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Isolation and Identification of Aldehyde Producing Methanol Utilizing Yeast

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메탄올 자화성 효모의 분리, 동정 및 Aldehyde 생산

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Abstract — *Hansenula nonfermentans* KYP-1 was selected and identified from 19 methanol utilizing yeasts isolated from soil samples by the enrichment culture technique. This strain showed a high cell concentration and a high aldehyde production. Aldehyde production was carried out in a resting cell system using methanol utilizing yeast as a biocatalyst. The molar yield of acetaldehyde was the highest among the aldehyde investigated, and the maximum amount of aldehyde was produced by cells obtained from a 40 hours' culture.

Since the discovery by Ogata *et al.* (1) of a yeast capable of growing on methanol as the sole source of carbon and energy, many methanol utilizing yeasts have been isolated from natural sources (2-4).

Interest in the cultivation of microorganisms on methanol has been stimulated by the potential importance of microbial protein as a food source or fodder material (5). The use of methanol for the production of cell mass offers great advantages, such as high solubility in water, relatively low cost, and high purity. Many investigations concerning methylotrophic fermentative production of SCP (6-10) or metabolites including amino acids (11, 13), vitamins (14, 15), ATP (16) and polysaccarides (17-19) have been performed. New processes using the biocatalytic functions of methylotrophs have also been attempted for the production of methyl ketones (20), acetone (21), formaldehyde (22, 23), and benzaldehyde (24).

Aldehyde, which has been used as a raw material for antiseptics, resins, pesticides, perfumes and other chemical products, is presently produced in chemical processes using metal catalysts at high temperature and pressure.

Biocatalytic use of microbial cells for the production of fundamental chemicals is attractive because, 1) the narrow specificity of the enzymatic reaction could reduce the amount of by-product, 2) milder reaction conditions than those of usual chemical processes could reduce energy costs, and 3) the biocatalytic process used instead of metal catalysts in chemical process might have advantages in environmental protection.

The alcohol oxidase (EC 1.1.3.13) and catalase (EC 1.1.11.6) of methanol utilizing yeast can be applied to the production of useful aldehyde from pri-

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mary alcohol. The alcohol are oxidized to corresponding aldehyde by alcohol oxidase, and catalase decomposes the toxic compound, H_2O_2, which is produced through the catalytic action of alcohol oxidase (25, 26).

In this investigation of the production of aldehyde using a methanol utilizing yeast as a biocatalyst, a methanol utilizing yeast which showed a high aldehyde production was isolated. The isolated methanol utilizing yeast was then identifided.

Materials and Methods

Medium

Since methanol is the sole source of carbon and energy for a methanol utilizing yeast, a methanol containing basal medium was used for the isolation, preservation and cultivation of methanol utilizing yeast. The medium contained in 1.0*l* of distilled water: methanol, 7.915 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, 1.0 g; thiamine, 1.0 mg; riboflavin, 1.0 mg; pyridoxine, 1.0 mg; nicotinic acid, 1.0 mg; pantothenic acid, 1.0 mg; ascorbic acid, 0.2 mg; biotin, 0.05 mg; folic acid, 0.01 mg; $CaCl_2$, 2.0 mg; $FeSO_4$, 1.0 mg; $CuSO_4$, 0.05 mg; H_3BO_3, 0.1 mg; $MnCl_2$, 0.4 mg, $ZnCl_2$, 0.4 mg; $CoCl_2$, 0.1 mg; and Na_2MoO_4, 0.1 mg. The pH was adjusted to 6.0 with 1 N NaOH.

Isolation

Methanol utilizing yeasts were isolated from 112 soil samples by an enrichment culture. A 0.5 g soil sample was added into 4.5 m*l* of methanol basal medium in a test tube (13×130 mm) and incubated with a reciprocal shaker at 160 rpm for 3 days at 28℃. The culture broth was then spread on an agar plate of the same medium and colonies formed were isolated. Then, yeasts were selected by microscopic morphological observation. Methanol utilizing yeasts were confirmed by a methanol concentration decrease in the liquid basal medium during cultivation. Among the selected methanol utilizing yeasts a strain which exibited the highest aldehyde production was selected.

Cultivation

One loopful of methanol utilizing yeast grown on an agar slant of the methanol basal medium was inoculated into 5 m*l* of the same liquid medium in a test tube (13×130 mm) as a preculture. It was cultured at 28℃ for 30 hours on a reciprocal shaker at 160 rpm. Then, the resultant culture broth was inoculated at a concentration of 3.0% (v/v) into a 500 m*l* flask containing 100 m*l* of medium. Cultivation was carried out at 28℃ and 160 rpm for 48 hours. Cells of methanol utilizing yeast grown on the methanol medium were used for aldehyde production after washing with 10 mM potassium phosphate buffer (pH 7.5).

Reaction conditions for the production of aldehyde

The production of aldehyde was examined in a resting cell system and a cell-free extract system. The reaction conditions of the resting cell system with whole cells were as follows: The reaction mixture contained 30 mg of washed cells, 1.0 M alcohol (methanol, ethanol, propanol and allyl alcohol), and 10 mM phosphate buffer (pH 7.5) in a final volume of 1.0 m*l*. It was placed into a test tube (20×100 mm). The reaction mixture was shaken reciprocally for 2 hours at 20℃ and 160 rpm, and the reaction was terminated by removing the cells by centrifugation. The resultant supernatant was subjected to gas chromatographic analysis.

The cell-free extract system for aldehyde production occured under the following reaction conditions: Thirty mg of washed cells were suspended in 1.0 m*l* of 10 mM phosphate buffer (pH 7.5), and the suspended cells were then disrupted at 4℃ by Cole-Parmer ultrasonicator. The cells and debris were removed by centrifugation at 6,000 rpm for 10 minutes at 4℃. The reaction was initiated by the addition of primary alcohol to the final concentration of 1.0 M into the cell free extract solution. The reaction mixture was incubated at 20℃ under reciprocal shaking at 160 rpm for 2 hours. The 0.1 m*l* reaction mixture was removed and put into 0.9 m*l* of 0.2 N HCl to stop the reaction. The resultant solution was subjected to gas chromatographic analysis.

Gas chromatographic analysis

Amounts of methanol and aldehyde were measured on a Varian Gas Chromatograph (Model 3700), equipped with a flame ionization detector. The column used for the quantitative analysis of aldehyde (formaldehyde, acetaldehyde, propionaldehyde and acrolein) and methanol was 10% carbowax 20 M on Chromosorb W-HP, 80/100 (column size; 2.0 m×2.0 mm ID,SS). The column temperature was maintained isothermally at 120℃. The injector and detector temperatures were 120℃ and 130℃, respectively. The flow rate of the carrier gas (N_2) was 35 m*l*/min, and the injection volume was 5 μ*l*. Integration and calibration of peak areas were performed with a Varian 4270 Integrator. Under the conditions, the retention times of methanol, formaldehyde, acetaldehyde, propionaldehyde and acrolein were 1.3, 0.9, 0.85, 1.0 and 1.1 minutes, respectively.

Identification

In order to identify the isolated methanol utilizing yeast, its principal physiological properties and morphological characteristics were analyzed according to the method of Kreger-Van Rij (27) and Barnett *et al.* (28).

Coenzyme Q was extracted, as reported by Yamada *et al.* (29), and was analyzed by HPTLC (Merck, 10×10 cm). A solvent system consisting of acetone-acetonitrile (80:20) was used, and detection was done under a UV lamp at a wavelength of 254 nm.

The composition of DNA bases (GC content) was determined by reversed-phase HPLC, according to the method of Tamaoka *et al.* (30). DNA was hydrolysed into nucleosides with nuclease P1 and bacterial alkaline phosphatase. The mixture of nucleosides was subjected to HPLC without further purification.

Results and Discussion

Isolation of a yeast capable of producing aldehyde

Nineteen methanol utilizing yeasts were isolated from soil samples. Methanol assimilation by yeasts was detected by three different methods: 1) growth on a methanol containing agar plate, 2) an increase of turbidity in a methanol containing liquid medium, and 3) determination of methanol consumption during cultivation. Four strains showing rapid growth and high cell concentrations in a methanol basal medium were analyzed for aldehyde production from alcohol.

Table 1 shows the amounts of aldehyde produced by methanol yeasts in a resting cell system and a cell-free extract system. The molar yield of acetaldehyde was the highest and that of formaldehyde was the lowest among the aldehyde analyzed. The substrate specificity of alcohol oxidase to methanol is known to be higher than the specificity to ethanol (31). Thus, the difference in molar yields between formaldehyde and acetaldehyde suggested that for-

Table 1. Amount of aldehyde produced by selected methanol yeasts

	Amount of aldehyde (mM)							
	Strain No. 1		Strain No. 2		Strain No. 3		Strain No. 4	
	R	C	R	C	R	C	R	C
Formaldehyde	33.6	36.3	–	21.7	45.2	45.8	47.6	49.6
Acetaldehyde	122.5	97.5	116.6	82.6	131.8	97.5	120.7	83.7
Propionaldehyde	93.4	56.7	90.8	58.2	122.0	73.4	87.5	53.1
Acrolein	85.2	38.9	97.0	49.3	88.8	50.8	82.4	36.2

Strains were grown in a methanol basal medium for 48 hours in batch culture. The reaction for aldehyde production was carried out with a cell conc. of 30 mg/m*l* and an alcohol conc. of 1.0 M for 120 min. Formaldehyde, acetaldehyde, propionaldehyde and acrolein were produced from methanol, ethanol, propanol and allyl alcohol, respectively.

R: Resting-cell system, C: Cell-free extract system

Fig. 1. Scanning electron micrograph of strain No. 3 (×8400).
Strain No. 3 was cultured in methanol basal medium for 48 hours at 28℃.

maldehyde was more inhibitory either to cells or to alcohol oxidase activity, than acetaldehyde.

The amount of aldehyde produced was larger in the resting cell system than in the cell-free extract system. *In vivo* alcohol oxidase and catalase are localized in special cell compartments, called peroxisomes, which are induced during growth of methanol utilizing yeasts on methanol (32). Peroxisomes may be destroyed in the cell-free extract system by cell disruption in a ultrasonicator. Therefore, the enzymes *in vitro* are less stable than in the resting cell system.

Strain No. 3 showed the highest aldehyde production among the selected methanol utilizing yeasts. Therefore, strain No. 3 was selected as an aldehyde producer, and the resting cell system was chosen for aldehyde production.

Identification of isolated yeast

A photomicrograph of strain No. 3 is shown in Fig. 1, and morphological properties of the strain are summarized in Table 2. After 3 days' culture on YM agar at 28℃ the cells were ellipsoidal shape and formed a singly, in pairs, or in small clusters. In YM liquid medium, a pellicle was absent and rings were rarely observed. The yeast reproduced by multilateral budding, mycelium was not formed in slide culture, and ascospores were observed by staining with malachite green. Based on morphological examination this strain was confirmed to be

Table 2. Mophological properties of strain No. 3

	Strain No.3
Shape and size of cell	ellipsoidal (1.3−2.2)×(2.0−3.0) μm
Growth in YM liquid medium* (after 3 days at 28℃)	pellicle formed
Growth on YM agar** (after 3 days at 28℃)	abundant growth slightly raised entirely smooth glistening butyrous
Characteristics of vegetative reproduction	
budding	multilateral budding
mycelium formation (slide culture)	not formed
spore formation	formed

*YM medium; peptone 5 g, yeast extract 3 g, glucose 10 g and distilled water 1,000 ml, pH 5 to 6.
**YM agar; peptone 5 g, yeast extract 3 g, glucose 10 g, agar 17 g and distilled water 1,000 ml.

an ascosporogenous yeast.

The quinone system of strain No. 3 was ubiquinone Q-7 and the GC content of the DNA was 45.6 mol%. Methanol utilizing yeasts are divided into four major groups by Komagata (33). The GC content of the DNA of Group 2 shows a rather broad distribution from 34.3 mol% (*Candida methanosorbosa*) to 56.3 mol% (*Candida entomophila*). The dominant ubiquinone of this group is Q-7, which is found in most species of the genera *Hansenula, Pichia* and *Candida*. Thus, strain No. 3, ascosporogenous yeast was considered to be a member of the genus *Hansenula or Pichia*.

The detailed taxonomical characteristics of the isolated yeast are shown in Tables 3, 4 and 5. Nitrate assimilation by this strain suggested that it belonged to the genus *Hansenula*. This yeast assimilated potassium nitrate, but no species of the genus *Pichia* assimilated nitrate as a nitrogen source.

Strain No. 3 showed similarity to *Hansenula nonfermentans* in the assimilation of carbon compounds, except trehalose and erythritol. Strain No.3 assimilated erythritol, but *H. nonfermentans* did not. Strain No. 3 did not ferment carbon compounds, unlike *H. nonfermentans*. This strain also showed the same results in additional physiological tests as the *H. nonfermentans*. Furthermore, the results of the

Table 3. Assimilation of carbon compounds

Compounds	No. 3	H*	Compounds	No. 3	H
D-galactose	−	−	Raffinose	−	−
L-sorbose	−	−	Melezitose	−	−
D-glucosamine	−	−	Inulin	−	−
D-ribose	+	+	Starch	−	−
D-xylose	−	V	Glycerol	+	V
L-arabinose	−	−	L-arabinitol	−	−
Sucrose	−	−	D-mannitol	+	+
L-rhamnose	+	V	Ribitol	+	+
Maltose	−	−	myo-Inositol	−	−
Trehalose	−	+	Erythritol	+	−
Cellobiose	+	+	D-gluconate	−	−
Salicin	+	+	D-glucuronate	−	−
Arbutin	+	+	Citrate	−	V
Melibiose	−	−	Methanol	+	+
Lactose	−	−	Ethanol	+	+

*H; *Hansenula nonfermentans*, V; variable

Table 4. Fermentation of carbon compounds

Compounds	No. 3	H*	Compounds	No. 3	H
D-glucose	−	−, D	Lactose	−	−
D-galactose	−	−	Cellobiose	−	−
Maltose	−	−	Melezitose	−	−
Sucrose	−	−	Raffinose	−	−
Trehalose	−	−	Inulin	−	−
Melibiose	−	−	Starch	−	−

*H; *Hansenula nonfermentans*, D; delayed

quinone system and the GC content of the DNA were consistent with those of *H. nonfermentans*.

Although some differences were found in the assimilation of carbon compounds, the physiological, morphological and other taxonomical characterestics of strain No. 3 were similar to those of *H. nonfermentans*. Therefore, this strain was named *Hansenula nonfermentans* KYP-1.

Aldehyde production

Aldehyde production was investigated with the cells of strain No. 3 obtained at different culture times (Fig. 2). The activity of cells for aldehyde production increased during the early exponential growth phase, reaching a maximum value at a culture time of 40 hours. When the cells entered the stationary growth phase, the amount of aldehyde produced sharply decreased. Similar patterns were shown

Table 5. Additional physiological properties

	Strain No. 3	*Hansenula nonfermentans*
Assimilation of		
nitrate	+	+
nitrite	−	V
L-lysine	+	+
creatinine	−	−
Growth in		
50% glucose medium	−	−
60% glucose medium	−	−
0.1% heximide	+	+
0.01% heximide	+	+
vitamin-free medium	−	−
Growth at		
25℃	+	+
37℃	+	+
42℃	−	−
Starch production	−	−
Production of acetic acid	−	−
Urea hydrolysis	−	−
Diazonium Blue B reaction	−	−
Coenzyme Q system	Q-7	Q-7
G+C mol%	45.6	45.3, 45.6

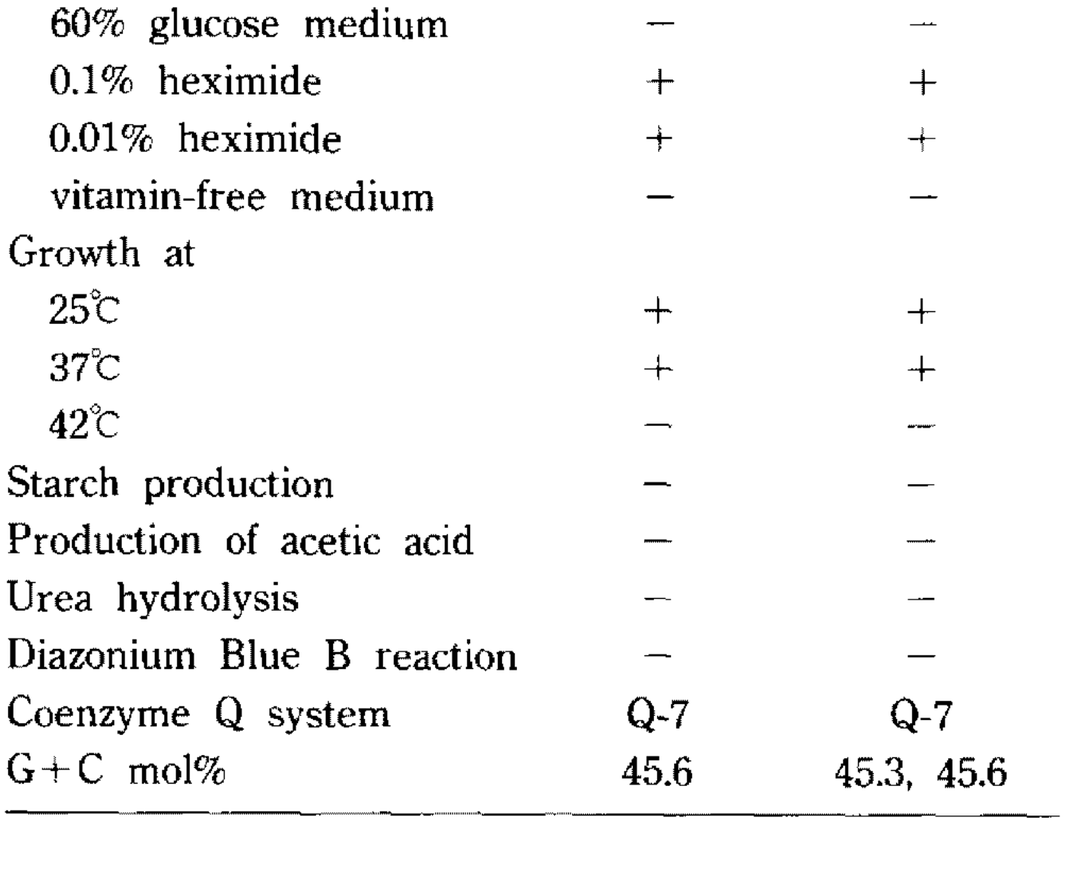

Fig. 2. Aldehyde production by cells of different culture times.

Cells were cultured in a methanol basal medium at 28℃ and stirred at 160 rpm. Reactions for aldehyde production were carried out with resting cell system. Cell conc.; ●, Formaldehyde; ○, Acetaldehyde; □, Propionaldehyde; ▲, Acrolein; ■

in the production of four other aldehyde, but the production of propionaldehyde and acrolein were more dependent on cell age than the production of formaldehyde and acetaldehyde.

요 약

토양으로부터 19균주의 메탄올 자화성 효모를 분리하였으며, 이들 중 높은 균체농도와 aldehyde 생산을 보인 균주를 선별 및 동정하여 *Hansenula nonfermentans* KYP-1으로 명명하였다.

Aldehyde 생산은 메탄올 자화성 효모의 균체를 생촉매로 하는 resting cell system에서 행하였으며, 조사된 aldehyde 가운데 acetaldehyde의 생산량이 가장 높았다. 최대의 aldehyde 생산은 40시간 배양한 균체를 생촉매로 이용하였을 때 얻어졌다.

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