Journal of the Taiwan Institute of Chemical Engineers 42 (2011) 492–497

Contents lists available at ScienceDirect

Journal of the Taiwan Institute of Chemical Engineers

journal homepage: www.elsevier.com/locate/jtice

Short communication

Decolorization of synthetic dyes by *Citrobacter amalonaticus* Y19

You-Kwan Oh [a,*], Eun-Hee Seol [b], Soonchul Park [a], Sunghoon Park [b,*]

[a] Bioenergy Research Center, Korea Institute of Energy Research, Jang-dong 71-2, Yuseong-gu, Daejeon 305-343, Republic of Korea
[b] Department of Chemical and Biochemical Engineering, Pusan National University, Busan 609-735, Republic of Korea

ARTICLE INFO

Article history:
Received 4 May 2010
Received in revised form 3 August 2010
Accepted 16 August 2010
Available online 26 November 2010

Keywords:
Decolorization
Synthetic dye
Azo dye
Black B
Citrobacter amalonaticus

ABSTRACT

Newly isolated *Citrobacter amalonaticus* Y19 could decolorize various synthetic dyes containing different chromogenic groups including azo bond (Crocein Orange G, New Coccine, Chromotrope FB, Congo Red, Remazol Black B), anthraquinone (Reactive blue 2) and indigo (Indigo Carmine). Y19's specific degradation of Black B, due to its industrial importance and high decolorization activity, was studied in detail. Y19's decolorization rate, under anaerobic conditions and with glucose as the carbon source, was enhanced as the temperature was increased from 25 to 40 °C or as the initial dye concentration was increased from 25 to 2000 mg/L. The highest decolorization rate was estimated to be 171 mg dye/g cell h for an initial dye concentration of 2000 mg/L. High-performance liquid chromatograph (HPLC) and mass spectrum analyses indicated that Black B was degraded by reductive cleavage of the azo bond. However, one of the degradation products exhibited spontaneous oxidation under the aerobic conditions, and this resulted in an approximately 10% re-development of Black B's original color intensity. This is the first report of newly isolated *C. amalonaticus* Y19's efficiency as an azo dye-degrading microbe.

1. Introduction

Over 7×10^5 metric tons of synthetic dyes are produced worldwide every year (Yu *et al.*, 2001). These dyes have different chemical structures to meet various coloring requirements. They are usually classified by their chromophores, such as azo, anthraquinone and indigo. Azo dyes constitute the largest group, including over 10,000 commercial dyestuffs. During the dyeing process, up to 15% of the dye is lost to wastewater (Pegga and Brown, 1986). Dyes in wastewater are not easily removed from effluents, and thus pose a particularly serious pollution problem (Oh *et al.*, 2004).

Azo dyes can be degraded biologically by bacteria or fungi (Chen, 2006; Glenn and Gold, 1983). Bacterial degradation of azo dyes often is initiated by an enzymatic cleavage of the azo bond. This reaction is catalyzed by azoreductase, and requires NAD(P)H as an electron donor (Chen, 2006). Bacterial cells generally grow fast, and their decolorization rate also is very high. In comparison, fungal degradation by such a strain as *Phanerochaete chrysosporium* is mediated by lignin peroxidase under aerobic conditions (Glenn and Gold, 1983). Since fungal cells grow slowly and decolorize at a correspondingly slow rate, their practical application to dye degradation processes has been rather limited.

The availability of efficient bacterial strains is central to the development of cost-effective decolorization processes. There have been many decolorization studies undertaken, most commonly with various mono- and di-azo dyes. For example, Chang and Kuo (2000) reported that mono-azo dye Reactive Red 22 was decolorized by *Escherichia coli* NO3 at the high specific decolorization rate of 100 mg dye/g cell h for an initial dye concentration of 2000 mg/L. Yu *et al.* (2001) reported that the specific dye decolorization activity of *Pseudomonas* sp. (isolated from an anaerobic–aerobic dyeing house wastewater treatment facility) on another mono-azo dye, Acid Violet 7, was 150 mg dye/g cell h for an initial dye concentration of 100 mg/L. Chen *et al.* (2003) showed that, with within 8 days, *Aeromonas hydrophila* (isolated from sludge samples) could decolorize 3000 mg/L of mono-azo Reactive Red 198 by more than 90%. There have been several reports on decolorization of di-azo dyes. However, for such dyes, especially the structurally complex reactive Remazol Black B (also known as Reactive Black 5), the results are somewhat controversial. Oxspring *et al.* (1996) and Sponza and Isik (2002) have claimed that decolorization of Reactive Black 5 by an upflow anaerobic filter or upflow anaerobic sludge blanket reactor is 90–95% for the inlet dye concentration of 100 mg/L. Contrarily, Ganesh *et al.* (1994) and Panswad *et al.* (2001) have reported that the efficiency is only around 50%, when the inlet dye concentration is 10–80 mg/L, for an anaerobic digester or anaerobic/aerobic sequential batch reactor. Such discrepancies among the literature data have yet to be clarified.

* Corresponding authors. Tel.: +82 42 8603697; fax: +82 42 8603739.
E-mail addresses: ykoh@kier.re.kr (Y.-K. Oh), parksh@pusan.ac.kr (S. Park).

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doi:10.1016/j.jtice.2010.08.008

We have been studying a new H_2-producing, chemoheterotrophic bacterium, *Citrobacter amalonaticus* Y19 (Kim *et al.*, 2003). This strain produces H_2 not only from carbon monoxide by a water-gas shift type of reaction, but also from many carbohydrates by anaerobic fermentation (Oh *et al.*, 2003). Y19 is a facultative anaerobe having the high specific growth rate on glucose of 0.76 h^{-1}, producing H_2 over wide ranges of pH (5–9) and temperature (25–40 °C). Y19 has also shown a high volumetric H_2 production rate of 16 mmol H_2/L-h, along with production stability, during prolonged continuous fermentation of more than 20 days. Therefore, Y19 has been considered to be a potentially useful strain for the production of H_2 from carbon monoxide and various organic substrates (Oh *et al.*, 2008).

The purpose of the present study was the evaluation of Y19 as a color degrader. Various synthetic dyes with different chromophores including mono-azo, di-azo, anthraquinone, and indigo, were examined. The degradation of Remazol Black B was studied in detail, since it is commercially important in Korea and its decolorization, by Y19, is very extensive. The effects of such parameters as pH, temperature, glucose concentration, and dye concentration on the decolorization rate were studied quantitatively. Additionally, the decolorization products of Black B were analyzed by HPLC and LC–MS.

2. Materials and methods

2.1. Synthetic dyes

Seven synthetic dyes (Table 1) of purities ranging from 50 to 85% were purchased from Aldrich (Milwaukee, USA) and used as delivered. For convenience, in this paper the trade names are used.

2.2. Microorganism and culture conditions

C. amalonaticus Y19 was isolated from a sludge digester (Kim *et al.*, 2003) and used throughout this study. PFN mineral salt medium (Oh *et al.*, 2003) fortified with a phosphate buffer (pH 7.0) of 100 mM and containing 3 g yeast extract/L and 10 g glucose/L (“glucose mineral salt medium,” hereafter) was employed in growing the Y19. Cultivation was performed at 35 °C in a gyratory incubator with a shaking speed of 250 rpm. A 165 ml serum bottle (working volume: 50 ml) was used. After inoculation, the bottle was flushed with argon (Ar) gas (99.999%) for 5 min to develop anaerobic conditions, after which it was sealed with a 12 mm-thick butyl rubber septum and aluminum cap. The detailed procedures have already been published (Oh *et al.*, 2003, 2008).

2.3. Dye decolorization

The dye decolorization and H_2 production were measured with cells grown in a glucose mineral salt medium containing various synthetic dyes. The dye concentration was 50 mg/L, if not specified. The cells were harvested during the late-exponential growth period, washed once with a buffer solution (pH 7.0, 100 mM phosphate buffer), and placed in a 38 ml serum vial (working volume: 10 ml) to the amount of 1.04–1.23 mg dry cell/ml. The vial was then sealed with the butyl rubber septum and aluminum cap. All of the procedures excepting centrifugation were conducted in an anaerobic chamber (100% Ar gas atmosphere; Bactron 1.5, Sheldon Manufacturing Inc., Oregon, USA), and all of the buffer solutions and media were carefully flushed with Ar gas before use. Dye decolorization was monitored for 2 h while the reaction vials were shaken at 100 strokes/min in a water bath at 35 °C (if not specified). The specific dye decolorization activity (mg dye/g cell h) was determined from a plot of dye concentration vs. time. The extents of dye decolorization were measured after 24 or 48 h incubation. Also, control experiments were carried out on autoclaved cells in the presence of the dye. The specific H_2 production activity was determined after 24 h incubation. In determining the pH dependence of the decolorization activity, several different buffer solutions were used, as follows (all in 100 mM): acetate buffer (pH 5.0), phosphate buffer (pH 6.0, 7.0, and 8.0), and Trizma base buffer (pH 9.0). The assays were performed in triplicate and the results were averaged.

2.4. Analyses

The cell concentrations were measured by a spectrophotometer (Lambda 20, PerkinElmer, Norwalk, CT, USA) (Oh *et al.*, 2003). The dye concentrations were determined spectrophotometrically at the visible absorbance maxima of the respective dyes (refer to Table 1) after removing the cells by centrifugation. The H_2 contents were measured by a gas chromatograph (GC) equipped with a thermal conductivity detector (Oh *et al.*, 2008). Black B and its degradation products were analyzed using a high-performance liquid chromatograph (HPLC, 1100 series, Agilent Technologies, Forster, CA, USA) equipped with a diode array detector (DAD) and a mass selective detector (MSD), after purifying the samples with a 0.45 μm disposable filter unit. A C_{18} (200 mm × 4.6 mm, particle size 5 μm) reverse-phase column (ODS-Hypersil, Agilent Technologies, Forster, CA, USA) also was employed. The mobile phase was changed as follows: water for the initial 10 min was varied linearly to a 9:1 (v/v) mixture of water–methanol for 40 min, and then varied linearly to pure methanol for the next 10 min. Always when water was used as the eluent, ammonium acetate (1 mM) was added to the water. The flowrate of the eluent was 0.3 ml/min, and

Table 1
Decolorization of synthetic dyes by *C. amalonaticus* Y19.

Dye[a]	Chromophore	λ_{max} (nm)	Specific dye decolorization activity (mg dye/g cell h)[b]	Decolorization extent (%)[c]	Relative H_2 production activity (%)[d]
Crocein Orange G	Mono-azo	482	5.57 ± 0.98	97.0 ± 0.1	98.6 ± 0.8
New Coccine	Mono-azo	506	6.12 ± 0.08	97.2 ± 0.3	98.4 ± 0.6
Chromotrope FB	Mono-azo	515	9.38 ± 0.17	88.1 ± 0.3	97.2 ± 1.2
Congo Red	Di-azo	497	10.7 ± 0.47	91.2 ± 3.3	94.4 ± 2.1
Remazol Black B	Di-azo	600	8.67 ± 0.23	98.5 ± 1.2	94.6 ± 4.1
Reactive Blue 2	Anthraquinone	607	3.75 ± 0.37	48.3 ± 0.9	99.1 ± 0.8
Indigo Carmine	Indigo	608	1.02 ± 0.16	12.5 ± 6.9	96.7 ± 1.1

The ± sign represents the standard deviation.
[a] The dye concentration was 50 mg/L.
[b] The initial decolorization rate during 2 h incubation was determined.
[c] The decolorization extent was determined after 48 h incubation.
[d] The H_2 production activity was determined after 24 h incubation and its relative activity was expressed as the % activity of the activity obtained without dye. 100% activity corresponds to 0.74 ± 0.07 mmol H_2/g cell h.