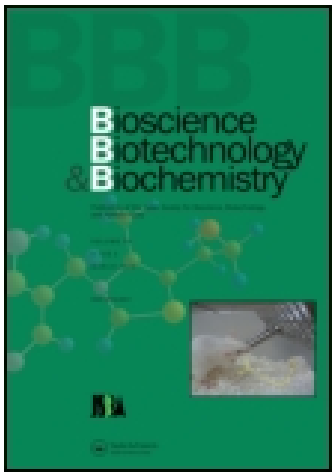

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The Biodegradation of Low-molecular-weight Urethane Compounds by a Strain of Exophiala jeanselmei

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The Biodegradation of Low-molecular-weight Urethane Compounds by a Strain of *Exophiala jeanselmei*

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To further analyze the biodegradation of polyurethane polymers, we investigated the biodegradation of low-molecular-weight *N*-tolylcarbamate model compounds with structures closely resembling the urethane linkages found in polyurethanes based on tolylene-diisocyanate (TDI). Soil microflora were screened for microorganisms that were able to utilize toluene-2,4-dicarbamic acid, diethyl ester (compound 1) as the sole source of carbon, and the soil fungus *Exophiala jeanselmei* strain REN-11A was selected as the most effective strain. Several *N*-tolylcarbamate compounds were used, and it was found that REN-11A was able to degrade compound 1, as well as the related compound toluene-2,6-dicarbamic acid, diethyl ester, very efficiently. Further investigation showed that compound 1 was biodegraded to tolylene-2,4-diamine *via* the aromatic amine intermediates carbamic acid, (3-amino-4-methylphenyl)-, ethyl ester and carbamic acid, (5-amino-2-methylphenyl)-, ethyl ester.

Key words: urethane; biodegradation; *Exophiala jeanselmei*

In recent years, the environmental impact of non-biodegradable waste polymer materials has become steadily more acute.[1,2] Because of the current impracticability of recycling synthetic polymers, however, the problem of disposal of discarded plastics can no longer be ignored. To alleviate this problem, we have been developing biodegradable polyurethane materials, for use in packaging, which can be decomposed by microorganisms in soil so they can be incorporated in the biocycle.

There are, however, comparatively few reports within the literature on the biodegradation of polyurethanes. One of the first systematic studies was by Darby and Kaplan,[3] who synthesized various types of polyurethanes, and showed that polyester-based polyurethanes are in general more susceptible to fungal attack than polyether polyurethanes. More recent work by Martens and Domsch[4] indicated that polyether polyurethanes (where the only hydrolyzable groups are the urethane linkages) are largely unaffected by fungal degradation, and that the urethane linkages in polyether or polyester polyurethanes are hydrolyzed only to a very limited extent. When the changes in composition of polyurethanes based on modified poly-D,L-lactic acid polyester polyols during fungal degradation were investigated by us, it was found that the polyester segments of these polyurethanes were selectively degraded; the segments derived from the polyisocyanate component of the polyurethane (which are linked by urethane and urea linkages) are more resistant to attack and remain in the residual material.[5]

Though the urethane groups in polyurethanes are not readily biodegraded, the biodegradation of urethane groups in low-molecular-weight urethane compounds has been extensively documented.[6-12] This research largely concerns the biodegradation of xenobiotic compounds that contain urethane groups, for example the *N*-methylcarbamate insecticides carbaryl and carbofuran,[6-7] and *N*-phenylcarbamate herbicides such as chloropropham.[9-12] It is important to note, however, that the structures of such xenobiotic compounds differ significantly from the structures of urethane linkages in polyurethanes. For example, the urethane groups in the most common type of polyurethane, based on tolylene diisocyanate (TDI), are of the *N*-tolylcarbamate type, with at least two carbamate substituents, as well as a methyl substituent, attached to the benzene ring.

The aim of our research was to obtain further information on the biodegradation of TDI-based polyurethanes, by investigating the biodegradation of low-molecular-weight urethane compounds with structures closely resembling the urethane linkages found in these polyurethanes. We screened for soil micro-organisms capable of utilizing the urethane compound toluene-2,4-dicarbamic acid, diethyl ester [compound **1**] as the sole source of carbon, and a strain of the "black yeast" *Exophiala jeanselmei*,[13] strain REN-11A, was selected as the most effective strain. This report describes the biodegradation of compound **1** and related *N*-tolylcarbamate compounds by *E. jeanselmei* strain REN-11A.

Materials and Methods

Synthesis of N-tolylcarbamate compounds. The chemical names, and physical and analytical data, of the *N*-tolylcarbamate compounds used in these experiments are given in Tables I and II.

Compounds **1** and **9** were synthesized by reacting tolylene-2,4-diisocyanate (Wako Ltd., Osaka, reagent grade) with excess ethanol or methanol in anhydrous $CHCl_3$. Compounds **2** and **3** were prepared by reacting tolylene-2,6-diamine (Lancaster Synthesis Ltd., UK, reagent grade) or toluene-3,4-diamine (Tokyo Kasei, reagent grade) with exceess ethylchlorocarbonate in $CHCl_3$. Compounds **4**, **5**, and **6** were prepared by reacting *o*-, *m*-, or *p*-toluidine, respectively with excess ethylchlorocarbonate in $CHCl_3$. Compounds **7** and **8** were synthesized by reacting 5-nitro-*o*-toluidine or 3-nitro-*p*-toluidine (Tokyo Kasei, reagent grade)

† Corresponding author.

Abbreviations: 2,4-TDA, tolylene-2,4-diamine; TDI, tolylene-diisocyanate.

Table I. HRMS Elemental Analysis and Melting Points of the *N*-Tolylcarbamate Compounds

Compound no.	Name	Formula	HRMS m/z (M^+)		mp (°C)
			Calcd.	Found	
1	Toluene-2,4-dicarbamic acid diethyl ester	$C_{13}H_{18}N_2O_4$	266.1266	266.1255	126
2	Toluene-2,6-dicarbamic acid diethyl ester	$C_{13}H_{18}N_2O_4$	266.1266	266.1260	153
3	Toluene-3,4-dicarbamic acid diethyl ester	$C_{13}H_{18}N_2O_4$	266.1266	266.1264	85.5
4	Carbamic acid, (2-methylphenyl),-ethyl ester	$C_{10}H_{13}NO_2$	179.0946	179.0943	35
5	Carbamic acid, (3-methylphenyl),-ethyl ester	$C_{10}H_{13}NO_2$	179.0946	179.0946	< −20
6	Carbamic acid, (4-methylphenyl),-ethyl ester	$C_{10}H_{13}NO_2$	179.0946	179.0950	45
7	Carbamic acid, (5-amino-2-methylphenyl),-ethyl ester	$C_{10}H_{14}N_2O_2$	194.1055	194.1044	91.5
8	Carbamic acid, (3-amino-4-methylphenyl),-ethyl ester	$C_{10}H_{14}N_2O_2$	194.1055	194.1108	89
9	Toluene-2,4-dicarbamic acid, dimethyl ester	$C_{11}H_{14}N_2O_4$	238.0954	238.0958	165

Melting points are uncorrected. Mass spectra were recorded on a JEOL JMS-AX 500 mass detector; ion source voltage, 70 eV; temperature, 230–240°C.

Table II. Spectral Data for the *N*-Tolylcarbamate Compounds

Compound no.	IR_{max} (KBr) cm^{-1}	^{1}H-NMR δ (ppm) (DMSO, TMS, 50°C) [J=Hz]	^{13}C-NMR δ (ppm) (DMSO, TMS, 50°C)
1	3335, 1698, 1537, 1252, 1064, 659	1.2 (t, 6H, J=7.0), 2.1 (s, 3H), 4.1–4.2 (q, 4H, J=6.8), 7.0 (d, 1H, J=8.4), 7.2 (d, 1H, J=8.1), 7.5 (d, 1H, J=2.2), 8.6 (s, 1H), 9.4 (s, 1H)	14.3, 16.8, 59.8, 60.0, 115.0, 125.5, 129.9, 136.4, 137.1, 153.4, 154.1
2	3298, 1694, 1522, 1238, 1044, 777	1.2–1.3 (t, 6H, J=7.2), 2.1 (s, 3H), 4.1 (q, 4H, J=7.0), 7.1 (s, 3H), 8.7 (s, 2H)	12.4, 14.4, 59.9, 122.1, 125.0, 127.4, 136.8, 154.3
3	3303, 1709, 1541, 1266, 1073, 774	1.2–1.3 (t, 6H, J=7.0), 2.3 (s, 3H), 4.1–4.2 (m, 4H), 6.9 (d, 1H, J=9.4), 7.3–7.4 (m, 2H), 8.5 (s, 2H)	14.2, 20.3, 60.2, 123.9, 124.7, 127.3, 130.0, 133.5, 153.9, 154.1
4	3299, 1694, 1588, 1456, 1244, 1073, 754, 654	1.2 (t, 3H, J=7.0), 2.2 (s, 3H), 4.1 (q, 2H, J=7.0), 7.0–7.1 (t, 1H, J=7.4), 7.2 (m, 2H), 7.3 (d, 1H, J=7.6), 8.7 (s, 1H)	14.3, 17.5, 59.9, 124.4, 124.5, 125.7, 130.0, 131.4, 136.3, 154.2
5	3322, 1615, 1227, 1175, 1069, 779	1.2 (t, 3H, J=7.2), 2.2 (s, 3H), 4.1–4.2 (q, 2H, J=7.1), 6.8 (d, 1H, J=7.6), 7.1 (t, 1H, J=7.7), 7.29 (s, 1H), 7.33 (s, 1H), 9.4 (s, 1H)	14.3, 20.9, 59.8, 115.4, 118.7, 122.8, 128.3, 137.6, 139.1, 153.4
6	3320, 1701, 1541, 1316, 1237, 1069, 818, 766, 681	1.2–1.3 (t, 3H, J=7.0), 2.2 (s, 3H), 4.1–4.2 (q, 2H, J=7.0), 7.1 (d, 2H, J=8.6), 7.3 (d, 2H, J=8.1), 9.3 (s, 1H)	14.3, 20.0, 59.7, 118.2, 128.8, 130.9, 136.5, 153.4
7	3432, 3339, 1713, 1592, 1464, 1237, 1202, 1061, 610	1.2 (t, 3H, J=7.0), 2.1 (s, 3H), 4.1–4.2 (q, 2H, J=7.0), 4.8 (s, 2H), 6.3–6.4 (q, 1H, J=7.8), 6.7 (d, 1H, J=1.9), 6.8 (d, 1H, J=8.1), 8.5 (s, 1H)	14.7, 16.9, 60.0, 110.7, 111.1, 118.5, 130.4, 136.7, 146.8, 154.4
8	3449, 3368, 3262, 1728, 1607, 1523, 1242, 1067, 791	1.2 (t, 3H, J=7.2), 2.0 (s, 3H), 4.1 (q, 2H, J=7.0), 4.7 (s, 2H), 6.6 (d, 1H, J=7.8), 6.79 (d, 1H, J=8.4), 6.84 (d, 1H, J=2.2), 9.2 (s, 1H)	14.5, 16.8, 59.8, 104.2, 106.9, 115.5, 129.8, 137.7, 146.6, 153.6
9	3266, 1717, 1694, 1603, 1547, 1244, 1071, 816	2.1 (s, 3H), 3.6 (s, 6H), 7.0 (d, 1H, J=8.4), 7.1 (d, 1H, J=7.8), 7.5 (d, 1H, J=2.2), 8.7 (s, 1H)	16.8, 51.3, 51.4, 114.9, 115.0, 125.5, 130.0, 136.3, 137.0, 153.8, 154.5

IR spectra were recorded on a Shimadzu DR8000 spectrometer; NMR spectra were recorded in DMSO at 50°C on a JEOL EX-270 spectrometer at 270 MHz.

with excess ethylchlorocarbonate (Tokyo Kasei, reagent grade) in DMF, followed by reduction of the nitro group to amine with $SnCl_2$/conc. HCl in ethanol. All compounds were dissolved in $CHCl_3$, washed with alkali water to extract residual aromatic amines, and then recrystallized twice (excepting compounds **4**, **5**, and **6**).

Isolation and identification of fungus. Microorganisms capable of utilizing compound **1** as the sole source of carbon were isolated from soil samples obtained from a polyurethane factory in Hyogo Prefecture, Japan, by enrichment culture and serial transfer in inorganic salts medium containing 0.1% compound **1**, 0.2% NH_4NO_3, 0.2% KH_2PO_4, 0.2% K_2HPO_4, 0.05% $MgSO_4$, 0.0005% NaCl, 0.0002% $ZnSO_4$, 0.0002% $FeSO_4$, and 0.0002% $MnSO_4$ at pH 6.2 and incubated with shaking at 27°C. The sole fungal strain obtained, strain REN-11A, was identified as *Exophiala jeanselmei* by the International Mycological Institute, UK. Two other strains of this species, strains ATCC 10224 and ATCC 16637, were obtained from the American Type Culture Collection.

Cultivation. *E. jeanselmei* strains were grown in Difco Sabouraud liquid medium (Difco, U.S.A.), for 6–8 days at 25°C in shaking culture, then the culture medium was sonicated to separate spores from the fungal hyphae. A suspension of spores was obtained by filtration of the culture medium followed by washing and resuspension in 0.85% saline. The inorganic salts medium used for the biodegradation experiments contained 0.1% NH_4NO_3, 0.07% KH_2PO_4, 0.07% K_2HPO_4, 0.05% $MgSO_4$, 0.001% NaCl, 0.0004% $ZnCl_2$, 0.0004% $FeSO_4$, 0.0002% $MnSO_4$, and 300 μM substrate urethane compound at pH 6.2; this medium was inoculated with the spore suspension to approximately 5×10^5 cfu/ml, then 100 ml of the medium was transferred to a 500-ml Sakaguchi flask and incubated with shaking at 25°C.

Isolation of metabolites. After 7 days of shaking culture at 25°C, the products of biodegradation of compound **1** were extracted in $CHCl_3$, concentrated 20-fold, and analyzed by GC-MS. Gas-chromatographs were recorded on a Hewlett-Packard 5890 Series II chromatogram; column OV-

17, 0.247 mm × 30 m × 0.25 μm, column temperature 50–200°C, 15°C/min, injection port temperature 240°C. Mass spectrograms were recorded on a JEOL JMS-AX 500 mass detector, ion source voltage 70 eV, temperature 230–240°C.

Measurement of urethane compounds. The concentrations of urethane compounds in the culture medium, after the fungal cells had been removed by centrifugation (12,000 rpm, 10 min), were measured using HPLC (Tosoh, model 8010): column, ODS 80-Ts (Tosoh); mobile phase, acetonitrile/water (ratio adjusted for each compound); flow rate, 0.8 ml/min.

Measurement of tolylene-2,4-diamine. The concentration of tolylene-2,4-diamine in the culture medium was measured using ion-exchange chromatography (Shimadzu): column, Shim-Pack IC-C1 (Shimadzu); mobile phase, (20 mM tartaric acid, 2 mM ethylenediamine, 1.5 mM nitric acid in water)/acetonitrile 10:1; flow rate 1.5 ml/min; detection, electron conductivity detector (Shimadzu model CDD 6A).

Results and Discussion

Screening for the optimal strain for biodegradation of compound **1**

After ten serial transfer cultures of the microflora of soil samples, 25 strains of microorganism were isolated. When these strains were individually cultured in inorganic salts medium containing 300 μM compound **1** as the sole carbon source, one fungal strain, REN-11A, was found to be able to rapidly assimilate compound **1**; this strain was identified as *Exophiala jeanselmei* (IMI number 362754).[13] The morphology and growth conditions of strain REN-11A were very similar to those of two other strains of *Exophiala jeanselmei*, ATCC 10224 and ATCC 16637. On Sabouraud agar, all three strains formed umbonate black-yeast-like colonies with olive-grey velvety upper surfaces. Hyphae were septate, 2–3 μm diameter, and conidia were 1 × 3 μm, exogenous and ellipsoidal. The pH and temperature growth ranges for these three strains were also similar (pH range 4–10, temperature range 13–34°C). However, strain REN-11A was slower growing than the two ATCC strains. Next, these three strains of *E. jeanselmei* were compared for their ability to biodegrade compound **1**. The results are shown in Table III; although all three *E. jeanselmei* strains were able to degrade compound **1**, strain REN-11A was found to be the most effective. Consequently, *E. jeanselmei* strain REN-11A was used in subsequent studies.

Substrate specificity

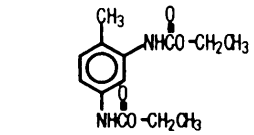
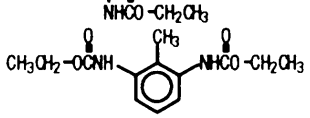
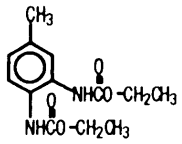
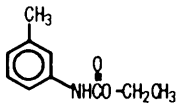
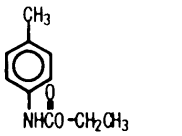
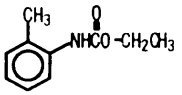
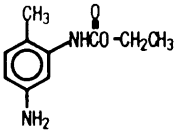
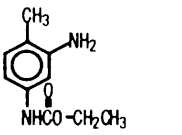
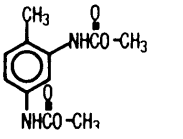
Table IV shows the percentage biodegradation of a variety of *N*-tolylcarbamate compounds after 6 days of cultivation of strain REN-11A. Comparison of the rates of biodegradation of the monourethane compounds, compounds **4**, **5**, and **6**, and also compounds **7** and **8**, showed that location of the urethane group *ortho* to the methyl group on the benzene ring increased the rate of biodegradation. In addition, in diurethane compounds (compounds **1**, **2**, and **3**), the presence of at least one *ortho* urethane group greatly increased the rate of biodegradation. Biodegradation of the methylcarbamate ester compound **9** was significantly slower than the biodegradation of the ethylcarbamate ester compound **1**. Aromatic amines liberated during biodegradation of all the above compounds were detected by GC-MS analysis (see below), confirming in each case that the urethane groups were cleaved by strain REN-11A. Non-microbial degradation of these compounds was found to be insignificant over this

Table III. Comparison of the Rates of Biodegradation of Toluene-2,4-dicarbamic Acid, Diethyl Ester [Compound **1**] by Three Strains of *Exophiala jeanselmei*

Exophiala jeanselmei strain	% Biodegradation of compound **1** after 5 days cultivation
REN-11A	100
ATCC 10224	69.7
ATCC 16637	68.1

Initial substrate concentration 300 μM, initial cell count of 5×10^5 cfu/ml, 25°C, shaking culture.

Table IV. Structure and Biodegradation Yields of Several *N*-Tolylcarbamate Compounds after 6 Days of Culture of *Exophiala jeanselmei* Strain REN-11A

Compound number	Structure	% Biodegradation of compound after 6 days cultivation
1		100
2		100
3		12.5
4		48.5
5		32.0
6		29.2
7		57.5
8		36.6
9		52.0

Initial substate concentration 300 μM, initial cell count of 5×10^5 cfu/ml, 25°C, shaking culture.

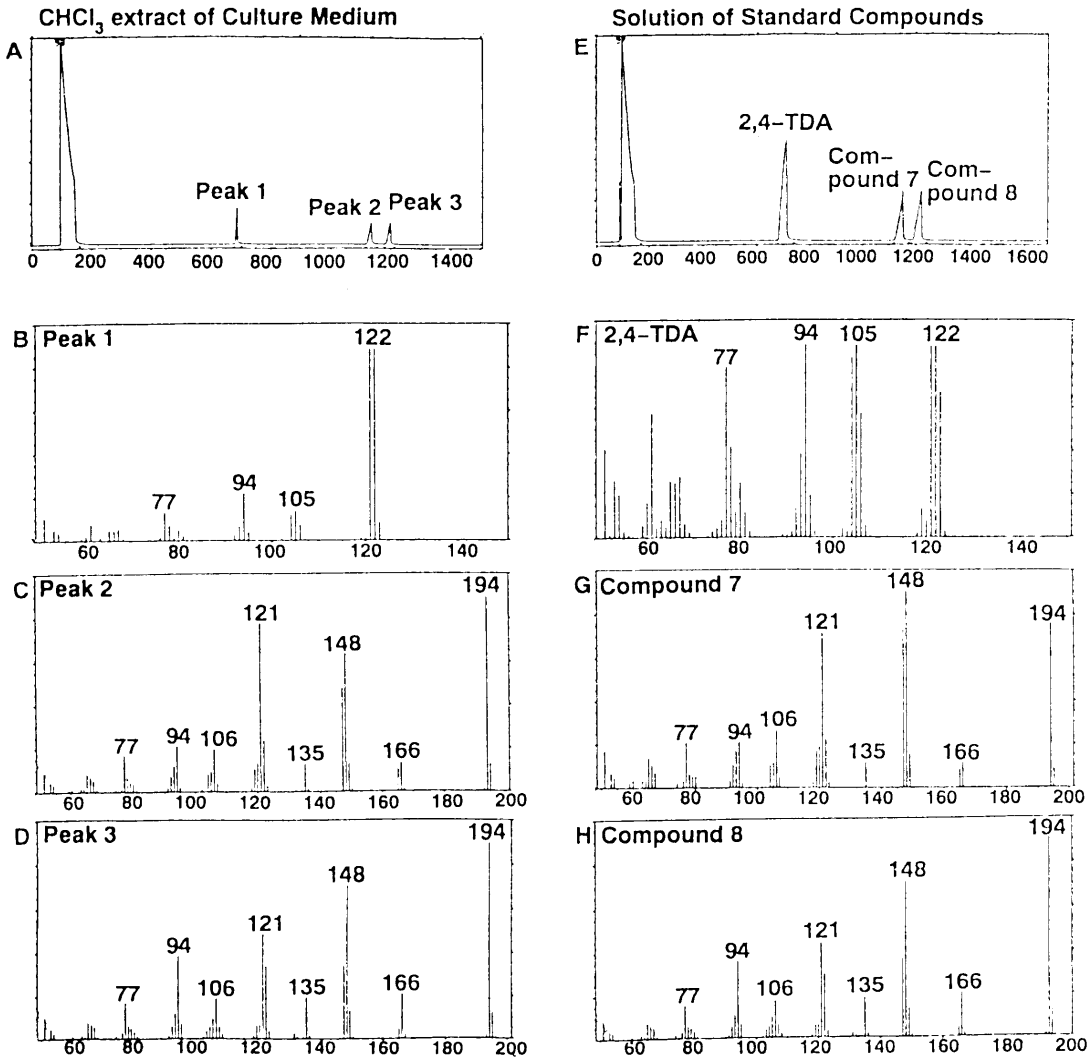


Fig. 1. GC-MS Analysis of the Products of Biodegradation of Toluene-2,4-dicarbamic Acid, Diethyl Ester (Compound **1**) after 7 Days of Culture of *Exophiala jeanselmei* Strain REN-11A.

(A) GC of $CHCl_3$ extract of the culture medium; (B) (D) mass spectra of the compounds detected in (A); (E) GC of $CHCl_3$ solution of: carbamic acid, (5-amino-2-methylphenyl)-, ethyl ester [compound **7**], carbamic acid, (3-amino-4-methylphenyl)-, ethyl ester [compound **8**], and toluene-2,4-diamine; (F) mass spectrum of toluene-2,4-diamine; (G) mass spectrum of compound **7**; (H) mass spectrum of compound **8**. Initial concentration compound **1** 300 μM, initial cell count of 5×10^5 cfu/ml, 25 C, shaking culture.

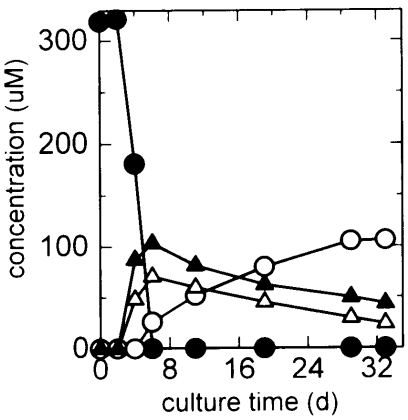


Fig. 2. The Decrease in the Amount of Compound **1** and the Accumulation of the Metabolites Compounds **7** and **8** and 2,4-TDA, during Growth of *Exophiala jeanselmei* Strain REN-11A.

Initial concentration compound **1** 310 μM, initial cell count of 5×10^5 cfu/ml, 25 C, shaking culture. (●), compound **1**; (▲), compound **7**; (△), compound **8**; (○), 2,4-TDA.

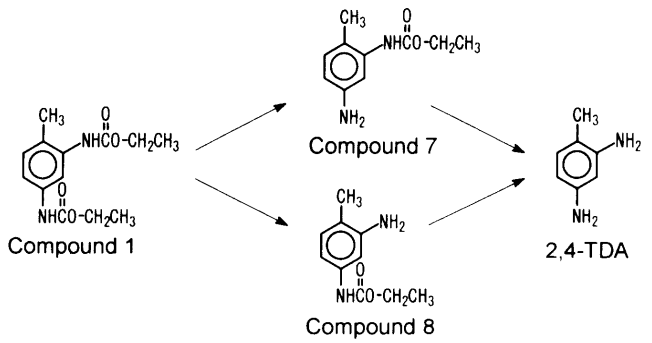


Fig. 3. Proposed Pathway for Biodegradation of Toluene-2,4-dicarbamic Acid, Diethyl Ester (Compound **1**) by *Exophiala jeanselmei* Strain REN-11A.

time.

Compound **1** is one of the most readily biodegraded of the above urethane compounds and also shows the most resemblance to the structure of urethane groups within TDI-based polyurethanes. We therefore restricted further research to the biodegradation of this compound.

Identification of metabolites of compound **1**

The results of GC-MS analysis of the products of biodegradation of compound **1** are shown in Fig. 1. The three products detected were identified, by comparison of the MS fragment pattern with standard compounds, as the aromatic amines (3-amino-4-methylphenyl)-carbamic acid, ethyl ester [compound **7**], (5-amino-2-methylphenyl)-carbamic acid, ethyl ester [compound **8**], and tolylene-2,4-diamine [2,4-TDA].

Metabolism of compound **1**

The decrease in the amount of compound **1** and the accumulation of the metabolites of compound **1**, the aromatic amines compounds **7** and **8** and 2,4-TDA, are shown in Fig. 2. Compound **1** was completely consumed within 6 days cultivation, and compounds **7** and **8** started to accumulate in the culture medium after 4 days; after 6 days 2,4-TDA accumulated concurrently with the decrease in compounds **7** and **8**. From this we propose that the major pathway for biodegradation of compound **1** by *E. jeanselmei* strain REN-11A is as shown in Fig. 3; compounds **7** and **8** are produced by hydrolysis of the *para* or *ortho* urethane group respectively of compound **1**, and tolylene-2,4-diamine is produced by subsequent hydrolysis of the remaining urethane group. However, the amounts of compounds **7** and **8** and 2,4-TDA that accumulated in the medium did not balance the amount of compound **1** consumed; this is possibly because compounds **7** and **8** and 2,4-TDA were metabolized by alternative pathways, or were converted to unidentified products by non-microbial processes such as oxidation.[14]

The exact site of hydrolysis of the urethane group, at the amide or ester bond, was not investigated in this study; however, cleavage at either site yields ethanol, which was used by *Exophiala jeanselmei* as an energy source for cell growth.

In this study, we demonstrated that a strain of soil micro-organism, *Exophiala jeanselmei*, was able to hydrolyze the urethane groups in tolylcarbamate compounds that resemble the urethane segments in TDI-based polyurethanes. Previous research by us suggested that biodegradation of polyurethanes prepared from TDI and modified poly-D,L-lactic acid produces compounds similar to compound **1**, after the ester groups of the polyurethane had been hydrolyzed.[5] Therefore, we hope to be able to utilize *E. jeanselmei* to achieve complete biodegradation of polyester-based polyurethanes.

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