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**KINETICS AND PROCESS DEVELOPMENT
OF
L-LACTIC ACID FERMENTATION**

(L-乳酸発酵の速度論とプロセス開発)

Hitomi Ohara

1993

PREFACE

In recent years, L-lactic acid is highlighted as an optically active compound that can be converted into functional materials. This suggests the great demand of L-lactic acid. Especially, biocompatible and biodegradable plastics made from polylactate have the greatest potential for expanding the market of lactic acid, if more economical fermentation processes can be developed. The most suitable method for the effective preparation of L-lactic acid is not chemical synthesis but rather fermentation using defined strains.

Utilization of a membrane to separate the product from the culture broth is a beneficial technique for the improvement of the fermentation process and, in addition, in-situ recovery of a cell-free product becomes possible. However, some problems still remain for long-term operation.

In this study, kinetics of lactic acid fermentation by *Streptococcus faecalis* was focused to be clarified in order to produce lactic acid efficiently. A novel membrane reactor called a filter-bed-type reactor was designed and tried to operate for a long period under the optimum conditions

calculated from the equations obtained by the kinetic experiments. The key to produce L-lactic acid of high optical purity was also searched in this study.

The kinetics and the bioreactor studied in this thesis will contribute to the fermentation system of many other microorganisms and products.

This thesis was submitted to the Faculty of Engineering, Osaka University as a doctoral thesis.

May, 1993

A handwritten signature in black ink, appearing to read 'Ohara', with a stylized, cursive script.

Hitomi Ohara

PUBLICATION LIST

- 1. Lactic Acid Production by Membrane Reactors.** Hitomi Ohara and Keiichiro Hiyama, *Enzyme engineering* 10, New York Academy of Sciences, ISBN 0-89766-625-9, p. 832-838 (1990).
- 2. Kinetic Analysis of Continuous and Batch Cultures with *Streptococcus faecalis*.** Hitomi Ohara, Keiichiro Hiyama, and Toshiomi Yoshida, *Biochemical Engineering For 2001*, Springer-Verlag, ISBN 4-431-70091-9, p. 198-200 (1992).
- 3. Non-competitive Product Inhibition in Lactic Acid Fermentation from Glucose.** Hitomi Ohara, Keiichiro Hiyama, and Toshiomi Yoshida, *Applied Microbiology and Biotechnology*, 36: 773-776 (1992).
- 4. Kinetics of Growth and Lactic Acid Production in Continuous and Batch Culture.** Hitomi Ohara, Keiichiro Hiyama, and Toshiomi Yoshida, *Applied Microbiology and Biotechnology*, 37: 544-548 (1992).

5. **Kinetic Study on pH Dependence of Growth and Death of *Streptococcus faecalis*.** Hitomi Ohara, Keiichiro Hiyama, and Toshiomi Yoshida, *Applied Microbiology and Biotechnology*, 38: 403-407 (1992).
6. **HPLC Monitoring System for Lactic Acid Fermentation.** Hitomi Ohara, Tetuo Hiraga, Isao Katasho, Toru Inuta, and Toshiomi Yoshida, *Journal of Fermentation and Bioengineering*, 75: 470-473 (1993).
7. **Lactic Acid Production by a Filter-Bed-Type Reactor.** Hitomi Ohara, Keiichiro Hiyama, and Toshiomi Yoshida, *Journal of Fermentation and Bioengineering*, 76: 73-75 (1993).
8. **A Higher Optical Purity of L-Lactic Acid Produced in *Streptococcus faecalis*.** Hitomi Ohara and Toshiomi Yoshida, *Applied Microbiology and Biotechnology*, 40: 258-260 (1993).

LIST OF KEY WORDS

Algorithm, Anaerobic cultivation, ATP yield, Automatic monitoring system, Batch culture, Cell yield, Chemostat culture, Complex medium, Computer, Cross-flow filter, Diammonium hydrogen phosphate, Dilution rate, Enzyme model equation, Filter-bed-type reactor, Filter paper, HPLC, Inhibition constant, Kinetics, L-Lactic acid, Luedeking-Piret model, Material balance, Membrane reactor, Microporus membrane filter, Non-competitive product inhibition, N₂ gas, Optical purity, Optical-resolution column, Optimization, pH dependence, Poly lactate, Reciprocal plots, Residual glucose, Saturation constant, Specific consumption rate of glucose, Specific death rate, Specific growth rate, Specific production rate of lactic acid, *Streptococcus faecalis*, Variable-volume sampler, Volumetric productivity.

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CHAPTER 1

General Introduction

1.1 Background of the work

Lactic acid acting as organic acid has many applications such as a pH-adjuster, a preservative, and so on, in pharmaceutical and food industries. However, lactic acid production through chemical synthesis has many disadvantages such as safety problems in the production process where acetaldehyde and prussic acid or acetaldehyde and carbon monoxide are used, and the yielding product consists of a racemic mixture of D- and L-lactic acid. FAO and WHO (1974) recommended that lactic acid used in infant foods or Ringer's solution should be L-lactic acid which is directly usable by the human body. L-lactic acid is also advocated more desirable than the racemic mixture for the

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use in general food additives as well as for that in infant foods. It has been reported that the adult suffered from D-lactic acidosis is caused by an accumulation of D-lactic acid and there is a possibility that the accumulation of D-lactic acid is toxic to the brain tissue where no D-2-hydroxy acid dehydrogenase is present (Oh et al. 1979). Thus, the lactic acid for Ringer's solution must be L-lactic acid of extremely high optical purity.

Currently, our daily life is deeply related to plastics in all respects. The conventional plastics, which are produced from fossil resources, petroleum, as raw materials, are very stable in the ordinary environment. Those fates, therefore, deviates from the normal circulatory cycle of materials on the earth, the plastics are accumulated in the ocean, on the sea bottom, in the ground, etc., and remain there for a very long time, influencing the environment in various ways. The incineration of the plastics produced from petroleum and increasing use of petroleum as an energy source, in addition, will raise the level of carbon dioxide which will in turn magnify the greenhouse effect on the earth. It will also cause environmental pollution due to the discharge of nitrogen oxides and sulfur oxides.

Lactic acid can be converted to a polyester because it contains both a hydroxyl and a carboxyl group. Polylactate has high strength, transparency, thermoplasticity, fabricability, and availability from renewable resources. This polymer is hydrolyzed to simple lactic acid which is metabolized by the bacteria in the ground. For that reason, lactic acid could become the source of an environmentally benign polymer. If lactic acid can be produced by fermentation at a low cost, there will be a great demand for it as the raw material of biodegradable plastics (Naude 1989).

The U.S. Feed Grains Council inform the following matter on the annual report in 1993. Output of corn in the United States is about 200 million tons and 40 million tons is exported. So far corn has been chiefly used for feed, and only 15% of it for industries. However, it is expected that corn will be increasingly used as fermentation materials for ethanol and lactic acid. In 1992, about 4.5 billion liters of ethanol were made from 10 million tons of corn, and an expected annual growth rate will be around 8% in the future. It is also expected that the quantity of production will be reach 8 billion to 8.5 billion liters in 2000. The ethanol is added to gasoline at a rate of about 10% as a clean energy

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source. Tax incentives are provided for the production of ethanol by the federal government. In 1992, the United States produced 30 million tons of general purpose plastics worth \$50 billion, and an expected annual average growth rate will be 10% in the future. In 1992, the output of biodegradable plastics was 2,300 tons estimated at \$5 million. Following the increase in the demand for fast food, legal regulations governing the food packing materials have become stricter in each state. Most of the legal regulations have been enacted at the request of consumers. It is estimated that the demand for biodegradable plastics will be 8% of the packages (500 to 600 thousand tons/year) by 1996. Polylactate has attracted special interest from the viewpoint of environmental preservation and a means of effective use of corn.

In Japan, under the guidance of Ministry of International Trade and Industry, the Biodegradable Plastics Society has been established in 1989 for the test and evaluation methods, technical development and propagation of biodegradable plastics. The biodegradable plastics that is being developed includes polylactate, microbial polymer of the polyhydroxyalkanoates represented by PHB, starch

product obtained through direct forming of starch, copolymer of ethylene glycol and succinic acid, polycaprolactone, etc., which are made from petroleum but which are relatively high degradability. The microbial polymer is expensive and hard to form. A gene level improvement in microorganisms and blending with other polymers are therefore in progress. Any starch product is lacking in water resisting property and its forming method is restricted. To solve these problems, studies such as chemical introduction of a hydrophobic group have been made. It is doubtful that the petroleum products are friendly to the environment. From the viewpoint of measures against surplus grain as well as biodegradable plastics, the intense interest has been shown toward the polylactate.

As shown in Fig. 1.1, both the microorganism-based production of biodegradable plastics and energy do not depend on fossil resources and have the advantage as to the material circulation in this system. With the photosynthesis used as the source of driving force, lactic acid is produced from plant materials through lactic acid fermentation to make polylactate, and the alcohol produced through alcohol fermentation is used to acquire the energy. The carbon

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dioxide released by decomposition and combustion is reused as the resource by means of photosynthesis. It is very interesting that lactic acid fermentation and alcohol fermentation which human beings has utilized since time immemorial govern the basis of this material circulation.

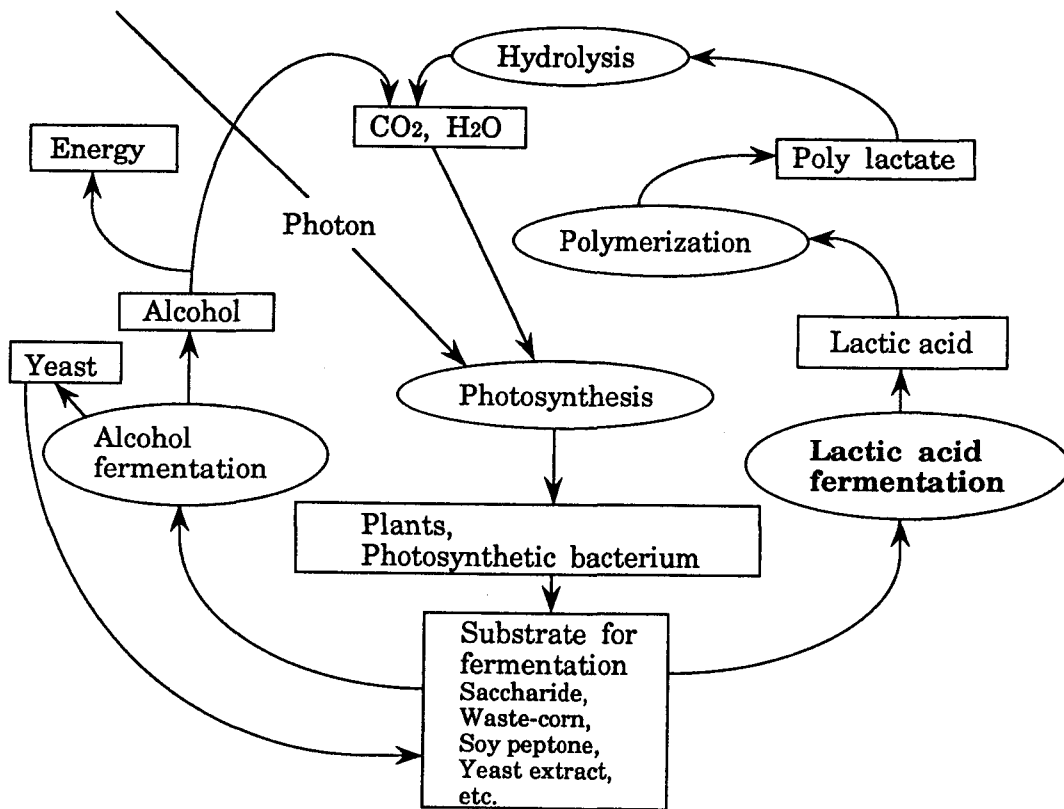


Fig. 1.1 Fermented products circulation in environment.

A racemic polymer and an L-lactic acid polymer differ from each other in the crystallinity and the melting point (Kulkarni 1971). Therefore, a high optical purity of L-lactic acid is required to secure the physical properties of the plastic. Also, as an optically active material, L-lactic acid is expected to be used for the liquid crystal production (Sato et al. 1990; Yoshinaga et al. 1990). These applications will increase the demand for L-lactic acid of a high optical purity. However, L-lactic acid will be produced in only fermentation where a defined strain of bacteria is used.

1.2 Historical perspective

As a traditional method of lactic acid production malt or cheese was put into starch or milk for spontaneous fermentation, and successively it was neutralized with calcium carbonate. This method was shown by Boutron and Frémy (1841). It is said that Avery in USA produced lactic acid with this method on an industrial base in 1881 (Prescott and Dunn 1949). Most of the fermented lactic acid in the world is currently produced with this method. This batch

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type method, however, has some problems, for example, requiring long fermentation time and difficulty of fermentation control. The pH electrodes suitable for fermentation have been put for practical use and some neutralizers such as NaOH and NH₄OH have also been examined. This made it possible for kinetic analysis of fermentation under the constant pH value.

There have been some reports (Pirt 1987; Edwards and Wilke 1968; Roy et al. 1987) on kinetic analysis of lactic acid fermentation by batch experiments. In batch experiments, however, it is difficult to estimate the small values of the saturation constants of Monod type equations, because batch experiments are usually carried out at higher concentrations of a limiting substrate. It is especially difficult to determine the type of inhibition for a fermentation where product inhibition takes place. In the chemostat culture, various parameters of fermentation can be estimated quantitatively because a stable steady-state is easily reached. However, chemostat culture should be carried out at various concentrations of the product to determine the type of inhibition and the saturation constants.

Many models such as the Luedeking-Piret model (Luedeking and Piret 1959a; Eq. 1.1) and modified ones (Rogers et al. 1978; Eq. 1.2) have been proposed as kinetic models for lactic acid fermentation.

$$\pi = \alpha\mu + \beta \quad (1.1)$$

$$\pi = \alpha\mu + \beta S \quad (1.2)$$

where π is the specific production rate of lactic acid, μ is the specific growth rate, α and β is the constants, and S is the glucose concentration.

These models are based on the material balance that the limiting substrate is distributed between cells and metabolites. The equations derived from these models are represented quite simply, consisting of two terms associated with growth and independent of growth. Although these equations can be easily applied to many systems, they are erroneous at a high dilution rate in the chemostat culture. Jørgensen and Nikolajsen (1987) added one term to them to take into account the effect of residual glucose. In their study, however, the product inhibition by lactic acid has not been considered. The equations of this model are too complicated for a practical application and the type of inhibitions have to be clarified. It was also pointed out that

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this equation is not suitable for the calculation of ATP yield at a low growth rate.

Recently, immobilization methods of bacteria have been developed for effective production of lactic acid. They include a method where bacteria immobilized in the carrageenan or calcium alginate gel (Stenroos et al. 1982), which is used for continuous production of lactic acid. In a view of industrial use, however, this method has many problems: weakness of the gel, leakage of biomass from the gel, etc. On the other hand, utilization of a membrane to separate the product from the culture is a potential technique for the improvement of the fermentation and, in addition, in-situ recovery of a cell-free product is possible. The production of lactic acid by a membrane reactor has been studied using a reactor of a dialysis type (Coulman et al. 1977; Stieber and Gerhardt 1981a, b), and cross-flow membrane type (Major and Bull 1989; Mehaia and Cheryan 1986; Vick Roy et al. 1982, 1983a, b) reactors. In the case of the dialysis type reactor, the driving force of the mass transfer depends on only diffusion, thus the productivity is limited by diffusibility of the product. Recently, the method of cross-flow filtration has been introduced to separate the fermentation products from

the cell suspension in a cell recycle system (Nakanishi et al. 1985; Nagata et al. 1989). However, the membrane is often clogged by the cells in a long-term operation. As another problem in practical production we should remind that a monitoring system is indispensable for effective lactic acid fermentation. However, the availability of sensor for industrial purpose is quite limited.

There are two types of lactic acid bacteria: homo-type where 2 moles lactic acid are produced from 1 mole glucose (Fig. 1.2a) and hetero-type where 1 mole lactic acid (Fig. 1.2b), 1 mole carbon dioxide, and 1 mole ethanol or acetic acid are produced from 1 mole glucose. The former has the metabolic pathway called "Embden-Meyerhof pathway" by which no loss of carbon source occurs and the latter includes the pathway of pentose phosphate cycle. Some glucose-grown cells of *Streptococcus faecalis* possess the enzymes of at least the oxidative portion of the pentose phosphate cycle (Sokatch 1960) as well as all of the enzymes of the Embden-Meyerhof pathway. However, all of the glucose degraded proceeded through the Embden-Meyerhof pathway with the stoichiometric accumulation of lactic acid. This is because fructose 1,6-diphosphate inhibit 6-phosphogluconate

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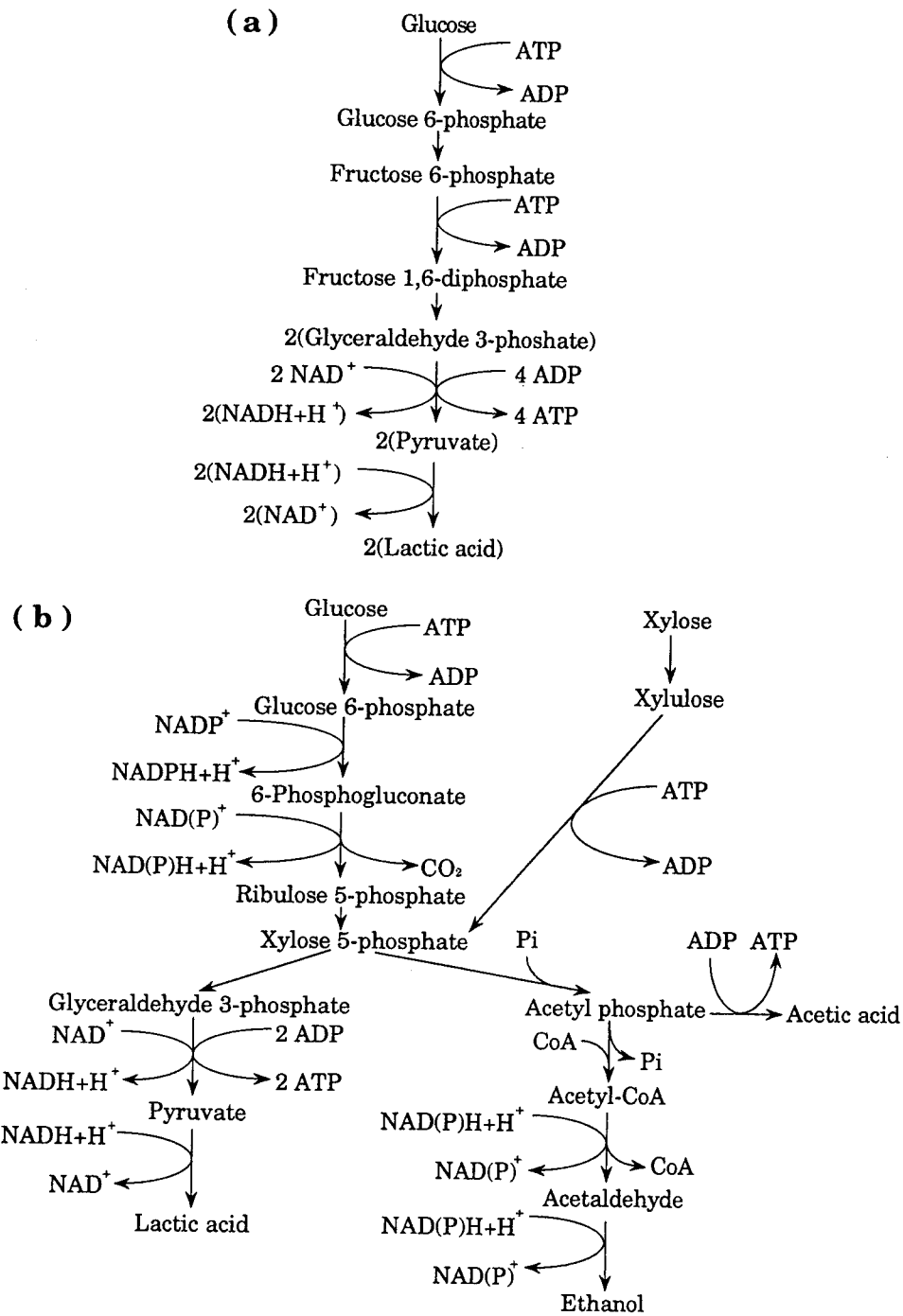


Fig. 1.2 Two types of metabolic pathway of glucose to lactic acid. (a) Homo-type. (b) Hetero-type.

dehydrogenase (Brown and Wittenberger 1971). On the other had, xylose is metabolized to lactic acid and ethanol in these strains (Fig. 1.2b). Another homo-type strain changes to hetero-type in high pH range (Kitahara 1966a). Therefore, for the effective production of lactic acid, it is necessary to select the proper strain, carbon source, and pH value.

The type of optical isomers of lactic acid produced mainly depends on the strain used, so that a proper strain must be selected to produce L-lactic acid. In addition, to produce the L-lactic acid of an extremely high optical purity, the medium composition must be carefully selected since it affects the optical purity in some cases (Kitahara 1966b).

1.3 Outline of the work

In this study, *Streptococcus faecalis*, a homo-lactic acid bacterium which mainly produces L-lactic acid, was used to elucidate the kinetics of lactic acid fermentation using the chemostat culture, and a novel membrane reactor and monitoring system were developed to realize the efficient production of lactic acid.

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Chapters 2 to 4 describe the lactic acid fermentation in terms of kinetics. Concretely, Chapter 2 shows the inhibition by the produced lactic acid against the production of lactic acid, the consumption of glucose, and the growth of the cell.

Chapter 3 shows the relationship between the growth of the cell and the production of lactic acid, and proposes the new equations for the relation. The ATP yield is discussed using this equation and the conventional Luedeking-Piret type equation.

Chapter 4 verifies that these relational equations are applicable to wide pH range and determines the parameters at each pH. This chapter also derives a new equation of the relation between pH values and the death rate of the cell at various lactic acid concentrations.

Chapter 5 introduces a new type of membrane reactor and describes the optimization of the reactor using the kinetic equations in Chapters 2, 3 and 4.

Chapter 6 describes an HPLC monitoring system for the fermentation.

Chapter 7 describes the optical purity of lactic acid. The relationship between the optical purity and the culture medium composition are examined here.

Finally, Chapter 8 summarizes this study and shows the subjects for future study.

CHAPTER 2

Non-competitive Product Inhibition in Lactic Acid Fermentation From Glucose

2.1 Introduction

Kinetic analysis of the fermentation is very important for the effective production of L-lactic acid. There have been some reports (Pirt 1987; Edwards and Wilke 1968; Roy et al. 1987) on kinetic analysis of lactic acid fermentation by batch experiments. Hanson and Tsao (1972) reported a kinetic analysis and the simulation of continuous fermentation using results obtained from batch experiments. In batch experiments, however, it is difficult to estimate the small values of saturation constants, because batch experiments are usually carried out at higher concentrations of a limiting substrate. It is especially difficult to determine the type of inhibition for a fermentation where product inhibition takes place, such as lactic acid fermentation.

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By the chemostat culture, various parameters for fermentation can be estimated quantitatively because a stable steady-state is easily reached. However, chemostat culture should be carried out at various concentrations of the product to determine the type of inhibition and the saturation constants. Therefore, it should be done at various concentrations of the limiting feed substrate to change the concentration of the product in the fermentor.

In this chapter, a chemostat culture was performed at various concentrations of glucose in the feed solution (at pH 7.0), where *Streptococcus faecalis*, an L-lactic-acid-producing homolactic acid fermentation bacterium, was cultured continuously, and the type of the inhibition by the product lactic acid was ascertained. The glucose concentrations in this study were chosen by reference to those used in the studies of high-performance membrane bioreactors (Vick Roy et al. 1983b; Mehaia and Cheryan 1985; Tanaka et al. 1988). Then the specific growth rate, the specific production rate of lactic acid, and the specific consumption rate of glucose are represented by Monod-type equations. These equations will contribute to the design of an efficient continuous process for lactic acid fermentation.

2.2 Materials and methods

2.2.1 Microorganism and medium

A strain of *S. faecalis* AHU 1256 was used in this study. The medium for cultivation of the strain contained 10 g/l of yeast extract (Difco, Detroit, Mich., USA), 10 g/l of Polypepton (Nihon Pharmaceutical, Tokyo, Japan), and various amounts (10, 20, 30 g/l) of D-glucose. With 1 M NaOH the pH value of the medium was adjusted to pH 7.0 before cultivation.

2.2.2 Continuous culture

The fermentation was performed in a 2-l stirred fermentor (TBR-2-3, Oriental Yeast, Tokyo, Japan) for continuous cultivation at 37°C and an agitation speed of 200 rpm, keeping the working volume at 1 l with microtube pumps for feed and effluent. In order to maintain anaerobic conditions the reactor was sparged with N₂ gas (20 ml/min). The pH value was controlled at pH 7.0 with 6 M NaOH during cultivation.

2.2.3 Analytical methods

The cell dry weight was determined from the optical density at 660 nm using a spectrophotometer (UV-240, Shimadzu, Kyoto, Japan) and a calibration curve for the relationship between dry weight and optical density in any culture phase (Rosenberger and Elsdén 1960). An aliquot of the culture broth was withdrawn after the feed solution had supplied five times more than the working volume. Then the cells were immediately removed from the culture broth with a membrane filter of 0.22- μm pore size to stop the reaction. The amounts of lactic acid and glucose were determined by HPLC (LC-6A, Shimadzu) with a differential refractometer (Shodex SE-11, Showa Denko, Tokyo, Japan). The analytical conditions were as follows: column, Shim-pack SCR-102H (Shimadzu); column temperature, 50°C; solvent for elution, distilled water which the pH was adjusted to 2.1 with perchloric acid; flow rate, 0.9 ml/min.

2.2.4 Calculations

Considering the material balance at the steady state in a chemostat, the specific consumption rate of glucose (v ; g glucose/g cell h), and the specific production rate of lactic

acid (π ; g lactic acid/g cell h), were calculated by Eqs. 2.1 and 2.2, respectively:

$$v = \frac{D(S_0 - S)}{X} \quad (2.1)$$

$$\pi = \frac{DP}{X} \quad (2.2)$$

where D is the dilution rate (h^{-1}); S_0 , the feed glucose concentration (g glucose/l); S , the effluent glucose concentration (g glucose/l); X , the cell concentration (g cell/l); and P , the effluent lactic acid concentration (g lactic acid/l).

2.3 Results and discussion

2.3.1 Chemostat culture

To examine the effect of lactic acid on the specific growth rate (μ), v , and π , a chemostat culture was performed with various concentrations of glucose (10, 20, and 30 g/l) in feed. The concentration of the limiting substrate should be a variable when the growth rate and the metabolic rate are represented by a Monod type equation. The results are summarized in Fig. 2.1. From Fig. 2.1 it was found that the the lactic acid concentration (P) and X increased proportionally with increase in the glucose consumed ($S_0 - S$) at any dilution rate.

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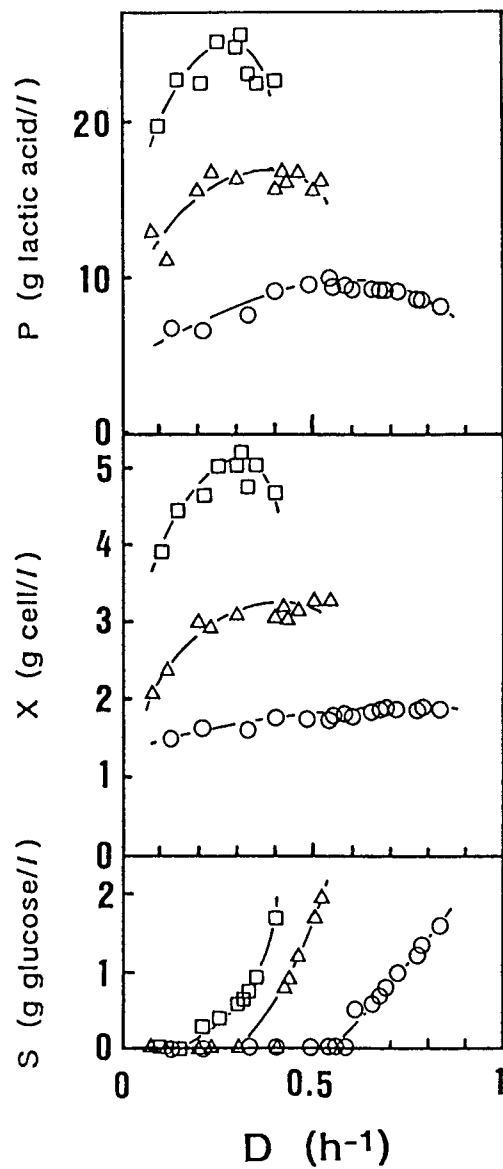


Fig. 2.1 Effect of dilution rate (D) on the concentration of lactic acid produced (P), cell mass (X), and glucose (S). Fermentation was performed at 37°C , and sparged with N_2 gas (20 ml/min). The pH was controlled at pH 7.0 with 6 M NaOH: \circ , feed glucose concentration (S_0) = 10 g/l; Δ , S_0 = 20 g/l; \square , S_0 = 30 g/l.

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The relationship between cell yield ($Y_{x/s}$) and lactic acid concentration at some typical dilution rates is shown in Fig. 2.2. The cell yield was independent of the concentration of extracellular lactic acid. This suggests that the ATP yield, which is defined under the anaerobic condition and with a complex medium (Bauchop and Elsdon 1960) as in this experiment, is not affected by the concentration of extracellular lactic acid.

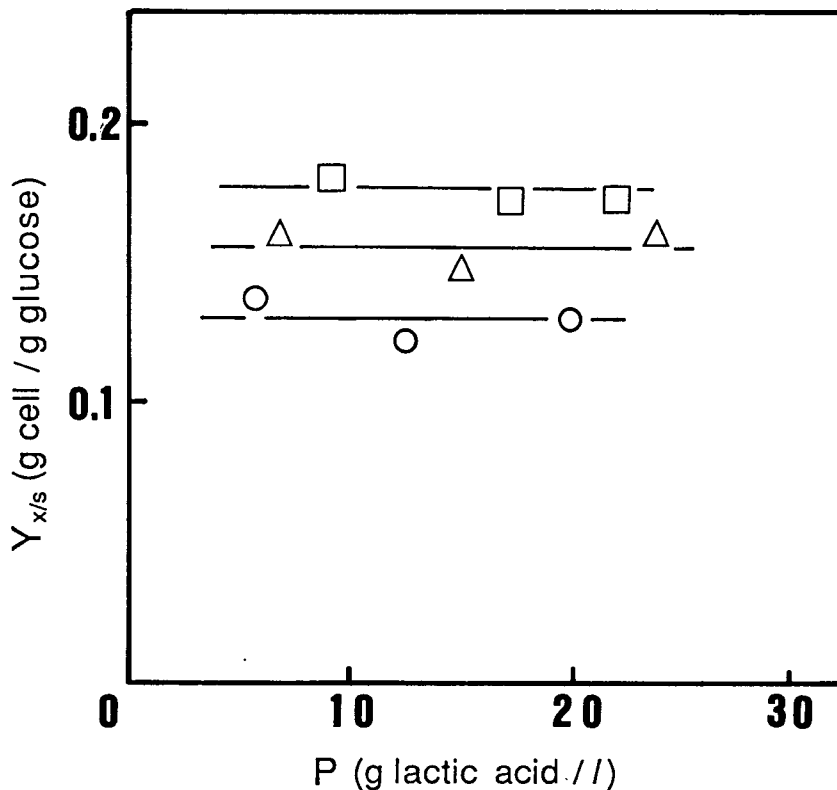


Fig. 2.2 Relationship between cell yield ($Y_{x/s}$) and P at three D values: \circ , $D = 0.1 \text{ h}^{-1}$; Δ , $D = 0.2 \text{ h}^{-1}$; \square , $D = 0.4 \text{ h}^{-1}$.

Yamane (1985) predicted that lactic acid fermentation by lactic acid bacteria may undergo non-competitive inhibition by lactic acid and the kinetic equation is expressed using an enzyme model. However, this hypothesis has not been proved. In another report, the kinetic equation of lactic acid fermentation, which was derived from batch culture experiments, was expressed by an uncompetitive enzyme model (Ishizaki and Ohta 1989a). However, uncertainty still remains as to how to distinguish between non-competitive and uncompetitive inhibition.

2.3.2 Type of product inhibition

The S/D values for each S_0 were plotted against S in Fig. 2.3a. The P values for each curve in Fig. 2.3 became almost constant. The P values for each S_0 values were as follows: $P = 8.8 (\pm 0.5) \text{ g/l}$, ($S_0 = 10 \text{ g/l}$); $P = 16.3 (\pm 0.3) \text{ g/l}$, ($S_0 = 20 \text{ g/l}$); $P = 24.1 (\pm 1.6) \text{ g/l}$, ($S_0 = 30 \text{ g/l}$). Then the three curves in Fig. 2.3a could be regarded as those for each P value. The curves cross at the same intersection on the horizontal axis. Therefore, the fermentation undergoes non-competitive inhibition by lactic acid. Thus, μ could be represented by Eq. 2.3.

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Fig. 2.3 Hofstee plots of S/D vs. S (a), $S/\text{specific consumption rate of glucose } (v)$ vs. S (b), and $S/\text{specific production rate } (\pi)$ vs. S (c); ○, $P = 8.8 (\pm 0.5) \text{ g/l}$, ($S_0 = 10 \text{ g/l}$); △, $P = 16.3 (\pm 0.3) \text{ g/l}$, ($S_0 = 20 \text{ g/l}$); □, $P = 24.1 (\pm 1.6) \text{ g/l}$, ($S_0 = 30 \text{ g/l}$).

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$$\mu = \mu_m \frac{S}{K_{s,\mu} + S} \frac{K_{p,\mu}}{K_{p,\mu} + P} \quad (2.3)$$

where μ_m is the maximum specific growth rate (h^{-1}).

From the value of the intersection in Fig. 2.3a, the saturation constant for specific growth rate ($K_{s,\mu}$) was determined as 0.22 g glucose/l. In the same manner as above, ν and π were found to undergo non-competitive inhibition by the product because both S/ν vs. S plots and S/π vs. S plots for each P value also showed the same intersections on the horizontal axis (Fig. 2.3b, 2.3c), respectively. Thus ν and π could be represented by Eqs. 2.4 and 2.5.

$$\nu = \nu_m \frac{S}{K_{s,\nu} + S} \frac{K_{p,\nu}}{K_{p,\nu} + P} \quad (2.4)$$

$$\pi = \pi_m \frac{S}{K_{s,\pi} + S} \frac{K_{p,\pi}}{K_{p,\pi} + P} \quad (2.5)$$

where ν_m is the maximum specific consumption rate (g glucose/g cell h) and π_m is the maximum specific production rate (g lactic acid/g cell h).

The saturation constants for specific consumption rate and specific production rate ($K_{s,\nu}$ and $K_{s,\pi}$, respectively) were also determined from the values of the intersections; $K_{s,\nu} = 0.22$ g glucose/l, $K_{s,\pi} = 0.22$ g glucose/l.

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It had been reported that when a strain of *S. faecalis* was cultured under aerobic conditions in a medium containing glucose as the limiting substrate, the cell yield increased more than that obtained under anaerobic conditions and acetic acid was produced because of occurring of oxidative phosphorylation (Smalley et al. 1968). However, this strain (*S. faecalis* AHU 1256) was cultured under anaerobic conditions in this experiment. Thus all the glucose was metabolized through the Embden-Meyerhof pathway to produce lactic acid, and the types of product inhibition coincided with those regarding the specific consumption rate and the specific production rate. The type of inhibition seemed to have also coincided with that of the specific growth rate because the strain was cultured in complex medium and the cell mass increased concomitant with the amount of ATP produced through the glycolysis.

2.3.3 Inhibition constants

To determine the value of the inhibition constant for the specific growth rate ($K_{p,\mu}$), the values of D , X , and P at $S = 0.5$, 0.7 , and 1.0 (g/l) were estimated from the curves in Fig. 2.1, respectively. Then the $K_{p,\mu}$ value was estimated

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according to the method of Cornish-Bowden (1974); $K_{p,\mu} = 9.5$ g lactic acid/l. The values of the inhibition constants for the specific consumption rate ($K_{p,v}$) and the specific production rate ($K_{p,\pi}$) were also determined in the same manner; $K_{p,v} = 11.0$ g lactic acid/l, $K_{p,\pi} = 5.5$ g lactic acid/l. Using these values and Eqs. 2.3, 2.4, and 2.5, μ_m , v_m , and π_m were calculated, as summarized in Table 2.1.

Although there has been a research to analyze the type of product inhibition in a batch culture (Ishizaki and Ohta 1989a), it is very difficult to distinguish between uncompetitive and non-competitive inhibition because S values in batch culture were too large compared with K_s value. In this chapter the chemostat culture was carried out at low concentrations of glucose in order to elucidate the type of product inhibition. To increase the efficiency of lactic acid fermentation the product lactic acid should be removed from the culture broth. Thus we have considered a novel membrane reactor (Ohara and Hiyama 1990) for continuous lactic acid fermentation.

Table 2.1 Various parameters obtained from the chemostat culture

Parameter Value			Unit
$K_{s,\mu}$	=	0.22	g glucose/l
$K_{s,\nu}$	=	0.22	g glucose/l
$K_{s,\pi}$	=	0.22	g glucose/l
$K_{p,\mu}$	=	9.5	g lactic acid/l
$K_{p,\nu}$	=	11.0	g lactic acid/l
$K_{p,\pi}$	=	5.5	g lactic acid/l
μ_m	=	1.6	h ⁻¹
ν_m	=	8.3	g glucose/g cell h
π_m	=	11.8	g lactic acid/g cell h

The saturation constant (K_s) and inhibition constant (K_p) values were estimated from Fig. 2.3 and by the method of Cornish-Bowden (1974), respectively. The values of the maximum specific growth rate (μ_m), the maximum specific consumption rate of glucose (ν_m), and the maximum specific production rate (π_m) were calculated using the values of K_s and K_p , and Eqs. 2.3, 2.4, and 2.5 (see text): $K_{s,\mu}$, $K_{p,\mu}$ for the specific growth rate; $K_{s,\nu}$, $K_{p,\nu}$ for the specific consumption rate; $K_{s,\pi}$, $K_{p,\pi}$ for the specific production rate.

2.4 Summary

A kinetic study regarding product inhibition in lactic acid fermentation by *Streptococcus faecalis*, which produces L-lactic acid, was performed in a chemostat at various feed concentrations of glucose (10, 20, and 30 g/l) at pH 7.0. Steady-state kinetic constants for the specific consumption rate of glucose and the specific production rate of lactic acid were determined at a residual glucose concentration below 2 g/l, which was accomplished in a chemostat. All the parameters, the specific growth rate, the specific consumption rate of glucose, and the specific production rate of lactic acid, were definitely related to non-competitive inhibition with regard to the concentration of the product, lactic acid.

CHAPTER 3

Kinetics

of Growth and Lactic Acid Production in Continuous and Batch Culture

3.1 Introduction

It is very important to analyze the fermentation processes kinetically and to construct a mathematical model to be used for the design, operation, and control of the fermentation processes. Many models such as the Luedeking-Piret model (Luedeking and Piret 1959a; Eq. 1.1) and modified ones (Rogers et al. 1978; Eq. 1.2) have been proposed as kinetic models for lactic acid fermentation. These models are based on the material balance that the limiting substrate is distributed between cells and metabolites. The equations derived from these models are represented quite simply, consisting of two terms associated with growth and independent of growth. Although these equations can be

easily applied to many systems, they are erroneous at a high dilution rate in the chemostat culture. Jørgensen and Nikolajsen (1987) added one term to them to take into account the effect of residual glucose. In their study, however, the product inhibition by lactic acid has not been considered. The equations of this model are too complicated for practical application and the type of inhibitions have to be clarified.

As described in Chapter 2, by using a chemostat we found that the types of product inhibitions on the specific growth rate, the specific consumption rate of glucose, and the specific production rate of lactic acid were all non-competitive when glucose was used as the limiting substrate for *Streptococcus faecalis*. In this chapter, the kinetics of lactic acid fermentation was discussed based on the results of Chapter 2 and batch culture experiment. Then the specific consumption rate of glucose and the specific production rate of lactic acid were expressed by simple equations as functions of the specific growth rate as a variable. These equations must be more useful than the Luedeking-Piret type equation (Eq. 1.1) for estimating ATP yield in batch culture and chemostat culture.

3.2 Materials and methods

3.2.1 Microorganism and medium

A strain of *S. faecalis* AHU 1256 was used. The medium for the cultivation contained 10 g/l of yeast extract (Difco, Detroit, Mich., USA), 10 g/l of Polypepton (Nihon Pharmaceutical, Tokyo, Japan), and 10 g/l of D-glucose.

3.2.2 Cultivation

The method of chemostat culture was described in section 2.2.2. Batch cultivation was performed in 2-l stirred fermentor with working volume of 1 l. The pH value was adjusted to pH 7.0 with 1 M NaOH before cultivation. During batch cultivation, the pH value was controlled at 7.0 by 6 M NaOH. The culture temperature was kept at 37°C with agitation at 200 rpm. In order to maintain anaerobic conditions the reactor was sparged with N₂ gas (20 ml/min).

3.2.3 Analytical methods

The cell dry weight and the amounts of lactic acid and glucose were determined by the same manner as section 2.2.3. In batch cultivation 1 ml of the culture broth was sampled at each time.

3.3 Results and discussion

3.3.1 Luedeking-Piret type kinetics

The results of the batch culture experiment is shown in Fig. 3.1. The change in the culture volume caused by addition of 6 M NaOH was negligible. The specific growth rate (μ ; h^{-1}), the specific consumption rate of glucose (v ; g

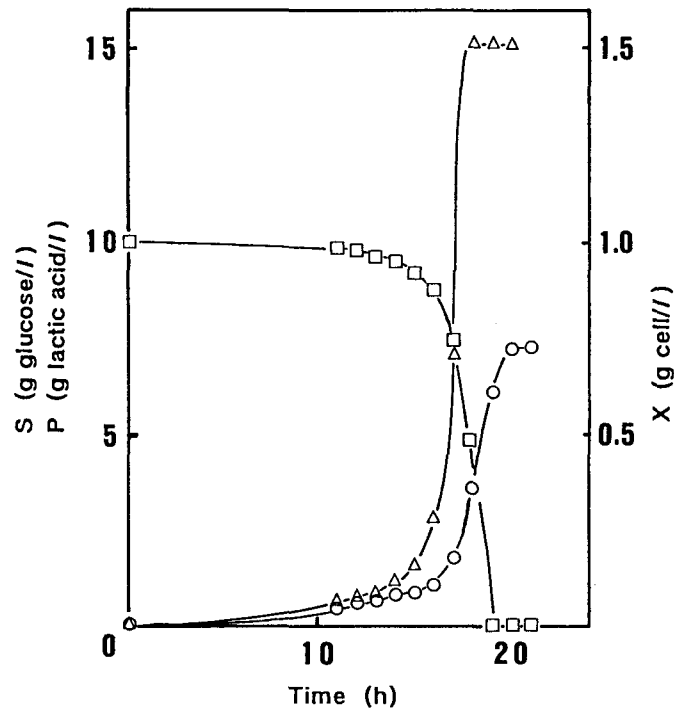


Fig. 3.1 Time course of fermentation in batch culture. Fermentation was performed at 37°C, and sparged with N₂ gas (20 ml/min). The pH was controlled at 7.0 with 6 M NaOH: ○, lactic acid; □, glucose; △, cell.

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glucose/g cell h), and the specific production rate of lactic acid (π ; g lactic acid/g cell h) were calculated from the differentials of glucose concentration (ΔS ; g glucose/l) and lactic acid concentration (ΔP ; g lactic acid/l) at each time as the following equations:

$$\mu = \frac{\ln(X_2/X_1)}{\Delta t} \quad (3.1)$$

$$v = -\frac{1}{X} \frac{\Delta S}{\Delta t} \quad (3.2)$$

$$\pi = \frac{1}{X} \frac{\Delta P}{\Delta t} \quad (3.3)$$

where X_1 and X_2 represent the cell concentration (g cell/l) before and after the cultivation for Δt hours. The values of v and π were plotted against the μ value to check whether the results fit to the Luedeking-Piret type equation (Fig. 3.2; Eq. 1.1). It indicates that there was a deviation from a linear relationship.

The result of the chemostat culture experiment is shown in Fig. 2.1. The values of v and π in chemostat culture were calculated by using Eqs. 2.1 and 2.2. In these equations, the value of dilution rate (D), is equal to μ because of the material balance in the chemostat culture.

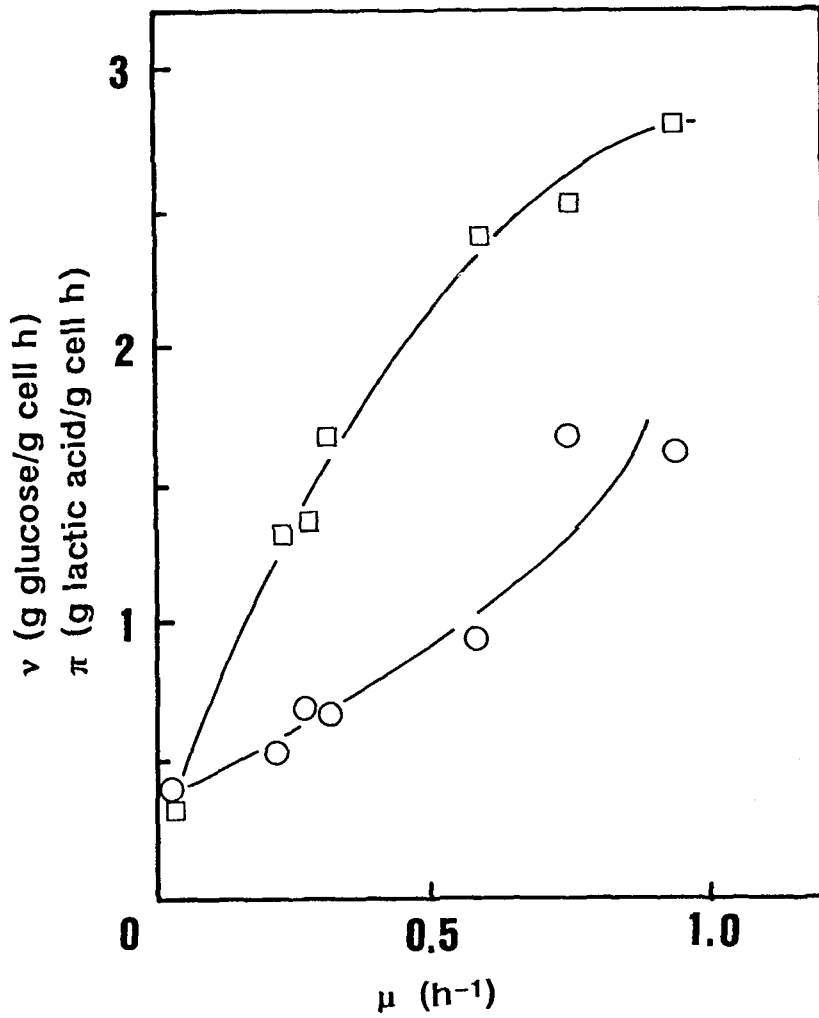


Fig. 3.2 Luedeking-Piret type plots of the specific consumption rate of glucose (v ; \square) and the specific production rate (π ; \circ) against the specific growth rate (μ) in batch culture.

The plots of D vs. v and D vs. π are shown in Fig. 3.3. A deviation from the linear relationships was also found in this experiment. The deviation started to appear at the dilution rate where residual glucose was detected and was very similar to that noted by Jørgensen and Nikolajsen (1987) in the chemostat cultivation using *S. cremoris* under glucose-limiting conditions.

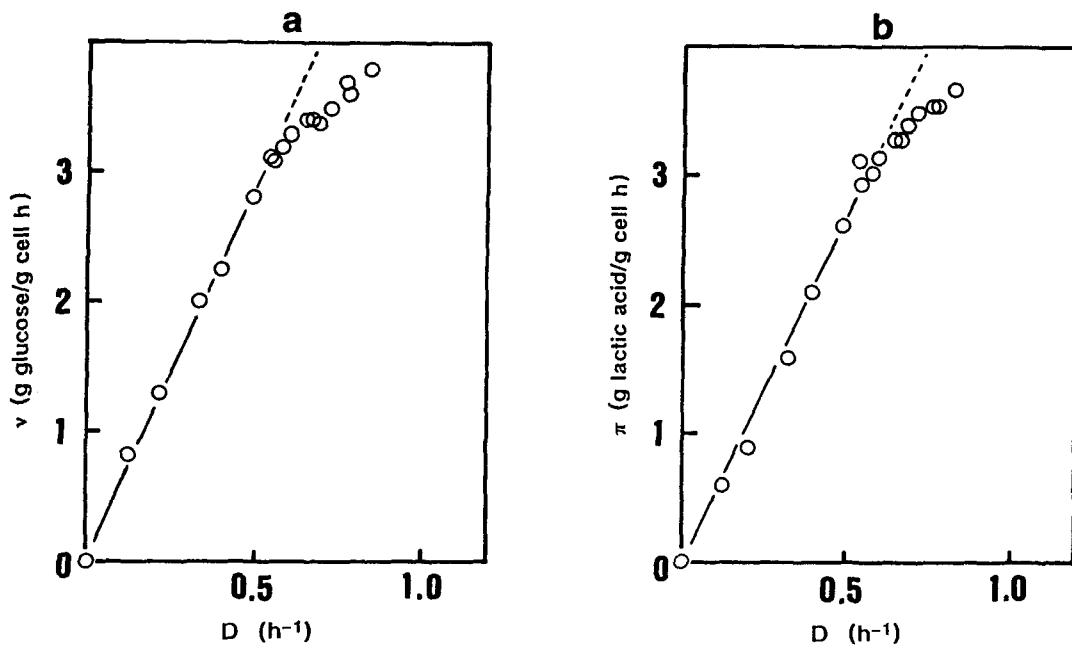


Fig. 3.3 Luedeking-Piret type plots of (a) the specific consumption rate of glucose (v ; \square) and (b) the specific production rate (π ; \circ) against dilution rate (D) in chemostat culture.

3.3.2 Reciprocal plots of batch culture data

The specific growth rate (μ), ν , and π were all subjected to non-competitive inhibition by the product, lactic acid as described in Chapter 2 (Eqs. 2.3, 2.4, and 2.5).

The values of $K_{s,\mu}$, $K_{s,\nu}$, and $K_{s,\pi}$ were far smaller (0.22 g glucose/l, all) (Table 2.1) than the values of S in batch culture experiments (4.9-10 g glucose/l). Therefore, in batch culture Eqs. 2.3, 2.4, and 2.5 are replaced by the following equations:

$$\mu = \frac{\mu_m K_{p,\mu}}{K_{p,\mu} + P} \quad (3.4)$$

$$\nu = \frac{\nu_m K_{p,\nu}}{K_{p,\nu} + P} \quad (3.5)$$

$$\pi = \frac{\pi_m K_{p,\pi}}{K_{p,\pi} + P} \quad (3.6)$$

Equation 3.7 is derived from Eqs. 3.4, 3.5, and Eq. 3.8 is derived from Eqs. 3.4 and 3.6:

$$\frac{1}{\nu} = \gamma_\nu \frac{1}{\mu} + \delta_\nu \quad (3.7)$$

$$\frac{1}{\pi} = \gamma_\pi \frac{1}{\mu} + \delta_\pi \quad (3.8)$$

where $\gamma_\nu = \mu_m K_{p,\mu} / \nu_m K_{p,\nu}$ (g cell/g glucose), $\delta_\nu = (K_{p,\nu} - K_{p,\mu}) / \nu_m K_{p,\nu}$ (g cell h/g glucose), $\gamma_\pi = \mu_m K_{p,\mu} / \pi_m K_{p,\pi}$ (g cell/g lactic acid),

$\delta_{\pi} = (K_{p,\pi} - K_{p,\mu}) / \pi_m K_{p,\pi}$ (g cell h/g lactic acid). According to Eqs. 3.7 and 3.8, the values of $1/\nu$ and $1/\pi$ were plotted against that of $1/\mu$ as shown in Fig. 3.4 and linear relationships were obtained, respectively.

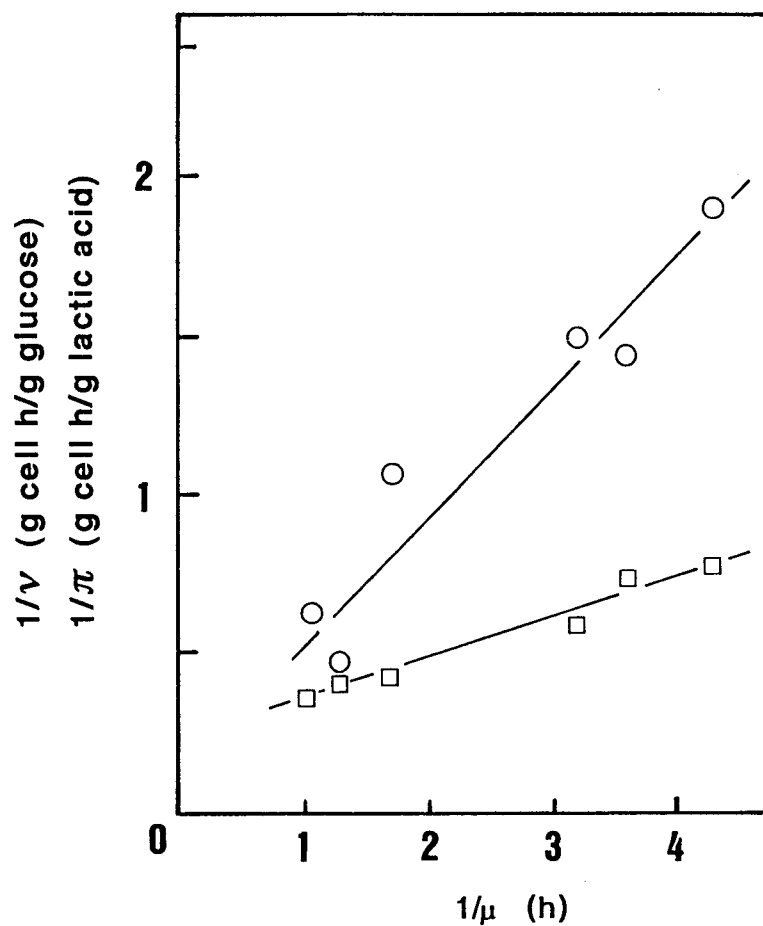


Fig. 3.4 Reciprocal plots of the specific consumption rate of glucose ($1/\nu$; \square) and the specific production rate ($1/\pi$; \circ) against that of the specific growth rate ($1/\mu$) in batch culture.

3.3.3 Reciprocal plots of chemostat culture data

In a chemostat culture, such an explanation is invalid because Eqs. 2.3, 2.4, and 2.5 are not applicable when the glucose is consumed almost completely, and Eqs. 3.4, 3.5, and 3.6 are not derived when the value of glucose concentration is comparable with the values of saturation constants. However, the linear relationships between $1/v$ and $1/\mu$ and between $1/\pi$ and $1/\mu$ were obtained as shown in Fig. 3.5. The validity of Eq. 3.7 is confirmed by consideration of the ATP yields as described in the following section.

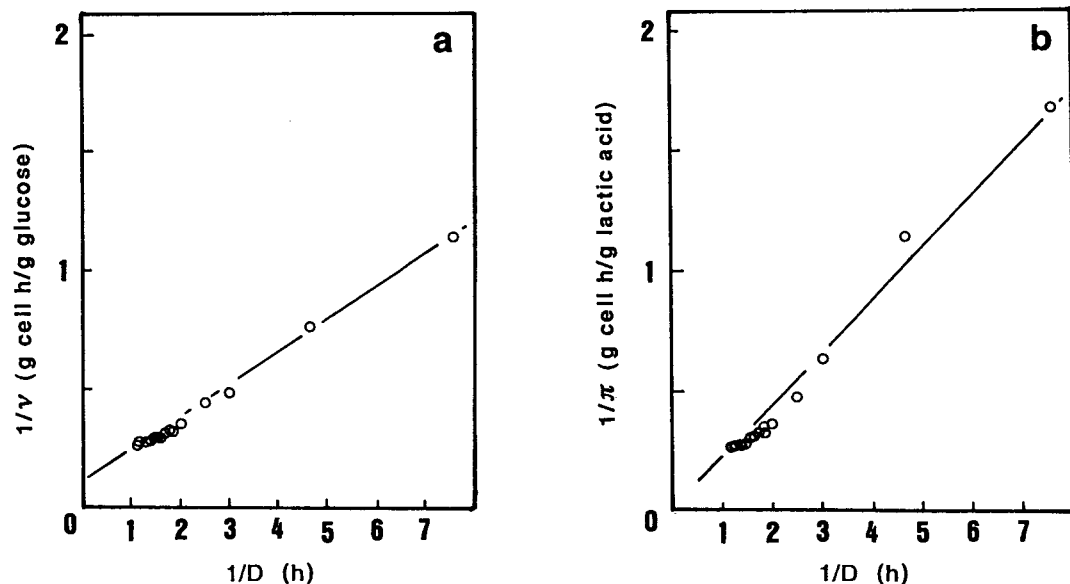


Fig. 3.5 Reciprocal plots of (a) the specific consumption rate of glucose ($1/v$) and (b) the specific production rate ($1/\pi$) against that of dilution rate ($1/D$) in chemostat culture.

3.3.4 Calculation of ATP yields

Equation 3.7 is more suitable for calculating ATP yield than the Luedeking-Piret type equation (Eq. 1.1). ATP yield was proposed by Bauchop and Elsdon (1960), and defined as $Y_{ATP} = \Delta X / \Delta ATP$ (g cell/mole ATP). They claimed that Y_{ATP} was nearly equal to 10 g cell/mole ATP, which was independent of the type of microorganisms. However, many values different from this empirical value have been reported. For lactic acid bacteria, Y_{ATP} is generally larger than the above value. For example, a Y_{ATP} of 32 g cell/mole ATP was reported for *S. faecalis* at the logarithmic phase (Forrest and Walker 1964), and 20 g cell/mole ATP for *Lactobacillus casei* (Vries et al. 1970). Since Y_{ATP} is related to μ , and lactic acid synthesis takes place through the Embden-Meyerhof pathway in anaerobic cultivation using a complex medium as in this experiments, 2 moles of ATP should be produced when 2 moles lactic acid is produced from 1 mole glucose ($Y_{A/S} = 2$; mole ATP/g glucose). Therefore, Y_{ATP} is expressed by Eq. 3.9:

$$Y_{ATP} = \frac{M_g}{Y_{A/S}} \frac{-\Delta X}{\Delta S} = \frac{M_g}{Y_{A/S}} \frac{\mu}{v} \quad (3.9)$$

where M_g represents the relative molecular weight of glucose

($M_g = 180$). The values of Y_{ATP} are determined by Eq. 3.9. Furthermore, Eq.3.10 is derived from Eqs. 3.7 and 3.9.

$$Y_{ATP} = \frac{M_g}{Y_{AS}}(\gamma_v + \delta_v\mu) \quad (3.10)$$

From this equation, it may be expected that Y_{ATP} is linearly correlated with μ . As shown in Fig. 3.6 the plots of Y_{ATP} vs. μ in batch and chemostat cultures showed a linear relationships, which proved that Eq. 3.7 is valid in both batch culture and chemostat culture. The validity of Eq. 3.10 was also confirmed by applying the data of published work (Major and Bull 1985) on *L. delbrueckii*. In Eq. 3.7, γ_v and δ_v can be regarded as constants because the value of Y_{ATP} only depends on μ when glucose is the limiting substrate (Fig. 2.2). On the other hand, Eq. 3.12 is derived from the conventional Eq. 3.11, which contains the maintenance coefficient (m ; g glucose/g cell h), and the cell yield (Y_G ; g cell/g glucose).

$$v = m + \frac{1}{Y_G}\mu \quad (3.11)$$

$$Y_{ATP} = \frac{M_g}{Y_{AS}} \frac{\mu}{m + \frac{1}{Y_G}\mu} \quad (3.12)$$

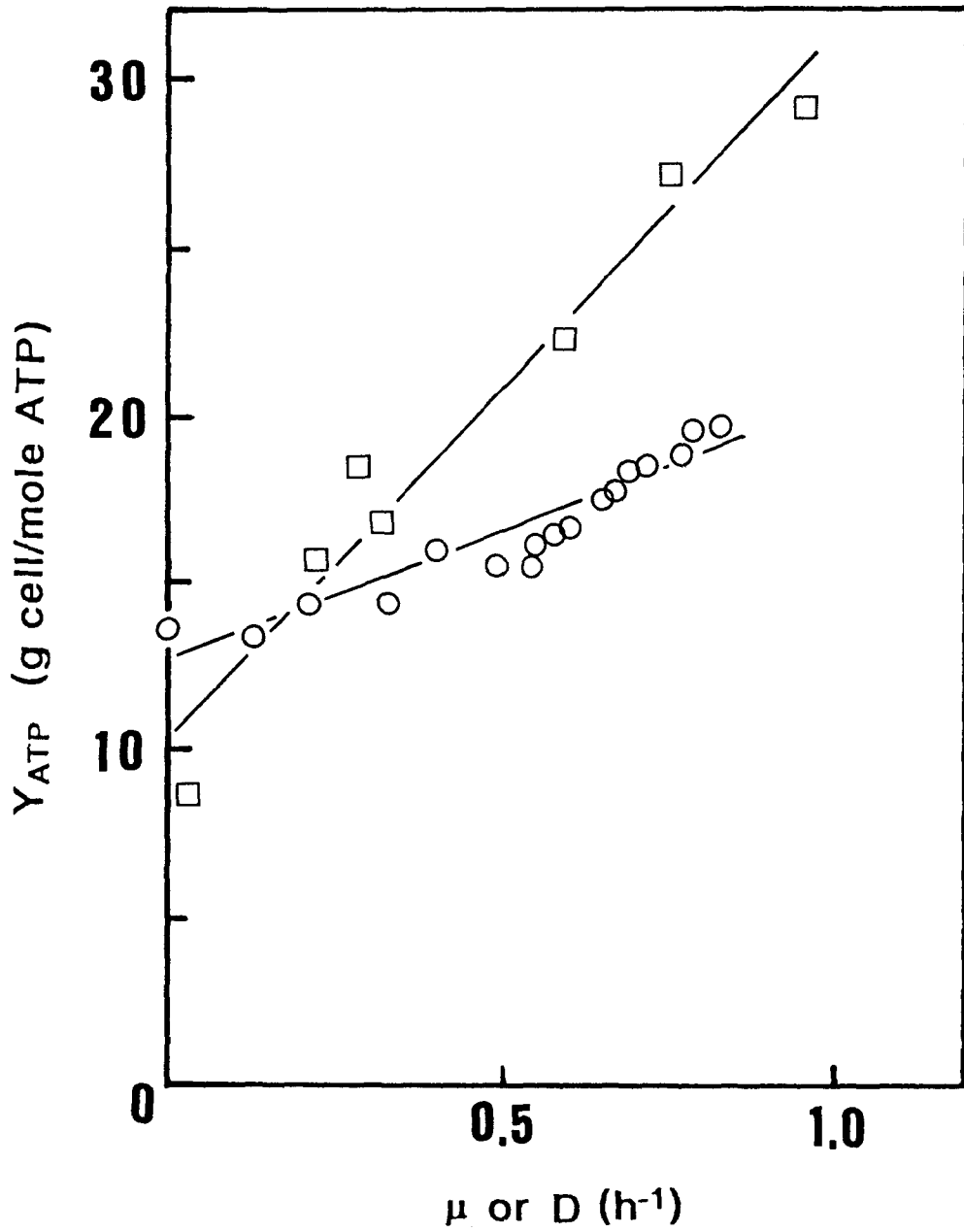


Fig. 3.6 Effect of the specific growth rate (μ) or dilution rate (D) on ATP yield (Y_{ATP}) in batch culture(\square), and chemostat culture(\circ).

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With Eq. 3.12, the plot of Y_{ATP} vs. μ should result in a curve that passes through the origin of the coordinate, which does not agree with the experimental results. A requirement condition for having a positive value of Y_{ATP} at $\mu = 0$, as shown in Fig. 3.6, is that ΔATP approaches zero with ΔX approaching zero. In the case of energy-coupled growth this requirement is satisfied because the growth rate is depends on the production rate of ATP. In addition, Y_{ATP} increased with the increase in μ , as observed here. In the case of energy-coupled growth, most ATP is used for anabolism, and about a three times larger amount of ATP is required for protein synthesis than for lipid or polysaccharide synthesis (Lehninger 1980).

As shown in Fig. 3.6, the increase in Y_{ATP} with that of the specific growth rate is presumably due to a decrease in cell protein content in contrast to the increase in the specific growth rate. On the other hand, in the case of non-energy-coupled growth, ΔATP is not equal to zero when ΔX approaches zero, because the step where the substrates are utilized for synthesis of the cell components is the rate-determining step. In this case, the plot of Y_{ATP} vs. μ is expected to form a curve that passes through the origin of

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the coordinate. It suggests that another equation may be valid for non-energy-coupled growth. In addition, since oxidative phosphorylation occurs in cultivation under aerobic conditions (Bryan-jones and Whittenbury 1969), glucose may be converted to organic acids other than lactic acid (Smalley et al. 1968). Therefore such a simple material balance is invalid, and Y_{ATP} becomes larger than that observed under anaerobic conditions (Mickelson 1972). Another model should be constructed to explain such cases.

In order to simulate the lactic acid fermentation in batch culture, the cell yield was calculated from the enzyme model equations of the specific consumption rate of glucose and the specific production rate of lactic acid (Ishizaki et al. 1989b), but this value can be calculated easily from Eq. 3.10. Furthermore, we have considered a novel membrane reactor (Chapter 5) for the continuous lactic acid fermentation; the equations in this chapter will be useful for discovering the optimum conditions of this reactor.

3.4 Summary

In a lactic acid fermentation by *Streptococcus faecalis*, the specific consumption rate of glucose (v) and the specific production rate of lactic acid (π) were represented by the following simple equations as functions of the specific growth rate (μ):

$$\frac{1}{v} = \gamma_v \frac{1}{\mu} + \delta_v$$

$$\frac{1}{\pi} = \gamma_\pi \frac{1}{\mu} + \delta_\pi$$

By use of data from a batch culture, these two equations were derived from enzyme kinetics of the product inhibition. These equations were successfully applied to the results of batch culture and chemostat culture. In addition, calculation of ATP yield by these equations agreed with the experimental results better than the conventional Luedeking-Piret type equation (Eq. 1.1), which includes two terms associated with growth and not with growth.

CHAPTER 4

Kinetic Study on pH Dependence of Growth and Death of *Streptococcus faecalis*

4.1 Introduction

The growth of microorganisms and their metabolic reactions are significantly influenced by the pH value of the culture media (Luedeking and Piret 1959a). In particular, the production of the metabolites by lactic acid bacteria depends on the pH value (Rhee and Pack 1980; Thomas et al. 1979) and they are usually cultivated under acidic conditions. In Chapter 2, chemostat cultivation of *Streptococcus faecalis* was carried out at pH 7.0 using glucose as the limiting substrate. The result showed that lactic acid non-competitively inhibited cell growth, lactic acid production, and glucose consumption. Subsequently in Chapter 3, the relationships between the specific growth rate and the specific production rate of lactic acid or the specific

consumption rate of glucose were determined. They were verified for their higher accuracy covering a wider range of the variation in the specific growth rate, comparing them with the Luedeking-Piret type equation (Luedeking and Piret 1959a; Eq. 1.1).

To understand the pH dependence of the various reaction rates, it is necessary to confirm whether these relationships are valid only at pH 7.0, or if similar relations are also found in a broader pH range. Therefore, the chemostat culture was made over a wide range of pH. It is very important to clarify the cell death rate for the efficient production of lactic acid. However, no one has reported on the influence of pH and the product, lactic acid, on the death rate of the cells.

4.2 Materials and methods

4.2.1 Microorganism and medium

A strain of *S. faecalis* AHU 1256 was used. The medium for the cultivation was the same as section 2.2.1. The pH value of the medium was adjusted to various values (pH 8.0, 7.0, 6.0, 5.5, 5.0) with 1 M NaOH or 1 M HCl solution before cultivation.

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4.2.2 Cultivation

Cultivation was performed in the same manner as section 2.2.2. The pH value was controlled at prescribed values by 6 M NaOH solution during cultivation.

4.2.3 Analytical methods

The cell dry weight and the amounts of lactic acid and glucose were determined by the same manner as section 2.2.3.

4.2.4 Cell death

Cells were washed with physiological saline and suspended in 0.2 M of phosphate buffer (pH 9.0, 8.0, 7.0, 6.0, 5.0, 4.0) including various amounts of lactic acid (0, 10, 20, 30 g/l). The culture was incubated at 37°C with shaking (60 rpm). The number of colonies were counted on an ager plate with bromcresol purple (Nissui, Tokyo, Japan) and estimated as the colony-forming unit (cfu).

4.3 Results and discussion

4.3.1 Effect of pH on glucose consumption and lactic acid production

The results of the chemostat culture at various pH values are shown in Fig. 4.1. The specific consumption rate of glucose (v ; g glucose/g cell h) and the specific production

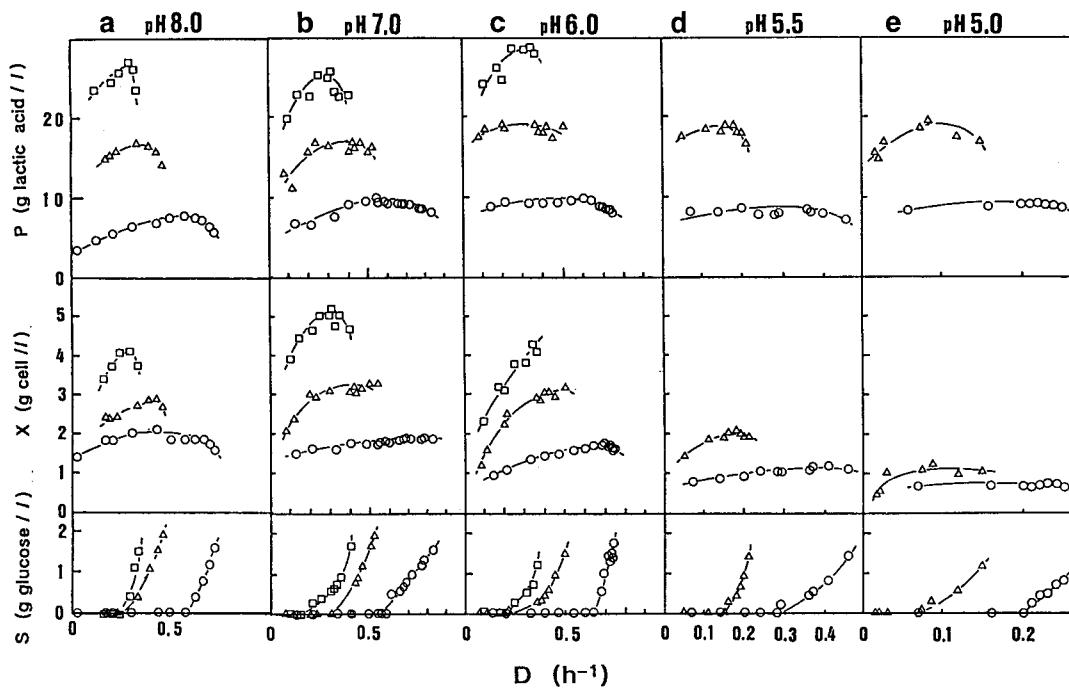


Fig. 4.1 Effect of dilution rate (D) on the concentration of lactic acid produced (P), cell mass (X), and glucose (S) at various pH values. The fermentation was performed at 37°C, and sparged with N₂ gas (20 ml/min). The pH was controlled with 6 M NaOH: ○, feed glucose concentration (S_0) = 10 g/l; Δ, S_0 = 20 g/l; □, S_0 = 30 g/l.

rate (π ; g lactic acid/ g cell h) were calculated from Eqs. 2.1 and 2.2. The types of product inhibition were determined in Chapter 2, and the relationships among ν , π , and μ were represented by Eqs. 3.7 and 3.8. Figure 4.2a shows the values of γ_ν and δ_ν whereas Fig. 4.2b shows those of γ_π and δ_π at various pH values.

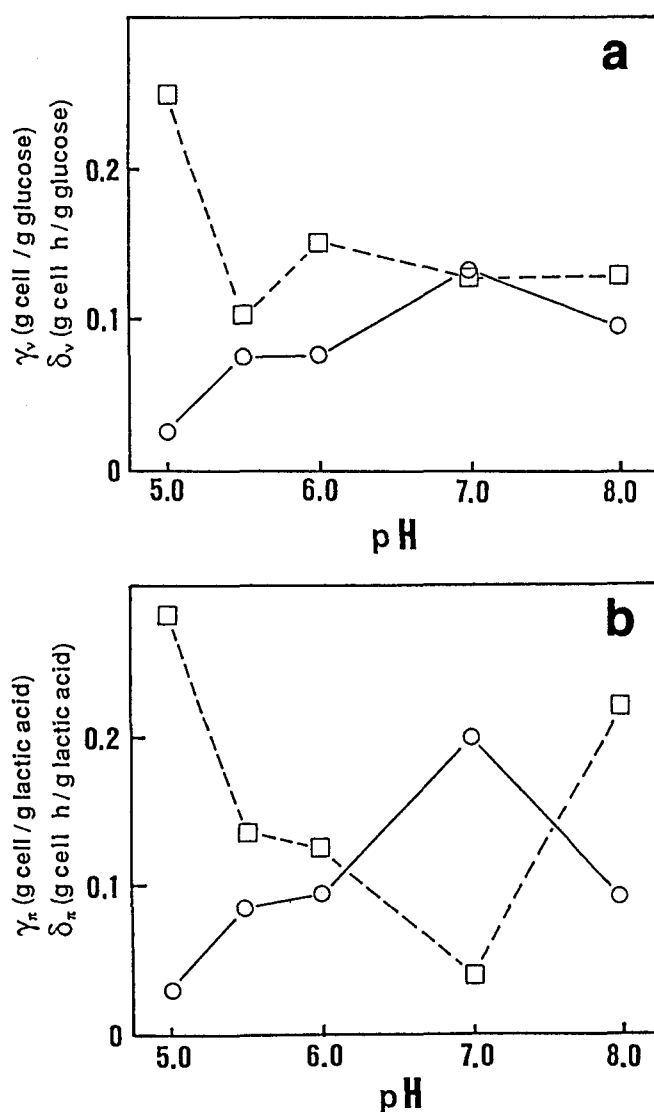


Fig. 4.2 (a) The values of γ_ν (g cell/g glucose) and δ_ν (g cell/h g glucose) at various pH values calculated from Eq. 3.7: \circ , γ_ν ; \square , δ_ν . (b) The values of γ_π (g cell/g lactic acid) and δ_π (g cell/h g lactic acid) at various pH values calculated from Eq. 3.8: \circ , γ_π ; \square , δ_π .

4.3.2 Product inhibition at various pH values

The steady state values of S/D were plotted against the steady state values of S for each S_0 as shown in Fig. 4.3. The P value varied in a limited range for each curve, as shown in Table 4.1. The values of the saturation constants for μ ($K_{s,\mu}$; g glucose/l) at various pH values were estimated from intersections on the horizontal axis of Fig. 4.3. To estimate the values of the inhibition constants for μ ($K_{p,\mu}$; g lactic acid/l), the values D , X , and P at $S = 0.5, 0.7$, and 1.0 g/l were estimated from the curves in Fig. 4.1, respectively. Then, $K_{p,\mu}$ values were determined according to the method of Cornish-Bowden (1974). The estimated values are listed in Table 4.2. It was reported in Chapter 2 that the product, lactic acid, non-competitively inhibited μ at pH 7.0. At each pH value tested, 8.0, 7.0, 6.0, 5.5, and 5.0, lactic acid also non-competitively inhibited μ as shown in Fig. 4.3.

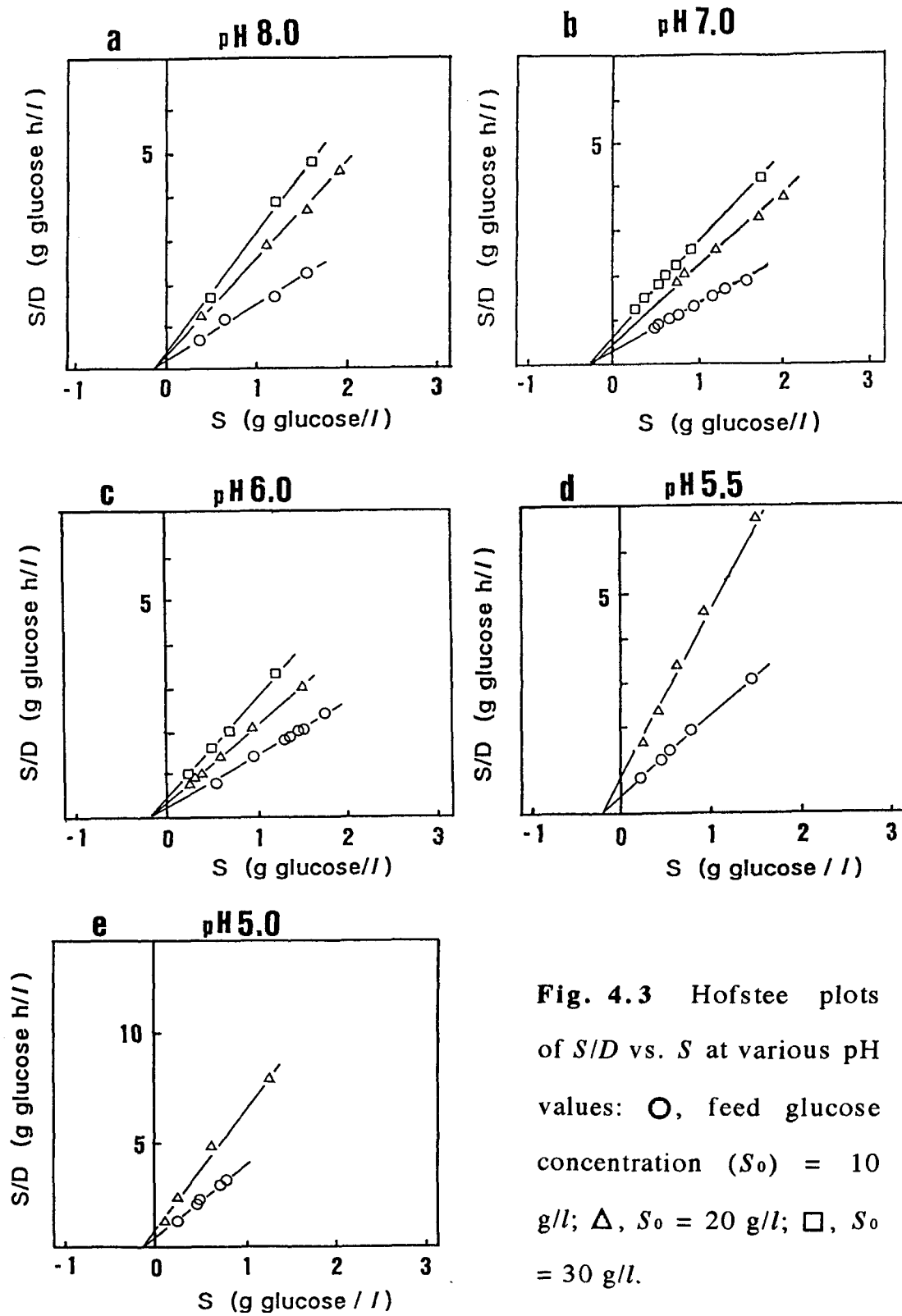


Fig. 4.3 Hofstee plots of S/D vs. S at various pH values: \bigcirc , feed glucose concentration (S_0) = 10 g/l; Δ , S_0 = 20 g/l; \square , S_0 = 30 g/l.

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Table 4.1 The mean value of lactic acid concentration (P) for each feed glucose concentration (S_0) and the variance

S_0 (g glucose/l)	Mean value of P (g lactic acid/l)				
	pH				
	5.0	5.5	6.0	7.0	8.0
10	9.2 ± 0.2	7.9 ± 0.6	8.6 ± 0.5	8.0 ± 0.5	6.7 ± 0.9
20	18.2 ± 1.4	17.8 ± 1.2	18.2 ± 0.8	16.3 ± 0.3	15.4 ± 1.4
30			28.2 ± 0.4	24.1 ± 1.6	25.3 ± 1.8

Table 4.2 The values of the saturation constant for the specific growth rates ($K_{s,\mu}$), and those of the inhibition constant for specific growth rate ($K_{p,\mu}$) at various pH values

pH	5.0	5.5	6.0	7.0	8.0	
$K_{s,\mu}$	0.13	0.20	0.18	0.22	0.15	(g glucose/l)
$K_{p,\mu}$	12	12	15	9.5	12	(g lactic acid/l)

The values of $K_{s,\mu}$ and $K_{p,\mu}$ were estimated from the intersections on the horizontal axis of Fig. 4.3 and by the method of Cornish-Bowden (1974), respectively.

4.3.3 Effect of pH on μ_m

The μ under non-competitive inhibition by lactic acid is expressed by Eq. 2.3. As carried out at pH 7.0 in Chapter 2, substituting $K_{s,\mu}$ and $K_{p,\mu}$ values, as well as the S , P , and μ values used to calculate them, into Eq. 2.3, the maximum specific growth rates ($\mu_m; h^{-1}$) were obtained and plotted against pH (Fig. 4.4). No kinetic model for the pH

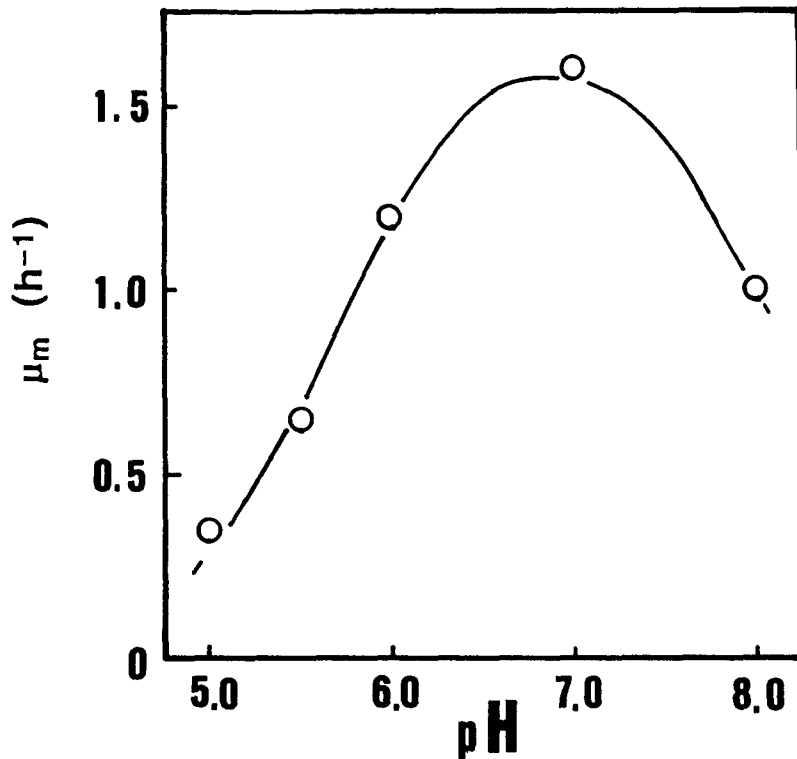
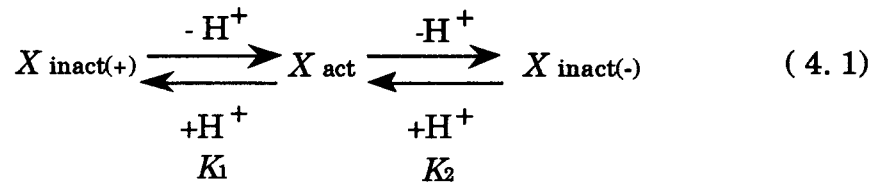


Fig. 4.4 The values of the maximum specific growth rate (μ_m) at various pH values. The solid line shows the curve of Eq. 4.3.

dependence of the maximum growth rate has yet been proposed. Referring to the model of the active and ionization sites in enzyme reactions (Bailey and Ollis 1986), we will discuss the kinetics of pH dependence of cell growth based on an analogy to the enzyme reaction. It is assumed that the active (X_{act}) and inactive type cells (X_{inact}) are interchangeable with each other in proton donating and accepting reactions. The reactions are expressed by Eq 4.1:



where K_1 and K_2 are the equilibrium constants expressed by the equations: $K_1 = X_{inact(+)} / X_{act} [H^+]$ (mole/l), and $K_2 = X_{act} / X_{inact(-)} [H^+]$ (mole/l), respectively. The total number of the cells (X_0) is defined by the equation: $X_0 = X_{act} + X_{inact(+)} + X_{inact(-)}$, and Eq. 4.2 is derived.

$$\frac{X_{act}}{X_0} = \frac{1}{1 + K_1[H^+] + \frac{1}{K_2[H^+]}} = \frac{1}{1 + K_1 10^{pH} + \frac{1}{K_2 10^{pH}}} \quad (4.2)$$

Therefore, μ_m is represented as follows;

$$\mu_m = \frac{K_3 X_{act}}{X_0} = \frac{K_3}{1 + K_1 10^{pH} + \frac{1}{K_2 10^{pH}}} \quad (4.3)$$

where K_3 (h^{-1}) is the constant. The values of K_1 , K_2 , and K_3 were determined by the least-squares method as follows: $K_1 = 5.1 \times 10^5$ mole/l, $K_2 = 1.3 \times 10^8$ mole/l, $K_3 = 1.8 \text{ h}^{-1}$. The curve of Eq. 4.3 is shown in Fig. 4.4 as a solid line. Thus, the μ_m at various pH values can be estimated by Eq. 4.3 whereas the μ at any substrate and product concentrations is expressed by Eq. 2.3. The specific consumption rate of glucose can be calculated from Eq. 3.7, and the π calculated from Eq. 3.8 by substituting the value of μ in these equations. The values of ATP yield and cell yield can be also calculated from Eq. 3.7 (section 3.3.4).

4.3.4 Cell death rate

A part of the cell is dead even in the growth phase, and the most important factor to this is lack of nutrients in the cell. Therefore, the experiment to determine cell death rate was carried out with physiological saline. The cell death rate is proportional to the number of viable cells. Due to the chain-forming nature of the organism, each chain of cells was counted as a single colony. The number of viable cells, however, is proportional to the cfu, because the number of cells in the chain does not change when the cell is not

growing, as in this experiment. Thus Eq. 4.4 is valid, and the values of the specific death rate can be estimated from the slopes of time vs. logarithm of cfu.

$$\frac{dX_c}{dt} = -K_d^P X_c \quad (4.4)$$

where K_d^P (h^{-1}) is the specific death rate in P g/l of lactic acid, and X_c (cfu) is the colony number. The values of the specific death rate without lactic acid (K_d^0 ; h^{-1}) are plotted against pH in Fig. 4.5, which shows that the death rate of the cell was

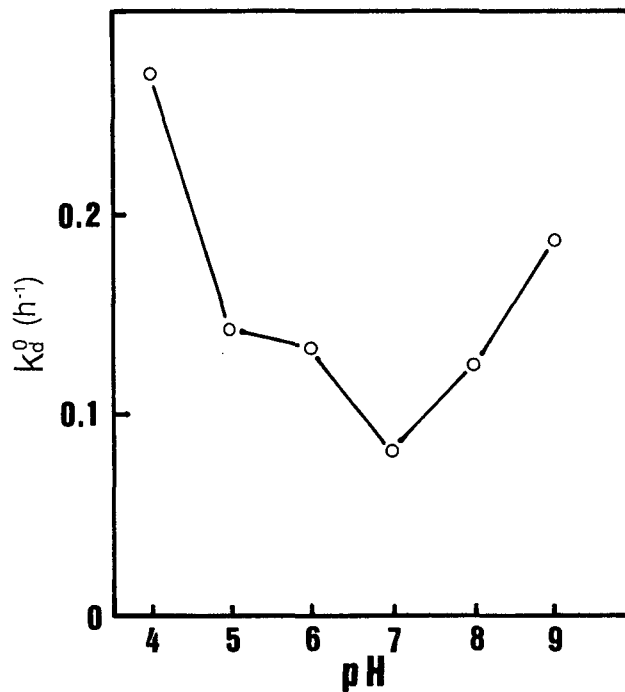


Fig. 4.5 The specific death rate without lactic acid (K_d^0) was plotted against pH. The circular symbols were estimated from the slopes of t vs. $\ln X_c$.

influenced by pH and lowest at pH 7.0. In the same manner as above, the values of the specific death rate at various pH and lactic acid concentrations were estimated, and P vs. K_d^P/K_d^0 is plotted in Fig. 4.6, which shows that cell death was accelerated by lactic acid at each pH tested.

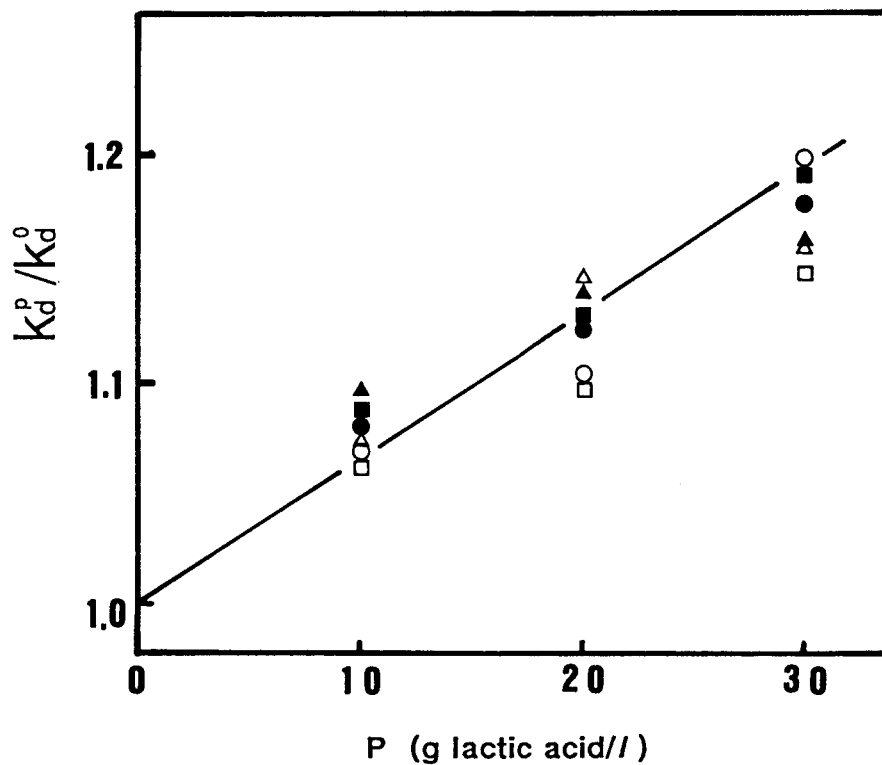


Fig. 4.6 The effect of lactic acid on the specific death rate (K_d^P) in P g/l of lactic acid. P vs. K_d^P/K_d^0 was plotted: \circ , pH 4.0; Δ , pH 5.0; \square , pH 6.0; \bullet , pH 7.0; \blacktriangle , pH 8.0; \blacksquare , pH 9.0.

In this chapter, it is confirmed that Eqs. 3.7 and 3.8 are valid, and the type of inhibition to the cell growth by lactic acid is a non-competitive over a broad pH range. Furthermore, it is revealed that the death rate of the cell was influenced by pH and lactic acid. Lactic acid fermentation can be analyzed and discussed more efficiently by using the equations in this chapter.

4.4 Summary

A chemostat culture was used for lactic acid fermentation with *Streptococcus faecalis* at various pH values (8.0, 7.0, 6.0, 5.5, 5.0) and glucose concentrations (10, 20, 30 g/l). At every pH value, the reciprocals of the specific consumption rate of glucose and the specific production rate of lactic acid were linearly correlated to the reciprocal of the specific growth rate. The product, lactic acid, caused non-competitive inhibition of the specific growth rate at every pH value. Moreover, it was found that the cell death rate was dependent on pH and lactic acid. The death rate was smallest at pH 7.0 and increased with increasing lactic acid concentration. The kinetic equations of growth and death are proposed in a broader pH range.

CHAPTER 5

Lactic Acid Production by a Filter-Bed-Type Reactor

5.1 Introduction

In lactic acid fermentation, there is marked product inhibition on the productivity of lactic acid (Friedman et al. 1970). If this inhibitory effect can be eliminated, an increase in productivity can be expected. Utilization of a membrane to separate the product from the culture is a potential technique for the improvement of the fermentation, which in addition, could allow in-situ recovery of a cell-free product. The production of lactic acid by a membrane reactor has been studied using reactors of the dialysis (Coulman et al. 1977; Stieber and Gerhardt 1981a, b), and cross-flow membrane types (Major and Bull 1989; Mehaia and Cheryan 1986; Vick Roy et al. 1982, 1983a, b). In the case of the dialysis-type reactor, the driving force of the mass transfer

depends only on diffusion, and thus the productivity is limited by the diffusibility of the product. Recently, cross-flow filtration was introduced to separate the fermentation products from the cell suspension in a cell recycle system (Nakanishi et al. 1985; Nagata et al. 1989). However, the membrane becomes clogged by cells in long-term operation. Therefore, a filter-bed-type reactor (FBR) was designed, with filter paper placed between two composite sets of membrane and porous support (Ohara and Hiyama 1990).

Chapter 2, dealing with a chemostat culture of *Streptococcus faecalis*, reported that lactic acid exhibited non-competitive inhibition of cell growth, lactic acid production, and glucose consumption. In Chapter 3, equations for the relationships between the specific growth rate and the specific production rate of lactic acid or the specific consumption rate of glucose were thus formulized. Further in Chapter 4, the various parameters of these equations and the cell death rate at various pH values were estimated. In this chapter, these equations are used to optimize the reactor operation.

5.2 Materials and methods

5.2.1 Microorganism and medium

A strain of *S. faecalis* AHU 1256 was used. The medium employed to produce lactic acid contained 10 g/l of yeast extract (Difco Laboratories, Detroit, USA), 10 g/l of Polypepton (Nihon Pharmaceutical, Tokyo, Japan), 20 g/l of D-glucose, and 35 g/l of dipotassium hydrogen phosphate (GYP medium). The pH of the medium was adjusted to 7.0 with 6 M HCl.

5.2.2 Design of the reactor

A cross-sectional view of the reactor is shown in Fig. 5.1. The cells were inoculated on to the filter paper (no.101, Advantec, Tokyo, Japan) and then placed in the ring (inner diameter, 70 mm; thickness, 0.6 mm). A microporous membrane filter made of polypropylene, with a pore size 0.2 μm wide 0.02 μm long (Duraguard #2400, Polyplastic Co., Tokyo, Japan), was placed on either side of the filter paper and fixed by isotropic porous supports made of polypropylene (diameter, 72 mm; thickness 0.7 mm; pore size, 0.05-0.1 mm) and holders. The porous support at the inlet side distributes

FILTER-BED-TYPE REACTOR

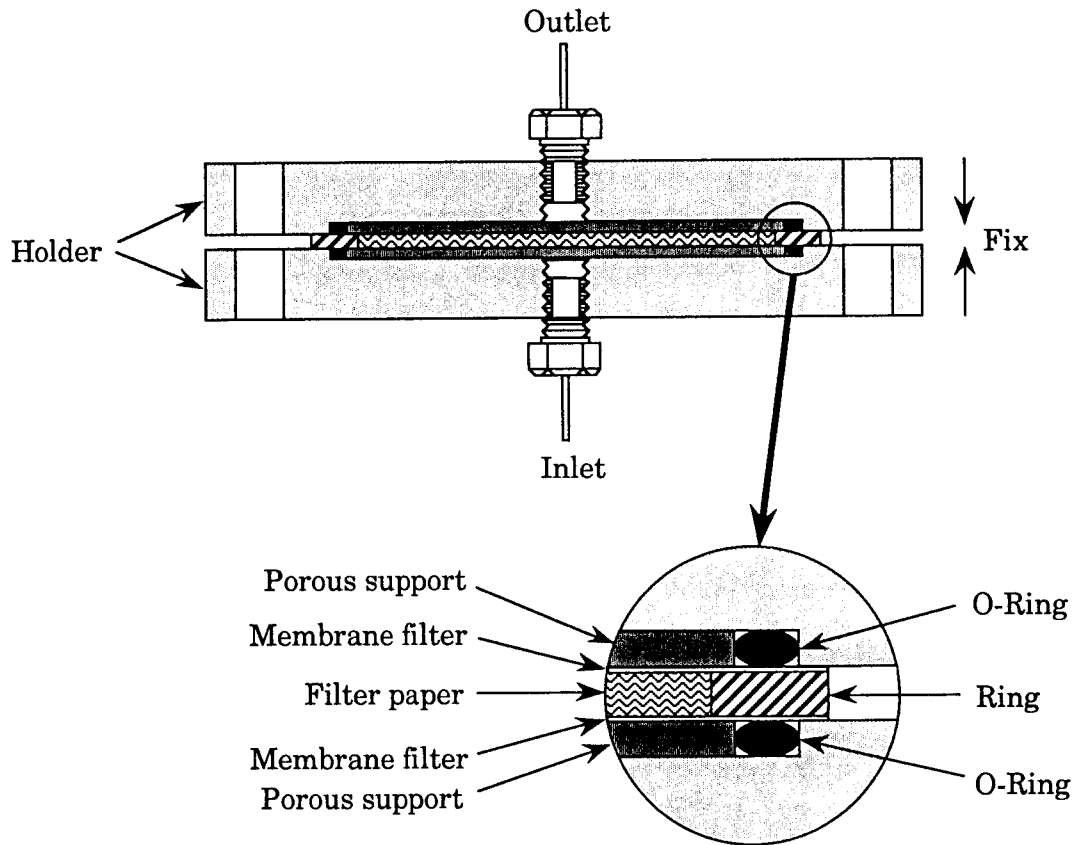


Fig. 5.1 Cross-sectional view of the filter-bed-type reactor. The filter paper was soaked in the culture broth and then set in the ring. The inner diameter of this ring was 70 mm, and the thickness 0.6 mm. The microporous membrane filter has a pore size 0.2 μm wide 0.02 μm long.

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the medium to the surface of the membrane, while that at outlet side collects the product from the membrane. The volume of the reactor was 2.31 (8.0, including the porous spacers) ml. The reactor was kept at 37°C and the medium was fed by a high pressure pump (LC-6A, Shimadzu, Kyoto, Japan) from the bottom of the reactor, avoiding the entry of air.

5.2.3 Analytical methods

The amounts of lactic acid and glucose were determined by the same manner as section 2.2.3.

5.3 Results and discussion

5.3.1 Glucose concentration

The pH of the medium decreased as the medium passed through the filter paper due to an increase in the concentration of produced lactic acid. Therefore, the strain was cultivated in a batch mode with GYP medium to clarify the relationship between the pH and the lactic acid concentration in the culture. The result is shown in Fig. 5.2. The pH dropped to 4.2 with the accumulation of lactic acid up

to a concentration of 18.0 g/l. For complete consumption of glucose in continuous production, the pH must be maintained at a suitable value. Thus a feed glucose concentration (S_0) of 15.0 g glucose /l was selected in the expectation from Fig. 5.2 that the pH value in the FBR would be kept above 4.4.

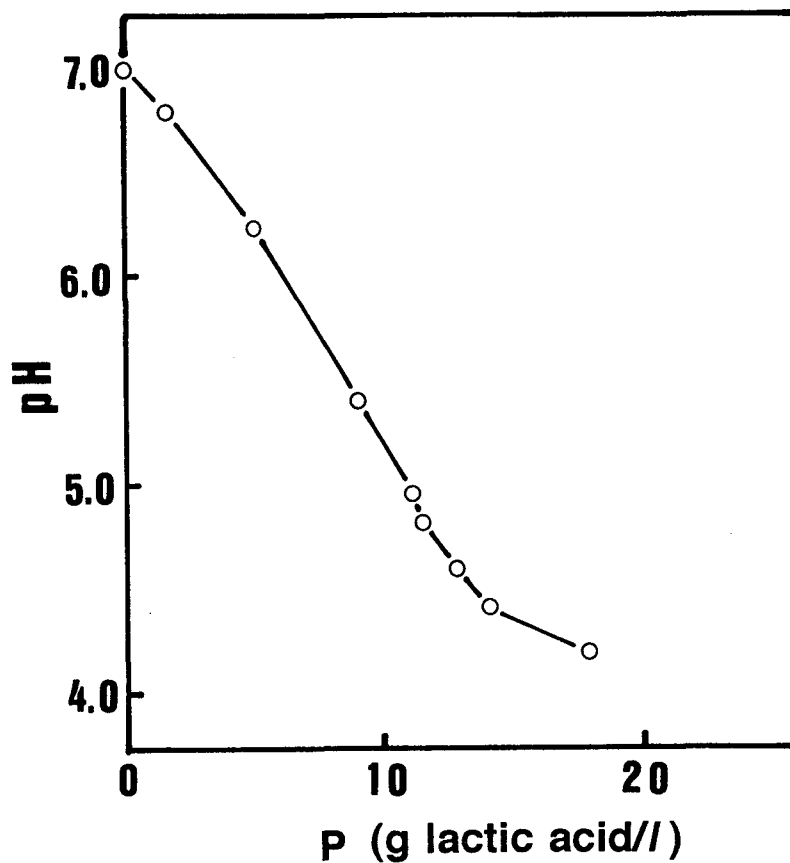


Fig. 5.2 Relationship between the pH value and lactic acid concentration in the culture of *S. faecalis* in GYP medium at 37°C.

5.3.2 Calculation of optimum flow rate

The optimum flow rate was chosen as the largest flow rate with which minimum glucose concentration is obtained at the outlet of FBR. The optimum flow rate for the FBR was calculated as follows. The FBR was divided into 50 sections vertically to the direction of the flow. Then the material balance of the cell, glucose, and lactic acid in each section were formulated, respectively. The cell mass balance for the n -th section is given by:

$$V \frac{dX_n}{dt} = FX_{n-1} - FX_n + V\mu_n X_n - VK_d^P X_n \quad (5.1)$$

where V is the volume of one section (4.62×10^{-2} ml); F , the flow rate (l/h); t , the time (h); X_n , the cell concentration (g cell/l); μ_n , the specific growth rate in the n -th section (h^{-1}); and K_d^P , the specific death rate at P g/l of lactic acid (h^{-1}) (Chapter 4). The substrate balance for the n -th section is given by:

$$V \frac{dS_n}{dt} = FS_{n-1} - FS_n + VX_n v_n \quad (5.2)$$

where S_n and v_n are the substrate concentration (g glucose/l) and the specific consumption rate of glucose in the n -th

section (g glucose/g cell h), respectively. The product balance for the n-th section is given by:

$$V \frac{dP_n}{dt} = FP_{n-1} - FP_n + VX_n\pi_n \quad (5.3)$$

where P_n and π_n stand for the product concentration (g lactic acid/l) and the specific production rate of lactic acid in the n-th section (g lactic acid/g cell h), respectively. At the steady state, the left hand sides of Eqs. 5.1, 5.2, and 5.3 become zero. Thus the following equations are obtained:

$$X_n = \frac{DX_{n-1}}{D - \mu_n + K_d^p} \quad (5.4)$$

$$S_n = S_{n-1} - \frac{X_n v_n}{D} \quad (5.5)$$

$$P_n = P_{n-1} + \frac{X_n \pi_n}{D} \quad (5.6)$$

where D is the dilution rate for one section (F/V ; h^{-1}). The optimum flow rate for the FBR was calculated with a personal computer according to the algorithm in Fig. 5.3 using Eqs. 5.4, 5.5, 5.6, and the equations in our previous chapter. The sources of these equations are summarized in Table 5.1. The calculation was done by a home-made C-program.

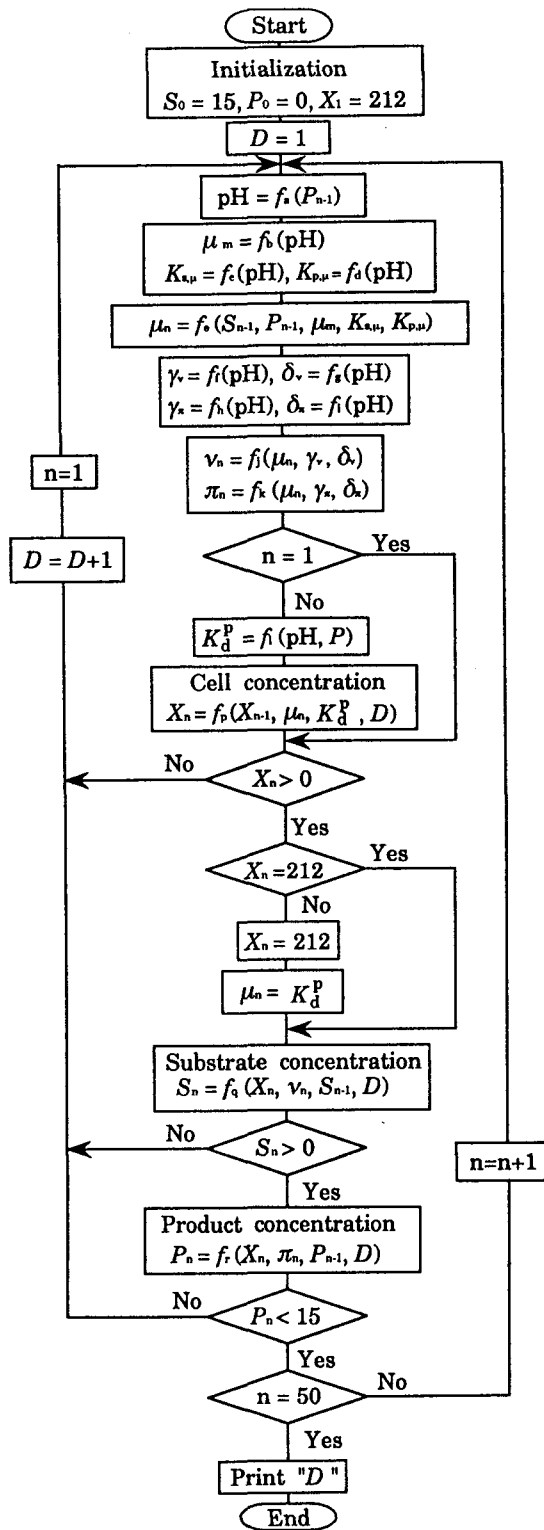


Fig. 5.3 Algorithm to calculate the critical flow rate of the complete consumption of glucose. The equations are shown in Table 5.1.

Table 5.1 List of equations used for calculation

Equation	Unit	Reference
f_a	pH	Fig. 5.2
$f_b = \mu_m = K_3 / \{1 + K_1 10^{\text{pH}} + 1 / (K_2 10^{\text{pH}})\}$	h^{-1}	Eq. 4.3
$K_1 = 5.1 \times 10^5$	mol/l	
$K_2 = 1.3 \times 10^8$	mol/l	
$K_3 = 1.8$	h^{-1}	
$f_c = K_{s,\mu}$	g glucose/l	Table 4.2
$f_d = K_{p,\mu}$	g lactic acid/l	Table 4.2
$f_e = \mu_n = \mu_m \{S_{n-1} / (K_{s,\mu} + S_{n-1})\} \{K_{p,\mu} / (K_{p,\mu} + P_{n-1})\}$	h^{-1}	Eq. 2.3
$f_f = \gamma_v$	g cell/g glucose	Fig. 4.2a
$f_g = \delta_v$	g cell h/g glucose	Fig. 4.2a
$f_h = \gamma_\pi$	g cell/g lactic acid	Fig. 4.2b
$f_i = \delta_\pi$	g cell h/g lactic acid	Fig. 4.2b
$f_j = v_n = \{\gamma_v (1/\mu_n) + \delta_v\}^{-1}$	g glucose/g cell h	Eq. 3.7
$f_k = \pi_n = \{\gamma_\pi (1/\mu_n) + \delta_\pi\}^{-1}$	g lactic acid/g cell h	Eq. 3.8
$f_l = K_d^p$	h^{-1}	Fig. 4.5 Fig. 4.6
$f_p = X_n = DX_n / (D - \mu_n + K_d^p)$	g cell/l	Eq. 5.4
$f_q = S_n = S_{n-1} - X_n v_n / D$	g glucose/l	Eq. 5.5
$f_r = P_n = P_{n-1} - X_n \pi_n / D$	g lactic acid/l	Eq. 5.6

A high-density cell layer is formed in the inlet section due to the gradients of glucose and lactic acid concentration between the two membranes, as in the test run (Ohara and Hiyama 1990) (Fig. 5.4). This packed cell volume at the inlet section was estimated by centrifuging (1200 G, 10 min) the

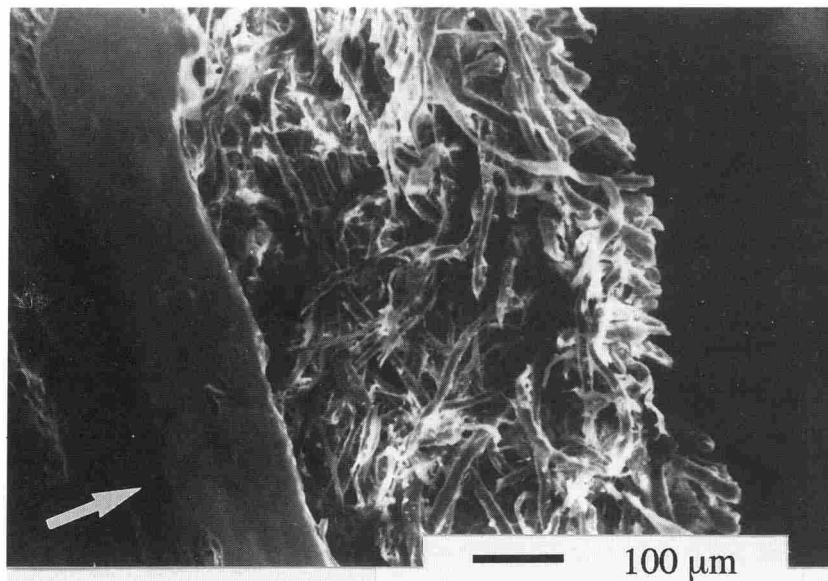


Fig. 5.4 Electron microscopic photograph of a cross-section of the cell layer and the filter paper. The white arrow indicates the direction of the feed flow. To preparing the objective of photograph the membrane filters were removed, and the cross-section of the cell layer and filter paper was prepared after drying. The length of the solid line at the bottom corresponds to 100 μm .

batch culture broth using GYP medium at the stationary phase. Then the dry cell weight at the inlet section was estimated ($X_1 = 212 \text{ g cell/l}$) and the cell density at each section was assumed under this value. The result of the calculation was $D = 255 \text{ h}^{-1}$ and $F = 11.8 \text{ ml/h}$ (converted value for total sections is 5.1 h^{-1}).

5.3.3 Operation of FBR

S. faecalis AHU 1256 was cultivated in the FBR. Various values of flow rates (6, 12, 18, 30, 60 ml/h) were used to determine the critical flow rate for complete glucose consumption. The pressure of the pump was less than 10 kgf/cm^2 at any flow rate. The results are shown in Fig. 5.5. Lactic acid was produced stably for two weeks. The volumetric productivity was 73.2 (including the porous supports in the reactor volume, this value was 21.2) g lactic acid/l h at the critical flow rate (12 ml/h). The calculated result for the optimum flow rate (11.8 ml/h) corresponded to this result.

Continuous fermentation of *Lactobacillus delbrueckii* has been described in other articles. A continuous stirred tank reactor (CSTR) was used by Luedeking and Piret (1959b) and

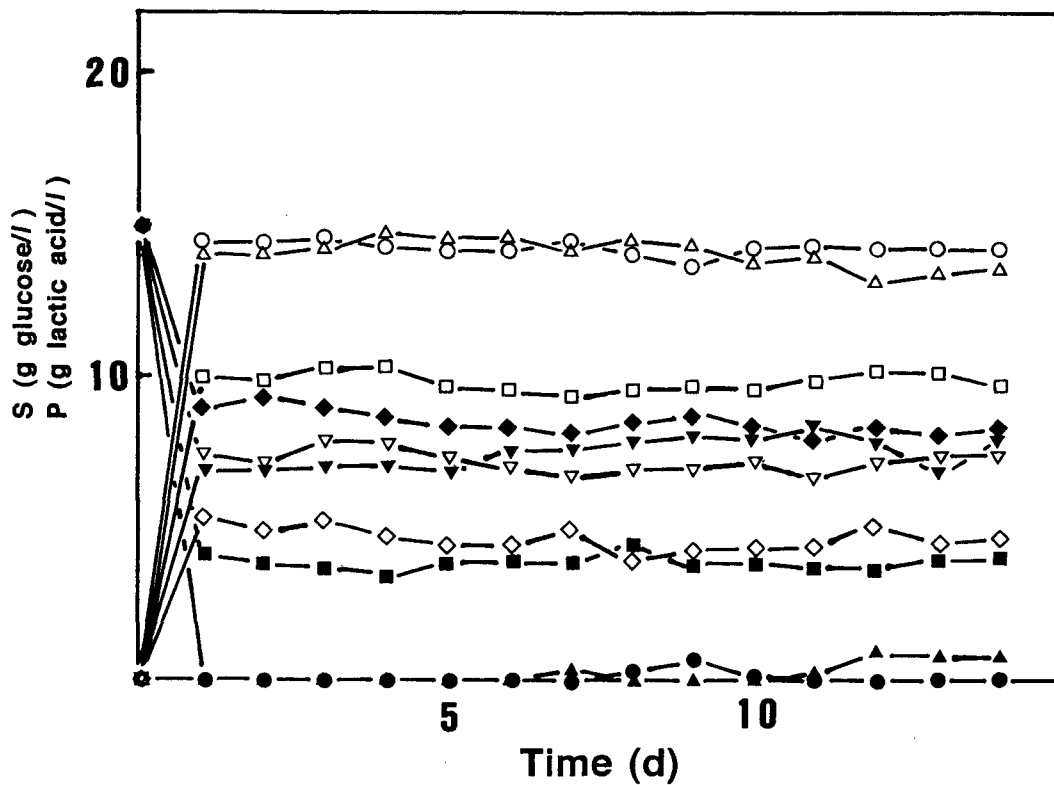


Fig. 5.5 Results of the cultivation in the filter-bed-type reactor. The medium was fed by a high pressure pump at various flow rates, and the reactor was incubated at 37°C. The open symbols represent the lactic acid concentration and the closed symbols the glucose concentration at various flow rates. Symbols: (○, ●), 6 ml/h; (△, ▲), 12 ml/h; (□, ■), 18 ml/h; (▽, ▼), 30 ml/h; (◇, ◆), 60 ml/h.

a volumetric productivity of 7 g lactic acid/l h was obtained. A batch reactor with dialysis was employed by Friedman and Gaden (1970) to yield a volumetric productivity of 3 g lactic acid/l h. Stenroos et al. (1982) immobilized the bacterium in calcium alginate beads and obtained a volumetric productivity of 3 g lactic acid/l h. Vick Roy et al. (1982) immobilized the bacteria in a hollow fiber fermentor; the productivity was 100 g lactic acid/l h, but only 4% of the glucose was converted. A CSTR with cell recycle through an ultrafiltration unit was also employed by Vick Roy et al. (1983b) and a volumetric productivity of 76 g lactic acid/l h was obtained. The productivity of the FBR would be improved by controlling the pH of the medium. To solve this problem, the membrane must be multi-layered or the medium must be recycled.

5.4 Summary

A novel membrane reactor, named a filter-bed-type reactor, was designed to produce lactic acid. The reactor, which was equipped with a membrane filter to immobilize cells of *Streptococcus faecalis*, was operated continuously for

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two weeks. The optimum flow rate was determined from calculations using kinetic equations developed in previous chapter. The calculated result was evaluated experimentally.

CHAPTER 6

HPLC Monitoring System for Lactic Acid Fermentation

6.1 Introduction

For effective production using microorganisms it is very important to monitor and control the fermentation process. Ryu and Humphrey (1973) selected the most important process variables affecting fermentation and the importance of these data has been recognized. However, sensors for industrial process are available only for limited variables such as temperature, pH, and dissolved oxygen. Attempts have been made to apply biosensors, such as an immobilized enzyme electrode (Shi et al. 1992; Durliat et al. 1979; Karube et al. 1980), an immobilized microorganism electrode (Suzuki and Karube 1979; Karube et al. 1979), or a fluorometric sensor (Beyeler et al. 1981) to the fermentation process for the measurement of saccharides, organic acids, amino acids, and cell concentration. However, the performance of such

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sensors has yet to provide good stability or sterilizability for industrial purposes. On the other hand, high performance liquid chromatography (HPLC) has merits in the analysis of thermally unstable samples, polar compounds, and polymers (Wilson 1985). In addition, HPLC can analyze several substances by a simple analysis, and can be easily applied to many different kinds of samples by changing the column and the detector. However, HPLC has a disadvantage when a sample includes microbial cells because cells clog the inside the column and cause serial damage to the packed resin, thereby shortening the life of the column. Thus, a cross-flow type ultrafiltration membrane is utilized to remove cells continuously for HPLC sample analysis. An automatic sampling system is needed not only for continuous but also for batch cultivation, since the process often takes a long time. However, a suitable automatic sampling system for HPLC analysis of the fermentation process does not yet exist. In this chapter, a variable-volume (7-100 μ l) automatic sampling system with a cross-flow filter was developed and applied to lactic acid fermentation on a pilot scale (1000 l) to measure lactic acid and glucose concentrations by HPLC at fixed time intervals.

6.2 Materials and methods

6.2.1 Monitoring system with cross flow filter

A schematic diagram of the stirred tank reactor and monitoring system is shown in Fig. 6.1. The volume of fermentor was 1200 l and sanitary ferrule-type pipe (Toyo Stainless Steel, Okayama, Japan; 1.5 and 2.0 inch) was used for piping. The pH value of the medium was measured (Ingold, InTrac-776, Zurich, Switzerland) and adjusted by a PID controller (DA, Chino, Tokyo, Japan). The filter unit (Filtron Sigma, Fuji Filter, Tokyo, Japan) has 0.46 m² of membrane made of polyether-sulfone and removes molecules larger than 10⁵ molecular weight. The culture broth was recycled (10 l/min) by a sine pump (SPS-15, Tokushu Kika Kogyo, Tokyo, Japan) and the sample was filtrated by the cross-flow filter to remove cells. Next, a 20 µl sample was taken by the automatic sampler and the amount of lactic acid and glucose were determined with a high performance liquid chromatograph (LC-6A, Shimadzu., Kyoto, Japan) with a differential refractometer (RID-6A, Shimadzu). Analytical conditions was the same as section 2.2.3.

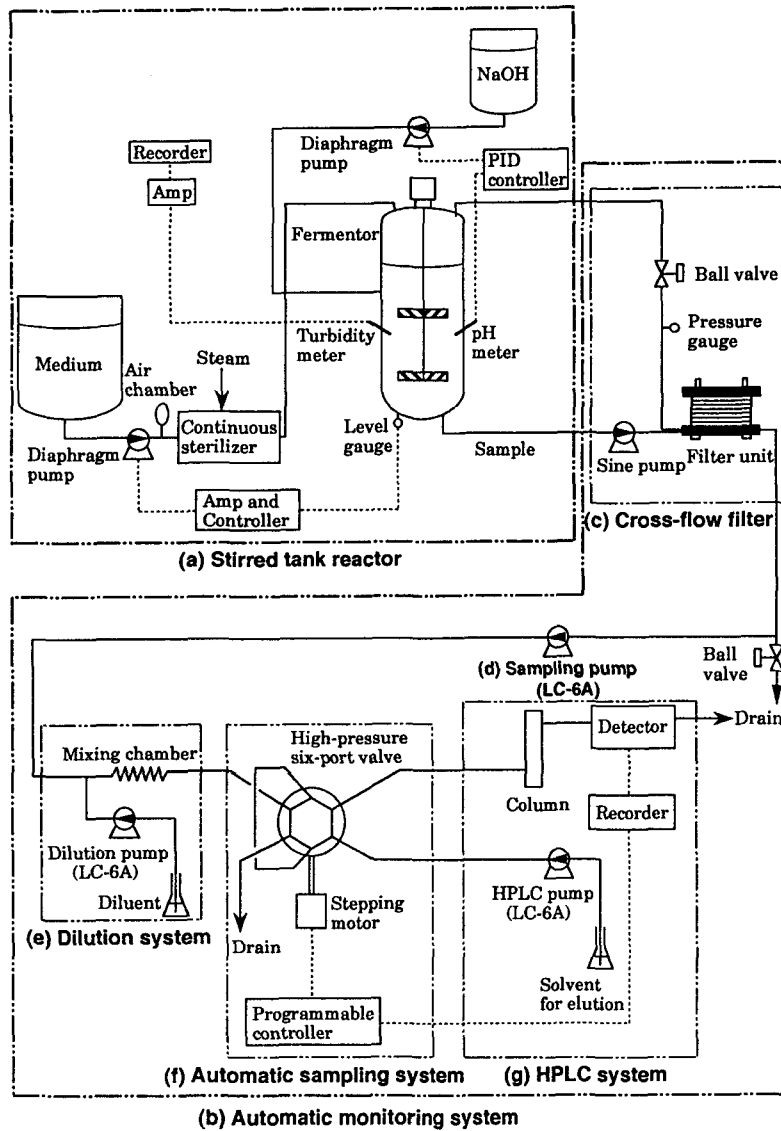


Fig. 6.1 Schematic diagram of stirred tank reactor for lactic acid fermentation (a), and automatic monitoring system (b). The automatic monitoring system consists of a cross-flow filter (c), sampling pump (d), dilution system (e), automatic sampling system (f), and HPLC system (g).

The following is a more detailed explanation of the components and their roles: a cross-flow filter which removed the cells from the culture; a sampling pump (LC-6A) to feed the sample (9.0 ml/min) to the automatic sampling system; a dilution system which diluted samples at a constant ratio with a diluent when the sample concentration was over the detecting range, that is, the dilution ratio depends on the flow rates of the dilution pump (LC-6A) and HPLC pump (LC-6A); an automatic sampling system in which a programmed amount of sample was fed to the HPLC system through a high-pressure six-port valve (FCV-2AH, Shimadzu) operated by a programmable controller; HPLC system, in which components of the sample were resolved by the column, by the detector, and the results recorded. The cell dry weight was determined by using a turbidity meter (SSB-50, Denkikagaku Keiki, Tokyo, Japan) and a calibration curve for the relationship between dry weight and turbidity.

6.2.2 Automatic sampling system

The principles of the automatic sampling system are shown in Fig.6.2. In the variable-volume sampler, the

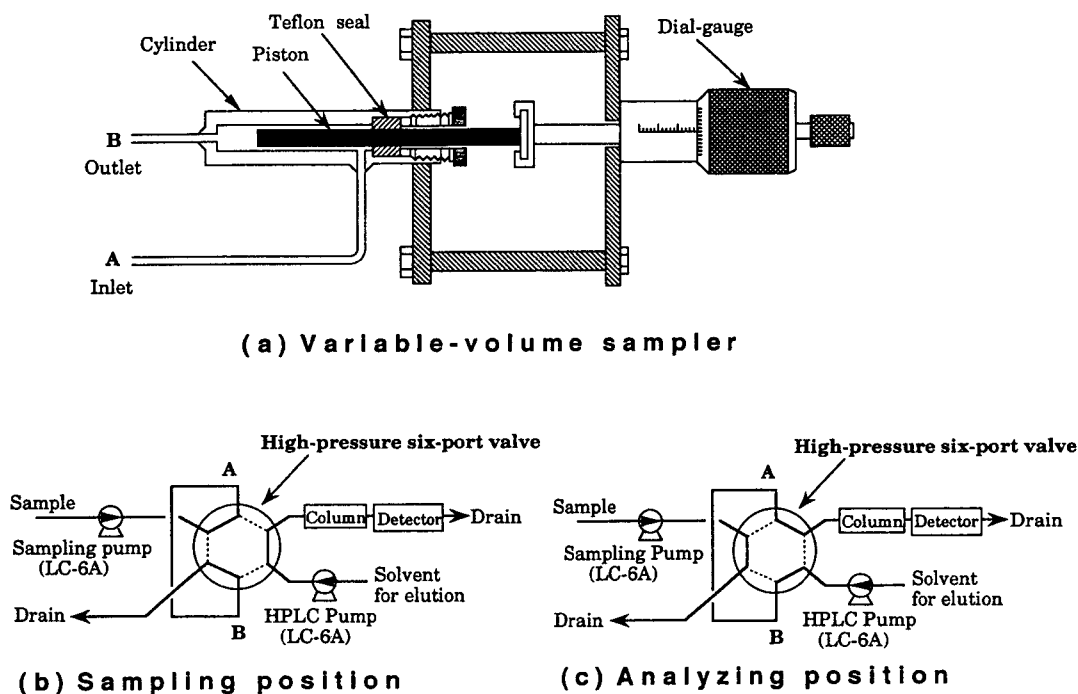


Fig. 6.2 The variable-volume sampler (a) controls the sample volume by the position of the piston, which is moved by rotating the dial-gauge. The inlet A and outlet B are respectively bound to the A and B ports of the high-pressure six-port valve. The high-pressure six-port valve is rotated by a stepping motor to change the flow to the sampling position (b) or the analyzing position (c). The solid lines indicate the flow of the sample and the solvent for elution. The broken lines indicate interrupted flow.

sample volume is determined by the position of the piston; this is moved by rotating the dial-gauge which utilizes a part of a micrometer. The high-pressure six-port valve was changed to the sampling or analyzing position by a stepping motor according to the programmed times. A photograph of the automatic sampling system is shown in Fig. 6.3.

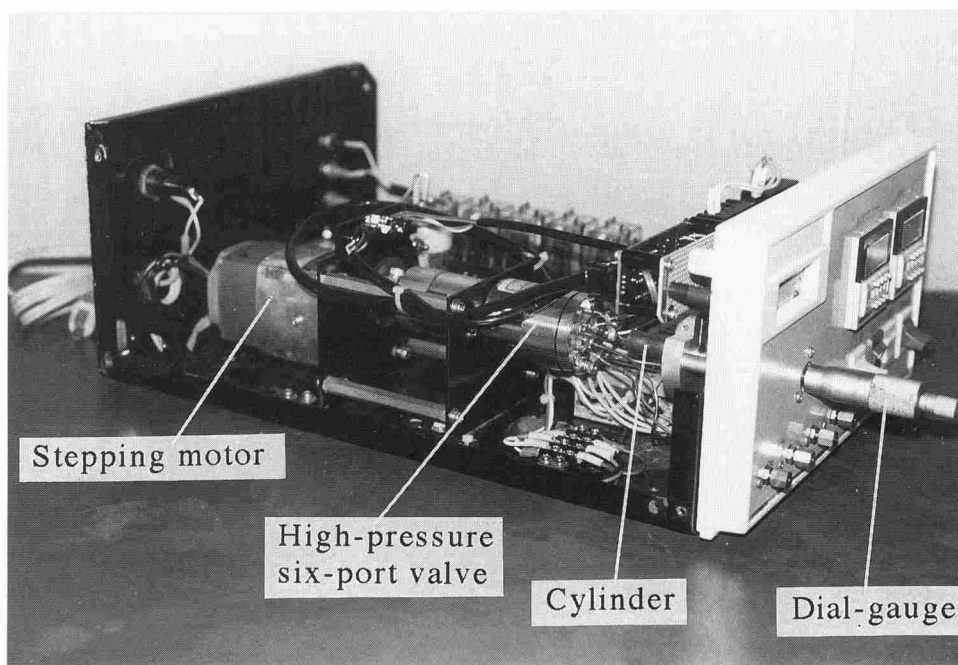


Fig. 6.3 Photograph of automatic sampling system.

6.2.3 Microorganism and medium

A strain of *S. faecalis* AHU 1256 was used. The seed culture was prepared as follows: 10 g/l of yeast extract (D-3, Nihon Pharmaceutical, Tokyo, Japan), 10 g/l of Polypepton (Nihon Pharmaceutical), 20 g/l of D-glucose, and 35 g/l of K_2HPO_4 . The 10 l of seed culture was cultivated for 12 hours at 37°C and then inoculated into the fermentor. The medium for the cultivation contained 10 g/l of yeast extract (D-3), 10 g/l of Polypepton (Nihon Pharmaceutical), 120 g/l of D-glucose, and 5 g/l of $(NH_2)_4HPO_4$.

6.2.4 Cultivation

The filter unit was autoclaved (121°C, 20 min) separately from the fermentor. The fermentor was sterilized by steam (121°C, 6 h) and the medium was sterilized by a direct-steam-mixing continuous sterilizer (140°C, 40 s) of our own design. The amount of condensed water was calculated beforehand and the concentration of the medium was chosen taking this water into account. Cultivation was performed at 37°C and the pH value of the medium was adjusted to pH 7.0 with 6 M NaOH. An agitation speed was 60 rpm with a working volume of 1000 l.

6.3 Results and discussion

Immediately after the inoculation, the lactic acid and glucose concentrations in the reactor was monitored. The retention times for glucose and lactic acid were about 6.8 and 9.5 min respectively. Therefore, the periods of the sampling and analyzing positions of the high-pressure six-port valve were both programmed to be 15 min and the monitoring procedure was conducted at intervals of 30 min. The result is shown in Fig. 6.4.

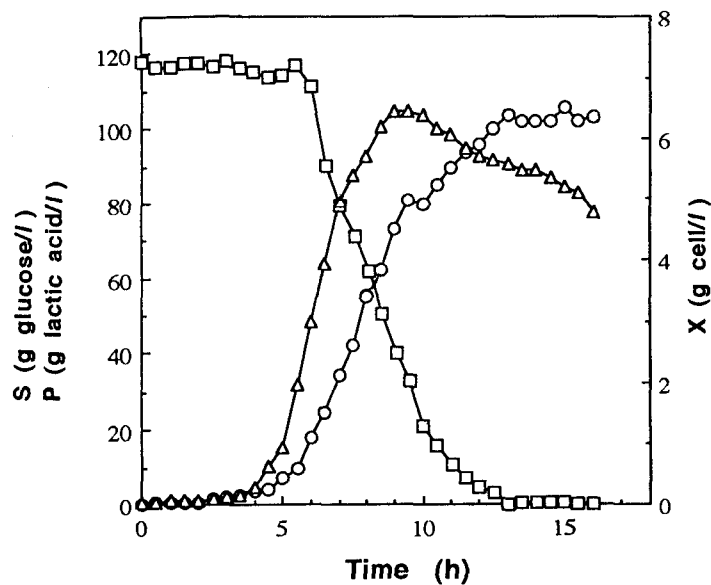


Fig. 6.4 Time courses of changes in lactic acid concentration (g lactic acid/l; \circ) and glucose concentration (g glucose/l; \square) measured by the automatic monitoring system, and cell concentration (g cell/l; Δ) measured by the turbidity meter.

In Chapter 2, we proposed the relationship between the specific growth rate and the specific production rate of lactic acid in continuous and batch culture to be Eq. 3.8. This equation had higher accuracy and a wider range of variation in the specific growth rate than the Luedeking-Piret type equation (Luedeking and Piret 1959a; Eq. 1.1). However, since these experiments were performed under relatively low glucose concentrations (10, 20, 30 g/l) and on a 1-l scale, an attempt was made to verify the validity of Eq. 3.8 at a higher glucose concentration and on a larger fermentor scale in this chapter. With Eqs. 3.1 and 3.3, the specific growth rate (μ ; h⁻¹) and the specific production rate of lactic acid (π ; g lactic acid/g cell h) were calculated employing the differential of the lactic acid concentration at each of the times in Fig. 6.4 without the decline phase (9.5-16 h). When the values of $1/\pi$ were plotted against the $1/\mu$ values to check whether the results fitted Eq. 3.8 (Fig. 6.5), a linear relationship was shown at this glucose concentration (120 g/l) and fermentor scale (1000 l).

The dilution system was not needed in the analysis of this fermentation. If necessary, the monitoring interval can be shortened by increasing the HPLC flow rate. The

fermentation process can be analyzed more easily and efficient control realized by using this system.

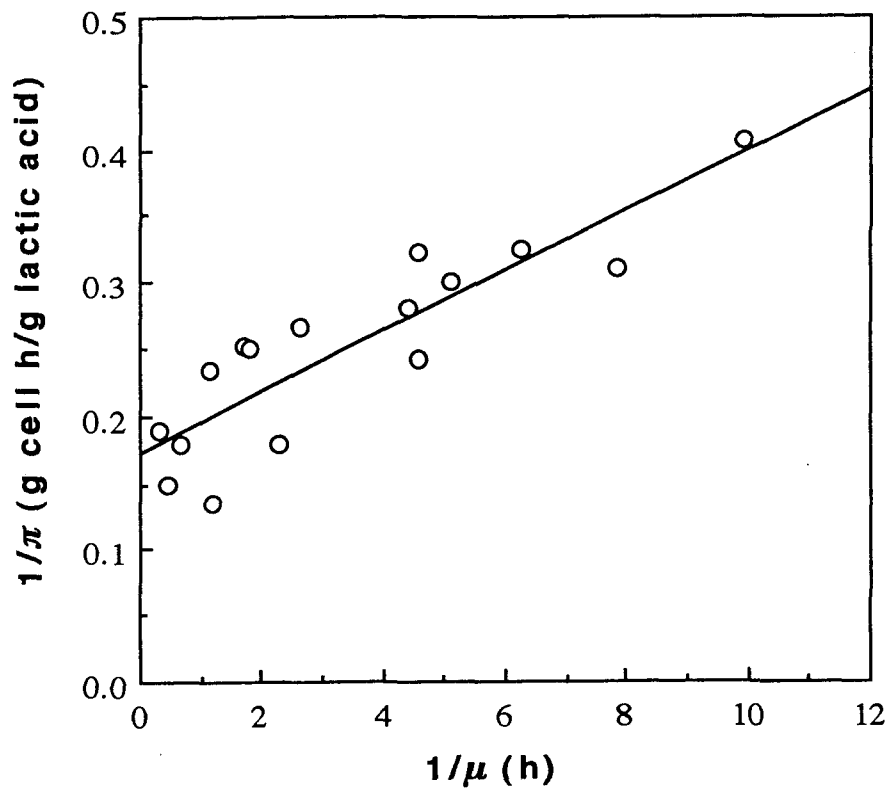


Fig. 6.5 Reciprocals of the specific production rate of glucose ($1/\pi$) against those of the specific growth rate ($1/\mu$).

6.4 Summary

An automatic monitoring system using HPLC was developed and applied to lactic acid fermentation on a pilot scale. The system consisted of a cross-flow filter, a dilution system, an automatic sampling system, and a high performance liquid chromatograph. The lactic acid and glucose concentrations were measured every 30 min. From these results the relationship between the specific growth rate and the specific production rate of lactic acid which we reported in Chapter 3 at glucose concentrations of 10, 20, and 30 g/l was verified at a higher concentration of glucose (120 g/l).

CHAPTER 7

A Higher Optical Purity of L-Lactic Acid

Produced in *Streptococcus faecalis*

7.1 Introduction

Lactic acid produced by chemical synthesis has the demerit in that the product yielded are composed of racemic D- and L-lactic acid. Polylactate, a new biodegradable plastic, has attracted the public interest lately (Naude 1989). The racemic polymer and the L-lactate polymer differ from each other in crystallinity and melting point (Kulkarni et al. 1971). High optical purity of L-lactic acid is therefore required to maintain the desired physical properties of the plastic. Furthermore, as an optically active material, L-lactic acid is expected to be used for the production of a liquid crystal (Sato et al. 1990; Yoshinaga et al. 1990) and for optically nonlinear materials. These applications will lead to the demand for much higher optical purity of L-lactic acid as well as infant food or Ringer's solution (FAO and WHO 1974).

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This chapter describes an improvement for higher optical purity of L-lactic acid formed by fermentation of *Streptococcus faecalis*.

7.2 Materials and methods

7.2.1 Microorganism and medium

A strain of *S. faecalis* AHU 1256 was used. The medium for the cultivation contained 10 g/l of yeast extract (Difco, Detroit, Mich., USA), 10 g/l of Polypepton (Nihon Pharmaceutical, Tokyo, Japan), 80 g/l of D-glucose, and various amounts of dipotassium hydrogen phosphate (K_2HPO_4 ; 0, 0.5, 1.0, 5.0, 35.0 g/l) or diammonium hydrogen phosphate ($(NH_4)_2HPO_4$; 0, 0.5, 1.0, 2.0, 5.0 g/l).

7.2.2 Cultivation

Cultivation was performed by the batch method in the same manner as section 3.2.2.

7.2.3 Analytical methods

The sample used in measuring the optical purity was filtered by a UF membrane (UFPI, Nihon Millipore, Tokyo, Japan) to remove molecules larger than 5000 molecular

weight. The optical purity of L-lactic acid was determined with an HPLC (LC-6A, Shimadzu, Kyoto, Japan) with a spectrophotometric detector (SPD-6AV, Shimadzu) at 254 nm. The analytical conditions were as follows: column, CRS10W (Mitsubishi Kasei, Tokyo, Japan); column temperature, 30 °C; solvent for elution, 2 mM CuSO₄ solution; flow rate, 0.5 ml/min. Optical purity (OP; %) was defined as follows: $OP = 100([L]-[D])/([L]+[D])$ where [L] denotes the concentration of L-lactic acid and [D] of D-lactic acid.

The cell dry weight and the amounts of lactic acid and glucose were determined by the method described in section 2.2.3. The fermentation time was determined by the detection of stopping the neutralizer feed pump.

7.3 Results and discussion

7.3.1 Neutralizer and dipotassium hydrogen phosphate

In many cases, K₂HPO₄ is added to the medium in order to supply phosphoric acid ion in lactic acid fermentation (Man et al. 1960). Thus, various concentrations of K₂HPO₄ (0, 0.5, 1.0, 5.0, 35.0 g/l) were added to the culture medium, and 6 M NH₄OH or 6 M NaOH were used to control the pH at 7.0. As

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shown in Table 7.1, the OP was improved from 97.1-97.3% 98.9-99.7% when 0.5-35.0 g/l of K_2HPO_4 was added. The was found to be higher when NH_4OH rather than $NaOH$ was used. This suggested that the phosphoric acid ion ammonium ion had an effect on the improvement of Thus, $(NH_4)_2HPO_4$ was added to the medium.

Table 7.1 Effect of neutralizer and dipotassium hydrogen phosphate on optical purity (%)

Neutralizer	Optical purity (%)				
	Dipotassium hydrogen phosphate (g/l)				
	0	0.5	1.0	5.0	35.0
NaOH	97.1	99.0	98.9	99.0	99.1
NH_4OH	97.3	99.7	99.7	99.7	99.7

The pH of the medium was controlled at 7.0 by 6 M $NaOH$ or NH_4OH .

7.3.2 Effect of diammonium hydrogen phosphate

Various concentrations of $(\text{NH}_4)_2\text{HPO}_4$ (0, 0.5, 1.0, 2.0, 5.0 g/l) were added to supply phosphoric acid ions and ammonium ions, and NaOH was used as a neutralizer. As shown in Fig. 7.1, the fermentation time was reduced from 130 h to 47 h

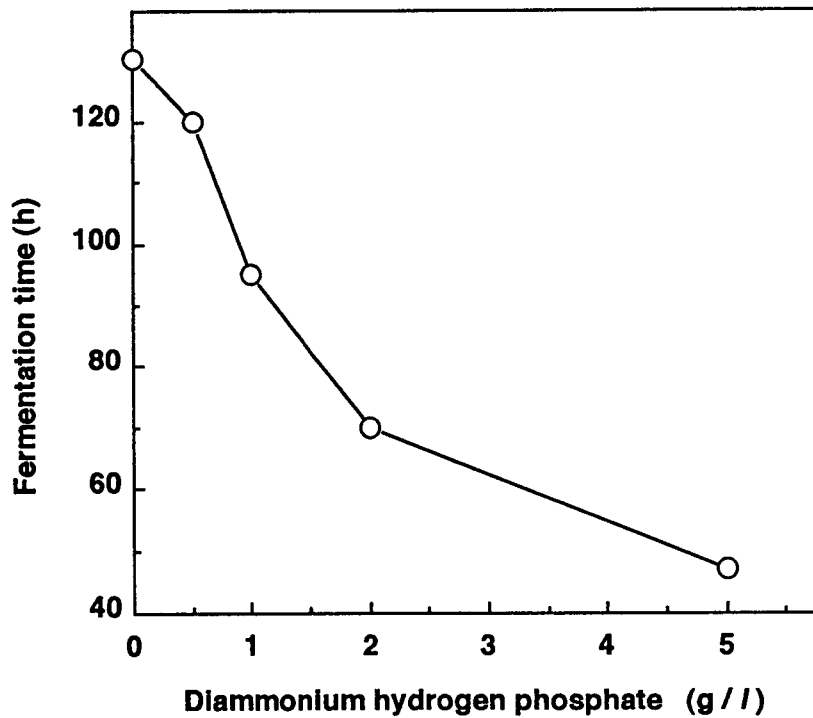


Fig. 7.1 Effect of diammonium hydrogen phosphate on fermentation time. Fermentation was performed at 37°C, and sparged with N_2 gas (20 ml/min). The pH was controlled at pH 7.0 with 6 M NaOH. Various concentrations of diammonium hydrogen phosphate (0, 0.5, 1.0, 2.0, 5.0 g/l) were added.

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when 5.0 g/l of $(\text{NH}_4)_2\text{HPO}_4$ was added. In addition, the OP was improved to 99.8% when 0.5 g/l of $(\text{NH}_4)_2\text{HPO}_4$ was added, as shown in Fig. 7.2.

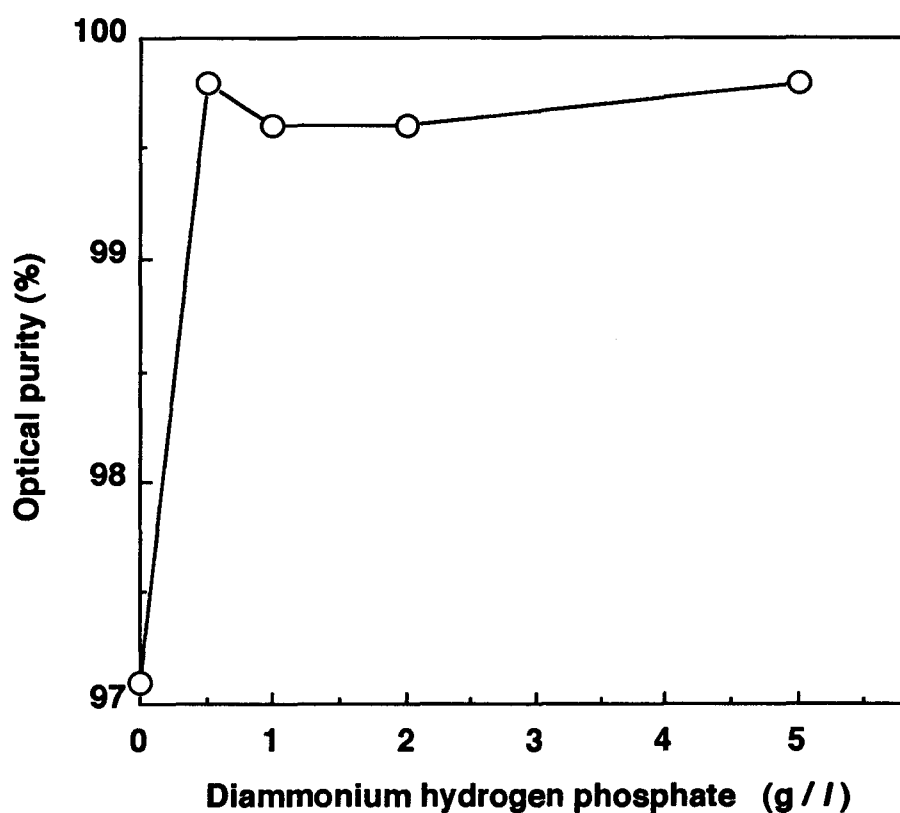


Fig. 7.2 Effect of diammonium hydrogen phosphate on optical purity. Various concentrations of diammonium hydrogen phosphate (0, 0.5, 1.0, 2.0, 5.0 g/l) were added. The optical purities were measured by HPLC.

The OP of lactic acid in the culture broth is usually measured by an increase in absorbance at 340 nm by reducing NAD to NADH with L-lactate dehydrogenase (L-LDH) or D-lactate dehydrogenase (D-LDH) (Okada et al. 1978). This measurement, however, provides inaccurate OP in the vicinity of 100%. This may have been caused by a small amount of L-LDH included in the D-LDH preparation and D-LDH in the L-LDH preparation. More accurate measurement has been achieved together with a development of optical-resolution column for HPLC. Olieman and Vries (1988) determined D- and L-lactic acid in fermented dairy products with HPLC. However, they did not mention the medium composition and bacterial strain. In this study, the OP was measured with an HPLC using an optical-resolution column that is different from theirs.

Figure 7.3 shows two chromatograms; one without addition of $(\text{NH}_4)_2\text{HPO}_4$ and one with addition of 0.5 g/l. The OP was 97.1% and 99.8%, respectively. These values were confirmed by measuring the OP of the fermented lactic acid purified by means of exchanging sodium ions for hydrogen ions by an ion-exchange resin (SK1B, Mitsubishi Kasei) and distillation at 3 mmHg and 140°C.

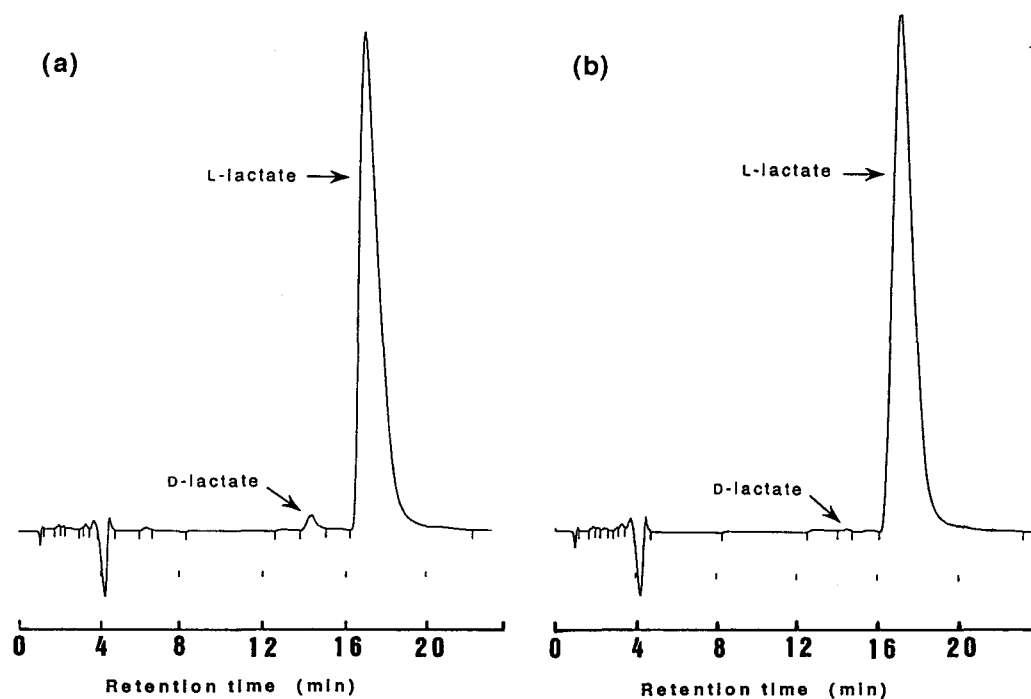


Fig. 7.3 HPLC analysis of fermentation broth without diammonium hydrogen phosphate (a), and with 0.5 g/l added (b). The optical purity is 97.1% (a) and 99.8% (b), respectively.

As shown in Fig. 7.4, the biomass concentration increased when $(\text{NH}_4)_2\text{HPO}_4$ was added. The lactic acid concentration, on the other hand, was almost constant (76-80 g/l). One possibility to explain the fact that the fermentation time was reduced by the addition of $(\text{NH}_4)_2\text{HPO}_4$, as shown in Fig. 7.1,

is a rapid increase in biomass concentration. The other possibility is that phosphoric acid, used in the recycling of ATP and ADP in the anabolic cycle, was fully supplied when $(\text{NH}_4)_2\text{HPO}_4$ was added. On the other hand, the fact that the OP was improved by the addition of a small amount of $(\text{NH}_4)_2\text{HPO}_4$ may be explained in terms of LDH or racemase,

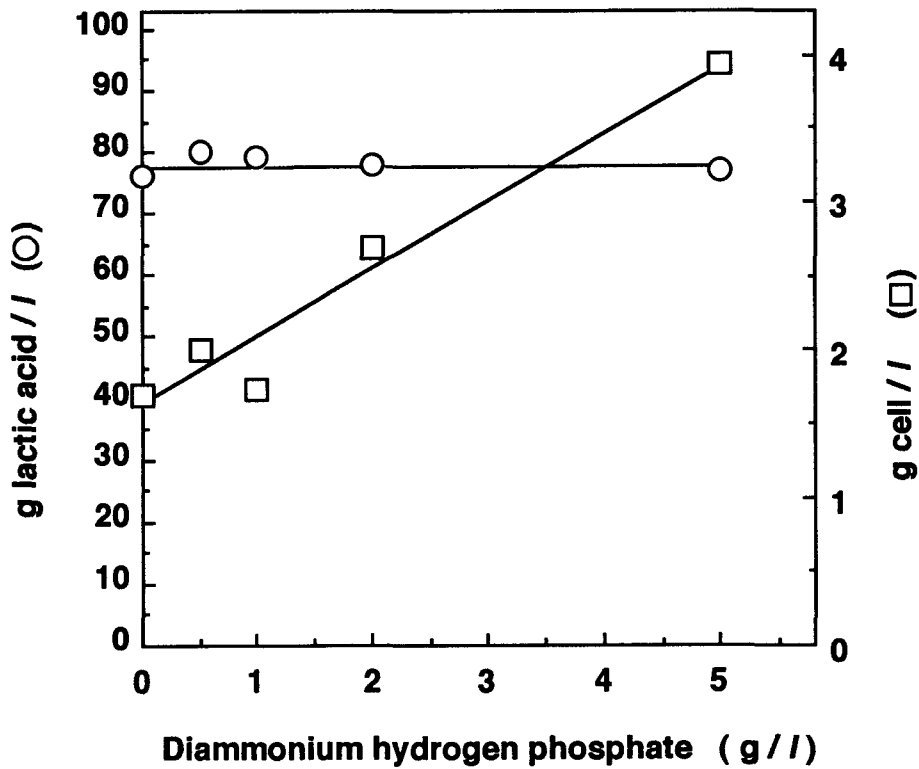


Fig. 7.4 Effect of diammonium hydrogen phosphate on the concentration of lactic acid (O) and on the concentration of cell mass (□).

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since pyruvate, the metabolite which precedes lactic acid in the Embden-Meyerhof pathway, is not optically active. In the extensive study of lactic acid bacteria performed by Kitahara (1966b), he pointed out the importance of medium composition for optical rotations of fermented lactic acid, for example nicotinic acid. However, he did not mention the effect of phosphoric acid in the medium.

In order to produce L-lactic acid of extremely high optical purity, the culture medium must be carefully selected. For example, corn steep liquor, which is often used for cultivation, includes a large amount of DL-lactic acid, thus reducing the optical purity. Yeast extract also includes a small amount of DL-lactic acid. However, the DL-lactic acid, included in the yeast extract, that was used in this study was 0.5% or less, which did not cause a noticeable reduction of optical purity when 10 g/l of yeast extract was added to the medium. Also, no lactic acid was detected in other materials such as Polypepton and no racemase activity was observed in the autoclaved (120°C, 20 min) yeast extract. From our experiments on a pilot scale (1000 l), the contamination of another microorganism often drastically decreased the optical purity.

7.4 Summary

In fermentation of lactic acid with *Streptococcus faecalis*, which produces mainly L-lactic acid, the optical purity of the L-lactic acid produced was improved from 97.1% to 99.8% by the addition of 0.5 g/l of diammonium hydrogen phosphate. The fermentation time was reduced from 130 h to 47 h by the improved method.

CHAPTER 8

General Conclusion and Future Perspective

8.1 General conclusion

The purpose of the study compiled in this thesis is elucidation of the kinetics of lactic acid fermentation and development of a novel membrane reactor for efficient production of L-lactic acid of extremely high optical purity, then optimizing the operation of this reactor using the obtained kinetic equations.

The essence of each chapter are as follows.

Chapter 1 described the background of this thesis as well as historical background.

Chapter 2 showed the cultivation of *Streptococcus faecalis*. This cultivation was performed using a chemostat at pH 7.0, 37°C various dilution rates, and various glucose concentrations (10, 20, 30 g/l). The use of chemostat

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enabled the cultivation in the region where the glucose concentration was as low as 2 g/l. As the result, it was proved clearly that produced lactic acid gave non-competitive inhibition to the specific growth rate, the specific consumption rate of glucose, and the specific production rate of lactic acid.

Chapter 3 revealed the following facts. Both reciprocals of the specific consumption rate of glucose and the specific production rate of lactic acid were linearly correlated with the reciprocal of the specific growth rate at pH 7.0. The double reciprocal equations thus obtained showed their higher accuracy covering a wider range of the variation in the specific growth rate, comparing with the Luedeking-Piret type equation. For the batch culture experiments, these relational equations were derived from the non-competitive inhibition type enzyme equations for the specific growth rate, the specific consumption rate of glucose, the specific production rate of lactic acid. In addition, the ATP yield was able to be calculated more accurately in both batch and chemostat culture by use of these relational equations compared with the use of conventional Luedeking-Piret type equation. This equation could be explained by introducing the

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concept of the components ratio of the cell rather than that of the conventional maintenance metabolism.

Chapter 4 showed that lactic acid induces the non-competitive inhibition against the specific growth rate, the specific consumption rate of glucose, and the specific production rate of lactic acid at wide range of pH 5.0 to pH 8.0 as well as pH 7.0. It was also proved that the reciprocals of the specific consumption rate of glucose and the specific production rate of lactic acid were linearly correlated with the reciprocal of the specific growth rate at wide pH range of pH 5.0 to 8.0. On the other hand, the death rate of the cell depended on pH and lactic acid concentration, and a new relational equations were proposed between pH value (4-9) and the death rate of the cells at various lactic acid concentrations (0-30 g/l).

Chapter 5 showed a novel membrane reactor, filter-bed-type reactor, which was designed by sandwiching a filter paper impregnated with the cell between two microporus membrane filters. The optimum rate of a feeding medium was calculated by using a personal computer based on the kinetic equations and kinetic constants showed in Chapters 2, 3, and 4. The reactor was operated under the calculated

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optimum conditions ($D = 5.1 \text{ h}^{-1}$). This reactor continued steady production of lactic acid for two weeks without clogging the membranes with cells, resulting high volumetric productivity (73.2 g lactic acid/l h).

Chapter 6 showed an automatic monitoring system using HPLC for lactic acid fermentation. The system consisted of a cross-flow filter, a dilution system, an automatic sampling system having a variable-volume sampler (7-100 ml), and a high performance liquid chromatograph. The lactic acid and glucose concentrations were measured every 30 min. From these results the double reciprocal equation shown in Chapter 3 at glucose concentrations of 10, 20, and 30 g/l was verified at a higher concentration of glucose (120 g/l).

Chapter 7 described about the optical purity of lactic acid. The strain of *Streptococcus faecalis* is known as L-lactic acid producer. But according to accurate measurement of optical purity using HPLC, this strain produced small amount of D-lactic acid. The optical purity of L-lactic acid produced was improved from 97.1% to 99.8% by addition of 0.5 g/l ammonium hydrogenphosphate.

In lactic acid fermentation, produced lactic acid gave non-competitive inhibition on the production of lactic acid

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(Chapter 2). If this inhibitory effect can be eliminated, an increase in productivity can be expected. Therefore, a filter-bed-type reactor, that removed the product continuously from the reactor, was designed (Chapter 5). In order to optimize this reactor, the new relational equations were formulized, because conventional Luedeking-Piret type equation deviated from a linear relationship in high dilution rate and was not suitable for calculation of ATP yield (Chapter 3). It was necessary to verify the validity of new relational equations in a broader pH range (Chapter 4), because the pH decreased as the medium passed through the reactor due to an increase in the concentration of produced lactic acid. The study of medium composition was also necessary to obtain extremely high optical purity of L-lactic acid (Chapter 7).

8.2 Future perspective

The subject for future study includes the scale up, reactor control, and purification of lactic acid. The scale up may be realized by using spiral or multi-layer membranes compactly.

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The reactor control is difficult, because the reactor is very thin in structure. However, the use of reactor connected in series and a monitoring system introduced in Chapter 6 between the reactors will be available.

The purification process is also important to produce lactic acid. The method of esterification with methanol which widely prevails today is for food or medicine. For production of chemical materials, it is necessary to select and develop an inexpensive method such as solvent extraction or electro dialysis which provides required purity.

The other subject is efficient production of polylactate. As conventional production of a high-molecular-weight polylactate, a cyclic dimer, lactide, must be made and purified in the process. Then, the lactide can be subjected to ring-opening polymerization to get a high-molecular-weight polylactate. If a high-molecular-weight polylactate can be made directly, the price of polylactate must be remarkably lower. This problem will be solved by a development of catalyst or chemical modification of lactic acid. From another point of view, a copolymer made from low-molecular-weight polylactate produced directly and another chemicals will be realized.

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In Japan, most of lactic acid is currently produced by a synthetic method. The reason why such a method is used is that Japan imposes import restrictions on starch to protect the domestic agriculture. These restrictions will be lifted when Uruguay round-multilateral trade negotiations are settled. In addition, many food-processing companies are saddled with the need to dispose of large volumes of food waste byproducts, such as potato peelings, cheese whey permeate and corn starch. These sources are available for lactic acid fermentation. Thereby allowing inexpensive lactic acid to be produced domestically. Globally, the current quantity of production of lactic acid is too small to use lactic acid as chemical materials. If polylactate attracts interest as a solution to plastic waste problems, the demand for inexpensive lactic acid will increase remarkably.

The lactic acid fermentation, which can be used for the production of chemical materials independent of fossil material and the production of foods or medicines as well, will get into the spotlight. Human beings must create a place where they can live in outer space in the space age to come. For this purpose, the reaction of microorganisms will be utilized. Whether environmental problems arising now on the

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earth can be solved with the help of microorganisms will be a touchstone of whether human beings can create a place where they can live in outer space in the forthcoming space age.

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