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Degradation of polyurethane by novel bacterial consortium isolated from soil

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Abstract - The present study describes the isolation of bacteria from soil with the ability to degrade plastic polyurethane (PU). Bacterial strains attached on the polyurethane film, after soil burial for 6 months, were isolated and identified as *Bacillus* sp. AF8, *Pseudomonas* sp AF9, *Micrococcus* sp. 10, *Arthrobacter* sp. AF11, and *Corynebacterium* sp. AF12. In plate assay, zones of hydrolysis were visualised around the bacterial colonies on mineral salt agar plates containing polyurethane as a sole carbon source. The results of the Sturm test for degradability showed more CO_2 production in the test than in control. The production of esterase was detected in the presence of polyurethane as a substrate. The Scanning Electron Microscopy and Fourier Transform Infrared Spectroscopy showed certain changes on the surface of PU film and formation of some new intermediate products after polymer breakdown.

Key words: polyurethane, biodegradation, scanning electron microscopy, Fourier transforms infrared spectroscopy.

INTRODUCTION

Many plastics are both physically and chemically robust and cause waste management problems (Bouwer, 1992). However, several families of plastics undergo biodegradation in the environment, and an understanding of how this degradation occurs may aid in the development of strategies to exploit these processes for waste management purposes. Microorganisms are responsible for the majority of plastic degradation (Bentham *et al*., 1987), and abiotic factors such as photodegradation or hydrolysis play a very minor role (Griffin, 1980; Woods, 1990). Plastics vulnerable to biodegradation include the polyhydroxyalkanoates, polycaprolactone, polylactic acid, polyvinyl chloride (Sabev *et al*., 2006a, 2006b), and polyester polyurethane (PU) (Cosgrove *et al*., 2007).

The polyurethanes are a diverse group of synthetic polymers that are used in a variety of industrial applications, including furniture, insulating foams, adhesives constructional materials, fibres, paddings, paints, and synthetic leather and rubber goods (Cosgrove *et al*., 2007; Howard, 2002). Structurally, polyurethane is the condensation product of polyisocyanate and polyol having intra-molecular urethane bonds (carbonate ester bond, -NHCOO-) (Sauders and Frisch, 1964). The presence of ester and urethane linkages in the backbone of PUs makes them susceptible to hydrolysis by enzymes secreted by microorganisms, releasing breakdown products which may act as a carbon source (Pathirana and Seal, 1985; Akutsu *et al*., 1998; Nakajima-Kambe *et al*., 1999).

Both PU-degrading fungi (Barratt *et al*., 2003; Sabev *et al*., 2006b) and bacteria (Kay *et al*., 1991; Howard *et al*., 1999) have been isolated from PU, indicating that there are potential reservoirs of PU-degrading organisms widespread in the environment. Crabbe *et al*. (1994) isolated four fungal species, *Curvularia senegalensis, Fusarium solani, Aureobasidium pullulans* and *Cladosporium* sp., from soil and found to degrade ester-based polyurethane. Sixteen different bacterial strains were isolated by Kay *et al*. (1991) with the ability to degrade PU. In another comprehensive study in Japan, PU was found to be metabolized as a sole carbon and nitrogen source by *Comamonas acidovorans* (Akutsu *et al*., 1998; Nakajima-Kambe *et al*., 1999).

Protease, urease, and esterase activities have been associated with the degradation of polyester PU by fungi and bacteria (Pathirana and Seal, 1984; Santerre *et al*., 1994; Nakajima-Kambe *et al*., 1999; Howard, 2002). Polyurethanase protease activities have been reported for *Pseudomonas fluorescens* and *Pseudomonas chlororaphis* (Howard and Blake, 1998; Ruiz *et al*., 1999), polyurethanase lipase activity has been detected in *Bacillus subtilis* strains (Rowe and Howard, 2002). Commercially-available *Candida rugosa* lipase was successfully used to biodegrade synthetic polyester polyurethane particles in an aqueous medium (Gautam *et al*., 2007). Polyurethanase esterase activities have been reported for *Corynebacterium* sp., *Comamonas acidovorans* TB-35, and *P. chlororaphis* (Kay *et al*., 1993; Nakajima-Kambe *et al*., 1997; Howard *et al*., 1999). Two kinds of polyurethane esterase were isolated and characterised (Allen *et al*., 1999; Howard *et al*., 1999; Vega *et al*., 1999). These were shown to be a cell-associated membrane bound PU-esterase and an extracellular PU-esterase. These two enzymes play different roles in polyurethane biodegradation. Membrane-bound esterase activity produced by the *Comamonas acidovorans* TB-35 strain, which is able to attack solid PU (Nakajima-Kambe *et al*., 1997; Akutsu *et al*., 1998), is the best characterised of these. The membrane bound PU-esterase provides cell-mediated access to the hydrophobic polyurethane surface. The extracellular PU-esterase sticks on the surface of the polyurethane. Under

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these enzymatic actions, bacteria could adhere to the surface of polyurethane and hydrolyse PU substrate to metabolites. Results obtained by Nakajima-Kambe *et al*. (1995) and Howard *et al*. (1999) indicated that the polyurethane biodegradation was due to the hydrolysis of ester bonds. Esterase can hydrolyse polyester chains in PU to diethylene glycol and adipic acid (Akutsu *et al*., 1998).

The present study aimed to isolate polyurethane degrading bacteria from the surface of PU film after soil burial and analysis of its degradation through FTIR, SEM and Sturm test. We also identified an inducible extracellular esterase activity which might be responsible for the polyurethanolytic activity.

MATERIALS AND METHODS

Material. Poly [4,4'-methylenebis (phenyl isocyanate)-alt-1,4-butanediol/poly (butylene adipate)] (Polyurethane, PU) (Sigma-Aldrich, GmbH, Germany) having 1.220 g/ml density and melting temperature about 190 °C, was used in the present study. Nutrient agar and Nutrient broth were also purchased from Sigma-Aldrich. Mineral salt media (M1) (g/l: K_2HPO_4 0.5, KH_2PO_4 0.04, NaCl 0.1, $CaCl_2{\cdot}2H_2O$ 0.002, $(NH_4)_2SO_4$ 0.2, $MgSO_4{\cdot}7H_2O$ 0.02, $FeSO_4$ 0.001, pH adjusted to 7.0) devoid of any carbon sources, was used for the degradation experiments. Agar 2% was added in solid media.

Isolation of polyurethane degrading microorganisms. Polyurethane degrading microorganisms were isolated from soil collected from the plastic waste disposal sites, Islamabad, Pakistan. Polyurethane films were prepared by conventional solvent casting technique, dissolving 0.5% (w/v) PU in tetrahydrofuran and pouring in Petri plates. The plates were kept in dark at room temperature; the films were detached from the plates, washed with sterilised distilled water and buried in soil for 6 months, at room temperature (30-35 °C), in a large pot amended with mineral salt solution (g/l: K_2HPO_4 0.5, KH_2PO_4 0.04, NaCl 0.1, $CaCl_2{\cdot}2H_2O$ 0.002, $(NH_4)_2SO_4$ 0.2, $MgSO_4{\cdot}7H_2O$ 0.02, $FeSO_4$ 0.001) to maintain the availability of trace elements and moisture and 10 g/l glucose was used as a co-metabolite. Structural changes in the polymer were analysed after 6 months of burial in soil. The film was taken out from the soil, washed with sterilised distilled water to remove the loosely attached material. Enriched bacterial consortium was obtained by culturing in minimal medium containing PU film as a sole carbon source.

Identification of selected isolates. Bacterial isolates showing clear zones of hydrolysis around their colonies as a result of degradation of PU were selected for further study. The bacterial isolates were then identified macroscopically (colony morphology, surface pigment, shape, size, margin, surface), microscopically (Gram staining, shape, cell arrangement, granulation, presence of spore, motility) and biochemically on the basis of Bergey's Manual of Determinative Bacteriology (Holt, 1993).

Biodegradation of polyurethane.

Plate assay. Polyurethane degrading microorganisms were isolated from the films by enrichment technique. They were further tested for their ability to degrade the polymer by clear zone test. For clear zone tests (Augusta *et al*., 1993) polyurethane plates were prepared with mineral salt medium (modified from Nishida and Tokiwa, 1993) so as to give 0.5% (w/v) as a final concentration of PU. The PU agar plates were inoculated with the bacterial consortium and incubated at 37 °C for 48 h. The plates were flooded with a 0.1% (w/v) Coomassie Brilliant blue R 250 solution in 40% (v/v) methanol and 10% (v/v) acetic acid for 20 min. The dye was then poured off, and the plates were flooded with 40% (v/v) methanol and 10% (v/v) acetic acid for 20 min. The clear zones of degradation were visualised in a blue back ground.

Sturm test. CO_2 evolved as a result of PU biodegradation was determined by Sturm Test (Müller *et al*., 1992). A consortium of five selected bacterial isolates was used as inoculum. Test flask contained 3g of PU pieces as substrate and inoculum (5%) in MSM. Whereas, control flask contained the inoculum in MSM without PU pieces. The test was performed at room temperature (35 °C) for 4 weeks with continuous stirring. After culturing, the change in biomass (CFU/ml) and the amount of CO_2 produced was calculated in the test and control flask gravimetrically. Evolution of CO_2 as a result of degradation of polymeric chain was trapped in the absorption flasks containing 1 M KOH. $BaCl_2$ solution (0.1 M) was added to the CO_2 containing KOH flasks and as a result precipitates of $BaCO_3$ (using CO_2 released from breakdown of polymer) were formed. CO_2 produced was calculated gravimetrically by measuring amount (g) of CO_2 precipitates evolved by addition of $BaCl_2$.

Esterase assay. Esterase activity was determined according to the method of Eggert *et al*. (2000). The *p*-nitrophenyl acetate (0.8 mM) was dissolved in 10 ml isopropanol and mixed with 90 ml of sodium phosphate buffer, pH 8, supplemented with sodium deoxycholic acid (207 mg) and gum arabic (100 mg). The final concentration of the substrate was 0.8 mM. About 0.1 ml of cell free supernatant was taken from Sturm test and mixed with 10 µl of 2 mM glycine (pH 11) and was added to 2.5 ml of the substrate emulsion. After 15 min of incubation at 37°C the absorbance at 410 nm was recorded. One unit of enzyme activity was defined as the amount of enzyme which forms 1 µmol of *p*-nitrophenol per min.

Fourier Transform Infrared Spectroscopy (FTIR). After incubation of PU films in liquid medium (Sturm test) for 1 month, PU films were analysed by Fourier Transform Infrared Spectroscopy (FTX 3000 MX Bio Rad Ex-CliburTm FTIR Series, USA) to detect the degradation on the basis of changes in the functional groups. The polymer pieces were mixed with KBr and made into tablets, which were fixed to the FTIR sample plate. A spectrum was taken at 400 to 4000 wave-numbers cm^{-1} for each sample.

Scanning electron microscopy (SEM). Scanning electron microscopy (LEO 440i, Leica, Bensheim, Germany) of PU films was done after 1 month incubation in Sturm test, in order to check for any changes in surface morphology. The PU films were washed thoroughly with sterilised distilled water, and the samples were mounted on the aluminium stubs by silver coating in vacuum. The images of the test samples were compared with those of the original untreated samples.

RESULTS AND DISCUSSION

The present study deals with the isolation of polyurethane degrading microorganisms from the soil, analysis of biodegradation by Sturm test, FTIR, SEM. Solvent casted PU films were buried in soil for a period of 6 months and then used for the isolation of PU degrading microorganisms.

Polyurethanes are considered to be comparatively susceptible to microbial degradation (Morton and Surman, 1994). In our

study, out of 12 bacterial isolates, having the ability to adhere to PU films after soil burial for 6 months, 5 were obtained through enrichment technique, utilising PU film as a sole carbon source. The adherence of *Bacillus* sp. on the polyurethane surface has also been reported by Robert *et al*. (1998). Kay *et al*. (1991) isolated 15 kinds of bacteria from polyester PU pieces following their burial in soil for 28 days.

In our study, plate assay was conducted to check the PU degrading ability of 5 different enriched bacterial isolates. The clear zones of hydrolysis around the bacterial colonies was observed after flooding the PU containing mineral salt agar plates with Coomassie blue R 250, indicating the production of polyurethanases. The detection of polyurethanase in a PU gel is based on the ability of enzymes to depolymerise the substrate. Thus by hydrolysing the substrate, the interaction of the Coomassie blue with the polyurethane is diminished resulting in a zone of clearing within a blue background (Howard and Hilliard, 1999).

The bacterial isolates were identified as *Bacillus* sp. AF8, *Pseudomonas* sp. AF9, *Micrococcus* sp. AF10, *Arthrobacter* sp. AF11 and *Corynebacterium* sp. AF12 (Table 1). Oceguera-Cervantes *et al*. (2007) isolated two bacterial strains identified as *Alicycliphillus* sp. Capable of degrading a commercial surface-coating PU as the carbon source. Four species of fungi, *Curvularia senegalensis, Fusarium solani, Aureobasidium pullulans* and *Cladosporium sp.* (Crabbe *et al*., 1994) and three of bacteria, *Pseudomonas chlororaphis, Comamonas acidovorans,* and *Pseudomonas fluorescens* (Nakajima-Kambe *et al*., 1995), were obtained from soil and found to degrade ester-based polyurethane.

In the present study, the biodegradation of PU was checked in liquid medium through Sturm test. In case of test, the total amount of CO_2 produced was 4.46 g/l, whereas, in control (without PU) it was 2.23 g/l (Table 2). The increase in CFU/ml and amount of CO_2 evolved in the test as compared to control reaction vessels indicated the increased activity of bacterial consortium against PU, i.e., its ability to utilise it as carbon and energy source. Sturm test has been used by many researchers to study the biodegradation of biodegradable polymers (Whitchurch and Terence, 2006), the aliphatic and aromatic compounds (Kim *et al*., 2001; Sturm, 1973).

TABLE 1 - Identification of polyurethane degrading bacterial strains

Characteristics	Strain				
	AF8	AF9	AF10	AF11	AF12
Colony					
Shape	Round	Round	Round	Round	Round
Size	Large	Large	Small	pinpoint	Large
Colour	White/pale	Pale	Yellow	Off white	Off-white
Surface	Dull, granular, wrinkled	Convex	Shinny	Convex	Raised
Margin	Irregular	Undulate	Entire	Entire	Undulate
Cell morphology					
Straight rod	+	+	-	+	+
Cocci	-	-	+	-	-
Gram stain	+	-	+	+	+
Cell arrangement	Short chains, single	Short chains, single	Irregular clusters	Short chains, single	Long club shaped, single
Spore	C	C	-	-	-
Motility	+	+	-	+	-
Granulation	+	+	-	-	-
Enzyme production					
Protease	+	+	-	+	+
Amylase	+	+	-	-	+
Lipase	+	+	-	+	+
Gelatinase	+	+	-	+	-
Carbohydrate fermentation					
Glucose	A/-	-/-	-/-	-	A/-
Fructose	A/-	-/-	-/-	-	-/-
Sucrose	A/-	-/-	-/-	-	A/-
Lactose	A/-	-/-	-/-	A/-	A/-
Raffinose	A/-	-/-	-/-	-	-/-
Mannose	A/-	-/-	-/-	-	-/-
Sorbitol	A/-	-/-	-/-	-	-/-
Urease	-	-	-	+	-
Nitrate reduction	+	+	+	-	-
Citrate	+	+	+	-	-
TSI	Y/Y	-	R/R	Y/Y	+
MR	-	-	+	-	-
VP	+	-	+	-	-
SIM	+	+	-	-	-
Oxidase	+	+	+	+	+
Catalase	+	+	+	-	-
Identified as (Holt, 1993)	*Bacillus* sp.	*Pseudomonas* sp.	*Micrococcus* sp.	*Arthrobacter* sp.	*Corynebacterium* sp.

Key: C, Central; A, Acid; Y, Yellow; R, Red.

TABLE 2 - Total viable count and gravimetric analysis of CO_2 evolution during breakdown of polyurethane by bacterial consortia as determined through Sturm tes

Sample	Before experiment (CFU/ml)	After experiment (CFU/ml)	Amount of CO_2 produced (g/l)
Test	4.5×10^9	9.0×10^9	4.46
Control	4.5×10^9	2.4×10^9	2.23

Polyester polyurethane (PU)-degrading enzyme, esterase, from *Pseudomonas fluorescens* (Vega *et al*., (1999) and *Pseudomonas chlororaphis* (Howard *et al*., 1999) was studied, that utilises polyester PU as the sole carbon source. In our study, the esterase activity, as determined by the method given by Eggert *et al*. (2000), was 12 U/ml at the end of Sturm test.

FTIR spectroscopy was performed to check the biodegradation of PU by the hydrolysis of the ester bonds. FTIR analysis of PU films at the end of Sturm test, showed decrease in peak from wavelength 2963 cm^{-1} (control) to 2957 cm^{-1} (test). The decrease indicated the cleavage of C-H bonds. The appearance of some new peaks (shown by C=C) and increase in already existing peaks, at the region of 1400-1600 cm^{-1}, indicating the formation of new intermediate products (Fig. 1). Similar mechanism of biodegradation was reported by Nakajima-Kambe *et al*. (1995) and Howard *et al*. (1999). FTIR analysis of PU films after microbial treatment for 1 month, showed the appearance of some new groups at the region of 1400-1600 cm^{-1}. The decrease in intensity of the IR absorption bands indicated that the N=C and C=O valency bonds of the isocyanate groups are broken first, followed by the splitting of urea groups (1613 cm^{-1}) (Filip, 1978). Pettit and Abbott (1975) were also of the opinion that the decomposition of urea units by release of ammonia contributes to the degradation of polyurethane. Sequentially the ester bonds of the urethane groups (H_2N-CO-OR) at 1715 cm^{-1} could be hydrolysed by the action of microbial esterases. Polyurethane breakdown products obtained by the action of *Corynebacterium* sp., were analysed by FTIR and reported that PU degradation was caused by the hydrolysis of ester bonds (Kay *et al*., 1993).

The surface morphology of the polyurethane film pieces analysed at the end of one month of Sturm test showed some changes such as pits, erosions and dark spots when analysed through SEM (Fig. 2). Whereas, surface cracking of polyester PU after fungal treatment was observed by Griffin (1980). The capacity of *Alicycliphilus* sp. to degrade PU was demonstrated by changes in the PU IR spectrum and by the numerous holes produced in solid PU observed by scanning electron microscopy after bacterial culture (Oceguera-Cervantes, 2007).

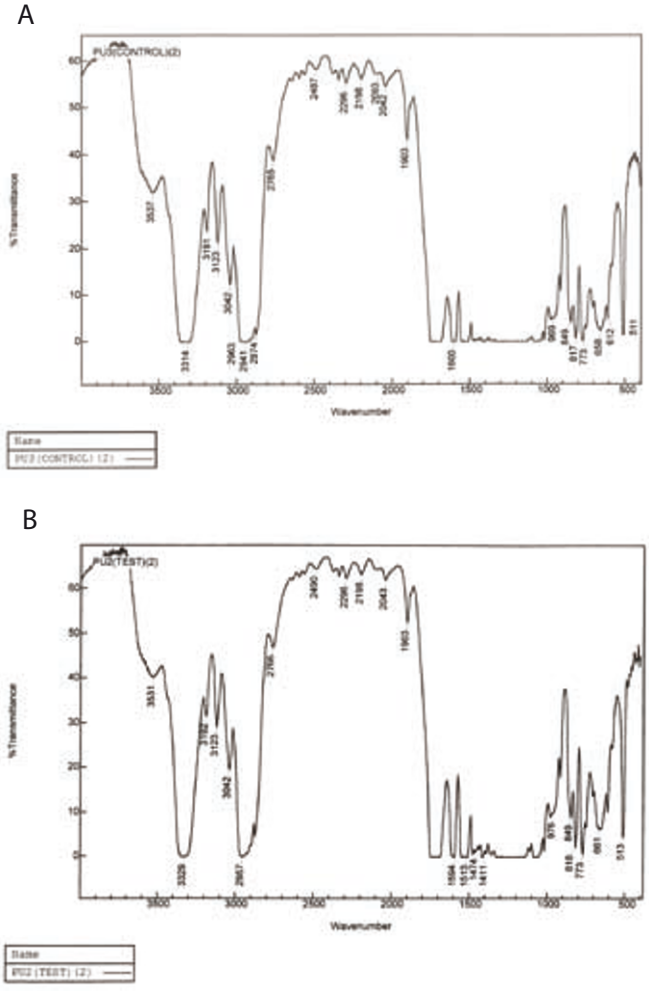

FIG. 1 - FTIR spectra of polyurethane film treated with bacterial consortia. A: control; B: test.

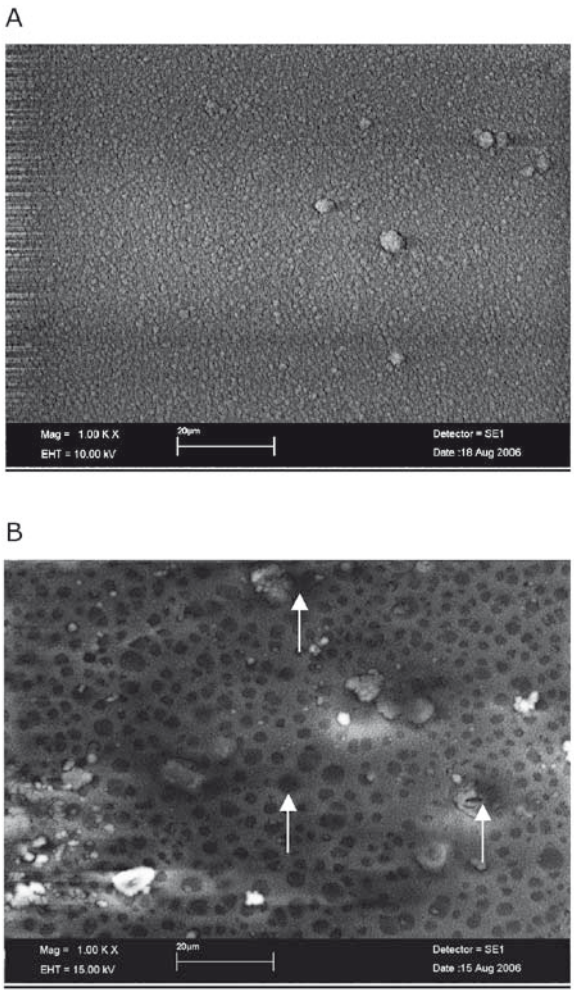

FIG. 2 - Scanning electron micrograph of polyurethane film, A: before soil burial; B: after soil burial for 6 months (arrows indicate the pits formation).

CONCLUSION

Bacteria with the ability to degrade polyurethane were isolated from the soil. It can be concluded that soil contains the potential candidates for bioremediation of plastic wastes.

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