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Biodegradation of polyethylene by some bacteria *Bacillus* isolated from marine sediments in Khanh Hoa province, Vietnam

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Abstract: *Marine pollution caused by plastic waste is a worldwide concern. Of which, plastic waste originating from Polyethylene (PE) accounts for the highest proportion. This study focuses on evaluating the biodegradation ability of PE plastic of some bacteria belonging to the genus Bacillus isolated from marine sediments collected in Khanh Hoa province, Vietnam. Four bacterial strains including: C1.2, C3.4, C13.1, C17.1 reduced the weight of PE substrate after 45 days of culture (compared to the initial) by (%): 4.5 ± 0.058[d], 3.6 ± 0.100[c], 6.4 ± 0.100[b], 6.7 ± 0.058[a], respectively, completely equivalent to the results of determining laccase enzyme activity (U/ml): 974.0 ±3.61[d], 649.3±3.06[c], 791.7±1.53[b], 1,206.0±3.61[a]. Scanning electron microscopy (SEM) images showed that bacteria created distinct cracks and dents on the surface of plastic pellets. The bacteria were then studied for a number of biological characteristics including colony and cell morphology, pH, temperature, salinity and PE substrate concentration limits, and identified through 16S rRNA gene sequencing. The four bacterial strains C1.2, C3.4, C13.1, C17.1 were closely related to species of the genus Bacillus including: Bacillus cereus, Bacillus amyloliquefaciens, Bacillus safensis, Bacillus megaterium. This study could be a premise for further studies on the treatment of saline PE plastic waste using marine bacteria.*

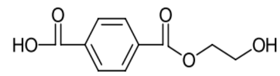
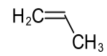
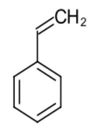
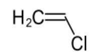
Keywords: *Bacillus; biodegradation; marine bacteria; polyethylene; waste plastic*

1. Introduction

Marine pollution caused by plastic waste is a worldwide concern. On average, the world generates about 300 million tons of plastic waste each year, with up to 8 million tons ending up in the oceans (Liang Y. et al., 2021). According to Plastic Europe, global plastics production climbed to 390.7 million tonnes in 2021. Of these, 352.3 million tonnes (90.20%) were fossil-derived plastics, while only 32.5 million tonnes (8.30%) and 5.9 million tonnes (1.50%) were post-consumer recycled plastics and bio-based plastics, respectively (Plastic – The Facts 2022). Vietnam is ranked fourth among the 20 countries with the largest total plastic waste discharged into the ocean. According to the 2022 plastic waste status report, the total volume of plastic waste generated was 2.93 million tons, with an increase rate of about 5% per year (Ministry of Natural Resources and Environment, 2024).

Plastic is a material prepared from macromolecular groups, including 6 types of monomers: Low-density polyethylene (LDPE), High-density polyethylene (HDPE), Polyethylene terephthalate (PET), Polypropylene (PP), Polystyrene (PS), Polyvinyl chloride (PVC) (Ramkumar M. et al., 2022). Each monomer has different structure and physical and chemical properties (Table 1). Of that, PE and PP are the two types of plastic with the highest consumption output (Figure 1). Accordingly, PE is one of the groups with the highest proportion in waste composition (Rafey A. et al., 2021).

Tab. 1. Summary of 6 types of plastic and some of their properties (Othman A. R. et al., 2021)

No	Polymer name	Monomer name	Monomer structure	Chemical formula	Density (g cm^3)
1	Low-density polyethylene (LDPE)	Ethene	$H_2C{=}CH_2$	C_2H_4	0.91–0.92
2	High-density polyethylene (HDPE)	Ethene	$H_2C{=}CH_2$	C_2H_4	0.93–0.97
3	Polyethylene terephthalate (PET)	Ethylene terephthalate		$C_{10}H_8O_4$	1.37–1.38
4	Polypropylene (PP)	Propylene		C_3H_6	0.89–0.92
5	Polystyrene (PS)	Styrene		C_8H_8	0.28–1.04
6	Polyvinyl chloride (PVC)	Vinyl chloride		C_2H_3Cl	1.10–1.47

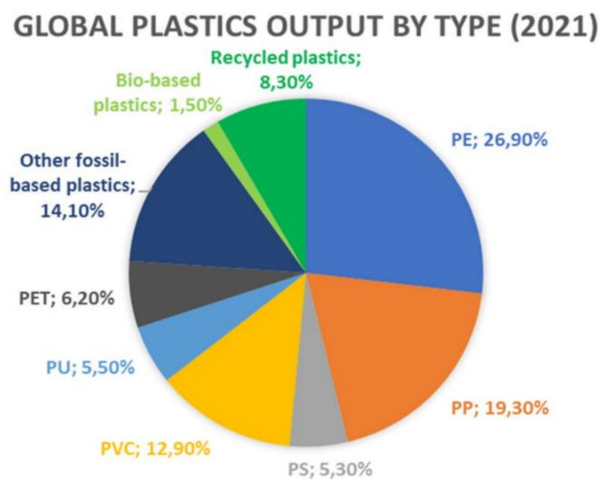


Fig. 1. Global plastic polymers production in 2021 (Plastic – The Facts 2022)

Many studies have demonstrated the ability of microorganisms to biodegrade plastic waste (Othman A. R. et al., 2021). Srikanth M. et al. (2022) evaluated the biodegradation potential of fungi for plastics. Some well-known species which show effective degradation on plastics are *Aspergillus nidulans, Aspergillus flavus, Aspergillus glaucus, Aspergillus oryzae, Aspergillus nomius, Penicillium griseofulvum, Bjerkandera adusta, Phanerochaete chrysosporium, Cladosporium cladosporioides, Pleurotus abalones, Pleurotus ostreatus, Agaricus bisporus* and *Pleurotus eryngii,* which use plastics as a food source for their growth (Srikanth M. et al., 2022). Additionally, in an assessment of current advances, challenges and strategies for enhancing the biodegradation of plastic waste, Yuehui H. et al (2024) have listed some plastic biodegrading bacteria such as: *Rhodococcus ruber, Ideonella sakaiensis, Pseudomonas sp., Bacillus sp., Mycobacterium sp., and Nocardia sp*. In which, some bacteria belonging to the genus *Bacillus* that can biodegrade PE have been identified (Yao Z. et al, 2022). The mechanism of plastic biodegradation in microorganisms can be divided into four steps: biological degradation, hydrolysis, biological assimilation, and mineralization. In which, enzymes play a key role in the entire biodegradation process (Othman A. R. et al., 2021). PE plastic degrading enzymes include: *laccase*, *cutinase*, *peroxidase*,…(Ghatge S. et al, 2020).

This study focused on evaluating the biodegradation ability of PE plastic of some bacteria belonging to the genus *Bacillus* isolated from marine sediments collected in Khanh Hoa province, Vietnam. The results of the study could be a premise for further studies on the treatment of saline PE plastic waste using marine bacteria.

2. Materials and methods

2.1. Materials

Four bacterial strains were isolated from marine sediments (Nha Trang, Khanh Hoa, Vietnam) including: C1.2, C3.4, C13.1, C17.1.

Chemical: Polyethylene (Sigma-Aldrich, code: 428043, form: pellets, melt index: 25 g/10 min (190°C/2.16kg), impact strength: 45.4 J/m (Izod, ASTM D 256, −50 °C, notched), density: 0.925 g/mL at 25°C), mineral salt medium (MSM) were used to study plastic degradation using a method described by Kanniahi et al. (2013), nutrient agar (NA) according to Emenike et al. (2016).

2.2. Methods

Reduced the weight of PE substrate

PE plastic pellets were cultured with bacteria in MSM medium. After 90 days, they were recovered by filtration. Next, they were washed with 70% ethanol and dried overnight at 50°C. Finally, they were weighed to determine the mass (Mohan et al., 2016). Each experiment was repeated three times and differences in mean values between experiments were noted at the 5% significance level. The polymer weight loss rate was determined to evaluate the degradability of the bacterial strains through the following formula (the initial weight of plastic beads was also determined in the same technique):

$$\text{Percentage weight loss} = \left(\frac{\text{Initial weight of polymer} - \text{Final weight of polymer}}{\text{Initial weight of polymer}}\right) \times 100. \quad (1)$$

Determining laccase enzyme activity

The enzyme reaction solution consisted of: 2.2 ml of 0.1M phosphate buffer pH 6.5; 0.3 ml of 0.216 mM syringaldazine solution in methanol, 0.5 ml of enzyme solution. Mix the reaction mixture well and measure the increase in absorbance at 530 nm of the reaction mixture compared with the control sample after each minute of reaction. The control reaction used deionized water instead of enzyme (Kumar R. et al., 2016). One unit of *laccase* activity is the amount of enzyme that in one minute at pH 6.5, 30°C converts 1 µmol of syringaldazine ($\varepsilon = 65\ mM^{-1}\ cm^{-1}$). Each experiment was repeated three times and differences in mean values between experiments were noted at the 5% significance level.

Scanning electron microscopy (SEM)

The surface of PE plastic pellets after 40 days of culture was photographed by SEM and compared with the control sample to evaluate the ability of bacterial strains to decompose the surface of PE plastic (Kang B. R. et al., 2019).

Physiological and biochemical characteristics

The bacterial strains were studied for some physiological and biochemical characteristics including: initial pH (investigated pH values of 4, 5, 6, 7, 8 and 9), temperature (investigated values - °C: 20, 25, 30, 37, 40, 44), salinity (investigated values - ‰: 10, 15, 20, 26, 35, 40), PE substrate concentration (investigated values - % w/v: 0.1, 0.3, 0.5, 0.7, 1.0). The procedure was as follows: Bacteria were cultured on MSM liquid medium with PE plastic as the sole carbon source and the investigated factors varied. Each measurement was repeated 3 times and the reliability of the measurements was determined. The optical density ($\lambda = 600$ nm) of the culture medium was determined after 3 weeks of culture at 37°C and 150 rpm (Auta H.S. et al, 2017) . The standard curve was constructed to show the linear relationship between cell density and optical density 600nm value (Michail S., 2005). Bacteria were grown in MSM medium, then diluted to different concentrations, measured the density ($\lambda = 600$ nm) and spread the plate on NA medium incubated at 37 °C for 48 hours, then counted the colonies (CFU). Using Microsoft Excel software to process the data, the standard curve results:

$$Log\left(\frac{CFU}{ml}\right) = 1.9791 + 8.2389 \quad (2)$$

In experiments to determine cell density, determine the optical density (λ = 600 nm) after the end of the culture period, based on the standard curve to deduce the cell density.

16S rRNA gene sequencing

DNA samples of the strains were extracted using TopPURE® genomic DNA extraction kit. Use specific primer pairs to amplify the 16S rRNA gene bacteria (27F - 5'AGAGTTTGATCMTGCCTCAG3', 1492R - 5'TACGGTTACCTTGTTACGACTT3') with recommended components and reaction cycle. PCR products are checked on agarose gel, then purified and sent for sequencing.

Statistical analysis

Statistical analysis of data was carried out using ANOVA from SPSS software 21.0.

3. Results and Discussion

Reduced the weight of PE substrate

The weight loss rate of the plastic pellets in the culture flask was recorded after 90 days of culture because this was the minimum time to detect weight differences (Montazer Z. et al, 2020). After 90 days, the weight loss rates recorded for the four strains C1.2, C3.4, C13.1, C17.1 were: 4.5%, 3.6%, 6.4%, 6.7%, respectively (Table 2). The statistically significant differences (a, b, c, d) in Table 2 demonstrate that the average substrate weight loss rates of the strains were different and strain C17.1 had the highest weight loss rate.This result was similar to the study of Auta H. S. et al. (2017) when evaluating the weight loss rates of PE plastic of the two bacterial strains *B. cereus* and *B. gottheilii* were 6.6%, 7.4% respectively (Auta H.S. et al, 2017). Harshvardhan K. and Jha K. (2013) recorded weight loss of 1%, 1.5%, and 1.75% for PE by *K. palustris*, *B. pumilus*, and *B. subtilis* isolated from pelagic water, respectively (Harshvardhan K. and Jha B., 2013).

Tab. 2. PE substrate weight reduction rate of strains.

Strains	**C1.2**	**C3.4**	**C13.1**	**C17.1**
PE substrate reduction (%)	4.5 ± 0.058^{d}	3.6 ± 0.100^{c}	6.4 ± 0.100^{b}	6.7 ± 0.058^{a}

Scanning electron microscopy

The surface of PE pellets in the culture flasks was photographed by SEM and compared with the control PE pellets to evaluate the degradation efficiency. It can be seen that the surface of the pellets after being cultured with bacteria appeared to have adhesions, forming small, concave and convex holes (Figure 2). Of which, C1.2, C3.4 and C17.1 were the 3 strains that created the most obvious cuts on the PE pellets surface.

The study of Kang B.R. et al. (2019) also evaluated the ability of *Pseudomonas* sp. to decompose PE plastic by SEM imaging of the surface of PE plastic pellets after 90 days of culture. The authors also suggested that the ability of microorganisms to decompose PE plastic is due to the activity of enzymes, including *laccase* (Kang B.R. et al., 2019).

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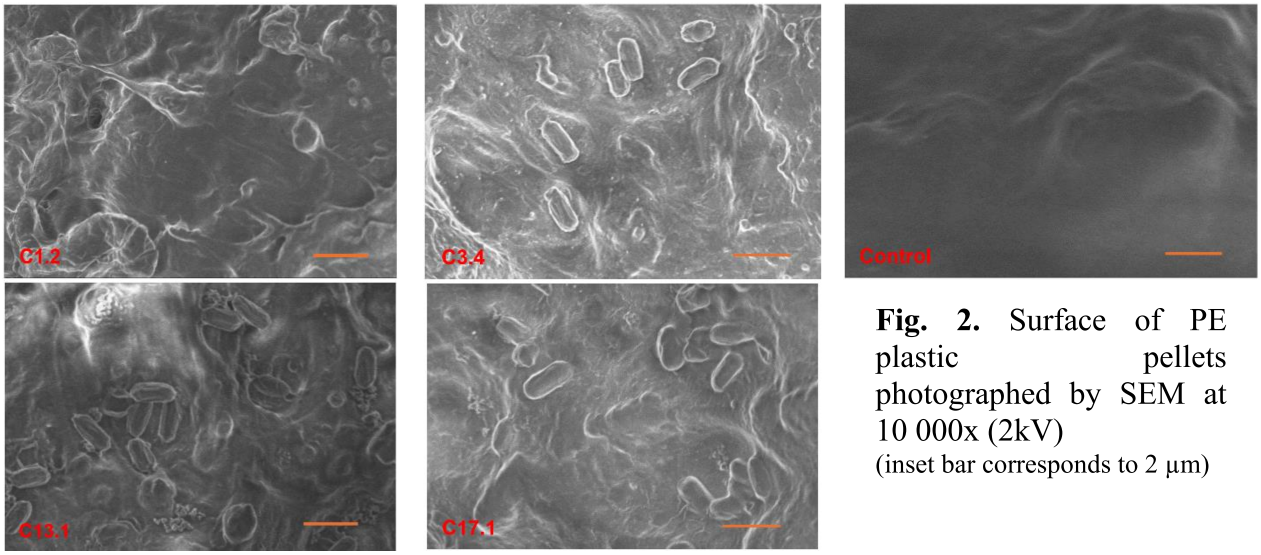


Fig. 2. Surface of PE plastic pellets photographed by SEM at 10 000x (2kV)
(inset bar corresponds to 2 µm)

Determining laccase enzyme activity

Along with evaluating the PE degradation ability of bacterial strains through SEM imaging of plastic pellet surfaces, bacterial strains were also evaluated for *laccase* enzyme activity to predict their PE degradation efficiency.

In 1993, bacterial *laccase* was first isolated from Azospirillum sp. distributed in the rhizosphere of rice plants. Subsequently, *laccase* was discovered in many different bacterial species, such as *Geobacterial, Staphylococcus, Lysinibacillus, Aquisalibacillus, Pseudomonas, Delfia, Enterobacter, Proteobacterium* and *Alteromonas*. Among them, the most characterized CotA *laccase*s are from *Bacillus*, such as *B. subtilis, B. pumilus, B. licheniformis, B. halodurans, Bacillus* sp. HR03… Bacterial *laccases* are not only common in Actinobacter but also found in α-, β- and γ-Proteobacteria (Yadav D. and Kudanga T., 2023).

In this study, the *laccase* enzyme activity of the bacterial strains was evaluated (Table 3). The statistically significant differences (a, b, c, d) in Table 3 indicate that the average *laccase* enzyme activities of the strains were different. Accordingly, the activity of strain C17.1 was the highest among the four bacterial strains (1,206.0±3.61 U/mL). This result was higher than the study of Muthukumarasamy P. N. et al (2015). The authors studied the production of *laccase* enzyme from agricultural waste using *Bacillus subtilis* MTCC 2414, the highest enzyme activity was 352.2 ± 4.32 U/mL (Muthukumarasamy P. N. et al, 2015).

Tab. 3. *Laccase* enzyme activity of strains

Strains	**C1.2**	**C3.4**	**C13.1**	**C17.1**
***Laccase* enzyme activity (U/mL)**	974.0 ± 3.61^{d}	649.3 ± 3.06^{c}	791.7 ± 1.53^{b}	$1{,}206.0 \pm 3.61^{a}$

Biological characteristics

Morphology of colonies and cells

Strains	**Morphology**		**Descriptions**
	Colonies	**Cells**	

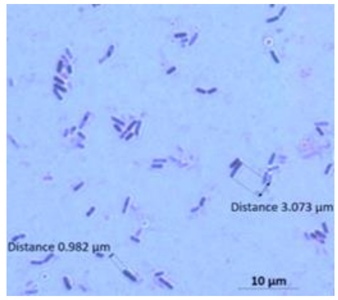
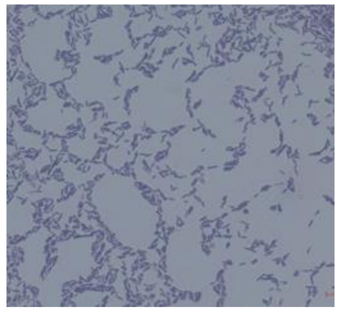
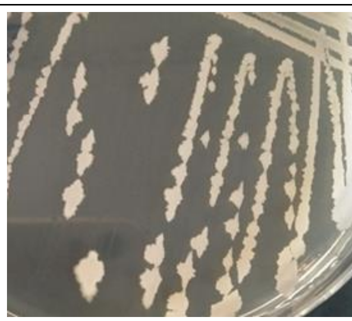
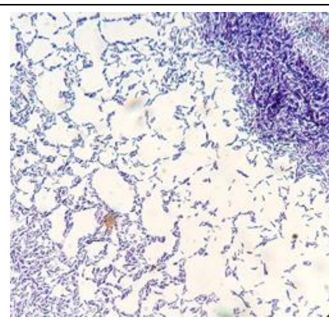
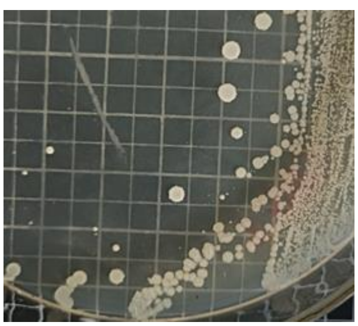
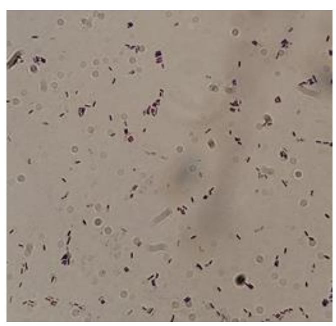
C1.2			Colonies: ivory white, smooth and slightly convex surface, smooth edges, not shiny. Cells: rod-shaped.
C3.4			Colonies: white, wrinkled surface, slightly porous, smooth edges. Cells: rod-shaped.
C13.1			Colonies: white, serrated edges, spreading growth. Cells: rod-shaped.
C17.1			Colonies: white, wrinkled edges, smooth, porous surface. Cells: rod-shaped

Fig. 3. Colony and cell morphology of bacterial strains and their descriptions.

Colony and cell morphology of bacterial strains were recorded after 3 days of culture on NA medium (Figure 3). Cells were photographed by microscope at 1000x magnification. All four bacterial strains had typical rod-shaped cells of Gram-positive bacteria while colony morphology varied. These morphological characteristics could be the basis for the identification of bacterial strains in the next research steps.

Physiological and biochemical characteristics

Four factors were considered to study the physiological and biochemical characteristics including: temperature, initial pH, salinity and PE substrate concentration. The evaluation results were based on the cell density determined at each investigated factor (Figure 4).

The bacterial strains had a wide range of growth parameters for all four factors examined. All bacterial strains grew optimally at 37°C and pH 7. This was also a typical characteristic of species belonging to the

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bacterial group (Michael T. M. et al, 2008). However, they differed in their tolerance to salinity and PE substrate concentration. The optimal salinity for growth of strains C1.2, C3.4, C13.1, C17.1 was 20‰, 15‰ and 26‰, respectively. Both strains C13.1 and C17.1 grew optimally at 26‰ salinity, however, strain C17.1 had a higher cell density, indicating that it had better growth ability than strain C13.1. Although there were differences in the optimal salinity tolerance of bacterial strains, the salinity range from 15‰ to 26‰ was also the typical salinity range of marine microorganisms (Castillo H. and Villafania M., 2024), specifically the bacterial strains isolated from marine sediments in this study. The PE substrate concentration at which the strains could grow optimally was the most interesting value because this could be the basis for the application of these bacterial strains in the treatment of saline plastic waste pollution in island areas. The results showed that the optimal PE substrate concentration for growth was 0.3%, 0.5% and 0.7% for strains C13.1, C1.2, C3.4 and C17.1, respectively. Among them, strain C17.1 coud grow well at the highest PE substrate concentration (0.7%) and this strain also had the highest cell density (about 1.3x10^8 CFU/ml), proving that this strain had the best PE degradation ability among the four isolated bacterial strains. In some similar studies, the PE substrate concentration at which microorganisms grow optimally is about 0.5% (Auta H.S. et al, 2017), therefore, the PE substrate concentration level of 0.7% could be considered a noteworthy high level. The research results on the optimal PE substrate concentration of the bacterial strains were also completely consistent with the research results on the substrate weight loss rate and *laccase* enzyme activity in this study.

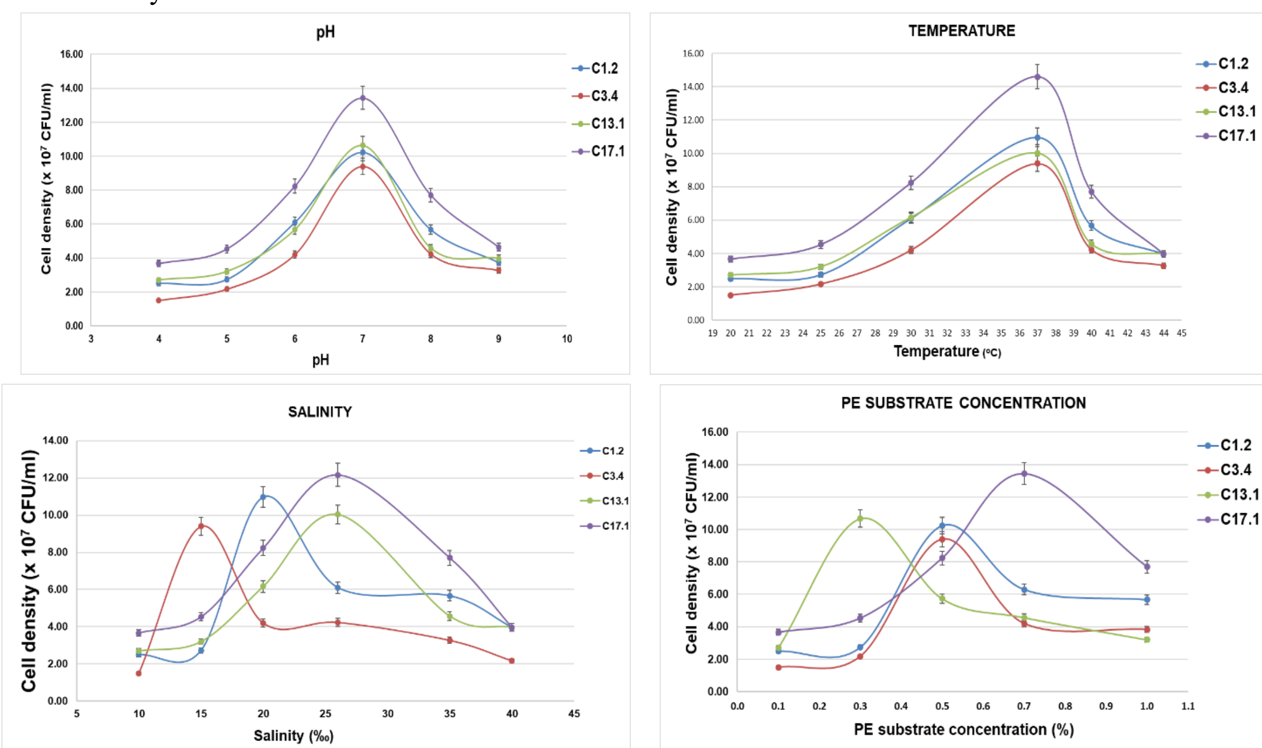

Fig. 4. Survey results of some physiological and biochemical characteristics of bacterial strains

16S rRNA gene sequencing

16S rRNA is a ribosomal subunit found in prokaryotes. It is approximately 1500 nucleotides in length and contains nine variable regions interspersed with conserved regions. 16S rRNA sequencing is a molecular biological method that helps identify bacteria to the genus or species level. Through this, the diversity of bacteria present in the environment can be compared. This method is especially meaningful for complex microbial communities or microbial communities distributed in environmental conditions that are difficult to access by conventional culture methods. (Weisburg W. G. et al., 1991). For bacteria, identification through colony and cell morphology or biological characteristics is unclear, 16S rRNA gene sequencing is a reliable tool for bacterial identification.

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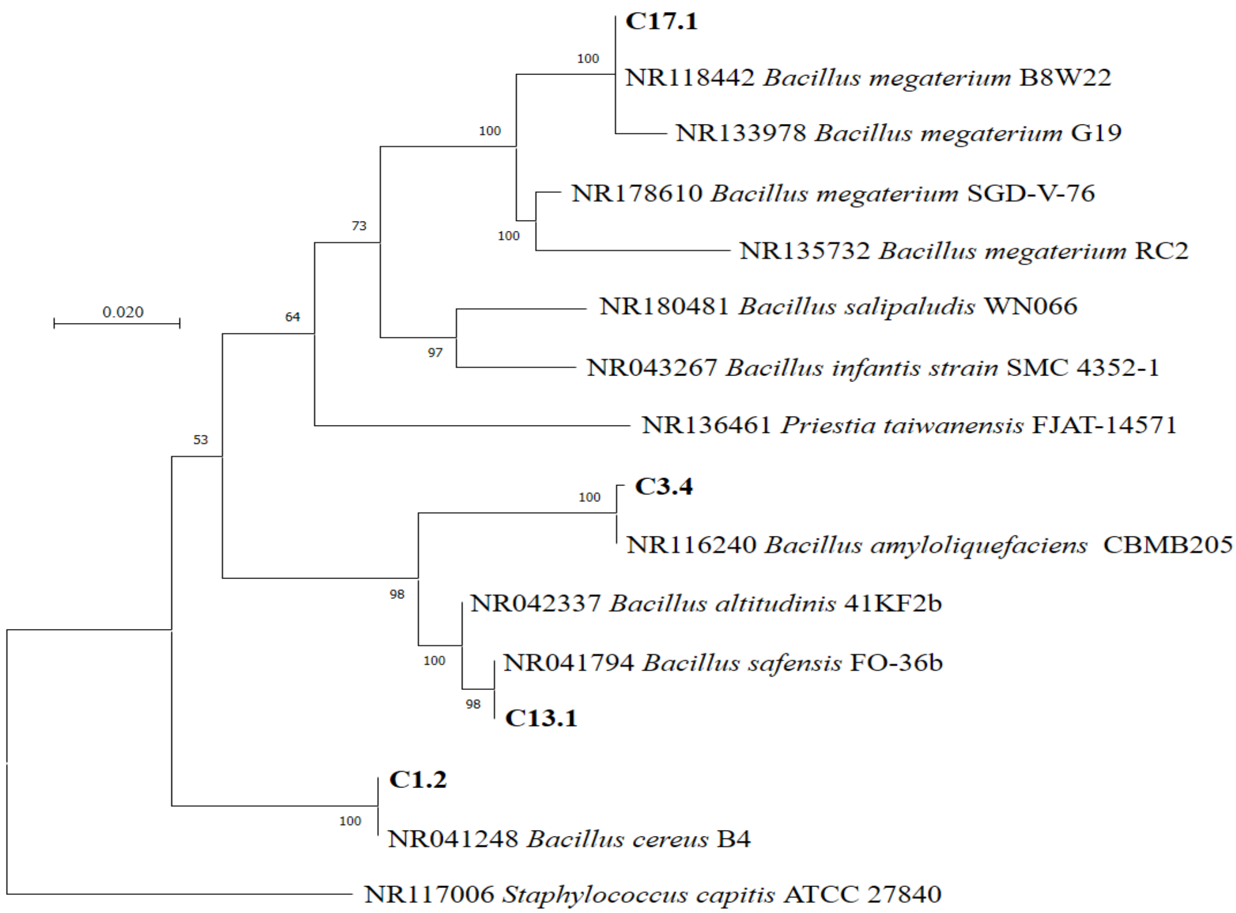

Fig. 5. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences of bacterial strains and related species in GenBank

(Unit = 0.020 Knuc in nucleotide sequence. Numbers shown at branch positions are bootstrap results for 1000 comparisons)

In this study, four strains were sequenced for 16S rRNA gene using primer pair 27F and 1492R. The sequences were then compared with data on GenBank to identify related species (Figure 5). Accordingly, the four bacterial strains were closely related to species of the genus *Bacillus*. In particular, strain C1.2 is closely related to *Bacillus cereus*, which is widely used in the treatment of PE plastic (Auta H.S. et al, 2017). Strain C17.1 was closely related to *Bacillus megaterium*, which is a marine bacteria species (Dhangdhariya J. H. et al, 2015). Strain C3.4 was closely related to *Bacillus amyloliquefaciens*, which had been published by Das M. P. and Kumar S. for its ability to degrade PE plastic (Das M. P. and Kumar S., 2014). Strain C13.1 was closely related to species *Bacillus safensis*. In addition, Cucini C. et al. (2020) published a number of bacterial species of the genus *Bacillus* that are capable of degrading many groups of plastics, including PE plastic (Cucini C. et al, 2020). However, to date, there has been no publication on the ability of bacteria *B. megaterium* and *B. safensis* to degrade PE plastic. Therefore, this study can be considered the first study on the ability of these bacteria to degrade PE plastic.

4. Conclusion

The study identified four species of bacteria belonging to the genus *Bacillus* isolated from marine sediments collected in Nha Trang (Khanh Hoa, Vietnam) that were capable of degrading PE plastic. Based on the 16S rRNA gene sequence, four strains C1.2, C3.4, C13.1, C17.1 were identified as closely related to the species: *Bacillus cereus, Bacillus amyloliquefaciens, Bacillus safensis, Bacillus megaterium*. Of which, strain C17.1 was the strain with the highest ability to reduce PE substrate weight and *laccase* enzyme activity. All four strains grew optimally at 37ºC and pH 7. In which, C17.1 was the strain with the best growth ability. Strains had the ability to biodegrade PE substrate at different optimal concentrations. Strain C17.1 grew optimally at PE concentration of 0.7%. The optimum salinity tolerance of the strains ranged from 15 ‰ to 26 ‰.

The results of the study are only the initial steps in evaluating the biodegradation ability of some bacteria belonging to the genus *Bacillus*. Based on this study, it is necessary to conduct more detailed and specific studies to accurately evaluate the ability and efficiency of plastic degradation of the isolated bacterial strains. Accordingly, it is possible to discuss their exploitation and use to decompose plastic waste, contributing to reducing environmental pollution caused by plastic waste in the future.

Acknowledgements

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