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Research article

Biodegradation of low-density polyethylene by *Microbulbifer hydrolyticus* IRE-31

Zhongyu Li [a], Ren Wei [b], Meixi Gao [a], Yanru Ren [a], Bo Yu [c], Kaili Nie [a], Haijun Xu [a], Luo Liu [a,*]

[a] *Beijing Bioprocess Key Laboratory, Beijing University of Chemical Technology, Beijing, 100029, PR China*
[b] *Institute of Biochemistry, University of Greifswald, Felix-Hausdorff-Straße 4, D-17487, Greifswald, Germany*
[c] *CAS Key Laboratory of Microbial Physiological and Metabolic Engineering, State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, Beijing, PR China*

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ABSTRACT

Polyethylene (PE) is one of the most widespread plastic materials. Nevertheless, due to its recalcitrance against biological degradation and the presence of toxic additives, landfilled and carelessly disposed PE products have caused serious pollution in the natural environments. In this work, we aimed to investigate the growth characteristics of *Microbulbifer hydrolyticus* IRE-31 and its application in the biological degradation of low-density PE. The IRE-31 strain was isolated from marine pulp mill wastes rich in lignin which is a natural complex polymer containing also saturated carbon-carbon bonds like in PE. Following 30 days cultivation of the IRE-31 strain, the biodegradation of linear low-density PE particles was evidenced clearly by morphological changes of the polymer surface monitored by scanning electron microscopy and the formation of additional carbonyl groups in the polymer chains indicated by Fourier transform infrared spectroscopy.

1. Introduction

Plastics are favored synthetic materials for many civil and industrial applications due to low cost, light weight, and good water resistance. Since 1950s, plastic products have gradually occupied every area of human society. However, due to the lack of effective waste management provisions and poor biodegradability of many petrochemical plastics, plastic waste has accumulated in the natural environments, causing serious pollution (Baeyens et al., 2010; Brems et al., 2012). The widespread use of plastic products has led to a large amount of plastic waste entering the marine environment where they can be gradually fragmented into micro-plastics of millimeter size and accumulate in the marine environment (Barnes et al., 2009). Due to their persistence in the marine environment, the impacts of micro-plastics affect marine wildlife as well as on human health, since they can enter food chain via ingestion by zooplankton (Thompson et al., 2009). Because of the small size and wide distribution of plastic particles, it is difficult to collect them in a concentrated manner and then remove them from aquatic bodies. Likewise, the rate at which microplastics enter the environment exceeds the clearance rate (Auta et al., 2017). Moreover, extended investigations on microbial degradation of plastics are urgently needed for further understanding of this complex process under both lab and natural conditions as well as for development of bioremediation process of synthetic polymers using microorganisms (Auta et al., 2017). Complete biodegradation requires that microbes can use synthetic polymers as sole source of carbon and energy (Caruso, 2015). However, microbial degradation and utilization of recalcitrant petrochemical plastics as sole carbon source has been so far not adequately evidenced (Wei and Zimmermann, 2017).

In recent years, microorganisms able to degrade synthetic polymers with carbon-carbon backbones have been increasingly reported. Gauri Singh (2016) proved that *Bacillus* sp. has great potential for degradation of PE. They isolated 15 bacteria from different sites and after primary and secondary screening *Bacillus* sp. shows maximum degradation. Similarly, Savoldelli et al. (2016) developed a novel approach to break down polystyrene, by utilizing a combination of thermal treatment and application of bacteria. The thermally pretreated polystyrene (PS) could be liquefied and partially degraded into oligomers, which had lower viscosity and thus facilitated the bacterial diffusion and resulted in more sufficient degradation by bacteria. They found that *P. putida* and *Salmonella* were the two most effective bacteria in the degradation of oligomer mixture derived from polystyrene. In addition, *P. putida* was able to break down the PS-derived mixture by 98-fold higher rate compared to the control. The cleavage of C–C bond in recalcitrant

* Corresponding author.
E-mail address: liuluo@mail.buct.edu.cn (L. Liu).

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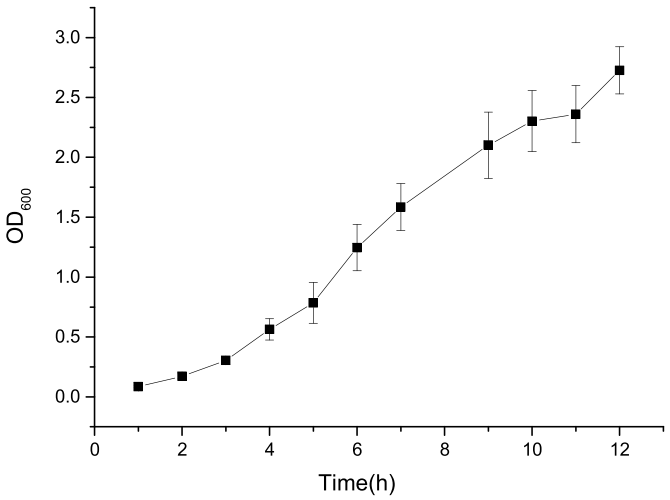

Fig. 1. Time course of OD_{600} during the first 12 h of incubation of *M. hydrolyticus* IRE-31.

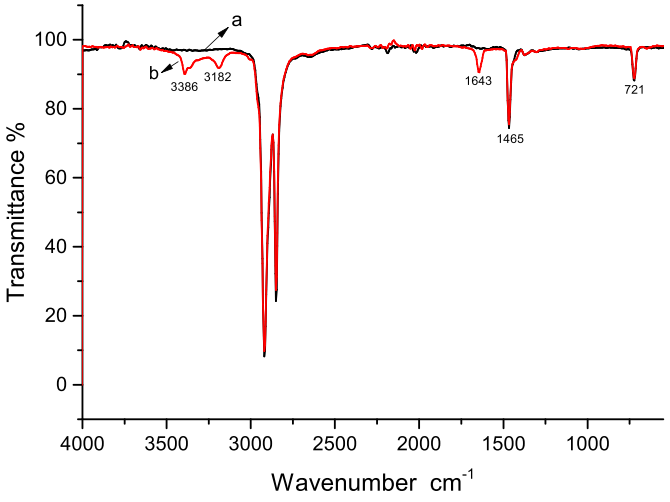

Fig. 2. Fourier transform infrared spectroscopy of **a** control LLDPE, **b** the IRE-31 strain treated LLDPE with copper sulfate for 30 days.

plastics has been recently suggested being contributed by the attack of free radicals (Xu et al., 2019). Sumathi et al. (2016) isolated a fungus *Cochliobolus* sp. from a soil sample where plastics were dumped and found that it can degrade low molecular weight polyvinyl chloride by producing laccase. They first used different carbon sources and copper sulfate to increase the laccase production. Xylose was found to be the most suited carbon source to boost the yield of laccase. Using a copper sulfate concentration of 350 μM showed that the production of laccase was increased. By culturing this strain using low molecular weight polyvinyl chloride as the sole carbon source for the strain, significant degradation could be demonstrated, however, after a long incubation period of one year. Krueger et al. (2017) investigated the ability of brown-rot fungus *Gloeophyllum trabeum* to attack PS through the quinone redox cycle-driven Fenton chemistry, and found that the low bioavailability and resistant backbone structures of PS were the major reasons for its overall poor biodegradation via bio-based Fenton chemistry.

Microbes living in neighborhood to marine plastic garbage has great potential in the development of future bioremediation process for plastics waste. The marine bacterium *Microbulbifer hydrolyticus* IRE-31 (ATCC 700072) was isolated from wastewater discharged from a lignin-rich pulp mill (Gonzalez et al., 1997). The IRE-31 strain is a Gram-negative, rod-shaped, absolutely aerobic marine bacterium. According to 16s rRNA analysis, the IRE-31 strain belongs to the γ-3 line of *Proteus* which can decompose cellulose, xylan, chitin, gelatin and Tween 80 by expressing hydrolytic enzymes (Gonzalez et al., 1997). The IRE-31 strain was speculated able to produce laccase, which has been considered a key enzyme for plastic degradation (Fujisawa et al., 2001). In this study, the potential of IRE-31 strain for PE degradation was characterized by exploring its growth conditions, the growth factors and optimal pH of the laccase activities. The degradation efficiency of linear low-density PE (LLDPE) by the IRE-31 strain was studied by scanning electron microscope (SEM) and Fourier transform infrared spectroscopy (FTIR).

2. Materials and methods

2.1. Polyethylene

Linear low-density polyethylene (LLDPE, DFDA-7042) particles, with density of 915–921 kg/m^3 and radius of about 2 mm were provided by Sinopec Group (Beijing, China).

2.2. Media and culture conditions

The PE-degrading bacterium *M. hydrolyticus* IRE-31 (ATCC 700072) was used in this study. Bacterial cultures of the IRE-31 strain (20 ml) were maintained at 37 °C in marine broth 2216 (BD; Difco, USA), either in 100 ml flasks incubated on a rotary shaker (220 rpm) or on marine broth 2216 agar plates. Another liquid medium consisting of 5.0 g/L tryptone, 1.0 g/L yeast extract, 40 g/L sodium chloride, 0.034 g/L strontium chloride was also used. The IRE-31 strain was stored in a mixture at 1:1 ratio of marine broth 2216 and 40% glycerol at −80 °C. Unless otherwise specified, all assays for evaluating the efficiency of bacterial degradation of LLDPE were conducted in marine broth 2216 (5.0 g/L tryptone, 1.0 g/L yeast extract, 0.1 g/L ferric citrate, 19.45 g/L sodium chloride, 5.9 g/L anhydrous magnesium chloride, 3.24 g/L sodium sulfate, 1.8 g/L calcium chloride, 0.55 g/L potassium chloride, 0.16 g/L sodium bicarbonate, 0.08 g/L potassium bromide, 34 mg/L strontium chloride, 22 mg/L boric acid, 2.4 mg/L sodium fluoride, 1.6 mg/L ammonium nitrate, 8.0 mg/L dipotassium hydrogen phosphate, 4.0 mg/L sodium silicate nonahydrate). Marine broth 2216 agar was prepared by adding 15 g of agar per liter. Plate cultures were incubated overnight at 37 °C for 12 h.

2.3. Determination of the growth curve

After incubating the IRE-31 strain seed liquid for 12 h, 400 μL of the seed liquid was respectively inoculated into 100 ml flasks labeled No. 1, 2, and 3 containing 20 ml of the liquid media. The inoculated flasks were placed in an incubation shaker and cultured in darkness at 37 °C and 200 rpm. Samples were taken every 1 h for a total of 12 times, and the removed samples were stored at 4 °C. The optical density was measured after all sampling was completed. The blank liquid media was used as a reference, and the optical density was measured at a wavelength of 600 nm. Sterile H_2O was used to dilute the to ensure that the determined OD_{600} is in the range of 0.1–0.6.

2.4. PE degradation

LLDPE particles (sphere, r = 2 mm) were washed twice with distilled water and then twice with 75% ethanol, followed by drying particles with filter paper. Each sample containing 10 LLDPE particles was placed in a beaker which was sealed with thin foil, and sterilized at 121 °C for 30 min by a high-pressure steam sterilizer. Pre-treated LLDPE particles were placed in flasks containing 20 ml bacterial culture with an OD_{600} of 0.6 both in the presence and the absence of 0.2 μmol/ml copper sulfate. As a reference, LLDPE particles were also incubated in culture medium

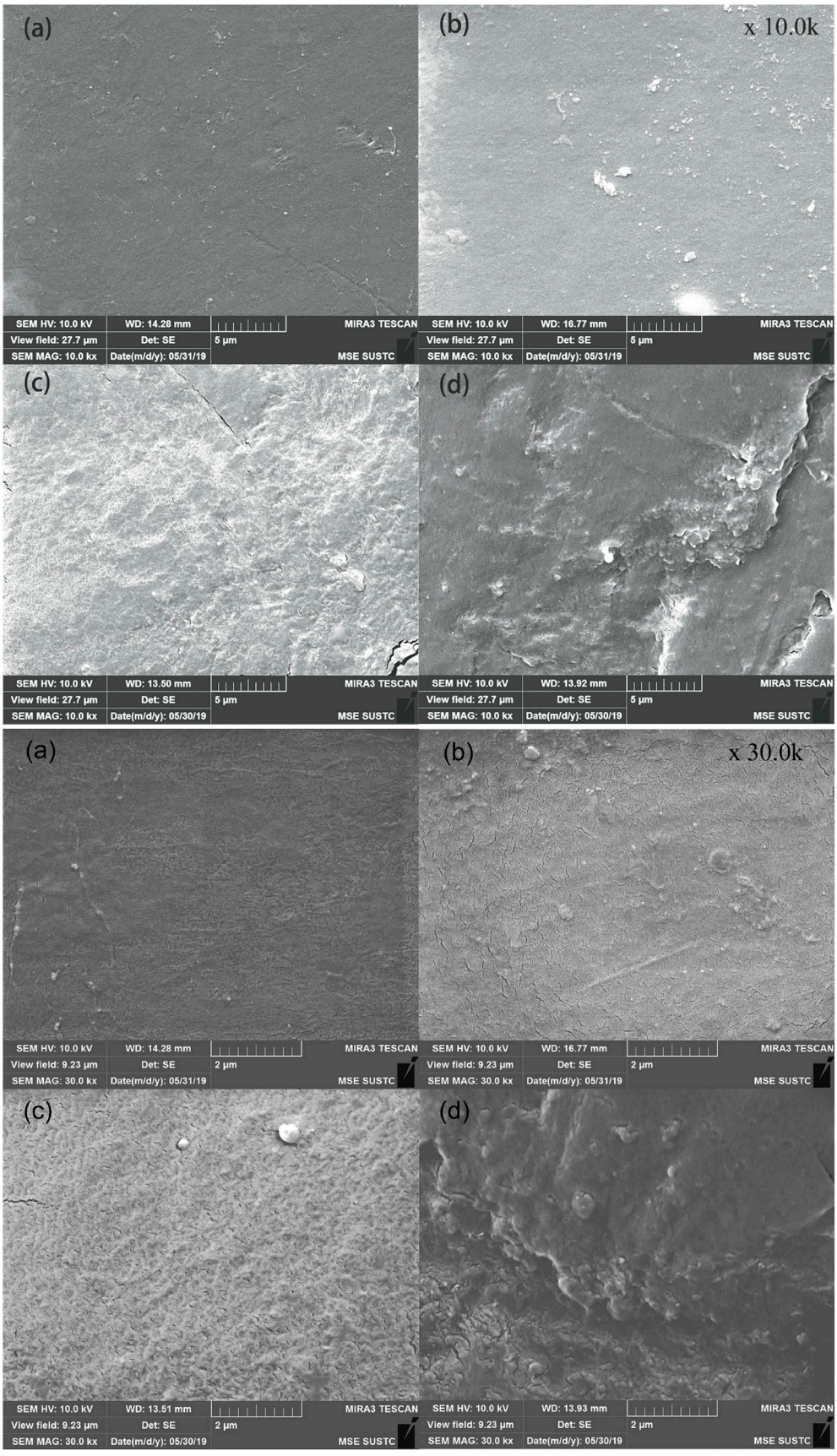

Fig. 3. SEM captures of LLDPE particles following 7 days of incubation or treatment under various conditions. a negative control, **b** UV-treated LLDPE, **c** the IRE-31 strain treated LLDPE and **d** the IRE-31 strain treated LLDPE in the presence of copper sulfate.

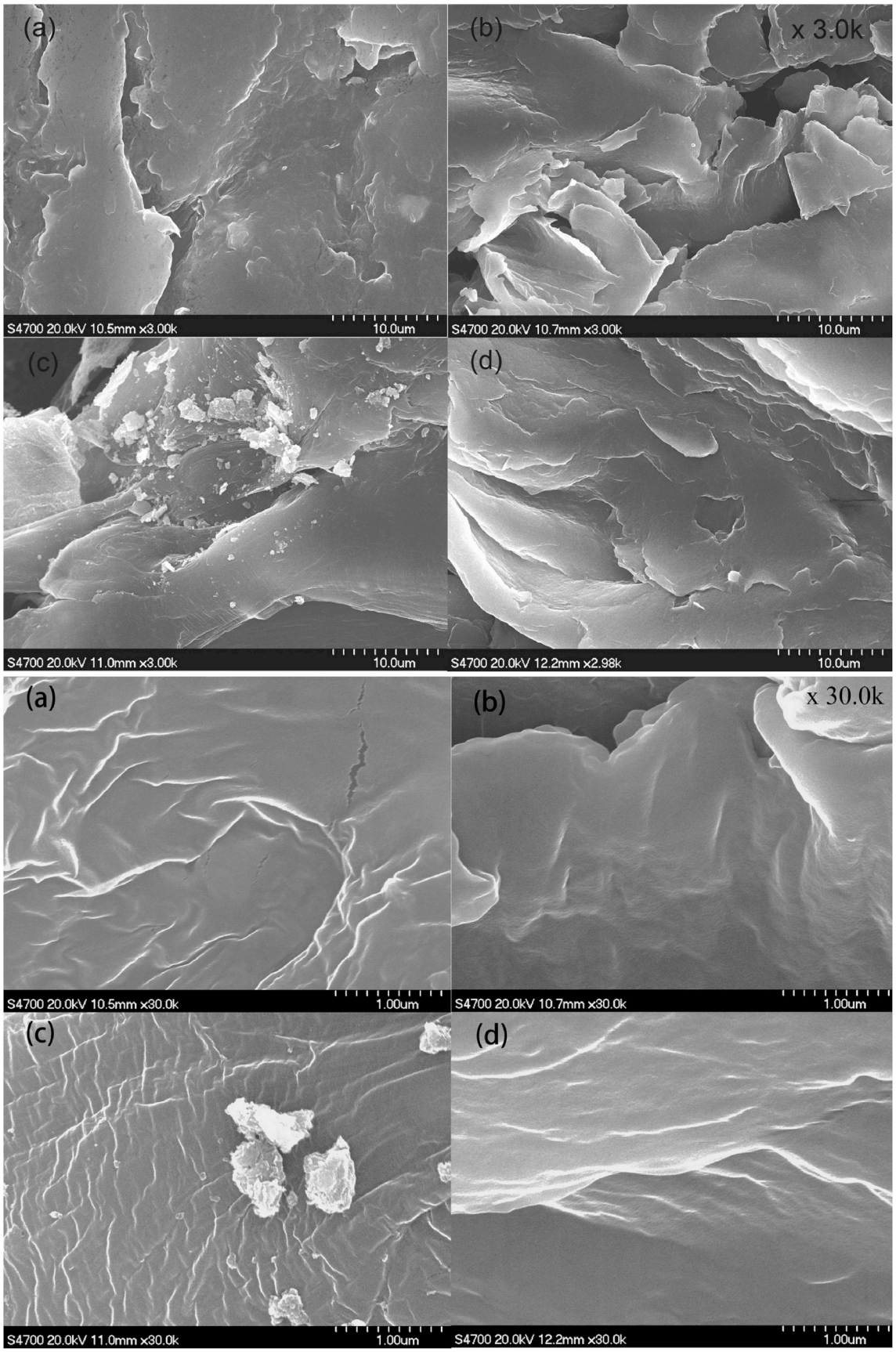

Fig. 4. SEM captures of LLDPE particles following 20 days of incubation or treatment under various conditions. **a** negative control, b UV-treated LLDPE, c the IRE-31 strain treated LLDPE and d the IRE-31 strain treated LLDPE in the presence of copper sulfate.

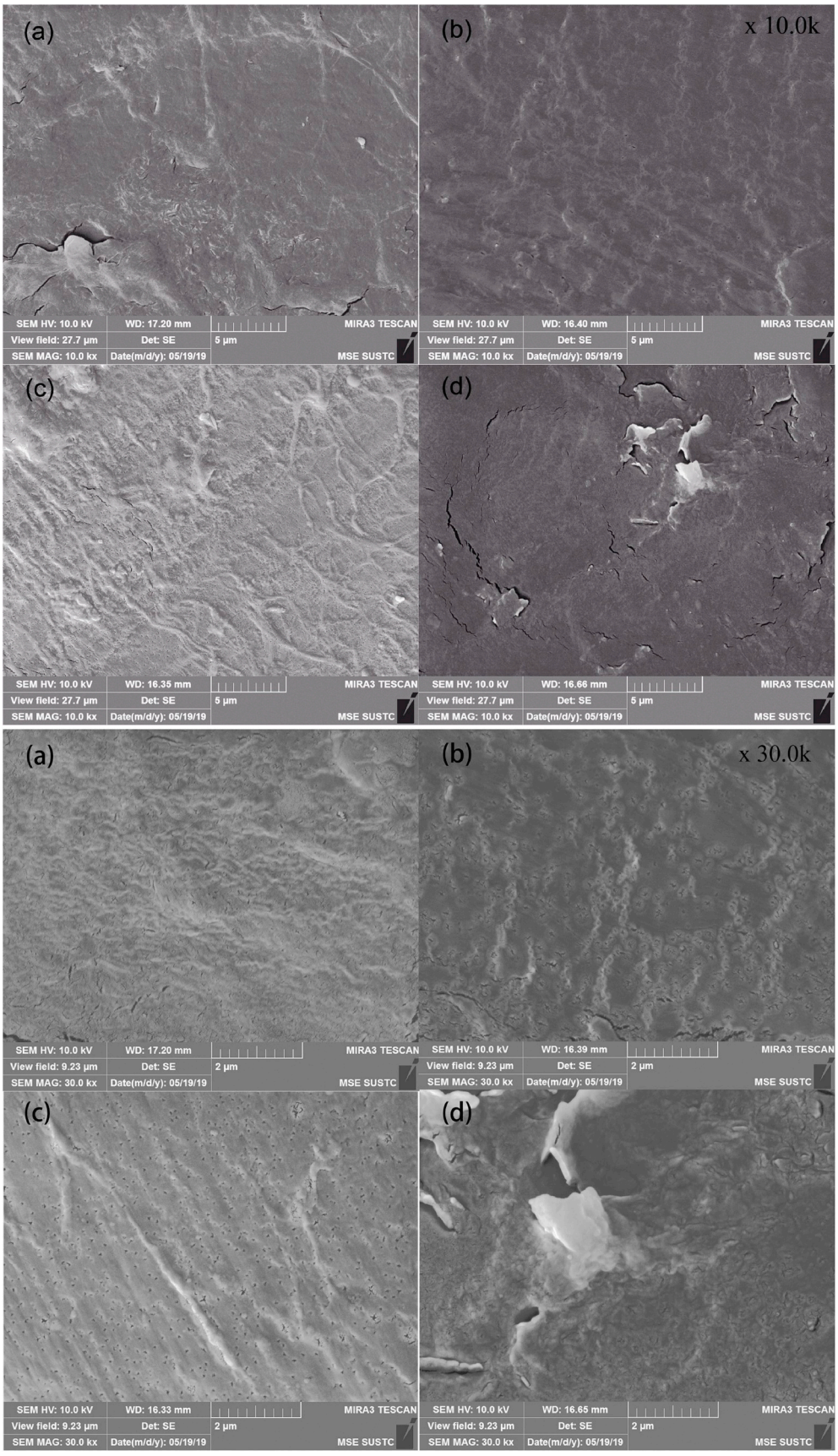

Fig. 5. SEM captures of LLDPE particles following 30 days of incubation or treatment under various conditions. a negative control, b UV-treated LLDPE, c the IRE-31 strain treated LLDPE and d the IRE-31 strain treated LLDPE in the presence of copper sulfate.

Table 1
M. hydrolyticus IRE-31 genome features. (no plasmid).

Characteristics	Chromosome
Size of genome	4,209,307
G + C content (%)	57.6%
Coding density (%)	84%
No. of genes	3545
No. of function assigned protein	2327
No. of hypothetical protein	1218
No. of RNA genes	78
No. of rRNA	12
No. of tRNA	66

without bacteria inoculation. All samples were incubated at 37 °C and 220 rpm and removed after 7-, 20-, and 30-days of cultivation, respectively, for subsequently analyses. In addition, pre-treated LLDPE particles exposed to ultraviolet radiation for 12 h per day were also sampled after 7-, 20-, and 30-days of treatment, respectively.

2.5. Analyses of LLDPE particles

The LLDPE particles were removed from the bacterial cultures at above-indicated incubation intervals by tweezers, washed twice with distilled water, and washed twice with 75% ethanol. Then the LLDPE particles were dried with clean paper towel and followed by incubation at 65 °C for 12 h. FTIR (Nicolet 6700, Thermo) can monitor the changes of PE in molecular structures and homogeneity as a result of degradation. Spectra in the frequency range of 7800–350 cm^{-1} were used at a resolution of 0.09 cm^{-1}. SEM (HITACHI S-4700 , Japan & TESCAN MIRA3 Czech Republic) was used to examine the surface morphology of the LLDPE. The LLDPE particles were fixed, and scanned by SEM with magnifications of 3000, 10,000, and 30,000, respectively.

2.6. Genome sequencing

The frozen glycerin bacteria IRE-31 were activated, and then inoculated 100 ml of medium placed in a 500 ml flask. The IRE-31 strain was cultured at 37 °C and 220 rpm for 7 days. Cell pellets yielded by centrifugation was sent to the Majorbio Cloud company (Shanghai, China; https://cloud.majorbio.com/) for genome sequencing. The IRE-31 strain genome was sequenced using a combination of PacBio RS and Illumina sequencing platforms. The Illumina data was used to evaluate the complexity of the genome. The complete genome sequence was assembled using both the PacBio reads and Illumina reads. Identification of predicted coding sequences (CDS) was performed using Glimmer version 3.02 (http://cbcb.umd.edu/software/glimmer/). SwissProt (http://uniprot.org), KEGG (http://www.genome.jp/kegg/), and COG (http://www.ncbi.nlm.nih.gov/COG) were used to perform functional annotation. In addition, Mummerplot (http://www.jcvi.org/cms/research/software/) was used to compare the genomics analysis.

3. Results and discussion

3.1. The growth curve of M. hydrolyticus IRE-31

As shown in Fig. 1, the population density of *M. hydrolyticus* IRE-31 increased slowly initially until 4 h and was followed by a rapid increase from 4 to 10 h, indicating that the strain entered the logarithmic growth phase in this time period. The growth curve began to stabilize at 10 h, indicating that population of bacteria approached gradually a stable period after 10 h of incubation.

3.2. Characterization of biodegraded LLDPE using FTIR

A previous study has indicated that the biodegradation of PE can be briefly divided into three steps (Albertsson et al., 1987): first, PE is broken down into oligomers, dimers or monomers through biological oxidation or by enzymes. Fatty acids are then formed as a result of polymer chain scission by further oxidative reactions. Finally, the fatty acids are metabolized to CO_2 and H_2O by the microorganisms. FTIR analysis demonstrated that the bacterium-treated PE formed new functional groups such as hydroxyl groups (3392 cm^{-1}) and carbonyl groups (1644 cm^{-1}), indicating the oxidation on the surface. This is consistent with previous studies (Aeutchelvi et al., 2008; Zhang et al., 2019), which indicated that the appearance of hydroxyl and carbonyl groups provided the evidence of bio-oxidation of PE and ultimately promoted the degradation of PE. In addition, the peak at 3195 cm^{-1} has been attributed to the C = C bond, which can be much easily broken down than the saturated C–C bond in PE. Interestingly, we did not observe the formation of ether groups (1100 cm^{-1}) and multiple peaks in the 1600-1800 cm^{-1} region. Furthermore, no esters and aromatics were found in the spectrum (Fig. 2) of bacterium-treated LLDPE samples, which is different from some previous reports (Zhang et al., 2019; Sarmah and Rout, 2019; Sowmya et al., 2014). This may indicate that in our study, the degradation of PE has a high product selectivity.

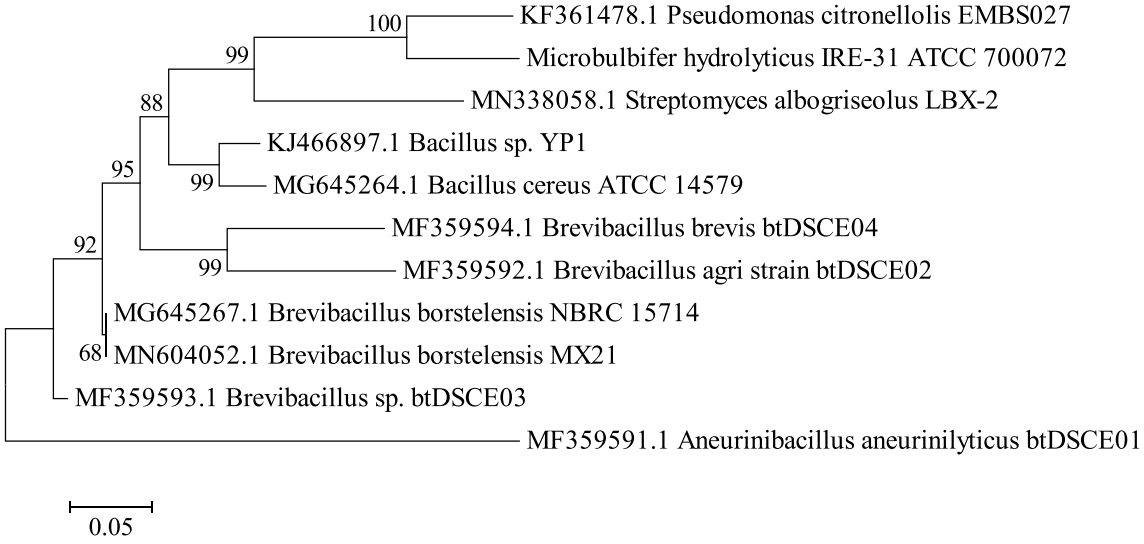

Fig. 6. 16 S rDNA-based phylogenetic analysis of *M. hydrolyticus* IRE-31 strain. The phylogenetic tree was constructed by the neighbor-joining method using MEGA. The scale bar shows 0.05 nucleotide exchanges per nucleotide.

3.3. Characterization of treated LLDPE using SEM

The surface morphology of LLDPE treated by the IRE-31 strain and UV-radiation captured by SEM following 7, 20 and 30 days of incubation or treatment were shown in Figs. 3, 4 and 5, respectively. As shown in Fig. 3, even after a short cultivation time of 7 days, uneven surface was observed with LLDPE incubation in the presence of IRE-31, suggesting the disruption of the plastic particles as a result of bacterial degradation. When copper sulfate was present, the surface destruction of treated LLDPE was more pronounced exhibiting obvious protrusion and crease. This observation indicated that copper-dependent enzyme, such as laccase, may be involved in biodegradation of PE, which is in agreement with previous literature (Santo et al., 2013). By comparison, control samples incubated in culture medium as well as UV-treated LLDPE showed similar surface structures without notable damage.

As illustrated in Fig. 4, after 20 days of incubation, compared with the relatively smooth surface of the control LLDPE, the IRE-31 strain treated LLDPE obviously showed the release of plastic debris, and the IRE-31 strain treated LLDPE with copper sulfate resulted in the formation of obvious holes as shown in Fig. 4d (below) with a magnification factor of 3000. However, the negative control and the UV-treated LLDPE showed still similar surface structures, suggesting that the damage degree caused by UV-radiation was not significant.

Similarly as described above, the negative control and UV-radiated LLDPE particles showed inadequate sign of surface degradation after 30 days of incubation or treatment. In comparison, the IRE-31 strain treated LLDPE particle in the presence of copper sulfate clearly showed the formation of surface cracks, further confirming the microbial degradation by *M. hydrolyticus* IRE-31.

Sarmah et al. (Sarmah and Rout, 2019) studied the degradation of PE with *Nostoc carneum* isolated from domestic sewage water. After co-culturing cyanobacteria and PE sheet for 6 weeks, they observed destruction in surface structures of the PE sheets where holes were formed. In comparison, we can clearly observe the formation of surface cracks in LLDPE particles following 30 days of incubation with *M. hydrolyticus* IRE-31, which was isolated from wastewater discharged from pulp mill, suggesting a potentially higher degradation ability against PE with our bacteria strain.

Devi et al. (2015) studied the degradation of high density PE (HDPE) using fungus *Aspergillus* spp. and could show destructed surface structure of HDPE film, to a similar extent as shown in our study, treated by the fungus *Aspergillus* spp.

3.4. Genome DNA sequencing and analysis

We sequenced the complete genome sequence of *M. hydrolyticus* IRE-31 (GenBank CP047491). Genome sequencing is helpful for discovering enzymes potentially involved in the degradation of PE. The complete IRE-31 strain genome contains a circular chromosome of 4,209,307 bp with a 57.6% G + C content (Table 1). A total of 3545 genes were predicted to be open reading frames (ORF). The number of specified and hypothetical proteins were identified by homological alignment of the genes with reference genes in the databases (NR, Swiss-Prot, Pfam, EggNOG, GO, KEGG). Total of 2995 genes were classified into 22 Clusters of Orthologous Groups (COG) categories. There were 66 tRNA genes, 20 type of tRNAs, 40 repeat sequences and 12 rRNA loci, respectively. The role of Gene Ontology (GO) annotation is to obtain standardized functional description information, which includes three aspects: biological process, molecular function, and cellular component. Based on GO annotation, ORFs were preferentially categorized to molecular functions (1664), followed by biological processes (1544) and cellular components (1001). Through the metabolic network of the IRE-31 strain analyzed by KEGG, 27 genes were found to be involved in xenobiotics biodegradation and metabolism.

3.5. Phylogenetic analysis of M. hydrolyticus IRE-31

The 16 S rRNA sequence of the IRE-31 strain showed the closest similarity (79.18%) with *Pseudomonas citronellolis* strain EMBS027. *Pseudomonas citronellolis* was isolated from collected municipal landfill soil and was confirmed to be a novel LDPE degrading *Pseudomonas* species by phylogenetic assessment (Bhatia et al., 2014). Fig. 6 shows the phylogenetic relationship of the IRE-31 strain with other strains that can degrade PE as so far reported. Recent study reported the degradation of PE with a bacterium *Streptomyces albogriseolus* LBX-2 isolated from soil (Shao et al., 2019). However, PE degradation by marine microbes is so far rarely reported. Thus, according to the results shown in this study, the marine strain IRE-31 fills this gap and will have great potential to facilitate the understanding of biodegradation pathways of plastic degradation in marine environments.

4. Conclusion

Plastic pollution has caused serious environmental crisis for marine ecological systems. Hence, it is of scientific interest to explore plastic degrading microorganisms in the ocean for further understanding of the plastic biodegradation there. In this study, a marine bacterial strain *M. hydrolyticus* IRE-31 was investigated with respect to the degradation of pretreated LLDPE particles. SEM captures of the surface structures of LLDPE particles illustrated the formation of cracks as a result of cultivation with the IRE-31 strain in the presence of copper ion for up to 30 days. It was speculated that copper-dependent enzymes may be involved in biodegradation of plastics, which is in agreement with previous literatures. FTIR analysis indicated the formation of additional hydroxyl- and carbonyl functional groups at the polymer surface, suggesting that oxidation has occurred there during the incubation with IRE-31, in line with previous studies regarding PE degrading bacteria. The phylogenetic analysis based on the alignment of 16 S rRNA sequences indicated its close relationship to the previously reported PE degrading bacterial strains. Oxidative reactions may be the initial step of the biodegradation of PE, of which the metabolic pathways are, however, subject of ongoing investigations. Nevertheless, the novel PE-degrading marine bacterium *M. hydrolyticus* IRE-31 described in this study may serve as additional microbial candidate for the future development of effective bioremediation processes.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Zhongyu Li: Data curation, Writing - original draft. **Ren Wei:** Conceptualization. **Meixi Gao:** Data curation. **Yanru Ren:** Data curation. **Bo Yu:** Writing - review & editing. **Kaili Nie:** Writing - review & editing. **Haijun Xu:** Writing - review & editing. **Luo Liu:** Conceptualization, Writing - original draft.

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