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To cite this article: Agung Primanto Murdanoto, Yasuyoshi Sakai, Langkah Sembiring, Yoshiki Tani & Nobuo Kato (1997) Ester Synthesis by NAD⁺-dependent Dehydrogenation of Hemiacetal: Production of Methyl Formate by Cells of Methylotrophic Yeasts, Bioscience, Biotechnology, and Biochemistry, 61:8, 1391-1393, DOI: <u>10.1271/bbb.61.1391</u>

To link to this article: <u>http://dx.doi.org/10.1271/bbb.61.1391</u>

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Note

Ester Synthesis by NAD⁺-dependent Dehydrogenation of Hemiacetal: Production of Methyl Formate by Cells of Methylotrophic Yeasts

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Received February 14, 1997

A water-soluble ester, methyl formate, was detected as a metabolite in the culture medium of methylotrophic yeasts. Methyl formate synthase, which catalyses NAD⁺-dependent dehydrogenation of the hemiacetal adduct of methanol and formaldehyde, catalyses the ester synthesis. The enzyme activity was induced on a methanol medium and was increased further by the addition of formaldehyde. In the reaction system using intact cells of Pichia methanolica AKU 4262, 135 mM (8.1 g/liter) methyl formate was produced from 2 M methanol. This is a new biological process for ester synthesis that couples spontaneous formation of hemiacetal and alcohol dehydrogenase.

Key words: formaldehyde oxidation; methanol; methyl formate; methyl formate synthase; Pichia methanolica

In methylotrophic yeasts, methanol is completely oxidized to CO2 through the sequential reactions catalyzed by alcohol oxidase (EC 1.1.3.13), formaldehyde dehydrogenase (EC 1.2.1.1), S-formyl glutathione hydrolase (EC 3.1.2.12), and formate dehydrogenase (EC 1.2.1.2).¹⁾ Recently, we found a considerable amount of methyl formate accumulation in the culture medium of methanol-grown yeasts,²⁾ and found that a novel mitochondrial enzyme, methyl formate synthase, is involved in the methyl formate formation.³⁾ This enzyme catalyzes NAD⁺-dependent dehydrogenation of the hemiacetal adduct of methanol and formaldehyde, leading to the formation of methyl formate, as in the following equation;

$CH_2(OH)CH_3 + NAD^+ = CH(O)OCH_3 + NADH + H^+$

This enzyme is a class 3 alcohol dehydrogenase that contains 2 atom of zinc per subunit and uses preferably an aliphatic long chain alcohol like octanol as well as hemiacetal for its substrate.³⁾ The enzyme was induced with methanol or formaldehyde and the ester accumulation in culture media was stimulated by the addition of formaldehyde to the culture medium. From these results, the enzyme is assumed to participate in a glutathione-independent pathway for formaldehyde oxidation in methylotrophic yeasts. The reaction catalyzed by NAD⁺-dependent dehydrogenase of a hemiacetal could be a novel strategy for ester-synthesis reaction in yeasts. Although several isozymes of alcohol O-acetyltransferase (EC 2.3.1.84) are thought to be responsible for the ester formation in brewing yeasts,⁴⁾ it may be possible that alcohol dehydrogenase is involved in ester synthesis during brewing processes.

Methyl formate is a starting substrate in ' C_1 chemistry,' which is synthesized from methanol under high temperature conditions using metal catalysts. The microbial process for the ester production is attractive from the viewpoint of 'cleaner production' which means energy-saving processes without harmful byproducts. The reverse reaction of lipase in a hydrophobic solvent is generally useful for ester production. However, such a process could not be applied to methyl formate synthesis, because the substrates and product are highly water-soluble. In this communication, we describe methyl formate production using methylotrophic yeast cells having high methyl formate synthase activity.

Methylotrophic yeasts were grown on a medium containing 1.5% methanol (MI medium) at 28°C on a reciprocal shaker.⁵⁾ As shown in Fig. 1, methyl formate and formaldehyde accumulated during the course of growth of Pichia methanolica AKU 4262. Methyl formate accumulated was identified by GC-MS, giving a molecular ion peak at m/z 60 and a spectrometric pattern that was identical to that of the authentic compound. High concentrations of methanol and formaldehyde are required for the reaction of methyl formate synthase, since the genuine substrate in vivo, the hemiacetal, is present in equilibrium with methanol and formaldehyde.³⁾ Therefore, the resting-cell system seems to be a more efficient process than the growing-cell system. A reaction mixture (10 ml), containing 100 mM potassium phosphate buffer, pH 7.0, 2.0 M methanol, and 50 mg (as dry cell weight, dcw) cells in a 30 ml-conical flask, was sealed with a rubber stopper to prevent evaporation of the ester. The reaction was done on a reciprocal shaker (120 rpm) is a water bath at 10°C. After an appropriate period, a portion of the reaction mixture was centrifuged (15,000 gat 4°C for 1 min), then the supernatant was subjected to gas chromatography for measurement of methyl formate and methanol as described previously.²⁾ Formaldehyde was measured by the method of Nash.⁶⁾

Preliminary screening for the methyl formate producer was done using 20 strains of methylotrophic yeasts from our collections in



Fig. 1. Course of Growth of P. methanolica on Methanol. The yeast was grown on MI medium. Symbols: growth (●), pH (○), methyl formate (\blacktriangle) , formaldehyde (\Box) , and methanol (\blacksquare) .

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Fig. 2. Effects of Methanol Concentration (A), pH (B), Cell Concentration (C), and Temperature (D) on Methyl Formate Production with Resting-cells of *P. methanolica*.

The standard reaction mixture is described in the text. The yeast cells used were grown on MI medium for 72 h. Buffers (100 mM) used; sodium acetate, pH 4.0 and 5.0; potassium phosphate, pH 6.0 and 7.0; Tris-Cl, pH 8.0; glycine-NaOH, pH 9.0.

the resting-cell reaction. The cells were grown on MI medium containing 1.5% methanol as a sole carbon source at 28°C for 72 h. Among them, P. methanolica AKU 4262 showed the highest productivity (21 mm after 24-h incubation), while the other yeasts accumulated the ester to some extent as follows; Candida sp. 1-B AKU 4622 (16.3 mm), P. pinus AKU 4259 (11.2 mm), and Candida sp. 25-A AKU 4621 (9.7 mM). From these, P. methanolica was selected for further investigation. Effects of several reaction conditions on the methyl formate accumulation are summarized in Fig. 2. The methanol concentration of the reaction mixture influenced ester accumulation significantly, the optimum concentration being 2.0 m. The optimum pH 7.0, agreed with that for activity of the purified methyl formate synthase.³⁾ The cell concentration of 5.0 dcw mg/ml was the best for the reaction. The optimum temperature (10°C) was much lower than that for the enzyme activity (25°C).³⁾ This is the same phenomenon as observed previously in the conversion of methanol to formaldehyde by C. boidinii.7) Reactions at a low temperature seem to minimize the inactivation of alcohol oxidase by formaldehyde and H₂O₂, and maximizes the solubility of oxygen that is required for alcohol oxidase, resulting in the improvement of formaldehyde production. In each case shown in Fig. 2, the maximum amount of methyl formate accumulated was 65 to 80 mm. No positive result for the ester production was obtained by the addition of a metal salt (1 to 10 mm) or an enzyme inhibitor (1 mm). Among them, α, α' -dipyridyl (10 mM) and HgCl₂ (1 mM), which are inhibitors of methyl formate synthase,3) inhibited about 35 and 100%, respectively, of the productivity under the standard conditions.

Next we examined the growth conditions to obtain cells having higher activity of methyl formate synthesis. The methyl formate



Fig. 3. Course of the Resting-cell Reaction for Methyl Formate Production.

The yeast cells used in a reaction were grown on MI medium (open symbols) or MI medium supplemented with 13.2 mM formaldehyde (closed symbols). The reaction conditions were given in the text. Symbols: methanol $(\triangle, \blacktriangle)$, methyl formate $(\bigcirc, \blacklozenge)$, and formaldehyde (\Box, \blacksquare) .

production was not observed for glucose-grown cells, since all of the enzymes required for the reaction, e.g., alcohol oxidase, catalase, and methyl formate synthase, were induced with methanol, but not with glucose. The methyl formate synthesis of the cell was increased by the addition of formaldehyde (1.3 to

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16.5 mM) to the culture medium, the specific activity of the cell-free extract (1.0 to 1.2 units/mg protein) being 3-fold higher than that of the cell grown on MI medium. Figure 3 shows the courses of reactions with the cells grown on the media containing 0 (medium MI) and 13.2 mM formaldehyde. In the case of the formaldehyde-induced cells, the maximum accumulation of methyl formate was 135 mM (8.1 g/liter) after 96-h incubation, at which point about 1 M methanol was consumed, and a small amount of formaldehyde (25 mM) was accumulated.

We had developed a process for the production of formaldehyde from methanol using several types of mutants of *C. boidinii.*^{8,9)} This comprises a rather simple enzyme system, which involves alcohol oxidase oxidizing methanol to formaldehyde, and catalase decomposing H_2O_2 . Compared to this process, the catalytic elements related to the methyl formate production from methanol are more complicated, requiring methyl formate synthase in addition to the formaldehyde-producing system and presumably, an NAD⁺ regenerating system. The latter step is likely the rate-limiting step in the methyl formate production, since energy pool of the cell becomes saturated as the reaction proceeds. In order to achieve higher production, an NAD⁺-generating system should be improved. The process described here, alcohol dehydrogenation of hemiacetal, introduces a new concept to microbial production processes for useful esters, and in principle, a similar reaction may be used for other ester synthesis.

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