FEEDING STRATEGIES IN RECOMBINANT HUMAN ERYTHROPOIETIN PRODUCTION BY *Pichia pastoris* FERMENTATION IN STIRRED-TANK BIOREACTOR

by

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LIST OF ABBREVIATIONS

Abbreviations

AOX	Alcohol oxidase
BMGY	Buffered glycerol-complex medium
BMMY	Buffered methanol complex
BFU-E	Burst forming unit erythroid cells
C	Concentration in the medium g L^{-1} or mol m^{-3}
CBB	G- 250 Coomassie brilliant blue G-250
CDW	Cell dry weight
CFU-E	Colony forming unit-erythroid
DMSO	Dimethyl sulpoxide
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
EDTA	Ethylene diaminetetraacetic acid
EPO	Human erythropoietin hormone
g	Relative centrifugal force (centrifugation)
hGH	Human growth hormone
HPLC	High pressure liquid chromatography
PCR	Polymerization chain reaction
Mut^+	Methanol utilization plus
Mut^-	Methanol utilization slow
N	Agitation rate min^{-1}
OD	Oxygen demand mol m^{-3} sec^{-1}
OD_{600}	Optical density at 600 nm

PAGE	Polyacrylamide gel electrophoresis
PBM	Phosphate buffer medium
PBS	Phosphate buffer saline
PMSF	Phenylmethylsulfonyl fluoride
rhuEPO	Recombinant human erythropoietin
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium Dodecyl sulphate polyacrylamide gel electrophoresis
TCA	Tricarboxylic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris [hydroxymethyl] aminomethane
Tween^{R} 20	Polyoxyethylene-sorbitan mono-laurate
YNB	Yeast nitrogen base
YPD	Yeast peptone dextrose
F	Flow rate mL h^{-1}
t	Cultivation time h
T	Temperature, °C
U	One unit of an enzyme
V	Volume of the bioreactor L
Y	Yield (overall) g g^{-1}

Greek Letters

ρ	Density g L^{-1}
μ_{set}	Desired specific growth rate h^{-1}
$\mu_{s,max}$	Maximum specific growth rate on sorbitol h^{-1}

Subscripts

0	Refers to initial condition
AOX	Refers to alcohol oxidase
G	Glycerol
M	Refers to methanol
O	Refers to oxygen
S	Refers to sorbitol or substrate
X	Refers to cell

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LIST OF PUBLICATION AND SEMINARS

Seminars

1. Production of recombinant human erythropoietin in *Pichia pastoris* fermentation.

2. Effect of feeding modes on biomass and rhuEPO production.

STRATEGI SUAPAN DALAM PENGHASILAN REKOMBINAN ERYTHROPOIETIN MANUSIA MELALUI *Pichia pastoris* FERMENTASI DALAM REAKTOR TANGKI TERANDUK

ABSTRAK

Pengunaan erythropietin atau EPO sebagai terapeutik dapat meningkatkan kualiti hidup di kalangan pesakit kanser, buah pinggang dan anemia. Mengetahui kepentingan EPO dan permintaan yang tinggi di pasaran biofarmaseutikal, rekombinan erythropoietin manusia (rhuEPO) telah dihasilkan secara genetik menggunakan *Pichia pastoris* strain GS115 2 (5) sebagai sel perumah melalui proses pengkulturan tiga-peringkat. Kultur pertama kali dikulturkan dalam medium penghasilan yang mengandungi gliserol sebagai sumber karbon (fasa I) sebelum memperolehi sel berkepadatan tinggi (fasa II) dan diaruhkan dengan metanol untuk penghasilan protein (fasa III). Formulasi medium dan peningkatan kultur sekelompok diselidiki sebelum fermentasi suap-kelompok. Pelbagai strategi pemakanan dan keadaan aruhan metanol yang mempengaruhi proses penghasilan protin juga dikaji. Sebuah proses penghasilan sel berkepekatan tinggi telah dihasilkan mengambil masa 60 jam untuk mencapai tujuh kali ganda sel dengan jumlah sel sebanyak 132.10 g/L menggunakan suap malar. Keadaan aruhan juga telah diperbaiki mengunakan suapan menaik secara berperingkat. Ia hanya mengambil masa 19 jam dari fasa aruhan untuk menghasilkan sejumlah 104.8 ng/L EPO dengan hasil produk per biojisim ($Y_{p/x}$) 1.19 ng g^{-1} berbanding dengan cara pengkulturan biasa 36 jam.

FEEDING STRATEGIES IN RECOMBINANT HUMAN ERYTHROPOIETIN PRODUCTION BY *Pichia pastoris* FERMENTATION IN STIRRED-TANK BIOREACTOR

ABSTRACT

Administration of erythropoietin or EPO as a therapeutic improves the quality of life in patients with cancer, renal and anemia. Knowing the importance of EPO and the high demand in the biopharmaceutical market, *Pichia pastoris* strains GS115 2 (5) has been genetically engineered to produce recombinant human erythropoietin (rhuEPO) from the synthetic gene using three-stage cultivation process. The culture was first grown in production medium containing glycerol as a carbon sources (phase I) prior to obtained high cell density culture (phase II) and was induced with methanol for protein production (phase III). Medium formulation and enhanced batch cultivation was investigated prior to fed-batch fermentation. Various feeding strategies and methanol induction conditions which highly influenced the production of protein were also investigated. A process of high cell density cultivation was developed which took 60 hours for the culture to reach seven fold of cells 132.10 g/L using constant flow rate. An improved induction condition was also developed using step wise increase feeding strategy. It took only 19 hours from the induction phase to produce a total of 104.8 ng/L of EPO with the yield of product over biomass ($Y_{p/x}$) 1.19 ng g^{-1} compared to the preliminary 36 hours.

CHAPTER 1.0: INTRODUCTION

Pichia pastoris is methylotrophic yeast capable of utilizing methanol as a sole carbon and energy source; it is a promising foreign protein expression host, offering numerous advantages. It requires simple molecular genetic manipulations. The high cell densities culture can be achieve on simple, inexpensive media (Thorpe *et al.*, 1999). In the controlled environment of a bioreactor, it is possible to achieve high cell densities of about 400 g/L cell fresh weight or 130 g/L cell dry weight, respectively with *P. pastoris* (Jahic *et al.,* 2002). Moreover, it has good secretion ability for proteins (Jahic *et al.*, 2006). More than 700 proteins have been expressed in *P. pastoris* (Zhang *et al.*, 2009). The most essential to the fast, inexpensive production in *P. pastoris* is the strong inducible AOX1 promoter which drives the expression of recombinant protein and is inducible in the presence of methanol.

Erythropoietin (EPO) is a glycoprotein hormone function as a red blood cell formation stimulator in bone marrows and also initiates hemoglobin production. It synthesized by interstitial fibroblasts in kidney during adult and liver by per sinusoidal cells during fetal and predominantly during adulthood (Ahmed *et al.*, 2002; Buemi *et al.*, 2005; Siren *et al.*, 2009). Administration of EPO as a therapeutic improved the quality of life in patients with cancer, renal and anemia.

Knowing the importance of EPO and the high demand in the biopharmaceutical market, *Pichia pastoris* strains GS115 2 (5) has been genetically engineered (Bustami *et al.*, 2009) to produce recombinant human erythropoietin (rhuEPO) from the synthetic gene. This transformed produce a strain with the phenotype of *hEPO*-Mut^{+} for an extracellular recombinant human erythropoietin.

Achieving high cell density has always been a vital step since the production of heterologous protein expression is frequently dictated by the cell density. In this research, the development of fermentation process for high cell density culture of recombinant *Pichia pastoris* has been carried out in shake flask and bench-top scale bioreactor system. Growth and production parameters such as selection of cultivation medium, carbon sources, methanol concentration, induction temperature as well as methanol feeding strategies were investigated in batch and fed-batch cultivation for high cell density culture and EPO production. An improved fermentation process for biomass and EPO production was developed from this research.

1.1 Motivation and scope of the project

Several studies of recombinant human erythropoietin production from cDNA using *Pichia pastoris* as a host has been reported and conducted by other researchers in other parts of the world such as Turkey and Indonesia (Bayraktar *et al.*, 2009; Calik *et al.*, 2009; Celik *et al.*, 2007; Celik *et al.*, 2010; Soyaslan and Calik, 2011) and a few has been conducted in Malaysia (Teh *et al.*, 2011). However none of synthetic recombinant human erythropoietin production has been reported in large scale fermentation.

The main focus of this project was to produce synthetic recombinant human erythropoietin from genetically engineered *Pichia pastoris* having a methanol utilization phenotypes *hEPO*-Mut$^+$ in a high cell density culture and to investigated the effect of feeding strategies on EPO production. The research began with shake flask experiments for the selection of cultivation medium, carbon sources and methanol induction concentration as well as induction temperature for enhanced EPO

production. In bench-top bioreactor studies, in order to get high cell density cultures, three phase fed-batch cultivation was performed which contained glycerol batch (GB), glycerol fed-batch (GFB), and methanol fed-batch phase (MFB). During the GFB phase, a predetermined limiting substrate was fed at a constant flow rate. In MFB phase, the effect of using different methanol feeding strategies on cell growth and rhuEPO production was investigated. Quantification of extracellular and intracellular EPO was done by using an accurate determination of cytokine protein concentration enzyme-linked immunosorbent assays (ELISA).

1.2 Objectives of the study

This work studies the development of a fermentation process for the production of human erythropoietin in *P. pastoris*. This is included in all of the objectives below:

1. To investigate the effect of glycerol and sorbitol as a carbon sources and methanol induction on biomass and EPO production as well as to obtain a high cell density culture in glycerol fed-batch phase.
2. To access different methanol feeding strategies in methanol fed-batch phase toward biomass and EPO production.

CHAPTER 2.0: LITERATURE REVIEW

2.1 Human erythropoietin (EPO)

The synthesis of red blood cells in our body is controlled by a glycoprotein hormone named erythropoietin, also known as erythropoetin or erthropoyetin (EPO). Erythropoietin is a protein signaling molecule or a cytokine for erythrocyte precursors in the bone marrow (Ahmet *et al.*, 2011; Siren *et al.*, 2009). Hematopoietin or human EPO is manufactured by interstitial fibroblasts in kidney. The molecular weight of EPO is 34 kDa (Atkins *et al.*, 1991; Lu *et al.*, 2006). Furthermore it is also produced in the liver by perisinusoidal cells during fetal period and predominant during adulthood. Besides regulating the making of red blood cell, EPO was also known to have association in brain response especially in neuronal injury and engaged in healing wound (Clavio *et al.*, 2004; Dordal and Goldwasser, 1982; Kao *et al.*, 2011; Nissenson, 1989; Youssoufian *et al.*, 1990).

2.1.1 Primary role in red blood cell production

The erythropoiesis process will never arise in the absence of EPO (Osterborg, 2000). In order to increase the production of the red blood cell, the kidney will directed toward CFU-E pro-erythroblast and basophilic erythroblast, subsets in the differentiation before manufacturing and generating erythropoietin under hypoxic conditions (Coleman and Brines, 2004). The survival of red blood cell progenitors in human bone marrow can be prolonged by erythropoietin which will defend these cells from programmed cells death process (Ferri and Grassi, 2010).

In addition, the development of erythroid linage from multipotent progenitors required erythropoietin which act as a primary erthropoietic factor to cooperate with multiple growth factor such as IL-3, IL-6, glucocorticoids, and SCF (Benz *et al.*, 1992; Soubasi *et al.*, 1999).

Erythropoietin receptor expression is initiated by the burst forming unit erythroid (BFU-E) cells, however the colony forming unit-erythroid (CFU-E), is entirely reliant on erythropoietin for further differentiation and is able to expresses in maximal density erythropoietin receptor (Teh *et al.*, 2011; Youssoufian *et al.*, 1993).

2.1.2 Secondary roles

Besides performing many primary important deeds, erythropoietin hormones also performed secondary action such as vasoconstriction-dependent hypertension, stimulating angiogenesis, and proliferation of smooth muscle fibers. In addition, it also can increase the adsorption of iron in blood by suppressing the hepcidin hormone (Haroon *et al.*, 2003). Furthermore, Chen *et al.* (2006a) has successfully detected about 100x of EPO in brain with hypoxic damage as a natural response. Due to this finding, the researchers believed that EPO can also affect neuronal protection during hypoxic conditions such as stroke (Chen *et al.*, 2006b; Lin *et al.*, 2008). Nevertheless, no studies on human subjects are reported at the present.

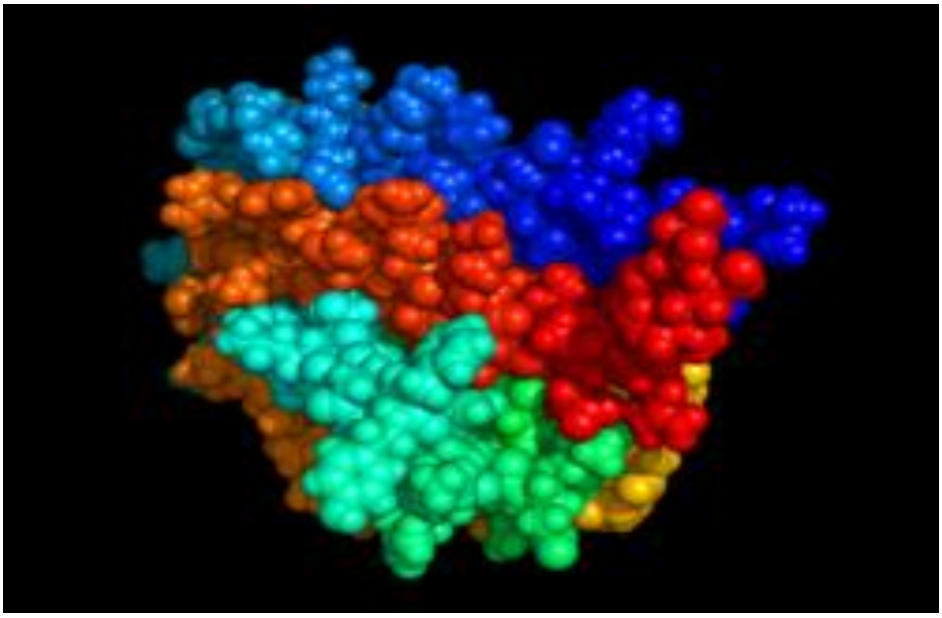

Figure 2.1: The structure of human erythropoietin adapted from European Molecular Biology Laboratory (http://www.ebi ac.uk/pdbe-srv/view/entry/1eer/taxonomy).

2.1.3 EPO gene structure

Erythropoietin is a glycoprotein hormone which comprised of 165 amino acids. The molecular mass is made up with 40% of carbohydrate (Rebollo *et al.*, 2003). The amino acid group of EPO is able to migrate from 18,398 to 30,000 Dalton. This process will merely occur in fully glycosylated form of EPO. The glycosylation of EPO does not affect the biological activity (Macdougall, 1999). Furthermore, the biological half-life of EPO molecule is only 8.5 hours. There are fours of the carbohydrate hydrate side chains bound with sialic acid residues. The hydrophobic polypeptide of EPO contains one O- and three N-linked sites (Wilson and Jolliffe, 1999). The glycosylation site of three complex types N-glycans is sited at positions 24, 38 and 83 asparagine residues and a mucin- type-O glycan located at

ser-126. In addition, the oligosaccharaide side chain is the site for processing and transportation of EPO from its cell (Mayeux *et al.*, 1994).

2.2 *Pichia pastoris*

Pichia pastoris is mesophilic yeast which is unicellular, having an oval shape approximately 1-5 μm wide and 5-30 μm long. *Pichia pastoris* are classified under the Kingdom *Fungi*, Division *Eumycota*, Subdivision *Ascomycotina*, Class *Hemoascomycetes*, Order *Endomycetales*, Family *Saccharomycetaceae* and Genus *Pichia* (Bayraktar *et al.*, 2009). The eukaryotic cell structure is generally possessed a thick polysaccharide cell wall. The yeast is classified as a facultative anaerobe based on its oxygen requirement (Tanaka *et al.*, 1986).

Yeast, capable of utilizing methanol as a sole carbon and energy source, is known as methylotrophic yeast (Tanaka *et al.*, 1986). Amongst many, only four genera were identified up to now; namely, *Hansenula*, *Pichia*, *Candida* and *Torulopsis* (Faber *et al.*, 1994). Phillips Petroleum was the first company who developed the protocols and cultivation media for *P. pastoris* in 1970s. The interest on *P. pastoris* has increased dramatically and at present, almost more than 700 of recombinant proteins have been produced by *P. pastoris* (Cregg *et al.*, 2000).

Pichia pastoris, as a promising foreign protein expression host, offers numerous advantages. It required simple molecular genetic manipulations. *Pichia pastoris* can be growth on simple, inexpensive media (Thorpe *et al.*, 1999). Moreover, it has good secretion ability for proteins (Jahic *et al.*, 2006). A detailed list showing advantages and disadvantages of *P. pastoris* is given in Table 2.1(Cregg *et al.*, 1999; Daly and Hearn, 2005; Macauley-Patrick *et al.*, 2005).

2.2.1 Metabolism of *Pichia pastoris*

According to the Invitrogen fermentation procedure Invitrogen, (2002) *P. pastoris* can be grown first on glycerol as a substrate rather then methanol for higher biomass yield and maximum specific growth rate. On the other hand, the protein expression is repressed during growth on glycerol (Cregg and Higgins, 1995).

In glycerol utilization pathways (Figure 2.2), the glycerol is phosphorylated to glycerol-3-phosphate through a glycerol kinase. The FAD-dependent glycerol-3-phosphate dehydrogenase is further oxidized the product to dihydroxyacetone phosphate, which will then entering the glycolytic pathway (Nevoigt and Stahl 1997). According to Nevoigt and Stahl, (1997) alternative pathways which use NAD-dependent glycerol dehydrogenase kinase and a dihydroxyacetone for glycerol dissimilation are performed by certain yeast only. The product of glycolysis is pyruvate. The pyruvate dehydrogenase will then oxidize the pyruvate to acetyl–CoA. Afterward, acetyl–CoA will enter the tricarboxylic acid (TCA) cycle, to form various metabolites which are crucial for the synthesis of cellular constituents such as amino acids, nucleic acids as well as cell wall components (Ren *et al.*, 2003). It is crucial to have better understanding *Pichia* metabolism for better control of growth and product formation.

Methanol metabolism emerged as an important pathway for the *P. pastoris* expression system (Dehoop *et al.*, 1991; Johnson *et al.*, 2001). The initial utilization reaction occurs in the peroxisomes and later in the cytoplasm (Macauley-Patrick *et al.*, 2005).The first step of methanol utilization pathway is catalyzes by the alcohol oxidase enzyme (AOX), which is responsible for the oxidation of methanol to formaldehyde and hydrogen peroxide (Figure 2.3). The catalase enzyme was then degraded the hydrogen peroxide formed in the first reaction to oxygen and water.

The formaldehyde which leaved the peroxisome was oxidized further to formate and carbon dioxide and was used as an energy source and for growth. Formaldehyde which still remain will be assimilated to form cell constituents in cyclic pathway and begin the reaction with formaldehyde and xylulose 5-monophosphate. The products of this reaction departed the peroxisome and enter the cytoplasmic pathway reproduced xylulose 5-monophosphate and other cell supplies (Charoenrat *et al.*, 2006; Cregg *et al.*, 2000).

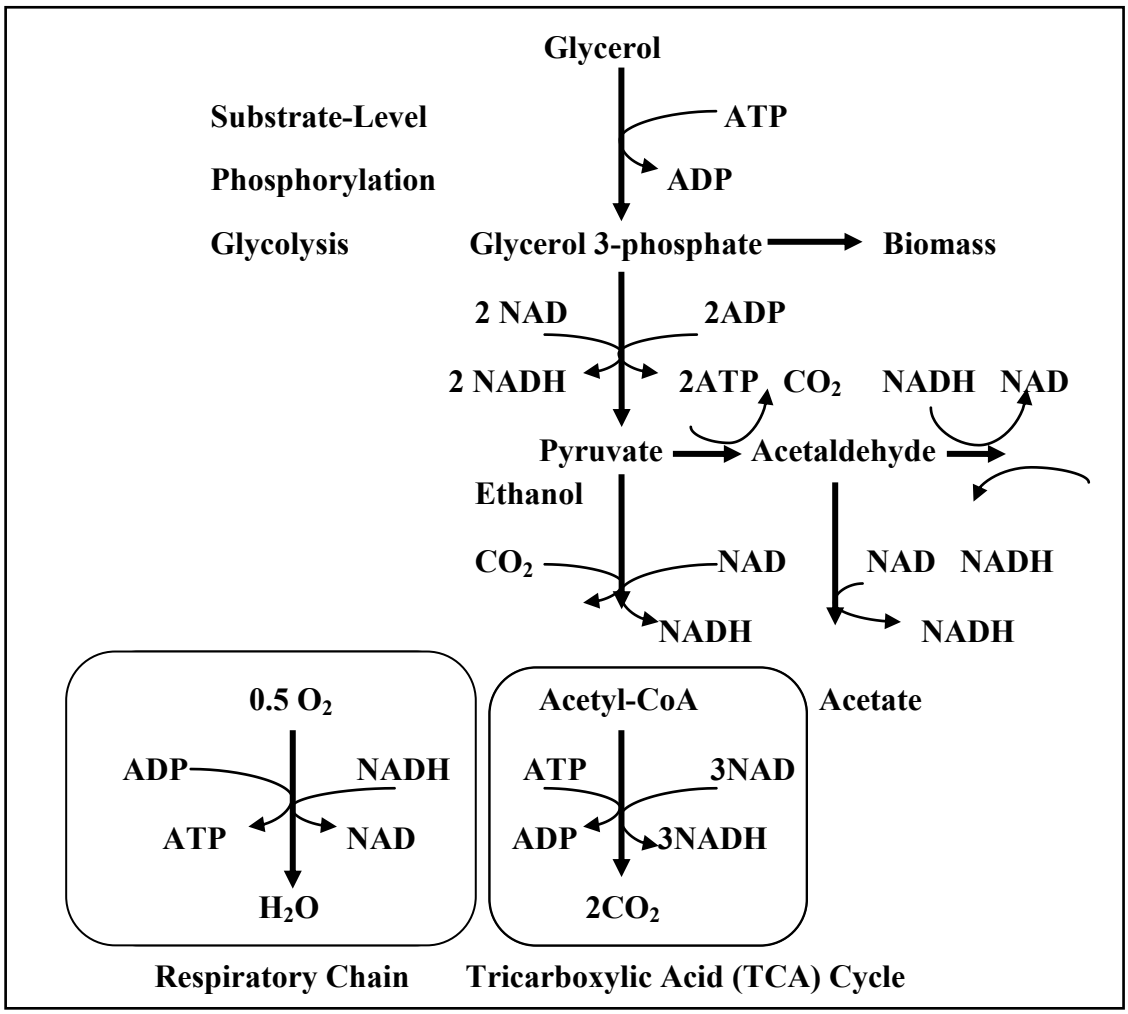

Figure 2.2: The glycerol catabolic pathway in *P. pastoris* (adapted from Ren *et al.* (2003).

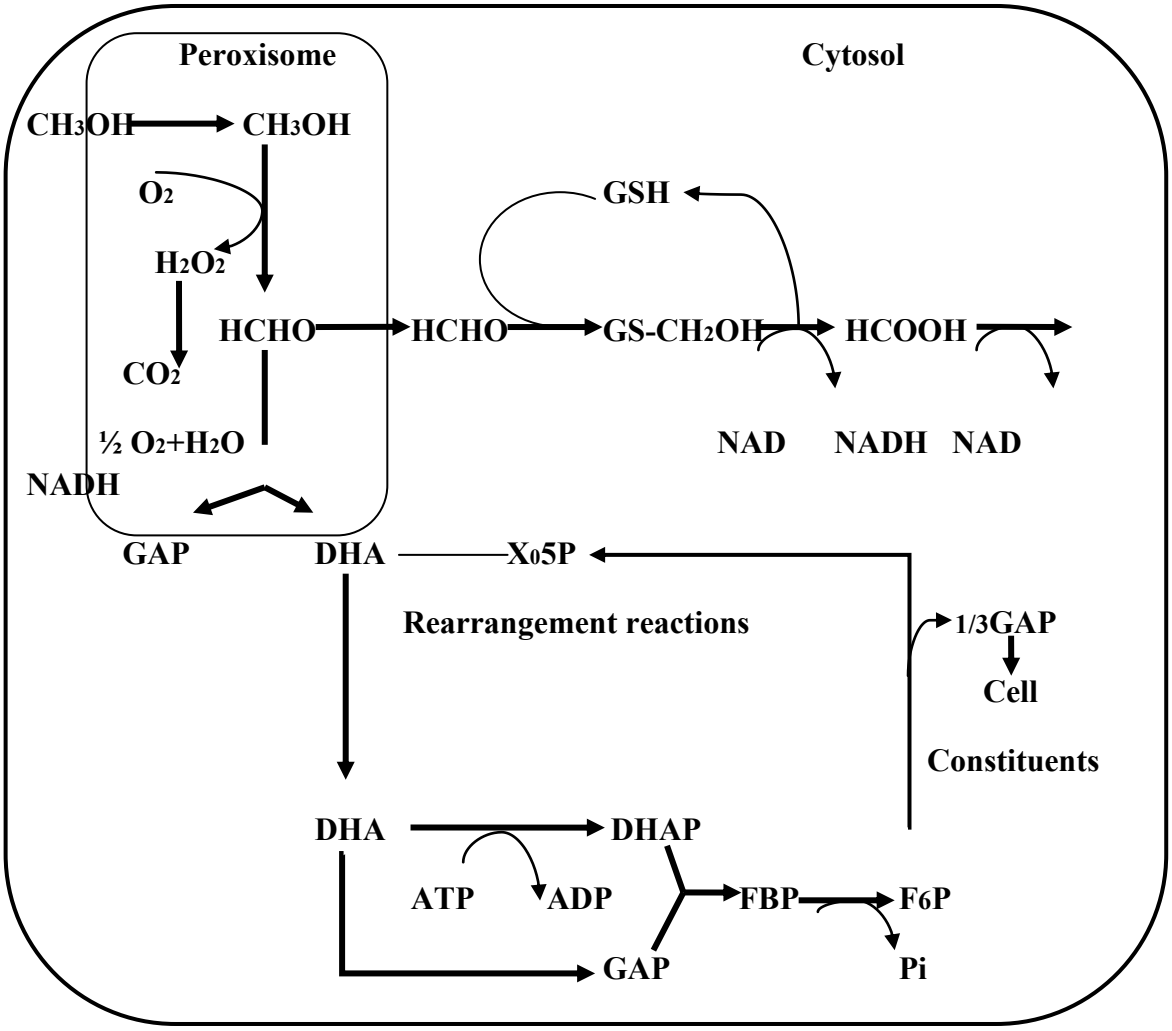

Figure 2.3: The methanol pathway in *P. pastoris*. Adapted from; (Bayraktar *et al.*, 2009; Cregg *et al.*, 1999; Ren *et al.*, 2003).

2.2.2 Expression system of *Pichia pastoris*

Pichia pastoris with AOX1 promoter should be grown on methanol for recombinant protein expression. In order to utilized methanol, several enzymes are required and these enzyme are only expressed when cells are grown on methanol (Cereghino *et al.*, 2002; Jahic *et al.*, 2006). When the cells are grown on other carbon sources such as glucose and glycerol, the AOX1 promoter is partially suppressed. However, the promoter can be strongly induced by methanol (Cereghino *et al.*, 2002; Jahic *et al.*, 2006). There are two types of alcohol oxidase genes producing alcohol oxidase which is AOX1 gene and AOX2 gene. Almost 85% of alcohol oxidase activity in the cell is modulated by AOX1 gene (Zhang *et al.*, 2002). On the other hand, the AOX2 gene is only capable generated 10–20 times less AOX activity than the AOX1 gene (Macauley-Patrick *et al.*, 2005). The total concentration of AOX promoter, in a methanol-limited fed-batch culture, reaches over 30% of total soluble protein.

Table 2.1: Advantages and disadvantages of *P. pastoris* adapted from Acik *et al.* (2009).

Advantages	Disadvantages
• High yield and productivity • Non pathogenic • Strong promoter (AOX1) • Chemically defined media-simple, inexpensive formulation • Broad pH range: 3- 7 • Ability of utilizing methanol • Preference for respiratory growth rather than fermentative, a major advantage relative *S. cerevisiae* • Stable production strains • Low purification cost • No endotoxin problem • Crabtree-negative • Hyper-glycosylation is not as much as in *S. cerevisiae*. • High levels of expression of intracellular and secreted proteins • Eukaryotic post-translational	• Potential of proteolysis, non-native glycosylation. • Long time for cell cultivation compared to bacteria • Monitoring methanol during a Process is difficult in order to induce AOX1 promoter. • Since methanol is a petrochemical substance, it may be unsuitable for use in the food industry and also storing of this in industrial scale is undesirable because it is a fire hazard.

Despite the fact that AOX1 is the most efficient promoter for expressing numerous foreign genes, the usage of methanol inducing gene expression may not be appropriate in some conditions, especially in food production, since methane, a petroleum-related compound is not suitable for human consumption (Boze *et al.*, 2001). Thus, the expressions of certain genes that do not rely on methanol or an alternative to the AOX1 promoter are more attractive such as GAP, FLD1, PEX8, and YPT1 promoters (Cereghino and Cregg, 2000).

2.2.3 Methanol utilization phenotypes of *Pichia pastoris*

The ability of *P. pastoris* on utilizing methanol can be divided into three phenotypes; *P. pastoris* plus phenotype, Mut^+, which comprised AOX1 and AOX2 genes and is able to growth on methanol at the wild-type rate. Jungo reported the maximum specific growth rate of Mut^+ on methanol is 0.14 h^{-1} (Jungo *et al.*, 2006).

The second type is methanol utilization slow phenotype, Mut^s, which has a dislocated AOX1 gene and only depends on the weaker AOX2 gene to metabolized methanol. The disruptions in the AOX1 gene take place during transformation of the expression vector. Deletion and substitution of AOX1 gene which is conducted by the expression cassette and marker gene acquired only 10 to 20 % of transformation actions. Therefore Mut^s merely relied on the weaker AOX2 gene for growth on methanol since its AOX1 gene is disrupted (Cregg *et al.*, 1987). The maximum specific growth rate of Mut^s on methanol is 0.04 h^{-1}(Bayraktar *et al.*, 2009). The large difference of specific growth rates for both phenotypes can be used to differentiate between the two transformed simply by cultivating these two phenotypes on methanol minimal medium (MM), whereby the Mut^s grew slower and only formed a small colony compared with the Mut^+. The last phenotype is methanol

utilization minus, Mut$^-$, which has both AOX1 and AOX2 genes dislocated, which makes it incapable grew on methanol. Yet, methanol is still required for the induction of recombinant protein expression (Macauley-Patrick *et al.*, 2005).

2.2.4 Proteolytic degradation in *Pichia Pastoris*

Pichia pastoris is one of the most desirable expression systems for producing foreign proteins. However, in high cell density cultures, proteolytic degradation is a concern (Kobayashi *et al.*, 2000b; Sinha *et al.*, 2003; Van den Ende *et al.*, 2005). Proteolysis in the production of recombinant protein can cause reduction of product yield, product degradation, loss of biological activities and product contamination due to intermediate degradation in downstream process (Macauley-Patrick *et al.*, 2005; Zhang *et al.*, 2007).

Proteases in *P. pastoris* are well characterized. It was reported that the most common type of protease released from lysed cell in the medium are extracellular proteases, cell-bound proteases and intracellular proteases (Jahic *et al.*, 2002; Macauley-Patrick *et al.*, 2005). The level of protease in yeast vacuoles can vary and highly depended on their nutrition (Hansen and Stougaard, 1997; Sinha *et al.*, 2005). In addition, according to Jahic *et al.* (2006) vacuolar protease is probably the major type of the proteases secreted by *P. pastoris* since the extracellular proteases was founded only at low level in the medium (Jahic *et al.*, 2006). Moreover, the vacuolar proteases do not necessitate ATP and have low substrate specificity so they can executed on any peptide bond in secreted protein structure, which result in protein degradation.

Most of the proteases presented in the medium are probably released from lysed cells (Jahic *et al.*, 2006). There are several factors which can lead to cell lysis

such as change of heat and pH, starvation or toxic chemicals since these factors stress the cells. In addition, proteins impairment due to oxidative stress and heat-shock responses were also elicited proteolytic response (Sweeney and Holoman, 2001; Zhang *et al.*, 2007).

2.3 Medium design and bioreactor operation parameters

The yield and the production of recombinant proteins are affected by various parameters. Medium composition and bioreactor operation parameters such as pH, temperature and oxygen transfer are the most vital since they can influence metabolic pathways and changing metabolic fluxes consequently generating miscellaneous effects on product formation especially in aerobic fermentation processes (Celik *et al.*, 2009).

2.3.1 Medium design

In bioprocess development, cultivation medium is the most crucial factor as cell growth and product formation is highly influenced by the uptake and conversion of nutrients component in the medium. According to Nielsen and Villadsen, (1994) the cultivation medium should contain carbon, nitrogen and energy sources. Furthermore, it should also contain all essential mineral required for growth and contain all necessary growth factors to ensure rapid growth and high yield of the desired product. In addition, the cultivation medium should always be readily available and stable. Finally, it should not pose any difficulty in downstream processing (Wang *et al.*, 2001).

The essential nutrients for cell growth can be divided into two groups, which can be classified based on the required concentrations of the nutrients in the medium. The first group is the macronutrients. Carbon, oxygen, nitrogen, hydrogen, sulfur, phosphorus and magnesium are the main macronutrients which are generally required at concentrations higher than 10^{-4} M. The second group is the micronutrients, with the required concentrations less than 10^{-4} M. Trace elements such as Ca, Cu, Fe, Na, Mn, Mo, Zn, and vitamins are some micronutrients and they are usually added to the culture medium as mineral salts (Fiecher *et al.,* 1984).

2.3.2 Carbon source

Carbon source play essential roles in cell growth and recombinant protein production. Most commonly used carbon sources for *Pichia pastoris* are methanol, glycerol, sorbitol, glucose, mannitol, trehalose, etc. (Brierley *et al.*, 1990; Inan and Meagher, 2001b; Sreekrishna *et al.*, 1997; Thorpe *et al.*, 1999). Methanol is not only used as a sole carbon source, but also used as an inducer of AOX1 to trigger the expression of recombinant protein (Celik *et al.*, 2008). Beside carbon sources, nitrogen is also required as an essential macronutrient. In *P. pastoris* fermentation protocol, the ammonium hydroxide is typically used as a nitrogen sources as well as pH controller to maintain the pH at the preferred level (Cos *et al.*, 2006a). According to Brankamp *et al.,* (1995) the use of ammonium ions as a nitrogen source throughout recombinant protein production is likely the most frequently used method for reducing proteolytic degradation due to nitrogen-limitation. In addition, ammonium ions can probably act as a substitute and competing substrate for proteases with lower substrate specificity (Brankamp *et al.*, 1995). On the contrary,

extension of lag phase was observed when a high ammonium concentration was used in the medium and thus inhibited the growth of the cells and protein production (Xie *et al.*, 2003; Yang *et al.*, 2004).

The major types of growth media can be divided into three categories; defined medium, which contain specific amounts of pure chemical compounds with known chemical compositions, semi-defined medium, and complex medium, containing natural compounds whose chemical composition is not exactly known (Shuler *et al.*, 1994). Complex media frequently contains an organic nitrogen source, essential minerals and different growth factors with difference in the composition (Nielsen and Villadsen, 1994).

In industrial applications, defined medium is more preferred in bioprocess due to the lower number of steps in subsequent downstream processing and effortless process validation (Macauley-Patrick *et al.*, 2005). Basalt salt medium (BSM) along with the trace salts (PTM1) is the most common medium used in high cell density fermentation for methylotrophic yeast. Even though it may not be the optimum medium for growth but it can still be considered as a standard medium due to its popularity. Nevertheless, it may possess some important problems, such as, precipitates and high ionic strength (Cereghino *et al.*, 2002; Cos *et al.*, 2006b).

2.3.3 pH

Microbial cells have a distinguished capability to sustain their intracellular pH at constant level, although with large fluctuation of pH in the medium, provided that, the energy demands for maintenance is only increase at the outflow, since Gibbs

free energy has to be used for maintaining the proton gradient across the cell membrane (Nielsen and Villadsen, 1994).

Microbial growth rate, the activity of enzymes, transport mechanisms and other extracellular and intracellular actions are mainly regulated by hydrogen ion (H^+) concentration which is also one of the most imperative bioreactor operation parameters.

Pichia pastoris can tolerate a broad pH, range between 3.0 and 7.0 and more preferable at pH around 3.5 to 5.5 as this range gave minimum effect on the growth rate and provide substantial alternative in adjusting the pH and can minimize proteases activity in the medium (Macauley-Patrick *et al.*, 2005; Sreekrishna *et al.*, 1997). The expression of recombinant protein and its stability robustly depends on the optimum pH value. Hence, different pH values were found to be favorable for different proteins (Macauley-Patrick *et al.*, 2005); for instance, the production of recombinant human erythropoietin was optimum at pH 6.0 when the protein was produce in Buffer minimal glycerol medium (BMGY) and pH 3.0 was found to be optimal in production of insulin-like growth factor-I and cytokine growth-blocking peptide (Brierley *et al.*, 1990; Calik *et al.*, 2010; Koganesawa *et al.*, 2002). In addition the production of recombinant ovine interferon was higher at pH 6.0 due to the substantial reduction of proteolytic activity and protein degradation however the proteolytic response increased at pH 5.0 with an average of 2836 U/mL protease or 1243.9 U of protease/mg protein in fermentation supernatant Kobayashi *et al.,* (2000) and Sinha *et al.,* (2003) observed that, during recombinant human serine albumin production, protease activity rapidly increased when the pH was set below pH 5.0 and was decrease at pH 5.6 and they has successfully minimizing the degradation

rate of human albumin serum or HAS at pH 5.6 (Kobayashi *et al.*, 2000b; Sinha *et al.*, 2003).

2.3.4 Temperature

Microorganisms do not have the capability to regulate their inner temperature. Hence, their internal temperature is always equal to their external temperature and all cellular biochemical reactions occurred directly depend on the external temperature. Beside the reaction rates, the metabolic regulation, nutritional requirements, biomass composition and product formation can also influenced by temperature. In order to inquire the optimum temperature, consideration should be taken for optimum growth temperature and product formation since both can be diverge. In addition, when temperature is elevated higher than the optimum temperature, the maintenance necessities of cells begin to amplify (Nielsen and Villadsen, 1994). Moreover, secondary and tertiary structures of the protein are also highly contingent on temperature since lower temperature facilitated to diminishing protein misfolding and able to generate more correctly folded proteins (Georgiou *et al.*, 1996).

The upshot of temperature on the maximum specific growth rate of a microorganism is equivalent to enzyme activity. Moreover, protein denaturation will rapidly increase when temperature is abruptly increases to a high level. In addition, the maximum specific growth rate is greatly increases the same way as in normal chemical rate constant whilst temperatures are lower (Nielsen and McCammon, 2003). According to Shay *et al.* (1983), the optimum temperature for growth of *P. pastoris* is 30 °C and temperatures higher than 32 °C can be detrimental to protein expression and may lead to cell death (Invitrogen, 2002). Temperatures above 30 °C are conducive to cell lysis and will elicit protease activity in fermentation media and

are not appropriate for the production of recombinant protein because high temperature will result in cell death (Inan *et al.*, 1999). Li *et al.* (2001) observed that, during the production of herring antifreeze proteins, decreasing the temperature from 30 ^{o}C to 23 ^{o}C improved the yield from 5.3 mg/L to 18.0 mg/L, and increased cell viability (Li *et al.*, 2001). Moreover, Hong *et al.* (2002) were successfully acquiring more active laccase when decreasing the production temperature from 30 ^{o}C to 20 ^{o}C. In addition, it is observed that, when the cultivation temperature is lowered, the rates of proteases activity were also decreased (Hong *et al.*, 2002; Li *et al.*, 2001; Macauley-Patrick *et al.*, 2005).

Zhang *et al.* (2007) was observed that, the stability of intracellular protein was ameliorate when the production temperature was around 25-27 °C as compared with 30°C. Thus, optimization of production temperature may also minimize degradation (Zhang *et al.*, 2007). However, it has to be remark that, many other cases where the production of recombinant proteins expressed by *P. pastoris* strains does not extensively influence by lowering production temperature below 30 °C (Curvers *et al.*, 2001; Hong *et al.*, 2002; Inan *et al.*, 1999; Kupcsulik and Sevella, 2005). In this study, temperature was kept at 30 °C during the entire process.

2.4 Fed-batch fermentations of *Pichia pastoris*

2.4.1 High cell density cultivation

In fermentation, fed-batch is the most preferred mode of cultivation as such system proficient for achieving high cell densities and easier to control (Chiruvolu *et al.*, 1998; Cos *et al.*, 2005a; Huy *et al.*, 2011; Li *et al.*, 2011b; Liu *et al.*, 2011a; Tang

et al., 2010; Zhang *et al.*, 2011). Typical fed-batch fermentation for *Pichia pastoris* consist of growth and production phase. In growth phase glycerol was commonly used to support the growth of the cell whereas in production phase methanol was used to induce protein expression. *P. pastoris* with AOX1 can be cultivated to three phases; glycerol batch phase (GB), glycerol fed-batch phase (GFB), methanol fed-batch (MFB) and in some cases additional methanol transition phase (MT) (Cos *et al.*, 2006b; Gurramkonda *et al.*, 2009; Potvin *et al.*, 2012; Tang *et al.*, 2010; Zhang *et al.*, 2001b).

In glycerol batch, glycerol will be included in define medium to culture the cell. Subsequently, when the substrate is depleted the dissolved oxygen (DO) will suddenly increase, the substrate will be feed at limited feed rate to further increase cell concentration. The end time of glycerol fed-batch is depends on the desired biomass. The maximum specific growth rate (μmax) of *P. pastoris* grew on glycerol is 0.18 h^{-1} (Cos *et al.*, 2005b; Liu *et al.*, 2011b). An optional methanol transition or transition phase can also be included between glycerol limited feeding (GFB) and induction phase (MFB), allowing the cells to adapt to methanol and synthesizing some of alcohol oxidase (AOX) enzyme to trigger the first step of methanol catabolism before utilize methanol. In addition, according to Zhang *et al.* (2001), the length of transition phase can be shorten by simultaneously feeding a limited amount of glycerol with methanol over 3 hour period and the feed rate of glycerol is set to ramp down linearly and at the same time the methanol will be continually added for additional 2 hours to accelerate the synthesis of AOX enzyme. The transition phase can be terminated when the dissolve oxygen start to decrease abruptly after adding several drop of methanol. The cell density in this phase should be around 5-10 g/L

due to the addition of glycerol and the length of the transition phase is between 5-6 hours (Minning *et al.*, 2001; Zhang *et al.*, 2001b). Finally, after the transition phase, the production phase can be initiated to produce the recombinant protein. The end time of MFB will be based on the desire biomass. The maximum specific growth rate (μmax) of *P. pastoris,* Mut^+ grew on methanol is 0.14 h^{-1} (Siegel and Brierley, 1989; Tang *et al.*, 2010).

2.4.2 Fed-batch process control strategies

In fed-batch fermentation, the main factor to control under AOX1 regulated system is methanol addition in production phase as the feed rate, the residual and the methanol concentration will directly affecting the rate of expression, proteolytic degradation, cell growth, cell lysis and oxygen transfer (Potvin *et al.*, 2012; Tang *et al.*, 2010). Numerous strategies has been proposed and implemented to control the addition of methanol to enhance protein production. The most frequent methanol control strategies are include constant specific growth-rate feeding (μ-stat), constant methanol concentration feeding, constant DO-based feeding (DO-stat), oxygen limited fed-batch (OLFB) and temperature-limited fed-batch (TLFB) (Khatri *et al.*, 2011; Tang *et al.*, 2010).

2.4.2.1 μ-stat control

In μ-stat control strategy, methanol feeding rate can be determined base on mass balance equations for maintaining a constant specific growth rate (μ) (Cos *et al.*, 2006b). Since most of the protein production processes are directly or indirectly related with cell growth, μ-stat is a good control strategy for process optimization,

does not required any on-line monitoring for parameters system (Ren *et al.*, 2003). A constant specific growth rate can be achieved by using methanol feed profile models devised (Tang *et al.*, 2010). Zhang *et al.* (2001b) was successfully developed a model which involved the combination of specific growth rate, methanol concentration, and specific methanol consumption rate. The μ_{max} calculated from their model was 0.08 h^{-1} however the highest level of protein production was attained at 0.03 h^{-1}. Furthermore, Ren *et al.* (2003) has reported a macrokinetic model for *P. pastoris* expressing recombinant human serum albumin based on stoichiometric balances to describe the cell growth and protein production. They have successfully maintained the μset by combining the linear and exponential feeding profiles (Khatri *et al.*, 2011).

Three methanol feeding strategies for production of mouse endostatin by *P. pastoris* were reported by Trinh *et al.* (2003) which involved comparison between methanol consumption, dissolved oxygen concentration and exponential feeding (Trinh *et al.*, 2003). Unlimited methanol was supplied in first two strategies and limited methanol was employed in exponential feeding with (μ_{set}) of 0.02 h^{-1}. The specific productivity was two times higher in limited methanol feeding compared to other strategies (Trinh *et al.*, 2003). Despite the fact that the limited methanol feeding is widely used but this strategy is not suited for recombinant protein which will easily get dissipated under these conditions (Jahic *et al.*, 2003; Khatri *et al.*, 2011; Trinh *et al.*, 2003; Zhou and Zhang, 2002).