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EXPRESSION OF PHYTASE FROM Mitsuokella jalaludinii IN HETEROLOGOUS HOSTS AND CHARACTERIZATION OF EXPRESSED PROTEIN FROM Escherichia coli

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EXPRESSION OF PHYTASE FROM *Mitsuokella jalaludinii* IN HETEROLOGOUS HOSTS AND CHARACTERIZATION OF EXPRESSED PROTEIN FROM *Escherichia coli*

By

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Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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EXPRESSION OF PHYTASE FROM *Mitsuokella jalaludinii* IN HETEROLOGOUS HOSTS, AND CHARACTERIZATION OF EXPRESSED PROTEIN FROM *Escherichia coli*

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Chairman: Assoc. Prof. Sieo Chin Chin, PhD

Faculty: Institute of Bioscience

Phytase is an enzyme which catalyzes the hydrolysis of phytate to release lower inositol phosphate and usable form of inorganic phosphate. It plays an important role in the breakdown of phytate present in the plant-based animal feed, especially poultry, fish, and swine which lack of natural phytase in their digestive systems to improve the bioavailability of phytate-phosphorus, nutritionally important minerals, and growth performance. In our laboratory, a strict anaerobic phytase-producing rumen bacterium, *Mitsuokella jalaludinii* was isolated and a comparative study on the freeze-dried active *M. jalaludinii* culture (FD-AMJC) and commercial Natuphos® phytase (phytase-N) revealed that the overall effect of feed conversion rate, phosphorus and calcium retention, dry matter digestibility and phosphorus excretion in the FD-AMJC was better than phytase-N. However, due to the strict anaerobic growth mode of *M. jalaludinii*, the mass production of the viable culture as feed supplement is restricted. In the present study, the 1.05 kb phytase gene which was previously cloned in pCR2.1TOPO plasmid was amplified by Polymearse Chain Reaction (PCR) and subcloned into *Pichia pastoris* expression vectors (pGAPZαC and pPIC6αA) and *Escherichia coli* expression vector (pET32a). The amplified gene sequence showed 99% similarity with native phytase gene. The p*GAPphy* and p*PICphy* were transformed into *Pichia pastoris* X-33 for extracellular expression, while the p*PETphy* was transformed into *E. coli* Rosetta-gami for intracellular expression of recombinant phytase. Expression was only observed in *E. coli* Rosetta-gami transformants but not *P. pastoris* transformants. Thus, only the recombinant phytase from *E. coli* Rosetta-gami was used for subsequent characterization. Based on the SDS-PAGE analysis, the size of the purified recombinant phytase expressed by *E. coli* Rosetta-gami was estimated to be 55 kDa by SDS-PAGE analysis. The purification by His-Trap one step purification column produced 1.8-fold higher yield than the unpurified fraction with the enzyme activity of 303 U/mg. The recombinant phytase exhibited its highest enzyme activity at 55 °C and pH 4.5. The enzyme was

stable at a broad pH range from 3.5 to 5.5 with more than 78% relative activity retained after 1 h exposure. The recombinant phytase was slightly inhibited by copper (5.9%) and EDTA (15.8%) and was greatly inhibited by ferum (76.6%) and zinc (78.5%) at 1.0 mM concentration. Nevertheless, calcium, potassium, and magnesium were found to enhance phytase activity with increment of activity to 148.8%, 118.3%, and 103.4%, respectively, at 1.0 mM concentration. Besides, the enzyme activity was found to be reduced when 0.05 to 10.0 mM phosphate was present in the reaction mixture. The digestive enzyme sensitivity analyses had revealed that the recombinant phytase was susceptible to pepsin proteolysis with only retention of 23.7% relative activity, but less affected to trypsin degradation with retention of 78.8% relative activity after pre-treatment of recombinant phytase with respective digestive enzymes at 37 °C for 1 h. The present findings showed that phytase from *Mitsuokella jalaludinii* can be expressed from *Escherichia coli* Rosetta-gami under IPTG induction and possesses moderate temperature profile, good pH tolerance, and pepsin stability, which indicates that with further study, the recombinant phytase can be a potential candidate as feed enzyme.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENGEKPRESAN FITASE DARIPADA *Mitsuokella jalaludinii* DALAM HOS HETEROLOGUS DAN PENCIRIAN PROTEIN DARIPADA *Escherichia coli*

Oleh

TAN WAN QIN

Disember 2013

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Fitase merupakan enzim yang terlibat dalam reaksi hidrolisis untuk menghasilkan inositol fosfat dan inorganik fosfat. Fitase memainkan peranan penting untuk memecahkan fitat yang biasanya berada dalam makanan haiwan monogastrik. Fitase biasanya ditambah ke dalam makanan ayam, ikan, dan babi yang kekurangan enzim ini dalam sistem penghadamanya untuk menigkatkan sumber fosforus semulajadi, mineral penting dan prestasi pertumbuhan haiwan. Di makmal kami, sejenis bakteria rumen anaerobik, *Mitsuokella jalaludinii*, yang dapat menghasilkan enzim fitase telah dipencilkan dalam kajian lepas. Perbandingan prestasi antara *M. jalaludinii* selepas pembekuan kering dengan komersial Natuphos® fitase menunjukkan prestasi *M. jalaludinii* aktif lebih baik dalam aspek peningkatan tenaga metabolisma, penyerapan zink, kuprum, dan protein. Namun, disebabkan ciri-ciri *M. jalaludinii* yang memerlukan keadaan anaerobik untuk pertumbuhan, penghasilan bakteria ini secara skala industri sangat terhad. Sehubungan itu, gen fitase daripada *M. jalaludinii* dipencil dan digunakan untuk menghasilkan fitase dalam hos rekombinan. Dalam kajian tersebut, amplifikasi gen fitase bersais 1.05 kb yang dipencil dan dikekalkan dalam plasmid pCR2.1-TOPO telah dijalankan dengan Reaksi Berantai Polimerase (PCR). Produk PCR diklon dalam vektor ekspresi, pGAPZαC , pPIC6αA, dan pET32a untuk menghasilkan enzim rekombinan dan keputusan penjujukan DNA menunjukkan gen fitase yang diklon mempunyai 99% persamaan dengan fitase gen asli daripada *M. jalaludinii*. Plasmid rekombinan p*GAPphy* dan p*PICphy* ditransformasi ke dalam *P. pastoris* X-33 untuk pengekspresan fitase secara ekstrasel. Sementara itu, p*PETphy* ditransformasi ke dalam *E. coli* Rosetta-gami untuk pengekspresan intrasel. Penghasilan fitase rekombinan hanya dikesan daripada klon *E.coli* Rosetta-gami dan bukan daripada *Pichia pastoris*. Sehubungan itu, hanya fitase rekombinan yang diekspres dalam sistem *E. coli* Rosetta-gami digunakan untuk pencirian. Fitase rekombinan yang dihasilkan oleh *E. coli* Rosetta-gami didapati bersaiz 55 kDa melalui analisis SDS-PAGE. Penulenan fitase rekombinan melalui His-Trap column meningkatkan produk sebanyak 1.8 kali ganda dengan

aktiviti enzim 303 U/mg. Aktiviti enzim didapati paling tinggi pada suhu 55 ˚C dan pH 4.5. Fitase rekombinan tersebut didapati stabil pada pH 3.5 hingga 5.5 dengan pengekalan aktiviti relatif melebihi 78%. Kuprum, EDTA, ferum, dan zink masing-masing didapati megurangkan enzim aktiviti sebanyak 5.9%, 15.8%, 76.6% dan 78.5% pada kepekatan 1.0 mM. Namun begitu, kalsium, kalium, dan magnesium didapati berkesan untuk meningkatkan aktiviti fitase sebanyak 148.8%, 118.3%, dan 103.4%. Enzim aktiviti fitase rekombinan didapati turun apabila 0.05 hingga 10.0 mM fosfat ditambah dan hanya 78.8% dan 23.7% aktiviti relatif kekal apabila fitase rekombinan dihadam dengan tripsin dan pepsin selama 1 jam pada suhu 37 °C. Permerhatian yang didapati dalam kajian ini menunjukkan enzim fitase daripada *Mitsuokella jalaludinii* dapat diekspres oleh *Escherichia coli* Rosetts-gami bawah induksi IPTG dan mempunyai profil suhu sederhana, toleransi baik terhadap pH dan pepsin. Ini menunjukkan dengan kajian lanjut, enzim fitase rekombinan mempunyai potensi sebagai calon enzim makanan haiwan.

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I certify that a Thesis Examination Committee has met on 26 December 2013 to conduct the final examination of Tan Wan Qin on her thesis entitled "Expression of Phytase From *Mitsuokella jalaludinii* In Heterologous Hosts and Characterization of Expressed Protein from *Escherichia coli*" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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This thesis was submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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Date:

DECLARATION

Declaration by graduate student

I hereby confirm that:

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APS	ammonium persulphate
ANNOVA	analysis of variance
BCIP	5-bromo-4-chloroindolyl phosphate
BSA	bovine serum albumin
bp	base pair
dH_2O	deionised distilled water
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
IPTG	isopropyl β-D-thiogalactopyranoside
LB	Luria Bertani
mM	milimolar
MWCO	molecular weight cut-off
NBT	nitro blue tetrazolium
OD_{600}	Optical density at 600 nm wavelength
OD_{820}	Optical density at 820 nm wavelength
PCR	Polymerase Chain Reaction
RE	restriction enzyme
RG	Rosetta-gami
r-phytase	recombinant phytase
SDS	sodium dodecyl sulfate
SOC	Super Optimal broth with Catabolite repression
Tris	tris[hydroxymethyl]aminomethane
U/µl	enzyme unit per microlitre
v/v	volume to volume
w/v	weight to volume

CHAPTER 1

INTRODUCTION

Phytate (*myo*-inositol hexakisphosphate) is a six-carbon ring compound bound with six phosphate groups (Lei and Stahl, 2001). It is widely distributed in plant legumes, cereals, and oilseeds and is the natural phosphorus reservoir of plants. When the plant materials are used as feed components for monogastric animals like broiler chickens, fish, and swine which lack of the ability to digest phytate, it forms complexes with the dietary important ions such as iron, magnesium, zinc, and calcium. This often results in reduced bioavailability of these ions in the animals. Besides, the interaction of phytate with intestinal digestive enzymes such as α-amylase, trypsin, and pepsin was also reported to affect enzymatic activity and digestibility of the feed (Greiner and Konietzny, 2006). Hence, efficient digestion of phytate is required in monogastric animals to avoid these negative effects.

Phytase is the enzyme which catalyzes the hydrolysis of phytate into its lower inositol phosphates and orthophosphate (Lei and Stahl, 2001). It is used as a feed enzyme supplement in plant-based animal feed to provide natural phytate-phosphorus through degradation of phytate in the feed. Supplementation of phytase in ruminant production is not as crucial as in monogastric animals as some of the ruminal microorganisms are able to produce phytase. Monogastric animals which are unable to degrade phytate require either the supplementation of inorganic phosphorus or phytase enzyme in their feed to ensure sufficient phosphorus for their growth. Incorporation of inorganic phosphorus is less favorable as unabsorbed phosphorus can be excreted through animals' manure that often leads to eutrophication and environment pollution (Lei and Stahl, 2001).

Phytase can be found in plants, microorganisms, and certain animal tissues. In a previous study in our laboratory, a phytase-producing bacterium, *Mitsuokella jalaludinii* was isolated from the rumen of local cattle (Lan *et al.*, 2002a). *Mitsuokella jalaludinii* is a novel rod-shaped, gram-negative, strict anaerobic bacterium. It produces a membrane-bound phytase enzyme with high enzyme activity between 55 °C to 60 °C and pH ranges from 4.0 to 5.0 (Lan *et al*., 2002a; 2002b). *In vivo* study showed that supplementation of active *M. jalaludinii* culture (AMJC) in the diet of broiler chickens increased weight gain, apparent metabolizable energy (AME), digestibility of dry matter (DM) and crude protein (CP). The bioavailability of minerals such as P, Ca, and Cu were also found to increase with the supplementation of active *M. jalaludinii* (Lan *et al*., 2002b). Besides, the performance of freeze-dried active *M. jalaludinii* culture (FD-AMJC) was also compared with the commercial Natuphos® phytase (phytase-N), in which the overall

LIST OF ABBREVIATIONS

APS	ammonium persulphate
ANNOVA	analysis of variance
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BSA	bovine serum albumin
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dH_2O	deionised distilled water
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
IPTG	isopropyl β-D-thiogalactopyranoside
LB	Luria Bertani
mM	milimolar
MWCO	molecular weight cut-off
NBT	nitro blue tetrazolium
OD_{600}	Optical density at 600 nm wavelength
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PCR	Polymerase Chain Reaction
RE	restriction enzyme
RG	Rosetta-gami
r-phytase	recombinant phytase
SDS	sodium dodecyl sulfate
SOC	Super Optimal broth with Catabolite repression
Tris	tris[hydroxymethyl]aminomethane
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effect of feed conversion rate, P and Ca retention, dry matter digestibility and phosphorus excretion were found to be better in the FD-AMJC than phytase-N. The superior characteristic of FD-AMJC in the improvement of the apparent metabolizable energy value of the diet, Cu and Zn retention and crude protein digestibility which was not observed in phytase-N supplementation has made FD-AMJC a potential candidate as commercial feed enzyme (Lan *et al*., 2012). However, as *M. jalaludinii* is a strict anaerobic bacterium, the production of phytase and maintenance of this bacterium would be tedious and non-economical. Thus, a better approach would be the production of this enzyme *via* recombinant expression system.

DNA recombinant technology allows various genes from different sources to be cloned and expressed in well-established commercial expression vectors and hosts for large scale production purposes. Yeast and *E. coli* have been extensively used and recognised for the expression of many genes including phytase gene. Rao *et al*. (2008) and Xiong *et al*. (2005) have sucessfully cloned, expressed, and characterized phytase from *Bacillus* and *Aspergillus* origin in *E. coli* and *P. pastoris*, respectively.

In the present study, the 1.05 kb phytase gene from *M. jalaludinii* which was previously isolated by Phang (2008) was subcloned and expressed in *P. pastoris* and *E. coli* expression systems. Expression from both hosts were analysed and properties of the recombinant phytase were studied. The specific objectives of this study were:

1) To subclone the phytase gene from *Mitsuokella jalaludinii* into yeast and *Escherichia coli* expression vectors.

2) To optimize the expression conditions of the recombinant phytase in different expression systems.

3) To study the properties of the recombinant phytase.

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