Cell Viability and Fatty Acids Composition of *Zymomonas mobilis* grown at different Concentrations of Ethanol

Kwon, Suk-Heum and Kye Joon Lee
Department of Microbiology, College of Natural Sciences, Seoul National University, Seoul, 151, Korea

Zymomonas mobilis 균체의 지방산 분포와 균의 생존성에 미치는 Ethanol 농도의 영향

권석흠 · 이계준
서울대학교 자연과학대학 미생물학과

ABSTRACT: The aim of the present studies was to analyze the physiological background of ethanol inhibition in *Zymomonas mobilis*. The experiments were carried out with a number of continuous culture to give steady state concentration of ethanol. The composition of fatty acids in the cells obtained from various conditions was analyzed and cell viability was also estimated. As results, it was found that vaccenic acid was the major fatty acid in the cell of *Z. mobilis* and the concentration was changed apparently to increase as increasing the concentration of ethanol produced from substrate utilization. Finally it was observed also that cell viability was decreased remarkably at the elevated ethanol concentration. Those changes might play important roles in the ethanol fermentation to give more complex phenomena observed at high concentration of ethanol.

KEY WORDS □ *Zymomonas mobilis*, fatty acids composition, cell viability, ethanol inhibition.

There have been many reports of inhibition in yeasts at increased ethanol concentrations including studies on the kinetics of ethanol inhibition (Baua and Wilke, 1977 ; Lee *et al.*, 1980 ; Brown *et al.*, 1981). Some of the proposed mechanisms for ethanol inhibition are follows: (1) inhibition of solute transport system (Thomas and Rose, 1979) (2) alteration in membrane fluidity and permeability (Novak *et al.*, 1981) (3) feedback inhibition on the key enzymes of the glycolytic pathway (Nagodawithana *et al.*, 1977).

Loss of viability in yeasts at high ethanol levels has been reported, although the effect was partially alleviated by the presence of dissolved oxygen (Cysewski and Wilke, 1977). The loss of viability may resulted from accumulation of ethanol within the cell and it has been reported that ethanol level within the cell can reach much higher concentration than in the external environment (Novak *et al.*, 1981).

Fermentation kinetics studies with various strains of *Zymomonas mobilis* have shown that linear inhibition kinetics occurred for the effects of ethanol on specific growth rate (μ) and specific ethanol production rate (q_p). However the threshold concentration of ethanol, from which the growth rate was inhibited, was much lower than that initiating the inhibition on ethanol production rate (Lee *et al.*, 1983).

The uncoupling growth was evident in *Z. mobilis*, although an appropriate mechanism has not been proposed.

In the present study, attempts have been tried to elucidate the mechanism of ethanol inhibition in the culture of *Z. mobilis*.

As the first step, the changes of fatty acids composition and cell viability were tested as functions of growth rate and ethanol concentration.

MATERIALS AND METHODS

Strain and media used

The strain used in the investigation was *Z. mobilis* ZM 4 (now designated to ATCC 31821) and the strain was obtained from Prof. P.L. Rogers, University of New South Wales. It was maintained by transferring to fresh agar slants containing 20 g/*l* glucose, 10 g/*l* yeast extract(Difco), and 20 g/*l* agar at pH 5.0 each week and storing at room temperature. The composition of the seed culture and fermentation media was: 20-250 g/*l* glucose, 10 g/*l* yeast extract, 1 g/*l* KH_2PO_4, 1 g/*l* $(NH_4)_2SO_4$, 0.5 g/*l* $MgSO_4 \cdot 7H_2O$. For 250 g/*l* glucose media, second seed culture was carried out with the same media of fermentation for adaption to high glucose concentration.

Experimental procedure

Z. mobilis was first propagated at 30℃ for 24 hr without agitation by transferring single colonies from the stock culture slant to 50 m*l* of pre-seed culture medium. Ten m*l* of the culture broth were then transferred to 90 m*l* of seed culture medium. After 12-20 hr incubation it was inoculated to 900 m*l* of fermentation medium in a 1 *l* fermentor (B. Broun model M). The culture was grown under non-aerated conditions at 30℃ and pH 5.0. To maintain a homogeneous culture, mild agitation was provided by a stirrer.

Analytical methods

Biomass(expressed as dry weight) was determined from the optical density at 660 nm using the uninoculated media as a blank. A turbidity-dry weight calibration curve was prepared from cells grown under similar conditions. The total glucose concentration was estimated on the supernatant after centrifugation, 4000xg, 10 min, using the denitrosalysilic acid method (Miller, 1959). For ethanol estimation, samples were distillated and analysed using a procedure developed by Sawer and Dixon(1968). Viable cell counts were carried out by plating an appropriately diluted culture broth onto the plates of the stock culture medium. The dilution of cells was done with saline solution and the liquid stock culture medium. The inoculated plates were incubated at 30℃ for two three days. The viable cell counts were carried out in quintuplicate.

Estimation of fatty acids

Total fatty acids of *Z. mobilis* were extracted by the method of Tornabene *et al.* (1982) as followed: cells harvested from 40 m*l* of culture broth (4,000 xg, 10 min) were washed twice with a phosphate buffer(pH 6.0, 0.1 M) then saponificated with 2 m*l* solution of 15% NaOH in 50% methanol by boiling at 100℃ for 30 min. The mixtures were reacted with 3 m*l* of 25% HCl-methanol at 85℃ for 10 min. The methylated fatty acids were then extracted with 2 m*l* of hexane-ethylacetate (1 : 1) mixture and the supernatants were treated with 5 m*l* of 0.3 N NaOH and centrifuged again then the clear supernatant was used as samples for gas-chromatography and mass-spectroscopy. Varian model 3700 gas chromatograph with a column packed diethylene glycol succinate 15% on Chromosorb was used under following conditions: temp; 180℃, injection temp; 210℃ FID temp; 210℃, carrier gas; He 20 m*l*/min.

RESULTS

Effect of growth rate

In order to know the effects to growth rate on the composition of fatty acids, continuous cultures were carried out using a rich medium containing 40 g/*l* of glucose. Dilution rates were varied from 0.05 to 0.45 h^{-1}. The steady-state concentration of ethanol, glucose,

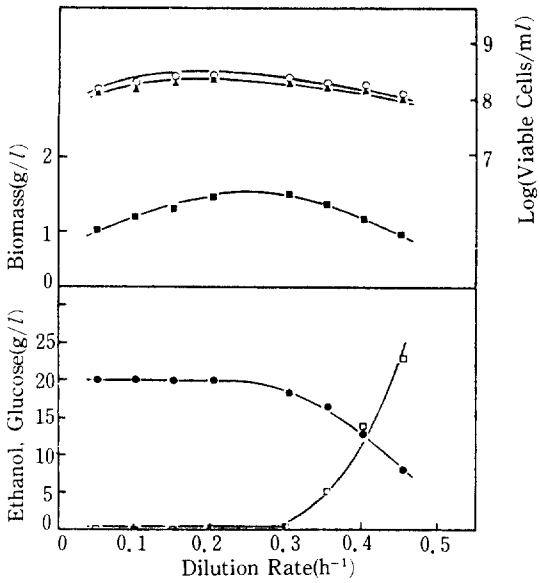

Fig.1. *Steady-state data of glucose, ethanol, biomass and viable cells for Z. mobilis ZM 4 with 40 g/l glucose medium (pH= 5.0, temp=30℃).*
□ : glucose, ● : ethanol, ■ : biomass,
△ : viable cells from saline solution,
○ : viable cells from rich medium

biomass and viable cell number, were shown in Fig.1. Relatively same number of viable cells were counted from the both diluted culture broth and the viable cells were very closely related to the biomass estimated as dried cell weight. It was evident that glucose limited chemostat was operated until dilution rate reached to $0.3\,h^{-1}$ and further increase of dilution rate over $0.3\,h^{-1}$ resulted in ethanol inhibited cultures. The ethanol concentrations varied from 19.0 g/l to 7.6 g/l as a function of dilution rate.

The profile of fatty acids extracted from the cell of different growth rate were shown in Table 1. Vaccenic acid ($C_{18:1}$) was the major component of the fatty acids extracted from the cell of *Z. mobilis*. It was interesting to note that the contents of lauric acid (C_{12}) was decreased remarkably as increasing the ethanol concentration, while the content of palmitic acid (C_{16}) was increased. However the contents of myristic acid (C_{14}) and vaccenic acid ($C_{18:1}$) were not changed in some extent. It was not clearly concluded that the changes of the fatty acids profile were solely affected by the ethanol concentration produced in the chemostat, since it was shown that the contents of lauric acid (C_{12}) and palmitic acid (C_{16}) were varied with the specific growth rate under constant ethanol concentration *viz.* 18.9-19.3 g/l. It was considered therfore to contemplate chemostat cultures at constant growth rate but the ethanol concentration should be varied.

Effects of ethanol concentration

A number of continuous cultures were carried using rich media containing 40, 60, 80 and 100 g/l of glucose. The experiments were attempted to elucidate the effects of ethanol on the fatty acid profile without any interferences. The specific growth rate was fixed to $0.25\,h^{-1}$ and the conditions were maintained to be constant except the ethanol concentrations in the culture. The ethanol concentrations were maintained steadily as 19.3, 26.5, 35.3 and

Table 1. *Effect of specific growth rate on the composition of fatty acids of Zymomonas mobilis grown on a rich medium containing 40 g/l glucose medium.*

Growth rate (h^{-1})	0.15	0.20	0.25	0.30	0.35	0.40
Ethanol conc. (g/l)	18.90	19.00	19.30	16.60	11.20	7.60
Fatty acids (%)						
Lauric acid	0.30	2.60	3.10	4.60	6.00	7.40
Myristic acid	31.10	32.70	32.50	32.10	33.10	33.10
Palmitic acid	13.00	9.90	9.80	9.30	8.70	7.40
Vaccenic acid	55.60	54.80	54.60	54.00	52.20	52.10

41.8 g/l by changing the input concentration of glucose from 40 g/l to 100 g/l. From the chemostat cultures, it was possible to show only the effects of ethanol.

From the data shown in Table 2, it was found that the profile of fatty acids was influenced apparently by the concentration of ethanol produced. It was clear also that the content of myristic acid was reduced as the ethanol concentration was increased, while vaccenic acid and palmitic acid were increased. And the content of lauric acid was very low at lower ethanol concentration then was not detected at higher ethanol concentration.

Table 2. *Effect of ethanol concentration on the composition of fatty acid of Zymomonas mobilis grown at specific growth rate 0.25 h^{-1}.*

Glucose conc. input (g/l)	40.0	60.0	80.0	100.8
Ethanol produced (g/l)	19.3	26.5	35.5	41.8
Fatty acids (%)				
Lauric acid	3.10	ND	ND	ND
Myristic acid	32.50	29.60	27.90	22.10
Palmitic acid	9.80	9.70	11.70	12.40
Vaccenic acid	54.60	59.50	60.30	64.80
Unknown		1.10		

ND: not detected

Viable cells at different ethanol concentration are shown in Fig.2. It was evident that cells grown at high ethanol concentration, *viz.* above 35.0 g/l, were more fragile to saline solution than those grown at low ethanol concentration. However it was very interest to note that both cells grown at low and high concentration of ethanol were not damaged when the liquid rich medium was used as a diluent.

Batch culture and cell viability

The data of dried cell weight and viable cell number calculated from batch cultures grown in rich media containing 20 g/l and 250 g/l

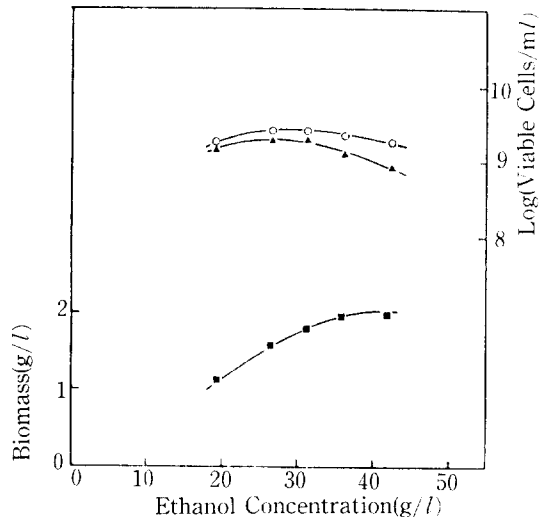

Fig.2. *Effects of ethanol concentration on the biomass formation and cell viability of Z. mobilis in continuous cultures with different concentration of glucose*

(■) : biomass, (▲) : viable cells from saline solution used for a diluent, (○) : viable cells in rich medium used for a diluent

glucose were designed to reveal both conditions of low and high concentration of ethanol. As shown in Fig.3, it was very clear that a good relationship between dried cell weight and viable cell number was observed at low ethanol concentration. However at elevated ethanol concentration the viable cell number was drastically dropped, although the biomass concentration was increased.

It was very interest to note that more viable cells were obtained when the liquid rich medium was used instead of saline solution (0.085% NaCl). The reason why the more cells were survived in the rich medium compared to the saline solution was not clear. But it is clear that the rich medium contains more organic compounds compared to the saline solution and that the membrane of cells would be altered by the ethanol concentration. It might indicate that slight changes in the cell membrane composition would result in the loss of cell viability. And the loss of viability could be compensated by the external nutrients, therefore, the cells diluted

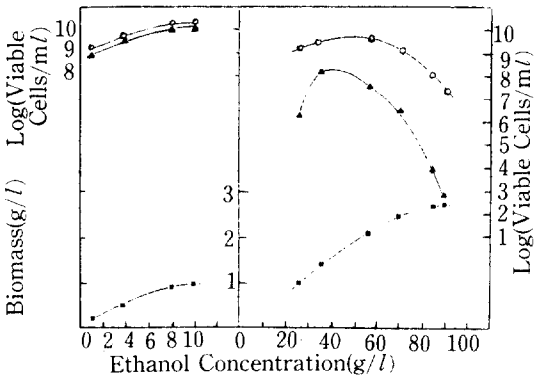

Fig.3. *Effects of ethanol concentration on the biomass formation and cell viability of Z. mobilis in batch cultures with 20 g/l glucose medium(A) and 200 g/l glucose medium(B).*

(■) : biomass; (▲) viable cells from saline soulution used for a diluent, (○) : viable cells from rich medium used for a diluent.

in rich medium were protected from loss of viability in some extent. However in the present experiments, it was not clear yet what would be implicated in the changes of the fatty acid profile and what would be resulted from the changes.

DISCUSSION

Ingram (1976) reported that ethanol molecules might be inserted in the hydrophobic region of fatty acid, middle part of the bilayer membrane, hence the fluidity of the membrane would be changed. The changes of the membrane fluidity could restrain the permeability of certain metabolite or inhibit the uptake of essential nutrient through the membrane.

Cary and Ingram (1983) reported again that glucose concentration had no major effect on the fatty acid composition of *Z. mobilis* but the extremely high levels of vaccenic acid in the membrane lipids may have been of evolutionary advantage for survival and competition in the presence of environmental stress *viz.* high concentration of glucose and ethanol. Bringer *et al.* (1985) suggested that the stability and permeability of the cytoplasmic membrane of *Z. mobilis* was regulated by variations in the distribution of fatty acids and that vaccenic acid is the most sensitive to increase as the ethanol concentration. Cary and Ingram (1983) concluded that ethanol would decrease the integrity of the primary permeability barrier of the cell by weakening the water lattice structure and decreasing the strength of hydrophobic interactions and/or by inserting the ethanol molecules within the hydrophobic core of membrane. These changes would promote membrane leakage and resulted in abnormal metabolism due to the loss of essential enzymes located in the hydrophobic core of the membrane. However increases in the chain lengths of monosaturated or branched-chain fatty acids would tend to compensate for the physical changes in membrane structure caused by the presence of ethanol.

The data presented in the current experiments indicated that the increases of the longer chain fatty acids (vaccenic acid and palmitic acid) was the compensating results of *Z. mobilis* faced upon to high concentration of ethanol. And it would be postulated also that the loss of cell viability at the elevated ethanol concentration was resulted from the changes of membrane permeability to leak some essential enzymes or metabolites, although the self compensating counteraction was evident in *Z. mobilis*.

From these observations, it was considered that the ethanol concentration played very important roles on the fatty acid composition, which might bring changes in the permeability of membrane, and that the slight changes of membrane permeability would influence on the cell viability.

적 요

*Zymomonas mobilis*에 관찰된 ethanol 저해작용의 원인을 규명하고저 회분 및 연속배양해서 얻은 균의 생존성과 균체내의 지방산 분포를 조사하였다. 그 결과 ethanol 농도의 변화는 균체의 지방산 분포에 직접적인 영향을 주었고 또한 균의 생존성과도 밀접한 연관이 있음을 알았다.

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REFERENCES

1. Barrow, K.D., J.G. Collins, P.L. Rogers and G.M. Smith, 1983 ; Lipid composition of an ethanol-tolerant strain of *Zymomonas mobilis, Biochimica et Biopysica Acta,* **753**, 324-330.
2. Bazua, C.D. and C.R. Wilke, 1977 : Ethanol effects on the kinetics of a continuous fermentation with *Saccharomyces cerevisiae, Biotech. Bioeng. Symp.* **7**, 105-118.
3. Bringer, S.T., Hartner, K. Poralla, and H. Sahm, 1985 ; Influence of ethanol on the hapanoid content and the fatty acid pattern in batch and continuous cultures of *Zymomonas mobilis, Arch. Microbiol.*, **140**, 312-316.
4. Cary, V.C. and L.O. Ingram, 1983 : Lipid composition of *Zymomonas mobilis* : Effects of ethanol and glucose, *J. Bacteriol.*, **154**, 1291-1300.
5. Cysewski, G.R. and C.R. Wilke, 1977 : Rapid ethanol fermentation using vacuum and cell recycle, *Biotech. Bioeng.*, **19**, 1125-1143.
6. Ingram, L.O., 1982 : Regulation of fatty acid composition in *Escherichia coli* : A proposed common mechanism for changes induces by ethanol, chaotropic, and a reduction of growth temperature, *J. Bacteriol.*, **149**, 166-172.
7. Lee, J.H., J.C. Williamson and P.L. Rogers 1981 : The effect of temperature on the kinetics of ethanol produced by *Saccharomyces uvarum Biotech. Letters.* **3**, 177-182.
8. Lee, K.J. and P.L. Rogers, 1983 : The fermentation kinetics of ethanol production by *Zymomonas mobilis, The Chemical Engineering Journal,* **27**, B31-38.
9. Miller, G.L., 1959 : Use of dinitrosalicylic acid reagent for determination of reducing sugar, *Anal. Chem.*, **31**, 426-428.
10. Nagodawithana, T.W., J.T. Whitt and A. J. Cutata, 1977 : Study of the feedback effect of ethanol on selected enzymes of the glycolytic pathway, *J. Am. Soc. Brew. Chem.*, **35**, 158-162.
11. Novak, M., P. Strehaiano, M. Moreno and G. Goma, 1981 : Alcoholic fermentation: On the inhibitory effect of ethanol, *Biotech. Bioeng.*, **23**, 201-212.
12. Sawyer, R. and E.J. Dixon, 1968 : The automatic determination of orgianl gravity of beer, Part II the determination of alcohol and gravity lost, *Analyst.*, **93**, 680-687.
13. 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.
14. Tornabene, T.G., G. Holzer, A.S. Bittner and K. Grohmann, 1982 : Characterization of the total extractable lipids of *Zymomonas mobilis var. mobilis, Can J. Microbiol.*, **28**, 1107-1116.

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