Study of Operational Strategies and Carbon Source Selection for the Production of Phytase using *Pichia pastoris*

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Abstract

The methylotrophic yeast *Pichia pastoris* has become an efficient expression system for heterologous protein production. Different methods have been studied to enhance cell growth as well as the production of products of interest. Two of the major strategies for improving the product or biomass yields are optimizing bioprocess controls and cultivation conditions.

In this work, the characteristics of this yeast system and of its different promoters are discussed, and the effect of operational strategies on cell growth and recombinant protein expression is also studied. The effect of different feeding strategies were studied and optimized for pGAP (glyceraldehyde-3-phosphate dehydrogenase)-regulated phytase production in *P. pastoris*. Alternative carbon sources were screened and the feasibility of using citric acid as a carbon source for recombinant protein production was also investigated. The effects of parameters such as the carbon source concentration and culture pH were studied using shake-flasks, and the effect of different feeding profiles on bioreactor performance was also investigated.

Three feeding strategies, Stepwise feeding, Exponential feeding and DO-stat feeding were tested and DO-stat was found to be more efficient and led to a high phytase activity. A modified DO-stat method was investigated to overcome the oxygen limited condition in the standard DO-stat method. For the carbon source, citric acid showed promise in improving phytase expression. Further experiments in bioreactors performed with the presence of certain amount of citric acid showed that less glycerol could be used to achieve the same level of phytase activity.

Résumé

La levure méthylotrophique *Pichia pastoris* est aujourd'hui considérée un des systèmes les plus efficaces pour la production de protéines recombinantes. Différentes méthodes sont disponibles afin d'améliorer les rendements de ces produits d'intérêt, et d'augmenter la croissance cellulaire dans ce système. Les stratégies principales utilisées pour accomplir ces objectifs sont l'optimisation des systèmes de contrôle, et l'optimisation des conditions de culture.

Le travail décrit dans cette œuvre discute les caractéristiques de la levure *P. pastoris,* les différents promoteurs utilisés dans ce système, et les effets qu'ont les différents paramètres d'opération sur la croissance cellulaire et la production de protéines. Les effets de différents modes d'alimentation furent également étudiés et optimisés pour l'expression de phytase par *P. pastoris* sous le contrôle d'un promoteur pGAP (glyceraldehyde-3-phosphate déhydrogénase). Des sources de carbone alternatives furent investiguées, et l'utilisation de l'acide citrique comme source de carbone fut étudiée en profondeur. Les effets des paramètres opérationnels, tels que la concentration d'acide citrique et le pH de la culture furent étudiés dans des cultures en flacons, et l'impact de différentes stratégies d'alimentation sur la performance de la culture fut aussi investigué dans des cultures en bioréacteur.

Trois stratégies d'alimentation, y compris l'alimentation par intervalle, l'alimentation exponentielle, et l'alimentation à concentration d'oxygène constante furent caractérisées, et il fut déterminé que cette dernière stratégie est la plus efficace en termes d'activité

enzymatique. Afin de minimiser les conditions dans lesquelles l'oxygène est limité dans les bioréacteurs, une nouvelle stratégie d'alimentation à oxygène constante fut utilisée. Les expériences en bioréacteur ont démontré qu'en ajoutant de l'acide citrique aux cultures, on peut produire autant de phytase en utilisant moins de glycérol que dans les cultures qui utilisent seulement le glycérol.

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List of Acronyms

Acronym	**Definition**
Promoters	
AOX	alcohol oxidase
GAP	glyceraldehydes-3-phosphate dehydrogenase
FLD	formaldehyde dehydrogenase
ICL	isocitrate lyase
TEF 1-α	translation elongation factor 1-α
Medium	
BSM	basalt salt medium
YPD	yeast extract-peptone-dextrose medium
MSM	minimal salt medium
m-MSM	modified MSM
Bioprocess control	
GBP	glycerol batch phase
GFBP	glycerol fed-batch phase
MIP	methanol induction phase
DO	dissolved oxygen
OD	optical density
DCW	dry cell weight
PA	Phytase acitivity
Terms	
$Y_{X/S}$	biomass yield based on the substrate (g[DCW]/g[S])
F	feeding rate (g/h)
x	Biomass concentration (g/L)
μ	the specific growth rate (h^{-1})
vvm	aeration rate, gas volume flow per unit of liquid volume per minute (volume per volume per minute)
FTU	Phytase unit
Others	
HPLC	high-performance liquid chromatography

Chapter 1

Introduction

Over the last few decades, DNA manipulation has developed as a powerful technology that is commonly used to express heterologous proteins in a variety of organisms. Since many of these recombinant proteins—such as industrial enzymes and pharmaceutical proteins—have vast commercial applications, countless studies have been completed or are underway that seek to investigate and improve efficiency and productivity of these systems.

Pichia pastoris has been adopted as a useful model for heterologous protein expression due to several of its advantageous characteristics, including its ability to grow in simple media and its potential to perform many eukaryotic modifications. The main contributor to the success of this strain is the powerful alcohol oxidase (AOX) promoters. Since methanol is required to induce the expression of proteins under pAOX control, alternative promoters were developed that eliminate the need to use and store large quantities of methanol, which is quite hazardous. One of these alternative promoters is glyceraldehyde-3-phosphate dehydrogenase (GAP), a strong constitutive promoter.

Most of the work aiming to improve the production of the proteins of interest is based on two processes, namely carbon source screening, and bioprocess control. However, little work has been done using the GAP system since it is relatively new. Moreover, optimal physico-chemical conditions vary based on different proteins and promoters, and little specific work has been done to examine phytase production in *Pichia pastoris* under pGAP control. In terms of bioprocessing, fed-batch cultivation is a method that can obtain high cell densities, and the feeding strategies used have a great impact on biomass and protein production. Few studies have been performed that compare different feeding methods, and optimal feeding strategies remain controversial with respect to various strains and proteins. For carbon sources, glycerol and glucose are still dominant for the fermentation of *Pichia pastoris*. Less expensive or higher performance carbon sources should be evaluated as alternatives with the objective of achieving lower cost or higher productivity fermentation systems.

Based on these considerations, this research consists of three parts, as follows:

1. The investigation of the use of *Pichia pastoris* as an expression system, including its performance under different promoters and systems, the physical and chemical conditions used for its cultivation, and the bioprocess controls used in bioreactors.
2. The study of the effects of bioreactor control strategies on phytase production in *Pichia pastoris*.
3. Testing the feasibility of using a novel carbon source, citric acid, for the production of phytase in *P. pastoris*, and to determine the optimal conditions for cell growth in flasks and bioreactors.

This research, divided into these three sections, aims to study the efficiency of a system for phytase production in *Pichia pastoris* under pGAP control, from the aspect of both bioprocess control and carbon source screening.

Chapter 2

Heterologous Protein Production in *Pichia pastoris*: A Review

2.1 Introduction

In the early 1970s, *Pichia pastoris* attracted attention as a promising source of single-cell protein (SCP), mostly because of its ability to use methanol as the sole carbon source. The media and protocol for growing *P. pastoris* in a continuous culture at high cell density (>130g/L dry cell weight (DCW)) were first developed by the Phillips Petroleum Company [1]. However, the appeal of using this source of SCP decreased in the 1970s because of the oil crisis that caused a rise in the cost of the carbon source, methanol [2].

In the following decade, the promoter and the gene correlating to this characteristic were isolated, and corresponding protocols were developed by the Phillips Petroleum Company, together with the Salk Institute Biotechnology/Industrial Associates, Inc. (SIBIA, La Jolla, CA), as they studied *P. pastoris* as an organism for foreign protein expression [2]. Since then, *Pichia pastoris* has become a successful platform for heterologous protein expression; by 2006, there were more than 500 proteins expressed using the *Pichia pastoris* system [3].

The methylotrophic yeast *Pichia pastoris* has been widely used in both basic laboratory research and industrial production [4]. It can grow on cheap and common media as well as on defined medium, at high cell density. Because of this characteristic, parameters such as pH, option and feed rate of carbon sources, and aeration, which will influence the growth of cells and the production of protein during the fermentation of *Pichia pastoris,* can easily be controlled [5]. Moreover, this yeast has the ability to correctly fold foreign proteins and perform post-translational modifications such as glycosylation and disulfide bond formation. Compared with the most common prokaryotic host for protein expression, *Escherichia coli, Pichia pastoris* is more suitable for expressing certain types of proteins, including enzymes. For example, expression vectors containing various cDNAs from a human foetal brain expression library for *P. pastoris* and *E. coli* were constructed by Lucking *et al.* and coworkers. From the 29 different clones with a correct reading frame that were identified, all were found to produce soluble protein in *Pichia pastoris,* while only 31% could produce soluble protein in the *E. coli* system [6]. The purification of the secreted recombinant proteins from other cellular components is simple as only low levels of the native protein are secreted in this organism [7]. The foreign proteins expressed in *P. pastoris* can be secreted through genetic modifications such as the addition of signal peptides, which considerably simplifies downstream product purification and recovery [8].

2.2 Promoters Used in the *P. pastoris* System

2.2.1 AOX Promoter

Forty years ago, the abilities of certain yeasts that used methanol as their sole carbon source were described by Ogata *et al.* [9]. The methanol-inducible promoter alcohol oxidase (pAOX) is, to a large extent, responsible for the widespread use of *P. pastoris* as an expression system. AOX is the first enzyme in the methanol utilization pathway, catalyzing the oxidation of methanol to formaldehyde and hydrogen peroxide [10], and its level can reach up to 35% of the total cellular protein (TCP) content grown with methanol as the sole carbon source [7, 11].

There are two genes that encode the enzyme alcohol oxidase in *P. pastoris*: AOX1 and AOX2. It has been reported that the former, the AOX1 promoter, is the most widely used of all the promoters available in *P. pastoris* [12], and accounts for up to 95% of the total expressed alcohol oxidase [4]. This strong promoter is therefore preferred for the expression of the recombinant proteins, even when there is only a single copy of the gene in the cassette [7, 13]. It is also responsible for the utilization of approximately 85% of the methanol in the SMD 1168 and GS115 strains, which contain one or more functional copies of the alcohol oxidase 1 gene [14]. These strains having a wild-type methanol utilization phenotype are recognized as Mut$^+$ [7]. In methanol cultivation, approximately 5% of poly(A)$^+$ RNA is from AOX1, but for the majority of the other carbon sources, such as glucose and ethanol, the expression under pAOX1 is undetectable [15, 16]. It appears that there are two mechanisms of regulation for the pAOX promoter: a repression/derepression mechanism plus an induction mechanism [2]. The presence of methanol can result in a high level of transcription of pAOX1, while the presence of repressing carbon sources does not result in such transcriptions [17]. Although high expression levels are achieved with methanol induction, methanol is a toxic substance and associated with considerable health and environmental concerns. The most common source of methanol is the petrochemical yield, and methanol is not a prime carbon source for the production of food and medicine [18]. In addition, the transportation and storage of large volumes of methanol are costly and dangerous.

The AOX2 gene codes for functional enzymes which have the same specific activity as AOX1, but with a much lower expression level and slower methanol consumption rate [7, 14, 19]. For these reasons, pAOX2 is considered a weak promoter. Strains that contain a non-functional AOX1 and rely on AOX2 are named “methanol utilization slow” (Mut^s). Since the AOX1 gene is deleted, the growth is dependent on the AOX2 expression [20]. In order to eliminate the hazard of using a large volume of methanol, in some research Mut^s strains have been applied as hosts for foreign protein expressions. Due to the lower expression levels of AOX, Mut^s strains can tolerate a lesser concentration of methanol and thus the growth of cells cannot reach as high of a level if grown only on methanol [14]. One strategy used for expressing proteins in Mut^s strains is the mixed-feed fermentation strategy. Feeding methanol and alternative carbon sources such as sorbitol or mannitol can support the growth of *P. pastoris* while inducing expression by the pAOX promoter [11, 19]. Some research was completed using the AOX2 promoter, and certain cases showed higher productivity in Mut^s strains. Hepatitis B Surface Antigen (HBsAg) was expressed in a single culture of the *P. pastoris* HBsAg expression strain, in a volume of 240 liters with the yield of 90 grams, which is sufficient for approximately 9 million doses of vaccine [21]. *Rhizopus oryzae* lipase was produced in Mut^s, and the specific productivity is 1.1-folds higher than when using Mut$^+$ [22].

Both AOX1 and AOX2 are deleted in methanol utilization minus (Mut^-) strains. Therefore, Mut^- strains cannot grow on methanol. Due to this strain's inability to utilize methanol, the use of alternative carbon sources for the growth and expression of heterologous proteins is required. Nonetheless, this strain's slow growth rate may be an advantage for the expression of certain products [4, 16, 21].

2.2.2 GAP Promoter

The glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter (pGAP) was first isolated by Waterham in 1997 [18], and has since been used for the expression of many heterologous proteins. Proteins regulated by the GAP promoter are expressed constitutively and therefore not require methanol for induction. As opposed to the pAOX system, the biomass and the expression of recombinant proteins occur simultaneously in the pGAP system [20]. The fermentation and cultivation of *P. pastoris* is therefore simpler as there is no requirement for the utilization and storage of methanol, and more suitable for the large-scale production of heterologous proteins and continuous cultures [23, 24].

Many recombinant proteins have been expressed by using the GAP promoter-driven *P. pastoris* system. The expression levels of different heterologous proteins vary greatly, and may be dependent on the properties of these specific proteins. Some research shows that higher protein yields can be obtained using the GAP promoter than with AOX promoter. For example, with the pGAP system, the expression level of *Yarrowia lipolytica* lipase LIP2 (YlLIP2) increased, and the culture time was shortened from 160 h to 8 h [25]. However, results vary from study to study. The maximum level of expression of the Hepatitis B surface Antigen (HBsAg) by a single copy of the pAOX1 integrant exceeded the level achieved by the single copy of the pGAP integrant [24].

The efficiency of expression of the recombinant protein may be influenced by the pGAP-regulated gene dosage. Vassileva and coworkers [24] isolated multi-copy clones of *P. pastoris* integrants and found that the expression level of the HBsAg is directly correlated to the gene dosage. Four copy clones achieve approximately four times higher yields of HBsAg than do single copy clones. On the other hand, a report showed that there was no effect on the expression of the human trypsinogen gene between different gene copy numbers in the pGAP system [26]. More research is required to determine which factors have an impact on the cell biomass and expression yields of the desired proteins.

2.2.3 Alternative Promoters

Many heterologous proteins have been expressed in *P. pastoris* under the control of different promoters. A summary of some proteins expressed using different promoters is shown in Table 2.1. In addition to the pAOX and pGAP promoters, there are other promoters that have been widely applied, such as pTEF1, pFLD1, pICL1, pYPT1, pPGK1 (3-phosphoglycerate kinase gene). pFLD1, a strong promoter, has been accepted as an alternative to pAOX1 [27]. The pTEF1 promoter, a constitutive promoter, has the strength comparable to that of pGAP [28]. The pYPT1 (a yeast GTP binding protein [29]) promoter and pPEX8 (a peroxisomal matrix protein involved in peroxisome biogenesis [30]) promoter are weak promoters and have not been widely applied for the expression of heterologous proteins. The majority of the research conducted thus far aims to achieve a maximum cell density or a higher expression of foreign proteins [2], which is why these weak promoters were not viable options. However, high-level expression of certain proteins expressed under the control of strong promoters may overwhelm the post-translational modification pathways of cells, making these weaker promoters more desirable in certain cases [31].

Table 2.1 Summary of some heterologous proteins expressed under the control of different promoter in *Pichia pastoris*

Series	Promoter	Inducer	Heterologous Proteins	Reference
Inducible	**pAOX**	methanol	Angiostatin	[32, 33]
			Anti-carcinoembryonic Antigen Single Chain Fv Antibody Fragment	[34]
			Hepatitis B Surface Antigen (HBsAg)	[21]
			Human Erythropoietin (rHuEPO)	[35, 36]
			Laccase	[37, 38]
			Ovine Pregnancy Recognition Hormone Interferon-τ (rOvIFN-T).	[39]
			Tetanus Toxin Fragment C	[13]
	pFLD	methanol or methylamine	B-lactamase	[27]
			Rhizopus oryzae Lipase	[40]
	pICL	ethanol	Dextranase	[41]
Constitutive	**pGAP**		Angiostatin	[42]
			Aqualysin I	[43]
			Hepatitis B Surface Antigen	[24]
			Human Granulocyte-macrophage Colony-Stimulating Factor (hGM-CSF)	[44]
			Human Serum Albumin (HSA)	[45]
			Streptokinase (SK)	[46]
			Vitellogenin	[47]
			Yarrowia lipolytica Lipase LIP2	[25]
	pTEF		CLLip	[28]
			Human Serum Albumin (HSA)	[45]
	pPGK1		α-amylase	[48]
	pYPT1		β-glucuronidase	[49]

2.2.3.1 Inducible Promoters

2.2.3.1.1 pFLD1 Promoter

The FLD1 gene encodes a glutathione-dependent formaldehyde dehydrogenase (FLD), which is a key enzyme in the methanol metabolic pathway as well as in the methylated amines metabolism in *P. pastoris* [50]. FLD also helps to protect the cell from the toxic effects caused by the accumulation of formaldehyde, which is a product of the methylamine metabolism [27]. This promoter can be induced by both methanol and methylamine. The induction of the recombinant protein in *P. pastoris* can be achieved by either using methylamine as a nitrogen source (inducible substance) with glucose or glycerol as a carbon source, or by using methanol as the sole carbon source and ammonium sulfate as the nitrogen source. One advantage of the pFLD1 system is the elimination of the hazards caused by the use of methanol as inducer, making it a good alternative promoter for certain heterologous proteins that require methanol-dependent expression [4, 27] .

Some recombinant proteins have been produced by using the pFLD1 system in *P. pastoris*. For instance, a *Rhizopus oryzae* lipase (ROL) has been expressed in *P. pastoris* using the FLD1 promoter [40]. This report, executed by Resina *et al*., showed that pFLD1 was as efficient as pAOX1. In addition, a methanol-free culture strategy has been achieved using the pFLD system, which makes it possible to obtain a high cell density culture both on a large and a small scale. Another study showed that the specific productivity of ROL was 3.4 times higher when using the pFLD1 system than when using the pAOX1-based system [22].

2.2.3.1.2 pICL1 Promoter

In 2003, the isocitrate lyase (ICL) gene was cloned and characterized from *Pichia pastoris*. The expression of the dextranase gene (*dexA*) from *Penicillium miniluteum* under pICL1 has also been reported [41]. According to this report, the expression of the ICL1 gene was induced by ethanol and repressed by glucose. Thus, the ICL1 promoter may be an alternative to the AOX1 promoter, especially when it comes to certain proteins that are cytotoxic and where concentration control is required. However, very few studies have been conducted and further research is needed with pICL1 in order to find the optimal promoter and protein formation conditions [51].

2.2.3.2 Constitutive Promoters

2.2.3.2.1 pTEF Promoter

The promoter for the translation elongation factor 1-α (TEF 1-α) was successfully isolated from *P. pastoris* in 2007, and has proven to be a constitutive promoter [28]. TEF1 is involved in the transportation of aminoacyl transfer RNA (tRNAs) to ribosome. The expression of a reporter gene, the lipase gene from *B. stearothermophilus* L1 fused to the CBD (CLLip), was studied to compare the strength of the pTEF promoter and the pGAP promoter. The results showed that the pTEF1 had tighter growth-associated expression characteristics than pGAP, and the expression of the reporter gene under the control of pTEF achieved two-fold higher than using pGAP in a carbon-limited fed-batch culture system. In addition, the expressions of human serum albumin (HSA) under pTEF and pGAP were achieved, and the highest HSA concentrations reached were 57.2 mgL^{-1} and 44.2 mgL^{-1} in the two systems respectively [45]. pTEF may become a good alternative to pGAP, with a wider choice of production conditions and simpler expression methods [28].

2.3 Culture Media and Operational Conditions

2.3.1 Effect of Culture Media

P. pastoris is ideal for large-scale production of recombinant proteins, one of the reasons being that the growth conditions and medium components are inexpensive and well defined [2]. The most common medium for the cultivation and fermentation of *P. pastoris* is the Basal Salt Medium (BSM) proposed by Invitrogen Co [52]. There are some alternative media developed by other researchers, such as the FM22 medium proposed by Stratton *et al*. [53]. The trace element solution PTM1 is usually added to the BSM medium during fermentation, and biotin, as a component of the PTM1 solution can act as growth factor [54]. Boze and co-workers found that the presence of vitamins and trace elements have a great impact on protein expression [55]. Some research projects studied the composition of media needed in order to maximize biomass or production levels of certain proteins. Liu *et al.* adopted a Plackett-Burman design to screen the medium composition of BSM for phytase production when grown on glycerol, and K_2SO_4, $CaSO_4•2H_2O$ and $MgSO_4•7H_2O$ were identified as having a significant effect on the productivity of the system [56]. Ghosalkar and co-workers had optimized a defined medium including basic carbon sources, mineral chemicals, and vitamin solutions, and under that medium a biomass of 11.25 g DCW/L was achieved [57].

The nitrogen source is one of the important parameters in the cultivation of *P. pastoris*. Commonly, ammonium hydroxide is used as the nitrogen source in BSM, and is also employed to adjust the pH during fermentation. The concentration of ammonia needs to be controlled, as high concentrations can inhibit cell growth and prolong the lag phase [4, 58]. Nitrogen starvation may occur during high cell density fermentation and have a negative influence on heterologous protein expression, but certain researchers have found that using ammonium sulfate may overcome this limitation [57]. Other nitrogen sources commonly used are yeast extract, peptone, and casamino acid [59]. Casamino acids added as a nitrogen source has the ability to minimize proteolysis and increase protein production [60-62].

2.3.2 Effect of Carbon Sources

P. pastoris, as a methylotrophic yeast, attracted attention for the expression of foreign proteins mostly because of its ability to utilize methanol. Methanol simultaneously acts as an inducer, driving the expression of the pAOX promoter, and as the sole source of carbon and energy. The concentration of methanol is important and needs to be monitored during the cultivation, as high levels of methanol are cytotoxic, while low levels may not be sufficient to initiate the induction of the promoter [2, 63]. Although *P. pastoris* can grow with a high protein expression level on methanol, the cell density and growth rate are both lower than on other carbon sources. Some strategies have previously been developed for the cultivation and fermentation of high concentrations of *P. pastoris* [64]. Commonly, cells are initially grown in a medium containing glucose or glycerol as a carbon source, in order to get high cell density. Paulová *et al*. reported that *P. pastoris* grown on glucose alone achieved 0.28 h^{-1} as the maximum specific growth rates, compared with 0.12 h^{-1} when grown on methanol [65].

Cells grown on glucose or glycerol can achieve high cell densities, which may contribute to the high-level expression of heterologous proteins. However, the production of proteins in *P. pastoris* under the control of pAOX1 is sensitive to the concentration of certain repressing carbon sources, even at low concentrations. The repressing non-C_1 carbon sources are members of either a glucose- or an ethanol-type family of compounds [66] and may fully repress the activity of pAOX1, which will have a negative effect on the expression of proteins [67]. The experiment conducted by Inan and Meagher tested the expression of β-galactosidase under four combinations of carbon sources: ethanol-glycerol, ethanol-methanol, methanol-glycerol, and ethanol-methanol-glycerol, and the results showed that the expression of the protein of interest occurred only when methanol was used [68]. This experiment also indicated that an ethanol concentration of 10 mg/L repressed the pAOX promoter in shake-flask cultures [68].

Since certain carbon sources showed repression of protein expression under an inducible promoter, some researchers therefore focused on investigating alternative non-repressing carbon sources. Sorbitol is widely accepted as a non-repressing carbon source for *P. pastoris*; it was suggested by Sreekrishna in 1997 [15] and further proven by Thorpe and coworkers [67]. Thorpe *et al*. compared glycerol/methanol and sorbitol/methanol mixed-feed fermentation strategies for the production of sea raven antifreeze protein (srAFP) using a Mut^s strain. The results showed that the level of protein expression on sorbitol/methanol was comparable to that on glycerol/methanol, and the presence of sorbitol in the broth was less repressive to pAOX [67]. Another experiment done by Inan and coworkers indicated that alamine, mannitol, and trehalose did not repress β-gal production and could be used as sole carbon sources, with methanol as an inducer [19]. Lactic acid has also been tested as a non-repressing carbon source and has a higher level of production of angiostatin than sorbitol [69]. The screening of non-repressing carbon sources may be helpful for large-scale production using the pAOX promoter, since the residual concentrations of non-repressing carbon sources do not repress expression as much as glucose does. Additionally, the study of the expression of proteins under non-repressing carbon sources using the Mut$^-$ strain had same, or even higher levels, compared to those obtained using the Mut$^+$ strain [68], which could eliminate the need to use methanol and avoid all of its hazardous properties.

For the constitutive promoter system, methanol is not required as an inducer, and the carbon source options are therefore greater. Some experiments have previously been conducted to test the performance of different carbon sources. Glycerol and glucose are the most commonly used carbon sources for the cultivation of *P. pastoris* under a constitutive promoter system. An experiment tested the effect of six carbon sources, including glucose, glycerol, methanol, sorbitol, oleic acid, and maltose, on *Yarrowia lipolytica* Lipase LIP2 (YlLIP2) production and cell density in flasks. The highest cell biomass, 15.9 g DCW/l, was achieved on glycerol and the second highest by glucose. However, in terms of the YlLIP2 activity, glucose was the carbon source with the highest activity, with 900 U/mL, while glycerol only reached 620 U/mL [25].

Thus, it is clear that the carbon source used may have an important influence on cell growth and on the expression of heterologous proteins. Based on the information above, different carbon sources may be preferable based on the main objective of the fermentation process, be it biomass accumulation or recombinant protein expression. Research has found that a small part of trypsinogen was retained in the cell when glucose was used as the carbon source under control of the pGAP promoter, while no intracellular trypsinogen was found when using methanol as the carbon source under control of the pAOX promoter [26]. The author indicated that this was substrate-dependent instead of promoter-dependent.

2.3.3 Effect of pH

The pH of the growth medium plays an important role in the fermentation process as it affects the growth rate of the culture, enzyme activity, the specific expression rate, the protease activity, and the stability of the expressed proteins [70]. *P. pastoris* can tolerate a broad pH range between 3.0 and 7.0, but processes typically maintain pH values between 5 and 6 [51]. A summary of the optimal pH reported for the expression of different proteins is shown in Table 2.2.

Table 2.2 Optimal pH values for the expression of different proteins in *P. pastoris*

Proteins	Optimal pH value or range	References
Cytokine growth-blocking peptide	3.0	[71]
Bovin enterokinase	4.0	[72]
Recombinant human erythropoietin (rHuEPO)	4.5	[36]
Human growth hormone (rhGH)	5.0	[70]
Recombinant Human serine albumin (HSA)	5.6	[73]
α-amylase	6.0	[74]
Mouse epidermal factor	6.0	[62]
Mini-proinsulin	6.3	[75]
Recombinant hookworm (Ancylostoma caninum) anticoagulant peptide (AcAP-5)	7	[76]
Yarrowia lipolytica Lipase LIP2 (YlLIP2)	6.5-7.5	[25]

Although a range of pH between 3 and 7 was reported to have almost no effect on cell growth, it can, however, have an effect on the secreted heterologous proteins [76]. Secreted proteins are often affected by various proteases secreted into the medium. Since proteases are secreted by the host strains or released following cell lysis, the optimal pH to reduce protease activity depends on the strains and on the characteristics of the recombinant proteins [77]. Generally, higher pH values decrease cell viability and may lower the stability or activity of recombinant products [20], and lower pH values may reduce the effect of the protease in the medium. The influence of the pH on the production of the recombinant human growth hormone (rhGH) was reported by Çalık [70]. It showed that the highest total protease concentrations were obtained when the pH was greater than 6.0, while the highest concentration of rhGH was achieved at a pH of 5.0. Another study showed similar results for the production of a humanized single-chain variable domain fragment antibody (A33scFv) [78]. The protein could not be detected after 48 h in pH 6.0 due to the high protease activity. However, some experiments showed that a high pH might be better for protein expression and a low pH may lead to higher protease activity. Work done by Inan and co-workers suggests that the optimum pH for anticoagulant peptide (AcAP-5) is between 6.8 and 7.0, a level at which proteolytic degradation could be minimized [76].

Most pH-optimization experiments are performed in shake-flasks. However, the optimal pH for shake-flask cultures may not directly apply to bioreactor-based fermentation. Zhu *et al.* reported that the optimum pH was 4.5 for the production of α-glucosidase in the shake-flask experiment, but for fermentation in bioreactors, the optimum pH was 6.5 [79].

Ammonium hydroxide is commonly used to control pH during fermentation. Yu *et al.* studied the impact of NH_4^+ on the production of *Rhizopus oryzae* lipase in *P. pastoris* [80]. Three concentrations, 400 mmol/L, 440 mmol/L and 500 mmol/L of NH_4^+ were tested, and the results showed that the highest dry cell weight was achieved at 500 mmol/L, while the highest lipase activity was obtained at 440 mmol/L of NH_4^+. They also indicated that an appropriate addition of NH_4^+ can improve cell growth as well as the expression of the proteins of interest. Moreover, protease activity decreased under higher NH_4^+. Research by Yang *et al.* suggests that a higher concentration of NH_4^+ leads to a lower degradation of Hir65 (one active fraction of hirudin) [58]. Based on the research described above, although different products were expressed, the optimal concentration of NH_4^+ was very similar in each case. This may be because the concentration of NH_4^+ has a similar influence on the production of different proteins.

2.3.4 Effect of Temperature

Temperature is an important parameter when it comes to cell cultivation, fermentation, and production because it influences cell growth, protein synthesis and stability, and enzyme activity. The optimal temperature for a higher productivity of recombinant proteins or higher levels of biomass in *P. pastoris* was studied. The typical cultivation temperature for *P. pastoris* is 30 °C. The expression of YlLIP2 in *P. pastoris* showed that lowering the cultivation temperature did not have any significant effect on the production of YlLIP2 or on cell biomass [25]. However, other research indicates that a lower temperature will increase the yield of proteins. Li and coworkers have shown that higher yields of herring antifreeze protein and cell viability were achieved at 23 °C compared to 30 °C [81]. Hong and coworkers improved the specific laccase activity, by decreasing the temperature from 30 °C to 20 °C [82]. Higher temperatures may lead to a poor stability of the recombinant protein, a release of more protease from lysed cells, and incorrect folding of some products. A 3-fold increase in the specific productivity of an antibody Fab fragment was obtained at a lower temperature, when the experiment was executed at different growth temperatures (20, 25, 30 °C) [83]. According to the author, the reason for the result is an increase in cell viability and a related decrease in proteolytic activity at lower temperatures. However, the research also found that other physiological parameters might be responsible for the higher productivity at lower temperatures as the proteolytic activity did not change following a temperature shift. Although the common method used to improve heterologous protein production is to lower temperature conditions, no detailed studies have been performed.

A method using a Temperature Limited Fed-Batch (TLFB) strategy was first described by Jahic *et al*. [84]. In their work, the TLFB was compared to methanol-limited fed-batch. Using the TLFB method, the protein yields were increased and no proteolytic degradation was detected.

2.4 Operational Strategies

Since various foreign proteins have been widely expressed using *P. pastoris* or other expression systems such as *Escherichia coli* and *Saccharomyces cerevisiae*, a lot of research was done to increase the production of the proteins of interest. A common technique used to improve productivity is to increase cell density, since the concentration of heterologous proteins is roughly proportional to cell density [4]. Fed-batch cultivation is a method employed to create high cell density and has been applied to produce heterologous proteins in bioreactors. Shake-flask experiments are a basic step and part of a preliminary study of a system, however, the highest biomass and protein concentration cannot be achieved due to physico-chemical limitations. Nutrient sufficiency, volume, pH changes, dissolved oxygen levels in the medium, and secondary or toxic by-products cannot be controlled or monitored during flask cultivations. Conducting experiments in bioreactors eliminate these limitations and these parameters can, in general, be controlled. Supplying nutrients during the fermentation process can also increase the biomass concentration compared to shake-flask or batch cultivations. Various feeding strategies were developed to obtain a higher biomass and greater protein productivity.

2.4.1 Operational Strategy for the pAOX1 Promoter

The expression of heterologous proteins in *P. pastoris* based on the pAOX1 promoter involves a three-stage scheme. In the first stage, *P. pastoris* is grown in a salt medium on a non-fermentable carbon source, such as glycerol. The second stage is initiated by adding the glycerol at a growth-limiting rate, once the glycerol from the first stage is depleted. The last stage is the induction phase, which is initiated by adding the inducer, in this case methanol [19]. This fermentation three-stage protocol is also referred to as a glycerol batch phase (GBP), a glycerol fed-batch phase (GFBP), and a methanol induction phase (MIP). It was developed by Invitrogen Co, who based their work on that of Brierley and coworkers [64].

The aim of GBP, the first stage, is to accumulate sufficient biomass before inducing the expression of the recombinant proteins, which is itself correlated to cell density [51]. The use of methanol as a sole carbon source can lead to a high level of production, but only a slow growth rate and cell yield [64]. Therefore, it is essential to apply other carbon sources, in order to obtain a high cell density. Glycerol is used as the growth substrate, and *P. pastoris* has a higher specific growth rate, up to 0.18 L/h [22], when grown on glycerol compared to growth on methanol, which is limited to 0.14 L/h [64]. The concentration of glycerol in this phase is usually initiated at around 40 g/L, and a glycerol concentration over this level can inhibit growth [51] and cause toxicity-related problems.

The depletion of glycerol is accompanied by a spike in the dissolved oxygen (DO), and the length of time required for this consumption depends on the initial density of cells [52]. Once the glycerol is consumed, the glycerol fed-batch phase starts, where glycerol is added to generate more biomass under limiting conditions, and to allow the derepression of the pAOX promoter [64]. In addition, a limited glycerol feed will facilitate the consumption of metabolites such as acetate and ethanol, which are generated during the batch phase and will inhibit the activity of the pAOX promoter during the induction phase [63]. Some authors recommend a constant glycerol feeding rate. The *Pichia* Fermentation Process Guidelines developed by Invitrogen suggest setting the initial feed rate at 18.15 mL/h/L of culture [52]. Another experiment expressing Human Chymotrypsinogen B (hCTRB) in a *Pichia* system used a constant feeding rate of 220 mL/h in 30 L of bioreactor [85]. Some researchers propose using an exponential feeding rate for the sole carbon source at this phase. Zhang *et al*. modeled the *Pichia* growth and production of the Heavy-Chain Fragment C of Botulinum Neurotoxin, Serotype A [BoNT/A(Hc)] using a glycerol fed-batch profile and achieved a high cell density of 410 g/L after the glycerol fed-batch phase, which lasted 22 h with a feed rate of 20 g/L/h (50% w/v glycerol) [63].

In GBP and GFBP, glycerol acts as the sole carbon source to support the growth of cells but the activity of the pAOX promoter is repressed and no recombinant protein is produced. During the methanol induction phase, methanol acts as the sole carbon source in addition to being the inducer. The feeding rate of methanol dictates the residual methanol concentration, specific growth rate, and the expression level of heterologous proteins [20, 54, 63]. In fed-batch or continuous cultivations, the methanol is maintained at a constant concentration, which has a positive effect on the production of foreign proteins during the induction phase [86]. The accumulation of methanol in the broth and the subsequent increase in the concentration of formaldehyde will have an inhibitory effect on cell growth [65], and a lower concentration may affect the induction phase. Thus, the level of methanol needs to be controlled during cultivation. The optimal methanol concentration varies based on each specific protein, but it typically ranges from 0.5-1.0% v/v in *P. pastoris* systems [14]. One example shows that a 5-fold higher volumetric laccase activity was obtained when methanol was kept at 0.5% instead of at 1.0%. The detrimental effect of methanol on the production of recombinant laccase may be attributed to lower laccase stability, a higher proteolytic activity, and a folding problem due to the higher growth rate of the culture when 1.0% methanol is maintained [82]. In addition, the length of the induction period also impacts recombinant protein yields. Plantz *et al.* found that 31-45 h after the methanol induction phase started, the production of rOvIFN-T decreased [3]. The operational conditions such as temperature, pH, medium composition, phenotype of the host strain, and the characteristics of the recombinant protein being expressed can all impact the optimal length and strength of the methanol induction phase [51].

During the fermentation of *P. pastoris*, an optional transition phase is generally adopted between the biomass build-up step and the induction of the pAOX promoter to help the cell adapt to the presence of methanol [20] and to favor the derepression of the pAOX promoter [51]. In this phase, a mixed-feeding of glycerol and methanol is used, and the glycerol concentration in the feed is gradually decreased, while the concentration of methanol is increased [63]. One of the benefits of the co-feeding strategy is the prevention of a rapid increase in the methanol concentration, which may cause damage to the cells [87]. In addition, this can reduce the cultivation time and increase the yield of heterologous protein expression [64, 88].

2.4.2 Mixed-Substrate Feeding

As a strategy to increase the biomass concentration and smooth the transition from a glycerol phase to an induction phase, mixed-substrate feeding is commonly used during the fermentation of Mut$^+$ *P. pastoris*. This method simply uses a mixed-carbon feed containing various substrates in addition to methanol [64].

Early in 1981, Eggling and coworkers showed that growing cells on mixed substrates can enhance the utilization of methanol [89]. In 1983, Hazeu *et al.* demonstrated that the addition of formic acid to a culture can increase the protein yield when grown on methanol by 16%-18% [90]. Additional research [91, 92] has also been conducted to study the influence of mixed substrates on productivity or cell yields.

Generally, this mixed-substrate feeding is used for the cultivation of Mut^s strains because of its lower sensitivity to residual methanol and its slow utilization. By mixing substrates with methanol, cells may simultaneously utilize substrates that support growth, while inducing the expression of recombinant proteins [67].

Glycerol is the most widely used co-substrate in the *P. pastoris* system, as it leads to good cell growth. The concentration of glycerol and the proportion of glycerol in the mixture are the main concerns when using such a mixed-feeding strategy, because a higher glycerol feeding rate may result in the accumulation of ethanol, which is a strong repressor for the pAOX1 promoter [76].

The general approach was developed by Brierley and coworkers, who attempted to produce Bovine Lysozyme in a Mut^s strain. The use of a 4:1 feed composition of glycerol and methanol produced a lower yield of lysozyme. A shorter induction time led to a 3-fold greater volumetric productivity compared to the original strategy when applied in the Mut^s strain. Further improvements were seen with a 2:1 (glycerol: methanol) mixed-feeding, where a 4-fold increase in volumetric productivity was obtained after 43 h [64].

Hellwig *et al.* investigated the effects of supplementing the feeding solution with glycerol while maintaining a constant concentration of methanol on the expression of single-chain antibodies in the Mut$^+$ strain. The maximum specific growth rate was obtained when the glycerol feeding rate was kept at 4.9 g/ (L*h), however, no product was detected. A lower specific growth rate was obtained when the glycerol feeding rate was maintained at 1.23 g/ (L*h), but the protein was obtained at a concentration of 25 g/L. The results suggest that glycerol shows negative effect on the expression of protein even at extremely low levels [93].

Woo *et al.* expressed bivalent anti-T-cell immunotoxin under the control of the pAOX1 promoter. The immunotoxin was secreted by adding glycerol to the methanol feed with a 4:1 methanol-glycerol ratio [94]. In this research, adding glycerol did not completely repress the expression of recombinant proteins, which may indicate that the presence of glycerol has varying effects, depending on the characteristics of the desired product.

Another reason to adopt mixed substrates is to test non-repressive carbon sources for the pAOX1 promoter. Inan and Meagher used this strategy to qualify a series of carbon sources as non-repressive to the production of recombinant proteins, including alanine, sorbitol, and mannitol [19]. Thorpe and coworkers further proved that sorbitol is a promising non-repressive carbon source by performing cultivations with a mixed substrate composed of sorbitol and methanol [67].

Glucose is a strongly repressing carbon source and is therefore not commonly used in research surrounding the fermentation of *Pichia*. However, it is still attractive to some because of its lower price when compared to the other carbon sources. The lower price is more appropriate for industrial scale-ups. Hang *et al.* worked with Mut^s to express recombinant phytase using glucose as a substrate. By co-feeding glucose and methanol during the fed-batch phase, they achieved a similar cell density to the one obtained when using glycerol and methanol (100 ± 8 g [DCW]/L and 100±7 g [DCW]/L, respectively). The glucose concentration is one of the important factors that affect biomass production. A higher specific growth rate and a lower specific ethanol formation rate were obtained at lower initial glucose concentrations, which may be due to weaker glucose repression

[95]. Paulová *et al.* and coworkers examined a mixture of glucose and methanol (60:40) using the Mut^+ strain to produce recombinant trypsinogen. The yield of biomass was 1.4 times larger than the yield obtained with methanol alone [65].

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Chapter 3

Study of the Effects of Feeding Strategies on Phytase Production in *Pichia pastoris*

3.1. Introduction

P. pastoris is considered a promising platform for recombinant protein production [1]. This methylotrophic yeast was first noticed approximately forty years ago for its ability to use methanol as a sole carbon source [2]. This system is of particular interest as it can grow in simple defined medium and achieve high cell densities [3], and can perform many of the post-translational modifications not possible in prokaryotic systems, including glycosylation, disulphide bond formation, and phosphorylation [4-6]. It also has the ability to secrete the recombinant proteins of interest into the culture broth, which simplifies the downstream purification and recovery process [4, 7].

The strong and tightly regulated promoter alcohol oxidase promoter (pAOX) has contributed to the success of this microorganism as a recombinant protein production platform. Alcohol oxidase is involved in the methanol assimilation pathway, and catalyzes the production of formaldehyde from methanol [1]. Strains are categorized into three types based on which alcohol oxidase genes are present: Mut^+, when the two genes are functional (AOX1 and AOX2); Mut^S when only AOX2 is functional, and Mut^- when both genes are inactivated. The Mut^+ strain is widely used because the pAOX1 promoter can lead to high expression levels [6]. The pAOX promoters are induced by methanol and repressed by glycerol, glucose, and other common carbon sources. The fed-batch fermentation protocol consists of three phases: the glycerol batch phase, the transition phase, and the methanol induction phase [8]. This protocol, developed by Brierley *et al.* [9] leads to high cell density cultures and high protein yields. For the pAOX1 promoter, residual glycerol concentrations need to be controlled due to its high susceptibility for repression, as even a low level of glycerol may interfere with protein expression [10]. Although pAOX systems are useful, they require the use of methanol, which is hazardous to handle, transport, and store. Mut^s strains, due to the weakness of the pAOX2 promoter, grow slowly and utilize methanol at a much lower rate, and usually lead to much lower protein yields than the Mut$^+$ strains [11]. However, certain types of protein production reportedly benefit from using the Mut^S strains [12]. A lot of research has been conducted to improve the use of Mut^S strains, for example by developing fermentation strategies [13-15] and screening non-repressing carbon sources [16, 17].

In recent years, constitutive promoters were developed to replace inducible promoters and avoid the use of methanol for heterologous protein production. The glyceraldehyde-3-phosphate dehydrogenase promoter (pGAP) was isolated in 1997 [18] and has since been used for the expression of many heterologous proteins in *P. pastoris* [19-23]. Some studies showed that the production of the proteins of interest using the pGAP promoter was achieved at a similar or even higher level compared to pAOX systems [24, 25]. By not using methanol as an inducer, the hazards associated with methanol are eliminated. The pGAP system therefore has greater potential for large-scale production and continuous fermentation [26].

Several studies aim to develop techniques to increase cell density and improve heterologous protein expression. Medium optimization and operational strategies are the two major components that can lead to an improvement in fermentation productivity. For medium optimization, carbon source screening and minimal salt optimization attracted most of the attention, and several works were completed on the subject [27-30]. Compared with inducible promoters, the constitutive pGAP promoter has more options for carbon sources and a variety have successfully been used in this system including glycerol, glucose, and oleic acid. Among these choices, glycerol and glucose are dominant [11, 18]. Feeding strategies have a critical effect on both the maximum cell density achievable and product yields. The common feeding methods can be divided into two types, namely with and without feedback control. The nutrient feeding rate is usually determined in advance for the feeding method without feedback control, whereas when using feedback control, the feeding rate changes according to various physical parameters over the course of the fermentation [31]. Some experiments were conducted to test the effect of the different methods on biomass and protein production. Lee and co-workers examined the effects of constant and variable substrate feeding rates using a DO-stat control strategy on the expression of α-amylase [32]. Yamawaki *et al*. compared two methods, DO-stat and methanol concentration control, on single-chain variable fragment antibody production [33]. Given the increasing use of *P. pastoris,* novel feeding methods were developed, for example applying model-based control [34-37] and combining two basic feeding methods [38]. However, it is difficult to determine which of the feeding strategies is best, especially when different products are expressed. Like optimal culture conditions, ideal control strategies vary based on the host strains and proteins produced.

In this study, three basic feeding strategies were evaluated. To the best of our knowledge, no studies have previously been performed that examine the influence of different nutrient feeding methods on pGAP-mediated phytase production in *P. pastoris*. In this work, the effects of various feeding methods on cell growth, phytase activity, and residual substrate concentrations were studied. Since the DO-stat method is common and easily controlled in research, a modified DO-stat method was developed and its feasibility was tested.

3.2 Materials and Methods

3.2.1 Yeast Strain and Chemicals

A strain of *P. pastoris* was kindly provided by Zell Technologies Inc., Canada. This strain expresses and secretes phytase under the control of a constitutive pGAPZα promoter. All chemicals were purchased from Fisher Scientific or Sigma-Aldrich.

3.2.2 Cultivation Medium

The strain was kept on YPD plates composed of 10 g/L yeast extract, 10 g/L peptone, 20 g/L dextrose, 20 g/L agar, and 100 µg/mL zeocin. A liquid YPD medium without zeocin was used for precultures. Distilled-deionized water was used for preparing the media.

A defined minimal salt medium (MSM) was used for the bioreactor experiments. The fermentation medium contained 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, 11.7 g/L magnesium sulfate, 2 mL/L PTM-4 trace elements solution, and 10 g/L glycerol as the carbon source. The PTM-4 trace elements solution consisted of 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 dihydrate, 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. A 30% (v/v) ammonium hydroxide (NH_4OH) solution was used to maintain the pH level during the fermentation and also served as a source of nitrogen. A 30% (w/v) glycerol solution with 12 mL/L PTM-4 was fed to the bioreactor at different feeding rates based on the different control strategies.

3.2.3 Inoculum Preparation and Batch Phase Cultivation

Single colonies were picked from the agar plate, transferred into the 50 mL liquid YPD medium and then cultivated in the shaker at 30 ˚C and 250 rpm. After 24 h, the culture was used to inoculate the 3 L NBS Bioflo 110 bioreactor (New Brunswick Scientific) containing 1 L of initial fermentation medium. All experiments were performed at 30 ˚C and pH 5.0, the latter of which was maintained by adding NH_4OH. The air flow rate and agitation rate were controlled at 1.5 LPM (1.5 v/v) and from 250 to 1200 rpm respectively, to keep the dissolved oxygen (DO) level above 20%. At approximately 20 h, the initial glycerol in the broth was depleted, at which point the feeding solution began to be fed to the bioreactor.

3.2.4 Glycerol Fed-Batch Cultivation with Various Feeding Strategies

The glycerol fed-batch phase is triggered when the initial glycerol in the fermentation broth is completely consumed, as identified by a spike in the DO level. Four feeding strategies were investigated:

i. *Stepwise increased feeding*: The feeding rate was increased stepwise from 1.73 g of glycerol per hour (20-36 h) to 2.59 (36-44 h) and 3.45 (44-50 h).

ii. *Exponential feeding*: The feeding rate was increased exponentially to allow cells to grow at a constant specific growth rate. The feeding rate was predetermined based on the following equation [31]:

$$F = \frac{\mu_{set}\, X_0 V_0 \exp(\mu_{set} t)}{Y_{X/S}} \qquad (1)$$

in which F is the feeding rate (g/h) of the solution at time t (h), X_0 and V_0 are the initial concentration of biomass (g/L) and initial volume of the medium in the bioreactor (L), $Y_{X/S}$ is the biomass yield based on the substrate (g[DCW]/g[S]) that is measured from the previous experiments and equal to 0.517 (data not shown), μ_{set} is the specific growth rate, set to 0.15 (h^{-1}).

iii. *Standard DO-stat*: The feeding was triggered by an increase in the level of dissolved oxygen in the medium. The agitation rate was automatically set by the primary control unit (PCU), between 250 rpm and 1200 rpm to maintain the DO level at 20%. When cells are added to the bioreactor, nutrients start being consumed, which requires oxygen. As the cell density increases, more oxygen is required, which causes the agitation rate to increase. When the concentration of nutrients decreases, cell growth slows down, which leads to a lower oxygen requirement. Once the nutrients are completely depleted, the DO level increases while the agitation rate decreases to 250 rpm. In this strategy, feeding was triggered when DO levels were higher than 30% (10% above the set point) and the agitation rate was kept at 250 rpm.

iv. *Modified DO-stat*: Compared to the standard DO-stat, the modified DO-stat introduced a new factor-agitation rate. In the standard DO-stat, feeding was triggered when the DO level was higher than the set point. However, the feeding start point was dictated by the interaction of the DO level and the agitation rate. When both the DO and the agitation rate were outside of the range of settings, the feeding phase began. The conditions of the control program are listed in Table 3.1.

Table 3.1 Operational conditions of modified DO-stat

Group	1	2	3
DO level	>30%	>30%	>30%
Agitation rate (rpm)	<450	<700	<900

3.2.5 Analytical Methods

Samples were taken every few hours to determine the biomass, the phytase activity in the supernatant, and the substrate concentration in the broth. Cell density was indicated by optical density (OD), which was measured spectrophotometrically at the 600 nm wavelength (Biochrom Ultrospec 60 double-beam spectrophotometer). The dry cell weight to OD ratio is 0.3715 g DCW per unit of OD (data shown in the appendix). Samples were centrifuged at 10,000 rpm for 10 min; the supernatant was then used to measure the phytase activity according to a phosphatase assay protocol described in other work [39]. The concentrations of glycerol and ethanol in the culture were monitored. To achieve this, culture supernatant was analyzed by an Agilent high-performance liquid chromatography (HPLC) unit with an Aminex HPX-87H column at 60 ˚C, with a flow rate of 0.6 mL/min, and 0.005 M sulfuric acid was used as the mobile phase.

3.3 Results and Discussion

3.3.1 Influence of Stepwise, Exponential and DO-Stat Feeding Methods on *P. pastoris* Growth and Phytase Activity

The feeding method is an important factor during fermentation, especially for cells grown in fed-batch processes. The feeding strategy selected provides nutrients to the cells through the feeding of a concentrated solution into the bioreactor, and different methods usually directly influence the final biomass concentration and protein expression. In this section, three basic feeding strategies commonly applied in different processes were investigated. Cells were initially grown in glycerol medium for 20 h, until the glycerol was totally consumed. The solution then started to be fed into the bioreactor. The first two methods investigated were the stepwise feeding method and the exponential feeding method. These two methods did not have feedback control, which means that the feeding rates were predetermined. The third method on the other hand, the DO-stat method, does involve feedback control. The feeding rate was changed or triggered at an exact moment in the fermentation based on the dissolved oxygen level in the medium. Final cell densities and phytase activities obtained using these three feeding methods are shown in Figure 3.1 and Table 3.2.

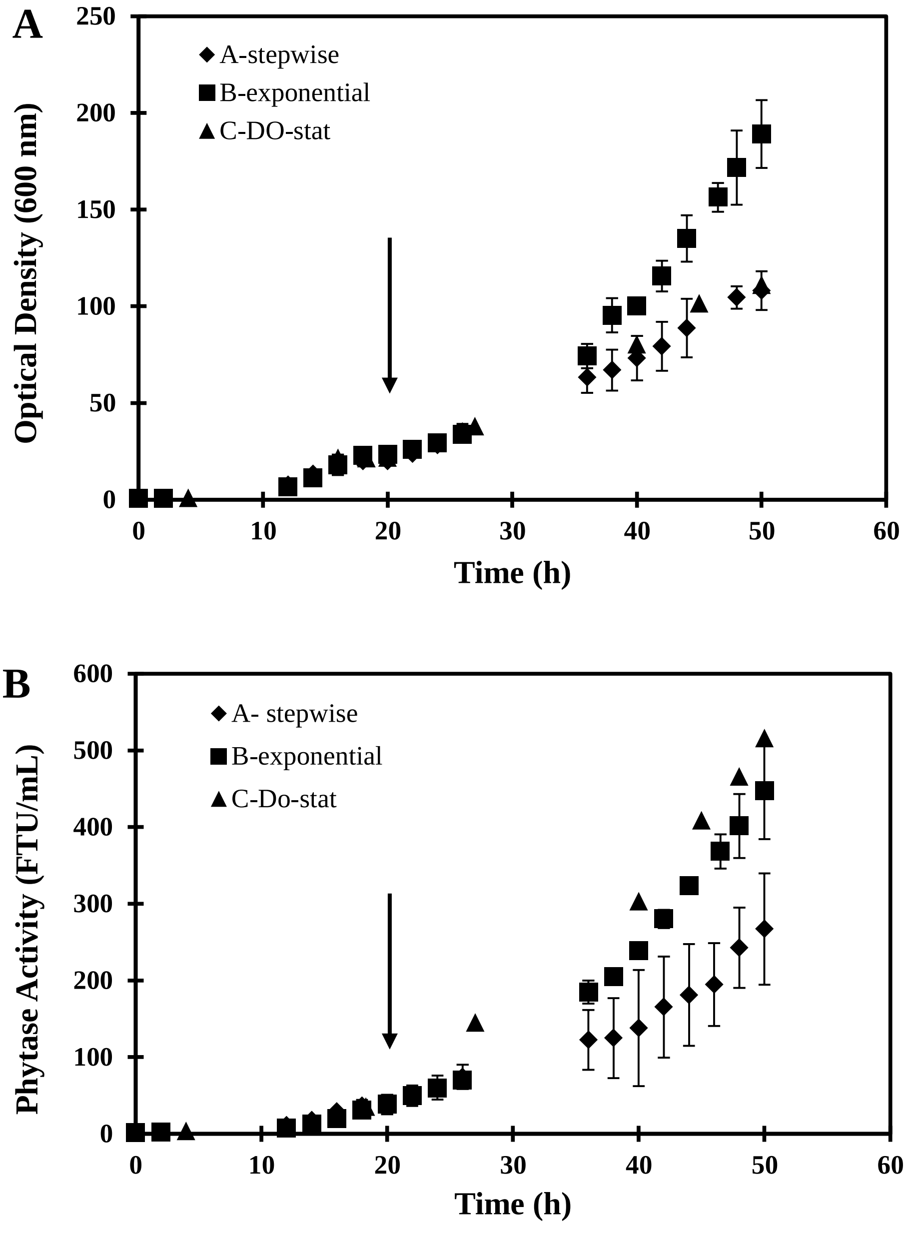

Figure 3.1 ***P. pastoris*** **performance under three basic feeding strategies in bioreactors.** A: optical density. B: phytase activity. Arrows indicated the point at which substrate feeding started. (average ± std dev, n=1 to 3)

Table 3.2 Summary of three feeding methods (average ± std dev, n=1 to 3)

		Feeding strategies		
		Stepwise feeding	Exponential feeding (set μ=0.08 h^{-1})	DO-stat
Overall	Duration (h)	50	50	50
	Maximum Cell Density (g [DCW] L^{-1})	40.15±0.42	70.26±6.52	41.24
	Maximum Phytase Activity (FTU mL^{-1})	267.11±72.64	447.35±63.25	515.82
Feeding phase	Duration (h)	30	30	30
	Glycerol Consumption (g)	69.07	128.51	93.21
	Maximum Specific Growth Rate (h^{-1})	0.055±0.003	0.071±0.006	0.056
	Yield (FTU mL^{-1} [phytase] g^{-1} [glycerol])	3.32±0.89	3.19±0.52	5.32
	Yield (FTU mL^{-1} [phytase] g^{-1} [DCW])	7.06±2.24	6.75±1.67	14.94

The maximum dry cell weight was 70.26 g/L, obtained using the exponential feeding method. The final biomass concentrations obtained using stepwise feeding method and DO-stat method were close, at 40.15 g/L and 41.24 g/L respectively. The maximum specific growth rates (0.0705 h^{-1}) in the feeding phase were obtained by applying exponential feeding, followed by the DO-stat (0.056 h^{-1}) and the stepwise method (0.055 h^{-1}). In terms of phytase expression, unlike the trends related to cell growth, the optimal feeding strategy (yielding 515.82 FTU/mL) was the DO-stat method. For stepwise feeding, the final biomass concentration, and phytase activity were lower than with the other two strategies. This result might be caused by the depletion of nutrients in the broth. The feeding rate was semi-empirical and might not have been able to provide the nutrients needed for optimal cell growth. Moreover, the feeding rate was kept constant for a certain period during fermentation and then increased every few hours. As the cell concentration increased during fermentation, the specific growth rate decreased because the nutrients were fed into the broth at a fixed feeding rate, which was unable to meet the increasing demand [40]. Since in most cases protein expression under the pGAP promoter depends on cell growth, the lower phytase activity observed might be due to the lower cell biomass concentration. Although this feeding method has its weakness, it is still commonly used in various processes [31, 32]. This method is simple and easy to control, especially to understand a new trait that was scaled-up for the first time using a bioreactor, and to investigate the effect of the feeding rates on cell growth.

The highest dry cell weight was obtained with the exponential feeding method. Exponential feeding is more efficient than stepwise feeding because of its accuracy. The specific growth rate can be predetermined and controlled by setting different μ based on an equation. The exponential feeding rate may allow the cells to grow exponentially, which leads to a high cell density, which is desirable for fermentation since a higher expression of the protein of interested can generally be achieved at higher cell density. This feeding strategy has been widely applied in *P. pastoris* fermentation [14, 41-43]. Cultures grown using this method had a higher growth rate, as shown in Figure 3.1A, and a similar trend for the phytase activity is displayed in Figure 3.1B. The final phytase activity, however, was not the highest among the three methods.

As opposed to the first two feeding methods, the third strategy used an indirect feedback control. The feeding is triggered by a spike in the DO level in the medium, which is caused by the depletion of nutrients. Thus, this method is suitable for fermentation processes requiring that specific components, especially the carbon source, at a specific level. In this experiment, the final biomass concentration was 41.25 g/L, which was close to the value obtained when using the stepwise feeding method. The reason for the lower biomass might be due to carbon limitation in the medium. When the nutrients in the broth were completely consumed, the solution did not start feeding into the bioreactor. Instead, the feeding was initiated when the DO was above the set point. However, the highest phytase activity (515.83 FTU/mL) was measured while using this feeding strategy, although the quantity of glycerol consumed was relatively low. The yield of phytase was 14.94 (FTU/mL phytase / (g/L cell)).

A study that compared the three feeding strategies on the production of recombinant mouse endostatin in *P. pastoris* showed different results [44]. The highest dry cell weight was achieved using the DO-stat method, while the maximum endostatin expression level was obtained during exponential feeding. These differences are likely due to the different promoter and carbon sources used in this experiment, which used a Mut$^+$ strain and methanol as the inducer/carbon source. In such experiments, methanol levels need to be controlled as higher methanol concentrations are toxic to cells. This can be achieved by using the DO-stat method, allowing cells to grow quickly and resulting in higher biomass. As a result, the lower level of methanol affects the activity of the pAOX promoter and leads to a lower expression of the desired protein. For exponential feeding, cell growth was depressed because the methanol level was higher, but the expression of protein was also higher due to greater pAOX promoter activity. In this work, glycerol was used as the carbon source, to which *P. pastoris* is much less sensitive than to methanol. The amount of glycerol in the broth during exponential feeding was not high enough to inhibit cell growth.

The residual substrate concentration must be monitored during fermentation, and glycerol, and its metabolic byproduct ethanol, were tracked in all experiments (full data not shown). In the three runs, glycerol concentrations remained near zero, but ethanol concentrations showed more variability. The ethanol level was lower (less than 0.15 g/L) while using the stepwise and DO-stat feeding strategies, while the concentration with exponential feeding was much higher at approximately 1.5 g/L. Ethanol is a common inhibitory byproduct during the cultivation of yeast, and is generally produced when the medium contains excess nutrients [45]. The high concentration of ethanol may explain the lower phytase activity measured, since the ethanol has been shown to repress the protein expression [46]. In this experiment, the feeding rate was calculated based on the specific growth rate of 0.08 h^{-1}. The commonly used growth rates depend on the strain of *P. pastoris* used. The specific growth rates used for Mut^+ strains, for example, are normally lower, since methanol is used as the carbon source. According to research by Zhang *et al.* [47], the maximum μ for these strains is 0.08 h^{-1}. In pGAP systems, which do not have to contend with methanol toxicity, μ is usually higher. A higher μ of 0.266 h^{-1} was achieved in a study done by Tang *et al.* using the pGAP promoter [41]. In this research, an accumulation of ethanol was observed using higher specific growth rates, suggesting they are not recommended for overall protein accumulation.

3.3.2 Modified DO-Stat Feeding

As discussed in the preceding section, DO-stat control was beneficial for the expression of phytase, even if it led to a lower maximum cell density, which could be improved. Further experiments were conducted to study the impact of different DO-stat parameters including the effect of the feeding rate in the fed-batch phase [32] and the optimal set point of DO [48]. According to the standard DO-stat control protocols for *Pichia* [49], the DO is controlled automatically by increasing the agitation rate during fermentation. After the substrate is depleted, DO control is turned off and the agitation rate is set at a certain level, and feeding is triggered when a spike in the level of DO is observed. Thus, the feeding is related to the dissolved oxygen and the agitation rate, the latter being kept constant during the fed-batch phase. There are some shortcomings to this method however. The constant agitation set point is critical for cell growth. Since cell density increases in the feeding phase, the constant air flow rate and the constant agitation rate may not be sufficient to support a high cell growth rate, as the oxygen requirements augment with increasing cell density. A high agitation set point may benefit cell growth, but it may also result in nutrient accumulation in the early stage of the fed-batch phase, during which the cell density is lower. A lower agitation set point can maintain the nutrient level in the medium but may cause a carbon source insufficiency in the later stages.

In this section, the agitation rate parameter was introduced. Agitation was not kept constant but instead controlled within a certain range. The feeding rate was not only determined by the dissolved oxygen, but also by the agitation rate. Figure 3.2 shows the time course of the agitation, the dissolved oxygen, and the feeding conditions during the fed-batch processes. The standard DO-stat control is displayed in Figure 3.2A. According to this strategy, the agitation rate was kept constant during the whole phase. Once the feeding was triggered, the dissolved oxygen level decreased, since a large amount of oxygen was needed to consume the carbon source. Before the next feeding, the DO level was much lower due to the fixed agitation. This leads to the inhibition of cell growth and oxygen transfer [7], and has a negative effect on protein synthesis [4]. In Figure 3.2B, the agitation rate is controlled using the modified DO-stat protocol. Once the carbon source was fed into the broth, the dissolved oxygen decreased. When the DO level was lower than the set point of 20%, the agitation rate automatically increased in order to maintain a dissolved oxygen level above 20%. Thus, oxygen limitation-associated inhibition was minimized.

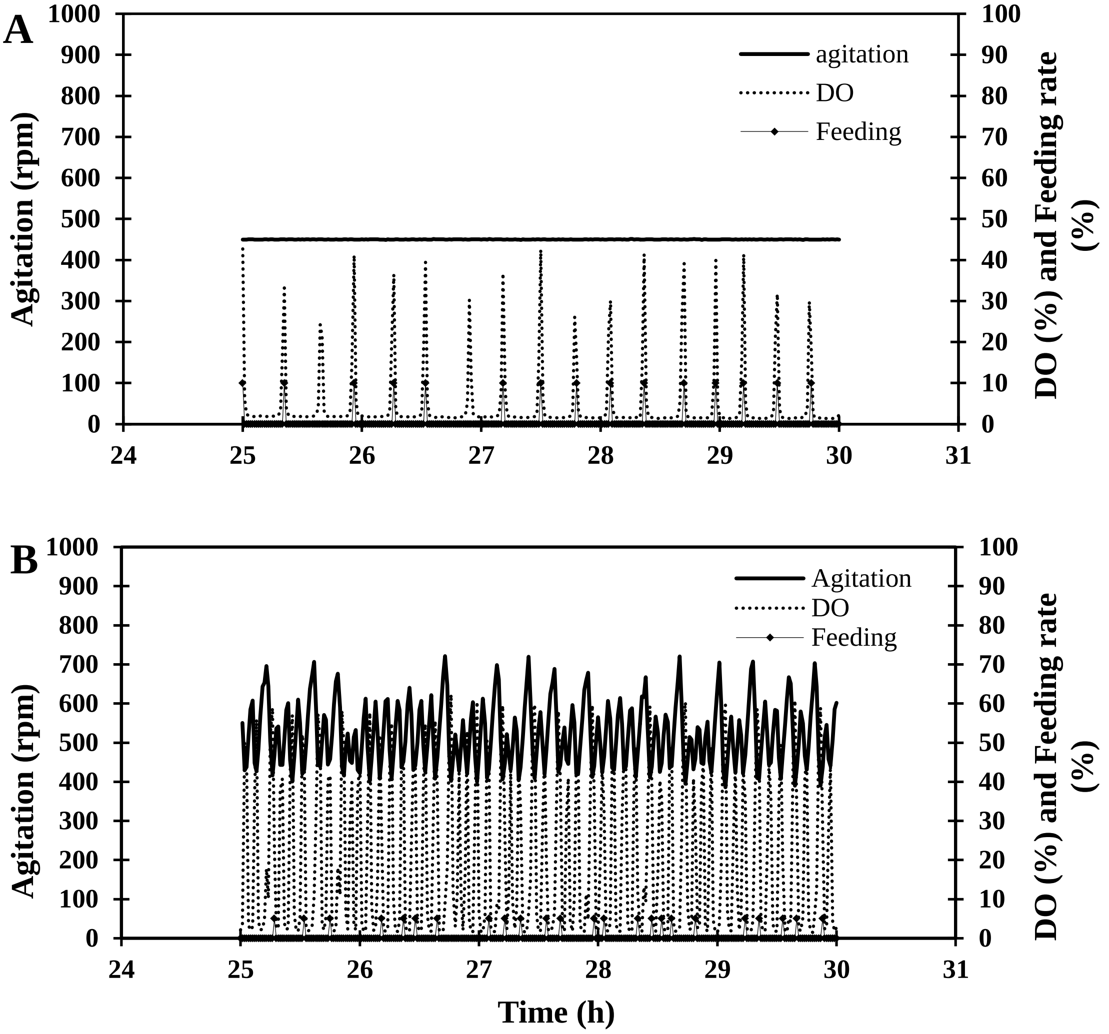

Figure 3.2 Time course of agitation rate, dissolved oxygen and feeding conditions between 25 and 30 h using standard DO-stat and modified DO-stat. A: agitation was kept constant at 450 rpm and feeding started when DO above 30%. B: agitation was controlled automatically and feeding was triggered by an agitation rate lower than 450 rpm and DO level above 30%.

A profile of the agitation rate during fermentation is shown in Figure 3.3. As opposed to the standard DO-stat, the agitation rate was controlled. It is important to note that the maximum agitation rate was set at 900 rpm (Figure 3.3D). In the early stage of fed-batch, cell density was low and the uptake of oxygen was slow; thus, the agitation rate was not able to reach the highest point of 900 rpm. If the agitation rate was set at 900 rpm, which is the case in the standard DO-stat, the oxygen requirement would have been overestimated and might have caused an excess of nutrients in the broth. Using the modified DO-stat protocol, the control of agitation was based on cell growth and the consumption of the carbon source, which varied throughout the whole fermentation. As seen in the early phase in modified DO-stat, the agitation rate was increasing with the need of cells instead of keeping constant. With the standard DO-stat, in the late stage of fed-batch the oxygen level might not have met the high demand caused by the increasing biomass, which could have resulted in a lower cell growth rate. Since the agitation level was able to adjust in the modified DO-stat control, the supply of oxygen was more efficient.

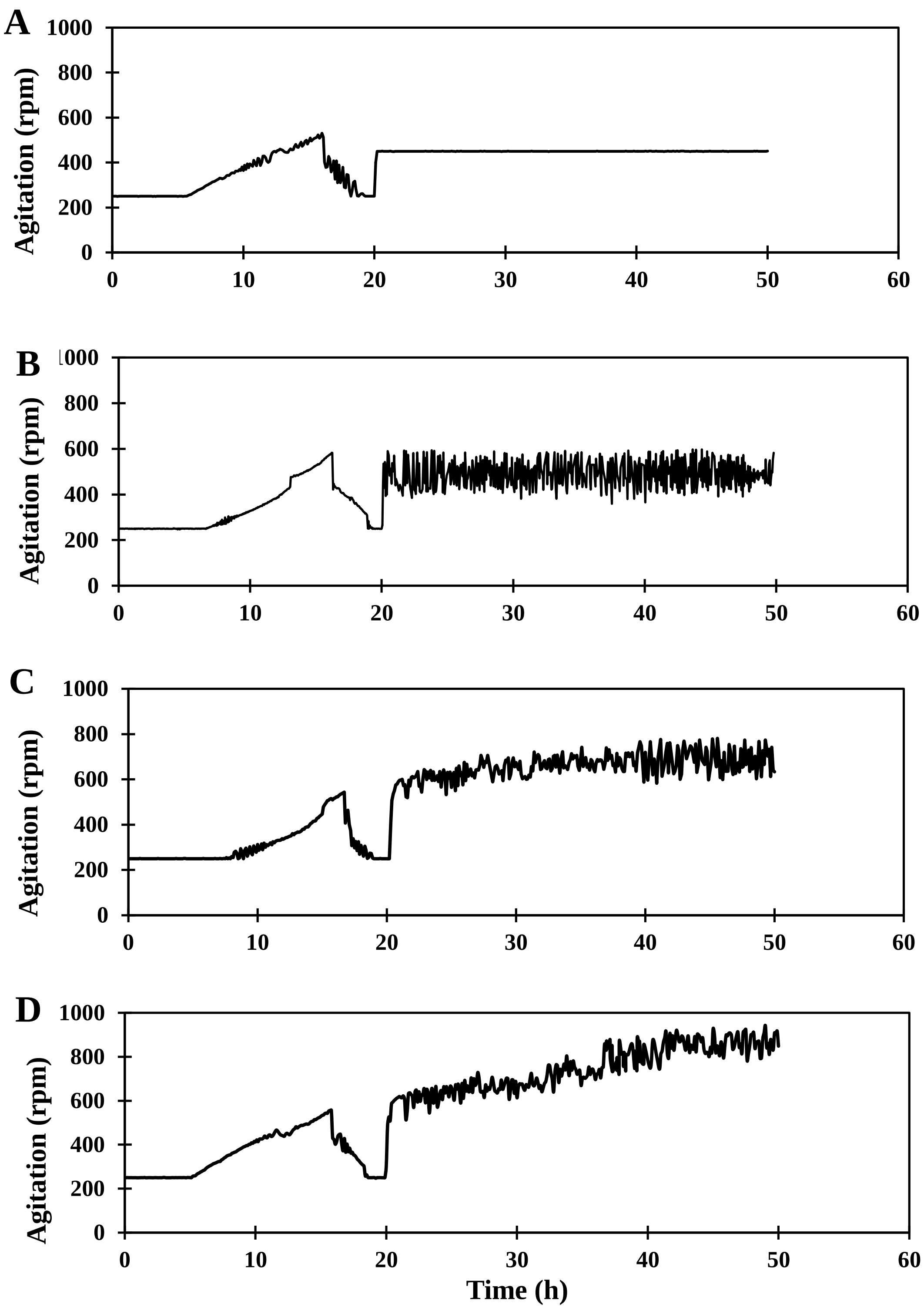

Figure 3.3 Agitation during fermentation. A: the standard DO-stat which kept agitation rate constant at 450 rpm. B,C,D: modified DO-stat which the agitation rate was controlled at B: 450 rpm; C:700 rpm; D: 900 rpm.

Culture performance using the modified DO-stat method is displayed in Figure 3.4 and Table 3.3. Cell growth and phytase activity exhibited a similar trend. Both the maximum biomass concentration (97 g/L) and phytase activity (1044.84 FTU/mL) were achieved when agitation was controlled automatically at 900 rpm. Increasing the set point of agitation was beneficial to cell growth and protein expression. In the early stage, the agitation rate was controlled automatically at a lower level based on cell growth, in order to prevent an accumulation of the carbon source. In the late stage, the agitation rate was increased to the higher set point, to supply more oxygen for cell growth. A lower agitation set point did not provide sufficient oxygen for the increasing biomass, especially in the late stage. When comparing the two methods, one setting a constant agitation rate of 450 rpm, and the other using agitation control at same level, higher cell growth rate and phytase activity were achieved with less glycerol.

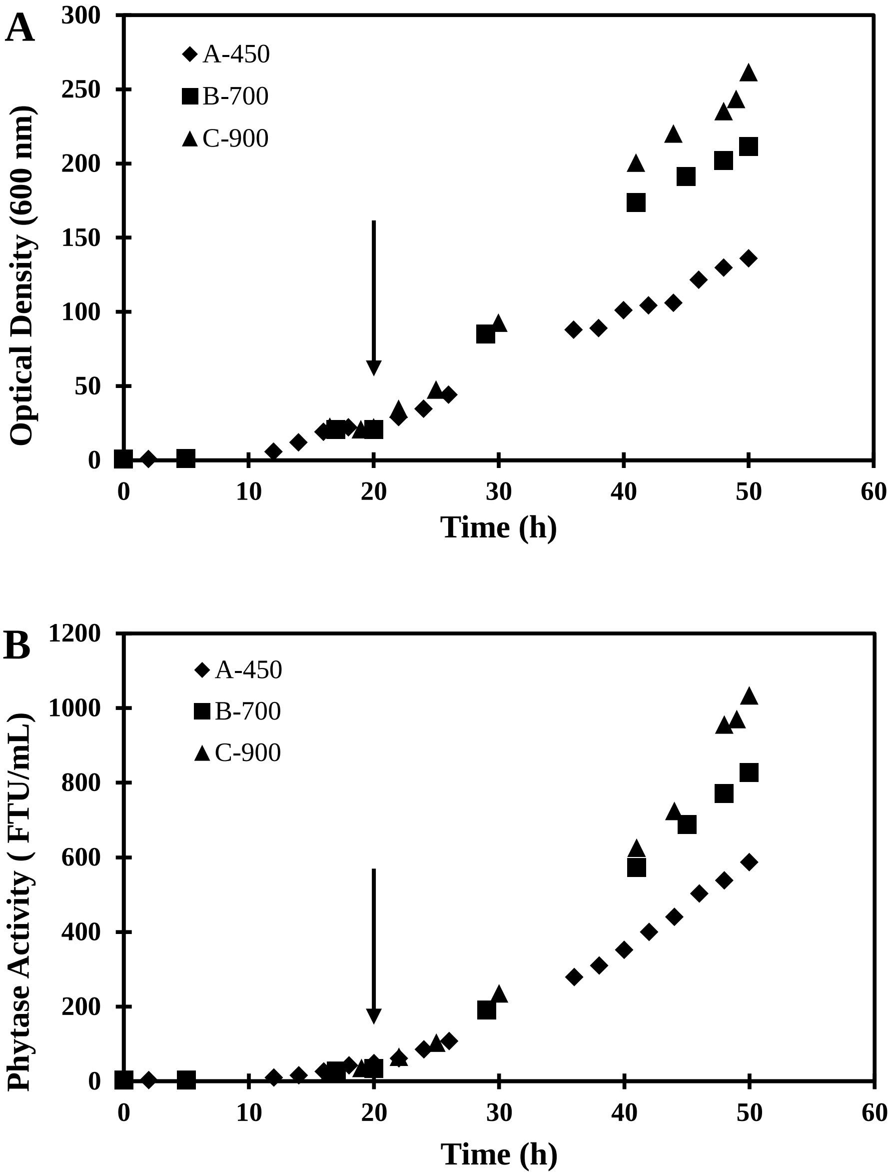

Figure 3.4 Profile of cell growth and phytase activity using modified DO-stat feeding method with different agitation rate. A: optical density B: phytase activity. Arrow indicated the time at which the feeding started based on different feeding strategies.

Table 3.3 Summary of the outcomes of the of modified DO-stat feeding methods

		Feeding strategies			
		Standard DO-stat	Agitation 450	Agitation 700	Agitation 900
Overall	Duration (h)	50	50	50	50
	Maximum Cell Density (g [DCW] L^{-1})	41.24	50.49	78.57	97.13
	Maximum Phytase Activity (FTU mL^{-1})	515.82	586.33	834.86	1044.84
Feeding phase	Duration (h)	30	30	30	30
	Glycerol Consumption (g)	93.21	87.0	171.9	178.5
	Specific Growth Rate (h^{-1})	0.0555	0.0596	0.0778	0.0824
	Yield (FTU mL^{-1} [phytase] g^{-1} [glycerol])	5.318	6.190	4.657	5.649
	Yield (FTU mL^{-1} [phytase] g^{-1} [DCW])	14.943	12.590	11.278	11.337

3.4 Conclusion

During fed-batch cultivation, feeding control has an impact on both cell growth and heterologous protein expression. Three widely used strategies were tested to express phytase in *P. pastoris*. Stepwise feeding is easy to control but leads to a lower production of cells and phytase. The exponential feeding strategy is a common method and results in a high biomass, but the highest phytase activity (515.82 FTU/mL) is achieved using the standard DO-stat feeding strategy consuming less glycerol. To overcome difficulties associated with an insufficient supply of oxygen, a modified DO-stat control method was used. The agitation rate was introduced into the control instead of keeping it constant. Using this method, higher biomass (50.49 g/L) and phytase production (586.33 FTU/mL) were obtained compared to standard DO-stat method when the agitation was controlled around 450 rpm in both experiments.

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Chapter 4

Citric Acid as a Carbon Source for Phytase Production in *Pichia pastoris*

4.1 Introduction

The methylotrophic yeast *Pichia pastoris* has been widely used as an expression system for the production of heterologous proteins. It can grow in simple conditions without the need for complex media or refined control systems, and can achieve high cell densities under these conditions [1]. Moreover, it has the ability to perform eukaryotic modifications including processing signal sequences, glycosylation, disulfide bond formation, and lipid addition [2, 3]. These features explain the popularity of this platform for the expression of proteins that require post-translational modifications, such as therapeutic enzymes and antibodies. *P. pastoris* has the ability to secrete expressed proteins, which can help simplify downstream processing such as purification [1, 4].

This yeast first attracted attention for its ability to use methanol as a sole carbon source [5, 6]. It can achieve this due to its expression of an alcohol oxidase (AOX) enzyme, which is responsible for the catalysis of the first reaction in the methanol utilization pathway [5, 7]. In a common fermentation procedure, methanol is used as the sole carbon source for *P. pastoris* (Mut$^+$) cultivation, as well as the inducer for any gene expressed under the control of the pAOX promoter [8]. However, since it is a flammable and toxic chemical, the transportation and storage of methanol is costly and it needs to be carefully handled. Additionally, Mut^+ strains are sensitive to methanol, and its concentration in the broth therefore needs to be monitored and controlled [9]. Certain improvements have been suggested to overcome the inconvenience of using methanol, Such as the set-up of a three-stage feeding scheme [10], or the development of other promoters [11-15].

Among these promoters, the glyceraldehyde-3-phosphate dehydrogenase (pGAP) promoter has become an increasingly attractive alternative for pAOX. This promoter was isolated and analyzed [16], and is applied as a constitutive promoter for heterologous protein expression in *P. pastoris* [17, 18]. Without using methanol as an inducer, potential hazards can be avoided and strains are therefore more suitable for applications in the lab or large-scale productions [19]. According to certain studies [17, 20, 21], cell growth and protein expression under the strains carrying pGAP were competitive or at times even more efficient than those using the pAOX system. However, cell growth and the expression level of the proteins of interest are both highly dependent on the carbon source, as no inducer is required [3].

The selection of the carbon source is one of the key factors for *P. pastoris* cultivation. In general, the carbon sources used for the fermentation of yeast are methanol, glucose, and glycerol. Methanol is required as a carbon source in the pAOX system of protein expression. However, because of the low growth rate during the methanol induction phase, the common fermentation protocol for this strain requires the introduction of glycerol or glucose during the first stage, in order to accumulate biomass. Alternatively, the use of mixed feeds is considered as another method for the cultivation of *P. pastoris*. In these feeding strategies, glycerol or glucose concentrations must be monitored and maintained at a lower level due to the repression of the pAOX promoter by non-C_1 carbon sources [10]. Some studies have identified non-repressive carbon sources to replace the use of glycerol and glucose, and thus far, sorbitol, mannitol, alanine, and trehalose were reported as non-repressing [8, 22]. For the pGAP system, the choice of the carbon source is less limited as there is no need to consider the repression of the promoter. However, glycerol and glucose still remain the dominant carbon sources for this system.

Citric acid can be used as a carbon and energy source for certain strains [23-26]. It is an organic acid and is commonly used in the food industry as a food additive and preservative. Research has found that citric acid has an inhibitory effect on the growth of microorganisms by lowering the pH and chelating divalent metal ions [27]. A study from Lawrence *et al.* showed that the growth of the yeast *Saccharomyces cerevisiae* might be inhibited by citric acid, but the production of glycerol, however, was increased [28]. Another study, investigating the effect of citrate on *Chlorella vulgaris,* found that higher biomass was achieved at a higher citrate level [29]. Citric acid was suggested as a carbon source for fatty acid synthesis. Higher citrate concentrations led to higher pigment concentrations during the production of astaxanthin in *Phaffia rhodozyma* [30]. The research concerned with the production of Aspergiolide A in *Aspergillus glaucus* also showed similar results: the production was increased by 80% by adding citric acid [31]. It should be noted that most research studying the use of citric acid was conducted under anaerobic conditions or using prokaryotic organisms, and very little work has been done to study the effect of citric acid on eukaryotic strains or on the expression of proteins.

In this study, the feasibility of using citric acid as a carbon source for *P. pastoris* was investigated, and its effect on cell growth and phytase production was analyzed. The optimal conditions such as the optimal pH and the ideal concentration of citric acid were studied using flask experiments. The operating parameters for bioreactors using citric acid were also studied, with the objective to achieve high cell biomass and phytase productivity.

4.2 Materials and Methods

4.2.1 Microorganism and Chemicals

P. pastoris expressing and secreting phytase under the control of the pGAP promoter was kindly provided by Zell Technologies Inc., Canada. A constitutive pGAPZα vector with a zeocin selection agent was used by this strain. All chemicals were purchased from Fisher Scientific or Sigma-Aldrich.

Agar yeast extract-peptone-dextrose (YPD) plates were used to store the *P. pastoris* strains. This solid medium contained 10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose, 20 g/L agar and 100 ug/mL zeocin. Distilled deionized water was used in the experiment.

4.2.2 Inoculum Preparation

Single colonies were taken from agar plates and transferred into flasks with 100 mL liquid YPD medium. The flasks were cultivated in a shaker (New Brunswick Scientific) for 24 h. The cultivation conditions in the shaker were maintained at 30 °C and 250 rpm. For the experiments, 5% (v/v) of the liquid medium containing cells was added as the inoculum.

4.2.3 Cultivation Conditions

For the flask experiments, two types of media were used, a YP-Carbon source and a minimal salt medium (MSM). The YP-Carbon source contains 10 g/L yeast extract, 20 g/L peptone and different concentrations of a selected carbon source, based on the experimental design.

MSM was composed of 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, 11.7 g/L magnesium sulfate, and 2 mL/L PMT-4 trace elements solution. The composition of the PMT-4 trace elements solution are: copper (II) 2.0 g/L sulfate pentahydrate, 0.08 g/L sodium iodide, 3.0 g/L manganese sulfate monohydrate, 0.2 g/L sodium molybdate dihydrate, 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. 15 g/L of glycerol was used as the carbon source, and other carbon sources were added based on the experimental design. Ammonium hydroxide was added, as required, to adjust the pH. All flasks were cultivated in the shaker, which was set to 30 °C and 250 rpm, for several days, and samples were taken every few hours.

4.2.4 Fermentation System

For fermentation, a 3 L BioFlo 110 bioreactor (New Brunswick Scientific) initially containing 1 L of modified MSM (m-MSM) medium and 10 g/L glycerol as the carbon source was inoculated with *P. pastoris*. Modified MSM has a recipe similar to that of MSM, but without potassium citrate and with an increase in the concentration of potassium sulfate from 14.33 g/L to 14.93 g/L to balance the potassium requirements. The temperature was kept at 30 °C, and the pH was monitored (pH probe, Mettler Toledo) and maintained at 5.0 by adding 30% ammonium hydroxide (NH_4OH). The air flow rate was set between 1.5 and 2.5 LPM (1.5-2 vvm), and the agitation rate was adjusted automatically from 250 to 1200 rpm to maintain a dissolved oxygen (DO) level above 20%. DO level was detected by a dissolved oxygen sensors (Mettler Toledo).

Four different compositions were used in the feeding solution as listed in Table 4.1. When the initial carbon source was consumed, which was indicated by a spike in the DO concentration, the solution started to feed into the bioreactor (16 h).

Table 4.1 Composition of feeding solutions for experimental runs

Effects of substrate concentration in feeding solutions				
Solution	1	2	3	4
Glycerol (g/L)	500	450	400	350
Citric acid (g/L)	0	52	104.2	156.4

To study the effect of cell status, three OD levels representing different timings were chosen as the standards for triggering the feeding. These details are listed in Table 4.2.

Table 4.2 Effect of Citric acid Timing for experimental runs

Effect of Citric acid Timing			
Adding citric acid solution when OD was achieved at	1	2	3
OD	72.5	90.5	147

4.2.5 Analysis

Samples were taken every few hours to measure the optical density (OD), the phytase activity, and the substrate concentration in the broth.

OD was determined at a 600 nm wavelength using a Biochrom Ultrospec double-beam spectrophotometer (Biochrom Ltd). For dry cell weight (DCW), 2 mL of broth was taken in a microtube, rinsed using distilled de-ionized water, and put into an oven for several days. It was weighed regularly until the weight remained the same (data shown in appendix A). The equivalence ratio was found to be 0.3715 g of DCW per 1 unit of OD at 600 nm. To measure phytase activity, samples were centrifuged at 10,000 rpm for 10 min. The supernatant was diluted as required, and the phytase activity was determined with a standard phosphatase assay protocol described previously [32].

The concentration of various compounds in the culture broth were analysed using an Agilent HPLC with an Aminex HPX 87-H column. Samples were also centrifuged at 10,000 rpm for 10 min and the supernatant was injected into the HPLC for analysis. Three components were monitored: glycerol, ethanol, and citric acid. For glycerol and ethanol, 0.05 mol sulfuric acid was used as the mobile phase, and the flow rate was set to 0.6 min/mL. The temperature of the column was 60 °C. To determine the concentration of citric acid, the same concentration and flow rate of the sulfuric acid was used, but the temperature was kept at 30 °C.

4.3 Results and Discussion

4.3.1 Citric Acid as the Sole Carbon Source

The use of citric acid as a carbon source for phytase production in *P. pastoris* has not previously been investigated. In this study, the possibility of using citric acid was tested, and the primary culture parameters such as the optimal substrate concentration and pH were investigated in shake-flasks. Once appropriate conditions for the use of citric acid as a carbon source were identified, other experiments were conducted to compare the efficiency and productivity of cultures using citric acid compared to the common carbon sources, glucose and glycerol. In this section, all of the experiments were performed in a YP-Carbon source medium. This complex medium can provide the full nutrient requirements for yeast to grow. The experiments were conducted in shake-flasks.

4.3.1.1 Effect of Citric Acid Concentration and pH on *Pichia pastoris* Growth

An experiment was carried out using citric acid as the carbon source, with concentrations ranging from 5 g/L to 10 g/L. The effect of the initial citric acid concentration on cell growth is shown in Figure 4.1A. The maximum biomass (OD_{600}=1.66) was obtained with 5 g/L citric acid, and with an increase of the initial citric acid concentration, cells grew more slowly and a lower final biomass concentration was achieved (OD_{600} 0.37, 0.31 and 0.22 corresponding to 10 g/L, 20 g/L and 40 g/L initial carbon source respectively). As the growth curve shows, the concentration of citric acid has a great impact on the final biomass concentration and on the specific growth rate of the culture.

The difference in biomass concentration may due to the pH in the medium. As citric acid is an organic acid, the pH level of the medium is associated to its concentration. A profile of the pH before and after the experiment is shown in Figure 4.1B. The increase in pH at the end of the experiment may have been caused by the consumption of the citric acid, since citric acid was the sole carbon source involved. With the increase in citric acid concentration, the initial pH levels dropped from 4.24 to 3.70, 3.26 and 2.83. The common pH levels for *P. pastoris* cultivation range between 3.0 and 7.0 [33, 34]. In this experiment, the pH for citric acid with 40 g/L already exceeded this limit. The pH from others concentrations, 20 g/L and 10 g/L, were still within the range, but were close to the lowest limit.

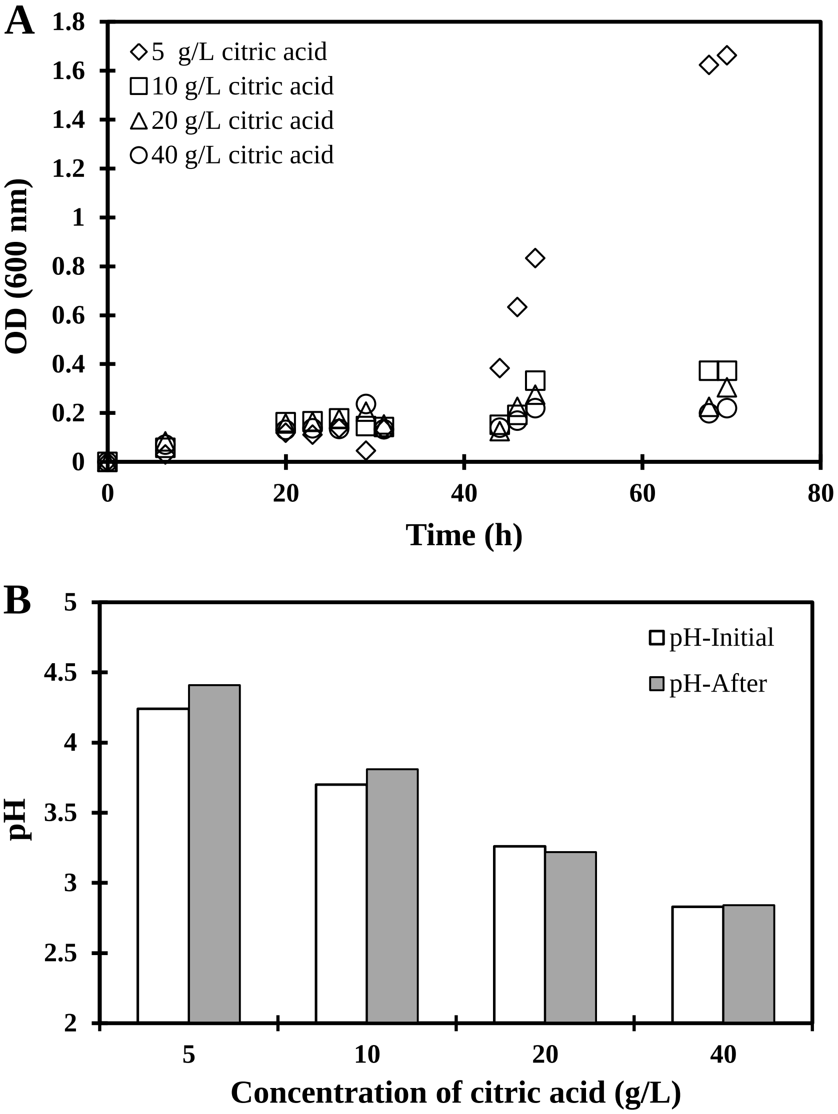

Figure 4.1 Cell growth and pH changes under different citric acid concentration. A Profile of cells growth; **B** Changes of pH before and after the experiments (0 and 70 h).

To study the effect of the pH on cell growth, the same initial citric acid concentration of 5 g/L was adopted. Ammonium hydroxide was added to adjust the pH to different levels, in increments of 0.5. The initial pH was controlled within the common range from 4.24 to 6.31, with an initial pH of 4.24 (without adding NH_4OH, as control) when 5 g/L citric acid was applied. The profile of cell growth is shown in Figure 4.2.

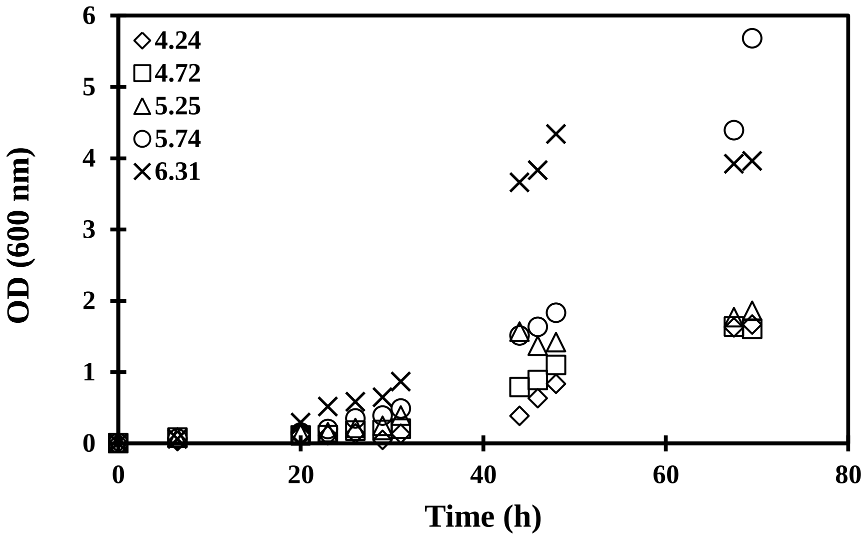

Figure 4.2 Cell growth using 5 g/L citric acid at various initial pH levels.

The results displayed the longer lag phase at lower pH. Cell growth at different pH levels started to differ after approximately 40 h. At higher pH, cells were already in the exponential phase, but at the lowest pH of 4.24, cells were still in the lag phase. The final biomass concentration increases with an increasing pH, which is compatible with results obtained by Soyaslan [35]. The maximum OD_{600} of pH 6.31 was achieved around 50 h, while the cells kept growing exponentially even after 65 h for pH 5.74. The reason for discontinued cell growth with a pH of 6.31 may be due to the large concentration of ammonium hydroxide in the medium; the cells were unable to tolerate this high pH level. The pH changes are shown in Figure 4.3. The increasing in pH following the experiment is likely due to the consumption of the citric acid. For pH 5.74 and pH 6.31, better performances were obtained, as indicated by a higher biomass and growth rate. Considering that the objective is to conduct experiments in bioreactors, it should be kept in mind that common applications in industry and higher pH levels may lead to a higher risk of contamination [36, 37]. A pH of 5.74 is therefore recommended as slightly better option between these two pH levels.

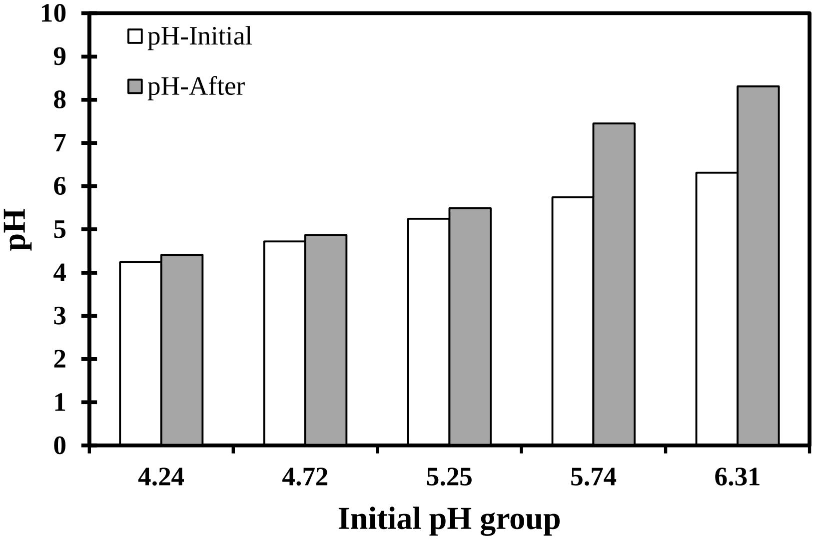

Figure 4.3 Changes of pH before and after cultivation using 5 g/L citric acid as the initial concentration of the carbon source.

As discussed above, the pH has a great impact on cell growth, the maximum biomass concentration, and the consumption rate of the carbon source. This is not true in all cases however. An experiment done by Charoenrat *et al*. to study the effect of pH showed that similar growth curves were obtained when using two pH levels (pH 5 and pH 6) [38]. Some articles also state that the pH has no obvious effect on the growth rate [9, 34, 39]. The variation in these conclusions may be due to the selection of the carbon source. The usual carbon sources for *P. pastoris* are glycerol, glucose, or methanol. Growth in a citric acid medium and using citric acid as the carbon source leads to changes in pH (from 6.31 to 8.31) in flask experiments, as opposed to other carbon sources whose consumption does not affect the pH and which therefore provide a stable growth environment.

To study the effect of the concentration of citric acid without considering the influence of pH, another experiment was needed. In this experiment, three different concentrations of citric acid were taken, from 5 g/L to 10 g/L, and the medium was adjusted to a pH of 6.0 using ammonium hydroxide at the beginning. The results are shown in Figure 4.4. When the effect of pH was eliminated, the concentration of citric acid had little impact on growth. The lag phase for three conditions was close. The final optical densities were 4.81±0.53, 5.69±0.29, and 5.59±0.39 respectively, with the increase in citric acid. This showed that these concentrations of citric acid are not at the limiting concentration since there was no growth inhibition at these concentrations. It also suggested that the concentration of citric acid up to 10 g/L do not have an effect on cell growth which is helpful for experiments in bioreactors.

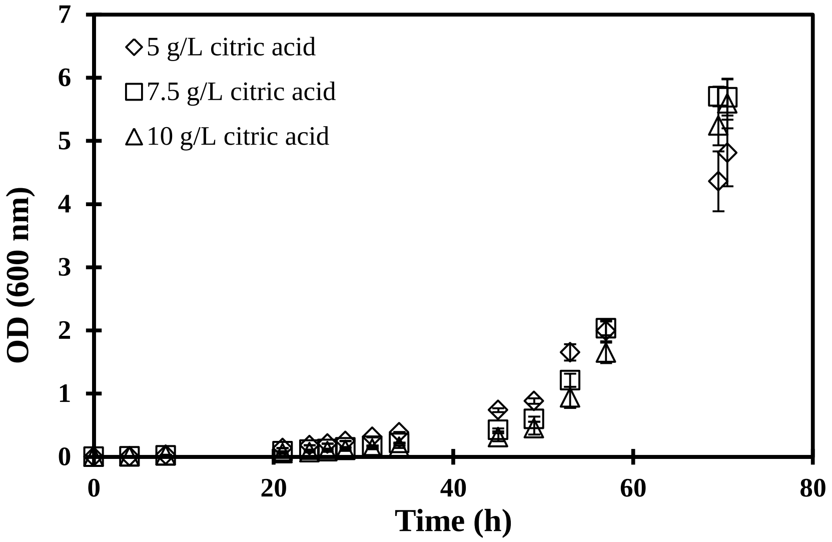

Figure 4.4 Effect of various concentrations of citric at the same pH on the growth of *P. pastoris*. (average ± std dev, n=3)

The concentration of citric acid has less of an impact on cell growth than the level of pH does. Various levels of pH affected the lag phase as well as the cell growth rate. Thus, pH was an important parameter to consider when using citric acid. In the further experiments conducted in the bioreactor, the pH was controlled at a stable level which can eliminate the effect of pH.

4.3.1.2 Comparison of Citric Acid, Glucose and Glycerol as Sole Carbon Sources

As shown in the previous section, *P. pastoris* cells can grow when using citric acid as the sole carbon source. In this section, the effect of citric acid on cell growth and phytase expression was examined and compared to the performance on glycerol and glucose. Three carbon sources of glycerol, glucose, and citric acid were tested, at three different concentrations, 5 g/L, 10 g/L, and 20 g/L, respectively. Since the concentration of citric acid has an effect on the pH, the media were adjusted at the same pH 5.0 using ammonium hydroxide. The results are shown in Figure 4.5 and Table 4.3.

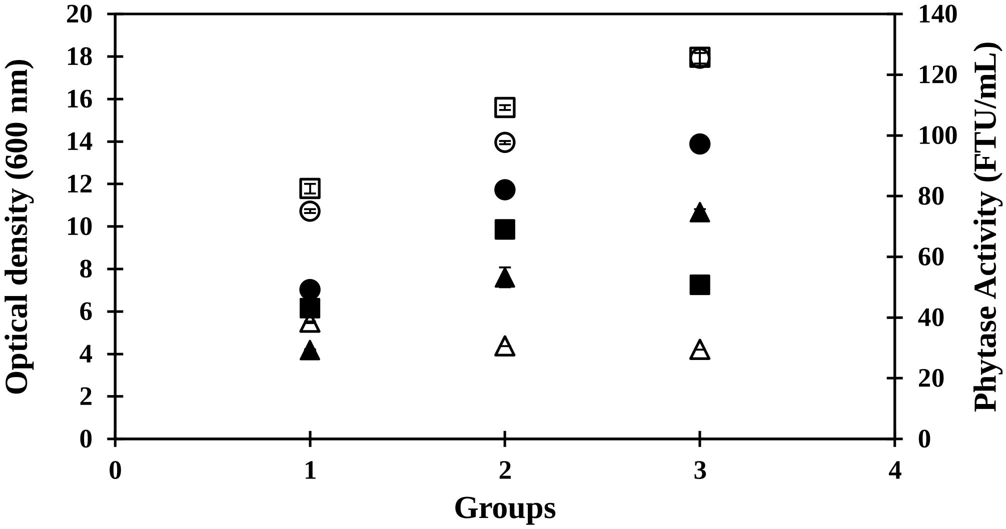

Figure 4.5 Comparison of carbon sources at three concentrations. Open symbols: optical density at 600 nm; Closed symbols: phytase activity. Circles: glucose; square: glycerol; triangle: citric acid. Group 1: 5 g/L carbon source; group 2: 10 g/L carbon source; 3: 20 g/L substrate. (average ± std dev, n= 3)

Table 4.3 Summary of parameters and results for the different carbon sources Results shown are the average of three runs. (average ± std dev, n=3)

Carbon Sources		**Concentration**		
		5 g/L	**10 g/L**	**20 g/L**
Glucose	Max. O.D	10.73±0.09	13.95±0.09	17.92±0.10
	Max. μ (h^{-1})	0.1475±0.0009	0.1922±0.0068	0.1917±0.0020
	Max. Phytase activity (FTU/mL)	49.20±1.27	82.05±1.01	97.18±1.27
	Yield $Y_{p/x}$ (FTU mL^{-1}/g cell L^{-1})	13.05±0.44	16.40±0.39	16.11±0.58
Glycerol	Max. O.D	11.78±0.23	15.60±0.07	17.97±0.25
	Max. μ (h^{-1})	0.1538±0.0028	0.1891±0.0054	0.1842±0.065
	Max. Phytase activity (FTU/mL)	43.11±0.35	69.02±3.04	50.83±0.61
	Yield $Y_{p/x}$ (FTU mL^{-1}/g cell L^{-1})	10.26±0.13	10.38±0.67	8.08±0.21
Citric Acid	Max. O.D	5.49±0.04	4.37±0.12	4.21±0.41
	Max. μ (h^{-1})	0.0414±0.0021	0.0181±0.0038	0.0426±0.0009
	Max. Phytase activity (FTU/mL)	29.32±0.25	52.26±3.27	74.76±0.93
	Yield $Y_{p/x}$ (FTU mL^{-1}/g cell L^{-1})	15.72±0.16	35.910±0.18	54.14±0.80

Using citric acid as the carbon source, the highest biomass concentration (OD of 5.49) was found when the concentration was 5 g/L, and the final biomass decreased with the increase in the concentration of citric acid. The reason for this result might be the use of ammonium hydroxide. Increasing the concentration of citric acid resulted in a lower pH in the medium. To balance the pH, more ammonium hydroxide was used at the higher concentration of citric acid. The length of the lag phase was increased, and cell growth was inhibited with the increased concentration of NH_4^+ [40]. However, the expression of phytase was not repressed.

Increased phytase activity was achieved at a higher citric acid concentration, and the maximum phytase activity was 74.76 FTU/mL using 20 g/L citric acid. Although this value was smaller than when using glucose, the yield of phytase was much higher using citric acid. It indicates clearly that the phytase expression was not repressed in the citric acid system, since the phytase activity per cell was threefold that of using glucose. Citric acid is therefore a promising carbon source for heterologous protein expression.

4.3.2 Citric Acid as Co-Carbon Source

The previous experiments suggested that citric acid can be an alternative carbon source for the production of phytase using *P. pastoris*. As shown above, the presence of citric acid will not repress the expression of phytase, but it may enhance its productivity. For biomass and cell growth, citric acid was less competitive than glucose and glycerol. Generally, the expression of pGAP is linked to cell growth, which can influence the production of phytase. The greatest challenge when using citric acid is to improve cell growth in the medium. Coupling citric acid with another dominant carbon source in the medium might help improve biomass and productivity.

For this set of experiments, glycerol was used as the first carbon source to allow cells to grow, in order to increase the biomass level. Citric acid was then added to encourage a high productivity of the proteins of interest. Since there are two carbon sources in one system, it is important to determine the sequence used by the cells to utilizing them, including the proper timing to introduce the secondary carbon source. In this section, the medium was a MSM medium instead of YP-Carbon source. These experiments were designed to gain preliminary results for the possibility of using citric acid as a secondary carbon source in tests performed in a bioreactor.

As yeast can tolerate a wider range of glycerol concentrations than glucose concentrations, glycerol was taken as the initial carbon source, with a 15 g/L concentration. To eliminate the decrease in the pH when citric acid is added to the broth, an addition of ammonia hydroxide is required. In order to study the effect of the concentration of citric acid, as well as the timing of adding it to the broth, two approaches were designed, as follows.

4.3.2.1 Addition of Citric Acid at the Beginning of Experiment

Two quantities of citric acid were added at the beginning, during the preparation of the medium. The performance of yeast is shown in Figure 4.6. The results show that with the presence of citric acid, the cells grew slowly. The difference in growth started to appear 14 h after the introduction of the citric acid, and the growth under citric acid was slowly compared to when only glycerol was used. The specific growth rate and the final biomass concentration were reduced with the increasing concentration of citric acid; the maximum OD_{600} were 17.49±0.30, 13.19±1.08 and 6.47±0.61 respectively. The profile of the phytase activity had a similar curve (data not shown).

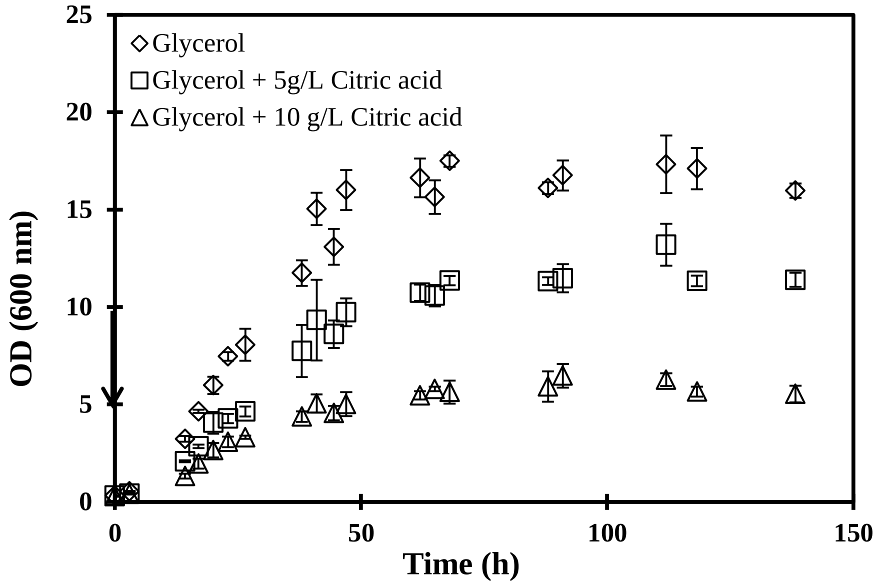

Figure 4.6 *Pichia pastoris* performance with citric acid added at the beginning of the experiment (0 h). (average ± std dev, n=3)

Cell growth was influenced by adding citric acid. Since the pH of the medium was kept at the same level by adding ammonium hydroxide, the effect of the pH was eliminated. The reason for the difference of cell growth might be the presence of citric acid, and associated the addition of NH_4^+ As reported, citric acid will inhibit cell growth by chelating the metal ions from the medium [41, 42]. With a higher amount of citric acid, final cell density was decreased, indicating that the utilization pathway of glycerol might be repressed.

4.3.2.2 Addition of Citric Acid during the Exponential Phase

Two concentrations of citric acid were added in the early stage of the exponential phase. Cell growth consumed 15 g/L glycerol as the first carbon source, and then different concentrations of citric acid or glycerol were added to the medium at 15 h. A certain amount of ammonia hydroxide was added at the same time, in order to balance the changes in pH. The performance of *P. pastoris* is shown in Figure 4.7.

The final OD_{600} were 16.15, 15.75±0.19, and 15.86±0.06, corresponding to glycerol only, with the addition of 5 g/L citric acid, and with 10 g/L citric acid respectively. The similar growth curves and close final biomass concentration showed that adding citric acid had little or no influence on yeast growth. The close final biomass concentration might be due to the consumption of glycerol. Since the cells coming from the inoculum were already adapted to the glycerol medium and the enzymes involved had been synthetized, the addition of citric acid after 15 h might not inhibit cell growth.

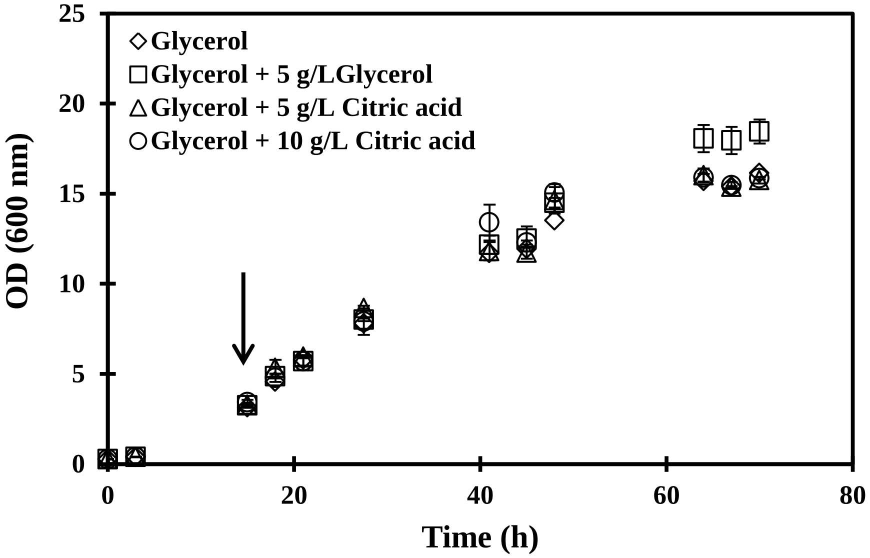

Figure 4.7 Profile of biomass (OD_{600}) with citric acid added at the beginning of exponential phase (15 h). (average ± std dev, n=3)

Usually, when the medium contains more than one carbon source, cells consume the dominant carbon source first. Once the first carbon source is depleted, cells start to use the secondary carbon source [43]. This phenomenon is known as *diauxic growth,* which was previously observed in other work [44, 45]. In this feeding profile, there was no sign of typical diauxic growth, even after 100 h when the stationary phase was achieved. According to the previous experiments, the lag phase for cells to adapt to the citric acid medium was approximately 40 h. Cells were already in the exponential phase or had started jumping to exponential phase, after approximately 40 h, based on the previous data.

The theory of diauxic growth did not fit the results, indicating that the cells might utilize glycerol and citric acid at the same time. In previous work the simultaneous utilization of different compounds was observed when cells were presented with a mixed medium [46-49]. The explanation needed to be supported by further results and the residual chemicals such as glycerol and citric acid would be measured in bioreactor experiments which is beneficial for understanding consumption of glycerol and citric acid.

Comparing these two scenarios, the timing of citric acid introduction into the system is critical to cell growth. The early addition of citric acid has a negative effect on cell growth and results in a lower growth rate and final biomass concentration. In comparison, the addition of citric acid after the cells adapted to the new medium does not inhibit cell growth.

4.3.3 Fermentation in a Bioreactor

Preliminary experiments showed that *P. pastoris* had the ability to use citric acid as a sole carbon source and that phytase could be expressed in a citric acid medium. The results indicated that the yield of phytase was higher in a citric acid medium than with glucose.

However, cell density was lower in citric acid. For the constitutive pGAP promoter, the expression of proteins of interest is higher linked to cell growth. The goal of the following experiments was to achieve high cell density with an increase of the yield of phytase. Thus, fed-batch cultivation fed with glycerol and citric acid were performed. Compared with the shake-flask experiments, higher cell density can easily be achieved in a bioreactor because the relative physico-chemical conditions can be monitored and controlled. Based on the results above, adding citric acid during the exponential phase might be a promising option for further experiments, especially for bioreactors, as it has a minimal negative effect on cell growth. Glycerol was used as the initial carbon source to guarantee cell growth and to eliminate the negative effect of citric acid. In this section, optimal feeding conditions were investigated.

The exponential feeding strategy was adopted in this section as it is simple but efficient. The exponential feeding rate can supply nutrients for cells to grow exponentially. Moreover, the feeding rate was calculated and applied to all the runs that could maintain a certain volume of the feeding solution during fermentation. Applying other feeding strategies such as pH-stat might cause differences in the consumption volume of the carbon source because these feeding methods have feedback control, and would therefore have been influenced by the conditions at that time. Using the exponential feeding method can eliminate the different feeding solution consumption rates in the various runs.

4.3.3.1 Effect of the Concentration of Citric Acid in the Feeding Solution

In the previous shake-flask experiment, *P. pastoris* growth using citric acid as a carbon source was proven. However, without further analysis, the utilization of citric acid is not well understood, and the utilization pattern for dominant carbon sources and citric acid still remains unknown. Another problem that needs to be solved is the optimal feeding concentration of citric acid. Thus, in this experiment, four different concentrations of citric acid blended with different levels of glycerol were tested.

Cell growth using the five feeding solutions is shown in Figure 4.8A. The maximum optical density of 313.93 was obtained when there was no citric acid in the solution. The increase of citric acid concentration in the feeding solution has a negative effect on cell growth. The final phytase activity under these feeding conditions is shown in Figure 4.8B; it displays a dissimilar trend with cell growth. There is no statistically difference in phytase activities when using 500 g/L glycerol and a mixed solution with 52 g/L citric acid and 450 g/L glycerol. The summary of the effects of the various concentrations of citric acid is shown in Table 4.4.

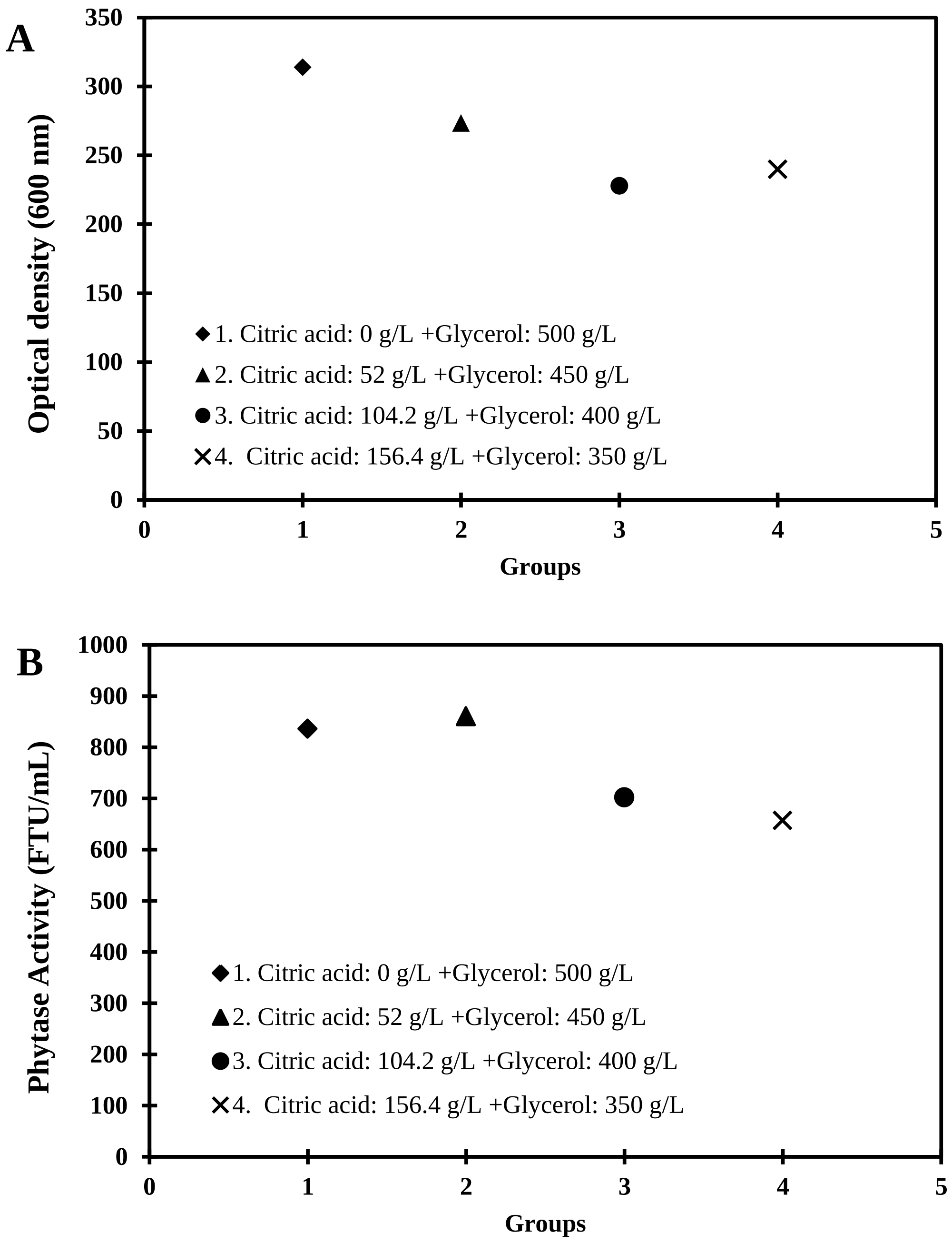

Figure 4.8 Maximun OD and phytase activity under various concentrations of citric acid and glycerol. A: optical density at 600 nm; B: phytase activity under four feeding solutions. (average, n=2 to 3)

Table 4.4 Summary of results under various compositions of glycerol and citric acid in the feeding solutions (average, n=2 to 3)

Citric acid (g/L)	**0**	**52**	**104.2**	**156.4**
Total hours (h)	40	40	40	40
Feeding hours (h)	24	24	24	24
Fed-in citric acid (g)		23.98	48.06	72.13
Residual citric acid (g)		17.42	28.61	58.68
Maximum OD (600nm)	313.93	273.4	227.75	239.75
Maximum phytase activity (FTU/mL)	836.21	860.22	701.97	656.83
Yield $Y_{p/DCW}$ (FTU mL^{-1} / g cell)	7.18	8.43	8.29	7.34
Fed phase μ (h^{-1})	0.126	0.120	0.116	0.116

In the presence of citric acid, cells grew slowly as predicted by the previous experiments in shake-flasks. However, these differences might be caused by the decreasing concentration of glycerol instead of by the inhibition of citric acid or NH_4^+. Since the concentration of glycerol was decreased, the growth rate might be reduced due to an insufficient dominant carbon source. Consuming citric acid as a carbon source for cell growth, the yield of biomass was lower than when using glycerol. Based on the previous results, the final biomass was doubled when using 5 g/L glycerol compared to when using citric acid. Compared with the control group containing 500 g/L glycerol and no citric acid, biomass was 12% less in the mixtures of 450 g glycerol and 52 g citric acid per liter feeding solution. This percentage was close to the difference in the concentration of glycerol, namely 10%. When the concentration of citric acid was higher than 104.2 g/L, the effect on cell growth was similar, since a similar biomass concentration was obtained at citric acid concentrations of 104.2 g/L and 156.4 g/L.

Another explanation for the low biomass concentration is the accumulation of ammonium hydroxide. With the increase in the concentration of citric acid in the feeding solution, more ammonium was required to maintain the pH in the broth.

Usually, the expression of heterologous proteins under the constitutive promoter pGAP is proportional to cell growth. Higher cell density leads to more protein expression. However, in this experiment, when using 500 g/L glycerol and a mixed solution with 52 g/L citric acid and 450 g/L glycerol, there is no statistical difference in phytase activities even with a lower cell density achieved at the second solution. This suggests that in a certain level of citric acid, the synthesis pathway for the proteins of interest was enhanced or the protease activity in the broth was decreased. Phytase activity decreased when the concentration of citric acid in the broth was higher than 104.2 g/L. It indicates citric acid might have negative effect on phytase activities when the concentration of citric acid is excess the limit.

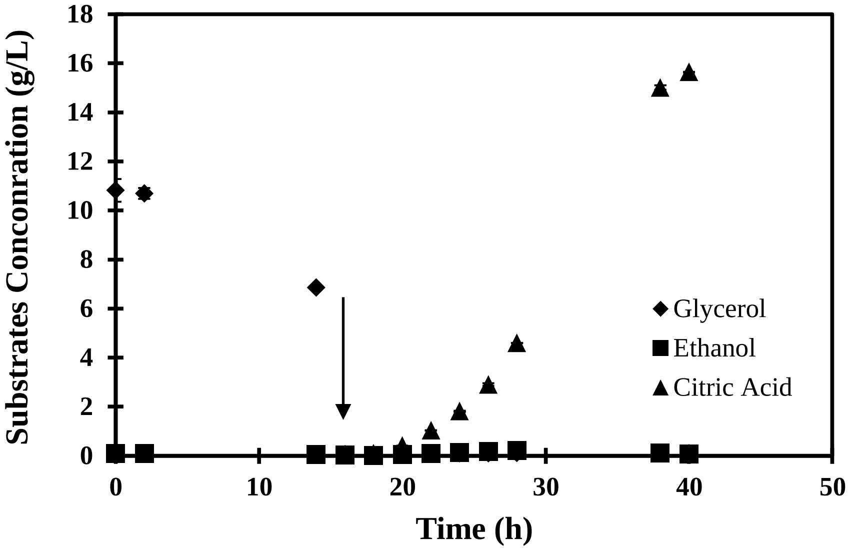

Figure 4.9 Substrate concentrations during the fermentation using feeding solution with 52 g/L citric acid. Arrow indicated the timing started to feed-in solution.

The effect of citric acid is not well understood as the research dedicated to the study of cell growth in citric acid is extremely limited. The citric acid that accumulated during fermentation is shown in Figure 4.9, and the residual concentration of citric acid is shown in Table 4.4. From the residual concentration, the cells did consume citric acid during the fermentation. However, the uptake of citric acid varied in different concentrations. For the 52 g/L concentration, approximately 6.56 g citric acid was used. When the feeding solution concentration increased to 104.2 g/L, 19.45 g citric acid was used, but the consumption of citric acid was decreased to 13.45 g when 156.4 g/L of citric acid solution was used. The uptake rate of citric acid was influenced by the solution concentration. The maximum consumption of citric acid was obtained using a 104.2 g/L citric acid solution. The maximum uptake rate of citric acid and the maximum phytase activity were not obtained in the same conditions. It suggests that the uptake rate of citric acid was not beneficial to phytase expression. Maintaining the uptake rate of citric acid within a certain level is another parameter needed to enhance phytase expression.

4.3.3.2 The Effect of Citric Acid Timing

As discussed for the flask experiments, the timing at which we add citric acid has an effect on cell growth and protein expression. The addition of citric acid during the exponential phase has no negative influence as opposed to adding it at the beginning of the experiment. In terms of the cultivation in a bioreactor, much of the fermentation aims to keep cells in the exponential phase so that the biomass can be accumulated at a high rate. In order to investigate the effect of introducing citric acid at different stages of the exponential phase, the feeding was started at different set points of OD. According to the previous data, the blended solution of 52 g/L citric acid and 450 g/L glycerol led to higher phytase activity and was therefore adopted.

The feeding was triggered when the OD reached 72, 91 and 147.4 for runs 1, 2 and 3 respectively. The final biomass concentration, phytase activity, and consumption of citric acid are displayed in Figure 4.10 and Table 4.5. The maximum OD was achieved when citric acid was introduced at a higher biomass. The results are similar to those of previous experiments. The addition of citric acid has a negative effect on cell growth since a higher biomass was obtained when less citric acid was presented in the system. However, phytase activity showed a different trend than cell growth. With the increase of the feeding hours or of the amount of citric acid, phytase activity improved. The results suggest that the phytase expression is affected by the length of time citric acid is in the system. The earlier the citric acid was added to the system, the higher the phytase activity achieved. The reason might be the lag phase for utilizing citric acid. Cells need to adapt to the environment when a new carbon source is added. Adding citric acid early can provide enough time for cells to respond to the citric acid, especially the substrates involved into the phytase expression, since adding citric acid in the medium is critical to phytase activity. However, based on the data obtained, additional runs would be necessary to get an idea of the variability of the results.

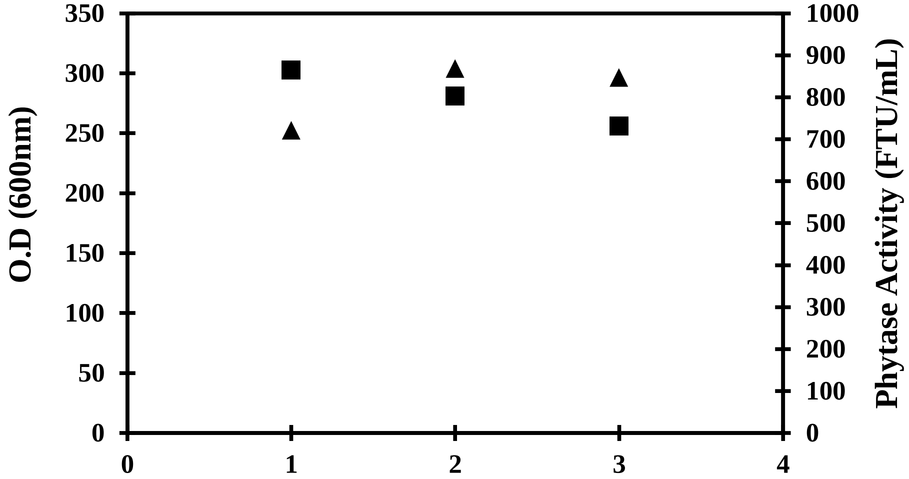

Figure 4.10 Optical density and phytase activity under various OD conditions as start of feeding. Closed triangle: O.D 600nm; Closed square: phytase activity. 1: Feeding started when OD was 72; 2: feeding stared when OD achieved 91; 3: feeding triggered when OD was 147.5.

Table 4.5 Results of effect of timing of adding citric acid

Feeding start: when OD	Total hours (h)	Feeding citric acid hours (h)	Total fed-in citric acid (g)	Residual citric acid (g)	Maximum OD	Maximum phytase activity (FTU/mL)
72	40	13.5	11.68	9.81	252.5	865.29
91	40	12	10.38	9.48	304	803.41
147.5	40	10	8.65	7.69	296.5	731.39

4.4 Conclusion

The carbon source has an impact on cell growth and heterologous protein expression in *P. pastoris*. The common carbon sources adopted for fermentation were sugars and glycerol. Alternative carbon sources should be considered to improve cell growth and protein expression, or even as a secondary carbon source to reduce the use of the dominant carbon source. Citric acid was examined as a promising carbon source. Using citric acid as the sole carbon source in flask experiments resulted in a lower biomass concentration but a high phytase yield compared with glycerol and glucose. The concentration and the pH have an influence on both cell growth and phytase activity. To improve the biomass and increase the total phytase activity, citric acid was adopted as a co-carbon source and fed into the system with glycerol. The flask experiment showed that introducing citric acid during the exponential phase had little effect on cell growth. Further experiments conducted in bioreactors displayed that the high phytase activity achieved using a mixed feeding solution of 52 g/L citric acid and 450 g/L glycerol was statistically identical to that of using a glycerol solution. Introducing citric acid in the system is promising for phytase activities. To achieve the same level of phytase activities, less amount of glycerol will be required by adding citric acid. Adding citric acid early may be beneficial for achieving high phytase activities.

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Chapter 5

Conclusions and Recommendations

5.1 Conclusions

Pichia pastoris has become a popular platform for expressing heterologous protein because of its various benefits including the ability of growing on simple culture media and under simple operational conditions, as well as its ability to perform post-translational modifications. Studies of the use of promoters, medium composition, cultural conditions and operational strategies aim to improve the performance of the expression system or the yields of heterologous proteins.

In this research, three basic feeding strategies were discussed and a new carbon source was tested. The main conclusions that can be drawn are the following:

- Constant feeding control, exponential feeding strategy and DO-stat control were compared in this research. The highest biomass was achieved using exponential feeding control, while the maximum phytase activity was obtained using DO-stat feeding strategies, which also had lower glycerol consumption.
- To eliminate the oxygen-limiting conditions, the agitation was introduced and coupled to the DO-stat method. Instead of keeping the agitation rate constant, a flexible agitation rate was used in the modified DO-stat feeding. The results showed the higher biomass and phytase activity were obtained when using the modified DO-stat feeding method.
- The feasibility of using citric acid as a carbon source was tested. Cells can grow on

citric acid as sole carbon source. Compared to the glycerol or glucose system, the cell grew slowly and the final biomass was low when using citric acid. However, yield of phytase (FTU mL^{-1} / g cell) was higher.

- Citric acid was tested as a secondary carbon source. Using glycerol as dominant carbon source to increase the biomass and then introducing citric acid to maintain or even improve the productivity of phytase. Using a mixed feeding solution with glycerol and citric acid, the phytase activity was similar compared to when using pure glycerol feeding. Thus, to achieve similar level of phytase activity, the usage of glycerol can be reduced by adding citric acid.

5.2 Recommendations

Based on the results obtained in this research, a few recommendations can be proposed for further study, as follows.

- For the feeding strategy, to improve the yields of heterologous proteins using DO-stat method, mathematical models or chemical online monitoring can be introduced in the setup. Since yeast-based expression systems are becoming increasingly important to industry, a lot of research found that combined the operational strategy with a model or using chemical online monitor can improve the understanding or controlling the fermentation procedure.
- Since the modified DO-stat method was shown to increase the phytase activity compared to using the traditional DO-stat method, it is interesting to study the detail of the difference of both methods. Based on the results gained in this research, the relationship amongst the agitation rate, oxygen level, feeding rate and the cell growth would deserve further study.

- Since citric acid was revealed to be a promsing carbon source for *P. pastoris*, experiments can be conducted to study the optimal cultivation conditions such as the optimal pH, temperature and concentration. Fermentation conditions have critical effects on the cell growth and protein expression. The relationship between pH, concentration of citric acid and the concentration of ammonium hydroxide could be examined. Experiments can be conducted to optimal the cultivation conditions for citric acid. For the bioreactor experiments, the control strategies could apply to improve the performance of the expression system.
- The effect of introducing citric acid on the metabolic pathways in yeast cells should be investigated. Due the limited research focusing on using citric acid as a carbon source, the influence of citric acid on the metabolism still remained little known.

Appendix A: Calibration for Ethanol, Citric Acid and Glycerol

Chemical compositions, including glycerol, ethanol, and citric acid, are the important indices or factors during fermentation; they were detected using an Agilent HPLC with an Aminex HPX-87H column. Before the experiment starts, a calibration curve and some basic information such as the retention time of each composition needed to be measured. Different concentrations of substrates were prepared and then injected into the HPLC. For glycerol and ethanol, 0.005 mol sulfuric acid with a flow rate of 0.6 mL/min was used as the mobile phase, and the column temperature was controlled at 50 ˚C. For the citric acid, the same concentration of sulfuric acid was used but with a flow rate of 0.4 mL/min and a column temperature of 30 ˚C.

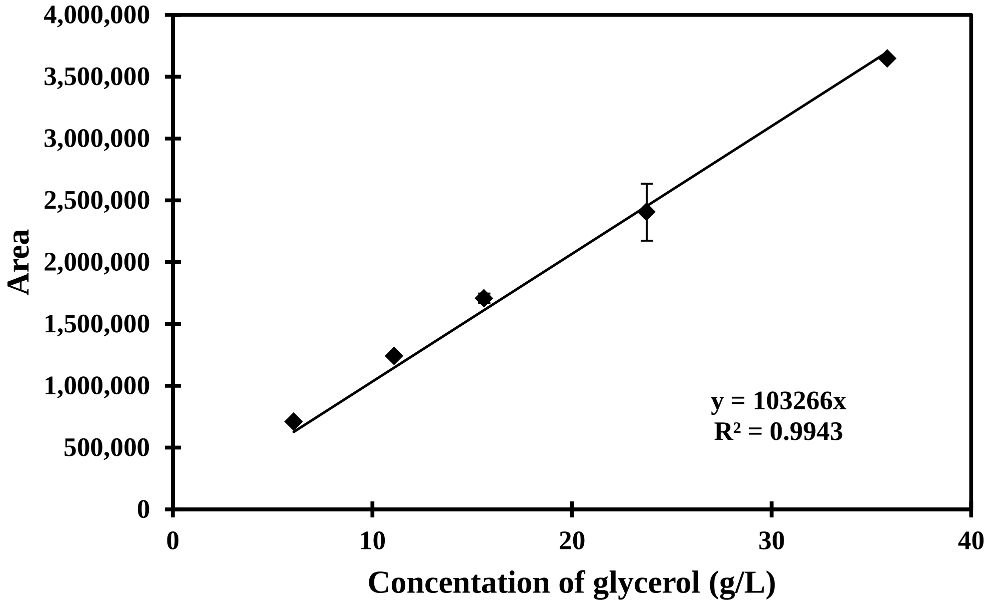

Figure A1 Correlation of Area and concentration of glycerol.

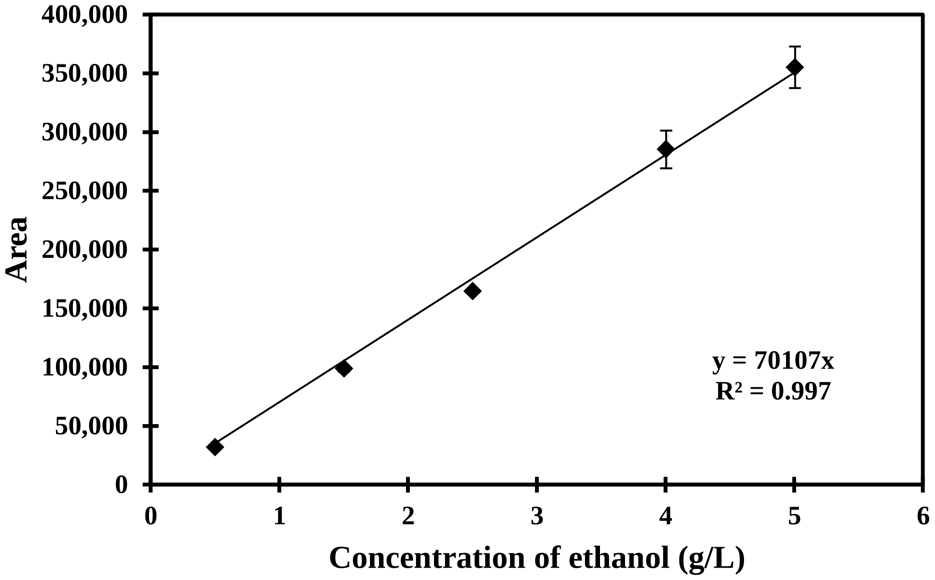

Figure A2 The relation between area and concentration of ethanol.

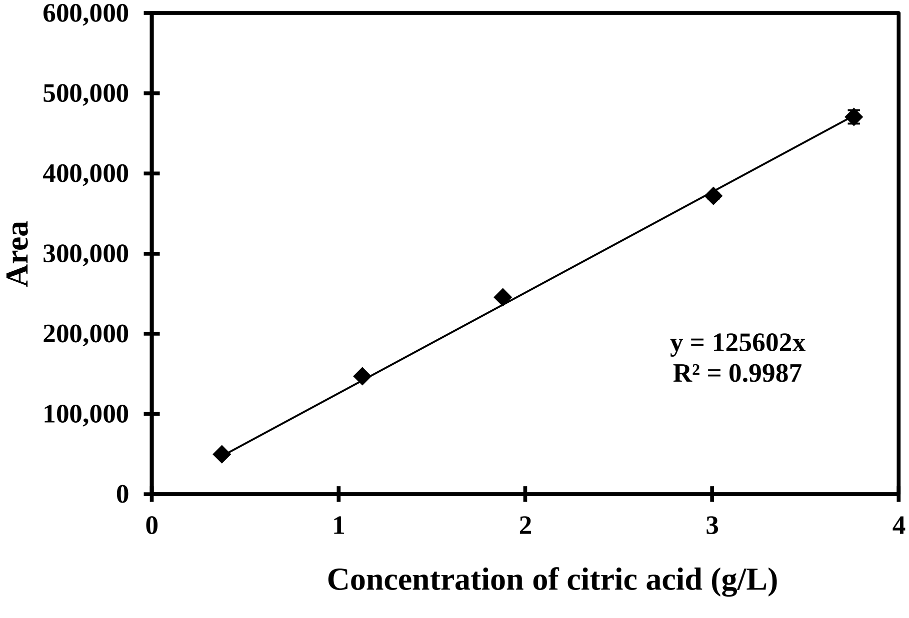

Figure A3 Correlation of area and concentration of citric acid.

Appendix B: Calibration for Dry Cell Weight

To determine the correlation of dry cell weight and optical density, an equivalence line was drawn. Cells were cultivated in the shaker, controlled at 30 ˚C and 250 rpm. Samples were taken every few hours to measure optical density. To gain the dry cell weight, 2 mL of broth were collected in the microtubes and centrifuged at 10,000 rpm for 10 min. The supernatant were poured out. The pellet was rinsed using distilled de-ionized water, and then put into an oven. The weight was measured every few hours until it stayed unchanged.

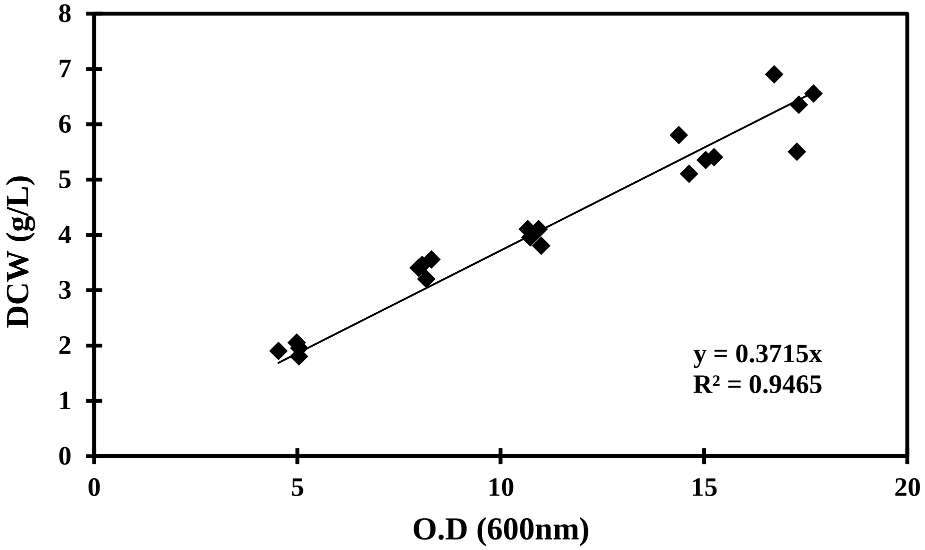

Figure B1 Correlation of dry cell weight and optical density (600 nm) for *P. Pastoris*.