MOLECULAR CLONING AND PRODUCTION OF RECOMBINANT PHYTASE FROM *BACILLUS SUBTILIS* ASUIA243 IN *PICHIA PASTORIS*

NOR SOLEHA MOHD DALI[1], TAMRIN NUGE[1], MOHD. HAFIDZ MAHAMAD MAIFIAH[1], FARIDAH YUSOF[1], ANIS SHOBIRIN MEOR HUSSIN[2], ABD-ELAZIEM FAROUK[3] AND HAMZAH MOHD. SALLEH[1]

[1]Bioprocess and Molecular Engineering Research Unit, Department of Biotechnology Engineering, International Islamic University Malaysia, Jalan Gombak, 53100 Kuala Lumpur, Malaysia.
[2]Department of Food Technology, Faculty of Science and Food Technology, 43400, University Putra Malaysia, Serdang, Selangor, Malaysia.
[3]Department of Biotechnology, Faculty of Science, Taif University, 21974 Taif, Al-Hawiayah, P. O. Box. 888, Saudi Arabia.

hamzah@iium.edu.my

ABSTRACT: Phytase gene obtained from *Bacillus subtilis* ASUIA243 was cloned into a medium vector and transformed into *E. coli*. Restriction enzyme digestion was conducted to get blunt-ended phytase gene and ligated into the *Pichia* expression vector, pPICZαA. The recombinant vector, pPICZαA-243HPp was then linearized with *Pme*I and transformed into *P. pastoris* strain X33. Screening for multi copy gene number of transformants was done by re-plating the selected colonies on increasing concentration of zeocin. One positive clone, X243HPp#2 was then grown in BMGY media as the starting culture, followed by induction in BMMY media for protein expression study. The supernatant was then analysed by SDS-PAGE and Western blot method to check the protein expression.

ABSTRAK: Gen fitase yang didapati daripada *Bacillus subtilis* ASUIA243 diklonkan sebagai vektor perantara dan berubah menjadi *E. coli*. Sekatan pencernaan enzim dijalankan untuk mendapatkan gen fitase berhujung tumpul dan diligatkan dengan vektor ekspresi *Pichia,* pPICZαA. Vektor rekombinan, pPICZαA-243HPp kemudian dilinearkan dengan *PmeI* dan berubah menjadi *P. pastoris* strain X33. Penyaringan untuk nombor gen berbilang salinan yang menjalani transformasi genetik dijalankan dengan menyalur semula koloni terpilih dengan penambahan kepekatan *zeocin*. Satu klon positif, X243HPp#2 kemudian dibiarkan hidup dalam perantara BMGY sebagai kultur permulaan, diikuti dengan aruhan dalam perantara BMMY untuk kajian penglahiran protein. Supernatan kemudian dikaji dengan SDS-PAGE dan kaedah sap Western untuk menyemak penglahiran protein.

KEYWORDS: *phytase; Bacillus subtilis; Pichia pastoris; gene cloning*

1. INTRODUCTION

Phytase (*myo*-inositolhexakiphosphate phosphohydrolase) catalyzes the hydrolysis of phytic acid (the principle storage form of phosphorus in legumes, cereals, oil seeds and nuts) to myo-inositol and inorganic phosphate. Phytic acid is a polyanionic chelating agent that forms complexes with several divalent cations of major nutritional importance such as Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, and Mn^{2+} [1]. Therefore, the catalytic activity of phytase on

phytic acid leads to the loss of ability of phytic acid to chelate metal ions. The animal feeds contain phytic acid which cannot be digested by monogastric animals because they have no or low level of phytase activity in their digestive tracks [2]. Inorganic phosphate is frequently added to their feed to facilitate optimal growth for these animals which leads to stimulation of algal blooms and euthrophication of surface water due to the high content of phytate and inorganic phosphate in the excretion of the animals [3]. Thus, supplementation of animal feeds with phytase increases the bioavailability of phosphorus and reduces the phosphorus pollution in the environment. Thus, the benefits of phytase are two-fold: saving the expensive and non-renewable inorganic phosphorus resource by reducing the need for its inclusion in animal diets and protecting the environment from pollution of excessive manure phosphorus runoff [4].

Recent research has shown that microbial sources are more promising for the production of phytases on a commercial level [5]. Most of the scientific work has been done on those originating from filamentous fungi such as *Aspergillus niger* [6,7], *Rhizopus oligosporus* [8] and *Cladosporium* species [9]. Other than these species, phytase from other microbes such as *Escherichia coli* [10], *Pseudomonas* species [11], *Klebsiella* species [12] and *Bacillus* species [13] were also identified.

Therefore in this research, a local wild type *Bacillus subtilis* strain ASUIA243 has been chosen as the source for phytase gene and the gene was cloned in an expression vector with strong promoter, pPICZαA. The recombinant vector was then transformed in the methylotrophic yeast, *Pichia pastoris* strain X33 for enzyme production. *P. pastoris* has been chosen as the production host because of the ability to produce the heterologous protein extracellularly, therefore eliminating the method for cell lysis, and the expressed proteins are generally hyperglycosylated [8].

2. MATERIALS AND METHODS

2.1 Strains, Plasmids and Media

The wild type *Bacillus subtilis* ASUIA243 strain was obtained from a previous study [14]. The pPICZαA vector, pCR®-Blunt II-TOPO® vector, *Pichia pastoris* strain X33 and One Shot® TOP10 competent cell were purchased from Invitrogen, USA. Low salt Luria-Bertani (LB) broth and agar were purchased from Becton, Dickinson and Company, USA. Yeast extract, peptone, dextrose and agar for YPDS, BMGY and BMMY media were purchased from Merck, Germany. Glycerol and methanol were purchased from Merck, Germany. Sorbitol was purchased from MP Biomedicals, France. Yeast Nitrogen Base (YNB) for BMGY and BMMY media was purchased from Laboratorios Conda, Spain.

2.2 Isolation of Phytase Gene

The plasmid DNA was isolated from the wild-type *B. subtilis* ASUIA243 by using QIAprep® Spin Miniprep Kit (Qiagen). The phytase gene was then amplified by Polymerase Chain Reaction (PCR). The oligonucleotide primers were designed based on the *B. subtilis* subsp. subtilis str. 168 complete genome obtained from NCBI database (GenBank accession no. *NP_389861.1*) The primer sequences are the following:

Bsnf5Pp (forward): 5'-GGTACCATGAAGGTTCCAAAAACAATGCTG-3'

Bsnr6Pp (reverse) : 5'-TCTAGATAGCCGTCAGAACGGTCT-3'

The underlined sequences in forward and reverse primers represent restriction enzyme site for *Kpn*I and *Xba*I, respectively.

The reaction mixture contained 10 µl of 5x Phusion HF Buffer (Finnzyme), 1 µl of each primer, 5 µl of plasmid DNA, 1 µl of 10 mM dNTPs, and 0.5 µl of PFU DNA polymerase (Finnzyme). PCR was conducted at a condition of 98 ^{o}C for 2 minutes, 30 cycles of 98 ^{o}C for 10 seconds, 55 ^{o}C for 10 seconds, and 72 ^{o}C for 10 seconds, followed by 72 ^{o}C for 10 minutes, and hold at 4 ^{o}C. Gel electrophoresis was done on the PCR products by using 0.8% agarose gel followed by gel purification using Gel Extraction Kit (Qiagen).

2.3 Cloning into pPICZαA Vector and Transformation into *P. pastoris*

The purified PCR products were cloned into pCR®-Blunt II-TOPO® as medium vector and transformed into competent One Shot® TOP10 *E. coli* according to the manufacturer's instructions. The transformants were plated out onto LB medium containing 50 µg/ml kanamycin and incubated overnight at 37 ^{o}C.

Next, single colonies were selected and grown at 250 rpm overnight at 37 ^{o}C in 10 ml LB broth containing 50 µg/ml kanamycin. The plasmids were extracted by using QIAprep® Spin Miniprep Kit (Qiagen) and digested with *Kpn*I and *Xba*I at 37^{o}C for 2.5 hours. The digestion product, 243HPp was purified by using Gel Extraction Kit (Qiagen).

The purified products were then ligated with expression vector, pPICZαA that has been already been digested with the same restriction enzymes. The ligation was conducted at 16 ^{o}C for 16 hours. Ligation product was then transformed into One Shot® TOP10 *E. coli* and plated on low salt LB medium containing 25 µg/ml zeocin (Invivogen, USA). The plates were incubated at 37 ^{o}C overnight. Single colonies were selected and grown in low salt LB broth containing 25 µg/ml zeocin at 37^{o}C, 250 rpm overnight. The plasmids pPICZαA-243HPp were extracted by using QIAprep® Spin Miniprep Kit (Qiagen) and digested with *Kpn*I and *Xba*I to confirm for positive clones. One positive clone, pPICZαA-243HPp#1 was chosen for sequencing analysis.

The recombinant plasmid pPICZαA-243HPp#1 was linearized with *Pme*I at 37^{o}C for 2.5 hours and transformed into the *Pichia pastoris* strain X33 (Mut^{+}) by chemical transformation according to the manufacturer's instructions. The cells were cultured on YPDS media (1 % yeast extract, 2 % peptone, 2 % dextrose, 2 % agar, 1 M sorbitol) containing 100 µg/ml zeocin at 30 ^{o}C for 3 days.

2.4 Screening for Multiple Copy of Gene

The positive colonies were plated again on YPDS agar containing different concentration of zeocin: 100 µg/ml, 500 µg/ml and 1000 µg/ml to screen for the clone with multiple gene copy number. The resistant colony at 1000 µg/ml was selected and transferred to other fresh agar plates. Colony PCR was conducted for a few selected colonies to determine the stable integration of the gene inside the host.

2.5 Expression of Recombinant Phytase Gene in *P. pastoris*

One clone from the multiple gene copy number screening was grown in a 250 ml shake flask containing 25 ml of BMGY media (1 % yeast extract, 2 % peptone, 1.34 % YNB, 4x10^{-5} % biotin, 1% glycerol, 100 mM potassium phosphate pH 6.0) at 30^{o}C and shaken at 250 rpm until the OD_{600nm} reached 2.0-6.0 (about 26-30 hours). Cells were harvested by centrifugation at 5000 x *g* at 4^{o}C for 5 minutes. The cells were resuspended to an OD_{600nm} of 1.0 in 100 ml BMMY (1% yeast extract, 2% peptone, 1.34% YNB, 4x10^{-5}% biotin, 0.5% methanol, 100mM potassium phosphate pH 6.0) at 30 ^{o}C for 4 days followed by induction with 0.5% methanol for every 24 hours. Samples (1 ml) were taken

at 24-hour intervals to analyse the protein expression. All these methods followed the manufacturer's instructions (Invitrogen, 2002).

2.6 Analysis of Expressed Recombinant Protein

Protein expression was analysed on 12% SDS polyacrylamide gel [15]. Two sets of gels were prepared where one set was subjected to Coomasie blue staining and the other set was subjected to Western blot analysis. Samples (20 µl) were loaded into each well of the gel.

For Western blot analysis, proteins separated by SDS-PAGE was transferred onto nitrocellulose membrane, blocked and incubated overnight with the primary antibody (1:5000). Afterwards, the membrane was washed with buffer and incubated with the polyclonal goat anti rabbit IgG-alkaline phosphatase conjugate used as the secondary antibody for another six hours. The membrane was incubate in a combination of NBT and BCIP (Bio-Rad Laboratories, USA) for colour development.

3. RESULTS AND DISCUSSION

3.1 Cloning and Transformation into *P. pastoris* Host

The *B. subtilis* ASUIA243 phytase gene was successfully amplified by using the designed primers, Bnsf5pp and Bsnr6Pp. The primers were designed in such that the N-terminal of the gene was in frame with the α-factor signal sequence and the C-terminal of the gene contained no stop codon to include the *c-myc* epitope and 6x-His tag. From the gel electrophoresis (Fig. 1), the bands were located at approximately 1,149bp which is the size of *B. subtilis* phytase gene according to the original DNA template obtained from the NCBI website.

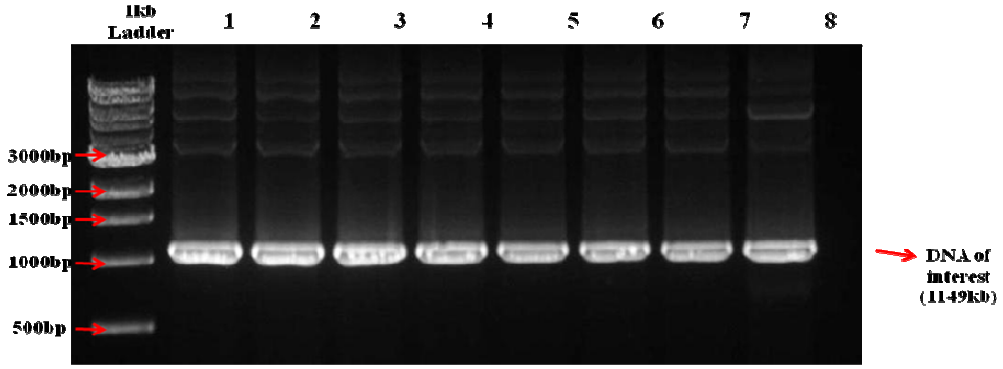

Fig. 1: Gel electrophoresis of *B. subtilis* ASUIA243 phytase gene by using Bsnf5Pp and Bsnr6Pp primers. The bands are located at approximately 1,149bp.

The phytase gene 243HPp was successfully ligated into the expression vector pPICZαA and transformed into the One Shot® TOP10 *E. coli* in order to get many copies of the recombinant plasmid. Re-screening was done to ensure that the clones were not false positives that caused by nonspecific binding. According to Dale and von Schantz (2002) [16], the re-screening of recombinant colonies is a common practice to pick a recombinant colony that is free from contaminating neighbours. The true positive colonies, pPICZαA-243HPp were then grown in low salt LB broth, plasmids were extracted and sent for sequencing (Fig. 2).

Fig. 2: Sequencing result of pPICZαA-243HPp shows that the phytase gene is in frame with the α-factor signal sequence (italic and green highlighted), *c-myc* epitope (blue highlighted) and the 6x-His tag (yellow highlighted).

The positive recombinant plasmids were linearized with *Pme*I before being transformed into the *P. pastoris* X33 competent cell. The linear vector DNAs can generate stable transformants of *P. pastoris* via homologous recombination between sequences shared by the vector and host genome [17]. By linearizing the recombinant vector at the unique site located in HIS4 region, Mut+ recombinants were generated [18]. About 38 colonies were selected for multiple gene copy screening, and among these 38 colonies, only 12 colonies contained multiple copy of gene while others were not grown on the 1000µg/ml Zeocin plate. Colony-PCR was performed on seven of the 12 colonies to determine stable integration of the gene inside the host genome (Fig. 3). One positive clone, X243HPp#2 was selected for shake-flask expression.

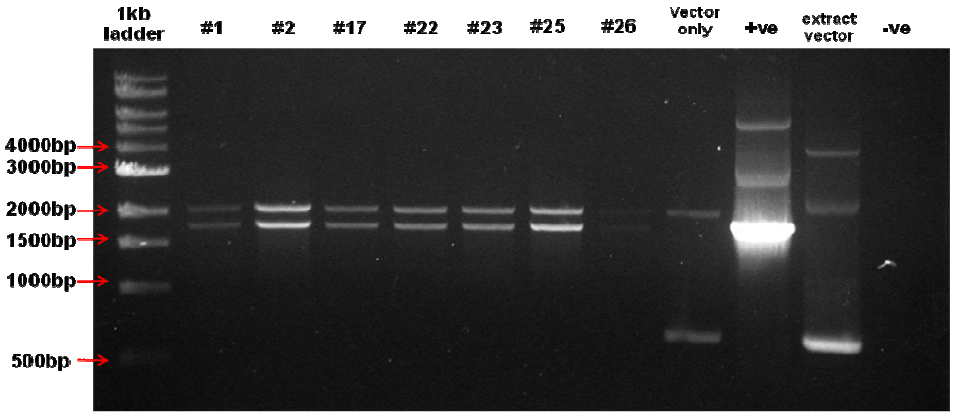

Fig. 3: Gel electrophoresis of colony-PCR. Number #1, #2, #17, #22, #23, #25 and #26 are the positive colonies from high copy selection.

Vector only is the host with pPICZαA only; +ve is the extracted recombinant plasmid with inserted gene; extract vector is the extracted pPICZαA; -ve is the mixture of PCR reagent with primers only. The result shows that all clones except #26 have stable integrated gene. The upper bands are located at appr. 2200bp while the lower bands are the inserted genes with parent plasmid PCR products (1149bp + 588bp = 1737bp). For the vector only, the result shows two bands, the upper one is the AOX1 gene (2200bp) and the lower one is the parent plasmid PCR product (588bp). For +ve, the band indicates the positive result for the gene of interest (1737bp).

3.2 Expression of Recombinant Phytase in *P. pastoris*

As shown in Fig. 4, there is no expected band appeared on the SDS-PAGE gel after 4 days of induction. The predicted size of phytase X243HPp#2 is about 45.02 kDa (including the *c-myc* epitope and 6x-His tag). Similarly, no bands were detected on the membrane for Western blot analysis (data now shown). This indicates that no protein was expressed during the induction. However, from the colony-PCR result, we can see that the gene was indeed integrated in the host genome and the DNA sequencing result confirmed that the phytase gene is in frame with the α-factor signal sequence, *c-myc* epitope and the 6x-His tag. These results showed that the phytase gene has correctly been inserted into the yeast genome.

Since there was no expressed protein detected on the SDS-PAGE gel and nitrocellulose membrane, there might be a few reasons that would explain this problem. There are many cases of protein expression failure but only fews were reported. Burt et al. [19] explained three main reasons for the lack of expression of biologically active proteins in heterologous system. Host cell lacking components for translation of recombinant protein, the heterologous protein being prone to proteolysis as it emerges from translation, and incomplete or improper folding of heterologous protein can be some of the reasons that lead to the failure of protein expression in heterologous system.

Sreekrishna et al. [20] listed two possible factors which influence protein expression in *Pichia* systems; untranslated regions in recombinant mRNA, and transcriptional and translational blocks. Since the phytase gene was correctly inserted into the yeast genome, there is also a possibility of excessive transcription. Gasser et al. [21] reported in their work that excessive transcription may cause cellular stress, such that the production rate of polypeptides can be rapid and interfere with correct protein folding. Only properly folded and assembled proteins can be exported within the yeast cell, modified and and

transported to the extracellular space [22]. Misfolded or aggregated proteins in the cell will be degraded inside the cell [23]. Accumulation of unfolded or misfolded proteins in the cell can also suppresses transcription of the secretory target protein [24].

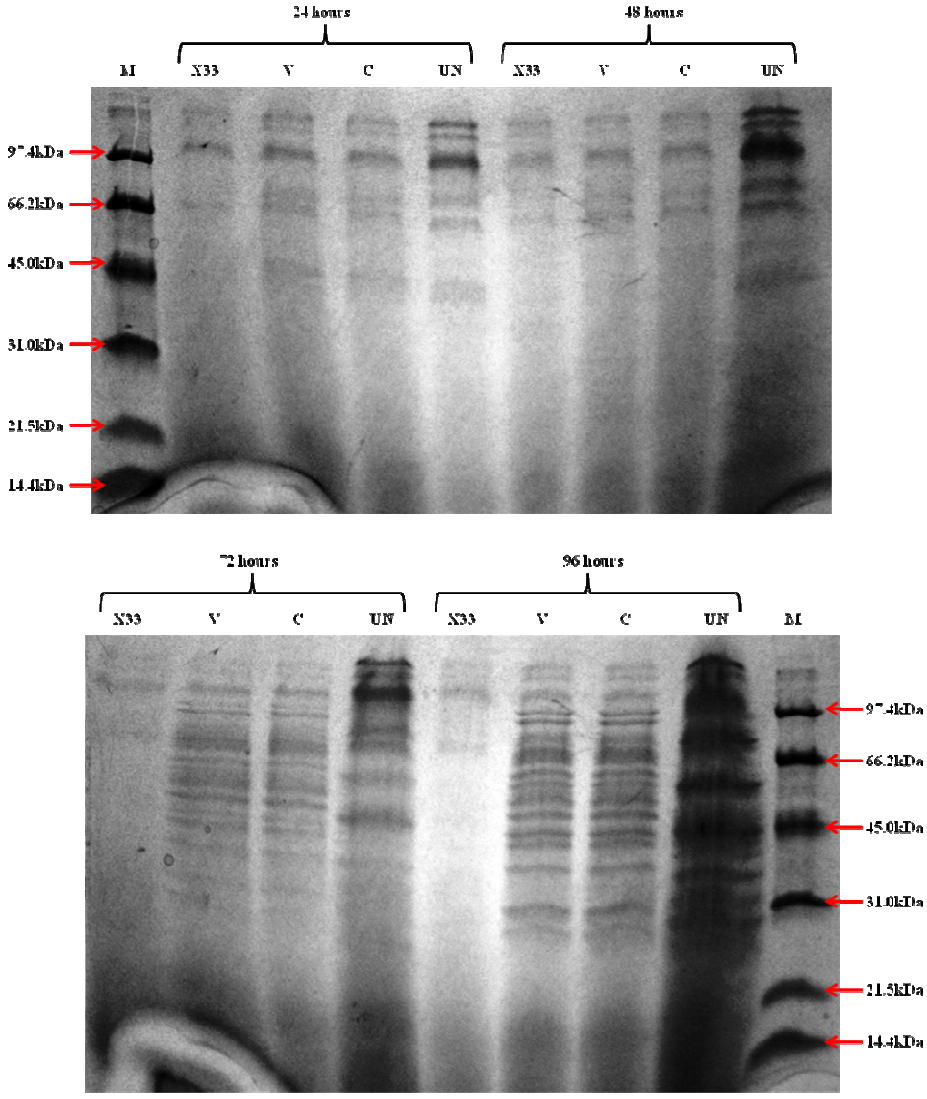

Fig. 4: SDS-PAGE result for induction of phytase X243HPp#2. X33: Untransformed host cell; V: *P. pastoris* X33 transformed with pPICZαA vector only; C: Multiple gene copy clone (X243HPp#2); UN: clone that was grown in media without methanol inducer; M: protein low range standard marker. There is no expected band appeared on the gel for all four days of induction.

There are many possibilities that can lead to the failure of protein expression in *Pichia pastoris*. Although only few have been reported, these can be the possible reasons for the problem in this work. Further investigation needs to be conducted. The process conditions and media compositions during the induction time can be other possible reasons for the failure of protein expression and these two conditions should be optimized. Romanos (1995) [25] reported that a critical factor that has long been recognized to affect induction efficiency is aeration of *P. pastoris* culture because in shake-flask inductions,

the tendency of cultures, especially Mut^+ strains, to become oxygen-limited is quite high. Besides that, changing the codons in the foreign gene that those of codons more frequently used by the yeast significantly improves the levels of expression of those foreign genes [26]. The A+T ratio of a foreign gene can be changed to one more similiar to the *P. pastoris* genome to improve expression [27].

Changing the expression vector to other alternative vectors is also possible to improve the protein expression. The pPICZαA vector contains AOX1 promoter which requires methanol as an inducer. When methanol is oxidized by the alcohol oxidase, the accumulation of the oxidized products, formaldehyde and hydrogen peroxide, can lead to toxicity for the host cell itself, which also can be one of the factors for protein expression failure [28, 29]. Therefore, changing to other alternative expression vector for protein secretion such as pGAPZα can be a better solution for this problem. This vector has the glyceraldehyde-3-phosphate (*GAP*) promoter which does not require methanol for induction, and it is not necessary to shift cultures from one carbon source to another, making strain growth and protein expression more convenient and straightforward [30].

4. CONCLUSION

Despite of the problem occurred, *P. pastoris* has become one of the favourite expression systems for the production of various proteins of interest. It is well known that *P. pastoris* secretes very little native protein, providing an advantageous means to recover foreign secreted protein from the supernatant. Other advantages of using *P. pastoris* expression system are ease of fermentation to high cell density, genetic stability and scale up without loss of yield [31], molecular genetic manipulations are simple and protocols are available, can be grown in a simple mineral salts medium [32], high eukaryotic protein modifications such as glycosylation, disulfide-bond formation and proteolytic processing [33].

In this research, the failure of protein expression can be further investigated. It is suggested that the process conditions and media compositions should be optimized to improve the cell growth and protein expression, since the phytase gene was integrated stably in the host genome. The same clone can be used for the optimization or the gene can be re-transformed into a new competent cell. Since there are many reports of successful production of heterologous phytase from various sources using *P. pastoris* expression system, it is possible that our phytase can also be expressed using this expression system after further investigations are conducted.

ACKNOWLEDGEMENT

This research work is funded by Biotech Research Project from the Ministry of Science, Technology and Innovation (MOSTI) Malaysia, (grant project no. 07-01-08-EIB011). The authors also thanked Dr. Farah Diba Abu Bakar for valuable discussions.

REFERENCES

[1] Harland, B. F., Oberleas, D. Phytic acid complex in feed ingredients. In: M. B. Coelho, E. T. Kornegay, (eds) Phytase in animal nutrition and waste management. 2nd rev edn. BASF, Mexico, 1999, 69-76.

[2] Schroder, B., Brever, G., Rodehutscord, M. Mechanisms of intestinal phosphorus absorption and availability of dietary phosphorus in pigs. Dtsch Tieraerztl Wochenschr., 1996, 103: 209-214.

[3] Oh, B. C., Choi, W. C., Park, S., Kim, Y. O., Oh, T. K. Biochemical properties and substrate specificities of alkaline and histidine acid phytases. Appl Microbiol Biotechnol., 2004, 63, 362-372.
[4] Xin G. L. and Porres, J. M. Phytase enzymology, applications, and biotechnology. Biotechnology Letters, 2003, 25: 1787-1794.
[5] Pandey, A., Szakacs, G., Soccol, C. R., Rodriguez-Leon, J. A., Soccol, V. T. Production, purification and properties of microbial phytases. Bioresource Technology., 2001, 77, 203-214.
[6] Ngo, T. X., Mai, T. H., Vu, N. T. Cloning and over expression of an *Aspergillus niger* XP phytase gene (phyA) in *Pichia pastoris*. World Academy of Science, Engineering and Technology, 2009, 56, 750-753.
[7] Soni, S. K., and Khire, J. M. Production and partial characterization of two types of phytase from *Aspergillus niger* NCIM 563 under submerged fermentation conditions." World J. Microbiol. Biotechnol., 2007, 23, 1585-1593.
[8] Casey, A., Walsh, G. Identification and characterization of a phytase of potential commercial interest. J. Biotechnol., 2004, 110, 313-322.
[9] Quan, C. S., Tian, W. J., Fan, S. D., Kikuchi, Y. I. Purification and properties of a low-molecular-weight phytase from Cladosporium sp. FP-I." J. Biosc. Bioeng., 2004, 97, 260-266.
[10] Greiner, R., Konietzny, U., Jany, K. D. Purification and characterization of two phytases from *Escherichia coli*. Arch. Biochem. Biophys., 1993, 303, 117-113.
[11] Cho, J. S., Lee, C. W., Kang, S. H., Lee, J. C., Bok, J. D., Moon, Y. S., Lee, H. G., Kim, S. C., Choi, Y. J. Purification and characterization of a phytase from *Pseudomonas syringae* MOK1. Curr. Microbiol., 2003, 47, 290-294.
[12] Sajidan, A., Farouk, A., Greiner, R., Jungblut, P., Mueller, E. C., Borriss, R. Molecular and physiological characterization of a 3-phytase from soil bacterium *Klebsiella* sp. ASR1." Appl. Microbiol. Biotechnol., 2004, 65: 110-118.
[13] Kerovuo, J., Lauraues, M., Nurminen, P., Kalkkinen, N., Apajalahti, J. Isolation, characterization, molecular gene cloning, and sequencing of a novel phytase from *Bacillus subtilis.* Appl. Environ. Microbiol., 1998, 64, 2079-2085.
[14] Anis Shobirin M. H., Farouk, A., Greiner, R. Potential phytate-degrading enzyme producing bacteria isolated from Malaysian maize plantation. African J. Biol., 2009, Vol. 8, no. 15, 3540-3546.
[15] Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriphage T4. Nature, 1970, 227, 680-685.
[16] Dale, J. W., and von Schantz, M. From Gene to Genomes: Concepts and Applications of DNA Technology. John Wiley & Sons Ltd., New York, 2002.
[17] Higgins, D. R. and Cregg, J. M. *Pichia* Protocols. Humana Press, New Jersey. Vol.10, 1998.
[18] Zou, L., Wang, H., Pan, X., Tian, G., Xie, Z., Wu, Q., Chen, H., Xie, T., and Yang, Z. Expression, purification and characterization of a phyAm-phyCs fusion phytase. Journal of Zhejiang University SCIENCE B, 2008, Vol. 9, No. 7, 536-545.
[19] Burt, T. R., Edavettal, S. C., Hall, J. P. and Mattern, M. R. Sumo fusion technology for difficult-to-express proteins. Protein Expr. Purif., 2005, Vol. 43, No. 1, 1-19.
[20] Sreekrishna, K., Brankamp, R. G., Kropp, K. E. Strategies for optimal synthesis and secretion of heterologous proteins in the methylotrophic yeast *Pichia pastoris*. Gene, 1997, Vol. 190, No. 1, 55-62.
[21] Gasser, B., Maurer, M., Gach, J., Kunert, R., and Mattanovich, D. Engineering of *Pichia pastoris* for improved production of antibody fragments." Biotechnol. Bioeng., 2006, 94, 353-361.
[22] Klausner, R. Architectural editing: determining the fate of newly synthesized membrane proteins. New Biol., 1989, 1, 3-8.
[23] Yoshida, H. ER stress and diseases. FEBS Journal, 2007, 274, 630-658.
[24] Schroder, M. and Kaufman, R. J. The mammalian unfolded protein response. Annu. Rev. Biochem., 2005, 74, 739-789.

[25] Romanos, M. A. Advances in the use of *Pichia pastoris* for high-level gene expression. Current Opinion in Biotechnology, 1995, 6, 527-533.
[26] Gurkan, C. and Ellar, D. J. Expression of the *Bacillus thuringiensis* Cy t2Aa1 toxin in Pichia pastoris using a synthetic gene construct." Biotechnol. Appl. Biochem., 2003, 38, 25-33.
[27] Cregg, J. M. Distinctions between *Pichia pastoris* and other expression system. In Methods in Molecular Biology: *Pichia* Protocols (ed) Cregg, J. M., Humana Press, New Jersey, p1-10, 2007.
[28] Couderc, R. and Baratti, J. Oxidation of methanol by the yeast, *Pichia pastoris*, purification and properties of the alcohol oxidase. Agric. Biol. Chem., 1980, 44, 2279-2289.
[29] Van der Klei, I. J., Bystrykh, L. V., Harder, W. Alcohol oxidase from *Hasenula polymorpha* CBS4732. Methods Enzymol., 1990, 188, 420-427.
[30] Li, P., Anumanthan, A., Gao, X., Ilangovan, K., Suzara, V. V., Düzgüneş, N., Renugopalakrishnan, V. Expression of recombinant protein in *Pichia pastoris*. Appl. Biochem. Biotechnol., 2007, 142, 105-124.
[31] Tuite, M. F., Clare, J. J., Romanos, M. A. Expressing cloned genes in the yeasts Saccharomyces cerevisiae and *Pichia pastoris*." In Protein Expression: A Practical Approach (eds) Higgins, S. J. and Hames, B. D., Oxford University Press, New York, 1999.
[32] Jahic, M., Veide, A., Charoenrat, T., Teeri, T., Enfors, S. Process technology for production and recovery of heterologous proteins in *Pichia pastoris*. Biotechnol. Prog., 2006, 22, 1465-1473.
[33] Cregg, J. M., Cereghino, L., Shi, J., Higgins, D. R. Recombinant protein expression in *Pichia pastoris*. Mol. Biotechnol., 2000, 16, 23-52.