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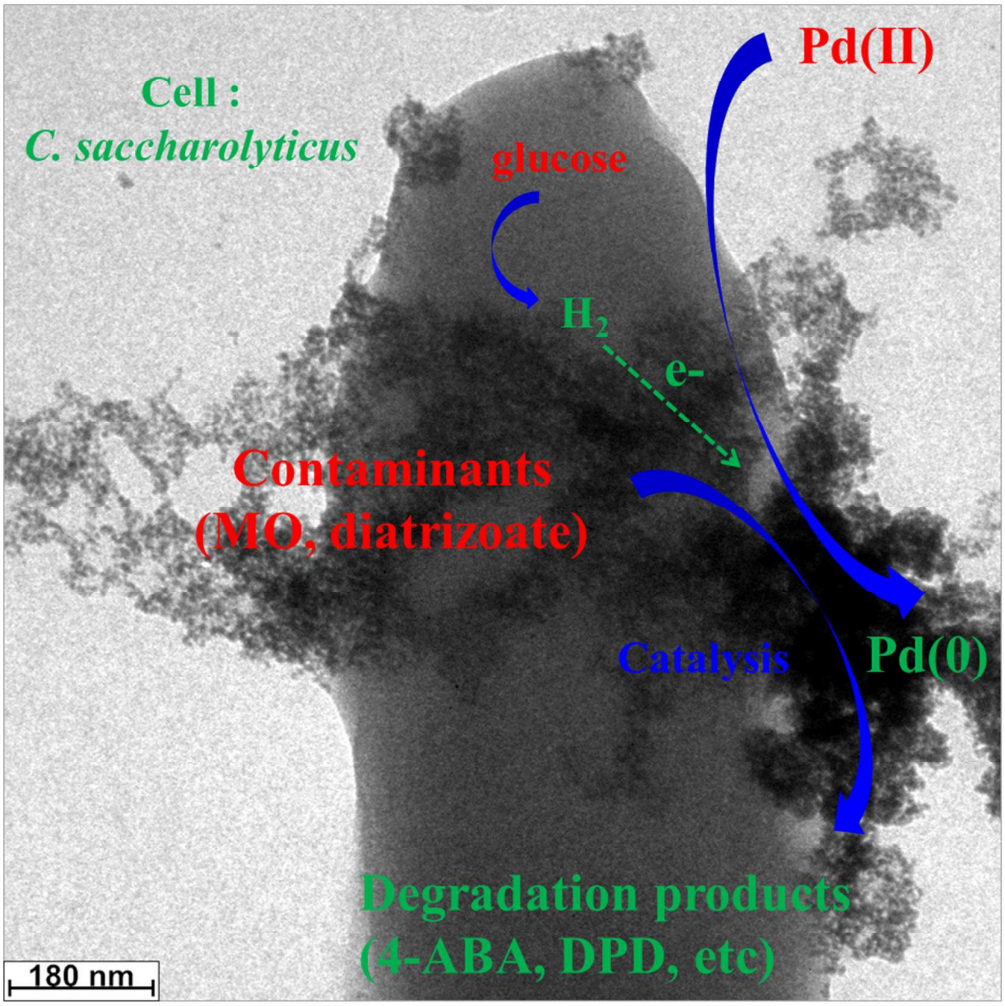
Graphical Abstract



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Palladium nanoparticles produced and dispersed by *Caldicellulosiruptor saccharolyticus* enhance the degradation of contaminants in water

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Abstract

This study focused on examining the general applicability of coupling bio-palladium (Pd) nanoparticle generation and bio-H_2 produced by *Caldicellulosiruptor saccharolyticus* for wastewater treatment under extreme thermophilic conditions. Na_2PdCl_4 was added to cell cultures to achieve a final Pd concentration of 50 mg/L. Methyl orange (MO) and diatrizoate were chosen as the contaminants in water. In the cultures with, and without, Pd added, MO (100 mg/L) was degraded within 30 min and in over 6 h, respectively. Diatrizoate (20 mg/L) was degraded within 10 min in Pd-added cultures. However, no diatrizoate degradation happened without Pd addition. The degradation rates were correlated positively with dissolved hydrogen generated by *C. saccharolyticus*. Furthermore, the catalytic actions of Pd(0) nanoparticles and cells were distinguished during the degradation process. MO was degraded under the combined action of Pd(0) and hydrogenase. However, Pd(0) was the essential catalyst, and hydrogenase had no effect on the deiodination of diatrizoate within 20 min. Pd(0) particles were dispersed well by the cells of *C. saccharolyticus* and showed a better catalytic activity than Pd(0) formed without cells. Dissolved hydrogen produced by *C. saccharolyticus* should be the perfect reduction equivalent for Pd formation and for reducing degradation. Therefore, Pd should be added to *C. saccharolyticus* cultures to enhance the degradation of contaminants in water.

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1. Introduction

Nanoparticles (NPs) of Pd(0) have a high catalytic activity in a standard hydrogenation reaction [1]. Conventional production methods of these NPs require the use of a series of toxic and expensive chemical agents, such as $NaBH_4$ and H_2. Both stabilizers and carrier materials are needed to prevent the particles from aggregating in a solvent. These materials would be released and pollute the environment, resulting in an overall increase in cost. However, without these materials, Pd(0) could be formed in bulk instead of the nanoscale. It is known that bulk Pd(0) has a lower catalytic activity compared to NPs [2]. Nanopalladium catalysts can also be synthesized by the precipitation of Pd on the surface of bacteria, leading to the production of biogenic Pd nanoparticles [3]. This synthesis process is considered a more 'green' or environment-friendly, low-cost technique. Hence, there is growing interest in synthesizing metal NPs by biological methods.

Bio-Pd(0) formation and hydrogenation reactions need the addition of an external electron donor, such as hydrogen, in the biosystem. Hydrogen is consistently identified as one of the most reactive electron donors. To avoid the safety concerns with the use of hydrogen and to reduce the cost of synthesis, many investigators prefer to use hydrogen produced by bacteria *in situ*. Thus, several strains of hydrogen-producing bacteria were investigated in the reduction of Pd(II) or other metals [4-6]. Hennebel et al. studied how bacteria could be used to produce Pd(0) under fermentative conditions. *In situ* hydrogen produced by bacteria was coupled to the formation of Pd(0). Then Pd(0) was tested for the ability to dehalogenate the

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recalcitrant aqueous pollutants, diatrizoate and trichloroethylene [6]. Those studies were all under mesophilic conditions. Whether extreme thermophilic bacteria have the same or better ability to reduce metals using *in situ* hydrogen is not known. How Pd(0) particles are formed and act under the extreme thermophilic conditions has also not been reported. Other studies have reported that the biochemical reaction rate of anaerobic digestion is higher at high temperatures [7, 8]. Thus, in this study, the degradation rate of contaminants and the catalytic activities of Pd(0) particles under extreme thermophilic conditions and mesophilic conditions were compared.

Caldicellulosiruptor saccharolyticus, isolated from thermal springs, is an extreme thermophilic fermentation bacterium with an optimum growth temperature of 70 ℃. *C. saccharolyticus* can use saccharides or polysaccharides for growth, and has high hydrogen yields (up to 3.5 mol H_2/mol glucose) [9]. More importantly, it contains Ni-Fe hydrogenase bound to the cytoplasmic membrane [10]. Ni-Fe hydrogenase is involved in both the uptake and release of H_2 [11]. When dissolved hydrogen is reduced by Ni-Fe hydrogenase, the generated electrons could 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]. Others have reported the extracellular iron reduction of neutral red by *E. coli*, mediated by hydrogen and with the aid of a hydrogenase [13]. With these properties (hydrogen production and hydrogenase), *C. saccharolyticus* is expected to produce Pd(0) from Pd(II).

In this study, the *in situ* application of hydrogen produced by *C. saccharolyticus* was investigated under extreme thermophilic conditions. Methyl orange (MO) and

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diatrizoate were chosen as the contaminants in water. This study aimed at getting Pd(0) out of Pd(II), and studying the catalysis in decolorization and deiodination. First, it was verified that Pd(0) was formed under the action of *in situ* hydrogen produced by *C. saccharolyticus*. Second, the effects of Pd(0) on enhancing decolorization and deiodination were studied. Third, the catalysis of Pd(0) and hydrogenase in the processes of decolorization and deiodination was distinguished. Furthermore, the role of cells in dispersion was confirmed.

2. Materials and methods

2.1. Microorganism and growth medium

Pure culture of *C. saccharolyticus* (DSM 8903) was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) and was cultivated in the DSM640-medium as described previously without supply of cysteine, trypticase and $FeCl_3$ [14]. Glucose was supplied as the carbon source.

2.2. Experimental setup

2.2.1. Batch experiment setup: with glucose

The experiments were conducted in 165-mL serum bottles with 60 mL DSM640 medium. After being purged with N_2 gas to ensure anaerobic conditions, the serum bottles were sealed with butyl rubber stoppers and aluminum caps, sterilized by autoclaving under 105 °C for 20 min and then incubated at 70 °C. After reaching 70 °C, each bottle was inoculated with the microbial culture, which was in its exponential growth phase, to a final optical density (OD_{620}) of 0.03. Two groups of

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experiments, one with Pd(II) added and the other without Pd(II), were conducted at the same time. The experiments were carried out in triplicate. Normally, a Na_2PdCl_4 stock solution (500 mg Pd/L) was used to achieve a final Pd(II) concentration of 50 mg/L. Then 100 mg/L of MO or 20 mg/L of diatrizoate were added into the two groups, respectively. When Pd(II) was reduced to Pd(0) through the action of hydrogen produced by *C. saccharolyticus*, the effect of Pd(0) was investigated.

2.2.2. Batch experiment setup: without glucose

To confirm the reducing action of hydrogen produced by *C. saccharolyticus,* and distinguish the catalytic action of Pd(0) and cells, glucose was removed from the medium. Hydrogen or nitrogen was bubbled into the DSM640 medium without glucose. Thus, four groups of experiments were conducted at the same time: (1) 60% H_2 and Pd(II); (2) 60% H_2, cells and Pd(II); (3) N_2 and Pd(II); (4) N_2, cells and Pd(II). After autoclaving, 50 mg/L of Pd(II) was added into the serum bottles. Thirty minutes later, cells in the logarithmic growth phase were centrifuged (6000×g for 8 min) and collected, and then re-suspended into the medium.

The difference in the catalytic activity between Pd(0) particles produced with and without cells was investigated in three groups: (1) H_2 and Pd(0); (2) H_2 and inactivated cells; (3) H_2, activated cells and Pd(0). The headspace contained 60% H_2 and 40% N_2. Harvested cells were washed with medium and added into the anaerobic serum bottles until the OD_{620} value was 1.0.

2.3. Pd reduction and nanopalladium observation

The concentration of Pd(II) was determined by atomic absorption spectrometry

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(AAS, Shimadzu AA-360, Kyoto, Japan). Palladium particles produced by hydrogen without cells were observed directly by scanning electron microscopy (SEM, JSM-6700F, JEOL Co., Japan). The palladium particles produced in the presence of cells were fixed in 5% glutaraldehyde for 12 h at 4 °C, and then dehydrated in increasing concentrations of ethanol (30%, 50%, 70%, 80%, 95% and 100%) for 15 min in each, and freeze-dried. After the samples were prepared, they were observed by SEM. Transmission electron microscopy combined with energy dispersive spectrometry (TEM-EDS) analysis was also performed to observe the morphology of the cells and particles, and their distribution.

2.4. Chemical analysis

Concentrations of residual MO and the intermediates of MO decolorization were determined using a HPLC system (LC-1100, Agilent Inc., USA) equipped with a UV detector and a Hypersil ODS C18 column. Methanol (solution A) and water with 0.1% (v/v) acetic acid and 0.1% (w/v) ammonium acetate (solution B) were used as the isocratic mobile phase. The gradient elution program was as follows: 5% (v/v) solution A and 95% (v/v) solution B at 0 min; 20% solution A and 80% solution B at 15 min; 100% solution A at 30 min; 5% solution A and 95% solution B at 38 min. The flow rate was 0.8 mL/min, the wavelength of the UV detector was set at 450 nm and 254 nm, and 20 μL of sample were injected for analysis.

The concentration of diatrizoate was monitored by HPLC (LC-1100, Agilent Inc., USA) equipped with a UV detector and a Hypersil ODS C18 column. Elution was performed isocratically at 25 °C and at a flow rate of 1 mL/min with 5% solvent A

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(100% acetonitrile) and 95% solvent B (0.1% formic acid). The injection volume was 50 μL, and the elution program ran within 15 min.

Hydrogen was sampled by a gas-tight syringe (SGE Analytical Science, Australia) and the hydrogen concentration was determined by a GC (Lunan model SP7890, CN) equipped with a thermal conductivity detector and a 1.5-m stainless steel column packed with a 5-Å molecular sieve. The temperatures of the injector, detector, and column were kept at 80, 100, and 50 °C, respectively. N_2 was used as the carrier gas.

3. Results and discussion

3.1. Pd nanoparticle formation

The reduction of Pd(II) and Pd(0) nanoparticle formation could be confirmed by the results of AAS, TEM images and EDS (Fig. 1). The results of AAS showed that Pd(II) disappeared completely from the solution (data not shown). The color change of the medium, from pale yellow to black, indicated that Pd(0) nanoparticles formed in the system. EDS analysis of the point marked in the TEM image indicated that Pd(0) was indeed formed in this system. This result also illustrated that hydrogen, as a reducing power produced by *C. saccharolyticus*, could reduce Pd(II) to Pd(0) via the action of hydrogenase, as reported by others [6, 12, 15]. Furthermore, the TEM image illustrated that Pd(0) particles were formed and that the diameter of the Pd(0) particles was around 10–20 nm. Most of the particles were distributed around the cells.

Initially, the Pd(II) ions spread over the cell culture including the extracellular polymeric substances (EPS) of *C. saccharolyticus*. EPS are high-molecular-weight

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polymers that are secreted by microorganisms into the surrounding environment [16]. Many particles could hide in the EPS. Hydrogen produced by *C. saccharolyticus* escaped from the cytomembrane, and then reached the EPS and the bulk solution. Then Pd(0) formed with the aid of hydrogen. It is possible that the EPS facilitated the dispersion of the Pd(0) particles. Thus the Pd(0) particles could disperse well without the addition of a chemical dispersant. The EPS prevented the aggregation of the Pd(0) particles. Conventional production methods of Pd(0) particles required the use of a series of toxic or expensive chemical agents, such as stabilizers and reductant [6, 17]. Thus, our study has shown that EPS can act as an environmental bio-dispersant, and that hydrogen is produced *in situ* by *C. saccharolyticus*.

3.2. Enhanced decolorization and dehalogenation

The *in situ* application of the Pd(0) particles and hydrogen produced by *C. saccharolyticus* was investigated in cases of decolorization and dehalogenation. MO (100 mg/L) was added into all the batches with and without Pd(0) particles. The rates of decolorization with Pd(0) particles were higher than that without Pd(0) particles (Fig. 2). MO was completely removed within 30 min in the group with Pd(0) particles. However, more than 6 h were required to completely remove 100 mg/L of MO in the group without Pd(0) particles. To eliminate the possible effect of adsorption by Pd(0) particles, the intermediates of MO decolorization were determined. 4-aminobenzenesulfonic acid (4-ABA) and N',N-dimethyl-p-phenylenediamine (DPD) were produced as the intermediates during the decolorization of MO. The concentrations of both 4-ABA and DPD increased with the decrease of MO (Fig. 2a,

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b). As shown in Fig. 2b, 100 mg/L of MO (0.30 mM) disappeared completely within 30 min, which corresponded to the stoichiometric appearance of 50 mg/L 4-ABA (0.29 mM) and 40 mg/L DPD (0.29 mM). This result demonstrated that MO was reduced rather than adsorbed by cells and Pd(0) particles.

The complete decolorization in the group without Pd(0) particles was realized with the combined action of hydrogen and hydrogenase. This phenomenon was confirmed in our previous study [18]. In the group with Pd(0) particles, the complete decolorization was the combined effects of hydrogen, hydrogenase and Pd(0) particles. The nanopalladium enhanced the decolorization of MO. MO decolorization driven by cells alone needed longer time as other researchers reported. For example, MO (100 mg/L) was completely removed within 7 h by *S. oneidensis* MR-1 [19]. A 95% decolorization by *P. luteola* was observed at 6 h and 9 h for 100 and 350 mg/L MO, respectively [20].

Pd(0) particles have been reported frequently as the catalyst of the reduction of halides, such as trichloroethylene (TCE), chlorophenols and diatrizoate [21-24]. This study chose diatrizoate as the halide to test the catalytic activity of Pd(0) particles in extreme thermophilic condition. As shown in Fig. 3, 20 mg/L of diatrizoate disappeared within 10 min in the two groups with Pd(0) particles during different time slot. However, in the group without Pd(0) particles diatrizoate was not reduced within 20 min. This result indicates that the catalytic activity of Pd(0) particles was essential in the deiodination, whereas cells had no catalytic effect. Furthermore, the rate of deiodination was slightly higher when the reaction of deiodination started at 20 h

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compared to 10 h. The time span, i.e. 10 and 20 h, was calculated from the inoculation time. The hydrogen content in the headspace was 16 mL at 20 h and 10 mL at 10 h. From the Michaelis-Menten model, it can be seen that the enzymatic catalytic reaction rate is positively related to the concentrations of catalyst and substrate. In this study, the catalyst was Pd(0) particles and the substrate was hydrogen. Thus, higher concentrations of hydrogen accelerated the rate of deiodination.

The rate of deiodination observed in this present study was higher than that reported in a study of *Citrobacter braakii* [6], which was also able to reduce Pd(II) to Pd(0) through hydrogen produced *in situ* under mesophilic conditions. The same amount of Pd(II) (50 mg/L) was added into both systems. More hydrogen (1.31 mmol vs. 0.6 mmol in our study) was produced under mesophilic conditions when the reaction of deiodination started. However, it took over 30 min to remove 20 mg/L of diatrizoate in the study of *C. braakii* [6], whereas in the present study it took only 10 min. It is likely that temperature played an important role in enhancing the deiodination process in our experiments. Thus, extreme thermophilic conditions should be beneficial to deiodination. It is also possible that the extreme thermophilic fermentation bacterium *C. saccharolyticus* performed better in coupling bio-Pd nanoparticle generation and bio-H_2 for the deiodination process than many mesophilic bacteria reported previously.

3.3. Catalysis of hydrogenase and/or Pd(0)

In our previous study, we found that hydrogen could decolorize MO with the action of hydrogenase [18]. Pd(0) enhanced the degradation of MO and also had the

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catalytic activity of hydrogenation, as in the deiodination of diatrizoate. What role did hydrogenase and Pd(0) play in, respectively, decolorization and deiodination? The role of hydrogenase and Pd(0) in decolorization and deiodination was determined in this study. Glucose was removed from the medium, and four groups of experiments were conducted at the same time. As shown in Fig. 4a, MO (50 mg/L) was removed completely within 30 min in the group with H_2, cells and Pd(0), as the concentration of MO was measured only at 30 min. The disappeared MO (50 mg/L) corresponded to the appearance of 29 mg/L 4-ABA and 23 mg/L DPD, indicating the completed degradation of MO, rather than adsorption by Pd(0). However, only 25 mg/L of MO was removed in the group with H_2 and cells within 30 min. In the group with N_2, cells and Pd(II), only 10 mg/L of MO was removed, which was a result of adsorption. In the group with N_2 and Pd(II), there was almost no reduction of MO. Pd(II) could not be reduced to Pd(0) in all the batches without hydrogen in our test. Thus, there was no catalytic activity of Pd(0) in both groups without hydrogen. It was speculated that about 20% MO was adsorbed by cells and 30% was catalyzed by hydrogenase, which was comparable to the catalysis of Pd(0) and hydrogenase within 30 min. Thus, Pd(0) greatly enhanced the decolorization of MO, and the catalytic activity of hydrogenase and the adsorption of cells could be instead by the result of the catalytic effect of Pd(0) particles, because the catalytic activity of Pd(0) particles was sufficiently powerful to completely remove 50 mg/L of MO within 30 min.

Fig. 4b shows that 20 mg/L of diatrizoate was removed within 5 min in the group with H_2, cells and Pd(0). Diatrizoate was not degraded in the groups without Pd(0)

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formation. About 3 mg/L of diatrizoate was removed in the group with N_2, cells and Pd(II), resulted from adsorption to cells within 20 min. This phenomenon indicated that Pd(0) was the essential catalyst, and that hydrogenase had no effect in the deiodination process within 20 min. In other studies, diatrizoate could be effectively degraded solely by Pd(0). For example, the removal of 20 mg/L diatrizoate by a 10 mg/L Pd suspension and 100% hydrogen was completed after 4 h [21]. However, in our study, 50 mg/L Pd and 60% hydrogen were added. The degradation rate was different, as the degradation rate of diatrizoate was related positively to the dosage of Pd(0) and hydrogen. Furthermore, the temperature were different in two studies. Compared with the catalytic effect in the batches with glucose in the previous section (Fig. 3), it can be seen that the catalytic effect was better in the batches without glucose in this section, because the content of hydrogen in the headspace in the batches with glucose was between 0.45 and 0.71 mM, and was around 2.68 mM in the batches without glucose. Hydrogen was filled artificially in this section, whereas hydrogen was produced by cells in the batches with glucose in the previous section. This could also be explained by the Michaelis-Menten model [18].

3.4 Dispersive action of cells on Pd(0) particles

The difference in size and catalytic activity between Pd(0) particles produced with and without cells was also investigated in this study. Cells were sterilized after Pd reduction to eliminate the effect of hydrogenase. This design aimed to confirm the dispersive action of cells. The Pd(0) particles aggregated together and formed a bulk in the system without dispersant (Fig. 5a, b). The diameter of the Pd(0) particles

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formed under this condition (chemical-Pd(0)) was between 1 and 2 μm. By contrast, in the system with the cells as a 'green' dispersant, the Pd(0) particles could be finely dispersed (Fig. 5c, d). The diameter of most Pd(0) particles formed in the presence of cells was under 100 nm. These Pd(0) particles were polyporous and homogeneous. Previous research on EPS offered a sufficient theoretical foundation for this study [25-28]; that is, the EPS of *C. saccharolyticus* could adsorb the Pd(0) particles and prevent them from aggregating together. The Pd(0) particles in the system with cells had a smaller size and a larger specific surface area compared with the Pd(0) particles in the system without cells. Thus the Pd(0) particles in the system with cells should theoretically have a higher catalytic activity. The following experiments with these systems, i.e. with and without cells, provided an answer.

MO was chosen as the contaminant to verify the catalytic activity of the Pd(0) particles in three groups. As shown in Fig. 6a, only 25 mg/L of MO was removed within 15 min in the group with H_2 and Pd(0) (Fig. 6a), and the tendency of the intermediates is shown clearly in Fig. 6b, indicating the degradation of MO. Fig. 6c shows that there were no intermediates detected in the group with H_2 and cells. This result indicates that reduced MO was not degraded but adsorbed by the cells. Not more than 20 mg/L of MO was adsorbed by cells within 30 min, which indicates that the adsorption by the cells played only a small role. However, 100 mg/L of MO was removed completely within 15 min in the group with H_2, cells and Pd(0), and the MO was degraded to 4-ABA and DPD (Fig. 6d). These results confirmed that Pd(0) particles formed in the group with cells had a higher catalytic activity in the

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decoloration of MO. The cells of *C. saccharolyticus* were 'green' dispersants in this system.

4. Conclusions

Pd(0) particles were produced from Pd(II) with *in situ* hydrogen generated by *C. saccharolyticus* during glucose fermentation. The degradation of MO and diatrizoate were both enhanced by Pd addition. The removal of MO was the result of the combined action of hydrogen, hydrogenase and Pd(0) particles. However, Pd(0) particles played an essential role in the removal of diatrizoate. Furthermore, the Pd(0) particles were well dispersed by cells of *C. saccharolyticus* and showed a better catalytic activity than chemical Pd(0) without dispersant. Generally speaking, the addition of Pd would enhance the degradation of contaminants in water.

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A list of figures

Fig. 1 a, b: TEM image of the Pd(0) particles and cells in *C. saccharolyticus* with glucose supplied; c: EDS analysis of the point marked in the TEM image.

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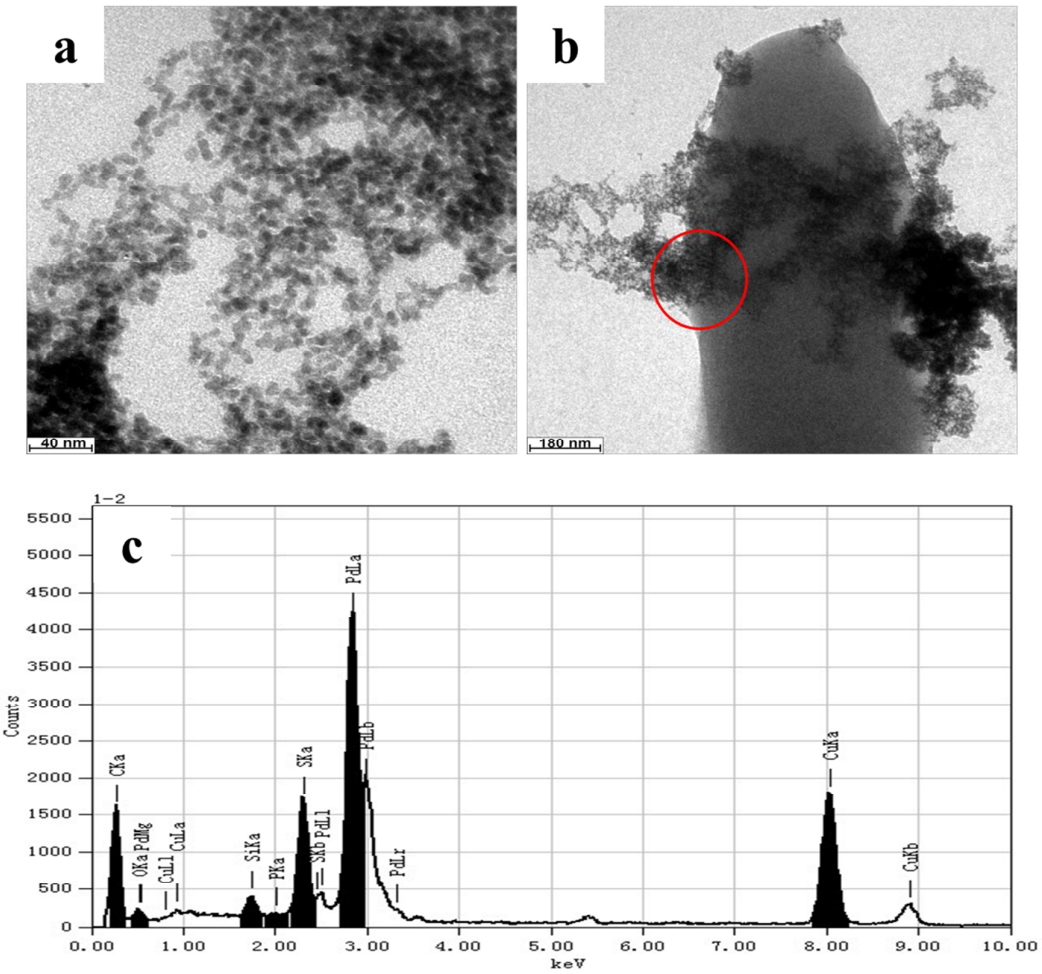

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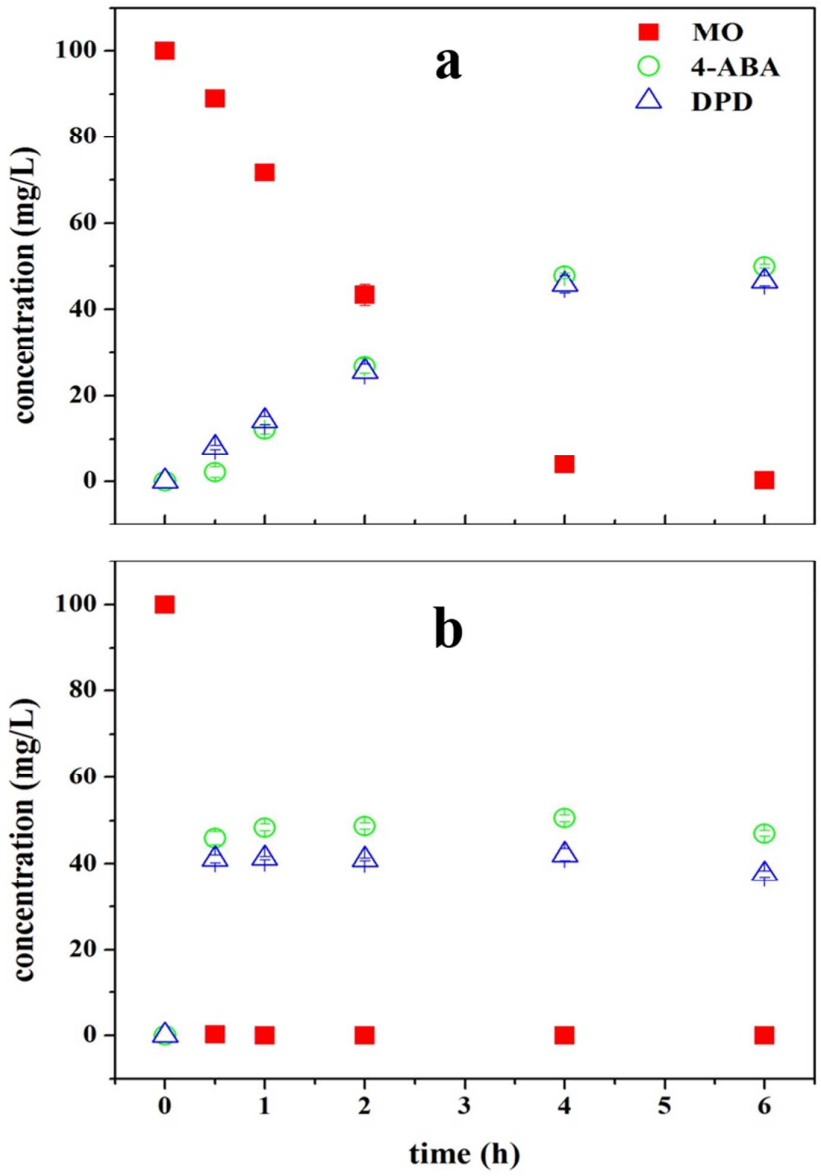

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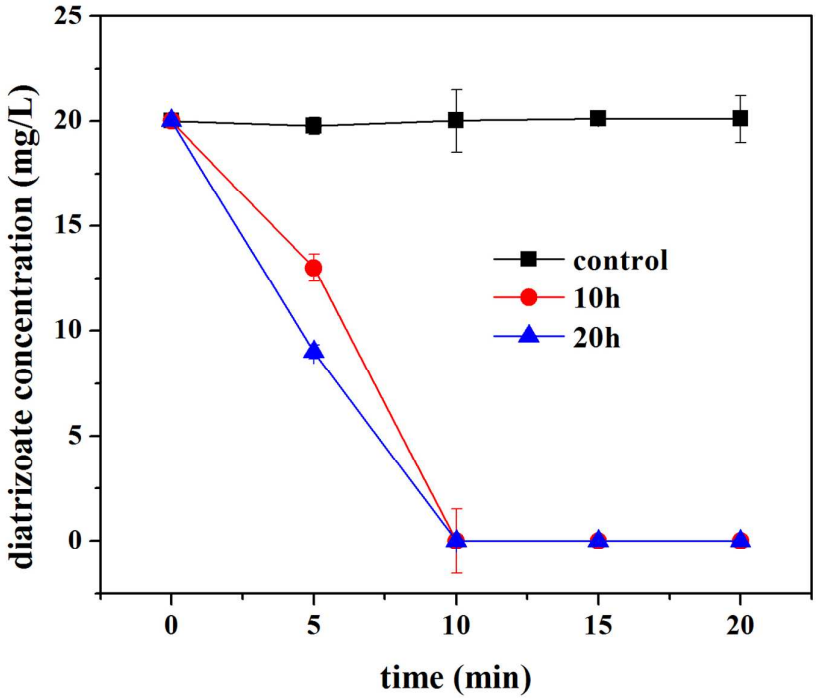

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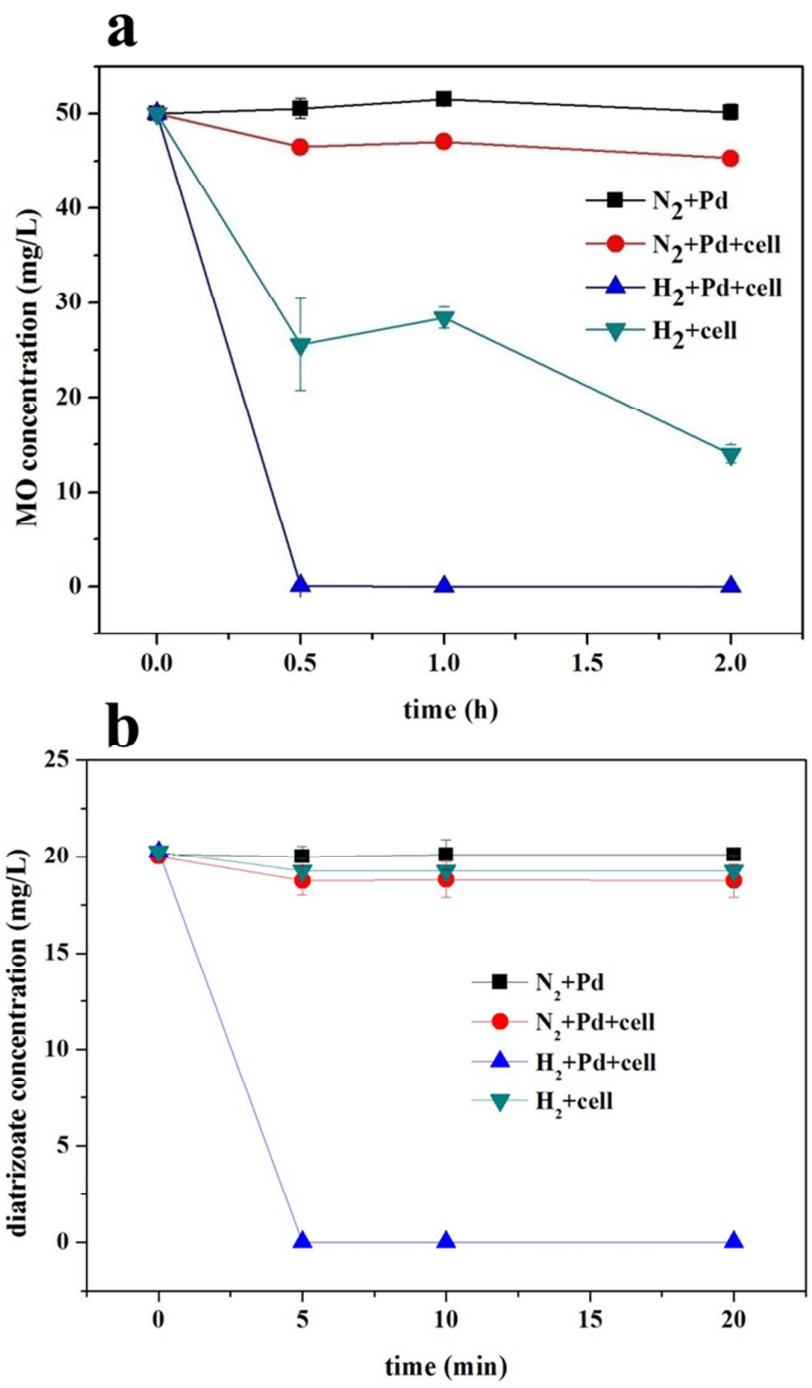

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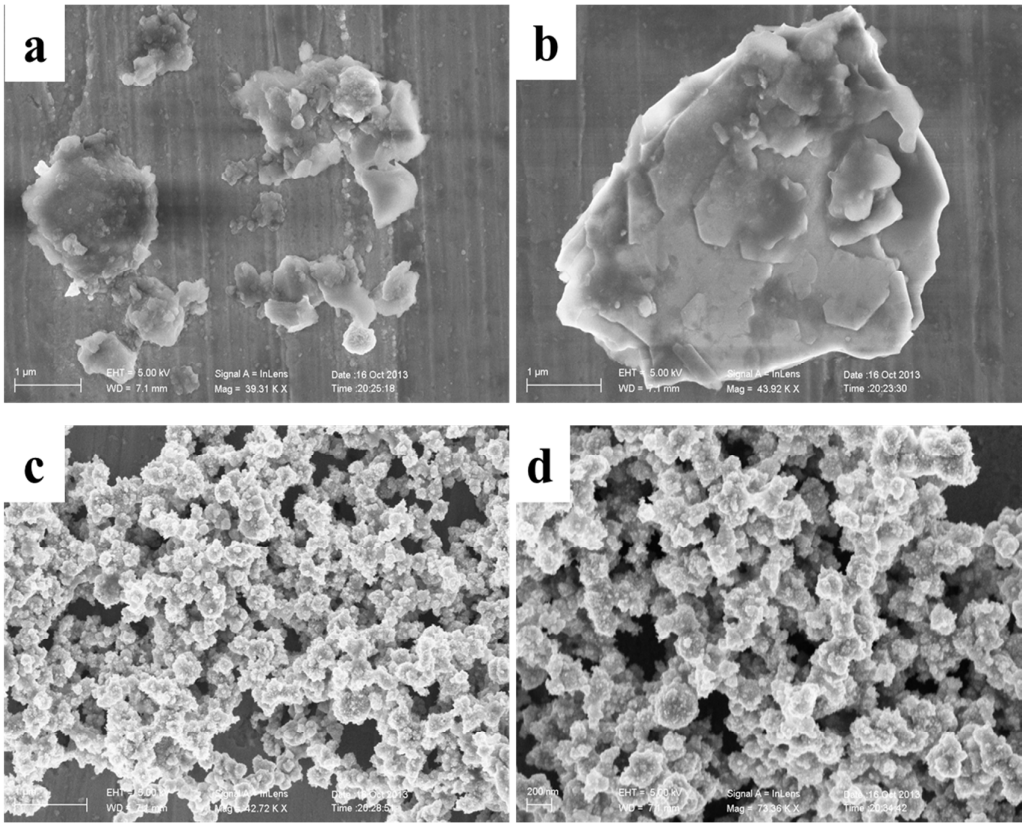

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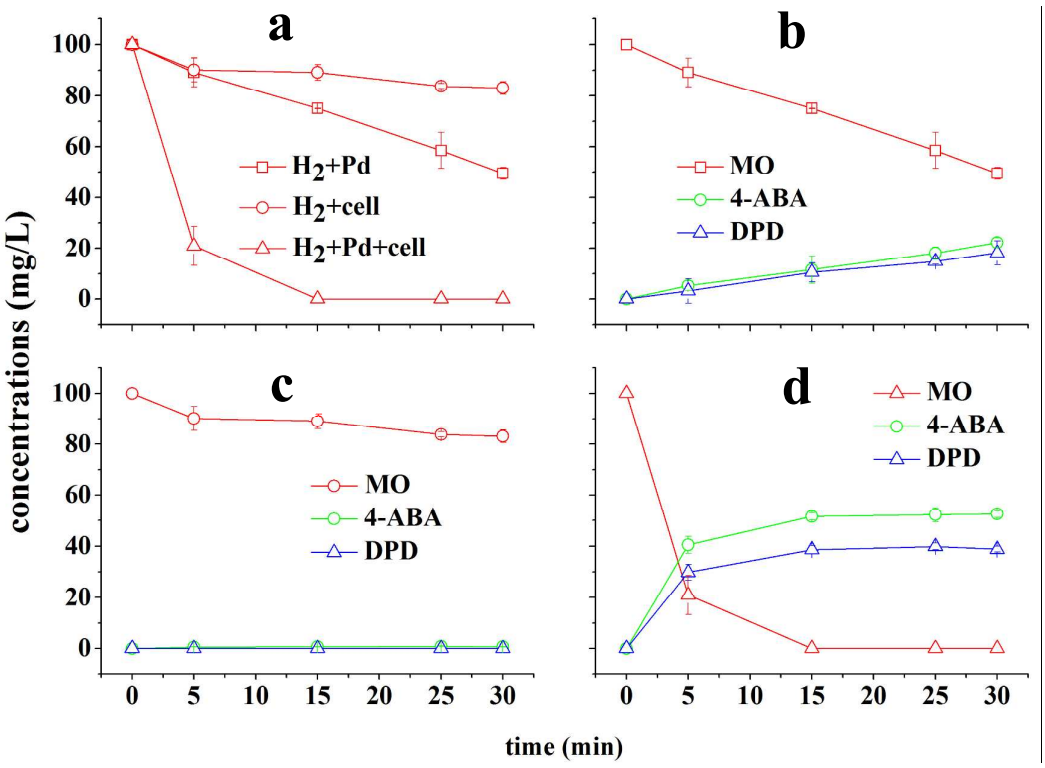

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