CHAPTER 3

RESULTS AND DISCUSSIONS

3.1  CLONING THE PHYTASE GENE IN P. PASTORIS

3.1.1  PCR Amplification of phytase (PhyA)

The polymerase chain reaction (PCR) is a scientific technique in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. The polymerase chain reaction was used to amplify the defined fragment size of phytase gene. The upstream primer 5’CCG GAA TTC CTG GCA GTC CCC GCC TCG AGA 3’ with the EcoRI restriction enzyme site and the downstream primer 5’ TAA AGC GGC CGC CTA TGC AAA ACA CTC CGC 3’ with NotI restriction enzyme site were used for amplification of gene from cDNA. Phytase gene (1347bp) was PCR amplified at 53°C from A. ficum cDNA with specific primers and the mixture was resolved in 1% agarose gel for size confirmation (Figure 3.1).

![Figure 3.1](image)

**Figure 3.1**  PCR amplification of 1.3 kb phytase gene from A. ficum cDNA
Lane1, 2: PCR products of amplified phytase gene
Lane M: 1kb DNA Ladder Marker
The phytase gene was obtained at-large quantity for further studies by generating multiple copies from the amplified gene as template.

3.1.2 **Restriction Digestion of Phytase Gene with EcoRI and NotI**

The phytase gene (1347 bp) was amplified with flanking ends containing *Eco*RI and *Not*I restriction sites. The enzymes cut the double-stranded DNA at specific recognition nucleotide sequences. Complete restriction digestion of phytase gene by *Eco*RI and *Not*I was analyzed in 1% agarose gel electrophoresis (Figure 3.2).

![Figure 3.2 Restriction digestion of phytase gene in 1% Agarose gel](image)

**Figure 3.2 Restriction digestion of phytase gene in 1% Agarose gel**

Lane M: 1kb DNA Ladder Marker

Lane 2, 3: *Eco*RI and *Not*I restricted phytase gene

3.1.3 **Purification of Phytase Gene after Restriction Digestion**

Purification of phytase gene is for general cleanup of oligonucleotides and enzymes of the restriction digestion reaction. The purified phytase gene which is 1.3kb size was analysed in 1% agarose gel electrophoresis (Figure 3.3).
3.1.4 Plasmid DNA Extraction of pPICZαA

The vector pPICZαA plasmid DNA was extracted for the ligation of phytase gene. The pPICZαA plasmid DNA was extracted using QIAGEN plasmid DNA extraction kit and further analysed by 1% agarose gel electrophoresis (Figure 3.4).
Plasmid DNA extraction of pPICZαA was extracted successfully and intense band of 3.6 kb size were observed in the agarose gel. Purification of pPICZαA after restriction digestion with *Eco*RI and *Not*I was confirmed in 1% agarose gel electrophoresis (Figure 3.5).

![Figure 3.5 Purification of pPICZαA after restriction digestion](image)

**Figure 3.5 Purification of pPICZαA after restriction digestion**

Lane M: 1kb DNA Ladder Marker  
Lanes1: 3.6 kb pPICZαA

### 3.1.5 Ligation of PICZαA and Phytase

The purified phytase gene and vector pPICZαA was ligated using T4 DNA ligase and the pPICZαA plasmid with phytase gene insert was named as pPICZαA-phytase.

The ligation mixture was then transformed into *E. coli* strain *DH5α* by calcium chloride method and transformants were selected on low salt LB agar with Zeocin antibiotic marker (25μg/ml). LB medium with salt
concentrations greater than 90 mM causes inactivation of Zeocin and so the NaCl concentration was taken as 5 g/l in low salt LB medium.

### 3.1.6 Lysate PCR

To confirm inserted DNA in bacterial colonies, colonies were picked, lysed, and analysed by PCR. The PCR confirmation reaction was done with phytase gene forward and reverse primers.

The positive colonies containing recombinant pPICZαA-phytase plasmid showed PCR amplification of phytase gene of size 1.347 kb in 1% agarose gel electrophoresis (Figure 3.6).

![Screening the positive E.coli transformants by lysate PCR](image_url)

**Figure 3.6 Screening the positive *E.coli* transformants by lysate PCR**

- Lane 1: Lysate PCR of recombinant phytase
- Lane M: 1 kb DNA ladder Marker
- Lane 2: Negative control pPICZαA
- Lane 3: Positive control (Phytase gene)
- Lane 4: pPICZαA
3.1.7 PCR Reaction for the Confirmation of Recombinant Phytase

From the positive *E. coli* transformants plasmid was extracted and confirmation of recombinant phytase gene was done by PCR reaction using *AOXI* forward and phytase reverse primer, and it was confirmed by 1% agarose gel electrophoresis.

The size of PCR amplified promoter and the phytase gene was 1.8 kb (Figure 3.7). Glycerol stock of the strain was made for large scale plasmid DNA extraction of pPICZαA-phytase.

![Figure 3.7 PCR reaction for the confirmation of recombinant phytase](image)

Figure 3.7 PCR reaction for the confirmation of recombinant phytase

Lanes 1, 2: PCR product of recombinant phytase using *AOXI* F and phytase gene R primers
Lane M: 1kb DNA Ladder Marker
3.1.8 Plasmid DNA Extraction of Phytase Gene

Plasmid of recombinant pPICZαA-phytase plasmid was extracted and it was confirmed by 1% agarose gel electrophoresis showing intense bands in Lane 1 and 2 respectively. The size of the recombinant pPICZαA-phytase plasmid was 4.9 kb (Figure 3.8).

![Figure 3.8 Plasmid DNA extraction of recombinant pPICZαA-phytase](image)

Lane 1, 2: Recombinant phytase plasmids  
Lane M: 1kb DNA Ladder Marker

3.1.9 Phytase DNA Sequencing

The phytase gene was sequenced using gene specific and promoter primers. The partial sequencing results were given in Figure 3.9.
3.1.10 Confirmation of the Presence of Gene by Restriction Digestion Analysis

Large scale purification of plasmid was carried out and linearized with NotI and ECoRI restriction enzyme individually and double digestion. Analysis on agarose gel electrophoresis is shown in Figure 3.10. Pop out of the gene from the plasmid was observed in the gel.

3.1.11 Linearization of Recombinant Phytase with PmeI

The linear plasmids have shown a higher efficiency of integration into Pichia genome and also generate stable transformants via homologous recombination in P. pastoris. The pPICZaA-phytase plasmid was linearized using the restriction enzyme PmeI. This enzyme is a unique restriction site present in the AOX1 promoter region.
The size of the linearized recombinant phytase by \textit{PmeI} enzyme was approximately 4.9 kb as shown in the 1% agarose gel picture (Figure 3.10).

\textbf{Figure 3.10} Linearization of recombinant pPICZ\textit{a}A-phytase plasmid with \textit{PmeI}

Lane M: 1 kb DNA ladder marker  
Lane 1: uncut recombinant phytase of plasmid DNA  
Lane 2: Linearized recombinant pPICZ\textit{a}A-phytase

\textbf{Figure 3.11} Confirmation of the presence of gene by restriction digestion of plasmid recombinant pPICZ\textit{a}A-phytase

Lane 1: Recombinant phytase gene restricted with \textit{EcoRI} and \textit{NotI} (1.347kb)  
Lane 2: 1kb DNA Ladder Marker  
Lane 3 and 4: recombinant phytase linearized with \textit{NotI} and \textit{EcoRI} individually (4.9kb size)  
Lane 5: Uncut pPICZ\textit{a}A-Phytase plasmid DNA  
Lane 6: pPICZ\textit{a}A linearised with \textit{EcoRI} and \textit{NotI}
3.1.12 Purification of Linearized Recombinant Phytase

Purification of recombinant pPICZαA-Phytase plasmid after linearization using \textit{PmeI} was done for the general cleanup of oligonucleotides and DNA from the restriction digestion reaction. Purification was generally done to avoid arcing during electroporation and improve the efficiency of transformation. The size of recombinant phytase was \( \sim 4.9 \text{kb} \) and this was confirmed after purification of linearized recombinant phytase with \textit{PmeI} (Figure 3.12).

![Figure 3.12 Purification of linearized recombinant phytase](image)

Lane1: Uncut pPICZαA-phytase plasmid DNA  
Lane M: 1kb DNA Ladder Marker  
Lane 2: Recombinant phytase gene restricted with \textit{EcoRI} and \textit{NotI} (1.347kb)  
Lane 3: Purified of recombinant phytase linearized with \textit{PmeI} (4.9kb size)

3.1.13 Construction of recombinant \textit{Pichia} clones

The recombinant plasmid pPICZαA-phytase and pPICZαA were linearized at the 5′ \textit{AOXI} region of the plasmid using the restriction enzymes \textit{PmeI}. If a plasmid is transformed without linearization, instead of gene
integration, the gene replacement between two homologous regions of the plasmid and host will occur. Since occurrence of this is of lower frequency and only single copy of the gene was integrated in the whole plasmid transformation, linearization of plasmid was preferred. Linear plasmids have shown a higher efficiency of integration into *Pichia* genome and also generate stable transformants of *Pichia pastoris* via homologous recombination between the vector and the *AOX1* locus within the genome and the integration of foreign DNA into *P. pastoris* genome by gene integration (Figure 3.13).

![Schematic showing integration of phytase gene in *P. pastoris* genome](image)

**Figure 3.13** Schematic showing integration of phytase gene in *P. pastoris* genome
The linearized plasmids were transformed into the fungal host *P. pastoris* GS115 by electroporation. The efficiency of electroporation could be judged from the value of time constant achieved with the electroporator and this was found to critically depend on the purity of the linearised vector and the proper washing of *Pichia* cells during preparation of electrocompetent cells. The recombinant phytase vector was transformed into *Pichia* GS115 host at voltage 1500V, capacitance 50 F and resistance 250 Ω at 10 to 11ms times constant. The transformants were selected at ZeoR (500mg/ml) concentration by electroporation.

3.1.14 Screening Transformed *P. pastoris* Strain in YPD Agar

After electroporation the cells were plated on YPD agar plate containing Zeocin (500 mg/ml) and incubated at 30°C for 2-3 days.

Single colonies were selected and patched in YPD agar plate. After incubation at 30°C for 2-3 days, the colonies were analysed for gene integration by PCR. Similarly the vector control i.e GS115 host containing the vector pPICZαA was also screened.

3.1.15 Genomic DNA Extraction of *P. pastoris* after Integration

The yeast DNA was extracted by the ‘smash and grab’ protocol using glass beads.

The intense bands in 1% agarose gel picture confirmed the genomic DNA extraction from *P. pastoris* from recombinant cells (Figure 3.14).
Figure 3.14 Genomic DNA extraction of *P. pastoris* after integration

Lane 1 to 10: *P. pastoris* genomic DNA extraction from *P. pastoris* transformants after integration  
Lane M: 1kb DNA Ladder Marker

3.1.16 Genomic DNA PCR for the Confirmation of Integration

PCR product of phytase gene was obtained from the template of the genomic DNA by using the phytase forward and reverses primers and confirmed in 1% agarose gel electrophoresis (Figure 3.15).
Figure 3.15 PCR for the confirmation of integration of Phytase gene in *Pichia pastoris*

Lane M: 1kb DNA Ladder Marker
Lane 1, 3, 4: Negative control GS115 host genomic DNA
Lane 2: Positive control recombinant phytase plasmid
Lane 5 to 10: PCR products of transformed colonies.

3.2 IMPACT OF GENE DOSAGE ON PHYTASE ENZYME PRODUCTION IN RECOMBINANT *P. PASTORIS*

*P. pastoris* genomic DNA was used as a template for real time PCR analysis. The qPCR was carried out with SYBR Green. The *pAOXI* was used as the target for amplification and *P. pastoris* host GS115 harbours a single
copy \textit{AOXI} gene which was used as a calibrator. The data was normalized to endogenous control ARG4 gene.

Figure 3.16 Gene copy number in different \textit{P.pastoris} transformants

Figure 3.17 Phytase enzyme production in different copy number \textit{P.pastoris} transformants
The n-fold changes of different recombinant *Pichia Pastoris* transformants showed that the transformed clone no.3 have increased copy number compare to other clones (Figure 3.16). The phytase enzyme production in these transformants was shown in Figure 3.17.

Gene copy number has been identified as a ‘rate-limiting’ step in the production of recombinant proteins from *P. pastoris* (Clare et al 1991). Increasing the number of copies of the expression cassette generally has the effect of increasing the amount of protein expressed (Clare et al 1991, Romanos 1995). *Pichia* cells expressing hepatitis B surface antigen (HbsAg) under the control of the constitutive GAP promoter were identified as undergoing spontaneous multiple gene insertions at a single locus. Multicopy clones containing up to four copies of the HbsAg gene were found to have a four-fold higher yield of HbsAg than those containing a single copy of the gene. No limitation at the transcriptional level was identified (Vassileva et al 2001). Thus, for this construct the relationship between copy number and product level was very simple. Mansur et al (2005) found a direct relationship between protein production in *Pichia pastoris* and the number of introduced synthetic genes of miniproinsulin, fused to the *Saccharomyces cerevisiae* pre-pro alpha factor used as secretion signal, and inserted between the alcohol oxidase 1 (*AOX1*) promoter and terminator sequences. This increased expression from 19 to 250 mg L$^{-1}$ when about 11 copies have been integrated. Zhu et al (2009) attempted to fully explore the expression potentials of *Pichia pastoris* for producing porcine insulin precursor through copy number optimization. In their study *P. pastoris* strains harbouring 0, 1, 3, 6, 12, 18, 29, 52 copies were generated. However, after 96 h methanol induction, a bell-shaped correlation curve was observed between gene dosage and protein yield, and the maximum expression level was achieved by a 12-copy strain.
Specific growth rate and methanol utilization capacity were found to decrease remarkably for high copy strains (>12 copies). Transcriptional analysis of KAR2 suggested higher copy strains were suffering more from ER stress. Increased copy number might reasonably be expected to exert a knock-on effect on transcription and translation, both of which may become rate-limiting due to a lack of resources, such as precursors and energy (Hohenblum 2004). However in Pichia it has been proposed that it is more likely that any limitations are due to post-translational events, such as folding within the endoplasmic reticulum (ER), membrane translocation and signal sequence processing (Hohenblum 2004). Sunga and Cregg (2004) also demonstrated that increasing the copy number of a β-galactosidase (β-gal) gene (lacZ), under the control of the FLD1 promoter, increased the relative activity of the enzyme proportionately. They reported a 17-fold increase in activity of β-gal when 22 copies of the lacZ gene were present, relative to the activity observed when a single copy of the gene was present.

In contrast to the work described above, where various researchers found a direct correlation between gene copy number and subsequent yield and activity of the gene product, Hohenblum et al (2004) did not note any increase in the expression of a recombinant human trypsinogen when the gene dosage was increased from one to three copies under the control of the GAP promoter. However, an increase in the expression level of this protein was observed using the AOX1 promoter when the gene copy number was increased to two, but levels of expression fell upon further copy number increases. Thus, the effect of gene copy number on expression is unpredictable. A practical solution to this problem is to examine the production level as a function of gene dosage.
Wyss et al (1999) did claim a greater expression of phytase achieved using *Hansenula polymorpha* featuring a highest copy number of the phytase gene gave best productivity. But our result from Real-time PCR showed that in *Pichia* the higher expression of the phytase gene (*PhA*) was observed in the medium copy number. Similar to this in *P.pastoris* a bell shaped curve was observed in the expression of insulin with varying copy number (Zhu et al 2009). This hyper expressing transformant B was selected for further studies.

### 3.3 SMALL-SCALE EXPRESSION OF PHYTASE

The expression of phytase was started in a shake flask by using the positive *Pichia pastoris* transformant B containing the phytase gene. The experiment was carried out by inoculating the clone into 3 ml YPD medium and incubated in shaker at 28°C. The culture was transferred into 50 ml YPG medium. Once the OD$_{600}$ reached between 20-25 the cells were pelleted and suspended in BMMY medium and unbuffered MMH medium separately. Uninduced sample was collected before induction with methanol 0.5% (250µl) for the first day and 1% for next 7 days. The samples were taken every 24 hrs and centrifuged, and the supernatant was used for further analysis.

The higher temperature expression (28°C) in a shaker flask with methanol induction alone was performed and the phytase was purified using acetone precipitation. Furthermore, its activity was calculated using inorganic phosphorus (*Pi*) liberated from sodium phytate per minute at 37°C (Figure 3.18). The Biomass increased to 20 OD in shake flask experiments and maximum phytase activity was observed after 6 days of induction. After 6 days the production did not increase.
Figure 3.18 Shake flask production of phytase by recombinant *P. pastoris*

The SDS-PAGE was used for checking the size of the phytase protein (Figure 3.19).

**Figure 3.19 SDS-PAGE analysis of phytase under different co-feeding induction**

M: Unstained protein Ladder Markers  
L5: Phytase protein before deglycosylation showing multiple bands between 66-118 kDa under methanol induction  
L6: Negative control with GS115-pPICZαA  
The SDS-PAGE analysis of the supernatant of induced samples showed smear of bands ranging from 66 to 118 kDa size. The expected size of phytase from \textit{A. ficcum} is 49 kDa and the protein has n- glycosylation sites. Since \textit{P. pastoris} can do post translational modification, it was suspected that the enzyme produced may be glycosylated. Analysing the gene sequence of \textit{A. ficcum} phytase gene, we found that it is having 9 potential N-Glycosylation sites (Asn X Ser/Thr) (Mellquist et al 1998).

It is common in \textit{P. pastoris} when a glycosylated protein is secreted, it will appear as a smear in SDS PAGE. Phytase was deglycosylated with \textit{PNGase} F enzyme to confirm the size of the protein (Meijin et al 2008, Casey et al 2004). The induced supernatant was analysed in SDS- PAGE before and after the \textit{PNGase} treatment, confirmed that the phytase was glycosylated and the deglycosylated protein was \textasciitilde 47 kDa in size.

Also for further glycosylation confirmation, the expressed phytase enzyme was analysed by PAS staining of the gel (Leach et al 1980, Fairbanks et al 1971).

The appearance of pink colored bands indicated the protein to be glycosylated at different experimental conditions (Figure 3.20).
Figure 3.20 PAS staining analysis of recombinant phytase

M: Unstained protein Marker
L1, L2: Shake flask sample stained with PAS after six days of induction
L3: Uninduced sample PAS staining
L4: PAS staining of induced sample after deglycosylation with PNGaseF (New England Biolabs)

The periodic acid Schiff (PAS) sugar staining, which identifies native phytase as a glycoprotein, failed to detect any residual carbohydrates attached to the protein after deglycosylation with PNGase F.

The PAS staining showed the presence of purple color smeared bands from ~ 47 to 118 kDa region. The appearance of pink colored bands indicated the protein is glycosylated and hence smear bands was obtained.

They are other reports on phytase production from Aspergillus ficuum NRRL 3135 to date (Kostrewa et al 1997) but analysis with Periodic Acid Schiff
(PAS) staining to identify the glycosylation level of phytase have not yet elucidated.

3.4 EVALUATION OF *PICHIA PASTORIS* EXPRESSED RECOMBINANT PHYTASE ENZYME PROPERTIES AND KINETIC PARAMETERS

The optimum temperature and pH of recombinant *Pichia pastoris* expressed phytase was evaluated over temperature range of 30 to 70°C and pH range of 2 to 9. The optimal temperature of produced phytase is 58°C when the reaction time was 10 minutes, and the optimum pH is 5.5 (Figure 3.21, Figure 3.22).

![Figure 3.21 Evaluation of optimum temperature for recombinant *Pichia pastoris* expressed phytase](image)

The only differences with other phytases are having two pH optimal (pH 2.5-5.5).
Figure 3.22 Evaluation of optimum pH for recombinant *Pichia pastoris* expressed phytase

The $\frac{K_{cat}}{K_m}$ which helps to measure how efficiently an enzyme converts a substrate into product, was calculated using the Lineweaver Burke plot (Figure 3.23):

Figure 3.23 Lineweaver Burke plot of recombinant *Pichia pastoris* expressed phytase
The phytase kinetic was determined by using different concentration of phytate as a substrate from 0.2 to 5 mM.

The $K_{cat}/K_m$ value of phytase showed high efficiency to convert a substrate into product and half time required for substrate consumption was reached after 3 minutes of reaction (Figure 3.24).

![Figure 3.24 The measurement of half time required for substrate consumption](image)

Phytase enzyme kinetic ($K_{cat}/K_m$) was $4.62 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$. The substrate decline from five mM to 0.2 mM and 50% reduction time was reached after 3 minutes of reaction time, which is slight different from a theoretical upper limit of $10^8$– $10^{10}\text{M}^{-1}\text{s}^{-1}$ (Stroppolo et al 2001, Brooks et al 2013). The enzymes working close to this are termed superefficient and also its reduction time is reaching after 2.5 minutes.

Various reports on phytase production showed the phytases with broader substrate specificity generally had low specific activities (Wyss et al 1999), despite the considerable economic interest, low yield and high cost of
enzyme production are the limiting factors for using phytase in monogastric animals.

3.5 HIGH CELL DENSITY FERMENTATION

3.5.1 Methanol Feeding during Induction

The bioreactors were started with the glycerol batch and fed batch phases at 28°C to increase the biomass then decrease to 24°C for the first batches, and the second batches were decreased to 20°C with 0.5-0.8 ml/l/h methanol feeding, and the PTM₁ was increased from 4.5 ml/l to 12 ml/l at rate of 2 ml/day/l. Both reactors were performed with addition of histidine 0.4 g/l, L-Glutamine 10µl/ml, and their pH was maintained at 4.8.

As the culture grows, the DO decrease and when the glycerol in the medium was completely consumed the DO rises sharply. This indicated the end of the initial batch phase, and the second step of glycerol 98% fed-batch was started (Figure 3.25).

![Figure 3.25 Growth curve analysis during Glycerol Batch and Fed-Batch Phases](image-url)
Phytase production was analysed under methanol induction along with the feeding of L- Histidine (0.4 g/l), PTM\textsubscript{1}: 4.5 ml/l and feeding of 2 ml/day/l.

The PTM\textsubscript{1} are metals elements required by living organisms to function properly and are depleted through the expenditure of energy by various metabolic processes of living organisms.

The biomass reached 67.7 g/l when the temperature was set to 28\textdegree C. The phytase activity was 2711 U/ml after 144h cultivation time (Figure 3.26).

![Figure 3.26 Profile of Fed Batch cultivation of Phytase producing recombinant \textit{P.pastoris} with methanol induction alone](image)

Three stage cultivation was carried out for the over expression of recombinant proteins in \textit{P.pastoris}. The cultivation was started with the carbon unlimited batch and carbon limited fed batch phases with glycerol as a carbon source and then induced with methanol for over expression of phytase.
In the conventional three stage cultivation the phytase activity increased to 2711 U/mL which is 10 fold more than the shake flask expression (384 U/mL). In three stage cultivation the biomass enhanced to 68 g/L due to limited carbon feeding which is 10 fold more than the shake flask cultivations in which building the biomass is limited by oxygen. In respect to physiology of the cells the recombinant protein production is connected to different cellular stresses. It is well established that the energy demand and cellular stresses are enhanced during the recombinant protein production rate.

Heterologous protein expression by *P. pastoris* using methanol as induction is an extremely oxygen-consuming process and induction at a lower temperature further intensifies oxygen supply requirement. Hence the use of a multi-carbon substrate mixture to replace pure methanol is preferred, since it could enhance both cell growth and targeted protein productivity. Methanol is a hazardous substance due to its high flammability and toxicity; also, cells growing on methanol have a very high oxygen consumption, which usually requires the addition of pure oxygen and another co-substrate to the culture, increasing the cost of the process and limiting the cultivation capacity at a high scale (Xiong et al 2005).

High cell density fermentation process for phytase production in *Pichia pastoris* under methanol and various nutritional supplements (like glutamine, alanine, mannitol and sorbitol) and change in the process conditions were carried out to augment the energy demand during the recombinant protein production.

### 3.5.2 Methanol/ L-Glutamine Feeding During Induction

Heyland et al (2011) in their study on carbon metabolism of *P.pastoris* reported supplementation of various amino acids resulted in the enhancement of recombinant protein production. In their study they reported
that glutamine supplementation enhances the recombinant protein production. They supplemented amino acids for constitutively expressing system and we have attempted supplementation of glutamine for alcohol oxidase inducible promoter system. Supplementation was made during methanol induction phase. In this supplementation of L-Glutamine (0.5g/day/l) we found the recombinant protein productivity enhanced by 15 % to 3171 U/ml (Figure 3.27).

**Figure 3.27** Profile of Fed Batch cultivation of Phytase producing recombinant *P. pastoris* with L-Glutamine/Methanol at 28°C

### 3.5.3 Temperature Limited Fed Batch

Jahic et al (2003) attempted temperature limited fed batch (TLFB) in which the methanol limitation during induction phase is replaced by temperature limitation during induction process.
In TLFB the productivity of the recombinant protein increased and also there was improvement in product quality due to reduced cell death and proteolytic activity. This strategy was attempted in the phytase production by reducing cultivation temperature from 28°C to 24°C and 20°C in the subsequent batches. In both the batches the recombinant protein production increased and the increase is more at 20°C.

The effect of temperature at 24°C increased the phytase activity to 3646 U/ml and the Biomass (OD 600 nm) levels were maintained around 28°C and the cultivation time decreased to 138 h (Figure 3.28). The biomass levels did not increase during induction phase at the reduced cultivation induction temperature.

Figure 3.28 Profile of Fed Batch cultivation of phytase producing recombinant P. pastoris with L- Glutamine/Methanol at 24°C
Furthermore, the temperature was decreased at 20°C for more analysis of phytase activity.

The cultivation induction temperature at 20°C showed further enhancement in the activity of phytase to 4026 U/ml. The low temperature decreased the biomass level to 52.5 g/l and OD₆₀₀nm 248 after 132h cultivation time (Figure 3.29).

![Graph showing Fed Batch cultivation of phytase producing recombinant P.pastoris with L-Glutamine/Methanol at 20°C](image)

**Figure 3.29 Profile of Fed Batch cultivation of phytase producing recombinant P.pastoris with L-Glutamine/Methanol at 20°C**

Recently the effect of temperature on the proteome of recombinant *P. pastoris* was studied by Dragosits et al 2009. In the production of antibody fragment they found that the reducing temperature to 24°C improved the antibody fragment by 2 fold and further reduction to 20°C improved that by three fold. They reported that at lower temperature during induction, the
folding stress decreased which enabled the increase in secretion of recombinant protein production.

The productivity and specific productivity rose gradually as the temperatures decreased (U/g/h) respectively (Figure 3.30, 3.31).

**Figure 3.30** Productivity of phytase enzyme during different cultivation temperature of recombinant *P. pastoris* cultivation

**Figure 3.31** Specific productivity of phytase enzyme during different cultivation temperature of recombinant *P. pastoris* cultivation
Since glutamine is an expensive amino acid and is used as both carbon and nitrogen source during induction phase, attempts were made to replace glutamine with other nutritional supplements such as alanine, mannitol, lactic acid and sorbitol in individual fed batch cultivations.

3.5.4 Methanol/L-Alanine Feeding During Induction

The method of induction was switched to co-feeding methanol/L-alanine (25 g/l), which increased the activity of phytase up to 144h of cultivation time.

The effect of L-alanine as nitrogen source and methanol as carbon source increased biomass to 53 g/l. When compared to higher temperature experiment, the biomass decreased as in the cultivation at 20°C. But the phytase activity increased to two times compared to methanol induction alone (Figure 3.36).

![Figure 3.32 Profile of Fed Batch cultivation of phytase producing recombinant *P.pastoris* with alanine/methanol at 20°C](image.png)
In this nutritional supplementation alanine enhanced the productivity by 2 fold than glutamine supplementation. The phytase activity enhanced to 8632 U/ml.

The Productivity (U/ml/h) and Specific Productivity (U/g/h) also increased during induction time).

Utilization of L-alanine as nitrogen source and energy improved phytase activity to around 308 fold increase compared to the wild type of phytase. Various possible mechanisms are reported in the literature for the enhancement of productivity by alanine. They are summarized below:

- Amino acid oxidase converts the L-alanine to pyruvate and pyruvate can be subsequently used for both energy and synthesis of cell material. Ammonia may be assimilated into amino acids via NADP-dependent glutamate dehydrogenase. Growth of *P. pastoris* on L-alanine led to excretion of ammonia, due to relatively low C/N ratio of this substrate. In contrast to utilization of alkylated mines, this ammonia excretion did not result in inhibition of growth, and alanine has been considered as non repressible carbon sources for *P. pastoris* (Sreekrishna et al 1997). L-Alanine, on the other hand, enters metabolism via acetyl-CoA, and its impact on regulation is largely unknown.

- The alternative reason for producing higher yield under methanol/L-alanine co-feeding strategy might happen around pyruvate, acetyl coenzyme A, glyoxylate, and α-ketoglutarate via increased levels of *ALT1, DAL7, PYC1, GDH2*, and *ADH5* and decreased levels of *GDH3, CIT2*, and *ACSI* transcripts (Usaite et al 2006) and also the physiological impact of L-alanine to the cell is to supply enough nitrogen source (Fu et al 2011).
addition, as mentioned in the literature of previous researchers, 30% of the total energy is given by L-alanine but a detailed mechanism behind this effect should be investigated.

3.5.5 The Methanol/Lactic Acid Feeding during Induction

Batches were carried out with cofeeding of methanol and lactic acid during induction phase. The Glycerol Batch Phase and Glycerol Fed Batch Phase last 56 h. After glycerol phases, feeding was switched to co-feeding with Lactic acid/methanol, which induced phytase expression until cultivation time.

The methanol/lactic acid co-feeding strategy increased phytase production a little higher compared to methanol/glutamine co-feeding strategy but lesser than the alanine feeding strategy. The phytase activity was achieved to 6740 U/ml, which is higher when compared to methanol alone used as inducer (Figure 3.33).

![Phytase activity (U/ml) during cultivation time on Methanol/Lactic acid co-feeding strategy](image)

Figure 3.33 Phytase activity (U/ml) during cultivation time on Methanol/Lactic acid co-feeding strategy
Supplementation of lactic acid improved the productivity to 2.4 fold increase compared to methanol induction alone. The lactic acid is a potential non-repressive carbon source for expression of foreign genes in *P. Pastoris* because the considerable amount of phytase production was reached with this substrate. The reduction degree and combustion heat of the carbon sources could support this productivity about the carbon source efficiency, since carbon source with different reduction degree and combustion heat is degraded and assimilated in different pathways, and thus displays different efficiencies for growth and product formation. The lactic acid in the presence of water degrades into the cell natural’s metabolites which are normally tolerated by the cells. Lactic acid helps to produce energy within the cells and was also demonstrated to possess antioxidant properties (Xie et al 2005).

### 3.5.6 Methanol/Mannitol Feeding during Induction

An alternative way for moderate expression is to use *AOX1* promoter and induction with mannitol cofeeding (2:20 (w/w) mannitol/methanol solution). When *P. pastoris* cells were grown on mannitol, phytase production using the *AOX1* promoter increased higher than in methanol-grown cells. Thus, mannitol appears to be a useful carbon source for expressions under *AOX1* promoter. The Methanol/Mannitol co-feeding strategy increased the phytase activity to 10280 U/ml as indicated by the following diagram (Figure 3.34).

Mannitol improved phytase activity to 10280 U/mL and their productivity is higher than alanine and lactic acid supplementation. Use of vector containing *AOX1* promoter inducible with methanol facilitates the expression of foreign genes, which helps to increase yield by using
Methanol/D-mannitol both as a carbon source for induction strategy (Werten et al 1999, Richter et al 2006, Xie et al 2008). D-mannitol provides a main source of energy, and it acts as a compatible solute and has multiple functions, including osmoregulation, storage, and regeneration of reducing power, and scavenging of reactive oxygen species.

![Figure 3.34 Phytase activity (U/ml) and OD<sub>600</sub> under D-Mannitol/Methanol co-feeding strategy at 20°C](image)

The most important physiologic functions of mannitol are the control of cell turgor by an increase in intracellular concentration at low water activity, as in hypertonic conditions (Yancey et al 1982; Davison; Reed 1985). Mannitol may also function as an antioxidant owing to its ability to scavenge free radicals (Tandon et al 2003; Yu et al 2003). Smirnoff & Cumbers (1989) showed that mannitol rescued the hydroxylation of salicylate and denaturation of malate dehydrogenase from the hydroxyl radical. The phosphorylative mannitol utilization pathway, which involves the action of a mannitol kinase
followed by an oxidative step catalysed by a mannitol-1-phosphate dehydrogenase, is poorly documented in *Pichia pastoris*. The only study realized so far concerning mannitol metabolism was done in the opportunistic pathogen *C. neoformans* (Niehaus & Flynn 1994; Suvarna et al 2000). Mannitol is the primary ingredient of Mannitol Salt Agar, a bacterial growth medium, and is used in others and mannitol acts as an osmotic laxative. Even though mannitol has a higher heat of solution than most sugar alcohols, its comparatively low solubility reduces the cooling effect usually, when mannitol is completely dissolved in a product, it induces a strong cooling effect (Smirnoff & Cumbes 1989, Jahic et al 2002, Mattanovich et al 2012, Jacobs et al 2009, Hamilton et al 2007).

### 3.5.7 Sorbitol/Methanol Feeding during Induction

The new strategy of sorbitol/methanol co-feeding was attempted at 20°C throughout the cultivation time in the next batches. After 45h, the induction time was started with sorbitol/methanol co-feeding (0.785 g/l/h and 1.437 g/l/h). When adopting sorbitol/ methanol co-feeding induction strategy, the DO was maintained at a moderate level during the induction phase without oxygen limitation. The most promising co-substrate to enhance the phytase activity in *Pichia Pastoris* was sorbitol; In high cell density fed batch cultivation at lower temperature under methanol and its co-substrate sorbitol it was observed that on 6th day of induction the phytase activity increased to 13250 U/mL (Figure 3.35, Figure 3.36).
Figure 3.35  Comparison of dry cell weight, DCW (g/l) and Optical Density (OD\textsubscript{600nm}) for phytase production under methanol and sorbitol co-induction

Figure 3.36  Phytase activity under methanol induction alone and sorbitol/methanol induction after Glycerol batch phase (GBF) and Glycerol Fed batch (GFB)
However, a deep understanding of the cellular physiology and the regulation of the \textit{AOXI} promoter, used to govern heterologous protein production under methanol/sorbitol co-feeding strategy is still limited. Jungo et al (2007) studied the co feeding of sorbitol with methanol and found that the oxygen consumption and heat production were less compared to methanol alone feeding in continuous cultures. The oxygen consumption is one of the critical factors in high cell density cultivations and it enhances the phytase production in the high cell density cultivation.

Although important partial explanations are that sorbitol weakens the accumulation of toxic metabolite-formaldehyde in fermentation by \textit{Pichia pastoris} (Mut\textsuperscript{+}) by repressing the formaldehyde dissimilatory pathway to glycine. Sorbitol is low-degree reductant, and a non-repressing carbon source for \textit{pAOXI} and energy supplier, reduced theoretical oxygen consumption rate, and increased energy/methanol utilization efficiency so that more methanol could be effectively used for phytase synthesis (Ramo´n et al 2007).

Additionally, sorbitol increase hyperosmolarity and more methanol can be utilized to allow proliferation of cell growth by changing the aldehyde group to a hydroxyl group.

Sorbitol is converted to fructose by sorbitol dehydrogenase; and the sorbitol dehydrogenase is an enzyme complex that participates in the citric acid cycle and together with aldose reductase. It provides also a way for the cell to produce fructose without using ATP (Jungo et al 2007).

Sorbitol dehydrogenase uses NAD\textsuperscript{+} as a cofactor; its reaction is the following:
Sorbitol + NAD$^+$ -- fructose + NADH + H$^+$

Co-feeding of sorbitol can reduce the oxidation flux in the peroxisome, leading to less oxygen consumption and heat production since the combustion enthalpy of sorbitol is much less than methanol. At the same time, sorbitol in the mixture produces energy through the TCA cycle and provides a carbon source for biomass synthesis (Ausubel 2005). The reduction in oxygen consumption and heat production rates is advantageous in high cell-density cultures using recombinant P. Pastoris strains, especially for the large-scale productions.

The L-Glutamine was used mostly through a whole optimization process because it supports the growth of cells that have high energy demands and synthesize large amounts of proteins and nucleic acids. It is also an alternative source of energy for fast dividing cells and cells that use glucose inefficiently.

3.6 REPEATED FED BATCH CULTIVATION WITH EXTERNAL SEPARATOR

Repeated fed batch phytase production at high cell density cultivation in Pichia pastoris under methanol/sorbitol cofeeding induction strategy increased the activity at around 49 times compare to methanol induction alone in the 4L reactor (Figure 3.37).
Figure 3.37 Repeated fed batch phytase activity (U/ml)

The productivity increased along with the cultivation time (Figure 3.38). The volume was maintained using the same medium used at the beginning. Thereafter once the fermentation reached a certain stage after which is not effective anymore, 90% of medium was removed from the vessel and replaced by fresh nutrient medium; here no culture was removed until the end of the batch. The decrease in volume resulted in an increase in the productivity.
Figure 3.38 Optic density and productivity of phytase from repeated fed batch

A cost effective repeated fed-batch process with methanol/sorbitol feed as carbon sources showed a significant increase in OD$_{600nm}$ around 5.6 times compare to methanol induction alone.

This method of continuous cultivation was carried out first to maintain volume by replacing the old medium by fresh nutrient medium, to maintain constant product quality and secondly to minimize by-products formation which is formed during methanol induction alone.