Study of a desulfurization process to convert dibenzothiophene to 2-hydroxybiphenyl by Rhodococcus rhodochrous NRRL (B-2149)

Aldo B. Soares Júnior, Yanne K. P. Gurgel, Bruna M. E. Chagas, Thyrone B. Domingos, Gorete R. Macedo and Everaldo S. Santos
Biochemical Engineering Laboratory, Chemical Engineering Department, Federal University of Rio Grande do Norte, Campus Universitário, 59072 – 970, Natal, Brazil

ABSTRACT

Actually, a great effort is being given to research on biodesulfurization processes, i.e., processes in which sulfur can be removed selectively from sulfur-compound moities without altering its British thermal unit. This effort relies on the fact that fossil fuels (coal and oil) contain organic sulfur compounds that are released after combustion to the environment mainly as SO_2 that causes acid rain becoming a potential pollutant. In this work we investigate the biodesulfurization of a model molecule that represents the main class of a group of recalcitrant compounds found in petroleum, Dibenzothiophene (DBT), to produce 2-Hydroxybiphenyl (2-HBP), a sulfur-free compound, by *Rhodococcus rhodochrous* (NRRL B-2149) using the 4S pathway. Experiments in which *R. rhodochrous* (NRRL B- 2149) was cultived during exponential growth phase using glucose and DBT as carbon and energy and sulfur sources, respectively, showed that the microorganism follows the 4S metabolic pathway in which DBT is converted to 2-HBP and sulfite. It was also showed that *R. rhodochrous* (NRRL B-2149) has cell-bounding surface active agents that that facilitates the emulsification of the apolar – water immiscible DBT.

1 INTRODUCTION

Biodesulfurization is a process in which microorganisms are used, under control conditions, to remove sulfur containing compounds from oil and coal. Biological and chemical-physical factors affect considerably heavy oil biodegradation as well as biotransformation such biodesulfurization (Setti *et al*., 1995). A variety of

microorganisms can use sulfur from aromatic hydrocarbons sources mainly bacteria such as *Mycobacterium sp*. (Srinivasaraghavan *et al*., 2006), *Rhodococcus erythropolis* (Oda and Otha, 2002) as well as *Microbacterium* Strain ZD-M2 (Li *et al*., 2005). Dibenzothiophene (DBT) has been used as the model compound (Figure 1) for sulfur hetero-cycles present in hydrodesulfurization treated fuel, *i.e.*, as a model that represents the so called recalcitrant compounds such as alkyled-thiophenes, benzonthiophenes, etc.

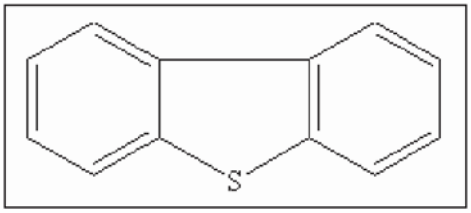

Figure 1. Dibenzothiophene.

Even though a significant number of microorganisms have been found to remove sulfur from DBT via a hydrocarbon degradative pathway like Kodama pathway (Kilbane and Jackowsky, 1992) such a means of sulfur removal involves the break of carbon-carbon bonds in the ring thus resulting in a reduction of fuel value. Therefore this route it is not interesting for reducing sulfur levels of compounds without loss its British termal unit. Among the microorganisms able to use sulfur from DBT, a small number have been shown to remove it via a specific-pathway so called «4S» pathway. *Rhodococcus erythropolis* IGTS8 as already shown follows «4S» pathway once this microorganism has three catabolic genes (*dsz*A, *dsz*B and *dsz*C) that are responsible for DBT desulfurization and that are clustered on a 120-kb linear plasmid. In this pathway (Figure 2), enzyme DszC catalyzes two consecutive mono-oxigenation reactions converting DBT to $DBTO_2$ (DBT-sulfone), in this case DszC uses NADH and $FMNH_2$ as cofactors. Following, a flavomonooxygenase (DszA) converts $DBTO_2$ to Hydroxyphenil benzene sulfonate (HPBS) that is converted to 2-Hydroxybiphenyl (2-HBP) and sulfite by a HPBS-desulfinase (DszB). 2-HBP is not further metabolized and accumulates in the medium. Inorganic sulfur released to the medium can be used by the strain as the sole source of sulfur. It is known that expression of the *dsz* gene cluster is strongly repressed by sulfate and sulfur-containing aminoacids.

On the other hand, microorganisms are known to produce a special classes of a molecules of high- and low-molecular weight called biosurfactants. Biosurfactants are molecules that have a hydrophilic portion, which may consist of mono-, oligo- or polissacharydes, amino acids or peptides or carboxylated or phosphate groups, and a hydrophobic portion composed, mainly, by saturated or unsaturated (hydroxyl) fatty

acids or fatty alcohols (Lang and Wullbrandt, 1999). These molecules reduce surface tension and Critical Micelle Dilution (CMD) in both aqueous solution and hydrocarbon mixtures. Investigation on influence of surface-active agent in cultivation medium using strain 1awq (further identified as *R. erythropolis*) has shown that there were no surfactant-like molecules produced in the culture medium (Feng *et al.*, 2006).

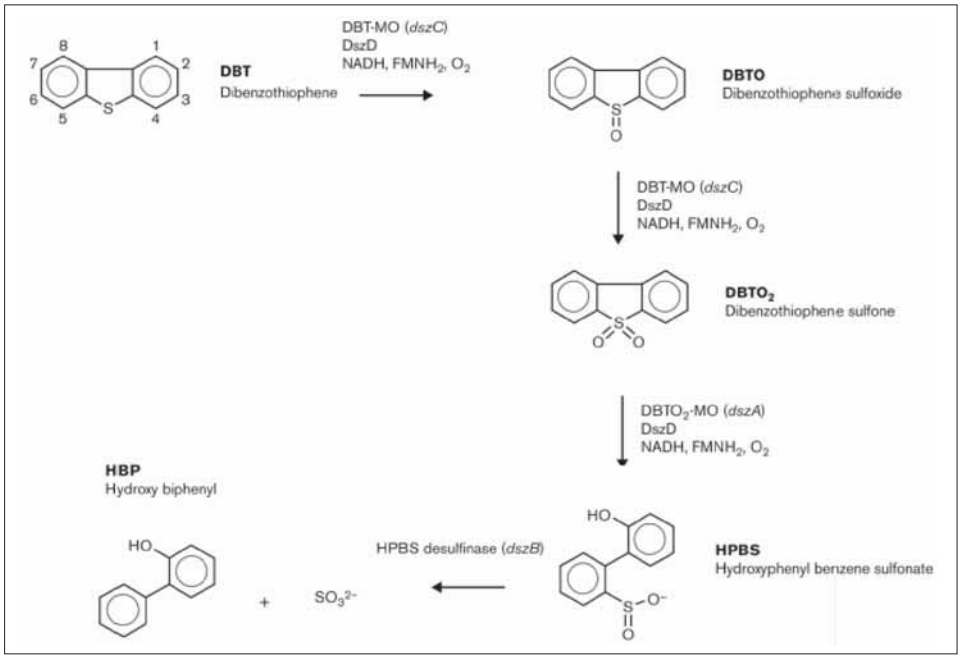

Figure 2. «4S» pathway for DBT desulfurization.

In this work we investigate the biodesulfurization of Dibenzothiophene (DBT), to produce 2-Hydroxybiphenyl (2-HBP), a sulfur-free compound, by *Rhodococcus rhodochrous* (NRRL B-2149) using the «4S» pathway.

2 MATERIALS AND METHODS

2.1 Microorganism and cultivation

The microorganism used was *Rhodococcus rhodochrous* (NRRL B - 2149) supplied by the National Center for Agricultural Utilization Research (USA). The strain was maintained at -20 °C in a vial containing a 10% glycerol solution. This strain is able to degrade cholesterol.

Pre-inoculum was prepared in LB medium (containing 1% tryptone, 0.5% yeast extract, and 1% NaCl) in which cells from the vial were incubated in a 50 mL Erlenmeyer flask in an orbital shaker during 12h at 150 rpm and 32 °C. For cultivation 10% (vol/vol) of *R. rhodochrous* (at exponential growth) was incubated in 50 mL of a 250 mL Erlenmeyer flask in an orbital shaker during 60 at 150 rpm and 32 °C using the following medium: $NaH_2PO_4 \cdot H_2O$, 4 g/L; K_2HPO_4, 4 g/L; $MgCl_2 \cdot 6H_2O$, 0.0245 g/L; $CaCl_2 \cdot 2H_2O$, 0.001 g/L; $FeCl_3 \cdot 6H_2O$, 0.001 g/L; NH_4Cl, 2 g/L; glucose, 20 g/L and the model compound DBT (0.2%). Due to its low water solubility DBT was diluted in ethanol. During cultivation samples were drawn for determination of cell concentration and after centrifugation supernatant was used to determine carbohydrate content, total protein, DBT and 2-HBP contents, pH, surface tension as well as critical micelle dilutions (CMD^{-1} and CMD^{-2}).

2.2 Carbohydrate determination

The reducing sugars formed during the cultivation were estimated spectrophotometrically at 600 nm by using the Dinitrosalicyclic acid (DNS) method with glucose as the standard.

2.3 Cell concentration

Biomass concentration (g/L) was estimated by optical density measurement at 600 nm using a standard curve.

2.4 Protein contents

Determination of total protein was carried out according to the Sedmak and Grossberg modified method as described by Santos (2001).

2.5 DBT and 2-HBF assays

The concentration of DBT and 2-HBP were analyzed by HPLC (Shimadzu, Japan), using a UV detector at 236 nm. A Shim-Pack C-18 column (150 mm × 4.6 mm, with 5 μm particles and 100Å pore size, Shimadzu) was used. The mobile phase was 50% of acetonitrile/water (ultra pure) with a flow rate of 1.0 mL/min and 25 °C temperature. The retention time of HBP was 7.4 min and 32 min, respectively.

2.6 pH measurement

pH measurement was carried out using a potenciometer (Model Digimed DM-21, Brazil).

2.7 Surface tension

Surface tension was determined with a Du Noüy Tensiometer (Central Scientific Company, USA) using the ring method.

2.8 Critical micelle dilutions (DCM^{-1} and DCM^{-2})

Critical micelle dilutions (CMD^{-1} and CMD^{-2}) were determined by measuring the surface tension of 10-times and 100-times diluted broth in distilled water at room temperature, respectively.

3 RESULTS AND DISCUSSION

3.1 Biodesulfurization of DBT by R. rhodochrous (NRRL B- 2149)

The capacity of biodesulfurization of DBT by *R. rhodochrous* (NRRL B- 2149) in a cultivation in which DBT was used as sulfur source has been investigated.

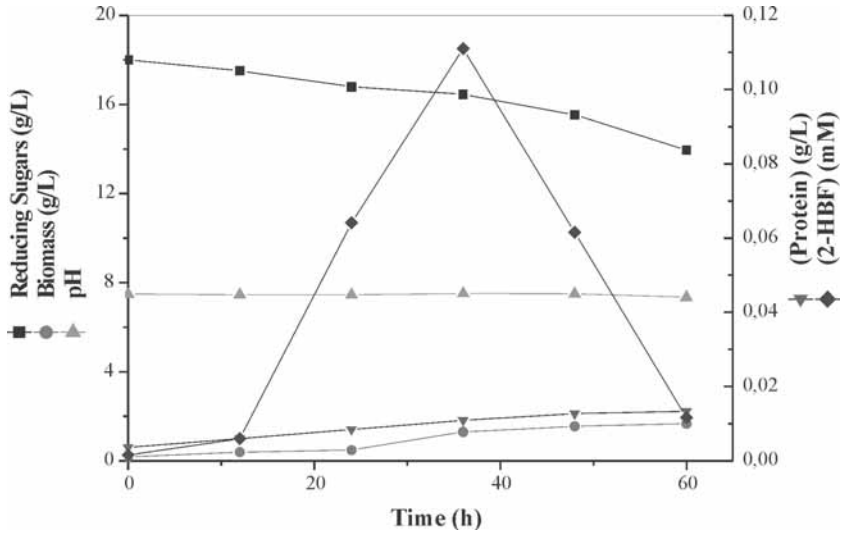

Figure 3. Biodesulfurization of DBT by *R. rhodochrous* (NRRL B- 2149).

According to Figure 3, it is possible to observe that *R. rhodochrous* (NRRL B-2149) is able to use the «4S» pathway in order to desulfurize DBT to produce 2-HBP. It can be seen that *R. rhodochrous* (NRRL B- 2149) grew during cultivation reaching about 1.7 g/L at 60 h without any significant change at cultivation pH. *Corybacterium sp* Strain SY1, a strain that can use the «4S» pathway, showed a 2-HBP (0.18 mM)

concentration maximum at 65 h (Omori *et al*., 1992). In our work maximum 2-HBP production occurred at 36 h (0.11 mM) suggesting that it can be used as an intermediary compound to another reaction. It is known that 2-HBP inhibits cells growth in the biodesulfurization process, for instance, 0.5 mM 2-HBP inhibited growth of *R. erythropolis* D-1 (Oshiro *et al*., 1996) while for *R. rhodochrous* (NRRL B- 2149) biomass stationary phase occurred at 0.1 mM. Reducing sugars measurement showed a reduction at glucose concentration reaching about 14.0 g/L at the cultivation end. Protein assay at supernatant (cell-free) cultivation showed a slightly increase in extracellular protein during cultivation time. In this case, about 0.013 g/L protein content was found at 60h.

3.2 Surface tension during DBT biodesulfurization

In order to observe any presence of surface-active agent during cultivation of DBT as a sulfur source by *R. rhodochrous* (NRRL B- 2149) surface tension of cell free culture medium as well as CMD^{-1} and CMD^{-2} were assayed.

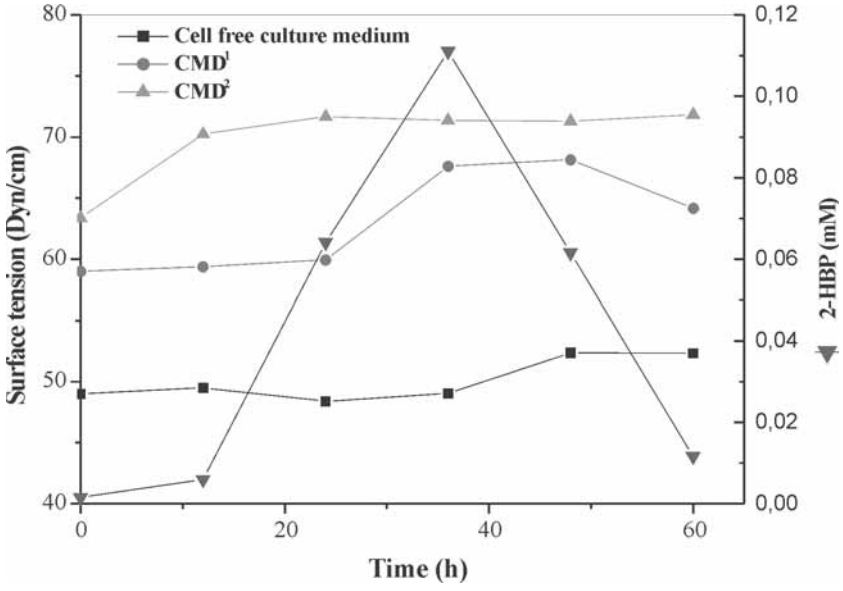

Figure 4. Surface tension during desulfurization of DBT by *R. rhodochrous* (NRRL B- 2149).

Figure 4 shows that *R. rhodochrous* (NRRL B- 2149) is not able to produce any surface-active agent in order to reduce the surface tension at cell free cultivation medium. However, a slightly increase on surface tension is observed for CMD^{-1} during

the period of increase of 2-HBP concentration in the cultivation. It is known that 2-HBP has a good solubility in water if compared to DBT. A significant surface tension occurred for CDM^{-2} during the initial time of cultivation after that surface tension approaches to the water surface tension, i.e., about 72 dyn/cm. Van Hamme and Ward (2001) found that *Rhodococcus sp.* Strain F9-D79 could not produce any biosurfactants at cultivation medium when growing on oil/water interface. However, Strain F9-D79 showed a reduction at surface tension for a non-free cell medium. Therefore it has been supposed that *R. rhodochrous* (NRRL B-2149) has cell-bounding surface active agents that that facilitate the emulsification of the apolar – water immiscible DBT since none significant surface-activity was observed at cell-free cultivation medium.

4 CONCLUSIONS

Experiments in which *R. rhodochrous* (NRRL B- 2149) was cultivated during exponential growth phase using glucose and DBT as carbon and energy and sulfur sources, respectively, showed that the microorganism follows the 4S metabolic pathway in which DBT is converted to 2-HBP. Maximum 2-HBP production occurred at 36 h (0.11 mM) suggesting that it can be used as an intermediary compound to another reaction. 2-HBP inhibited cells growth at 0.1 mM. It was also showed that *R. rhodochrous* (NRRL B-2149) has cell-bounding surface active agents that that facilitates the emulsification of the apolar – water immiscible DBT.

5 ACKNOWLEDGEMENTS

Authors thank CNPq-CTPETRO (Brazil) for financial supporting (Project 504859-04/8).

References

Feng, J., Zeng, Y., Ma, C., Cai, X., Zhang, Q., Tong, M., Yu, B. and Xu, P. (2006) The surfactant tween 80 enhances biodesulfurization. *Appl. Environ. Microbiol.* 72(11): 7390-7393.

Kilbane, J.J. and Jackoswki, F. (1992) Biodesulfurization of water soluble coal-derived material by *Rhodococcus rhodochrous* IGTS8. *Biotechnol. Bioeng.* 40(9): 1107-1114.

Lang, S. and Wullbrandt, D. (1999) Rhamnose lipids – biosynthesis microbial production and application potential. *Appl. Microbiol. Biotechnol*. 51(1): 22-32.

Li, W., Zhang, Y., Wang, M.D. and Shi, Y. (2005) Biodesulfurization of dibenzothiophene and other organic sulfur compounds by a newly isolated *Microbacterium* Strain ZD-M2. *FEMS Microb. Lett.* 247: 45-50.

Oda, S. and Ohta, H. (2002) Biodesulfurization of dibenzothiophene with *Rhodococcus erythropolis* ATCC 53968 and its mutant in an interface bioreactor. *Jour. Biosc. Bioeng.* 94(5): 474-477.

Omori, T., Mona, L., Saiki, Y. and Kodama, T. (1992) Biodesulfurization of dibenzothiophene by *Corynebacterium* Strain SY1. *Appl. Environ. Microbiol.* 58(3): 911-915.

Oshiro, T., Suzuki, K. and Izumi, Y. (1996) Regulation of dibenzothiophene degrading enzyme activity of *Rhodococcus erythropolis* D-1, *J. Ferment. Bioeng.* 81(2): 121-124.

Santos, E. S., Guiradello, R. and Franco, T.T. (2002) Preparative chromatography of xylanase using expanded bed adsorption. *J. Chromat.* 944(1): 217-224.

Setti, L., Lanzatini, G. and Pifferi, P.G. (1995) Dibenzothiophene biodegradation by a *Pseudomonas sp.* in model solutions. *Proc. Biochem.* 30(8): 721-728.

Srinivasaraghavan, K., Sarma, P.M. and Lal, B. (2006) Comparative analysis of phenotypic and genotypic characteristic of two desulfurizing bacterial strains, *Mycobacterium phlei* SM1201-1 and *Mycobacterium phlei* SM1201-1 GTIS10. *Lett. Appl.. Microbiol.* 42(5): 483-489.

Van Hamme, J.D. and Ward, O.P. (2001) Physical and metabolic interactions of *Pseudomonas sp.* Strain JA5-B45 and *Rhodococcus sp.* strain F9-D79 during growth on crude oil and effect of a chemical surfactant on them. *Appl. Environ. Microbiol.* 67 (10): 4874-4879.