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journal homepage: www.elsevier.com/locate/envpolBiodegradation of polyester polyurethane by *Aspergillus tubingensis*[☆]

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ABSTRACT

The xenobiotic nature and lack of degradability of polymeric materials has resulted in vast levels of environmental pollution and numerous health hazards. Different strategies have been developed and still more research is being in progress to reduce the impact of these polymeric materials. This work aimed to isolate and characterize polyester polyurethane (PU) degrading fungi from the soil of a general city waste disposal site in Islamabad, Pakistan. A novel PU degrading fungus was isolated from soil and identified as *Aspergillus tubingensis* on the basis of colony morphology, macro- and micro-morphology, molecular and phylogenetic analyses. The PU degrading ability of the fungus was tested in three different ways in the presence of 2% glucose: (a) on SDA agar plate, (b) in liquid MSM, and (c) after burial in soil. Our results indicated that this strain of *A. tubingensis* was capable of degrading PU. Using scanning electron microscopy (SEM), we were able to visually confirm that the mycelium of *A. tubingensis* colonized the PU material, causing surface degradation and scarring. The formation or breakage of chemical bonds during the biodegradation process of PU was confirmed using Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) spectroscopy. The biodegradation of PU was higher when plate culture method was employed, followed by the liquid culture method and soil burial technique. Notably, after two months in liquid medium, the PU film was totally degraded into smaller pieces. Based on a comprehensive literature search, it can be stated that this is the first report showing *A. tubingensis* capable of degrading PU. This work provides insight into the role of *A. tubingensis* towards solving the dilemma of PU wastes through biodegradation.

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1. Introduction

Tremendous increase in the production and use of various

manmade polymers has become a huge threat to the environment due to their unabated disposal. The polymeric materials do not decompose easily and their xenobiotic nature marks them a rising problem all around the globe (Álvarez-Barragán et al., 2016). Various commonly used synthetic plastics are polyethylene, polypropylene, polystyrene, polyester polyurethane (PU), polyvinylchloride (PVC), polyethylene terephthalate (PET), polytetrafluoroethylene (PTFE), nylon and polycarbonate. PU is a polymer used in various applications such as manufacturing of tyres, gaskets, bumpers, fibers, plastic foam, synthetic leathers, jackets, adhesive, paints, sponges and cushions, rubber goods, in automobiles and

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refrigerator insulation etc. all of which lack degradability.

The estimated annual production of PU around the world is approximately 8 million tons (Mt) which tends to increase by 4–5% every year (Matsumura et al., 2006). According to Caudron (2003), the PU foam waste generated in Europe is about 100,00 t/year, and Cregut et al., 2013 estimated that the total PU waste generated per year in Europe ranges from 2.1 to 3.6 Mt. The non-degradable nature and high persistence of these materials lead to increase in soil surface littering, smothering of water resources and dirtying the landfills (Zafar et al., 2013; Álvarez-Barragán et al., 2016). Polyurethane is synthesized from two monomers, a diol and a highly toxic and reactive diisocyanate, derived from a toxic substance phosgene, in the presence of some additives. The resulting urethane polymer is generally resistant to biodegradation (Tokiwa, 2002; Howard, 2002). On the other hand, reprocessing of PU produces toxic residues such as aldehydes, ammonia, cyanide, isocyanates, nitrogen oxides and vinyl chloride which are proven hazardous for human health, and responsible for disruption in ecological processes and environmental pollution. Hence, there is a need to search new alternative means to solve the problems posed by PU waste accumulation.

Numerous studies have reported that PUs are vulnerable to fungal attack (Barratt et al., 2003; Krasowska et al., 2012; Mathur and Prasad, 2012; Zafar et al., 2013, 2014). These and other studies have mentioned that the polyester polyurethanes (PUs) were more vulnerable to fungal biodegradation than any other type, while the polyether polyurethanes were not vulnerable to fungal attacks. The structure, type and position of different chemical groups, cross linkers and the molecular orientations of different types of PU determine their degree of degradation by fungus (Kim and Kim, 1998; Umare and Chandure, 2008). The involvement of soil fungal community in the biodegradation of PU has been reported by Cosgrove et al. (2007). The results of the Impranil treated PU buried in soil showed that very few species have the ability to grow on the surface of PU. PU was highly vulnerable to degradation by losing its tensile strength up to 100%, and the PU degrading fungal communities were found to be severely influenced by the soil type. *Geomyces pannorum* was identified to be the most abundant organism from the surface of PU buried in acidic soil, while a *Phoma* sp. was detected to be the major colonizing species in neutral soils (Cosgrove et al., 2007). Similarly, the pH, temperature, carbon source availability and composition could also affect the growth of fungus on the surface of PU.

Although various fungi and bacteria responsible for the biodegradation of PU have been identified, but the actual mechanism underlying the degradation is not clearly known. Various studies have revealed that some enzymatic mechanisms are involved in microbial degradation of PU (Loredo-Treviño et al., 2012; Mahajan and Gupta, 2015). Enzymes such as esterase and urethane hydrolase have been isolated from the fungi *Aspergillus terreus* and *Chaetomium globosum* (Boubendir, 1993). An extracellular and membrane bounded lipase has been characterized for the urethane bond hydrolysis by many researchers (Cregut et al., 2013).

It is concluded from the above discussion, that there are a vast number of fungi could have the ability to colonize the surface of PU, but remains undiscovered. Therefore, a thorough search for PU degrading fungi, testing for degradation in different ways, and determining their enzymatic activities would present an interesting insight into PU waste management.

The aim of this work was: a) isolation and identification of a PU degrading fungi from soil, b) biodegradation test of PU on SDA culture plate, in liquid MSM, and in soil burial and c) determination of the effect of pH, temperature, surfactants, and extra carbon sources on PU degrading enzymes (esterases and lipases) activities.

2. Materials and methods

2.1. Sample collection

Soil samples were collected in sterilized polyethylene bags from the dumping area, in Sector H-10, Islamabad, Pakistan. The pebbles, plastics, and wood pieces were removed and the clean soil samples were shifted to big pots (sterilized) and kept at room temperature in dark.

2.2. Preparation of polyurethane films

Polyester polyurethane beads (Aldrich Chemical Company, Inc. USA) (1 g) were dissolved in 100 ml of tetrahydrofuran (Pan-reacQuímica, SA) and sonicated for 30 min in 250 ml flask. The PU solution was then poured into four Petri dishes and allowed to solidify for 48 h in a desiccator at room temperature. Dried PU layers were carefully removed from Petri dishes and stored at room temperature (RT).

2.3. Isolation of polyurethane degrading fungus

Five films of polyurethane were buried about 4–6 inches deep vertically in the soil pot and kept at RT in dark. About 2 g of glucose was mixed in 200 ml of mineral salt medium (MSM) consisting of K_2HPO_4 (0.5 g), KH_2PO_4 (0.04 g), NaCl (0.1 g), $CaCl_2 \cdot 2H_2O$ (0.002 g), $(NH_4)_2SO_4$ (0.2 g), $MgSO_4 \cdot 7H_2O$ (0.02 g), $FeSO_4$ (0.001 g), autoclaved and after cooling added into the soil pot. After one-month time interval, one of the PU film buried in soil was washed gently with sterilized dH_2O water and shifted to SDA containing Petri dish and incubated at 37 °C for one week. The fungi which can colonize the PU surface were re-cultured on a separate SDA plate, incubated at 37 °C for 20 days (Fig. 1-A). The plates and PU sheets were observed through SEM and ATR-FTIR.

2.4. Identification of polyurethane degrading fungus

The fungal strain purified on agar plate was identified on the basis of microscopic and macroscopic examination, colony morphology and phylogenetic analyses (White et al., 1990; Thompson et al., 1997; Swofford, 2004). The microscopic examination for the structure of conidia, hyphae, conidial head, and conidiophores were done using a Nikon 80i compound microscope with a Canon camera mounted on the top. To observe the surface features of spores at higher magnification pieces of the culture were cut out and mounted for cryomicroscopy in the SEM. Biospin Fungus Genomic DNA Extraction Kit-BSC14S1 (BioFlux, P.R. China) was used to extract DNA from fresh mycelium of the fungus. The PCR was carried out using the primers (ITS4: 5' TCCTCCGCTTATTGATATGC 3'), and (ITS5: 5' GGAAGTAAAAGTCGTAACAAGG 3') following White et al. (1990). The sequences for each strain were aligned using ClustalX (Thompson et al., 1997) and to get maximum sequence similarity the alignment was manually adjusted. The gaps between nucleotides were considered as missing data and using PAUP* 4.0b10, phylogenetic analyses were performed (Swofford, 2004). Phylograms were figured in FigTree v. 1.4 and edited in Microsoft power point (2007) and Adobe illustrator CS3 (Adobe Systems Inc., USA).

2.5. Polyurethane biodegradation tests

2.5.1. Biodegradation test in the soil

The films of polyurethane buried vertically in the soil pots in dark at room temperature were maintained for four-month interval of time. After four-month, one of the polyurethane film buried in

the soil was washed with sterilized distilled water gently, and the colonies (fungal) developed on the PU surface were photographed and stored for ATR-FTIR and SEM analysis at room temperature.

2.6. *Aspergillus tubingensis* biodegradation tests

2.6.1. Culture plate technique

The fungus *Aspergillus tubingensis* was tested for biodegradation on SDA Petri plate. The plate was inoculated with *A. tubingensis* and covered by a sterilized PU film. The plate was placed in an incubator at 37 °C for four days. After four days of incubation, when the fungal growth appeared on the surface of PU film, it was shifted to MSM agar Petri plate and incubated at 37 °C. Photographs were taken from the test samples. Moreover, SEM and ATR-FTIR analyses of the test samples and control were performed.

2.6.2. Biodegradation in liquid medium

Two percent (4g) glucose was added to 200 ml mineral salt medium (MSM) in 500 ml flask having polyurethane films previously sterilized. The flask was incubated at 37 °C in shaker incubator at 150 rpm for three weeks after inoculation with 2 ml spore suspension of concentration of 1×10^5 spores/ml. After 20 days of incubation, the films were washed with sterilized distilled water and analyzed for degradative changes by ATR-FTIR and SEM as well as photographs. The remaining test sample was maintained for two months on room temperature in the same flask. The loss of tensile strength and the degradation were also photographed.

2.7. Optimization of PU degrading enzymes

As esterase and lipase are the two well-known enzymes responsible for the biodegradation of PU. Hence, the effect of temperature, pH, additional carbon sources, and surfactants on the biodegradation of PU, and growth of fungus were studied by measuring the esterase and lipase activities in broth culture.

2.7.1. Effect of pH

Four PU films (0.25 g) after sterilizing by exposure to ultra violet radiation for 2 min and were added into 250 ml flasks having 200 ml MSM, with different pH values of 5, 7, and 9. The flasks

were inoculated with 1% (v/v) spore suspension (1×10^5 spores/ml) of *A. tubingensis*, except the control. All the flasks were incubated at 37 °C at 150 rpm for a period of one month. Five milliliter (5 ml) of samples were collected as a crude enzyme, at zero time and after every week for testing enzyme activity (lipase and esterase).

2.7.2. Effect of extra carbon source

Four PU films were added to each flask containing 200 ml liquid MSM with two grams of various carbon sources such as glucose, fructose, galactose, maltose, and glycerol. The flasks were incubated at 37 °C (150 rpm) for a period of one month after inoculation with 2 ml spore suspension (*A. tubingensis*) of concentration of 1×10^5 spores/ml, except the control. To determine lipase and esterase enzyme activities, 5 ml of the samples were collected at the zero time, and after every week, for a period of one month.

2.7.3. Effect of surfactants

Four milliliter (2% v/v) surfactants (Tween 20 and Tween 80) were added to 250 ml flasks, having 200 ml MSM with pH 7. Sterilized PU films (0.25 g) were added to the flasks. Another flask having the same composition with no surfactants was used as control. All the flasks were inoculated with (1% v/v) spore suspension of concentration of 1×10^5 spore/ml and incubated at 37 °C at 150 rpm for a period of one month. Five milliliter (5 ml) of samples were collected at zero time and then after every week to test the activity of enzymes.

2.7.4. Effect of temperature

Three flasks containing MSM (200 ml), four (0.25g) sterilized PU films and 1% inoculum (1×10^5 spore/ml) were incubated at 30 °C, 37 °C and 40 °C for a period of one month at 150 rpm. One another flask was used as control having all other constituents except spore's suspension. Samples (5 ml) were collected from all the four flasks on weekly basis starting from zero time and after every seven days for a period of one month.

2.8. Lipase and esterase activities assays

The samples were centrifuged and the cell free supernatant was

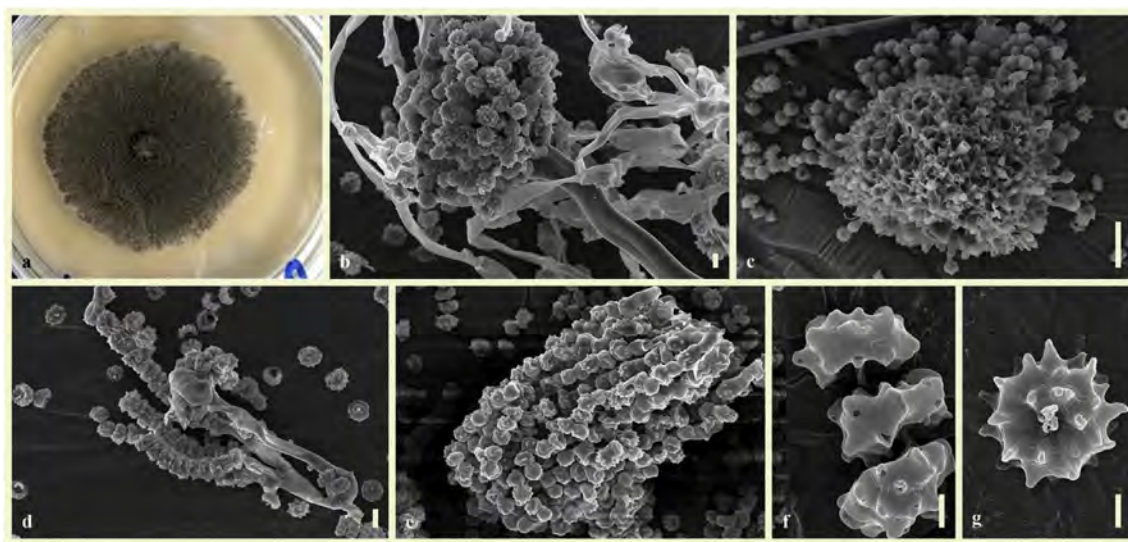


Fig. 1. *Aspergillus tubingensis* (a) culture on MEA after 20 days, SEM images of (b) hyphae, stalk and sporangium (c) Immature sporophores on sporangium, (d) Conidia and sporangium with bilayer structure, (e) Conidial chains (cantente) on sporangium, (f) Conidia showing lobatereticulate ornamentation and equatorial furrow flanked by lobate crests, (g) a single conidium. Scale bars: A = 70 mm culture plate, B = 2 μm, C = 10 μm, D = 3 μm, E = 3 μm, F = 1 μm, and G = 1 μm.

used to detect the activities of the hydrolytic enzymes (lipases and esterases).

Lipase and esterase activities were determined as previously described (Lesuisse et al., 1993). The *p*-nitrophenyl laurate and *p*-nitrophenyl acetate were used as a substrate and the quantity of *p*-nitrophenol production was used as enzyme activity (U/ml) for lipase and esterase, respectively. About 880 μ L of reaction mixture (0.1M potassium phosphate buffer pH 8.0, 0.2% sodium deoxycholate, 0.1% gum Arabic) was taken in a small tube, added 20 μ L crude enzyme extract and incubated for three minute at 37 °C. The reaction was started by adding 100 μ L (8 mM) substrate (8.0 mM *p*-nitrophenyl laurate (lipase) or 8.0 mM *p*-nitrophenyl acetate (esterase) solubilized in isopropanol) and terminated by adding 0.5 ml (3 M) hydrochloric acid. The suspension formed was centrifuged for ten min at 10,000 rpm. Supernatant (333 μ L) was taken in a separate tube and 1 ml (2 M) NaOH was added. Using spectrophotometer, the light absorbance was measured at wave length of 420 nm. Using know concentrations of *p*-nitrophenol the standard curve was prepared. The unit of activity was defined as the amount of enzyme that hydrolyzes 1 μ M substrate in 1 min.

3. Results

3.1. Identification of PU degrading fungus

The fungal strain was identified as *Aspergillus tubingensis* on the basis of colony morphology (Fig. 1-A), macroscopic and microscopic examination (Fig. 1B–D), and phylogenetic analyses (White et al., 1990; Lesuisse et al., 1993; Swofford, 2004) (Fig. 2).

Thirty-three ITS sequences of the genus *Aspergillus* section Nigri were retrieved from GenBank. The sequence set composed of six *A. tubingensis* sequences, 27 sequences of *Aspergillus* in section Nigri, and the sequence of our isolate HKAS No. 93727 (GeneBank accession No. KX165340). The Maximum Parsimony analyses showed our isolate was positioned among the group of *A. tubingensis* with 100% bootstrap support (Fig. 2), which indicating it is a strain of *A. tubingensis*.

3.1.1. Taxonomy

Aspergillus tubingensis Mosseray, La Cellule 43: 245 (1934) (Fig. 1A–D).

Synonymy: \equiv *Aspergillus niger* var. *tubingensis* (Mosseray) Kozak., Mycol. Pap. 161: 112 (1989)

Colonies (Fig. 1-A) attaining a diameter of 35 mm at 37 °C after 20 days on malt extract agar (MEA); generally, densely covered with conidial heads; grayish brown (8G3; Kornerup and Wanscher, 1978) or between grayish brown and dark brown (8G4); reverse white to cream. **Sexual morph** *Ascocarps* produced within stromata, globose to ellipsoidal but often straight sided where adjacent to other ascocarps. *Asci* globose to broadly ellipsoidal, containing eight inordinately arranged ascospores, 18.5–23.5 μ m high, \times 14.5–19.5 μ m diam. ($n = 50$). *Ascospores* (Fig. 1-E) oblate, lobate reticulate with an equatorial furrow flanked by lobate crests, hyaline to pale brown, globose to subglobose in face view, 6.5–8.5 \times 5.5–7.5 μ m ($n = 50$). **Asexual morph** *Stipes* 500–2400 μ m high, 10–20 μ m diam. near vesicle, light brown, smooth (Fig. 1B). *Conidial heads* biseriate, rarely uniseriate, radiate, commonly splitting into columns, up to 700 μ m (Fig. 1C–E). *Conidia* 3.2–4.3 μ m, globose to subglobose, tuberculate to aculeate with ridges (Fig. 1-F and G).

Material examined: Pakistan, Islamabad Capital of Pakistan, Islamabad City, UTM-N 3,734,759.34, UTM-E 322,552.71; alt. 604 m; 6 March, 2008; from the soil of plastics waste disposal area; Sehroon Khan (HMAS: 93727).

3.2. Biodegradation of PU on MSM agar plate

Aspergillus tubingensis was grown on SDA plate and a sterilized PU film was placed over it as shown in Fig. 3-A. It was incubated for four days until the fungal growth appeared on the surface of PU films and were then shifted to MSM agar plates (Fig. 3-B). The fungal growth on the surface of PU film was observed and the inoculated area of the PU film was totally degraded making large holes in that areas (Fig. 3-C & D). The visible changes (erosions, blackening, and holes) on the surface of PU film were observed (Fig. 3-G & H) showing biodegradation. The results of SEM showed roughness on the surface of PU film with pores and crakes in some areas were due to the hyphal penetration and growth (Fig. 3-G & H). In contrast, there were no surface degradations on the control PU films as seen in Fig. 3-E & F.

ATR-FTIR analysis of the fungus treated PU films on MSM agar plate showed few changes in the spectra as compared to control. The band in the test spectrum at 3271.9 cm^{-1} was shifted to 3325.4 cm^{-1} in control spectrum and the test spectrum band was broader than control spectrum (Fig. S1). Another band at 2919.3 cm^{-1} in test spectrum was shifted to 2954.2 cm^{-1} in control spectrum. The disappearance of sharp band in the test spectrum at 1725.8 cm^{-1} was observed in control spectrum. The appearance of characteristic band at 1632.0 cm^{-1} was present in test spectrum which was absent in control spectrum (Fig. S1). The appearance and disappearance of new bands are a sign of biodegradation.

3.3. Biodegradation in liquid medium

The ability of *A. tubingensis* to degrade PU films (Fig. 4-A) was checked in liquid MSM having 2% glucose. The fungus was found to adhere to the surface of PU films (Fig. 4-D) and folded the PU films (Fig. 4-B & C) by having high growth over the surface. The hyphae became penetrated in the surface of the film resulting in crakes (Fig. 4-D). The SEM results showed that there was a thick hyphal mass covered the surface of the PU film after one month of incubation in liquid MSM flask. The pores and cracks in the surface of PU film were clearly visible (Fig. 4-D). After two months of incubation in the MSM along with 2% glucose, the PU film was degraded into small pieces (Fig. 4-E). This result was very interesting with almost 90% degradation of the PU film. The PU film was observed to dissolved in the MSM (Fig. 4-E). ATR-FTIR analysis of the microbial treated samples of PU film after one month of incubation in liquid MSM showed few changes in the spectrum as compared to the control (no exposure to microorganisms) (Fig. S2). The band in the spectrum for hydrogen bonded NH was at 3321.3 cm^{-1} in the sample spectrum, while the same band was at 3327.3 cm^{-1} in the control spectrum. Similarly, another band was observed in the test spectrum on the shoulder of the band at 3321 cm^{-1} , which was absent in the control spectrum, which indicated the formation of some new product due to biodegradation. The absorption band for aliphatic CH was observed at 2954.8 cm^{-1} in the test spectrum while that was at 2954.0 cm^{-1} in the control spectrum. Similarly, a small band was observed on shoulder of band at 2954.8 cm^{-1} in the sample spectrum, while it was absent in the control spectrum. It is assumed that it was because of the formation of new aliphatic CH bond which may be occur due to hydrogenation of double bond in the chain. The band at 1726 cm^{-1} for urethane carbonyl group (C=O) persisted in both the test and control spectra. The strong band at 1218 cm^{-1} , assigned to C-O-C also was observed in the control as well as in the test spectrum. It can be concluded that a rupture of C-O-C does not take place at that point. A characteristic band of medium intensity at 1643.6 cm^{-1} assigned for NH deformation appeared in the test spectrum which was not present in control spectrum (Fig. 5). These changes showed that the PU film

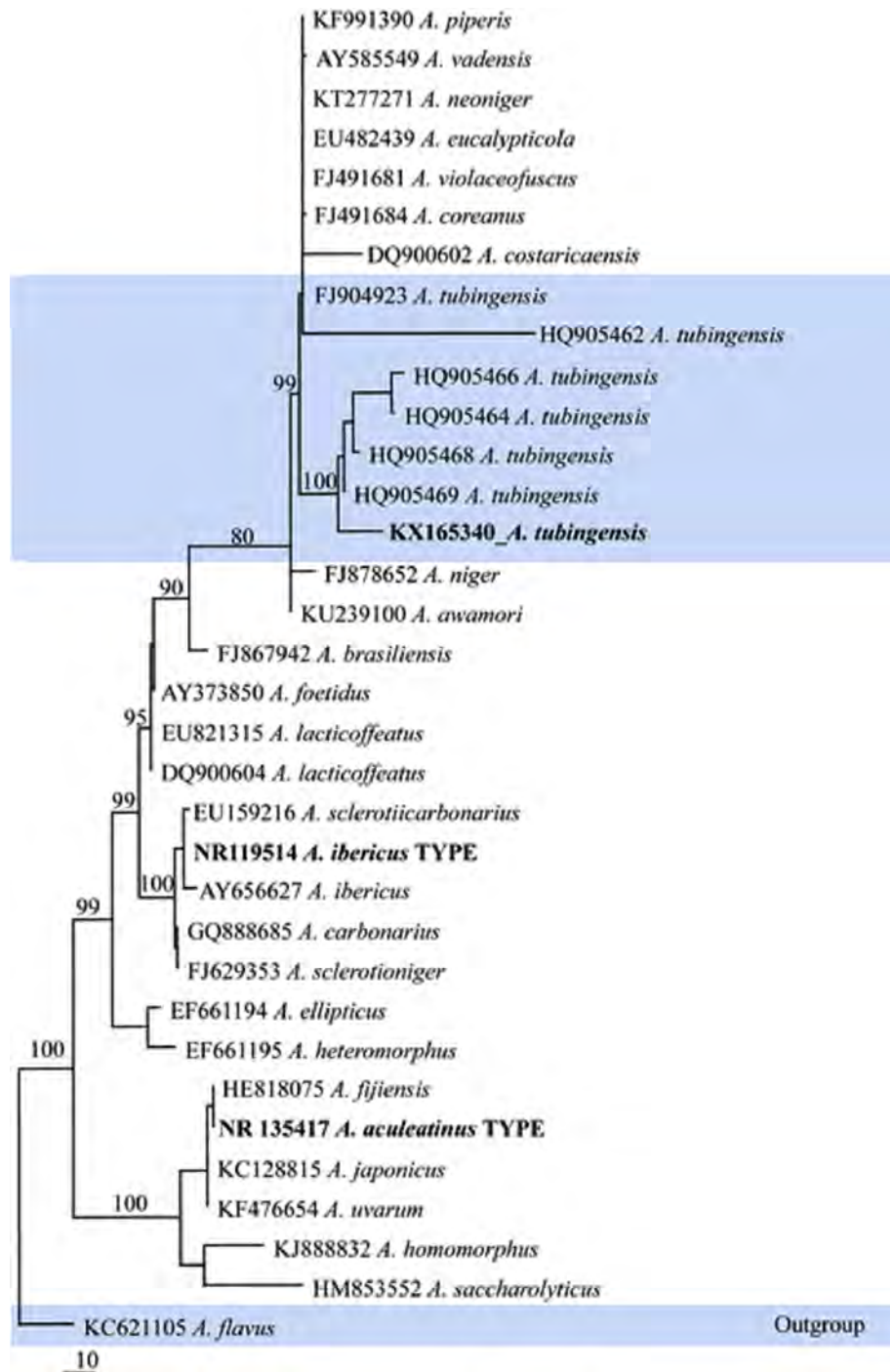


Fig. 2. The MP tree obtained based on phylogenetic analysis of ITS sequence data of *Aspergillus* section *Nigri*. Numbers above branches are bootstrap values. Only values above 70% are indicated. Type strains and our strain are in bold.

was degraded by the fungal enzymes through the breakdown of some bonds and formation of others. This breakdown and formation of bonds could be a sign of degradation.

3.4. Soil burial biodegradation test of PU films

PU films buried in the soil for four months were recovered every month each and were stored for SEM and ATR-FTIR analysis at room temperature in dark. PU films buried in soil for two months (Fig. 5-B) was found eroded and the biofilm formation was also observed

on the surface while observed under SEM. After four months of soil burial, the biofilm formation was visible on the surface of PU film along with the discoloration and other structural changes (Fig. 5-C). Its SEM results gave us the clear picture of the cracked and biodegraded PU film. The small holes, erosion, cracks, loss of tensile strength, and the roughness of the surface were clearly observed (Fig. 5-D).

PU films were analyzed by ATR-FTIR spectra after four-months soil burial. The results showed that in case of control spectrum, the band at 1725 cm^{-1} was absent which was present in the sample

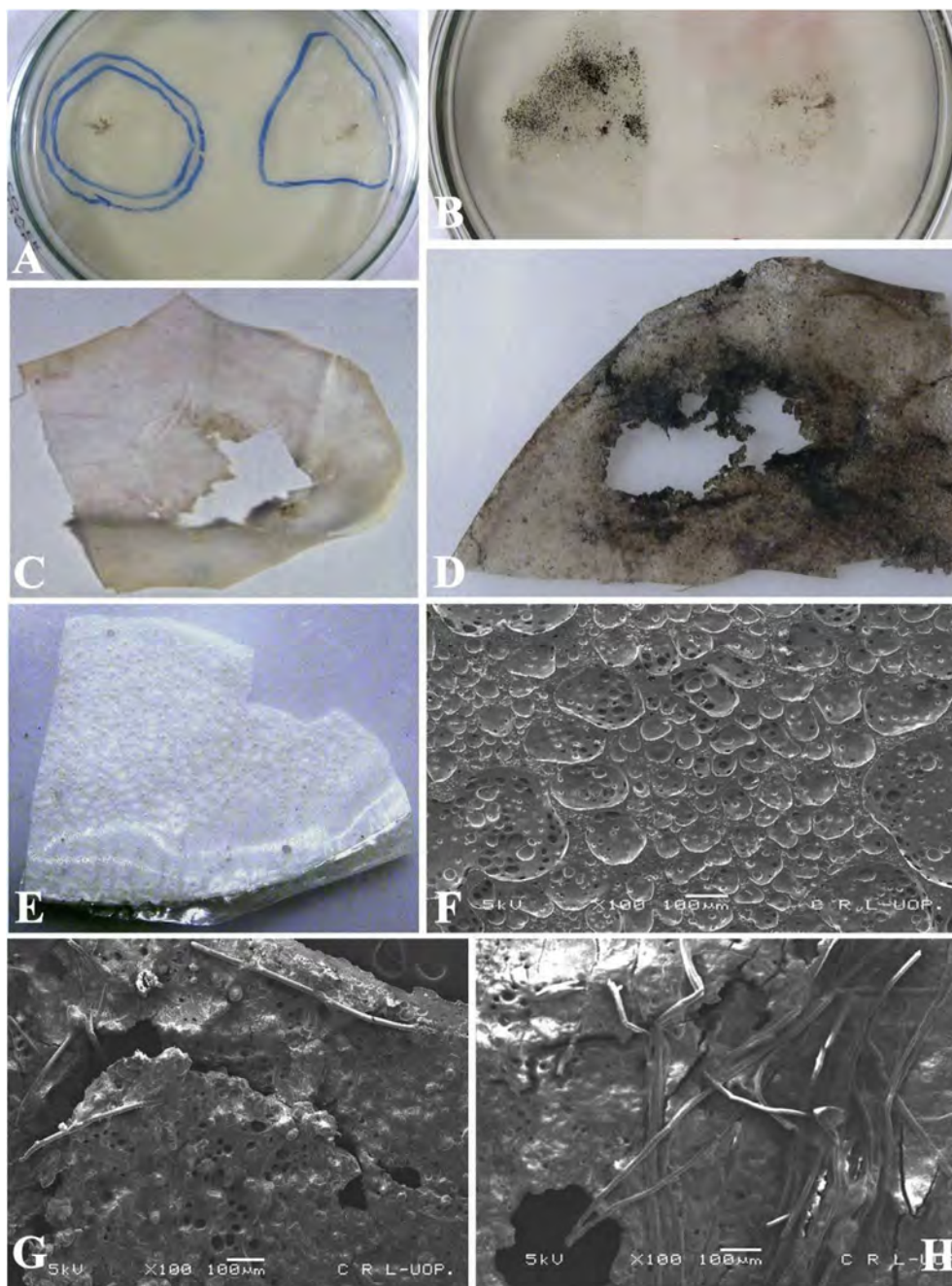


Fig. 3. Growth of *Aspergillus tubingensis* on the surface of PU, (A) SDA plate inoculated with *A. tubingensis* and covered by PU sterilized films, (B) the PU films shifted to MSM agar plate after 4 days of incubation, and (C) PU film recovered from MSM agar plate after three weeks, (D) degraded PU film incubated on SDA plate for two weeks, (E) control to the degraded PU film D, (F) SEM of the control E, (G and H) SEM of the test D.

spectrum which attributed to incompletely hydrogen bonded urea carbonyl (Fig. S3). Similarly, the band at 2917 cm^{-1} shifted to 2955.2 cm^{-1} in the sample spectrum, which showed the formation of C-H bonds (Fig. S3).

3.5. Esterase and lipase optimization

Effect of various environmental factors on the activities of the enzymes esterase and lipase produced by *A. tubingensis* was studied. Enzymes production in relation to various carbon sources was studied by adding different carbon sources (2% w/v) to MSM along with PU films. Results indicated that enzymes production

was enhanced when sucrose was used as the substrate (Fig. 6). Among the surfactants, increased esterase production was observed in the presence of Tween 80 while in case of lipase, increased enzyme production was observed in the presence of Tween 80 (Fig. 7).

The optimum pH required for maximum activity of esterase and lipase is shown in the Fig. 8. Maximum esterase activity was recorded at pH 7 while lipase showed maximum activity at pH 5. (Fig. 8). At pH 9 after 8 days of incubation, the enzyme activities started to decrease (Fig. 8). The optimum temperature for both the enzymes produced by *A. tubingensis* was 37°C . Above and below this temperature, the enzymes production was low (Fig. 9).

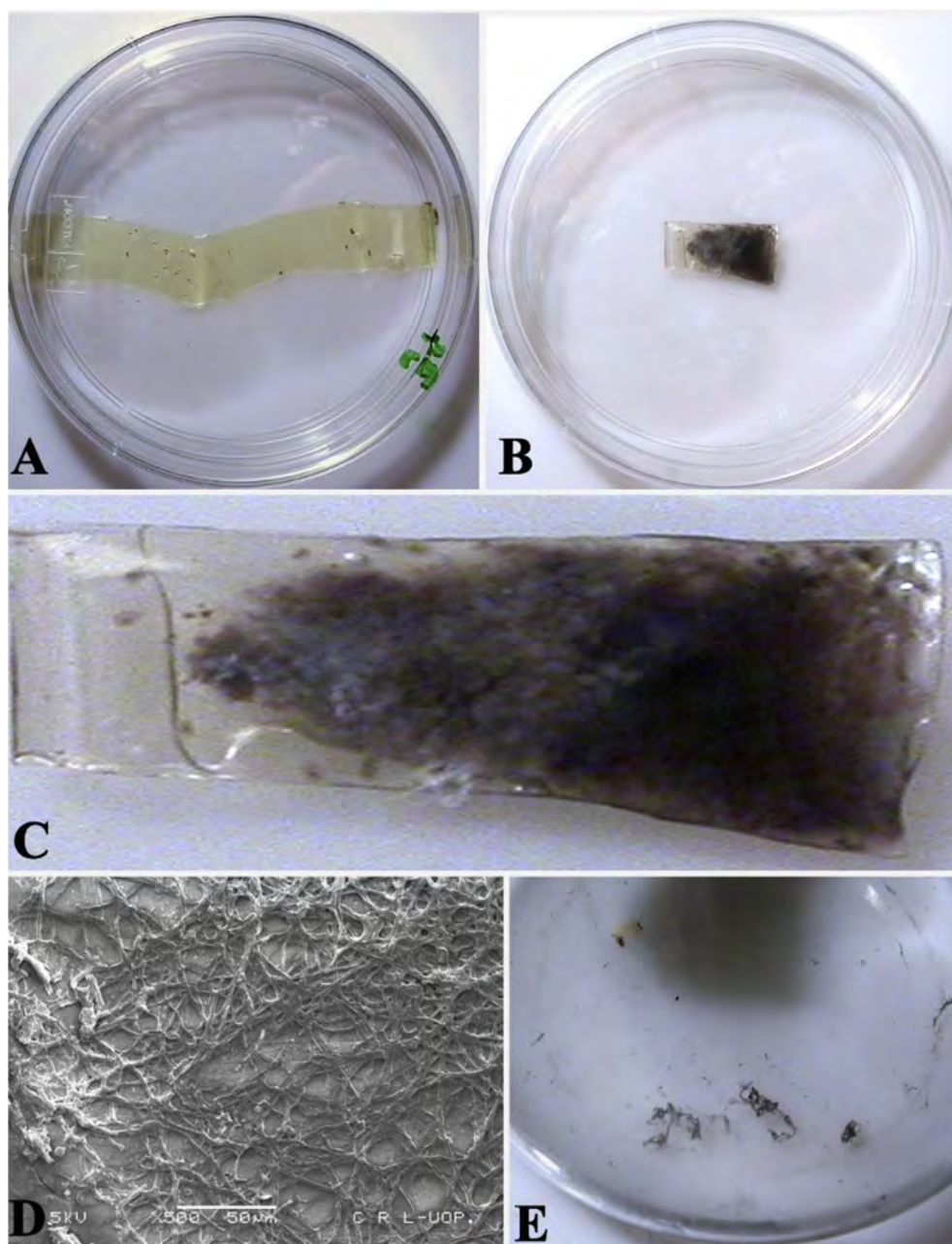


Fig. 4. *Aspergillus tubingensis* on the surface of PU incubated in liquid MSM (2% glucose) for three weeks. (A) sterilized PU film, (B & C) PU film after 21 days of incubation in liquid MSM with 2% glucose, (D) growth of *Aspergillus tubingensis* on the surface of PU film, and (E) the dissolved PU film in the MSM medium after two months.

4. Discussion

In the present study, we used PU for bating the fungal strain from soil of dumping area. Previously, *Geomyces pannorum* and *Phoma* sp. have been identified as the dominant fungi, which can biodegrade PU in soil (Cosgrove et al., 2007). The identification of the isolated strain in the present work was based on phenological and molecular techniques, and the identified strain was found to be *Aspergillus tubingensis*. It was found that this is the first study reporting *A. tubingensis* to be related with *in situ* biodegradation of PU. Similarly, it is also acknowledged by Barratt et al. (2003) that fungi were mainly responsible microorganism for PU degradation as compared to bacteria in the soil. Darby and Kaplan (1968) synthesized two kinds of PU and tested their degradability by using

A. niger, *A. flavus*, *A. vesicolor*, *Penicillium funiculosum*, *Aureobasidium pullulaus*, *Trichoderma* sp., *Chyetaotium globosum*). This literature supports the results of the present work and shows that other *Aspergillus* spp. also have the capacity to degrade PU. In the present study, in the preliminary screening of the PU degrading fungi on SDA plate, many bacterial colonies were observed, but we limited our work to fungi and re-cultured the fungi only. Later, we worked on only one species called as *A. tubingensis*. PU degradation by fungi, bacteria and their enzymes with polyurethanase activities have been previously reported but many of them have focused on PU degradation by bacteria only (Kay et al., 1991; Akutsu et al., 1998; Howard et al., 1999). Therefore, there is a need to search for PU degrading fungi.

The first step in PU degradation is the attachment or adhesion of

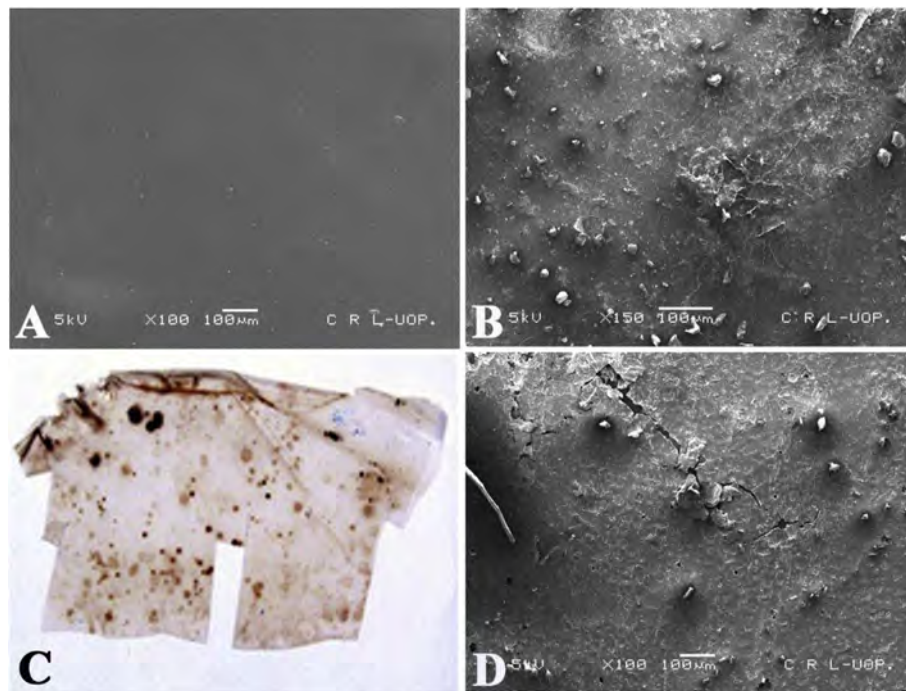


Fig. 5. Scanning Electron Micrographs of Polyurethane films buried in soil for four months: (A) SEM of control PU film, (B) SEM of PU film buried in soil for one month, (C) photograph of PU film buried in soil for four months, (D) SEM of polyurethane film buried in soil for four months.

bacterial or fungal spore to the surface of PU. Bos et al. (1999) showed that a hydrophobic interaction is responsible for the adhesion of microorganisms to the surface of buried PU which could be influenced by environmental conditions (Smits et al., 2003). This demonstrated that the environmental condition such as temperature, humidity, carbon source availability in the soil etc. are of the preliminary importance in the biodegradation of PU. In the present study, the efficiency of biodegradations was higher on SDA agar plate than in liquid MSM. Where the carbon/nitrogen sources for the growth of fungus was limited or more possibly the growth of the fungus in liquid medium is slower because of the osmotic pressure of the MSM on the hyphae of the fungus. Similarly, the growth of the fungus on the surface of PU film in liquid MSM was higher at 37 °C and the degradation was slower at temperatures below or above 37 °C (Figs. 4 and 9). This shows that temperature of the environment where the process of degradation takes place plays an important role in biodegradation.

The second step in the biodegradation of PU is the growth of mycelia on the surface of PU film followed by the third step of secretion of enzymes. The growth of *A. tubingensis* (Fig. 3-D, G and H, Fig. 4 C, D, and E) can be clearly seen and during their growth on the surface of PU, the fungus secreted different types of enzymes to degrade the PU films. The action of enzymes along with the mechanical force of the hyphae resulted in the biodegradation of PU films. This degradation can be accessed both physically and chemically. In our present work we have accessed the physically with the help of SEM and ordinary camera photographs while to access the chemical changes such as bond formation or cleavage, we used ATR-FTIR analysis. According to Dale and Squirrell (1990), the discoloration, loss of tensile strength and cracking detected in PU buried in soil are the characteristic signs of biodegradation of PU due to soil burial. In this study, on the surface of the PU film, there were discoloration, spots, and cracking (Fig. 5-B, C & D). The cracks appeared on the surface of PU is due to soil burial which allows the dispersion of fungal mycelium into the plastic. In our study, the

penetration of hyphae into the holes in the PU and the cracks were observed (Fig. 5-D). The results of this study coincide to the previous finding as reviewed in Howard (2002). Similar physical and chemical changes were observed in the PU film exposed to *A. tubingensis* on SDA plate within a time period of four days (Fig. 3 and Fig S1). SEM analysis of PU films showed the physical changes such as pits, holes, and erosions (Fig. 3). Similar physical and chemical changes in the PU film were observed in liquid MSM experiment (Figs. 4 and 6). The hyphal growth over the surface of PU film, with cracks and penetration of the hyphae in pores reveal biodegradation of the PU film (Fig. 3-G & H). The tensile strength and transparency of the PU film were different from that of control. The rate of tensile strength, discoloration, and biofilm formation on the PU was directly proportional to the time of burial in the soil with 2% glucose containing MSM. Previous studies have reported similar modifications on the surface after the colonization of microorganisms on the low-density polyethylene films (Matsunaga and Whitney, 2000; Bonhomme et al., 2003). When the PU films were buried for four months, the color of the PU films were changed to brown with many dots and became brittle. According to Woods (1990), soil burial of PU films for longer time resulted in loss of tensile strength (60%), and the PU films became very brittle due to microbial degradation. In our study, the loss of tensile strength of the PU film can be seen in the SEM of soil burial (Fig. 5-C & D), in liquid MSM (Fig. 4-D & E), and on the SDA plate (Fig. 3-G & H). More interestingly, after two months of incubation in liquid MSM the PU completely lost the tensile strength and were dissolved into small pieces (Fig. 4-E). The third step after attachment of the fungus to the surface of PU film is the secretion of different enzymes to hydrolyze various bonds. To access those chemical changes, we used ATR- FTIR spectrophotometry, a common technique used to follow chemical changes (in their functional groups) during polymer degradation. This technique was used previously by a number studies to determine chemical changes in the test sample as compared to control (Gonon et al., 2001; Bokria and Schlick, 2002;

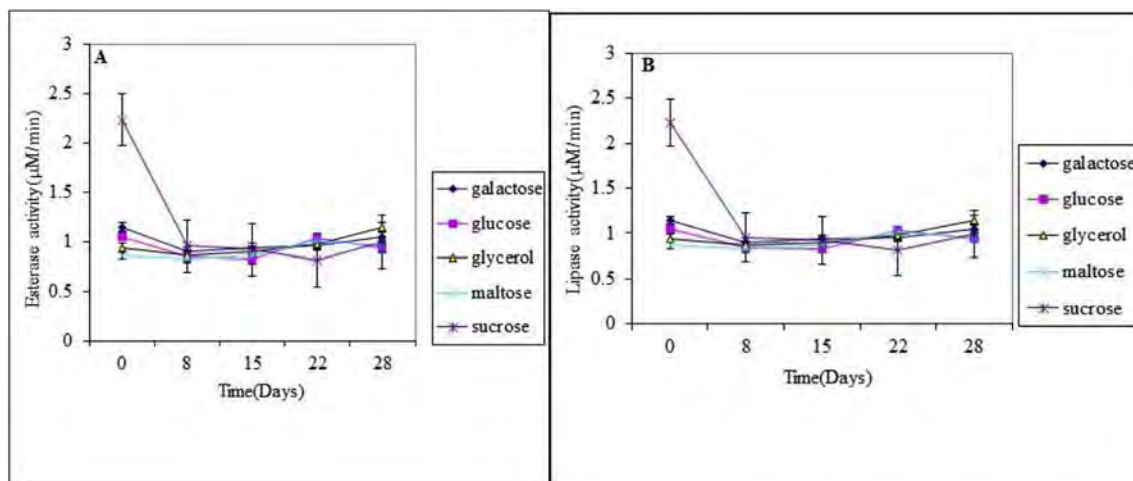


Fig. 6. Effect of different carbon source on production of: A) Esterase and B) Lipase by *Aspergillus tubingensis*.

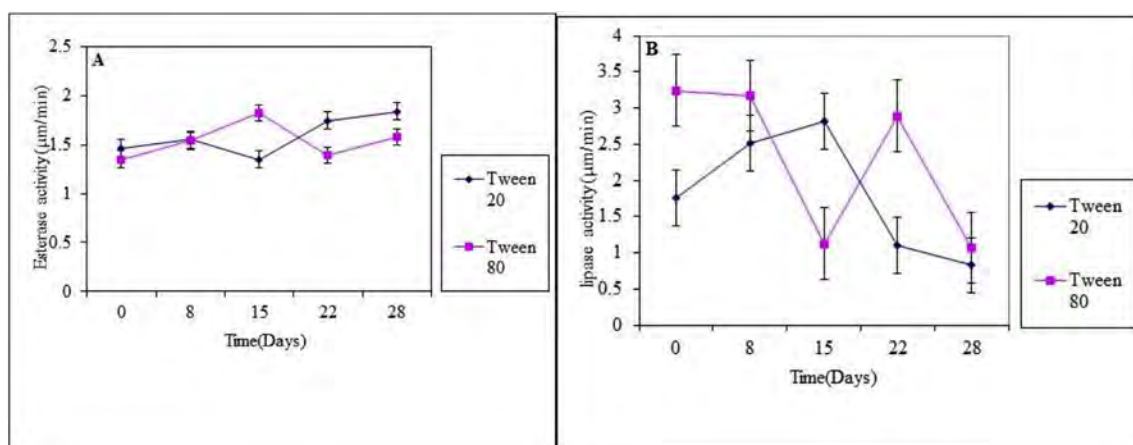


Fig. 7. Effect of Tween 20 and Tween 80 on the production of: A) esterase and B) lipase by *Aspergillus tubingensis*.

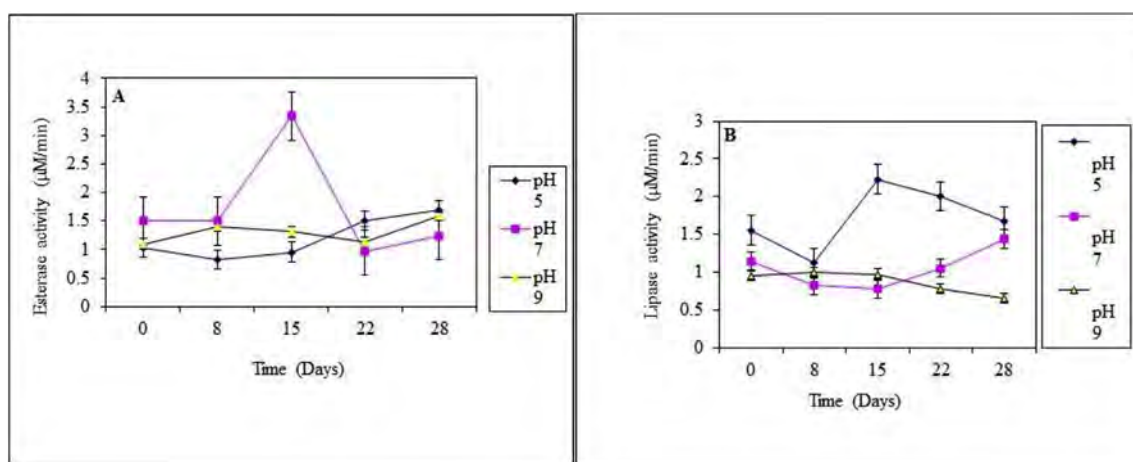


Fig. 8. Effect of pH on the production of: A) esterase and B) lipase by *A. tubingensis*.

Mailhot et al., 2005). In the present study, the ATR-FTIR analysis of the PU film, incubated with *A. tubingensis* on MSM agar plate, in liquid MSM as well as PU film buried in soil showed several changes in the spectra of the test as compared to control spectrum (Figs. S1,

S2, S3). This shifting or absence of the band in the test spectra implies the enzymatic hydrolysis of groups that brought about changes in the chemical structure of PU. The disappearance of urethane carbonyl group ($\text{C}=\text{O}$) was a clear evidence of

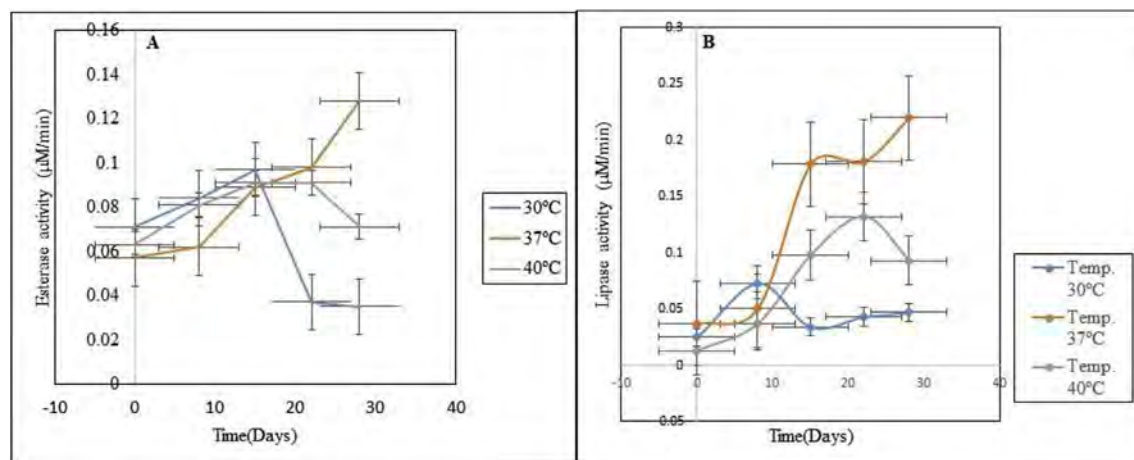


Fig. 9. Effect of temperature on the production on the production of: A) lipase and B) esterase by *A. tubingensis*.

biodegradation. Similarly, the disappearance of (CH₂)_n band (1164.4 cm⁻¹ and 1136.3 cm⁻¹) in the test spectrum declared the rupture of the polymeric chain. A characteristic band of medium intensity at 1643.6 cm⁻¹ assigned for NH deformation appeared in the test spectrum which was not present in control spectrum (Fig. 5). Another striking feature observed in the test spectra was the presence of characteristic C-O-C band at 1218 cm⁻¹ that showed that ester bonds were not hydrolyzed. A small band at the shoulder of band at 2954.8 cm⁻¹ indicated the hydrogenation of double bond in the chain. Loredó-Treviño et al. (2012) have presented similar results previously whereby, the decrement in the carbonyl signals at 1540 and 1260 cm⁻¹ were observed. In another experiment, the decrease in carbonyl signal and C-N-H signal at 1729 cm⁻¹ and 1540 and 1261 cm⁻¹ respectively, observed in the FTIR spectrum were attributed to the fungal enzymatic hydrolysis of the urethane groups (Álvarez-Barragán et al., 2016).

Increase in growth of fungus and some structural changes were observed in the case of treated PE (<http://www.dasma.dlsu.edu.ph/offices/ufro/sinnag/jaeintf.htm>). FTIR spectroscopy was used to confirm that the mechanism of PU biodegradation or the hydrolysis of the ester bond in PU. Nakajima et al. (1995) and Howard et al. (1999) previously did similar work and the results indicated that polyurethane biodegradation was due to hydrolysis of ester bonds. Kay et al. (1991) reported that the PU degradation was caused by the hydrolysis of ester bond while the PU breakdown products were analyzed by FTIR.

The fourth important step in biodegradation is the amount of enzymes produced by the microbe that are responsible for the degradation of PU films. It depends on a number of factors such as pH of the medium, carbon source availability, nature of substrate, the microorganism its capacity to produce hydrolyzing enzymes, the temperature of the environment, humidity etc. Abou Zeid (2001) demonstrated that the nature of polymer, degrading microorganism and the environmental condition (e.g. nutrient supply, temperature and pH) effect the rate of biodegradation. Therefore, in the present study, we tested different temperature, pH, carbon source and surfactants for esterase and lipases enzymes activities in MSM inoculated *A. tubingensis*. Previously a number of studies have characterized hydrolyzing enzymes with PU degrading abilities (Akutsu et al. 1998; Nomura et al., 1998; Howard and Blake, 1999; Howard, 2002; Howard et al., 2001).

In the present study, the optimum temperature of the enzymes produced by *A. tubingensis* was found to be 37 °C. Studies have mentioned that at high depths the biodegradability activities of microorganisms decrease due to low temperature and low levels of

humidity (Franzleubbers et al., 2001; Urgan-Demirtas et al., 2007). After temperature, the second abiotic factor that can affect biodegradation is the pH of the medium/environment. Therefore, on the optimum temperature of 37 °C, the enzymes activities at different pH were determined. The result showed that at pH 9, the esterase activity increases for the first ten days, then decreased gradually until the end of the month. Whereas, the lipase activities were maximum at pH 5 up to the first two weeks and then decreased gradually (Fig. 8-A & B). Similarly, in another study the supplementation of glucose, arabinose and fructose lowered the degradation level and no affect was observed in case of lactose supplementation into the medium (Manna and Paul, 2000). Therefore, in the present study, we assessed the effect of five different carbon sources such as galactose, glucose, glycerol, maltose, and sucrose on the production of esterase and lipase during degradation of PU by *A. tubingensis*. Lipase activities in the presence of glycerol and polyurethane was maximum (1.14 μM/min). Whereas, in the presence of galactose, glucose and sucrose increased esterase activities were observed (Fig. 6-A & B). Maximum enzyme activities were observed in the presence of sucrose (Fig. 6-A & B).

Although, Tween 80 (2%) has earlier been reported to increase the production of lipase from *Bacillus* sp. (Handelsman and Shoham, 1994). Hence, the effect of surfactants (Tween 20 and Tween 80) on the biodegradation of polyurethane was tested and lipase activities in the presence of Tween 20 and Tween 80 were observed to increase till the end of 2nd week and then gradually decreased by the end of the month. Whereas, the esterase activities increased in the presence of Tween 20 and Tween 80 in the 1st week of incubation, and then the activities were observed to decrease in case of Tween 20, whereas, increased in case of Tween 80 in the 3rd week (Fig. 7-A and B). The decrease in the enzyme production is assumed to the tendency of the species to produce the highest concentration and then gradually decreases with time. This could be also explained in other way as the surfactants may have repression effect on the gene responsible for the enzyme production. In our studies, the esterase and lipase optimization studies helped to determine the optimal environmental conditions for *A. tubingensis* to better understand its PU degrading ability. Our results suggests that similar conditions could be optimized for large-scale biodegradation of polymer wastes in dumping sites.

5. Conclusions

In the present study, we isolated and identified *Aspergillus*

tubingensis from the soil burial PU film. Literature review revealed that this is the first report that *A. tubingensis* degrades PU. PU biodegradation was tested in three different methods and the results showed that *A. tubingensis* could grow on the surface of PU film, and have biodegradation capabilities such as surface cracking, erosion, pore formation or loss in tensile strength. These physical and chemical changes were observed by SEM and ATR-FTIR analyses. The whole biodegradation process was completed in three steps as attachment or adhesion of the fungus to the surface of PU film, hyphal growth and propagation on the surface of PU, and secretion of PU degrading enzymes. The biodegradation of PU was higher on SDA agar plate than MSM and soil burial. It was assumed that the high biodegradation is due to the availability of nutrients and high growth and enzyme secretion on SDA culture plate. The process was highly dependent on pH, temperature, and carbon source (in the medium). The finding of this work could be optimized for large-scale biodegradation of PU and other polymer wastes. However, further research on molecular basis is required to reconfirm the high enzyme activities and the mechanism involved in biodegradations pathways.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.envpol.2017.03.012>.

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