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Studies on the Production of Yeast

(Part. Ⅱ) Yeasts Utilizing Methanol as Sole Carbon Source.

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酵母의 生産에 關한 硏究

(第二報) Methanol 資化性酵母에 關하여

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Abstract

Seven strains of methanol assimilating yeasts were isolated from soil enriched with tetracycline. Among them two better growing strains were selected and partially identified as species belonging to genus *Candida*. The both *Candida* 1-B and 25-A, grew best under conditions of pH 5.0 and 28°C.

The optimal methol concentration in the medium was found to be 1%, Whereas the organism, could grow up to the 4% level.

Biotin was required by the organisms for growth and organic nitrogen sources raised the rate of growth. The yield of biomass per unit weight of consumed methanol after 96 hours were 36.9% by *Candida* 1-B and 39.2% by 25-A.

Introduction

Certain method of producing single cell protein by the organisms utilizing C1 compounds such as methanol and methane as a carbon source has been studied in many labories.[1,9,11,12,21,22] Recently, the interest in methanol as a fermentation substrate has increased since methanol can be produced from natural gas at low cost in large scale plants, and has good properties such as water-soluble and easy nandling. The isolation of yeasts capable of growing on methanol has been reported by Ogata et al[1] and by other investigators.[7,8,10,13,21] In this paper, the results of isolation and taxonomical studies of new yeasts and their culture conditions in methanol are described.

Material and Methods

1) Microorganisms

The strains used throughout the study were methanol assimilating yeasts isolated from 150 samples of soil and sewage in Korea using enrichment techniques.

2) Media

Medium A; Medium A is consisted of methanol 20*ml*, $(NH_4)SO_4$ 6gr, KH_2PO_4 1gr, K_2HPO_4 1gr,

$MgSO_4 \cdot 7H_2O$ 0.5gr, $MnSO_4 \cdot 4\text{-}6H_2O$ 0.01gr, vitamin mixture[2] 1ml, and tetracycline[13] 50mg in 1000ml of tap water.

The pH of the medium was adjusted to 6.0.

Medium B; Medium B is consisted of methanol 20ml, $(NH_4)_2SO_4$ 6gr, KH_2PO_4 1gr, K_2HPO_4 1gr, $MgSO_4 \cdot 7H_2O$ 0.5gr, $MnSO_4 \cdot 4\text{-}6H_2O$ 0.01gr, vitamin mixture 1ml, and bromphenol blue 1.5mg in 1000ml of tap water. The pH of the medium was as same as medium A. Methanol was added without sterilization after autoclaving other components.

3) Isolation and culture conditions

Two grams of soil or sewage were added to 10ml of medium A in a test tube and incubated with reciprocal shaking for 7 days at 28°C. After several subcultures, methanol assimilation ability was detected by an increase in tubidity and a change of color of the culture broth. The methanol assimilating yeasts were isolated by the usual monocolony isolation method from the tubes which showed abundant growth. Cultivation were carried out in 500ml baffle flask containing 50ml of medium B on a rotary shaker (205 revolutions per min) at 28°C for 2 to 4 days.

4) Methanol determination.

Methanol was analyzed by a colorimetric method[17] using chromotrophic acid after oxidation with $KMnO_4$. A standard curve was prepared from volumetric quantities of methanol.

5) Cell concentration.

Cell growth was measured by the optical density and calculated from a standard curve estimated dry cell weight.

Results and Discussion

1. Taxonomic characterization of isolated strains.

From soil and sewage samples, 7 strains assimilating methanol were isolated by a methanol enrichment culture. Among them, two strains 1-B and 25-A which showed the most abundant growth in methanol medium were selected. Table 1, 2, 3, 4, 5 and 6 show taxonomic characteristics of the two strains of methanol assimilating yeast. The vegetative cells of the two yeasts are shown in Fig. 1 and Fig. 2, respectively. These esults suggest that

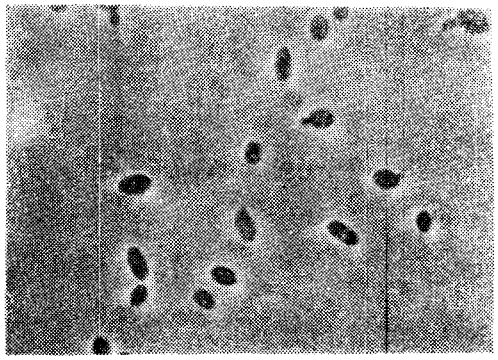

Fig. 1. Photomicrograph of *Candida* 25-A grown for48hr in YM medium at 28°C.

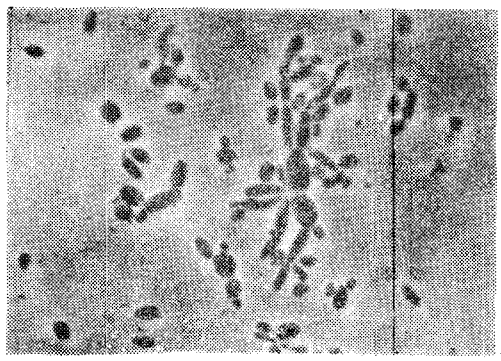

Fig. 2. Photomicrograph of *Candida* 1-B grown 48hr in YM medium at 28°C.

the two yeasts belong to the genus *Candida* sp. Any differences of the taxonomical properties were not found between the strain of 1-B and that of 25-A except cell size and maximum temperature of growth. There were no species identical with these yeasts in the system of Lodder.[16] Although some

Table 1. Morphological properties of metanol assimilating yeasts, 1-B and 25-A.

	1-B	25-A
Shape	oval or ellipsoid	oval or ellipsoid
Size	$(3-4) \times (5-10)\mu$	$(2-3) \times (4-7)\mu$
Reproduction	multilateral	multilateral
Pseudomycelium	formed	formed
Ascospore	not formed	not formed

Table 2. Macromorphological characteristics of methanol assimilating yeasts, 1-B and 25-A.

	1-B	25-A
Growth	abundant	abundant
Shape	raised, undulate	raised, undulate
Color	milky white	milky white
Surface	butyrous	butyrous

Table 3. Physiological properties of methanol assimilating yeasts, 1-B and 25-A.

	1-B	25-A
Optimum pH for growth	5	5
Optimum temperature for growth	28°C	28°C
pH available for growth	2 to 8	2 to 8
Maximum temperature for growth	45°C	40°C
Assimilating of KNO_3	positive	positive
Coagulation of milk	negative	negative
Reduction of Litmus	positive	positive
Sodium chloride tolerance	10%	10%
Gelatin hydrolysis	negative	negative
Production of carotenoid pigments	negative	negative
Production of starch-like substances	negative	negative
Splitting of arbutin	negative	negative
Vitamin stimulating growth	biotin	biotin

Table 4. Assimilation of carbon compound

Compounds	1-B	25-A
Glucose	+	+
Galactose	−	−
Maltose	−	−
Saccharose	+	+
Lactose	−	−
D-Xylose	+	+
Glycerol	+	+
Ethanol	+	+
Formaldehyde	−	−
Formate	−	−
Acetate	+	+
L-sorbose	+	+
Trehalose	−	−
D-Ribose	+	+
Erythritol	+	+
D-Manitol	+	+
Inulin	+	+
Soluole starch	−	−
Salicin	−	−
DL-Lactic acid	+	+

Table 5. Assimilation of nitrogen compounds

Compounds	1-B	25-A
Peptone	+	+
Ammoniumsulfate	+	+
Asparagine	+	+
Urea	−	−

Table 6. Fermentation of carbon compounds

Compounds	1-B	25-A
Glucose	+	+
Galactose	−	−
Saccharose	+	+
Maltose	−	−
Lactose	−	−
Raffinose	−	−

Table 7. Comparision of physiological characteristics of the isolated yeasts with *C. utilis*

Assimilation of carbon compounds	*C. utilis*	Isolated yeasts
Maltose	+	−
Sucrose	+	+
D-Ribose	−	+
Erythritol	−	+
L-Sorbose	−	+
Salicin	+	−
Inulin	+	+

of biochemical properties of these yeasts are similar to those of *Candida utilis*, differences were found in the properties as follow;

The isolates assimilate L-sorbose, erythritol and D-ribose, but *Candida utilis* does not have the ability to assimilate them.

And the former do not assimilate maltose and salicin but the later can assimilate them. Moreover, it was found that there are considerable differences vitamin requirement for growth. (Table 7)

2. Growth characteristics of two methanol assimilating yeasts.

1) Optimum pH

This was determined in the medium B by pH control culture with aqueous ammonia. These organisms grew over the range of pH 2.0 to 8.0. The optimum pH of two strains were observed at pH 5.0(Fig. 3).

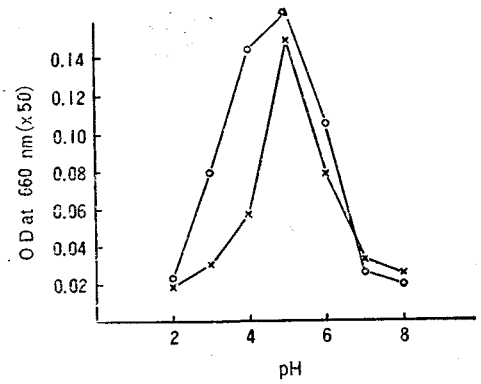

Fig. 3. Effect of pH on the growth of *Candida* 25-A and *Candida* 1-B. The growth in the medium B having different pH values was determined after shaking for 48 hours at 28°C.
—○— *Candida* 25-A —×— *Candida* 1-B

2) Optimum temperature

A growth test at various temperatures was carried out with a temperature gradient rotary shaker. The results are shown in Fig. 4. The optimum temperature of two strains was observed at 28°C.

3) Methanol concentration

Fig. 5 shows the effect of methanol concentration of growth of two organisms in the medium B. It was found that yeasts could grow in the medium containing as much as4.0% methanol and optimum concentration of methanol was 1.0%.

4) Growth factors.

Vitamin requirements of the two methanol assimilating yeasts were studied. Table 8 and 9 show that biotin was required for the growth of both yeasts, but was not observed to be the essential growth factors. Ogata et al[3] and Yokote et al[13] repored that thiamine was required for the growth of methanol assimilating yeasts, Kloeckera sp. and Torulopsis sp. But the yeasts of this study did not require thiamine for their growth.

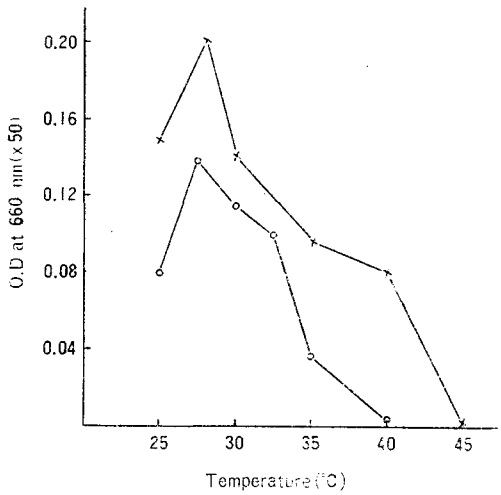

Fig. 4. Effect of temperature on the growth of the two isolates. The growth was determined after shaking for 48 hours at different temperature.
—○— *Candida* 25-A —X— *Candida* 1-B

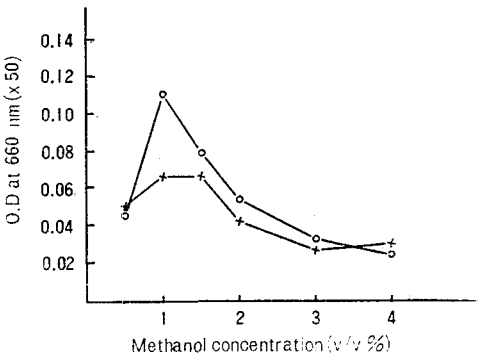

Fig. 5. Effect of methanol concentration on the growth of the two isolates. the growth in the medium B having different concentration of methanol was determined after shaking for 48 hours at 28°C.
—○— *Candida* 25-A —×— *Candida* 1-B

Table 8. Vitamin requirements for growth of *Candida* 1-B

Vitamins	Growth (OD 660nm×50)			
	Added to minimum medium		Removd from complete medium	
	Incubation time(hr)			
	24	48	24	48
None	0. 005	0. 030	0. 028	0. 140
Thiamine HCl	0. 018	0. 043	0. 018	0. 158
Riboflavine	0. 015	0. 043	0. 017	0. 209
Pyridoxine HCl	0. 005	0. 031	0. 020	0. 156
Panthothenate	0. 008	0. 050	0. 018	0. 098
Nicotinic acid	0. 009	0. 042	0. 018	0. 172
Folic acid	0. 013	0. 061	0. 025	0. 096
p-aminobenzoic acid	0. 014	0. 041	0. 024	0. 127
Biotin	0. 020	0. 147	0. 016	0. 025

Table 9. Vitamin requirements for growth of *Candida* 25-A

Vitamins	Growth (OD660nm×50)			
	Added to minimum medium		Removed from complete medium	
	Incubation time(hr)			
	24	48	24	48
None	0. 006	0. 028	0. 008	0. 076
Thiamine HCl	0. 008	0. 044	0. 015	0. 092
Riboflavine	0. 006	0. 038	0. 014	0. 072
Pyridoxine HCl	0. 006	0. 043	0. 009	0. 064
Panthothenate	0. 005	0. 045	0. 010	0. 078
Nicotinic acid	0. 012	0. 028	0. 010	0. 079
Folic acid	0. 009	0. 044	0. 014	0. 058
p-aminobenzoic acid	0. 009	0. 014	0. 009	0. 082
Biotin	0. 015	0. 162	0. 005	0. 027

5) Organic nitrogen sources.

The effect of the addition of organic nitrogen sources such as yeast extract, corn steep liquor aad beef extract, on growth in a methanol medium was also examined. The results are shown in Fig. 6 All the tested organic nitrogen sources stimluated the growth of strains in a methanol medium. The most effective growth was observed on the addition of yeast extract. However, some differences at optimum concentration between Candida 1-B and Candida 25-A were seen. Optimum concentrations of yeast extract were 0. 3% for *Candida* 1-B and 0. 2% for *Candida* 25-A. Yokote et al[18] suggested that organic nitrogen sources such as corn steep liquor increased the growth of yeast in a methanol medium. The most effective growth was obtained on the addition of yeast extract in our study, but it seems to be desirable to replace with corn steep liquor in the biomass production because of its low cost.

6) Time course of biomass production

The biomass production was carried out in the culture medium containing 0. 3% yeast extract. The results are shown in Fig. 7. The yields of biomass per weight of consumed methanol were 36. 9% by *Candida* 1-B and 39. 2% by *Candida*

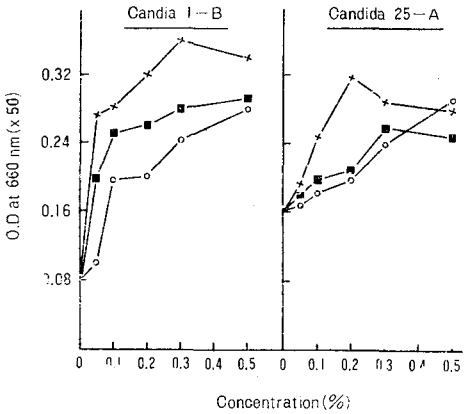

Fig. 6. Effect of organic nitrogen sources and their concentration on the growth of the two isolates. The growth gn the medium B Difco yeast extract, Difco beef extract and corn steep liquor (Kumsung Industry Co.) were determined after shaking for 48 hours at 28°C.
—×— Yeast extract, —■— CSL, —○— Beef extract

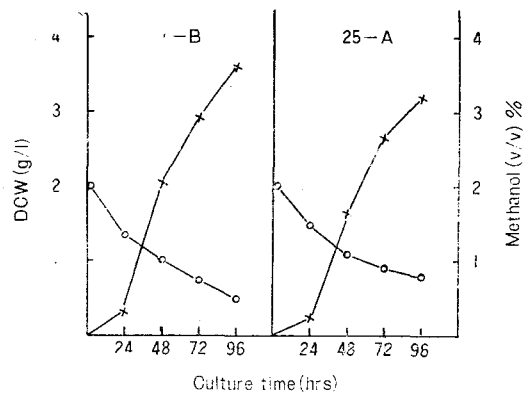

Fig. 7. The growth and substrates utilization pattern of the two isolates during the culture of 96 hours period. The organisms were grown in medium B shaking at 205 rev/min rate.
—×— dry cell weight, —○— methanol concentration (v/v%)

25-A.

3. Comparision of Candida 1-B and Candida 25-A with the previously reported methanol-a ssimila-

Table 10. The Comparison of characteristics of methanol-assimilating yeasts

	Kloeckera No 2201 Ogata[3]	*T. methanovescense* Oki etal[6]	*C. methanolica* Oki etal[5]	*Candida* N-17 Tomura[10]	*Saccharomyces* H-1 Tomura[10]	*T. methanosorbosa* KY12001 Yokote etal[20]	*T. methanodomercqui* Ky 12002 Yokote etal[20]	*Canadida* 1-B Isolated yeast	*Candida* 25-A Isolated yeast
Reproduction	budding, polar	Multilateral	Multilateral	Multilateral	Multilateral	Multilateral	Multilateral	Multlateral	Multilateral
Pseudomycelium	−	−	+	+	−	−	−	+	+
Ascospore	−	−	−	−	+	−	−	−	−
Protuberance	−	−	−	−	+			−	−
Assimilation of C and N-compounds									
Glucose		+	+	+	+	+	+	+	+
Sucrose		−	−	+	+	−	−	+	+
Ethanol	+	+	+	+	−	+	+	+	+
Glycerol		−	+	+	+			+	+
Acetate				+	−	−	+	+	+
Nitrate	+	+	+	+	−	+	+	+	+
Vitamin requirement									
Biotin	−	+	+	−	−	+	+	−	−
Thiamine	+	+	+	+	+	+	+	+	+

ting yeasts.

Table 10 shows a comparision of the principal properties of these isolates with the previously reported methanol assimilating yeasts. It indicates that these two isolates seem to be similar to *Candida* N-17 which was isolated by Tomura et al. [10] These yeasts were quite different from *Candida methanolica*[6] in the assimilation of carbon sources such as trehalose, solublestarch, salicin and vitamin requirement.

約 要

注油所 工場周邊의 下水吧 土壤으로부터 Methanol 資化性 酵母 7 株를 分離하고 그中 生育이 旺盛한 菌株 2 株에 對하여 分類學的 諸性質을 檢討한 結果 兩株는 모두 *Candida* 속으로 同定되었다. 兩株는 모두 ethanol 資化性을 가지고 生育에는 Biotin을 要求하였으며 培養特性은 最適溫度 28°C, 最適 pH5.0이고 methanol 濃度 1%에서 增殖이 菌體收率은 對消費 methanol에 對하여 35~40%에 達하였다.

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