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# Screening For Methanol-Utilizing Yeasts Having Methyl Formate-Producing Activity

# Seleksi Khamir Pengguna Metanol yang Mampu Menghasilkan Metil Format

**by:**
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**ABSTRACT**
Methanol-utilizing yeasts were screened for methyl formate- producing activity. Eleven strains out of twenty strains of yeasts tested were found to have the activity. *Pichia methanolica* showed the highest activity, followed by *Candida sp.* N-16, *Candida sp.* 1-B, *Pichia pinus*, and *Candida sp.* 25-A, respectively. Methyl formate was found to accumulate in the cultural media of those strains during the course of the their growth in methanol- containing media. Along with the methyl formate-producing activity it was found that resting cells also had a hydrolyzing activity against methyl formate. Cultivation and reaction conditions were optimized with respect to methyl formate production. The methyl formate-producing activity was strongly induced by methanol but weakly induced by glycerol. On the other hand, the methyl formate-producing activity was not induced by ethanol and glucose. However, methyl formate-hydrolyzing activity was induced by methanol, glycerol, glucose, and ethanol although with different intensities.

***Key words*** **:** methanol yeast - methyl formate

**ABSTRAK**
Telah dilakukan seleksi pada dua puluh strain khamir pengguna metanol untuk mendapatkan strain yang mampu menghasilkan metil format. Dari dua puluh strain khamir yang diuji didapatkan sebelas strain khamir penghasil metil format. *Pichia methanolica* mempunyai aktivitas terbesar, lalu diikuti oleh *Candida sp.* N-16, *Candida sp.* 1-B, *Pichia pinus* dan *Candida sp.* 25-A. Di samping kemampuan menghasilkan metil format ternyata khamir tersebut juga mempunyai aktivitas menghidrolisis metil format. Ditemukan pula bahwa metil format terakumulasi dalam cairan kultur ke empat strain khamir tersebut apabila ditumbuhkan dalam medium yang mengandung metanol. Kondisi kultivasi dan kondisi reaksi pembentukan metil format dioptimasi dalam hubungannya dengan produktivitas metil format. Hasil penelitian

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menunjukkan bahwa kemampuan menghasilkan metil format sangat terinduksi oleh metanol dan sedikit terinduksi oleh gliserol tetapi tidak terinduksi oleh etanol dan glukosa. Di lain pihak, aktivitas menghidrolisis metil format ternyata diinduksi oleh metanol, gliserol, etanol, maupun glukosa, meskipun dalam intensitas yang berbeda.

***Kata kunci*** : khamir metanol - metil format

## Introduction

Methyl formate is presently synthesized from methanol, using metal catalysts at high temperature and high pressure (1,2). This chemical is particularly important in C-1 chemical industry being used as a raw material for producing carbon monooxide of high purity, methyl formamide and acetic acid (2). On the other side, carbon monooxide is a most versatile C-1 building block for the synthesis of a wide variety of chemicals (3). In the field of preservation, methyl formate is also used as fumigant or larvacide for tobacco, dried fruits and cereals (5). However, there is no information concerning the production of methyl formate by microorganisms. Tani *et al.* (1985) reported that methyl formate was detectable in the reaction mixture of formaldehyde production by a mutant strain of methanol-utilizing yeast, *Candida boidinii* S2. Based on that finding, research on methyl formate production from methanol was conducted.

Screening for methanol-utilizing yeasts having methyl formate-producing activity was carried out. As many as twenty strains of methanol yeasts were screened for methyl formate production from methanol, using resting-cell system. Cultivation and reaction conditions were optimazed with respect to methyl formate-producing and hydrolyzing activity.

## Materials and Methods

### *Chemicals*

All reagents were purchased from commercial sources and used without further purification.

### *Microorganism*

Strains of methanol-utilizing yeasts preserved in Laboratory of Fermentation and Metabolic Regulation, Faculty of Agriculture, Kyoto

University (Japan) were screened for methyl formate production from methanol, using resting-cell system.

Basal medium for cultivation of methanol-utilizing yeasts composed of 7.63 g $NH_4Cl$, 2.81 g $KH_2PO_4$, 0.59 g $MgSO_4.7H_2O$, 55 mg $CaCl_2.2H_2O$, 37.5 mg $FeCl_3.6H_2O$, 0.45 g $EDTA_2Na$, 10 ml metal solution, 1 ml vitamin solution, 7.5 ml of methanol in 1000 ml of distilled water. pH was adjusted to 6.0 by addition of 2 N NaOH. Methanol was added after autoclaving or just before inoculation. *Vitamin solution* composed of 5 mg biotin, 500 mg thiamnie in 100 ml distilled water and storaged at - 20°C. *Metal solution* composed of 0,85 g $MnSO_4.3H_2O$, 1.1 g $ZnSO_4.7H_2O$, 0.2 g $CuSO_4.5H_2O$, 0.14 g $CoCl_2.2H_2O$, 0.13 g $Na_2MoO_4.2H_2O$, 0.2 g $H_3BO_3$, 0.03 g in 500 ml distilled water and storaged at 4°C. For slant culture 2% of agar was added into basal medium.

*Cultivation*

Each strain was inoculated into 5 ml basal medium containing 2% of ethanol, in 16.5-mm i.d test tube, incubated at 28°C for 3 days with reciprocal shaking at 240 rpm. After the full growth, each of 10 ml of seed culture was inoculated into 500 ml basal medium in 3-l shaking flask containing 1.5% methanol as a sole carbon and energy source, incubated at 28°C for 4 days with reciprocal shaking at 100 rpm. Growth was followed by spectrophotometer at the absorbance of 610 nm. In the case of other carbon source, 500 ml of basal medium contained 1.5% glyserol, ethanol, and glucose, instead of methanol. Cells were harvested by centrifugation at 3830 x g, 4°C for 15 min, washed twice with 100 mM of potassium phosphate buffer, pH 7.0. Washed cells were subjected to methyl formate-producing activity assay system.

*Assay system for methyl formate production*

For the assay of methyl formate, stocked cell suspension in 100 mM potassium phosphate buffer, pH 6.0, was routinely used. A total volume of 10 ml of reaction mixture in a 30-ml conical flask,. containing 100 mM of potassium phosphate buffer, pH 6.0, 500 mM of methanol, and 3.5 g-d.c.w/l of washed cells, incubated on reciprocal shaker at 10°C. The flask was sealed with rubber stopper in order to minimize evaporation. At certain interval time, 1 ml of reaction mixture was taken and

centrifuged at 3830 x g, 4°C, for 1 min to remove cells and the resultant supernatant was subjected to Gas-liquid chromatographic analysis for methyl formate determination.

*Gas-liquid chromatographic analysis*

Methanol and methyl fomate concentration in reaction mixture were determined by a Shimadzu Gas-Liquid Chromatography GC-7A 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 temperaure was kept at 190°C. Carrier gas was $N_2$ at a flow rate of 50 ml/min. The amount of methanol and metyl formate were automatically calculated by means of calibration program which had been constructed with the authentic sample. A typical gasliquid chromatogram is shown in *Fig. 1.*

*Growth and methyl formate accumulation in cultural medium*

Inoculum was prepared by inoculation of 100 ml of basal medium in a 500-ml shaking flask containing 2% of ethanol, incubated at 28°C for 3 days with reciprocal shaking at 100 rpm. After the full growth, 10 ml of seed culture was inoculated into 500 ml of basal medium containing 1.5% methanol as a sole carbon and energy source in a 2-l shaking flask incubated on reciprocal shaker at 100 rpm, 28°C. Growth was followed spectrophotometrically at the absorbance of 610 nm. Methyl formate accumullation and remaining methanol concentration were determined by gas liquid chromatographic analysis. The measurement was continued until stationary growth phase was completed.

*Assay of methyl formate-hydrolyzing activity*

Ten milliliter of reaction mixture in a 30-ml conical flask, containing 100 mM of potassium phosphate, pH 6.0, 3.5 g-d.c.w/l of washed cells and 20 mM of methyl formate, incubated at 10°C for 60 min. Remaining concentration of methyl formate was determined by gas-liquid chromatigraphic analysis. pH of reaction mixture was performed at 6.0 since it was found that methyl formate was most stable at this pH, when incubated at 4°C or 28°C for 17 h (*Fig.2*). Methylformate-hydrolizing activity was expressed in mmole methyl formate hydrolized per min after correction by control which was run without cells.

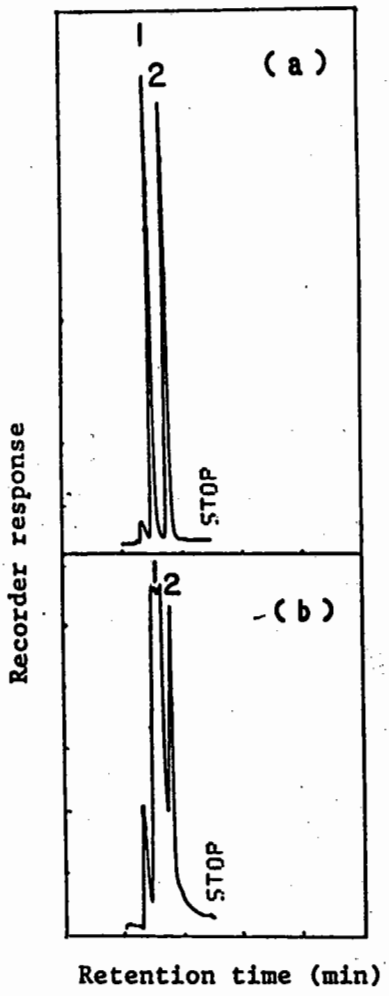


Fig. 1. Typical gas-liquid chromatogram of methyl formate production. Conditions of gas-liquid chromatographic analysis by Shimadzu GC-7A were as described in Materials and Methods. (a) authentic sample (b) sample obtained from reaction mixture of methyl formate production (1) methanol (2) methyl formate.

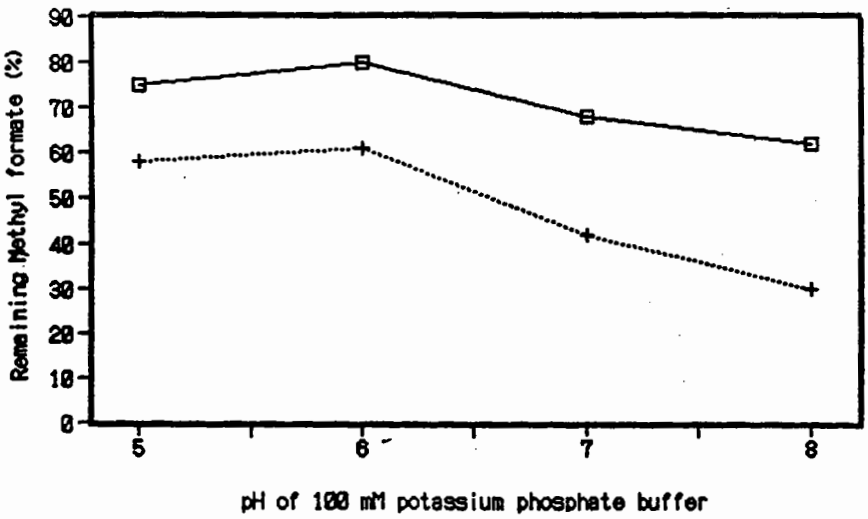


Fig. 2. Effect of pH and temperature on methyl formate stability. Methyl formate solutions in 100 mM potassium phosphate buffer were incubated at indicated temperature for 17 h without agitation. (□) incubated at 10°C (+) incubated at 28°C.

## Results And Discussion

### *Screening for methyl formate producer*

Results of screening are summarized in *Table 1.* Among twenty strains of methanol-utilizing yeasts tested, it was found that eleven strains could produce methyl formate from methanol in standard assay system after incubated for 8, 16 and 24 h. The productivity varied from 0.1 to 21.5 mM after 24 h incubation time.

*Pichia methanolica* showed the highest productivity, produced 21.5 mM methyl fromate from 500 mM of methanol. *Candida sp.* N-16 was the second, produced 16,3 mM, followed by *Candida sp.* 1.B, *Pichia pinus,* and *Candida sp.* 25-A, produced 11.2 mM, 9.7 mM and 8.9 mM, respectively. *Candida boidinii* S2 which was firstly found to have activity of producing methyl formate during the course of formaldehyde production from methanol showed only a relatively low activity in this result.

*Pichia methanolica, Candida sp.* N-16, *Candida sp.* 1-B and *Pichia pinus* were subjected to the subsequent screening.

**Table 1. Methyl formate production by methanol-utilizing yeasts.**

| Methanol-utilizing yeasts | Methyl formate produced (m mol/l) (8h) | (16 h) | (24 h) |
|---|---|---|---|
| *Candida boidinii* | 0 | 0 | 0 |
| *Candida* sp. *N-16* | 0 | 19.3 | 16.3 |
| *Candida boidinii* S2 | 7.0 | 2.7 | 2.9 |
| *Candida* sp. 25-A | 7.0 | 7.8 | 8.9 |
| *Candida* sp. 1- B | 7.6 | 15.0 | 11.2 |
| *Candida methanolica* | 6.6 | 5.6 | 3.0 |
| *Candida boidinii* S2 SA-051 | 2.6 | 1.3 | 1.3 |
| *Candida succiphila* | 0 | 0 | 0 |
| *Candida boidinii* No. 2201 | 0 | 0 | 0 |
| *Candida boidinii* S2 AOU-1 | 3.2 | 2.9 | 2.9 |
| *Hansenula polymorpha* | 0 | 0 | 0 |
| *Hansenula ofunaensis* | 1.0 | 1.4 | 1.1 |
| *Pichia trephalophila* IFO 1282 | 0.9 | 1.3 | 1.9 |
| *Pichia methanolica* Y-1023 | 3.1 | 14.0 | 21.5 |
| *Pichia pastoris* | 0 | 0 | 0 |
| *Pichia pinus* | 7.3 | 7.6 | 9.7 |
| *Saccharomyces* sp | 0 | 0 | 0 |
| *Torulopsis nagoyaensis* | 0 | 0 | 0 |
| *Torulopsis methanolovescens* | 0 | 0 | 0 |
| *Torulopsis* sp. | 0 | 0 | 0 |

*Growth and methyl formate formation in cultural medium*

In attempt to know whether methyl formate is accumulated in cultural medium of methanol-utilizing yeasts, *P. methanolica, Candida sp.* N-16, *Candida sp.* 1-B and *P. pinus* were grown on 1.5% methanol as sole carbon and energy source. Remaining concentration of methanol and the accumulation of methyl formate in cultural medium were measured from lag phase to stationnary phase of growth.

Growth was followed spectrophotometrically at the absorbance of 610 nm. Figure 3a shows that methyl formate accumulated in cultural

medium. At mid-log phase of growth, methyl formate reached the maximum level of 1.5 mM and then decreased along with the decrease of methanol concentration.

The other three strains (*Fig. 3b, 3c and 3d*) also showed the same characteristics of methyl formate accumulation and reached the maximum level in a range of 1.0 to 2.1 mM of methyl formate. However, in comparison of the accumulation pattern among the four strains, showing that only in the case of *P. methanolica* the maximum level was reached at mid-log phase of growth. In the case of *Candida* sp. 1-B, *Candida* sp. N-16 and *P. pinus*, the maximum levels were reached at earlier phase of growth compared to *P. methanolica*. These data indicate that methyl formate accumulation is probably influenced by available methanol concentration in the cultural medium. The accumulation peak of methyl formate in the cultural medium of *P. methanolica* was about 200 mM. On the other side, accumulation peaks in the cultural medium of *Candida* sp. 1-B, *Candida* sp. N-16, and *P. pinus* occured when methanol concentration were about 300 mM. The difference of accumulation peaks may attributed to enzymes responsible for methyl formate formation from methanyol. Therefore, it might be that in *Candida* sp. 1-B, *Candida* sp. N-16, and *P. pinus*, enzymes are synthetized at the earlier phase of growth than that of *P. methanolica*.

*Cultivation conditions*

*Candida* sp. 1-B was used in the study of effect of carbon source on methyl formate-producing activity. As it can be seen in *Fig. 4*, methanol was much more stronger inducer for methyl formate-producing activity. It is interesting to obeserve that methanol showed a strong induction on methyl formate-producing activity. This characteristic clearly indicated by the facts that glucose and ethanol-grown cells were induced to produce methyl formate after 10 h of reaction time in a reaction mixture containing 500 mM of methanol.

Conditions of the resting cell-reaction system were optimized with respect to methyl formate production from methanol. *Candida* sp. 1-B, otherwise indicated, was used throughout the investigation.

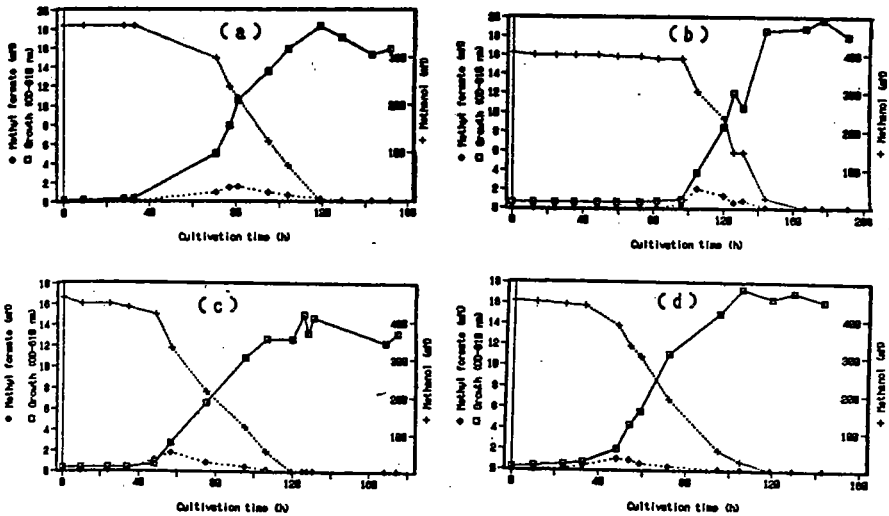


Fig. 3. Growth and methyl formate formation in cultural media of methanol-utilizing yeasts. Each strain was cultivated in the basal medium described in Materials and Methods, containing 1.5% methanol as a sole carbon and energy source. (a) *P. methanolica* (b) *Candida* sp. 1-B (c) Candida sp. N-16 and (d) *P. pinus*.

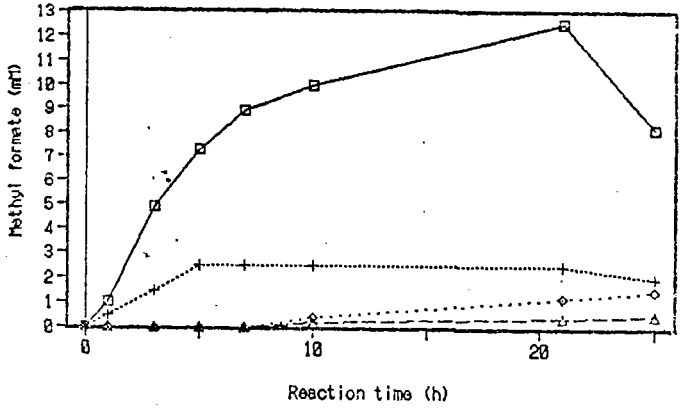


Fig. 4. Effect of carbon source on methyl formate-producing activity of *Candida* sp. 1-B. Cultivation was carried out in the basal medium described in Materials and Methods, containing 1.5% methanol, glycerol, ethanol or glucose, respectively. Harvested cells were subjected to the standard assay system of methyl format production. (□) methanol-grown cells (+) glycerol-grown cells (◇) glucose-grown and (Δ) ethanol-grown cells.

## *1. Effect of cell concertration*

The effect of cell concertration on methyl formate-producing activity was examined. The activity was optimum at cell concentration of 7.3 g-d.c.w/l (*Fig. 5*). At cell concentrations higher than this point, a significant decrease of the activity was observed. The decrease of activity at higher concentration of the cell possibly due to the presence of methyl formate- hydrolizing activity within the cell.

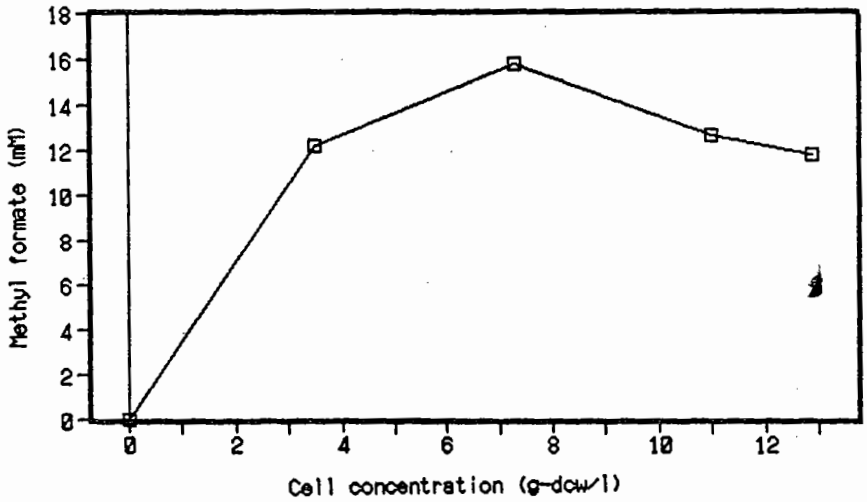


Fig. 5. Effect of cell concentration on methyl formate- producing activity of *Candida* sp. 1-B. Reaction mixture was carried out as described in Materials and Methods except for cell concentration.

## *2. Effect of methanol concentration*

The activity increased within the increase of methanol concentration (*Fig. 6*). This fact implies that methyl formate-producing activity was directly influenced by methanol concentration as it is in accordance with the results of methyl formate accumulation in the cultural medium during the course of growth.

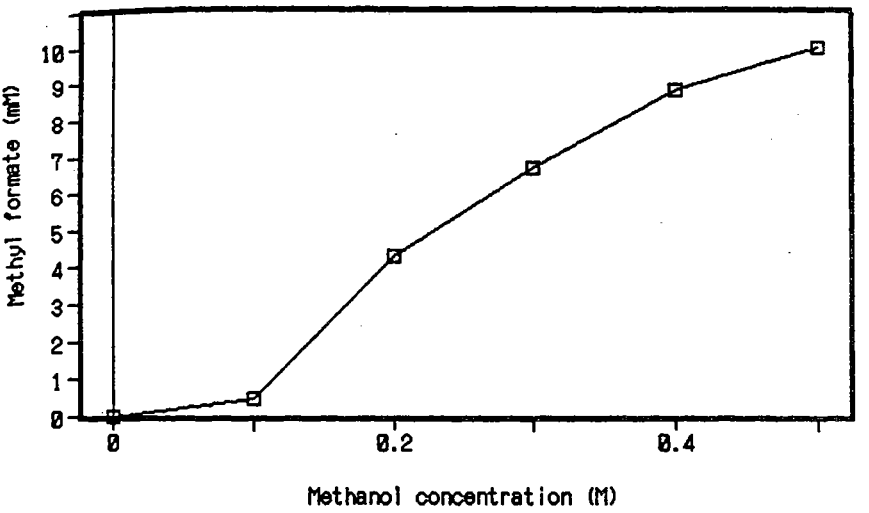


Fig. 6. Effect of methanol concentration on methyl formate- producing activity of *Candida* sp. 1-B. Reaction mixture was carried out as described in Materials and Methods, except for methanol concentration.

## *3. Effect of pH*

The effect of pH on methyl pormate-producing activity was studied with *Candida* sp. 1-B and *P. methanolica*. Various pHs of 100 mM of potassium phosphate buffer were used in reaction mixtures. Figure 7 shows that pH 7.0 was the best for *P. methanolica* while in the case of *Candida* sp. 1-B the activity increased within the increase of pH and leveled off from pH 7.0 to 8.0. Although methyl formate was most stable at pH 6.0, the optimum pH for production seem to be rather different since the appearance methyl formate concentration in the reaction mixture was a net result between producing and hydrolyzing activities.

### *Methyl formate-hydrolyzing activity*

Since a significant decrease of methyl formate-producing activity was obeserved at cell concentration higher than 7.3 g d.c.w/l during the course of reaction conditions optimization, it is valuable to study the ability of cells to hydrolyze methyl formate. *Candida* sp. 1-B was used throughout this investigation.

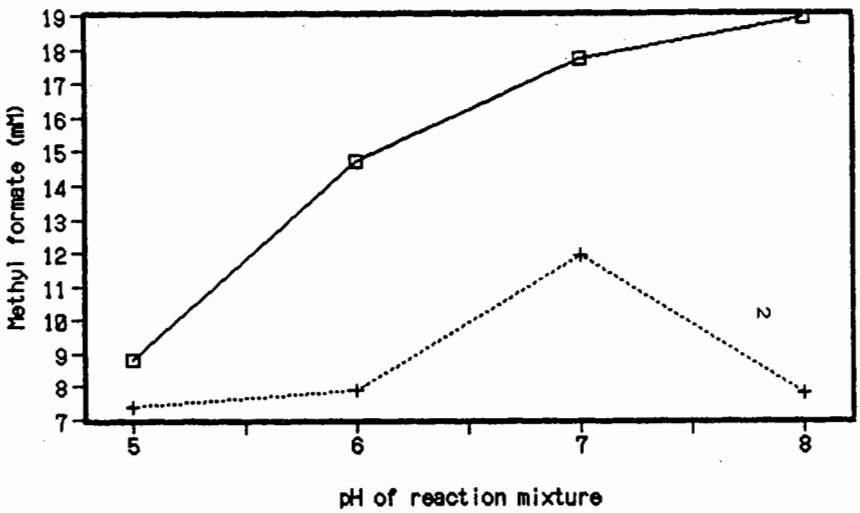


Fig. 7. Effect of pH on methyl formate-producing activity. Reaction mixture were carried out in various pHs of 100 mM potassium phosphate buffer. (□) *Candida* sp.1-B (+) *P. methanolica.*

## *1. Methyl formate-hydrolyzing activity of washed cells*

Depending on pH and temperture, methyl formate underwent hydrolysis in water solution. Methyl formate was found to be most stable at pH 6.0 during 17 h of incubation time at 10°C or 28°C *(Fig. 2)*. The rate of hydrolysis was also higher at 28°C than that of 10°C. However, the rate of hydrolysis could be significantly accelerated by resting cells of *Candida sp.* 1-B. This hydrolyzing activity was demonstrated in *Fig. 8.* After 2 h of reaction time, methyl formate was hydrolyzed by cells about 50%. This value was corrected by control experiment which was run without cells.

## *2. Effect of cell concentration*

Hydrolysis rate of methyl formate increased within the increase of cell concentration *(Fig. 9)*. Based on this result it may be correct to suggest that the decrease of producing activity at concentration higher than 7.3 g-d.c.w./l was due to the fact that cells possess methyl formate-hydrolyzing activity as well as producing activity. It is still not clear whether both activities attributed to the same or different enzyme.

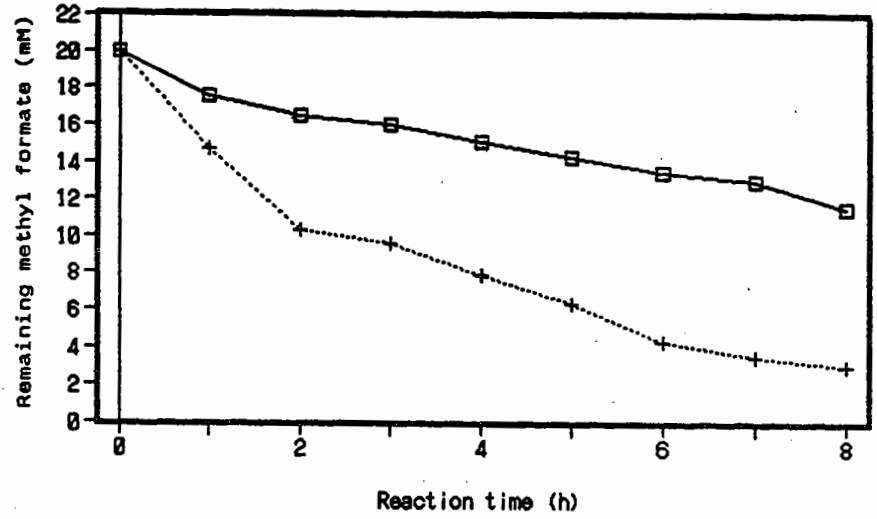


Fig. 8. Time course of methyl formate-hydrolysis by resting cells of *Candida* sp. 1-B. Reaction was carried out as described in Materials and Methods (□) control (without cells) and (+) containing 3.5 g-dcw/l of washed cells

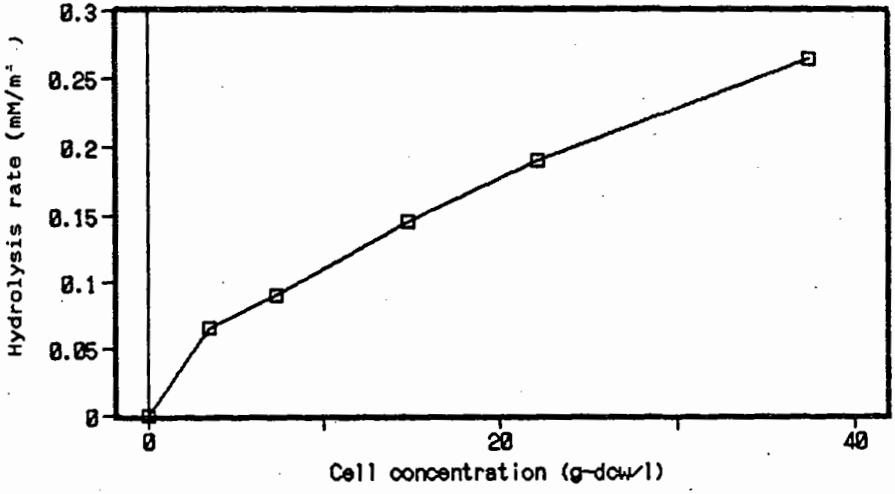


Fig. 9. Effect of cell concentration on methyl formate- hydrolyzing activity of *Candida* sp. 1-B. Reaction were performed in 10 ml of reaction mixture, containing 20 mM of methyl formate, 100 mM of potassium phosphate buffer, pH 6, incubated on water bath reciprocal shaker at 10°C for 1 h. The values of hydrolysis rates were corrected by control experiments which were run without cells.

### *3. Effect of substrate concentration*

Methyl formate-hydrolyzing activity was directly dependent upon substrate concentration up to 150 mM. At substrate concentration above this point, activity leveled off (*Fig. 10*), indicating substrate saturation. Such a characteristic expressed an enzyme- catalyzed reaction, and therefore the hydrolisis of methyl formate probably due to esterase activity of thes yeast.

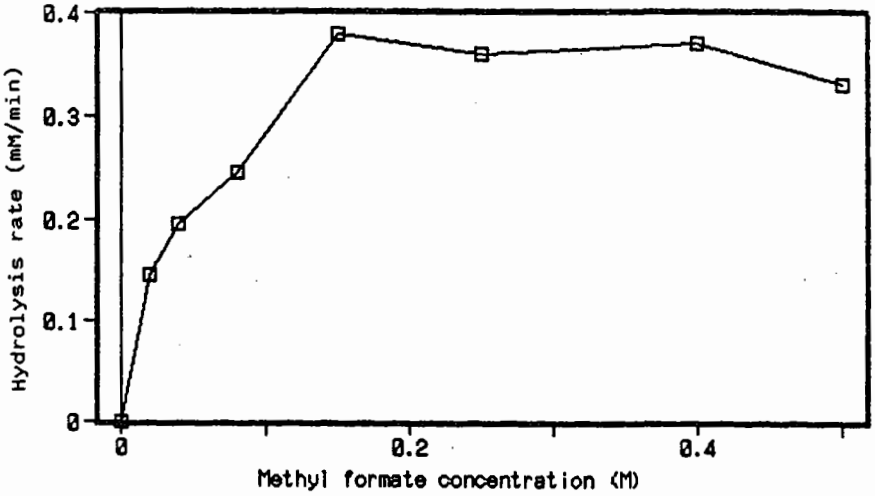


Fig. 10. Effect of substrate concentration on methyl formate- hydrolyzing activity of *Candida* sp. 1-B. Reaction was carried out in 10 ml of reaction mixture, containing 100 mM of potassium phosphate buffer, pH 6, 14.8 g-dcw/l of washed cells, incubated on water bath reciprocal shaker at 10°C for 1 h.

### *4. Effect of carbon source*

In attemp to explain whether the hydrolyzing activity is aslo induced by methanol, *Candida* sp. 1-B was cultivated on 1.5% methanol, glycerol ethanol and glucose, respectively. Results are summarized in *Fig. 11*. Even though the hydrolyzing activity of methanol-grown cells was higher than glycerol, glucose and ethanol grown cells, the activity was not merely induced by methanol, since glycerol, glucose and ethanol-grown cells also possessed the hydrolyzing activities. Rather different from

methyl formate-producing activity which was strongly induced by methanol, and slightly induced by glycerol, but was not induced by ethanol and glucose. On the other side, hydrolyzing activity was not only induced by methanol but also by glycerol, glucose and ethanol, though in a lower intensity. Based on these observations, it can be suggested that methyl formate- producing activity and methyl formate-hydrolyzing activity attributed to different enzymes.

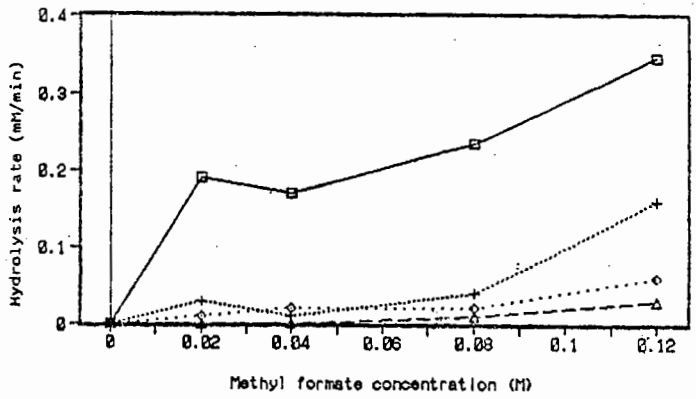


Fig. 11. Effect of carbon source on methyl formate-hydrolyzing activity of *Candida* sp. 1-B Cultivation was carried out in the basal medium as described in Materials and Methods, containing 1.5% of methanol, glycerol, glucose or ethanol, respectively, as a carbon source. Harvested cells were subjected to the assay system of methyl formate- hydrolyzing activity. Reaction mixture were performed in 10 ml of 100 mM potassium phosphate buffer, pH 6.0, containing 3.5 g-dcw/l of washed cells and various concentration of methyl formate, incubated at 10°C for 1 h. ( □ ) methanol-grown celss (+) glycerol-grown cells ( ◇ ) glucose-grown cells and (△) ethanol-grown cells.

## *Selection of strain*

Based on the results of screening and time course of methyl formate produciction (*Fig. 12*) then *Pichia methanolica* was selected as the best methyl formate producer.

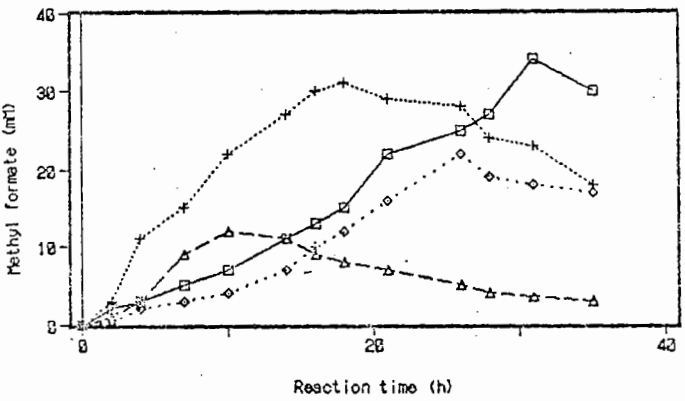


Fig. 12. Time course of methyl formate production. Reaction were carried out in 10 ml of reaction mixture, containing 100 mM of potassium phosphate buffer, pH 7.0, 500 mM of methanol, and 7.3 g dcw/l of washed cells, incubated on water bath reciprocal shaker at 10°C. (◇) *P. methanolica* (+) *Candida sp.* 1-B. (△) *Candida* sp. N-16 and (□) *P. pinus*.

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