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Original Article

Characterization of the decolorizing activity of azo dyes by *Bacillus subtilis* azoreductase AzoR1

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Abstract

The product of the *Bacillus subtilis* gene *azoR1* is annotated as a putative azoreductase, production of which is induced in response to thiol-reactive compounds. Here we report on the decolorization of four azo dyes by azoreductase activity. The ability of overexpressed AzoR1 strain ORB7106 to catalyze decolorization of azo dyes was investigated on agar plates and in liquid cultures. The decolorization efficacy of a mutant, ORB7106, which has lost negative control of *azoR1* expression, was significantly better than the parental strain (JH642), providing evidence that the *azoR1* gene product is an azoreductase with the ability to decolorize azo dyes. ORB7106 showed 40-98% azo dye decolorization within 48 h. The bacterium exhibited a remarkable color removal capability over a wide range of Methyl Red concentrations (10-200 mg l^{-1}), pH (5-9) and temperatures (25-45°C). A significant increase in the decolorization activity was observed after induction of AzoR1 production. The results suggested that AzoR1 production could be induced by Methyl Red. The decolorizing activity is primarily associated with the cytosolic fraction and requires NADH as a cofactor.

Keywords: *Bacillus subtilis*, AzoR1, decolorization, azoreductase, Methyl Red

1. Introduction

Azo dyes are characterized by the presence of one or more R1-N=N-R2 bonds and are synthetic organic colorants that are widely used in textiles, foods, industry, printing, tattooing, cosmetics and in clinical settings (Stolz, 2001; Suzuki *et al.*, 2001; Chen *et al.*, 2005). They are regarded as pollutants once they are released into the environment (Suzuki *et al.*, 2001). Azo dyes are usually recalcitrant to conventional wastewater treatment. Several physico-chemical methods such as adsorption, chemical treatment and ion pair extractions have been adopted and proven to be costly while producing large amounts of sludge. More studies are now focused on methods involving biological treatments (Song *et al.*, 2003; Chen, 2006). Many microorganisms such as bacteria, fungi and yeast have been observed to decolorize azo dyes by biosorption and biodegradation (Mabrouk and Yusef, 2008; Gou *et al.*, 2009). Therefore, the biological degradation of these dyes by microorganisms has potential advantages in developing decolorizing bio-treatment of wastewater (Suzuki *et al.*, 2001; Ooi *et al.*, 2007; Gou *et al.*, 2009).

Azoreductase catalyzes the NAD(P)H-dependent reduction of azo compounds to the corresponding amines, which involves cleavage of the azo linkages (-N=N-), resulting in azo dye degradation (Nakayama *et al.*, 1983; Chen, 2006). Although these enzymes reduced certain types of azo dyes, some dyes were not degraded efficiently. To establish biological wastewater treatment system for azo dye removal, it is advantageous to screen for microorganisms that express azoreductases with broad substrate specificities.

The Gram-positive bacterium *Bacillus subtilis* is exposed to a variety of toxic and antimicrobial compounds in the soil, which induce general and specific stress responses

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in growing cells. The *yocJ* (*azoR1*) gene, which is regulated by the novel DUF24/MarR-type repressor, YodB, encodes a putative FMN-dependent NADH-azoreductase and is also referred to as AzoR1 according to Swiss-Prot database annotations (Accession No. O35022) (Leelakriangsak *et al.*, 2008). As a part of the stress response, the *azoR1* gene is induced after exposure to thiol-reactive compounds (Leelakriangsak *et al.*, 2008). Both microarray and proteomic analyses confirmed increased levels of *azoR1* transcription and AzoR1 protein synthesis in *yodB* mutant cells (Leelakriangsak *et al.*, 2008). Moreover, the redox-sensing mechanism of YodB repressor in response to diamide and quinones has been proposed (Chi *et al.*, 2010). A detailed molecular mechanism for azoreductase-encoding *azoR1* gene expression in *B. subtilis* remains to be elucidated. Therefore, characterization of azo dye degradation activity *in vivo* is performed in this study.

In this study, we report the decolorization of azo dyes by ORB7106, which possesses a deletion of the *yodB* gene and constitutively expresses *azoR1* and produces elevated levels of azoreductase AzoR1, and compare decolorizing activity with that of its wild type parent, *B. subtilis* strain JH642. The effect of pH and temperature on decolorization of tested dyes is reported. We also report the identification of the main cellular location of decolorizing activity. Finally, the induction of dye decolorization when cells are treated with Methyl Red (MR) was demonstrated.

2. Materials and Methods

2.1 Bacterial strains and growth conditions

B. subtilis strains used in this study were a parental strain JH642 (*trpC2 pheA1*) and *azoR1* overexpression strain ORB7106 (*trpC2 pheA1 amyE::azoR1-lacZ pcm::tet yodB::cat*) (Leelakriangsak *et al.*, 2008). In brief, ORB7106 was constructed by transformation of *azoR1-lacZ* fusion strain with chromosomal DNA of *yodB* disruption strain. Cells were cultured at 37°C in Difco sporulation medium (DSM) with shaking at 200 rpm. Antibiotics were added with the following concentration when required: chloramphenicol (Cm), 5 mg ml^{-1} and erythromycin/lincomycin (Erm/Ln), 1 mg ml^{-1}, 25 mg ml^{-1}.

2.2 Azo dyes

Azo dyes used in this study were Azobenzene (AZ, Sigma), Methyl Red (MR, Sigma), Orange G (OG, Fluka) and Congo Red (CR, Merck) (Figure 1). The wavelengths of maximum absorption of azo compounds used in this study were 380, 430, 480 and 490 nm for AZ, MR, OG and CR, respectively (Sugiura *et al.*, 2006). The effect of dye concentration on decolorization performance of *B. subtilis* was studied over a range of 10, 50, 100 and 200 mg l^{-1}.

2.3 Plate assay

JH642 and ORB7106 were grown in DSM at 37°C and shaking at 200 rpm for 12 h. The same concentration of cells (approximate $5x10^7$ CFU/ml) was plated by spot inoculation technique (5 ml each spot) into DSM agar plates, which were supplemented with different concentrations of azo dyes (10, 50, 100 and 200 mg l^{-1}) and at different values of pH (5, 7 and 9). The plates were incubated at various temperatures (25°C, 30°C, 37°C, 40°C and 45°C) for 48 h. The clear zone around bacterial colonies indicated the ability of the organism to reduce azo dyes. At least three independent experiments were performed with the unspotted plate as a control.

2.4 Decolorization assay

Cells were precultured at 37°C in DSM liquid until stationary phase. Precultured cells were transferred at 1:100 dilution to DSM liquid supplemented with different concentrations of azo dyes. The DSM supplemented with the azo dye was used as control under light exposure to compare abiotic color loss during the experiment. The effect of pH (5, 7 and 9), with the same incubation temperature of 37°C, and temperatures (25°C, 37°C and 45°C), with the same pH of 7, on azo dye-degrading activity was determined under static conditions (non-shaking condition). Samples were collected every 4 h for 48 h. The cells were harvested by centrifugation at 5,000 rpm for 5 min. The supernatants were used to assay azo dye reduction by measuring residual absorption at the appropriate wavelength for each azo dye. An UV-visible spectrophotometer (Jenway, model6405, UK) was used for absorbance measurement. Percent decolorization was calculated as follows (Telke *et al.*, 2010).

$$\text{Decolorization (\%)} = \frac{(\text{Initial absorbance}) - (\text{Observed absorbance})}{(\text{Initial absorbance})} \times 100$$

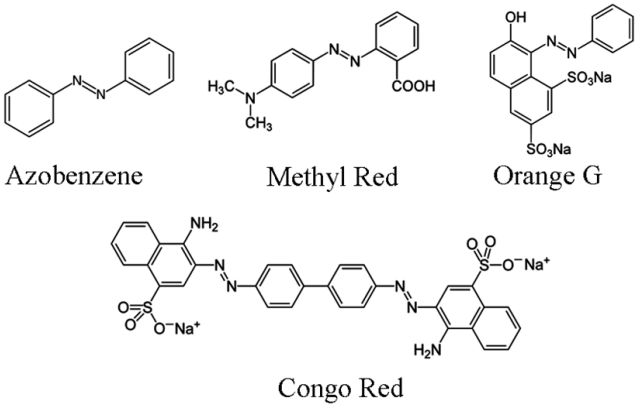

Figure 1. Chemical structures of azo dyes used in this study.

2.5 Intracellular and extracellular enzymatic activity

ORB7106 cells were grown at 37°C for 24 h. The samples (10 ml) were centrifuged at 5,000 rpm for 5 min. The supernatant fraction was filtered through a 0.45 mm sterile syringe filter (Millipore) and the resulting filtrate was designated as the extracellular fraction for the assay of extracellular enzymatic activity. The pellets were washed with 50 mM phosphate buffer (pH 7) and then frozen at -20°C until they were used. The frozen pellets were resuspended in 50 mM phosphate buffer (pH 7) containing 0.1 g ml^{-1} of lysozyme then were incubated at 37°C for 20 min. The solution was centrifuged at 5,000 rpm for 5 min and the supernatant was filtered through 0.45 mm sterile syringe filter (Millipore). The supernatant containing the cytosolic proteins was analyzed for the assay of intracellular enzymatic activity. The protein concentration was determined by the method of Bradford (Bradford, 1976), with bovine serum albumin as a standard.

Enzyme assays were carried out in cuvettes (path length = 1 cm) with a total volume of 1 mL to determine azoreductase activity. Cell fractions prepared above were mixed with 100 µl of MR (500 mg l^{-1}), resulting in a final concentration of 50 mg l^{-1}. The reaction was started by the addition of 4 µl or 8 µl of NADH (50 mM) giving a final concentration of 200 µM or 400 µM respectively. In all assay procedures, the blank contained all components except protein extract. The 0.1 g ml^{-1} lysozyme in phosphate buffer was added instead of protein extract in the blank. Enzymatic activity was detected by following the disappearance of MR (ε = 8.5 mM^{-1} cm^{-1}) at its maximum absorbance wavelength (430 nm). One unit of enzyme activity was defined as the amount of enzyme required to reduce 1 µmol of MR per min under the assay condition.

2.6 Induction of enzyme by Methyl Red (MR)

JH642 cells were cultured with or without 50 mg l^{-1} MR 37°C with shaking for 24 h. Samples were collected at 6, 12 and 24 h by centrifugation at 5,000 rpm for 5 min. The pellets were washed with 50 mM phosphate buffer (pH 7) and incubated with 0.1 g ml^{-1} lysozyme at 37°C for 20 min. After centrifugation, the supernatant was filtrated then used for enzyme assay.

2.7 Statistical analyses

All experiments were performed at least 3 times. All results were presented as either mean ± SEM (standard error of mean) or mean ± SD (standard deviation). The statistical '*t*' test was used to evaluate significant differences between groups at a 0.05 significance level. The multiple comparisons among treatments were made using ANOVA with Tukey's multiple comparison test.

3. Results and Discussion

3.1 Decolorization by plate assay

To investigate the effect of pH and temperature on azo dye decolorization activity by ORB7106, a plate assay was used, which provided a simple and rapid screening method. ORB7106 could grow well in the presence of all 4 azo dyes (AZ, MR, OG and CR) and in all tested concentrations (10, 50, 100 and 200 mg l^{-1}). Bacterial decolorization ability was confirmed by the clear halo formed around each colony by plate assay within 48 h. The bacterium was able to decolorize aerobically MR and CR whereas no clear zone was observed on plates containing AZ and OG (Figure 2). MR and CR were efficiently decolorized over a wide range of pH (5-9) and temperatures (25-40°C) (Figure 2B and D). However, as the dye concentration increased on agar plates, a decline in color removal was observed. Differences in the decolorization characteristics for the individual dyes were found. Not only did a clear halo form around the colony of the CR plate but also a reddish colony appearance was observed (Figure 2D), suggesting the adsorption of dye into the cell mass. However, plates containing AZ obtained a clear color

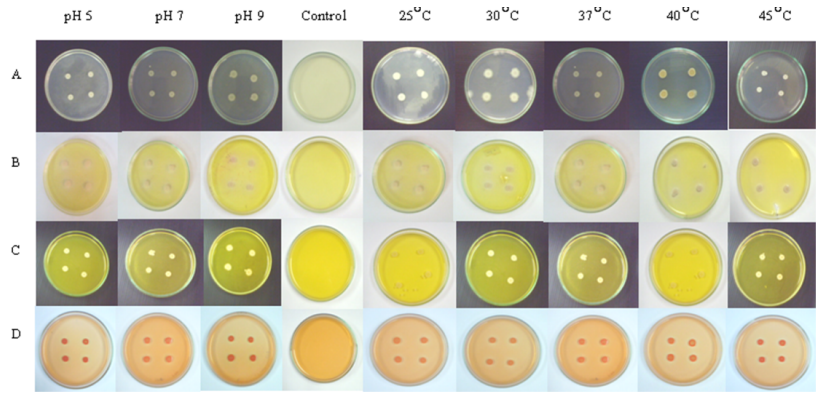

Figure 2. Effect of pH (5, 7 and 9) and temperature (25°C, 30°C, 37°C, 40°C and 45°C) on azo dyes decolorization. ORB7106 cells were spotted on DSM agar plates containing 50 mg l^{-1} azo dyes and incubated for 48 h. (A) Azobenzene, (B) Methyl Red, (C) Orange G and (D) Congo Red.

which was an unidentifiable clear zone, even at 200 mg l^{-1} concentration. Therefore, further investigations were carried out to determine azo dye-degrading activity by measuring the decrease in optical density of each dye and by calculating the percent decolorization.

3.2 Effect of AzoR1 overexpression on decolorization of dyes

To evaluate the level of azo dye-reducing activity of AzoR1, dye decolorization by JH642 and ORB7106 was performed and analyzed quantitatively. According to reported literatures, azo dyes are synthetic compounds which are extremely stable to exposure to light and biodegradation (Stolz, 2001; Pandey *et al.*, 2007). In this study, decolorization was not observed significantly in the control samples, which confirmed that all the tested dyes were stable to exposure to light and biodegradation. In 48 h, more than 89% color removal was observed in incubations containing MR and CR whereas the color of the remaining dyes (AZ and OG) was removed to a lesser extent in the ORB7106 cultures (Tables 1, 2). Among the tested dyes, MR appeared to be the most susceptible to degradation by both ORB7106 and JH642. This study showed that the smallest azo compound, AZ, was not a good substrate for AzoR1 activity. AZ might be more difficult to be transported into bacterial cells due to the absence of functional group of AZ. Recent studies showed that the functional groups and their relative position (e.g. *ortho*, *meta*, *para*) in the proximity of azo linkages could influence the susceptibility to biodecolorization (Hsueh and Chen, 2007, Hsueh and Chen, 2008, Hsueh *et al.*, 2009). The charged group near the azo bond on OG may have significantly hindered the decolorization efficiency as observed in the study reported herein. However, the carboxyl group at the *ortho* position in MR likely contributed to efficient decolorization by AzoR1 and/or other enzymes. In contrast, MR was not reduced by *Pseudomonas luteola* (Hsueh and Chen, 2007). A 95% decolorization efficiency of CR by *P. luteola* was reported recently (Hsueh and Chen, 2007), but no decolorization of AZ and CR was observed by these strains of *Bacillus* sp. OY1-2, *Xanthomonas* sp. NR25-2 and *Pseudomonas* sp. PR41-1 that produce azo-dye-degrading enzymes (Sugiura *et al.*, 1999). Therefore, azoreductases from different bacterial species possess different substrate specificities.

Statistical analysis of decolorization activity by *t*-test showed that dye decolorization under different conditions (pH, temperatures, incubation times and dye concentrations) by ORB7106 was significantly different from that of JH642 as shown in Table 1. This indicates that the observed dye decolorization is mainly due to degradation by AzoR1. Therefore, it was chosen for further statistical analyses.

3.3 Effect of pH and temperature on azo dye-degrading activity

To investigate the role of environmental factors on enzyme activity, the percent of decolorization was measured under different culture conditions, including varying pH (5-9), temperatures (25°C-45°C), dye concentrations (10-200 mg l^{-1}) over several time intervals (8-48 h). At 45°C, decolorization performance of ORB7106 using AZ, MR, OG and CR as substrates was higher (statistically significant $p<0.05$) than that at 25°C (Table 2). AZ, MR and CR decolorization efficiency was not affected by different pH (5-9). However, the best OG removal was observed at pH 7 which differed significantly from reactions at pH 5 and 9. Monitoring of the pH indicated little significant change throughout the course of the decolorizing tests. As the dye concentration increased in the culture medium, a decline in color removal was exhibited (Figure 3). Moreover, the time required for decolorization increased with increasing dye concentrations. This might be attributed to the toxicity of dye to bacterial cells through the inhibition of metabolic activity, saturation of the cells with dye products, inactivation of transport system by the dye or the blockage of active site of azoreductase enzymes by the dye molecules (Mabrouk and Yusef, 2008). Similar growth patterns were observed under all conditions (data not shown). Growth reached a stationary phase within 24-30 h then sharply declined. Cultures containing 200 mg l^{-1} of AZ and OG decreased cell mass 1000 fold, whereas MR and CR caused a 3-fold reduction in biomass of both strains (data not shown). In addition, all tested dyes showed no inhibition of cell growth during 24 h of decolorization. These observations showed the correlation between decolorization rate and biomass. Growth inhibition was observed after 24 h

Table 1. Decolorization of azo dyes by JH642 and ORB7106 in DSM broth

Strains	Decolorization (%)			
	Azobenzene	Methyl Red	Orange G	Congo Red
JH642	9.13±0.31*	47.08±1.40*	3.55±0.17*	24.97±0.60*
ORB7106	15.26±0.57	68.26±1.17	8.97±0.57	60.60±1.31

Values are mean±SEM of three independent replicates under all conditions (pH 5-7, Temperature 25-45ºC, dye concentration 10-200 mg l^{-1} and incubation time 8-48 h). Number of samples (N) =360 (*$P<0.05$ compared with different strains by *t*'test)

Table 2. Decolorization of dyes at different treatments by ORB7106 (mean±SEM)

Treatments		N	Decolorization (%)			
			Azobenzene	Methyl Red	Orange G	Congo Red
Temperature	25°C	72	9.61±0.80^{a}	62.41±3.00^{a}	6.83±0.81^{a}	54.24±2.89^{a}
	37°C	216	16.33±0.79^{bc}	66.80±1.41^{ab}	5.16±0.32^{a}	59.16±1.78^{a}
	45°C	72	17.68±1.17^{c}	78.49±2.27^{b}	22.54±1.82^{b}	71.28±2.05^{b}
pH	5	72	16.59±1.30	66.94±2.22	2.84±0.34^{a}	58.07±3.05
	7	216	14.50±0.71	70.30±1.56	12.54±0.84^{b}	62.44±1.61
	9	72	16.19±1.37	63.47±2.59	4.38±0.46^{a}	57.62±3.23
Concentration (mg l^{-1})	10	90	24.32±1.40^{a}	75.07±2.10^{a}	15.20±1.30^{a}	67.75±2.32^{ab}
	50	90	16.44±1.01^{b}	70.23±2.33^{ab}	10.66±1.18^{bc}	63.66±2.43^{bcd}
	100	90	11.40±0.69^{c}	66.27±2.27^{bc}	7.69±1.05^{c}	58.22±2.69^{de}
	200	90	8.87±0.51^{c}	61.49±2.41^{c}	2.32±0.22^{d}	52.77±2.79^{e}
Incubation time (h)	8	60	4.89±0.42^{a}	38.17±1.99^{a}	3.01±0.50^{ab}	27.19±1.52^{a}
	12	60	8.69±0.59^{ab}	52.05±1.87^{b}	5.74±0.90^{bc}	40.01±1.90^{b}
	16	60	12.87±0.89^{b}	64.31±1.52^{c}	7.79±1.10^{bcd}	54.20±1.59^{c}
	20	60	17.71±1.18^{c}	74.55±1.19^{d}	10.49±1.43^{cde}	69.06±1.31^{d}
	24	60	21.72±1.34^{cd}	87.12±0.84^{e}	12.15±1.58^{cd}	83.93±1.09^{e}
	48	60	25.67±1.46^{d}	93.38±0.52^{f}	14.52±1.86^{d}	89.22±0.57^{e}

Mean values with the same letter in the same column and treatment are not significantly different from each other (ANOVA, Tukey's multiple comparison test, $P<0.05$). N = number of samples

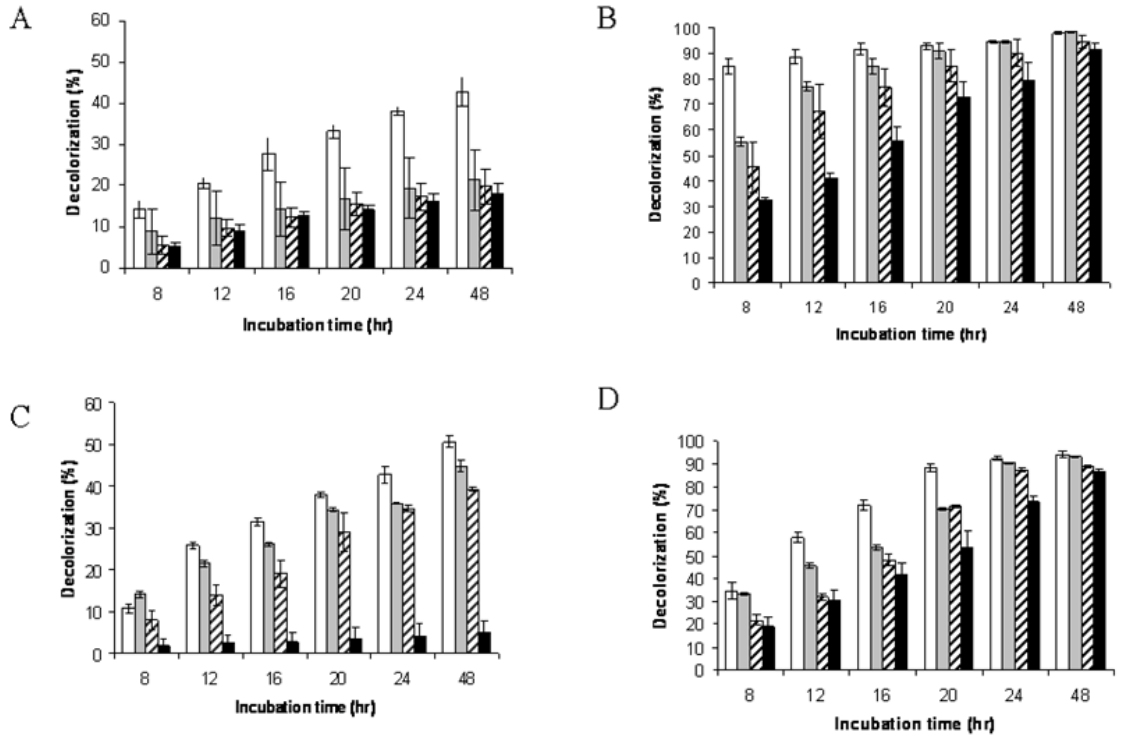

Figure 3. Time-courses of decolorization by ORB7106 at pH 7, 45C and different dye concentrations (10 mg l^{-1}, white bar; 50 mg l^{-1}, grey bars; 100 mg l^{-1}, striped bars and 200 mg l^{-1}, black bars) in DSM broth. (A) Azobenzene, (B) Methyl Red, (C) Orange G, (D) Congo Red.
Values shown are the mean of three independent experiments. Error bars represent the standard deviation.

possibly due to nutrient depletion and accumulation of toxic aromatic amine intermediates. Interestingly, expression of AzoR1 enhanced survival of ORB7106 when compared to JH642. It has been reported that azoreductases are involved in enhancing survival and the acceleration of azo dye decolorization (Liu *et al.*, 2008; Liu *et al.*, 2009). This confirmed that AzoR1 could function to detoxify and decolorize azo dyes.

The decolorization efficiency was strikingly reduced more than 7-fold when OG concentration increased from 10 mg l^{-1} to 200 mg l^{-1} (Table 2 and Figure 3C). After 48 h incubation, decolorization of AZ, MR, OG and CR was up to 26%, 93%, 15% and 89% respectively (Table 2). Decolorization of AZ at concentration of 10 mg l^{-1} was up to 43% at 45°C (Figure 3A). 50 mg l^{-1} of MR was efficiently removed up to 98% at 45°C (Figure 3B). 50% decolorization of 10 mg l^{-1} OG was observed at 45°C (Figure 3C). Maximal efficiency of 10 mg l^{-1} CR (94%) removal was achieved within 48 h at 45°C (Figure 3D). It is worth mentioning that the bacterial biomass exhibited a reddish color in DSM broth after CR decolorization, which corresponded to the result of the plate assay experiments. Biodecolorization of dyes could be due to biosorption to microbial cells or to biodegradation (Deng *et al.*, 2008; Gou *et al.*, 2009). The above observations demonstrated that CR was decolorized as a result of biosorption and degradation by AzoR1. The results suggest that CR could be transported across the cell membrane, thereby enhancing the decolorization performance by ORB7106. The residual adsorptive dye could be effectively extracted from the biomass by using methanol. The comparison and relationship of CR biosorption and biodegradation efficiency between JH642 and ORB7106 are in progress.

3.4 Enzymatic activity in the cell fractions

In recent years, there has been an increasing interest in the bioremediation of pollutants using bacteria that have the potential to degrade or mineralize several azo dyes under a variety of environment conditions (Pandey *et al.*, 2007). There are several reports that have suggested the efficacy of bacterial bioremediation using fungi and actinomycetes (Stolz, 2001; Telke *et al.*, 2010). It is now known that bacterial decolorization is achieved using various redox enzymes, including laccase, azoreductase and NADH-DCIP reductase (Stolz, 2001; Telke *et al.*, 2010; Parshetti *et al.*, 2010). Reductive cleavage of the -N=N- linkage is the initial step of the bacterial degradation of azo dyes and yields aromatic amines which are further degraded through multiple-step bioconversion (Stolz, 2001; Pandey *et al.*, 2007). Reduction may involve different mechanisms catalyzed by enzymes, or by reactions with low molecular weight redox mediators. Moreover, the location of the reactions can be either intracellular or extracellular (Pandey *et al.*, 2007; Dafale *et al.*, 2008; Telke *et al.*, 2010). In this study, two different sets of experiments were performed to understand whether decolorization was due to intracellular or extracellular enzymatic activity. At 24 h culture time, the ORB7106 cell-free extract from the pellet biomass showed more than 65% decolorization of MR (Figure 4A). However, the cell-free supernatant showed only 9-14% decolorization of MR. The decolorization of MR was significantly increased by the introduction of intracellular enzymes to the reaction. Moreover, the enzymatic activity requires NADH as cofactor not NADPH. Different NADH concentrations (200 mM and 400 mM) did not statistically affect the decolorization efficiencies (Figure 4A). Using the intracellular fraction, MR underwent a higher rate of degradation of more than 3-fold compared to extracellular fraction at a concentration of dye of 50 mg l^{-1} (Figure 4B). The above observation demonstrated that MR was decolorized by an intracellular activity in the cells overexpressing AzoR1.

3.5 Induction of enzyme production

Previous results showed that both *azoR1* transcript and AzoR1 protein synthesis are induced in response to oxidative/electrophile stress caused by the treatment with

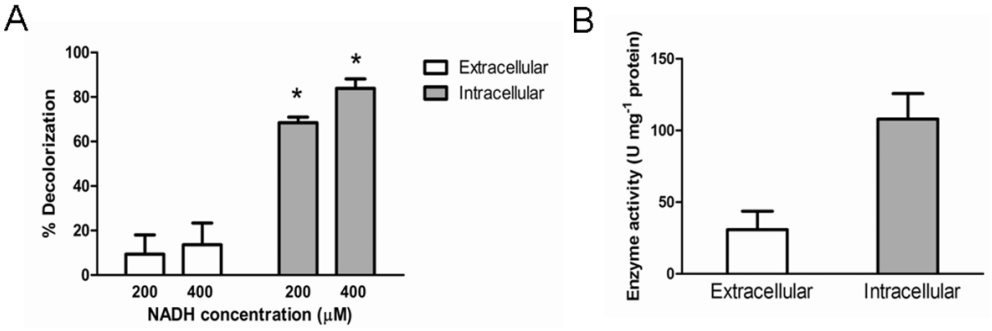

Figure 4. Cell fraction decolorizing test. ORB7106 cells were cultured in DSM broth at 37°C pH 7 for 24 h. The cell fraction decolorizing test was operated by adding 50 mg l^{-1} Methyl Red (MR) and NADH then measuring the MR reduction rate. (A) MR decolorization by extracellular fraction and intracellular fraction in the presence of NADH 200-400 mM. (B) Enzyme activity from extracellular and intracellular fraction showing the amount of enzyme required to reduce 1 mmol of MR per min per mg proteins in the presence of 400 mM NADH. Data are mean ± SEM. Difference between means were assessed using *t*'test (two-tailed) at $P<0.05$ (asterisk). All assays were done in duplicate.

catechol, 2-methylhydroquinone and diamide (Leelakriangsak *et al.*, 2008). To investigate the induction of azoreductase by MR, cells of strain JH642 were grown in medium with or without MR under shaking conditions. The percent decolorization increased significantly with increasing culture time of JH642 for both conditions. It was observed that under non-induced condition, the culture exhibited only 40% decolorization as compared to 71% of decolorization found upon inducing conditions and an incubation period of 24 h (Table 3). Therefore, the decolorization efficiency was increased up to 1.8-fold when cells were induced by MR. Statistical analyses showed the difference in decolorization efficiency for both conditions at each time point (Table 3). The longer culture time was significantly different in decolorization performance as observed for the induced condition. Decolorization rate was increased from 28% to 51 % and 71% when inducing JH642 with MR for 6, 12 and 24 h respectively (Table 3). However, 12 and 24 h culture times exhibited no statistical difference for uninduced culture (Table 3). At 24 h, enzymatic activity was detected as 7.8 U mg^{-1} protein for the induced condition, which was significantly different from the uninduced condition (4.3 U mg^{-1} protein) (Table 3). This experiment suggests that more enzyme production took place in the presence of MR. In addition to oxidative stress, MR could induce both *azoR1* transcript and AzoR1 production as shown in experiments using *azoR1-lacZ* fusion (Leelakriangsak, M. and Phongdara, A., unpublished data) and enzyme assays after induction of JH642 with MR (Table 3). From the thiol-disulfide switch mechanism of YodB (Chi *et al.*, 2010), MR could oxidize YodB and lead to derepression of transcription of the YodB regulon genes, of which *azoR1* is a member. However, *azoR1* could not be induced by CR (Leelakriangsak, M. and Borisut, S., unpublished data). Compared to JH642 in Table 1, ORB7106 exhibited 2.4-fold and 1.5-fold decolorization rate of MR and CR respectively. The purified AzoR1 could reduce CR slightly *in vitro* (Leelakriangsak, M. and Phongdara, A., unpublished data). Therefore, the majority of CR decolorization is likely to be due to biosorption.

4. Conclusion

The study described herein has confirmed the function of AzoR1. The production of *azoR1* is induced by the tested azo dyes. In contrast to AzoR1, AzoR2 protein synthesis is not induced by diamide (azo-compound), which was reported previously by Leelakriangsak *et al*. (2008). Therefore, AzoR1 is likley to be responsible for reduction of the tested azo dyes. In our study, decolorizing activity for the tested dye (MR) is contributed by the cytoplasmic fraction. We demonstrated that the cytoplasmic component for MR degradation is a NADH-dependent protein, which could endure the presence of oxygen. AzoR1 exhibits efficiency in the dye degradation at pH 5-9 and 25-45°C under anoxic conditions (static culture). Considering chemical structure effects, MR and CR are more readily transported into cellular compartment to be substrates for biodegradation, compared to other dyes. Results of this research provide evidence for the potential application of ORB7106 in the biological treatment of azo dyes contamination. Future studies will elucidate whether the aromatic amine intermediates are formed during decolorization, as many aromatic amines are toxic. The mechanism of dye decolorization by AzoR1 is still not clear. The reduction may function with other enzyme systems or require other redox mediators.

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Table 3. Decolorization efficiency after induction JH642 by Methyl Red at different time intervals of incubation

Incubation time (h)	No Induced	Induced
6	$14.15\pm5.19^{a}_{x}$	$28.00\pm1.28^{a}_{x}$
12	$30.96\pm4.60^{b}_{x}$	$51.41\pm2.89^{b}_{y}$
24	$39.78\pm1.79^{b}_{x}$ (4.31±0.19)	$71.07\pm2.30^{c}_{y}$ (7.78±0.25)*

Values are mean of % decolorization ± SEM from three independent replicates of intracellular fraction. Mean values with different superscript letters (a, b and c) in the same column are significantly different, while mean values with different subscript letters (x and y) in the same row are significantly different (ANOVA, Tukey's multiple comparison test, $P<0.05$)

Values in parenthesis are mean of enzyme activity (U mg^{-1} protein) SEM from three independent experiments of intracellular fraction. Differences between means were assessed using *t*'test (two-tailed) at $P<0.05$ (*)

References

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry. 72, 248-254.

Chen, H. 2006. Recent advances in azo dye degrading enzyme research. Current Protein and Peptide Science. 7, 101-111.

Chen, H., Hopper, S. L. and Cerniglia, C. E. 2005. Biochemical and molecular characterization of an azoreductase from *Staphylococcus aureus*, a tetrameric NADPH-dependent flavoprotein. Microbiology. 151, 1433-1441.

Chi, B. K., Albrecht, D., Gronau, K., Becher, D., Hecker, M. and Antelmann, H. 2010. The redox-sensing regulator YodB senses quinones and diamide via a thiol-disulfide switch in *Bacillus subtilis*. Proteomics. 10, 3155-3164.

Dafale, N., Rao, N. N., Meshram, S. U. and Wate, S. R. 2008. Decolorization of azo dyes and simulated dye bath wastewater using acclimatized microbial consortium-biostimulation and halo tolerance. Bioresource Technology. 99, 2552-2558.

Deng, D., Guo, J., Zeng, G. and Sun, G. 2008. Decolorization of antharaquinone, triphenylmethane and azo dyes by a new isolated *Bacillus cereus* strain DC11. International Biodeterioration & Biodegradation. 62, 263-269.

Gou, M., Qu, Y., Zhou, J., Ma, F. and Tan, L. 2009. Azo dye decolorization by a new fungal isolate, *Penicillium* sp. QQ and fungal-bacterial cocultures. Journal of Hazardous Materials. 170, 314-319.

Hsueh, C. C. and Chen, B. Y. 2007. Comparative study on reaction selectivity of azo dye decolorization by *Pseudomonas luteola*. Journal of Hazardous Materials. 141, 842-849.

Hsueh, C. C. and Chen, B. Y. 2008. Exploring effects of chemical structure on azo dye decolorization characteristics by *Pseudomonas luteola*. Journal of Hazardous Materials. 154, 703-710.

Hsueh, C. C., Chen, B. Y. and Yen, C. Y. 2009. Understanding effects of chemical structure on azo dye decolorization characteristics by *Aeromonas hydrophila*. Journal of Hazardous Materials. 167, 995-1001.

Leelakriangsak, M., Huyen, N. T., Towe, S., van Duy, N., Becher, D., Hecker, M., Antelmann, H. and Zuber, P. 2008. Regulation of quinone detoxification by the thiol stress sensing DUF24/MarR-like repressor, YodB in *Bacillus subtilis*. Molecular Microbiology. 67, 1108-1124.

Liu, G., Zhou, J., Jin, R., Zhou, M., Wang, J., Lu, H. and Qu, Y. 2008. Enhancing survival of *Escherichia coli* by expression of azoreductase AZR possessing quinone reductase activity. Applied Microbiology Biotechnology. 80, 409-416.

Liu, G., Zhou, J., Wang, J., Zhou, M., Lu, H. and Jin, R. 2009. Acceleration of azo dye decolorization by using quinone reductase activity of azoreductase and quinone redox mediator. Bioresource Technology. 100, 2791-2795.

Mabrouk, M. E. M. and Yusef, H. H. 2008. Decolorization of Fast Red by *Bacillis subtilis* HM. Journal of Applied Sciences Research. 4, 262-269.

Nakayama, T., Kimura, T., Kodama, M. and Nagata, C. 1983. Generation of hydrogen peroxide and superoxide anion from active metabolites of naphthylamines and aminoazo dyes: its possible role in carcinogenesis. Carcinogenesis. 4, 765-769.

Ooi, T., Shibata, T., Sato, R., Ohno, H., Kinoshita, S., Thuoc, T. L. and Taguchi, S. 2007. An azoreductase, aerobic NADH-dependent flavoprotein discovered from *Bacillus* sp.: functional expression and enzymatic characterization. Applied Microbiology Biotechnology. 75, 377-386.

Pandey, A., Singh, P. and Iyengar, L. 2007. Bacterial decolorization and degradation of azo dyes. International Biodeterioration & Biodegradation. 59, 73-84.

Parshetti, G. K., Telke, A. A., Kalyani, D. C. and Govindwar, S. P. 2010. Decolorization and detoxification of sulfonated azo dye methyl orange by *Kocuria rosea* MTCC 1532. Journal of Hazardous Materials. 176, 503-509.

Song, Z. Y., Zhou, J. T., Wang, J., Yan, B. and Du, C. H. 2003. Decolorization of azo dyes by *Rhodobacter sphaeroides*. Biotechnology Letters. 25, 1815-1818.

Stolz, A. 2001. Basic and applied aspects in the microbial degradation of azo dyes. Applied Microbiology Biotechnology. 56, 69-80.

Sugiura, W., Miyashita, T., Yokoyama, T. and Arai, M. 1999. Isolation of azo-dye-degrading microorganisms and their application to white discharge printing of fabric. Journal of Bioscience Bioengineering. 88, 577-581.

Sugiura, W., Yoda, T., Matsuba, T., Tanaka, Y. and Suzuki, Y. 2006. Expression and characterization of the genes ncoding azoreductases from *Bacillus subtilis* and *Geobacillus stearothermophilus*. Bioscience Biotechnology Biochemistry. 70, 1655-1665.

Suzuki, Y., Yoda, T., Ruhul, A. and Sugiura, W. 2001. Molecular cloning and characterization of the gene coding for azoreductase from *Bacillus* sp. OY1-2 isolated from soil. Journal of Biological Chemistry. 276, 9059-9065.

Telke, A. A., Joshi, S. M., Jadhav, S. U., Tamboli, D. P. and Govindwar, S. P. 2010. Decolorization and detoxification of Congo red and textile industry effluent by an isolated bacterium *Pseudomonas* sp. SU-EBT. Biodegradation. 21, 283-296.