J. BIOL. ENVIRON. SCI.,
2020, 14(42), 107-114

Original Research Article

Analysis by Scanning Electron Microscopy of Polyethylene Terephthalate and Nylon Biodegradation Abilities of *Bacillus* sp. Strains Isolated From Soil

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Received: 26.11.2020; Accepted: 07.12.2020; Published Online: 25.12.2020

ABSTRACT

Plastic pollution, the aggregation of synthetic plastic products in the environment generates serious problems for wildlife, habitats and the human population. Plastic accumulates at ocean, creating areas called the seventh continent, a mass of plastic garbage. Plastic wastes are disposed of by recycling, burying and incineration. However, these also have disadvantages. They are developing bioplastics as new solutions to plastic problems. Other alternative solutions may be microorganisms. Compared to other conventional technologies, it is extremely inexpensive and efficient in terms of cost and simplicity because it is based on the capabilities of microorganisms. In this study, we have reported the degradation abilities of *B. subtilis* and *B. cereus* strains on polyethylene terephthalate (PET, water bottle) and nylon (plastic bag). In the nylon medium with *Bacillus subtilis* ET18 and *Bacillus cereus* ET30 was observed red pigment formation. All bacteria showed biofilm formation in the presence of nylon. The surface morphology changes of the PET and nylon were determined by light microscope and SEM. The bacteria used in this study were found to biodegrade nylon more easily than PET. The activities of lipase, protease and α-amylase were determined. Among the enzymes, lipase was detected in the presence of both PET and nylon.

Keywords: *Bacillus*, PET, nylon, biodegradation, SEM, enzyme

INTRODUCTION

A plastic material consists of numerous synthetic or semi-synthetic organic material. During the past 100 years synthetic plastic materials are used in lots application, for instance packaging, automobiles, toys, furniture and buildings. The worldwide production of plastics reached 359 million metric tons in 2018 (Garside 2019). It is foreseen that global plastic production will fold in two over the next 20 years and just about quadruple till 2050 (Neufeld *et al.* 2016). The deposit of these products has been led to increasing amounts of plastic pollution around the world. As plastic and microplastics are comprised of major toxic chemicals such as polychlorinated biphenyls, phthalates and bisphenol A, it has big harmful potential for the environment in land and water pollution (Bryant *et al.* 2016). Therefore, their effect cause serious problems related to human health and also danger for plankton, aquatic birds and fishes (Comăniță *et al.* 2016). A very small amount (9%) of waste plastic was recycled, and most of it was left to land or sea (Geyer *et al.* 2017).

The degradability of plastics can be biodegradation, photodegradation, environmental erosion and thermal degradation. Biodegradation is the cleavage of the compounds into smaller organic substances by microbial organisms. The microbial biodegradation of plastics has been reported in various studies. Some bacteria and fungi are involved in the degradation synthetic and natural plastics. The most important bacterial and fungal strains are *Bacillus, Actinomycetes, Thermoactinomycetes, Nocardia, Streptomyces, Pseudomonas, Klebsiella, Brevibacillus, Penicillium, Fusarium and Aspergillus* (Chaisu 2016). During biodegradation, exoenzymes synthesized by microorganisms catalyze the breakdown of complex polymers to form smaller short-chain products such as oligomers, dimers, monomers, and thus descend to small enough to pass through the semipermeable cell membrane. These products can then be used as carbon and energy sources (Dussud and Ghiglione 2014). Some significant extracellular enzymes responsible for degradation of plastics are urease, protease, depolymerase, lipase, dehydratase, esterase, dehydrogenase, manganese peroxidase and cutinase (Urbanek *et al.* 2018). Bacteria either die or return to normal population levels after converting hazardous waste into harmless by-products. Thus, ecological balance is not disturbed. In this work, we have reported the biodegradation capacity of four *Bacillus* sp. strains on PET (water bottle) and nylon (plastic bag). The surface morphology of plastics was imaged by scanning electron microscopy (SEM) before and after biodegradation.

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MATERIALS AND METHODS

PET and nylon sources

PET bottle and nylon bag obtained from markets were used. Both samples were cut into about 1cm^2 pieces. They were sterilized for 30 minutes with 70% ethanol, and a few times washed with sterile distilled water before use.

Strains and culture conditions

The four *Bacillus* sp. strains were isolated from agricultural soils of Turkey (Demirkan *et al.* 2019). *Bacillus* sp. bacteria for the ability of PET and nylon biodegradability were grown in Bushnell Hass (BH) broth medium containing (g L -1) KH_2PO_4 1.0, K_2HPO_4 1.0, NH_4NO_3 1.0 , $MgSO_4.7H_2O$ 0.2 , $FeCl_3$ 0.05 , $CaCl_2$ 0.02 (pH 7.0) and after that 0.1% glucose was added as supplementary for easy adaptation of the bacteria to the medium (Bushnell and Haas 1941). Biodegradation treatments were performed with 3.5% PET and nylon pieces which added to the medium under sterile conditions. The precultures of bacteria were inoculated in LB (Luria-Bertani) medium for overnight, inoculated at a concentration of 5% in BH medium and incubated at 37 °C and 150 rpm for 1 month.

Identification of *Bacillus* sp. strain using 16S rRNA sequencing

Bacillus genomic DNA was extracted for the bacterial identification and phylogenetic analysis (Qbiogene, Montreal, PQ, Canada). The sequence analysis was performed using ABI 3100 Genetic Analyzer (Applied Biosystems, USA). The obtained sequences were checked with the GenBank database (NCBI) by BLAST. The 16S rRNA gene sequences of four strains were aligned with other *Bacillus* species by CLUSTAL W program (Thompson *et al.* 1994). The phylogenetic analysis was done by MEGA 6.0 software, the tree was created using the neighbor-joining method (Saitou and Nei 1987).

Surface characterization by light microscope and SEM

Light microscope (Nikon Eclipse E1000) was used to screen PET and nylon samples. The bacterial biomass residue was gently removed with distilled water from samples, and samples were checked for under microscope. The surface morphology changes were determined by Scanning Electron Microscopy (SEM). SEM studies were done by Carl Zeiss AG-EVO 40 S, and accordingly images were taken. PET and nylon untreated with bacteria were used as control.

Some extracellular enzyme activities

The activities of lipase, protease and α-amylase enzymes were determined. α-amylase activity was measured according to Yoo *et al.* (1987). One unit of amylase activity was defined as the amount of enzyme that hydrolyzes 1 mg starch (0.1% w v -1) under experimental conditions. For, lipase activity, titrimetric assay was performed for measuring the lipolytic activity. One-unit lipase activity was defined as the amount of enzyme which releases 1µmol of fatty acid under experimental conditions (Sugihara *et al.* 1991). Total protease activity was determined using modification of Anson method. One-unit protease activity was defined as the amount of enzyme which release 1 µg mL-1 tyrosine under experimental conditions (Keay and Wildi 1970).

RESULTS AND DISCUSSION

Plastic products might be attractive due to some characteristics features. However, when they were not recycled properly, they can become problem on land, in waterways and in oceans. Recently more ecological and environmentalist approach, the microbial degradation method is used instead of traditional methods (physical degradation method, chemical degradation method). Biodegredation by microorganisms has low cost, high efficiency and efficient degradation effect (Huang 2018). Yoshida *et al.* (2016) have been reported a new bacterium, *Ideonella sakaiensis* 201-F6 which degrades PET. Later, many investigators discovered new microorganisms that could break PET and nylon. The most important bacterial and fungal strains are *Bacillus,*

Pseudomonas, Klebsiella, Actinomycetes, Nocardia, Streptomyces, Pseudomonas, Brevibacillus, Thermoactinomycetes, Fusarium, Penicillium and *Aspergillus* species (Chaisu 2016).

In our previous study, we isolated *Bacillus* sp. strains which degrade gasoline and diesel from petroleum non-contaminated soil samples in Turkey (Demirkan *et al.* 2019). Four best isolates were chosen and identified regarding to 16S rRNA gene sequence analysis. The three isolates showed 99% sequence identity with *Bacillus cereus* while one isolate shared 99% with *Bacillus subtilis*. These were named as *Bacillus subtilis* ET18, *Bacillus cereus* ET30, *Bacillus cereus* ET106 and *Bacillus cereus* ET202 (Figure 1).

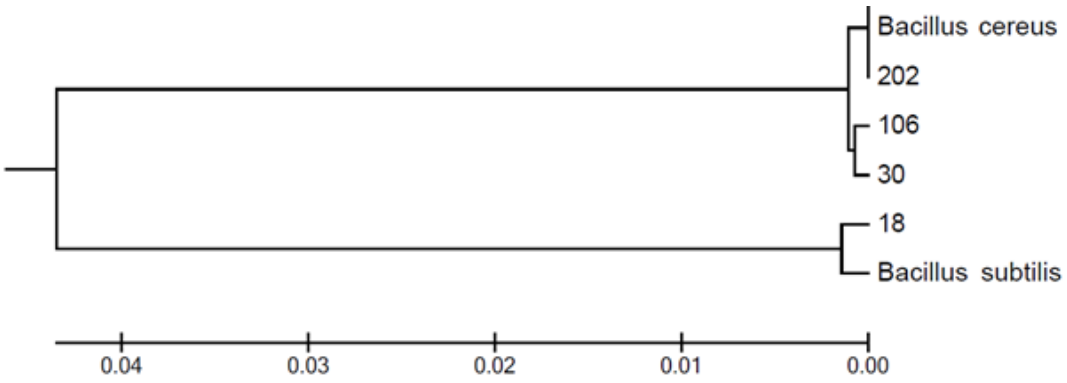

Figure 1. Phylogenetic relationship between *Bacillus* sp. strains regarding to their 16S rRNA gene sequence analysis. Bar, 0.01 changes per nucleotide position.

Pigment formation was increased and others showed a milky coloured growing (Figure 2). Odusanya *et al.* (2013) showed *Serratia marcescens* is able to biodegrading polyethylene plastic (LLDPE) and producing red pigment and this may act a role in degradation. However, in this study, while *Bacillus subtilis* ET18 and *Bacillus cereus* ET30 showed red pigment formation in the presence of nylon, others did not in the presence of nylon and PET. Therefore, pigmentation may its own characteristic property of the bacteria, biodegradation may not have an affinity. On the other hand, in the presence of nylon, biofilm formation was observed. Especially, ET30 and ET106 strains formed dense biofilm. However, biofilm formation was not observed in the presence of PET. Table 1 shows red pigment and biofilm forming strains.

Table 1. Red pigment, biofilm-formation and enzyme activity results of strains in the presence of nylon and PET.

Strains	Plastics	Red pigmentation	Biofilm	Amylase activity (IU mL -1)	Protease activity (IU mL -1)	Lipase activity (IU mL -1)
ET18	Nylon/PET	+/-	+/-	1/0	0/0	19/14
ET30	Nylon/PET	+/-	+/-	13/0	0/0	16/15
ET106	Nylon/PET	-/-	+/-	14/0	0/0	14/15
ET202	Nylon/PET	-/-	+/-	0/0	0/0	17/14
ET202	As a single carbon source contains 3.5% gasoline.	-	+	0	0	15
ET202	As a single carbon source contains 0.1% glucose.	+	+	84	89	20

there is (+), none (-)

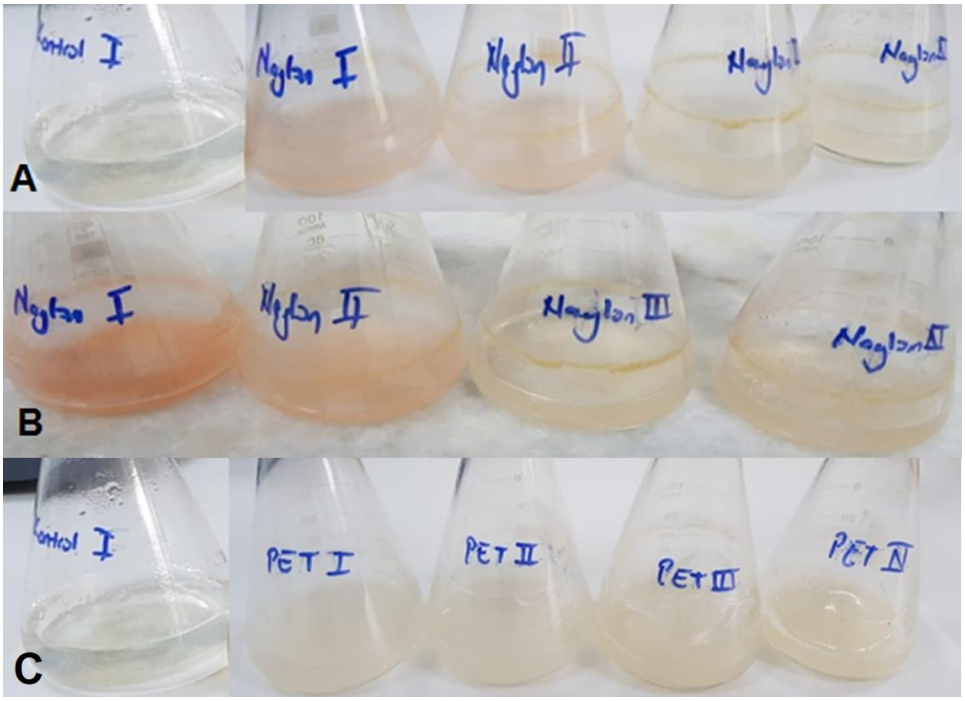

Figure 2. Red pigment formation after 15 days (A) and 30 days (B) in the presence of nylon. Control I (growth medium), nylon I (ET18), nylon II (ET30), nylon III (ET106), nylon IV (ETC202). Milky colour formation after 30 days (C). Control I (growth medium), PET I (ET18), PET II (ET30), PET III (ET106), PET VI (ET202).

The PET and nylon samples, treated and untreated with bacteria (control), were prescreened as morphologically under light microscopy and the variation in properties of nylon and PET after the treatment with bacteria for 1 month was determined (Figure 3). The nylon and PET surface of control was smooth. Only PET surface showed small visible black points. All four strains had the same effect on the surface of nylon and PET. It was observed that the outer structure of nylon was damaged, has ruptures. On the other hand, deeper damage was obtained on PET surface. That could be the reason that PET has a thicker structure than nylon.

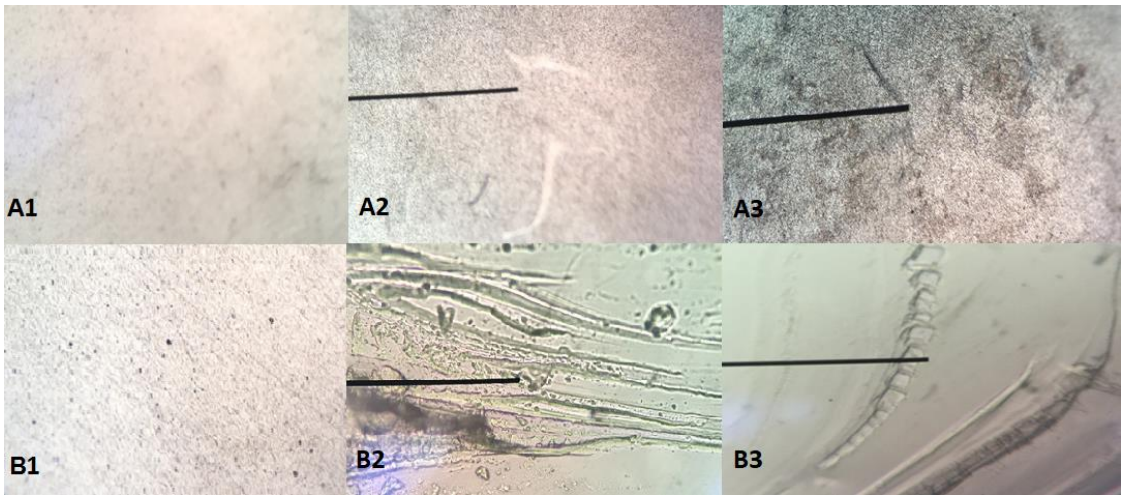

Figure 3. Light microscopy images of nylon (A) and PET (B) samples treated and untreated (control) with bacteria. Image of untreated (control) and treated nylon surfaces under light microscope (A). nylon (control) untreated with strains (A1), nylon treated with strain (A2, A3). Image of untreated (control) and treated PET surfaces under light microscope (B). PET (control) untreated (B1), PET treated with strain (B2, B3).

SEM images of control and bacteria treated nylon were shown in Figure 4A. Nylon had some small cracks, holes and pits when compared with control. All bacteria formed biofilms on the nylon surface. Many grooves, deep pits and degradations were also obtained on nylon surface. Bacteria were found embedded in nylon biofilm. There were deep holes on the nylon surfaces in samples inoculated with ET30 and ET106 strains. These indicated that biodegradation by all bacteria has occurred. Bacteria in the presence of PET showed different degradations. The ET18 and ET30 strains showed surface rupture (Figure 4B), while others showed deep ruptures and deformations. Damage of nylon samples was more than PET. This might be due to biofilm formation which was appeared on the nylon surface by all bacteria strains. Due to the characteristic feature of nylon, bacteria hold on to the nylon surface better and, biodegradation occurred. No biofilm formation was observed on the PET surface on SEM images. The rigid structure of PET could be the reason. The movement of bacteria may be restricted on PET surface. Similar result was reported by Webb (2012) that bacterial attachment on PET surfaces is considerably limited. According to our results, it can be said that biofilm formation facilitates biodegradation. The bacteria attacked better to the nylon surface by forming biofilm on the nylon surface. The nylon surface allowed forming biofilm. By changing the surface of the PET, biofilm formation can be stimulated and bacteria could attack to the PET surface better.

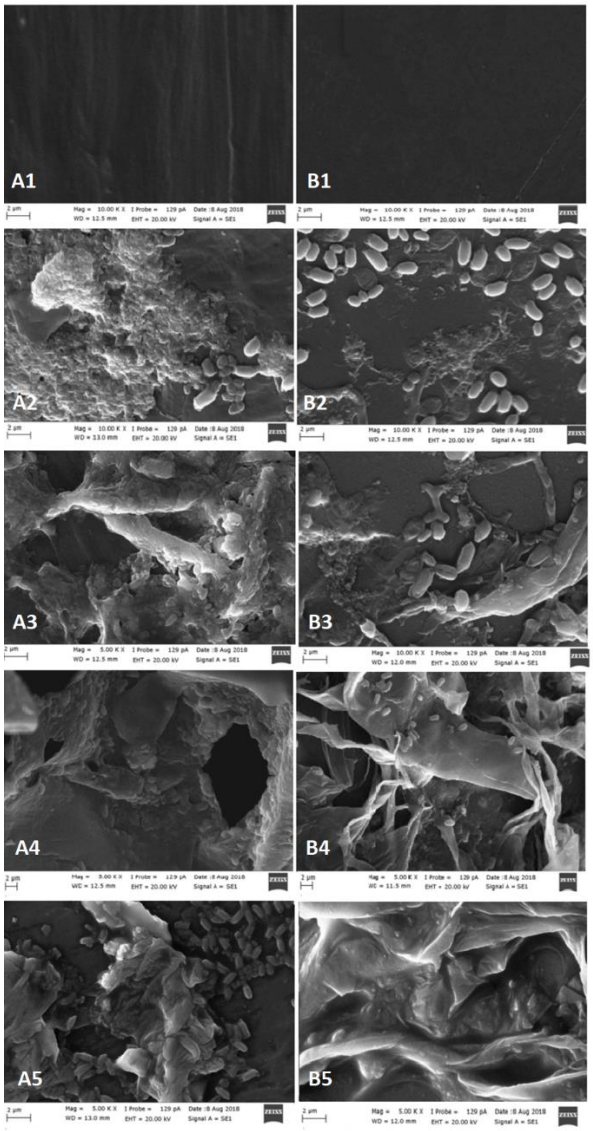

Figure 4. Surface morphologies of the nylon (A) and PET (B) imaged by SEM after 30 days treatment with strains. A1: nylon control, B1: PET control untreatment with strains. A2 and B2 indicate the samples treated with ET18, A3 and B3 with ET30, A4 and B4 with ET106, A5 and B5 with ET202.

Some scientists showed biodegradation of a wide range of polymers using different microorganisms. *Pseudomonas, Cellulosimicrobium, Bacillus, Lysinibacillus, Brevibacillus* and *Aspergillus* were comprised as different plastic degraders (Muhonja *et al.* 2018). *Aspergillus terreus* AF5, *Penicillium sp.* AF6 *and Fusarium sp.* AF4 have been attacked on Polyethylene sheets during of ten months (Shah *et al.* 2008). It was also demonstrated of ability of *Aspergillus niger* and *Bacillus subtilis* on polyethylene. *Bacillus cereus, Pseudomonas sp., Bacillus subtilis* were shown to degrade Polyethylene carry bags. LDPE biodegradation by *Bacillus* and *Aspergillus* was reported by Esmaeili *et al.* (2013). A novel thermophilic *Bacillus* sp. BCBT21 strain which degrades different kinds of plastic bags has been isolated from Vietnam soil (Dang *et al.* 2018). PVC powder, high density polyethylene (HDPE) and low density polyethylene (LDPE) powder are metabolized by several microorganisms such as *Listeria* sp., *Aspergillus* sp., *Penicillium* sp, *P. aeruginosa, Micrococcus* sp., *Bacillus* sp. and *Vibrio* sp., *P. putida, P. aeruginosa* and *P. syringae* (Kyaw *et al.* 2012), *A. niger*, Streptomyces KU1, *Streptomyces* KU5, Streptomyces KU6, *Streptomyces* KU8 (Usha *et al.* 2011); *A. terreus* MF12. Polyurethane is degraded by *Actinetobacter gerneri* P7, *Fusarium solani, Aureobasidium pullulans* sp (Shimao 2001). Polycaprolactone (PCL) is degraded by *Alcaligenes faecalis* and *Clostridium botulinum*. Polylactic acid (PLA) degradation by a *Bacillus brevis* and fungi *Fusarium moniliforme* and *Penicillium roqueforti* was reported (Ghosh *et al.* 2013). Some bacteria and fungi that degrade the nylon have been isolated. Marine bacteria were shown to biodegrade nylon in mineral salt medium at pH 7.5 and 35 °C (Sudhakar *et al.* 2007). *Anoxybacillus rupiensis* Ir3 (JQ912241) have been cultivated on the nylon, and bacteria were found to break down nylon (Mahdi *et al.* 2016). The biodegradation mechanics of PET have been reported.

Microbial enzymes play an important role in plastic biodegradation. Several enzymes have been reported to be involved in biodegradation such as manganese peroxidase from *Phanerochaete chrysosporium* in the presence of Polyethylene; cutinase from *Fusarium* in the presence of PCL; unknown enzyme from *Amycolaptosis* sp. in the presence of Polylactic acid (PLA); cutinase from *A. oryzae* in the presence of Polybutylene succinate (PBS); urease from *Trichoderma* sp. in the presence of Polyurethane (Trevino and Garcia 2011); protease from *Aspergillus niger* in the presence of PCL; Lipase from *Rhizopus delemar* in the presence of PCL serine hydrolase from *Pestalotiopsis microspora* in the presence of Polyurethane (Tokiwa and Calabia 2009).

In this study, the activities of lipase, α-amylase and protease were analyzed. As shown in Table 1, all strains showed only lipase enzyme activity in the presence of nylon and PET. Amylase activity was obtained with 30 and 106 strains in the presence of nylon, whereas no activity was observed in the others. Protease activity was not detected. The bacteria may have degrade with another enzyme or unknown enzyme in the presence of PET. In this study, it was shown that lipase enzyme may have a role in biodegradation of nylon and PET. Dang *et al.* (2018) reported that chitinase, xylanase, protease, CMCase and lipase from *Bacillus* might playing a substantial role in plastic biodegradation. The biodegradation of plastic or polyolefin by enzymes also was reported in some research (Koitabashi *et al.* 2016). The enzymes related with the biodegradation (e.g., PET hydrolase and tannase) are typically serine hydrolases, e.g. carboxylesterases (EC 3.1.1.1), cutinases (EC 3.1.1.74), and lipases (EC 3.1.1.3) (Danso *et al.*, 2018). Lipase catalysed biodegradation of poly(hydroxybutyrateco-valerate) (PHBV) has been indicated to occur preferably in the amorphous area of the polymer, exposing polymer crystals. Gong *et al.* (2018) has been reported that alkaline conditions for enzyme activity is more efficient for biodegradation of PET.

SDS-PAGE electrophoresis was done to show the protein profile, and 2 bands were obtained. Gels images not shown because the bands were faint.

CONCLUSIONS

The discovery of new potential plastic degradable microorganisms has become an important goal. The bacteria used in this study were found to biodegrade nylon more easily than PET. The observation of biofilm formation in the presence of nylon showed that the bacteria develops by holding onto the nylon. Since lipase enzyme activity was obtained in the presence of both pet and nylon, it is thought that lipase enzyme may play a role in biological degradation. These strains seemed to have potential for bioremediation of nylon.

ACKNOWLEDGEMENTS

We would like to express our appreciation to Bursa Uludag University Scientific Research Project Commission, which supported this study HDP(F)2016/57.

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