IDENTIFICATION OF "METHYL FORMATE-PRODUCING ENZYME" IN CELL-FREE EXTRACT OF *Pichia methanolica*

Indentifikasi "enzim pembentuk metil format" dari ekstrak sel *Pichia methanolica*

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ABSTRACT

Cell-free synthesis of methyl formate by extract of *Pichia methanolica* was investigated. It was observed that the addition of formaldehyde could increase the methyl formate-producing activity compared to control experiment which contained methanol as a sole substrate for methyl formate production. On the other hand, the addition of formic acid, formate, and S-formylglutathione or GSH did not increase the activity. Methyl formate-producing activity disappeared upon gel filtration of the cell-free extract on Sephadex G-25. However, the full activity was recovered with the addition of NAD^+. These results indicated that methyl formate-producing enzyme was present in cell-free extract. This enzyme was found to be responsible for methyl formate formation from methanol and formaldehyde when its cofactor (NAD^+) was available. Based on this finding, the standard assay system for methyl formate-producing enzyme could be constructed. Purification of methyl formate-producing enzyme from cell-free extract was possible due to the availability of the standard assay system constructed in this experiment.

Key words : identification; methyl formate-producing enzyme

ABSTRAK

Penelitian mengenai pembentukan metil format oleh ekstrak *Pichia methanolica* telah dilakukan. Ditemukan bahwa penambahan formaldehid dapat meningkatkan aktivitas pembentukan metil format bila dibandingkan dengan kontrol yang mengandung metanol sebagai satu-satunya substrat untuk membentuk metil format. Di lain pihak, penambahan asam format, garam format, dan S-formylglutation atau GSH tidak mempengaruhi

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aktivitas pembentukan metil format. Ekstrak sel yang telah dilewatkan melalui kromatografi gel Sephadex G-25 kehilangan kemampuan membentuk metil format. Namun, kemampuan membentuk metil format dapat dipulihkan dengan penambahan NAD^+. Hal ini menunjukkan bahwa enzim pembentuk metil format terdapat dalam estrak sel dan enzim inilah yang bertanggung jawab terhadap pembentukan metil format dari metanol dan formaldehid apabila tersedia NAD^+ yang berfungsi sebagai kofaktornya. Hasil temuan ini memungkinkan dibuatnya sistem pengukuran aktivitas enzim pembentuk metil format. Hal ini membuka kemungkinan untuk melakukan purifikasi enzim pembentuk metil format dari esktrak sel.

Kata kunci : identifikasi; enzim pembentuk metil format

INTRODUCTION

The principal reaction of methanol dissimilation in methanol-utilizing yeasts involves alcohol oxidase-catalase system in peroxisomes, and formaldehyde dehydrogenase, *S*-formylglutathione hydrolase and formate dehydrogenase in cytoplasm (Tani, 1984). The enzymes which are responsible for methanol oxidation to CO_2 through formaldehyde and formate have been purified and characterized in several strains of methanol-utilizing yeasts. Alcohol oxidase was first found in the cell-free extract of *Kloeckera* sp. No.2201 and characterized by Tani *et al*(1972). Formaldehyde dehydrogenase of *Kloeckera* sp. NO.2201 (Ogata *et al.*, 1975) *Candida boidinii* (Schutte *et al.*, 1976) *Pichia* sp. NRRL-Y-11328 (Patel *et al.*, 1983), and *Hansenula polymorpha* (Van Dijken *et al.*, 1976) were also purified and characterized. Subsequently, *S*-formylglutathione hydrolase was reported to be present in *C. boidinii* (Neben *et al.*, 1980) and *Kloeckera* sp. No.2201 (Kato *et al.*, 1980). Finally, formate dehydrogenase which takes a part in the oxidation of formate to CO_2 was discovered in the strains of *Kloeckera* sp. No.2201 (Kato *et al.*, 1974) *Pichia pastoris* (Allais *et al.*, 1983), *C. boidinii* (Schutte *et al.*, 1976) and *H. polymorpha* (Van Dijken *et al.*, 1976). Based on those findings, the dissimilatory sequence of methanol in methanol-utilizing yeasts has been established.

Ester formation by yeast has been reported by several researchers (Davies *et al.*, 1951; Peel, 1951; Tabachnick & Joslyn, 1953). Since ester formation during beer fermentation is important due to its great effect on the flavor quality of alcoholic beverages (Yamakawa *et al.*, 1978), the mechanism of acetate ester formation has been investigated extensively. The accepted theory on the mechanism of ester formation by brewers' yeast is based largely on the Nordstrom's work (Nordstrom 1961; Nordstrom 1962a; Nordstrom 1962b) who proposed that the biosynthesis of acetate esters are occurring through the

esterification of alcohols with acetyl-CoA in the presence of an ester-synthesizing enzyme which by some authors is called alcohol acyltransferase (Yamakawa *et al.*, 1978; Yamauchi *et al.*, 1989a; Yamauchi *et al.*, 1989b) or alcohol acetyl transferase (Yoshioka & Yokotsuka, 1981). The proposal was supported with evidence by Howard and Anderson (Howard & Anderson, 1976) who found that ethyl acetate was formed from ethanol and acetyl-CoA by the sediment prepared at 105,000 x *g* from cell-free preparations of brewer's yeast. Nevertheless, other reports (Schermer *et al.*, 1976; Yamakawa *et al.*, 1978) showed that the formation of ethyl acetate could also be occurring through the reverse reaction of esterase. Thus, depending on esters formed and the strains of organisms involved, both of these mechanisms are possibly responsible for ester biosynthesis in yeast. Yet, there is no available information concerning methyl formate formation by yeast.

Since it was found that methyl formate could be produced from methanol by cell suspension of methanol-utilizing yeasts (Sembiring, 1991) it becomes necessary to identify the enzyme responsible for this ester formation from methanol. Therefore, the study of cell-free system formation of methyl formate was conducted. Identification of substrates and cofactor requirement of "methyl formate-producing enzyme" in cell-free extract of *Pichia methanolica* are described in this report. On the basis of the results of this study, the assay system for the enzyme was constructed. This assay system will be very useful for the purification of the enzyme.

MATERIALS AND METHODS

Chemicals

Formaldehyde solutions were prepared by heating aqueous solution of paraformaldehyde at 120°C for 30 to 60 min. *S*-formylglutathione was enzymatically synthesized as described by Van Dijken (1976). Formaldehyde dehydrogenase of yeast and lactate dehydrogenase of pig heart were purchased from Oriental, Yeast Co., Ltd., Japan. All other chemicals were commercial products of analytical grade and used without further purification.

Microorganism and cultivation

P. methanolica was used throughout these studies. A pre-culture (5 ml) of basal medium (Sembiring, 1991) containing 2%(v/v) ethanol was inoculated and incubated on reciprocal shaker at 28°C for 3 days. Main culture were performed by inoculation of 500 ml of basal medium containing 1.5%(v/v) methanol, with 10 ml of full growth pre-culture then incubated on reciprocal shaker at 28°C for 4

days. Cells were harvested by centrifugation at 3830 x *g*, 4°C for 15 min and washed twice with 100 mM of potassium phosphate buffer, pH 7.0. Washed cells are suspended in 100 mM potassium phosphate buffer, pH 7.0, containing 5 mM of dithiothreitol (DTT). The cell suspension was stored at 4°C until used.

Preparation of cell-free extract

A given amount of cell suspension was subjected to KUBOTA Insonator Model 200M at 2MHz, 4°C for 30 min. Cell debris was removed by centrifugation at 14,490 x*g*, 4°C for 20 min. The clear supernatant was used as cell-free extract.

Determination of protein content

The protein content of cell-free extract and fractions of Sephadex G-25 were determined by the method of Bradford (1976) with a Laboratory Kit of BIO-RAD PROTEIN ASSAY. Bovine serum Albumine was used as a protein standard.

Assay system of "methyl formate-producing activity"

Cell-free extract or fractions after Sephadex G-25 gel filtration was added to the assay mixture of 1.0 ml, containing 100 mM of potassium phosphate buffer, pH 7.0, 1000 mM of methanol, incubated in water bath-reciprocal shaker at 15°C for 1 h. Methyl formate-producing activity is expressed as the amount of methyl formate produced at this assay system conditions. Formadehyde was determined according to Nash method (Nash, 1953)

Gas-liquid chromatographic analysis

Methyl formate concentration in the assay mixture was determined by a Shimadzu Gas-Liquid Chromatography GC7-A equipped with a Flame Ionization Detector (FID). The glass spiral column, Porapak Q 2.1 m, 3 mm i.d., stationary phase was used. Column temperature was maintained isothermally at 170°C. Injection temperature was kept at 190°C. Carrier gas was N_2 at a flow rate of 50 ml/min. Two microlitre sample was injected for each assay. The amount of methyl formate was automatically calculated by means of calibration program which had been constructed with the authentic sample.

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Sephadex G-25 gel filtration

The Sephadex G-25 was packed into column (2 x 30 cm) and equilibrated with 100 mM potassium phosphate buffer, pH 7.0. The samples (3 ml of cell-free extract) were applied into the column and then the buffer was allowed to flow at a rate of 1 ml per min. Fractions equal to 3-ml were collected and subjected to methyl formate-producing activity assay system.

Addition of various compounds

Effects of various compounds were investigated in attempt to identify the substrate(s) required for methyl formate formation. Methanol was always included as one of the required substrates with the assumption that the substrate in question should react with methanol in order to produce methyl formate in the presence of "methyl formate-producing enzyme".

RESULTS AND DISCUSSION

Optimization of methanol concentration

Methanol concentration was optimized in cell-free system with respect to methyl formate-producing activity. The result in Fig. 1 shows that the activity increased with the increament of methanol concentration up to the concentration of 1000 mM and then leveled off towards higher concentration. The optimum concentration was therefore 1000 mM. This value is two times lower than the optimum concentration of methanol in cell suspension system. The difference might be due to the sensitivity of "methyl formate-producing enzyme" against methanol since in cell-free system the enzyme is directly subjected to high concentration of methanol.

In the case of cell suspension system, the available concentration of methanol in the cell is probably lower than the apparent one since methanol has to enter the cell in order to be in contact with the enzyme. Further more, methanol or its intermediates such as formaldehyde might changes the permeability of cell membrane and so to decrease the rate of methanol diffusion into the cell.

Effect of various compounds

The effect of various compounds on methyl formate-producing activity was studied in attempt to identify the substrate(s) required for methyl formate production catalyzed by an enzyme in question which is tentatively called "methyl formate-producing enzyme". Among the additives used, it was found that only formaldehyde could enhanced the activity. The other additives such as

formic acid, formate, S-formylglutathione, GSH did not show any effect (results are not presented). Thus, it was hypothesized that "methyl formate-producing enzyme" requires methanol and formaldehyde to produce methyl formate.

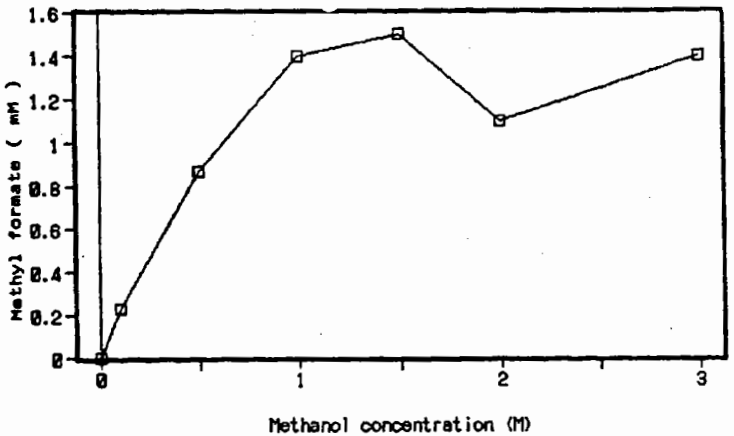

Fig. 1. Effect of methanol concentration on methyl formate-producing activity. Activity was determined with the standard assay system with 0.975 mg/ml of protein (cell-free extract) and various concentration of methanol

Effect of formaldehyde addition

Formaldehyde is directly produced from methanol oxidation by alcohol oxidase in methanol-utilizing yeasts. This compound plays an important role in methanol metabolism since it serves as a key substrate in methanol dissimilation as well as assimilation. Pertaining to methyl formate production, it was reported that in chemical synthesis this ester can be synthesized from methanol and formaldehyde or through the dimerization of formaldehyde (Aim, 1982).

Based on those possibilities, it was thought that methyl formate formation in methanol-utilizing yeasts may also occur through a reaction between methanol and formaldehyde since formaldehyde concentration must be kept at low level due to its toxicity. Such hypothesis can of course be tested by the addition of formaldehyde to cell-free system which already contained methanol as a substrate for methyl formate production.

The result of the experiment presented in Fig.2 seems to support the hypothesis. It was observed that methyl formate-producing activity surprisingly

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increased with the increament of formaldehyde concentration. At the concentration of 60 mM of formaldehyde, the production of methyl formate increased by 30 times compared to control experiment which contained methanol as a sole substrate. This result clearly suggested that methanol and formaldehyde may have served as the substrates for "methyl formate-producing enzyme" in methyl formate formation. Thus, formaldehyde produced from methanol oxidation by alcohol oxidase might have acted as the second substrate when methyl formate was produced in the reaction mixture containing methanol as a sole substrate. In essence, methyl formate production from methanol was therefore directly dependent on alcohol oxidase since it can supply formaldehyde from the oxidation of methanol.

In so far, there is no report concerning ester-synthesizing enzyme that can produce ester from aldehyde and alcohol. The fact that formaldehyde and methanol can be the substrate for methyl formate formation may contribute to the regulation mechanism of formaldehyde concentration during the course of methanol oxidation by methanol-utilizing yeasts.

The conversion of formaldehyde to a less toxic compound such as methyl formate seems important to yeast since methyl formate can then be decomposed through hydrolysis by esterase to produce methanol and formic acid. Methanol will undergoes oxidation catalyzed by alcohol oxidase while formic acid can be oxidized to CO_2 with the help of formate dehydrogenase.

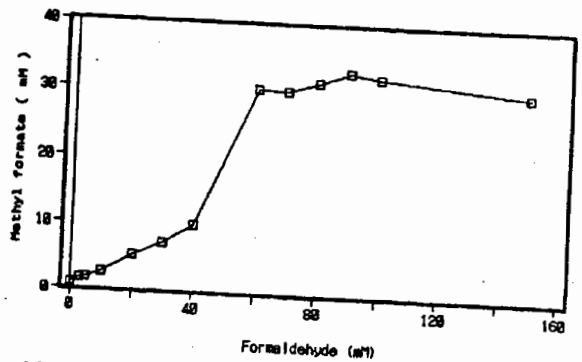

Fig. 2. Effect of formaldeldehyde addtion on methyl formate-producing activity Activity was measured in 1 ml reaction mixture containing 100 mM of potassium phosphate buffer, pH 7.0, 1000 mM of methanol, 4.27 mg/ml of protein (cell-free extract) and various concentration of formaldehyde incubated on water bath reciprocal shaker at 15°C for 1 h. Methyl formate produced was determined by gas-liquid chromatographic analysis as described in Materials and Methods.

Since it was found that methanol and formaldehyde could serve as the substrates for methyl formate production, their concentrations were optimized with respect to methyl formate-producing activity. Figure 3 shows that the activity was directly dependent on methanol and formaldehyde concentrations.

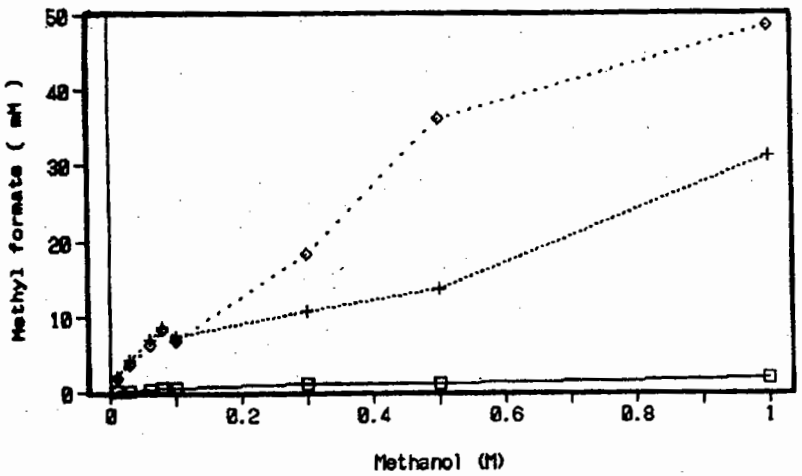

Fig. 3. Effect of methanol and formaldehyde concentration on methyl formate-producing activity. Activity was determined with the standard assay system with 1.62 mg/ml of protein (cell-free extract) and various concentration of methanol and formaldehyde. (□) contained 10 mM; (+) 60 mM; and (◇) 100 mM of formaldehyde

Gel filtration on Sephadex G-25

Cell free extract was applied to Sephadex G-25 column chromatography. Fractions obtained were then subjected to methyl formate-producing activity assay system containing 1000 mM of methanol, 60 mM of formaldehyde. The elusion pattern on Sephadex G-25 is shown in Fig. 4.

It was astounding to find that fractions of Sephadex G-25 were devoid of methyl formate-producing activity measured in the reaction mixture containing methanol and formaldehyde. In fact, the same activity was observed in the cell-free extract before gel filtration. However, full activity could be recovered upon NAD^+ addition about 1 mM as it can be seen in Table I. On the other hand, only a slight recovery of the activity was observed when 1 mM of $NADP^+$ was

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added. This may be due to the impurity of $NADP^+$ used, meaning that the presence of NAD^+ in $NADP^+$ might caused slight recovery of the activity. It was also found that FAD^+ could not replace NAD^+. Thus, those results evidently demonstrated that "methyl formate-producing enzyme" requires NAD^+ for its activity which probably had lost during the course of gel filtration on Sephadex G-25 due to the low molecular weight of NAD^+.

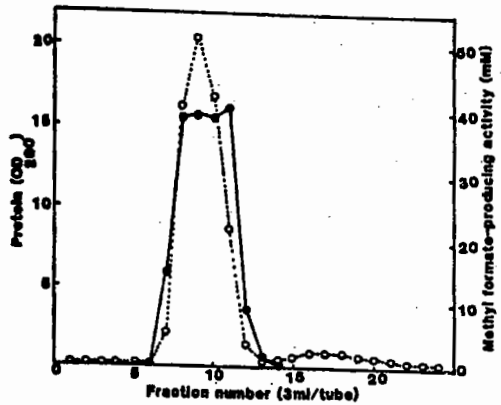

Fig. 4. Elution pattern of Sephadex G-25 gel filtration colum chromatography. Sephadex G-25 was packed into a column (2x30 cm) and equilibrated with 100 mM potassium phosphate buffer, pH 7.0. The sample (3 ml of cell-free extract) was applied into the column and then the buffer was allowed to flow at a rate of 1 ml per min and 3 ml fractions were collated. (o) protein absorbance at 280 nm (●) methyl formate-producing activity.

Table I. Cofactor* requirement of "methyl formate-producing enzyme".

Cofactor	Concentration (mM)	Methyl formate-producing activity (mM)
None (control)	0 (wihtout addition)	0
NAD+	1	38.98
NADP+	1	0.60
FAD+	1	0

*) Cofactor was added to one ml of reaction mixture containing 100 mM of potassium phosphate buffer, pH 7.0, 1000 mM of methanol, 60 mM of formaldehyde and 2 mg/ml of protein (fraction No. 11 of Sephadex G-25), incubated in water bath reciprocal shaker at 15°C for 1 h. Methyl formate-producing activity was measured as described in Materials and Methods.

On the basis of those finding, it is possible to construct a complete assay system for "methyl formate-producing enzyme". The availability of reliable assay system can led to the purification of the enzyme responsible for methyl formate formation from methanol and formaldehyde. The purification of the enzyme will be very useful in order to understand in more detail of methanol metabolism in methanol-utilizing yeasts.

Effect of enzyme stabilizer on the activity

The significant loss of the activity was observed when cell-free extract was stored at 4°C for 24 h. The study of stabilizing effect of several enzyme stabilizer on the activity was conducted. Enzyme stabilizer was added into 1 ml of cell-free extract and incubated at 4°C for 24 h. The remaining activity was measured upon the completion of incubation time. Among the enzyme stabilizers tested (Table II) it was discovered that DTT at the concentration of 5 mM could preserve the activity about 87.5% followed by mercaptoethanol at the concentration of 10 mM which caused the activity to remain as much as 64.7%.

Table II. Effect of enzyme stabilizers[a] on the stability of methyl formate-producing activity.[b]

Enzyme astabilizer	Concentration	Activity (%)
Control (None)	0 mM	7.75
Dithiothreitol (DDT)	5 mM	87.56
2-Mercaptoethanol	10 mM	64.68
Cysteine	10 mM	18.32
Ascorbic acid	10 mM	3.80
DMSO	7 %	8.94
Polyvinypolypyrrolidone	5 %	10.23
BSA	5 %	8.94
Sucrose	20 %	12.27
D-Sorbitol	20 %	17.88
Glycerol	20 %	15.51
Glucose	20 %	7.60

a) The effects of enzyme stabilizer were studied in 1 ml of cell- free extract contained each of stabilizer as much as indicated concentration, incubated at 4°C for 24 h. The remaining activity was determined in 1 ml of reaction mixture containing 100 mM of potassium phosphate, pH 7.0, 1000 mM of methanol, 60 mM of formaldehyde, and 1.28 mg/ml of protein (cell-free extract), incubated in water bath reciprocal shaker at 15°C for 1 h. Methyl formate-producing activity was measured as described in Materials and Methods.

b) Incubated at 4°C for 24 h

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The effect of DTT is in agreement with the result obtained during the course of methyl formate production from methanol by cell suspension in which 3 mM of DTT could slightly increase the productivity of methyl formate.

The stabilizing effect of DTT is very important during the course of purification since it can stabilize the activity along the time-consuming process. Therefore, 5 mM of DTT was always included in the potassium phosphate buffer used throughout the investigations.

Determination of initial velocity

Various concentration of protein (cell-free extract) was added to 1 ml of reaction mixture containing 100 mM of potassium phosphate buffer, pH 7.0, 1000 mM of methanol, and 60 mM of formaldehyde, incubated in water bath reciprocal shaker at 15°C.

At certain interval time, methyl formate produced was determined by gas-liquid chromatographic analysis. It was learned that the initial velocity appeared to be directly dependent on the protein concentration (Fig.5). The relationship between protein amount and methyl formate-producing activity indicated the presence of "methyl formate-producing enzyme" in the cell-free extract.

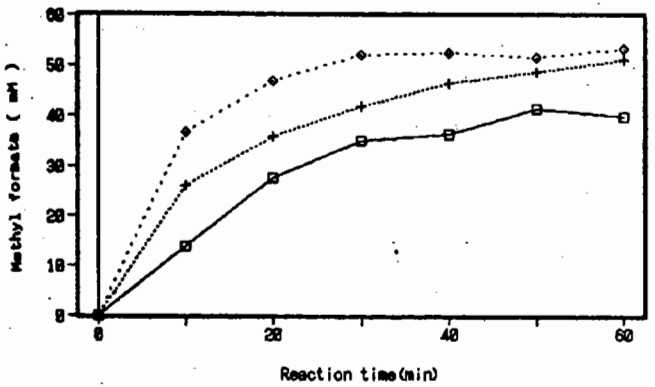

Fig. 5. Effect of protein concentration on methyl formate-producing activity
Methyl formate-producing activity was measured in 1 ml reaction mixture containing 100 mM of potassium phosphate buffer, pH 7.0, 1000 mM of methanol, 60 mM of formaldehyde, incubated on water bath reciprocal shaker at 15°C. Methyl formate produced was determined by gas-liquid chromatographic analysis as described in Materials and Methods. (□) contained 0.3 mg/ml (+) 0.6 mg/ml and (◇) 1.2 mg/ml of protein (cell-free extract).

CONCLUSION

The results of this experiment strongly suggested that methyl formate formation in methanol-utilizing yeast is catalyzed by an enzyme which is tentatively called "methyl formate-producing enzyme". The substrate for methyl formate formation were found to be methanol and formaldehyde. Further more, the fact that the activity was directly affected by NAD^+ strongly supported the existence of "methyl formate-producing enzyme". Therefore, the hypothesis proposed that methyl formate is enzymatically produced from methanol and formaldehyde is most likely to be true. This finding has led to the possibility of constructing a complete assay system for methyl formate-producing activity and therefore opened the possibility to purify the enzyme. The purification of the enzyme is very useful in attempt to achieve a more complete elucidation of methanol metabolism in methanol-utilizing yeasts.

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