การผลิตโปรตีนเซลล์เดียวจากกากส่าด้วยวิธีการหมักแบบกึ่งต่อเนื่อง โดยยีสต์ Saccharomyces cerevisiae

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SINGLE CELL PROTEIN PRODUCTION FROM STILLAGE BY SACCHAROMYCES CEREVISIAE USING FED-BATCH FERMENTATION

Miss Thasanaprapha Lertsremongkol

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Engineering in Chemical Engineering

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การบำบัดน้ำเสียเป็นปัญหาสำคัญประการหนึ่งของอุตสาหกรรมการหมักเอทานอล เนื่องจากน้ำหมักประกอบด้วยสารอินทรีย์ในปริมาณสูงจากทั้งเซลล์ยีสต์และน้ำตาลที่เหลือจากการหมัก การบำบัดน้ำกากส่า(น้ำเสียหลังกลั่นแยกเอทานอล)ก่อนปล่อยสู่สิ่งแวดล้อมตามข้อกำหนดจึง ค่อนข้างยุ่งยาก เพื่อที่จะลดจำนวนน้ำกากส่าที่จะต้องบำบัด งานวิจัยนี้จึงศึกษาถึงการนำน้ำกากส่าจากกระบวนการหมักเอทานอลจากกากน้ำตาลไปใช้ในการผลิตโปรตีนเซลล์เดียวโดยใช้ยีสต์ *Saccharomyces cerevisiae* M30 โดยตัวแปรสำหรับการศึกษาในระบบการหมักแบบกะคือ ความเข้มข้นของน้ำตาลเริ่มต้นในระบบ, สัดส่วนการแทนที่น้ำด้วยกากส่า, ค่าความเป็นกรด-เบส และความเข้มข้นของแอมโมเนียมซัลเฟต ค่าความเป็นกรด-เบสและความเข้มข้นของแอมโมเนียมซัลเฟตที่เหมาะสมในการผลิตมวลเซลล์คือ 4.5 และ 0.10%(มวลต่อปริมาตร) ตามลำดับ พบว่าในการผลิตเซลล์ *Saccharomyces cerevisiae* M30 ที่ความเข้มข้นน้ำตาลเริ่มต้น 6-10% (มวลต่อปริมาตร)สามารถใช้น้ำกากส่าทดแทนน้ำได้ 40-60% โดยปริมาตร โดยไม่พบการยับยั้งการผลิตเซลล์เมื่อแทนที่น้ำด้วยกากส่า 0-40% โดยปริมาตร จากการศึกษาการเพิ่มอัตราการผลิตเซลล์โดยการป้อนสารอาหารแบบเป็นช่วงๆ พบว่าการหมักแบบกึ่งต่อเนื่องขั้นตอนเดียวและแบบกึ่งต่อเนื่อง 2 ขั้นตอนสามารถเพิ่มปริมาณเซลล์แห้งเป็น 1.3 และ 2.1 เท่าตามลำดับเมื่อเทียบกับกระบวนการหมักแบบกะ โดยอัตราการผลิตเซลล์แห้งในการเลี้ยงเซลล์แบบกะ, แบบกึ่งต่อเนื่องขั้นตอนเดียวและแบบกึ่งต่อเนื่อง 2 ขั้นตอนเป็น 0.12, 0.17 และ 0.21 กรัมต่อลิตรต่อชั่วโมงตามลำดับ จากผลการศึกษาชี้ให้เห็นความเป็นไปได้ในการนำน้ำกากส่าจากกระบวนการหมักเอทานอลมาใช้ในกระบวนการผลิตโปรตีนเซลล์เดียว

ภาควิชา วิศวกรรมเคมี ลายมือชื่อนิสิต.....
สาขาวิชา วิศวกรรมเคมี ลายมือชื่ออาจารย์ที่ปรึกษา.....
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THASANAPRAPHA LERTSREMONGKOL: SINGLE CELL PROTEIN PRODUCTION FROM STILLAGE BY *SACCHAROMYCES CEREVISIAE* USING FED-BATCH FERMENTATION THESIS ADVISOR: ASSISTANT PROFESSOR MUENDUEN PHISALAPHONG, Ph.D., 61 pp.

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Waste treatment was one of the important problems in ethanol fermentation plants. Due to the very high organic matter of yeast cell and sugar residue in the fermentation broth, treatment of the stillage was found difficult to meet the regulatory discharge limits. To reduce the amount of stillage for the waste treatment, re-use of cane molasses alcohol stillage in single cell protein production using *Saccharomyces cerevisiae M30* was investigated. The system variables in batch fermentation were investigated including pH, ammonium sulfate concentration, initial sugar concentration, and proportion of stillage replacement. The optimal initial pH and ammonium sulfate concentration for cell production was 4.5 and 0.10% (w/v), respectively. It was found that in the production of biomass by *Saccharomyces cerevisiae* M30 with initial total inverse sugar 6-10 % w/v, it was viable to replace 40-60 % of total fresh water by cane molasses alcohol stillage. No inhibition effect due to the substitution of 0-40% stillage was observed. To achieve high mass concentration, cultivation of cell at stepwise addition of medium was used to optimize the cell production. Using single fed batch culture and repeated fed batch, biomass accumulation was increased by a factor of 1.3 and 2.1, respectively, relative to the batch culture. The biomass productivities from batch, single fed- batch culture and repeated fed- batch, were 0.12, 0.17 and 0.21 g/l.h, respectively. The results indicated that there exists a good potential for the use of alcohol stillage to replace of fresh water for single cell protein production.

Department Chemical Engineering Student's signature .....

Field of study Chemical Engineering Advisor's signature .....

Academic year 2004

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Chapter 1
INTRODUCTION

1.1 Background

To day, the number of ethanol manufacturing industries have been rapidly increasing in many countries because of the deficit and uncertain cost of petroleum fuel and the pollution problems arisen from groundwater contamination octane booster (MTBE, methyl-tertry-butyl-ether). The government of Thailand supports the use of ethanol as gasohol projects. It permits to build eight ethanol manufacturing factories which give overall potential about 1.5 millon liters of ethanol per day. Ten percent of ethanol is blended with benzene in order to substitute the octane booster.

The consequence of increasing ethanol manufacturing industries is an increase of stillage which is the waste from distillation process after the removal of ethanol. One liter of ethanol gives 10-14 liters of stillage. Therefore, increasing in ethanol production will also require effective solution for stillage management.

The production and characteristics of stillage are variable and depend on feedstocks aspects of ethanol production. Feedstocks yielding higher amounts of ethanol appear to also produce higher amount of stillage chemical oxygen demand (COD). The ethanol yield of cane molasses is 2.52 L/kg of feed stock and the COD yield is 1.33 kg/L of ethanol .The cane molasses stillage exhibits the highest levels of biochemical oxygen demand (BOD), COD (45g/L of BOD and 113 g/L of COD) and organic material. (Barnes and Halbert, 1979). The high COD, BOD and organic material in stillage can cause an environmental problem.

There are many solutions for the stillage treatment such as Physical / mechanical seperation that recovers and removes suspended solids containing yeast and other materials. The mechanical treatment is a technology, which requires future processing including evaporation and/or membrane separation. The other common treatments are aerobic and/or anaerobic treatments. Today, most of the stillage is treated by using

wastewater treatment processes with or without aeration. These treatment processes waste a lot of energy and require high operating cost.

Researches on single cell protein production from alcohol and organic waste, such as waste from paper manufacture, molasses from sugar manufacture and whey from milk manufacture have been reported. The single cell protein production from the waste is suggested to be an alternative way to reduce an environmental problem. The COD of the treated wastewater is expected to be reduced from the use of dissolved organic compounds by microorganism as substrates for cell growth. For the single cell protein production, the single cell should not only be nutritious, but should also pass all toxicity tests to be commercialized as food product. Yeasts are generally best accepted for the safety consideration.

In this research study the optimal condition of single cell protein production by flocculated yeast, *Saccharomyces cerevisiae* M30 using cane molasses stillage as substrate is investigated. This process not only reduces the amount of stillage waste but the product could also become an alternative source of protein for animal or human in the future.

1.2 Objective

To investigate the optimal condition for single cell protein production by *Saccharomyces cerevisiae* M30 using cane molasses stillage as a substrate.

1.3 Scope of work

1. Batch experiments were performed in 500-mL shaking flasks to investigate the effect of initial sugar concentration, proportion of stillage replacement, pH and ammonium sulfate concentration fermentation on cell production.
2. Molasses and sugar cane stillage were used as substrates in all experiments.
3. Batch and fed-batch experiments were performed in 1-L fermenter to determine the effects of substrate feeding strategy.

1.4 Expected benefits

The study will provide more understanding on the effects of sugar concentration, proportion of stillage replacement and feeding strategy on single cell protein production. The information obtained from the study can be applied as an alternative process to reduce the amount of stillage from ethanol production plants.

Chapter 2
BACKGROUNDS AND LITERATURE REVIEWS

2.1 Yeast production

Yeast can grow under 2 conditions, aerobic (presence oxygen) or anarobic (absence of oxygen). In anarobic condition, yeast grows very slowly and the sugar that supports either fermentation or growth is used mainly to produce alcohol and carbon dioxide. Only small amount of sugar is used for cell maintanance. In aerobic condition of *Saccharomyces cerevisiae* (Jones K.D. and Kompala D.S.,1999), which supplied with glucose as a carbon and energy source, after a initial lag phase which little growth occurs, yeast attains its highest specific growth rate, with exponential increasing in cell number, and ethanol production via the well-know fermentation pathway (see Figure 2.1)

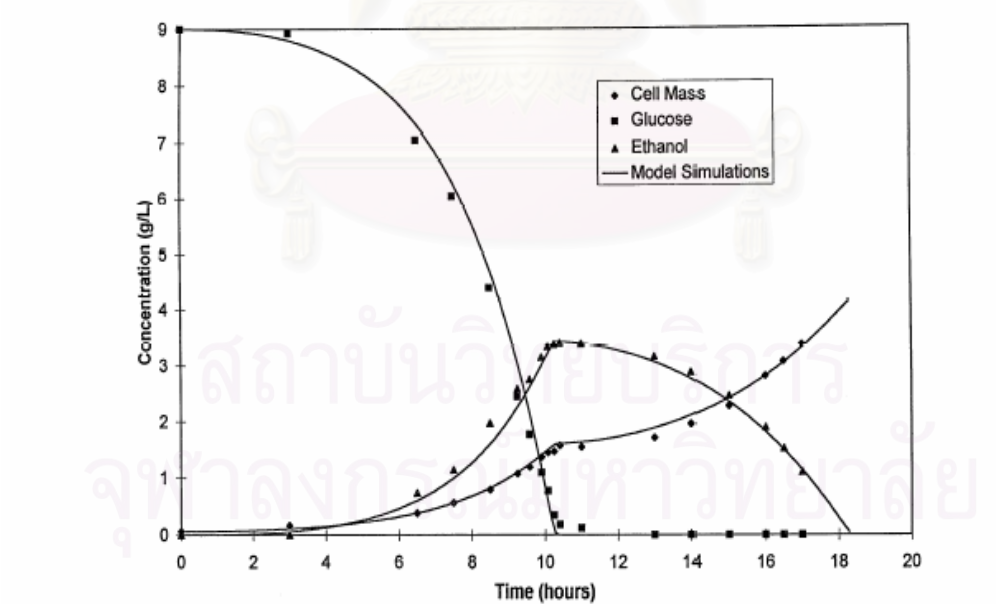


Figure 2.1. Cybernetic model simulations and experiment data from von Meyenburg (1969) for cell mass, glucose, ethanol concentration in aerobic batch culture of *S.cerevisiae*.

The maximum growth rate from the fermentation is about 0.45 h-1, with a low cell mass yield of 0.15 g per g glucose. During this lag phase, yeast undergoes a switch-over in its enzymatic make-up, synthesizing new enzyme to consume the ethanol produced as a by-product of fermentation. Gradually, a second exponential growth phase begins, during which the available ethanol is consumed via an oxidative pathway. This second growth rate is much slower than that of the earlier fermentattion but the cell mass yield is much higher during ethanol oxidation. The maximum growth rate from ethanol oxidation is about 0.20 h-1, with a high cell mass yield of 0.65 g per g ethanol. All of the carbon consumed via the ethanol oxidation pathway is used for the productioon of cell mass. The other metabolic pathway for glucose consumption has been seen in continuous culture experiments. At the low dilution rates where the residual glucose concentration in the chemostat is below about 50 mg l^{-1}, yeast consumes glucose through and oxidative pathway. The glucose oxidation pathway is controlled and inhibited at high concentration of glucose. The cell mass yield for this oxidative pathway, which is predominant at low dilution rates, has been estimated at about 0.50 g per g glucose. The cell mass yield via this pathway is much higher than that of fermentation because glucose is consumed primarily for the production of cell mass.

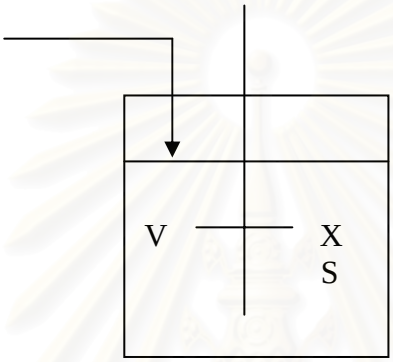
2.2 Single cell protein

Single cell protein (SCP) is the protein extracted from microorganism. Microorganisms grow as single or filamentous individuals rather than as complex multicellular organisms like plants or animal cell.

There are many reasons why microbes are possible choices for SCP production, some of which include: 1) microorganisms have rapidly growth rate under the optimum conditions, 2) microorganisms are more easily modified genetically than plants and animals, 3)microorganisms have approximately high protein content and the nutritional value of the protein is good , 4) microorganisms can be grown in vast numbers in relatively small continuous fermentation process using relatively small land area and are also inpendent of climate, and 5) microorganisms can grow on a wide rang of raw materials, in particular low value wastes, and can also use plant-derived cellulose. Algae, fungi and bacteria are main sources of microbial protein that can be utilized as SCP. Fungal species are cultured on different substrates, mostly cheap wastes from different sources have varying compoisition of hemicellulose, cellulose and lignin. Some sources of lignocellulose material are wood from angiosperms and gymnosperms, grasses, leaves, waste from paper manufacture, sugarcane bagsses and other agricultural waste. Many fungal species are used as protein-rich food. Most popular among them are the yeast species *Candida, Hansenula, Pitchia, Torulopsis* and *Saccharomyces*. (Anupama and Ravindra, 2000) Yeasts are generally best accepted by consumers because they are rarely toxic or pathogenic and can be used in human diets. Their protein content ranges from 35 to 50 percent and contains essential amino acid such as lysine (6 to 9 %), tryptophan and threonine. They are also rich in vitamins (B group), and their nucleic acid content ranges from 4 to 10%. The other adventage is yeasts sizing which larger than bacteria, so they are facilitated to separation. (Boze, 1999)

2.3 Batch fermentation

Batch fermentation is the fermentation in a close system by limiting substrate. The substrate is feeding only once time at the beginning of the fermentation process. The nutrient and product are removed at the ending of the fermentation process. From the batch system,



Mass balance for the batch fermentation:

Substrate balance: $$\frac{dS}{dt} = -\frac{\mu X}{Y_{x/s}} \qquad (1)$$

Cell balance: $$\frac{dX}{dt} = \mu X \qquad (2)$$

When

X = cell concentration [g/L]

t = time [hr]

μ = specific growth rate [hr^{-1}]

S = substrate concentration [g/L]

$Y_{x/s}$ = yield coefficient for cell on substrate

= $\frac{\text{cell concentration change}}{\text{substrate concentration change}}$

V = volume of vessel [L]

The microbial growth in the batch fermentation can be divided in five phase; a lag phase, an exponential phase, a deceration phase, a stationary phase and a declining phase. During the lag phase, microoganisms spend time for suitable adaptation to substrate. (Fig 2.2)

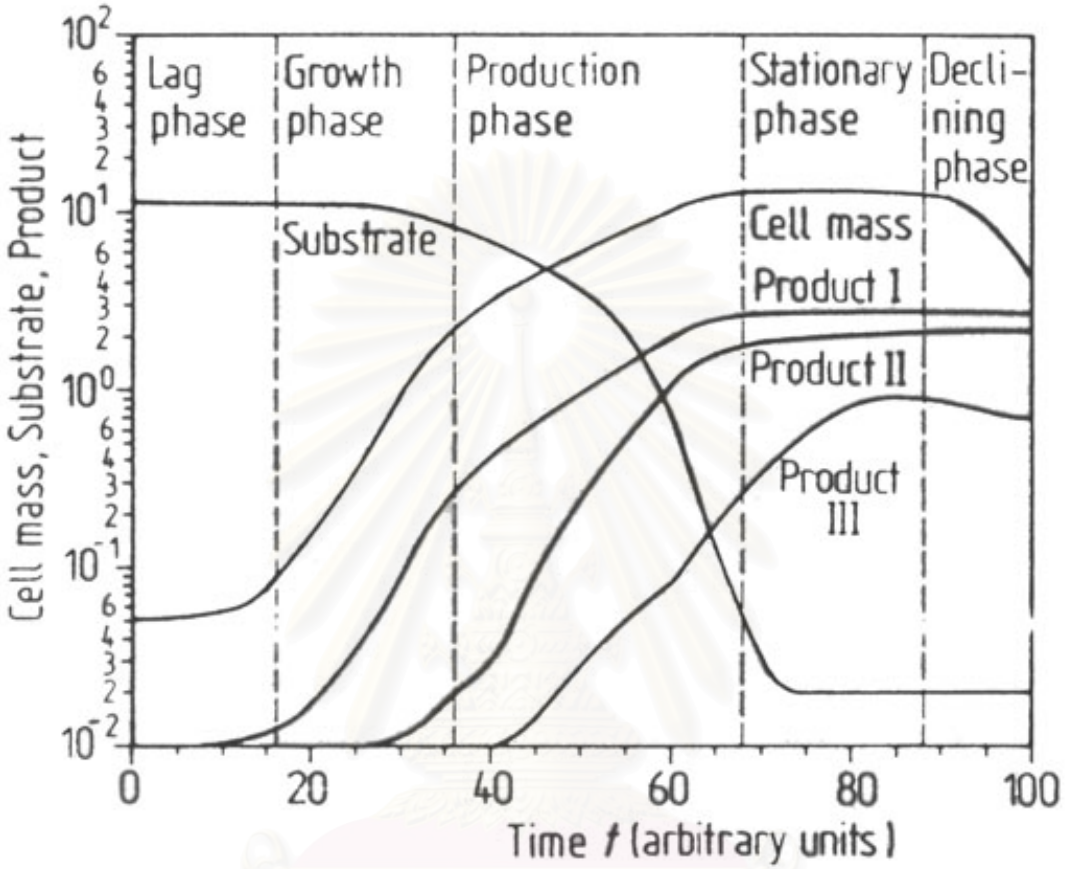


Figure 2.2 Principal time course of cell mass, substrate and product concentration for different types of fermentation

Source: Kehm et al., 2001

After that the pattern of microbial growth becomes an exponential curve. The maximum specific growth rate occurs in this phase. The growth pattern can be written as equation (2)

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

The integration of the equation (2) gives,

$$X_t = X_0 e^{\mu t} \qquad (3)$$

when

X_0 = initial cell concentration [g/L]

X_t = cell concentration [g/L]

e = natural logarithm

Taking natural logarithm of the equation (3) gives,

$$\ln X_t = \ln X_o + \mu t \qquad (4)$$

From the equation (4), the microbial concentration increases with increasing time. In the later stage, many factors not suitable such as the limit of substrate and the increase of microbial toxic, lead to stationary phase. This can be followed by a declining phase in which the cell mass decreases due to lysis and endogenous metabolism (Fig. 2.2 and Fig. 2.3).

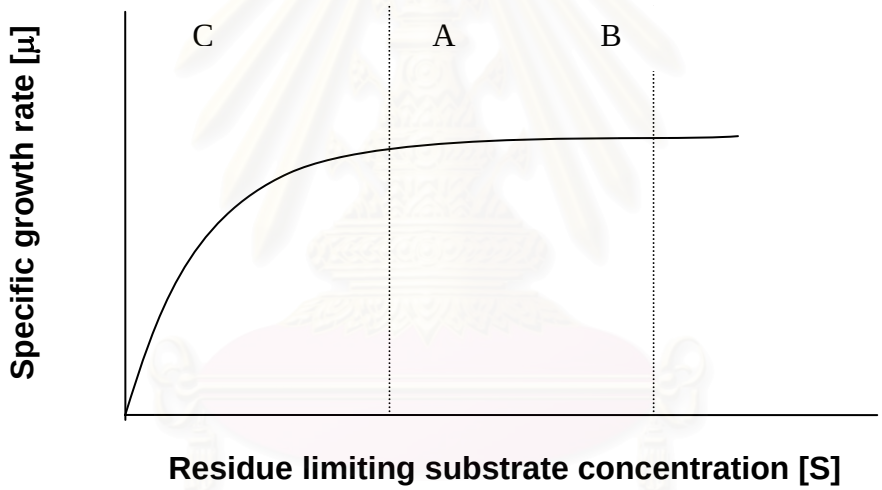


Figure 2. 3 the effect of initial substrate concentration to the maximum specific growth rate (Sanbury and whitaker, 1984)

The specific growth rate (μ) on the limiting substrate concentration (S) could be proposed by Monod. The Monod equation states that,

$$\mu = \frac{\mu_{\max} S}{K_s + S} \qquad (5)$$

However, the microbial growth rate can decrease by the effect of product inhibition, which can represent by Hinshelwood equation.

$$\mu = \frac{\mu_{\max} S}{K_s + S}(1 - K_p P) \tag{6}$$

Biorol et al, (1998) used Hinshelwood equation to explain the growth of immobilized *Saccharomyces cerevisiae* for ethanol fermentation.

Wang and sheu (2000) proposed an explanation of the growth of *Saccharomyces cerevisiae* for ethanol production in batch fermentation by the effects of limiting substrate (sugar) and inhibitory effect of substrate and product (ethanol).

$$\mu = \left(\frac{\mu_{\max} S}{K'_s + S + S^2 / K'_{SI}}\right)\left(\frac{K'_p}{K'_p + P + P^2 / K'_{PI}}\right) \tag{7}$$

where

μ_{max} = maximum specific growth rate [hr^{-1}]

K'_s = subtrate utilization constant [g/L]

K'_{si} = substrate inhibition constant [g/L]

K'_P = product utilization constant [g/L]

K'_{PI} = product inhibition constant [g/L]

2.4 Fed-batch fermentation

In fed-batch fermentation, the limiting substrate is feeded into the system during the fermentation time. The volume in the fermenter increases with the fermentation time. The basic approaches to the fed-batch fermentation can be divied into fixed volume fed- batch and variable volume fed-batch.

2.4.1 Fixed volume fed-batch

In this type, the limiting substrate is fed without diluting the culture. The cultutre volume can also be maintained pratically constant by feeding the growth limiting substrate in undiluted form, for example, as a very concentrate liquid. Alternatively, the substrate can be added by dialysis.

A certain type of extended fed-batch, the cyclic fed-batch culture, for fixed volume systems refers to a periodic withdrawal of portion of the culture and use of the residual culture as the starting point for a further fed-batch process.

2.4.2 Variable volume fed-batch

A variable volume fed-batch is one in which the volume changes with the fermentation time due to substrate feed. The volume changes depend on the requirements, limitations and objective of the operator.

The feed can be provided according to one of the following options:

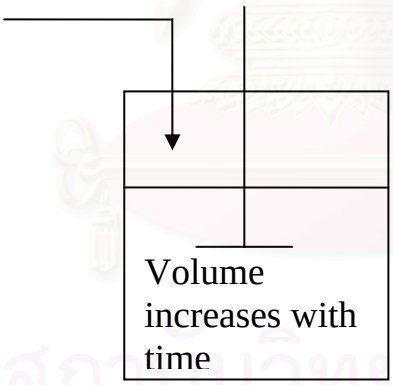
1. the same medium used in the batch mode is added
2. the limiting substrate concentration as same as the initial concentration medium is added
3. the very concentrated solution of the limiting substrate is added at a rate less than (1) and (2).

This type can still be further classified as repeated fed-batch process or cyclic fed-batch culture, and single fed-batch process.

Repeated fed-batch process means that once the fermentation reaches a certain stage after which is not effective anymore, a quantity of culture is removed from the vessel and replaced by fresh nutrient medium. The decrease in volume results in an increase in the specific growth rate, followed by a gradual decrease as the quisi-steady state is established.

A single fed-batch process refers to the type of fed-batch which supplementary growth medium is added during the fermentation and the culture is removed at the end of the fermentation.

The balance of the system can be written as following:



Mass balance

Substrate balance: $\frac{d(SV)}{dt} = FS_f - \frac{\mu XV}{Y_{x/s}}$ (8)

From equation (8), gives

$$S\frac{dV}{dt}+V\frac{dS}{dt}=FS_f-\frac{\mu XV}{Y_{x/s}} \quad (9)$$

volume in the vessel increases with time

$$V=V_0+Ft \quad (10)$$

when V_0 and F is constant, differentiate equation (10) give

$$\frac{dV}{dt}=F \quad (11)$$

substitute equation (11) in equation (9), give

$$SF+V\frac{dS}{dt}=FS_f-\frac{\mu XV}{Y_{x/s}} \quad (12)$$

$$\frac{dS}{dt}=\frac{F}{V}(S_f-S)-\frac{\mu X}{Y_{x/s}} \quad (13)$$

cell balance: $\frac{d(XV)}{dt}=\mu XV$ (14)

$$V\frac{dX}{dt}+X\frac{dV}{dt}=\mu XV \quad (15)$$

substitute equation (11) in equation (15), give

$$V\frac{dX}{dt}+XF=\mu XV \quad (16)$$

$$\frac{dX}{dt}=-\frac{FX}{V}+\mu XV \quad (17)$$

when

X_0 = initial cell concentration [g/L]

X = cell concentration [g/L]

t = time [hr]

μ = specific growth rate [hr^{-1}]

S_f = substrate concentration in feed [g/L]

S = substrate concentration in the vessel [g/L]

F = feed flow rate [L/hr]

$Y_{x/s}$ = yield coefficient for cell on substrate [-]

V = volume of the veesel [L]

V_0 = initial volume of the vessel [L]

Feeding method in fed batch culture can be divided following:

- Without feedback control

  - Constant feeding

    Feeding nutrient at perdeterminded (constant) rate. The specific growth rate continuously decreases.

  - Increased feeding

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

  - Exponential feeding

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

- With feedback control

  - Indirect feedback control

    - DO-state

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

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

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

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

➢ 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).

Advantages of fed-batch culture.

1. Can produce high cell densities due to extension of working time (particularly important in the production of growth-associated products).
2. Could be applied for controlled conditions in the provision of substrates during the fermentation, particularly regarding the concentration of specific substrates as for ex. the carbon source.

3. Can be applied to control over the production of by-products or catabolite repression effects due to limited provision of substrates solely required for product formation.
4. The mode of operation can overcome and control deviations in the organism's growth pattern[1] as found in batch fermentation.
5. Can justify the mode of operation for fermentations leading with toxic substrates (cells can only metabolize a certain quantity at a time) or low solubility compounds.
6. Can increase of antibiotic-marked plasmid stability by providing the correspondent antibiotic during the time span of the fermentation
7. No additional special piece of equipment is required as compared with the batch fermentation mode of operation.

2.6 Literature reviews

Bajbai and Bajbai (1995) studied the used of prehydrolysate liquor generated from a rayon pulp mill for the production of sigle cell protein using *Candida utilis* TCRDC-Y7 and *Paecilomyces variotii* TCRDC-MII cultures by repeated fed batch fermentation. The repeated fed batch gave up to 75 % higher biomass production when compared with simple batch fermentation. The optimum ratio of the volume drawn out to the total volume was 0.25 for the maximum biomass, for *Candida utilis* TCRDC-Y7 172.4g/L and for *Paecilomyces variotii* TCRDC-MII 196.3g/L at 196 hr.

Ahmad and Holland (1995) studied the growth kinetics of single cell protein from *Candida utilis* in batch fermentation. The system variables were investigated including agitation speed, glucose concentration, air flow rate, inoculum dosage and temperature. The maximum specific growth rate was found to increase with agitation, temperature and inoculum dosage. At the optimum condition, the maximum specific growth rate was 0.95 h^{-1}. (500 rpm,

34 ^{o}C) It was found that the lag phase of the curve increased with initial sugar concentration and decreased with increasing inoculum dosage and agitation speed.

Korz et al.(1995) studied simple fed batch technique for high cell density cultivation of Escherichia coli. Glucose and glycerol were used as carbon source. A pre-determined feeding strategy was chosen to maintain carbon-limited growth using a defined medium. The specific growth rate was controlled to less than specfic growth rate that caused the formation of acetic acid. They found that the optimal specific growth rate was 0.12 to 0.14 h^{-1}. Cell concentration of 128 and 148 g/L dry cell weight per liter (g/L DCW) were obtained using glucose or glycerol as carbon source, respectively.

Konlani et al. (1996) studied the single cell protein production from *Candida Krusei* SO1 and *Saccharomyces sp*. LK3G using batch and fed batch fermentation. Glucose and sorghum hydrolysate were used as substrates. The results obtained with synthetic glucosse showed better growth parameter under fed-batch conditions than under batch conditions.Biomass yield (Yx/s) was increased by as much as 64% for *Candida Krusei* SO1 and 70% for *Saccharomyces sp*. LK3G . Volumetric cell productivity was also improved for both yeasts: 0.75 g/l.h in fed batch conditions against 0.68 g/l.h in batch conditions for *Candida Krusei* SO1 and 0.63 g/l.h against 0.56 g/l.h for *Saccharomyces sp*. LK3G. The protein content for both obtained from using batch and fed batch conditions was 47-50%. However, the ratio of protein content from using glucose medium was higher than that from sorghum hydrolysate medium.

Chanda and Chakrabarti (1996) compared the growth of *Saccharomyces cerevisiae NCIM* 3095, *Torula utilis* NCIM 3055 and *Candida lipolytical* NCIM 3229 in leaf juice without protein of 4 plants: turnip, mustard, radish and cauliflower. The growth of saccharomyces cerevisiae NCIM 3095 was best in radish while for *Torula utilis* NCIM 3055 and *Candida lipolytical* NCIM 3229, turnip was slightly better. The protein content was 45.6%, 54.3%

and 50.5% for *Saccharomyces cerevisiae NCIM* 3095, *Torula utilis* NCIM 3055 and *Candida lipolytical* NCIM 3229, respectively.

Rhishpal and Philip (1998) determined the optimum conditions of single cell production from 4 marine yeasts: *Candida* M10, *Candida* M15, *Rhodotorula* M23 and Rhodotorula M28 using shaking flasks. The prawn-shell waste was used as substrate. Nacl concentration and pH were chosen as the system variable. Two percent NaCl was found to be optimum for *Rhodotorula* M23 and *Candida* M15, whereas 2.5 % NaCl was optimum for *Candida* M10 and 1% for Rhodotorula M28.The optimum pH of *Candida* M15, *Rhodotorula* M23 and *Rhodotorula* M28 was pH 5. *Candida* M10 exhibited a wide pH tolerance from 4 to 10 with very little difference growth. The protein content of the final products varied from 60.6 to 70.4 %. The protein enrichment was found to be maximum with *Candida* M15 and minimum with *Rhodotorula* M28.

Choi and Park (1999) investigated the possibility of culturing an osmotolerant yeast (*Pichia guilliermondii*) using waste brine from kimchi factory as a substrate for production of single cell protein. The system variable included NaCl concentration, pH, temperature, organic material addition ($(NH_4)_2SO_4$ and K_2HPO_4). They found that the optimal pH was 4.0 and optimal temperature was 30 ^oC. The final cell mass increased proportionally with amount of added organic material. The maximum cell yield was 0.69 g of dry cells per liter; contain 40% of protent content.

Shojaosadati et al. (1999) determined the optimal conditions for single cell protein (SCP) production and COD reduction of sugar beet stillage is specified for species of *Hansenula* in continuous culture. They found that the optimal dilution rate was 0.12 h^{-1}, temperature range 32-36 ^oC. The condition without additing nitrogen and phosphorus source gave 5.7 g / dm^3 , 31% COD reduction and 39.6% protein content of biomass.On the contrary, the condition of additing of nitrogen and phosphorus source gave 8.5 g/dm3 of biomass ,37.5 % COD reduction and 50.6% of protein content. The final effluent of single cell protein production was recycled to fermentation stage, the result represent 70% reduction of stillage volume.

Kim and Lee (2000) compared the mass production of *Rhodopseudomonas palustris in* batch, fed batch and contineous fermentation. For fed batch fermentation, the feeding was divided to 3 feeding pattern (linear ,exponential and sigmoidal). The optimal agitation speed and malate concentration were 300 rpm and 0.2 % in the modified MYC medium, respectively. In batch fermentations of *R. palustris*, the maximum number of viable cell was 1.1 x 10^{10}cfu/ml with 2.65 g l^{-1} of DCW, and maximum specific growth rate and biomass productivity were estimate to be 0.12 h^{-1} and 55 mg l^{-1} h^{-1}, respectively. Crude protein content of *R. palustris* was about 72-74%. The biomass productivity from fed- batch experiments were foud to be 50, 47 and 49 mg l^{-1} h^{-1} for linear, exponential and sigmoidal feeding strategy, respectively. The maximum biomass productivity was found to be 112 mg l^{-1} h^{-1} in chemostat. Compare to the growth in batch culture, continuous fermentation yield two times higher biomass productivity.

Chapter 3
METERIALS AND METHODS

3.1 Microorganism

Saccharomyces cerevisiae M30, a flocculating yeast, kindly provided by the laboratory of Assoc. Prof. Savithree Limthong (department of microbioology, Kasetsart University, Bangkok, Thailand) is used for the single cell protein production in this study.

3.2 Apparatus

- 1-L Fermenter Biostat Q, Germany
- Centrifuge model Kubota 5100 of Kubota Corporation, Japan
- Spectrophotometer model Spectronic 20 Genesys of Spectronic Instrument, USA
- Laminar flow model VS-124 of ISSCO, USA
- Autoclave model HL24ADY of Hirayama Manufacturing Corporation, Tokyo, Japan
- pH meter of Mettler Tolledo, Switzerland
- Hot air oven model ULM 500 of Menmert, Germany
- Gas chromatography

3.3 Chemicals

- Hydrochloric acids [HCl], Merck, Germany
- Sulfuric acids [H_2SO_4], Merck, Germany
- Sodium hydroxide [NaOH], Carlo Erba, Italy
- Ammonium sulfate [$(NH_4)_2SO_4$], Carlo Erba, Italy
- Potassium sodium tartrate [$KNaC_4H_4O_6 \bullet 4H_2O$], Carlo Erba, Italy
- 3,5-dinitrosalicylic acids [$(C_9H_9N_2^+)(C_7H_3N_2O_7^-)$], Fluka chemical, Switzerland
- Ethyl alcohol anhydrous for HPLC 99.8% (v/v), Italmar Co., Ltd., France
- Potato dextrose agar (PDA), Becton, Dickinson and Company, France

- Molasses
- Molasses stillage
- HPLC water
- Reverse osmosis water

3.4 Experimental Methods

3.4.1 Inoculums

Stock cultures are stored in a PDA agar slant. Precultures are prepared by transferring a stock culture to 100 mL of prepared medium in 500 mL Erlenmeyer flask and incubated at 33 °C for 20 hours before transferred to main culture. The medium for the inoculum contained 0.05% ammonium sulfate and 5% inverse sugar from molasses mash and are adjusted pH to 5.0. The prepared medium is sterilized at 121°C for 20 minutes.

Figure 3.1 *Saccharomyces cerevisiae* M30 on Potato dextrose agar slant.

Figure 3.2 *Saccharomyces cerevisiae* M30 in 500 mL Erlenmeyer flasks containing 250 mL of the prepared fermentation media

3.4.2 Batch fermentation

Batch fermentation was carried out in shaking flasks and 1 L-fermenter. In shaking flask, the fermentation was conducted in 500 ml shaking flask with the working volume of 200 ml at room temperature and 150 rpm of agitation speeds. The inoculume was 5% of the working volume .The initial sugar concentration, proportion of stillage replacement, pH and ammonium sulfate concentration are the variables in batch fermentation. First, the initial sugar concentration was varied from 2% to 16% reducing sugar (2%, 4%, 8%, 10%, 12%, 14% and 16% reducing sugar). The optimal initial sugar was used in the next experiment. Second, the proportion of stillage replacement was varied from 0 % to 100 % v/v (0%, 20%, 40%, 60%, 80% and 100%v/v) (volume of molasses stillage / working volume). The experiment with the optimal initial sugar concentration and proportion of stillage replacement from the earlier experiments was carried out in the presence of initial pH at 4, 4.5 and 5 controlled by 3 M HCL and 3M NaOH. To investigate the effect of Ammonium sulfate concentratio, Ammonium sulfate concentrations range from 0% to

0.3% w/v (0%, 0.05%, 0.10%, 0.15%, 0.20% and 0.30% w/v) were added in the culture medium to determine the optimal concentration. From the experimental results, the initial sugar concentration, proportion of stillage replacement, pH and ammonium sulfate concentration was selected and used for the batch fermentation and fed-batch fermentation in 1-Litre fermenter.

3.4.3 Fed-batch fermentation

Fed-batch fermentation was carried out in 1-Litre fermenter with the initial working volume of 550 ml and feeding volume of 200 ml at 35 ^{o}C and 250 rpm of agitation speeds. The pH was controlled at 4.5 by 3 M H_2SO_4 and 3M NaOH. Fed-batch fermentation was divided into 2 parts. First, the feeding pattern was pulse injection. After 12 hour of fermentation, the feeding medium was fed into the fermenter every 6 hours. The initial sugar in the fermenter was 5% reducing sugar. The sugar concentration in the feed was varied from 6% to 10% reducing sugar (6%, 8% and 10% reducing sugar). The optimal feeding sugar concentration was used in the repeated fed-batch concentration. During the fermentation, the fermentation broth was taken out for 5 ml every 6 hours for 48 hours for cell concentration, sugar and ethanol analyses

Second experiment was carried out in 1-Litre fermenter with initial working volume 550 ml and feeding volume 200 ml at 35 ^{o}C. The initial sugar in the fermenter was 5% reducing sugar. The initial agitation speed was 250 rpm. When the pO_2 value decreaed below 15 % saturated of oxygen, the agitation speeds increased to 300, 350, 400 and 500 rpm, respectively. The pH was controlled at 4.5 by 3 M H_2SO_4 and 3M NaOH. After 24 hour of fermentation, 500 ml of fermentation broth was withdrawn from the fermenter and 350 ml of fresh medium was added into the fermenter. After 12 hours of fermentation, the feeding medium was fed into the fermenter every 1 hour. During the fermentation, the medium was taken out 5 ml every 6 hours for cell concentration, sugar and ethanol analyses

Figure 3.3 Fed-batch fermentation in 1- Litre fermenter, Biostat Q.

3.5 Analytical methods

3.5.1 Cell concentration

Cell concentration is determined by cell dry weight determination. For this method, a 3-mL sample of the fermentation broth is centrifuged at 3,000 rpm for 10 minutes. The cell pellet is resuspended in 0.1 normal HCl and washed twice with distilled water and then dried for 48 hr, at 60°C and weighed.

3.5.2 Ethanol concentration

Concentrations of ethanol are determined by a gas chromatography system using a Shimadzu Model GC $7A_G$ equipped with a flame ionization detector. A column 2 m x 0.125 packed with Porapak Q 80-100 mesh is used with N_2 as carrier gas. The injector temperature is 280°C, and the detector temperature is 300°C.

3.5.3 Reducing sugar concentration

To measure the amount of sugar in sample, a 1 mL of sample solution is hydrolyzed in 1M HCI at 100°C for 10 minutes, neutralized with 20% NaOH solution and determined for reducing sugar content by using modified dinitrosalicylic acid method (See appendix).

3.5.4 Protein content

Protein content is reported as total nitrogen by kjeldahl method. (Factor is 6.25)

3.5.5 Chemical oxygen demand (COD)

COD of the samples is determined by Open refluxes method, 5220B, which is a standard method for the examination of water and wastewater (1998).

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Batch fermentation

The effects of initial sugar concentration, proportion of stillage replacement, pH and amonium sulfate concentration on the study of the re-use of stillage for single cell production by *Saccharomyces cerevisiae* M30 was determined in the 500- mL shaking flasks.

4.1.1 Initial reducing sugar concentration

The initial reducing sugar concentration in fermentation medium was varied from 2% to 16% (w/v) (2%, 4%, 8%, 10%, 12%, 14% and 16% (w/v)). The experiment was carried out to determine the effect of initial sugar concentration on the cell production. At the high initial sugar concentration between 12%, 14% and 16% w/v, there is not a significant difference of cell production because of the substrate inhibition effect on the oxidation pathway. Whereas, at the lower initial sugar concentration from 2% to 10% w/v, the cell growth rate increased with increasing initial sugar concentration. (See Fig. 4.1)

Yeasts can use sugar either by anaerobic fermentation or oxidation. For high concentration of sugar in the fermentation growth media, yeast would use sugar through the anaerobic fermentation pathway and produce ethanol even if the supply of oxygen is adequate. (Jones K.D. and Kompala D.S., 1999) In contrast, at the low substrate concentration, yeast consumes glucose to oxidative pathway and produce more cell mass. The cell mass yield via the oxidation pathway is much higher than that of the fermentation because glucose is consumed primarily for the production of cell mass. From this study, the optimal initial sugar concentration range for cell growth was 6-10% w/v (3.58 g/ L, 4.32 g/L and 4.45 g/L for 6% w/v, 8%w/v and 10% w/v with cell mass yield ($Y_{X/S}$) 0.053, 0.052, 0.051, respectively)

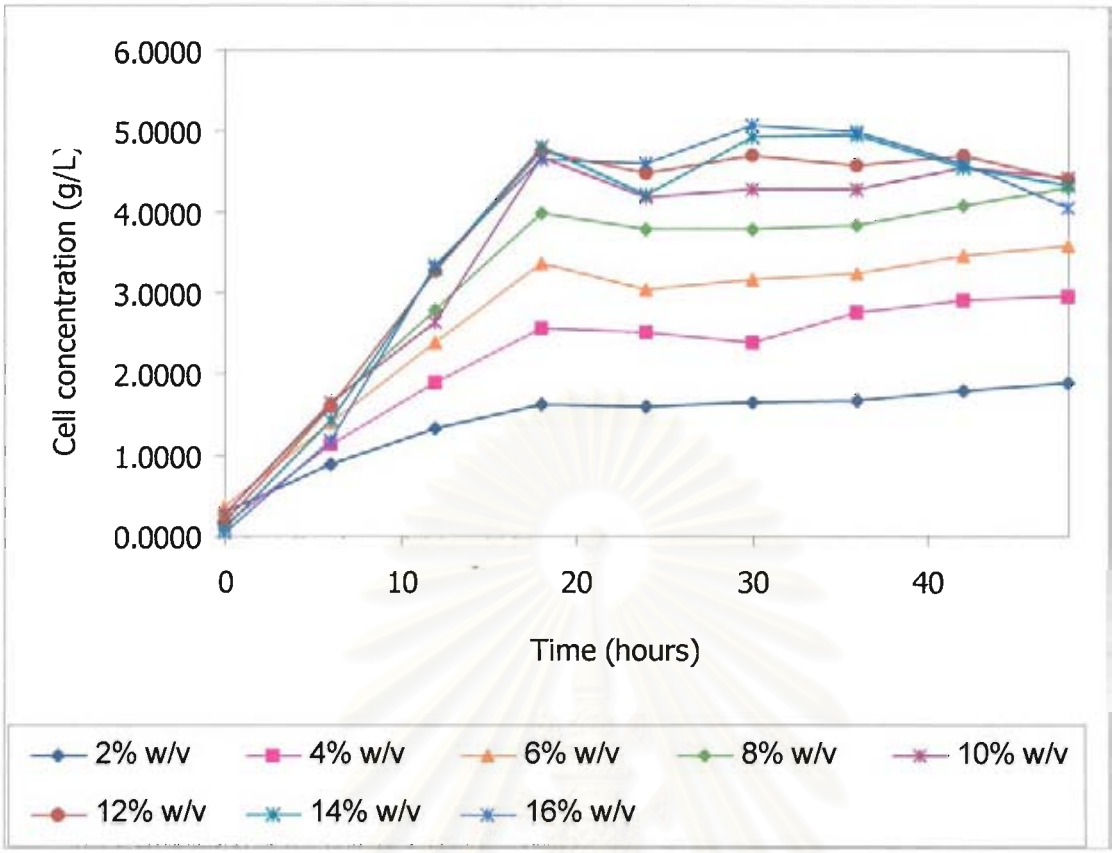


Figure 4.1 (a) Influence of initial reducing sugar concentration on cell growth.

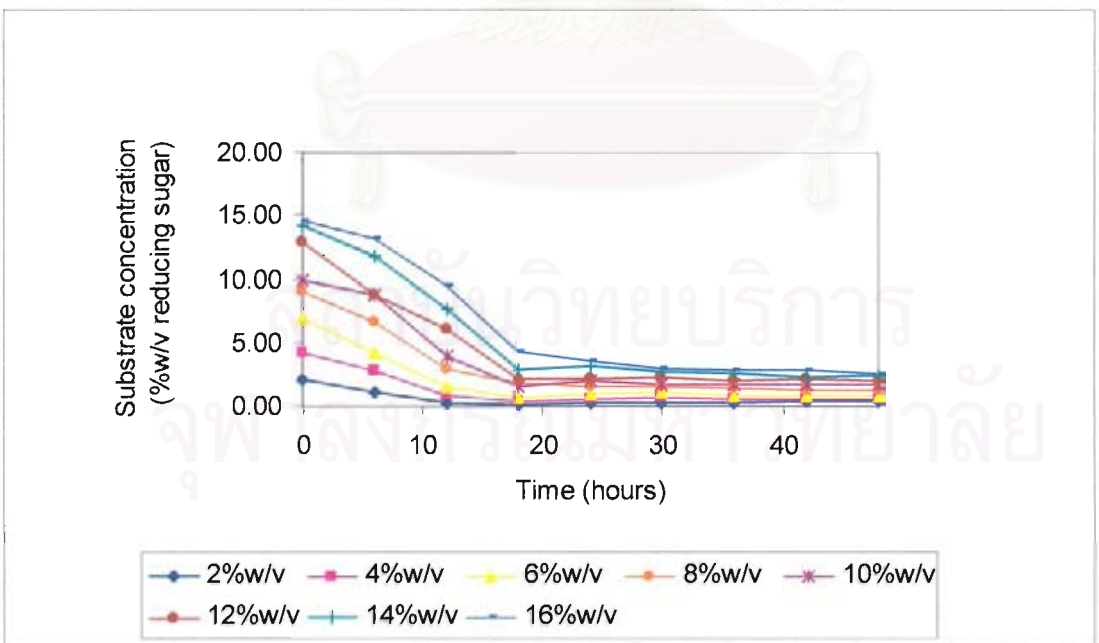


Figure 4.1 (b) Influence of initial reducing sugar concentration on sugar consumption

4.1.2 Stillage replacement

In this study, re-use of cane molasses alcohol stillage as replacement of fresh water for single cell protein production was investigated. Two values of the initial sugar concentrations (6% and 8% w/v), which were the values at the optimal range from the previous study was used for the preparation of the fermentation medium with the proportion of molasses alcohol stillage varied from 0 % to 100 % v/v (0%, 20%, 40%, 60%, 80% and 100%v/v) (volume of molasses stillage / medium volume).

At the end of fermentation, culture in 10% of initial sugar concentration medium gave higher cell concentration than 6% substrate concentration for all proportion of stillage replacement (fig. 4.2 and fig. 4.5). The residual sugar was 1-2.5 % (w/v) depended on the initial sugar concentration and % stillage replacement as shown in fig. 4.3 and fig. 4.4. However, the results revealed the lower yield for cell mass production at 10% initial sugar concentration compared with at 6% initial sugar concentration. For 10% initial sugar concentration, the residual sugar concentration value was nearly the same as the residual sugar in the molasses stillage (2-2.7% w/v), whereas for 6% initial sugar concentration, the residual sugar was less (1.2-1.7 % w/v). More inhibition of stillage replacement was observed at 10% initial sugar concentration compared to the effects on growth at 6% initial sugar concentration, particularly at 40% stillage replacement more than 60%. At 100% v/v stillage (without molasses), the cell growth was inhibited by the limiting of substrate because of the low sugar concentration in stillage. (2.5 % w/v reducing sugar)

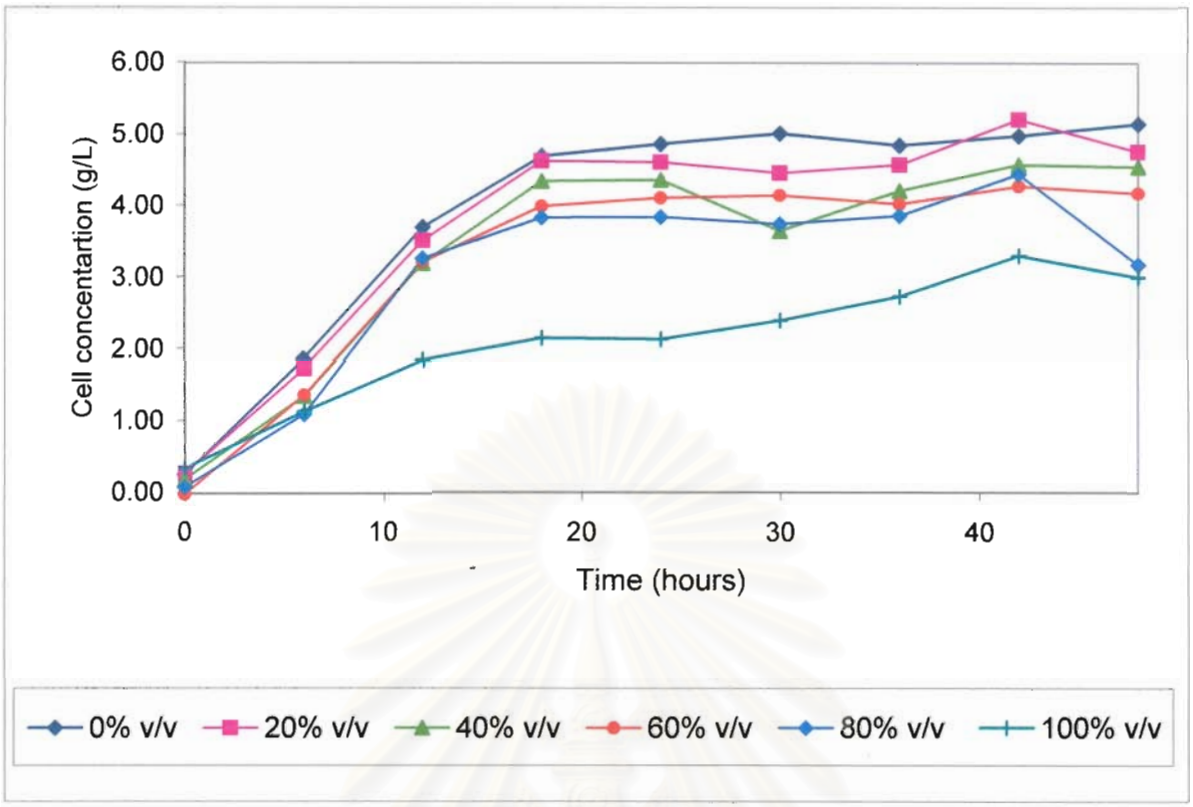


Figure 4.2 Influence of stillage replacement (% v/v) on cell growth in the fermentation at 10 % (w/v) initial sugar concentration.

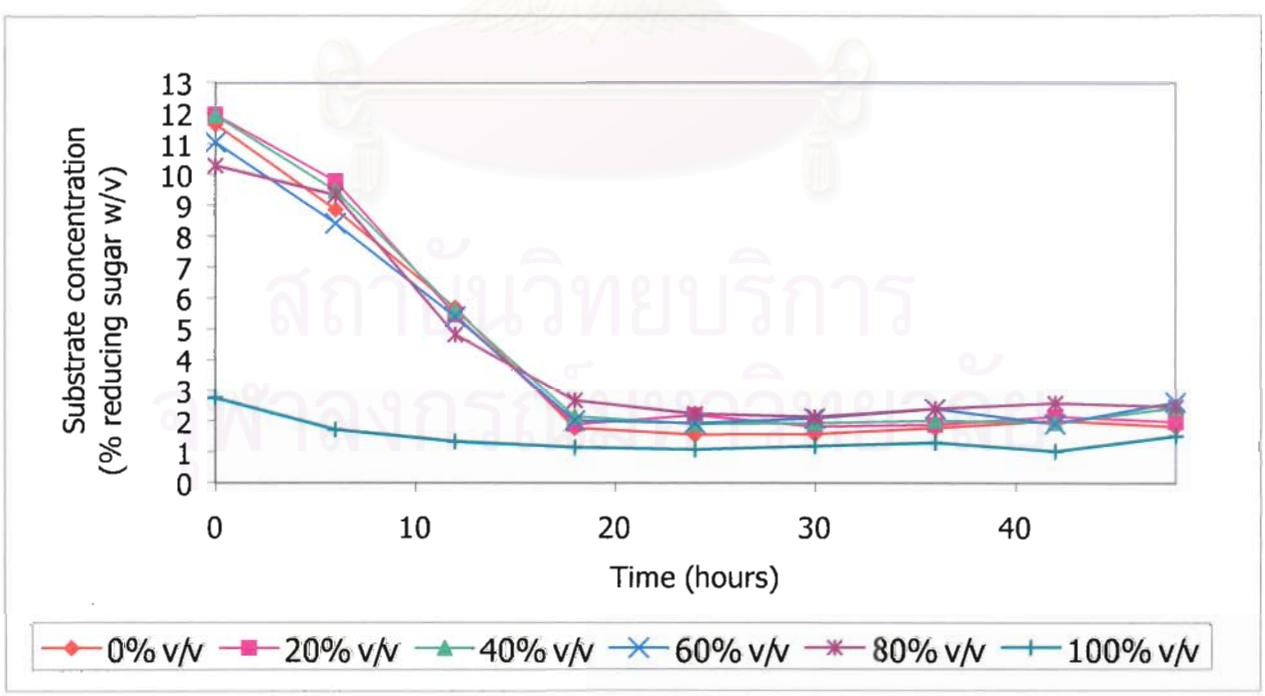


Figure 4.3 Influence of stillage replacement (% v/v) on sugar consumption in the fermentation at 10 % (w/v) initial sugar concentration.

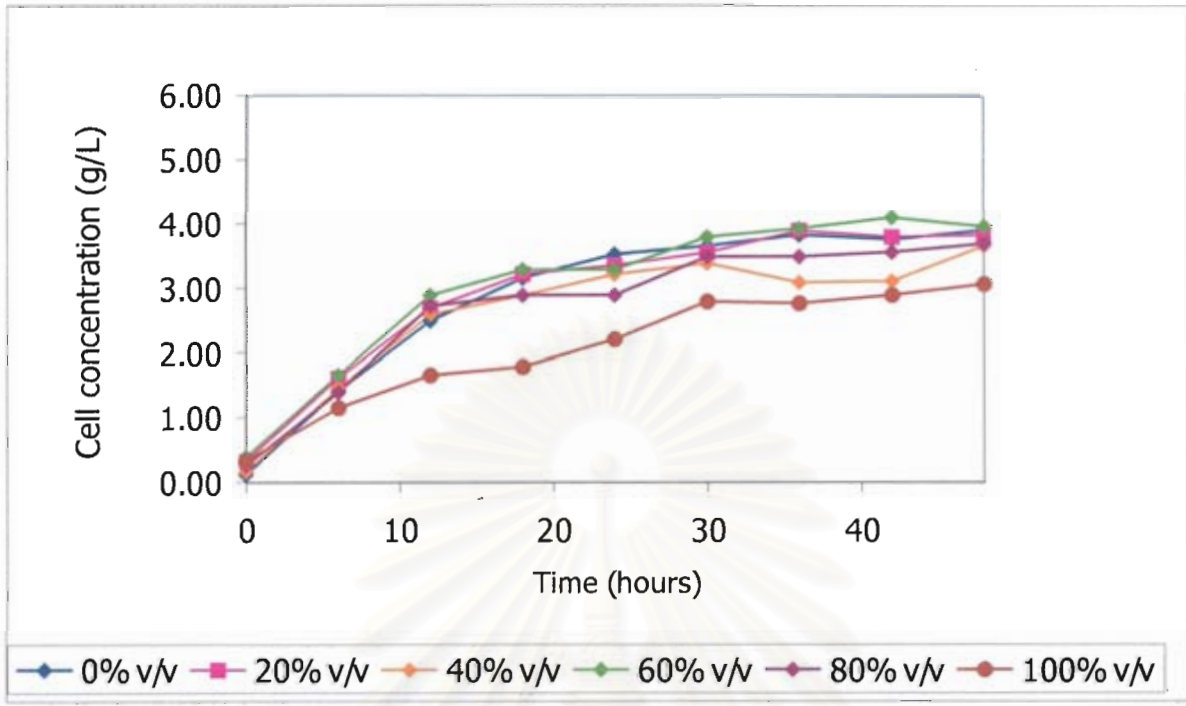


Figure 4.4 Influence of stillage replacement (%v/v) on cell growth in the fermentation at 6% (w/v) initial sugar concentration.

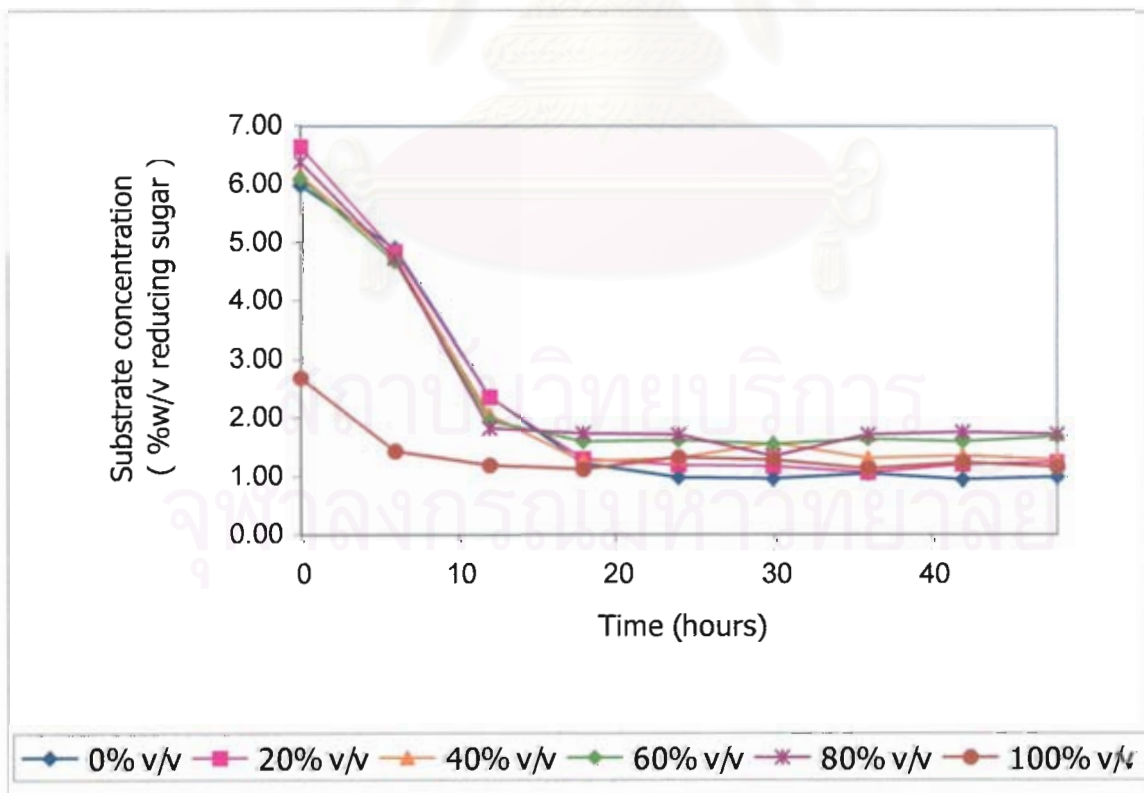


Figure 4.5 Influence of stillage replacement (%v/v) on sugar consumption in the fermentation at 6% (w/v) initial sugar concentration.

4.1.3 pH

This experiment was performed to determine the influence of various initial pH values on the growth of *S. cerevisiae* M30 in medium with 6% (w/v) of initial sugar concentration and 60% v/v of stillage replacement. The initial pH value was varied from 4.0, 4.5 and 5.

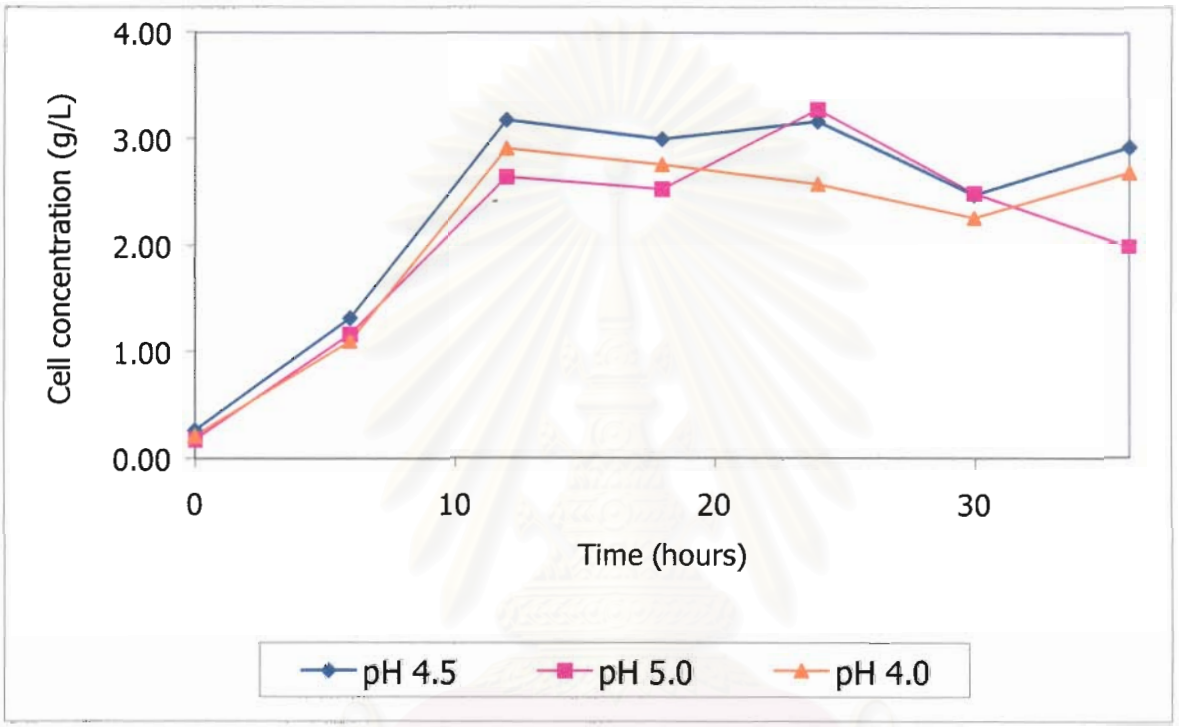


Figure. 4.6 Effect of pH on cell growth in the fermentation with 60% (v/v) stillage replacement at 6% (w/v) initial sugar concentration.

The effect of various initial pH values from 4.0 - 5.0 on the growth of *S. cerevisiae* M30 was shown in Fig 4.6. Maximum cell mass production was found at pH 4.5. After 18 hour of cultivation, the cell concentrations at initial pH values: 4.5, 4 and 5.0 were 3.00, 2.76 and 2.53 g/L, respectively. At the optimal pH, the cell growth rate and cell mass yield were about 10% higher than those at initial pH 4.0 or 5.0. A slightly difference of residual sugar was observed at various initial pH. (See Fig. 4.7)

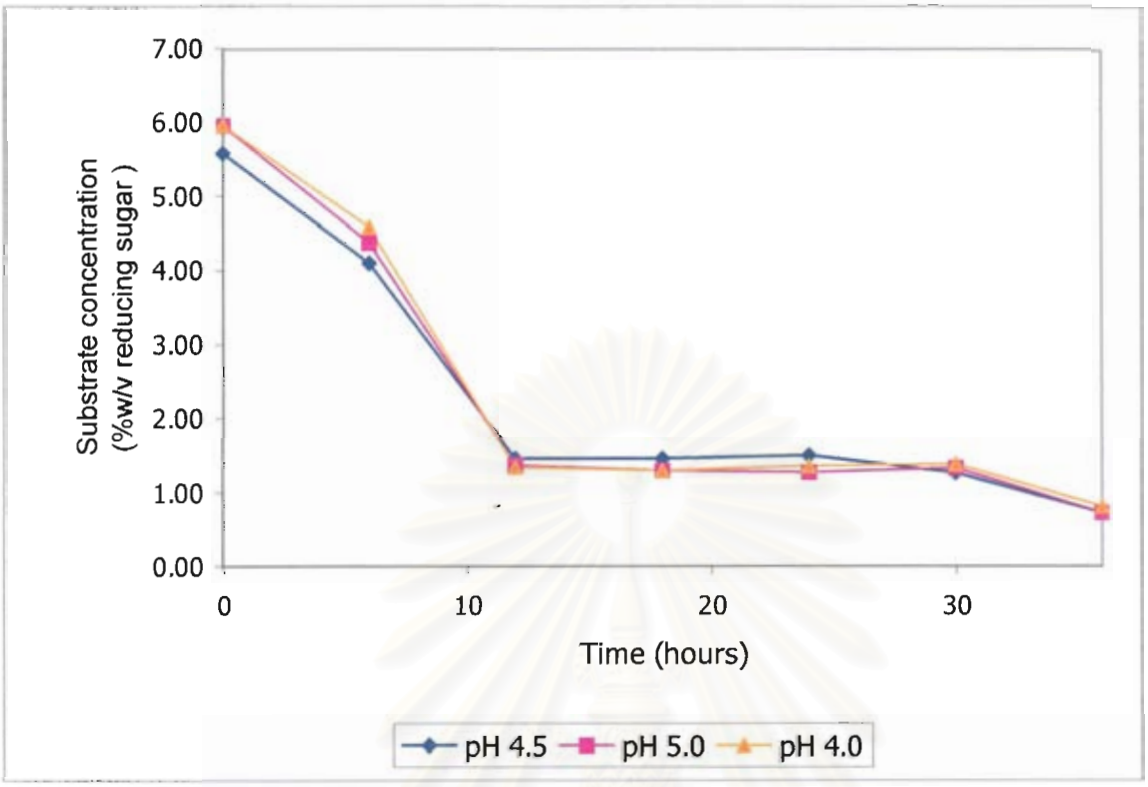


Figure 4.7 Effect of pH on sugar consumption in the fermentation with 60% (v/v) stillage replacement at 6% (w/v) initial sugar concentration.

Cell and substrate concentration obtained from the fermentation at initial pH 4.5, 6% w/v of initial sugar concentration with various proportion of stillage replacement were shown in Fig. 4.8 and Fig. 4.9, respectively. No significant difference in cell yield and growth was observed from the stillage replacement 0-60%. Inhibition effect of stillage replacement 80-100% was clearly detected, particularly at 100 % stillage replacement. Based on the experimental results, the optimum range for molasses stillage replacement of fresh water was 40 to 60%v/v.

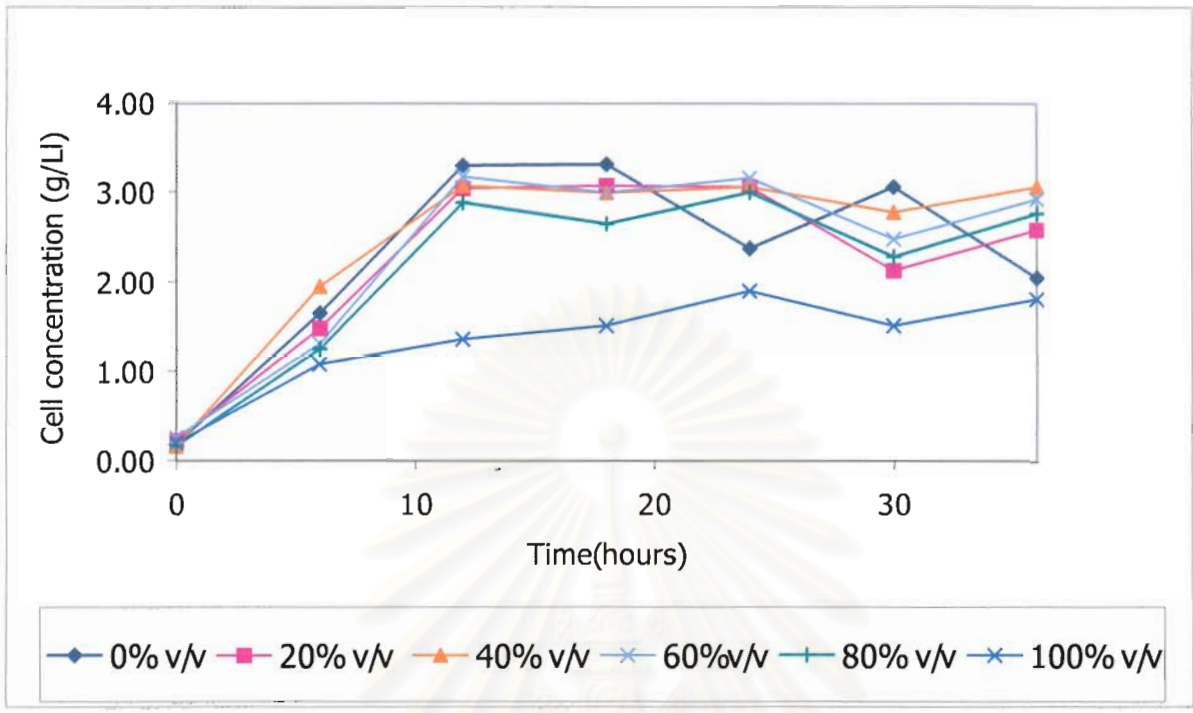


Figure 4.8 Effect of stillage replacements (% v/v) on cell growth in the fermentation at 6% (w/v) initial sugar concentration, pH 4.5.

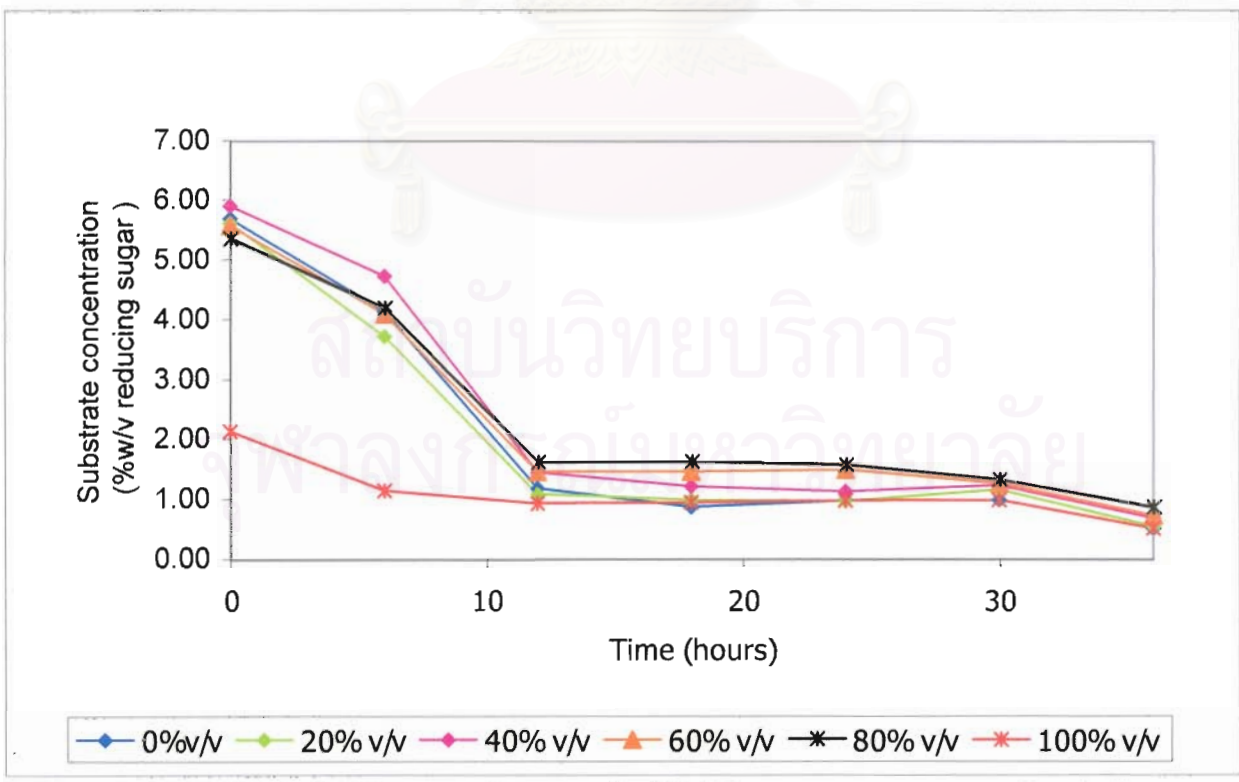


Figure 4.9 Effect of stillage replacements (%v/v) on sugar consumption in the fermentation at 6%(w/v) initial sugar concentration, pH 4.5.

4.1.4 Ammonium sulfate concentration

Nitrogen is essential in protein synthesis and as a component of cell wall polymers. Ammonium is most commonly used because of its price and ease of use. Ammonium sulfate, therefore, was used as the adding nitrogen source for the system. In this study, ammonium sulfate concentration was varied from 0% to 0.2% w/v (0%, 0.05%, 0.10%, 0.15% and 0.20% w/v). Between 0% to 0.10% w/v, the cell concentration increased with the increasing amonium sulfate concentration. Whereas, no significant difference in cell concentration was observed from the addition of ammonium sulfate from 0.15% to 0.20% w/v (See Fig. 4.10)

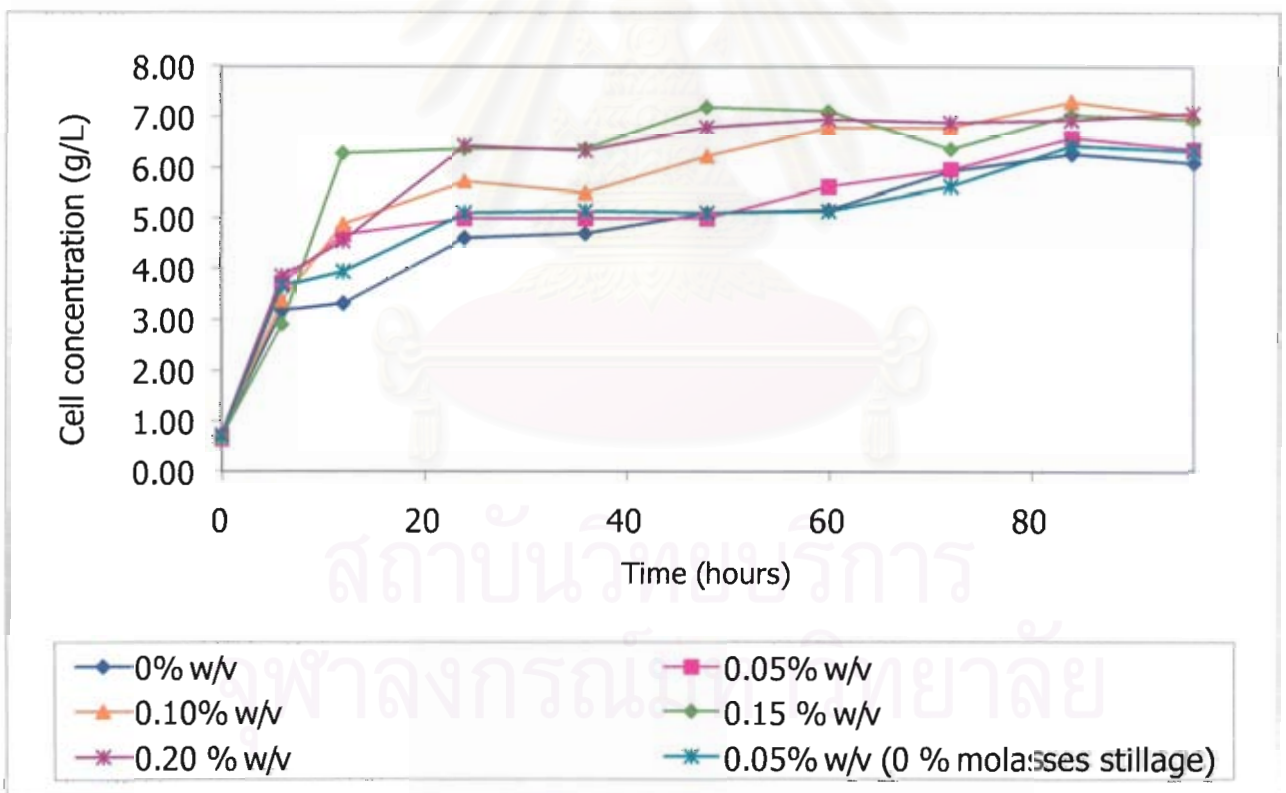


Figure 4.10 Effect of ammonium sulfate concentration on cell growth at 6% w/v of initial sugar concentration, 40%v/v stillage replacement and pH 4.5.

However, darkening of cell color was observed progressively with increasing ammonium sulfate concentration (Fig. 4.11). The observation was probably due to the more absorption of dark pigment from the mixture of molasses-stillage on cell wall at high ammonium sulfate concentration, particularly at the addition of ammonium sulfate more than 0.10%. Therefore, the optimal ammonium sulfate concentration chosen from the growth rate and desirable color of cell was 0.10%.

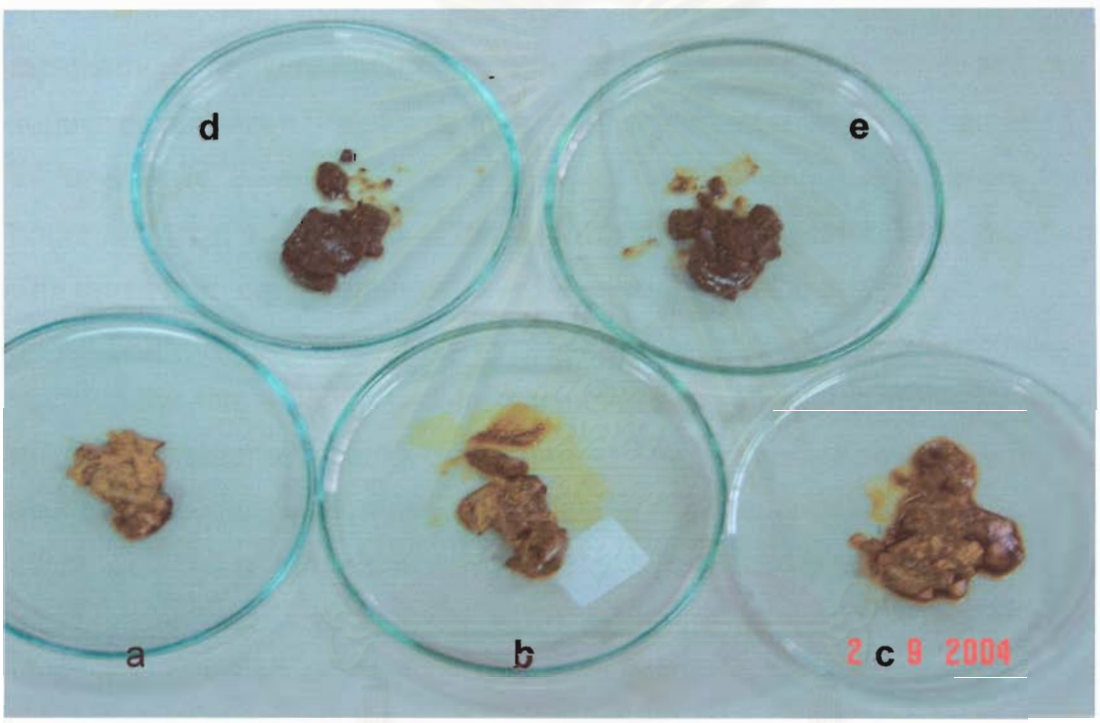


Figure 4.11 Effect of ammonium sulfate concentration on the cell color; a) 0 %, b) 0.05%, c) 0.10%, d) 0.15% and e) 0.20% w/v of ammonium sulfate.

4.2 Fed-batch fermentation

Based on the experimental results in the shaking flask, the initial sugar concentration, % stillage replacement, pH and ammonium concentration were chosen for batch fermentation and fed-batch fermentation in 1-Litre fermenter.

4.2.1 Simple fed-batch fermentation

The objective of this study was to determine the effect of feed concentration on the cell production. The fermentation in the fed batch started from initial working volume of 550 ml of 5% reducing sugar with 40% stillage replacement medium. Feed concentration was ranging from 6% to 8% w/v of sugar concentration. Feed was inject to the fermenter as pulse pattern (40 ml for 6% w/v, 30 ml for 8%w/v and 24 ml for 10% w/v) every 6 hours for 48 hours and then at 72 hours of fermentation time. The results were compared with those from batch fermentation of 7% initial reducing sugar.

From the previous study in batch fermentation, if the concentration of sugar in the fermentation growth medium is high, the yeast would use sugar to produce ethanol instead of cell mass. This problem could be solved by adding the sugar solution slowly throughout the fermentation process. In this study, fed-batch fermentation gave higher cell concentration than batch fermentation (Fig. 4.12 and Fig. 4.13). Fed-batch fermentation could lessen substrate inhibition and by product production. In addition, compared with batch fermentation, fed-batch fermentation can be considered as a high inoculum system. Cell mass yield, cell production can largely be affected from the concentration of biomass, sugar, oxygen and ethanol in the system. In the production of yeast, it is important to maximize both the cell mass yield and cell productivity. Sugar could be the limiting substrate of cell growth and as the same time, could be an inhibitor of the oxidation pathway. The results revealed that fed-batch culture feeding with 10% (w/v) reducing sugar to control sugar level between 1-2 % (w/v) gave the maximum cell concentration compared with the feeding of 6% w/v and 8% (w/v) sugar concentration. At the end of the fermentation, cell concentrations from the batch and fed batch with feeding of 6%, 8% and 10% w/v reducing sugar were 8.84, 10.32, 10.54 and 11.76 g/L, respectively (Fig. 4.12).

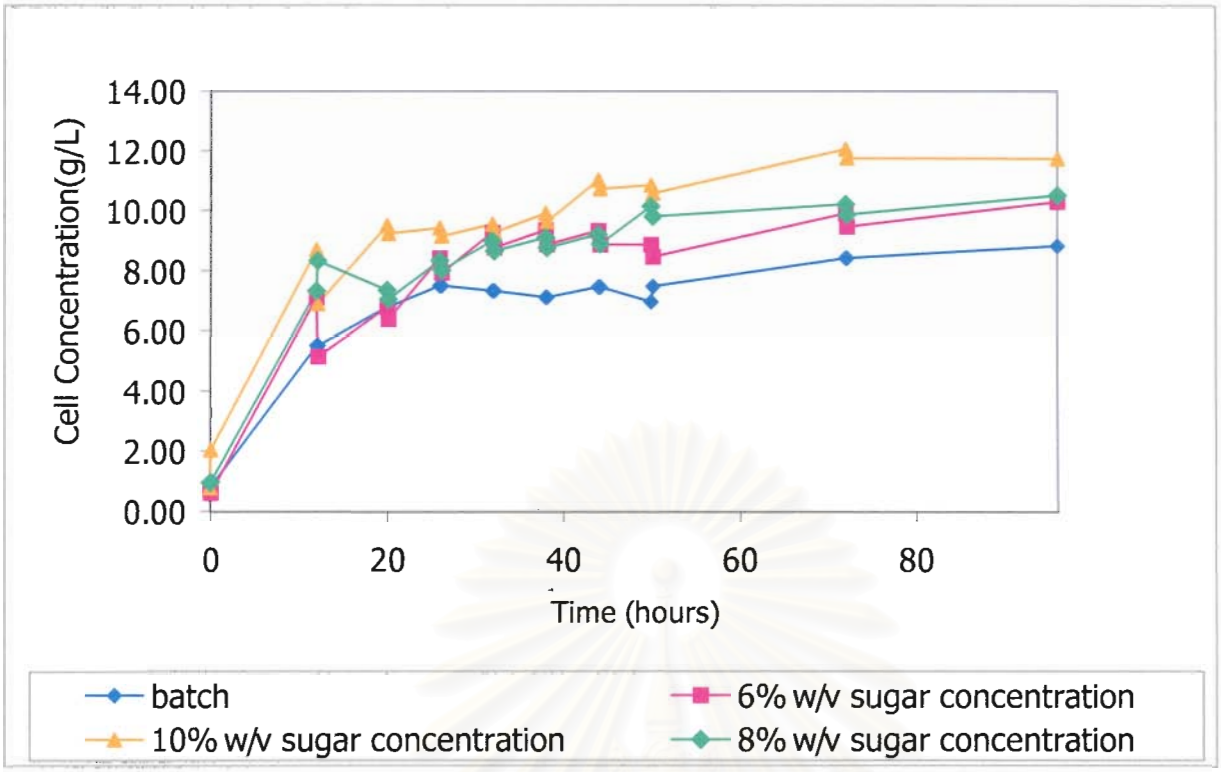


Figure 4.12 Effect of feeding with various sugar concentrations on cell growth in fed-batch fermentations.

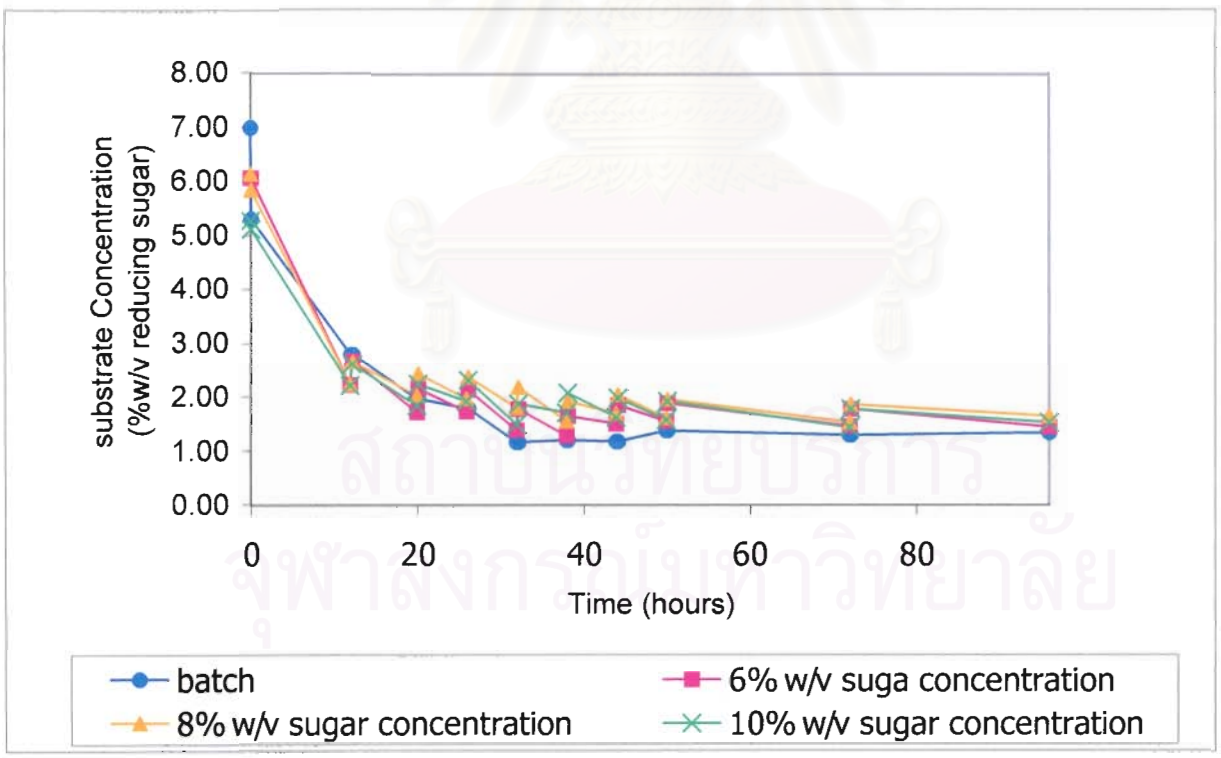


Figure 4.13 Effect of feeding with various sugar concentrations on sugar consumption in fed-batch fermentations.

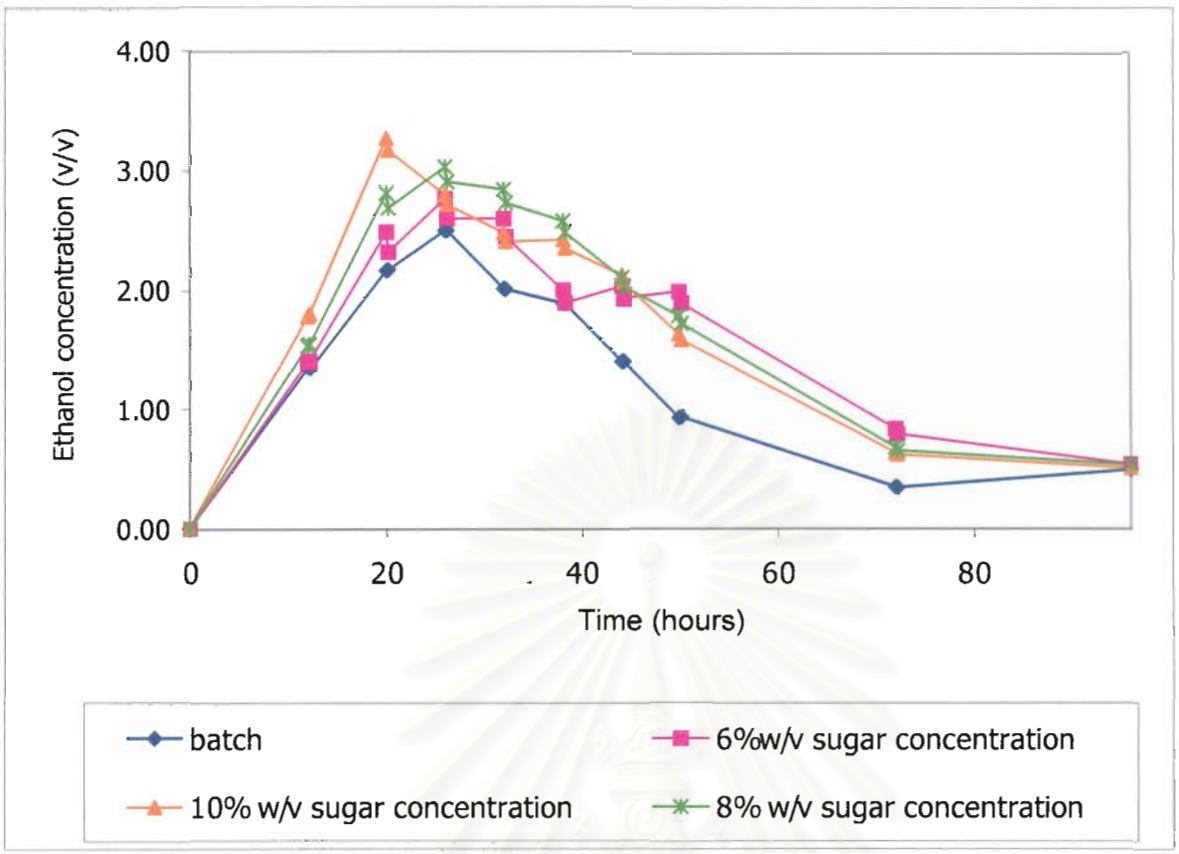


Figure 4.14 Ethanol profiles in the batch and fed-batch fermentations with various sugar concentrations in feed.

Ethanol concentration increased with time up to 3% (v/v) in the first 20 hours of the fermentation due to the high sugar concentration in the fermentation medium at the initial working volume of the processes. After that the ethanol concentrations continuously decreased through the end of the fermentation. After 20 hours of the fermentation, with the addition of feeding the yeasts used sugar fast enough so that sugar concentration level in the system was controlled between 1-2% (w/v). For the system with adequate supply of oxygen and low sugar concentration, yeast consumed ethanol as another C-source for the oxidation pathway.

4.2.2 Repeated fed-batch fermentation

In this study, the experiment was carried out to compare between repeated fed-batch with 40% v/v and 60% stillage replacement medium. The fermentation in the fed batch started from initial working volume of 500 ml of 5% reducing sugar with 40% and 60% v/v stillage replacement medium. Feed of 10 % (w/v) sugar concentration (40% and 60% stillage replacement) was injected to the fermenter as pulse pattern every 1 hour for 48 hours. Then 550 ml of the upper portion of fermentation broth was drawn out leaving 150 ml concentrated broth in the fermentor and 350 ml of fresh steriled medium was added. After 12 hours of the second fed-batch cultivation, feed of 10 % (w/v) sugar concentration (40% and 60% stillage replacement) was injected to the system again every 1 hour for 24 hour. Sample was taken every 6 hours for cell mass, sugar and ethanol analysts. The profiles of cell, residual sugar and ethanol concentration for the first cycle and second cycle of the repeated fed-batch were shown in Fig. 4.15, 4.16 and 4.17, respectively.

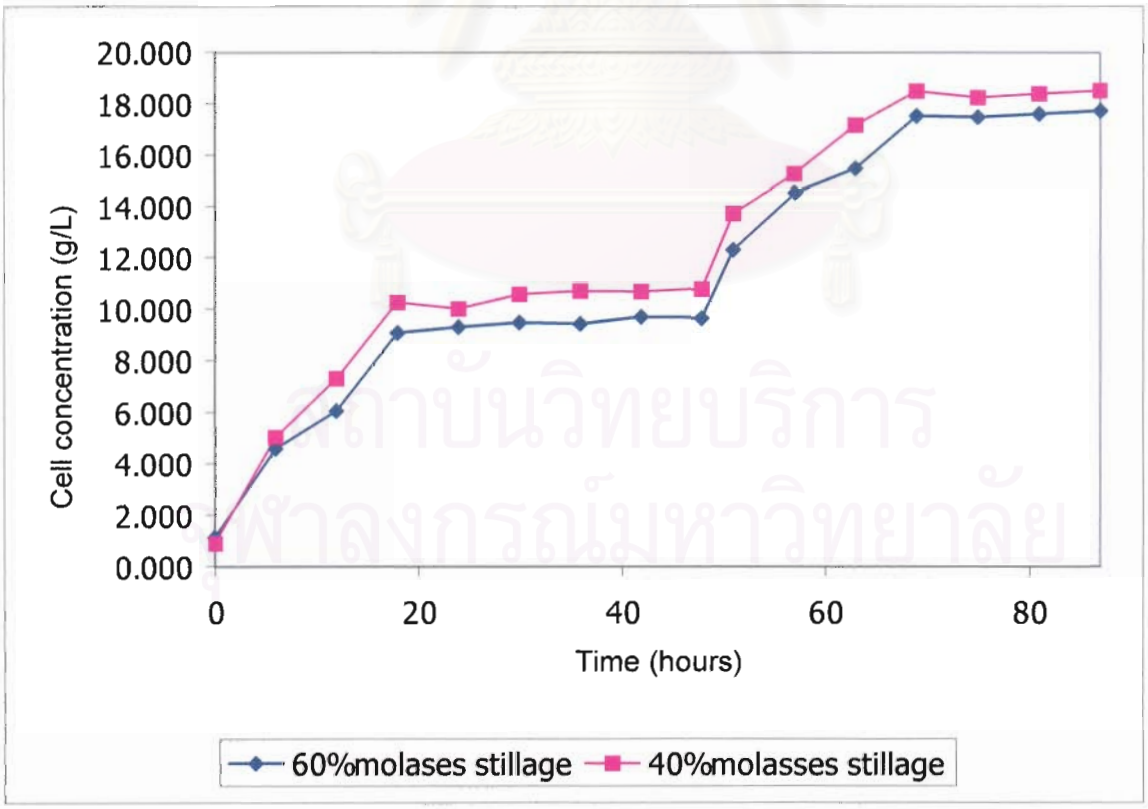


Figure 4.15 Cell concentration profiles in the repeated fed-batch fermentation.

The maximum cell concentration of 40% v/v and 60% v/v stillage replacement was 18.53 g/L and 17.75 g/L, respectively. The results revealed that the repeated fed-batch fermentation gave 58% higher cell concentration over the simple fed-batch. Bajbaiand Bajbai (1995) found that the repeated fed-batch gave 65.7% higher cell concentration over the batch for *Candida utilis* TCRDC-Y7 and 75% for *Paecilomyces variotii* TCRDC-M11.

Compared with the medium of 40% v/v stillage replacement, the results showed slightly negative effect of 60% v/v stillage replacement. With 60% V/V replacement, slightly decrease of cell growth and ethanol formation and the more residual sugar was observed.

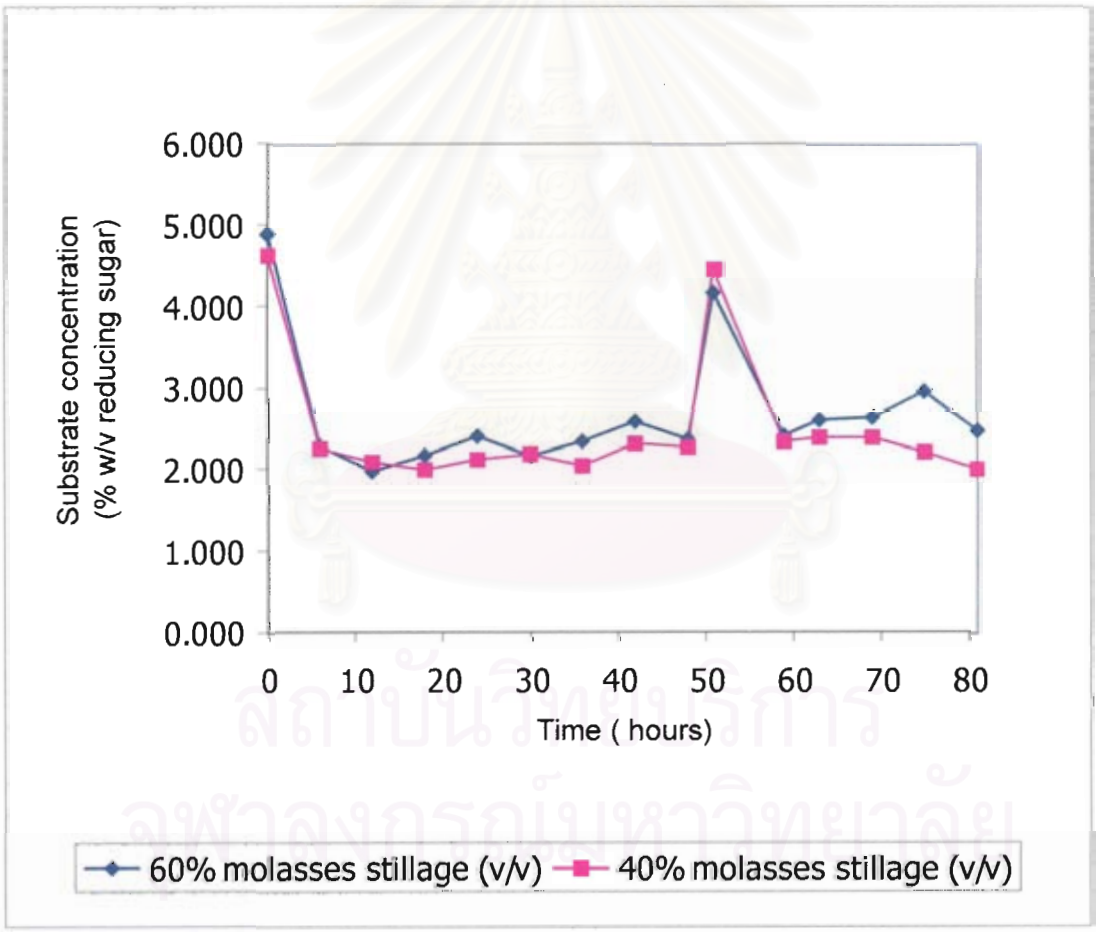


Figure 4.16 Residual sugar concentration profiles in the repeated fed-batch fermentation

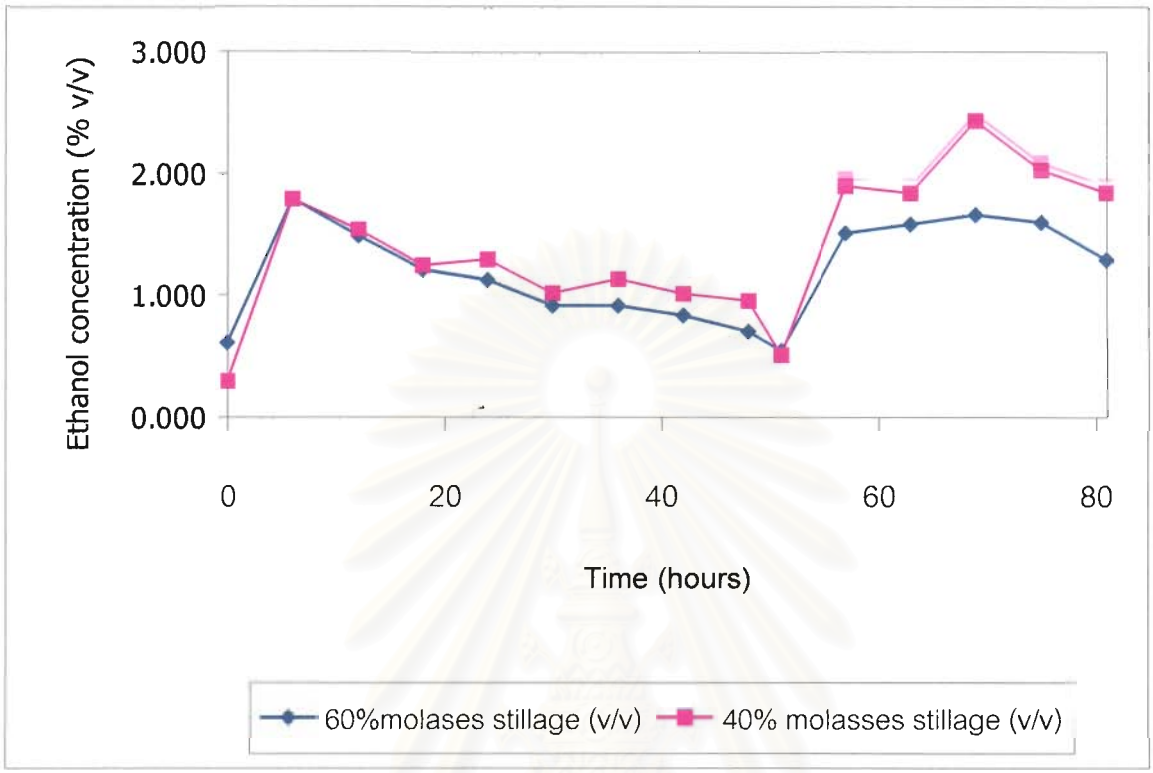


Figure 4.17 Ethanol concentration profiles in the repeated fed-batch fermentation.

In this study, ethanol formation was less than the previous simple fed-batch fermentation but with the same trend. The ethanol concentration increased during the high sugar concentration period and decreased after the sugar concentration in the system was maintained at low level (less than 2% (w/v)).

In fed-batch and repeated fed-batch fermentation, the growth can retard by the oxygen limitation. The oxygen- limited state for long period may cause some damage to the microorganism and may cause growth retardation.

4.3 COD and Protein content

Chemical oxygen demand (COD) of molasses was 1,109 g/L and COD of molasses stillage was 138 g/L. COD of the fermentation broth after the separation of cell mass was 115 g/L.

Percent of protein content of cell mass was 38 %.

Chapter 5

CONCLUSIONS AND RECOMMENTATIONS

5.1 Conclusions

The investigation of the optimal condition for biomass production from cane molasses alcohol stillage by *Saccharomyces cerevisiae* M30 was carried out in 500 ml shaking flasks at 33-35° C. The maximum cell concentration was obtained at pH 4.5 and the optimal ammonium sulfate concentration was 0.1% w/v. At initial sugar concentration from 2-10% w/v, the cell production rate increased with increasing initial sugar concentration. However, increasing of initial sugar concentration from 10-16% w/v did not enhance cell production but resulted in the decrease of cell mass yield. At initial sugar concentration 6-10%, the experimental results revealed that it was viable to use stillage replacement up to 40-60% of the total fresh water requirements without significant effect on cell growth. The result from the study was summarized in Table 5.1.1.

Table 5.1.1 The optimal conditions in shaking flask cultivation

Parameters	Ranges of study	Optimum	constraints
Initial sugar concentration (% w/v)	2-16	6-10	- At initial pH 5.0 - At room temperature (33-35 °C)
Stillage replacement (%v/v)	0-100	0-60	-At the initial sugar concentration of 6 and 10 %w/v without pH control -At 6%w/v of initial sugar and pH 4.5
pH	4.0-5.0	4.5	At 6% w/v of initial sugar concentration and 60% v/v of stillage replacement
Ammonium sulfate concentration (%w/v)	0.00-0.30	0.10	At 6% w/v of initial sugar concentration, 40%v/v stillage replacement, and pH 4.5

To improve biomass yield, adding the medium as pulse pattern throughout the fermentation process was applied with the variation of sugar concentration in the feeding. The results revealed that single fed-batch culture feeding with 10% (w/v) reducing sugar to control sugar level between 1-2 % (w/v) gave the maximum cell concentration compared with the feeding of 6% w/v and 8% (w/v) sugar concentration. At the end of the fermentation, cell concentrations from the batch and fed batch with feeding of 6%, 8% and 10% w/v reducing sugar were 8.84, 10.32, 10.54 and 11.76 g/L, respectively. Using single fed batch culture and repeated fed batch, biomass accumulation was increased by a factor of 1.3 and 2.1, respectively, relative to the batch culture. The biomass productivities from batch, single fed- batch culture and repeated fed- batch, were 0.12, 0.17 and 0.21 g/l.h, respectively. The results indicated that there exists a good potential for the use of alcohol stillage to replace of fresh water for single cell protein production. The results from the studies of the single fed-batch and repeated fed-batch were summarized in Table 5.1.2 and Table 5.1.3, respectively.

Table 5.1.2 The summary of the results from the single fed-batch cultivation

Sugar concentration in Feed stream (%w/v)	Attain maximal cell Concentration (g/L)	Yield of cell Yx/s	Improved cell productivity compare to batch cultivation (%)
6	10.32	0.14	16.74
8	10.54	0.15	19.23
10	11.76	0.17	33.03
Control (batch)	8.84	0.12	

.

Table 5.1.3. The summary of the results from the repeated fed-batch cultivation

Stillage replacement (%v/v)	Attained maximal cell concentration(g/L)	Yield of cell Yx/s	Improved cell productivity compare to batch cultivation (%)
40	18.53	0.22	78.57
60	17.75	0.21	64.29

5.2 Recommendations

For the further study, we suggest that:

1. The feed pattern in fed-batch fermentation should be optimized following the growth and sugar consumption during the fermentation.
2. The effect of scale up on the optimal conditions should be studied.
3. Oxygen could be the limiting substrate for the high cell-density cultivation; therefore the improved aeration rate system is required.
4. A kinetic and mass transfer model should be developed to optimize the process.

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APPENDICES

APPENDIX A

Reducing sugar concentration analysis

In this study, the sugar concentration analysis is modified from Dinitrosalicylic acid reagent Method as following.

Preparation the DNS reagent

1. Dissolve 1.6 gram of NaOH in 20 mL of distilled water.
2. Slowly add 1 gram of 3,5-dinitrosalicylic acid and stir until the solution is homogeneous.
3. Dilute the solution by adding 50 mL of distilled water.
4. Add 30 gram of sodium potassium tartrate and mix thoroughly.
5. Adjust the volume to 100 mL by adding distilled water.
6. Keep the prepared DNS solution in a brown bottle for 3 days.

Standard curve for DNS method

1. Dry sucrose in an oven at 100-105°C for 2 hours and then cool down the dried sucrose in a desiccator.
2. Dissolve 20 gram of dried sucrose in 100 mL of distilled water by using a volumetric flask.
3. Prepare the sucrose solution in the different concentration as shown in Table A.1.

Table A.1 Standard sucrose dilution

No.	Standard sucrose solution (mL)	Distilled water (mL)	Sucrose concentration (g/10mL)
1	0	10	0
2	2	8	4
3	4	6	8
4	6	4	12
5	8	2	16
6	10	0	20

4. Mix 1 mL of the prepared sucrose solution in 25 mL tube.
5. Blend 0.5 mL of conc. HCl (33%v/v) and hydrolyze sucrose in boil water for 10 minutes
6. Stop the reaction by placing in ice bath.
7. Add 0.5 mL of 20%v/v NaOH and mix.

8. Centrifuge the sample at 3000 rpm for 10 minutes to separate undissolved solid.
9. Mix 0.1 mL of the supernatant with 1 mL DNS reagent in 25 mL tubes.
10. Boil the sample in water bath for 10 minutes and place in ice bath to stop the reaction.
11. Add 10 mL of distilled water and mix thoroughly.
12. Measure absorbance at 520 nm by using sample no. 1 in the table A.1.
13. Plot the absorbance versus sucrose concentration.

Residual sugars concentration

The samples obtained from experiments are analyzed as the same procedure as the standard sample and quantified base on the standard curve. The calculation of sugars concentration as shown the follow:

$$\text{Sample sugar concentration (g/100ml.)} = \frac{\text{Absorbance}_{520}}{\text{Standard RDS slope}} \qquad \text{------- (A.1)}$$

APPENDIX B

Table B.1 Time-course of cell concentration during batch fermentation in shaking flasks with initial reducing sugar ranging from 2 to 16 % w/v

Time (hours)	Cell concentration (g/L)							
	2%	4%	6%	8%	10%	12%	14%	16%
0	0.30	0.15	0.37	0.28	0.27	0.18	0.10	0.05
6	0.90	1.15	1.42	1.63	1.65	1.60	1.43	1.18
12	1.33	1.92	2.42	2.80	2.65	3.27	3.30	3.35
18	1.63	2.58	3.38	4.00	4.68	4.77	4.80	4.65
24	1.60	2.53	3.05	3.78	4.18	4.48	4.22	4.62
30	1.67	2.42	3.18	3.80	4.30	4.70	4.93	5.08
36	1.68	2.77	3.25	3.83	4.28	4.58	4.95	5.02
42	1.80	2.92	3.47	4.08	4.55	4.72	4.55	4.60
48	1.90	2.98	3.58	4.32	4.45	4.42	4.33	4.07

Table B.2 Time-course of substrate concentration during batch fermentation in shaking flasks with initial reducing sugar ranging from 2 to 16 % w/v

Time (hours)	Substrate concentration (%w/v reducing sugar)							
	2%	4%	6%	8%	10%	12%	14%	16%
0	2.08	4.23	6.95	9.06	9.90	12.90	14.23	14.62
6	1.15	2.84	4.28	6.62	8.77	8.86	11.82	13.18
12	0.33	0.90	1.50	2.97	3.96	6.16	7.64	9.49
18	0.18	0.42	0.73	1.80	1.52	2.20	2.82	4.31
24	0.27	0.59	0.97	1.59	1.96	2.18	3.06	3.55
30	0.29	0.66	1.13	1.55	1.76	2.21	2.64	2.99
36	0.34	0.59	0.90	1.35	1.75	2.01	2.52	2.82
42	0.36	0.61	0.92	1.26	1.67	2.09	2.32	2.82
48	0.37	0.62	0.87	1.25	1.67	1.94	2.35	2.57

Table B.3 Time-course of cell concentration during batch fermentation in shaking flasks with stillage replacement from 0 to 100 % v/v at 10% w/v initial sugar concentration

Time (hours)	Cell concentration (g/l)					
	0%	20%	40%	60%	80%	100%
0	0.27	0.28	0.20	0.00	0.10	0.30
6	1.87	1.73	1.35	1.37	1.10	1.27
12	3.70	3.52	3.20	3.22	3.27	1.78
18	4.70	4.63	4.35	4.00	3.84	2.07
24	4.87	4.62	4.37	4.12	3.85	2.28
30	5.02	4.47	3.65	4.15	3.75	2.50
36	4.85	4.58	4.22	4.03	3.87	2.70
42	4.98	5.22	4.58	4.28	4.45	3.20
48	5.15	4.77	4.55	4.18	3.18	3.02

Table B.4 Time-course of substrate concentration during batch fermentation in shaking flasks with ranging stillage replacement from 0 to 100 % v/v at 10% w/v initial sugar concentration

Time	Substrate concentration (% w/v reducing sugar)					
(hours)	0%	20%	40%	60%	80%	100%
0	11.67	11.99	11.95	11.08	10.32	2.77
6	8.89	9.81	9.50	8.44	9.36	1.44
12	5.72	5.50	5.65	5.43	4.85	1.29
18	1.80	1.91	2.17	2.04	2.69	1.10
24	1.58	2.21	1.92	1.95	2.25	1.05
30	1.60	1.84	1.95	2.11	2.15	1.11
36	1.80	1.90	2.04	2.42	2.42	1.10
42	2.04	2.17	1.98	1.93	2.61	1.43
48	1.84	2.01	2.46	2.65	2.49	1.22

Table B.5 Time course of cell concentration during batch fermentation in shaking flasks with ranging stillage replacement from 0 to 100 % v/v at 6% w/v initial sugar concentration

Time (hours)	Cell concentration (g/l)					
	0%	20%	40%	60%	80%	100%
0	0.13	0.33	0.17	0.40	0.27	0.33
6	1.43	1.63	1.47	1.67	1.40	1.17
12	2.50	2.70	2.60	2.90	2.73	1.67
18	3.17	3.23	2.90	3.30	2.90	1.80
24	3.53	3.37	3.23	3.30	2.90	2.22
30	3.67	3.57	3.40	3.80	3.50	2.80
36	3.83	3.90	3.10	3.93	3.50	2.77
42	3.77	3.80	3.12	4.10	3.57	2.90
48	3.90	3.83	3.67	3.97	3.70	3.07

Table B.6 Time course of substrate concentration during batch fermentation in shaking flasks with ranging stillage replacement from 0 to 100 % v/v at 6% w/v initial sugar concentration.

Time	Substrate concentration (%w/v reducing sugar)					
(hours)	0%	20%	40%	60%	80%	100%
0	6.00	6.66	6.18	6.11	6.40	2.70
6	4.91	4.85	4.77	4.68	4.74	1.44
12	2.38	2.37	2.07	1.96	1.83	1.20
18	1.23	1.31	1.27	1.60	1.75	1.13
24	0.98	1.20	1.32	1.63	1.73	1.33
30	0.97	1.19	1.58	1.57	1.36	1.29
36	1.05	1.07	1.32	1.66	1.73	1.14
42	0.95	1.22	1.36	1.61	1.77	1.25
48	1.00	1.26	1.30	1.70	1.74	1.17

Table B.7 Time-course of cell concentration during batch fermentation in shaking flasks with pH ranging from 4.0 to 5.0 at 60% v/v stillage replacement

Time (hours)	Cell concentration (g/L)		
	pH 4	pH 4.5	pH 5
0	0.22	0.27	0.18
6	1.10	1.32	1.17
12	2.92	3.18	2.65
18	2.77	3.00	2.53
24	2.58	3.17	3.28
30	2.27	2.48	2.50
36	2.70	2.93	2.00

Table B.8 Time-course of substrate concentration during batch fermentation in shaking flasks with pH ranging from 4 to 5 at 60% v/v of stillage replacement.

Time (hours)	Substrate concentration (% w/v reducing sugar)		
	pH 4	pH 4.5	pH 5
0	5.97	5.59	5.97
6	4.60	4.11	4.40
12	1.35	1.47	1.37
18	1.31	1.46	1.31
24	1.37	1.50	1.28
30	1.39	1.27	1.34
36	0.82	0.74	0.74

Table B.9 Time-course of cell concentration during batch fermentation in shaking flasks with stillage replacement varied from 0-100% (v/v) and 6% (w/v) initial sugar concentration at pH 4.5

Time	Cell concentration (g/L)					
(hours)	0%	20%	40%	60%	80%	100%
0	0.18	0.23	0.17	0.27	0.17	0.20
6	1.65	1.48	1.95	1.32	1.25	1.08
12	3.30	3.05	3.08	3.18	2.88	1.37
18	3.32	3.08	3.00	3.00	2.65	1.52
24	2.38	3.07	3.07	3.17	3.00	1.90
30	3.07	2.13	2.78	2.48	2.28	1.52
36	2.05	2.58	3.07	2.93	2.77	1.81

Table B.10 Time-course of substrate concentration during batch fermentation in shaking flasks with stillage replacement varied from 0-100% (v/v) and 6% (w/v) initial sugar concentration at pH 4.5

Time (hours)	Substrate concentration (%w/v reducing sugar)					
	0%	20%	40%	60%	80%	100%
0	5.71	5.63	5.91	5.59	5.36	2.15
6	4.15	3.74	4.75	4.11	4.19	1.15
12	1.19	1.09	1.45	1.47	1.61	0.95
18	0.87	1.00	1.22	1.46	1.61	0.96
24	0.98	0.97	1.13	1.50	1.56	0.98
30	1.00	1.16	1.24	1.27	1.31	1.00
36	0.52	0.56	0.70	0.74	0.86	0.52

Table B.11 Time-course of cell concentration during batch fermentation in shaking flasks with ammonium concentration ranging from 0-2% w/v

Time (hours)	Ammonium sulfate concentration (% w / v)						
	40% stillage replacement (v / v)						0% stillage replacement
	0.00	0.05	0.10	0.15	0.20	0.30	0.05
0	0.72	0.66	0.71	0.68	0.75	0.65	0.70
6	3.20	3.73	3.40	2.93	3.87	4.13	3.67
12	3.34	4.70	4.90	6.30	4.57	5.97	3.97
24	4.60	5.00	5.73	6.37	6.43	6.43	5.10
36	4.70	5.00	5.50	6.37	6.33	6.60	5.13
48	5.10	5.00	6.23	7.20	6.80	7.07	5.10
60	5.17	5.63	6.80	7.13	6.97	7.00	5.13
72	5.93	5.97	6.80	6.37	6.90	7.23	5.63
84	6.27	6.60	7.32	7.05	6.95	7.54	6.44
96	6.10	6.37	7.03	6.97	7.09	7.40	6.32

Table B.12 Time-course of cell concentration during batch and fed-batch fermentation in 1 L- fermenter with various feeding concentration

Time (hours)	Cell concentration (g/L)			
	batch	fed-batch		
		6% w/v	8%w/v	10%w/v
0	0.58	0.76	0.98	0.82
0.1	0.86	0.64	1.00	2.08
12	5.54	7.14	7.36	8.72
12.2	5.54	5.18	8.35	6.92
20	6.82	6.82	7.38	9.54
20.2	6.82	6.40	7.08	9.25
26	7.52	8.44	8.36	9.44
26.2	7.52	7.95	8.03	9.16
32	7.36	9.30	9.02	9.58
32.2	7.36	8.79	8.68	9.31
38	7.14	9.38	9.12	9.94
38.2	7.14	8.90	8.79	9.66
44	7.48	9.36	9.24	11.04
44.2	7.48	8.90	8.91	10.74
50	7.00	8.90	10.18	10.88
50.2	7.50	8.48	9.83	10.59
72	8.44	9.94	10.24	12.08
72.2	8.44	9.50	9.90	11.77
96	8.84	10.32	10.54	11.76

Table B.13 Time-course of substrate concentration during batch and fed-batch fermentation in 1 L- fermenter with various feeding concentration.

Time (hours)	Substrate concentration (%w/v reducing sugar)			
	batch	fed-batch		
		6% w/v	8%w/v	10%w/v
0	7.00	6.08	5.29	6.16
0.1	5.32	6.08	5.13	5.86
12	2.82	2.24	2.22	2.25
12.2	2.82	2.70	2.63	2.68
20	1.97	1.73	1.85	2.03
20.2	1.97	2.16	2.24	2.44
26	1.80	1.74	1.93	1.98
26.2	1.80	2.15	2.32	2.37
32	1.17	1.40	1.50	1.81
32.2	1.17	1.78	1.88	2.19
38	1.21	1.29	1.72	1.56
38.2	1.21	1.66	2.09	1.93
44	1.19	1.51	1.64	1.70
44.2	1.19	1.86	1.99	2.05
50	1.39	1.56	1.58	1.63
50.2	1.39	1.89	1.93	1.97
72	1.30	1.47	1.45	1.54
72.2	1.30	1.79	1.79	1.87
96	1.34	1.46	1.54	1.66

Table B.14 Time-course of ethanol concentration during batch and fed-batch fermentation in 1 L- fermenter with various feeding concentration.

Time (hours)	Ethanol concentration (%v/v)			
	batch	fed-batch		
		6% w/v	8%w/v	10%w/v
0	0.01	0.01	0.02	0.02
12	1.35	1.41	1.80	1.54
12.2	1.35	1.42	1.81	1.56
20	2.18	2.50	3.28	2.82
20.2	2.18	2.33	3.18	2.70
26	2.51	2.78	2.81	3.04
26.2	2.51	2.61	2.73	2.92
32	2.02	2.61	2.49	2.85
32.2	2.02	2.46	2.42	2.74
38	1.90	2.01	2.43	2.59
38.2	1.90	1.90	2.36	2.49
44	1.42	2.05	2.14	2.12
44.2	1.42	1.94	2.08	2.04
50	0.94	1.99	1.64	1.79
50.2	0.94	1.90	1.59	1.72
72	0.35	0.84	0.64	0.69
72.2	0.35	0.80	0.63	0.66
96	0.50	0.55	0.51	0.54

Table B.15 Time-course of cell concentration during repeated fed-batch fermentation in 1 L- fermenter with various proportion of stillage replacement

Time (hours)	Cell concentration (g/L)	
	60% stillage replacement	40% stillage replacement
0	1.14	0.92
6	4.60	5.04
12	6.06	7.32
18	9.10	10.28
24	9.32	10.04
30	9.50	10.62
36	9.45	10.74
42	9.72	10.70
48	9.70	10.82
51	12.35	13.77
57	14.56	15.30
63	15.50	17.20
69	17.54	18.52
75	17.50	18.25
81	17.62	18.40
87	17.75	18.53

Table B.16 Time-course of substrate concentration during repeated fed-batch fermentation in 1 L- fermenter with various proportion of stillage replacement.

Time (hours)	Substrate concentration (%w/v reducing sugar)	
	60% stillage replacement	40% stillage replacement
0	4.90	4.63
6	2.29	2.26
12	1.98	2.10
18	2.18	2.00
24	2.41	2.13
30	2.17	2.19
36	2.35	2.05
42	2.59	2.33
48	2.37	2.28
51	4.18	4.47
57	2.42	2.35
63	2.60	2.40
69	2.64	2.40
75	2.96	2.21
81	2.48	2.00
87	1.92	1.54

Table B.17 Time-course of ethanol concentration during repeated fed-batch fermentation in 1 L- fermenter with various proportion of stillage replacement.

Time (hours)	Ethanol concentration (% v/v)	
	60% stillage replacement	40% stillage replacement
0	0.62	0.31
6	1.80	1.80
12	1.49	1.54
18	1.21	1.25
24	1.13	1.29
30	0.92	1.02
36	0.92	1.14
42	0.84	1.02
48	0.71	0.96
51	0.55	0.52
57	1.51	1.90
63	1.59	1.84
69	1.67	2.44
75	1.60	2.03
81	1.30	1.85
87	1.16	1.64

BIOGRAPHY

Miss Thasanaprapha Lertsremongkol was born on 26th February, 1979 in Bangkok. She finished her secondary school from Suankularb Witthayalai Nonthaburi School in March, 1997. After that, she studied in the major of Food Technology in Faculty of Science at Chulalongkorn University. She continued her further study in Master's degree in Chemical Engineering at Chulalongkorn University. She participated in the Biochemical Engineering Research Group and achieved his Master's degree in September, 2004.