for LDPE degrading bacterial isolation. By considering the above aspects, the present study aims on the isolation and identification of the soil bacteria, which is capable of degrading LDPE and to study its degrading efficiency under laboratory scale.

Experimental Procedures:

Sample Collection:

The soil samples were collected from plastic waste dumped site Thengapattanam, Kanyakumari District, Tamil Nadu. At a depth of 5 cm from the ground, soil was collected, sealed in a sterile ziplock cover and transferred to the lab immediately.

Isolation of Polythene Degrading Bacteria:

According to the method proposed by (Hadad *et al*., 2005) isolation of low-density polythene (LDPE) degrading bacterial strains was performed. Synthetic medium (NH_4NO_3: 1.0g; $MgSO_4.7H_2O$: 0.2g; K_2HPO_4: 1.0g; $CaCl_2.2H_2O$: 0.1g; KCl: 0.15g; yeast extract: 0.1g; $FeSO_4.6H_2O$: 1.0 mg; $ZnSO_4.7H_2O$: 1.0 mg and $MnSO_4$: 1.0 mg in 1000 ml distilled water) is used for the culture of bacterial strains. 1gm of soil sample was added as a inoculum source in 100 ml SM medium. Fresh untreated low-density polythene films were brought and cut into small pieces of each 1×1 cm, weighed the films at 300 mg/ml. Then the films were disinfected in 70% ethanol and kept in laminar air flow chamber to air dry for 15 min and was added to the SM medium. At 37°C, the medium was then incubated in a rotary shaker for 7 days. Once for every 7 days sub-cultures made up to 35 days (Gilan *et al.,* 2004).

After the sub-culture the enriched culture broth was spread on the agar plates and incubated at 37°C. Based on the size of the clear zone LDPE degrading bacterial strains were selected (Sriyapai *et al.,* 2018).

Identification of Polythene Degrading Bacteria:

By following the Bergey's manual of systemic biology (Sneath, 1986) the polythene degrading bacteria strains were identified on the basis of morphological, cultural and biochemical characteristics.

In-Vitro Biodegrading Assay:

The LDPE degrading efficiency of the isolated bacterial strains was studied individually in the in-vitro biodegradation assay. The bacterial strains were inoculated separately in a synthetic medium containing polythene as a carbon source at 30°C for 30 days in a rotary shaker (Ariba Begum *et al.,* 2015).

Growth Curve of the Bacterial Isolate:

In nutrient broth the bacterial isolates were grown overnight until the culture attained log phase. 10% of this log phase culture was inoculated in 50 ml synthetic medium containing polythene films. The untreated LDPE films in the non-inoculated minimal broth were maintained as control under similar conditions. At several intervals (Day 0, 3, 7, 10, 14, 17, 21, 24, 28 and 30) the culture broth was determined to spectrophotometric analysis (Kavitha *et al.,* 2014).

Quantification of bacterial biomass:

The quantitative estimation of bacterial biomass upon treated polyethylene was performed according to the method proposed by Gilan *et al*., (2004). Pieces taken from polyethylene film colonized in SM were washed briefly in water, then in 5 ml of 0.5 N NaOH and agitated overnight on rotary shaker. The suspension was centrifuged, the supernatant was saved and the pellet was subjected to the same procedure once again. The two supernatants were combined, the protein concentration was determined in each supernatant using Bovine serum albumin as standard according to Lowry *et al.*, (1951) and the values were then combined.

Determination of the Weight of Reduced LDPE:

Bacterial Bio-films colonizing the polythene surface was removed by treating with 2%

(v/v) aqueous sodium dodecyl sulfate (SDS) solution for 4 hours followed by rinsing with distilled water (Usha *et al*., 2011)

$$\text{Weight Reduction } (\%) = (W_0\text{-}W_t) \times 100$$

Fourier Transform Infrared Spectroscopy:

The untreated and treated LDPE films after incubation with bacterial isolates were analyzed using Fourier Transform Infrared Spectroscopy (FTIR). Formation of new functional groups or changes in the amount of existing functional groups in the polythene films was analyzed (Divyalakshmi and Subhashini, 2016).

Molecular characterization of the selected bacterial strains:

The genomic DNA was isolated from the selected bacterial strain and run under agarose gel electrophoresis. Sequenced the bacterial strains and the obtained sequence was compared with 16 rRNA database of NCBI using the BLAST program (Altschul *et al*., 1997). Phylogenetic tree was constructed using MEGA 11 software (Tamura *et al*., 2007) by the neighbor-joining method (Saitou and Nei, 1987).

Results and Discussion:

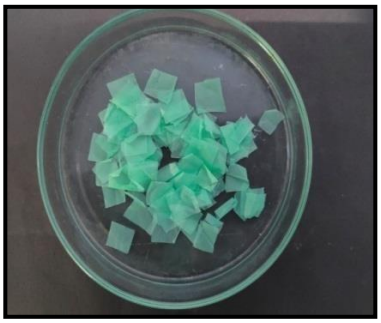


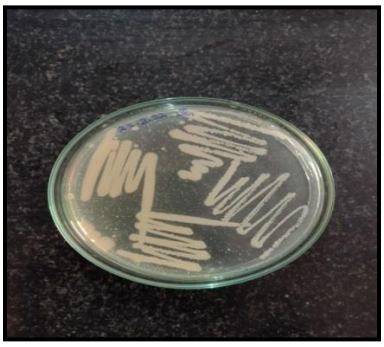
Fig 1: Plate displaying low density polythene sheet

Fig 2: Incubation for 30 days

JE1

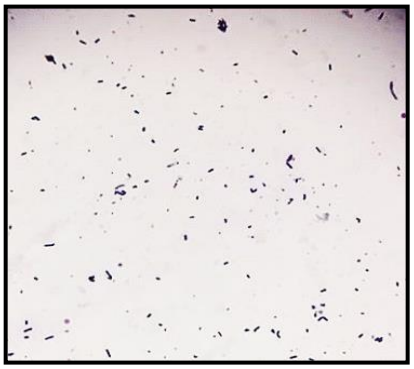
JE2

JE3

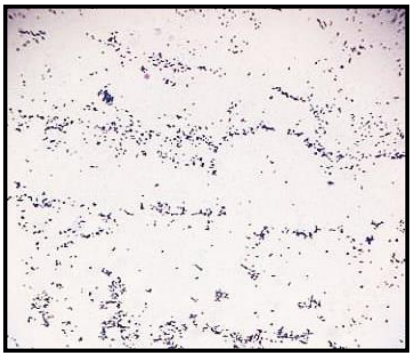


JE4

Fig 3: Plate Showing isolated four bacterial strains

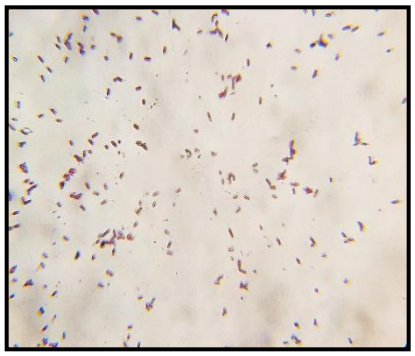


JE1

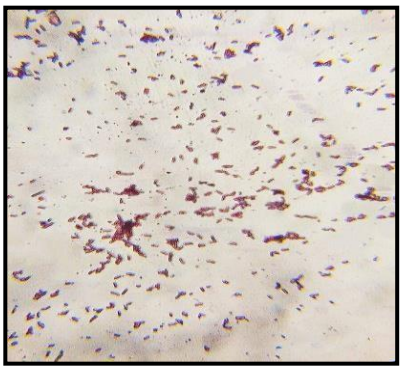


JE4

Fig: 4 Microscopic observations of Gram positive bacteria



JE2



JE4

Fig 5: Microscopic observations of Gram Negative Bacteria

Table 1: Biochemical Characteristics of the isolates

DESCRIPTION	**INFERENCE**			
	JE1	**JE2**	**JE3**	**JE4**
Colony morphology	Whitish creamy opaque, jagged edges and rough colonies	Whitish creamy, large, opaque and flat colonies	Whitish, smooth edges, convex surface and rough colonies	Greyish white, large, wavy edges and granular colonies
MICROSCOPIC EXAMINATION				
Gram's staining	+	–	–	+
Spore staining	+	–	–	+
BIOCHEMICAL TESTS				
Motility test	+	+	+	+
Catalase test	+	+	+	+
Indole production test	–	–	–	–
Methyl red test	–	–	+	–
Voges- proskeur	+	–	+	+
Starch hydrolysis	+	–	–	+

Gelatin liquefaction	+	+	+	–

Casein hydrolysis	+	+	+	+
Hydrogen sulphide test	+	–	+	+
TSI test	–	–	+	–
Carbohydrate fermentation test				
Sucrose	+	–	–	+
Glucose	+	+	–	+
Dextrose	–	+	+	+

(+) indicates Positive, (-) indicates Negative

Fig 6: Biodegradation of LDPE film

Table 2: Growth Curve studies

Name of the isolates	No. of days of incubation								
	3	**7**	**10**	**14**	**17**	**21**	**24**	**28**	**30**
JE1	0.12	0.32	0.16	0.26	0.21	0.27	0.16	0.13	0.12
JE2	0.10	0.23	0.14	0.20	0.18	0.11	0.17	0.12	0.09
JE3	0.07	0.20	0.12	0.19	0.16	0.18	0.13	0.11	0.10

JE4	0.09	0.24	0.16	0.20	0.21	0.16	0.12	0.10	0.13

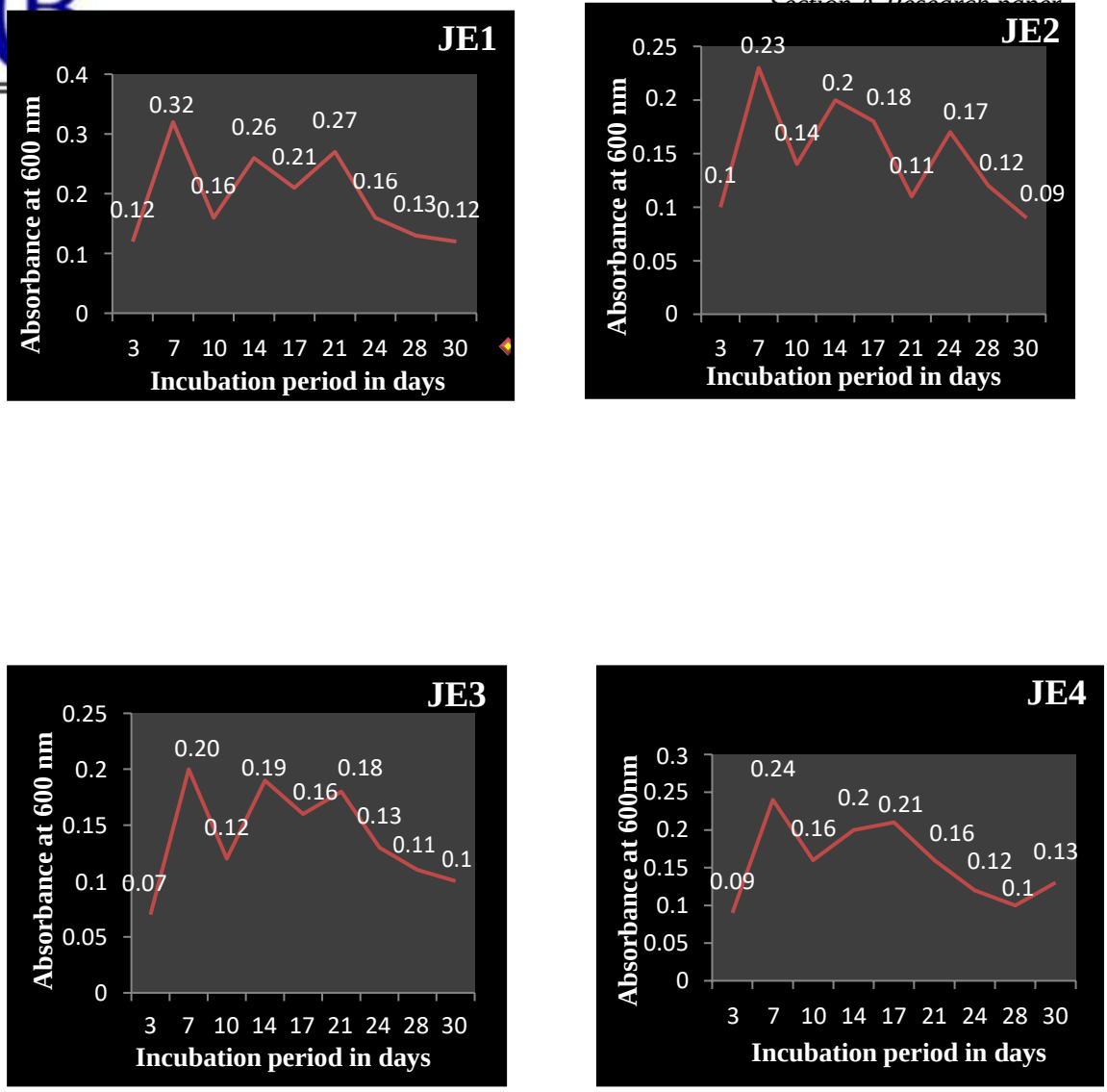


Fig 7: Growth curve of the bacterial isolates

Table 3: Estimation of Protein- Standard

Sl. No	Concentration of BSA (µg)	OD values at 670 nm
1	1	0.07
2	2	0.12
3	3	0.21
4	4	0.29

5	5	0.39

6	6	0.50
7	7	0.58
8	8	0.62

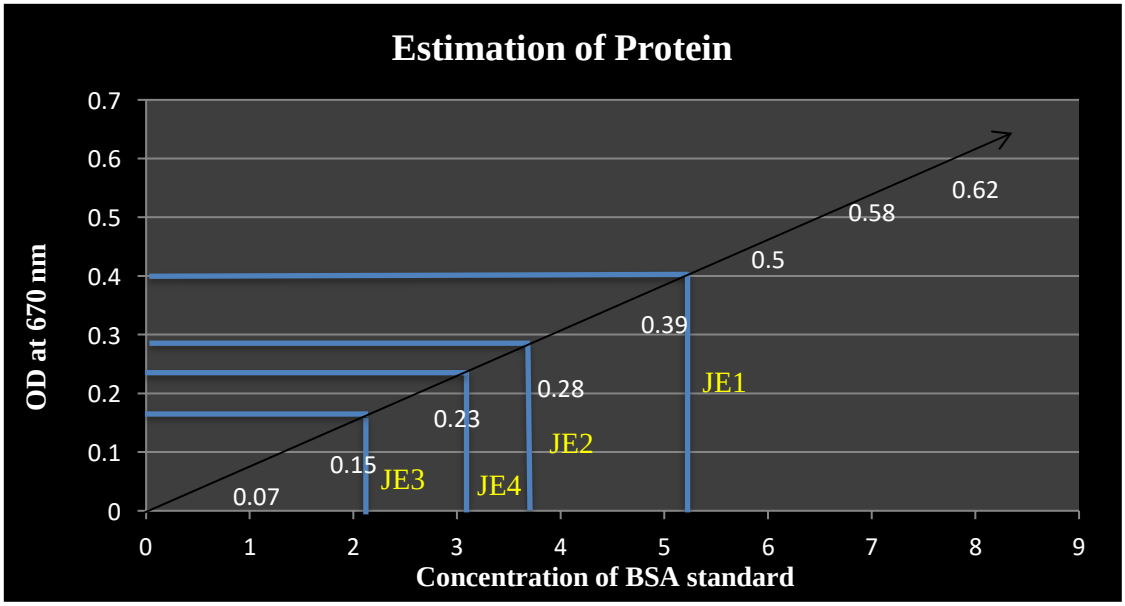


Fig 8: Estimation of Protein – standard

Table 4: Quantification of Bacterial Biomass

Bacterial isolates	Biofilm protein content ($\mu g\ mg^{-1}$ polyethylene)
JE1	5.22
JE2	3.72
JE3	2.15
JE4	3.12

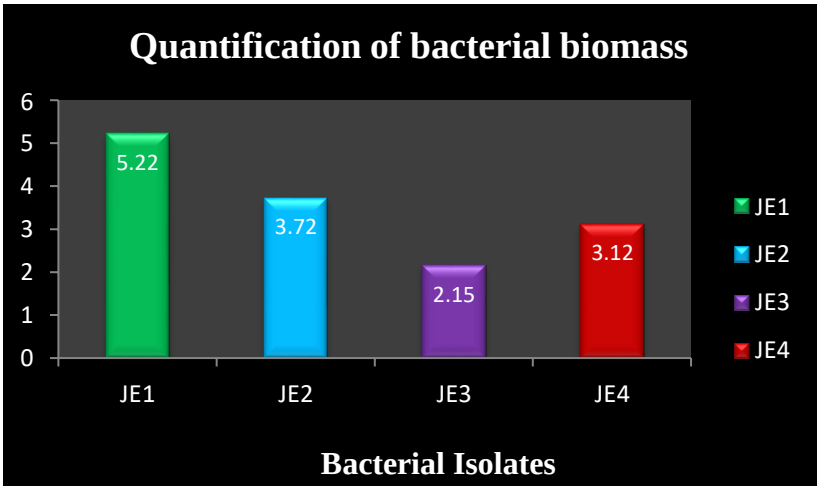


Fig 9: Quantification of bacterial biomass

Table: 5 Weight reduction of LDPE Films in 30 days' time period

	Weight reduction after 30 days (g)	**Weight loss percentage (%)**
JE1	0.293	0.7
JE2	0.295	0.5
JE3	0.298	0.2
JE4	0.297	0.3

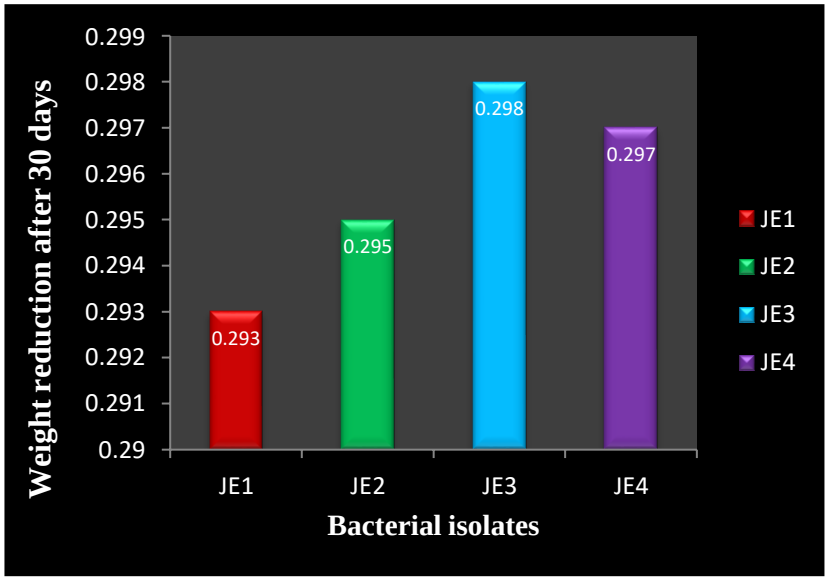


Fig 10: Weight loss percentage of the LDPE films

Table 6: Characterization peak values of Untreated LDPE

Sl. No	Wave Number (cm^{-1})	Bond	Functional group
1	2914.44	- C-H Stretch	Alkanes
2	2848.86	H-C=O: C-H stretch	Aldehydes
3	2366.66	H-C=O: C-H stretch	Aldehydes
4	1462.04	-C-H Bend	Alkenes
5	1020.34	=C-H Bend	Alkenes
6	875.68	-C=O Stretch	Ketones, Aldehyde
7	719.45	-C=O Stretch	Ketones, Aldehyde

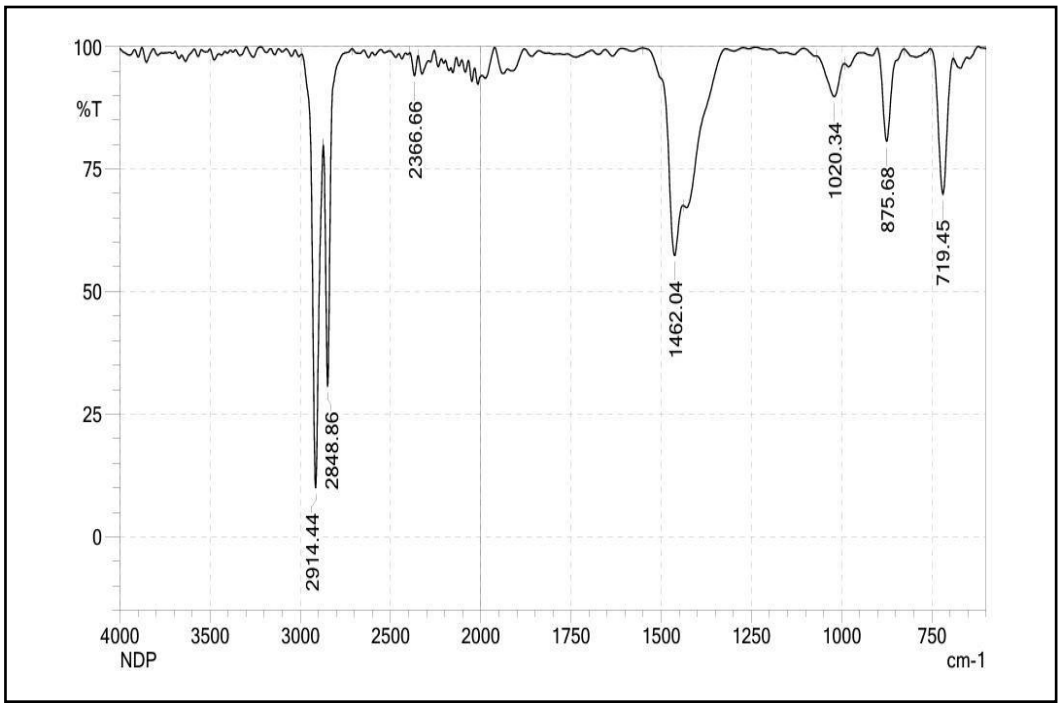


Fig 11: FTIR spectra of untreated LDPE

Table 7: Characterization peak values of Treated LDPE

Sl. No	Wave Number (cm^{-1})	Bond	Functional group
1	2914.44	- C-H Stretch	Alkanes
2	2848.86	H-C=O: C-H stretch	Aldehydes
3	2181.49	C≡***C***	Alkyne (modified)
4	1938.46	C-H Bend	Alkene (new)
5	1463.97	-C-H Bend	Alkene (modified)
6	1022.27	=C-H Bend	Alkene (modified)
7	875.68	-C=O Stretch	Ketones, Aldehyde

8	719.45	-C=O Stretch	Ketones, Aldehyde

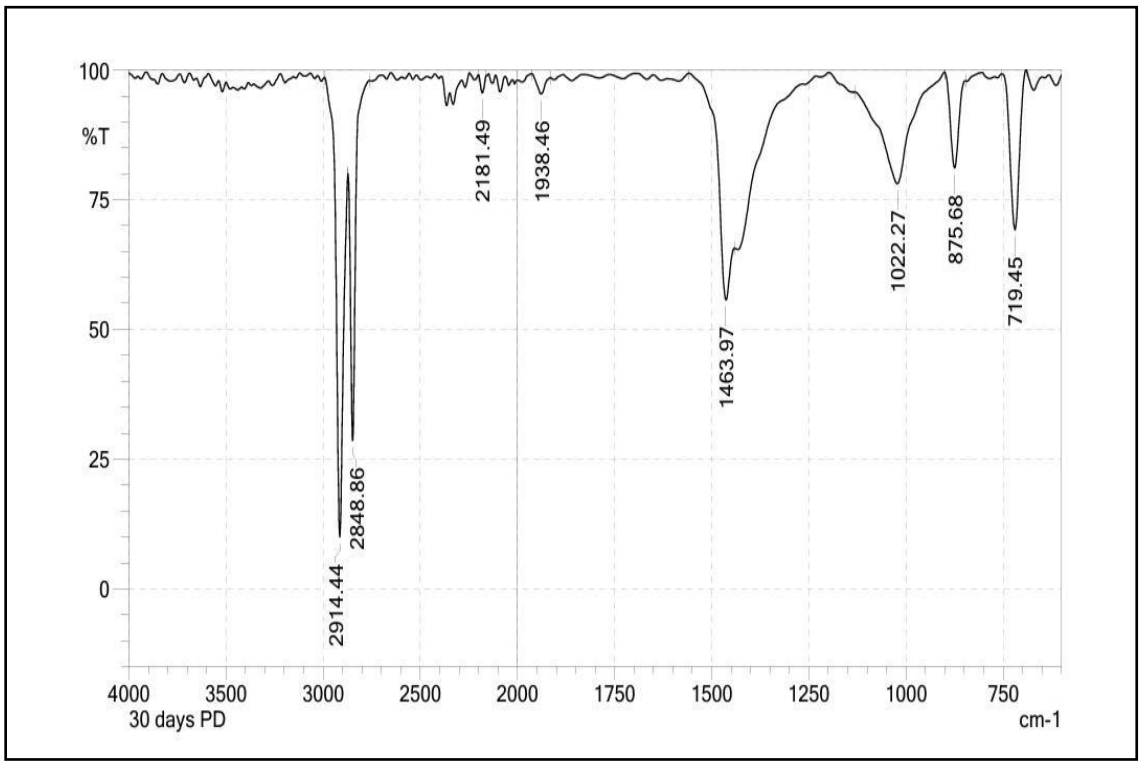


Fig 12: FTIR spectra of LDPE treated with JE1

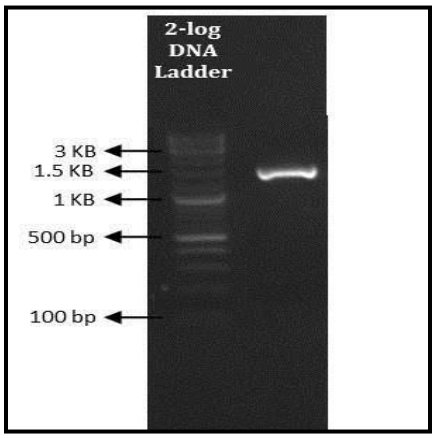


Fig 13: Agarose gel electrophoresis

6/1/22, 2:13 PM Bacillus subtilis strain SJJMPSM1 16S ribosomal RNA gene, partial sequ - Nucleotide - NCBI

An official website of the United States government
Here's how you know

Log in

Nucleotide

GenBank

Bacillus subtilis strain SJJMPSM1 16S ribosomal RNA gene, partial sequence

GenBank: ON544027.1

FASTA Graphics

Go to:

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  ORGANISM  Bacillus subtilis
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REFERENCE   1  (bases 1 to 449)
  AUTHORS   Jency,S., Sasi,P. and Mahitha,S.
  TITLE     Isolation and molecular characterisation of Plastic degrading
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  JOURNAL   Unpublished
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  AUTHORS   Jency,S., Sasi,P. and Mahitha,S.
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Fig 14: 16S rRNA SEQUENCE, SJJMPSM1, *Bacillus subtilis*, ACCESSION NO: ON544027

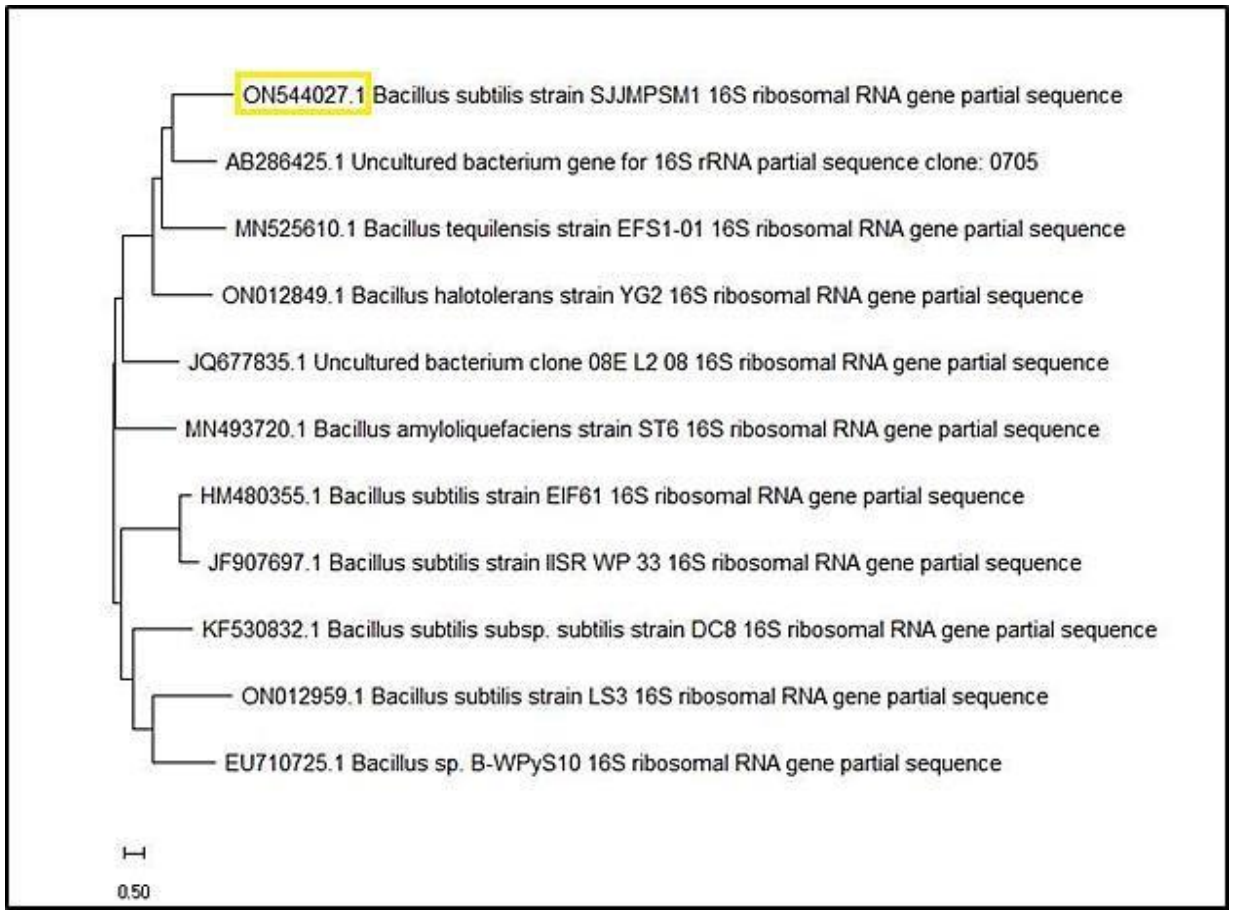

Fig 16: Phylogenetic Tree of SJJMPSM1, *Bacillus subtilis* ACCESSION NO: ON544027

The excessive use of plastics has created a great impact for the past few years. The need for biodegradable plastic and biodegradation of already existing plastics has been increasing lately (Sowmya *et al*., 2014). As microorganisms have not yet been fully evolved to synthesize enzymes to degrade artificial plastics such as polyethylene, waste plastics discarded in nature sometimes persist for centuries as xenobiotics and arouse public concern (Jeon and Kim, 2016).

The soil sample for the current study was chosen as plastic dumped sites for prolonged period of time. It was assumed that those soil samples would have plastic degrading strains (Vignesh *et al*., 2016). Serially diluted the soil sample and inoculated in SM medium with fresh Low density plastic films (Fig: 1). Bacteria which have the ability to sustain in this medium will degrade and utilize the plastic films. After incubation period of 30 days (Fig: 2), weight reduction

in plastic films was noticed. So, further from this, the colonies were spread on to nutrient agar plates and individual colonies isolated. A total of four bacterial strains were selected from the

individual colonies. The indigenous four strains were designated as JE1, JE2, JE3 & JE4 (Fig: 3). The isolated bacterial strains were identified according to their morphological, staining and Biochemical characteristics. When gram's staining was performed, it was found that the strains JE1 & JE4 are Gram positive (Fig: 4) and the strain JE2 & JE3 Gram negative (Fig: 5). Biochemical characterizations were performed for the isolates and are tabulated in (Table: 1).

The four isolated bacterial strains were subjected to secondary screening to evaluate their efficacy of polyethylene degradation (Kathiresan, 2003). Individually these colonies were inoculated in SM medium containing fresh LDPE films with bacterial strains and incubated for about thirty days in a rotary shaker (Fig: 6). Growth of the bacterial strains in the SM was studied throughout the incubation period of the biodegradation assay.

An aliquot from each of the four bacterial strains were taken at regular intervals of 3, 7, 10, 14, 17, 21, 24, 28 and 30 days of incubation for quantification of bacterial cells by measurement of OD at 600 nm. The growth rate of the isolated bacterial strains in SM containing polyethylene as sole carbon source are presented in (Table: 2). According to (Agustien *et al.,* 2016) normal bacterial growth in media containing plastic polymers and some elements of nitrogen can indicate that bacteria are able to use carbon elements from polymers to meet their carbon requirements in their metabolic processes and microorganisms will secrete a catalytic enzyme that can damage the structure of the polymer. The turbidity of the LDPE supplemented SM increased with time of incubation and reached the maximum at the end of seven days of inoculation with the isolated bacterial strains as evident from (Fig: 8). When necessary the fresh medium was supplemented to maintain the organism in their efficient logarithmic growth phase. Control was maintained with LDPE films in the microbe free medium. The growth rate of JE1 (0.32 at day 7) was found to be more when compared to the growth rate of all other strains at all the sampling periods (Fig: 8). (Chatterjee *et al*., 2010) reported in their study that when fresh mineral medium and sterile water were added to compensate the effects of natural evaporation, there might be slow increase of the turbidity due to the increase in the concentration of LDPE fragmented by bacterial action.

By standard techniques such as direct cell-counting or plating it was impossible to estimate the population density of bacteria, since the bacterial biofilm was strongly attached to the

polyethylene surface. Therefore, it was estimated by determination of protein concentration (Table: 3, Fig: 8). The measurement of protein content of the biofilm of the bacterial strains is an

indirect estimation of bacteria attached to surface of LDPE films (Kavitha *et al*., 2014). In the present study, the protein content was measured and it was found to be 5.22 μg mg^{-1} PE for JE1, 3.72 μg mg^{-1} PE for JE2, 2.15 μg mg^{-1} PE for JE3 and 3.12 μg mg^{-1} PE for JE4 respectively (Table: 4, Fig: 9). During incubation, the increase in protein content is reflected by an increase in surface attached biomass. This might suggest that biomass on LDPE films is proliferating continuously (Harshvardhan and Jha, 2013).

Selected bacterial strains were further tested after 30 days to check polythene degradation with subsequent bacterial inoculation under laboratory condition by weight reduction method. Due to accumulation microorganisms that grow within the polymer lead to an increase in weight, whereas a loss of polymer integrity leads to weight loss (Sudhakar *et al*., 2008). The strain JE1 showed highest weight loss percentage of 0.7% indicating maximum degradation capacity. JE3 showed lowest weight loss percentage of only 0.2% indicative of minimal degrading capacity. JE2 and JE4 recorded average degrading capacity which is shown in (Table: 5, Fig: 10). So JE1 had good efficiency of degradation and used for further study. According to (Gnanavei *et* al., 2012), each microbe has different characteristic, so degradation ability possessed will be variated between one microbe to with another. Microbe different characteristic includes types of enzyme produced for biodegradation process that helped in polymer degradation. (Kathiresan and Bingham, 2001) reported that bacteria caused the biodegradation ranging from 2.19 to 20.54% for polythene and 0.56 to 8.16% for plastics. This may be due to the thickness of the polythene that is 5-times thinner that the plastics.

In this study the changes in the polythene structure with bacterial inoculation before and after biodegradation was analyzed by Fourier Transform Infrared Spectroscopy (Shimadzu) in the frequency range of 4000 – 750 cm^{-1}. The FTIR spectra of the untreated and treated LDPE film incubated with bacterial isolate for 30 days in liquid SM media containing polythene as sole carbon source are shown in (Untreated – Table: 6, Fig: 11. Treated – Table: 7, Fig: 12).

In untreated LDPE spectra the band at 1462.04 cm^{-1} corresponds to -CH-H indicating alkanes was slightly modified to 1463.97 cm^{-1} in treated LDPE. The corresponding peak in untreated LDPE at 1020.34 cm^{-1} =CH bend was alkenes group and it was modified as 1022.27 cm^{-}

[1] =CH bend in treated LDPE. The characteristic band 2848.86 cm^{-1} corresponds to H-C=O: C-H Stretch which indicated the functional group of aldehydes. This was similar in both treated and

untreated LDPE spectra. The peak at 875.68 cm^{-1} for both treated and untreated LDPE is indicative of –C=O stretch and they were ketones and aldehyde groups. The native bands at 719.45 cm^{-1} corresponds to –C=O stretch of ketones and aldehydes and it remained unchanged for untreated and treated LDPE. The native band at 1020.34 cm^{-1} was increased to 1022.27 cm^{-1} in the FT-IR spectra of LDPE film inoculated with bacterial isolate JE1. This was in accordance with the (Das and Kumar, 2015). Also, the FT-IR spectra of LDPE film inoculated with JE1 showed broadening of the band in the range of 2181.49 cm^{-1} and 1463.97 cm^{-1} which indicate the presence of more than one oxidation products. A new band was formed at 1938.46 cm^{-1} in the FTIR spectra of LDPE with bacterial isolate JE1 (Fig: 13). This was attributed to oxidation of polyethylene. Thus, the FTIR spectra of LDPE biodegraded by Isolate JE1 gave conclusive evidence of the oxidation of polyethylene with the addition of carbonyl group and presence of more than one oxidation products in their spectra. (Patil R. C., 2018) concluded that isolated strains are solely dependent on plastic for its carbon source. FTIR spectra confirm the biodegradation of polymer as some changes are seen in surface of polythene.

To identify the bacteria DNA isolation (Fig: 13), PCR followed by 16 S rRNA gene sequencing was done. The 16SrRNA sequence obtained was run under BLAST N and it revealed that the strain JE1 as ***Bacillus subtilis***. Nearly 99% identity and homology was found with *Bacillus* strains. The obtained sequence was submitted in GENBANK and the given ACCESSION NUMBER is **ON544072** (Fig: 14). Phylogenetic analysis was done through MEGA X software through neighborhood joining method and it revealed that the strain JE1 showed a very close resemblance to *Bacillus* species (Fig: 16).

Conclusion:

The overall investigation can be concluded that *Bacillus subtilis* exhibited significant polythene degradation ability and in the near future, *Bacillus subtilis* can be used to reduce the quantity of plastic waste, which is rapidly accumulating in the natural environment. Although the present study has proved the biodegradation of polythene and complete biodegradation of polythene could be achieved after a prolonged exposure to the organism.

Statements & Declarations:

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Contribution by authors:

All the authors contributed equally to this research work, including conceptualization, design and discussion. Data collection and experimental procedures were carried out by Ms Jency S.J. Data analysis, interpretation of results, discussion and conclusion was collectively done by all the authors.

Data availability:

The 16SrRNA gene sequence is submitted in NCBI GENBANK database and found under the accession number MZ363886.

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