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Research Article

BIODEGRADATION OF LOWDENSITY POLYETHYLENE (LDPE) BY *PSEUDOMONAS AERUGINOSA* STRAIN P9 ISOLATED FROM POULTRY DROPPINGS

Jain Ankit[1*], Jogi Joycee[2], Chhabra Daljeet[3], Shukla Supriya[4], Aich Ranjit[5], Gangil Rakhi[6], Sikrodia Ravi[6], Sharda Rakesh[6], Patidar K Mukesh[7]

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ABSTRACT: This study aimed to isolate bacteria from poultry droppings for the degradation of low-density polyethylene (LDPE). Poultry droppings were collected from the Instructional Livestock Farm Complex (ILFC), Veterinary College, Mhow, Madhya Pradesh, India. LDPE films were treated with UV rays for 48 hours and used for the study. Four isolates were identified by Gram's staining. In M9, K9, P9, and E9; one was identified as Gram-positive, and 3 were found as Gram-negative bacteria. Screening of bacteria for LDPE biodegradation was done by clear zone formation. Out of 4 isolates, P9 was identified as LDPE degrading bacteria. LDPE degradation was analyzed by gravimetric analysis using the weight loss method, the functional group was analyzed by Fourier transform infrared spectroscopy, and the surface was analyzed by scanning electron microscopy. LDPE films with isolate P9 showed significant weight loss (16.98%±0.47) after 60 days. FT-IR analysis showed a decrease (58.8%) in carbonyl index and changes in functional group vibrations indicating LDPE degradation. SEM image examination revealed bacterial colonization, biofilm formation, surface erosions, cracks, and pit formation on the surface of LDPE which was indicative of the degradation of LDPE film. Further, the identification of isolate P9 was done by BD Phoenix M50 automated identification system and 16S rRNA sequencing. Sequencing and phylogenetic analysis of the P9 isolate showed 99.76% similarity with *Pseudomonas aeruginosa* (OR921266.1). Our findings underscore the potential of strain P9 from poultry droppings to efficiently utilize LDPE as a sole carbon source, thereby contributing to its degradation. This is the first report of isolating LDPE-degrading bacteria from poultry droppings. It is crucial to analyze the poultry gut microbiota for plastic degradation since this discovery highlights the need for further investigation.

Keywords: *Pseudomonas aeruginosa*, Poultry droppings, Biodegradation, LDPE, SEM, FT-IR.

INTRODUCTION

Polyethylene is a large environmental pollutant. It was non-biodegradable and required to degrade it. Large numbers of animals die due to ingestion of plastic material annually. Stray cattle also badly suffer due to the ingestion of plastics. Approximately 1.5 million marine animals died due to blockage of air passage and 1 million birds died due to ingestion of plastics annually [1, 2]. The use of physical and chemical methods to degrade plastic wastes results in the emission of polychlorinated biphenyls, polycyclic aromatic hydrocarbons, dioxins, and furans gases leading to health problems and environmental pollution [3]. Recently, microorganisms have focused on the environment friendly disposal of plastic waste [4]. Plastic biodegradation is a surface erosion process as the enzymes that break down plastics usually act only on the surface. Biodegradation can be enhanced by exposing polyethylene (PE) to UV, thermal, or chemical oxidation. Bacteria can also contribute to the process by creating a biofilm on the plastic surface that damages the surface and modifies its functional groups [5, 6, 7].

[1,2,3,6]Department of Veterinary Microbiology, [4]Department of Veterinary Pathology, [5]Department of Veterinary Biochemistry, College of Veterinary Science and Animal Husbandry, Mhow, Madhya Pradesh, India.
[7]Department of Biotechnology, Medi-Caps University, Indore, Madhya Pradesh, India.
**Corresponding author. e-mail: ankitjain271@gmail.com*

Recently isolation of polyethylene-degrading microbes from gut microflora from wax worms and larvae suggests that microbes isolated from the gut can also degrade polyethylene [7]. Thus, gut microflora can also be a new source for developing microbial populations that can degrade polyethylene and other plastics. In our study, we isolated *Pseudomonas aeruginosa* strain P9 from poultry droppings and assessed its biodegradation capabilities.

MATERIAL AND METHODS

Selection of LDPE

For the biodegradation experiment, LDPE was selected based on the classification of plastic reported by Mariotti *et al.* (2019) [8]. Fifty-micron thick LDPE sheet was selected for the biodegradation study which was obtained from computer wrapping. The thickness was measured by a micrometer caliper (Mitutoyo Absolute Digimatic Caliper Series 500, Japan). LDPE was treated with benzene for 30 minutes to remove any plasticizers, coloring agents, or fillers [9], followed by, washing manually with absolute ethanol, from cotton wool. Rinsed it several times with distilled water for the removal of dirt and other organic particles. Which adhered to the surface. Then allow it to dry for 30 minutes. LDPE sheet was used for the preparation of films and LDPE powder. Further, these LDPE film and powder was used as the sole carbon source for the growth of bacteria.

Preparation of LDPE films and LDPE powder

LDPE was cut into 1.5 x 1.5 cm^2 and sterilized as described by Lee *et al.* (1991), Nanda *et al.*(2010), and Yao *et al.* (2022) [10, 11, 12]. Subsequently, LDPE films were dried at 60ºC in a hot air oven for one hour. UV light exposure (365 nm) (UV tech, Cambridge) was given to these LDPE films for 48 hours as described by Lee *et al.* (1991) for photo-oxidation [10].

Further, each LDPE films were weighed separately (Denver Instrument PI- 214.3 Analytical Balance 210g). The dried films were stored in a desiccator for further use.

The LDPE powder was prepared as described by Bhatia *et al.* (2014) and Ghosh (2023) with some modifications [13, 14]. This LDPE powder was stored at room temperature.

Sample collection

One gram of poultry droppings was collected from the Instructional Livestock Farm Complex (ILFC), Veterinary College, Mhow, Madhya Pradesh (Latitude 22.5892751, Longitude 75.7829186) in a sterile flask. Then transported on ice to the laboratory.

Enrichment of LDPE-degrading bacteria

Mineral salt medium (MSM) broth was prepared as per Novo *et al.* (2023) [17]. One gram of poultry dropping was inoculated in 50 ml MSM broth with 0.2% LDPE powder. It was incubated aerobically at 120 rpm for 30 days in a shaker incubator at 37ºC for the enrichment of LDPE-degrading bacteria. Gram staining was performed to confirm bacterial growth in MSM broth.

MSM agar plates were prepared as described by Nademo *et al.* (2023) by adding 1.5% agar [17]. After 30 days of enrichment, one ml of enriched broth was inoculated on MSM agar plates containing 1.5x1.5 cm^2 LDPE films and 0.2% LDPE powder as the only carbon source for bacterial growth. Plates were incubated aerobically for 60 days at 37ºC. The gross morphological characteristics of the colonies after 60 days were observed and confirmed by Gram staining. Further, these isolates were characterized biochemically. These bacteria were inoculated on nutrient agar and incubated aerobically at 37ºC for 1-2 days for isolation.

Selection of LDPE degrading bacteria

Isolates were screened for bio-degradation of LDPE by the clear zone method as described by Rana and Rana [15, 16, 17]. Bacterial isolates were inoculated on MSM agar containing 0.2% polyethylene glycol and incubated aerobically at 37ºC for 14 days. After 14 days of incubation, 0.1% Coomassie brilliant blue solution was poured on plates for staining, then de-stained with a solution of 40% (v/v) methanol and 10% (v/v) acetic acid to visualize a clear zone around the colony. The clear zone-forming bacteria was selected for the degradation of polyethylene. That isolate was selected for the LDPE degradation experiment.

Identification of LDPE degrading bacteria

LDPE degrading bacteria was confirmed by BD Phoenix M50 automated identification system at the Dept. of Vet. Microbiology, COVS and A.H., Jabalpur, M.P. Further, confirmation of isolate was done based on Sanger sequencing of conserved 16S rRNA gene sequence by sending isolates to Barcode Biosciences, Bangalore, Karnataka, India.

Submission of sequence to National Centre for Biotechnology Information (NCBI) and analysis of sequence

The cured sequences of the open reading frame (orf) were submitted to NCBI and an accession number

was obtained. Based on 16S rRNA sequences, a phylogenetic tree was drawn by Mega 11 [18] considering 1,000 bootstrap values.

Experimental setup of biodegradation of LDPE in MSM agar

Eight MSM agar plates were prepared including 4 control plates. Each plate contained 4 LDPE films (LDPE 1-4). Bacterial isolate selected from clear zone analysis was used for experimentation. The bacterial isolate was compared with a standard 0.5 McFarland, and 4 MSM agar plates were inoculated and left for 15, 30, 45, and 60 days. The incubation was done aerobically at 37?C. Controls were incubated for the same duration.

Collection of LDPE film after incubation for gravimetric analysis, functional group analysis, and surface analysis

LDPE films numbered 1 to 3 were removed from MSM agar plates after a 15, 30, 45, and 60-day incubation period. The films were washed with 2% SDS solution for 30 min., followed by a 70% ethanol sol. for 30 minutes, then with distilled water for 30 minutes, to remove the biomass. Then the films were dried at 60ºC for 1 hr and then weighed for gravimetric analysis [12, 17, 20]. The gravimetric analysis was done as per this given formula :

Weight loss (%) = [(Initial weight – Final weight)/ Initial weight] × 100 [17]

Here, are the initial weight of LDPE films after being exposed to UV rays and the final LDPE film after incubation, respectively. The plates were discarded with proper bio-safety precautions after taking observations at each incubation interval.

LDPE films No. 3 and 4 were processed for the surface degradation; and visualization of bacterial attachment and bio-film formation, respectively under scanning electron microscopy as described by Harshvardhan and Jha (2013) [20]

The dried LDPE No. 1 of each plate was stored in the laboratory for further use after gravimetric analysis. The dried LDPE No. 2, 3, and 4 after 60 days of incubation from inoculated and un-inoculated (control) were sent for functional group analysis by FT-IR (Bruker, Germany, 3000), surface erosion; and visualization of bacterial growth and bio-film formation, respectively by Field emission scanning electron microscopy (FE-SEM) (Carl Zeiss, Germany) to SAIF, IIT, Mumbai, Maharashtra, India after gravimetric analysis; and compared results with their respective controls.

A decrease in the weight of LDPE film in gravimetric analysis, the presence of bacteria and bio-film, holes, cracks, groove formation, and surface erosions by surface analysis and change in functional groups, the shift of functional groups, new peaks formation, or alterations in existing peaks, an increase or decrease in carbonyl index were indicative for microbial activity and LDPE degradation.

RESULTS AND DISCUSSION

MSM agar was used for experimenting with biodegradation of LDPE. It does not contain any carbon or energy source for the growth of bacteria [21]. In this experiment, UV-treated LDPE was added as the sole carbon and energy source for bacterial growth. UV rays are required for the photo-oxidation of LDPE that enables function group and carbon to bacteria for growth. UV ray treatment of LDPE enhances the availability of carbon for the growth of bacteria from LDPE [22].

Isolation of LDPE degrading bacteria

After enrichment and inoculation of enriched broth into MSM agar for 60 days, four different colonies were observed on the surface of LDPE films named M9, K9, P9, and E9. Out of these four colonies, one was Gram-positive and 4 were found Gram-negative bacteria. Details of bacterial isolates are presented in Table 1.

On perusal of the literature, no study was conducted for the isolation of LDPE-degrading bacteria from poultry droppings. However, the isolation of LDPE-degrading bacteria was done by Skariyachan *et al.* (2021) from cow dung [23]. They reported *Enterobacter cloacaenov*. bt DSCE02 and *Pseudomonas aeruginosa* nov. bt DSCE-CD03. which was isolated from cow dung as an LDPE degrader. Gupta *et al.* (2022) isolated an HDPE-degrading bacteria, from the fecal matter of a cow [24]. The bacteria was identified as *Micrococcus luteus* CGK112.

Screening through clear zone analysis

Four isolates M9, P9, K9, and E9 were inoculated for 14 days on MSM agar containing 0.2% PEG. Among the four isolates, only P9 showed the presence of a transparent zone around the colonies. The other isolates did not show any zone formation. The diameter of the clear zone around P9 was measured. Which was from 0.2 to 0.5 mm according to the growth of a single colony (streaked on the plate). Based on the

Table 1. Details of bacterial isolates studied.

Name of Isolate	Colony characteristics	Gram Staining Result	Catalase test	Oxidase test	IMViC			
					Indole test	Methyl Red test	Voges - Proskauer test	Citrate Utilization test
M9	Brown pinpoint colonies	Gram Positive cocci	+	-	-	-	-	+
P9	White pinpoint colonies	Gram Negative rods	+	+	-	-	-	+
K9	Brown pinpoint colonies	Gram Negative coccobacilli	+	-	+	+	-	-
E9	Brown pinpoint colonies	Gram Negative coccobacilli	+	-	+	+	-	-

clear zone analysis P9 was seen as a potential LDPE degrader by observing clear zones around colonies (Fig. 1). Rana and Rana (2020) screened 28 isolates and identified 2 isolates *viz.*, PDBH1 and PDBM 2 as potential degrader of LDPE based on minimum (+), moderate (++), maximum (+++) and no zone of clearance (-) [16]. Nademo *et al.* (2023) screened 60 isolated and used 14 isolates for the LDPE degradation experiment [17].

Identification of bacterial isolates degrade LDPE polymer

The bacterial isolates were identified as *Pseudomonas aeruginosa* strain P9 by BD Phoenix M50 automated identification system (Identification No. 429351503693) and confirmed by 16S rRNA sequencing with universal forward and reverse primers. These primers were 27F and 1492R, respectively. The sequence of isolate P9 showed 99.76% similarity with *Pseudomonas aeruginosa* strain ZM130.

Submission of sequence and sequence analysis

The obtained sequence was cured using bio-edit software with a 100% query sequence of 829bp. The sequence was submitted to NCBI and obtained Accession No. OR921266.1

Further, phylogenetic trees were drawn by Mega 11 [18] based on nucleic acid sequences of 16S rRNA, following the alignment of the orf sequences using the Neighbour-joining method considering 1,000 bootstrap values. The BLASTn search result and phylogenetic analysis revealed a high degree of homology between the study sequence and other subject sequences. Which were available in the NCBI database (Fig. 2).

Determination of LDPE degradation by bacterial isolates

The LDPE degradation was characterized by gravimetric analysis, functional group analysis, and visualization of bio-film; and surface erosions.

Table 2. Initial weight (Iw), final weight (Fw), weight loss (Wl) and weight loss (% Mean) of LDPE films by *Pseudomonas aeruginosa* P9 at different incubation periods in MSM agar.

Incubation period (Days)	Iw (mg)	Fw (mg)	Wl (mg)	Weight loss (% mean)
60	66.66±6.64	55.33±5.48	11.33±1.20	16.98%±0.47^{a}
45	55.66±2.72	48.33±3.38	7.33±0.66	13.34±1.76^{b}
30	60.00±2.88	55.66±2.90	4.33±0.33	7.25±0.63^{c}
15	54.66±1.33	52.66±1.45	2.00±0.57	3.66±1.03^{d}

The mean of % WL with different superscripts differs significantly ($p<0.05$).

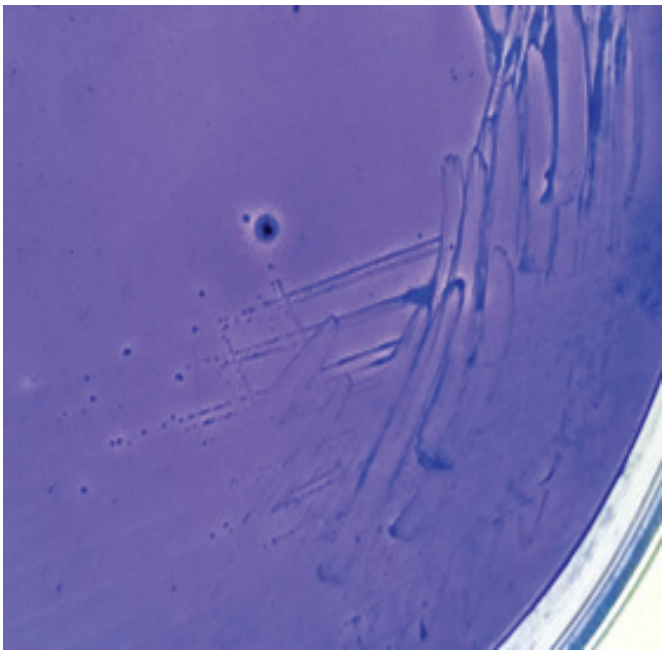

Fig. 1. Clear zone formation around bacterial isolate P9.

Gravimetric analysis

A significant increase ($p<0.05$) in mean weight loss (%) of LDPE films was seen in MSM agar media by *Pseudomonas aeruginosa* P9 at 15, 30, 45, and 60 days of the incubation period. It was found that the degradation of LDPE done by *Pseudomonas aeruginosa* strain P9 was increasing significantly with an increase in time interval. The mean weight loss (%) at 15 days of incubation was (3.66±1.03); at 30 days (7.25±0.63); at 45 days (13.34±1.76) and the highest degradation was seen at 60 days of incubation (16.98±0.47). The data shows that *Pseudomonas aeruginosa* P9 could degrade LDPE films actively in MSM agar with an increase in the time interval (Table 2 and Fig. 3). No weight loss was seen in control groups.

Ogunbayo *et al.* (2019) reported a 7.2 % weight loss of pure water sachet (plastic) sample in the mineral salt medium broth when *Pseudomonas* species were incubated for 60 days at 37ºC aerobically, in a rotary shaker at 120 rpm [25]. Kyaw *et al.* (2012)

Fig. 2. Phylogenetic relationship of *Pseudomonas aeruginosa* strain P9 16S rRNA gene (Accession No. OR921266.1) from different species using MEGA 11 following the alignment of the orf sequences using Neighbour-joining method.

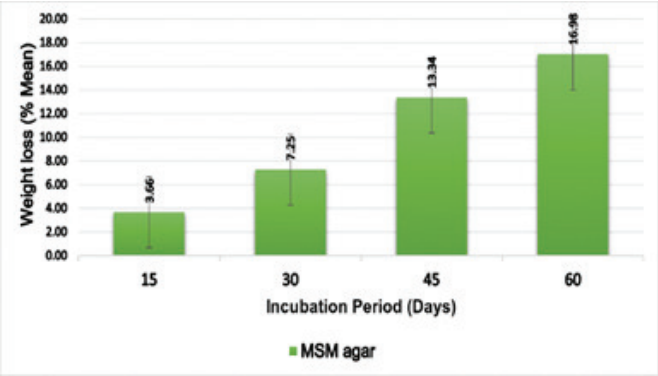

Fig. 3. Mean weight loss (%) of LDPE films with *Pseudomonas aeruginosa* P9 in MSM agar at different incubation period.

reported weight loss of LDPE after 120 days. The 20% loss by *Pseudomonas aeruginosa* (PAO1) (B1), 11% by *Pseudomonas aeruginosa* (ATCC-B2), 9% by *Pseudomonas putida* (B3) and 11.3% by *Pseudomonas syringae* (B4). The negative control showed 0.3% weight loss [4].

Functional group analysis

Functional groups were analyzed at 4000-400 per centimeter at a resolution of 4 per centimeter. The spectrum was plotted at a rate of transmittance (%) versus wave number (centimeter), which was analyzed against the controls. The FT-IR spectrum of the UV-treated control LDPE film served as a baseline. It represented the LDPE film without any microbial exposure. Peaks in this spectrum corresponded to the inherent vibrational modes of LDPE (Fig. 4).

The FT-IR spectrum of the LDPE film exposed to *Pseudomonas aeruginosa* P9 reflected changes induced by microbial activity. The analysis was done in a spectrum for shifts, new peaks, or alterations in existing peaks. Specific bands indicated interactions between the bacteria and the LDPE polymer.

There was an increase in intensity of the transmittance peak (cm-1) at 3082.08, 2736. 84 and 2638.48; and a decrease in intensity of transmittance peak at 2935.50, 2898.85, 2877.64, 2862.21, and 2846.78 represents a change in the symmetrical stretching vibration peak of the alkyl - CH_2 group. The decrease in transmittance intensity of 2221.87 and

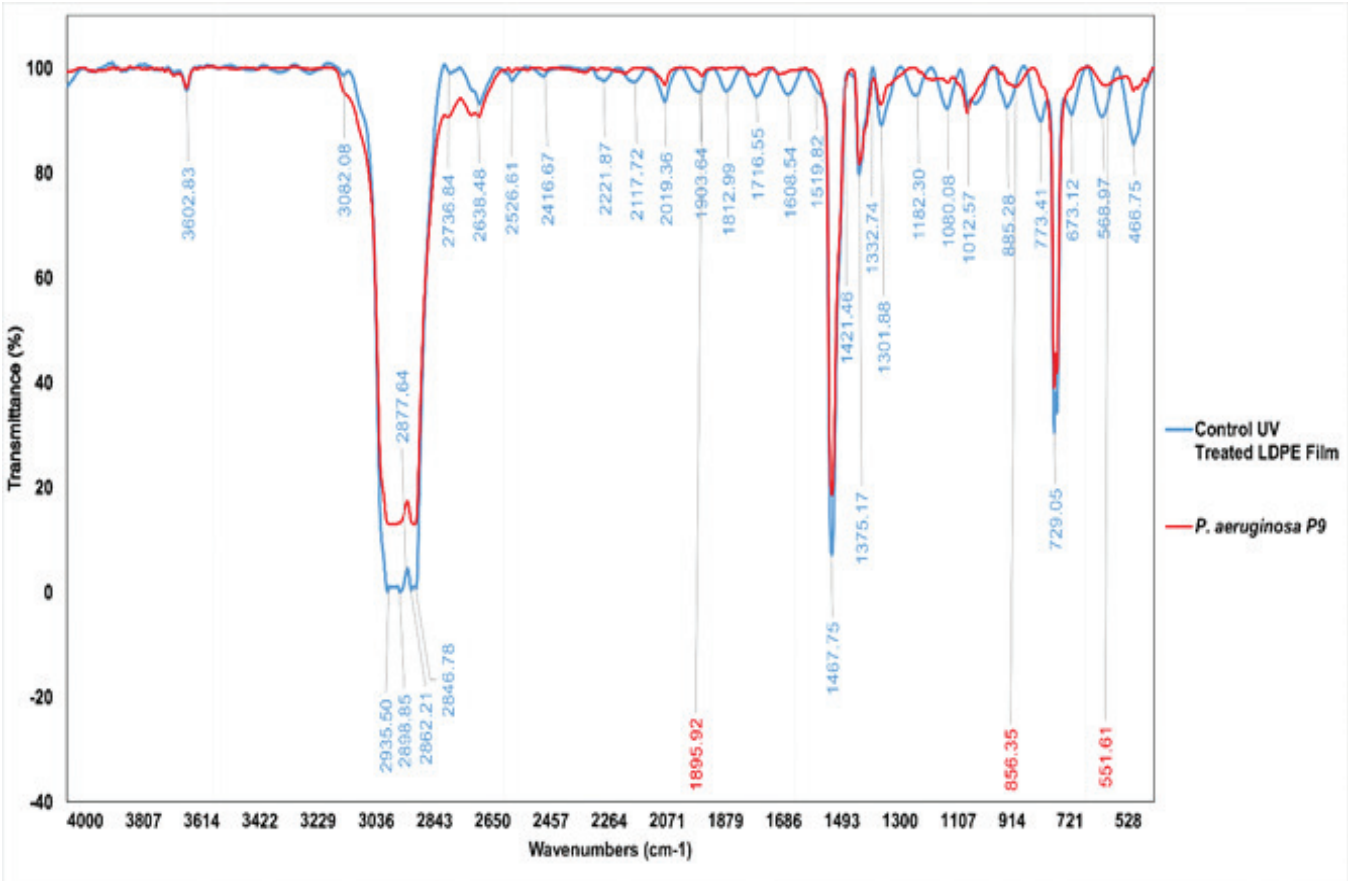

Fig. 4. FT-IR analysis of LDPE incubated with *Pseudomonas aeruginosa* P9 incubated at 37°C in MSM agar for 60 days control UV-treated LDPE without bacteria.

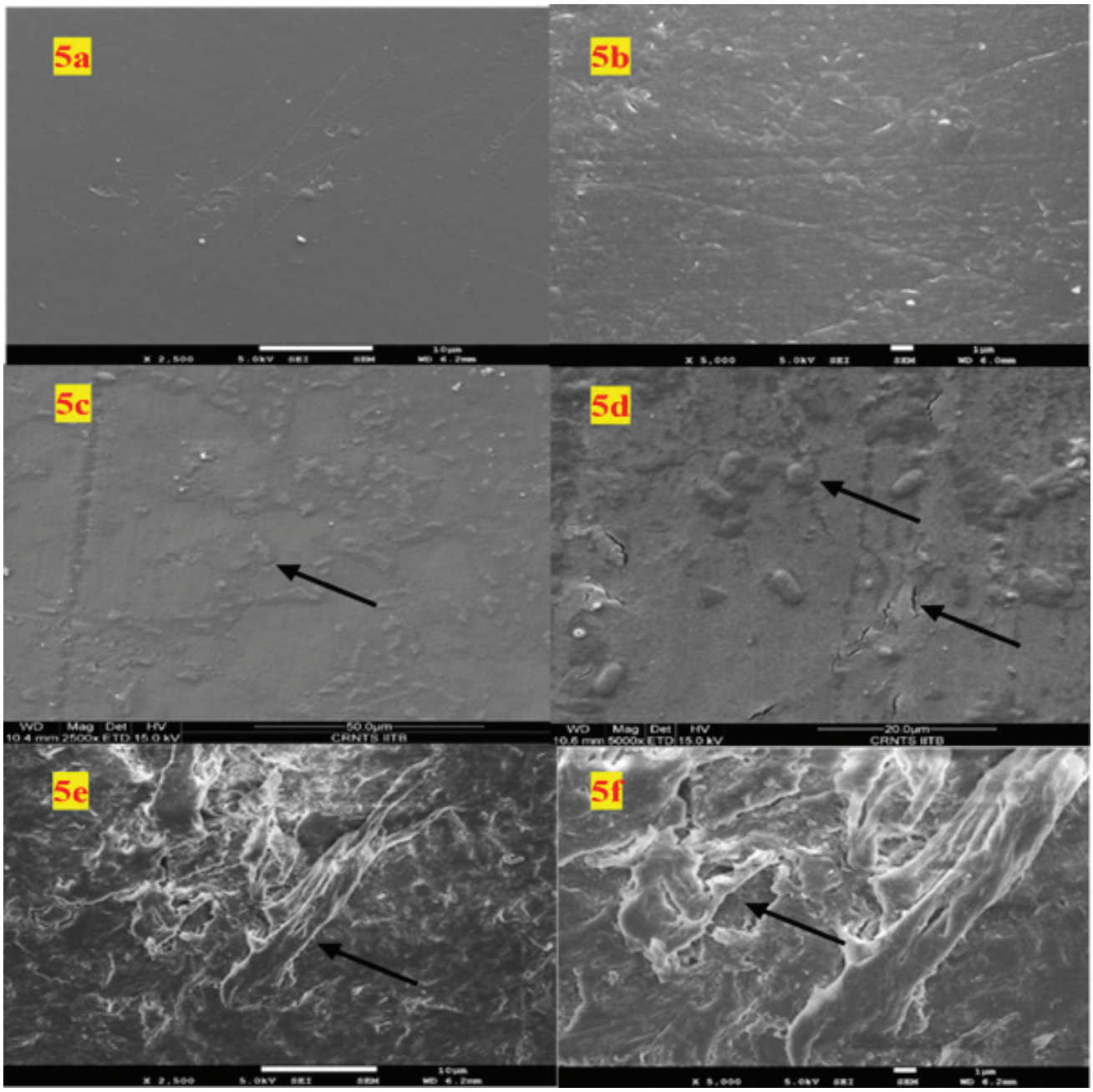

Fig. 5. Scanning electron micrograph of LDPE films. [(From left to right): 5a depicts control LDPE (2500x); 5b depicts control LDPE, 5c depicts bacterial colonization and biofilm of *P. aeruginosa* strain P9 (2500x); 5d depicts bacterial colonization and biofilm of *P. aeruginosa* strain P9, 5e depicts surface erosions, cracks, and pit formation after 60 days of incubation with *P. aeruginosa* strain P9 (2500x); 5f depicts surface erosions, cracks, and pit formation after 60 days of incubation with *P. aeruginosa* strain P9 (5000x)]

2117.72 represents cleavage of the alkyne (C≡C) bond stretching vibrations. The decrease in transmittance peaks at 2019.36 and 1903.64 representing C-H bond vibrations. A decrease in transmittance peaks at 1812.99 and 1714.62 represents cleavage of the carbonyl bond (C=O). The decrease in transmittance peak at 1608.54, 1519.82, and 1467.75 represents cleavage of conjugated C=C double bond. The decrease in transmittance peak at 1375.17 was related to the scissoring motion pattern of CH_2 bending. The decrease in transmittance peaks at 1301.88 the cleavage of ordered group vibrations. A decrease in transmittance peaks at 1182.30 and 1080.08 suggested cleavage of C-O-C bond stretching vibration in the LDPE structure. A decrease in transmittance peak at 773.41, 729.05, 673.12, and 466.75 represented cleavage of linear alkane chain (-CH_2 rocking). A shift

and decrease in intensity of transmittance peak from 1903.64 to 1895.92, from 885.28 to 856.35, and from 568.97 to 551.61 were seen. These vibrational changes in bands of spectra suggested that structural changes for the LDPE film during the degradation (Fig. 4).

Anantharam *et al.* (2018) analyzed the LDPE degradation efficiency of control unmuted *Pseudomonas putida* and mutated *Pseudomonas putida* with UV irradiation for 60 minutes. After 60 days of incubation of LDPE with control and mutated organisms, they found stretched peaks of O-H, C=C, and C-H at wavelengths (cm-1) of 1417.49 to 1454.29, 1641.53 and 2988.57, respectively [26].

The carbonyl index (CI) is a valuable parameter used to monitor oxidation reactions in polyethylene (PE). It specifically focuses on changes in the carbonyl band (C=O) observed in Fourier transform infrared (FT-IR) spectra [27]. In the FT-IR spectral analysis control and degraded LDPE showed transmittance intensity at 1467 nm was 8.63 and 19.33, respectively; and control and degraded LDPE intensity at 1716 nm were 94.589 and 98.82, respectively. The carbonyl index was calculated by the formula [27] - Carbonyl Bond Index (KCBI) = I1716/I1467.The carbonyl index decreased by 5.87%, indicating bacterial cleavage of carbonyl bonds and energy utilization through β-oxidation.

Harshvardhan and Jha (2013) also reported that the carbonyl index of the polyethylene increased after incubation with the bacterial isolates, indicating the degradation and modification of the polymer by the enzymatic activity of the bacteria [20]. Gupta *et al.* (2022) reported an increase in the carbonyl index attributed to the metabolic activity of bacterial bio-film formation [24].

Surface analysis

LDPE No. 4 was examined for bacterial growth and bio-film formation. Examination of scanning electron micrograph at 2500x and 5000x magnification (Fig. 5a, b, c, and d) showed colonization and biofilm formation of *Pseudomonas aeruginosa* P9. Notably, the LDPE surface displays signs of surface erosion in the form of cracks around bacterial colonies indicative of degradation. In the control group, no bacterial presence was observed, and the LDPE surface remained smooth.

LDPE No. 3 was examined at 2500x and 5000x for surface erosion and compared with control (Fig. 5e and 5f). Scanning electron micrograph showed surface erosions, groove, and pit formation which was indicative of degradation of LDPE film.

Zhang *et al.* (2022) also reported the degradation of plastic by changes in surface structure. Like cracks. holes, roughening, and microbial colonies over the surface [28]. Gupta and Devi (2020) analyzed the surface of LDPE after biological treatment with *Pseudomonas aeruginosa* ISJ14 for 60 days. They found surface erosions, cracks, and pit formation through SEM analysis [24].

CONCLUSION

The study focuses on the degradation of LDPE using *Pseudomonas aeruginosa* strain P9, which was isolated from poultry droppings. The study found that *Pseudomonas aeruginosa* strain P9 is highly effective in breaking down LDPE, especially when exposed to UV treatment. Moreover, the strain forms a bio-film on the LDPE surface, which further enhances the degradation process. These results highlight the importance of exploring diverse microbial communities in the search for effective biodegrading microorganisms. The study found that strain P9, isolated from poultry droppings, can efficiently use LDPE as the sole carbon source, contributing to its degradation. This is the first report of isolating LDPE-degrading bacteria from poultry droppings, which calls for further investigation on poultry gut microbiota for plastic degradation.

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