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Production of (*S*,*S*)-Ethylenediamine-*N*,*N*′-disuccinic Acid from Ethylenediamine and Fumaric Acid by Bacteria.

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We tried to produce (*S*,*S*)-ethylenediamine-*N*,*N*′-disuccinic acid [(*S*,*S*)-EDDS] with the aid of microorganisms that can degrade the compound. A reaction mixture consisting of ethylenediamine and fumaric acid was incubated with the bacteria isolated from soil and sludge, and we found that several kinds of bacteria such as *Sphingomonas, Brevundimonas, Pseudomonas*, and *Acidovorax* produced (*S*,*S*)-EDDS. The optimum pH and the temperature for the production was pH 7.5 and 35°C for *Acidovorax* sp. TNT149 and the bacterial cells produced 90 mmoles of (*S*,*S*)-EDDS from 200 mmoles of ethylenediamine and 350 mmoles of fumaric acid under these reaction condition.

Key words: ethylenediamine-*N*,*N*′-disuccinic acid; (*S*,*S*)-EDDS; *Acidovorax* sp.; fumaric acid; ethylenediamine

Chelating agents are used in a variety of industrial fields, such as for producing pulp, textiles, cosmetics, photographic supplies, and detergents. Ethylenediaminetetraacetic acid (EDTA) is a chelating agent that is most extensively used. But because of its resistance to biodegradation, EDTA might remain in the environment for a long time.[1-6] Recently, Ginkel *et al.* showed that EDTA was biodegraded by activated sludge at a high rate when the treatment facility was operated under alkaline conditions.[7] But the operation of a water treatment facility at high pH might be restricted. Therefore the development of a biodegradable chelating agent alternative to EDTA is still expected.

We found and showed in a previous paper that the (*S*,*S*)-isomer of EDDS was biodegradable but its (*R*,*R*)-isomer was not.[8] A mixture of (*R*,*R*)-, (*S*,*S*)-, (*R*,*S*)-EDDS isomers (mixture EDDS) with the ratio of 1:1:2 could be synthesized chemically from ethylenediamine and maleic acid,[9] while the single enantiomer (*S*,*S*)-EDDS could be prepared from 1,2-dibromoethane and L-aspartic acid.[10]

Nishikiori *et al.* reported previously that 1.7 g of (*S*,*S*)-EDDS was obtained from 10 liters of culture broth of *Actinomycetes*, showing the possibility of microbial production of the compound.[11,12] Zwicker *et al.* demonstrated that *Amycolatopsis orientalis* produced 20 g/l of (*S*,*S*)-EDDS after 1,000 hours of cultivation.[13]

In this study, we will demonstrate a novel procedure to produce (*S*,*S*)-EDDS from ethylenediamine and fumaric acid with the aid of microorganisms.

Materials and Methods

Chemicals. A mixture of EDDS were prepared by the method of Kezenrian *et al.*[9] Tetra-*n*-butylammonium hydroxide was obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Yeast extracts were purchased from Difco laboratories (Detroit MI USA), Polypepton was purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan) and all other chemicals were of analytical grade and purchased from Kanto Chemicals Co., Inc. (Tokyo, Japan).

Isolation of microorganism. EDDS-degrading bacteria were isolated from soil and sludge on a agar medium with 0.2% mixture EDDS as a sole carbon source. The composition of the medium was as follows: 21.8 mg K_2HPO_4, 8.5 mg KH_2PO_4, 44.6 mg $Na_2HPO_4 \cdot 12H_2O$, 1.7 mg NH_4Cl, 22.5 mg $MgSO_4$, 116.3 mg $CaCl_2$, 0.3 mg $FeCl_3 \cdot 6H_2O$, 2 g mixture EDDS, and 20 g agar, in 1,000 ml distilled water at pH 7.0. Small amounts of water and soil samples and activated sludges were collected from various areas and their suspensions in 50 mM sterilized phosphate buffer pH 7.5, were prepared and equal amounts of them were spread out on the agar medium. Colonies that appeared on the agar plates were picked up at random after 10 days of cultivation at 30°C. The isolated bacteria were purified on GYP agar medium[7] with 0.2% mixture EDDS and 2% agar. The GYP medium consisted of 2.72 g KH_2PO_4, 4.26 g Na_2HPO_4, 2 g glucose, 1 g yeast extracts, 0.5 g polypepton, 1.7 g $MgSO_4 \cdot 7H_2O$, 2 g mixture EDDS in 1,000 ml distilled water supplemented with 5 ml of metal solution. The metal solution contained $MgCl_2 \cdot 6H_2O$ (81.32 g/l), $CaCl_2$ (7.77 g/l), $MnSO_4 \cdot 4H_2O$ (0.755 g/l), $FeCl_3 \cdot 6H_2O$ (0.127 g/l), and $ZnSO_4$ (0.052 g/l).

Cultivation of the isolated bacteria. The isolated bacteria were cultivated on the GYP agar medium with 0.2% mixture EDDS. A loop of the isolates on the GYP agar media were inoculated into 10 ml of the GYP liquid medium in a Monod's tube and shaken at 30°C for 3 days. The bacterial cells were collected by centrifugation at 3,500 rpm, for 30 min and washed twice with 50 mM

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phosphate buffer (pH 7.5). Cells were suspended in 1 ml of the same buffer solution, and provided for the following experiments.

Production of (S,S)-EDDS. One milliliter of the cell suspension was added to 1 ml of reaction mixture consisting of 400 mM ethylenediamine and 400 mM fumaric acid in 50 mM phosphate buffer, pH 7.5, and incubated with gentle shaking at 30°C for 24 hours. Cell density of the reaction mixtures ranged from 0.5 to 0.6 g dry cell/l, and 100 μl of 1 N HCl was added to stop the reaction. The supernatants of the reaction mixtures were prepared by centrifugation at 14,000 rpm, for 10 min, and were used for HPLC analysis.

HPLC analysis. After an addition of 0.1 ml of 50 mM $CuSO_4$ solution (pH 1.5) to 1 ml of the supernatant, EDDS concentration in the mixtures were measured by HPLC, using a Gulliver high pressure liquid chromatograph PU-980 (Japan Spectroscopic Co., Ltd., Tokyo, Japan). The running conditions were: column, Shodex ODSpak F-511 (Showa Denko Co., Ltd., Tokyo, Japan); column temperature, 40°C; eluent, 10 mM tetra-*n*-butylammonium hydroxide including 0.4 mM $CuSO_4$ in 50 mM phosphoric acid (pH 2.0–2.3); flow rate, 1.0 ml/min; detector wavelength, 254 nm.[8]

Results

Production of (S,S)-EDDS by the isolated bacteria

Three hundred and forty-one colonies grew on the agar medium containing the mixture of EDDS as a sole carbon source. They were cultured for 3 days in the GYP liquid medium with 0.2% mixture EDDS and the cells were used to examine (*S,S*)-EDDS production (See Materials and Methods). One hundred and thirty colonies produced a material showing the same retention time as that of (*S,S*)-EDDS [(*R,R*)-EDDS was also eluted at the same retention time]. Fig. 1-A shows a chromatogram of a standard solution composed of 1 mM mixture EDDS and 25 mM fumaric acid. The peak I, peak II, and peak III correspond to (*R,S*)-EDDS, a mixture of (*S,S*)- and (*R,R*)-EDDS, and fumaric acid, respectively.

Figure 1-B shows the results of the 24-h incubation in 200 mM of ethylenediamine and 200 mM of fumaric acid with the strain No.TNT149. Peak II and peak III showed the retention time that of (*S,S*)-EDDS [or (*R,R*)-EDDS] and fumaric acid, respectively. It was confirmed clearly by NMR analysis (data not shown) and HPLC analysis using an optical resolution column[8] that the material eluted as peak II is (*S,S*)-EDDS.

Identification of isolated strains that produce (S,S)-EDDS

Isolated bacteria that produce (*S,S*)-EDDS from ethylenediamine and fumaric acid were identified according to Bergey's manual of determinative bacteriology 9th edition[14] and Bergey's manual of systematic bacteriology.[15] Characteristics of the isolated strains of TN28, TN30, TN131, and TNT149 are summarized in Table 1 and were identified taxonomically as the genera *Sphingomonas, Brevundimonas, Pseudomonas*, and *Acidovorax*, respectively. (*S,S*)-EDDS productivities of these bacteria are presented in Table 2.

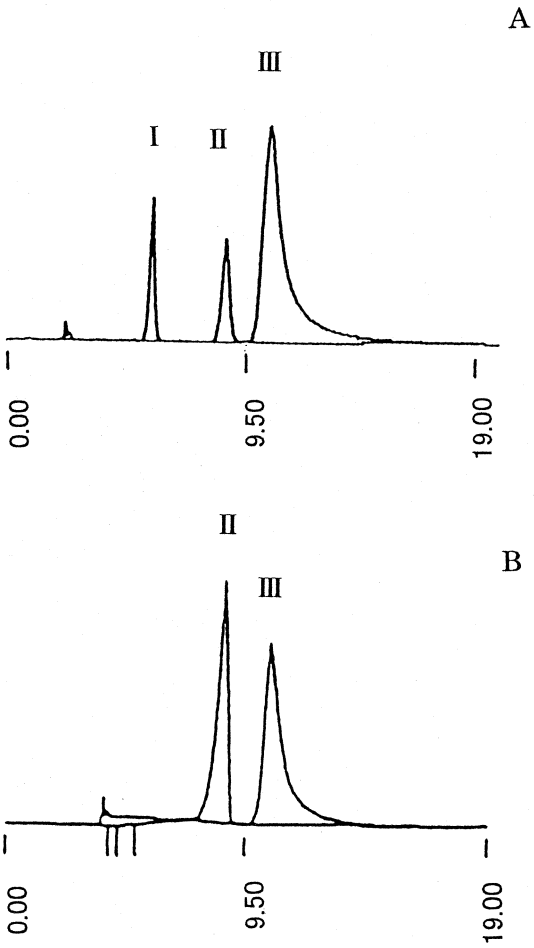

Fig. 1. HPLC Chromatograms of Mixture EDDS, Fumaric Acid, and the Reaction Mixture.

A: Elution profile of standard 1 mM mixture EDDS and 25 mM fumaric acid. (*R,S*)-EDDS, (*S,S*)-and (*R,R*)-EDDS and fumaric acid were detected as peak I, peak II, peak III, respectively.

B: Production of (*S,S*)-EDDS or (*R,R*)-EDDS in the reaction mixture. The reaction mixture was incubated with the strain TNT 149 for 24 hours. (See Materials and Methods)

(S,S)-EDDS production by Acidovorax sp TNT149

The consumption of fumaric acid, and (*S,S*)-EDDS production is shown in Fig. 2. After 24 h of incubation 159 mM of fumaric acid was consumed and 71 mM of EDDS appeared. The ratio of ethylenediamine to fumaric acid that used for EDDS synthesis was 1:2.2. The conversion ratio of fumaric acid to (*S,S*)-EDDS was calculated as 89% and no (*R,S*)-isomer was produced under these reaction conditions. The bacterial cell density was not changed during the incubation. The bacterial cells produced (*S,S*)-EDDS at 20.7 g/l and the value approximately corresponded to 76 g of (*S,S*)-EDDS per 1 g of the bacterial dry cells.

A higher concentration of the product was obtained when a higher initial concentration of fumaric acid was present in the reaction mixture (data not shown). For ex-

Table 1. Characteristics of Strains TN28, TN30, TN131, and TNT149

	Isolated bacterial strain			
	TN28	TN30	TN131	TNT149
Morphology	bacilliform	bacilliform	bacilliform	bacilliform
Gram staining	−	−	−	−
Spore	−	−	−	−
Morbility	+	+	+	−
Flagella	single polar	single polar	single polar	
Require for free oxygen	aerobic	aerobic	aerobic	aerobic
Oxidase	+	+	+	+
Catalase	+	+	+	+
OF test	−	−	−	o (slow)
Color of colony	yellow	NP[1)]	yellow	NP[1)]
Formation of fluorescent pigment	−	−	+	
Carotenoid pigment				−
Accumulation of PHB		+		+
Growth at 40°C				−
Cleavage of protocatenuate			*meta* form	
Quinone system	Q-10	Q-10	Q-9	Q-8
Reduction of nitrate	−	+	+	−
Indole formation	−	−	−	
Fermentation of glucose	−	−	−	
Arginine dehydrolase	−	−	−	
Urea decomposition	−	−	−	
Esculin decomposition	+	−	−	
Gelatin liquefaction	−	−	−	−
Starch decomposition				−
PNPG	−	−	−	
Utilization				
Glucose	+	−	−	+
Fructose				+
Maltose	+	−	−	
L-Arabinose	−	−	−	+
Sucrose				−
Fucose				−
D-Mannose	+	−	−	
D-Mannitol	−	−	−	
N-Acetyl-D-glucosamine	+	−	−	
Potassium gluconate	−	+	−	
Sodium malonate				−
Sodium glycolate				+
p-Hydroxybenzoic acid				+
2-ketogluconic acid				−
n-Capric acid	−	−	+	
Adipic acid	−	+	−	−
dl-Malic acid	+	−	+	
Sodium citrate	−	+	+	
Phenyl acetate	−	−	−	
Ethanol				−
Testosterone				−
GC content (mol%)				65
	Sphingomonas	*Brevundimonas*	*Pseudomonas*	*Acidovorax*

[1)] NP: no characteristic color.

Table 2. *S,S*-EDDS Productivities of Isolated Strains TN28, TN30, TN131, and TNT149

Bacterial cells were incubated in a reaction mixture composed of 200 mM ethylenediamine and 200 mM fumaric acid in 50 mM phosphate buffer (pH 7.5) at 30°C for 24 hours (Materials and Methods).

Bacterial strain	Concentration of *S,S*-EDDS produced (mM)
Sphingomonas sp. TN28	1.7
Brevundimonas sp. TN30	1.1
Pseudomonas sp. TN131	0.3
Acidovorax sp. TNT149	71.3

ample, 90 mM (26.3 g/l) of (*S,S*)-EDDS was produced from 350 mM of fumaric acid with a fixed ethylenediamine concentration at 200 mM during the 24 h of incubation. The conversion ratio of fumaric acid to EDDS was 75%. This observation shows that the equilibrium of the reaction shifted in the direction of EDDS synthesis, if higher concentration of substrates were used. Further productivity of (*S,S*)-EDDS at higher concentrations of the substrate was not tested, because of the low solubility of fumaric acid. (*S,S*)-EDDS was not produced when

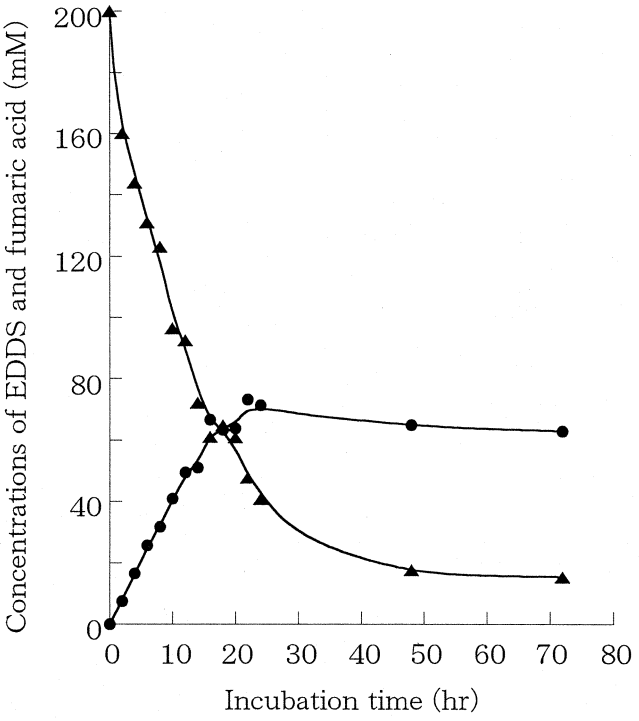

Fig. 2. Course of EDDS Production and Fumaric Acid Consumption.

Strain TNT149 was incubated with the reaction mixture containing 200 mM ethylenediamine and 200 mM fumaric acid at 30°C, pH 7.0. The concentrations of EDDS (●) and fumaric acid (▲) were followed by HPLC.

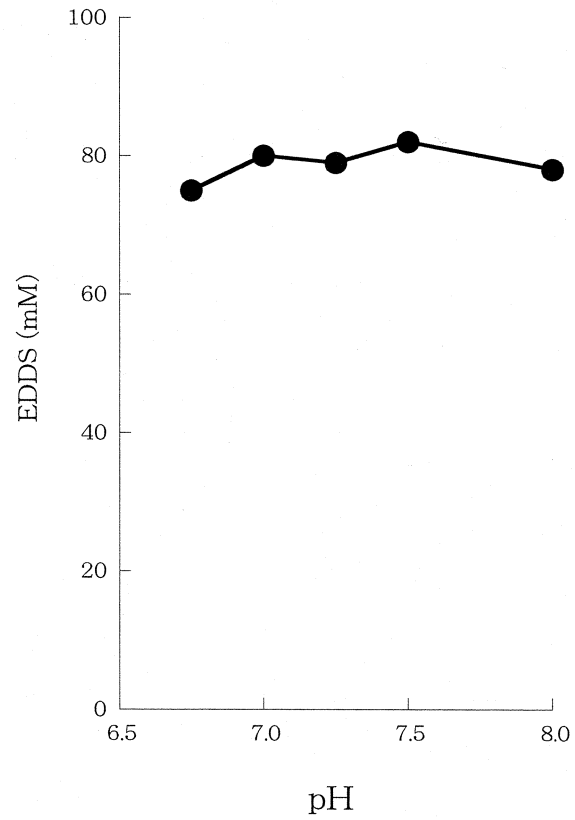

Fig. 3. Effects of pH on the EDDS Production.

Strain TNT149 was incubated in the reaction mixture for 24 hours with various pH at 30°C.

maleic acid was used as the substrate instead of fumaric acid.

Optimum temperature and pH for the production of (S,S)-EDDS

The optimum conditions for (*S*,*S*)-EDDS production by *Acidovorax* sp TNT149 were then investigated. The strain was cultivated in GYP liquid medium and the cells were harvested and washed as was shown in Materials and Methods. The cells were incubated under various reaction conditions with the pH at 6.5–8.0 and the temperatures at 25, 30, 35, and 40°C. Only a small difference of the productivity was observed in the pH range tested (Fig. 3). The maximum production of (*S*,*S*)-EDDS was obtained at 35°C (Fig. 4).

Discussion

Resting cells of EDDS-degrading bacteria catalyzed the synthesis of (*S*,*S*)-EDDS from ethylenediamine and fumaric acid (Fig. 5) in a reversible and stereo-specific manner.

We expected to obtain the (*S*,*S*)-isomer as a major product because the EDDS-degrading bacteria decomposed the (*S*,*S*)-isomer preferably to other isomers. We also found that the isolates belonging to several genera produced (*S*,*S*)-isomer with high optical purity.

One hundred and thirty strains among 341 bacteria that grew on EDDS as a sole carbon source, produced (*S*,*S*)-EDDS, having high optical purity. The absence of

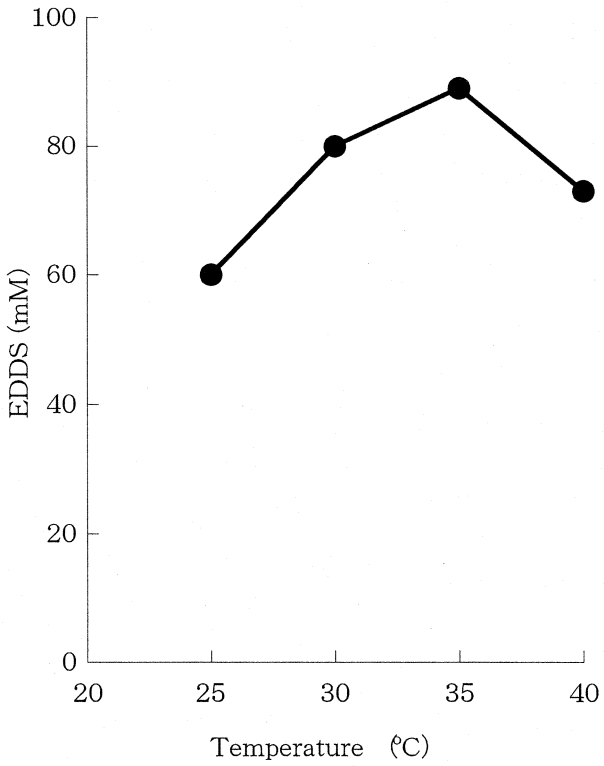

Fig. 4. Effects of Temperature on the EDDS Production.

Strain TNT149 was incubated in the reaction mixture at pH 7.5, for 24 hours.

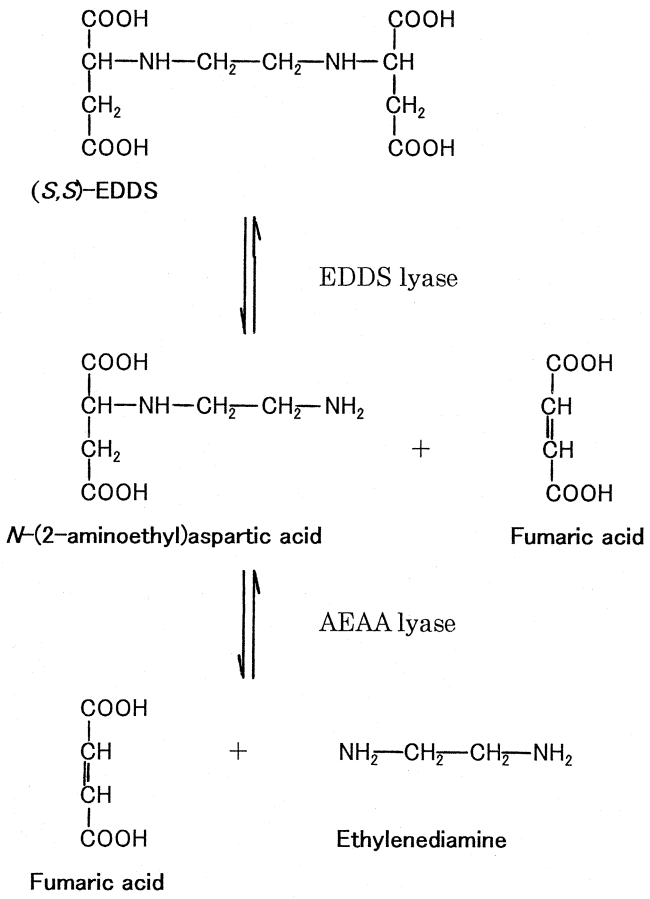

Fig. 5. Assumed Route of (*S,S*)-EDDS Formation by EDDS Degrading Bacteria.

(*S,S*)-EDDS productivity of the strains which also have ability to grow on EDDS might be attributed to the presence of an irreversible enzyme, lack of a lyase catalyzing cleavage of *N*-(2-aminoethyl) aspartic acid to ethylenediamine and fumaric acid, or lack of cell surface permeability to EDDS.

One of these assumptions is supported by the facts reported by M. Witschel *et al.*, that a purified (*S,S*)-EDDS lyase catalyzed (*S,S*)-EDDS synthesis from *N*-(2-aminoethyl)aspartic acid and fumaric acid, but did not catalyze the formation of EDDS from ethylenediamine and fumaric acid.[16]

References

1) Belley, R. T., Lauff, J. J., and Goodhue, C. T., Degradation of ethylenediaminetetraacetic acid by microbial populations from an aerated lagoon. *Appl. Microbiol.*, **29**, 787–794 (1975).
2) Lauff, J. J., Steele, D. B., Coodgan, L. A., and Breitfeller, J. M., Degradation of the ferric chelate of EDTA by a pure culture of an *Agrobacterium sp. Appl.Environ.Microbiol.*, **56**, 3346–3353 (1990).
3) Nortemann, B., Total degradation of EDTA by mixed culture and a bacterial isolate. *Appl. Environ. Microbiol.*, **58**, 671–676 (1992).
4) Egli, T., (An)aerobic breakdown of chelating agents used in household detergents. *Microbiol. Sci.*, **5**, 36–41 (1988).
5) Tiedje, J. M., Influence of environmental parameters on EDTA biodegradation in soils and sediments. *J. Environ. Qual.*, **6**, 21–26 (1977).
6) Thom, N. S., and Agg, R. A., The breakdown of synthetic organic compounds in biological processes. *Proc. R. Soc. Lond. B.*, **189**, 347–357 (1975).
7) Ginkel, C. G., Vandenbroucke, K. L., and Stroo, C. A., Biological removal of EDTA in conventional activated-sludge plants operated under alkaline conditions. *Bioresource Technology*, **59**, 151–155 (1997).
8) Takahashi, R., Fujimoto, N., Suzuki, M., and Endo, T., Biodegradabilities of ethylenediamine-*N,N'*-disuccinic acid (EDDS) and other chelating agents. *Biosci. Biotechnol. Biochem.*, **61**, 1957–1959 (1997).
9) Kezerian, C., and Ramsey, W. M., Bis-adduction products and methods of preparing same. U.S. Patent 3158635 (Nov. 24, 1964).
10) Neal, J. A., and Rose, N. J., Stereospecific ligands and their complexes, I. A cobalt (III) complex of ethylenediaminedisuccinic acid, *Inorg. Chem.*, **7**, 2405–2412 (1968).
11) Nishikiori, T. Okuyama, A., Naganawa, H., Takita, T., Hamada, M., Takeuchi, T., Aoyagi, T., and Umezawa, H., Production by actinomycetes of (*S,S*)-*N,N*-ethylenediaminedisuccinic acid, an inhibitor of phospholipase C. *J. Antibiotics*, **37**, 426–427 (1984).
12) Goodfellow, M., Brow, A. B., Cai, J., Chun, J. J., and Collins, M. D., *Amycolatopsis japonicum* sp. nov., an actinomycete producing (*S,S*)-*N,N'*-ethylenediamine-disuccinic acid. *System. Appl. Microbiol.*, **20**, 78–74 (1997).
13) Zwicker, N., Theobald, U., Zahner, H., and Fiedler, H-P., Optimization of fermentation conditions for the production of ethylene-diamine-disuccinic acid by *Amycolatopsis orientalis. J. Ind. Microbiol. Biotech.*, **19**, 280–285 (1997).
14) Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T., and Williams, S. T., *Bergey's Manual of Determinative Bacteriology*, Ninth Edition, Williams & Wilkins, Baltimore, MD 21202, U.S.A. (1994).
15) Krieg, N. R., and Holt, J. G., *Bergey's Manual of Systematic Bacteriology*, Vol. 1, Williams & Wilkins, Baltimore, MD 21202, U.S.A. (1984).
16) Witschel, M., and Egli, T., Purification and characterization of a lyase from EDTA-degrading bacterial strain DSM 9103 that catalyzes the splitting of [*S,S*]-ethylenediaminedisuccinate, a structural isomer of EDTA. *Biodegradation*, **8**, 419–428 (1998).