CLONING AND EXPRESSION OF *Phy2* GENE IN *Escherichia coli*

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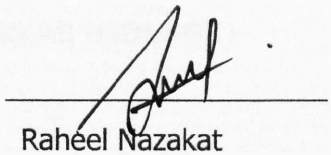

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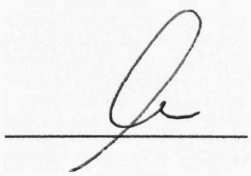

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ABSTRACT

One particular bacterium that demonstrates high phytase activity is *Mitsuokella jalaludinii*. However, the anaerobic nature of the bacterium prohibits its mass-production. Cloning and expression of the phytase gene into aerobes microorganism is essential in overcoming this problem. Thus, this study aims in isolating the *Phy2* gene of *Mitsuokella jalaludinii* followed by cloning and expression in *Escherichia coli*. The *Phy2* gene was isolated from the genomic DNA of *Mitsuokella jalaludinii* through polymerase chain reaction using PhyF and PhyR primers. PCR product of 1127 base pairs containing the probable promoter sites, *Phy2* coding region and putative terminator site was then inserted into pJET1.2/blunt cloning vector in the direction downstream of the T7 promoter. It was used to transform chemically-competent *Escherichia coli* strain DH5α. Growth of transformants was seen on LB agar containing 50 µg/ml ampicillin. The recombinant clone was then confirmed by *Bgl II* restriction digestion. Two fragments were seen; one representing the plasmid (2974 base pairs) while the other one represents the *Phy2* gene construct (1127 base pairs). The orientation of the insert was confirmed through PCR by using pJET1.2 forward sequencing primer and PhyR reverse primer. Screening of phytase activity among the recombinant clones was carried out qualitatively by growing the clones on LB agar plate supplemented with ampicillin and 2% sodium phytate. Clear zone was seen in the area where recombinant *Escherichia coli* DH5α carrying the *Phy2* construct grew. Meanwhile, no halo zone was seen for those clones carrying control PCR product as insert. This revealed that only those clones with *Phy2* gene construct exhibit phytase activity as the enzyme was expressed from the multi-copied vector. It can be concluded that the transcription of *Phy2* gene initializes from the gene's own promoter because *Escherichia coli* DH5α lacks the gene that encodes for T7 RNA polymerase. However, the identified candidate promoter sites were only tentative. Prior to its industrial application, further purification and characterization of the crude phytase will be required to determine its functionality and efficacy in reducing phytic acid content in animals' feed.

ABSTRAK

PENGKLONAN DAN EKSPRESI GEN Phy2 DALAM Escherichia coli

Salah satu bakteria yang menunjukkan aktiviti enzim fitase yang tinggi adalah Mitsuokella jalaludinii. Namun, sifat bakteria ini yang anaerobik menghalang ia daripada dihasilkan secara besar-besaran untuk tujuan pasaran. Bagi mengatasi masalah ini, pengklonan dan ekspresi gen bagi fitase di dalam mikroorganisma lain yang bersifat aerobik adalah penting. Oleh itu, tujuan kajian ini dilakukan adalah untuk mengasingkan gen Phy2 milik Mitsuokella jalaludinii dan diikuti dengan pengklonan dan ekspresi gen tersebut di dalam Escherichia coli. Gen Phy2 diasingkan daripada genomik DNA milik Mitsuokella jalaludinii melalui kaedah tindakbals berantai polymerase menggunakan primer PhyF dan PhyR. Produk dengan saiz 1127 pasangan bes yang mana terkandungnya tapak promoter, gen Phy2 dan jujukan tapak pemberhentian kemudiannya dimasukkan ke dalam agen pengklonan pJET1.2/b pada kedudukan selepas tapak promoter T7. Ia seterusnya ditransformasikan ke dalam Escherichia coli DH5α. Sel rekombinan daripada proses transformasi kelihatan tumbuh pada piring petri yang mengandungi media Luria Bertani dan 50 µg/ml ampicillin. Status sel rekombinan itu kemudiannya disahkan melalui tindakbalas pencernaan enzim BglII. Dua cebisan DNA kelihatan selepas pencernaan enzim di mana salah satu cebisan mewakili plasmid (2974 pasangan bes) manakala cebisan yang satu lagi mewakili gen Phy2 (1127 pasangan bes). Kedudukan gen Phy2 di dalam plasmid kemudiannya disahkan melalui tindakbalas berantai polymerase menggunakan primer 'pJET1.2 forward' dan PhyR. Saringan bagi aktiviti enzim fitase di kalangan sel rekombinan ditentukan secara kualitatif dengan menggunakan piring petri yang mengandungi media Luria Bertani, ampicillin dan 2% natrium fitate. Zon halo kelihatan pada kawasan tumbuhnya sel rekombinan Escherichia coli DH5α yang membawa gen Phy2. Manakala, tiada zon halo ditemui pada kawasan di mana tumbuhnya sel rekombinan Escherichia coli DH5α yang mengandungi plasmid tanpa gen Phy2. Hal ini menunjukkan bahawa hanya sel rekombinan yang membawa gen Phy2 akan menghasilkan enzim fitase di mana ekspresi bagi enzim tersebut disumbangkan oleh agen pengklonan yang dimasukkan. Memandangkan Escherichia coli DH5α tidak mempunyai gen yang menghasilkan T7 RNA polymerase, maka boleh disimpulkan bahawa transkripsi bagi gen Phy2 tersebut bermula dari tapak promoter milik Mitsuokella jalaludinii yang diklonkan bersama-sama gen tersebut. Tetapi, identiti bagi tapak promoter ini masih lagi tidak diketahui. Sebelum enzim rekombinan ini boleh dipasarkan, terdapat banyak lagi kajian yang perlu dilakukan untuk mengenalpasti ciri-ciri yang ada pada enzim tersebut termasuk keberkesanannya dalam mencernakan fitate dalam makanan haiwan.

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ABBREVIATIONS

AMJC	-	Active *Mitsuokella jalaludinii* culture
ATP	-	Adenine triphosphate
AP	-	Alkaline phytase
BLAST	-	Basic Local Alignment Searching Tool
$CaCl_2$	-	Calcium chloride
CAI	-	Codon adaptation index
ε	-	Epsilon
EDTA	-	Ethylenediaminetetraacetic acid
FD-AMJC	-	Freeze-dried active *Mitsuokella jalaludinii* culture
GI	-	Gastrointestinal
HAP	-	Histidine acid phosphatase
P_i	-	Inorganic phosphate
IUPAC-IUB	-	International Union of Pure and Applied Chemistry and International Union of Pure Biochemistry
LB	-	Luria-Bertani
NaOH	-	Sodium hydroxide
NCBI	-	National Centre for Biotechnology Institute
ORF	-	Open reading frame
ρ	-	Para
PYG	-	Peptone / yeast extract / glucose
FTU kg^{-1}	-	Phytase unit per kilogram
PCR	-	Polymerase chain reaction
RBS	-	Ribosomal binding site
rpm	-	Radius per minute
SDS	-	Sodium dodecyl sulfate
TAE	-	Tris-acetate-EDTA
TE	-	Tri-ethylenediaminetetraacetic acif
U kg^{-1}	-	Unit per kilogram
U/ml	-	Unit per milliliter

CHAPTER 1

INTRODUCTION

1.1 Research Background

The preeminent storage form of phosphorus in standard poultry and swine diets is phytic acid. It is also known as *myo*-inositol hexakisphosphate and comprises about 70% of the entire phosphorus found in cereal grains, oilseeds, legumes, corns and soya beans. The mentioned plants are the prime elements in the feeds of poultry and swine (Reddy *et al.*, 1982; Al-Asheh and Duvnjak, 1995; Lan *et al.*, 2002a). It has been reported previously that the total phosphorus within the animal feed is enough for the growth of poultry, provided that the phosphorus is fully utilized *in vivo* (Lan *et al.*, 2002a). However, due to inadequate gastrointestinal enzyme, most monogastric animals (such as pigs, poultry, fish, etc.) are unable to hydrolyze the phytic acid and therefore, incapable of liberating free phosphorus for absorptions (Common, 1989).

The ineptitude of monogastric animals to hydrolyze the phytic acid completely causes several problems. For instances, supplementation of inorganic phosphate (which is expensive) is needed to compensate with the inability of the monogastric animals to utilize phytic acid. The supplementation of inorganic phosphate in the animal feeds is crucial to meet the animals' dietary requirements. Furthermore, the unutilized phytate-phosphorus increases the concentration of phosphorus in the animals' excreta, and this will indirectly contribute to serious phosphorus pollution problems (Pen *et al.*, 1993; Volfova *et al.*, 1994). Cyanobacterial blooms, hypoxia and death of marine animals have been reported to occur due to the eutrophication of the rivers by excessive phosphorus (Mallin, 2000; Naqvi *et al.*, 2000). In addition, phytic acid has been well-known for its anti-nutritive activity. It is well document that phytic acid is capable of binding with protein to formed insoluble phytate-protein complexes. This complexing, which

occurs at a broad range of pH, resulted in lower protein digestibility (Carnovale *et al.*, 1988). Additionally, the bioavailability of certain nutrient minerals (such as zinc, calcium and iron) is lower due to the capacity of phytic acid to ionically chelate these elements (Erdman, 1979). It has been reported previously that phytic acid also has the capability of inhibiting the activity of both trypsin and pepsin (Singh and Kirkorian, 1982).

One of the approaches in dealing with these problems involves the mixing of phytase enzyme (*myo*-inositol hexakisphosphate phosphohydrolase) with the animals' feed. Even though degradation of phytic acid by chemical method has been introduced, it is not well accepted due to its higher cost as well as its capability of deteriorating the nutritional value of animal feeds (Pandey *et al.*, 2001). Therefore, phytase enzyme has remains as the method of choice in handling with these problems, as it can hydrolyzed phytic acid into more beneficial inorganic phosphate, inositol and inositol mono- and penta- phosphates (Pasamontes *et al.*, 1997). Phytase, first discovered by Suzuki *et al.* in 1907, is found in various sources, which include microorganisms such as fungi (Ullah, 1988; Segueilha *et al.*, 1993; Mitchell *et al.*, 1997) and bacteria (Shimizu, 1992; Yoon *et al.*, 1996; Greiner *et al.*, 1997). It is also found in plants (Gibson and Ullah, 1998; Hubel and Beck; 1996) and animals (Maga, 1982). Of all the sources mentioned, microbial phytase had attracted major interest from researchers all over the world. Numerous microorganisms have been identified to exhibit phytase activity. The incorporation of microbial phytase into animals' feed has shown to enhance inorganic phosphate availability in broiler chickens (Ravindran *et al.*, 2001; Lan *et al.*, 2002a). Moreover, the inclusion of phytase improved the bioavailability of nutritionally important minerals such as calcium (Sebastian *et al.*, 1996), zinc (Yi *et al.*, 1996) and copper (Sebastian *et al.*, 1996). It is also shown to reduce the presence of phosphate in broiler chickens' manure (Lan *et al.*, 2002a).

In the early days, the search of essential phytase is concentrated on finding microorganisms that generate high phytase activity. Initially, phytase of fungal origin has been exploited as an additive in the animals' feed. The discovery of *phyA* gene in *Aspergillus (ficuum) niger* NRRL 3135 (Mullaney *et al.*, 1991) and

its consecutive cloning and expression (Van Hartingsveldt *et al.*, 1993) has led to the first globally commercialized phytase, Natuphos® to meet with the market demands. However, successive exploration of bacterial phytase, which is high-caliber as compared to fungal phytase, has led to the expansion of a new generation of phytase (Lei *et al.*, 2013). Lan *et al.* (2002b) has successfully isolated *Mitsuokella jalaludinii*, a high producing phytase bacterium from the rumen of cattle. Subsequent purification and characterization of the isolated phytase revealed that this enzyme had favorable activity of increasing the bioavailability of inorganic phosphorus and other essential trace elements in non-ruminants feed (Lan *et al.*, 2011). Nevertheless, the *Mitsuokella jalaludinii* is an anaerobic bacterium and therefore, need rigid growth conditions for it to be mass produced. One of the possible solutions for this problem is to clone and express the phytase gene in other microorganisms, which are aerobe or facultative anaerobe in nature without compromising its existing phytase activity. The whole genome of *Mitsuokella jalaludinii* has been sequenced and the gene responsible for the expression of phytase, *Phy2*, has been identified through gene annotation of the genome. As such, the need for cloning and further expression of the *Mitsuokella jalaludinii* phytase is essential to identify its potential use. Through this study, reproducible and effective protocols for cloning and expression of *Mitsuokella jalaludinii Phy2* gene in *Escherichia coli* will be developed. The phytase produced by the recombinant *Escherichia coli* could be potentially applied as a feed additive to improve the nutritional quality of farm animals' diet.

1.2 Objectives

The objectives of this research are:

1. To isolate the phytase gene (*Phy2*) from the genome of *Mitsuokella jalaludinii*.
2. To clone and express the *Phy2* gene in *Escherichia coli* DH5α.
3. To determine the presence of phytase activity in recombinant *Escherichia coli* DH5α.

1.3 Research Approach

The early stage of this research involves the isolation of *Phy2* gene by polymerase chain reaction (PCR). Primers, which are specific to the upstream and downstream regions of the gene, are constructed. This is followed by insertion of the isolated genes into cloning vector and subsequent transformation of competent *Escherichia coli* DH5α. The phytase activity of recombinant *Escherichia coli* DH5α is then determined by using the phytase assay agar plates.

CHAPTER 2

LITERATURE REVIEW

2.1 Phytic Acid - The Reservoir of Phosphorus

The significance of phosphorus to living organisms is without doubt quite essential since it participates in critical enzymatic reaction, as well as cellular transport mechanisms. In fact, phosphorus involves in the formation of DNA backbone by acting as molecular glue that holds the DNA together. The native form of phosphorus is abundantly found in water and soil. However, the uptake of phosphorus by humans and animals is mainly occurred through the consumption of plant-based feeds since the plant can absorb phosphorus from nature. Numerous plant tissues such as cereals, oilseeds, legumes, corns and soya beans stored phosphorus in the form of phytic acid (Reddy *et al.*, 1982; Al-Asheh and Duvnjak, 1995; Lan *et al.*, 2002a). Chemically known as *myo*-inositol (1, 2, 3, 4, 5, 6) hexakisphosphoric acid, phytic acid is the hexaphosphoric ester of the hexahydric cyclic alcohol meso-inositol (Kumar *et al.*, 2010). It exists naturally and develops during the ripening period of seeds and cereal grains (Loewus, 2002). The molecular formula of phytic acid is $C_6H_{18}O_{24}P_6$, and its molecular mass is 660.04 g mol^{-1} (Kumar *et al.*, 2010). The formula structure of phytic acid is shown in Figure 2.1.

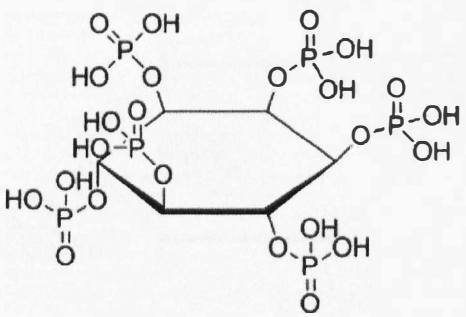

Figure 2.1: Molecular structure of phytic acid. (Source: Kumar *et al.*, 2010)

The salt form of phytic acid is known as phytate or *myo*-inositol (1, 2, 3, 4, 5, 6) hexakisphosphate. It exists predominantly in the form of IP6. Apart from IP6, a small portion of phytate exists in the form of *myo*-inositol penta- (IP5), tetra- (IP4) and triphosphate (IP3) in nature. However, the isomeric state of these phytate is unknown (Konietzny *et al.*, 2006). It exists in nature by complexing with mono- or divalent cations, such as K^+, Mg^{2+}, Ca^{2+}, etc (Kumar *et al.*, 2010). Since it exists as polydentate ligand (chelator agent), phytate able to bind to more than one coordination site of the metal atoms. In fact, phosphates on the inositol ring capable of binding up to 12 protons in total (Bohn *et al.*, 2008). An example of phytate structure is shown in Figure 2.2.

Phosphorus within the phytate is not readily available to monogastric animals. It is due to the inability of the mammals to hydrolyze the phytate within their gastrointestinal tract (Holm *et al.*, 2002). It has been reported previously that broiler chickens are incapable of completely digesting phytate within the standard poultry diets, containing about 10 g kg^{-1} Ca. Due to the absence of specific endogenous enzyme (phytase), the broiler chickens unable to fully hydrolyze the insoluble Ca-phytate complexes that develop *de novo* within its GI tract (Lan *et al.*, 2011). Selle and Ravindran (2008) have reported that only limited phosphorus is absorbed by swine when given phytate diet. This is due to the poor dephosphorylation process in its small intestine. As a result, large fractions of the undigested phytate passed through the digestive tract, with the phosphorus unutilized, and excreted in the manure as waste.

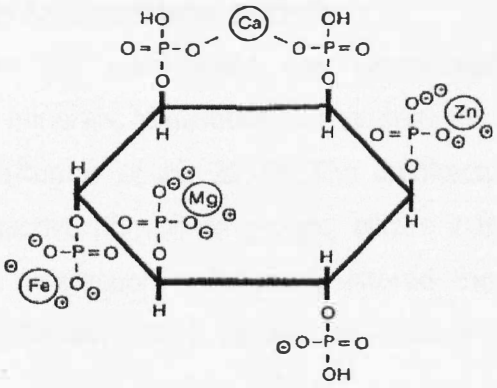

Figure 2.2: Molecular structure of phytate. (Source: Kumar *et al.*, 2010)

2.2 Phytate and Its Eutrophication Effect

The implementation of plant-based feeds such as the soya bean meal in the swine and poultry industries inflicted global ecological dilemma. The shifting of animal-based rations to the plant-based feeds occurred because of the lower cost of the plant-based diets (Lei *et al.*, 2013). Since the phosphorus is attached to phytate within this diet, the ability of simple-stomached animals (such as swine and poultry) to utilize this element is doubtful. As a result, most of the undigested phosphorus is excreted into the animal's manure. The excreted phosphorus will eventually be absorbed into the water bodies (e.g.: lakes, swamps, rivers, etc.) and this phenomenon results in eutrophication. It has been reported previously that the pollution of water bodies by phosphorus causes Cyanobacteria blooms, hypoxia as well as death of marine animals (Mallin, 2000; Naqvi *et al.*, 2000).

Increase in the death of finfish and shellfish has been reported, and it is believed to be contributed by the presence of dinoflagellates, *Pfiesteria picicida*. There is strong evidence demonstrating the effect of high concentration of inorganic phosphorus on the lethal activity of these dinoflagellates (Burkholder and Glasgow, 1998). In fact, high density of fish kills on the east coast of United States in the early 1990s by *Pfiesteria picicida* has evoked anxiety among the public. In order to comprehend with this effect, steps have been taken to decrease the phosphorus concentration in animal excreta (Burkholder and Glasgow, 1998). This displays how serious the implication of phytate-phosphorus towards the environment.

2.3 Phytate and Its Anti-nutritive Activity

Presence of phytate in the plant-based diet contributed negatively to the availability of essential minerals, digestibility of protein as well as utilization of carbohydrate and lipid (Kumar *et al.*, 2010). The architecture of phytate itself, which consists of six reactive phosphate groups, allows it to interact with these nutrient elements. This interaction unfavorably altered the utilization of these compounds by animals (Raboy, 2001), as well as humans (Kumar *et al.*, 2010) leading to malnutrition.

2.3.1 Interaction of Phytate with Essential Minerals

The consumption of phytate by both animals and humans exhibit a detrimental impact on the absorption of certain essential minerals. The structure of phytate allowed it to interact and subsequently, chelates metal cations to form insoluble complexes. This insoluble metal cation-phytate complex is not readily absorbed by the gastrointestinal tract of simple-stomached animals and therefore, lessens the bioavailability of essential minerals (Kumar *et al.*, 2010). The number of phosphate groups and its distribution on the *myo*-inositol ring allow phytate to attach to divalent or trivalent metal cations (Konietzny *et al.*, 2006). A number of factors have been reported to influence the stability and solubility of cation-phytate complexes such as the molar ratio of phytate to metal cation, pH value, types of individual cation and presence of other essential trace elements (Oberleas, 1983). pH value acts as the most substantial factor affecting the solubility (Cheryan *et al.*, 1983). The solubility of the complexes is much higher at low pH values (Torre *et al.*, 1991).

Some of the essential minerals that are inversely related to phytate includes zinc, iron, calcium, magnesium, manganese and copper (Lopez *et al.*, 2002; Konietzny and Greiner, 2003). However, among all the nutrient elements mentioned, zinc (Zn^{2+}) is the most adversely affected (Lopez *et al.*, 2002; Lonnerdal, 2002). Phytate reduces the availability of Zn^{2+} in gastrointestinal tract by producing insoluble complex upon binding with it. It is also reported that phytate unfavorably disturb the homeostasis of Zn^{2+} (Oberleas, 1983). Furthermore, calcium (Ca^{2+}) increased the precipitation of phytate when presence simultaneously with Zn^{2+}. This happens due to high affinity binding of calcium-bound phytate and Zn^{2+} (Hardy, 1998).

Brune *et al.* (1992) has revealed that the absorption of non-heme iron (Fe^{2+}/Fe^{3+}) is strongly inhibited in the presence of phytate. The bioavailability of calcium is also reduced in the presence of phytate. However, the malabsorption of calcium is less obvious as compared to Zn^{2+} and Fe^{2+}/Fe^{3+} (Lopez *et al.*, 2002). This is due to the presence of normal flora within the colon which assists in dephosphorylating the calcium-bound phosphate, thus releasing the Ca^{2+} for absorption (Sandstrom *et al.*, 1990). Even though the effect is less pronounced,