academicJournals

Vol. 13(21), pp. 2181-2187, 21 May, 2014
DOI: 10.5897/AJB2013.12986
Article Number: D59DBC144758
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Co-feeding strategy to enhance phytase production in *Pichia pastoris*

Ndayambaje, Jean Bernard and S. Meenakshisundaram*

Centre for Biotechnology, Anna University, Chennai, India.

Received 29 June, 2013; Accepted 2 May, 2014

Process techniques of the methylotrophic yeast *Pichia pastoris* for the production and recovery of heterologous phytase proteins has developed in last 20 years. High expression levels using methanol as induction have been made in the quality of recombinant proteins in the fermenter culture and in the quality of the protein product. This allowed rapidly *P. pastoris* to become the system of choice for the expression of recombinant proteins in yeast. The experimental designs, the methanol/L-alanine co-feeding strategy and optimization of phytase production by *P. pastoris* supported by the optimum levels of variables and lower temperature expression produced high level of phytase activity which could be scaled up to produce phytase for food additives at industrial level. An overall phytase activity was 8632 U/ml, this means 332 fold increase compare to the wild type of phytase. This work demonstrates not only the impact of α-factor prepro secretion signal and efficiency of methanol/L-alanine co-induction strategy for phytase production by recombinant *P. pastoris* Mut$^+$ strains, but also shows new insights for the expression of bioproduct at lower temperature.

Key words: Phytase, gene expression, *Pichia pastoris*, process optimization, co-feeding strategy, Deglycosylation.

INTRODUCTION

The phosphate (Phytic acid) which is released from non ruminant animals becomes pollutant to the environment and as far as the phytase enzyme for its degradation is needed as food additive (Yu et al., 2012; Xiong et al., 2006; Haefner et al. 2005). For a long time, methanol is used not only as energy and carbon source but also as an inducer of recombinant protein expression (McKinney et al., 2004), and long ago, at high concentrations, it inhibits growth (Zhang et al., 2000). The Glycerol Batch Phase and Glycerol Fed Batch are first fed to the culture to increase biomass concentration and then the culture is switched to methanol to increase productivity, cell density and also to reduce the induction time. However, the optimal level of protein expression is not achievable with methanol induction alone, due to a partial repression of the *AOX*1 promoter (Faber et al., 2005) and nitrogen source limitation (Callewaert et al., 2001); that is why co-feeding strategy has been applied. The proteolysis of the secreted products and cell death in the high cell density bioreactor cultures is the main limitations when the enzyme is expressed at higher temperature (Li et al., 2006; Pakkanen et al., 2003; Lee et al., 2005; Porro et al., 2005). The expression at lower temperature (20°C) is favorable for efficient heterologous protein expression,

*Corresponding author. E-mail: meenakshi@annauniv.edu. Tel: +91-44-2235 8365, 2254 2298. Fax: +91-44-2235 0299.

and the targeted protein production, specific methanol consumption rate, as well as specific alcohol oxidase (*AOX*1) and its facility in performing many post-translational modifications (Macauley-Patrick et al., 2005).

Therefore, this study focused on lower temperature expression of phytase in *Pichia pastoris* GS115 under co-feeding strategy of methanol/alanine at small scale expression and large scale fermentation. Naturally, the absence of nitrogen is often a limited factor for growing the yeast for enzyme expression at small scale and high cell density fermentation (Heyland, 2010; Fu et al., 2011; Sohn et al., 2010; Sola et al., 2007; Celik et al., 2009). *P. Pastoris* GS115, a Mut^{s} strains have been found to be useful for production of recombinant protein (Mullaney and Ullah, 2005; Gellissen, 2000; Yang et al., 2004). Through the use of an expression plasmid that contains an α-factor secretory signal sequence, the heterologous proteins are able to be secreted into the medium (Feist et al., 2009). In this work the methanol/alanine induction strategy was applied in 4 L NBS fermenter at 28, 24, and 20°C and the extracellular phytase enzyme was analyzed. The interpretation of the results from methanol/alanine induction strategy was compared with the methanol induction alone together with previously higher temperature expression of phytase by understanding the nitrogen metabolism and the mechanism of energy regeneration. The results and relevant data might be useful for alternative phytase production at industrial level for food additives.

MATERIALS AND METHODS

Bacterial strain and reagents

Aspergillus ficuum NRRL 3135 has been sequenced by TIGR (The Institute for Genomic Research) and was cultivated on Czapek Dox broth and agar, which has the following composition (g/l): sucrose 30, sodium nitrate 2, dipotassium phosphate 1, magnesium sulphate 0.5, potassium chloride 0.5, ferrous sulphate 0.01, agar 15 and the final pH (at 25°C) 7.3±0.2. Restriction enzymes (*EcoRI* and *NotI*) were purchased from NEB. The DNA gel-extraction kit and the plasmid extraction spin mini-prep kit were bought from Qiagen (Germany). Media components such as yeast extract, bacto peptone, bacto tryptone and agar were obtained from Himedia (India). The chemical reagents were obtained from Merck or SRL (India). The *P. pastoris* expression kit was obtained from Invitrogen (SanDiego, CA). Plasmid *pPICZαA*, which contains *AOX1* promoter, alpha-factor prepro secretion signal and a *Zeocin* selectable resistance marker was used as a yeast. *Escherichia coli* shuttle vector for recombinant phytase expression. *E. coli* Top 10F' was used for construction and propagation of the expression vector. The *P. pastoris* strain GS115 (*his4*) was used for the expression of phytase from the *A. ficuum* strain.

Preparation of mRNA and cDNA construction by reverse transcription polymerase chain reaction (RT-PCR)

Isolation of total RNA from A. ficuum NRRL 3135

A. ficuum was grown in a 250 ml baffled flask containing a 50 ml of Czapek Dox broth medium. After 3 days of incubation at 30°C and 200 rpm in shaker flask, the spores of *A. ficuum* were harvested for the extraction of total RNA using the RNeasy Mini Kit from QIAGEN Company with minor modification and the total RNA quantitation assessment was performed with Nano Drop 2000/2000c from (JH BIO Innovations Pvt. Ltd/Thermo scientific, India).

mRNA preparation

The mRNA were performed using T7-Oligo (dT) Promoter Primer; PCR primer to capture the poly (A) tail and 5' SMART IV Oligonucleotide); oligo (dT) linker-primers according to the invitrogen's instructions kit.

cDNA construction

PCR-based gene amplifications were performed using Phusion-HF reaction mix (Finnzymes) and screening PCR reactions were performed using Red-Taq Ready Master mix (BioRad) using the following 20 µl volume reaction; Total RNA (5 µl), oligo (dT) (2 µl), dNTP mix (4 µl), Nuclease free water (4 µl), 5X Buffer RT (2 µl), DTT (1 µl), RNase free water (1 µl), Supercript II RNase-H.RT (1 µl). The phytase gene was amplified using the following primers: The upstream primer 5'CCG GAA TTC CTG GCA GTC CCC GCC TCG AGA 3' with *EcoR*I restriction enzyme site and the downstream primer 5' TAA AGC GGC CGC CTA TGC AAA ACA CTC CGC 3' with *Not*I restriction enzyme site. The ligation mixture was transformed into competent *E. coli* DH5α. The transformants were grown in Luria Bertani broth with low salt concentrations (LB-LS) plates then supplemented with *Zeocin* (1 µg/mL). The clone that contained the PCR product was verified by restriction enzyme digestion, agarose gel electrophoresis, and sequencing.

Shake flask expression study of phytase

Shake flask expression of the transformed *P. pastoris* GS115 clones were inoculated in 250 ml conical flasks with the YPG complex media in duplicates. The shake flasks containing 50 ml of the media were inoculated with 1 ml of transformed *P. pastoris* culture, and grown into 3 mL YPD medium and incubated first at 28°C, and then decreased to 20°C. The culture was transferred into 50 mL YPG medium under *Zeocin* resistance. Once the OD_{600} reached between 20 and 25; the cells were pelleted and suspended in BMMY (YNB-700 µl, Biotin-14 µl, 100 mM potassium phosphate pH 6.0 to 700 µl and distilled water - 7 ml) and later in unbuffered MMH medium. Initially, uninduced sample was taken and induction was carried out with methanol/L-alanine (0.5% for the first day, 1% for subsequent day; 0.3925, 0.7185 g/l/h, respectively for subsequent days till 7 days). The samples were taken and centrifuged 10,000 X *g* for 10 min at 4°C and the supernatant was taken for further analysis. From these clones, highly expressed clone was chosen for reactor studies.

Fermentation strategy

Transformed *P. pastoris* GS115 was first cultured in a 500 ml shaker flask containing 100 ml BSM at 28°C until an OD_{600} value of around 35 had been reached. For high cell-density fermentation, *P. pastoris* GS115, the seed culture equal to 400 ml was added into a NBS fermentor (NBS BioFlo 415 Benchtop SIP fermentor) containing sterilized 4 L of BSM. The components of 1 L BSM medium: $CaSO_4 \cdot 2H_2O$ - 0.46 g/l; K_2SO4 - 9,1 g/l; $MgSO_4 \cdot 7H_2O$ - 7.45 g/l; KOH - 2.06 g/l; Glycerol - 40 g/l; H_3PO_4 - 26.7 ml/l; Histidine, 0.4 g/l. After sterilization and cooling down to 28°C for getting enough biomass, temperature was kept to 20°C throughout the cultivation

time and pH of the medium was adjusted to 4.8 with 25% ammonium hydroxide and 6 ml PTM_1 trace salts/liter of basal salts medium was added aseptically. Composition of PTM_1 solution: $CuSO_4$ - 6 g/l; NaI - 0.08 g/l; $MnSO_4$ - 3 g/l; NaMo - 0.2 g/l; H_3BO_3 - 0.02 g/l; $CoCl_2$ - 0.5 g/l; ZnCl - 20 g/l; $FeSO_4$ - 65 g/l; H_2SO_4 - 5 ml/l; Biotin - 0.2 g/l (Blumhoff et al., 2013). The stirring speed, airflow and the pH were monitored according to the following conditions: Temperature (28 to 20°C), Dissolved oxygen (>20%), Methanol for carbon source and L-alanine as nitrogen source, pH (4.8) was adjusted using 25% NH_4OH and 88% H_3PO_4, agitation (200 to 800 rpm), aeration (0.1 to 1.0 vvm for glass fermenters), antifoam (the minimum PPG is needed to eliminate foam). Another 40 g/l glycerol was fed to the reactor to increase the biomass before the induction phase. Cells were collected by centrifugation and resuspended in 1.5 ml tubes for further analysis. Feeding medium for induction: Pure methanol (0.5 to 8 ml/l/h, PTM1:4.5 ml/l at rate of 2 ml/L/day, L-alanine co-feeding rates: 0.3925 and 0.7185 g/L/h. Samples were taken periodically throughout the fermentation time for phytase assay and protein analyses.

Phytase assay

Phytase activity was determined according to the report of Bae et al. (1999) with minor modification. Briefly, 75 µl of enzyme solution was incubated with 300 µl substrate solution (1.5 mM sodium phytate in 0.1 M sodium acetate buffer, pH 5.0) at 37°C for 20 min. The reaction was stopped by adding a volume of 375 µl of 5% (w/v) trichloroacetic acid. The released inorganic phosphate was analyzed by adding 375 µl of a coloring reagent (freshly prepared by mixing four volumes of 1.5% (w/v) ammonium molybdate in a 5.5% (v/v) sulfuric acid solution and one volume of a 2.7% (w/v) ferrous sulfate solution) and the solution's absorbance at 595 nm was measured using a Versamax microplate reader. The activity of the strain was analyzed where "one phytase unit was defined as the activity that releases 1 µmol of inorganic phosphorus (*Pi*) from sodium phytate per minute at 37°C". One strain was selected because of its high activity and SDS-PAGE was used to check the size of the phytase protein.

Digestion of glycopeptides with PNGase F

The peptide-N4-(N-acetyl-β-glucosaminyl)-asparagine amidase F cleaves selectively N-glycans from the asparagine rest of the peptide (Hanson et al., 2009). The reaction was started by denaturing glycoprotein buffer with 0.5% SDS, 40 mM DTT at 100°C for 10 min. After an addition of NP-40 and G7 reaction buffer, two-fold dilutions of *PNGase F* were added and the reaction mix was incubited for 1 h at 37°C and the separation of reaction products were visualized by SDS-PAGE.

RESULTS

Amplification and cDNA construction by RT-PCR

The amplification of 1.3 kb phytase gene was resolved by 1% agarose gel. The *EcoR*I and *Not*I both cut the double-stranded DNA at specific recognition nucleotide sequences. Ligation PCR was performed with T4 DNA ligase, *AOX*1 forward and phytase reverse primers were used for the confirmation PCR reaction. Phytase gene 1.3 kb has been sent to synergy lab for sequencing and it was compared with previously isolated phytase genes. This confirmed that our recombinant phytase has been successfully inserted into chromosome of *P. pastoris* GS115.

Small scale expression and high cell density fermentation

The phytase activity in shaker flask using BMMY medium under methanol/L-alanine induction was 298 U/ml and it increased gradually up to 148 h cultivation time. The inoculum for high cell density fermentation was prepared from glycerol stocks maintained at -20°C. The frozen cells were thawed and inoculated in 3 ml test tube YPG medium. After 24 h, 1 ml culture was inoculated into 20 ml YPG medium in a sterile 100 ml flask and grown for 48 h. Both test tube and flask were kept in incubator at 28°C and 200 rpm. The inoculum equals to 20 ml was transferred aseptically to 200 ml sterile Basal salts medium in 500 ml Erlenmeyer flasks. The culture was grown for 36 h at 28°C and 200 rpm until the inoculum OD_{600} reached approximately 30. The fermenter was inoculated with 200 ml culture from shake flasks and the biomass concentrations were monitored with time. As the culture grew, the DO decreased and when the glycerol in the medium was completely consumed the DO rose sharply. This indicated the end of the initial batch phase and the second step of glycerol 98% fed-batch was started; after 45 h, it reached OD_{600} = 217. The optical density growth curve and the activity were found to be proportional during the induction time. The methanol/L-alanine co-feeding strategy increased the phytase activity to 8632 U/ml (Figure 1) compare to 2711 U/ml activity of phytase obtained under cells grown on methanol induction alone.

The productivity increased to 233 (U/ml/h) and it was obtained from the ratio of the activity and the induction time hours and the specific productivity arose to a level of 1058 (U/g/h) and it was calculated from a thousand productivity to biomass (Figure 2). The effect of L-alanine as nitrogen source and methanol as carbon source increased biomass to 53 g/l. The biomass decreased compared to that obtained at higher temperature expression and it affected positively the phytase concentration because it increased remarkably to 13.6 mg/ml after 144 h cultivation time (Figure 3).

SDS-PAGE analysis

The cells were pelleted and suspended in BMMY (YNB-700 µl, Biotin-14 µl, 100 mM potassium phosphate, pH 6.0 to 700 µl and distilled water – 7 ml) medium. The phytase was induced by methanol/L-Alanine (0.5% for first day and 1%, 0.3925 and 0.7185 g/L/h, respectively for subsequent days till 7 days). Due to the heavy glycolsylation, the expressed phytase was found to have molecular sizes of around 120, 116, and 66 kDa and this showed that phytase is a highly glycosylated proteins

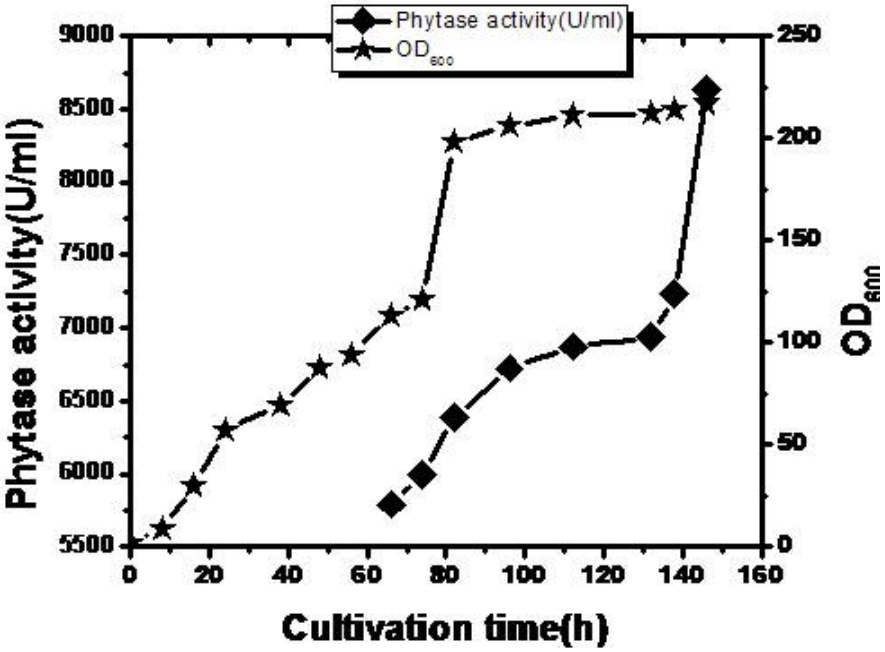

Figure 1. Phytase activity and OD_{600nm} during cultivation time on methanol/L-alanine co-feeding strategy. The Glycerol Batch Phase and Glycerol Fed Batch last 48 h. After glycerol phases, feeding was switched to co-feeding methanol/L-alanine, which induced phytase expression, then the activity and expression level of phytase were increased up to 146 h cultivation time.

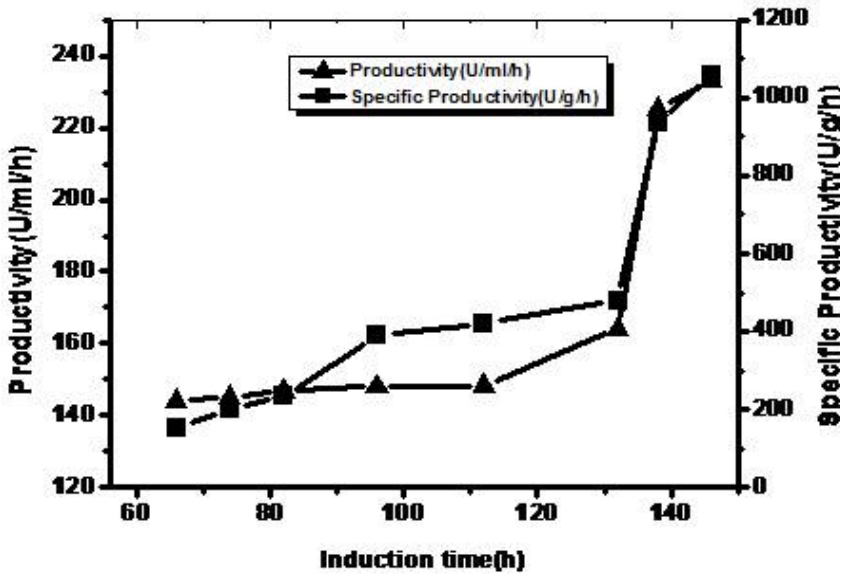

Figure 2. Productivity (U/ml/h) and Specific Productivity (U/g/h) during induction time of phytase production under methanol/L-alanine at 20°C.

(Figure 4).

DISCUSSION

Isolation of phytase gene (*phyA*) from *A. ficuum* NRRL 313, construction of recombinant *pPICZαA*-Phytase, integration and expression of phytase into *P. pastoris* GS115 was successful. Different reactors were run in different conditions at lower temperature expression of phytase (20°C) some reactors with nitrogen and others without nitrogen sources and this confirmed that the nitrogen source was one of the limiting factors for growth in all the

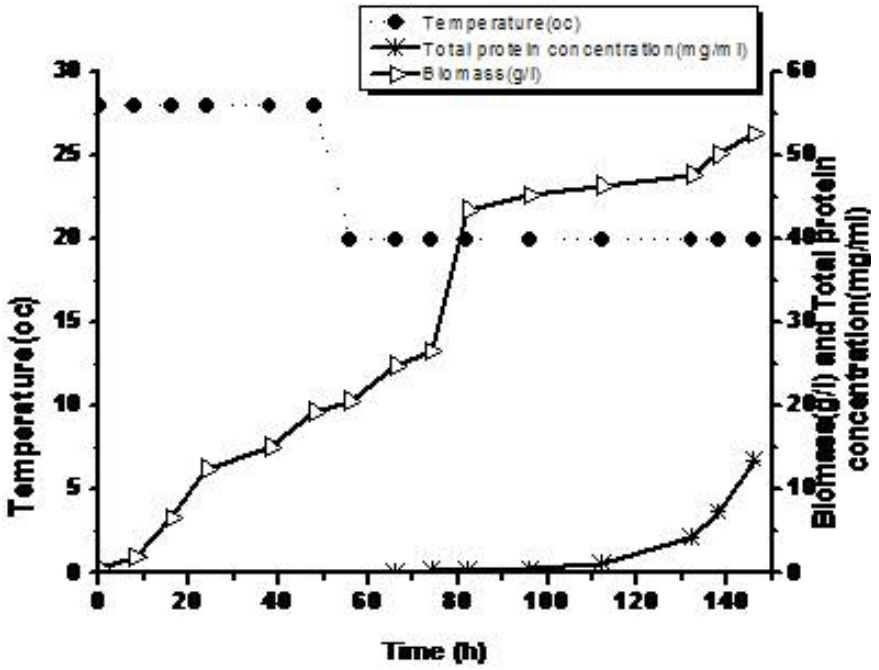

Figure 3. Biomass (g/l), Temperature (°C) for the time of Glycerol Batch Phase and Glycerol Fed Batch; Phytase concentration (mg/ml) under methanol/L-alanine co-feeding induction strategy.

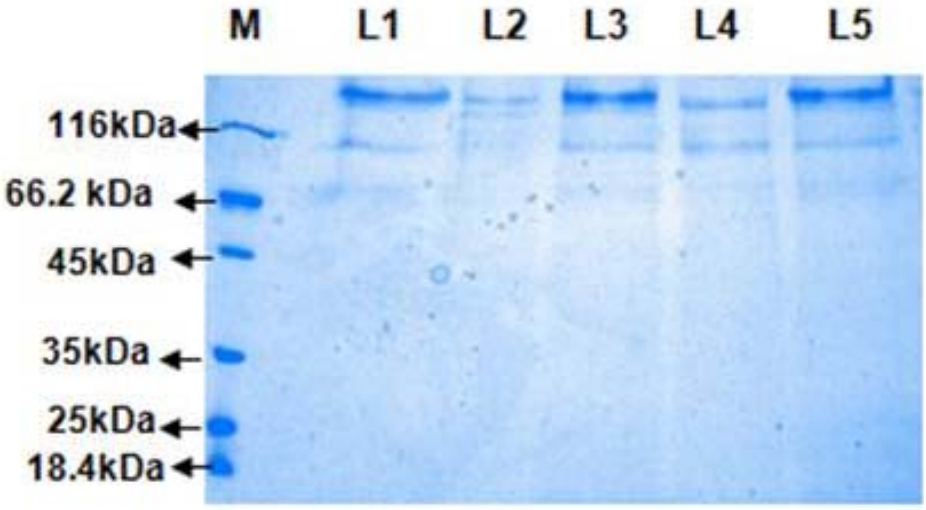

Figure 4. SDS-PAGE analysis of recombinant phytase expressed in BMMY medium. M: Unstained protein Ladder Markers. (L1, L2, L3, L4, L5): Phytase protein before deglycosylation showing multiple bands. **Note:** The molecular weight of phytase protein after deglycosylation with *PNGase* F was approximately 45 kDa as determined by SDS-PAGE (Figure 5).

continuous cultures. Cells grown under methanol/L-alanine limitations had high activity compared to cells grown on methanol feeding alone. The mechanism of methanol/L-alanine co-feeding strategy increased the enzyme activity to 8632 U/ml under the control of *AOX1* promoter and α-factor signal peptide meaning three times com-pared to methanol induction alone in the fermenter and 29 times from the shake flask expression. The alternative reason for producing higher yield under methanol/L-alanine co-feeding strategy might happen around pyruvate, acetyl co-

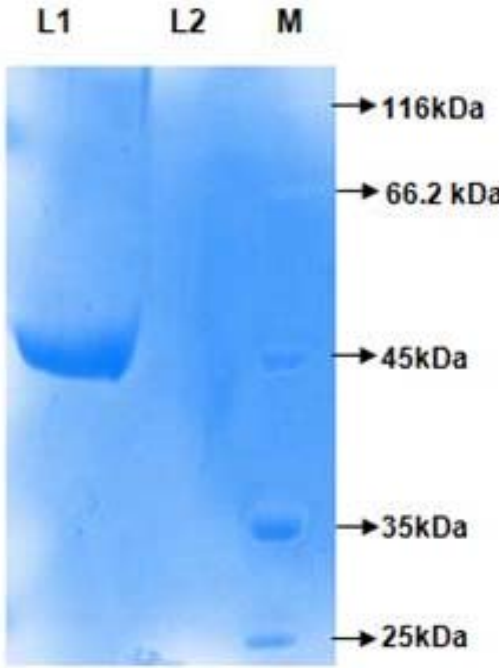

Figure 5. L1: Optimized Phytase protein after deglycosylation with PNGaseF (New England Biolabs). L2: Negative control with GS115-*pPICZαA*. M: Unstained protein ladder markers.

enzyme A, glyoxylate, and α-ketoglutarate via increased levels of *ALT1, DAL7, PYC1, GDH2,* and *ADH5* and decreased levels of *GDH3*, *CIT2*, and *ACS1* transcripts (Usaite et al., 2006) and also the physiological impacts of L-alanine to the cell is to supply enough nitrogen source (Fu et al., 2013).

The novel phytase that has a high extracellular activity and other characterizations (for example, temperature stability, wide pH optima, etc.), which are necessary for the commercial utilization using the methylotrophic yeast, *P. pastoris* is now available to be scaled up to the industrial level for food additives. The wide range of promoters available, as well as selectable markers, secretion signals, methods for coping with proteases and a better understanding of glycosylation patterns, are powerful to *P. pastoris* as a high efficient expression system available. The well-defined process protocols and some degree of process optimization are required to achieve maximum production of the heterologous protein. In fact, yield and activity are often dependent upon the parameters of the culture vessel (pH, temperature and O_2 availability), and they are also dependent on the residual L-Alanine and methanol concentrations. These factors can be closely monitored to ensure the exact conditions required. *P. pastoris* is able to add both O-linked and N-linked carbohydrate moieties to secreted proteins (Wang et al., 2005); the reason why phytase presented different bands size but after deglycosylation, the exact protein size of phyatse (*phyA*) became 45 kDa.

However, the induction at low temperature minimized extracellular proteolysis (Heyland, 2010) though it led to a high operation cost but the activity and protein concentration increased; and the toxicity of methanol was reduced compared to the induction at higher temperature and methanol feeding alone because there was too much methanol consumption. The cost of methanol and its hazardous substance is very high due to its high flammability and toxicity (Zou et al., 2006); additionally, cells growing on methanol have a very high oxygen consumption, which usually requires the addition of pure oxygen to the culture, increasing the cost of the process and limiting the cultivation capacity at high scale. Methanol was a cheaper and readily available substrate at the time the *P. pastoris* system was developed (Jahic et al., 2002). However, the wide range of applications in the field of protein production developed over the years have revealed the need for a more controllable, less volatile, and less flammable substrate for induction.

Conflict of Interests

The author(s) have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors would like to express thanks to the Government of Rwanda, ICCR and Anna University, India for their encouragement to accomplish this work.

REFERENCES

Bae HD, Yanke LJ, Cheng KJ, Selinger LB (1999). A novel staining method for detecting phytase activity. J. Microbiol. Methods 39(1): 17-22.

Blumhoff M, Steiger MG, Marx H, Mattanovich D, Sauer M (2013). Six novel constitutive promoters for metabolic engineering of *Aspergillus niger*. Appl. Microbiol. Biotechnol. 97(1): 259-267.

Callewaert N, Laroy W, Cadirgi H (2001). Use of HDEL-tagged *Trichoderma reesei* mannosyl oligosaccharide 1, 2-α-D-mannosidase for N-glycan engineering in *Pichia pastoris*. FEBS Lett. 503(2): 173-178.

Celik E, Calik P, Oliver SG (2009). Fed-batch methanol feeding strategy for recombinant protein production by *Pichia pastoris* in the presence of co-substrate sorbitol. Yeast 26:473-484.

Faber KN, Harder W, Ab G, Veenhuis M (2005). Methylotrophic yeasts as factories for the production of foreign proteins. Yeast 11(14): 1331-1344.

Feist AM, Herrgard MJ, Thiele I, Reed JL, Palsson BO (2009). Reconstruction of biochemical networks in microorganisms. Nat. Rev. Microbiol. 7:129-143.

Fu XY, Zhao W, Xiong AS, Tian YS, Peng RH (2011). High expression of recombinant *Streptomyces* sp. S38 xylanase in *Pichia pastoris* by codon optimization and analysis of its biochemical properties. Mol. Biol. Rep. 38: 4991–4997.

Gellissen G (2000). Heterologous protein production in methylotrophic yeasts. Appl. Microbiol. Biotechnol. 54(6):741-750.

Haefner, S., A. Knietsch, E. Scholten, J. Braun, M. Lohscheidt, and O. Zelder (2005). Biotechnological production and applications of phytases. Appl. Microbiol. Biotechnol. 68**:**588-597.

Hanson SR, Culyba EK, Hsu TL, Wong CH, Kelly JW, Powers ET (2009). The core trisaccharide of an N-linked glycoprotein intrinsically accelerates folding and enhances stability. Proc. Natl. Acad. Sci. USA 106(9): 3131-3136.

Heyland J (2010). Quantitative physiology of *Pichia pastoris* during glucose-limited high-cell density fed-batch cultivation for recombinant protein production. Biotechnol. Bioeng. 107(2): 357-368.

Jahic M, Rotticci-Mulder M, Martinelle M, Hult K, Enfors SO (2002). Modeling of growth and energy metabolism of *Pichia pastoris* producing a fusion protein. Bioprocess Biosyst. Eng. 24: 385-393.

Lee CCW, Tina G, Wong DWS (2005). An episomal expression vector for screening mutant gene libraries in *Pichia pastoris*. Plasmid 54(1): 80-85.

Li H, Sethuraman N, Stadheim TA, Zha D, Prinz B, Ballew N, Bobrowicz P, Choi BK, Cook WJ, Cukan M, Houston-Cummings NR, Davidson R, Gong B, Hamilton SR, Hoopes JP, Jiang Y, Kim N, Mansfield R, Nett JH, Rios S, Strawbridge R, Wildt S, Gerngross TU (2006). Optimization of humanized IgGs in glycoengineered *Pichia pastoris*. Nat. Biotechnol. 24(2): 210-215.

Macauley-Patrick S, Fazenda ML, McNeil B, Harvey LM (2005). Heterologous protein production using the *Pichia pastoris* expression system. Yeast 22(4): 249-270.

McKinney J, Knappskog PM, Pereira J (2004). Expression and purification of human tryptophan hydroxylase from *Escherichia coli* and *Pichia pastoris*. Protein Expr. Purif. 33(2): 185-194.

Mullaney EJ, Ullah AH (2005). Conservation of cysteine residues in fungal histine acid phytases. Biochem. Biophys. Res. Commun. 328: 312-179.

Pakkanen O, Haemaelaeinen ER, Kivirikko KI, Myllyharju J (2003). Assembly of stable human type I and III collagen molecules from hydroxylated recombinant chains in the yeast *Pichia pastoris* Effect of an engineered C-terminal oligomerization domain foldon. J. Biol. Chem. 278(34): 32478-32483.

Porro D, Sauer M, Branduardi P, Mattanovich D (2005). Recombinant protein production in yeasts. Mol. Biotechnol. 31(3): 245-259.

Sohn SB, Graf AB, Kim TY, Gasser B, Maurer M, Ferrer P, Mattanovich D, Lee SY (2010). Genome-scale metabolic model of methylotrophic yeast *Pichia pastoris* and its use for in silico analysis of heterologous protein production. Biotechnol J. 5(7):705-15.

Sola A, Jouhten P, Maaheimo H, Sanchez-Ferrando F, Szyperski T, Ferrer P (2007). Metabolic flux profiling of *Pichia pastoris* grown on glycerol/methanol mixtures in chemostat cultures at low and high dilution rates. Microbiology 153:281-290.

Usaite R, Patil KR, Grotkjær T, Nielsen J, Regenberg B (2006). Global transcriptional and physiological responses of *Saccharomyces cerevisiae* to ammonium, L-alanine, or L-glutamine limitation. Appl. Environ. Microbiol. 72(9): 6194-6203.

Wang HN, Wu Q, Zhao HX, Chen H, Liu P (2005). Secretory expression of *Bacillus subtilis* phytase phyC in *Pichia pastoris*. J. Zhejiang Univ. (Agric. Life Sci.) 31:621-627.

Xiong AS, Yao QH, Peng RH, Zhang Z, Xu F, Liu JG, Han PL, Chen JM (2006). High level expression of a synthetic gene encoding *Peniophora lycii* phytase in methylotrophic yeast *Pichia pastoris*. Appl. Microbiol. Biotechnol.72: 1039-1047.

Yang G, Zhou H, Lu Y, Lin Y, Zhou S (2004). Comparing expression of different forms of human DNA topoisomerase I in *Pichia pastoris*. Enzyme Microb. Technol. 34(2): 139-146.

Yu S, Cowieson AJ, Gilbert C, Plumstead P, Dalsgaard S (2012). Interactions of phytate and myo-inositol phosphate esters (IP1-5) including IP5 isomers with dietary protein and iron and inhibition of pepsin. J. Anim. Sci. 90: 1824-1832.

Zhang W, Inan M, Meagher MM (2000). Fermentation strategies for recombinant protein expression in the methylotrophic yeast *Pichia pastoris*. Biotechnol. Bioproc. Eng. 5: 275–287.

Zou LK, Wang HN, Pan X, Xie T, Wu Q, Xie ZW, Zhou WR (2006). Design and expression of synthetic phyC gene encoding the neutral phytase in *Pichia pastoris*. Acta Biochim. Biophys. Sin. (Shanghai) 38:803-811.