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Research Article

Enzyme Activity and Biodegradation Potentials of *Serratia marcescens* Cell-Free Extract on Polyethylene Terephthalate Sheet

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Abstract

Microbial strains with potential hydrolytic enzymes are excellent hosts for the biodegradation of polyethylene terephthalate (PET). However, knowledge of the best working conditions for enzymatic actions is essential to suggest the most suitable condition for the bio-transformation process. In this research, PETase and lipase produced by *Serratia marcescens* under PET exposure were screened and their physicochemical properties were evaluated. Furthermore, the biodegradation efficiency of the cell-free extract produced from *S. marcescens* on a PET sheet was studied via scanning electron microscope (SEM) and Fourier transform infrared spectrophotometer (FTIR). The result showed maximum production of PETase activity (8.80 U mL^{-1}) and lipase activity (7.48 U mL^{-1}) by *S. marcescens* on the sixteenth and tenth day of cultivation. The physicochemical study showed that they were active and stable over a wide range of pH (5.0–11.0) and temperature (30 °C–80 °C), with optimum PETase activity at pH 7.0 and 50 °C. At the same time, lipase had optimum activity at pH 9.0 and 60 °C. PETase activity was inhibited by Ca^{2+} and Cu^{2+} and significantly enhanced when exposed to Mg^{2+} and Ba^{2+} while lipase activity was significantly enhanced with the addition of Ca^{2+} and Mg^{2+}. However, EDTA chelated the activities of both enzymes. The SEM profile of the PET sheet surface after enzymatic degradation revealed the presence of cracks while FTIR spectra of the PET sheet showed the creation, disappearance, and shiftings of peaks at different wavelengths when compared with control. In conclusion, this study shows the capacity of *S. marcescens* to produce PETase and lipase and reveals the cell-free extract to hold great prospects that can be utilized for industrial and domestic remediation of PET wastes to achieve a cleaner environment.

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Introduction

The indiscriminate usage of plastics and their several derivatives coupled with improper disposal management has resulted in the widespread of plastic wastes in earthy and aqua surroundings. Plastics are ranked as the most enormously used commodity of the 21st century [1]. Betwixt the numerous examples of plastics, polyethylene terephthalate (PET) is the most prevalent due to its wide usage in packaging industries as elucidated by its chemical composition. Polyethylene terephthalate is synthesized through the condensation reaction of ethylene glycol (EG) and terephthalic acid (TPA) [2]. The PET is used to produce plastic plates, bottles, jars, and films [3]. The demand for PET polymer in packaging industries is about 18 million tons out of about 279 million tons produced annually [4] however, only 28 % of PET produced is recycled into films, fiber, sheets, and bottles [4] while others are either incinerated or abandoned in landfill grounds in the environment. These methods are not cost-effective and environmentally friendly as PET discarded

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in the environment threatens various terrestrial and aquatic forms of life. Amidst these remediation methods, microbial and enzymatic remediation of PET has been identified as the most efficient and eco-friendly strategies for PET waste management [5].

Microbial and enzymatic remediation techniques not only sustainably manage PET wastes but also produce environmentally friendly end products [2]. PET hydrolases which are generally referred to as PETase are mostly serine hydrolases and they have been disclosed to play a pivotal role in the enzymatic remediation of PET wastes [6]. These serine hydrolases comprising cutinases (EC 3.1.1.74), lipases (EC 3.1.1.3), and carboxylesterases (EC 3.1.1.1) possess an / -hydrolase fold and catalytic triad composed of serine, histidine, and aspartate residue [6]. They also have numerous disulfide bonds due to the presence of cysteine residues in their active sites which enhances their thermal stability and binding affinity to PET. Previous studies have identified these enzymes to be produced only by selected microorganisms involved in hydrolyzing PET into monomeric forms such as TPA and EG [7], unfortunately out of the selected microorganisms only a few bacteria have been reported to have PET hydrolase activities most of which are gram-positive [6].

Serratia marcescens is a gram-negative bacterium appertained to the Enterobacteriaceae family [8]; It is found in water, soil, insects, and plant surfaces and has been reported to produce enzymes such as lipase, laccase, amylase, manganese, cellulase, amylase, chitinase, and protease [9]. There have also been reports that the bacterium degraded the recalcitrant polymer, polyethylene [10–11]. Cognizance of the prime operating conditions for *S. marcescens* and enzyme activities (such as PETase and serine hydrolases) produced by *S. marcescens* are essential for the development of maximum PET waste management and biodegradation system unfortunately, the physicochemical properties of the bacterium and PET hydrolases produced by *S. marcescens* under PET exposure are limited. Furthermore, there has not been any report on the biodegradation potentials of *S. marcescens* and its cell-free extract on PET.

Hence, in this study, S. marcescens was screened for the production of PETase and lipase under PET exposure and the physicochemical properties of the enzymes were determined. The biodegradation efficiency of the resulting cell-free extract produced from *S. marcescens* on a PET sheet was also elucidated and assessed through analytical techniques such as scanning electron microscopy (SEM) and Fourier transform infrared spectrophotometer (FTIR).

Materials and methods

1) Preparation of PET sheet

The PET bottles of the same brand were purchased from an indigenous market in Akure, Ondo State, Nigeria. The PET bottles were verified by checking the manufacturers label placed on them and were confirmed as PET by the Department of Chemistry, Elizade University, Ondo State, Nigeria. The PET bottles were manually minced after which they were milled and manually sieved through a 0.6 mm sieve to obtain uniform fine particles [12]. The PET bottles and PET powder were decontaminated with distilled water and 90% ethanol for 40 min [12].

2) Screening and preliminary identification of bacteria

Soil samples were obtained from a selected dump precinct in Akure, Ondo State, Nigeria where plastic wastes were dominant. One gram of unsullied grime was shaved from the top of various plastic polymers located at disparate soil depths (between 5–10 cm) and conveyed to the laboratory. Isolation and screening were carried out by culture technique [13] and a clear zone was determined through the method of Usha et al. [14]. About 1 g of the grime sample was dissolved in an Erlenmeyer flask containing 50 mL sterile enrichment broth and was placed in a water bath shaker at 37 °C, pH 6.0 and 200 rpm for 4 weeks. The enrichment broth media contained; KCl (0.2% w/v) $MgSO_4$ (0.04% w/v), $(NH_4)_2SO_4$ (0.2% w/v), $NaNO_3$ (0.2% w/v), yeast extract (0.02% w/v) and 2 g of PET powder dissolved in 1,000 mL sterile H_2O [13]. The enrichment broth media was autoclaved at 121 °C for 20 min. After 4 weeks of incubation 10-1 dilution was done. Then, 1 mL of the soil was added into a test tube containing 9 mL of enrichment broth media to obtain 10-1 dilution. Afterward, 0.1 mL of the diluted soil was taken and added to the enrichment agar using the pour plate method. Two replicas were maintained and incubated at 37 °C and pH 6.0 for 2–7 d. The enrichment agar contained; $NaNO_3$ (0.2% w/v), KCl (0.2% w/v) $MgSO_4$ (0.04% w/v), $(NH_4)_2SO_4$ (0.2% w/v), yeast extract (0.02% w/v), 2 g PET powder, and 12 g agar dissolved in 1000 mL sterile H_2O. The enrichment agar was autoclaved at 121 °C for 20 min. The bacterium with the largest clear zone was selected for cell morphology and biochemistry characterization and further studies. The bacterium was initially identified using Gram staining and Vitek 2 Identification System according to the manufacturer's instructions (VITEK 2 Compact, Biomerieux, France).

3) Molecular identification of the bacterium

Total genomic DNA was isolated from the selected bacterium culture and grown for 24 h using the Bacterial Genomic DNA Purification Kit (GeneMark) following the manufacturer's instructions. The 16S rRNA gene was amplified using universal primers (forward primer (27F 5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer (1492R 5′-GGTTACCTTGTTAC GACTT-3′) [15]. Polymerase chain reaction (PCR) was performed on a Prime thermal cycler in, the UK, using Taq DNA polymerase. Thirty (30) cycles of amplification were carried out with the template DNA. The PCR reaction was performed in the following sequence: denaturation at 94 °C for 4 min, primer annealing at 56 °C for 1min, primer extension at 72 °C for 1 min, and final extension at 72 °C for 8 min. Finally, the PCR product was pictured via electrophoresis on a 1% agarose gel. About 1500 bp amplicon and a 1.5 kbp DNA ladder from New English Biolabs (NEB) were used and, sequencing was done using the Sanger sequencing technology (Applied Biosystems, India). The 16S rRNA gene sequence of the bacterium was evaluated using the BLAST program on the GenBank database of the National Center for Biotechnology Information (NCBI) and the best-matched organism with a valid name was retrieved.

4) Production of PET hydrolases

Seed inoculum was primed by growing a loopful of slant culture of *S. marcescens* in 200 mL aseptic nutrient broth containing peptone (2.0 % w/v), NaCl (2.0 % w/v), beef extract (0.60 % w/v) and yeast extract (0.60 % w/v) at pH 6.0, 37°C and 180 rpm for 24 h in a shaking incubator (Stuart, UK). Four hundred (400 mL) PET-based medium (PBM) was prepared by introducing 1% PET powder as the sole carbon source into mineral salt medium containing NH_4NO_3 (0.04 % w/v), KH_2PO_4 (0.04 % w/v), $KH_2PO_4{\cdot}12H_2O$ (0.04 % w/v), NaCl (0.16 % w/v), KCl (0.16 % w/v), $CaCl_2{\cdot}2H_2O$ (0.02 % w/v), $MgSO_4$ (0.04 % w/v) and $FeSO_4{\cdot}7H_2O$ (0.0004 % w/v). Thereafter, five percent (5 % v/v) inoculum was obtained from the seed culture and transferred to the PBM. The experiment set was replicated twice and incubated at 37 °C, pH 7.0, and 180 rpm, for 30 days to study the growth and enzyme production by *S. marcescens*. A fresh culture of *S. marcescens* was inoculated into freshly prepared PBM for the production of PETase and lipase. The culture was incubated at 37 °C, pH 7.0, and 180 rpm. At the end of the cultivation, the broths were centrifuged at 10,000 rpm for 20 min at 4 C. Clear supernatants were retrieved and PETase and lipase activities were determined. Thereafter, the supernatants were used for PETase and lipase characterization studies.

5) PETase assay

PETase activity was spectrophotometrically determined through the hydrolysis of p-nitrophenyl acetate (pNPA into acetate and p-nitrophenol [16]. The enzyme assay was performed at room temperature in a buffer containing 0.1 M Tris-HCl pH (7.5) and 90 mM NaCl, with less than 4% v/v dimethyl sulfoxide from 0.5 mM pNPA stock solution. The crude supernatant (25 µL) was incubated with 190 µL substrate mixture for 15 min at room temperature before initiation of the enzymatic reaction. p-nitrophenolate production was assessed at 405 nm.

6) Lipase assay

Lipase activity was evaluated using the substrate mixture containing 0.5 mM p-NP palmitate (C16) in methanol, 50 mM Tris-HCl buffer (pH 8), and 0.1% Triton X-100 [17]. The reaction mixture contained 200 µL of substrate mixture and 20 µL of the crude supernatants incubated at 37 °C for 1 h. Enzyme activity was investigated by determining the release of p-nitrophenol palmitate at 425 nm.

7) Protein content determination

Protein concentration was evaluated with bovine serum albumin (BSA) being the standard [18]. The assay was made up of a diluted dye reagent pipetted into the sample solution. The solution was later incubated for 15 min at room temperature for color development and the absorbance values were recorded at 595 nm against the blank solution. PETase and lipase-specific activities were expressed as U mL^{-1} protein.

8) Characterization of PET hydrolases from *S. marcescens*

8.1) Effect of pH on rnzyme PET hydrolases activity and stability

The effect of pH on PETase and lipase activities was assessed over a range of pH 3.0–11.0 (glycine-HCl (pH 3.0), sodium acetate (pH 5.0), Tris-HCl (pH 7.0) and glycine-NaOH (pH 9.0 and 11.0) using the earlier described standard enzyme activity assay. In other to determine pH stability, PET hydrolases were incubated for 24 h with different buffer solutions and their residual activities were assessed using the described standard enzyme activity assay.

8.2) Effect of temperature on PET hydrolase activity and stability

PETase and lipase activities were evaluated by incubating the assay mixture at 30 °C to 80 °C for 35 min after which enzyme activities were recorded. Enzyme stability at the selected temperature was assessed at 30-minute intervals for 3 h of incubation and residual

activity was determined using the described standard enzyme activity assay.

8.3) Effect of metal ions and EDTA on PET hydrolase activity

Metallic chlorides of selected concentrations (1, 5, and 10 mM) were used to investigate the effects of some cations (Ca^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Hg^{2+}) on PETase and lipase activities. Ethylene diamine tetraacetic acid (EDTA) was also prepared in varying concentrations (5 and 10 mM) to determine the effect of this metal-chelating agent on the crude enzymes. Each metallic chloride was added to the reaction mixture and incubated for 30 minat the obtained optimum temperature and pH of the enzyme, thereafter enzyme activities were determined.

9) Biodegradation studies of PET using cell-free extract

Pre-weighed 0.5 g PET sheets (3 cm × 2 cm) sterilized by soaking in 90% (v/v) ethanol and rinsed with sterile distilled water were used for this study. Fresh *S. marcescens* culture was prepared, and 5 % inoculum of *S. marcescens* was introduced into PBM incubated at 37 °C and 180 rpm for two weeks. At the end of the cultivation period, the broth was centrifuged at 10,000 rpm for 20 min at 4 °C and clear supernatant was retrieved. PETase and lipase activities were assayed and confirmed in the clear supernatant using the earlier described methods and were later used as the cell-free extract of *S. marcescens*. The pre-weighed PET sheet was then introduced into a conical flask containing 100 mL sterile nutrient medium and 5 % *S. marcescens* cell-free extract was added. The control contained pre-weighed PET sheets in a conical flask containing only 100 mL of sterile nutrient medium. The flasks were firmly corked with aseptic cotton wool covered with aluminum foil and incubated at pH 7.0 and 50 °C in a shaking incubator at 180 rpm for 90 d. All experimental setups were in triplicate.

9.1) Assessment of biodegradation Potentials of *S. marcescens* cell-free extract

The macroscopic examination was carried out on the control and biodegraded PET sheet using a Zeiss Evo MA variable pressure SEM (Carl Zeiss STM AG, Germany) [19]. Furthermore, an investigation with FTIR spectrophotometer was used to investigate bond variations in the structure of the biodegraded PET sheet compared to bonds in the structure of the un-degraded PET sheet (control). At the end of the experiment, PET sheets were retrieved and washed with several milliliters of aseptic distilled water. The PET sheets were air-dried overnight until a constant weight was obtained and they were subsequently analyzed using a Shimadzu FTIR spectrophotometer [19].

10) Statistical Analysis

Results are recorded as mean±standard deviation. The data were analyzed using a one-way analysis of variance. The mean values were correlated with the Duncan test and a statistical package for social sciences (SPSS) by IBM version 16 was used.

Results and discussion

1) Microbial growth on PET

Twelve bacteria (OZLS 1 – OZLS 12) were isolated from the soil samples (Table 1). Bacteria have been reported to be isolated from different sources and remarkably, those isolated from soil samples obtained from plastic dump sites have been reported to be effective in plastic biodegradation processes. Furthermore, bacteria isolated from dumpsite soil samples have been revealed to be potent in PET biodegradation [12, 20–21]. Notably, only 5 (OZLS-3, OZLS-5, OZLS-8, OZLS-10, and OZLS -12) of the 12 bacteria demonstrated abilities to grow and degrade PET as shown in Table 1. The bacteria grew on PET because they have abilities to utilize PET solely as their source of carbon for growth. Also, this proves that not all bacteria isolated from a plastic dump site have the potential to bio-mineralize plastic, especially PET [22]. Interestingly, OZLS-3 (a red-pigmented bacterium) displayed the highest clear zone (1.3 cm) and level of capability to grow on PET as shown in Table 1. This could be attributed to OZLS-3's ability to develop unique genes to maximally adapt to the PET environment and to produce enzymes essential for the biomineralization of PET polymer [23]. The results of the morphology and biochemical characterization studies of OZLS 3 are presented in Table 2. The result showed that OZLS-3 is a gram-negative fermentative rod bacterium with optimum growth at 37 °C and pH 7.0. Although earlier studies have reported the bio-degradation of PET by bacteria, however, most of the reported strains were gram positive bacteria [6]. Furthermore, the growth of OZLS-3 was highest at 37 °C and pH 7.0 because the sampling site was a dung site where temperatures are generally ambient with a varying pH of 6.5–7.5 [10].

Table 1 Screening for growth abilities and zone of clearance of the isolated bacteria on PET

Bacterial isolates	Growth on PET	Zone of clearance on PET (cm)
OZLS-1	-	-
OZLS-2	-	-
OZLS-3	++	1.3
OZLS-4	-	-
OZLS-5	++	0.8
OZLS-6	-	-
OZLS-7	-	-
OZLS-8	++	0.9
OZLS-9	-	-
OZLS-10	++	0.7
OZLS-11	-	-
OZLS-12	++	0.6

Note: ++ signifies growth on PET while – signifies no growth on PET

Table 2 Morphology and biochemical characterization of OZLS-3

Fermentation test	Result
Fructose	+
Arabinose	
Urease	-
Lactose	+
Glucose	+
Galactose	+
Mannose	-
Maltose	+
Reactivity test	**Result**
Oxidase	-
Indole production	-
Catalase	-
Hydrogen sulfide	+
Casein reaction	-
Gram's reaction	-ve rod
Nitrogen reduction	-
Temperature optimum	37 °C
pH optimum	6.5

2) Molecular identification of the bacterium

The 16S rRNA sequence of OZLS-3 revealed 97.16 % similarity with *Serratia marcescens*. The unique nucleotide sequence obtained for OZLS 3 was deposited to NCBI Genbank and given the accession number OM 302181. A previous study by Brandon et al. [24] isolated *Serratia marcescens* that can bio-mineralize polystyrene (PS) from the gut of *Tenebrio molitor*. *Serratia spp.* isolated from the gut of *Galleria mellonella* L was also reported to degrade polyethylene (PE) [25] and, Awasthi et al. [26] also degraded PE with *Serratia marcescens* isolated from wastewater.

3) Screening and production of PET hydrolases by *Serratia marcescens*

PETase was optimally produced by *S. marcescens* on the sixteenth day of cultivation with a yield of 8.80 U mL^{-1} while lipase was maximally produced by *S. marcescens* on the tenth day (7.48 U mL^{-1}) (Figure 1). PETase and lipase production increased maximally during the log phase of *S. marcescens* growth. The maximum production of the enzymes in the microbial log phase depicts the ability of *S. marcescens* to feed on PET for growth. Previous studies by Pathak et al. [27] and Mohanan et al. [28] revealed extracellular enzymes produced by organisms in response to their growth phases are effective in bio-deterioration of the backbones of polymers with parse ester bonds, such as PET.

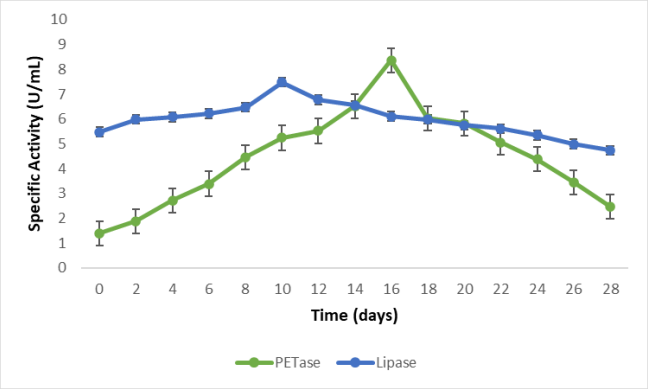

Figure 1 Production of PETase and lipase from *S. marcescens* during PET biodegradation. Error bars represent mean±standard deviation.

PETase (3.1.1.101) an enzyme detected from *I. sakaiensis* 201-F6 by Yoshida et al. [29] has been revealed to be principal in PET degradation [30–31]. Lipase has also been shown to be involved in the bioremediation of different pollutants and recalcitrant plastic polymers such as PET. In this study, *S. marcescens* produced PETase and lipase concurrently which are marker enzymes for PET degradation, showing the biodegradative potentials of *S. marcescens* and its ability to produce several enzymes including the PET hydrolases. Previous studies by Cahyani et al. [9] and Gong et al. [32] also revealed *S. marcescens* to have abilities to produce different extracellular enzymes with biodegradative efficiencies.

4) Physicochemical properties of hydrolytic rnzymes from *S. marcescens*

4.1) Effect of pH on PET hydrolase sctivity and dtability

The pH profile of PETase activity from *S. marcescens* showed that PETase was brisk in a wide range of pH (pH 3.0 to pH 11.0). However, optimum PETase activity was found at pH 7.0 which declined to 58.2 % at pH 11.0. While at pH 3.0; about 75.2 % of utmost PETase activity was noted, and increased to 83.9 %, at pH 5.0. (Figure 2). On enzyme stability, the produced PETase exhibited optimum stability at pH 7.0 with about 87% residual activity when incubated for 180 min at room temperature. 41.7 %, 63.6 %, 69.5 %, and 45 % residual activities were obtained at pH 3.0, pH 5.0, pH 9.0, and pH 11.0 respectively (Figure 3a). This suggests that the *S. marcescens* produced neutrophilic PETase.

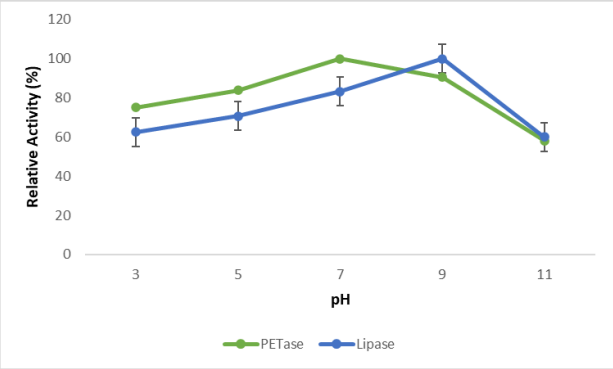

Figure 2 Effect of pH on the activity of PETase and Lipase produced by *S. marcescens* PET degradation. Error bars represent mean±standard deviation.

Earlier studies have also reported that constructive PET hydrolysis occurs under exposure to PETase with expansive activity in acidic and alkaline pH [3, 33]. At pH 9.0 lipase from *S. marcescens* showed optimum activity (100%) (Figure 2), and a further decrease in pH did not cause the deactivation of the enzyme activity (81%, 62.5 %, 70.8 %, and 60 % relative activities were documented at pH 7, pH 3, pH 5 and pH 11). Lipase was stable between pH 3.0–pH 9.0 and maximum residual activity was observed at pH 9.0 when incubated for 2 hours (Figure 3b). However, it retained more than 50% residual activity at pH 11.0. This shows that *S. marcescens* produced alkaline lipase. In previous studies, bacteria lipases have also been reported to be typically efficacious in an alkaline medium [12, 34].

4.2) Effect of Temperature on Activity and Stability of PET Hydrolase

The maximum temperature for PETase activity from *S. marcescens* was 50 °C Figure 4. PETase activity was accelerated with an increase in temperature from 30 °C to 50 °C while the PETase activity was abated above 50 °C. The enzyme exhibited 88.4 % of optimum activity at 60 °C and still retained 65 % of its optimum activity at 80 °C. A report by Gamerith et al. [35] on PETase isolated from *Pseudomonas mendocina* also showed PETase activity that was optimum at 50 °C. Likewise, Herrero et al. [36], revealed PETase with optimum activity at 50 °C. The increase in PETase activity with increasing temperature may be because elevated temperature results in excellent remediation of PET, as it generates pliable and smooth ingress to ester bonds present in the PET structure. Moreover, the enzyme reserved about 90 % of its activity at 50 °C while 78 % and 54 % residual activities were observed at 30 °C and 80 °C respectively. (Figure 5a).

The activity of lipase from *S. marcescens* showed a gradual increase in temperature from 30 °C to 60 °C where its activity was optimum (Figure 4) and reserved 52 % of its optimum activity at 80 °C. This shows that it is more active within this temperature range. Regarding thermal stability lipase from *S. marcescens* was most stable at 50 °C with 81 % residual activity however, it showed high stability over the temperature ranges 30 °C to 80 °C. 54 % residual activity was reserved at 80 °C after 3 hours of incubation. (Figure 5b). High temperatures have been revealed to influence the enzyme permeability of PET membranes during the biodegradation process [34].

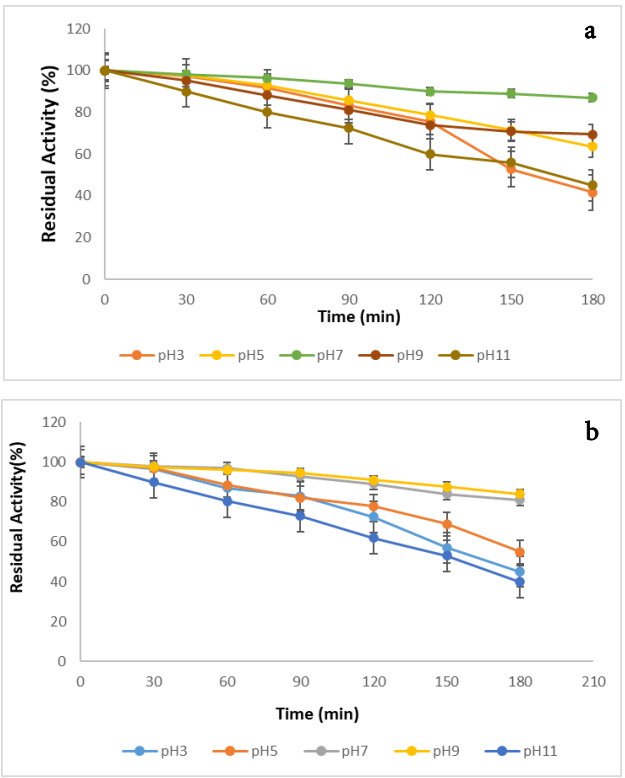

Figure 3 Effect of pH on the stability of (a) PETase and (b) lipase produced by *S. marcescens* during PET degradation. Error bars represent mean±standard deviation.

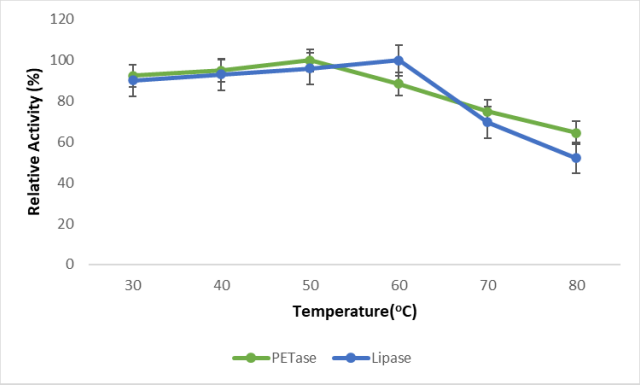

Figure 4 Effect of temperature on the activity of PETase and lipase produced by *S. marcescens* during PET degradation. Error bars represent mean±standard deviation.

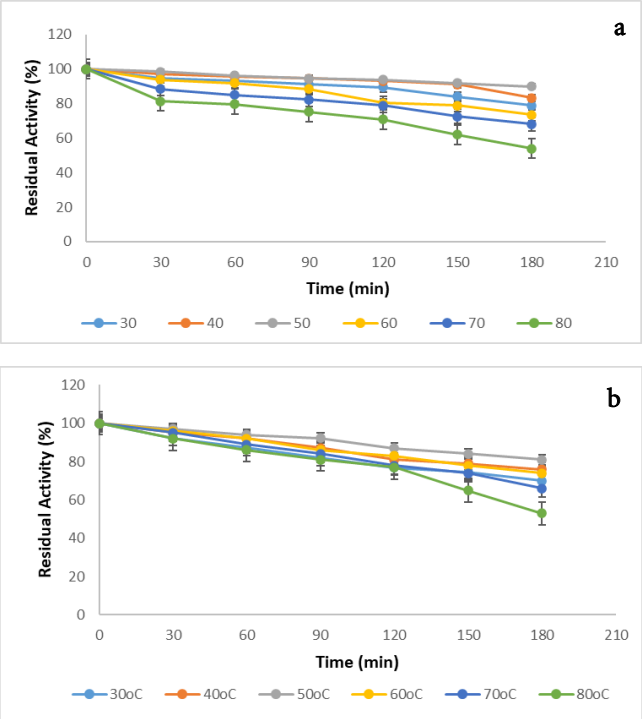

Figure 5 Effect of temperature on the stability of (a) PETase and (b) lipase produced by *S. marcescens* during PET degradation. Error bars represent mean± standard deviation.

Gururaj et al. [37] also reported lipase produced from *Pseudomonas aeruginosa* EF2 with optimum activity at 50 °C and 25% activity was observed at 70 °C. This result indicates the thermophilic properties of PETase and lipase produced from *S. marcescens* which makes the organism and enzymes suitable for adoption in PET bioremediation and for other industrial purposes.

4.3) Effect of metal ions and EDTA on PET hHydrolase activity

PETase activity increased significantly ($p<0.05$) in contiguity with 5 mM Ba^{2+} Mg^{2+} and Hg^{2+} and relative activities of 105.6±0.03 %, 104.1±0.02% and, 103±0.03% were obtained respectively. Eighty-nine percent (89± 0.01 %), 60.3±0.03 %, and 71.4±0.01 % reduced relative activities were observed when Ca^{2+}, Cu^{2+}, and Mn^{2+} were added to the reaction mixture. Likewise, a 45±0.01 % significant reduction in PETase activity was observed upon the addition of EDTA. Figure 6a. At the inclusion of 10 mM metal ions, Ba^{2+} Mg^{2+} and Hg^{2} significantly enhanced the PETase activity by 105.7± 0.02 %, 111.6± 0.03 %, and 107±0.03 %. The increase in enzyme activity when Ba^{2+} Mg^{2+} and Hg^{2} were added to the reaction mixture shows that these metal ions are cofactors of PETase with abilities to respond to specific residues of amino acid in the active site of PETase thereby causing conformational alterations and increase in PETase activity. Earlier studies by Then et al. [38] and Maurya et al. [3] have shown PETase activity that was enhanced in the presence of Ba^{2+} Mg^{2+} and Hg^{2+}. The reduction in PETase activity when EDTA was added, reveals EDTA as a potential inhibitor of PETase. EDTA bonded to the metal ions present in the active site of PETase limiting its affinity for substrates and this resulted in decreased activity.

Lipase from *S. marcescens* displayed 114±0.03 % and 116.8±0.04 % significant increase in activities ($p<0.05$) in the presence of 5 mM Ca^{2+} and Mg^{2+} (Figure 6b). Significant reduction in lipase activity was observed in contiguity with Ba^{2+}, Mn^{2+}, Cu^{2+}, and Hg^{2+} (88±0.03 %, 80±0.01 %, 77.2 ±0.03 % and 72±0.02 % relative activities were recorded respectively). Lipase activity also reduced significantly ($p<0.05$) in the presence of EDTA (19± 0.04 %). Ca^{2+} and Mg^{2+} have been reported to increase lipase activity and thermal stability due to the presence of their binding sites in lipase active sites [39]. At 10 mM inclusion of Ca^{2+} and Mg^{2}, there was a significant increase in the original activity of lipase. Ca^{2+} and Mg^{2+} enhanced the enzyme activity by 128±0.03 % and 125 ±0.03 %. There was a significant reduction in lipase activity when EDTA was added, showing EDTA as also an inhibitor of lipase. Studies by Mobarak et al. [40] and Gururaj et al. [37] reported that metal ions such as Ca^{2+} and Mg^{2+} adapt lipase solubility through ionized fatty acids deportment at its interface thus enhancing it catalytic potentials of lipase.

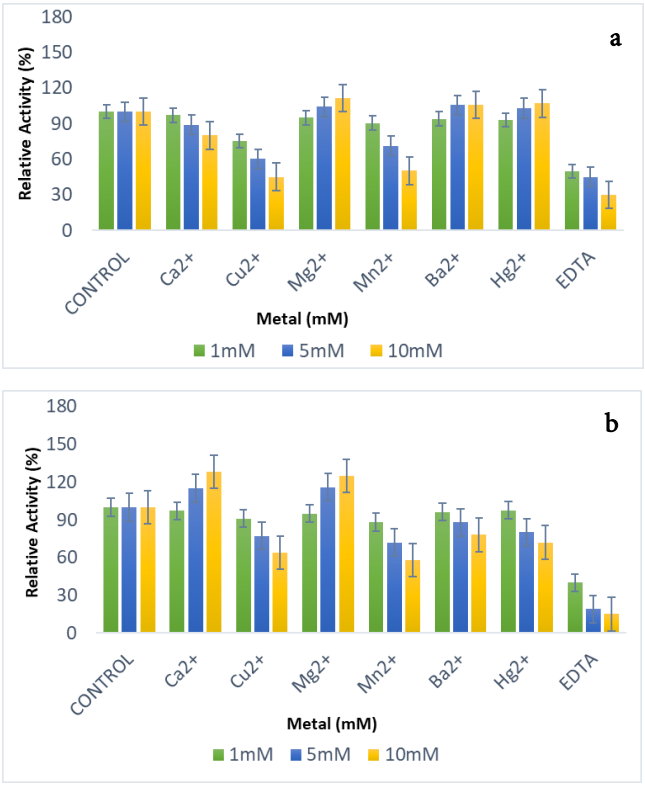

Figure 6 Effect of metal ions and EDTA on the activity of (a) PETase and (b) lipase produced by *S. marcescens* during PET degradation. Error bars represent Mean ± standard deviation.

5) Assessment of PET biodegradation potential of cell-free extract from *S. marcescens*

The SEM profile of the PET sheet surface after biodegradation showed breakage and erosion of the PET surface. It also revealed the presence of cracks, grooves, and scrapings on the PET sheet (Figures 7a and b). The micrograph confirms the degradation efficiency of cell-free extract produced from *S. marcescens* on a PET sheet. Studies by Azeko et al. [12] also showed the presence of scrapings and cracks on polyethylene sheet degradation using cell-free extract produced from *S. marcescens*. The result shows the bio-deterioration abilities of cell-free extract produced from *S. marcescens*.

The FTIR spectra of PET sheet exposed to cell-free extract produced from *S. marcescens* showed the creation of new peaks at wavelength range 1872–1959 corresponding to anhydride of carbonyl of the amide; 2258-2395 corresponding to cyanates asymmetric (-OCN) stretching vibration when compared with control (Figure 8). There was also a disappearance of secondary amine N-H stretching vibration at 1,579; C=O of ketone stretching vibration was formed at 1,722 wavelength and 3553 wavelengths corresponding to internally bonded O-H stretch also disappeared when compared with control. There was a shift to the right at wavelength 432 corresponding to C-H out-of-plane bending vibration of the substituted amide group, 1,342 corresponding to C-H bending vibrations of the methylene alkyl group to 426 and 1,340 when compared with control.

Peaks were observed at a wavelength range of 1,250–1,500 corresponding to compounds with aromatic rings, amines, and alcohol respectively. Analysis of the polyethylene terephthalate spectral figures indicates that the new peaks formed are intermediate products of the biodegradation of polyethylene terephthalate. A previous study by Gunawan et al. [41] revealed the appearance of an absorption band around 1,714 cm^{-1}, which could be assigned to the C=O stretching vibration of a ketone group that increased in intensity with an increased degradation period. Ren et al. [42] also displayed new peaks at the wavelength range of 1,250–1,500 corresponding to compounds with aromatic rings, amines, and alcohol respectively during PET biodegradation. Likewise, Pinto et al. [43] confirmed the appearance of an absorption band around 1,714 cm^{-1}, which could be assigned to the C=O stretching vibration of a ketone group that increased in intensity with an increase in the period of PET degradation. The new peaks seen in PET spectra indicate the formation of intermediate products of PET biodegradation. Additional bands were also seen, indicating the formation of more than one oxidation product.

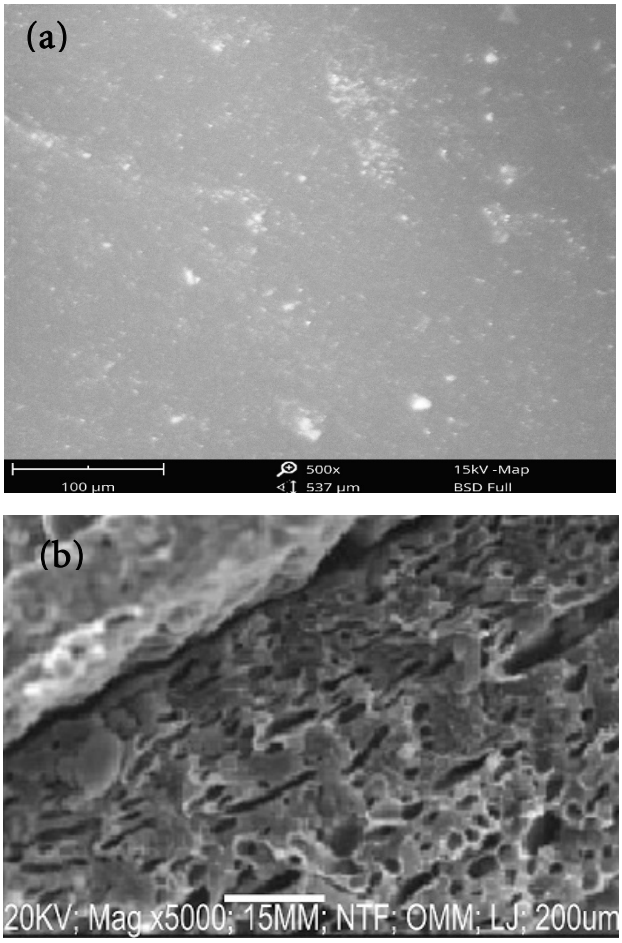

Figure 7 SEM micrograph of (a) control and (b) PET exposed to cell-free extract produced from *S. marcescens*.

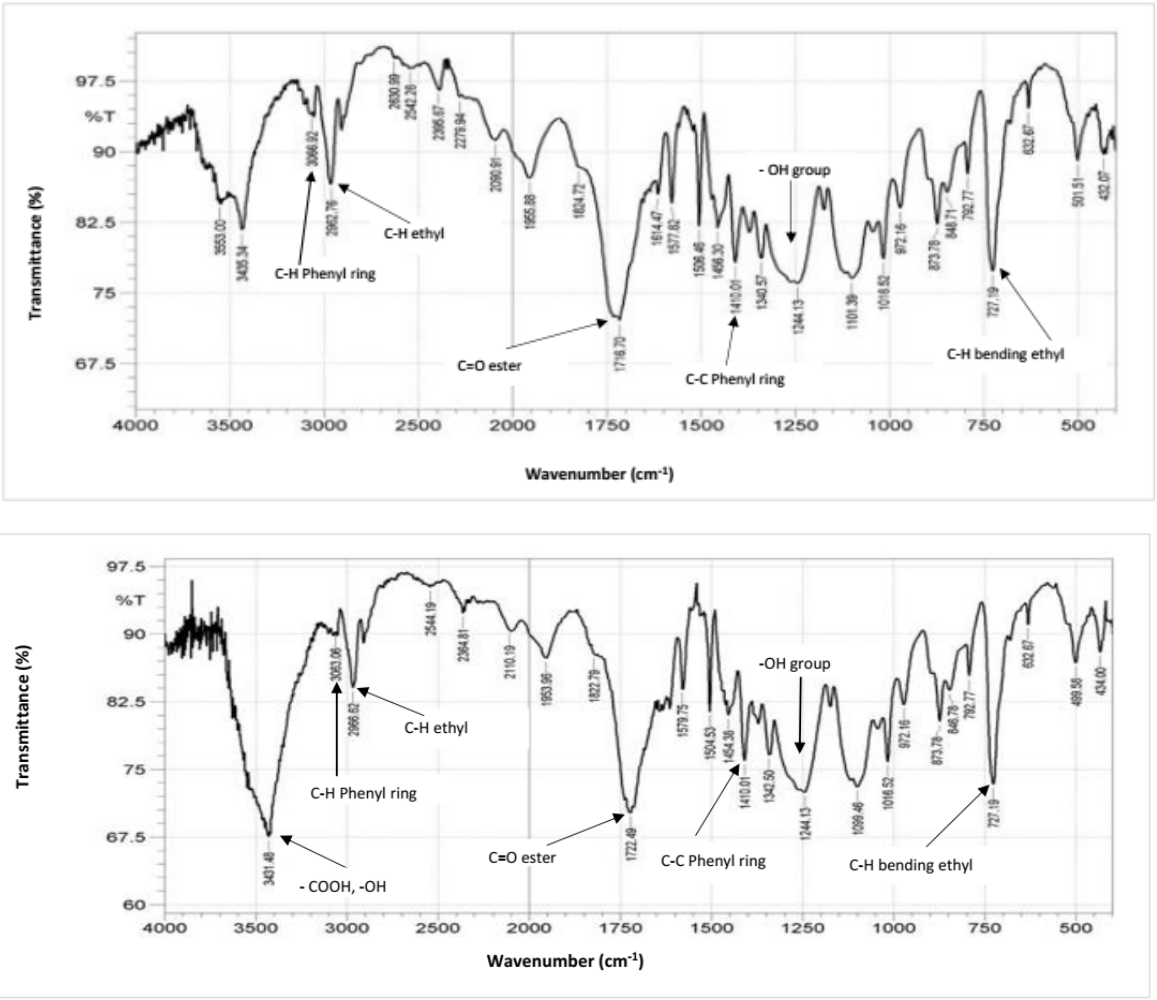

Figure 8 FTIR analysis of (a) untreated PET sheet (control) and (b) PET sheet exposed to cell-free extract produced from *S. marcescens*.

Conclusion

In this study, *S. marcescens* in PET-based media produced thermostable PETase and lipase with activity and stability over a wide range of temperatures (30 °C–80 °C) and pH (5.0–11.0). PETase activity was optimum at 50 °C and pH 7.0 while lipase had optimum activity at 60 °C and pH 9.0. The addition of Mg^{2+} and Ba^{2+} enhanced PETase activity while Ca^{2+} and Mg^{2+} increased lipase activity, however, EDTA chelated the activities of both enzymes. Furthermore, the cell-free extract produced from *S. marcescens* at 50 °C and pH 7.0 was used to biodegrade 0.5 g of PET sheet over 90 days. Cracks, pits, and alterations in PET functional groups were observed in the PET sheet. In conclusion, *S. marcescens* and *S. marcescens* cell-free extract produced thermostable extracellular enzymes with activity over a wide range of pH and temperature with potential applications in PET waste management and a variety of industrial processes. Sequel to this, further studies on the genetic engineering of *Serratia marcescens* to decipher the resident catabolic genes to improve the PET degradation efficiency are considered. This will invariably assist in developing cost-effective and efficient protocols for industrial and domestic biodegradation of PET waste.

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References

[1] Koshti, R., Mehta, L., Samarth, N. Biological recycling of polyethylene terephthalate: A mini-review. Journal of Polymer and Environment, 2018, 26, 3520–3529.

[2] Hiraga, K., Taniguchi, I., Yoshida, S., Kimura, Y., Oda, K. Biodegradation of waste PET: A sustainable solution for dealing with plastic pollution. Applied Microbiology and Biotechnology, 2019, 20, 49365.

[3] Maurya, A., Amrik, O., Bhattacharya, B., Sunil, K.K. Enzymatic remediation of polyethylene terephthalate (PET)–based polymers for effective management of plastic wastes: An Overview. Frontiers in

Bioengineering and Biotechnology, 2020, 34, 307–318.

[4] Taniguchi, I., Yoshida, S., Hiraga, K., Miyamoto, K., Kimura, Y., Oda, K. Biodegradation of PET: Current status and application aspects. Catalyst, 2019, 9, 4089–4105.

[5] Yan, Z., Jianbo, L., Huinong, C., Hui, N., Anfeng, X., Luhong, H. Characterization of a new and thermostable esterase from a meta-genomic library. Microbiological research, 2020, 16(9), 589–597.

[6] Danso, D., Jennifer, C., Wolfgang, R.S. Plastics: Microbial degradation, environmental and biotechnological Perspectives. Applied Environment and Microbiology, 2019, 32, 411–423.

[7] Billinger, S., Oeser, T., Birkemeyer, C., Zimmermann, W. Hydrolysis of cyclic poly (ethylene terephthalate) trimers by a carboxyl-esterase from *Thermobifida fusca* KW3. Applied Microbiology and Biotechnology, 2020, 87, 1753–1764.

[8] Kahrarian, Z., Saeed, K., Ahmad, T. A review of the production of biosurfactant and pigmented prodigyosin by bacteria *Serrassia marsseces*. Asian Journal of Research in Medicine and Medical Science, 2019, 1(1), 18–24.

[9] Cahyani, P., Wijanarka, A., Budi, R. Aktivitas Spesifik Selulase *Serratia Marcescens* Dengan Variasi Konsentrasi Amonium Sulfat ($(Nh_4)_2So_4$). Journal of Biology, 2017, 6(2), 41–49.

[10] Mohanan, N., Zahra, M., Parveen, K.S., David, B.L. Microbial and enzymatic degradation of synthetic plastics. Frontiers in Microbiology, 2020, 25, 234–245.

[11] Susilowali, S., Yamato, S., Kanaya, E., Kim, J.J., Koga, Y., Takano, K., Kanaya, S. Isolation of a novel cutinase homolog with polyethylene terephthalate-degrading activity from leaf-branch compost by using a metagenomic approach. Applied Environment and Microbiology, 2018, 78, 1556–1562.

[12] Azeko, S.T., Etuk-Udo, G.A., Odusanya, O.S., Malatesta, K., Anuku, N., Soboyejo, W.O. Biodegradation of linear low-density polyethylene by Serratia marcescens subsp. marcescens and its cell-free extracts. Waste and Biomass Valorization, 2015, 6, 1047–1057.

[13] Burd, D. Plastic not fantastic. 2008. [Online]. Available from: http//:wwsef.uwalerlou.ca [Accessed 15 December 2013].

[14] Usha, R., Sangeetha, T., Palaniswamy, M. Screening of polyethylene degrading microorganisms from garbage soil. Libyan Agriculture Research Center Journal Internasional, 2011, 2(4), 200–204.

[15] Olukunle, O.F. Molecular identification of crude oil-degrading bacteria and screening for catechol 2, 3 dioxygenase (C23O) gene. Biotechnology Journal International, 2019, 23(4), 1–14.

[16] Tobias-Fecker, F., Waku, T., Tanaka, N., Kawai, K. Active site flexibility as a hallmark for efficient PET degradation by I. sakaiensis PETase. Biophysical Journal, 2018, 114, 1302–1312.

[17] Ramnatha, D., Eiteljoerg, I., Trotscha, E., Fred, G. Identification of lipolytic enzymes isolated from bacteria indigenous to Eucalyptus wood species for application in the pulping industry. Biotechnology Reports, 2017, 15, 114–124.

[18] Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 1976, 72(1–2), 248–254.

[19] Ibrahim, B., Boke, H., Cizer, O. Assessment of material use about climate in historical buildings. Building and Environment, 2009, 42, 970–978.

[20] Yan, Z., Jacob, N.P, Bekir, E.E, Zheng, G. Biodegradation of polyethylene and polystyrene: From microbial deterioration to enzyme discovery. Biotechnology Advances Elsevier, 2022, 60, 107991.

[21] Zuriash, M.N., Nurelegne, T.S., Mesfin, T.G. Isolation and screening of low-density polyethylene (LDPE) bags degrading bacteria from Addis Ababa municipal solid waste disposal site. Annals of Microbiology, 2023, 73, 6.

[22] Kawinthip, W., Pitchaya, P., Narin, B., Nawatch, S., Suwanna, K.B. Potential bacteria isolated from contaminated sites as bio-degraders of various types of plastic, International Journal of Environmental Science and Development, 2023, 14(6), 348–354.

[23] Kaushal, K.B., Khatri, M., Arya, S.K. Recent insight into enzymatic degradation of plastics prevalent in the environment: A mini-review, Journal of Cleaner Engineering and Technology, 2021, 2, 100083.

[24] Brandon, S.H., Gao, R., Tian, D., Ning, S.S., Yang, J., Zhou, W.M., Wu, C.S. Criddle biodegradation of polyethylene and plastic mixtures in mealworms (larvae of Tenebrio molitor) and effects on the gut microbiome. Environmental Science and Technology, 2018, 52(11), 6526–6533.

[25] Lou, P., Ekaterina, S.S., Yang, B., Lu, B., Liu, N. Ren, P.F.X., …, Xing, O. Biodegradation of polyethylene and polystyrene by greater wax moth larvae (*Galleria mellonella* L.) and the effect of co-diet supplementation on the core gut microbiome. Environmental Science Technology, 2020, 54(5), 2821–2831.

[26] Awasthi, P., Srivastava, P., Singh, D., Tiwary, P.K. Mishra Biodegradation of thermally treated high-density polyethylene (HDPE) by Klebsiella pneumoniae CH001 3. Biotechnology, 2017, 7(5), 332.

[27] Pathak, V.M., Navneet. Review on the current status of polymer degradation: A microbial approach. Bioresources and Bioprocessing, 2017, 4, 15–26.

[28] Mohanan, N., Zahra, M., Parveen, K. Sharmaand, D.B.L. Microbial and enzymatic degradation of synthetic plastics. Frontiers in Microbiology, 2020, 32,123–135.

[29] Yoshida, S., Hiraga, K., Takehana, T., Taniguchi, I., Yamaji, H., Maeda, Y., …, Oda, K. A bacterium that degrades and assimilates poly (ethylene terephthalate). National Centre for Biotechnology Information, 2016, 351, 1196–1199.

[30] Fecker, T., Galaz-Davison, P., Engelberger, F., Narui, Y., Sotomayor, M., Parra, L. P. Active site flexibility as a hallmark for efficient PET degradation by I. sakaiensis PETase. Biophysics Journal, 2018, 114, 1302–1312.

[31] Kawai, F., Kawabata, T., Oda, M. Current knowledge on enzymatic PET degradation and its possible application to waste stream management and other fields. Applied Microbiology and Biotechnology, 2019, 103, 4253–4268.

[32] Gong, J., Kong, T., Li, Y., Li, Q., Li, Z., Zhang, J. Biodegradation of microplastic derived from poly (ethylene terephthalate) with bacterial whole-cell biocatalysts. Polymers, 2018, 10(12), 1326.

[33] Elif, D., Baran, E.G.,Tuba, S. AnsBacillus sp. Strains isolated from soil. Journal of Biology and Environmental Science, 2020, 14(42), 107–114.

[34] Leelatulasi, S., Raja, N.Z., Raja, A.R., Abu, B.S., Fairolniza, M., Shariff, P.C., …, Mohd Shukuri, M.A. Isolation, characterization, and lipase production of a cold-adapted bacterial strain *Pseudomonas* sp. LSK25 isolated from Signy Island, Antarctica. Molecules, 2019, 24, 715–727.

[35] Gamerith, C., Vastano, M., Ghorbanpour, S.M., Zitzenbacher, S., Ribitsch, D., Zumstein, M.T. Enzymatic degradation of aromatic and aliphatic polyesters by P. pastoris expressed cutinase 1 from *Thermobifida cellulosilytica*. Frontiers in Microbiology, 2017, 8,938.

[36] Herrero, B.T., Roberts, T.K., Lucas, S. An overview on biodegradation of polystyrene and modified polystyrene: The microbial approach. Critical Review in Biotechnology, 2018, 38, 308–320.

[37] Gururaj, P., Subramanian, R., Ganesan, N.D., Pennathur, G. Process optimization for production and purification of a thermostable, organic solvent tolerant lipase from Acinetobacter sp. AU07. Brazilian Journal of Microbiology, 2016, 47, 647–657.

[38] Then, J., Wei, R., Oeser, T., Barth, M., Belisario-Ferrari, M.R., Schmidt, J., Zimmermann, W. Ca^{2+} and Mg^{2+} binding site engineering increases the degradation of polyethylene terephthalate films by polyester hydrolases from *Thermobifida fusca*. Biotechnology Journal, 2015, 10, 592–598.

[39] Ghasemi, S., Rasoul, A.G., Kazemi, M.H., Zarrini, M.K.M. Isolation and characterization of some moderately halophilic bacteria with lipase activity. Microbiology, 2015, 80, 483–487.

[40] Mobarak, E.D., Soichiro, F., Toshiki, H., Young, C.C. Enzymatic degradation of polyethylene terephthalate by hydrolytic enzymes. Journal of Microbiology, 2015, 15, 11–21.

[41] Gunawan, N.R., Tessman, M., Schreiman, A.C., Simkovsky, R., Samoylov, A.A., Neelakantan, N. K. Rapid biodegradation of renewable polyurethane foams with identification of associated microorganisms and decomposition products. Bioresource Technology, 2020, 11, 100–112.

[42] Ren, L., Men, L., Zhang, Z., Guan, F., Tian, J., Wang, B., …, Zhang, W. Biodegradation of polyethylene by Enterobacter sp. D1 from the guts of wax moth *Galleria mellonella*. International Journal of Environmental Resource in Public Health, 2019, 16, 1941

[43] Pinto, A.V., Ferreira, P., Neves, R.P., Fernandes, P.A., Ramos, M.J. and Magalhaes, A.L. Reaction mechanism of MHETase, a PET gegrading enzyme. American Academy of Science Catalyst, 2021, 11, 10416–10428.