Biodegradation (2013) 24:865–877
DOI 10.1007/s10532-013-9634-5

ORIGINAL PAPER

Degradation of polyester polyurethane by a newly isolated soil bacterium, *Bacillus subtilis* strain MZA-75

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Received: 8 December 2012 / Accepted: 18 March 2013 / Published online: 28 March 2013

Abstract A polyurethane (PU) degrading bacterial strain MZA-75 was isolated from soil through enrichment technique. The bacterium was identified through 16S rRNA gene sequencing, the phylogenetic analysis indicated the strain MZA-75 belonged to genus *Bacillus* having maximum similarity with *Bacillus subtilis* strain JBE0016. The degradation of PU films by strain MZA-75 in mineral salt medium (MSM) was analyzed by scanning electron microscopy (SEM), fourier transform infra-red spectroscopy (FT-IR) and gel permeation chromatography (GPC). SEM revealed the appearance of widespread cracks on the surface. FTIR spectrum showed decrease in ester functional group. Increase in polydispersity index was observed in GPC, which indicates chain scission as a result of microbial treatment. CO_2 evolution and cell growth increased when PU was used as carbon source in MSM in Sturm test. Increase in both cell associated and extracellular esterases was observed in the presence of PU indicated by *p*-Nitrophenyl acetate (*p*NPA) hydrolysis assay. Analysis of cell free supernatant by gas chromatography–mass spectrometry (GC–MS) revealed that 1,4-butanediol and adipic acid monomers were produced. *Bacillus subtilis* strain MZA-75 can degrade the soft segment of polyester polyurethane, unfortunately no information about the fate of hard segment could be obtained. Growth of strain MZA-75 in the presence of these metabolites indicated mineralization of ester hydrolysis products into CO_2 and H_2O.

Keywords Polyester polyurethane · Biodegradation · *Bacillus subtilis* · Scanning electron microscopy · Fourier transform infra-red spectroscopy · Gas chromatography–mass spectrometry

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Introduction

Polyurethane (PU) is the condensation product of polyisocyanate and polyol having intra-molecular urethane bonds (carbonate ester bond, –NHCOO–). It is a diverse group of synthetic polymers, commonly utilized as a constituent material in many industries including furniture, coating, construction materials, fibers, and paints (Sauders and Frisch 1964). Since PU is such a versatile polymer, its production continues to rise worldwide. The global market for PU was estimated at 13,650.00 kilo tons in 2010 which is expected to reach 17,946.20 kilo tons by 2016, growing at a compound annual growth rate (CAGR)

of 4.7 % from 2011 to 2016. In terms of revenue, it was estimated at US$33,033 Mn in 2010 which is expected to reach US$55479.68 Mn by 2016, growing at a CAGR of 6.8 % from 2011 to 2016 (www.plastemart.com 25th July 2012) but this has resulted in an increased environmental burden through the generation of plastic waste.

Lack of biodegradability of plastics in landfills lead to the depletion of landfill sites, and aggravated the problem of littering and surface water pollution (Russell et al. 2011; Mukherjee et al. 2011; Rowe and Howard 2002). One process that is being developed for the degradation of plastics involves bioremediation. Both PU-degrading fungi (Barratt et al. 2003; Bentham et al. 1987; Sabev et al. 2006) and bacteria (Akutsu et al. 1998; Howard et al. 1999; Kay et al. 1991) have been isolated from PU, indicating that there are potential reservoirs of PU-degrading organisms widespread in the environment. A number of fungal species such as *Curvularia senegalensis*, *Fusarium solani*, *Aureobasidium pullulans*, and *Cladosporium* sp., were isolated from soil capable of degrading ester-based polyurethane (Crabbe et al. 1994). Kay et al. (1991) reported 16 different bacterial strains which can degrade PU. *Comamonas acidovorans* strain TB-35 has been recovered by Nakajima and co-workers which can utilize PU as a sole carbon and nitrogen (Nakajima-Kambe et al. 1995; Akutsu et al. 1998). Literature survey reveals that PU biodegradation reported so far was due to ester bond hydrolysis (Nakajima-Kambe et al. 1995; Howard et al. 1999). The growth of *Pseudomonas chlororaphis*, *C. acidovorans*, and *Pseudomonas fluorescens* has been previously studied, in order to purify and characterize polyurethane esterase enzyme. A polyurethane degrading enzyme has recently been reported from a newly isolated *Acinetobacter gerneri* strain P7 from soil (Howard et al. 2012). Both cell associated and extracellular polyurethane esterases from different organisms have been found to catalyze polyurethane degradation (Howard et al. 1999; Allen et al. 1999; Vega et al. 1999) these two enzymes play different roles in polyurethane biodegradation. The cell associated PU-esterase due to hydrophobic interactions gives access to the cells to polyurethane surface while the extracellular PU-esterase bind to the surface of the polyurethane. These enzymatic actions help the bacteria to stick to the surface of polyurethane and hydrolyze it into metabolites.

In order to develop bioremediation techniques for hazardous and refractory waste, such as plastics, knowledge of how these compounds are metabolized by existing organisms, an investigation of new organisms, and the development of novel metabolic capabilities using genetic engineering is needed. A basic understanding of the biological processes that lead to chemical degradation will add in the development of new bioremediation techniques (Shannon and Unterman 1993) and new biodegradable products with lesser environmental impact (Lu et al. 2004; Hoang et al. 2007; Ashby and Solaiman 2008). In this study we isolated a polyurethane degrading bacterium from soil. The esterase assay revealed induction of both extracellular and cell associated polyurethane esterases by using polyurethane as carbon source. PU degradation products were also analyzed through gas chromatography mass spectrometry (GC–MS).

Materials and methods

Materials

Polyurethane {poly[4,4′-methylene-bis(phenyl isocyanate)-alt-1,4-butanediol/poly (butylene adipate)]} was obtained in the form of pellets from Sigma-Aldrich, GmbH, Germany. Tetrahydrofuran (THF) was obtained from Panreac Quimica, SA. All the other reagents were commercial products of the highest grade available. 1 g of polyurethane pellets were dissolved in 100 ml of THF and poured into 4 clean glass petri dishes in equal amounts to prepare thin films of polyurethane. The THF was allowed to evaporate slowly by placing the covered petri dishes in desiccator.

Isolation of polyurethane degrading bacterial strain

Soil samples were collected from dumping area of Islamabad, Pakistan in sterilized polyethylene bags. One gram of soil was incubated in nutrient broth for 24 h at 37 °C. 10 ml of this culture was inoculated in 90 ml of fresh Minimal Salts Medium (MSM) [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] containing 250 mg of PU film pieces and incubated at 37 °C in shaker incubator

at 150 rpm for 1 week. Treated PU pieces were shifted to fresh MSM along with 10 ml of the culture after week incubation and allowed to incubate in shaker incubator under the same conditions for another week. This procedure was repeated four times. Viable cell count was performed each time before shifting of polyurethane films into fresh MSM. Culture showing best growth in the presence of polyurethane was selected for further studies.

Identification of the isolated bacterial strain

The bacterial strain was identified through colony morphology, microscopic examination, and biochemical characteristics. Gram staining, morphology and motility were observed by phase contrast microscope (Olympus Co., Ltd., Tokyo, Japan). Some standard physiological and biochemical properties, e.g., oxidase and catalase production, lipid, starch and casein hydrolysis, gelatin liquefaction, and nitrate reduction, including other enzymatic activities and acid production from carbohydrates, were determined by conventional methods (Smibert and Krieg 1981).

Phylogenetic analysis

16S rRNA gene sequencing was performed for the identification of isolates. The full length gene was amplified by PCR using bacterial primers 27F′ and 1494R′. 20 µl PCR reaction mixture consisted of template DNA 1 µl, 10× PCR buffer 2 µl, deoxynucleoside triphosphate (dNTP) mix 2 µl, forward and reverse primer 2 µl each, Ex taq DNA polymerase (Takara Shuzo, Otsu) 0.5 µl and distilled water 10.5 µl. At first, template DNA was denatured by incubating the reaction mixture at 96 °C for 4 min. Then 35 amplification cycles were performed at 94 °C for 45 s, 55 °C for 60 s, and 72 °C for 60 s. Reaction mixture was further incubated for 7 min at 72 °C. DNA fragments amplified were about 1,481 bps in the case of bacteria. A positive control (*E. coli* genomic DNA) and a negative control (without template DNA) were included in the PCR. The PCR product was purified from unincorporated PCR primers and dNTPs by using Montage PCR Clean up kit (Millipore). The purified PCR products of approximately 1,481 bps were sequenced by using 2 primers, 518F′ and 800R′. Sequencing was performed by using Big Dye terminator cycle sequencing kit v.3.1 (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA) at the Macrogen, Inc., Seoul, Korea.

Polyurethane degradation assay

Analysis for PU film degradation by strain MZA-75

The capability of the strain MZA-75 to degrade PU was analyzed by tracking changes in surface morphology, modifications in chemical bonds as well as molecular size of the PU through scanning electron microscopy (SEM), Fourier transform infra-red (FT-IR) spectroscopy and gel permeation chromatography (GPC), respectively. Small pieces of PU film (approx. 2 × 2 cm) were sterilized by autoclaving at 121 °C for 15 min in 250 ml conical flask containing 100 ml MSM. It was inoculated with the cells of MZA-75 and allowed to incubate in shaker incubator at 37 °C and 100 rpm for 4 weeks. Similar culture without polyurethane film pieces was taken as biotic control while polyurethane pieces in the same amount of MSM without bacterial culture was taken as abiotic control. The experiment was run in triplicate. The growth in test vessel was compared with that of control by measuring the absorbance at 600 nm. The experiment was terminated at the end of 4th week and PU films were recovered for analysis through SEM, FT-IR and GPC.

Scanning electron microscopy of PU film pieces Changes in the surface features of PU films, as a result of microbial treatment, were tracked by analysis through scanning electron microscopy (JSM 5910, Jeol, Japan). Samples were mounted on the copper stubs with gold paint after thorough washing with sterile distilled water. Gold coating was carried out in vacuum by evaporation in order to make the samples conducting. The images of the test samples were compared with those of abiotic control.

Fourier transform infrared spectroscopy (FT-IR) analysis FT-IR was employed for detection of changes in the functional groups in the chemical structure of PU film as a result of incubation with MZA-75. After pasting the polymer pieces on FTIR sample plate, a spectrum was taken in single at 500–4,000 wave-numbers cm^{-1} for each sample and compared with that of abiotic control.

Gel permeation chromatography (GPC) analysis of the treated polyurethane films 1 % (w/v) solution of PU films in THF was prepared and analyzed by Agilent PL Gel 5 µm 50 A, 300 × 7.5 mm GPC column. Flow rate was maintained at 1 ml/min. Refractive index detector was used for detection. Calibration curve of polystyrene standard was used for calculation of polydispersity and relative molecular weight.

Carbon dioxide (CO_2) evolution test (Sturm test)

Carbon dioxide (CO_2) evolved as a result of degradation of PU by strain MZA-75 was trapped and compared to the amount evolved in case of biotic control in Sturm test under similar conditions. 500 mg of PU film pieces were added to culture bottle (test bottle) containing 300 ml of MSM. Both test and biotic control bottles were inoculated with overnight grown culture up to 0.07 OD_{600}. Sterilized air was pretreated to remove dissolved CO_2 by passing it through pretreatment chamber consisting of two bottles having KOH solution (3 M). The test and control bottles were stirred continuously by placing them on the magnetic stirrer. The test was performed at room temperature (30 °C) for 4 weeks. After 4 weeks of culturing, the change in viable cell count and the amount of CO_2 produced was calculated in the test and control bottles. CO_2 evolved as a result of PU utilization was trapped in the absorption bottles containing KOH (1 M). Barium chloride solution (0.1 M) was added to the CO_2 containing KOH bottles and as a result precipitates of barium carbonate were formed. CO_2 was calculated out gravimetrically from the CO_3 precipitates evolved by addition of $BaCl_2$. Difference in the amount of precipitates in the test and control was determined (Muller et al. 1992).

Esterase activity assay

Strain MZA-75 was inoculated in 9 ml of liquid MSM with 0.5 % peptone and incubated at 37 °C in shaker incubator for 16 h. The culture was centrifuged at 8,000 rpm for 10 min at 4 °C and cell pellet was separated from supernatant. The pellet was washed with Tris HCl buffer (pH7) twice and then suspended in the same. Esterase assay for both supernatant and pellet was done using the method of Kanwar et al. (2005), using *p*NPA as substrate for esterase. To investigate about the cell associated esterases, 0.2 % of *N*,*N*-Bis(3-D-gluconamidopropyl) deoxycholamide (deoxy-BIGCHAP, Dojin Chem. Co., Japan), a surfactant, was added to the culture broth, and was mixed for 1 h by shaking and then centrifuged. The cell free supernatant was assayed for esterase activity. Protein concentration was determined as described by Lowry et al. (1951).

Analysis of ester hydrolysis products by GC–MS

300 ml of MSM was dispensed in 1 l Erlenmeyer flask. 500 mg of polyurethane film pieces were put in the flask. The flask was tightly plugged and sterilized by autoclaving at 121 °C for 15 min. After autoclaving, the flask was inoculated with MZA-75 up to OD_{600} (0.07). Both biotic and abiotic controls were set up in the similar fashion. The experiment was run in triplicate. 50 ml of sample was taken at zero time and then all three sets were shifted to shaker incubator at 37 °C and 100 rpm. All sets were sampled after every 7 days. Samples were centrifuged at 8,000 rpm for 10 min at 4 °C. The supernatant was preserved at −20 °C while the pellet was discarded. The samples were acidified up to pH 2.0 after thawing and left for 2 h before extraction. The sample was extracted with three volumes (40, 20, 20 ml) of ethyl acetate, the extracts were combined, dried over anhydrous sodium sulfate, concentrated by rotary evaporation, and reduced further to a volume of 50 µl under a stream of N_2. The extracts were derivatized with *N*,*O*-bis(trimethylsilyl)trifluoro acetamide (BSTFA) (Pierce Chemical Co., Rockford, IL) prior to analyses of the resulting compounds on an Agilent 6890 model gas chromatograph (GC) coupled with an Agilent model 5973 mass spectrometer (MS). Derivatized components were separated on HP-5 ms capillary column (30 m × 0.25 mm inner diameter × 0.25 µm film, J&W Scientific, Folsom, CA) using temperature programming as follows: The initial temperature of 80 °C was kept for 5 min and then ramped it up at the rate of 10 °C per minute up to a maximum of 230 °C. The identification of methyl esters of butanediol and adipic acid was done by comparison of the GC–MS profiles to authentic standards purchased from Sigma-Aldrich (St. Louis, MO) or the National Institute of Standards and Technology (NIST) Mass Spectral Library, version 2.0a.

Growth measurement in the presence of 1,4-butandiol and adipic acid

5 mM concentration of 1,4-butanediol and adipic acid was taken in 5 ml of MSM in separate test tubes. Tubes were inoculated with strain MZA-75 and incubated at 37 °C. The experiment was set up in triplicate. Abs_{600} was recorded at 24 h interval. A sample tube from each set was photographed to demonstrate the ability of strain MZA-75 to utilize 1,4-butanediol and adipic acid as a source of carbon and energy.

Statistical analysis

The experiments were done in triplicate. Student's *t* test and two way anova analysis were done using graph-pad prism version 5.01. *P* value of 0.05 was set as a level of significance. The data are expressed as mean standard errors.

Results

Isolation of polyurethane degrading microorganisms

About 6 different colonies were observed when serially diluted soil suspension was spread on nutrient agar plates and allowed to grow for 24 h at 37 °C. After enrichment for 1 month, only one bacterial strain was found to have the ability to grow in the presence of PU as a sole source of carbon in MSM, designated as strain MZA-75.

Identification of the bacterial strain

Characterization and phylogenetic analysis of strain MZA-75

Strain MZA-75 is a gram positive spore forming bacillus which could grow in the presence of 6.5 % NaCl while no growth was observed on or above 55 °C. The morphological and biochemical characteristics are mentioned in detail in Table 1. The sequencing results showed a total of 1,481 nucleotides of 16S rRNA of strain MZA-75 were used for identification. The sequence was aligned with reference sequences obtained from NCBI GeneBank. The phylogenetic analysis of the 16S rRNA sequence revealed that the strain MZA-75 belong to genus *Bacillus* having 99 % similarity with several strains of *Bacillus subtilis* but the closest one is *B. subtilis* JBE0016 (FJ982665) based on maximum score (Fig. 1). The nucleotide sequence reported here can be obtained from NCBI nucleotide sequence database under accession number HM101166.

Polyurethane degradation assay

Analysis for changes in PU film

Scanning electron microscopy (SEM) SEM of PU film pieces was performed in order to confirm alteration in the physical structure of PU film as a

Table 1 Characterization of isolated strain MZA-75

Characteristics	Results
Colony morphology	
Shape	Round
Size	Large
Color	White
Surface	Dull
Margin	Entire
Cell morphology	Bacillus
Gram staining	+
Spore formation	Central
Biochemical tests	
Casein hydrolysis	+
Starch hydrolysis	+
Lipid hydrolysis	+
Gelatin liquefaction	+
Oxidase	+
Catalase	+
Nitrate reduction	+
Citrate utilization	+
Voges Proskauer	+
Methyl red	−
Urease test	−
Carbohydrate fermentation	
Glucose	+
Fructose	+
Sucrose	+
Mannose	+
Raffinose	+
Lactose	+
Sorbitol	+

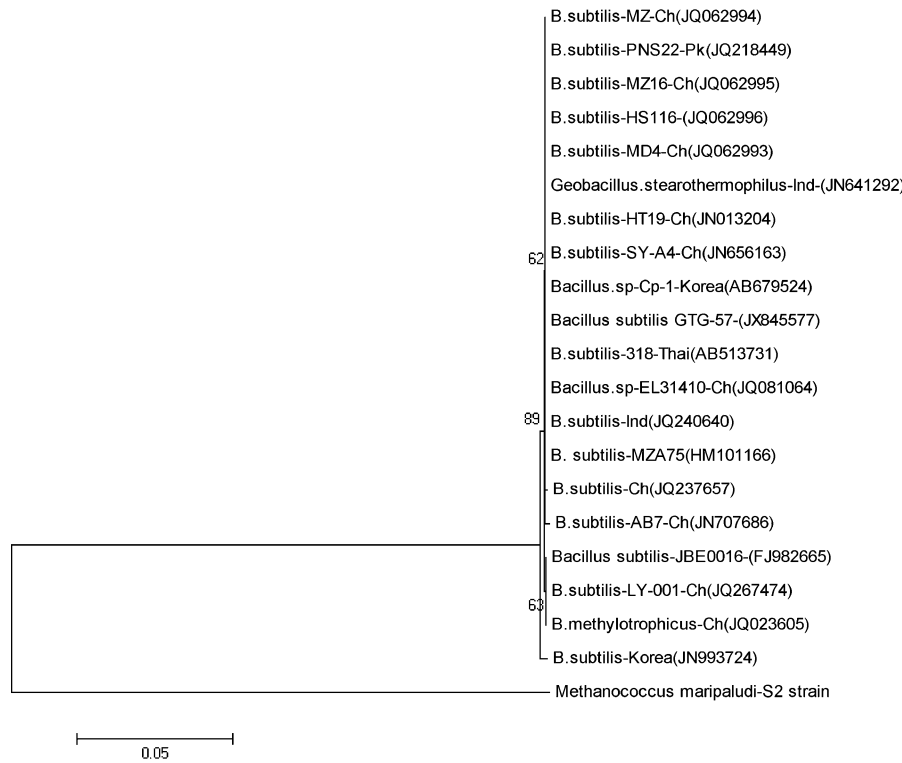

Fig. 1 Neighbor joining phylogenetic tree showing the position of isolate MZA-75 to other strains of *B. subtilis*. Accession numbers of the sequences used in this study are shown in *parentheses* after the strain designation. *Numbers* at *nodes* are percentage bootstrap values based on 1,000 replications. *Bar* 0.05 substitutions per nucleotide position

result of treatment with strain MZA-75. Figure 2b and c indicates the appearance of widespread cracks on the surface of PU film treated with *B. subtilis* strain MZA-75 for 1 month as compared to the untreated control (Fig. 2a).

FTIR spectroscopy The peak at 1,725 cm^{-1} representing carbonyl group of esters (Fig 3a) almost disappeared in the FTIR spectrum of test sample (Fig. 3b). The peaks at 1,164.9 and 1,136.9 cm^{-1} represent C–O stretching of the ester functionality, are disappeared in test samples. Both these indicate that ester hydrolysis took place as a result of microbial treatment.

GPC analysis Gel permeation chromatography (GPC) was conducted to evaluate changes in the molecular weight of the PU after treatment with *B. subtilis* strain MZA-75 for 4 weeks. Chromatogram of the treated PU film reveals an increase in polydispersity index as compared to that of untreated control. The polydispersity index increased from 1.369 (Fig. 4a) to 1.679 (Fig. 4b). The weight average molecular weight (Mw) and number average molecular weight (Mn) decreased from 48,762 to 48,152 and 35,616 to 28,667 respectively, after treatment with strain MZA-75. These results show that microbial treatment resulted in the cleavage of long chain polyester polyurethane molecules to fragments of relatively smaller molecular weight.

CO_2 production and cell growth coupled to polyurethane degradation

Indirect measurement of the capacity of MZA-75 to mineralize PU was quantified by estimating the amount of CO_2 evolved and number of viable cells count over the course of experiment. Polyurethane containing media inoculated with MZA-75 accumulated more CO_2 (7.62 g/l) ($P < 0.001$) than biotic control without polyurethane (3.5 g/l) ($P < 0.001$). Culture containing polyurethane increased approximately 4-log fold in cell numbers whereas no growth was observed in case of biotic control without polyurethane (Table 2).

Effect of polyurethane on esterase production

Esterase activity was detected both in cell free supernatant as well as in cell suspension. Extracellular

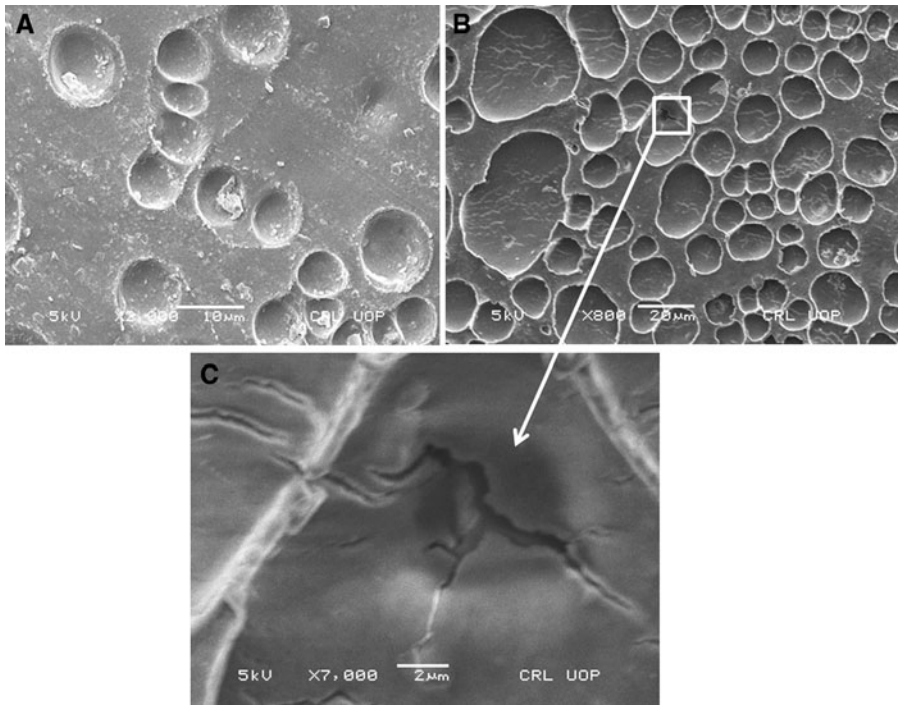

Fig. 2 SEM of PU films pieces after incubation with *B. subtilis* MZA-75 for 1 month. **a** Abiotic control with no surface change, **b** shows widespread cracks on the surface of PU film, **c** *encircled area* in "**b**" is magnified to have a clear view of the cracks

esterase activity increased steadily from 0.182 mM/min/mg on day one to 0.329 mM/min/mg ($P > 0.05$) on day 16 and then reached its maximum i.e., 0.494 mM/min/mg ($P < 0.001$) on day 24 in the presence of PU used as carbon source, while no increase in esterase activity was observed in the absence of PU (Fig. 5). Similarly, cell associated esterase activity dropped from 0.519 mM/min/mg on day one to 0.336 mM/min/mg ($P > 0.05$) on day 4 and then reached its maximum i.e., 1.210 mM/min/mg ($P < 0.0001$) on day 20 in the presence of PU (Fig. 6).

Detection of metabolic end products from polyurethane biodegradation

The monomers released as a result of degradation of PU by *B. subtilis* MZA-75, were analyzed by GC–MS. Two new peaks at retention times 13.1 and 18.19 min were observed in the samples drawn after 1 week incubation, which correspond to 1,4-butanediol and adipic acid respectively, after comparison with the standard chromatograms (Fig. 7).

Growth of MZA-75 on 1,4-butandiol and adipic acid

Strain MZA-75 could utilize the ester hydrolysis products of polyester PU by growing significantly faster in the presence of 1,4-butandiol and adipic acid, as indicated by a gradual increase in optical density (OD_{600}) at 37 °C within 48 h. Whereas no growth was observed in case of biotic control with no carbon source (Fig. 8). The difference between growth in the presence and absence of metabolites (1,4-butanediol and adipic acid) was extremely significant statistically with ($P < 0.001$).

Discussion

Current work focuses on the isolation of microorganisms capable of degrading polyester polyurethane by enrichment culture technique from local soil microflora. Soil samples were taken from solid waste dumping site in Islamabad Pakistan. The technique

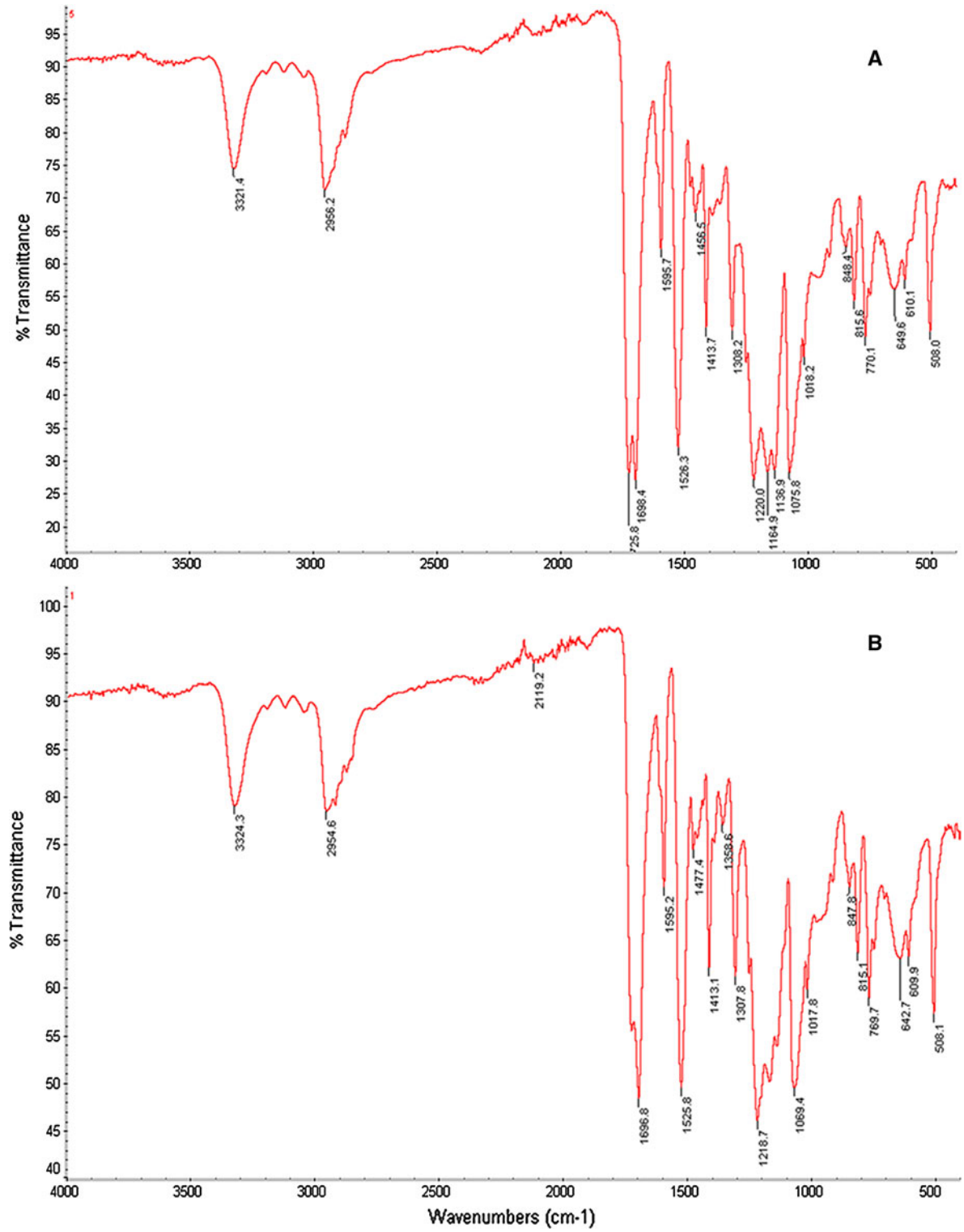

Fig. 3 FT-IR spectra of PU film pieces after incubation with strain MZA-75 for 1 month. **a** Abiotic control showing peaks at 1,725 cm^{-1} wave *number* corresponding to ester functional groups, **b** the peak at 1,725 cm^{-1} has disappeared which corresponds to degradation of ester linkage

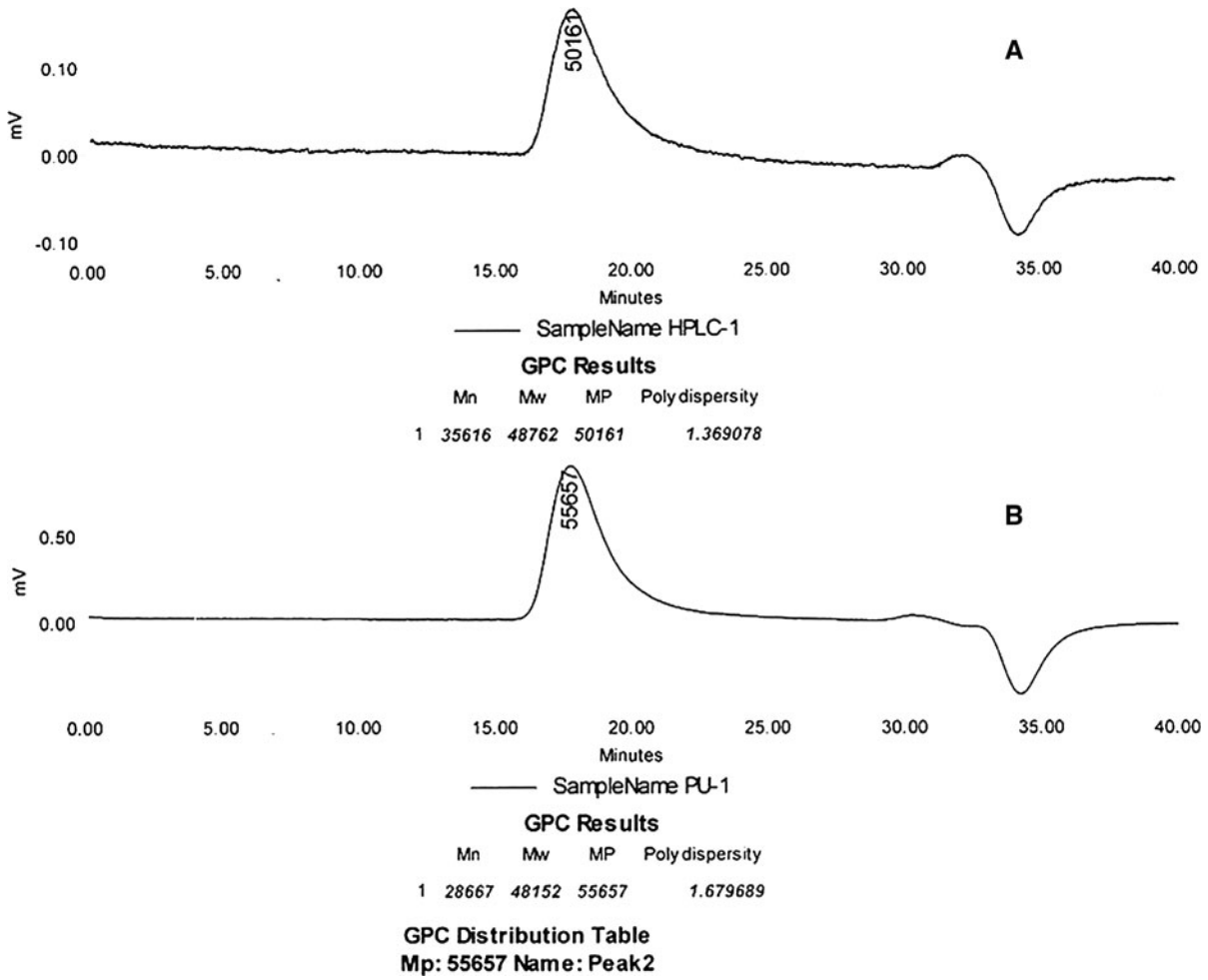

Fig. 4 GPC chromatograms of PU film pieces after incubation with strain MZA-75 for 1 month. **a** Abiotic control, **b** decrease in polydispersity and weight average molecular weight and number average molecular weight in treated samples

Table 2 Growth of MZA-75 and CO_2 evolution in the presence (test) and absence (control) of PU

	CFU/ml* at day 1	SD	CFU/ml after 30 days	SD	CO_2 evolved (g/l)	SD
Control	11×10^6	1	2.6×10^7	1.52	3.5	0.5
Test	10×10^6	1.73	6.6×10^{11}	1.52	7.62	0.54

CFU/ml colony forming units/ml, *SD* Standard deviation

of using contaminated soil for isolation of microorganisms with a particular metabolic capability has been previously used several times. Usha et al. (2011) isolated polyethylene degrading bacterial, fungal and streptomyces species from garbage soil. Mangrove soil has also been found to be a rich source of microorganisms degrading polyethylene (Kathiresan 2003). The isolated bacterium MZA-75 was identified as *B. subtilis* type strain, by morphological and biochemical characterization and comparative 16S rRNA sequence analysis. Rowe and Howard (2002) also isolated a *B. subtilis* strain from soil which could degrade PU and identified it by 16S rRNA sequencing. Besides this, a number species of Bacillus genus have

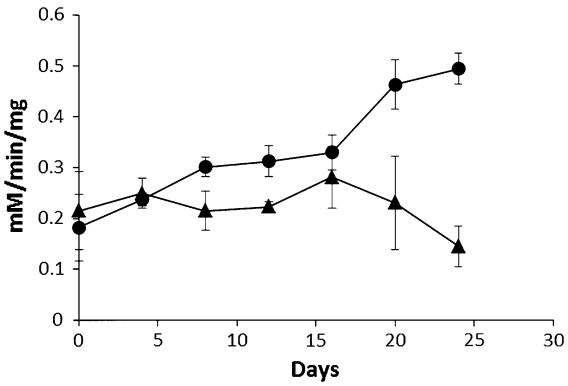

Fig. 5 Specific activity of extracellular esterases measured at 5 days intervals. A gradual increase in activity was observed in the presence of PU as sole carbon source till day 24 (*circle*). Esterase activity was low in the absence of PU (*triangle*). The results revealed that PU acts as an inducer for production of esterases

also been reported in degradation of other aromatic compounds. Perreault et al. (2012) reported biotransformation of 2,4-dinitroanisole by a *Bacillus* sp. G-12, which degraded 2,4-dinitroanisole via reduction and further acetylation. A *B. subtilis* strain RKJ 700 has recently been isolated from soil which can degrade a nitro-aromatic compound 4-chloro-2-nitrophenol into a number of metabolites (Arora 2012). Al-Sharidah et al. (2000) isolated hydrocarbon degrading *B. subtilis* from soil taken from crude oil contaminated sites.

Polyurethane degradation was tested by evaluating changes in surface morphology and chemistry of the exposed PU films through SEM and FT-IR respectively. Fourier transformed infra-red (FT-IR)

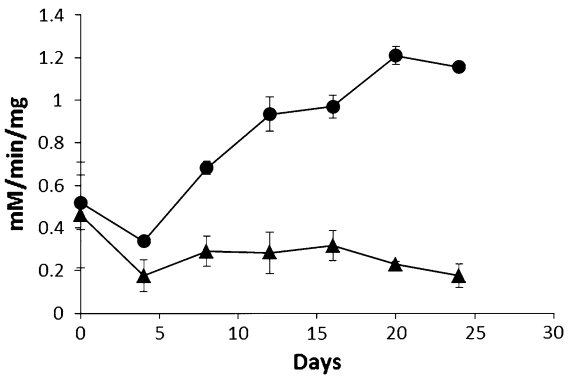

Fig. 6 Specific activity of extracellular esterases measured at 5 days intervals. A gradual increase in activity was observed in the presence of PU as sole carbon source till day 20 (*circle*). Esterase activity was low in the absence of PU (*triangle*)

spectroscopy is a non-destructive micro-analytical spectroscopic analysis that involves the study of molecular vibrations. It was observed that microbial treatment resulted in the appearance of widespread cracks and holes on the surface of treated PU films and hydrolysis of ester functionality into carboxylic acids as evident from a peak at 1,725 cm^{-1} in the FT-IR spectrum of the treated PU films. FT-IR spectrum of the treated PU films supports the idea of involvement of microbial esterases in the degradation of polyester polyurethane. Russell et al. (2011) investigated the fungal degradation of PU samples of Impranil DLN by using FT-IR and observed gradual decline in the peak representing ester functionality (1,735 cm^{-1}) with gradual loss of opacity in the PU supplemented culture broth. The appearance of cracks and holes on the treated PU surface is a visual evidence of biodegradation (Gautam et al. 2007). Akutsu et al. (1998) observed similar changes in the SEM photographs; however, they used purified PU esterase to degrade solid PU pieces in their experiment. Shah et al. (2008) used a consortium of *Bacillus* sp. strain AF8, *Pseudomonas* sp. strain AF9, *Micrococcus* sp. strain 10, *Arthrobacter* sp. strain AF11, and *Corynebacterium* sp. strain AF12 against PU film pieces in mineral salt medium. SEM and FTIR results indicated both morphological and chemical changes in the PU film.

Gel permeation chromatography (GPC) is a valuable aid to the study of polymer degradation. A standard GPC process is usually the best approach to compare the molecular weight distribution of polymers known to be of the same chemical type and structure (chain branching). So we used this technique to find out whether microbial treatment of the PU films results in changes in the molecular weight distribution or not. GPC analysis revealed an increase in polydispersity index and decrease in both weight average molecular weight (Mw) and number average molecular weight of the treated PU films, which is an evidence of changes in the molecular weight distribution of PU or biodegradation as a result of treatment with *B. subtilis* strain MZA-75. These results also indicate that the degradation carried out by strain MZA-75 is not limited to the surface of the films. Christenson et al. (2006) employed SEM, FT-IR and GPC to evaluate the response of commercial polyether polyurethane and polycarbonate polyurethane to cholesterol esterase and found that the treatment resulted in small loss of surface soft segments.

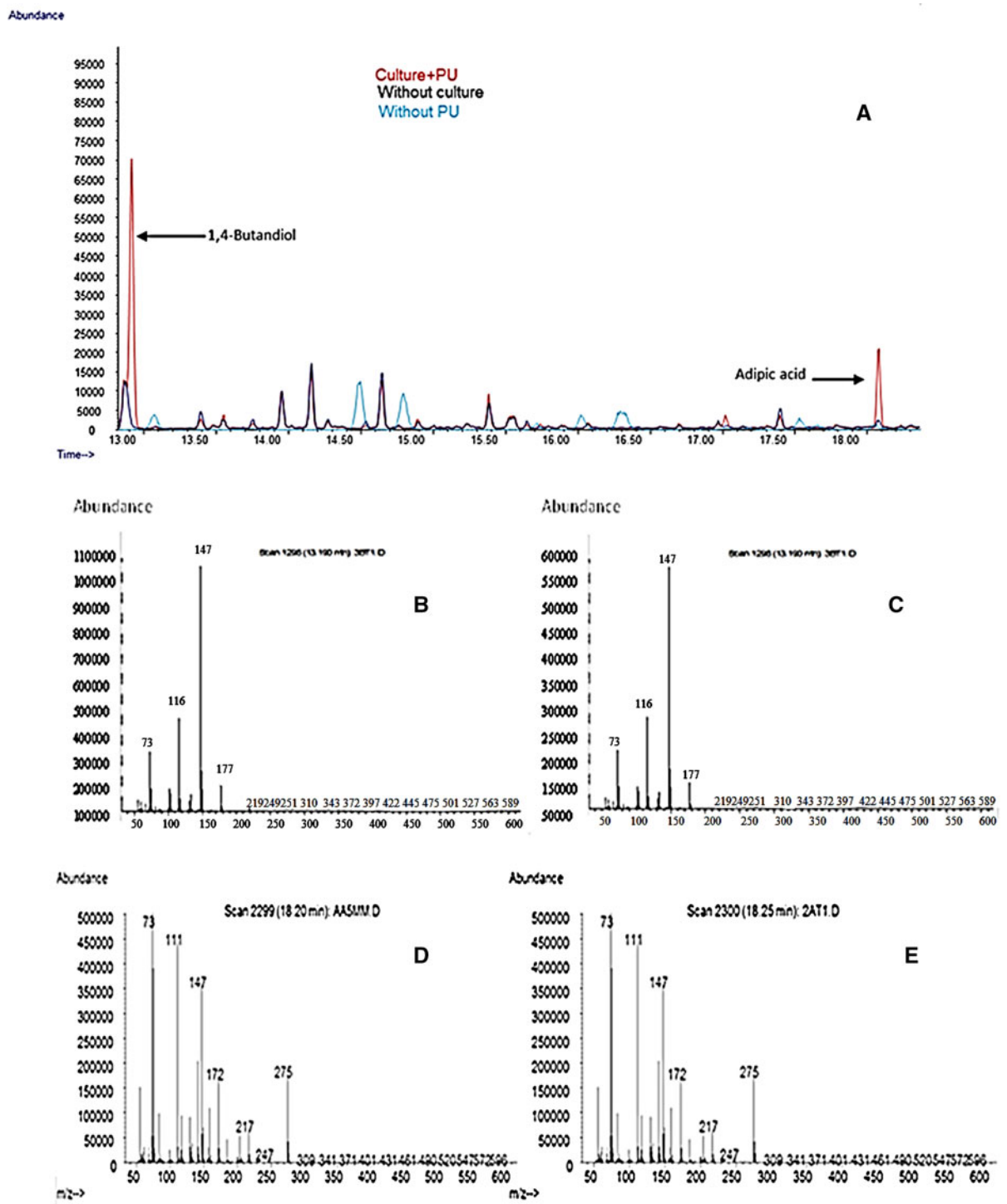

Fig. 7 GC–MS chromatogram of PU samples treated by strain MZA-75. **a** Chromatogram overlay of culture + PU (*red line*), abiotic control (without MZA-75) (*black line*), biotic control (without PU) (*blue line*). 1,4-Butanediol (13.4 min) and adipic acid (18.5 min) peaks are represented by arrows in culture + PU chromatogram. **b**, **d** Mass spectrum of 1,4-butanediol and adipic acid standard, respectively. **c**, **e** 1,4-Butanediol and adipic acid extracted from PU samples treated by *Bacillu subtilis* MZA-75, respectively. (Color figure online)

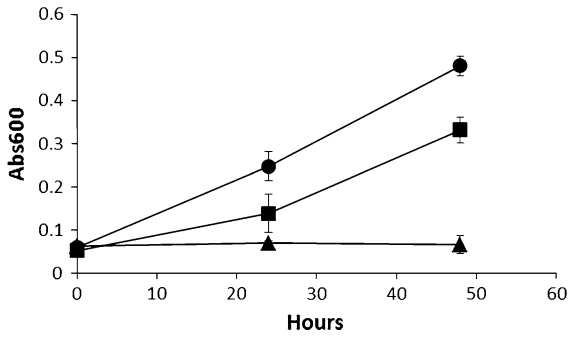

Fig. 8 Growth of *B. subtilis* strain MZA-75 based on changes in Abs_{600} with time in MSM (*triangle*), MSM supplemented with 1,4-butanediol (*circle*) and MSM with adipic acid (*cube*)

Generally, biodegradation occurs in two steps, in first step chain cleavage occurs and polymers are converted into their corresponding oligomers and monomers known as depolymerization. This is followed by mineralization in which monomers and oligomers formed are sufficiently smaller in size and are transported to the cytoplasm of cells of the microorganisms and get completely mineralized. This is the process in which various byproducts such as CO_2, water, methane and other inorganic substances are formed depending on whether the process is aerobic or anaerobic. The ability of aerobic microorganisms to mineralize polymeric substances can be traced by entrapment of CO_2 evolved as a result of mineralization by using Sturm test. In the current study, the amount of CO_2 evolved in the test vessel with PU as the sole carbon source, was greater than the control vessel without PU, this provides evidence that *B. subtilis* MZA-75 is mineralizing the polymer. Previously we observed similar results when we employed Sturm test to evaluate PU mineralization capability of the consortium isolated from soil (Shah et al. 2008).

Bacillus subtilis strain MZA-75 hydrolyzed the polyester portion of polymer into 1,4-butanediol and adipic acid as identified by GC–MS analysis of the cell free supernatant. Nakajima-Kambe et al. (1997) detected ester hydrolysis product i.e., adipic acid and diethylene glycol during polyurethane degradation using GC–MS but was unable to elucidate the effect of polyurethanolytic enzymes on the polyisocyanate portion of the polyester PU. Strain MZA-75 showed rise in both extracellular and cell associated esterase activity when grown in the presence of PU in minimal salt medium. Russell et al. (2011) reported the capability of endophytic fungus *Pestalotiopsis microspora* to produce extracellular PU degrading serine hydrolase enzyme. Strain MZA-75 cells associated esterases hydrolysed the esters of PU into corresponding monomers i.e., 1,4-butanediol and adipic acid. *Comamonas acidovorans* TB-35 produce both cell free and cell bound esterases and its polyester polyurethane degrading ability is because of cell bound esterase having a molecular weight of 62 kDa (Nakajima-Kambe et al. 1997; Akutsu et al. 1998). One possible rationale for this may be that cell bound esterase is depolymerizing the polymer into suitable size that can be transported inside cells, and then cellular enzymes monomerize it to be used as carbon source.

Conclusions

Bacillus subtilis MZA-75 employs both extracellular and cell associated esterases to utilize polyester PU as carbon source. Degradation as a result of MZA-75 treatment manifests in the form of both changes in the surface morphology and chemistry. Changes in polydispersity index indicates decrease in the molecular weight of polymer chain. The organism not only degrades the polyester diol portion constituting soft segment of the polyester polyurethane and produce degradation metabolites i.e., 1,4-butanediol and adipic acid, but also utilize these intermediates as carbon source to mineralize the polymer. Further study on the mechanism of degradation of polyester polyurethane by strain MZA-75 is required for its effective application in polyurethane waste management and as a tool in the process of biochemical monomerization for purified monomer recycling.

Acknowledgments We are thankful to Higher Education Commission of Pakistan and University of Oklahoma, USA, for providing funds and utilization of lab facilities to accomplish this research, respectively.

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