

1-1-2011

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Recommended Citation

IBRAHIM, IBRAHIM N.; MARAQA, ANWAR; HAMEED, KHALID M.; SAADOUN, ISMAIL M.; and MASWADEH, HAMZAH M. (2011) "Assessment of potential plastic-degrading fungi in Jordanian habitats," *Turkish Journal of Biology*: Vol. 35: No. 5, Article 3. <https://doi.org/10.3906/biy-0901-9>
Available at: <https://journals.tubitak.gov.tr/biology/vol35/iss5/3>

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Assessment of potential plastic-degrading fungi in Jordanian habitats

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Received: 06.01.2009

Abstract: Out of 70 fungal isolates recovered from soil, wall paints (Latex), and pieces of plastic debris from different habitats in Jordan and plastic shields of street light posts on campus, 35 isolates showed varied potential to degrade polyester-polyurethane (PS-PUR). Six of these isolates (*Fusarium solani*, *Alternaria solani*, *Spicaria* spp., *Aspergillus fumigatus*, *Aspergillus terreus*, and *Aspergillus flavus*) were selected on the basis of their growth rates on basal salt media amended with PS-PUR as the sole source of carbon. Isolates were further evaluated utilizing 3 different methods: 1) direct plating, 2) clear zone in a 2-layered agar media, and 3) liquid shaking culture. These isolates caused significant weight loss in the PS-PUR blocks in the shaken cultures, reaching up to 100% in case of the isolate *Fusarium solani*. The petri dish test method revealed a maximum degradation activity achieved by the isolate *Aspergillus flavus*, which caused 94% loss in weight of PS-PUR pieces. However, only 4 isolates (*Fusarium solani*, *Spicaria* spp., *Alternaria solani*, and *Aspergillus flavus*) yielded positive results of biodegradation, indicated by clear zones created due to PS-PUR hydrolysis in 2-layered agar culture plate media. Out of the 6 fungal isolates reported here, 2 novel organisms have not been previously reported, *Alternaria solani* and *Spicaria* spp. Overall, these findings helped identify predominant as well as novel fungi in Jordanian habitats that play a key role in PS-PUR degradation.

Key words: Polyurethane (PUR), *Fusarium solani*, *Alternaria solani*, *Spicaria* spp., *Aspergillus fumigatus*, *Aspergillus terreus*, *Aspergillus flavus*

Ürdün'e özgü habitatlardaki potansiyel plastik-indirgeyici mantarların değerlendirilmesi

Özet: Ürdün'deki farklı habitatlardan topraktan, duvar boyalarından (Lateks) ve plastik döküntü parçalarından ve kampüsteki caddenin elektrik direklerindeki plastik kaplamalarından toplanan 70 mantar mikrobiyal izolatın arasından, 35 izolat, poliester-poliüretanın (PS-PUR) bozunması için değişken potansiyel göstermiştir. Bu izolatların altısı (*Fusarium solani*, *Alternaria solani*, *Spicaria* spp., *Aspergillus fumigatus*, *Aspergillus terreus*, ve *Aspergillus flavus*) tek karbon kaynağı olarak PS-PUR'lu değişik bazal tuz ortamlarında büyüme oranlarına dayanarak seçilmiştir. İzolatlar, daha sonra üç farklı metottan faydalanılarak değerlendirilmiştir: 1) direkt kaplama 2) iki tabakalı agar ortamında açık zon ve 3) sıvı çalkalama kültürü. Bu izolatlar çalkalama kültürlerdeki PS-PUR bloklarında önemli ağırlık kayıplarına sebep olmuştur; *Fusarium*

solani izolatinın olması halinde ağırlık kaybı %100'e ulaşmıştır. Petri kabı test metoduyla maksimum bozunma aktivitesinin, PS-PUR parçalarının ağırlığında %94 kayba sebep olan *Aspergillus flavus* izolatu ile elde edildiği belirlenmiştir. Ancak sadece dört izolat (*Fusarium solani*, *Spicaria* spp., *Alternaria solani* ve *Aspergillus flavus*) iki tabakalı agar kültürü kaplama ortamında, PS-PUR hidrolizinden dolayı ortaya çıkan açık zonlarla belirlenen pozitif biyolojik bozunma sonuçlarını vermiştir. Burada rapor edilen altı mantar izolatinın arasından iki yeni organizma, *Alternaria solani* ve *Spicaria* spp. daha önce rapor edilmemiştir. Sonuçta bu bulgular, Ürdün habitatlarında bulunan ve PS-PUR bozunmasında anahtar rol oynayan hem predominant mantarların, hem de yeni mantarların tanımlanmasına yardımcı olmuştur.

Anahtar sözcükler: Poliüretan (PUR), *Fusarium solani*, *Alternaria solani*, *Spicaria* spp., *Aspergillus fumigatus*, *Aspergillus terreus*, *Aspergillus flavus*

Introduction

Plastics were developed as strong, lightweight, durable, and bioinert materials and have been applied to a wide range of commodities, including some related to medical and pharmaceutical applications. They have replaced many natural materials, such as metals, woods, and gravel, and have been used as delivery vehicles for some drugs. In particular, polyurethanes (PUs) are a complex group of synthetic polymers used in many industrial and medical applications. Their bioinertness and resistance to deterioration has raised appreciable ecological concerns about their increased production and accumulation in the environment. Many plastics are not biodegradable, but some PUs are well known to undergo biodegradation (1). In fact, it has been shown that the susceptibility of PU to microbial degradation is highly dependent on the chemical structure of its constituents (2). Polyurethane is formed by the condensation of a polyol and a poly- or di-isocyanate.

The phenomenon of PU biodegradation has been exploited in an attempt to reduce PU build up in the environment as well as problems related to PU disposal. Many previous studies investigated soil-microorganisms' ability to degrade soil-buried polyester PU. The majority of these studies isolated more fungi than bacteria as key players in PU degradation using extracellular enzymes such as ureases, proteases, and esterases (3-10). Still, there is no record of exploration of the local habitats of Jordan for PU-degrading fungi. Thus, the aims of this study were to isolate and identify the predominant polyester PU (PS-PU) degrading fungi from different Jordanian habitats.

Materials and methods

The polymer

Sheets (5 × 20 cm) of PS-PUR were supplied by Dr. Toshiaki Nakajima-Kambe from the Institute of Applied Biochemistry, University of Tsukuba, Ibaraki-Japan, and used as a source of carbon and nitrogen in growth media.

Isolation of test microorganisms

Test fungi were isolated from different sources: 1) soil, 2) wall paint (latex) coated with polyurethane, and 3) plastic waste from different habitats in Jordan, by various techniques dependent on each specific case.

Isolation from soil

Fungi with a potential to degrade plastic were trapped from soil by using cubes (3 mm³) of PS-PUR as a bait buried inside soil samples (1,10). Soil samples were collected from different habitats in Northern Jordan (9 from Tayba, 11 from Samar, 2 from Hawarah, 1 from J.U.S.T, 12 from Irbid, Mafrak, and Ramtha) that have been exposed to plastic debris and hydrocarbon contamination for a long time. Soil samples were collected from the upper 10 cm layer, and stored in plastic bags in the laboratory at room temperature. They were then sieved through a 2 mm pore size sieve [Retsch, Germany] to remove large debris. The sieved soil was used for screening purposes to isolate PS-PUR utilizing fungi by the enrichment technique (1). 0.2 g test soil was added to 50 mL glass vial containing 5 mL of basal medium. Basal medium was prepared as previously described by Nakajima-Kambe (1) and Şahin et al. (11) with a slight modification: 2 g KH₂PO₄ [BDH, England]; 7 g

K_2HPO_4 , [BDH, England]; 1 g NH_4NO_3 , [BDH, England]; 0.1 g $MgSO_4 \cdot 7H_2O$, [Peking, China]; 1 mg $ZnSO_4 \cdot 7H_2O$, [BDH, England]; 0.1 mg $CuSO_4 \cdot 7H_2O$, [BDH, England]; 10 mg $FeSO_4 \cdot 7H_2O$, [Dualigens, India]; 2 mg $MnSO_4 \cdot 4 \cdot 6H_2O$, [Fluka, Switzerland], and trace amount of thiamine-HCl [Janssan chemica, Belgium] were dissolved in 1 L of water with a final pH adjustment to 7.2 by adding lactic acid [CHEMLAB CO., England] or NaOH [Frutarom, UK].

The direct isolation process was carried out by adding 1 g of soil to 9 mL of sterile distilled water in a test tube to yield a 10-fold dilution. Next, a series of 10-fold dilutions were made in which 1 mL of each dilution was cultured on potato dextrose agar (PDA) [Himedia, India]. Pure cultures were finally obtained by selecting a single colony of growth from highly diluted cultures.

Isolation from wall paint (latex)

Samples of wall paint (latex) showing spots of black growth during winter were collected. Swabs from this growth were applied on a PDA plate for fungal isolation. The microbial growth on these media was subcultured several times by culture dilution as described above until a pure culture was obtained.

Isolation from plastic waste

Plastic waste pieces buried inside old garbage piles were obtained and washed several times with sterile distilled water and then placed in a test tube with 10 mL of sterile distilled water for 15 min. Serial dilution and plating were performed as described previously, and pure culture was obtained. Direct isolation was also carried out from a heavy black growth observed on plastic shields of street lamp posts on the Jordan University of Science and Technology (JUST) campus. The black surface was scraped using a razor blade, and the scraped material was cultured directly to PDA and subcultured several times until pure culture was obtained.

Isolating microorganisms able to grow on PS-PUR pieces

The selection was performed as described by Nakajima-Kambe (1), using the liquid shaking culture test method. In brief, cubes of PS-PUR (about 25 mg) were added to a small vial containing 5 mL of basal

medium prepared as previously described. Spore suspensions of test isolates were prepared by suspending scraped mycelium within 8 mm diameter of fungal growth in a vial containing 5 mL of normal saline (0.85% NaCl w/v). The test isolate (0.1 mL of spore suspension) was added to the test medium, properly stoppered and incubated at 30 °C on a rotating shaker (120 oscillations /min) for 3 weeks. The test isolate, which was grown on a PS-PUR surface, was selected for further studies. Growth was rated visually on a scale of 0 to 4 as follows: 0 = no growth; 1 = trace of growth (visible under microscope but not to the naked eye); 2 = light growth; 3 = moderate growth; 4 = heavy growth (10).

Biodegradation experiments

The selected isolates were tested for their PS-PUR biodegradation potential by different methods:

Liquid shaking culture test methods

A cube-shaped PS-PUR (about 25 mg) was added to a small vial (50 mL) containing 5 mL of basal medium, sterilized by autoclave at 121 °C for 20 min. The test isolate (0.1 mL of spore suspension) was added to the test medium, properly stoppered, and incubated at 30 °C with reciprocal shaking (120 oscillations/min) for 3 weeks. PS-PUR degradation was monitored by measuring the weight of PS-PUR before and after incubation. At the end of the incubation period, the PS-PUR pieces were taken out and washed several times with distilled water. Next, they were dried overnight at 80 °C and the change in PS-PUR weight was determined as $D \text{ weight loss} = \text{Initial weight} - \text{Final weight}$ (8).

Petri dish test method

Basal medium agar was prepared by adding 15 g of agar to 1 L of basal medium, followed by autoclaving for 20 min and pouring into petri dishes. Sterilized PS-PUR pieces were overlaid on the medium surface, and each test isolate (1.0 mL of spore suspension) was added over the PS-PUR pieces. After incubating at 30 °C for 3 weeks, the PS-PUR pieces were taken out and washed several times with distilled water. They were next dried over night at 80 °C and the change in weight of the PS-PUR was determined as $D \text{ weight loss} = \text{Initial weight} - \text{Final weight}$ (12).

Clear zone test method

Two agar media layers were poured onto 10 cm plates as follow: the lower layer (15 mL) contained basal medium agar while the upper layer (10 mL) contained a polymer suspension (4 g/L suspended polymer powder and 14 g/L agar [Himedia, India]). Each test isolate (5 mL of spore suspension) was added to the center of the plate and incubated at 30 °C. The colony mean diameter (CMD) and the clear zone diameter were measured and recorded after 14 days of incubation (13).

Identification of the isolated test fungi

The selected fungi were identified according to the protocols of taxonomy used by fungal cultural collections (14). This included Riddel's slide culture technique (15) to examine the vegetative and non-sexual reproductive structures of these fungi. In addition to the macroscopic colony morphology grown on PDA for 1 week at 28 °C, the texture, color, and both surface and reverse sides were taken into consideration. Spores and conidial heads, as well as the characteristics of the surface and reverse sides of each colony, were photographed by the Department of Applied Biological Sciences.

Results and discussion

This protocol lead to the discovery of 35 fungal isolates with the potential to degrade PS-PUR as determined by growth rates achieved on basal medium amended with PS-PUR as the sole source of carbon (Table 1). Meanwhile, 70 fungi isolates were isolated as follows: 44 from soil directly by serial dilution method, 15 from a plastic waste sheet buried in soil for an extended period, 3 from lamp shields from the JUST campus, Irbid, Jordan, and 8 from a wall paint (Latex) site with moldy growth (spots showing black growth during the winter). These isolates were examined for degradation potential against PS-PUR. Of these, only 21 were able to grow in basal medium supplemented with polyester PUR.

Among all isolates employed in the screening program, only 6 fungal isolates showed degradation potential on the 3 test methods (Table 2). The fungal isolates were tentatively identified according to the protocols of taxonomy used by fungal cultural collection (14). The isolates belong to the genera *Fusarium*, *Alternaria*, *Spicaria*, and *Aspergillus*, and were further identified to their species level as *Fusarium solani*, *Alternaria solani*, *Aspergillus terreus*, *Aspergillus fumigatus*, and *Aspergillus flavus*.

Table 1. Growth rating of fungi isolated and collected for screening experiments.

| Isolation Habitat/Site code and Growth rating | | | | | |
|---|----------------|---------------------------|---------------|--------------|---------------|
| Wall paint | | Plastic sheet or fragment | | Soil samples | |
| Isolate code* | Growth rating* | Isolate code | Growth rating | Isolate code | Growth rating |
| Fp- 1 | 2 | FopI-1 | 1 | FsH-3 | 2# |
| Fp- 2 | 1 | FopI-2 | 3# | FsH-4 | 2 |
| Fp- 3 | 3 | FopI-3 | 1 | FsH-11 | 3 |
| Fp- 4 | 3 | FopI-4 | 2# | FsM-12 | 3 |
| Fp- 5 | 2 | FopII-4 | 2 | FsM- 6 | 4# |
| Fp- 6 | 2 | - | - | FsH- 8 | 3# |
| Fp- 7 | 4# | - | - | FsJ-2 | 4 |
| Fp- 8 | 2 | - | - | FsH-5 | 4 |

Fp-#; wall paint from Hawarah, FopI-#; plastic sheet-I from Hawarah, FopII-#; plastic sheet-II from JUST, FsH-#; soil from Hawarah, FsM-#; soil from Tayba, FsJ-#; soil from JUST.

**Growth rating was done on an arbitrary scale of 0-4, where 0 = no growth, 1 = trace of growth (visible under microscope but not visible to naked eye), 2 = light growth, 3 = moderate growth, 4 = heavy growth (Darby and Kaplan, 1968). Growth was rated after 14 days of incubation.

Table 2. Fungal isolates used in the present investigation based on their morphological characteristics.

| Isolate Code | Fungi name |
|--------------|------------------------------|
| FsM-6 | <i>Fusarium solani</i> |
| Fp-7 | <i>Spicaria</i> spp. |
| FsH-3 | <i>Alternaria solani</i> |
| FsH-8 | <i>Aspergillus terreus</i> |
| FopI-2 | <i>Aspergillus flavus</i> |
| FopI-4 | <i>Aspergillus fumigatus</i> |

The results in Table 3 show the activity produced by the 3 different culturing techniques. On the shaking liquid culture technique, only 6 isolates out of 21 were shown to cause a significant weight loss of the PS-PUR blocks. The FsM-6 isolate, shown to be the most active isolate, caused 100% weight loss of PS-PUR blocks, while the Fp-7 isolate caused only 12.7% weight loss.

The results of the petri dish test method indicated that only 6 isolates showed degradation activity (Table 3). The maximum degradation activity was achieved by the isolate FopI-2, which caused 94% loss in weight of PUR pieces, and the isolate Fp-7 showed a minimal activity of about 22.9%.

Increased attention has been devoted to biological degradation as a friendly solution for plastic accumulation and environmental pollution. This problem and its curtailment are becoming more significant in Jordan, but have not been researched yet. PUR as a plastic polymer receives special attention from many researchers due to its biodegradation resistance. Although previous studies have reported the degradation of PS-PUR by fungi, no one has thus far reported that fungi can utilize PUR as the sole source of carbon (9). Therefore, the pioneering work described here is aimed at the exploration and assessment of Jordanian habitats for PS-PUR degrading fungi.

The preliminary screening test of soil samples for the presence of indigenous plastic degrading microorganisms yielded no significant findings during one month of incubation. Such a result could be attributed to the presence of a consortium of microorganisms in the soil. That is, the inhibition of plastic-degrading microorganism growth could be due to competition with non-degrading microorganisms, thus masking the former's plastic degradation potential. Moreover, soil may be carbon-rich with simple organic compounds that could be more readily favored by microorganisms as the main

Table 3. Weight loss of PS-PUR blocks used as a sole carbon source in shaking liquid culture, petri dish direct plating, and the clear zone diameter indicating the activity of the tested isolates to degrade PS-PUR.

| Codes of Fungi | Shaking liquid culture technique | | Petri dish test technique | | Clear zone diameter method. | |
|------------------------------|------------------------------------|---------------|----------------------------------|---------------|-----------------------------|---------------------------|
| | Δ Weight Loss (initial-final) (mg) | % weight loss | Weight Loss (initial-final) (mg) | % weight loss | Diameter of clear zone (mm) | Colony mean diameter (mm) |
| <i>F. solani</i> . (FsM-6) | 27.5 ± 0.3* | 100% | 17.4 ± 0.3 | 72.5% | 70 ± 1 | 80 ± 1 |
| <i>Spicaria</i> spp. (Fp-7) | 2.80 ± 0.3 | 12.7% | 5.5 ± 0.4 | 22.9% | 60 ± 1 | 70 ± 1 |
| <i>A. solani</i> . (FsH-3) | 23.7 ± 3.9 | 71.8% | 19.8 ± 0.4 | 63.6% | 58 ± 1 | 62 ± 1 |
| <i>A. terreus</i> (FsH-8) | 6.00 ± 0.3 | 26.1% | 11.6 ± 1.8 | 58.0% | - | 80 ± 1 |
| <i>A. flavus</i> (FopI-2) | 7.70 ± 0.9 | 40.5% | 25.5 ± 2.3 | 94.8% | 28 ± 1 | 80 ± 1 |
| <i>A. fumigatus</i> (FopI-4) | 9.30 ± 1.4 | 43.5% | 7.9 ± 2.3 | 39.5% | - | 30 ± 1 |
| Control | 0.70 ± 0.1 | < 1% | 0.7 ± 0.1 | <1% | - | - |

*Results are the means of 3 replicates, represented as: mean ± standard deviation (M ± SD)

source of energy over more complicated compounds such as plastic polymers. Furthermore, most of the soil samples were characterized by a high of clay mineral content, thus possibly contributing to some of the negative results obtained. Filip et al. (8) demonstrated that in the presence of clay, the decomposition of solid PUR is inhibited possibly due to the adsorption of exoenzymes. Therefore, the direct isolation of soil fungi to determine their degradation ability was adopted in order to obtain microorganisms with PS-PUR degradation potential.

However, only 6 isolates identified to the genus and species level demonstrated significant degradation potential when evaluated by 3 different recommended test methods (Table 2). These included the petri dish test method, the liquid shaking culture method, and the clear zone method (Table 3). Our results are clearly in agreement with previous studies (8,12,13), as these isolates caused significant weight loss in the PS-PUR blocks placed in the shaken cultures, even reaching up to 100% in the case of the *Fusarium solani* isolate. On the other hand, the isolate *Spicaria* spp. caused only 12.7% weight loss in those blocks. The petri dish test method further confirmed the results obtained by the liquid shaking culture technique, as the same trend of biodegradation activity by those fungi was observed (Table 3). The maximum degradation activity was achieved by the *Aspergillus flavus* isolate, which caused 94% weight loss in PS-PUR pieces. A minimal activity of about 22.9% was observed in the *Spicaria* spp. isolate. Furthermore, only 4 isolates (*Fusarium solani*, *Spicaria* spp., *Alternaria solani* and *Aspergillus flavus*) yielded positive results of biodegradation as indicated

by clear zones (70 mm, 60 mm, 58 mm, and 28 mm, respectively) created due to PS-PUR hydrolysis in 2-layered agar culture plate media.

Four species out of the 6 fungal isolates dealt with in the present investigation were previously reported by other researchers as biodegraders of polyurethane, namely, *Aspergillus flavus* (10), *Aspergillus fumigatus* (16,17), *Aspergillus terreus* (18,19), and *Fusarium solani* (16,20-22). Isolates of those species were also reported as active biodegrading organisms to pesticides applied to soil (11). Two other species, *Alternaria solani* and *Spicaria* spp. were not previously reported as PS-PUR degrading fungi. We have chosen 2 isolates, *F. solani* and *A. solani*, for further studies since they showed the highest degradation activity observed on all 3 methods utilized in exploring the biodegradation of PS-PUR.

Acknowledgments

The authors acknowledge the financial support provided by the Deanship of Graduate Studies, Jordan University of Science and Technology, to this research work.

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