[J. Ferment. Technol., Vol. 52, No. 4, p. 201~209, 1974]

Yeasts Utilizing Methanol as a Sole Carbon Source

Yasuharu Yokote, Masahiro Sugimoto,
and Shigeo Abe*

Tokyo Research Laboratory, Kyowa Hakko Kogyo Co., Ltd. Machida, Tokyo

Abstract

Seven species of methanol assimilating yeasts were isolated from samples of soil by enrichment techniques using tetracycline. Among them, two new species; *Torulopsis methanosorbosa* ABE *et* YOKOTE sp. nov. (KY 12001, FERM-P NO 1208, ATCC 20361) and *Torulopsis methanodomercqii* ABE *et* YOKOTE sp. nov. (KY 12002, FERM-P NO 1978) were selected and characterized. *T. methanosorbosa* could grow in as much as 5% methanol concentration, and grew well in 1%. The maximum production rate of biomass was observed at 30°C and pH 5.5 to 6.0, using a semi-synthetic medium supplemented with methanol, ammonium chloride, vitamins, salts and 0.1% of corn steep liquor. A mean generation time was estimated to be 4.0 hr. The corresponding specific growth rate was 0.092 hr^{-1}. The cell composition was as follows: crude protein 47.4 to 57.1%, nucleic acid 2.82 to 3.36% and glutathione 0.2%.

Introduction

Many bacteria utilizing methanol and methane have been isolated and their mechanism of oxidation of C_1 compounds studied.[1] With the advent of large scale production of low cost methanol from natural gas, interest in methanol as a fermentation substrate has increased. The present and future cost structure, the solubility, the ease of handling, and the purity of methanol are the factors which make methanol an attractive raw material for single-cell protein production. As a consequence, the isolation of yeasts capable of growing on methanol has recently been reported by Ogata *et al.*[2] and by other investigators.[3~8]

In this paper, the results of isolation and taxonomical studies of new yeasts and their culture conditions in methanol are described.

Materials and Methods

Microorganisms Methanol utilizing yeasts were isolated from 850 samples of soil in Japan using enrichment techniques. Thirty one strains of hydrocarbon utilizing yeasts and 125 strains of stock cultures preserved in our laboratory were also used for a screening test.

Media The media used for isolation, purification, and production culture are shown in Table 1.

Isolation and culture conditions Three g of soil were added to 5 ml of medium A in a test tube and incubated with reciprocal shaking for 5 days at 30°C. After several subcultures, methanol assimilation ability was detected by an increase in turbidity of the culture broth. The methanol assimilating yeasts were isolated by the usual monocolony isolation method from the tubes which showed abundant growth. Growth studies were carries out in shaking flasks or in 5 *l* jar fermenters. The reciprocal shakers were run at a rate of 120 rpm at 30°C. Jar fermenters were operated with an agitation of 600 rpm, an aeration of 1 vvm, and pH control with aqueous ammonia.

* Present address: Department of Agricultural Chemistry, University of Kyoto Prefecture.

Table 1. Composition of methanol media.

Medium	A	B
Methanol[a]	2.0% (v/v)	1.0
NH_4Cl	0.4% (w/v)	0.4
KH_2PO_4	0.1	0.1
K_2HPO_4	0.1	0.1
$MgSO_4 \cdot 7H_2O$	0.05	0.05
$FeSO_4 \cdot 7H_2O$	0.001	0.001
$MnSO_4 \cdot 4 \sim 6H_2O$	0.001	0.001
Vitamine mixture[b]	1 ml/*l*	1
Corn steep liquor	—%	0.1
Achromycin[c]	175 mg/*l*	—

a Methanol was added without sterilization after autoclaving other components.
b Contained thiamine-HCl 1,000 mg, riboflavine 1,000 mg, pyridoxine 1,000 mg, nicotinic acid 1,000 mg, *p*-aminobenzoic acid 200 mg, folic acid 10 mg and biotin 10 mg in 1 *l*.
c Contained 50 mg of tetracycline and 125 mg of ascorbic acid.

Identification of yeasts The taxonomical studies of new isolates were conducted in accordance with the methods of Lodder and Kreger-Van Rij[9] and Iizuka and Goto[10] and the results were discussed according to the system of Lodder.[11]

Method of analysis Methanol was analyzed by a colorimetric method using chromotrophic acid after oxidation with $KMnO_4$. A standard curve was prepared from volumetric quantities of methanol. Protein was estimated by the nitrogen content obtained by the micro-Kjeldahl method. Cell concentration was measured by the optical density at 660_{nm} and calculated from a standard curve estimated gravimetrically. RNA and DNA were measured by the method of Shmidt-Thannhauser.[12] Glutathione was extracted from yeast by incubating the yeast in dilute sulfuric acid at 40°C for 1 hr and assayed by the 5,5′-dithiobis-(2-nitrobenzoic acid) method.[13]

Results

Isolation and identification of methanol-assimilating yeasts From soil samples, 24 strains assimilating methanol were isolated by a methanol enrichment culture. On the other hand, none of the 156 strains of stock cultures of our laboratory showed any growth in a methanol medium. These 24 strains reproduced by multilateral budding and

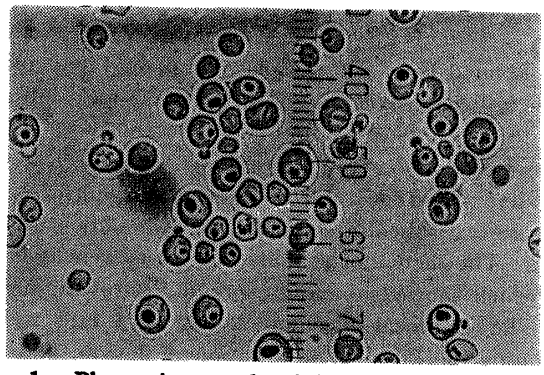

Fig. 1. Photomicrograph of *T. methanosorbosa* KY 12001 grown for 48 hr in YM medium at 25°C. Ten divisions show 10 μm.

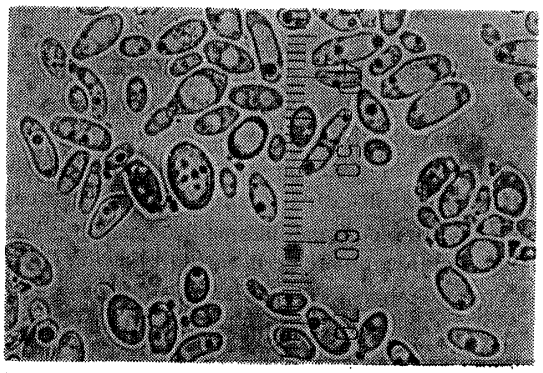

Fig. 2. Photomicrograph of *T. methanodomercqii* KY 12002 grown for 48 hr in YM medium at 25°C. Ten divisions show 10 μm.

did not form ascospores, teliospores, ballistospores or arthrospores. They were confirmed to be asporogenous yeasts. According to the morphological and physiological studies, these 24 yeasts were classified into 7 species, 5 of which were identified as *Candida boidinii*, *Torulopsis torresii*, *Torulopsis bovina*, *Torulopsis anatomiae* and *Torulopsis cantarellii*. The other two strains which showed abundant growth in a methanol medium were not identified with any known species and were named *Torulopsis methanosorbosa* ABE *et* YOKOTE sp. nov. (KY 12001, FERM-P NO 1208, ATCC 20361) and *Torulopsis methanodomercqii* ABE *et* YOKOTE sp. nov. (KY 12002, FERM-P NO 1978). Photomicrographs of *T. methanosorbosa* KY 12001 and *T. methanodomercqii* KY 12002 are shown in Fig. 1 and Fig. 2 respectively.

Taxonomical characteristics of new species The detailed taxonomical characteristics of the two species are shown in Table 2, Table 3, Table 4 and Table 5. There were no species identical to these yeasts in the system of Lodder.[11] Although some of the physiological and morphological properties of KY 12001 and KY 12002 seemed to be similar to those of *Torulopsis nitratophila* and *Torulopsis domercqii* respectively, some significant differences were found in the fermentation and the assimilation of carbon sources, and the maximum temperature and vitamin requirement for the growth. They were regarded as new species of *Torulopsis* and we propose to name them *Torulopsis methanosorbosa* and *Torulopsis methanodomercqii* because they utilize methanol as their sole carbon source.

***Torulopsis methanosorbosa* ABE *et* YOKOTE sp. nov.**

Type: *T. methanosorbosa* sp. nov. KY 12001 (FERM-P NO 1208, ATCC 20361)

Cultura in extracto maltio: 3 dies ad 25°C, cellulae globose, (2.5–3) × (3–4) μm, singulae aut binae. Pellicula non formatur. Cultura in YM-agaro: Cultura in striis

Table 2. Morphological properties of *T. methanosorbosa* KY 12001 and *T. methanodomercqii* KY 12002

	T. methanosorbosa KY 12001	*T. methanodomercqii* KY 12002
Shape and size of cell	globose (2.5–3) × (3–4) μm	long oval (2–3) × (5–8) μm
Growth in YM liquid medium (after 3 days at 25°C)	pellicle not formed	pellicle not formed
Growth on YM agar (after 3 days at 25°C)	abundant growth slightly raised entirely smooth glistening butyrous	abundant growth slightly raised entirely smooth glistening butyrous
Characteristics of vegetative reproduction		
budding	multilateral budding	multilateral budding
mycelium formation (slide culture)	not formed	not formed
spore formation	none	none
Macromorphological characteristics (after 50 days at 25°C)		
size	1.7 cm	2.5 cm
shape	raised, irregularly round	raised, irregularly round
color	cretaceus	yellowish white
surface	butyrous	butyrous

YM medium: peptone 5 g, yeast extract 3 g, malt extract 3 g, glucose 10 g and distilled water 1,000 ml pH 5 to 6.[10]

post 3 dies ad 25°C cremea, nitida, mucosa, elevata et laevis. Cultura in lamina vitrea: Pseudomycelium non formatur. Fermentatio: D-glucosum et D-mannosum fermentatur. Assimilatio carbo-compositorum: D-glucosum, L-arobinosum, D-xylosum, D-mannosum, D-galactosum, L-rhamnosum, D-fructosum, L-sorbosum, trehalosum, melezitosum (valde exiguum), arbutinum (valde exiguum), D-sorbitolum, D-mannitolum, erythritolum, ado-

Table 3. Assimilation of carbon compounds

Compounds	*T. methanosorbosa* KY 12001	*T. methanodomercqii* KY 12002	Compounds	*T. methanosorbosa* KY 12002	*T. methanodomercqii* KY 12002
D-Arabinose	−	−	D-Mannitol	+	+
L-Arabinose	+	−	Inositol	−	−
D-Ribose	−	+	Dextrin	−	−
D-Xylose	+	+	Dulsit	−	−
D-Glucose	+	+	Erythrit	+	+
D-Mannose	+	+	Adonit	+	+
D-Galactose	+	−	Malic acid	+	+
L-Rhamnose	+	−	Succinic acid	+	+
D-Fructose	+	+	2-Keto-D-gluconate	−	−
L-Sorbose	+	+	Lactic acid	+	+
Maltose	−	−	Gluconic acid	−	±
Sucrose	−	−	Acetic acid	−	+
Lactose	−	−	Pyruvic acid	+	+
Melibiose	−	−	Formic acid	−	−
Cellobiose	−	−	Methanol	+	+
Trehalose	+	−	Ethanol	+	+
Raffinose	−	−	*n*-Propanol	−	−
Melezitose	±	−	*i*-Propanol	−	−
α-Methylglucoside	−	−	*n*-Buthanol	−	−
Arbutin	±	−	Methylamine·HCl	−	−
Starch	−	−	Ethylamine·HCl	−	−
D-Sorbitol	+	+	*n*-Paraffin	−	−

Table 4. Fermentation of carbon compounds

Compounds	*T. methanosorbosa* KY 12001	*T. methanodomercqii* KY 12002
D-Glucose	+	+
D-Mannose	+	+
Maltose	−	−
Cellobiose	−	−
Melibiose	−	−
Galactose	−	−
Sucrose	−	−
Lactose	−	−
Raffinose	−	−
Melezitose	−	−
Inulin	−	−

Table 5. Physiological properties.

	T. methanosorbosa KY 12001	*T. methanodomercqii* KY 12002
Assimilation of potassium nitrate	Positive	Positive
Splitting of fat	Negative	Negative
Vitamin stimulating growth	Biotin Thiamine-HCl	Biotin Thiamine-HCl
Production of carotenoid pigments	Negative	Negative
Production of starch-like compounds	Negagive	Negative
Production of excess acids	Negative	Negative
Gelatin liquefaction	Negative	Negative
Optimum temperature for growth	30 to 37°C	28 to 32°C
Optimum pH for growth	5 to 7	5 to 7

nitolum, acidum malicum, acidum succinium, acidum lacticum et acidum pyruvicum assimilantur at non D-arabinosum, D-ribosum, maltosum, sucrosum, lactosum, melibiosum, cellobiosum, raffinosum α-glucosidum methylum, amylum, inositolum, dulsitolum, acidum gluconicum et acidum aceticum. Et etiam methanolum, fons carbonarius, utitur. Nitras kalicus assimilatur. Biotinae et thiaminae necessariae ad crescentiam. Materia amylo similis non producitur. Maxima temperatura crescentiar: 30—37°C. Habitat in terra, Sakai, Japonia.

***Torulopsis methanodomercqii* ABE *et* YOKOTE sp. nov.**

Type: *T. methanodomercqii* sp. nov. KY 12002 (FERM-P NO 1978)

Cultura in extracto maltio: 3 dies ad 25°C, cellulae ovatae, longovatae aut longae, (2–3) × (5–8) μm, gingulae aut binate. Cultura in YM-agaro: Cultura in striis post 3 dies ad 25°C cremea, mucosa, elevata et laevis. Cultura in lamina vitrea: Pseudomycelium non formatur. Fermentatio: D-glucosum et D-mannosum fermentatur. Assimilatio carbo-compositorum: D-glucosum, D-ribosum, D-xylosum, D-mannosum, D-fructosum, L-sorbosum, D-sorbitolum, D-mannitolum, erythritolum, adonitolum, acidum malicum, acidum succinicum, acidum lacticum, acidum gluconicum (valde exiguum), acidum aceticum et acidum pyruvicum assimilatur at non DL-arabinosum, D-galactosum, L-rhamnosum, maltosum, suctosum, lactosum, melibiosum, cellobiosum, trehalosum, raffinosum, melezitosum, α-glucosidum methylum, arbutinum, amylum, inositolum et dulsitolum. Et etiam methanolum, fons carbonarius, utitur. Nitras kalicus assimilatur. Biotinae et thiaminae necessariae ad crescentiam. Materia anylo similis non producitur. Maxima temperatura crescentiar: 28～32°C. Habitat in terra, Sakai, Japonia.

Growth characteristics of *Torulopsis methanosorbosa* KY 12001 The most abundant growth in a methanol medium was shown by *Torulopsis methanosorbosa* KY 12001. The growth characteristics of the strain are as follows:

Optimum pH This was determined in the medium B by pH control culture with aqueous ammonia. The organism grew over the range of pH 5.0 to 6.5 with an optimum at pH 5.5 to 6.0. The growth curves at various pH in a 5 *l* jar fermenter are shown in Fig. 3.

Optimum temperature A growth test at various temperatures was carried out with a temperature gradient incubator model-TN-3 (Toyo Kagaku Sangyo Co., Ltd.). It was found that the yeast grew over the range of 25 to 40°C, well at 37°C. (Fig. 4). Jar fermenter experiments determined that the optimum temperature for the growth was 30°C and the

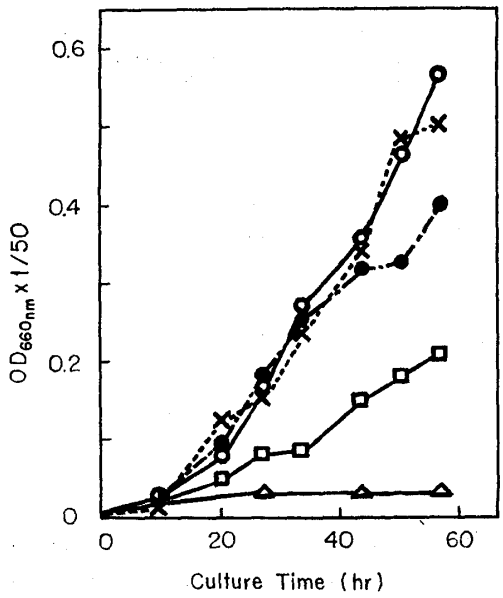

Fig. 3. Effect of pH on growth of *T. methanosorbosa* KY 12001.
The culture was grown in medium B in 5 *l* jar fermenter with pH control with aqueous ammonia. 30°C, 600 rpm.
—●— pH 5.0, --×-- pH 5.5,
—○— pH 6.0, —□— pH 6.5,
—△— pH 7.0.

subsequent experiments were carried out at 30°C.

Methanol concentration The effect of methanol concentration on growth was studied in a 5 *l* jar fermenter. Methanol was fed every 24 hr and the pH was maintained at 5.5. Growth was delayed in the medium containing more than 1% methanol, but the yeast could grow in the medium containing as much as 5% methanol. Below these concentrations a mean generation time of 5 to 6 hr was normally observed. (Fig. 5).

Growth factors Vitamin requirements were investigated. The results are shown in Table 6. Biotin and thiamine-HCl were effective for the growth of the yeast, but were not observed to be the essential growth factors. (Fig. 6). Rather they were determined to be the growth stimulative factors. The influence of the addition of other substances on the growth rate in a methanol medium was also examined. Many compounds suitable for

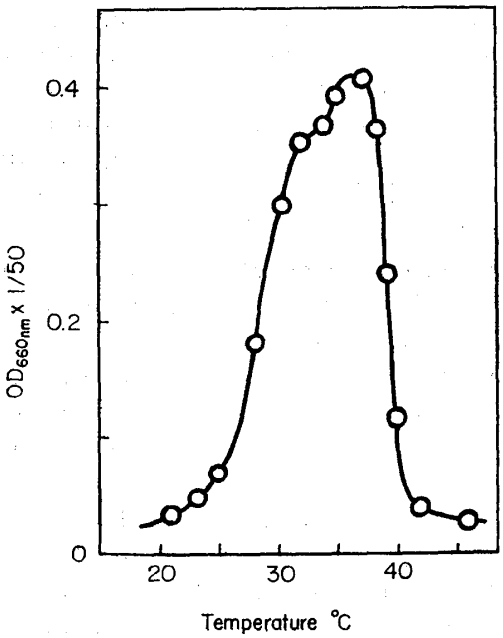

Fig. 4. Effect of temperature on growth of *T. methanosorbosa* KY 12001.
A temperature gradient incubator model-TN-3 was used. Ten ml of medium A in tube with silicon foam rubber stopper, 50 rpm, temperature 21 to 46°C, and 2 days culture.

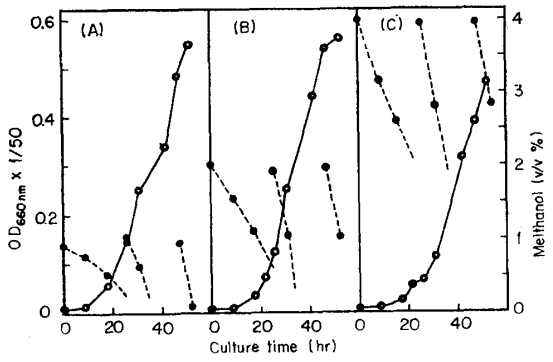

Fig. 5. Methanol concentration and growth rate of *T. methanosorbosa* KY 12001. Growth curves in 5 *l* jar fermenter with methanol concentration of 1% (A), 2% (B) and 4% (C) are shown.
—○— growth,
--●-- methanol concentration.
Methanol was added at intervals of 24 hr, pH 5.5, 30°C 600 ppm, 1 vvm.

single-cell protein production were tested at a concentration of 0.1%. Effective growth was observed on the addition of corn steep liquor.

Growth on other substrates Many compounds were examined as sole sources of carbon and energy at a concentration of 0.1%. Table 3 shows the results of cultivation for 3 days in shaking test tubes. Satisfactory growth was observed on methanol, ethanol, glycerol, glucose, L-arabinose, D-mannose, D-galactose, L-rhamnose, D-fructose and L-sorbose. No growth was found in the presence of propanol, butanol, alcoholamine, or formaldehyde. Neither was growth observed when the yeast was incubated in liquid or solid media without a carbon source other than that contained in an atmosphere of methane-air (1 : 1 v, v).

Specific growth rate Using the medium B, modified to contain about 1% of methanol, maintained by micropump feeding, and with 0.2% of corn steep liquor, culture experiments were performed in a 5 *l* jar fermenter at a temperature of 30°C and a pH of 5.5 maintained with aqueous ammonia. Methanol in the medium and effluent methanol gas recovered in a cooling chamber were determined by the method using chromotrophic acid. The course of the fermentation is shown in Fig. 7. The maximum specific growth rate and the mass doubling time were determined 0.092 hr^{-1} and 4.0 hr respectively. Seventeen g per *l* of biomass was obtained after 47 hr of fermentation.

Composition of methanol-grown cells of *T. methanosorbosa* KY 12001

The cells grown on methanol had a nitrogen content of 7.6 to 9.18%. From this data the

Table 6. Vitamin requirement for growth of *T. methanosorbosa* KY 12001

Vitamins	Growth (OD_{660nm})			
	Added to minimum medium		Removed from complete medium	
	Incubation time (hr)			
	38	62	38	62
None	0.20	0.90	1.90	2.04
Thiamine-HCl	0.25	2.15	0.76	1.90
Riboflavine	0.15	1.62	1.94	2.15
Pyridoxine-HCl	0.20	1.32	1.73	2.14
Pantothenate	0.33	1.29	1.59	2.11
Nicotinic acid	0.22	0.96	1.64	2.10
Folic acid	0.56	1.57	1.65	2.12
p-Aminobenzoic acid	0.54	1.75	1.66	2.10
Biotin	0.46	2.00	0.88	1.76

Shaking culture was carried out in a test tube with a silicon foam rubber stopper.
Medium A, without Achromycin, was used as the complete medium.

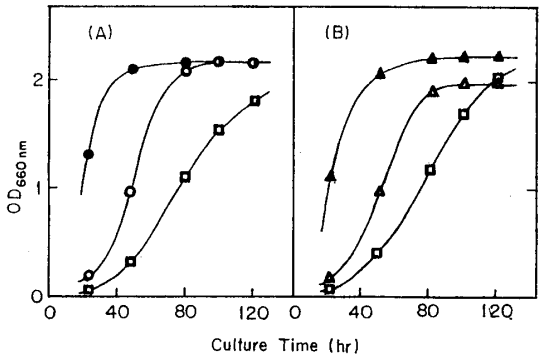

Fig. 6. Effect of biotin and thiamine-HCl on growth of *T. methanosorbosa* KY 12001.
The effect of biotin (A) and of thiamine-HCl (B) were examined by the addition of the vitamins to the basal medium. Basal medium: methanol 1%, NH_4Cl 0.4%, KH_2PO_4 0.1%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, CSL ash 10 mg/*l*.
—□— : biotin 0, thiamine-HCl 0 μg/*l*
—○— : biotin 0, thiamine-HCl 400 μg,*l*
—●— : biotin 0.1 to 10, thiamine-HCl 400 μg/*l*
—△— : biotin 10, thiamine-HCl 10 μg/*l*
—▲— : biotin 10, thiamine-HCl 20 to 400 μg/*l*

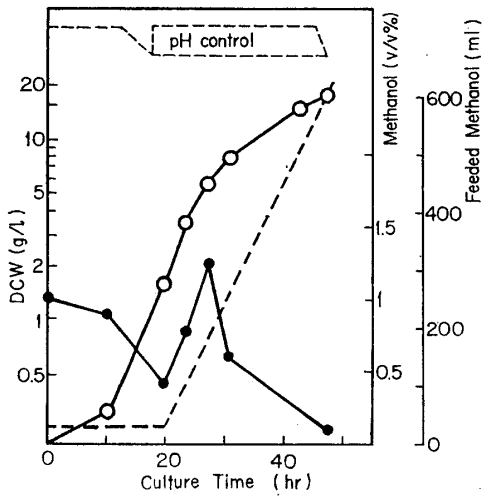

Fig. 7. Growth course of *T. methanosorbosa* KY 12001 in methanol medium.
Methanol was fed in 5 *l* jar fermenter by micro-pump. 30°C, 600 rpm, 1 vvm, pH maintained at 5.5 with 28% aqueous ammonia, inoculum size 10%.
—○— : dry cell weight,
—●— : methanol concentration,
---- : ml of added methanol.

crude protein was estimated to be 47.4 to 57.1%. Nucleic acid and glutathione contents were assayed to be 2.82 to 3.36% and 0.2% respectively.

Discussion

Recently methanol has drawn interest as an economically attractive raw material for industrial fermentations. Many methanol assimilating microorganisms have been reported, but the excellent methanol-utilizers have been restricted to gram-negative bacteria. The high incidence of bacteria capable of utilizing methanol appears to be the result of the techniques used for enrichment and isolation. For this reason, in the present study, the isolation was carried out using a medium of low pH supplemented with tetracycline. The low pH and the addition of tetracycline provided preferential selection of yeasts over bacteria.

The protein and nucleic acid contents of *Torulopsis methanosorbosa* KY 12001 are considered to be superior to those of the yeasts reported by many other authors.[2~8] Cell composition is of prime concern in the production of single-cell protein. In particular, high protein, low nucleic acid, low carbohydrate and low lipid contents are desired. A high cellular yield per unit weight of substrate utilized is most important, particularly if biomass production is desired. But in the present experiment, a careful mass balance between methanol and cells was not obtained. *T. methanosorbosa* KY 12001 is able to grow in the medium containing 5% methanol, whereas methanol is toxic to other organisms at this concentration. Harrington and Kallio[14] working with *Pseudomonas methanoria*, have reported that NAD and glutathione are involved in the mechanism of methanol oxidation. However, in the present study the glutathione content of *T. methanosorbosa* KY 12001 grown in a methanol medium was no greater than that of other yeasts cultivated in a glucose medium.[15]

References

1) Cooney, C. L., Levine, D. W.: *Adv. Appl. Microbiol.* **15**, 337 (1972).
2) Ogata, K., Nishikawa, H., Ohsugi, M., Tochikura, T.: *J. Ferment. Technol.*, **48**, 389 (1970). *Agr. Biol. Chem.*, **33**, 1519 (1969). *Arg. Biol. Chem.*, **48**, 470 (1970).
3) Arof, A.: *Proc. Ann. Conv. Sugar Technol. Assoc.* (India), **42**, 36 (1968).
4) Cooney, C. L., Levine, D. W.: *Adv. Appl. Microbiol.*, **15**, 344 (1972).
5) Asthana, H., Humphrey, A. E., Moritz, V.: *Biotechnol. Bioeng.*, **13**, 923 (1972).
6) Oki, T., Kouno, K., Kitai, A., Ozaki, A.: *J. Gen. Appl. Microbiol.*, **18**, 295 (1972). (Abstr.) *Annual Meet. Agr. Chem. Soc. Japan*, p. 344 (1970).
7) Kanzaki, F., Tomura, K.: (Abstr.) *Annual Meet. Agr. Chem. Soc. Japan*, p. 345 (1970).
8) Tomura, K., Uragami, T., Kanbayashi, A.: (Abstr.) *Annual Meet. Agr. Chem. Soc. Japan*, p. 133 (1971).
9) Lodder, J., Kreger-Van Rij, N. J. W.: *The Yeast, a Taxonomic Study*, North-Holland Publ. Co., Amsterdam (1952).
10) Iizuka, H., Goto, S.: *A Taxonomy of Yeast* (in Japanese), University of Tokyo Press, Tokyo (1969).
11) Lodder, J. ed.: *The Yeast, a Taxonomic Study*, North-Holland Publ. Co., Amsterdam-London (1970).
12) Schmidt, C., Thannhauser, S. J.: *J. Biol. Chem.*, **161**, 83 (1945).
13) Ellman, G. L.: *Arch. Biochem. Biophys.* **82**, 70 (1959).
14) Harrington, A. A., Kallio, R. E.: *Can. J. Microbiol.*, **6**, 11 (1960).
15) Hashitani, Y. (Ed.): *Koubo-Gaku* (*Science of Yeasts*) (in Japanese), Iwanami Publ. Co., Tokyo (1960).

(Received October 15, 1973)