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Degradation of low density polyethylene by *Bacillus* species

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Abstract

Since its invention, polyethylene (PE) has brought many conveniences to human production and life. In recent years, however, environmental pollution and threats to human health caused by insufficient PE recycling have attracted widespread attention. Biodegradation is a potential solution for preventing PE pollution. In this study, *Bacillus subtilis* and *Bacillus licheniformis*, which are widespread in the environment, were examined for their PE degradation abilities. Biodegradation of low-density polyethylene (LDPE) was assessed by weight loss, Fourier transform infrared spectroscopy (FTIR), and high performance liquid chromatography (HPLC) analyses. Weight losses of 3.49% and 2.83% were observed for samples exposed to strains *B. subtilis* ATCC6051 and *B. licheniformis* ATCC14580 for 30 days. Optical microscopy revealed obvious structural changes, such as cracks, pits, and roughness, on the surfaces of the microorganism-exposed LDPE sheets. Oxidation of the LDPE sheet surfaces was also demonstrated by the FTIR-based observation of carbon-unsaturated, –OH, –NO, –C=C, and –C–O bonds. These results support the notion that *B. subtilis* ATCC6051 and *B. licheniformis* ATCC14580 can degrade PE and could potentially be used as PE-biodegrading microorganisms. Further research is needed to examine potential relevant degradation mechanisms, such as those involving key enzymes.

Keywords: Polyethylene, Biodegradation, LDPE, Environmental pollution, *Bacillus*

Introduction

Plastics were invented in the 1850s and replaced commonly used materials such as glass, metal, and wood. In current, plastic materials are widely used in all aspects of human production and life, due to their low manufacturing cost, good durability, and high strength [1]. The most widely used plastics are polyethylene (PE), polyethylene terephthalate (PET), polychlorinated vinyl (PVC), polypropylene (PP), polystyrene (PS), and polyurethane (PU) [10, 33, 37].

Among them, PE has the highest yield, more than 100 million tons each year globally [10]. PE-based materials are used in various industries, including transportation, construction, agriculture, machine building, and packaging [14]. However, a large proportion of PE products are not subjected to proper disposal after use. Published statistics indicate that less than 20% of PE waste is recycled each year [3]. In the natural environment, the accumulation of large amounts of PE waste severely impacts animals, plants, and microorganisms on land [25]. Consequently, PE waste flows into the ocean and affects marine ecology via toxicity exerted on organisms through consumption and suffocation [23, 30].

Conventional disposal methods for PE waste include landfilling, thermal treatment, and chemical treatment [17, 31, 35, 52, 58]. The management and recycling of waste through these traditional methods has been improved to a certain extent over time. However, such methods may cause secondary pollution of the environment. For example, burning PE waste can cause air pollution and excessive emissions of greenhouse gases. Toxic compounds released from PE waste can

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eventually be consumed by the human body through the food chain, threatening human health [4, 36].

Given some of the shortcomings of traditional methods, the advantages of recycling and degrading PE waste by biological methods have gained increasing interest [1]. Biodegradation has the advantages of low cost, strong operability, and low risk for releasing toxic fumes and/or harmful compounds to the environment. In recent years, researchers have isolated microorganisms with PE degradation potential from landfills, seawater, soil, and other sources [13, 20, 40, 46, 55]. For example, *Anabaena spiroides*, *Bacillus* sp., *Lysinibacillus* sp., *Pseudomonas* sp., and *Aspergillus flavus* were identified as good candidate strains [15, 20, 21, 29, 40, 43, 55, 57]. Invertebrates can also degrade PE, such as *Tenebrio molitor*, *Galleria mellonella*, *Achroia grisella*, and *Lumbriculus variegatus* [5, 28, 34, 45].

In this study, we selected five kinds of *Bacillus* species, which are widely present in natural soil, as potential strains to degrade LDPE. Their biodegradation of LDPE was assessed by weight loss, high performance liquid chromatography (HPLC), and attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) analyses. Furthermore, we identified some relevant chemical bond changes in LDPE.

Materials and methods

Materials

LDPE film was obtained from Goodfellow (9002884, Huntingdon, England). The chemical composition of the product was further characterized using FT-IR IS50 (Thermo Fisher, Waltham, USA). Bacterial cells were cultured in Luria Bertani (LB) broth composed of sodium chloride (10 g/l), tryptone (10 g/l), and yeast extract (5 g/l). The pH of LB broth was adjusted to 7.4 with NaOH. In the assay for microbial degradation of LDPE, mineral salt medium, Bushnell-Haas (BH) broth was used. BH broth contained K_2HPO_4 (1 g/l), KH_2PO_4 (1 g/l), NH_4NO_3 (1 g/l), $CaCl_2$ (0.02 g/l), $MgSO_4$ (0.20 g/l), and $FeCl_3$ (0.05 g/l): pH was adjusted to 7 with NaOH [51]. Organic solvents such as acetone, chloroform, and ethanol were used for sample processing and analysis (Daejung, Siheung, Korea).

Microbial strains

Microbial strains used in this study were obtained from Korean Collection for Type Cultures (KCTC): *Bacillus subtilis* ATCC6051, *Bacillus licheniformis* ATCC14580, *Bacillus pumilus* ATCC7061, *Bacillus amyloliquefaciens* ATCC23350 and *Bacillus velezensis* KCTC13012.

LDPE film

Polythene film was cut into approximately square pieces with a dimension of 3 × 3 cm. The cut pieces were soaked in 70% ethanol solution for 30 min and washed with sterile distilled water. Subsequently, the PE sheets were dried at 60 °C for 1 h and weighed separately. The dried LDPE film samples were stored in glass desiccators until further use.

LDPE biodegradation in flasks

To see if PE could be decomposed by bacterial strains, *Bacillus* strains were cultured in 250 ml flasks containing 100 ml of BH broth along with LDPE film (3 × 3 cm) [27]. For inoculum preparation, 10 ml of overnight-culture *Bacillus* cells were collected by centrifugation at 12,000 rpm for 2 min at 4 °C, and resuspended in distilled water. The same process was further repeated twice times to remove LB medium. The final cells were inoculated into 100 ml BH broth and incubated at 37 °C with constant shaking at 135 rpm. LDPE film in BH broth without bacterial inoculation was used as a negative control, which was incubated under the same conditions as the sample group. As a blank, the LDPE film was used as received without culturing. All experiments were performed independently in triplicate.

Determination of cell counts during LDPE biodegradation

Once every 2 days, the absorbance of culture broth was measured at 600 nm (OD600). In addition, the number of viable cells was also determined by the colony counting method. For determination of colony counts, 100 μl of culture broth was spread on LB plates. Then, plates were incubated at 37 °C for 24 h. Viable cell counts were expressed in colony forming units (CFU) per milliliter.

Determination of weight loss

Once every 10 days, the LDPE film was taken from the cultures and washed with 2% sodium dodecyl sulfate (SDS) solution for 30 min, 70% ethanol solution for 30 min, and distilled water for 30 min in turn to remove the bacterial biomass. Then, the film was dried at 60 °C for 1 h and weighed. The weight loss of LDPE was calculated by using Eq. 1.

$$\text{Weight loss (\%)} = \frac{\text{initial weight} - \text{final weight}}{\text{initial weight}} \times 100\%. \tag{1}$$

Optical microscopic analysis

Surfaces and edges of LDPE film were observed using an optical microscope. Optical microscopic analysis was performed with LDPE film specimens, indicating the blank (as-received), the negative control cultured without microbe, and the samples cultured with bacterial cells, at 1000 × magnification.

High-performance liquid chromatography (HPLC) analysis

Supernatant taken from the 30-day culture was filtered and applied to HPLC, Agilent 1100 series (Agilent Technologies, CA, USA) equipped with the RI-101 refractive index detector (Shodex, Denmark) [32, 44]. The flow rate was controlled at 500 µl per minute through a MetaCarb 87H column (Agilent Technologies, USA) at 25 °C using 5 mmol/l sulfuric acid as a single mobile phase.

Attenuated total reflection-Fourier transform infrared spectroscopy analysis

Samples were analyzed in the same conditions in attenuated total reflection (ATR) mode using an FT-IR IS50 (Thermo Fisher, Waltham, USA) [50]. All LDPE film specimens, including blank (as-received), negative control, and samples, were washed with 2% SDS, 70% ethanol, and distilled water sequentially. The PE specimens from the negative control was incubated under the same conditions as the sample group. In the final step, the LDPE film specimens were fully dried. IR spectra of all specimens were recorded in the 4000–400 cm^{-1} range at room temperature [38]. There were no significant differences between the three scans of each specimen.

Results and discussion

Growth of *Bacillus* cells in the medium containing LDPE as a sole carbon source

In this study, as potential strains to degrade LDPE, we selected five kinds of *Bacillus* species, including *B. subtilis* ATCC6051, *B. licheniformis* ATCC14580, *B. pumilus* ATCC7061, *B. amyloliquefaciens* ATCC23350 and *B. velezensis* KCTC13012. The five *Bacillus* strains were cultured for 30 days at 37 °C in the mineral salt medium supplemented with PE film fragments for the preliminary screening. As a result, *B. subtilis* ATCC6051 and *B. licheniformis* ATCC14580 showed cell growth by absorbance measurement at 600 nm after 30 days of culture, while the other strains didn't (data not shown).

Thus, for these two strains *B. subtilis* and *B. licheniformis*, the bacterial culture was monitored for 30 days with a 2-day sampling interval to see the detailed growth profiles in the salt medium supplemented with LDPE as the sole carbon source (Fig. 1a, b). Immediately after inoculation with *B. subtilis* and *B. licheniformis*, the absorbance values were similar, at 0.10 and 0.11, respectively. These values dropped sharply over the next 6 days (Fig. 1a, b). We speculated that this might reflect the hysteresis of *Bacillus* strains when using LDPE as the only carbon and energy source, due to the removal of the original medium during inoculation. However, viable count analysis revealed that the viable cell counts decreased significantly only in the first 2 days, from 1.29×10^4 CFU/ml (*B. subtilis*) and 5.62×10^4 CFU/ml (*B. licheniformis*) at the time of inoculation to 9.77×10^2 CFU/ml and 4.67×10^3 CFU/ml at day 2 post-inoculation, respectively (Fig. 1c, d). Thereafter, the trend flattened. This difference was presumed to reflect that the mineral salt medium contains a small amount of precipitate, leading to a pronounced decrease in absorbance caused by its early bacterial consumption [47]. For *B. subtilis*, bacterial growth significantly increased starting at day 6 of culture; the growth rate reached a plateau at day 12 and remained at that level to day 30 (Fig. 1a). The OD600 value reached the highest point of 0.062 at day 14. The viable cell counts of *B. subtilis* also showed the same patterns, reaching 1.95×10^4 CFU/ml at day 16 (Fig. 1c). Comparison of the results for each strain revealed that the absorbance of *B. licheniformis* showed a longer lag time than that of *B. subtilis*, and *B. subtilis* showed a stronger increase in viable cells in the presence of LDPE as the sole carbon source (Fig. 1b, d).

The average weight loss of LDPE films was determined every 10 days during incubation with *B. subtilis* or *B. licheniformis* (Fig. 2). Cultures without inoculation of microorganisms were incubated as negative controls. The weight of the LDPE film steadily decreased over 30 days in the presence of the tested *Bacillus* strains, while that of the negative control was almost unchanged (Fig. 2). After 30 days, LDPE weight losses of 3.49% and 2.83% were observed for films exposed to *B. subtilis* and *B. licheniformis*, respectively. The weight losses of LDPE and the increases in viable cell counts indicate that both *Bacillus* strains utilized the polymer as a carbon source for growth. Meanwhile, when the bacteria entered the stationary phase (12–30 days), LDPE was still being gradually degraded. At this time, the *Bacillus* strains may be in a viable but not culturable (VBNC) state, which is a survival state exhibited in response to adverse growth conditions [48]. Interestingly, the VBNC state of bacteria was reported to have a positive effect on the degradation of LDPE [12]. To determine the degradation status of LDPE in our experimental setting during this period, further examination in combination with more methods would be required.

In earlier reports, *Bacillus* sp. SM1 generated an 18.9% weight loss in LDPE sheets within 180 days [3]. Harshvardhan and coworkers reported 1.50% and 1.75% of LDPE weight loss due to a 30-day exposure to *B. pumilis* M27 and *B. subtilis* H1584, respectively [19]. *Bacillus* sp. ISJ55 isolated from plastic-contaminated soil reduced the weight of LDPE by 1.50% at 60 days [18]. In another study, 3.5% and 10% weight losses were reported

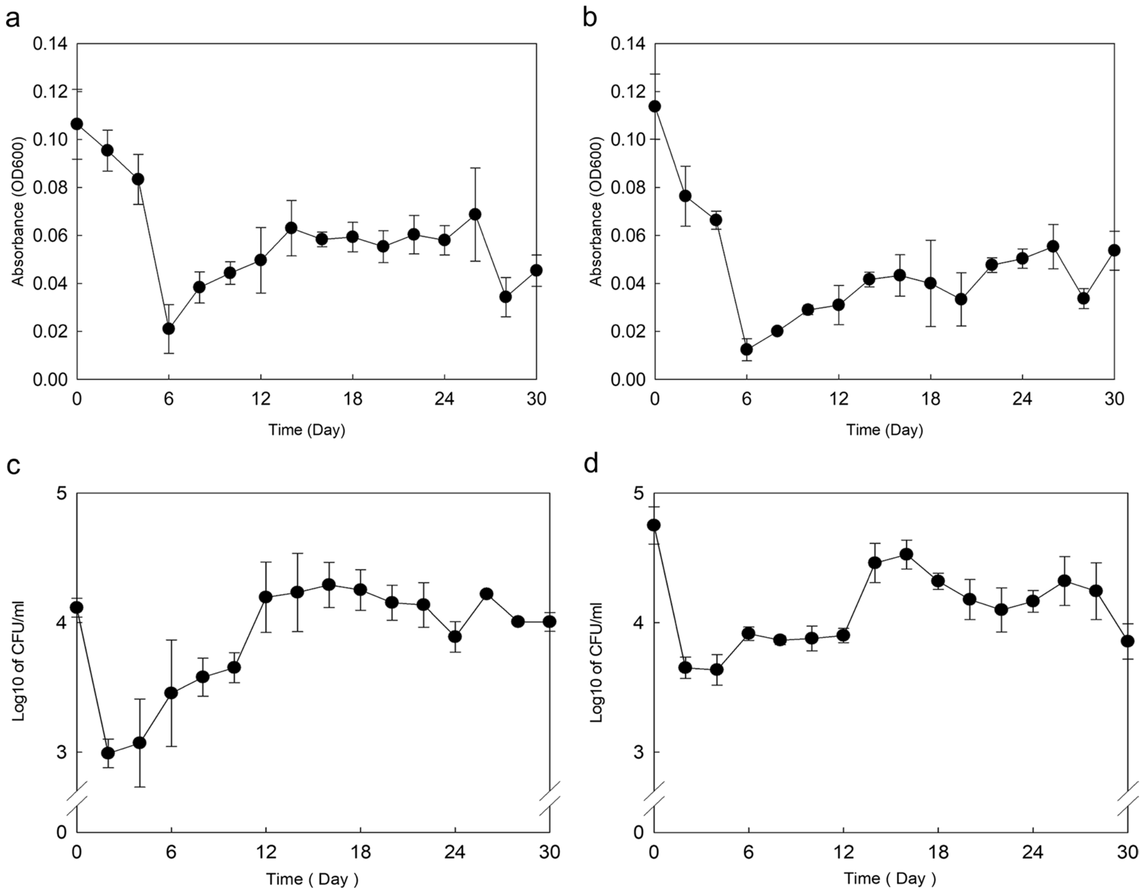

Fig. 1 Growth profiles of *B. subtilis* and *B. licheniformis* strains in a minimal salt medium supplemented with LDPE as a sole carbon source. **a**, **c** *B. subtilis*; **b**, **d** *B. licheniformis*; **a**, **b** cell density at 600 nm (OD600); and **c**, **d** viable cell count as CFU/ml. All experiments were performed independently in triplicate

in HDPE and LDPE degradation tests using *B. sphaericus* strain over 1 year [49].

When seeking to degrade plastics in the natural environment, it may be more effective to employ a microbial community composed of various microorganisms rather than using a single strain. In a recent study, Gao and Sun suggested an artificial community containing *Idiomarina* sp., *Marinobacter* sp., and *Exiguobacterium* sp. to degrade PE effectively [13]. In this aspect, future development of *B. subtilis* within microbial communities and further studies on the interaction of various enzymes in *B. subtilis* during degradation could be needed.

LDPE morphology

To assess the morphology of the LDPE film specimens after 30 days of incubation, the films were washed and dried. Polymer samples were analyzed using an optical microscope (Fig. 3). Freshly prepared LDPE film without any treatment was observed as a blank (Fig. 3a, e). In the comparisons with the blank, after 30 days of incubation, there was no noticeable change on the surface of the negative control group (Fig. 3b). However, the surfaces of LDPE samples cultured with *B. subtilis* (Fig. 3c) or *B. licheniformis* (Fig. 3d) were roughened and exhibited some cracks. We speculate that these changes occurred because the bacteria formed a biofilm on the LDPE surface.

Microscopic observation of the LDPE film edges (Fig. 3e–h) revealed that after 30 days of culture, the edges of negative control LDPE (Fig. 3f) were more rounded and flatter than those of the blank (Fig. 3e). This suggests that the sample experienced friction during the culture process, however the data on weight loss indicate that this friction was insufficient to significantly decrease the weight of the LDPE film (Fig. 2). In contrast, fine cracks with peeling were observed at the edges of LDPE cultured with *B. subtilis* (Fig. 3g) or *B. licheniformis* (Fig. 3h). Taken together, these results indicate that LDPE is degraded to a certain extent by the two *Bacillus* strains tested in this study.

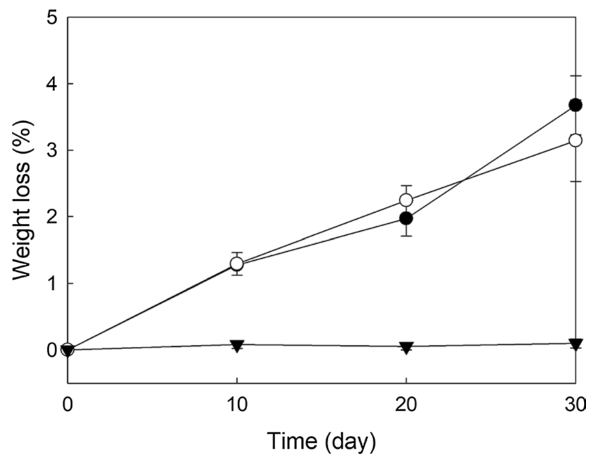

Fig. 2 Weight loss of LDPE film samples. Symbols are: filled circle, LDPE samples incubated with *B. subtilis*; open circle, LDPE samples incubated with *B. licheniformis*; and filled triangle, negative control, indicating LDPE specimens incubated in the flask in which bacterial cells were not inoculated. All experiments were performed independently in triplicate. See the Methods section for the details on calculating the weight loss of LDPE film samples

LDPE decomposition products

Because PE can be structurally classified as a hydrocarbon, it might follow the terminal oxidation, double terminal oxidation, or subterminal oxidation metabolic pathway. PE molecules that undergo one of the above processes are eventually carboxylated and structurally similar to fatty acids upon carboxylation [26]. Thus, we hypothesized that low-molecular-weight organic acids would be produced from the decomposition of LDPE. To determine the decomposition products of LDPE, supernatants taken from 30-day cultures of the two *Bacillus* strains were subjected to HPLC analysis (Fig. 4). The *Bacillus*-inoculated samples each revealed three distinct new peaks, found at 21.8, 24.5, and 29.7 min (Fig. 4). Identification for these peaks was not conducted in this study, but it seems that the peak at 21.8 min indicates butyrate, based on retention time of the standard. We were also unable to confirm the component of the other two peaks due to a lack of standards. More specifically, after 30 days of culture, the peak at 21.8 min (estimated as butyrate) was detectable in both bacteria-containing culture supernatants (Fig. 4b, c) but not the negative control (Fig. 4a). Alkane oxidase and laccase, present in *Bacillus* species, are predicted to be involved in the degradation of PE [22]. Polyethylene molecules are converted to alcohols by the action of monooxygenases, and these alcohols are further oxidized to aldehydes by alcohol dehydrogenase [7]. The aldehydes are converted to fatty acids by aldehyde dehydrogenases [11] and, finally, the fatty acids are metabolized through the β-oxidation pathway and finally converted into CO_2 and energy [6, 8]. Butyrate estimate (the peak at 21.8 min) in HPLC analysis, as one of the typical volatile lower fatty acids, indicates that *Bacillus* strains can utilize LDPE as the sole carbon source for metabolic activities to produce organic acids and other products, further verifying that *Bacillus* can degrade LDPE.

Surface functional groups on LDPE

The analysis of surface functional groups can be used as an indicator of PE degradation [2]. In this study, the functional groups on LDPE were determined by ATR-FTIR (Fig. 5). All four LDPE specimens, including blank, negative control, two samples incubated with *B. subtilis* or *B. licheniformis*, exhibited peaks at wavelengths

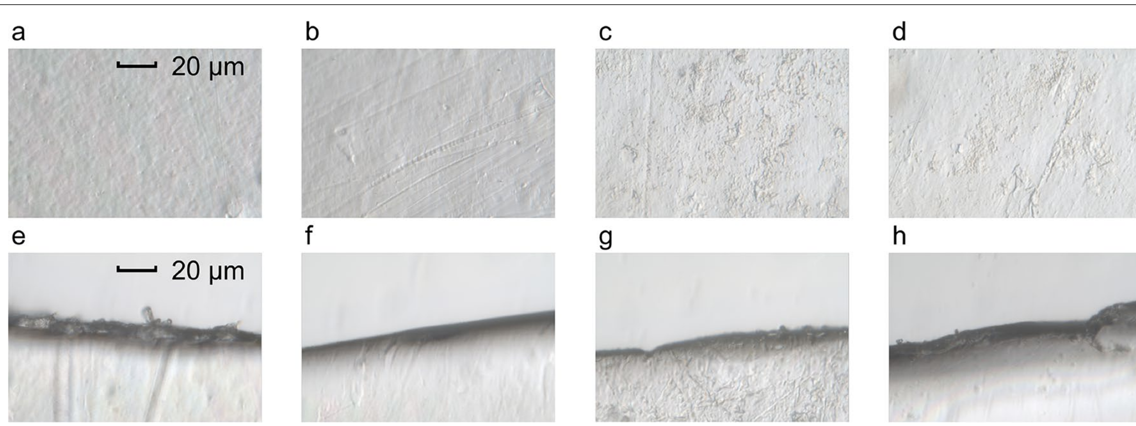

Fig. 3 Morphologies of LDPE film under the optical microscope. For morphological analysis, all LDPE specimens were taken after 30-day incubation except for blank (fresh film). Surfaces (**a**–**d**) and edges (**e**–**h**) were analyzed separately. **a**, **e** Blank group, indicating fresh LDPE film; **b**, **f** negative control, indicating LDPE specimens incubated in a flask in which bacterial cells were not inoculated; **c**, **g** LDPE film incubated with *B. subtilis*; and **d**, **h** LDPE film incubated with *B. licheniformis*

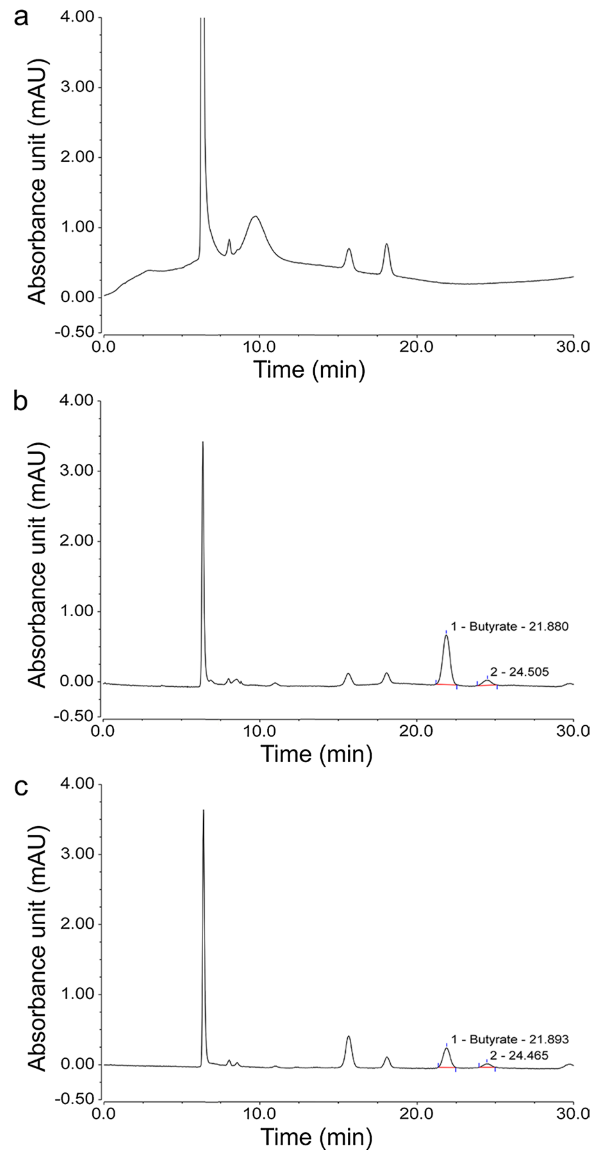

Fig. 4 Determination of LDPE decomposition products using HPLC. For HPLC analysis, the broth was taken after 30-day culture. **a** In the negative control, the LDPE specimens were incubated in a flask without bacteria; **b**, **c** LDPE samples were incubated with **b** *B. subtilis* and **c** *B. licheniformis*

of 700 cm^{-1}, 1390 cm^{-1}, 1485 cm^{-1}, 2851 cm^{-1}, and 2922 cm^{-1}, which indicated C–H, $-CH_3$ (methyl, C–H asymmetric/symmetric bend), $=CH_2$ (methylene, C–H bend), $=CH_2$ (methylene, C–H asymmetric/symmetric stretch) and $=CH_2$ (methylene, C–H asymmetric/symmetric stretch) bonds, respectively [9, 24].

There were a number of peak differences between negative control (or blank) and LDPE samples incubated with bacterial strains, as follows: On the surface of LDPE incubated with *B. subtilis*, a new peak was observed at 1030.65 cm^{-1} (Fig. 5), indicating C–O stretching and the presence of alcohol, carboxylic acid, ester, and ether groups [53]. This phenomenon was also reported in LDPE biodegradation using *B. siamensis* [16].

On the other hand, the LDPE incubated with *B. subtilis* also exhibited a new peak at 1538.86 cm^{-1} indicating N–O stretching. In recent studies for PE degradation, it has been reported that the N–O stretching was found in PE samples [39, 41, 50]. For example, it has been reported that the corrosive gas NO_x can cause PE degradation [39]. Furthermore, nitric oxide synthase (NOS), ubiquitous in *Bacillus*, can generate nitric oxide gas in an enzymatic reaction, which is thought to be responsible for the formation of nitro groups on LDPE [41]. Another study speculated that bacteria might secrete nitro and surfactants [50]. Although the partial pressure of NO_x produced by *Bacillus* strains during culture was much lower than the reaction conditions of Oluwoye and coworkers' study, the NO_x-induced LDPE free radical reaction deserves further investigation.

Additional new peaks at 1646.10 cm^{-1} and 3281.24 cm^{-1} were observed in LDPE incubated with *B. subtilis*, suggesting the existence of C=C stretching and an –OH group, respectively. These results indicate that the *B. subtilis*-exposed LDPE underwent oxidation under our experimental conditions. Hydroxylation is generally considered to be an important step in PE biodegradation, because an hydroxyl group is necessary for the formation of carbonyl groups [54], which can be converted into esters for eventual cleavage by lipase or esterase [56].

Finally, LDPE incubated with *B. subtilis* exhibited decreases in the peaks at 2851 cm^{-1} and 2922 cm^{-1}, indicating the weakening of $=CH_2$ stretching. In a previous study on the biodegradation of LDPE, similar results were obtained using *Acinetobacter baumannii* [42].

The results obtained for surface functional groups of LDPE incubated with *B. licheniformis* were consistent with those obtained for LDPE incubated with *B. subtilis*, but of a lesser degree (Fig. 5). Collectively, these findings on the changes in surface functional groups support the notion that LDPE films are degraded by the two *Bacillus* strains tested herein. In this study, we tested two *Bacillus* strains, *B. subtilis* ATCC6051 and *B. licheniformis* ATCC14580, and reveal that they both exhibit potential for the colonization and biodegradation of LDPE. *B. subtilis* could form biofilms on untreated LDPE films and grow using PE as the sole carbon source. After 30 days, it effectively degraded 3.49% of the input LDPE. The LDPE degradation ability of *B. licheniformis* was slightly lower than that of *B. subtilis*, effectively degraded by 2.83%. This is the highest rate reported so far for LDPE degradation using a single *Bacillus* strain. Morphological changes

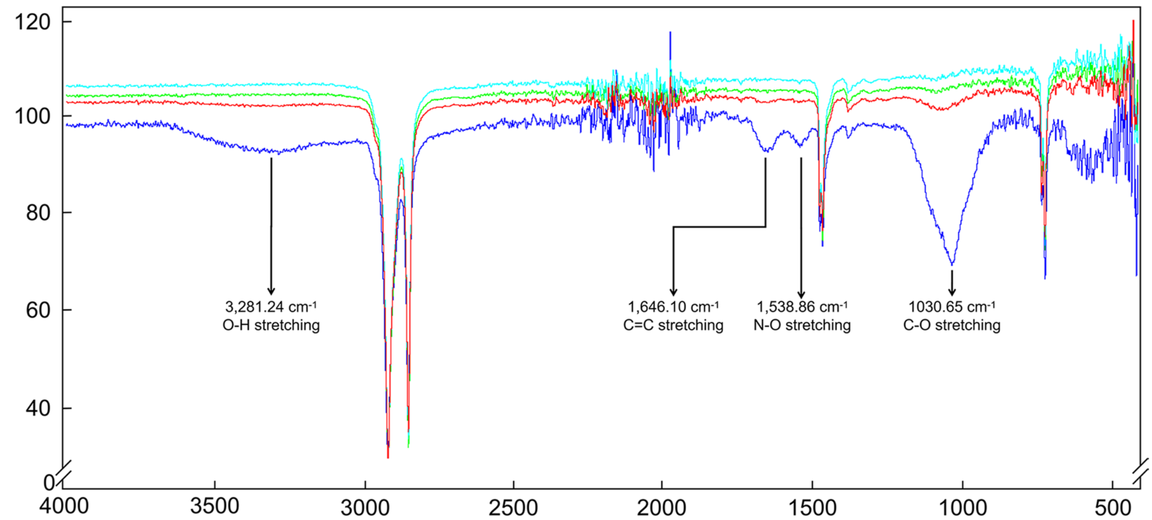

Fig. 5 ATR-FTIR spectra of LDPE film. For ATR-FTIR analysis, all LDPE specimens were taken after 30-day incubation except for blank (fresh film). The colors indicate: blank (fresh LDPE film; cyan); negative control (LDPE specimens incubated in a flask without bacteria; yellow-green); LDPE film samples incubated with *B. subtilis* (blue) or *B. licheniformis* (red)

seen by microscopic observations, differences in peaks on HPLC analysis, changes in peak intensities on ATR-FTIR, and the generation of new absorption peaks all confirmed the microbial-induced degradation of the PE film. Biofilm development and PE film degradation were observed by incubating LDPE films with each microbe as the sole carbon source. The study highlights that *B. subtilis* and *B. licheniformis*, the most common bacteria in soil, can be candidate microorganisms for bioremediation of plastic pollution. Various enzymes or microbial communities can be developed for polymer degradation. It will be a safe and environmentally friendly method. However, as aerobic bacteria, *Bacillus* has obvious limitations for plastic degradation in anaerobic environments (such as deep soil, and inside landfills), and it is necessary to further develop microbial communities composed of various microorganisms to cope with complex natural environments.

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Author contributions
Project design, YSJ; experiment, ZY; analysis, ZY, HJS, and YSJ; writing-original draft, ZY and YSJ; writing-review and editing, ZY and YSJ. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests
The authors declare that they have no competing interests.

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