Journal of Microbiology and Biotechnology
Vol. 1, No. 1, 6-11, 1991

Effect of Oxygen and Unsaturated Fatty Acids on the Ethanol Tolerance of Yeast Strains

RYU, YEON WOO* AND HEANG WOOK JANG

Department of Biotechnology, College of Engineering, Ajou University, Suwon 441-749, Korea

Received 2 February 1991 / Accepted 14 March 1991

This study deals with investigation of the ethanol tolerance of yeast strains with respect to fatty acid composition and intracelluar ethanol concentration during alcohol fermentation. The cell viabilities and fermentation abilities of *Saccharomyces cerevisiae* and *Kluyveromyces fragilis* were improved by aeration and addition of unsaturated fatty acids into growth medium. Aeration decreases the accumulation of ethanol, while increases unsaturated fatty acid contents inside yeast cells. Thus it was found that oxygen and unsaturated fatty acids play decisive roles in the increase of ethanol tolerance of yeasts.

In the fuel alcohol production, it is favorable to produce high content of alcohol for high productivity. However the cell growth and ethanol production in alcohol fermentation by yeasts are inhibited by ethanol produced, resulting in the decrease of the yeast cell viability (4, 16). The inhibitions are partially induced by noncompetitive feedback inhibition by intracellular ethanol on phosphoglycerate kinase, pyruvate decarboxylase and hexokinase (13, 17). Moreover the increase in apparent intracellular ethanol content was reflected in a fall in alcohol dehydrogenase activity (16), and denaturation of hexokinase was shown to correlate well with measured intracellular ethanol concentration (19).

The ethanol tolerance of yeasts depends on the fermentation conditions and cellular characterization of the organisms used. The fermentation conditions for obtaining the ethanol tolerance are achieved by aeration, lowering the fermentation temperature, and adding unsaturated fatty acids in the medium (10, 11).

Oxygen is utilized by yeast for two purpose: as an ultimate electron acceptor in aerobic respiration (1, 20) and as a growth factor (5, 22). In particular, oxygen is participating in the synthesis of unsaturated fatty acids, sterol and its precursors necessary for cellular membrane (8, 9, 22). The ethanol tolerance of yeasts is increased by the high content of unsaturated fatty acids due to higher ethanol diffusion rate into the medium (28). Therefore, the cell growth rate, ethanol production rate and cell viability are increased by aeration (10, 11). As a result aeration increases the ethanol tolerance of yeasts (6, 16).

The ethanol tolerance of yeasts is also increased by medium supplements, such as ergosterol and unsaturated fatty acids (2, 3, 5). The possibility that the well-known ability of unsaturated fatty acids to moderate ethanol toxicity might be due to enhanced rates of ethanol leakage from the cell (23). Especially, intracellular ethanol concentration was consistently lower when linoleic acid was employed, suggesting that the rate of ethanol efflux might be higher in linoleyl-residue enriched cells (23, 28).

Hence, this study was undertaken to investigate the effect of oxygen and unsaturated fatty acids in a fermentation medium on the ethanol tolerance of yeast strains by measuring fatty acid composition and intracellular ethanol concentration in batch alcohol fermentations.

MATERIALS AND METHODS

Organisms

Saccharomyces cerevisiae STV 89, reported to produce high content ethanol (24), and *Kluyveromyces fragilis* CBS 397 obtained from Centraalbureau voor Schimmel Culture (Netherland) were used. Strains were maintained on slants containing 1% yeast extract, 1%

*Corresponding author
Key words: Intracellular ethanol, ethanol tolerance, unsaturated fatty acids

peptone, 2% glucose and 1.5% agar at 4℃.

Inocula were prepared by growing the organisms in medium containing 10% glucose, 0.5% peptone and 0.5 % yeast extract for 16 hr with agitation at 30℃.

Fermentation Medium

The fermentation medium consisted of 20% glucose, 0.5% yeast extract, 0.5% peptone, 0.5% KH_2PO_4, 0.2% $(NH_4)_2SO_4$ and 0.04% $MgSO_4 \cdot 7H_2O$. pH was adjusted to 5.0. The stock solution of lipid complex was prepared by dissolving 12 g of ergosterol in 96 m*l* absolute ethanol and then adding 2 m*l* linoleic acid and 2 m*l* oleic acid. This mixture was flushed with nitrogen and added into the medium at the ratio of 5 m*l* per one liter medium.

Fermentation

Fermentations were carried out in 20 *l* jar fermentor (Bioengineering Co., Swiss) by loading 16 *l* fermentation medium with agitation of 300 rpm at 30℃. Aerobic and strictly anaerobic conditions were maintained by supplying 0.16 vvm of air and 0.01 vvm of nitrogen gas without air, respectively.

Analytical Methods

Glucose concentration was measured as reducing sugar using DNS methods (14). Cell mass concentration was determined by measuring the optical density at 540 nm and was expressed in grams (dry weight) of cells per liter by referring to a calibration curve showing a linear correlation between optical density and dry cell weight. Cell viability was determined by counting the fraction of viable cells over the total cells (over 500 cells counted) using the methylene blue staining method of McDonald (12).

Ethanol concentration was measured by gas chromatography using G3800 (Yamaco, Japan) with a flame ionization detector. A column (2.0 m by 2.0 mm) packed with Chromosorb W/AW (60~80 mesh) impregnated with 10% Carbowax 20 M. The temperatures of injector and detector were 250℃ and the column oven was operated isothermally at 90℃. The internal standard was used with 2% (v/v) n-butanol.

For the determination of intracellular ethanol concentration, the fermentation broth (500 m*l*) was immediately centrifuged at 4,000 rpm for 20 min in a refrigerated centrifuge. The volume of supernatant was correctly measured at 30℃ for determining packed cell volume and retained for extracellular ethanol analysis. The sediment was resuspended in distilled water of three volumes of packed cell. The ethanol analysis in supernatant and cell suspension was carried out using the gas chromatography mentioned above. Intracellular ethanol concentration (P_i) was determined from the formula, $P_i = (P_cV_c - P_eV_e)/V_{cell}$ where P_c=ethanol concentration of cell suspension, V_c=volume of cell suspension, P_e=extracellular ethanol concentration, V_e=extracellular volume in packed cell and V_{cell}=volume of cells in samples which was assumed as 3 μl per mg dry weight of intact viable cells (18, 21).

Fatty Acid Analysis

Lipids were extracted from freeze-dried cells by the method of Taylor and Parks (26) except that methanol and 0.9% NaCl were used instead of methyl sulfoxide and 2 M KCl, respectively. Fatty acids were analyzed as their methyl ester derivatives. Methyl esters of fatty acids were formed from the dry total lipids by refluxing for 10 min with anhydrous methanol containing 14% (w/v) of BF_3 by method of Morrision and Smith (15). Fatty acid methyl esters were then extracted with n-hexane. The n-hexane extract was concentrated by a stream of nitrogen. The fatty acid composition of methyl esters was determined by a Hewlett-Packard G-5890 gas chromatography with a flame ionization detector and a stainless steel column (3 mm×3 m) of 10% Silar-7CP on 100~120 mesh Chromosorb W-HP. The injector and detector temperatures were 260℃ and 290℃, respectively, and the column oven was operated at 185℃ initial to 220℃ final at rate of 1.3℃ per minute. Peak areas were determined by integrator.

RESULTS AND DISCUSSION

Alcohol fermentations were carried out to study the effect of oxygen and lipid complex (ergosterol, linoleic and oleic acids) added on the ethanol tolerance of *S. cerevisiae* and *K. fragilis*. The various kinetic parameters in

Table 1. Various kinetic parameters in different fermentation conditions

Fermentation conditions	*Saccharomyces cerevisiae*				*Kluyveromyces fragilis*			
	$\mu_{max}(hr^{-1})$	$X_m(g/l)$	$P(g/l \cdot hr)$	$P_m(g/l)$	$\mu_{max}(hr^{-1})$	$X_m(g/l)$	$P(g/l \cdot hr)$	$P_m(g/l)$
Aerobic	0.384	11.80(26)	3.24	97.1(30)	0.347	9.97(38)	3.35	87.0(26)
Strictly anaerobic	0.226	6.69(72)	0.93	74.5(80)	0.054	1.23(56)	0.63	55.2(88)
Strictly anaerobic with lipids	0.300	8.26(48)	1.45	81.2(56)	0.309	4.65(40)	1.09	72.6(72)

μ_{max}: Maximum specific growth rate, P: Overall ethanol productivity.
X_m: Maximum cell concentration; P_m: Maximum ethanol concentration.
The numbers in parenthesis indicate the fermentation time (hr) at the maximum cell and ethanol concentration.

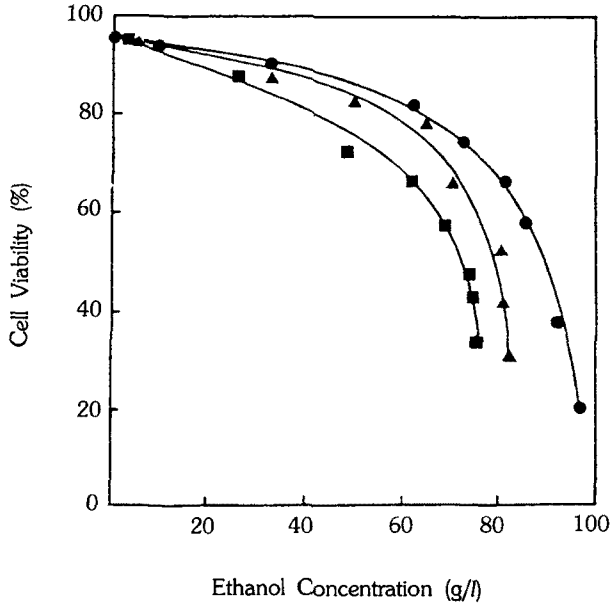

Fig. 1. Cell viability of *S. cerevisiae* STV 89 as function of ethanol concentration in medium under aerobic (●), anaerobic (■) and lipid supplemented anaerobic (▲) conditions.

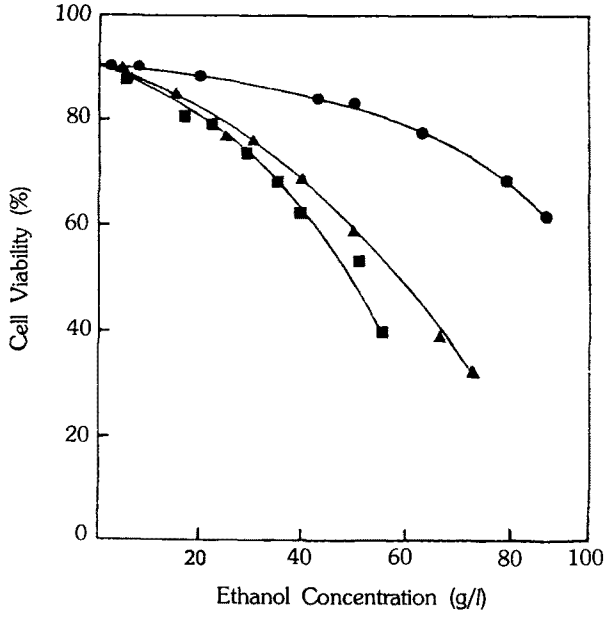

Fig. 2. Cell viability of *K. fragilis* CBS 397 as function of ethanol concentration in medium under aerobic (●), anaerobic (■) and lipid supplemented anaerobic (▲) conditions.

Table 2. Fatty acid composition of *S. cerevisiae* STV 89 grown aerobically, anaerobically and anaerobically with lipid supplements (ergosterol, oleic and linoleic adis)

Culture conditions	Culture time(hr)	Fatty acid composition(%)										Sum(%)	
		$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:0}$	Saturated	Unsaturated
Aerobic	9	–	0.1	0.8	16.0	11.0	5.4	27.4	25.4	13.9	–	22.3	77.7
	22	–	0.2	0.4	8.6	9.4	8.1	35.1	19.2	16.9	2.1	19.4	80.6
	30	–	0.3	0.6	10.2	14.9	8.2	41.1	24.7	–	–	19.3	80.7
Anaerobic	16	0.5	–	2.2	16.2	13.0	11.4	18.8	20.3	12.7	4.9	35.2	64.8
	24	–	0.4	3.7	23.2	12.4	11.9	13.2	14.2	13.6	7.4	46.6	53.4
	56	0.2	0.6	6.1	20.9	11.0	12.3	14.9	14.7	12.6	6.7	46.8	53.2
Anaerobic with	16	0.2	0.3	1.9	14.6	7.9	7.6	29.4	38.1	–	–	24.6	75.4
ergosterol, oleic	24	–	0.1	1.6	15.6	7.4	13.6	28.3	33.4	–	–	30.9	69.1
and linoleic acids	56	–	0.2	1.6	16.7	6.4	13.5	30.2	31.4	–	–	32.0	68.0

Table 3. Fatty acid composition of *K. fragilis* CBS 397 grown aerobically, anaerobically and anaerobically with lipid supplements (ergosterol, oleic and linoleic acids)

Culture conditions	Culture time(hr)	Fatty acid composition(%)										Sum(%)	
		$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:0}$	Saturated	Unsaturated
Aerobic	9	–	1.2	2.0	16.5	40.4	4.4	31.2	2.8	–	1.5	25.6	74.4
	22	0.5	0.7	0.8	14.4	20.3	10.1	30.5	22.7	–	–	26.5	73.5
	30	0.1	0.8	0.7	12.9	27.8	9.2	32.8	15.7	–	–	23.7	76.3
Anaerobic	16	8.4	8.2	9.5	32.5	9.8	11.1	7.4	3.8	4.3	5.0	74.7	25.3
	24	11.7	11.0	8.6	31.6	8.3	15.7	8.2	4.9	–	–	78.6	21.4
	56	8.4	15.7	10.7	34.6	6.7	17.6	6.3	–	–	–	87.0	13.0
Anaerobic with	16	–	3.2	9.5	25.1	7.5	10.7	25.0	19.0	–	–	48.5	51.5
ergosterol, oleic	24	0.1	0.9	1.6	9.9	7.2	17.5	23.0	23.3	8.6	7.9	37.9	62.1
and linoleic acids	56	–	1.6	1.3	9.9	3.1	19.6	34.7	28.7	–	1.1	33.5	66.5

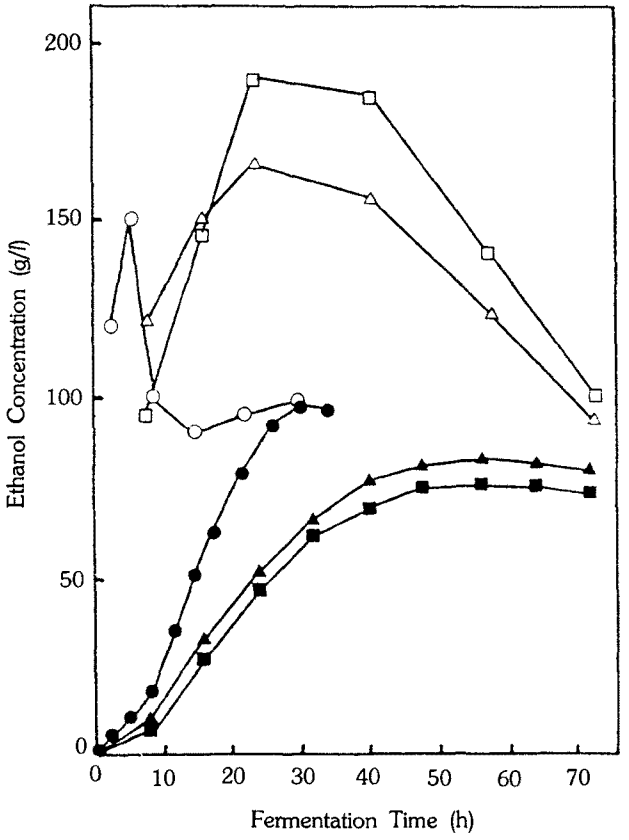

Fig. 3. Accumulation of extracellular (●, ■, ▲) and intracellular (○, □, △) ethanol during the fermentation time of *S. cerevisiae* STV 89 in aerobic (●, ○), anaerobic (■, □) and lipid supplemented anaerobic (▲, △) conditions.

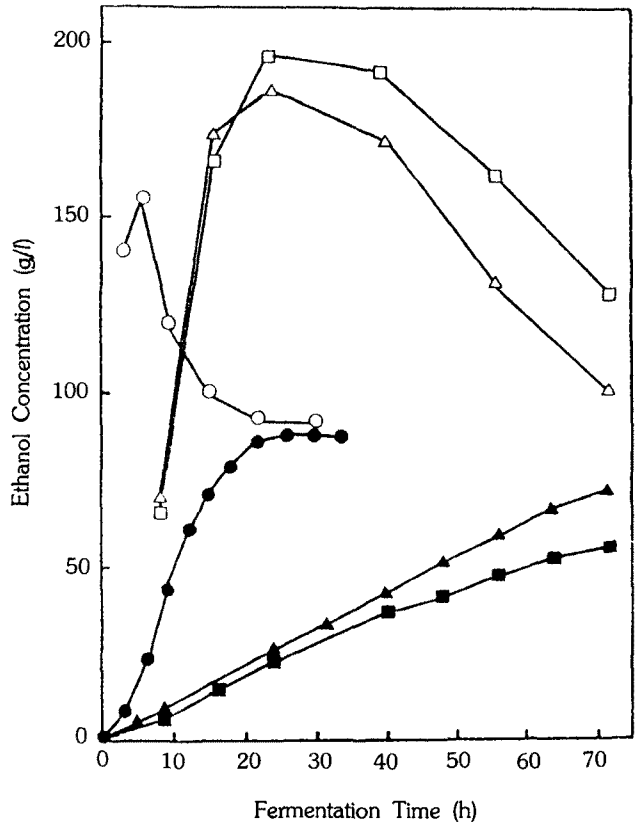

Fig. 4. Accumulation of extracellular (●, ■, ▲) and intracellular (○, □, △) ethanol during the fermentation time of *K. fragilis* CBS 397 in aerobic (○, ●), anaerobic (■, □) and lipid supplemented anaerobic (▲, △) conditions.

aerobic, anaerobic and lipid supplemented anaerobic conditions were shown in Table 1.

The cell growth rate and ethanol production rate were stimulated by aeration. The cell mass and ethanol production were also substantially improved by aeration. With respect to strains, the maximum specific growth rate and overall ethanol productivity of *K. fragilis* under aerated condition were 6.4 fold and 5.3 fold higher than those under anaerobic condition, although those of *S. cerevisiae* were increased 1.7 times and 3.5 times by aeration. Under aerobic condition, the maximum ethanol concentration (P_m) reached at 97.1 g/l and ethanol yield was 0.486 g-ethanol/g-glucose in *S. cerevisiae*, whereas in *K. fragilis*, P_m reached at 87.0 g/l and ethanol yield was 0.435 g-ethanol/g-glucose. These values slightly lower than those in *S. cerevisiae*, because *K. fragilis* utilized the ethanol for its growth at late stage of fermentation as reported by Guiraud *et al.* (7). The addition of ergosterol, linoleic and oleic acids also improved the cell growth and ethanol production of *S. cerevisiae* and *K. fragilis* as reported by Janssens *et al.* (8) and Ryu *et al.* (25).

The cell viabilities of *S. cerevisiae* (Fig. 1) and *K. fragilis* (Fig. 2) have been substantially improved by aeration and addition of ergosterol, linoleic and oleic acids.

As a result, the cell growth and ethanol production rate, cell viability were enhanced by aeration and supplemented lipids. It was suggested that the oxygen participates in the synthesis of sterol, unsaturated fatty acids and its precursors necessary for cellular membranes (8, 9, 22). The ergosterol and unsaturated fatty acids also act directly as precursors of the lipid synthesis of cellular membranes (8, 27). Hence, the enrichment of unsaturated fatty acids of cell membrane improved cell viability in yeasts (5, 8, 22).

To investigate the factors affecting ethanol tolerance, qualitative fatty acid profiles were made from *S. cerevisiae* and *K. fragilis* cultures harvested after growth under aerobic, anaerobic growth, and lipid supplemented anaerobic conditions.

In fatty acid composition of *S. cerevisiae* (Table 2) grown under aerobic condition, the content of unsatura-

ted fatty acid primarily linoleic acid ($C_{18:2}$) and oleic acid ($C_{18:1}$) was about 4 times higher than that of saturated fatty acid. The anaerobically grown cells contained slightly higher level of unsaturated fatty acid, although palmitic aicd ($C_{16:0}$) was contained in the highest level. In the lipid-supplemented anaerobic cultures containing exogeneous linoleic and oleic acids represented 34% and 29% in average of these fatty acids, respectively, which are significant increases compared with the anaerobic cultures.

In fatty acid composition of *K. fragilis* (Table 3), the aerobically grown cells contained predominantly unsaturated fatty acids (74.7%), primarily palmitoleic acid ($C_{16:1}$), oleic acid and linoleic acid. Meanwhile, tne content of saturated fatty acid under anaerobically grown cell was approximately 4 fold higher than that of unsaturated fatty acid. Especially, palmitic aicd ($C_{16:0}$) was the highest content. The content of unsaturated fatty acid was also markedly increased by the supplement of oleic and linoleic acids and ergosterol.

As a result, the content of unsaturated fatty acid of *S. cerevisiae* and *K. fragilis* was increased 1.4 fold and 3.8 fold by aeration, respectively. The content unsaturated fatty acids of *S. cerevisiae* was slightly higher than that of *K. fragilis*. Thus it was found that the stimulation of cell growth and ethanol production is owing to the increase of unsaturated fatty acid content by aeration.

The intracellular ethanol concentration of *S. cerevisiae* during the fermentation was shown in Fig. 3. Under aerobic condition, the intracellular ethanol was rapidly accumulated due to higher ethanol production rate than ethanol diffusion into the medium during an early fermentation stage. After reaching up to a maximum intracellular ethanol concentration, the accumulation of ethanol in cells was rapidly decreased up to extracellular level in the late fermentation stage, resulting from the higher rate of ethanol diffusion into medium than that of ethanol prodution.

Under anaerobic condition, however, the accumulation of ethanol in cells slowly increased up to about 190 g/l in 30 hr of fermentation and thereafter slowly decreased up to the extracellular level. The intracellular ethanol concentration of *K. fragilis* was shown in Fig. 4. The intracellular ethanol concentration of *K. fragilis* showed almost same profiles as *S. cerevisiae* regardless of fermentation mode. The maximum intracellular ethanol concentration of *K. fragilis* was slightly higher than that of *S. cerevisiae* under same fermentation conditions.

From these results, it was suggested that the cell growth rate ethanol production rate, and cell viability were increased by high content of unsaturated fatty acids, especially linoleic acid, showing moderate ethanol toxicity due to enhanced rate of ethanol diffusion into medium (23, 28) under aerobic condition. Thus, it was concluded that oxygen and unsaturated fatty acids take decisive roles in the increase of ethanol tolerance of yeast strains.

Acknowledgement

This study was supported by a research grant (1988~1989) from the Korea Science and Engineering Foundation, for which authors are very grateful.

REFERENCES

1. **Akbar, M.D., P.A.D. Ricard, and F.J. Moss.** 1974. Response of the adenosine phosphate pool level to changes in the catabolic pattern of *Saccharomyces cerevisiae*. *Biotechnol. Bioeng.* **16**: 455-474.
2. **Andreasen, A.A. and T.J.B. Stier.** 1953. Anaerobic nutrition of *Saccharomyces cerevisiae*. I. Ergosterol requirement for growth in a defined medium. *J. Cell. Comp. Physiol.* **41**: 23-26.
3. **Andreasen, A.A. and T.J.B. Stier.** 1954. Anaerobic nutrition of *Saccharomyces cerevisiae*. II. Unsaturated fatty acid requirement for growth in a defined medium. *J. Cell. Comp. Physiol.* **43**: 271-282.
4. **Bazua, C.D. and C.R. Wilke.** 1977. Ethanol effects on the kinetics of a continuous fermentations with *Saccharomyces cerevisiae*. *Biotechnol. Bioeng. Symp.* **7**: 105-118.
5. **Cysewski, G.R.,** 1976. Fermentation kinetics and process economics for the production of ethanol. *Ph.D. Dissertation*, University of Berkley.
6. **Cysewski, G.R. and C.R. Wilke.** 1977. Rapid ethanol fermentation using vacuum and cell recycle. *Biotechnol. Bioeng.* **19**: 1125-1143.
7. **Guiraud, J.P., J.M. Caillaud, and P. Galzy.** 1982. Optimization of alcohol production from Jerusalem Artichokes. *Eur. J. Appl. Microbiol. Biotechnol.* **14**: 81-85.
8. **Janssens, J.H., H. Burris, A. Woodward, and R.B. Bailey.** 1983. Lipid-enhanced ethanol production by *Kluyveromyces fragilis*. *Appl. Environ. Microbiol.* **45**: 598-602.
9. **Jollow, D., G.M. Kellerman, and A.W. Linnane.** 1968. The biogenesis of mitochodria III. The lipid composition of aerobically and anaerobically grown *Saccharomyces cerevisiae* as related to the membrane systems of the cells. *J. Cell. Biol.* **37**: 221-230.
10. **Kim, H.J. and Y.W. Ryu.** 1989. The conditions affecting ethanol tolerance of yeast strains in alcohol fermentation -Study on the fermentation temperature and substrate type. *Kor. J. Biotechnol. Bioeng.* **4**: 167-171.
11. **Kim, H.J., H.W. Jang, and Y.W. Ryu.** 1989. The conditions affecting ethanol tolerance of yeast strains in alcohol fermentation -Study on the aeration and lipid addition. *Kor. J. Biotechnol. Bioeng.* **4**: 172-176.
12. **McDonald, V.R.** 1963. Direct microscopic technique to detect viable yeast cells in pasteurized orange drink. *J. Food Sci.* **28**: 135-139.

13. **Miller, D.G., K. Griffiths-Smith, E. Algar, and R.K. Scopes.** 1982. Activity and stability of glycolytic enzymes in the presence of ethanol. *Biotechnol. Lett.* **4**: 601-605.
14. **Miller, G.L.,** 1959. Use of dinitrosalicylic acid reagent for determination of reduing sugar. *Anal. Chem.* **31**: 426-428.
15. **Morrison, W.R. and L.M. Smith.** 1964. Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride-methanol. *J. Lipid Res.* **5**: 600-608.
16. **Nagodawithana, T.W. and K.H. Steinkraus.** 1976. Influence of the rate of ethanol production and accumulation on the viability of *Saccharomyces cerevisiae* in "Rapid fermentation". *Appl. Environ. Microbiol.* **31**: 158-162.
17. **Nagodawithana, T.W., J.T. Whitt, and A.J. Cutaia.** 1977. Study on the feedback effect of ethanol on selected enzymes of the glycolytic pathway. *J. Amer. Soc. Brew. Chem.* **35**, 179-183.
18. **Navarro, J.M. and G. Durand.** 1978. Fermentation alcoholique: Influence de la temperature sur l'accumulation d'alcohol dans les cellules de levure. *Ann. Microbiol. (Inst. Pasteur)* **129B**: 215-224.
19. **Navarro, J.M. and J.D. Finck.** 1982. Evolution de l'activity "hexokinase" de *Saccharomyces uvarum* fermentant le saccharose. *Cell. Mol. Biol.* **28**: 85-90.
20. **Oura, E.** 1974. Effect of aeration intensity on the biochemical composition of baker's yeast. II. Activities of the oxidative enzymes. *Biotechnol. Bioeng.* **16**: 1213-1225.
21. **Pamment, N.B. and G. Dasari.** 1989. Intracellular ethanol concentration and its estimation, p. 147-192. *In* N. van Uden (ed.) *Alcohol toxicity in Yeast and Bacteria*, CRC Press, Inc., Florida.
22. **Rogers, P.J. and P.R. Stewart.** 1973. Mitochondrial and peroxisomal contributions to the energy metabolism of *Saccharomyces cerevisiae* in continuous culture. *J. Gen. Microbiol.* **79**: 205-217.
23. **Rose, A.H. and M.J. Beavan.** 1981. End-product tolerance and ethanol, p.513-531. *In* A. Hollaender (ed.), *Trends in the Biology of Fermentations for Fuel and Chemicals, Basic life Sci.*, Vol. **18**, Plenum Press, New York.
24. **Ryu, Y.W. and J.J. Kwon.** 1982. Effect of fermentation temperature on the production of high content alcohol. *Kor. J. Microbiol.* **20**: 67-72.
25. **Ryu, Y.W., C. Kim, and S.I. Kim.** 1988. A study on the factors for improving the ethanol production from Jelusalem Artichoke by *Kluyveromyces fragilis*. *Kor. J. Chem. Eng.* **5**: 1-4.
26. **Taylor, F.R. and L.W. Parks.** 1978. Metabolic interconversion of free sterols and steryl esters in *Saccharomyces cerevisiae*. *J. Bacteriol.* **136**: 531-537.
27. **Thomas, D.S., J.A. Hossack, and A.H. Rose.** 1978. Plasma-membrane lipid composition and ethanol tolerance in *Saccharomyces cerevisiae*. *Arch. Microbiol.* **117**: 239-245.
28. **Thomas, D.S. and A.H. Rose.** 1979. Inhibitory effect of ethanol on growth and solute accumulation by *Saccharomyces cerevisiae* as affected by plasma-membrane lipid composition. *Arch. Microbiol.* **122**: 49-55.