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# Decolorization of Remazol Brilliant Blue R using a novel acyltransferase-ISCO (*in situ* chemical oxidation) coupled system

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ABSTRACT

The feasibility of a novel perhydrolase-ISCO (*in situ* chemical oxidation) coupled technology for decolorization process of dye effluent was investigated. All the tested synthetic dyes, Malachite green chloride, Indigo Carmine, Rhodamine B, Remazol Brilliant Blue R (RBBR) and Orange I, could be decolorized using the novel process technology. In the RBBR effluent model, the optimized parameters for decolorization process using free acyltransferase as biocatalyst were as follows: 4 U/mL acyltransferase dosage, 40:1 molar ratio of ethyl acetate to hydrogen peroxide, and 80 mg/L RBBR original concentration in 20 mmol/L disodium hydrogen phosphate-citrate buffer, pH5.0. Under the optimal parameters for RBBR decolorization process, the maximum decolorization rate reached 81.11% at 30 °C for 6 h. The decolorization rate could be further increased to 95.08% in 10 min when acyltransferase CLEAs (Crossed-Linked Enzyme Aggregates) was used as biocatalyst. In the continuous batch process for RBBR decolorization, acyltransferase E-CLEAs (Encapsulated CLEAs with polyvinyl alcohol) could be recycled at least 6 times.


## 1. Introduction

During the past decades, China's textile industry have played an important role for the rapid economic growth, which was accompanied with an extensive use of various synthetic dyes, including Remazol Brilliant Blue R (RBBR), Orange I, Malachite Green Chloride, Indigo Carmine, and Rhodamine B. More than 24.55 hundred million tons dyeing effluent per year without any pre-treatment was directly discharged into rivers or lakes, which resulted in serious environmental concerns [1,2].

Compared with traditional physicochemical treatment technology for dyeing effluent, biological treatment technology has been given much attention due to lower sludge production, environmental friendliness, and sustainability [3,4]. *Ganoderma* sp., *Pseudomonas luteola,* and *Aspergillus fumigatus* can efficiently biodegradate, bioadsorb, or biotransform various dyes from dyeing effluent [5–7]. Cocultures of different dye-degradation strains can further improve the degradation efficiency [8–10].

Enzyme-based dye decolorization process is another green treatment technology. The best-known dye-decolorizing enzyme is laccase (EC 1.10.3.2). Laccase oxidizes phenolic and non-phenolic compounds, and requires only nontoxic, eco-friendly molecular oxygen (air) as a co-substrate [11,12]. In the RBBR effluent model, the decolorization rate reached 96% with only 0.2 U/mL dosage of the magnetic cross-linked laccase-aggregate [13]. The biotransformation products of RBBR show less phytotoxicity than the dye itself [14]. However, the catalytic activity of most laccases is highly depended on the mediator system, ABTS (2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)). A process without the use of ABTS would be environmentally benefical [15]. Moreover, biocatalysts with strict substrate selectivity impose a narrow spectrum of dyes that can be degraded. On the other hand, application in large scale of the enzymatic-decolorization technology is also hampered by a high production cost.

In recent years, a novel technology, the combined enzymatic-chemical process technology, displayed high decolorization rate and operational stability [16]. For example, peracid, one kind of strong chemical oxidizer, can efficiently decolorize various of dyes [17]. However, the chemical synthesis technology for peracid involves two steps, harsh reaction conditions and hazardous catalysts. Moreover, peracid solution at low concentration does auto-hydrolyze, which results in a short shelf half-life [18].

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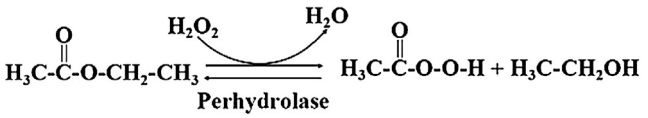


**Fig. 1.** The formulation of peracetic acid synthesis catalyzed by the perhydrolase.

In addition, peracid solutions at high concentration (over 70%, *v*/*v*) would explode, which makes it hard to store and expensive to transport [19]. It was desirable to prepare peracid *in situ* using a green technology so that the above problems could be solved. Excitingly, several perhydrolases catalyze peracid synthesis in the presence of $H_2O_2$ and a corresponding acyl donor [20–22] (Fig. 1). The peracid generated by the perhydrolase catalysis is then used as the oxidizer to directly act on the targeted substances *in situ*. This process routine was known as perhydrolase-ISCO (*in situ* chemical oxidation) coupled technology, which decreases the accumulation of peracid in the reaction system, and avoided the occurrence of peracid explosion and perhydrolase deactivation. The perhydrolase-ISCO coupled technology is an environmentally friendly alternative [23]. In previous work, perhydrolase-ISCO technology was for example applied in the field of fabrics destaining, pulp bleaching, organic synthesis, lignin removal from biomass [24–26].

All perhydrolases identified so far belong to the same α/β-hydrolase fold [22,26]. Instead of the natural activity, perhydrolysis activity is a promiscuous activity from α/β-hydrolase because of the reactive nature of peracids and low natural concentrations of hydrogen peroxide in natural environment [19]. Among the known promiscuous α/β-hydrolases with perhydrolysis activity, acyltransferase from *Mycobacterium smegmatis* displays the highest perhydrolysis activity, which is 50 fold over hydrolysis activity [27]. In the present research, we investigated the feasibility of the acyltransferase-ISCO coupled technology to be used in a RBBR decolorization process *in situ*.

## 2. Materials and methods

### 2.1. Chemicals and biochemistry reagents

Monochlorodimedone was purchased from Alfa-Aesar Company. Remazol Brilliant Blue R was purchased from Heowns Biochemical Technology Co., Ltd (China). HisTrap HP affinity chromatography column (5 mL) was purchased from GE Healthcare Life Sciences. Dimethoxyethane, glutaraldehyde, polyvinyl alcohol (1750 ± 50), ethyl acetate, sodium bromide, hydrogen peroxide, orange I, malachite green chloride, indigo carmine, and rhodamine B were of analytical grade and purchased from Sinopharm Chemical reagent Co. Ltd (China). Solution I and solution Π of peracetic acid were purchased from Xilong Chemical reagent Co. Ltd (China).

High-fidelity DNA polymerases, restriction enzymes, $T_4$-DNA ligases, PCR purification kits, the DNA Gel-Extraction Kits, DNA markers, and protein markers were purchased from Takara Biotechnology Co. Ltd (Dalian, China). Primers synthesis and DNA sequencing was completed by Sangon Biotechnology Co. Ltd (Shanghai, Beijing). Kanamycin was purchased from Beijing dingguo changsheng biotechnology Co. Ltd (Beijing, China).

### 2.2. Construction of the recombinant Escherichia coli *BL21(DE3)* for acyltransferase production

Based on the known amino acid residue sequence of acyltransferase from *M. smegmatis* (Protein Data Bank database under the accession No. 2Q0Q) [27] and the genomic DNA sequence of *M. smegmatis* NCTC8159, the encoding region of acyltransferase was mined (gene locus in the GenBank database: LN831039.1). The full-length acyltransferase gene sequence (named as *act* gene) was then artificially synthesized by Sangon Biotech Shanghai Co. Ltd. and incorporated the *Nco* I/*Xho* I restriction site at the 5′-terminal and 3′-terminal of the *act* gene, respectively. The *Nco* I/*Xho* I-digested *act* gene was then ligated into the *Nco* I/*Xho* I-digested plasmid pET28a. The resulting plasmid was designated pET28a-*act*. The sequence of the acyltransferase encoding region was further confirmed by DNA sequencing. The recombinant plasmid pET28a-*act* was then transformed into *E. coli* BL21(De3). The recombinant *E. coli* BL21(De3)/pET28a-*act* was deposited in the center of industrial culture collection of FJNU and the collection number was FJNU IM1705.

### 2.3. Production and purification of acyltransferase

The acyltransferase-producing *Escherichia coli* BL21(DE3) was inoculated into LB liquid medium supplemented with 50 μg/mL kanamycin. The acyltransferase gene was induced to be expressed in *Escherichia coli* BL21(DE3) by adding IPTG to 1 mmol/L when the cell density ($OD_{600}$) of *Escherichia coli* reached 0.6. The induction cultivation was continued 16 h at 30 °C and then the cells were harvested by centrifugation.

The cell pellet was resuspended in the following buffer: 20 mmol/L pH 7.4 $NaH_2PO_4$—$Na_2HPO_4$, 20 mmol/L imidazole, 500 mmol/L NaCl and then disrupted by ultrasonication. The cell lysate was centrifuged at 10625 × *g* for 20 min at 4 °C, and the supernatant was recovered and purified by affinity chromatography (HisTrap HP, 5 mL) according to the instructions of the manufacturer (GE healthcare).

### 2.4. Determination of perhydrolysis activity

Homogeneity of the purified acyltransferase was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The protein concentration was assayed using the method of Bradford, with bovine serum albumin as the standard [28]. The perhydrolysis activity was quantitatively determined by the spectrophotometric assay method using monochlorodimedone as the substrate, where the amount of enzyme added was adjusted to give a linear dependence of the reaction rate to enzyme concentration at 40 °C [23]. The reaction mixture for perhydrolysis activity assay constituted 20 mmol/L $NaH_2PO_4$-$Na_2HPO_4$ buffer (pH 7.4), 817.16 mmol/L ethyl acetate, 149 mmol/L sodium bromide, 0.047 mmol/L monochlorodimedone, and 0.01 mol/L hydrogen peroxide. The reaction was initiated by the addition of hydrogen peroxide to the reaction mixture. The kinetics was detected at 290 nm and 40 °C. Under the above conditions, the extinction coefficient ($\varepsilon_{290}$) of monochlorodimedone was $2.03 \times 10^4$ L/mol cm. One unit of perhydrolysis activity was defined as the amount of enzyme that produced 1 μmol of the brominated monochlorodimedone, per min.

### 2.5. Dyes spectrum range determination of perhydrolase-ISCO coupled technology

Five different textile dyes, including Orange I, Malachite Green Chloride, Indigo Carmine, Rhodamine B and RBBR, were tested in the present research to evaluate the feasibility of the acyltransferase-ISCO coupled technology for dyeing effluent treatment. Hydrogen peroxide tolerance of various textile dyes was tested in the concentration range from 0.005 mol/L-0.1 mol/L at 30 °C for 6 h. Peracetic acid tolerance of the textile dyes was tested at the final concentration of 5 (*vol*/*vol*). Peracetic acid was instantly synthesized as soon as it would be used according to the product manual. Solution I and solution Π of peracetic acid were blended at the ration of 5:4 (*vol*/*vol*), and 20% (*vol*/*vol*) peracetic acid could