PRODUCTION OF RECOMBINANT PHYTASE IN *PSEUDOMONAS PUTIDA* CARRYING *ESCHERICHIA COLI APPA*

JETNAPA TECHAWIPARAT

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Thesis

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PRODUCTION OF RECOMBINANT PHYTASE IN *PSEUDOMONAS PUTIDA* CARRYING THE *ESCHERICHIA COLI GENE (APPA)*

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Abstract

Phytase is important for animal feed supplementation. Previous work showed that *Escherichia coli* K12 produced phytase but could not secrete it. Later, the gene coding for phytase was cloned and transformed into *Pseudomonas putida* which could secrete the enzyme and utilize simple materials as a nutrient source. Thus, it was necessary to find out the general properties of phytase and its digestion efficiency as well as the best developing medium.

Pseudomonas putida carrying *Escherichia coli* gene (*appA*) [P(EappA)] was studied for extracellular phytase production. General properties of P(EappA) phytase were determined by using culture supernatant produced in Luria Bertani medium. Crude phytase was found to be most stable at pH 4 and 8 when stored for two hours at 4^{o}C, and at pH 4 and 10 when stored at 37^{o}C. It was most stable at 45^{o}C. The optimal condition for its activity was obtained at pH 4, 55 ^{o}C. P(EappA) phytase was sensitive to trypsin, taurocholic acid, deoxycholic acid and propionic acid. Nonetheless, its activity was enhanced when stored with lactic acid. As to the effect of ions, P(EappA) phytase was stable when stored in Na^{+}, K^{+}, Mn^{2+} and Co^{+} but was not stable when stored in Ca^{2+}, NH_4^{+}, Cu^{2+}, Fe^{2+} and Zn^{2+}. In addition, the enzyme activity was enhanced when stored in Mg^{2+}, although it was insensitive to EDTA. In developing the medium, P(EappA) phytase was found to be best produced in medium composed of Phytase Screening Medium with phytate omitted and supplemented with 10% soybean meal, 0.1% yeast extract and 0.1% citric acid. In preparation of this medium, glucose was added prior to sterilization. Cultivation of P(EappA) in 500-ml of this medium showed that P(EappA) growth entered log phase at 12 hours and reached maximum growth at the 18 hours with a growth rate at 0.1 OD_{600}/hr. Phytase production was concomitant with growth with a rate at 6.32 U/hr. After 48 hours, the phytase activity obtained was 177.62 U/ml. The level of reducing sugar decreased slowly during the first 30 hours and then dropped rapidly when growth reached the stationary phase. The level of pH did not show vigorous change throughout the cultivation. P(EappA) also contained other enzyme activities: amylase, cellulase, xylanase and endoglucanase. P(EappA) phytase was concentrated and stored in three forms: solution, air-dried and wet precipitates, which retained ≥80% of activity after a 28-day storage. When each form of enzyme was mixed in chicken feed, the air-dried form was the most stable. As to digestion efficiency, the enzyme helped to increase the phosphate and protein contents in chicken feed, wheat bran and soybean meal but not in rice bran, while the level of reducing sugar contents in all materials tested did not significantly increase. Soymilk digestion under the condition used in this study was unsuccessful.

KEY WORDS: *PSEUDOMONAS PUTIDA*/ PHYTASE/ *ESCHERICHIA COLI APPA*
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การผลิตเอนไซม์ไฟเทสจาก *Pseudomonas putida* ที่มียีน *appA* จาก *Escherichia coli*
(PRODUCTION OF RECOMBINANT PHYTASE IN *Pseudomonas putida* CARRYING *Escherichia coli appA*)

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บทคัดย่อ

Pseudomonas putida ที่มียีน *appA* จาก *Escherichia coli* [P(EappA)] ผลิตเอนไซม์ไฟเทสแบบ extracellular นำมาเลี้ยงในอาหารเลี้ยงเชื้อ LB เพื่อศึกษาคุณสมบัติเอนไซม์ ไฟเทสของP(EappA) มีความคงทนต่อความเป็นกรดและด่างที่อุณหภูมิ 4^{o}C ที่ pH 4 และ 8และเปลี่ยนระดับความคงทนมาที่ pH 4 และ 10 ที่อุณหภูมิ 37^{o}C และมีความคงทนสูงสุดที่อุณหภูมิ 45^{o}C เอนไซม์มีประสิทธิภาพการทำงานสูงสุดที่ pH 4.5 และอุณหภูมิ 55^{o}C ไฟเทสลดความคงทนในสภาวะที่มี trypsin, taurocholic acid, deoxycholic acid และ propionic acid แต่คงทนในสภาวะที่มี lactic acid ไฟเทสมีความคงทนในสภาวะที่มี Na^{+}, K^{+}, Mn^{2+} และ Co^{+}แต่เสียความคงทนเมื่อมี Ca^{2+}, NH_4^{+}, Cu^{2+}, Fe^{2+} และ Zn^{2+} ส่วน Mg^{2+}เพิ่มความสามารถการทำงานของเอนไซม์และ EDTA ไม่มีผลกระทบต่อไฟเทส เมื่อพัฒนาสูตรอาหารพบว่าอาหารชนิด SMYC ประกอบด้วย ส่วนประกอบของ PSM (Kerovuo *et al*., 1998)ที่ไม่เติม phytate และเติม 10% กากถั่วเหลือง, 0.1% yeast extract และ 0.1% citric acidและเติมน้ำตาลกลูโคสก่อนฆ่าเชื้อให้ความสามารถการทำงานของไฟเทสสูงสุด นำ SMYC 500 มิลลิลิตรมาเลี้ยง P(EappA)พบว่าใน 12 ชั่วโมงอัตราการเจริญเข้าสู่ log phaseและสู่จุดสูงสุดในชั่วโมงที่ 18โดยมีอัตราการเจริญเท่ากับ 0.1 OD_{600}/hr และความสามารการทำงานที่ 6.32 U/ml เมื่อครบ 48 ชั่วโมงความสามารถการทำงานของเอนไซม์เท่ากับ 177.62 U/ml ปริมาณน้ำตาลรีดิวซิ่งลดลงอย่างช้าใน 30 ชั่วโมงแรกและลดอย่างรวดเร็วเมื่อเข้าสู่ stationary phase ส่วนค่า pH ไม่มีการเปลี่ยนแปลง ใน P(EappA)พบเอนไซม์ชนิดอื่นเช่น amylase, cellulase, xylanase และ endoglucanase ทำไฟเทสให้เข้มข้นและเก็บในรูปของสารละลาย, ตะกอนแห้ง และตะกอนเปียกพบว่าทั้งสามชนิดมีความสามารถการทำงานคงเหลือมากกว่า 80%หลังจากเก็บ 28 วันและเอนไซม์ชนิดตะกอนแห้งมีความคงทนสูงสุดเมื่อผสมกับอาหารสัตว์ ประสิทธิภาพการย่อยโดยไฟเทสเพิ่มปริมาณฟอสเฟตและโปรตีนในอาหารเลี้ยงไก่, รำข้าวสาลี และกากถั่วเหลืองแต่ไม่พบในรำข้าวเจ้า ส่วนปริมาณน้ำตาลรีดิวซิ่งไม่เพิ่มขึ้นอย่างมีนัยสำคัญ การย่อยน้ำนมถั่วเหลืองของการศึกษานี้ไม่ประสบผลสำเร็จ

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^{o}C	degree celcius
EDTA	Ethylene diamine tetraacetic acid
et al.	Et. Alii (latin), and others
etc	Et cetera (latin), other things
g	gram
hr	hour
i.e.	id est
l	liter
M	molar
µg	microgram
µl	microlitre
µmole	micromole
mg	milligram
ml	milliliter
mM	millimolar
min	minute
nm	nanometer
N	normality
OD	optical density
rpm	revolution per min
sp.	species
Tween-20	polyxyethylene sorbitane monooleate
Tween-80	polyxyethylene sorbitane monooleate
U	unit
v/v	volume by volume
w/v	weight by volume

CHAPTER I
INTRODUCTION

Cereals, legumes and oilseeds are composed in feed stuffs. These materials contain phytic acid as the major storage form of phosphorus (Dvorakova, 1998). Phytic acid, which is chemically myo-inositol hexakis-dihydrogen phosphate (Vohra and Satyanarayana, 2003), typically represents about 75% of seed total phosphorus (Cosgrove, 1980). The salt form of phytic acid is called phytate, and this form is representing 50 to 80% of phosphorus in plant origin food (Harland and Morris, 1995). Phytic acid plays many important roles in the plants; e.g. energy store, competition for ATP, complexation of multivalent cations and regulation of an inorganic phosphate level (Dvorakova, 1998). In contrary, it is considered to be an antinutritional factor for human and monogastric animals. Because of forming complexes with some multivalent ions, thus, these complexes interfere the absorption of important trace elements. Moreover, phytic acid binds to protein and makes them more resistant to proteolytic digestion (Dvorakova, 1998). In addition, phytate form that is not utilized would be excreted leading to high phosphorus pollution to the environment (Eeckhout and De Paepe, 1994).

In 1907, Suzuki *et al*. discovered phytase, myo-inositol-hexakisphosphate phosphohydrolase, in the course of rice bran hydrolyzing studies (Liu *et al*.,1998). This enzyme was found in rice bran which catalyzed the hydrolysis of phytic acid to inositol and inorganic phosphorus (Nagai and Funahashi, 1962). Phytase was also found in other sources; i.e. animal tissues and microorganisms. Although, phytic acid in agricultural products could be decreased by using chemical methods, the cost is generally expensive, and chemicals affect the product quality (Pandey *et al*., 2001). Thus, phytase was chosen for phytic acid treatment. *Aspergillus niger* phytase was found to efficiently degrade phytic acid presented in the broiler's feed (Qian *et al*., 1996). This fungal phytase was then studied for production. Besides, the genera *Mucor*, *Penicillium* and *Rhizopus* have also been tested for phytase production

(Gargova *et al*., 1997; Howson and Davis, 1983). For yeast, it was only few studies that have been reported on the phytase; i.e. *Saccharomyces cerevisiae* and *Schwanniomyces castellii* (Lambrechts *et al*., 1993; Nayini and Markakis, 1984; Segueilha *et al*., 1992). Nevertheless, the bacterial sources are also a focus of interest for phytase production. These are from *Bacillus subtilis* (Powar and Jaganathan, 1982; Shimizu, 1992), *Escherichia coli* (Griner et al., 1993), *Pseudomonas sp*. (Irving and Cosgrove, 1971) and *Klebsiella sp*. (Shah and Parekh, 1990). Moreover, genetic engineering has been introduced as a method to improve the phytase gene expression which is beneficial for the large-scale expression of phytase (Kim *et al*., 1999). For application, the main important role of phytase is used for elimination of phytic acid contained in feed and food. In addition, the preparation of myo-inositol phosphate as a tool for biochemical analytical application is also performed.

The aim of this study is to produce the recombinant phytase in *P. putida* that carried *E. coli appA* [P(EappA)] for studying its characteristics prior to using it as feed supplement.

Objectives:

1. To study the general properties of P(EappA) phytase.
2. To develop a low-cost medium for the bacterial growth and phytase production.
3. To formulate the enzyme.
4. To determine the digestion efficiency of the formulated P(EappA) phytase.

CHAPETER II
LITERATURE REVIEW

Phytic acid

Phytic acid or *myo*-inositol 1,2,3,4,5,6-hexakisphosphate (IP6) is the major storage form of phosphorus in plants, particularly, cereal grains and legumes (Reddy *et al.*, 1982). It typically represents approximately 75%-80% of the total phosphorus found in nature (Oh *et al*., 2001). The molecular formula of phytic acid is $C_6H_{18}O_{24}P_6$, and its molecular weight is 660.0 (Sigma-Aldrich, Co.) (Figure 1). The salt form of phytic acid is phytate, and its calcium and magnesium salts are phytin.

Phytate plays a role as primary source of inositol and phosphorus in plant seeds (Vohra ans Satyanarayana, 2003). The total phosphorus (P), phytate-P and phytic acid contents of common poultry feedstuffs are presented in Table 1. Phytate stored in dormant seed is used in the energy storage and synthesis of ATP during germination (Dvorakova, 1998; Eeckhout and De Paepe, 1994). For inositol phosphate, it is an important constituent in phospholipid presents in animal and plant tissues (Cason and Anderson, 1938). It plays a crucial role in signaling processes (Michell, 1975; Berridge and Irvine, 1984; Samanta *et al*., 1993; Dasgupta *et al*., 1996) especially transportation of substances and secondary messengers involved in signal transduction in plant cells (Eeckhout and De Paepe, 1994).

However, phytate is an antinutritional factor for monogastric animals; i.e. human, pigs and chickens. Phytate chelates cations and binds minerals; i.e. Ca^{2+} (Heaney *et al*., 1991), Zn^{2+} (Sandstrom and Sandberg, 1992; Lonnerdal *et al*., 1988) and Fe^{2+} (Hallberg, 1989). It makes these materials to unavailable for absorption in the gastrointestinal tract. The intake of large amounts of food rich in phytate may cause several deficiency symptoms (Torre et al., 1991). Moreover, phytate reduces digestibility of protein, starch and lipid. It complexes with proteins to make them less soluble, and then, protein resists to proteolysis (Dvorakova, 1998). Phytate also inhibits digestive enzymes; i.e. amylase, pepsin and trypsin (Dvorakova, 1998). Furthermore, the unadsorbed phosphorus is released to the environment through

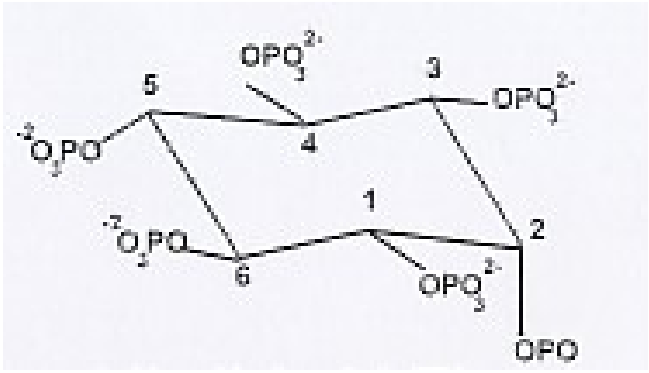

Figure 1 Molecular structure of phytic acid

Table 1 Total P and phytate P of common poultry feedstuffs (Tyagi *et al*., 1998)

Ingredients	Total P (%)	Phytate P (%)	% of total P
Cereals/millets			
Maize	0.39	0.25	64
Rice	0.15	0.09	60
Wheat	0.44	0.27	61
Sorghum	0.30	0.22	73
Barley	0.33	0.20	61
Bajra	0.31	0.23	74
Oilseeds meals			
Groundnut meal	0.60	0.46	77
Soybean meal	0.88	0.56	64
Cotton seed meal	0.93	0.786	82
Sunflower meal	0.90	0.45	51

animal faecal excretion. It enhances the accumulation of phosphorus in the environment and causes high cost for phosphorus supplementation in animal feed.

Phytase

Phytase (*myo*-inositol hexakisphosphate phosphohydrolase) belongs to the group of phosphoric monoester hydrolases, and it catalyzes the hydrolysis of phytic acid or phytate to inorganic monophosphate and lower phosphoric esters of *myo*-inositol or in some cases to free *myo*-inositol (Dvorakova, 1998). The research on phytase has expanded considerably since it was discovered (Suzuki et al., 1907) during the study on rice bran hydrolysis.

There are two phytases as classified by Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBNB) in consultation with the IUPAC-IUBNB Joint commission on biochemical Nomenclature (JCBN):

1. EC 3.1.3.8
   Recommended name: 3-phytase
   Systematic name: myo-inositol-hexakisphosphate 3-phosphohydrolase
   Other name(s): phytase, phytate 3-phosphatase
2. EC 3.1.3.26
   Recommended name: 6-phytase
   Systematic name: myo-inositol-hexakisphosphate 6-phosphohydrolase
   Other name(s): phytase, phytate 6-phosphatase

Two phytases are different at the attacking position in the phytate molecule. The 3-phytase (EC 3.1.3.8) attacks phytate at the 3-position first while the 6-phytase (EC 3.1.3.26) does so at the 6-position first. Thus, the first step reaction products from hydrolysis by the two types of phytase were different as shown below.

3-phytase reaction :

Myo-inositol hexakisphosphate + $H_2O \rightarrow$ D-myo-inositol 1,2,4,5,6-pentakisphosphate + orthophosphate.

6-phytase reaction:

Myo-inositol hexakisphosphate + $H_2O \rightarrow$ D-myo-inositol 1,2,3,4,5-pentakisphosphate + orthophosphate.

3-phytase is the typical type found in microorganisms while 6-phytase is so for plants (Johnson and Tate, 1969; Cosgrove, 1969, 1970) (Figure 2).

In theory, the inositol pentakisphosphate can rebind to the enzyme, releasing a further phosphate group and the inositol tetrakisphosphate (Vohra and Satyanarayana, 2003). Measurement of phosphate resulted from phytase reaction can be done by using the combined molybdate and vanadate reagents or so-called molybdovanadate reagent (Engelen *et al*., 1994). With the addition of this reagent, the enzymatic reaction was terminated and the color developed. The final result can be detected colorimetrically.

Source of phytase

Phytase can be isolated from a number of sources including plants, animals and microorganisms (Pandey *et al*., 2001). In plants, phytase can be found in cereals, legumes, seeds and pollens. In animals, phytase exists in the erythrocytes and plasma of various species of vertebrates and in the mammalian small intestine (Cooper and Gowing, 1983; Martin and Luque, 1985; Zyta, 1992). Several microorganisms including bacteria, yeast and fungi also have phytase-producing abilities. For recent research, it has been shown that microbial sources are more promising sources for the commercial production of phytase.

Plant sources

Phytase has been reported in rice (*Oryza sativa*), wheat (*Triticum aestivum*), maize (*Zea mays*), soybeans, corn seeds, lettuces, dwarf beans, mung beans, fab bean, rye (*Secale cereale*) and other legumes or oil seeds (Chang, 1967; Eskin and Wiebe, 1983; Gibson and Ullah, 1990; Laboure *et al*., 1993). In germinating seeds or pollens, the phytase seems responsible for phytin degradation (Greene *et al*., 1975). Suzuki *et al*. (1907) were the first investigators to make a preparation of phytase. They detected activity in rice and wheat bran and isolated inositol as a product of the

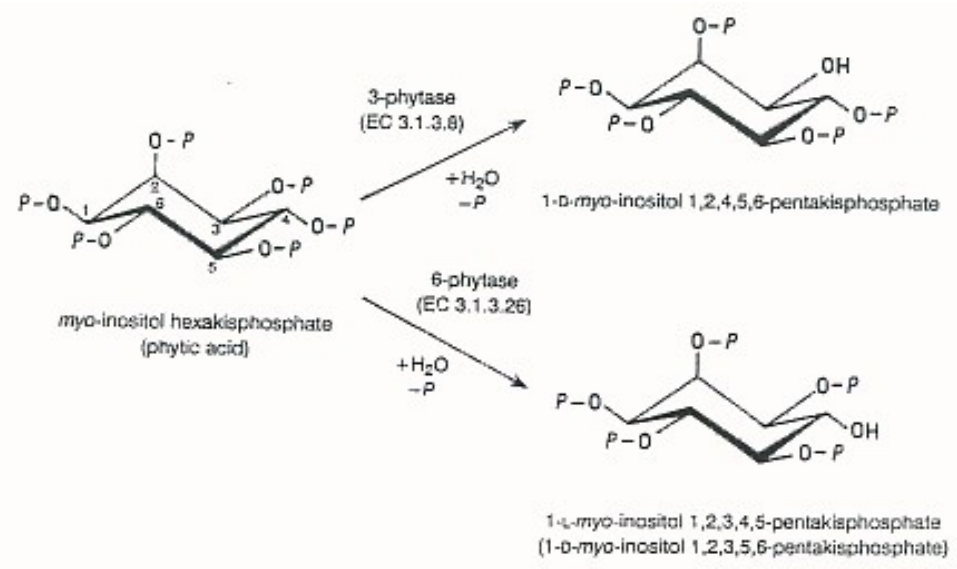

Figure 2 The general enzymatic reaction of 3- and 6-phytase.

reaction. A number of plant seed phytase has been purified. Soybean phytase was purified by ammonium sulfate precipitation and three column chromatographic steps, and it showed the optimal activity at 55^{o}C and pH 4.5-4.8 and had 60 kDa of molecular weight (Gibson and Ullah, 1990; Sutardi and Buckle, 1986). Laboure *et al*. (1993) purified and characterized phytase from germinating maize seedlings, and maize phytase presented the optimum at pH 4.8 and 55^{o}C with 60 kDa of molecular weight. Furthermore, cDNA coding for this maize phytase has been cloned (Maugenest *et al*., 1997). This would allow the isolation of corresponding genes and the study of their regulation during germination to be done. Konietzny *et al*. (1995) identified the phytase from germinating spelt. They found that it behaved like a monomeric protein with a molecular mass of about 68 kDa and showed a broad substrate specificity. Optimal degradation of phytate with spelt phytase was around pH 6.0 at temperature of 45^{o}C. Phytase from scallion leaves had also been purified, and its maximum activity was obtained at pH 5.5 and 51^{o}C (Mullaney *et al*., 2000).

Animal sources

Animal phytase has been discovered in calf liver and blood as reported by Mc Collum and Hart (1908). It was also detected in blood of lower vertebrates; i.e. birds, reptiles, fish and sea turtle (Rapoport *et al*., 1941). Patwardhan (1937) first noted phytate hydrolysis in rat intestine. Then, phytase activity was also observed in other animal intestines, such as pig, sheep and cow (Spitzer and Phillips, 1972). Phytase of the mammalian intestine has been suggested to be the same enzyme as alkaline phosphatase, but little is known about the structure of its subunits (Liu *et al*., 1998). The normal human small intestine has a limited ability to digest undergraded phytate (Igbal *et al*., 1994). It does not seem to play a significant role in phytate digestion. However, ruminants can digest phytate through the action of phytase produced by microbial flora in the rumen. The inorganic phosphate produced by splitting of phytate is utilized by both the microbial flora and ruminant host (Vohra and Satyanarayana, 2003).

Microbial sources

Several phytase-producing microorganisms have been examined (Table 2). Over 200 fungal isolates belonging to the genera *Aspergillus*, *Penicillium*, *Mucor* and *Rhizopus* have been tested for phytase production (Liu *et al*., 1998). All isolates produced active extracellular phytase. Two strains of *Aspergillus* sp., *A. niger* and *A. ficuum*, have most commonly been employed for their commercial production (Pandey et al., 2001). However, the most active fungal phytase producer was *A. niger*. *A. niger* NRRL 3135 produced two different phytases, one with pH optimum at pH 5.0, and the other was highly active at pH 2.5, when the assay were performed with phytate as substrate. They showed the same of optimal temperature, at 60^{o}C. Later, these enzymes were designated PhyA and PhyB, respectively (Ullah and Cummins, 1987, 1988; Ullah, 1988a, b; Chelius and Wodzinski, 1994; Ullah and Phillippy, 1994). However, the second phytase (PhyB), which was presented the pH optimum at pH 2.5, was first characterized as an acid phosphatase, when the assay was performed with *p*-nitrophenyl phosphate as substrate (Ehrlich *et al*., 1993). *A. ficuum* NRRL 3135 also produced phytase by solid state fermentation (SSF) using canola meal as the substrate (Ebune *et al.*, 1995). It showed the optima at pH 4.5 and 55 - 60^{o}C (Howson and Davis, 1983; Irving and Cosgrove, 1974; Ullah and Gibson, 1987). Besides, a thermophilic *Aspergilllus sp*. was cultivated for thermostable phytase production. *Thermomyces lanuginosus* exhibited optimum activity at 65^{o}C and pH of 6.0 (Berka *et al*., 1998). Another thermophilic fungus, *Sporotrichum thermophile* produced phytase optimally at 45^{o}C and at pH 6.0 (Ghosh, 1997). Phytase was also detected in whole cells in the fungi; i.e. *A. clavatus* J239, *A. flavipes* Fla.A-14, *A. flavus*, *A. nidulans* QM-329, *A. niger* NRRL 67, *A. niger* P330, *A. oryzae* QM228, *A. phoenicus* QM329, *A. repens* QM-44C, *A. terreus* Fla.C-93, *A. tamari* J1008, *Mucor spp*., *Penicillium spp*. P-320 and *Rhizopus spp*. (Casida, 1959).

In the case of yeast, extracellular phytase has been reported for *Schwanniomyces castellii* (Segueilha *et al*., 1992) and *Arxula adeninivorans* (Sano *et al*., 1999). *S. castellii* phytase was a glycoprotein with an estimated glycosylation rate of 31% which is similar to the phytase of *A. ficuum*. The optimum temperature for activity of *S. castellii* phytase was 77^{o}C, and the enzyme was stable for 1 hr at 74^{o}C. For *A. adeninivorans* phytase, it was reported to be one of the rare cases of yeast

capable of using phytate as carbon and phosphate sources. *Pichia spartinae* and *P. rhodanensis* showed the highest level of phytase activity (Nakamura *et al*., 2000). Among these two species, *P. spartinae* phytase exhibited the optimal temperature for reaction at 75 to 80^{o}C, whereas that of *P. rhodanensis* did so at 70 to 75^{o}C. The optimum pHs were 3.6 to 5.5 and 4.5 to 5.0, respectively (Nakamura *et al*., 2000). *P. anomala* phytase was found in the cellular fraction. The optimum temperature and pH were at 60^{o}C and 4.0, respectively. In addition, it exhibited a broad substrate specificity and was thermostable and acid tolerant (Vohra and Satyanarayana, 2002).

Phytase has also been found in many bacteria; i.e. *Bacillus subtilis* (Powar and Jagannathan, 1982; Shimizu, 1992), *Escherichia coli* (Greiner *et al*., 1993), *Pseudomonas sp*. (Irving and Cosgrove, 1971) and *Klebsiella sp*. (Shah and Parekh, 1990). Generally, the enzyme was prepared by a typical ammonium sulfate precipitation or an ultrafiltration step followed by ion-exchange and gel filtration chromatographies (Liu *et al*., 1998). For example, *E. coli* phytases were purified over 16,000 fold with a four chromatographic purification protocol (Greiner *et al*., 1993), and *B. subtilis* phytase expressed a 322-fold purified protein that was obtained from the culture supernatant after three gel filtrations through a Sephadex G-100column. *E. coli* contains cell associated phytases while those belong to *B. subtilis*, *Lactobacillus amylovorus* and *Enterobacter sp*.4 are extracellular enzymes (Yoon *et al*., 1996).

Production of microbial phytase

Screening and assay

Screening for phytase producing microorganism has been carried out aiming at isolation of bacteria, fungi and yeast that produce extracellular phytase from soil samples. It was found that fungi metabolized phosphorus more effective than bacteria (Lissitskaya *et al*., 1999). Howson and Davis (1983) reported a survey of microorganism for the activity to produce a suitable extracellular phytase. They employed the rapeseed meal as an indicator to measure for the phytate reduction during fermentation. Volfova *et al*. (1994) tested on 132 microorganisms isolated from soil and decayed fruits for their phytase activity. This was done on solid medium containing hexacalcium phytate, which made media opaque. They found that all

Table 2 List of microorganism producing phytases

Microorganisms	Location of the enzyme	References
Bacteria		
Aerobacter aerogenes	Cell bound	Greaves *et al*., 1967
B. amyloliquefaciens	Extracellular	Ha *et al*., 1999
B. subtilis	Extracellular	Kerovuo *et al*., 1998
Bacillus sp., DS11	Extracellular	Kim *et al*., 1998
Bacillus subtilis	Extracellular	Powar and Jagannathan, 1982
*Enterobacter sp.*4	Extracellular	Yoon *et al*., 1996
Escherichia coli	Cell bound	Greiner *et al*., 1993
Klebsiella aerogenes	Cell bound	Tambe *et al*., 1994
K. oxytoca MO-3	Cell bound	Jareonkitmongkol *et al*., 1997
Lactobacillus amylovorus	Extracellular	Sreeramulu *et al*., 1996
Mitsuokella multiacidus	Cell bound	Yanke *et al*., 1998
Pseudomonas sp.	Cell bound	Irving and Cosgrove, 1971
Selenomonas ruminantium	Cell bound	Yanke *et al*., 1999
Fungi		
Aspergillus amstelodami	Extracellular	Howson and Davis, 1983
A. chevalieri	Extracellular	Howson and Davis, 1983
A. candidus	Extracellular	Howson and Davis, 1983
A. niger syn A. ficuum	Extracellular	Howson and Davis, 1983
A. flavus	Extracellular	Shieh and Ware, 1968
A. niger	Extracellular	Skowronski, 1978
A. niger	Extracellular	Shieh and Ware, 1968
A. repens	Extracellular	Howson and Davis, 1983
A. syndowi	Extracellular	Howson and Davis, 1983
A. terreus	Extracellular	Yamada *et al*., 1968
A. versicolor	Extracellular	Howson and Davis, 1983
A. wentii	Extracellular	Howson and Davis, 1983
A. fumigatus	Extracellular	Pasamontes *et al*., 1997

Table 2 (continued) List of microorganism producing phytases

Microorganisms	Location of the enzyme	References
A. carbonarius	Extracellular	Al-Asheh and Duvnjak, 1994
A. carneus	Extracellular	Ghareib, 1990
Aspergillus terreus 9A1	Extracellular	Mitchell *et al*., 1997
Botrytis cinerea	Extracellular	Howson and Davis, 1983
Emericella nidulans	Extracellular	Psamontes *et al*., 1997
Geotrichum candidum	Extracellular	Howson and Davis, 1983
Mucor piriformis	Extracellular	Howson and Davis, 1983
M. racemosus	Extracellular	Howson and Davis, 1983
Myceliophthora thermophila	Extracellular	Mitchell et al., 1997
Penicillium sp.	Extracellular	Shieh and Ware, 1968
Rhizopus oryzae	Extracellular	Howson and Davis, 1983
R. oligosporus	Extracellular	Howson and Davis, 1983
R. stolonifer	Extracellular	Howson and Davis, 1983
Sporotrichum thermophile	Extracellular	Ghosh, 1997
Talaromyces thermophilus	Extracellular	Pasamontes *et al*., 1997
Thermomyces lanuginosus	Extracellular	Berka *et al*., 1998
A. clavatus	Cell bound	Casida, 1959
A. flavipes Fla.A-14	Cell bound	Casida, 1959
A. flavus	Cell bound	Casida, 1959
A. nidulans QM-329	Cell bound	Casida, 1959
A. niger NRRL 67	Cell bound	Casida, 1959
A. niger P330	Cell bound	Casida, 1959
A. oryzae QM228	Cell bound	Casida, 1959
A. phoenicus QM329	Cell bound	Casida, 1959
A. repes QM-44C	Cell bound	Casida, 1959
A. terreus Fla.C-93	Cell bound	Casida, 1959
A. tamari J1008	Cell bound	Casida, 1959
Mucor spp.	Cell bound	Casida, 1959

Table 2 (continued) List of microorganism producing phytases

Microorganisms	Location of the enzyme	References
Penicillium spp. P-320	Cell bound	Casida, 1959
Rhizopus spp.	Cell bound	Casida, 1959
Yeast		
Arxula adeninivorans	Extracellular	Sano *et al*., 1999
Candida spp.	Extracellular	Nakamura *et al*., 2000
Clavispora lusitaniae	Extracellular	Nakamura *et al*., 2000
Debaryomyces yamadae	Extracellular	Nakamura *et al*., 2000
Hanseniaspora lactis	Extracellular	Nakamura *et al*., 2000
Metchnikowia pulcherrima	Extracellular	Nakamura *et al*., 2000
Pichia anomala	Extracellular	Nakamura *et al*., 2000
P. anomala	Cell bound	Vohra and Satyanarayana, 2001
Pichia spp.	Extracellular	Nakamura *et al*., 2000
Sachharomyces cerevisiae	Cell bound	Howson and Davis, 1983
S. cerevisiae	Extracellular	Nakamura *et al*., 2000
S. kluyveri	Extracellular	Nakamura *et al*., 2000
Schwanniomyces	Extracellular	Segueilha *et al*., 1992
occidentalis	Extracellular	Nakamura *et al*., 2000
Torulaspora delbrueckii	Extracellular	Nakamura *et al*., 2000
T. globosa	Extracellular	Nakamura *et al*., 2000
T. pretoriensis	Extracellular	Nakamura *et al*., 2000

isolates that intensively produced extracellular phytase were of fungi origin. For yeast, Sano *et al*. (1999) screened from approximately 1,200 yeast strains in CBS collection for the strains that could grow on medium containing phytate as a sole source of carbon and phosphate. From such a screening, only *Arxula adeninivirans* was found to grow on the phytate medium and exhibited high activity of phytase. For bacteria, 334 strains of 22 species of anaerobic ruminal bacteria were screened for phytase activity using the modified Scott and Dehority (Scott and Dohority, 1965) medium containing sodium phytate from corn (Yanke et al., 1998). The strains showing phytase activity were those belong to *Selenomonas ruminantium*, *Megasphaera elsdenii*, *Prevotella riminicola*, *Mitsuokella multiacidus* and *Treponema sp*. From another group of researchers, 438 soil bacteria were isolated for their capability to grow on selective medium containing sodium inositol hexakisphosphate (Richardson and Hadobas, 1997). Among these, 4 strains were identified as *Pseudomonas sp*.

Determination of microbial phytase activity was based on the ability to release inorganic orthrophosphate from the hydrolysis of sodium phytate at pH 5.5. One unit of activity was the amount of enzyme that released 1 μmole of inorganic phosphate in 1 min (Engelen *et al*., 1994). Alternatively, reversed phase C_{18} high performance liquid chromatography (HPLC) method was developed for separation and quantitative determination of phytic acid and lower inositol phosphates with respect to the phytase-catalyzing reaction (Burbano et al., 1995).

Production parameters

1. Physical conditions

The most important physical parameters for growth and phytase production are pH, temperature, agitation, dissolved oxygen and pressure. Most of the microorganisms capable of producing phytase are mesophiles with optimum temperatures for the production line in the range of 25^{o}C to 37^{o}C (Table 3). The optimal pHs for phytase production from both bacteria and fungi were in the range between pH 5.0 and 7.0 (Table 3). Aeration and agitation are important for keeping the medium composition, microbial cells and oxygen uniformly distributed. These parameters vary among different organisms. For example, in *S. castellii* cultivation was carried out with aeration at 1 VVM and agitation at 600 rev/min (Segueilha *et al*., 1992).

Table 3 Production of phytases by microorganisms

Microorganisms	Opt. Temp. (°C)	Opt. pH	Inducible/ constitutive	References
Bacteria				
Aerobacter aerogenes	27	6.8	Constitutive	Greaves *et al*., 1967
Bacillus sp. DS11	37	6.5	Constitutive	Kim *et al*., 1998
Bacillus subtilis	30	6.5	Inducible	Powar and Jagannathan, 1982
*Enterobacter sp.*4	39	5.5	Inducible	Yoon *et al*., 1996
Escherichia coli	37	7.5	Constitutive	Greiner *et al*., 1993
Klebsiella aerogenes	30		Inducible	Tambe *et al*., 1994
Lactobacillus amylovolus	37	6	Inducible	Sreeramulu *et al*., 1996
Pseudomonas sp.	25		Constitutive	Irving and Cosgrove, 1971
Selenomonas ruminantium	39		Constitutive	Yanke *et al*., 1999
Yeast				
Arxula adeninivorans	28	5.5	Constitutive	Sano *et al*., 1999
Pichia rhodanensis	28	6	Constitutive	Nakamura *et al*., 2000
P. spartinae	28	6	Constitutive	Nakamura *et al*., 2000
Schwanniomyces castellii	30	6.5	Constitutive	Segueilha *et al*., 1992
Fungi				
Aspergillus carneus	30	6	Constitutive	Ghareib, 1990
A. carbonarius (SSF)	30		Constitutive	Al Asheh and Duvnjak, 1995
A. ficuum NRRl 3135	27	5	Constitutive	Shieh and Ware, 1968
A. niger	30	5	Constitutive	Shieh and Ware, 1968
Rhizopus oligosporus	25	5.5	Constitutive	Howson and Davis, 1983

In the case of fungi, *A. ficuum* NRRL 3135 and *A. niger*, phytase production was carried out at 270 rev/min agitation (Shieh and Ware, 1968). Phytase was maximally produced by *Bacillus* sp. DS11 with shaking at 230 rpm (Kim *et al*., 1998). However, the lower agitation speed of 180 rpm was optimum for *B. subtilis* phytase production in a fed-batch culture (Kerovuo *et al*., 2000).

2. Nutrients

2.1 Carbon source

Glucose has been a common carbon source for the use as substrate for phytase production (Vohra and Satyanarayana, 2003). In bacteria, 1% of glucose was optimal for *Lactobacillus amylovorus* (Sreeramulu et al., 1996) and *Enterobacter sp.*4 producing phytase. Glucose at 2% concentration was suitable for *B. subtilis* producing phytase (Kerovuo *et al*., 1998). Other kinds of carbon sources have also been reported. For examples, wheat bran at 6% concentration was used as carbon source for phytase production in *Bacillus sp*. DS11 (Kim *et al*., 1998), while myo-inositol at 0.2% concentration was used for phytase production from *Pseudomonas sp*. (Irving and Cosgrove, 1971). The highest phytase production from *Klebsiella aerogenes* was reported when it was cultivated in the medium containing 2% sodium phytate as carbon source (Tambe *et al*., 1994).

For yeast, *Arxula adeninivorans* produced high phytase activity in theyeast peptone medium containing 2% galactose in place of glucose (Sano *et al*., 1999). Galactose at 1% was also found to be a preferred carbon source for phytase production from *Schwanniomyces castellii* (Segueilha *et al*., 1992).

In fungi, when simple sugars; i.e. glucose and sucrose, were used as sole carbon source, mycelial pellets were formed and low yield of phytase were observed in *A. niger* NRRL 3135. Replacing sugars with corn meal caused dispersed mycelial growth and higher enzyme yield (Shieh and Ware, 1968). For *A. ficuum*, supplementation of 5.2% canola meal to the medium with glucose as carbon source increased phytase production (Ebune *et al*., 1995).

For glucose or fructose as sole carbon source, high concentration of glucose up to 5.2% was found to promote phytase synthesis from *A.*

ficuum in SSF (Ebune *et al*., 1995). However, higher glucose concentration; i.e. 9.8 and 17.8%, showed an adverse effect (Vohra and Satyanarayana, 2003).

2.2 Nitrogen source

Both organic and inorganic forms of nitrogen have been used extensively for the production of phytase. For organic nitrogen sources, peptone was used for phytase production from *Aerobacter aerogenes* (Greaves et al., 1967) and *K. oxytoca* (Jareonkitmongkol *et a*l., 1997). In the case of the *K. oxytoca* phytase production, the medium was also supplemented with 1% yeast extract (Jareonkitmongkol *et a*l., 1997). For *A. adeninivorans*, 1% each of yeast extract and peptone served as good nitrogen sources (Sano *et al*., 1999). For the inorganic nitrogen sources, ammonium sulfate at 1% (w/v) was used for phytase production from *Pseudomonas sp*. (Irving and Cosgrove, 1971), *Enterobacter sp*.4 (Yoon et al., 1996) and *S. castellii* (Lambrechts *et al*., 1992). In some cases, both forms of nitrogen sources were used; e.g. casein hydrolysate at 1% and $(NH_4)_2SO_4$ at 0.1% were used together as nitrogen sources for phytase production from *B. subtilis* (Powar and Jagannathan, 1967).

2.3 Requirement of trace elements and vitamins

Trace elements and vitamins are required to promote growth and phytase production from yeast (Galzy, 1964; Segueilha *et al*., 1992). However, these were not needed for phytase production from either bacteria; i.e. *B. subtilis* (Powar and Jagannathan, 1967), *Bacillus sp*. DS11 (Kim *et al*., 1998), *E. coli* (Greiner *et al*., 1993), or from fungi; i.e. *A. niger* ATCC 9142 and *A. ficuum* NRRL 3135 (Shieh and Ware, 1968).

Factors effecting microbial phytase production

1. Effect of phosphorus concentration

The most commonly used phosphorus sources for phytase production are KH_2PO_4 and K_2HPO_4. Medium containing 0.05% KH_2PO_4 and 0.04% K_2HPO_4 was used for cultivation of *Bacillus sp*. DS11 (Kim *et al*., 1998). For *B. subtilis*, 0.06% sodium phytate was added to medium with 0.1% KH_2PO_4. In *S.*

castellii, 0.06% sodium phytate was used as a sole phosphorus source for phytase production (Segueilha *et al*., 1992). In most cases, the presence of inorganic phosphate inhibited phytase synthesis as often found in several fungi and yeast (Shieh and Ware, 1968; Nayini and Markakis, 1984; Yamada *et al*., 1968). High phosphorus content in corn grains, an ingredient of a complex medium, repressed fungal *A. ficuum* NRRL 3135 phytase production (Shieh and Ware, 1968). The specific activity of phytase from *A. niger* AbZ4 in molasses medium increased by 7.3-fold after elimination of phosphate from the medium (Zyta and Gogol, 2002). The negative effect of high phosphorus on phytase synthesis was also reported for *A. ficuum* (Howson and Davis, 1983; Han and Gallagher, 1987; Ullah and Gibson, 1987).

2. Effect of surfactant

Sometimes surfactants can help resolving problem of pellet formation in fungal cultures. For example, in the case of *A. niger* NRRL 3135, mycelial pellets was formed and the enzyme activity was low (Shieh and Ware, 1968; Han and Gallagher, 1987). Addition of surfactant; i.e. 0.5% (v/v) sodium oleate, promoted the fungal growth with dispersed pellets and high yield of phytase (Han and Gallagher, 1987). Similarly, sodium oleate promoted growth and phytase production from *A. carbonarius* by solid substrate fermentation using canola meal as substrate (Al Asheh and Duvnjak, 1995). Tween-80 was also found to increase phytase production and hydrolysis of phytic acid, while Triton X-100 had a negative effect on these processes (Ebune *et al*., 1995).

3. Effect of medium ingredients

The source and optimal concentration of nutrients are important factors for growth and phytase production. Carbon is the first source that has high influence for metabolite production. In the case of fungi, *A. ficuum* NRRL 3135 was found to use glucose and sucrose as sources of carbon (Shieh and Ware, 1968). The fungal mycelia were found to form pellet and low yields of phytase were observed. Thus, corn meal was tried using as a carbon source, which gave dispersed mycelium growth and higher enzyme yield. However, the phosphorus content in corn meal suppressed the phytase production in *A. ficuum* NRRL 3135. Thus, the feed grade

cornstarch and dextrin were used successfully for limiting the orthophosphate content in the medium. Although the fungal growth was at a slightly sub-optimal level, the fungal phytase production in such a medium was maximum. For some yeast; e.g. *Candida krusei* WZ-001 (Quan et al., 2001), it exhibited a high growth rate when 5% of either glycerol or fructose was used as carbon source. However, the phytase activity per gram of wet cells was lower than that obtained when 5% glucose was a sole carbon source. For soil isolate, *Pseudomonas spp*. CCAR152, it was shown to be able to utilize arabinose as a carbon source for growth but could not directly utilize Na-IHP for phytase production (Richardson and Hadobas, 1997). However, the nonfluorescent strains of *Pseudomonas spp*.; i.e. CCAR31 and CCAR60, could utilize arabinose as their carbon sources and showed promoted growth and high level of Na-IHP utilization for phytase production.

In the case of nitrogen source, *A. terreus* was found to prefer using ammonium ions over nitrate and urea for its phytase production (Howson and Davis, 1983). In addition, $(NH_4)_2SO_4$ and $NH_4H_2PO_3$ were found to be the best nitrogen sources in the medium for subsequent phytase production from *A. terreus* (Howson and Davis, 1983). For *C. krusei* WZ-001, the suitable nitrogen source for its phytase production was polypepton at 0.7% concentration (Quan *et al*., 2001). When polypepton was presented at greater than 12%, it favored growth but not phytase production.

Characterization of phytases

Temperature

In animal feed industry, phytase with thermotolerant property is preferred since the pelleting process involves a step of exposure to high temperature up to 80 - 85^{o}C for a few seconds (Vohra and Satyanarayana, 2003). Some bacterial phytases were found to have optimal temperature for activities at relatively high levels. For examples, phytases from *B. subtilis* (Powar and Jagannathan, 1982), *E. coli* (Greiner *et al*., 1993), *Klebsiella aerogenes* (Tambe *et al*., 1994), *Enterobacter sp*.4 (yoon *et al*., 1996), *K. oxytoca* MO-3 (Jareonkitmongkol *et al*., 1997) and *Selenomonas ruminantium* (Yanke *et al*., 1999) were optimally active at temperatures ranging from 50 to 60^{o}C. *Bacillus sp*. DS11 phytase showed maximum activity at

70°C (Kim *et al*., 1998). These phytases would be able to tolerate high level of temperature for a few seconds during the pelleting process in feed production. In the contrary, phytase of *Aerobacater aerogenes* had an optimal temperature for its activity at a lower level of 25°C, and this kind of phytase could only be used for pretreatment of raw material prior to the feed formulation process (Greaves *et al*., 1967).

In the case of yeast, phytase from *S. castellii* was optimally active at 77°C (Segueilha *et al*., 1992) while that from *Arxula adeninivorans* showed maximum activity at 75°C (Sano *et al*., 1999). *Pichia rhodanensis* and *P. spartinae* phytases worked best at 70 to 80°C (Nakamura *et al*., 2000).

Phytases from *A. fumigatus* and *A. niger* NRRL 3135 showed maximum activity at 37°C (Pasamontes *et al*., 1997) and 55°C (Howson and Davis., 1983), respectively. For the thermophilic fungi, the optimum temperatures for activity of *Thermomyces lanuginosus* phytase was 65°C (Berka *et al*., 1998) and that of *Sporotrichum thermophile* was 45°C (Ghosh, 1997). These optimum temperatures were relatively low when compared to those from other thermophilic microorganisms. However, such levels of temperature were in the thermophilic range (Table 4).

pH

Different microbial phytases showed different level of optimum pH for their activities. For bacteria, their optimum pHs were ranging from 6.5 to 7.5 (Vohra and Satyanarayana, 2003). Phytases from *A. aerogenes* (Greaves *et al*., 1967), *Pseudomonas sp*. (Irving and Cosgrove, 1971), *E. coli* (Greiner *et al*., 1993), *S. ruminantium* (Yanke *et al*., 1999) and *L. amylovorus* (Sreeramulu *et al*., 1996) showed the optimum pH in the range between 4 and 5.5. Phytases that worked well at higher pH were also found, for examples, those from *Entrobacter sp*.4 (Yoon *et al*., 1996) and *Bacillus sp.* DS11 (Kim *et al*., 1998) exhibited the optimal pH for activity in the neutral range; i.e. pH 7.0 to 7.5. In the case of fungi, *A. niger* NRRL 3135 produced two different phytases; i.e. PhyA with optimum pH at 2.5-5.5 and PhyB with that at 2.0 (Howson and Davis, 1983). Phytases from *T. lanuginosus* and *A. fumigatus* were found to be most active at pH 6.0 to 6.5 (Berka *et al*., 1998; Pasamontes *et al.*, 1992) while those from *S. castellii* and *A. adeninivorans* were done so at pH 4.4 and 4.5, respectively (Segueilha *et al*., 1992) (Table 4).

All yeast strains with extracellular phytase activity were found to be most active at the pH range of 4.0 to 5.0 as measured at optimal temperatures of 50 to 60^{o}C (Nakamura *et al*., 2000). Nevertheless, many strains produced phytases with the optimal pH for activity at pH 3.0 to 4.0 when observed at 37^{o}C. These cases showed that the level of optimum pH for activity of protein was affected by the level of temperature of the reaction (Vohra and Satyanarayana, 2003).

Effect of metal ions

Phytases from different microorganisms required different metal ions for their activities. For *B. subtilis* phytase, EDTA removed metal ions from the enzyme resulted as a complete inactivation of the activity (Kerovuo *et al*., 2000). This loss of enzymatic activity is most likely due to a conformational change in the absent of Ca^{2+}. The enzyme was partially restored to their active forms when Ca^{2+} was added to the storage buffer. In addition, Ca^{2+} was required for *B. subtilis* phytase active function (Kerovuo *et al*., 2000).

Nontheless, some kinds of cations were found to inhibit phytase activity at different concentrations depending on nature of the ions (Segueilha *et al*., 1992). The enzyme from *A. ficuum* (Ullah and Cummins, 1998), *K. terrigena* (Greiner *et al*., 1997) and *E. coli* (Greiner *et al*., 1993) were inhibited when the reaction mixture contains 5 mM Fe^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+} and Hg^{2+}. Similarly, *S. ruminantium* phytase was strongly inhibited by these ions (Yanke *et al*., 1999). In *Bacillus sp*. DS11, were strongly inhibited by EDTA, Cd^{2+} and Mn^{2+} and moderately inhibited by Hg^{2+}, Mg^{2+}, Ba^{2+} and Cu^{2+} at 5 mM (Kim *et al*., 1998).

Effect of substrate

Some phytases show broad substrate specificity with the highest affinity for phytate. For example, phytase of *Bacillus sp*. DS11 was very specific for phytate and had little or no activity on phosphate esters; i.e. *p*-nitrophenyl phosphate, ATP, ADP, AMP, β-glycerophosphate, sodium pyrophosphate and α-naphthylphosphate (Kim *et al*., 1998). Similarly, phytase from *Pseudomonas sp*. did not show activity toward inorganic pyrophosphate, β-glycerophosphate, ADP or AMP, and activity against *p*-nitrophenyl phosphate was 14% of that on phytate (Irving and

Cosgrove, 1971). However, some phytases were highly specific to only phytate substrate; such as those from *A. niger* and *A. terreus* CBS (Wyss *et al*., 1999). For some other fungi; i.e. *A. fumigatus*, *E. nidulans* and *M. thermophila* showed that their phytases exhibited broad substrate specificity (Wyss *et al*., 1999). For yeast, *S. castellii* has been reported that its phytase showed low substrate specificity, and its *Km* value for phytate was the lowest. In addition, the *Km* value for glucose-1-phosphate, glucose-6-phosphate, *p*-nitrophenyl phosphate and ATP were at high levels (Segueilha *et al*., 1992). The *Km* values of some published phytases are given in Table 4.

Thermostability

Thermostability is considered to be an important criteria in phytase selection for industrial application. The enzyme should be able to withstand temperatures of 60^{o}C to 90^{o}C, which is carried out for feed pelleting process (Vohra and Satyanarayana, 2003). Phytases are normally thermostable enzymes that are active in a broad pH range. *A. fumigatus* phytase was stable at high temperature up to 100^{o}C for over a period of 20 min, with a loss of only 10% of the initial enzymatic activity (Pasamontes *et al*., 1997). However, phytase from *A. fumigatus* was denatured at temperatures between 50^{o}C and 70^{o}C. After heat denature, once the temperature was dropped, *A. fumigatus* phytase could completely refold into a native-like, fully active conformation (Wyss et al., 1998). On the contrary, the conformational change of *A. niger* phytase due to high temperature was of irreversible type. Its enzymatic activity was lost up to 70 to 80% (Wyss *et al*., 1998).

Phytase from *E. coli* retained only 24% activity when exposed to 60^{o}C for 1 hr. Its activity was completely lost at 70^{o}C (Vohra and Satyanarayana, 2003). Similarly, the *Enterobacter sp*.4 phytase activity was undetectable after 20 hr storage at 70^{o}C to 80^{o}C (Yoon *et al*., 1996). In some cases, ions can help increasing the stability of enzyme towards high temperature. For example, *Bacillus sp.* DS11 phytase was stable when incubated at high temperatures up to 90^{o}C in the presence of 5 mM $CaCl_2$, but almost completely lost its activity at temperature above 50^{o}C in the absence of $CaCl_2$ (Kim *et al*., 1998).

Table 4 Physico-chemical properties of purified phytases

Microorganisms	T opt (^{o}C)	pH	*Km* (mM)	Mol. Wt. (kDa)	References
Bacteria					
Aerobacter aerogenes	25	4-5	0.135	-	Greaves *et al*., 1967
Bacillus sp. DS11	70	7.0	0.55	-	Kim *et al*., 1998
B. subtilis	55	7.0	0.4	36.5	Powar and Jagannathan, 1982
*Enterobacter sp.*4	50	7-7.5	-	-	Yoon *et al*., 1996
E. coli	55	4.5	0.13	42	Greiner *et al*., 1993
Klebsiella aerogenes	60	4.5-5.2	0.11	700	Tambe *et al*., 1994
K. oxytoca MO-3	55	5-6	-	-	Jareonkitmongkol *et al*., 1997
Pseudomonas sp.	40	5.5	0.016	-	Irving and Cosgrove, 1971
S. ruminantium	50-55	4-4.5	-	46	Yanke *et al*., 1999
Fungi					
A. carneus	40	5.6	-	-	Ghareib, 1990
A. carbonarius	53	4.7	-	-	Al Asheh and Duvnjak, 1994
A. niger 92	55	5.0	0.44	100	Dvorokava *et al*., 1997
A. terreus	70	4.5	-	214	Yamada *et al*., 1968
A. niger NRRL 3135	58	2.5; 5.0	0.04	85-100	Ullah and Gibson, 1987
Yeast					
Arxula adeninivorans	75-80	4.5	0.25	-	Sano *et al*., 1999
P. anomala	60	4	0.20	64	Vohra and Satyanarayana, 2002
P. rhodanensis	70-75	4-4.5	0.25	-	Nakamura *et al*., 2000

Application of phytases

Animal feed industry

Plant raw materials including cereals, legumes, oil seeds, pollens and nuts are used for animal diet production. These materials contain phytic acid or phytate as their major storage of phosphorus (Reddy *et al*., 1982). However, this form of phosphorus is unusable by animals like porcine and poultry which lack of phytate digesting enzyme (Nelson, 1967) Furthermore, phytate chelates several important minerals; i.e. Fe^{2+}, Cu^{2+} and Zn^{2+}, and makes them unavailable to be assimilated in animal gastrointestinal tract. For phytate that is not absorbed, it is disposed with the feces to the environment (Mullaney *et al*., 2000). The phosphorus released is transported into the water bodies causing eutrophication which results in oxygen depletion due to excessive algal growth (Bail and Satyanarayana, 2001).

The problem caused by phytate can be resolved by the addition of microbial phytase to the animal feed which contain phytate-rich cereal diet (Nelson, 1967). The enzyme minimizes the need for supplementation of inorganic phosphorus due to improving the utilization of organic phosphorus in poultry. Furthermore, the reduction of the excretion of phosphorus in manure is occurred (Mohanna and Nys, 1999). Apart from hydrolyzing phytate, and the addition of phytase to feed at 250 to 1000 U/kg can fully replace phosphorus supplementation to the animal feed formular (Golovan *et al*., 2001).

Nevertheless, the use of phytase as a feed additive is limited by the enzyme cost and the loss of activity during the feed pelleting process at high temperatures up to 80^{o}C or during storage (Vohra and Satyanarayana, 2003). *A. niger* phytase was denatured at high temperature during the feed pelleting process (65^{o}C to 95^{o}C). Although the enzyme was added after pelleting process, the production cost was still high (Mullaney *et al*., 2000). Such a problem could be overcome by using thermotolerant phytase. For example, *A. fumigatus* phytase incubated at 90^{o}C or even 100 ^{o}C for 20 min resulted in only a minor loss of activity (10%), and after exposure to 90^{o}C for 120 min, 70% of the initial activity still remained (Pasamontes *et al*., 1997). Although, *M. thermophila* (Mitchell *et al*., 1997) and *T. lanuginosus* (Berka *et al*., 1998) are thermophilic fungi, their phytase are fully inactivated by a high-temperature incubation. Therefore, from the characteristic property of thermophilic phytase gene,

A. fumigatus phytase was cloned and expressed in *A. niger* NW205 (Pasamontes *et al*., 1997) and *Pichia pastoris* (Rodriguez *et al*., 2000), the transformant phytase still showed the thermostability at 65^{o}C and 90^{o}C. Moreover, the use of compounds to enhance thermostability of phytase has been investigated. Salts and polyols have been used to increase the thermostability of enzymes (Lamosa *et al*., 2000). For example, calcium is reported to enhance the heat tolerance of *Bacillus sp.* DS11 (Kim *et al*., 1998). Immobilization of *E. coli* phytase (Greiner and Konietzny, 1996) and glycosylation of *A. niger* phytase expressed in *S. cerevisiae* (Han *et al*., 1999) contributed to the increase in the thermostability of phytase. Moreover, protein engineering has also been used to increase phytase thermostability; i.e. the replacement of proline with hydrophilic amino acids (Glu, Lys and Thr) (Watanbe and Suzuki, 1998) and introducing disulfide bonds (Scott and Steven, 2000).

In the case of pH, the enzyme is improved to be optimally active in the pH range prevalent in the digestive tract. Site-directed mutagenesis confirmed that a replacement of Gly-277 and Tyr-282 of *A. fumigatus* phytase with the corresponding residues of *A. niger* phytase which were Lys and His, respectively, gave a phytase with a pH optimum changed from pH 2.8 to 3.4 (Tomschy et al., 2002).

The enzyme from microorganisms can cause allergic response. Phytase is an occupational allergen that can cause specific IgE immune response (Vohra and Satyanarayana, 2003). Bio-Feed® Phytase from Novo Nordisk is available in a coated granulated form; this is a dust-free product that offers several advantages over the powdered enzyme (Mullaney *et al*., 2000).

The Alko Co. (Finland) as well as Altech (USA) and *BASF* (USA) started the industrial scale production of phytase marketed under the names Finase, Allzyme phytase and Natuphos, respectively. These marketed enzymes were successfully utilized in feed applications. Finase added to a corn-soybean pig diet converted one-third of the unavailable phosphorus to an available form (Cormwell *et al.*, 1993). Similarly, the addition of the Allzyme and Natuphos to pig and chicken diets also indicated that phytase was efficacious in improving the bioavailability of phytate phosphorus for pigs (Cormwell *et al*., 1995a, b; Yi *et al*., 1996; O'Quinn *et al*., 1997) and broilers (Yi *et al*., 1997).

Growth rate and feed conversion ratio on the low-phosphorus diets containing microbial phytase were comparable to or even better than the untreated diets. The addition of microbial phytase to diets for growing pigs increased the apparent absorbability of phosphorus by 24%. The amount of phosphorus in the feces was 35% lower (Simons et al., 1990). A new phytase was expressed in yeast. Among several species of yeast, *Saccharomyces cerevisiae* is the ideal yeast for phytase production (Panda et al., 1998). When its phytase was fed to weanling pigs, their bioavailability of phytate phosphorus was improved. This phytase was found to be as effective as Natuphos at the inclusion level of 700 or 1200 U/kg of a phosphorus-deficient, corn-soybean meal diet in improving phytate-phosphorus utilization of young pigs (Stahl et al., 2000).

In the late twentieth century, sales of phytase as an animal feed additive were estimated to be $500 million and growing (Abelson, 1999). The evaluation of the market for this feed additive can be attributed, and the need of phytase is led to a means for its commercial development (Vohra and Satyanarayana, 2003).

Food industry

The plant origin food contains phytic acid or phytate that can cause the unavailability of nutrient; i.e. trace elements, vitamins and protein in human. Simell *et al.* (1989) used Finase S phytase for the preparation of phytate-free soy protein isolates being more suitable at low pH (pH 3) than the control protein. The Finase phytase can also be used in the production of phytate free soybean milk. Anno *et al.* (1985) eliminated phytate from soybean milk using wheat phytase from Sigma Co. Furthermore, Khare *et al*., (1994) successfully hydrolyzed soy milk phytate using immobilized wheat phytase. Extracellular phytase from *A. ficuum* NRRL 3135 was used for dephosphorylation in legume products (Shieh *et al*., 1969). *A. niger* phytase caused the loss of 78% phytate from soybean meal when incubated together for 15 min (Han, 1988). In addition, a crude preparation of intracellular acid phosphatase rich in phytase obtained from *A. niger* caused nearly complete dephosphorylation of protein isolates from soybean in a few hours (Zyta *et al*., 1989).

However, the dietary phytase is inactivated at high temperatures, especially those for cooking. It also caused a poor mineral absorption. Thus, *A. niger*

phytase was tried adding to flour containing wheat bran which helped increasing iron absorption in humans (Sandberg *et al*., 1996). The use of phytase was suggested for producing low phytin bread. In commercial whole wheat bread, molds phytases were added during the bread making, and then, phytate could be almost completely eliminated (Knorr *et al*., 1981). Caransa *et al*. (1988) reported that microbial phytase could accelerate the process of steeping required in the wet milling of corn, thereby improving the properties of corn steep liquor.

Potential in aquaculture

Several studies have been conducted on the use of soybean meal or other plant meals in aquaculture (Mwachireya *et al*., 1999). By substituting a more expensive protein source; i.e. menhaden fish meal, with lower-cost plant protein, a significant cost reduction could be achieved. As in poultry and hogs, fish also lacks an adequate digestive enzyme to effectively utilize the phytate phosphorus in plant origin feed. Thus, the phytate phosphorus excretes into the water. Therefore, phytase has been evaluated as a means to increase the use of low cost plant meals in the aquaculture industry, and thereby, controlling phosphorus at the acceptable levels in water. Several fish feeding studies have documented the potential value of phytase in diets containing high levels of plant feed stuffs (Robinson *et al*, 1996; Mwachireya *et al*., 1999). The fish diet supplemented with microbial phytase could increase the availability of phosphorus in plant ingredients to fish (Cain and Garling, 1995; Eya and Lovell, 1997; Li and Robinson, 1997; Sugiura *et al*., 2001). Phytase is applied to pellets in fish feed after extrusion for remaining activity during feed production (Hardy and Gatlin III, 2002). However, the important of phosphorus absorption are influenced by phytase level and sources of ingredients, and the reduction P excretion is reported to range from 30% to 80% (Kornegay, 2001).

CHAPTER III
MATERIALS AND METHODS

Media and solutions

All solution and media were prepared with distilled water and sterilized by autoclaving at 121°C, 15 lb/in^2, unless stated otherwise. Glassware, eppendorf tubes and pipettes were also sterilized by autoclaving.

Culture media were products from Difco. Co., (Michigan, U.S.A.). Soybean meal, rice bran, wheat bran and soybean grain were purchased locally. The chemicals and solvents used for preparation of media and reagents were from Merck Co., (Darmstadt, Germany), Sigma Chemical Co., (St. Louis, Mo, U.S.A.) and APS Ajax Finechem Co., (Auburn, NSW, Australia).

Media

1. **Luria Bertani Broth (LB)**

   1.0% (w/v) tryptone, 0.5% (w/v) yeast extract and 0.5% (w/v) NaCl in distilled water.

2. **Luria Bertani Agar (LA)**

   Luria bertani broth supplemented with 1.5% (w/v) bacto agar.

3. **Nutrient Broth (NB)**

   0.8% (w/v) nutrient broth dissolved in distilled water.

4. **Nutrient Agar (NA)**

   Nutrient broth supplemented with 1.5% (w/v) bacto agar.

5. **Phytase Screening Medium Broth (PSM) (Kerovuo *et al.*, 1998)**

   2% (w/v) glucose, 0.4% (w/v) sodium phytate, 0.2% (w/v) $CaCl_2$,

0.05% (w/v) KCl, 0.05% (w/v) $MgSO_4.7H_2O$, 0.5% (w/v) NH_4NO_3, 0.001% (w/v) $FeSO_4.7H_2O$ and 0.001% (w/v) $MnSO_4.H_2O$ in distilled water.

6. Solutions for Minimal P Medium (Leisinger *et al.*, 1972)

Solution A: 7.3% (w/v) Na_2HPO_4, 3.2% (w/v) KH_2PO_4

Solution B: 2.1% (w/v) $MgSO_4$

Solution D: 0.2% (w/v) $FeSO_4$, one drop of concentrated HCl in sterile distilled water

$(NH_4)_2SO_4$ solution: 5% (w/v) $(NH_4)_2SO_4$

50% Glucose solution: 50% (w/v) D-glucose in distilled water, sterilized by autoclaving at 110°C for 10 min

7. Minimal Medium (MMP) (Leisinger *et al.*, 1972)

2% (v/v) solution A, 2% (v/v) solution B, 2% (v/v) $(NH_4)_2SO_4$ solution, 0.2% (v/v) solution D and 2% (v/v) 50% glucose solution in sterile water.

8. Rice bran extract

10% (w/v) rice bran in distilled water was autoclaved and then centrifuged at 10,000 rpm for 10 min. The supernatant was collected for used as rice bran extract.

9. Wheat bran extract

10% (w/v) wheat bran in distilled water was autoclaved and then centrifuged at 10,000 rpm for 10 min. The supernatant was collected for used as wheat bran extract.

10. Soybean meal extract

10% (w/v) soybean meal in distilled water was autoclaved and then centrifuged at 10,000 rpm for 10 min. The supernatant was collected for used as soybean meal bran extract.

Solutions

1. Antibiotics

Tetracycline: stock solution was prepared as 15 mg/ml solution in distilled water and used at a concentration of 15 μg per ml 0f media.

2. Soymilk

500 g of soybean in 1.5 liter of distilled water was boiled for 30 min and then filtered with squeezing through cheese-cloth.

3. Solution for Colorimetric Phytase Assay

3.1 Substrate Solution for Phytase Assay

0.84 g sodium phytate in 90 ml of the buffer solution, adjusted to pH 5.5 with 100% acetic acid and volume adjusted to 100 ml with distilled water.

3.2 Buffer Solution

0.176 ml of 100% acetic acid, 30.02 g sodium acetate trihydrate, 0.0147g $CaCl_2.2H_2O$, 90 ml distilled water, adjusted to pH 5.5 with 100% acetic acid and adjusted volume to 100 ml with distilled water.

3.3 Color Stop Mix Reagent

25 ml ammonium molybdate solution [10 g $H_{24}MO_7N_6O_{24}.4H_2O$, 90 ml distilled water, 1 ml ammonium solution 25% and adjusted to 100 ml with distilled water] was mixed with 25 ml ammonium vanadate solution [0.235 g NH_4VO_3, 40 ml distilled water at 60°C, added 2 ml nitric acid (70 ml 65% nitric acid and 130 ml distilled water) and adjusted to 100 ml with distilled water], stirred while slowly added 16.5 ml 65% nitric acid, cooled to room temperature and adjusted to 100 ml with distilled water.

4. Solution for Colorimetric Lipase Assay

4.1 Solution A

0.062 g of *p*-nitrophenylpalmitate (*p*-NPP) in 10 ml of 2-propanol which was sonicated for 2 min before use.

4.2 Solution B

0.4% (w/v) Triton X-100 and 0.1% (w/v) gum arabic in 50 mM tris-HCl (pH 8.0).

5. Solution for Colorimetric Amylase Assay

5.1 Substrate Solution

0.2% (w/v) soluble starch in distilled water.

5.2 0.15 M Acetate Buffer pH 5.0

Mixing 14.8 ml of 0.2 M acetic acid solution (11.55 ml 100% acetic acid in 1,000 ml distilled water) with 35.2 ml of 0.2 M sodium acetate solution (27.2 g $C_2H_3O_2Na.3H_2O$ in 1,000 ml distilled water) and adjusted volume to 75 ml with distilled water.

5.3 DNS Solution

2.5 g dinitrosalicylic acid in 50 ml of 2 N NaOH, 75.9 g sodium potassium tartate and adjusted to 250 ml with distilled water. Stored in a light-protected bottle at room temperature.

6. Solution for Colorimetric Endoglucanase Assay

6.1 Substrate Solution

0.3% (w/v) β-glucan in 50 mM sodium phosphate buffer pH 6.8

7. Solution for Colorimetric Cellulase Assay

7.1 Substrate solution

1% (w/v) carboxymethylcellulose (CMC) in 50 mM sodiumphosphate buffer pH 6.8.

8. Solution for Colorimetric Xylanase Assay

8.1 Substrate Solution

1% (w/v) xylan in 50 mM sodium phosphate buffer pH 6.8.

9. Solution for Colorimetric Protease Assay

9.1 Substrate Solution

1.5 g casein in 20 ml 0.1 N NaOH (4g NaOH in 1,000 ml distilled water) was gently heated and stirred in order to melt casein, cooled to room temperature. pH was adjusted to pH 7.0 with 0.1 M HCl or 0.1 N NaOH. 50 ml of 50 mM sodium phosphate buffer pH 7.0 was added and volume was adjusted to 100 ml with distilled water.

9.2 0.05 M Sodium Phosphate Buffer pH 7.0

39 ml 0.2 M NaH_2PO_4 solution (156.01 g NaH_2PO_4 in 1,000 ml distilled water) was mixed with 61 ml 0.2 M Na_2HPO_4 solution (141.96 g Na_2HPO_4 in 1,000 ml distilled water) and adjusted to 400 ml with distilled water.

9.3 0.4 M Trichloroacetic acid Solution (TCA)

65.356 g TCA in 1,000 ml distilled water.

9.4 0.4 M Na_2CO_3 Solution

42.396 g Na_2CO_3 in 1,000 ml distilled water.

9.5 Folin Reagent

1:1 diluted solution in distilled water was freshly prepared before use.

10. 0.1 M Tris-HCl Buffer, pH 7.0

500 ml of 0.2 M Tris –base solution (24.238 g $C_4H_{11}NO_3$ in 1,000 ml distilled water) was adjusted to pH 7.0 with 6 N HCl.

11. 0.1 M Tris-HCl Buffer, pH 8.0

500 ml of 0.2 M Tris –base solution (24.238 g $C_4H_{11}NO_3$ in 1,000 ml distilled water) was adjusted to pH 8.0 with 6 N HCl.

12. 0.1 M Tris-HCl Buffer, pH 9.0

500 ml of 0.2 M Tris –base solution (24.238 g $C_4H_{11}NO_3$ in 1,000 ml distilled water) was adjusted to pH 9.0 with 6 N HCl.

13. 0.1 M Acetate Buffer, pH 3.6

46.30 ml of 0.2 M acetic acid solution and 3.70 ml of 0.2 M sodium acetate solution are mixed.

14. 0.05 M Acetate Buffer, pH 4.0

20.55 ml of 0.2 M acetic acid solution and 4.50 ml of 0.2 M sodium acetate solution are mixed.

15. 0.1 M Acetate Buffer, pH 5.6

4.80 ml of 0.2 M acetic acid solution and 45.2 ml of 0.2 M sodium acetate solution are mixed.

16. 0.1 M Glycine-HCl Buffer solution, pH 2.2

25 ml of 0.2 M glycine solution (15.01 g $C_2H_5NO_2$ in 1,000 ml distilled water) was mixed with 22.0 ml of 0.2 N HCl to obtain the required pH 2.2.

17. 0.1 M Glycine-HCl Buffer solution, pH 3.6

25 ml of 0.2 M glycine solution (15.01 g $C_2H_5NO_2$ in 1,000 ml distilled water) was mixed with 2.5 ml of 0.2 N HCl to obtain the required pH 3.6.

18. Bradford Solution

18.1 Bradford Stock Solution

100 ml 95% ethanol, 200 ml 85% phosphoric acid and 200 mg Coomassie Blue G. The solution was stored at room temperature.

18.2 Bradford Working Buffer

Mixed 25 ml distilled water, 15 ml 95% ethanol, 30 ml 88% phosphoric acid and 30 ml Bradford stock solution and filtrated through Whatman No. 1 paper, stored at room temperature in a light-protected bottle.

Methods

1. **Cultivation of *Pseudomonas putida* carrying *Escherichia coli app*A [P(EappA)]**

Cultivation of *Pseudomonas putida* carrying *Escherichia coli app*A on solid medium was carried out at 30°C. Liquid cultures were grown with 200 rpm aeration on a platform shaker at 30°C for 48 hr.

2. **Measurement of Cell growth**

Bacterial growth was determined by measuring the optical density at 600 nm.

3. **Preparation of Crude Enzyme**

Culture broth was centrifuged and the supernatant was collected to determine the phytase activity.

4. **Ammonium Sulfate Precipitation**

280.50 gm of ammonium sulfate was slowly added with continuously stirring to 500ml of crude enzyme to give 80% saturation. This was carried out in an ice bath. After all salt was added, the solution was left stirring for 2 hr under the same condition. The protein precipitated was collected after centrifugation at 10,000 g for 60 min.

5. **Quantitative Phytase Assay**

The quantitative assay method used in this study was done following Engelen *et al.*, 1994. The reaction mixture contained 0.5 ml of enzyme sample and 1 ml of substrate solution. The reaction was carried out at 37°C for 60 min and stopped by adding 1 ml freshly prepared color reagent. The developed color from the phytase activity was determined for optical density at 415 nm. One unit was the amount of enzyme that released 1 μmole of inorganic phosphate in 1 min.

6. Quantitative Amylase Assay

Quantitative amylase assay was done following Bernfeld (1995). The reaction mixture contained 0.5 ml of enzyme sample, 1 ml of 0.15 M acetate buffer pH 5.5 and 1.5 ml of substrate solution. The mixture was incubated at 40°C for 15 min. The reaction mixture was taken to determine reducing sugar by modification of DNS method. Using 1 ml of enzyme sample and 1 ml of DNS solution did modification of DNS. The mixture was incubated at 100°C for 5 min and cooled immediately. The mixture was added with 10 ml of distilled water. The color developed was determined at optical density 520 nm. The amount of reducing sugar was determined based on the OD_{520} value of a standard glucose solution. One unit was the amount μmole of glucose released per min of 1 ml enzyme.

7. Quantitative Lipase Assay

Quantitative lipase assay was done following Dharmsthiti *et al.* (1998). The reagent consisted of 1 part of solution A and 9 parts of solution B. Both the composition of solution A and solution B were previously described as the solution for colorimetric lipase assay. The reaction reagent was prepared freshly before the detection. A 200 μl of the appropriate diluted enzyme solution was added to 1800 μl reaction reagent. The reaction was carried out at 37°C for 15 min. The color developed was determined by spectrophotometry at 410 nm. One unit of lipase activity was defined as 1.0 μmole of *p*-NP released from *p*-NPP per min.

8. Quantitative Xylanase Assay

Quantitative xylanase assay was done following Bailey *et al.*, 1992. The reaction mixture contained 100 μl of enzyme sample and 900 μl substrate solution. The mixture was incubated at 50°C for 20 min. The reaction was stopped and developed color by DNS method. DNS method was done following Miller *et al.*, 1959. The reaction mixture contained 1 ml of sample and 3 ml of DNS solution. The reaction mixture was incubated at 100°C for 5 min. The sample was cooled immediately in ice bath. The color intensity was determined spectrophotometrically at optical density 540 nm. The amount of reducing sugar determined was based on the OD_{540} value of a standard glucose solution. One unit was the amount μmole of glucose released per min of 1 ml enzyme sample.

9. Quantitative Endoglucanase Assay

Quantitative endoglucanase assay was done following Munir *et al.*, 1999. The reaction mixture contained 100 μl of enzyme sample and 900 μl of substrate solution. The mixture was incubated at 50°C for 20 min. The reaction was stopped and color developed by DNS method. DNS method was done following Miller *et al.*, 1959. The reaction mixture contained 1 ml of sample and 3 ml of DNS solution. The reaction mixture was incubated at 100°C for 5 min. The sample was cooled immediately in ice bath. The color intensity was determined spectrophotometrically at optical density 540 nm. The amount of reducing sugar was determined based on the OD_{540} value of a standard glucose solution. One unit was the amount μmole of glucose released per min of 1 ml of enzyme sample.

10. Quantitative Protease Assay

Quantitative protease assay was done following Anson (1938). The reaction mixture contained 1 ml of enzyme sample and 1 ml of substrate solution. The mixture was incubated at 40°C for 15 min. The reaction was stopped by adding with 2 ml of TCA and filtrated pass through filter paper (Whatman No. 1). Then, 0.5 ml of filtrate was transferred to a glass tube, 2.5 ml of Na_2CO_3 solution and 0.5 ml folin reagent were added. The mixture was incubated at room temperature for 10 min. The

color developed was determined by spectrophotometry at 660 nm. One unit was the amount μmole of L-tryptone released per min of 1 ml enzyme sample.

11. Quantitative Cellulase Assay

Quantitative cellulase assay was done following Ye *et al*., 2001. The reaction mixture contained 200 μl of enzyme sample and 200 μl of substrate solution. The mixture was incubated at 37°C for 15 min. Reducing sugar produced by this reaction was measured according to the DNS method. DNS method was done following Miller *et al*., 1959. The reaction mixture contained 1 ml of sample and 3 ml of DNS solution. The reaction mixture was incubated at 100°C for 5 min. The sample was cooled immediately in ice bath. The color intensity was determined spectrophotometrically at optical density 540 nm. The amount of reducing sugar was determined based on the OD_{540} value of a standard glucose solution. One unit was the amount μmole of glucose released per min of 1 ml of enzyme sample.

12. Determination of Phosphate Concentration

Phosphate concentration was determined by following Engelen *et al*., 1994. The reaction mixture contained 1 ml of sample was added with 1 ml of freshly prepared color reagent. The developed color was determined for optical density at 415 nm. The amount of phosphate was calculated based in a standard KH_2PO_4 solution.

13. Determination of Protein Concentration

Protein concentration was determined by following Bearden (1978), Bradford (1976) and Read and Northcote (1981). A clear glass tube containing 200 μl of sample was added with 2 ml of Bradford solution. The developed color was determined at optical density 595 nm. The amount of protein was calculated based on a standard BSA solution.

14. Determination of Reducing Sugar Concentration

Reducing sugar concentration was determined by modification of DNS method following Miller *et al.*, 1959. The reaction mixture contained 1 ml of sample was added with 1 ml of DNS solution. The mixture was incubated at 100°C for 5 min and cooled immediately. The mixture was added with 10 ml of distilled water. The developed color was determined at optical density 520 nm. The amount of reducing sugar was calculated based on the OD_{520} value of a standard glucose solution.

15. Concentrated enzyme preparation and Enzyme Storage

The concentrated enzyme from ammonium sulfate precipitation was prepared into 3 forms: 1.) resuspension form which was the ammonium sulfate precipitated protein resuspending in the least volume of 0.05 M acetate buffer, pH 5.6, 2.) air-dried enzyme precipitate form which was the ammonium sulfate precipitated protein drying by dried air at room temperature and 3.) wet enzyme precipitate from which was the ammonium sulfate precipitated protein collecting by centrifugation after precipitation.

The concentrated enzyme precipitate into 3 steps: resuspended enzyme, air-dried enzyme and wet enzyme precipitates. These were stored at 4°C and 25°C for 4 weeks. The enzyme activity was determined every week by using the method of Engelen *et al.*, 1994.

16. Formulation of phytase for animal feed supplement

Phytase was formulated as feed-based formulae. Chicken feed was mixed with the concentrated enzyme at 500 units of enzyme per 1 kg of feed. The concentrated phytase was prepared as 3 forms; i.e. concentrate enzyme suspension, air-dried enzyme and wet enzyme precipitates. The feed-based enzyme formulae were stored at 4°C and 25°C for 4 weeks. The remaining phytase activity was determined weekly by using the method of Engelen *et el.*,1994.

17. Feed and Agricultural Waste Digestion

Chicken feed, rice bran, wheat bran and soybean meal were mixed with crude enzyme. The supplementation was at 500 units of enzyme per 1 kg of materials. The formulated materials were added with 20 ml 0.05 M acetate buffer pH 4.0 and bile acids (trypsin, taurochloric acid and deoxycholic acid), lactic acid and propionic acid. The digestion condition was at 37°C with 200 rpm agitation for 60 min. Then, the digested materials were determined for phosphate, protein and reducing sugar content .

18. Soymilk Digestion

The soymilk was mixed with crude enzyme at 500 units of enzyme per 1 liter of soymilk. The mixture was carried out in the digestion condition at 37°C with 200 rpm agitation for 60 min. After that, the digested soymilk was determined for phosphate, protein and reducing sugar content.

CHAPTER IV
RESULTS

1. Determination of general properties of crude phytase from *Pseudomonas putida* carrying *Escherichia coli app*A [P(EappA)]

From the previous study, *Escherichia coli appA* gene from *Escherichia coli* K12 was cloned by polymerase chain reaction method (PCR) and carried on pBBRT plasmid, which was a broad host range cloning vector (Kovach *et al*., 1995). Then, pBBRT carrying *Escherichia coli appA* was transformed into *Pseudomonas putida*. For *Pseudomonas putida*, it showed the phytase activity at the undetectable level which was less than 0.01 U/ml (data not shown).

The preliminary study on general properties of crude P(EappA) phytase which was prepared from a 24-hr culture grown at 30°C with 200 rpm agitation in LB + 15μg/ml tetracycline. Cells were separated by centrifugation and the supernatant was collected as crude extracellular phytase for further study.

1.1 Effect of pH on the phytase activity and stability

Optimum pH for the activity of P(EappA) phytase were determined by adjusting the pH of the reaction mixture to various levels. This was done using 0.1 M glycine-HCl buffer for pH 3.0 and 3.6, 0.1 M acetate buffer for pH 3.6-6.0 and 0.1 M tris-HCl buffer for pH 6.0-10.0. Changing the kind of buffer was necessary due to the limitation of the buffing capacity of each buffer. However, the composition of different buffers could have different effects on the enzyme activity. Therefore, upon changing the kind of buffer used, the enzyme activity at the same pH point under the condition of different buffers had to be determined. The result indicated that the maximum activity was obtained at pH 4.0 (Figure 3).

For the effect of pH on the stability of phytase, it was determined by storing P(EappA) phytase under various pHs at two temperatures; i.e. 4°C and 37°C. The samples were taken at various time intervals to determine for the remaining activity under standard condition. The result was expressed as the remaining phytase

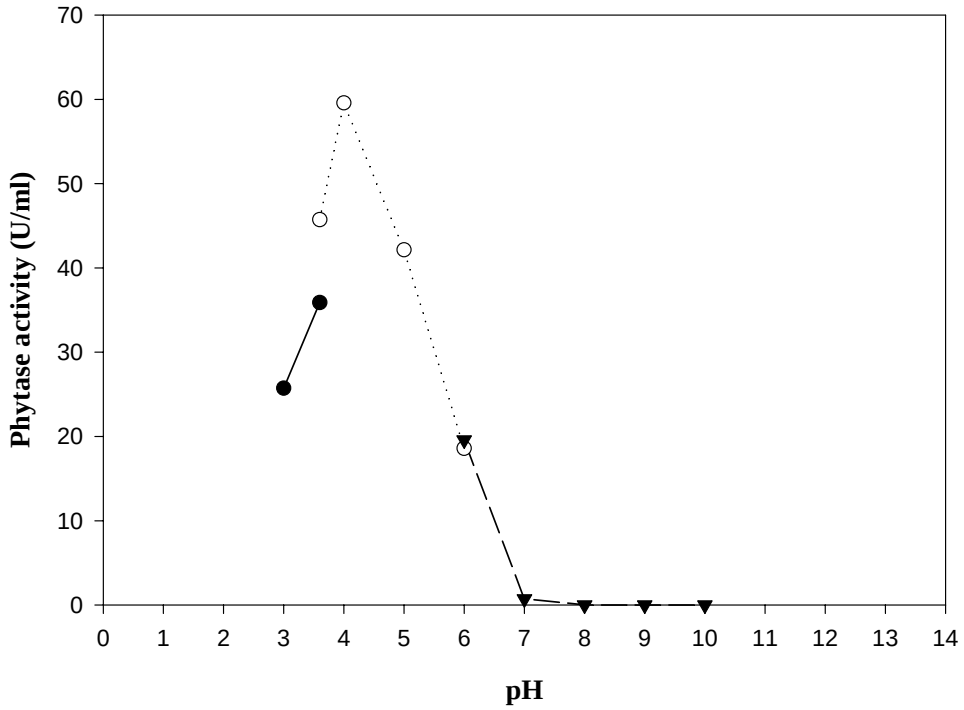

Figure 3 Effect of pH on phytase activity of crude enzyme from P(EappA). Phytase activity was measured by using the standard phytase assay method at various pHs. Glycine-HCl buffer pH 3.0 and 3.6 (●), acetate buffer pH 3.6-6.0 (○) and tris-HCl buffer pH 6.0-10.0 (▼) at a final concentration of 0.1 M were used as buffer in the reaction mixture. A unit was the amount of enzyme that released 1 μmole of inorganic phosphate in 1 min.

activity compared to that at time zero. It was shown that when stored at 4^{o}C, the enzyme was stable at 2 pH levels; i.e. pH 4 and pH 8, where the remaining activities were 98% and 118%, respectively, after the 2-hr storage (Figure 4). This could be reflecting the presence of 2 phytases in the recombinant strain. For the storage at 37^{o}C, after 2-hr, the remaining activity at pH 4 was unchanged while that at pH 8 was decreased to less than 60% (Figure 5). This could be due to the unstability of one of the two phytases. However, storing the enzyme at this temperature under pH10, there was another activity peak raised up. This indicated that one of these two phytases was more stable at such pH when stored at 37^{o}C.

1.2 **Effect of temperature on the phytase activity and stability**

In the case of optimum temperature, the P(EappA) phytase was determined by carrying out the reaction at various temperature from 30°C to 70°C. The enzyme showed maximum activity at 55°C (Figure 6).

For the effect of temperature on the stability of P(EappA) phytase, the samples were stored at various temperatures and samples were taken at various time intervals to determine for the remaining activity under the standard condition. The enzyme activity was highly stable at 45°C. This could be indicating that a long-term storage at 45^{o}C could activate the enzyme activity as the remaining activity raised up to nearly 250% of that of the original. Nonetheless, the remaining activities after 2-hr storage at 50°C and 55°C were decreased and reached to the undetectable level when stored at 60°C to 70°C (Figure 7).

1.3 Effect of trypsin, taurocholic acid and deoxycholic acid on the phytase stability

In order to use this enzyme as feed additive, study on the effect of trypsin, taurocholic acid and deoxycholic acid on the phytase stability was determined. These substances are some of those present in the animal digestive tracts. The study was done by storing the crude enzyme aliquots in the presence of 1% (w/v) of each substance at4^{o}C and 37^{o}C. Samples were taken at various time intervals to determine for the remaining activities.

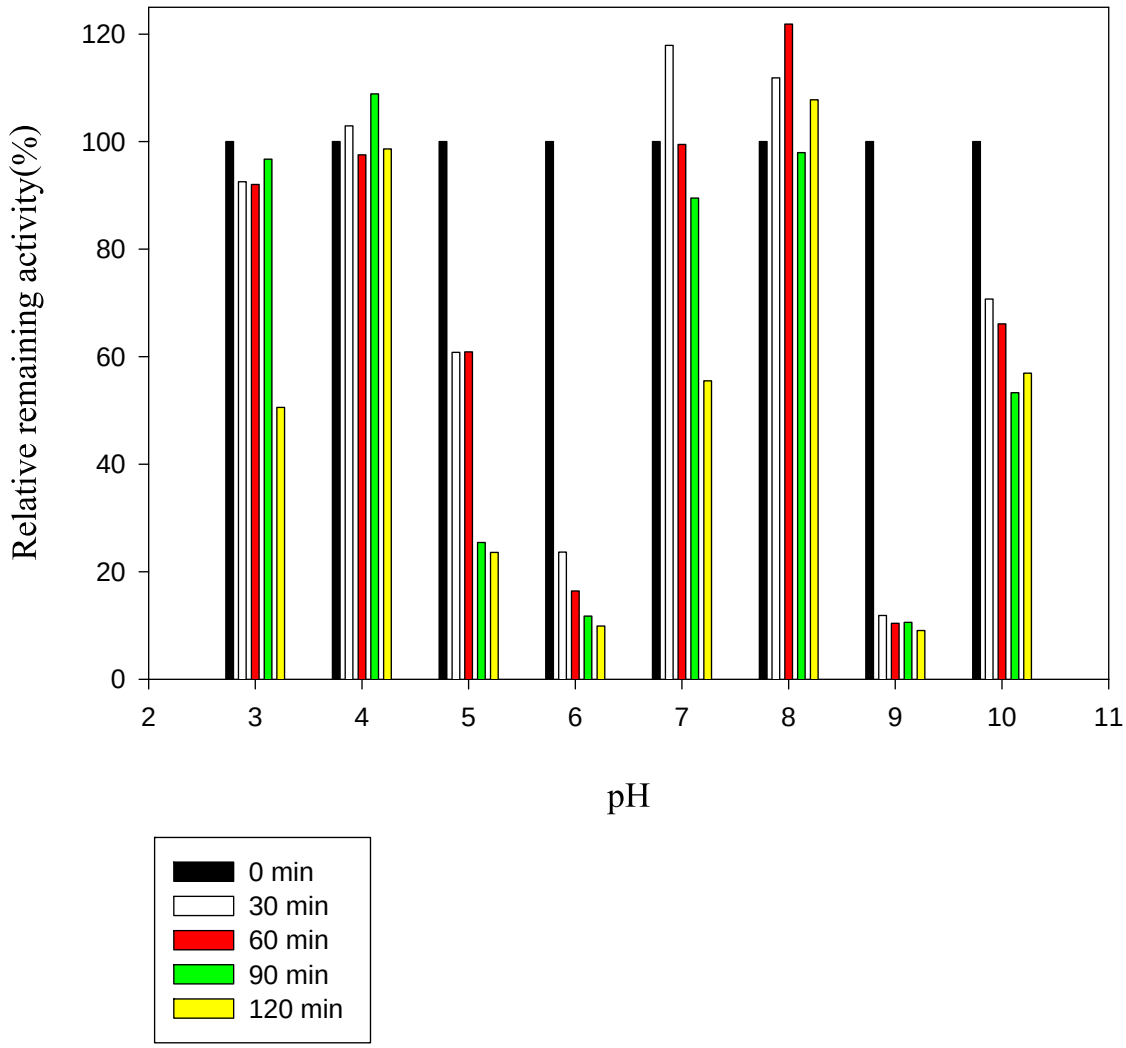

Figure 4 Effect of pH on the stability of crude phytase from P(EappA). P(EappA) phytase was incubated for 2-hr storage at various pHs, at 4°C. Glycine-HCl buffer pH 3.0 and 3.6, acetate buffer pH 3.6-6.0 and tris-HCl buffer pH 6.0-10.0 at a final concentration of 0.1 M were used as buffer in the reaction mixture. Phytase activity was measured by using the standard phytase assay. The remaining activity was expressed as the relative value to that of initial activity (100% is 44.78 U/ml).

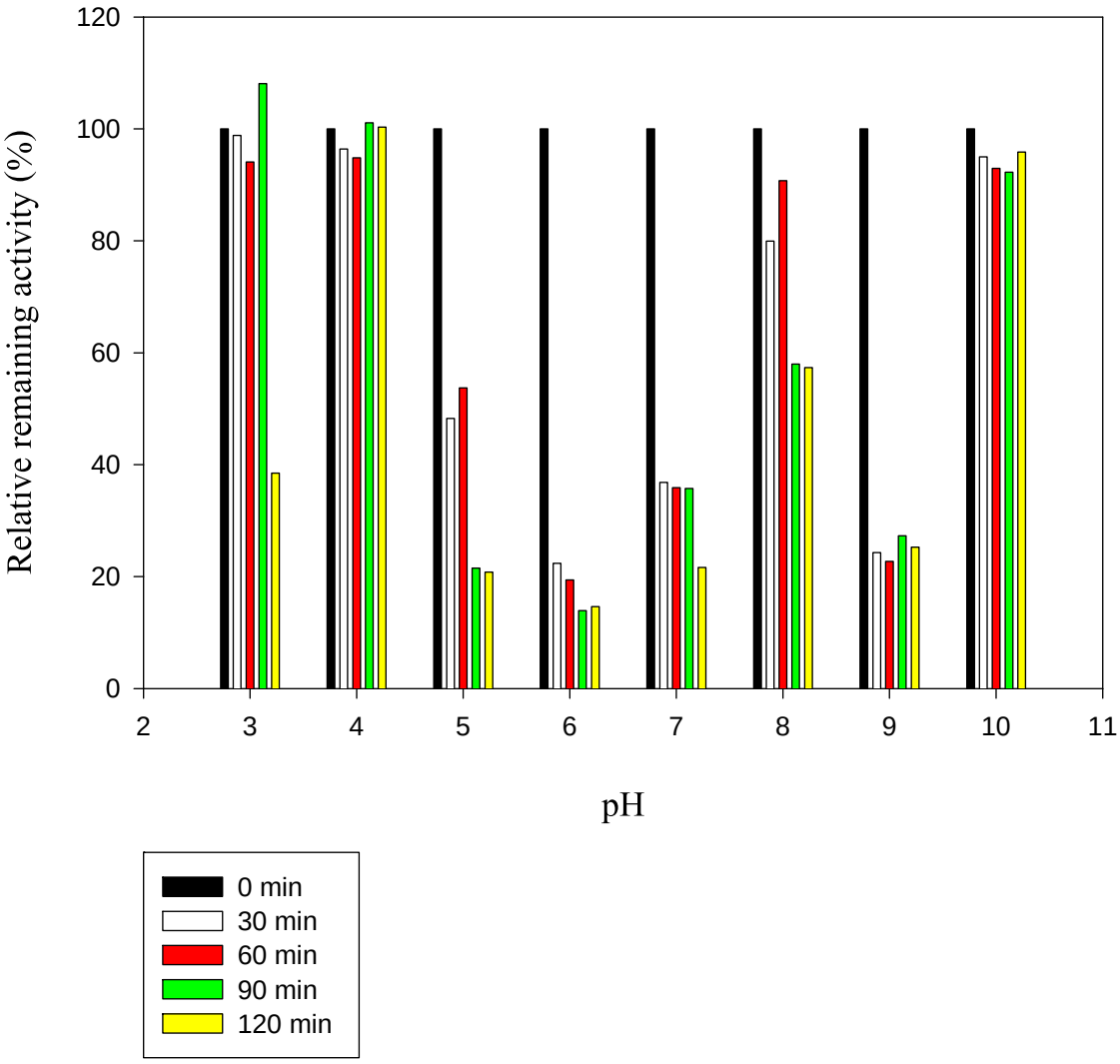

Figure 5 Effect of pH on the stability of crude phytase from P(EappA). P(EappA) phytase was incubated for 2-hr storage at various pHs, at 37°C. Glycine-HCl buffer pH 3.0 and 3.6, acetate buffer pH 3.6-6.0 and tris-HCl buffer pH 6.0-10.0 at a final concentration of 0.1 M were used as buffer in the reaction mixture. Phytase activity was measured by using the standard phytase assay. The remaining activity was expressed as the relative value to that of initial activity (100% is 44.78 U/ml).

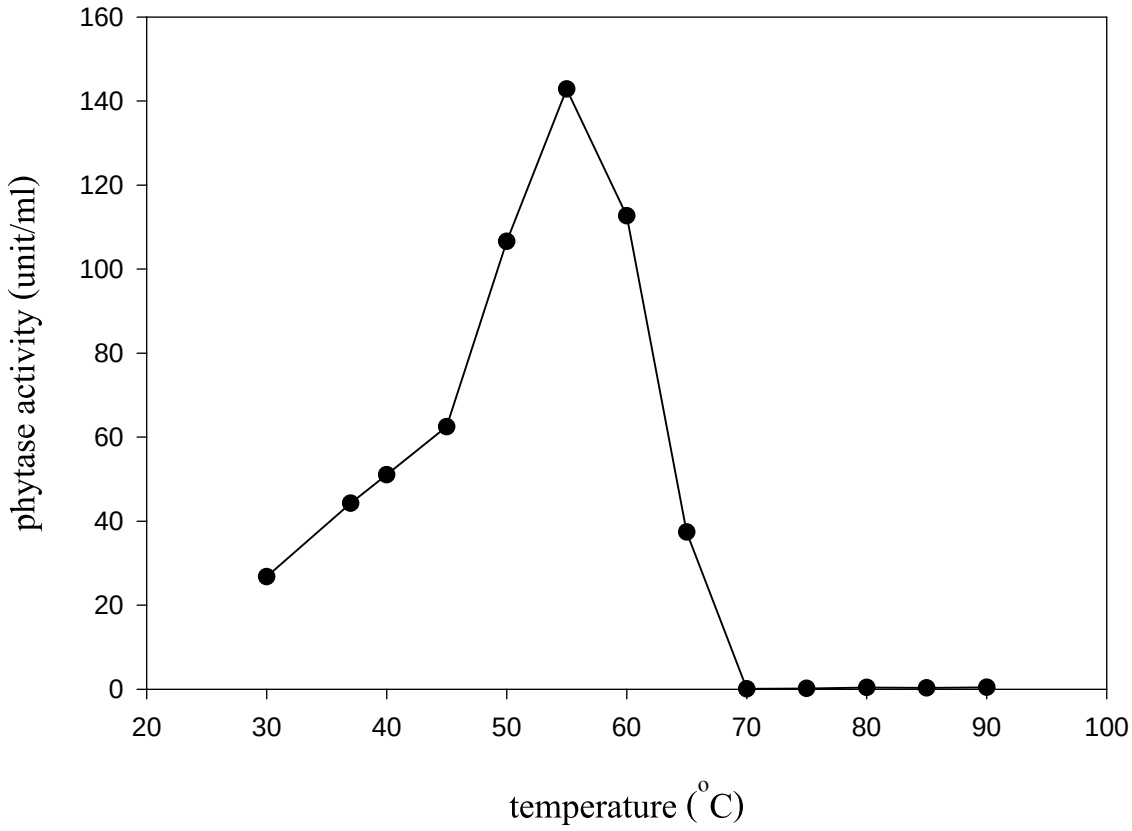

Figure 6 Effect of temperature on the activity of crude phytase from P(EappA). Phytase activity was determined by using the standard phytase assay method at various temperatures. A unit was the amount of enzyme that released 1μmole of inorganic phosphate in 1 min.

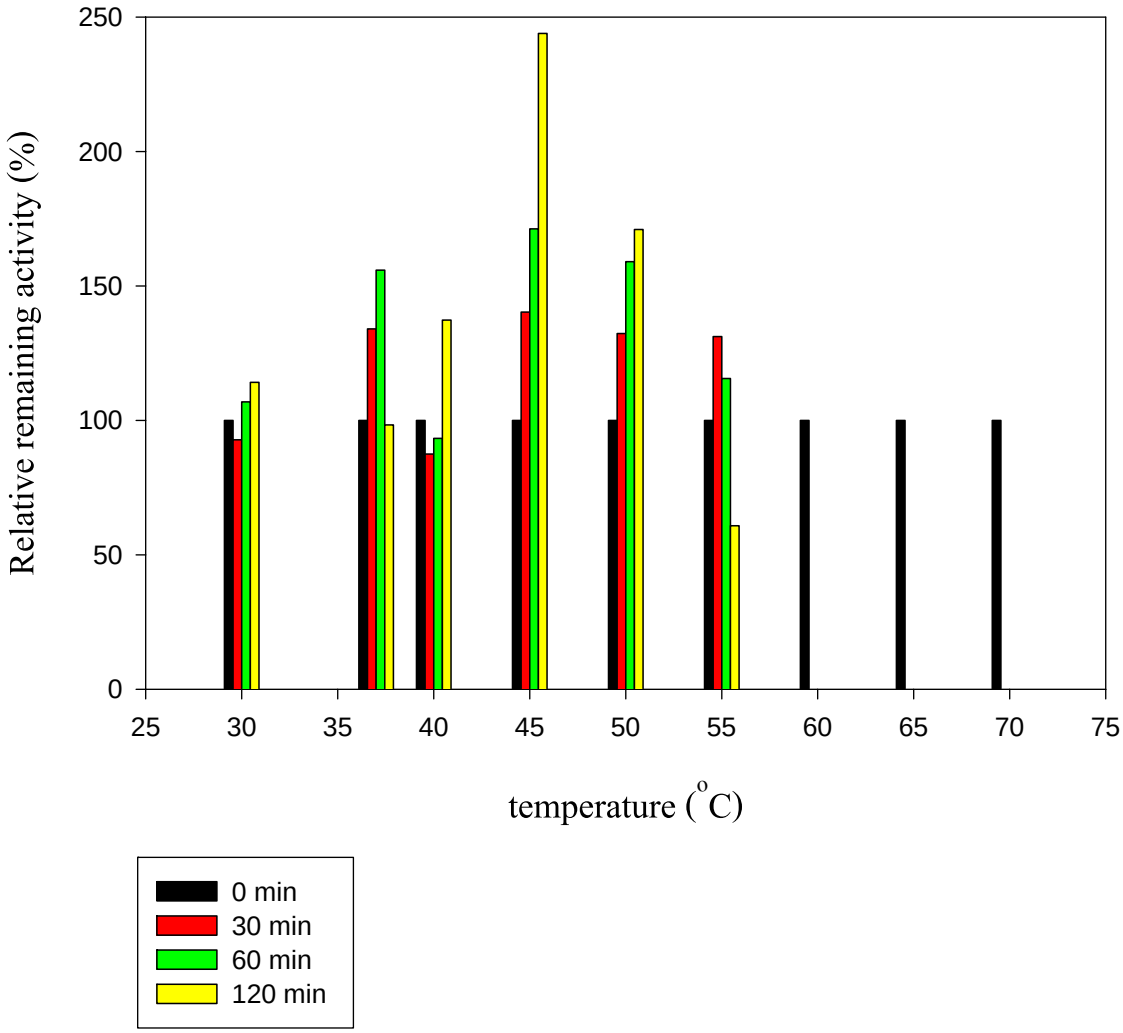

Figure 7 Effect of temperature on the phytase stability of crude enzyme from P(EappA). P(EappA) phytase was incubated for 2-hr at various temperatures. Phytase activity was measured by using the standard assay method. The remaining activity was expressed as the relative value to that of initial activity (100% is 44.78 U/ml).

The results were expressed as the remaining activity based on that at time zero as 100%. It was found that the enzyme was sensitive to all the solution tested when stored at either 4°C or 37°C (Figure 8 and 9). This indicated that the enzyme could only be used for pretreatment of the raw materials prior to the feed formulation process.

1.4 Effect of propionic acid and lactic acid on the phytase stability

As it has been reported that some organic acids could be used to enhance the efficiency of phytate digestion and promote the improvement of the mineral adsorption in the digestive tract (Hohler and Pallauf, 1993; Ravindran and Kornegay, 1993). Thus, the effect of lactic acid and propionic acid on the phytase stability was evaluated. It was carried out by storing the crude enzyme in the presence of the two acids, each at two levels of concentrations; i.e. 0.1% and 1.0% (v/v). The samples were prepared in 2 sets; one was stored at 4°C, and the other was at 37°C. Samples were taken at various time intervals to determine for the remaining phytase activities. The result showed that the remaining enzyme activities decreased when stored with propionic acid at either concentration at both storage temperatures. On the contrary, when stored with lactic acid under every condition tested, the remaining activities of enzyme were enhanced (Figure 10 and 11).

1.5 Effect of metal ions and EDTA on phytase stability

The effect of metal ions and EDTA on phytase stability were determined since it has been reported that some metal ions could enhance phytase activity and stability (Dvorakova *et al.*, 1997; Greiner *et al.*, 1993). The crude enzyme was incubated in the presence of 1 mM each of Na^{+}, K^{+}, Ca^{2+}, NH_4^{+}, Mg^{2+}, Mn^{2+}, Co^{+}, Zn^{2+}, Cu^{2+}, Fe^{2+} and EDTA at 4°C and 37°C for 2 hr. The effect of metal ions and EDTA on phytase stability was evaluated as the percentage of the relative remaining activity. It was found that P(EappA) phytase was insensitive to EDTA. This indicated the ion-independent

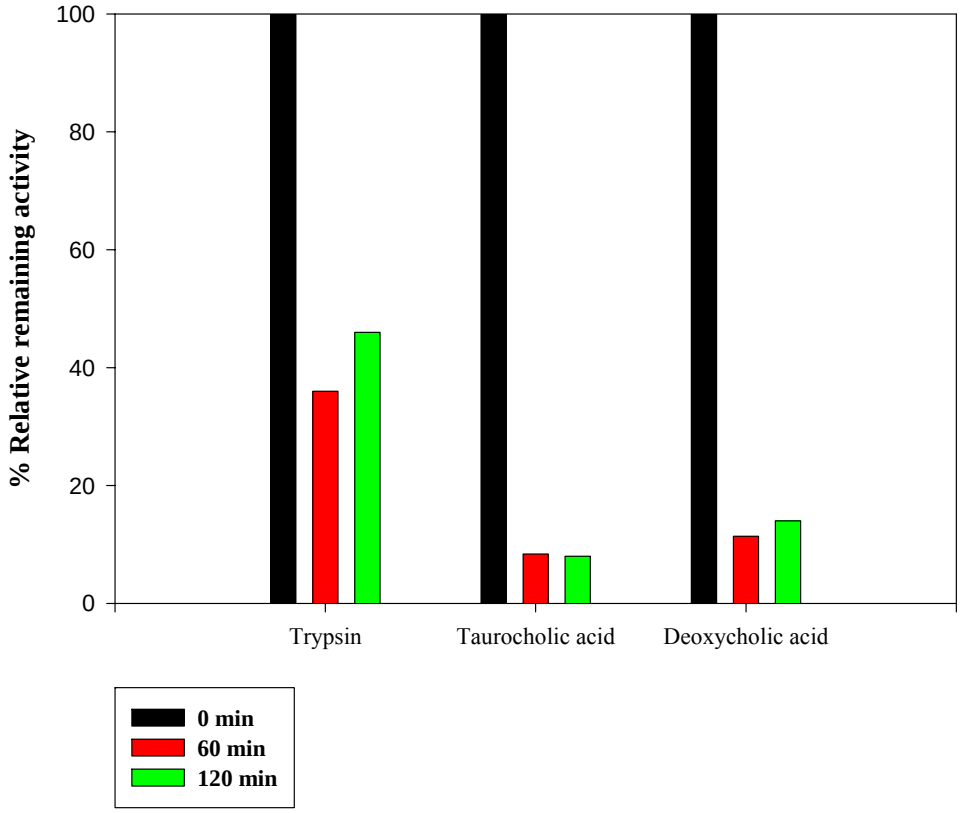

Figure 8 Effect of trypsin, taurocholic acid and deoxycholic acid on the phytase activity of crude enzyme from P(EappA). Phytase was incubated with 1.0% (w/v) trypsin, taurocholic acid and deoxycholic acid, samples were separated in each solution, at 4°C for 2hr. Samples were taken to determine the remaining activity under the standard condition. The remaining activity was expressed as the relative value to that of initial activity (100% is 44.78 U/ml).

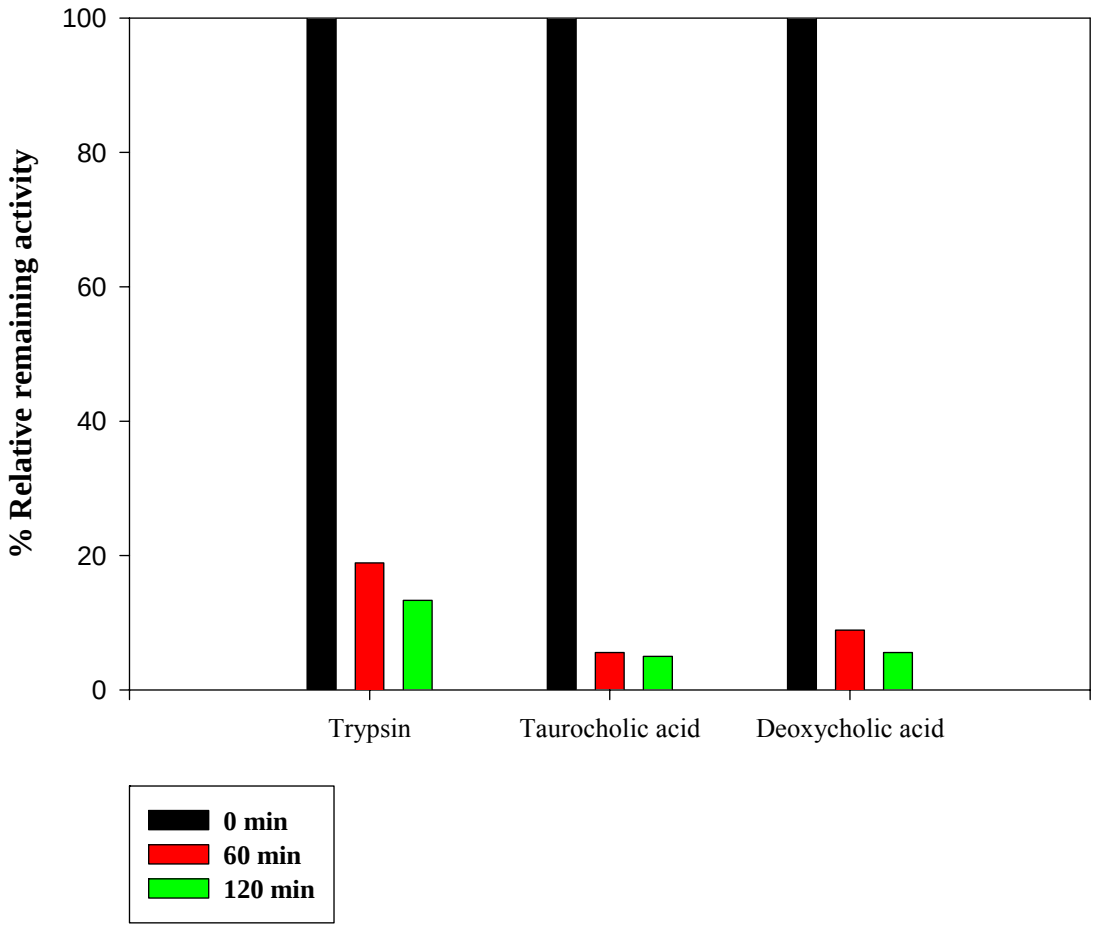

Figure 9 Effect of trypsin, taurocholic acid and deoxycholic acid on the phytase activity of crude enzyme from P(EappA). Phytase was incubated with 1.0% (w/v) trypsin, taurocholic acid and deoxycholic acid, samples were separated in each solution, at 37°C for 2hr. Samples were taken to determine the remaining activity under the standard condition. The remaining activity was expressed as the relative value to that of initial activity (100% is 44.78 U/ml).

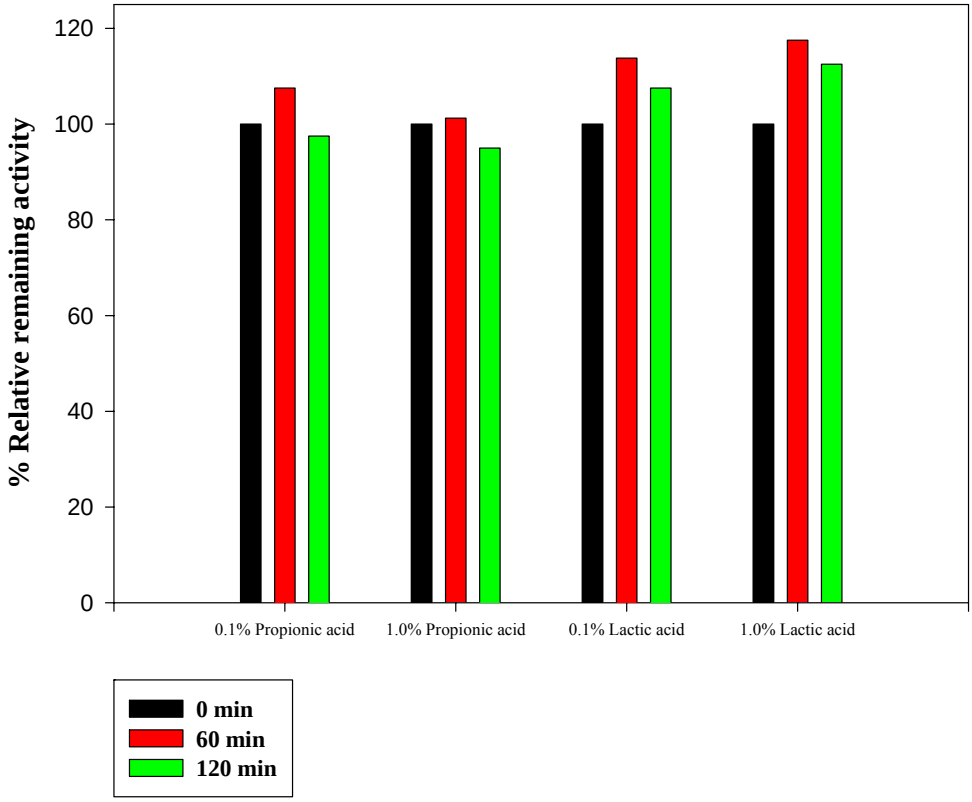

Figure 10 Effect of propionic acid and lactic acid on the phytase stability of crude enzyme from P(EappA). P(EappA) phytase was incubated at 4°C for 2 hr at 0.1% and 1.0% (v/v) of propionic acid and lactic acid. The remaining activity was expressed as the relative to that of the initial activity (100% is 44.78 U/ml).

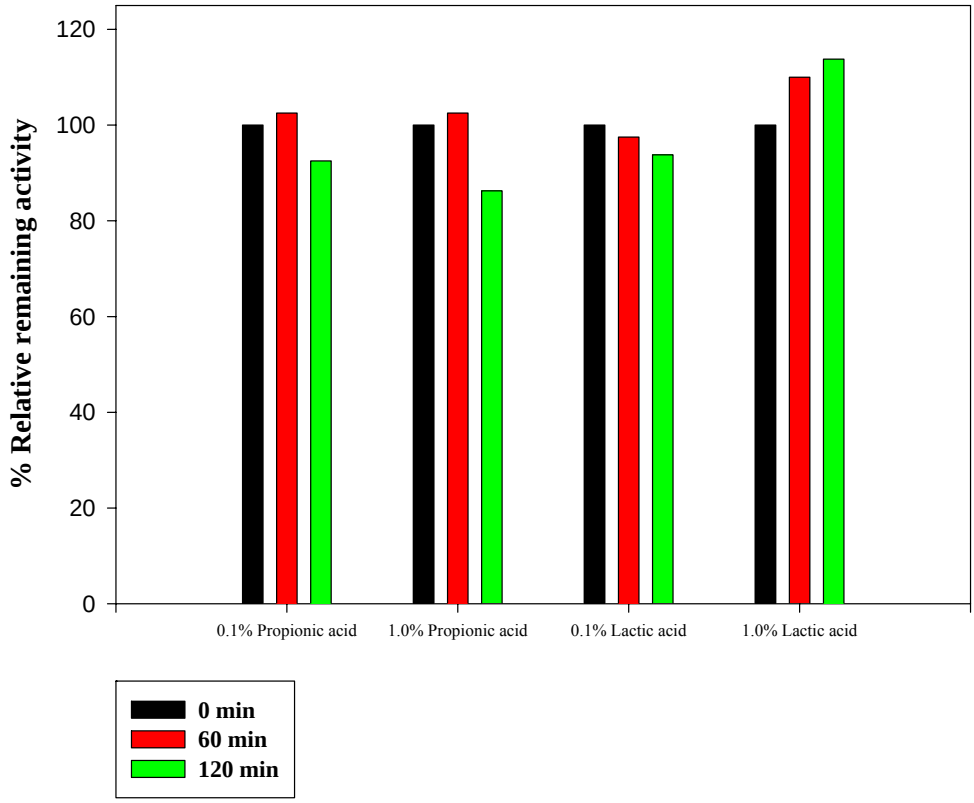

Figure 11 Effect of propionic acid and lactic acid on the phytase stability of crude enzyme from P(EappA). P(EappA) phytase was incubated at 37°C for 2 hr at 0.1% and 1.0% (v/v) of propionic acid and lactic acid. The remaining activity was expressed as the relative to that of the initial activity (100% is 44.78 U/ml).

property of the enzyme. Nonetheless, the enzyme activity was markedly increased when stored with Mg^{2+} at either temperature. In addition, the enzyme was stable towards Na^{+}, K^{+}, Mn^{2+} and Co^{+} but unstable towards Ca^{2+}, NH_4^{+}, Cu^{2+}, Fe^{2+} and Zn^{2+}, at both temperatures (Figure 12 and 13).

2. Media development for phytase production from P(EappA)

To reduce the production cost of enzyme, a reduction in raw material cost would be one strategy. Therefore, an aim to search for low cost material for growth and phytase production from P(EappA) was performed. Throughout the medium development process, all the media tested were added with 15 μg/ml tetracycline for preventing the lost of the plasmid in P(EappA). Samples were taken at 24-hr and 48-hr of incubation and determined for growth and phytase activity. The medium development was first carried out based on two kinds of minimal media, namely, MMP and PSM. This was also compared to that obtained from LB as a control. It was found that PSM could support a better growth and phytase production than MMP (Table 5). Taking out phytate from PSM caused increasing in both growth and phytase production from P(EappA). Replacing phytate with other kinds of inducers; i.e. 10% extracts of rice bran, wheat bran and soybean meal, were tried. It was found that 10% soybean meal extract could promote the highest phytase activity. It even enhanced phytase activity to a higher level than that obtained from the LB culture. The P(EappA) phytase activity obtained from the 48-hr culture in PSM-phytate + 10% soybean meal (SM) was 143.99 U/ml. This was, then, referred as the medium no.1 which was used for further medium development.

Although, the medium no.1 (PSM + 10% soybean meal) expressed the highest phytase activity, it still showed low level of growth. Attempt was carried out in order to enhance growth by adding yeast extract (YE) as the nitrogen and vitamin sources. So, the medium no.1 was added with various concentrations of yeast extract; i.e. 0.1%, 0.5%, 1.0% and 1.5% (w/v). The result showed that yeast extract at a concentration of 1% (w/v) was enough to give a better growth and enzyme production. The highest levels of phytase activities from the cultures with 0.1% and 0.5% yeast extract were found at 24-hr; i.e

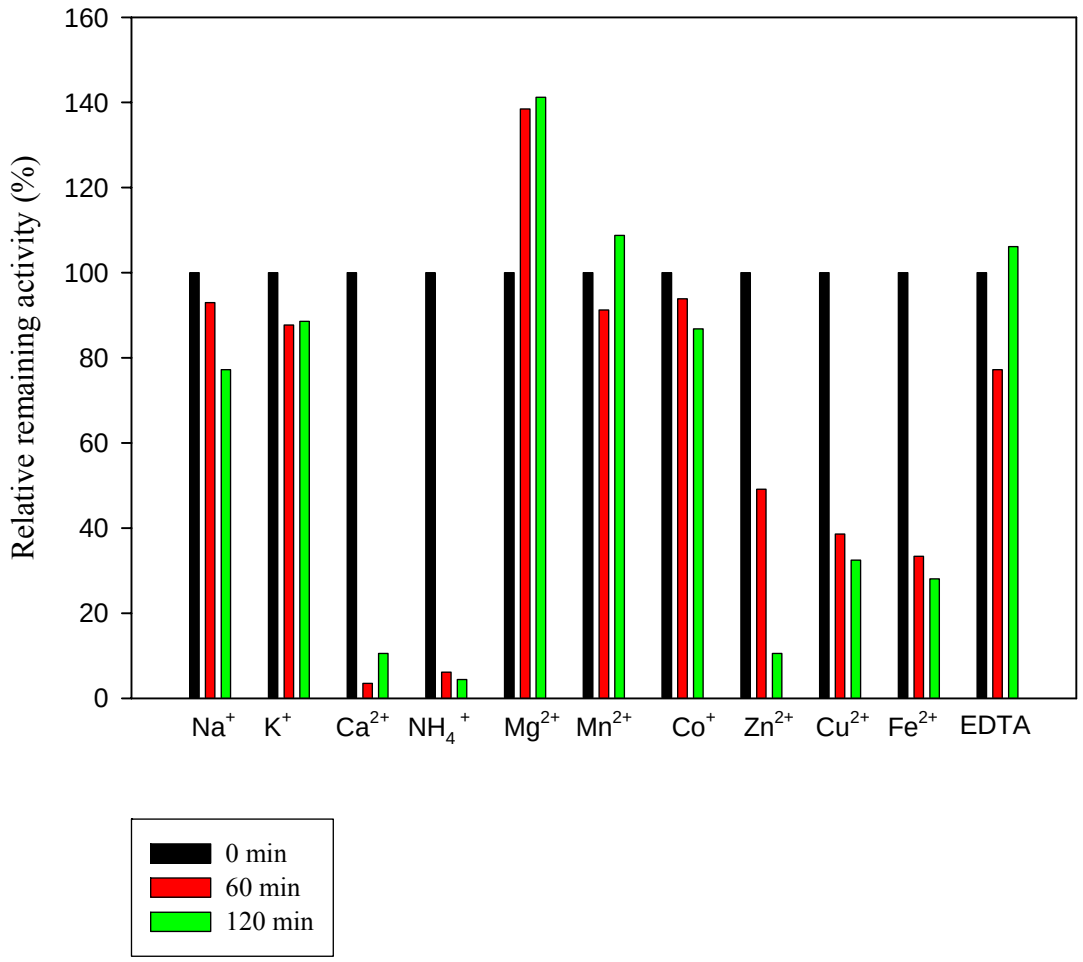

Figure 12 Effect of metal ions and EDTA on the stability of crude P(EappA) phytase. The enzyme was incubated in the presence of 1 mM of each Na^{+}, K^{+}, Ca^{2+}, NH_4^{+}, Mg^{2+}, Mn^{2+}, Co^{+}, Zn^{2+}, Cu^{2+}, Fe^{2+} and EDTA at 4°C for 2-hr. The remaining activity was expressed as the relative value to that of the initial activity. The phytase activity under the condition without ions at time zero was 44.78 U/ml (100%).

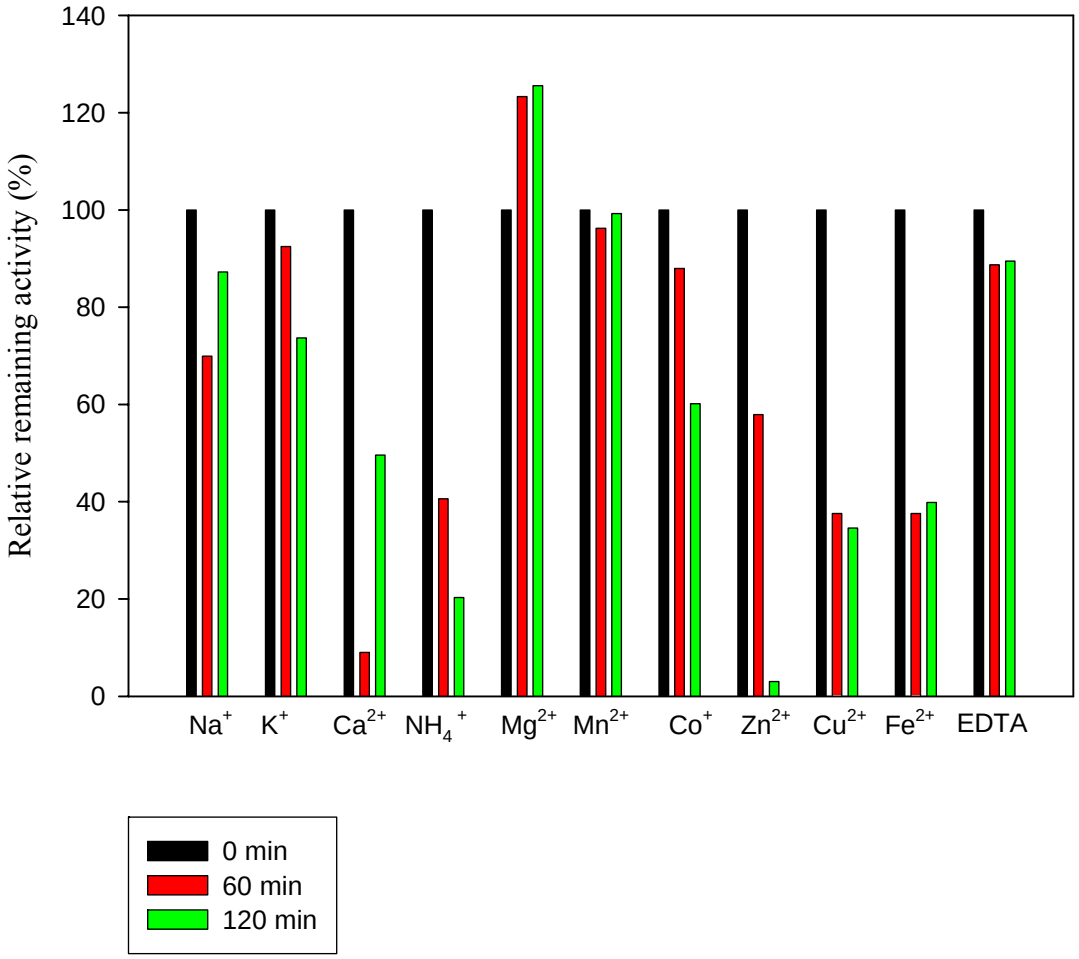

Figure 13 Effect of metal ions and EDTA on the stability of crude P(EappA) phytase. The enzyme was incubated in the presence of 1 mM of each Na^{+}, K^{+}, Ca^{2+}, NH_4^{+}, Mg^{2+}, Mn^{2+}, Co^{+}, Zn^{2+}, Cu^{2+}, Fe^{2+} and EDTA at 37°C for 2-hr. The remaining activity was expressed as the relative value to that of the initial activity. The phytase activity under the condition without ions at time zero was 44.78 U/ml (100%).

Table 5 Growth and phytase production of P(EappA) in various media.

Media	Growth (OD_{600})		Phytase activity(U/ml)	
	24 hr	48 hr	24 hr	48 hr
LB	3.13	3.22	20.57	62.68
MMP	1.15	0.34	1.19	0.07
PSM	0.02	0.2	0.28	1.09
PSM-phytate	0.01	0.05	0.02	0.01
PSM-phytate +10% rice bran	0.07	1.14	UD[1]	UD[1]
PSM-phytate +10% wheat bran	0.3	0.16	15.55	74.53
PSM-phytate +10% soybean meal (no.1)	0.21	0.29	88.78	143.99

[1]UD: Undetectable (Phytase activity less than 0.01 U/ml).

160.99 U/ml and 161.17 U/ml, respectively (Table 6). Higher concentration of yeast extract did not enhance any higher growth and phytase production. Thus, this was the medium no.2 (PSM-phytate + 10% soybean meal medium with 0.1%(w/v) yeast extract) and was used for further development.

In the medium no.2, a proper kind of carbon source was investigated. In the absence of glucose, the culture could still grow and produce phytase to a certain level. This indicated that the culture could have gained carbon from other sources in the medium composition as well. Replacing glucose with 1% molasses, a slightly higher activity but still be lower than that with glucose as carbon source was obtained. Thus, glucose was retained as a medium composition. In order to ease the medium preparation process, instead of autoclaving glucose separately from other medium composition as it was done before, glucose was tried included to the medium prior to autoclaving. The result showed that the medium no.2 which included glucose with other medium composition and autoclaved altogether gave even higher growth and phytase activity than that from the medium no.2 with separately autoclaved glucose (Table 7). Thus, sterilization of the glucose together with or separately from the other medium compositions did not make much differences to either growth or phytase production from P(EappA). Therefore, this was called the medium no.3, which was the medium no.2 which glucose was autoclaved together with other medium composition, and was used to the further development.

To further enhance the phytase productivity, the effect of surfactants; i.e. Tween 20 and Tween 80, and citric acid was investigated. The medium no.3 was added with Tween-20 and Tween-80, each of which at 2 levels of concentration; i.e. 0.1% and 1.0% (v/v). Addition of citric acid was also tested in another set of the medium no.3 at 2 concentrations; i.e. 0.1% and 1.0% (w/v). Samples were taken at 24 and 48-hr of cultivation and determined for growth rate and phytase activity. The result showed that the medium no.3 added with 1.0% (v/v) Tween-20 showed the highest growth and phytase activity, when comparing to that with Tween-80 and that without the addition.However, among all medium tested, the medium no.3 added 0.1% (w/v) citric acid expressed the highest phytase activity (Table 8). Thus, this was called the medium no.4, which was the medium no.3 + 0.1% (w/v) citric acid, and was used for the further development.

Table 6 Growth and phytase production of P(EappA) in PSM-phytate +10% SM with various concentrations of yeast extract

Media	Growth (OD_{600})		Phytase activity(U/ml)	
	24 hr	48 hr	24 hr	48 hr
no.1[1]	0.03	0.36	147.53	133.15
no.1 +0.1% YE (no.2)	0.59	0.49	160.99	150.75
no.1 +0.5% YE	0.86	0.82	161.17	138.64
no.1 +1.0% YE	0.95	0.89	122.22	147.63
no.1 +1.5% YE	0.35	0.34	147.69	148.58

[1]no.1 medium: PSM- phytate +10% soybean meal extract

Table 7 Effect of glucose adding time on growth and phytase production of P(EappA)

Media	Growth (OD_{600})		Phytase activity (U/ml)	
	24 hr	48 hr	24 hr	48 hr
no.2[1] (with glucose sterilized separately)	0.88	2.14	22.91	160.99
no.2 with glucose omitted	0.52	0.78	77.47	83.74
no.2 but replacing glucose with 1% malasses	0.01	0.01	48.77	92.80
no.2 included glucose before sterilization (no.3)	1.20	7.79	100.12	165.00

[1]no.2 medium: PSM –phytate +10% soybean meal extract +0.1% YE

Table 8 Effect of surfactant and citric acid on growth and phytase production of P(EappA)

Media	Growth (OD_{600})		Phytase activity(U/ml)	
	24 hr	48 hr	24 hr	48 hr
no.3[1]	5.58	5.79	77.65	161.00
no.3 +0.1% Tween 20	5.02	6.26	61.03	151.62
no.3 +1.0% Tween 20	5.24	7.08	33.33	173.86
no.3 +0.1% Tween 80	6.91	6.83	62.93	170.23
no.3 +1.0% Tween 80	5.81	9.52	84.88	150.62
no.3 +0.1% citric acid (no.4)	0	3.06	41.62	177.48
no.3 +1.0% citric acid	0.02	1.31	0.35	0.51

[1]no.3 medium : PSM –phytate +10% soybean meal +0.1% YE and included glucose before sterilization

As it has been reported that some kinds of starch or flour could further enhance phytase production from microorganisms (Gibson, 1987). Thus, in this experiment, rice flour, corn flour, tapioca starch and soluble starch, each was tried using as the inducer for growth and phytase production from P(EappA). For the starch property, it can form starch granule which does not dissolve in water. As in granulated form, the starch can not provide any nutrient for bacterial adsorption. So to ensure the dissolving of starch and homogenously dispersed in the medium solution, 1% of each kind of starch or flour was pre-heated before sterilization until gelatinized. This helped preventing the starch from granulation. After sterilization, the cultivation was carried out as the usual method and samples were taken at 24 and 48 hr for determination of growth and phytase production. The result showed that the medium no.4 containing 1% of tapioca starch expressed the highest activity at 24 and 48 hr; i.e. 129.39 and 179.62 U/ml, respectively. However, it was not significantly different from that obtained from the medium no.4 by itself. Therefore, it was considered not necessary to use tapioca starch as an additional inducer in this medium (Table9).

Up to this study, the medium no.4 that promoted the bacterial growth and phytase production was composing of PSM which phytate was omitted and, then, supplemented with 10% soybean meal extract, 0.1% yeast extract and 0.1% citric acid with glucose was included in the medium prior to sterilization. This medium was name SMYC medium.

Kinetics of growth, phytase production, reducing sugar content and pH were investigated. This was done in SMYC medium prepared as 500 ml medium in 1-liter flask. The culture condition was at 30°C with 200 rpm agitation. Samples were taken at every 3 hr during the first of 24-hr, and then, were done at every 6 hr until reaching to 48-hr of cultivation. These were for the determination of growth, phytase production, reducing sugar content and pH. For the result, it showed that P(EappA) slowly grew during the first 10 hr, and then rapidly raised up to at 12 hr which it entered the log phase growth. The growth rate during this phase was 0.1 OD_{600}/hr. The maximum growth was reached at the 18-hr and entered the stationary phase. In the case of phytase production, the phytase activity rapidly increased concomitant with growth, at the production rate of 6.32 U/hr, and leveled off at the 18-hr of cultivation.

Table 9 Effect of starch as an inducer on growth and phytase production of P(EappA)

Media	Growth(OD_{600})		Phytase activity(U/ml)	
	24 hr	48 hr	24 hr	48 hr
no.4[1]	0.22	13.9	41.6	177.50
no.4+1.0% corn flour	0.02	6.61	61.66	90.69
no.4+1.0% rice flour	0.02	6.33	78.00	40.75
no.4+1.0% tapioca starch	0.04	9.20	129.39	179.62
no.4+1.0% soluble starch	0.25	10.96	119.93	27.46

[1]no.4 medium: PSM –phytate +10% soybean meal +0.1% YE and included glucose before sterilization with 0.1% citric acid

After that, the activity increased again at the 36-hr at lower rate of 3.77 U/hr until 48 hr which was the end of cultivation. For the reducing sugar content, it was decreased slowly in the first 30 hr, and it was rapidly decreased when the growth rate and phytase activity rate came to a stable phase. The pH value of the culture did not show any vigorous changes during cultivation. At the completion of cultivation, the phytase activity reached 177.62 U/ml (Figure 14).

3. Other enzyme activities in the SMYC culture supernatant of P(EappA)

The presence of other enzyme activities were determined from the crude enzyme which was the culture supernatant of P(EappA) grown in SMYC medium. The culture condition was done at 30^{o}C with 200 rpm agitation for 48 hr. The enzyme activities determined were amylase, protease, lipase, cellulase, xylanase and endoglucanase. There were high amylase and cellulase activities, which were 69.50 kU/ml and 83.10 kU/ml, respectively. For xylanase and endoglucanase, they were presented at lower activities than the two previous enzymes; i.e. 31.70 kU/ml and 31.20 kU/ml, respectively. Protease and lipase activities were not detected**.**

4. The formulation and storage of P(EappA) phytase produced from SMYC medium

Prior to formulation, the enzyme had to be concentrated. This can be done by a few methods. However, the easiest one would be precipitation. Protein precipitation can be done by a few methods; i.e. ammonium sulfate precipitation, organic solvent precipitation and poly (ethylene glycol) [PEG] precipitation (Ingham, 1984; Kaufman, 1971; Scopes, 1982). The first two methods are mostly chosen for this purpose. However, organic solvent precipitation often causes protein denaturation while the ammonium sulfate precipitation is a much milder process (Scopes, 1982). Moreover, ammonium sulfate is a cheaper and safer chemical than organic solvent. Thus, ammonium sulfate precipitation was a method of choice for concentration of P(EappA) phytase. All of the 500 ml supernatant from the P(EappA) culture in SMYC medium was precipitated by 80% saturation of ammonium sulfate. The condition for precipitation was at 0°C to 4^{o}C.The concentrated protein was collected

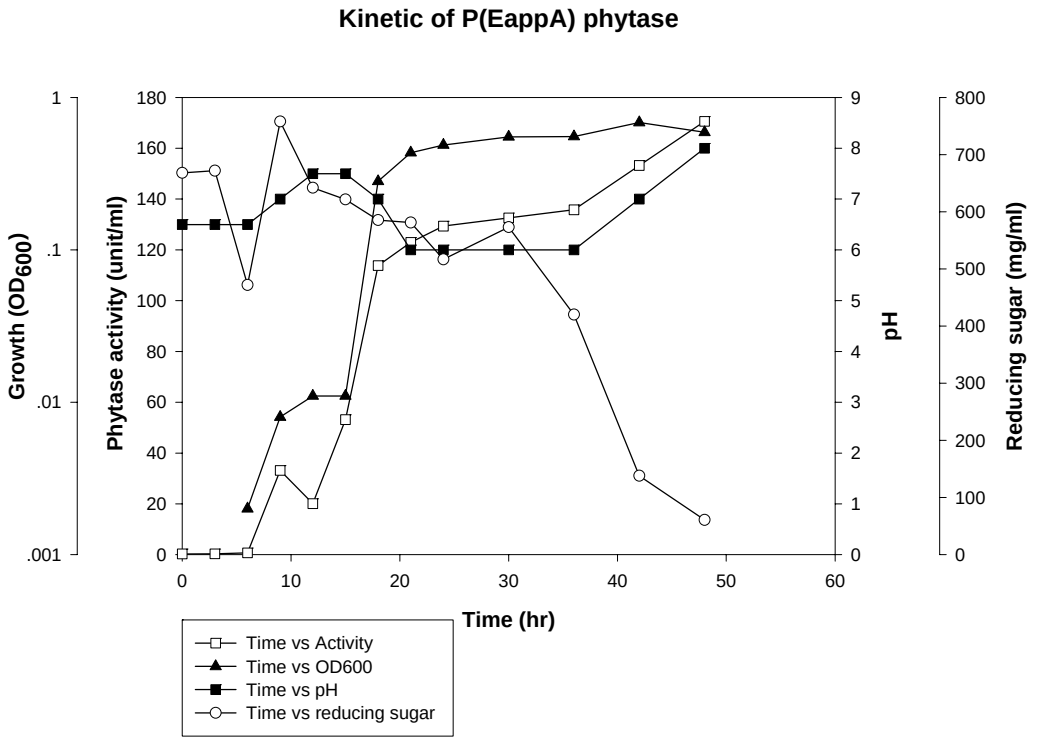

Figure 14 Growth rate, phytase activity, reducing sugar content and pH determinations of P(EappA) in SMYC medium.

and determined the total protein and phytase activity by comparing with the crude enzyme. The result showed that the total protein was decreased from 7.86 mg/ml in crude enzyme to 2.66 mg/ml in concentrated enzyme. For the enzyme activity, the phytase activity of concentrated enzyme was 197.40 U/ml, which was at 1.11 times higher than that the pre-concentrated enzyme (Table 10). Moreover the specific activity of enzyme was enhanced from 22.60 U/mg in crude enzyme to 74.21 U/mg in concentrated enzyme. It was at 3.28 times higher than that the pre-concentrated enzyme.

After protein concentration by ammonium sulfate precipitation, the concentrated enzyme was studied for the effect of types of the enzyme precipitate and temperature on P(EappA) phytase activity. The concentrated enzyme was distributed into 3 parts for preparation of the enzyme into 3 forms: 1) resuspension form which was the ammonium sulfate precipitated protein resuspending in 8 ml of 0.05 M acetate buffer, pH 5.6, 2) air-dried enzyme precipitate form; and 3) wet enzyme precipitate form. Each form was further divided into 2 portions; one was stored at 4°C, and the other was at 25°C, for 28 days. Samples were taken once every 7 days to determine the phytase activity. Before the storage, samples from every form and condition were determined the activity at the time of zero as controls. For the resuspension form, the activity of enzyme at 4°C was decreased from 191.40 U/ml to 129.05 U/ml at the last week of storage. However, the enzyme activity at 25°C was increased from 191.40 to 208.51 U/ml at the last week of the storage. This increase in the enzyme activity could be due to evaporation of water from the enzyme suspension. For the air-dried precipitate form, the phytase activity at both temperatures was decreased from 168.82 U/ml to 159.38 U/ml when stored at 4°C and to 154.38 U/ml when did at 25°C on the last week of storage. These decreasing activities between two storage temperatures were not significantly different, and they still expressed the relative remaining activity up to 90% even when it got to the last week. For the wet precipitate form, the phytase activity at 4°C was decreased from 103.39 U/ml to 84.9U/ml which was 82.15% remaining activity. However, the activity at 25°C was increased from 103.39 U/ml to 133.14 U/ml at the last week of storage. This, again, could be due to evaporation of the moisture content from the wet precipitate (Figure 15).

5. Feed – based phytase formulation

The three forms of concentrated phytase; i.e. resuspension, air-dried precipitate and wet precipitate were further formulated as feed-based form. The efficiency for each of concentrated enzyme was evaluated as the enzyme activity. The enzyme was mixed with chicken feed to obtain 500 units of enzyme per 1 kg of feed. The control sample was prepared by mixing chicken feed with distilled water to obtain the same level of moisture content. Samples were stored at 4°C and 25°C and were taken to determine the phytase activity which affect to the chicken feed, once every 7 days until 28-day of storage. For the formulation with the enzyme resuspension, the phytase activity of the samples stored at 4^{o}C increased during the first week and then sharply decreased to approximately 50% of the original activity. In the third week, the activity was unchanged and then raised up to about 15 U/g at the last week. For the same formulae but stored at 25°C, the activity reduced by approximately 50% during the first week and then stayed with slightly fluctuated until the last week of storage. In the case of feed mixed with the air-dried precipitate form, the activity of this formulae either during storage at 4^{o}C or 25^{o}C showed the relatively stable activity. The activities under either storage temperatures were up and down slightly until they got to 7.51 U/g feed for the sample at 4^{o}C and to 9.12 U/g feed for that at 25^{o}C in the last week of storage. For the feed mixed with the wet precipitate formulae, the activity decreased from 20.68 U/g feed to 11.60 U/g feed when stored at 4^{o}C and to 8.36 U/g feed when stored at 25^{o}C (Figure 16).

Table 10 Summary of the concentration of P(EappA) phytase in SMYC medium.

Type of enzymes	Protein concentration (mg/ml)	Phytase activity (U/ml)	Specific activity (U/mg)
Crude enzyme	7.86	177.62	22.60
Concentrated enzyme	2.66	197.40	74.21

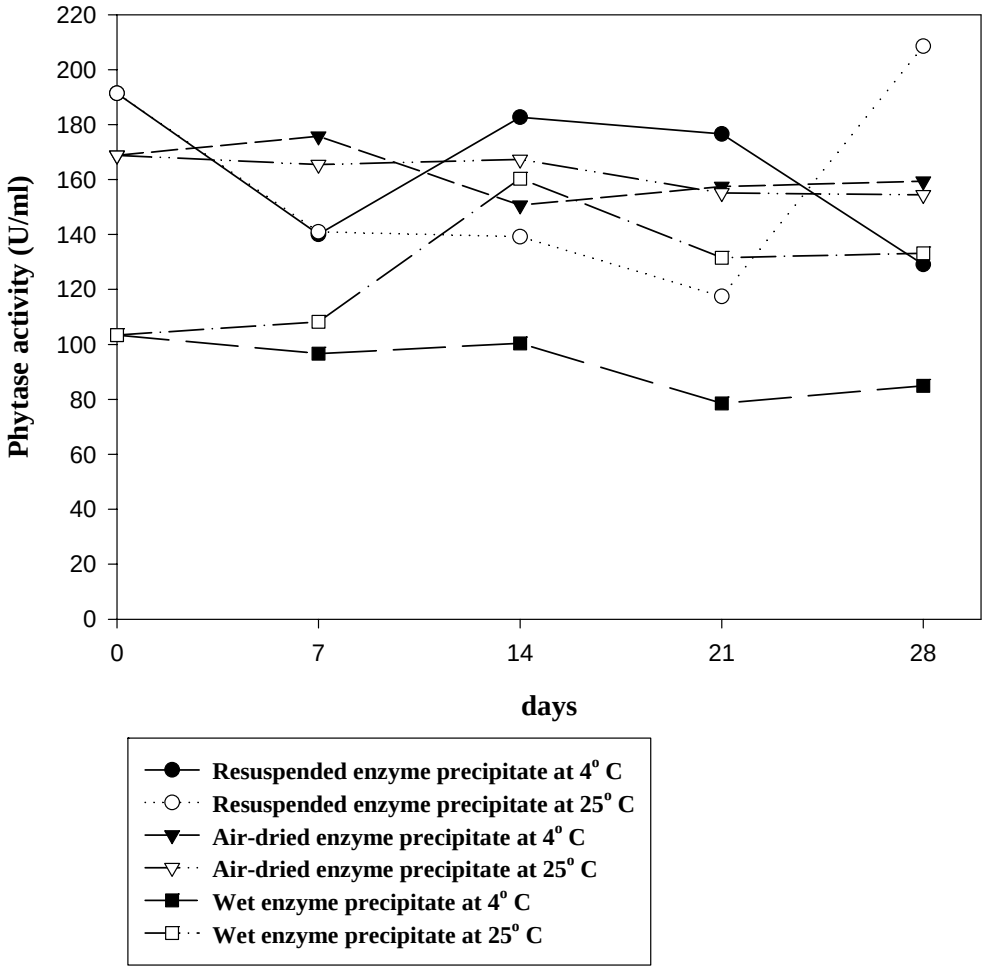

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Figure 15 The remaining activity of various forms of P(EappA) phytase activity during 28 days storage at 4°C and 25°C (see the method for 3 forms of concentrated P(EappA) phytase preparation in Chapter III, materials and methods).

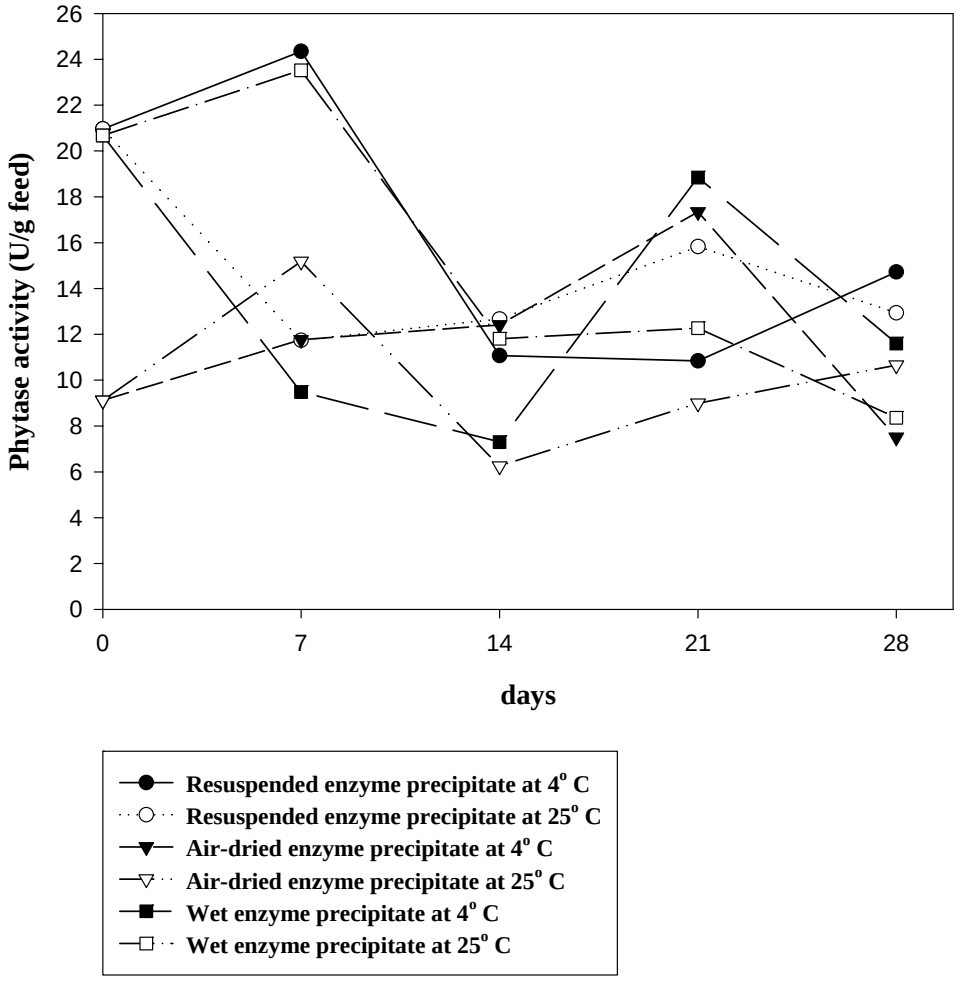

Figure 16 The phytase activities of various feed-based P(EappA) phytase forms during 28 day storage at 4°C and 25°C. The feed-based formula was prepared by mixing feed with various forms of the concentrated enzyme; i.e. enzyme precipitate resuspension, air-dried enzyme and wet enzyme precipitates. The control samples at both temperatures showed an average activity of 7.0 U/ml throughout the incubation and the data were omitted from this graph to reduce the confusion due to too many lines.

6. Digestion efficiency of P(EappA) phytase

This experiment was carried out to determine the digestion efficiency of crude P(EappA) phytase on various phytate containing materials; i.e. chicken feed and agricultural by-products which were used as raw materials for animal feed production; i.e. rice bran, wheat bran and soybean meal. Each of the phytate containing materials was mixed with crude P(EappA) phytase to obtain 500 units of enzyme per 1 kg of material. The control sample for each digestion was the material mixed with distilled water. After adding enzyme, the control and experimental samples were added with 20ml of 0.05 M acetate buffer pH 4.0 which was approximately the same level of that of chicken digestive tract (Gillespie, 1987) and some other young animals' stomach (Simell *et al.*, 1989). In addition, 1% each of trypsin, taurocholic acid, deoxycholic acid, lactic acid and propionic acid were added into the mixture. Then, all the samples were incubated at 37°C with 200 rpm agitation for 1 hr. The result was expressed as the value of phosphate, protein and reducing sugar contents. In digesting the chicken feed, it showed that the phosphate content was increased from the undetectable level found in the control sample to 16.89 μg/g feed in the experimental sample (Table 11). However, the protein and reducing sugar contents in the chicken feed were not significantly different between the two sets of sample. For rice bran digestion, the result showed that P(EappA) phytase could not help increasing the content of phosphate, protein and sugar to be available for animal consumption.

Different results were obtained from digesting wheat bran and soybean meal. The phosphate content in wheat bran was increased from 86 μg/g to 144.8 μg/g material while that in soybean meal did from the undetectable level to 42.3 μg/g. The protein content was increased from 79.8 μg/g to 116.8 μg/g of wheat bran and from 37.4 μg/g to 50.7 μg/g of soybean meal. However, the reducing sugar contents were not significantly different between the control and the experimental samples for both materials (Table 11).

7. Soymilk digestion

Soymilk has become a popular kind of healthy drink. It is replacing cow milk as a source of calcium. It also contains high protein content. However, the consumption of these nutritions was restricted due to the presence of an antinutritional factor, phytic acid or phytate (Khare *et al.*, 1994). This experiment was carried out to determine if P(EappA) phytase could help digesting phytate and, thus, releasing the phytate-bound nutrient to be available for adsorption. Soymilk was prepared as described in materials and methods session and mixed with crude P(EappA) phytase that was prepared from SMYC medium. The enzyme was added at 500 units enzyme per 1 liter soymilk. The control sample was soymilk added with distilled water, replacing the crude enzyme. The reaction was done at 37°C with 200 rpm agitation for 1 hr. The phosphate, protein and reducing sugar contents were determined. However, P(EappA) phytase at the concentration tested in this experiment was failed to increase the contents of phosphate, protein and reducing sugar in soymilk to detectable levels (Table 12). Higher concentration of the phytase might help improving the function. Alternatively, the condition for soymilk digestion with P(EappA) phytase might have to be optimized. This should have to be done in the future study.

Table 11 The phosphate, protein and reducing sugar contents in feed and phytate containing raw materials for feed formulation; i.e. rice bran, wheat bran and soybean meal before and after digestion with P(EappA) phytase.

Materials	P(EappA) phytase[1]	Phosphate content (μg/g material)	Protein content (μg/g material)	Reducing sugar content (mg/g material)
Feed	-	UD[2]	79.31	1.48
	+	16.89	76.85	1.40
Rice bran	-	UD	31.04	0.50
	+	UD	34.48	0.44
Wheat bran	-	85.97	79.80	2.48
	+	144.80	116.75	3.16
Soybean meal	-	UD	37.44	0.46
	+	42.26	50.74	0.70

[1]+: added (the experimental sample); -: not added (the control sample)

[2]UD: Undetectable (the value less than 1 μg/g of materials)

Table 12 The phosphate, protein and reducing sugar contents from soymilk digestion by crude P(EappA) phytase

P(EappA) phytase[1]	Phosphate content (mg/ml soymilk)	Protein content (mg/ml soymilk)	Reducing sugar content (mg/ml soymilk)
-	UD[2]	9.88	50.40
+	UD	10.27	56.40

[1]+: added (the experimental sample); -: not added (the control sample)

[2]UD: Undetectable (the value less than 1 μg/g of soymilk)

CHAPTER V
DISCUSSION

The general properties of crude P(EappA) phytase produced in LB medium with 15 µg/ml of tetracycline were investigated. Crude P(EappA) phytase exhibited the optimum pH for activity at 4.0, and it was completely inactivated at pH higher than 7.0. Similar results were found in phytase from *E. coli* K12 (ATCC 33965) (Greiner *et al*., 1993; Golovan *et al*., 1999), which activity was decreased when kept under acidic condition. In this study, crude P(EappA) phytase was stable at 2 pH levels; i.e. pH 4.0 and pH 8.0 when stored at 4^{o}C for 2 hr. However, when the 2-hr storage was carried out at 37^{o}C, the remaining activity at pH 4.0 was remained unchanged while that at pH 8.0 was decreased to less than 60%. Nonetheless, there was another activity peak raised up at pH 10.0 when stored at 37^{o}C. These could be due to the presence of two phytases in the recombinant strain, and one of these two phytases may be unstable when stored at different temperatures. It might be an important temperature-pH interaction in that at higher temperature, and the enzyme was more stable at higher pH value (Golovan *et al*., 1999). However, the alkali condition of the buffer might be the cause of phytic acid breaking down. Thus, P(EappA) phytase was presented the second pH stability at pH 8 and 10 for both storage temperatures, 4^{o}C and 37^{o}C, respectively. The pH optimum was within the range of the normal pH in monogastric stomach (Clemens *et al*., 1975).

The optimal temperature for the crude P(EappA) phytase activity was at 55^{o}C, and the activity was sharply decreased at above 60^{o}C. The result was corresponded with that reported by Greiner *et al*. (1993) and Rodriguez *et al*. (1999). Nevertheless, this temperature optimum was different from that reported by Golovan *et al*. (1999) which was at 45^{o}C. After 2-hr storage at 50^{o}C and 55^{o}C, the activities were decreased and reached to the undetectable level when stored at 60^{o}C to 70^{o}C. These were similar to the stability of *E. coli* K12 (ATCC 33965) phytase (Greiner *et al*., 1993). This crude enzyme might be suitable for pre-treatment of feed since it could not tolerate the high

temperature of 80^{o}C-90^{o}C in the feed pelleting process (Vohra and Satyanarayana, 2003).

Study on the effect of trypsin, taurocholic acid and deoxycholic acid on the phytase stability was determined due to the intention of using this enzyme as feed additive. Trypsin has been reported to degrade phytase in stomach and small intestine, resulting in the need for a relative high level of phytase supplementation in animal diets (Jongbloed *et al*., 1992). In this study, it was found that crude P(EappA) phytase was sensitive to trypsin. Rodriguez *et al*. (1999) and Golovan *et al*. (1999) reported that *E. coli* phytase was somewhat also sensitive to trypsin. In that case, there were also other monogastric enzymes that have influences on phytase activity; i.e. chymotrypsin, elastase (Ruckebusch *et al*., 1991) and pepsin (Golovan *et al*., 1999). In the case of taurocholic acid and deoxycholic acid, it was found that P(EappA) phytase was also sensitive to these bile acids. These results indicated that the enzyme could only be used for pre-treatment of the raw materials prior to the feed formulation process.

Organic acids have been reported to promote some improvements in the gastrointestinal tract digestibility of minerals (Hohler and Pallauf, 1993; Ravindran and Kornegay, 1993). Complexes are formed among organic acids and various cations; i.e. minerals, and these may result in increased intestinal absorption of minerals (Ravindran and Kornegay, 1993). Kemme *et al*. (1999) showed that supplementation of a grower-finisher diet with 3% lactic acid not only had a positive effect on the absorpability of inorganic phosphate, but also a synergistic interactive effect with phytase. The use of propionic acid for treatment of grains to prevent the development of mold has proven to be effective (Gillespie, 1987). For this study, effects of lactic acid and propionic acid on the phytase stability were determined. It was found that the remaining enzyme activity was enhanced when stored in 0.1% and 1.0% (w/v) of lactic acid while the remaining enzyme activity was slightly decreased when store in 0.1% and 1.0% (w/v) of propionic acid, both that at 4^{o}C and 37^{o}C for 2-hr incubation. Moreover, the lower gastric pH did not enhance the efficacy of microbial phytase (Kornegay, 2001). Thus, feedstuffs could be supplemented with lactic acid and phytase as pre-treatment. Furthermore, other fungal inhibitor might be

investigated to replace propionic acid for preventing mold development. It is important that such a antifungal agent should not effect the efficacy of phytase.

In the case of the metal ion effect, it was shown that the crude P(EappA) phytase activity was markedly increased when stored with Mg^{2+}. Nonetheless, it was insensitive to EDTA at both 4°C and 37°C. This EDTA effect was similar to that reported for *E. coli* K12 (ATCC 33965) phytase (Greiner *et al*., 1993). In addition, the enzyme was stable towards Na^{+}, K^{+}, Mn^{2+} and Co^{2+} but unstable towards Ca^{2+}, NH_4^{+}, Cu^{2+}, Fe^{2+} and Zn^{2+}, at either temperature. Effects of Ca^{2+}, Fe^{2+} and Zn^{2+} on P(EappA) phytase were similar to the commercial phytase; i.e. Natuphos (Gist-Brocades, Delft, The Netherlands), while that of Mg^{2+} and Mn^{2+} were different (Maenz *et al*., 1999). Golovan *et al*. (1999) reported that *E. coli* phytase was unaffected by Na^{+}, K^{+} and NH_4^{+} at a concentration of 100mM for each salt; namely, NaCl, KCl, NH_4Cl, Na_2SO_4 and K_2SO_4.

For the media development, it was investigated for reducing the production cost of P(EappA) phytase. Reduction in raw material cost would be one strategy. The medium development was first carried out based on two kinds of minimal media, namely, MMP (Leisinger *et al*., 1972) and PSM (Kerovuo *et al*., 1998). It was found that PSM was the most suitable for using as basic medium for medium development. Inorganic phosphate in MMP medium did not indeed induce the phytase production (Popanich *et al*., 2003), while sodium phytate, a sole source of phosphate, was found to induce the phytase production in PSM (Kerovuo *et al*., 1998). However, Popanich et al., (2003) had included phosphate solution in the medium formulation as it was found to help buffering the medium. Sodium phytate which could not induce the P(EappA) phytase production in PSM was replaced by other kinds of inducers; i.e. 10% extracts of either rice bran, wheat bran or soybean meal. It showed that PSM with 10% soybean meal extract could promote the highest phytase activity. Among these inducers, soybean meal had the highest level of phytate (Tyagi *et al*., 1998), and it could be at the appropriate level for inducing the phytase production. However, other bacteria; for example, soil bacterium strain PHO1, its phytase production was induced by both soybean meal and rice bran extracts (Popanich *et al*., 2003). For fungal phytase production, different fungal strains could be induced by different cereal grains, bran or seed and seed meal (Gibson, 1987). For instant, wheat bran was used

for production of phytase from *A. niger* (Popagianni *et al*., 2000; Nagashima *et al*., 1999) and canola meal was done for the production from *A. ficuum* (Nair *et al*., 1990). In the case of yeast, *Schwaniniomyces castellii* used wheat bran for phytase production (Segueilha *et al*., 1993). PSM without phytate in 10% soybean meal extract was investigated for phytase production. Nonetheless, it was still expressed the low level of growth. Thus, yeast extract was added as additional sources of nitrogen and vitamin in the medium. Although, 1.0% of yeast extract gave high growth at 48-hr, its phytase activity was lower than that did by 0.1% of yeast extract. As the medium development was aimed at the high phytase production, and thus, 0.1% of yeast extract, which gave satisfied level of growth and high level of phytase activity, was chosen to supplement to PSM+10% soybean meal extract medium. This was different from that for *Klebsiella aerogenes* (Jareonkitmongkol *et al*., 1997) which was supplemented with 1.0% yeast extract for phytase production. However, the *Candida krusei* WZ-001 phytase production was enhanced when its growth medium was supplemented with 0.02% yeast extract (Quan *et al*., 2001). Other bacteria, such as *Aerobacter aerogenes*, peptone as a nitrogen source could promote its phytase production (Greaves *et al*., 1967).

For a proper kind of carbon source, it was found that glucose had to be retained as a carbon source since it gave higher level of growth and phytase production from P(EappA) than that when it was omitted or when it was replaced by molasses. *E. coli* phytase was also found to be produced in the medium containing glucose as a sole source of carbon (Greiner and Jany, 1991; Jia *et al*., 1998; Kretz, 1999; Sunitha *et al*., 1999; Wyss *et al*., 1999; Golovan *et al*., 2000; Lim *et al*., 2000). For other carbon sources, Richardson and Hadobas (1997) reported that nonfluorescent strains, CCAR31 and CCAR60, of *Pseudomonas* sp. used arabinose as a carbon source, and they showed rapid growth and decrease in the Na-IHP concentration. *C.krusei* WZ-001 used fructose as a sole source of carbon, and gave high level of growth and phytase production (Quan *et al*., 2001). However, when it was cultivated with glucose, the lower level of phytase production was expressed. Additionally, the preparation of glucose in the medium for P(EappA) cultivation, sterilization of glucose separately or together with other compositions did not make significantly differences to either its growth or phytase production.

Surfactants have been reported to effect on growth rate and enzyme production from fungi (Reese and Maguire, 1969; Han and Gallagher, 1987; Amtual *et al*., 1988; Al-Asheh and Davnjak, 1994; Ebune *et al*., 1995). Some surfactants are very useful in terms of biology and biochemistry and have been used in biotechnology for improving the yields of a number of enzymes produced by fermentation (Sukan *et al*., 1989). For this study, Tween-20 and Tween-80 were added to the latest developed medium (PSM+10% soybean meal extract+0.1% yeast extract). It was found that 1.0% (v/v) Tween-20 showed the highest growth and phytase activity. However, El-Batal and Karen (2001) reported that Tween-80 enhanced the maximum of phytase activity in *A. niger*. Al-Asheh and Duvnjak (1994) and Ebune *et al*. (1995) also obtained high phytase production from *A. carbonarius* or *A. ficuum* grown on solid state fermentation media containing the surfactants. It was suggested that the surfactants caused an abundant biomass growth, and altered the cell permeability, which resulted in a high release of the enzyme. In the case of citric acid, addition of 0.1% (w/v) citric acid to the medium gave higher phytase activity than from that supplemented with surfactants. Citric acid acted as the pH controller that helped to preventing the pH change during cultivation (Merck, Co.). Thus, it was added to the PSM-phytate+10% soybean extract+0.1% yeast extract.

The effect of starch and flour on growth and phytase production was determined. It has been reported that some kinds of starch or flour could further enhance phytase production from microorganisms (Gibson, 1987). In this study, rice flour, corn flour, tapioca starch and soluble starch, all were tried out as inducers for growth and phytase production. It was found that tapioca starch added in the latest developed medium showed the highest activity. However, the medium without any flour or starch showed high phytase activity, and it was significantly different from the medium supplemented with tapioca starch. Therefore, it was not considered necessary to use tapioca starch as an additional inducer in this medium. Moreover, phytic acid from soybean meal extract may effect the starch digestion of P(EappA) by inhibiting its amylase enzyme (Thomson and Yoon, 1984).

From the medium development, the medium that promoted good growth and phytase production from P(EappA) was composing of PSM composition which phytase was omitted, then, supplemented with 10% soybean meal extract, 0.1% yeast

extract and 0.1% citric acid with glucose was included in the medium prior to sterilization. This medium was named SMYC medium. Then, it was used for investigating the kinetics of growth, phytase production, reducing sugar content and pH. It showed that P(EappA) slowly grew during the first 10 hr and then, rapidly raised up at the 12-hr which it entered the log phase growth. The maximum growth was reached at the 18-hr and entered the stationary phase. Moreover, P(EappA) phytase rapidly increased concomitant with growth and leveled-off at the 18-hr of cultivation. Phytase activity increased again at the 36-hr but at a lower rate until the end of 48 hr, which the cultivation was terminated. Nevertheless, Greiner *et al*. (1993) reported that *E. coli* K12 (ATCC 33965) expressed the increasing level of phytase activity during the stationary phase at the 8-hr of cultivation. A further increase of the activity was obtained after a shift from aerobic to anaerobic conditions. However, P(EappA) was cultivated only in aerobic condition. For the reducing sugar content, it rapidly decreased when growth and phytase activity came to the stationary phase. The result was similar to that of *E. coli* BL21 (DE3) cultivated by fed-batch fermentation (Golovan *et al*., 1999). The pH values of the culture did not show any vigorous change during cultivation.

The presence of other enzyme activities was determined from the crude enzyme which was the culture supernatant of P(EappA) grown in SMYC medium. There were high amylase and cellulose activities, which were 69.50 kU/ml and 83.10 kU/ml, respectively. For xylanase and endoglucanase, they were presented at lower activities than those two previous enzymes; i.e. 31.70 kU/ml and 31.20 kU/ml, respectively. Protease and lipase activities were not detected. Amylase, cellulose, xylanase and endoglucanase are normally used in animal feed industry to improve feed digestion, weight gain and feed efficiency (Coenzyme, 2003). Doses of enzymes used for feed supplementation were 100-200 U/kg for phytase, 100,000-200,000 U/kg for endoglucanase, 320,000 U/kg for cellulose, 10,500,000 U/kg for amylase and 550,000 U/kg for xylanase (Coenzyme, 2003). The presence of these enzymes in crude P(EappA) extract could contribute partly to the amount of enzyme supplement to feed leading to reduction in cost for the feed production.

Prior to formulation, crude P(EappA) had to be concentrated. Ammonium sulfate precipitation was a method of choice for concentrating P(EappA) phytase. This

was a cheaper and safer method than others, namely, organic solvent precipitation and poly (ethylene glycol) (PEG) precipitation (Scopes, 1982). Crude P(EappA) phytase produced in SMYC medium was concentrated by using ammonium sulfate precipitation at 80% saturation. It was found that the total protein was decreased from 7.86 mg/ml in crude enzyme to 2.66 mg/ml in concentrated enzyme. The phytase activity of concentrated enzyme was 197.40 U/ml, which was at 1.11 times higher than that the crude enzyme. In addition, the specific activity of concentrated enzyme was 74.21 U/mg protein, which was also increased from the crude enzyme at 3.28 times. This purification fold was similar to the *E. coli* phytase that was purified by ammonium sulfate precipitation, which its activity increased by 3 times (Greiner *et al*., 1993). However, the specific activity of P(EappA) purified phytase was higher than that of *E. coli* purified phytase, 1.5 U/mg protein. After protein concentration, the concentrated enzyme was distributed into 3 parts, each of which was prepared in different forms: 1.) resuspension form which was the ammonium sulfate precipitated protein resuspending in 0.05 mM acetate buffer, pH 5.6; 2.) air-dried precipitate form; and, 3.) wet precipitate form. It was found that the form of air-dried enzyme precipitate still expressed the relative remaining activity up to 90% when stored at either 4^{o}C or 25^{o}C for 28 days. The remaining activity of the resuspension form and the wet precipitate form were up to 60% when stored at 4^{o}C, for 28 days. However, their remaining activities were increased to more than 100% when stored at 25^{o}C for 28 days. It could be due to evaporation of the moisture content from these formulas.

The three forms of concentrated enzyme; i.e. resuspension, air-dried precipitate and wet precipitate, were formulated as feed-based form. For the formulation with the enzyme resuspension, the P(EappA) phytase activity when stored at 4^{o}C increased during the first week and then sharply decreased to the remaining activity of about 50%. Moreover, the activity raised up at the last week. For the same formulae but store at 25^{o}C, the activity reduced by approximately 50% during the first week and then stayed with slightly fluctuated until the last week of storage. In the case of air-dried precipitate form, the activity of sample showed the relative stable activity at both 4^{o}C and 25^{o}C. For the wet precipitate formulae, the activity decreased approximately 50% when stored both at 4^{o}C and 25^{o}C. These three forms were not stable during storage. This problem could be solved by enhancing the enzyme stabilization which might be

done by protein engineering and chemical modification (O'Fagain, 1995; 1997; 2003). This would require amino acid sequence analysis of P(EappA) phytase which would have to be carried out in the future study. Alternatively, P(EappA) phytase might be modified by chemical crosslinking of the glycan chains in the enzyme molecules to form phytase oligomers (Brugger *et al*., 2001).

The digestion efficiency of crude P(EappA) phytase on various kinds of phytate containing materials used for animal feed production. These included pre-mixed chicken feed and agricultural by-products; i.e. rice bran, wheat bran and soybean meal. In the chicken feed digestion, phosphate content was markedly increased while protein and reducing sugar contents were not significantly different between the control and experimental samples. Both phosphate and protein contents in wheat bran and soybean meal were increased when compared to both of control samples. However, reducing sugar contents in both samples were not significantly different from those in the control and experimental samples. For rice bran, there was no increase the content of phosphate, protein and reducing sugar. After hydrolyzing phytate by phytase, protein was free from the protein-phytate complex (Dvorakova, 1998). Thus, the increasing of protein content after digestion of wheat bran and soybean meal was due to the presence of the available protein after the phytate degradation. In the case of reducing sugar content, this was determined in order to find out if the available starchy carbohydrate was increased after phytate digestion. The rationale for this experiment was that when starch was freed from the starch-phytate complex, it would be digested by any carbohydrase enzymes present in the P(EappA) enzyme extract; i.e. amylase, cellulose, xylanase and endoglucanase, leading to an increased content of reducing sugar. However, there was no significant changing to be detected. This might be due to the fact that there was not much starch-phytate complex formed in the raw material prior to the enzyme treatment. Alternatively, the activities of carbohydrases in the extract were not sufficient for digesting the available starch.

Soymilk has become popular due to its good nutritional value. It contains calcium and high protein content. However, the consumption of these nutrients was restricted due to the presence of an antinutritional factor in soy bean, phytic acid (Khare et al., 1994). The digestion efficiency of crude P(EappA) phytase on soymilk was determined. It showed that crude P(EappA) phytase was failed to increased the

contents of phosphate, protein and reducing sugar in soy milk to detectable levels. The high concentration of phytase and the long term of digestion period might help improving the digestion efficiency. Moreover, the condition for soymilk digestion with P(EappA) phytase might have to be optimized.

For the future studies on P(EappA) phytase, many experiment can be done such as;

1.) Purification and characterization the purified P(EappA) phytase and compare to that of the crude phytase.
2.) Amino acid sequence analysis should be done to obtain a better understanding on the primary structure of the enzyme. The data could be beneficial for further improvement of enzyme property through protein engineering.
3.) Toxicity of enzyme should be studied for using as a food additive.
4.) Supplementation of phytase in feed should be studied on the animal growth performance.

CHAPTER VI
CONCLUSION

1. The general properties of crude P(EappA) phytase produced in LB medium with 15 µg/ml of tetracycline were investigated. It exhibited the optimum pH and temperature of pH 4.0 and 55^{o}C, respectively. P(EappA) phytase was stable at 2 pH levels; i.e. pH 4.0 and pH 8.0 when stored at 4^{o}C and its remaining activities were 98% and 118%, respectively, after the 2-hr. For the 2-hr storage at 37^{o}C, the remaining activity at pH 4.0 was unchanged while that at pH 8.0 was decreased to less than 60%. However, there was another activity peak raised up, when stored at this temperature and pH 10.0. P(EappA) phytase was highly stable at 45^{o}C, and the remaining activity was raised up to nearly 250%. This enzyme was sensitive to trypsin, taurocholic acid, deoxycholic acid and propionic acid. Nevertheless, lactic acid was found to enhance phytase activity, at both 4^{o}C and 37^{o}C. In addition, P(EappA) phytase activity was markedly increased when stored with Mg^{2+}, but it was insensitive to EDTA at either temperature. The enzyme was stable towards Na^{+}, K^{+}, Mn^{2+} and Co^{2+} but unstable towards Ca^{2+}, NH_4^{+}, Cu^{2+}, Fe^{2+} and Zn^{2+}, at both temperatures.
2. The developed medium that promoted the P(EappA) growth and its phytase production was composing of PSM (Kerovuo *et al*., 1998) which phytate was omitted. Then, it was supplemented with 10% soybean meal extract, 0.1% yeast extract and 0.1% citric acid with glucose was included in the medium prior to sterilization. This medium was named SMYC medium.
3. Kinetic of growth, phytase production, reducing sugar content and pH were investigated by growing P(EappA) in SMYC medium. The log phase was reached at the first 12-hr, and the growth rate was 0.1 OD_{600}/hr in this phase. The maximum growth was reached at the 18-hr with the enzyme production rate of 6.32 U/hr. The activity increased again at the 36-hr at a lower rate of 3.77 U/hr until the 48-hr of cultivation. The phytase activity reached 177.62

U/ml at the completion of cultivation. The sugar content was slowly decreased in the first 30-hr and rapidly decreased when the growth rate and phytase activity rate came to a stable phase. The pH value of the culture did not show any vigorous changes during cultivation.

4. The presence of other enzyme was determined. There were high amylase and cellulose activities, which were 69.50 kU/ml and 83.10 kU/ml, respectively. Moreover, xylanase and endoglucanase activities were presented at 31.70 kU/ml and 31.20 kU/ml, respectively. Protease and lipase activities were not detected.
5. The crude P(EappA) phytase produced in SMYC medium was concentrated by using ammonium sulfate precipitation, 80% saturation. After precipitation, the protein concentration was decreased from 7.86 mg/ml in crude enzyme to 2.66 mg/ml in concentrated enzyme. The phytase activity of concentrated enzyme was 197.40 U/ml, which was at 1.11 times higher than that the pre-concentrated enzyme. In addition, the specific activity of concentrated enzyme was 74.21 U/mg protein, which was also at 3.28 times higher than that the crude enzyme.
6. The concentrated enzyme was distributed into 3 parts of preparation of the enzyme into 3 forms: 1.) resuspension form which was the ammonium sulfate precipitated protein resuspending in 0.05 mM acetate buffer, pH 5.6, 2.) air-dried precipitate form and 3.) wet precipitate form. The air-dried enzyme precipitate form still expressed the relative remaining activity up to 90% at both storage temperatures, 4^{o}C and 25^{o}C, for 28 days.
7. The three forms of concentrated enzyme; i.e. resuspension, air-dried precipitate and wet precipitate were formulated as feed-based form. The efficiency for each of the concentrated enzyme was evaluated as the enzyme activity. For the formulation with the enzyme resuspension, the phytase activity of the sample stored at 4^{o}C increasing during the first week and then sharply decreased to the remaining activity about 50%. Furthermore, the activity raised up to about 15 U/g at the last week. For the same formulae but stored at 25^{o}C, the activity reduced by approximately 50% during the first week and then stayed with slightly fluctuated until the last week of storage. In the case of air-dried

precipitate form, the activity of samples showed the relatively stable activity at both 4°C and 25°C. Their activities were 7.5 U/g feed for the sample at 4°C and 9.12 U/g feed for that at 25°C in the last week of storage. For the wet precipitate formulae, the activity decreased from 20.68 U/g feed to 11.60 U/g feed when stored at 4°C and to 8.36 u/g feed when stored at 25°C.

8. The digestion efficiency of crude P(EappA) phytase on various kinds of phytate containing materials; i.e. chicken feed and agricultural by-products; i.e. rice bran, wheat bran and soybean meal. The result was expressed as the value of phosphate, protein and reducing sugar contents. In the chicken feed, the phosphate was increased from the undetectable level found in the control sample to 16.89 µg/g feed in the experimental sample. However, the protein and reducing sugar contents were not significantly different between the two sets of sample. The phosphate content in wheat bran was increased from 86 µg/g to 144.8 µg/g materials while that in soybean meal was from the undetectable level to 42.3 µg/g material. The protein content was increased from 79.8 µg/g to 116.8 µg/g of wheat bran and from 37.4 µg/g to 50.7 µg/g of soybean meal. However, the reducing sugar contents were not significantly different between the control and the experimental samples for both materials. In the case of rice bran digestion, there were no increasing the content of phosphate, protein and sugar.
9. The digestion efficiency of crude P(EappA) phytase on the soymilk was determined. The enzyme was failed to increase the contents of phosphate, protein and reducing sugar contents in soymilk to detectable levels. Further study has to be carried out for this application.

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