Microbial Conversion of Petro-sulfur Compounds

Part II. Culture Conditions of Dibenzothiophene-Utilizing Bacteria*

By Shigeru NAKATANI, Toshio AKASAKI, Koki KODAMA**,
Yasuji MINODA** and Koichi YAMADA**

Central Research Institute of Electric Power Industry, Abiko, Chiba Prefecture

**Department of Agricultural Chemistry, Faculty of Agriculture,
The University of Tokyo

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In order to enhance the efficiency converting dibenzothiophene (DBT) into water-soluble compounds by DBT-utilizing bacteria, the following subjects were studied: comparison of activities of isolated strains, their mixed cultivation, DBT dosing, nitrogen sources, substrate concentration, pH, temperature, and oxygen supply. Light oil solution of DBT (5%) was used as substrate. The conversion efficiency was calculated by the amount of sulfur accumulated in the aqueous layer of culture broth.

The conversion ratio of 40% was attained by the mixed culture of *Pseudomonas abikonensis* and *Ps. jianii* under the following conditions; medium, DBT 4.6 g dissolved in 87.8 g of light oil, meat extract 4.0 g, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 9.5 g, KH_2PO_4 1.4 g, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 0.2 g, distilled water 1000 ml; pH, 6.9 to 7.3 (M/30 phosphate buffer); oxygen supply, 50 ml broth 500 ml flask, shake culture at 220 rpm; inoculum size, 10%; temperature, 28°C; and period of cultivation, 3 days.

As reported in the previous paper,¹⁾ six strains of bacteria capable of converting DBT into water-soluble forms were isolated. The work of isolation of DBT-utilizing bacteria was continued further, and three bacterial strains, DDE 27-2, DDE 27-3 and DDE 27-4, were newly obtained.

This report deals with the taxonomic studies and the culture conditions of the new isolates.

MATERIALS AND METHODS

Microorganisms. Three strains of bacteria DDE 27-2, DDE 27-3 and DDE 27-4, were used in addition to six strains of the DBT-utilizing bacteria reported in the previous paper (DDC 327, DDE 188, DDE 206,

Ps. abikonensis DDA 109, and *Ps. jianii* DDC 279 and DDE 27-1).

Cultivation. One to five ml of seed culture, having been precultured in submerged broth for 2 days, was inoculated into 50 ml of the medium. Incubation was carried out at 28°C on a rotary shaker (220 rpm, 7 cm dia.) or a reciprocal shaker (110

FIG. 1. Preparation of Sample for Sulfur Determination.

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1) K. Yamada, Y. Minoda, K. Kodama, S. Nakatani, and T. Akasaki, *Agr. Biol. Chem.*, **32**, 840 (1968).

strokes per min, 7 cm amplitude) for 3 to 10 days using 500 ml flask unless otherwise stated. The growth was measured by optical density at 660 m μ .

Determination of sulfur. The conversion efficiency was calculated by the amount of sulfur accumulated in the aqueous layer of culture broth. After cultivation broth was treated as shown in Fig. 1. One part of dried culture filtrate was combusted in a quartz combustion tube,²⁾ and sulfate resulted was determined by titration with barium perchlorate solution.³⁾

RESULTS AND DISCUSSION

Taxonomic studies of new isolates

Three bacterial strains, DDE 27-2, DDE 27-3 and DDE 27-4, were isolated newly from the enrichment culture,¹⁾ from which *Ps. jianii* DDE 27-1 had been isolated. The new isolates, pertaining to *Ps. jianii* as shown in Table I, differ each other in ability to convert DBT into water-soluble form.

TABLE I. DESCRIPTIVE CHART OF THE ISOLATED MICROORGANISMS

	<i>Ps. jianii</i>	New isolates	
	DDE 27-1	DDE 27-2	DDE 27-3, DDE 27-4
Shape	Rods	Short rods	Rods
Size (μ)	$0.5 \times 1.0 \sim 1.5$	$0.6 \times 1.2 \sim 1.4$	$0.6 \times 1.4 \sim 1.8$
Flagella	Polar (tuft)	Polar (tuft)	Polar (tuft)
Gram-stain	Negative	Negative	Negative
Pigmentation	No pigmentation	No pigmentation	No pigmentation
Glutamate agar slant	Growth moderate	Growth moderate	Growth moderate
Nutrient gelatin stab	No liquefaction	No liquefaction	No liquefaction
Litmus milk		Unchanged	Slowly coagulated
B. C. P. milk	Slightly acid, slowly coagulated	Slightly alkaline	Acid, coagulated
NO ₃ →NO ₂	(+)	(+)	(+)
Nitrate respiration	(-)	(-)	(-)
H ₂ S production	(+)	(+)	(+)
Indol production	(-)	(-)	(-)
Hydrolysis of starch	(-)	(-)	(-)
V-P test	(-)	(-)	(-)
Production of acid from			
glycerol	(-)	(-)	(-)
arabinose	(+)		
xylose	(+)	(+)	(+)
glucose	(+)	(+)	(+)
fructose	(-)		
sucrose	(-)	(-)	(-)
lactose	(+)	(+)	(+)
maltose	(+)		
starch	(-)	(-)	(-)
Assimilation			
glucose	(+)	(+)	(+)
gluconate	(+)	(+)	(+)
citrate	(+)	(+)	(+)
succinate	(+)	(+)	(+)
<i>p</i> -OH-benzoate	(+)	(+)	(+)
Growth at			
30°C	(+)	(+)	(+)
37°C	(±)		
42°C	(-)	(-)	(-)

2) Japanese Industrial Standard K 2541, "Method of Test for Sulphur in Petroleum Products by Quartz-tube Combustion", Japanese Standards Association, 1963.

3) S. Kinoshita and K. Hozumi, *Japan Analyst*, **14**, 352 (1965).

Determination of sulfur content by optical density

As shown in the previous paper, the visible

FIG. 2. The Relationship between Optical Density and Sulfur Content of Aqueous Layer in Culture Broth.

absorption spectrum of culture broth had two absorption maxima at 395 mμ and 470 mμ. The relation between sulfur content and optical density at 395 mμ of the aqueous layer in broth is shown in Fig. 2. It is clear that there is correlation between them (correlation coefficient, $r=0.93$). Hence, the sulfur concentration of culture filtrate was estimated

TABLE II. DECOMPOSITION OF DBT DISSOLVED IN LIGHT OIL

Substrate	Amount of substrate per 500 ml flask		Amount of sulfur accumulated in aqueous layer (mg/100 ml)
	DBT	Light oil	
DBT*	0.25 g		13
Light oil**		5 ml	4
Light oil solution of DBT	0.21 g	5 ml	22

* sulfur content: 17.15%

** sulfur content: 0.74%

Basal medium: $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 9.5 g, KH_2PO_4 1.4 g, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 0.2 g, meat extract 4.0 g, distilled water 1000 ml, pH 7.3; 45 ml per 500 ml flask.

FIG. 3. Activities Converting Substrate-sulfur into Aqueous-sulfur of Isolated Strains and Their Mixed Cultures.

spectrophotometrically with the factor $E_{1cm}^{1\%}$ (at 395 m μ) = 2.35×10^3 .

Decomposition of DBT dissolved in light oil

DBT, solid and water-insoluble at room temperature, was added to culture medium after dissolving in light oil to increase the interface of the substrate. The experimental results are given in Table II. Higher conversion efficiency was obtained with light oil solution of DBT than with powdered DBT. DBT, therefore, was used as substrate for further experiments after it was dissolved in light oil.

Comparison of activities of isolated cultures

The conversion efficiency of DBT was examined for the selection of microorganisms by their single or mixed use. The experimental results are given in Fig. 3. The activities of four other strains, DDC 327, DDE 188, DDE 206, and *Ps. jianii* DDC 279, were almost same to that of *Ps. jianii* DDE 27-1, whereas *Ps. jianii* DDE 27-4 did not convert DBT into water-soluble form. The

good results were obtained by the mixed culture of DDE 27-2 and DDE 27-3; DDE 27-2 and DDA 109; DDE 27-2 DDE 27-3 and DDA 109; DDE 27-1, DDE 27-2, DDE 27-3, and DDA 109; and also by the single culture of DDE 27-2. The mixture of DDE 27-1, DDE 27-2, DDE 27-3, and DDA 109, accordingly, was used for further experiments.

Decrease of activity was observed in the case of mixed culture including DDE 27-1. The mixed culture of DDE 27-1, DDE 27-2, DDE 27-3, and DDA 109, however, did not decrease the activity. The reason of this exception has not yet been fully elucidated.

Effect of culture conditions

1) **Nitrogen sources.** In order to find out the suitable nitrogen source for the conversion of DBT, many kinds of nitrogen sources were examined. Figure 4 denotes the conversion efficiency after 5 days of incubation. Meat extract was chosen as a favorable nitrogen source and its optimum concentration was examined as presented in Fig. 5. The best

FIG. 4. Effect of Nitrogen Sources on the Conversion of DBT.

Basal medium: DBT 4.6 g dissolved in 87.8 g of light oil, Na₂HPO₄·12H₂O 9.5 g, KH₂PO₄ 1.4 g, MgCl₂·6H₂O 0.2 g, meat ext. 0.1 g, distilled water 1000 ml, pH 7.3.

FIG. 5. Effect of Meat Extract Concentration on the Conversion of DBT.

Basal medium: same as Fig. 4.

conversion efficiency occurred at the concentration of 0.4%.

2) **Substrate concentration.** The light oil solution of DBT was added to the medium to give such concentrations as 1, 2, 5, 10, 20, 50, and 70% (w/v) in the total volume of 50 ml.

FIG. 6. Effect of Substrate Concentration on the Conversion of DBT.

Medium: DBT 4.6 g dissolved in 87.8 g of light oil, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 9.5 g, KH_2PO_4 1.4 g, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 0.2 g, meat ext. 4.0 g, distilled water 1000 ml, pH 7.3.

■: Aq.-sulfur (mg)/500 ml-flask
□: Conversion ratio

Figure 6 shows the conversion ratio of DBT and the amount of aqueous sulfur accumulated in the broth after 5 days of incubation. The lower the substrate concentration was, the higher the percentage of conversion became. As substrate concentration was increased, the amount of aqueous sulfur in the broth increased and reached to the maximum at 5 to 10% (w/v) of the substrate. It consequently, was employed in the concentration of 10% for further experiments.

3) **pH.** The effect of initial pH on the conversion efficiency of DBT was examined

FIG. 7. Effect of pH on the Conversion of DBT.

Medium: DBT 4.6 g dissolved in 87.8 g of light oil, KH_2PO_4 0.5 g, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 0.2 g, meat ext. 4.0g, distilled water 1000 ml. pH was adjusted to the values as indicated with NaOH or HCl.

as shown in Fig. 7. The initial pH of 9.1 was suitable for the highest accumulation of aqueous sulfur. Since the initial pH of 9.1 dropped to 7.2 after cultivation, another experiment was carried out using m/30 phosphate buffer. The solution of m/30 KH_2PO_4 and m/30 Na_2HPO_4 were mixed duly to provide the medium to various pH values. The experimental results are presented in Fig. 8. The initial pH of 7.3 (final pH, 6.9) appeared to be optimum for the conversion of DBT in 5 days of incubation.

FIG. 8. Effect of pH Buffered with M/30 Phosphate Solution on the Conversion of DBT.

Medium: DBT 4.6 g dissolved in 87.8 g light oil, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 0.4 g to 11.2 g, KH_2PO_4 0.8 g to 4.9 g, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 0.2 g, meat ext. 4.0 g, distilled water 1000 ml.

FIG. 9. Effect of Temperature on the Conversion of DBT.

Medium: same as Fig. 6.

□: Growth (OD at 660 mμ)
■: Conversion ratio

4) **Temperature.** The effect of temperature was investigated. The results obtained are shown in Fig. 9. The activity was higher at 28°C than 24°C and 32°C. The optimum temperature for DBT-utilization and for growth were same in this culture.

5) **Oxygen supply.** The effect of oxygen supply on the conversion efficiency was investigated by the shaking culture with the medium of 20 ml to 100 ml in a 500 ml flask. The highest conversion of DBT was obtained

with 20 ml of the medium (Fig. 10). This result suggests that vigorous aeration is favorable for the conversion of DBT.

Time course of cultivation. Figure 11 shows the time course of cultivation by the mixed culture of *Ps. abikonensis*, *Ps. jianii* DDE 27-1, DDE 27-2 and DDE 27-3. Aqueous sulfur was formed in a relatively short time, and the highest level (conversion ratio 40%) was at-

FIG. 10. Effect of Aeration on the Conversion of DBT.

Medium: DBT 4.6 g dissolved in 87.8 g of light oil, KH_2PO_4 0.5 g, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 0.2 g, meat ext. 4.0 g, distilled water 1000 ml, pH 9.0.

FIG. 11. Time Course of Cultivation.

Medium: same as Fig. 6.

---●---: Conversion ratio,
—○—: OD at 395 mμ,
---▲---: Growth (OD at 660 mμ),
—△—: OD at 470 mμ

tained on the third day. Optical density at 470 m μ reached a peak on the second day and thereafter decreased. The curve of cell growth had not similar tendency to that of conversion of DBT.

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