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Effects of nicotinamide and riboflavin on the biodesulfurization activity of dibenzothiophene by *Rhodococcus erythropolis* USTB-03

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Abstract

Rhodococcus erythropolis USTB-03 is a promising bacterial strain for the biodesulfurization of dibenzothiophene (DBT) via a sulfur-specific pathway in which DBT is converted to 2-hydroxybiphenyl (2HBP) as an end product. The effects of nicotinamide and riboflavin on the sulfur specific activity (SA) of DBT biodesulfurization by *R. erythropolis* USTB-03 were investigated. Both nicotinamide and riboflavin were found to enhance the expression of SA, which was not previously reported. When *R. erythropolis* USTB-03 was grown on a medium containing nicotinamide of 10.0 mmol or riboflavin of 50.0 μmol, SA was raised from 68.0 or so to more than 130 mmol 2HBP/(kg dry cells·h). When *R. erythropolis* USTB-03 was grown in the presence of both nicotinamide of 5.0 mmol and riboflavin of 25.0 μmol, SA was further increased to 159.0 mmol 2HBP/(kg dry cells·h). It is suggested that the biological synthesis of reduced form of flavin mononucleotide (FMNH2), an essential coenzyme for the activities of biodesulfurization enzyme Dsz C and A, might be enhanced by nicotinamide and riboflavin, which was responsible for the increased SA of *R. erythropolis* USTB-03.

Key words: biodesulfurization; dibenzothiophene; nicotinamide; *Rhodococcus erythropolis* USTB-03; riboflavin

Introduction

Combustion of sulfur-rich fossil fuels leads to the release of sulfur oxides that causes air pollution and acid rain, which has become a major environmental problem. The sulfur content ranges from 0.05% to 5.0% in crude oil and from 0.3% to 6.0% in coal (Shennan, 1996; Wang and Krawiec, 1994). On average, organic and inorganic sulfurs are present in equal amounts in coal, while the majority of sulfur compounds in liquid petroleum fuels are organic (Li *et al*., 1996). Although chemical-physical processes can be used to remove inorganic sulfur from crude fuels, organic sulfur in dibenzothiophene (DBT), a widely used model of organic sulfur compound, remains in petroleum and coal even after these processes (Gray *et al*., 1996). To overcome this problem, some promising bacterial strains are identified to remove sulfur from DBT through the sulfur-specific pathway, which is capable of obtaining nutritional sulfur from DBT and producing 2-hydroxybiphenyl (2HBP) as the final product without causing oxidative loss of fuel carbon, which is the most efficient means for the desulfurization of DBT (Yan *et al*., 2000; Furuya *et al*., 2001; Kirimura *et al*., 2002; Li *et al*., 2005; Ishii *et al*., 2005; Yang and Marison, 2005; Gunam *et al*., 2006).

In the sulfur-specific pathway for the conversion of DBT to 2-HBP, four enzymes are involved in a four-step biochemical reaction: DBT monooxygenase (Dsz C) catalyses two consecutive monooxygenation reactions involving the conversion of DBT to DBT-sulfone ($DBTO_2$). Subsequently, a second flavomonooxygenase (Dsz A) is responsible for the conversion of $DBTO_2$ to 2-2′-hydroxyphenyl benzene sulfinate (HPBS), and Dsz B, a desulfinase, catalyzes the final step involving the conversion of HPBS to 2HBP (Yan *et al*., 2000). Reduced form of flavin mononucleotide (FMNH2) is required for the activities of both Dsz C and A, and a enzyme flavin reductase (Dsz D) is needed for the conversion of FMN to FMNH2 using NADH as the reducing agent (Lei and Tu, 1996; Matsubara *et al*., 2001; Gray and Mrachko, 2003). Overexpression of Dsz D enhances the overall rate of desulfurization, which can be further induced up to 100-fold when flavin reductase is added to the biological reaction mixture, suggesting that the supply of reduced flavin is a rate-limiting step in the sulfur-specific pathway (Gray *et al*., 1996; Galan *et al*., 2000; Reichmuth *et al*., 2000; Ohshiro *et al*., 1995).

A significant stumbling block to the commercialization of biodesulfurization is the low specific activity (SA) for removing sulfur from organic compounds (Borgne and Quintero, 2003). If microorganisms are to be used as non-growing biocatalysts, they need to be grown in a separate process and SA of biodesulfurization is a crucial indicator for efficiency. Previously, high SA of 135.5 mmol

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2HBP/(kg dry cells·h) and cell concentration of 37 g dry cells/L of *Rhodococcus erythropolis* KA2-5-1 were found in a fed-batch fermentation culture, when ethanol as the sole carbon and energy source was maintained at 1.0 g/L under the control of a semiconductor gas sensor (Yan *et al*., 2000). The genes for biodesulfurization enzymes have been cloned, sequenced and engineered from a variety of microorganisms (Ishii *et al*., 2000; Borole *et al*., 2002; Nomura *et al*., 2005); however, little information was provided on how to increase SA of biodesulfurization through the metabolic promotion of FMNH2 and the production of reduced form of nicotinamide-adenine dinucleotide (NADH) .

In this study, the effects of nicotinamide and riboflavin on the biodesulfurization activity of DBT by *R. erythropolis* USTB-03 were investigated. It showed that addition of nicotinamide of 5.0 mmol and riboflavin of 25.0 μmol to the culture medium of *R. erythropolis* USTB-03 induced SA more than two-fold, from 69.0 to 159.0 mmol 2HBP/(kg dry cells·h). Further exploration of this process is warranted to obtain high SA of biodesulfurization for industrial application.

1 Materials and methods

1.1 Strain and chemicals

Rhodococcus erythropolis USTB-03 used was previously isolated from the contaminated soil at the petroleum refining company in Zhanjiang City, China (Wei *et al*., 2005). DBT and 2HBP were purchased from Wako Pure Chemical Industries, Japan. Nicotinamide and riboflavin were obtained from Sigma Chemical Co., USA.

1.2 Medium and culture conditions

The sulfur-free basal medium was the same as previously described (Izumi *et al*., 1994). Glucose of 15 g/L and DBT of 50 mg/L were used as carbon and sulfur sources, respectively. Both medium and all experimental utensils were sterilized at 124°C for 20 min. The solution of 1.0 mol nicotinamide or riboflavin filtered with a 0.22-μm embrane was added in the sterilized medium on a clean bench. The strain was grown in 100 ml-flasks containing 20 ml inoculated liquid medium. The liquid culture was shaken at the rate of 200 r/min at 30°C.

1.3 Analytical methods

Growth was measured turbid metrically at 680 nm (OD_{680}). To measure DBT and 2HBP concentrations in the medium, the culture broth was taken and directly extracted with *n*-hexane by shaking for 1.0 min on a vortex. After centrifugation at 12,000 r/min for 5 min, the supernatant of the *n*-hexane extract was collected and injected into a HPLC (Shimadzu LC-10ATVP, Japan) with a UV Diode Array Detector at 240 nm using a SB-C18 (4.6 × 250 mm, 5 μm) column from Agilent. The mobile phase was 95% (*V/V*) methanol-water solution and the flow-rate was 1.0 ml/min. The calibration curves were established between the peak areas and the known concentrations of DBT and 2HBP in *n*-hexane, respectively, which were used to calculate the amount of DBT and 2HBP by peak area from unknown samples. The data presented here were the average of three measurements and their standard deviations were less than 10%.

1.4 Specific activity

Samples from the culture medium were taken and centrifuged at 12,000 r/min for 5 min. After discarding the supernatant, cells remained at the bottom of the tube were washed twice with 50 mmol phosphate buffer solution (PB, pH 7.0). The washed cells mixed with DBT of 100 mg/L in PB of 10 ml were directly used to measure SA. As a control, the cell suspension was mixed with 10 μl of 12 mol hydrochloric acid to stop the reaction. The reaction was maintained at 30°C for 1 h with a shake rate of 200 r/min, and 10 μl of 12 mol hydrochloric acid was added to stop the reaction. The measurement procedures of DBT and 2HBP were the same as those in the analytical methods described above. SA was expressed as the amount (mmol) of 2HBP produced per kg-dry cells per hour. The linear relationship between OD_{680} and dry weight concentrations of *R. erythropolis* USTB-03 was established as: dry weight concentration (g/L) = 0.22 OD_{680} – 0.15, which was used to calculate the dry weight of cells based on OD at 680 nm.

2 Results

2.1 Determination of DBT and 2HBP on HPLC

Figure 1 is the HPLC chromatogram and scanning profiles of standard DBT and 2HBP of 15 mg/L in *n*-hexane, and the retention time of 2HBP and DBT were 3.1 and 5.3 min, respectively (Fig.1a). The scanning profiles of DBT and 2HBP peaks in the wavelength range from 200 to 300 nm confirmed that the maximum absorbance of DBT and 2HBP were at 232 (Fig.1b) and 245 nm (Fig.1c), respectively. To measure DBT and 2HBP simultaneously,

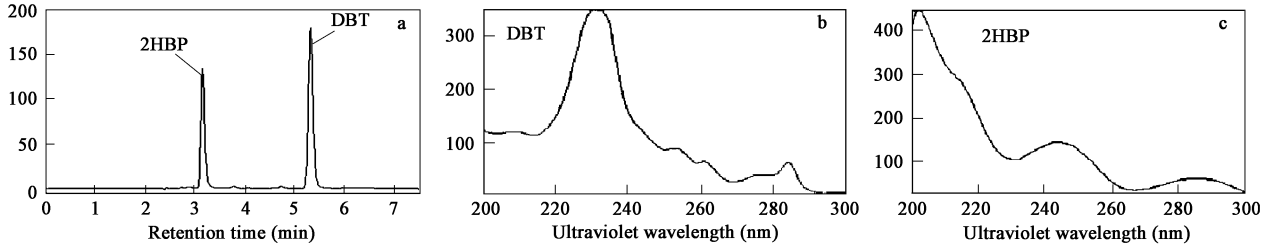

Fig. 1 HPLC chromatograms and scanning profiles of standard 2HBP and DBT of 15 mg/L in *n*-hexane. (a) HPLC chromatograms of 2HBP and DBT; (b) scanning profile of DBT in the ultraviolet wavelength from 200 to 300 nm; (c) scanning profile of 2HBP in the ultraviolet wavelength from 200 to 300 nm.

the detection wavelength of 240 nm was used for the quantification of DBT and 2HBP. Using this method, the detection limits for 2HBP and DBT were found to be 0.4 and 0.3 mg/L, respectively. The calibration curves in the range from 0 to 50 mg/L were established as: DBT (mg/L) = 6.88 peak area (10^6) – 0.47 (R^2 = 0.994); 2HBP (mg/L) = 1.31 peak area (10^5) – 0.61 (R^2 = 0.991), which were used to calculate the concentrations of DBT and 2HBP based on their peak areas.

2.2 Effect of nicotinamide on the growth and SA of *R. erythropolis* USTB-03

Figure 2a shows the effect of nicotinamide on the bacterial growth rate. The growth of *R. erythropolis* USTB-03 at exponential phase of day 2 was gradually inhibited with the increase of nicotinamide from 0 to 20 mmol. However, both the decline of DBT and the production of 2HBP were slightly induced in the presence of nicotinamide (Fig. 2b). SA of *R. erythropolis* USTB-03 on day 4 was also improved when it was grown on nicotinamide (Fig.2c). Compared to that of 67.0 mmol 2HBP/(kg dry cells·h) in control, the maximum SA of 130.2 mmol 2HBP/(kg dry cells·h) was observed when USTB-03 was cultured in the presence of 10 mmol nicotinamide (Fig.2c).

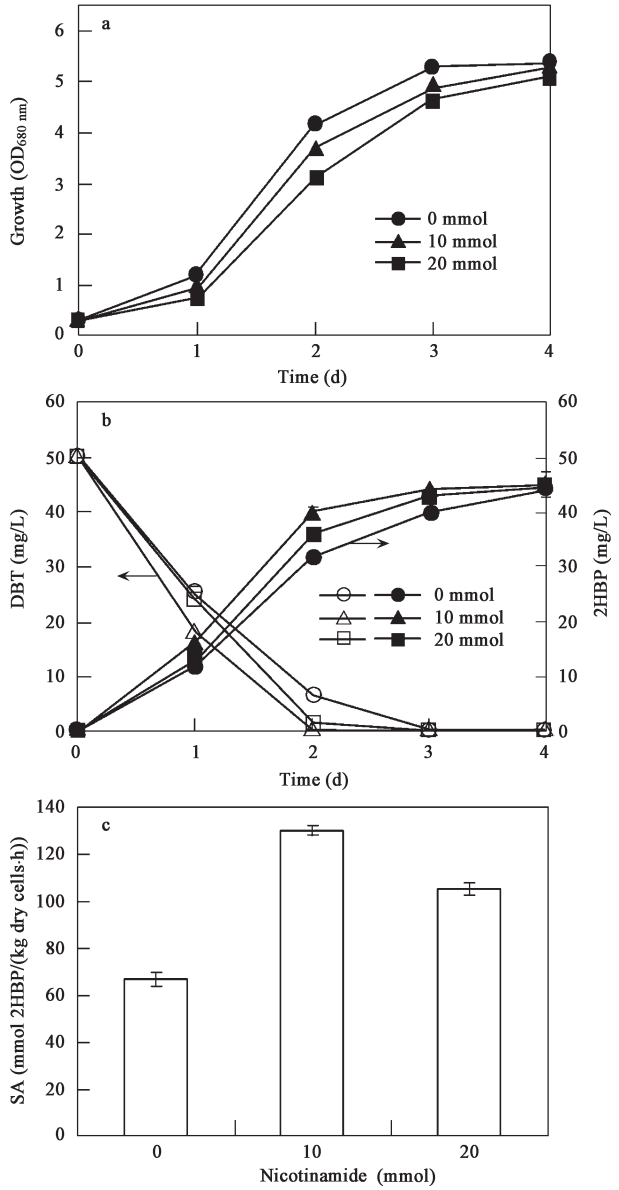

Fig. 2 Effect of nicotinamide on the growth and SA of *R. erythropolis* USTB-03. (a) effect of nicotinamide on the growth of *R. erythropolis* USTB-03; (b) removal of DBT (blank symbols) and the production of 2HBP (black symbols); (c) effect of nicotinamide on SA of *R. erythropolis* USTB-03.

2.3 Effect of riboflavin on the growth and SA of *R. erythropolis* USTB-03

Similar to that of nicotinamide, Fig.3a demonstrates that the growth of *R. erythropolis* USTB-03 was slightly repressed by riboflavin in the range from 0 to 100 μmol, but the reduction of DBT and the production of 2HBP were enhanced (Fig.3b). The maximum SA of 135.0 compared with 68.6 mmol 2HBP/(kg dry cells·h) in control was achieved when *R. erythropolis* USTB-03 was grown on 50 μmol riboflavin (Fig.3c).

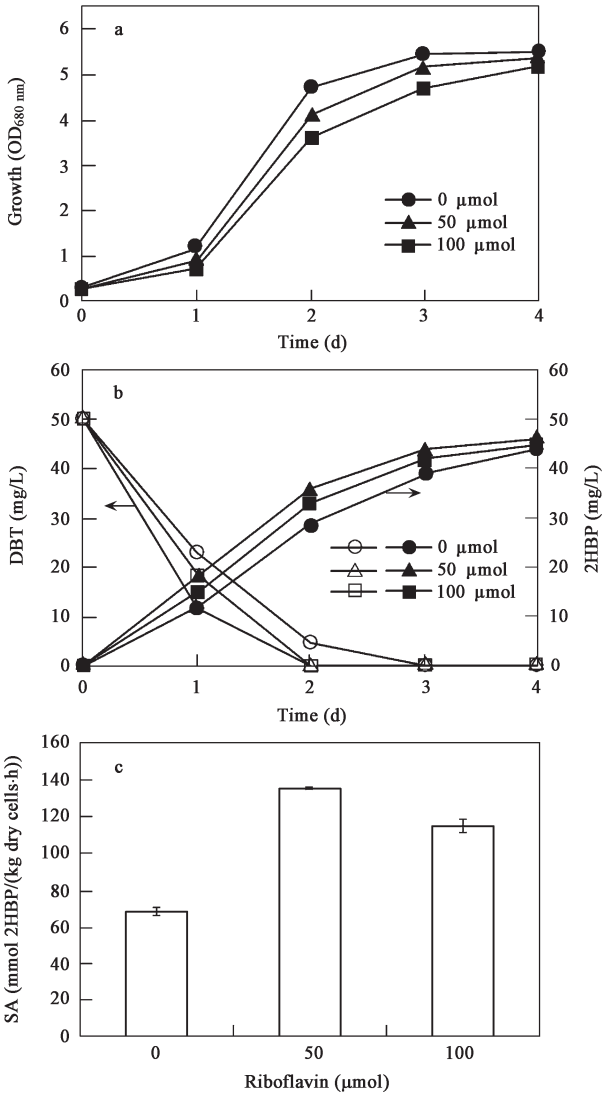

Fig. 3 Effect of riboflavin on the growth and SA of *R. erythropolis* USTB-03. (a) effect of riboflavin on the growth of *R. erythropolis* USTB-03; (b) removal of DBT (blank symbols) and the production of 2HBP (black symbols); (c) effect of riboflavin on SA of *R. erythropolis* USTB-03.

2.4 Effect of nicotinamide and riboflavin on the growth and SA of *R. erythropolis* USTB-03

Figure 4a shows that the growth of *R. erythropolis* USTB-03 at the exponential phase of day 2 was apparently inhibited by nicotinamide and riboflavin; however, the growth rate at stationary growth phases on day 4 was almost independent of nicotinamide and riboflavin. The reduction of DBT and the augment of 2HBP were also promoted when nicotinamide and riboflavin were simultaneously added to the culture medium (Fig.4b). The maximum SA of 159.0 mmol 2HBP/(kg dry cells·h) was achieved when *R. erythropolis* USTB-03 was cultured with initial nicotinamide of 5 mmol and riboflavin of 25 μmol (Fig.4c).

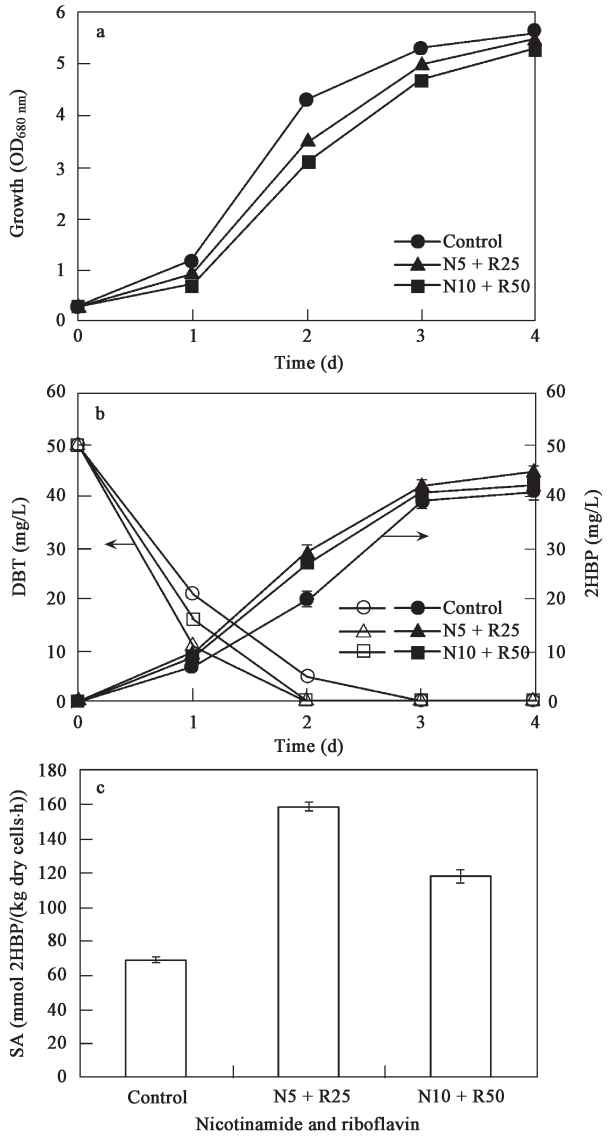

Fig. 4 Effects of nicotinamide and riboflavin on the growth and SA of *R. erythropolis* USTB-03. (a) effects of nicotinamide and riboflavin on the growth of *R. erythropolis* USTB-03; (b) removal of DBT (blank symbols) and the production of 2HBP (black symbols); (c) effects of nicotinamide and riboflavin on SA of *R. erythropolis* USTB-03. Control: nicotinamide 0 mmol and riboflavin 0 μmol; N5+R25: nicotinamide 5 mmol and riboflavin 25 μmol; N10+R50: nicotinamide 10 mmol and riboflavin 50 μmol.

3 Discussion

3.1 Determination of DBT and 2HBP on HPLC

Quantitative measurement of DBT and 2HBP were previously achieved using GC. In this study, we report a method of measurement using HPLC (Fig.1). The maximum absorbance of DBT and 2HBP were found to be at the wavelengths of 232 and 245 nm, respectively. The wavelength of 240 nm was chosen to determine both DBT and 2HBP simultaneously, which ensured the veracious detection of DBT and 2HBP throughout the experiment.

3.2 Effects of nicotinamide and riboflavin on the growth of *R. erythropolis* USTB-03

Results showed that the growth (OD_{680}) of *R. erythropolis* USTB-03 at exponential phases of day 2 were gradually repressed with the increase in the initial concentrations of nicotinamide and riboflavin; however, the final OD_{680} at stationary phases of day 4 remained almost independent of nicotinamide or riboflavin (Figs.2a, 3a, and 4a). Although the exponential growth of *R. erythropolis* USTB-03 was apparently delayed, the biomass obtained at stationary phase of day 4 was hardly affected (Figs.2a, 3a, and 4a), which ensured to obtain the the enough biomass of R. erythropolis USTB-03 for the in the biodesulfurization of DBT.

3.3 Effects of nicotinamide and riboflavin on SA of *R. erythropolis* USTB-03

Both the reduction of DBT and the production of 2HBP were enhanced (Figs.2b, 3b and 4b), although the growth of *R. erythropolis* USTB-03 was slightly inhibited by nicotinamide and riboflavin (Figs.2a, 3a and 4a). Addition of nicotinamide or riboflavin was found to obviously promote SA of *R. erythropolis* USTB-03 (Figs.2c and 3c). The current maximum SA of 159.0 mmol 2HBP/(kg dry cells·h), in comparison with 135.0 mmol 2HBP/(kg dry cells·h) in our previous report (Yan *et al*., 2000), was *et al*., 2000), was achieved when *R. erythropolis* USTB-03 was grown on both nicotinamide of 5 mmol and riboflavin of 25 μmol (Fig.4c). In addition to the enhancement of SA by genetic engineering was widely studied (Ishii *et al*., 2000; Borole *et al*., 2002; Nomura *et al.*, 2005), it could also be achieved by the metabolic supplementation using nicotinamide and riboflavin in the culture medium.

In the sulfur-specific pathway, the first step involving the conversion of DBT to DBT sulfoxide is the rate-limiting step (Yan *et al.*, 2000). Both enzyme Dsz C and D are involved in catalyzing this step of biochemical reaction. Because Dsz D, responsible for the conversion of FMN to FMNH2 using NADH as the reducing agent, is required for the activity of Dsz C. Both NADH and FMNH2 play vital roles in the biosulfurization of DBT (Lei and Tu, 1996; Matsubara *et al.*, 2001; Gray and Mrachko, 2003; Furuya *et al*., 2005). As nicotinamide and riboflavin are the precursors of NAD and FMN, it may easily be converted to NADH and FMNH2, respectively. So both the removal of DBT and the production of 2HBP were

enhanced during the growths of *R. erythropolis* USTB-03 when nicotinamide and riboflavin were added to the culture medium (Figs.2b, 3b and 4b). SA of *R. erythropolis* USTB-03 grown on nicotinamide or riboflavin was increased from 68 or so to more than 100 mmol 2HBP/(kg dry cells·h) (Figs.2c and 3c), and the maximum SA of 159.0 mmol 2HBP/(kg dry cells·h) was further achieved when *R. erythropolis* USTB-03 was cultured in the presence of both 10 mmol nicotinamide and 25 μmol riboflavin (Fig.4c).

For the production of NADH from nicotinamide, Evans *et al*. (2002) found that nicotinamide of 50 μmol was the dominant precursor and NAD levels in the cell rose well above normal levels. Reduction of NAD to NADH by *S. cerevisiae* during assimilation of biomass from glucose was reported by Bakker *et al*. (2001). Because the conversion of ethanol to acetaldehyde catalyzed by alcohol dehydrogenase might promote the production of NADH from NAD, the increase in SA of *R. erythropolis* KA2-5-1 was indicated (Yan *et al.*, 2000). For the formation of FMNH2, Bauer *et al*. (2003) reported that the conversion of riboflavin to FMN was catalyzed by riboflavin kinase, which was further converted to FMNH2 using NADH as the reducing agent (Gray *et al*., 1996; Lei and Tu, 1996; Matsubara *et al*., 2001; Furuya *et al*., 2005; Bauer *et al.*, 2003). Here we suggest that the production of FMNH2 as a key coenzyme for the activities of Dsz C and A can be promoted by NADH and FMN that can be enhanced by NAD and riboflavin, and NAD is easily produced by nicotinamide, which is responsible for the increased SA of *R. erythropolis* USTB-03.

Although microbial biodesulfurization from unprocessed fuel may be useful for environmental protection, the low efficiency may block commercialization of biodesulfurization process. Here we report that the improvement of a crucial indicator SA of *R. erythropolis* USTB-03 can be attained by adding suitable amounts of nicotinamide and riboflavin in culture medium. Using little amount of riboflavin and nicotinamide as precursors of FMNH2 and NADH, SA of *R. erythropolis* USTB-03 is much increased, which will be very useful in the cultivation of *R. erythropolis* USTB-03 with high SA for the effective biodesulfurization of DBT in practical application.

4 Conclusions

The effects of nicotinamide and riboflavin on the biodesulfurization activity of DBT by *R. erythropolis* USTB-03 were investigated in this study. Both the removal of DBT and the production of 2HBP by *R. erythropolis* USTB-03 were apparently enhanced by the addition of suitable concentrations of nicotinamide and riboflavin into the culture medium. SA of 68.0 mmol 2HBP/(kg dry cells·h) increased to 130.2 or 135.0 mmol 2HBP/(kg dry cells·h) at initial nicotinamide concentration of 10 mmol or riboflavin of 50 μmol, respectively. The maximal SA of 159.0 mmol 2HBP/(kg dry cells·h) was obtained when *R. erythropolis* USTB-03 was grown on both nicotinamide of 5 mmol and riboflavin of 25 μmol. We hypothesize that addition of nicotinamide and riboflavin may promote the biological synthesis of FMNH2, an essential coenzyme for Dsz C and A, which is very important in the increase of SA of *R. erythropolis* USTB-03 for the effective biodesulfurization of DBT in practical application.

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