Biodecolorization of a food azo dye by the deep sea *Dermacoccus abyssi* MT1.1T strain from the Mariana Trench

Weeranuch Lang\(^a\), Sarote Sirisansaneeyakul\(^b,c,*\), Lígia O. Martins\(^d\), Lukana Ngiswara\(^a\), Nobuo Sakairi\(^e\), Wasu Pathom-aree\(^f\), Masayuki Okuyama\(^a\), Haruhide Mori\(^a\), Atsuo Kimura\(^a,++\)

\(^a\) Research Faculty of Agriculture, Hokkaido University, Sapporo 060-8589, Japan
\(^b\) Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Bangkok 10900, Thailand
\(^c\) Centre for Advanced Studies in Tropical Natural Resources, National Research University-Kasetsart University, Bangkok 10900, Thailand
\(^d\) Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Deiras 2780-157, Portugal
\(^e\) Graduate School of Environmental Science, Hokkaido University, Sapporo 060-0810, Japan
\(^f\) Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand

**A B S T R A C T**

This study reports the characterization of the ability of *Dermacoccus* spp. isolated from the deepest point of the world’s oceans for azo dye decolorization. A detailed investigation of *Dermacoccus abyssi* MT1.1\(^T\) with respect to the azoreductase activity and enzymatic mechanism as well as the potential role of the bacterial strain for biocleaning of industrial dye baths is reported. Resting cells with oxygen-insensitive azoreductase resulted in the rapid decolorization of the polysulfonated dye Brilliant Black BN (BBN) which is a common food colorant. The highest specific decolorization rate \((v)\) was found at 50 °C with a moderately thermal tolerance for over 1 h. Kinetic analysis showed the high rates and strong affinity of the enzymatic system for the dye with a \(V_{\text{max}} = 137\) mg/g cell/h and a \(K_m = 19\) mg/L. The degradation of BBN produces an initial orange intermediate, 8-amino-5-((4-sulfonatophenyl)diazenyl)naphthalene-2-sulfonic acid, identified by mass spectrometry which is later converted to 4-aminobenzene sulfonic acid. Nearly 80% of the maximum \(v\) is possible achieved in resting cell treatment with the salinity increased up to 5.0% NaCl in reaction media. Therefore, this bacterial system has potential for dye decolorization bioprocesses occurring at high temperature and salt concentrations e.g. for cleaning dye-containing saline wastewaters.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

Synthetic dyes are widely applied in industries such as food, leather and textile. The structural variety in the chromophoric groups of these compounds, like azo, triphenylmethane and phthalocyanine, leads to a high recalcitrance to degradation by the natural microflora present in effluents. Azo dyes are aromatic compounds characterized by one or more azo bonds \((R_1-N=N-R_2)\) that are not found in nature. Their degradation by bacteria occurs typically in anaerobic conditions and is initiated by the cleavage of the azo bonds in azoreductase-catalyzed reductions (Zimmermann et al., 1982). In contrast, the products created by breaking of the \(N=N\) bond, the so-called aromatic amines, are easily degraded under aerobic biological conditions (Haug et al., 1991; Coughlin et al., 2003). Therefore the bottleneck of the biodecolorization process using conventional methods (e.g. activated sludge) is considered to be the first step of azo dye degradation. The resistance for biological degradation of textile dye baths is due to the presence, in addition to dyes, of a mixture of organic and inorganic additive chemicals, like salt, acids or alkaline compounds which leads to the need for screening microorganisms that are able to handle these harsh conditions under aerobic conditions for example a robust bacterial system producing oxygen-tolerant azoreductases.

Aquatic ecosystems have barely been studied for bioremediation purposes but are thought to represent a potential interesting source of new isolates suitable for the treatment of certain wastewaters.
Their living conditions, and hence possible physiological adaptations, may better fit the unfavorable conditions for processing effluents in industrial dye-containing effluents (D’Souza et al., 2006). Exploring the potential for dye decolorization and investigating the possibility of using these organisms as industrial dye decontaminants is therefore the main purpose of the present investigation.

Indigenous *Dermacoccus abyssi* MT1.1T, *Dermacoccus barathri* MT2.1T and *Dermacoccus profundi* MT2.2T were isolated from a sediment sample, collected from the deepest region of the world’s oceans, the Mariana Trench in the north-west Pacific Ocean [Challenger Deep (10,898 m): 11°19.911’, N142°12.372’ E] by the unmanned submersible *Kaiko*, and proposed as novel actinobacterial species (Pathom-aree et al., 2006a, 2006b). They are Gram-positive, aerobic cocci with an ability to grow at 10°C and in the presence of 10% (w/v) NaCl, while *Dermacoccus nishinomiyaensis* (originating from the human skin) does not show these properties. Interestingly, the G+C content of the three species sourced from the Mariana Trench was 65.2, 66.8 and 69.1 mol%, respectively (Pathom-aree et al., 2006a,b) which is a characteristic property of thermophilic strains. These isolates originally live in an energy-poor and oxygen-deficient habitat, and their need for a high electron transfer activity for ATP synthesis may explain their ability for the reductive cleavage of different compounds such as azo dyes with an energetic purpose. Therefore, investigations were undertaken to assess the potential of the new actinomycetes strains, in particular *D. abyssi* MT1.1T. The dye used was Brilliant Black BN (BBN), a food colorant that appears to cause some allergic or intolerance reactions but is allowed to be used in United Kingdom and other European countries with a maximum limit of 100 mg/L for fish paste and crustacean, precooked crustacean and smoked fish, but not in the USA. The possible enzymatic mechanisms involved in the BBN decolorization process and the potential use of *D. abyssi* MT1.1T cells in biocleaning of dye-contaminated waters is also reported.

### 2. Materials and methods

#### 2.1. Dyestuffs and chemicals

Acid Red 88 (AR), BBN, Brilliant Yellow (BY) and Methyl Red (MR) sodium salts were purchased from Sigma–Aldrich (St. Louis, MO, USA). Brilliant Crocein MOO (BC) and Congo Red (CR) were purchased from Wako Pure Chemical Industries Ltd. (Tokyo, Japan). Ponceau S (PS), 4-aminobenzene sulfonic acid and other required chemicals were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). The structures of tested dyes are shown in Fig. 1 and they were used without further purification. All chemicals used were analytical grade and the highest purity available.

#### 2.2. Microorganisms and preparation of resting cell suspension

MT1.1T, MT2.1T and MT2.2T designated as type strains of *Dermacoccus* spp. isolated from the Mariana Trench (Pathom-aree et al., 2006a,b) and are maintained in glycerol suspensions (20% v/v) at 0°C, were streaked in glucose-yeast extract (GYE) agar and grown at 30°C for 48 h (Gordon and Mihm, 1962). The cells were subsequently transferred to a fresh 250 mL-GYE broth using baffled Erlenmeyer flasks without addition of dye. The culture was harvested by centrifugation from the early-stationary phase with an OD<sub>600</sub> of approximately 13 after 48 h of growth. These cells were suspended in 20 mM Tris HCl (pH 7.6) and used in resting cell assays (i.e. whole cells not undergoing division also described as non-growing cells). The specific growth rate, $\mu$, of cell cultures was calculated from:

$$
\mu = \frac{\ln(C_{t_2}/C_{t_1})}{t_2 - t_1}, \quad t_2 > t_1
$$

(1)

![Fig. 1. Structure of azo dyes used in this study.](image-url)
where $C_1$ and $C_2$ are biomasses (g dry weight/L) at the different time points ($t_1$ and $t_2$), respectively.

### 2.3. Screening for dye decolorization

Seven synthetic azo dyes, containing one or two azo linkages, were used to assess the decolorization ability of the deep sea isolates. All decolorization experiments were performed in 10 mL polypropylene tubes ($1.3 \times 10 \text{ cm}$) with caps unless otherwise stated. Cells were grown in 10 mL-tubes (up to a 5.20–5.38 g/L final cell dry weight) before adding dyes at a final concentration of 100 mg/L. The tubes were gently inverted a few times and incubated at 30 °C without shaking. Samples (200 μL) were taken periodically during 0–3 h, immediately boiled for 5 min in a dry hot bath and then centrifuged (13,000g, 10 min). The supernatant was then measured for residual dye concentration as described in Section 2.7. The dissolved oxygen (DO) and oxidation–reduction potential (ORP) levels were monitored during the 3 h period of incubation and found to be in the range of 0.12 to 0.03 mg/L and 30 to ~320 mV, respectively. In these screening experiments, the solution pH was not adjusted but the initial pH was typically 7.3.

### 2.4. Preparation of cell-free extracts and enzymatic assays

Cells of MT111T were prepared according to the procedure described in Section 2.2 suspended in 20 mM Tris–HCl (pH 7.6) and disrupted in a French Press. Cell debris was removed by centrifugation (13,000g, 1 h, 4 °C). n-Dodecyl-β-D-maltopyranoside at a concentration twice per milligram of crude protein was added to the supernatant (1 mL) in order to dissolve the membrane-bound enzymes and centrifuged again. The supernatant (1 mL) was then measured for residual dye concentration as described in Section 2.7. The dissolved oxygen (DO) and oxidation–reduction potential (ORP) levels were monitored during the 3 h period of incubation and found to be in the range of 0.12 to 0.03 mg/L and 30 to ~320 mV, respectively. In these screening experiments, the solution pH was not adjusted but the initial pH was typically 7.3.

### 2.5. Environmental and operation factors

In order to assess the optimal conditions of enzyme activity, the temperature was varied between 10 and 60 °C and the pH between 1.8 and 9.0 (50 mM Britton–Robinson buffer) using cell free extracts. At the optimal conditions of temperature and pH, the optimal salinity (0–10% NaCl) was determined and the kinetic parameters measured by varying the initial BBN concentration (0–80 mg/L).

Dye decolorization assays were carried out using resting cell suspensions (described above in Section 2.2) at different temperatures (10, 20, 30, 35, 40, 50 and 60 °C) and pH values (5.0, 6.0, 7.0, 7.5, 8.0, and 9.0) (in 50 mM sodium phosphate buffer, PBS)). Further physicochemical factors such as the salinity (0, 1, 2.5, 5, 7.5, and 10% NaCl) and dye concentration (50–2000 mg/L) were also investigated at 50 °C and pH 5.0 (50 mM PBS buffer).

Time course and adsorption isotherm experiments were carried out using resting and heat-killed cell suspensions in 50 mM PBS (pH 5.0) with initial BBN concentrations of 50, 100, 250, 500, 1000, 1500 and 2000 mg/L for the resting cells (described in Section 2.2) and 8, 10, 25, 50, 100, and 250 mg/L for the heat-killed cells (the resting cells described in Section 2.2, with boiled at 100 °C for 5 min). Samples were taken at designated intervals, centrifuged (13,000g, 10 min, 4 °C) and the residual dye concentration was determined. The amount of dye uptake, $q$, was calculated as:

$$q = \frac{(S_0 - S_e)V}{W}$$

where $S_0$ and $S_e$ are the initial and equilibrium solution concentrations (mg/L), respectively, $V$ is the volume of the solution (L) and $W$ is the weight of the dry cells used (g).

For assessing the effect of cyclic addition of dyes in fed-batch experiments, the resting cells prepared in Section 2.2 were mixed with 1-volume of PBS buffer (50 mM, pH 5.0). BBN stock solution was added to reaction mixtures at a final concentration of 100 mg/L and re-added every further 1 h of incubation (4 batch cycles at 35 °C and 3 cycles at 50 °C). Residual BBN concentration and the presence of intermediate and products of reaction were monitored in supernatant aliquots taken periodically.

### 2.6. Oxygen and ORP measurement

The oxygen dependence was determined using a portable dissolved oxygen/oxidation-reduction potential (DO/ORP) meter (DM-32P, DKK-TOA Corporation, Tokyo, Japan). The device was calibrated using sodium sulfite solution (0% O2 saturation) and aerated Na2SO3 solution (100% O2 saturation). The volume of the reaction vessel was 250 mL and decolorization experiments were performed without a cap. The reaction mixtures were flushed with pure nitrogen gas or air sparging for 5 min before the addition of BBN.

### 2.7. Extraction and analysis of dye decolorization products

The products of BBN decolorization after 30 min of reaction were extracted five times with 1-volume of 1-butanol. The solvent layers were combined and dried in a rotary evaporator at 40 °C. The residue was dissolved in 50% methanol and filtered through a 0.2 μm polytetrafluoroethylene filter (Advantec®, Tokyo, Japan). The extracted samples were separated using high-performance liquid chromatography (HPLC) using a Jasco (Tokyo, Japan) apparatus equipped with a Mightysil RP-18 GP Aqua column (150 × 4.6 mm; Kanto Chemical Co. Inc., Tokyo, Japan) and a guard column at 40 °C with a linear gradient of the mobile phase A, methanol:acetoniitrile:water 3:3:4 (v/v/v) solution was used consist of 5% (w/v) ammonium acetate (AcONH4) and mobile phase B, AcONH4 solution (5%, w/v). Samples resulting from reactions of BBN decolorization were filtered through a 0.45 μm syringe filter unit (Millipore Millex-QHV, Tokyo, Japan) and directly injected in an HPLC without extraction. Peaks were detected by a UV–visible detector at 254 nm and compared with 4-aminoazobenzene sulfonic acid, injected as a chemical standard. Mass spectrometry was performed with an electro-spray ionization source (ESI) in negative mode.

### 2.8. Phytotoxicity studies

To verify the toxicity of BBN and its enzymatic intermediates or products of reaction, a phytotoxicity test was performed at 0–1000 mg/L BBN and 30–150 mg/L for the two resulting intermediates/products. These were prepared separately using resting cells incubated with BBN for 30 min or 2 h, maximizing the yield of C4-asba or C4._asba, respectively. These suspensions were centrifuged, extracted using the procedure described above, and used as samples for the phytotoxicity tests. Ten seeds of Arabidopsis thaliana were sterilized using 1% sodium hypochlorite, transferred into a 12-well flat-bottomed plate containing MGRL medium and mixed with the samples. The control sets contained distilled water instead of...
samples. The plants were incubated at 22 °C with 100 mE/m²/s lighting to provide the optimal growth conditions. Seed germination (%) and the length of roots were recorded after 7 days.

2.9. Experimental parameters

The color in the reaction supernatants was measured at the absorbance maxima of the dyes using a Multiskan ascent microplate photometer (model H-12, Thermo Fisher Scientific, Helsinki, Finland) and compared to a calibration curve of dye concentration versus optical density. The decolorization efficiency (%DE) is defined as the percentage of initial dye removed from the reaction mixtures during 3 h, as given by the following equation:

\[
\text{%DE} = \left( \frac{S_0 - S}{S_0} \right) \times 100
\]

(3)

where \(S_0\) and \(S\) are the initial and residual dye concentrations, respectively. All assays were conducted in duplicate with cell-free extracts as a blank control.

The specific decolorization rate (\(\nu_s, \text{mg/g cell/h}\)) was calculated from the slope of the initial linear region of the dye concentration versus the time curve and normalized with the biomass concentration:

\[
\nu_s = \frac{1}{C} \frac{dS}{dt}
\]

(4)

where \(C\) is the biomass concentration (g dry weight/L) and \(t\) is the incubation time (h). Since cell growth during the decolorization period was limited under static conditions, \(C\) was assumed to be a constant and calculated from the initial biomass loading. Half time (\(t_{1/2}\)) is defined as the time (min) needed for removing half of the initial amount of dye from the reaction mixtures during the decolorization process:

\[
t_{1/2} = \left( \frac{0.693}{k} \right) 60
\]

(5)

The activation energy \((E_a)\) of the BBN decolorization was determined using the Arrhenius Equation (6) and following the Equation (7):

\[
E_a = A_0 \exp \left( -\frac{E_a}{RT} \right)
\]

(6)

\[
\ln k = \ln A_0 - \frac{E_a}{RT}
\]

(7)

where \(k\) is a pseudo-first order kinetic rate constant which does not consider substrate or electron acceptor limitation, and is deduced from a nonlinear curve fitting. \(A_0\) is the frequency factor that shares the same units as \(k\), \(E_a\) is the activation energy (kJ/mol), \(R\) is the universal gas constant (8.314 kJ/mol/K), and \(T\) is the absolute temperature (K). Michaelis–Menten constants \((V_{\text{max}}\) and \(K_m\) of the BBN decolorization were determined using the equation:

\[
\nu_s = \frac{V_{\text{max}} S_0}{K_m + S_0}
\]

(8)

where \(V_{\text{max}}\) is the maximum specific decolorization rate (U/g crude protein or mg/g cell/h) and \(K_m\) is the half-saturation constant mg of dye/L.

Langmuir and Freundlich isotherms were used to describe the adsorption equilibrium of the dye adsorption to the biomass in resting or heat-killed cells i.e. to investigate the background of the enzymatic color removal. The Langmuir model (Langmuir, 1915), valid for mono-layer sorption onto a surface with a finite number of identical sites, used is:

\[
q = \frac{q_{\text{max}} b S}{1 + b S}
\]

(9)

where \(q_{\text{max}}\) is the maximal adsorption capacity (mg/g dry cell) and \(b\) is the Langmuir constant (mg/L). The Freundlich model was applied (Freundlich, 1906), based on sorption on a heterogeneous surface, as follows:

\[
q = k_f S^{1/n}
\]

(10)

where \(k_f\) and \(n\) are Freundlich constant characteristics of the system and determined using nonlinear regression in the Microsoft® Excel software package (Microsoft Corporation, USA). The kinetic expression with the best fit to the experimental data was determined using the coefficient of correlation, \(R^2\) (Kumar, 2007), defined as:

\[
R^2 = \frac{\sum (X_{\text{cal}} - X_{\text{exp}})^2}{\sum (X_{\text{cal}} - X_{\text{exp}})^2 + \sum (X_{\text{cal}} - X_{\text{exp}})^2}
\]

(11)

where \(X_{\text{cal}}\) is the calculated variable, \(X_{\text{exp}}\) is experimental data and \(X_{\text{exp}}\) is the average of the experimental data. A high value of \(R^2\) implies a causal relationship between the variables.

2.10. Statistics

Data were analysed by one-way analysis of variance with a least significant difference test using the SPSS for Windows software package (version 9.0, SPSS Inc., 1989–1999).

3. Results and discussion

3.1. Screening process for dye decolorization

Dermacoccus spp. strains MT1.1T, MT2.1T and MT2.2T grown aerobically in GYE medium with \(\mu = 0.24, 0.22\) and 0.17 h⁻¹ respectively, were harvested in the early stationary phase. The color of cells became dense yellow but this does not interfere with the dye measurement in the supernatants as the color remains attached to the cells. This is possibly related with the high oxidative stress cultivation conditions that might induce the production of quinones or flavoproteins. Respiratory quinones are assumed to take part in protection of the microbial cells against the toxic effects of free radicals and reactive oxygen species arising from electron reductions. Fig. 2 shows the results of the dye decolorization screening, with the half times listed in Table 1.

All strains tested exhibit remarkable decolorization efficiency (DE), nearly 100% after 3 h, for MR, which has a simple dye structure with low molecular mass and no aromatic sulfonate groups (Fig. 2A). MT1.1T is among the three strains the one that leads to the fastest decolorization rates of MR with a \(t_{60} = 7\) min (Table 1) and the highest measured \(\nu_s = 136\) mg/g cell/h (Fig. 2B). Recently, the metabolism of azo dyes by human skin microflora i.e. Staphylococcus, Corynebacterium, Micrococcus, Dermacoccus and Kocuria spp. has been reported by Stingley et al. (2010). Among these organisms, the species nearest the dermacocii used here, D. nishimiyanausis, reduced MR by 74% (initial concentration, 50 μM or 14.6 mg/L) in 24 h.

The five dyes with twoazo bonds used in this study contains at least one sulfonate group (Table 1). The results showed that BBN, which is highly polar and was the largest dye molecule tested with
four sulfonate groups, was decolorized by the resting cells of MT1.1\textsuperscript{T} within 1 h (t\textsubscript{50} = 26 min) showing a v\textsubscript{50} of 37 mg/g cell/h (Fig. 2B). Moreover, MT1.1\textsuperscript{T} is also able to decolorize other large dye molecules (PS and BC) up to 80% or more after 3 h-incubation (Fig. 2A). The differences observed in the decolorization results might be explained by the structural features of the different enzyme substrates. BY which is hardly degraded by the strains used, with the lowest values of DE and v\textsubscript{50} does not contain an electron-withdrawing sulfo (SO\textsubscript{3}\textsuperscript{-}) group at the 7-position of the naphthol ring, which is for example present in BBN, PS and BC. Based on these results strain, MT1.1\textsuperscript{T} and BBN were selected for further decolorization studies.

3.2. Decolorization of BBN by cell-free extracts and resting cells of MT1.1\textsuperscript{T}

In order to clarify the enzymatic systems involved in decolorization in D. abyssi MT1.1\textsuperscript{T}, we used cell-free extracts and resting cells of MT1.1\textsuperscript{T}. The requirement of both FMN and NADPH for decolorization using cell-free extracts implied the presence of an azoreductase activity. The maximal decolorization occurs at pH 2.0 and 50 °C (results not shown). The determined apparent V\textsubscript{max} and K\textsubscript{M} values for BBN decolorization are 54.3 U/g protein and 54.6 mg/L, respectively. The activity of azoreductase decreases 94, 83, 78 and 73% with addition of 1, 2.5, 5 and 10% of NaCl, respectively, to the reaction mixtures.

The effect of temperature on the decolorization of BBN by resting cells was also examined between 10 and 60 °C (Fig. 3A). The optimum temperature was also found at 50 °C and the v\textsubscript{50} (68 mg/g cell/h) being three times faster than that at 30 °C and the v\textsubscript{50} dropped dramatically when the temperature exceeded 60 °C. The activation energy (E\textsubscript{a}) is 48.5 kJ/mol (Fig. 3B), which is well agreeable with E\textsubscript{a} = 48.8 kJ/mol of Remazol Black B (two azo bonds) decolorization using activated bacterial consortium reported by Dafale et al. (2008). The activation energy (E\textsubscript{a}), is usually determined within 16.7–83.7 kJ/mol range (Shuler and Kargi, 2002).

The effect of pH on BBN decolorization was examined by varying the pH between 5.0 and 9.0, at 50 °C, since textile dyes in effluents are mostly found at high pH (Ali et al., 2006). The highest measured rate occurred at pH 5.0, v\textsubscript{50} = 120 mg/g cell/h (Fig. 3C). This pH as compared with the highest values of pH tested most probably facilitates dye adsorption to the biomass surface allowing further biodegradation. Usually dyes are metabolically independently adsorbed onto the cell surfaces by chemical or physical interactions (Vijayaraghavan and Yun, 2008). To evaluate the effect of the cell surface, dye decolorization assays were performed using resting and heat-killed cells at various dye concentrations. We found that the Langmuir model better fit the experimental data for both the resting and heat-killed cells (R\textsuperscript{2} > 0.95, as shown in Table 2) than the Freundlich model (data not shown). The monolayer saturation capacity (q\textsubscript{max} determined by Equation (9) showed a biomass capacity of 11 mg/g dead cells, nearly 30 times lower than the q\textsubscript{max} of resting cells (Table 2). This indicated that the enzymatic mechanisms leading to dye decolorization in D. abyssi MT1.1\textsuperscript{T} include the adsorption of dye molecules onto structurally intact membranes through the most likely formation of a monolayer.

The correlation between v\textsubscript{50} and the initial BBN concentration is well represented by Michaelis–Menten model (R\textsuperscript{2} = 0.98, Fig 3D). The values for the apparent V\textsubscript{max} and K\textsubscript{M} estimated from the experimental data using Equation (8) were 137 mg/g cell/h and 19 mg/L, respectively. These results indicate faster rates and stronger affinity of MT1.1\textsuperscript{T} resting cells for BBN in comparison to data previously reported in the literature, i.e. V\textsubscript{max} = 15.97 mg/g cell/h and K\textsubscript{M} = 85.7 mg/L for RB5 using a bacterial consortium (Dafale et al., 2008) and V\textsubscript{max} = 36.5 mg/g cell/h and K\textsubscript{M} = 300.1 mg/L for Reactive Red 22 decolorization by Pseudomonas luteola (Chen and Lin, 2007).

Cyclic batch operations performed by stepwise addition of dyes showed that at 35 °C, the relative v\textsubscript{50} and t\textsubscript{50} was almost constant in the 4-cycles even if the v\textsubscript{50} slightly increased in the second cycle due to the presence of residual BBN in the reaction mixture (Table 3). The cell viability remained close to 85% during the static incubation period of 3 h as measured by the change in total viable counts on GYE agar following incubation at 30 °C for 3 days. At 50 °C, the value of v\textsubscript{50} decreased slightly to 90% in the second cycle and dropped to 60% in the third cycle. The t\textsubscript{50} was slightly prolonged after two cycles (Table 3). The cell viability of 9.6 × 10\textsuperscript{6} CFU/mL decreased to 4.1 × 10\textsuperscript{6} CFU/mL at 3 h-incubation. Cell viability decreased exponentially with time at 50 °C, following a first-order reaction (Dunn et al., 1992) resulting in:

\[
C = C_0 e^{-k_d t} \tag{12}
\]

where C\textsubscript{0} and C are the biomass concentration (g dry weight/L) initially, and after treatment at t; k\textsubscript{d} is the specific deactivation
constant ($h^{-1}$), $k_d$ was found to be $1.9 \ h^{-1}$ by nonlinear curve fitting of the experimental data and Equation (12) with a resultant $R^2$ value of $0.95$. The viability decreased to 50% when the cells were incubated at 22 min and further decreased to 6% after 1 h indicating that the resting cells of MT1.1T were relatively thermal labile at $50 \ ^\circ C$. A constant accumulation of circa $30 \ mg/L$ of the orange compound intermediate and the final product of degradation, 4-aminobenzene sulfonic acid was found.

### 3.3. Decolorization in different NaCl concentrations

The moderately halophilic bacteria grow optimally within the range of $0.5$–$2.5 \ M$ NaCl ($2.9$–$14.6\%$, w/v). However at sodium chloride concentrations above $0.3\%$ cause generally moderate inhibition of bacterial activities (De Baere et al., 1984). In the current study, the additions of $1.0$–$2.5\%$ (w/v) NaCl in GYE medium lead to $v_s$ value for BBN decolorization, similar to that in the absence of NaCl (Table 4). The presence of $1.0\%$ (w/v) NaCl was however found as the optimum for BBN decolorization. Further increase in the concentration of NaCl up to $5\%$ (w/v) resulted in $v_s$ values which are still $80\%$ of the maximum possible for BBN decolorization. MT1.1T strain is more saline tolerant than *Bacillus* sp. AK1 and *Lysinibacillus* sp. AK2 isolated from soil samples of the dyeing industry and are capable of decolorizing $99\%$ Metanil Yellow in the presence of $1\%$ NaCl (Anjaneya et al., 2011).

### 3.4. Proposed mechanisms for decolorization of BBN by MT1.1T and phytotoxicity studies

Samples of the reaction mixtures were taken at appropriate times and were analyzed by HPLC and UV–vis spectrophotometry for dye substrates and products of degradation (Figs. 4A and B). The HPLC showed a peak ($RT = 25.97 \ min$; $\lambda_{max} = 570 \ nm$) corresponding to BBN which vanished after $30 \ min$ of incubation. Peak b, the orange intermediate ($B_{orange}$, $RT = 24.04 \ min$; $\lambda_{max} = 490 \ nm$) is immediately produced at the beginning of the reaction, but

---

**Table 2**

<table>
<thead>
<tr>
<th>Biomass</th>
<th>$q_{max}$ (mg/g cell)</th>
<th>$b$ (mg/L)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat-killed cells</td>
<td>11</td>
<td>0.02</td>
<td>0.98</td>
</tr>
<tr>
<td>Resting cells</td>
<td>339</td>
<td>0.02</td>
<td>0.96</td>
</tr>
</tbody>
</table>

The resting cells was prepared as described in Section 2.2, when boiled at $100 \ ^\circ C$ for $5 \ min$ was to be used as heat-killed cells (see Section 2.5). The kinetic parameters from Langmuir isotherm, Eq. (9) were determined using nonlinear regression (Microsoft® Excel, USA) with the best fit using $R^2$ by Eq. (11).

**Table 3**

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Cycle no.</th>
<th>Relative $v_s$ (%)</th>
<th>$t_{1/2}$ (min)</th>
<th>DE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>1</td>
<td>100</td>
<td>26 ± 1</td>
<td>93 ± 1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>122 ± 5</td>
<td>20 ± 0</td>
<td>83 ± 0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>105 ± 2</td>
<td>27 ± 1</td>
<td>71 ± 1</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>104 ± 5</td>
<td>26 ± 4</td>
<td>61 ± 2</td>
</tr>
<tr>
<td>50</td>
<td>1</td>
<td>100</td>
<td>8 ± 0</td>
<td>100 ± 0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>90 ± 2</td>
<td>8 ± 0</td>
<td>100 ± 0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>60 ± 1</td>
<td>10 ± 1</td>
<td>63 ± 1</td>
</tr>
</tbody>
</table>

---

*BBN with $100 \ mg/L$ was added every $1 \ h$-incubation cycle.

*DE was determined by $3 \ h$-incubation.

*The resting cells and repeated batch decolorization were described in Section 2.2 and 2.5, respectively.*
vanished after 1 h, most likely due to its conversion to a product corresponding to peak c (C4-absa, RT = 5.40 min, colorless). Furthermore, the peak c retention time from HPLC was identical to the standard aromatic amine, 4-aminobenzene sulfonic acid, which concentration was stable when plotted against time (Fig. 4C). It is reasonable to consider that the MT1.1T cells catalyze a two-step decolorization process due to the different reactivities of the two azo bonds in BBN. A decolorization mechanism (Fig. 5) is proposed as follows:

\[
BBN \xrightarrow{k_A} B_{orange} + C_1 \xrightarrow{k_B} C_{4-absa} + C_1 + C_2
\]  

(13)

where \(k_A\) and \(k_B\) are first order rate constants of the proposed chain reaction, \(C_1\) and \(C_2\) are two undetectable unknown intermediates with proposed structures shown in Fig. 5.

The first step of degradation corresponds to the reduction of one azo bond localized between the naphthalene rings, which later is decomposed to form two aromatic naphthal compounds represented by \(B_{orange}\) and compound \(C_1\) with \(k_A = 6.0 \text{ h}^{-1} (R^2 = 0.95)\) (Fig. 5). Then, \(B_{orange}\) was further decomposed at its azo link localized between the naphthalene ring and the benzene ring to form two additional aromatic compounds with \(k_B = 5.5 \text{ h}^{-1} (R^2 = 0.84)\), represented by \(C_{4-absa}\) (a stable product) and compound \(C_2\). Then, \(C_{4-absa}\) corresponds to the final reduced product of BBN that is not degraded further by the enzymatic systems under study. For identification of the \(B_{orange}\) compound,

\begin{table}
\centering
\caption{Influence of salinity on BBN decolorization by the resting cells of MT1.1T.}
\begin{tabular}{lcccc}
\hline
NaCl (%) & Relative \(v_o\) (%) & \(t_{1/2}\) (min) & DE (%) \\
\hline
0 & 100 & 8 ± 1 & 100 ± 0 \\
1.0 & 108 ± 6 & 8 ± 0 & 100 ± 0 \\
2.5 & 100 ± 4 & 8 ± 0 & 100 ± 0 \\
5.0 & 83 ± 1 & 14 ± 0 & 84 ± 0 \\
7.5 & 51 ± 3 & 71 ± 2 & 54 ± 0 \\
10.0 & 34 ± 5 & 187 ± 29 & 32 ± 4 \\
\hline
\end{tabular}
\footnotesize{\textsuperscript{a} The resting cells prepared was described in Section 2.2. \\
\textsuperscript{b} \(v_o\) at the first cycle was taken as 100%. \\
\textsuperscript{c} DE was determined by 3 h-incubation.}
\end{table}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig4}
\caption{Analysis of BBN decolorization products by (A) HPLC from 1 min to 3 h. (B) Typical UV–visible spectra scanned at 0 min (---), 1 min (--; --), 30 min (--.--), and 3 h incubation (-----). (C) Residual concentrations during BBN decolorization: a – BBN; b – RT24 intermediate; and c – 4-aminobenzene sulfonic acid, and (D) ESI-MS analysis of orange intermediate.}
\end{figure}
supernatant samples after 30 min-incubation were extracted with 1-butanol, collected by preparative HPLC at a retention time of 24.04 min and then analyzed by ESI-MS to identify the structure. The signal spectra of the B orange in Fig. 4D showed three fragment ions of $m/z$ 203 \([\text{M} – 2\text{H}]^{+}\), 406 \([\text{M} – \text{H}]^{+}\) and 428 \([\text{M} + \text{Na} – 2\text{H}]^{+}\) that were ascribed to 8-amino-5-((4-sulfonatophenyl)diazenyl)naphthalene-2-sulfonic acid \((m/z = 407.39)\). Based on the results of HPLC and MS, the mechanism for BBN decolorization proposed is presented in Fig. 5. B orange and C4-absa could be detected by HPLC, but the naphthol-based compounds \((C_1\) and \(C_2)\) containing a sulfo group at the 7-position could not. It appears that the enzyme localized at the cell membrane has a preference for the naphthol ring substituted with sulfo groups at the 7-position and translocates the intermediate compounds into the cells after degradation.

The toxicity tests on \(A.\ thaliana\) revealed that the microbial degradation of BBN results in products that are more toxic than the dye itself (Table 5). Therefore, further treatment is recommended for permissible safe disposal of the treated dye solutions before releasing it into main watercourses and agricultural fields. A possibility is the design of multi-step enzymatic processes using laccase as the second catalysts after azoreductase; laccases uses aromatic amines as substrates releasing non-toxic oligomers and polymers (Mendes et al., 2011).

### Table 5

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mg/L)</th>
<th>Germination (%)</th>
<th>Root elongation(^*) (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>100</td>
<td>0.23 ± 0.12</td>
</tr>
<tr>
<td>BBN</td>
<td>100</td>
<td>100</td>
<td>0.59 ± 0.22</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>100</td>
<td>0.52 ± 0.19</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>100</td>
<td>0.38 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>100</td>
<td>0.29 ± 0.11</td>
</tr>
<tr>
<td>B orange</td>
<td>30</td>
<td>100</td>
<td>0.31 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>50</td>
<td>0.34 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>50</td>
<td>0.28 ± 0.13</td>
</tr>
<tr>
<td>C4-absa</td>
<td>30</td>
<td>100</td>
<td>0.10 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>80</td>
<td>0.10 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>0</td>
<td>–</td>
</tr>
</tbody>
</table>

\(^*\) Root elongation presented as mean ± SD, \(n = 10\).
amount of orange intermediate was substantially decreased within 1 h for both conditions indicating that oxygen is rapidly depleted under static conditions by resting cell metabolism resulting in anaerobic conditions. Under oxidative stress conditions prepared by sparging air (Fig. 6C), BBN was decolorized at a slightly lower rate, $v_i = 97 \pm 0$ mg/L, but total decolorization was also achieved in the first hour of incubation, with values of ORP and DO in the range of $\sim 74$ to $\sim 161$ mV and 0.06–0.86 mg/L, respectively. In addition, after 1 h of incubation the ORP values increased noticeably to approximately 0 mV and reached 3 mV in after 2 h, presumably after 1 h of incubation the ORP values increased noticeably to approximately 0 mV and reached 3 mV in after 2 h, presumably leading to conditions that result in critical inhibition of the decolorization reaction by oxygen. From a kinetics viewpoint, it seems clear that the second reaction, Borange converting to C4-absa is most inhibited by the increased oxygen tension in reaction mixtures, which might relate to enzyme competition between reduction of oxygen and dye.

4. Conclusions

This work is the first to explore the special characteristics of the deep sea D. abyssi MT1.1³ bacteria isolated from the Challenger Deep sediment of the Mariana Trench for dye decolorization. The maximal rates for dye decolorization occurs at 50 °C, exhibiting thermal tolerance for up to 1 h, with complete degradation of 250 mg/L of BBN in reaction conditions up to 5.0% NaCl. The bacteria show oxygen-tolerant azoreductase activity, with a preference for the cleavage of the azo dyes around the naphthol ring substituted with a sulfo group at the 7-position. Further studies will focus in the cloning of azoreductase genes, design of efficient multi-step enzymatic in reaction mixtures supplemented with nutrients in order to improve cell viability and using immobilized recombinant resting cells expressing azoreductases and laccases leads to decolorization and detoxification of real dye-containing saline wastewaters.

Acknowledgement

The authors wish to thank Dr. Shinji Wakuta, Research Faculty of Agriculture, Hokkaido University for his guidance in the phytotoxicity test and Sónia Mendes, Instituto de Tecnologia Química e Biologia, Universidade Nova de Lisboa for the technical support in the enzymatic analysis.

References


