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A Physiological Study on Growth and Dibenzothiophene (DBT) Desulfurization Characteristics of *Gordonia* sp. CYKS1

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Abstract−Physiological characteristics of DBT desulfurization and cell growth of *Gordonia* sp. CYKS1 were investigated. It exhibited a preference to ethanol in a medium containing two carbon sources, ethanol and one of the carbohydrates used, glucose, sucrose, maltose, and galactose although it consumed both carbon sources simultaneously. Cell growth on ethanol or glucose followed the Monod kinetics. The optimal range of pH for the desulfurization of DBT and the cell growth was 7 to 8. The desulfurization rate decreased about 30% at pH 6, and no significant desulfurization or cell growth was observed at pH 5. As the initial DBT concentration increased up to 1.5 mM, the desulfurization rate also increased while no significant changes in the growth rate were observed. The maximum desulfurization rate was 12.50 μmol L^{-1} h^{-1} at an initial DBT concentration of 1.5 mM. Cell growth and desulfurization activity were severely inhibited by the presence of 2-hydroxybiphenyl (2-HBP). When 0.05 mM of 2-HBP was added at the beginning, both of the desulfurization rate and cell growth rate decreased about 20%. It was found that cell growth and desulfurization were completely inhibited in the presence of 2-HBP at 0.15 mM or a higher concentration. The inhibition by 2,2'-dihydroxybiphenyl (DHBP) was less severe than 2-HBP. About 80% of desulfurization activity was retained in the presence of 2,2'-DHBP at 0.4 mM.

Key words: Microbial Desulfurization, *Gordonia* sp., Carbon Source Selection

INTRODUCTION

Significant progress in biocatalytic desulfurization (BDS) of petroleum-base fuel oils has been made in recent years. Dibenzothiophene (DBT) and its alkylated derivatives comprise a major group of organic sulfur compounds in fuel oils. They are known to be recalcitrant against hydrodesulfurization (HDS). DBT has been generally accepted as a model heterocyclic organic sulfur compound in most BDS researches. It has been reported that *Gordonia* sp. CYKS1, *Nocardia* Strain CYKS2, *Rhodococcus rhodochrous* IGTS8, *Rhodococcus erythropolis* D-1, *Corynebacterium* sp. SY1 could selectively remove sulfur atom from DBT under aerobic conditions, without destroying the carbon skeleton into low-carbon-number hydrocarbons [Chang et al., 1998; Gallagher et al., 1993; Izumi et al., 1994; Kayser et al., 1993; Oshiro et al., 1995; Piddington et al., 1995; Rhee et al., 1998]. The selective removal of sulfur from organic compounds by these strains is quite desirable from the practical viewpoint of fuel oils desulfurization, for valuable combustible carbons in the oil are retained without being lost as water-soluble forms to the aqueous phase in contact with the oil. Alkylated DBT can be desulfurized by *Paenibacillus* [Konishi et al., 1997], *Mycobacterium* sp., *Pseudomonas* sp. [Nekodzuka et al., 1997], *Arthrobacter* sp. [Lee et al., 1995] and *Pseudomonas* sp. [Kropp et al., 1997].

In this study, the growth and DBT desulfurization characteristics of *Gordonia* sp. CYKS1 that had been screened by our group [Rhee et al., 1998] were investigated. Growth patterns for two different carbon sources and the effect of DBT concentration on the growth and desulfurization activity were investigated. Inhibitory effects of 2-HBP and sulfate, two dead-end metabolites of DBT, were assessed. The effects of pH were also examined.

MATERIALS AND METHODS

1. Strain

The strain used in this study was *Gordonia* sp. CYKS1 (KCTC 0431BP) [Rhee et al., 1998].

2. Medium

The minimal salt medium (MSM) used in this study was a sulfur-free medium as previously described [Chang et al., 1998]. The relatively high content of mono- and di- basic phosphates in the MSM provided a good pH buffering capacity. As the major carbon source and sulfur source, respectively, 10 g L^{-1} of glucose and 0.3 mM of DBT was supplemented. For the addition of an accurate amount of DBT which is practically non-soluble in water, a stock solution (100 mM DBT in ethanol) was used. Stock solutions (100 mM in ethanol) were also used in the cases of 2-HBP and 2,2'-DHBP addition. To examine the cell growth and desulfurization activity on different pHs, phosphate buffers with different pH values were used.

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3. Culture Conditions

All experiments were carried out in 250-mL Erlenmeyer flasks containing 50 mL of MSM, at 30 °C and 180 strokes per minute in a reciprocal-shaking incubator. The inoculums size was 1% (v/v). The cultures were carried out for 72 hours. All the experiments were done in triplicate.

4. Analytical Methods

Cell concentration was determined by measuring the optical density (OD) of culture broth at 600 nm (Spectronic 20, Milton Roy Co., USA). A linear relationship between OD and dry cell mass was obtained when the optical density lay between zero to 0.5. One optical density unit was equivalent to 0.57 g-dry cell weight per liter. Liquid-liquid extraction for quantitative analysis of residual DBT and its metabolites was carried out by using ethyl acetate as the extractant. After the extraction, 10 μL of ethyl acetate layer was analyzed by using a reverse-phase high performance liquid chromatograph (HPLC) equipped with a UV-VIS detector (Waters 486, Waters, USA) and a Nova-Pak C_{18} column (3.9×150 mm, Waters, USA). The UV-VIS detector was set at 280 nm. The mobile phase was pure methanol and its flow rate was 0.6 mL/min. Glucose was analyzed with a glucose analyzer (YSI 2700, YSI, USA). Ethanol was analyzed by a gas chromatograph equipped with a flame ionization detector (FID) (5890 Series II, Hewlett Packard, USA). A Supelcowax 10 TM column (30 m, 0.32 mm, 25 μm, Supelco, USA) was used.

5. Chemicals

DBT, 2-HBP and other organic sulfur compounds of reagent grade were purchased from Sigma Co. (St. Louis, USA). All other chemicals were of analytical grade. Solvents for extraction and HPLC analysis were of LC grade and purchased from Merck Co. (Darmstadt, Germany).

RESULTS AND DISCUSSION

1. Effects of Ethanol Added with DBT

As mentioned earlier, we used ethanol to dissolve DBT before its addition to the culture medium. Thus, the medium inevitably contained a certain amount of ethanol. It was reported that the addition of DBT dissolved in ethanol provided more rapid growth and desulfurization than DBT powder only [Setti et al., 1995]. For this reason, the effects of ethanol on cell growth and desulfurization activity were investigated. As shown in Fig. 1, *Gordonia* sp. CYKS1 (CYKS1 hereafter) could grow on ethanol as well as glucose. The growth rate on ethanol was observed to be higher than that on glucose.

When CYKS1 was cultivated in a medium containing both glucose and ethanol, ethanol was consumed more readily than glucose although they were consumed simultaneously (Fig. 2). Similar results were observed when glucose was replaced by sucrose, maltose, or galactose (data not shown). Ethanol was consumed first in all these cases. About 0.07 mM of DBT was desulfurized while ethanol was predominantly consumed. As the consumption of glucose became significant, DBT concentration started to decrease more rapidly, and then leveled off at a concentration of about 0.06 mM. The decreased rates of growth and desulfurization were considered to be due to the accumulation of 2-HBP, which will be discussed later.

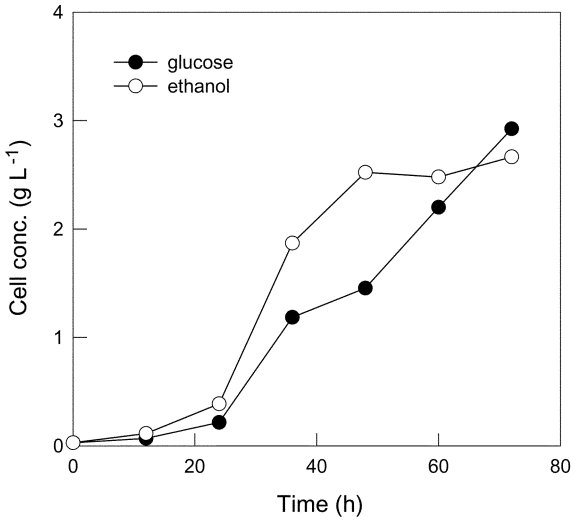

Fig. 1. Growth of *Gordonia* sp. CYKS1 on either of glucose and ethanol (Initial concentration of DBT is 0.3 mM, and both glucose and ethanol concentrations are 10 g/L).

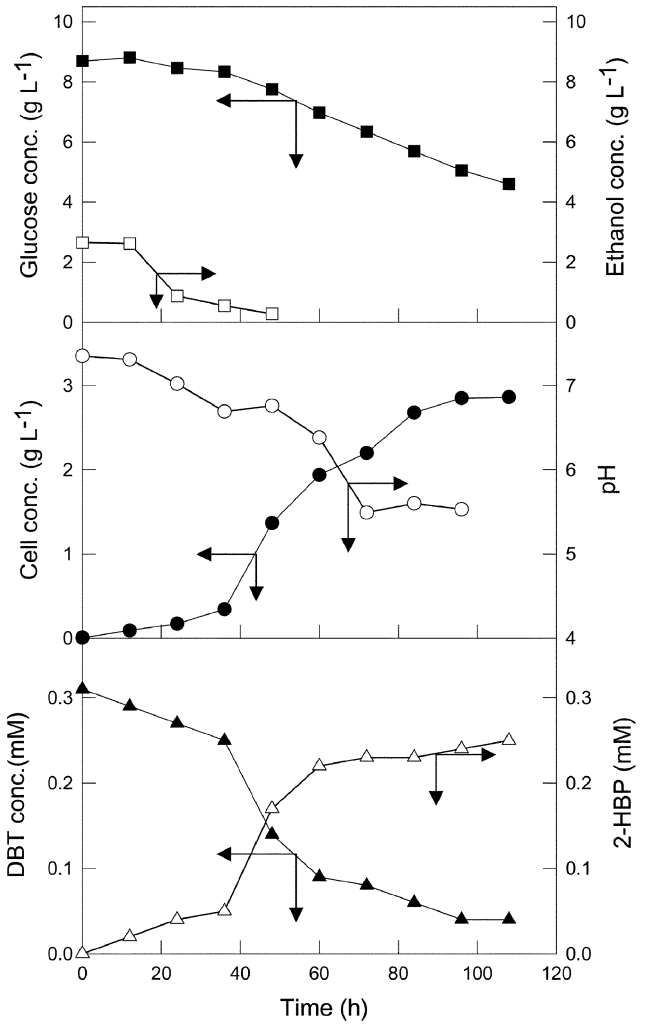

Fig. 2. Diauxic growth of *Gordonia* sp. CYKS1 in a medium containing glucose and ethanol.

Table 1. Initial rate data of cell growth and DBT desulfurization at different initial ethanol concentrations

Ethanol concentration (g L^{-1})	DBT desulfurization rate (μmol L^{-1} h^{-1})	Specific growth rate (h^{-1})
3	1.71±0.12	0.06±0.01
5	2.93±0.46	0.10±0.02
10	2.90±0.32	0.16±0.02
15	3.83±0.31	0.17±0.03
20	4.02±0.80	0.18±0.03

All the cultures were done in a medium containing 0.3 mM DBT.

To investigate effects of ethanol in more detail, 3 to 20 g/L of ethanol was added to the medium as a sole carbon source. The cell growth and desulfurization rates increased as the ethanol concentration was increased as shown in Table 1. The desulfurization rate increased about 2.5 times (from 1.71 to 4.02 μ-mol L^{-1} h^{-1}) as the ethanol concentration increased from 3 to 20 g/L.

The growth rate on ethanol could be well represented by the Monod equation. The maximum specific growth rate (μ_m) and the saturation constant (K_m) were estimated to be 0.20 h^{-1} and 2.76 g L^{-1}, respectively. The cell growth on glucose could be represented by the Monod equation also, and μ_m and K_m were 0.08 h^{-1} and 6.29 g L^{-1}, respectively.

The active utilization of ethanol by CYKS1 is a desirable physiological nature of biocatalysts for fuel oil desulfurization. In one of the diesel desulfurization processes under development, ethanol is used as de-emulsifier to promote phase separation for the recovery of desulfurized diesel oil and microbial cells from the very stable emulsion from the reactor [Choi et al., 2003]. After the phase separation, the major portion of the added ethanol remains in the aqueous phase containing microbial cells. It can serve as a good carbon source for the growth of the recovered cells to be recycled to the reactor, replacing, at least partially, the carbon source requirements.

2. Effects of pH

To determine the optimal pH for cell growth and desulfurization activity of CYKS1, the effects of the initial pH were investigated. A good cell growth, and thus desulfurization activity, was observed at pH 7-8. It had been observed that the desulfurization activity of CYKS1 was directly related to the growth rate (data not shown). When the pH was 7.0, about 0.25 mM of DBT was desulfurized in 72 hours and the cell concentration reached 2.74 g L^{-1} as shown in Fig. 3. In the case of pH 8.0, similar patterns of cell growth, DBT desulfurization, and 2-HBP production to the case of pH 7.0 were observed. Both the cell growth and the desulfurization activity were decreased as pH was decreased below 7.0. When the pH was 6.0, only 0.15 mM of DBT was desulfurized and the final cell concentration was 0.97 g L^{-1}. When the pH was below 6.0, neither a significant desulfurization of DBT nor an observable cell growth occurred.

3. Effects of DBT Concentration

DBT is intrinsically a xenobiotic compound and there have been several reports addressing that a high concentration of DBT had inhibition effects on cell growth and desulfurization activity. The effect of the initial DBT or the amount of DBT added is shown in Fig. 4. The initial DBT concentration was varied in a range from

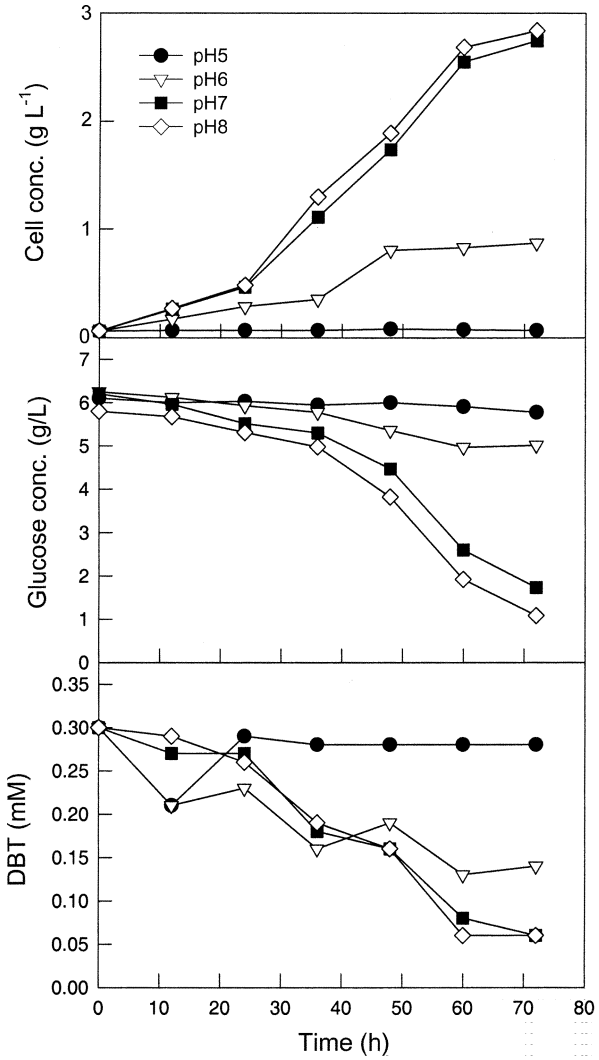

Fig. 3. Growth and desulfurization characteristics of *Gordonia* sp. for different initial pHs.

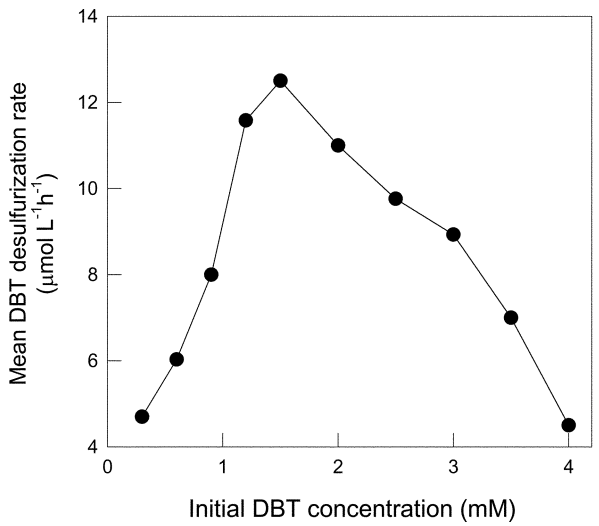

Fig. 4. Effect of initial DBT concentration on DBT desulfurization rate (The initial glucose concentration is 10 g/L).

0.3 to 4.0 mM. The desulfurization rate was observed to be strongly affected by DBT concentration. The desulfurization rate increased as the initial DBT concentration increased up to 1.5 mM reaching a maximum value of 12.5 μmol L^{-1} h^{-1} which is about 2.7 times higher than that at 0.3 mM (4.7 μmol L^{-1} h^{-1}). But, the increase in the initial DBT concentration above 1.5 mM resulted in a lower desulfurization rate. When 4.0 mM of DBT was added, the rate was only 4.5 μmol L^{-1} h^{-1}. Such an inhibitory effect of DBT has been reported elsewhere also [Oshiro et al., 1995; Setti et al., 1996]. It is known that only water-soluble compounds play an inhibitory role on cell growth or enzyme activities. DBT has an extremely low solubility to water and expected to precipitate on being added to the medium; thus DBT in solid form in the medium was expected to be have negligible effects, if any, on desulfurization activity and cell growth. However, most microbial strains with DBT desulfurization activity are known to secrete some biosurfactants to solubilize and thus enhance the bioavailability of DBT. If a strain secretes a sufficient amount of biosurfactants, the effects of the amount of DBT added would not be limited by its intrinsic solubility to water. In this rationale, CYKS1 seems to have secreted biosurfactants to solubilize DBT added. When 5 (v/v%) of a surfactant, Tween-80, known to be benign to cells was added with 0.3 mM of DBT, the desulfurization rate and cell growth rate increased as shown in Fig. 5. With the addition of Tween-80, the cell growth and desulfurization rates were 0.08 h^{-1} and 5.90 μmol L^{-1} h^{-1}, respectively, while 0.06 h^{-1} and 4.70 μmol L^{-1} h^{-1} without it (the control). This result implies that the addition of the surfactant enhanced the desulfurization of DBT by increasing their availability. Recombinant Pseudomonas strains with an enhanced desulfurization activity have been developed which can produce a large amount of biosurfactants [Gallado et al., 1997]. In oil desulfurization processes, the production of a large amount biosurfactants by biocatalyst would enhance the bioavailability of organic sulfur compounds in fuel oils and at the same time increase the reaction rate by improving the contact between the oil and aqueous phases. At this point, however, it should be noted that the biosurfactants could cause the formation of excessively stable emulsion and thus may cause serious phase separation problems in real processes.

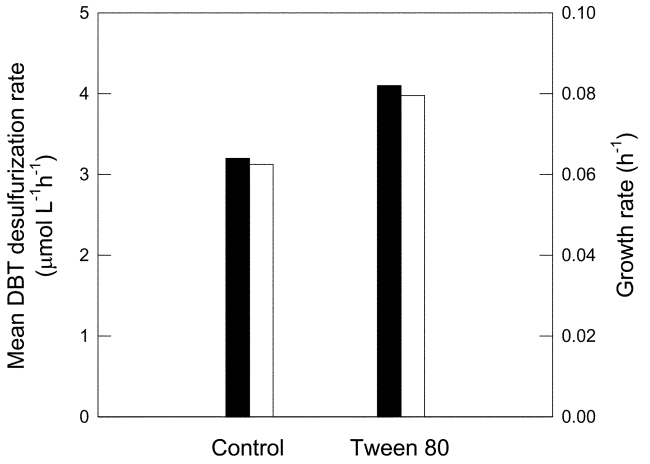

Fig. 5. Effects of surfactant addition on cell growth and DBT desulfurization (The initial concentrations of DBT and glucose are 0.3 mM and 10 g/L, respectively).

4. Inhibition of Growth and Desulfurization by 2-HBP and 2,2'-DHBP

Inhibitions of cell growth and desulfurization activity by the end products from DBT desulfurization, 2-HBP and sulfate, are known to be severe. Most of the strains previously reported which desulfurized DBT via sulfur-specific pathway were observed to be inhibited by sulfate or other organic sulfur compounds [Kayser et al., 1993; Oshiro et al., 1995].

The desulfurization activity of CYKS1 was also strongly inhib-

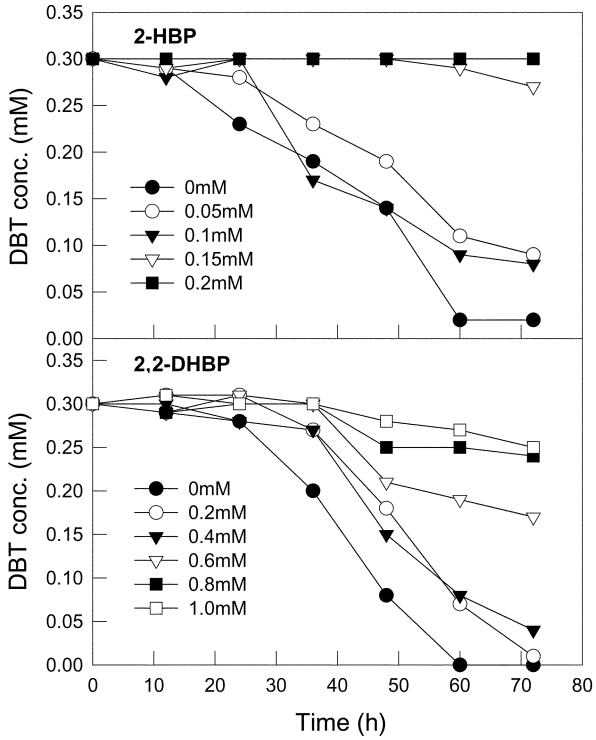

Fig. 6. Inhibition of desulfurization activity by 2-HBP and 2,2'-DHBP.

Table 2. Initial rate data of cell growth and DBT desulfurization in the presence of 2-HBP and 2,2'-DHBP

Concentration (mM)		DBT desulfurization rate (μmol L^{-1} h^{-1})	Specific growth rate (h^{-1})
2-HBP	0	4.70±0.46	0.08±0.01
	0.05	3.25±0.16	0.08±0.01
	0.10	3.43±0.27	0.09±0.01
	0.15	a	a
	0.20	a	a
2,2'-DHBP	0	4.70±0.46	0.08±0.01
	0.20	3.89±0.45	0.09±0.02
	0.40	3.93±0.34	0.06±0.01
	0.60	1.88±0.17	0.01±0.01
	0.80	a	a
	1.00	a	a

a: completely inhibited.

All cultures were carried out in a medium containing 10 g L^{-1} glucose and 0.3 mM of DBT.

ited by 2-HBP as shown in Fig. 6-(a). Desulfurization activity and cell growth were only slightly inhibited when the initial 2-HBP concentration was 0.1 mM or below. The strain CYKS1 maintained about 90% of desulfurization rate compared with the control (no addition of 2-HBP) when the initial 2-HBP concentration was 0.1 mM. However, when the initial 2-HBP concentration was 0.15 mM or higher, no significant cell growth and desulfurization of DBT were observed.

The desulfurization activity of CYKS1 was also found to be inhibited by 2,2'-DHBP, which was an analog of 2-HBP. However, the inhibition by 2,2'-DHBP was less severe than that by 2-HBP as shown in Fig. 6-(b). Most of the added DBT (0.3 mM) was desulfurized in 72 hours in the presence of 0.2 mM of 2,2'-DHBP. About 0.25 mM of DBT was desulfurized when 0.4 mM of 2,2'-DHBP was added. However, no significant desulfurization of DBT was observed in the presence of 2,2'-DHBP at 0.8 mM.

The desulfurization activity of various microbial strains capable of desulfurizing DBT or its derivatives is reported to be inhibited in the enzyme level by biphenyl-derivatives such as biphenyl (BP), 2-HBP and 2,2'-DHBP [Oshiro et al., 1995, 1997; Setti et al., 1996]. The cell growth of CYKS1 was also inhibited by the 2-HBP even in the presence of sulfates (data not shown) implying that 2-HBP not only inhibited the enzymes involved in desulfurization but also certain enzymes for cell growth.

5. Inhibition of Desulfurization by Sulfate

The desulfurization activity of CYKS1 was inhibited by the presence of sodium sulfate as summarized in Table 3. By the addition of sulfate, the growth rate was significantly enhanced, while the desulfurization activity of the strain CYKS1 was strongly inhibited. When 0.07 mM of sulfate was initially added, only about 0.02 mM of DBT was desulfurized in 72 hours, while 0.27 mM of DBT was desulfurized in the case of the control in which only DBT was the sole sulfur source. The desulfurization rate at 0.07 mM of sulfate was 3.13 μmol L^{-1} h^{-1} being about 70% of that (4.70 μmol L^{-1} h^{-1}) obtained in the control. No significant desulfurization of DBT occurred when the initial sulfate concentration was above 0.7 mM.

It is known that DBT desulfurization activity is inhibited by sulfate in almost all DBT-desulfurizing strains [Kayser et al., 1993; Oshiro et al., 1994, 1996]. The inhibition of DBT desulfurization activity by sulfate is considered to be a gene-level regulation. The expression of *dsz* genes that are involved in desulfurization of DBT is strongly repressed by sulfates [Li et al., 1996]. The removal of this feedback regulation is of great importance from a process viewpoint.

Table 3. Cell growth and DBT desulfurization at various sodium sulfate concentrations

Sulfate concentration (mM)	Desulfurized DBT (mM) in 60 hours	Initial rate of DBT desulfurization (μmol L^{-1} h^{-1})	Initial rate of growth (h^{-1})
0	0.270	4.7±0.46	0.08±0.01
0.07	0.225	3.13±0.15	0.12±0.02
0.7	0.024	0.34±0.05	0.16±0.03
1.4	0.009	0.13±0.03	0.17±0.05
3.5	0.008	0.11±0.03	0.15±0.03

All cultures were carried out in a medium containing 10 g L^{-1} glucose and 0.3 mM DBT.

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