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Biocatalytic Production of Aldehyde by a Methanol Utilizing Yeast, *Hansenula nonfermentans* KYP-1 Grown in Methanol-limited Continuous Culture

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Aldehyde production by cells of a methanol utilizing yeast, *Hansenula nonfermentans* KYP-1 was improved when they were grown in a methanol-limited continuous culture, in comparison with cells grown in a batch culture. A higher cell yield was also obtained in continuous culture than in batch culture. This could be due to the fact that a lower methanol concentration was maintained in the jar fermentor to minimize growth inhibition by methanol. A maximum cell productivity of 0.219 $g \cdot liter^{-1} \cdot hr^{-1}$ and a cell yield of 47% were obtained at dilution rates of 0.1 hr^{-1} and 0.06 hr^{-1}, respectively. The greatest amount of aldehyde was measured at a dilution rate of 0.08 hr^{-1}. Under optimum reaction conditions, 915.7 mM of acetaldehyde was produced from 1.5 M ethanol after 21 hours' reaction, with a conversion rate of 61%. Propionaldehyde and acrolein were produced with conversion rates of 32.7% and 44%, respectively.

Methanol utilizing yeasts would be useful as biocatalysts for the production of aldehyde from primary alcohol in a low energy consuming process (3, 8, 10). However, constant preparation of fully active cells for the production of aldehyde by batch culture is difficult because of the instability of the culture, in which momentarily changing conditions in the medium and culture effect cell growth.

The methanol-limited continuous culture of methanol utilizing yeasts has been studied in respect to SCP production (6), regulation of alcohol oxidase (12), and formaldehyde production (7). Van Dijken *et al.* (12) reported that cells of *Hansenula polymorpha*, grown in a methanol-limited chemostat culture at a low dilution rate, contained alcohol oxidase amounting to as much as 20% of total soluble proteins. Thus, an improvement of aldehyde production can be expected for a chemostat culture, which provides fully active cells easily and continuously under optimum culture conditions. Furthermore, growth inhibition by methanol can be minimized by a methanol-limited chemostat so that cell yield will be increased. These improvements are important if the preparation cost of catalytic cells is considered, especially in the production of cheap materials such as aldehyde.

In a previous study, aldehyde-producing methanol utilizing yeast was isolated and identified as *Hansenula nonfermentans* KYP-1. In this investigation, a methanol-limited continuous culture was established to obtain appropriate cells of *H. nonfermentans* KYP-1 for aldehyde production. Aldehyde production by whole cells grown in continuous culture was then characterized and optimized.

MATERIALS AND METHODS

Strain and Medium

The isolated methanol utilizing yeast, *H. nonfermentans* KYP-1 was used in this study. The methanol medium was composed of methanol, 7.9 g; ammonium sulfate, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; K_2HPO_4, 1.0 g; KH_2PO_4, 1.0 g; NaCl, 0.1 g; and a vitamin mixture solution

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(4) concentrated by 1000 times, 1 ml/l. The pH was adjusted to 6.0 with 1 N NaOH.

Cultivation

Flask culture was performed with a 500 ml flask containing 100 ml of medium at 28℃, stirred at 160 rpm for 48 hours. Continuous culture was carried out at 28℃ in a BIOSTAT-M 2l jar fermentor(B.Braun Co.). The fermentor working volume was controlled at 1 liter by an overflow tube. Feed methanol was set at 0.7% (v/v), corresponding to 5.54 g/l. The pH was kept at 5.3 by automatic adjustment with 1 N NaOH. The impeller speed was 300 rpm and the aeration rate was 1 vvm. Steady state was defined as stability of cell concentration over a time period equal to at least four times turnover of the working volume.

Aldehyde Production

The reaction for aldehyde production was performed with a 50ml flask under conditions of a resting cell system. The 10ml reaction mixture solution contained 300 mg of continuously cultured cells as dry cell weight in a 10 mM phosphate buffer (pH 7.5). The reaction was initiated by adding 1.0 M alcohol. The reaction mixture was shaken reciprocally at 20℃ and 160 rpm for 2 hours, unless otherwise stated. The reaction was terminated by removing the cells by centrifugation, and the amount of aldehyde was measured by gas chromatographic analysis.

Gas Chromatographic Analysis

The amounts of aldehyde and methanol were measured on a Varian Gas chromatograph (Model 3700). The column used for this analysis was 10% Carbowax 20 M on Chromosorb W-HP(2.0 m×2.0 mm ID,SS). The column, injector, and detector temperatures were set at 120℃, 120℃ and 130℃, respectively. Nitrogen gas was used as a carrier at a flow rate of 35 ml/min.

Assay of Alcohol Oxidase Activity

The activity of alcohol oxidase was measured colorimetrically by the method of Couderc and Baratti (2). The reaction mixture contained 14 μmoles of methanol, 140 μmoles of potassium phosphate buffer (pH 7.5), and 0.1 ml of enzyme solution in a total volume of 1.5 ml. Incubation was carried out at 37℃ for 10 minutes and the reaction was terminated by the addition of 0.1 ml of 4 N HCl. Then, a chromotrophic acid solution (0.4% chromotrophic acid in 67% H_2SO_4) was added to the reaction mixture and the mixture was then placed in boiling water for 10 minutes. The optical density of the reaction mixture was measured on a DU-64 Spectrophotometer (Beckman) at 580 nm. The reading was corrected for the blank, which was obtained from the reaction mixture without methanol. One unit was defined as the amount of enzyme forming 1 μmole of formaldehyde in 1 minute. A standard curve was prepared using a standard solution of formaldehyde. The optical density was linearly proportional to 20 μM to 100 μM formaldehyde.

Assay of Catalase Activity

The decomposition of H_2O_2 can be confirmed directly by a decrease in absorbance at 240 nm. The difference in absorbance values (ΔA_{240}) per unit time reflects catalase activity (1). The standard reaction mixture of 1.0 ml, containing 30 mM hydrogen peroxide in a 50 mM phosphate buffer (pH 7.0), was pre-incubated at 25℃ for 5 minutes. The reaction was initiated by the addition of 2.0 ml of enzyme solution to a final volume of 3.0 ml. The reaction was performed in a cuvette of 10 mm light path for 15 seconds and the absorbance decrease was measured with a spectrophotometer at 240 nm. The blank contained the enzyme solution, and a phosphate buffer instead of a substrate. The amount of enzyme units was calculated as:

$$\text{Number of Units} = \frac{2.3}{\Delta t} \times \log \frac{A_1}{A_2} \times 10^3$$

where Δt is a time interval of 15 sec; A_1 is A_{240} at t=0; A_2 is A_{240} at t=15 sec.

Cell Mass Analysis

Cell mass was expressed as dry cell weight. The cell pellet was washed twice with distilled water and dried in a predried aluminum weighing dish for 12 hours at 105℃. An optical density of 1.0 at 660 nm corresponded to 0.36 g of cell mass per liter of broth.

RESULTS AND DISCUSSION

Effect of Methanol Concentration on Growth

The effect of the methanol concentration in the feed medium on growth was examined at a dilution rate of 0.04 hr^{-1}, pH 5.3 at 28℃. As shown in Fig. 1, the cell concentration in the jar fermentor was proportionally increased with an increase in feed methanol concentration, up to 0.7% (v/v). Residual methanol concentration was not detected in this range. Methanol-limited growth was then confirmed by a methanol pulse in the feed methanol at a concentration of 0.7% (v/v). Above this range, methanol residue and growth inhibition were observed. Therefore, the feed methanol concentration was set at a concentration of 0.7% (v/v).

Effect of Dilution Rate

As shown in Fig. 2, a maximum cell concentration of 2.63 g/l was obtained at a dilution rate of 0.06 hr^{-1}, and no residual methanol was detected for dilution rates less than 0.08 hr^{-1}. At dilution rates greater than 0.1 hr^{-1}, the cell concentration decreased with an increase in dilution rate, and the residual methanol concentration increased with an increase in the dilution rate. The maxi-

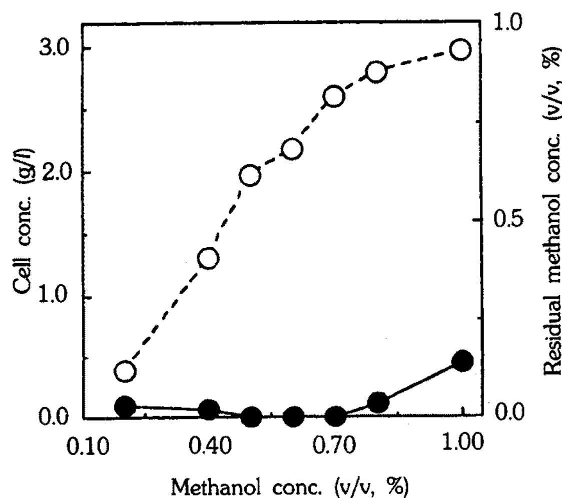

Fig. 1. Effect of methanol concentration on growth.
Cell and residual methanol concentrations were measured in culture broth at a dilution rate of 0.04 hr^{-1}.
Cell conc.; ○, Residual methanol conc.; ●

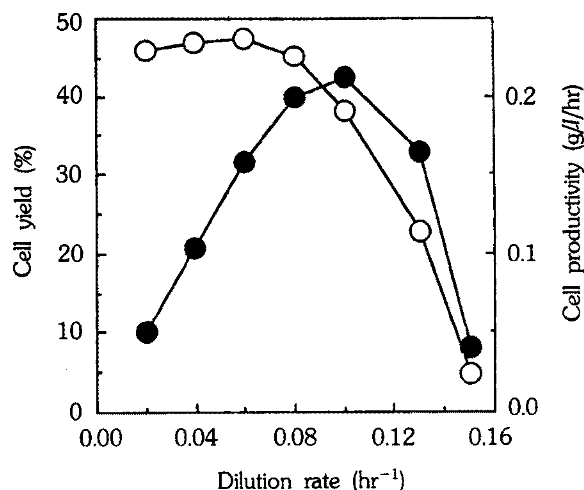

Fig. 3. Effect of dilution rate on cell yield and cell productivity.
Cell yield; ○, Cell productivity; ●

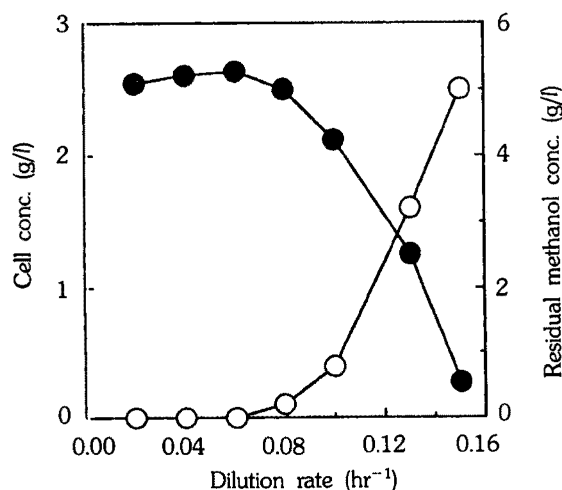

Fig. 2. Steady state behavior in methanol-limited continuous culture.
The methanol concentration in the feed is 0.7% (v/v).
Cell conc.; ●, Residual methanol conc.; ○

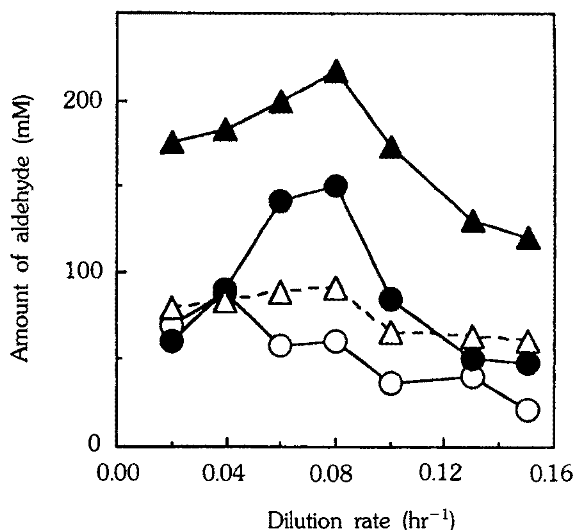

Fig. 4. Effect of dilution rate on the aldehyde production.
The reaction for aldehyde production was carried out with a cell concentration of 30 mg/ml at 20℃ for 120 min.
Formaldehyde; ○, Acetaldehyde; ▲, Propionaldehyde; ●, Acrolein; △

mum dilution rate (μ_{max}) was determined to be 0.155 hr^{-1}.

Cell productivity reached a maximum of 0.219 $g \cdot liter^{-1} \cdot hr^{-1}$ at a dilution rate of 0.1 hr^{-1} (Fig. 3). A maximum cell yield of 47% was obtained at a dilution rate of 0.06 hr^{-1}. The maximum cell productivity and cell yield of *H. nonfermentans* KYP-1 were as good as those of other yeasts studied for SCP production by several workers (5, 6, 7, 12).

Aldehyde production by cells grown with different dilution rates was measured (Fig. 4). The highest productions of acetaldehyde, propionaldehyde and acrolein were showed by cells grown at a dilution rate of 0.08 hr^{-1}, but formaldehyde production reached a maximum at a dilution rate of 0.04 hr^{-1}. The amount of aldehyde produced gradually decreased as the dilution rate increased past 0.08 hr^{-1}. Biocatalytic cells were obtained at a dilution rate of 0.08 hr^{-1}.

To investigate the enzymatic basis for the optimum

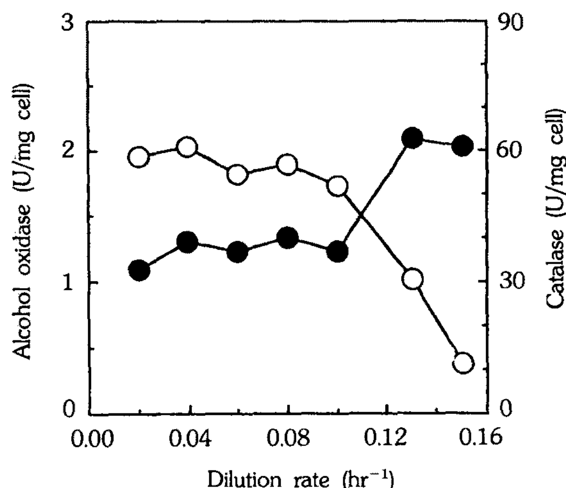

Fig. 5. Effect of dilution rate on activities of alcohol oxidase and catalase.
Alcohol oxidase; ○, Catalase; ●

dilution rate of aldehyde production, activities of alcohol oxidase and catalase were measured at different dilution rates (Fig. 5). The activity of alcohol oxidase per mg of cells was kept constant below 0.08 hr^{-1}, and then gradually decreased with an increase in dilution rate beyond 0.1 hr^{-1}. Catalase activity slightly increased as the dilution rate increased up to 0.08 hr^{-1}, and sharply increased above dilution rate of 0.1 hr^{-1}. The optimum dilution rate for aldehyde production, 0.08 hr^{-1}, is attributed to the effects of alcohol oxidase activity and the peroxidative activity of catalase.

A higher cell yield was obtained in continuous culture than in batch culture. This can be due to the fact that a lower methanol concentration was maintained in the jar fermentor to minimize growth inhibition by methanol. The production of aldehyde was obviously improved by continuous culture, especially the 65% increase in acetaldehyde production. Improved aldehyde production was reflected in higher activities of alcohol oxidase and catalase in continuously cultured cells than in batch cultured cells (Table 1).

Reaction Conditions of Aldehyde Production

The reaction conditions for the production of aldehyde (acetaldehyde, propionaldehyde, acrolein) were optimized using cells grown at a dilution rate of 0.08 hr^{-1}.

Effect of pH

The effect of pH on aldehyde production was measured using 0.1 M citrate buffer (pH 4~6), phosphate buffer (pH 6~8), and borate buffer (pH 8~10). As shown in Fig. 6, the greatest amount of acetaldehyde and acrolein production in the reaction mixture was obtained at an initial pH of 7.5, but the greatest production of propionaldehyde was obtained in a reaction mixture at pH 9.0. Reactions for production of acetaldehyde and acrolein were carried out with a phosphate buffer at pH 7.5. Propionaldehyde production used a borate buffer at pH 9.0.

Table 1. Characteristics of batch and continuous cultures.

Characteristics	Batch[1] culture	Continuous[2] culture
Cell concentration (g/*l*)	3.13	2.50
Cell yield (%)	39	45
Production of aldehyde (mM)		
Formaldehyde	46.2	61.0
Acetaldehyde	139.8	216.6
Propionaldehyde	123.0	150.1
Acrolein	90.1	91.5
Enzyme activity (U/mg·cell)		
Alcohol oxidase	1.787	1.900
Catalase	32.3	40.0

[1]Batch culture was performed in a methanol medium at 28℃ and stirred at 160 rpm for 48 hours. Aldehyde production was measured with cells obtained from a 40 hours culture time.
[2]Continuous culture was carried out with a feed methanol concentration of 0.7% (v/v) at a dilution rate of 0.08 hr^{-1}.

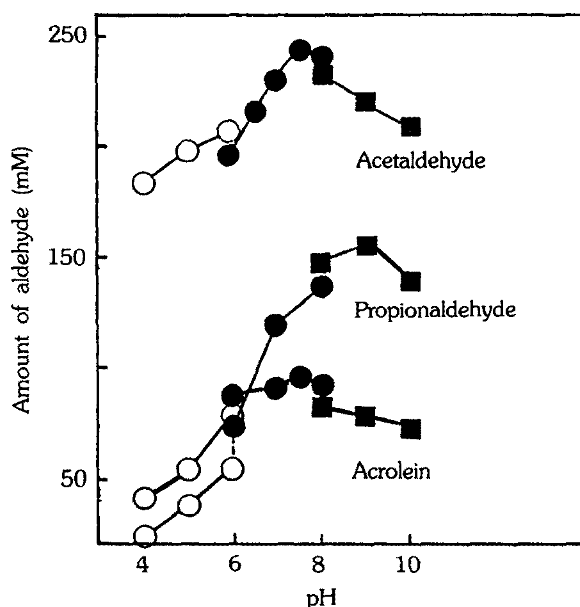

Fig. 6. Effect of pH on the aldehyde production.
The reaction was performed with a cell concentration of 30 mg/m*l* at 20℃ for 120 min.
Citrate buffer (pH 4~6); ○, Phosphate buffer (pH 6~8); ●, Borate buffer (pH 8~10); ■

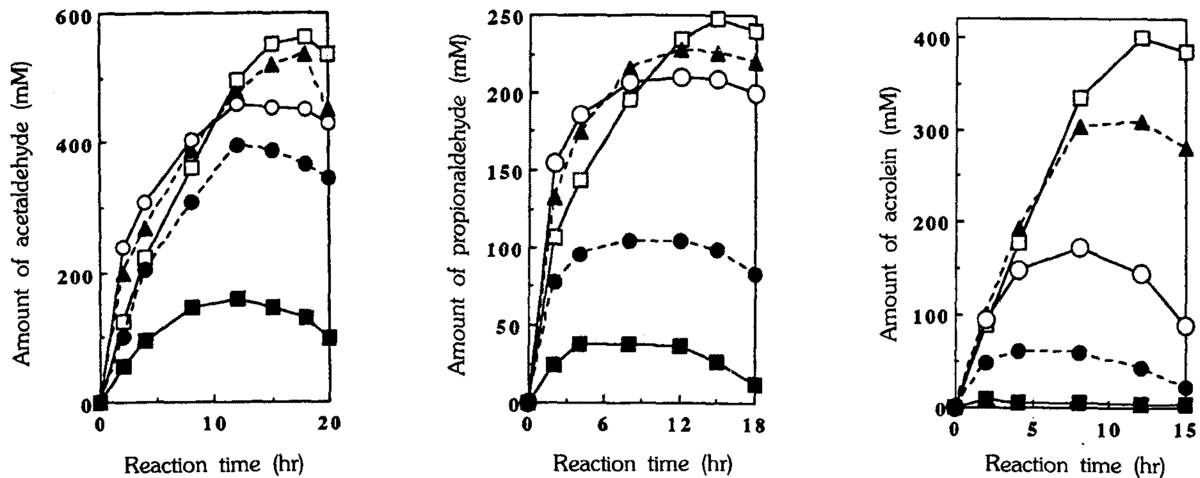

Fig. 7. Effect of temperature on the aldehyde production.
The reaction for the production of acetaldehyde, propionaldehyde and acrolein were performed with an initial pH of 7.5, 9.0 and 7.5, respectively. Other conditions are described in MATERIALS AND METHODS.
4℃; □, 10℃; ▲, 20℃; ○, 30℃; ●, 40℃; ■

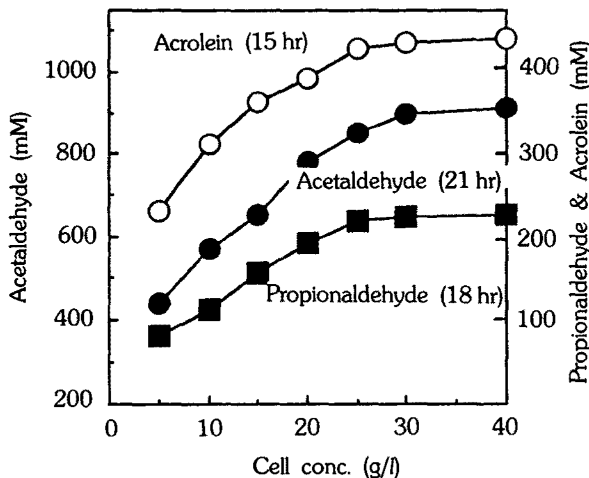

Fig. 8. Effect of cell concentration on aldehyde production.
The aldehyde was produced from 1.0 M alcohol at 4℃.

Effect of Temperature

As shown in Fig. 7, similar patterns were observed for the production of acetaldehyde, propionaldehyde and acrolein at different temperatures. The highest initial rate of aldehyde production was obtained at 20℃, but the maximum amount of aldehyde produced was at 4℃. A greater amount of aldehyde was obtained at a lower temperature than the optimum temperature for the activity of alcohol oxidase. The activity of cells for aldehyde production was relatively depressed by the reaction products, hydrogen peroxide and aldehyde. Alcohol oxidase is susceptible to hydrogen peroxide which masks the catalytic sulfhydryl group of the enzyme (9). Aldehyde might inactivate the enzyme by forming a Schiff's base with the amino groups of the enzyme (11). These inhibitory non-catalytic reactions can be suppressed by lowering the reaction temperature. Furthermore, the vaporization of aldehyde would be reduced at low temperature. Therefore, reactions for aldehyde production were perfomed at 4℃.

Effect of Cell Concentration

The amount of produced acetaldehyde increased with an increase in cell concentration, up to 30 mg/ml (Fig. 8). In the production of propionaldehyde and acrolein, a greater amount of aldehyde was obtained with a higher cell concentration, up to 25 mg/ml.

Effect of Alcohol Concentration

The effect of alcohol (ethanol, propanol and allyl alcohol) concentration on the production of corresponding aldehyde is shown in Fig. 9. A higher concentration of acetaldehyde was obtained with a higher initial concentration of ethanol, up to 1.5 M, and 915.7 mM of acetaldehyde was produced after 21 hours, with a conversion rate of 61%. The greatest amount of propionaldehyde was produced with 1.25 M propanol in 18 hours reaction time, but the highest conversion rate, 32.7%, was obtained with 0.75 M propanol. When the concentration of allyl alcohol was 1.0 M, 440 mM of acrolein was produced with a conversion rate of 44% in a reaction time of 15 hours.

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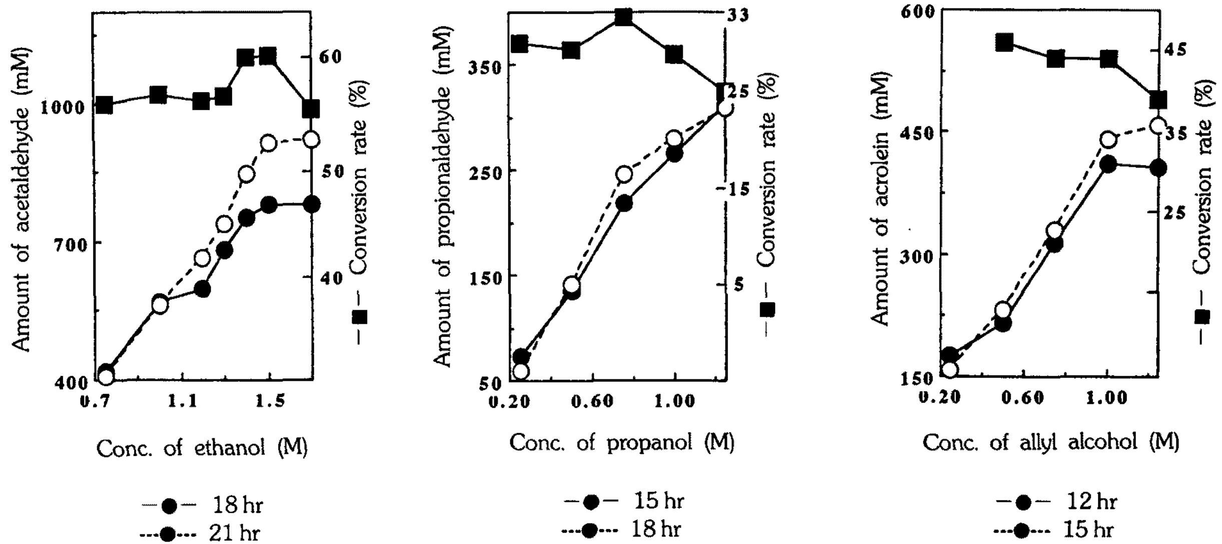

Fig. 9. Effect of alcohol concentration on aldehyde production.
The reaction was carried out under the optimized conditions.

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