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Degradation of Ferric EDTA by *Burkhol cepacia*

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Abstract

EDTA, the target compound of this study from the effluent of secondary biotreatment units, can be biodegraded by special microorganisms. So far, there are three species of microorganisms—*Agrobacterium*, Gram-negative *BNCI*, and *DSM9103*—that can degrade EDTA and are published in the literature. We have successfully isolated a bacterial strain that can degrade EDTA. It was identified as *Burkhol cepacia*, an aerobic species, elliptically shaped with a length of 5–15 µm. The growth medium contains 1000 mg/L of ferric-EDTA as carbon source, 750 mg/L of $(NH_4)_2SO_4$ + $(NH_2)_2CO$ as nitrogen source, and 1000 mg/L of KH_2PO_4 as phosphorus source, and mineral factors such as Fe and Mg. Incubated at pH 7.0, 30°C, and 150 rpm on a shaker for 15 d, the average specific growth rate of this microbe is 0.135 d^{-1}, which shows that the respective degradation efficiency of Fe-EDTA and Cu-EDTA is 90 and 75% individually.

Index Entries: EDTA; biodegradation; *Burkhol cepacia*.

Introduction

EDTA, widely used in industries and households, is an aminopolycarboxylic acid compound *(1)*. It can chelate with metal ions to form a stable and soluble chelate compound. It is used in areas such as industrial washing, detergent production, photo industries, medical, textile industries, and paper mills. During 1987–1988, Western European countries used 28,000 t of EDTA. In 1994, Western European countries, the United States, and Japan produced 169,000 t of EDTA *(2,3)*, diethylenetriaminepentaacetate, and nitrilotriacetate, but mostly EDTA. EDTA causes radioactive

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elements and heavy metals to dissolve in drinking water, which harms human health, or enters the human body by bioaccumulation through the food chain in the environment *(4–8)*.

Because of the soluble and nonvolatile properties of EDTA, and its low biodegradability, high levels of it are found in wastewater *(9,10)*. The environmental pollution problem caused by EDTA has become a serious concern *(11)*. It can be degraded by physical, chemical, or biologic means, such as electrodialysis, photolysis, thermolysis, adsorption by activated sludge, and biodegradation. Obviously biodegradation is more economical and has less of an impact on the environment *(12)*.

A report by Van Ginkel et al. *(8)* showed that there are only three species—*Agrobacterium*, Gram-negative *BNCI*, and *DSM9103*—that can degrade EDTA. However, EDTA degradation is slow in the environment *(13,14)*. EDTA can be degraded by a special bacterial strain, with the proper nutrition and environment *(2,15–17)*. Effectively solving the high chemical oxygen demand (COD) problem caused by EDTA effluent from biologic treatment, as well as isolating and screening the EDTA-degrading strain locally, is the focus of the present study.

Materials and Methods

Chemicals and Basal Medium

Sodium ferric-EDTA, disodium copper-EDTA, NH_2CONH_2, $CoCl_2$, $NaMoO_4$, and $FeSO_4 \cdot 7H_2O$ were purchased from Katayama. All of these chemicals are reagent grade.

All growth media contained mineral salt in the following concentrations: 1000 mg/L of ferric-EDTA, 250 mg/L of CH_3COOK, 2000 mg/L of $(NH_2)_2CO+(NH_4)_2SO_4$ (ratio of 1:1), 1000 mg/L of KH_2PO_4, 100 mg/L of NaCl, 25 mg/L of $CaCl_2$, 20 mg/L of $FeSO_4 \cdot 7H_2O$, 100 mg/L of $MgSO_4 \cdot 7H_2O$, 10 mg/L of $ZnSO_4 \cdot 6H_2O$, 0.5 mg/L of $NaMoO_4 \cdot 2H_2O$. Ferric-EDTA was used as major carbon source and CH_3COOK as cometabolic carbon source. Urea and ammonium sulfate were used as the major nitrogen source.

Isolation and Screening of Microbial Strain

Samples of sludge were obtained from wastewater stream that had contained ferric-EDTA for a long period of time. The enrichment culture method was used to isolate and screen the EDTA-degrading strain. An enrichment medium of 200 mL was inoculated with 1 g of the sample and incubated at 30°C for 10–15 d. Growth from this initial enrichment was placed on an agar plate medium and incubated at 30°C until the growth was observed. To select colonies that used ferric-EDTA as the carbon source, colonies from these plates were repeatedly transferred and tested for growth on agar media. Five percent (v/v) of these cultures that are used as seed were transferred to liquid media contained in a flask. The liquid culture medium containing 1000 mg/L of ferric-EDTA as carbon source and

phosphate buffer in basal medium at pH 7.0 was incubated at 30°C on a shaker at 150 rpm. Sampling was performed every 12 h, and cell growth was monitored by a spectrophotometer at 660 nm (OD_{660}).

Strain Counting and Identification

After growth took place in ferric-EDTA medium, cells were harvested by centrifuging at 17,300*g* for 5 min, with a rotation speed of 12,000 rpm (Model CR22E; Hitachi, Japan). As an approximate measurement of growth, the $OD_{660\ nm}$ was measured with a spectrophotometer (Model UVIKON930; Kontron, Italy) after incubation. Strain identification was done by biochemical assay using an API 20 NE test kit (Biomerieux, Marcy l'Etoile, France).

Measurement of COD, Total Organic Carbon, and EDTA

High-performance liquid chromatography analysis was performed to determine EDTA concentration *(18)*. The column was 250 × 4.6 mm (Spherisorb C18). Sample peaks were separated by isocratic elution at 1.0 mL/min where 0.05 *M* sodium acetate was used as the mobile phase and buffer solution. The pH was adjusted to 4.5 by adding acetic acid. EDTA concentration was detected by ultraviolet spectrophotometer at 254 nm. COD and total organic carbon (TOC) were analyzed according to Standard Methods for the Examination of Water and Wastewater *(19)*. These were published by R.O.C. Environmental Protection Agency (COD Method, NIEA W515.53A; TOC Method, NIEA W530.50C).

Morphology of EDTA Degradation Strain

The morphology of the EDTA degradation strain YL-6 was observed by scanning electron microscope. The sample was pretreated in the following procedure. Samples containing EDTA degradation strain were mixed with 2.5% glutaraldehyde and fixed for 2.5 h at 4°C. The samples were centrifuged at 17,300*g* for 10 min. Then the supernatant fluid was decanted, and the samples were washed with 0.1 *M* phosphate buffer solution (pH 7.0) three times, 10 min each time. After flushing, samples were dehydrated with alcohol eight times. Each dehydration procedure lasted 10 min, and the alcohol concentration was 30, 50, 70, 90, 95, 100, 100, and 100%. After the entire procedure was finished, a few samples were dried to critical point. Then they were shadowed with gold containing 60% Au and 40% Pd for 90 s. Finally, the samples were observed by a scanning electron microscope (JEOL JSM-5410 LV).

Composition of Basal Medium and Growth Conditions

To determine the optimum growth conditions for the EDTA degradation strain, we modified the composition of the basal medium and growth conditions. Microbial growth and EDTA degradation efficiency were examined. The concentrations of carbon source, nitrogen salt (urea +

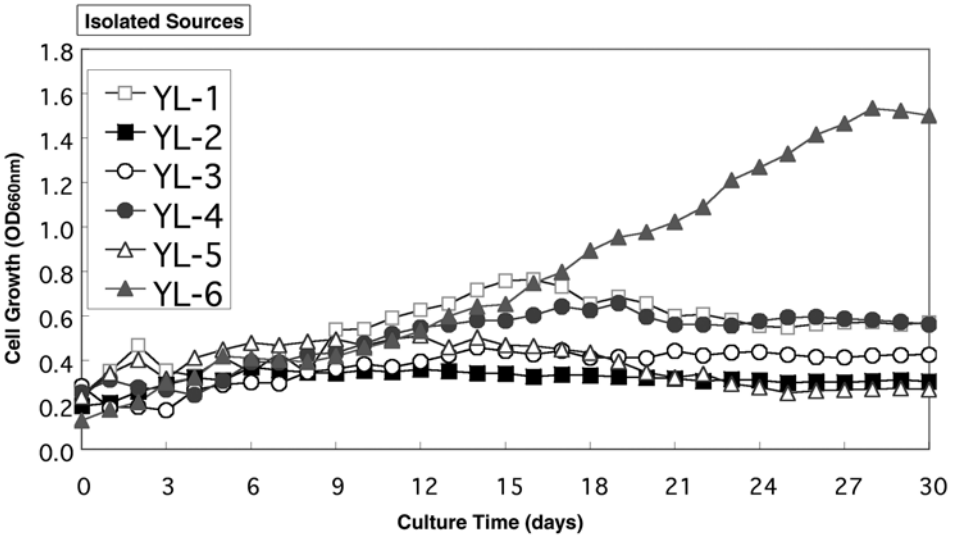

Fig. 1. Accumulation of different isolated sources in Fe-EDTA medium.

ammonium sulfate), and phosphate were changed. Growth conditions, pH, and temperature were also investigated.

Results and Discussion

Isolation and Screening of EDTA Degradation Strain

The isolated strain was obtained from the Taiwan environment. It was enriched by Fe-EDTA at pH 7.0, 30°C, and 150 rpm for 30 d, and the strain YL-6, which has the greatest degradation capability, was isolated.

The growth curve is shown in Fig. 1. After accumulation, purification, and isolation, strain YL-6 was incubated on a shaker running at 150 rpm at pH 7.0 and 30°C with 1000 mg/L of ferric-EDTA as the major carbon source. The growth curve is shown in Fig. 2. Clearly, the culture time was shortened from 28 d to 15 d, and the average specific growth rate was raised from 0.09 to 0.11 d^{-1}. In addition, COD decreased from the initial 684 to 249 mg/L. Obviously, the adaptation in the ferric-EDTA significantly promoted the strain growth rates and EDTA degradation efficiency.

Identification of Strain YL-6

Since the color of Gram staining was red, strain YL-6 was Gram negative. The oxidase test showed a dark-color colony, which means oxidase exists. The catalase test revealed O_2 generation, which proves catalase exists. Through Gram staining, the oxidase test, and the catalase test, YL-6 was found to be an aerobic species. The *B. cepacia* in Fig. 3, taken by a scanning electron microscope at ×1500, is elliptical and 5–15 μm in length. The isolated culture was identified as *B. cepacia* using the API 20 NE test kit (99% probability).

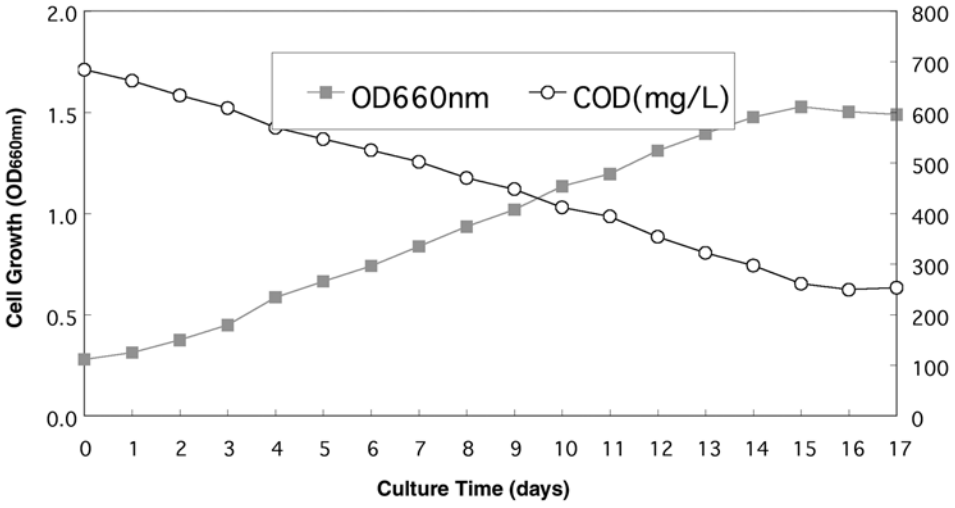

Fig. 2. Growth and COD degradation of YL-6 strain.

Fig. 3. Morphology of *B. cepacia* YL-6 by scanning electron microscope.

Composition of Culture Media and Growth Conditions

The isolated bacteria were cultivated in batch culture with ferric-EDTA media. EDTA is the major source of carbon, which is definitely needed for EDTA removal by strain YL-6. Ferric-EDTA as the major carbon source was added at 0, 500, 750, 1000, 1500, 2000, and 3000 mg/L in liquid media. The growth of *B. cepacia*, as measured by average specific growth rate (μ_a), in a shake flask with media at different carbon source concentrations at pH 7.0

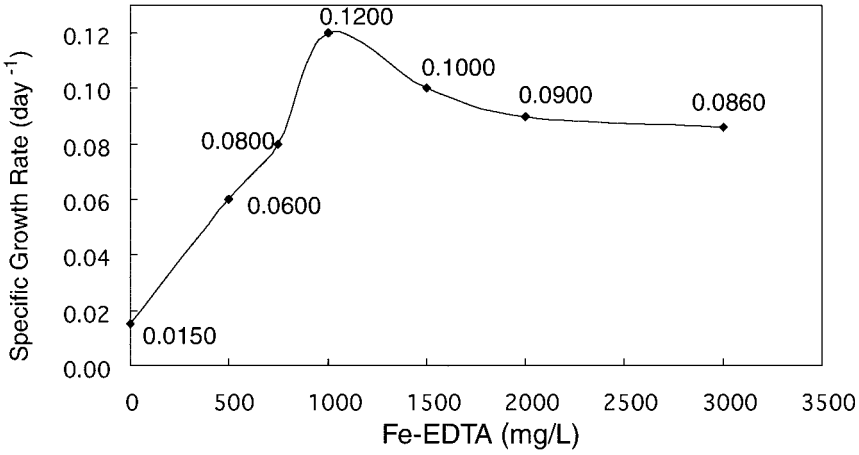

Fig. 4. Specific growth rate of YL-6 in media of different Fe-EDTA concentrations.

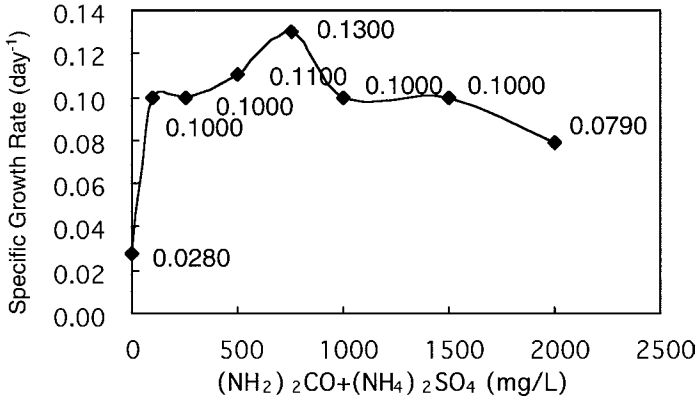

Fig. 5. Specific growth rate of YL-6 in media of different concentrations of nitrogen salt.

and 30°C, is illustrated in Fig. 4. The value of μ_a was 0.06–0.12 d^{-1}. The highest was 0.12 d^{-1}, when the addition of ferric-EDTA was 1000 mg/L. The addition of ferric-EDTA >1000 mg/L did not promote the value of μ_a. Hence, the optimum ferric-EDTA concentration was 1000 mg/L during the course of this experiment.

Urea and ammonium were used as the nitrogen source with the ratio of 1:1 for keeping the media solution at neutral. The media were prepared with different nitrogen source concentrations of 0, 100, 250, 500, 750, 1000, 1500, and 2000 mg/L, respectively, and then YL-6 was incubated at pH 7.0, 30°C, and 150 rpm on a shaker. Figure 5 presents the results of the experiment. At the beginning, without addition of nitrogen salt, the growth of *B. cepacia* was very low (μ_a = 0.028 d^{-1}). The value of μ_a was increased and reached a maximum value of 0.13 d^{-1}, when the addition of nitrogen salt was 750 mg/L. When the nitrogen salt concentration was >750 mg/L, the value of μ_a decreased slowly.

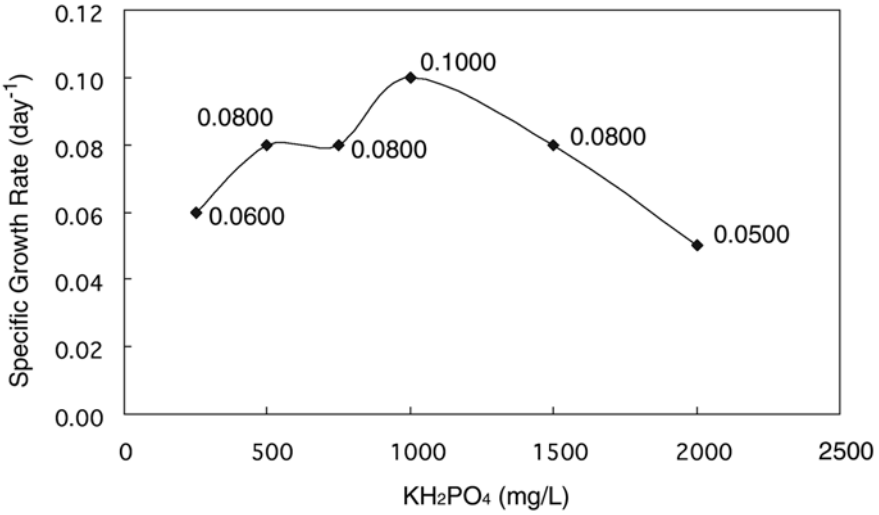

Fig. 6. Specific growth rate of YL-6 in media of different concentrations of phosphorous salt.

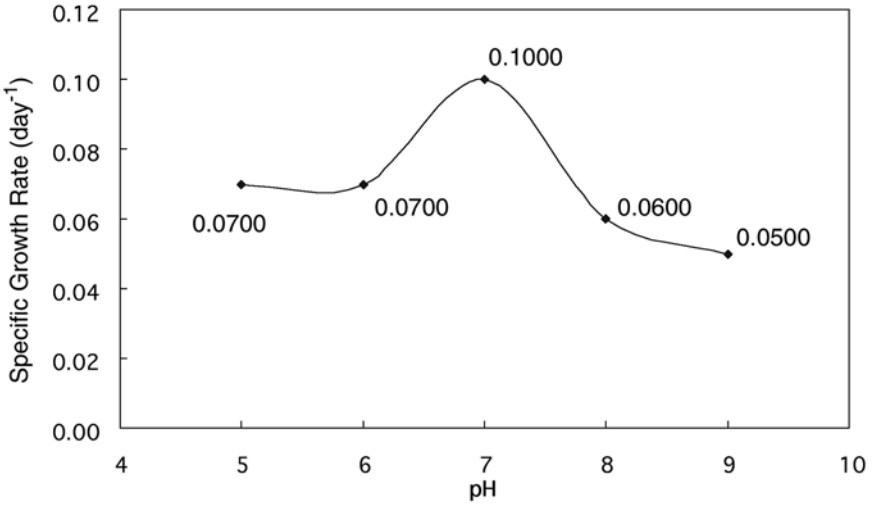

Fig. 7. Specific growth rate of YL-6 at different pHs.

The isolated YL-6 was added to the culture media containing 1000 mg/L of ferric-EDTA with different phosphorous concentrations from 0 to 2000 mg/L and incubated for 15 d at pH 7.0, 30°C, and 150 rpm on a shaker. The value of μ_a after incubation is shown in Fig. 6. The maximum value of μ_a was 0.10 d^{-1}, when the phosphorous concentration was 1000 mg/L. The value of μ_a decreased when the media contained >1000 mg/L of phosphorous salt.

Since pH variation is known to affect growth of a microorganism, a pH range of 5.0–9.0 was tested in order to investigate the impact on EDTA degradation. Figure 7 shows the μ_a value of *B. cepacia* at different

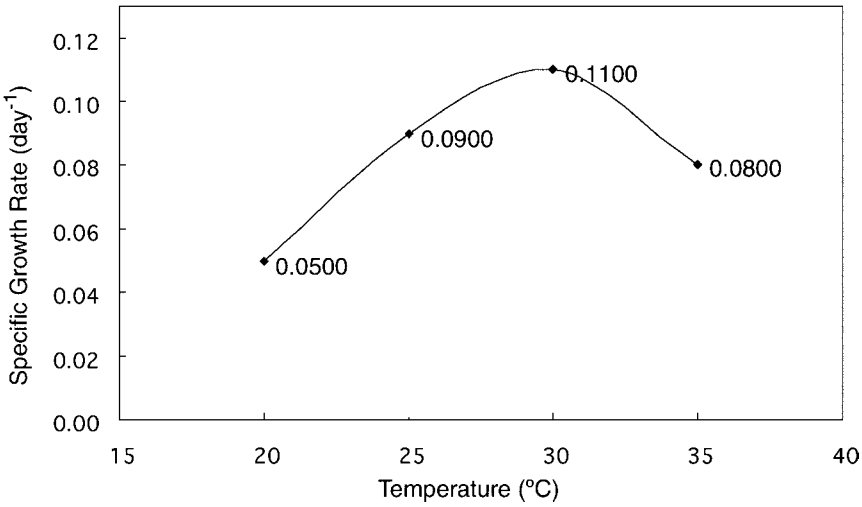

Fig. 8. Specific growth rate of YL-6 at different temperatures.

pHs. The value of μ_a was highest when the pH was 7.0. When the pH was >7.0, the growth rate decreased appreciably. Similar studies were performed for the growth temperature. The culture media contained 1000 mg/L of ferric-EDTA, 750 mg/L of nitrogen salt, and 1000 mg/L of phosphorous salt. Incubation was performed at pH 7.0 and 150 rpm on a shaker at different temperatures (20, 25, 30, and 35°C, respectively). Figure 8 shows the results of the experiment. When the temperature was controlled at 30°C, the maximum μ_a was 0.11 d^{-1}, which means that *B. cepacia* was more active at 30°C.

EDTA Degradation by B. cepacia

Ferric-EDTA Degradation Test

From the culture media composition and growth conditions test, we obtained information about growth conditions for *B. cepacia*. In this experiment, the culture medium was prepared with 1000 mg/L of ferric-EDTA as the carbon source, 750 mg/L of $(NH_2)_2CO$ + $(NH_4)_2SO_4$ as the nitrogen source, and 1000 mg/L of KH_2PO_4 as the phosphorous source, and then it was incubated at 30°C and pH 7.0 on a shaker running at 150 rpm. Figure 9 shows the growth of *B. cepacia* and the degradation rate of ferric-EDTA. The maximum $OD_{660\,nm}$ was 2.3 and the μ_a was 0.13 d^{-1} after 15 d of incubation. The concentration of EDTA was degraded from 977 to 95 mg/L. The degradation rate for EDTA was about 90%. At the same time, the COD and TOC of the solution had decreased by 77 and 67%, respectively.

Cu-EDTA Degradation Test

The mixed-culture Gram-negative *BNC1* biodegraded the metal-EDTA complexes slowly. The biodegradability order was shown to be Fe > Cu > Co > Ni > Cd *(12)*. Along with other reports, this shows that

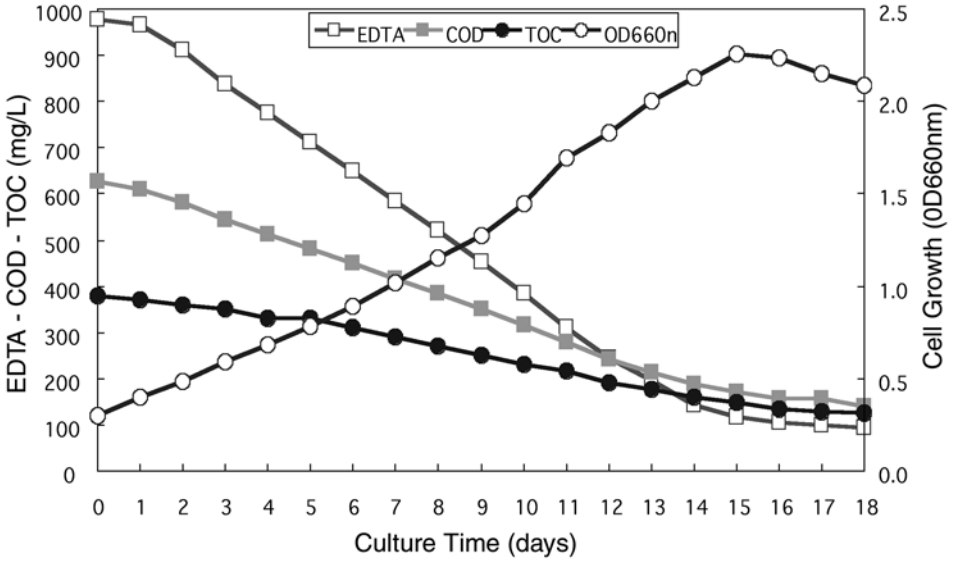

Fig. 9. Degradation of Fe-EDTA by *B. cepacia* YL-6.

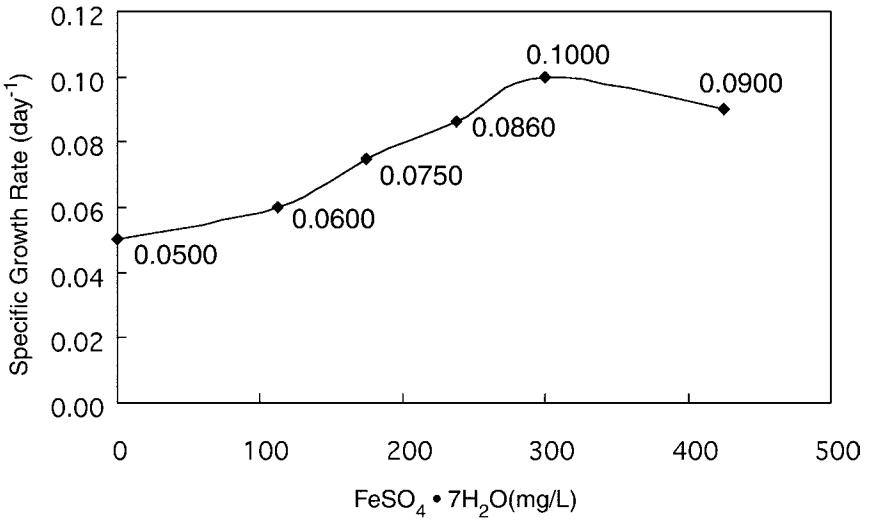

Fig. 10. Specific growth rate of YL-6 in Cu-EDTA media with different ferrous salt concentrations.

Fe-EDTA is the most readily biodegraded complex *(20,21)*. To investigate the growth conditions of *B. cepacia* with Cu-EDTA as the major carbon source, the 1000 mg/L of ferric-EDTA was replaced with 500 mg/L of Cu-EDTA and 0–425 mg/L of $FeSO_4 \cdot 7H_2O$. The culture medium was incubated at pH 7.0, 30°C, and 150 rpm on a shaker. Figure 10 shows the value of μ_a of *B. cepacia* at different ferrous concentrations in the 500 mg/L Cu-EDTA solution. The μ_a of *B. cepacia* with the addition of 300 mg/L of

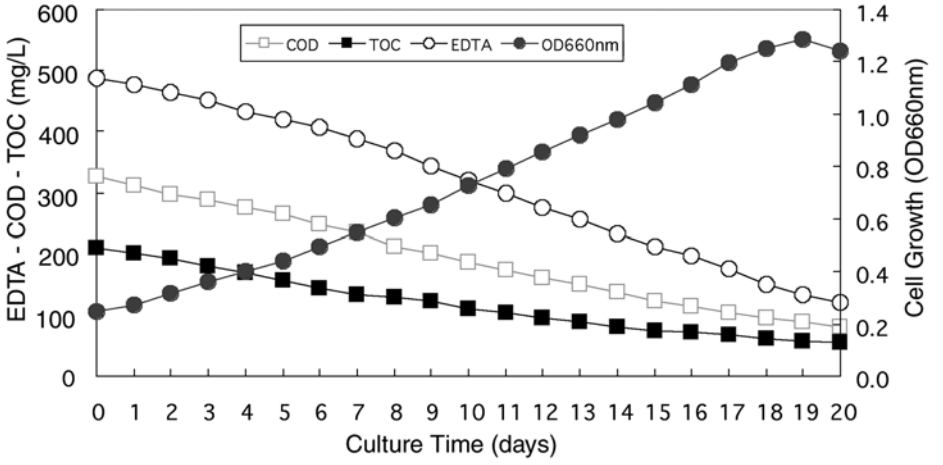

Fig. 11. Degradation of Cu-EDTA by *B. cepacia* YL-6.

$FeSO_4 \cdot 7H_2O$ was 0.10 d^{-1}, higher than that without the addition of ferrous salt. The value of μ_a of *B. cepacia* was obviously enhanced when ferrous salt was added to the Cu-EDTA solution to replace the copper ions. When the molar ratio of Cu^{+2} to Fe^{+2} was 1:1, *B. cepacia* showed the greatest growth.

In the incubation without ferrous salt addition, there was a 2 to 3 d lag phase. When ferrous salt was added, the lag phase disappeared. Figure 11 shows that after 20 d of incubation, the EDTA concentration decreased from the initial 487 to 121 mg/L; the degradation efficiency was about 75%. Similarly, COD removal was 75% (from 326 to 80 mg/L), whereas TOC removal was 64% (from 210 to 75 mg/L). The lower EDTA degradation rate resulted from chelation with copper ion, which has a lower biodegradability than ferric ions. Besides, the heavy metal inhibits the growth of *B. cepacia*.

Conclusion

This research aimed at isolating an EDTA degradation microorganism from the environment of Taiwan. We successfully isolated an EDTA-degrading bacterial strain that was identified as *B. cepacia* via an API 20 NE test kit. Through Gram staining, oxidase test, and catalase test, *B. cepacia* was found to be an aerobic species, elliptically shaped with a length of 5–15 μm. The optimal culture conditions of *B. cepacia* were determined to be 1000 mg/L of Fe-EDTA as the major carbon source, 750 mg/L of $(NH_2)_2CO$ + $(NH_4)_2SO_4$ as the major nitrogen source, and 1000 mg/L of KH_2PO_4 as the phosphorous source. Incubation was done at 30°C, pH 7.0, and the specific growth rate was 0.135 d^{-1}. After 15 d of incubation, *B. cepacia* degraded 90% of EDTA, 77% of COD, and 67% of TOC. For degradation testing of Cu-EDTA, 300 mg/L of $FeSO_4 \cdot 7H_2O$ was added to 500 mg/L of Cu-EDTA solution. It degraded 75% of EDTA, 75% of COD, and 64% of TOC. From the results, it is evident that the biodegradability of Fe-EDTA is higher than that of Cu-EDTA, which has also been reported by other researchers *(12)*.

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