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Isolation and identification of microorganisms for polyurethane degradation

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ABSTRACT

Plastic wastes decomposition has been a pressing environmental problem worldwide. In this study, polyurethane (PUR), a thermoset plastic was tested for biodegradation by polyurethane-degrading microorganisms that were isolated from a dumpsite at Kamishihoro, Tokachi, Obihiro, Japan. Actinomycetes were the most abundant microorganisms from soil samples. From the 65 isolated microbial species, 16 possessed polyurethane-degrading ability. These isolates exhibited clearing zones on Yeast-extract salts + Agar and gelatin with polyurethane (YES-AG + PUR). The PUR-degrading isolates were characterized and identified based on their DNA sequence patterns. Some isolates belong to the same genus or species. They were *Bacillus chitinolyticus* (B03, B04, B07), *Streptomyces* spp. (B13, B19, C13a, C15, C17a, C17b), *Pseudomonas* sp. (B20), *Bacillus pumilus* (B21), *Streptomyces cuspidosporus* (C10b, C18, C19) and *Pseudallesscheria baydii* (F04, F07). *Streptomyces* sp. coded as C13a, with base sequence homology of 99.7% with *Streptomyces albogriseolus,* was believed to produce the highest amount of both exo- and endo-polyurethanases. This was demonstrated by the widest clearing zones when broth and cell-bound supernatants were inoculated into the YES-AG + PUR plate.

Keywords: Thermoset plastic, Actinomycetes, Coomassie Blue, Biodegradation

INTRODUCTION

Plastics are one of the most abundant solid wastes in the Philippines. They are considered major environmental pollutants since they are non-biodegradable due to the complexity of their compositions.

Practically, recycling of plastics has been identified as one of the solutions to

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the problem. About more than 50% of wastes from the developed countries are now being recycled (Child 2004). However, developing countries in Asia, like the Philippines, lag behind on waste recycling due to ineffective policies and lack of awareness of waste recycling processes (Jereme et al 2015). Most of the municipal solid wastes on developing countries contain potential recyclable materials of about 80% of the total wastes (Habitat International 1994). However, this method is labor-intensive and expensive considering the voluminous plastics to be sorted out each day. Furthermore, unless new and novel products can be produced from such wastes, this may worsen the problem of pollution if recycled products are still in complex form when disposed and remain non-biodegradable.

Plastics, in general, are either thermoplastics or thermoset. The thermoplastics are made up of the long and sole carbon chain, which provides resistance to degradation or hydrolytic cleavage of chemical bonds. Thus, they are considered as non-biodegradable plastics. Because of hydrophobicity, water repellency, high molecular weight and the absence of functional groups recognizable by microbial enzyme systems, thermoplastics (particularly polyethylene) are found resistant to biological action (Chiellini et al 2003). Furthermore, the presence of antioxidants in thermoplastics provides environmental stability of the polymer (Eggins et al 1971). Some researchers made attempts to make thermoplastics biodegradable: thermoplastics are blended with starch and antioxidants are reduced. However, without the addition of a suitable pro-oxidant, biodegradation merely causes the removal of starch and leave behind shorter chains of unmodified polyolefins (Zheng et al 2005). The addition of starch to polyolefins modifies their structures. In starch blends, there is a continuous starch phase that is favorable to microorganisms under the catalytic action of alpha- and beta- amylase enzymes. The continuous starch is then removed, resulting in porous polyolefin starch blends. In the presence of pro-oxidants, the degradation of the polyolefin matrix is accelerated.

Thermoset plastics are characterized by a highly cross-linked structure of heteroatoms which are believed to be responsible for their susceptibility to biodegradation (Zheng et al 2005). Thermoset plastics have not been considered useful, but recently, because of environmental pollution caused by plastic wastes, thermoset plastics are now seriously considered for various applications. Among the thermoset plastics, polyurethane (PUR) is recently gaining much attention in researches because of its increasing importance in industry, commerce, agriculture and medicine. The use of polyurethane has increased because of its high durability and resistance to degradation (Rowe & Howard 2002). Polyurethanes are found almost everywhere. They are components of rubber adhesives, water-blown flexible foams, elastomers, coatings, fibers, foams in furniture, beddings, automobile cushions, thermal insulation on commercial vehicles and ships and medical devices (Ulrich 1983). This polymer represents the first industrially important and commercially useful culmination of an old line of organic research in isocyanates (Howard et al 1999). The polyurethane is suitable for varied applications due to its specific mechanical, physical, biological and chemical properties which can further be enhanced to suit its intended use (Akindoyo et al 2016).

Development of protocol to degrade the polymeric materials through microbial action has been identified as one of the most effective solutions in solving

environmental pollution. In this study, therefore, microorganisms were isolated from dumpsites, screened for polyurethane degradation and identified.

MATERIALS AND METHODS

Isolation and Purification of Microorganisms from Dumpsite Soil

Thirty bags of randomly selected soil samples (approximately 200g each) from the dumpsite soil in Kamishihoro, Tokachi, Obihiro, Japan were collected and brought to the laboratory for microbial isolation. The isolates were purified using appropriate growth medium for each type of microorganisms. The isolation media were sodium caseinate agar (SCA) for Actinomycetes, Potato Dextrose Agar (PDA) for fungi and Nutrient Agar (NA) for bacteria. The pure cultures were stored in 20% glycerol at -80°C until use.

Screening of Microorganisms for Polyurethane Degradation

The microorganisms were screened for their abilities to degrade polyurethane following the method described by Crabbe et al (1994) and Howard et al (1999) with some modifications.

Preparation of basal medium and polyurethane substrate. Yeast-extract salts (YES) medium was used for qualitative screening of microorganisms for their abilities to degrade polyurethane. A step-wise combination of four stock solutions was prepared (Table 1).

Table 1. Yeast-Extract Salts Medium

Stock Solution	Composition
A (50x phosphate buffer, pH7.4 (Dash & Cudworth 2001)	197mL KH_2PO_4 (9.10g L^{-1}) 803mL Na_2HPO_4 (9.48g L^{-1})
B (100x magnesium sulfate)	$MgSO_4.7H_2O$ (50g L^{-1})
C (1000x trace metals)	$MnCl_2.4H_2O$, 2000.0mg L^{-1} $CuCl_2.2H_2O$, 28.0mg L^{-1} $ZnCl_2$, 22.0mg L^{-1} $CaCl_2.6H_2O$, 40.0mg L^{-1} $Na_2MoO_4.2H_2O$, 26.0mg L^{-1} $FeCl_3.6H_2O$, 150mg L^{-1}
D (500x yeast extract)	Yeast Extract (10.0g L^{-1})

Preparation of Yeast-extract salts + Agar and gelatin with polyurethane (YES-AG + PUR). A 20mL portion of stock solution A was added to 970mL distilled water. Purified agar (1.5% w/v), $(NH_4)_2SO_4$ (0.5g) and gelatin (4.0g) were added, and the solution was heated to dissolve the agar. The solution was autoclaved at 121°C for 20min and allowed to cool to 50°C. While the temperature of solution A was maintained at 50°C, 10mL of filter-sterilized solution B was added with constant stirring. Likewise, 1mL of filter-sterilized solution C and 2mL of solution D were then added with constant stirring. The sequence of each step and the temperature were strictly followed to prevent the formation of insoluble phosphates. The main

substrate was added in the prepared solution in the form of a milky white, liquid PUR 860 from Daiichi Kogyo Seiyaku, Superflex Company, Japan. PUR 860 is an ester/aromatic isocyanate-based, water-dispersed solution. The prepared YES-AG + PUR was dispensed in plates at about 20mL each and the agar was allowed to solidify. The agar plates were stored at 4°C if not immediately used.

Inoculation of microorganisms on the YES-AG + PUR plate. Bacteria and *Actinomycetes* were streaked into the YES-AG + PUR plate. Since fungi grow fast and spread profusely in the agar plate, they were simply spot inoculated once in the center of the agar plate. Using the mouth of a sterilized Pasteur pipet, 8mm diameter plug of the fungal isolate was dug from a confluent surface growth of isolate on a PDA plate. Using a sterile stainless steel spatula, the surface growth was separated from the underlying agar plug and inoculated into the center of YES-AG + PUR plate.

Testing of enzyme activities of the isolates. The top-five isolates that exhibited clearing in YES-AG + PUR plates were grown in YES broth with PUR 860 under an agitated condition at 120rpm at 30°C for seven days. The fermented broth was centrifuged at 10,000xg for 10min and the supernatant was separated from the cell pellets. The cell pellets were stored at –20°C for one day. The supernatant was filtered using 0.45µm cellulose acetate. The presence of PUR-degrading enzymes in the filtered supernatant µ was tested by inoculating 100µL into 6mm wells dug in YES-AG plates with PUR 860. A rapid method of detecting PUR-degrading enzymes by Howard and Hilliard (1999) was used. The plates were incubated for 18 to 20h at 37°C and then flooded with 0.1% (w/v) Coomassie blue R-250 solution in 40% (v/v) methanol and 10% (v/v) acetic acid for 20min. The Coomassie blue solution was decanted and the plates were flooded with 40% (v/v) methanol and 10% (v/v) acetic acid for 20min. PUR-degrading enzymes developed clear zones around the well.

After one day of freezing the cell pellets, 10x (w/v) of 20mM potassium phosphate buffer with 0.2% N, N^1-bis(3-D-gluconamidopropyl) deoxycholamide (DEOXY BIG CHAP) was added and the mixture was stirred vigorously for 30min at room temperature. The mixture was then centrifuged at 10,000xg for 10min and the supernatant was filtered using 0.45µm cellulose acetate. The filtered cell-bound supernatant was assayed for PUR-degrading enzyme in 6mm wells dug in YES-AG plate.

Selection, characterization and identification of polyurethane-degrading microorganisms. The 16 isolates that exhibited PUR-degrading abilities were characterized taxonomically through DNA sequencing analysis. DNA from tough-to-lyse fungi was isolated following the more recent, simple and rapid isolation method described in ZR Fungal/Bacterial Kit (Zymo Research Corporation 2007). The DNA from fungi were extracted and ITS region was amplified using primer pairs of ITS1/ITS4 in the following conditions: 3min at 94°C, 40 cycles of 30s at 94°C, 30s at 50°C, 1min at 72°C and one final step of 10min at 72°C. For bacteria, the primers used were rD1 and fD1 and amplified at the following conditions; 3min at 93°C, 45 cycles of 30s at 93°C, 15s at 50°C, 1.5min at 72°C and one final step of 7min at 72°C.

The amplified products were purified and mixed with primers and the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) containing a fluorescent dye. Then an extension reaction was carried out by PCR following the procedure recommended by the manufacturer. The

fragments were sequenced in both directions using an ABI PRISM 3100 DNA Sequencing System, according to the manufacturer's instructions. The sequences obtained were compiled and compared with those in the GenBank databases through the National Center for Biotechnology Information (NCBI), using BLAST-search.

RESULTS AND DISCUSSION

A total of 65 microbial strains were isolated from the soil in a dumpsite in Kamishihoro, Japan (Table 2). Actinomycetes were found the most abundant, followed by other types of bacteria and then fungi. Actinomycetes are the most abundant organisms in the soil which inhibit the growth of plant pathogens, decompose complex mixtures of polymer and produce extracellular enzymes for crop production (Bhatti et al 2019). From the isolated and purified species, only 16 isolates exhibited positive activity on PUR. Of these 16 isolates, there were 7 Actinomycetes, 7 bacteria and 2 fungal species. Likewise, an actinomycete species, identified as *Streptomyces rochei*, was found efficient in degrading plastics particularly polyethylene bags and bottles (Nakei 2015).

Table 2. Total number of isolates exhibiting polyurethane degradation

Types of microorganisms	Total Number	Number of microorganisms with positive activity on PUR
Bacteria		
Actinomycetes	27	7
Other types of Bacteria	23	7
Fungi	15	2
Total	65	16

The PUR-degrading ability was demonstrated by the formation of clearing zones on YES-AG + PUR Plate. Figure 1 clearly shows the clearing and non-clearing zones that were formed by the PUR-degrading and non-PUR-degrading isolates, respectively. The formation of clearing zones was enhanced with Coomassie blue which acts as an indicator for PUR degradation by the isolate in YES-AG + PUR Medium. The detection of the polyurethane-degrading enzyme in the agar medium is based on the ability of enzymes to depolymerize the substrate. Consequently, upon hydrolysis of the substrate, the interaction of the Coomassie blue with the polyurethane is reduced, resulting in a clear zone within a blue background (Howard & Hilliard 1999).

The biodegradation of polyurethane is initiated by surface erosion process by microbial enzymes, which eventually produces water-soluble intermediates that can be assimilated by microbial cells (Muller et al 2001). The chemical process that occurs during biodegradation is divided into two categories, namely, (1) assimilatory process, in which constituents of the plastics serve as sources of nutrients to organisms and (2) dissimilatory processes, in which the plastics are not used as a source of carbon but maybe chemically damaged by corrosive substances secreted by an organism living on detritus lying on the plastics (Eggins et al 1971).

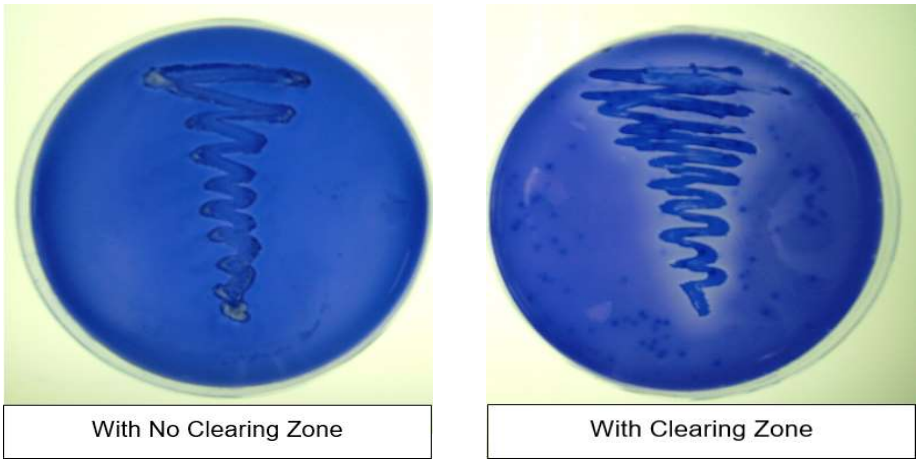

Figure 1. Isolates showing non-clearing and clearing zones on YES-AG Agar Plate

The PUR-degrading isolates were identified base on their DNA sequence patterns. Table 3 presents the identification of the 16 PUR-degrading isolates. They were *Bacillus chitinolyticus* (B03, B04, B07), *Streptomyces* spp. (B13, B19, C13a, C15, C17a, C17b), *Pseudomonas* sp. (B20), *Bacillus pumilus* (B21), *Streptomyces cuspidosporus* (C10b, C18, C19) and *Pseudallesscheria baydii* (F04, F07).

Table 3. Identification of the isolates

Isolate Code	Name/Identification
Actinomycetes	
C10b, C18, C19	*Streptomyces cuspidosporus*
B13, B19, C13a, C15, C17a, C17b	*Streptomyces* spp.
Bacteria	
B20	*Pseudomonas* sp.
B21	*Bacillus pumilus*
B03, B04. B07	*Bacillus chitinolyticus*
Fungi	
F04b, F07	*Pseudallesscheria baydii*

The measurement of clearing zones of the top 5 PUR-degrading microorganisms is presented in Figure 3. From the 5 isolates, 4 belonged to *Streptomyces* spp., while 1 isolate was identified as *Bacillus pumilus*. Although C17a (*Streptomyces* sp.) formed the widest clearing zone, it did not exhibit the clearest zone. In general, C13a, which also belongs to *Streptomyces* sp., consistently formed wider and clearer zones than the others, both in broth and cell-bound supernatant. The strain C13a is closely related to *Streptomyces albogriseolus* with base sequence homology of 99.7%.

Isolation and identification of microorganisms

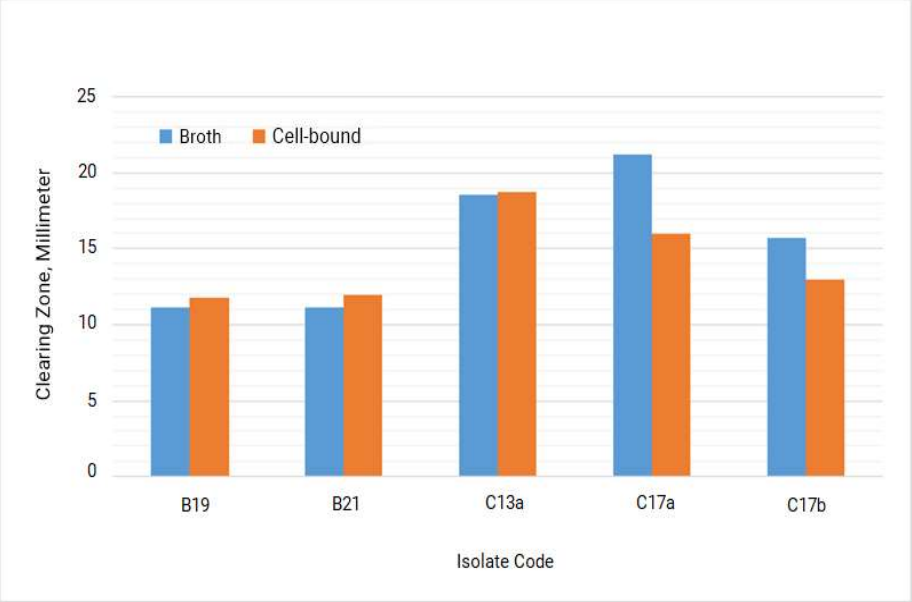

Figure 2. Clearing zones (mm) of the top PUR-degrading microorganisms in YES-AG + PUR plates

As reported by Crabbe et al (1994) and Howard et al (1999), the PUR is degraded by the combined activities of endo-polyurethanase (alpha) and exo-polyurethanase (beta), which formed incomplete and complete clearing zones, respectively. It is possible, therefore, that both endo- and exo-polyurethanase are produced by C13a in both broth and cell-bound supernatant. Incomplete or indistinct clearing zones formed by other isolates might have been brought about by the activity of endo-polyurethanase only. Vigorous stirring and centrifugation of the cells in the presence of DEOXY BIG CHAP ,to dislodge cell-bound enzyme, released the endo-polyurethanase from within the cells. Polyurethanes, especially the polyester type, possess many ester bonds that can be hydrolyzed (Nakajima-Kambe et al 1999). The process involves endo-type depolymerization.

Based on the results of this study, C13a strain has proven to be a very potential isolate for polyurethane degradation and believed to produce both endo- and exo-polyurethanases. Undoubtedly, the use of this microorganism in solving the country's problem on solid wastes, particularly plastics, is very promising. Further tests to confirm its identification can be done and this strain can also be tested in other types of plastics that are commonly found in solid wastes.

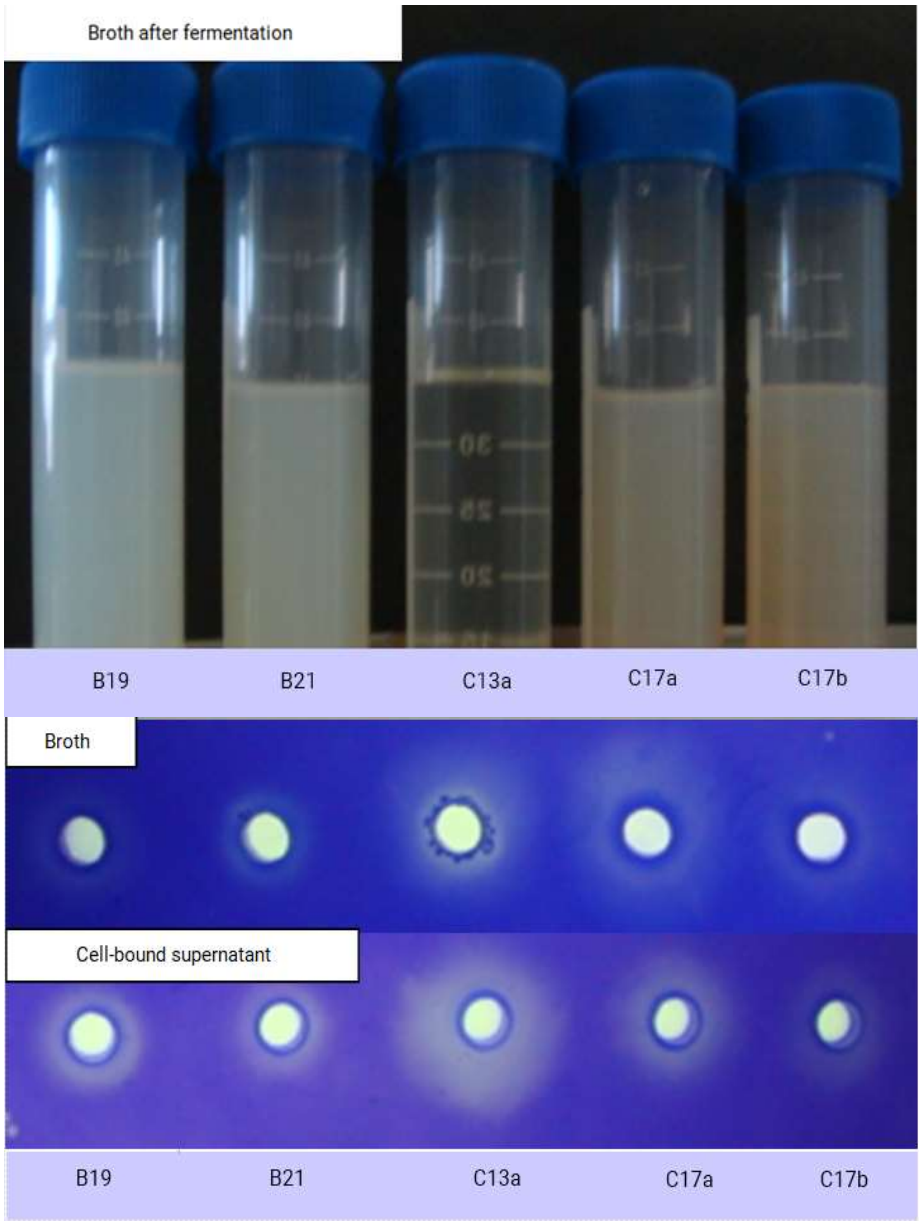

Figure 3. Formation of clearing zones in broth and cell-bound supernatant by the top five PUR-degrading isolates

CONCLUSION AND RECOMMENDATION

Actinomycetes belonging to *Streptomyces* sp. with base sequence homology of 99.7% with *Streptomyces albogriseolus,* possessed the highest PUR-degrading

ability among the microbial isolates from a dumpsite soil in Kamishihoro, Japan. The isolate was believed to produce endo- and exo-polyurethanase, as shown by clearing zones when both fermented broth and cell-bound supernatant were inoculated in YES-AG + PUR. Optimization of conditions for increased production of these enzymes is recommended, considering the voluminous thermoset plastics being disposed daily. The identification of this microorganism needs further confirmation and it can also be tested in other kinds of plastic wastes.

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