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Short communication

Integrating a light-driven coenzyme regeneration system by expression of an alcohol dehydrogenase in phototrophic bacteria for synthesis of chiral alcohol

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ABSTRACT

Herein, we reported that *Rhodobacter sphaeroides* (*R. sphaeroides*) can be engineered by heterologous expression of an alcohol dehydrogenase (*adh*) from *Leifsonia* sp. to build a light-driven cofactor regeneration system for synthesis of chiral alcohol. The model substrate, 3′-chloroacetophenone, can be reduced by the engineered *R. sphaeroides* to (*R*)-1-(3-chlorophenyl) ethanol with an enantiomeric excess (e.e.) value of more than 99% in an *n*-hexane/aqueous biphasic media. This system, which is fully controlled by light, exhibited potential power to be an alternative cofactor regeneration platform for cheap synthesis of various chiral alcohols via the cloning other oxidoreductases with diverse characteristics.

1. Introduction

With regard to biocatalytic production of chiral alcohols, extensive studies have focused on developing environment-friendly, efficient, and repeatable ways for asymmetric reduction (Nestl et al., 2014; Tao and Xu, 2009). NAD(P)H-dependent oxidoreductases with high chemo-, regio-, and enantio-selectivity have been widely employed for preparing chiral alcohols from ketones, either independently or carried out by a whole-cell catalytic system (van der Donk and Zhao, 2003). Oxidoreductases meeting demands of application can be obtained and/or evolved with preferable catalytic properties by genome mining-based screening and characterizations and/or directed evolution. However, efficient regeneration of costly NAD(P)H in an oxidoreductase-catalyzed process is the key to large-scale application (Zhao and van der Donk, 2003).

Cofactor recycling by coupled-substrate method has been proved to be an efficient and practical way for reduction of ketone in lab or in industry (Faber and Johnson, 1996; Goldberg et al., 2007), demonstrating that alcohol dehydrogenases, with isopropanol as a co-substrate, can be used to bioreduction process efficiently with high activity and yield. An alternative method is coupled-enzyme approach, in which NAD(P)H regeneration system is constructed by co-expression (Weckbecker and Hummel, 2004) or fusion expression (Kataoka et al., 2003; Torres Pazmiño et al., 2008; Gao et al., 2014) of primary enzyme (responsible for primary chiral reaction) and auxiliary enzyme (responsible for regeneration of NAD(P)H that drives primary reaction) as whole-cell biocatalyst. In this system, uncontrollable expression of different enzymes in the cell may restrict the performance of primary and auxiliary enzymes (Gao et al., 2014; Clair et al., 2000). NAD(P)H regeneration involved the preparation of combined cross-linked enzyme aggregates (combi-CLEAs) of alcohol dehydrogenase (*adh)* and D-glucose dehydrogenase (*gdh)* was developed to precisely fine-tuning the ratio of primary and auxiliary enzyme (Ning et al., 2014). Similarly, co-immobilization of primary enzyme and auxiliary enzyme onto the same carrier can achieve higher cofactor recycling efficiency with improved transfer efficiency (Mazid and Laidler, 1982). These systems, with improved maneuverability, needs glucose and cofactor supplementation in reaction system to activate regeneration (Ning et al., 2014; Mazid and Laidler, 1982). Light used as driving force for regeneration of cofactor in living microorganism, was proposed for enzyme catalyzed oxidation and reduction (Huisman et al., 2010). Using ethylenediaminetetraacetic acid (EDTA) as electro donor, a cofactor regeneration system was developed by using light to drive enantioselective Baeyer-Villiger oxidations in vitro (Hollmann et al., 2007). In addition to tedious enzyme preparation, undesired oxygen-dependent uncoupling reaction and low electro transfer may limit this light-driven system's application.

Phototrophic bacteria, with photosynthetic regeneration center, where $NADP^+/NAD^+$ is reduced to NADPH/NADH and transported to

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wherever the reduction reaction occurs (Ind et al., 2009; Williams et al., 1983; Drews and Dawes, 2013), is increasingly considered as a highly potential candidate to be engineered as light-driven whole-cell biocatalyst. Nonetheless, few studies have focused on exploiting genetically engineered phototrophic bacteria for asymmetric reduction of ketones (Huisman et al., 2010). When driven by light, cyanobacterium (*Synechococcus* sp. PCC 7942), employed as whole-cell biocatalyst with no additional NAD(P)H or auxiliary enzyme, reduced aryl methyl ketone to (*S*)-alcohol with 90% yield and 99% *enantiomeric excess* (e.e.) value (Nakamura and Yamanaka, 2002). Recently, coupling of intracellularly expressed NADPH-dependent oxidoreductases with natural photosynthesis system of *Synechococcus* sp. PCC 6803 was demonstrated to be a feasible strategy to power an enzyme mediated redox reaction with the aid of light, which gave a 99% e.e. and 80% yield of 2-methylsuccinimide from reduction of prochiral 2-methylmaleimide (Köninger et al., 2016). Having a membrane light-harvesting protein, phototrophic bacteria capture energy from visible light and activate electron transfer for reduction.

Based on the aforementioned experimental demonstrations, we hypothesize that other wild-type phototrophic bacterium, equipped with more sophisticated genetic manipulation tools, is also capable of supplying adequate NADPH/NADH for the reduction reaction by an endogenous light-driven NAD(P)H regeneration system. Here, we developed a phototrophic bacterium-based whole-cell catalyst, which harbors a selected *adh*, to test our concept.

Owing to its unique physiological properties, *R. sphaeroides* is a valuable host for investigating the function of photosynthesis-associated membrane proteins, phototrophic bacterial metabolism, and bioenergetics (Ind et al., 2009; Williams et al., 1983; Drews and Dawes, 2013). Several inducible expression plasmids are available for the expression of heterologous proteins in *R. sphaeroides*, such as pRKSK1 (Hunter et al., 1994), pRECTX (Pasternak et al., 1999), and pIND4 (Ind et al., 2009).

An *adh* from *Leifsonia* sp. (L*sadh*) (Inoue et al., 2005a), which had been validated as a NADH-dependent biocatalyst for enantioselective synthesis of (*R*)-alcohol (Inoue et al., 2005b), was heterologously expressed in a phototrophic bacterium by using *Escherichia coli-R. sphaeroides* shutter plasmid pIND4 (Scheme 1) (Ind et al., 2009). The resultant recombinant *R. sphaeroides* cells with the endogenous light-driven NAD(P)H regeneration system were then tested for their performance in synthesizing a model compound by reducing 3′-chloroacetophenone to (*R*)-1-(3-chlorophenyl)ethanol (Scheme 1).

2. Construction of expression plasmid

pIND4 was chosen owing to its superior segregational instability and a modified promoter. L*sadh* gene was cloned in pIND4, and the recombinant plasmid was conjugationally transformed into *R. sphaeroides* JPA1336 (see Supplementary content). To confirm the occurrence of the transformation, PCR was performed using specific primers designed to clone the full and partial sequence of L*sadh* coding gene (756 base pairs) and plasmid (595 base pairs), respectively. The results in Fig. S1 indicate that both empty vector and recombinant pIND4-L*sadh* plasmid are transformed into *R. sphaeroides* JPA1336, and bands of the full-length L*sadh* gene and the partial plasmid sequence could be clearly detected at their corresponding positions (Fig. S1). The negative control experiment was performed using recombinant *R. sphaeroides* JPA1336-pIND4 cells as templates for colony PCR. The results show no corresponding band of the L*sadh* gene (Fig. S1), while the band of the partial sequence of the empty vector is observed. These results validate the efficiency of the transformation method used in this study. Fig. S2 shows the morphology of the *R. sphaeroides* JPA1336-pIND4-L*sadh* colony formed on the RS plate (see Supplementary content) containing selective antibiotics, after cultivation for 24 h at 34 °C, as observed with 8-W fluorescent lamps.

3. Optimization of culturing and reaction conditions

In recombinant *R. sphaeroides* JPA1336 cells, an IPTG-inducible promoter regulates L*sadh* expression. We investigated the effects of induction conditions on the whole-cell enzyme activity. Instead of adding extra NAD(P)H, we used sodium acetate and sodium thiosulfate as the electron-donor and hydrogen donor, respectively, in the reaction system. The results in Fig. 1A and B indicates that inducer concentration and duration of induction substantially affect the whole-cell enzyme activity. The whole-cell enzyme activity increases with an increase in IPTG concentration; the highest enzyme activity was attained at an IPTG concentration of 0.8 mM (Fig. 1A). Evidently, the whole-cell enzyme activity decreases rapidly with increasing induction time; 4 h was ascertained to be the optimum time to collect the cells for subsequent biotransformation after adding IPTG (Fig. 1B). Both high IPTG concentration and increased induction time can result in a decrease in the whole-cell enzyme activity, suggesting that IPTG is toxic to L*sadh*-expressing bacterial cells to a certain degree.

Under optimized conditions, recombinant *R. sphaeroides* JPA1336-pIND4-L*sadh* cells were cultured with or without IPTG induction to verify the presence of the L*sadh* gene. The bacterial culture was centrifuged, and supernatants and precipitates were analyzed by SDS-PAGE (Fig. S3). Compared to the negative control (non-induced recombinant cells), the recombinant cells showed no difference in the L*sadh* band, possibly due to the low expression level of L*sadh* in *R. sphaeroides* JPA1336. Western blotting with higher detection sensitivity was performed to detect the His-tag attached to the C-terminal of L*sadh* (Fig. S3). As expected, a faint band is observed in lane 4 (corresponding to the supernatant of induced recombinant cells), while no remarkable band was observed in the negative control (non-induced cells) (lane 3).

The whole-cell catalytic activity is also influenced by the reaction temperature and pH. As shown in Fig. 1C, the maximum enzyme activity of whole cell was reached at a temperature of 35 °C, which close to the optimum cultivation temperature for the bacterium (34 °C) but different from the maximum enzyme activity of the L*sadh* as determined *in vitro* (Inoue et al., 2005a, 2005b). This temperature may be more suitable for the metabolic activity of resting-cell, thereby maintaining stable NAD(P)H regeneration. The results in Fig. 1D indicate the high sensitivity of the whole-cell catalyst to pH variation, as catalytic activity was barely maintained at pH < 4 or > 10; the optimum reaction pH was 7.0 when 0.1 M Tris–HCl buffer was used. The reaction conditions required for the optimum catalytic performance of the whole-cell catalyst were similar to those characteristic of *R. sphaeroides* and L*sadh* (Inoue et al., 2005a, 2005b).

4. Optimization of substrate concentration in two reacion systems

In selected reaction systems (Tris–HCl buffer and *n*-hexane/Tris–HCl buffer), we investigated the effect of substrate concentration on the whole-cell enzyme activity, to determine the behavior of the developed whole-cell catalyst. The results are shown in Fig. 2. Compared to a single aqueous reaction system, the whole-cell catalyst can tolerate high substrate concentrations in an aqueous-organic biphasic system. The whole-cell enzyme activity in the biphasic reaction system was retained at approximately 20% at a substrate concentration of 300 mM (Fig. 2B), while it could hardly be detected in a single aqueous reaction system at the same substrate concentration (Fig. 2A). The aqueous-organic biphasic system is an established reaction system to facilitate the recovery of products, alleviate the whole-cell toxicity, increase the solubility of substrates or products, and regulate the thermodynamic equilibrium (Zhu et al., 2015). *n*-hexane, a widely used organic phase in biphasic systems, can dissolve products or substrates and can act as a substrate reservoir to avoid direct contact of the substrate with the cells. Compared to a single aqueous reaction system, the *n*-hexane biphasic reaction system alleviates substrate inhibition to a variable degree (Fig. 2). The optimum substrate concentration obtained