Purification and Characterization of the Phytase Produced by *Burkholderia* sp. Strain a13 Isolated from the Aquatic Environment

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ABREVIATIONS

ATP : Adenosine triphosphate

Arg : Arginine

Asp : Aspartic acid

BPP : β-Propeler phosphatase

BSA : Bovine serum albumin

CBB : Coomassie brilliant blue

Cys : Cysteine

D.W. : Deionized water

DTT : (±)-dithiothreitol

Da : Daltons

EDTA : Ethylene diamine tetra acetic acid

EGTA : Ethylene glycol tetra acetic acid

Glu : Glucose

Glu : Glutamic acid

HAP : Histidine acid phosphatase

His : Histidine

IAA : Iodoacetic acid

IP : Inositol phosphate

IP_6 : Phytic acid/ *myo*-inositol hexakisphosphate

IPTG : Isopropyl β-D-1-thiogalactopyranoside

K_{av} : Relative elution volume

MES : 2-(N-morpholino)ethanesulfonic acid

MOPS : 3-(N-morpholino)propanesulfonic acid

ORF : Open reading frame

PA : Phytic acid

PAP : Purple acid phosphatase

PCR : Polymerase chain reaction

PMSF : Phenylmethylsulphonyl fluoride

PTP : Protein tyrosine-like phosphatase

PVDF : Polyvinylidene fluoride

Pi : Inorganic phosphate

RBS : Ribosome binding site

SDS-PAGE : Sodium dodecyl sulfate polyacrylamide gel electrophoresis

TCA :Tricarboxylic acid

TRIS : Tris(hydroxymethyl)aminomethane

WT : Wild type

bp : Base pairs

***p*-NPP** : *para*-Nitrophenylphosphate

ABSTRACT

Phytases (*myo*-inositol 1,2,3,4,5,6-hexakisphosphate-phosphohydrolase) are a special group of phosphatases which are capable of hydrolyzing phytic acid to a series of lower phosphate esters of *myo*-inositol and phosphate. Phytic acid is the principal storage form of phosphorus in plant tissues and is specially concentrated in grains and seeds, where it can represent more than 70% of total phosphorous contents. Ruminant animals partially absorb phosphorous from phytic acid by the action of phytase-producing bacteria colonized in their rumens, but, as monogastric animals lack phytases in their gastrointestinal tract, they cannot utilize or degrade phytic acid present in grain-based feed. Phytic acid is commonly referred to as an anti-nutritional factor due to its chelation of divalent cations properties and also for causing protein aggregation and pepsin inhibition, affecting nutrient utilization of monogastric animals. Moreover, the lack of phytases in the gastrointestinal tract can cause the release of undigested phytic acid into the environment, which can be hydrolyzed resulting in eutrophication or algal bloom. In order to reduce environmental impact caused, as well as to enhance the nutritional value of feed for more efficient animal production, phytases have been largely applied to the farming of poultry, swine, and fish. More recently, new biotechnological applications for phytases have been also explored, especially in food processing, soil amendment and production of pharmacologically important lower *myo*-inositol phosphates.

The objective of this research was to screen for novel phytase-producing microorganisms from the aquatic environment, and proceed with the biochemical characterization of the enzyme, giving an insight of its biological role to the microorganism and also contribute to the knowledge of phytases, encouraging its application.

In the present research, ten phytase-producing bacteria were isolated from fish intestinal contents and mud of Lake Kasumigaura by enrichment culture using a minimum medium containing phytic acid as the sole phosphorous source. Strain a13 showed the highest phytase activity in comparison to other isolated strains. It was classified in the genus *Burkholderia*, with the closest related species being *B. soli* GP-25-8 (98.9% identity). Since there is no reported phytase from bacteria of the *Burkholderia* genus, strain a13 was selected for purification and biochemical characterization of its enzyme. Enzyme production by strain a13 was demonstrated to be induced by phytic acid but not repressed by the presence of excess amount of phosphate;

instead, the production was strongly repressed by the addition of glucose and moderately by *myo*-inositol. It seems likely that strain a13 produces phytase to utilize phytic acid as a carbon source, after degrading it to *myo*-inositol, rather than utilizing it as a phosphorous source, which contrast with the majority of other phytase-producers. The purified enzyme had a molecular mass of 44 kDa and a phytase activity of 174 μmol $min^{-1}mg^{-1}$ (U). The optimal temperature and pH were at 45-55°C and 4.5, respectively. The enzyme was quite stable at 4°C, as more than 90% of the activity was maintained after one year. It also showed a good stability at 30°C, loosing less than 20% of its activity after five days of incubation. Strain a13 phytase showed broad substrate specificity, being able to hydrolyze other phosphorylated compounds, but the highest activity was observed with phytic acid. The activity of this enzyme was inhibited by thiol-acting agents, such as Zn^{2+}, Cu^{2+}, Hg^{2+}, and iodoacetic acid, and activated weakly by the disulfide bridge-reducing agents, DTT and β-mercaptoethanol, clearly indicating the requirement of free thiol group(s) of Cys residue(s) for the activity. Genetic cloning revealed that the mature portion of this enzyme consists of 428 amino acids with a calculated molecular mass of 46 kDa. The amino acid sequence showed the highest similarity to the phytase produced by *Hafnia alvei* with 48% identity; it also contained histidine acid phosphatase (HAP) motifs (RHGXRXP and HD), indicating the classification of this enzyme in the HAP phytase family. Two potential ATG codons were found in-frame: one at 34 amino acid upstream and the other at 45 amino acid upstream from the N-terminus of the purified enzyme. The previous one was preceded by a putative ribosome binding site (GAGTG) and a putative promoter sequence (-35; TGAACA, -10; AATATT). For that reason, the putative translational initiation codon seemed to exist 34 amino acid upstream from the N-terminus of the purified phytase. The cloned gene was successfully expressed in *E. coli* from its putative initiation codon, showing that the gene actually encodes the phytase. No phytase activity was observed when the mature enzyme gene (with addition of the initiation codon) was cloned in *E. coli*, suggesting that the upstream region may play an important role in enzyme maturation. Commercial carp fish feed and gold fish feed were treated using 4.2 U of the native enzyme. After treatment, the amount of soluble inorganic phosphate increased 62% and 51%, respectively, showing the possibility of using the phytase purified from *Burkholderia* sp. a13 for pretreatment of fish or animal feed.

Keywords: *Burkholderia* sp. a13; catabolite repression; enzymatic characterization; genetic cloning; phytase

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CHAPTER I - INTRODUCTION

Section I - Phosphorous Management and the World Scenario

The element phosphorous, which is essential for life, is involved in the synthesis of very important molecules to biological systems, such as ATP and DNA. In higher complexity animals the element is essential for promotion of bone and tissue development. In plants phosphorous can improve root growth, consequently ameliorating the assimilation of nutrients, culminating in faster production. Therefore, inorganic phosphate obtained from phosphate rocks have been largely applied into food production, especially at agriculture and animal farming activities, as fertilizers and diet supplementation, respectively. For this reason the access to this very valuable resource is indispensable for those basic economic activities in the modern society. However, the biogeochemical cycling of the element phosphorous is a very slow process in nature, thus phosphates can be considered as a non-renewable resource (Van Vuuren et al., 2010). World phosphate rock reserves are not equally distributed over the planet, and only five countries are responsible for detaining almost 90% of the total world phosphate rock reserves (Bhavsar and Khire, 2014). In this way, countries with scarce phosphate reserves, as Japan, or growing economies such as India, are almost completely dependent on the importation of phosphorous for utilizing it in agriculture and animal farming. Scientific reports indicate an increasing demand for phosphate rocks, which is used to supply the agriculture and animal farming industry. Those reports also estimate that if the increasing demand for this mineral continues with same rates, it is expected that the world phosphate rock reserves will be completely depleted in 50-100 years (Cordell et al., 2009). Consequently, increasing extraction and production costs of inorganic phosphate is expected (Van Vuuren et al., 2010). The problems mentioned above generate a global rising interest in technologies able to promote reduction, reuse and recycling of the element phosphorous. One of those technologies that might comply with this international interest is the application of phytases, which is discussed on the next sections of this chapter.

Section II - Phytic Acid

1-2-1 Phytic Acid

Phytic acid is refereed in the scientific literature as the primary storage form of phosphorous in plant tissues and is considered an important source of *myo*-inositol, contributing to plant growth and seedling development (Raboy, 2002; Jorquera et al., 2008; Ali et al., 2013). This molecule was firstly isolated from non-starch grains from various plant seeds by Hartig in 1855, and years later, with extensive chemical and structural analysis, the molecule of phytic acid was unveild (Fig 1-2-1). The official nomenclature for phytic acid has been determined by IUPAC-IUB (1968) as "*myo*-inositol 1, 2, 3, 4, 5, 6 hexakis (dihydrogen) phosphate".

1-2-2 Occurrence of Phytic Acid

Due to its nutritional relevance to plant metabolism, phytic acid is usually abundant in seeds and grains, typically representing approximately 75% of total phosphorous and more than 80% of soluble *myo*-inositol phosphates (Dorsch et al., 2003; Cao et al., 2007). In cereals largely used for human and animal consumption such as wheat grain and corn grain, the phytic acid molecule is responsible for 71.6% of total phosphorous contents for both seeds, while in some by-products of seed this proportion tend to rise, reaching values as high as 79.5% for rice bran (Cao et al., 2007). Although phytic acid is mainly encountered in seeds rather than other plant tissues, its location inside of the seed can vary depending on the plant species. In corn grains, phytic acid is accumulated at the germ portion of the kernel, which contains almost 90% of the phytic acid, on the other hand, wheat kernel contains only 0.32% of the phytic acid, which is concentrated at the external part of this seed. In rice phytic acid is primarily found on the outer layers of the grain. Specifically, 2% of the outside kernel of rice was found to contain 23 times more phytic acid than the intact kernel (Maga, 1982). Vucenik & Shamsuddiny (2003) reported the presence of phytic acid inside of mammalian cells in small concentration, and also in the form of lower-inositol phosphates, which are important for regulating vital cellular functions and cellular

activities. Phytic acid is also reported to be present in high amounts in soil, where it tends to accumulate, representing approximately 20-50% of the total soil organic phosphorous (Richardson et al., 2001)

1-2-3 Chemical Properties of Phytic Acid

Phytic acid molecule is characterized by a *myo*-inositol core in which six phosphate groups are performing a phosphoester ligation. Structurally the most stable configuration for the molecule of phytic acid is in the chair conformation, in which five phosphate groups adopt an equatorial orientation, while only one phosphate group is axially oriented. As an international recommendation by the Nomenclature Committee of the International Union of Biochemistry (NC-IUB, 1988), The axial phosphate group pointing upwards is numbered as the phosphate group number two and the other phosphate groups are subsequently numbered in an anticlockwise orientation (Fig. 1-2-1). Phytic acid carries in its structure twelve protons at the phosphate groups. Titration and ^{31}P NMR spectroscopy experiments have determined the dissociation constants (pKa) values of those protons, showing that at a neutral condition, six of those twelve protons in the phytic acid molecule are strongly dissociated with pKa around 1.84, two are weak acid (with pKa of 6.30), and four are weakly associated (at pKa of 9.70) (Costello *et al.* 1976). Those characteristics show that *myo*-inositol hexakisphosphate molecule contains several levels of negative charge in a wide range of pH. For this reason, phytic acid molecule act as a very strong chelation agent, showing special affinity for divalent cations like Ca^{2+}, Mg^{2+} and Zn^{2+}. Also, under acidic conditions where most of the proteins are positively charged, the negative charges of phytic acid can complex with positively charged parts of peptides and proteins, causing protein aggregation (Suhairin et al., 2010; Yu et al., 2012). When phosphate groups are removed from the phytic acid molecule (generating lower-inositol phosphates), the binding strength becomes progressively lower and the solubility of complexing molecules with phytic acid increases. (Kumar et al., 2011).

1-2-4 Anti-Nutritional Effects of Phytic Acid

Although phytic acid is important for plants as a phosphorous and energy storage, and also in the control of cellular processes (Dorsch et al., 2003). From an animal nutrition point of view, phytic acid is considered as an anti-nutritional compound due to its strong chelating properties promoted by the negative charges at a broad range of physiological pHs. As indicated on sub-section 1-2-3, phytic acid molecule shows very strong chelating properties of divalent minerals such as Ca^{2+}, Mg^{2+}, Zn^{2+} and Fe^{2+} (Afinah et al., 2010). Therefore, the interactions between phytic acid and metal ions in the homeostasis of animals and the problems generated by this interaction have been extensively described at the scientific literature.

The high concentration of phytic acid in the diet can form insoluble complexes with metal ions reducing its bioavailability and absorption. One example of the formation of those complexes leading to deleterious anti-calcifying effects was reported by Mellanby (1949) in which the complex phytic acid-Ca^{2+} develop rickets in puppies. Also, zinc deficiency symptoms caused by a high concentration of phytic acid in the diet, have been studied in pigs, where it caused parakeratosis (Oberleas et al., 1962). High amounts of phytic acid in the diet was also reported to cause less absorption of zinc in rats, and the addition of 1% phytic acid to an egg albumin diet significantly reduced the absorption of copper and manganese (Davies and Nightingale, 1975). Hurrell (2004) reported the negative effects of phytic acid on bioavailability and absorption of iron. Simpson & Wise (1990) have reported that the mixture of two different cations raises the interaction between the molecule of phytic acid and metals, forming complexes which precipitate increasing absorption problems.

There is strong evidence that phytic acid interactions with proteins negatively affect digestion (Harland and Morris, 1995; Suhairin et al., 2010; Yu et al., 2012). Phytic acid, which is negatively charged over all the physiological pH range of the gastro-intestinal tract, can interact with positively charged proteins causing the formation of phytic acid-protein aggregates, which can compromise the accessibility of digestion proteases to proteins from the diet, resulting in inefficient protein digestion (Knuckles, 1985). Yu et al. (2012) reported that phytic acid presence on the stomach also caused pepsin inhibition, which might compormise digestability of proteins and absorption of aminoacids. Phytic acid molecule

complexed with copper ions has been reported to cause more than 95% decrease in activity of carboxypeptidase A by exchanging Cu^{2+} with Zn^{2+} in enzyme molecules at pH 7.5 (Martin, 1989). It was reported that phytic acid can also inhibit amylopsin, and amylase which might interfere with the digestibility and absorption of lipid and starch (Selle and Ravindran, 2007).

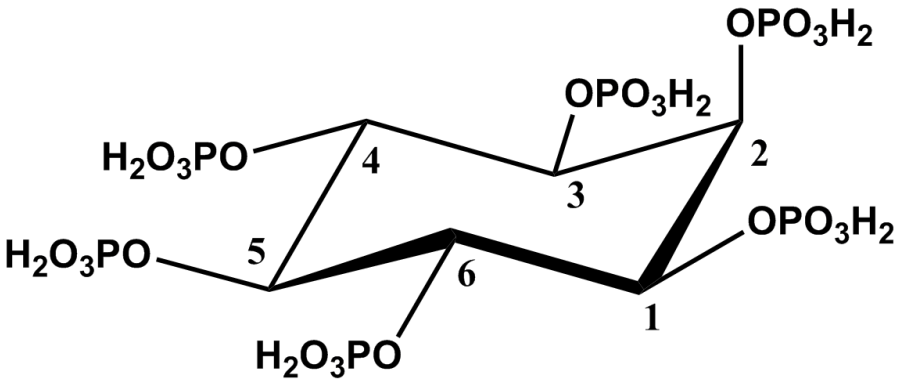

Fig. 1-2-1. Phytic acid (*myo*-inositol 1, 2, 3, 4, 5, 6 hexakisphosphate) structure.

Section III - Phytases

1-3-1 Definition, Characteristics and Sources

Phytases are a special class of phosphatases that sequentially hydrolyze phytic acid to less-phosphorylated *myo*-inositol, which release inorganic phosphates. This class of enzyme is widely distributed in nature and has been isolated from several sources, including plants, animals, and microorganisms (Suhairin et al., 2010). While phytases are produced by microorganism and plants to utilize phytic acid as a source of phosphorous (Konietzny and Greiner, 2004), the phytases isolated from animal cells is believed to be involved in cell-signaling pathways, regulating the pool of inositol phosphates, because of their several folds lower activity (Iqba et al., 1994). For instance bacterial and fungal phytases cannot complete hydrolize the molecule of phytic acid into *myo*-inositol, generaly producing as the final product *myo*-inositol 2-monophosphate. This occurs because of a pronounced stereospecificity and a strong preference for equatorial phosphate groups, while they are virtually unable to cleave axial phosphate groups. However, the combination with other phosphatases into the reaction is capable to promote production of *myo*-inositol (Wyss et al., 1999). Despite the fact that all phytases are able to hydrolize phytic acid, not all of those enzymes are structurally similar neither do they hydrolyze phosphate groups with the same mechanism (Mullaney and Ullah, 2003). For that reason, phytases have been classified into different families, according to their catalytic mechanism, or into different classes, according to the first dephosphorylation position of the molecule of phytic acid.

1-3-2 Families of Phytases

Through the analysis of sequence similarity and the catalytic mechanism, phytases are classified into four families: Histidine acid phosphatase (HAP) family, β-propeller phytase family, Purple acid phosphatase (PAP) family and Protein tyrosine phosphatase-like (PTP) family, which is also refereed as cysteine phytases (Mullaney and Ullah, 2003; Puhl et al., 2007). In bacteria, strains of the genus *Bacillus* produce β-propeller phytases (Kerovuo et al., 1998), and many strains of *Enterobacteriaceae* produce

HAP phytases (Greiner et al., 1993; Sajidan et al., 2004). More recently, PTP-like phytase was found in *Megasphaera elsdenii* (Puhl et al., 2009). To date, PAP phytases haven't been isolated from bacterial sources, despite several plants showed to contain enzymes belonging to this family (Dionisio et al., 2011).

Histidine Acid Phosphatases

The most well-known and extensively reported family of phytases belongs to the histidine acid phosphatases (HAP) family. This family of phytases has been isolated from bacteria, fungi and plants (Mullaney and Ullah, 2003). This family of phytases have as representants of well biochemicaly-characterized enzymes, appA and phyA, produced by *Escherichia coli* (Greiner et al., 1993) and *Aspergillus niger* (van Hartingsveldt et al., 1993), respectively. All HAP phytases share a common active site motif, RHGXRXP, near the N-terminal and have a two-step mechanism in the hydrolysis of phosphomonoesters (Mullaney and Ullah, 2003). Oh et al., (2004) describes the proposed mechanism for phosphomonoester hydrolysis as the histidine residue in the conserved motif, RHGXRXP, serving as a nucleophile in the formation of a covalent phosphohistidine intermediate and the aspartic acid residue of the C-terminal conserved HD motif serving as a proton donor to the oxygen atom of the scissile phosphomonoester bond. All the members of this family of phytases share the same characteristic optimal pH in the acidic range. However, there are many histidine acid phosphatases that have little or no activity against phytic acid, functioning as simple phosphatases (Cho et al., 2006). The difference between histidine acid phosphatases and HAP phytases remains in the larger structure of the active site pocket containing amino acids positively charged, which support the orientation, and is able to comport the bigger molecule of phytic acid (Tomschy et al., 2002).

β-Propeller phosphatases

β-Propeller phosphatases (BPPs) were firstly found and cloned from *Bacillus* genus of bacteria, and a three-dimensional model of its molecule displays a basic form similar to a propeller with six blades.

The dependence on binding Ca^{2+} for thermostability and catalytic activity makes this type of phytase unique among others. Also, calcium ions facilitate the binding by creating a favorable electrostatic environment (Mullaney and Ullah, 2003). The enzyme reaction appears to act through a direct attack of the metal-bridging water molecule on the phosphorous atom of phytate and the subsequent stabilization of the pentavalent transition state by the bound calcium ions (Chu et al., 2004). For this reason the BPP have a characteristic optimum pH into the neutral to alkaline range and are strongly inhibited by Ca^{2+} chelants, such as EDTA and EGTA. They also can only hydrolyze three of the six available phosphate groups from the phytic acid molecule (Kerovuo et al., 1998). The most important isolated bacteria producing a phytases belonging to the BPP family is *Bacillus subtilis* (Kerovuo et al., 1998) and *Serratia sp.* (Zhang et al., 2011).

Cysteine Phytases

Cysteine phytases are the newest discovered family of phytases, which was firstly isolated and reported from a rumen colonizing bacteria, *Selenomonas ruminantium* JY35 (Yanke et al., 1999). More recently a cysteine phosphatase was isolated form *Megasphaera elsdenii* (Puhl et al., 2009), also a bacteria colonizing the rumen of animals, suggesting that this family of phytases is related to this kind of environment. This new phytase is structurally distinct from the four known classes of phytases and is characterized by the presence of the protein tyrosine phosphatase (PTP) active site signature sequence of HCXXGXGRT (Nakashima *et al.* 2007). Chu et al. (2004) proposed that the mechanism for this family of phytases consist in an initial binding of phytate to the active-site pocket, which is mainly facilitated by the interactions between the basic charges in the pocket and the negatively charged groups on phytate. The phytic acid molecule is then oriented to the productive position by hydrogen bonds between its phosphate group and amide NH groups of amino acids localized in the P loop of the enzyme. Then, the thiol anion of a Cys residue attacks the phosphate group at the 5^{th} position of the phytic acid molecule, forming a cysteinyl-phosphate trigonal-bipyrimidal pentavalent intermediate. Finally, the release of *myo*-inositol pentakisphosphate follows the donation of a proton by and Asp residue. This family of enzymes requires a

Cys residue for the activity, which explains the strong inhibition by thiol-acting reagents. Also, cysteine phytases are known to be activated by Pb^{2+}, though the mechanism of activation is not completely understood (Yanke et al., 1999).

Purple Acid Phosphatases

Purple acid phosphatases (PAPs) are metallohydrolases that bind two metal ions in the active center. One of the ions is usually iron III, while the second metal in plant PAPs can be zinc, manganese, or iron II. The ions are responsible for a specific absorption into the visible spectrum, thus conferring the enzyme with a pink to purple color (Dionisio et al., 2011). Their characteristic pink or purple color derives from a charge transfer transition between a Tyr residue and the "chromophoric" ferric ion in the binuclear Fe(III)-Me(II) center, where the metal (Me) is iron, zinc, or manganese (Schenk et al., 1999). PAP proteins are also characterized by seven conserved amino acid residues (indicated in bold) in the five conserved motifs **D**XG, G**D**XX**Y**, G**N**H(D/E), VXX**H**, and G**H**X**H**, which are involved in the coordination of the dimetal nuclear center (Li, 2002). Searches of genomic databases have revealed PAP-like sequences in plants, mammals, fungi, and bacteria. However, GmPhy is the only known PAP-phytase reported to have significant phytase activity. Also this family of phytase is likely restricted to plants, and has never been reported to be isolated from microorganisms. The lower catalytic activity of GmPhy may be necessary during germination in soybeans because the germination process requires a steady breakdown of phytate over a period of several days (Mullaney & Ullah, 2003).

1-3-3 Classes of Phytases

The International Union of Biochemists and The International Union of Pure and Applied Chemistry (IUPAC) currently recognize, depending on the position on the inositol ring where the dephosphorylation is initiated, three classes of phytase enzymes: 3-phytase (EC 3.1.3.8), 4-phytase, also refereed as 6-phytase (EC 3.1.3.26) and 5-phytase (EC 3.1.3.72). Bacterial, fungal, plant and animal

phytases have been classified into the class of 3- and 4- phytases(Greiner et al., 1993; van Hartingsveldt et al., 1993; Dionisio et al., 2011). However, very few 5-phytases have been identified to date, with the majority of them being reported to be produced by plants (Barrientos, 1994).

Section IV - Application of Phytases

The recent "phosphate crisis" term that have been recurrently mentioned into the scientific literature (Cho et al., 2006; Cordell et al., 2009; Van Vuuren et al., 2010) is generating a global rising interest in technologies related to the better management of phosphorous. One of the technologies that might contribute to a better usage of phosphorous is the application of phytases. Historically, phytases have been largely used at animal farming to reduce the environmental impact caused, as well as to enhance the nutritional value of feed, for a more efficient animal production. More recently, new biotechnological applications for phytases have also been explored, especially in food processing, soil amendment and production of pharmacologically important lower *myo*-inositol phosphates. The different applications for phytases are detailed below.

1-4-1 Animal Nutrition

The constant increase in world population triggers a higher demand for food production, especially the production of food with high nutrition values and high economic value. In this way the demand for animal farming products have being growing in developing countries, while is evolving to stagnation although at high levels in developed countries (Thornton, 2010). Recently, animal farming industry is facing many changes, which have also been generating many issues. This type of economic activity requires a massive input of feed concentrates which contains as a major compound grains, that also contain a high concentration of phytic acid. As discussed on the previous sections, phytic acid are known for the strong chelation of divalent minerals such as Ca^{2+}, Mg^{2+}, Zn^{2+} and Fe^{2+}, which are important minerals for animal growth. Phytic acid has also a potential for binding positively charged proteins, amino acids, and multivalent cations or minerals in foods, resulting in complexes that are insoluble, difficult to hydrolyze during digestion, and thus, typically are nutritionally less available for absorption (Afinah *et al.* 2010). Ruminant animals partially absorb phytic acid by the action of phytase-producing bacteria colonized in their rumens (Nakashima et al., 2007), but, as monogastric animals lack phytic

acid-degrading enzymes in their gastrointestinal tract (Bikker et al., 2012), they cannot utilize or degrade phytic acid, which causes decreased mineral and protein absorption. The high phytic acid content of the feed could impact the growth of monogastric animals, reducing the efficiency of poultry, swine, and fish production. Moreover, in dense animal farming areas, high levels of phytic acid excreted by monogastric animals might spread into the environment from surface runoff, leading to excessive nutrient loading into surface water and promoting cyanobacteria blooms, hypoxia, death of aquatic animals and eutrophication (Gupta et al., 2013). The above mentioned problems, allied with requirements for better animal feed, environmental protection and reductions of feed cost, have prompted the fast development of phytases research and their application into the animal farming of poultry, swine, and fish, which constitute the major application for phytases today.

1-4-2 Food Processing

The application of phytases at the food industry has been considered recently, because of the high concentrations of phytic acid in grain-based food products (Cao et al., 2007). Haros et al. (2001) reported that supplementation of an exogenous phytase to different bread formulations, shortened the fermentation period for all of them. Moreover, there was a considerable increase in the specific bread volume, which represents an improvement in the crumb texture. For Japanese sake production, the use of a mold (*Aspergillus orizae*) with higher phytase activities at the *koji* preparation was able to improve the alcoholic fermentation by yeast, reaching higher percentages of ethanol. This result suggested that phosphorous is a limitating nutrient at the fermentation process in sake production (Fujita et al., 2001).

1-4-3 Soil Amendment

Phosphorus deficiency in soil is a major issue for crop production. Phytic acid is also reported to be present in high amounts in soil, representing approximately 20-50% of the total soil organic phosphorous (Richardson et al., 2001). However, the uptake of this soil organic phosphorous by plants is

very poor, thus phytases have been also considered for application into agriculture recently. Singh and Satyanarayana (2009) reported that a compost prepared by addition of native microflora of wheat straw together with a phytase producing mold of *Sporotrichum thermophile* promoted the growth of plants in comparison to the control group. The authors also report that the isolated phytase, as well as the mold, were able to promote the growth of wheat seedlings, improving several physiological characteristics in comparison to the respective control groups. Those approaches can be applied as a strategy for improvement of the production, minimizing the use of fertilizers in agriculture.

1-4-4 Production of Pharmacologically Active Lower-Inositol-Phosphates

Several lower-inositol-phosphates have been reported to have pharmacological activity in mammals. The molecule of *myo*-inositol-1,2,6-trisphosphate (PP56) have been reported to possess anti-inflammatory effects on animal models for inflammation (Claxson et al., 1990), also it showed to ameliorate the diabetic condition in rats (Carrington et al., 1993). Maffucci (2005) reported that inositol pentakisphosphate could promote anti-angiogenic and consequently antitumoral effects, through the inhibition of the phosphatidylinositol 3-kinase/akt pathway. Through the use of immobilized phytase from *E. coli*, some important lower-inositol-phosphates were prepared, such as inositol 1,2,3,4,5-pentakisphosphate, inositol 2,3,4,5-tetrakisphosphate, inositol 2,4,5-triphosphate and inositol 2,5-biphosphate (Greiner and Konietzny, 1996). In this way, the use of specific phytases towards the production of those lower-*myo*-inositol phosphates might contribute to the pharmaceutical industry, since their production by chemical approach is very costly.

CHAPTER II - SCREENING AND IDENTIFICATION OF PHYTASE PRODUCING STRAINS

Section I - Introduction

Phytase is widely distributed in nature and has been isolated from several sources, including plants, animals, and microorganisms (Suhairin et al., 2010). The potential applications of phytases in animal farming, food industry, agriculture and pharmaceutical industry, were previously discussed in Chapter I, and emphasize the demand for the enzyme. In order to satisfy the needs of each industry with a phytase having desired properties, an ongoing interest in isolation of microorganisms for production of novel phytases have encouraged the biochemical characterization of novel phytases obtained from microorganisms. The main objective of this work was to isolate a phytase-producing strain that shows an enzyme with high activity and unique biochemical properties, which to date haven't been described yet on the scientific literature. The application of this novel enzyme, especially to animal farming, is expected to improve production and also reduce the environmental impact as discussed previously on Chapter I. In order to isolate a bacterial strain showing those characteristics, a special attention was given to carp production using net cages in Lake Kasumigaura. Historically, this model of fish farming has been extensively explored in the Lake, and was responsible for more than half of the total farmed production in Japan. Since the feed commonly used for freshwater fish farming is grain-based, its phytic acid contents can accumulate on areas of intensive production, which allow phytic acid degrading bacteria to flourish in this kind of environment. Although some phytase-producing strains have been reported to be isolated from the aquatic environment (Cheng and Lim, 2006), majorly phytase-producing microorganisms have been reported to be isolated from terrestrial samples (Popanich, 2003; Hussin et al., 2007; Kumar et al., 2013). In this way, the screening of phytase-producing strains from the aquatic environment might be an interesting source for obtaining novel phytase-producing strains. For those reasons, samples of mud and fish intestinal contents from areas adjacent to net cage culture of carp in Lake Kasumigaura were screened for phytase producing strains. As a screening strategy, enrichment cultivation using phytic acid as the sole

phosphate source was performed. Finally, the isolated strains showing high phytase activity were identified by their 16S rRNA gene sequence.

Section II - Material and Methods

2-2-1 Enrichment Culture

Samples of fish intestinal contents and mud from the Lake Kasumigaura were obtained and screened by enrichment culture, containing sodium phytate as the sole phosphorous source. A modified Davis Minimal Medium (Davis, 1949) named as the CG medium (described in Table 2-2-1) was used for cultivation. One gram of sample was inoculated into 100 mL of CG medium containing 2 g L^{-1} of $CaCl_2 \cdot 2H_2O$ for precipitation of free inorganic phosphate in the medium and incubated at 30ºC with agitation for 48 h. Then, the culture was transferred to a fresh medium with a concentration of 1% (v/v) every 12 h and this step was repeated twice. The last culture was properly diluted and plated onto agar plates of CG medium. The plates were incubated for 48 hours at 30 °C and the obtained colonies were single isolated and transferred to a master plate.

2-2-2 Phosphate Concentration and Phytase Activity Assay

The phosphate concentration standard curve was obtained by a modification of the colorimetric method described by Fiske & Subbarow (1925). Solutions of KH_2PO_4, ranging from the concentration of 0.1 mM to 1.0 mM, dissolved in 0.1 M acetate buffer pH 5.0 were prepared. 150 µL of the prepared solutions were mixed with same amount of a color reagent described below, and the absorbance at 620 nm was measured with a microplate reader (DTX-880 Multimode Detector, Beckman Coulter, USA). The color reagent is freshly prepared by mixing four volumes of 1.5% (w/v) ammonium molybdate in a 5.5% (v/v) sulfuric acid solution and one volume of a 2.7% (w/v) ferrous sulfate solution. The increasing concentrations of KH_2PO_4 against the obtained absorbance were plotted in a graphic and the phosphate concentration curve was obtained by linear regression (Fig. 2-2-1).

2-2-3 Phytase-producers Screening Using Agar Plates

For screening of phytase producing bacteria on agar plates, the formation of halos was observed by cultivation in CG medium with the sodium phytate content replaced by insoluble calcium phytate salt. The microorganisms obtained from the enrichment culture and single isolated in the master plate were transferred to the CG calcium phytate agar medium and incubated for 48 h, 30 °C. Insoluble calcium phytate salt was prepared from sodium phytate as described by Shieh & Ware (1968). Thirty grams of phytic acid sodium salt hydrate was dissolved in 250 ml of 0.15 N HCl, and undissolved impurities were removed by filtration. The clear supernatant fluid was neutralized with 25% (w/v) NaOH solution, and 110 ml of 10% (w/v) $FeCl_3$ was added. Iron phytate was recovered by centrifugation and washed three times with 800 ml of 0.15 N HCl to remove ferric phosphate. The white iron phytate was resuspended in deionized water, and portions of 5% (w/v) NaOH solution were added until no additional brown precipitate formed. The supernatant liquid was adjusted to pH 7.0, and portions of 20% (w/v) calcium acetate were added until no further precipitate formed. The calcium phytate was washed three times with 500 ml of methanol and air-dried to a white powder.

In order to differentiate halo formation by phytase activity from halo formation of organic acid production by colonies, a treatment described by Bae et al. (1999) was performed. For confirmation of the effectiveness of this methodology a CG calcium phytate agar plate was produced with two perforations. In one perforation, 20 µL of commercially available phytase solution was added, in the other perforation 20 µL of 0.1 mM lactic acid solution was added, and the plate was incubated for 6 hours at 37 °C. The treatment described by Bae et al. (1999) was performed for the control plate and the cultivated plates. The cultivated colonies were washed from the agar surface with deionized water and the petri dishes were flooded with 2% (w/v) aqueous cobalt chloride solution. After incubation at room temperature the cobalt chloride solution was replaced with freshly prepared solution containing equal volumes of 6.25% (w/v) aqueous ammonium molybdate solution and 0.42% (w/v) ammonium vanadate solution. Following 5 min incubation, the ammonium molybdate/ammonium vanadate solution was removed and the plates were examined for zones of clearing.

2-2-4 Phytase-producers Screening Using Liquid Culture

Single isolated colonies obtained from the enrichment culture were cultivated in 10 mL of CG medium for 48 h and cells were harvested by centrifugation at 4,400 × g for 10 min at 4°C. Then, the supernatant was assayed for phosphate concentration and, the strains that showed an increase in phosphate concentration, compared with the medium without bacterial inoculation, were selected as candidates.

The harvested cell pellet of the candidates was resuspended in 500 µL of 0.1M sodium acetate buffer, pH 5.0. The cells suspensions were sonicated in low temperature at 20 KHz, 220 W in a UCW-201 sonicator (Tosyo Electric Co., Ltd.). The sonicated product was centrifuged at 4 °C, 20,000 × g for 15 min. The supernatant was collected and filtered with a Millex – GV PVDF 0.22 µm syringe filter (Millipore, Carrigtwohill, CO.) and it was defined as the cell free extract (soluble fraction). The pellet obtained from centrifugation of the sonicated cells was resuspended in 500 µL of 0.1M sodium acetate buffer, pH 5.0. and was defined as the insoluble fraction. All the obtained candidates were assayed for phytase activity and measured with the cell-free extract and culture supernatant. The strains showing higher activities were assayed for total phytase activity among the soluble fraction, insoluble fraction and culture supernatant, for determination of the enzyme localization.

2-2-5 Phytase Activity Assay

Phytase assay was performed by measuring the liberation of free orthophosphate. A sample of 6 µL of enzyme solution was mixed with 84 µL of substrate solution containing 2 mM phytic acid dipotassium salt in 0.1 M sodium acetate buffer, pH 5.0, and incubated at 37 °C for 15 min. Then, the reaction was stopped by adding 90 µL of 10% trichloroacetic acid and the released orthophosphate was measured by mixing 150 µL aliquot of the reaction mixture with 150 µL of freshly prepared ammonium heptamolybdate reagent, as described previously (Fiske and Subbarow, 1925). Absorbance at 620 nm was

measured. One unit of enzyme activity was defined as the amount of enzyme which produced 1 μmol of orthophosphate per min. under the assay condition. The specific activity was expressed in units of enzyme activity per milligram of protein. Protein concentration was measured by the method described by Bradford (1976).

2-2-6 Strains Identification

The colonies were identified by the determination of their 16S rRNA gene sequence. The Obtained colonies were picked from the master plate and dissolved in 40 μL of autoclaved deionized water. One microliter of this solution was used as the template for colony-direct PCR. 16S rRNA gene was amplified with 10F and 1500R primers described in Table 2-2-2. PCR amplification consisted of an initial denaturation at 95 °C for 9 min., then 35 cycles of 20 sec. at 95 °C, 30 sec. at 50 °C, and 2 min. at 72 °C. The amplified DNA fragments were purified with an UltraClean 15 DNA Purification Kit (Nippon Gene, Tokyo) and sequenced using a CEQ DTCS-Quick Standard kit (Beckman Coulter, Fullerton, CA, USA) with the primers listed in Table 2-2-2. DNA was sequenced using a CEQ-2000XL sequence analyzer (Beckman Coulter, Fullerton, CA USA). The closest species were assigned on the basis of 16S rRNA gene sequence comparison with the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic tree was drawn using the maximum likelihood method with the MEGA6 package. The sequence of *Neisseria polysaccharea* was used as an out group. Bootstrap values were calculated from 1000 repeats and those greater than 50% are shown at branch points. The bar represents 0.01 substitution

Table 2-2-1. Composition of the CG medium (g).

Glucose	2
$MgSO_4$	0.2
$(NH_4)_2SO_4$	2
Sodium citrate	1
Yeast extract	0.1
$FeSO_4 \cdot 7H_2O$	0.01
$MnSO_4 \cdot 4H_2O$	0.01
Sodium phytate*	5
	1L (pH 5.8)

* sodium phytate was sterilized separately by filtration and added to the medium

Table 2-2-2. Primers used for amplification and sequencing of the 16s rRNA gene.

Primer	Sequence (5’-3’)
10F	AGAGTTTGATCCTGGCTCAG
500R	GTATTACCGCGGCTGCTGCTGG
519F	CAGCMGCCGCGGTAAT
800R	CATCGTTTACGGCGTGGAC
1000F	GTCCCGCAACGAGCGCAAC
1500R	GGTTACCTTGTTACGACTT

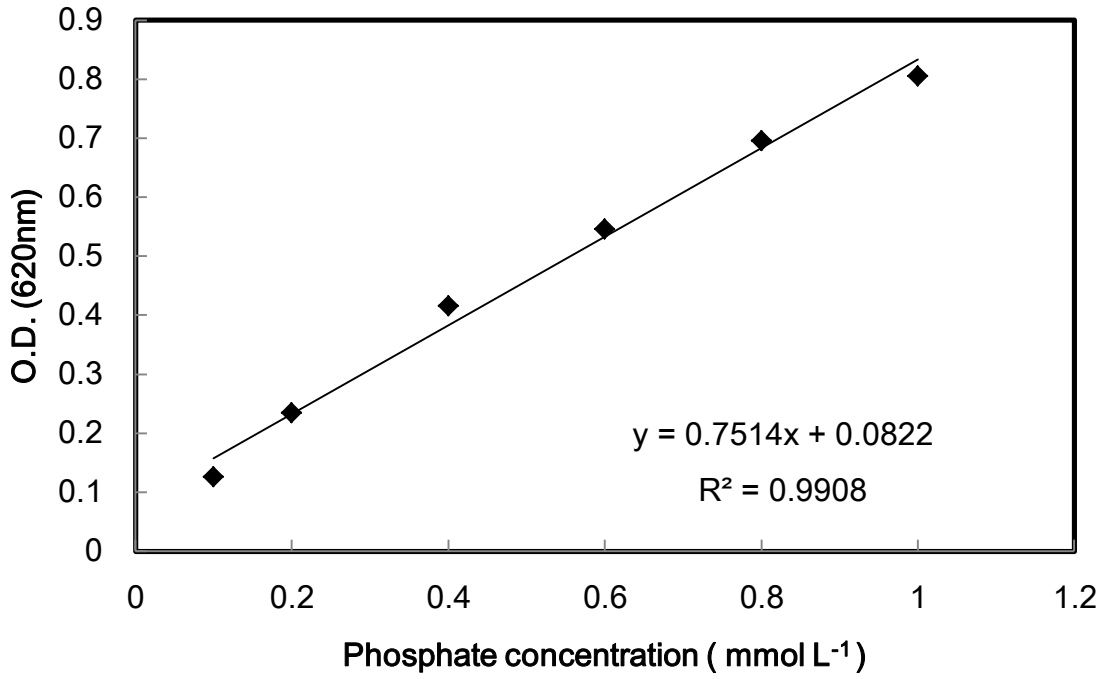

Fig. 2-2-1. Phosphate concentration standard curve.

Section III - Results

2-3-1 Enrichment Cultivation and Strain Selection by Halo Formation.

First, Samples of mud and fish intestinal contents obtained from areas surrounding net cage carp culture areas in Lake Kasumigaura were used for enrichment cultivation in CG medium, containing phytic acid as the sole phosphorous source. After the last step of enrichment cultivation, the liquid culture was properly diluted and spread over agar plates of the CG medium. After the incubation of the agar plates, around 300 colonies were obtained. Among the obtained colonies, 84 colonies were selected by morphological differences; single isolated and organized in a master plate. In order to narrow the selection of colonies able to produce phytase, two screening methodologies were performed: Screening using agar plates containing insoluble calcium phytate; Screening performed in liquid culture medium.

The first screening methodology tested consisted in the examination of halo formation by colonies cultivated in an agar plates containing insoluble calcium phytate. This plate received a treatment for a differential staining proposed by Bae et al., (1999), which allow visual distinction between phytase presence or organic acid production of microorganisms. This methodology was tested with a control plate, which was incubated with a commercial phytase and an acidic solution of lactic acid. Prior to the differential staining treatment, the insoluble calcium phytate was solubilized by the acid and was degraded by the action of the enzyme, leading to the formation of two clear zones. However, after performing the differential staining treatment, only the clear zone formed by the acid solution was reconstituted by re-precipitation of the acid-solubilized phytic acid, leaving the clear zone formed by enzyme action intact (Fig. 2-3-1). This result shows the effectiveness of the differential staining methodology, which was applied to confirm the phytase production by the 84 colonies obtained from enrichment cultivation. Although some colonies cultivated in the agar plate containing insoluble calcium phytate showed large clear zones prior to the differential staining, after the treatment, no clear zones could be observed for none of the colonies (Fig. 2-3-2). However, later analysis of phytase activity into the cell-free extract of some of those colonies indicated the presence of the enzyme.

2-3-2 Liquid Culture Screening and Phytase Activity Confirmation.

Since phytase activity couldn't be confirmed using the differential staining methodology, a different approach was performed. This approach consisted in the cultivation of the isolated colonies in the CG liquid medium and the supernatant was assayed for phosphate concentration. The strains showing an increase in phosphate concentration, compared with the medium without bacterial inoculation, were selected as candidates. From this screening, I selected 10 candidates which were assayed for phytase activity using the cell-free extract and the culture supernatant of those strains. Among those candidates, no phytase activity was detected at the culture supernatant, thus enzyme activity could be detected at the cell-free extract. The highest activity was obtained for strain a13 which showed a phytase activity of 605 $nmol.min^{-1}.mg^{-1}$ and was isolated from the mud of the Lake Kasumigaura, the second and the third highest activity was obtained for strain a16 and a17 respectively which were isolated form fish intestinal contents, all the other strains showed an enzyme activity lower than 100 $nmol\ min^{-1}\ mg^{-1}$ and were isolated from the mud of Lake Kasumigaura (Fig. 2-3-3). Enzyme localization was investigated for the strains with highest phytase activity. Total enzyme activity was measured at the culture supernatant, soluble and insoluble fractions. The majority of the phytase activity was detected at the soluble fraction of the strains a13, a16 and a17 (Fig. 2-3-4).

2-3-3 16S rRNA Gene Sequence Analysis of Phytase-Producing Strains.

All obtained strains showing phytase activity had the 16S rRNA gene sequence partially determined (around 500 bases) and the BLAST search of those sequences revealed that almost all strains have a similarity with bacteria of the *Klebsiella* genus. With the exception of strains bp21 and a13, that showed a close relation with the bacteria of the *Raoultella* and *Burkholderia* genus respectively (Table 2-3-1). The strains showing a phytase activity higher than 100 $nmol.min^{-1}.mg^{-1}$ had their complete 16s rRNA gene sequence elucidated. Phylogenetic analysis based on 16S rRNA gene sequences confirmed that strain a13 was included in the cluster of the genus *Burkholderia* (Fig. 2-3-5), with the closest related species being *Burkholderia soli* GP-25-8 which showed 98.9% identity (Table 2-3-1). Based on this

finding, I concluded that strain a13 should be classified in the genus *Burkholderia*, and the obtained strain was named as *Burkholderia* sp. Strain a13 and deposited at the JCM culture (JCM 30421). The nucleotide sequence data of the 16S rRNA was deposited into GenBank/EMBL/DDBJ databases under accession number LC002813.

Table 2-3-1. 16S rRNA gene sequence analysis from the isolated strains.

ID	Identity (%)	Coverage (bp)	Similar strain
a13	98.90	1437	*Burkholderia soli* strain GP25-8
a16	99.23	1435	*Klebsiella variicola* strain R25
a17	97.40	1465	*Klebsiella oxytoca* strain IARI-NIAW2-11
bp3	99.10	565	*Klebsiella* sp. SW81
bp15	98.50	665	*Klebsiella oxytoca* strain LF-1
bp21	98.77	570	*Raoultella ornithinolytica* strain B18
bp7	99.79	482	*Klebsiella* sp. SW81
bp10	99.42	525	*Klebsiella* sp. SW81
br14	99.43	535	*Klebsiella* sp. SW81
bp14	91.59	585	*Klebsiella oxytoca* strain XJU-1

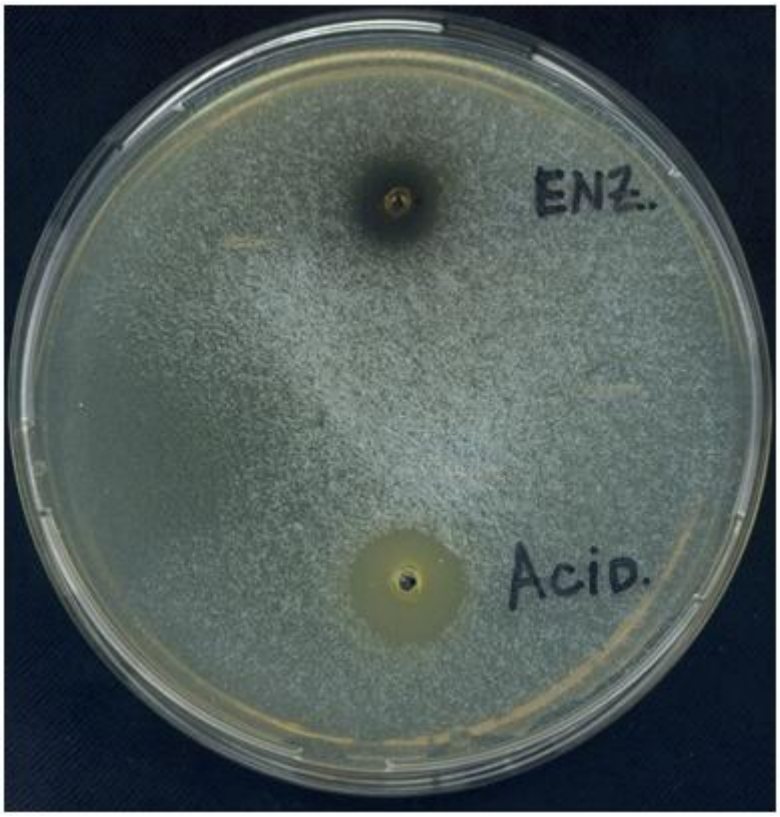

Fig. 2-3-1. Diferential phytase staining of clear zones formed by enzymatic activity and acid solubilization. (Enz) Represent a commercially available phytase clear zone. (Acid) Represent clear zone formed by acid solubilization.

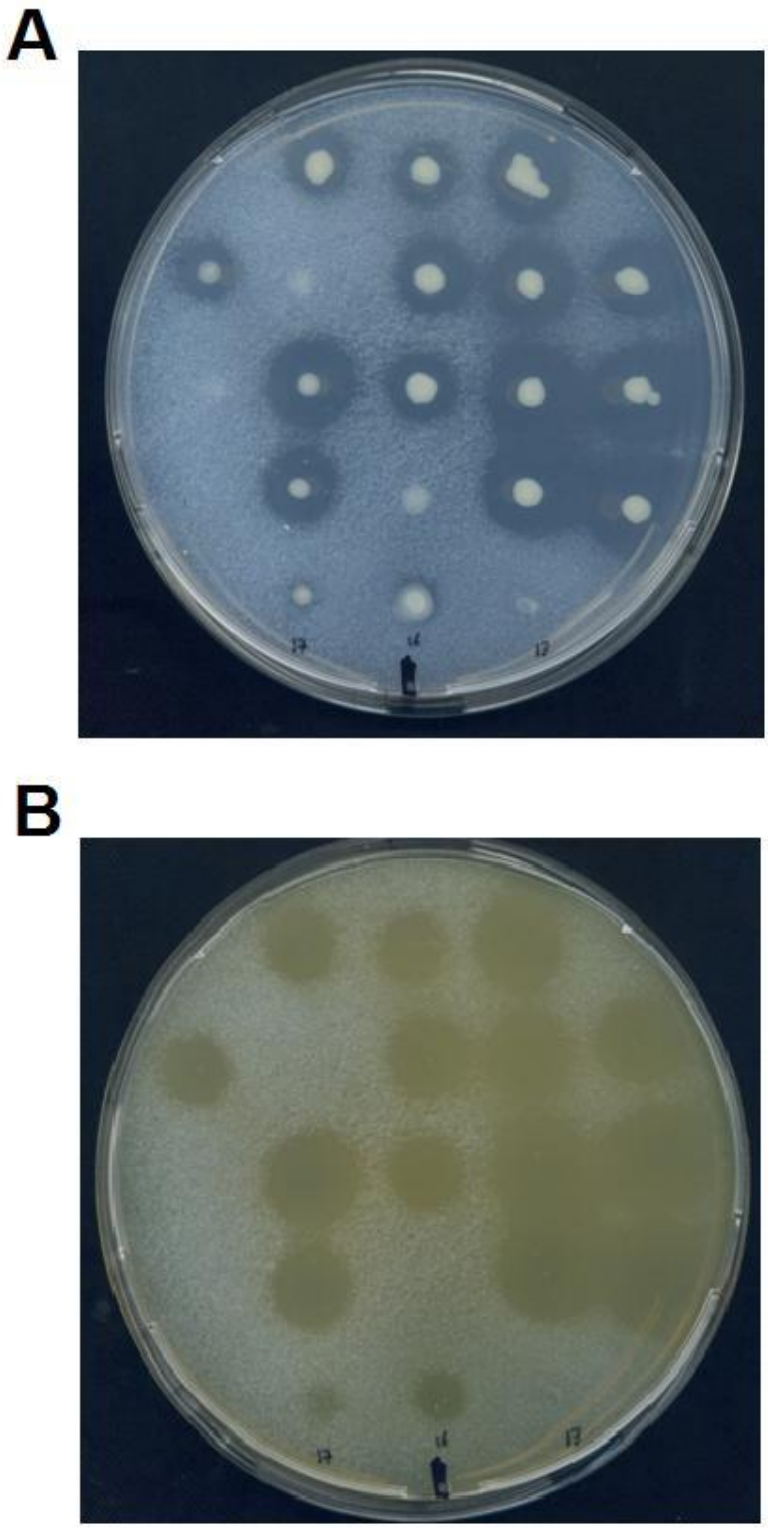

Fig. 2-3-2. Formation of halos by microorganisms before and after phytase differential staining. (A) Before phytase differential staining, (B) After phytase differential staining.

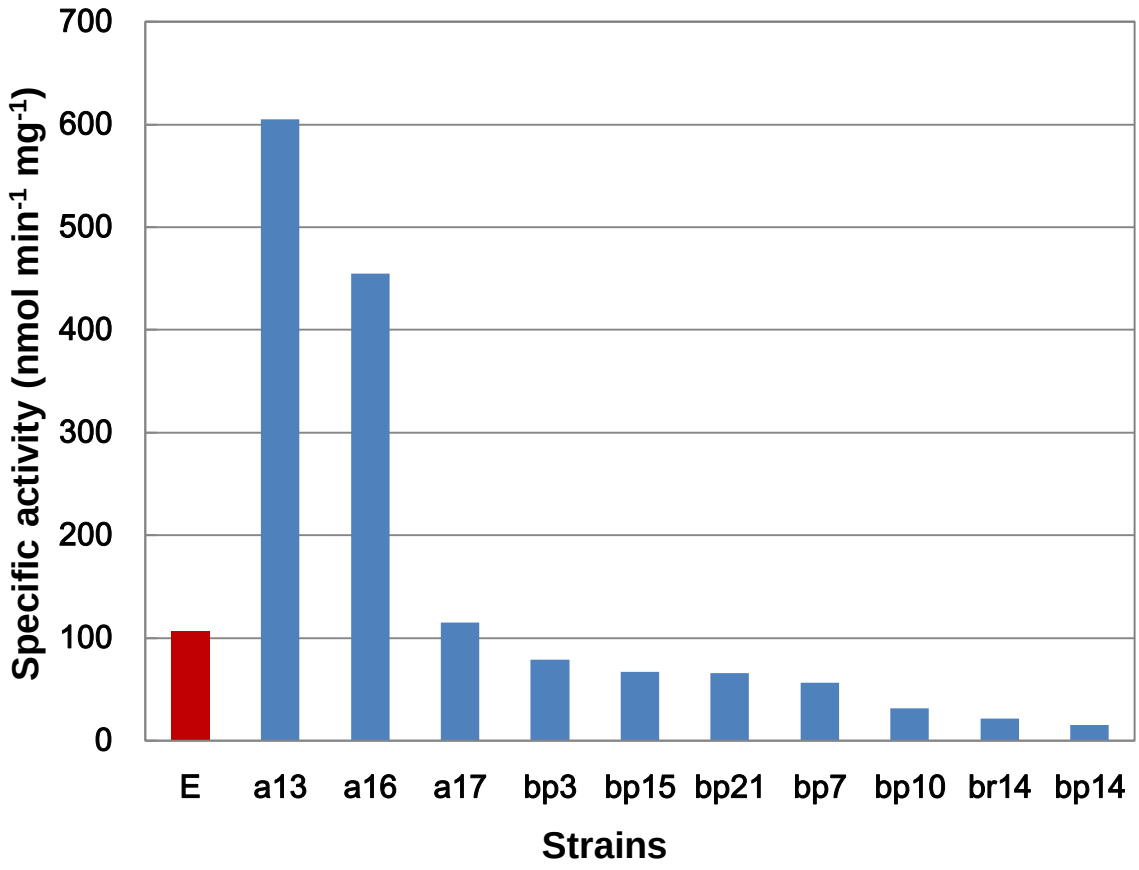

Fig. 2-3-3. Phytase specific activity of the cell-free extract of isolated strains in comparison to a commercially available phytase. Blue bars indicate activity of strains, (E) indicate activity of the commercial phytase.

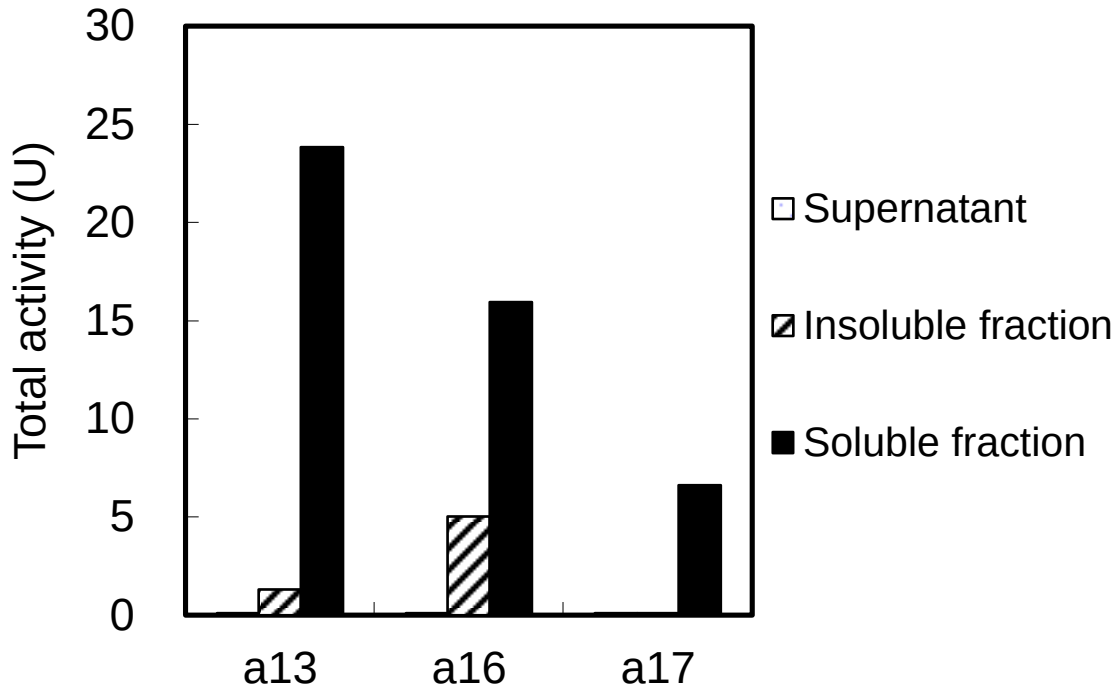

Fig. 2-3-4. Total phytase activity for different cell fractions and culture supernatant.

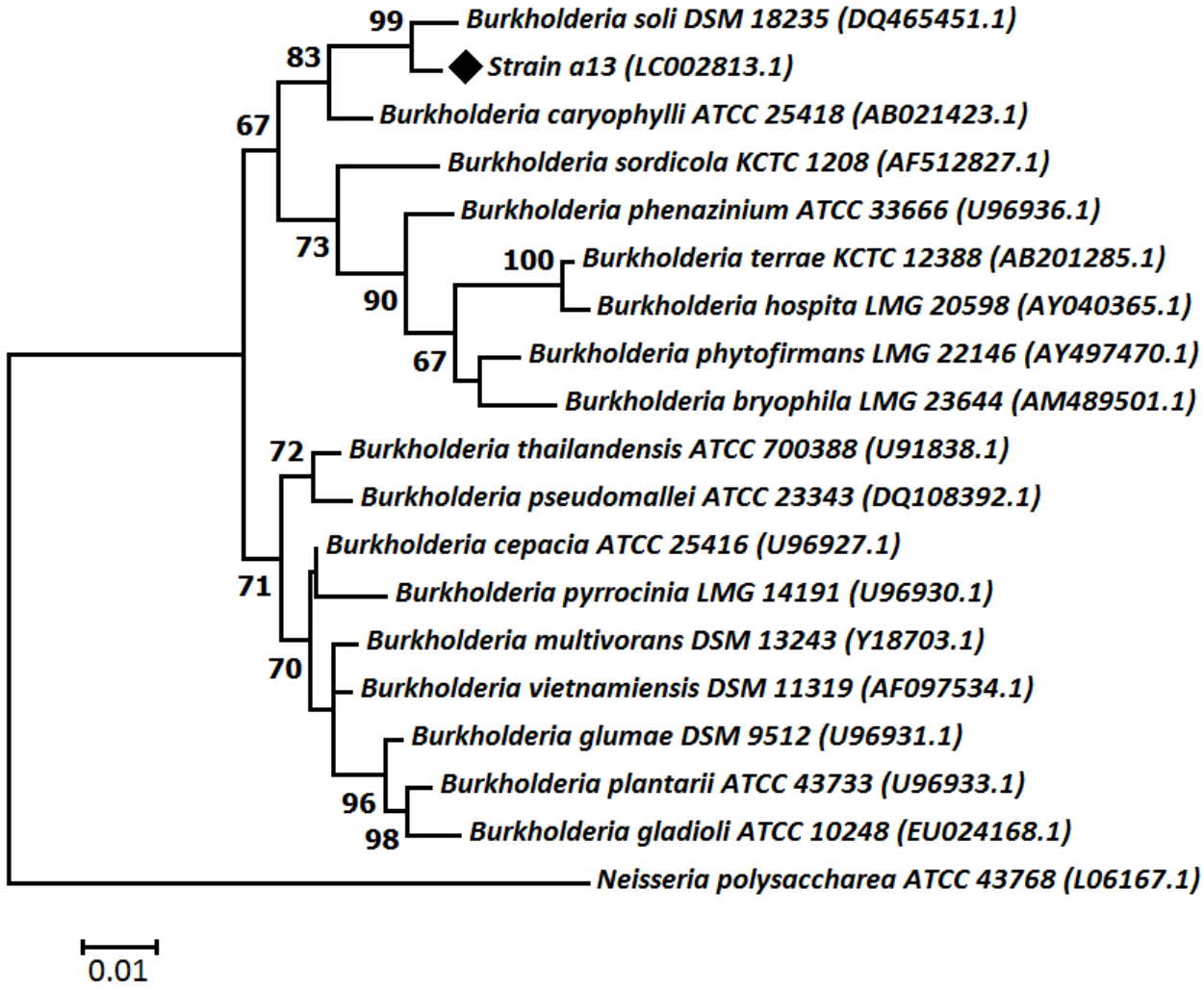

Fig. 2-3-5. Phylogenetic tree based on 16S rRNA gene sequences of strain a13 and type strains of the genus *Burkholderia*.

Section IV - Discussion

Phytic acid is one of the major organic phosphorus compounds in nature and is very stable in soils. Although a substantial amount of phytic acid is carried from terrestrial to aquatic systems, in aquatic system its presence is a minor component of organic phosphorous. The reason for this difference when compared to terrestrial environment implies the rapid hydrolysis of phytic acid under aquatic conditions (Cheng and Lim, 2006). Microorganism from the aquatic environment plays an important role in the conversion of phytic acid into lower inositol phosphate and phytate-phosphorous cycling in this environment (Lim et al. 2007). Although some phytases have been reported in microorganism isolated from the aquatic environment (Li et al., 2008b; Nam et al., 2014), the vast majority of reports are centered on phytase-producing microorganism isolated from the terrestrial environment, which suggests that the aquatic environment is less explored by researches as a source of phytase-producing microorganism. Taking in consideration the relevance and presence of phytic acid degrading bacteria in the aquatic environment, together with the possibility that grain-based feed used for net cage carp production in Lake Kasumigaura might represent a considerable load of phytic acid to the aquatic environment, allowing those bacteria to flourish around net cages environment, I decided to screen for phytase-producing bacteria from samples of mud and fish intestinal contents of those areas, raising the possibility of obtaining a novel strain with high phytic acid hydrolysis ability.

Through enrichment cultivation using phytic acid as a sole phosphate source, ten phytase-producing bacteria could be successfully isolated from the aquatic environment of Lake Kasumigaura. Enrichment cultivation is an effective strategy to obtain the desired microorganism by the fact that this method is selective to microorganisms possessing specific characteristics (in this case, the microorganisms able to hydrolyze phytic acid), however it is important to consider the limitations of enrichment cultivation as a screening technique, because it also can lead to the selection of false positive strains (strains without phytase production) that can be present at the end of the cultivation. In the screening of phytase-producing bacteria here described, enrichment cultivation could lead to growth of undesired false positive strains, due to phosphate contamination from the sample (mud or fish intestinal

contents), metabolite exchanges between bacterial strains present on the sample, or either by a lower purity of chemicals used, which might containing traces of free phosphate allowing bacterial growth. In order to reduce such possibilities, the CG medium used for screening was supplied with calcium chloride, which precipitate the free orthophosphate into insoluble calcium phosphate. Also for minimizing metabolites exchange between bacterial strains present on the sample, the incubation time was rather short (12 hours), lowering the possibility that a strain without phytase production ability would use the products of phytic acid hydrolysis promoted by a phytase-producing strain.

After enrichment cultivation, two different methods were used for selection of microorganisms and confirmation of their phytase production. The first method tested was the analysis of halo formation on agar plates, which received a phytase differential staining. The phytase differential staining described on the Section III of this chapter, is based on the principle of repreciptation of calcium phytate solubilized by organic acids. By this staining technique, it was possible to differentiate weather halo formation occurred by enzyme hydrolysis or organic acid presence, which was shown to be effective on the control experiment (Fig. 2-3-1). When bacterial strains obtained from enrichment cultivation were tested with the same methodology, halo formation couldn't be observed for none of the strains, because of the difficult differentiation between acid promoted clear zones from enzyme promoted clear zones (Fig. 2-3-2). However, liquid medium screening latterly tested, demonstrated that at least 10 strains possessed phytase activity (Fig. 2-3-3). The disparity between those results could be explained by the fact that the phytase activity couldn't be detected at the culture supernatant of the tested strains. The internal localization of the enzyme might lead to the formation of a very small clear zone, causing a difficult visualization for confirmation of enzyme activity by this methodology. Indeed, the differential staining methodology proposed by Bae et al. (1999) was used to isolate phytase-producing ruminal bacteria as *Selemonas Ruminantium*, which was reported to possess a phytase with a localization at the outer membrane (D'Silva et al., 2000). To date, the phytases produced by the *Bacillus* and the *Enterobacter* genus are reported to be excreted (Vohra and Satyanarayana, 2003), while the majority of the bacterial phytases are intracellular enzymes.

Since it wasn't possible to confirm phytase activity on agar plates, another strategy to narrow the number of candidates was adopted. This strategy consisted in measuring the concentration of free inorganic phosphate in the liquid medium. Since the minimal medium used contain sodium phytate as the sole phosphorous source, a rise of the concentration of inorganic phosphate into the liquid medium represents a hydrolysis reaction of phytic acid, which is a probable indicator that the microorganism is producing a phytase. Through this approach, the cultured strains showing a rise of the concentration of free inorganic phosphate in the medium were selected for determination of the phytase activity in the cell-free extract and the culture supernatant. Ten candidates were compared for phytase specific activity and some of them showed even a higher specific activity when compared to a commercial phytase labeled as "E" (Fig. 2-3-3). In this way the strains a13, a16 and a17 showing the highest specific activity were investigated to determine the localization of the enzyme. For all three strains, the highest total activity was obtained at the soluble fraction (Fig. 2-3-4), indicating the internal localization of the phytase produced by those strains.

The partial 16S rRNA gene sequence analysis of the phytase-producing isolates, revealed that almost all strains have a similarity with bacteria of the *Klebsiella* genus. With the exception of bp21 and a13 strains, that showed a close relation with the bacteria of the *Raoultella* and *Burkholderia* genus respectively (Table 2-3-1). *Routella* and *Klebsiella* genus of bacteria were previously reported as phytase producers, and the enzymes have already been characterized (Escobin-Mopera et al., 2012; Greiner et al., 1997). Among the isolates, strain a13 showed the highest phytase activity and was included in the cluster of the *Burkholderia* genus (Fig. 2-3-5). The genus *Burkholderia* comprises species isolated from a wide range of ecological niches including soil, water, human, plant, and clinical samples (Compant et al., 2008). Several reports have indicated a strong association of species from the genus *Burkholderia* within the rhizosphere of plants (Compant et al., 2008; Oliveira et al., 2009), and have suggested that their presence could contribute to plant growth by liberating phosphates from soil organic compounds (Unno et al., 2005). Although some reports have also suggested that some strains of the genus *Burkholderia* possess phytate-degrading ability (Unno et al., 2005; Weisskopf et al., 2011; Hayatsu, 2013), to date, there are no reports on purification and biochemical characterization of phytases from this genus. Strain a13, showing

the highest phytase activity was determined as belonging to the *Burkholderia* genus. The further study of its phytase could be considered important in two aspects: First, the biochemical characterization could help determine if the enzyme is suitable for a future application. Second, the better understanding of this novel enzyme produced by strain a13 could contribute to the knowledge of phytases, and unveil its role to strain a13 metabolism.

CHAPTER III - CONDITIONS FOR ENZYME PRODUCTION

Section I - Introduction

In most microorganisms, phytases are reported to be an inducible enzyme and its expression is subjected to a complex regulation, which is not controlled uniformly among different microorganisms (Liu et al., 1998). Although reports about isolation and characterization of phytases from different microorganisms have been increasing recently, there is a lack of detailed reports about regulation and production of phytases on those microorganisms. To date, phytase production by bacterial strains was investigated in detail only in *Escherichia coli* (Touati et al., 1987; Greiner et al., 1993) and *Raoultella terrigena* (Greiner et al., 1997), which indicate that more profound studies about phytase production and expression regulation are expected.

In this chapter, I investigated the conditions for production of *Burkholderia* sp. strain a13 phytase. This information is relevant for understanding if the enzyme is inducible or constitutive, and how this production is regulated in strain a13, giving an insight of its role to the cell physiology. The knowledge about the conditions for phytase production in strain a13 was used to improve the yield of enzyme production, which facilitates the later purification of the enzyme produced by this strain. At the first experiment, Strain a13 was grown in a modified CG medium (CG') which was supplemented with different phosphate and carbon sources and enzyme production was analyzed over cultivation time. From this experiment, it was possible to determine the peak of enzyme production, but little information was obtained about the regulation of enzyme production by this experimental condition. In this way, in order to address the physiological role of the phytase to strain a13 a different experimental approach was taken. The bacterium was grown at a modified CG medium (CG'') containing a neutral carbon source (sodium citrate). Citrate, which is a substrate of the TCA cycle, is known as a neutral carbon source because it has neither an inducing nor a repressing effect, allowing strain a13 to grow using it as a carbon source. The effect of carbon and phosphorous sources over phytase production was studied through later addition of glucose, *myo*-inositol, phytic acid and sodium phosphate to this medium.

Section II - Material and Methods

3-2-1 Phytase Production

Enzyme production was tested in the modified CG' medium with addition of different carbon and phosphorous sources before bacterial inoculation. The CG' medium consisted of the same composition of the CG medium previously described at the Chapter II (Table 2-2-1), but sodium phytate was removed. Four variants of the CG' were prepared by addition of different phosphate and carbon source. The first variation had addition of 65 mM sodium phosphate pH 5.8 (CG' + Pi); The second variation had addition of 5 $g.L^{-1}$ sodium phytate (CG' + PA); The third had addition of 5 $g.L^{-1}$ sodium phytate with 65 mM sodium phosphate pH 5.8 (CG' + PA + Pi); The last variant was done with addition of 5 $g.L^{-1}$ sodium phytate, 65 mM sodium phosphate pH 5.8 and 8 g of glucose added after 8 hours of incubation (CG' + PA + Pi + Glu). All media described had the final pH adjusted to 5.8. A start culture composed of each respective medium was incubated for 24 hours at 30 °C with agitation. The main culture was inoculated to a final O.D. of 0.03, and incubated at 30 °C in a shaker at 120 rpm. The culture cell growth was monitored by absorbance measurement at 600 nm. Enzyme activity at the cell-free extract was measured by sampling the culture at 6, 15, 24 and 48 hours. Phytic acid content in the culture supernatant was measured by a colorimetric method described by Latta and Eskin (1980). Two-hundred micro liters of Wade reagent was added to 600 µl of the sample properly diluted in distilled water, and the transmittance was measured at 500 nm, using 600 $mg.L^{-1}$ of a phytic acid sodium salt solution as blank. The phytic acid standard curve was obtained using a series of standard solutions ranging from 10-75 $mg.L^{-1}$ phytic acid in distilled water. Increasing concentrations of phytic acid against the transmittance were plotted in a graphic, and the phytic acid concentration standard curve was obtained by linear regression (Fig. 3-2-1). Glucose content of culture supernatant was measured with AutoKit Glucose (439-90901, Wako Pure Chemical Industries, Ltd., Osaka, Japan). Glucose and phytic acid concentration of culture supernatant were measured periodically.

3-2-2 Phytase Production in Neutral Carbon Source Containing Medium

To test the effect of carbon source and inorganic phosphate over phyatse production, strain a13 was grown with a neutral carbon source. The CG''- phytic acid and the CG''- phosphate medium were prepared as shown in (Table 3-2-1). Sodium citrate and a low amount of glucose were added as the main carbon source and to support initial growth, respectively. One percent of an overnight culture of strain a13, grown at 30 °C in a nutrient rich medium containing (per liter) 8 g of nutrient broth (Difco Laboratories ltd., Detroit, USA), 5 g of yeast extract (Difco Laboratories), 3 g of NaCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, was inoculated to the above media and the cultures were incubated at 30 °C with 120 rpm of agitation. After 8 h of incubation, glucose content of culture supernatant was measured with AutoKit Glucose (Wako Pure Chemical, Japan) for confirmation of total consumption of this carbon source. After 10 h of incubation, 5 $g.L^{-1}$ of phytic acid only, or together with 5 $g.L^{-1}$ of glucose or *myo*-inositol, was added to the culture of CG''-phosphate medium and 65 mM (final concentration) sodium phosphate, pH 5.8, was added to the culture of CG''-phytic acid medium. Then, phytase activity and cell growth, monitored by the absorbance at 600 nm, were periodically measured.

3-2-3 Utilization of Phytic Acid and myo-Inositol by Strain a13

Strain a13 was grown in a minimal medium containing phytic acid or *myo*-inositol as the sole carbon source. This medium was composed by 0.2 g of $MgSO_4$, 2 g of $(NH_4)_2SO_4$, 0.1 g of yeast extract (Difco Laboratories), 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.01 g of $MnSO_4 \cdot 4H_2O$, 65 mM sodium phosphate pH 5.8 and 5g of *myo*-inositol or 5 g of sodium phytate (sodium phytate was sterilized separately by filtration and added to the medium). One percent of an overnight culture of strain a13, grown at 30 °C in a nutrient rich medium containing (per liter) 8 g of nutrient broth (Difco Laboratories), 5 g of yeast extract (Difco Laboratories), 3 g of NaCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, was inoculated to the above media and the cultures were incubated at 30 °C with 120 rpm of agitation. Then, cell growth was monitored by the absorbance at 600 nm periodically.

Table 3-2-1. Composition of the neutral carbon source media

CG'' - Phosphate medium (g)	
Sodium citrate	3
Glucose	1
$(NH_4)_2SO_4$	2
$MgSO_4$	0.2
Yeast extract	0.1
$FeSO_4 \cdot 7H_2O$	0.01
$MnSO_4 \cdot 4H_2O$	0.01
Sodium phosphate	9
	1L (pH 5.8)

CG'' - Phytic acid medium (g)	
Sodium citrate	3
Glucose	1
$(NH_4)_2SO_4$	2
$MgSO_4$	0.2
Yeast extract	0.1
$FeSO_4 \cdot 7H_2O$	0.01
$MnSO_4 \cdot 4H_2O$	0.01
Sodium phytate*	5
	1L (pH 5.8)

* sodium phytate was sterilized separately by filtration and added to the medium

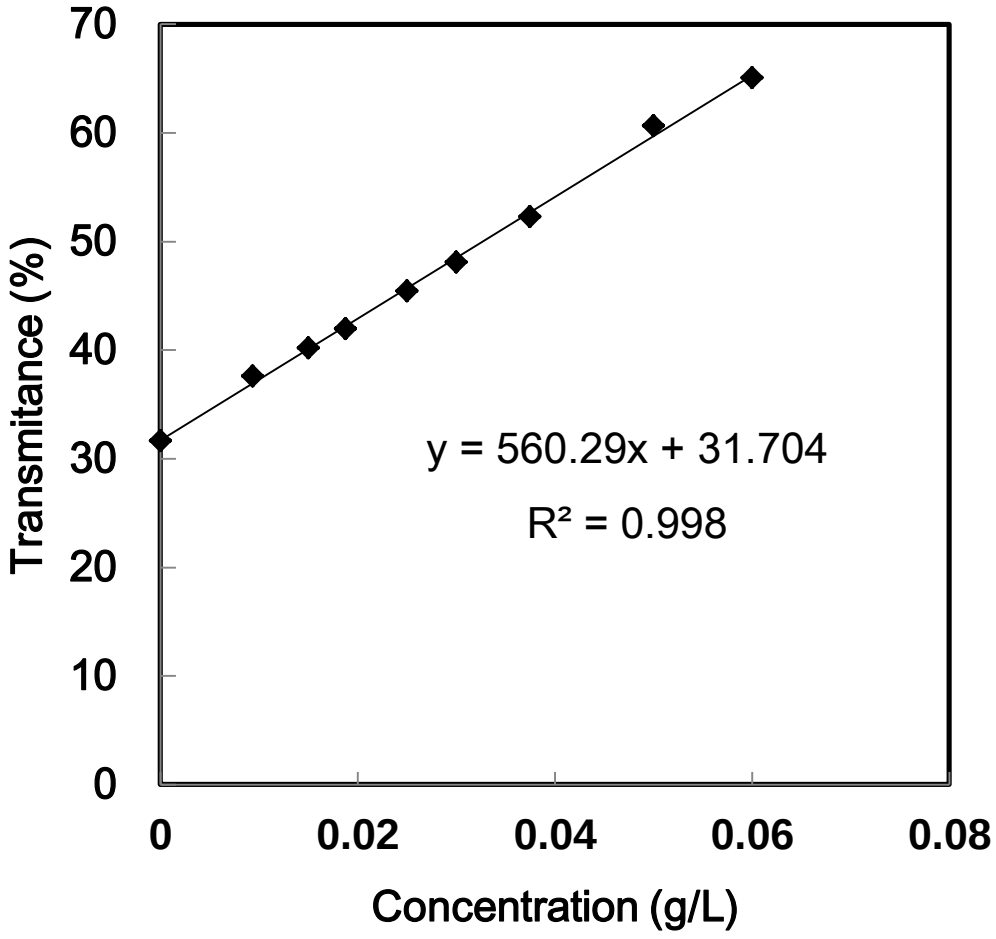

Fig. 3-2-1. Phytic acid concentration standard curve.

Section III - Results

3-3-1 Phytase Production

In order to evaluate the production of the phytase in media containing different compositions of carbon and phosphorous source, as well to determine the optimal timing for enzyme production, strain a13 was incubated in four variants of the CG' medium (CG' + Pi; CG' + PA; CG' + PA + Pi; CG' + PA + Pi + Glu). Phytase activity, which was represented by bars in the graphic, was only detected when a13 strain was incubated in media containing phytic acid in its composition (light-gray, dark-grey, black bars), but no activity could be detected for the cultivation in media containing sodium phosphate as the sole phosphorous source (white bars) (Fig. 3-3-1C). Although phytase activity was measured at the exponential and stationary phase of the cultures, enzyme activity could only be detected after the culture enters in the stationary phase, being only detected after 15 hours of cultivation. The highest phytase activity was obtained after 48 hours of incubation in the medium containing phytic acid as the sole phosphorous source (light-gray bars). Addition of inorganic phosphate to this medium (represented by dark-gray bars) didn't show to strongly affect enzyme activity, which had similar production. However, the addition of glucose (black bars) completely repressed enzyme production at 15 and 24 hours of cultivation, which showed a shift in its production, being only detected after 48 hours (Fig. 3-3-1C). When phytic acid contents of the medium were measured, the cultures of CG' + PA (circles) and CG' + PA + Pi (triangles) showed fast consumption of phytic acid as soon as the culture reach the stationary phase, which is consistent with their phytase activity that was detected after 15 hours of incubation (Fig. 3-3-1B, C). A lower phytic acid consumption was observed for the culture that had addition of glucose after 8 hours of incubation (diamonds), which also showed lower and shifted phytase production (Fig. 3-3-1B, C). On the other hand, this addition of glucose after 8 hours of cultivation, showed to strongly promote the cell growth of this culture, which was able to reach an O.D. of 9.3 in comparison to the other cultures containing phytic acid (circles and triangles) that reached a final O.D. of only 3.2 (Fig. 3-3-1A). The lowest growth was observed for CG' + Pi culture (squares) which showed an O.D. of 2.7 at the

stationary phase (Fig. 3-3-1A). All cultures showed a complete consumption of glucose at 12 hours, with exception for the culture that had addition of glucose after 8 hours (diamonds), that just consumed all the glucose after 24 hours (Fig. 3-3-1C). Those results suggest the inducible production of strain a13 phytase by phytic acid. It also suggests a correlation between glucose starvation and enzyme production. However, in order to address this correlation and determine if the enzyme is regulated by a catabolite repression, further experimentations are needed. The analysis of phytase production in response to a later addition of different carbon and/or phosphorous source to a medium without the interference of inducers or repressors would help determine this correlation.

3-3-2 Effect of Carbon and Phosphorous Source Over Enzyme Production

To examine the conditions for the phytase production, as well to address the physiological role of the phytase, strain a13 was cultured in minimal medium based on CG medium (Table 3-2-1), in which a neutral carbon source was used for growth. Initially, the effect of carbon sources over phytase activity was analyzed (Fig. 3-3-2A). Strain a13 was incubated for 10 h in the CG”- phosphate medium, which contained sodium phosphate as the phosphorous source. Then phytic acid, with or without glucose or *myo*-inositol, was added to the medium, and the phytase activity was measured. As a result, in the absence of phytic acid, the phytase activity (represented by closed symbols) was not detected throughout culturing time, but when phytic acid was added to the medium, the activity was detected after 2 h and reached maximum at 8 h after addition (Fig. 3-3-2A). Addition of glucose or *myo*-inositol, together with phytic acid, inhibited the production of the phytase, though the growth of the cells was improved substantially (open symbol). Glucose inhibited the phytase production almost completely: at 8 h after the addition, the activity was about 7.7% of that without addition of glucose and, in the case of *myo*-inositol addition, the activity was about 20%. These results indicate that, in strain a13, the phytase production is induced by the addition of phytic acid and this induction is controlled by a catabolite repression. The effect of phosphorous source over phytase activity was analyzed by incubation of strain a13 at the CG”- Phytic acid medium (Fig. 3-3-2B). Phytic acid is generally considered as a phosphorous source and phytase

production had been shown to occur during phosphorous starvation in some bacteria (Greiner et al., 1993; Voigt et al., 2006); however, in the case of strain a13, addition of enough amount of phosphate to the culture in the CG''-phytic acid medium did not affect the phytase production (Fig. 3-3-2B), indicating that the production is insensitive to phosphate availability. It seems likely that a13 strain produces the enzyme in order to use phytic acid as a carbon source. For that reason, it's important to demonstrate that strain a13 is able to grow in a medium containing phytic acid as the sole carbon source. Indeed, when strain a13 was grown in a media containing phytic acid or *myo*-inositol as the sole carbon source, it was able to grow, showing a final O.D. of 3.75 for *myo*-inositol and 1.24 for phytic acid (Fig 3-3-3).

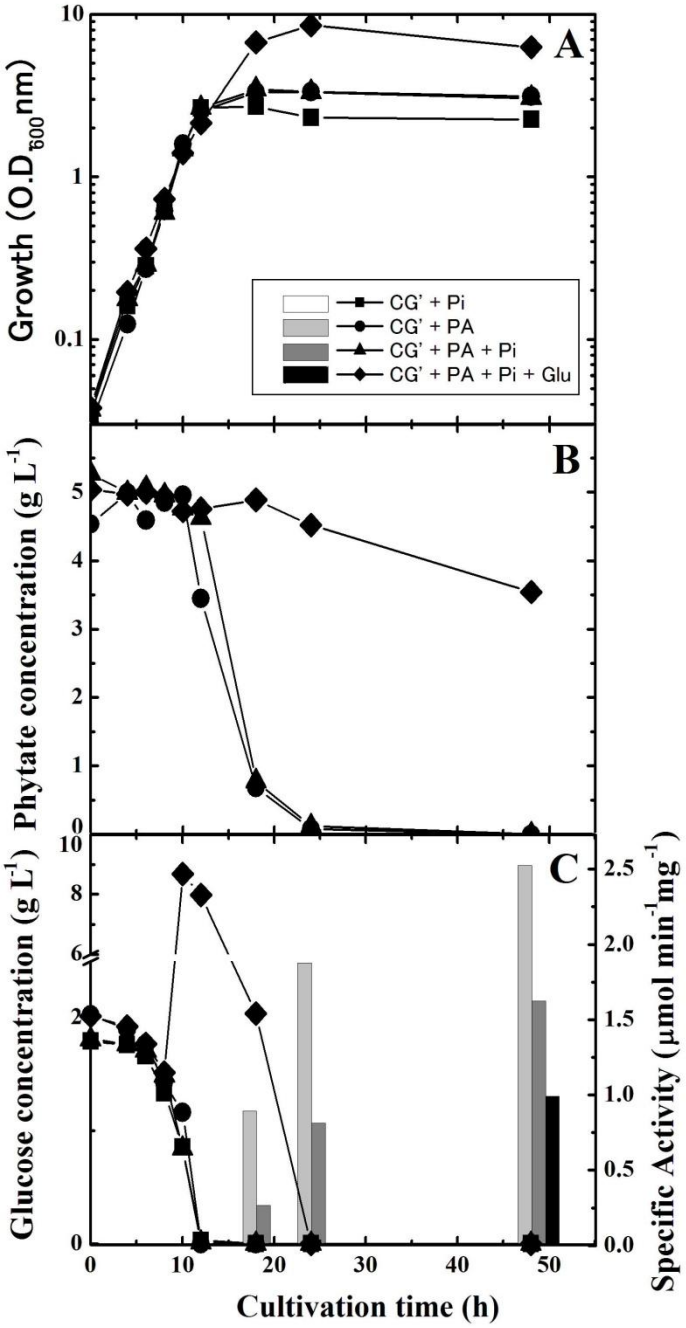

Fig. 3-3-1. Strain a13 phytase production by cultivation in modified CG' medium with addition of different carbon and/or phosphorous sources. Supplementation with inorganic phosphate was indicated by squares or white bars; phytic acid by circles or light gray bars; phytic acid and inorganic phosphate by triangles or dark grey bars; phytic acid, sodium phosphate and later glucose at 8h of cultivation is represented by diamonds or black bars. (A) Cell growth monitored by optical density at 600 nm. (B) Phytic acid concentration at culture supernatant. (C) glucose concentration at culture supernatant (lines) and phytase activity in the cell-free extract (bars).

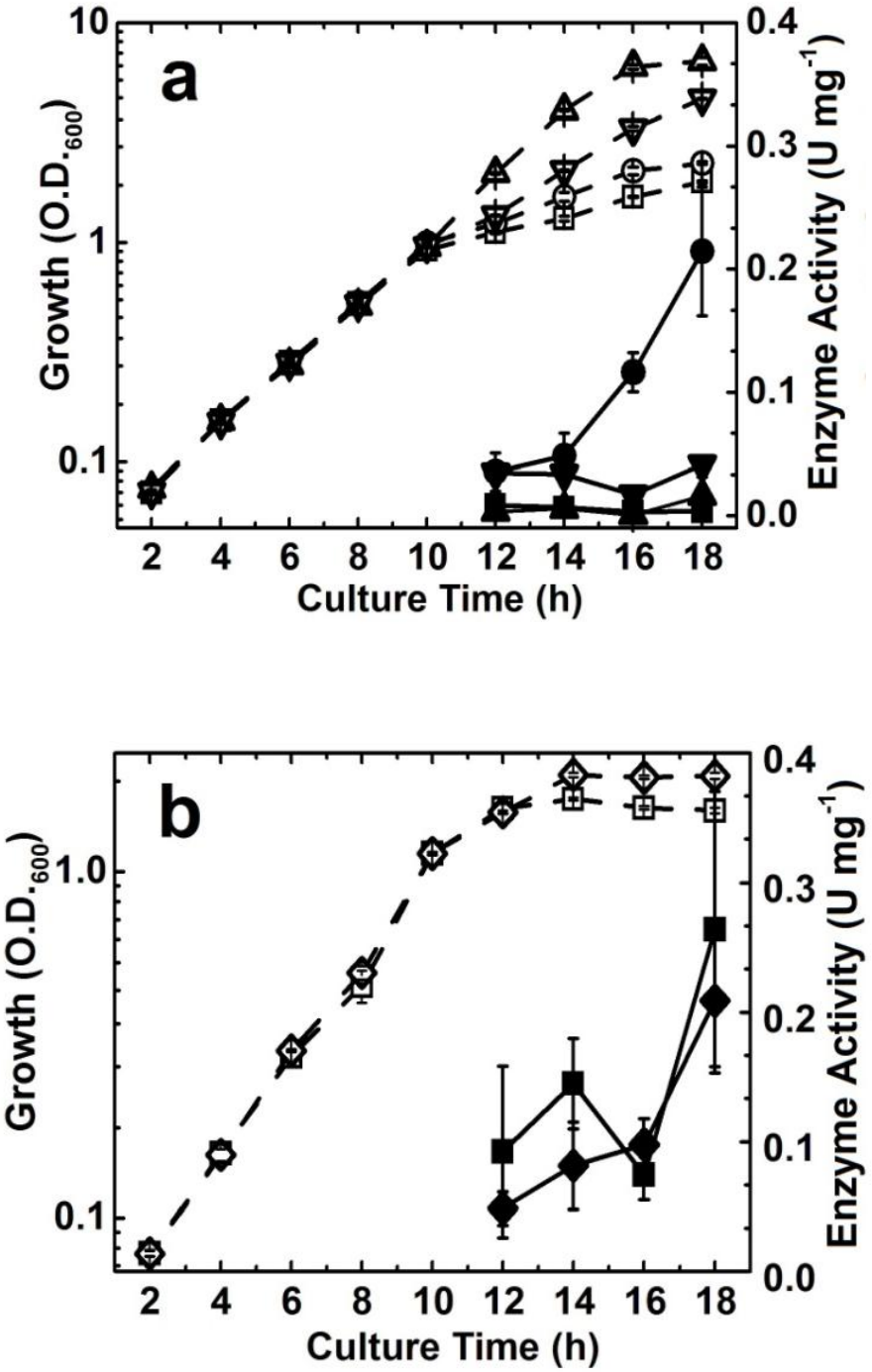

Fig. 3-3-2. Effect of phytic acid, carbon sources, and phosphate addition on the phytase production of strain a13. Strain a13 was cultured in CG'-phosphate (a) and CG'-phytic acid (b) media, and at 10 h after cultivation, phytic acid (circles), phytic acid + glucose (up triangles), phytic acid + *myo*-inositol (down triangles), and phosphate buffer (diamonds) were added. Squares indicate the cultures without any addition. Open and filled symbols indicate cell growth monitored by optical density at 600 nm and the phytase activity in the cell-free extract, respectively. The cultures were conducted in triplicate and average ± S.D. are shown

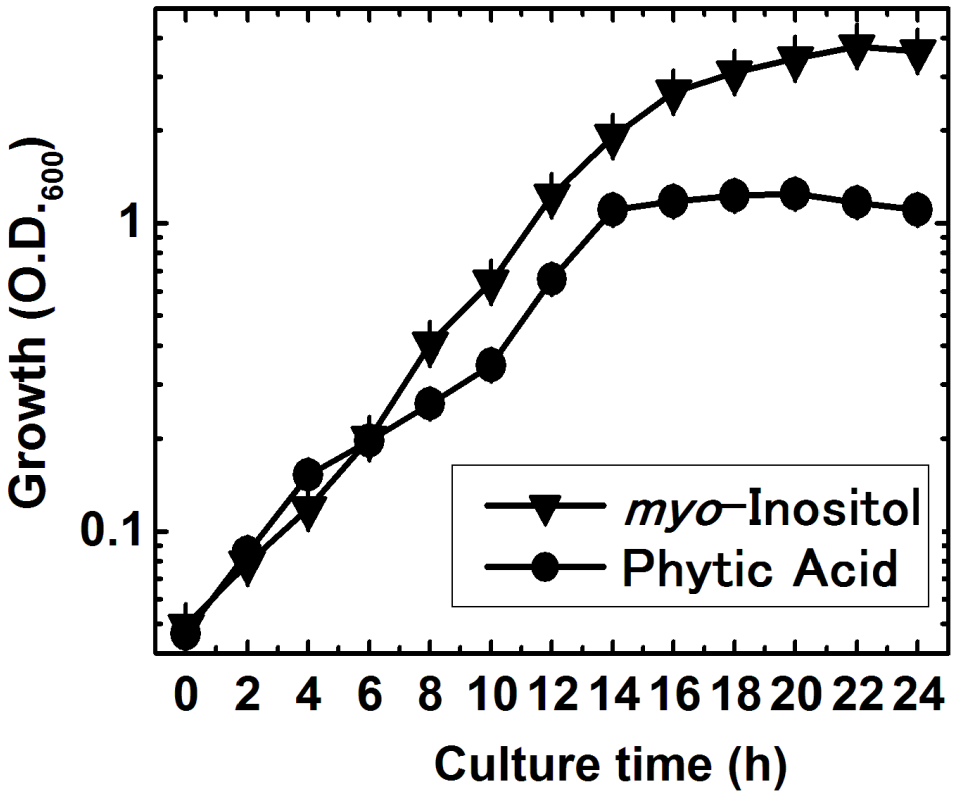

Fig. 3-3-3. Growth curve of strain a13 cultivated on minimal medium with phytic acid (circles) or *myo*-Inositol (down triangles) as the sole carbon source.

Section IV - Discussion

Phytase production was tested by incubation of strain a13 in the modified CG' medium, which was supplemented with different carbon and phosphorous sources. The highest activity (about 2.5 U mg^{-1}) was obtained after 48 hours of incubation at the medium supplemented with phytic acid only (Fig. 3-3-1C). In this way, future incubations of strain a13 with the objective to produce the enzyme should consider this culture condition for higher enzyme productivity. A higher yield of enzyme production is a desired property, which might facilitate the future enzyme purification process. The only culture that didn't showed any phytase activity was the culture containing sodium phosphate as the sole phosphorous source. On the other hand, addition of phytic acid to the cultures showed to induce enzyme production, suggesting that the phytase is inducible rather than an constitutive enzyme (Fig. 3-3-1C). One interesting result obtained by this experiment is the fact that phytase activity was only detected after the glucose content of the media is completely consumed (around 12 hours of cultivation). Later addition of an extra-amount of glucose to the medium caused a shift of the enzyme production which couldn't be detected at the expected cultivation time (15 or 24 hours), but it was only detected after the glucose was completely consumed, which occurred around 24 hours of cultivation. Also, addition of inorganic phosphate to the medium didn't show to strongly affect the enzyme activity, which was produced on the same cultivation timing and with relative same levels of production in comparison to the culture without supplementation of inorganic phosphate (CG' + PA)(Fig. 3-3-1C). The timing of phytase production by strain a13 is accompanied by the consumption of phytic acid from the media, which became fast as soon as the levels of glucose dropped and when the enzyme levels start to rise, indicating that the enzyme is responsible for this consumption. The supplementation with extra-amount of glucose to the medium seemed also to change this phytic acid consumption velocity, which was consumed slowly (Fig. 3-3-1B). Taken together, those results suggest that the phytase production is repressed by the carbon source and is possibly involved in the utilization of phytic acid as a carbon source, rather than a phosphorous source. However, the experimental conditions of this first experiment weren't ideal and deterministic for this type of regulation. At those experimental conditions, glucose was added together with phytic acid from the

beginning of the incubation. In this way, if glucose is actually working as a repressor, a possible induction promoted by addition of phytic acid to the medium can't be detected at the first stages of the culture. This generates a problem, because it creates a very large gap between induction caused by phytic acid and enzyme activity detection that just occurred at later stages of the culture. This problem generates a difficulty in isolation of other variables that might also be causing this enzyme production, like a nutrient or energy limitation. Another important factor to analyze that wasn't tested by this experimental condition is the effect of *myo*-inositol over enzyme production. If strain a13 is able to use phytic acid as a carbon source, it is most probable that the phytic acid molecule is hydrolyzed to produce *myo*-inositol which might be used by the strain. For that reason the effect of *myo*-inositol over the enzyme production might be an important data to reveal the role of the phytase to strain a13 metabolism.

In order to countermeasure the experimental problems of the first experiment and to determine which type of regulation is taking place for the phytase produced by strain a13, a new experimental approach was adopted. This experimental approach consisted of the incubation of strain a13 in a medium containing a neutral carbon source (sodium citrate) which doesn't act as a repressor. Different from the first experiment, the effect of carbon sources (glucose and *myo*-inositol) as well the effect of inorganic phosphate was tested by measuring phytase activity right after this supplementation, during bacterial growth, which minimizes interference of other variables. By those experimental conditions, the induction or the inhibition of enzyme activity became clear, because it could be detected right after the addition of the different carbon or phosphate sources. Generally, phytase-producing microorganisms, with the exception of *Raoultela terrigena* and the rumen bacteria, have a tight inhibition on enzyme synthesis by inorganic phosphate levels (Konietzny and Greiner, 2004). In *E. coli*, enzyme synthesis occurs upon entry into the stationary phase and under anaerobic conditions, when cells are starved for inorganic phosphate, while carbon, nitrogen, and sulfur limitation are ineffective (Touati et al., 1987; Greiner et al., 1993). In rumen bacteria, phytase production has been induced by phytic acid and, in the case of *R. terrigena*, the production was induced by carbon source starvation, as well as induction by phytic acid (Greiner et al., 1997). In contrast to the majority of other phytase producers, the enzyme production by strain a13 was

induced by phytic acid but not repressed by the presence of excess amount of phosphate; instead, the production was strongly repressed by the addition of glucose and moderately by *myo*-inositol (Fig. 3-3-2). It seems likely that strain a13 produces phytase to utilize phytic acid as a carbon source, after degrading it to *myo*-inositol, rather than utilizing it as a phosphorous source. The observation that this strain can grow with phytic acid and *myo*-inositol as a sole carbon source strengthens this conclusion (Fig. 3-3-3). In fact, the utilization of *myo*-inositol as a sole carbon source have already been reported for *Bacillus subtilis* (Yoshida et al., 1997), *Enterobacter aerogenes* (Berman and Magasanik, 1966), *Sinorhizobium meliloti* (Galbraith et al., 1998) and *Corynebacterium glutamicum* (Krings et al., 2006).

The metabolic pathway of the catabolism of *myo*-inositol in *Bacillus subtilis* have already been studied in detail at the genetic and also protein level (Yoshida et al., 2008). The *iolABCDEFGHIJ* operon of *Bacillus subtilis* is responsible for *myo*-inositol catabolism involving multiple and stepwise reactions that were represented at Fig. 3-4-1. As a result *B. subtilis* is able to promote the conversion of *myo*-inositol to yield one acetyl-CoA molecule, which provide the cell with energy upon its utilization in the TCA cycle; one molecule of dihydroxyacetone phosphate, which can be rapidly and reversibly isomerized to glyceraldehyde 3-phosphate entering into the glycolysis metabolic pathway; and one CO_2 molecule. All those conversions results in acquisition of two NADH molecules and the consumption of an ATP molecule (Yoshida et al., 2008). The role of the *iolABCDEFGHIJ* operon genes are very important to *B. subtillis*, conferring the ability to synthesize enzymes for utilization of *myo*-inositol as a carbon source. This ability is very important to *B. subtillis*, because it can confer advantages in the obtainment of energy from different sources. Since strain a13 is also able to grow with *myo*-inositol as the sole carbon source (Fig. 3-3-3), it is interesting to determine how conserved the *iolABCDEFGHIJ* operon genes would be in the genome of *Burkholderia* sp. strain a13. Unfortunately, strain a13 genome as well its closest related species (*Burkholderia soli*) doesn't have their genomic information available to date. In this case, the closest related species to strain a13, that have the genome accessible in databases was *Burkholderia phytofirmans*. A search of ortholog genes of the *iolABCDEFGHIJ* operon in the genome of *Burkholderia phytofirmans* revealed some genes with considerable similarity (Table 3-4-1). The highest similarity was

found for BPYT_2812 (47.6%) which was annotated as "*myo*-inositol catabolism *iol*B domain-containing protein". On the immediate upstream sequence from this gene was found BPYT_2811, BPYT_2810 and BPYT_2819 genes, which showed homology with *iol*E, *iol*D and *iol*C of *B. subtillis* respectively, suggesting that a species of the *Burkholderia* genus might have conserved some of the genes that are involved in the catabolism of *myo*-inositol, at least for *B.subtilis*. The search of those *iol* genes on other species of the *Burkholderia* genus (*B. cepacia*) showed even a higher identity (58.2%) to the *iol*G gene of *B. subtillis* (data not shown), indicating important differences among species of the *Burkholderia* genus. Nowadays, a large number of bacteria have genes annotated *iol* in their genome sequence, but the annotation is only based on sequence similarity to *B. subtilis iol* genes, as relatively few studies have been done to demonstrate the participation of the deduced *iol* genes in *myo*-inositol catabolism. In the future, determining if strain a13 also have on its genome, genes similar to those of the *iol* operon, as well more detailed studies at the protein level for the catabolism of *myo*-inositol might be of great value to determine if those genes are really involved in this catabolism. Also, the production of a phytase knockout gene of strain a13 would prove the ability to use phytic-acid as a carbon source, as this knockout strain is expected to grow with *myo*-inositol as a carbon source, but lose its ability to hydrolyze phytic acid becoming unable to grow in a minimal medium containing phytic acid as the sole carbon source.

Table 3-4-1. Sequence homology of *Bacillus subtilis iol*ABCDEFGHIJ operon genes with the corresponding genes of *Burkholderia phytofirmans* PsJN.

Locus tag	Annotation	Gene	Identity (%)
Bphyt_2804	Inositol 2-dehydrogenase	*iol*G	26.3
Bphyt_2811	Xylose isomerase	*iol*E	37.1
Bphyt_2810	Thiamine pyrophosphate protein central region	*iol*D	40.4
Bphyt_2812	*myo*-inositol catabolism *Iol*B domain-containing protein	*iol*B	47.6
Bphyt_2809	PfkB domain-containing protein	*iol*C	29.0
Bphyt_3275	Fructose-1,6-bisphosphate aldolase	*iol*J	32.4

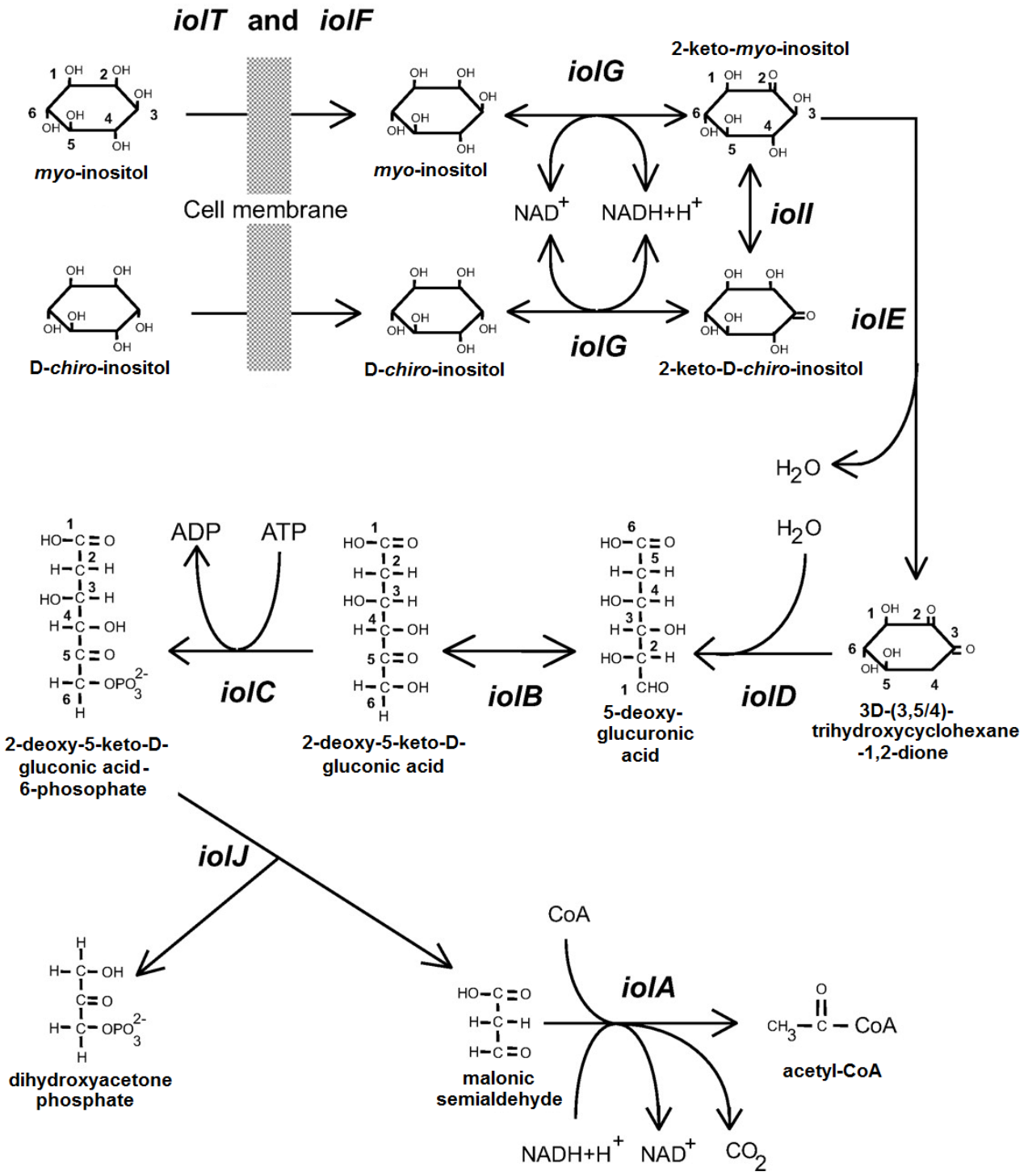

Fig. 3-4-1. *myo*-Inositol catabolic pathway and functional activities of the B. subtilis *iol* genes. Adapted from (Yoshida et al., 2008).

CHAPTER IV - ENZYME PURIFICATION AND BIOCHEMICAL CHARACTERIZATION

Section I - Introduction

Performing the biochemical characterization of phytases is a crucial step to determine if the enzyme properties are suitable for an application and also it is very important to generate data for comparison with other enzymes, contributing to the general knowledge of the phytases. The biochemical characterization of the enzyme can provide important information about the mechanism of the phytase, which to date is divided into four different families, according to their mechanism of action. The first family comprehends the most common phytases, the histidine acid phosphatase (HAP-Phytase) family, which is characterized by the optimal activity at the acidic pH range. This enzyme have a two-step mechanism involving the formation of a covalent phospho-histidine intermediate, then an aspartic acid residue act donating a proton to the oxygen of the phosphomonoester bond, completing the dephosphorilation reaction without the requirement of metals or co-factors. The second family is represented by the β-propeler phosphatases (BPP-Phytases). BPP-phytases are characterized for the optimal activity at the alkaline pH and also by the requirement of a Ca^{2+} group for enzyme activity, being strongly inhibited by addition of chelating agents like EDTA. Another family of phytases is represented by the cysteine phytases (also referred as protein tyrosine phosphatase [PTP]-like phytases), this family have been reported only to rumen bacterial isolates. Cysteine phytases catalyze the removal of a phosphate group attached to a tyrosine residue, using a cysteinyl-phosphate enzyme intermediate, in this way its activity is very inhibited by thiol-acting reagents. Also, cysteine phytases are known to be activated by Pb^{2+}, though the mechanism of activation is not completely understood. The last known family of phytases is the purple acid phosphatases (PAP). The PAPs are metallohydrolases that bind two metal ions in the active center. One of the ions is usually iron III, while the second metal in plant PAPs can be zinc, manganese, or iron II. In order to perform the biochemical characterization of the phytase produced by strain a13, the enzyme was purified to homogeneity. The optimal pH and temperature, effect of ion metals and enzyme modulators, enzyme kinetics, stability and applicability in fish feed treatment was tested.

Section II - Material and Methods

4-2-1 Cell-free Extract Preparation

A starter culture of strain a13 was prepared by inoculation of the bacteria from an agar culture of the CG medium to a 500 mL Erlenmeyer flask containing 100 mL of liquid CG medium, which was incubated with agitation for 24 h at 30 °C. The starter culture was inoculated to 6 L of CG medium (separated in 4 Erlenmeyer's flasks of 5 L) at final concentration of 1% and the strain was further cultivated for 48 h with agitation of 120 rpm. Then, the cells were harvested by centrifugation at 4,400 × g for 10 min. at 4 °C. The supernatant was carefully discarded, and the cell pellet was resuspended in 120 mL of 30 mM sodium acetate buffer, pH 4.5 containing 0.5 mM PMSF and 1 mM EDTA, and disrupted by sonication in low temperature at 20 Khz, 220 W with a UCW-201 sonicator (Tosyo Electric, Yokohama, Japan). The supernatant was obtained by centrifugation at 4 °C, 120,000 × g for 15 min and was filtered with a Millex-GV PVDF 0.22 mm syringe filter (Millipore, Carrigtwohill, CO.) and it was defined as the cell-free extract.

4-2-2 Protein Purification

The cell free-extract prepared in 4-2-1 was applied to an open column of ToyoPearl CM-650M (Tosoh Bioscience, Tokyo, Japan; ϕ 3.5 cm x d 4.5 cm) pre-equilibrated with 30 mM sodium acetate buffer, pH 4.5. Proteins were eluted with a linear gradient of 100-500 mM NaCl in the above buffer and the active fractions were pooled and ammonium sulfate was added to a final concentration of 1 M. The sample was applied to an ÄKTA purifier system (Amersham Pharmacia Biotech, USA) equipped with a HiTrap Butyl FF 5ml column (GE Healthcare Life Sciences, USA), pre-equilibrated with 1 M ammonium sulfate in 30 mM sodium acetate buffer, pH 4.5, and proteins were eluted with a linear gradient of 1-0 M ammonium sulfate in the above buffer. The active fractions were pooled, and dialyzed against the same buffer without addition of ammonium sulfate, and applied to a Mono S HR 5/5 column. After the proteins were eluted with a linear gradient of 0-500 mM NaCl, the fractions containing the phytase activity were concentrated

with ultrafiltration, using an Amicon Ultra-30 Centrifugal Filter (Millipore, Billerica, MA) and the buffer was exchanged to 30 mM Tris-HCl Buffer, pH 8.5. Then, the sample was applied to a Mono Q HR 5/5 column, pre-equilibrated with 30 mM Tris-HCl Buffer, pH 8.5, and the proteins were eluted with a linear gradient of 0-500 mM of NaCl in 30 mM Tris-HCl buffer, pH 8.5. Total protein concentration was measured by the method described by Bradford (1976).

4-2-3 SDS-PAGE

SDS-PAGE was performed for all purification steps by the method described by Laemmli (1970) in a 12.5% polyacrilamide gel. The gels were stained overnight by a solution containing: 2.5 g of coomassie brilliant blue R-250, 450 mL of methanol, 100 mL of acetic acid, 450 mL of deionized water. The gel was discolored by the same solution without addition of coomassie brilliant blue R-250 until the background become clear. The protein size calibration curve was obtained by plotting the molecular mass of the protein standard [(Precision Plus Protein Pre-stained Standards (Bio-Rad, Richmond, CA.)]in logarithmic scale against the relative mobility. The molecular mass was calculated by measuring the relative mobility of the purified enzyme in the same gel, and the protein size was calculated based on the obtained SDS-PAGE protein size standard curve (Fig. 4-2-1).

4-2-4 Gel Filtration Protein Size Measurement

A Superdex 200 10/30 column (Amersham Pharmacia Biotech, Inc. Piscataway, NJ) was attached to ÄKTApurifier system (Amersham Pharmacia Biotech) and the column was equilibrated with 3 column volumes of sodium acetate buffer 30 mM, pH 4.5, 300 mM NaCl. 200 μL of a protein standards mixture [Gel Filtration Calibration Kit LMW, HMW (GE Healthcare Bio-sciences AB, Uppsala, Sweden)] containing aldolase (158 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa), ribonuclease A (13 kDa) and aprotinin (6 kDa) was injected and eluted with a flow rate of 0.5 mL min^{-1}. The void volume of the column was calculated by injection of dextran blue with detection at 620 nm. A calibration curve was

performed by plotting the relative elution volume (K_{av}) against log of the protein molecular mass 200 μL of the purified phytase solution (0.04 mg mL^{-1}) was injected to the system at a flow rate of 0.5 mL min^{-1}. The protein size was calculated based on the obtained gel filtration protein size standard curve (Fig 4-2-2).

4-2-5 Optimal Temperature and pH

Optimum temperature was determined by measuring the activity of the purified enzyme in a substrate solution of 2 mM potassium phytate in 100 mM sodium acetate buffer, pH 4.5, at different temperatures, ranging from 10 to 80 °C. Optimal pH was determined by measuring the purified enzyme activity at 50 °C in the substrate solution of 2 mM potassium phytate in 0.1 M Glycine–HCl (pH 2-3.5); 0.1 M Sodium acetate (3.5-5.5); 0.1 M MES–HCl (5.5-6.5); 0.1 M MOPS–NaOH (6.5-7.5). The activity was measured in triplicate.

4-2-6 Effect of Metal Ions and Enzyme Modulators

The effect of metal ions was performed by measuring the enzyme activity at optimal conditions (pH 4.5, 50 °C) in addition of metal ions or enzyme modulators. Concentrate solutions of 50 mM of salts of metal ions ($AlCl_3$, $CaCl_2$, $CoCl_2$, $CuSO_4$, $FeCl_2$, $FeCl_3$, $Hg(NO_3)_2$, KCl, LiCl, $MgCl_2$, $MnCl_2$, $NiCl_2$, $Pb(NO_3)_2$, $SrCl_2$ and $ZnCl_2$) in 0.1 M sodium acetate buffer pH 4.5 were prepared. Also, concentrate solutions of 10 mM of dithiothreitol, 2-mercaptoethanol, EDTA, EGTA, iodoacetic acid and PMSF were prepared. The concentrate solutions were mixed with the enzyme solution to form a final concentration of 1 mM. Then the substrate solution was added to start the enzyme reaction on optimal conditions. For the control group, the enzyme solution was mixed with 0.1 M sodium acetate buffer pH 4.5. The activity measurements were obtained for tree independent experiments.

4-2-7 Enzyme Kinetics

Kinetic properties of the purified enzyme were obtained by adding a known concentration of substrate to the enzyme, and then the initial reaction rate was determined for that concentration of substrate. For enzyme reactions, it was used substrates of 0.01-2.0 mM phytic acid, 0.1-40 mM *p*-nitrophenylphosphate (*p*-NPP), 0.1-40 mM glycerol-3-phosphate, 1.5-60 mM glucose-6-phosphate, and 0.1-20 mM ATP. The K_m and k_{cat} values were calculated by fitting the results with the basic Michaelis-Menten equation, using the fitting tool of Origin v6.1 (OriginLab Corporation, North Hampton, MA).

4-2-8 Enzyme Stability

Enzyme stability determination was performed using a concentrated solution of the purified enzyme (0.46 mg mL^{-1}) in 0.1 M sodium acetate buffer solution pH 4.5. The enzyme was stored at room temperature (30 °C) and low-temperature (4 °C). For the incubation at room temperature, enzyme activity was measured for a period of five days, every 24 hours. For the incubation at low temperature the enzyme activity was measured two times, after incubation for 16 months and after incubation for 22 months, all measurements were conducted in triplicate.

4-2-9 Applicability in Fish Feed Treatment

Commercially available carp fish and gold fish feed samples were treated with the purified phytase. Samples were grind down and 0.1 g of the fish feed was suspended in 1 mL of 0.1 M sodium acetate buffer, pH 4.5. Five micro liters of the purified enzyme solution was added to the suspension (4.5 U per gram of sample) and the suspension was incubated at 50 °C for 90 minutes. A suspension of the samples without addition of enzyme solution was used as control. After incubation, the samples were centrifuged at 120,000 × g for 15 min, and the supernatant was collected for measurement of the soluble phosphate concentration. Phosphate concentration was measured as described in the sub-section 2-2-2. The results are expressed for three independent experiments.

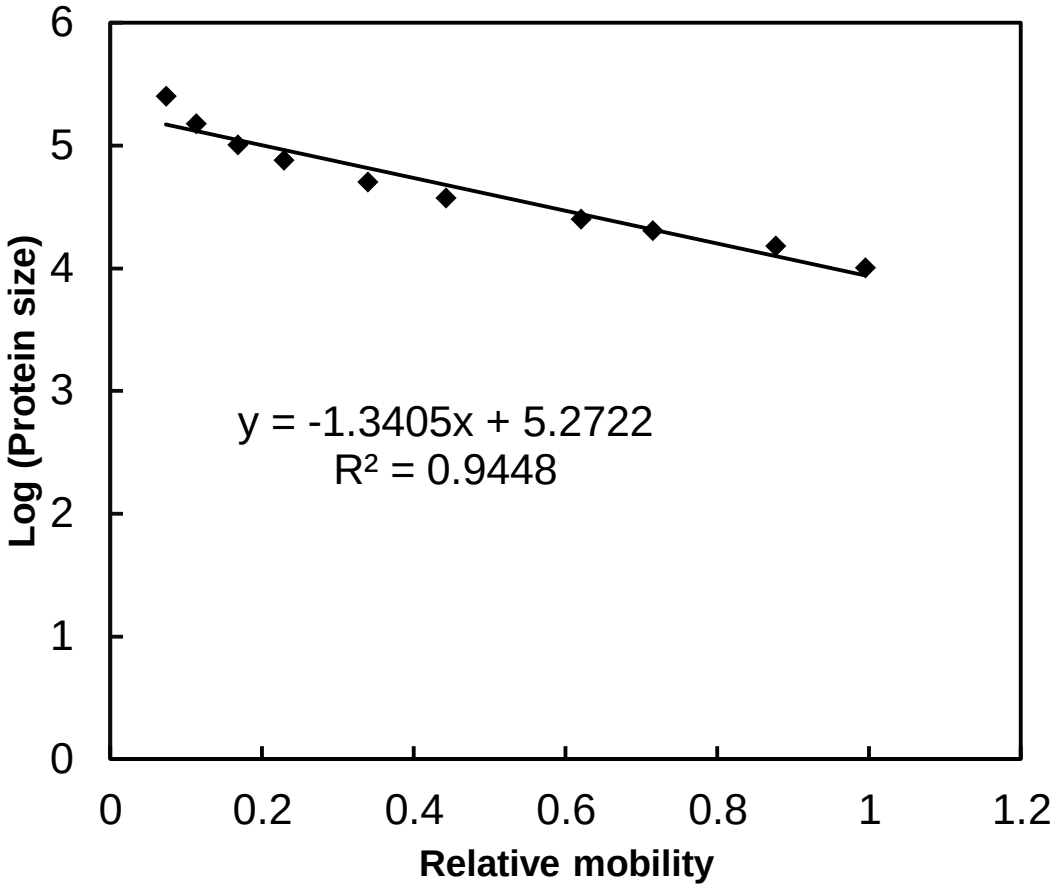

Fig. 4-2-1. SDS-PAGE protein size standard curve

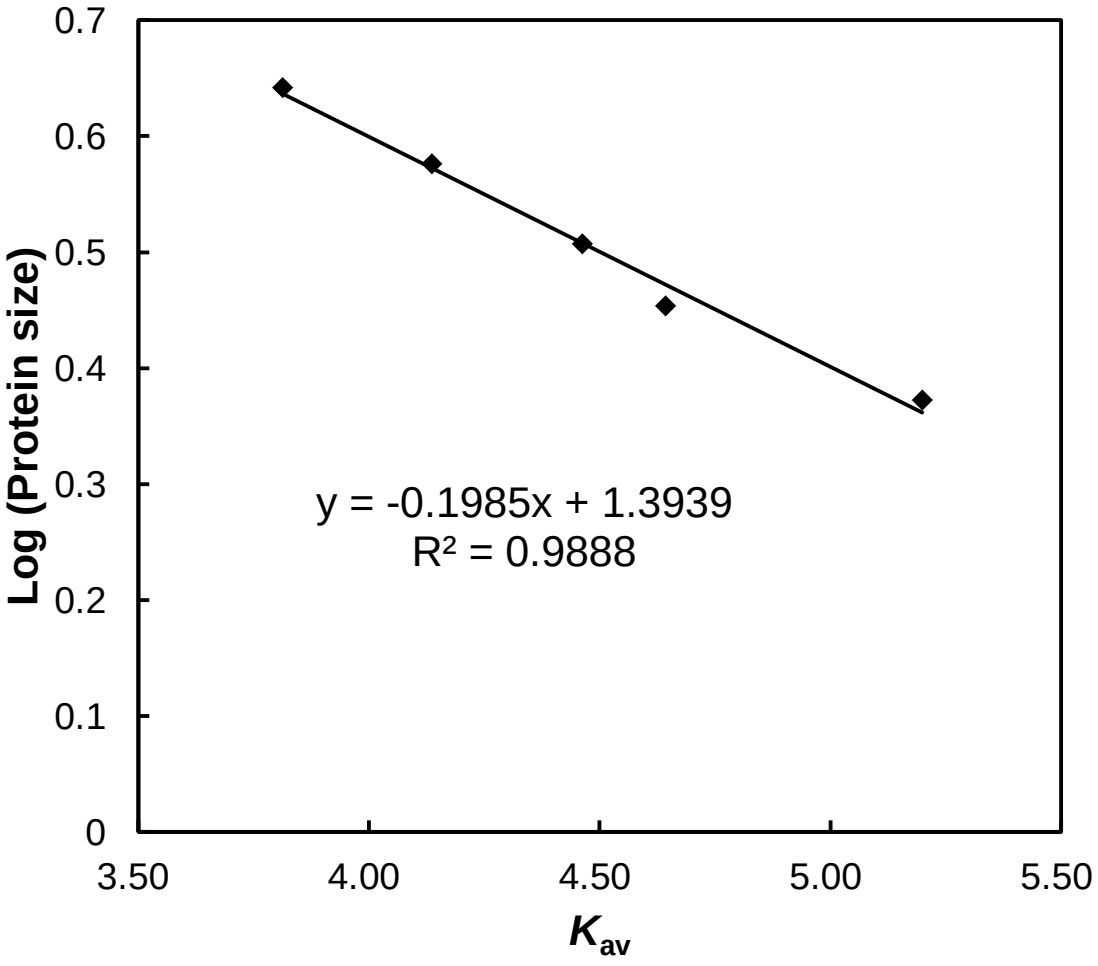

Fig. 4-2-2. Gel-filtration protein size standard curve.

Section III - Results

4-3-1 Strain a13 Phytase Purification and Biochemical Properties

The phytase was successfully purified to homogeneity from the cell-free extract of a13 strain by four-steps of chromatography, showing a single band, which had a calculated protein size of 43 kDa on SDS-PAGE (Fig. 4-3-1). The enzyme specific activity had an increase of 109 fold after purification, showing a final phytase specific activity of 174.1 U mg^{-1}, while the global purification process showed an yield of 32.1% (Table 4-3-1). Gel-filtration analysis of the purified protein showed a single peak with an elution volume from the column of 15.1 mL, which correspond to a relative elution volume (K_{av}) of 0.476, showing a calculated size of 44 kDa (data not shown). Taken together, the results of SDS-PAGE showing a single band with a size of 43 kDa over denaturation condition, and the result from the gel filtration showing a protein size of 44 kDa, indicate that the phytase produced by strain a13 is a monomer. The optimal temperature and pH conditions were at 45-55 °C and 4.5, respectively. The enzyme showed a large temperature working range in comparison with other bacterial phytases with a relative activity above 50% for the temperatures between 30-60 °C, it also showed an acidic working range of pH 3-6 (Fig. 4-3-2). Phytase stability was evaluated for room temperature and low temperature storage, and showed good stability at 30 °C as the enzyme maintained 80% of activity after an incubation of 5 days (Fig. 4-3-3). The enzyme was quite stable at 4 °C, as more than 85% of the activity was maintained after 22 months of incubation (Table 4-3-2). The effect of metal ions and other enzyme inhibitors on the phytase activity was tested and the activity was strongly inhibited by Cu^{2+}, Zn^{2+}, and Hg^{2+} and partially inhibited by Al^{3+}, Fe^{2+}, Fe^{3+}, Pb^{2+}, and iodoacetic acid. Strong enzyme activation was detected for addition of EDTA and EGTA. Inhibition by Cu^{2+}, Zn^{2+}, Hg^{2+}, and iodoacetic acid may indicate the requirement of a thiol group of a Cys residue for enzyme activity. A slight activation by addition of 2-mercaptoethanol and dithiothreitol supports this assumption (Table 4-3-3). Kinetic analyses revealed that the enzyme has broad substrate specificity, including *p*-nitrophenyl phosphtate and ATP, but the highest catalytic efficiency (k_{cat}/K_m) was observed with phytic acid (Table 4-3-4). These results indicate that this enzyme has a preference for the hydrolysis of

phytic acid instead of other phosphorylated compounds, suggesting that the enzyme is working as a phytase instead of an acid phosphatase.

4-3-2 Application of the Purified Phytase in Fish Feed Treatment

Although phytases have been reported for uses in several different applications, as previously discussed in Chapter I, to date the most preeminent and commercially explored application for phytases is into the animal farming industry. Therefore, it is important to determine if the purified phytase from strain a13 is able to increase the soluble inorganic phosphate concentration of animal feed by treatment. Generally, fresh water fish feed is grain based, in this way the application of phytases into the aquaculture had been recently explored. In aquaculture, phosphorous management is considered critical because high phosphorous concentrations are the principal cause of eutrophication in the aquatic environment (Correll, 1999). For that reason, two commercially available fresh water fish feed (carp fish feed and gold fish feed) was treated with 4.5 U of the purified phytase per gram of feed. The treatment for 90 minutes under optimal conditions, could promote an increase of the soluble inorganic phosphate concentration of 61% and 56% for the carp fish feed and gold fish feed respectively (Fig. 4-3-4).

Table 4-3-1. Purification summary of strain a13 phytase.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Fold
Crude Extract	450.3	704.2	1.6	100.0	1
CM-650M	26.4	656.0	24.8	93.2	15.5
Hi-Trap Butyl FF	8.0	254.2	31.7	36.1	19.8
Mono S HR 5/5	1.5	252.4	172.9	35.8	108.1
Mono Q HR 5/5	1.3	226.3	174.1	32.1	108.8

Table 4-3-2. Enzyme stability at low temperature storage (4 °C)

Measurement date (YYYY/MM/DD)	Incubation period (Months)	Phytase activity [a] ($U\ mg^{-1}$)	Residual activity (%)
2012/12/18	0	172.8 ± 2.3	100
2014/04/21	16	162.2 ± 4.6	93.9
2014/10/30	22	154.4 ± 9.3	89.3

[a] The assays were conducted in triplicate, and averages ± S.D. are shown.

Table 4-3-3. Effect of metal ions and enzyme modulators over phytase activity.

Metal ion or modulator (1 mM)	Relative activity (%)[a]
Control	100 ± 0.4
Al^{3+}	65.9 ± 3.8
Ca^{2+}	109.8 ± 2.5
Co^{2+}	95.5 ± 1.1
Cu^{2+}	0.6 ± 0.1
Fe^{2+}	37.7 ± 6.8
Fe^{3+}	32.5 ± 5.6
Hg^{2+}	0.1 ± 0.1
K^{+}	106.2 ± 6.7
Li^{+}	105.1 ± 2.0
Mg^{2+}	97.8 ± 3.8
Mn^{2+}	106.7 ± 3.3
Ni^{2+}	83.2 ± 4.7
Pb^{2+}	30.5 ± 2.1
Sr^{2+}	98.4 ± 2.4
Zn^{2+}	0.6 ± 0.2
dithiothreitol	117.3 ± 10.2
2-mercaptoethanol	120.6 ± 16.8
EDTA	177.8 ± 8.7
EGTA	161.2 ± 2.5
iodoacetic acid	30.3 ± 4.5
PMSF	101.6 ± 0.5

[a] The assays were conducted in triplicate, and averages ± S.D. are shown.

Table 4-3-4. Kinetic parameters of the phytase.

Substrate	K_m (mM)[a]	k_{cat} (s^{-1})[a]	k_{cat}/K_m (M^{-1} s^{-1})
Phytic Acid	0.42 ± 0.04	236.2 ± 8.5	5.6×10^5
p-NPP	3.61 ± 0.19	154.2 ± 2.8	4.3×10^4
ATP	0.36 ± 0.16	48.2 ± 5.8	1.3×10^5
Glucose-6-Phosphate	22.42 ± 5.58	38.6 ± 5.0	1.7×10^3
Glycerol-3-Phosphate	12.67 ± 0.81	53.4 ± 1.5	4.2×10^3

[a] The assays were conducted in triplicate, and averages ± S.D. are shown.

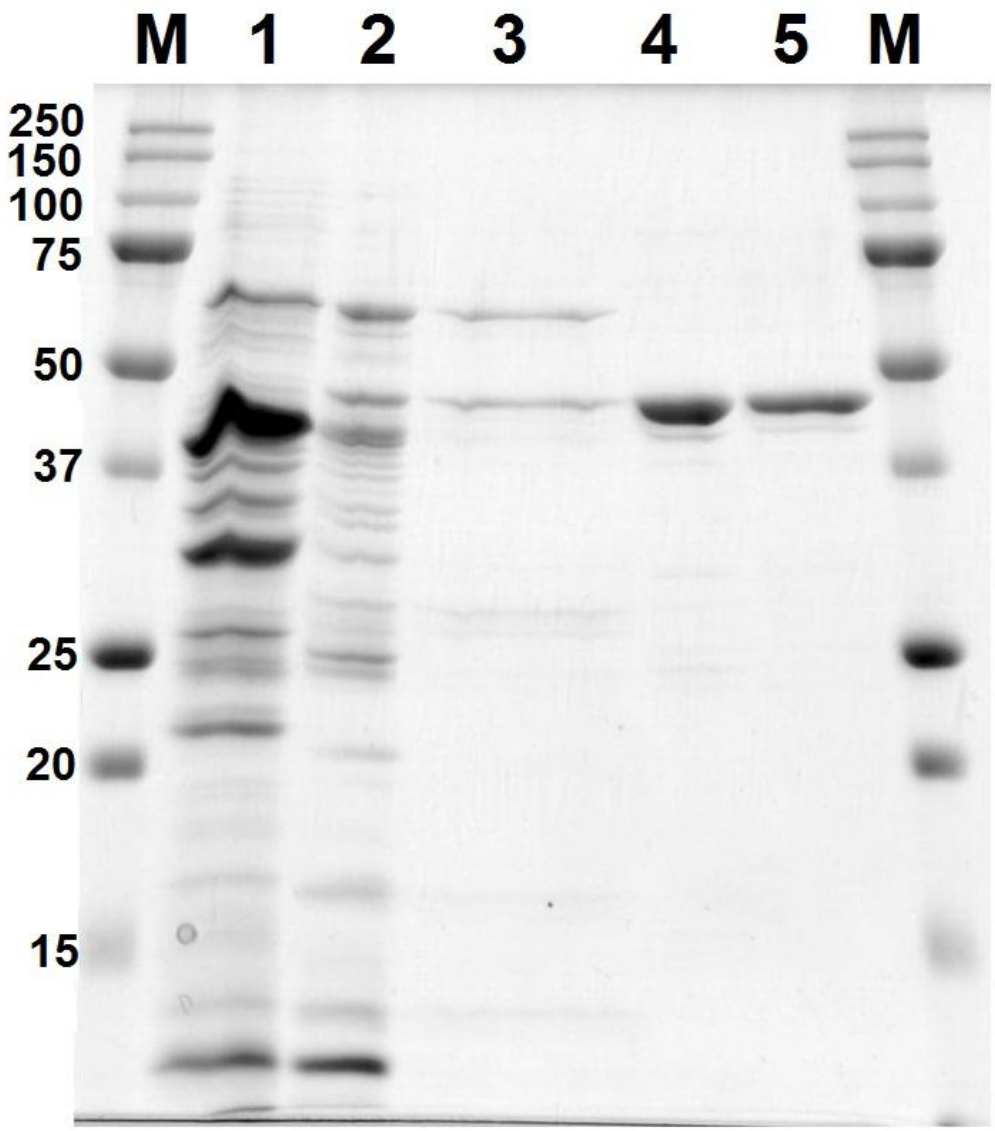

Fig. 4-3-1. SDS-PAGE of purification steps of the phytase. Lane M, molecular marker standards (kDa); 1, crude extracts; 2, after Toyopearl CM-650M; 3, after Hi-Trap Butyl FF; 4, after Mono S HR 5/5; and 5, after Mono Q HR 5/5 chromatography.

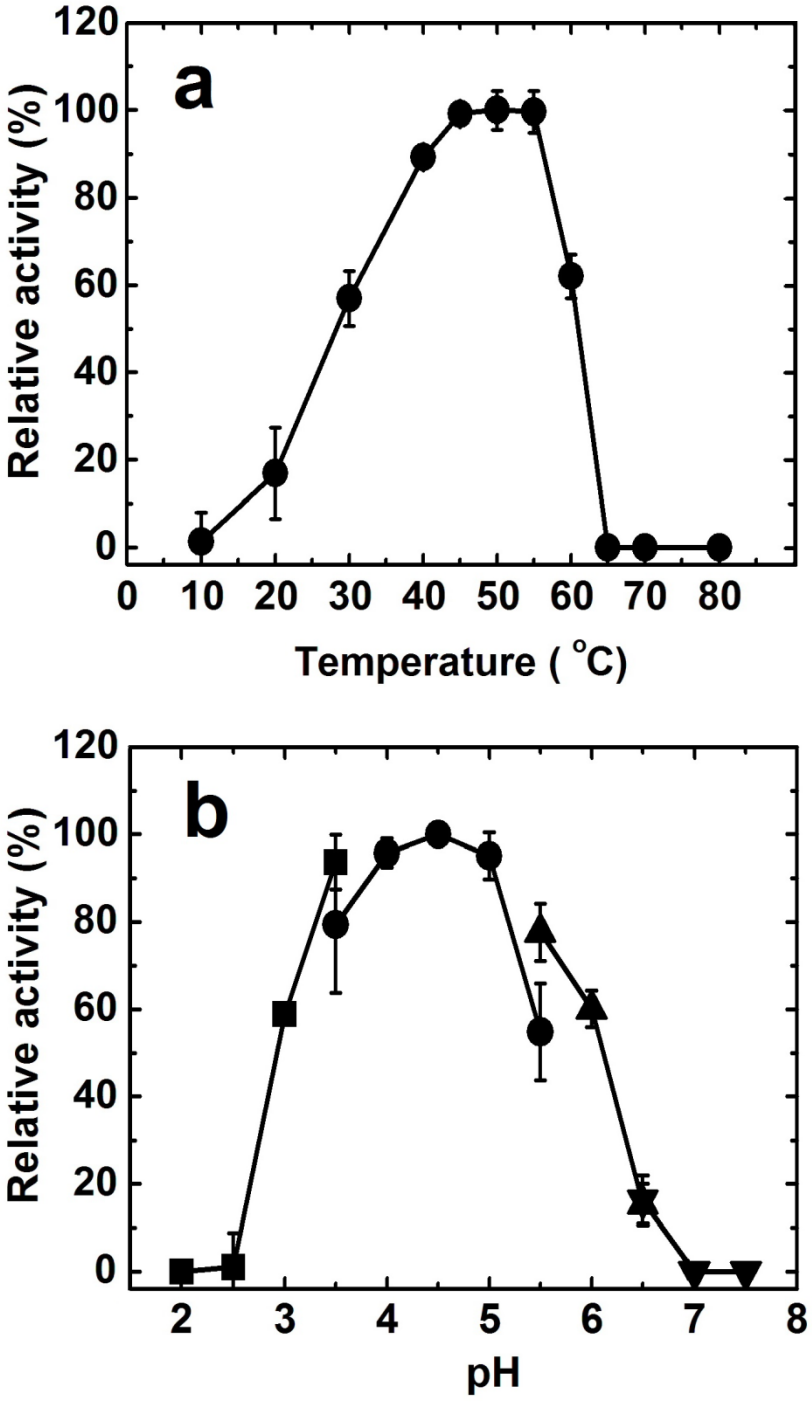

Fig. 4-3-2. Optimal temperature (a) and pH profile (b) of the purified phytase. The buffers used are 0.1 M Glycine–HCl (squares); 0.1 M Sodium acetate (circles); 0.1 M MES–HCl (up triangles); 0.1 M MOPS–NaOH (down triangles). The activity was measured in triplicate and the average ± S. D. are shown

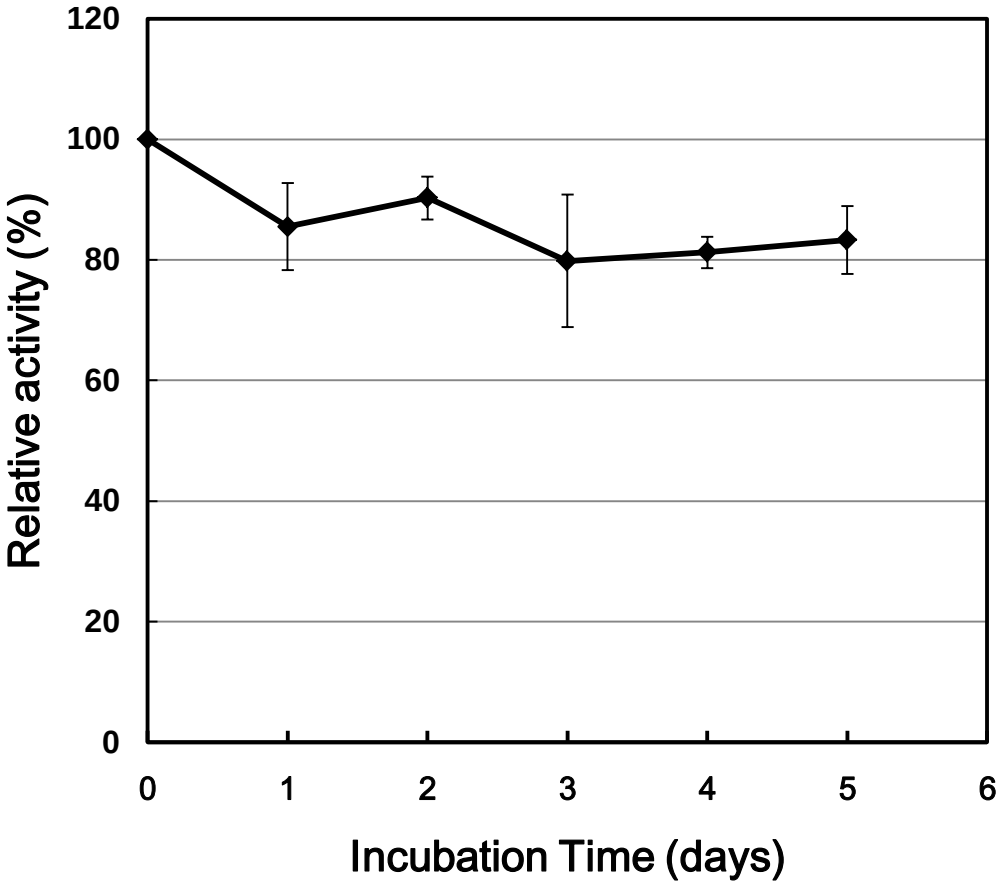

Fig. 4-3-3. Enzyme stability of the purified phytase incubated at 30 °C. The activity was measured in triplicate and the average ± S. D. are shown

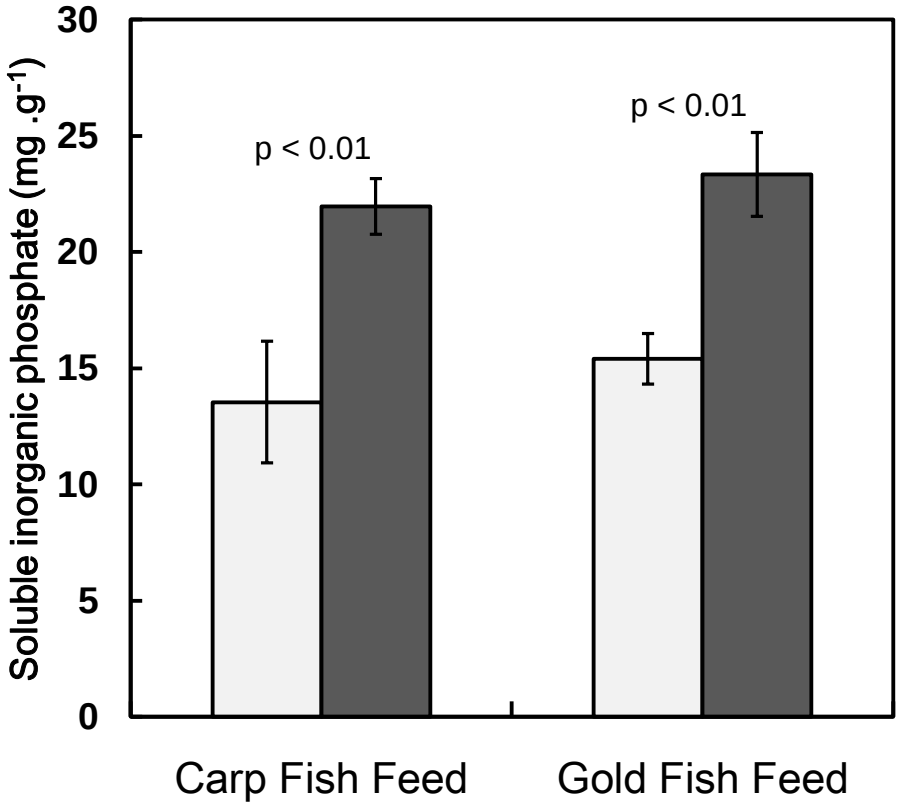

Fig. 4-3-4. Soluble inorganic phosphate concentration of carp and gold fish feed before and after phytase treatment. White bars indicate the soluble inorganic phosphate before phytase treatment, Gray bars indicate soluble inorganic phosphate after treatment with 4.5 U of the purified phytase of strain a13.

Section IV - Discussion

Because phytase is a term used to describe an enzyme that hydrolyzes phosphomonoester bonds from phytic acid this terminology is generic and comprises four different families of enzymes (Mullaney and Ullah, 2003), the biochemical characteristics between phytases can divert largely according to the families that the enzyme belongs. However, some biochemical properties are conserved among phytases, like optimal temperature, which is generally high for the majority of phytases, or optimal pH which is in the acidic range for the majority of phytases with exception of those belonging to the β-propeller phytase family, that tend to have an optimum pH in the neutral to basic range. In this way, the comparison of biochemical properties of the enzyme with previously characterized enzymes is important to give an insight about what family the enzyme belongs and also suggest a possible application for the enzyme. In order to compare the phytase produced by strain a13 with other phytases, the enzyme was successfully purified from the cell-free extract by four steps of ion-exchange and hydrophobic interaction chromatography (Table 4-3-1; Fig. 4-3-1), and its biochemical characterization was performed.

The purified protein was revealed as a monomer enzyme with a size of 44 kDa (Fig. 4-3-1). Generally The protein size of phytases show a great variation, with the biggest phytase of 700 kDa and the smallest one of 12.8 kDa reported to be produced by the same microorganism, *Enterobacter aerogenes* (Tambe et al., 1994). The size of 44 kDa of the phytase produced by strain a13 can be considered relatively small, since the majority of phytases have a reported size bigger than 50 kDa. The enzyme also showed a similar size with other bacterial phytases. Generally, bacterial strains produce smaller phytases when compared to fungal sources, independent of the family of the enzyme. In this way, at the same size range of strain a13 phytase, there is the enzyme produced by *Bacillus subtilis*, 41 kDa (Farhat et al., 2008); *Escherichia coli*, 43 kDa (Greiner et al., 1993) and *Yersinia intermedia*, 44 kDa (Huang et al., 2008); while the smallest phytase produced by a fungal source have a reported size of 49.2 kDa, which is produced by *Aspergillus niger* (Zhao et al., 2007). The purified enzyme showed a phytase activity of 174.1 U mg^{-1}. The highest phytase activity reported to date is the enzyme produced by *Yersinia*

intermedia, which showed a phytase activity of 3960 U mg^{-1} (Huang et al., 2006). In comparison to the enzyme produced by *Y. intermedia* the purified phytase from strain a13 might be considered an enzyme with low activity, however several phytases with activity lower than 100 U mg^{-1} had already been reported (Jareonkitmongkol et al., 1997; Watanabe et al., 2009; Azeke et al., 2010), also a commercialized phytase produced by *Aspergillus ficuum*, was reported to have a phytase activity of 150.1 U mg^{-1}, lower than the one produced by strain a13 (Zhang et al., 2010). Those comparisons indicate that the enzyme produced by *Burkholderia* sp. strain a13 can be considered high, which is a much desired property for several applications. The phytase produced by strain a13 also showed broad substrate specificity (Table 4-3-4). This is a common characteristic to the majority of phytases, which can hydrolyze other phosphorylated compounds not structurally similar to phytic acid (Konietzny and Greiner, 2002). Broad substrate specificity was reported by Wyss (1999) to be more advantageous for application in feed treatment, when compared to more specific phytases. Those characteristics support the possible application of strain a13 phytase for feed treatment.

Although *Burkholderia* sp. strain a13 showed a relatively high activity in comparison with other phytases, the production by this strain is considered low, since only 117.3 U of phytase are obtained from the cell-free extract of strain a13 cultivated in one liter of CG medium under optimal conditions. In comparison with other phytase producing microorganisms, a strain of *Kodamaea ohmeri* cultivated under optimal conditions for phytase production was able to produce 557.9 U of per liter of culture, within 72 h of fermentation at the shake flask level (Li et al., 2008a). In this way, further efforts should be done to obtain higher yields of phytase production by strain a13, which is also a desired characteristic to use this novel isolated enzyme for a commercial application. In future, over-expression of the enzyme in a heterogeneous system might contribute with the improvement of phytase production.

Strain a13 phytase showed an optimum temperature and pH of 45-55°C and 4.5 respectively (Fig. 4-3-2). This pH and temperature profile is very similar to several phytases, and suggests that the enzyme produced by strain a13 doesn't belong to the β-propeller phytase family because of the optimum pH detected at the acidic range. One important characteristic of the phytase produced by strain a13 is the

broad temperature working range of the enzyme, which shows to have a remaining activity of more than 50% between 30 °C and 60 °C. When compared to other bacterial phytases, strain a13 phytase showed advantages, working well in lower temperatures. For example, the phytase of *E. coli*, that have an optimal temperature of 55 °C, shows only 35% of its activity when the reaction is proceeded at 37 °C. The application of phytases in aquaculture as a feed supplement, requires enzymes with high activity at temperatures, generally lower than 28 °C, because of the physiology of fishes which are poikilotherms. Also the possibility to pretreat the feed using the phytase incubated at room temperature is a desired characteristic for application. In this way, isolation of phytases capable to work at lower temperatures is also object of interest for applications. For example, the phytase produced by *Erwinia carotovora* var. *carotovota* ACCC 10276 , showed strong phytase activity at lower temperatures, retaining over 24% activity at 5 °C, and for that reason it was reported as a good candidate for application in aquaculture. On the other hand, the enzyme produced by this strain was very sensitive to high temperatures, losing more than 96% of activity at 60 °C (Huang, 2009). Strain a13 showed good activity at 30 °C an also some activity at 20 °C, the enzyme also had good activity for higher temperatures as it showed a relative activity of more than 60% at 60 °C (Fig. 4-3-2). The enzyme showed also high stability, since it lost less than 20% of its activity after incubation for 5 days at 30 °C (Fig. 4-3-3), and it also remained stable for more than a year and a half, losing only 11% of its activity when it was incubated at 4 °C (Table 4-3-2). For comparison, the purified phytase from the bacterium *Pantoea agglomerans* was shown to produce a phytase with a half-life of 10 days for storage at room temperature, and to lose 50% of its activity when stored at 4 °C for the period of 6 months (Greiner, 2004). Also the purified native enzyme produced by *Aspergillus niger,* was reported to have a remaining activity of 88.9%, 84.25%, 57.5% after incubation in 4 °C for 30 days, 60 days and 180 days respectively. The results obtained for the purified phytase of strain a13 show that the enzyme has high storage stability in comparison to other phytases. It's important also to mention that the enzyme was simply stored in a sodium acetate buffer solution, and that this stability can be further increased during storage by utilization of protein stabilizers, preservatives and salts. In this way, the high stable enzyme still has potential for improvements of its shelf-life, and in the future, further

experimentation about the stability of the enzyme is needed. The high stability of this phytase at 4 °C may provide some advantages, such as facilitating the long-term storage of this enzyme in solution, encouraging a commercial application of the enzyme. The main advantage of producing liquid formulations over powder formulations is that the enzyme can be easily and promptly used on its application, reducing the risk of mismanage due to error in dilutions, which is a very recurrent characteristic of powder formulations.The application of phytases in aquaculture is one of the several counter measures to reduce phosphorous excretion where it can compromise water quality. Also, the use of phytases can improve the production of fish, which can absorb the inorganic phosphate released from phytic acid origin in the diet. Therefore, in order to test if the phytase produced by strain a13 was effective in the treatment of the feed, two types of feed commonly given to fish were treated with 4.5 U per gram, and a rise in inorganic phosphate concentration of 61% and 56% for the carp fish feed and gold fish feed respectively was detected (Fig. 4-3-4). This result is in good agreement with a report of application of a phytase to soybean based carp fish feed. In this report, an increase around 20% and 40% of phytate originated inorganic phosphate was released by addition of 0.5 U and 1 U of enzyme respectively (Schäfer, 1995). The characteristics of the enzyme produced by strain a13, which is able to hydrolyze phytic acid in lower temperatures, have a high stability for the long-term storage and ability to successfully treat grain-based fish feed, suggest that the enzyme have good properties for application as a liquid formulation for pretreatment of fish feed. In the future, studies of the enzyme stability to proteolysis and also experiments to determine the ability to release inorganic phosphate from phytic acid in the gastro-intestinal tract of fishes might also support the application of this enzyme as a feed supplement for aquaculture.

According to the biochemical characteristics of the enzyme produced by strain a13, it is possible to presume which family of phytase the enzyme belongs. According to the pH profile in the acidic range, the enzyme is unlikely classified into the β-propeller phytase family, which generally have phytases with an neutral to alkaline working range (Kerovuo et al., 1998; Zhang et al., 2011), also the members of this family of phytases are known to have a requirement of a Ca^{2+} for the activity, therefore the activity of

β-propeller phytase is strongly inhibited in the presence of chelants like EDTA and EGTA. Contrary to that, strain a13 phytase activity was induced by the presence of chelants (Table 4-3-3), indicating that the enzyme likely belong to other families. Activation by EDTA have already been reported for HAP phytases like the phytase produced by *Aspergillus niger, Klebsiella pneumoniae and Escherichia coli*, though the mechanism for this activation is still unknown (Vats and Banerjee, 2005; Escobin-Mopera et al., 2012; Tai et al., 2013). Interestingly the phytase activity was shown to be affected by thiol-acting agents, such as Zn^{2+}, Cu^{2+}, Hg^{2+}, iodoacetic acid and activated weakly by DTT and β-mercaptoethanol (Table 4-3-3). The activity of some other HAP phytases of bacterial origin were also inhibited by Cu^{2+} and Zn^{2+}, though the level of inhibition was different (Cho et al., 2003; Huang et al., 2006; Fu et al., 2008). The inhibiton caused by thiol-acting metals and a specific alkilant chemical of cysteines thiol group (iodoacetic acid), together with the activation promoted by the disulfide bridge-reducing agents (DTT and β-mercaptoethanol), clearly indicate the requirement of free thiol group(s) of Cys residue(s) for the enzyme activity. The family of cysteine phytases, have been reported to contain a conserved cysteine residue in the active site of the enzyme which is necessary for phytase activity (Huang et al., 2011). This property suggest the classification of the phytase produced by strain a13 in the cysteine phytase family, however cysteine phytases are reported to be activated by Pb^{2+} (Yanke et al., 1999), while the phytase produced by strain a13 is strongly inhibited by this cation. Strain a13 phytase is also unlikely to belong to the purple acid phosphatase family, because the purified enzyme didn't show any absorption for the visible spectrum (data not shown). Enzymes belonging to this family have a requirement of iron III at the active site, which confers the protein a specific pink to purple color (Li et al., 2011). By the characteristics described above, the enzyme produced by *Burkholderia* sp. strain a13 is supposed to be classified into the cysteine phytase family or the HAP phytase family, but only genetic cloning and the verification of the conserved motifs for those families would address the classification of the phytase.

CHAPTER V - STRAIN A13 PHYTASE GENE CLONING AND PROTEIN PRIMARY STRUCTURE STUDY

Section I - Introduction

The genes encoding phytases have been cloned from several organisms, such as plants, fungi, and bacteria (Singh et al., 2014). Genetic cloning of phytases is of great importance, because it can provide information about the primary structure of the enzyme, which can be used for comparison with other phytases, allowing to verify in which family the enzyme is included. Some families of phytase have specific sequence motifs characterizing them. The most common family of phytases is the HAP phytases, which share an active site sequence motif (RHGXRXP) near the N-terminal of the protein, this family also show a motif near the C-terminal of the protein (HD) which constitute the active site (Mullaney and Ullah, 2003). Another family of phytases with a well characterized sequence motif is the family of cysteine phytases (PTP- like phytases) which share the conserved motif HCXXGXXR, also present on the active site of those phytases members (Puhl et al., 2008). On the previous chapter, the biochemical characterization results of the phytase indicated that the enzyme had similar properties of HAP phytases, like the activation by EDTA and inhibition by Pb^{+2}, but also shared properties of cysteine phytases like the inhibition by IAA and thiol-acting agents. Therefore, genetic cloning of the enzyme and the determination of its primary structure would be a decisive factor to include the phytase in one of those families, or even determine a novel phytase family for this newly isolated enzyme. Also, the identification of the phytase gene would be the first step to perform genetic manipulations of the phytase gene allowing the expression of the enzyme in an heterogeneous system, possibly increasing the phytase production yield. In this chapter the phytase gene was identified by an approach using degenerate primers, based on the protein amino acid sequence, and the ORF containing the phytase gene was determined by inverse PCR and direct sequencing from the genome of strain a13. From the obtained phytase gene sequence it was performed the primary structure analysis of the enzyme, which allowed to discuss a possible link of the observed biochemical characteristic with the enzyme structure.

Section II - Material and Methods

5-2-1 Protein Digestion with Endoproteinases

Digestion of the mature phytase purified protein was performed with V8 Protease (Wako Pure Chemical Industries Ltd., Japan), Lysyl Endopeptidase (Wako Pure Chemical Industries Ltd. Japan) and Endoproteinase Asp-N (Takara Bio Inc., Japan). For the digestion using V-8 endoproteinase, the purified phytase was buffer exchanged with 150 mM Tris-HCl buffer pH 7.8 with 1M of urea by ultrafiltration using an Amicon Ultra-30 centrifugal filter (Millipore, Billerica, MA). A concentrated solution of the V-8 endoproteinase diluted in the same buffer was added to the phytase solution to a ration of 1:10 in mass. The protein was digested at 30 °C for 6 h or overnight. The same procedure was performed with BSA as a positive control. For the digestion using Lys-C and Asp-N endoproteinase, the phytase was buffer exchanged with 200 mM ammonium bicarbonate buffer, pH 8.0 with 4M of urea by ultrafiltration using an Amicon Ultra-30 centrifugal filter (Millipore, Billerica, MA). Then, DTT was added to solution to a final concentration of 5 mM and the enzyme was heat treated at 50 °C for 15 minutes. After the heat treatment, the phytase solution was diluted two times with D.W. and the endoproteinases Lys-C and Asp-N concentrated solutions were added to the substrate solution to a ratio of 1:25 and 1:50 in mass respectively. The digestion was performed overnight by incubation at 37 °C. All reactions were stopped by addition of 1 mM of PMSF.

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5-2-2 Semi-dry Blotting

Protein samples were separated by SDS-PAGE and then transferred to a PVDF membrane by semi-dry blotting. A PVDF membrane with a size of 8.5 x 9 cm was washed in methanol for 30 second and then hydrated for 1 hour at the buffer solution containing 25 mM Tris and 5% methanol for one hour. Two blotting papers were soaked at a buffer solution containing 300 mM Tris and 5% methanol and then set at the transferring positive electrode. Over those, a blotting paper soaked on the same buffer used for hydration of the membrane was set, and over it the PVDF membrane was placed. The SDS-PAGE gel

containing the separated protein samples was placed carefully over the membrane and over it was placed another blotting paper soaked with the same buffer. Finally, one blotting paper soaked in a buffer containing 25 mM Tris, 40 mM aminocaproic acid and 5% mehtanol was set at the top. The transfer was held at constant amperage of 150 mA for 45 minutes. The membrane was stained briefly by a diluted CBB staining solution containing 0.001% CBB, 10% acetic acid and 30% methanol. Unstaining was performed by soaking the membrane for 15 minutes in a solution containing 10% acetic acid and 50% methanol.

5-2-4 Protein Thiol-pyridyl-ethylation and N-terminal Amino Acid Sequencing

As a preparation of the samples for Edman degradation aminoacid sequence, the membranes of PVDF containing the protein samples was treated by Thiol-pyridyl-ethylation for protection of the thiol groups. The cut membrane pieces were inserted in tubes and were wet with small amount of ethanol. Then 300 µL of a reducing buffer of 8M Gunidine-HCl, pH 8.5 containing 0.5M Tris, 0.3% sodium EDTA, 5% acetonitrile, 1 mg DTT was added to the tube containing the PVDF membrane. The oxygen of the tube was exchanged with nitrogen and the tube was incubated for denaturation of the protein at room temperature for one hour. After incubation 1.5 µL of 4-vinylpyridine was added to the tube, which was agitated for 20 minutes. Then the solution was discharged and the membrane was washed several times with D.W. and a 2% acetonitrile solution to remove impurities. Finally the membrane was dried and the samples respective N-terminal amino acid sequences were analyzed with a Procise Sequencing System, Model 491cLC (Applied Biosystems, Foster city, CA) by Creative Research Institution, Hokkaido University.

5-2-5 Genomic DNA Extraction (miniprep)

One milliliter of an overnight culture of strain a13 in CG medium was harvested by centrifugation at 4,400 × g for 10 min. at 4 °C. The supernatant was carefully discarded, and the cell pellet

was resuspended in 360 µL of TE buffer with addition of 1µL and 40µL of an 1 $mg.mL^{-1}$ and 10 $mg.mL^{-1}$ stock solution of RNAse A (Sigma-Aldrich, USA) and lyzozyme (Sigma-Aldrich, USA) solution respectively. The resuspended cells were incubated at 37 °C for 15 minutes and then 50 µL of a 10% SDS solution was added and the suspension was vigorously agitated for 5 minutes. One hundred micro liters of 5M $NaClO_4$ solution was added and the DNA was purified by several phenol-chloroform purification process. After discharging the phenol-chloroform fraction, 800 µL of pure ethanol was added carefully and the DNA precipitated at the interphase was pick up with a Pasteur pipet and let it dry. The DNA stick at the glass was rinsed up with 70% ethanol and then dissolved in 50 µL of TE buffer. DNA purity and concentration was determined spectrophotometricaly.

5-2-6 Degenerate PCR Amplification and Semi-Nested PCR

PCR amplification from the genome of strain a13 was performed by utilization of the primers nter1-F, nter2-F and intS1-R listed on Table 5-2-1 with GoTaq® Green Master Mix (Promega, USA). A mixture containing 1µg of the genomic template was used with forward and reverse primers added to a final concentration of 1.5 µM. As a control for unspecific PCR amplification products a reaction mixture containing only the forward or the reverse primer was also performed. PCR amplification consisted of an initial denaturation at 94°C for 2 min, 35 cycles of 98°C for 20 sec, 55°C for 30 sec, 72°C for 1 min and 30 sec. Than a final incubation at 72°C for 10 min. The obtained PCR amplification products were separate by 1% agarose gels. The specific bands were purified with UltraClean 15 DNA Purification Kit (Nippon Gene, Japan) and utilized as templates for a semi-nested PCR after one thousand fold dilution in D.W.. For the second amplification, the primer set nter2-f and intS1-R was used, with the same PCR conditions of the first amplification. The obtained product was purified with UltraClean 15 DNA Purification Kit (Nippon Gene, Japan) and used for TA cloning and sequencing.

5-2-7 TA Cloning and Gene sequencing

The resultant fragments of the PCR amplification were used for ligation in pGEM-T Easy vector (Promega, USA). The reaction contained 5 µl of 2X Rapid Ligation Buffer, 1 µl of pGEM-T Easy Vector, 4 µl of PCR product, 1 µl of T4 DNA Ligase (Promega, USA). After incubation for 6 hours in 16 °C the sample was mixed with JM109 competent cells and the plasmid was inserted by heat-shock. After recovery culture incubation for 1.5 hours at 37 °C the cells were spread into LB/ampicillin/IPTG/X-Gal plates and in incubated overnight. White colonies were pick up and utilized for a colony-PCR using the M13 universal primers for amplification of a fragment containing the insert. The resultant DNA fragmented was sequenced with the universal primer M13, using a CEQ8000XL DNA sequencer (Beckman Coulter).

5-2-8 Genomic DNA Digestion and Inverse PCR Amplification

Based on the sequence obtained containing part of the phytase gene, two primer sets, phy-inv1 and phy-inv2, were designed with a separation of 507 and 518 bp respectively. *Eco*RI, *Pst*I and *Bam*HI, *Sca*I, *Stu*I, *Not*I overnight digested strain a13 genomic DNA was purified by ethanol precipitation and self-ligated with T4 DNA Ligase (Promega, USA) at 16 °C overnight. The ligation product was 200 fold diluted with D.W. and used for inverse PCR, which consisted of an initial denaturation at 94°C for 3 min, 35 cycles of 94°C for 30 sec, 52°C for 30 sec, 72°C for 3 min and a final incubation at 72°C for 7 min. The amplified 1 kb and 1.5 kb fragment from the digested genomic DNA with *Bam*HI and *Stu*I respectively was again cloned into pGEM-T Easy and sequenced with M13 universal primers. Based on the obtained sequence, the phytase ORF of 1,492 bp was amplified from strain a13 genomic DNA with primers a13phy-orf-F and a13phy-orf-R, and directly sequenced with sequencing primers listed in Table 5-2-1.

Table 5-2-1. List of primers used for phytase gene genetic cloning

Primer	Sequence (5'-3')
Degenerate primers used for identification of the phytase gene	
nter1-F	GARACNGCNCCNGCNACNGC
nter2-F	GAYATHGCICCNMGRCCNGAY
intS1-R	TCNGCCATNAGNGGNGTNCC
Primers used for inverse PCR	
phy-inv1-F	TTCTTGCTCGAGTATGCCCA
phy-inv1-R	TAATACCCGCCCATGAGACC
phy-inv2-F	TCTCCGAAGTCTTCTTGCTCG
phy-inv2-R	CGTAATACCCGCCCATGAGAC
Primers used for amplification and sequencing of the phytase gene	
M13-Forward	GTTTTCCCAGTCACGAC
M13-Reverse	CAGGAAACAGCTATGAC
a13phy-orf-F	ACCAACAACCAGGTGGC
a13phy-orf-R	AGGCTCCGTCCTGTCTTTGTC
a13phy-520-F	GTCGATCAACGCACGCGC
a13phy-912-F	TTCTTGCTCGAGTATGCCCA
a13phy-1328-F	TTATCAGTCGCTTGCGCAG
a13phy-853-R	TAGGGTCGATTTGCACGCG
a13phy-520-R	TAATACCCGCCCATGAGACC

Section III - Results

5-3-1 Phytase N-Terminal and Internal Amino Acid Sequences

In order to identify the phytase gene of *Burkholderia* sp. strain a13, an approach using degenerate primers was taken. Therefore, in order to design such primers it was important to obtain information from the protein sequence, which is encoded by the gene. For this reason, the purified native enzyme was blotted to a PVDF membrane to determine its N-terminal amino acid sequence by Edman degradation (Fig 5-3-1). Also, in order to determine internal amino acid sequences of the phytase, the purified enzyme was digested with three different endoproteinases, and after a separation of the fragments by SDS-PAGE, the obtained peptides amino acid sequences were also analyzed by Edman degradation. First the protein was digested with *Staphylococcus aureus* V-8 endoproteinase, however this endoproteinase was unable to hydrolyze strain a13 phytase which remained intact in the gel with a size of 44 kDa, even when longer times of incubation was performed (Fig. 5-3-2). Although smaller size bands than 44 kDa were visualized at the gel, they couldn't be confirmed to be fragments of the phytase protein because the V-8 endoproteinase used at this experiment, contained several impurities, which caused the formation of those bands. On the other hand, when a control reaction was done with BSA as a substrate to the V-8 endoproteinase, the protein was successfully fragmented, indicating that the endoproteinase was active and was able to digest BSA (data not shown). Since the phytase wasn't successfully digested by V-8 endoproteinase, different endoproteinases (Lys-C from *Achromobacter lyticus* and Asp-N from *Pseudomonas fragi*) were used for digestion of the protein. The enzyme was almost completely digested by those endoproteinases, which produced several fragments that were blotted to PVDF membrane for sequencing (Fig 5-3-3). Digestion by Asp-N endoproteinase gave rise to only one fragment with a size of 24.9 kDa, however no sequence could be obtained from this fragment as the amount of protein was insufficient for a good reading by Edman degradation. On the other hand, digestion with Lys-C produced seven visible fragments, from which four of them were successfully sequenced (Table 5-3-1). From the N-terminal of the protein, a sequence of 39 amino acids was obtained. Based on this sequence, two

forward degenerate primers (nter1-F and nter2-F) were designed. From the sequence of the fragment with 15.1 kDa size a reverse degenerate primer was designed (intS1-R).

5-3-2 Genetic Cloning of Strain a13 Phytase

Based on the determined sequences of the mature protein and one of the internal peptide (Table 5-3-1), degenerate primer sets were designed as listed in Table 5-2-1 and then used for semi-nested PCR. As the mature protein had a molecular mass of 44 kDa and the internal peptide used for primer design had a molecular mass of 15.1 kDa, a possible PCR amplification product would have an expected size of less than 900 bp. The first PCR amplification was performed with the outer primer set, ntr1-F and intS1-R, resulting in two amplification products indicated as A and B (Fig. 5-3-4). When the outer primer ntr2-F was used as the forward primer for the first amplification, a single specific band with similar, but slightly lower size than product A was obtained, suggesting that product A might contain a part of the phytase gene sequence instead of product B (Fig. 5-3-4). Then, for improvement of the amplification specificity, a semi-nested PCR was performed using the outer forward primer (ntr2-F) with both amplification products from the first reaction (A and B) as templates. As a result of this semi-nested PCR, only when A was used as a template, a product with a size of 810 bp was obtained, while B didn't resulted in any amplification product (Fig. 5-3-5). The resultant fragment of 810 bp was confirmed by cloning and sequencing to contain a part of the phytase gene sequence. Then in order to reveal the unknown part of the gene sequence and obtain the complete phytase ORF, the digested genome of strain a13 with *Eco*RI, *Pst*I and *Bam*HI was self-ligated and used as templates for inverse PCR, which gave rise to two amplification products, both with a size of 900 bp (Fig. 5-3-6). Those products, which were cloned in *E. coli*, revealed part of the unknown sequence of the phytase, however, the stop codon of the phytase gene couldn't be identified, because the gene contained a *Bam*HI restriction site in its sequence. In order to countermeasure this problem, the *Bam*HI was replaced with *Stu*I, and the same procedure was repeated and an amplification product of 1,600 bp was obtained (Fig. 5-3-7). After sequencing this inverse PCR amplification product, the complete phytase ORF was identified, and lately sequenced directly from the genome of strain a13.

The phytase genes of strain a13 was deposited into GenBank/EMBL/DDBJ databases under accession numbers LC002814.

5-3-3 a13 Strain Phytase Primary Structure Analysis

From the genome of *Burkholderia* sp. strain a13, a PCR product of 1,492 bp containing the phytase ORF was amplified and sequenced (Fig. 5-3-8). The mature region of the phytase ORF consists of 1,287 bp. It encodes 428 amino acids, starting from a glutamic acid residue (indicated by the arrow) and the calculated molecular mass, based on the protein sequence is 46 kDa, which is in agreement with the molecular mass of the purified protein (44 kDa). Upstream of the mature region, two potential ATG codons, one at 34 amino acid upstream and the other at 45 amino acid upstream, were found in-frame. The former one was preceded by a putative ribosome binding site (RBS; GAGTG) and a putative promoter sequence (-35; TGAACA, -10; AATATT), similar to the consensus sequence recognized by the *E. coli* σ^{70} RNA polymerase (Harley and Reynolds, 1987); therefore it was concluded that the phytase gene was translated from the ATG codon at 34 amino acid upstream of the mature enzyme, which encodes a methionine (indicated in the circle) (Fig. 5-3-8). This N–terminal region contains three Arg residues followed by a stretch of hydrophobic residues, showing a similarity to a secretory signal sequence.

A BLAST search based on the amino acid sequence of the phytase showed the highest identity of 64% with a gene annotated as phosphoanhydride phosphorylase from *Burkholderia* sp. TJI49 (accession No., EGD02589.1) and, among the biochemically-characterized phytases, the enzyme showed the highest similarity of 48% identity with *Hafnia alvei* phytase (accession No., WP_004090479.1); it also showed moderate similarity with several cloned phytases of bacterial origin, especially from *Enterobacteriaceae*. The amino acid sequence of this enzyme contains motifs of RHGXRXP at the N-terminal region and HD at the C-terminal region, found in the histidine acid phosphatase (HAP) family (indicated in yellow)(Fig. 5-3-9) . From this result, it was concluded that the phytase from strain a13 belongs to the family of HAP phytases, which contains the majority of the known phytases.

Table 5-3-1. Strain a13 phytase N-terminal and internal amino acid sequences and designed degenerate primers.

Sample size (kDa)	Amino acid sequence[a]	Designed Primer
Native enzyme		
44	ETAPATARDIAPRPDLQLESVVIVSRHGVRS	nter1-F / nter2-F
Fragments obtained from digestion with Lys-C endoproteinase		
25	SPYCAHDRPGAECRFSALAN	-
15.1	GTPLMADILGALTQSATG	intS1-R
14.9	DWPTWPVQPGELTERGA	-
14.7	TPYLASRKGTPLMA	-

[a] underline indicates the position of primers designed

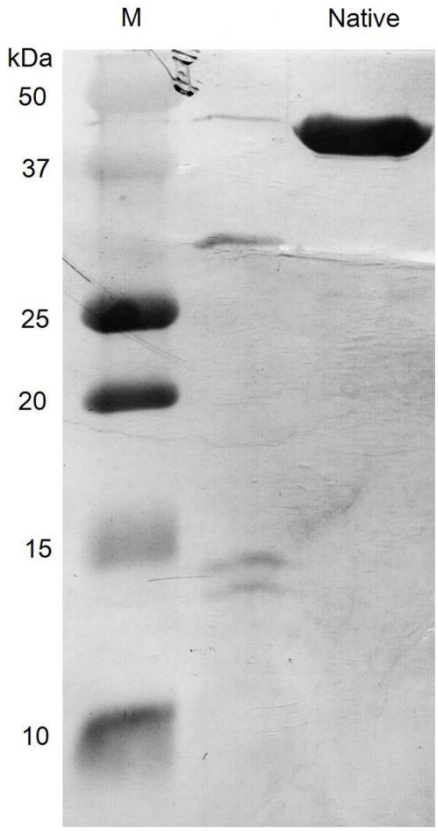

Fig. 5-3-1. PVDF membrane stained by CBB, containing strain a13 native phytase. Lane M, molecular marker standards (kDa); Lane Native, native purified protein.

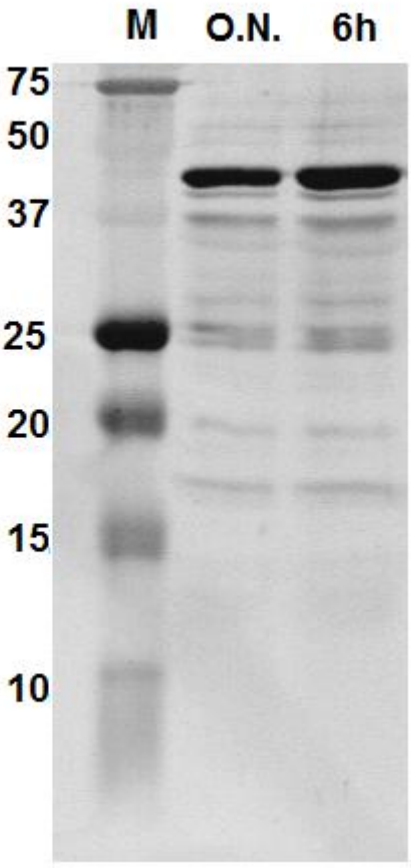

Fig. 5-3-2. PVDF membrane stained by CBB, containing strain a13 native phytase after digestion with V-8 endoproteinase. Lane M, molecular marker standards (kDa); Lane O.N., digestion products after an overnight incubation of the purified enzyme with V-8 endoproteinase ; Lane 6h, digestion products of the purified phytase after 6 hours of incubation with V-8 endoproteinase

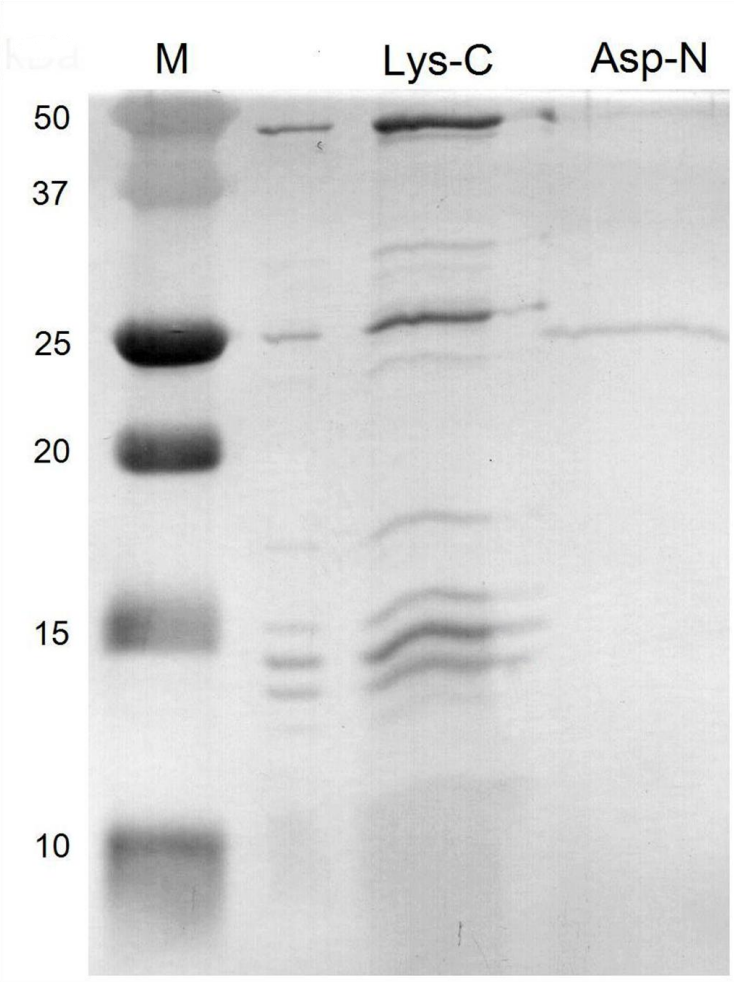

Fig. 5-3-3. PVDF membrane stained by CBB, containing strain a13 native phytase fragmentation products after digestion with Lys-C and Asp-N endoproteinase. Lane M, molecular marker standards (kDa); Lane Lys-C, digestion products after an overnight incubation of the purified enzyme with Lys-C endoproteinase ; Lane Asp-N, digestion products after an overnight incubation of the purified enzyme with Asp-N endoproteinase.

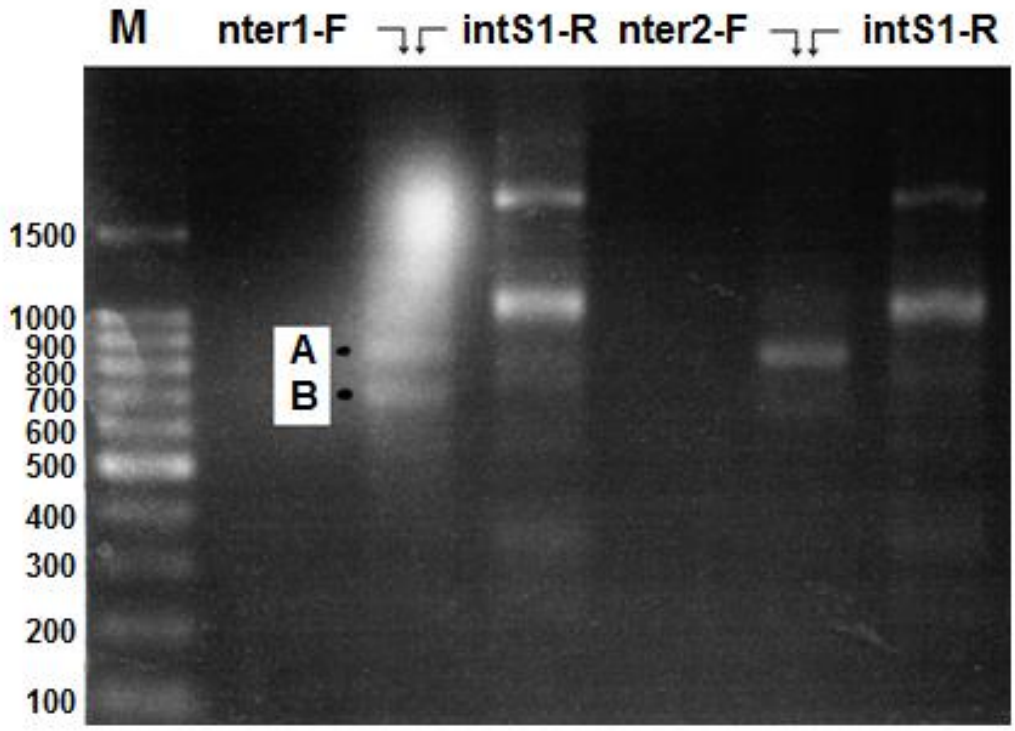

Fig. 5-3-4. PCR Amplification products obtained from the genome of strain a13 using degenerate primers. Control reaction containing only the forward or reverse primers are indicated above the lane by the primer identification. Arrows indicate forward and reverse primers used for amplification of the partial phytase gene. A and B indicate the recovered PCR amplification products. Lane M denotes molecular mass markers (bp).

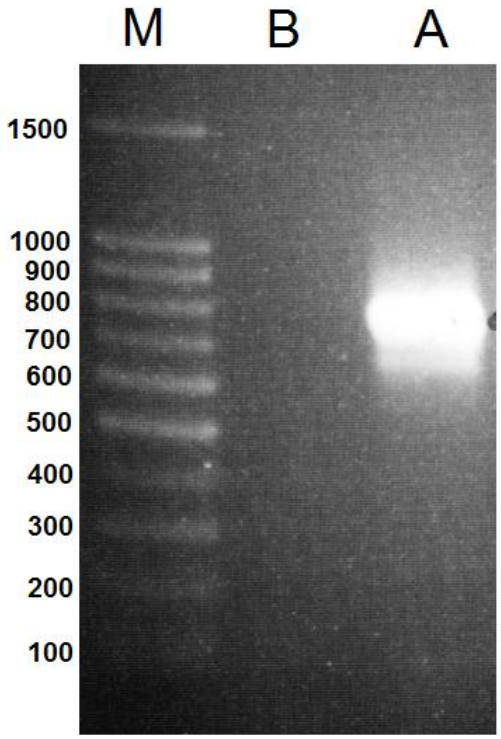

Fig. 5-3-5. Semi nested-PCR amplification products using the primer set: nter2-F and intS1-R. Lane A show the PCR product obtained when the previous PCR product A was used as a template; Lane B show the PCR result by the use of previous PCR product B as a template . Lane M denotes molecular mass markers (bp).

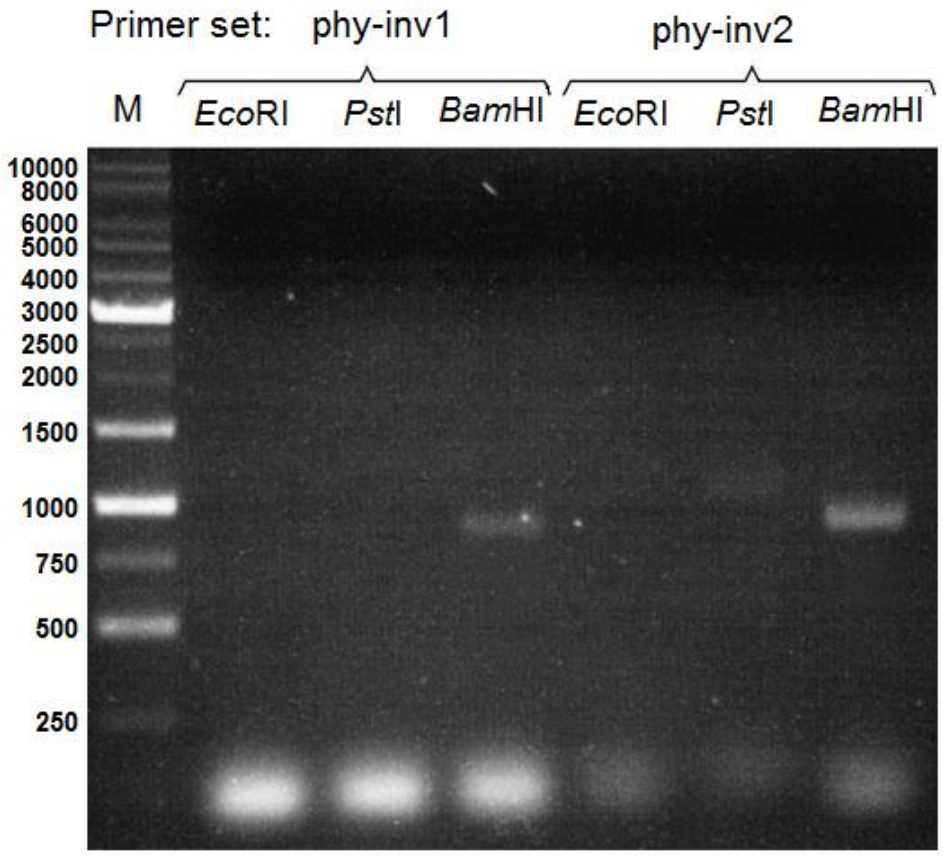

Fig. 5-3-6. Inverse PCR amplification products obtained from the self-ligated genome of strain a13 digested with *Eco*RI, *Pst*I and *Bam*HI. Primers set phy-inv1 and phy-inv2 were designed based on the known phytase ORF sequence with a separation of 507 and 518 bp respectively. Lane M denotes molecular mass markers (bp).

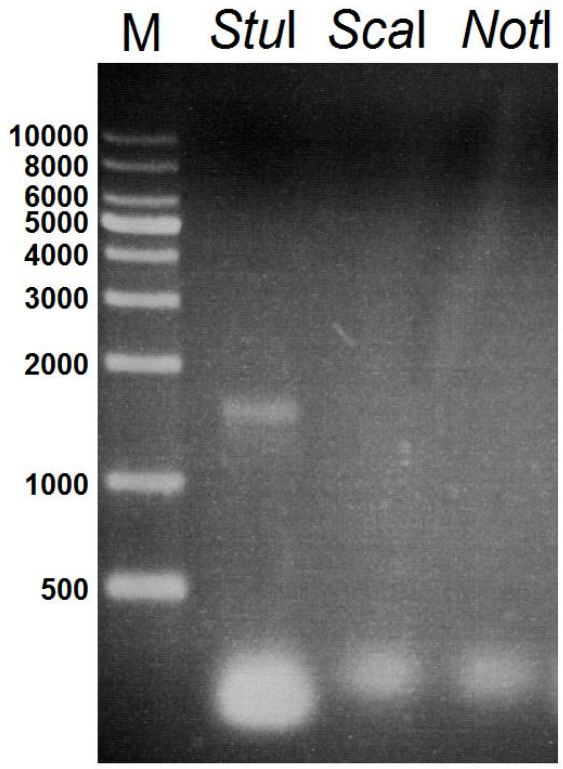

Fig. 5-3-7. Inverse PCR amplification products obtained from the self-ligated genome of strain a13 digested with *Stu*I, *Sca*I and *Not*I. Primer set phy-inv2 was used for the reaction. Lane M denotes molecular mass markers (bp).

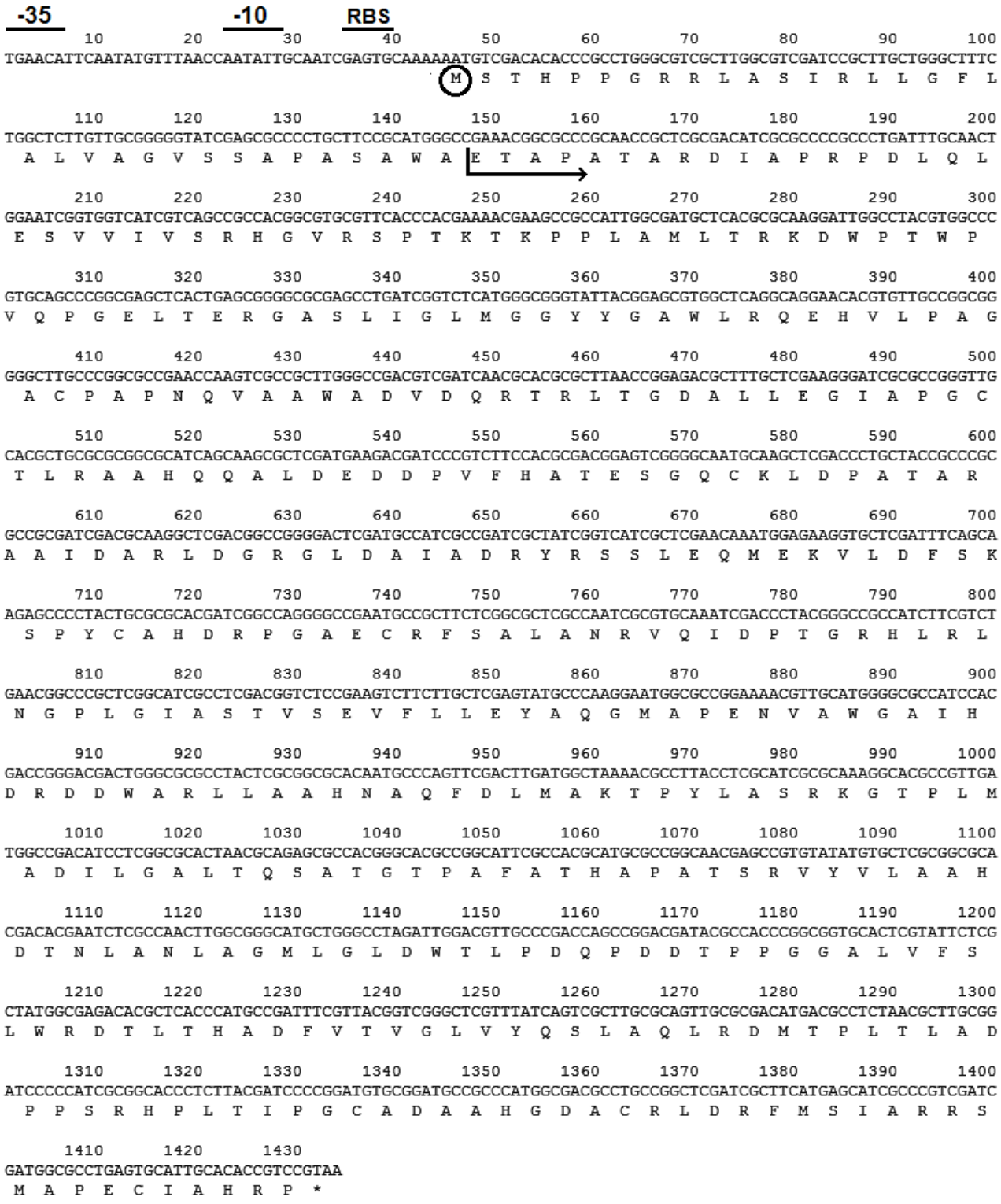

Fig. 5-3-8. Strain a13 phytase ORF with the upstream sequence and the translated protein sequence. N-terminal amino acid of the mature phytase of strain a13 is indicated by the arrow. The putative initiation codon ATG, encoding a methionine is indicated by the circle. The putative RBS and promoter sequences (-10 and -35) are indicated by lines.

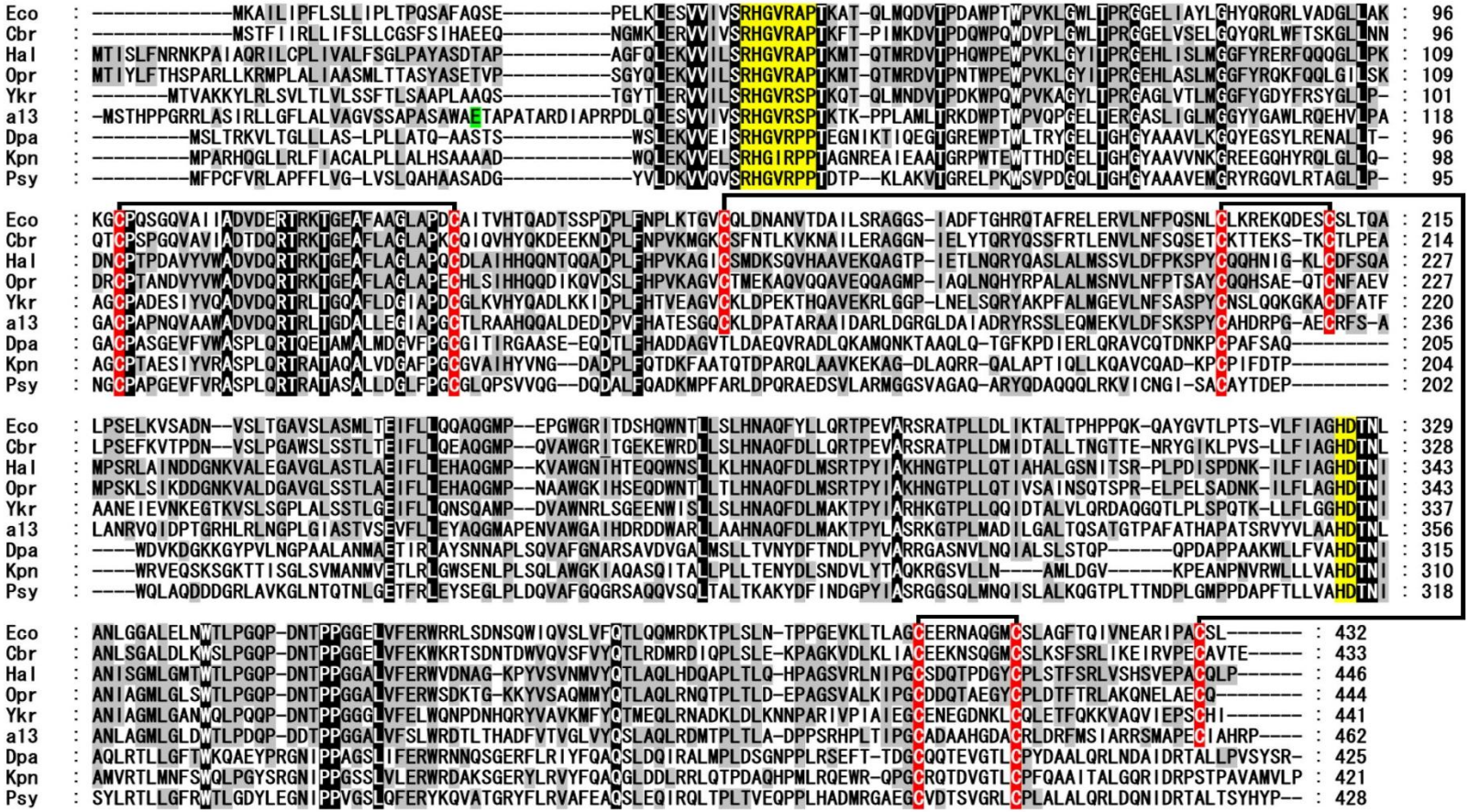

Fig. 5-3-9. Alignment of the amino acid sequences of phytases from *Enterobacteriaceae*. Eco, *Escherichia coli* (PPA_ECOLI); Cbr, *Citrobacter braakii* (Q2VY22_CITBR); Hal, *Hafnia alvei* (H9TUK5_HAFAL); Opr, *Obesumbacterium proteus* (Q6TAQ8_9ENTR); Ykr, *Yesrsinia kristensenii* (H9TUK6_YERKR); a13, *Burkholderia* sp. a13; Dpa, *Dickeya paradisiaca* (B4XT21_9ENTR); Kpn, *Klebsiella pneumoniae* (Q84CN9_KLEPN); and Psy, *Pseudomonas syringae* (Q8GD20_PSESX). Conserved amino acids are shaded, of which those conserved among all the phytases are in black. The HAP phytase motifs are shown with yellow-shades. The conserved Cys residues are shaded in red, and lines indicate the positions of disulfide bridges in the 3D structure of H. *alvei* phytase. The N-terminal amino acid of the mature phytase of strain a13 is shown in green

Section IV - Discussion

The phytase gene of *Burkholderia* sp. strain a13 was successfully identified through the use of degenerate primers, which were designed based on the protein sequence information of the N-terminal and one internal peptide. For obtaining the internal amino acid sequence, the purified mature protein was digested with specific endoproteinases. Interestingly, the mature phytase was successfully digested with Lys-C and Asp-N endoproteinases, though the protein couldn't be digested by V-8 endoproteinase under those experimental conditions (Fig. 5-3-2). The *Staphylococcus aureus* V-8 endoproteinase is a serine proteinase which selectively cleaves peptide bonds C-terminal to glutamic acid residues. The phytase protein sequence revealed that the mature enzyme had 15 Glu residues in its constitution (Fig. 5-3-8), and the V-8 digestion prediction, revealed that even the complete hydrolysis of all susceptible peptide bonds would form at least a product with 19.21 kDa in size. Since the protein couldn't be digested by this protease, maintaining its mass of 44 kDa; It was supposed that few glutamic acids were exposed at the surface of the protein, compromising the activity of the V-8 endoproteinase, or might be producing only products lower than the limit of detection of 8 kDa by this experiment. The incubation of the protein under stronger denaturizing conditions, might improve the digestibility of the V-8 endoproteinase.

Genetic cloning revealed an upstream sequence from the glutamic acid of the mature protein. This sequence contained one ATG codon, which was considered the putative initiation codon, encoding a methionin 34 amino acids from the glutamic acid (Fig. 5-3-8). Since the purified enzyme from the cell-free extract didn't contain this upstream sequence, it is certain that the phytase is passing through a maturation process after synthesis. Although the role of this upstream sequence for the enzyme maturation is still unknown, it is speculated that this sequence is possible working as a signal peptide because of its characteristics with other reported signal peptides (Nakajima et al., 1999). In order to address the function of this upstream sequence further studies are needed, like the expression of the phytase containing or not the upstream sequence. Also the development of genetic manipulation and expression system of strain a13 would be important to investigate the role of the upstream sequence. If this sequence is working as a

secretory signal sequence to the periplasm of strain a13, a colicin V reporter assay and western blot analysis, as described by Flannagan (2007) would be determinant for understanding its biological role.

Strain a13 phytase sequence showed the highest similarity of 48% identity with *Hafnia alvei* phytase, it also revealed the characteristic histidine acid phosphatase motifs in the N-terminal and C-terminal of the protein (Fig. 5-3-9). For that reason, it was concluded that the phytase from strain a13 belongs to the family of HAP phytases. Indeed, in chapter IV it was discussed that the biochemical properties of the enzyme were very similar to other HAP phytases, however expressive differences, specially the importance of reduced form of Cys residue (s) for the enzyme activity were also discussed. The alignment showed that the mature portion of this enzyme contains eight Cys residues, of which five are conserved in bacterial HAP phytases examined, and the rest three are conserved in six phytases, including *H. alvei* phytase. In the crystal structure of *H. alvei* phytase (Ariza et al., 2013), it has been reported that the eight conserved Cys residues formed four disulfide bridges with each other (Fig. 5-3-9). The catalytic mechanism proposed for the HAP phytases includes the involvement of the His residue in the RHGXRXP motif for formation of a covalent phosphohistidine intermediate and the Asp residue in the HD motif as a proton donor (Mullaney and Ullah, 2003), but none of the Cys residues are involved in the catalysis. I speculate that, if the strain a13 phytase also has four disulfide bridges, one (or more) of them may need to be reduced to obtain the enzyme activity, at least for this enzyme. It should be noted that, in the structure of *H. alvei* phytase, none of the four disulfide bridges are located in the proximity of the catalytic center, and the closest Cys residue had a calculated distance of 15.9 Å from the active site, which preclude a direct interaction of this residue with the substrate (Fig 5-4-1). In the future further investigations by performing site specific mutagenesis of the phytase produced by strain a13 may clarify this point.

Site specific mutagenesis studies targeting Cys residues of the HAP phytases produced by *Aspergillus niger* NRRL 3135, and *Escherichia coli* have already been reported (Rodriguez et al., 2000; Berkmen et al., 2005; Mullaney et al., 2010). Generally those studies focus on the production of a phytase with different biochemical properties, like altered pH and temperature profiles, in order to meet the needs

for application. The mutations C215S and C264G over the *Aspergillus niger* phytase, shifted the optimum temperature of the enzyme to 37°C, making it more suitable for application, but the overall activity dropped for all mutations studied. On the other hand, *E. coli* phytase C200N mutant had an increase in phytase activity (54%). The crystal structure of *E. coli* phytase shows that the Cys-200 residue have a distance of 23.3 Å from the catalytic site, which similarly to *H. alvei*, preclude a direct interaction of this residue with the active site (Fig. 5-4-2). The author theorized that when the G helix and the GH loop of the protein do not contain the disulfide bond Cys-200/Cys-210, the α-domain may become slightly more flexible, resulting a positive modulation on the catalytic efficiency of the enzyme (Rodriguez et al., 2000). A later study, focusing on the disulphide-bonds of *E. coli* phytase showed that the double consecutive dissulphide-bond mutant C200S/C210S had an improvement in thermo stability and even a higher activation (124%) than the single mutant when compared to the WT phytase (Berkmen et al., 2005). In agreement with this result, *E. coli* phytase have already been reported to be activated by glutathione, a thiol reducing agent (Tai et al., 2013). Interestingly, the alignment result of phytases show that the *E. coli* Cys-200 residue is conserved for all HAP phytases, which correspond to the Cys-223 in *Burkholderia* sp. strain a13 phytase (Fig. 5-3-9). By those results, Cys-223 of strain a13 phytase is likely promoting the enzyme activity, however I cannot assume which one of the Cys residues are responsible for this phenomenon. Further investigation by conducting site specific mutagenesis of all cysteine residues of the phytase produced by strain a13 may clarify this point.

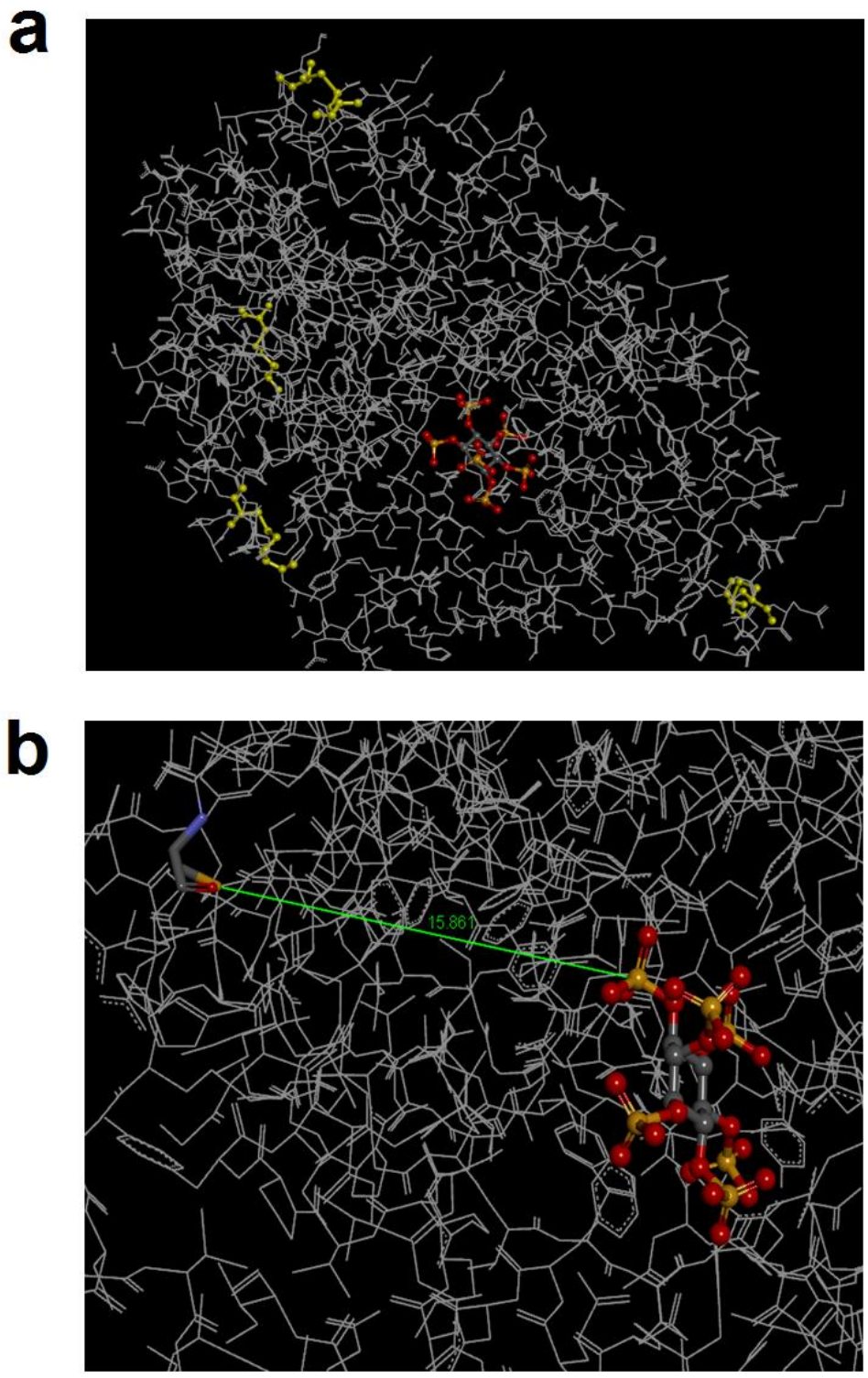

Fig. 5-4-1. *Hafnia Alvei* phytase crystal structure in complex with *myo*-inositol hexakissulphate representation. (a) Cys residues represented in yellow and the ligand molecule of *myo*-inositol hexakissulphate at the catalytic center represented with ball-stick model. (b) Distance measurement from the thiol group of the closest Cys residue to the sulphate group of the ligand.

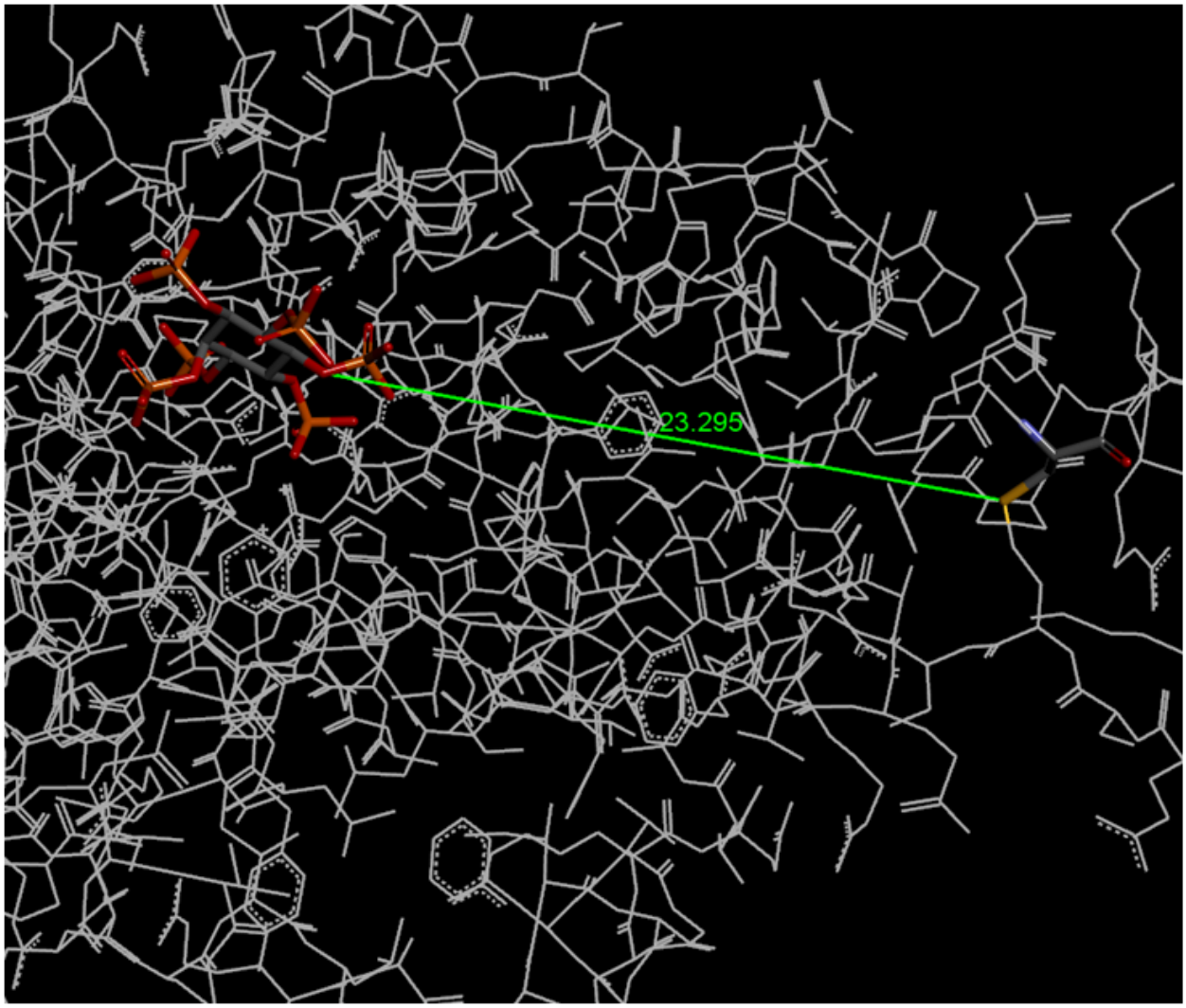

Fig. 5-4-2. *Escherichia coli* AppA phytase crystal structure in complex with *myo*-inositol hexakissulphate, showing the caluculated distance between the thiol group of Cys-200 residue to the sulphate group of the ligand.

CHAPTER VI - RECOMBINANT ENZYME EXPRESSION

Section I - Introduction

Overexpression of genes in heterogeneous system is an important tool to easily obtain protein products with higher yields. Genetic manipulation also allows the production of poly-histidine-tagged proteins, which can substantially diminish the chromatographic steps, facilitating the purification of target protein using affinity chromatography columns. As previously discussed in chapter IV, a higher productivity of phytase by the microorganism is also an important factor for considering the application of the enzyme into a commercial scale. For that reason, in this chapter, the newly identified phytase gene from *Burkholderia* sp. strain a13 was cloned and expressed in *Escherichia coli*, with the objective to obtain higher amounts of the enzyme and facilitate its purification. Also, the primary structure study of the protein, which was discussed on the previous chapter, revealed an upstream sequence with similarity to a secretory signal sequence. Genetic manipulation techniques allow the overexpression of the phytase gene of strain a13, containing or not this upstream sequence, which might be an important tool for obtaining information about the function of this upstream sequence. To date, there is several reports of phytase genes possessing sequences similar to a signal sequence at their N-termini (Sajidan et al., 2004; Tamayo-Ramos et al., 2012; Ariza et al., 2013), but the function of those sequences is usually not discussed on those reports, which rises the interest about the function of this upstream sequence to the strain a13 phytase. Also, it is important to mention that the cellular machinery of *E. coli*, which is classified into the class of *Gammaproteobacteria,* is expected to be very different when compared to the cellular machinery of strain a13 that is classified into the class of *Betaproteobacteria*. Both classes of bacteria are reported to contain the periplasmic space in its constitution (Flannagan et al., 2007; Hernández-Montes et al., 2012), though differences in the cellular machinery might impose a barrier into the analysis of the actual function of this putative secretory signal sequence. In the future, development of an expression system in *Burkholderia* sp. strain a13 might be important to determine the function of this upstream sequence precisely.

Section II - Material and Methods

6-2-1 Construction of Expression Vectors

For insertion of the phytase gene into expression vector pET22b(+), the mature region of the phytase ORF and the one starting from the putative initiation codon, the ATG codon 34 amino acid upstream of the mature region, were PCR-amplified with primer sets listed in Table 6-2-1 of pET22-NdeI-E and pET22-EcorI, pET22-NdeI-M and pET22-EcorI, respectively. The PCR was performed with PrimeSTAR HS DNA Polymerase (Takara Bio Inc., Japan), which consisted of an initial denaturation at 98°C for 1 minute and 30 seconds, 35 cycles of 98°C for 10 seconds, 55°C for 5 seconds, 72°C for 1 minute and 30 seconds. The PCR fragments thus obtained were digested with *Nde*1 and *Eco*R1 and cloned into respective sites of pET22b(+) by T4 DNA Ligase (Promega, USA), giving rise to pET22b-Ephy and pET22b-Mphy. For the constructs pET28a-Ephy and pET28a-Mphy, exactly the same procedure was repeated, but the primers used were pET28-NdeI-E and pET28-EcorI, pET28-NdeI-M and pET28-EcorI, and the pET28b(+) was used as the vector. In these constructs, the 6 x His-tag in the vector was attached to the C-termini or to the N-termini of the cloned ORFs in pET22b(+) and pET28a(+), respectively. The constructed plasmids pET22b-Mphy and pET22b-Ephy, also pET22b(+), as the control group, were used to transform competent cells of *E. coli* BL21 (DE3). The constructed plasmids pET28a-Mphy and the control vector pET28a(+), were used to transform competent cells of *E. coli* BL21 (DE3), *E. coli* OverExpress C41(DE3), *E. coli* HMS174 (DE3), *E. coli* BL21 CodonPlus(DE3) RIPL, *E. coli* BL21 Star (DE3), *E. coli* SHuffle T7 (DE3), while pET28a-Ephy was used to transform *E. coli* BL21 (DE3).

6-2-2 Recombinant Phytase Expression and Cell Fractions

For enzyme production, an overnight culture of the strain harboring the plasmid was inoculated at 1% to a fresh LB medium containing adequate concentration of the respective selection antibiotic and cultured at 37°C. When the O.D. of the culture reached 0.6, IPTG was added at a final concentration of

0.5 mM, and the culture was continued further at 30°C for 6 h. For promotion of proper protein folding from the construct pET28a-Mphy in *E. coli* HMS174 (DE3), induction was performed at 18 °C, with addition of IPTG to a final concentration of 0.05 mM, and the culture was continued further for 15 h. Then the cells from the cultures were harvested by centrifugation at 5000 x g for 10 min and the cell pellets were resuspended in 50 mM Tris-HCl buffer pH 7.5 with 100 mM NaCl and 0.5 mM PMSF, and disrupted by sonication in low temperature at 20 Khz, 220 W with a UCW-201 sonicator (Tosyo Electric, Japan). The supernatant was obtained by centrifugation at 4 °C, 120,000 × g for 15 min and was filtered with a Millex-GV PVDF 0.22 mm syringe filter (Millipore, Germany) and was defined as the soluble fraction. The remaining cell debris pellet was defined as the insoluble fraction.

6-2-3 His-Tagged Protein Purification

The soluble fractions containing the recombinant phytase produced by pET22b-Mphy and pET28a-Mphy constructs were applied to an ÄKTA purifier system (Amersham Pharmacia Biotech, USA) equipped with a HisTrap excel 1 ml column (GE Healthcare Life Sciences, USA), pre-equilibrated with 50 mM Tris-HCl buffer pH 7.5, and proteins were eluted with a linear gradient of 0-1 M imidazole in the above buffer. For the purification of the protein from the insoluble fraction of pET22b-Ephy, the cell debris pellet was solubilized by addition of 1 mL of 8M urea and 50 mM Imidazole in 50 mM Tris-HCl buffer pH 7.5, and proteins were eluted with a linear gradient of 0-1 M imidazole in the above buffer.

6-2-4 Chaperone Gene Co-expression

Competent cells of *E. coli* HMS174 (DE3) harboring the plasmid pET28a-Mphy were prepared and used for transformation by heat-shock with chaperone gene expression plasmids pG-KJE8, pGro7, pKJE7, pG-Tf2 and pTf16. The resulting transformation strains were used for over-expression of the phytase with co-expression of the chaperone. An overnight culture of the strain was inoculated at 1% to a fresh LB medium containing 50 γ of kanamycin and 35 γ of cloramphenicol and cultured at 37°C. At the

same time of inoculation, chaperone expression was induced by addition of a final concentration of 0.5 $mg.mL^{-1}$ of L-arabinose for the strain harboring pGro7, pKJE7 and pTf16. For the strain harboring pG-Tf2, induction was done with a final concentration of 5 $mg.mL^{-1}$ of tetracycline. For the strain harboring pG-KJE8, induction was done with a final concentration of 5 $mg.mL^{-1}$ of tetracycline and 0.5 $mg.mL^{-1}$ of L-arabinose. When the O.D. of the culture reached 0.6, IPTG was added at a final concentration of 0.5 mM, and the culture was continued further at 30°C for 6 h. Then the cells from the cultures were harvested by centrifugation at 5000 x g for 10 min and the prepared soluble and insoluble fractions were used for SDS-PAGE and phytase activity measurement.

Table 6-2-1. Primers used for construction of phytase expression plasmids

Primer	Sequence (5'-3')[a]
pET22-NdeI-M	CGACACA*CATATG*TCGACACACCCGCCTGG
pET22-NdeI-E	CGACACA*CATATG*GAAACGGCGCCCGCAACC
pET22-EcoRI	GC*GAATTC*GGCGGACGGTGTGCAATGCACTC
pET28-NdeI-M	CGACACA*CATATG*TCGACACACCCGCCTG
pET28-NdeI-E	CGACACA*CATATG*GAAACGGCGCCCGCAAC
pET28-EcoRI	GC*GAATTC*TTACGGACGGTGTGCAATGC

[a] Restriction sites introduced are shown in italics.

Section III - Results

6-3-1 Recombinant Phytase Expression and His-Tagged Protein Purification

First, the expression of the cloned phytase gene was tested in *E. coli* using two different C-terminal 6×His-tagged constructs (pET22b-M and pET22b-E) as described in Section II of this chapter. In the case of pET22b-Ephy containing the mature portion of the phytase gene, enzyme activity was not detected. With the pET22b-Mphy construct containing the phytase gene from the putative initiation codon, a rather weak but distinct phytase activity of 0.183 U mg^{-1} was detected in the cell-free extract, whereas a control strain harboring the vector plasmid did not show any phytase activity (Table 6-3-1). Although phytase activity was detected at the soluble fraction of pET22b-Mphy, the SDS-PAGE of this fraction didn't showed a difference in band-pattern between the constructs and the control strain, indicating a low production of the enzyme, which correlates to the low activity obtained for pET22b-Mphy (Fig. 6-3-1). Purification of the enzyme produced by this strain by affinity chromatography wasn't effective because the enzyme adhesion to the column was very poor and the activity was detected before the beginning of the imidazole gradient (Fig. 6-3-2a). Then, the insoluble fraction of pET22b-Ephy was treated with 8M of urea and a specific band with a similar size to the native phytase (44 kDa) was successfully purified by affinity chromatography (Fig. 6-3-3), suggesting that pET22b-Ephy expressed protein is encountered at the inclusion bodies of the cell. Attempts to refold the urea-denatured protein by dialysis didn't restored phytase activity, which remained undetected for this construct. In order to successfully purify the protein by affinity chromatography from the soluble fraction, two different N-terminal 6 × His-tagged constructs (pET28a-Mphy and pET28a-Ephy) were produced. Similarly to the previous results, only the construct containing the phytase gene from the putative initiation codon (pET28a-Mphy) showed phytase activity at the soluble fraction, but no enzyme activity was detected for the construct containing the mature portion of the phytase gene (Table 6-3-1). Although enzyme activity of the soluble fraction of pET28a-Mphy had a slight improvement in comparison to pET22b-Mphy, the SDS-PAGE of the soluble fraction didn't showed specific bands of the expected protein size of 44 kDa at the soluble fraction (Fig. 6-3-4),

indicating that production of the phytase by this strain is still low. Purification of the His-tagged phytase from the soluble fraction of this strain by affinity chromatography encountered the same problems as the previous one (Fig. 6-3-2b).

6-3-2 Recombinant Phytase Expression Optimization in E. coli

Although phytase activity was detected for the constructs containing the phytase gene from the putative initiation codon, the enzyme activity was rather weaek showing an phytase activity of 0.183 U mg^{-1} and 0.590 U mg^{-1} for pET22b-Mphy and pET28a-Mphy, respectively. The purification of a protein by affinity chromatography from the insoluble fraction of pET22b-Ephy construct with similar size to the native enzyme, also suggest that the enzyme is possible not properly folding in the heterogeneous expression system. In this sub-section, efforts to obtain higher phytase activity at the heterogeneous expression system are presented. First the introduction of the plasmid pET28a-MPhy and its expression in different hosts of *E. coli* were tested. The introduction of the plasmid in HMS174 (DE3) and protein expression in this host promoted a slightly improvement in phytase activity, which showed a value of 0.717 U mg^{-1} (Table 6-3-2). On the SDS-PAGE analysis of the soluble fractions of all strains it wasn't possible to confirm specific recombinant phytase bands, around 44 kDa when compared to their control strains harboring the expression vector (Fig. 6-3-5). However, when the insoluble fraction was analyzed by SDS-PAGE, specific strong bands with similar size to the native enzyme were detected for some of the constructs containing the phytase gene, like HMS174 (DE3), BL21 CodonPlus(DE3) RIPL and SHuffle T7. This result suggest that the enzyme is not properly folding, which leads the major portion of the enzyme to have as destination the inclusion bodies of the cell. Induction of the gene expression in lower concentrations of IPTG (0.05 mM) and at lower incubation temperatures (18 °C) didn't improved phytase expression in HMS174 (DE3) host as the enzyme activity wasn't substantially changed (0.567 U mg^{-1}). In this case, co-expression of the phytase gene with chaperone genes was tested. Unfortunately, co-expression of chaperone genes didn't contribute substantially to the enzyme activity, which remained at the same levels (Table 6-3-3).

Table 6-3-1. Phytase activity of the cell-free extract of different phytase constructs overexpressed in *E. coli* BL21 (DE3).

Construct	Phytase activity (U mg^{-1})
pET22b(+)	-
pET22b-Mphy	0.183
pET22b-Ephy	-
pET28a(+)	-
pET28a-Mphy	0.590
pET28a-Ephy	-

Table 6-3-2. Phytase activity of the cell-free extract of the construct pET28a-Mphy overexpressed in different *E. coli* hosts.

E. coli expression host	Phytase activity (U mg^{-1})
BL21 (DE3)	0.590
OverExpress C41(DE3)	-
HMS174 (DE3)	0.717
BL21 CodonPlus(DE3) RIPL	0.483
BL21 Star (DE3)	0.125
SHuffle T7 (DE3)	-

Table 6-3-3. Phytase activity of the construct pET28a-Mphy co-expressed with the indicated chaperones in *E. coli* HMS174 (DE3)

Chaperone plasmid	Phytase activity (U mg^{-1})
pG-KJE8	0.682
pGro7	0.583
pKJE7	0.553
pG-Tf2	0.652
pTf16	0.724

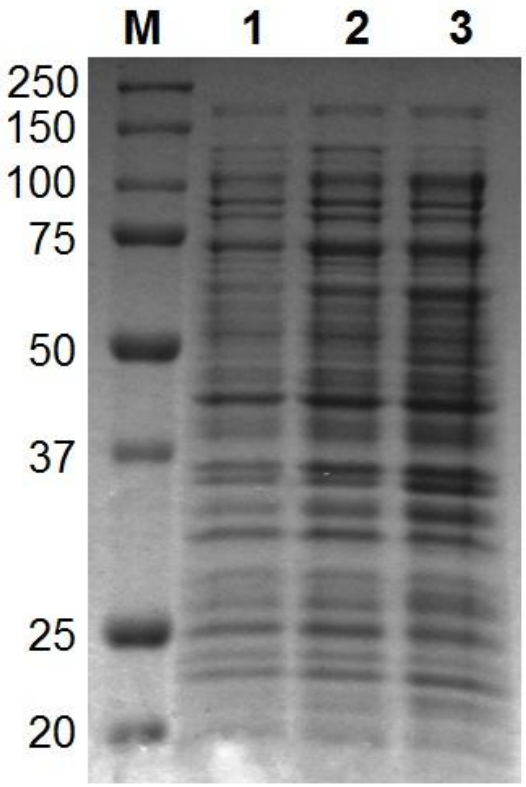

Fig. 6-3-1. SDS-PAGE of the soluble fractions of the recombinant strains of *E. coli* harboring pET22b-Ephy, pET22b-Mphy and the control vector. Lane M, molecular marker standards (kDa); Lane 1, control strain harboring the vector plasmid pET22b(+); Lane 2, pET22a-Mphy construct containing the phytase gene from the putative initiation codon; Lane 3, pET22b-Ephy containing the mature portion of the phytase gene.

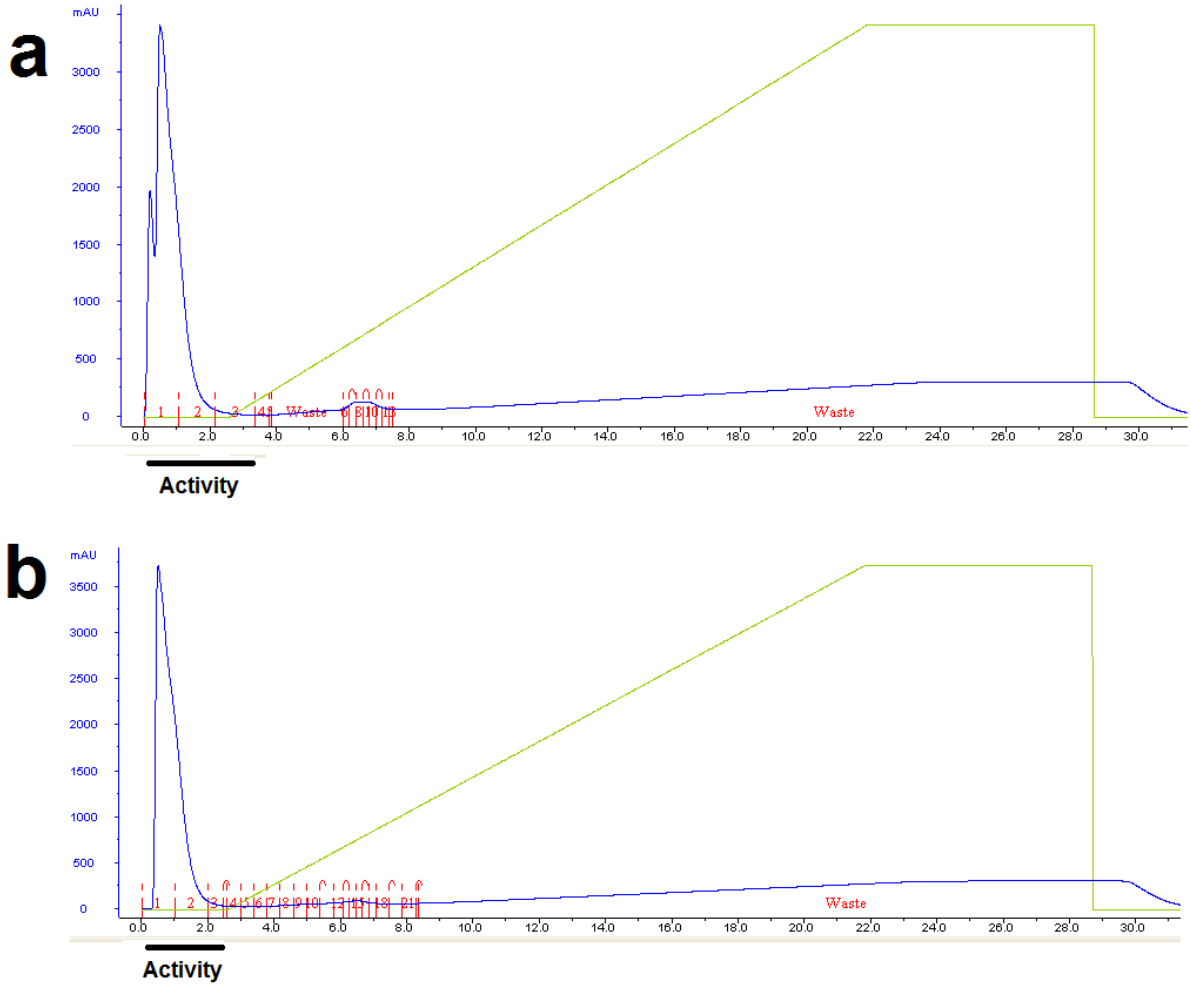

Fig. 6-3-2. Affinity chromatography purification profile from the cell-free extract of constructs showing phytase activity. Blue lines indicate absorbance at 280 nm; green lines indicate imidazol gradient profile; colected fractions are indicated in red; black bar under the fractions indicate fractions with positive detection for phytase activity. (a) Soluble fraction of pET22b-Mphy. (b) Soluble fraction of pET28a-Mphy

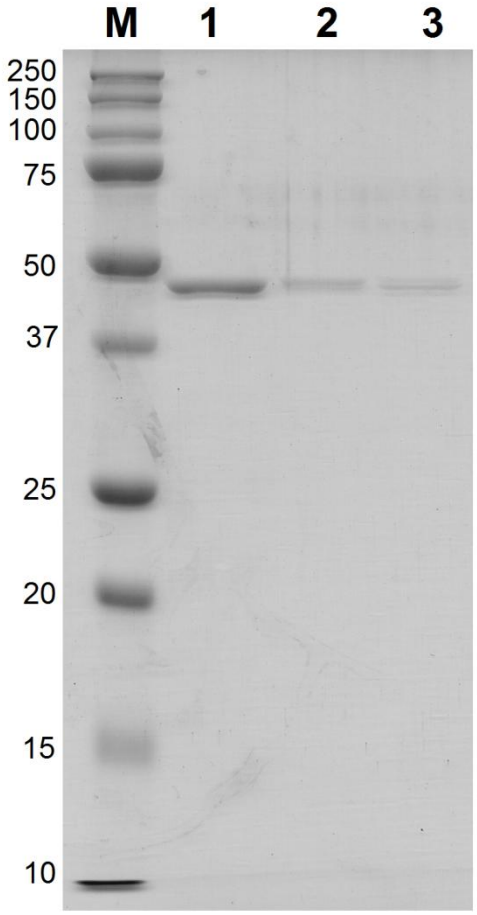

Fig. 6-3-3. SDS-PAGE of the protein purified by affinity chromatography form the insoluble fraction of the construct pET22b-Ephy treated with 8M of urea. Lane M, molecular marker standards (kDa); Lanes 1, 2 and 3 represents fractions obtained from the purification process.

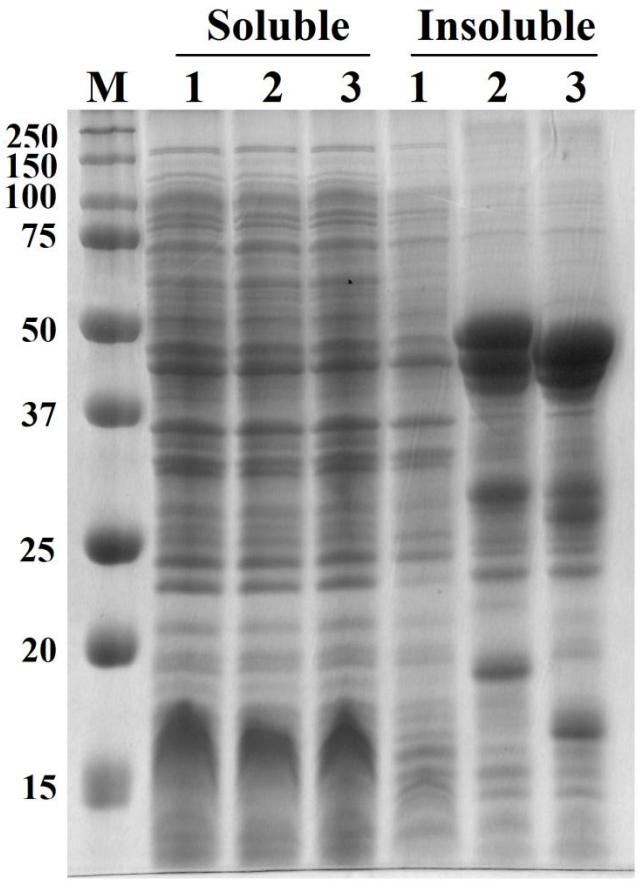

Fig. 6-3-4. SDS-PAGE of the soluble and insoluble fractions of the recombinant strains of *E. coli* harboring pET28a-Ephy, pET28a-Mphy and the control vector. Lane M, molecular marker standards (kDa); Lane 1, control strain harboring the vector plasmid pET28a(+); Lane 2, pET28a-Mphy construct containing the phytase gene from the putative initiation codon; Lane 3, pET28a-Ephy containing the mature portion of the phytase gene.

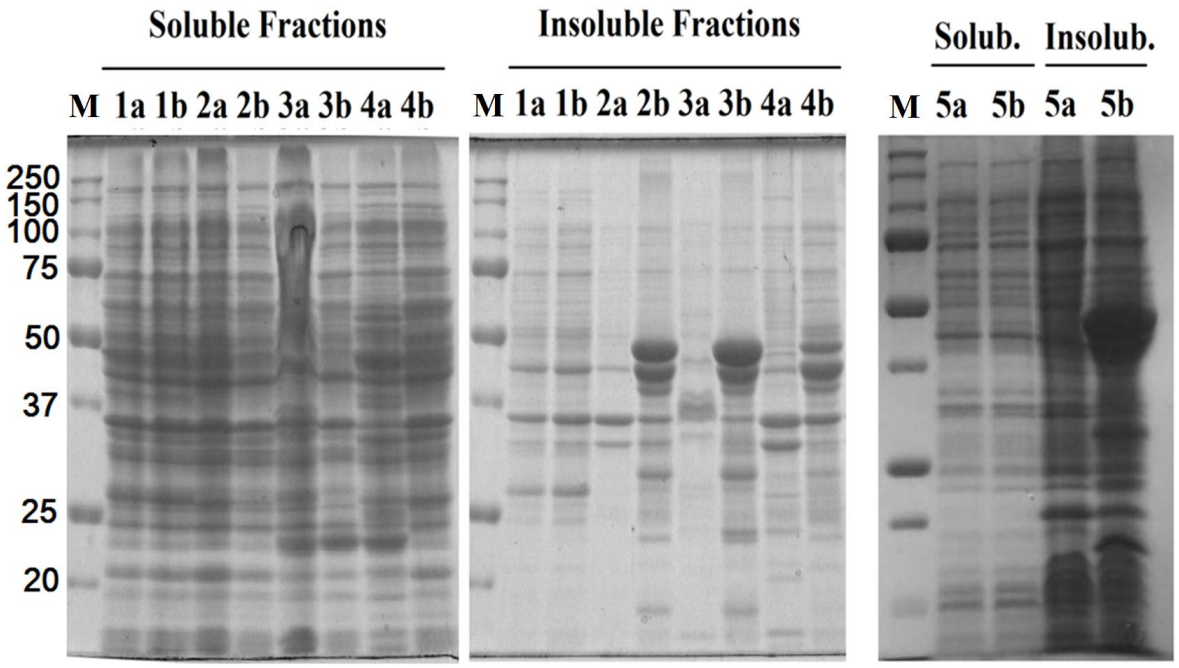

Fig. 6-3-5. SDS-PAGE of the soluble and insoluble fractions of the construct pET28a-Mphy expressed in different *E. coli* hosts. Lane M, Molecular markers. Letters indicate the plasmid: a, pET28a(+); b, pET28a-Mphy. Lane numbers indicate *E. coli* cell hosts: 1, OverExpress C41(DE3); 2, HMS174 (DE3); 3, BL21 CodonPlus(DE3) RIPL; 4, BL21 Star (DE3); 5, SHuffle T7 (DE3)

Section IV - Discussion

In this chapter, the cloned gene from *Burkholderia* sp. strain a13 containing its putative initiation codon was successfully expressed in *Escherichia coli*, showing that the gene actually encodes the phytase. As previously discussed in chapter IV, the native phytase was purified from the cell-free extract of strain a13, and its N-terminal started with a glutamic acid. However, as discussed in Chapter V, the phytase ORF contained a similar sequence to the secretory signal sequence at its upstream portion from the glutamic acid. Some of the bacterial HAP phytases, like the phytase produced by *E. coli* and *Pantoea agglomerans*, were reported to be produced in the periplasmic space in their original hosts (Greiner et al., 1993; Greiner, 2004) and several phytase genes are reported to possess sequences similar to the signal sequence at their N-termini (Sajidan et al., 2004; Tamayo-Ramos et al., 2012; Ariza et al., 2013). Although phytase activity could not be detected in the cultural broth of strain a13, it may be possible that the phytase is secreted into the periplasmic space in strain a13. The genus *Burkholderia* is classified in the class *Betaproteobacteria*, and the HtrA protease of *B. cenocapacia* was reported to be localized in the periplasmic space of this bacterium (Flannagan et al., 2007), indicating that strain a13 also possesses a periplasmic space. In order to obtain a proof of the phytase protein exportation to the periplasmic space of strain a13, further experimentations are required, and the methodology used by Flannagan (2007), which performed a colicin V reported assay to clarify this point, might be an interesting experimental way to follow. The only obstacle for following the same methodology is the necessity to develop an expression system of strain a13. If the phytase synthesized by strain a13 is transported to the periplasmic space, supposedly the dephosphorylation of the molecule of phytic acid also occurs at this compartment, which raises the question of how the phytic acid molecule is incorporated by the cell. Generally, the bacterial outer membrane serves as a permeability barrier to prevent the entry of noxious compounds and at the same time to allow the influx of nutrient molecules (Nikaido, 2003). The outer membrane, in comparison to the cytoplasmic membrane can be considered more permissive because of the existence of nonspecific porin channels, which allow the diffusion of small nutrients to the periplasmic space and consequently to the cell. One possible transportation pathway for the phytic acid molecule to the periplasmic space of

strain a13 is supposedly through those nonspecific porin channels. Unfortunately, to date there is no detailed reports focused on phytic acid transportation in bacterial cells, which remains obscure. The only report found mentioning the transportation of phytic acid into a bacterial cell, suggested that a TonB-dependent transporter gene of *Citromicrobium bathyomarinum* JL354 was possibly involved with phytic acid assimilation (Tang et al., 2012). Possible assimilation pathways for phytic acid molecule to the periplasmic space of strain a13 would be based only in speculations. Therefore, in the future, studies considering the transportation of phytic acid to the periplasm would be important to clarify this mechanism, not only for strain a13, but also for other bacterial phytases that have already been reported to be localized at the periplasmic space.

The overexpression analysis of the enzyme in *E. coli* showed that the phytase activity was detected only when the enzyme was produced within the upstream N-terminal region (Table 6-3-1). It may be possible that this region plays an important role in enzyme maturation and promotion of proper folding, because the construct containing the mature form of the phytase, showed to produce the protein at the insoluble fraction (Fig. 6-3-3; 6-3-4), suggesting that the protein is not properly folding, which might be the principal factor for the lack of phytase activity. Although the construct containing the putative initiation codon showed phytase activity at the soluble fraction, the obtained activity was relatively weak and the SDS-PAGE data indicated that the protein production was rather low for those constructs (Fig. 6-3-1; 6-3-4). An explanation for this low productivity of the enzyme is that a portion of the produced protein is possibly incorrectly folded, ending up incorporated at the inclusion bodies of *E. coli*. The SDS-PAGE of the insoluble fraction of the different hosts shows the presence of specific strong bands, with a size around 44 kDa (Fig. 6-4-2), which support this notion. Co-expression of chaperone genes weren't effective to promote a substantial change in activity, which remained at the same low levels.

Higher levels of production are desirable for application of phytases and also to facilitate the enzyme purification, lowering the production costs. Despite the constructs containing the phytase gene had attached at the C-termini and the N-termini the 6 x His-tag from the vector, unfortunately the

produced enzyme could not be purified by affinity chromatography, because of the poor absorption to the column. Possibly, the enzyme C-terminal region is not exposed to the surface of the protein since it contains the His-Glu motif which is involved into the formation of the catalytic site of the HAP phytases. For the N-terminal his-tagged recombinant protein, the purification by affinity chromatography also wasn't effective. This region of the protein also contains the HAP phytase motif (RHGXRXP) that is involved into the formation of the catalytic site, which might be responsible for the internalization of the poly-histidine-tag. Perhaps the putative signal peptide sequence of the produced protein is recognized and removed by the *E. coli* cellular machinery, which would also lead to a protein without the poly-histidine-tag. Further experimentations are needed to determine those possibilities.

Taken together, the over-expression of strain a13 phytase gene in *E. coli* is not favorable, due to possibly incorrect folding process of the enzyme. Indeed, production of several recombinant HAP phytases in *E. coli* also faced similar problems, and for this reason different expression systems were used for production of the recombinant protein. As an example, for the production of the recombinant bacterial HAP phytases, the yeast *Pichia pastoris* and the fungus *Aspergillus oryzae* were used as host strains for production of recombinant phytases from *Yersinia intermedia* (Huang et al., 2006) and *Hafnia alvei* (Ariza et al., 2013), respectively. In the case of *Dickya paradisiaca* phytase, the *E. coli* pET system was successfully used, but the level of production was rather low (Gu et al., 2009). In order to produce the recombinant phytase in a large amount, further examination is required, such as the use of the yeast expression system.

CONCLUSION

The element phosphorous, which is essential for life, is involved in the synthesis of very important molecules to biological systems, such as ATP and DNA. Therefore, inorganic phosphate has been largely applied into agriculture and animal farming, as fertilizers and diet supplementation, respectively. However, the biogeochemical cycling of the element phosphorous is a very slow process in nature, thus is considered as a non-renewable valuable resource. World phosphate rocks reserves are expected to be completely depleted in 50-100 years, which generate a global rising interest in technologies able to promote reduction, reuse and recycling of the element phosphorous. One of those technologies that might comply with this international interest is the application of phytases. Phytic acid, which is the substrate of phytases, is characterized by a *myo*-inositol core in which six phosphate groups are performing a phosphoester ligation. This phosphate rich molecule is highly present in plant tissues, where it role as a storage form of phosphorous for plants. From an animal nutrition point of view, phytic acid is considered as an anti-nutritional compound, which is highly present in grain-based animal feed and is non-absorbed by monogastric animals, causing problems such as impaired animal growth and eutrophication. As previously discussed in chapter I, phytases have been largely applied to the farming of poultry, swine and fish, in order to reduce environmental impact caused by excretion of phytic acid, as well as to enhance the nutritional value of feed for more efficient animal production. More recently, new biotechnological applications for phytases have also been explored, especially in food processing, soil amendment and production of pharmacologically important lower *myo*-inositol phosphates. Those potential applications of phytases emphasize the demand for the enzyme, which generate an ongoing interest in isolation of microorganisms for production of novel phytases. In order to meet those requirements of isolation and biochemical characterization of novel phytases with unique biochemical properties, this research work started from the screening of a novel phytase producing microorganism, from the aquatic environment, with high phytase activity.

In Chapter II, Samples of mud and fish intestinal contents from areas adjacent to net cage culture

of carp in Lake Kasumigaura were screened for phytase producing strains by enrichment cultivation at the minimal CG liquid media containing phytic acid as a sole phosphorous source. By a subsequent screening approach consisting in the cultivation of the obtained strains in the CG liquid medium and the measurement of supernatant phosphate concentration increase, ten phytase producing candidates were successfully obtained. The highest phytase activity was obtained for the cell-free extract of strain a13, and later phylogenetic analysis based on 16S rRNA gene sequences confirmed that strain a13 was included in the cluster of the genus *Burkholderia*. Interestingly, to date there is no reports on purification and biochemical characterization of phytases from this genus. Therefore, further study of its phytase could be considered of extreme importance to contribute not only to the application of phytases, but also to the scientific literature of phytases. It was expected that the biochemical characterization of the phytase produced by strain a13 could help determine if the enzyme contain suitable properties for future applications. Also, the better understanding of this novel enzyme could contribute to the knowledge of phytases, and unveil its role to strain a13 metabolism. For those reasons, strain a13 was selected as the subject of further analysis on the subsequent chapters.

In Chapter III, An approach to examine the conditions for the phytase production, as well to address the physiological role of the produced phytase was taken. Strain a13 was cultured in minimal medium containing a neutral carbon source that was used for growth. The effect of different carbon and phosphate sources over enzyme production was verified by this experimental approach. The knowledge about the conditions for phytase production in strain a13 was used to improve the yield of enzyme production, which facilitates the later purification of the enzyme produced by this strain. Remarkably, addition of enough amount of phosphate to the culture did not affect the phytase production, indicating that the enzyme production in strain a13 is insensitive to phosphate availability. This result contrasts with the majority of phytase-producing strains, which have a tight inhibition of enzyme synthesis by inorganic phosphate levels. In this chapter, substantial data was provided to indicate that strain a13 likely produces phytase to utilize phytic acid as a carbon source, after degrading it to *myo*-inositol, rather than utilizing it as a phosphorous source. The observation that this strain can grow with phytic acid and *myo*-inositol as a sole

carbon source supported this conclusion. Also a search of ortholog genes of the *iolABCDEFGHIJ* operon of *Bacillus subtilis*, in the genome of *Burkholderia phytofirmans* revealed some genes with considerable similarity, suggesting that species of the *Burkholderia* genus might have some ortholog genes that are involved in the catabolism of *myo*-inositol in *B. subtilis*.

In Chapter IV, The phytase was successfully purified to homogeneity from the cell-free extract of a13 strain by four-steps of chromatography. The enzyme specific activity had an increase of 109 fold, showing a final phytase specific activity of 174.1 U mg^{-1}. This activity could be considered high because of the existence of several reports of phytases with activity lower than one hundred units, and also the existence of a commercially available phytase produced by *Aspergillus ficum*, which produces a phytase with 150 units, support this notion. The purified enzyme was used to perform its biochemical characterization, which provided valuable information for comparison with other phytases, and determine a possible application to the enzyme. The analysis of the biochemical properties of the enzyme allowed previewing the possible classification of the phytase family. According to the pH profile, in the acidic range, activation by the presence of metal chelants (EDTA and EGTA) and inhibition by Pb^{2+}, it was suggested that the enzyme belonged to the HAP phytase family. However, concomitant results of inhibition by thiol-acting agents, such as Zn^{2+}, Cu^{2+}, Hg^{2+}, iodoacetic acid and activation by disulfide bridge-reducing agents, such as DTT and β-mercaptoethanol, clearly indicated the requirement of reduced form of Cys residue (s) for enzyme activity, also suggesting the classification of the enzyme into the cysteine phytase family. The resolution of those conflicting data was achieved after the genetic cloning of the enzyme in chapter V. The characteristics of the phytase, unveiled an enzyme that was able to hydrolyze phytic acid in lower temperatures, with broad substrate specificity. The enzyme also showed high stability for the long-term storage and ability to successfully treat grain-based fish feed. In an application point of view, those characteristics suggest that the enzyme have good properties for application as a liquid formulation for pretreatment of fish or animal feed.

In Chapter V, the phytase gene was identified by an approach using degenerate primers, based on the amino acid sequence of the N-terminal and one internal amino acid sequence of the enzyme. The ORF

containing the phytase gene was determined by inverse PCR and direct sequencing from the genome of strain a13. It was demonstrated that the mature region of the phytase ORF consisted of 1,287 bp, which encodes 428 amino acids. It was shown that the amino acid sequence of the enzyme contained motifs of RHGXRXP at the N-terminal region and HD at the C-terminal region, found in the HAP phytase family. Therefore, it was concluded that the phytase produced by strain a13 belongs to the family of HAP phytases, not belonging to the cysteine phytase family, as it was cogitated in chapter IV. This family embraces the majority of phytases, however, In chapter IV, strain a13 phytase had some intriguing differences in biochemical properties when compared to the majority of HAP phytases, such as the importance of reduced form of Cys residue (s) for the enzyme activity, which is not essential for HAP phytases activity mechanism. It was shown that the highest similarity of strain a13 phytase with other well biochemically-characterized phytases was of 48% identity with *Hafnia alvei* phytase. Genetic cloning also revealed an upstream sequence from the glutamic acid of the mature protein, which contained one ATG codon that was considered as the putative initiation codon, encoding a methionine 34 amino acids upstream from the glutamic acid. This N–terminal region showed similarity to a secretory signal sequence, because of the presence of three Arg residues followed by a stretch of hydrophobic residues. Also, the primary structure study revealed that the mature portion of this enzyme contained eight Cys residues, which all of them are conserved in *H. alvei* phytase. This result was very intriguing because the reported crystal structure of *H. alvei* phytase showed that the eight conserved Cys residues form four disulfide bridges with each other, generating conflicting result to the findings of strain a13 phytase. It was speculated that the Cys-223 of strain a13 phytase was responsible for the activity, since this residue is also conserved in *E. coli* phytase, and site specific mutagenesis reports of *E. coli* phytase showed that the mutant C200N had an increase in phytase activity, which is also corroborated by biochemical-characterization data of the enzyme produced by *E. coli*, that show activation by glutathione.

Finally, in Chapter VI, the overexpression of the cloned phytase gene was tested in *E. coli* using two different constructs: one containing the mature portion of the phytase gene, and the other containing the phytase gene from the putative initiation codon. It was demonstrated that the phytase activity was

detected only when the recombinant enzyme was produced within the upstream N-terminal region, which suggests the possibility that this region is playing an important role in enzyme maturation and promotion of proper folding. The construct containing the mature form of the phytase produced the protein into the insoluble fraction, which might constitute the principal cause for the lack of phytase activity. Although the construct containing the putative initiation codon showed phytase activity at the soluble fraction, the obtained activity was weak and SDS-PAGE data indicated that the protein production was rather low. Attempts to improve phytase activity with co-expression of chaperones or changes of the expression host didn't contribute to substantial improvements of phytase activity.

In a future prospect, it will be important to determine how many, and which phosphate groups the enzyme is able to hydrolyze from the molecule of phytic acid, in order to determine the class of the phytase produced by strain a13. If the phytase is found to manage only partial hydrolysis of the molecule of phytic acid, there is a high possibility that other enzyme(s), such as alkaline phosphatases, are also involved in the subsequent dephosphorylation of the lower-inositol phosphate products. Those subsequent dephosphorylation reactions would lead the formation of *myo*-inositol as a final product that was demonstrated to be utilized as a carbon source by strain a13. Therefore, it is also important to demonstrate that strain a13 is able to convert phytic acid into *myo*-inositol. The production of a phytase-knockout strain of *Burkholderia* sp. strain a13 would be essential to determine the importance of the phytase to this process, since it is expected that the phytase-knockout strain would grow up with *myo*-inositol as a carbon source, but lose its ability to hydrolyze phytic acid, becoming unable to grow in a minimal medium containing phytic acid as the sole carbon source. More detailed studies at the genomic and protein level for the catabolism of *myo*-inositol might be of great value to determine this pathway in strain a13 and perhaps, it would clarify the presence of similar pathways in other bacteria of the *Burkholderia* genus. I speculate that the ability to use phytic acid as a carbon source might confer strain a13, survival advantages on an environment lacking easily available carbon compounds. Phytic acid is reported to be present in high amount in soils and the ability to use it as carbon and energy source could help *Burkholderia* sp. strain a13 to thrive in this kind of environment. Therefore, the knowledge obtained by

this research work, might also contribute to future studies analyzing the interactions between the bacteria and the environment. Strain a13 phytase showed biochemical characteristics suggesting that the enzyme have good properties for application as a liquid formulation for pretreatment of fish or animal feed. In the future, further analysis of the enzyme stability to proteolysis together with experiments to determine the ability to release inorganic phosphate from phytic acid inside the gastro-intestinal tract of fishes might support the application of this enzyme as a feed supplement for aquaculture. However, lower production of the phytase by strain a13 might constitute a barrier for applications. In this way, further developments for improvement of enzyme production are essential for promoting the commercial application of this novel phytase. Primary structure analysis and overexpression of the phytase gene in *E. coli* indicated that the mature phytase sequence contained an upstream sequence that is important to enzyme maturation. This sequence is possibly involved in the exportation of the enzyme to the periplasmic space of strain a13. In the future, development of an expression system in *Burkholderia* sp. strain a13 might be important to determine the function of this upstream sequence precisely, by the use of a colicin V reporter assay.

The results thus obtained by this research work about the phytase produced by *Burkholderia* sp. strain a13, isolated from the aquatic environment were compiled and generated a scientific publication in The Journal of General and Applied Microbiology (Graminho et al., in press), that I hope it will contribute to the knowledge of phytases and also encourage its application.

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Tsukuba, January 2015

Eduardo Rezende Graminho

"Nobody can go back and start a new beginning,

but anyone can start today and make a new ending."

Chico Xavier 1910 - 2002

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