ISOLATION AND CHARACTERIZATION OF POLYURETHANE-DEGRADING MICROORGANISMS

A Thesis

by

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ABSTRACT

Polyester polyurethanes (PUR) are one of most versatile synthetic polymers with various applications. However, tremendous quantities of end-of-life PUR is problematic and has caused negative impacts on the environment and health of ecological systems. Due to their environmental pollution, development of biodegradation technology is needed. In this work, PUR degrading microorganisms were isolated from the sediment in Galveston Bay and local landfill in Texas. One bacterial and two fungal strains degrading PUR were isolated. The bacterium belonged to *Pseudomonas* sp. and the fungi were identified as *Purpureocillium* sp. and *Coniochaeta* sp. The degradation efficiency of culture of *Pseudomonas* sp. with culture supernatant of *Purpureocillium* sp. against PUR was recorded to identify their relationship towards degradation for 42 days. An extracellular esterase activity of culture (PGB+PLI) was 12.3 U L^{-1} on day 22 maintaining 7 times higher activity than that of pure culture. Pits on surface and porous structure of degraded PUR were observed through scanning electron microscopy (SEM). Furthermore, a peak at 1,731 cm^{-1} corresponding ester linkage decreased most in culture (PGB+PLI). Intensity belonging to degradation product, diethylene glycol (DEG) and adipic acid (AA), was detected higher in culture (PGB+PLI) compared to pure culture, through Gas Chromatography (GC) with Flame Ionization Detection (FID).

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CHAPTER I

INTRODUCTION AND OBJECTIVES

Introduction

Plastics have become essential materials for industry and our everyday life, in part, due to their low cost and useful properties such as strength, lightness, and durability that are suitable for wide applications.[1] At least 300 million metric tons of plastics are produced yearly worldwide and its accumulation has become a global crisis due to its adverse effects on public health, ecosystem, and environment.[2-4] Managing such a tremendous amount of end-of-life plastic waste is a great challenge. Current approach includes landfill and incineration. Landfill resulted in terrestrial pollution and incineration required high energy input and led emission of toxic compounds and carcinogens, like carbon monoxide, hydrogen cyanide and nitrogen oxides.[1, 5] On the other hand, biodegradation of plastics is a more attractive alternative. However, biodegradation of plastics is slow in the environment, and little is known about the microorganisms capable of degrading plastics.

Polyurethanes (PUR) are the sixth largest of the plastics produced globally, accounting for 8% of the total produced plastics.[6] Common PUR-based products are elastomers, adhesives and coatings. PUR are also produced as either soft or rigid foams, and they are found in paint, insulation materials, and tires.[7, 8] The structure of PUR is generally organized with two types of segments, hard and soft segments, corresponding to polyisocyanate and polyol parts, respectively. Several studies have suggested that PUR biodegradation could be hydrolysis by microbial attack on the urethane and hydrogen bonds in the soft segment followed by degradation of the hard segments.[9, 10]

Biological degradation of PUR has been intensively studied.[7, 11] Both bacteria such as *Pseudomonas* species and fungi such as *Aspergillus, Pennicilium, Plectosphaerella,* and *Neonectria* have been reported to degrade PUR.[12-15] Interestingly, bacterial degradation contributes to significant break in the PUR structures, while fungal degradation only made minor modifications of the PUR structure.[10] Accordingly, it has been suggested that multiple microbial strains are needed to enhance biodegradation of PUR.[16-18] Common fungal enzymes such as peroxidase and laccase have been shown to attack PUR by nonspecific oxidation, and bacterial esterases and lipases are able to cleave urethane bonds in the structures of PUR.[7, 13, 19, 20]

However, our knowledge of PUR biodegradation is generally derived from single fungal or bacterial strain, and little is known about the effects of multiple microbial strains on PUR biodegradation, particularly the interactions between fungal and bacterial strains during PUR degradation.[21] To this end, this study is to fill the knowledge gap described above.

Goal, objectives, and hypotheses

The overall goal of this research is to understand the mechanisms of PUR biodegradation in the environment where fungal and bacterial strains are commonly co-mingled. We hypothesize that both fungal and bacterial strains can use PUR as carbon and/or nitrogen source. To access the carbon and/or nitrogen in PUR, both fungal and bacterial strains express different extracellular enzymes to degrade PUR, resulting in different extent of degradation. As such, one can enhance PUR degradation by selecting a

proper culture of fungal and bacterial strains. The objective of this study is to determine synergistic or competitive relationship between fungal and bacterial strains during PUR degradation. Figure 1 illustrates the overall technical methods in this study.

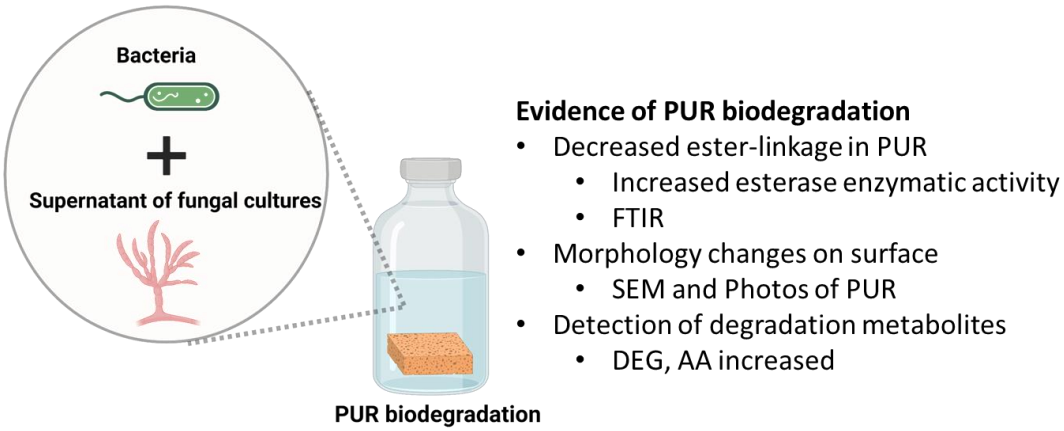


Figure 1. Overview of this study (created with Biorender.com).

Three tasks are proposed to test the hypothesis. They are

Task 1: Isolate microorganisms capable of degrading PUR from the environment.

Approach: Isolate PUR-degrading strains by using a model polyester PUR substrate, Impranil® DLN-SD. Impranil® DLN-SD is used as either carbon or nitrogen sources in the experimental setup. Isolates are identified based on 16S rRNA, and ITS region sequences for bacterial and fungal strain, respectively.

Task 2: Identify enzymes responsible for PUR degradation.

Approach: Determine activities of laccase, peroxidases, esterases, and lipases produced by isolates during degradation of Impranil® DLN-SD and PUR foams.

Task 3: Examine synergistic or competitive relationship between fungal and bacterial strains on PUR biodegradation.

Approach: Determine if PUR biodegradation can be enhanced by using different combinations of fungal enzymes and bacterial cultures. Assess extent of PUR degradation based on time course measurements of Fourier transform infra-red spectroscopy (FTIR), Gas Chromatography coupled with Flame Ionization Detection (GC-FID) and scanning electron microscopy (SEM).

CHAPTER II

LITERATURE REVIEW

Polyurethanes

Chemical and physical properties of Polyurethanes

Polyurethanes (PUR) are known as versatile plastics that can be extensively used in our daily lives. Characteristics PUR have is versatility and recalcitrance so that they are produced worldwide, and the uses commonly are found in foams, textile, and paint. PUR can be synthesized through the condensation of an isocyanate with an alcohol, producing heterogeneous structures.[22] The general structure of PUR is not specified, but it is commonly organized with the hard segments based on polyisocyanates and the soft segments consisted of polyols in which monomer contains a urethane moiety.[7] Polyisocyanates play a critical role in the synthesis of PUR by reacting with the hydroxyl groups of polyols. Indeed, polyisocyanates serve as a building block providing hardness and immobility to PUR whereas polyol compounds produce resiliency and elongation properties.[9, 10] Due to the chemical characteristics of the hard segment, PUR is poorly soluble in commonly used solvent such as acetone, ethanol, and methanol. Moreover, fire resistance is conferred since the hard segments are recalcitrant to changes in pH and temperature.[23] On the other hand, polyol compounds provide resilience, tensile strength, and adsorption, comprising the larger portion as the soft segment. The soft segments are sensitive to biodegradation resulting hydrolysis.[24, 25] Therefore, the relative ratio of polyisocyanates and polyols determines chemical and physical properties of PUR showing susceptibility and resistance to degradation.[7] Also, the general properties of PUR are

derived from the R groups in the structure and the functional groups contain urethane, aromatic, urea, ether, and ester.[10] By adding polyfunctional compounds, PUR exhibit different features. The most common additives are polyols, isocyanate, catalysts, pigments, and fillers.[8] Chemical and structural compositions determine linear or branched structures of PUR with varying tensile strength.[26]

Environmental effects of polyurethane wastes

Plastics are now essential to our lives and thus, the enormous increase in polymer production and amounts of plastics threaten human health and the environment. Approximately 60% of plastic production have been remained after waste management such as recycling and incineration.[27] The waste management has not maintained the same rate of the production, leading environmental contamination.[28] Among synthetic polymers, the annual production of PUR ranks sixth exceeding 22 million tons per year.[8, 29] Due to the high durability, PUR waste has resulted accumulation in landfills. Moreover, PUR with additives to enhance chain extension and to avoid biodegradation can release toxic and hazardous substances, such as dibutyl tin dilaurate (DBTDL) in the environment.[30] PUR products such as foams are commonly utilized in cosmetic and pharmaceutical industries as small fragment. Weathering and exposure to sunlight on the waste of small fragment cause the formation of smaller pieces, which are known as micro- and nano-plastics.[31] Those smaller particles can be a significant threat to our health by entering the food chain and intestines.[2] Notably, degradation of synthetic polymers by microorganisms and enzymes is highly promising for recycling and waste management by

depolymerization and mineralization.[32, 33] To accomplish efficient biodegradation of PUR, it is necessary to explore novel organisms with capability to degrade PUR. Understanding of accurate mechanisms will also help the growth of innovative biodegradation technologies.[12]

PUR degradation

Fungal PUR degradation

PUR degraders can be found in some fungal and bacterial strains isolated from the soil contaminated by plastics, such as landfills and marine environment.[15, 34, 35] Numerous studies found that PUR is susceptible to fungal attack.[36, 37] PUR largely containing polyester are easily subjected to fungal attack. Majority of the studies are related to strains from soil.[38, 39] Degradation is mainly occurred by fungi in the genera *Aspergillus*, *Penicillium*, *Cladosporium*, *Pestalotiopsis*, and *Fusarium*.[10, 21, 38, 40] In particular, researchers revealed that fungi which form filamentous structure are capable to grow on various organic compound. Rather than yeast, filamentous fungi are known to have capability to degrade PUR. Filaments from fungi can penetrate PUR resulting cracking. Abiotic effect by fungal attack can mostly cause minor changes of the properties. This process represents general PUR degradation contributing to complete biodegradation. Degradation by filamentous fungi is more efficient than bacterial degradation since they colonize the compound with and secrete abundant enzymes involved in degradation.[9, 41, 42] However, the mechanism of fungal degradation are not fully studied.[10, 21] Most kinds of

PUR such as polyester, polyether, thermoplastic PUR, and PUR foams were studied with fungal strains.[42]

Bacterial PUR degradation

A wide range of bacterial strains are capable to utilize polyester PUR as a carbon, and nitrogen source for living. There are differences in PUR degradation between bacteria and fungi. While fungal degradation on PUR generally exhibits an abiotic degradation, bacterial degradation leads major breaks in the structure of PUR leading a site-directed specific attack.[10] The bacterial growth on PUR induces cleavage in the structure.[41, 43] Gram-negative *Betaproteobacteria* have been identified to be most relevant class to PUR degradation.[4] The genus known to have biodegradability for PUR are *Bacillus*, *Acinetobacter*, and *Comamonas*.[44-47] They can degrade PUR as a source of carbon. Bacteria may reveal decreasing rate of the level of degradation when PUR is supplemented as a carbon and nitrogen source.[48] The degradation by *Bacillus* may require the adherence of bacteria on to the polymer leading the floc.[49] Several studies reported that *Comamonas acidovorans* use an extracellular membrane bound esterase for polyester PUR degradation.[47]

Enzymes involved in PUR degradation

Polyurethane biodegradation can be accomplished by several enzymes known as cutinases, lipases, laccases, peroxidases, proteases, and ureases. However, it can be mainly described by urethanases and esterases.[50] The bacterial enzymes generally are found to

express intrinsically, however, the enzymes derived from fungi are contributed by substrate induction.[20, 48] This difference implicates that particular mechanisms are involved in PUR biodegradation. Moreover, it is expected that there are specific enzymes from numerous microorganisms.[10, 45] The enzymes degrading PUR can be divided into two categories, which are secreted or membrane-bound.[20] During initial stage of biodegradation for PUR, membrane-bound enzymes may adhere on the surface of PUR so that the urethane bond can be broken through hydrolysis. As a result, related metabolites are released into the medium.[51] Compared to the membrane-bound enzymes, secreted enzymes are damaging the PUR surface facilitating biodegradation via increase of the surface area. One of the main enzyme classes is esterase which hydrolyzes the ester bonds in the soft region in PUR releasing carboxylic acid and alcohol end-groups. The other enzymes known as amidases and proteases are shown to hydrolyze peptide or amide bonds through hydrolysis of urethane bonds in PUR structure.[52, 53] Oxidoreductases such as laccase or lignin peroxidase can be applied for degradation where hydrolytic enzymes are no longer effective. Among them, laccase is a multicopper oxidase used due to its versatility. It can be applied to degradation of polymers, bioremediation, and bleaching.[54] Several studies already reported that laccase is associated with degradation of PUR.[19]

Interaction between fungal and bacterial enzymes during biodegradation

Microbial communities can be defined as groups of microorganisms sharing a common living space. Interactions occur between the microorganisms in the presence of mutualism, predation, or competition.[55] Degradation of synthetic polymers or organic

pollutants by microbial communities may be more effective by rather than single strains. It has been demonstrated that the members in the community can utilize the metabolites from the degradation process of PE.[56] Recent study investigated comparative analysis of PUR degradation by fungi and bacteria. This study revealed that weight losses of PUR increased when fungi and bacteria were cultured together.[16] The degradation of organic pollutants such as the benzo(a) pyrene, and phenol has been studied for microbial communities consisting of fungi and bacteria. Generally, it has been demonstrated that biodegradation could be enhanced by the consortia.[57-59] However, there are few studies exploring synergistic effect between fungi and bacteria during degradation of synthetic polymers, PUR. Thus, our understanding of synergistic biodegradation process is still limited.

CHAPTER III

EXPERIMENTAL SECTION

Chemicals

Impranil® DLN-SD (designated as Impranil) was obtained from Covestro, Leverkusen, Germany, which as anionic aliphatic polyester-polyurethane dispersion. *p*-nitrophenyl acetate (*p*-NPA, 98%) was purchased from Sigma-Aldrich, St. Louis, MO, USA. PUR foams, makeup sponge, were purchased from a local market. Commercial artificial seawater was obtained from Instant Ocean, Blacksburg, VA, USA. N, O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) were purchased from Pierce Biotechnology Inc., Rockford, IL, USA. Diethylene glycol (99%) and adipic acid (99%) was purchased from Sigma-Aldrich, St. Louis, MO, USA.

Culture media

For cultivation for PUR degradation, fungal strains and bacteria were grown in ammonium mineral salt medium (AMS) or N-free mineral salt medium (N-free MS) with a minor modification.[60] One liter of AMS contained 0.776 g of $(NH_4)SO_4$, 0.866 g of Na_2HPO_4, 0.171 g of K_2SO_4, 0.0370 g of $MgSO_4 \cdot 7H_2O$, 0.0121 g of $CaSO_4 \cdot 2H_2O$, 0.0222 g of $FeSO_4 \cdot 7H_2O$, 0.0002 g of KI, 0.0006 g of $ZnSO_4 \cdot 7H_2O$, 0.0003 g of $MnSO_4$, 0.0001 g of H_3BO_3, 0.0011 g of $CoSO_4$, 0.001 g of $CoSO_4$, vitamin mixture (0.1%; 0.05% thiamine-HCl, 0.03% p-aminobenzoic acid, 0.01% panthothenate, 0.005% biotin, and 0.05% vitamin B_{12}), and 0.1 g of H_2SO_4 with pH of approximately 7.5. To prepare one liter of N-free MS, 5.4 g of sodium succinate hexahydrate were additionally supplemented into AMS

medium and $(NH_4)SO_4$ was not included in the culture medium. Commercial artificial seawater at 50% was supplemented into all the medium (approximately 18 g l^{-1}).

Isolation and identification of polyurethane-degrading microorganims

Sediment samples were collected from location (referred as G2) in the Galveston Bay on 09/09/2017 and from local landfill on 09/05/2018.[61] Impranil was used to isolate PUR-degrading microorganisms from the samples. Impranil was used to isolate PUR-degrading microorganisms from the samples. Enrichment cultures from the soil samples were grown on the solid media containing N-free MS with Impranil as carbon and nitrogen sources. It was expected that microorganisms degrading Impranil would produce clearing halo on the media. Fungal strains capable to grow on the Impranil plates were isolated by transferring the fungal hyphae and spores and cultured in Sabouraud Dextrose Broth (SDB). Single colonies forming degradation halos on the Impranil plates were isolated and cultured in R2A (Reasoner's 2A) broth. DNA was extracted from the isolated strains capable to degrade PUR by using commercial soil genomic DNA kits (MP Biomedicals FastDNA SPIN Kit for Soil). Approximately 100 ng of DNA was used to amplify the internal transcribed spacer (ITS) region of fungal strains and 16S rRNA gene of bacterial strains with PCR Master Mix from Promega. The primers used for ITS sequencing were ITS1 (5'-TCCGTAGGTGAACCTGCGGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3').[12] On the other hand, bacterial primers were 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'- GGTTACCTTGTTACGACTT-3'). Sequences were analyzed by comparing to known 16S rRNA gene and ITS region

sequences in database of NCBI using the BLAST program. Phylogenetic analyses of aligned sequences were performed using MEGA version 11.0.10 through neighbor-joining method with 1000 bootstraps.[62]

Impranil degradation tests

The fungal and bacterial isolates were grown in SDB and R2A, respectively. They were inoculated on plates containing the mineral salt medium with Impranil at 0.6% as carbon or nitrogen sources at 30 °C. To determine their utilization of Impranil, clearance of the substrate was observed. The strains capable to degrade the substrate exhibit a zone of clearance ('halo') on the plates as a result of degradation by enzymatic activities.[12] The cultures with Impranil as carbon or nitrogen sources were monitored for changes in the functional groups. Each fungal and bacterial cultures were washed to remove all residual medium prior to inoculation. Approximately 100 mg of fungal material and 0.8 of optical density (OD_{600}) of bacteria were cultured in 20 ml glass bottles with 5 ml of AMS or N-free MS with Impranil at 0.6%, respectively. Each fungal and bacterial culture was collected after 2 days. Identical cultures containing approximately 60 mg of fungal material and optical density (OD_{600}) of 0.3 were monitored for gradual changes in enzyme activities. All tests were conducted in duplicate. Fungal culture was collected on day 0, 4 and 12. Similarly, bacterial culture was analyzed on day 0, 14 and 22. Uninoculated bottles were used as controls. All bottles were incubated at 30 °C by shaking at 150 rpm.

Degradation of PUR foam

PUR foams were washed with DI water and then dried in vacuum overnight at room temperature (RT). These plastic pieces were treated with oxygen plasma for 5 min at RT by a plasma cleaner (Harrick PDC 32G). The treatment was carried out with a RF power of 18 W and a working pressure about 600 mTorr. They were cut to approximately 5 × 5 mm and thickness of 2 mm and autoclaved. Each piece of PUR foams was introduced into 120 ml glass bottle containing 15 ml of sterile N-free MS medium. To explore synergistic or competitive relationship between fungal and bacterial enzymes for PUR degradation, four identical glass bottles were used; Three of them were inoculated with bacterial strain (approximately 0.1 of OD_{600}) and one is not inoculated as abiotic control. Fungal cultures were initially grown on Impranil as carbon or nitrogen sources for approximately 3 weeks were 0.22 μm syringe filtered. Seven and half milliliters of the supernatant was supplemented into two culture bottles among three bacterial cultures, one with or without PUR foams, in a final volume of 15 ml as a parallel set. The bacterial culture, which was not supplemented with supernatant, were used to determine PUR degradation by pure culture. All bottles were incubated at 30 °C by shaking at 150 rpm. pH values in cultures were monitored to determine chemical changes and cell growth based on optical density were measured at OD_{585}.

Esterase activity assay

Liquid samples collected periodically from Impranil or PUR foam degradation tests were used for determining esterase enzyme activity as described by Musidlowska-

Persson et al.[63] They were centrifuged 13,000 × g for 15 min, and the supernatant was prepared. Extracellular esterase activity was evaluated by hydrolysis of *p*-NPA at 410 nm (ε_{410} = 17100 M^{-1} cm^{-1} for pH 8.0). The reaction mixture contained 0.25 ml of the supernatant, 0.75 ml of sodium phosphate buffer (pH 8), and 1 mM of *p*-NPA (125 mM stock solution dissolved in ethanol) in a final volume of 1 ml. One unit (U) of esterase activity was expressed by the amount of enzyme releasing 1 μmol of *p*-nitrophenol per minute at 25°C.[64]

Scanning electron microscopy (SEM)

PUR foams were collected from liquid culture medium after the completion of incubation time (42 days). The foams were washed out twice with deionized water and then vacuum dried at room temperature. Dried PUR foams were coated with gold by TedPella Cressington 108 before analyzed by Jeol JCM-5000 Neoscope.

Fourier transform infrared (FTIR) spectroscopy

FTIR spectroscopy (Bruker Alpha-platinum) was conducted to observe the evidence of degradation of PUR substrate, such as Impranil and PUR foams. The FTIR spectra were collected at 4 cm^{-1} resolution and 16 scans in absorbance mode equipped with an attenuated total reflectance (ATR diamond crystal). PUR foams were collected and washed with deionized water at the end of degradation and an atmospheric background was collected before analysis. Spectra were collected to deionized water as a background.[12]

Analysis of degradation products

During the 42-day incubation period, 5 ml of liquid cultures were collected on day 0, day 22, and day 42 to analyze degradation by-products such as diethylene glycol (DEG) and adipic acid (AA). Prior to extraction with ethyl acetate, the liquid cultures were centrifuged at 4,300 rpm for 10 min. Supernatants were then 0.22 μm syringe filtered and acidified to pH 2 adding concentrated HCl. Extraction was conducted twice with ethyl acetate. The solvent phases were pooled, dehydrated over anhydrous sodium sulphate, and evaporated to dryness at room temperature. The extracts were reconstituted in 100 μl ethyl acetate and derivatized with 100 μl of BSTFA + 1% TMCS at room temperature for 15 min prior to analysis. Concentrations of DEG and AA were analyzed by Agilent 6890N Gas Chromatography (GC, Agilent 6890N) / Flame Ionization Detection (FID) equipped with a J&W122-5532G capillary column and 1 μl was injected[46, 65, 66]: 80 °C initial for 1 min, 250 °C for final, and then increased it at the rate of 16 °C per minute. Calibration curves of DEG and AA in ethyl acetate were created to determine the concentration in the samples.

CHAPTER IV

RESULTS AND DISCUSSION

Identification of Impranil- and PUR- degrading microorganisms

Three strains including filamentous fungus (designated as PLI), yeast-like fungus (designated LLI), and bacterium (designated as PGB) showed a 'halo' in Impranil plates as carbon and nitrogen sources after 12, 11, and 58 days of growth, respectively (Fig. 2). Each phylogenetic relationship involving the isolates are shown in Fig. 3 and Fig. 4. The nucleotide sequence of PLI, LLI, and PGB corresponding 16S rRNA or ITS region can be obtained from NCBI nucleotide sequence database under accession number OM995897, OM995896, and OM995894. Fungal strains and bacterial strain were belonging to *Purpureocillium* sp., *Coniochaeta* sp., and *Pseudomonas* sp., closely related to known plastic degrader such as *Purpureocillium* sp. (MN962646), *Coniochaeta* sp. (MW073470) and Pseudomonas fluorescens Pf0-1 (NC_007492), respectively (Table 1-2).[67-69] Esterase activity of fungal PUR degraders, PLI and LLI, under the same condition was shown to be similar (data not shown). Thus, among three of them, *Purpureocillium* sp. and *Pseudomonas* sp. were selected to examine relation between fungal and bacterial strain toward degradation.

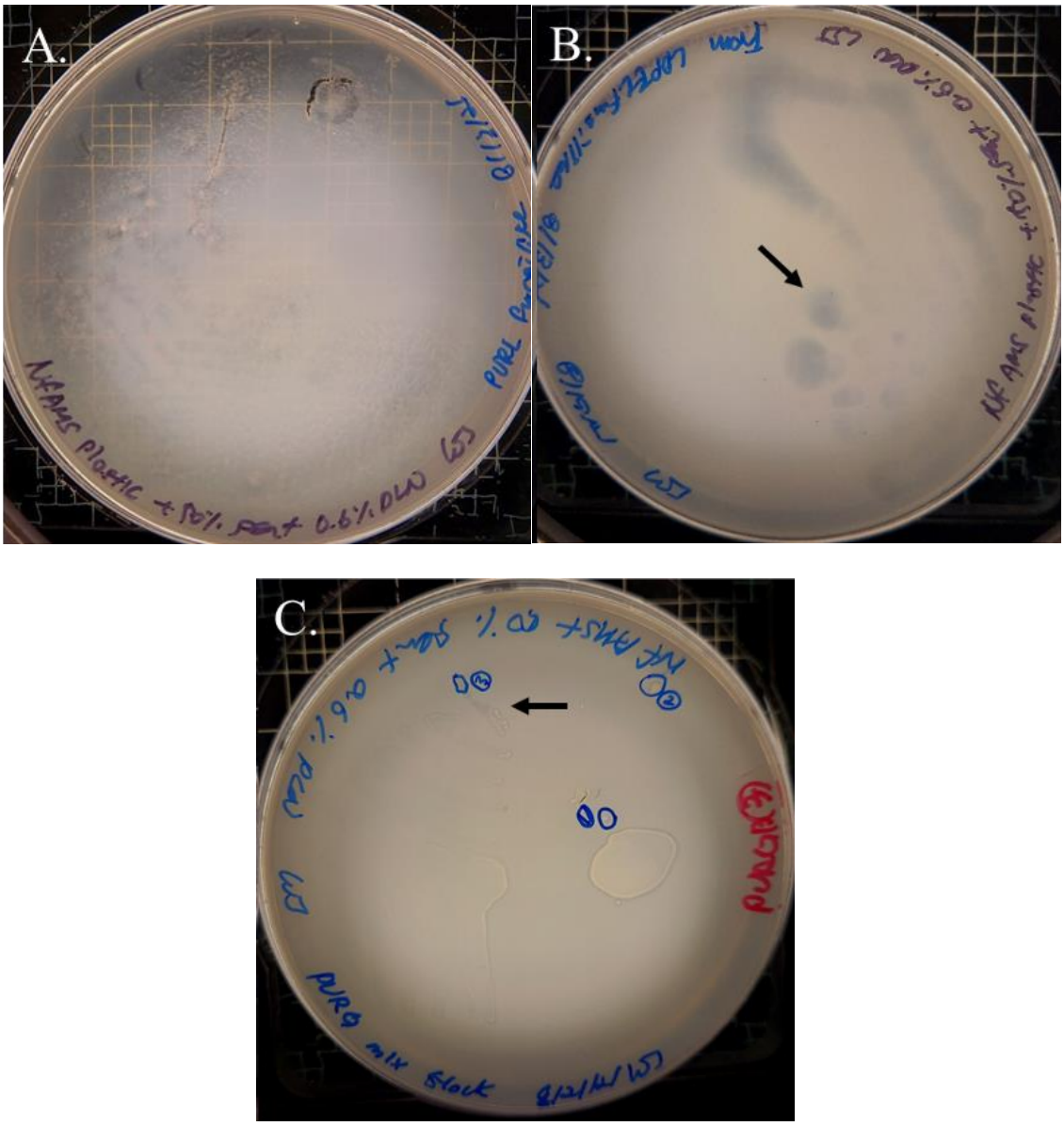


Figure 2. Initial screening using Impranil as carbon and nitrogen sources. (A) *Purpureocillium* sp. after 12-day incubation. (B) *Coniochaeta* sp. after 11-day incubation. (C) *Pseudomonas* sp. after 58-day incubation.

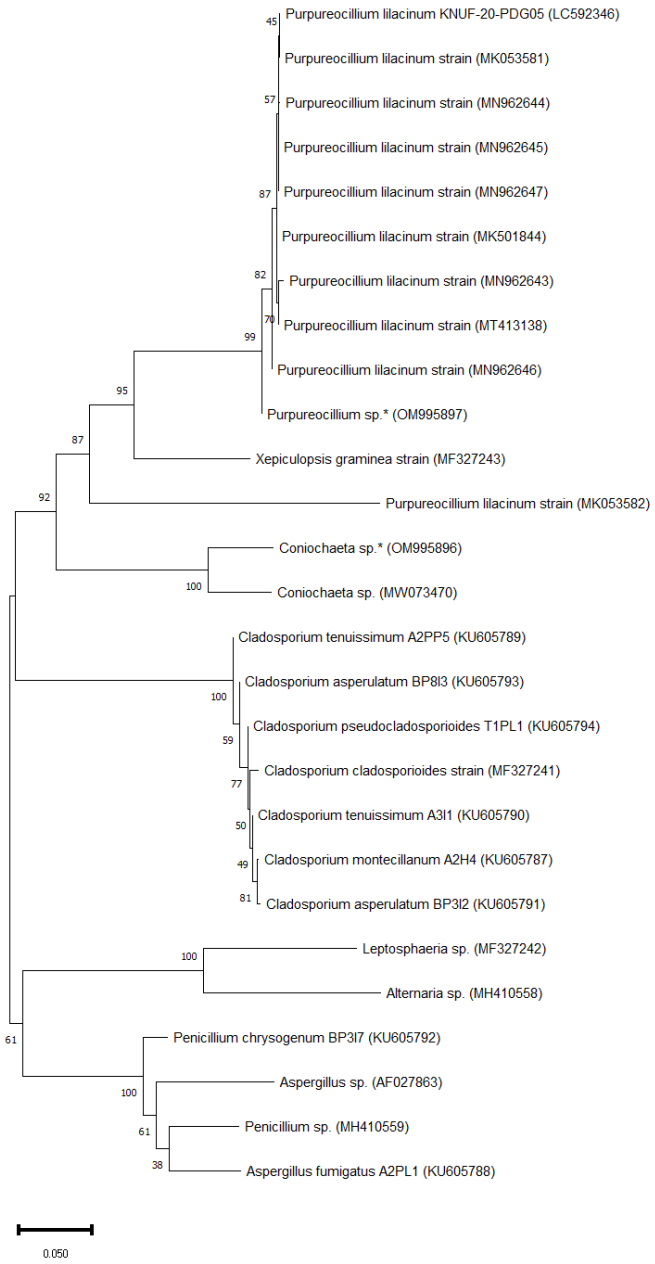


Figure 3. A phylogenetic tree showing relationship between fungal strains (PLI, LLI) and related polymer-degrading fungal strains. The phylogenetic tree was generated using neighbor-joining method. Values at branch nodes indicates as percentage bootstrap values based on 1,000 replicates. Evolutionary distances are computed through the maximum composite likelihood method and represent substitutions per nucleotide position. NCBI nucleotide accession numbers for each sequence are described in parentheses. An asterisk identifies the fungal strains degrading PUR in this study.

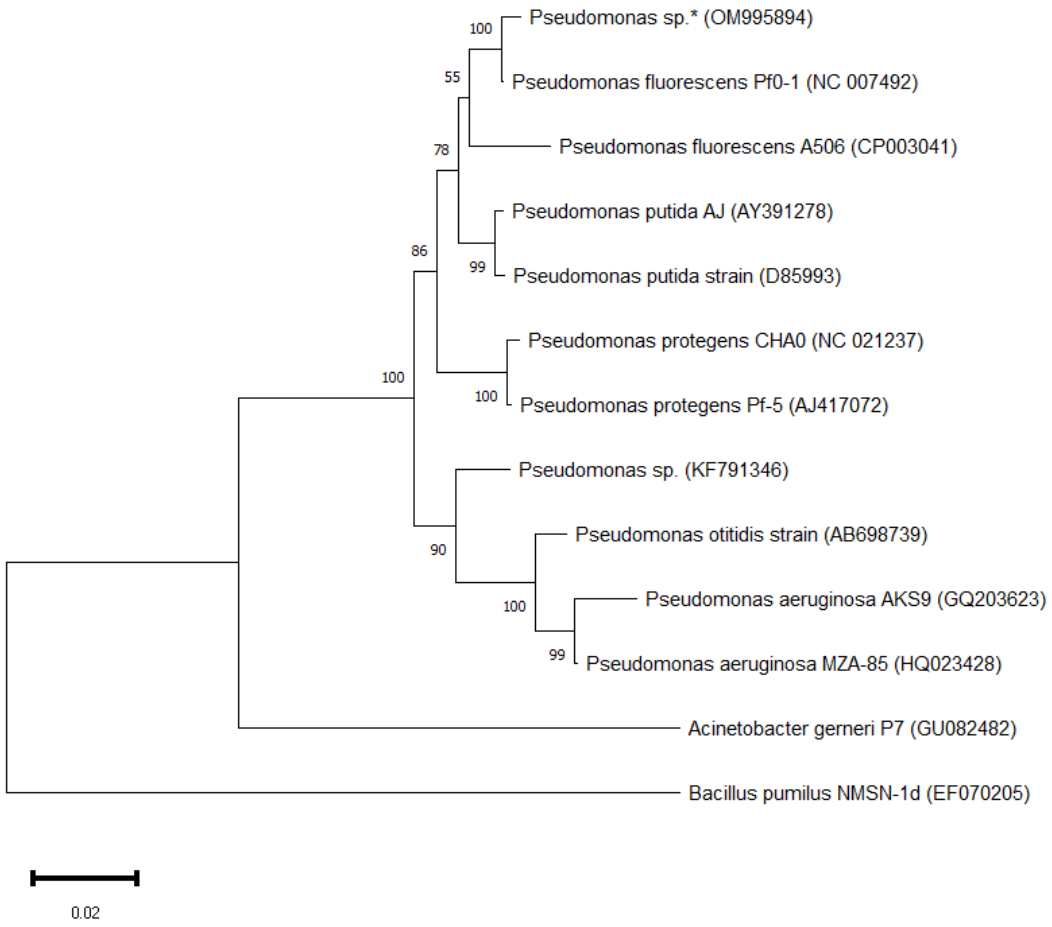


Figure 4. A phylogenetic tree showing relationship between bacterial strain (PGB) and related *Pseudomonas* species. The phylogenetic tree was generated using neighbor-joining method. Values at branch nodes indicates as percentage bootstrap values based on 1,000 replicates. Evolutionary distances are computed through the maximum composite likelihood method and represent substitutions per nucleotide position. NCBI nucleotide accession numbers for each sequence are described in parentheses. An asterisk identifies the bacterial strain degrading PUR.

Table 1 Polymer-degrading fungal strains. Polymers include polyurethane (PUR), polylactic acid (PLA), low density polyethylene (LDPE) and Polycarbonate (PC).

Species and/or strains	Substrates	Accession number	References
Cladosporium cladosporioides	PUR	MF327241	Brunner et al., 2018[15]
Leptosphaeria sp.	PUR	MF327242	
Xepiculopsis graminea	PUR	MF327243	
Alternaria sp. P2a1	PUR	MH410558	Magnin et al., 2019[42], Peterson et al., 2017[70]
Penicillium sp. MMP3b	PUR	MH410559	
Aspergillus sp. MMP3c1	PUR	AF027863	
Penicillium chrysogenum BP3I7	PUR	KU605792	Álvarez-Barragán et al., 2016[34]
Aspergillus fumigatus A2PL1	PUR	KU605788	
Cladosporium pseudocladosporioides T1PL1	PUR	KU605794	
Cladosporium tenuissimum A2PP5	PUR	KU605789	
Cladosporium asperulatum BP8I3	PUR	KU605793	
Cladosporium tenuissimum A3I1	PUR	KU605790	
Cladosporium montecillanum A2H4	PUR	KU605787	
Cladosporium asperulatum BP3I2	PUR	KU605791	
Purpureocillium lilacinum KNUF-20-PDG05	PLA	LC592346	Lee et al., 2021[71]
Purpureocillium lilacinum strains	LDPE	MK053581	Spina et al., 2021[67]
		MK053582	
		MK501844	
		MN962643	
		MN962644	
		MN962645	
		MN962646	
		MN962647	
		MT413138	
Coniochaeta sp.	PC	MW073470	Palermo et al., 2021[68]

Table 2 Polymer-degrading bacterial strains. Polymers include polyurethane (PUR), polyethylene (PE) and polystyrene (PS).

Species and/or strains	Substrates	Accession number	References
Pseudomonas protegens Pf-5	PUR	AJ417072	Biffinger et al., 2014[72]
Pseudomonas aeruginosa MZA-85	PUR	HQ023428	Shah et al. 2013[46]
Pseudomonas putida ATCC 17484	PUR	D85993	El-Sayed et al., 1996[73]
Pseudomonas protegens CHA0	PUR	NC_021237	Hung et al.,2016[74]
Pseudomonas fluorescens A506	PUR	CP003041	
Pseudomonas fluorescens Pf0-1	PUR	NC_007492	
Pseudomonas aeruginosa AKS9	PUR	GQ203623	Mukherjee et al., 2011[75]
Pseudomonas otitidis strain	PUR	AB698739	Peng et al., 2014[76]
Acinetobacter gerneri P7	PUR	GU082482	Howard et al., 2012[45]
Bacillus pumilus NMSN-1d	PUR	EF070205	Nair and Kumar, 2007[77]
Pseudomonas sp. E4	PE	KF791346	Yoon et al., 2012[78]
Pseudomonas putida AJ	PS	AY391278	Danko et al., 2004[79]

Degradation of Impranil

Impranil screening (solid and liquid culture)

The ability of the isolates, *Pseudomonas* sp. and *Purpureocillium* sp., to degrade Impranil was conducted by growth on mineral salt medium with Impranil 0.6% as a carbon or a nitrogen source. After 2 days at 30°C, *Purpureocillium* sp. showed distinct 'halo' in the plates with Impranil as the sole carbon source, whereas 'halo' was observed on the plates with Impranil as nitrogen or carbon source by *Pseudomonas* sp. were observed on the plates (Fig. 5). 'Halo' was not visible near the inoculi on the plates in the presence of Impranil as nitrogen sources by *Purpureocillium* sp., however, its growth was observed. On the other hand, all isolates, fungal and bacterial strain, were able to degrade Impranil in liquid mineral salt medium within 2 days with displaying the changes of the liquid cultures from milky white to almost opaque (Fig. 6). Almost all of the Impranil as carbon sources in liquid culture were degraded by *Purpureocillium* sp. as described in Fig. 6.

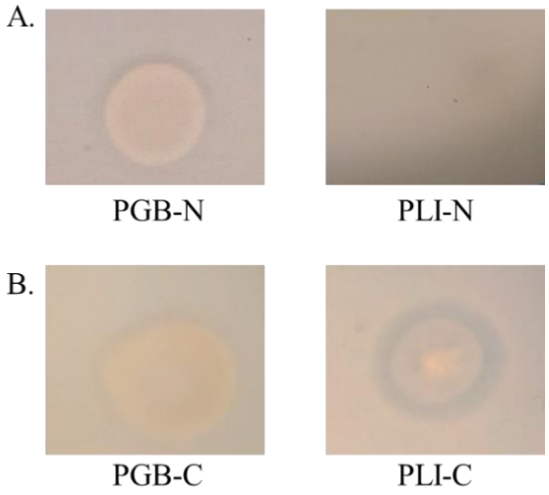


Figure 5. Degradation of PUR in Petri dishes by bacterial and fungal strains in presence of Impranil as nitrogen or carbon source after 2 days. (A) *Pseudomonas* sp. (left and designated as PGB-N) and *Purpureocillium* sp. (right and designated as PLI-N) degrading Impranil as a nitrogen source. (B) *Pseudomonas* sp. (left and designated as PGB-C) and *Purpureocillium* sp. (right and designated as PLI-N) degrading Impranil as a carbon source.

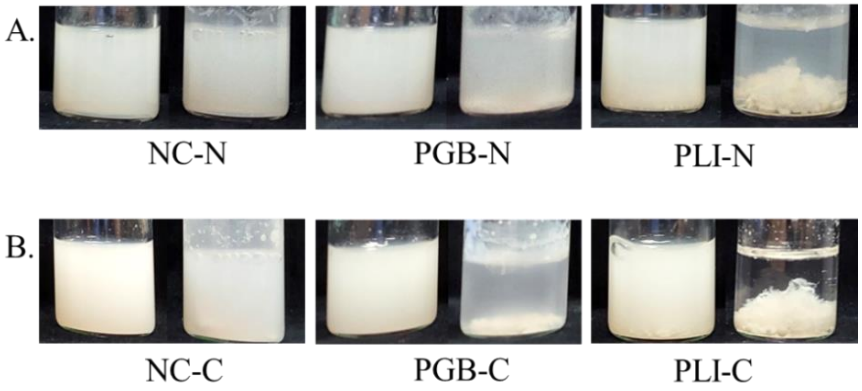


Figure 6. Degradation of PUR in liquid medium by bacterial and fungal strains in presence of Impranil as nitrogen or carbon source after 2 days. (A) Control (left), *Pseudomonas* sp., *and Purpureocillium* sp. (right) degrading Impranil as a nitrogen source. (B) Control (left), *Pseudomonas* sp., *and Purpureocillium* sp. (right) degrading Impranil as a carbon source.

FTIR analysis

FTIR analyses of the liquid cultures containing Impranil were performed by ATR-FTIR after 2 days of incubation (Fig. 7). Inoculated samples inoculated by fungal strain with Impranil as nitrogen or carbon sources displayed a reduction resulting disappearance of the peak at 1,731 cm^{-1} related to the ester fraction.[50] The peak was disappeared when Impranil was supplemented as carbon sources, however, slight intensity of the peak remained in the liquid culture with Impranil as nitrogen sources. The peak at 1,640 cm^{-1} representing C=O-stretch in inoculated vials was attributed to amide or protein from the isolates.[80, 81]

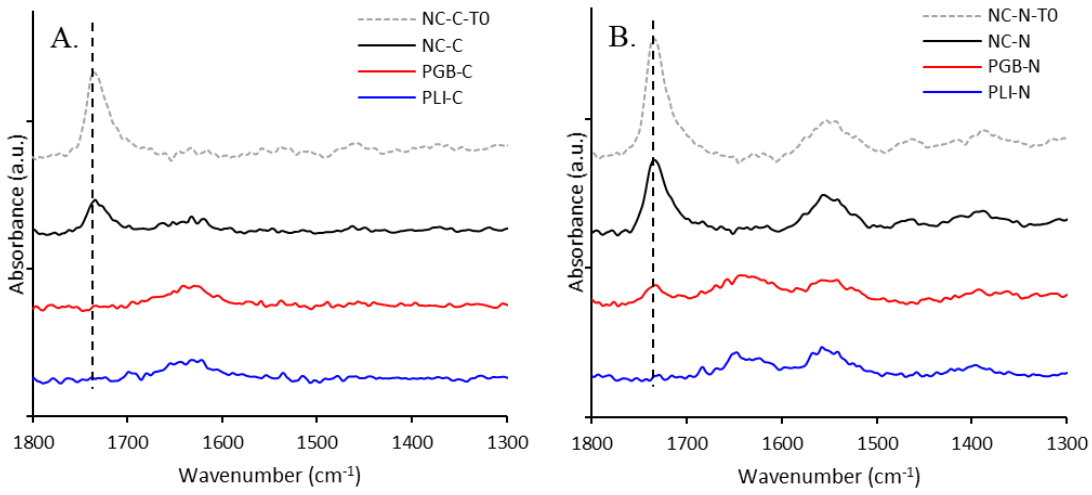


Figure 7. ATR-FTIR spectra of Impranil degraded by abiotic control on day 0 (*dash line*) and on day 2 (*black line*), *Pseudomonas* sp. (*red line*) and *Purpureocillium* sp. (*blue line*) on day 2. (A) Degradation in the presence of Impranil as a carbon source. (B) Degradation in the presence of Impranil as a nitrogen source. The peak denoted by a dash line at 1,731 cm^{-1} corresponding the ester linkage almost disappeared after 2 days.

Enzyme activities detected in supernatant

Extracellular esterase activity was monitored in culture supernatant on day 0, 4 and 12 for *Purpureocillium* sp. and day 0, 14 and 22 for *Pseudomonas* sp. (Table 3, Fig. 8). The activity of the bacterial strain in the presence of Impranil as nitrogen or carbon sources showed both 1.2 U. L^{-1} on the 14th day. Similarly, fungal strain on the 12th day the culture supernatant exhibited 1.6 and 10.6 U. L^{-1} in the presence of Impranil as nitrogen source or carbon source, respectively. Steady increase in activity of fungal strain was only observed in the presence of Impranil as carbon source.

Table 3. Time course of extracellular esterase activities of bacterial and fungal strains in presence of Impranil as nitrogen (designated as PGB-N and PLI-N, respectively) or carbon source (designated as PGB-C and PLI-C, respectively). A gradual increase in activity was detected in fungal strain with Impranil as carbon source.

Day	Activity (U.L^{-1})		Day	Activity (U.L^{-1})	
	PLI-N	PLI-C		PGB-N	PGB-C
0	-	-	**0**	-	-
4	1.3 ± 0.4	6.1 ± 3.3	**14**	1.2 ± 0.7	1.2
12	1.6 ± 1.6	10.6 ± 3.2	**22**	-	-

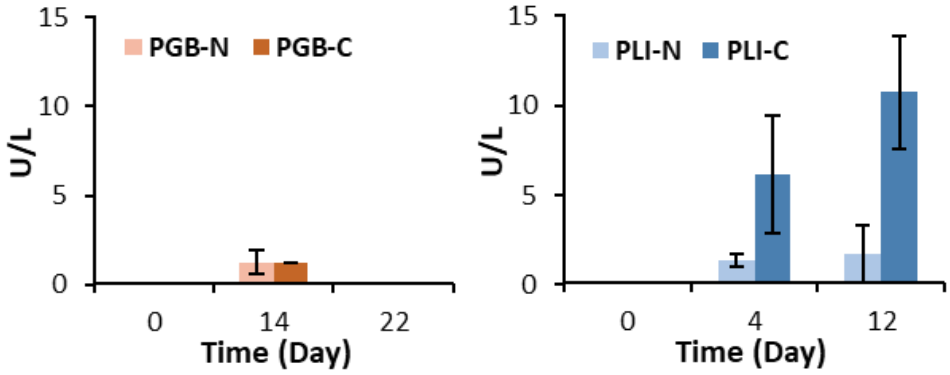


Figure 8. Time course of extracellular esterase activities of bacterial and fungal strains in presence of Impranil as nitrogen.

PUR foam degradation

Bacterial growth, pH, and esterase activity

In order to explore the relationship between the fungus and the bacterium during PUR foam degradation, the bacterial growth, pH, and extracellular activity was recorded. Cultures were replenished with mineral salt medium except nitrogen sources on day 22. Abiotic control (designated as NC) was recorded to see the effect of only chemical in the medium, while biotic control (designated as PC) was observed to determine any effect of fungal supernatant on growth of PGB. *Pseudomonas* sp. could grow in the presence of PUR foam as nitrogen sources (Fig. 9). The bacterial cultures containing the supernatant (PGB+PLI) was detected as faster growth compared to pure culture, however, pure culture reached similar OD_{585} on day 42.

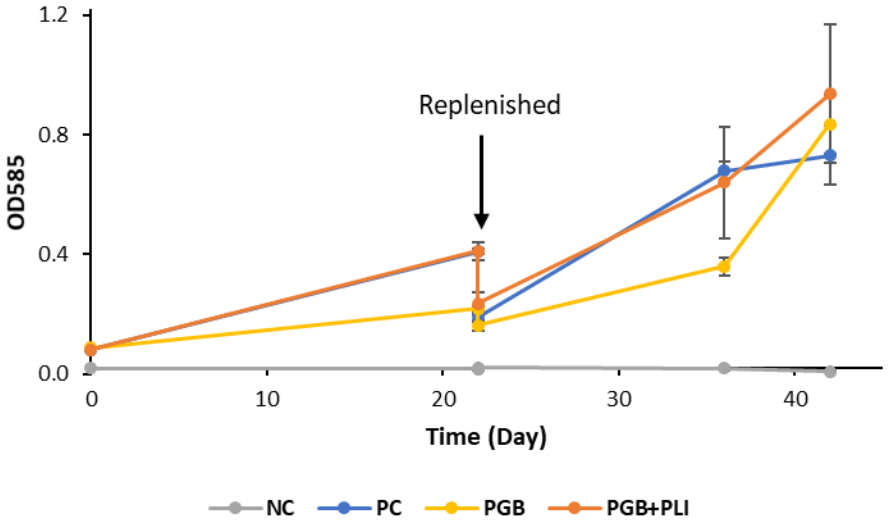


Figure 9. Changes in Abs_{585} against time course. Abiotic control (NC), *Pseudomonas* sp. (PGB), and *Pseudomonas* sp. with fungal supernatant (PGB+PLI) in MSM with PUR foam as nitrogen sources (PGB). biotic control containing *Pseudomonas* sp. with fungal supernatant (PC).

As a result of growth, pH in the inoculated vials steadily increased to reach approximately 8.5 as shown in Fig. 10. In the abiotic control vial, the pH value did not change. A similar result was reported by Gautam et al. in a study related PUR foam by *Pseudomonas* sp.[66] Increment in pH was attributed to the metabolites produced from PUR foam biodegradation. It is noted that pH in the biotic control increased. The increment of pH is because fungal supernatant was induced with Impranil, thus, containing metabolites involved with biodegradation of Impranil by PLI.

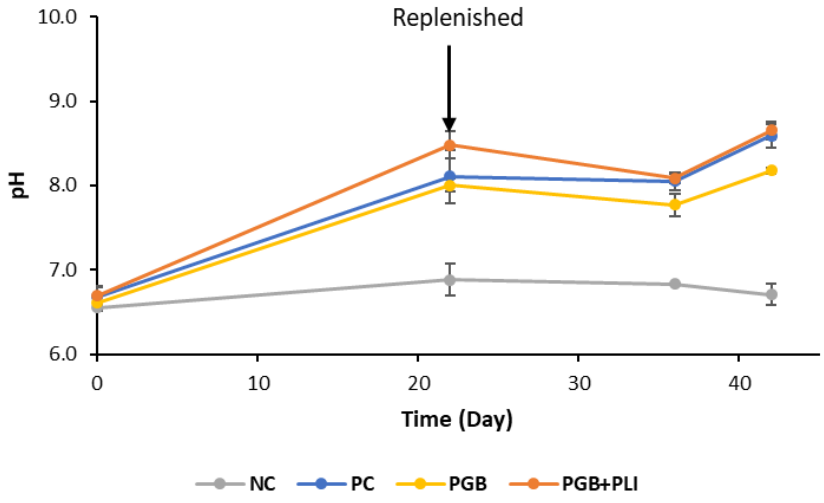


Figure 10. Time course changes in pH.

A gradual increase in extracellular esterase of all bacterial cultures was observed during initial bacterial growth from day 1 to day 22, then the activities decreased until day 42, the end of the experiment. Activity in pure culture was almost negligible from day 0 to day 22 (from 0.6 to 1.8 U L^{-1}) compared to the bacterial cultures supplemented with fungal supernatant. No esterase activity was detected in cultures with sole bacterial strain

from day 36 to the end of experiment. It is noteworthy that the activity in culture (PGB+PLI) with PUR foam maintained longer until the experiment ended (Table 4 and Fig. 11). The activity of culture (PGB+PLI) was approximately 7 times higher than that of pure culture on day 22. Furthermore, it was 8 times higher than that of biotic control on day 36. The activity was only observed in culture (PGB+PLI) as 3 U L^{-1} on day 42. Esterase activity in the biotic control (PC) is recorded due to the metabolites of Impranil degradation in the fungal supernatant. This is consistent with increment of pH in the control. However, the activity in the control was almost disappeared on day 36 (0.6 U L^{-1}). In the abiotic control vial, esterase activity was not detected during the test.

Table 4. Extracellular esterase activities measured for 42 days.

Day	**Activity (U.L^{-1})**			
	NC	**PC**	**PGB**	**PGB+PLI**
0	0.0	7.1 ± 0.1	0.6 ± 0.4	7.1 ± 0.1
22	0.0	11.5 ± 1.9	1.8 ± 0.6	12.3 ±2.5
36	0.0	0.6 ± 0.4	0.0	4.7 ±0.2
42	0.0	0.0	0.0	3.0

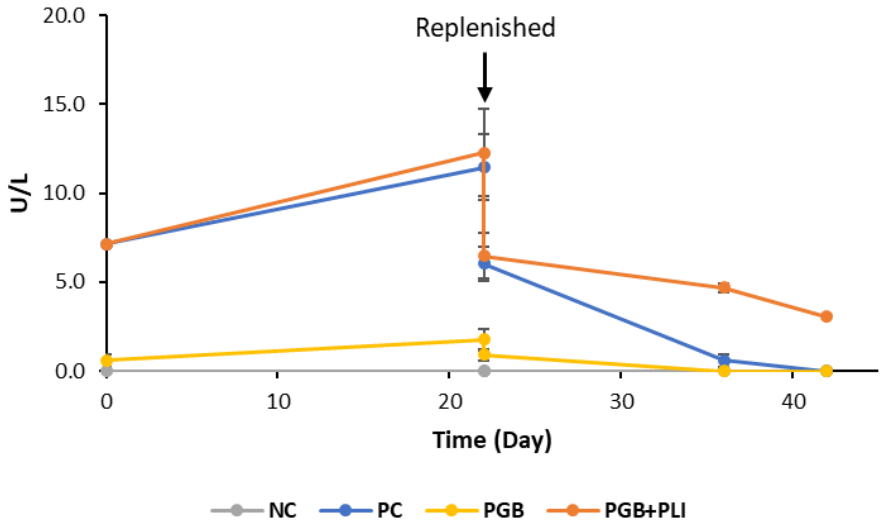


Figure 11. Extracellular esterase activities measured for 42 days.

FTIR analysis

Structural changes on surfaces of PUR foams were determined using FTIR analysis. It is noted that there is no change in abiotic control (NC) on day 42 compared to day 0 indicating chemicals in the medium has no effect on PUR degradation. (Fig. 12). On the other hand, the intensity of the peak at 1,731 cm^{-1} PUR foam of culture (PGB+PLI) decreased more than that of pure culture (PGB) representing that degradation efficiency has been enhanced by culture (PGB+PLI) (Fig. 13).[34] A peak at 3,325 cm^{-1} assigned to the N-H linkage to became wider due to a broad peak at 3,600-3,200 cm^{-1} derived from biofilm exopolysaccharides.[80, 82] Decrements in the intensity of the peaks at 1,535 and 1,225 cm^{-1} representing C-N-H linkage in the urethane bond was found in the inoculated vials (Appendix).[83]

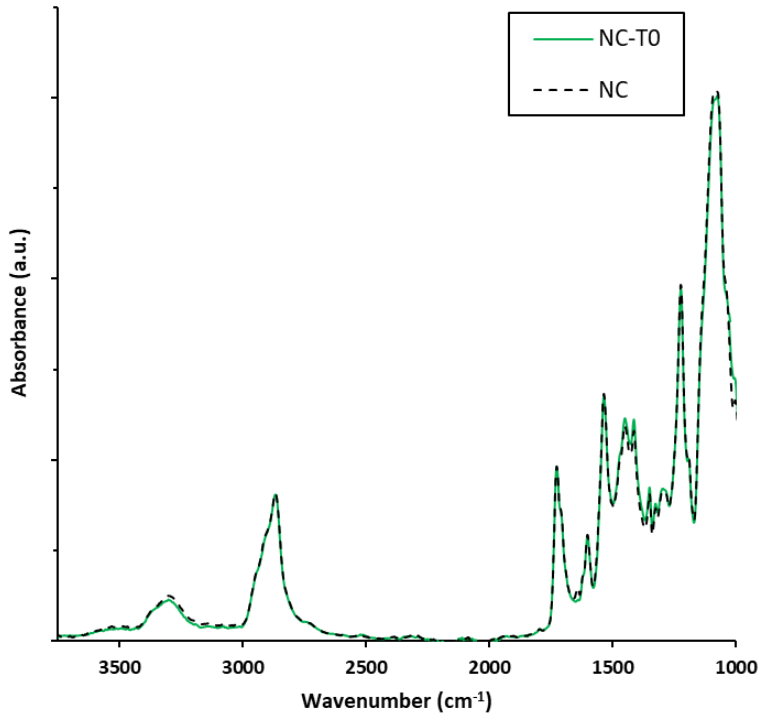


Figure 12. ATR-FTIR spectra of PUR foams as nitrogen sources for abiotic control on day 0 (*green line*) and on day 42 (*dash line*).

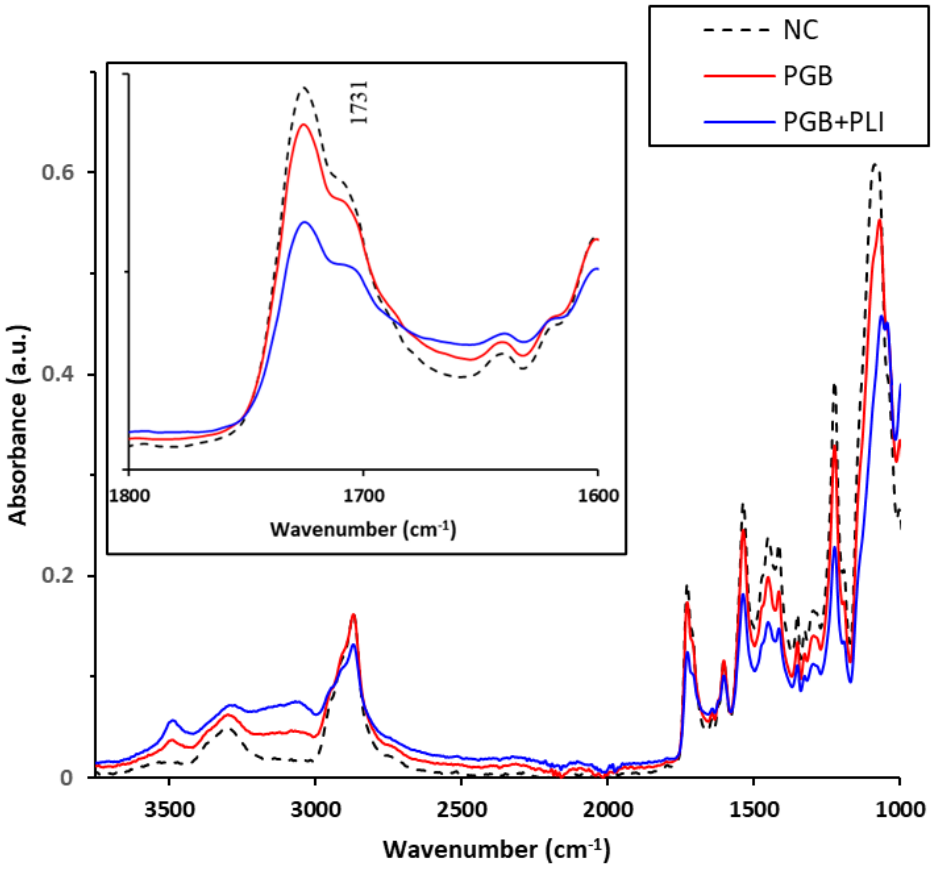


Figure 13. ATR-FTIR spectra of PUR foams as nitrogen sources after 42-day cultivation in media with abiotic control (*dash line*), *Pseudomonas* sp. (*red line*), and *Pseudomonas* sp. with fungal supernatant (*blue line*)

SEM

Changes in the shape and structure of PUR foam were investigated. Analysis showed the alterations in physical properties of PUR foams, such as size and color, after 42 days of incubation with bacterial strain compared to untreated control (Fig. 14). Evidence of biodegradation by cultures is found in SEM micrographs, in which large number of the adherence of bacterial strain to the surface of PUR foam of pure culture and culture (PGB+PLI) can be found, whereas no change was observed in abiotic control (Fig. 14A). The foam with culture (PGB+PLI) was more degraded than the foam with sole bacterial strain displaying widespread cracks and rough surfaces.

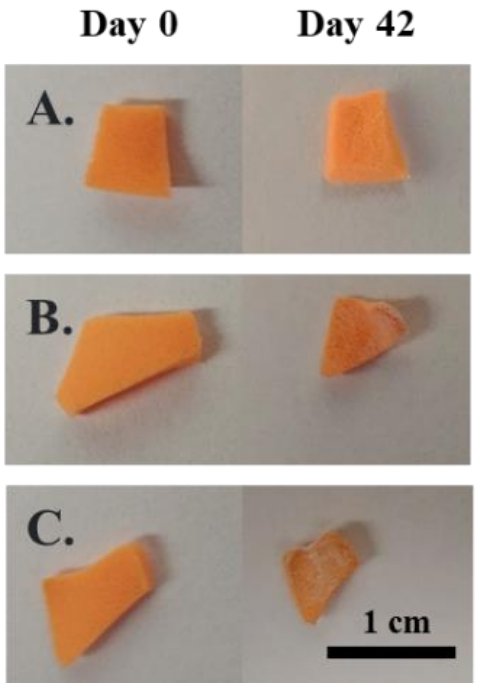
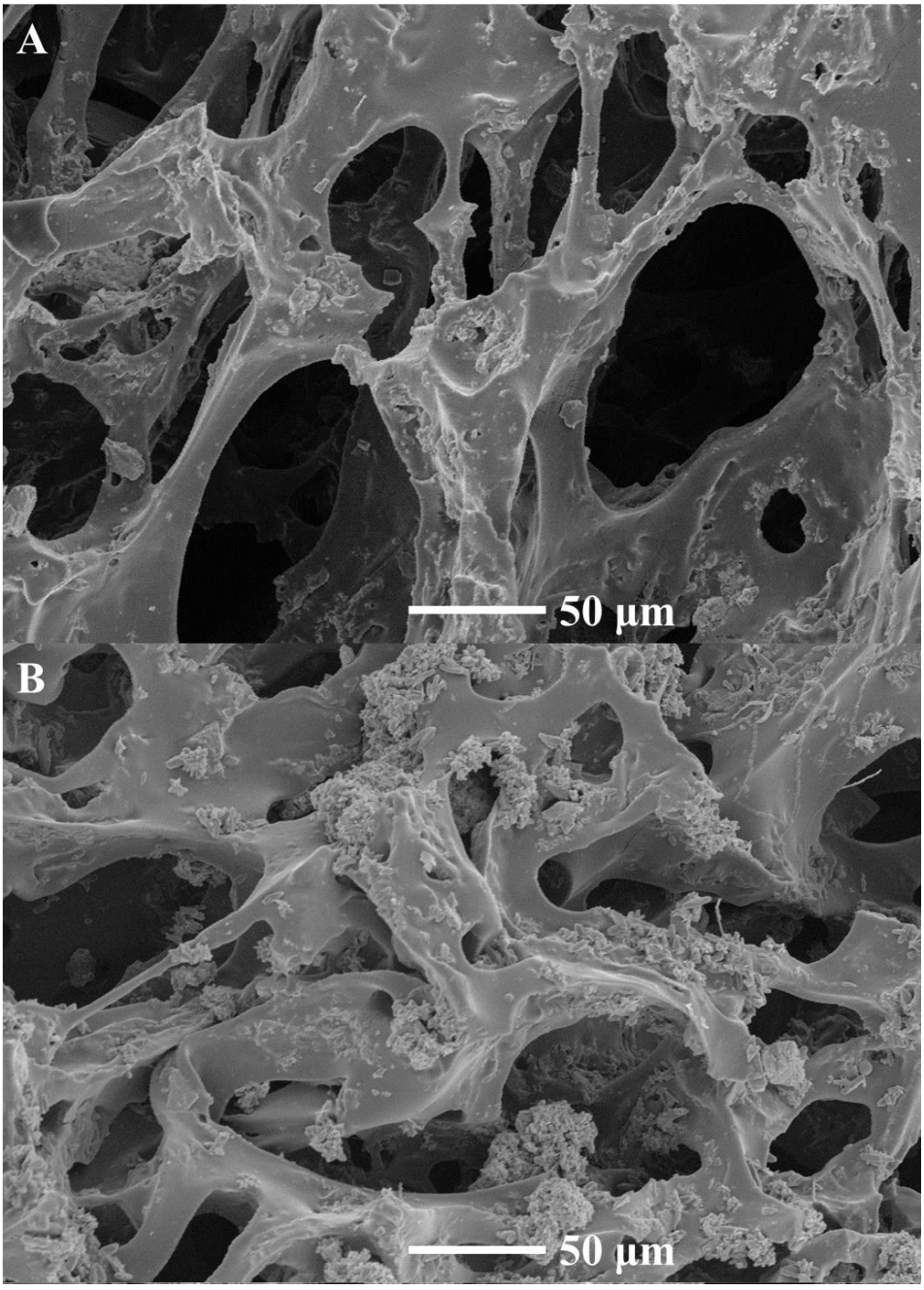


Figure 14. Biodegradation of PUR foams by *Pseudomonas* sp. after 42-day cultivation (A) Control foam. (B) PUR foam degradation by *Pseudomonas* sp. (C) PUR foam degradation by culture (PGB+PLI).


A
50 µm
B
50 µm

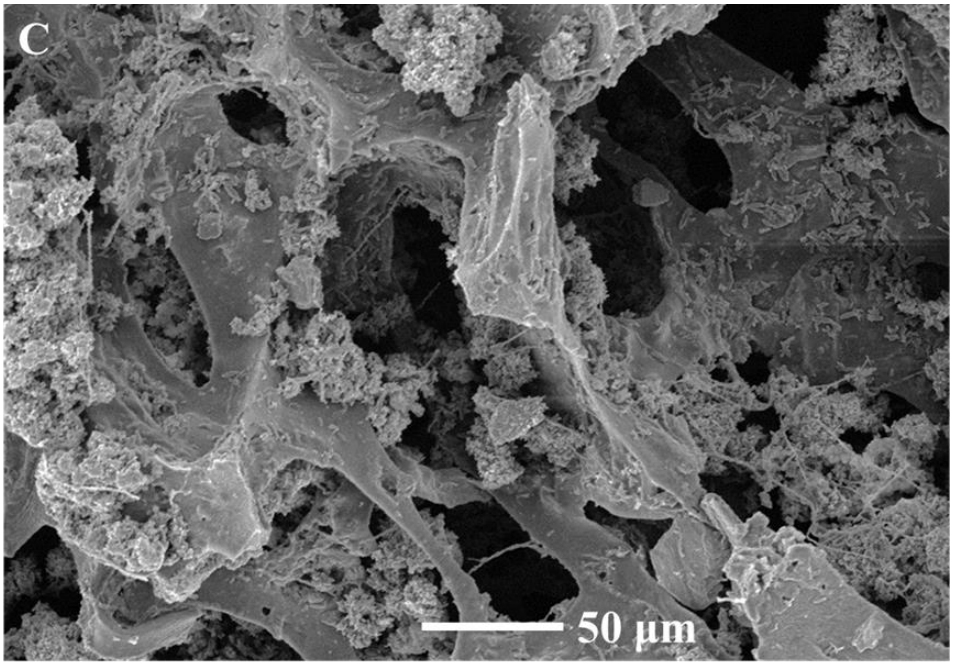


Figure 15. Scanning electron micrographs of PUR foams degraded by bacterial culture. (A) Control foam. (B) PUR foam degradation by *Pseudomonas* sp. (C) PUR foam degradation by culture (PGB+PLI).

Metabolites from PUR degradation

The monomers produced from degradation of PUR foam by bacterial strain were determined by GC with FID. Two peaks corresponding diethylene glycol (designated as DEG), and adipic acid (designated as AA) were detected at retention time of 13.8 min and 17.9 min in treated vials, respectively. Peak area belonging to DEG in culture (PGB+PLI) was detected highest among the samples on day 22, resulting 3 times and 0.1 times higher than that of bacterial pure culture (PGB) and biotic control (PC), respectively (Fig. 16). DEG in culture (PGB+PLI) maintained greatest level among the samples until the end of the experiment (day 42), which is approximately 3.5 times higher than biotic control.

AA was observed in culture supplemented with fungal supernatant displaying high peak area due to the metabolites from Impranil on day 0 (Fig. 17). However, the peak area of AA of culture (PGB+PLI) and biotic control decreased during 42-day incubation. The high decrement of AA in biotic control was observed (0.6-fold) on day 42 compared to day 22. However, it is recorded that AA maintained the same level (0.02-fold decreased) during the 20-day incubation period in culture (PGB+PLI). Actually, the peak area assigned to AA in culture (PGB+PLI) is 5 times higher than that of biotic control on day 42. Peaks corresponding both of DEG and AA in pure culture and abiotic control were almost negligible.

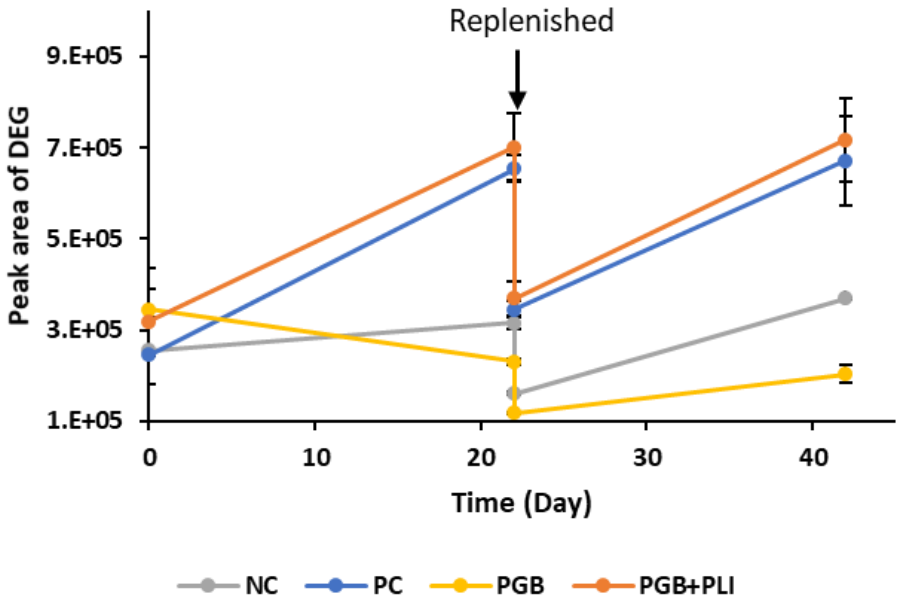


Figure 16. Changes in peak area assigned to diethylene glycol (DEG) against time course. Abiotic control (NC), *Pseudomonas* sp. (PGB), and *Pseudomonas* sp. with fungal supernatant (PGB+PLI) in MSM with PUR foam as nitrogen sources (PGB). biotic control containing *Pseudomonas* sp. with fungal supernatant (PC).

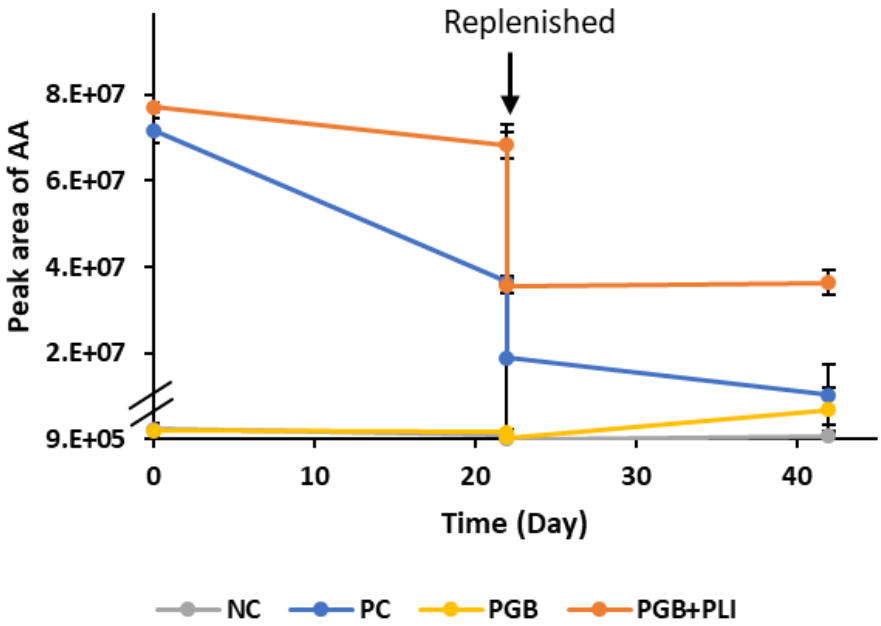


Figure 17. Changes in peak area assigned to adipic acid (AA) against time course. Abiotic control (NC), *Pseudomonas* sp. (PGB), and *Pseudomonas* sp. with fungal supernatant (PGB+PLI) in MSM with PUR foam as nitrogen sources (PGB). biotic control containing *Pseudomonas* sp. with fungal supernatant (PC).

Discussion

Polyurethanes (PUR) are extensively used as a wide array of products in modern life due to their versatility. However, accumulation after the end of their life and increasing production poses threat to the environment. PUR degradation by microbial and enzymatic means is a promising solution to pollution problems. PUR waste can be depolymerized into higher value monomers, or completely degraded into carbon dioxide, water, and biomass via mineralization.[84] However, biodegradation of PUR is generally slow and it takes enormous time.[85] It is necessary to improve the rate and efficiency of PUR degradation.

Several studies reported PUR degradation by fungi and bacteria since the 1980s [86]. The mechanism of fungal degradation is not fully studied, and it is generally known that fungi can mostly cause minor changes of the properties contributing complete degradation.[10, 21] Degradation by filamentous fungi is more efficient than bacterial degradation since they colonize the compound with and secrete abundant enzymes involved in degradation.[9, 41, 42] While fungal degradation on PUR generally exhibits an abiotic degradation, bacterial degradation leads major breaks in the structure of PUR leading a site-directed specific attack.[10, 41, 43] However, although fungi and bacteria may have difference in their capability to degrade PUR, very few have been reported to utilized both their culture related to PUR degradation. PUR biodegradation studies mainly focused on utilizing pure cultures.[41, 47, 48] Only a few studies reported degradation of PUR utilizing both fungal and bacterial cultures providing limited information such as changes in weight loss and tensile strength.[16, 87] Thus, current study employed cultures of isolated bacterial strain (*Pseudomonas* sp.) and fungal strain (*Purpureocillium* sp.) to make the development of PUR degradation. This study focused on understanding the mechanisms of PUR biodegradation in soil where fungal and bacterial strains are found living together and proper cultures for degradation.

In this study, three isolates including fungal and bacterial strains capable to degrade PUR are found to be *Pseudomonas* sp., *Coniochaeta* sp., and *Purpureocillium lilacinum* type strain. Seawater at 50% was supplemented into the medium used for isolation to simulate the condition of saline area (landfill and bay), which PUR-degrading microorganisms were originated from.[88] Recent study reported that *Purpureocillium*

lilacinum caused strong oxidation and changes in the PE film utilizing it as carbon source.[67] Although PE and PUR have different structural characteristics, *Purpureocillum lilacinum* is found to trigger oxidative transformation which can be preferable for polymer degradation based on reference. Lastly, *Coniochaeta* sp. have deteriorated CD-R (Compact Discs Recordable) composed of a polycarbonate plastic substrate.[68] Strain shown to close to our *Pseudomonas* sp., such as *P. chlororaphis*, has been reported to degrade Impranil and PUR foam and to express esterase during degradation.[66, 89] PUR deterioration has been monitored with *P. fluorescens* under various nutrient condition.[90]

PUR degradation ability of *Pseudomonas* sp. and *Purpureocillium* sp. was determined by using PUR dispersion, Impranil, before PUR foam test. As a result, *Pseudomonas* sp. utilized Impranil as carbon or nitrogen sources, revealing that the esterase activities of the culture supernatant on day 14 were measured to be both 1.2 U $.L^{-1}$,which are observed similarly in other works.[66, 91] Extracellular esterase activity of the fungal strain was detected higher (10-fold) than the bacterial isolate during the period of approximate 2-week incubation in the presence of Impranil as carbon sources. This result is consistent with Impranil screening in solid culture. *Purpureocillium* sp. exhibited a distinct 'halo' on Impranil plates supplemented as carbon sources compared to *Pseudomonas* sp. PUR Degradation by filamentous fungi is found to be more effective than bacterial degradation since they can colonize and secrete abundant enzymes involved in degradation.[41, 42, 92] Both *Purpureocillium* sp. and *Pseudomonas* sp. using Impranil as a nitrogen source have low extracellular esterase activities, resulting a decrease in the level of degradation. In several studies, nitrogen source generally was provided in to the

medium to prevent abolishment of degradation.[89, 93, 94] However, even though low enzyme activity was exhibited from both degraders in presence of Impranil as a carbon source, the peak at 1,725 cm^{-1} assigned to ester linkage in PUR disappeared due to high accessibility between extracellular enzymes and water-soluble substrate.[95] The peak in the bacterial culture containing Impranil as nitrogen source was slightly remained compared to that of fungal strain. Based on these results of halo appearance and esterase levels, *Purpureocillium* sp. was considered as efficient PUR degrader compared to *Pseudomonas* sp.

Incubation of fungi with polymers can enhance secretion of enzymes responsible to hydrolyze polymers. Fungi including *Aspergillus* sp., *Fusarium* sp., and *Lanatonectria* sp. were cultivated with aliphatic and aromatic polyesters have shown increased activity toward model substrate of polymers, producing degradation by-products.[96] Fernandes et al. demonstrated experimental results on thermoplastic polyurethanes with a consortium of both *Aspergillus niger* and *Pseudomonas aeruginosa*. The consortium after 30 days showed that the weight loss improved significantly, providing a synergistic activity between fungal and bacterial strains during degradation.[16] In this study, *Purpureocillium* sp. was incubated with a model PUR, Impranil to induce enzymes active on PUR to prepare supernatant. Cultures containing *Pseudomonas* sp. culture with the fungal supernatant was evaluated toward degradation of PUR foams. Mostly, PUR biodegradation by bacteria is slow using PUR as a carbon and nitrogen source, thus, PUR foams were supplemented as a nitrogen source with succinate as a carbon source in this

study.[47] Effect of succinate as a carbon source was demonstrated in PUR degradation by *Pseudomonas* sp.[69]

Esterase activities in samples supplemented with the supernatant, such as biotic control (PC) and culture (PGB+PLI), were shown to increase on day 22 compared to day 0. Even though activities from both exhibited comparable, however, that of culture (PGB+PLI) were 7% higher than PC sample since esterase was produced by the bacteria and the fungal enzymes in the supernatant were preferably active on PUR foams due to substrate specificities.[97] Moreover, esterase activity in culture (PGB+PLI) was detected 7 times higher than pure culture with sole bacteria on the day. Based on the results that esterase activity in culture (PGB+PLI) remained most longer by the end of the experiment confirms that supplementing cultures with fungal supernatant might be beneficial for degradation of PUR.

FTIR Analysis of changes in PUR foams degraded by microorganisms has been studied in several papers.[12, 98] The analysis allows understanding of mechanisms for PUR degradation. Carbonyl signal (1,731 cm^{-1}) of PUR foams attacked by culture (PGB+PLI) showed more evident decrements compared to that of pure bacterial culture and biotic control, suggesting degradation of ester bonds and urethane groups in the structure. Degradation of urethane groups can be also observed in changes of the peaks at 1,535 and 1225 cm^{-1} corresponding the C-N-H linkage.[50] Moreover, spectrum around at 3,600-3,200 cm^{-1} in culture (PGB+PLI) was most widely developed due to biofilm attributed to *Pseudomonas* sp. adherence to the surface of PUR foams. These results provide evidence of active PUR degradation in culture (PGB+PLI).[80, 82]

Some enzymes possess some properties allowing themselves to attach on the surface of insoluble substrates such PUR.[99, 100] Thus, PUR degradation by enzymes has been proposed as two-step degradation process. Enzymes responsible to degrade PUR first adhere on the surface followed by hydrolyzing the ester bonds in the structure.[20] In this study, large number of cell attachment and rough surface were observed in PUR foam in culture (PGB+PLI) by SEM analysis. The appearance of holes and cracks contributed by adherence indicated that *Pseudomonas* sp. in the culture utilized esterase enzymes leading degradation of PUR via hydrolysis.

A possible pathway of PUR was reported suggesting ester hydrolysis products such as diethylene glycol (DEG), adipic acid (AA), and trimethylol propane.[65] In our study, diethylene glycol (DEG) and adipic acid (AA) were selectively analyzed to determine the levels of degradation of PUR foams. PUR degradation study with *Pseudomonas chlororaphis*, one of close strains to our bacterial degrader, have reported that DEG concentration increased gradually during the incubation period.[66] In our study, the amount of DEG in culture (PGB+PLI) with PUR foams as the sole nitrogen source was greatest among other conditions. Even though DEG in the culture was a comparable level to that of biotic control (PC), however, the peak area of DEG was slightly higher (7%) than that of PC, with 3.5 times higher than that of pure culture on day 42. These results can be described that fungal supernatant in culture (PGB+PLI) attacked PUR foams to breakdown the molecular structures facilitating the access of *Pseudomonas* sp. to initiate further degradation. On the other hand, changes of AA, which is one of metabolites produced from PUR degradation, was observed differently in our experimental results.

The ability of PUR degrader, *Pseudomonas aeruginosa*, to utilize monomer of PUR such as 1,4-butanediol and adipic acid as a source of carbon was reported.[46] In our study, extensive amount of AA in fungal supernatant derived from Impranil induction was detected on day 0. AA in vials of culture (PGB+PLI) and PC decreased from day 0 to day 22. Increase of AA was only observed in the culture on day 42, suggesting that culture (PGB+PLI) might have accomplished greater degradation.

CHAPTER V

CONCLUSIONS AND FUTURE STUDIES

Summary and conclusions

Polyurethanes (PUR) are polymers synthesized from various precursors and one of most widely used plastics. PUR are extensively used due to their chemical and physical properties. Their resistance and resilience lead them to various applications in industries and customer products, such as packaging, apparel, and construction. The advantages of these polymers cause a significant cause due to increasing production. Moreover, PUR are slowly degraded leading accumulation to the environment. Although there have been several studies on PUR degradation by fungal and bacterial strains since the 1980s, few research on PUR degradation by cultures containing multiple strains has been conducted. This was the first study using fungal supernatant in bacterial degradation of PUR to demonstrate synergistic relationship between fungal and bacterial strains.

In this study, *Pseudomonas* sp. and *Purpureocillium* sp. able to utilize PUR as carbon and nitrogen sources were isolated from the environment. Both strains were able to grow on Impranil displaying clearance in liquid medium as nitrogen and carbon sources. It is noted that *Purpureocillium* sp showed significant clearance in the presence of Impranil as carbon sources. Since it was demonstrated that there were differences in their degrading behavior on PUR, thus we had the hypothesis that cultures with the two strains can enhance PUR biodegradation by employing a wide variety of enzymes.

Purpureocillium sp. were cultivated in liquid medium with Impranil as nitrogen and carbon sources prior to filtration to harvest supernatant for PUR foam experiment.

PUR foams degradation by cultivation of bacteria with fungal supernatant was analyzed for a period of 42 days. Extracellular esterase activity of culture (PGB+PLI) maintained greatest among the samples degrading PUR foam to be porous and tough surfaces. Changes in structure and chemistry in PUR foams were monitored by FTIR analysis. Higher biofilm development and significant decrease in ester linkage of PUR foams cultivated with culture (PGB+PLI) indicates successful PUR degradation process. Accordingly, increase in amount of degradation metabolites, i.e. DEG and AA, were detected in culture (PGB+PLI). Thus, we could confirm that application of culture with bacterial PUR degrader supplemented fungal supernatant can make PUR degradation more efficient.

Future studies

In this study, three different strains, bacterial and fungal species, were isolated from the sediment and were found to degrade PUR. It was demonstrated that a defined mixed culture containing two strains, using as culture of *Pseudomonas* sp. and supernatant of *Purpureocillium* sp. can enhance PUR degradation. However, the results of this study remained question future studies. Below are suggestions for future studies required our understanding of PUR degradation by culture employing bacterial and fungal strains.

Two isolates revealed degradation of Impranil, however, extracellular esterase activity from *Pseudomonas* sp. were significant low than that of fungal strain. Although it was an acceptable level compared to other studies involving esterase of *Pseudomonas* sp.,

measurement of specific activity and identification of other potential enzymes are also needed to elucidate enzymatic pathway for PUR degradation.

Degradation of PUR foam with culture (PGB+PLI) was carried under nitrogen-limiting condition, but fungal strain expressed increasing esterase activity under carbon-limiting condition in liquid medium with Impranil. Thus, different condition such as PUR foam as carbon sources can be performed to develop a method for selecting appropriate cultures of the two strains. In this study, fungal cultures were filtered to harvest cell-free supernatant to prevent competition between strains. However, in the environment, various strains are actually co-mingled together. Thus, direct cultivation containing actual fungal and bacterial cultures can be carried to understand the mechanisms of PUR degradation in the soil.

Further studies on the metabolic relationship between PUR degraders is required for application of cultures for PUR degradation.

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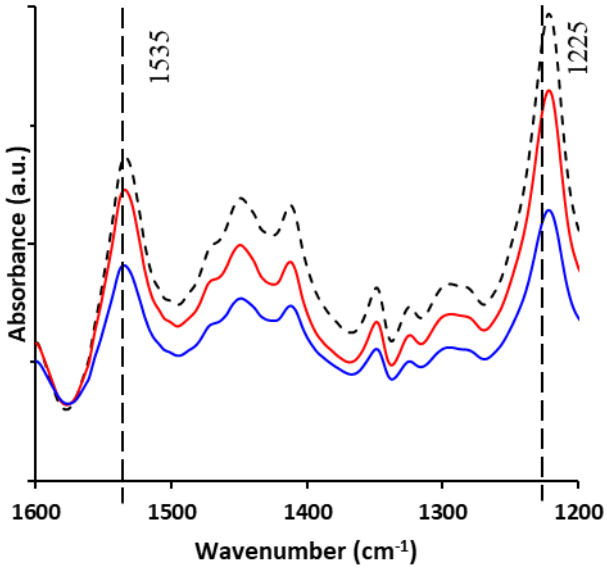
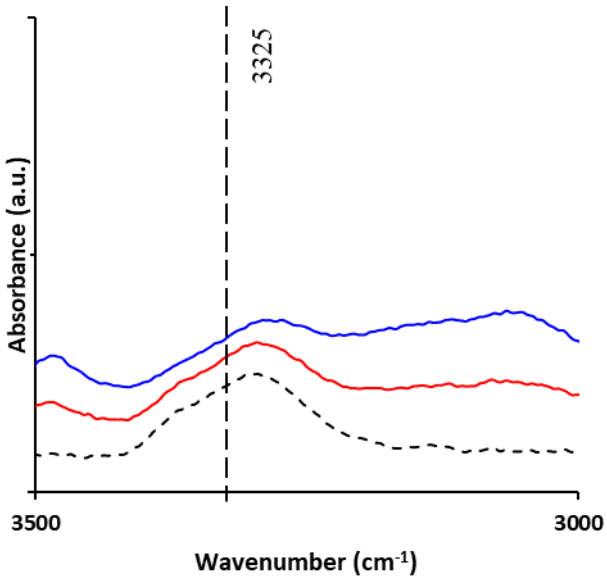
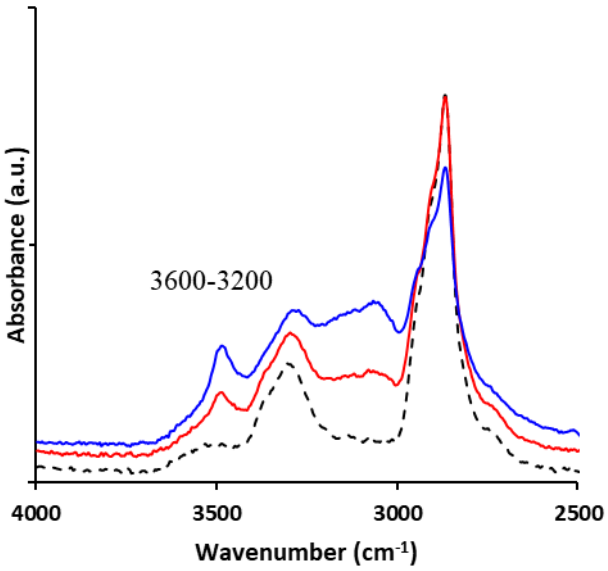
APPENDIX

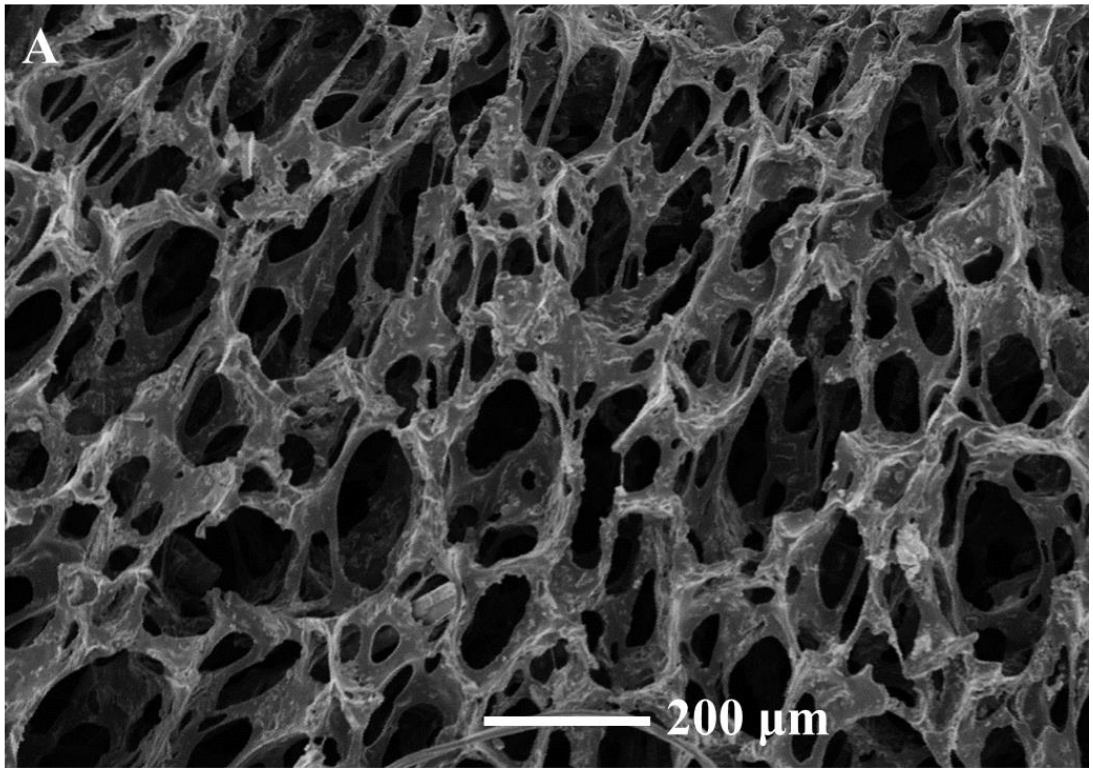
ATR-FTIR spectra of PUR foams as nitrogen sources after 42-day cultivation in media with abiotic control (*dash line*), *Pseudomonas* sp. (*red line*), and *Pseudomonas* sp. with fungal supernatant (*blue line*)

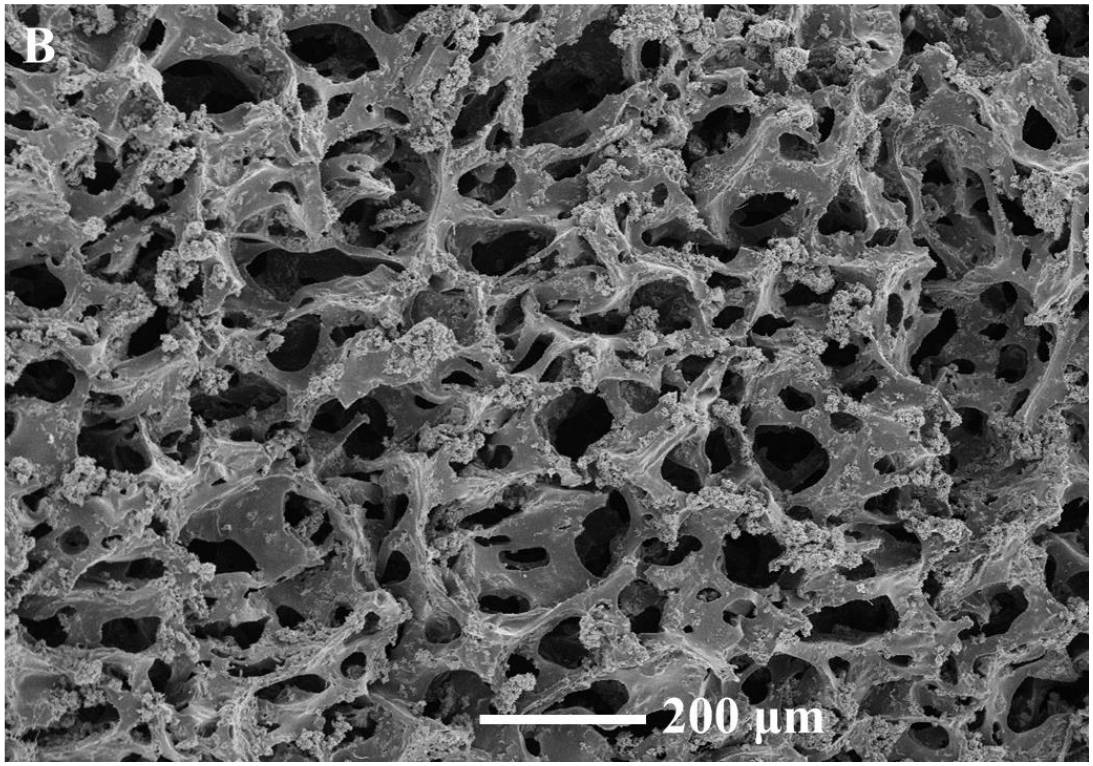


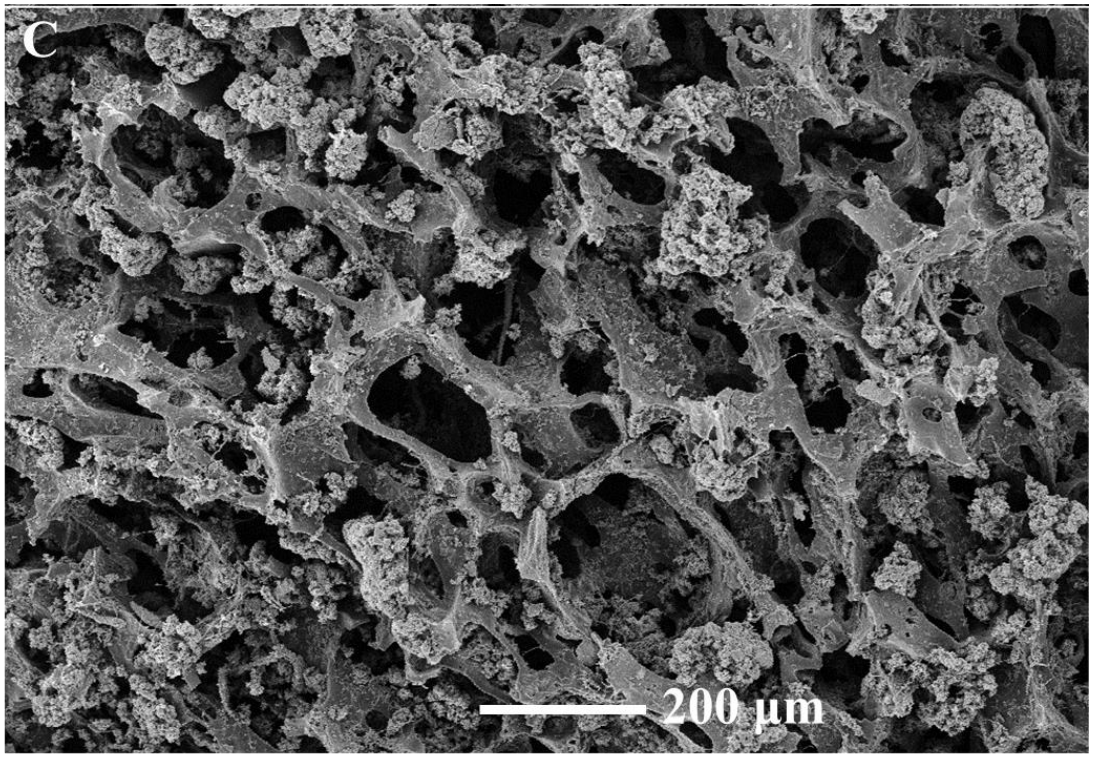




Scanning electron micrographs of PUR foams degraded by bacterial culture. (A) Control foam. (B) PUR foam degradation by *Pseudomonas* sp. (C) PUR foam degradation by culture (PGB+PLI).




B
200 µm


C
200 µm