International Biodeterioration & Biodegradation 86 (2014) 343–348

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

# Taguchi optimization of dibenzothiophene biodesulfurization by *Rhodococcus erythropolis* R1 immobilized cells in a biphasic system

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ARTICLE INFO

*Article history:*
Received 28 January 2013
Received in revised form
22 September 2013
Accepted 8 October 2013
Available online 13 November 2013

*Keywords:*
Biodesulfurization
Immobilization
Dibenzothiophene
Taguchi approach
Nano γ-$Al_2O_3$

ABSTRACT

Organic sulfur components of the petroleum are too resistant to be removed by the conventional desulfurization processes. This study aimed to investigate the removal of dibenzothiophene (DBT) as an organic sulfur compound, from the oily phase by a bioprocess employing the immobilized cells. *Rhodococcus erythropolis* R1 cells were encapsulated in calcium alginate beads by considering factors such as the alginate concentration, size of the beads, the concentration of surfactants and γ-$Al_2O_3$ nano particles for optimizing biodesulfurization (BDS) via Taguchi approach. The impact of two cofactor precursors (nicotinamide and riboflavin) on the long term BDS efficiency was also examined. The results indicated that the optimum factor levels for the bigger is better criterion could be achieved at 20% (w/w) of γ-$Al_2O_3$ nano particles, alginate beads size equal to 1.5 mm, 1% (w/v) of the alginate and 0.5% (v/v) of span 80. The related statistical analysis showed that the concentration of γ-$Al_2O_3$ nano particles was the most significant factor in the BDS process. Moreover, the addition of nicotinamide and riboflavin significantly decreased the biocatalytic inactivation of the immobilized cells system after successive operational steps enhancing the BDS efficiency by more than 30% after four steps. It can be concluded that a combination of the nano γ-$Al_2O_3$ particles with alginate immobilized cells could be very effective in biodesulfurization process.



## 1. Introduction

The extensive consumption of sulfur-rich fossil fuels can lead to the release of a number of harmful chemicals such as the sulfur oxides which in turn, can result in severe environmental problems including air pollution and acid rain. In fact, a major part of the petroleum sulfur content consisting of organic compounds which are hard to separate through conventional methods are considered as one of the major problems in crude oil refining (Tailleur et al., 2005). For instance, it is reported that some organic components such as dibenzothiophene (DBT) remain in the oil even after desulfurization processes (Gray et al., 1996). As a remedy, several effective bioprocesses have been developed based on the ability of a few bacterial strains such as *Rhodoccocus* species which can remove sulfur from organic compounds like the DBT and produce 2-hydroxybiphenyl (2-HBP) as the final product without causing oxidative loss of fuel carbon. Although bioprocesses have been shown to be promising in organic desulfurization, there are still certain problems within the system which may hinder their large scale application. For example, using free cells in biodesulfurization can (BDS) lead to the formation of a two phase oil/water mixture with suspended cells requiring cost intensive unit operations e.g. centrifugation at the downstream of the process. In addition, there is a possibility to have cell contaminations at the final products (Guo et al., 2006).

To address the problem, immobilization methods have frequently been used in the industrial processes. Clearly, compared to the free cells, immobilization has inherent advantages including enhanced stability of the system, easy separation of cells, minimizing or eliminating the cell contaminations in the products, convenient recovery and re-use of cells which enable their frequent use in the process (Zhang et al., 2010). Several immobilization methods have been suggested for industrial applications such as the encapsulation of the cells in semi permeable membranes or their entrapment within polymeric structures (Martinsen et al., 1989; Biria et al., 2008). Alginate is one of the unique biopolymers that can be employed for the encapsulation of cells and enzymes which has found extensive applications in industrial processes because of the advantages like biocompatibility, simplicity and low cost, (Blandino et al., 1999).

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http://dx.doi.org/10.1016/j.ibiod.2013.10.006

There are a few important factors affecting the rate of desulfurization in an immobilized cells system such as the concentration of alginate (Zhang et al., 2010), the size of beads, the presence of surfactants (Li et al., 2008) and the application of nano γ-$Al_2O_3$ particles in the system (Zhang et al., 2011). Evidently, maximum BDS efficiency can be achieved by setting the parameters at their optimized values. Consequently, Taguchi method was used in this study to optimize the important parameters and to increase the BDS efficiency of the immobilized cells in oil/water biphasic system.

Among many advantages of immobilization, re-usability of cells in successive reaction steps is of great importance. However, BDS activity of the entrapped cells will decrease after each step as a result of the reduction of cofactors such as $NADH_2$ and $FMNH_2$ in the 4S-pathway (YAN et al., 2008). Moreover, the entrapment technique itself often leads to a decrease in biocatalytic activity (Karsten and Simon, 1993). As a consequence, to reduce the loss of BDS efficiency because of the immobilization, the influence of two cofactor precursors (i.e. nicotinamide and riboflavin as precursors of $NADH_2$ and $FMNH_2$ respectively) was also studied and viability of encapsulated cells was measured after each reaction cycle by flow cytometry.

## 2. Materials and methods

### 2.1. Chemicals

Sodium alginate, methanol (HPLC grade), Rhodamin 123 and nano γ-$Al_2O_3$ were purchased from Sigma Chemical Co., DBT and n-tetradecane were purchased from Merck. 2-HBP, span 80 and tween 80 were prepared from Fluka Chemical Co. All other chemicals were analytical grade and commercially available.

### 2.2. Bacterial strain and growth condition

*Rhodococcus erythropolis* R1 (NCBI GenBank Accession No. GU570564) was used in desulfurization experiments. This strain which has a high capability in the conversion of DBT to 2-HBP, was previously isolated from an oil-contaminated soil sample (Etemadifar et al., 2008). It was cultured in basal salt medium (BSM) supplemented with 0.3 mM DBT as the sole sulfur source. Cell cultivation was carried out in a 1000 ml flask containing 200 ml of BSM medium on an orbital shaker incubator (n-biotech,inc) at 180 rpm and 30 °C. The BSM had the following composition: $Na_2HPO_4.7H_2O$ 8 g $l^{-1}$, $KH_2PO_4$ 4 g $l^{-1}$, $NH_4Cl$ 2 g $l^{-1}$, $MgCl_2$ 0.2 g $l^{-1}$, $FeCl_3$ 0.001 g $l^{-1}$, $CaCl_2$ 0.001 g $l^{-1}$ and glucose 15 g $l^{-1}$ as carbon source.

### 2.3. Entrapment of bacteria

Bacterial cells were harvested after 72 h by centrifugation at 7000 rpm for 10 min and were washed several times with 0.9% w/v NaCl solution in order to remove the residual DBT from the cell walls surfaces. Finally, the harvested cells were resuspended in the same brine solution.

Sodium alginate solutions were prepared through slow dissolving of the alginate powder in water to concentrations equal to 2%, 4% and 6% w/v. The mixture was continuously stirred to avoid formation of precipitates. Next, alginate solutions were diluted by addition of equal volumes of the cell suspension to reduce the concentration values to 1%, 2% and 3% respectively. The target concentration of surfactants (tween 80 and span 80) was 0.5% (v/v) and the volume ratio of cell mass to nano γ-$Al_2O_3$ particles was 5:1 (w/w). The obtained alginate- cell suspension mixture was added drop-wise to a 0.2 M $CaCl_2$ solution using a peristaltic pump and a magnetic stirring to prevent droplet aggregation. The gelation time was restricted to 1 h and afterward, beads were washed twice with modified BSM (MBSM) and kept in MBSM supplemented with 0.1 mM DBT in order to preserve the bacteria in the active form. In the MBSM composition, the phosphate concentration was reduced to 0.1 of the BSM to hinder the dissolution of alginate beads, while the other components were identical to those of the BSM.

### 2.4. BDS media condition

The biphasic media consisted of MBSM (aqueous phase) and n-tetradecane (organic phase) in a 2:1 ratio and 1 mmol/L DBT as the sulfur source. The volumes of nicotinamide and riboflavin were 10 mmol/L and 40 μmol/L respectively. The BDS experiments were carried out in 100 ml flasks at 30 °C on an orbital shaker at 180 rpm (n-biotech,inc). The incubation time of DBT utilization and 2-HBP production was 20 h. Subsequently, the beads were isolated, washed and suspended in MBSM supplemented with 0.1 mM DBT and stored in refrigerator at 4 °C for next reactions.

### 2.5. Design of experiment

Taguchi method has generally been adopted to optimize the design variables in a timely manner and at lower costs (Taguchi, 1986). It can be used to manage the system by a set of factors at different levels, to facilitate identifying influential individual factors and their relationships and finally to establish their performance at the optimum levels by utilizing a few selected experimental sets (Das Mohapatra et al., 2009). In this approach, an orthogonal array of the controllable factors (inner array) is crossed with a separate orthogonal array for the noise factors (outer array). Each run in the inner array would be performed for all of the combinations in the outer array. A signal to noise (S/N) ratio which summarizes the mean and variance information is defined and data analysis would be carried out for this ratio using ANOVA to determine the optimum conditions as well as the contribution of each factor in the experimental results (Biria and Balouchi, 2013). There are three applicable types of (S/N) ratio depending on the optimization criteria: (1) lower is better (LB), (2) nominal is better (NB), and (3) higher is better (HB). The purpose of this study was to maximize the BDS activity, therefore, the S/N ratio with HB characteristics was utilized, which was calculated using the below equation:

$$\frac{S}{N} = -10\log_{10}\left[\frac{1}{n}\sum\left(\frac{1}{X_{BDS}}\right)^2\right] \quad (1)$$

In this equation, $n$ represents the number of repetitions and $X_{BDS}$ is the results of experimental measurements. For BDS activity, the $X_{BDS}$ is given by:

$$X_{BDS} = \frac{C_{2-HBP'20}}{C_{DBTO}} \times 100 \quad (2)$$

where $C_{DBT0}$ is the initial concentration of DBT (mM) and $C_{2\text{-}HBP'20}$ (mM) is the 2-HBP concentration after 20 h. All statistical experimental designs and analytical results were carried out using Minitab 16 software for Windows.

Four parameters with a critical effect on BDS activity were selected to be optimized in alginate capsules. The factors and their levels are shown in Table 1. For three parameters in three levels ($3^3$) and one parameter in two levels ($2^1$) Taguchi has been suggested an L18 orthogonal array with a layout of $2^1 \times 3^3$ which indicating 18 experimental runs in duplicate (Table 2). The effects of factor levels on the BDS efficiency were determined employing analysis of variance (ANOVA) and the statistically significant factors were