Role of pH in Biodesulfurization of Dibenzothiophene

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Abstract- **Removal of sulfur from the fossil fuel has gained a huge attention in the recent years because of the harmful impacts of sulfur dioxide and strict rules on their permissible sulfur content. Compared to other desulfurization techniques biodesulfurization has the ability to remove recalcitrant organosulfur compounds such as Dibenzothiophene (DBT) at moderate temperature without affecting the octane number. Its commercial implementation suffers inherent limitations of bacterial growth inhibition and low efficiency. This study was carried out on maintenance of constant pH of the growth medium and its effect on desulfurization efficiency. *Rhodococcuserythropolis* MTCC 3951 was allowed to grow in 0.75 mM DBT supplemented Basal Salt Medium. This strain was found to exhibit a drop in pH of the growth medium from 7.2 to 6.3 in its course of growth for 10 days. To maintain a constant pH of growth medium at 7.2 addition of Na_2CO_3 was done intermittently. After stable pH was maintained culture exhibited a shift in death phase by 3 days and increase in percentage degradation of DBT by 1.1 fold. Hence maintaining a constant pH of the growth medium may resolve the problem of bacterial growth inhibition and in turn increase the desulfurization efficiency.**

***Keywords:** Biodesulfurization, DBT (Dibenzothiophene), organosulfur, octane number, Na_2CO_3*

I. INTRODUCTION

Biodesulfurization is a process of removal of sulfur compounds, specifically organic sulfur by employing microbes. Importance of this method lies in its potency to degrade generally recalcitrant organic sulfur compounds such as Dibenzothiophene [17]. This degradation ability is attributed to dsz ABC gene products. Most commonly used microbe for degradation of DBT is *Rhodococcuserythropolis*[4],[18]. This organism follows 4S pathway of degradation mechanism, in which DBT is converted to the end product 2-Hydroxybiphenyl [5],[9],[19]. A lot of advantages are offered by biodesulfurization over other conventional methods [2],[4],[12]-[14],[16]. In spite of the different advantages it faces a lot of challenges like low desulfurization rate, enzyme inhibition by 2- HBP, inhibition in the presence of other sulfur sources, decreased mass transfer and economic considerations during reactor design, which need to be taken care during commercializing this technique [11]. There is no individual enzyme assay for dsz proteins. In addition to this the molecular mechanism is also not clearly explained. In almost all the coal mines the percentage of sulfur is very high and the dependence of energy sector upon fossil fuels is also high. This results into higher concentration of sulfur dioxide release in the atmosphere. This rising concentration of sulfur dioxide has become alarming.Therefore government has imposed strict rules on sulfur emissions.

Till date various means have been utilized to improve the efficiency of biodesulfurization like screening of novel high desulfurizing strains, cloning dsz ABC genes in solvent tolerant species, genetic manipulations like removal of overlap in dszoperon improved reactor designs, employing iron nanoparticles for desulfurization and etc. [1],[7]-[8]. Still decreased desulfurization rate remains as a major concern. Hence it is very important to find missing links which can give us some insight into the inherent problems and provide solutions to improve the efficiency of biodesulfurization.

The present study revolved around pH as a key factor to increase the desulfurization efficiency. The main objectives of this study are (i) to check if there is a change in pH profile during the course of growth (ii) to survey if change in pH profile has an impact on growth and desulfurization efficiency and (iii) to study the effect of maintaining constant pH of growth medium on desulfurization efficiency of *Rhodococcuserythropolis*.

II. MATERIALS AND METHODS

A. Bacterial Strain

Rhodococcuserythropolis MTCC 3951 (lyophilized culture) was obtained from the Microbial Type Culture Collection and Gene Bank, Chandigarh, India. A Loop full of lyophilized culture was taken and inoculated aseptically in 5 mL of Medium 3 (prescribed in MTCC Catalogue). The composition of the Medium 3 was 1.0 g of beef extract, 2.0 g of yeast extract, 5.0 g of peptone and 5.0 g of NaCl per 1000 mL of deionized water. Culture tubes were taken in duplicate with respective control. These culture tubes were incubated at 30 °C till the cells reached mid logarithmic phase. Then, a loop full of culture was taken and streaked on Luria Bertini agar medium and incubated at 30 °C for three days. Plates in which isolated colonies were obtained were picked up using a sterile tooth pick and inoculated into 10 mL of LB medium. The glycerol stocks of the cultures were maintained after the attainment of logarithmic phase and stored at -70 °C for future use.

B. Selective Enrichment Of The Culture

Revived *Rhodococcuserythropolis* was inoculated in DBT supplemented sulfur free medium i.e. Basal salt medium (BSM). The medium composition for BSM was 4.0 g of KH_2PO_4, 4.0 g of Na_2HPO_4, 2.0 g of NH_4NO_3, 0.2 g of $MgCl_2$, 0.001g of $CaCl_2$, 0.001 g of $FeCl_3$ and 5.0 g of Glucose per 1000 mL of deionized water[15],[20].DBT was added as the sole sulfur source in increasing concentration of 0.1 - 0.75 mM. The organism was incubated at 30 ˚C and 120 rpm.

C. Desulfurization Assay

*1) HPLC Assay:*The concentration of DBT in the supernatant was determined by Waters 1525 binary HPLC pump [10] using a C18 column (150 mm × 4.5 μm) with waters 2487 dual λ absorbance detector. At intervals of 24 hours, 10 ml of culture was taken and extracted with double the volume of ethyl acetate. After phase separation lower phase containing the cell biomass is discarded and top layer is concentrated to about 0.5 mL to 1 mL. About 10 μL of sample was injected using Rheodyne manual injector. The chromatogram was developed using a mobile phase of HPLC grade Methanol: water (4:1) at a flow rate of 1mL/min. Run time for each sample was 30 minutes. Retention time of each signal was recorded at a wavelength of 220 nm and the data was processed with Empower software. The commercial DBT from Sigma, USA (Molecular weight; 184.26 g) was used as standard. DBT quantification was performed by reference to standard curves with a series of dilution of pure DBT.

2) Gibb's Assay: Gibb's assay was performed to find the amount of 2-Hydroxy Biphenyl (2-HBP) formed. The 2-HBP liberated as end product of 4S pathway when DBT is desulfurized can be quantified by Gibb's assay [6]. 2 mL of culture medium was taken and subjected to centrifugation at 5000 rpm for 5 minutes. To 1 ml of the supernatant obtained, 200 μL of sodium bicarbonate was added to maintain the pH 8.0. Then 20 μl of Gibb's reagent (0.1 g in 10 mL of ethanol) was added to it. It was vortexed and when allowed to stand for 30 minutes led to full colour development. The amount of 2-HBP (mM) generated was calculated from 2-HBP generated standard graph. Hydroxyl group containing aromatic compounds i.e. 2-HBP react with Gibb's reagent (2,6-Dichloroquinone-4-Chloroimide) at an alkaline pH to form a blue colored complex that can be measured calorimetrically at 610 nm.

D. Growth Characteristics

Rhodococcuserythropolis was cultivated in 100 mL of BSM medium in 250 mL flask with a final concentration of 0.75 mM DBT concentration and 0.5 g of glucose as the carbon source. Temperature and rpm conditions were 30 °C and 120 rpm respectively. Aliquots of 1 mL were regularly taken for 10 days to measure the O.D. at 600 nm. As a control BSM medium without inoculated organism was taken. Growth characteristics study was performed for both with and without intermittent addition of Na_2CO_3. Each study was performed in triplicate. In order to study the effect of maintaining constant pH on desulfurization efficiency, pH of the medium was monitored each day and drop wise addition of 0.5 mM Na_2CO_3 was performed during the experimental period in order to maintain the pH of 7.2.

III. RESULTS AND DISCUSSION

A. Selective Enrichment Of The Culture

Lyophilized *Rhodococcuserythropolis* was revived in the Medium 3 successfully. After revival, the organism showed the ability to grow with maximum 0.75 mM of DBT supplemented Basal Salt Medium. Hence the concentration of DBT was maintained as 0.75 mM in the Basal Salt Medium for all the studies.

B. Growth Profile And pH

Figure 1 shows the growth curve of *Rhodococcuserythropolis* in Basal salt medium supplemented with 0.75 mM DBT as the sole sulfur source and glucose (5g/L) as the carbon source. The organism was allowed to grow for a period of 10 days. In its course of growth for 10 days, it was observed that there was a drop in pH from 7.2 to 6.3 in the culture medium i.e. a shift towards relatively acidic side. Similar pattern of drop in pH was obtained during the growth profile by reference [3].

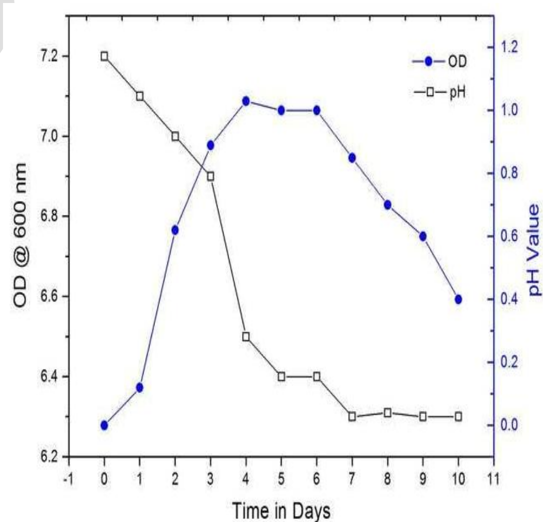

Fig. 1 Growth pattern and change in pH

C. Desulfurization Assay

The organism was grown in 0.75 mM DBT and culture supernatant was checked for the amount of DBT degraded and 2-HBP produced at an interval of 24 hours.

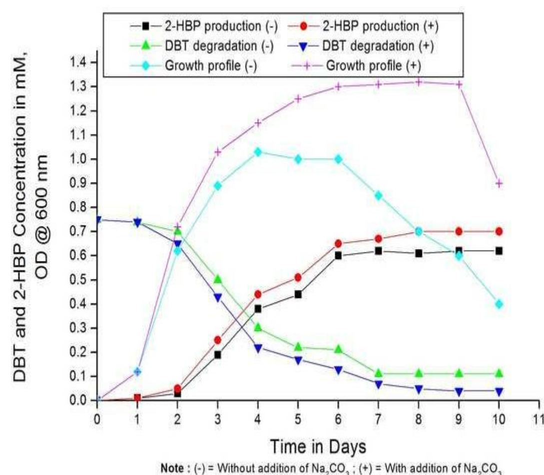

Fig. 2 Desulfurization assay and growth profile of *RhodococcusErythropolis* with and without addition of Na_2CO_3.

In a time course of 10 days 85% of DBT was degraded. Rate of DBT degradation was found to be highest on 3rd and 4th day. Approximately 33% of DBT was degraded by the 3rd day. Also 2-HBP produced during the course of reaction was quantified by Gibb's assay. Rate of 2-HBP production was the highest on 3rd and 4th day. About 30% of 2-HBP was produced by the end of 3rd day (Figure 2). The medium was maintained at constant pH 7.2 by drop wise addition of 0.5 M Na_2CO_3. After maintaining the pH of the growth medium at 7.2 there was a shift in the death phase of the organism from 7th day to 10th day. The organism showed higher percentage degradation for DBT (94.6%) compared to before (85.33%). Also the concentration of 2-HBP produced showed an increase (0.7 mM) compared to the medium without adjusting pH (0.61 mM) as shown in Fig. 2.

IV. CONCLUSION

From the above study it is concluded that the shift in pH during the growth profile acts as a major hindrance in desulfurization of DBT. However if a constant stable pH is maintained towards the relatively alkaline side the organism retains its viability for a longer time thereby increasing the desulfurization.

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REFERENCES

[1]. Ansari, F., Grigoriev,P., Libor,S., Tothill, I.E., Ramsden, J.J., (2008). DBT Degradation Enhancement by Decorating *Rhodococcuserythropolis* IGST8 With Magnetic Fe_3O_4 Nanoparticles. BiotechnolBioeng 102:1505-1512

[2]. Chen, H., Zhang, W., Chen, J., Cai, Y., Li, W., (2008). Desulfurization of various organic sulfur compounds and the mixture of DBT + 4,6-DMDBT by *Mycobacterium sp.* ZD-19. BioresourTechnol 99:3630–3634

[3]. Etemadifar, Z., Emtiazi, G., Christofi, N., (2008). Enhanced Desulfurization Activity in Protoplast Transformed *Rhodococcuserythropolis*. Am Eurasian J Agric Environ Sci 3:795-801

[4]. Izumi, Y., Ohshiro, T., Ogino, H., Hine, Y., Shimao, M., (1994).Selective desulfurization of dibenzothiophene by *Rhodococcuserythropolis*.D-1. Appl Environ Microbiol 60:223–226

[5]. Kayser, K.J., Bielaga-Jones, B.A.,Jackowski, K., Odusan, O., Kilbane, J.J., (1993). Utilization of organosulfur compounds by axenic and mixed cell cultures of *Rhodococcusrhodochrous* IGTS8. J Gen Microbiol 139:3123–3129

[6]. Kilbane, J.J., (1989). Desulfurization of coal: the microbial solution. Trends Biotechnol 7: 97-101.

[7]. Kilbane, J.J., (2006). Microbial biocatalyst developments to upgrade fossil fuels.CurrOpinBiotechnol 17:305-314.

[8]. Li, G., Ma, T., Li, S., Li, H., Liang, F., Liu, R., (2007).Improvement of dibenzothiophene desulfurization activity by removing gene overlap in the dsz operon.BiosciBiotechnolBiochem 71:849-854

[9]. Li, W., Wang, M.D., Chen, H., Chen, J.M., Shi, Y., (2006).Biodesulfurization of dibenzothiophene by growing cells of *Gordonia sp.* in batch cultures. BiotechnolLett 28: 1175–1179

[10]. Ma, T., Li, G., Li, J., Liang, F., Liu, R., (2006). Desulfurization of dibenzothiophene by *Bacillus subtilis* recombinants carrying dszABC and dszD genes.BiotechnolLett 28:1095–1100

[11]. Mohebali, G., Ball,A.S., (2008). Biocatalytic desulfurization (BDS) of petrodiesel fuels. Microbiology 154:2169–2183

[12]. Monticello, D., (2000).Biodesulfurization and the upgrading of petroleum distillates.CurrOpinBiotechnol 11:540–546

[13]. Ohshiro, T., Izumi, Y., (2002).Desulfurization of fossil fuels. In: Bitton G (Ed) Encyclopedia of Environmental Microbiology, Vol. II. Wiley and Sons, New York, pp 1041–1051

[14]. Oldfield, C., Pogrebinsky, O., Simmonds, J., Olson, E.S., Kulpa, C. F., (1997).Elucidation of the metabolic pathway for dibenzothiophene desulfurization by *Rhodococcus sp.* IGTS8 (ATCC53968). Microbiology 143:2961–2973

[15]. Raheb, J., Memari, B., Hajipour, M.J., (2011). Gene-manipulated Desulfurizing Strain *Pseudomonas putida* Reduced Energy Consuming in the Biodesulfurization Process. Energ Source Part A 33: 2018–2026

[16]. Setti, L., Lanzarini, G.,Pifferi, P., (1997).Whole cell biocatalysis for an oil desulfurization process. Fuel Process Technol 52:145–153

[17]. Shavandi, M., Sadeghizadeh, M., Zomorodipour, A.,Khajeh, K., (2009).Biodesulfurization of dibenzothiophene by recombinant *Gordoniaalkanivorans* RIPI90A.BioresourTechnol 100: 475-479

[18]. Singer, M.E.V., Finnerty, W.R., (1988). Construction of an *Escherichia coli-Rhodococcus*shuttle vector and plasmid transformation in *Rhodococcusspp*. J Bacteriol 170:638-645

[19]. Soleimani, M., Bassi, A.,Margaritis, A., (2007). Biodesulfurization of refractory organic sulfurcompounds in fossil fuels.BiotechnolAdv 25:570-596.

[20]. Yu, B.,Xu, P., Shi, Q., Ma1, C., (2006). Deep Desulfurization of Diesel Oil and Crude Oils by a Newly Isolated *Rhodococcuserythropolis* Strain. ApplEnviron Microbiol 72: 54-58