Desulfurization of Dibenzothiophene by *Bacillus* K10

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From soil samples of Mae Moh lignite mine, Lamphang, Thailand; 342 bacterial strains capable of growing in medium containing dibenzothiophene (the representative of organic sulfur compound in lignite coal) as sole sulfur source (SFMM-DBT) were isolated. Only one isolated strain, *Bacillus* K10, degraded dibenzothiophene via a sulfur-specific metabolic pathway (4S pathway) which removed only sulfur from the molecule of dibenzothiophene. Growth of *Bacillus* K10 in SFMM-DBT was stimulated by increasing the amount of yeast extract added or addition of casein in place of yeast extract. Analysis of the culture filtrate revealed the presence of 2-hydroxybiphenyl, indicated that dibenzothiophene was degrading via the 4S pathway only when not more than 0.005% (w/v) yeast extract or 0.1-2.0% (w/v) casein was added. *Bacillus* K10 desulfurized 1.9 mg/l of dibenzothiophene when grown in SFMM-DBT medium containing 10 mg/l dibenzothiophene supplemented with 0.2% (w/v) casein in place of yeast extract at 30°C with shaking at 200 rpm for 3 days. The activity of the dibenzothiophene degrading enzyme of *Bacillus* K10 was found to be activated by NADH.

Key words : Dibenzothiophene, *Bacillus*, organic sulfur compound

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การกำจัดกำมะถันจากไดเบนโซไธโอฟีนโดย *Bacillus* K10

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จากตัวอย่างดินบริเวณเหมืองถ่านลิกไนท์ อ.แม่เมาะ จ.ลำปาง สามารถแยกแบคทีเรียที่เจริญได้ในอาหารเลี้ยงเชื้อที่มีไดเบนโซไธโอฟีน (DBT) ซึ่งใช้เป็นตัวแทนของกำมะถันอินทรีย์ในถ่านลิกไนท์ เป็นแหล่งกำมะถันเพียงอย่างเดียว (SFMM-DBT) ได้ 342 สายพันธุ์ เป็นแบคทีเรียที่ย่อยสลายไดเบนโซไธโอฟีนด้วยวิถี 4S หรือวิถีที่ย่อยสลายเอาเฉพาะโมเลกุลกำมะถันออกจากโมเลกุลไดเบนโซไธโอฟีนเพียง 1 สายพันธุ์ คือ *Bacillus* K10 การเพิ่มปริมาณสารสกัดจากยีสต์หรือใช้เคซีนแทนสารสกัดจากยีสต์ในอาหารเลี้ยงเชื้อ SFMM-DBT ทำให้การเจริญของ *Bacillus* K10 เพิ่มมากขึ้น ผลการวิเคราะห์น้ำเลี้ยงเชื้อ พบ 2-ไฮดรอกซีไบฟีนิลซึ่งเป็นสารที่บ่งชี้ว่าแบคทีเรียย่อยสลายไดเบนโซไธโอฟีนโดยวิถี 4S เฉพาะเมื่อเติมสารสกัดจากยีสต์ปริมาณไม่เกิน 0.005% (น้ำหนัก/ปริมาตร) หรือเคซีนปริมาณ 0.1-2.0% (น้ำหนัก/ปริมาตร) ในอาหารเลี้ยงเชื้อ SFMM-DBT *Bacillus* K10 สามารถย่อยสลาย 1.9 มิลลิกรัม/ลิตรของไดเบนโซไธโอฟีนเมื่อเพาะเลี้ยงไว้ในอาหารเลี้ยงเชื้อ SFMM-DBT ที่เติมไดเบนโซไธโอฟีน ปริมาณ 10 มิลลิกรัม/ลิตร และเติมเคซีนปริมาณ 0.2% (น้ำหนัก/ปริมาตร) แทนสารสกัดจากยีสต์ที่อุณหภูมิ 30 องศาเซลเซียส บนเครื่องเขย่าความเร็ว 200 รอบต่อนาที เป็นเวลา 3 วัน พบว่า NADH มีผลทำให้กิจกรรมของเอนไซม์ย่อยสลายไดเบนโซไธโอฟีน ของ *Bacillus* K10 สูงขึ้น

คำสำคัญ ไดเบนโซไธโอฟีน *Bacillus* กำมะถันอินทรีย์

INTRODUCTION

Abundant resources of lignite coal exist in Thailand. Efforts have thus been made to utilize it for fueling electricity. However, consideration has to be given to its sulfur content. Burning coal that contains sulfur generates sulfur oxide (SOx), especially air pollution of sulfur dioxide, that is harmful to human beings and animals. It also causes acid rain, as sulfur dioxide reacts with rain water to yield bisulfite and hydrogen ions. Bisulfite oxidation results in sulfate and hydrogen ions. Rain water with a high content of hydrogen ions is defined as acid rain.[1]

There are two kinds of sulfur contamination in coal, i.e., organic sulfur and inorganic sulfur. For organic sulfur, the sulfur molecule is in itself a part of the coal molecular structure. Among the desulfurization processes for the precombustion step of coal, biological method is the most efficient method to remove organic sulfur.[2] Organic sulfur contamination in coal is mostly found as dibenzothiophene (DBT) and its derivatives. Therefore, dibenzothiophene is used as an organic sulfur model compound to isolate microorganisms capable of degrading organic sulfur. The bacteria *Sulfolobus acidocaldarius,*[3] *Pseudomonas putida,*[4] *Rhodococcus rhodochrous*[5] and *Corynebacterium* sp.[6] were reported as dibenzothiophene degrading strains. Bacteria degrade dibenzothiophene by two mechanisms[7]:1) Carbon-destructive metabolic pathway, the mechanism by which carbon-carbon bonds in the benzene ring of dibenzothiophene is destroyed. The degraded structure of dibenzothiophene results in loss of calorimetric value of coal; 2) Sulfur-specific metabolic pathway or 4S pathway, the mechanism by which only the sulfur-carbon bond of dibenzothiophene are destroyed. The sulfur molecule is removed from dibenzothiophene without destruction of other part of the molecule. The 4S pathway is the desired pathway for dibenzothiophene degradation in coal because the calorimetric value of coal is not lost. The intermediates of dibenzothiophene degradation via the 4S pathway by *Corynebacterium* sp. was elucidated as shown in Figure 1.[6] Analysis of 2-hydroxybiphenyl (2-HBP), the final product of dibenzothiophene degradation via 4S pathway, in cell-free culture containing dibenzothiophene as sole sulfur source could be a method to screen for bacteria capable of degrading dibenzothiophene via 4S pathway.

Figure 1. Schematic representation of microbial metabolism of dibenzothiophene via the sulfur-specific pathway.

The objective of this work is to screen for bacteria capable of degrading dibenzothio-

phene via 4S pathway, and to study some factors affecting the efficiency of bacteria to degrade dibenzothiophene.

MATERIALS AND METHODS

Bacterial isolation

Soil samples (148 samples) from Mae Moh lignite mine, Lamphang, Thailand were screened for dibenzothiophene (DBT) degrading bacteria by a selective method using a sulfur-free mineral medium (SFMM) [3] containing Na_2HPO_4 (0.22%), KH_2PO_4 (0.08%), NH_4NO_3 (0.3%), $FeCl_3$. $6H_2O$ (0.001%), $CaCl_2$.$2H_2O$ (0.001%), $MgCl_2$. $7H_2O$ (0.001%), glucose (1.0%) and yeast extract (0.005%) supplemented with DBT 0.05% (w/v), pH 7.0 and incubated at 30°C with shaking (200 rpm) for 4 days. Cultures were consecutively transferred to fresh SFMM-DBT medium at 1% inoculum and incubated at the same condition given above 3 times. Purification of culture was done by a streak plate method using SFMM-DBT agar medium. Identification of bacterial genus was done by the method of Cowan.[8]

Analysis of dibenzothiophene degradation products.

Bacteria capable of growing in medium containg DBT as sole sulfur source were grown in 10 ml of SFMM-DBT at 30°C, 200 rpm for 3 days. The cell free cultures obtained after centrifugation at 5,000 g, 4°C for 15 min were adjusted to pH 2.0 by addition of 6 N HCl, and then 5 ml was loaded into Sep-Pak C 18 column (Waters, Millipore Corp., USA) and eluted by 1 ml of absolute ethanol. The filtrate (30 µl) was analysed by thin layer chromatography[3] using silica gel plate (0.25 mm thick) containing a fluorescent compound (TLC aluminium sheets Lilica gel $60F_{254}$; Merck, USA). Elution was made by benzene : dioxane: acetic acid at a ratio of 60 : 25 : 4. The degradative products obtained was visualized under UV light at 254 nm. A 2-hydroxybiphenyl (2-HBP) (Aldrich Chemical Co. Ltd., USA) solution at 1.0% (w/v) in methanol (5 µl) was used as the standard. The cell free culture containing tentatively 2-HBP in degradative product by thin layer chromatography analysis was reconfirmed for the existing of 2-HBP by gas chromatography [5]. Cell free culture (200 ml) grown in the SFMM-DBT medium was adjusted to pH 2.0 by addition of 6 N HCl, and then extracted by 0.8 vol. of ethyl acetate. The ethyl acetate fraction was concentrated to 1 ml by a rotary vacumm evaporator at 40°C and consecutively concentrated to 100 µl by evaporation under nitrogen gas. A fraction (1 µl) was analysed by gas chromatography (Shimadzu, Japan), with a Silicone OV-17 column, 5% chromosorb, an flame ionization detector, an injector port temperature of 220°C, an oven temperature of 140-200°C, a temperature increase rate of 4°C/min, and a nitrogen gas flow rate of 50 ml/min

Optim*al condition for dibenzothiophene degradation*.

Cells were grown in SFMM medium containing 10 mg/l DBT. The concentration of each SFMM medium composition was varied and incubated at 30°C, 200 pm for 4 days. Cell growth was monitored by optical density at 660 nm (OD_{660}) and decreased in DBT was analysed by gas chromatography.[5] Casein was used in place of yeast extract in some experiments.

Effect of NADH on dibenzothiophene degradation activity.

Cells grown in SFMM-medium containing 0.2% (w/v) casein in place of yeast extract and 10 mg/l DBT at 30°C, 200 rpm for 3 days were harvested by centrifugation at 4,000 g, 4°C for 20 min and then washed once by 0.85% (w/v) NaCl. The washed cells (2 g) suspended in 4 ml of 100 mM potassium phosphate buffer pH 7.0 were broken by ultrasonication at 0°C for 10 min (at 2 min intervals). The supernatant obtained after centrifugation at 5,000 g, 4°C

for 30 min was used as crude enzyme. Dibenzothiophene degradation activity was measured by the method of Izumi, et al..[9] The reaction mixture (0.5 ml) containing 100 mM potassium phosphate buffer (pH 7.0), 1.0% (w/v) DBT in 0.1 mM dimethylformamide and 1.5 mg protein of crude enzyme were incubated at 30°C for 60 min. NADH was added in some experiments at 5 mM final concentration. The reaction was stopped by addition of 50 µl of 1 N HCl and then 400 µl of ethyl acetate was added. The upper layer of ethyl acetate fraction was collected and centrifuged at 9,200 g, room temperature for 10 min. The resulting supernatant was concentrated to 100 µl by evaporation under nitrogen gas and the remaining DBT was analysed by gas chromatography.

Figure 2. Thin layer chromatography showing 2-hydroxybiphenyl (2-HBP) as the degradative product of dibenzothiophene by *Bacillus* K10.

RESULTS AND DISCUSSIONS

Bacteria degraded dibenzothiophene via the 4S pathway

A total of 342 bacterial strains capable of growing in media containing DBT as the sole sulfur source were obtained from a selective method by inoculation of 148 soil samples from Mae Moh lignite mine, Lamphang, Thailand into SFMM-DBT medium which contained DBT as sole sulfur source. Among the above bacterial strains obtained, only one strain, designated K10, revealed the presence of 2-HBP in cell free culture as analyzed by thin layer chromatography (Figure 2) and gas chromatography (Figure 3), indicated that DBT was degraded via 4S pathway. The R_f value of 2-HBP from the thin layer chromatogram was 0.63. The DBT degradative products of bacterial strain K10 are shown in Figure 3A and the product eluted at the retention time of 9.31 was identified as 2-HBP. Morphological and biochemical characterization of bacterial strain K10 indicated that it was *Bacillus* sp. (Table 1). This is the first report about capability of *Bacillus* sp to desulfurize DBT via 4S pathway. The spore forming character of *Bacillus* sp. made them tolerant to the environmental stress and suitable for desulfurization of DBT in lignite coal under uncontrolled conditions.

Figure 3. Gas Chromatography of degradative products of dibenzothiophene by Bacillus K10 showing 2-hydroxybiphenyl (2-HBP) (A), and of standard 2-HBP(B).

Table 1. **Morphological and biochemical characteristics of bacterial strain K10.**

Characterization test	
Colony grown on nutrient agar medium	spherical with 0.5-1.0 mm diameter, white, turbid, convex, filamentous margin
Cell shape	rod shape
Gram staining	Gram-positive
Spore staining	spore forming
Endospore	oval shape at the cell terminal
Catalase test	positive
Oxidase test	negative
Oxidation-fermentation test	oxidation
Production of acid from glucose	positive

Optimal condition for dibenzothiophene degradation

Yeast extract was a growth limiting factor of *Bacillus* K10 grown in SFMM-DBT medium. Casein, in place of yeast extract in SFMM-DBT medium, can stimulate growth of *Bacillus* K10 in SFMM-DBT medium (Figure 4). The concentration of yeast extract or casein in SFMM-DBT medium at higher than 0.005% or 2.0% (w/v), respectively, inhibited DBT degradation by *Bacillus* K10 as shown in Table 2. Maximal DBT degraded

was 1.9 mg/l when *Bacillus* K10 grew in SFMM-DBT medium containing 0.2% (w/v) casein and 10 mg/l DBT after 3 days of shaking at 200 rpm, 30°C (Table 3). The inhibitory effect of a high concent-ration of yeast extract or casein on DBT degradation by *Bacillus* K10 might be the result of their sulfur form and content which is more suitable for growth than DBT or their metabolic intermediate inhibited DBT degrading enzyme activity. A 2, 2′-dihydroxybiphenyl, an analog of 2-HBP, was reported as inhibitor of DBT degrading enzyme activity of *Rhodococcus erythropolis* D-1.[10]

Table 2. Effect of yeast extract and casein concentration in SFMM-DBT medium on dibenzothiophene (DBT) degradation by *Bacillus* K10.[a)]

Organic nitrogen source	concentration (%)	amount of DBT degraded (mg/l)
Yeast extract	0	-
	0.005	0.43
	0.01	-
Casein	0.01	-
	0.1	1.1
	0.2	1.9
	0.5	1.5
	1.0	1.3
	2.0	0.9
	3.0	-

a) Cells were grown as detailed in Materials and Methods.

Table 3. Effect of cultivation time on dibenzothiophene (DBT) degradation by *Bacillus* K10.

Cultivation time[a)] (days)	amount of DBT degraded (mg/l)
1	0.7
2	1.6
3	1.9
4	1.9

a) Cells were grown in SFMM-DBT medium containing 0.2% (w/v) casein and 10 mg/l DBT with shaking at 200 rpm, 30° C.

Effect of NADH on dibenzothiophene degradation activity

As shown in Table 4, NADH activated the DBT degrading activity in cell-free extract of *Bacillus* K10. Oshiro, et al.[11] reported that the enzymes in cell-free extract of *Rhodococcus erythropolis* D-1 grown in a medium containing DBT as the sole sulfur source were able to degrade DBT to 2-HBP. And NADH, the co-factor of an enzyme in the oxidoreductase group, was necessary for the activity of the DBT degrading enzyme.

The optimal concentration of NADH for DBT degrading activity reported was 5 mM. Our results support the work of Oshiro, et al., [11] NADH also activated the DBT degrading activity in cell-free extract of *Bacillus* K10.

Figure 4. Effect of yeast extract and casein concentration in sulfur free mineral medium (SFMM) containing 10 mg/l dibenzothiophene (DBT) on growth of *Bacillus* K10.
Cells were grown at 30°C, 200 rpm for 4 days. Symbol used :
(A) Yeast extract concentration: 0% (✧), 0.005% (◆), 0.01% (❍), 0.025% (●), 0.05% (Δ)
(B) Casein concentration: 0.01%(❍), 0.1%(▲), 0.2%(□). 1.0%(■), 2. (+), 3.0% (∗), control, 0.005% yeast extract. (◆)

Table 4. Effect of NADH on dibenzothiophene (DBT) degrading activity in cell-free extract of *Bacillus* K10.

Experiments	incubation time (min)	relative amount of remaining DBT (%)
Control :	0	100

without cell extract and NADH	30	100
	60	100
cell extract without NADH	0	100
	30	93
	60	78
cell extract with 5 mM NADH	0	100
	30	6
	60	2

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