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Note

Cofactor Recycling for Selective Enzymatic Biotransformation of Cinnamaldehyde to Cinnamyl Alcohol

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The enzymatic, selective hydrogenation of cinnamaldehyde to cinnamyl alcohol is reported here. Yeast alcohol dehydrogenase was used in a substrate-coupled process with cofactor recycling. Both 100% selectivity and aldehyde conversion were achieved within 3 h. The reaction took place under very mild conditions, in the absence of toxic organic solvent. The overall process proved inexpensive and deserves further optimization studies in order to evaluate industrial applications.

Key words: alcohol dehydrogenase; cinnamaldehyde; cinnamyl alcohol; cofactor recycling; hydrogenation

Cinnamaldehyde (CMA) selective hydrogenation to cinnamyl alcohol (CMO) is an important challenge in the perfume and flavor industries.[1,2] However, side reactions leading to unwanted products are common by conventional methods.[3] Therefore selectivity is the main goal to be reached in CMA hydrogenation. Many catalysts have been described,[4–6] involving different inorganic catalysts. Those approaches however do not show acceptable selectivity, and use extreme operational conditions and hazardous solvents and chemicals. This usually leads to serious economic concerns.

In order to overcome these drawbacks, we propose the use of commercial, inexpensive, NAD-dependent, alcohol dehydrogenase (ADH) from the yeast *Saccharomyces cerevisiae*, whose action towards cinnamaldehyde have been described.[7] ADHs are inhibited by high NAD^+ concentrations;[8] moreover this is a rather expensive reagent. Accordingly, its stoichiometric use during CMA reduction appears not to be affordable. In this perspective, the goal of this study was the development of a cofactor recycling catalytic system in which ADH reduces CMA to CMO (oxidizing NADH to NAD^+). The NAD^+ produced was then recycled by the same enzyme at the expense of ethanol, as suggested previously.[7] This acted as co-solvent for both CMA and CMO, and was present in large excess to force the whole process toward CMA reduction (Fig. 1A). Also, elimination of the obtained dehydrogenation product acetaldehyde drove CMA reduction toward completion. As two reactions have to occur at the same time, the two reactions were studied separately to determine the optimum conditions for each, and then operational conditions at which both can occur simultaneously were assessed.

ADH activity determination and preliminary operational characterization were carried out by spectrophotometric assay. In a 3-ml cuvette, 18 ADH E.U. (Enzyme Units, one unit defined as the enzyme amount capable of transforming one micromole of substrate per min) was incubated at 25 °C in the presence of 25 mM buffer, 0.25 mM NADH, and 1 mM CMA. The decrease in absorbance at 340 nm was recorded using a Cary 50 UV-Vis spectrophotometer Varian (NADH $\varepsilon_{340} = 6.22 \times 10^3\ \mathrm{M}^{-1}\,\mathrm{cm}^{-1}$). NADH and CMA concentrations were changed over a proper range for determination of Michaelis-Menten kinetic parameters.

Although reduction of CMA by NADH exhibits a pH optimum at about 6, whereas oxidation of ethanol and 2-propanol is best accomplished at pH about 9, we have found that pH 7 represents a satisfactory compromise, taking into account that ethanol was always present in large excess. Moreover, the chosen conditions ensured optimal durability for ADH (no significant activity loss up to 24 h).

ADH activity was tested after 1 h of storage at various temperatures (ranging between 20 and 60 °C). The results showed that up to 30 °C, ADH retained all initial catalytic activity. Only 18% of the activity was lost at 40 °C. Almost no activity was detected after incubation at 50 °C and 60 °C (30 and 0% residual activity respectively). Moreover, since prolonged incubation of the enzyme at 30 °C caused no significant inactivation, in the perspective of the development of an industrial process in which all ADH catalytic activity can be exploited, 30 °C was chosen as the operational temperature. In order to exploit maximum ADH activity, kinetic constants needed to be determined: the K_M values for NAD^+, ethanol, and 2-propanol are already known,[9] while those for CMA and NADH were found to be 0.46 and 0.071 mM respectively. Accordingly, the initial concentrations of CMA and cofactor were chosen only slightly higher than the respective K_M values. The initial 1 mM CMA and 0.1 mM cofactor were then used.

CMA hydrogenation reactions were performed in a mechanically stirred glass reactor equipped with temperature and pH controls (Fig. 1B). In a final volume of

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Abbreviations: ADH, alcohol dehydrogenase (E.C. 1.1.1.1); CMA, cinnamaldehyde 2-(E)-3-phenyl-2-propenal; CMO, cinnamyl alcohol, 2-(E)-3-phenyl-2-propen-1-ol

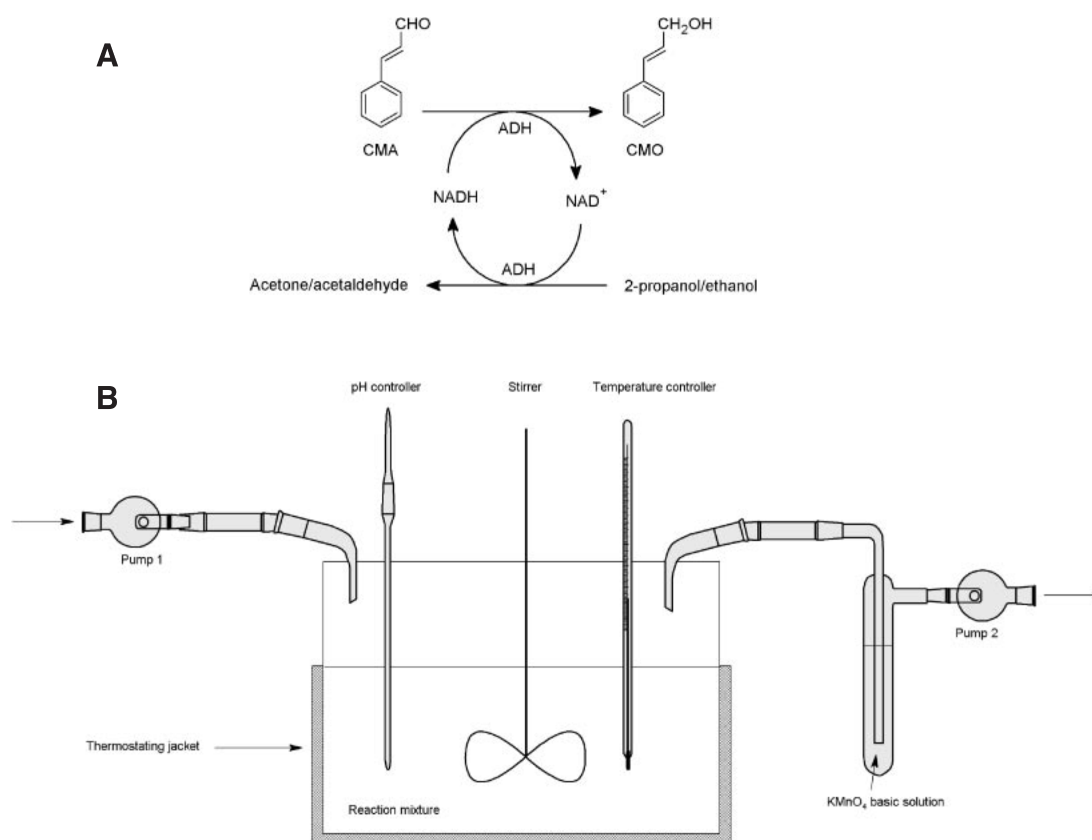

Fig. 1. NAD-Mediated CMA Hydrogenation to CMO by Ethanol (2-propanol) (A) and Scheme of the Used Reactor (B).

1 liter, 6,000 ADH E.U. was incubated at 30 °C in the presence of 1 mM CMA, 25 mM sodium phosphate buffer pH 7, 0.1 mM NAD^+, and 8% v/v ethanol. In view of the low boiling point of acetaldehyde (21 °C), the system was simply fluxed with an air stream, and the acetaldehyde vapor bubbled in 0.1 M $KMnO_4$ solution in 0.3 M NaOH to oxidize it to harmless sodium acetate.

Identification of the products of enzymatic activity was carried out with a System Gold apparatus (Beckman, Fullerton, CA) equipped with an UV-Vis detector module. The column used for chromatographic separations was Ascentis RP-C18 (250 mm × 4.6 mm i.d., 5 µm particle size), purchased from Sigma-Aldrich (St. Louis, MO). The samples were deproteinized with H_3PO_4 before injection. Separation of the compounds was achieved with 0.085% H_3PO_4 in water (solvent A) and 95% v/v acetonitrile in 0.085% v/v H_3PO_4 (solvent B) as mobile phases. Chromatographic conditions: initial isocratic elution, 10% B for 4 min, followed by a gradient phase, $10 \rightarrow 90\%$ B, in 5 min at a 1 ml min^{-1} flow rate. The detector was set at 268 nm.

Analysis of the reaction products, dissolved in CH_2Cl_2 and dried with anhydrous Na_2SO_4, was conducted using an HP 5980 gas-chromatograph connected with an HP 5971A mass spectrometer. The measurements were carried out by working in electronic impact at 70 eV with a source temperature of 100 °C. Gas-chromatographic separation was performed with an HP 5–MS column (length 30 m, inner diameter 0.25 mm, film thickness 0.25 µm). Analysis conditions: initial temperature 120 °C for 15 min, then 10 °C min^{-1} gradient until 250 °C. This temperature was held for 5 min.

Reactant concentrations, pH, and temperature optimum conditions were determined, and then cofactor recycling reaction was performed, using ethanol as low MW alcohol. The results in terms of CMA conversion and CMO production are reported in Fig. 2. CMA was almost completely reduced after only 3 h, as the only product detectable by both HPLC/UV and GC/MS was CMO (100% selectivity).

In the presence of 2-propanol, similar results were obtained, but at the same time CMA conversion was lower (85%) and CMO production was accordingly lower (although, even in this case, selectivity was 100%). Therefore ethanol was chosen as the low MW alcohol for this study.

Since ethanol was changed to toxic acetaldehyde during the reaction, the latter was easily stripped out from reaction mixture by an air stream, and bubbled into an alkaline $KMnO_4$ solution which oxidize it to harmless acetate anions.

Since NAD^+ is expensive, decreasing of the cofactor concentration has also been attempted. With 0.1 and 0.05 mM NAD^+, almost the same value of CMA conversion was obtained (99%), while decreasing NAD^+ to 0.01 mM led to a slightly lower conversion (73%). In all cases the CMO selectivity was 100%. These results proved effective cofactor recycling, and are very for the development of an economical affordable industrial process of CMO production, since it appears feasible to reduce noticeably the cofactor amount employed without significantly affecting reaction yield.

As stated above, ADH maintained its whole activity until 30 °C. At 40 °C a small activity drop appeared, while at higher temperatures the enzyme was almost

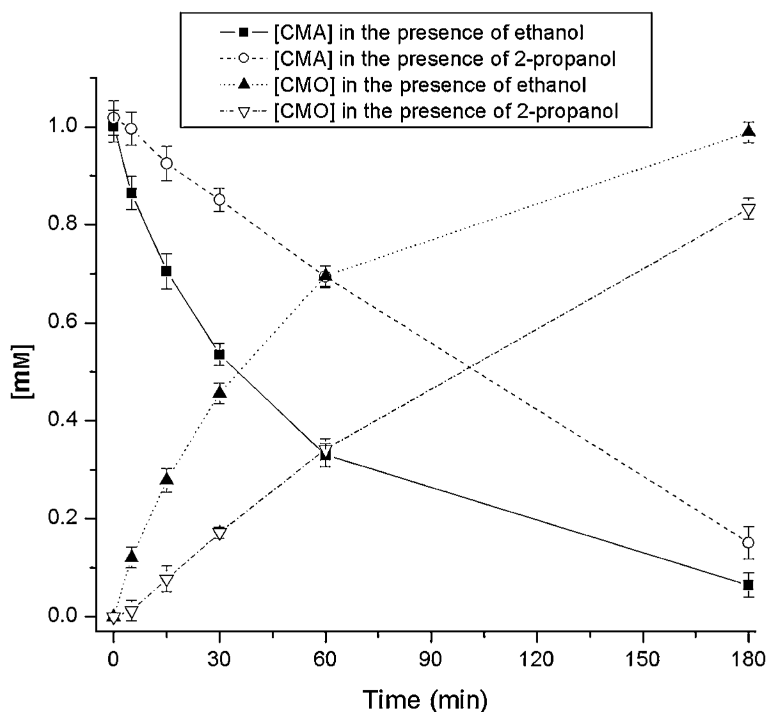

Fig. 2. CMA Conversion and CMO Production in the Presence of Ethanol and of 2-Propanol.
In a 1-liter stirred tank reactor, 6,000 ADH E.U. was incubated at 30 °C in 25 mM sodium phosphate buffer, pH 7, 1 mM CMA, 0.1 mM NAD^+, and 8% v/v ethanol or 2-propanol (n = 3).

completely inactivated. Accordingly, the 30–40 °C range was more carefully investigated in order to evaluate both negative (enzyme inactivation) and positive (reaction rate enhancement) effects of temperature on the process. Working at 30, 35, and 40 °C, no significant modification was detected in terms of CMA conversion or CMO production. This suggests that the positive effect on the reaction rate of rising temperature is countered by enzyme inactivation. In this perspective, raising the temperature to over 30 °C appears not to be a profitable approach.

For industry, the CMO amount produced per time unit will be a key factor to determine the economical feasibility of the whole process. Increasing initial CMA amounts were tested, ranging between 1 and 10 mM. Doubling CMA from 1 to 2 mM led to 88% conversion, as opposed to 99% (in 3 h); with 10 mM CMA, only 15% conversion was detected. Even if in all cases CMO selectivity was 100%, the produced alcohol amounts were rather different: 0.99, 1.70, and 1.51 mmol of CMO respectively were obtained. Such behavior suggests substrate inhibition mediated by CMA concentrations far from the K_M value (0.46 mM). This does not allow for excessively increasing the starting CMA concentration, since the process yield might be greatly affected. Thus 1–2 mM can be identified as the ideal starting CMA concentration.

Further studies are in progress in order to optimize the reaction conditions. In particular, a promising strategy is ADH immobilization, in order to allow complete exploitation of its catalytic activity.

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