Degradation of the Herbicides
Thiobencarb, Butachlor and Molinate
by a Newly Isolated Aspergillus niger

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(Received February 5, 2004; Accepted April 24, 2004)

Thiobencarb ([S-4-chlorobenzyl diethylthiocarbamate] is a thio-
carbamate herbicide used for weed control in rice production. After screening, a strain able to degrade thiobencarb, identified as Aspergillus niger van Tieghem, was successfully isolated. Opti-
mal conditions for thiobencarb degradation in culture were at pH 5.5 and 30°C. The strain also showed the ability to degrade the herbicides molinate and butachlor. © Pesticide Science Society of Japan

Keywords: thiobencarb, Aspergillus sp., molinate, butachlor.

INTRODUCTION

Thiobencarb ([S-4-chlorobenzyl diethylthiocarbamate) is a thio-
carbamate herbicide widely used for weed control in paddy fields. Studies have documented that thiobencarb and molinate exist, seasonally, in rivers in Japan.1 It has been reported that thiobencarb was presumably degraded by molds and/or aerobic bacteria from the fact that it was rapidly degraded under oxid-
avie conditions.2,3) The authors carried out screening of micro-
organisms able to degrade the herbicide thiobencarb. Degrada-
tion of three other herbicides by the isolated strain was also investigated. Ametryn (2-(ethylamino)-4-(isopropylamino)-
6-(methylthio)-s-triazine) is a triazine herbicide. Molinate (S-
ethylazepine-1-carbothioate) is another thiocarbamate herbicide, and butachlor (N-butoxymethyl-2-chloro-2',6'-diethylacetanilide) is an acetonilide herbicide. Both are used in paddy fields.

MATERIALS AND METHODS

1. Isolation and Cultivation of Microorganisms

Screening was carried out from nearly hundred soil samples taken from several origins (paddy fields, orchard and mountain areas, crop fields, feces). Some bacterial strains stored in our lab were also tested for thiobencarb degradability (Moraxella ovis, Ralstonia sp., Pseudomonas putida). The enrichment culture was conducted in test tubes containing 5 ml of media (per liter: 0.1 M NaH2PO4, 60 ml; 0.1 M KH2PO4, 40 ml; CaCl2-2H2O, 10 mg; FeCl3-6H2O, 10 mg; MgSO4-7H2O, 200 mg, yeast extract, 25 mg; thiobencarb, 50 mg; pH 7.0) and inoculated with 0.1 g of soil sample, of feces, or with the liquid culture broth of the bacteria strains. The tubes were shaken in a reciprocating shaker (300 cy-
cles per minute) in the dark at 30°C. After 7 days of cultivation, if turbid, a 0.2 ml sample was taken and diluted in fresh medium. Subsequent identical transfers were performed five times every 7 days. Once a strain was isolated, stock cultures of the organism (a fungus) were maintained on agar slants with Czapek-Dox medium containing 25 ppm thiobencarb.

2. Degradation of Herbicides

The medium for further cultivations was Czapek-Dox containing 100 ppm Tween 20, and citric acid/Na3 citrate (for pH 3.5, 4.5 and 5.5) or phosphoric acid buffer (for pH 6.5 and 7.5), except where otherwise stated. In studies of thiobencarb degradation in Cza-
pek-Dox media containing different sucrose or NaN3 concentrations, their concentrations ranged between 0, 1, 5, 15, and 30 g/l; or 0, 0.01, 0.1, 0.5, and 2 g/l, respectively. Spores of the isolated strain were harvested from 15-day-old agar slants and suspended in sterile 0.1% Tween 80 solution. The strain was cul-
tivated, per duplicate, in 500-ml Erlenmeyer flasks with baffles containing 50 ml of medium and inoculated with the strain (10⁶ spores/l). The flasks were shaken on a rotary shaker at 100 rpm and 30°C, and contained 20 ppm thiobencarb, except where other-
wise stated. All data presented are the average of results ob-
tained from at least two independent measurements. In the stud-
ies for thiobencarb degradation flasks containing the medium, they were autoclaved at 121°C for 15 min prior to the addition of herbicide. Thiobencarb, in an acetone solution, was filter sterilized (0.2 µm filter, Millipore, France) and added to the sterilized media. For the studies on degradation of ametryn, molinate, and butachlor, each herbicide in a methanol solution was added to the media prior to autoclave sterilization. Herbicide concentrations were 100 ppm for ametryn and molinate, and 20 ppm for butachlor.

3. Analytical Methods

Aliquots of the culture broth were taken periodically and herbi-
cides extracted with an equal volume of ethyl acetate. After cen-
trifugation at 3000 rpm for 5 min, aliquots of the solvent layer were analyzed by gas chromatography (model GC-14B, Shimadzu, Japan) equipped with an FID and 5% silicone 4 DC2000. Chromosorb W HP column was used. For thiobencarb, ametryn and butachlor analysis the chromatographic conditions were as follows: nitrogen flow, 60 ml min⁻¹; hydrogen flow, 50 ml min⁻¹; air flow, 50 ml min⁻¹; and for temperatures: column oven, 200°C; injection, 220°C; detector, 220°C. For molinate analysis, condi-
tions were the same as mentioned above, though the column oven, injection and detector temperatures were 180°C, 200°C,
and 200°C, respectively. The amount of biomass was determined gravimetrically after the cells were collected by paper filtration and dried at 105°C to a constant weight. Dry weight of biomass was measured at 36 hr of initiated cultivation in all cases, except where otherwise stated.

**RESULTS AND DISCUSSION**

1. *Isolation of Herbicide-Degrading Organism*

As result of screening for microorganisms able to degrade the herbicide thiobencarb, a filamentous fungus strain, from a mixture of rat feces and named strain CRN, was successfully isolated and identified as *Aspergillus niger* van Tieghem by the Centraalbureau voor Schimmelcultures in the Netherlands.

2. *Optimal Degradation Conditions*

Studies on the optimal conditions for thiobencarb degradation by the isolated strain were carried out considering the variables pH, temperature, and agitation speed. The induction period for thiobencarb degradation was shorter at pH 5.5. A greater increase in biomass was monitored at lower pH (0.83, 0.41, and 0.36 g/l at pH 3.5, 4.5 and 5.5 respectively, and at 48 hr of initiated cultivation 0.24 and 0.14 g/l at pH 6.5 and 7.5). However, regarding herbicide degradation, the optimal pH was considered to be pH 5.5. Considering the solubility in water of thiobencarb at 25°C is 30 mg/l, four different initial concentrations of thiobencarb in media were studied (28, 20, 12 and 6 ppm). Degradation by *A. niger* strain CRN occurred in a similar way for all the studied concentrations, with no considerable difference.

In fixing agitation at 100 rpm and monitoring the increase of dry weight of biomass in media at different temperatures (25, 30, 35, and 40°C), the cell growth increased concomitantly as the temperature was increased (0.1, 0.4, 1.0, and 3.0 g/l respectively). Figure 1A shows the degraded thiobencarb related to a gram of cell dry weight at 36 hr. The isolated strain showed the peak of degradation at 30°C. In fixing temperature at 30°C and monitoring the increase of biomass in media at different agitation conditions (100, 125, 150, and 200 rpm) at 36 hr of initiated cultivation (Fig. 1B), cell growth was 0.4, 0.4, 0.6, and 0.7 g/l respectively. However, the difference in biomass was not as such in the case of increased temperature. For the lower agitation conditions no considerable difference was observed on the thiobencarb degradation, though at 150 rpm it was slightly enhanced. Higher agitation was concomitant with decreased degradation. We studied, as well, the combined case of 35°C and 150 rpm of agitation. Under this condition, a synergistic effect of temperature and agitation on the growth of strain was observed, the dry weight of biomass after 36 hr of initiated cultivation was 2.5 g/l. However, it did not affect the rate of degraded thiobencarb per gram of cell dry weight.

The degradation of thiobencarb in Czapek-Dox media containing either different sucrose or NaNO₃ concentrations was studied.
(Fig. 2). Flasks were shaken at 150 rpm. As the sucrose concentration in media was higher, the degradation rate was increased, and the time to enter to stationary phase was shorter. At sucrose concentrations of 30 and 15 g/l, and NaNO₃ concentrations of 0.5 and 2 g/l, no considerable difference was observed in the disappearance of thiobencarb. Sucrose and nitrogen concentrations at 1 g/l and 0.01 g/l respectively resulted in considerable removal of thiobencarb from the media in 18 and 16 hr, respectively. In the absence of sucrose or NaNO₃, a decrease of thiobencarb was also observed probably due to absorption of the herbicide into the fungal cells. Further studies on the metabolism of thiobencarb in the cell, e.g. isolation and purification of metabolites, may clarify the degradation pathway of the herbicide.

3. Degradation of Molinate and Butachlor

*A. niger* strain CRN was also tested by introducing three other herbicides into the cultivation media. It was capable of degrading molinate and butachlor in medium containing sucrose as the main carbon source. Ametryn degradation was not detected after 7 days of cultivation under the stated conditions. The degradation curves of the different herbicides are shown in Fig. 3.

Abe and Kuwatsuka identified a *Corynebacterium* sp., which decomposed thiobencarb.⁴ However, to our knowledge, this is the first report on degradation of the herbicide thiobencarb by *A. niger* or any fungus. Degradation by *A. niger* of several contaminant compounds such as phenanthrene and pyrene, chlorinated derivatives of phenoxyacetic acid and benzoic acid, and organophosphonates herbicides has been reported.⁵⁻⁷

**REFERENCES**