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ORIGINAL ARTICLE

Polyester-polyurethane Biodegradation by *Alternaria Solani*, Isolated from Northern Jordan

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ABSTRACT

Alternaria solani isolate number Ss1-3, isolated from soil sample from northern Jordan was found to be able to utilize the polyester-polyurethane (PS-PUR) as sole source of carbon and nitrogen. After three weeks of incubation at pH7 and 30 °C, *A. solani* caused 65.8% loss in weight, 24.7% reduction in tensile strength at break, and 28.2% decrease in percentage elongation at break, of PS-PUR substrate blocks. There was conspicuous microscopic cracking of the PS-PUR surface. Penetrating fungal hyphae through the PS-PUR blocks was envisaged, initially in the reduction in the number of -CH₂- groups and increases in the number of -OH groups of PS-PUR, and as ascertained by Fourier transform-Infrared (FT-IR) spectrophotometer. The detection of acid and amine fractions by thin-layer chromatography (TLC) in the purified culture filtrate (PCF), which is comparable to that in the acid hydrolyzed PS-PUR, was attributed to the biological hydrolysis, by the fungus, of diisocyanate segment in this polymer. Adipic acid, diethylene glycol, and trimethylolpropane were probably the main degradation products released from the polyester segment in the PCF and detected by high performance liquid chromatography (HPLC) when PS-PUR was utilized as a source of carbon. This is further supported by protease activity of *A. solani*

Key words: *Alternaria solani*, polyester-polyurethane, Polyurethane, Degradation

Introduction

Plastics were developed as strong, lightweight, durable and bioinert materials and have been applied to a wide range of commodities. They replaced many natural materials, such as metals, woods, and gravel. However, their bioinertness and resistance to deterioration raised appreciable ecological concerns, about the increase in production, and accumulation of the plastic wastes. Biodegradation and mineralization

of plastic wastes have been given an increasing attention.

Polyurethane, which is synthesized from polyols and polyisocyanates, are widely used plastics as raw materials in various industries, and they are resistant to biodegradation. However, they were intensively investigated for their susceptibility attack by fungi that are capable of producing extracellular enzymes such as ureases, proteases, and esterases[5,8,6,7]. Therefore, polyester-polyurethane (PS-PUR) could be

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used as a model substrate in searching the local habitat for potential plastic-degrading fungi.

Materials and methods

I-The Polymer:

Sheets (5 x 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 the growing media.

II- The isolate:

The test isolate (*Alternaria solani* Number Ss.1-3) was isolated from soil sample collected from northern Jordan as described in previous work (Ibrahim, *et al.*, Manuscript in preparation).

III-Biodegradation experiments:

The isolate, *A. solani* Number Ss.1-3, investigated tested for its PS-PUR biodegradation activities by liquid shaking culture test as described by Nakajima-Kambe, *et al.*, [10]. Measuring the weight loss and other physical properties in this substrate before and after incubation as well as the breakdown residues of this polymer monitored degradation steps of PS-PUR.

IV-Characterization of the selected test fungi:

The colony mean diameter (CMD) measurements after a specific incubation period [17] on PDA medium were used as the criterion to study the effects of temperature and pH on the growth rates of the test fungi.

V- Assessment of biodegradation potential of the isolated fungi:

a- Change in the color, tensile strength, and the elongation at break of the treated PS-PUR pieces:

PS-PUR sample pieces about 6 mm X 0.6 mm X 0.3 mm in dimensions were carefully cut. Individual test pieces were placed in 250-ml flasks to which 50 ml of basal salts medium were added. Each flask was inoculated with 1 ml of spore suspension then incubated at 30 °C for 3 weeks. At the end of the incubation period, the test pieces were removed, washed with distilled water then dried for overnight at 80 °C. The tensile test was estimated on an Instron Tensile Tester (model 1195) using a cross head speed of 100 mm/min according to Pathirana and Seal, [14] before and after the incubation. The change in the color was also observed.

b- Compound light microscopic studies:

The morphological changes of PS-PUR associated with the degradation were observed under compound light microscope and recorded by microphotography.

c- FT-IR spectrophotometer studies:

Due to the fact that PS-PUR is not soluble in any standard organic solvent suitable for use in FT-IR analysis, the following method of preparing the treated and non-treated plastic (PS-PUR) for FT-IR spectrum determination was used. Scrapings from those samples were ground using mortar and pestle and then incorporated with Potassium bromide (KBr), and then made into KBr-discs. Those discs were used in obtaining spectra of the bio-degraded and non-treated PS-PUR, using FT-IR spectrophotometer [Nicolet, Impact410, USA] [15]. The spectra of those samples were recorded over a range of 4000-600 cm⁻¹ with an average of 100 scans and a resolution of 4 cm⁻¹ against an air background.

d- TLC studies:

TLC was used to examine chemical changes during the degradation of PS-PUR by the test isolate. The hard PUR was acid hydrolyzed with 3N HCl solution as described by Pathirana and Seal, [15]. The hydrolyzed extract was evaporated in a fume-cupboard at room temperature. The precipitated solids were dissolved in dilute HCl solution (0.01N HCl) and a TLC was run on silica gel 60 F254 [Riedel-deHaen, Germany] plates using the developing solvent system of n-butanol / acetic acid / water in a 4/1/5 ratio, respectively [15]. The amine was detected under UV light [Model UV-56, USA] [15] as a bright blue fluorescent spot, which later on, turned to purple due to oxidation. The acid was detected by spraying with bromocresol green indicator [15]. The acid gave a yellow spot on a blue background. The alcohol portion was not detected. The precipitated powders of PCF were dissolved in dilute HCl solution (0.01 N HCl) and a TLC was run and compared with the acid hydrolysed non-degraded PUR.

e- HPLC studies:

The PS-PUR breakdown products were analyzed by HPLC. The cultured broth was centrifuged at a speed of 3,000 rpm/min for 10 min, and the cell-free supernatant was collected. The cell-free supernatant of the test fungi and corresponding control were placed in capped vial, and sent to the Institute of Applied Biochemistry, University of Tsukuba, Ibaraki-Japan (Dr. Toshiaki Nakajima-Kambe) for

HPLC analysis. The samples were analyzed by HPLC system equipped with a refractive index detector. Aliquots of 20ml cell free supernatant were injected under the following conditions; Aminex HP-87H column, 300x 7.8 mm, (BIO-RAD); column temperature, 30°C; solvent, 0.1N H₂SO₄ (at a rate flow equal 0.8ml/min);[10].

VI-Determination of optimal pH and temperature for the degradation activities of the test isolate:

The pH value of the basal medium was adjusted to 4, 5, 6, 7 and 8 using HCl 20% w/v and NaOH (0.1N). The degradation experiment was performed as previously described, and incubated at 20 °C, 25 °C, 30 °C, 35 °C, and 40 °C.

VII-The effect of media composition on the degradation activities of the test isolates:

a- The effect of nitrogen source:

Four nitrogen forms namely; NH₄Cl (2g/L), NaNO₃ (2g/L), NH₄NO₃, (1g/L) and Urea (1g/L) were investigated. The basal salts medium was prepared as previously described, without nitrogen source, or supplemented with NH₄Cl (2g/L), NaNO₃ (2g/L), NH₄NO₃, (1g/L) and Urea (1g/L) as sources for nitrogen.

b- The effect of Glucose and Yeast extract:

Glucose and yeast extract were supplemented in the concentrations of 0.1 %, 0.5%, and 1% on a w/v basis into the basal salts medium.

VIII-The effect of some competitive substrates on the degradation activities of the test isolates:

Gelatin [Himedia, India], urea, and polyethylene adipate [ACROS, USA]) were added at the concentrations of 0.1 %, 0.5%, and 1% on a w/v basis into the basal salts medium as substrate competitive.

Optimal condition and media compositions were collectively used in order to determine the maximum degradation activity of the isolated fungi.

IX-Preliminary examination of enzymatic activities of the test isolate:

a- Extracellular enzymatic activity:

Protease, esterase, and urease enzymes production by the fungi was studied on solidified media. The method used was a modification of Rautela and Cowlings[16] test tube method, and as it was re-described by Pathirana and Seal[13]. The

depth of the clear zone or color change that developed beneath the growing culture provided a visual measurement of enzyme activities on a continuous, cumulative basis.

b- Extracellular enzymatic activity in broth culture:

The extracellular enzymatic activity of fungi was detected in plates containing the same media used for fungal study. Wells of 8 mm in diameter were cut and charged with 100 mL of the PCF. Plates were incubated at 30 °C for 2 days and zones of clearance or color change were visualized as indicator of enzymatic activities.

Results and discussions

I-Characterization of the selected test fungi:

Results of the colony mean diameter (CMD) of *A. solani* Number Ss 1-3 with respect to temperature and pH showed that this isolate grew well in wide range of temperatures of 20° C to 35° C and a range in of pH 4 to 8. However, the optimum conditions were 25° C and pH 7.

II-Assessment of the biodegradation potential of the isolated fungi:

a- Assessment upon change in physical properties of PS-PUR:

Result revealed that decrease in tensile strength (24.7%) and elongation (28.2%) at break was caused by *A. solani*. In addition, *A. solani* caused weight loss of the PS-PUR as substrate by 68.9%. Result shows that the PS-PUR block was changed from colorless to brown by *A. solani* (Plate.1).

b- Assessment via Microscopic examination:

Microscopic examination of the untreated PS-PUR blocks indicated no sign of breakdown (Plate 1). However, degradation of these blocks by three test organisms was evident by forming of cracking on the lamellae between the foam cells and destruction of the foam structure. Furthermore, there are fungal hyphae growing around the PS-PUR blocks, which were used as a substrate.

c- Chemical change assessment upon FT-IR spectrometric studies:

Fig 1 shows that the untreated (control) polymer exhibits a peak due to C-H stretch at (2950 cm⁻¹) was relatively greater than the N-H stretch at (3350 cm⁻¹). However, the C-H stretch was relatively smaller than the peak due to N-H stretch for

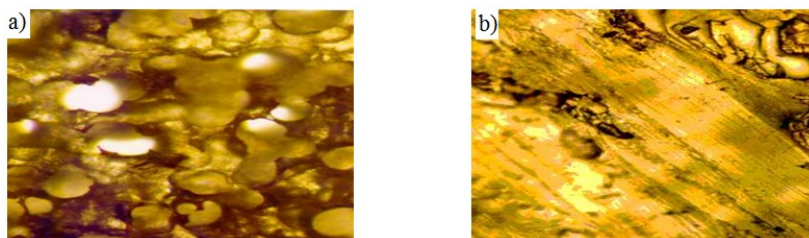


Plate 1: Photomicrographs showing affected PS-PUR after 21 days of incubation at 30 °C supplemented as sole carbon source in basal media by a-*A. solani*, and b- undegraded control PS-PUR.

degraded polymer. When the IR spectra of the peak from test isolate at wave numbers of 1455, 1387, and 1183 cm^{-1} were compared with that of the untreated (control) polymer, they were relatively smaller and not identical (Fig 1).

d- Assessment of chemical change and breakdown by product determination by TLC studies:

Unfortunately, chromatographic comparison between the acid hydrolyzed of control and treated PS-PUR (utilized by the test isolate as carbon source) revealed the presence of three bright blue and one yellow spots. In contrast, when the precipitated powder from cell-free supernatant was compared chromatographically with the chemically hydrolyzed (acid hydrolysis) products, one yellow spot parallel to the yellow spots of acid hydrolyzed was appeared. Furthermore, bright blue spots, 2 in case of *A. solani*, and only one in case of the control (non-inoculated broth) parallel to the three blue spots that appeared in case of acid hydrolyzed. However, new one blue bright spot was appeared in all cases but not in the control (uninoculated broth).

III-HPLC analysis of water-soluble PS-PUR breakdown products:

Metabolites resulting from the degradation of PS-PUR by, *A. solani* were studied after one week of incubation at 30 °C (Fig 2). Two small peaks (P-1, and P-4) and two relatively large peaks (P-2, and P-3) were detected after one week. The retention time of these peaks (P-1), (P-2), and (P-3), corresponded to those of authentic adipic acid, diethylene glycol, and trimethylolpropane, respectively.

These peaks were not found in uninoculated control containing PUR. In all cases, the medium ingredient was detected as a large peak (P-5). HPLC analysis indicated that adipic acid, diethylene glycol, and trimethylolpropane were the main degradation by-product of the PS-PUR by *A. solani*. Peak 4 (P-4) was not identified.

IV-Determination of the optimal degradation conditions:

The degradation course of the PS-PUR by *A. solani* was investigated, and optimal condition for PS-PUR hydrolysis by test isolates was elucidated at different temperature and pH.

Results showed that *A. solani* was capable to hydrolyze the PS-PUR at a pH range between 4 and 8. However, *A. solani* had prominent degradation activity at pH 7.

In addition, *A. solani* was found to be able to hydrolyze the PS-PUR at a temperature range between 20 °C and 35 °C with optimal hydrolytic temperature of 30 °C with no significant activity at 35 °C and 40 °C.

VIII-The effect of media composition on the degradation activity:

The effects of different concentrations of glucose and yeast extract on degradation activity of the isolate are given in Fig 3. Results indicated that the presence of glucose repressed the degradation process. The presence of yeast extract at a concentration of (0.1%-1%) in basal media caused reduction in the degradation activity of *A. solani*. Moreover, in general, more than 60% of the degradation activity of these isolates was reduced when the nitrogen source was absent. Data revealed that *A. solani* can utilize all nitrogen sources provided, but it slightly prefers ammonium nitrate (NH_4NO_3) over the other nitrogen sources (Na_2NO_3 , NH_4Cl , and urea).

V-The effects of some competitive substrates on the degradation activity of the test isolate:

Fig 4 summarize the results of weight loss of PS-PUR when the competitive substrates were introduced in to the original basal medium. As gelatin, polyester (polyethylene adipate) and urea were added to the basal medium, the weight losses

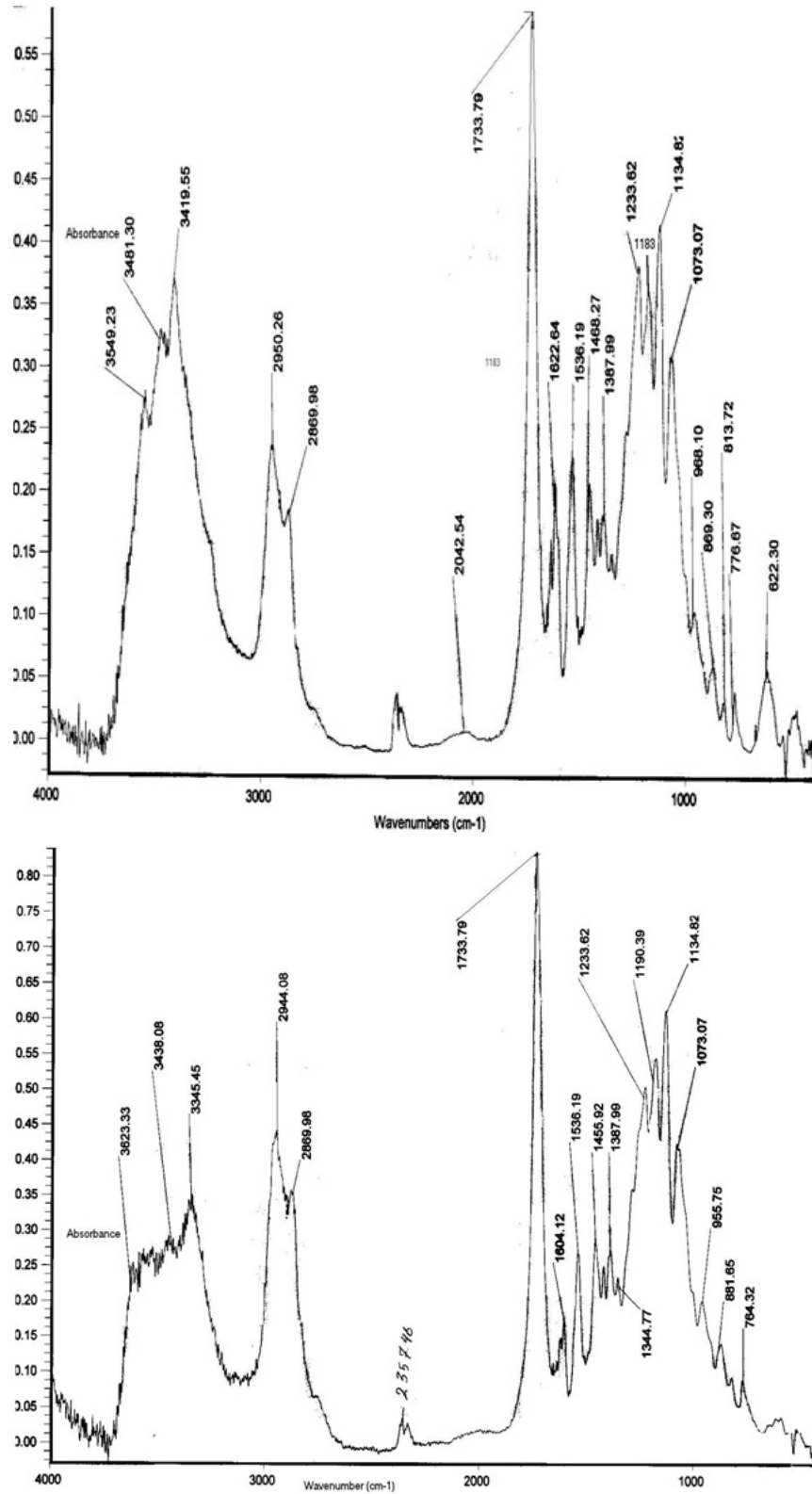


Fig. 1: Comparative FT-IR spectrum of the PS-PUR a- *A. solani*, and b- control (untreated PS-PUR).

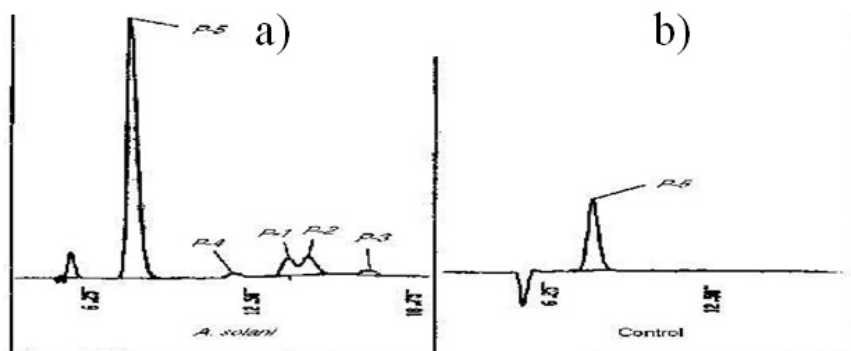


Fig. 2: HPLC-refractive index spectra of the degradation by-product of PS-PUR by test isolate after one week., A- *A. solani*, B- control.

Note: P-1; Adipic acid, P-2; Diethylene glycol, P-3; Trimethylol propane, P-4; Unidentified, P-5; Media ingredient.

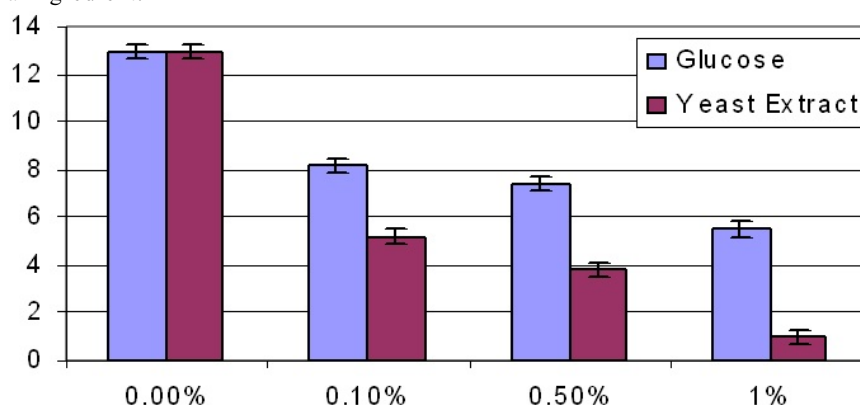


Fig. 3: Effect of media composition on the PS-PUR biodegradation by *A. solani*.

*Results are the means of three replicates. Error bars indicate the standard error of the mean.

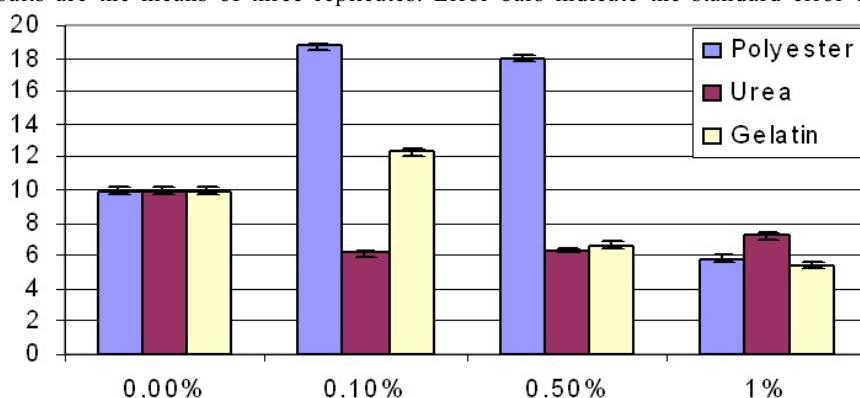


Fig. 4: Effect of competitive substrates on the PS-PUR biodegradation by *A. solani*.

*Results are the means of three replicates. Error bars indicate the standard error of the mean.

of PS-PUR media were relatively changed. *A. solani* showed increase in the relative weight loss of polyester medium containing low concentrations (0.1% and 0.5%) of polyethylene adipate, but showed

reduction in the relative weight loss at high concentration (1%). However, the presence of urea at a concentration of 0.1% to 1.0 % caused reduction in relative weight loss. When gelatin is added to the

medium at low (0.1%) and high (1%) concentration, *A. solani* showed an increase and decrease in relative weight losses, respectively. *A. solani* showed a significant increase in relative weight loss when the optimal condition and concentration of the tested substrates were collectively utilized (Table 1).

Table 1: The degradation activity of *A. solani* at optimal condition.

Media	Weight loss in the PS-PUR cubes in mg.
Basal medium	29.0 \pm 2.2
Optimal condition	44.8 \pm 4.4

*Results are means of three replicates, expressed as (M \pm SD).

VI -Preliminary examination of enzymatic activities of the test isolate:

The enzymatic activity was detected by clearing and/ or color change for protease, esterase, and urease activities. *A. solani* showed low urease activity, moderately protease and esterase activity. Examination of the enzymatic activity of the PCF revealed the presence of protease and absence of urease and esterase activity.

Discussion:

Accelerated attention has been focused at biological degradation as friendly solution for plastic accumulation and environmental pollution. This problem and its curtailment are becoming more significant in Jordan, but it is not researched, yet. However, there were indications of degradation of PS-PUR by fungi. But, it was not, yet, reported that fungi can utilize PUR as the sole source of carbon[12].

A. solani was not previously reported as PS-PUR degrading fungi. The assessment of this isolate potentials and its degradation impact upon PS-PUR was accomplished by utilizing several test methods. Thus, it was demonstrated that *A. solani* caused a noticeable decrease in tensile strength and elongation at break, and in the weight of the PS-PUR cubes after three weeks of incubation inside culture media supplemented with PS-PUR as sole source of carbon. This was comparable to many previous reports[14,2,9]. However, the degree of weight losses is more indicative than the decreases in both tensile strength and elongation at break. Such finding suggests the involvement of exo-acting enzymes that was previously proposed by Wales and Sagar,[18]. The action of an exo-acting enzymes, which removes successive monomer units from the chain ends, resulting in a disproportionate weight loss relative to the effect on tensile strength[18]. It was also found

that the microscopic cracking on PS-PUR surface is good evidence to the susceptibility of the PS-PUR to be attacked by this isolate.

Results of the FT-IR analyses supported chemical change in the PS-PUR block after the exposure to the test isolate. The relative reduction of the C-H stretch for the treated block indicated a loss of $-\text{CH}_2-$ groups from the PS-PUR. The diacid and most probably the dialcohol contribute to the major portion of $-\text{CH}_2-$ of PS-PUR. Therefore, the relative reduction of the intensity of C-H stretch during the degradation indicated a loss of diacid and dialcohol groups from the polymer. This is in agreement with Pathirana and Seal,[15] and David and Staley,[4] findings as they documented the release of diacid and dialcohols from PS-PUR hydrolysis. Moreover, the reduction in the peak at 1370 cm^{-1} revealed the reduction in the $-\text{CH}_2-$ of the PS-PUR, and so supports the previous explanation. Increases in the peak at 1230 cm^{-1} could be attributed to the formation of the hydroxyl groups of degradation products. Anderson, *et al*, [1] reported the same results.

Comparison of TLC results of the culture filtrates from different treatments revealed that the presence of one yellow spots (acid fraction) comparable to a spot obtained from acid hydrolyzed PS-PUR, suggesting presence of diacid in the culture broth but not in the control. This result is another indication of PS-PUR degradation, and it is comparable to that achieved by Pathirana and Seal[15]. Furthermore, at least two blue bright spots were detected from culture filtrates found comparable to two spots from acid hydrolyzed PS-PUR. This indicates the presence of molecules containing amines (amines fraction) in the treated samples but was not found in the control, indicating the hydrolysis of the urethane part of the PS-PUR during the degradation process.

Finally, using the HPLC technique, adipic acid, diethylene glycol, was found as the main metabolites of test fungi. In addition, trimethylolpropane, which is used in PS-PUR synthesis to form the crosslinked and branch also, was detected in this investigation as was detected by Nakajima-Kambe[10,11]. These by-products represent the diacid and dialcohol, which were proposed based upon of the hydrolysis of ester bond as a mechanism of the PUR degradation. The amine fraction, which was noted by TLC, was explained as a result of the hydrolysis of the urethane bond. In fact, Nakajima-Kambe[11] used GC-MS to detect the diamine (Diaminotoluene) in the alkaline broth culture of *C. acidovorans*. However, in all previously reported cases[14,3], fungi could degrade polyester-type PUR only if they are

provided with additional utilizable nutrients. In contrast, *A. solani* has been considered to be able to utilize polyester-PUR as sole carbon and nitrogen sources. Results indicate that presence of glucose reduces the degradation as it is readily available to be utilized as simple carbon source. On the other hand, YE inhibited the degradation activity of *A. solani*, which possesses protease activity in its PCF. In general, absence of nitrogen basal media reduces the degradation activity of all isolates to about more than 60%, because of limitation of nitrogen sources derived from urethane bonds. However, *A. solani* was able to utilize NaNO_3 , NH_4Cl , Urea, and NH_4NO_3 as nitrogen source.

The effects of the included competitive substrates in the medium indicated that the presence of low (0.1%) concentration of polyester and gelatin increased the degradation activity, which may be due to induction of protease enzyme which is proved to be the PS-PUR degrading enzyme. However, low concentration of gelatin and polyester had a positive effect on the PS-PUR degradation activity of *A. solani*. These results confirmed an inductive nature of protease production i.e. the fungal genes responsible for the synthesis of these enzymes, proteins, become switched on as the substrate is found, which could be PS-PUR plastic in this case. Hence, *A. solani* urease positive, so it can utilize urea and this explains the reduction in its degradation activity in the presence of urea in the media. Pathirana and Seal[14] achieved comparable results when they noted that complete media delayed the degradation of PS-PUR during the 28 days incubation. Results of the present investigation lead to the correlation between the production of protease, esterase, and urease enzymes to degrade PS-PUR such as what is demonstrated in the PCF when protease activity was detected for *A. solani*. These findings are in complete harmony with what is published about involvement of extracellular enzymes produced from fungi in PS-PUR degradation. Furthermore, the chemical structure of the PS-PUR by its nature suggests its susceptibility to proteases, esterases, and ureases enzymes. It is well documented that esterase and protease are mainly non-specific enzyme[15] and they are similar in their mode of action and overlap in their substrate specificity. Which may be due, in some cases, to an indiscriminative bond-break mechanism which will act on any ester or amide that can gain close contact with the enzyme activity center[15]. According to that, it is acceptable to correlate the protease activity of *A. solani* to its PS-PUR biodegradation potential, confirming that ester bond hydrolysis as main

mechanism of PS-PUR degradation during this investigation.

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