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Recent advances in enzymatic oxidation of alcohols

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Enzymatic alcohol oxidation plays an important role in chemical synthesis. In the past two years, new alcohol oxidation enzymes were developed through genome-mining and protein engineering, such as new copper radical oxidases with broad substrate scope, alcohol dehydrogenases with altered cofactor preference and a flavin-dependent alcohol oxidase with enhanced oxygen coupling. New cofactor recycling methods were reported for alcohol dehydrogenase-catalyzed oxidation with photocatalyst and coupled glutaredoxin-glutathione reductase as promising examples. Different alcohol oxidation systems were used for the oxidation of primary and secondary alcohols, especially in the cascade conversion of alcohols to lactones, lactams, chiral amines, chiral alcohols and hydroxyketones. Among them, biocatalyst with low enantioselectivity demonstrated an interesting feature for complete conversion of racemic secondary alcohols through non-enantioselective oxidation.

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Introduction

Alcohol oxidation to ketones or aldehydes is an important reaction in organic synthesis as the products are essential building blocks for many valuable chemicals [1,2]. Chemical oxidations of alcohols typically require the use of toxic metal catalysts and generate problematic by-products. A recently developed organocatalyst, TEMPO (2,2,6,6-tetramethylpiperidinyloxyl), oxidizes many alcohols, polyols, and carbohydrates with copper or aerobic oxygen as oxidant. However, such systems are of low catalytic selectivity and less active towards secondary alcohols [1].

Compared with chemical methods, enzymatic oxidation of alcohols is conducted under mild conditions without using any toxic reagents [3,4]. It often shows high catalytic specificity and selectivity and produces less waste, and thus, has been the primary choice for green oxidation of alcohols. Alcohol dehydrogenases (ADHs) and alcohol oxidases (AOx) are the most commonly-used enzymes for the oxidation. ADHs catalyze the oxidation with $NAD(P)^+$ as the electron acceptor [3]. As the cofactor is chemically unstable and expensive, a cofactor recycling system is required for practical application. Unlike ADHs, AOx use molecular oxygen as a green oxidant and only produce H_2O_2 by-product [2,4]. In practice, an *in situ* H_2O_2 removal by catalase or peroxidase is often employed with good efficiency ($k_{cat} = 10^6$) to avoid enzyme deactivation [5]. Peroxidases and oxygenases are occasionally used to catalyze alcohol oxidations but received little development due to the narrow substrate scope or low substrate specificity [6,7].

In recent years, discovery and development of practical enzymatic alcohol oxidation has attracted tremendous interest with particular focus on three sections. Firstly, new enzymes were developed with improved substrate specificity, catalytic selectivity and stability. This was done through bioinformatics-based genome-mining as well as protein engineering of existing enzymes. Secondly, new $NAD(P)^+$ recycling systems were developed for ADH-catalyzed oxidation with coupled substrate or coupled catalyst in order to increase the catalytic efficiency and reduce production cost. Finally, enzymatic alcohol oxidation was combined with other enzymatic or chemical catalysis to perform cascade transformation, enabling one-pot synthesis *in vitro* or even *in vivo* with an engineered host cell co-expressing all of the required enzymes. Such cascade transformation could avoid the isolation of the intermediates and minimize the generation of waste, providing many practical syntheses [8••,9].

In view of these developments, this review highlights recent advances in the discovery and development of new alcohol oxidation enzymes through genome-mining and protein engineering, new cofactor regeneration systems for ADH-catalyzed oxidation and the application of enzymatic oxidation of primary and secondary alcohols in biopreparation of lactones, lactams, chiral amines, alcohols, hydroxyacids, amino acids and amino alcohols. The detailed classification and collection of alcohol oxidation enzymes, enzyme substrate scope and catalytic mechanisms have been thoroughly reviewed elsewhere [2–4,10,11], thus will not be covered by this review.

Discovery and development of new ADHs and AOx for alcohol oxidation

Enzyme discovery through genome-mining

With the advances in many genome projects, genome-mining has become a routine for efficient enzyme-hunting. A typical procedure for genome-mining consists of the identification and preparation of enzyme candidates and subsequent test for target catalysis. Through this method, many new ADHs and AOx were discovered in the past two years, including several alcohol dehydrogenases [12–16], lactate dehydrogenases [17], hydroxysteroid dehydrogenases [18], glucose-methanol-choline (GMC) type oxidases [19–23] and a vanillyl alcohol oxidase (VAO) that showed rare activity towards carbohydrates [24•]. Those new enzymes, however, tend to have overlapping substrate scopes with existing enzymes [2], probably due to the considerable sequence similarity from the sequence-based enzyme discovery. Furthermore, some enzymes were only tested for their natural substrates to piece out enzyme's biochemical function without testing for the catalysis with practical interest [14,17].

The most significant development in this field was the discovery of two novel Cu^{2+} radical oxidases (CROs) from *Colletotrichum* spp. [25••]. CROs are alcohol oxidases with a conserved copper-radical catalytic site that coordinates with a crosslinked Tyr-Cys, 2 His and a Tyr residue. The Cu^{2+} radical transfers electron from alcohol substrate to molecular oxygen oxidant, hence, CROs do not require any organic cofactor, making them an ideal choice for green alcohol oxidation [4]. However, the number of CROs is very limited and galactose oxidase (GO) had been the only CRO developed for alcohol oxidation. The wild type GO had relatively narrow substrate scope, catalyzing D-galactose, D-talose and substituted benzyl alcohols (Scheme 1i) [26]. Directed evolution was conducted to create variants with expanded substrate range. For instance, the variant M_{3-5} has been widely used for the oxidation of secondary alcohols [27], glycoprotein [28], 5-hydroxymethylfurfural [29] and amino alcohols [30].

Through the analysis of *Colletotrichum* genomes, two new CROs (CgrAOx and CglAOx) were identified and investigated. Although both enzymes had similar active sites with GO, they showed broad and unique substrate ranges, catalyzing the oxidation of over 20 aliphatic and aromatic alcohols with high catalytic efficiency (Scheme 1i). For instance, the CgrAOx had k_{cat}/K_m for the oxidation of non-activated C_2–C_7 alcohols ranging from 8.6×10^3 s^{-1} M^{-1} to 5.3×10^5 s^{-1} M^{-1}, more competent than other AOx enzymes [25••]. Moreover, the results demonstrated that a coordinated Tyr radical could also oxidize non-activated alcohols, which would inspire further development of small-molecule oxidation catalyst.

Another new CRO (GlxA) was recently identified from *Streptomyces lividans* [31]. The enzyme had a distinctive protein structure compared with other CROs and catalyzed 4 alcohol substrates with highest activity towards glycolaldehyde (k_{cat}/K_m = 1.2 s^{-1} M^{-1}). The limited substrate range and catalytic activity was probably attributed to the buried active site. Structure-based rational design would be able to broaden the substrate range in future studies.

Generally, the alcohol oxidation enzymes are among those of the highest sequence diversity, which may affect the accuracy of sequence-based genome-mining [32]. Structure modeling of putative enzymes could hence be used to provide a better guide for the identification of new alcohol oxidation enzymes. For example, 10 genomes of wood-rotting fungi were searched for GMC type oxidases. The structures of 98% enzyme candidates (94/95) were predicted to identify the presence of catalytic histidine residue of the oxidation enzyme [33].

Enzyme development through protein engineering

Protein engineering is another method often used to tailor enzyme catalysts for given catalysis. Mimicking the natural evolution process, it incorporates different mutagenesis methods to create enzyme variants and certain selection force to yield those with desired catalytic performance. For the alcohol oxidation enzymes, the method was successfully used to improve the enzyme stability [34], catalytic activity [35•], stereo-selectivity [36], and expand the substrate scope [37,30].

In a recent study, a signal peptide was fused into an aryl alcohol oxidase (AAO) from *Pleurotus eryngii* to enable extracellular expression of the enzyme. Region-focused protein engineering was then performed, and a H91N variant was obtained with enhanced expression, giving 96 folds of total activity increase [34]. On the other hand, substrate binding could be designed from well-established enzyme structures, as demonstrated in the engineering of 5-hydroxymethylfurfural oxidase (HMFO) for the oxidation of 5-formyl-2-furancarboxylic acid [35•]. In the wild type enzyme, this oxidation was not efficient and limited the production of 2,5-furandicarboxylic acid. Two key mutations (V367R and W466F) were designed to increase substrate binding affinity and facilitate a correct binding pose with the aldehyde group (*gem*-diol) correctly orientated. This boosted the catalytic efficiency (k_{cat}/K_m = 2.2 S^{-1} mM^{-1}) by 1000 times and the acid production by 3.7 times. A similar strategy was also applied to engineer the substrate scope of chitooligosaccharide oxidase of *Fusarium graminearum* to cover non-natural substrates. The strict substrate specificity of the enzyme towards N-acetyl-D-glucosamine was successfully altered to accept other carbohydrates [37].

Structure-guided protein engineering was also used to tweak the electron transfer for the alcohol oxidation