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Decolorization by *Caldicellulosiruptor saccharolyticus* with dissolved hydrogen under extreme thermophilic conditions

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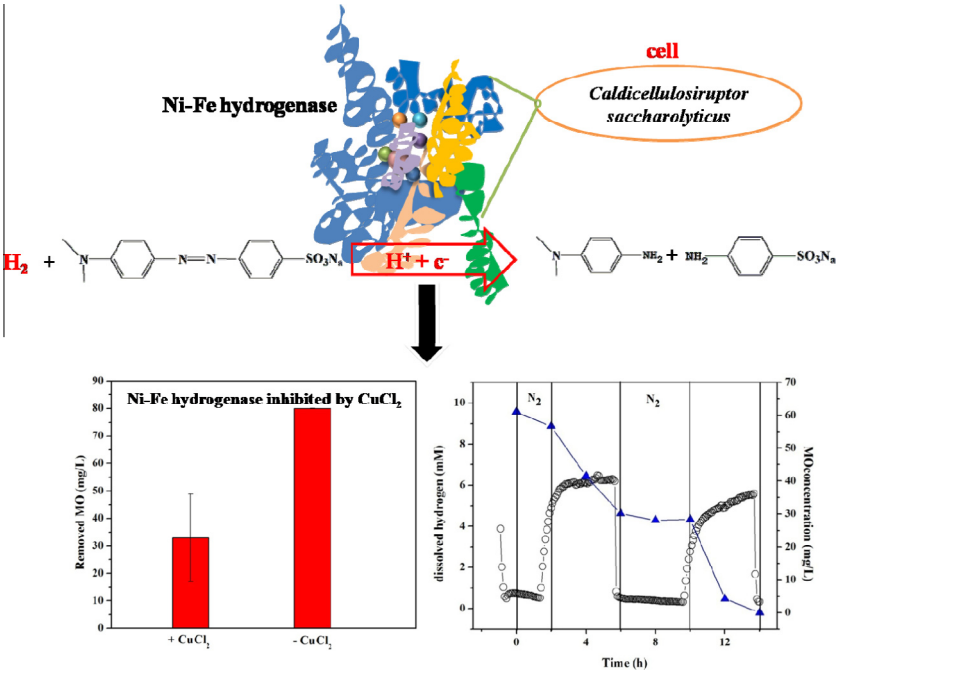
HIGHLIGHTS

- *Caldicellulosiruptor saccharolyticus* could degrade methyl orange effectively.
- The decolorization happened enzymatically via dissolved hydrogen (DH).
- The decolorization rate was positively related to DH and Ni–Fe hydrogenase.
- Competition for hydrogen existed between decolorization and glucose fermentation.

GRAPHICAL ABSTRACT



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ABSTRACT

This study examined the decolorization ability of *Caldicellulosiruptor saccharolyticus* isolated from a thermal spring at the optimum growth temperature of 70 °C. This study demonstrated, for the first time, *C. saccharolyticus* could effectively degrade methyl orange (MO) to 4-aminobenzenesulfonic acid (4-ABA) and *N*′,*N*-dimethyl-*p*-phenylenediamine (DPD) with dissolved hydrogen (DH) as the reducing equivalent. The decolorization reaction was catalyzed by Ni–Fe hydrogenase and the reaction rate was correlated positively to the concentration of DH. At high concentrations of DH the decolorization rates were 6.65 and 7.08 mg/L/h, which decreased to 2.16 and 0.88 mg/L/h after purging with N_2. Furthermore, the addition of MO decreased the yield of ethanol via glucose fermentation owing to the limited reducing

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Caldicellulosiruptor saccharolyticus
Dissolved hydrogen
Fermentation

equivalents. We suggest the competition for hydrogen between the reduction of azo dyes and hydrogenotrophic methanogenesis processes might exist in mixed culture fermentation.

1. Introduction

The majority of widely used dyes are azo dyes, which can be decolorized by bacteria under anaerobic conditions [1,2]. The azo dye is reduced by the reducing equivalents produced during metabolic processes, including glucose fermentation; most azo dye decolorization processes are associated closely with fermentation [3,4]. In those processes, the reducing equivalents are transferred to the azo dye directly or indirectly, and interact with the azo dyes chemically or enzymatically. For example, sulfide produced via sulfate respiration by sulfate reducing bacteria can decolorize azo dyes chemically [3,5]. The enzymes involved in the decolorization process include unspecific or specific azoreductases and hydrogenases, e.g. Ni–Fe hydrogenase [6–9]. The results of earlier studies suggested the reduced flavins generated by flavin reductases are responsible for the unspecific reduction of azo dyes [7]. And a recent study suggested the electron transport chain of MtrA-MtrB-MtrC might be a major avenue for delivering electrons from the outer membrane surface to the azo dyes [10]. There is no report, however, on how the hydrogenase is involved specifically in the dye decolorization process [8].

Ni–Fe hydrogenase is involved in both uptake and release of H_2 [11]. When dissolved hydrogen (DH) is reduced by Ni–Fe hydrogenase, the electrons generated can be transferred to other electron acceptors, such as heavy metals. For example, the reduction of Pd by *Escherichia coli* is catalyzed by three hydrogenases [12]. Another study reported extracellular iron reduction of neutral red by *E. coli*, mediated by H_2 and with the aid of hydrogenases [9]. H_2, a common intermediate product of fermentation, is a good reductant and is consumed for the reduction of heavy metals with the aid of Ni–Fe hydrogenase. Thus it might be involved in the decolorization of azo dyes in the same way. The function of hydrogenase in the decolorization process, however, is not fully understood [8,13]. By contrast, H_2 supersaturation is widely observed in mixed culture fermentation [14,15]; thus, DH in liquid is more meaningful than H_2 in the headspace to represent hydrogen for the metabolic process.

Caldicellulosiruptor saccharolyticus isolated from a thermal spring is an extreme thermophilic fermentation bacterium with an optimum growth temperature of 70 °C. *C. saccharolyticus* can use (poly)saccharides for growth and has high yields of H_2 (up to 3.5 mol H_2/mol glucose) [16]. More importantly, it contains Ni–Fe hydrogenase bound onto the cell membrane [17]. With these properties, *C. saccharolyticus* is expected to decolorize azo dyes; however, this has not been reported. More importantly, although most textile wastewater is discharged at high temperature (50–80 °C), only a few studies have investigated the azo dye reduction under thermophilic conditions [3,4]. And how the biodegradation proceeds in situ in a naturally high-temperature environment, such as a thermal spring, should be elucidated. As extreme thermophilic fermentation (70 °C) can offer the advantages of high substrate degradation rate, high yield of H_2 and efficient heat utilization of wastewater, it is important to study the decolorization ability of *C. saccharolyticus* under extreme thermophilic conditions.

The objective of this study was to examine the decolorization of azo dyes by *C. saccharolyticus* during glucose fermentation at 70 °C and to elucidate the underlying mechanism. Methyl orange (MO), was chosen as a typical azo dye. The removal of MO by *C. saccharolyticus* was determined first and then the reaction of DH with MO was studied. Last, the mechanism of decolorization of MO by *C. saccharolyticus* was analyzed. The results of this study will enhance our understanding of the decolorization of azo dyes, especially by the enzymatically catalyzed process.

2. Materials and methods

2.1. Microorganism and growth medium

A pure culture of *C. saccharolyticus* (DSM 8903) was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) and cultured in DSM640-medium without cysteine, trypticase and $FeCl_3$.

2.2. Experimental setup

2.2.1. Batch experiment setup

Anaerobic decolorization of MO by *C. saccharolyticus* was tested in 165 mL serum bottles containing 60 mL DSM640-medium. Two sets of experiments were done, with different concentrations of glucose (1 and 4 g/L). In the 4 g/L glucose experiment, different concentrations of MO (50, 100 and 200 mg/L) were tested. After purging with N_2 gas to ensure anaerobic conditions within the bottles, they were sealed with butyl rubber stoppers and aluminum caps, autoclaved at 105 °C for 20 min and then incubated at 70 °C. After the temperature reached 70 °C, each bottle was inoculated with bacteria in the exponential growth phase to a final optical density at 620 nm (OD_{620}) of 0.03. MO was added when OD_{620} reached 0.2 after incubation for 10 h. Each experiment was done in triplicate.

2.2.2. H_2 effects in MO decolorization

In order to verify whether H_2 was the electron donor for MO reduction, hydrogen was supplied instead of glucose. The anaerobic serum bottles containing 30 mL medium were purged with a 3:2 (v/v) H_2/CO_2 mixture. *C. saccharolyticus* cells in the exponential growth phase were harvested by centrifugation at 6000×*g* for 8 min. Harvested cells were washed with medium and added into the anaerobic serum bottles until the OD_{620} value was 1.0. MO (50 mg/L) was added into a bottle with 30 mL cell suspension and $CuCl_2$ was added (final concentration 0.1 mM) as a Ni–Fe hydrogenase inhibitor.

All experiments involving DH were done in a reactor monitored by a membrane inlet mass spectrometer (MIMS; Hiden HPR-40 DSA, UK). The total volume of the reactor was 3.0 L and the working volume was 2.0 L.

2.3. Analysis

Concentrations of DH were determined by MIMS, which was calibrated by a standard calibration method [14]. Briefly, the recorded mass to charge (m/z) ratio of H_2 was 2 for the MIMS signal. The exact H_2 partial pressures were determined by gas chromatography (GC) when the MIMS signals in liquid were stable and the standard calibration curve between H_2 partial pressures and MIMS signals was obtained. H_2 was sampled with a gas-tight syringe (SGE Analytical Science, Australia) and the concentration