Biodesulfuration of DBT in model oil by resting cell of Pseudomonas *putida* CECT5279. Process enhancement
Proceedings of European Congress of Chemical Engineering (ECCE-6)
Copenhagen, 16-20 September 2007

# Biodesulfurization of DBT in model oil by resting cell of *Pseudomonas putida* CECT5279: Process enhancement

R. Alonso del Aguila, K. Boltes, P. Leton, A. Rodríguez, R. Rosal, J.A. Perdigón and Eloy Garcia-Calvo

*Department of Chemical Engineering, University of Alcala , CP-28871 Alcala de Henares-Madrid, Spain*

## Abstract

Biodesulfurization (BDS) can be used as complementary technology to traditional hydrodesulfurization (HDS) process for sulfur reduction in fossil fuel. One of the mayor problems of BDS is the lower desulfurization yield obtained due to the low solubility of organosulfur compounds in water phase where microorganisms survive. In this work, the use of chemical surfactants largely increase the process yield conducted in resting cell operation mode.

Keywords: biodesulfurization, dibenzothiophene, resting cell, surfactant.

## 1. Introduction

Sulfur dioxide emission through fossil fuel combustion is the mayor contributor to the acid rain and air pollution (Monticello, 2000) for that reason governments are increasing their attention on the reduction of the limit of the sulphur content in oil fractions after refining. For environmental protection, the sulfur in diesel oil will have to be lower than 10 ppm by 2010 in European Union and the USA.

By biodesulfurization (BDS) is possible to remove recalcitrant compounds to conventional hydrodesulfurization (HDS), usually heterocyclic organosulfur compounds like dibenzothiophene (DBT) (Rhee et.al., 1998). Several aerobic bacterial strains have been used to selective desulfurization of DBT via the so-called 4S pathway (Fig. 1). The 4S route is a multi-enzymatic oxidative system of four consecutive reactions which convert DBT in dibenzothiophene sulfoxide (DBTO) and in dibenzothiophene sulfone ($DBTO_2$). The sulfone is transformed in 2-(2-hydroxibiphenyl)-benzenesulfinate (HBPS) and then in 2-hydroxybiphenyl (2HBP), as the final product free of sulfur, and sulfate.
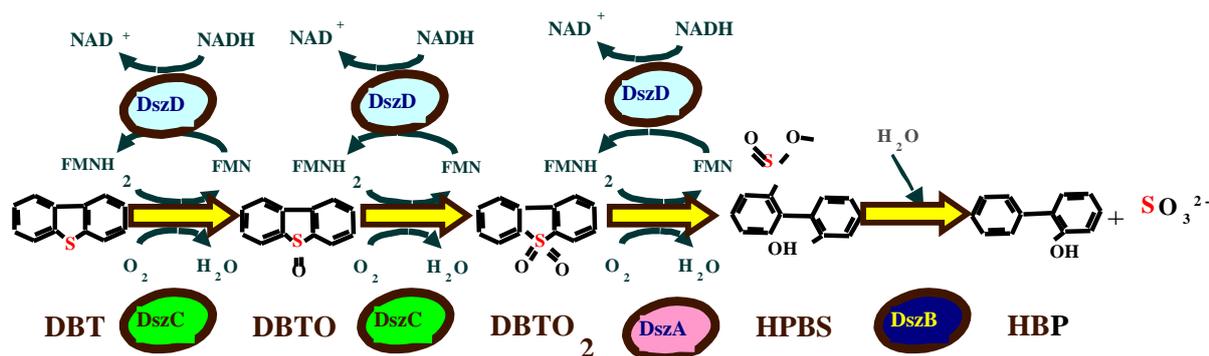
Figure 1.The oxidative metabolic 4S-pathway for DBT desulfurization.

In this work, biodesulfurization of DBT in a model oil solution were conducted by resting cells of *Pseudomonas putida* CECT 5279, a GMO. This bacterium carries the genes dszABC from *Rhodococcus erytropolis* IGTS8 and the gene hpac from *Escherichia coli*, these genes are implicated in the biodesulfurization process (Galán, et al., 2000).

*P. putida* is a Gram negative bacterium. The outer membrane of Gram negative microorganisms consist of a lipid bilayer with an outer layer of lipopolysaccharide and protein. This structure is surrounding a thin layer of peptidoglycan and the cytoplasmatic membrane that imparts more affinity of the *P. putida* with aqueous phase. In addition, the cell wall structure of Gram negative microorganisms: multi-layer and thicker cell wall, may limit the DBT transport into the cell due to the low permeability to organic compounds.

The solubility of DBT, a highly hydrophobic compound and their capability to be transported into bacterial cells, is probably the rate limiting step of the biodesulfurization process in biphasic liquid medium (Marcelis et al, 2003). For these reasons, the efficiency of biodesulfurization process largely depends on the bioavailability of the DBT in the aqueous phase, where the microorganism exists.

One way to promote the contact between DBT (organic phase) and microorganism (aqueous phase) is adding co-solvent in culture broth as well as surfactants. It is know that surfactants can increase the solubility of hydrophobic substances in water as was reported by Déziel et.al, 1999 and van Hamme and Ward, 2001., by the formation of reversed micelles that increase the surface of the liquid interface and consequently can improving the transport of DBT to inside cell media. The combination of these two effects: use of co-solvent and surfactant, may enhance the process yield.

Several assays were conducted to study the effect of the addition of ethanol as co-solvent and the addition of a chemical surfactant like Tween 85 on the desulfurization yield. Tween 85 is a poly (oxyethylene) sorbitan trioleate, non- ionic, biodegradable surfactant with a hydrophilic/lipophilic balance (HLB) 11, as well as

an oil-in-water emulsifier. That indicates that it can form reversed micelles in the water middle due to its high hydrophobic activity.

## 2. Materials and methods

The biocatalyst *Pseudomonas putida* CECT5279 was grown in a 2 liter commercial fermentor (Biostat B- Braun) using a standard protocol defined with basal salts medium, L- glutamic acid as carbon source and $MgSO_4$ as sulfur source (Martin et al.,2004). The temperature (30ºC), agitation rate (250 rpm) and air flow (1L/Lmin) was automatically controlled.

Cell growth was monitored spectrophotometrically at a wavelength of 600 nm. Cells were collected during the late logarithmic growth phase by centrifugation at 7000 rpm, 20ºC for 10 min. The harvested cells were re-suspended in a saline serum - glycerol solution and stored at –80 ºC (biomass stock) until use.

Resting cells assays were conducted, by adding different fraction of Tween 85, in Erlenmeyer flasks. Incubation in orbital shaker at 30ºC and 250 rpm of agitation speed, during 24 hours was performed. In all cases the liquid medium contains 50 mg/L of DBT dissolved in hexadecane (organic phase) and HEPES buffer at pH 8 (aqueous phase) in 1:1 volume proportion. Ethanol was added in 0.5% v/v proportion. Biomass concentration of 15 g/L of dry cell was used in all assays.

Emulsions were separated by centrifugation to determine DBT and resulting products concentrations. The organic and aqueous phases were analyzed by HPLC, equipped with a diode-array detector. For organic phase analysis, the next conditions were used: UV at 278 nm, a Kromasil C18 5 µm (150X4.6mm) column with the mobile phase consisting in 45% water - 55% acetonitrile.

The aqueous phase analysis was performed using a Supelcosil C8 column of 3 µm (150X4.6mm), the eluent consist in 50% v/v water and 50% v/v acetonitrile. DBT, DBTO, and $DBTO_2$ were measuring at 239 nm but 2HBP was detected at 210 nm. A flow rate of 1.0 L/L min was used for both methods.

## 3. Results and conclusions

The parameter to be maximized is the desulfurization yield ($X_{BDS}$), which has been defined as the percentage of DBT initial concentration converted in 2HBP in 24 hours, according to the following equation:

$$X_{BDS} = \frac{C_{HBP'24}}{C_{DBT_0}} x100$$

To study the effects of the addition of ethanol and Tween 85 on the desulfurization yield, several assays have been performed. Initially it was evaluated the enhancement of the process due to the co-solvent and surfactant added separately to the culture broth. Here, the fraction of co-solvent or surfactant has been used in the

same proportion between 0.2 – 1 % volumes. In Figure 2, it is showed than the *P. putida* capability of desulfurization achieved increase with the use of ethanol or Tween 85, in 7% and 18%, respectively.
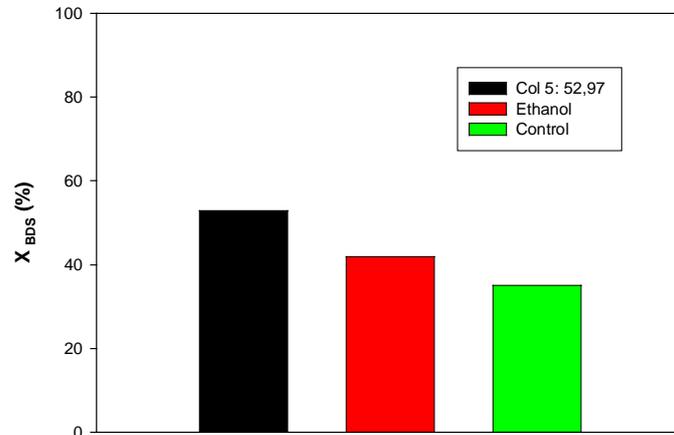


Figure 2. Experimental results of $X_{DBS}$ with the addition of co-solvent and surfactant.

Due to the obtained enhancement reached by this two additional components added individually, it appear necessary to study the synergistic effects of both of them. A systematic experimental assay was planed to optimize the doses of ethanol and Tween 85 separately (data not shown). Results indicate that 0.5 % v/v of ethanol and 1 % v/v of Tween 85 were the optimum proportion of each ones to maximize the process yield.

In a next step, of series of assays was performed varying the Tween 85 proportion to be mixed with 0.5 % of co-solvent. The obtained yields ($X_{BDS}$) in presence of different fractions of Tween 85 are presented in Figure 3.
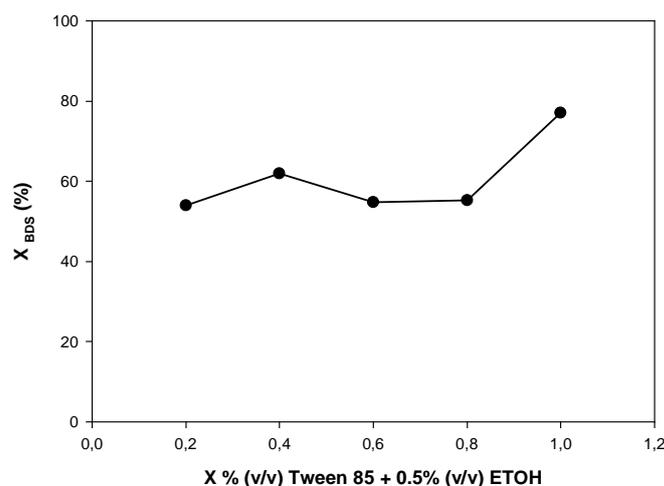


Figure 3. Experimental yield of desulfurization obtained with the addition of different fractions of Tween 85.

As can be observe, the value of DBT conversion has been kept constant between 0.2 and 0.8% (v/v) Tween 85 fractions. Nevertheless, the maximum value of desulfurization is reached with the addition of 1 % (v/v) of Tween 85 fraction, obtaining 77.07 % value of $X_{BDS}$, being a very highest value compared with the other assays when only Tween 85 or ethanol was added in their optimum dosage.

Analysis of the obtained results permits to conclude that the additions of ethanol like co-solvent or Tween 85 as surfactant increase the organosulfur compounds solubility in the aqueous phase by different mechanisms. But the synergic effect is more relevant.

Probably the combination of co-solvent and surfactant improves the transport of the organosulfur compounds from the organic to the aqueous phase by different pathways. The ethanol principally acts as co-solvent increasing the compounds solubility in watery phase, but Tween 85 can change the outer membrane structure, with the formation of pores in the cellular wall (Van Hamme et al.2006), and it facilitates the entry of organic compounds into the cell. On the other hand, Tween 85 can form reversed micelles with the solution of DBT in hexadecane to which the microorganisms have not direct access.

The sum of effects results in a greater process yield enhancement, which obviously was reached optimizing the dosage of each compound to causes minimal alterations in cell membrane structure.

**Acknowledgements**

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