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Original Article

Isolation of Aryl Acylamidase-Producing Soil Bacteria and Some Properties of the Extracellular Enzymes*

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Soil bacteria producing aryl acylamidases extracellularly were isolated by a convenient method capable of detecting the activity directly on an agar plate where colonies developed, by overlaying successively the agar layers containing substrate acetanilide (AAN) and reagents for coloration by aniline (AN) released. The detection ratio of colonies producing the enzymes was 6% in Bouillon medium and 13% in a minimal medium containing AAN as a sole carbon source. In a liquid culture of the most active bacterium, A-1, 69% of the activity existed in the supernatant of broth and 29% was remained with sonicated cell debris. The activity of this extracellular enzyme(s) remarkably increased under preincubation with AAN. Among the extracellular enzymes from active colonies isolated with Bouillon medium and the minimal medium, 60% and 42% of the enzymes, respectively, were highly activated by AAN. This substrate activation, at least by AAN, seemed fairly general for extracellular aryl acylamidases originated from soil bacteria.

INTRODUCTION

While many microbial hydrolases are essentially intracellular enzymes, extracellular ones are also found in soils associated with cell debris and soil colloids.[1] In our previous paper,[2] we have shown that such extracellular hydrolases in soil may play an important role in degrading pesticides and preserving the environment. The general conception of extracellular enzymes[1,3] and their involvement in pesticide degradation[4] have been already reviewed, but studies on related microbial extracellular hydrolases seem still insufficient. In our studies, microbial aryl acylamidases were taken up because enzymes of bacteria[5-16] and fungi[17-24] had a broad substrate specificity and their activities were easily induced by various pesticidal acylanilides, carbamates or urea derivatives. This paper reports the isolation of active soil bacteria producing aryl acylamidases and some properties of the extracellular enzymes.

MATERIALS AND METHODS

1. *Chemicals and Materials*

Acetanilide (AAN), *N*-(1-naphthyl)ethylenediamine dihydrochloride (NED) and tubing-seamless 20/32-inch cellophane for dialysis (cellophane tube) were purchased from Wako Pure Chemical. Ehrlich's meat extract and peptone were purchased from Kyokuto Seiyaku Kogyo. Other chemicals and materials were purchased from Wako Pure Chemical or Nakarai Chemicals. Aniline (AN)

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for calibration was used after distillation.

2. Soils

Soils were collected from the surface (5 cm in depth) of different paddy or upland fields and the A horizon of different forest soils at Shimane University and the neighborhood. The soils were air-dried, sieved with a 2-mm screen and mixed thoroughly before use.

3. Media

Bouillon medium: 10 g of Ehrlich's meat extract, 10 g of peptone and 5 g of NaCl were dissolved in 1 *l* of distilled water.

Minimal medium supplemented with AAN as a sole carbon source: 3.4 g of KH_2PO_4, 2.32 g of Na_3PO_4, 1.0 g of $(NH_4)_2SO_4$, 0.2 ml of 5000 ppm AAN aqueous solution and 10.0 ml of mineral solution were dissolved in 1 *l* of distilled water. The mineral solution contained 3.0 g of $MgSO_4$, 0.5 g of $MnSO_4$, 1.0 g of NaCl, 0.1 g of $CaCl_2$, 0.1 g of $ZnSO_4 \cdot 7H_2O$, 10 mg of $K_2Al_2(SO_4)_4 \cdot 24H_2O$, 10 mg of H_3BO_4 and 10 mg of $Na_2MoO_4 \cdot 2H_2O$ in 1 *l* of distilled water.

Agar plates for bacterial isolation were prepared by dissolving 1.5 to 2.0% of agar into Bouillon or minimal medium.

4. Isolation of Active Bacterial Colonies

Bacteria were isolated by a conventional soil dilution plate method. One-milliliter portions of serial dilutions from 1 g of each soil were mixed with 9 ml of Bouillon or minimal agar medium liquefied at 45°C in a 9-cm petri dish and incubated at 30°C for 5 days.

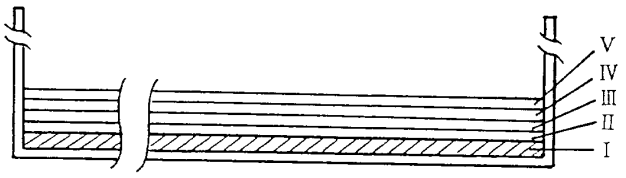

Fig. 1 Multiple agar layers method for detection of bacterial colonies producing aryl acylamidases.

I: Soil dilution agar plate developing colonies (thickness; *ca.* 1.7 mm), II, III, IV and V: Agar layers containing substrate AAN, $NaNO_2$, sulfamic acid and NED, respectively (each thickness; *ca.* 1 mm).

Active colonies were detected by a multiple agar layers method in which agar layers containing AAN and reagents were overlaid successively for coloration by released AN by modifying the diazotization and coupling method,[25] as shown in Fig. 1. To an agar plate where bacterial colonies were developing, 1 ml of 37 mM (5000 ppm) ANN and 5 ml of 2% agar (liquefied at 50°C) were added and mixed gently to overlay a homogeneous layer. The plate was then incubated at 30°C for 3 to 4 hr. In a similar manner, the layer of 1 ml of 0.2% $NaNO_2$ in 20% tartaric acid and 5 ml of 2% agar, after 10 min, the layer of 1 ml of 0.2% sulfamic acid in 20% tartaric acid and 5 ml of 2% agar, and after another 15 min, the layer of 1 ml of 0.2% NED in 20% tartaric acid and 5 ml of 2% agar were overlaid on the plate, which was left standing at room temperature until an appropriate color (purplish red) developed around active colonies.

The active bacterial colonies isolated were stored on Bouillon agar slants at 5°C.

5. Liquid Culture and Crude Enzyme Preparation

Active bacteria were inoculated in 10 ml of Bouillon medium and precultured on standing at 30°C for 3 to 5 days. Precultured broth (10 ml) was added into 250 or 2500 ml of Bouillon medium in a 500-ml flat bottom flask or a 5-*l* Erlenmeyer flask, and the broth was incubated on standing at 30°C for 9 days with occasional stirring. The bacterial growth was followed by measuring the absorbance at 550 nm. Crude enzyme preparation was made up by centrifuging the broth at 8000 rpm at 0°C for 20 min, followed by dialysis against water in order to remove inhibitory substances; each 10 ml of the supernatant packed in a cellophane tube was dialyzed against 2 *l* of distilled water at 5°C for 24 hr with gentle stirring. This inner solution was employed as crude enzyme preparation.

6. Assay of Aryl Acylamidase Activity

AAN was used as a substrate, and AN released during incubation for 2.0 to 6.0 hr was determined by the diazotization and coupling method[25] as follows: One milliliter of the crude enzyme preparation was mixed with 1.0

ml of 0.1 M Na-phosphate buffer (pH 7.4) and 2.0 ml of 37 mM AAN in a test tube, and the reaction mixture was incubated at 25°C or 30°C for a definite time with reciprocal shaking. To the mixture, 1.0 ml of 1 N HCl, 4.2 ml of distilled water and 0.2 ml of 2% $NaNO_2$ were added. After standing for 5 min with occasional stirring, 0.2 ml of 10% sulfamic acid was added, and the mixture was left standing for 10 min with occasional stirring. After 0.4 ml of 1% NED was added, the mixture was allowed to stand for another 2.0 hr. Released AN was estimated colorimetrically by measuring the absorbance at 550 nm.

7. Localization of Enzyme Activity

Ten milliliters of liquid-cultured broth was centrifuged at 15,000 rpm at 0°C for 15 min. The precipitated cells were redispersed in 10 ml of distilled water and destructed 10 times for 1 min each at 38 kHz with an ultrasonication apparatus (Kaijo Denki, T-A 4280). The suspension was centrifuged again, and the precipitate was redispersed in 10 ml of distilled water. Enzyme activity in each supernatant and suspension was assayed as mentioned above.

8. Activation of Crude Enzymes

Ten milliliters of the crude enzyme preparation packed into a cellophane tube was immersed into 2 *l* of 18.5 mM AAN at 5°C for 96 hr with gentle stirring. The inner solution was dialyzed twice against 2 *l* of distilled water at 5°C for 12 hr each with gentle stirring in order to remove AAN and/or AN. It was later used as activated enzyme preparation.

RESULTS AND DISCUSSION

1. Isolation of Active Bacteria

In order to detect active bacterial colonies capable of producing aryl acylamidases, a multiple agar layers method was devised. The zone around active colonies turned purplish red. This method was found very convenient and advantageous for isolating active colonies producing enzymes extracellularly, although coloration by AN excreted from cells might be partly included.

Isolation was done by using Bouillon medium and minimal medium containing AAN as a sole carbon source. The detection ratios of active colonies against the total colonies are shown in Table 1. Although the number of total colonies was greater in the Bouillon medium than in the minimal medium, the detection ratios in the latter were approximately twice those in the former (*ca.* 13% vs. *ca.* 6%). Active bacteria were present in almost all the soils. Several colonies showing relatively high activity were stored on Bouillon medium slants at about 5°C.

2. Enzyme Excretion from Bacterial Cells

By employing the most active bacterium A-1, enzyme production and localization in a liquid culture were examined in Bouillon medium and minimal medium.

Table 1 Detection ratio of active bacterial colonies capable of hydrolyzing AAN as a substrate.

Soil	Active colonies/Total colonies (%)							
	Bouillon medium				Minimal medium[a)]			
	1	2	3	Av.	1	2	3	Av.
Paddy soil (1)	6.5	8.5	4.1	6.4	14.3	27.7	17.2	19.7
(2)	12.5	11.1	2.6	8.7	22.2	20.0	11.8	18.0
Upland soil (1)	2.5	0	11.1	4.5	6.3	11.8	5.5	7.9
(2)	7.4	5.3	0	4.2	7.4	14.3	4.2	8.6
Forest soil (1)	13.8	3.1	7.4	8.1	14.8	21.4	9.1	15.3
(2)	2.9	7.3	1.9	4.0	4.2	7.7	6.3	6.1
Total av.				6.0				12.6

a) Containing 1.0 ppm AAN as a sole carbon source and mineral salts.

Both bacterial growth and enzyme production were satisfactory in the Bouillon medium, while those in the minimal medium were poor. Enzyme activity in a cultured broth, however, increased remarkably by dialyzing the broth against water or phosphate buffer (pH 7.4) with or without mercaptoethanol, suggesting that one or another inhibitory substance might exist in the broth, although it was left unidentified. The activity reached maximum after dialyzing for 24 hr and decreased thereafter, as shown in Fig. 2. The activity before and after dialysis is shown in Table 2. The broth supernatant after dialysis against water was employed as crude enzyme preparation thereafter.

The bacterium A-1 was cultured in Bouillon medium. The bacterial growth in the broth and the activity of crude enzyme preparation are shown in Fig. 3. The enzyme was excreted from bacterial cells into the broth, and the activity reached nearly equilibrium on the 9th day although the bacterial growth declined.

Activity distribution in the broth supernatant and cells was examined in order to confirm the localization of enzyme(s); 69% of the activity existed in the supernatant and *ca.* 29% remained with the sonicated cell debris, as shown in Table 3. These results suggested that the enzyme might exist originally associated with the cell wall, although it was easily excreted from living cells and released from lysed cells.

3. *Properties of Enzyme from Bacterium A-1*

The A-1 was found to belong taxonomically to the Coryneform group of bacteria. The details will be presented in our next paper.

Crude enzyme preparation from bacterium A-1 was prepared by dialyzing a liquid-cultured broth against water and stored at about 5°C. Although an induction period in enzyme

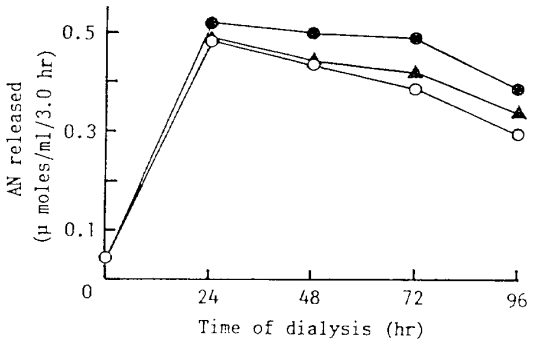

Fig. 2 Effect of dialysis with a cellophane tube on the enzyme activity in a liquid culture broth of bacterium A-1.

Dialyzed against distilled water (○), 0.02 M phosphate buffer (pH 7.4) (●), and 0.02 M phosphate buffer (pH 7.4) containing 10 mM mercaptoethanol (▲).

Table 2 Increase of the enzyme activity in a liquid culture broth[a] by dialyzing against water.

Active colony	Activity (Released AN, μmol/ml/3 hr)	
	Before dialysis	After dialysis
A-1	0.04	0.90
A-2	0.01	0.86
A-3	0.01	0.76
A-4	0.02	0.62

[a] Preculture broths were employed.

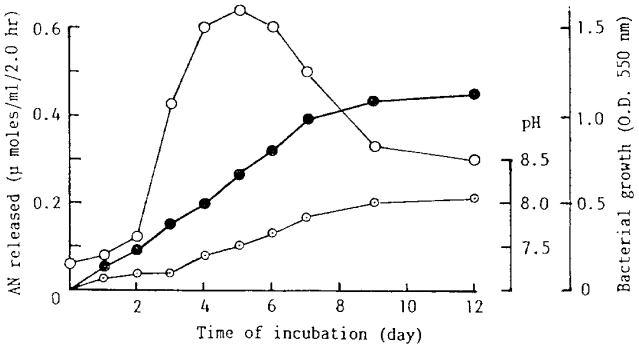

Fig. 3 Growth of bacterium A-1 and the enzyme activity excreted into broth in a liquid culture with Bouillon medium.

○: Growth of bacterium A-1. ⊙: pH of the cultured broth. ●: Enzyme activity (released AN, μmol/ml/2 hr) in the broth supernatant after dialysis against water.

Table 3 Localization of the enzyme in a liquid culture of bacterium A-1.

Part	Activity[a]	(Ratio, %)
Supernatant of broth	4.86	(69.0)
Bacterial cells	1.28	(31.0)
Sonicated cell debris	1.24	(28.7)
Extract from cells	0.10	(2.6)

[a] Released AN, μmol/ml/6 hr.

reaction was found to be 1 to 2 hr, there was a good linear correlation between the amount of added enzyme preparation and the amount of AN released during the 5-hr incubation.

The optimal pH and temperature in enzyme reaction were about 7.0 and 25°C, respectively. The enzyme(s) was stable in the pH range of 6 to 8.5, but unstable in incubation (1 hr) at over 30°C; about three-fourths of the activity was lost at 35°C. The effects of metal ions and EDTA on the activity are shown in Table 4. The enzyme(s) was not affected by Mn^{2+} but strongly inhibited by Co^{2+}, Mg^{2+} and Fe^{2+}. Among sulfhydryl inhibitors, Ag^{2+} was very effective, while Cu^{2+} only slightly. EDTA, a chelating agent, was also very inhibitory, suggesting that a certain metal ion might play an important role in enzyme reaction. Effects of cations such as Li^{+}, K^{+} and Ca^{2+} were not so remarkable.

The induction period in enzyme reaction did not disappear in optimal conditions, but it shortened when the enzyme(s) were preincubated with substrate AAN (9.25 mM) at room temperature for 6 hr. The activity remarkably increased.

This substrate activation was followed by preincubation of the enzyme(s) with a higher concentration of AAN at a lower temperature; the enzyme solution was packed with 18.5 mM (2500 ppm) AAN in a cellophane tube and immersed in 18.5 mM AAN solution at 5°C with gentle stirring, and the activity was assayed after dialysis against water to remove AAN. For comparison, enzyme solution without AAN was packed and immersed in a same

Table 4 Effect of metal ions and EDTA on the activity of crude enzyme A-1.

Chemical	Relative activity (%)	
	Concentration	
	0.25 mM	2.5 mM
None	100	100
$MnCl_2$	100	104
$ZnSO_4$	89	16
$CoCl_2$	3	2
$MgCl_2$	7	7
$FeCl_2$	8	5
$AgNO_3$	3	5
$CuCl_2$	89	85
KCl	100	122
$CaCl_2$	89	96
Li_2SO_4	85	78
EDTA	3	3

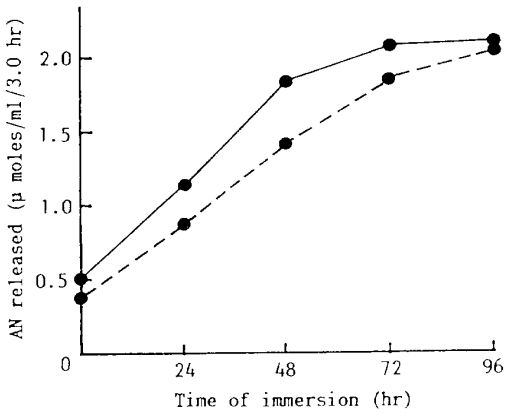

Fig. 4 Activation of the enzyme from bacterium A-1 with substrate AAN.

A crude enzyme preparation with (—●—) or without (– –●– –) 18.5 mM AAN was packed in a cellophane tube and immersed in 18.5 mM AAN solution at 5°C with gentle stirring. Activity was assayed after dialysis against water for removing AAN.

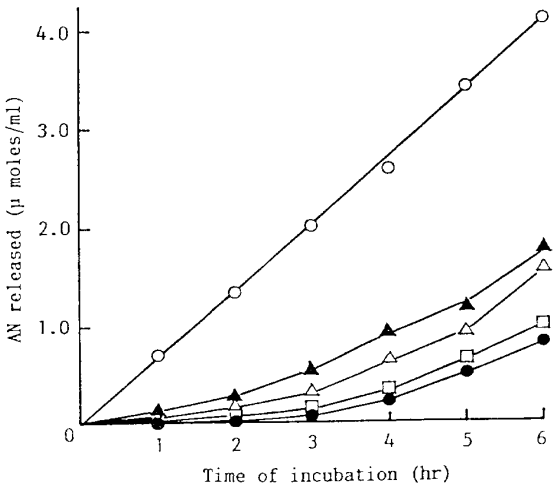

Fig. 5 Effect of substrate AAN and metabolites AN and/or Na-acetate on activity of the enzyme from bacterium A-1.

A crude enzyme preparation (●) was packed in a cellophane tube and immersed in 18.5 mM solution of AAN (○), AN (▲), Na-acetate (△) or both AN and Na-acetate (□) for 96 hr at 5°C with gentle stirring. Activity was assayed after dialysis against water for removing AAN.

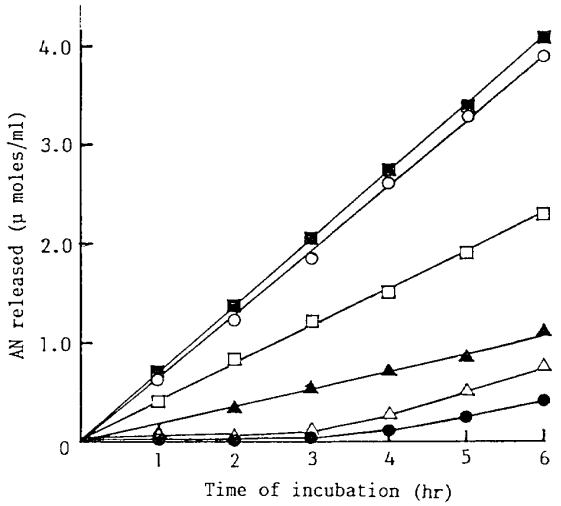

Fig. 6 Influence of AAN concentration on the activation of the enzyme from bacterium A-1.

A crude enzyme preparation packed in a cellophane tube was immersed in AAN solutions of 0 (●), 0.74 (△), 3.7 (▲), 7.4 (□), 18.5 (○) and 37.0 mM (■) for 76 hr with gentle stirring.

manner as above. The activity equivalently reached maximum after preincubation for 96 hr in both cases, as shown in Fig. 4, and so the latter method was employed thereafter. The enzyme activity remarkably increased after activation treatment, and the lag time disappeared completely, as shown in Fig. 5.

The effects of AN and/or Na-acetate (18.5 mM) as metabolites were minor (Fig. 5). Moreover, the effect of AAN concentration on activation reached nearly maximum at 18.5 mM, as shown in Fig. 6.

4. *Substrate Activation of Enzymes from Soil Bacteria*

Since it became of interest whether the above-mentioned substrate activation occurs generally for aryl acylamidases originated from soil bacteria, the active bacterial colonies were isolated again from various soils for activation test with crude enzyme preparations.

Table 5 Typical patterns for the activation with substrate AAN on the crude enzymes from active soil bacteria.

Type[c]	Isolates from Bouillon medium				Isolates from minimal medium[a]			
	Colony No.[d]	Activity[b] before(*a*)	Activity[b] after(*b*) activation	(*b*/*a*)[e]	Colony No.[d]	Activity[b] before(*a*)	Activity[b] after(*b*) activation	(*b*/*a*)[e]
I	3 P	0.07	6.25	91.0	7 P	0.18	6.40	35.5
	19 U	0.08	2.70	33.7	15 U	0.03	1.94	64.7
	30 F	0.09	3.10	34.5	28 F	0.09	3.74	41.6
I′	10 P	0.32	3.15	9.9	19 U	0.18	1.38	7.7
	18 U	0.25	2.10	8.4	17 U	0.27	2.04	7.6
	27 F	0.25	2.00	8.0	31 F	0.33	2.50	7.6
II	2 P	1.13	4.10	3.6	11 P	1.06	1.50	1.4
	8 P	1.80	2.10	1.2	12 U	0.76	1.80	2.4
	11 U	0.83	2.70	3.3	29 F	0.97	1.74	1.8
III	9 P	0.19	0.44	2.3	2 P	0.05	0.14	2.9
	14 U	0.19	0.60	3.2	5 P	0.14	0.31	2.2
	29 F	0.37	0.74	2.0	14 U	0.17	0.58	3.4

[a] Containing 0.1 ppm AAN and mineral salts.
[b] Released AN, μmol/ml/5.5 hr.
[c] I: Low initial activity & extremely high activation ratio, I′: Low initial activity & remarkably high activation ratio, II: Rather high initial activity & low activation ratio, III: Low initial activity & low activation ratio.
[d] P: Paddy soil, U: Upland soil, F: Forest soil.
[e] Activation ratio (-fold).

Table 6 Appearance ratios of different types of the activation with substrate AAN in the crude enzymes from active soil bacteria.

Medium for isolation of bacteria	Type of the activation			
	I	I′	II	III
Bouillon medium	38	22	13	27%
Minimal medium	17	25	25	33
Parameters for the type classification				
Initial activity[a]	—	—	>0.5	<0.5
Activation ratio[b]	>10	10–5	<5	<5

[a] Released AN, μmol/ml/5.5 hr.
[b] -fold.

All enzymes were activated more or less by AAN, and the activation patterns were classified into four types as shown in Table 5. Type I was characterized by bacteria excreting enzymes with a low initial activity and an extremely high activation ratio and type I′, with a low initial activity and a remarkably high activation ratio. Type II belonged to bacteria excreting enzymes with a fairly high initial activity and a low activation ratio and type III, low in both parameters.

Appearance ratios of each type of bacterial colonies are in Table 6, in which tentative parameter values for the classification are shown. At least, enzymes which belong to types I and I′ seemed to be activated by AAN. Total appearance ratios of types I and I′ reached 60% among isolates with the Bouillon medium and 42% among ones with the minimal medium.

Our study showed: a large number of bacteria extracellularly producing aryl acylamidases similar to those from bacterium A-1 were present in soil; in other words, substrate AAN generally activated extracellular enzymes from soil bacteria. The mechanism of activation may be the induced-fit of the enzymes to the substrate.

REFERENCES

1) R. G. Burns: *Soil Biol. Biochem.* **14**, 423 (1982)
2) T. Nakamura, K. Mochida, Y. Ozoe, S. Ukawa, M. Sakai & S. Mitugi: *J. Pesticide Sci.* **15**, 593 (1990)
3) J. Skujins: *Crit. Rev. Microbiol.* **4**, 383 (1976)
4) R. G. Burns & J. A. Edwards: *Pestic. Sci.* **11**, 506 (1980)
5) P. C. Kearney: *J. Agric. Food Chem.* **13**, 561 (1965)
6) P. C. Kearney & D. D. Kaufman: *Science* **147**, 740 (1965)
7) P. R. Wallnöfer & J. Bader; *Appl. Microbiol.* **19**, 714 (1970)
8) G. Engelhardt, P. R. Wallnöfer & R. Plapp: *Appl. Microbiol.* **22**, 284 (1971)
9) G. Engelhardt, P. R. Wallnöfer & R. Plapp: *Appl. Microbiol.* **26**, 709 (1973)
10) D. D. Kaufman & J. Blake: *Soil Biol. Biochem.* **5**, 297 (1973)
11) J. Alt & K. Krisch: *J. Gen. Microbiol.* **87**, 260 (1975)
12) J. Alt, E. Heymann & K. Krisch: *Eur. J. Biochem.* **53**, 357 (1975)
13) P. M. Hammond, C. P. Price & M. D. Scawen: *Eur. J. Biochem.* **132**, 651 (1983)
14) D. Vega, J. Bastide & C. Coste: *Soil Biol. Biochem.* **17**, 541 (1985)
15) J. L. Marty, T. Khafif, D. Vega & J. Bastide: *Soil Biol. Biochem.* **18**, 649 (1986)
16) J. L. Marty & J. Vouges: *Agric. Biol. Chem.* **51**, 3287 (1987)
17) N. E. Sharabi & L. M. Bordeleau: *Appl. Microbiol.* **18**, 369 (1969)
18) R. P. Lanzilotta & D. Pramer: *Appl. Microbiol.* **19**, 301 (1970)
19) R. P. Lanzilotta & D. Pramer: *Appl. Microbiol.* **19**, 307 (1970)
20) S. J. L. Wright & A. Forey: *Soil Biol. Biochem.* **4**, 207 (1972)
21) D. D. Kaufman & J. Blake: *Soil Biol. Biochem.* **5**, 297 (1973)
22) J. Blake & D. D. Kaufman: *Pestic. Biochem. Physiol.* **5**, 305 (1975)
23) P. R. Wallnöfer, G. Tillmanns & G. Engelhardt: *Pestic. Biochem. Physiol.* **7**, 481 (1977)
24) A. Hiramatsu, S. Yasumoto, O. Komada & T. Akatsuka: *Agric. Biol. Chem.* **46**, 1751 (1982)
25) T. Nakamura: *J. Agric. Chem. Soc. Jpn.* **39**, 227 (1965) (in Japanese)

要　　約

Aryl acylamidase 産生土壌細菌の分離および産生菌体外酵素の性質

中村利家，持田和男，李　文新，尾添嘉久

Aryl acylamidases を産生する土壌細菌を，まずコロニー形成培地上に基質 acetanilide (AAN) を含む寒天層を重ねて培養し，次いで生成 aniline (AN) の反応・

呈色用試薬類を含む寒天層を逐次重層する簡便法で分離した．活性を示すコロニーの検出率は，ブイヨン培地では6%であったが，炭素源としてANNのみを含む最少培地では13%であった．最大活性を示したA-1菌の液体培養で，活性の69%が培地中に存在し，29%が破壊菌体膜中に残ることを確かめた．その菌体外酵素の性質を検討する過程で，基質ANNによる前処理で活性が著しく増大されることがわかった．また，ブイヨンおよび最少培地で再分離した活性細菌のそれぞれ60および42%からの菌体外酵素がAAN前処理で顕著な活性増大を示した．土壌細菌由来の aryl acylamidases では，この基質活性化はかなり一般的な現象と思われた．

* 土壌 aryl acylamidases に関する研究（第1報）