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Original Article

Degradation Pathways of Isouron by Soil Perfusion and Isolated Yeast

Mamoru OZAKI, Yoshio HAYASE, Shinobu KOBAYASHI and Shozo KUWATSUKA*

Aburahi Laboratories, Shionogi Research Laboratories, Shionogi & Co., Ltd., Koka-cho, Koka-gun, Shiga 520–34, Japan
**Faculty of Agriculture, Nagoya University, Chikusa-ku, Nagoya 464, Japan*

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The microbial degradation of 3-(5-*tert*-butyl-3-isoxazolyl)-1,1-dimethylurea (isouron) was investigated by the soil perfusion technique using a soil collected from an upland field. Isouron was degraded within four weeks in the perfusion culture. During the course of isouron degradation, three new compounds were detected, and these were identified as 1-(1-amino-4,4-dimethyl-3-oxo-1-pentenyl)-3,3-dimethylurea, 1,1-dimethyl-3-(2-pivaloylacetyl)-urea, and 4-amino-6-*tert*-butyl-2*H*-1, 3-oxazin-2-one. The same metabolites were also detected in the liquid culture containing a small amount of soil under standing conditions, but not detected under shaking conditions. A strain of yeast capable of degrading isouron was isolated from the soil perfusate and was identified to be *Hansenula saturnus*. New metabolic pathways of isouron were proposed from the results.

INTRODUCTION

A new urea herbicide with isoxazole ring, isouron[3-(5-*tert*-butyl-3-isoxazolyl)-1,1-dimethylurea] has been developed by Shionogi & Co., Ltd., Osaka, Japan. This herbicide has excellent activity against ranges of broadleaved and grass weeds by both pre- and post-emergence application at a low dose.[1,2] We have studied the fate and behavior of the chemical in soil. Ozaki & Hayase demonstrated that isouron and its related compounds were metabolized by a soil fungus, *Rhizoctonia solani*, and that the main degradation reactions were hydroxylation of the *tert*-butyl group at isoxazole ring and demethylation of the urea side chain.[3] From the results we suggested the degradation pathways of isouron in soil.

In the present study, other new degradation pathways were found through the identification of three new metabolites of isouron in the enrichment culture of isouron using a soil perfusion technique, and a microorganism responsible for the degradation was identified to be *Hansenula saturnus*.

MATERIALS AND METHODS

1. *Chemicals*

Isouron and 5-*tert*-butyl-3-aminoisoxazole were synthesized in Shionogi Research Laboratories.[4] 1-(1-Amino-4,4-dimethyl-3-oxo-1-pentenyl)-3,3-dimethylurea (**A**) was synthesized by hydrogenation of isouron with 10% Pd-C in 95% ethanol at room temperature. 1,1-Dimethyl-3-(2-pivaloylacetyl)-urea (**B**) was prepared by hydrolysis of isouron with 0.1 N HCl at 55°C for 90 min. 4-Amino-6-*tert*-butyl-2*H*-1,3-oxazin-2-one (**C**) was obtained by hydrogenation of 5-*tert*-butyl-3-aminoisoxazole with 10% Pd-C in 95% ethanol followed by treatment with 1,1′-carbonyldiimidazole at room temperature.

2. *Soil and Perfusion Method*

Soil was collected from the upland field in our laboratories, crushed and passed through

a 5 mm sieve. The properties of the soil were as follows; clay mineral: vermiculite, texture: CL, clay content: 16.90%, pH (H_2O and KCl): 6.3 and 4.9, respectively, organic matter: 20.83 %, C.E.C: 45.70 meq/100 g soil, and maximum water holding capacity: 75.0%. Soil perfusion method was similar to that of Collins.[5] Fifty grams of the soil was packed in the column of the apparatus, and 300 ml of a mineral medium consisting of 0.1% $(NH_4)_2SO_4$, 0.02% KH_2PO_4, 0.16% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.01% NaCl, 0.001% $FeSO_4 \cdot 7H_2O$ and 0.05% isouron, adjusted to pH 7.0, was circulated at the rate of 120 drops per min for a month at room temperature.

3. Analysis of the Perfusate

One milliliter of the perfusate was periodically collected during the soil perfusion and extracted with one ml of benzene–ethyl acetate (3 : 1). The extract solution was concentrated and dried *in vacuo* at 40°C. The concentrate was subjected to thin-layer chromatography (TLC) using silica gel G with fluorescent indicator (Merck, 0.25 mm thick). Ethyl ether was used for the developing solvent. Isouron and its metabolites were detected by spraying a solution of 0.1% 4-dimethylaminocinnamaldehyde in ethanol acidified with HCl, then heating on an electric heater, or by placing under a UV lamp. Isouron and its metabolites were determined from the diameter of the spots and the shade of its color on TLC plate.

4. Isolation and Characterization of Metabolites

The perfusate was extracted with ethyl acetate, concentrated *in vacuo* and charged as a zone on the original line of silica gel G plate (2.0 mm thick) and developed with ethyl ether. Each area of silica gel of metabolites detected under UV light was collected and extracted three times with ethyl acetate. The extracts were concentrated and dried *in vacuo* at 40°C. The crude materials obtained were recrystallized from a small amount of ethyl acetate.

Mass spectra of the metabolites were obtained using a Hitachi mass spectrometer, Type M-68, at 70 eV of ionization potential using a direct insertion probe. The NMR spectra were determined in $CDCl_3$ containing tetramethylsilane as an internal standard on a Varian EM-36 spectrometer at 60 MHz.

5. Isolation and Identification of Isouron-degrading Microorganisms

Microorganisms capable of degrading isouron were isolated from the perfusate by the dilution method using the aforementioned mineral medium with 1.5% agar. The colonies developed on the agar plate were picked up and transferred to the nutrient agar slant (1.0% beef extract, 1.0% polypeptone, 0.5% NaCl, 1.5% agar, pH 7.2) for bacteria, to potato-dextrose agar slant (decoction extracted from 200 g of potato with 1000 ml of water at 121°C for 20 min, 20 g of glucose and 15 g of agar in 1000 ml of total volume of water) for fungi, and to Sabourauds' agar slant (4.0% glucose, 1.0% polypeptone, 1.5% agar) for yeast. To examine the isouron degradation by the isolated microorganisms, each isolate was inoculated into a test tube (18.0×1.5 cm, i.d.) containing 5 ml of the mineral medium and incubated without shaking at 28°C for a month. Isouron and its metabolites in the medium were analyzed by TLC as described above. To study the degradation by high concentration of cell suspension of yeast isolated, 3-day old cells grown in the Sabourauds' liquid medium were harvested by centrifugation, washed twice with saline, and resuspended in the mineral medium to give 20 mg dry weight of cells per ml. The cell suspension was incubated at 28°C on a reciprocal shaker at 250 rpm or without shaking. Isouron and its metabolites in culture medium were analyzed by TLC. The identification of the yeast was performed by the method proposed by Lodder.[6]

RESULTS

1. Isouron Degradation in an Enrichment Culture and Isolation of the Metabolites

From the enrichment culture of the soil perfusion for isouron degradation, a metabolite having UV-absorption was detected on TLC plate after 19 days of incubation (Fig. 1). Isouron almost disappeared within 4 weeks in the perfusate. Finally 3 main metabolites (metabolite **A**, **B**, **C** in Fig. 1) were found on the plate under UV lamp. These metabolites

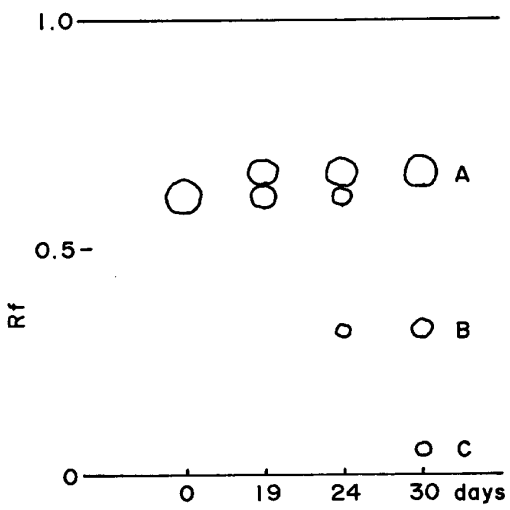

Fig. 1 Thin-layer chromatogram of isouron metabolites in perfusate at 0, 19, 24, and 30 days after incubation.

A, **B** and **C** in the figure: metabolites from isouron. The TLC was developed with ethyl ether.

were isolated from the perfusate by TLC. Metabolite **A** was white crystals, mp 147–148°C, analytical value: C 56.27, H 9.09, N 19.68, O 14.96, mass (*m/z*): 213 (M^+), calcd. for $C_{10}H_{19}N_3O_2$: C 56.32, H 8.98, N 19.70, O 15.00. This compound has UV-absorption spectrum characterized by two peaks at 225 nm (ϵ in methanol, 10,670) and 304 nm (ϵ in methanol, 27,300). The ^{1}H-NMR spectrum of metabolite **A** is shown in Fig. 2. From these data, metabolite **A** was identified as 1-(1-amino-4,4-dimethyl-3-oxo-1-pentenyl)-3,3-dimethylurea. This compound may be formed by reductive cleavage of the isoxazole ring of isouron. Metabolite **B** was oily liquid, analytical value: C 55.98, H 8.50, N. 13.06, O 22.45, MW 214, calcd. for $C_{10}H_{18}N_2O_3$: C 56.07, H 8.41, N 13.08, O 22.43. The ^{1}H-NMR spectrum of metabolite **B** is shown in Fig. 3. The lack of the peak at 14.1 ppm indicated the hydrolysis of the amino group in metabolite **A**. Metabolite **B** was consequently identified as 1,1-dimethyl-3-(2-pivaloylacetyl)-urea. Metabolite **C** was white crystals, mp 245–248°C, analytical value: C 56.74, H 7.04, N 16.55, O 19.67, MW 168, calcd. for $C_8H_{12}N_2O_2$: C 57.14, H 7.14, N16.67, O 19.05. The ^{1}H- and ^{13}C-NMR spectra are shown in Fig. 4. From these results, metabolite **C** was identified as 4-amino-6-*tert*-butyl-2*H*-1,3-oxazine-2-one, representing the metabolic product derived from metabolite **A** by intramolecular elimination of dimethylamine. The same metabolites were also detected in the liquid culture containing soil. The conditions were as follows: a small amount of soil was put in a test tube containing 5 ml of the mineral medium, and the test tube was incubated at 28°C for a month with or without shaking. TLC of the culture medium showed that 3 metabolites were produced in the medium only under standing conditions, but not with shaking.

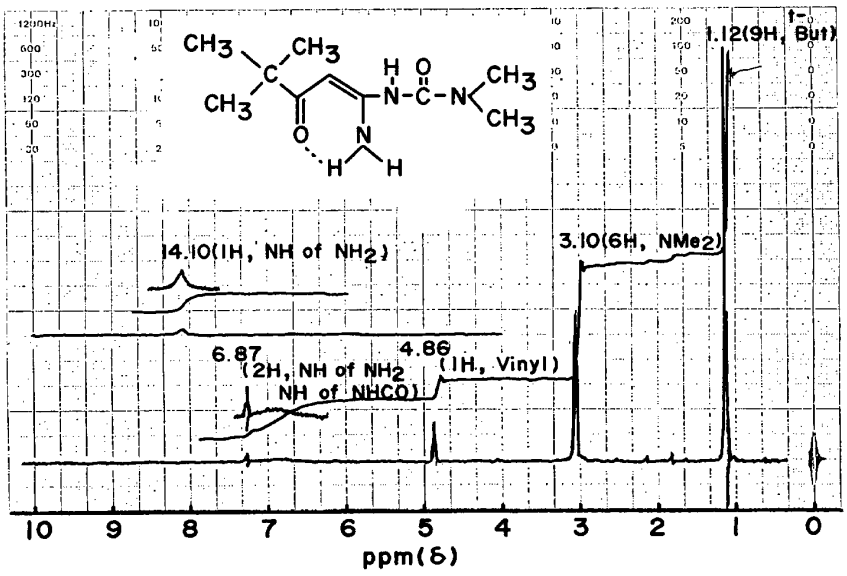

Fig. 2 ^{1}H-NMR spectrum of metabolite **A** (solvent: $CDCl_3$).

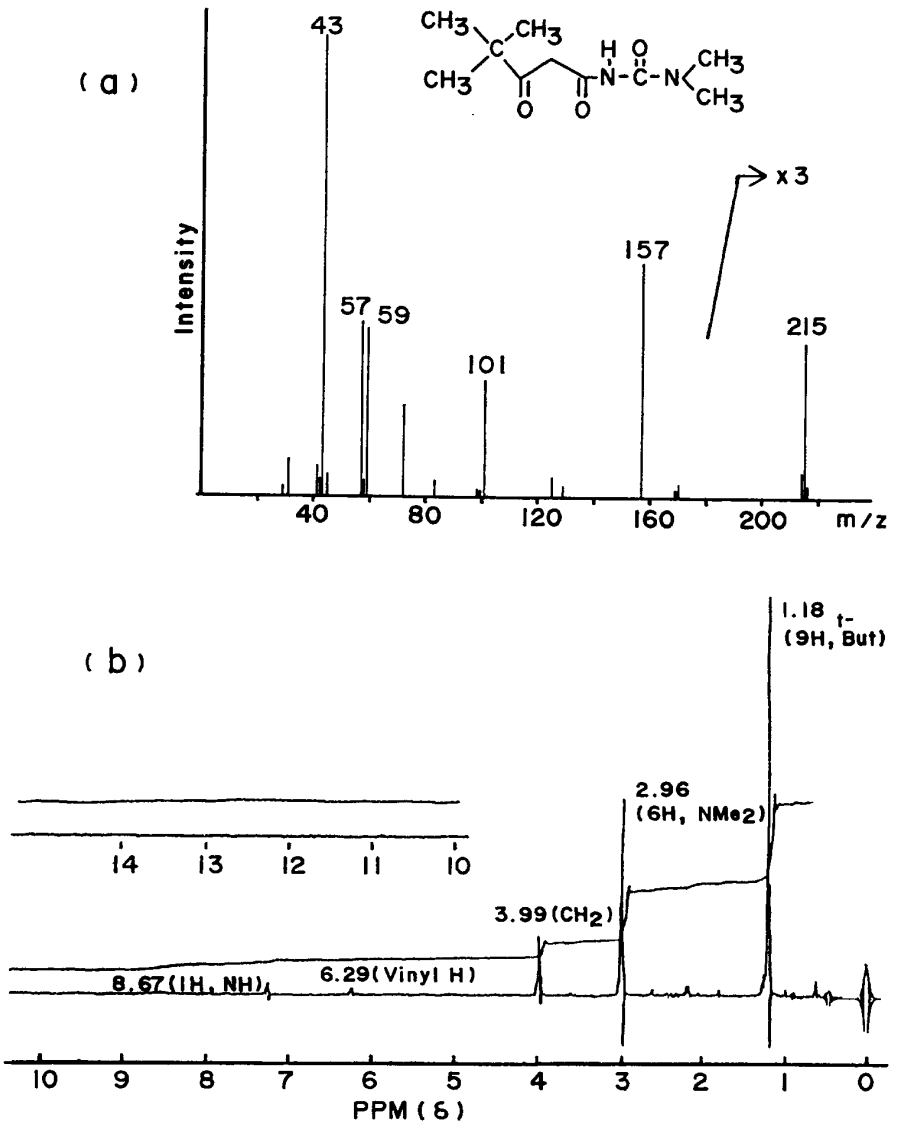

Fig. 3 Mass spectrum (a) and ^{1}H-NMR spectrum (b) of metabolite **B**.

2. Isolation and Identification of Isouron-degrading Microorganisms

Various colonies were developed on the agar mineral medium inoculated with the perfusate. Among the colonies, five strains of bacteria, two of fungi and three of yeasts were isolated as pure culture. Only a strain of yeast (No. E-2-4 isolate) had the ability to degrade isouron, but the degradability was weak in spite of the sufficient growth of the isolate in the mineral medium. Then, the isouron degradation was examined in the high concentration of cell suspension of No. E-2-4 isolate in the mineral medium (20 mg dry wt/ml) incubated at 28°C. After 2 weeks of incubation, the isouron degradation and the production of its metabolites were observed using the TLC technique. Mass spectrum of a metabolite separated from the culture medium was identical with that of the authentic compound **A** (Fig. 5). It was concluded that the strain of yeast, No. E-2-4 isolate, took part in reductive cleavage of isouron, which was a new degradation process of isouron, by soil perfusion. The cells of No. E-2-4 isolate were round to oval (size: 4–5 by 4.5–6.0 μm), and multilateral budding. The colony on malt extract agar was cretaceous and grayish white. The isolate formed faint pseudomycelia on corn meal agar. The spore was saturn-shaped. Creeping pellicle was formed in a liquid culture. Glucose and sucrose were fermented by the isolate, but galactose, maltose and lactose were not fermented at all. The isolate fermented one-

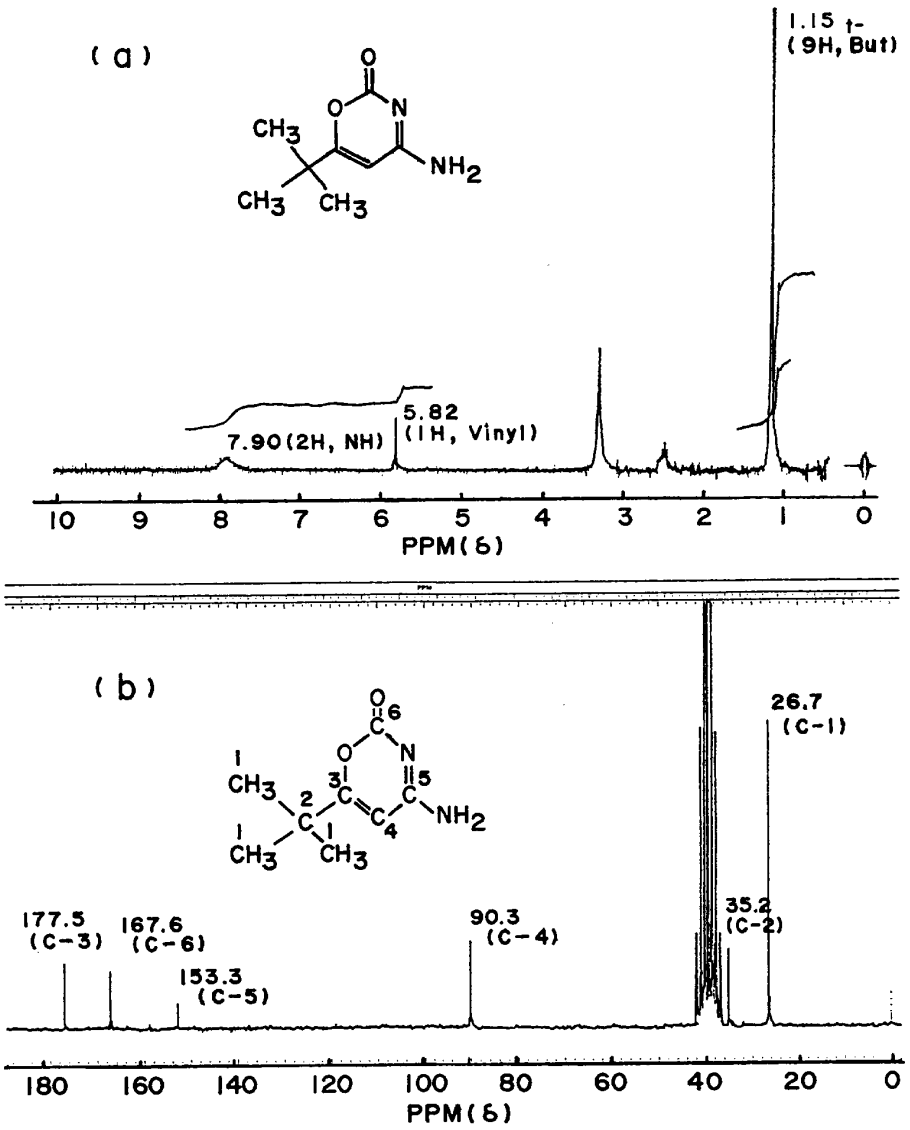

Fig. 4 ^{1}H- (a) and ^{13}C-NMR (b) spectra of metabolite **C** (solvent: d_6-DMSO).

third of raffinose. Glucose and sucrose were assimilated, but galactose, maltose and lactose were not. Ethanol was assimilated as a carbon source. Nitrate was reduced to nitrite. Ester was produced. From these results, No. E-2-4 isolate was identified as *Hansenula saturnus* on the basis of the description by Lodder.[6]

DISCUSSION

We previously showed that *Rhizoctonia solani* degraded easily isouron in a medium under shaking.[3] The degradation occurred by demethylation of the urea side chain and hydroxylation of the *tert*-butyl group at the isoxazole ring. The former reaction is well known in the degradation of phenylurea herbicides.[7-9] The latter was reported in the metabolism of the herbicide tebuthiuron with thiadiazole ring in animals.[10]

In this study, the reductive cleavage of isouron in the enrichment culture was confirmed. Isouron was metabolized to its reductive cleaved derivative (compound **A** in Fig. 5) by addition of two hydrogen atoms. This compound was further degraded to two compounds (**B** and **C** in Fig. 5) by deamination of compound **A** and by ring-closure with urea carbon after dedimethylamination of the urea side chain, respectively. As shown in Fig. 5, the degradation pathways of isouron were suggested from the results obtained. These findings indicate that isouron is degraded by quite different pathways from that indicated in our previous report.[3] The reductive cleav-

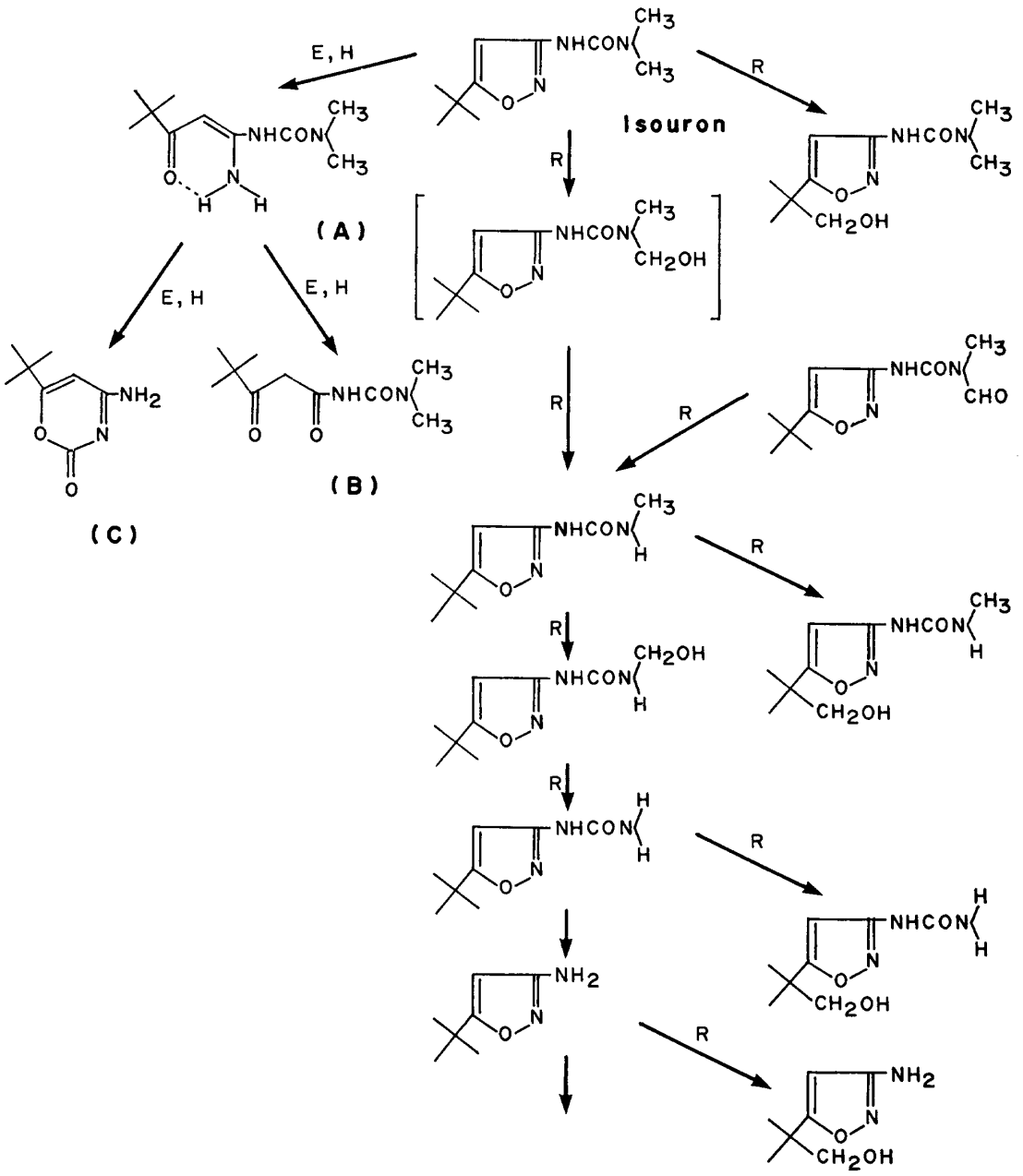

Fig. 5 Proposed degradation pathways of isouron.
R: degradation by *Rhizoctonia solani*,
E: degradation by an enrichment culture,
H: degradation by *Hansenula saturnus*.

age may be one of the important degradation processes in the metabolism of isouron, for it is possible that isouron is transferred into simple compounds more quickly through the reaction. A similar reaction has been already reported in the metabolism of the fungicide hymexazole in soil.[11] The new metabolites found in the perfusion culture were also recognized in the enriched liquid culture containing soil under standing but not under shaking conditions. Further, a strain of yeast (No. E-2-4 isolate) was isolated as an isouron-degradable microorganism and identified as *Hansenula saturnus*. Isouron was degraded by this strain when it was incubated in the mineral medium only under standing but not under shaking conditions. These results suggest that the reductive cleavage by microbial action hardly occur under fully aerobic conditions. Soil perfusion technique is a method in which

the solution with substrate is circulated against soil aggregates under aeration. Therefore, the soil in the column is in fairly oxidative conditions. However, our results demonstrate that the reductive reaction occurred in the perfusion. The solution in the soil could not pass enough after a long incubation period, probably due to altered structure of soil aggregates by various factors. Thus it may be that the soil could occur in reductive state. If we shall take this state into consideration, the reductive cleavage of isouron can occur in the soil perfusion. For these results obtained in the laboratory we suggest that isouron applied in the field soil may be metabolized to simple compounds through the reductive cleavage of isoxazole ring under semi-anaerobic conditions which are commonly distributed in soil, and through the hydroxylation of urea side chain and *tert*-butyl group at the isoxazole ring under aerobic conditions.

Hansenula saturnus degraded isouron to 1-(1-amino-4,4-dimethyl-3-oxo-1-pentenyl)-3,3-dimethylurea when the high concentration of cells of the organism were incubated with isouron under standing conditions. Isouron, however, was not metabolized thoroughly. It seems that other species of microbes in addition to *H. saturnus* may take part in the remarkable degradation of isouron which was observed in an enrichment culture.

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要　　約

土壌環流および分離した酵母によるイソウロンの分解経路

尾崎　守，早瀬善男，小林　忍，鍬塚昭三

畑地土壌を用いて土壌環流によるイソウロン [3-(5-*tert*-butyl-3-isoxazolyl)-1,1-dimethylurea] の微生物分解を検討した．4週間の培養でイソウロンは消失した．3種の分解物が環流液中に蓄積していることを薄層クロマトグラフィにより確認した．これらの分解物を単離しそれぞれ 1-(1-amino-4,4-dimethyl-3-oxo-1-pentenyl)-3,3-dimethylurea, 1,1-dimethyl-3-(2-pivaloylacetyl)-urea, 4-amino-6-*tert*-butyl-2*H*-1,3-oxazin-2-one と同定した．またこれらと同じ分解物が少量の土壌を添加した液体培地の静置培養でもイソウロンから生成することを認めたが，振盪培養ではその現象は認められなかった．なおイソウロン分解能を有する酵母を環流液より分離し *Hansenula saturnus* と同定した．以上の結果より還元的開裂によるイソウロンの新分解経路を推定した．