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Enhanced bio-decolorization of 1-amino-4-bromoanthraquinone-2-sulfonic acid by *Sphingomonas xenophaga* with nutrient amendment

Hong Lu, Xiaofan Guan, Jing Wang, Jiti Zhou, Haikun Zhang*

Key Laboratory of Industrial Ecology and Environmental Engineering (Ministry of Education), School of Environmental Science and Technology, Dalian University of Technology, Dalian 116024, China. E-mail: lvhonghj@163.com

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ABSTRACT

Bacterial decolorization of anthraquinone dye intermediates is a slow process under aerobic conditions. To speed up the process, in the present study, effects of various nutrients on 1-amino-4-bromoanthraquinone-2-sulfonic acid (ABAS) decolorization by *Sphingomonas xenophaga* QYY were investigated. The results showed that peptone, yeast extract and casamino acid amendments promoted ABAS bio-decolorization. In particular, the addition of peptone and casamino acids could improve the decolorization activity of strain QYY. Further experiments showed that L-proline had a more significant accelerating effect on ABAS decolorization compared with other amino acids. L-Proline not only supported cell growth, but also significantly increased the decolorization activity of strain QYY. Membrane proteins of strain QYY exhibited ABAS decolorization activities in the presence of L-proline or reduced nicotinamide adenine dinucleotide, while this behavior was not observed in the presence of other amino acids. Moreover, the positive correlation between L-proline concentration and the decolorization activity of membrane proteins was observed, indicating that L-proline plays an important role in ABAS decolorization. The above findings provide us not only a novel insight into bacterial ABAS decolorization, but also an L-proline-supplemented bioaugmentation strategy for enhancing ABAS bio-decolorization.

Introduction

Plenty of xenobiotic compounds have been synthesized and extensively applied in various industries (Bonnet et al., 2008; Fenner et al., 2013; Husain, 2006). The improper treatments of industrial effluents containing these compounds are undesirable due to their toxicity, carcinogenicity and mutagenicity. Among these compounds, the treatments of synthetic dyes have been widely studied (Keharia and Madamwar, 2003; Novotný et al., 2011). In particular, the degradation of anthraquinone dyes and their intermediates has received much attention (Lee et al., 2004; Wang et al., 2011). However, they are very resistant to degradation due to their fused aromatic structures, which remain colored for a long time (Fan et al., 2009; Lee et al., 2004).

Compared with physical and chemical methods, microbial degradation and decolorization is seen as an environment-friendly and cost-effective method. It has been reported that many fungi are capable of degrading anthraquinone dyes and their intermediates (Andleeb et al., 2012; Kaushik and Malik, 2009; Levin et al., 2010). Moreover, various nutrient (carbon and nitrogen sources, micronutrients) amendments can accelerate the degradation of these anthraquinone compounds by inducing enzymes including laccase and peroxidase and/or supporting cell growth (Levin et al., 2010). However, it was difficult for bacteria to

* *Corresponding author*. E-mail: jwangcn@sina.com (Jing Wang).

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degrade anthraquinone dyes and their intermediates under aerobic conditions. So far, only several strains were found to be capable of degrading anthraquinone dye intermediates including 1-amino-4-bromoanthraquinone-2-sulfonic acid (ABAS). Although the isolated *Sphingomonas* strains could cleave anthraquinone ring of ABAS (Fan et al., 2009; Qu et al., 2005), its decolorization and degradation is a slow process. Moreover, the inhibition of bacterial activities was observed in the presence of a high ABAS concentration (Fan et al., 2009; Qu et al., 2005). Fan et al. (2009) ever reported that ABAS bio-decolorization could be accelerated by the addition of glucose, starch and sucrose. Further research still needs to be done to investigate the effects of other nutrients and to reveal the reasons for the enhanced ABAS bio-decolorization by nutrient amendments.

In the present study, *Sphingomonas xenophaga* QYY was selected because strain QYY can grow with ABAS as sole carbon and nitrogen sources (Qu et al., 2005). The effects of various nutrients including carbon and nitrogen sources, micronutrients on ABAS decolorization by strain QYY and related decolorization enzymes were investigated.

1. Materials and methods

1.1. Chemicals

1-Amino-4-bromoanthraquinone-2-sulfonic acid (ABAS, Fig. S1) was kindly presented by Dye Synthesis Laboratory of Dalian University of Technology, Dalian, China. Kinds of amino acids were purchased from Solarbio (Beijing) Technology Co. Ltd. (China). NADH was obtained from Sigma-Aldrich (Shanghai) Trading Co. Ltd. (China). All other chemicals used were of the highest analytical grade.

1.2. Strain and culture conditions

S. xenophaga QYY (16S rDNA GenBank accession number AY611716) was isolated from the sludge samples of ABAS producing workshop of Zhaoyuan Chemical Plant, Shandong, China. Strain QYY was cultured aerobically at 30°C in ABAS-LB medium containing 10 g/L Bactotryptone, 5 g/L Bactoyeast extract, 10 g/L NaCl, and 0.05 g/L ABAS (pH 7.2). Strain QYY can grow with ABAS as the sole carbon and nitrogen sources in a defined basal salt medium (DBSM), which contained 1.3 g/L NaH_2PO_4, 2 g/L K_2HPO_4, and 0.0025 g/L $FeCl_3$ (Lu et al., 2008; Qu et al., 2005).

1.3. Effects of additional nutrients on bio-decolorization

Strain QYY grown in ABAS-LB medium for two days was harvested by centrifugation (10 min, 10,000 ×*g*) and washed twice with 10 mmol/L phosphate buffer (pH 7.0). Then cell pellets were added into 100 mL DBSM containing 100 mg/L ABAS and cultured at 30°C with 150 r/min. The effects of different carbon and nitrogen sources (each nutrient, 500 mg/L) on ABAS decolorization were studied. These carbon and nitrogen sources include yeast extract, peptone, glucose, fructose, sucrose, urea and ammonium nitrate. Additionally, the effects of micronutrients including mixed vitamins (Balch et al., 1979), casamino acids (500 mg/L) and various amino acids (20 mg/L of each amino acid) on the decolorization were also investigated. Amino acids used above were divided into four groups based on Søensen's classification (Søensen et al., 2002). Amino acid mixture 1 (A1) contains L-leucine, L-glutamic acid, L-lysine, L-proline and L-valine. Amino acid mixture 2 (A2) contains L-aspartic acid, L-alanine, L-isoleucine L-phenylalanine and L-threonine. Amino acid mixture 3 (A3) contains L-arginine, L-serine, L-histidine, L-glycine and L-methionine. Amino acid mixture 4 (A4) contains L-cysteine, L-tryptophan, L-tyrosine and L-cystine. All experiments were performed in triplicate. Samples were taken at regular intervals and cell growth was monitored at an optical density of 660 nm (OD_{660}). Then the samples were centrifuged at 10,000 ×*g* for 10 min and the obtained supernatants were used for the determination of ABAS concentration at its maximum absorption wavelength (485 nm) using a UV–vis spectrophotometer (V-560 model, JASCO Corporation, Japan). The efficiency of ABAS decolorization (*D*, %) was calculated by Eq. (1):

$$D = (C_i - C_r)/C_i \times 100\% \quad (1)$$

where C_i (mg/L) and C_r (mg/L) are the initial and residual concentrations, respectively.

1.4. ABAS decolorization by resting cells

Strain QYY was grown in ABAS-LB medium until ABAS was completely decolorized. Then cells were harvested by centrifugation (10 min, 10,000 ×*g*) and washed twice with 10 mmol/L phosphate buffer (pH 7.0). Resting cell experiments were conducted in sterile 50-mL serum bottles containing 10 mmol/L phosphate buffer (pH 7.0). Then ABAS (10–70 mg/L), L-proline (20 mg/L) and cells (10 mg/mL, wet weight) were added into reaction systems. The control experiment without L-proline was also done. The decolorization of ABAS was detected after incubation of 30 min. The experiments were performed in triplicate. The decolorization rate (R_{dye}, mg/(L·min)) was calculated by Eq. (2):

$$R_{dye} = (C_i - C_t)/t \quad (2)$$

where, C_t (mg/L) is the residual concentration at *t* time; *t* (min) is reaction time.

The correlation between the decolorization rate and ABAS concentration was described by Eq. (3):

$$R_{dye} = R_{dye,max} C_{dye}/(K_m + C_{dye}) \quad (3)$$

where $R_{dye,max}$ (mg/(L·min)) and K_m (mg/L) are the maximum decolorization rate and Michaelis constant, respectively; and C_{dye} (mg/L) represents ABAS concentration.

1.5. Preparation of membrane isolation and soluble cell extracts

After strain QYY was cultured in 600 mL ABAS-LB medium (6 × 100 mL) for two days, cells were harvested by centrifugation (10 min, 10,000 ×*g*) and washed twice with 10 mmol/L phosphate buffer (pH 7.0). Then cell pellets were added into 600 mL DBSM (6 × 100 mL) containing 100 mg/L ABAS and cultured at 30°C with 150 r/min. After complete decolorization, cells were harvested by centrifugation (10 min, 10,000 ×*g*) and washed twice with 10 mmol/L phosphate buffer (pH 7.5). All

subsequent steps were carried out at 4°C unless otherwise stated. The obtained cells (4 g, wet weight) were suspended in a cold phosphate buffer (10 mmol/L, pH 7.5) containing 20% glycerol, 1 mmol/L dithiothreitol and 2 mmol/L EDTA. The cell suspension was sonicated in an ice bath with a Ultrasonic processor (Model CPX 750, Cole-Parmer Instrument Co. Ltd., IL, USA) at 225 W for 30 min. Unbroken cells were removed by centrifugation at 10,000 ×*g* for 15 min. The crude extracts were then treated by ultracentrifugation at 150,000 ×*g* for 2.0 hr. The obtained precipitation was suspended in 10 mmol/L phosphate buffer (pH 7.5) and used as a mixture of membrane proteins. The supernatant was used as a mixture of cytoplasmic and periplasmic proteins. The protein concentration of each fraction was determined by the modified Lowry protein assay, using bovine serum albumin as the standard.

1.6. Preparation of periplasmic proteins

Cells of strain QYY were cultured as described above (Section 1.5). The obtained cell pellets (4 g, wet weight) were washed and suspended in a cold Tris–HCl buffer (10 mmol/L, pH 7.5) containing 20% sucrose. The cell suspension was centrifuged at 10,000 ×*g* for 10 min, and then the pellets were placed on ice. All isolation steps were carried out at 4°C, unless otherwise indicated. The periplasmic proteins were prepared using the method described by Koshland and Botstein (1980).

1.7. Analysis of ABAS decolorization activity

NADH-mediated decolorization of ABAS by cell different fractions was carried out. The total volume of reaction mixture was 2.0 mL, which contained 1.56 mL of 10 mmol/L phosphate buffer (pH 7.5), 0.02 mL of 0.26 mmol/L ABAS, 0.4 mL proteins of cell different fractions mentioned above and 0.02 mL of 10 mmol/L NADH. Effects of L-proline (2.3–11.5 mg/L) on ABAS decolorization were also investigated in the absence of NADH in triplicate. The decreasing absorbance of ABAS (ε_{485} = 6.4 L/(mmol·cm)) was monitored spectrophotometrically on line at 485 nm at 30°C for 10 min. ABAS decolorization activity (V, nmol/(mg_{pro}·min)) was determined using Eq. (4):

$$V = (A_i - A_r) \times 10^6 / (\varepsilon_{485} \cdot b \cdot m_{Pro} \cdot t) \quad (4)$$

where A_i and A_r are the initial and residual absorbance values at zero and t time, respectively; b (cm) is optical path length of cuvette; and m_{pro} (mg/L) is protein content.

1.8. LC–MS analysis

The decolorization products of ABAS were identified using Agilent 6410B triple quad LC–MS (Agilent Technologies Co. Ltd., CA, USA) with Eclipse plus C18 (2.1 × 150 mm, 5 μm, Agilent Technologies Co. Ltd., CA, USA). The mobile phase was composed of methanol and water containing 0.1% (*m*/*m*) acetic acid. The elution program began with 15% (V/V) methanol for 5 min, then linearly increased to 90% over 15 min and then remained for 5 min. The flow rate was 0.25 mL/min. MS was performed under the following conditions: electrospray ionization (ESI-) source, CDL temperature 300°C, block temperature 200°C, detector pressure 1.6 V, scan mode.

2. Results and discussion

2.1. Effect of different nutrient amendments on bio-decolorization

Effects of various nutrients on ABAS bio-decolorization are shown in Fig. 1. It is obvious that peptone was the most effective nutrient for promoting ABAS bio-decolorization, followed by yeast extract. The addition of the two nutrients resulted in the decolorization of over 96% ABAS in 10 hr. In comparison, only 5.8% ABAS was removed without nutrient amendment (Fig. 1). Because yeast extract is typically rich in carbohydrate (4%–13%), nitrogen (8%–2%) and microbial growth stimulants including amino acids (3.0%–5.2%) and vitamins (Wu et al., 2011), more biomass was observed in yeast extract-amended system than that in peptone-amended system. To investigate the function of specific ingredients in yeast extract and peptone used for ABAS bio-decolorization, different kinds of carbon and nitrogen sources and micronutrients, respectively, were added into DBSM. As shown in Fig. 1, extra urea and ammonium nitrate as nitrogen sources did not promote ABAS bio-decolorization. Our previous studies showed that strain QYY can use ABAS as a sole nitrogen source (Qu et al., 2005). Thus, it seems that nitrogen in ABAS meets the metabolic need of strain QYY during ABAS decolorization. Different from additional nitrogen sources, glucose, fructose and sucrose as additional carbon sources could enhance ABAS decolorization. Fig. 1 shows that ABAS bio-decolorization was more significantly enhanced by glucose amendment compared with fructose and sucrose due to the faster growth of strain QYY in glucose-supplemented

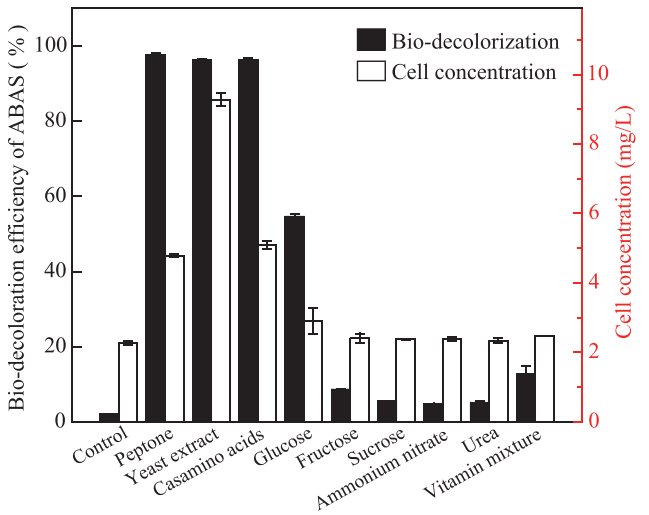

Fig. 1 – Effect of additional nutrients on ABAS bio-decolorization by *Sphingomonas xenophaga* QYY in 10 hr. Experimental conditions: the concentrations of all additional nutrients concentrations were 500 mg/L except vitamin solution (2.8 mg/L of total concentration). ABAS, 1-amino-4-bromoanthraquinone-2-sulfonic acid.

system. This behavior was similar with previous reports that additional glucose could promote the growth of cells, resulting in the enhanced biodegradation of refractory compounds (Schukat et al., 1983; Tobajas et al., 2012).

Additionally, the degradation rates of xenobiotic compounds could be also enhanced by micronutrients including vitamins and amino acids, which were not adequately synthesized by microbial metabolism (Jonssona and Östberg, 2011; Östberg et al., 2007; Wu et al., 2011). In our experiments, additional mixed vitamins only slightly increased ABAS bio-decolorization rate, while casamino acid amendment resulted in the significantly enhanced decolorization of ABAS. The accelerating effect of casamino acids was similar to that of peptone, indicating that some specific amino acids might play important roles during ABAS bio-decolorization.

2.2. Effect of different amino acids on bio-decolorization

Amino acids were divided into four groups (A1, A2, A3 and A4) according to Sørensen's classification (Søensen et al., 2002). Fig. 2 shows that A1, A2 and A3 amendments promoted ABAS bio-decolorization. In fact, they could serve as nitrogen sources to promote cell growth, resulting in the increased decolorization rate of ABAS. However, combined with the results obtained from the experiments with ammonium nitrate and urea supplements, it was thought that amino acids might play more roles in ABAS decolorization. Among four groups above, A1 and A2 were more effective amino acid mixtures for accelerating ABAS decolorization compared with A3. Thus, the effect of each amino acid in A1 and A2 on ABAS bio-decolorization was investigated. The results showed that except for L-lysine and L-phenylalanine, the remaining amino acids in A1 and A2 could promote ABAS bio-decolorization at various levels. In particular, the addition of L-proline resulted in 72% ABAS decolorization, which was over twice higher than

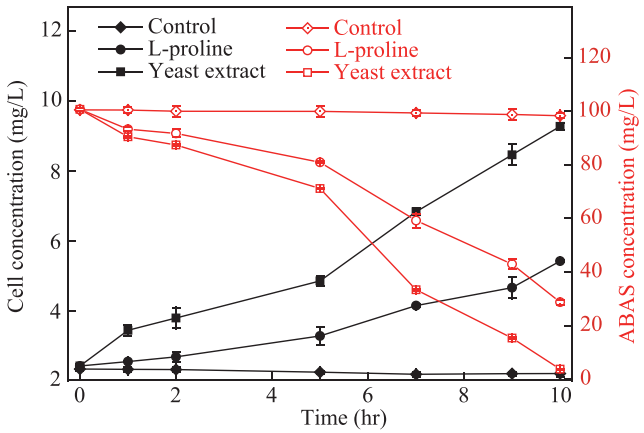

Fig. 3 – Time course of ABAS bio-decolorization and cell growth with L-proline and yeast extract amendments. Experimental conditions: the concentrations of L-proline and yeast extract were 20 and 500 mg/L, respectively. ABAS, 1-amino-4-bromoanthraquinone-2-sulfonic acid

those in other amino acid-amended systems. To our knowledge, it was first found that a single amino acid significantly promoted ABAS decolorization. Meanwhile, it was observed that the effect of L-proline on ABAS bio-decolorization was even larger than that of G1 containing L-proline, indicating that other amino acids might inhibit the accelerating effect of L-proline on ABAS bio-decolorization.

L-Proline plays several roles in microbial, plant and animal cells. It has been reported that L-proline can stabilize proteins and prevent loss of viability during osmotic stress, drought, freezing, UV radiations and exposure to toxic heavy metals (Bach and Takagi, 2013; Çelekli et al., 2013). Additionally, L-proline as an antioxidant in oxidative stress metabolism can also remove free

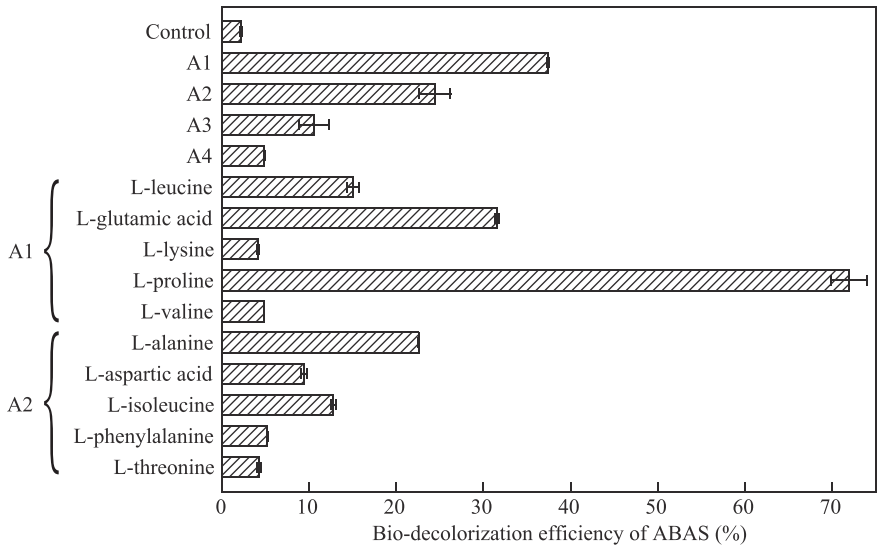

Fig. 2 – Effect of amino acids on ABAS bio-decolorization by strain QYY in 10 hr. Experimental conditions: concentration of each amino acid in each group or separated amendment was 20 mg/L; A1, A2, A3 and A4 represent amino acid mixtures; A1 contains L-leucine, L-glutamic acid, L-lysine, L-proline and L-valine; A2 contains L-aspartic acid, L-alanine, L-isoleucine, L-phenylalanine and L-threonine; A3 contains L-arginine, L-serine, L-histidine, L-glycine and L-methionine; A4 contains L-cysteine, L-tryptophan, L-tyrosine and L-cystine. ABAS, 1-amino-4-bromoanthraquinone-2-sulfonic acid.

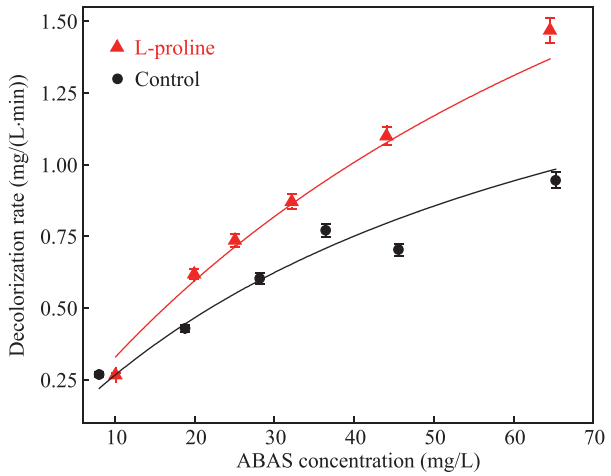

Fig. 4 – Effect of L-proline (20 mg/L) on ABAS decolorization by resting cells. ABAS, 1-amino-4-bromoanthraquinone-2-sulfonic acid

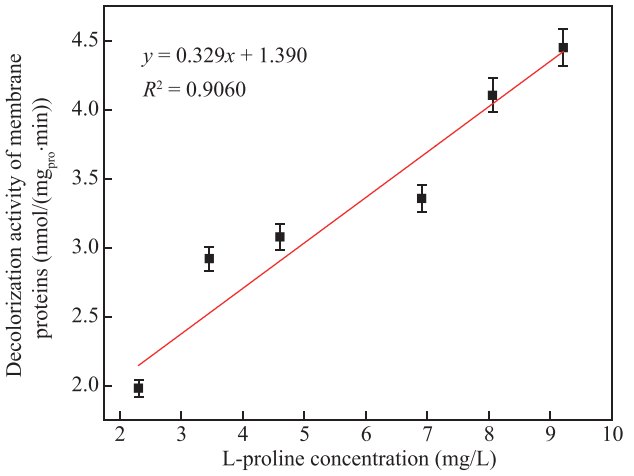

Fig. 5 – Relationship between L-proline concentration and decolorization activity of membrane proteins.

radicals (Bach and Takagi, 2013; Kaul et al., 2008). In the present study, ABAS is toxic to strain QYY. Perhaps L-proline could play a role in protecting strain QYY from ABAS toxicity. Further studies (Fig. 3) showed that L-proline amendment resulted in the faster growth of strain QYY compared with the control without L-proline. It was different from the report of Östberg et al. (2007) that amino acid amendment mainly shortened a lag time for the biodegradation of *n*-hexadecane. More importantly, the average decolorization rate of ABAS in L-proline-amended system (1.84 mg/(mg cell·hr)) was higher than that in yeast extract-amended system (1.66 mg/(mg cell·hr)). This indicates that L-proline can not only promote cell growth, but also enhance the decolorization activity of strain QYY, which was also supported by our resting cell experiments. Fig. 4 shows that the correlation between ABAS decolorization rate and its concentration could be described by Michaelis–Menten kinetics. The predicted $R_{dye,max}$ and K_m values in L-proline-amended systems were 3.28 mg/(L·min) and 90.30 mg/L, respectively. Compared with control systems without L-proline ($R_{dye,max}$ = 1.94 mg/(L·min), K_m = 63.77 mg/L), the increase of $R_{dye,max}/K_m$ ratio indicates that L-proline amendment enhances the decolorization activity of related enzymes in strain QYY. This behavior was also observed in previous reports that some specific amino acids, including L-phenylalanine, L-glutamic acid and L-proline, increased bacterial degradation activities (Wu et al., 2011; Ronen et al., 2005). Moreover, Wu et al. (2011) reported that the enhanced degradation rate resulted from the increase of cell protein concentration and cytochrome P450 activity. Thus, it is necessary to investigate the effect of L-proline on ABAS decolorization enzymes in strain QYY.

2.3. *Effect of L-proline on the oxygenases responsible for ABAS decolorization*

Most of oxygenases involved in the decolorization and degradation of anthraquinone dyes and their intermediates were found in fungi (Kim et al., 2012; Hofrichter et al., 2010; Hsu et al., 2012). For bacteria, few studies were reported. Our previous study only showed that the major enzyme(s) responsible for the decolorization of ABAS and 1-aminoanthraquinone-2-sulfonic acid is NADH-dependent oxygenase(s) in strain QYY (Lu et al., 2008). Moreover, ABAS decolorization enzyme(s) was very unstable and completely lost its activity after storage overnight at 4°C. In the present study, cellular location of the above oxygenases involved in ABAS decolorization was investigated. The results (Table 1) showed that ABAS-decolorization activities of cytoplasmic and periplasmic proteins were not detected, while membrane proteins displayed ABAS decolorization activities in the presence of NADH as a cofactor. It is interesting that membrane proteins also showed similar ABAS decolorization activities (Table 1), when L-proline replaced NADH. However,

Table 1 – ABAS[a] decolorization by different fractions from cells of strain QYY.

Different fractions	Decolorization activity (nmol/(mg_{pro}·min))	
	0.1 mmol/L NADH	0.1 mmol/L L-proline[b]
Mixture of membrane and intracellular soluble	0.556 ± 0.024	0.513 ± 0.026
Cytoplasmic proteins	0	0
Membrane proteins	7.181 ± 0.259	5.859 ± 0.293
Periplasmic proteins	0	0

[a] ABAS, 1-amino-4-bromoanthraquinone-2-sulfonic acid.
[b] The concentration of additional L-proline was about 11.5 mg/L.

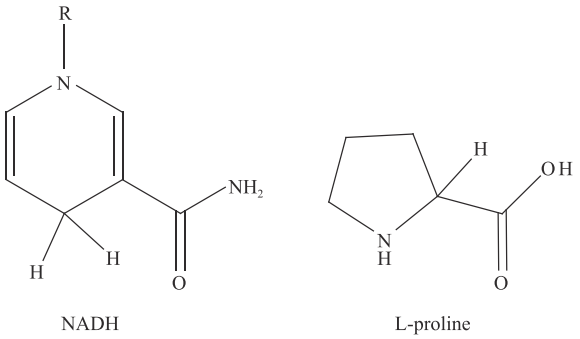

Fig. 6 – Chemical structures of NADH and L-proline.

this phenomenon was not observed in the presence of other amino acids, perhaps that other amino acids, including L-glutamic acid, L-alanine and L-leucine, play major roles in supporting cell growth. Further studies showed that L-proline concentration is positively correlated with the decolorization activity of membrane proteins (Fig. 5).

HPLC-MS analysis demonstrated that the products (2-amino-3-hydroxy-5-bromobenzenesulfonic acid and 2-amino-4-hydroxy-5-bromobenzenesulfonic acid) from ABAS decolorization catalyzed by membrane proteins in the presence of L-proline were the same with those using NADH as a cofactor (Fig. S2). Moreover, the decolorization products described above were also consistent with those from ABAS decolorization catalyzed by whole cells (Lu et al., 2008; Fan et al., 2008), indicating that L-proline is closely related with anthraquinone ring cleavage enzymes located in membrane fraction. Fig. 6 shows that there is a carbonyl group connected with a nitrogen-containing heterocyclic ring in the chemical structures of L-proline and NADH, suggesting that there are some similarities between these two compounds. Perhaps that L-proline could be used as a cofactor of anthraquinone ring cleavage enzyme(s) during ABAS decolorization. For the treatment of ABAS-containing wastewater, our previous experiments demonstrated that bioaugmented system with strain QYY exhibited better decolorization performance compared with non-augmented systems (Qu et al., 2009). Combined with the above results, systems augmented with L-proline and strain QYY will provide a new possibility for accelerating the treatment of ABAS-containing wastewater.

3. Conclusions

ABAS bio-decolorization by strain QYY could be significantly enhanced by the addition of peptone, yeast extract and casamino acids. Further studies showed that L-proline had a more significantly accelerating effect on ABAS decolorization compared with other amino acids, which was attributed to the enhanced cell growth rate and decolorization activity of strain QYY. In the presence of L-proline, membrane proteins displayed ABAS decolorization activities, indicating that L-proline is closely related with anthraquinone ring cleavage enzyme(s). The above findings provide us not only a novel insight into bacterial ABAS decolorization, but also an L-proline-supplemented bioaugmentation strategy for enhancing ABAS bio-decolorization.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jes.2014.05.041.

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