Full Paper

Performance of *Burkholderia multivorans* CCA53 for ethyl red degradation

(Received August 8, 2019; Accepted November 5, 2019; J-STAGE Advance publication date: June 5, 2020)

Hironaga Akita,^{1,*} Shinji Fujimoto,¹ Keisuke Wada,¹ Noriyo Takeda,¹ Yuki Iwasaki,¹
Tatsuya Fujii,¹ and Akinori Matsushika^{1,2}

¹ Research Institute for Sustainable Chemistry, National Institute of Advanced Industrial Science and Technology (AIST),
3-11-32 Kagamiyama, Higashi-Hiroshima, Hiroshima 739-0046, Japan

² Graduate School of Advanced Sciences of Matter, Hiroshima University,
1-3-1 Kagamiyama, Higashi-Hiroshima, Hiroshima 739-8530, Japan

The discharge of industrial dyes and their breakdown products are often environmentally harmful. Here, we describe a biodegradation method using *Burkholderia multivorans* CCA53, which exhibits a capacity to degrade azo dyes, particularly ethyl red. Under the optimized culture conditions, 100 μ M ethyl red was degraded more than 99% after incubation for 8 h. Real-time PCR analysis of *azoR1* and *azoR2*, encoding two azoreductases, revealed that transcription level of these genes is enhanced at early phase under the optimized conditions. For a more practical approach, hydrolysates were prepared from eucalyptus or Japanese cedar chips or rice straw, and rice straw hydrolysate was used as the best medium for ethyl red biodegradation. Under those conditions, ethyl red was also degraded with high efficiency (>91%). We have thus constructed a potentially economical method for the biodegradation of ethyl red.

Key Words: azoreductase; biodegradation; *Burkholderia multivorans*; ethyl red; hydrolysate; real time-PCR analysis

Introduction

A variety of dyes are used extensively to color textiles, foods, cosmetics, plastics, and numerous other consumer products. At present, more than 800,000 tons of dyes are produced annually worldwide. As a result of this widespread usage, there occurs a significant discharge of dyes into the natural environment, which is undesirable because the dyes and their breakdown products are often genotoxic,

mutagenic, and/or carcinogenic in both animals (including humans) and plants. For example, Shinka et al. (1995) showed that the likelihood of cancerogenesis in factory workers is proportional to the duration of their dye exposure. Similar findings were reported for factory workers at a Chinese manufacturer (Bi et al., 1992), Italian dye-stuff workers (Piolatto et al., 1991), and Japanese kimono painters (Morikawa et al., 1997). In addition, factory workers showed a 12% higher mortality rate than the general population (Montanaro et al., 1997). Thus, it is necessary to degrade harmful dye products to harmless forms before discharging them into the natural environment.

The current treatment methods for degrading dye-contaminated waste are constructed based on chemical processes. For example, Fenton's reagent is used to degrade both soluble and insoluble dyes (Pak and Chang, 1999). With photochemical methods, reactive oxygen species, including hydrogen peroxide and reactive radicals, are used to degrade dyes to CO₂ and H₂O (Oancea and Meltzer, 2014). In addition, physical processes are also useful for dye degradation. Membrane filtration can continuously separate most dyes from an effluent (Xu and Lebrun, 1999). Adsorption and ion exchange methods are very effective for removing both anionic and cationic dyes (Rafatullah et al., 2010). Although these methods have been applied in the textile industry, their costs are high and they require considerable energy consumption. Moreover, the byproducts of these reactions, which can include halides, metals, and/or chloride ions, are themselves toxic. For these reasons, microbial degradation has been studied for years to establish more effective and economical methods of degrading dyes. Several of the developed microbial methods have shown an ability to completely eliminate certain dyes. For example, textile dye waste containing reactive black RC, reactive yellow HF2-GL, reactive

*Corresponding author: Hironaga Akita, Research Institute for Sustainable Chemistry, National Institute of Advanced Industrial Science and Technology (AIST), 3-11-32 Kagamiyama, Higashi-Hiroshima, Hiroshima 739-0046, Japan.

Tel: +81 82-493-6848 Fax: +81 82-420-8291 E-mail: h-akita@aist.go.jp

None of the authors of this manuscript has any financial or personal relationship with other people or organizations that could inappropriately influence their work.

blue BGFN, reactive black B-150, and reactive red A-6BF can be decolorized using *Aspergillus fumigatus* XC6 (Jin et al., 2007). In addition, industrial effluent containing mainly scarlet RR is decolorized using a bacterial-yeast consortium with *Brevibacillus laterosporus* MTCC 2298 and *Galactomyces geotrichum* MTCC 1360 (Kurade et al., 2012). But although these methods show excellent decolorization rates, they require reagent-grade carbon, nitrogen, and mineral sources. For practical use, however, it would be preferable if that is unnecessary because reagent-grade materials are expensive.

Burkholderia multivorans species are currently being used as biocatalysts. For example, *B. multivorans* NG1 is used to degrade various aromatic polycyclic hydrocarbons, including naphthalene, *n*-hexadecane, *n*-octadecane, *n*-nonadecane, 1-methylnaphthalene and pyrene in liquids (Mohanty and Mukherji, 2013). In our previous study, *Burkholderia* sp. CCA53 was isolated from leaf soil (Akita et al., 2016b) and a draft genome sequence of this strain was determined (Akita et al., 2016a). Ultimately, the strain was identified as *B. multivorans* CCA53 based on its phylogenetic, phenotypic and biochemical characteristics (Akita et al., 2017). Notably, *B. multivorans* CCA53 exhibited a capacity for lignin degradation (Akita et al., 2016b). *B. multivorans* CCA53 is capable of growing on lignin as the sole carbon source, which entails cleavage of several main building blocks to assimilate lignin-associated aromatic monomers (Akita et al., 2016b). Several dyes contain aromatic amines, and have structures similar to lignin-associated aromatic monomers. Thus, we consider that *B. multivorans* CCA53 has a potential for dye degradation.

Here, we report the degradation of dyes using *B. multivorans* CCA53. After testing the degradative capacity of *B. multivorans* CCA53 with 13 kinds of dyes, the degradation of ethyl red was assessed. Moreover, real-time (RT)-PCR was performed to establish a correlation between ethyl red degradation and the transcription of azoreductase genes. As part of the development of practical applications, we also investigated the biodegradation of ethyl red using hydrolysate prepared from rice straw.

Materials and Methods

Screening for dye-degradation capacity. *B. multivorans* CCA53 was previously deposited in the HUT culture collection under strain number HUT-8135. Bordeaux S, bromothymol blue, congo red, cresol red, curcumin, ethyl red, fluorescein, methyl orange, methyl red, orange II, rhodamine B, thymol blue, and trypan blue were purchased from Fujifilm Wako Chemicals (Osaka, Japan) or Tokyo Chemical Industry (Tokyo, Japan). To confirm dye-degradation capacity, *B. multivorans* CCA53 was aerobically grown on LB plates (pH 7.0) containing 100 μ M each dye for 3 days at 30°C.

Optimization of the culture conditions for ethyl red degradation. Ethyl red degradation was performed in a flask with a working volume of 20 mL. After *B. multivorans* CCA53 was pregrown overnight, the pre-culture was diluted to OD₆₀₀ = 0.05 with fresh LB medium containing 100 μ M ethyl red, and then the culture was incubated un-

Table 1. Nucleotide primers used for the quantitative RT-PCR.

Primer	Nucleotide sequence
<i>azoR1</i> -fwd	5'-GCGCGCCGATGTACAACCTC-3'
<i>azoR1</i> -rev	5'-CGTAGATGAAGCTCACGTCGG-3'
<i>azoR2</i> -fwd	5'-TCTTCATATTGATTTCAGCCCGCG-3'
<i>azoR2</i> -rev	5'-ATCCACGTCTTGAAGCCTGAC-3'
<i>gyrB</i> -fwd	5'-GAGTTCCTGCTCGAAACGCC-3'
<i>gyrB</i> -rev	5'-CGACTTCGCCGGATCCTTCT-3'

der several conditions. The OD₆₀₀ was measured by monitoring the difference between the cellular and cell-free turbidity values using an Eppendorf BioSpectrometer (Eppendorf, Hamburg, Germany). Ethyl red was quantified according to the method of Tomic-Carruthers et al. (2013). Degradation efficiency was expressed as the percentage ratio of the decolorized dye concentration to the initial concentration using the following equation: Degradation efficiency (%) = (Initial dye concentration – Residual dye concentration)/Initial dye concentration $\times$ 100.

The standard culture conditions were as follows: culture pH, 7.0; culture temperature, 30°C; flask rotation speed, 180 rpm. The effects of culture temperature and initial culture pH on ethyl red degradation were examined at pH 6.5–9.0 and 25–50°C, respectively.

Quantitative RT-PCR analysis. *B. multivorans* CCA53 was cultured aerobically in LB medium containing 100 μ M ethyl red under the standard or optimized culture conditions. The cells were harvested by centrifugation (15,000 $\times$ g, 15 min), and their total RNAs were extracted using NucleoSpin RNA (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. The collected total RNAs (1 μ g) were converted to cDNAs using a ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan). RT-PCR analysis was then performed using a Chromo4 Four-Color Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with MJ OpticonMonnitor Analysis Software ver. 3.1 (Bio-Rad Laboratories).

The *azoR1* and *azoR2* gene sequences of strain CCA6 are available in the GenBank/EMBL/DDBJ databases under accession numbers BDDJ01000001 and BDDJ01000002, respectively. The specific primers were designed using the Primer3 web server (Untergasser et al., 2012), and their sequences are shown in Table 1. RT-PCR analysis was carried out as previously described with some modifications (Rahman et al., 2009). The relative transcription ratio was normalized to the *gyrB* (β subunit of DNA topoisomerase) gene.

Phylogenetic analysis of azoreductase. Multiple alignment and construction of a neighbor-joining phylogenetic tree (Saitou and Nei, 1987) were performed using CLUSTAL W ver. 1.83 (Thompson et al., 1994) with the Tamura-Nei model (1993). All sequences were then compared with reference sequences available in the GenBank/EMBL/DDBJ databases using BLAST.

Preparation of hydrolysate from lignocellulosic biomass. Lignocellulosic biomass, including eucalyptus or Japanese cedar chips or rice straw, was used as the raw material for the preparation of hydrolysate. After milling each sample

Fig. 1. Effects of culture pH and temperature on the efficiency of ethyl red degradation.

B. multivorans CCA53 was cultivated for 8 h in LB medium containing 100 μ M ethyl red. Shown are the effects of culture pH (A) and temperature (B) on the OD₆₀₀ (open bars) and the degradation efficiency (gray bars). Error bars indicate SE ($n = 3$).

using a cutter mill (P-15; Fritsch Japan, Kanagawa, Japan), the resulting powders were separated into <3 mm particles by sieving. Thereafter, hydrothermal pretreatment was carried out according to the method of Fujimoto et al. (2018) with some modifications. The reaction mixture (50 mL) used for enzymatic saccharification contained 1 M acetate buffer (pH 5.0), 0.25 g/mL Acremonium cellulase (Meiji Seika Pharma, Tokyo, Japan), 0.20 g/mL Optimash BG (Genencor International, Rochester, NY, USA). After incubation for 72 h at 50°C, the reaction mixture was purified by filtration. The resulting solution was supplemented with corn steep liquor (Sigma-Aldrich, Saint Louis, MO, USA) to 1% of the final concentration and then used as the hydrolysate.

Quantification of sugars and other carbohydrates. After clarifying the culture by centrifugation and filtration, several kinds of sugars in the supernatant were quantified by HPLC (LC-10AD VP; Shimadzu, Kyoto, Japan) using an Aminex HPX-87H column (Bio-Rad Laboratories). The chromatographic conditions were as follows: mobile phase, ultrapure water; flow rate, 0.6 mL/min; and column oven temperature, 80°C. Sugar was detected using a refractive index indicator.

Furfural and 5-hydroxymethyl furfural were also analyzed by HPLC (LC-2000Plus; Jasco, Tokyo, Japan) using a Shodex SUGAR SH1821 column (Showa Denko, Tokyo, Japan). The chromatographic conditions were as follows: mobile phase, 2 mM H₂SO₄; flow rate, 0.6 mL/min; and column oven temperature, 60°C. These carbohydrates were also detected using a refractive index indicator.

Results and Discussion

Determination and optimization of the degradative capacity for ethyl red

To confirm its capacity to degrade dye, *B. multivorans* CCA53 was plated onto LB plates containing each tested dye. After incubation for 48 h at 30°C, decolorization of ethyl red was confirmed (Supplementary Fig. 1). Moreover, their decolorization proceeded with the time elapse. Decolorization of orange II, methyl red, and congo red was also confirmed after 72 h (data not shown). There was no decolorization of bordeaux S, bromothymol blue, cresol

Fig. 2. Efficiency of ethyl red degradation under the standard (culture pH, 7.0; culture temperature, 30°C) and optimized (culture pH, 7.5; culture temperature, 45°C) culture conditions.

B. multivorans CCA53 was cultured in LB medium containing 100 μ M ethyl red. Shown are the time-dependent changes in the OD₆₀₀ (circles) and the efficiency of ethyl red degradation (squares) under the standard (open symbols) and optimized (filled symbols) culture conditions. Error bars indicate SE ($n = 3$).

red, curcumin, fluorescein, methyl orange, rhodamine B, thymol blue, or trypan blue. These results demonstrated that *B. multivorans* CCA53 has the capacity to degrade mono azo dyes. Azo dye has one or more azo bonds (R₁-N=N-R₂), and contains a few linkages linking aromatic rings and/or functional groups. In the textile industry, more than 80% of the dyes used are azo dyes. Importantly, during the dyeing process, approximately 10% of azo dye does not bind to the fiber, and the unfixed dye is released into the natural environment (Asad et al., 2007). Thus, the establishment of an effective method for azo dye degradation is important.

We initially evaluated the effect of ethyl red concentrations (100–500 μ M). Under the standard conditions, *B. multivorans* CCA53 was capable of growth in LB medium containing 100 μ M ethyl red. However, a high concentration of ethyl red was unfavorable for the growth of *B. multivorans* CCA53, which was strongly inhibited by adding more than 200 μ M ethyl red. Thus, we used 100 μ M ethyl red for the degradation test. In our previous study, we found that the growth of *B. multivorans* CCA53 was affected by culture pH and temperature (Akita et al., 2017). Thus, the effects of culture pH and temperature for ethyl red degradation were evaluated. After *B. multivorans* CCA53 was incubated for 8 h, the maximum degradation efficiency was achieved at pH 7.5 (Fig. 1A). When the effect of culture temperature was investigated, the maximum degradation efficiency was obtained at 45°C (Fig. 1B). In conclusion, the optimized culture conditions for the degradation of ethyl red were a culture pH of 7.5 and a culture temperature of 45°C. Under the standard conditions, *B. multivorans* CCA53 decomposed 80% of ethyl red after incubation for 12 h, and the degradation efficiency reached >99% after incubation for 20 h (Fig. 2). Under the optimized conditions, by contrast, 84% of ethyl red was decomposed after incubation for 6 h, and the degradation efficiency was >99% after incubation for only 8 h (Fig. 2). The growth rate of *B. multivorans* CCA53 was also improved under the optimized culture conditions (Fig. 2). In contrast, the degradation of ethyl red did not occur

Fig. 3. Relative transcription levels of *azoR1* and *azoR2* under the standard and optimized culture conditions.

Bars depict changes in the relative gene transcription at the indicated times without 100 μ M ethyl red (white) or under the standard (light grey) and optimized (dark grey) culture conditions. transcription levels are shown relative to that of *gyrB*, which served as an internal control. Error bars indicate SE ($n = 3$).

without *B. multivorans* CCA53.

The culture pH is especially important for industrial bacterial processes. Fungi and yeast exhibit high biodegradation activities at an acidic pH (Sen et al., 2016), but the degradation rate is lower at a neutral or an alkaline pH, which means that strict pH control is required to maintain effective culture conditions. By contrast, the activities of *B. multivorans* CCA53 were higher at pH 6.5–8.0, which is an advantage for practical utilization (Fig. 1). For ethyl red degradation using *B. multivorans* CCA53, we considered the culture temperature to be the most important factor. The maximum rate of dye degradation is generally related to the optimized culture temperature for each microbial species (Bardi and Marzona, 2010; Khan et al., 2013), but there were discrepancies with *B. multivorans* CCA53. For example, when *B. multivorans* CCA53 was cultured in Nutrient Broth without ethyl red, the maximum growth rate was achieved at 20°C (Akita et al., 2017). The reason for this was unclear, but we speculate that azoreductase activity is enhanced at that temperature. In fact, however, mesophilic bacterial azoreductases from *Bacillus* sp. B29 (Ooi et al., 2009), *Bacillus* sp. OY1-2 (Sugiura et al., 2006), and *Rhodococcus opacus* 1CP (Qi et al., 2016) exhibit maximum activity at 45–55°C. In the draft genome of *B. multivorans* CCA53, two azoreductase genes, designated *azoR1* and *azoR2*, are annotated. The amino acid sequences of the two encoded enzymes show 36.7% identity. To confirm the relationships between the dye-degradation capacity of *B. multivorans* CCA53 and the transcription of each azoreductase gene, we next performed quantitative RT-PCR.

Consideration on the degradative capacity of *B. multivorans* CCA53

Microorganisms often accomplish dye biodegradation through the expression of several degradative enzymes. Actinomycete species are known to produce extracellular peroxidase, which catalyzes the removal of a hydrogen atom from the hydroxyl group of dyes (Sarkar et al., 2017). Laccase is mainly produced by white-rot fungi. This enzyme catalyzes the oxidation of various phenolic and aro-

matic compounds in the presence of molecular oxygen. Azoreductase catalyzes the reductive cleavage of azo bonds, which enables it to play an important role in bacterial dye degradation. Finally, those degradants are further degraded into simpler compounds (Stolz, 2001). Within the draft genome sequence of *B. multivorans* CCA53, two kinds of genes encoding azoreductase are confirmed. Thus, we next performed quantitative RT-PCR to investigate the transcription levels of *azoR1* and *azoR2* in *B. multivorans* CCA53 under the standard or optimized conditions (Fig. 3).

When *B. multivorans* CCA53 was cultured in LB medium without 100 μ M ethyl red under the standard conditions, the transcription levels of *azoR1* and *azoR2* at 4–8 h were 1.5–2.5 and 0.38–0.40, respectively (Fig. 3). Under the standard conditions, the transcription levels of the two genes increased with the time in culture, and the highest transcription levels were detected after 12 h. Under the optimized conditions, transcription of *azoR1* and *azoR2* was enhanced at early phase, and their maximum levels were reached at 8 and 4 h, respectively. Although *azoR1* was stably expressed under both conditions, the transcription level of *azoR2* was clearly enhanced at early time under the optimized conditions. These results demonstrated that ethyl red adding induced the transcription level of *azoR1* and *azoR2*, and the culture condition was effected by their transcription levels. This suggests that transcription mechanism of *azoR1* and *azoR2* may be similar to that of *B. multivorans* ATCC 17616 (Denman et al., 2014). When *B. multivorans* ATCC 17616 was cultured in mannitol-rich media, the transcription levels of genes related to sugar metabolism are also enhanced.

To further appraise the biodegradative capacity of *B. multivorans* CCA53, we constructed a neighbor-joining phylogenetic tree based on the amino acid sequences of several azoreductases (Fig. 4). Within the constructed tree, AzoR1 and AzoR2 from *B. multivorans* CCA53 showed a clear branching from close azoreductases. The sequence of *B. multivorans* CCA53 AzoR1 showed similarity to azoreductases from *Klebsiella pneumoniae* subsp. *pneumoniae* HS11286 (48.5%), *Shigella dysenteriae* Sd197 (48.5%), *Pseudomonas aeruginosa* PAO1 AzoR3 (46.9%) and *Escherichia coli* JM109 (46.8%). AzoR2 exhibited similarities of 44.3%, 43.1% and 42.7% its closest relatives from *Pantoea agglomerans* TX10, *Halomonas elongata* DSM 2581 and *Shewanella oneidensis* MR-1, respectively. The *P. agglomerans* enzyme shows the greatest specific activity toward methyl red (Moutaouakkil et al., 2003). More than 80% of its activity was detected at temperatures between 15 and 50°C or at pHs between 6.5 and 7.5. Similar characteristics were also observed with the azoreductase from *E. coli* (Cui et al., 2012). To determine the enzymatic characteristics of the *azoR1* and *azoR2* gene products, we are now planning to express each gene in recombinant *E. coli* cells. The results will be described elsewhere as the next stage of our study.

Overall, these results indicate that transcription of *azoR1* and *azoR2* was induced by the addition of ethyl red, and their transcription levels were markedly accelerated at early times under the optimized conditions. In addition to the greater transcription levels of *azoR1* and *azoR2*, the

Fig. 4. Phylogenetic tree constructed through analysis of the amino acid sequences of azoreductase showing the relationships between *B. multivorans* CCA53 and other bacteria.

The bar indicates a 0.1% amino acid residue substitution rate.

Table 2. Concentrations of sugar and aldehyde in each hydrolysate.

Hydrolysate	Incubation time (h)	Glucose (mM)	Arabinose (mM)	Galactose (mM)	Mannose (mM)	Xylose (mM)	Furfural (mM)	5-Hydroxymethyl furfural (mM)
Eucalyptus	0	173	0	1.67	10.9	1.27	12.5	2.42
	8	168 ± 6.30	0	1.61 ± 0.490	0	0.971 ± 0.486	12.3 ± 0.105	2.25 ± 0.0281
Japanese cedar	0	70.7	9.07	14.0	27.9	18.5	N.D.*	N.D.
	8	66.3 ± 0.189	8.55 ± 0.0913	13.0 ± 0.0634	28.0 ± 0.0806	17.1 ± 0.0695	N.D.	N.D.
Rice straw	0	10.5	2.23	14.2	2.20	15.7	N.D.	N.D.
	8	5.28 ± 0.0444	0.750 ± 0.189	6.01 ± 0.166	1.83 ± 0.0153	12.9 ± 0.318	N.D.	N.D.
	12	5.26 ± 0.0501	0.705 ± 0.154	5.83 ± 0.147	1.56 ± 0.166	12.6 ± 0.517	N.D.	N.D.

*N.D. = not detected.

degradation activities of AzoR1 and AzoR2 may also be enhanced under the optimized conditions. Thus, the degradation efficiency of ethyl red was enhanced under the optimized conditions.

When ethyl red is degraded by azoreductase, anthranilate and *N,N*-diethyl-1,4-phenylenediamine are generated. Although *B. cepacia* decomposes anthranilate to catechol by anthranilate 1,2-dioxygenase (Chang et al., 2003), this gene is not found in the draft genome sequence of *B. multivorans* CCA53 (Akita et al., 2016a). *N,N*-Dimethyl-1,4-phenylenediamine, which has a similar structure to *N,N*-diethyl-1,4-phenylenediamine, is demethylated to 1,4-phenylenediamine in azo dye-degrading bacteria (Yemashova and Kalyuzhnyi, 2006). However, 1,4-phe-

nylenediamine is not further degraded and exists as an end product. These results suggest that *B. multivorans* CCA53 may lack the metabolic pathway for the complete degradation of ethyl red.

Hydrolysate from lignocellulosic biomass

A broad range of assimilated carbon sources is a key characteristic of *B. multivorans* CCA53. When its carbon source utilization was characterized, it was found that *B. multivorans* CCA53 assimilates at least 27 different carbon sources (Akita et al., 2017). Tons of lignocellulosic biomass resources are generated every year in the agricultural and forest industries. Moreover, for example, fermentation of lignocellulosic biomass resources to bio-etha-

Fig. 5. Effect of lignocellulosic hydrolysates on the efficiency of ethyl red degradation.

Each hydrolysate was prepared from lignocellulosic biomass: Eu, eucalyptus; Jc, Japanese cedar; Rs, rice straw. *B. multivorans* CCA53 was cultivated for 8 h in each hydrolysate containing 100 μ M ethyl red. Open and gray bars depict the OD₆₀₀ and the degradation efficiency, respectively. Error bars indicate SE ($n = 3$).

nol is now established on a global scale and is commercially available (Furkan and Remzi, 2015). To establish an economical biodegradation method, LB medium should be replaced with lignocellulosic biomass-based hydrolysates. When hydrolysate is prepared from lignocellulosic biomass, several sugars are extracted from cellulose and hemicellulose through enzymatic saccharification. Unfortunately, several inhibitors are also generated during the hydrothermal process. Several inhibitors suppress microbial growth, interfere with subsequent fermentation, and ultimately decrease productivity (Jönsson et al., 2013). It is, therefore, necessary to reduce the generation ratio of fermentation inhibitors. We previously established a useful method for preparing hydrolysate from eucalyptus chips (Fujimoto et al., 2018). Using the method with some modifications, we prepared hydrolysates from eucalyptus and Japanese cedar chips as well as rice straw (Table 2). Each of the hydrolysates contained a variety of sugars, including glucose, arabinose, galactose, mannose, and xylose. These components were consistent with those observed by Thalagala et al. (2009), which confirms that our method is an effective means of preparing hydrolysate from the aforementioned materials. In addition, the concentration of several inhibitors was low enough (<12.5 mM) for us to perform ethyl red degradation using each of the hydrolysates as the medium.

Ethyl red degradation using lignocellulosic hydrolysate

When ethyl red degradation was performed, the highest degradation efficiency and OD₆₀₀ were observed after incubation for 8 h with hydrolysate prepared from rice straw (Fig. 5). We consider the lower degradation efficiency seen with the other two hydrolysates was caused by the higher sugar concentration and the presence of aldehyde inhibitors. Although glucose is the best carbon source for bacterial growth, high glucose concentrations inhibit their growth (Kazana et al., 1995). In fact, OD₆₀₀ values were reduced in the presence of higher glucose concentrations

Fig. 6. Efficiency of ethyl red degradation using hydrolysate prepared from rice straw.

B. multivorans CCA53 was cultured in the hydrolysate containing 100 μ M ethyl red under the optimized culture conditions. Shown are the time-dependent changes in the OD₆₀₀ (circles) and the efficiency of ethyl red degradation (squares). Error bars indicate SE ($n = 3$).

(Table 2). In addition, two aldehyde inhibitors, furfural, and 5-hydroxymethyl furfural, also inhibited bacterial growth. These compounds cause DNA damage and inhibit several enzymes in the glycolytic pathway, which leads to a decrease in cell growth (Monlau et al., 2014). When hydrolysates were prepared from each lignocellulosic biomass, furfural and 5-hydroxymethyl furfural were present only in the hydrolysate prepared from eucalyptus. This likely explains why the lowest degradation efficiency was obtained with the eucalyptus hydrolysate (Table 2). We also confirmed the time-dependent changes in the degradation efficiency with hydrolysate from rice straw. We found that >91% of the degradation efficiency was achieved after incubation for 12 h, with consumption of 8.37 mM galactose, 5.24 mM glucose, 3.1 mM xylose, 1.53 mM arabinose and 0.64 mM mannose (Fig. 6 and Table 2).

In the present study, we developed a potentially economical method for degrading ethyl red using *B. multivorans* CCA53. This method has several noteworthy features. First, the mesophilic bacterium *B. multivorans* CCA53 exhibits the highest activity at 45°C, and it requires no intricate culture conditions. These characteristics would be expected to decrease utility costs, even with large scale preparations. Second, *B. multivorans* CCA53 is capable of simultaneous assimilation of several carbon sources. Carbon catabolite repression, which leads to selective carbon-source usage, is seen with several industrially useful bacteria, including *Corynebacterium glutamicum* and *Escherichia coli* (Görke and Stülke, 2008). Release from carbon catabolite repression is needed to enhance productivity. It appears this carbon catabolite repression is weak in *B. multivorans* CCA53, which is advantageous for fermentation with lignocellulosic hydrolysate and may encourage the promotion of green sustainable chemistry. Third, *B. multivorans* CCA53 is not genetically modified. When using genetically modified bacteria in industry, it is necessary to follow the regulatory guidelines for recombinant DNA experiments, such as the

Cartagena Protocol. These guidelines need not be applied to *B. multivorans* CCA53. On the other hand, a weakness of our method is the need to block the release of *B. multivorans* CCA53 into the atmosphere. Several *B. multivorans* species cause Cepacia syndrome in cystic fibrosis patients, which leads to pneumonic illness (Blackburn et al., 2004). With our method, therefore, a fermentation tank equipped with a high efficiency particulate air filter will be necessary to avoid infection by *B. multivorans* CCA53.

Conclusion

In this study, we have assessed ethyl red degradation using *B. multivorans* CCA53. This is the first report of an azo dye degradation method using a *B. multivorans* species. After investigating the effects of culture pH and temperature on ethyl red degradation, the optimized culture conditions were a culture pH of 7.5 and a culture temperature of 45°C. Under the optimized conditions, the efficiency of ethyl red degradation exceeded 99% after incubation for 8 h. Using quantitative RT-PCR and comparative sequence analysis, we investigated a correlation between ethyl red degradation and transcription of azoreductase genes. As the result, *B. multivorans* CCA53 would achieve its degradative capacity with two azoreductases, their activities may be enhanced under the optimized culture conditions. For a more practical approach, we performed ethyl red degradation using three kinds of hydrolysates as the medium. With hydrolysate prepared from rice straw, a degradation efficiency of >91% was confirmed after incubation for 12 h. We have thus constructed a potentially economical method for the biodegradation of ethyl red.

Acknowledgments

We are grateful to all members of the Bio-conversion Research Group at our Institute [Research Institute for Sustainable Chemistry, National Institute of Advanced Industrial Science and Technology (AIST)] for their technical assistance and valuable discussion.

Conflict of Interest

The authors declare that they have no conflict of interest.

Supplementary Materials

Supplementary figure is available in our J-STAGE site (<http://www.jstage.jst.go.jp/browse/jgam>).

References

- Akita, H., Kimura, Z., Yusoff, M. Z. M., Nakashima, N., and Hoshino, T. (2016a) Draft genome sequence of *Burkholderia* sp. strain CCA53, isolated from leaf soil. *Genome Announc.*, **4**, e00630-16.
- Akita, H., Kimura, Z., Yusoff, M. Z. M., Nakashima, N., and Hoshino, T. (2016b) Isolation and characterization of *Burkholderia* sp. strain CCA53 exhibiting ligninolytic potential. *SpringerPlus*, **5**, 596.
- Akita, H., Kimura, Z., Yusoff, M. Z. M., Nakashima, N., and Hoshino, T. (2017) Identification and characterization of *Burkholderia multivorans* CCA53. *BMC Res. Notes*, **10**, 249.
- Asad, S., Amoozegar, M. A., Pourbabae, A. A., Sarbolouki, M. N., and Dastgheib, S. M. (2007) Decolorization of textile azo dyes by newly isolated halophilic and halotolerant bacteria. *Bioresour. Technol.*, **98**, 2082–2088.
- Bardi, L. and Marzona, M. (2010) Factors affecting the complete mineralization of azo dyes. In *Biodegradation of Azo Dyes*, ed. by Erkurt, H. A., Springer, Heidelberg, pp. 195–210.
- Bi, W., Hayes, R. B., Feng, P., Qi, Y., You, X. et al. (1992) Mortality and incidence of bladder cancer in benzidine-exposed workers in China. *Am. J. Ind. Med.*, **21**, 481–489.
- Blackburn, L., Brownlee, K., Conway, S., and Denton, M. (2004) 'Cepacia syndrome' with *Burkholderia multivorans*, 9 years after initial colonization. *J. Cyst. Fibros.*, **3**, 133–134.
- Chang, H. K., Mohseni, P., and Zylstra, G. J. (2003) Characterization and regulation of the genes for a novel anthranilate 1,2-dioxygenase from *Burkholderia cepacia* DBO1. *J. Bacteriol.*, **185**, 5871–5881.
- Cui, D., Li, G., Zhao, D., Gu, X., Wang, C. et al. (2012) Purification and characterization of an azoreductase from *Escherichia coli* CD-2 possessing quinone reductase activity. *Process Biochem.*, **47**, 544–549.
- Denman, C. C., Robinson, M. T., Sass, A. M., Mahenthiralingam, E., and Brown, A. R. (2014) Growth on mannitol-rich media elicits a genome-wide transcriptional response in *Burkholderia multivorans* that impacts on multiple virulence traits in an exopolysaccharide-independent manner. *Microbiology*, **160**, 187–197.
- Fujimoto, S., Innoue, S., and Yoshida, M. (2018) High solid concentrations during the hydrothermal pretreatment of eucalyptus accelerate hemicellulose decomposition and subsequent enzymatic glucose production. *Bioresour. Technol. Rep.*, **4**, 16–20.
- Furkan, H. I. and Remzi, B. (2015) Lignocellulosic biomass: a sustainable platform for the production of bio-based chemicals and polymers. *Polym. Chem.*, **6**, 4497–4559.
- Görke, B., and Stülke, J. (2008) Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. *Nat. Rev. Microbiol.*, **6**, 613–624.
- Jin, X. C., Liu, G. Q., Xu, Z. H., and Tao, W. Y. (2007) Decolorization of a dye industry effluent by *Aspergillus fumigatus* XC6. *Appl. Microbiol. Biotechnol.*, **74**, 239–243.
- Jönsson, L. J., Alriksson, B., and Nilvebrant, N. O. (2013) Bioconversion of lignocellulose: inhibitors and detoxification. *Biotechnol. Biofuels*, **6**, 16.
- Kazana, D., Çamurdanb, A., and Hortaçsu, A. (1995) The effect of glucose concentration on the growth rate and some intracellular components of a recombinant *E. coli* culture. *Process Biochem.*, **30**, 269–273.
- Khan, R., Bhawana, P., and Fulekar, M. H. (2013) Microbial decolorization and degradation of synthetic dyes: a review. *Rev. Environ. Sci. Bio.*, **12**, 75–97.
- Kurade, M. B., Waghmode, T. R., Kagalkar, A. N., and Govindwar, S. P. (2012) Decolorization of textile industry effluent containing disperse dye Scarlet RR by a newly developed bacterial-yeast consortium BL-GG. *Chem. Eng. J.*, **184**, 33–41.
- Mohanty, S. and Mukherji, S. (2013) Surfactant aided biodegradation of NAPLs by *Burkholderia multivorans*: comparison between Triton X-100 and rhamnolipid JBR-515. *Colloid. Surface. B*, **102**, 644–652.
- Monlau, F., Sambusiti, C., Barakat, A., Quéméneur, M., Trably, E. et al. (2014) Do furanic and phenolic compounds of lignocellulosic and algae biomass hydrolyzate inhibit anaerobic mixed cultures? A comprehensive review. *Biotechnol. Adv.*, **32**, 934–951.
- Montanaro, F., Ceppi, M., Demers, P. A., Puntoni, R., and Bonassi, S. (1997) Mortality in a cohort of tannery workers. *Occup. Environ. Med.*, **54**, 588–591.
- Morikawa, Y., Shiomi, K., Ishihara, Y., and Matsuura, N. (1997) Triple primary cancers involving kidney, urinary bladder, and liver in a dye worker. *Am. J. Ind. Med.*, **31**, 44–49.
- Moutaouakkil, A., Zeroual, Y., Zohra Dzayri, F., Talbi, M., Lee, K. et al. (2003) Purification and partial characterization of azoreductase from *Enterobacter agglomerans*. *Arch. Biochem. Biophys.*, **413**, 139–146.
- Oancea, P. and Meltzer, V. (2014) Kinetics of tartrazine photodegradation by UV/H₂O₂ in aqueous solution. *Chem. Pap.*, **68**, 105–111.
- Ooi, T., Shibata, T., Matsumoto, K., Kinoshita, S., and Taguchi, S. (2009) Comparative enzymatic analysis of azoreductases from *Bacillus* sp. B29. *Biosci. Biotechnol. Biochem.*, **73**, 1209–1211.
- Pak, D. and Chang, W. (1999) Decolorizing dye wastewater with low temperature catalytic oxidation. *Water Sci. Technol.*, **40**, 115–121.

- Piolatto, G., Negri, E., La Vecchia, C., Pira, E., Decarli, A. et al. (1991) Bladder cancer mortality of workers exposed to aromatic amines: an updated analysis. *Br. J. Cancer*, **63**, 457–459.
- Qi, J., Schlömann, M., and Tischler, D. (2016) Biochemical characterization of an azoreductase from *Rhodococcus opacus* 1CP possessing methyl red degradation ability. *J. Mol. Catal. B Enzym.*, **130**, 9–17.
- Rafatullah, M., Sulaiman, O., Hashim, R., and Ahmad, A. (2010) Adsorption of methylene blue on low-cost adsorbents: a review. *J. Hazard Mater.*, **15**, 70–80.
- Rahman, Z., Shida, Y., Furukawa, T., Suzuki, Y., Okada, H. et al. (2009) Evaluation and characterization of *Trichoderma reesei* cellulase and xylanase promoters. *Appl. Microbiol. Biotechnol.*, **82**, 899–908.
- Saitou, N. and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*, **4**, 406–425.
- Sarkar, S., Banerjee, A., Halder, U., Biswas, R., and Bandopadhyay, R. (2017) Degradation of synthetic azo dyes of textile industry: a sustainable approach using microbial enzymes. *Water Conserv. Sci. Eng.*, **2**, 121–131.
- Sen, S. K., Raut, S., Bandyopadhyay, P., and Raut, S. (2016) Fungal decolouration and degradation of azo dyes: A review. *Fungal Biol. Rev.*, **30**, 112–113.
- Shinka, T., Miyai, M., Sawada, Y., Inagaki, T., and Okawa, T. (1995) Factors affecting the occurrence of urothelial tumors in dye workers exposed to aromatic amines. *Int. J. Urol.*, **2**, 243–248.
- Stolz, A. (2001) Basic and applied aspects in the microbial degradation of azo dyes. *Appl. Microbiol. Biotechnol.*, **56**, 69–80.
- Sugiura, W., Yoda, T., Matsuba, T., Tanaka, Y., and Suzuki, Y. (2006) Expression and characterization of the genes encoding azoreductases from *Bacillus subtilis* and *Geobacillus stearothermophilus*. *Biosci. Biotechnol. Biochem.*, **70**, 1655–1665.
- Tamura, K. and Nei, M. (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol.*, **10**, 512–526.
- Thalagala, T. A. T. P., Kodama, S., Mishima, T., Isono, N., Furujyo, A. et al. (2009) Study on ethanol fermentation using D-glucose rich fractions obtained from lignocelluloses by a two-step extraction with sulfuric acid and *Issatchenkia orientalis* MF 121. *J. Appl. Glycosci.*, **56**, 7–11.
- Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.*, **22**, 4673–4680.
- Tomic-Carruthers, N., Mahoney, N., Walters, M., Claus, J., and Tang, G. (2013) HPLC method for analysis of red dye from marked *Pectinophora gossypiella* (Lepidoptera: Gelechiidae). *Fla. Entomol.*, **96**, 1574–1578.
- Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C. et al. (2012) Primer3—new capabilities and interfaces. *Nucleic Acids Res.*, **40**, e115.
- Xu, Y. and Lebrun, R. E. (1999) Treatment of textile dye plant effluent by nanofiltration membrane. *Sep. Sci. Technol.*, **34**, 2501–2519.
- Yemashova, N. and Kalyuzhnyi, S. (2006) Microbial conversion of selected azo dyes and their breakdown products. *Water Sci. Technol.*, **53**, 163–171.