Biodesulfurization of Dibenzothiophene by a Newly Isolated Thermophilic Bacteria Strain

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ABSTRACT: *Microbiological analyses of soil chronically exposed to petroleum complex compounds of some oil springs in south of Iran resulted in isolation and purification of a new native thermophilic strain which is capable to desulfurize petroleum sulfur compounds by 4-S mechanism. Dibenzothiophene (DBT) was selected as a complex sulfur compound model and many experiments were done to identify the metabolic pathway. The results of these experiments show that DBT is ultimately converted to 2-hydroxybiphenyl (2-HBP) and sulfite. This is a special metabolic pathway in that there is no effect on the carbon skeleton of organic compounds and would be ideal for desulfurization to upgrade the petroleum products because it keeps the remaining hydrocarbon molecules fully active as energy sources without any loss of their thermal units. At the next step, some physical and chemical properties of main culture were optimized as follows: 6gr/lit glucose, 4gr/lit ammonium chloride, 0.15 mM DBT, pH= 7 and temperature= 45°C. During 6 days, growing cells of this microorganism can convert 87.5% of DBT in 250 ml flask. At last, kinetic analysis has been done and Michaelis-Menten equation qualified. Equation parameters V_{max} and K_M calculated and data led to 0.548 mM h^{-1} and 0.458 mM, respectively.*

KEY WORDS: *Dibenzothiophene, Thermophilic strain, Biodesulfurization, Complex sulfur compounds, Michaelis-Menten equation.*

INTRODUCTION

Release of sulfur-oxides to the atmosphere through combustion of petroleum and it's distillates causes serious environmental problems such as acid rain and world deforestation .For instance, their sulfur content will have to be as low as 10 or 15 ppm (w/w) by 2011 in Europe and in US while the initial sulfur content of crude oils typically varies from 0.04 to 6 wt.% [1].

Hydrodesulfurization (HDS) processes used in the refinery industry can not completely remove some heterocyclic organosulfur compounds such as

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1021-9986/06/3/65 7/$/2.70

dibenzothiophene (DBT) present in middle distillates and biodesulfurization (BDS) has been considered as a possible alternative to HDS of diesel oils[2,16]. Several aerobic strains able to specifically oxidize covalently-bound sulfur of DBT without breaking carbon–carbon bonds have been isolated. DBT is ultimately converted to 2-hydroxybiphenyl (2-HBP) and sulfite and the metabolic pathway followed has been elucidated [3].

Four genes are involved in this multienzymatic biodegradation process which is called 4-S metabolic mechanism: (i) dszC encoding a DBT-monooxygenase responsible for the first two oxidations of DBT to sulfoxide and then to sulfone; (ii) dszA encoding a DBT-sulfone monooxygenase oxidizing DBT-sulfone to 2-hydroxybiphenyl-2-sulfinic acid; (iii) dszB encoding a 2-hydroxybiphenyl sulfinate desulfinase that converts HBP-sulfinate to 2-hydroxybiphenyl and sulfite ; and (iv) dszD, encoding a NADH-flavin mononucleotide oxido-reductase supplying FMNH2 needed for the first three oxidations [1].

Some mesophilic DBT-desulfurizing microorganisms have been isolated to date, for example *R. erythropolis* D-1[4] , *Rhodococcus* sp. IGTS8 [5,6], *R. erythropolis* KA2-5-1[7,8], *Mycobacterium* sp. G3 [9] and *Rhodococcus* sp. Strain P32C1 [10].

Since these DBT-desulfurizing bacteria are meso-philic, the DBT-desulfurizing ability is high only around 30°C and decreases at higher temperatures. Diesel oil, however, following HDS is at a temperature much higher than 30°C, and a cooling system is necessary for the practical use of these DBT-desulfurizing bacteria. If biodesulfurization can be performed around 45°C and higher, it would be unnecessary to cool the HDS-treated diesel oil to ambient temperatures. In addition, contamination by undesirable bacteria which affect the biodesulfurization process would be avoided at 45°C and higher. For these reasons, biodesulfurization under high-temperature conditions is more advantageous than that by mesophilic microorganisms [11, 13].

By these viewpoints, some thermophilic DBT-desulfurizing microorganisms have been isolated to date, for example *Bacillus subtilis* WU-S2B [11], *Mycobacterium phlei* WU-F1 [12], *Paenibacillus. Bacillus* [13], *Paenibacillus* sp. All-2 [14]. In the table 1 a list of previously isolated strains has been gathered. Those data are for growing cells and it must be mentioned that the reported yields for resting cells are higher than that of growing cells. In this paper, we describe the desulfurization of DBT, by a moderately thermophilic bacterium which is newly isolated from polluted soils in the south of Iran through screening. This is the first reported themophilic native microorganism that can be used in the practical plants. At the next step, we examined the effects of some chemical and physical parameters on the desulfurizing ability of this microorganism and evaluated the kinetic patterns to maximize production of biomass. The practical process of biodesufurization includes two microbial steps. First, we need to produce high amounts of biomass in the fermentors to inoculum to the two phase bioreactors where next microbial step happens. Then, in two phase bioreactors, desulfurization will happen and growth of microorganism is not important so far. Also in the literatures is mentioned that the optimal condition for producing high amounts of biomass is not far away from the optimal condition of achieving highly active microorganism.

MATERIALS AND METHODS

Chemicals

DBT (Merck, Germany), Dibenzothiophene sulfone (Aldrich, Milwaukee, Wis., USA), 2-HBP (Wako, Osaka, Japan). All other chemicals were of analytical grade, commercially available and used without further purification.

Analytical methods

The cell concentration was determined from the optical density at 660 nm (OD_{660}). For this microorganism, a linear relationship between OD and dry cells weight was obtained in the range from 0.06 to 1.2 (absorbance); gr dry cells (gr/l) = 1.8 OD + 0.069. DBT and its product 2-hydroxybiphenyl (2-HBP) were measured using high-performance liquid chromatography (Dual λ Absorbance Detector , type 2487; Waters, Milford, USA) equipped with a μ Bondapack™ C_{18} column (4.6×250mm cartridge; Waters, Ireland). The mobile phase was acetonitrile-water (1:1, v/v) and the flow rate was 1.5 ml/min. 2 ml of culture broth or reaction mixture was acidified to pH 2.0 with 6 M HC1 and extracted with 2 ml ethyl acetate. Samples were mixed and after centrifugation (2000 rpm, 20 min), the ethyl acetate layer was filtered through a

0.22 μ m Millex® membrane filter (Bedford, USA), and 20 μ l of filtrate was used for HPLC analysis. After the separation of compounds in the HPLC column the absorbance of the effluent solution was continuously measured mainly at 280 nm. The amounts of DBT and other compounds were calculated from standard calibration curves. Also in the first steps of isolation, because of the ease of use Gibbs assay [3], a special colorimetric method for determination of 2-HBP, was used to detect 2-HBP. With this test we can measure the amount of 2-HBP with a low error with respect to the HPLC results but in a shorter time. Also this method is a nice way to detect the presence of 2-HBP at the first steps of isolation of microorganisms where we are looking to opt 2-HBP producing microorganism.

Isolation and cultivation of DBT- desulfurizing microorganisms

Microorganisms able to utilize DBT as a sole sulfur source were isolated from various oil polluted soils, containing a high content of sulfur in the south of Iran and from Tehran refinery sites. Samples from the same origin were mixed. Soil aliquots were used to inoculate Sulfur-free Medium A [15] with DBT as a sulfur source and 6 g l^{-1} glucose as carbon source .The composition of the medium A is presented in table 2. Samples were incubated in 250 ml flasks contain 50 ml culture, on an orbital shaker (180 rpm, 45 °C). DBT was dissolved in ethanol and added to sterilized medium from stock solution (27 mM) to achieve 0.57 mM concentration in culture. Successive transfers in the same conditions achieved enrichments in microbial microorganisms using organic sulfur of DBT. After at least three subcultivations of the initial culture, repeated streaking on solid A medium containing 15 g l^{-1} agar were carried out to obtain isolated colonies. Growth of the purified microorganisms with DBT as a sulfur source was verified in liquid media. The microorganisms were then stored in 50% glycerol at −80 °C.

RESULTS AND DISCUSSION

Microorganism primary properties

To isolate DBT-desulfurizing microorganisms, many oil polluted soils and oil sludge samples were collected as sources of microorganisms. A small amount of each sample was suspended in distilled water at an appropriate concentration, and 1 ml of this suspension was inoculated into a 250 ml flask containing 50 ml of A medium with DBT at 45°C for 3-5 d. Then, aliquots of turbid cultures were transferred into fresh medium. After 3 sub cultivations, the culture broth was appropriately diluted with distilled water and spread onto solid medium plates. After cultivation at 45°C for 3-5 d, colonies formed on the plates were again inoculated into liquid A medium with DBT. After purification of microorganisms we achieved 12 microorganisms. We inoculated these microorganisms to 250 ml medium A in the erlenmeyer flasks. During this time we tested medium composition regarding production of 2-HBP. We used Gibbs assay and HPLC to detect 2-HBP in the medium. Microorganisms that use DBT but didn't produce 2-HBP were eliminated and finally one bacterium, which could desulfurize DBT at 45°C, was isolated. At the next step, as previously mentioned, we needed to optimize culture condition to achieve high amounts of biomass. Hereupon, optimum initial DBT concentration for growth was found to be 0.15 mM and it was demonstrated that the growth of microorganism was inhibited by DBT concentrations above 0.6 mM. This microorganism is a rod-shaped bacterium with the approximately length of 1.0-2.5 μm. Table 3 shows other primary properties of isolated microorganism. Other optimum points are as follows: Optimum concentrations of glucose and NH_4Cl for growth of this microorganism were determined to be 6gr/lit for glucose and 4gr/lit for NH_4Cl. (Fig. 1) Also the optimum temperature and pH for growth were 45°C and 7, respectively. According to Fig. 2 growth of microorganism was inhibited completely in pH lower than 5. More examinations about the microorganism has been carried out by IROST and microorganism was categorized in *bacillus* genus.

Microorganism proficiency in biodesulfurization

Newly isolated microorganism can use DBT as sulfur source and decrease 87.5% of DBT through 6 days and produce 2-HBP according to Fig. 3. In the first two days of cultivation, microorganism shows very low 2-HBP production and at the end of 6 days, conversion of DBT to 2-HBP does not proceed stochiometrically. The reason for such treatment maybe the existence of some intermediate products which are mentioned in metabolic pathway and accumulate in the media. It should be noted

Table 1: List of microorganism used for biodesuforization with their reported activity at the growing state.

Reported Data	Strain Name
DBT > 95% conversion to 2-HBP. Attacks alkylated DBT's model compounds: ±25% conversion, in diesel fuel: ±11% conversion. Converts BT analogous to the 4S-pathway. [13]	*Paenibacillus* sp. A11-2 Thermophilic (55ºC)
This BT desulfurizing microorganism can assimilate various alkyl BT's, but cannot use DBT as the sole sulfur source. When genetically modified, strain T09 also utilized alkylated BT's and DBT's.[2]	*Rhodococcus* Strain T09
Desulfurizes naphthothiophene (80% in 7 days) and benzothiophene (57% in 5 days) starting from a sulfur concentration of 0.27 mM. [2]	*Rhodococcus* Strain WU-K2R
Attacks alkyl DBT's in distillates of the diesel range. Diluted diesel fuel with 20 ppm S: approx. 30% efficiency at a VFO (volume fraction oil) of 0.1%. Diluted light cycle oil with 669±40 ppm S is reduced to 56±4 ppm S, at a VFO of 2%.[2]	*Rhodococcus* Strain ECRD-1
DBT: 0.28 mg S/ (L dispersion. h) at a VFO of 10%. In case of 0.3 wt% S Diesel fuel: 0.91mg S/(L dispersion . h); 20% efficiency at a VFO of 10% 0.99mg S/(L dispersion . h); 33% efficiency at a VFO of 5%[2]	*Nocardia* Strain CYSK2
Desulfurizes BT (but not DBT), during growth a phenolic compound accumulates. [2]	*Gordona* sp. Strain 213E
Batch reaction wit DBT $\mu = 0.153\ h^{-1}$ 2-HBP production: 1.8 $\mu M.h^{-1}$ $\mu_{max} = 0.235\ h^{-1}$[2]	*R. erythropolis* N1-36

Table 2: Composition of Medium A used at the different steps of cultivation of microorganism.

Main Media	(gr)amount	Metal solution	(gr)Amount	Vitamin mixture	(mg)Amount
K_2HPO_4	4	Na_2MoO_4	0.1	Calcium pantothenate	400
KH_2PO_4	0.5	$FeCl_2.4H_2O$	0.5	Inositol	200
NH_4Cl	1	$ZnCl_2$	0.5	Niacin	400
$MgCl_2.6H_2O$	0.2	$MnCl_2.4H_2O$	0.5	Pyridoxine hydrochloride	400
$CaCl_2$	0.02	$CuCl_2$	0.05	P-Aminobenzoic acid	200
NaCl	0.01	$Na_2WO_4.2H_2O$	0.05	Cyanocobalamin	0.5
Metal solution	10ml	Distilled Water	1000ml	Distilled Water	1000ml
Vitamin mixture	1ml				
Distilled Water	1000ml				

Table 3: Some primary properties of isolated microorganism.

Spore	Dimension(micrometer)	shape	Catalase	Oxidase	Gram	Test name
Forms spore	1.5-3.5	Rod-shaped	+	-	+	Result

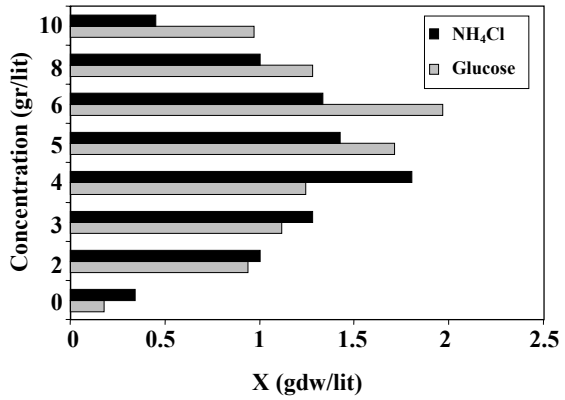

Fig. 1: Optimum concentrations of glucose and NH_4Cl to achieve maximum cell concentration after 6 days cultivation.

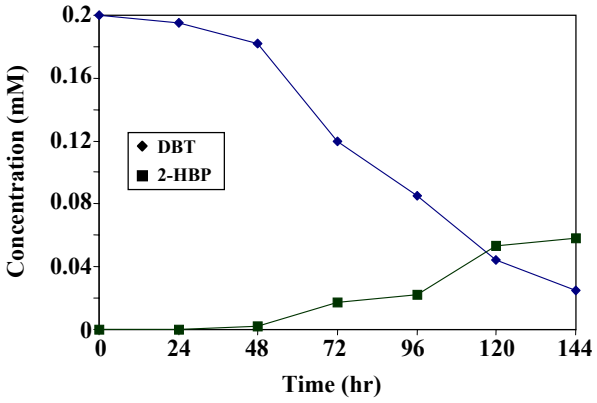

Fig. 3: Conversion of DBT to 2-HBP during 6 days cultivation.

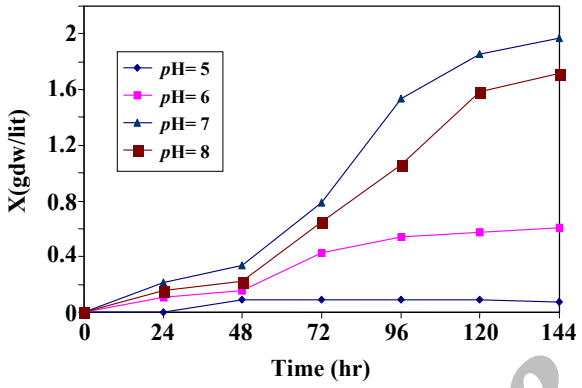

Fig. 2: Optimum growth of strain at initial pH=7 and decreasing of strain growth in other initial pH's.

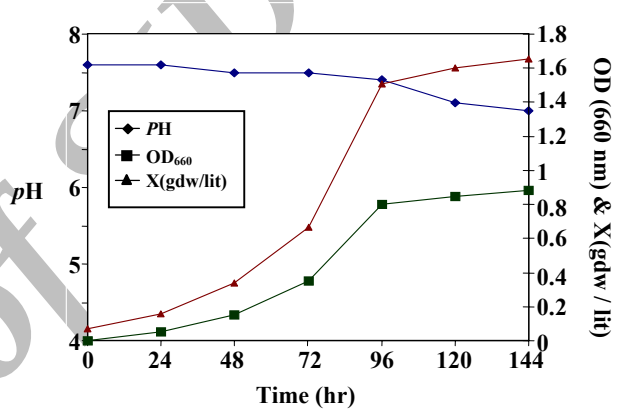

Fig. 4: pH variation in media and growth of microorganism during 6 days cultivation.

that we can reach to stochiometric amounts of 2-HBP by increasing the plotting reaction time. Microorganism activities during growth in terms of DBT disappearance (nM DBT/gdw/hr) and 2-HBP appearance (nM 2-HBP/ gdw/hr) were calculated. Derived data show that microorganism activity reaches the maximum activity of 5.13 nM DBT/gdw/hr for DBT disappearance and 1.24 nM 2-HBP/gdw/hr for 2-HBP production at its logarithmic growth phase.

Some peaks obtained from HPLC were investigated and these peaks demonstrate that during growth of microorganism, DBT concentration is reduced and accordingly 2-HBP is produced. We examined the variation in pH and cell density during growing phase.

According to Fig. 4 pH decrease is negligible and initial pH=7.6 at the end of cultivation time reach 7.0. This microorganism can not grow in pH lower than 5.We also find out that the variation in pH during the growth of microorganism depends on components of media. (Data not shown) In cultures with high buffer capacity, pH variation is low and in those with lower buffer capacity, we can see higher difference between initial and final pH. Obtained linear equation was used in Fig. 4 to convert OD_{660} to dry cell weight.

The evaluation of kinetic patterns

To analyze the desulfurization pattern we probed the reaction in 5 liter jar fermentor. Samples were harvested during logarithmic growth phase with 2 hr intervals (Δt) and were analyzed. Biodesulfurization was carried out in 45°C and aeration rate of 2.5 VVM. By plotting $1/\mu$ vs. 1/S and obtaining moderately linear line we qualified the Michaelis-Menten equation as found in *R. erythropolis* KA2-5-1 [17] and *Pseudomonas delafieldii* R-8 [18], as follows:

$$\mu = \frac{dS}{dt} = \frac{V_{max} \times S}{K_M + S} \leftrightarrow \frac{1}{\mu} = \frac{1}{V_{max}} \times \frac{1}{S} + \frac{1}{V_{max}}$$

And μ was calculated approximately as follows:

$$\mu = \frac{1}{X} \times \frac{\Delta X}{\Delta t}$$

Where S is the concentration of DBT (mM) $\overline{X}$ is the average cell concentration between intervals, V_{max} the limiting maximal velocity (mM h^{-1}) and K_M is the Michaelis constant (mM). The values of rate constants, the limiting maximal velocity (V_{max}) and Michaelis constant (K_M), for desulfurization of DBT were fitted as 0.548 mM h^{-1} and 0.458 mM, respectively.

CONCLUSIONS

In this research for the first time in Iran a moderately thermophilic microorganism has been isolated and has been categorized in *bacillus* species. This microorganism can grow in 45°C and use DBT in a special metabolic pathway in the way that there is no effect on the carbon skeleton of organo sulfur compounds.

This microorganism can reach the concentration of 1.7 gr/lit of biomass in 250 ml erlens. These results are for growing cells of microorganism and this microorganism in the fermentors must be grown to the amount required for the two phase bioreactors. The results have shown that at the DBT concentrations larger than 0.15 mM microorganism growth will be suppressed.

Newly isolated microorganism can use DBT as sulfur source not carbon source and decrease 87.5% of DBT through 6 days and produce 2-HBP. The growth curve and material balance show that some DBT component has been disappeared. It seems that this amount of DBT presents in the medium as the intermediate compounds. Also the optimum temperature and pH for growth were 45°C and 7, respectively. Growth of microorganism was inhibited completely in pH lower than 5.

Acknowledgment

We appreciate the financial support of project by Iranian central oil fields company (ICOF) to complete a part of this work. We also wish to thank Miss Zahra Ghobadi, Mr. Salamat and Mr. Shams for their assistance in the isolation of new microorganism.

Received : 3rd February 2005 ; Accepted : 26th December 2005

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