EFFICIENT CULTIVATION OF *Kluyveromyces lactis* IN HIGH CELL DENSITY CULTURE IN FED-BATCH CULTIVATION SYSTEM

MOHD SHAFIQ B MOHD SUEB

A dissertation submitted in fulfillment of the
requirements for the award of degree of
Master of Engineering (Bioprocess)

Faculty of Chemical Engineering
Universiti Teknologi Malaysia

JUN 2012

PERPUSTAKAAN
UNIVERSITI MALAYSIA PAHANG
No. Perolehan 067354
Tarikh 11 OCT 2012
No. Panggilan TP 248.65 .L59 S53 2012

ABSTRACT

Kluyveromyces lactis is beneficial and well-known yeast due to its status of GRAS that has made the yeast as a vital microorganism for the subject of studies and also the applications of industry such as a possible source of single-cell protein with expected probiotic properties, oligonucleotide-derived flavour enhancers and lactic acid. In addition, High Cell Density Cultivation (HCDC) of *K. lactis* has been scientifically and biotechnologically important trend in improving microbial mass and product formation substantially. More importantly, fed-batch strategy has been identified as another effective mean to increase the yield by preventing substrate limitation or inhibition through maintaining medium substrate concentration at a low level during cultivation. In this study, there were two system cultivations have been employed which was batch cultivation and fed-batch cultivation. Further studies have been done under batch cultivation on the aeration effect and dissolved O_2, DO. The results showed that 1.0 v/v/min aeration rate gave relatively high cell dry weight, CDW, 18.6 g/L. On the contrary, the results from DO stat value of 60% showed substantial increment with CDW of 28.7 g/L. As for the fed-batch study, constant feeding rate strategy has been applied with two different feeding substrates i.e complete media and mono-lactate. The CDW harvested for complete media had showed remarkable result, 59.75 g/L as compared to CDW produced from lactose as a sole feeding substrate, 44.75 g/L.

ABSTRAK

Kluyveromyces lactis merupakan yis yang dilihat bermanfaat dan sudah dikenali, mempunyai status GRAS telah menjadikannya mikroorganisma yang penting dalam subjek kajian dan juga aplikasi industri seperti probiotik yang terkandung dalam sumber protein sel-ringkas, penyedap rasa oligonukleotid dan asid laktik. Tambaham pula, Pengkulturan Sel Berketumpatan Tinggi oleh *K. lactis* secara saintifik dan bioteknologi merupakan kaedah yang penting dalam mempertingkatkan biojisim dan produk secara berkesan. Tidak hanya sekadar itu, strategi fed-batch telah dikenalpasti sebagai suatu kaedah yang efektif bagi meningkatkan hasil dengan menghalang kekurangan substrat atau ketidakcukupan melalui pengekalan kepekatan media substrat di tahap rendah semasa pengkulturan. Dalam kajian ini, terdapat dua jenis sistem pengkulturan yang telah digunapakai iaitu pengkulturan *batch* dan *fed-batch*. Kajian lanjut telah dijalankan bagi pengkulturan batch terhadap kesan pengudaraan dan keterlarutan O_2.Kajian mendapati bagi kesan pengudaraan, 1.0 v/v/min telah menunjukkan berat sel kering yang tinggi iaitu 18.6 g/L. Manakala bagi keterlarutan O_2 pula, keputusan menunjukkan keterlarutan O_2 pada tahap 60% telah memberikan nilai yang tinggi bagi berat sel kering iaitu 28.7 g/L. Bagi kajian yang dijalankan terhadap pengkulturan *fed-batch*, strategi penambahan substrat berkadar tetap telah digunakan untuk 2 jenis penambahan substrat yang berbeza iaitu media penuh dan mono-laktos. Berat sel kering yang diperolehi melalui penambahan media penuh menujukkan hasil di luar jangkauan iaitu 59.85 g/L berbanding berat sel kering yang diperolehi daripada penambahan laktos sebagai substrat sahaja, iaitu 44.75 g/L.

TABLE OF CONTENTS

CHAPTER	TITLE	PAGE
	DECLARATION	ii
	DEDICATION	iii
	ACKNOWLEDGEMENT	iv
	ABSTRACT	v
	ABSTRAK	vi
	TABLE OF CONTENTS	vii
	LIST OF TABLES	xi
	LIST OF FIGURES	xiii
	LIST OF ABBREVIATIONS	xiv
	LIST OF SYMBOLS	xv
	LIST OF APPENDICES	xvi
1	INTRODUCTION	1
	1.1 Background of the Study	1
	1.2 Problem Statements	4
	1.3 Objective of the Study	4
	1.4 Scope of the Study	5

CHAPTER	TITLE	PAGE

2	**LITERATURE REVIEW**	6
	2.1 *Kluyveromyces sp.*	6
	2.1.1 *Kluyveromyces lactis*	6
	2.1.2 Properties of *Kluyveromyces lactis*	8
	2.2 Industrial Use	9
	2.3 High Cell Density Cultivation	10
	2.3.1 HCDC of Microorganism	10
	2.3.2 HCDC Bioreactors	11
	2.4 Effect of Fermentation Parameters on *K. Lactis*	11
	2.4.1 Effect of Temperature	11
	2.4.2 Effect of pH	13
	2.4.3 Effect of Agitation	14
	2.4.4 Effect of Dissolved Oxygen	15
	2.4.5 Effect of Incubation Period	17
	2.5 Effect of Medium Composition	18
	2.5.1 Effect of Carbon Source	20
	2.5.2 Effect of Nitrogen and Other Macronutrients	23
	2.5.3 Effect of Micronutrients and Vitamins	25
	2.6 Fermentation Mode of Operations	26
	2.6.1 Fed-Batch Cultivation	26
	2.7 Fed-Batch Feeding Strategies	28

CHAPTER	TITLE	PAGE
3	**METHODOLOGY**	30
	3.1 Strain	30
	3.1.1 Working cell culture	30
	3.1.2 Inoculation procedure	31
	3.2 Cultivation media	31
	3.2.1 Shake flask cultivation	32
	3.2.2 Bioreactor cultivation	32
	3.2.2.1 Different aeration rate	33
	3.2.2.2 Different dissolved oxygen	33
	3.2.2.3 Fed batch strategy	34
	3.3 Analytical method	34
	3.3.1 Determination of cell dry weight	34
	3.3.2 Determination of pH	35
	3.3.3 Determination of lactose consumption	35
	3.3.4 Determination of lactic acid concentration	36
4	**RESULTS & DISCUSSION**	37
	4.1 Growth of *Kluyveromyces lactis* in shake flask	37
	4.2 Cultivation of *K. lactis* in 16-L stirred tank bioreactor in batch culture	40
	4.2.1 Effect of aeration rate on the kinetics of *K. lactis* cell mass production during batch cultivation in 16-L stirred tank bioreactor	41

4.2.2 Effect of dissolve oxygen in batch cultivation on kinetics of cell growth of *K. lactis* cultivation in 16-L stirred tank bioreactor 49
4.3 The effect of fed-batch cultivation strategy on high cell density production 55
4.4 Overview of efficient cultivation of *K. lactis* in high cell density culture 61

5 **CONCLUSION & RECOMMENDATIONS** 64

REFERENCES 66
Appendix A 79
Appendix B 81

LIST OF TABLES

TABLE NO.	TITLE	PAGE
2.1	Temperature suggested for *K. lactis* fermentation by various authors	12
2.2	Comparison of lactic acid fermentation by bacterial, yeast and filamentous fungal cultures	22
2.3	Effect of medium composition on extracellular α-galactosidase production by *K. lactis* MSK110-MIRK in aerobically chemostat culture at 30°C, dilution rate of 0.10/h and pH 5	24
4.1	Kinetic parameters of cell growth in shake flask	38
4.2	Kinetic parameters of cell growth in bioreactor aeration rate 0.5 v/v/min	42
4.3	Kinetic parameters of cell growth in bioreactor aeration rate 1.0 v/v/min	44
4.4	Kinetic parameters of cell growth in bioreactor aeration rate 2.0 v/v/min	47
4.5	Kinetic parameters of cell growth in bioreactor at controlled DO stat value of 20%	49
4.6	Kinetic parameters of cell growth in bioreactor at controlled DO stat value of 40%	51

4.7	Kinetic parameters of cell growth in bioreactor at controlled DO stat value of 60%	53
4.8	Feeding media components with 5 times concentration	56
4.9	Kinetic parameters of cell growth of fed-batch cultivation in bioreactor using complete feeding	57
4.10	Kinetic parameters of cell growth of fed-batch cultivation in bioreactor using mono-lactose feeding	59
4.11	Overview of kinetic parameters of *K. lactis* for different strategies of cultivations	63

LIST OF FIGURES

FIGURE NO.	TITLE	PAGE
2.1	Equation based on the stoichiometry for growth and product formation	18
4.1	Kinetics of cell growth, lactose consumption and lactic acid production in shake flask culture	39
4.2	Cultivations in bioreactor aeration rate 0.5 v/v/min	43
4.3	Cultivations in bioreactor at aeration rate 1.0 v/v/min	45
4.4	Cultivation in bioreactor at aeration rate 2.0 v/v/min	48
4.5	Batch cultivation in bioreactor at controlled DO stat value of 20%	50
4.6	Batch cultivation in bioreactor at controlled DO stat value of 40%	52
4.7	Batch cultivation in bioreactor at controlled DO stat value of 60%	54
4.8	Fed-batch cultivation in bioreactor using complete medium feeding	58
4.9	Fed-batch cultivation in bioreactor using mono-lactate feeding	60

LIST OF ABBREVIATIONS

GRAS	- Generally Regarded As Safe
MSUR	- Maximum Substrate Uptake Rate
DO	- Dissolved Oxygen
OD	- Optical Density
HCDC	- High Cell Density Culture
STR	- Stirred Tank Reactor
FDA	- Food and Drug Administrative
CO_2	- Carbon Dioxide
H_2O	- Water
EPA	- Eicosapentaenoic acid
NaOH	- Sodium Hydroxide
H_2SO_4	- Sulphuric Acid

LIST OF SYMBOLS

μ	-	cell specific growth rate (h^{-1})
μ_{max}	-	max cell specific growth rate (h^{-1})
$Y_{x/s}$	-	biomass/substrate yield (g/g)
q_s	-	specific substrate consumption rate (h^{-1})
S	-	substrate concentration in medium (g substrate/ g medium)
S_i	-	substrate concentration in feeding solution (g substrate/g feed) or (g/L)
F	-	substrate feed rate (g/L/h)
V_0	-	initial culture volume (L)
X_0	-	initial cell concentration (g/L)
t	-	culture time (h)

LIST OF APPENDICES

APPENDIX.	TITLE	PAGE
A		79
A1	Lactose concentration standard curve	79
A2	Lactic acid standard curve	80
B	Equipments & Instruments	81
B1	Spectrophotometer (HACH, Loveland, CO., USA).	81
B2	Centrifuge machine (Hettich, ZENTRIFUGEN, Germany)	82
B3	pH meter(TOLEDO, Delta 320 pH Meter, Switzerland)	82
B4	Autoclave (Hirayama, HICLAVA HV-110, Japan)	83
B5	Mass Balance (Precisa, XT 220A, Switzerland)	83
B6	Rotary shaker (Innova 4080, New Brunswick, NJ, USA)	84
B7	16.0L Stirred Tank Bioreactor (BioEngineering, Wald, Switzerland)	84

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Kluyveromyces lactis is viewed as fascinating yeast which has became the studies for decades of its distinctiveness. This has been stressed by many researchers that *K. lactis* is safe to be used in many food industry purposes (Bonekamp and Oosterom 1994). Based on its status as Generally Regarded As Safe (GRAS) according to Food and Drug Association (FDA), it made the yeast as one of the most important microorganism for both conventional and modern applications of industry which includes β-galactosidase production and biomass production for probiotic applications. Recently, *K. lactis* has been utilized commercially for host in the production of heterologous protein in pharmaceutical applications (van Ooyen *et al.*, 2006). Due to that reason, *K. lactis* is recognized as a spectacular microorganism over other yeast expression system which includes easy genetic manipulation and the availability of a fully sequenced genome (Dujon *et al.*, 2004). More recently, *K. lactis* has become a potential source of single-cell protein (Ghaly *et al.*, 2005) with putative probiotic

characteristics (Kumura *et al.*, 2004), as well as oligonucleotide-derived flavor enhancers (Belem and Lee, 1998) and lactic acid (Porro *et al.*, 1999)

High-cell-density cultivation has been known to enhance biomass production as well as product formation significantly. In order to maximize the volumetric productivity, high cell density culture (HCDC) has become the prerequisite for the cultivation in many bioprocesses related to recombinant and non-recombinant products formation. Moreover, Lee and Chang, (1993) have stressed the importance of high cell density culture that would makes downstream processing easier associated with lower waste production. In order to achieve HCDC, good knowledge about the strain used is required since this process is not generic and highly specific for each strain based on its physiological characteristics and growth kinetics. Thus, to optimize HCDC cultivation strategy, further studies are usually required based on the type of strain used (Riesenberg and Guthke, 1999). Nutrient(s) limitation particularly in carbon source such as glucose, glycerol, methanol and others could make the microbial HCDC possible to be done under proper feeding conditions.

Attempts to enhance scaling up production of heterologous protein have become the main focus in the study of cultivation *K. lactis*. To achieve HCDC, fed-batch cultivation strategy optimization is required. HCDC in fed-batch cultivation can be referred as a successful technique for overcoming cellular regulatory mechanisms like crabtree effect, catabolite expression, and product inhibition would improve the productivity for both the homologous and heterologous protein. By maintaining medium substrate concentration at low level during cultivation, substrate limitation or inhibition can be avoided that would lead to a high cell density (Wen *et al.*, 2002).

High substrate consumption showed the fast growth to high cell densities and high product formation. Since both overfeeding and underfeeding of nutrient is unfavorable to cell growth and product formation, development of a suitable feeding strategy is vital in fed-batch cultivation (Lee *et al.*, 1999). Consequently, in order to develop fed-batch strategies without growth limitation, a well-balanced substrate feeding with direct feedback control of the carbon source concentration is required (Riesenberg and Guthke, 1999). They have also reported that there are two principle strategies for the control of the nutrient feed; open-loop control which is used to achieve a certain predetermine feeding profile and closed-loop control which is used for physicochemical and environmental parameters such as temperature, pH, foam, agitation etc. are widely applied to design feeding strategy.

The main goal of the present study is to maximize the cell mass production of the probiotic yeast *K. lactis* in semi-industrial scale production using different bioprocess strategies. However, the approach to be taken to fulfill the purpose is through the efficient cultivation method of fed-batch in High Cell Density Culture. This would include the roles of oxygen supply to the fermentation process by studying the effect of different aeration rate. Besides that, different strategies of substrate feeding would also become the main interests in this study in order to identify the most suitable strategy for high cell mass production of this type of yeast.

1.2 Problem Statements

It is significant to develop a cultivation system that would allow production of required product to a high concentration as well as high productivity and yield. Thus, to enhance the production of biomass yield of *K. lactis*, two steps of bioprocess optimization are required. First, by developing a suitable cultivation strategy for high cell mass production during cell cultivation in bioreactor culture. This could be achieved by studying the effect of the key biochemical engineering parameters in this process. Second, to design an effective fed-batch cultivation strategy to promote cell growth in high cell density culture with minimal byproduct(s) formation. In addition, the ultimate intention in any production is to have low-cost of production in terms of raw materials, process, utilities and others. Consequently, by having efficient strategies that could improve the yield, this matter could be achieved in parallel.

1.3 Objective of the Study

The objective of this study is to maximize *K. lactis* biomass production through efficient cultivation system of fed batch in High Cell Density Culture.

1.4 Scope of the Study

Hence, in order to accomplish the objectives of this study, the following research scopes have been identified

1. Effect of different aeration rate on cell growth kinetics in batch culture
2. Cultivation of cells under different DO stat cultures
3. Design of proper feeding strategy for High Cell Density Culture by
    - Constant feeding strategy of substrate (complete medium addition) concomitant with keeping DO at constant level.
    - Constant feeding strategy of substrate (carbon source addition) concomitant with keeping DO at constant level

CHAPTER 2

LITERATURE REVIEW

2.1 *Kluyveromyces sp.*

2.1.1 *Kluyveromyces lactis*

Kluyveromyces lactis is a yeast strain of different industrial and research applications. The name of this yeast comes from its capability to assimilate lactose and convert it into lactic acid. Besides that, Food and Drug Administration (FDA) has declared *K. lactis* as one of safe organisms and given GRAS status (Generally regard as safe) for different industrial applications. The FDA confirms that: "*K. lactis* is a normal, even necessary component of many cultured dairy products" and that "no reports of toxicity or pathogencity have ever been associated with the presence of *K. lactis* in food" (Randolph, 1984).

There are many publications that have reported the successful use of *K. lactis* in different types of industries. According to van Ooyen *et al*, (2006), *K. lactis* is excellent host for recombinant chymosin production in large scale and it was the first protein from higher eukaryote origin to be expressed in single cellular organisms. Merico *et al.* (2004) have also reported that *K. lactis* is commercially used to produce lactase- and lactose-free milk and also potential source of production of α-galactosidase in the food industry. On the other hand, Swinkels *et al.* (1993) reported that the genetically modified *K. lactis* has been used to produce human serum albumin, human interleukin-1beta, and hepatitis-B surface antigen and other biopharmaceutically important compounds.

As reported by Van der Walt (1970), *K. lactis* was initially known as *Saccharomyces lactis*. However, due to the unable cross-breed with the yeast *S. cerevisiae,* it was re-classified under the genus *Kluyveromyces* in 1965 and renamed *K. lactis.* The yeast was then placed at the Central Bureau voor Schimmelcultures in The Netherlands. Generally, the shorter name *K. lactis* is used and the classification of the species became as followed:

Family:	Saccharomycetaceae
Subfamily:	Saccharomycetoideae
Genus:	*Kluyveromyces*
Species:	*K. marxianus* var. *lactis*

2.1.2 Properties *of Kluyveromyces lactis*

It have been reported by many authors that *Kluyveromyces lactis* is as essential as *Kluyveromyces marxianus* due to lactose metabolize ability (van der Walt., 1970; Wésolowski *et al.,* 1996). It is amenable to genetic studies, having four spores in an evanescent ascus, so that single-spore cultures can be easily obtained which is slightly smaller than those of *S. cerevisiae.*

K. lactis is a type of yeast which does not have some less favorable characteristics that *S. cerevisiea* might have such as hyperglycosylation of excreted recombinant proteins (Innis, 1989), a limited protein-secretion capacity (Kingsman *et al.,* 1987) and a Crabtree-positive physiology (Petrik *et al.*, 1983; Postma *et al.*, 1988). According to Suleau *et al.* (2006), *K. lactis* is generally described as a strict aerobic yeast and Crabtree-negative yeast.

K. lactis can be grown on numerous of lactose, e.g., cheese whey, for food or fodder yeasts or as a source of β-galactosidase. However, it have been reported that this type of yeast can grow in high cell density culture using fed-batch cultivation strategy (up to more than 100 g dry cell weight per liter) in corn steep liquor/glucose medium (Wésolowski *et al.*, 1996). As known, due to non-pathogenic for human, this strain can also be used as a host for production of heterologous proteins whereby *K. lactis* possesses effective ability to synthesize and secrete as such protein which allows using chymosin expressed in this yeast in the food industry (Starovoitova *et al.*, 2006).

2.2 Industrial Use

Traditionally, this type of strain was successfully used for many years for production of intracellular and extracellular enzymes by many industries. However, DSM Food Specialities, Delft, Netherland is considered as one of the pioneer companies who used this type of yeast for different metabolites production in industrial scale (Asia Pasific Food Industry, 2001). This has been also reported by Van den Berg *et al.* (1990) who studied the production and secretion of milk clotting enzyme bovine prochymosin by *K. lactis*. On the other hand, the native intracellular enzyme lactase expressed by *K. lactis* is produced on an industrial scale and is sold under the trade name Maxilact^{TM} (DSM Food Specialities, Delft, The Netherlands). Consequently, people who are lactose-intolerant to use milk based products require this GRAS enzyme which is generally used in milk products to degrade milk sugar (lactose). Ultimately, the protocols for large-scale fermentation and downstream processing have been established for isolation of the enzyme directly from *K. lactis* cells (van Ooyen *et al.*, 2006)

Besides biotechnological potential of excellent protein synthesizing capability of *K. lactis*, there are many other potential applications for this type of yeast. According to Bonekamp & Oosterom, (1994), during the Biafra war in 1960s, *K. lactis* was used as a baby food component. Furthermore, Laloux *et al.* (1991) have reported that *K. lactis* produces commercially native enzymes like inulinase. On the other hand, other researchers reported the potential commercial production of native enzymes of the wild type strain such as phospholipase B (Oishi *et al.*, 1999) and chitinase (Colussi *et al.*, 2005).

2.3 High Cell Density Cultivation

High cell density cultivation was first established historically for cultivation of yeast cells for in different processes such as: backers/fodder yeast production, single cell protein and ethanol production (Suzuki *et al.*,1987). This cell cultivation technique was further applied for production of high dense cultures of other mesophilic unicellular microorgtanisms and widely applied for different metabolites production. Moreover, HCDC was also applied for cultivation of filamentous microorganisms such as streptomycetes for high volumetric antibiotic production (Suzuki *et al.*,1987). Furthermore, this technique was also used for cultivation of higher microorganisms such as mammalian and plant cells to improve culture productivity.

2.3.1 HCDC of Microorganism

There are limited microorganisms have been effectively cultivated under High-cell-density cultivations (HCDC) such as some bacteria, a few extremophilic archea and a certain number of yeast. Additional biotechnological study is required to implement HCDC for microorganism that has different physiological features as well as to have optimum process. The presence of on-line coupling of current monitoring practices will give better details on the dense cultivations which includes intrinsic fluorescence spectroscopy, fluorescence-activated cell sorting and flow cytometry and also *in situ* microscopy of HCDC.

2.3.2 HCDC Bioreactors

As for HCDC in industry, simple stirred-tank reactor (STR) with fed-batch operation is preferable due to its simplicity, tendency for high productivity, suitability for fermentation robustness and lastly for its availability of this type of bioreactor in industries.

2.4 Effects of Fermentation Parameters on *K. lactis*

2.4.1 Effect of Temperature

Temperature is a vital aspect that influences the microbial growth kinetics and different metabolite production based on its direct effect on the enzymatic systems involved in all metabolic pathways. It is well-known that enzymes are most active and proceed and their maximal rates at optimum temperature. On the contrary, below and above optimal temperature the reaction rate is decreased which causes imbalance in overall cell metabolism. Temperatures range between 28 and 30°C is usually considered as optimal for cultivation of *K. lactis* as reported by many authors. As for the production of enzyme production, Ramirez-Matheus and Rivas (2003) have reported that 30.3°C was the optimal temperature for β-D-galactosidase production by *K. lactis*. It was found