DEVELOPMENT OF HIGH CELL DENSITY RECOMBINANT *PICHIA PASTORIS* CULTURE FOR HUMAN GROWTH HORMONE PRODUCTION

LEE YOEU CHEK

UNIVERSITI SAINS MALAYSIA

2008

DEVELOPMENT OF HIGH CELL DENSITY RECOMBINANT *PICHIA PASTORIS* CULTURE FOR HUMAN GROWTH HORMONE PRODUCTION

By

LEE YOEU CHEK

Thesis submitted in fulfillment of the requirements for the degree of Master of Science

October 2008

ACKNOWLEDGEMENT

Firstly, I offer my sincerest gratitude to my supervisor, Dr. Ahmad Ramli Mohd. Yahya for his invaluable guidance and support. With his enthusiasm and patience, he provided encouragement, sound advice, good teaching, good company, and lots of good ideas throughout my research; I would have been lost without him.

I am deeply grateful to my co-supervisor, Assoc. Prof. Dr. Mohd. Azizan Mohd. Noor, for his detailed and constructive comments, and for his important support throughout this work. Also, I would like to gratefully acknowledge the support of Dr. Amirul Al-Ashraf Abdullah for stimulating suggestions and encouragement throughout the research and thesis writing.

I warmly thank Hui Ling, Danley, Bee Tien, Shifa, Syida, Koe, Mang Ling, Guat Siew, Shima, Rahayu, Fahima, Mr. Sekaran and kak Mizah for all their help and valuable support. In addition, I wish to thank Chin Yee, Ain, Chay Hoon, Mei Wah, Shuh Ling, Anusiah, Shaikh, and Fishah. Without them the research would not have been possible. I would also like to thank all my friends from Lab 318, Lab 218, Lab 414 and Lab 409 for their co-operations for the use of laboratories facilities.

The financial support under MOSTI Pasca scholarship and Graduate Assistant Scheme are gratefully acknowledged. I am grateful to Julian, Shun Win and Leong Hong for their support.

Last but not least, I am forever indebted to my beloved family and Samantha for their understanding, endless patience, unfailing support and encouragement when it was most required. To them I dedicate this thesis.

Lee Yoeu Chek
October 2008

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PHOTOS

LIST OF ABBREVIATIONS

BMGY	buffered glycerol-complex medium
BMMY	buffered methanol-complex medium
CBB	G-250 Coomassie brilliant blue G-250
DMSO	dimethyl sulphoxide
ECL	electrochemiluminescence
EDTA	ethylene diaminetetraacetic acid
g	relative centrifugal force (centrifugation)
Mut^+	methanol utilization plus
Mut^s	methanol utilization slow
OD	optical density
OD_{600}	optical density at 600nm
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
PMSF	phenylmethylsulfonyl fluoride
rhGH	recombinant human Growth Hormone
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris[hydroxymethyl]aminomethane
Tween® 20	polyoxyethylene-sorbitan mono-laurate
YNB	yeast nitrogen base
YPD	yeast peptone dextrose

PEMBANGUNAN KULTUR *PICHIA PASTORIS* REKOMBINAN BERKEPEKATAN TINGGI UNTUK PENGHASILAN HORMON PERTUMBUHAN MANUSIA

ABSTRAK

Pichia pastoris telah digunakan sebagai perumah yang unggul untuk menghasilkan lebih daripada 400 jenis protein heterologus kegunaan bidang akademik dan industri. Klon *Pichia pastoris* GS 115 2(5) yang digunakan dalam kajian ini berfenotip Mut^+ dan telah diselitkan dengan gen penghasilan hormon pertumbuhan manusia rekombinan (rhGH) secara intrasel. Kajian ini memberi tumpuan kepada pembangunan protokol fermentasi untuk menghasilkan kultur *Pichia pastoris* berkepekatan tinggi. Kajian terhadap formulasi media kultur dan pengkulturan sekelompok telah dijalankan sebelum memasuki pengkulturan suapan sekelompok. Kesan pelbagai strategi suapan dan pengaruhan metanol terhadap penghasilan biojisim dan rhGH juga dikaji. Hormon pertumbuhan manusia rekombinan yang dihasilkan ditulenkan dengan menggunakan kromatografi penukaran ion. Satu protokol pemfermentasian yang menghasilkan biojisim tertinggi sebanyak 120 g/L dan kadar pertumbuhan spesifik maksimum sebanyak 0.133 h^{-1} telah dicapai. Satu lagi protokol pemfermentasian yang menghasilkan rhGH dalam masa paling singkat juga dicapai. Hanya 39 jam diperlukan dari inokulasi sehingga tahap penghasilan rhGH, ia memendekkan masa pengkulturan sebanyak 50% dibandingkan dengan cara pengkulturan biasa iaitu selama 72 jam. Satu keadaan pengaruhan yang menghasilkan rhGH tertinggi sebanyak 2.4 mg rhGH / L genangan sel juga dicapai. Sebanyak 349.5 μg rhGH telah ditulenkan dari jumlah 2418.8 μg dengan memberikan nilai hasil akhir sebanyak 14.5% dan faktor penulenan 1.5.

DEVELOPMENT OF HIGH CELL DENSITY RECOMBINANT *PICHIA PASTORIS* CULTURE FOR HUMAN GROWTH HORMONE PRODUCTION

ABSTRACT

Methylotrophic yeast, *Pichia pastoris* has been used as an excellent host to produce more than 400 recombinant heterologous proteins of academic and industrial interests. *Pichia pastoris* strain used in this study was GS 115 2(5) with the phenotype of Mut^+, which has been genetically modified to produce intracellular recombinant human growth hormone (rhGH). In this research, the development of fermentation protocols for high cell density culture of recombinant *Pichia pastoris* was carried out. Medium formulation and improved batch cultivation was investigated prior to fed batch fermentation. Various feeding strategies and methanol induction conditions on biomass and rHGH production were also studied. Ion exchange chromatography was performed to purify the rhGH produced. A protocol of the high cell density culture was developed which gave the highest dry cell weight of 120 g/L with the maximum specific growth rate of 0.133 h^{-1}. A protocol that produces rHGH at the shortest time requirement was also obtained. It took only 39 hours from the inoculation to reach the rhGH production stage, which was almost 50% shorter time required compared to the preliminary 72 h. An enhanced induction condition for rhGH production was also developed where a yield of 2.4 mg of rhGH /L of lysate was achieved. A total of 349.5 µg of rhGH was purified from the total of 2418.8 µg of rhGH, which give the final yield of 14.5% and the purification factor of 1.5.

1.0 INTRODUCTION

Pichia pastoris is methylotrophic yeast that can assimilate methanol as sole carbon and energy source through a highly inducible methanol utilization pathway. It has been used as an excellent host system to produce more than 400 recombinant prokaryotes, eukaryotes, and viruses proteins of academic and industrial interests (Cereghino and Cregg, 2000). *Pichia pastoris* provides several advantages as a heterologous protein expression system compared to the conventional host systems. One of the major characteristic is the prolific growth rate and ability to reach extremely high cell densities up to 130g/L dry weight (Zhang *et al.,* 2004).

Human growth hormone (hGH) is a hormone produced and secreted by the anterior pituitary gland throughout a person lifetime. It is known to be critical for tissue repair, muscle growth, healing, brain and sexual function, physical and mental health, bone strength, energy and metabolism (Catzel *et al.,* 2003). Virtually, all organs and systems of human body are dependent on hGH for growth, development and functioning appropriately.

Knowing the importance of hGH to humanity and the high demand in biopharmaceutical market, *Pichia pastoris* strain GS115 2(5) has been genetically modified to produce recombinant human growth hormone (rhGH). This transformation produces a strain with the phenotype of Mut^+ which produces intracellular recombinant human growth hormone (rhGH) (Loh, 2005).

Achieving high cell density is always a crucial step since the production of heterologous protein often influenced by the cell density. In this research, the development of fermentation protocols for high cell density culture of recombinant *Pichia pastoris* was carried out in shake flask and bench scale fermenter system. Growth parameters such as media formulation and cultivation conditions were studied

in batch and fed-batch culture to achieve the major objective of reaching high cell density culture and rhGH production. Several fed-batch strategies were carried out to investigate its effects on biomass and rhGH production. Effects of the inducer concentration on the rhGH productivity were also examined. An improved fermentation protocol for biomass and rhGH production was developed with the results from this research.

1.1 Research Objectives

The development of fermentation protocols for high cell density culture of recombinant *Pichia pastoris* was carried out. The objectives were:

i. To develop the medium formulation and batch cultivation condition for high cell density culture of recombinant *Pichia pastoris.*

ii. To investigate the effect of glycerol feeding and methanol induction on biomass and rhGH production.

iii. To obtain a culture protocol and feeding strategy for high cell density culture and rhGH production.

iv. To perform ion exchange chromatography and purify the rhGH produced.

v. To detect and quantify rhGH produced by fermentation.

2.0 LITERATURE REVIEW

2.1 *Pichia pastoris*

Pichia pastoris, a methylotrophic yeast that can assimilate methanol as the sole carbon and energy source through its highly inducible methanol utilization pathway (Lee *et al.,* 2003b). It is well known to be an out-standing host system for heterologous protein production since the isolation and identification of its tightly regulated alcohol oxidase gene (*AOX*) (Chen *et al.* 1997). It has been chosen as one of the major host system due to the excellent performance in the production of either secreted or intracellular proteins of academic and industrial researchers (Cereghino and Cregg,1999).

This excellent host system has been used to produce more than 400 heterologous proteins of prokaryotes, eukaryotes, and viruses ranging from human endostatin to spider dragline silk protein (Cereghino and Cregg, 2000). The concentration of foreign proteins expressed by *Pichia pastoris* was reported in the range of 1 mg/L to 12 g/L of culture volume (Li *et al.,* 2001). Most of the proteins are expressed at levels of more than 1 g/L of culture medium (Cereghino and Cregg, 2000), which is comparatively higher than other host system such as bacterial, insect or mammalian systems (Romanos, 1995).

Pichia pastoris is known to grow over a wide pH range, from 3 to 7, with minor effect on the growth rate. Inan and co-workers (1999) reported that *Pichia pastoris* grew slowly on nutrient agar plates at 30^{o}C. It takes 3 - 7 days to form a milky white colony of 5 mm in diameter.

Methylotrophic yeast of the genera of *Candida* and *Pichia* shared an almost similar methanol metabolic pathway. Most of the enzymes are compartmentalized in methanol induced microbodies such as peroxisomes and cytoplasm (Veenhuis *et al.,*

1983). *Pichia pastoris* has the ability to utilize glycerol, ethanol or acetate as a carbon source like reported in other yeasts.

2.1.1 Advantages of *Pichia pastoris*

Pichia pastoris provides several advantages as a heterologous protein expression system. It has a prolific growth rate and is able to produce extremely high cell densities on a defined basal salt medium, often achieving 130 g/L dry weight (Zhang *et al.,* 2004). *Pichia pastoris* can be grown to high cell densities of more than 400 g/L (wet weight) on comparatively simple and defined medium with glycerol as carbon source (Cereghino *et al.,* 2002). Scale up of high cell density culture for *Pichia* is an easy and achievable task which often results in enormous product yields on volumetric basis (Romanos, 1995).

In contrast to the prokaryotic or other eukaryotic expression systems, *Pichia pastoris* expression system becomes an excellent choice due to the sub-cellular organelles, such as endoplasmic reticulum and Golgi apparatus, which facilitate the post-translational modification abilities (Zhou *et al.,* 2006). Consequently, complex recombinant proteins produced in *Pichia* expression system does not require additional *in vitro* unfolding and refolding process as in other expression systems.

The glycosylation machinery of *Pichia pastoris* might not be exactly the same as mammalian cells; however, it allows heterologous production of functional mammalian proteins especially when glycosylation is the compulsory procedure for the proper folding or biological activities. Yeast cells were found to have the ability to target the same types of N-glycosylation site like eukaryotic cell systems (Asami *et al*., 2000). One of the common problems of yeast as host cell is the over-glycosylation of target protein. However, according to Silvia and co-workers (2003),

the over-glycosylation of human growth hormone did not affect its function and binding sites.

In addition, the presence of alcohol oxidase gene (*AOX*) is also a major advantage that makes *Pichia* as the excellent host system (Cregg *et al.,* 1989). The inducibility and the ability to repress foreign protein transcription is a special advantage of *Pichia* system. This expression system becomes a suitable host for proteins which are toxic to the host cells (as many heterologous proteins are when accumulated at high concentrations) (Tschopp *et al.,* 1987; Lin Cereghino *et al.,* 2002; Diatloff *et al.,* 2006). The heterologous proteins expression can be repressed in the biomass accumulation stage and induced at the protein production stage only.

Pichia pastoris expression system provides an easy and simple cultivation procedures as compared to other eukaryotic expression systems. It is able to produce high expression level of heterologous proteins (extracellularly or intracellularly) into a simple and inexpensive culture medium (Zhou *et al.,* 2006). The endogenous proteins produced in *Pichia pastoris* are lower as compared to the conventional *Saccharomyces cerevisiae* system (Ascacio-Martinez and Barrera-Saldana, 2004). The extremely high recombinant proteins production and comparatively low native proteins secretion make the protein separation process much easier (Romanos, 1995), which is a major advantage for subsequent protein purification steps (Shi *et al.,* 2003; Aloulou *et al.,* 2006).

Furthermore, *Pichia pastoris* genome can be integrated with single or multi-copy of expression plasmids at specific sites. The genetic content of wild type and recombinant *Pichia* are very stable as the heterologous proteins are inserted into chromosomes with homologous recombination and not kept in the form of plasmids. Genetic stability and scale-up without loss of yield is necessary to achieve the over-

production of heterologous proteins (Romanos, 1995). This host and expression system is now available as a kit from Invitrogen Corporation (Carlsbad, CA, USA). Detailed procedures and precautions were attached together with the kits; users can easily achieve the targeted goal by following the step-by-step guideline.

The ability of *Pichia pastoris* to grow in a medium containing methanol reduces the risk of microbial contaminations (Cereghino and Cregg, 1999). No toxic cell wall pyrogens (as found in *Escherichia coli*) or oncogenic and viral nucleic acids (as found in mammalian cells) were found in heterologous proteins produced by *Pichia pastoris* (Romanos, 1995).

Yeasts do not produce toxins that are normally active by oral route. Documentation of *Pichia pastoris* toxigenic effects was not found in the extensive literature databases (Pariza and Johnson, 2001). *Pichia pastoris* has been classified as Biosafety Level 1 (BL-1), a group of well-characterized microorganisms which will not causing sickness in healthy human adults and need minimal safety attention in handling and storage (Center for Disease Control, 1999).

Many human genes such as Insulin-like growth factor-1, Amyloid precursor protein, Insulin, Leukemia inhibitory factor and etc. (Lin Cereghino and Cregg, 2000) have been expressed in *Pichia pastoris* for pharmaceutical purpose and they fulfill the standard of several safety examinations. Toxicity studies were done and approved in animal feed (including pathogenicity study in mice, acute and sub acute oral toxicity study in rats, and two generation teratology study in rats). The FDA reported that *Pichia pastoris* is neither pathogenic nor toxigenic (FDA, 1993).

2.1.2 Alcohol oxidase gene (*AOX*)

The genome of *Pichia pastoris* contains two copies of alcohol oxidase (*AOX*) gene i.e. *AOX* 1 and *AOX* 2. The *AOX* 1 promoter regulates 85% of alcohol oxidase activity and commonly used to control heterologous protein expression (Damasceno *et al.,* 2004). The *AOX* 2 gene played only a minor role in the total alcohol oxidase activity regulation (Cregg *et al.,* 1989).

AOX catalyzes the oxidation of methanol to formaldehyde and hydrogen peroxide, which is the first step in methanol metabolic pathway. It is then responsible in the degradation of hydrogen peroxide to form oxygen and water in peroxisome after its sequesteration with catalase. Some of the formaldehydes leave peroxisome and further oxidized by cytoplasmic dehydrogenase to form formate and carbon dioxide. These reactions are the main energy output of the methanol metabolic pathway (Ellis *et al.,* 1985; Lee *et al.,* 2003a).

The expression of alcohol oxidase is partially repressed in the medium containing excess non-methanol carbon sources such as glucose and glycerol. However, the promoter is induced more than 1000-fold in medium containing methanol as the sole carbon source (Thorpe *et al.,* 1999; d'Anjou and Daugulis 2000). Recombinant proteins that placed under the regulation of *AOX* 1 promoter can be permitted to be expressed at high levels via methanol induction (Chiruvolu *et al.,* 1997).

Typically, the methanol regulation is found to be similar with alternative carbon source pathways in many microorganisms. Interestingly, ethanol as a small alcohol and gluconeogenic carbon source like methanol is reported to repress transcription of *AOX* and other methanol pathways. In the logical physiological perspective, *AOX* might oxidize ethanol nearly as readily as methanol, which cause

the accumulation of acetaldehyde in peroxisome and become a major catastrophe for cells. However, this critical phenomenon was not found in the record of extended literature for *Pichia pastoris*. How the cellular regulatory machinery is able to recognize and differentiate these similar compounds still remains a mystery (Lin Cereghino *et al.,* 2006).

2.1.3 Methanol utilization (Mut) phenotypes

There are three phenotypes in recombinant *Pichia pastoris* strains, based on their ability to utilize methanol: Mut^+ (methanol utilization plus, wild type *AOX*, where both alcohol oxidase genes, *AOX* 1 and *AOX* 2 are remained), Mut^s (methanol utilization slow, where only *AOX* 2, which is responsible for 15% of the *AOX* activity, is intact), and Mut^- (methanol utilization minus, in which both *AOX* 1 and *AOX* 2 are impaired). The Mut^s strains exhibit slower growth on methanol than Mut^+ strains due to deletion of *AOX* 1 gene. The lower levels of alcohol oxidase produced from *AOX* 2 gene are not sufficient to oxidize methanol and causing growth limitation (Inan *et al.,* 1999).

Mut^+ strains have a higher oxygen demand which is more often in causing oxygen limiting conditions in the culture (Files *et al.,* 2001). In contrast, Mut^s strains has the less possibility to become oxygen-limited (Romanos 1995). It utilize less methanol and may reach higher expression levels of heterologous proteins than the Mut^+ strains (Xie *et al.,* 2005). But, protein expression with Mut^s strains requires long induction period (approximately 100 hours) for maximal expression due to the low *AOX* 2 activity (Files *et al.,* 2001).

2.2 Human growth hormone (hGH)

Human growth hormone (hGH) or somatotropin is a small, single chain peptide. It is formed by 191 residues which 50% of the residues are in helical conformation (Catzel *et al.,* 2003). It is a hormone produced and secreted by the anterior pituitary gland throughout a person lifetime. Human growth hormone is responsible for many human body functions such as growth, development, immunity, and metabolism (Catzel *et al.,* 2003). It is needed for tissue repair, muscle growth, healing, brain and sexual function, physical and mental health, bone strength and energy metabolism. Virtually, all organs and systems of human body are dependent on hGH for growth, development and functioning appropriately (Growth Hormone Explanation (2006) [Online] [Accessed 16th July 2008], available from World Wide Web: Http://www.21stcenturyhgh.com/hgh-research-explanation.htm).

The main function of growth hormone is to stimulate the secretion of IGF-1 by liver and other tissues. IGF-1 is the major component to enhance rapid reproduction and differentiation of chondrocytes (cartilage cells) and resulting in bone growth. On the other hand, IGF-1 also stimulates the differentiation and proliferation of myoblasts, amino acid utilization and protein production in muscle and other parts of the human body (Growth Hormone Explanation (2006) [Online] [Accessed 16th July 2008], available from World Wide Web: Http://www.21stcenturyhgh.com/hgh-research-explanation.htm).

The first medical application of human growth hormone is the use of exogenous hGH from human pituitary glands for growth hormone deficiency treatment. Pituitary-extracted hGH was then become the major treatment for most of the growth hormone deficiency cases. However, the application of pituitary-derived

hGH was abruptly banned when hGH extraction process was found to related to the Creutzfeld–Jakob disease (CJD) in 1985 (Catzel *et al.,* 2003).

Nowadays, hGH is used as the treatment for children growth retardation such as short stature caused by insufficient growth hormone secretion (Tae *et al.,* 2005). The hormone has also been used as a remediation for Turner's syndrome and chronic renal insufficiency (Leitner *et al.,* 2004). Besides, hGH is used as a therapy for adults growth hormone deficiency and in the management of HIV-related waste and cachexia (Chen *et al.,* 2004).

Human growth hormone formed as a mixture of peptides with major physical component (22 kDa) and minor component (20 kDa). The minor component is formed as the result of deletion of 15 amino acid residues (32–46) from the 22 kDa form. There are also variants of 45 kDa and 24 kDa hGH form, derived from the aggregation or additional amino acids in 22 kDa compartment (Catzel *et al.,* 2003).

Nowadays, synthetic human growth hormone is produced with recombinant DNA technology. It is a 191 amino acid polypeptide (22 kDa) with an amino acid sequence and two internal disulphide bridges which is identical to the major component of growth hormone extracted from human pituitary (Chen *et al.,* 2004).

Purification of recombinant human growth hormone (rhGH) is commonly performed with the combinations of adsorption chromatographies, including ion exchange, hydrophobic interaction, and metal–chelate which followed by gel filtration for protein separation according to protein size and removal of aggregated compounds. The overall rhGH recovery is found to be greatly influenced by the efficiency of solubilization and refolding processes (Catzel *et al.,* 2003).

2.3 High cell density culture

Pichia pastoris is well characterized budding yeast that has a prolific growth rate (Cino 1996). It is possible to achieve extreme high cell density (up to 130 g/L of dry cell weight) with a comparatively low cost glycerol basal salts medium (Zhang *et al.,* 2004). *Pichia pastoris* prefers respiratory metabolism mode over a fermentative mode (Lin Cereghino *et al.,* 2002). Hence, the tendency of ethanol accumulation rarely happened in *Pichia pastoris* culture and thus minimized the risk of ethanol accumulation as an inhibitor of cell growth (Sunga and Cregg, 2004).

Optimization of fermentation process to achieve high cell density can be carried out by either modifying one growth parameter at a time or changing a series of factors at the same time and investigate the interactions among the factors (Thiry and Cingolani, 2002). The optimization process is always a crucial step since the yield of heterologous protein production often affected directly by culture density of the host cells (Inan and Meagher, 2001).

Optimization of *Pichia pastoris* is usually initiated with the investigation of growth parameters in shake flask system. Some of the growth parameters such as medium formulation can be pre-optimized in shake flask system prior to the practical in fermenter. The production of biomass and desired protein is then further optimized in fermenter where the effect of pH, aeration, agitation and feeding strategies were investigated. This step always resulted in a significant improved total recombinant protein production due to the high cell density achieved by cultivation in fermenter (Fantoni *et al.,* 2007).

Despite the advantages obtained from high cell densities, it actually brings some critical problems to the culture. Firstly, oxygen transfer is a major limiting factor especially in the large scale fermenter. This problem can be solved by

providing oxygen enriched air into the fermenter but results in the increase of production costs and create a safety risk. Secondly, high cell density cultures are often susceptible to develop some level of stress on the cells. This may lead to a decreased productivity, decreased viability and increased cell lysis. The increase of cell lysis increase the difficulties of purification process with the release of intracellular proteins into supernatant (Hohenblum *et al.,* 2003). Efforts to get a balance point of achieving high cell density culture and minimize its disadvantages are necessary in order to obtain high expression level of the particular desired proteins.

2.3.1 Carbon sources

Several carbon sources have been used to generate *Pichia pastoris* biomass, such as glycerol, methanol, ethanol, glucose, sorbitol, mannitol and etc. (Sreekrishna *et al.,* 1997; Inan and Meagher, 2001; Lee, 2005; Xie *et al.,* 2005). Growth on glycerol or glucose alone allows high cell growth rate without expression of the foreign gene. Methanol is needed to induce the expression of *AOX* 1 promoter. Culture with methanol as the sole carbon source produces a high product yield, however, the low growth rate and low biomass production on methanol lowered the overall productivity (Thorpe *et al.,* 1999).

Research has found that a diauxic growth was observed when there are two or more carbon sources the culture medium. In the culture with a mixture of ethanol and glycerol, utilization of ethanol was started after glycerol consumption. A transient accumulation of acetate was observed after the depletion of glycerol. Then, growth was supported with acetate as carbon source. However, medium with glycerol and methanol mixture do not showed in a complete diauxic curve. Methanol utilization started before glycerol depletion. This indicates that glycerol does not repress the

synthesis of methanol utilizing enzymes as strong as ethanol since methanol utilizing enzymes was expressed before the complete exhaustion of glycerol (Inan and Meagher, 2001).

In the case of all three carbon sources (glycerol, ethanol and methanol) presence in the medium, the order of carbon source consumption is glycerol, ethanol, acetate (produced from the ethanol consumption) and methanol (Inan and Meagher, 2001). Glycerol is preferred over ethanol and methanol, ethanol being preferred over methanol.

As reported by Lee (2005), cultivation of *Pichia pastoris* GS115 2(5) in glycerol gave the highest biomass production compared to methanol, sorbitol, glucose and mannitol. While some researchers found that *Pichia pastoris* was not able to utilize xylose and lactate, while acetate repress the growth of *Pichia pastoris* especially on the phenotype of Mut^+ or Mut^- (Inan and Meagher, 2001).

2.3.1.1 Glycerol and its metabolic pathways

Glycerol is found to be partially repress the *AOX* 1 gene promoter, however, it is still considered as the most widely used carbon source in *Pichia pastoris* fermentations (Xie *et al.,* 2005). High cell density culture required high glycerol feeding rates in the fed-batch phase but high concentrations of glycerol may inhibit the cell growth (Lee *et al.,* 2003a).

The theoretical biomass yield ($Y_{x/s}$) of glycerol is 0.50 g dry cell weight / g substrate (Boze *et al.,* 2001) and higher specific glycerol consumption rate (at the average of 0.10g / g dry cell weight / h) can be observed with high glycerol feed rates (Lee *et al.,* 2003a). Jungo and co-workers, (2007), reported that the maximal specific growth rate of *Pichia pastoris* on glycerol as sole carbon source is 0.24 h^{-1}; however,

the maximal specific growth rate achieved in this study is only half of the value reported here.

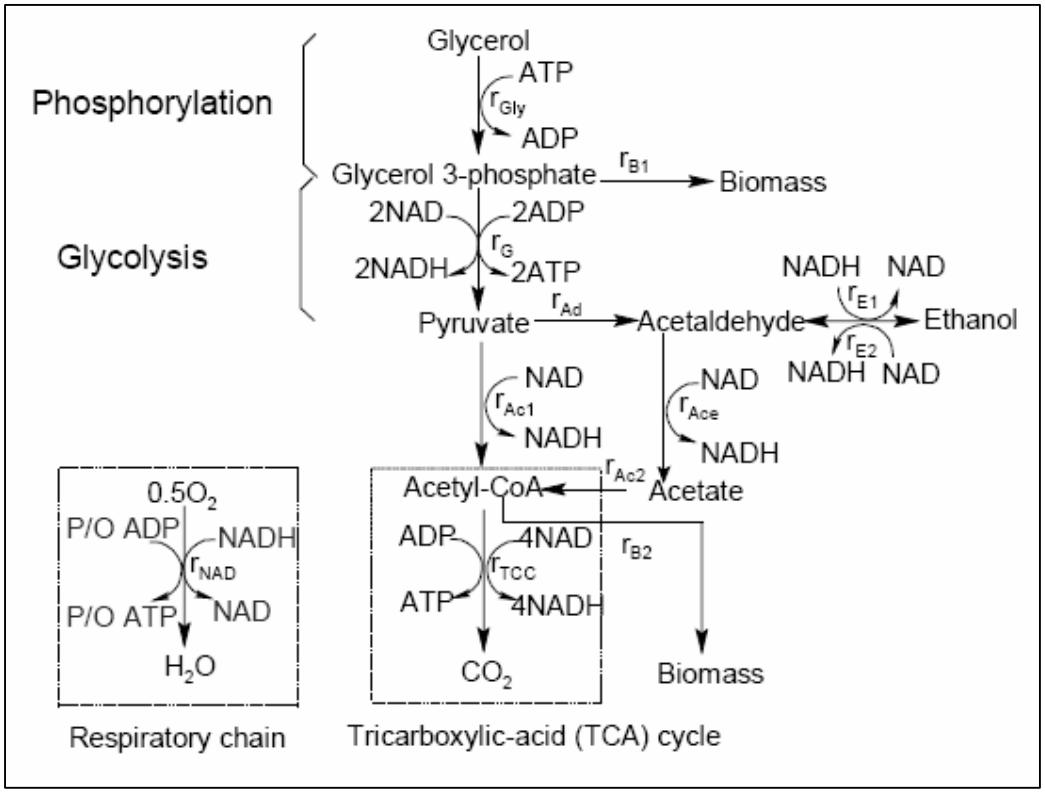

Fig 1.1: Metabolic pathways of glycerol in *Pichia pastoris* (Figure adapted from Ren *et al.*, 2003).

Fig 1.1 is the simplified metabolic pathways of glycerol (Ren *et al.,* 2003). The pathway starts with phosphorylation of glycerol by glycerol kinase to produce glycerol-3-phosphate (G3P). G3P is then further oxidized to dihydroxyacetone phosphate and pyruvate in glycolysis process by FAD-dependent glycerol-3-phosphate dehydrogenase. Further oxidation of pyruvate by pyruvate dehydrogenase results in the formation of acetyl–CoA and used in the tri-carboxylic acid (TCA) cycle. TCA cycle is the main part to produce metabolites for cellular components such as amino acids, nucleic acids and cell wall synthesis (Ren *et al.,* 2003). Most of the

energy sources needed for cell growth and maintenance (in the form of ATP and NADH) were generated in the TCA cycle from acetyl-CoA while a small portion might come from G3P.

Ethanol might be produced as the result of respiratory capacity limitations or glycolytic flux (Sonnleitner and Kaeppeli, 1986; Lei *et al*., 2001). Pyruvate is converted to acetaldehyde by pyruvate decarboxylase and then oxidized to ethanol by alcohol dehydrogenase. But, ethanol may also be used as substrate when it was changed to acetaldehyde by alcohol dehydrogenase, then to acetate by acetaldehyde dehydrogenase. Finally, acetate is converted back to acetyl–CoA by acetyl–CoA synthetase and enter the TCA cycle (Ren *et al.,* 2003).

2.3.1.2 Methanol and its metabolic pathways

Pichia pastoris has the ability to assimilate methanol as sole carbon and energy source (Sunga and Cregg, 2004). It contains an alcohol oxidase (*AOX*) enzyme that catalyzes the oxidation of methanol which eventually results in the production of carbon dioxide and energy (Zhang *et al.,* 2003).

The '*Pichia* Fermentation Process Guidelines' from Invitrogen Co. (San Diego, CA) suggested two different empirical methanol feeding strategies which are widely used in *Pichia pastoris* fermentation. The first method is based on the dissolved oxygen (DO) spike while the second involve preprogrammed linear methanol feed rates to maintain a very low methanol concentration in the medium. The choice of feeding strategy and specific regulatory parameters such as methanol feed rate are dependent on the strains methanol-utilization ability phenotype and characteristic of the recombinant protein (Cregg *et al.,* 1993; Minning *et al.,* 2001).

Generally, maintaining excess methanol in the culture is able to achieve higher recombinant protein expression than conventional methods of methanol-limited conditions (Brady *et al.,* 2001). The higher expression levels might be contributed by the increase of cell growth rate or higher induction possibility of *AOX*1 promoter in high methanol concentration.

The increase of methanol concentration in the medium causes an increase in oxygen uptake, carbon dioxide production and heat production rate. These rates were found to be higher in cultures on methanol than glycerol. However, biomass production was lower with the increase of methanol concentration in the medium, due to the lower biomass yield of methanol compared to glycerol (Jungo *et al.,* 2007).

The theoretical biomass yield ($Y_{x/s}$) on methanol is 0.30 g dry cell weight / g substrate (Boze *et al.,* 2001) and the highest reported value of maximal specific growth rate for *Pichia pastoris* grown on methanol as the sole carbon source is 0.14 h^{-1}. Culture with methanol medium produces a notably large amount of heat during the fermentation process. A rapid and efficient cooling system is required especially in large scale fermenter where heat transfer was dependent solely on the reactor wall. Inefficient heat removal will cause the increase of reactor temperature which bring significant effect on the productivity and highly affect the quality of recombinant protein produced (Jungo *et al.,* 2007).

Methanol evaporates rapidly. The evaporation rate of methanol was 5.9 times of butyl acetate at room temperature (Material Data Safety Sheet (MSDS): methyl alcohol [Online] [Accessed 16th July 2008], available from World Wide Web: http://www.bu.edu/es/labsafety/ESMSDSs/MSMethanol.html). It is difficult to control methanol concentrations within an optimal range especially in small and micro scale cultures. Moreover, methanol metabolism utilizes oxygen at a high rate.

The inefficient aeration limits the availability of oxygen, which might affect recombinant protein expression especially in small and micro scale culture conditions (Resina *et al.*, 2004).

Although methanol is needed for *AOX* 1 promoter induction, however, it is a potential fire hazard and may not be suitable for the production of food products or human consumption products (Cereghino and Cregg,1999).

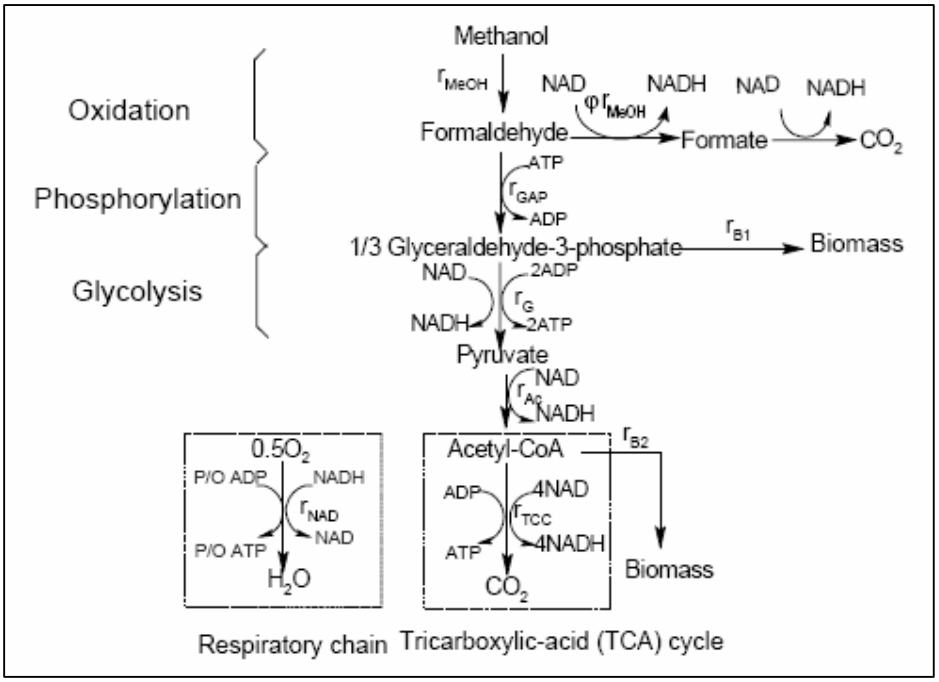

Fig 1.2: Metabolic pathways of methanol in *Pichia pastoris* (Figure adapted from Ren *et al.*, 2003).

Fig 1.2 shows the simplified metabolic pathways of methanol in *Pichia pastoris*. First, methanol is oxidized to form formaldehyde and hydrogen peroxide by alcohol oxidase. Formaldehyde then enters both dissimilatory and assimilatory pathways (Cereghino and Cregg, 2000).

In the dissimilatory pathway, some of the formaldehyde is oxidized to formate and carbon dioxide by formaldehyde dehydrogenase (FLD) and formate dehydrogenase (FDH) respectively, which produce energy in the form of NADH.

In the assimilatory pathway, formaldehyde is metabolized by condensation of formaldehyde with xylulose-5-monophosphate, catalyzed by the peroxisomal enzyme dihydroxyacetone synthase to form glyceraldehyde-3-phosphate (GAP), which later enters the TCA cycle (Cereghino and Cregg, 2000). Similar to the glycerol metabolic pathways, biomass formation was assumed to come from GAP and acetyl–CoA in the TCA cycle (Cereghino and Cregg, 2000; Jahic *et al.,* 2003).

2.3.2 pH

Pichia pastoris is known to tolerate a pH range of 3–7 (Damasceno *et al.,* 2004) but cannot grow at pHs below pH 2.2 (Thiry and Cingolani, 2002). Investigation on medium pH is critical for yeast-secreting protein because they can grow in a wide range of pHs. The choice of pH depends on the stability of the recombinant protein expressed. It has been reported that pH 5 is optimal for cell metabolism and biomass production as oxygen consumption rate is higher at that pH (Cino, 1996).

Cultivation of *Pichia pastoris* at low pH increases the expression of recombinant proteins by reducing protease degradation (Damasceno *et al.,* 2004). Protease activity in shake flask cultures of *Pichia pastoris* is drastically reduced at pH 3 (Shi *et al.,* 2003). Maintaining culture at pH 3 or lower in methanol-induction phase to inhibit neutral proteases is one of the efficient approach to protect the product from proteolysis (Murasugi and Tohma-Aiba, 2003; Mattanovich *et al.,* 2004).

On the other hand, optimum production pH varies for each recombinant protein which is dependent on the product biochemical properties. Low range values of pH 3.0 to 5.0 is found to be effective for gelatins and HIV-1 envelope proteins (Ohya *et al.,* 2002) while low acidic condition of pH 5.6 to 6.0 is suitable for human serum albumin and mouse epidermal growth factor (Ohya *et al.,* 2002). However, low fermentation pH decreases the viability of *Pichia pastoris*. A decreased yield in biomass at pH 3.0 was observed compared to cultures at pH 5.0 (Hohenblum *et al.,* 2003). The viability of cells decreased drastically when low culture pH was reached and the problem became severe during prolonged fermentation period. Critical stresses from high acidic environment often leading to cell death. The decrease of cell viability causes the release of host intracellular proteases level into supernatant and eventually causing a decreased productivity.

A low pH may offer higher protein expression but it may also lead to the increase of cell death. One of the common approaches applied in *Pichia pastoris* fermentation is to maintain optimum pH for cell growth (pH 5.0) at biomass production stage while the pH is then adjusted to the optimum production value for desired proteins at induction stage.

2.3.3 Temperature

Pichia pastoris is a psychrotrophic microorganism that can grow at a temperature as low as 12°C (Jahic *et al.,* 2003). Elevated culture temperature always lead to cell death and causing cell lysis. As a result, intracellular protease is released into the culture media (Inan *et al.,* 1999).

Temperature may be a critical factor to reduce protease activity and minimizing product proteolysis. Low culture temperature might increase cell viability

and possibly reducing proteolytic degradation of the desired proteins (Li *et al.,* 2001). Some researchers lowered culture temperature during the induction phase achieve a four-fold increase of production yield (Whittaker and Whittaker, 2000, Thiry and Cingolani, 2002).

In addition, methanol is found to be more toxic at 30°C compared to the lower environment temperature (Jahic *et al.,* 2003). This is important especially when culturing *Pichia* strain with Mut^+ phenotype, which is more likely to be sensitive to overdoses methanol and become poisoned.

2.3.4 Oxygen and aeration factors

Oxygen is one of the critical and major requirements for aerobic fermentations. Stirred tank fermenter becomes the most commonly used fermenter due to the effectiveness and reliability of providing a comparatively high dissolved oxygen level. It is the most well-received reactor type especially for shear tolerant cultures (Liang and Yuan, 2006).

Dissolved oxygen level in a particular culture is usually regulated by the agitation and aeration rates. Aeration rates provide the initial volume of air bubbles to the system whereas agitation rates facilitate oxygen and substrate distribution in the culture (Crolla and Kennedy, 2004). However, increased agitation will create shear force on cell walls and the cell-insoluble substrate interface which results in a decreasing cell viability. The agitation rate should be regulated appropriately for optimum product production and also to cut down the operational costs (Crolla and Kennedy, 2004).

Aeration is a critical factor that affects the growth rate and induction efficiency of *Pichia* cultures significantly especially for Mut^+ strains, which tends to

become oxygen-limited. A consistently increase (5-10 fold) in yield was observed when the culture was switched from shake flasks system to fermenter due to the better aeration environment (Romanos, 1995). *Pichia pastoris* culture required comparatively high oxygen demand as described in the glycerol and methanol metabolic pathways (Ren *et al.,* 2003). Oxygen was used by alcohol oxygenase as a substrate and involved in cellular electron transportation; hence, dissolved oxygen (DO) concentration in the medium should be maintained at high levels throughout methanol utilization metabolism (Shi *et al.,* 2003).

More oxygen is needed for energy and maintenance pathway than for the anabolic flux (Jahic *et al.,* 2003). Dissolved oxygen is a common limiting factor if a high growth rate is reached in fed-batch processes. It is crucial to avoid oxygen limitation in methylotrophic yeast culture so that cells remain in respiratory metabolism to avoid methanol accumulation in the broth and methanol toxicity.

A number of approaches have been done to increase and maintain DO level in the medium. These efforts include the supplementation of oxygen gas and the use of microbubble dispersions (MBD) strategy (Zhang *et al.,* 2003; Damasceno *et al.,* 2004). The increase of cultivation pressure is useful to increase DO concentration but at the same time, it also raise up dissolved carbon dioxide concentration in the culture.

Oxygen mass transfer is often occurred extensive of the given cell density and causing oxygen limitation in high cell density culture. Oxygen transfer capacity of fermenters is always unable to support the oxygen metabolic demand at high cell density. Thus, oxygen availability should be taken into consideration upon the selection of feeding strategy (Oliveira *et al.,* 2005).

A mass transfer coefficient can be defined with mass balance of species in the fermenter. An assumption of oxygen transfer from gas to liquid phase is controlled

by the liquid film surrounding air bubbles was made (Mavituna and Sinclair, 1985). The rate of oxygen transfer is given by:

$$\mathbf{OTR = k_L a\ (C^*\text{-}C_L)} \qquad \mathbf{(1)}$$

Where OTR = Oxygen transfer rate,

$k_L a$ = volumetric oxygen transfer coefficient (h^{-1}),

C* = saturated DO concentration,

C_L = actual DO concentration in the liquid.

2.4 Fermentation and feeding strategy

2.4.1 Fermenter and shake flask culture

Fermentation process of *Pichia pastoris* generally starts in shake flask system before transferring to larger volume fermenter. Shake flask system is considered as sub-optimal condition due to the lack of data recorder and regulatory controller (Romanos, 1995). Some minor adjustment on the culture parameters were needed during the shift from a shake flask system to a small fermenter or from a small fermenter to a large volume fermenter (Lin Cereghino *et al.*, 2002).

Pichia is able to achieve extremely high cell densities (130 g/L dry cell weight) in the fully equipped fermenter where culture parameters such as pH, aeration, temperature and carbon source feed rate were controlled. Protein level in fermenter culture is generally much higher (up to 140%) compared to culture in shake flask (Jahic *et al.*, 2003). Switching from shake flask system to fermenter might increase the dissolved oxygen (DO) levels by increasing agitation, air flow, or supplementation of pure oxygen in the gas inlet.

Substrate limitation can also be minimized with the fed-batch strategy in fermenter. Growth limiting nutrients can be provided to the culture from time to time

as the replacement of exhausted substrates and maintained at a particular concentration. Methanol concentration can also be maintained at the level of just enough for protein synthesis while preventing accumulation to toxicity level (Cino, 1996).

Cultivation in fermenter system is found to match the high oxygen demand of *Pichia pastoris.* An optimal growth and induction condition can be achieved when culture parameters and feeding rate were well controlled. Accumulations of substrate or by-products were minimized in the fermenter system. Optimization of biomass production to achieve high cell density culture is utmost importance for recombinant protein production especially for growth-related products (Cereghino *et al.,* 2002).

2.4.2 Three stage high cell density fermentation scheme

Standard high cell density fed-batch cultures for recombinant *Pichia pastoris* strains are usually performed in three steps. Cells are first batch cultured in salt medium with a non-fermentable carbon source (most commonly glycerol) to achieve unlimited growth and biomass accumulation (Inan and Meagher, 2001). The *AOX* 1 promoter is repressed and recombinant protein is not produced at this stage (Chen *et al.,* 1997).

The second phase (*AOX* 1 derepression phase) is started upon the depletion of initial glycerol. Glycerol is provided in a fed-batch mode to further increase the biomass concentration and derepress the cells for methanol induction (Cereghino *et al.,* 2002). The second phase is important to ensure that high cell density is achieved and cells were primed prior to the induction phase (Lee *et al.,* 2003a). This transition phase played a key role to derepress the enzymes involved in methanol metabolic pathway gradually and helps to reduce time needed for cells to adapt to methanol.

This transition phase is found to be a compulsory step in order to shorten the time required for methanol adaptation (Chiruvolu *et al.,* 1997).

The third phase is the induction phase by adding methanol at a slow rate, which helps the culture to adapt to methanol and induces the synthesis of recombinant protein (Inan and Meagher, 2001; Cereghino *et al.,* 2002; Lee *et al.,* 2003a). The methanol feed rate is then increased gradually to achieve the level of sufficient for cell induction but lower than the concentration of toxicity (Cereghino *et al.,* 2002). The *AOX* 1 promoter is induced by methanol and recombinant protein is produced in this phase.

2.4.3 Fed batch and feeding strategy

Fermentation can be performed in batch, fed-batch or continuous mode. Batch cultivation is simple and robust but always suffered from low biomass production. Continuous culture is rarely practiced in pharmaceutical product due to the high mutation and contamination risks. Fed batch fermentation is a strategy in between batch and continuous culture and it seems to be the most effective approach to achieve high cell density culture (Thiry and Cingolani, 2002). Fed-batch culture is initiated with a batch culture and fed continuously or sequentially without removing the culture medium (Stanbury and Whitaker, 1995).

Fed batch culture offers many advantages compared to batch and continuous cultures. Conventional batch culture suffers from low biomass and product yield due to the high initial substrate concentration which causes substrate and product inhibition (Ding and Tan, 2006). Fed-batch strategy seems to be an ideal solution to overcome the inhibitions. Fed batch fermentation allows the concentration of limiting substrate in the culture broth to be adjusted precisely at constant low level that