แบบจำลองทางจลนพลศาสตร์สำหรับการผลิตสาร 1,3-โพรเพนไดออลจากการหมักกลีเซอรอล โดย *Clostridium butyricum* DSM 5431 ในถังหมักแบบกึ่งต่อเนื่อง

นางสาว ณัฐวดี เหล่าศิริลือชาไกล

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KINETIC MODEL FOR 1,3-PROPANEDIOL PRODUCTION FROM GLYCEROL FERMENTATION BY *Clostridium butyricum* DSM 5431 IN FED-BATCH FERMENTER

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A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	KINETIC MODEL FOR 1,3-PROPANEDIOL PRODUCTION FROM GLYCEROL FERMENTATION BY *Clostridium butyricum* DSM 5431 IN FED-BATCH FERMENTER
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ณัฐวดี เหล่าศิริลือชาไกล : แบบจำลองทางจลนพลศาสตร์สำหรับการผลิตสาร 1,3-โพรเพนไดออล จากการหมักกลีเซอรอลโดย *Clostridium butyricum* DSM 5431 ในถังหมักแบบกึ่งต่อเนื่อง (KINETIC MODEL FOR 1,3-PROPANEDIOL PRODUCTION FROM GLYCEROL FERMENTATION BY *Clostridium butyricum* DSM 5431 IN FED-BATCH FERMENTER) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร. สีรุ้ง ปรีชานนท์, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.ดร. เหมือนเดือน พิศาลพงศ์, 115 หน้า.

งานวิจัยนี้มีวัตถุประสงค์เพื่อพัฒนาแบบจำลองทางจลนพลศาสตร์ของเชื้อจุลินทรีย์*Clostridium butyricum* DSM 5431 สำหรับการผลิตสาร 1,3-โพรเพนไดออลจากกลีเซอรอลในถังหมักแบบกึ่งต่อเนื่อง ซึ่งในส่วนแรกของงานวิจัยได้ศึกษาถึงผลของความเข้มข้นของกลีเซอรอลเริ่มต้น (60-120 กรัมต่อลิตร) ที่มีต่อการผลิตสาร 1,3-โพรเพนไดออล และการเจริญของเชื้อจุลินทรีย์ในถังหมักแบบไม่ต่อเนื่องขนาด 1 ลิตรที่อุณหภูมิ 33 องศาเซลเซียสและพีเอช 7.0 พบว่าความเข้มข้นสูงสุดของสาร 1,3-โพรเพนไดออล (44.75 กรัมต่อลิตร) ซึ่งมีค่าผลได้ของผลิตภัณฑ์เท่ากับ 0.77 ได้จากการหมักที่ความเข้มข้นของกลีเซอรอลที่เหมาะสมที่สุด 80 กรัมต่อลิตร ผลการยับยั้งของกลีเซอรอล พบที่ความเข้มข้นมากกว่าเท่ากับ 100 กรัมต่อลิตร ผลการทดลองภายใต้สภาวะการหมักเดียวกันในถังหมักแบบไม่ต่อเนื่องขนาด 15 ลิตร แสดงให้เห็นว่าความเข้มข้นสูงสุดของสาร 1,3-โพรเพนไดออล คือ 42.89 กรัมต่อลิตร ซึ่งมีค่าผลได้ของผลิตภัณฑ์เท่ากับ 0.71 แบบจำลองทางจลนพลศาสตร์ของเชื้อจุลินทรีย์ที่ได้เสนอในงานวิจัยนี้ซึ่งถูกพัฒนาขึ้นโดยใช้โปรแกรม MATLAB (เวอร์ชั่น 2007a) แสดงรูปแบบการยับยั้งของกลีเซอรอล 1,3-โพรเพนไดออลและกรดบิวทาริกต่อการเจริญเติบโตของเชื้อจุลินทรีย์ *Clostridium butyricum* DSM 5431 ดังสมการ

$$\mu = 0.3786\left(\frac{C_{Gly}}{C_{Gly}+0.7629}\right)\left(1-\frac{C_{Gly}}{181.7}\right)\left(1-\frac{C_{PD}}{66.4}\right)\left(1-\frac{C_{HBu}}{11.1}\right)$$

การป้อนสารแบบคงที่ถูกพบจากแบบจำลองการแก้ปัญหาทางคณิตศาสตร์ว่าเป็นวิธีการที่เหมาะสมที่สุดสำหรับอัตราการผลิตสาร 1,3-โพรเพนไดออลในการหมักแบบกึ่งต่อเนื่อง การหมักแบบกึ่งต่อเนื่องโดยใช้ความเข้มข้นของกลีเซอรอลเริ่มต้น 80 กรัมต่อลิตรและความเข้มข้นของกลีเซอรอลในสายป้อน 120 กรัมต่อลิตรที่อัตราการป้อน 0.353 ลิตรต่อชั่วโมง ถูกทดลองขึ้นเพื่อทดสอบความแม่นยำของแบบจำลอง ค่าเบี่ยงเบนเฉลี่ยระหว่างผลที่ได้จากการทำนายกับผลที่ได้จากการทดลอง 2.77 เปอร์เซ็นต์แสดงให้เห็นความแม่นยำสูงของแบบจำลอง ความเข้มข้นสูงสุดของสาร 1,3-โพรเพนไดออลที่ได้จากถังหมักแบบกึ่งต่อเนื่องขนาด 15 ลิตร คือ 52.29 กรัมต่อลิตร การหมักแบบกึ่งต่อเนื่องให้ความเข้มข้นของสาร 1,3-โพรเพนไดออลสูงกว่าการหมักแบบไม่ต่อเนื่องอย่างชัดเจน 39.27 เปอร์เซ็นต์ ผลที่ได้พิสูจน์ให้เห็นว่าการผลิตสาร 1,3-โพรเพนไดออลในการหมักแบบกึ่งต่อเนื่องดีกว่าการหมักแบบไม่ต่อเนื่อง

ภาควิชา	วิศวกรรมเคมี	ลายมือชื่อนิสิต.....
สาขาวิชา	วิศวกรรมเคมี	ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....
ปีการศึกษา	2551	ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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NATTAWADEE LAOSIRILURCHAKAI: KINETIC MODEL FOR 1,3-PROPANEDIOL PRODUCTION FROM GLYCEROL FERMENTATION BY *Clostridium butyricum* DSM 5431 IN FED-BATCH FERMENTER. THESIS PRINCIPAL ADVISOR: ASSOC. PROF. SEEROONG PRICHANONT, Ph.D., THESIS CO-ADVISOR: ASSOC. PROF. MUENDUEN PHISALAPHONG, Ph.D., 115 pp.

This study aims at developing the kinetic model of *Clostridium butyricum* DSM 5431 for 1,3-propanediol production from glycerol in fed-batch fermenter. The first part of the study was to investigate effects of initial glycerol concentration (60-120 g/l) on 1,3-propanediol production and cell growth in 1 litre batch fermenter under 33 °C and pH 7.0. The maximum 1,3-propanediol concentration (44.75 g/l) with the production yield of 0.77 (mol/mol) was obtained from fermentation at optimal initial glycerol concentration of 80 g/l. The inhibition effect of glycerol was observed at concentration equaled to or higher than 100 g/l. Experimental results under the same fermentation conditions in a 15 litre batch fermenter revealed the maximum 1,3-propanediol concentration of 42.89 g/l with the production yield of 0.71 (mol/mol). The proposed cell model developed using MATLAB program (version 2007a) indicated multiple inhibition effects of glycerol, 1,3-propanediol, and butyric acid on *Clostridium butyricum* DSM 5431 growth as the following equation:

$$\mu = 0.3786\left(\frac{C_{Gly}}{C_{Gly}+0.7629}\right)\left(1-\frac{C_{Gly}}{181.7}\right)\left(1-\frac{C_{PD}}{66.4}\right)\left(1-\frac{C_{HBu}}{11.1}\right)$$

Constant feeding was found by mathematical simulation to be the most suitable strategy for 1,3-propanediol productivity in fed-batch fermentation. Fed-batch experiment with 80 g/l of initial glycerol concentration and 120 g/l of glycerol concentration in feed with a constant feed rate of 0.353 l/hr was carried out to test the model accuracy. The deviation between simulated and experimental results of 2.77% was obtained demonstrating high model accuracy. The highest 1,3-propanediol concentration obtained in 15 litre fed-batch fermenter was 52.29 g/l. Fed-batch fermentation apparently gave 1,3-propanediol concentration 39.27% higher than batch fermentation. This proved that production of 1,3-propanediol is more favorable in fed-batch than batch fermentation.

Department	Chemical Engineering	Student's signature……...
Field of study	Chemical Engineering	Principal Advisor's signature……
Academic year	2008	Co-advisor's signature……...

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NOMENCLATURE

C_{PD} is the 1,3-propanediol concentration [g/l].

C^{*}_{PD} is the maximum 1,3-propanediol concentration above which cells cannot grow due to 1,3-propanediol inhibition [g/l].

C_{Gly} is the glycerol concentration [g/l].

C^{*}_{Gly} is the maximum glycerol concentration above which cells cannot grow due to glycerol inhibition [g/l].

C_{HBu} is the butyric acid concentration [g/l].

C^{*}_{HBu} is the maximum butyric acid concentration above which cells cannot grow due to butyric acid inhibition [g/l].

C_{HAc} is the acetic acid concentration [g/l].

C^{*}_{HAc} is the maximum acetic acid concentration above which cells cannot grow due to acetic acid inhibition [g/l].

C_{EtOH} is the ethanol concentration [g/l].

C^{*}_{EtOH} is the maximum ethanol concentration above which cells cannot grow due to ethanol inhibition [g/l].

D_i is the impeller diameter [m].

D_t is the fermenter diameter [m].

F is the substrate feed rate [l/hr].

g is the gravitational constant [m/s^2].

H is the liquid depth [m].

J is the objective function.

K_d is the constant for cell death rate [hr^{-1}].

K_p is a constant having the value of the product concentration at 50% growth inhibition [g/l].

K_S is a constant having the value of the limiting substrate concentration at which the specific growth rate is half its maximum value [g/l].

M_C is the maintenance coefficient [g substrate consumed/g cell $\cdot$ hr].

N is the impeller rotation speed [rev/s].

P is the power input [J/s or W].

Q is the volumetric gas flow rate [m^3/s].

r_P is the product formation rate [g/l $\cdot$ hr].

r_S is the substrate consumption rate [g/l $\cdot$ hr].

S_f is the substrate concentration in feed [g/l].

S is the substrate concentration in reactor [g/l].

t is the time [hr].

t_M is the experimentally observed molecular mixing time [s].

V_0 is the initial volume of substrate [l].

V is the reactor volume [l].

X_0 is the initial cell concentration [g/l].

X is the cell concentration at time t [g/l].

Y_{ATP} is the energetic yield of biomass [g biomass/mol ATP].

$Y_{Pi/S}$ is the yield coefficient (mass product produced/mass substrate consumed).

$Y_{X/S}$ is the yield coefficient (mass cell produced/mass substrate consumed).

$y_{experiment}$ is the experimental value.

$y_{estimate}$ is the optimization value.

μ is the specific growth rate [hr^{-1}].

μ is the viscosity [kg/m·s].

μ_{max} is the maximum specific growth rate [hr^{-1}].

α is the constant for growth associate product.

β is the constant for non-growth associate product [hr^{-1}].

ρ is the specific production rate [g product produced/g cell · hr].

ρ is the fluid density [kg/m^3].

υ is the specific consumption rate [g substrate consumed/g cell · hr].

υ_{tip} is the impeller tip speed [m/s].

CHAPTER I

INTRODUCTION

1.1 Motivation

Nowadays, because of the rising price of traditional fuel and the increasing demand of fuel, the demand of alternative fuel such as biodiesel has increased. This leads to glycerol surplus in the world market because glycerol is formed as a main by-product in biodiesel production. With the production of 10 kg ester fuel from rapeseed oil in biodiesel production process, 1 kg of glycerol becomes remainder (Deckwer, 1995; Meesters et al., 1996). Therefore, it is essentially advantageous to develop a technology that converts a large volume of glycerol into products of high value, for example, to 1,3-propanediol which has a wide range of potential uses, particularly as a monomer for the production of biodegradable polymers such as polytrimethylene terephthalate (PTT) and as an intermediate for the synthesis of heterocyclic compounds. In addition, 1,3-propanediol can be used as a solvent and additive for lubricants.

There are two methods of producing 1,3-propanediol (Cho et al., 2006). One is the chemical method that 1,3-propanediol (petroleum-based PDO) produced by chemical synthesis using ethylene or propylene derived from petroleum (Biebl et al., 1992). And the other is the biotechnological method that 1,3-propanediol (bio-based PDO) produced through the metabolism pathway by microorganisms (Gunzel et al., 1991; Deckwer, 1995; Barbirato et al., 1995; Menzel et al., 1997; Reimann et al., 1998; Biebl et al., 1999; Papanikolaou et al., 2000; Seraphim et al., 2000). However, the biotechnological method is preferable since it is better for environmental protection and its raw material is cheaper than the chemical method.

In biotechnological method, the substrate for producing 1,3-propanediol by microbial metabolism is glycerol, and the cost for the microbial production of 1,3-propanediol, including product recovery and purification, can be estimated roughly from the relationship as the following (Deckwer, 1995):

Price of 1,3-propanediol ($) $= 1 + 2 \times$ (price of glycerol)

1,3-propanediol can be produced from glycerol fermentation by using the microorganisms such as *Klebsiella pneumoniae*, *Clostridium butyricum*, *Citrobacter freundii*, and *Enterobacter agglomerans* (Forsberg, 1987; Homann et al., 1990; Gunzel et al., 1991; Abbad-andaloussi, 1995; Barbirato et al., 1995; Deckwer, 1995; Menzel et al., 1997; Abbad-andaloussi, 1998; Reimann et al., 1998; Biebl et al., 1999; Papanikolaou et al., 2000). Different microorganisms require different nutrients, produce 1,3-propanediol at varied rates, and form different types of by-products. Among these, *Clostridium butyricum* DSM 5431 and *Klebsiella pneumoniae* DSM 2026 have been found to convert glycerol to 1,3-propanediol at relatively high yields and productivities. However, according to its safety record *Clostridium butyricum* DSM 5431 is preferred for potential industrial use in the production of 1,3-propanediol from glycerol (Saint-Amans et al., 1994; Barbirato et al., 1995; Petitdemange et al., 1995; Himmi, 1998).

The production of 1,3-propanediol from glycerol using *Clostridium butyricum* DSM 5431 has been developed in our laboratory by Pullsirisombat (2007). The optimum conditions for the batch production were found to be 80 g/l initial glycerol concentration, 33 °C, and pH 7.0 under anaerobic conditions. The maximum product concentration obtained after 33 hours was 41.37 g/l with the yield of 0.76 mol 1,3-propanediol/mol glycerol.

Since glycerol inhibition starts at concentrations higher than 80 g/l, fed-batch fermentation is an obvious choice for prolonging 1,3-propanediol production using proper feeding strategy to control substrate concentration under inhibition level. This project, therefore, aims at investigating proper feeding strategy of glycerol in fed-batch fermentation by *Clostridium butyricum* DSM 5431 based on the developed kinetic model of *Clostridium butyricum* DSM 5431.

1.2 Objectives

1. To develop the kinetic model for 1,3-propanediol production from glycerol fermentation by using *Clostridium butyricum* DSM 5431 in a fed-batch fermenter.
2. To increase 1,3-propanediol productivity by determining proper feeding strategy for 1,3-propanediol production in fed-batch fermentation.

1.3 Scope of work

1. Search and study the relevant information.
2. Experiment : batch fermentation in a 1 litre and 15 litre fermenter and study the concentration profiles of cell, substrate, product, and by-products.
3. Develop the kinetic model from 1 litre and 15 litre batch fermentation.
4. Determine the proper feeding strategy of fed-batch fermentation from mathematical simulation using productivity as criteria.
5. Experiment : fed-batch fermentation in a 15 litre fermenter by using the proper feeding strategy.
6. Test the accuracy of the model.

1.4 Expected benefits

1. To add value of glycerol by using *Clostridium butyricum* DSM 5431 to produce 1,3-propanediol which has a higher value.
2. Obtain proper feeding strategy for fed-batch fermentation.
3. Obtain the kinetic model of *Clostridium butyricum* DSM 5431 in a fed-batch fermenter in order to give an approach for scale-up 1,3-propanediol production process.

CHAPTER II

THEORY

In order to develop a kinetic model for 1,3-propanediol production from glycerol fermentation by using *Clostridium butyricum* DSM 5431 in fed-batch fermentation, we should understand the biochemical pathways of glycerol fermentation of *Clostridium butyricum*, the reaction rate models of cell growth, substrate consumption and product formation. Finally, it is necessary to understand mass balance equations developed for fed-batch fermentation. These topics are, therefore, included in this chapter.

2.1 Microbial conversion of glycerol

The biochemical pathways of glycerol fermentation to produce 1,3-propanediol under anaerobic condition take place via the following reactions (see Figure 2.1): after glycerol enters the cell by diffusion, one portion of glycerol is oxidized to dihydroxyacetone by an NAD-dependent glycerol dehydrogenase and subsequently converted to pyruvate in the glycolysis pathway, whereas the rest of glycerol is dehydrated to 3-hydroxypropionaldehyde by vitamin B_{12}-dependent dehydratase. The product of the dehydration reaction, 3-hydroxypropionaldehyde (3-HPA), is reduced to 1,3-propanediol by an NAD-dependent oxidoreductase. The physiological role of the 1,3-propanediol pathway is to regenerate the reducing equivalents (e.g., $NADH_2$) which are released from the formation of dihydroxyacetone and during the oxidation of glyceraldehydephosphate to bis-phosphoglycerate. The cleavage of pyruvate to acetyl-CoA and CO_2 is considered to be carried out by the enzyme pyruvate: ferredoxin oxidoreductase. Under normal culture conditions acetate and butyrate are the main fermentation products of pyruvate. The physiological role of acetate and butyrate pathways are to generate the energy (ATP) for growth of the microorganism.

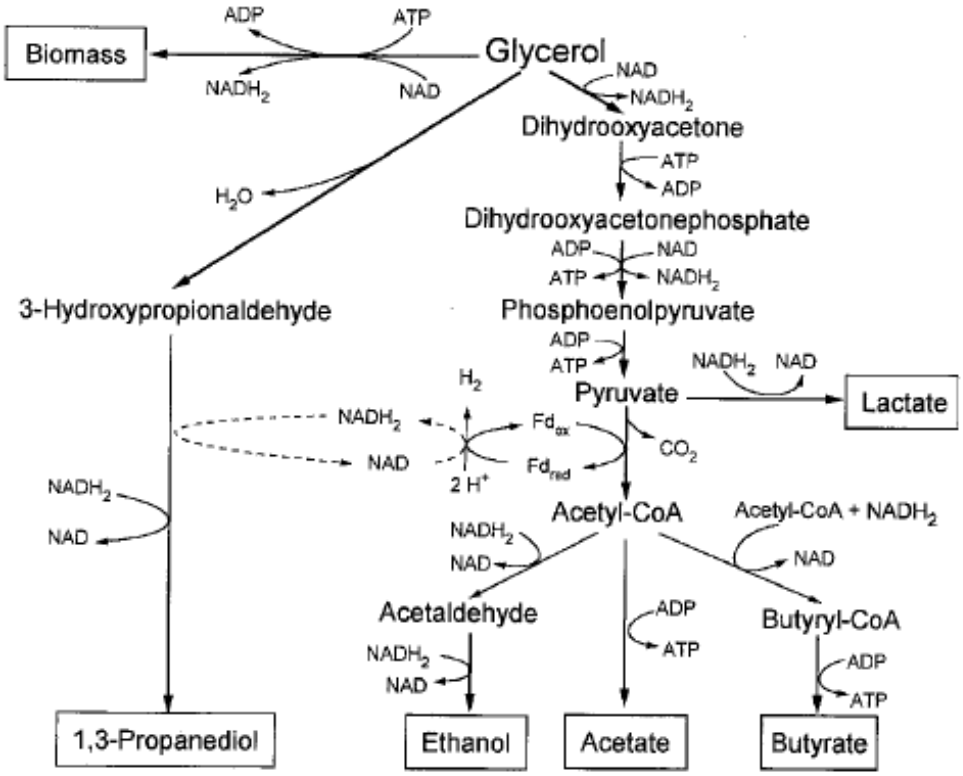


Figure 2.1 Biochemical pathways of anaerobic glycerol fermentation of *Clostridium butyricum* (Zeng, 1996)

The yield of 1,3-propanediol depends on the combination and stoichiometry of the reductive and oxidative pathways. The maximum of 1,3-propanediol production is obtained when butyrate and hydrogen were not produced and acetate was generated as the sole by-product (Zeng, 1996).

The reactions of the different metabolites formation in the glycerol fermentation by *Clostridium butyricum* can be written as the following:

Acetate formation (Zeng, 1996).

$$C_3H_8O_3 \longrightarrow C_2H_4O_2 + 2NADH_2 + 2ATP + CO_2 + H_2 - H_2O \quad (2.1)$$

Butyrate formation (Zeng, 1996).

$$2C_3H_8O_3 \longrightarrow C_4H_8O_2 + 2NADH_2 + 3ATP + 2CO_2 + 2H_2 \quad (2.2)$$

1,3-propanediol formation (Zeng, 1996).

$$C_3H_8O_3 + NADH_2 \longrightarrow C_3H_8O_2 + H_2O \quad (2.3)$$

Biomass formation (Zeng, 1996).

$$4C_3H_8O_3 + 3NH_3 + 3\frac{101}{Y_{ATP}}ATP \longrightarrow 3C_4H_7O_2N + 8[H] + 6H_2O \qquad (2.4)$$

where $C_4H_7O_2N$ is the elemental composition of biomass (Herbert, 1971) and corresponds to a molecular biomass weight (M_G) of 101 g/mol. For simplicity, Y_{ATP} is taken here as 8.6 g/mol ATP. In equation (2.4), [H] represents one mole of hydrogen atom which is equivalent to 1/2 mole $NADH_2$. Thus, equation (2.4) becomes as follows (Zeng,1996):

$$4C_3H_8O_3 + 3NH_3 + 35ATP \longrightarrow 3C_4H_7O_2N + 4\ NADH_2 + 6H_2O \qquad (2.5)$$

2.2 Reaction rate models

2.2.1 Growth rate model

The simplest of growth model which demonstrates the relationship between the specific growth rate (μ) and a substrate concentration is the model developed by Jacques Monod in 1942. The Monod equation states that,

$$\mu = \frac{\mu_{max} S}{K_S + S} \qquad (2.6)$$

Other related forms of growth model have been proposed which in particular instances give better fits to experimental data. For example, Teissier, Moser, and Contois suggest the following models:

Teissier: $\mu = \mu_{max}\left(1 - e^{-S/K_s}\right)$ (2.7)

Moser: $\mu = \mu_{max}\left(1 - K_S S^{-\lambda}\right)^{-1}$ (2.8)

Contois: $\mu = \mu_{max}\frac{S}{Bx + S}$ (2.9)

Furthermore, the specific growth rate may be inhibited by medium constituents such as substrate or product. In case of initial substrate inhibition, it is generally found experimentally that the rate of growth decreases at high values of the initial substrate concentration (S_0) due to the influence of ionic strength, osmotic pressure, or overloading of membrane transport system. In this case, the growth model can be written as follows:

$$\mu = \mu_{max} \frac{S}{K_S + K_S S_0 + S} \tag{2.10}$$

In case of product inhibition, the growth model can be written as follows:

$$\mu = \mu_{max} \left(\frac{S}{K_S + S} \right) \left(\frac{K_p}{K_p + C_p} \right) \tag{2.11}$$

or $$\mu = \mu_{max} \left(\frac{S}{K_S + S} \right) \left(1 - \frac{C_p}{C_{pm}} \right)^n \tag{2.12}$$

In case of multiple substrates inhibition, the growth model can be written as follows:

$$\mu = \mu_{max} \frac{S_1}{K_1 + S_1} \frac{S_2}{K_2 + S_2} \ldots \tag{2.13}$$

or $$\mu = \mu_{max} \sum_{i=1}^{m} \left(\frac{S_i}{K_i + S_i} \right) \tag{2.14}$$

2.2.2 Substrate consumption rate model

To optimize the performance of a bioprocess, and especially to monitor and control the environmental conditions, it is necessary to assess quantitatively the influences of substrate concentration. Therefore, a kinetic model for substrate consumption is needed.

The overall substrate consumption rate is the sum of the substrate consumption rates that are used for cell growth, cell maintenance, and product (or by-products) formation. The form of the overall substrate consumption rate can be written as follows:

$$r_S = \frac{\mu X}{Y_{X/S}} + M_c X + \sum \frac{r_{Pi}}{Y_{Pi/S}} \quad (2.15)$$

2.2.3 Product formation rate model

Based on the product is formed as a result of primary metabolic function of the cell or from secondary metabolic function of the cell, the models of product formation rate can be divided as follows:

- Growth associate production rate is the rate of product formation that parallels with the growth of the cell population. This type of products are generally primary metabolites which are produced during the cell growth. The growth associate production rate can be written as the following:

$$r_P = \alpha(\mu X) \quad (2.16)$$

- Non-growth associate production rate is the rate of product formation that does not depend on the growth of the cell population. This type of products are secondary metabolites which are generally produced at the end of the exponential phase such as antibiotics and vitamin. The non-growth associate production rate can be written as the following:

$$r_P = \beta X \quad (2.17)$$

- Mixed-growth associate production rate is the rate of product formation between the two types of production rate above. The mixed-growth associate production rate can be written as the following:

$$r_P = \alpha(\mu X) + \beta X \quad (2.18)$$

2.3 Specific rates

2.3.1 Specific growth rate (μ)

The specific growth rate represents the rate at which the individual cells divide in the exponential growth phase. The specific growth rate can be calculated from the following equation:

$$\frac{dX}{dt} = \mu X \tag{2.19}$$

$$\int_{X_0}^{X} \frac{1}{X} \cdot dX = \int_{t_0}^{t} \mu \cdot dt \tag{2.20}$$

$$\ln X = \mu(t - t_0) + \ln X_0 \tag{2.21}$$

Using the equation (2.21), if plot ln X versus $t-t_0$, it can obtain the specific growth rate as the slope of the plot.

2.3.2 Specific consumption rate (υ)

The specific consumption rate represents the substrate consumption rate at which the individual cells consume in the exponential growth phase**.** The specific consumption rate can be calculated from the following equation:

$$r_S = - \upsilon \cdot X \tag{2.22}$$

$$\frac{dS}{dt} = -\upsilon \cdot X \tag{2.23}$$

$$\int_{S_0}^{S} dS = \int_{t_0}^{t} -\upsilon \cdot X \cdot dt \tag{2.24}$$

$$S = -\upsilon \int_{t_0}^{t} X \cdot dt + S_0 \tag{2.25}$$

Using the equation (2.25), if plot S versus $\int_{t_0}^{t} X \cdot dt$, it can obtain the specific consumption rate as the slope of the plot.

2.3.3 Specific production rate (ρ)

The specific production rate represents the product formation rate at which the individual cells produce in the exponential growth phase**.** The specific production rate can be calculated from the following equation:

$$r_P = \frac{dP}{dt} = \rho \cdot X \tag{2.26}$$

$$\int_{P_0}^{P} dP = \int_{t_0}^{t} \rho \cdot X \cdot dt \tag{2.27}$$

$$P = \rho \int_{t_0}^{t} X \cdot dt + P_0 \tag{2.28}$$

Using the equation (2.28), if plot P versus $\int_{t_0}^{t} X \cdot dt$, it can obtain the specific production rate as the slope of the plot.

2.4 Batch fermentation

Batch fermentation is a simplest type of fermenter operation. In this mode, the fermenter is filled with medium when started and the fermentation is allowed to proceed but no fresh medium is added to the culture during the fermentation. When cells are grown in a batch fermenter, the cell growth cycle can be divided into a number of distinct phases (see Figure 2.2):

- The lag phase is a period of adaptation to growth in a new environment, and involves the synthesis of required enzymes for growth in this environment. The length of the lag phase is variable and depends on the previous growth history of the cells.
- The exponential or log phase is a period of balanced or steady state growth during which the synthesis of all cell constituents increases at a constant rate so that the cell population doubles, and continues to double at regular intervals.
- The stationary phase occurs when all the cells have stopped dividing or when viable cells have reached equilibrium with dead cells, that is, with the rate of death. The cells remain viable for long periods in this phase with endogenous

metabolism. The population usually enters the stationary phase as the result of the depletion of some essential nutrient, the formation of toxic products, or a change in the physical environment.

- The death phase occurs when cell growth has either stopped or is so slow that the rate of cell death exceeds the rate of cell growth.

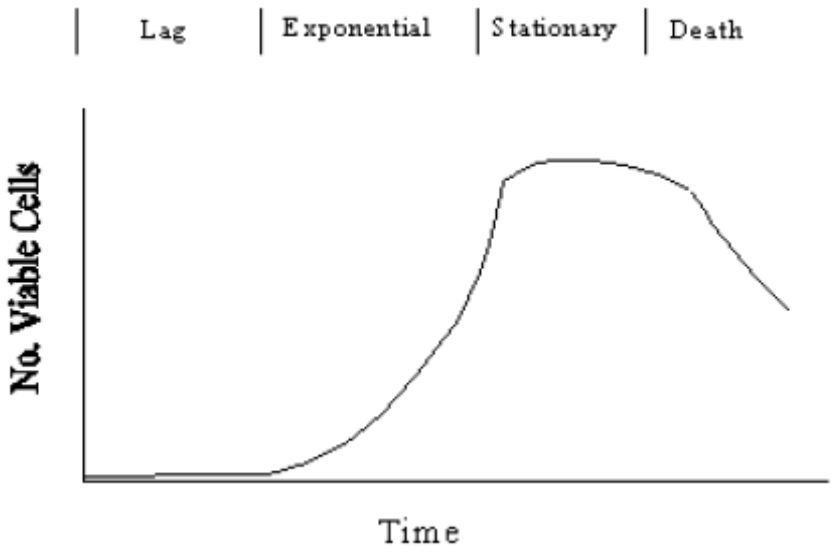


Figure 2.2 Cell growth cycle in batch fermentation (http://www.rocw.raifoundation.org/biotechnology/ BTechbiotech/bio-process-engg/lecture-notes/lecture-12.pdf)

There is no substrate feed or product withdrawal from the process in batch fermentation, then the mass balance equations can be written as the following:

Cell balance:

Cell accumulation rate = Cell growth rate - Cell death rate

$$\frac{dX}{dt} = r_X - r_d = \mu X - K_d X \tag{2.29}$$

Substrate balance:

Substrate accumulation rate = Substrate consumption rate

$$\frac{dS}{dt} = -r_S = -\frac{\mu X}{Y_{X/S}} - M_C X - \sum \frac{r_{Pi}}{Y_{Pi/S}} \tag{2.30}$$

Product balance:

Product accumulation rate = Product formation rate

$$\frac{dP}{dt} = r_P = \alpha(\mu X) + \beta X \qquad (2.31)$$

2.5 Fed-batch fermentation

Fed-batch fermentation is a production technique in between batch and continuous fermentation. The main advantages of the fed-batch fermentation are the possibilities to control both reaction rate and metabolic reactions by substrate feeding rate. The fermentation is at first started as a batch process with a small amount of biomass and substrate in the fermenter. The substrate feed is started when most of the initial added substrate has been consumed and is added during the fermentation, but no culture is removed until the end of the fermentation (see Figure 2.3). The inlet substrate feed should be as concentrated as possible to minimize dilution and to avoid process limitation caused by the reactor size. The culture volume in fed-batch fermentation varies with the fermentation time due to the substrate feed and no removing of culture during the fermentation. This type of fermentation enables the maintaining of a low substrate concentration during fermentation in order to avoid substrate inhibition and extend a working time, which is necessary for achieving a high product formation rate.

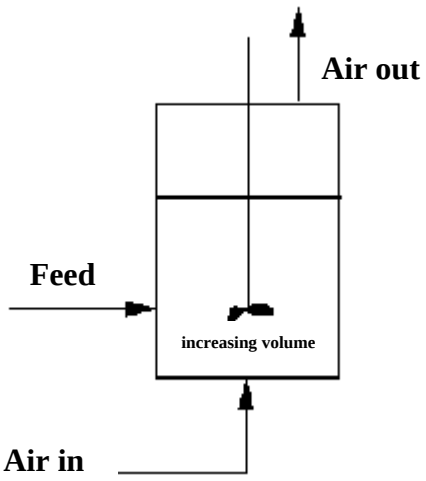


Figure 2.3 Fed-batch operation mode (www.gl.umbc.edu/~gferre1/ fermentor.gif)

In fed-batch fermentation, the culture volume increases with time. Then, the mass balance equations can be written as the following:

Cell balance:

Cell accumulation rate = - Feed in rate + Cell growth rate - Cell death rate

$$\frac{dX}{dt} = -\frac{F}{V}X + r_X - r_d \tag{2.32}$$

Substrate balance:

Substrate accumulation rate = Feed in rate - Substrate consumption rate

$$\frac{dS}{dt} = \frac{F}{V}(S_f - S) - r_S \tag{2.33}$$

Product balance:

Product accumulation rate = - Feed in rate + Product formation rate

$$\frac{dP}{dt} = -\frac{F}{V}P + r_P \tag{2.34}$$

The feeding strategy of fed-batch fermentation can be written as follows:

Constant feeding:

$$F = \frac{\upsilon X_0 V_0}{S_f} \tag{2.35}$$

Increased feeding:

$$F = at + b \quad \text{where a, b = constant} \tag{2.36}$$

Exponential feeding:

$$F = \frac{\upsilon X_0 V_0 e^{\mu t}}{(S_f - S)} \tag{2.37}$$

2.5.1 Feeding strategy in fed-batch fermentation can be divided as follows:

1. Without feedback control

1.1 Constant feeding

Feeding nutrient at a constant rate. The specific growth rate continuously decreases.

1.2 Increased feeding

Feeding nutrient at an increasing rate (gradual, stepwise or linear). The decrease in specific growth rate can be compensated.

1.3 Exponential feeding

Feeding nutrient at an exponential rate. Constant specific growth rate can be achieved.

2. With feedback control

2.1 Indirect feedback control

- DO-stat

Feeding nutrient when there is a rise in the concentration of dissolved oxygen (DO), which results from depletion of the substrate.

- pH-stat

Feeding nutrient when there is a rise in pH as a result of depletion of the principal carbon source.

- Cell concentration

The nutrient feeding rate is determined from the cell concentration, which is measured on-line using a laser turbidimeter.

- Carbon dioxide evolution rate (CER)

  This is an estimated on-line using a mass spectrometer to control nutrient feeding. The CER is proportional to the consumption rate of the carbon source. This method is most frequently used to control the specific growth rate.

2.2 Direct feedback control

- Substrate concentration control

  Nutrient feeding is directly controlled by the concentration of the principal carbon source (e.g. an on-line glucose analyzer is used to control glucose concentration in the fermenter).

2.6 Scale-up fermentation

Because the fermentation process are concerned with the complex interactions of biological, chemical and physical factors, therefore, there are a number of steps involved in studying the fermentation process. To properly investigate the fermentation and to be able to predict the effects that those factors play on fermentation, the process will initially be studied in laboratories using shake-flasks and laboratory scale fermenters. If the process looks commercially successful, the process will be scaled-up to a pilot scale process. The pilot scale studies will determine the commercial viability of the process and if successful the process will be scaled up to industrial or commercial scale (see Figure 2.4).

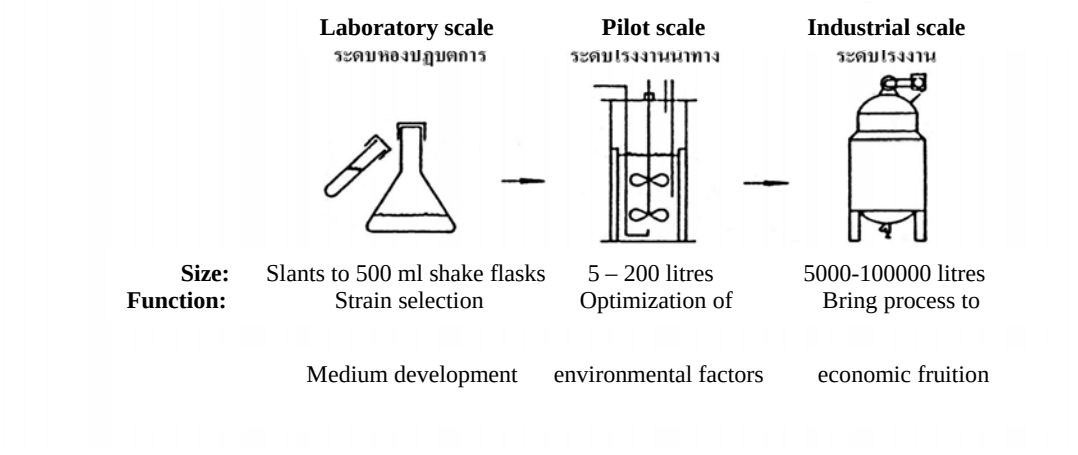


Figure 2.4 Scale-up process from laboratory scale to industrial scale (Wang et al., 1979)

Generally, the scale-up criteria that are used in the fermentation process are collected as follows:

- Constant power input per unit volume (P/V) implies constant gas transfer rate.
- Constant gas flow rate per unit liquid volume (Q/V) implies constant liquid circulation rate inside the fermenter.
- Constant impeller tip speed (ND_i) implies constant shear.
- Constant impeller rotation speed (N) implies constant mixing time.
- Constant Reynolds number ($\frac{\rho N D_i^2}{\mu}$) implies geometrically similar flow patterns.

However, the application of the above scale-up criteria is frequently up to the designer to rely on judgment and experience in the scale-up process.

2.6.1 Scale-up based on constant power input per unit volume

For the manipulation of an agitated system, an important factor is the required power to drive the impeller. Since the power required for a given system cannot be predicted theoretically, empirical correlations have been developed to predict the power required. The flow patterns can be correlated with the impeller Reynolds number (Re), defined as:

$$Re = \frac{\rho N D_i^2}{\mu} \tag{2.38}$$

The flow is laminar in the fermenter for Re < 10, turbulent for Re > 10^4, and for a range between 10 and 10^4, the flow is transitional, being turbulent at the impeller and laminar in remote parts of the fermenter.

Nongassed power input (P) is related to fluid density (ρ), fluid viscosity (μ), impeller rotation speed (N), and impeller diameter (D_i) by plots of power number (N_p) versus Re (see Figure 2.5). The power number is

$$N_p = \frac{P}{\rho N^3 D_i^5} \tag{2.39}$$

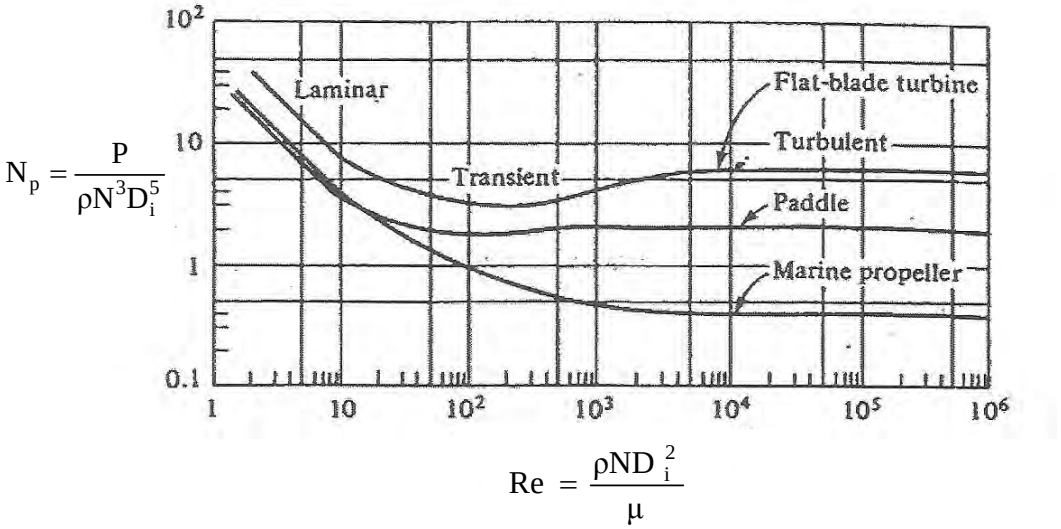


Figure 2.5 Power correlation for various impeller (Rushton et al., 1950)

Upon gassing the power input requirements can drop by as much as 60-65% of the nongassed power input demand. Therefore, gassed power input (P_g) is calculated from the graph that was plotted between ratio of gassed power input and nongassed power input (P_g/P) versus aeration number (N_a) (see Figure 2.6). The aeration number is

$$N_a = \frac{Q}{N D_i^3} \tag{2.40}$$

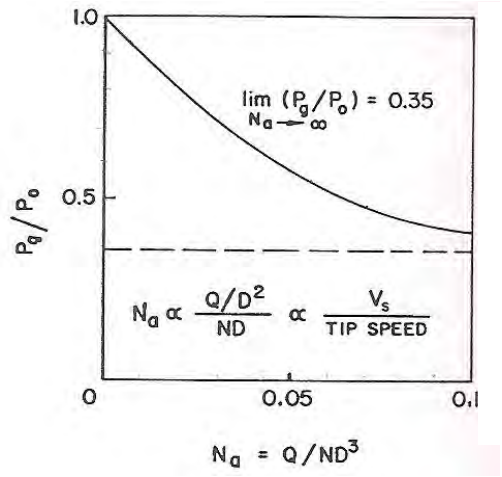


Figure 2.6 Effect of gassing rate on agitated power input (Ohyama and Endoh, 1955)

2.6.2 Scale-up based on constant impeller rotation speed

In order to operate the fermentation process with constant mixing time as a scale-up criteria, it need to control the larger vessel as the same impeller rotation speed as in the small vessel. Figure 2.7 shows a correlation of mixing time for a turbine agitator. The dimensionless mixing factor (f_T) is defined as

$$f_t = t_M \frac{\left(ND_i^2\right)^{2/3} g^{1/6} D_i^{1/2}}{H^{1/2} D_t^{3/2}} \tag{2.41}$$

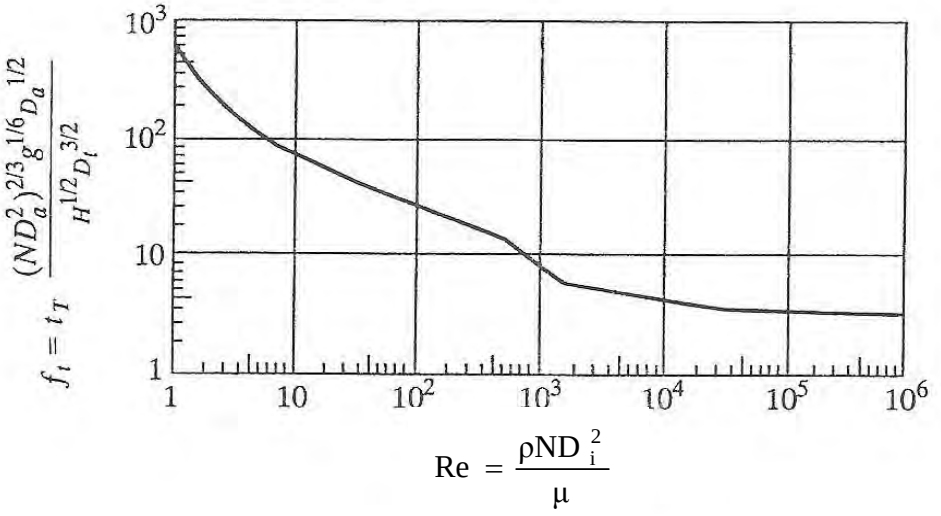


Figure 2.7 Correlation of mixing time for miscible liquids (Norwood and Metzner, 1960)

For Re > 1000, since f_t is approximately constant, then $t_M N^{2/3}$ is constant. For scale-up from vessel 1 to another size vessel 2 with geometric and kinematic similarity, the mixing times are related by

$$\frac{t_{M1}}{t_{M2}} = \frac{N_2}{N_1} \tag{2.42}$$

2.6.3 Scale-up based on constant impeller tip speed

In the cultivation of sensitive cells, one factor to concern is the shear rate that can affect cells. For scale-up the larger vessel, in order to control shear rate at the same value as in the small vessel, it need to calculate the absolute value of the impeller tip speed. The impeller tip speed is

$$v_{tip} = 2\pi ND_i \tag{2.43}$$

2.7 Optimization problem (Genetic algorithm)

The genetic algorithm is a method for solving both constrained and unconstrained optimization problems that is based on natural selection. The genetic algorithm repeatedly modifies a population of individual solutions. At each step, the genetic algorithm selects individuals at random from the current population to be parents and uses them to produce the children for the next generation. The genetic algorithm usually selects individuals that have better fitness values as parents. The genetic algorithm can apply to solve a variety of optimization problems that are not well suited for standard optimization algorithms, including problems in which the objective function is discontinuous, nondifferentiable, or highly nonlinear.

The genetic algorithm uses three main types of rules at each step to create the next generation from the current population:

- Selection rules select the individuals in the current generation with the best fitness values as parents. These individuals automatically survive to the next generation, called elite children.

- Crossover rules combine the vectors of a pair of parents in the current population to form children for the next generation, called crossover children.

- Mutation rules apply random changes the genes of individual parents to form children for the next generation, called mutation children.

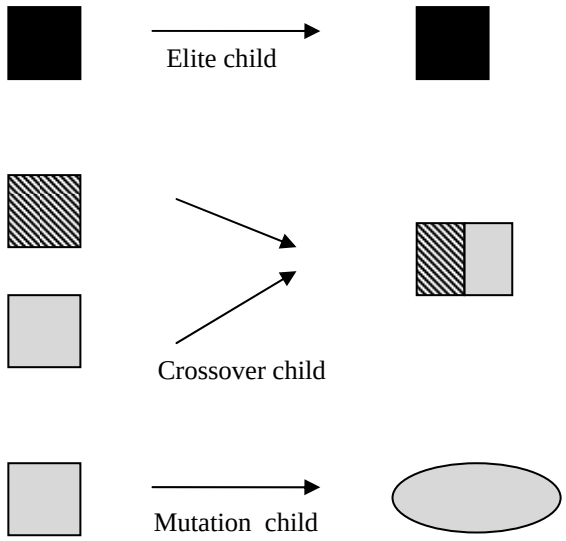


Figure 2.8 The schematic diagram illustrates the three types of children

The following outline summarizes how the genetic algorithm works:

1. The algorithm begins by creating a random initial population.

2. The algorithm then creates a sequence of new populations. At each step, the algorithm uses the individuals in the current generation to create the next population. To create the new population, the algorithm performs the following steps:

- Scores each member of the current population by computing its fitness value.

- Scales the raw fitness scores to convert them into a more usable range of values.

- Selects members, called parents, based on their fitness.

- Some of the individuals in the current population that have higher fitness are chosen as elite. These elite individuals are passed to the next population.

- Produces children from the parents. Children are produced either by making random changes to a single parent, or by combining the vector entries of a pair of parents.

- Replaces the current population with the children to form the next generation.

3. The algorithm stops when one of the stopping criteria is met.

The objective function of mathematical model for optimize the parameters is the minimum sum square error between the experimental values and the optimization values. It can be written as following:

$$\min J = \sum_{i=1}^{n} \left(y_{experiment} - y_{estimate} \right)^2 \tag{2.44}$$

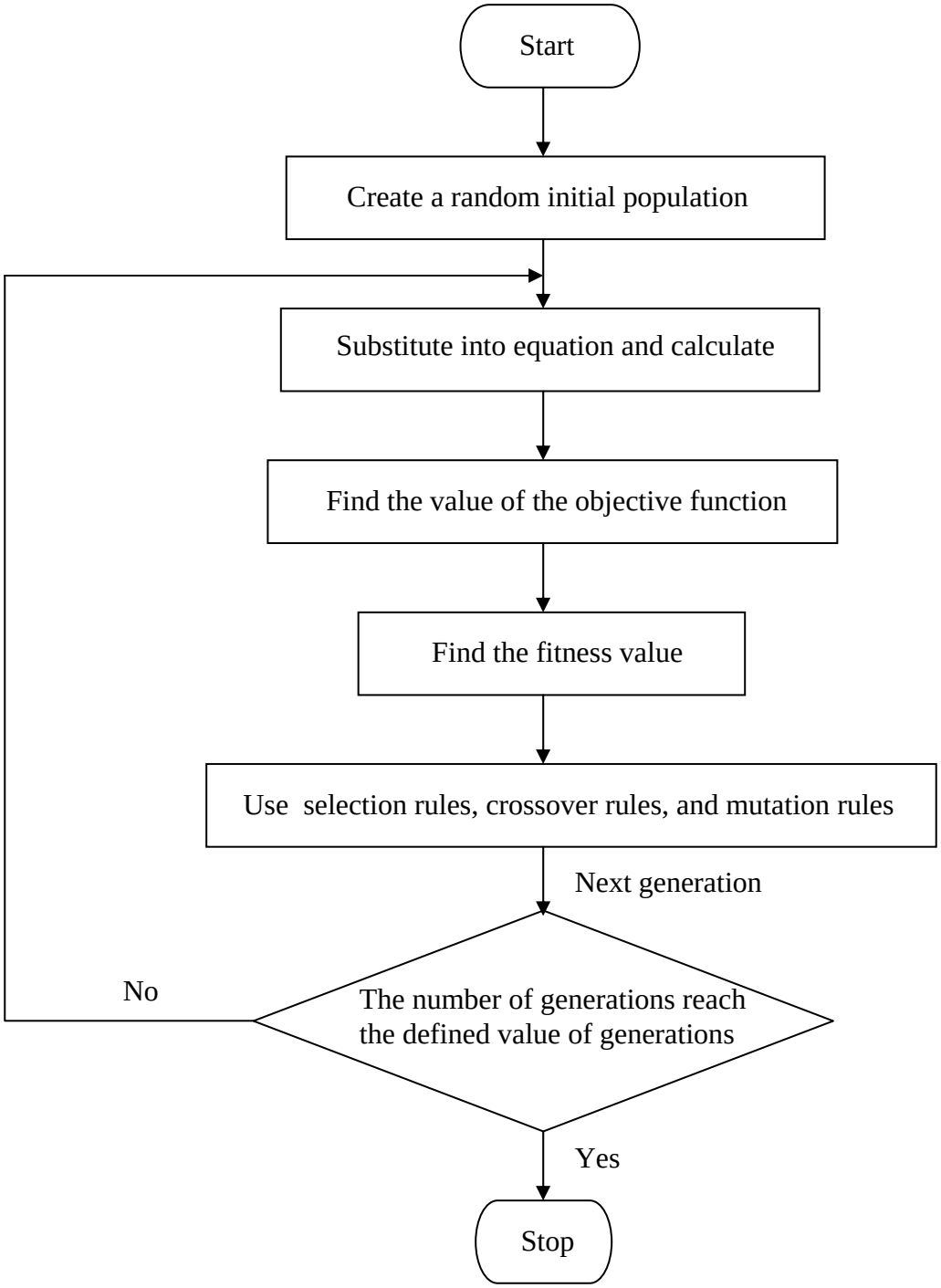


Figure 2.9 The genetic algorithm works

CHAPTER III

LITERATURE REVIEW

The study of kinetic model for 1,3-propanediol production from glycerol fermentation in fed-batch fermenter requires many aspects of information regarding microorganism strain, fermentation process and productivity for 1,3-propanediol production, key parameters affecting 1,3-propanediol production by *Clostridium butyricum* DSM 5431 and including the study of kinetic model.

3.1 Microorganism strain, fermentation process and productivity

Nowadays, fermentation of glycerol has been a matter of increasing interest since the product, 1,3-propanediol, is a high performance polyester such as polytrimethylene terephthalate (PTT). In addition, 1,3-propanediol has been necessarily used as a chemical intermediate in the productions of polyester, polyether, and polyurethane. Polytrimethylene terephthalate (PTT) produced from 1,3-propanediol has served as a raw material for fibers and textile industries because of its excellent physical properties.

Many microorganisms are capable of anaerobic metabolism using glycerol as a sole substrate to produce 1,3-propanediol. They include enterobacteria of the genera *Klebsiella* (*K. pneumoniae), Enterobacter* (*E. agglomerans*) and *Citrobacter* (*C. freundii*), *Lactobacillus* (*L. brevis and L. buchneri*) and clostridia of *C. butyricum* and *C. pasteurianum* (SchuÈtz et al., 1984; Forsberg, 1987; Homann et al., 1990; Dabrock et al., 1992; Biebl et al., 1992; Barbirato et al., 1995). However, in view of the high fermentation yield and productivity, *Clostridium butyricum* DSM 5431 and *Klebsiella pneumoniae* DSM 2026 are widely used in the production of 1,3-propanediol from glycerol.

There are many articles available about the glycerol fermentation for 1,3-propanediol production in batch, fed-batch, and continuous fermentations (Gunzel et al., 1991; Biebl et al., 1991; Zeng et al., 1994; Deckwer, 1995; Barbirato et al., 1995; Reimann et al., 1996; Menzel et al., 1997; Reimann et al., 1998; Biebl et al.,

1999; Papanikolaou et al., 2000). Different microorganisms produce 1,3-propanediol at varied rates in different types of fermentation (see Table 3.1). In batch and fed-batch fermentations, a maximum 1,3-propanediol concentration of about 50-60 g/l was achieved for both *Clostridium butyricum* and *Klebsiella pneumoniae* (Gunzel et al., 1991; Deckwer, 1995; Reimann et al., 1996; Chen et al., 2003). In continuous fermentation, *Clostridium butyricum* achieved so far only half of the maximum 1,3-propanediol concentration obtained with *Klebsiella pneumoniae* (Reimann et al., 1998; Menzel et al., 1997). However, substrate and product inhibition are the main limiting factors for the microbial production of 1,3-propanediol. Product inhibition can be decreased by selection of more product tolerant strains. Such mutants of *Clostridium butyricum* DSM 5431 have been developed (Abbad-Andaloussi et al., 1995). Substrate inhibition can be decreased by an appropriate feeding strategy which keeps the concentration of critical substrates at a low, but not limiting level. One possibility to decrease substrate inhibition is the fermentation in continuous culture, another is fed-batch fermentation.

In fed-batch fermentation, to achieve glycerol consumption in quantities up to 100 g/l, Günzel et al. (1991) applied an intermittent substrate addition during fermentation of *Clostridium butyricum* DSM 5431 at pH 7.0, 35 °C by starting with initial glycerol concentration of 20 g/l and feeding glycerol into the reactor in amount a concentration of 20 g/l. This was done 4 times giving a final 1,3-propanediol concentration of 50 g/l at productivity of 2.70 g/l·hr. As well as Abbad-andaloussi et al. (1995) who used an intermittent substrate addition during glycerol fermentation of *Clostridium butyricum* DSM 5431 at pH 7.0, 32 °C by starting with initial glycerol concentration of 62 g/l and feeding glycerol into the reactor in amount a concentration of 20 g/l. This was done 5 times giving a final 1,3-propanediol concentration of 44.5 g/l at productivity of 0.66 g/l·hr. While Reimann et al. (1996) developed a continuous substrate feeding for 1,3-propanediol production by *Clostridium butyricum* DSM 5431 at pH 7.0, 35 °C in which the glycerol feeding (the glycerol concentration was about 60 g/l) was controlled by the alkali consumption. The fermentation was started with the initial glycerol concentration of 20 g/l and the substrate feeding was started as soon as three quarters of the initial substrate was used up. The final 1,3-propanediol concentration that was obtained from this study was 47.5 g/l and productivity of

2.40 g/l·hr. Moreover, Deckwer (1995) studied the fed-batch fermentation at pH 7.0, 35 °C and controlled an appropriate feeding from the pH set point. The fermentation was started with the initial glycerol concentration of 20 g/l and giving a final 1,3-propanediol concentration of 56.0 g/l at productivity of 2.20 g/l·hr.

Table 3.1 Production of 1,3-propanediol by different strains of bacteria and different types of fermentation

Microorganism	1,3-propanediol concentration (g/l)	Yield (mol/mol)	Productivity (g/l·hr)	Fermentation type	Reference
Clostridium butyricum DSM 5431	25.0	0.70	2.20	Batch	Günzel et al. (1991)
Clostridium butyricum DSM 5431	24.9	0.62	-	Batch	Petitdemange et al. (1995)
Clostridium butyricum DSM 5431	13.8	0.78	1.15	Batch	Biebl et al. (1995)
Clostridium butyricum DSM 5431	31.2	0.61	0.98	Batch	Abbad-andaloussi et al. (1995)
Clostridium butyricum DSM 5431	41.4	0.76	1.30	Batch	Pullsirisombat et al. (2007)
Clostridium butyricum DSM 5431	50.0	0.61	2.70	Fed-batch	Gunzel et al. (1991)
Clostridium butyricum DSM 5431	56.0	0.62	2.20	Fed-batch	Deckwer (1995)
Clostridium butyricum DSM 5431	44.5	0.62	0.66	Fed-batch	Abbad-andaloussi et al. (1995)
Clostridium butyricum DSM 5431	47.5	0.62	2.40	Fed-batch	Reimann et al. (1996)
Clostridium butyricum DSM 5431	26.6	0.58	13.30	Continuous	Reimann et al. (1998)

Table 3.1 (continue) Production of 1,3-propanediol by different strains of bacteria and different types of fermentation

Microorganism	1,3-propanediol concentration (g/l)	Yield (mol/mol)	Productivity (g/l·hr)	Fermentation type	Reference
Klebsiella pneumoniae DSM2026	43.2	0.54	2.30	Batch	Tag (1990)
Klebsiella pneumoniae DSM2026	22.3	0.53	1.68	Batch	Homann et al. (1990)
Klebsiella pneumoniae DSM2026	56.0	0.45	2.30	Fed-batch	Deckwer (1995)
Klebsiella pneumoniae DSM2026	59.5	0.54	1.57	Fed-batch	Chen et al. (2003)
Klebsiella pneumoniae DSM2026	48.5	0.63	4.90	Continuous	Menzel et al. (1997)
Clostridium butyricum CNCM 1211	63.7	0.64	-	Batch	Colin et al. (2000)
Clostridium butyricum CNCM 1211	52.9	0.64	1.89	Batch	Barbirato et al. (1998)
Clostridium butyricum CNCM 1211	65.4	0.66	1.67	Batch	Himmi et al. (1999)
Clostridium butyricum F2b	26.1	0.69	5.50	Continuous	Papanikolaou et al. (2000)
Clostridium butyricum VPI 3266	29.7	0.62	2.98	Continuous	Gonzalez-Pajuelo et al. (2004)

Table 3.1 (continue) Production of 1,3-propanediol by different strains of bacteria and different types of fermentation

Microorganism	1,3-propanediol concentration (g/l)	Yield (mol/mol)	Productivity (g/l·hr)	Fermentation type	Reference
Citrobacter freundii K2	26.0	0.64	1.37	Batch	Homann et al. (1990)
Enterobacter agglomerans CNCM 1210	25.9	0.56	0.99	Batch	Barbirato et al. (1998)

3.2 Key parameters affecting 1,3-propanediol production by *Clostridium butyricum* DSM 5431

Key affecting parameters for the production of 1,3-propanediol under anaerobic processes are concentrations of substrate and products, pH, and temperature.

3.2.1 Influences of substrate and product concentrations

Substrate and product inhibitions can affect the growth of microorganisms and productivity of 1,3-propanediol. Therefore, it is necessary to measure the levels of substrate and product concentrations that inhibit the growth of microorganisms. Products of this fermentation include 1,3-propanediol which is the main product and by-products such as butyric acid and acetic acid.

3.2.1.1 Influence of substrate

Glycerol is the substrate for 1,3-propanediol production by microorganism. Deckwer (1995) found that *Klebsiella pneumoniae* DSM 2026 is more glycerol tolerant than *Clostridium butyricum* DSM 5431 and the initial glycerol concentration should not be higher than about 50 g/l in order to avoid substrate inhibition. However, Biebl (1991) measured the influence of glycerol of *Clostridium butyricum* DSM 5431 in pH-auxostat culture at pH 6.5, 32 °C and showed that the appreciable inhibition by glycerol was found only above a concentration of 80 g/l (97 g/l in the culture corresponding to 122 g/l in the feed, growth and medium request ceased entirely). Furthermore, Biebl (1991) reported that the effect of glycerol inhibition of *Clostridium butyricum* DSM 5431 increased the cultivation time and reduce 1,3-propanediol productivity while the final product concentrations did not differ. This result is in accordance with the finding of Gunzel et al. (1991) who reported that growth of *Clostridium butyricum* DSM 5431 was inhibited with increasing concentration of glycerol. In addition, Gunzel et al. (1991) suggested that cultivations should be carried out in fed-batch operation if glycerol consumption in quantities up to 100 g/l is required. Recently, Pullsirisombat (2007) has studied the optimal glycerol concentration using *Clostridium butyricum* DSM 5431 in batch fermentation at pH 7.0

and 33 °C with initial glycerol concentration in range 40-130 g/l and reported that the substrate inhibition occurred at glycerol concentration 100 to 130 g/l.

3.2.1.2 Influence of product and by-products

Biebl (1991) studied growth inhibition of *Clostridium butyricum* DSM 5431 with respect to 1,3-propanediol, butyric acid, and acetic acid concentrations by adding 1,3-propanediol, butyric acid, or acetic acid separately to a culture that grew near its maximal specific rate in pH-auxostat culture at pH 6.5 and 32 °C. The experimental result showed that 1,3-propanediol was the least toxic product compared to the other substance that were added into the culture because *Clostridium butyricum* DSM 5431 could tolerate 1,3-propanediol concentration up to 60 g/l. While butyric acid and acetic acid caused stronger inhibition than 1,3-propanediol because total growth inhibition occurred when 19 g/l of butyric acid concentration or 27 g/l of acetic acid concentration were added to the culture.

3.2.2 Influence of fermentation conditions

3.2.2.1 Influence of pH

An essential regulatory parameter on cell growth and product formation is the pH. Therefore, it is necessary to examine the optimal pH for 1,3-propanediol production by microorganisms. Gunzel et al. (1991) studied the influence of pH in the range of pH 6.0-8.0 in batch fermentation with initial glycerol concentration 20 g/l by *Clostridium butyricum* DSM 5431 and discovered the range of pH 6.6-7.0 that leads to high 1,3-propanediol concentration and productivity. However, in our research, pH 7.0 was used for 1,3-propanediol production from glycerol fermentation by *Clostridium butyricum* DSM 5431 as reported in many researches (Deckwer, 1995; Abbad-andaloussi et al., 1995; Petitdemange et al., 1995; Reimann et al., 1996; Reimann et al., 1998; Pullsirisombat, 2007).

3.2.2.2 Influence of temperature

Temperature is the necessary parameter on cell growth and product formation as well as the pH. Gunzel et al. (1991) studied the influence of temperature in the range of 30-37 °C from batch fermentation with initial glycerol concentration 50 g/l by *Clostridium butyricum* DSM 5431 and found that the highest 1,3-propanediol productivity was obtained at 35 °C. However, various temperatures such as 32 °C (Biebl, 1991; Abbad-andaloussi et al., 1995; Petitdemange et al., 1995), 33 °C (Biebl et al.,1995; Pullsirisombat, 2007), and 35 °C (Gunzel et al., 1991; Deckwer, 1995; Reimann et al., 1996; Reimann et al., 1998) were used for 1,3-propanediol production from glycerol fermentation by *Clostridium butyricum* DSM 5431.

3.3 Previous work in our laboratory

In our laboratory, Pullsirisombat et al. (2007) investigated the effect of substrate inhibition on cell growth and 1,3-propanediol production by varying the initial glycerol concentrations from 40-130 g/l in shake flask at 33 °C, and pH 7.0 under anaerobic condition and found that the optimal initial glycerol concentration was 80 g/l, whereas the substrate inhibition was apparent from 100 to 130 g/l. Furthermore, Pullsirisombat et al. (2007) studied the 1,3-propanediol production in a fermenter with 80 g/l initial glycerol concentration and reported that glycerol was used up in 33 hours and converted to 1,3-propanediol at maximum concentration of 41.37 g/l, a yield of 0.76 mol 1,3-propanediol/mol glycerol and productivity of 1.3 g/l·hr.

3.4 Kinetic model

The kinetic model that was proposed in all previous researches (Zeng et al., 1994; Zeng, 1996; Menzel et al., 1997; Xiu et al., 2004; Gao et al., 2005; Gao et al., 2006; Shen et al., 2007; Wang et al., 2007) to describe cell growth is collected in Table 3.2. However, its can be written in one form as the following:

$$\mu = \mu_{max}\left(\frac{C_S}{C_S + K_S}\right)\left[\Pi\left(1-\left(\frac{C_{Pi}}{C^*_{Pi}}\right)\right)\right] \quad (3.1)$$

3.4.1 The critical concentrations of related substances

The critical concentration is the concentration of substance that inhibits the growth of microorganisms. Its can be estimated from the kinetic model. The previous researches about these studies are collected as follows:

Zeng et al. (1994) studied the growth inhibition of 1,3-propanediol production by *Clostridium butyricum* DSM 5431 in chemostat cultures without addition of inhibitor with the same medium as that of Biebl (1991) and similar culture conditions (pH 7.0, 35 °C) by Gunzel et al. (1991) and calculated the critical concentrations of all substances by using a Nelder-Mead optimization algorithm included in the software Microsoft Excel. The critical concentrations were 181.7 g/l for glycerol, 66.4 g/l for 1,3-propanediol, 21.8 g/l for acetic acid and 11.1 g/l for butyric acid. The results from this study were compared with the experimental results of Biebl (1991). The level of 1,3-propanediol concentration that inhibited the growth of *Clostridium butyricum* DSM 5431 closed to that found from Biebl (1991) 's result while a much lower values for butyric acid and acetic acid were obtained. Butyric acid produced by the microorganisms appeared to have an inhibitory potential 2 to 3 times more than butyric acid added externally. The same seem to apply for acetic acid. In addition, Zeng et al. (1994) modified a growth model which was applicable to *Clostridium butyricum* DSM 5431 and *Klebsiella pneumoniae* DSM 2026 grown under a variety of conditions. The estimated critical concentrations from this model were 0.35 g/l for undissociated acetic acid, 10.1 g/l for total butyric acid, 16.6 g/l for ethanol, 71.4 g/l for 1,3-propanediol and 187.6 g/l for glycerol. For *Klebsiella pneumoniae* DSM 2026, Menzel et al. (1997) estimated the critical concentrations of all substances in continuous culture by using culture medium compositions and cultivation conditions (pH 7.0 and 37 °C) with the same as that of Solomon et al. (1994) and (Zeng et al. (1993). The reported critical concentrations were 46.8 g/l for acetic acid (0.26 g/l for undissociated acetic acid), 18.3 g/l for ethanol, 68.2 g/l for 1,3-propanediol and 185.1 g/l for glycerol.

3.4.2 Optimum operating conditions

Xiu et al. (2004) optimized the conditions of batch and continuous fermentations by *Klebsiella pneumoniae* DSM 2026 on the basis of highest volumetric productivity of 1,3-propanediol. Their mathematical model was based on growth kinetics of multiple inhibitions and the metabolic overflow of substrate consumption and product formation. They found the optimal initial glycerol concentration to be 88.3 g/l with a given inoculation of 0.1 g biomass/l for the batch culture leading to the highest volumetric productivity (about 4 g/l·hr) of 1,3-propanediol. For continuous cultures, the optimal dilution rate and initial glycerol concentration in feed were 0.29 hr^{-1} and 67.3 g/l, respectively. The productivity was 8.66 g/l·hr which was more than twice the productivity of an optimal batch culture. They proposed two-stage continuous process in which the first stage was operated at the optimal conditions and the second one was used to consume the residual glycerol in the first one. A comparison between computational and experimental results of the concentration, yield and productivity of 1,3-propanediol showed that the estimated values were higher than the most of continuous fermentation result.

3.4.3 Nonlinear dynamical system

A comparison between experimental data and computational result of the concentration, yield and productivity of 1,3-propanediol showed that most of continuous fermentation data were lower than or approached to the calculated values in the previous works, for example, the errors of substrate ’s values reach 50% in the study of Zeng (1994). Therefore, Gao et al. (2005) constructed a parameter identification model for the system in continuous glycerol fermentations by *Klebsiella pneumoniae* to decrease the errors.

In fed-batch fermentation by *Klebsiella pneumoniae*, the process of adding glycerol and alkali is not continuous, so the process characterized by continuous models is not fit for the actual process. Therefore, Gao et al. (2006) studied a nonlinear impulsive differential equation of the fed-batch glycerol fermentation process and did some theoretical and numerical analysis. Moreover, Wang et al. (2007) applied the impulsive differential equations to the fed-batch fermentation and established a parameter identification model for the impulsive system by introducing *Powell* direct search algorithm into genetic algorithm as a operator like crossover and

mutation operator. And because of most researches considered the fermentation process as only growth period, which neglected the distinctive impact of cells during different phases such as lag phase, exponential phase and stationary phase. To take the different performance, dynamic behavior of these three phases and the sudden jump of substrate into consideration, Shen et al. (2007) proposed the multi-stage system and established the bilevel system to identify its sensitive parameters during different phases. The properties of the solutions for the nonlinear multi-stage dynamical system were investigated and identifiability of the parameters was proved. In addition, in this study, an optimal algorithm was constructed to obtain the optimal solution of the identification model.

Table 3.2 Growth models under different conditions

Microorganism	Model	Reference
Clostridium butyricum DSM 5431	$\mu = \mu_{max}\left(\frac{C_{Gly}}{C_{Gly}+K_S}\right)\left(1-\frac{C_{HAc}}{C^*_{HAc}}\right)\left(1-\frac{C_{HBu}}{C^*_{HBu}}\right)\left(1-\frac{C_{PD}}{C^*_{PD}}\right)\left(1-\frac{C_{Gly}}{C^*_{Gly}}\right)$	Zeng et al. (1994)
Clostridium butyricum DSM 5431 *Klebsiella pneumoniae* DSM 2026	$\mu = \frac{\mu^*_{max}}{1+\frac{H^+}{K_H}+\frac{K_{OH}}{H^+}}\left(\frac{C_{Gly}}{C_{Gly}+K_S}\right)\left(1-\frac{C_{HAc}}{C^*_{HAc}}\right)\left(1-\frac{C_{HBu}}{C^*_{HBu}}\right)\left(1-\frac{C_{PD}}{C^*_{PD}}\right)\left(1-\frac{C_{Gly}}{C^*_{Gly}}\right)\left(1-\frac{C_{EtOH}}{C^*_{EtOH}}\right)$	Zeng et al. (1994)
Clostridium butyricum DSM 5431	$\mu = \mu_{max}\left(1-\frac{C_{HAc}}{C^*_{HAc}}\right)\left(1-\frac{C_{PD}}{C^*_{PD}}\right)$	Zeng (1996)
Klebsiella pneumoniae DSM 2026	$\mu = \mu_{max}\left(\frac{C_{Gly}}{C_{Gly}+K_S}\right)\left(1-\frac{C_{HAc}}{C^*_{HAc}}\right)\left(1-\frac{C_{EtOH}}{C^*_{EtOH}}\right)\left(1-\frac{C_{PD}}{C^*_{PD}}\right)\left(1-\frac{C_{Gly}}{C^*_{Gly}}\right)$ $\mu = \mu_{max}\left(1-\frac{C_{HAc}}{C^*_{HAc}}\right)\left(1-\frac{C_{PD}}{C^*_{PD}}\right)$	Menzel et al. (1997)

Table 3.2 (continue) Growth models under different conditions

Microorganism	Model	Reference
Klebsiella pneumoniae DSM 2026	$\mu = \mu_{max}\left(\frac{C_{Gly}}{C_{Gly}+K_S}\right)\left(1-\frac{C_{Gly}}{C^*_{Gly}}\right)\left(1-\frac{C_{PD}}{C^*_{PD}}\right)\left(1-\frac{C_{HAc}}{C^*_{HAc}}\right)\left(1-\frac{C_{EtOH}}{C^*_{EtOH}}\right)$	Xiu et al. (2004), Gao et al. (2005), Gao et al. (2006), Shen et al. (2007), Wang et al. (2007)

CHARTER IV

MATERIALS AND METHODS

4.1 Equipments and chemicals

4.1.1 Equipments

Laminar flow (VS-124, ISSCO , U.S.A.)

Centrifuger (Kubota 5100 apan, Kubota Corporation, Japan)

Vortex mixer (G-560E, Scientific Industries, Inc., U.S.A.)

Spectrophotometer (UV-2450, Shimadzu, Japan)

Autoclave (SS-325, TOMY, Japan)

Hot Air Oven (ULM 500, Memmert, Germany)

Fermenter 1 litre (Biostat Q® , B Braun Biotech International, Germany)

Fermenter 15 litre (Biostat C® , B Braun Biotech International, Germany)

pH Meter (MP220, Mettler Toledo, Switzerland)

4.1.2 Chemicals

$CaCl_2.2H_2O$ (APS Finechem, Australia[1])

$CaCO_3$ (Ajax Finechem, Australia[1])

$CoCl_2.2H_2O$ (APS Ajax Finechem, Australia[1])

$CuCl_2.2H_2O$ (Ajax Finechem, Australia[1])

$FeSO_4.7H_2O$ (APS, Australia[2])

H_3BO_3 (Merck, Germany[1])

HCl (Ajax Finechem, New Zealand)

HCl 37 % (J.T. Baker, Thailand)

K_2HPO_4 (Ajax Finechem, Australia[1])

KH_2PO_4 (Ajax Finechem, Australia[1])

$MnCl_2.4H_2O$ (Ajax laboratory chemicals, Australia[1])

$MgSO_4.7H_2O$ (APS, Australia[1])

$Na_2MoO_4.2H_2O$ (APS Ajax Finechem, Australia[1])

NaOH (Ajax Finechem, Australia[1])

$(NH_4)_2SO_4$ (APS, Australia[1])

$NiCl_2.6H_2O$ (Ajax Finechem, Australia[1])

$ZnCl_2$ (APS, Australia[1])

Acetic acid (J.T. Baker, U.S.A.[2])

Antifoam (Ajax Finechem, Australia)

Butyric acid (Laboratory Rasayan, India[2])

Ethanol (VWR, CE[1])

Glycerol (Fisher Scientific UK Limited, UK[1])

1,3-propanediol (Acros Organics, U.S.A.[2])

Reinforced Clostridial Medium (Himedia , India[2])

Yeast extract (Himedia, India[2])

Remark : [1]Analytical reagent

[2]Laboratory reagent

4.2 Microorganism

A bacterial strain of *Clostridium butyricum* DSM 5431 was used in this study. This strain was obtained from American Type Culture Collection (ATCC) BAA-557™.

4.3 Experimental procedures

4.3.1 Fermentation in a 1-L fermenter

4.3.1.1 Preparation of inoculum

Stock culture was stored at -20 °C in Reinforced Clostridial Medium (RCM) that was mixed with 10% glycerol with a ratio 1:1. For inoculum preparation, RCM medium was purged with nitrogen for 5 minutes before being sterilized in an autoclave for 15 minutes at 121°C. After sterilization, the medium was again purged with nitrogen for 5 minutes. Stock culture in amounts of 1 ml was transferred to 9 ml RCM medium in glass tube (10% inoculum by volume), heat shocked at 80°C for 10 min, and incubated at 33°C for 18 hours under anaerobic condition without agitation.

4.3.1.2 Cultivation in preculture medium

Preculture medium (the carbon source of the preculture was 20 g/l of pure glycerol) was purged with nitrogen for 5 minutes before being sterilized in an autoclave for 15 minutes at 121°C before use and was purged with nitrogen for 5 minutes again after sterilization. RCM medium in amounts of 2 ml was transferred to 18 ml preculture medium in glass tube (10% inoculum by volume) and incubated at 33°C for 20 hours under anaerobic condition without agitation.

4.3.1.3 Fermentation in culture medium

- Batch fermentaion

Batch cultures (the carbon source of the culture was pure glycerol as required 60-120 g/l) were carried out in a 1-L glass fermenter (see Figure 4.1) filled with 560 ml of culture medium and inoculated with 140 ml of preculture medium (20% inoculum by volume). In order to ascertain the anaerobiosis during the fermentation,

nitrogen gas, at a rate of 0.1 vvm, was infused into the culture medium. The agitation speed was controlled at 100 rpm and the pH was adjusted to 7.0 by automatic addition of 4 M NaOH and 1 M HCl. The incubation temperature was 33°C. Samples were taken every 3 hours during the fermentation time of 48 hours.

Figure 4.1 Fermenter 1 litre

4.3.2 Fermentation in a 15-L fermenter

4.3.2.1 Preparation of inoculum

Stock culture was stored at -20 °C in Reinforced Clostridial Medium (RCM) that was mixed with 10% glycerol with a ratio 1:1. For inoculum preparation, RCM medium was purged with nitrogen for 5 minutes before being sterilized in an autoclave for 15 minutes at 121°C. After sterilization, the medium was again purged with nitrogen for 5 minutes. Stock culture in amounts of 1 ml was transferred to 9 ml RCM medium in glass tube (10% inoculum by volume), heat shocked at 80°C for 10 min, and incubated at 33°C for 18 hours under anaerobic condition without agitation.

4.3.2.2 Cultivation in preculture medium

Preculture medium (the carbon source of the preculture was 20 g/l of pure glycerol) was purged with nitrogen for 5 minutes before being sterilized in an autoclave for 15 minutes at 121°C before use and was purged with nitrogen for 15 minutes again after sterilization. RCM medium in amounts of 30 ml was transferred to 270 ml preculture medium in shake flask (10% inoculum by volume) and incubated at 33°C for 20 hours under anaerobic condition without agitation.

4.3.2.3 Fermentation in culture medium

- Batch fermentaion

Batch culture (the carbon source of the culture was 80 g/l of pure glycerol) was carried out in a 15-L fermenter (see Figure 4.2) filled with 8400 ml of culture medium and inoculated with 2100 ml of preculture medium (20% inoculum by volume). In order to ascertain the anaerobiosis during the fermentation, nitrogen gas, at a rate of 0.1 vvm, was infused into the culture medium. The agitation speed was controlled at 100 rpm and the pH was adjusted to 7.0 by automatic addition of 4 M NaOH and 1 M HCl. The incubation temperature was 33°C. Samples were taken every 3 hours during the fermentation time of 48 hours.

- Fed-batch fermentation

Fermentation was initiated as batch fermentation which had a working volume of 7.5 litres (50% of total volume). Batch culture (the carbon source of the culture was 80 g/l of pure glycerol) was carried out in a 15-L fermenter filled with 6000 ml of culture medium and inoculated with 1500 ml of preculture medium (20% inoculum by volume). After 21 hr, it was converted to fed-batch fermentation by adding concentrated glycerol feed (the glycerol concentration was 120 g/l) at a constant feed rate of 0.353 l/hr. Feeding was stopped at 34 hr (time of full volume (12 litres, 80% of total volume)) and residual glycerol in the fermentation broth was allowed to ferment by batch fermentation. In order to ascertain the anaerobiosis during the fermentation, nitrogen gas, at a rate of 0.1 vvm, was infused into the culture medium. The agitation speed was controlled at 100 rpm and the pH was adjusted to 7.0 by automatic addition of 4 M NaOH and 1 M HCl. The incubation

temperature was 33°C. Samples were taken every 3 hours during the fermentation time of 60 hours.

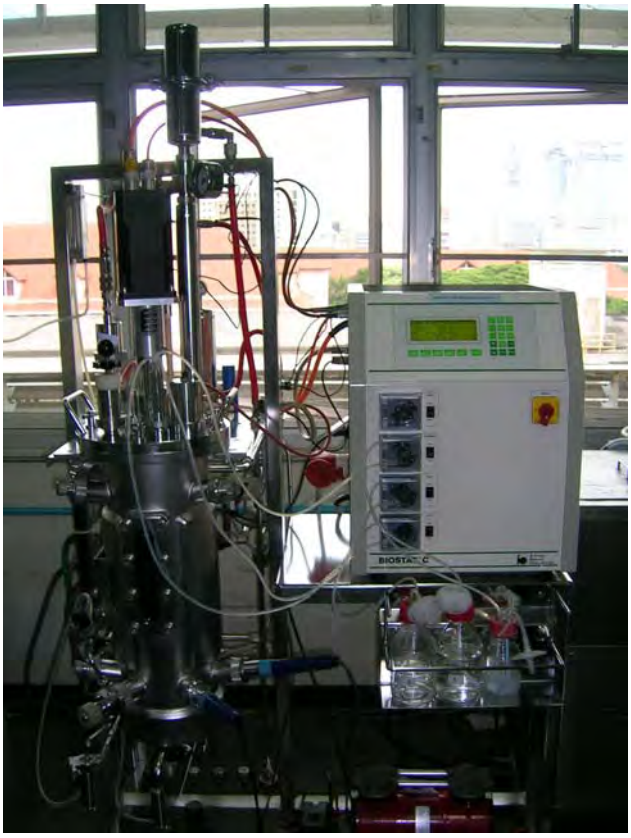


Figure 4.2 Fermenter 15 litre

4.3.3 Analytical methods

4.3.3.1 Estimation of the biomass concentration

Biomass concentration was estimated by measuring both optical density and cell dry weight. Optical density (OD) was measured at 650 nm with a spectrophotometer. In order to determine the dry weight of cells, 20 ml of samples were removed from the culture, centrifuged (2000 rpm, 15 min), and the supernatant was discarded. Cell pellets were resuspended in distillated water, centrifuged again and washed with distilled water. Drying of samples were performed at 80°C for 24 hours or to until a constant weight was obtained, and then weighed. In this study, the cell dry weights were weighed from only one weight. The biomass concentrations

were estimated from the average of three measurement of OD which had coefficient of variation $\left(\frac{SD}{\bar{X}} \times 100\%\right)$ of 4.45%.

4.3.3.2 Estimation of the density

In order to measure the density of culture medium, 10 ml of samples were removed from the culture, weighed, and then recorded. The density was calculated from the following equation:

$$\text{density(g/l)} = \frac{\text{weight of culture medium (g)} \times 10^3}{\text{volume of culture medium(l)}}$$

4.3.3.3 Measurement of the viscosity

A U-tube viscometer (size 150, CANNON, USA) which was conformed specification to ASTM D 446 and ISO 3105 was used in this study. The viscometer constant was 0.035 (cSt/s).

In order to determine the viscosity of culture medium, 10 ml of samples were removed from the culture, and poured into the viscometer. The viscosity was calculated from the recorded time that the culture medium moved through the capillary tube (efflux time) from the following equation:

Kinematic viscosity (cSt) = viscometer constant (cSt/s) $\times$ efflux time (s)

$$\text{Viscosity}\left(\frac{\text{cSt} \cdot \text{g}}{\text{l}}\right) = \text{Kinematic viscosity (cSt)} \times \text{density (g/l)}$$

4.3.3.4 Determination of substrate and products

Substrate (glycerol) and fermentation products (1,3-propanediol, butyric acid and acetic acid) were separated and quantified by using HPLC (LC-3A, Shimadzu, Japan) equipped with refractive index Detector (RI). A column (Lichrocart C18) with length of 250 mm and outer diameters of 4 mm was used. Operating conditions were 20 mM H_3PO_4 as a mobile phase at a flow rate of 1.2 ml/min and column temperature at room temperature. The samples were injected with volume of 30 μl.

The other product, ethanol, was measured by gas chromatography (GC-7AG, Shimadzu, Japan) equipped with Flame Ionization Detector (FID). A column with length of 2 m, outer diameter of 3.3 mm, and packed with Porapak Q 80-100 mesh was used in collaboration with N_2 as carrier gas. The flow rate of N_2 was 50 ml/min. The oven and detector temperatures were 190°C and 240°C, respectively. The samples were injected with a volume of 1 µl at the injection temperature of 240°C. The substrate and product concentrations were measured from only one injection.

4.3.3.5 Scanning Electron Microscopy (SEM)

Cells from each of fermentation time were sputter-coated by gold and examined using scanning electron microscope (JSM-5410LV, JEOL, Japan).

CHAPTER V

RESULTS AND DISCUSSION

In order to develop a kinetic model for 1,3-propanediol production from glycerol fermentation by using *Clostridium butyricum* DSM 5431 in fed-batch fermentation, firstly, the effect of initial glycerol concentration in 1-L batch fermenter was investigated. The kinetic model for 1,3-propanediol production from glycerol using *Clostridium butyricum* DSM 5431 in a 1-L batch fermenter at the optimal initial glycerol concentration was developed. Then, the batch fermentation was scaled-up to a 15-L fermenter. In addition, the developed kinetic model for 1,3-propanediol production obtained from the 1-L batch was used and readjusted to fit results obtained from a 15-L batch fermenter. The developed kinetic model in a 15-L batch fermenter was further used to determine the proper feeding strategy for 1,3-propanediol production in a 15-L fed-batch fermenter. The model was finally verified with the fed-batch experiment.

5.1 Effect of initial glycerol concentration

Since glycerol, in high concentrations, is considered as an inhibitor of microbial growth, substrate inhibition level for *Clostridium butyricum* DSM 5431 should be investigated. The batch fermentations of *Clostridium butyricum* DSM 5431 under anaerobic condition were primarily performed with different initial glycerol concentrations of 60-120 g/l in a 1-L glass fermenter. The incubation temperature was controlled at 33°C and pH of 7.0. The time-course of glycerol batch fermentation at different initial glycerol concentrations are shown in Figure 5.1 and Figure 5.2.

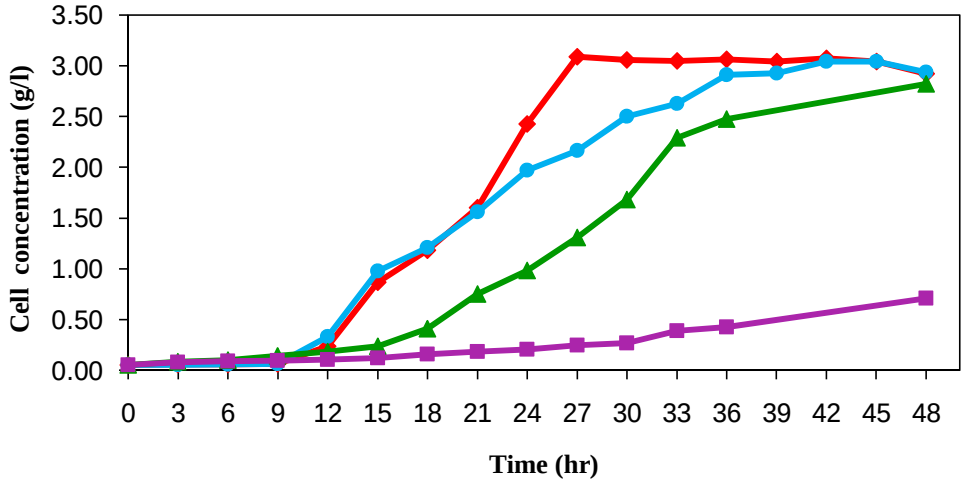


Figure 5.1 Cell concentration profile at different initial glycerol concentrations in 1-L batch fermenter under 33 °C, pH 7.0, and 100 rpm: ♦ , 60 g/l; ● , 80 g/l ; ▲ , 100 g/l; ■ , 120 g/l

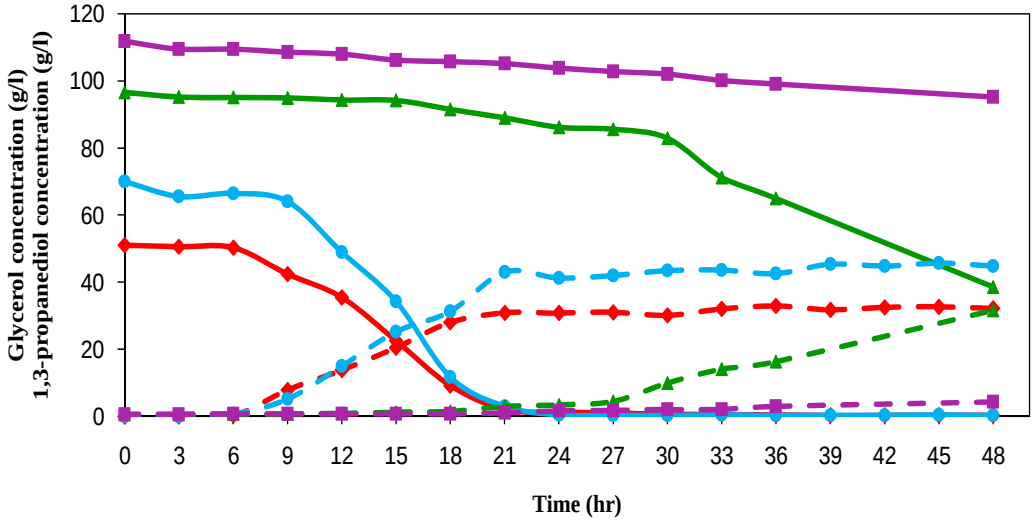


Figure 5.2 Glycerol and 1,3-propanediol concentration profiles at different initial glycerol concentrations in 1-L batch fermenter under 33 °C, pH 7.0, and 100 rpm : ♦, 60 g/l; ●, 80 g/l; ▲, 100 g/l; ■, 120 g/l

Comparison of specific rates obtained from various initial glycerol concentrations is shown in Figure 5.3.

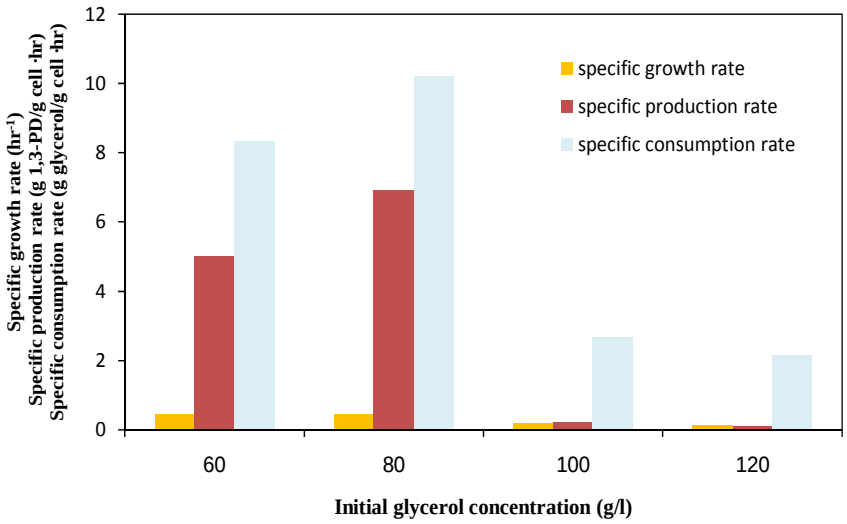


Figure 5.3 Effect of initial glycerol concentration on specific rates in 1-L batch fermenter under 33 °C, pH 7.0, and 100 rpm

Table 5.1 Yields and final 1,3-propanediol concentrations (at 48 hours) at different initial glycerol concentrations in 1-L batch fermenter under 33 °C, pH 7.0, and 100 rpm

Initial Glycerol concentration (g/l)	$Y_{P/S}$ (mol/mol)	$Y_{X/S}$ (g/g)	$Y_{P/X}$ (g/g)	Final 1,3-propanediol concentration (g/l)
60	0.76	0.056	11.17	32.06
80	0.77	0.042	15.52	44.75
100	0.64	0.047	11.19	31.60
120	0.26	0.039	5.48	4.19

It was found that initial glycerol concentrations of 60, 80, 100, and 120 g/l were converted respectively to 32.06, 44.75, 31.60, and 4.19 g/l of 1,3-propanediol with the conversion yields of 0.76, 0.77, 0.64 and 0.27 mol 1,3-propanediol/mol glycerol at 48 hours (see Table 5.1). In order to compare experimental with theoretical yields, equations (5.1), and (5.2) are considered. Zeng (1996) proposed these two equations for two different pathways of 1,3-propanediol production. The one is when acetic acid is the sole by-product (Eq. 5.1), the other is for butyric acid (Eq. 5.2).

$$78C_3H_8O_3 + 3NH_3 \longrightarrow 3C_4H_7O_2N + 17.5C_2H_4O_2 + 56.5C_3H_8O_2 + 17.5CO_2 \quad (5.1)$$

$$78.2C_3H_8O_3 + 3NH_3 \longrightarrow 3C_4H_7O_2N + 11.7C_4H_8O_2 + 50.8C_3H_8O_2 + 23.4CO_2 \quad (5.2)$$

Judging from the stoichiometries, $Y_{P/S}$ from acetic and butyric acid pathways could be determined at 0.72 and 0.65, respectively. This means that our experimental yield value (0.77) was at its maximum. Deviations from the theoretical yields were only around 7% and 15% which were probably due to some experimental errors. The production pathways, in our case, were a mixed between both pathways demonstrated in equations (5.1), and (5.2) (results will be shown in Section 5.2). Table 5.2 demonstrates comparison of final 1,3-propanediol concentration and $Y_{P/S}$ from various studies. Results from our experiments are comparable to or higher than other works. Especially in terms of final 1,3-propanediol concentration which we could accumulate up to a satisfying value of 44.7 g/l.

Table 5.2 Yields and final 1,3-propanediol concentrations from various studies

Reference	Final 1,3-propanediol concentration (g/l)	$Y_{P/S}$ (mol/mol)
Günzel et al. (1991)	25.0	0.70
Petitdemange et al. (1995)	24.9	0.62
Biebl et al. (1995)	13.8	0.78
Abbad-andaloussi et al. (1995)	31.2	0.61
Pullsirisombat et al. (2007)	41.4	0.76
This study	44.7	0.77

According to effect of initial glycerol concentration on 1,3-propanediol production shown above, the maximum specific rates, $Y_{P/S}$, and final 1,3-propanediol concentration were observed at initial glycerol concentration 80 g/l. Therefore, it can be concluded from the experimental results that the optimal initial glycerol concentration for cell growth and 1,3-propanediol production was found to be 80 g/l and glycerol could obviously inhibit cell growth at concentration levels equal to or higher than 100 g/l. This is in contrast to the finding of Deckwer (1995) who reported that substrate inhibition occurred when the initial glycerol concentration was higher than 50 g/l. On the other hand, our results agree well with the observation of Biebl (1991) who found that the appreciable inhibition by glycerol was found only above a concentration of 80 g/l.

5.2 Kinetic model development from 1-L batch experimental results

Based on this experiment, batch fermentation in 1-L fermenter was performed at optimal initial glycerol concentration of 80 g/l under 33 °C, pH 7.0, and 100 rpm. The experimental results are shown in Figure 5.4.

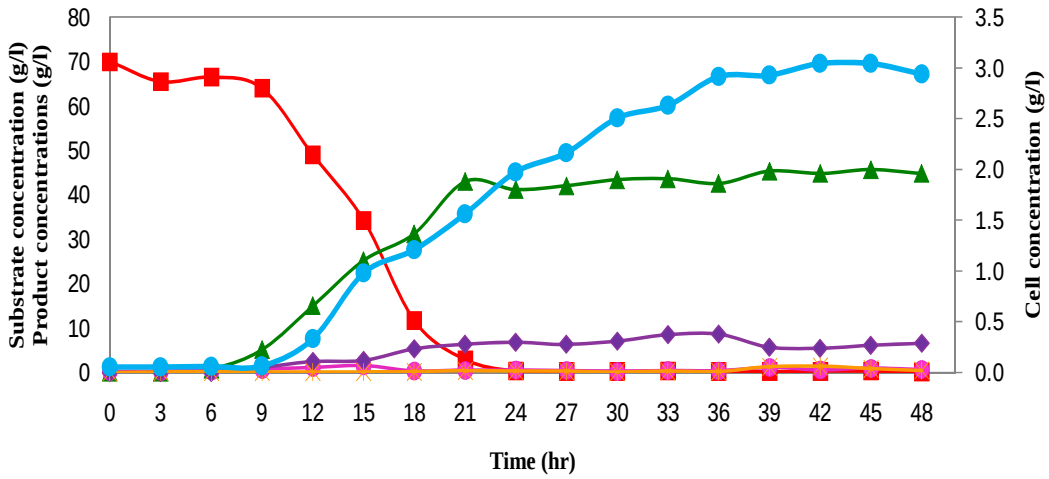


Figure 5.4 Time-course of batch fermentation at 80 g/l of initial glycerol concentration in 1-L fermenter under 33 °C, pH 7.0, and 100 rpm: ■, Glycerol; ▲, 1,3-propanediol; ♦, Butyric acid; ●, Acetic acid; ∗, Ethanol; ●, Cell

So as to develop the kinetic model, the by-product concentration profiles such as butyric acid, acetic acid, and ethanol in batch fermentation at optimal initial glycerol concentration were investigated. The main by-product found in this batch fermentation was butyric acid, with little acetic acid (0.66 g/l) and ethanol (0.46 g/l) accumulation. These results differed from the other results of 1,3-propanediol production from glycerol by *Clostridium butyricum* DSM 5431 which had butyric acid and acetic acid as the main by-products (Biebl, 1991; Günzel et al, 1991; Zeng et al., 1994; Abbad-andaloussi et al., 1995; Petitdemange et al., 1995). It may be explained that cells in this study required a lot of energy (ATP) for growth in these environmental conditions. Hence, cells produced a high amount of butyric acid because the butyric acid pathway renders higher ATP than the acetic acid pathway.

Therefore, the kinetic model for 1,3-propanediol production in batch fermentation of optimal initial glycerol concentration in 1-L fermenter was further developed by using mathematical simulation. According to the previous reports, the cell model indicated multiple inhibition effects of glycerol, 1,3-propanediol, butyric acid, and acetic acid on *Clostridium butyricum* DSM 5431 growth. Due to trace amount of acetic acid which was very lower than the 21.8 g/l of critical concentration (Zeng et al., 1994), the acetic acid concentration was neglected from the model. Therefore, the most suitable cell model in this study indicated multiple inhibition

effects of glycerol, 1,3-propanediol, and butyric acid on *Clostridium butyricum* DSM 5431 growth. The critical concentrations of glycerol, 1,3-propanediol, and butyric acid were 181.7 g/l, 66.4 g/l, and 11.1 g/l, respectively (Zeng et al., 1994). The growth model and the mass balance equations that were used in mathematical simulation can be written as follows:

$$\mu = \mu_{max}\left(\frac{C_{Gly}}{C_{Gly}+K_S}\right)\left(1-\frac{C_{Gly}}{C^*_{Gly}}\right)\left(1-\frac{C_{PD}}{C^*_{PD}}\right)\left(1-\frac{C_{HBu}}{C^*_{HBu}}\right) \tag{5.3}$$

$$\frac{dX}{dt} = \mu X - K_d X \tag{5.4}$$

$$\frac{dC_{Gly}}{dt} = -\frac{\mu X}{Y_{X/S}} - M_C X - \frac{\alpha_{PD}\mu X + \beta_{PD} X}{Y_{PD/S}} - \frac{\alpha_{HBu}\mu X + \beta_{HBu} X}{Y_{HBu}} \tag{5.5}$$

$$\frac{dC_{1,3\text{-}PD}}{dt} = \alpha_{PD}\mu X + \beta_{PD} X \tag{5.6}$$

$$\frac{dC_{HBu}}{dt} = \alpha_{HBu}\mu X + \beta_{HBu} X \tag{5.7}$$

In this study, the mass balance equations of 1,3-propanediol and butyric acid production rate were used in mixed-growth associated form because this form is the general form of product formation rate. However, because the growth model and 4 differential equations of mass balance had 11 unknown parameters, these unknown parameters must be firstly optimized to find their values by using genetic algorithm (GA) in MATLAB program (version 2007a). The objective function for optimizing the parameters that guessed initially from the lower boundary of the constraints was the minimum sum square error between the experimental results and the optimization results. The optimization values of their parameters in growth model and mass balance equations are given in Table 5.3. Complete definitions of all parameters are described in nomenclature and a source code of MATLAB program is provided in Appendix D .

Table 5.3 Kinetic parameters and optimization values in 1-L batch fermenter

Kinetic parameter	Constraint	Optimization value
μ_{max}	0.13 - 0.40	0.3933
K_S	0.5 – 0.9	0.5731
K_d	0 – 0.001	0.0003
$Y_{X/S}$	0.88 – 1.0	0.8811
M_C	0 – 0.04	0.0274
$Y_{1,3\text{-PD}/S}$	0.8 – 0.9	0.8022
$Y_{HBu/S}$	0.9 – 1.0	0.9006
$\alpha_{1,3\text{-PD}}$	13 – 16	15.8975
α_{HBu}	2.8 – 3.2	2.8076
$\beta_{1,3\text{-PD}}$	0.005 – 0.008	0.0065
β_{HBu}	0.004 – 0.006	0.0042

By optimizing the values of all parameters, it is suggested that 1,3-propanediol and butyric were obviously growth associated products because the constant for growth associate products ($\alpha_{1,3\text{-PD}}$, α_{HBu}) were noticeably higher than the constant for non-growth associated products ($\beta_{1,3\text{-PD}}$, β_{HBu}). Furthermore, the optimized value of μ_{max} in this study was lower than the optimized value of Zeng et al. (1994) who reported that μ_{max} was 0.67 hr^{-1}.

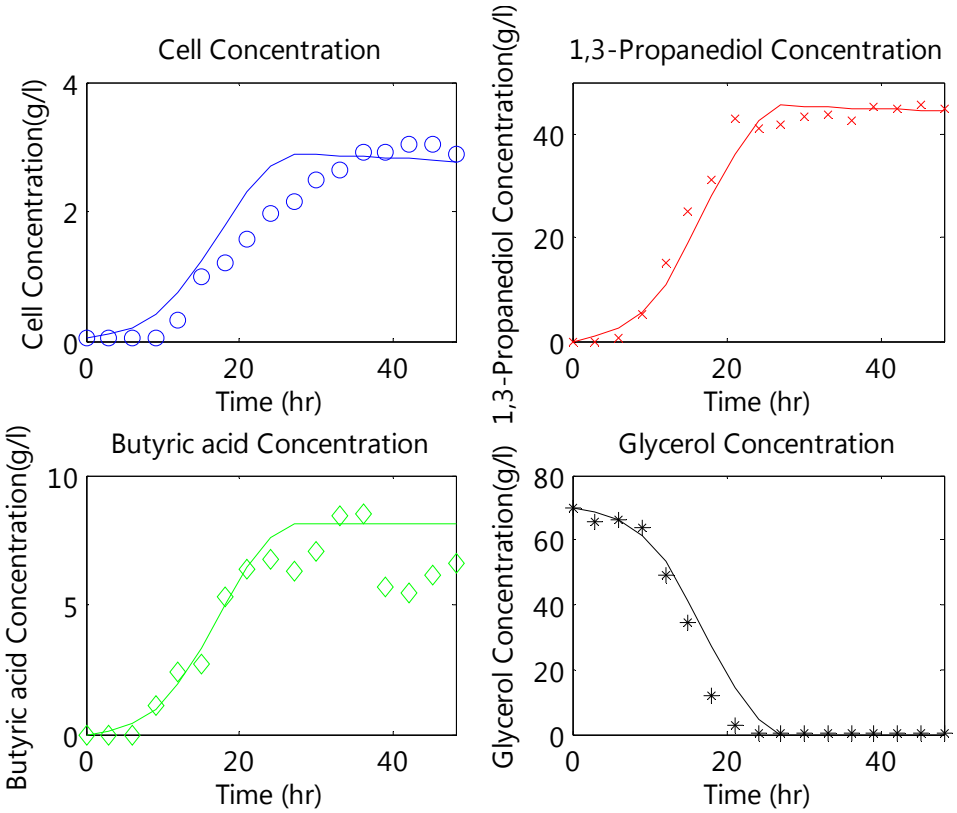


Figure 5.5 Comparison of experimental results (○,x,◊,∗) and simulated results (-) in 1-L batch fermenter

The comparison of the experimental results and the simulated results which were simulated from the parameter values that were optimized from MATLAB program are also shown in Figure 5.5. It is evident from the figure that the simulated results of cell, glycerol, 1,3-propanediol, and butyric acid concentrations fitted the experimental results with a deviation of 8.0%, 2.9%, 2.6%, and 9.0% respectively (see Appendix C), while an average deviation is 5.6%. This suggests that the simulated results of biomass, glycerol, 1,3-propanediol, and butyric acid concentration were very close to the experimental results. Furthermore, the simulated results demonstrated that 1,3-propanediol concentration can be estimated from this growth model.

5.3 Scale-up of a batch fermentation from 1 L to 15-L

Before determining a proper feeding strategy of 15-L fed-batch fermentation, the study of scale-up batch fermentation from 1-L to 15-L fermenter was first necessarily investigated. Since Pullsirisombat et al. (2007) found that the production of 1,3-propanediol by *Clostridium butyricum* DSM 5431 required strictly anaerobic conditions, nitrogen transfer in the fermenter should be the key factor for scale-up consideration. Therefore, power input per unit liquid volume (P/V), and gas flow rate per unit liquid volume (Q/V) were primarily used as scale-up criteria. The calculated impeller rotation speed for operating 15-L fermenter as same P/V and Q/V as in 1-L fermenter (see Appendix C) was 48 rpm. Therefore, batch fermentation in 15-L fermenter was performed at initial glycerol concentration of 80 g/l under 33 °C, pH 7.0, and 48 rpm. The experimental results are shown in Figure 5.6.

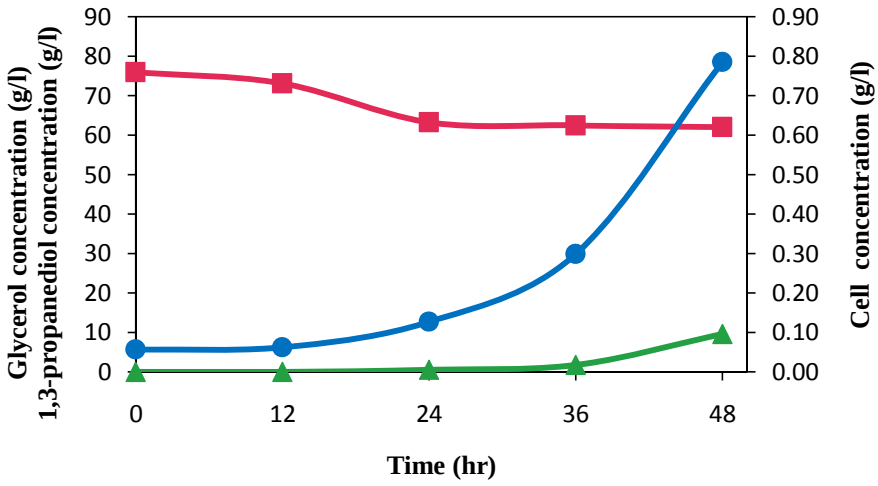
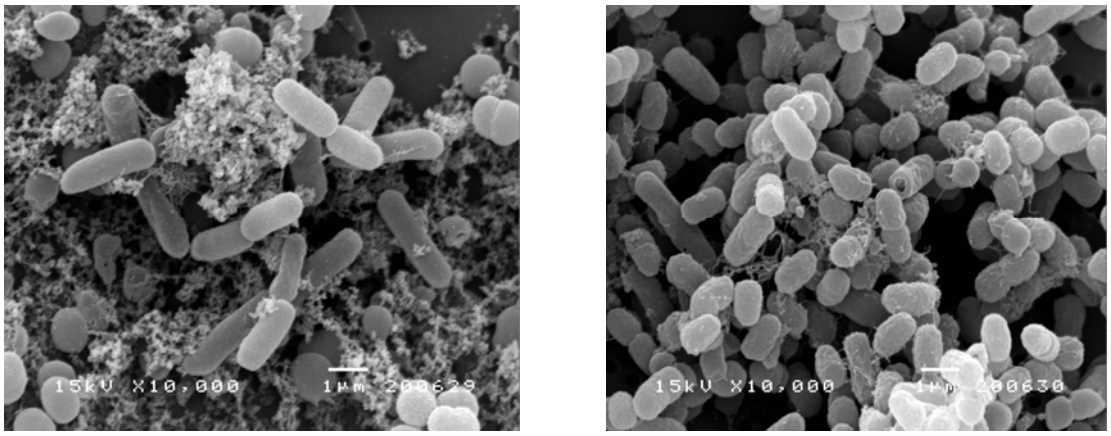


Figure 5.6 Time-course of batch fermentation at 80 g/l of initial glycerol concentration in 15-L fermenter under 33 °C, pH 7.0, and 100 rpm: ●, Cell; ■, Glycerol; ▲, 1,3-propanediol

When these results were compared with the experimental results in 1-L fermenter, it can be noticed that in these conditions not only 1,3-propanediol slowly produced and glycerol belatedly consumed but also cell slowly grown. Therefore, it can be approximately concluded that these conditions did not suitable for cell growth.

However, there are many possible factors that may impact on cell, for example, shear rate or pH effect. Thus, the analysis of morphological changes of *Clostridium butyricum* DSM 5431 in these environments by scanning electron microscopy (SEM) was carried on.



(a) (b)

Figure 5.7 SEM photographs of *Clostridium butyricum* DSM 5431 cells at initial glycerol concentrations of 80 g/l in 15-L batch fermenter under 33 °C, pH 7.0, and 48 rpm: (a) 0 hour; (b) 48 hours

The morphological changes of *Clostridium butyricum* DSM 5431 were obviously observed from SEM photographs. The cells were healthy and appeared normal rod shape at 0 hour (Figure 5.7 a). In contrast, the normal rod shape was lost and the cell lengths decreased with increasing culture time (Figure 5.7 b). In other word, the cells showed a segmented aspect in which individual segments were shorter at 48 hours.

Therefore, the previous researches about the affecting factor of morphological changes of cells were studied. However, Jan et al. (2001) reported that the morphology of *Propionibacterium freudenreichii* cells which were rod shape, gram-positive, and incubated at low pH showed acid stress adaptation in which individual cells were shorter. Since glycerol fermentation produced merely acid as

by-products and the morphological changes of *Clostridium butyricum* DSM 5431 cells as same as *Propionibacterium freudenreichii* cells, thus, pH was considered to be the affecting factor of *Clostridium butyricum* DSM 5431 cells. This illustrates that the scale-up problem was the mixing problem which occurred from the inconsistent distribution of pH in fermenter.

Thus, it should find a new scale-up criteria to operate 1,3-propanediol production with Q/V in 15-L batch fermenter. The various scale-up criteria and its calculated impeller rotation speeds are listed in Table 5.4.

Table 5.4 Impeller rotation speeds at various scale-up criteria

Scale-up criteria	**Impeller rotation speed**	
	Fermenter 1-L	**Fermenter 15-L**
Constant P/V	100 rpm	48 rpm
Constant N	100 rpm	100 rpm
Constant Re	100 rpm	14 rpm
Constant tip speed (ND_i)	100 rpm	38 rpm

Because the calculated impeller rotation speeds of the other criteria were lower than the previous criteria (constant P/V), therefore, the only choice of scale-up criteria was constant impeller rotation speed (N) which implied constant mixing time. Therefore, batch fermentation in 15-L fermenter was performed at initial glycerol concentration of 80 g/l under 33 °C, pH 7.0, and 100 rpm. The experimental results are shown in Figure 5.8. Furthermore, the final 1,3-propanediol concentration, yields (at 48 hours), and specific rates in 15-L batch fermenter are also shown in Table 5.5.

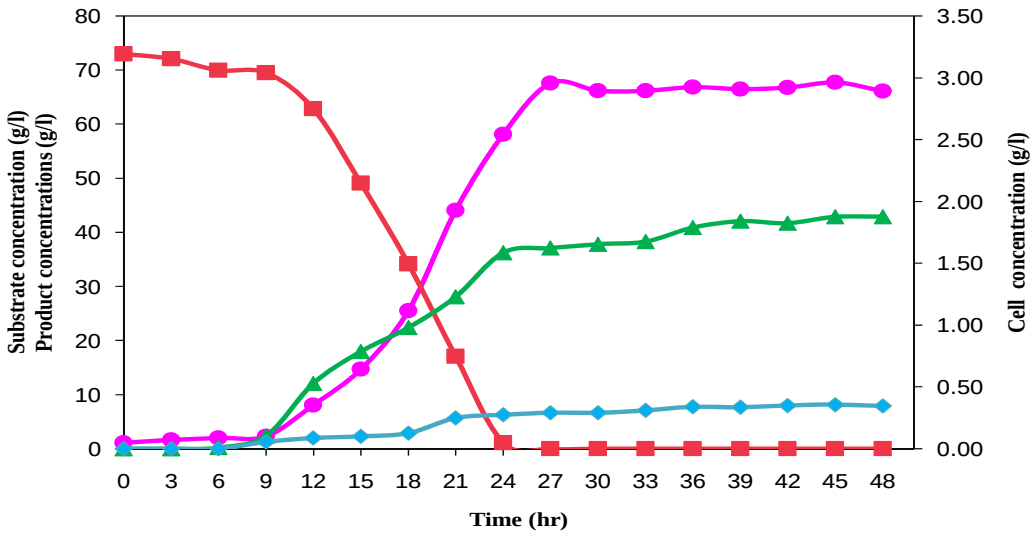
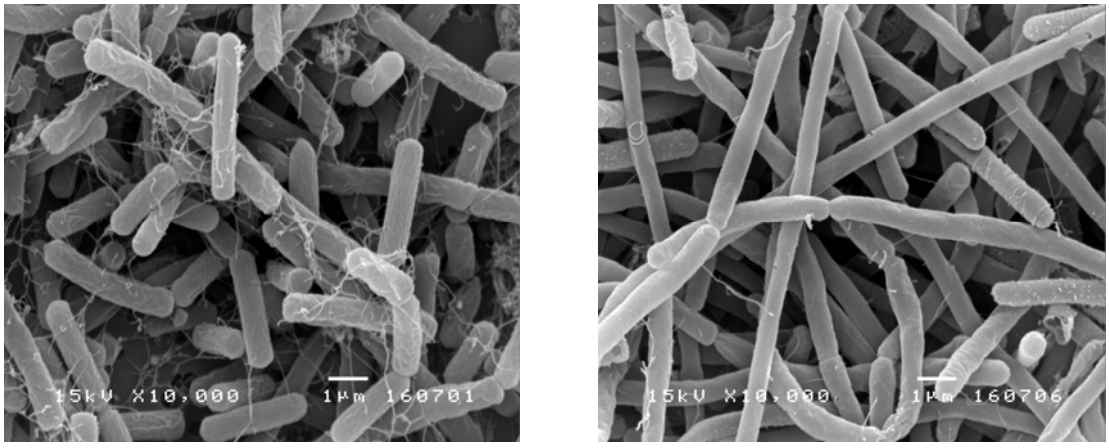


Figure 5.8 Time-course of batch fermentation at initial glycerol concentration of 80 g/l in 15-L fermenter under 33 °C, pH 7.0, and 100 rpm: ●, Cell; ■, Glycerol; ▲, 1,3-propanediol; ♦, Butyric acid

Table 5.5 Final 1,3-propanediol concentration, yields (at 48 hours), and specific rates in 15-L batch fermenter under 33 °C, pH 7.0, and 100 rpm

μ **(hr^{-1})**	**ρ** $\left(\frac{\text{g 1,3 – PD}}{\text{g cell} \cdot \text{hr}}\right)$	**υ** $\left(\frac{\text{g glycerol}}{\text{g cell} \cdot \text{hr}}\right)$	**$Y_{P/S}$ (mol/mol)**	**$Y_{X/S}$ (g/g)**	**$Y_{P/X}$ (g/g)**	**Final 1,3-propanediol concentration (g/l)**
0.31	6.67	9.41	0.71	0.039	15.11	42.89

Since the 1,3-propanediol concentration and kinetic parameters in this experiment did not practically differ from the previous results in 1-L batch fermenter, therefore, these scale-up criteria were finally the appropriate criteria for scale-up 1,3-propanediol production. However, the morphological changes of *Clostridium butyricum* DSM 5431 in these environments by SEM were verified again.



(a) (b)

Figure 5.9 SEM photographs of *Clostridium butyricum* DSM 5431 cells at initial glycerol concentrations of 80 g/l in 15-L batch fermenter under 33 °C, pH 7.0, and 100 rpm: (a) 0 hour; (b) 48 hours

The morphological changes of *Clostridium butyricum* DSM 5431 were evidently different from the former study. When increased cultivation time, the cell lengths elongated to filamentous cells (Figure 5.9). However, it can be assumed that the cell elongation might cause by the accumulation of high product concentration. This presumption was in accordance to the finding of Biebl (1991).

5.4 Kinetic model development from 15-L batch experimental results

The development of kinetic model was further developed in 15-L batch experimental results. The growth model, the critical concentrations, and the mass balance equations that were used in 15-L mathematical simulation were similarly used in 1-L fermenter (see Section 5.2).

Also, the 11 unknown parameters must be firstly optimized to find their values by using GA in MATLAB program. The previous constraints of each parameter were applied to optimize these parameters. The optimization values in growth model and mass balance equations are given in Table 5.6.

Table 5.6 Kinetic parameters and optimization values in 15-L batch fermenter

Kinetic parameter	Constraint	Optimization value
μ_{max}	0.13 - 0.40	0.3933
K_S	0.5 – 0.9	0.5217
K_d	0 – 0.001	0.0003
$Y_{X/S}$	0.88 – 1.0	0.9043
M_C	0 – 0.04	0.0389
$Y_{1,3\text{-PD}/S}$	0.8 – 0.9	0.8022
$Y_{HBu/S}$	0.9 – 1.0	0.9006
$\alpha_{1,3\text{-PD}}$	13 – 16	14.2210
α_{HBu}	2.8 – 3.2	2.8268
$\beta_{1,3\text{-PD}}$	0.005 – 0.008	0.0052
β_{HBu}	0.004 – 0.006	0.0058

Comparison of the experimental results and the simulated results from MATLAB program are also shown in Figure 5.10.

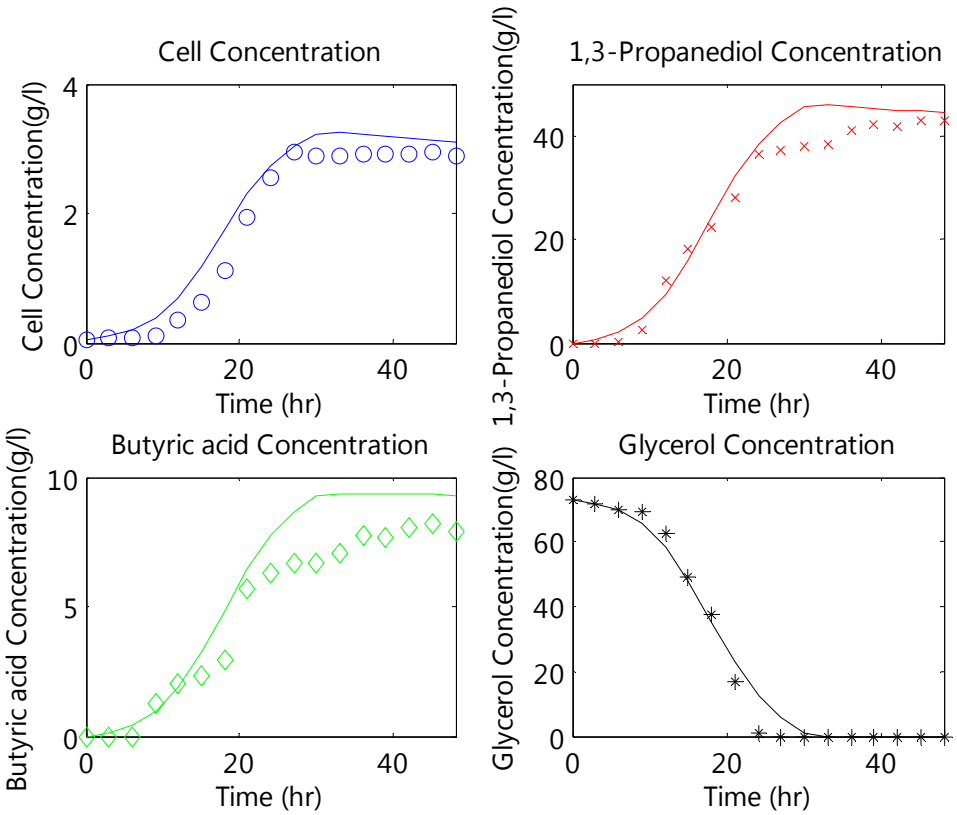


Figure 5.10 Comparison of experimental results (○,x,◊,∗) and simulated results (-) in 15-L batch fermenter

Since the simulated result of butyric acid concentration was relatively more than the experimental result, therefore, the first assumption was there were some constraint related to butyric acid did not proper. In this view point, we would firstly decrease the constraint of yield butyric acid on glycerol. The new optimization values in growth model and mass balance equations are given in Table 5.7.

Table 5.7 Kinetic parameters and optimization values in 15-L batch fermenter when decreasing the constraint of $Y_{HBu/S}$

Kinetic parameter	Constraint	Optimization value
μ_{max}	0.13 - 0.40	0.3786
K_S	0.5 – 0.9	0.7629
K_d	0 – 0.001	0.0003
$Y_{X/S}$	0.88 – 1.0	0.9002
M_C	0 – 0.04	0.0016
$Y_{1,3\text{-}PD/S}$	0.8 – 0.9	0.8065
$Y_{HBu/S}$	0.43 - 0.5	0.4304
$\alpha_{1,3\text{-}PD}$	13 – 16	14.2210
α_{HBu}	2.8 – 3.2	2.8089
$\beta_{1,3\text{-}PD}$	0.005 – 0.008	0.0079
β_{HBu}	0.004 – 0.006	0.0040

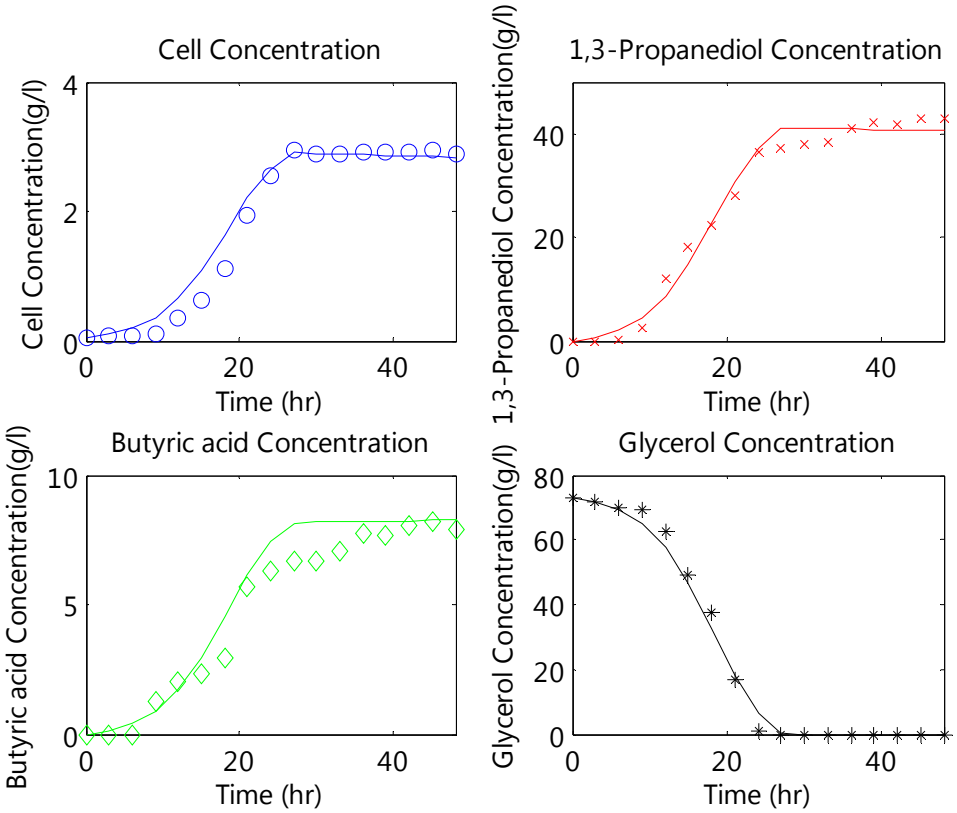


Figure 5.11 Comparison of experimental results (○,x,◊,∗) and simulated results (-) in 15-L batch fermenter when decreasing the constraint of $Y_{HBu/S}$

Based on decreasing the constraint of yield butyric acid on glycerol, it can be noticed that the simulated results of cell, glycerol, 1,3-propanediol, and butyric acid concentrations well fitted with the experimental results (see Figure 5.11). Furthermore, the calculated deviation between the simulated results and the experimental results showed that the simulated results of cell, glycerol, 1,3-propanediol, and butyric acid concentrations fitted the experimental results with the deviation of 1.8%, 0.4%, 1.8%, and 2.8% respectively, while the average deviation is 1.7%. This suggests that the cell model which was proposed in this study was already suitable for determining the proper feeding strategy of fed-batch fermentation for 1,3-propanediol production. Therefore, fed-batch fermentation in 15-L fermenter was continually studied.

5.5 Kinetic model development from 15-L fed-batch experimental results

5.5.1 Determination of a appropriate feeding strategy

In order to achieve the higher 1,3-propanediol productivities and prevent substrate inhibition phenomena by maintaining substrate concentration at a low value, fed-batch fermentation was the obvious technique to be employed. In this study, the fermentation is at first started as a batch process, fed medium according to fed-batch process and allowed to ferment residual glycerol by batch process after time of full volume until glycerol was completely consumed.

Due to the requirement of the proper feeding strategy to give the highest 1,3-propanediol productivity, three feeding strategies were compared by using mathematical simulation in MATLAB program. The first strategy was the constant feeding which substrate was fed into fermenter at a constant rate. The second strategy was the increasing feeding which substrate was fed into fermenter at an increasing rate (linear with time). The last strategy was the exponential feeding which substrate was fed into fermenter at an exponential rate. The equations of all feeding strategies can be written as follows:

Constant feeding: $$F = \frac{\upsilon X_0 V_0}{C_{Gly,feed}} \tag{5.8}$$

Increased feeding: $$F = at + b \tag{5.9}$$

Exponential feeding: $$F = \frac{\upsilon X_0 V_0 e^{\mu t}}{(C_{Gly,feed} - C_{Gly})} \tag{5.10}$$

The growth model and the mass balance equations that were used in mathematical simulation in fed-batch fermentation can be written as follows:

$$\mu = \mu_{max}\left(\frac{C_{Gly}}{C_{Gly} + K_S}\right)\left(1 - \frac{C_{Gly}}{C^*_{Gly}}\right)\left(1 - \frac{C_{PD}}{C^*_{PD}}\right)\left(1 - \frac{C_{HBu}}{C^*_{HBu}}\right) \tag{5.11}$$

$$\frac{dX}{dt} = -\frac{F}{V}X + \mu X - K_d X \tag{5.12}$$

$$\frac{dC_{Gly}}{dt} = \frac{F}{V}\left(C_{Gly,feed} - C_{Gly}\right) - \frac{\mu X}{Y_{X/S}} - M_C X - \frac{\alpha_{PD}\mu X + \beta_{PD} X}{Y_{PD/S}} - \frac{\alpha_{HBu}\mu X + \beta_{HBu} X}{Y_{HBu}} \quad (5.13)$$

$$\frac{dC_{1,3\text{-}PD}}{dt} = -\frac{F}{V} C_{1,3-PD} + \alpha_{PD}\mu X + \beta_{PD} X \quad (5.14)$$

$$\frac{dC_{HBu}}{dt} = -\frac{F}{V} C_{HBu} + \alpha_{HBu}\mu X + \beta_{HBu} X \quad (5.15)$$

$$\frac{dV}{dt} = F \quad (5.16)$$

Based on the previous experimental results in 15-L batch fermenter, at 21 hours was considered to be a suitable beginning time of feeding because cells almost grew in the end of exponential growth phase and glycerol was almost completely consumed. Therefore, the kinetic parameters in this time were also calculated in order to determine each feeding rate. The calculated specific growth rate and specific consumption rate were 0.137 hr^{-1} and 2.934 g glycerol/g cell$\cdot$hr, respectively.

The appropriate concentration of glycerol in feed was also looked up (see Appendix C). From varying of glycerol concentrations in feed, it can be found that glycerol should be 120 g/l so as to give the highest 1,3-propanediol concentration and the complete consumption of glycerol.

Furthermore, because this study aims at providing the increased feeding rate between constant feeding rate and exponential feeding rate, thus, the examined values of a and b were 0.05 and corresponding to constant feeding rate respectively (see Appendix C).

Owing to the best comparing of 1,3-propanediol concentrations and productivities, the feeding rates at starting time should have an equal value (see Figure 5.12). However, because the different feeding rates resulted in a different time of full volume, these brought about the various productivities. Therefore, the comparison of three feeding strategies was started by using 1,3-propanediol productivity at time of full volume as a criteria. The simulated results in fed-batch fermentation using glycerol concentration in feed of 120 g/l are also shown in Figure 5.13 -5.15.

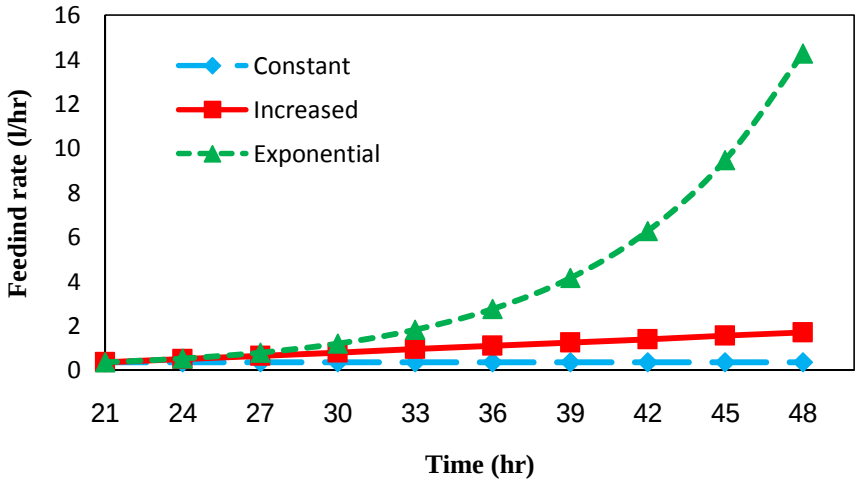


Figure 5.12 Feeding rates of different feeding strategies in fed-batch fermentation using glycerol concentration in feed of 120 g/l

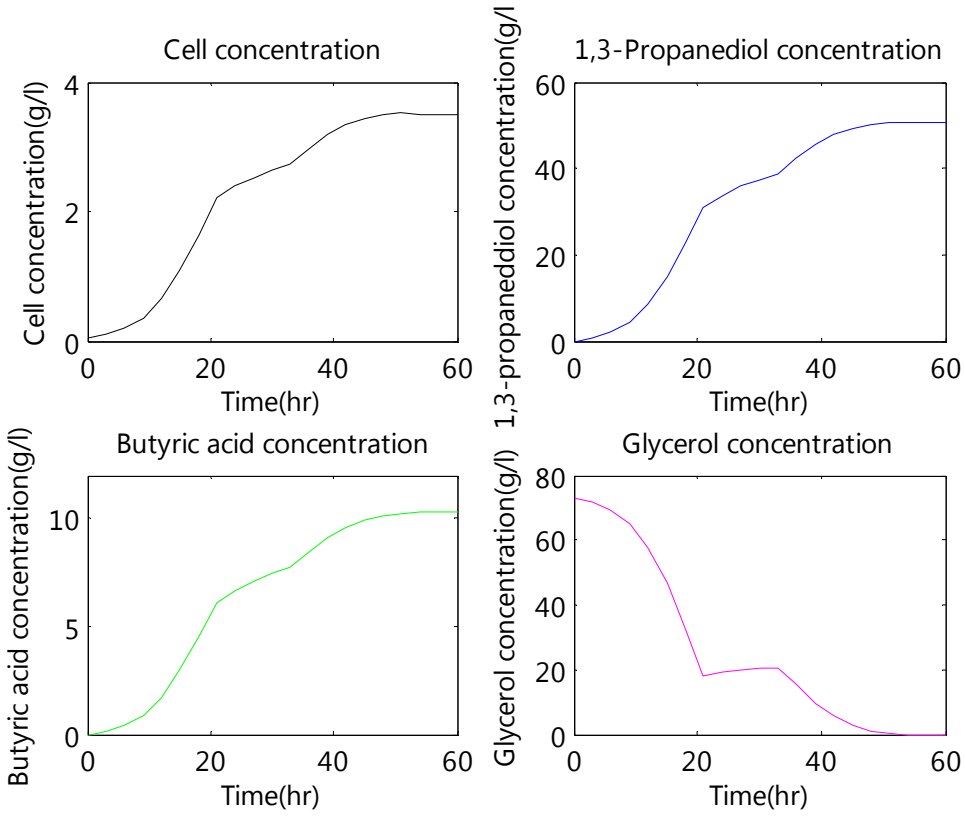


Figure 5.13 The simulated results at glycerol concentration in feed of 120 g/l using constant feeding strategy

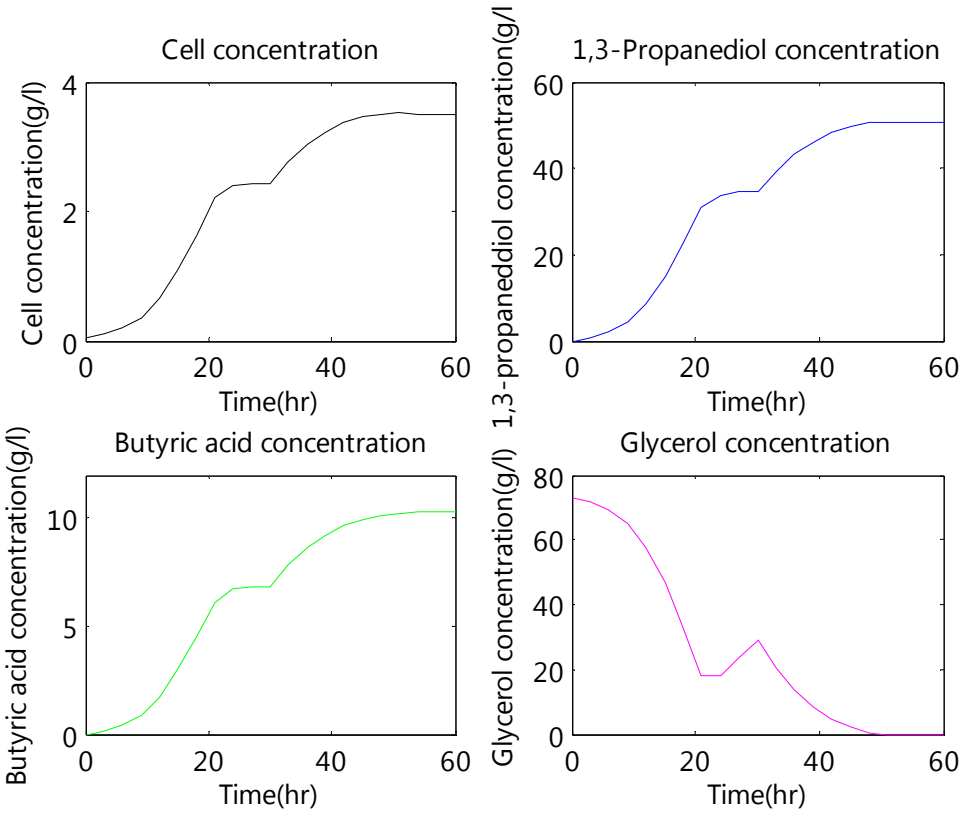


Figure 5.14 The simulated results at glycerol concentration in feed of 120 g/l using increased feeding strategy

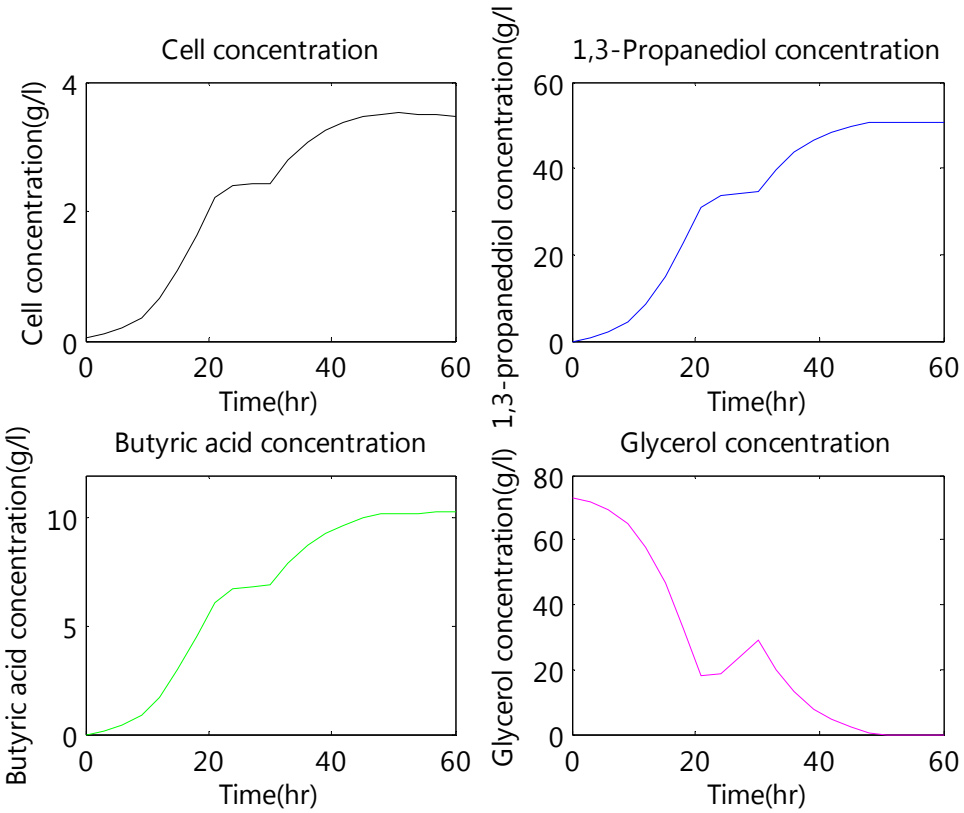


Figure 5.15 The simulated results at glycerol concentration in feed of 120 g/l using exponential feeding strategy

Table 5.8 The simulated 1,3-propanediol concentrations and productivities at time of full volume using glycerol concentration in feed of 120 g/l

Feeding strategy	**1,3-propanediol concentration (g/l)**	**Productivity (g/l · hr)**
Constant	39.45	1.160
Increased	34.66	1.155
Exponential	34.49	1.150

Since the constant feeding strategy obtained the highest 1,3-propanediol concentration and productivity (see Table 5.8), therefore, this strategy was considered to be a suitable feeding strategy for fed-batch experiment in 15-L fermenter.

5.5.2 Experimental results in 15-L fed-batch fermenter

Fed-batch fermentation using constant feeding strategy in 15-L fermenter was performed at initial glycerol concentration of 80 g/l and glycerol concentration in feed of 120 g/l with a constant feed rate of 0.353 l/hr under 33 °C, pH 7.0, and 100 rpm. Feeding was started at 21 hours and stopped at 34 hr (time of full volume). In order to achieve more 1,3-propanediol concentration, the residual glycerol was allowed to ferment by batch process after 34 hours until glycerol completely consumed. The experimental results are shown in Figure 5.16. Furthermore, final 1,3-propanediol concentration, yields (at 60 hours), and specific rates are also shown in Table 5.9.

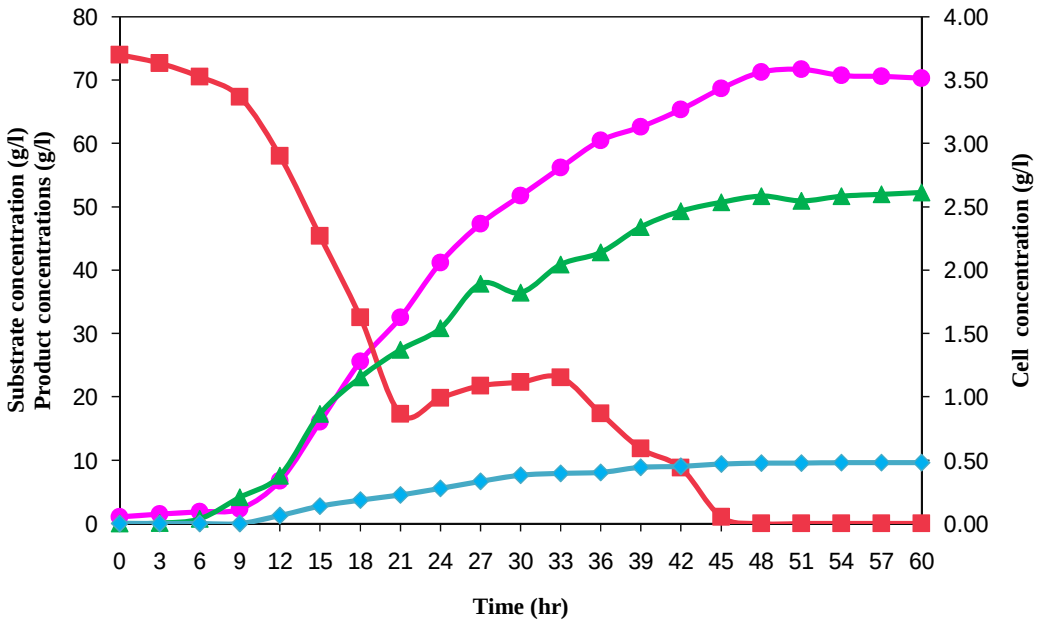
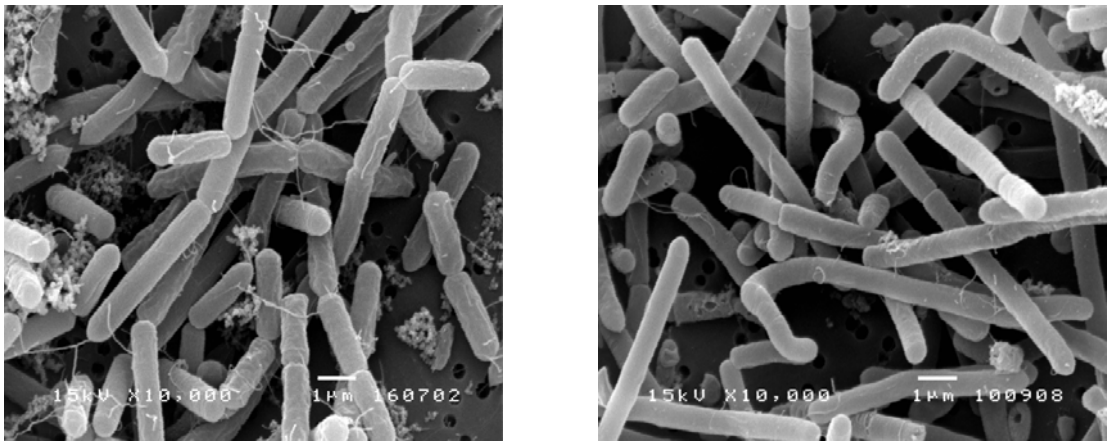


Figure 5.16 Time-course of fed-batch fermentation at initial glycerol concentration of 80 g/l and glycerol concentration in feed of 120 g/l in 15-L fermenter under 33 °C, pH 7.0, and 100 rpm: ●, Cell; ■, Glycerol; ▲, 1,3-propanediol; ♦, Butyric acid

Table 5.9 Final 1,3-propanediol concentrations, yields (at 60 hours), and specific rates in 15-L fed-batch fermenter under 33 °C, pH 7.0, and 100 rpm

μ **(hr^{-1})**	**ρ** $\left(\frac{g\,1,3-PD}{g\,cell\cdot hr}\right)$	**υ** $\left(\frac{g\,glycerol}{g\,cell\cdot hr}\right)$	**$Y_{P/S}$** **(mol/mol)**	**$Y_{X/S}$** **(g/g)**	**$Y_{P/X}$** **(g/g)**	**Final 1,3-propanediol concentration (g/l)**
0.32	5.38	8.54	0.43	0.023	15.11	52.29



(a) (b)

Figure 5.17 SEM photographs of *Clostridium butyricum* DSM 5431 cells at initial glycerol concentrations of 80 g/l in 15-L fed-batch fermenter under 33 °C, pH 7.0, and 100 rpm: (a) 0 hour; (b) 48 hours

Due to the assumption which elongated cells may be problem for prolong the 1,3-propanediol production in fed-batch fermentation, therefore, the morphological changes of cells in these environments were also verified again by SEM. Figure 5.17 confirms that there was no severe elongated problem of cells as the elongated cells in these conditions could produce 1,3-propanediol up to a satisfying value of 52.29 g/l.

5.5.3 Model verification

Model verification was performed in order to test the model accuracy. The comparison of experimental results and simulated results are shown in Figure 5.18.

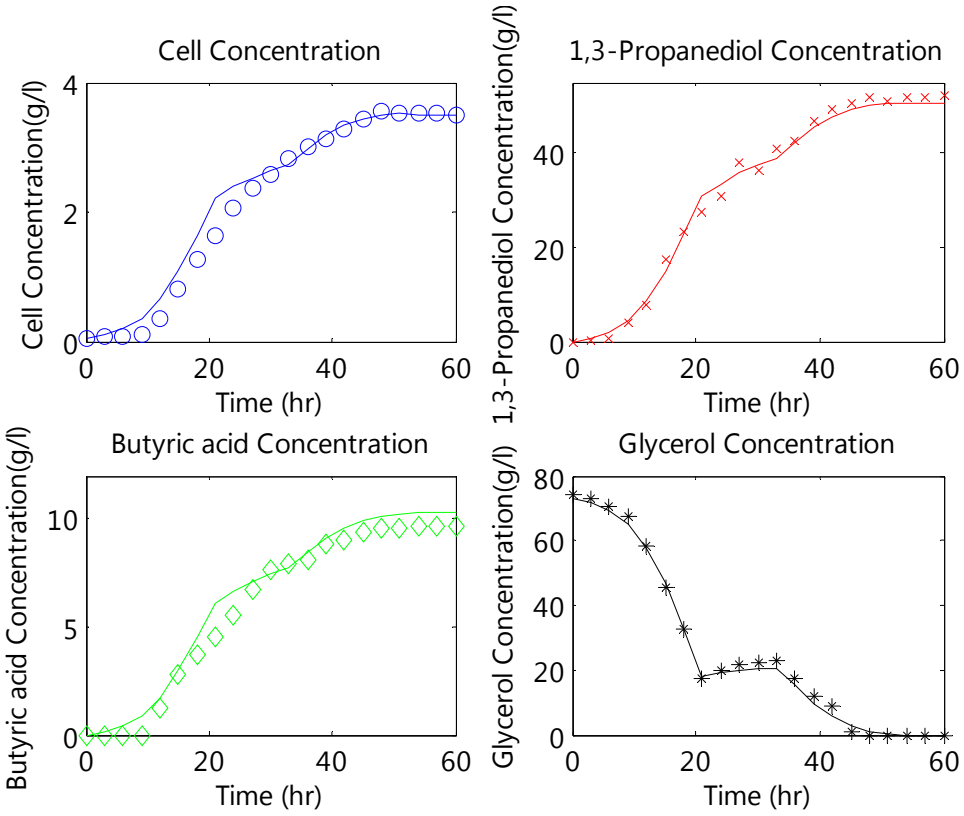


Figure 5.18 Comparison of experimental results (○,x,◊,∗) and simulated results (-) in 15-L fed-batch fermenter

The calculated deviation between the simulated results and the experimental results showed that the simulated results of cell, glycerol, 1,3-propanediol, and butyric acid concentrations fitted the experimental results with a deviation of 1.4%, 0.3%, 0.6%, and 1.2%, respectively, while an average deviation is 1.0%. This verified that the proposed cell model was absolutely appropriate model for determining the achievable 1,3-propanediol concentration from glycerol using *Clostridium butyricum* DSM 5431.

5.5.4 Suggestion a suitable mode for operation

Finally, in order to evaluate the proper mode of operation, therefore, the comparison of batch and fed-batch results was further studied. The final 1,3-propanediol concentrations, yields, and productivities obtained from both fermentations were shown in Table 5.10.

Table 5.10 Comparison of batch and fed-batch results in 15-L fermenter

Fermentation	1,3-propanediol concentration (g/l)	Yield (mol/mol)	Productivity (g/l · hr)
Batch	37.10	0.62	1.37
Fed-batch (Full volume)	44.07	0.42	1.30
Fed-batch (Depleted glycerol)	51.67	0.43	1.08

Although the productivities of fed-batch fermentation in both cases did not higher than batch fermentation as expected, the achievable 1,3-propanediol concentration of fed-batch fermentation at time of depleted glycerol was significantly 39.27% higher than batch fermentation. The final 1,3-propanediol concentration of fed-batch fermentation in this study was comparable to or higher than the previous report (Gunzel et al., 1991; Abbad-andaloussi et al., 1995; Reimann et al. 1996). Therefore, this means that fed-batch fermentation was a suitable mode of operation than batch because of the more achieving 1,3-propanediol concentration and the easy approach of 1,3-propanediol purification due to the more distinction of product concentrations in culture medium.

CHAPTER VI
CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

6.1.1 Batch fermentation

1. Glycerol was distinctively found to inhibit cell growth and 1,3-propanediol production at concentration levels equal to or higher than 100 g/l.

2. **1-L fermenter under 33 °C and pH 7.0 :** maximum 1,3-propanediol concentration (44.75 g/l) with the conversion yield of 0.77 mol/mol was achieved when cells were cultivated with optimal initial glycerol concentration of 80 g/l .

3. **15-L fermenter under 33 °C and pH 7.0 :** maximum 1,3-propanediol concentration (42.89 g/l) with the conversion yield of 0.71 mol/mol was achieved when cells were cultivated with optimal initial glycerol concentration of 80 g/l.

6.1.2 Kinetic model development

1. The most suitable cell model indicated multiple inhibition effects of glycerol, 1,3-propanediol, and butyric acid on *Clostridium butyricum* DSM 5431 growth.

$$\mu = \mu_{max}\left(\frac{C_{Gly}}{C_{Gly}+K_S}\right)\left(1-\frac{C_{Gly}}{C^*_{Gly}}\right)\left(1-\frac{C_{PD}}{C^*_{PD}}\right)\left(1-\frac{C_{HBu}}{C^*_{HBu}}\right)$$

In this study, it was found that μ_{max} and K_S were 0.3786 hr^{-1} and 0.7629 g/l while C^*_{Gly}, C^*_{PD}, and C^*_{HBu} were literaturely determined at 181.7 g/l, 66.4 g/l, and 11.1 g/l, respectively. Therefore, the proposed cell model in this study can be written as follows:

$$\mu = 0.3786\left(\frac{C_{Gly}}{C_{Gly}+0.7629}\right)\left(1-\frac{C_{Gly}}{181.7}\right)\left(1-\frac{C_{PD}}{66.4}\right)\left(1-\frac{C_{HBu}}{11.1}\right)$$

2. The proposed cell model was of high accuracy with the deviation of only 2.77% between simulated and experimental results.

6.1.3 Operating strategy for 1,3-propanediol production

1. Comparison of three feeding strategies using productivity as a criteria suggested that the proper feeding strategy for this particular system in fed-batch fermentation is a constant feed with a feed rate of 0.353 l/hr.

2. Fed-batch fermentation was revealed to be a suitable mode of operation and 1,3-propanediol concentration of 39.27% higher than batch fermentation was successfully obtained.

6.2 Recommendations

1. The potential use of industrial glycerol in this system should be evaluated in the future.
2. Based on the positive outcome gained in this study, the application of developed cell model for fermentation in other processes such as continuous should be considered.
3. From a biotechnological point of view, 1,3-propanediol production from glycerol by clostridia will now need further development in the field of strain improvement in order to improve the 1,3-propanediol yield.

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APPENDICES

APPENDIX A

EXPERIMENTAL DATA

A-1 Experimental data of batch fermentations

A-1.1 Data of cell, substrate, and product concentration at 60 g/l of initial glycerol concentration under 33 °C, pH 7.0, and 100 rpm in 1-L fermenter

Time (hr)	Cell concentration (g/l)	1,3-propanediol concentration (g/l)	Glycerol concentration (g/l)
0	0.052	0.00	50.97
3	0.058	0.00	50.54
6	0.060	0.18	50.26
9	0.061	7.87	42.39
12	0.236	13.69	35.45
15	0.867	20.41	22.42
18	1.180	27.90	9.07
21	1.601	30.84	2.04
24	2.426	30.74	1.20
27	3.086	31.01	1.02
30	3.058	30.05	0.60
33	3.045	31.99	0.46
36	3.064	32.82	0.44
39	3.043	31.73	0.18
42	3.071	32.42	0.23
45	3.040	32.62	0.20
48	2.922	32.06	0.23

A-1.2 Data of cell, substrate, and product concentrations at 80 g/l of initial glycerol concentration under 33 °C, pH 7.0, and 100 rpm in 1-L fermenter

Time (hr)	Cell concentration (g/l)	1,3-propanediol concentration (g/l)	Glycerol concentration (g/l)	Butyric acid concentration (g/l)	Acetic acid concentration (g/l)
0	0.053	0.00	69.95	0.00	0.20
3	0.053	0.00	65.46	0.00	0.24
6	0.057	0.73	66.45	0.00	0.39
9	0.064	5.09	63.99	1.14	0.74
12	0.329	14.99	48.95	2.42	1.13
15	0.975	25.15	34.22	2.70	1.53
18	1.208	31.20	11.71	5.28	0.34
21	1.558	42.96	2.88	6.36	0.48
24	1.972	41.15	0.31	6.79	0.54
27	2.163	41.96	0.20	6.26	0.43
30	2.502	43.36	0.18	7.04	0.26
33	2.629	43.54	0.31	8.42	0.44
36	2.912	42.48	0.23	8.53	0.37
39	2.924	45.27	0.19	5.66	1.13
42	3.039	44.73	0.28	5.42	0.55
45	3.039	45.58	0.33	6.13	0.95
48	2.936	44.75	0.17	6.60	0.66

A-1.3 Data of cell, substrate, and product concentration at 100 g/l of initial glycerol concentration under 33 °C, pH 7.0, and 100 rpm in 1-L fermenter

Time (hr)	Cell concentration (g/l)	1,3-propanediol concentration (g/l)	Glycerol concentration (g/l)
0	0.052	0.59	96.51
3	0.087	0.60	95.18
6	0.100	0.61	95.02
9	0.143	0.63	94.87
12	0.184	0.79	94.27
15	0.234	1.16	94.17
18	0.408	1.36	91.52
21	0.751	2.90	88.97
24	0.980	3.37	86.15
27	1.310	4.44	85.52
30	1.679	9.85	82.89
33	2.292	14.03	71.19
36	2.474	16.18	64.88
48	2.823	31.60	38.46

A-1.4 Data of cell, substrate, and product concentration at 120 g/l of initial glycerol concentration under 33 °C, pH 7.0, and 100 rpm in 1-L fermenter

Time (hr)	Cell concentration (g/l)	1,3-propanediol concentration (g/l)	Glycerol concentration (g/l)
0	0.052	0.59	111.75
3	0.076	0.60	109.49
6	0.091	0.62	109.45
9	0.094	0.63	108.48
12	0.103	0.64	107.91
15	0.123	0.67	106.11
18	0.155	0.71	105.68
21	0.183	1.00	105.05
24	0.206	1.58	103.81
27	0.245	1.76	102.79
30	0.269	1.98	101.98
33	0.389	2.08	100.04
36	0.424	2.96	99.04
48	0.709	4.19	95.19

A-1.5 Data of cell, substrate, and product concentration at 80 g/l of initial glycerol concentration under 33 °C, pH 7.0, and 48 rpm in 15-L fermenter

Time (hr)	Cell concentration (g/l)	1,3-propanediol concentration (g/l)	Glycerol concentration (g/l)
0	0.056	0.00	75.88
12	0.062	0.00	73.07
24	0.127	0.51	63.23
36	0.298	1.74	62.49
48	0.785	9.53	62.02

A-1.6 Data of cell, substrate, and product concentrations at 80 g/l of initial glycerol concentration under 33 °C, pH 7.0, and 100 rpm in 15-L fermenter

Time (hr)	Cell concentration (g/l)	1,3-propanediol concentration (g/l)	Glycerol concentration (g/l)	Butyric acid concentration (g/l)
0	0.051	0.00	72.98	0.00
3	0.073	0.00	72.06	0.00
6	0.089	0.21	69.96	0.00
9	0.100	2.40	69.48	1.26
12	0.354	12.10	62.87	1.99
15	0.643	17.97	49.11	2.30
18	1.117	22.42	34.18	2.91
21	1.927	28.07	17.06	5.69
24	2.543	36.26	1.16	6.28
27	2.955	37.10	0.00	6.70
30	2.895	37.80	0.00	6.68
33	2.893	38.27	0.00	7.09
36	2.923	40.87	0.00	7.78
39	2.907	42.08	0.00	7.67
42	2.920	41.71	0.00	8.02
45	2.961	42.86	0.00	8.18
48	2.890	42.89	0.00	7.91

A-2 Experimental data of fed-batch fermentation

A-2.1 Data of cell, substrate, and product concentrations at 80 g/l of initial glycerol concentration and 120 g/l of glycerol concentration in feed under 33 °C, pH 7.0, and 100 rpm in 15-L fermenter

Time (hr)	Cell concentration (g/l)	1,3-propanediol concentration (g/l)	Glycerol concentration (g/l)	Butyric acid concentration (g/l)
0	0.054	0.00	73.96	0.00
3	0.076	0.07	72.69	0.00
6	0.093	0.75	70.48	0.00
9	0.115	4.14	67.33	0.00
12	0.338	7.57	58.06	1.26
15	0.801	17.35	45.42	2.75
18	1.281	23.11	32.54	3.75
21	1.628	27.46	17.32	4.53
24	2.060	30.86	19.86	5.58
27	2.366	37.87	21.75	6.69
30	2.587	36.44	22.32	7.65
33	2.811	40.91	23.05	7.91
36	3.025	42.78	17.36	8.10
39	3.132	46.82	11.85	8.87
42	3.268	49.30	8.84	9.00
45	3.435	50.69	1.02	9.39
48	3.564	51.67	0.00	9.53
51	3.538	50.93	0.00	9.57
54	3.535	51.69	0.00	9.63
57	3.528	51.98	0.00	9.63
60	3.514	52.29	0.00	9.65

APPENDIX B

FORMULAE OF FERMENTATIVE MEDIUM

There were 3 varieties of medium used in this study. One was for 1,3-propanediol production and the other was designated for cell cultivation. The main component of the medium was glycerol which was used as carbon and energy source for *Clostridium butyricum* DSM 5431. For 1 litre of medium solution, the ingredient of each medium are listed as follows:

1. Reinforced Clostridial Medium (RCM medium)

RCM medium	38.0 g

2. Preculture medium

K_2HPO_4	3.4 g
KH_2PO_4	1.3 g
$(NH_4)_2SO_4$	2.0 g
$MgSO_4.7H_2O$	0.2 g
$CaCl_2.2H_2O$	0.02 g
$CaCO_3$	2.0 g
Yeast extract	1.0 g
Glycerol	l20 g
Trace element solution	1 ml
Fe solution	2 ml

3. Fermentation medium

K_2HPO_4	1.0 g
KH_2PO_4	0.5 g
$(NH_4)_2SO_4$/100 g Glycerol	5.0 g
$MgSO_4.7H_2O$	0.2 g
$CaCl_2.2H_2O$	0.02 g
Yeast extract	1.0 g
Glycerol	as required 60-l20 g

Trace element solution	1 ml
Fe solution	1 ml

The compositions of trace element solution and Fe solution per litre are detailed as follows:

1. Trace element solution

$ZnCl_2$	70 mg
$MnCl_2.4H_2O$	0.1 g
H_3BO_3	60 mg
$CoCl_2.2H_2O$	0.2 g
$CuCl_2.2H_2O$	20 mg
$NiCl_2.6H_2O$	25 mg
$Na_2MoO_4.2H_2O$	35 mg
HCl (37%)	0.9 ml

2. Fe solution

$FeSO_4.7H_2O$	5 g
HCl (37%)	4 ml

APPENDIX C

CALCULATION OF PARAMETERS

C-1 Calculation of specific growth rate (μ)

The specific growth rate can be calculated from the following equation:

$$\frac{dX}{dt} = \mu X \tag{C-1}$$

$$\int_{X_0}^{X} \frac{1}{X} \cdot dX = \int_{t_0}^{t} \mu \cdot dt \tag{C-2}$$

$$\ln X = \mu(t - t_0) + \ln X_0 \tag{C-3}$$

Using the equation (C-3), if plot ln X versus $t-t_0$, it can obtain the specific growth rate as the slope of the plot.

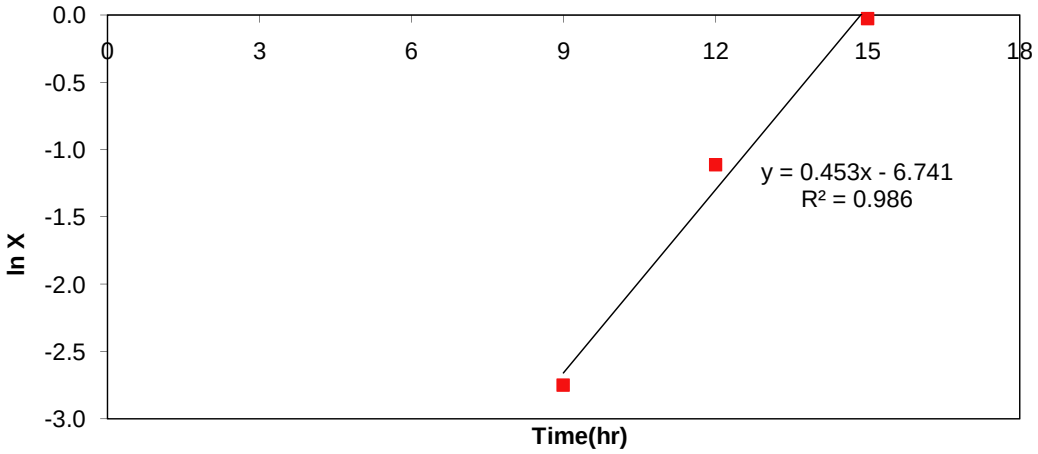


Figure C-1 Correlation between ln of cell concentration and time

C-2 Calculation of specific consumption rate (υ)

The specific consumption rate can be calculated from the following equation:

$$\frac{dS}{dt} = -\upsilon \cdot X \qquad \text{(C-4)}$$

$$\int_{S_0}^{S} dS = \int_{t_0}^{t} -\upsilon \cdot X \cdot dt \qquad \text{(C-5)}$$

$$S = -\upsilon \int_{t_0}^{t} X \cdot dt + S_0 \qquad \text{(C-6)}$$

Using the equation (C-6), if plot S versus $\int_{t_0}^{t} X \cdot dt$, it can obtain the specific consumption rate as the slope of the plot.

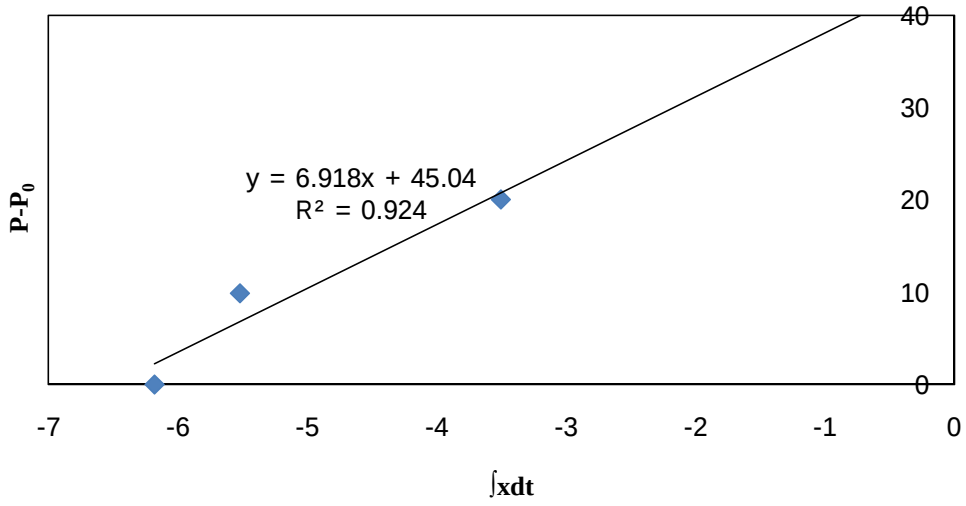


Figure C-2 Correlation between product concentration and integration of cell concentration

C-3 Calculation of specific production rate (ρ)

The specific production rate can be calculated from the following equation:

$$r_P = \frac{dP}{dt} = \rho \cdot X \qquad \text{(C-7)}$$

$$\int_{P_0}^{P} dP = \int_{t_0}^{t} \rho \cdot X \cdot dt \qquad \text{(C-8)}$$

$$P = \rho \int_{t_0}^{t} X \cdot dt + P_0 \qquad \text{(C-9)}$$

Using the equation (C-9), if plot P versus $\int_{t_0}^{t} X \cdot dt$, it can obtain the specific production rate as the slope of the plot.

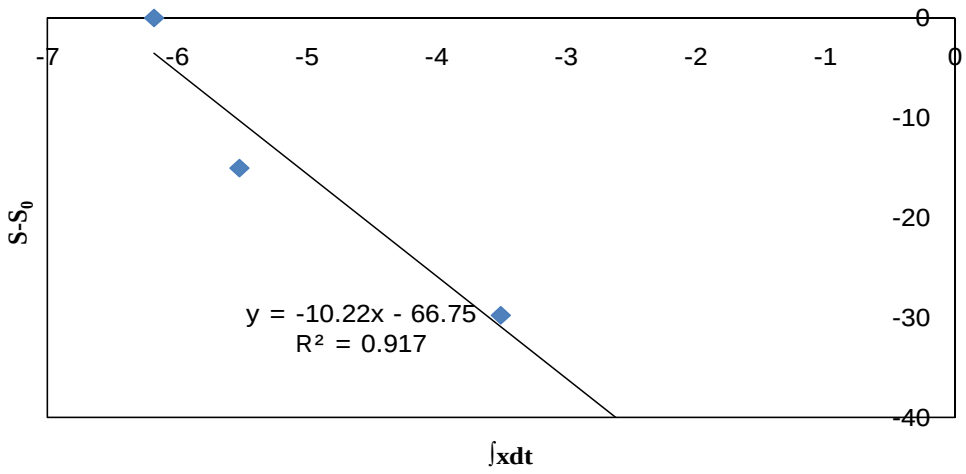


Figure C-3 Correlation between substrate concentration and integration of cell concentration

C-4 Calculation of yields

The yields of product on substrate, cell on substrate, and product on cell can be determined from the following equation:

$$Y_{P/S} = \frac{\Delta P}{\Delta S} \quad \text{(C-10)}$$

$$Y_{X/S} = \frac{\Delta X}{\Delta S} \quad \text{(C-11)}$$

$$Y_{P/X} = \frac{\Delta P}{\Delta X} \quad \text{(C-12)}$$

C-5 Calculation of gas flow rate per unit liquid volume (Q/V)

The volumetric gas flow rate per unit liquid volume can be calculated from the following equation:

$$\frac{Q}{V} = \frac{\text{gas transfer rate (l/min)}}{\text{volume of culture medium (l)}} \quad \text{(C-13)}$$

C-6 Calculation of power input per unit volume (P/V)

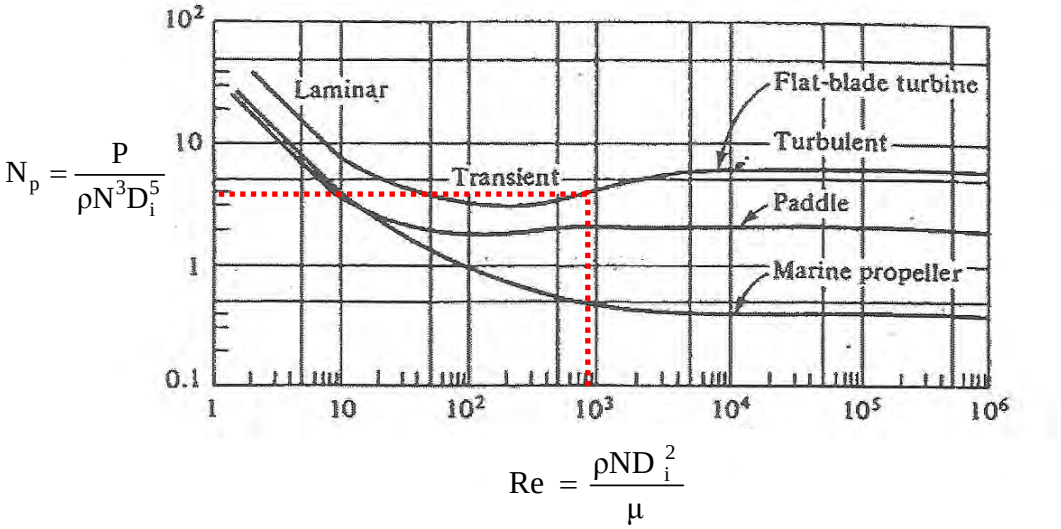
The required data from experiment (1-L fermenter)

- Diameter of impeller (D_i) = 0.028 m
- Agitation speed (N) = 100 rpm = 1.67 rps

Time (hr)	Density (g/l)	Viscosity (kg/m·s)
0	1024.41	0.00143
12	1017.14	0.00153
24	994.52	0.00143
36	996.09	0.00147
48	1015.82	0.00156
Average	1009.60	0.00148

Calculating Reynolds number, gives:

$$\mathrm{Re} = \frac{\rho N D_i^2}{\mu} = \frac{(1009.60)(1.67)(0.028)^2}{0.00148} = 893 \qquad \text{(C-14)}$$



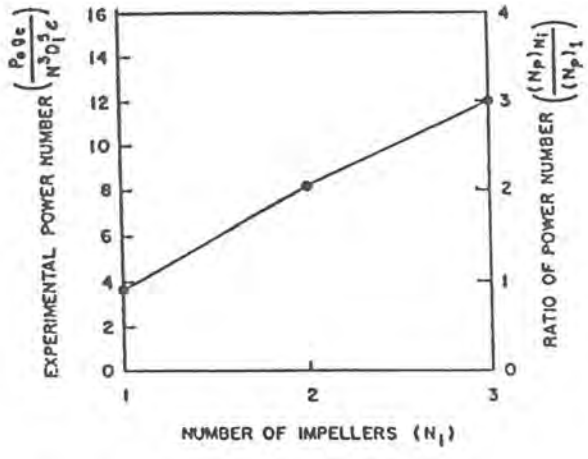
Because the type of impeller in this study was flat-blade turbine, therefore, the power number that was found from graph was 3.8.

Substituting known values and solving for ungassed power input (P) from the following equation:

$$N_p = \frac{P}{\rho N^3 D_i^5} \qquad \text{(C-15)}$$

$$P = (3.8)(1009.60)(1.67)^3(0.028)^5 = 3.08\times10^{-4}\ \mathrm{W}$$

The ungassed power input must then be estimated by the relationship given in the following graph:



Since the fermenter in this study had 2 impellers, therefore, the ratio of power number was 2. Then, gives:

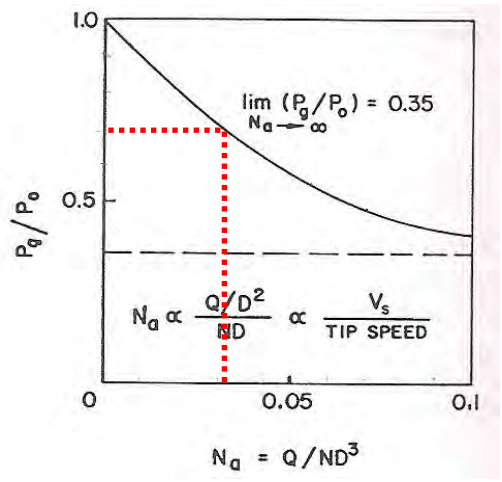
$$P = (3.08\times10^{-4})(2) = 6.16\times10^{-4}\ W$$

In this study, gas flow rate per unit liquid volume was 0.1 vvm and the volume of culture medium was 700 ml. Substituting known values and then gives:

$$Q = (0.1)(0.0007) = 7\times10^{-5}\ m^3/min$$

Calculating aeration number (N_a), gives:

$$N_a = \frac{Q}{ND_i^3} = \frac{7\times10^{-5}}{(1.67)(0.028)^3} = 0.032 \qquad \text{(C-16)}$$



Estimation of gassed power input could be obtained from the following relationship:

$$\text{gassing factor} = \frac{P_g}{P} \qquad \text{(C-17)}$$

$$P_g = (6.16\times10^{-4})(0.7) = 4.31\times10^{-4}\ W$$

Owing to the volume of culture medium in this study was 0.7 litre. Therefore, the power input per unit volume was

$$\frac{P_g}{V} = \frac{4.31\times10^{-4}}{0.0007} = 0.62\ W/m^3$$

C-7 Determination of proper glycerol concentration in feed

In order to determine the proper glycerol concentration in feed, glycerol concentrations (100–130 g/l) were varied by using mathematical simulation. The concentration profiles of cell, glycerol, 1,3-propanediol and butyric acid at different glycerol concentrations in feed are also shown in Figure C-4 – C-7.

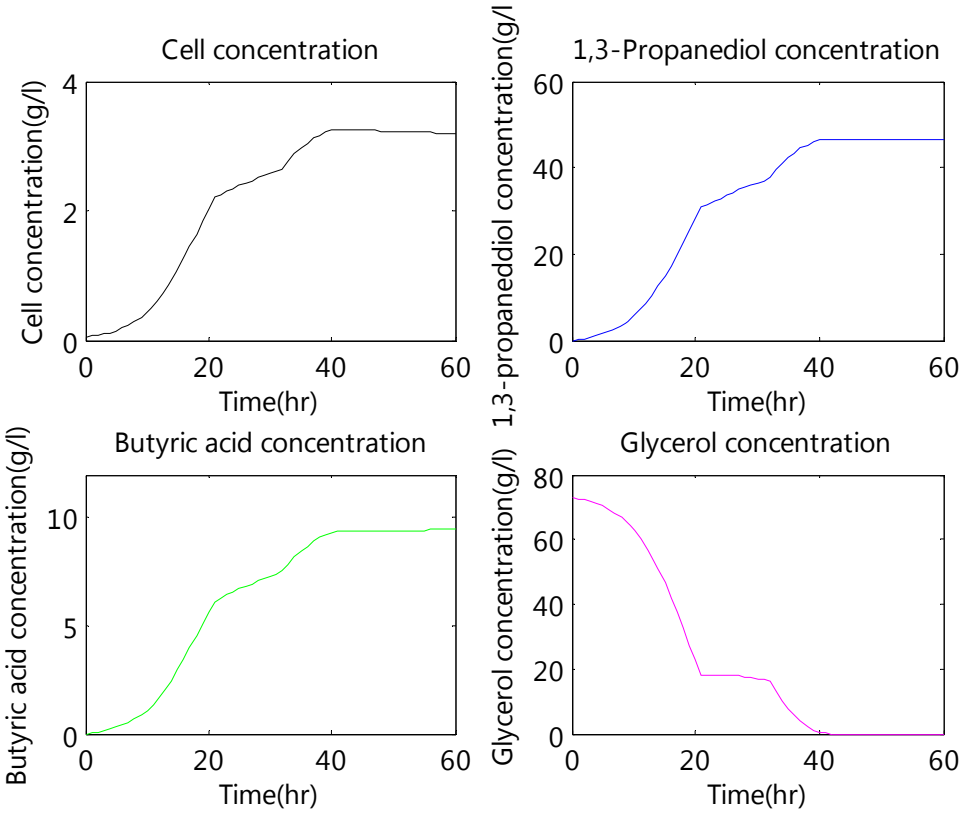


Figure C-4 The simulated profiles of cell, glycerol, 1,3-propanediol and butyric acid at glycerol concentrations in feed of 100 g/l using constant feeding strategy

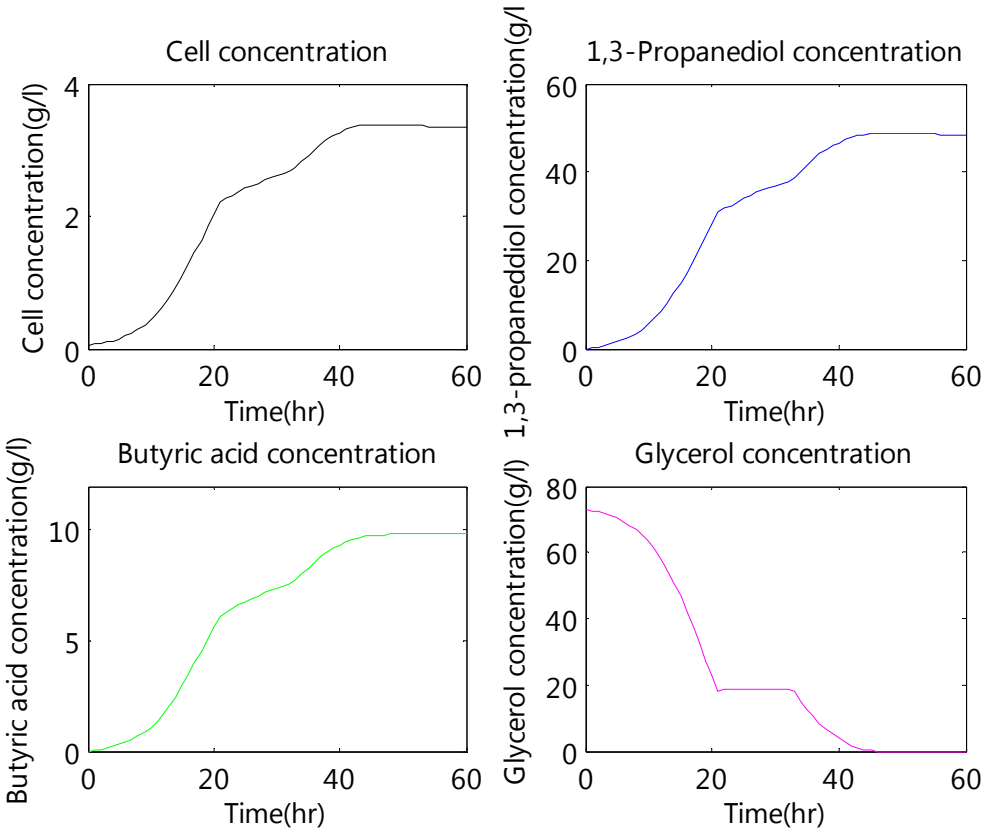


Figure C-5 The simulated profiles of cell, glycerol, 1,3-propanediol and butyric acid at glycerol concentrations in feed of 110 g/l using constant feeding strategy

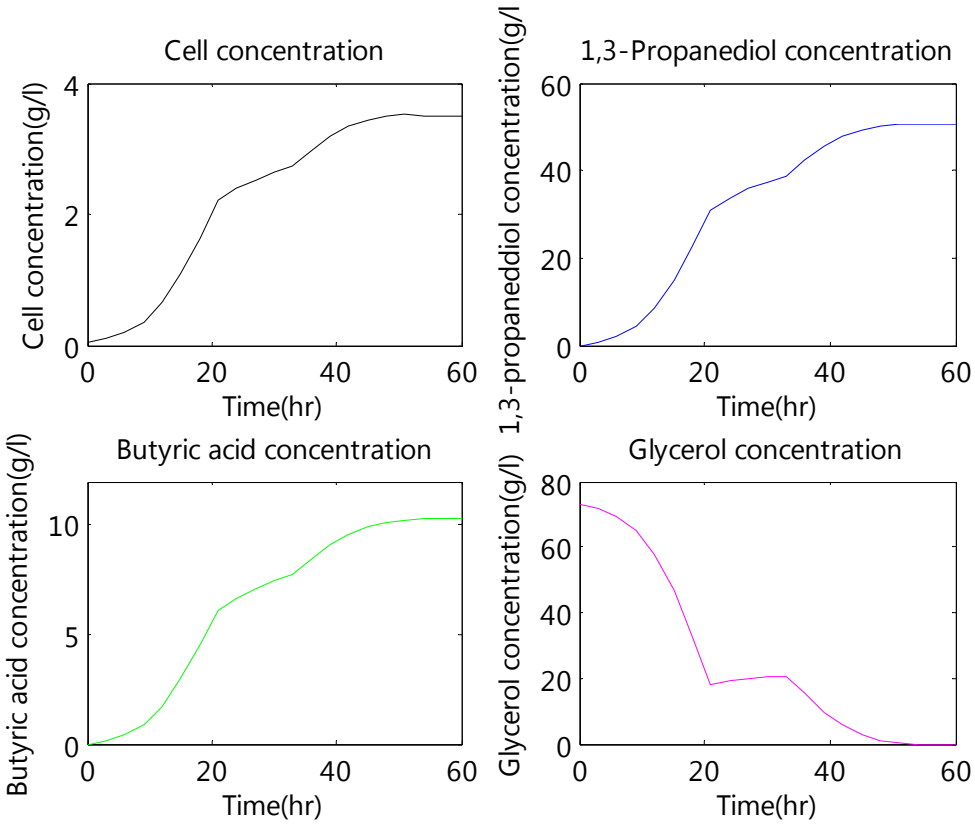


Figure C-6 The simulated profiles of cell, glycerol, 1,3-propanediol and butyric acid at glycerol concentrations in feed of 120 g/l using constant feeding strategy

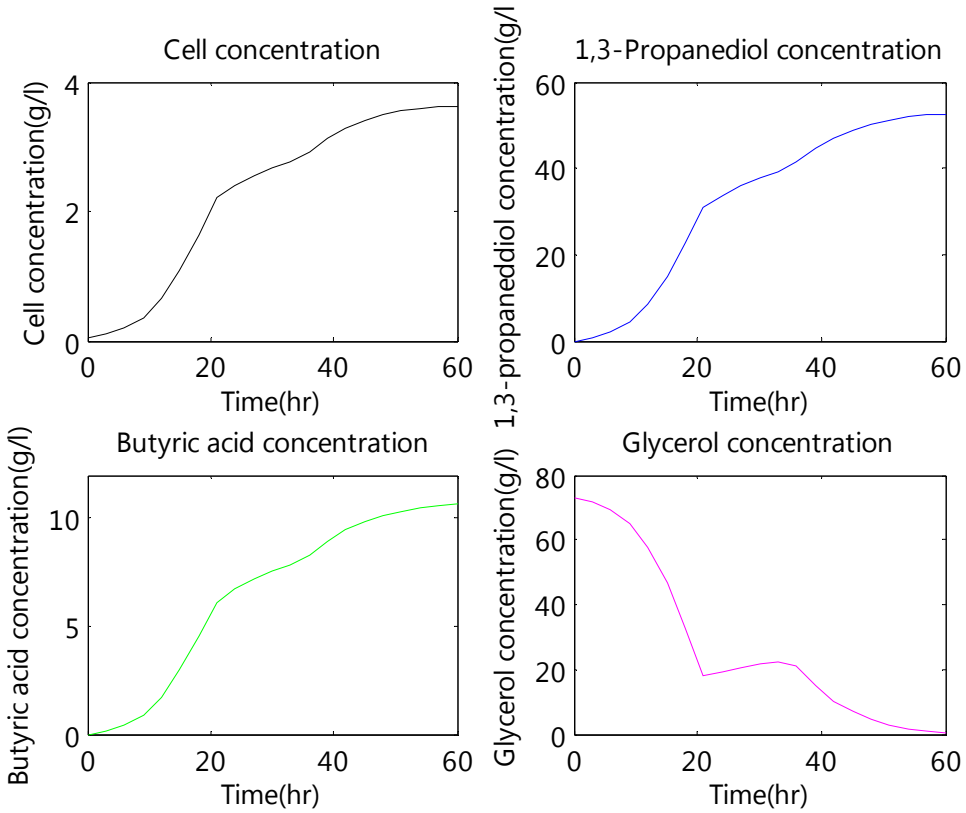


Figure C-7 The simulated profiles of cell, glycerol, 1,3-propanediol and butyric acid at glycerol concentrations in feed of 130 g/l using constant feeding strategy

C-8 Determination of constant parameters (a and b)

Since this study purposed that the increased feeding rate should be between constant feeding rate and exponential feeding rate, therefore, the suitable constant parameters of a and b in the increased feeding strategy were found**.**

The constant value of b was corresponding to constant feeding rate. While the constant value of a was found by varying values (0.01-0.15). The proper value of parameter a was 0.05 because this value would give the increased feeding rate between the constant feeding rate and exponential feeding rate. The concentration profiles of cell, glycerol, 1,3-propanediol and butyric acid at different values of parameter a are also shown in Table C-1.

Table C-1 Feeding rates of various feeding strategies in fed-batch fermentation using glycerol concentration in feed of 120 g/l

Time (hr)	Feeding rate (l/hr)					
	Constant	Increased (a=0.01)	Increased (a=0.05)	Increased (a=0.1)	Increased (a=0.15)	Exponential
21	0.353	0.353	0.353	0.353	0.353	0.353
24	0.353	0.383	0.503	0.653	0.803	0.533
27	0.353	0.413	0.653	0.953	1.253	0.804
30	0.353	0.443	0.803	1.253	1.703	1.213
33	0.353	0.473	0.953	1.553	2.153	1.829
36	0.353	0.503	1.103	1.853	2.603	2.759
39	0.353	0.533	1.253	2.153	3.053	4.161
42	0.353	0.563	1.403	2.453	3.503	6.276
45	0.353	0.593	1.553	2.753	3.953	9.466
48	0.353	0.623	1.703	3.053	4.403	14.278

C-9 Calculation of deviation

The example of calculated deviation is shown in Figure C-8. If plot the experimental data versus the simulated data, the deviation can be calculated by using the equation below:

Deviation = $100 - (R^2 \times 100) = 100 - (0.971 \times 100) = 2.9\%$

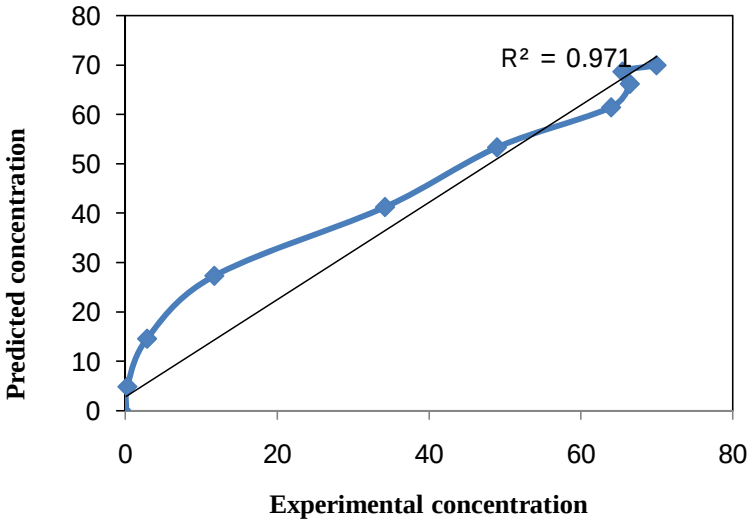


Figure C-8 Calculation of deviation

APPENDIX D

SOURCE CODE OF MATLAB

D-1 Source code of batch fermentation in 1-L fermenter

```
function dC = GLY(t,C,P)

CPD = 66.4;
CBu = 11.1;
CGly = 181.7;
S = C(4);
U = P(11)*(S/(S+P(2)))*(1-(C(2)/CPD))*((1-(C(3)/CBu)))*(1-(C(4)/CGly));

dC = zeros(4,1);
dC(1) = (U-P(1))*C(1);
dC(2) = ((P(3)*U)+P(4))*C(1);
dC(3) = ((P(5)*U)+P(6))*C(1);
dC(4) = -((U/P(7))+(P(9))+(((P(3)*U)+P(4))/P(8))+(((P(5)*U)+P(6))/P(10)))*C(1);


function F = OFGLY(P)

global data_50

time  = [0 3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48];
x0 = [0.05  0  0  69.95];
[t,C]  = ode45(@GLY,time,x0,[ ],P);

FF  = (data_50-C).^2;

FFF = [FF(:,1);FF(:,2);FF(:,3);FF(:,4)];
F = sum(sum(FFF));
```

```
global data_50

data_50 =  [0.05      0      0   69.95;
            0.05      0      0   65.46;
            0.06   0.73      0   66.45;
            0.06   5.09   1.14   63.99;
            0.33  14.99   2.42   48.95;
            0.98  25.15   2.70   34.22;
            1.21  31.20   5.28   11.71;
            1.56  42.96   6.36    2.88;
            1.97  41.15   6.79    0.31;
            2.16  41.96   6.26    0.20;
            2.50  43.36   7.04    0.18;
            2.63  43.54   8.42    0.31;
            2.91  42.48   8.53    0.23;
            2.92  45.27   5.66    0.19;
            3.04  44.73   5.42    0.28;
            3.04  45.58   6.13    0.33;
            2.94  44.75   6.60    0.17];

rand('state', 71);
randn('state', 59);

A = [ ];
beq = [ ];
b  = [ ];
Aeq = [ ];
lb = [0 0.5 13 0.005 2.8 0.004 0.88 0.8 0 0.9 0.13];
ub = [0.001 0.9 16 0.008 3.2 0.006 1 0.9 0.04 1 0.4];

nonlcon = [ ];

Range = [0; 1;];

options = gaoptimset('Generations',1000,'PopulationSize',100, 'PlotFcns',
@gaplotbestf, 'Display','iter');

[P FVAL reason output] = ga(@OFGLY,11,A,b,Aeq,beq,lb,ub,nonlcon,options);

disp('Kinetic parameters obtained:')
P
```

```
x0 = [0.05  0  0  69.95];

Data_50 =  [0.05       0       0   69.95     0;
            0.05       0       0   65.46     3;
            0.06    0.73       0   66.45     6;
            0.06    5.09    1.14   63.99     9;
            0.33   14.99    2.42   48.95    12;
            0.98   25.15    2.70   34.22    15;
            1.21   31.20    5.28   11.71    18;
            1.56   42.96    6.36    2.88    21;
            1.97   41.15    6.79    0.31    24;
            2.16   41.96    6.26    0.20    27;
            2.50   43.36    7.04    0.18    30;
            2.63   43.54    8.42    0.31    33;
            2.91   42.48    8.53    0.23    36;
            2.92   45.27    5.66    0.19    39;
            3.04   44.73    5.42    0.28    42;
            3.04   45.58    6.13    0.33    45;
            2.90   44.75    6.60    0.17    48];

[t,C] = ode45(@GLY,[0 3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48],x0,[ ],P);

figure
subplot(2,2,1);
plot (t,C(:,1),'b-',Data_50(:,5),Data_50(:,1),'bo');axis ([0 48 0 4]);
title ('Cell Concentration');
xlabel('Time (hr)'),ylabel('Cell Concentration(g/l)')
subplot(2,2,2);
plot (t,C(:,2),'r-',Data_50(:,5),Data_50(:,2),'rx');axis ([0 48 0 50]);
title ('1,3-Propanediol Concentration');
xlabel('Time (hr)'),ylabel('1,3-Propanediol Concentration(g/l)')
subplot (2,2,3);
plot (t,C(:,3),'g-',Data_50(:,5),Data_50(:,3),'gd');axis ([0 48 0 10]);
title ('Butyric acid Concentration');
xlabel('Time (hr)'),ylabel('Butyric acid Concentration(g/l)')
subplot (2,2,4);
plot (t,C(:,4),'k-',Data_50(:,5),Data_50(:,4),'k*');axis ([0 48 0 80]);
title ('Glycerol Concentration');
xlabel('Time (hr)'),ylabel('Glycerol Concentration(g/l)')
```

D-2 Source code of batch fermentation in 15-L fermenter

```
function dC = GLY(t,C,P)

CPD = 66.4;
CBu = 11.1;
CGly = 181.7;
S = C(4);
U = P(11)*(S/(S+P(2)))*(1-(C(2)/CPD))*((1-(C(3)/CBu)))*(1-(C(4)/CGly));

dC = zeros(4,1);
dC(1) = (U-P(1))*C(1);
dC(2) = ((P(3)*U)+P(4))*C(1);
dC(3) = ((P(5)*U)+P(6))*C(1);
dC(4) = -((U/P(7))+(P(9))+(((P(3)*U)+P(4))/P(8))+(((P(5)*U)+P(6))/P(10)))*C(1);


function F = OFGLY(P)

global data_50

time = [0 3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48];
x0  = [0.05  0  0  72.98];
[t,C]  = ode45(@GLY,time,x0,[ ],P);

FF  = (data_50-C).^2;

FFF = [FF(:,1);FF(:,2);FF(:,3);FF(:,4)];
F = sum(sum(FFF));


global data_50

data_50  =  [0.05      0      0   72.98;
             0.07      0      0   72.06;
             0.09   0.21      0   69.96;
             0.10   2.40   1.26   69.48;
             0.35  12.10   1.99   62.87;
             0.64  17.97   2.30   49.11;
             1.12  22.42   2.91   34.18;
             1.93  28.07   5.69   17.06;
             2.54  36.26   6.28    1.16;
             2.96  37.10   6.70       0;
             2.90  37.80   6.68       0;
             2.90  38.27   7.09       0;
             2.92  40.87   7.78       0;
             2.91  42.08   7.67       0;
             2.92  41.71   8.02       0;
             2.96  42.86   8.18       0;
             2.89  42.89   7.91       0];
```

```
rand('state', 71);
randn('state', 59);

A = [ ];
beq = [ ];
b = [ ];
Aeq = [ ];
lb = [0 0.5 13 0.005 2.8 0.004 0.88 0.8 0 0.43 0.13];
ub = [0.001 0.9 16 0.008 3.2 0.006 1 0.9 0.04 0.5 0.4];

nonlcon = [ ];

Range = [0; 1;];

options = gaoptimset('Generations',1000,'PopulationSize',100, 'PlotFcns',
@gaplotbestf, 'Display','iter');

[P FVAL reason output] = ga(@OFGLY,11,A,b,Aeq,beq,lb,ub,nonlcon,options);

disp('Kinetic parameters obtained:')
P


x0  = [0.05  0  0  72.98];

Data_50  =  [0.05       0       0   72.98     0;
             0.07       0       0   72.06     3;
             0.09    0.21       0   69.96     6;
             0.10    2.40    1.26   69.48     9;
             0.35   12.10    1.99   62.87    12;
             0.64   17.97    2.30   49.11    15;
             1.12   22.42    2.91   37.42    18;
             1.93   28.07    5.69   17.06    21;
             2.54   36.26    6.28    1.16    24;
             2.96   37.10    6.70       0    27;
             2.90   37.80    6.68       0    30;
             2.90   38.27    7.09       0    33;
             2.92   40.87    7.78       0    36;
             2.91   42.08    7.67       0    39;
             2.92   41.71    8.02       0    42;
             2.96   42.86    8.18       0    45;
             2.89   42.89    7.91       0    48];

[t,C] = ode45(@GLY,[0 3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48],x0,[ ],P);

figure
subplot(2,2,1);
plot (t,C(:,1),'b-',Data_50(:,5),Data_50(:,1),'bo');axis ([0 48 0 4]);
title ('Cell Concentration');
xlabel('Time (hr)'),ylabel('Cell Concentration(g/l)')
```

```
subplot(2,2,2);
plot (t,C(:,2),'r-',Data_50(:,5),Data_50(:,2),'rx');axis ([0 48 0 50]);
title ('1,3-Propanediol Concentration');
xlabel('Time (hr)'),ylabel('1,3-Propanediol Concentration(g/l)')
subplot (2,2,3);
plot (t,C(:,3),'g-',Data_50(:,5),Data_50(:,3),'gd');axis ([0 48 0 10]);
title ('Butyric acid Concentration');
xlabel('Time (hr)'),ylabel('Butyric acid Concentration(g/l)')
subplot (2,2,4);
plot (t,C(:,4),'k-',Data_50(:,5),Data_50(:,4),'k*');axis ([0 48 0 80]);
title ('Glycerol Concentration');
xlabel('Time (hr)'),ylabel('Glycerol Concentration(g/l)')
```

D-3 Source code of fed-batch fermentation in 15-L fermenter

```
function dy = fedbatch(t,y,FF)

Umax = 0.3786;
Ks = 0.7629;
Kd = 0.0003;
alphaPD = 14.2210;
betaPD = 0.0079;
alphaBu = 2.8089;
betaBu = 0.0040;
Mc = 0.0016;
Yxc = 0.9002;
Ypc = 0.8065;
Ybc = 0.4304;
Glyfeed = 120;


model = Umax*(y(4)/(y(4)+Ks))*(1-(y(2)/66.4))*((1-(y(3)/11.1)))*(1-(y(4)/181.7));

if t<21 ;
   F = FF(1);
else t>=21
   if    y(5)<12;
         F = FF(2);
   else F = FF(1);
   end
end
```

```
dy = zeros(5,1);

dy(1)  =  -(F/y(5))*y(1) + model*y(1) - Kd*y(1);
dy(2)  =  -(F/y(5))*y(2) + alphaPD*model*y(1) + betaPD*y(1) ;
dy(3)  =  -(F/y(5))*y(3) + alphaBu*model*y(1) + betaBu*y(1) ;
dy(4)  =  (F/y(5)*(Glyfeed - y(4))) - (model/Yxc)*y(1) - Mc*y(1) - ((betaPD*y(1)) +
(alphaPD*model*y(1)))/Ypc - ((betaBu*y(1)) + (alphaBu*model*y(1)))/Ybc;
dy(5)  =  F;


x0 = [0.05  0   0  72.98  7.5];
FF = [0  0.353];

Data_51  =  [0.05       0       0    73.96     0;
             0.08    0.07       0    72.69     3;
             0.09    0.75       0    70.48     6;
             0.11    4.14       0    67.33     9;
             0.34    7.57    1.26    58.06    12;
             0.80   17.35    2.75    45.42    15;
             1.28   23.11    3.75    32.54    18;
             1.63   27.46    4.53    17.32    21;
             2.06   30.86    5.58    19.86    24;
             2.37   37.87    6.69    21.75    27;
             2.59   36.44    7.65    22.32    30;
             2.81   40.91    7.91    23.05    33;
             3.02   42.78    8.10    17.36    36;
             3.13   46.82    8.87    11.85    39;
             3.27   49.30    9.00     8.84    42;
             3.43   50.69    9.39     1.02    45;
             3.56   51.67    9.53        0    48;
             3.54   50.93    9.57        0    51;
             3.54   51.69    9.63        0    54;
             3.53   51.98    9.63        0    57;
             3.51   52.29    9.65        0    60];


[t,C]   = ode45(@fedbatch,[0:3:60],x0,[ ],FF)

figure
subplot(2,2,1);
plot (t,C(:,1),'b-',Data_51(:,5),Data_51(:,1),'bo');axis ([0 60 0 4]);
title ('Cell Concentration');
xlabel('Time (hr)'),ylabel('Cell Concentration(g/l)')
subplot(2,2,2);
plot (t,C(:,2),'r-',Data_51(:,5),Data_51(:,2),'rx');axis ([0 60 0 55]);
title ('1,3-Propanediol Concentration');
xlabel('Time (hr)'),ylabel('1,3-Propanediol Concentration(g/l)')
```

```
subplot(2,2,3);
plot (t,C(:,3),'g-',Data_51(:,5),Data_51(:,3),'gd');axis ([0 60 0 12]);
title ('Butyric acid Concentration');
xlabel('Time (hr)'),ylabel('Butyric acid Concentration(g/l)')
subplot(2,2,4);
plot (t,C(:,4),'k-',Data_51(:,5),Data_51(:,4),'k*');axis ([0 60 0 80]);
title ('Glycerol Concentration');
xlabel('Time (hr)'),ylabel('Glycerol Concentration(g/l)')
```

APPENDIX E

LIST OF PUBLICATION

Nattawadee Laosirilurchakai, Muenduen Phisalaphong, Veerapat Tantayakom, Phatthanon Prasitchoke, and Seeroong Prichanont, “Kinetic model for 1,3-propanediol production from glycerol fermentation by *Clostridium butyricum* DSM 5431 in batch fermenter”, Extended Abstract for 12th Asian Pacific Confederation of Chemical Engineering Congress (APPChE) 2008 – Chemical Engineering in Sustainable Development, China, 4-6 August 2008, Paper ID Vol.4-109.

12th Asia Pacific Confederation of Chemical Engineering Congress
August 3-6, 2008
Dalian, China
http://apcche2008.ecust.edu.cn/
E-mail: apcche2008@ecust.edu.cn

To: Nattawadee Laosirilurchakai

Date: May 8, 2008

Dear Sir or Madam,

This is to confirm that you have attended and given an oral presentation at the 12th Asia Pacific Confederation of Chemical Engineering Congress (APCChE 2008) held in Dalian, China from August 3rd to 6th, 2008.

Yours sincerely,

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Kinetic model for 1,3-propanediol production from glycerol fermentation by *Clostridium butyricum* DSM 5431 in batch fermenter

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ABSTRACT

1,3-propanediol (1,3-PD) has a wide range of potential uses, particularly as a monomer for the production of biodegradable polymers such as polytrimethylene terephthalate (PTT) and as an intermediate for the synthesis of heterocyclic compounds. 1,3-PD is a typical product from anaerobic fermentation of glycerol, a main by-product in biodiesel production processes. In this study, 1,3-PD production by *Clostridium butyricum* DSM 5431 was investigated in batch culture using different initial glycerol concentrations 60-120 g l^{-1}. The optimum conditions in 1 litre batch fermenter were found to be 80 g l^{-1} initial glycerol concentration, 33 °C, and pH 7.0. The maximum product concentration obtained was 44.75 g l^{-1} with the production yield of 0.77 (mol/mol). The inhibition effect of glycerol on the growth and productivity was observed at concentration higher than 80 g l^{-1}. The kinetic model for 1,3-PD production in batch fermentation was further developed. The most suitable cell model was the specific growth rate model which indicated multiple inhibition effects of glycerol, 1,3-PD, and butyric acid on *Clostridium butyricum* DSM 5431 growth. The model is useful for applications in fed-batch fermentation where substrate concentration can be controlled to prolong the production.

Keywords: 1,3-propanediol, glycerol, *Clostridium butyricum* DSM 5431, substrate inhibition, kinetic model

INTRODUCTION

Nowadays, because of the rising price of traditional fuel and the increasing demand of fuel, the demand of alternative fuel such as biodiesel has increased. This leads to glycerol surplus in the world market because glycerol is formed as a main by-product in biodiesel production. The conversion to biodiesel fuel produces glycerol about 10% by weight [1,2]. Therefore, it is essentially advantageous to develop a technology that converts a large volume of glycerol into products of high value, for example, to 1,3-propanediol (1,3-PD) which is a useful compound for polymer industries, especially for producing biodegradable polymers such as polytrimethylene terephthalate (PTT). This compound has mainly been produced by both chemical and biotechnological routes. However, the biotechnological method is preferable since it is better for environmental protection, relatively high theoretical molar yield, and its raw material is cheaper than the chemical method [3,4,5,6,7].

1,3-PD can be produced from fermentation of glycerol under anaerobic condition by many microorganisms such as *Klebsiella pneumoniae*, *Clostridium butyricum*, *Citrobacter freundii*, and *Enterobacter agglomerans* [3,6,8,9,10,11,12,13,14,15,16,17,18,19,20,21]. However, according to its safety record and relatively high yields and productivities *Clostridium butyricum* DSM 5431 is preferred for potential industrial use in the production of 1,3-PD from glycerol [15,22].

In order to estimate the productivity of 1,3-PD in batch fermentation, the study of kinetic model of *Clostridium butyricum* DSM 5431 is necessary. To date there is no report on growth model of *Clostridium butyricum* DSM 5431 with mass balance equations in batch fermentation. Therefore, this study aims at developing the kinetic model of *Clostridium butyricum* DSM 5431 in a batch fermenter at optimal initial glycerol concentration. The developed model will be useful for the development of a scale-up process.

MATERIALS AND METHODS

Microorganism and medium

Clostridium butyricum DSM 5431, obtained from American Type Culture Collection (ATCC) BAA-557™, was used in this study. The composition of the preculture medium per liter was: 3.4 g K_2HPO_4; 1.3 g KH_2PO_4; 2 g $(NH_4)_2SO_4$; 0.2 g $MgSO_4.7H_2O$; 0.02 g $CaCl_2.2H_2O$; 2 g $CaCO_3$; 1 g yeast extract; 20 g glycerol; 1 ml trace element solution; 2 ml Fe solution. The composition of the fermentation medium per liter was: 1 g K_2HPO_4; 0.5 g KH_2PO_4; 5 g/100 g glycerol $(NH_4)_2SO_4$; 0.2 g $MgSO_4.7H_2O$; 0.02 g $CaCl_2.2H_2O$; 1 g yeast extract; as required 60-120 g glycerol; 1 ml trace element solution; 1 ml Fe solution. The Fe solution per liter consisted of: 5 g $FeSO_4.7H_2O$; 4 ml HCl (37%). The trace element solution per liter consisted of: 70 mg $ZnCl_2$; 0.1 g $MnCl_2.4H_2O$; 60 mg H_3BO_3; 0.2 g $CoCl_2.2H_2O$; 20 mg $CuCl_2.2H_2O$; 25 mg $NiCl_2.6H_2O$; 35 mg $Na_2MoO_4.2H_2O$; 0.9 ml HCl (37%).

Bioreactor experiments

Cells were cultivated in Reinforced Clostridial Medium (RCM) for 18 hours. Before starting experiments, a heat-shock (80°C for 10 min) was conducted in order to stimulate germination of *Clostridium butyricum* DSM 5431 spores. The preculture was carried out in a glass tubes, filled with 18 ml of medium and inoculated with 2 ml of RCM medium (10% inoculum by volume), and incubated for 20 hours. Batch cultures were carried out in a 1-L glass fermenter (Biostat Q®, B Braun Biotech International, Germany) filled with 560 ml of culture medium and inoculated with 140 ml of preculture medium (20% inoculum by volume). In order to ascertain the anaerobiosis during the fermentation, nitrogen gas at a rate of 0.1 vvm was infused into the culture medium. The agitation speed was controlled at 100 rpm and the pH was adjusted to 7.0 by automatic addition of 4 M NaOH. The incubation temperature was 33°C. Samples were taken every 3 hours during the fermentation time of 48 hours.

Analytical methods

Biomass concentrations were measured as optical density (UV-2450, SHIMADZU, Japan) at 650 nm, and correlated directly with cell dry weight. The determination of glycerol, 1,3-PD, butyric acid, and acetic acid was carried out by HPLC (LC-3A, SHIMADZU, Japan). The other product, ethanol, was measured by GC (GC-7AG, SHIMADZU, Japan).

Simulation method

The kinetic model of *Clostridium butyricum* DSM 5431 for 1,3-PD production in batch fermentation was developed using a MATLAB program. The mathematical simulation was formulated using a non-linear programming and the optimization formulation was solved by minimum sum square error method.

RESULTS AND DISCUSSION

Effect of initial glycerol concentration

The batch fermentations of *Clostridium butyricum* DSM 5431 under anaerobic condition were primarily performed with different initial glycerol concentrations 60-120 g l^{-1} in a 1-L glass fermenter. The experimental results are shown in Table 1.

Table 1 Specific growth rate, specific production rate, specific consumption rate, molar yields, and final 1,3-PD concentrations (at 48 hours) of batch fermentation at different initial glycerol concentration

Glycerol concentration (g l^{-1})	μ (hr^{-1})	ρ ($\frac{g\,1,3-PD}{g\,cell\cdot hr}$)	υ ($\frac{g\,glycerol}{g\,cell\cdot hr}$)	$Y_{P/S}$	$Y_{X/S}$	$Y_{P/X}$	Final 1,3-PD concentration (g l^{-1})
60	0.44	5.03	8.33	0.76	0.056	11.17	32.06
80	0.45	6.92	10.22	0.77	0.042	15.52	44.75
100	0.19	0.20	2.66	0.64	0.047	11.19	31.60
120	0.12	0.11	2.15	0.26	0.039	5.48	4.19

Table 1 also shows the specific growth rate, specific production rate and specific consumption rate which were evaluated in the exponential growth phase. According to effect of initial glycerol concentration on 1,3-PD production shown above, the maximum specific growth rate, specific production rate, molar yield, and final 1,3-PD concentration were observed at initial glycerol concentration 80 g l^{-1}. Therefore, it can be concluded from the experimental results that the optimal initial glycerol concentration for cell growth and 1,3-PD production was found to be 80 g l^{-1} and glycerol could obviously inhibit cell growth at concentration levels higher than 80 g l^{-1}. This is in contrast to the finding of Deckwer [12] who reported that substrate inhibition occurred when the initial

glycerol concentration higher than 50 g l^{-1}, on the other hand, agrees well with the observation of Biebl [23] who found that the appreciable inhibition by glycerol was found only above a concentration of 80 g l^{-1}. In addition, the specific growth rate at optimal initial glycerol concentration (0.453 hr^{-1}) in this study was higher than in the results of Papanikolaou et al. [20] who reported that the maximum specific growth rate was about 0.39-0.43 hr^{-1}.

Batch fermentation at optimal initial glycerol concentration

Based on this experiment, batch fermentations in 1-L fermenter were performed at optimal initial glycerol concentration of 80 g l^{-1} under pH of 7.0, and temperature of 33 °C. The experimental results are shown in Fig. 1. The lag time of cell growth was about 9 hours. The concentration of 1,3-PD reached up to maximum when glycerol was almost completely consumed in just 21 hours. These results reveal that the highest 1,3-PD yield achieved in this study was higher than that in the previous reports [3,11,14,18]. Furthermore, the by-product concentration profiles such as butyric acid, acetic acid, and ethanol in batch fermentation at optimal initial glycerol concentration were investigated in order to develop the kinetic model. The main by-product that found in this batch fermentation was butyric acid, with little acetic acid and ethanol accumulation. The shape of butyric acid and acetic acid concentration profiles suggest that butyric acid and acetic acid may be consumed for cell growth after glycerol exhausted because cell could grow over even though glycerol depleted. This is consistent with the observations of Günzel et al. [3] that acetic acid was consumed when the 1,3-PD concentration profile passed its inflection point.

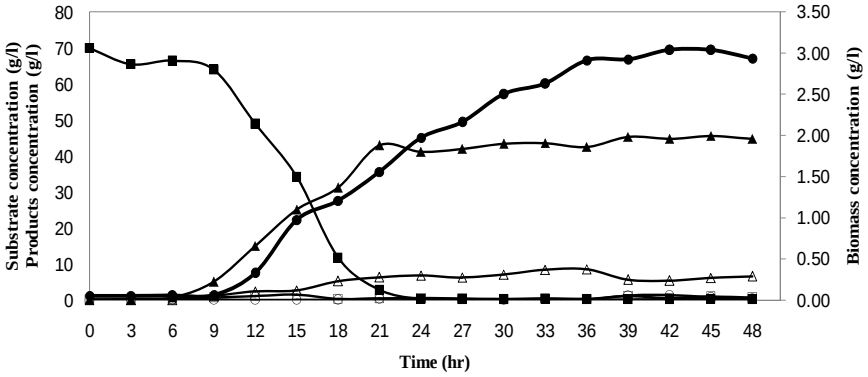

Fig. 1 Time-course of batch fermentation by *Clostridium butyricum* DSM 5431 at 80 g l^{-1} of initial glycerol concentration: ■, Glycerol; ▲, 1,3-PD; Δ, Butyric acid; □, Acetic acid; ○, Ethanol; ●, Biomass

Kinetic model development

The kinetic model for 1,3-PD production in batch fermentation of optimal initial glycerol concentration was developed by using mathematical simulation. Due to trace amounts of acetic acid and ethanol accumulation in this batch fermentation, the concentrations of acetic acid and ethanol were neglected from the model. According to the previous reports, the cell model was only specific growth rate model which indicated multiple inhibition effects of glycerol, 1,3-PD, butyric acid, and acetic acid on *Clostridium butyricum* DSM 5431 growth while the most suitable cell model in this study was the specific growth rate model which indicated multiple inhibition effects of glycerol, 1,3-PD, and butyric acid on *Clostridium butyricum* DSM 5431 growth. The critical concentrations of glycerol, 1,3-PD, and butyric acid were 181.7 g l^{-1}, 66.4 g l^{-1}, and 11.1 g l^{-1}, respectively [10]. The growth model and the mass balance equations that were used in mathematical simulation can be written as follows:

$$\mu = \mu_{max}\left(\frac{C_{Gly}}{C_{Gly}+K_S}\right)\left(1-\frac{C_{Gly}}{C^*_{Gly}}\right)\left(1-\frac{C_{1,3\text{-PD}}}{C^*_{1,3\text{-PD}}}\right)\left(1-\frac{C_{HBu}}{C^*_{HBu}}\right)$$

$$\frac{dX}{dt} = \mu X - K_d X$$

$$\frac{dS}{dt} = -\frac{\mu X}{Y_{X/S}} - M_c X - \sum \frac{r_{Pi}}{Y_{Pi/S}}$$

$$\frac{dPi}{dt} = \alpha_i \mu X + \beta_i X$$

The kinetic parameters and the optimization values of each term in growth model and mass balance equations are shown in Table 2 and the simulation results are shown in Fig. 2. The simulation results of biomass, glycerol, 1,3-PD, and butyric acid concentrations fit the experimental results with a deviation of 7.6%, 3.0%, 2.7%, and 9.1%, respectively, while an average deviation is 5.6%. This suggests that the simulation results of biomass, glycerol, 1,3-PD, and butyric acid concentrations were very close to the experimental results. Furthermore, the simulation results demonstrated that 1,3-PD concentration can be estimated from this growth model.

Table 2 Kinetic parameters and optimization values in growth model and mass balance equations of batch fermentation

Kinetic parameter	μ_{max}	K_S	K_d	$Y_{X/S}$	M_c	$Y_{1,3\text{-PD/S}}$	$Y_{HBu/S}$	$\alpha_{1,3\text{-PD}}$	α_{HBu}	$\beta_{1,3\text{-PD}}$	β_{HBu}
Optimization value	0.39	0.53	0	0.90	0.027	0.81	0.90	15.90	2.81	0.007	0.004

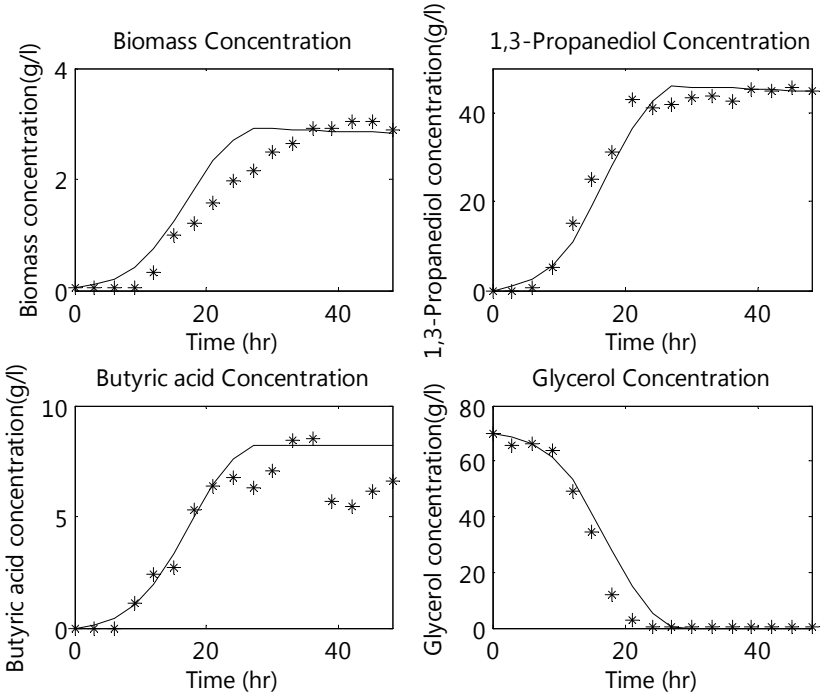

Fig. 2 Comparison of experimental results (*) and simulation results (-)

CONCLUSION

This study indicated that the influence of the initial glycerol concentration on cell growth and 1,3-PD production was significant. Maximum 1,3-PD concentration (44.75 g l^{-1}) was achieved when the cells were cultivated with initial glycerol concentration 80 g l^{-1} in batch fermentation. Comparison of the experimental results and the simulation results showed that the simulation results were very close to the experimental results with an average deviation of 5.6%. These results reveal that the specific growth rate model reported here satisfactorily. The achievable concentration of 1,3-PD in batch fermentation can be predicted from this model. Results of this study provide useful information to apply for prolonging the 1,3-PD production in fed-batch fermentation.

NOMENCLATURE

C_{Gly} is the glycerol concentration [g l^{-1}].
$C_{1,3\text{-PD}}$ is the 1,3-PD concentration [g l^{-1}].
C_{HBu} is the butyric acid concentration [g l^{-1}].
C^*_{Gly} is the maximum glycerol concentration above which cells cannot grow due to glycerol inhibition [g l^{-1}].

$C^{*}_{1,3\text{-PD}}$ is the maximum 1,3-PD concentration above which cells cannot grow due to 1,3-PD inhibition [g l^{-1}].
C^{*}_{HBu} is the maximum butyric acid concentration above which cells cannot grow due to butyric acid inhibition [g l^{-1}].
K_d is the specific death rate [hr^{-1}].
K_S is the constant having the value of the limiting substrate concentration at which the specific growth rate is half its maximum value [g l^{-1}].
M_c is the maintenance coefficient [g substrate consumed g $cell^{-1}$ hr^{-1}].
Pi is the product concentration [g l^{-1}].
r_{Pi} is the product formation rate [g l^{-1} hr^{-1}].
S is the substrate concentration [g l^{-1}].
t is the time [hr].
X is the cell concentration [g l^{-1}].
$Y_{P/S}$ is the yield coefficient (mass product produced/mass substrate consumed).
$Y_{P/X}$ is the yield coefficient (mass product produced/mass cell produced).
$Y_{X/S}$ is the yield coefficient (mass cell produced/mass substrate consumed).
μ is the specific growth rate [hr^{-1}].
μ_{max} is the maximum specific growth rate [hr^{-1}].
α_i is the constant for growth associate product.
β_i is the constant for non-growth associate product [hr^{-1}].
ρ is the specific production rate [hr^{-1}].
υ is the specific consumption rate [hr^{-1}].
i = 1,2 where represents 1,3-PD and butyric acid (HBu).

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