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The green and effective oxidation of alcohols to carboxylic acids with molecular oxygen via biocatalytic reaction

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Abstract

A clean and effective alcohol oxidizing system using three enzymes has been developed. Regeneration of NAD^+ by NADH oxidase with molecular oxygen enabled to oxidize alcohols to carboxylic acids in good yield under mild conditions (25 °C, 1 atm).
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Oxidation of alcohols is one of the most important and essential processes in organic chemistry. Many synthetic methods have been reported so far. However, hitherto known methods have some drawbacks as the industrial processes from the environmental point of view, because they require a stoichiometric amount of toxic metal ions, harmful organic solvents, or a large amount of energy consumption with high pressures and/or high temperature. Therefore, development of the catalytic process of alcohol oxidation under mild conditions is essential for green and sustainable chemistry. Recently, several catalytic methods have been reported, such as aerobic oxidation using palladium complexes,[1] ruthenium complex,[2] and platinum catalyst.[3] However, the metal-independent oxidations of alcohols are very few. Thus we tried to construct a biocatalytic system for alcohol oxidation. Much attention has been paid to biocatalysts for these years as environmentally friendly and sustainable catalysts, because they are biosynthesized from recyclable biomaterials and are biodegradable. Some biocatalytic methods for the oxidation of alcohol using whole cells and isolated enzyme were reported.[4] As the representative examples, the enantioselective oxidation of 2-phenylpropanol by *Acetobacter aceti*[5] and the oxidation of primary alcohols to aldehyde by *Gluconobacter oxydans*[6] have been reported. In these cases, intact cells were used as the oxidizing reagents. On the other hand, the enantioselective oxidation of amines[7,8] and secondary alcohols using enzymes[9] have also been reported. However, the enzymatic oxidation of primary alcohols to carboxylic acids, which can be applicable to a broad range of alcohols, has not been studied enough. Recently, we have isolated *Brevibacterium* sp. KU 1309 from soil, which grows on the medium containing 2-phenylethanol as the sole carbon source. This microorganism oxidized various primary and secondary alcohols to the corresponding carboxylic acids and ketones, respectively.[10] NAD^+ dependent 2-phenylethanol dehydrogenase (PEDH) was purified from this strain and characterized.[11] This enzyme is presumed to be responsible for the abovementioned oxidation by the intact cells, because it exhibited similar activity to a wide range of aromatic and aliphatic alcohols. In addition, NAD^+ dependent phenylacetaldehyde dehydrogenase (PADH) was also purified from the same microorganism.[12] PEDH and PADH are expected to be useful enzymes because of their exceptionally broad substrate specificities. For this type of enzymatic oxidation to proceed effectively, the cooperation of an NAD^+-regenerating system is essential. NADH oxidase was reported as

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effective regeneration system of NAD^+.[13] Thus, we employed the NADH oxidase (NOX) isolated from *Lactobacillus brevis*.[14] NOX from *L. brevis* catalyzes oxidation of NADH to NAD^+ with molecular oxygen and produces H_2O. The family of NOX is divided into two types according to the reduction of oxygen such as H_2O-producing or H_2O_2-producing. H_2O-producing NOX is suitable for isolated enzymatic reaction, because high concentration of H_2O_2 is harmful for enzymes. Herein, we would like to report that we have accomplished a green and highly effective oxidation system of alcohols using the above three enzymes (Scheme 1). 2-Phenylethanol was oxidized to phenylacetaldehyde by PEDH, which in turn was further oxidized to phenylacetate by PADH. The resulting reduced form cofactor NADH was oxidized with molecular oxygen to regenerate NAD^+ by the aid of NOX. PEDH and PADH were used as partially purified enzyme from *Brevibacterium* sp. KU1309. NOX was isolated from recombinant *Escherichia coli* BL21 (DE3) overexpressing the NOX transformed by plasmid containing nox gene under the control of T7 promoter (see Supplementary data). Thus, we achieved the oxidation of alcohols (5–10 mM) forming H_2O as the only by-product under normal pressure (1 atm) and at room temperature (25 °C).

Generally, the rate of enzymatic reaction depends on the pH of the medium, because each enzyme exhibits its own charge distribution. These pH dependencies are especially important in the case of biotransformation using plural enzymes. Accordingly, pH profiles of PEDH, PADH, and NOX were examined first (described in detail in Supplementary data). PEDH and PADH prefer basic conditions. On the other hand, although NOX showed its highest activity at pH 6, it also exhibited a maximum activity of about 30% at pH 9. Consequently, the pH range in which the three enzymes showed their activities at the same time was revealed to be between 8 and 10. Thus, in all three enzymatic reactions were performed at once in this pH range and the results are summarized in Table 1. When the excess amount of NOX was added to the system, the reaction proceeded most effectively at pH 9. Because NOX was easily obtained in large amount from *E. coli* BL21 (DE3) transformed by the plasmid vector containing nox gene, it was not difficult to use a relatively large amount of this enzyme.

The concentration of NAD^+ is an extremely important factor from the standpoint of the efficiency of reaction

Table 1
The total effect of pH and NAD^+ concentration on the combined three enzymatic reactions

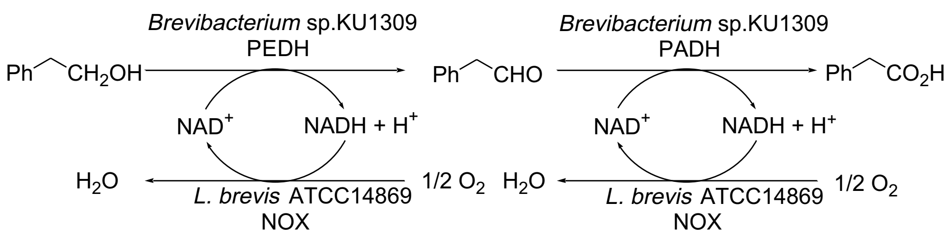
Ph⌒CH2OH (**1**, 10 mM) —[PEDH, PADH, NOX; NAD^+; buffer; 25 °C, 12 hr]→ Ph⌒CO_2H (**2**)

Entry	pH	$[NAD^+]$ (mM)	Yield of **2**[a] (%)
1	8	1	62
2	9	1	86
3	10	1	12
4	9	0	5
5	9	0.1	34
6	9	0.5	84
7	9	10.0	5

[a] The details of reaction condition and determination of yields were described in the part of Supplementary data.

and the cost of the procedure. The conversion of 2-phenylethanol to phenylacetate requires 2 equiv of NAD^+. However, oxidation reaction proceeded smoothly and quantitatively when only 5 mol % of NAD^+ was used in the presence of NOX, because molecular oxygen could be utilized as the final electron acceptor (Table 1). On the other hand, alcohol was not oxidized in the absence of NOX. Neither was phenylacetate detected after incubation of phenylacetaldehyde under the same conditions, but without enzymes. In the absence of PADH, the oxidation of alcohol did not proceed. Thus, it was confirmed that the oxidation of the aldehyde was an enzyme-catalyzed reaction. On the contrary, the oxidation reaction did not proceed in the presence of high concentration of NAD^+ (10 mM). Under high concentration of NAD^+, the absorbance at 340 nm, which corresponds to NADH, did not increase regardless of the presence of 2-phenylethanol and PEDH. Thus we considered that PEDH was inhibited by a large amount of NAD^+. In this way, the efficiency of the NAD^+ regenerating system is the key to achieve the oxidation of alcohols in high yields.

Utilizing this reaction system, various primary alcohols were oxidized to the corresponding carboxylic acids in good yields (Table 2), which are generally prone to be further degraded via β-oxidation pathway in the case of whole-cell reaction. Although the yields of the products were less than 100% in some cases, other by-products were not detected in the reaction mixture. The yields of



Scheme 1. Three enzymatic oxidation reactions of alcohol to carboxylic acid via molecular oxygen.

Table 2
Enzymatic oxidation of alcohols coupled with NADH oxidase

$$R{-}CH_2OH \xrightarrow[\text{buffer, 25 °C, 12 hr}]{\text{PEDH, PADH, NOX; } NAD^+ \text{ (1mM)}} R{-}CO_2H$$

Entry	Substrate[a]	Product	Yield (%)
1	CH_2OH	CO_2H	86
2	CH_2OH	CO_2H	74
3	CH_2OH	CO_2H	87
4	O CH_2OH	O CO_2H	35
5	CH_2OH	CO_2H	86
6	CH_2OH	CO_2H	60
7	CH_2OH	CO_2H	57

[a] The concentration of each substrate was 5 mM.

carboxylic acids were determined by GC after extraction with diisopropylether. The reason for the yields not being quantitative would be due to the insufficient efficiency of extraction. For example, while phenylacetate was obtained in 71% yield from 2-phenylethanol by the reaction with intact cells of *Brevibacterium* sp. KU1309, the yield of the acid increased to 86% by the application of this system, because the enzymes as well as cofactors required for the metabolism of the carboxylic acids were excluded in the in vitro reactions. The successful transformation of 2-phenylethanol to phenylacetate demonstrates the usefulness of this system, because the successful conversion of this type of compounds has been only limited.

In summary, we developed a novel approach to the oxidation of alcohol using biocatalyst. This reaction proceeds at room temperature (25 °C) and under the atmospheric pressures (1 atm). Using an NAD^+ regenerating system, the required amount of the expensive cofactor could be greatly reduced. Because the final electron acceptor is molecular oxygen and the only by-product is water, this system can be considered as green and sustainable. The preparation of PEDH and PADH in large scale utilizing genetic and protein technology is expected to enable this oxidizing system to be applied to the industrial process.

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Supplementary data

Supplementary data (preparation of PEDH, PADH, and NOX and general experimental procedure) associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2007.12.032.

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