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Studies on C_1-Compounds-Utilizing Microorganisms in Marine Environments-II

Isolation and Characterization of Methanol-Utilizing Yeasts from Coastal Water and Sediments

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A number of methanol-utilizing yeasts were isolated from coastal water and sediments by five isolation methods. According to their morphological and physiological characteristics these strains were found to belong to *Candida boidinii*, *Candida tropicalis*, *Torulopsis sonorensis* and *Torulopsis* spp., respectively.

It was indicated that the most effective method for the isoation of various methanol-utilizing yeasts involved the use of a density gradients centrifugation, separating yeasts from suspended matter in seawater.

Key words: methanol, marine yeast, isolation method, density gradient, centrifugation.

Since HUTTON and ZOBELL (1949) frist indicated the distribution of methane-oxidizing bacteria in marine environments, reports concerning C_1-compounds-utilizing bacteria have demonstrated that these bacteria are not only widespread in marine environments but also possess the physiological characteristics of marine bacteria. (KIMURA *et al.* 1977, YAMAMOTO *et al.* 1978, 1980)

While, a number of studies on marine yeasts have shown that the yeasts are normal inhabitants in oceans and estuaries (FELL *et al.* 1960, MEYERS *et al.* 1967, AHEARN *et al.* 1968), UDEN and FELL(1968) indicated that dominant yeasts in open sea areas possessed wide assimilative versatility relative to terrestrial strains. Attempts were made, therefore, to isolate methanol-utilizing yeasts from coastal water and sediments by various methods. It seems that methanol-utilizing yeasts may occur in very limited number in nature. Accordingly the procedures adopted in the separation of microorganisms from much seawater could be of importance in the isolation of methanol-utilizing yeasts. The present paper describes the isolation of methanol-utilizing yeasts from coastal water and sediments by various methods and the taxonomic characteristics of the isolated yeasts.

Materials and Methods

Source of inoculum Coastal water samples and sediments were collected mainly along the coast of Mie Prefecture. Sediment samples were obtained by the use of a KK core sampler [the original design was reported by KIMATA (1960)] equipped with a plastic tube of a 5cm inside diameter.

Media The composition of the media used in this investigation are summarized in Table 1.

Table 1. Composition of media.

Medium I			Medium II		
$(NH_4)_2SO_4$	2.0	g	$(NH_4)_2SO_4$	2.0	g
KH_2PO_4	1.0	g	K_2HPO_4	1.0	g
$MgSO_4 \cdot 7H_2O$	0.5	g	KH_2PO_4	0.5	g
$FeSO_4 \cdot 7H_2O$	0.1	g	$MgSO_4 \cdot 7H_2O$	0.2	g
Yeast extract	0.2	g	NaCl	25.0	g
Aureomycin	0.1	g	$FeSO_4 \cdot 7H_2O$	5.0	mg
Chloromycetin	0.02	g	$CaCl_2 \cdot 2H_2O$	5.0	mg
Streptmycin sulfate	0.02	g	Thyamin·HCl	100	μg
Methanol (75%) *	10.0	ml	Biotin	10	μg
Seawater	500	ml	Cyanocobalamin	1	μg
Tap water	500	ml	Methanol (75%) *	15	ml
pH 5.5			Tap water	1,000	ml
			pH 6.0		

* Methanol was added after sterilization of the other components of the medium.

Isolation In order to isolate methanol-utilizing yeasts, five isolation methods (designated A, B, C, D and E) were performed in the following manner.

Isolation Method A: Approximately 40 ml of seawater sample were added to 10 ml of five-fold concentrated Medium I in a 500ml flask, and incubated at 25 ℃ on a reciprocal shaker for 3 to 10 days. The turbid broth was streaked on the agar plate of Medium I, and incubated at 25 ℃ for a week. The colonies on the agar plate were transfered to agar slants of Medium II.

Isolation Method B: Five liters each of seawater samples were adjusted to pH 4.5 with 6N HCl after addition of 50ml methanol and 0.4 g $(NH_4)H_2PO_4$, and incubated at 20–25 ℃ for 2–5 days. Yeasts were then collected in a membrane filter (Nuclepore membrane: pore size 1.0 μ, Nomura Micro Science Co., Ltd.)by filtration from 1 liter of the incubated samples. The membrane filter was put into a flask containing 20 ml of Medium II and incubated at 25 ℃ on a reciprocal shaker for 3–7 days. After this first enrichment culture, 0.2 ml of the culture broth was inoculated into the same medium and the second enrichment culture was repeated by the same method and then selective isolation was carried out by platting out on the Medium II.

Isolation Method C: The suspended materials were collected from 120 liters seawater by continuous flow centrifugation and introduced into 100 ml of the Medium II. Enrichment culture was repeated twice and selective isolation on the agar plate medium in a manner similar to Isolation Method B.

Isolation Method D: The suspended materials were collected from seawater by continuous flow centrifugation and fractionated by a density gradient centrifugation in the following manner. Approximately 1 g of the suspended material was resuspended in 10 ml of sterile 2.5 % NaCl solution and the aliquot of 1 ml was applied on top of a discontinuous Ficoll density gradient consisting of 0.5 ml each of 40, 35, 30, 25, 20 and 15 % (w/w) of Ficoll solution. After 20 min of centrifugation at 5,000 g, yeasts located between the surface of the gradient and the bands located from 15 % Ficoll (ρ=1.05 g/ml) to 30 % Ficoll (ρ=1.12 g/ml) were collected. The suspended materials in the bands were washed with Medium II and the pure isolation on the agar plate of Medium II was repeated twice. The colonies isolated purely were transferred to agar slants of Medium II.

Isolation Method E: For the isolation from sediment samples, one gram of mud was put into 20 ml of Medium II, and the enrichment culture and the pure isolation were carried out in a similar manner as in Isolation Method B.

Identification of the isolated strains The taxonomical studies of the yeast were carried out according to the methods of LODDER *et al.* (1970) and IIZUKA and GOTO (1969), and results were discussed according to the system of LODDER *et al.* (1970) and BARNETT *et al.* (1979).

Results and Discussion

Screening test of methanol-utilizing yeasts

The results of the isolation from seawater and sediments are shown in Table 2. Although

Table 2. Screening test of methanol-utilizing yeasts

Isolation method	Type of samples	No. of samples	Amount of sample for test	No. of isolated strains
A	Seawaters	53	0.04 ℓ	0
B	Seawaters	42	5 ℓ	82
C	Seawaters	2	120 ℓ	2
D	Seawaters	10	12 ℓ	38
E	Muds	40	1 g	0

methanol-utilizing yeasts were not isolated by Isolation Methods A and E, one hundred and twenty-two yeasts were collected by using Isolation Methods B, C and D. It was suggested that methanol-utilizing yeasts are very rare in the coastal environments and that such screening of yeasts requires a large quantity of seawater.

Separation of yeasts by a density gradient centrifugation

In the experiment of Isolation Method D, a density gradient centrifugation was used to separate yeasts from the suspended substance collected from a large quantity of seawater. Preliminary recovery test of several species of yeasts, *Saccharomyces cerevisiae*, *Candida tropicalis* and *Rhodotorula* sp., were carried out by centrifuging into density gradients of Ficoll. When the yeast mixture with suspended matter centrifuged into 30 % Ficoll solution ($\rho = 1.12$ g/ml), the yeasts banded near the top of the 30 % Ficoll solution, while the greater amount of suspended matter precipitated under the soluton. Consequently yeasts were isolated from the band located between the 15 % Ficoll solution ($\rho = 1.05$ g/ml) and the 30 % Ficoll sulution.

Identification

Of the isolated strains, twelve typical ones were identified. These strains reproduced by multilateral budding and did not form true mycelium, teliospores, ballistospores or arthrospores. Their morphological properities, biochemical characteristics, physiological characteristics and assimilation of carbon compounds are shown in Tables 3, 4 and 5. Five

Table 3. Morphological properties of isolated methanol-unilizing yeasts

Strain No.	Isolation method	Pellicle (YM Medium)	Shape	Size (μg)	Vegetative reproduction	Formation of ascospore	Formation of pseudomycelium
YA−10	B	Creeping	Oval	(2−4)×(6−7)	M. budding*	−	+
YA−15	B	Creeping	Oval	(2−4)×(7−9)	M. budding	−	+
YB−16	B	Wrinkled	Oval, Round	(2−4)×(5−9)	M. budding	−	+
YC−3	B	Heavy	Oval	(2−4)×(7−10)	M. budding	−	+
TY−1	C	−	Oval, Round	(2−6)×(7−11)	M. budding	−	−
TY−2	C	−	Oval	(3−5)×(7−11)	M. budding	−	+
NY−15	D	Ring	Oval, Round	(2−4)×(5−7)	M. budding	−	−
NY−26	D	Ring	Oval, Round	(3−5)×(5−7)	M. budding	−	−
NY−12	D	Ring	Oval, Round	(2−4)×(4−5)	M. budding	−	−
NY−25	D	Ring	Round	(3−5)	M. budding	−	−
NY−28	D	Ring	Oval, Round	(2−3)×(3−5)	M. budding	−	−
NY−36	D	Wrinkled	Oval, Elongate	(3−5)×(6−10)	M. budding	−	+

*M. budding : multilateral budding

strains, YA−10, YA−15, YB−16, YC−3, NY−36, were asporogenous, had oval or round shapes, formed pseudomycelium, fermented glucose and assimilated nitrate. According to these morphological and physiological characteristics, these five strains were identical to *Candida boidinii*. However one of five strains, NY−36, assimilated succinic acid and was the only one which differed from the system of LODDER (1970). Strain TY−2 was asporogenous, had an oval shape, formed well-developed pseudomycelium, fermented glucose, galactose, sucrose and maltose, and did not assimilate nitrate. Thus, this strain was identified as *Candida tropicalis*. Strain NY−15 was asporogenous, had an oval or round shape, did

Table 4. Biochemical and physiological characteristics of isolated methanol-utilizing yeasts

	Isolated strains											
	YA-10	YA-15	YB-16	YC-3	TY-1	TY-2	NY-15	NY-26	NY-12	NY-25	NY-28	NY-36
Formation of carotenoid pigment	−	−	−	−	−	−	−	−	−	−	−	−
Liquefaction of gelatin	−	−	−	−	−	−	−	−	−	−	−	−
Production of starch-like compounds	−	−	−	−	−	−	−	−	−	−	−	−
Splitting of glucoside	−	−	−	−	−	−	W	W	+	−	+	−
Production of excess acid	−	−	−	−	−	−	−	−	−	−	−	−
Urease test	−	−	−	−	−	−	−	−	+	−	+	−
Assimilation of potassium nitrate	+	+	+	+	−	−	−	−	−	+	−	+
Vitamin stimulating growth	Bi	Bi	Bi	Bi	Bi	Bi	Bi	Bi,Th	Bi	Bi	Bi	Bi
Maximum growth temperature	35–37℃	35–37℃	35–37℃	33–35℃	41–43℃	43–45℃	41–43℃	30–33℃	41–43℃	37–39℃	41–43℃	37–39℃
NaCl tolerance	10–12%	10–12%	12–15%	10–12%	12–15%	12–15%	10–12%	8–10%	12–15%	8–10%	12–15%	12–15%
Fermentation of cabohydrates												
Glucose	+	+	+	+	+	+	+	+	+	+	+	+
Galactose	−	−	−	−	−	+	−	−	−	−	−	−
Sucrose	−	−	−	−	−	+	−	−	−	−	+	−
Maltose	−	−	−	−	−	+	−	−	−	−	+	−
Lactose	−	−	−	−	−	−	−	−	−	−	−	−

* Codes in table : + positive ; − negative ; W week ; Bi biotin ; Th thiamin.

not form pseudomycelium, fermented glucose, and did not assimilate nitrate. This strain was identified as *Torulopsis sonorensis*. Strain NY−26 was asporogenous, had an oval or round shape, did not form pseudomycelium, fermented glucose and required biotin and thiamin for their growth. Their morphological and physiological characteristics were in fair agreement with those of *Pichia pinus* except formation of ascospore. However, we judged it to belong to *Torulopsis* sp., because ascospore of the strain could not be observed. The morphological and physiological properties of strain TY−1 seemed to be similar to those of *Torulopsis sonorensis*, but significant differences were found in the assimilation ability of L-sorbose, D-xylose and succinic acid. The strains of NY−12, NY−25 and NY−28, respectively, seemed to belong to the genus *Torulopsis*, however it did not coincide with any species of *Torulopsis* described by BARNETT *et al.* (1979).

The results of identification of the isolated strain are summarized in Table 6. In our attempts to isolate the methanol-utilizing yeasts from seawater, we found that we were able to isolate the yeasts which seemed to be very rare by continuous flow centrifugation and obtain more various species of yeast by making use of a density gradient centrifugation.

Table 5. Assimilation of carbon compounds by isolated methanol-utilizing yeasts

Compounds	Isolated strains											
	YA-10	YA-15	YB-16	YC-3	TY-1	TY-2	NY-15	NY-26	NY-12	NY-25	NY-28	NY-36
Glucose	+	+	+	+	+	+	+	+	+	+	+	+
Galactose	−	−	−	−	−	+	−	−	+	−	+	−
Sucrose	−	−	−	−	−	+	−	−	+	+	+	−
Maltose	−	−	−	−	−	+	−	−	+	+	+	−
Lactose	−	−	−	−	−	−	−	−	−	+	+	−
Ethanol	+	+	+	+	W	+	+	+	+	+	+	+
Raffinose	−	−	−	−	−	−	−	−	+	−	+	−
L-Arabinose	−	−	−	−	W	W	+	+	+	−	+	−
D-Arabinose	−	−	−	−	−	−	NT	NT	NT	NT	NT	NT
D-Ribose	+	+	+	+	−	−	+	+	+	−	+	+
Rhamnose	−	−	−	−	−	−	−	−	−	−	+	−
L-Sorbose	−	−	−	−	+	+	−	−	−	+	−	−
Cellobiose	−	−	−	−	+	+	+	+	+	+	+	−
Trehalose	−	−	−	−	−	+	−	+	+	+	+	−
Melibiose	−	−	−	−	−	−	−	−	−	−	+	−
Melezitose	−	−	−	−	−	+	−	−	+	+	+	−
Inulin	−	−	−	−	−	−	−	−	−	−	−	−
Soluble starch	−	−	−	−	−	+	−	−	−	−	+	−
D-Xylose	+	+	+	+	−	+	+	+	+	−	+	+
m-Erythritol	+	+	+	+	−	−	−	+	−	−	+	+
Glycerol	+	+	+	+	−	−	−	+	+	+	+	+
Ribitol	+	+	+	+	+	+	+	+	+	+	+	+
Galactitol	−	−	−	−	−	−	−	−	−	−	+	−
D-Mannitol	+	+	+	+	+	+	+	+	+	+	+	+
D-Glucitol	+	+	+	+	+	+	+	+	+	+	+	+
Inositol	−	−	−	−	−	−	−	−	−	−	+	−
Salicin	−	−	−	−	+	+	+	+	+	+	+	−
α-Methyl-D-glucoside	−	−	−	−	−	+	−	−	−	+	+	−
K-D-Gluconate	−	−	−	−	−	+	+	+	+	+	+	−
Ca 2-keto-D-gluconate	−	−	−	−	−	+	−	−	±	−	+	−
DL-Lactic acid	+	+	+	+	+	+	−	−	−	−	+	+
Succinic acid	−	−	−	−	−	+	+	+	+	+	+	+
Citric acid	−	−	−	−	−	+	−	−	−	−	−	−

Codes in table: + positive; − negative; W week utilisation; NT not tested.

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Table 6. Yeasts isolated by the various methods from seawater

Taxon	Strains	Isolation method		
		B	C	D
Candida boidinii	YB−16,NY−36,YA−10	82	0	1
Candida Tropicalis	TY−2	0	1	0
Torulopsis sonorensis	NY−15	0	0	30
Torulopsis sp.	TY−1	0	1	0
Torulopsis sp.	NY−12	0	0	2
Torulopsis sp.	NY−25	0	0	1
Torulopsis sp.	NY−28	0	0	1
Torulopsis sp.(*Pichia pinus*)*	NY−26	0	0	3
Total No. of strains		82	2	38

* These strains were seemed to be similar to *Pichia pinus* except asporogenous property.

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