

Optimization of nicotinamide and riboflavin in the biodesulfurization of dibenzothiophene using response surface methodology

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Abstract

Introduction: Dibenzothiophene (DBT) is a sulfuric compound and resistant to Hydrodesulfurization process. *Rhodococcus erythropolis* R1, a previously isolated bacterial strain, is capable to bioconversion of DBT to 2-hydroxybiphenyl (2-HBP)

Materials and methods: The effect of nicotinamide (precursor of NAD) and riboflavin (precursor of FMN) on DBT biodesulfurization and growth rate by this strain was studied using Gibbs assay and turbidimetric assay respectively. The level of cofactor precursors were optimized using response surface methodology (RSM)

Results: Analyses showed that both nicotinamide and riboflavin were statistically significant and could enhance the biodesulfurization rate of DBT by induction of *dsz* operon. The optimum level of nicotinamide and riboflavin was obtained at 10.67 mM and 34.2 μ M respectively

Discussion and conclusion: In spite of increasing in BDS, the addition of these cofactor precursors led to decreased growth rate and biomass production due to limited effect of produced 2-HBP.

Key words: *Rhodococcus erythropolis*, Nicotinamide, Riboflavin, Dibenzothiophene

Introduction

Sulfur is the third most plentiful element of crude oil after carbon and hydrogen (1). Consumption of sulfur-rich fossil fuels leads to releasing sulfur oxides and therefore creates severe air pollution and acid rain. The major part of petroleum sulfur compounds are organic compounds and one of the main problems of oil refining is its refinement from crude oil (2). Although the physical-chemical process can remove inorganic sulfur, organic sulfur compounds such as dibenzothiophene (DBT) remain in oil after this process (3) and these recalcitrant compounds can be removed by biodesulfurization (BDS). Due to specificity and cost-effectiveness, BDS can be an alternative or complementary process for hydrodesulfurization (HDS) technology (4). DBT is often used as a model of heterocyclic sulfuric compounds in biodesulfurization studies. Some of bacterial strains can remove the sulfur of DBT via 4S-pathway and because selective attacking C-S bounds, does not affect the combustion value of petroleum (5- 7). Four enzymes are involved in 4S-pathway: first DBT monooxygenase (Dsz C) oxidizes DBT to DBT-sulfone (DBTO₂). In the second step, flavomonooxygenase (Dsz A) catalyze transformation of DBTO₂ to 2-2'-hydroxyphenyl benzene sulfinate (HPBS) and in the final step, HPBS is desulfinated by Dsz B to produce 2-hydroxybiphenyl (2-HBP) as a final product (8). Three catabolic genes, *dszA*, B, C, are clustered in *dsz* operon. Dsz D, a flavinreductase, prepares FMNH₂ (reduced form of flavin mononucleotide) required for oxygenase reactions via oxidation of NADH (reduced form of nicotinamide-adenine dinucleotide) and is a chromosomal gene (9). Previous study showed that the addition of riboflavin and nicotinamide as precursor of FMNH₂ and NADH in culture medium, led to increasing the BDS activity of *Rhodococcus erythropolis* USTB-03 (10).

In this study, the optimization of riboflavin and nicotinamide using Response Surface Methodology (RSM) was performed and their effects on BDS and growth of *Rhodococcus erythropolis* R1 were investigated. In ordinary optimization procedures, a parameter is changed and others are invariant. This procedure is time consuming and also the impact of a parameter on other parameters is not clear. Statistical optimization is an alternative approach that rapidly screens a set of factors at different levels and reflects the role of each factor and interaction between them. RSM is a mathematical-statistical technique for condition optimization based on the multivariate non-linear model and is useful to evaluate the affecting factors (11).

Materials and methods

Chemicals

DBT was purchased from Merck. Riboflavin and nicotinamide were purchased from Sigma. 2-HBP was prepared from FlukaChemika Co. All other chemicals were analytical grade and commercially available.

Bacterial strain and growth condition

Rhodococcus erythropolis R1 (GU570564), a capable strain in the desulfurizing DBT to 2-HBP was previously isolated from oil-contaminated soil (12) and 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 250 mL flask containing 50 mL of BSM medium at 30°C on a rotary shaker operated at 180 rpm (n-biotech, inc). The BSM contain following composition: Na₂HPO₄·7H₂O 8 g/l, KH₂PO₄ 4 g/l, NH₄Cl 2 g/l, MgCl₂ 0.2 g/l, FeCl₃ 0.001 g/l, CaCl₂ 0.001 g/l and glucose 15 g/l as carbon source.

The stock solution of riboflavin (10 mM) was prepared by adding 0.376 g of riboflavin in 100 mL of distilled water and

for preparing stock solution of nicotinamide (1 M), 12 g of nicotinamide was dissolved in 100 mL of distilled water. Different amount of these stock solutions were filtered and inoculated to sterile medium culture.

Analytical methods

Cell density was measured by the absorbance at 600 nm (OD_{600}) (spectronic21D Milton Roy). Desulfurization activity was monitored using the Gibb's reagent (2,6-dichloroquinone-4-chloroimide). Gibb's reagent reacts with aromatic hydroxyl groups such as 2-HBP at a pH of 8.0 and forms a blue-colored complex that can be monitored by spectrophotometer at 610 nm. The Gibb's assay was performed as follows: Microbial culture was centrifuged at 7000 rpm for 10 min and 1 mL of supernatant was placed in a clean test tube and its pH was adjusted to 8.0 by adding 200 μ l $NaHCO_3$ 1M and then 20 μ l of Gibb's reagent (10 mM) was added. The solution was allowed to incubate for 30 min at room temperature to produce full-color development and then absorbance of the solution was measured at 610 nm (spectronic21D Milton Roy). The BDS activity (X_{BDS}) of the cells was determined as the percentage of desulfurization according to the following equation:

$$X_{BDS} = \frac{C_{2-HBP}}{C_{DBT0}} \times 100$$

Where C_{DBT0} is the initial concentration of DBT (mM) and C_{2-HBP} is the 2-HBP concentration (mM) after a certain time.

Results

Statistical analysis

The variable interaction can be simultaneously investigated by response surface model. Coded values of experimental variables are shown in Table 1.

Table 1- Coded values of experimental variables for growth rate and X_{BDS}

Independent variables	Min	-1	0	+1	Max
X_1 : Nicotinamide concentration (mM)	1.8	5	12.5	20	23.11
X_2 : Riboflavin concentration (μ M)	15.5	30	65	100	114.5

A quadratic polynomial equation was established to recognize the relationship between 2-HBP production of growing cells and variables based on the experimental results of CCD (Table 2).

Table 2- Response surface Central Composite Design (CCD)

Run	X_1	X_2	X_{BDS}	growth
1	0	0	2.35	2.55
2	1	-1	2.04	2.43
3	-1	-1	2.38	2.81
4	0	0	2.41	2.59
5	0	0	2.44	2.54
6	Max	0	1.77	2.24
7	0	0	2.44	2.54
8	0	Max	2.16	2.47
9	1	1	1.84	2.25
10	Min	0	2.07	2.59
11	-1	1	2.04	2.62
12	0	0	2.37	2.55
13	0	Min	2.4	2.68

The model of coded units is calculated using:

$$Y = \beta_0 + \sum_{i=1}^3 \beta_i X_i + \sum_{i=1}^3 \beta_{ii} X_i^2 + \sum_{i=1}^2 \sum_{j=i+1}^3 \beta_{ij} X_i X_j$$

Where Y is the predicted response, X_i is the variable β_0 is constant, β_i is the linear effect, β_{ii} is the quadratic effect, and β_{ij} is the interaction effect.

Biodesulfurization activity

For X_{BDS} , the model of coded units after removing non significant parameters can be expressed as:

$$Y = 2.4 - 0.12 X_1 - 0.11 X_2 - 0.25 X_1^2 - 0.067 X_2^2$$

Where Y is the response value (%), X_1 is nicotinamide (mM) and X_2 is riboflavin (μM). Positive and negative sign before terms indicates synergistic and antagonistic effect respectively (13). The equation indicates a quadratic linear relationship between variables and X_{BDS} . As can be seen in ANOVA Table (Table 3), the model F-value is 62.35. The greater model F value implies that the model is significant. There is only a 0.01% chance that a model F-value could occur due to noise. In addition, the value of $\text{Prob} > F$ less than 0.05

indicates model terms are significant. In this case X_1 , X_2 , X_1^2 and X_2^2 are significant model terms. There is a 32.49% chance that a Lack of Fit F-value this large could occur due to noise. The Pred R-Squared of 0.8995 is in reasonable agreement with the Adj R-Squared of 0.9624. Adeq Precision measures the signal to noise ratio. The ratio greater than 4 is desirable. Our ratio of 22.082 indicates an adequate signal. Thus, this model could be used to navigate the design space satisfactorily. According to the present model, both factors are significant model terms.

Table 3- Analysis of Variance (ANOVA)

Source of variance	df	X_{BDS}			Growth		
		Mean square	F value	P value	Mean square	F value	P value
Model	5	0.13	62.35	<0.0001	0.056	31.11	0.0001
X_1	1	0.12	55.60	0.0001	0.19	107.39	<0.0001
X_2	1	0.097	46.24	0.0003	0.056	30.82	0.0009
X_1X_2	1	4.900E-003	2.34	0.1696	2.500E-005	0.014	0.9096
X_1^2	1	0.43	203.43	0.0001	0.026	14.53	0.0066
X_2^2	1	0.031	15.5	0.0061	2.413E-003	1.34	0.2854
Residual	7	2.090E-003			1.804E-003		
Lack of fit	3	2.651E-003	1.59	0.3249	3.636E-003	8.46	0.0332
Pure error	4	1.670E-003			4.300E-004		

Growth: $R^2 = 0.9569$ R (adjusted) = 0.9262
 X_{BDS} : $R^2 = 0.9780$ R (adjusted) = 0.9624

Growth rate

In growth rate, the model of coded units after removing non significant parameters can be stated as:

$$Y = 2.55 - 0.16 X_1 - 0.083 X_2 - 0.061 X_1^2$$

Where Y is the response value (OD_{600}), X_1 is nicotinamide (mM) and X_2 is riboflavin (μM). The ANOVA for growth rate by resting cells is shown in Table 3. The Model F-value is 31.11 and the $\text{Prob} > F$ is less than 0.05, both imply the model and model terms are significant (Table 3). In this case, X_1 , X_2 and X_1^2 are significant model terms. The Lack of Fit F-value of 8.46 indicates that the Lack of Fit is significant. There is 3.32% chance that Lack of Fit F-value occurs due to noise. The Pred R-Squared of 0.7263 is in reasonable agreement the Adj R-Squared of

0.9262. The ratio greater than 4 is desirable. Our ratio of 18.769 indicates an adequate signal. Thus, this model could be used to navigate the design space satisfactorily. According to the present model, both factors are significant model terms.

Discussion and conclusion

The response surface and its contour plot at the base can represent the regression model developed to investigate the interaction between factors and specify the optimum level of each factor. The interaction of two independent factors can be shown by each response surface and a contour plot. The fitted surface and contour plots between nicotinamide and riboflavin are presented in Fig.1 for X_{BDS} and Fig. 2

for growth rate.

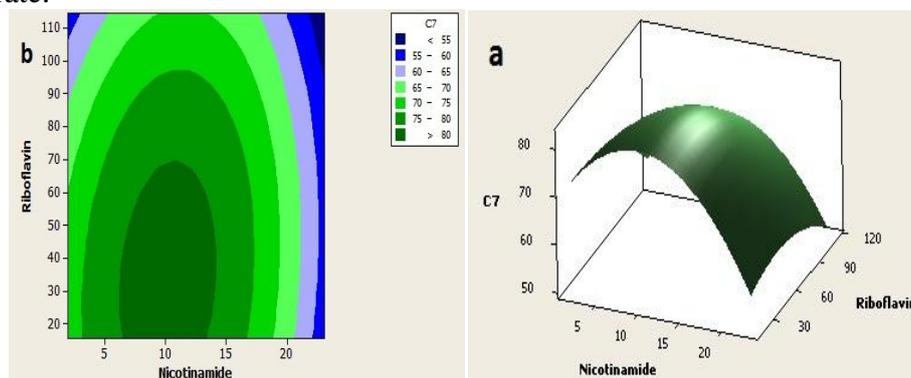


Fig. 1- Response surface (a) and contour plot (b) of biodesulfurization of DBT after the addition of cofactor precursors (C7 is the X_{BDS}).

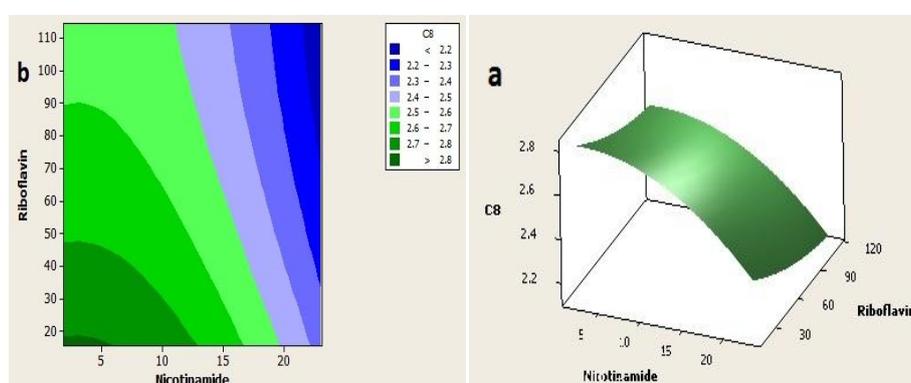


Fig. 2- Response surface (a) and contour plot (b) of bacterial growth rate after the addition of cofactor precursors (C8 is the OD_{600}).

The rate limiting step of 4S-pathway is the first step that DBT is converted to DBT-sulfone. Two enzymes are involved in this step; Dsz C catalyses the reaction and Dsz D prepares the FMNH₂ required for activity of Dsz C by oxidizing NADH (14). Evans et al (15) showed that nicotinamide is the dominant precursor of NAD production. Bauer et al (16) showed that riboflavin can be converted to FMN by riboflavin kinase. Hai et al (10) indicated that adding nicotinamide and riboflavin enhanced the biodesulfurization of DBT by *Rhodococcus erythropolis* USTB-03. The results of this study showed that addition of nicotinamide and riboflavin to medium culture led to increasing in biodesulfurization activity of *R. erythropolis* R1. The optimum level of nicotinamide and riboflavin was 10.67 mM

and 34.2 μ M respectively, but adding too much of these cofactor precursors caused decrease in BDS activity (Fig.1). This reducing in X_{BDS} was due to decreasing the growth rate. Fig.2 shows the cell density of *R. erythropolis* R1 after 48 h in the presence of different amounts of nicotinamide and riboflavin and as can be seen, by increasing the volume of these cofactor precursors in medium culture, the growth rate of cells was reduced. Reduction in growth rate is probably due to increasing in biodesulfurization and 2-HBP production. As has been previously reported, increasing the volume of 2-HBP, limits the growth of cells. In addition, inoculation of nicotinamide and riboflavin at the maximum level in BSM medium reduced the pH from 7 to 6.2 that can affect the growth rate of cells.

In spite of decreasing growth rate, results showed that both cofactor precursors can enhance the biodesulfurization of DBT and production of 2-HBP as a final product. Thus, it can be concluded that by the addition of nicotinamide and riboflavin at the optimum level in culture medium, biodesulfurization of DBT can be improved.

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بهینه سازی میزان نیکوتین آمید و ریوفلاوین در سولفورزدایی از دی بنزوتیوفن به روش آماری سطح پاسخ

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چکیده

مقدمه: رودوکوکوس اریتروپولیس R1 یک سویه باکتریایی با توانایی سولفورزدایی دی بنزوتیوفن و تولید ۲-هیدروکسی بی فنیل است.

مواد و روش‌ها: در این مطالعه، تاثیر نیکوتین آمید و ریوفلاوین بر میزان سولفورزدایی و میزان رشد سلولی در این باکتری بررسی شد. میزان فاکتورها به وسیله روش آماری سطح پاسخ بهینه سازی شد.

نتایج: بررسی‌ها نشان داد که تأثیر نیکوتین آمید و ریوفلاوین از نظر آماری معنی دار است و هر دو فاکتور موجب افزایش میزان سولفورزدایی شدند. میزان بهینه نیکوتین آمید و ریوفلاوین به ترتیب ۱۰/۶۷ میلی مولار و ۳۴/۲ میکرومولار به دست آمد.

بحث و نتیجه‌گیری: افزایش این کوفاکتورها به محیط کشت، موجب کاهش میزان رشد شد که این کاهش رشد به علت تاثیر مهاری ۲-هیدروکسی بی فنیل تولید شده به عنوان محصول نهایی سولفورزدایی است.

واژه‌های کلیدی: رودوکوکوس اریتروپولیس، نیکوتین آمید، ریوفلاوین، دی بنزوتیوفن

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