KOR. JOUR. MICROBIOL. June. 1985 p. 101-106 Vol. 23, No. 2

Effect of Ethanol Concentration on the Rates of Cell Growth and Ethanol Production in *Zymomonas mobilis*

Lee, Kye-Joon,
Suk-Heum Kwon, P.L. Rogers*
Department of Microbiology, Seoul National University, Seoul 151, Korea
**School of Biotechnolog, University of New South Wales, Australia*

醱酵 Ethanol농도가 *Zymomonas mobilis*의 菌体成長과 Ethanol生成速度에 미치는 영향

권석흠 · 이계준 · P. L. Rogers
서울대학교 자연과학대학 미생물학과
뉴사우스웨일즈대학 생물공학과

The effects of ethanol on the specific rates of growth and ethanol production were found to be threshold and linear inhibition. The degree of inhibition was more apparent on the specific growth rate while ethanol production was continued even the growth was ceased. The nature of uncoupling between the growth (anabolism) and ethanol production (catabolism) was clearly observed under high concentration of ethanol. The uncoupling indicated that ethanol concentration plays a great role in maintenance energy coefficient.

Different mechanisms have been proposed for the effect of ethanol inhibition on the rates of microbial growth and production formation with various strains of yeasts. These include models with linear inhibition (Ghose and Tyagi, 1979, Lee *et al.*, 1980), linear inhibition with a threshold concentration (Holzberg *et al.*, 1967), non-competitive enzyme inhibition models (Yarovenko and Nakhmanovich 1973, Novak *et al.*, 1981), exponential inhibition (Aiba *et al.*, 1969) and empirical relationships (Bazua and Wilke, 1977). In the development model with *Zymomonas mobilis*. It was found that linear inhibition kinetics occurred for the effect of ethanol on specific growth rate (μ) and specific ethanol production rate (q_s). However the threshold concentrations and the miximum ethanol concentrations were different in the two cases. Good agreement was found between the model predictions and experimental data with the model (Lee and Rogers 1983). At the present study the ethanol inhibition patterns have been demonstrated with continuous culture using high glucose medium. The nature of different degree of ethanol inhibition appeared in μ and q_s has also discussed with the concepts of uncoupling and maintenance energy coefficient.

MATERIALS AND METHODS

Zymomonas mobilis ZM4 (cp4, *de* Lima *et al.* 1970 , now designated ATCC 31821, Lee *et al.*, 1980a) was used in this studies. Maintenance of the strain and seed culture procedures have been described in earlier publications (Rogers *et al.*, 1979, Lee *et al.*, 1979) Continuous culture experiments were carried out in a nonaerated 1 liter fermentor controlled at pH 5.0 and 30°C. A vibromix unit was used to provide mild agitation (Lee *et al.*, 1979). Analytical methods for biomass, glucose and ethrnol concentrations have been

reported previously (Rogers *et al.*, 1979).

RESULTS AND DISCUSSION

Glucose-limited Continuous Culture

As can be seen in Figure 1 it is clear that in the region where glucose limitation occurred, the specific glucose uptake rate (q_s) increased linearly with the increase in dilution rate (μ) (phase I). This indicated that close coupling existed between q_s and μ. Under glucose limitation, the concentration of glucose acted to control the catabolic activity of *Z. mobilis*. The maximum values of q_s were reached at high dilution rates when there was a relatively high concentration of glucose in the culture broth. Belaich *et al.* (1968) reported that the maximum catabolic activity of *Z. mobilis* was limited by the maximal rates of reaction of the catabolic enzymes. A similar observation was made by Belaich *et al.* (1972) who evaluated the kinetic parameters of *Z. mobilis* ATCC 10988 growing in pantothenate-limited cultures and reported that catabolic activity in *Z. mobilis* was largely independent of growth rate and the nature of the culture media.

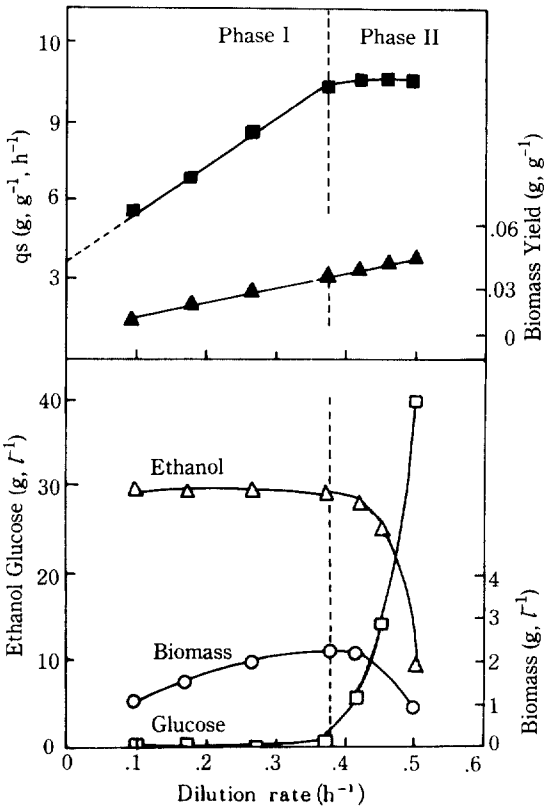

Fig. 1. Steady state data and kinetic parameters for *Z. mobilis* ZM4 with 60 g, l^{-1} glucose medium (pH = 5.0, T = 30°C).

In terms of the mechanisms of glucose uptake Romano *et al.* (1979) have shown experimentally that *Z. mobilis* lacks the phospho-enolpyruvate (PEP): glucose phosphotransferase system. An active transport system for glucose is therefore not present. These findings make it likely that glucose uptake by *Z. mobilis* occurs either by passive diffusion or by facilitated diffusion.

Glucose excess continuous culture

It is clear again that q_s and μ were no longer closely coupled when glucose was in excess and the culture became ethanol-limited (Phase II in Figure 1). It was considered that for the conditions of excess glucose, the specific growth rate of the culture was controlled by ethanol concentration. In other word the culture functioned as a product-limited system instead of the usual chemostat operation.

Oscillations of glucose, biomass and ethanol in continuous culture with 200g l^{-1}. glucose medium at dilution rate 0.1 h^{-1} are shown in Figure 2. The maximum ethanol concentration was about 80-85g l^{-1} and the minimum was about 40g l^{-1}. The oscillations were sustained over 400 hours operation and it was not possible to be steady-state. These oscillations were found where the growth rate was controlled essentially by the level of ethanol. The continuous culture system then functioned as a product-limited culture with glucose concentrations being in excess. The oscillations resulted from the dynamic responese of the culture to higher concentrations of ethanol. An increase in ethanol above a steady-state level caused a fall in growth rate, which have rise to a decline in biomass concentration and ultimately a fall in ethanol concentration. Perpetuation of this cycle of events gave rise to sestained oscillations. Such behaviour has been observed for continuous culture studies following the addition of metabolic inhibitors and inhibition products (Dean and Rogers,

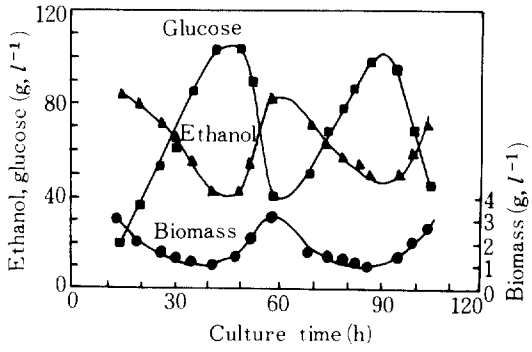

Fig. 2. Oscillations in *Z. mobilis ZM4* culture with 200 g, l^{-1} glucose medium, at dilution rate is of 0.1 h^{-1} (pH = 5.0, T = 30°C).

1967: Zines and Rogers. 1970. 1971: Gray and Rogers, 1971 a, b).

Effect of ethanol inhibition on fermentation kinetics

The effect of ethanol inhibition on the specific growth rate and specific glucose uptake rate are shown in Figure 3. The data were orginally obtained from a number of continuous culture using 60,000, 135 and 170g, l^{-1} glucose media (Lee *et al.*, (1980), Figure 3 shows that the specific growth rates were more strongly influenced by ethanol than were the specific rate of glucose uptake (ethanol production), although there were threshold concentration of ethanol below which inhibitions on μ and q_s did not occur. It is evident that the growth (μ) was more apparently inhibited by ethanol at much lower concentration (viz. 20g, l^{-1} while the glucose uptake rate (q_s) maintained its maximum rate until the ethanol concentration exceeded 60g, l^{-1}. It is worth also to note that there existed an ethanol concentration, viz. 85g, l^{-1}, from which growth no longer occurrred but for which glucose uptake and ethanol production were apparent. As can be seen in Figure 3, μ and q_s were influenced differently by increasing ethanol levels. No inhibition of either occurred up to 20g, l^{-1}, μ showed a linear decline with increasing ethanol concentrations while q_s was unaffected up 60g, l^{-1} ethanol therefore a significant uncoupling between μ and q_s occurred.

In modelling studies, good agreement was found between the model predictions and experimental data by describing in terms of threshold and linear inhibition function (Lee and Rogers 1983). Those observation provides further support that uncoupling of growth (anabolism) and ethanol production (catabolism) in *Z. mobilis* can be initiated by a number of factors inculuding high ethanol levels.

Ethanol concentration and the nature of uncoupling

In the present experiments with *Z. mobilis* there was a considerable excess of glucose in the media (beyond that required for cell growth) and this factor, combined with the effects of ethanol inhibition, appears to have resulted in significant uncoupling of catabolism and anabolism (Phase II in Figure 1). The relatively high concentrations of ethanol and glucose were both likely to cause inhibiton and changes in osmotic pressure resulting in increased maintenance energy requirements.

Z. mobilis ZM4 exhibited a considerable high maintenance energy coefficient (m) which was calculated with the Equation as postulated by pirt (1965). It was interested to note that *Z. mobilis* used a relatively high proportion of energy for maintenance. (Lee *el al.* 1980; Fieschko and Humphrey, 1983 Beyer *et. al.* 1984) Senez, 1962). The ratio (m/q_s) determined from glucose-limited chemostat experiments is plotted as a function of dilution rate and showed in Figure 4

Other authors have reported relatively high values for anaerobically grown cells. Stouthamer and Bettenhausen (1973) reported that when *Klebsiella aerogenes* grew anaerobically in tryptophan-

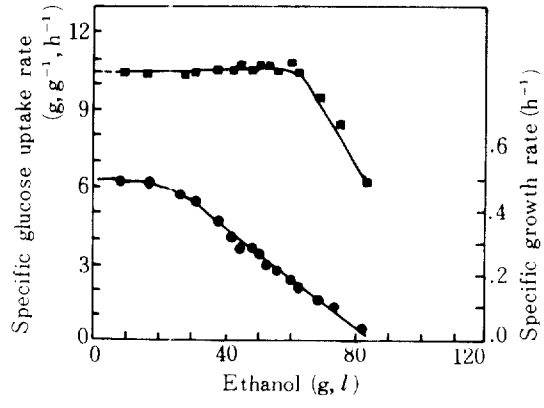

Fig. 3. Effect of ethanol on the specific growth rate and glucose uptake of *Z. mobilis ZM4.*

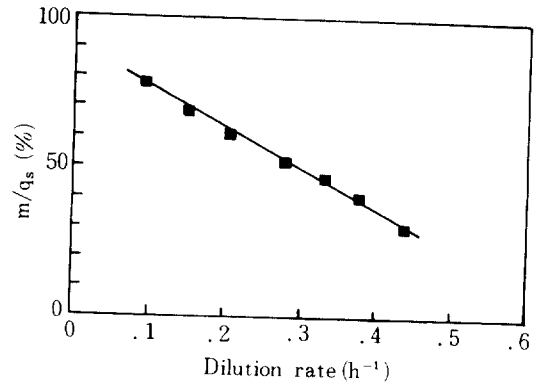

Fig. 4. Effect of dilution rate on ratio of maintenance energy coefficient to specific glucose uptake rate under the same conditions.

limited culture with excess glucose, the maintenance energy coefficient accounted for 90% of total energy produced at growth rate of $0.1h^{-1}$ It has been found that the maintenance energy coefficient can be influenced by the particular nutrient limiting growth (Neijssel and Tempest, 1979) and by the presence of growth inhibitory substances (Stouthamer, 1976). Environmental conditions have been shown also to significantly influence the maintenance energy coefficient (Pirt, 1975; Harrison, 1978; Van Unden an Madeira-Lopes, 1976). Furthermore, an increase in osmotic pressure may result in high maintenance energy requirements (Watson, 1970).

For coupled catabolism (energy-generating reactions) and anabolism (energy-consuming reactions) an equation, based on utilization of energy source as postulated by Pirt (1965), can be written:

$$q_s = \frac{\mu}{Y^{max}_{x/s}} + m_s \quad \text{Eqn. 1}$$

where $Y^{max}_{x/s}$ is the maximum biomass yield when m = 0 (g of biomass/g of energy source)
m_s is the maintenance energy coefficient (g of energy source/g of biomass/h)

Stouthermer and Bettenhausen (1973) proposed balance of energy utilization in terms of ATP production and utilization, viz:

$$q_{ATP} = \frac{\mu}{Y^{max}_{ATP}} + m_{ATP} \quad \text{Eqn. 2}$$

where q_{ATP} is the specific rate of ATP production (mol ATP/g of biomass/h)
10.5g/mol. M_{ATP} is maintenance energy coefficient expressed in terms of ATP requirements (mol of ATP/g of biomass/h)

The concept of maintenance energy has been the subject of considerable discussion as to whether the maintenance energy is constant or dependent on growth rate and culture conditions (Pirt, 1965, 1975; Stouthamer and Bettenhausen, 1973: Hempling and Mainzer, 1975; Stouthamer and Bettenhausen, 1975; De Kwaadsteniet *et. al., 1976,* Harrison, *1976, 1978;* Stouthamer, *1977;* Tempest, *1978;* Roels and Kossen, *1978;* Esener *et al.,* 1981).

적 요

발효액중에 생성된 에탄올의 농도가 균의 성장 및 에탄올 생성속도에 미치는 영향을 연속발효법으로 연구하였다. 그 결과 생성된 에탄올의 농도 $20gl^{-1}$에부터 균의 비성장속도가 급속히 저하되나 에탄올의 생성속도는 에탄올 농도가 60 g, l^{-1}이상이 되어야 저해되는 것을 알았다. 이러한 현상은 ***Zymomas mobilis*** 균에서 특이하게 보고되었던 균성장(Anabolism)과 당대사(catabolism)사이에의 연결에 분리가 일어나는 소위 uncoupling현상이 에탄올 농도에 크게 영향을 받는 것으로 판단되었다.

사 사

본 논문은 1984년도 한국과학재단지원 연구과제인 "***Zymomonas mobilis***내의 에탄올 저해작용기작에 관한 연구"의 일부결과입니다. 지원해주신 관계재단과 여러분께 감사드립니다.

REFERENCES

1) Aiba (1969): Kinetics of Product Inhibiton in Alcohol Fermentation-Temperature Effect in the "Sake" Brewing, *Biotech, Bioeng.*, **11,** 1985-1287.
2) Belaich, J. P., J. C. Senez and M. Murgier (1968): Microcalorimetric Study of Glucose Permeation in Microbial Cells, *J. Bacterial,* **95.** 1750-1757.
3) Belaich, J. P: A. Belaich and P. Simonpietri (1972): Uncoupling in Bacterial Growth: Effect of Pantothenate Starvation on Growth of *zymomonas mobilis., J. Gen. Microbiol.,* **70,** 179-185.
4) Beyerler, W., P. L. Rogers and A. Fiechter (1984): A simple technique for the direct determination of maintenance energy coefficient: An exampl with *Zymomonas mobilis., Appl. Microbiol. Biotechnol.*, **19,** 277-280.
5) de Lima, G. O., De Araujo, I. E. Schumcher and E. C. Da Silva (1970): Estudos de Microorganismos: antagonistas presentes nas besides fermentadas usadas pelo povo do Recife, *Rev. Inst. Antibiot. Univ. Recife* **10,** 3-15
6) De Kwaadsteiniet, J. W., J. C. Jager and A. H. Stouthamer (1976): A Quantitative Description of Heterotrophic Growth in Microorganisms, *J. Theor. Biol.*, **57,** 103-120.
7) Dean, A. C. R. and P. L. Rogers (1967): The Action of Drugs on Aerobacter *Aerogenes* in Various System of Continuous Culture, *Biochim. Biophys. Acta.*, **£;** 280-292.
8) Esener, A. A., J. A. Roels and N. W. F. Kossen (1981): Comment on the Descritption of the Maintenance Metabolism during Anagerobic Growth with Product Formation, *Biotech. Letters,* **3,** 15-20.
9) Fieschko J. and A. E. Humphrey (1983): Effect of temperature and ethanol concentration on the maintenance and yield coefficient of *zymomonas mobilis, Biotechnol. Bioeng.* **25,** 1655-1660.
10) Ghose, T. K. and R. D. Tyagi (1979): Rapid Ethanol Fermention of Cellulose Hydrolysate. II. Product and Substrate Inhibition and Optimization of Fermentor Design, *Biotech. Bioeng.*, **21,** 1401-1420.
11) Gray, P. P. and P. L. Rogers (1971 a): I. Cytochrome Oscillations in Continuous Culture-Action of Chloramphenical on *Saccharomyces carlsbergensis* and *Candida utilis, Biochim. Biophys.* Acta., 230, 393-400.
12) Gray, P. P. and P. L. Rogers (1971 b): II. Mathematical Simulation of Cytochrome Oscillations, *Biochim. Biophys.* Acta., 230, 401-410.
13) Harrison, D. D. F. (1978): Efficiency of Microbial Growth, In *"Companion to Microbiology"*, Bull, A. T. and P. M. Meadow ed., Longman, London, pp. 155-182.
14) Hempling, W. P. and S. E. Mainzer (1975): Effects of Varing the Carbon Limiting Growth on Yield and Maintenance Characteristics of *Escherichia coli* in Continuous Culture, *J. Bacteriol.*, **23,** 1076-1087.
15) Holzberg, I., R. K. Finn. and K. H. Steinkraus (1967): A Kinetic Study of Alcoholic Fermentation of Grape Juice, *Biotech. Bioeng.* **9,** 413-427.
16) Lee, J. H., D. Williamson and P. L. Rogers (1980): The Effect of Temperature on the kinetics of Ethanol Production by *Saccharomyces uvarum, Biotechnol. Letters,* **2,** 141-146.
17) Lee, K. J., D. E. Tribe and P. L. Rogers (1979): Ethanol Production by *Zymomonas mobilis* in Continuous Culture at High glucose Concentration., *Biotechnol. Letters.* **1,** 421-426.
18) Lee, K J., D. E. Tribe and P. L. Rogers (1980a): Kinetic Studies on a Highly Productive Strain of *Zymomonas mobilis., Biotechnol. Letters,* **2,** 339-344
19) Lee, K. J., N. L. Skotnicki, D. E. Tribe and P.L. Rogers (1981): The Effect of Temperature on the Kinetics of Ethanol Production by Strains of *Zymomonas mobilis, Biotechnol. Letters,* **3** 291-296.
20) Lee, K. J. and P. L. Rogers (1983): The Fermentation Kinetics of Ethanol Production

by *Zymomonas mobilis*, *The Biochemical Engineering J.* **27.** 31B-38B

21) Neijssel, O. M. and D. W. Tempest (1979): The Physiology of Metabolic Over-Production, In *"Microbial Technology-Current State and Furture Prospects"* Bull A. T. ed., Academy Prss, New York, pp53-72

22) Novak, M., P. Strehaiano, M. Moreno and G. Goma (1981): Alcoholic Fermentation: On the Inhibitory Effect of Ethanol, *Biotechnol. Bioeng.*, **23,** 201-212.

23) Pirt, S. J. (1965): The Maintenance Energy of Bacteria in Growing Culture, *Proc. Roy. Soc.* **B, 163,**224-231.

24. Pirt, S. J. (1975): Energy and Carbon Requirement, In *"Principle of Microbe and Cell Cultivation"*, Blackwell Scientivic Publication, Oxford, London, pp63-80.

25. Romano, A. H., J. D. Trifone and M. Brustolon (1979): Distribution of the phosphotransferase, *J. Bacteriol.*, **139,** 93-97.

26) Rogers, P. L., Lee, K. J. and Tribe, D. E. (1979): Kinetics of Production by *Zymomonas mobilis* at High Sugar Concentration, *Biotechnol. Letters;* **1,** 165-170.

27) Senez, J. C. (1962): Some Considerations on Energetics of Bacterial Growth, *Bacterial. Review,* **26,** 95-107.

28) Stouthamer, A. H. (1976): Yield Studies in Micro-organims. Durham, England, Meadowfield Press.

29) Stouthamer, A. H. (1977): Energetic Aspects of the Growth of Microorganisms, *Sym. Soc. Gen. Microbiol.*, **27,** 285-315

30) Stouthamer, A. H. and C. Bettenhaussen (1973): Utilization of Energy for Growth and Maintenance in Continuous and Batch Cultures of Microorganisms, *Biochim. Biophys. Acta.*, **301,** 53-70.

31) Stouthamer, A. H. and C. Bettenhaussen (1975): Determination of the Efficiency of Oxidative Phosphorylation in Continuous Cultures of Aerobacter aerogenes; *Arch. Microbiol.*, **103,** 187-192.

32) Tempest, D. W. (1978): The Biochemical Significance of Micro Growth Yields- A Reassesment, *Trend. in Biochem. Sci.*, **2,** 180-185.

33) Van Unden, N. and A. Madeira Lopes (1976): Yield and Maintenance Relations of Yeast Growth in the Chemostat at Superoptinal Temperatures, *Biotech. Bioeng.*, **18,** 791-804.

34) Watson, T. G. (1970): Effects of Sodium Chloride on Steady-State Growth and Metabolism of Saccharomyces cerevisiae, *J. Gen. Microbiol.*, **64,** 91-99.

35) Yarovenko, V. L. and B. M. Nakmanovich (1973): Kinetics of Product Synthesis in Continuous Alcohol Fermentation, *Biotech. Bioeng. Symp.*, **4,** 115-116.

36) Zines; D. O. and P. L. Rogers (1970): The Effect of Ethanol on Continuous Culture Stability, *Biotech. Bioeng.* **12,** 561-575.

37) Zines, D. O. and P. L. Rogers (1971): A Chemostat Study of Ethanol Inhibition, *Biotech. Bioeng.*, **13,** 293-308.

(Received March 3, 1985)