

Biodegradation of an *s*-triazine herbicide, simazine

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Abstract

Two types of simazine-degrading microorganisms were isolated from soil samples where the herbicide had been spread with the enrichment culture using simazine as the sole carbon and nitrogen source.

A fungal strain, DS6F, identified as *Penicillium steckii*, is the first simazine-degrading fungus ever reported.

Simazine was gradually degraded by *P. steckii* DS6F in the mineral medium containing simazine (initial concentration: 50 mg/l) and 25 mg/l of yeast extract. The rate of simazine degradation was improved when assimilable carbon sources were added into the medium, and the reduction rate of 53% was obtained after 5 days of cultivation at 30°C when glucose was added into the basal medium.

The bacterial strain, N5C, identified as *Moraxella (Branhamella) ovis*, was newly isolated and proven to degrade simazine more effectively than strain, DS6F, and 200 mg/l of simazine disappeared almost completely within 5 days at 35°C.

M. (Branhamella) ovis could also degrade another *s*-triazine herbicide, atrazine, at the similar reduction rate as simazine. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Simazine (2-chloro-4, 6-bis (ethylamino)-1,3,5-triazine, CAT), is an *s*-triazine herbicide, and since 1955, it has been extensively used worldwide as a germination controller of the broad-leaved weeds in corn, soybean, peanuts, potato, garlic, orchard and mulberry fields. As a result of its widespread use over the last 40 years, and because of its hardly biodegradable structure, CAT accumulated abundantly in the environments [1–3].

Thus, CAT caused contamination of both surface and groundwater. Although the use of CAT is now forbidden in many countries, the detection of CAT and other *s*-triazine derivatives has even recently been reported in several countries [4–10].

The reports concerning the microbial degradation of CAT are quite little, and the microorganisms ever isolated from soil were limited to bacteria.

Among them, *Rhodococcus corallinus* was reported to have the ability of transforming deethylsimazine by dehalogenation, dealkylation and hydroxylation, but the bacterium could not cleave the triazine ring [1]. Mixed culture system containing *Rhodococcus* strains screened for their ability of degrading atrazine was observed to degrade not only atrazine but also CAT [3].

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However, none of the pure strains isolated from the mixed culture showed the ability of metabolizing CAT and other *s*-triazine herbicides, except atrazine.

Concerning the fungi, some atrazine-related compounds, except CAT, were reported to be degraded by soil fungi [11–13]; no purified strains were ever reported to degrade CAT.

In this paper, we describe the isolation of microorganisms capable of degrading CAT with the enrichment culture method and also the growth characteristics of the isolated fungal strain, DS6F, and bacterial strain, N5C.

Optimal conditions for the biodegradation of CAT by those strains are also investigated.

2. Results

2.1. Isolation and characterization of microorganisms

After the repeated enrichment culture and isolation procedure, several strains of bacteria and only one pure fungal strain, named DS6F, were obtained.

These strains were proven to have the activity of degrading and growing on CAT as the sole source of carbon and nitrogen.

Bacterial strain, N5C, was finally selected for its high growth rate on CAT. Fungal strain, DS6F, was obtained from an apple orchard soil sample in Japan.

Strain N5C was identified as *Moraxella (Branhamella) ovis* by NCIMB Japan, and strain DS6F was identified as *Penicillium steckii* Zaleski by Centraalbureau Voor Schimmelcultures, the Netherlands, by their various typical characteristics.

2.2. CAT degradation by the cells of *P. steckii* strain, DS6F

Although *P. steckii* DS6F can grow on simazine as the sole source of carbon and nitrogen, the fungus showed better growth when a small amount of yeast extract was added in the mineral medium as a growth factor. So, in the further experiments, we used the medium containing yeast extract (25 mg/l) as the basal medium. As shown in Fig. 1, CAT degradation rate was remarkably stimulated by the addition of

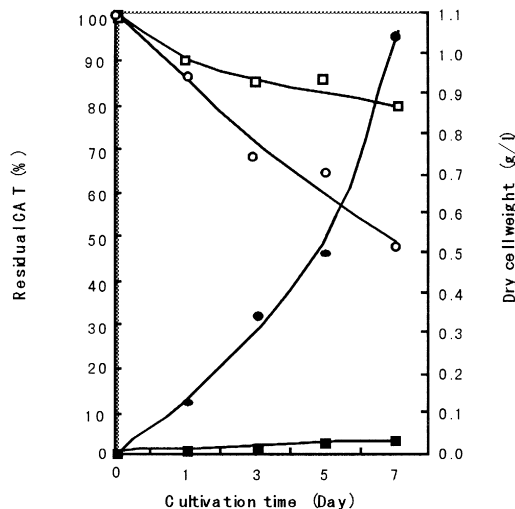


Fig. 1. Growth curve of strain in DS6F in SY (■), CzaS (●) media and CAT degradation in SY (□), CzaS (○) media.

other carbon sources such as glucose into the basal medium.

Precultured spores of strain DS6F on Czapek-Dox medium were cultivated for 5 days in CzaS medium (pH 4–8) in order to clarify the effects of pH on CAT degradation. Fig. 2 shows that CAT was degraded most rapidly at pH 6–8 by the fungal strain, although the optimal pH for growth was 5.5, and almost 78% of the initial CAT disappeared in 5 days of cultivation at pH 7.

Effects of temperature on CAT degradation by strain DS6F were tested with growing cells in CzaS medium for 5 days. Cell growth was best at 30°C and CAT was degraded well at 25–30°C (Fig. 3).

2.3. CAT degradation by the cells of *M. ovis* strain, N5C

The cells of strain N5C precultured in LB broth were inoculated into the SC medium and cultivated for 96 h at 30°C.

Fig. 4 shows that the bacteria reached the full growth within 72 h and at that time, CAT was completely degraded. Strain N5C was cultivated for 3 days at various pH values between 4 and 8 in the SC medium containing CAT at an initial concentration of 50 mg/l (Fig. 5).

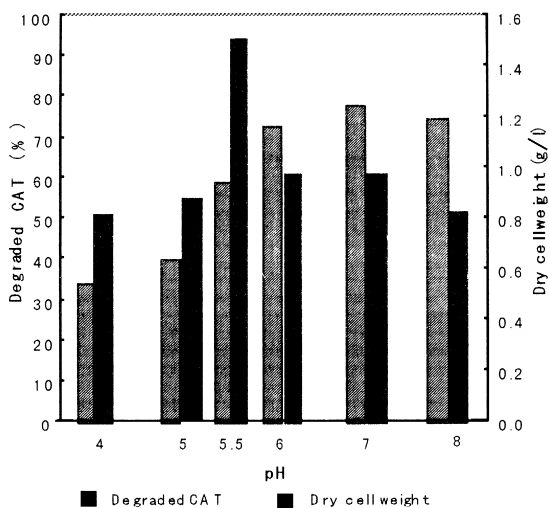


Fig. 2. Growth and degradation of CAT by strain in DS6F at various pH values.

Fig. 5 shows that the bacteria grow well at high pH, but pH values around 5 are suitable for CAT degradation by the strain. The strain, N5C (OD_{610} : 2), degraded almost 100% of CAT within 24 h by the resting cell reaction system at pH 5 (data not shown).

Strain N5C grows well at 25–40°C and the effects of temperature on CAT degradation were tested in

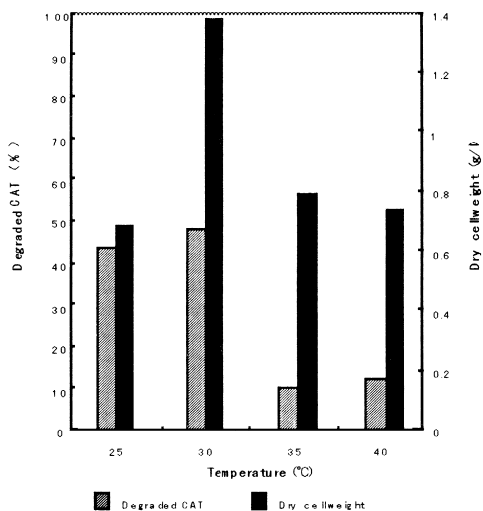


Fig. 3. Growth and degradation of CAT by strain in DS6F at various temperatures.

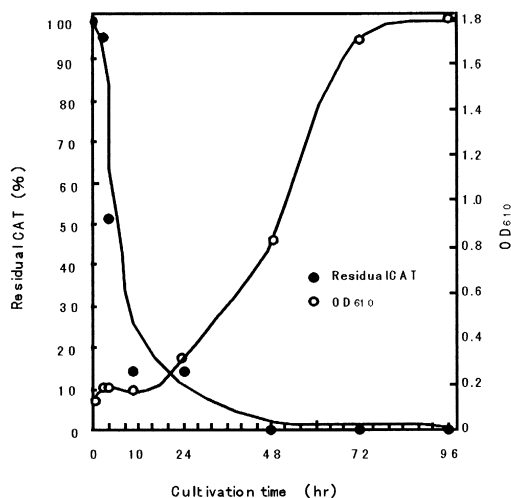


Fig. 4. Growth curve and CAT degradation by strain N5C.

the SC medium. Both the growth and CAT degradation rates were optimal at 35°C and almost 63% of the initial CAT was degraded at 35°C for 3 days (Fig. 6).

2.4. Degradation of CAT by successive addition

Strain N5C precultured in LB medium (OD_{610} : 0.2) was inoculated in 100 ml of SC medium (initial

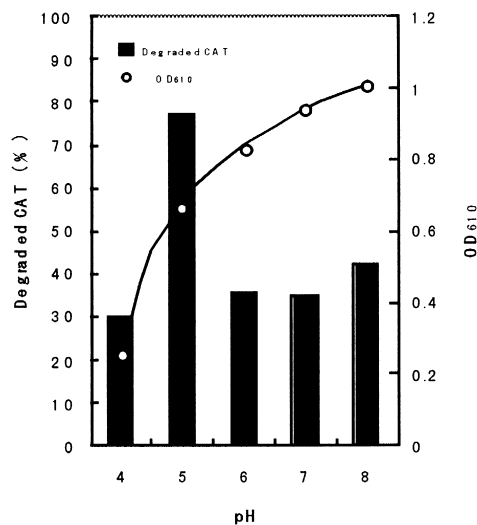


Fig. 5. Growth and degradation of CAT by strain N5C at various pH values.

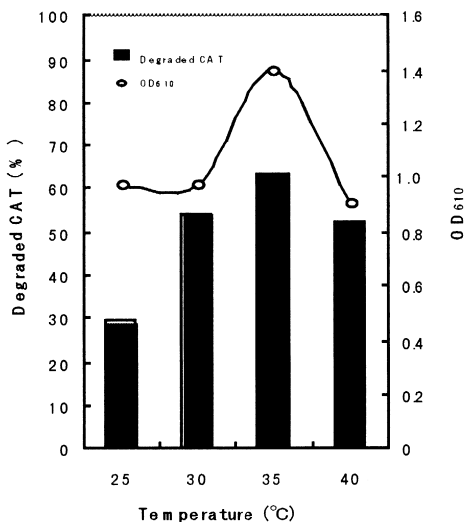


Fig. 6. Growth and degradation of CAT by strain N5C at various temperatures.

CAT; 50 mg/l). One milligram of CAT (methanol solution) was added every 1 or 2 days into the culture broth for 8 days (Fig. 7). As shown in Fig. 7, a total of 10 mg of added CAT was completely degraded within 10 days of cultivation. Although the degradation of CAT continued even at 10 days, the

addition of CAT was stopped because the decreasing rate of CAT became gradually slower after 6 days of cultivation.

2.5. Degradation of CAT in soil by strain DS6F

In order to examine the possibility of practical use of the isolated fungus, CAT degradation was tested in soil environment. After the soil samples (water-holding capacity, 16%; and added with 100 mg/kg CAT) were sterilized, the soil was inoculated with spores of strain DS6F at the concentration of 8×10^5 spores/g, and 1 kg each of soil sample was put into a beaker, covered with aluminum foil and incubated at 25°C. After the static incubation, 10 g aliquots were sampled and extracted with dichloromethane and analysed for CAT by gas chromatography (GC).

The rate of CAT degradation by fungal treatment was about 45.5% in 5 weeks as compared with the control sample.

2.6. Degradation of atrazine by strain N5C

Strains N5C and DS6F were tested for the atrazine-degrading activity. Bacterial strain, N5C, was proven to degrade atrazine at the almost equal rate as

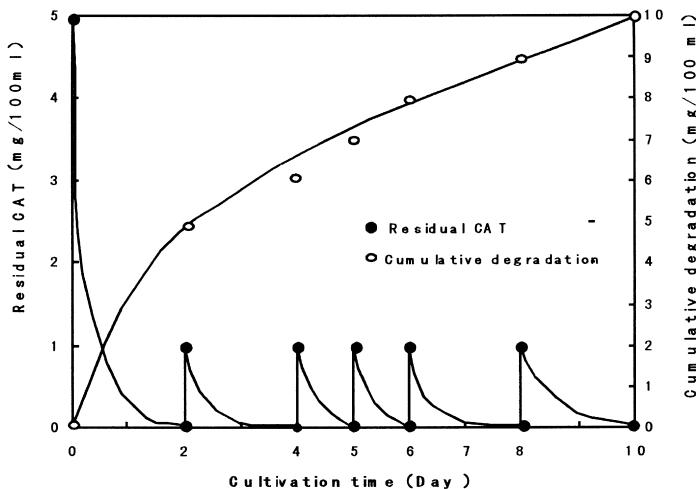


Fig. 7. Degradation of CAT by successive addition.

simazine. Whereas, fungal strain, DS6F, could hardly degrade atrazine (data not shown).

3. Discussion

A CAT-degrading fungus was isolated for the first time from a soil sample treated with the herbicide using the enrichment culture method. The fungus was discovered simultaneously with bacterial isolates on the culture plate for the isolation of CAT-degrading bacteria.

One of the isolated bacterial strain, *M. (Branhamella) ovis* N5C, was proven to have the much stronger CAT-degrading activity than the fungal strain, DS6F.

Although both strains were obtained on the screening medium containing CAT as the sole source of carbon and nitrogen, their abilities for CAT degradation were enhanced by adding other carbon sources in the medium. A similar phenomenon was reported by Giardina et al. [14] and Bheki and Khan [15]. Moreover, the presence of other organic or inorganic nitrogen sources stimulated cell growth of both strains, but had little effect on the degradation rate of CAT (data not shown). The optimal pH and temperature conditions for CAT degradation were quite moderate for both strains.

These results show that these strains are advantageous for the degradation of CAT in the natural environments, where carbon and nitrogen compounds are rich and pH and temperature conditions are moderate.

The experiments using the fungal strain, DS6F, for the degradation of CAT in soil proved the possibility of bioremediation of polluted soil environments.

The characteristics of bacterial strain, N5C, capable of degrading atrazine showed that not only the limited kinds of pollutants but the similar kinds of their derivatives can be degraded by using these bacteria.

During the cultivation, we detected other compounds with the decrease of CAT amount by GC analysis. We have not yet determined the possible intermediate compounds present in the medium, but it must be useful for the elucidation of CAT degrada-

tion pathway to know the structure of those compounds.

4. Experimental

4.1. Enrichment cultures and isolation of microorganisms

Soil samples were collected from various areas which have a history of sprinkled CAT. Each soil sample (0.1 g) was inoculated into 5 ml of SY medium and incubated on a reciprocating shaker at 130 rpm and 30°C. SY medium (1 l) contained 0.1 M phosphate buffer (pH 7.0); 100 ml, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 200 mg, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 10 mg, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; 10 mg, yeast extract; 25 mg, and 50 mg of CAT (as methanol solution). After 1 week cultivation, 0.2 ml of the culture broth was inoculated into 5 ml of the new SY liquid medium and was incubated for another week. The enrichment culture was repeated at least five times.

After repeating the enrichment culture, the culture broth was inoculated on basal medium plates. The colonies that appeared on the plates were picked up, purified with several cultivation media and stocked. In case of fungal strain, the spores that formed on a colony were picked up and purified.

4.2. Cultivation medium

SY medium was used as the basal medium for strain N5C, and it was added with 1 g/l of glycerol in further experiments for cultural conditions (SC medium). For the experiments on the cultural conditions for strain DS6F, Czapek-Dox medium was used as the basal medium and 50 mg/l of CAT was added (CzaS medium).

4.3. CAT degradation by growing cultures

The degradation of CAT was tested with a 30-ml test tube containing 5–10 ml of SC medium for N5C and CzaS medium for DS6F. Preculture was performed on Czapek-Dox solid medium in order to

obtain enough amount of spores of fungal strain, DS6F, while LB broth was used in case of bacterial strain, N5C. Precultured spores or cells were inoculated and incubated on a reciprocating shaker at 130 rpm and 30–35°C. The cell growth was determined by OD₆₁₀ (for strain N5C) or dry cell weight (for strain DS6F).

4.4. Analysis of CAT

The samples were taken at the appropriate reaction time intervals and the residual CAT in the culture broth was extracted with dichloromethane and the concentration of CAT was determined by gas chromatography (FID, Shimadzu GC 14B, Kyoto, Japan). The analytical condition of GC was as follows:

- Injection port temperature: 220°C
- Detection temperature: 220°C
- Column temperature: 200°C
- N₂ flow rate: 60 ml/min
- H₂ flow rate: 50 ml/min
- Column: 2.1 m glass column, packed with Chromosorb WHP (5% Silicone DC 200).

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