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Bio degrading ability of organo-sulphur compound of a newly isolated microbe *Bacillus sp.* KS1 from the oil contaminated Soil

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ABSTRACT

The techniques used to reduce the amount of sulphur in fossil fuels before combustion, include physical, chemical and biological processes (Biodesufurization).Generally the biological process is based upon the breakdown of organo-sulfur compounds by potential microorganism having many advantages over the conventional chemical processes, which is performed under mild conditions with no harmful reaction products without affecting the octane number of the fuels. In this context a dibenzothiophene (DBT) degrading bacteria, which can use DBT as the sole source of sulphur was screened from soil contaminated with crude oil collected at the site of Chennai Petroleum Corporation Limited's refinery in Manali, Tamilnadu, India. This gram-positive, aerobatic (rod shaped) bacteria with the ability to desulfurize DBT was identified as Bacillus sp. and designated as strain KS1. HPLC analysis and Gibbs assay were performed to determine the quantity of desulfurized product 2-Hydroxybiphenyl (2-HBP) in which, its concentration was increased to 0.11mM along with the degradation of DBT over a period of 7 days and the desulfurization trait was expressed at increasing levels during the exponential growth phase of the microbe and then declined in stationary phase. This isolate did not grow on Thiophene-2-carboxyllic acid taking as the sulphur source. The desulfurization activity of KS1 resting cells in a two-phase system was higher than that of in aqueous media.

Key Words: Biodesufurization, Organo-sulfur, DBT, Gibbs assay, 2-HBP, Degradation.

INTRODUCTION

Sulphur dioxide (SO_2) during combustion of fossil fuels like coal, petrol, diesel oils and other fuels released into the atmosphere creating a serious environmental hazard like air pollution, smog, acid rain and respiratory problems for human beings. To overcome this critical condition, it is very necessary to produce large amounts of low sulphur containing petroleum and coal without affecting its calorific values [1]. Therefore, desulfurization is important tools in petroleum processing. Biodesulfurization using microbial catalysts capable of desulfurizing DBT and their alkylated compounds is suitable for this purpose [2].

Presently hydrodesulphurization processes mainly used in the so many oil refinery industry can not completely remove organo-sulphur compounds such as dibenzothiophene (DBT), hence biodesulfurization has been considered as a possible alternative for this. Several types of microbial strains able to specifically oxidize and break covalently bound sulphur of DBT without breaking carbon–carbon bonds have been reported in many literature and in this process DBT is ultimately converted to 2-hydroxybiphenyl (HBP) and sulphite by 4S path ways [3,4] .

In the molecular biology aspect, biodesulfurization activity is shown due to presence of dsz operon A,B,C and an accessory gene dsz D [4] . In this multienzymatic biodegradation process, four genes are involved which is called 4-S metabolic mechanism. Dibenzothiophene (DBT) is the model compound for biodesulfurization since it is the most recalcitrant compound for the conventional processes of desulfurization and is a major constituent of almost all fossil fuels like coal and petroleum [5].

Generally, the desulfurization of fuel is carried out by hydrodesulphurization process which is carried out at high temperature (450°C) and very high hydrogen pressure (150–200 atm). CoMo and NiMo are used as metal catalysts for this process. Deep desulfurization is associated with fuel being in a reactor for a long time [6, 7]. All of the above process characteristics lead to a high cost of products. Additional and fundamental drawback of hydrodesulphurization process is the hydrogenation of olefins which reduces the octane number that decreases the quality of the petroleum fuel but on the contrary biodesulfurization process is carried out at normal temperature and pressure, does not require hydrogen, and proceeds with high selectivity without affecting octane number and not producing undesirable products [8, 9].

In this paper, newly we describe the isolation and characterization of the desulfurizing bacterium *Bacillus sp.* KS1 from oil contaminated fields of oil refinery regions (Chennai Petroleum Corporation Limited's refinery, Tamilnadu, India) which has broader substrate specificity for DBT and can be able to desulfurize the organo-sulphur successfully.

MATERIALS AND METHODS

Isolation and cultivation of DBT desulphurizing microorganism

Microorganisms which can utilize DBT as a major sulphur source have been isolated from various oil contaminated soils from the different site of Chennai Petroleum Corporation Limited's refinery in Manali, near Chennai, Tamilnadu state, India. Soil aliquots (0.1gm) were used to inoculate Sulphur-free Medium A which consists of K_2HPO_4; 4 gm, KH_2PO_4; 0.5 gm, NH_4Cl; 1 gm, $MgCl_2.6H_2O$;0.2 gm, $CaCl_2$; 0.02gm, NaCl; 0.01gm, Metal solution; 10 ml, Vitamin mixture; 1 ml, with DBT (25Mm/Litre in ethanol) as a sulphur source and 6 g/litre glucose as carbon source [10] .The metal solution (Na_2MoO; 0.1 gm, $FeCl_2.4H_2O$; 0.5 gm, $ZnCl_2$; 0.5 gm, $CuCl_2$; 0.05 gm, $Na_2WO_4.2H_2O$; 0.05gm) and vitamin solution (Calcium pantothenate; 400 mg, Inositol; 200 mg, Niacin; 400 mg, Pyridoxine hydrochloride; 400 mg, P-Aminobenzoic acid; 200 mg, Cyanocobalamin; 0.5 mg) were prepared per 1000 ml of distilled water before preparation of the medium A and were added [11]. Samples were incubated in 250 ml flasks containing 50 ml media, on an orbital shaker (180 rpm, 45 °C). DBT was added after the sterilization of the medium. After five times sub cultivations of the initial culture, repeated streaking on the same media with 17 g/litre agar were carried out to obtain isolated colonies. The morphological analysis and biochemical test (Table:-1) for the isolated microbe was done. The growth and cultivation of the purified and isolated microorganism with DBT as a sulphur source was detected in liquid media. The selection of strain among the isolated strains was based on the

high desulfurization activity and the higher production rate of 2-hydroxyl biphenyl from DBT [12].

Estimation of DBT and 2-HBP
Measurement of metabolic by-product after degradation of DBT by the isolated strain was performed using Gibbs reagent (2,6-dichloroquinone-4-chloroimide) as follows. After centrifugation (2000 rpm for 10 min) of bacterial cultures the supernatant (250 µl) was taken and mixed with 30 µl of 1 M $NaHCO_3$ (pH 8.0). After that 25 µl of Gibbs reagent (0.1% in ethanol) was added and the culture broth was gently agitated in the room temperature for a period of 20 min .Finally the absorbance was then measured at 595 nm which is proportional to the amount of HBP produced in the culture [13].

The amount of degraded DBT was measured using HPLC (Dual λ Absorbance Detector) in specific time intervals with mobile phase acetonitrile-water (1:1, v/v) and the flow rate was 1.5 ml/min. 2 ml of culture broth was acidified by adding 6 M HCl to make the final pH of the reaction mixture is equal to 2 for its extraction with 2 ml of ethyl acetate. After the separation of compounds in the HPLC column the absorbance of the effluent mixture was measured at 280 nm. The amounts of DBT and 2-HBP were calculated from standard calibration curves [11].

Study of desulfurization activity in aqueous and Bi-phasic systems
50 ml of resuspended resting cells (10 gm dry cells/litre) in potassium phosphate buffer (0.1 M, pH 7.2) with 3 mM of DBT as sulphur source was used for the aqueous desulfurization process .On the other hand to investigate the ability of the isolated strain to desulfurize the DBT in oil itself , a biphasic system containing 25 ml of potassium phosphate buffer (0.1 M, pH 7.2) along with 25 ml of n-tetradecane (The hydrocarbons present in petroleum) was employed [14,15]. The desulfurization experiments were carried out in at 30 °C with 200 rpm. Samples were collected at each 2-hours interval for the estimation of DBT and 2-HBP by HPLC.

Various organo-sulphur compound specificity of strain
Different types of sulphur sources like benzothiphene, dibenzothiophene (DBT), thiophene, thiophene-2-carboxyllic acid, dimethyle sulfoxide, 5,5'-dithiobis (2-nitrobenzoic acid) and designed medium (Mixture of the all the above components) were inoculated to the media containing the microbe and the growth of the strain was analysed by taking the OD at 660 nm.

RESULTS AND DISCUSSION

Isolation and identification of DBT-desulphurizing microorganism
Total six microorganisms that were able to use DBT as the sole source of sulphur were isolated from 40 different oil contaminated soil samples out of which one strain designated as KS1 having DBT-utilizing ability was obtained. According to 'Bergey's manual of systematic bacteriology' and biochemical testing this strain was tentatively identified as the genus *Bacillus* [16].The results are noted in the Table:-1.The size of the isolated strain KS1 was found to be 0.9 ~1.3×0.8 ~ 1.1µm under bright field microscopy.

Growth and DBT degradation study of strain KS1
Figure:-1 shows the time course of DBT degradation with production of 2-HBP by *Bacillus sp.* KS1. This strain showed maximum growth at 168 hours of cultivation, and at this point of time the turbidity at 660 nm was found to be 0.24. After 168 hours of growth, the concentrations of DBT and 2-HBP in the culture medium were found to be 0.1mM and 0.11mM respectively. The concentration of DBT was decreasing while that of 2-HBP was increased along with the growth

of the *bacillus sp.* KS1 over the period of 7 days. Increasing concentration of DBT doesn't increase 2-HBP probably due to the fact that HBP produced is not efficiently discharged. Lag phase increased with increasing concentration of DBT while growth rate decreased which shows bacterium follows substrate inhibition type kinetics.

Table -1: Results of bio chemical test to identify the isolated strain

Characteristics	***Bacillus* strain**
Gram staining	gram-positive (rod shaped)
Colony morphology	The margin is undulate, with circular form and flat elevation
Cell size (µm)	0.9 ~1.3×0.8 ~ 1.1
Oxygen requirements	Aerobatic in nature
Catalase reactions	Catalase positive
Indole Test	Negative for indole production.
Reactions in glucose fermentation broth	Cannot ferment glucose.
Reactions in lactose fermentation broth	Unable to ferment lactose.
Starch hydrolysis	All the starch in the medium near the microbe has been hydrolyzed by extracellular amylases.
Nitrate Broth reactions	Can reduce nitrate to nitrite
Motility Test	Motile in nature
Butanediol fermentation	Positive

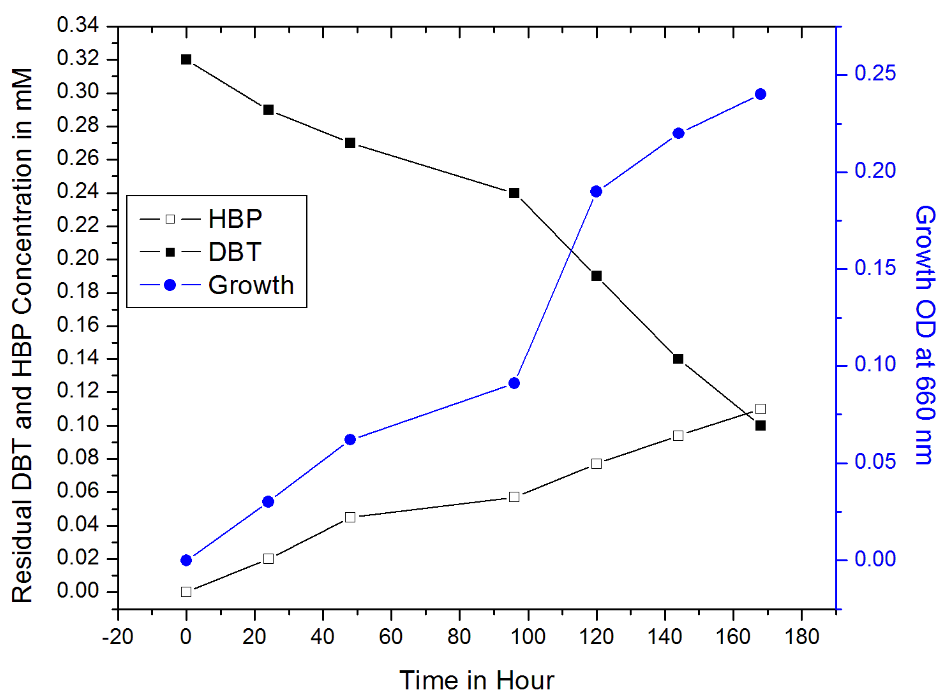

Fig.1: Dibenzothiophene (DBT) degradation (filled cubes) and residual production of 2-HBP (open cubes) during growth (filled circles) of *Bacillus sp.* KS1.

Strain KS1 grew well on DBT, thiophene, 5,5'-di thio bis (2-nitrobenzoic acid), dimethylsulfoxide and designed medium but growth on thiophene-2-carboxyllic acid was not considerable ($OD^{660} < 0.1$). The range of substrates utilized as sulphur sources by KS1 is similar to that of some reported desulphurising strains [17,18]. These results are shown in figure 2.

Various organo-sulphur compound specificity of strain KS1

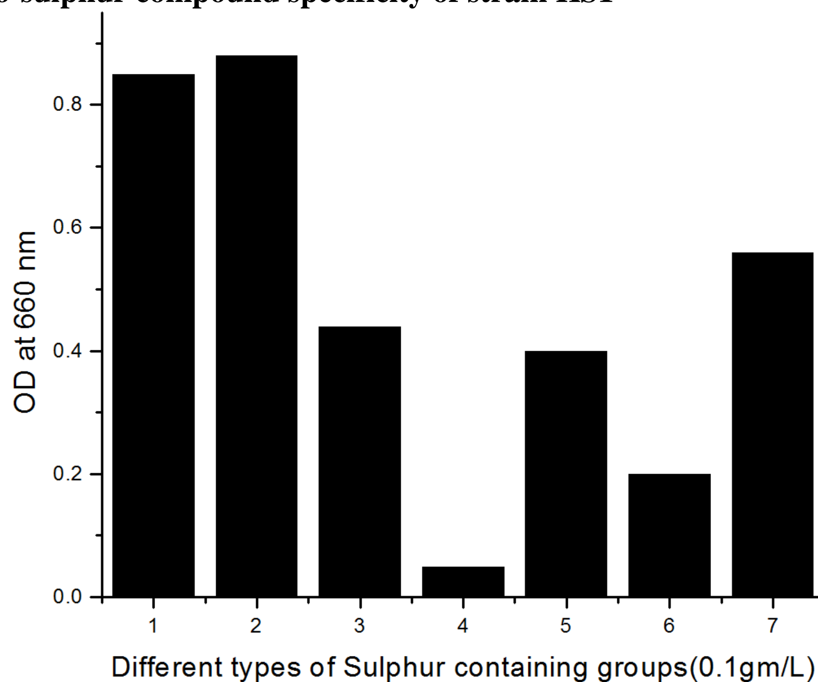

Fig.2: Study of Sulphur compound specificity for the strain KS1 and its growth at 660 nm over a period of 16 hours

1. Benzothiphene; 2. DBT; 3.Thiophene; 4.Thiophene-2-carboxyllic acid; 5.Dimethyle sulfoxide; 6. 5,5'-di thio bis(2-nitro benzoic acid); 7. Designed medium (mixture of all the above organo-sulphur compounds).

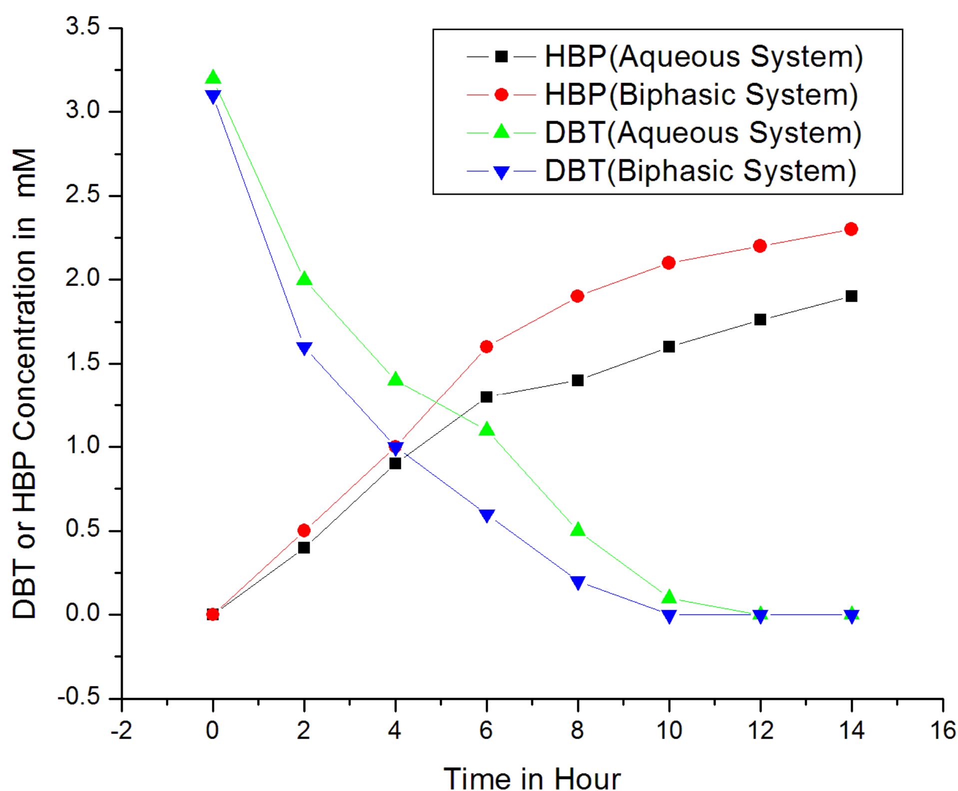

Fig.3: A common plot for the DBT degradation and residual 2-HBP production in both aqueous and Biphasic systems

Desulphurization assay in aqueous and Biphasic systems

In both bio desulphurization process , the highest desulphurization activities were observed in the beginning 2 hours of studies and approximately 40% of DBT was utilized by the microbe during this time. The DBT was metabolized by the resting cells in both aqueous and biphasic desulfurizing systems within a time period of 14 hours. The desulphurization activity of KS1 cells in a biphasic system is higher than in aqueous media which has been indicated in Fig.3. This may be due to the fact that in biphasic system, 2-HBP accumulates in the oil phase and does not harm the cells [19].Moreover the dissolution of DBT in tetradecane which increases the chance of DBT contact with the cells [20].

CONCLUSION

Newly isolated microorganism can use DBT as sulphur source and decreases 68.75% of DBT over a period of 7 days and produce 2-HBP. The isolate is presumptively identified as *Bacillus sp.* strain KS1. Further investigation of the mechanism of desulphurization and genetic and enzymatic aspects of the metabolic pathways used by this organism need to be carried out. Desulphurization was also detected in the assay using resting cells or immobilized cells both in aqueous and biphasic systems indicating the possibility of the commercial use of strain KS1. Moreover, the ability of strain KS1 to desulfurize other sulphur organic compounds along with DBT suggests the usefulness of this strain for further genetic improvement of its desulphurization rate.

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