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Biodesulfurization of dibenzothiophene by Shewanella putrefaciens NCIMB 8768

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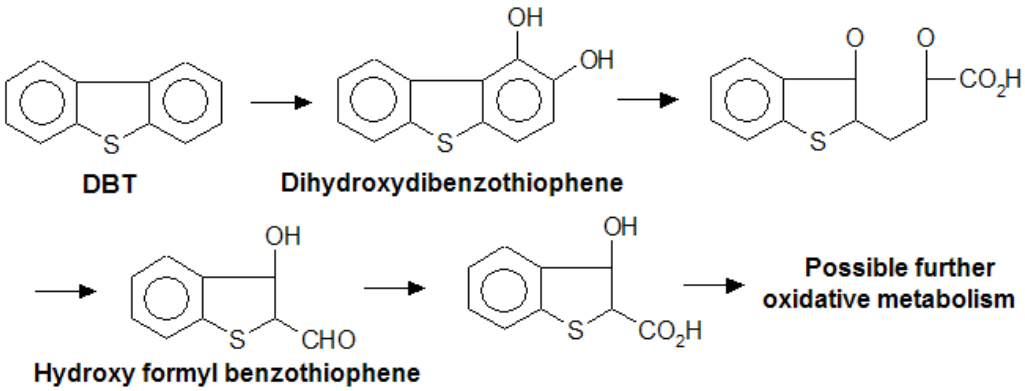
The desulfurization ability of *Shewanella putrefaciens* strain NCIMB 8768 was studied and its activity profile was compared with the widely studied strain *Rhodococcus erythropolis* strain IGTS8. Dibenzothiophene (DBT) is a recalcitrant thiophenic component of fossil fuels especially among diesel blend stocks. DBT in basic salt medium (BSM) at a final concentration of 0.3, 0.6 and 0.9 mM was supplied to the microbes as the sole sulfur source. Experimental results showed that *S. putrefaciens,* similar to other biodesulfurization organisms, converted DBT to the end product 2-hydroxybiphenyl (HBP), as detected by the Gibbs assay and HPLC. Cells cultivated in medium containing 0.3 mM of DBT showed the highest desulfurization activity, with a maximum specific production rate 43.5 mmol/L of HBP.

Keywords: biodesulfurization, dibenzothiophene, *Shewanella putrefaciens.*

1. INTRODUCTION

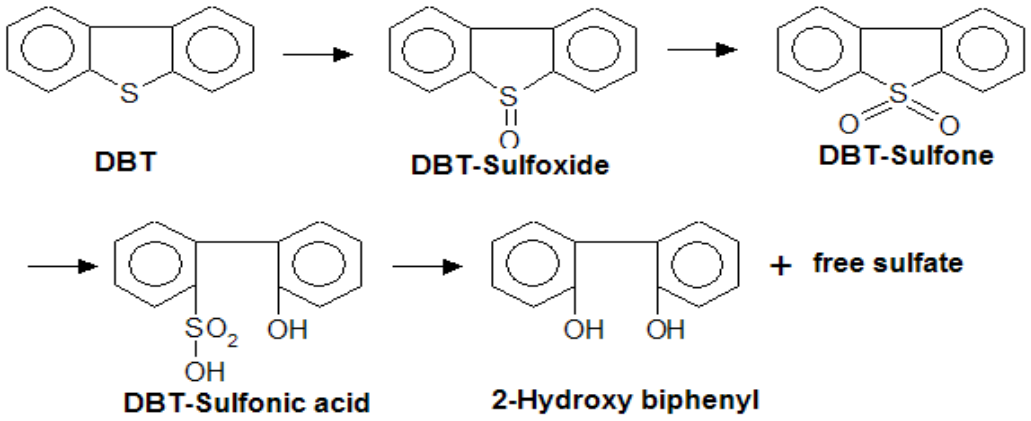
Clean fuel research, including into improvement of the processes of desulfurization and dearomatization, has become an important focus of environmental catalysis studies worldwide. Sulfur contained in diesel fuel is an environmental concern because the sulfur is converted to SO*x* during combustion, which not only contributes to acid rain, but also poisons the catalytic converters now widely installed for exhaust emission treatment. The problem of sulfur removal has become more apparent due to the high sulfur contents in crude oils and the low limit of sulfur content in finished fuel products specified by regulatory authorities. The Environmental Protection Agency of the United States (EPA) had set a target to reduce the sulfur content of diesel fuel from 500 ppm to 15 ppm for the year 2006 and 10 ppm will become the maximum content for sulfur by 2008 (Yang et al., 2005). Thiols, sulphides and thiophenes are readily removed by hydrodesulfurization (HDS), but up to 70% of the sulfur in petroleum is found as DBT and substituted (methylated) DBTs, which are particularly recalcitrant to HDS treatment compared with mercaptans and sulfides (Yang et al., 2005; Le Borgne et al., 2003). Microbiological methods to desulfurize hydrocarbon streams offer a potentially attractive alternative to traditional chemical engineering methods. Biological processes require relatively mild conditions (low pressures and low temperatures), which could be a major advantage of biodesulfurization. Therefore most studies on biodesulfurization (BDS) have focused on the removal of thiophenic sulfur compounds, of which DBT is widely accepted to be representative. DBT is indeed considered to be particularly relevant as a model compound for the forms of thiophenic sulfur found in fossil fuels, such as crude oils, coals or bitumen of particular geographic origins, and the various refining intermediates and fuel products manufactured therefrom (Olson, 1998). Various bacteria are able to metabolize DBT. In the 1970s, Kodama

showed that microörganisms could attack DBT without removing sulfur (Scheme 1). This microbial transformation of DBT by the Kodama pathway involves cleavage of one of DBT's aromatic rings so it has not been commercially developed because it did not remove the sulfur atom from the heterocyclic compound, and moreover led to a decrease in the fuel's calorific value through the oxidation of benzene ring.



Scheme 1. Kodama pathway for the degradation of DBT. The product, hydroxyl formyl benzothiophene, retains the sulfur moiety (Kodama et al., 1973).

Another biological pathway that is of interest to researchers involves four enzymatic steps and is therefore called the 4S pathway (Kilbane 1989). This pathway (Scheme 2) is more interesting for practical applications, since sulfur removal takes place without destruction of the aromatic rings.



Scheme 2. 4S pathway for DBT desulfurization by *R. erythropolis* IGTS8. The DBT desulfurization pathway results in the formation of 2-hydroxybiphenyl (HBP) and sulfate.

In a previous investigation, it has been found thatusing the bacterium *S. putrefaciens* strain NCIMB 8768 in clay desulfurization, sulfur odour was reduced, and the use of this bacterium for coal desulfurization has been examined (Prayuenyong, 2001). The present study builds on that previous work to study in more detail the desulfurization ability of the bacterium *S. putrefaciens*, and to compare its biodesulfurization activity profile with that of the widely studied *Rhodococcus erythropolis* strain IGTS8.

2. MATERIALS AND METHODS

2.1 Chemicals

Nutrient agar and broth were from Oxoid (UK), dibenzothiophene (DBT) (99%),dimethyl sulfoxide, 2-hydroxybiphenyl (HBP), Gibbs reagent and all other chemicals were from Fisher Scientific (UK). Water was highly purified by ion exchange and reverse osmosis in an Elga installation.

2.2 Bacterial strains

Shewanella putrefaciens (NCIMB 8768) was purchased from National Collections of Industrial and Marine Bacteria Ltd (Aberdeen, UK). *Rhodococcus erythropolis* IGTS8 (ATCC 53968) from American Type Culture Collections (Virginia, USA) was used for comparison.

2.3 Medium and growth conditions

Basic salt medium (BSM), a sulfur-free medium containing 2.44 g KH2PO4, 5.47 g Na2HPO4, 2.00 g NH4Cl, 0.2 g MgCl2.6H2O, 0.001 g CaCl2.2H2O, 0.001 g FeCl3.6H2O, 0.004 g MnCl2.4H2O and 1.84 g glycerol per litre of deionized water, was used. DBT dissolved in ethanol was added as the sole sulfur source to give final concentrations of 0.3, 0.6 and 0.9 mM. Cells were cultivated in 250 mL flasks containing 100 mL of medium on a rotary shaker operated at 100 rpm for one week at 30 °C.

2.4 Resting cell reaction

Cells were grown until the mid-exponential growth phase and harvested by centrifugation (Hettick-EBA 20- Germany) at 6000 rpm for 15 min. The cell pellets were washed twice with Ringer's solution. The cells were then resuspended in the same solution, giving $A600 = 1.0$, and used on the day of harvesting. One mL of inoculum was added to 250 mL flasks containing 100 mL of BSM with 0.3 mM of DBT-ethanol solution and incubated at 30 °C (shaking at 100 rpm).

2.5 Detection methods

Growth was monitored via optical density at 600 nm; the concentration of cells was determined from the linear relationship between OD600 and cell concentration (Stanier, 1976) while dry

weights were determined after washing cultures three times with Ringer's solution and drying in a 100 °C oven until a constant weight was obtained. Determination of HBP and DBT was carried out by Gibb's assay and high-performance liquid chromatography (HPLC). All experiments were done in triplicate.

2.5.1 Gibb's assay

Desulfurization activity was monitored using the Gibb's reagent (2,6-dichloroquinone-4-chloroimide) to detect the 2-hydroxybiphenyl (HBP) produced from DBT. Gibb's reagent reacts with the aromatic hydroxyl groups at pH 8 to form a blue complex that can be monitored spectrophotometrically at 610 nm after 30 min incubation at room temperature (Kayser et al., 1993). Briefly the assay consists of the following steps: aliquots (5 mL) of culture broth are centrifuged (1200 rpm, 10 min) to remove cells. The supernatant (2 mL) was then transferred to an Eppendorf tube. 0.1 g Gibb's reagent was dissolved in 10 mL absolute ethanol in a test tube, and 20 μL were added to each Eppendorf tube containing 2.0 mL supernatant adjusted to pH 8. The assays were incubated at 30 °C for 30 minutes to complete the colour development. The absorbance of the supernatant was determined at 610 nm and converted to HBP concentration based on a standard curve (Monticello, 1993).

2.5.2 HPLC

The concentration of DBT and HBP were analysed by high-performance liquid chromatography using a Shimadzu HPLC Model LC-10AD VP equipped with a Nova Pak phenyl column (3.9 × 150 mm) together with a guard column. Elution was isocratic with 60% acetonitrile and 40% water at 1.5 mL min–1, and detection used a 117 UV detector fixed at wavelength = 233 nm. The

mobile phase, a mixture of HPLC grade water and acetonitrile, was sonicated for 10 minutes and further degassed with helium before use.

3. RESULTS AND DISCUSSION

Biodesulfurization activity comparison: both strains grew in BSM+DBT and produced HBP DBT degradation (Figure 1). The DBT **concentration** was halved after 3 days incubation. HBP analysis confirmed that similarly to *R. erythropolis, S. putrefaciens* desulfurized DBT through the selective cleavage of the C-S bonds, resulting in the accumulation of HBP. The detection of HBP when *Shewanella* utilized DBT as the sole sulfur source suggests that the DBT-desulfurizing pathway is the so-called 4S pathway as proposed by Kilbane et al. (1992) for degradation using *Rhodococcus*.

This type of reaction is desirable for practical desulfurization since the sulfur atom is removed without C-C bond cleavage, that is, without loss of calorific energy. The amounts of HBP produced and DBT degraded of *R. erythropolis* were not quite equivalent however (Figure 1) (Kayser et al., 1993).

It has been reported that HBP is toxic to bacterial cells; hence biodesulfurization will be inhibited by accumulation of HBP (Zhang et al., 2005). This is a complicating factor in our batch experiments, i.e. that the end product HBP is somewhat inhibitory to cell growth and possibly therefore to desulfurization activity.

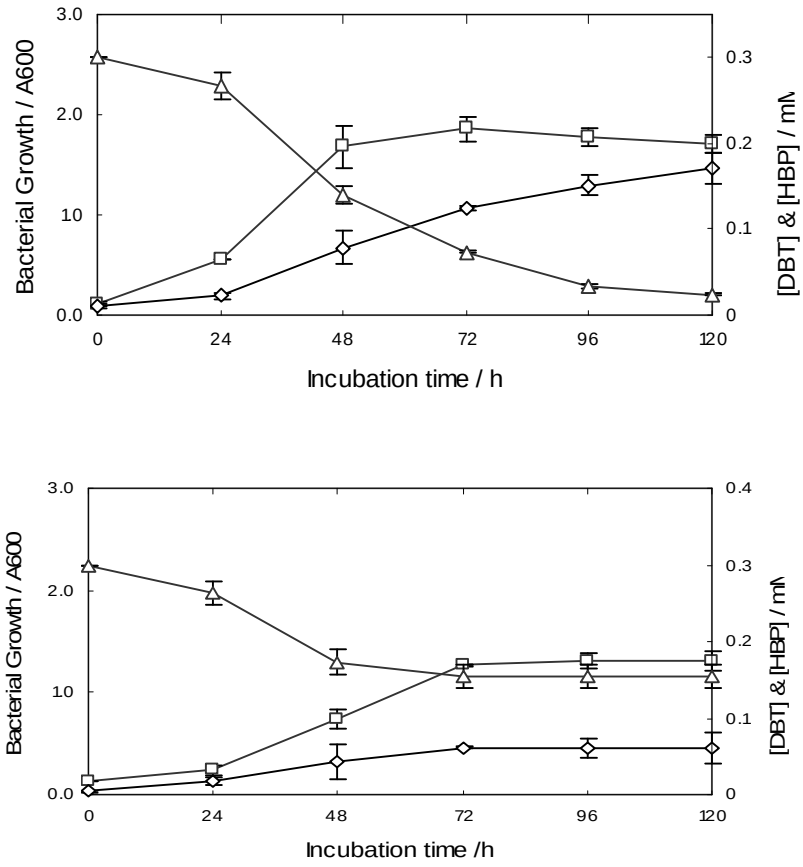


Figure 1. DBT desulfurization by (A) *R. erythropolice* IGTS8, and (B) *S. putrefaciens* NCIMB in DBT at a concentration of 0.3 mM as the sole sulfur source. Key: □, bacterial growth; □, concentration of HBP; □, concentation of DBT.

Effect of DBT concentration (Figure 2): *S. putrefaciens* could only grow in BSM medium with DBT at an initial concentration of 0.3 mM as the sole sulfur source. At higher concentrations, bacterial growth was inhibited, presumably because the bacteria could not tolerate the high concentration of what is actually a bacteriotoxin.

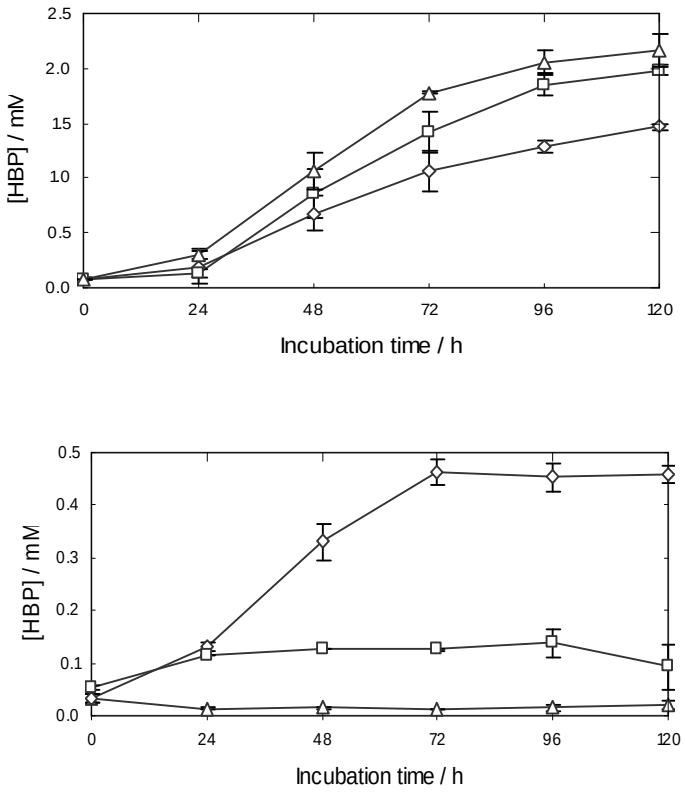


Figure 2. Production of HBP by (A) *R. erythropolis* and (B) *S. putrefaciens* at different concentrations of DBT. Key: □, 0.3 mM; □, 0.6 mM; □, 0.9 mM.

4. CONCLUSION

Shewanella putrefaciens showed desulfurization activity and detection of HBP in the culture medium is evidence that this strain desulfurized DBT via the 4S pathway. Therefore, *S. putrefaciens* is a potentially useful desulfurizing bacterium. However, regarding its effectiveness we consider it necessary to confirm what is the rate-limiting step in DBT desulfurization by *S. putrefaciens*, as a first step towards determining whether the genes encoding the DBT desulfurizing enzymes of *Shewanella* are different from those of other desulfurizating bacteria such as *R. erythropolis* IGTS8.

Moreover, more experiments need to be carried out to investigate parameters like pH and carbon source and enriched media on the growth of the bacteria and their desulfurization activity.

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