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Study of Growth of *Rhodococcus erythropolis* (MTCC 1526) and Biotransformation of Dibenzothiophene into Hydroxylbiphenyl at Shake Flask Level.

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ABSTRACT

Combustion of sulfur containing fossil fuels releases a large amount of noxious gaseous in the environment which are not ecofriendly and contributor to acid rain. Due to toxicity and governmental regulations for limit of sulfur content in oil, petroleum industries are making efforts to generate low sulfur fuels. Common method of sulfur removal from oil is hydrodesulfurization but costly. Nowadays biodesulfurization is being used as alternative enzyme-catalyzed process to remove sulfur which is environment friendly and uses. Biological desulfurization (BDS) requires mild reaction conditions, low cost and put low impact on the environment. Dibenzothiophene (DBT) is form of sulfur which is present abundantly in fuel. Usually and microbes employ 4S pathway for conversion of DBT into hydroxybiphenyl (HBP). In this research work Gram's positive bacterium *Rhodococcus erythropolis* was used to catalyze conversion of DBT into HBP. Growth conditions in terms of temperature and pH were optimized and found pH 7.0 and 30^{o}C suitable for growth of bacterium. In these conditions conversion of DBT into HBP was analyzed and found that at 2.0mM addition of DBT into culture medium and 67.55% conversion into HBP was observed.

Keywords: Fossil fuel; Dibenzothiphene; Hydroxybiphenyl; *Rhodococcus erythropolis*; Bioconversion; Desulfurization

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INTRODUCTION

Nature has maintained vast diversity of microbial world which are habitant in diversity of environmental conditions. Microbes present near different crude oil reservoirs have adapted themselves through course of evolution and maturation and microbes are gifted to transform and utilize complex chemicals available in crude oil. During evolution, microorganisms have developed diversity of metabolic reactions regulated by enzymes. Microbes have capacity to biotransform a range of natural and synthetic compounds (xenobiotics) due to these enzymes [1,2]. This extraordinary metabolic biotransformation capability of microorganisms has enabled microbes to be explored in petroleum industry to improve the quality of crude oils by reducing sulphur content.

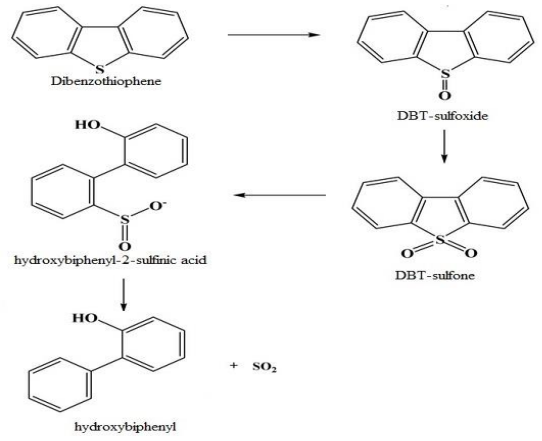
Sulfur is recognized as the most abundant component in crude oil behind carbon and hydrogen [3]. The average sulfur in crude oil ranges from 0.03 to 7.89 mass percentage [4]. The two forms of sulfur compounds inorganic and organic are present in crude oil. Examples of Inorganic sulfur are elemental sulfur, hydrogen sulfide and pyrite which can be present in dissolved or suspended form [5]. Examples of organic sulfur compounds present in crude oil are thiols, sulfides and thiophenic. Requirement of advancement in the processes of desulfurization and dearomatization of diesel has become essential in environmental concern worldwide. Sulfur is abundantly present in crude oil, predominantly in the form of heterocyclic thiophene [6] and is associated with environmental pollution as combustion of sulfur produce SOx, which is major contributor to acid rain. Including this regulatory authorities have allowed very low limit of sulfur content in petroleum fuel. Thus the sulfur removal from crude oil becomes very necessary.

Desulfurization is removal of sulfur from crude oil or petroleum. The chemical form of sulfur present in oil directly affects removal process. The desulfurization of aliphatic sulfur compounds like sulfides and thiols, is considered easier than desulfurization of aromatic sulfur compounds like thiophenics [7].

The common approach of sulfur removal is hydrodesulfurization (HDS) but it is energy intensive, costly and not suitable for fuel having aromatic sulfur compounds like 4,6-dimethyldibenzothiophene (4,6-DMDBT) [8]. An alternative technique termed as biodesulfurization (BDS) has been developed by investigators, which has been emerged as potent alternative technology due to selectivity, energy efficient, environment friendly and requirement of moderate reaction conditions [9,10]. BDS utilize microbial metabolic machinery of biotransformation for conversion of aromatic sulfur present in crude oil and it is suggested that microbial system is equipped with metabolic mechinary to desulfurize a wide range of organic sulfur compounds available in crude oil fractions [11,12].

Dibenzothiophene (DBT) is thiophenic sulfur compound and is widely accepted as representative of sulfur in crude oil as it is reported that 50-95% of the sulfur in crude oil is constituted by thiophenic sulfur and alkylated derivatives of DBT [9]. Researchers have used the removal of DBT to analyze the capacity of biodesulfurization (BDS).

Figure 1: Process of dibenzothiophene (DBT) degradation into hydroxybyphenyl (HBP). DBT is degraded into HBP though step by step conversion subsequently into DBT-sulfoxide, DBT-sulfonate, HBSA and HBP



The biocatalytic process of biodesulfurization of DBT is recognized as 4S pathway [11], in which four S oxidized chemical intermediates and four moles of NADH are involved. This pathway converts DBT into hydroxybiphenyl (2-HBP). In this bioconversion process DBT is first converted into DBT-sulfoxide (DBTO) and finally to DBT-sulfone ($DBTO_2$). $DBTO_2$ is converted into 2-hydroxybiphenyl-2-sulfinic acid (HBPS) through DBT-sulfone monooxygenase (DszA), catalyzed reaction [13,14]. HBPS is converted into 2-HBP by HBPS desulfinase (DszB) catalyzed process and the sulfur is released from the hydrocarbon in the form of sulfite (Fig.1) [15,16]. In this article author have analyzed the biotransformation capability of *Rhodococcus erythropolis* of Dibenzothiophene (DBT) into 2-hydroxybiphenyl (HBP).

MATERIALS AND METHODS

The chemicals used in this study were AR grade and procured from HiMedia (Mumbai, India), SRL (Mumbai, India) and Merck (Germany). All culture media were procured from HiMedia (Mumbai, India). The chemicals and culture medium components used in the experiments were selected from chemicals having least inorganic sulfur as impurity.

Inoculum, growth medium and culture conditions

Rhodococcus erythropolis (MTCC-1526) procured from Microbial Type Culture Collection Chandigarh, India in lyophilized form. Bacterial strain was inoculated into nutrient agar medium comprising beef extract (10.0 g/L), yeast extract (20.0 g/L), Peptone (5.0 g/L), NaCl (5.0 g/L), Agar (15.0 g/L) of double distilled water. pH of culture medium was adjusted 7.2 before sterilization. *Rhodococcus erythropolis* was grown and maintained on agar plates for further experiments (modified protocol of Izumi et al., 2004; Davoodi-Dehaghani et al., 2010) [17,18].

The inoculum for experiments was prepared by aseptically transferring a loop full of culture from the nutrient media agar plates into 50 mL nutrient broth. After this, inoculated broth was kept for 24 h at 30°C in an orbital shaker at 200 rpm. DBT was dissolved in ethanol at different concentrations and added to a sterilized medium as the only sulfur source.

Determination of growth

The growth of bacteria was determined by turbidity measurement using UV-Vis spectrophotometer (Labindia 3000+, Mumbai, India) by measuring optical density (OD) at 600 nm. In this experiment effect of temperature**,** pH and shaking rate were monitored for growth of bacteria. Growth of bacteria was measured at interval of 2 h. For optimization of bacterial growth the temperature range was selected at 25, 30 and 35°C, pH range was selected at 6, 7, and 8. For temperature optimization study pH 7.0 was selected for optimized temperature was used in pH optimization study.

Analysis of biomass

Broth was centrifuged at 3000 rpm for 10 min. Supernatants were poured into measuring cylinder and volume of supernatant was subtracted from total broth volume. This subtraction was calculated as PCV. Cells were washed three times by 0.85% (w/v) NaCl (normal saline) and kept at 60^{o}C for 24 h for drying in hot air oven [19].

Toxicity study of DBT to Bacterial Growth

The growth of Rhodococcus erythropolis was performed in 250 mL Erlenmeyer flasks containing 100 mL of nutrient broth in DBT concentrations (Control, 0.5mM, 1.0mM and 2.0 mM), and using 1% of inoculum of 24 h old culture. The flasks were kept in incubator (orbital shaker) at 200 rpm and at 30°C temperature. All experiments were performed in triplicate. Each 4 h, aliquots were removed to determine the growth of culture by measuring OD at 600nm using un-inoculated media as blank. For study of amount of DBT and HBP, the absorption of known concentrations of DBT and HBP is measured.

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Analysis of degradation of DBT

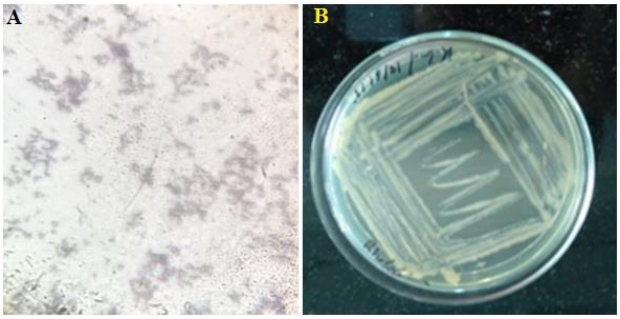
To analyze the biodesulfurization activity (conversion of DBT into 2-hydroxybiphenyl) produced from DBT) of bacterial strain Gibb's reagent (2,6-dichloroquinone-4 chloroimide) was used. At alkaline pH 8.0, Gibb's reagent binds with the aromatic hydroxyl groups and a complex of blue color is formed which was monitored spectrophotometrically at 580 nm (modified methods of Monticillo, 2000; Kayser et al., 1993) [13,20]. Bacterial supernatants of different concentration of DBT were diluted 2.5 times using double distilled water and analyzed for presence HBP.

RESULTS AND DISCUSSION

Revival and growth of bacterial strain

Rhodococcus erythropolis strain was revived in nutrient agar medium and after 24 h incubation, it was observed visible bacterial colonies on agar plates. The morphologically bacterial colonies appeared creamy and shiny. Bacterial culture was successfully transferred further for experiments and analyzed by Gram's stain. It was observed that *Rhodococcus erythropolis* is Gram's positive bacterium (Fig. 2).

Figure 2: Gram's staining of *Rhodococcus erythropolis* and growth on agar plate. Morphology of bacterium is observed creamy shine colonies and Gram's positive



Optimization of growth conditions

Physical conditions (temperature and pH) for growth of *Rhodococcus erythropolis* were optimized and production of biomass was analyzed. The temperature range selected for optimization of bacterial growth was 25°C, 30°C and 35°C and pH of 6, 7 and 8 was used. Broth medium was inoculated by 1% inoculum and kept for incubation at selected temperature. The bacterial growth was measured by measuring optical density at 600nm with in interval of 2 h and biomass was calculated at different temperatures. In this study it was found that *Rhodococcus erythropolis* showed growth at all the temperatures but maximum growth was observed at 30°C. Growth curve of these temperatures was prepared (Fig. 3). For biomass study 48 h incubation was performed. It was found that at 25°C biomass produced was 6.18 g/L, at 30°C, 8.84 g/L biomass was obtained while at 35°C, 7.32 g/L biomass was obtained. Growth rate were also calculated at these temperatures and it was found that at 25°C growth rate was 0.129, at 30°C growth rate was 0.184 and at 35°C growth rate was 0.1525.

Figure 3: Growth curve of *Rhodococcus erythropolis* at different temperature

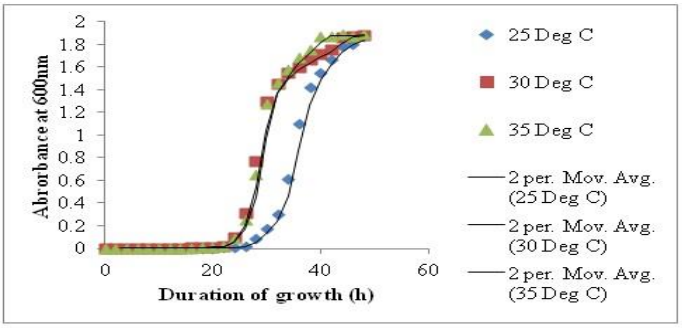
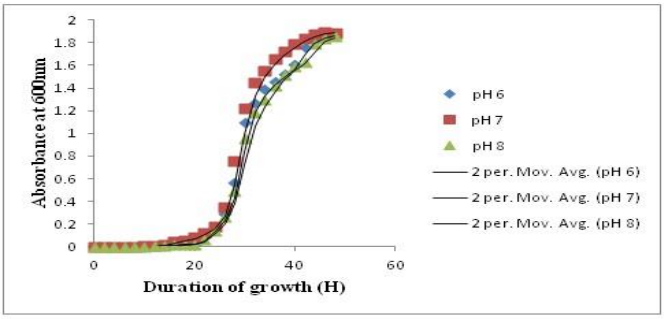


Figure 4: Growth curve of *Rhodococcus erythropolis* at different pH



Bacterial growth was also analyzed at different pH range at pH 6, 7, and 8. For this study bacterial culture was inoculated into nutrient medium and incubated at 30°C. The growth was measured by measuring optical density at 600nm after interval of 2 h. *Rhodococcus erythropolis* survived at all pH but at pH 7.0 maximum growth was observed. Growth curve (Fig.4) and biomass was obtained and analyzed. At pH 6.0 biomass obtained was 6.57 g/L, at pH 7.0 biomass obtained was 8.65 g/L and at pH 8.0 biomass obtained was 7.11 g/L. For further study pH 7.0 was maintained in culture medium and temperature at 30°C was used for incubation.

Toxicity study of DBT to bacterial growth

Aim of this study was to analyze the impact (toxicity if any) of DBT on the growth of Rhodococcus erythropolis. DBT at different concentrations was added to culture medium and incubated at 30°C and interesting results were obtained. Growth was analyzed by measuring optical density at 600nm. It was observed that DBT at 0.5mM enhanced the growth of Rhodococcus erythropolis as compared with control but increasing concentration of DBT was reducing growth of bacteria in compare to each other (Table 1). It was observed that still at 2.0 mM concentration DBT is enhancing growth of Rhodococcus erythropolis but it can be concluded that DBT is toxic at 2mM concentration but it is showing more growth than control.

Table 1: Effect of DBT on bacteria growth at different concentration

Concentration of DBT	OD (600nm)		
	20 hrs	24 hrs	48 hrs
00	0.046	0.164	1.404
0.5mM	0.176	0.308	1.569
1mM	0.153	0.123	1.550
1.5mM	0.141	0.093	1.484
2.0mM	0.120	0.064	1.456

Experiments were carried in triplicate and each data point is mean value

Table 2: Conversion of DBT into HBP

Culture with different Concentration of DBT(mM)	Optical Density (600 nm)	Conc. of HBP produced (mM)	Dilution factor	Conc. of HBP produced (mM)	% conversion
00 (Control)	0.00	00	2.5	00	00
0.5	0.041	$10.7*10^{-2}$	2.5	$26.7*10^{-2}$	50.35
1.0	0.067	$23.5*10^{-2}$	2.5	$58.75*10^{-2}$	58.82
1.5	0.110	$38.1*10^{-2}$	2.5	$95.25*10^{-2}$	63.52
2.0	0.171	$88.49*10^{-2}$	2.5	$221.1*10^{-2}$	67.55

Experiments were carried in triplicate and each data point is mean value

Conversion of DBT into HBP

Rhodococcus erythropolis biocatalyze the conversion of DBT into HBP. In this study the amount of HBP produced in culture medium was measured by spectrophotometric method by measuring absorbance at

580nm. Increasing concentration of HBP was used for standard curve preparation and amount of DBT converted into HBP was measured further using this standard. In this experiment different concentrations of DBT were added to culture medium and after overnight incubation production of HBP were analyzed. It was found that at varying concentration of DBT, the amount of HBP is different (Table 2). It was interesting to observe that conversion of DBT into HBP increases with concentration (2.0mM) of DBT while same concentration (2.0mM) showed inhibitory effect in compare to 0.5mM concentration. Previous reports stated that at 0.125mM concentration of DBT was biotransformed in 2 days [17].

CONCLUSION

Microbes are playing very important role in nature such as maintaining of biogeochemical cycles, control of pollution by transformation of many xenobiotics and synthesis of many valuable metabolites. Sulfur in fuel is contributes to air pollution by making harmful gases and acid rain hence desulfurization of diesel is important for environmental concern. Common processes reported for desulfurization is Hydrodesulfurization (HDS) but it is cost effective and requires energy in large amount. It also has limitation of not effective on aromatic sulfur compounds. Now a days biodesulfurization (BDS) using microbes is being recognized as potent alternative to HDS [21].

In this research work we investigated role of *Rhodococcus erythropolis* in desulfurization. For this work we first optimized growth temperature 30^{o}C and pH 7.0 and studied bioconversion of DBT into HBP. In optimized conditions we found 50.35% conversion of 0.5 mM DBT into HBP and 67.55% conversion at 2.0mM of DBT. Toxicity of DBT against *Rhodococcus erythropolis* was also studied and we found that DBT in low concentration enhances the growth but at higher concentration (2.0 mM) it reduced the growth of *Rhodococcus erythropolis.*

Future Prospective

There are various ways for desulfurization of oils based on sulfur content, viscosity, refractory nature of sulfur compounds etc. and boiling point of oil [7] Biodesulfurization of diesel using *Rhodococcus erythropolis* is a promising approach in the direction of green energy and environment protection in compare to conventional hydro desulfurization. Although BDS is recommended approach for desulfurization, but for more efficiency microbes with high sulfur specificity need to be isolated and identified. We obtained 67.55% bioconversion of 2.0mM DBT into HBP in optimized the growth conditions but there is always a possibility to get better yield by optimizing media constituents. With this, mutants of *Rhodococcus erythropolis* may produce better biotransformation. Through isolation and screening of new species from oil fields researchers may provide new strains with capacity of desulfurization.

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