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

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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 (dibenzothiophene 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 rhodocrous*

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 HDS 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-hexadecan) 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 in a solid minimal salt medium (MSM) with the following composition ($g.l^{-1}$): NH_4NO_3 (1.0), KH_2PO_4 (2.0), K_2HPO_4 (7.0), $MgCl.6H_2O$ (2.0), $CaCl_2.6H_2O$ (0.1), $FeCl_2.6H_2O$ (0.1), $MNCl_2.6H_2O$ (0.1), $ZNCl_2$ (0.1), glucose (25.0) and agar (15.0). For cell growth and desulfurization experiments, liquid medium had the same composition except agar. Carbon source and sulfur sources were added as specified below.

Growth of *R. erythropolis* on different carbon sources

Growth of *R. erythropolis* on various carbon sources was investigated in 500 ml shake flasks, each containing 150 ml of MSM supplemented with 25 or 50 $g.l^{-1}$ of glucose, ethanol or a mixture of 25 $g.l^{-1}$ of each, as carbon source. Incubation was carried out at 30°C, pH 7 and 200 rpm. Samples were taken at regular intervals and analyzed for cell growth and residual glucose and ethanol.

Bioreactor

The VRICR previously described [11,12] was used with a slight modification. It consists of two stacks of immobilized biomass units (IBUs) mounted on a rotating shaft. These stacks were separated by a gap to allow insertion of electrodes and sampling. The shaft was driven by an external motor and extended into double-walled vessel. Each IBU had a core of polyurethane foam disc (97% porosity) between two perforated stainless steel sheets. The medium was pumped at the bottom of the bioreactor and the effluent was drawn off through outlet pipes at different heights, up the bioreactor. The total volume of the bioreactor was 1500 ml. For pH control, the alkaline solution was added via an opening at the top of the bioreactor located between the two

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stacks 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 O_2.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, supplementary 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-chloroimide 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 (Biochro. 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 $FMNH_2$ 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.l^{-1}. The bioreactor was rotated at 0, 15 and 30 and rpm. Aeration rate was manipulated to insure OTR of 90.0 mMO2.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 ($M_{f/i}$) 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 $M_{f/I}$ 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%.

Cultivation time (h)	Carbon sources				
	Glucose		Ethanol		Glucose + Ethanol
	25 g.l^{-1}	50 g.l^{-1}	25 g.l^{-1}	50 g.l^{-1}	25 g.l^{-1} + 25 g.l^{-1}
0.0	0.020	0.025	0.020	0.025	0.025
5.0	0.026	0.045	0.400	0.065	0.255
10.0	0.045	0.095	0.845	0.185	0.750
24.0	0.350	0.650	1.440	0.270	2.300
36.0	0.655	1.300	1.335	0.555	3.325
40.0	1.220	2.425	1.415	0.755	3.235
48.0	1.212	2.400	1.395	0.800	3.200
54.0	1.189	2.550	1.402	0.795	3.300
60.0	1.195	2.475	1.365	0.805	3.255

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

Rotation speed (rpm)	Carbon source utilization (%)		Immobilized cells (g.l^{-1})	Free cells (g.l^{-1})	$M_{f/i}$* (%)
	Glucose	Ethanol			
0	94.8	100.0	85.40	2.35	2.75
15	100.0	100.0	70.36	15.03	21.43
30	20.13	50.45	25.06	9.24	36.87

*$M_{f/i}$ is the mass ratio of free cells to immobilized cells.

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

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 $M_{f/I}$ 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 BDS 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 $M_{f/I}$ 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; $M_{f/I}$ value and

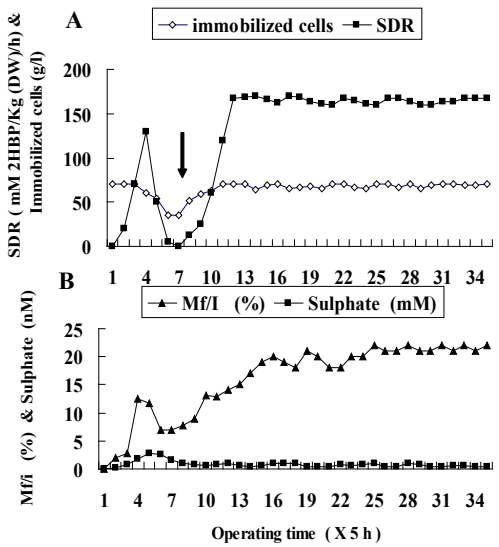

Figure 1: Performance of the VRICR of *R.erythropolis* during the second stage of operation. The arrow indicates feeding with MSM supplemented with carbon and sulfur sources.

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 $M_{f/I}$ 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, $M_{f/I}$ 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

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	-	95.1	48
Matsushita et al., 2001	Fed-batch using free suspended cells	ethanol	24.3	18.2	10
Yoshikawa et al., 2002	Fed-batch using free suspended cells	ethanol	20.0	111.1	89
Konoshi et al., 2005	Fed-batch using free suspended cells	ethanol	20.0	200.0 (5.0)*	89
This work	Continuous using immobilized cells	glucose + ethanol	70.0	110.5 (36.5)*	120

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

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

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.

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