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Process Engineering of *Pichia pastoris* Cultivation for the Production of Phytase with GAP Promoter

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Process Engineering of *Pichia pastoris* Cultivation for the Production of a Phytase with GAP Promoter

By

Shuiquan Tang

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ABSTRACT

Pichia pastoris (*P. pastoris*) is a popular host for recombinant protein production. In this work, phytase production with *P. pastoris* under GAP promoter was studied. In the first part of this study, feasibility of using crude glycerol from biodiesel production as the sole carbon source for *P. pastoris* cultivation was investigated. Although growth inhibition was found in batch cultivations stated with high concentration of biodiesel glycerol, efficient high cell density production was realized by fed-batch cultivation started with a low concentration of biodiesel glycerol. The second part of this work focused on the study of growth kinetics with continuous cultivations under different dilution rates. The influences of dilution rate upon cell growth and phytase production were revealed and characterized with empirical equations. Based on these results, a simple kinetic model was established and model parameters were estimated mainly based on these results as well. Good agreement with experimental results was found when this model was applied to the prediction of cell growth and phytase production in regular fed-batch cultivation processes.

RÉSUMÉ

Pichia pastoris (*P. pastoris*) est un hôte populaire pour la production de protéine recombinante. Dans ce travail, la production de phytase avec le *P. pastoris* sous contrôle du promoteur GAP a été étudiée. Dans la première partie de cette étude, la faisabilité d'utiliser du glycérol brut provenant d'une production de biodiesel, comme unique source de carbone pour la culture de *P. pastoris,* a été examinée. Bien que de l'inhibition de croissance ait été constatée dans les cultures batch à hautes concentrations de glycérol de biodiesel, une production efficiente de haute densité cellulaire a été accomplie en culture fed-batch, démarrée avec une basse concentration de glycérol de biodiesel. La deuxième partie de cette étude s'est concentrée sur l'examen de la cinétique de croissance en mode continu avec différents taux de dilutions. Les influences du taux de dilution sur la croissance cellulaire et la production de phytase ont été constatées et caractérisées par des équations empiriques. Sur la base de ces résultats, un simple modèle cinétique a été établi et les paramètres du modèle ont été estimés principalement sur la base de ces mêmes résultats. Il y a bonne concordance avec les résultats expérimentaux quand le modèle est appliqué à la prédiction de croissance cellulaire et la production de phytase dans des procédés de cultures fed-batch réguliers.

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CHAPTER 1

Bioprocess Engineering of Heterologous Protein Production in *Pichia pastoris*: a Review

Abstract

*Pichia pastoris*has been widely used for the production of heterologous protein, for both academic and industrial purpose. This expression system is powerful but also complicated. The expression level and productivity have been found to be controlled by many factors: factors determined by the expressed protein (such as codon usage and GC content), factors determined by the host strain (such as the selection of promoter, gene copy number and strain phenotypes) and factors in cultivation levels. In this article, efforts are mainly focused on the review of recent development of process engineering with respect to factors in cultivation level. Contents will cover: medium selection, cultivation condition optimization, control of proteolytic degradation, process monitoring, process control, et al. In addition, recent development of using an alternative promoter, GAP, will be reviewed.

1.1 Introduction

Pichia pastoris(*P. pastoris*) is one of the most cited methylotrophic strains, which are capable of growing on methanol. The studies of such methylotrophic strains date back to the early 70's of last century and stem from the interest of producing single cell protein (SCP) from methanol [1]. Although the economics of SCP production from methanol were proved unfavourable later, *P. pastoris* tended to become a powerful system for heterologous protein production.

The success of *P. pastoris* as a host for foreign protein production is mainly attributed to the existence of a strong promoter, the promoter of alcohol oxidase 1 (AOX1). The gene and promoter for alcohol oxidase (AOX) were isolated by Salk Institute

Biotechnology/Industrial Associates Inc. (SIBIA, La Jolla, CA, USA), who then generated vectors and strains for the molecular manipulation of this microorganism[1]. Alcohol oxidase is the first enzyme of the inducible methanol utilization pathway and it accounts up to 35% of the total protein in cells when growing upon methanol [2]. Nevertheless, when growing on glucose, ethanol or glycerol, the expression of the enzyme is undetectable [2].

Nowadays more than 500 proteins have been cloned and expressed with the system of *P. pastoris* [3]. Integration of many advantages makes *P. pastoris* a successful system for heterologous protein production in terms of either academic or industrial purposes: 1. the existence of a strong and inducible promoter, AOX1; 2. the capability to secrete foreign protein extracellularly, simplifying downstream recovery; 3. the capability to perform eukaryotic post-translational modification such as glycosylation and proteolytic processing, which makes it more attractive for the expression of human proteins than prokaryotic expression systems such as *E. coli*; 4. the ability to grow upon defined medium and achieve high cell density; 5. a strong preference for respiratory (versus fermentative) growth; 6. the relative simplicity of molecular manipulation and the availability of the expression system as a commercialized kit (Invitrogen Co.).

There are two genes that code for AOX in *P. pastoris*: AOX1 and AOX2. Although both genes encode equally functional AOX enzymes, the enzyme of AOX1 accounts for more than 90% of total AOX enzymes due to the relative strength of its promoter [3]. Therefore, foreign protein production is often expressed under the control of AOX1 promoter, although high expression using AOX2 promoter has been reported as well [4].

AOX1 promoter is the one most commonly used for *P. pastoris* expression system, there are some conditions on which this promoter may not be suitable. For example, the promoter is not suitable for the production of food products since methanol is derived from petroleum-related compounds. The biggest disadvantage of AOX1 promoter is that it requires the addition of methanol, which is a potential fire hazard and a big concern for large-scale application.

Other promoters have been used to replace AOX1 to avoid the utilization of methanol. The glyceraldehide 3-phosphate dehydrogenase (GAP) promoter is one of them. GAP promoter was first isolated by Waterham in 1997 [5] and has been used to express many heterologous proteins. With the promoter, foreign proteins are expressed constitutively with glucose or glycerol as substrate. In some cases, the expression level with GAP promoter was found to be higher than AOX1 promoter [3]. FLD1 gene encodes for an enzyme that plays an important role in methanol metabolism and in methylated amines metabolism. The FLD1 promoter was strongly induced by either methanol or methylamine. Other alternative promoters can be AOX2 [4], PEX8 [6], YPT1 [7] and ICL1 [8].

All *P. pastoris* expression strains are derived from NRRL-Y 11430; for example, *P. pastoris* GS115 (his4) was generated by introducing nitrosoguanidine mutagenesis to NRRL Y-11430 [9]. With regard to ability of methanol utilization, there are three phenotypes of *P. pastoris*: Mut^+, Mut^s and Mut^-. Mut^+ indicactes that the strain can grow on methanol at the wild-type rate. Mut^s strains have a deletion of AOX1 gene, but have an intact AOX2 gene; so Mut^s strains can still grow on methanol although with a lower growth rate. Foreign protein expression in Mut^s strains is sometimes better than in Mut^+ strains [10, 11, 12]. In addition, Mut^s strains require less amount of methanol and oxygen for growth and induction and, therefore, do not have some problems of large scale fermentation, such as oxygen limitation. Mut^- strains have both AOX genes deleted and are unable to grow on methanol. Some strains of *P. pastoris* are genetic-engineered to delete some of their protease genes with the purpose of reducing proteolytic degradation. These strains are protease-deficient strains, such as SMD1163 (his4 pep4 prb1), SMD1165 (his4 prb1) and SMD 1168 (his4 pep4). Utilization of such strains was proved to be an effective way to reduce proteolytic degradation of some expressed proteins [13, 14].

The production of heterologous protein is a very complex process, which is influenced by many factors of both gene level and cultivation level. Gene level factors can be further divided into two groups: foreign protein specific and host strain specific. Hundreds and thousands of examples of heterologous proteins expression present a feature that the expression level at least partly depends on the expressed protein itself. Optimization of foreign protein specific factors is becoming a regular practice, such as optimization of codon usage, GC content, protease reaction sites and potential glycosylation site of the foreign gene. *P. pastoris*' preferential codons of the 20 amino acids was determined by

Sinclair and Choy and their optimization of codon usage and GC content of human glucocerebrosidase led to 10.6- and 7.5-fold improvement of expression level respectively [15]. Host strain specific factors, such as promoter, gene copy number and secretion signal peptide, have dramatic influences on the expression level as well. More detailed information can be referred to the review by Macauley-Patrick and coworkers [1]. In this article, a review will be provided focused on the optimization of cultivation level factors. Cultivation level issues covered include: nutritional factors, cultivation conditions, control of proteolytic degradation, process monitoring, process control, operational mode et al.

1.2 Nutritional consideration

One advantage of using *P. pastoris* for recombinant protein production is its capability of high cell density cultivation on defined medium. This is of significant importance for the production of proteins that have critical requirement on the production cost, such as phytase, which is mainly used as feed additive for animals. Furthermore, using defined medium can prevent potential prion contamination in complex medium containing animal-derived products. However, several journal papers reported that using complex medium enhanced protein production or inhibit proteolytic degradation, which we will discuss in 'Control of proteolytic degradation' section. The selection of medium also depends on the operation mode of cell culture. For example, the medium used in the continuous cultivation is usually different from that in the fed-batch cultivation.

The most commonly used defined medium is the basal salt medium (BSM) proposed by Invitrogen Co. [16]. This medium is mainly used for the fed-batch cultivation to achieve high-cell-density culture. It is considered a standard one, but it has some important problems like precipitation, unbalanced composition and high ionic strength, etc [3]. In the literature, a variety of alternative media have been described, such as FM22 formulated by Stratton and Coworkers [17]. Table 1.1 compared the elemental composition of a variety of media.

Table 1.1 A variety of media for high cell density cultivation of *P. pastoris*

Medium	N	P	S	Mg	Ca	K	Trace Salts	Ref.
	mmol/L							
BSM	-	390.2	172.1	60.6	6.9	282.9	PTM1	16
BSM1	-	190.0	171.2	60.6	6.1	398.5	PTM1	21
BSM2	-	394.6	195.4	85.3	6.7	280.6	YTM	84
FM21	-	511.5	224.8	79.3	8.8	389.6	PTM1*	79
FM22	75.8	315.4	173.5	47.6	5.8	479.8	PTM4	65
PBM	-	205.6	89.8	31.7	3.5	155.6	PTM1	20
LSM	-	261.8	42.7	15.2	1.3	70.7	PTM1	95
Hellwig	-	62.1	26.8	9.4	1.0	44.2	PTM1	24
D'anjou	303.0	88.2	170.6	19.1	2.4	88.2	N.N.	3
Zhao[1]	60.6	29.4	173.1	38.2	3.4	238.6	N.N.	114
Maurer[1]	187.9	93.9	2.0	2.0	0.1	12.1	PTM1	112

[1] Additional salts were supplemented in feeding solution during fed-batch.

The major problem with these media is the precipitation occurring when pH is above 5.5 [18], which makes the actual concentration of dissolved salts uncertain and the measurement of optical density inaccurate. The precipitation is caused by the reaction between phosphate and magnesium, calcium ions or other metal ions from the trace salt solution. Several ways have been proposed to prevent precipitation. The most obvious way is to use complex medium which provides an organic phosphorus source. Zamost reported that phosphate precipitation in a defined medium for *Pichia methanolica* can be inhibited by the addition of citric acid [19]. Zhang et al. reported that replacement of phosphate in BSM with glycerophosphate improved the solubility of the solution greatly and proved that glycerophosphate can be employed as a reliable phosphorus source for both cell growth and recombinant protein production for *P. pastoris* [18].

Nitrogen source is an essential component in medium. The lack of nitrogen can result in increased proteolytic activity and degradation of target protein [20]. Over-accumulation of nitrogen is detrimental as well; it can inhibit cell growth and enlarge the lag phase [21]. In the bioreactor cultivation, pH can be automatically controlled with the adding of ammonium hydroxide, which serves as a major nitrogen source in most cases. That is why there is no nitrogen source present in the formula of BSM, and only 1.06g/L nitrogen in FM22. However, nitrogen addition from ammonium hydroxide for pH control might not be enough to support cell growth when cell density is really high [3]. Therefore, additional nitrogen supplement might be necessary.

As shown in Table 1.1, nearly every defined medium is accompanied by a formula of trace salts containing Fe, Cu, Zn, Mn, Co, biotin, etc. For example, PTM1 is with BSM and PTM4 is with FM22. However, the requirement of actual amount of trace salts for cell growth and protein production is not well studied. In the literature, the concentration of trace salts applied varies very much in different formulas. Boze and coworkers demonstrated that the addition of seven vitamins and two trace salts into BSM medium allows optimal biomass and recombinant protein production [22]. Bradley et al. studied the effect of different metal ions upon biomass and Recombinant β–Galactosidase production in *P. pastoris* cultivation [23]. It was found that magnesium and zinc were both required to support cell growth of *P. pastoris* GS115, while supplementation with calcium, cobalt, iron, manganese, iodine, boron, and molybdenum were not required to sustain cell mass, but they influenced the yield of Recombinant β–Galactosidase.

A reduced salt medium containing approximately one eighth the salt concentrations of the BSM medium was used by Hellwig et al. for the production of Single-Chain Antibody with *P. pastoris* [24]. Surprisingly, with such a diluted medium, no negative effect upon cell growth or final cell density was observed. Is BSM too concentrated? Unfortunately, no report was found in the literature to deal with optimization of defined medium for the fed-batch cultivation of *P. pastoris*. There is only one paper reporting of optimization of a defined medium, but it is in batch mode. Ghosalkar and coworkers reported a medium optimization using response surface methodology with regard to the concentration of magnesium sulfate, phosphate, trance salt and vitamin in a defined medium [25]. An optimized medium is obtained with respect to maximum biomass production; however, this formula should be only applicable in batch cultivation or continuous cultivation because all experiments were completed in batch mode.

Under the promoter of AOX1, the combination of glycerol and methanol is the dominant carbon source, with glycerol the carbon source in biomass accumulation phase (there are reports of using glucose for biomass production as well [26]) and methanol the carbon source and inducer in protein production phase. Under another popular promoter, GAP, the mainly used carbon source is glucose or glycerol. The concentration of glycerol in the initial medium is usually 40 g/L as proposed by *pichia* fermentation process guideline from Invitrogen [16]. Tang and coworkers compared the batch cultivations with glycerol and glucose at similar initial concentration of about 60g/L [27]. In this case, glycerol has a much higher yield of biomass with no fermentative by-products produced, while in the case of glucose, quite an amount of fermentative by-products, such as ethanol and acetate, were found resulting in a low yield of biomass. It can be concluded that for *P. pastoris*, glycerol is a less fermentative substrate while glucose is not. Therefore, when growing *P. pastoris* with glucose, the residual glucose concentration should be controlled critically to prevent fermentative metabolism.

Glucose and glycerol have long been used in the cultivation of industrial microorganisms. Historically, glucose has a priority due to lower price. However, in recent years, with the rapid development of biodiesel production, the price of glycerol has decreased dramatically, since glycerol is the main by-product of biodiesel production (10%) [28]. It is estimated that the price of crude glycerol from biodiesel production could become stable at as low as 0.05$/lb [29]. Two reports have been found to study the possibility of substituting pure glycerol with such crude glycerol from biodiesel production as cultivation carbon source for *P. pastoris* culture. In the comparative experiments with flask cultivation, Celik and coworkers showed that the performance of canola oil-derive crude glycerol is even better than that of pure glycerol for biomass production phase in inductive cultivation with AOX1 promoter [30]. Tang and coworkers also successfully applied such crude glycerol to the cultivation of *P. pastoris* for the constitutive expression of phytase using GAP promoter in a 7.5 L fermenter, and efficient phytase production under high cell density condition was achieved [27].

It is noted that cell growth is slow in the methanol-induction phase under the promoter of AOX1. Researchers have found that co-feeding other carbon sources with methanol can improve cell growth rate and the final production of recombinant protein. Co-feeding mixed carbon sources will be discussed later.

1.3 Cultivation condition

Temperature is a very important factor, which affects the growth of yeasts and recombinant protein production. It is well known that the optimal growth temperature for

P. pastoris is 30°C and when temperature is above 32 °C, the growth decays quickly [3]. Lower induction temperature was found to be an effective way to enhance foreign protein production, which we will discuss later in the section of 'Control of proteolytic degradation'. Moderate decrease of cultivation temperature did not inhibit cell growth, since cell growth was already very slow in the induction phase [31]. Interestingly, Wu and coworkers found that cells grew much faster when induction temperature was at 15°C and 20°C compared to 30°C and this can be explained by the finding that intracellular AOX activity is higher at lower temperature. Similar results were found by Sirén and coworkers when using a low-temperature fed-batch strategy [32]. The temperature used in the biomass production phase upon glycerol or glucose is relatively fixed, usually at 30°C.

P. pastoris is capable of growing in a wide range of pH from 3 to 7 [33]. Different values of pH have little effect on cell growth; however it might have a great influence on the production of recombinant protein, because both the stability of the foreign protein and the extracellular protease activity are found to be affected by pH [20, 34]. Therefore, manipulating pH is another way to reduce proteolytic degradation, while we will discuss in the chapter of 'Control of proteolytic degradation'. As mentioned earlier, defined media such as BSM have precipitation problem when pH is higher than 5.5. So when higher pH is needed, potential effects caused by precipitation (e.g. nutrients depletion and OD reading interference) should be considered.

During large scale high cell density cultivations of microorganisms, one of the most limiting parameters is the maximum oxygen transfer rate, especially in the case of *P. pastoris*. Fermentative by-products, such as ethanol, will be produced when *P. pastoris* is growing on glycerol or glucose and not enough oxygen is supplied according to our experience. In the induction phase, due to characteristic of methanol metabolism, oxygen requirement does not diminish. Reversely, oxygen requirement in methanol metabolism is known to be about three times greater than in glycerol metabolism [35]. And some studies indicated that oxygen limitation negatively affected the expression of foreign proteins [36]. Therefore, it is generally assumed that sufficient level of dissolved oxygen is a priority during the production phase. To find a balance between dissolved oxygen concentration and high productivity, often pure oxygen is supplemented for oxygen enrichment in air supply. Otherwise, cell density will be limited or growth limiting strategy needs to be implemented. However, oxygen enrichment with pure oxygen is not cost effective for industrial applications. Many researchers have been working on alternative methods to solve oxygen limitation.

Oxygen transfer rate (OTR) can be described by the following equation.

$$OTR = K_{La}(C^{*} - C) \qquad (1)$$

Generally, there are two kinds of ways to enhance oxygen transfer: to increase oxygen transfer coefficient K_{La}, and to increase the driving force $C^{*} - C$. A common strategy to increase K_{La} is increasing agitation rate. In some commercial bioreactor control systems, accurate and stable DO control is usually realized with a controller by adjusting agitation rate. K_{La} can be increased by the selection of agitation impeller as well. It is said that pure oxygen supplementation is usually needed to operate the bioreactor at biomass concentrations higher than 150 g kg-1 and specific growth rates greater than 0.03 (1/h). Jenzsch and coworkers successfully ruled out the use of pure oxygen in this situation by impeller improvement: the standard Rushton turbines were replaced by a set of lower power number impellers with improved air handling capabilities [37].

Increasing total pressure and oxygen enrichment in the air supply are another two common ways to enhance oxygen transportation. They both increase the equilibrium oxygen concentration in the water C^{*} and, therefore, increase the driving force. Traditionally, oxygen enrichment is done by mixing air with pure oxygen, which is not cost effective. An alternative oxygen enrichment strategy is to use polysulphone membrane to separate regular air into nitrogen-enriched retentate and oxygen-enriched permeate [38]. An oxygen enrichment membrane system using polysulphone membrane capable of generating 38% oxygen partial pressure was used to increase oxygen supply for *P. pastoris* cultivation producing elastase inhibiting peptide [39]. Another way to increase the driving force is to reduce oxygen concentration in the broth C. Based on this consideration, oxygen-limited fed-batch process (OLFB) was employed by some researchers [40, 41, 42]. In this strategy, residual methanol concentration is usually kept constant at an appropriate level by feed-back controller, while allowing the dissolved oxygen concentration to decrease to a low level, often close to or equal to zero.

Charoenrat and coworkers used this strategy, and oxygen transfer rate was increased by 35% [41]. Charoenrat and coworkers compared the performance of this strategy with a methanol limited fed-batch strategy (MLFB), DO-stat and found that in OLFB the oxygen uptake rate was about 40% higher than that in the MLFB, resulting in 40% higher of total methanol consumption and finally higher final biomass and protein production [42].

1.4 Process monitoring

In order to achieve proper process control, e.g. to make the process proceed in predetermined route to maximize productivity, a pre-conditional thing is the ability to determine if the process state deviates to abnormal state. To do so, online or offline measurement of state variables is needed. Highly non-linearity of bioprocess evolution emphasizes more and more demand on online monitoring of key process variables, for the purpose of more accurate and efficient process control and optimization. In the following, we will review recent development on the in-situ measurement of key process variables for *P. pastoris* cultivation.

1.4.1 Methanol monitoring

In the induction phase of *P. pastoris* cultivation, methanol concentration in the broth has vital influence on cell growth and protein production. High level of methanol concentration can be toxic to cells [43], while low level usually results in slow cell growth and may not enough for efficient AOX induction [36]. Presently, coupling online methanol sensor with feed-back controller has enabled the accurate and reliable control of methanol concentration at constant levels throughout the induction phase.

Some online techniques are simply based on modification of typical off-line analysis systems, such as HPLC and gas chromatography, i.e. combining them with automatic sampling and sample pretreatment. The Sequential Injection Analysis is an example, with capability to measure seven samples per hour with 4% standard deviation [44]. However, these methods create large time delay and can be expensive. In addition, continuous extraction of samples increases contamination probability.

Currently, a common method for methanol monitoring is using an organic vapour sensor, which is usually placed in off-gas. Based on liquid gas equilibrium, this method can be used to measure methanol concentration in the broth, although it requires non-linear calibration and has long response time (ca. 5 min.) [45]. Such methanol sensors have become commercially available and relatively inexpensive. Application of this method has been studied in several papers [24, 46-53].

Fourier Transform Mid-Infrared Spectroscopy (FTIR) proved to be an efficient tool for off-line measurements of methanol concentration in *Pichia* cultivations [54]. Schenk and coworkers applied FTIR to online analysis of methanol concentration in the broth: during cultivation, broth was continuously pumped from the reactor bottom, passing through a noninvasive FTIR/ATR cell for data acquisition, and then going back to the reactor [45]. Off-line HPLC control analysis showed a good agreement with on-line FTIR data, with standard error of prediction values <0.12 g/L.

1.4.2 Protein production monitoring

Methods for qualitative or quantitative determination of the production of recombinant protein include bioactivity assay, ELISA, SDS-PAGE and western blot coupled with band density scanning. However, all of these methods are time-consuming, not to mention in-situ measurement. Moreover, some of them have special requirements. For example, a mature and reliable procedure is required for bioactivity assay and specific antibodies are required for western blot. An alternative method for protein production monitoring has been proposed: fusing green fluorescence protein (GFP) to heterologous protein as a signal protein. Cha and coworkers applied this strategy to intracellular expression Human interleukin-2 (hIL-2) with *P. pastoris* and demonstrated that the use of GFP fusion facilitated identification of fusion protein expression and, more importantly, the quantification of hIL-2 [55]. Zhang and Yang used the same strategy for the intracellular production of glutathione *S*-transferase (GST) and monitored the expression by using a 96-well microtiter plate and simple fluorescent scanning [56]. However, in the secreted-expression system of *P. pastoris*, quantitative relation between GFP fluorescence and target protein level was not observed [57]. Presently, no report of using this strategy for the monitoring of protein production at high cell density cultivation of *P. pastoris* was found.

1.4.3 Biomass monitoring

Biomass concentration is a direct indication of the state of cell growth. But in-situ measurement of biomass concentration still remains a challenge despite that many methods have been invented and studied for decades. Such methods include dielectric spectroscopy, optical density, infrared spectroscopy, and fluorescence. More detailed information about these techniques can be found in a review by Kiviharju and coworkers [58]. Multi-wavelength fluorescence spectroscopy is a promising method to monitor bioprocess because it is capable of detecting multiple variables at the same time. Surribas and coworkers applied multi-wavelength fluorescence spectroscopy combined with PLS to the monitor of the fed-batch cultivation of *P. pastoris* producing Rhizopus oryzae lipase [59]. Quantitative prediction of biomass and substrate evolution profile was made in both biomass accumulation phase and induction phase. The production of heterologous protein was satisfactorily estimated in the exponential growth phase but not in the stationary phase when proteolytic degradation occurred.

If direct measurement of biomass concentration is not available, indirect estimation can be achieved by model-based prediction. Classical mechanistic models based on mass balances are not always the best choice for this purpose, because they require the detailed knowledge about the process dynamics and biochemical reaction kinetics, which are not always available. In that case, data-driven models (such as artificial neural networks) might be more applicable. With inputs such as the oxygen and the carbon dioxide concentrations in the off gas, these data-driven models can provide very accurate biomass estimation [60].

1.5 Control of proteolytic degradation

There is no doubt that one of the largest defects of *P. pastoris* expression system is the existence of proteolysis. The seriousness of proteolysis varies with respect to the specific foreign protein. Some researchers may not even notice the existence of proteolysis in their system, while in some cases, proteolysis can be so serious that no intact recombinant protein is present.

Generally, proteases responsible for the degradation of secreted recombinant protein come from extracellular proteases, cell bound proteases and intracellular proteases. The mechanism underlying the production of extracellular proteases in *P. pastoris* is not well studied. Sinha and coworkers detected bioactivity of four prominent vacuolar proteases, PrA, PrB, CpY and aminopeptidase in the supernatant of *P. pastoris* culture [61]. They also found that intracellular or extracellular protease levels and cell mortality were higher using methanol as carbon source than using glycerol. Therefore, methanol metabolism is responsible for release or misdirection of proteases of vacuolar origin to the fermentation medium, causing degradation of secreted proteins. It is generally assumed that high mortality of cells during induction period is caused by oxidative stress imposed by reactive oxygen species (ROS), such as H_2O_2, an intermediate of methanol metabolism. Xiao and coworkers applied a means of Flow cytometry to test cell viability and intracellular ROS levels of samples taken across the whole process of *P. pastoris* fed-batch cultivation and the loss of cell viability in induction phase was found to be correlated with accumulation of intracellular ROS level [62, 63]. Thus, the whole picture may be: continuous utilization of methanol causes accumulation of intracellular ROS; high oxidative stress elicits cell damage and cell lysis, which results in the release of intracellular proteases, mainly of vocuolar origin.

Total protease activity in broth supernatant can be measured by some methods using casein as substrate [64, 20]. Several reports showed the evolution of total protease activity throughout the whole fed-batch cultivation [20, 31, 61, 63]. Wang and coworkers reported a profile: there was no observed protease activity during initial growth on glycerol; upon transition of cells from glycerol growth phase to methanol induction phase, protease activity spiked; the observed protease activity finally dropped down to the baseline over the time course of induction [65]. But Wu and coworkers had a different result: the level of total protease activity increased with fermentation time throughout the whole process [31]. Kobayashi and coworkers found that there was no protease activity detected in the broth until about 100 h, after which a dramatic increase in protease activity and substantial degradation of target protein were recorded [20]. Further examination of concentration of medium components suggested that this phenomenon was caused by nitrogen starvation [20].

The hazards of proteolytic degradation are straightforward, the reduction of product yield or product bioactivity and contamination of the product by degradation intermediates. A lot of strategies have been studied to eliminate or reduce proteolytic degradation. One of the most effective way is to use protease-deficient strains, such as SMD1165 (his4 pep4 prb1), SMD1163 (his4 pep4) and SMD1168 (his4 pep4). Proeteolytic degradation can also be alleviated by modifying the amino acid sequence of the expressed protein. Zhang and coworkers reported that changing the N-terminal sequence protects recombinant Plasmodium falciparum circumsporozoite protein from degradation in *P. pastoris* [66]. Here we will focus more on strategies at cultivation level.

1.5.1 pH

Manipulating pH is an effective tool to reduce the proteolytic degradation of heterologous protein. However, some findings of the effects of pH upon proteolytic degradation are controversial. Two articles reported that at pH 6.0 the proteolytic activity in the supernatant and foreign protein degradation was substantially reduced compared with lower pH [20, 61]. However, Wang and coworkers found that the background protease activity is lower at lower pH [65]. To be noted, Wang and coworkers also found that the optimum pH for MSP3 production is 6.8, although the protease activity in supernatant is high compared to lower pH of 3.0-6.0. This means that the improvement of MSP3 production at pH of 6.8 has little to do with protease activity. As an important factor, pH is always chosen to be optimized.

1.5.2 Temperature

A lot researches pointed out that culture temperature has great effect on the production of recombinant protein as well, especially the temperature during induction phase. Low induction temperature strategy has been proved to be worth trying for optimization purposes. Li and coworkers decreased culture temperature from 30°C to 23°C, leading to 3 folds improvement in the production of herring antifreeze protein [67]. The fact that low induction temperature improves the production of recombinant protein may be explained by: lower temperature increases the stability of recombinant protein or inhibits protease activity. Jahic and coworkers proved that lower induction temperature decreases protease activity and cell lysis (which leads to the release of intracellular proteases) [34].

Similar observation was obtained by Wu and coworkers [31]. By applying a temperature limited fed-batch, Jahic and coworkers improved target protein production by 100% and no proteolytic degradation was observed [68]. A low temperature induction strategy was found to be effective in the production of Nef protein, although the protease activity under lower temperature induction was similar to that under regular temperature of 30°C [32]. Thus, it is possible that low induction temperature improves protein production by inhibiting protease activity, not by reducing protease production. However, this strategy is not always effective. Wang and coworkers found that lower induction temperature did not improve MSP3 production, although its production was affected by adjusting pH [65].

1.5.3 Nutritional supplementation

Although capability of growing upon defined medium, such as BSM, is an advantage of *P. pastoris* system, severe degradation of heterologous protein is usually found to follow, especially in high cell density situation. Depletion of certain nutritional component is a possible cause of increased proteolytic activity. Kobayashi and coworkers found that a dramatic increase in protease activity in the later phase of induction was caused by nitrogen depletion when using a defined medium and such phenomenon was overcome by increasing the initial nitrogen source concentration [20]. Enhanced protein production was often observed by using of complex medium or supplementation of amino acid-rich components (e.g. peptone, casamino acid) [69, 70, 71, 72]. Using complex nitrogen source can not only prevent nitrogen depletion, but inhibit protease activity by acting as excess competing substrates. As we discussed previously, methanol metabolism tends to leave cells in the oxidative stress, which may account for reduced cell viability and the release of intracellular proteases. Xiao and coworkers found that by adding an antioxidant, ascorbic acid, during the induction phase, cell mortality and proteolysis of target protein, can be reduced significantly [63].

1.5.4 Inhibitors supplementation

Another way to reduce proteolysis is to add protease inhibitors. Shi et al. identified the presence of aspartic, cysteine and serine-type proteases in *P. pastoris* culture producing a single-chain antibody [72]. The addition of a serine protease inhibitor resulted in 53% reduction of total protease activity and 30% reduction was obtained when an aspartic protease inhibitor was used. The effect of more inhibitors (PMSF + EDTA + E-64 + Pepstatin) and their combination was studied by Sinha et al. in the production of recombinant ovine interferon-H with *P. pastoris* [61]. It was found that total protease activity was reduced by 78% when adding PMSF (1mM), by 45% when adding EDTA (1mM) and a combination of EDTA (1mM) and PMSF (1mM) can reduce protease activity by 94.2%. However, addition of protease inhibitors may not be applicable in industrial applications.

1.6 Fed-batch cultivation under AOX1 promoter

Generally, there are three operational modes for microorganism cultivation: batch, fed-batch and continuous. Regular batch cultivation is easy to operate, but has a low productivity. Fed-batch cultivation is the most commonly used method for *P. pastoris* cultivation, because it can achieve very high cell density and it is relatively easier to control than continuous cultivation. Fed-batch process under AOX1 promoter can be divided into three phase: a glycerol batch phase, a glycerol fed-batch phase and finally a methanol induction phase.

The combination of the first two phases is also called biomass production phase because the main purpose of them is to obtain adequate biomass before methanol induction. Glycerol batch phase usually starts with 40g/L glycerol, because higher glycerol concentration may inhibit cell growth [3]. Glycerol batch phase takes about 24 hours. After the consumption of initial glycerol, indicated by a sudden increase in the dissolved oxygen, glycerol fed-batch phase starts. Constant rate of glycerol feeding can be used as suggested in the Invitrogen *pichia* fermentation protocol [16]. Exponential feeding profile can be used to get exponential growth and avoid glycerol accumulation. Cell growth of *P. pastoris* upon glycerol is supposed to be independent of AOX phenotypes and is much faster than growth upon methanol. Meanwhile, in the first two phases, the activity of AOX1 promoter is completely depressed at the presence of only glycerol. Thus, no products were induced. The length of glycerol fed-batch depends on the amount of glycerol to be input or in other word, the biomass concentration prior to methanol induction phase. Consumption of more glycerol takes more time, but higher biomass concentration may result in higher protein production rate.

Between biomass production phase and methanol induction phase, a transitional phase can be employed to smooth cells adaptation. Some researchers recommended co-feeding glycerol and methanol for a certain period of time, during which the content of glycerol decreases while methanol's increases [36, 47, 73]. Jungo and coworkers proved that after growth on glycerol use of mixed feeds of glycerol and methanol allowed faster induction of alcohol oxidase and faster adaptation of cellular metabolism than with a feed containing methanol as sole carbon source [74]. Chen and coworkers found that replacement of culture medium with fresh medium prior to methanol induction significantly improved phytase expression, probably because of the removal of repressing glycerol and metabolic wastes [75].

In this phase, high concentration of methanol solution can be used while pure methanol is often used directly. This is the phase when foreign protein production is induced. Nearly all state variables are found to be related with protein production or proteolytic degradation. An important topic in this phase is the control of methanol feeding, which also dictates residual methanol concentration and specific growth rate. Subsequently, the methanol feeding strategy is one of the most important factors for maximizing heterologous protein production [3]. The length of induction phase depends on specific conditions. Bradly and coworkers found that the production of recombinant protein is not only associated with specific growth rate, but also with the induction time upon methanol [76]. They found that the production of rOvIFN-τ started to decrease after a certain period of induction time, referred as decoupling time, which was independent of initial cell density before induction.

1.7 Modeling of cultivation process

Modeling is an important tool in process engineering. It not only enhances our understanding of the process mechanism, but also plays a vital role in process analysis, control and optimization. Growth kinetics is an important topic in such bioprocess as *P. pastoris* cultivation, especially the growth kinetics upon methanol. The Monod equation is the most commonly used to describe growth kinetics. However, the resulting Monod's equation described in literature is quite different. For example, the value of maximum specific growth rate $\mu_{\max}$ ranges from 0.046 to 0.16 [3].

It is generally accepted that growth inhibition is observed at high methanol concentration. Zhang and coworkers [47] found that the specific growth rate (μ) upon methanol increased with the increase of methanol concentration (S), until a critical level was reached, and further increase of methanol concentration led to decline of specific growth rate. They fit these data to an uncompetitive inhibition growth model as followed:

$$\mu = \frac{\mu_{\max} S}{k_s + S + S^2 / K_I} \tag{2}$$

Based on critical level of methanol concentration, cell growth can be divided into two phase: growth limited region (μ increases with S) and growth inhibited region (μ increases with S).

Jahic and coworkers formulated a kinetic model to describe both cell growth and oxygen consumption in fed-batch cultivation of *P. pastoris* [77]. In this model, specific methanol uptake rate was obtained directly based on methanol concentration with Monod's Equation and specific growth rate was derived by the difference between specific methanol uptake rate and maintenance. To relate oxygen consumption to growth kinetics, the flux of methanol was divided into two branches, one for cell growth, and the other for energy production; different oxygen consumption rate was assigned to each branch.

For simplicity, constant cell composition or state is assumed, i.e. unstructured model is used. But for more accurate description of cell's non-linear behaviour, structured model can be employed. A simple structured model was proposed by Li and coworkers for the modeling of fed-batch cultivation of *P. pastoris* to produce IDSHr protein [78]. In this model, biomass is divided into two parts, viable fraction and dead fraction. The viable fraction is further divided into 3 departments: intracellular substrate, peroxisomes and the remaining. The main characteristic of this model is to assume that the cell viability loss and generation of protease is caused by the oxidative stress phenomena. It is supposed that specific rates of cell viability loss and proteolytic activity generation are proportional to specific methanol uptake rate.

Taking into consideration metabolic pathways of glycerol and methanol, a macrokinetic model for *P. pastoris* is established by Ren and coworkers [79]. In this work, the main frame of the stoichiometric model for *S. cerevisiae* was applied for *P. pastoris*. Balances of carbon source, ATP and NADH, were taken into account during glycerol and methanol metabolism. The specific uptake rates of glycerol and methanol took the form of Monod's equation. In order to model the lag phase of growing on glycerol, a metabolic regulator model was used. Similar regulator model was also used for the description the initial lag phase for protein production.

The morphological feature of cell growth of *P. pastoris* is similar to that of *S. cerevisiae*, both undergoing budding. Experimental data indicated that heterologous proteins produced by *S. cerevisiae* were mainly secreted during the late G2 and M phases of growth [80]. Although the relationship between foreign protein production and cell cycle phase in *P. pastoris* is uncertain, Jia and Yuan have proposed a cell cycle model to model the budding cell distribution during fed-batch cultivation of *P. pastoris* on glycerol [80]. Experimental results showed that budding cell fraction increased with the increase of specific growth rate and this phenomenon was simulated well by the cell cycle model coupled with a macrokinetic model and a bioreactor model. However, this model was unable to describe cell cycle distribution in the methanol induction phase, when multi-bud phenomenon was observed.

Cultivation conditions such as pH, temperature and dissolved oxygen level have significant influences on cell growth and protein production as well. Some researchers have constructed models to describe such underlying relations in *P. pastoris* cultivation. Kupcsulik and Sevella formulated an empirical parabolic statistical model to describe the relation between specific product formation rate and pH and temperature [81]:

$$\mu_P = \mu_{P_0} \frac{K_1[H^+]}{K_1K_2 + K_1[H^+] + [H^+]^2} \left\{ a \cdot \exp(\frac{-\Delta G_1}{RT}) - b \cdot \exp(\frac{-\Delta G_2}{RT}) \right\} \qquad (3)$$

Parameters in this model were evaluated by fitting to experimental results. The optimal condition predicted by the model is pH 5.64 and temperature 20.24°C.

Liang and Yuan established an oxygen transfer model for recombinant protein production by *P. pastoris* upon glycerol and methanol [82]. This model relates the oxygen transfer rate to the agitation speed, aeration rate, dissolved O_2 concentration, medium volume and medium temperature, while the oxygen uptake rate is determined by a macrokinetic model proposed by Ren et al. [79]. Assuming that oxygen transfer rate is in balance with oxygen uptake rate, this model is able to estimate biomass concentration given agitation speed, aeration rate, dissolved oxygen level and other state variables. Excellent prediction can be achieved by coupling the model with a rolling updating approach of the parameters.

In some cases, data-driven models, such as artificial neural network, are more applicable than classic mechanical models that are based on mass balances. Data-driven models are capable of predicting process variables with global measurement variables such as the oxygen and the carbon dioxide concentrations in the off gas. Jin and coworkers developed a artificial neural network pattern recognition (ANNPR) model for the online adaptive control of methanol feeding based on DO and pH measurements in phytase production using *P. pastoris* [26]. The model can predict the state of the process state: "substrate starvation" or "substrate in excess" and a coupled controller then adjusts feeding rate correspondingly. Experimental results showed that the model-based controlling leads to an approximate three-fold stable increase in phytase production compared with those obtained by the traditional DO-Stat method and the online methanol electrode-based on–off control strategy.

1.8 Fed-batch control strategies

Methanol is the substrate for cell growth and the inducer for heterologous protein production in induction phase, which is often characterized with high cell density and intensive oxygen consumption. The control of methanol feeding is the most important and the most commonly studied issue in the process engineering of *pichia* cultivation, since it has direct influences on production & proteolytic degradation of heterologous protein, cell growth & cell lysis, and the balance of oxygen transfer. A lot of strategies have been proposed and implemented to control the feeding of methanol with the purpose of maximizing protein production or enhancing process reproducibility. Methanol feeding strategies include: DO-stat, constant specific growth rate feeding (μ-stat), constant methanol concentration feeding (CMCF), oxygen limited fed-batch (OLFB), and temperature limited fed-batch (TLFB).

1.8.1 DO-stat

Constant or step feeding strategy is proposed in *Picha* Fermentation Process Guideline provided by Invitrogen Co. [16]. According to the description, feeding of 100% methanol starts at 3.6mL (per liter of initial volume) for 2-4 hours, then switches to 7.3mL for 2 hours and finally set to 10.6mL throughout the process. The advantage of constant feeding strategy is its simplicity. But its problems are also straightforward: it can lead to over-accumulation of methanol concentration to a toxic level or it can go to the other end, severe starvation.

Generally, unlimited methanol supply in high cell density situation will lead to oxygen limitation if oxygen enrichment is not applied. It was reported that oxygen limitation negatively affects foreign protein [36] and dissolved oxygen level was critical for cell to grow to high cell density [46]. Therefore, on one side, different methods was employed to accelerate oxygen transfer as we discussed earlier; on the other side, cell growth was limited in order to avoid oxygen limitation. An operational way is to limit cell growth by controlling methanol feeding rate to keep dissolved oxygen concentration constant, i.e. DO-stat control.

In early applications of this method, methanol feeding was controlled manually based on DO profile. For example, Rodríguez Jiménez and coworkers applied DO-stat control to the cultivation of a Mut^s strain of *P. pastoris*, producing dextranase [83]. They adjusted the methanol flow in order to maintain the decreasing tendency of the DO between 20% and 15% of saturation. Similar manual control strategy was applied by Woo and coworkers to the production of the short version of human leukotactin-1 (shLkn-1) using *P. pastoris* [84]: methanol feeding rate was reduced when the low limit of DO profile reaches the low set point; reversely, when the low limit is well above the low set point, the feeding rate was increased. Lim and coworkers reported another DO-stat control strategy for the production of rGuamerin [85]: when the DO was below DO_{low}, methanol feeding rate was decreased by 0.5%, and when DO was above DO_{up}, methanol feeding rate was increased by 1%; these loops were executed every 30 s.

Manual control of methanol feeding to realize DO-stat is tedious and severe fluctuation of DO profile was often observed [85]. Some authors applied simple "on-off" controller based on DO-stat: feeding of glycerol or methanol commenced when the DO tension increased to the set value and continued until the DO tension decreased to the set value [86]. The performance of such on-off DO controller is often not satisfactory. In order to improve the performance of DO-stat control, automatic controllers based on feed-back control have been developed. Olivereira et al. employed an adaptive dissolved oxygen control to adjust glycerol feeding in fed-batch cultivation of *P. pastoris* [87]. Chung designed a feed-back controller in theory and discussed the tuning of controller based on Bode stability criterion [88].

With DO-stat control, methanol feeding is not sufficient and cells sometimes experience substrate starvation. It is observed that residual methanol concentration fluctuated below 0.5g/L with DO-stat control [89]. Therefore, DO-stat strategy often results in slow cell growth and lower productivity. DO-stat strategy has another problem: if an inhibitory level of methanol is reached, a sharp increase in dissolved oxygen will be observed; however, DO-stat controller will make a response by increasing the feeding rate further, resulting in a vicious circle.

1.8.2 Constant specific growth rate feeding (μ-stat)

In order to limit cell growth and maintain DO, the feeding of methanol can be adjusted according to a predetermined feeding profile, usually designed to keep specific growth rate (μ) constant. Maintaining constant μ will not only enhance process reproducibility, but also facilitate the study of the effects of μ. The maximum specific growth (μ_M) is about 0.14 for Mut$^+$ *P. pastoris* [90] and about 0.015 for Mut^s *P. pastoris* [91] when growing upon methanol. The effect of μ upon protein production has been studied by many researchers with chemostat and fed-batch process. Chemostat cultivation indicated that heterologous protein production is considered to be mainly growth associated. Highest volumetric productivity of foreign protein is usually observed at high dilution rate, while highest yield of recombinant proteins is often achieved at low dilution rate [3]. To maintain cell growth at constant μ in fed-batch process, a regular method is to design a feeding rate profile derived based on kinetic models. Zhang and coworkers constructed an unstructured kinetic model with maximum specific growth rate $\mu_m = 0.0709$ 1/h and based on the model, an exponential methanol feeing profile was designed to keep cell growth below μm [47]. Maximum protein yield was achieved at $\mu = 0.0267$ 1/h. With the assistance of a macrokinetic model developed previously, Ren and Yuan studied the effects of μ in fed-batch cultivation for the production of recombinant human serum albumin [92]. They found that the most productive value of μ was between 0.005 and 0.006, which yielded protein higher than 5g/L at 160h. Furthermore, they demonstrated that the strategy of μ-stat was better than constant feeding. Sinha and coworkers modeled cell growth upon methanol with a substrate-feed equation for the production of recombinant ovine interferon-τ and use the model for effective control of the process [93]. The optimal μ was found to be 0.025 (1/h).

As we know, μ-stat is an open loop control strategy. The major problem with this strategy is the robustness and stability of process. With a different initial condition or a big disturbance during the process, over-accumulation of methanol could occur. Furthermore, to increase the robustness of this method, μ is usually controlled at a set point fairly below μ_m, resulting in a low productivity.

1.8.3 Constant methanol concentration feeding (CMCF)

DO-stat and μ-stat often results in limited cell growth or methanol limited fed-batch process. However, the methanol concentration is not measured online. Obviously, to improve process stability and robustness, online monitoring and control of residual methanol concentration is required. Moreover, methanol concentration has dramatic effect on cell growth and protein production. Since methanol induces protein production, complete depletion is not desired in the induction phase; however, attention should be paid to avoid methanol accumulation to inhibitory levels. Inhibitory level of methanol reported in the literature varies from 3.7g/L [46] to 20g/L [17]. Optimum methanol concentration for protein productivity varies as well: 2g/L [47], 3.5g/L [48] and 1g/L (Mut^s phenotype) [91].

As we described earlier, several methods have been successfully used in the online monitoring of methanol concentration. To control methanol concentration, different closed-loop control coupling with specific methanol sensor has been used. Some work has been published using simple "on-off" controller [3]. However, the performance of such controller is poor since it always results in fluctuation of methanol concentration [94]. Some authors showed that inconstancy of methanol concentration has negative effects, as higher productivity was achieved with constant methanol concentration than with fluctuating concentration [50, 73].

Zhang and coworkers applied the classic PID control algorithm to improve the control of methanol concentration in *P. pastoris* growth upon methanol [94]. The three control parameters, K_C (controller gain), τ_I (integral time constant) and τ_D (derivative time constant) were tuned with frequency response analysis and Bode stability criteria. In the tuning process, optimal settings of K_C, τ_I and τ_D depend on the specified value of biomass concentration and broth volume. Fortunately, according to the stability rule derived from Bode stability criteria, the control system with optimum settings derived from early state will still be stable as the fed-batch fermentation goes on. The robustness of the controller and stability of the settings were confirmed when it was applied to the fermentation processes with four strains of *P. pastoris*, each producing a different foreign protein. Cos and coworkers designed a predictive control algorithm coupled with a PI feed-back controller to control methanol concentration in the cultivation of a Mut^s strain of *P. pastoris* [91]. The predictive model requires the first time-derivative of methanol concentration as an input. The control parameters of the PI controller, K_P (Proportional gain) and τ_I (integral time constant) were given and fixed throughout the cultivation. However, the tuning process of the two parameters was not provided and their influence on control stability was not discussed either.

1.8.4 Temperature limited fed-batch (TLFB)

Surribas and coworkers showed that during ROL production with Mut$^+$ phenotype, methanol non-limited concentrations should be kept in the induction phase to obtain satisfactory yields and productivities [95]. However, rapid cell growth and early oxygen limitation can be expected under this condition. Unlike DO-stat and μ-stat, there is another control strategy which can keep both oxygen and methanol concentrations at sufficient levels, that is, temperature limited fed-batch (OLFB). Its principle is: methanol concentration was controlled at constant level with the same methods in CMCF control, and when oxygen limitation occurs, culture temperature was adjusted to maintain DO at the set point. In this strategy, cell growth is limited by temperature not by methanol or dissolved oxygen. The extra benefit of this strategy is that lowering culture temperature usually helps to inhibit proteolytic degradation as we described in the chapter of 'Control of proteolytic degradation'. Jahic and coworkers were the first to apply this strategy [68]. Constant methanol concentration of 300mg/L was maintained until temperature dropped 12 °C, which is the system limit. After that temperature was fixed and DO was maintained by lowering the feeding rate. Compared with ordinary DO-stat control, this TLFB strategy resulted in 100% improvement of foreign protein production, lower cell mortality and complete inhibition of proteolytic degradation. It was interesting that the specific alcohol oxidase (AOX) activity in the TLFB process was 3.5 times higher than in the DO-stat. Sirén and coworker applied TLFB strategy for the production of the HIV-1 Nef (negative factor) with *P. pastoris*, also resulting in great reduction of proteolytic degradation [32].

1.8.5 Oxygen limited fed-batch (OLFB)

It is generally believed that oxygen limitation is not desirable in the induction phase. Actually, cell responses are not well studied when growing *P. pastoris* upon methanol. In the studies of Lin and Cregg [36], oxygen limitation negatively affected the expression of foreign proteins. Trentmann et al. [96] found that the recombinant scFv protein quality and productivity were higher in the oxygen limited processes than in methanol limited processes. While avoiding oxygen limitation has been given priority, successful protein production has also been achieved under oxygen-depleted conditions [24].

Oxygen limited fed-batch (OLFB) is a modification of CMCF. In OLFB, residual methanol concentration is kept constant like in CMCF, but DO is not controlled with any measure. Thus in OLFB, DO always drops to 0% due to oxygen limitation, which increases the driving force of oxygen transfer. This feature makes OLFB very attractive for industrial application. Charoenrat and coworkers compared OLFB with DO-stat in the cultivation of *P. pastoris* for the production of recombinant Thai Rosewood β-glucosidase [42]. Due to the higher driving force for oxygen transfer in the OLFB, the oxygen and methanol consumption rates were about 40% higher in the DO-stat control. It was found that the obligately aerobic *P. pastoris* did not respond to severe oxygen limitation with major negative responses but just increased maintenance demand.

Compared with DO-stat control, OLFB resulted in higher cell density and higher protein production. The feasibility of OLFB is further confirmed by Khatri and Hoffmann [40, 97]. They also found that the production of foreign protein increased with the increase of methanol concentration in OLFB.

1.8.6 Comparison of different strategies

The selection of methanol feeding control strategies shows a firm dependency on the specific system, including the strain, the foreign protein, the equiopment, etc. Following are some cases that compared the performance of different strategies in the same system.

Trinh and coworkers evaluated three different strategies for recombinant endostatin production [51]: 1. CMCF at 3.0g/L methanol; 2. DO-stat; 3. μ-stat at μ=0.02 1/h. Total protein production was similar in all three strategies but the final protein concentration in the third strategy is much higher due to lower methanol addition. So in this case, μ-stat is more efficient.

Yamawaki and coworkers compared two feeding strategy in the Production of Single-Chain Variable Fragment Antibody (scFv) [86]: DO-stat and CMCF at 3.9g/L methanol. A higher concentration of scFv was obtained with CMCF than with DO-stat and increasing residual methanol concentration led to decrease of scFv production. However, the highest scFv production rate was obtained by applying a hybrid strategy of DO-stat and CMCF: use CMCF at 15.7g/L methanol in the first five hours, followed by DO-stat.

Surribas and coworkers evaluated three methanol feeding strategies for the Production of a Rhizopus oryzae lipase from *P. pastoris* [95]: 1. CMCF at 3.0g/L methanol, followed by DO-stat in response to oxygen limitation; 2. TLFB; 3. CMCF at 3.0g/L methanol, followed by TLFB. The best production, volumetric productivity and specific productivity were reached with Strategy 1, however important operational problems (oxygen supply and excessive foam formation) and a more contaminated product due to high cell lysis were detected. Strategy 2 resulted in lowest volumetric productivity despite great improvement of cell variability. Operational problems and product purity shortcomings were minimized with Strategy 3.

1.9 Continuous cultivation

Continuous cultivation is an alternative to fed-batch cultivation in order to achieve high productivity. Continuous cultivation can be divided into three phases as well: a glycerol batch phase, a glycerol fed-batch phase and finally a continuous feeding phase. The first two phases are the same with those in the fed-batch cultivation. In the third phase, continuous input of feeding substrate and salt medium and continuous withdrawal of broth are performed in the same time to keep broth volume constant. Due to elongated protein production time and reduction of down time, continuous cultivation often has a higher volumetric productivity as compared to fed-batch cultivation. Furthermore, continuous cultivation provides more uniform product quality and enhanced process reproducibility, and reduces the exposure of product to proteases. In addition, given enough time, continuous cultivation will reach certain steady state, at which the composition of the broth will remain constant. This feature makes continuous cultivation an effective tool to study cell growth and protein production.

The most important process parameter in continuous cultivation is dilution rate, which is equal to specific growth rate at steady states. Many studies on continuous cultivation aimed to reveal the relationship between protein production and dilution rate at constant feeding medium. For either Mut^{s} strains [98] or Mut^{+} strains[86, 99], it is generally found that final foreign protein concentration decreases with the increase of dilution rate, while volumetric productivity of foreign protein increase with the increase of dilution rate. However, exceptions were found: the maximum volumetric productivity was found at a moderate value of dilution rate $D = 0.0333$ (1/h) in the continuous cultivation for the production of interferon-τ [100]. Nakano and coworkers found that the volumetric productivity of α-amylase decreased with increases in dilution rate, when the continuous feeding was controlled by DO-stat [101].

Besides dilution rate, the production of foreign protein is supposed to be affected by cell density as well. However, this effect is not examined in most of published work. Zhang and coworkers used continuous cultivation to study the influence of cell density upon protein production for the first time [100]. According to their results, protein production was strongly and jointly affected by both dilution rate and cell density. With surface response methodology, the maximum volumetric productivity was found at cell density of 328.9 g/L (WCW) and dilution rate of 0.0333 (1/h).

In protein production phase, cell growth upon methanol as the sole carbon source is very slow, especially for Mut^s phenotype. One way to improve cell growth rate and protein productivity is to use mixed substrate co-feeding as in fed-batch cultivation. However, Boze and coworkers found that using sorbitol and methanol as mixed substrate in continuous cultivation did not necessarily increase protein yield and productivity compared with using methanol only [22].

In terms of volumetric productivity, the advantage of continuous cultivation was confirmed by several studies. Boze and coworkers, who studied the production of porcine follicle-stimulating hormone (rFSH) with continuous cultivation of a Mut^s strain, obtained a rFSH concentration and a productivity of respectively 3.7 and 6.4 times higher than those in fed-batch cultivation [22]. D'Anjou and Daugulis demonstrated that the continuous cultivation of a Mut$^+$ yielded a higher productivity than that with a fed-batch system for the production of a sea raven antifreeze protein [98]. Curves and coworkers found that the productivity of Chymotripsinogen B using a Mut$^+$ strain in continuous cultivation at a dilution rate of 0.072 (1/h) is 4.7 times higher than that in fed-batch [102].

For continuous cultivation, GAP expression system may be a better choice. Goodrick and coworkers described the first report of continuous cultivation of *P. pastoris* for the production of h-chitinase under GAP promoter. Compared to fed-batch system, the continuous one provided not only five to six folds enhancement in protein production rates but also a more intact product [103]. They proved that GAP system enabled longer production period: high level production of foreign protein lasted for 30 days. Their further studies also proved the feasibility of using this system in large scale applications [104].

Another application of continuous cultivation is to serve as a platform to study cell growth and protein production metabolism. Solà and coworkers used continuous cultivation to metabolic flux profiling of *P. pastoris* grown on glycerol/methanol mixtures at low and high dilution rates [105]. Jungo and coworkers found that the deficiency of biotin led to wash-out phenomena in continuous cultivation, proving the need of biotin for cell growth of *P. pastoris* [106]. Jungo and coworkers applied a transient continuous cultivation methodology to obtain the best ratio of sorbitol and methanol in mixed substrate, which was then applied to the fed-batch process [107].

1.10 GAP promoter

Constitutive expression of heterologous proteins can be realized by using the glyceraldehide 3-phosphate dehydrogenase (GAP) promoter in *P. pastoris* upon glucose or glycerol. In recent years, this promoter is becoming more and more popular. Compared with AOX1 expression system, GAP promoter has some attractive advantages. Firstly, it rules out the use of methanol, which is potentially hazardous in aerobic production environment. The hazard and cost associated with the storage and delivery of methanol in large volume is also a big concern for large scale production [103]. Unlike AOX1 system, with which protein production in induction phase under controlled conditions is required, GAP expression system does not require such critical control [108]. Stable and efficient continuous cultivation using GAP promoter can be achieved with longer production period. Goodrick and coworkers established a continuous cultivation with GAP expression system and efficient expression of foreign protein lasted for one month [103].

In general, the fed-batch cultivation of *P. pastoris* under GAP promoter can be divided into two phases: batch phase and fed-batch phase. Glucose or glycerol is usually chosen as the sole carbon source. There is no absolute conclusion about which one is better: some found that glucose is better [109, 110]; others found that glycerol is better [111, 27]. To be noted, these comparisons were done either in shake flasks or batch mode with different media. Formulas of defined salt medium in this system can be referred to Table 1.1. In the fed-batch phase, the feeding solution is generally high concentration substrate solution supplemented with trace salts, while some authors also put essential salts into feeding solution [112].

Some studies have been done to study the kinetics of cell growth and protein production under this promoter. Khasa and coworkers studied the kinetics of this system with continuous cultivation of *P. pastoris* expressing human granulocyte-macrophage colony stimulating factor (hGM-CSF) [113]. They found that the concentration of target protein and cell biomass increased with the increase of dilution rate and therefore, specific protein production (r_P) rate increased with dilution rate (D) as well, and more aggressively than linear-increase. Notably, their medium used was not defined medium, but YPDCA medium, a complex medium. According to their findings, cell growth should be controlled close to maximum specific growth rate to achieve maximum production and maximum productivity. Similar continuous cultivation was done by Maurer and coworkers and they also found r_P increased with D, but less aggressively than linear-increase [112]. The relationship between r_P and D can be fit to an equation like Monod's equation. Zhao and coworkers used μ-stat feeding strategy to study the fed-batch cultivation of *P. pastoris* expressing *Candida rugosa* lipase with GAP promoter [114]. They found that a relatively low μ (0.15) induced a high lipase activity peak, although the maximum μ value (0.25) brought to maximum cell density and lipase activity.

Foreign protein production under GAP promoter was also found to be affected by culture condition, such as pH, temperature and dissolved oxygen level. Zhao and coworkers found that the optimal pH and temperature for the production of *Candida rugosa* lipase were 6.5 and 26°C [114]. Pal and coworkers found that the optimal pH and temperature for the production of hGM-CSF were 5 and 30°C [110]. Chang and coworkers performed culture condition optimization with surface response methodology and found the best condition for optimum protein production is: temperature 24.2°C, glucose concentration 1.9%, yeast extract 1.5%, and pH 7.6 [115]. By applying hypoxic conditions to continuous cultivation of *P. pastoris* expressing an antibody Fab fragment under the GAP promoter, Baumann and coworkers obtained a 2.5-fold increase of the specific productivity (q_P) under low oxygen supply [116]. Meanwhile, biomass production decreased and ethanol was produced, indicating a shift from oxidative to oxido-fermentative conditions. Based on these results, they designed a feeding strategy, using a fed-batch controller to control the feeding of glucose by maintaining the ethanol concentration at approximately 1.0% (v/v). Using this strategy, the volumetric production rate was increased by 2.3-fold.

So far, many heterologous proteins have been successfully expressed under GAP promoter and the level of expression has been shown to vary significantly depending on the expressed protein. Several researchers have also reported that GAP promoter is more efficient than AOX1 promoter for protein expression [117, 5, 109]. However, other authors reported AOX1 is more efficient [7, 118].

Combination of AOX1 and GAP promoters to express protein has been proved to be an effective way to increase expression level [119, 1209, 121]. In this method, the cell host is sequentially transformed with 2 kinds of vectors, containing AOX1 promoter and GAP promoter respectively. The resulting strain is then capable of expressing constitutively and inductively. As we know, the expression of foreign protein is determined by many factors. Thus, in some cases, when the fermentation process is not optimized, the comparison of effects of different promoter is not quite persuadable.

1.11 Concluding remarks

The selection of medium is of vital importance to the success of microorganism cultivation. However, few reports have been found to study the optimization of medium for the culture of *P. pastoris*, or the influences of medium components. One barrier to do medium optimization for *P. pastoris* cultivation is that meaningful experiments should be done at high cell density level, requiring tremendous devotion of both money and efforts. This fact leads to the existence of a variety of media in the literature, although BSM plus PTM1 is most commonly used. In addition, since these media are mainly designed for the fed-batch cultivation under AOX1 promoter, medium optimization is needed to application of continuous cultivation and cultivation processes with other promoters.

P. pastoris is capable of rapid growth upon a wide range of cultivation conditions in terms of pH, temperature or DO. However, optimal conditions for recombinant protein production are relatively limited, since cultivation conditions have significant influences upon not only the expression of foreign proteins but also their stability, which, in the case of *P. pastoris* cultivation, is greatly affected by proteolytic degradation. A lot of studies have been found concerning the control of proteolytic degradation by different methods, most often adjusting pH and temperature. However, the influences of these methods showed high dependency upon the expressed protein. It is generally believed that aerobic condition is required for the cultivation of *P. pastoris*. The truth is that the effects of DO upon cell growth and recombinant protein production are not intensively studied. Some researchers found that oxygen limited fed-batch was a good methanol feeding strategy under AOX1 promoter. Some researchers also found that hypoxic condition (with low oxygen supply) led to great improvement of productivity under GAP promoter.

Process monitoring is the basis of effective process control. Despite great technology development in the past, biomass monitoring still remains a challenge, especially in high cell density situation, like the culture of *P. pastoris*. Most dramatic progress of process monitoring for *P. pastoris* cultivation is the development of methanol monitoring. Commercialization of methanol sensors has enabled the online measurement of methanol concentration in the broth and realized the control of constant methanol concentration coupled with feed-back controllers. Online monitoring of protein expression during fermentation processes is still impossible, although co-expression of green fluorenscence protein was proved to be effective in the monitor of target protein expression in small scale.

Different models have been reported in the modeling of *P. pastoris* cultivation under AOX1 promoter, like kinetic models and data-driven models, unstructured and structured models. However, few reports are concerning the modeling of recombinant protein expression under constitutive promoter, GAP.

Methanol feeding strategy is the most important factor to the expression of recombinant proteins, and is the one most commonly studied in the control of fed-batch process under AOX1 promoter. Several methods have been reported: DO-stat, μ-stat, CMCF, TLFB and OLFB. Each method has its own advantage and disadvantage and their performance showed great dependency upon the expressed protein as well.

There are a lot of studies concerning continuous cultivation of *P. pastoris*. Generally, under AOX1 promoter, the volumetric productivity increases with the increase of specific growth rate, while the yield of recombinant protein decreases with the increase of specific growth rate. However, exceptions were found in some cases. In addition, the production of foreign protein is affected by cell density as well. For the application of continuous cultivation, GAP promoter seems to be more suitable, for continuous expression of foreign protein under GAP promoter can last as long as one month.

Although GAP promoter is becoming more and more popular, as an alternative to AOX1 promoter, studies on the cultivation with this promoter are far from enough. Medium and cultivation conditions for cultivation with GAP promoter are usually borrowed directly from those with AOX1 promoter, although their differences are obvious. The control of substrate feeding with GAP promoter should be easier than that with AOX1 promoter, but few reports studied the effect of different feeding strategies. Kinetic data about cell growth and protein production with this promoter are limited as well. For effective process control of recombinant protein production under GAP promoter, more intensive studies will be needed.

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CHAPTER 2

Pichia pastoris Fermentation for Phytase Production Using Crude Glycerol from Biodiesel Production as the Sole Carbon Source

Abstract

Efficient utilization of crude glycerol, a byproduct from biodiesel production, could bring significant economic and environmental benefits. In this work, such a low-grade glycerol was used as the sole carbon source in phytase production with recombinant *Pichia pastoris* (*P. pastoris*) possessing a pGAP – based constitutive expression vector. In batch and fed-batch modes, the effects of important cultivation conditions were investigated using both analytical and biodiesel glycerols. The following factors were analyzed: initial glycerol concentration, dissolved oxygen level and the effect of feeding strategy. Significant cell growth inhibition was observed in batch fermentation when initial crude glycerol concentration was as high as 70 g/L. However, such inhibition was overcome in fed-batch mode by starting the cultivation with a lower crude glycerol level. Finally, cell densities and phytase activity levels of as high as 146 g (dry cell weight)/L broth and 1125 U/mL supernatant were achieved in the fed-batch fermentation with crude biodiesel glycerol. The study has proven the potential of using crude glycerol from biodiesel production as the carbon source for industrial scale phytase mass production in high cell density fermentation with recombinant *P. pastoris*.

Key words: Crude glycerol, biodiesel, phytase, *pichia pastoris*, high cell density fermentation.

2.1 Introduction

The surging price of fossil hydrocarbons and increasing concerns about the environment have led to the rapid growth of biodiesel production worldwide. It is estimated that the annual production capacity of biodiesel in the US alone will reach 2.24 billion gallons in 2008 [1]. Glycerol is the main, by-product of the conversion of vegetable oils into biodiesel, comprising approximately 10% by mass of the vegetable oil fed to the system [2]. The increased production of biodiesel has caused a sudden increase in production of glycerol creating a glut in the glycerol market [3]. The price of high purity glycerol in the United States plunged from US$1.00/lb in 1995 to less than US$0.40/lb in 2005 [4] and it is suggested that the price of crude glycerol might become stable at as low as $0.05/lb [5]. It is believed that refining crude glycerol to high purities is too costly and energy-intensive; therefore, it is urgent to discover innovative utilizations for crude glycerol that will make biodiesel production more profitable and sustainable. Researchers around the world are currently looking at the thermal, chemical, and biological conversion of crude glycerol to a variety of value-added products. One application that has been evaluated is the potential of using crude glycerol from biodiesel in animal feeds [6]. Another application for crude glycerol that is currently being investigated is the fermentation of glycerol to 1-3-propanediol, an intermediate compound for the synthesis of polymers used in cosmetics, foods, lubricants, and medicines [7, 8].

Glycerol has long been used as a major carbon source in culture medium for the cultivation of microorganisms in industrial fermentation. Less expensive carbon sources, such as glucose, have limited the use of glycerol in fermentations. Now that glycerol has decreased in price, as a result of a dramatic increase in biodiesel production, its use as a carbon source needs to be re-evaluated. If feasible, large scale cultivation in industry will create a substantial demand for glycerol. Phytase is an important industrial enzyme and is used as an animal feed additive in diets largely for swine and poultry, and to some extent for fish [9]. The whole market volume of phytase was estimated to be in the range of 150 million euro [10]. Adding phytase to the diets of monogastric animals can improve the uptake of phosphate from phytate, therefore effectively improving nutrient digestion and reducing the need of supplementation of external phosphate in their diets.

Furthermore, the breakdown of phytate liberates chelated minerals such as calcium, magnesium, iron, and zinc. Meanwhile, the release of phosphate from phytate will reduce the pollution caused by undigested phytate remaining in animal feces. The promising application of phytase in conjunction with environmental concerns has led to studies concerning phytase technology and phytase production.

P. pastoris has become a popular host for the expression and mass production of industrial enzymes, including phytase. Traditionally, *P. pastoris* cultivation is performed in fed-batch fermentation using the methanol-inducible system, an AOX1 – based expression system. In this system, excessive accumulation of methanol suppresses cell growth, making process control very difficult [11]. Another strong expression system, pGAP – based system, is reported to produce protein at a comparative level to the AOX1 – based system, although the level appeared to vary depending on the protein being expressed and the carbon source used for cell growth [12]. For the pGAP – based system, foreign protein was expressed constitutively without induction using methanol, which is costly and hazardous to handle in large volumes [13].

This research will study the feasibility of using crude glycerol as the sole carbon source for *P. pastoris* cultivation using a pGAP – based expression vector. The goal is to propose a new way to use crude glycerol from biodiesel, a waste product, to produce economically useful products on a large-scale.

2.2 Materials and Method

2.2.1 Yeast Strain and Chemicals

The recombinant yeast strain for the production of foreign phytase was kindly provided by Zell Technologies Inc., Canada. The host yeast is the methyltrophic yeast *P. pastoris*, which harbors a constitutive pGAPZα vector for phytase production. Phytase is expressed constitutively and exported to the broth supernatant and does not precipitate.

Analytical grade methanol and glycerol were purchased from Fisher Scientific, Canada. Crude biodiesel glycerol was kindly donated by Integrity Biofuels, Indiana, USA. This glycerol sample was taken from the product of the transesterification reaction right after the separation of biodiesel and the neutralization of base catalyst, sodium hydroxide.

Simple centrifugation was used to pre-treat the glycerol and after removing the solid phase, mainly sodium sulfate, the pre-treated glycerol was used as a cultivation substrate without further treatment.

2.2.2 Cultivation medium

YPD medium (12.5 g/L Yeast extract, 25g/L Difco peptone, 15 g/L glucose, 0.4 mg/L biotin, and 0.1mol/L potassium phosphate, pH = 6.0) was used as a starter medium for inoculum preparation. To grow the yeast in a 7.5 L bioreactor (New Brunswick, Bioflo 110), minimum salt medium (MSM) was used. The medium contains 2.1 g/L potassium citrate, 1.0 g/L calcium sulfate-dihydrate, 42.9 g/L potassium phosphate monobasic, 5.17 g/L ammonium sulfate, 14.33 g/L potassium sulfate, 11.7g/L magnesium sulfate heptahydrate, and 2.0 mL/L trace salt solution. The trace salt solution contains 2.0 g/L Copper (II) sulfate pentahydrate, 0.08 g/L sodium iodide, 3.0 g/L manganese sulfate monohydrate, 0.2 g/L sodium molybdate dehydrate, 0.02 g/L boric acid, 0.5 g/L cobalt chloride, 7 g/L zinc chloride, 22 g/L ferrous sulfate pentahydrate, and 0.2 g/L biotin.

2.2.3 Fermentation conditions

Glycerol stocks of the strain were streaked onto a YPD zeocin agar plate and incubated at 30°C until colonies appeared. Individual colonies were then picked and inoculated into 100mL of liquid YPD medium and cultivated in a shaker (30 °C, 250 rpm) for 24 hours to create the innoculum for the bioreactor. The innoculum was then sterilely poured directly into an autoclaved bioreactor containing medium. All runs were performed at 30 °C and pH 5.0, which was controlled by addition of an ammonium hydroxide solution. Air flow rate was maintained at approximately 5 L/min throughout all batches. Agitation was automatically adjusted to control the level of dissolved oxygen unless otherwise specified. For fed-batch fermentations, the feeding was controlled to prevent over-accumulation of glycerol and to maintain the desired level of dissolved oxygen once the agitation reached its maximum level. A DO-Stat was used to control the feeding rate. During the feeding phase of all fed-batch fermentations, the residual glycerol concentration was found to be below 0.2 g/L.

2.2.4 Analytical methods

Chemical composition analyses were done using a Waters HPLC with a Shodex SH-1011 column (with eluent 0.005 mol/L sulfuric acid, flow rate 1.0 mL/min, column temperature 50 °C, and reflective index detector). Residual glucose concentrations in the broth were determined using a YSI 2730 biochemical analyzer (YSI Incorporated, USA). Cell density was determined by measuring both optical density at 600 nm (OD600) and dry cell weight (DCW). Phytase protein production was monitored by performing a phosphatase activity assay based on the phosphatase activity of phytase. The procedure of the phosphatase assay was based on a protocol from Sigma [14] with some modifications. Disodium nitrophenyl phosphate (Sigma) was used as the substrate. For calibration, a phytase standard from Zell Technologies Inc. with an activity of 41261 U/g was used. One unit of phytase activity (U) is defined as the amount of enzyme that catalyzes the release of 1.0 micromole of inorganic phosphate per minute from 5.1 mmol/L sodium phytase in pH 5.5 buffer at 37 °C.

2.3 Results and Discussion

2.3.1 Comparison of using three different C-sources as substrate

The expression level of heterologous protein by *P. pastoris* with pGAP is reported to be dependent on the foreign protein itself and the carbon source used for cell growth [12]. In this study, parallel experiments were conducted to observe the influence of three different carbon sources most commonly used for *P. pastoris* cultivation: analytical grade glucose, glycerol, and methanol. The cultivations were done in shake flasks with 1.5% (w/v) initial carbon source in MSM medium. To obtain reliable data for each carbon source, triplicate experiments were run concurrently at 30 °C and 200 rpm. The results are displayed in Figure 2.1. The data shown takes the average of the results of the three identical batches. Error bars are included, where possible but most are too small to be displayed.

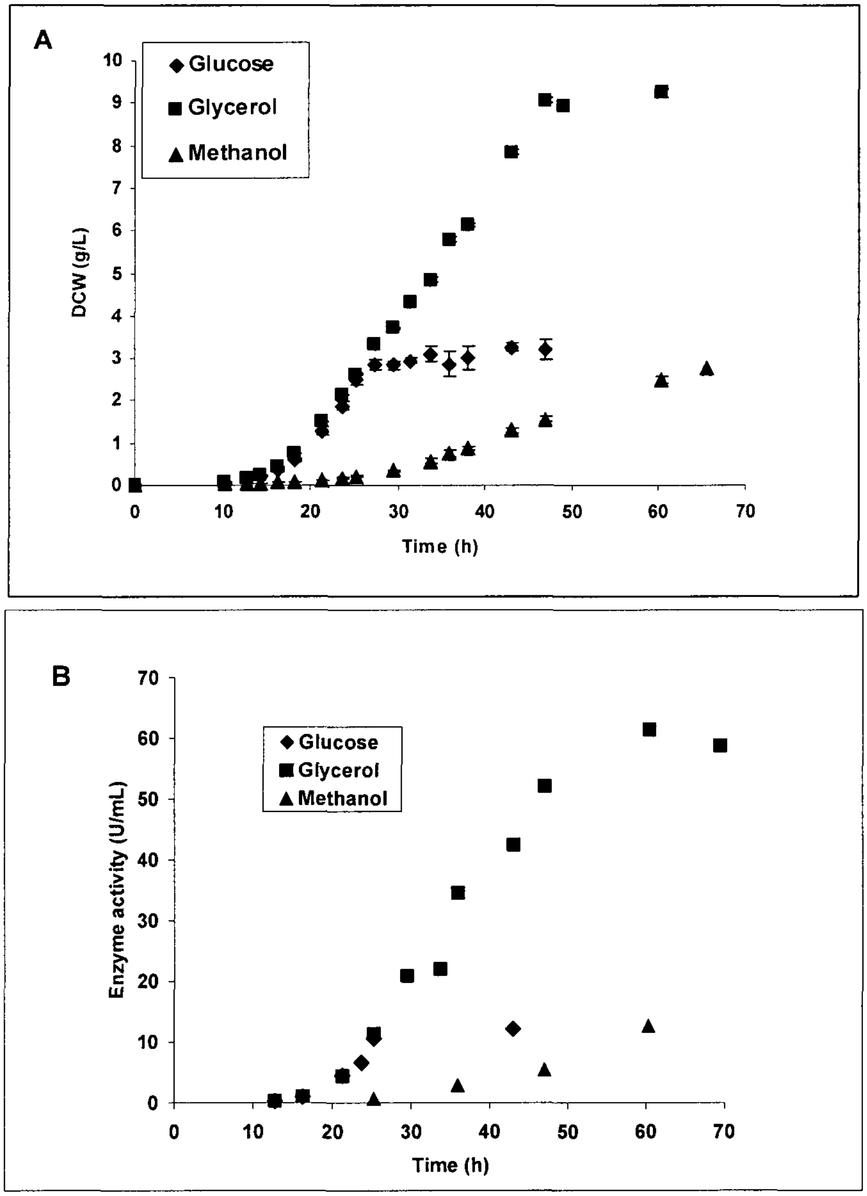


Figure 2.1 Shake flask cultivation with glucose, glycerol and methanol. A: cell growth profile (OD600). B: phytase production profile.

In similar flask experiments done by Waterman et al. (1997) and Sears et al. (1998), glucose gave the best performance [15, 16]. However, according to the results displayed in Figure 2.1, it is obvious that glycerol outperformed the other two carbon sources in terms of both protein expression and cell growth. In the first 25 hours the cell growth and protein expression profiles were similar for glycerol and glucose. After this period, a plateau of cell growth and protein production was observed in the case of glucose, while cell growth using glycerol continued. With glycerol as a carbon source, a final cell density of approximately 18 (OD_{600}) and a protein level of approximately 60 U/mL (enzyme activity per volume of cell-free broth) were obtained, both of which are much higher than values achieved with glucose.

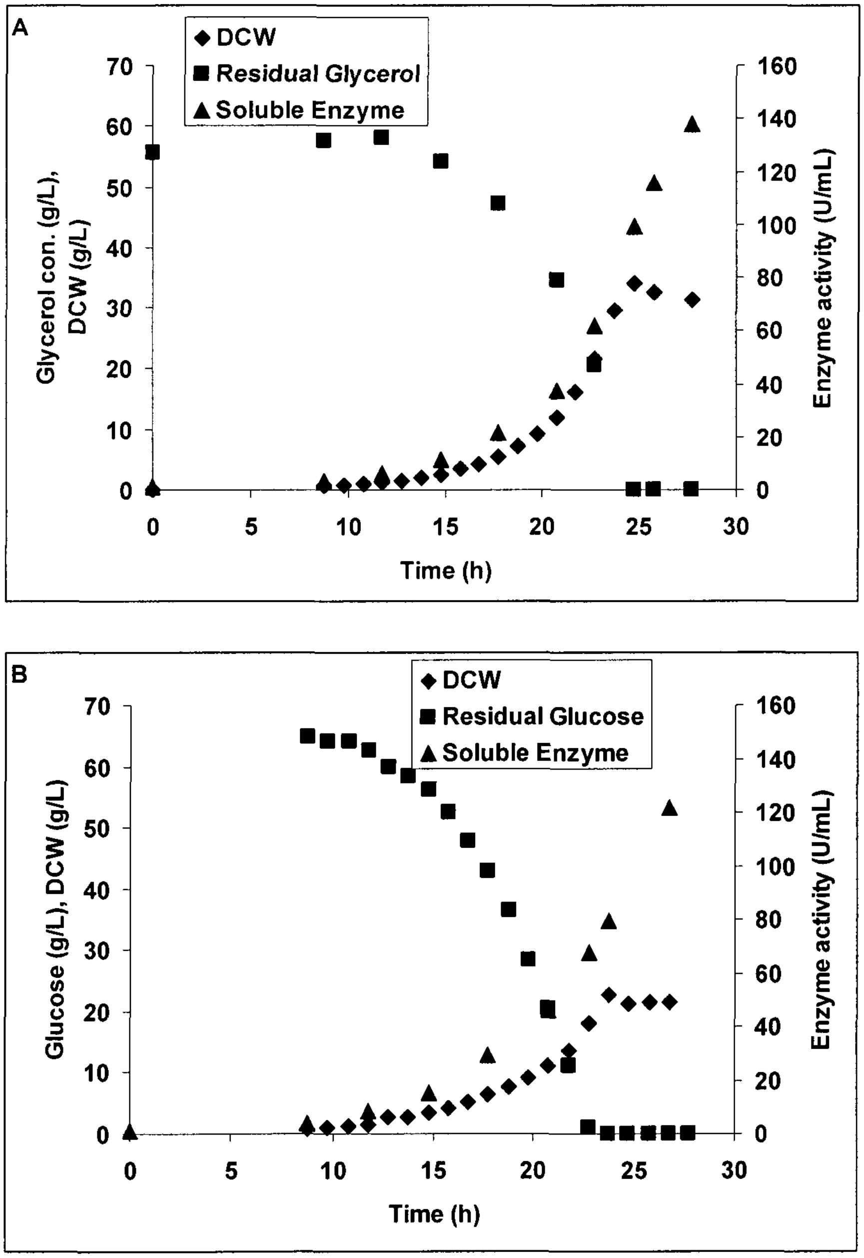


Figure 2.2 Profiles of batch cultivation performed in the 7.5 L bioreactor using glucose and glycerol. A: glycerol. B: glucose.

Table 2.1 Summary of the results of batch fermentation performed in 7.5 L bioreactor using glucose and glycerol

Carbon source	Total mass of carbon source (g)	DCW (g/L)	Final enzyme activity (U/mL)	Maximum specific growth rate (h^{-1})	Biomass per carbon source (g/g)	Product per carbon source (U/g)
Glycerol	120	31.1	137.8	0.266	0.507	1971
Glucose	120	21.6	121.6	0.203	0.35	1785

Batch experiments were conducted in 7.5 L bioreactors to study the effect of carbon source further. Only analytical grade glycerol and glucose were used and the results are shown in Figure 2.2 and Table 2.1. The initial carbon source concentration in MSM medium was approximately 60 g/L, the broth pH was controlled at 5, and the dissolved oxygen level was maintained above 30 % throughout the whole experiment.

Compared to the shake flasks, there was an increase in biomass and protein production using glucose as a carbon source which might be attributed to the completely aerobic environment in the bioreactors. Nevertheless, glycerol still performed better than glucose as a carbon source with maximum specific growth rate 31 % higher, biomass yield 45 % higher, and protein product yield 10 % higher. Notably in this situation, the yield of biomass with glucose consumption is only 0.35 g (DCW)/g, even though the cultivation environment was totally aerobic throughout the whole process. This suggested that glucose might have undertaken fermentative metabolism under aerobic conditions as well. This hypothesis was confirmed by the HPLC chromatogram of the broth supernatant (Figure 2.3B) taken at the end of the batch run with glucose as the sole carbon source. As shown in Figure 2.3B, in addition to the two peaks representing sulfate and phosphate, the major salt components in the medium, there are five more peaks due to five different metabolites. Two ordinary by-products, acetate and ethanol, are found among them, with concentrations of 2.3 g/L and 3.5 g/L respectively. Solà et al. (2004) studied the metabolism of *P. pastoris* with glucose as the sole carbon source in continuous fermentation and found that no fermentative by-products were detected for a residual glucose level of 0.5 g/L under aerobic conditions [17]. The reason for different observations might be due to the higher initial glucose concentration used. The production of fermentative by-products like ethanol or acetic acid not only depletes the carbon source, it also introduces the potential of inhibiting cell growth. It is reasonable to infer that a lower specific growth rate with glucose might have resulted from inhibitive effects caused by fermentative by-products. In contrast, no detectable by-products were present in the case of glycerol. As such, the metabolism of glycerol in *P. pastoris* in this situation can be described as fully aerobic, even at high glycerol concentrations, agreeing with the shared opinion that glycerol is a less fermentative carbon source than glucose.

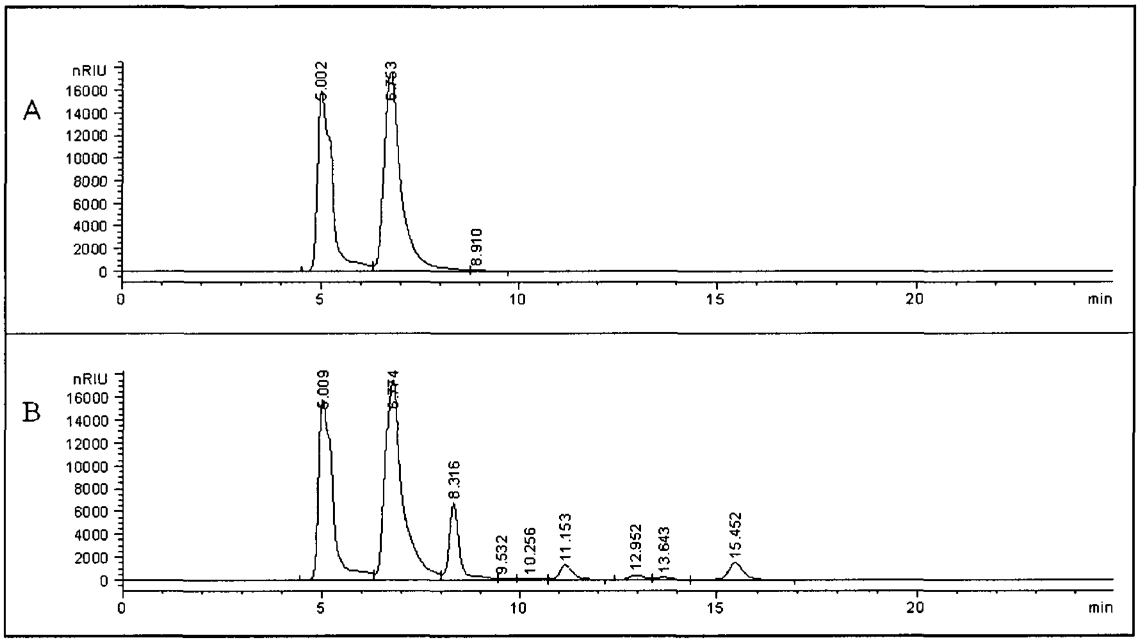
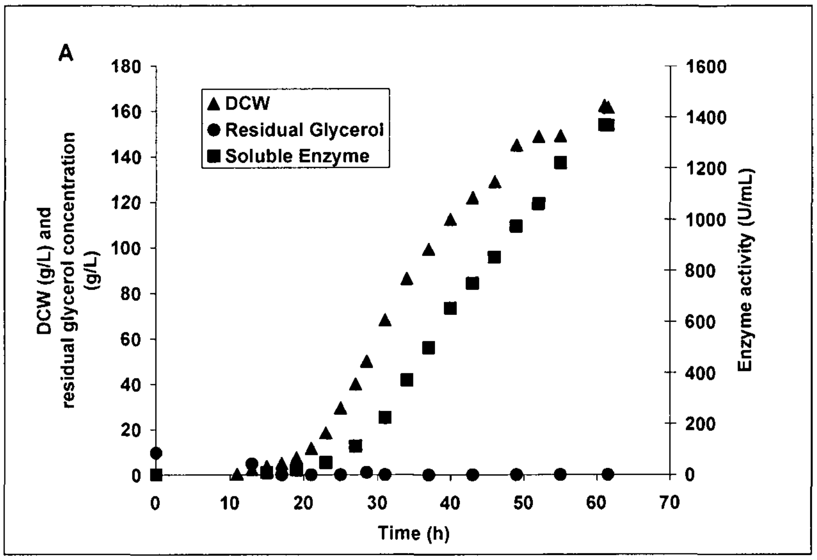


Figure 2.3 HPLC analysis of broth supernatant in 7.5 L bioreactor. Samples are taken at the end of the batch cultivation using glucose and glycerol. A: glycerol. B: glucose. Peak identities: 5.0 (retention time), sulfates; 6.7, phosphate; 11.1, acetate; 15.4, ethanol.

2.3.2 Effect of glycerol concentration and dissolved oxygen

Two initial concentrations of glycerol were chosen and their effects on the whole fed-batch fermentation were tested, with the total glycerol consumption the same. In these runs, the feeding of 60 % (w/v) analytical grade glycerol was started after the initial glycerol was exhausted. The glycerol feed rate was controlled in order to maintain the dissolved oxygen level above 30 % with maximum agitation. For an initial glycerol



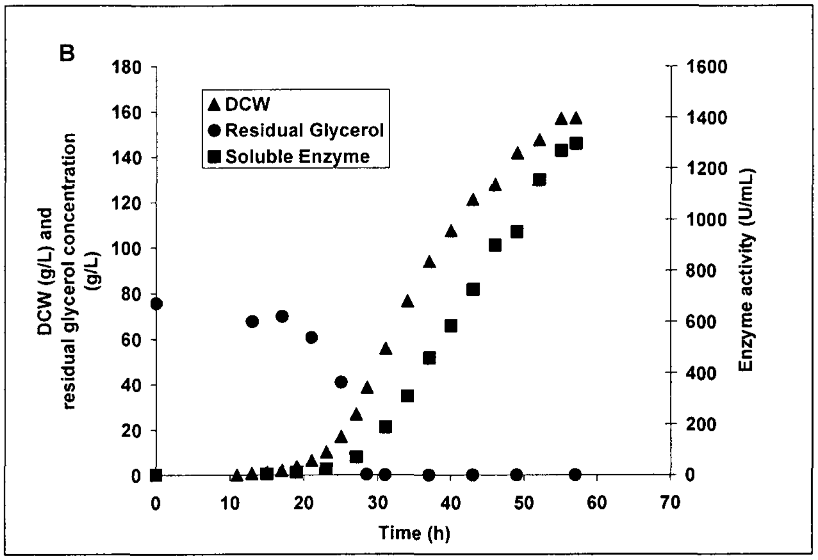


Figure 2.4 Profiles of fed-batch fermentation started with different glycerol concentration A: 10 g/L. B: 70 g/L.

Table 2.2 Comparison of the outputs of fed-batches started with two different glycerol levels

Initial glycerol Concentration (g/L)	Total glycerol input (g)	Biomass per glycerol (g/g)	Product per glycerol (U/g)
10	960	0.505	1847
70	960	0.508	1873

concentration of 10 g/L, the feed rate followed an exponential profile before the agitation speed reached maximum. The results are shown in Figure 2.4 as well as Table 2.2. Despite the difference in initial glycerol concentration, the final yields of biomass and enzyme production are quite similar and consistent to the values obtained in batch fermentation. HPLC analysis of broth samples withdrawn throughout the whole process indicated no by-product peaks. The metabolism of glycerol under aerobic conditions produced no detrimental by-product accumulation, even at a high concentration (70 g/L), consistent with what was found in the bioreactor batch fermentations. Thus, it is feasible to start fed-batch cultivations with a high initial glycerol concentration, resulting in a more efficient fermentation.

The dissolved oxygen level is critical in aerobic cultivations, especially in the case of large scale high cell density production where the cost of compressed air becomes a significant factor. During the feeding phase of fed-batch cultivation, the effects of three levels of dissolved oxygen were studied and the results are summarized in Table 2.3. The yields of biomass and phytase using glycerol as the feed source showed little variation among the three batches. Although the yield of biomass seems to decrease slightly with a decrease in dissolved oxygen level, the difference is insignificant. No detectable by-products were found in the HPLC chromatograms of the broth supernatant samples from the three runs. Although maintaining at least 30 % dissolved oxygen is recommended for the cultivation of *P. pastoris*, the results show that using glycerol as a carbon source *P. pastoris* undergo aerobic metabolism with dissolved oxygen levels as low as 10 %.

Table 2.3 Comparison of the outputs of fed-batches maintained in different dissolved oxygen levels in the feeding phase

Dissolved oxygen level (%)	Total glycerol input (g)	Biomass per glycerol (g/g)	Product per glycerol (U/g)
30	960	0.508	1872
20	960	0.506	1921
10	960	0.501	1823

2.3.3 Performance of biodiesel glycerol as the carbon source

Crude biodiesel glycerol samples, from a stock provided by Integrity Biofuels, were pretreated by centrifugation under 10,000 RPM for 15 minutes. This was effective in removing approximately 85% of the sodium sulfate particles from the original samples. Pretreated glycerol was used directly to prepare aqueous glycerol solutions for use as the carbon source in *P. pastoris* cultivation. Chemical compositions of the glycerol before and after pretreatment were analyzed by HPLC. The analytical results are summarized in Table 2.4. According to the results, the crude biodiesel glycerol is a mixture consisting primarily of glycerol, sodium sulfate, methanol, and water. Glycerol is the primary by-product in the conversion of lipids to biodiesel through transesterification reactions catalyzed by acids or bases such as sodium hydroxide [18]. Sodium sulfate is the product of the neutralization of sodium hydroxide with sulfuric acid. As indicated in Table 2.4, approximately 85% of the sodium sulfate was removed and the resulting supernatant was a transparent yellow liquid containing approximately 80.4 % glycerol and 3.9 % sulfate. No methanol was detected in the supernatant. Water and other impurities accounted for approximately 16 % of the total weight. Other impurities include fats, proteins, and metal ions; their concentrations differ with respect to parent source oil feedstock [19].

Table 2.4 Chemical compositions of crude and pretreated biodiesel glycerol

Sample	Glycerol (%, w/w)	Methanol (%, w/w)	Sodium sulfate (%, w/w)	Others (%, w/w)
Crude glycerol	67.5 ± 3.2	1.5 ± 0.2	25.8 ± 2.7	5.2 ± 1.0
Pretreated glycerol	80.4 ± 1.4		3.9 ± 0.4	15.7 ± 1.0

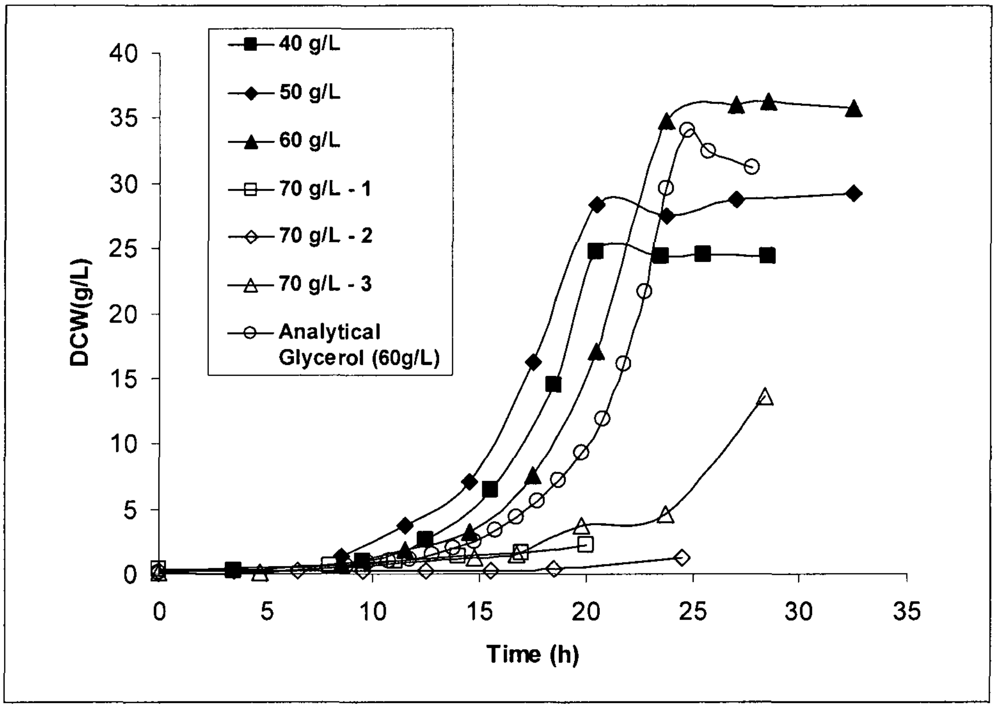


Figure 2.5 Cell growth profiles of batch fermentations with different initial concentrations of pretreated biodiesel glycerol

Without further treatment, this pretreated biodiesel glycerol supernatant was used in the batch cultivation of *P. pastoris*. Four levels of initial glycerol concentration were studied: 40 g/L, 50 g/L, 60 g/L, and 70 g/L, as shown in Figure 2.5. For the first three concentration levels, cells grew in similar profiles, and the maximum specific growth rates were 0.263 h^{-1}, 0.266 h^{-1}, and 0.260 h^{-1} respectively. However, at an initial glycerol concentration of 70 g/L, cells grew at an extremely low rate. To confirm this result the batch was repeated twice, but no significant changes were observed. It can therefore be concluded that in the case of an initial concentration of 70 g/L crude glycerol, cell growth was inhibited by certain impurities in the pretreated biodiesel glycerol. Interestingly, at a slightly lower initial concentration of 60 g/L crude glycerol, such inhibition was found to be insignificant. According to our previous results, an initial concentration of 70 g/L analytical grade glycerol had no inhibitive effects on cell growth. The small amount of sulfate present in the MSM medium is unlikely to be detrimental; therefore, the inhibitory substances are likely to be other impurities residing in crude glycerol which are yet unknown.

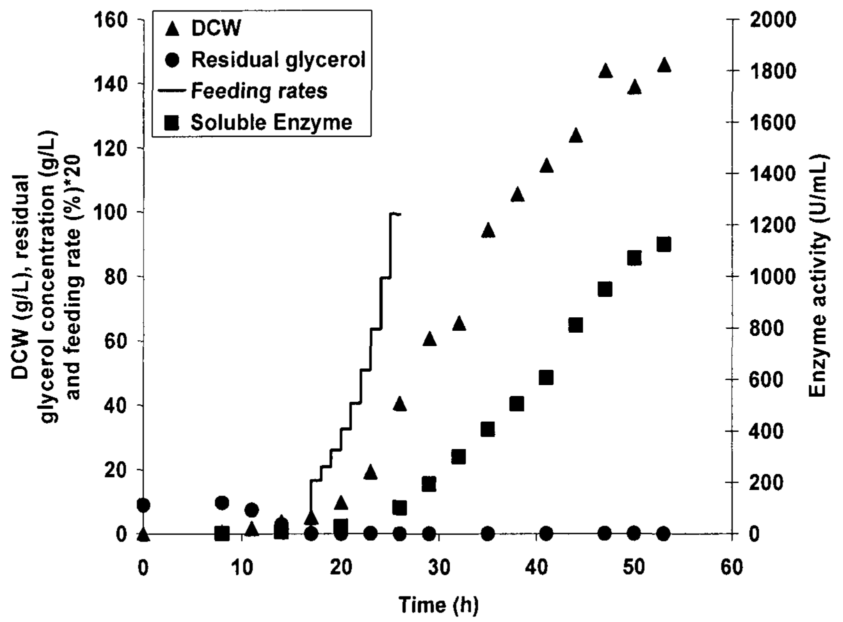


Figure 2.6 Profiles of fed-batch cultivation using pretreated biodiesel glycerol.

On the application side, the aim of this work was to utilize crude glycerol to achieve high cell density production of phytase. A fed-batch run was conducted with the feeding of a 60 % glycerol solution made with pretreated biodiesel glycerol. Fearing that the inhibition seen in the batch mode would reoccur, this fed-batch cultivation was initiated with pretreated biodiesel glycerol at a concentration of 10 g/L. After the consumption of the initial glycerol, exponential feeding of 60 % biodiesel glycerol followed until agitation reached maximum, after which the feeding was controlled to maintain the dissolved oxygen level above 20 %. The result of this batch is shown in Figure 2.6. After 53 hours of cultivation, a final biomass concentration of 146 g/L (DCW) and a final phytase concentration within the broth supernatant of 1125 U/L were achieved. In total, 1247 grams of pretreated crude glycerol was consumed. The yields of biomass and protein are 0.508 g (CDW)/g glycerol and 1900 U/g glycerol respectively, which are similar to those obtained from the fed-batches using glycerol of analytical grade. The inhibition shown in Figure 2.5, observed in the batch cultivation with an initial biodiesel glycerol concentration of 70 g/L, did not occur in fed-batch cultivation. The reason is unknown. Perhaps in fed-batch mode, where nutrients and possible hazardous impurities are introduced gradually, the inhibitive effect can be greatly reduced or eliminated.

2.4 Conclusions

In batch cultivations performed in both shake flasks and 7.5 L bioreactors, analytical grade glycerol outperformed analytical grade methanol and glucose, in terms of cell growth rate, biomass yield, and phytase production. Under aerobic conditions, the metabolism of glycerol adopted aerobic respiration at concentrations as high as 70 g/L. This was confirmed by the fact that no fermentative by-product was detected in the broth supernatant. The finding agrees with the shared opinion that glycerol is a less fermentative carbon source. In fed-batch cultivations with analytical grade glycerol, the effects of initial glycerol concentration and dissolved oxygen level were studied. It was found that aerobic respiration was maintained under initial glycerol concentrations as high as 70 g/L and dissolved oxygen levels as low as 10%. Therefore, glycerol should be a better carbon source than glucose and allows for more flexible control in fed-batch *P. pastoris* cultivation. In the batch cultivation with pretreated biodiesel glycerol, growth inhibition was observed when the initial glycerol concentration was 70 g/L. This suggested the existence of detrimental impurities in the pretreated biodiesel glycerol, which are still to be determined. To address the inhibition caused by crude glycerol, a fed-batch cultivation was performed using an initial crude glycerol concentration of 10 g/L. An exponential feeding profile ensued to maintain rapid cell growth and to prevent excessive glycerol accumulation. With this lower initial crude glycerol concentration, no obvious inhibition was observed. A final cell density of 146 g/L (DCW) and a final phytase production of 1125 U/L (enzyme activity per cell-free broth) were achieved within 53 hours of cultivation. These results demonstrate the potential of using crude glycerol as the sole carbon source in high cell density cultivation of *P. pastoris* for the mass production of phytase and possibly other protein products.

2.5 Acknowledgements

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CHAPTER 3

Conclusions

With decades of development, *P. pastoris* has become a popular host for the expression and mass production of heterologous protein. The success of this system is attributed to many advantages it has, one of which is the existence of strong promoters, such as AOX1. The expression level of heterologous protein using this system depends on many factors both of gene level and cultivation level. At the beginning of this work, a literature review was presented, focused on the studies of factors at cultivation level. The review tried to cover all relative aspects in the cultivation of *P. pastoris*, including medium selection, cultivation condition, process monitoring, process control, trouble-shooting measures to deal with proteolytic degradation and oxygen limitation. Although significant efforts were tried to extract possible regulations and mechanism within *P. pastoris* cultivation, more commonly found are controversies or irregularities among different researchers' results. For example, for the control of proteolytic degradation, some found that decreasing pH reduced the degradation of target protein and the activity of background protease activity. However, others had completely opposite results. Such facts, on the other hand, corroborate the complexity of this system.

GAP promoter is another powerful expression motor of *P. pastoris* expression system. Unlike AOX1 promoter, GAP promoter circumvents the use of methanol and expresses foreign proteins constitutively. It has become a potential substitute to AOX1 promoter, especially in large scale application in industry. In Chapter 2, we investigated the feasibility of utilizing biodiesel-originated crude glycerol as the substrate for the production of a Phytase protein with *P. pastoris* under GAP promoter. The prosperity of biodiesel production in recent years has created a glut of crude glycerol, the major by-product of biodiesel production, leading to the dramatic slash of glycerol price. Exploring the market of glycerol is of significant importance to development of biodiesel. Our work aims at both providing a new application of crude glycerol and reducing the cost of *P. pastoris* cultivation. The results turned out to be encouraging. In batch cultivations, we found that glycerol was better than glucose in terms of either biomass production or phytase production. Although in some batch cultivation using biodiesel glycerol, growth inhibition was found when initial substrate concentration is as high as 70g/L, we successfully realized high cell density production of phytase in fed-batch cultivation mode by starting with lower initial concentration of biodiesel glcyerol. The potential utilization of biodiesel glycerol for the production of heterologou protein with *P. pastoris* under GAP promoter was proved.

APPENDIX

Kinetic study of phytase production with *Pichia pastoris* cultivation under GAP promoter in chemostat and modeling

Abstract

A phytase protein was expressed constitutively with a strain of *Pichia pastoris* under GAP promoter. Phytase production and cell growth of the strain was studied in batch, fed-batch and continuous processes. In batch cultivation, glycerol outperformed glucose as sole carbon source, while in continuous cultivation glucose presented better performance in terms of both specific phytase formation rate and yield of phytase. In the continuous study concerning the effects of dissolved oxygen level, phytase production decreased with the decrease of dissolved oxygen level, while cell growth had little change. Moreover, continuous studies upon glycerol and glucose also revealed that both phytase production and biomass production were greatly influenced by specific growth rate or dilution rate. Specific phytase formation rate increased linearly with dilution rate; specific substrate uptake rate increased with dilution rate as well, but more aggressively than linear increase. Based on kinetic studies in continuous cultivation, a simple kinetic model was established. With parameters mainly estimated with the results in continuous cultivation, the model gave very good performance in the prediction of fed-batch cultivation processes, since model prediction fitted fairly well with experimental results in three different fed-batch processes.

Key words: kinetic modeling, GAP promoter, continuous cultivation, *Pichia pastoris*

Nomenclature

X	Biomass production, gDCW
P	Phytase production, kU
V	Total broth volume, L
V_S	Volume of broth supernatant, L
C_X	Concentration of biomass, (gDCW)/L
C_P	Concentration of phytase in the supernatant, kU/L
F	Feeding rate of glucose solution, L/h
C_S	Concentration of glucose solution, 600g/L
f_{NH_3}	Feeding rate of ammonia hydroxide solution, L/h
f_e	Evaporation rate, L/h
f_P	Sampling rate, L/h
μ	Specific growth rate, 1/h
K_C	Specific volume of biomass, 0.0033L/(gDCW).
R_S	Glucose feeding rate, g/h
q_s	Specific gluose uptake rate, g/(gDCW)/h
K_u	Model parameter, g·h/(gDCW)
$Y_{S/X}$	Model parameter, g/(gDCW)
m	Model parameter, g/(gDCW)/h
$Y_{P/X}$	Model parameter, kU/(gDCW)
Y_m	Model parameter, kU/(gDCW)/h
K_P	Model parameter, (gDCW)/L

3.1 Introduction

Nowadays more than 500 proteins have been cloned and expressed with the system of *Pichia pastoris* (*P. pastoris*) [1]. *P. pastoris* has many advantages being a host for the expression of heterologous proteins, such as the capability to secrete foreign protein extracellularly, simplifying downstream recovery, the capability to perform eukaryotic post-translational modification such as glycosylation and proteolytic processing and the ability to grow upon defined medium and achieve high cell density.

For *P. pastoris* , the methanol-inductive promoter, AOX1 promoter is most commonly used. However, this promoter requires the use of methanol, which is toxic and flammable, and is a big concern for large scale applications [2]. To eliminate the use of methanol, alternative promoters have been invented. The constitutive promoter, GAP promoter, is a promising substitute, especially in large scale industrial applications [2]. The promoter was first isolated by Waterham in 1997 [3] and has been used successfully to express many heterologous proteins. With the promoter, foreign proteins are expressed constitutively with glucose or glycerol as substrate. In some cases, the expression level with GAP promoter was found to be higher than that with AOX1 promoter [1].

Contradictory to the increasing popularity of GAP promoter, only a few papers reported kinetics and models of this system. To enhance our understanding of heterologous protein production and cell growth under this promoter, we utilized continuous cultivation to study kinetics of this system under a defined medium. The target protein was a recombinant phytase, the activity of which can be monitored with a stable procedure. A simple kinetic model was established based on the kinetic studies performed with continuous cultivation.

3.2 Materials and Methods

3.2.1 Yeast Strain and Media

The host yeast is the methylotrophic yeast, *P. pstoris*, which harbors a constitutive vector pGAP-α for phytase production. Phytase is expressed constitutively and exported to the broth supernatant. For strain maintenance and inoculums preparation, Minimum Salt

Medium (MSM) was used with the supplementation of a trace salt medium [4]. MSM medium was used for fed-batch cultivation. Half MSM (concentrations of all medium components are in half except trace salts) was used for the continuous cultivation.

3.2.2 Fed-batch and Continuous cultivation

Fed-batch cultivation was performed similarly as we did in a previous work [4]. Continuous cultivation was performed in a 3.0L bioreactor (NBS Bioflo 110) with a working volume of 2.0L. Initialized with a regular fed-batch process, continuous cultivation was realized with continuous withdrawal of liquid broth from a fixed level to keep the liquid volume constant. Steady states were achieved in about 3 volume changes. All the runs were performed at 30°C and pH 5.0, which was controlled by feeding an ammonium hydroxide solution. Air flow rate was kept constant at 3.0L/min throughout all processes. Agitation was cascaded to the control of dissolved oxygen level if not specified. Two common substrates were studied in the continuous cultivation of *P. pastoris* , glucose and glycerol, in the concentration of 40g/L. To investigate the case of high cell density situation with continuous cultivation, 350g/L glucose was used.

3.2.3 Analytical methods

Chemical composition analyses of broth supernatant were done using an Agilent HPLC system with a Shodex SH-1011 column (with eluent 0.005 mol/L sulfuric acid, flow rate 1.0 mL/min, column temperature 50 °C) and a refractive index detector. Residual glucose concentrations in the broth were also determined by a YSI 2730 biochemical analyzer (YSI Incorporated, USA). Cell density was determined by measuring optical density at 600nm (OD600), and dry cell weight was found to be proportional to OD600 with a ratio of 0.387 (data not shown). Phytase protein production was monitored by performing a phosphatase activity assay based on the phosphatase activity of phytase [4].

3.3 Kinetic Model Description

This kinetic model was mainly designed to model *P. pastoris* cultivation in fed-batch operation mode. In fed-batch cultivation upon glucose, cell growth should be limited to avoid substrate accumulation or oxygen limitation. Therefore, residual glucose concentration was assumed to be negligible and the uptake rate of glucose is equal to the feeding rate of glucose.

$$R_S = F \cdot C_S \tag{1}$$

According to our results in continuous cultivation, specific glucose uptake rate increased with the increase of dilution rate and their relationship can be simulated by a second order polynomial regression. Therefore, following equation for specific glucose uptake rate was designed.

$$q_s = K_u \cdot \mu^2 + Y_{S/X} \cdot \mu + m \tag{2}$$

The uptake rate of glucose is then used for cell growth and maintenance.

$$R_S = q_s X \tag{3}$$

As we will discuss later, the specific phytase production rate was found to be proportional to specific growth rate and decreased with the increase of cell density. Thus, the following equation was designed to account for the effects of both specific growth rate and cell density level.

$$\frac{dP}{dt} = (Y_{P/X} \cdot \mu + Y_m) \frac{K_P}{X/V + K_P} X \tag{4}$$

For cell growth,

$$\frac{dX}{dt} = \mu X \tag{5}$$

For broth volume,

$$\frac{dV}{dt} = F + f_{NH_3} - f_e - f_P \tag{6}$$

where f_{NH_3}, f_e and f_P account for volume changes caused by the addition of ammonia, evaporation and sampling respectively.

In the situation of high cell density cultivation, a great part of broth volume is occupied by cell biomass. The following equation is to account for this and to determine the volume of broth supernatant.

$$V_S = V - K_C X \tag{7}$$

where K_C is equal to 0.0033L/(gDCW).

The concentration of phytase protein (in the supernatant), cell biomass can then be expressed as followed:

$$C_P = P / V_S \tag{8}$$

$$C_X = X / V \tag{9}$$

3.4 Results and Discussion

3.4.1 Batch cultivation

Constitutive expression of heterologous proteins under GAP promoter is greatly influenced by the selection of carbon source. Several studies have demonstrated that glucose and glycerol are productive carbon sources for foreign protein production under GAP promoter, but there is no agreement about which one is better: some found that glucose was better [5, 6]; others found that glycerol was better [4, 7]. In our previous study [4], we compared the performance of glycerol and glucose in batch cultivation with initial concentration of about 60g/L with MSM medium and glycerol was found to be better in this situation. To give more insights of guidance in the design of continuous cultivation and for better comparison, we designed similar experiments with initial concentration of 40g/L glycerol and glucose in Half MSM medium. The results were depicted in Figure 3.1 (a) and (b). For more definite comparison, key results were summarized in Table 3.1.

The evolutions of cell biomass and substrate concentration are similar to our previous results [4]. Again, the yield of biomass upon glucose is much lower than that upon glycerol. Previously, we detected the production of fermentative by-products in the case of glucose. In this study, this phenomenon is better described with the evolutions of ethanol and acetate concentrations. Interestingly, the evolution of ethanol is different from that of acetate. Ethanol was accumulated with the consumption of glucose from the beginning, and the decrease of ethanol concentration was found at nearly the same time with the depletion of residual glucose. Acetate was not detected until the time when glucose is completely consumed and its concentration increased thereafter with the decrease of ethanol concentration. As we will show later, in the case of continuous cultivation upon glucose where glucose concentration was out of detection or below 3g/L, ethanol production was not found. Thus, high residual concentration of glucose should account for the accumulation of ethanol in batch cultivation. The production of ethanol was also found in other cases. Baumann and coworkers found that oxygen limitation led to the production of ethanol by *P. pastoris* [8]. According to our observations, acetate appeared not to be the direct by-product of glucose metabolism under this condition but associated with the consumption of ethanol. Unfortunately, we found no papers in the literature reported the production of acetate in the cultivation of *P. pastoris* .

Table 0.1 Comparison of glucose and glycerol in batch cultivation

	Carbon source amount	Final volume	OD600	Final enzyme activity	μ_{max}	Yield of biomass	Yield of enzyme
	g	L		U/mL	1/h	g/g	kU/g
Glucose	60	1.45	20	65.5	0.268	0.187	1.54
Glycerol	60	1.47	42.3	70.9	0.239	0.401	1.64

Maximum specific growth $\mu_{\max}$ is another factor to compare the performance of carbon source. To get $\mu_{\max}$, linear regression was employed upon data taken from the middle of cell growth profile as shown in Figure 3.2. The first data point was above the trend line for both cases, indicating the existence of a slight lag phase at the beginning. Cell grew exponentially at constant specific growth rate throughout the cultivation upon glycerol until the depletion of glycerol. A little difference was found in the case of glucose: exponential cell growth upon glucose slowed down after 21^{st} hour when there were still about 14 g/L of glucose left. Finally, $\mu_{\max}$ upon glucose was found to be higher than that upon glycerol.

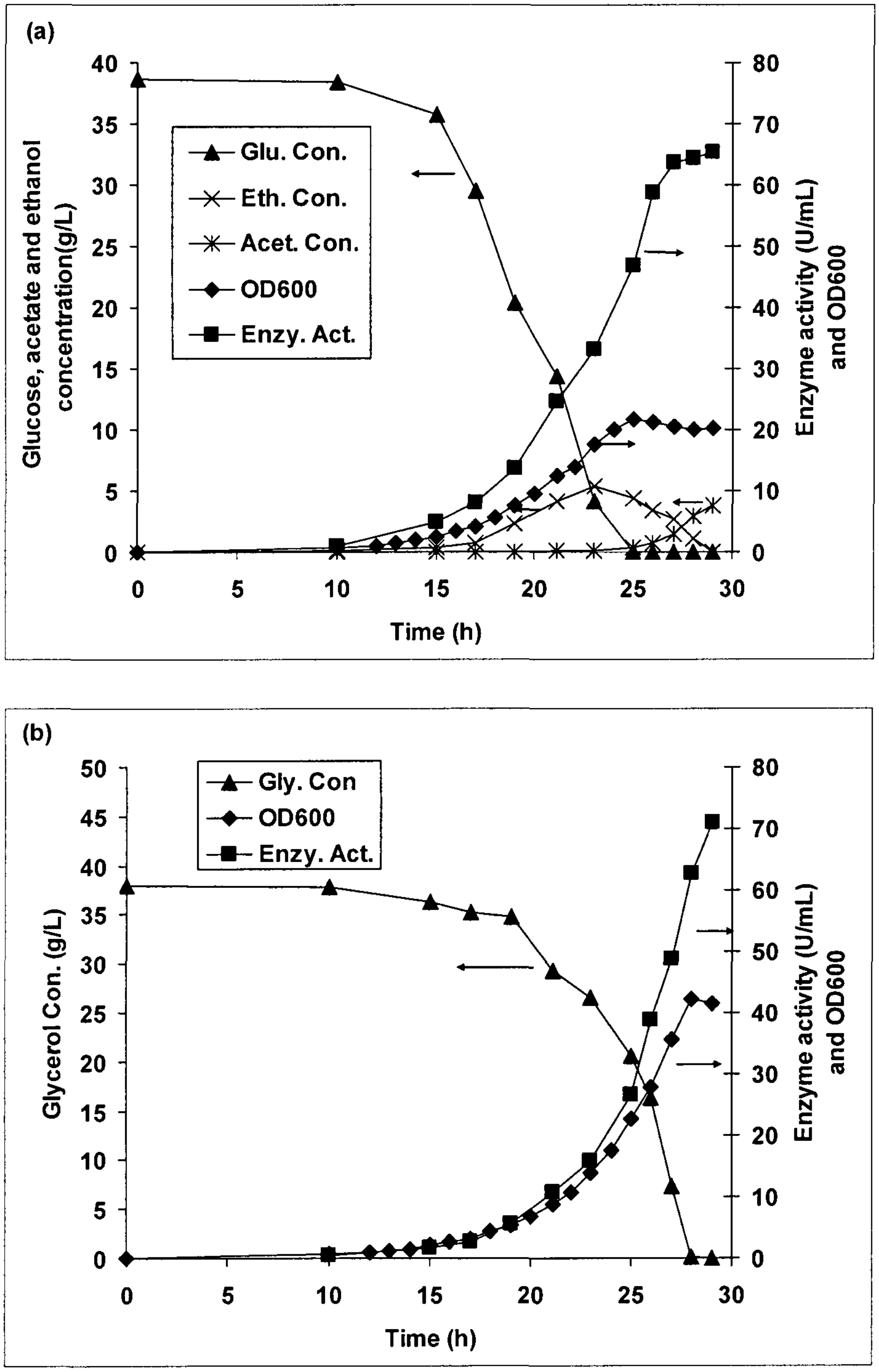


Figure 0.1 Batch cultivation profiles upon glucose (a) and glycerol (b)

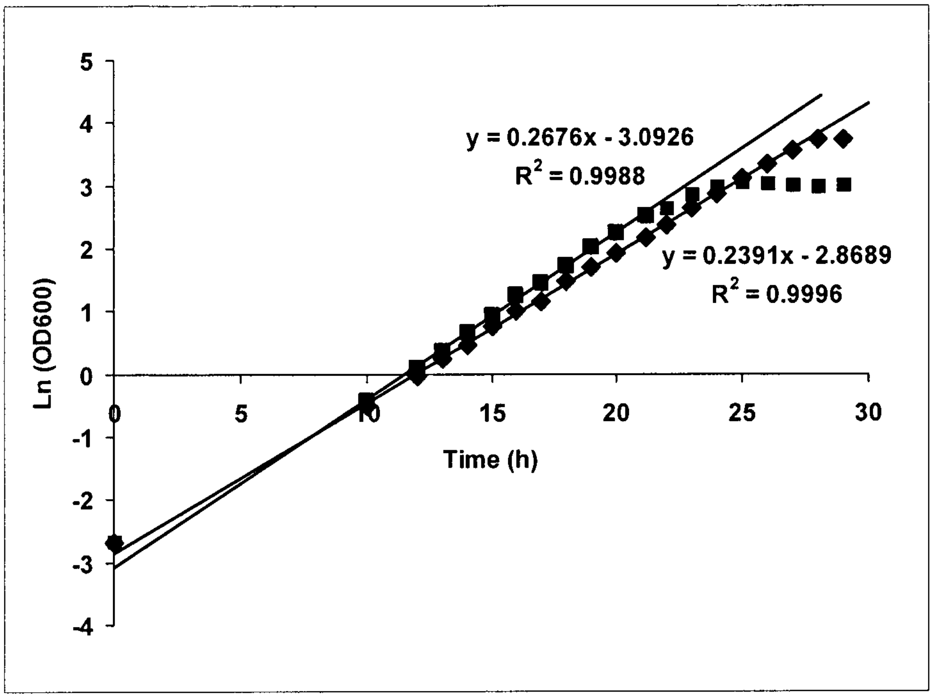


Figure 0.2 Linear regression of cell growth upon glycerol (solid diamond ◆) and glucose (solid square ■) in batch mode

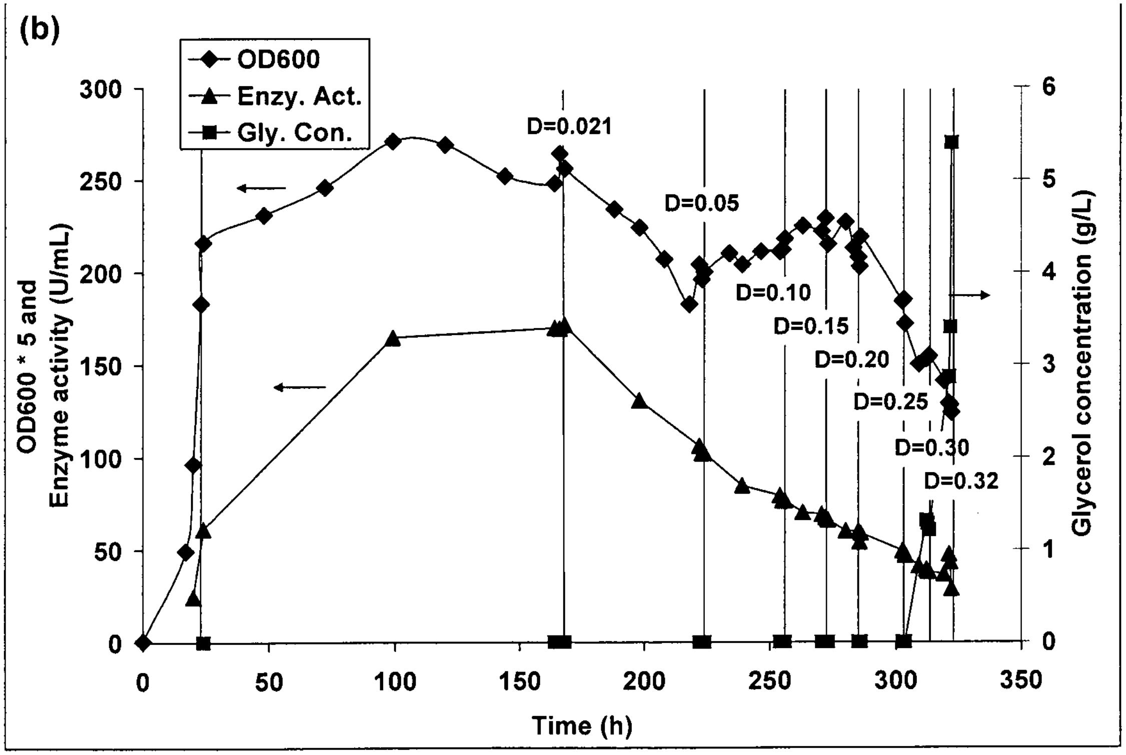
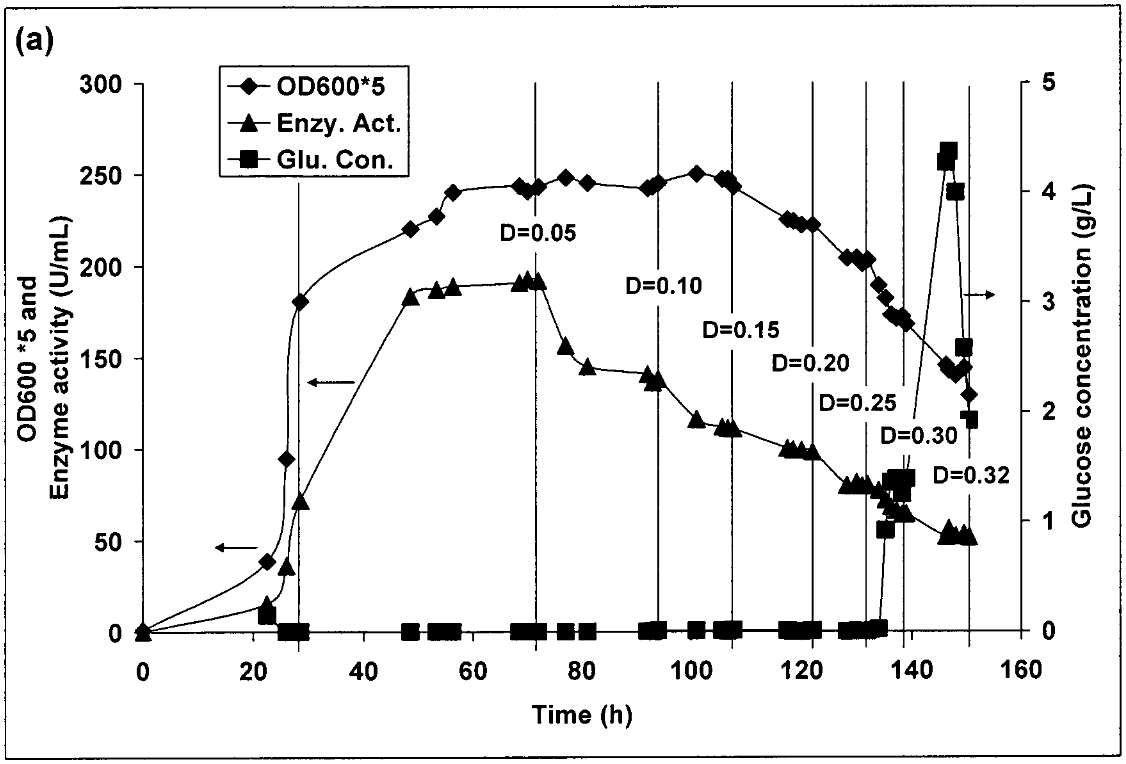


Figure 0.3 Time profiles of kinetic studies in chemostat upon glucose (a) and glycerol (b) in 40g/L


(a)
OD600 *5 and Enzyme activity (U/mL)
300
250
200
150
100
50
0
OD600*5
Enzy. Act.
Glu. Con.
D=0.05
D=0.10
D=0.15
D=0.20
D=0.25
D=0.30
D=0.32
Glucose concentration (g/L)
5
4
3
2
1
0
0
20
40
60
80
100
120
140
160
Time (h)

3.4.2 Kinetic study of *P. pastoris* cultivation with chemostat

Continuous cultivation is an effective tool to study cell growth and protein production, especially with respect to growth kinetics. We designed a continuous cultivation process with 40g/L glucose or glycerol in the feeding solution to study the influence of dilution rate upon cell growth and protein production. The process started with a low dilution rate and covered a wide range of dilution rate: 0.05 to 0.32 (1/h) upon glucose and 0.022 to 0.32 (1/h) upon glycerol. State variable such as OD600, residual substrate concentration, phytase activity, dissolved oxygen level (DO), agitation rate were monitored and analyzed to determine if steady state had been achieved. Generally, certain steady state was achieved in 2.5-3 volume changes. The results were shown in Figure 3.3 (a) and (b). As shown in Figure 3.3 (a) and (b), we found that both cell growth and phytase production were significantly influenced by dilution rate. Growing upon glucose, highest cell density and highest phytase activity were found at lowest dilution rates. The linear increase of dilution rate led to steady and consistent decrease of phytase activity. Although increasing dilution rate resulted in the decrease of biomass production in general, the evolution of biomass was different from that of foreign protein. In Figure 3.3 (a), we found that biomass concentration remained almost constant at 49 in OD600 for the first 3 dilution rates: 0.05 (1/h), 0.10 (1/h) and 0.15 (1/h). Increasing dilution rate from 0.15 (1/h) to 0.20 (1/h) and then 0.25 (1/h) led to stable decrease in biomass concentration, but, surprisingly, still no residual glucose or other by-products were detected in the broth. Such decrease in the yield of biomass indicated that a greater proportion of glucose was directed to energy metabolism or cell maintenance at higher dilution rates. Further increase of dilution rate resulted in dramatic decrease of the yield of biomass and accumulation of residual glucose, indicating that specific growth rate was close to its maximum level $\mu_{\max}$. At dilution rate of 0.30 (1/h), system was stable at OD600 33.6 and glucose concentration 1.39g/L. In addition, the HPLC analysis also detected the existence of an unknown by-product with peak area close to that of glucose (data not shown). When dilution rate was set to 0.32 (1/h), steady state could not be achieved any more. Therefore, $\mu_{\max}$ of *P. pastoris* growing upon glucose should be between 0.30 (1/h) and 0.32 (1/h).

The time profiles upon glycerol were very similar to that upon glucose, except for some details in the evolution of biomass production. Highest cell density of 50 (OD600) was observed at $\mu = 0.021(1/h)$. Then the yield of biomass decreased obviously at $\mu = 0.05(1/h)$, followed by gradual increase till $\mu = 0.15(1/h)$. After that, dramatic decrease in the yield of biomass was observed as in the case of glucose. |The $\mu_{\max}$ of *P. pastoris* growing upon glycerol should be between 0.30 (1/h) and 0.32 (1/h) as well.

In continuous cultivation upon both glucose and glycerol, $\mu_{\max}$ is obviously higher than in batch cultivation. This can be explained by the fact that in batch cultivation substrate concentration is mainly at very high level, which might inhibit cell growth. The value of $\mu_{\max}$ in continuous cultivation (between 0.30 and 0.32 (1/h)) is the highest compared to results of other researchers growing *P. pastoris* upon glucose or glycerol. The value of $\mu_{\max}$ reported in the literature were generally between 0.2 and 0.25 (1/h), such as 0.2 [9], 0.22 [10], 0.23 [11] and 0.247 [12]. The differences of $\mu_{\max}$ probably stem from the use of the different media and different measurement method. For example, Pal Khasa and coworkers obtained a value of 0.22 (1/h) in batch cultivation upon a complex medium YPDCA [10]. Ghosalkar and coworkers obtained a value of 0.23 (1/h) derived from results in continuous cultivation with Monod's equation and the medium they used was a defined medium they optimized [12].

As we showed earlier, the yield of biomass and the yield of phytase upon glycerol are both higher than upon glucose in batch cultivation. However, the result was completely opposite in the case of continuous cultivation. For any specific value of dilution rate, the yield of biomass and the yield of phytase upon glucose were higher than upon glycerol as shown in Figure 3.4. For example, at a moderate value of D = 0.15 (1/h), upon glucose the yield of biomass is 15% higher and the yield of phytase is 66% higher. Obviously, the results from batch cultivation in bioreactors or flasks might be misleading to determine the selection of carbon source, although that is the way used by many researchers [4-7].

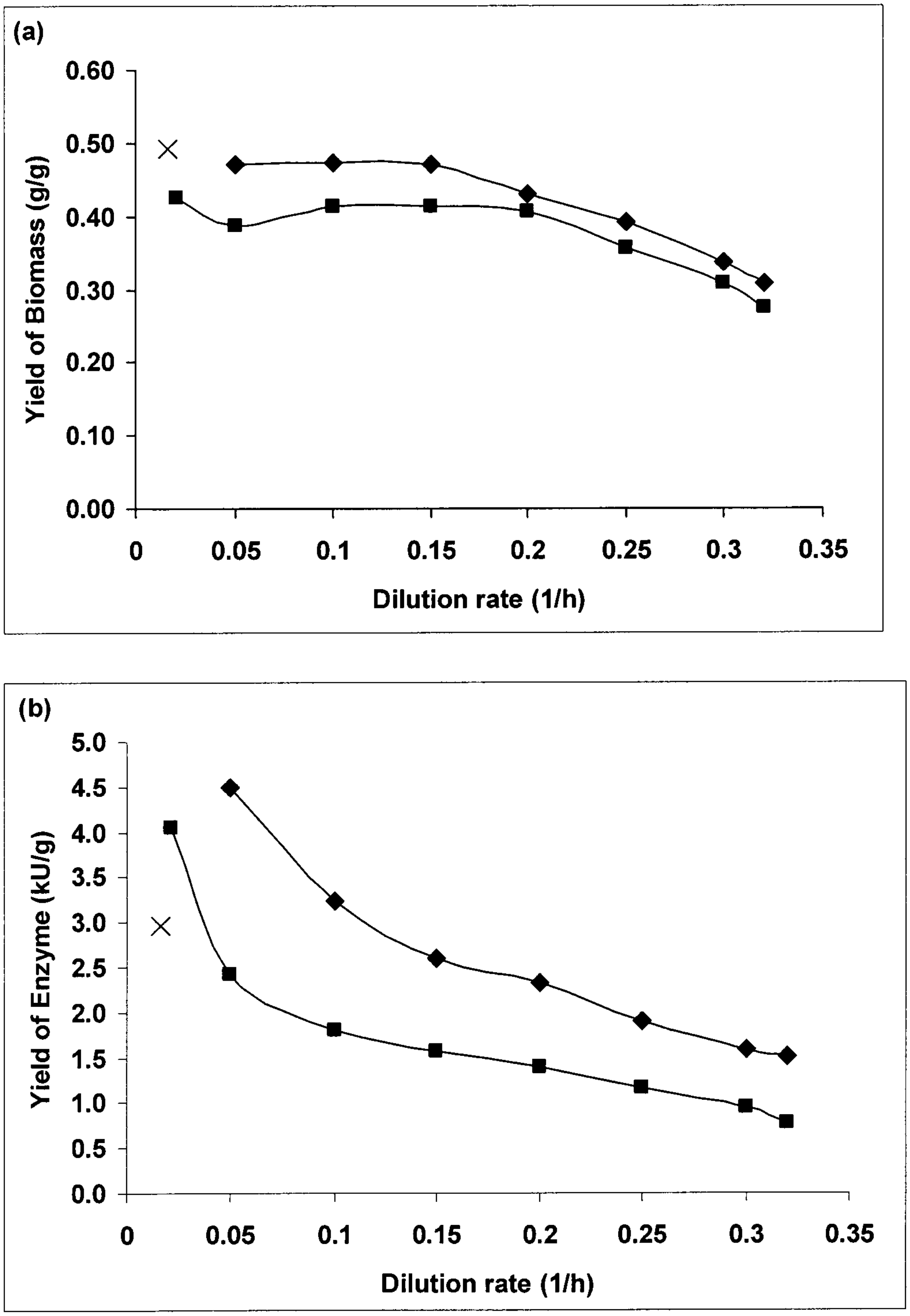


Figure 0.4 Results of kinetic study in Chemostat with respect to yield of biomass (a) and yield of enzyme (b) (glucose in 40g/L, solid diamond ◆; glycerol in 40g/L, solid square ■; glucose in 350g/L, cross ×)

Kinetic influence of heterologous protein production under AOX1 promoter is more widely studied than under GAP promoter. Although there were some disagreements, it was found that under AOX1 promoter highest protein yield was generally obtained at lower μ , while higher μ usually led to higher protein production rate [1]. This is consistent with our results as shown in Figure 3.4(b) and 3.5(a), although our system is under GAP promoter. However, other researchers found different results using GAP promoter. In continuous cultivation of *P. pastoris* expressing human granulocyte-macrophage colony stimulating factor (hGM-CSF) under GAP promoter, Khasa and coworkers found that both the yield of biomass and the yield of target protein increase with the increase of dilution rate, completely opposite to our results [10]. In continuous cultivation of *P. pastoris* expressing anti-HIV antibody 2F5 under GAP promoter, Maurer and coworker found that the yield of target protein decreased with the increase of dilution rate, while the yield of biomass increased with the increase of dilution rate [9]. Notably, in addition to the difference of target protein, in the two cases mentioned above, different media were also used compared to our condition. This might account for the differences of results.

Another way to express the influences of dilution rate upon cell growth and protein yield is in terms of specific protein formation rate and specific substrate uptake rate. As shown in Figure 3.5, upon glycerol and glucose, specific phytase production rate is in linear relationship with dilution rate, while specific substrate uptake rate is not. The two relationships can be simulated well with linear and polynomial regression.

3.4.3 Continuous cultivation in high cell density

One advantage of GAP promoter expression is that it is more favourable to the application of continuous cultivation to increase protein production rate [1]. Goodrick and coworkers proved that GAP system enabled longer production period than AOX1 system: high level production of foreign protein lasted for 30 days [2]. To study the feasibility of using continuous cultivation in high cell density, we performed a continuous cultivation with 350g/L glucose in half MSM medium. The resulting cell density was as high as 446 of OD600 as shown in Figure 3.6. However, under such high cell density, due to the limitation of oxygen supply (no pure oxygen was supplied), the highest

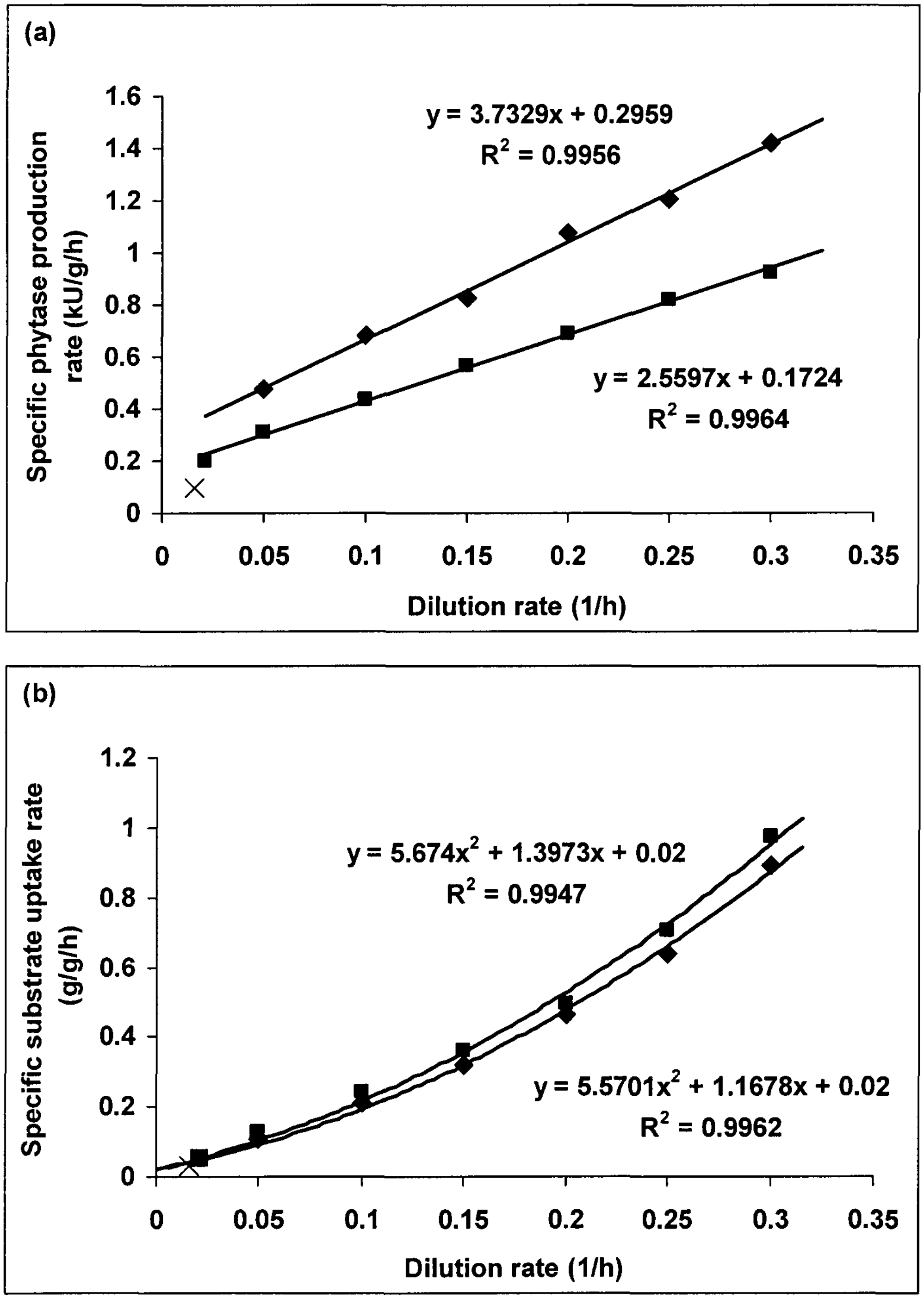


Figure 0.5 Results of kinetic study in Chemostat with respect to specific phytase production rate (a) and specific substrate uptake rate (b) (glucose in 40g/L, solid diamond ◆; glycerol in 40g/L, solid square ■; glucose in 350g/L, cross ×)

dilution rate can only be maintained at about 0.016 (1/h). At this condition, the yield of biomass upon glucose was 0.493 g/g, similar to the values at lower dilution rates in continuous cultivation with 40g/L glucose, as shown in Figure 3.4 (a). However, the yield of phytase upon glucose was as high as the value at $\mu = 0.05$ (1/h), although its μ was much lower, as shown in Figure 3.4 (b). The corresponding specific phytase production rate is much lower than expected as well, as indicated in Figure 3.5(a). The resulting volumetric productivity in this continuous cultivation system was only similar to that obtained in regular fed-batch cultivation. The reasons underlying in the resulted differences in continuous cultivation in high cell density and low cell density were not determined. There could be several possibilities. It might be just the effect of cell density. Zhang and coworkers found that protein yield was strongly and jointly affected by both dilution rate and cell density in the cultivation of *P. pastoris* for continuous production of foreign protein under AOX1 promoter [13]. Alternatively, such high cell density can result in the depletion of certain nutritional components necessary for foreign protein production. Our lab will conduct more experiments to study the influence of cell density in the future.

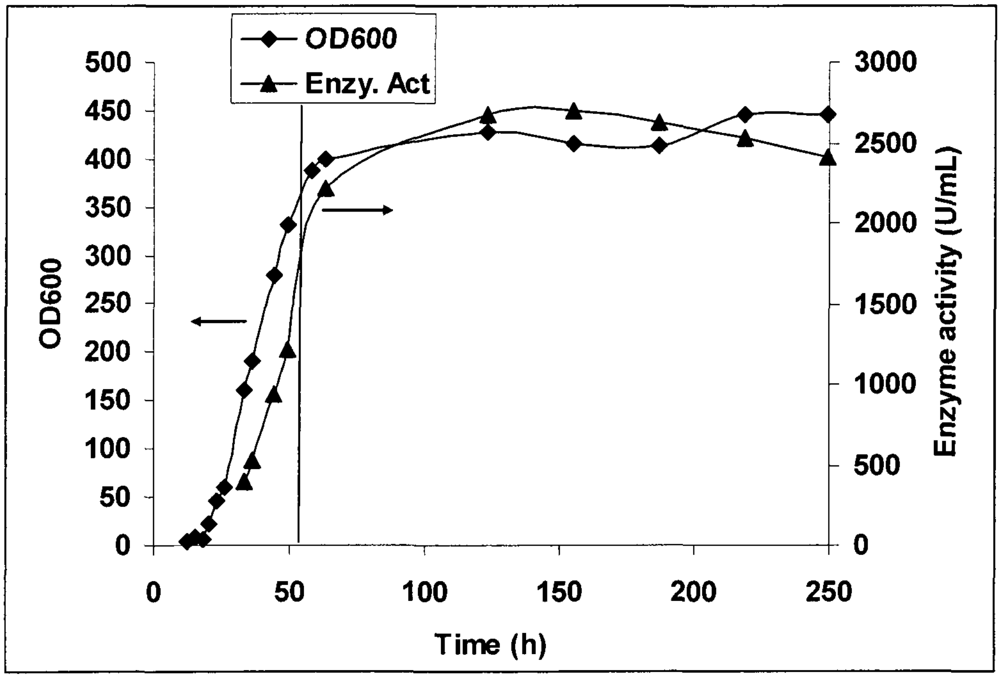


Figure 0.6 Continuous cultivation with 350g/L glucose

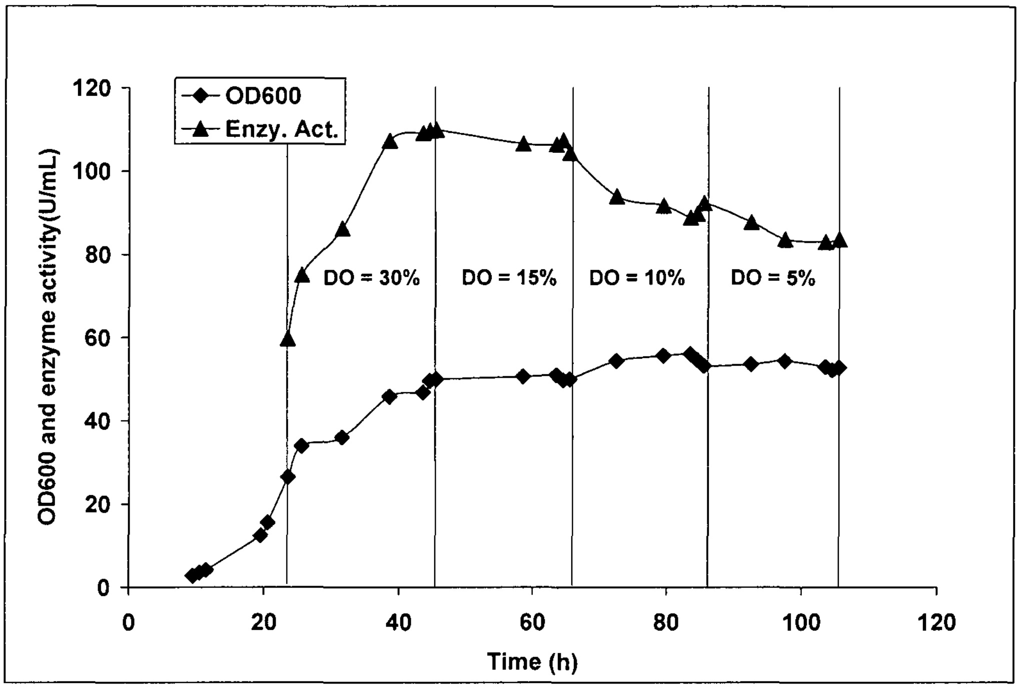


Figure 0.7 Continuous cultivation with 40g/L glucose and μ = 0.15 (1/h) under different DO

3.4.4 The effect of DO in continuous cultivation

DO is an important state variable in the control of fermentation. For the aerobic cultivation of *P. pastoris* , DO is generally supposed to be kept high. However, influences of DO upon cell growth and foreign protein production under GAP promoter were not well studied. Here, we used continuous cultivation with 40g/L glucose and μ = 0.15 (1/h) under different levels of DO to study its effects. The results are shown in Figure 3.7. From the figure, we found that in the range of 5% to 30% DO had little effect on cell growth, but negative impact on phytase production. When DO decreased from 30% to 5%, the production of enzyme decreased by nearly 25%. To be noted, in our experiments, no fermentative by-product (such as ethanol) was detected for DO as low as 5%. It can be concluded that our cultivation still remained in aerobic domain and in this situation higher DO level was more favourable. By applying hypoxic conditions to continuous cultivation of *P. pastoris* expressing an antibody Fab fragment under the GAP promoter, Baumann and coworkers obtained a 2.5-fold increase of the specific productivity at low oxygen supply[8]. In their experiments under hypoxic conditions, oxygen supply was greatly limited and fermentative ethanol was produced. We are conducting similar experiments in the future to see if we can have similar results under such condition. But according to our present results, oxygen limitation is detrimental to protein production.

3.4.5 Kinetic Modeling

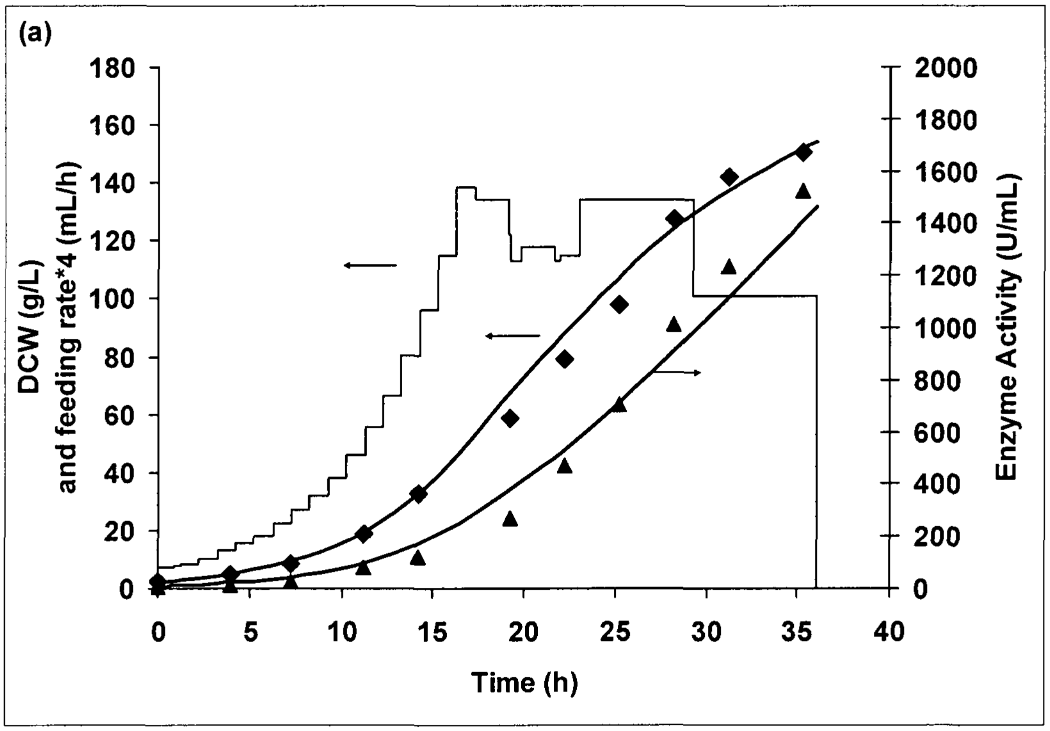
Although there were plenty of journal papers having reported modeling of cell growth and recombinant protein production in *P. pastoris*, most of them dealt with the expression system under AOX1 promoter, few under GAP promoter. As far as we know, there is only one paper reporting the modeling of the process under GAP promoter, i.e. the one by Maurer and coworkers [9]. For modeling, they assumed linear relationship between q_s (specific substrate uptake rate) and μ and utilized a Monod's type equation to relate q_p (specific product formation rate) to μ. Both of the two assumptions are different from our observations in continuous cultivation as shown in Figure 3.5.

Based on our findings in continuous cultivation, a simple kinetic model was constructed as described in the section of "Kinetic Model Description". In this model, there are six parameters needed to be estimated, that is, K_u, $Y_{S/X}$, m, $Y_{P/X}$, Y_m, and K_P. The values of K_u, $Y_{S/X}$ and m were directly estimated with regression of data from continuous cultivation of glucose as shown in Figure 3.5 (b). The other three parameters account for the description of phytase production. $Y_{P/X}$ and Y_m were estimated from linear regression of data from continuous cultivation as shown in Figure 3.5 (a). In Equation (4), the term of $\frac{K_P}{X/V+K_P}$ aims to account for the negative effect of cell density upon phytase production. K_P was estimated with previous experimental data of fed-batch cultivation, equal to 150g/L. If the term of $\frac{K_P}{X/V+K_P}$ is deleted, then model parameters are estimated completely based on continuous cultivation results. However, overestimation of phytase production was found when using this simplified model without the term to predict results in fed-batch cultivation. This indicates the difference of phytase production kinetics in continuous cultivation and fed-batch cultivation. The estimated values of the six parameters were listed in Table 3.2.

Table 0.2 Estimation of model parameters

Parameters	K_u	$Y_{S/X}$	m	$Y_{P/X}$	Y_m	K_P
Estimation	5.570	1.168	0.020	3.733	0.296	150

With the estimated parameters listed in Table 3.2, the model was used to predict cell growth and phytase production in fed-batch cultivation under limited glucose feeding. Three batches of data of fed-batch cultivation were used to test the model's predictive performance with the results shown in Figure 3.8. As shown in the Figure 3.8, model prediction fits pretty well with experimental results, indicating the powerful capacity of the model.



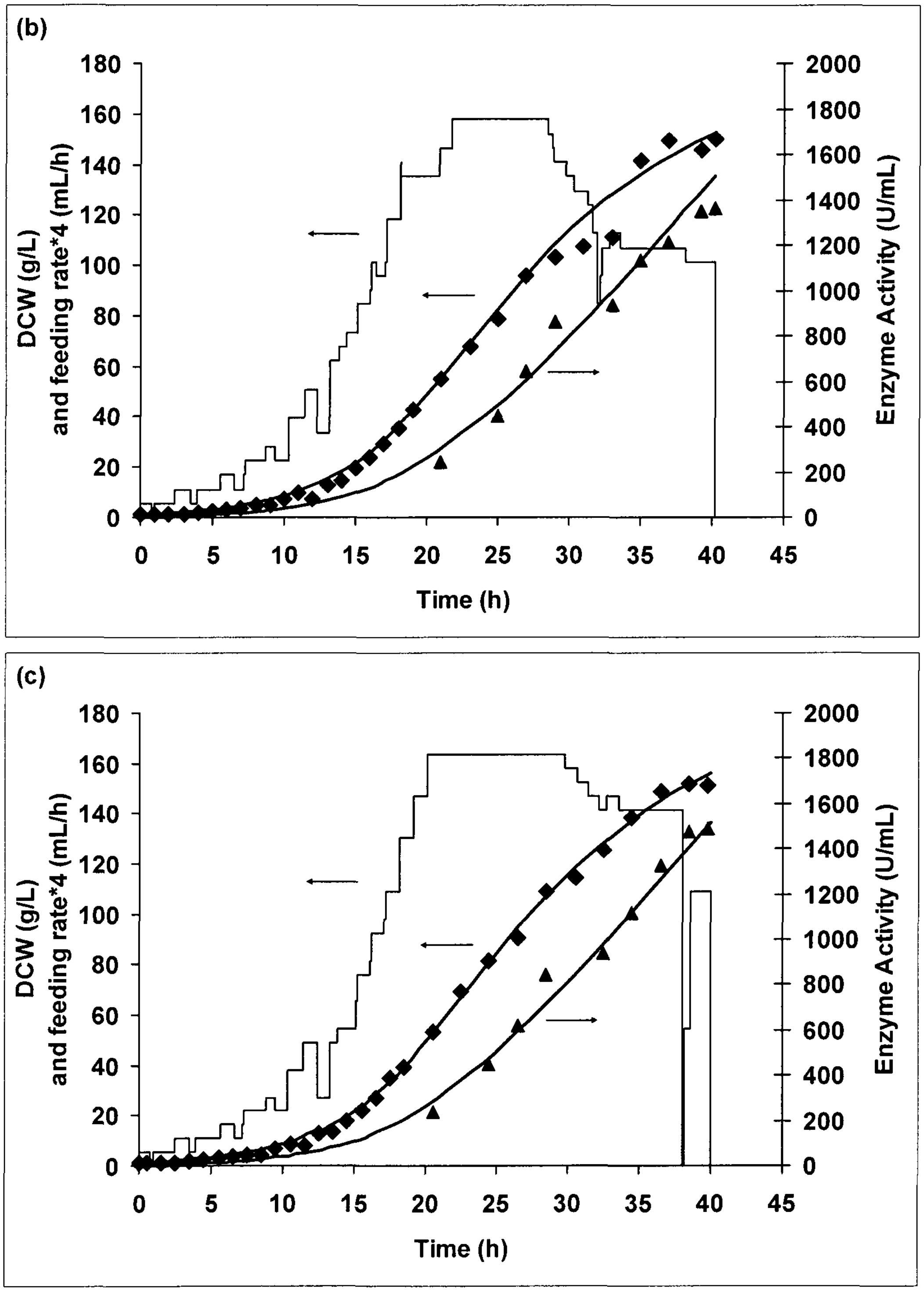


Figure 0.8 Comparison of experimental results with model prediction in three fed-batch cultivations (a), (b) and (c): DCW, solid diamond ◆; enzyme activity, solid triangle ▲; substrate feeding rate, thin line; model prediction, thick line.

3.5 Conclusions

We investigated phytase production with the cultivation of *P. pastoris* in batch, continuous and fed-batch processes. In comparative batch cultivations upon glucose and glycerol, phytase production with glycerol is slightly higher than with glucose, while the yield of biomass with glucose is much lower than with glycerol. This result was consistent with the detection of fermentative by-products (ethanol and acetate) in the case of glucose. In continuous cultivation, however, the results were completely opposite, since glucose outperformed glycerol in terms of either the yield of phytase or the yield of biomass. Furthermore, kinetic studies in continuous cultivation revealed that both cell growth and phytase production under GAP promoter were greatly influenced by specific growth rate or dilution rate. Specific phytase formation rate increased linearly with dilution rate; specific substrate uptake rate increased with dilution rate as well, but more aggressively than linear increase. In continuous cultivation of 350g/L glucose, high cell density cultivation was realized, but the yield of phytase was much lower than expected, indicating the potential negative influence of cell density upon phytase production. We also performed continuous cultivation to study the effect of DO and found that phytase production decreased significantly with the decrease of DO, but little impact was found upon biomass production in the range of DO: from 30% to 5%. Based on results in kinetic studies with continuous cultivation, we established a simple kinetic model to describe cell growth and phytase production. With model parameters mainly estimated with data from continuous cultivation, the model gave excellent performance in the prediction of both cell growth and phytase production in fed-batch processes, as compared to experimental data of three different processes.

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