Integrated Two-Stage Process for Biodesulfurization of Model Oil by Vertical Rotating Immobilized Cell Reactor with the Bacterium *Rhodococcus erythropolis*

Gamil A. Amin  
Taif University, Taif, Saudi Arabia

**Abstract**
A single vertical rotating immobilized cell reactor (VRICR) with the bacterium *R. erythropolis*, as a biocatalyst, was developed and used for investigation of biodesulfurization process with its two successive stages of cell growth and desulfurization activity. With a rotation speed of 15 rpm and oxygen transfer rate of 90 mM O$_2$.l$^{-1}$.h$^{-1}$, immobilized cell concentration of up to 70.0 g.l$^{-1}$ was achieved during the first stage and further used, in the second, to carry out a stable continuous desulfurization of model oil (dibenzo thiophene in hexadecane). A steady state with specific desulfurization rate as high as 167 mM 2HBP.Kg$^{-1}$.h$^{-1}$ and sulfur removal efficiency of 100% were maintained for more than 120 h. The proposed integrated biodesulfurization process utilizing the VRICR has the potential to lower operating costs and support possibilities of commercial application at the expense of Hydrodesulfurization process currently employed.

**Keywords:** Biodesulfurization; immobilized cells; bioreactor; *Rhodococcus rhodoculus*

**Introduction**
The sulfur content of crude oil can vary from 0.03 to 7.89 % (w/w) [1]. Sulfur emission through fossil fuel combustion is a major cause of acid rain and air pollution [2]. Many governments have recognized the problems and decided to reduce sulfur emissions through legislation. Hydrodesulfurization (HDS) process, operating at high-pressure and high-temperature, is currently employed to remove sulfur from fossil fuels. Recently, Biodesulfurization (BDS) of fuels through microbial activities has been shown to be a potential alternative to HDS, since BDS cannot remove the heterocyclic organo-sulfur compounds such as dibenzothiophene (DBT) [3] which represent about 70% of the sulfur in fossil fuels. A number of microorganisms, particularly *Rhodococcus* [4], *Bacillus* [5], *Arthrobacter* [6], *Gordonia* [7] and *Pseudomonas* [2] species have been found to metabolize DBT as a source of sulfur by cleaving the C–S bond of DBT via a sulfur-specific pathway (4S pathway) without affecting the carbon skeleton. In order to compete successfully with HDS, BDS process with a suitable biocatalytic design has to be developed. In literature, very few investigations on BDS process designs and operating costs have been reported but with little success. Lee et al., [8] investigated diesel oil desulfurization in a combination of air/lift/stirred-tank reactor using cells of *Gordonia nitida* CYKS1. An air-lift reactor was used to minimize energy costs [9].

Recently, Immobilized cells have been reported for increased volumetric reaction rates and lower operating costs for BDS [8,10] mainly due to the utilization of high cell concentrations of the biocatalyst and increased transport rate of sulfur-containing substances into biocatalytic cells.

In this study the feasibility of using a vertical rotating immobilized cell reactor (VRICR) with the bacterium *R. erythropolis* to carry out BDS of a model oil (BDT in n-hexadecane) was investigated. Performance of such bioreactor over long-term operation was also studied.

**Materials and Methods**

**Microorganisms and culture media**

The bacterium *R. erythropolis* ATCC 53968, was purchased from ATCC and used in this study. It was maintained as a stock culture at 4°C and used in this study. It was maintained as a stock culture at 4°C.
stokes of IBUs. Fermentation temperature was maintained at 30°C. A filter sterilized air stream was introduced at the bottom of the bioreactor and adjusted in order to achieve an oxygen transfer rate (OTR) of 90.0 mM O₂.l⁻¹.h⁻¹ of liquid volume. To increase efficiency of aeration, the air stream was distributed into small bubbles using a sparging system.

**Immobilization technique**

The VRICR was charged with glucose solution and sterilized at 121°C for 15 min. After cooling, supplemental growth medium (separately sterilized) was added aseptically to the bioreactor. After 90 min, the content of the bioreactor was discharged leaving only a thin layer of the medium on polyurethane foam surfaces. An actively growing 24-h-old culture of the bacterium *R. erythropolis* was centrifuged at 4500 x g for 10 min and the biomass was introduced into the bioreactor as an inoculum. The bioreactor was then allowed to stand for 10 h to allow cell attachment to the polyurethane foams. A complete growth MSM was then pumped into the bioreactor.

**Analytical methods**

Cell concentration in polyurethane foams (immobilized cells), or in bioreactor effluent (free cells) and residual glucose and ethanol were estimated as described by Amin [11]. Sulphate in effluent was determined by turbidimetric standard method [13]. The OTR was determined by direct measurement method described by Hirose [14]. The oxygen content in inlet and outlet gas streams was measured using oxygen analyzers. Then, the exact OTR was directly obtained by multiplying the difference in oxygen content by aeration rate, taking into consideration the gas low.

**Gibb’s assay**

Gibb’s assay was used in order to detect the presence of 2-HBP as the end product of BDS in model oil. The Gibb’s reagent was prepared by dissolving 10 mg of 2,6 dichloroquinone-4-chlorimide in 1 ml of ethanol [15]. Then, 50 µl was added to 1 ml samples previously adjusted to pH 8. The reaction mixture was incubated at 30°C for 1 h. Positive reactions developed purple color. Control cultures with no added DBT showed no color development. The absorbance of the reaction mixture was determined at 595 nm (Biochrom. Model Biowave, UK). Quantification of OD values was performed by reference to standard curve plotted, with a series of dilutions of pure 2-HBP (0 to 10 mg.L⁻¹).

**High-performance Liquid Chromatography (HPLC)**

HPLC (type LC-10A; shimadzu, Kyoto, Japan) was also used to detect and quantify DBT and 2-HBP. It was equipped with a Puresil c18 column (Water, Milford, MA, USA) [16].

**Results**

**Effect of carbon source on cell growth of the bacterium *R. erythropolis* grown as suspended cell culture**

Ethanol and glucose have been reported as proper carbon sources for BDS by different bacterial strains [17,18]. Therefore, growth of *R. erythropolis* on the two carbon sources and on a mixture of both was investigated. The results are depicted in Table 1. Clearly, *R. erythropolis* could grow faster on ethanol. In MSM supplemented with 25 g.L⁻¹, the maximum biomass concentrations of 1.4 g.L⁻¹ was obtained from ethanol after 24 h whereas 1.2 g.L⁻¹ from glucose after 40 h. Glucose at higher concentration (50 g.L⁻¹) supported more growth but not ethanol. Only 0.80 g.L⁻¹ of biomass was recovered from cultivation on 50 g.L⁻¹ of ethanol compared with up to 2.5 g.L⁻¹ from glucose. Obviously, high concentration of ethanol inhibited growth and multiplication of *R. erythropolis*. Similar findings were reported by Wang and Krawiec [17] and Honda et al. [18].

Beside being effective carbon source at low concentration, ethanol was suggested to provide NADH+ + H⁺ required for conversion of FMN to FMNH₂ which is a co-enzyme for the activities of desulfurization enzymes [19]. Therefore, cell growth on mixture of low concentration of both ethanol and glucose were studied. As shown in Table 1, *R. erythropolis* grew well under such conditions and up to 3.3 g.L⁻¹ of biomass was obtained after 36 h. Thus, this mixture was used in subsequent experiments as the appropriate carbon source for BDS by *R. erythropolis*.

**Production of high cell concentration of *R. erythropolis* in polyurethane foams**

In order to enhance attachment of bacterial cells into polyurethane foams, the VRICR was inoculated with *R. erythropolis* as described in the Materials and Methods section. It was firstly fed with MSM containing 25 g.L⁻¹ of each of glucose and ethanol at a high feed rate of 320 ml.h⁻¹. The bioreactor was rotated at 0, 15 and 30 and rpm. Aeration rate was manipulated to insure OTR of 90.0 mM.O₂.L⁻¹.h⁻¹. Samples were taken at regular intervals and analyzed for free and immobilized cells and residual carbon source. The results are shown in Table 1. It is clear that rotation speed had a pronounced effect on biomass concentration in polyurethane foams. Cultivation without rotation resulted in a steady increase in immobilized cells; 80.2 g.L⁻¹ were found after 5 days. However, incomplete utilization of carbon source, mainly glucose, was observed, most probably due to cell over-growth into polyurethane foams and limitation in mass transfer for both oxygen and nutrients. The mass ratio of free to immobilized cells (Mf/i) was only 2.8%, which reflects an extremely slow growth rate of immobilized biomass similar to those obtained previously with the bacterium *Zymomonas mobilis* [11]. With the highest rotation speed (30 rpm), cell leakage from polyurethane foams into surrounding medium was probably higher than cell growth in polyurethane foams. The concentration of immobilized cells decreased dramatically after reaching a maximum value to attain only 12.0 g. L⁻¹ at the end of attachment period (Table 2). The highest Mf/i value of 36.9% was reached under such conditions, and a steady state was never reached. Ethanol was completely utilized whereas glucose utilization was only 20%.

---

**Table 1:** Cell growth (DW, g.l⁻¹) of the bacterium *R. erythropolis* in MSM supplemented with different carbon sources at various concentrations.

| Cultivation time (h) | Glucose (g.L⁻¹) | Ethanol (g.L⁻¹) | Glucose + Ethanol (g.L⁻¹) |
|----------------------|----------------|----------------|--------------------------|
| 0.0                  | 0.020          | 0.025          | 0.020                    |
| 5.0                  | 0.026          | 0.049          | 0.040                    |
| 10.0                 | 0.045          | 0.095          | 0.084                    |
| 24.0                 | 0.350          | 0.650          | 0.440                    |
| 36.0                 | 0.655          | 1.300          | 1.335                    |
| 40.0                 | 1.220          | 2.425          | 1.415                    |
| 48.0                 | 1.212          | 2.400          | 1.385                    |
| 54.0                 | 1.189          | 2.550          | 1.402                    |
| 60.0                 | 1.195          | 2.475          | 1.365                    |

**Table 2:** Performance of VRICR of *R. erythropolis* during the first week of operation (cell attachment period) at various rotation speeds.

| Rotation speed (rpm) | Carbon source utilization (%) | Immobilized cells (g.L⁻¹) | Free cells (g.L⁻¹) | Mf/i (%) |
|----------------------|-------------------------------|---------------------------|-------------------|----------|
| 0                    | 94.8                          | 85.40                     | 2.35              | 2.75     |
| 15                   | 100.0                         | 70.36                     | 15.03             | 21.43    |
| 30                   | 20.19                         | 25.06                     | 9.24              | 36.87    |

Mf/i is the mass ratio of free cells to immobilized cells.
Steady state with complete utilization of both carbon sources were achieved only at rotation speed of 15 rpm. Immobilized cell concentration of 69.8 g l\(^{-1}\) was maintained. Under such conditions, growth of immobilized *R. erythropolis* cells was apparently balanced by cell leakage into surrounding medium with *M*\(_{\text{f}}\) of 21.4%. In earlier publications, a similar growth pattern was observed with the bacterium *Z. mobilis* [12] and *Corynebacterium glutamicum* [11], but with relatively lower immobilized cell concentrations of 25 and 46 g l\(^{-1}\) respectively. Thus, *R. erythropolis* appears to attach more successfully to polyurethane foams.

### Continuous BDS of model oil by VRICR of *R. erythropolis*

In most previous studies on biodesulfurization activity using high cell density of biocatalyst [19,20] a separate two-stage process was employed; one for cell growth and the second for induction and biodesulfurization activity. In order to simplify the process and lower operating costs, the previously prepared VRICR harboring high cell density of *R. erythropolis* was tested for conducting the two stages in a single reactor. The bioreactor was fed with 0.1 M phosphate buffer at 320 ml l\(^{-1}\) in order to remove non-attached cells and all attached metabolites particularly sulphate. To initiate the second stage, a model oil mixture with a volume ratio of oil phase and aqueous phase of 1:6 containing 11.68 mM DBT in hexadecane was fed into the bioreactor at 320 ml h\(^{-1}\). As shown in Figure 1, 2-HBP as the final end product of mercaptans was detected in bioreactor effluent after 5 h indicating a successful induction of Dsz-enzymes in resting (non-growing) immobilized cells. The highest desulfurization activity of 132.0 mM 2-HBP kg\(^{-1}\) dry cells h\(^{-1}\) was recorded at 15.0 h, and then decreased dramatically (Figure 1A).

This period of reactor operation was also coordinated by accumulation of sulphate (Figure 1B) which might have caused inhibition of cell growth and desulfurization activity. Such inhibition has been reported by other investigators [21,22]. It was also noticed that cell leakage from polyurethane foams in absence of cell growth has taken place as M\(_{\text{f}}\)/I increased to reach a high value of 12.1% and immobilized cell concentration decreased to 35 g l\(^{-1}\) at 24 h (Figure 1B). This could be due to additional inhibition of cell growth caused by lack of nutrients [23]. These results indicate that maintaining a stable continuous desulfurization activity of immobilized resting cells is impossible most probably due to the need for a continuous supply of Dsz-enzymes and cofactors in order to compensate for a possible loss of these enzymes and cofactors under operational condition employed. This finding is in good agreement with those obtained by Shan et al. [22], but disagree with Kirimura et al. [5]. Such discrepancy could be attributed to differences in fermentation techniques and type of biocatalyst used. The latter researchers used batch cultivation for carrying out biodesulfurization of DBT by the bacterium *Bacillus subtilis*.

Therefore, a complete rich MSM containing 25 g l\(^{-1}\) of each of glucose and ethanol as carbon source and 11.68 mM DBT in hexadecane as nitrogen source was fed into the bioreactor at 35 h. Activity of the bioreactor was gradually restored; *M*\(_{\text{f}}\)/I value and concentration of sulphate decreased whereas immobilized cells grew actively and its concentration increased back to its original level. Again, 2-HBP was detected in bioreactor effluent at 40 h and a steady increase in its concentration was recorded (Figure 1A). This was coordinated with an increase in *M*\(_{\text{f}}\) values indicating an increase in cell growth rate of immobilized cells. Then, a steady state was reached at 55 h with a maximum cell concentration of 70 g l\(^{-1}\), complete removal efficiency of sulfur, *M*\(_{\text{f}}\) of 22.5% and specific desulfurization activity of 166.86 mM 2-HBP kg\(^{-1}\) dry cells h\(^{-1}\) for 120 h more.

The results obtained using VRICR compare very favorably with those reported by other researchers (Table 3) for both specific biodesulfurization rate and stability of operation over long period of time. Konishi et al. [20] obtained higher specific biodesulfurization rate. However, the VRICR has longevity of operation, final biomass concentration and volumetric BDS activity of 1.4, 3.5 and 7.3 times higher respectively. Certainly, this will be of great importance when such process is to be considered for industrial implementation.

#### Conclusion

The obtained results confirm the feasibility of using the VRICR of the bacterium *R. erythropolis* for an efficient long-term continuous BDS of a model oil. Compared to free-suspended cell reactors utilized for BDS processes, VRICR has many advantages. Its scale-up is simple, since only IBUs are to be constructed and mounted into the presently existing shaft in the conventional bioreactors. Secondly, the open structure of the polyurethane foams together with the good internal configurations of the IBUs permit simple and direct contact between BTD in oil phase, dissolved oxygen and immobilized cells and hence

---

**Table 3:** Comparative data for biodesulfurization of sulfur-containing model oil by *R. erythropolis*.

| References | Cultivation technique | Carbon source | **Biomass** (g l\(^{-1}\)) | **Specific BDS rate** (mM 2-HBP. Kg\(^{-1}\).h\(^{-1}\)) | **Longevity** (h) |
|------------|-----------------------|---------------|--------------------------|---------------------------------|-----------------|
| Yan et al., 2000 | Fed-batch using free suspended cells | ethanol | 24.3 | 18.2 | 10 |
| Matsushita et al., 2001 | Fed-batch using free suspended cells | ethanol | 20.0 | 111.5 | 89 |
| Yoshikawa et al., 2002 | Fed-batch using free suspended cells | ethanol | 20.0 | 200.0 (9.0) | 89 |
| Konishi et al., 2005 | Fed-batch using free suspended cells | ethanol | 70.0 | 110.5 (36.5) | 120 |

* Values between brackets are the overall volumetric BDS activities of the process (mM 2HBP h\(^{-1}\))

---

**Legend:**

- SDR: sulfide desulfurization rate
- M/I: metabolite index
- DBT: dibenzothiophene
- 2-HBP: 2-mercaptobenzothiazole
- MSM: mineral salts medium
- BTD: biorefinery distillates
- BDES: biodesulfurization
- VRICR: Vertical Rotating Immobilized Cell Reactor
- IBUs: immobilized biocatalytic units

---

**Figure 1:** Performance of the VRICR of *R. erythropolis* during the second stage of operation. The arrow indicates feeding with MSM supplemented with carbon and sulfur sources.
a rapid and efficient BDS process. Finally, cell over-growth within polyurethane foams can be effectively eliminated so as a relatively smaller but enough concentrations of actively growing immobilized cells are maintained and used to sustain long-term BDS process. Work in progress in order to optimize operating cultural and environmental parameters to further desulfurize higher concentration of DBT and other sulfur-containing petroleum products.

Acknowledgement

The authors are grateful to Department of Academic Affairs, Taif University, K.S.A. for the financial support for this work.

References

1. Kilbane J, Le Borgne S (2004) Petroleum biorefining: the selective removal of sulfur, nitrogen and metals. In Petroleum Biotechnology. Developments and perspectives, R. Vazquez-Duhalt & R. Quintero-Ramirez (eds). The Netherlands: Elsevier pp29-65.
2. Jamshid R, Javad H, Babak M (2010) Increasing of Biodesulfurization Activity of Newly Recombinant Pseudomonas Aeruginosa ATCC 9027 by Cloning the Flavin Reductase Gene. Inter J Biotechnol Biochem 6: 219-229.
3. Sumedha B, Sharma K (2010) Biodesulfurization of dibenzothiophene, its alkylated derivatives and crude oil by a newly isolated strain Pantoea agglomerans D23W3. Biochem Eng J 50: 104-109.
4. Izumi Y, Oshiro T, Ogino H, Hine Y, Shimao M (1994) Selective desulfurization of dibenzothiophene by R. erythropolis D-1. Appl Environ Microbiol 60: 223-226.
5. Krimura K, Furuya T, Nishii Y, Ishii Y, Kino K, et al. (2000) Biodesulfurization of dibenzothiophene and its derivatives through selective cleavage of carbon-sulfur bonds by a moderately thermophilic bacterium B. subtilis WU-SEB. J Biosci Bioeng 91: 262-266.
6. Seo S, Keum S, Cho K, Li X (2006) Degradation of dibenzothiophene and carbazole by Arthrobacter sp. P1-1. In Biodeter Biodegr 58: 36-43.
7. Li W, Wang M, Chen H, Chen J, Shi Y (2006) Biodesulfurization of dibenzothiophene by growing cells of Gordonia sp. in batch cultures. Biotechnol Lett 28: 1175-1179.
8. Lee I, Bae H, Ryu H, Cho K, Chang Y (2005) Biocatalytic desulfurization of diesel oil in an air-lift reactor with immobilized Gordonia nitida CYKS1 cells. Biotechnol. Prog 21: 781-785.
9. Mohebali G, Andrew S (2008) Biocatalytic desulfurization (BDS) of petroleum fuels. Microbiol 154: 2169-2183.
10. Dinamarca A, Ibacache-Quiroga C, Baeza P, Galvez S, Villarroel M, et al. (2010) Biodesulfurization of gas oil using inorganic supports biomodified with metabolically active cells immobilized by adsorption. Bioresour Technol 101: 375-8.
11. Amin G (1994) Continuous production of glutamic acid by Vertical Rotating Immobilized Cell Reactor of the bacterium Corynebacterium glutamicum. Bioresource Technology 47: 113-119.
12. Amin G, Doelle W (1989) Vertical Rotating Immobilized Cell Reactor of the bacterium Z. mobilis for stable long-term continuous ethanol production. Biotech Techniques 3: 5-100.
13. American Public Health Association (APHA) (1992) Standard Methods for the Examination of Water and Wastewater, 18th edition. Washington DC, USA.
14. Hirose Y (1972) Oxygen transfer. In K. Yamada et al. [eds.]. The microbial production of amino acids. Wiley p203-226.
15. Quintana M, Didion C, Dalton H (1997) Colorimetric method for a rapid detection of oxygenated aromatic biotransformation products. Biotechnol Tech 11: 585-587.
16. Monticello J, Fennerty R (1985) Microbial desulfurization of fossil fuels. Ann Rev Microbiol 39: 371-389.
17. Wang P, Krawiec S (1996) Kinetic analysis of desulfurization of dibenzothiophene by R. erythropolis in batch and fed-batch cultures. Appl Environ Microbiol 62: 1670-1675.
18. Honda H, Sugiyama H, Saito I, Kobayashi T (1998) High cell density culture of R. rhodochrous by pH-stat feeding and dibenzothiophene degradation. J Ferment Bioeng 86: 334-338.
19. Yan H, Kirshimoto M, Omasa T, Kakatka Y, Suga K, et al. (2000) Increase in desulfurization activity of R. erythropolis KA2-5-1 using ethanol feeding. J Biosci Bioeng 89 : 361-366.
20. Konishi Y, Kishimoto M, Omasa T, Kakatka Y, Shioya S, et al. (2005) Effect of sulfur sources on specific desulfurization activity of R. erythropolis KA2-5-1 in exponential-fed batch culture. J Biosci Bioeng 99: 259-263.
21. Ohshiro T, Hirata T, Izumi Y (1995) Microbial desulfurization of dibenzothiophene in the presence of hydrocarbon. Appl Microbiol Biotechnol 44: 249-252.
22. Shan G, Zhang H, Xing J, Chen G, Li W, et al. (2006) Biodesulfurization of hydrodesulfurized diesel oil with Pseudomonas delafIELDi R-8 from high density culture. Biochem. Eng J 27: 305-309.
23. Chang H, Kim J, Lee H, Cho KS, Rye W, et al. (2001) Production of a desulfurization biocatalyst by two-stage fermentation and its application for the treatment of model and diesel oils. Biotechnol Prog 17: 876-880.