Mycobiology 31(4): 191-195 (2003)

Distribution of Alcohol-tolerant Microfungi in Paddy Field Soils

Soon-Young Choi*

Department of Biological Science, College of Science, Sookmyung Women's University, Seoul 140-742, Republic of Korea
(Received November 12, 2003)

Ethanol treatment method was attempted for the selective isolation of ethanol-tolerant fungi from two sites of rice paddy fields around Seoul area. The vertical and seasonal fluctuation of the fungal population were also investigated. The ethanol-tolerant fungi were *Talaromyces stipitatus*, *T. flavus* var. *flavus*, *T. helicus* var. *major*, *Eupenicillium javanicum*, *Emericellopsis terricolor*, *Pseudourotium zonatum*, *Aspergillus flavus*, *Cladosporium cladosporioides*, *Penicillium frequentans*, *P. janthinellum*, and *P. verruculosum*. The most dominant species isolated by this method was *T. stipitatus*. It was found that the numbers of fungal species and colony forming units (CFUs) of ethanol-tolerant fungi were higher in Ascomycota than in Deuteromycota. A particular tendency appeared the highest CFUs in autumn, but lower in spring and winter. *T. stipitatus* was the dominant species of ethanol tolerant microfungi. This result would suggest that membrane lipid composition of ethanol-tolerant fungi isolated from the soils may play on important role in the ethanol tolerance.

KEYWORDS: Ethanol-tolerant microfungi, Paddy field soils, Selective isolation

Several isolation methods of soil fungi have been attempted for study of soil microfungal flora (Tokumasu, 1974; Furuya and Naito, 1979; Min *et al.*, 1981). Effective isolations of soil fungi by treatment of temperature, sodium acetate, and ethanol were used for excluding mesophilic fungi (Apinis, 1963; Tokumasu, 1974; Furuya and Naito, 1979).

Thermophilic and thermotolerant fungi were isolated by the heat incubation method at 42°C from soils of paddy fields in Japan. Also seasonal fluctuation and distribution were investigated with dominant species (Ito *et al.*, 1981).

Min *et al.* (1987) reported that two kinds of heat treatment methods for the selection of soil fungi were applied in order to eliminate the ubiquitous fungi from soil samples. The incubation method at 42°C and heat treatment at 72°C were adopted to isolate the thermotolerant and thermophilic fungi, respectively. However the major population of paddy field soils was mesophile and only a few were thermotolerant and thermophilic fungi.

Especially, Furuya and Naito (1979) reported that four species of *Ascodesmis* were isolated from soils of Japan, Malaysia, Thailand and Philippines by means of an effective isolation technique. Isolation frequency of *Ascodesmis* was extremely increased with sodium acetate, suggesting that this might be attributable to stimulative effect of sodium acetate for ascospore germination of the species.

In this experiment, the selective isolation of ethanol-tolerant fungi was attempted using paddy field soils. It is well known that ethanol is known to fluidize yeast membranes (Mishra and Prasad, 1988). Membrane lipids are modulators of membrane fluidity (Russell, 1989), and are considered to play an essential role in the ethanol tolerance of *S. cerevisiae* (Mishra and Kaur, 1991; Sajbidor, 1997).

In this work, the ethanol treatment method for the selection of soil fungi was applied to eliminate the common fungi from soil samples. The incubation method with ethanol at the final concentration of 50% was adopted to isolate the ethanol-tolerant microfungi. We describe fungal species and distribution of the ethanol-tolerant fungi in paddy field soils in Korea.

Materials and Methods

Sampling sites. Two sites in the rice paddy fields at Yugkog-dong, Buchun in Kyunggido and Shinwon-dong in Seoul were selected in this experiment (Min *et al.*, 1981). Yugkog-dong is located at the southwest of Seoul and Shinwon-dong in the southeast of Seoul. These two sites are one of the typical rice fields in Seoul area.

Sampling method. Soil samples were collected once every three months from the sites from April 1999 to January 2000. Soil samples were collected from each site of paddy field using a sterilized stainless-steel soil sampler (70 cm×3 cm) equipped with narrow side windows in order to confirm soil samples. Soil samples were divided into three parts representing depths of 0~10 cm (upper layer), 10~20 cm (middle layer) and 20~30 cm (lower layer), respectively, from the soil surface.

Dilution method. Three soil suspensions were prepared as follows; one gram of soil from the upper layer, two

*Corresponding author <E-mail: sychoi@sookmyung.ac.kr>

grams from the middle layer and three grams from the lower layer, respectively. Each soil sample was added to 5 *ml* of sterilized water in test tube and shaked thoroughly. The aliquots were referred as soil suspension. An aliquot of soil suspension (0.2 *ml*) was spreaded on malt extract yeast extract agar (MYA) plate.

Isolation of ethanol tolerant microfungi. For the isolation of the alcohol tolerant microfungi without the most mesophilic fungi, the incubation method with ethanol treatment for 15 minutes was applied in this experiment.

One gram of a soil sample (1 g) was suspended in 5 *ml* of sterilized water, then 0.2 *ml* of ethanol (99.9%) was added to the soil suspension (0.2 *ml*). The mixture of soil and ethanol was incubated at 24°C for 15 min, and then 0.2 *ml* aliquot was spread on MYA plate amended with tetracycline (50 mg/*ml*) and incubated at 24°C. Fungal colonies appeared on the plates were transferred into a MYA slant medium and incubated at 24°C. Fungal identification was done on cultures of potato dextrose, Czapek and malt extract agar media.

Results

The isolation of fungi species and investigation of fungal distribution using ethanol treatment method was attempted, which could exclude the most common soil fungi from two sites of paddy fields around Seoul area.

Isolation of ethanol-tolerant microfungi. The results of these investigations are summarized in Table 1. In this experiment, 12 species in 7 genera were identified and classified, comprising 6 species of Ascomycota, and 6 species of Deuteromycota. Species of basidiomycota species were not isolated. The common species in two sites were *Talaromycota stipitatus*, *T. flavus* var. *flavus*, *Eupenicillicum javanicum*, *Cladosporicum clodosporioides* and *Penicillium verruculusum*.

Table 1. Fungal species isolated from paddy fields by ethanol treatment method

Fungal species	Site	
Ascomycota		
*Talaromyces stipitatus**	Y[a]	S[b]
T. flavus var. *flavus**	Y	S
T. helicus var. *major*	Y	
*Eupenicillium javanicum**	Y	S
Emericellopsis terricolor	Y	
Pseudourotium zonatum	Y	
Deuteromycota	Y	
*Aspergillus flavus**	Y	
*Cladosporium cladosporioides**	Y	S
*Penicillium frequentans**	Y	
P. janthinellum	Y	
*P. verruculosum**	Y	S
P. sp.		
Total	11	5

[a]Y=Yugkog-dong.
[b]S: Shinwon-dong.
*: Thermotolerant species (Min *et al.*, 1987).

Table 2. Colony forming units of ethanol-tolerant microfungi in paddy field soils

Species	CFU per gram of soil
Talaromyces stipitatus	1,845
T. flavus var. *flavus*	52
T. helicus var. *major*	16
Pseudourotium zonatum	29
Eupenicillium javanicum	9
Penicillium verruculosum	34
P. janthinellum	15
P. frequentans	14
Penicillium. sp.	2
Aspergillus flavus	1
Emericellopsis terricolor	2
Cladosporium cladosporioides	6
Total	2,025

CFU : Colony forming unit.

Total number of fungal species isolated in Yugkog-dong site was more than those of Shinwon-dong site from the soil samples through four seasons.

Distribution of ethanol-tolerant microfungi of paddy field soils were shown in Table 2. The CFUs of *T. stipitatus* was 1,845 per gram of soil, followed by *T. flavus* var *flavus*, *Penicillium verruculusum*, *Pseudourotium zonatum*, and *T. helicus* var. *major*.

It was also found that the numbers of fungal species and total CFUs of ethanol tolerant fungi in Ascomycota were more than that of Deutromycota (Table 2).

Vertical distribution of fungal flora. For the vertical distribution of the fungal population of ethanol-tolerant microfungi at Yugkog-dong site as shown in Table 3, the numbers of CFUs in the soil of the middle layer (5 cm) was relatively higher than the lower layer (15 cm) and the upper layer (25 cm) throughout four seasons.

However, at Shinwon-dong site shown in Table 4, the fungal distribution at depth of soil layer was the highest level in the soil of the lower layer, followed by the middle, and the upper layer. There results do not coincide with the tendency of fungal distribution of common microfungi which had been investigated at the same sites (Min *et al.*, 1981).

Seasonal distribution of ethanol-tolerant fungi. Seasonal distribution in fungal colonies at two sites shows in Figs. 1 and 2. In general, the distribution of ethanol-tolerant fungi seems to be the same tendency to all three layers with the exception of autumn and winter seasons in

Table 3. Seasonal distribution of alcohol-tolerant microfungi from paddy field soils at Yugkog-dong site

Season	April			July			Oct.			Jan.			Sub-total			Total
Layer / Fungal species	A[a]	B	C	A	B	C	A	B	C	A	B	C	A	B	C	
Ascomycotina																
Talaromyces stipitatus	53[b]	58	42	45	100	31	42	91	46	33	44	76	173	293	195	661
T. flavus var. *flavus*		1		3	1					5	6	2	8	8	2	18
T. helicus var. *major*										8	6	2	8	6	2	16
Eupenicillium javanicum		2										5		2	5	7
E. terricolor												1			1	1
Pseudourotium zonatum	7	17	5										7	17	5	29
Deuteromycotina																
Aspergillus flavus	1								1				1		1	2
Cladosporium cladosporioides									1						1	1
Penicillium frequentans							3	9	2				3	9	2	14
P. janthinellum							2		9				2		9	11
P. verruculosum							7	1	1	2	1	1	9	2	2	13
Total	61	78	47	48	101	31	54	101	60	48	57	87	211	337	225	773

[a]A : upper layer (5 cm), B : middle layer (15 cm), C : lower layer (25 cm).
[b]Numbers represent colony forming units per gram of soil.

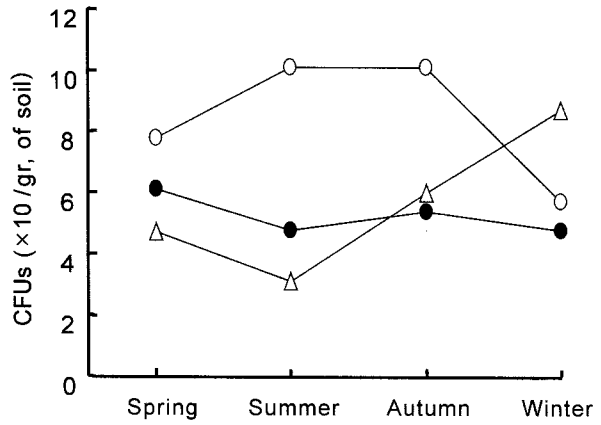

Fig. 1. Seasonal fluctuation of ethanol tolerant microfungi at Yugkog-dong site. ●: upper layer, ○: middle layer, △: lower layer.

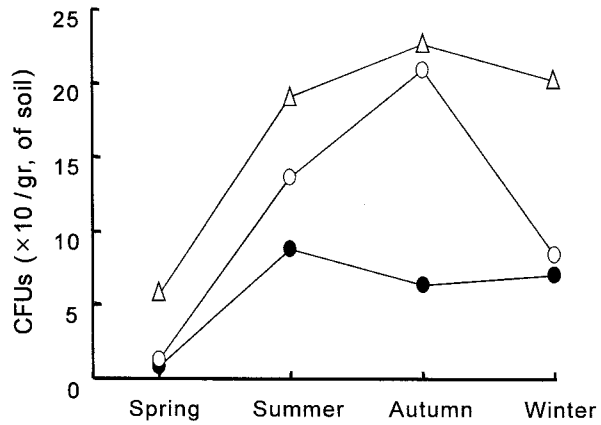

Fig. 2. Seasonal fluctuation of ethanol tolerant microfungi at Shinwon-dong site. ●: upper layer, ○: middle layer, △: lower layer.

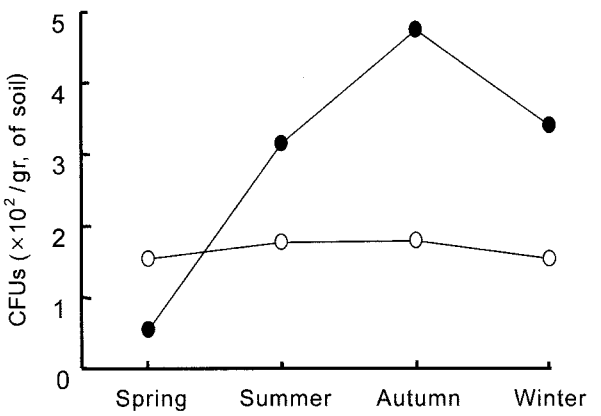

Fig. 3. Seasonal variation of Talaromyces stipitatus (○: Yugkog-dong site, ●: Shinwon-dong site).

Yugkog-dong site. A particular tendency appeared the highest density of CFUs in autumn, but lower in spring and winter seasons.

The highest distribution of the fungal population in autumn season is suggested that the autumn provides not only good temperature, but also suitable water content in paddy field soils for fungal growth, in relation to roots of rice plants. However, in winter season, the soil is frozen up to 90cm depth due to cold temperature.

Seasonal distribution of dominant species, *Talaromyces stipitatus*. As shown in Tables 3 and 4, it was found that the dominant species of ethanol-tolerant microfungi was *T. stipitatus*.

Seasonal distribution of CFUs of dominant species, *T. stipitatus* from two sites, Yugkog-dong and Shinwon-dong, was showed in Fig. 1. This seasonal distribution of

Table 4. Seasonal distribution of alcohol-tolerant fungi from paddy field soils at Shinwon-dong site

Season	Apil			July			Oct.			Jan.			Sub-total			Total
Layer / Fungal species	A[a]	B	C	A	B	C	A	B	C	A	B	C	A	B	C	
Ascomycotina																
Talaromyces stipitatus	8	11	35	88	136	91	40	208	227	64	77	199	200	432	552	1,184
T. flavus var. *flavus*			16							7	8	3	7	8	19	34
Eupenicillium javanicum			2												2	2
Deuteromycotina																
Cladosporium cladosporioides			4						1						5	5
Penicillium verruculosum								24	1					24	1	25
Penicillium sp.		2												2		2
Total	8	13	57	88	136	191	64	210	227	71	85	202	207	466	579	1,252

[a]A : upper layer (5 cm), B : middle layer (15 cm), C : lower layer (25 cm).
[b]Numbers represent colony forming units per gram of soil.

the dominant species, *T. stipitatus* at Shinwon-dong showed remarkably higher in autumn than that of winter, summer, and spring. It was found that *T. stipitatus* was also a dominant species of thermotolerant and thermophilic fungi as has been reported by Min *et al.* (1987).

Discussion

From the results of vertical distribution of ethanol-tolerant fungi at two sites, ethanol-tolerant fungi were distributed at the lower or the middle layer more than those of mesophilic microfungi. It may suggest that ethanol-tolerant fungal species with cell membrane containing high unsaturated fatty acids are not aerobic microorganism (Mishra and Kaur, 1991; Sajbidor, 1997).

Dutta *et al.* (1964) and Min *et al.* (1981) had pointed out a seasonal variation in fungal flora with different stages of rice cultivation. This tendency of seasonal fluctuation of mesophilic fungal flora is in good agreement with our results in this experiment.

Ethanol is well known as an inhibitor of fungal growth. It has been reported to damage mitochondrial DNA in yeast cells (Ibeas and Jimenez, 1997) and to cause inactivation of some enzymes (Augustin *et al.*, 1965). Nevertheless, some strains of the yeast show ethanol tolerance and can adapt to high concentration of ethanol (Alexandre *et al.*, 1994; Ghareib *et al.*, 1988). Many studies have reported the alteration of cellular lipid composition in response to ethanol exposure (Beaven *et al.*, 1982; Ingram, 1976; Mishra and Prasad, 1989; Swan and Watson, 1999).

Chi and Arneborg (1999) reported that the more ethanol-tolerant strain contained a higher ergosterol/phospholipid ratio, a higher proportion of phosphatidylcholine, a high incorporation of long-chain fatty acids, and a slightly higher proportion of unsaturated fatty acids in total phospholipids. They suggested that these results showed a clean relationship between the lipid composition and the ethanol tolerance of yeast.

The degree of fatty acid unsaturation is considered to play a major role in the ethanol tolerance of yeast (Mishra and Kaur, 1991). In addition; most studies show that the ethanol-tolerance of yeast correlates with an increased degree of fatty acid unsaturation (Chi and Arneborg, 1999; Ghareib *et al.*, 1988).

Our results show clearly that ethanol-tolerant microfungi are *T. stipitatus*, *T. helicus* var. *major*, *T. flavus* var *flavus*, *Eupenicillium javanicum*, *Emericellopsis terricolor*, and *Pseudourotium zonatumin*, in Ascomycota.

Especially taking into consideration of dominant species of ethanol-tolerant fungi, it is assumed that *T. stipitatus* in this experiment would contained more long-chain fatty acids and the high degree of unstaturation of fatty acids in lipid composition indicated by Chi and Arneborg (1999).

Acknowledgement

This research was supported by the Sookmyung Women's University Research Grants 2002.

References

Alexandre, H., Rousseaux, I. and Charpentier, C. 1994. Ethanol adaptation mechanism in *Saccharomyces cerevisiae*. *Biotech. Appl. Biochem.* **20**: 173-183.

Apinis, A. E. 1963. Occurrence of thermophilous microfungi in certain alluvial soils near Nottingham. *Nova Hedwigia* **5**(2): 57-78.

Augustin, H. W., Kopperschlager, G., Steffen, H. and Hofmann, E. 1965. Hexokinase as limiting factor of anaerobic glucose consumption of *Saccharomyces carlsbergensis* NCYC74. *Biochim. Biophys. Acta* **110**: 437-439.

Beaven, M. J., Charpentier, C. and Rose, A. H. 1982. Production and tolerance of ethanol in relation to phospholipid fatty-acyl composition in *Saccharomyces cerevisiae* NCYC. *J. Gen. Microbiol.* **128**: 1447-1455

Chi, Z. and Arneborg, N. 1999. Relationship between lipid com-

position, frequence of ethanol-induced respiratory deficient mutants, and ethanol tolerance in *Saccharomyces cerevisiae*. *J. Applied. Microbiol.* **86**: 1047-1052.

Dutta, B. G., Ghosh, G. R., Orr, G. F. and Kuehn, H. H. 1964. Soil fungi from Orissa (India). *Mycologia* **56**: 153-157.

Furuya, K. and Naito, A. 1979. An effective method for isolation of *Ascodesmis* from soil. *Trans. Mycol. Soc. Japan* **20**: 171-175.

Ghareib, M., Youssef, K. A. and Khalil, A. A. 1988. Ethanol tolerance of *Saccharomyces cerevisiae* and its relation to lipid content and composition. *Folia Microbiol.* **33**: 447-452.

Ibeas, J. I. and Jimenez, J. 1997. Mitochondrial DNA loss caused by ethanol in *Saccharomyces* flor yeasts. *Appl. Envrion. Microbiol.* **63**: 7-12

Ingram, L. O. 1976. Adaptation of membrane lipids to alcohols. *J. Bacteriol.* **125**: 670-678

Ito, T., Ueda, M. and Yokoyama, T. 1981. Thermophilic and thermotolerant fungi in paddy field soils. *IFO Res. Comm.* **10**: 20-32.

Min, K. H., Ito, T. and Yokoyama, T. 1981. Fungus flora of paddy fields in Korea. I. Fungal distribution of paddy fields. *Kor. J. Microbiol.* **19**: 153-162.

Min, K. H., Ito, T. and Yokoyama, T. 1987. Fungus flora of paddy fields in Korea. . Filamentous fungi isolated by heat treatment. *Kor. J. Microbiol.* **15**: 187-195.

Mishra, P. and Kaur, S. 1991. Lipids as modulators of ethanol tolerance in yeast. *Appl. Microbiol. Biotech.* **34**: 697-702.

_____ and Prasad, R. 1988. Role of phospholipid head groups in ethanol tolerance of *Saccharomyces cerevisiae*. *Jour. Gen. Microbiol.* **134**: 3205-3211.

_____ and _____. 1989. Relationship between ethanol tolerance and fatty acyl composition of *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* **30**: 294-298.

Russell, N. J. 1989. Functions of lipids: Structural roles and membrane functions. In Microbial Lipids, Vol. 2 ed. Ratledge, C. and Wilkinson, S. G. pp. 279-365.

Tokumasu, S. 1974. A study of evaluation of methods for the isolation of soil fungi. *Trans. Mycol. Soc. Japan.* **15**: 135-146.

Sajbidor, J. 1997. Effect of some environmental factors on the content and composition of microbial membrane lipids. *Crit. Rev. Biotech.* **17**: 87-103.

Swan, T. M. and Watson, K. 1999. Stress tolerance in yeast lipid mutants; membrane lipids influence tolerance to heat and ethanol independently of heat shock proteins and trehalose. *Can. J. Microbiol.* **45**: 472-479.