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"BIOTECHNOLOGICAL AGRONOMIC STRATEGIES FOR IMPROVING ORGANIC P CYCLING IN ANDISOLS USING CATTLE MANURE TREATED WITH PHYTASES STABILIZED IN NANOCLAYS"

DANIEL MENEZES BLACKBURN

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Ph.D. Dissertation

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Esta tesis fue realizada bajo la supervisión de la Directora de Tesis, Dra. MARIA DE LA LUZ MORA GIL (UFRO) y por los Co-Directores de Tesis, Dr. MILKO JORQUERA TAPIA (UFRO) y Dra. MARIA A. RAO (UNINA –It), y ha sido aprobada por los miembros de la comisión examinadora.

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"To see a world in a grain of sand,
and a heaven in a wild flower,
hold infinity in the palm of your hand,
and eternity in an hour"
William Blake - Auguries of Innocence

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Abstract

Cattle manures have high phytate (inositol hexakisphosphate) content and are a significant source of phytate input to soil systems. These manures contain undigested phytate in high amounts, corresponding from 8.6 to 54% of its total organic phosphorus (P). Phytate added to soils is highly unavailable to plants and microorganisms, and accumulate mainly due to its interaction with soil particles. This soil phytate is very recalcitrant, and is the most unavailable of all soil P compounds. On the other hand, other authors have pointed out that phytase incubation with cattle manure can increase inorganic P concentration up to 32,7% of total P. The use of unspecific phytase allows the hydrolysis of phytate fraction as well as other monoester P, which increase the effectiveness of the biotechnology proposed herein. Bacteria phytase have the highest pH optima, which may increase P hydrolysis under alkaline conditions. Our working group has isolated phytate utilizing bacteria (PUB) that can be promising for use in manure P-phytate recovery treatments. In this work, we have assessed the feasibility of the phytase treatment of cattle manure and Andisols with phytases immobilized in allophanic nanoclays and phytase producing bacteria, and the possible use of these treated manure as improved P fertilizers in volcanic soil crops.

We studied the phytase activity of twelve bacteria strains in order to evaluate their potential use in manure P-phytate hydrolysis biotechnological applications. Except for *Ochrobactrum* sp. C95 and *Bacillus* sp. N1-19NA, all the studied bacteria strains showed a cell-associated phytase activity. Most of the identified phytases were active at acid pHs, and only *Bacillus* sp. MQH-19 and Bacillus sp. MQH-15 showed alkaline phytase activity. Among the studied native bacteria strains, *Bacillus* sp. MQH-19 was the only one presenting extracellular alkaline phytase activity, with capacity of increasing extracellular Pi when grown in liquid PSM medium. This phytase

producing bacteria strain was selected to be tested in manure amendment treatments for increasing the availability of organic P pool to plants.

The stabilization of the activity of two commercial microbial phytases (*Aspergillus niger* and *Escherichia coli*) after immobilization on nanoclays was studied to establish optimal conditions for their immobilization. Synthetic allophane, synthetic iron-coated allophanes and natural montmorillonite were chosen as solid supports for phytase immobilization. Phytase immobilization patterns at different pH values were strongly dependent on both enzyme and support characteristics. After immobilization, the residual activity of both phytases was higher under acidic conditions than under alkaline conditions. Immobilization of phytases increased their thermal stability and improved resistance to proteolysis, particularly on iron-coated allophane (6% iron oxide), which showed activation energy (Ea) and activation enthalpy ($\Delta H^{\#}$) similar to free enzymes.

Two Andisols and two stabilized cattle manures were selected for incubations with phytases due to their contrasting Olsen P. A series of liquid (controlled pH and temperature) and solid incubations were performed to assess the phytase labile P under optimal conditions and conditions similar to natural ones, respectively. The resulting treated waste, soil and manure-soil mixture from solid incubations were used for performing a greenhouse plant growth and P availability study. Additionally, a bacterium producing an alkaline phytase (*Bacillus* sp. MQH-19) was used as an inoculant for the treatment of a cattle waste. The treatment of an Andisol and cattle manure with phytase immobilized in allophane coated with 6% of iron oxide resulted in a significant increase in inorganic P. To the case of direct treatment of manure without pH adjustment, inoculation with PUB was more effective in increasing inorganic P. Finally, the phytase treatment of cattle manure affected wheat growth (dry weight), shoot P concentration and

content positively, when grown in an Andisol fertilized with this treated manure. However, bacteria inoculation caused no significant impact on plant growth and P nutrition under the same conditions.

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CHAPTER 1

INTRODUCTION AND OBJECTIVES

1. INTRODUCTION AND OBJECTIVES

1.1. Introduction

In many soils, organic phosphorus (Po) pool is very abundant, usually accounting from 40 to 80% of total phosphorus (P) (Dalal, 1977; Tsai & Rosseto, 1992). In soils of southern Chile, this content averages over 60% of total P, corresponding to 1,618 mg kg^{-1} in cultivated soils and 1,147 mg kg^{-1} in uncultivated soils. Among the Po forms, phytate is the most abundant P form in soils (Borie & Rubio, 2003; Tsai & Rosseto, 1992; Turner et al., 2002). Although soil phytate pool construction can occur in natural environments, its accumulation is significantly enhanced by both inorganic P fertilization (George et al., 2007a) and organic fertilization with phytate rich organic wastes (Dao, 2004). Technologies and practices directed to access this important P pool for crop nutrition are of great interest in modern agriculture, since phosphate fertilizers are produced from a non-renewable and limited mineral resource (Abelson, 1999; Richardson et al., 2007).

The Po pool is only available to plants after phosphate is released to soil solution by phosphatases (Richardson et al., 2005; Turner et al., 2002). These enzymes are responsible for the release of orthophosphate from organic phosphate (Stevenson & Cole, 1999). Among all phosphatase types, phytases are of special interest because they are capable of initiating the cleavage of phosphate groups from the phytate molecule (Mullaney & Ullah, 2003) making phosphate bioavailable (George et al., 2007b). Significant extracellular phytase activity is not naturally produced by plants, therefore, phytase activity in soils must be attributed mainly to phytase producing microorganisms (Richardson, 2001; Richardson et al., 2001). Given that phytate is the most abundant and recalcitrant form of organic P in soils and animal organic wastes used as soil

amendments, understanding of the catalytic behaviour of phytases in these complex media is of strategic importance for developing biotechnologies to improve plant P nutrition.

1.2. Hypotheses

i) The immobilization of phytase enzymes from *Aspergillus niger* and *Escherichia coli* in nanoclays will enhance organic P hydrolysis of cattle manure and Andisols, due to its effect on enzyme protection against biological and enzymatic degradation;

ii) Total organic P hydrolysis of manure and Andisols is higher under phytase enzyme treatment in comparison with β-propeller phytase producing bacteria inoculation treatments.

iii) The use of phytase treated cattle manure as a biofertilizer will increase P uptake and plant growth in P deficient Andisols.

1.3. General objective

To evaluate the phytase treatment of cattle manure as potential biofertilizer for improving phosphorus plant nutrition in Andisols.

1.4. Specific objectives

SO1: To characterize phytase activities of β-propeller phytase producing bacteria isolated in previous and select the most appropriate for the focused biotechnological application.

SO2: To design support-phytase system and test the effect of enzyme immobilization on its residual activity, stability and catalytic properties.

SO3: To test the effect of immobilized phytase, free phytase and β-propeller phytase producing bacteria inoculation on organic P hydrolysis of cattle manure under laboratory conditions.

SO4: To test the effect of the treated cattle manure on plant growth and P acquisition of *Triticum aestivum*, under greenhouse experiment conditions.

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CHAPTER 2

THEORETICAL BACKGROUND - PHYTASES AND PHYTASE-LABILE ORGANIC PHOSPHORUS IN MANURES AND SOILS

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2. THEORETICAL BACKGROUND – PHYTASES AND PHYTASE-LABILE ORGANIC PHOSPHORUS IN MANURES AND SOILS

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2.1 Abstract

Organic phosphorus (Po) hydrolysis by microbial phytases has extensively been considered in diverse biotechnological applications, including environmental protection, agricultural, and animal and human nutrition. Here, we have reviewed the available information on the content of phytase-labile Po in manures and soils, as well as the environmental factors and enzyme properties affecting catalytic behaviour of phytases in these environments. In addition, we have critically analyzed the present and possible future biotechnological approaches for using phytases to access phytate Po pool present in soils and manures for plant nutrition, with the concomitant reduction of runoff P in the environment. This paper also summarizes the results of previous investigations on animal organic wastes and soil incubations with phytases, and the characteristics of known phytases that may influence their behaviour in these environments. The adopted approach will help to select enzymes and microorganisms for future site-specific biotechnological applications.

2.2 Introdution -Phytate pool and phytase labile organic p in manures and soil

Inositol phosphates are the family of phosphoric esters of inositol (hexahydroxycyclohexane) (Turner et al., 2002b). There are 63 different possible inositol phosphates (with a constant orientation of the phosphate residues) or more if considering possible pyrophosphate attached moieties (Irvine & Schell, 2001). However, a more limited number of these compounds are actually found in nature. The number of phosphate groups bound to inositol ring can vary up to 6, and IP6 is the most abundant form in soils accounting for more than 80% of total inositol phosphates (Turner et al., 2002b). Due to its six orthophosphate moieties phytate (see below), it is highly a reactive molecule, with molecular weight of 660 g/mol and 12 hydrogen donors in its structure (PubChem, 2009).

Phosphorylated inositols of higher order can occur in cells by formation of pyrophosphate residues (Irvine & Schell, 2001; Lee et al., 2008) and may eventually be released into organic wastes and soils. Four stereoisomers are known to occur in soils, which in order of abundance are: *myo, scyllo*, d-*chiro* and *neo* inositol phosphate. The *myo* stereoisomer of inositol phosphates (phytates) are the most abundant form, representing up to 90% of total soil IP6 (Turner et al., 2002b). For more detailed information of the molecular structure of inositol phosphates see Irvine and Schell (2001) and Turner et al. (2002b). The term phytate is often found in reference to all metal ion derivatives of *myo* inositol hexakisphosphates, and it is commonly abbreviated as IP6, InsP6, or IHP.

Phytate can be synthesized by plants and microorganisms (Lee et al., 2008); however, plants are the main source of phytate in soil environments (Turner et al., 2002b). Indeed, plants accumulate phytate in grains/seeds, in concentrations up to 80% of the total seed P (Bieleski, 1973; Bohn et al., 2008; Lott et al., 2001), as P reserve for seedling growth (Bieleski, 1973; Bohn et al., 2008; Lott et al., 2001), This plant phytate can be found in other tissues in lower concentrations, participating in molecular signalling and other biochemical reactions (Brinch-

Pedersen et al., 2002; Turner et al., 2002b). Phytate may also have an antioxidant role in plants in preventing iron-driven lipid peroxidation through the chelation of Fe^{2+} and slowing down browning and putrefaction processes by inhibiting polyphenol oxidase (Graf et al., 1987). P fixing in plant phytate reaches nearly 51 million metric tons annually, which corresponds to 65% of the elemental P consumption in fertilizers (Lott et al., 2001). Thus, plant deposition is considered an important input to soil phytate pool (Turner et al., 2002b).

2.3 Hydrolysable phytate content in animal wastes

Data of phytate content in manures must be considered with caution, since strong variations are observed among different methodologies and extraction methods (He et al., 2007; He et al., 2004a). Methods using enzymatic hydrolysis seem to be very useful for the determination of the bioavailability of different organic P fractions. The use of the enzyme phytase to assess organic hydrolysable P was first proposed for soil solution samples (Pant et al., 1994; Shand & Smith, 1997). Zhongqi He and Thanh Dao, both from USDA-ARS, have proposed very interesting methodologies for assessing hydrolysable monoester and phytate-like P in animal manures and slurries (Dao, 2004b; Dao, 2003; Dao et al., 2006; Dao & Hoang, 2008; He et al., 2007; He et al., 2009a; He & Honeycutt, 2001; He et al., 2003). These methodologies include use of organic polydentate chelating agents (like EDTA), successive extractions and use of different enzymes for optimizing the determination procedure and assessing different bioactive organic P forms. Enzymatic methodologies can also be used for evaluating hydrolysable organic P compounds in soils (Dao, 2004a; He et al., 2004a; He et al., 2004b), feeds (Megazyme, 2007) and natural waters (McKelvie et al., 1995). Although enzymatic methodologies are very promising, some further standardization may still be required before they can be applied in large scale for marketable applications.

Organic P, hydrolysable by phytases, or phytase labile organic phosphorus ($P_{Phy\text{-}lab}$) is inferred by the increase of inorganic P after the incubation of samples with phytases. These methodologies often use phytases with broad substrate specificity, and may be measuring hydrolysis of other monoester P different from phytate. In some of the reviewed literature, phytases and non-phytase phosphatase enzymes are combined to assess hydrolysable phytate-like organic P ($P_{IP6\text{-}like}$). The $P_{IP6\text{-}like}$ is defined as the difference between Pi released through the incubations of samples with phytases and non-phytase phosphatases, and the Pi released by non-phytase phosphatases alone. Hydrolysable $P_{IP6\text{-}like}$ and $P_{Phy\text{-}lab}$ are resumed in Table 2.1.

Monogastric animals like poultry, swine and fish are incapable of utilizing phosphate from the phytate present in feeds, and even ruminants are not capable of mineralizing the entire phytate P, especially in high phytate grain-based diets (Brinch-Pedersen et al., 2002; Godoy et al., 2005; Godoy & Meschy, 2001). These high phytate animal wastes can also be an important source of phytate input in some agro-ecosystems (Fuentes et al., 2006; Lott et al., 2001).

A fraction of the phytate in ruminant diets is converted to inorganic P in the rumen; a reduction in phytate percentage from 32% in feed to 18% of total P in dairy faeces was verified by Toor et al (2005). Although phytases from cattle intestine mucosa and from rumen microflora are capable of hydrolyzing phytate, a significant amount of phytate passes undigested (Dao, 2007). This phytate persistence in ruminant manures may be due to precipitation mechanisms of IP6 with counterions (Dao, 2003), rapid rate of feed passage through digestive trait and high phytate concentration in grain-based diets (Dao, 2007). Lack of effective phytate hydrolysis in animal digestive trait may also induce directly animal nutritional disorders such as inhibition of protein digestion and anemia, due to the complexation of proteins and iron by IP6 molecules (Cheryan & Rackis, 1980; Erdman, 1979).

Table 2.1. Phytase hydrolysable P and phytate like P in different animal wastes and under different experimental conditions.

	Phytase hydrolysable P			Phytate like P			Experimental conditions			
	$\%P_{tot}$	$\%P_o$	mgP kg^{-1}	$\%P_{tot}$	$\%P_o$	mgP kg^{-1}	N	Enzyme	Extraction	
Dairy cattle manure										
(He & Honeycutt, 2001)	9.4	12	168.5	6.7	8.6	121	1	A, b, c, d	Sequential H_2O, $NaHCO_3$, NaOH	
(Dao, 2003)	-	-	698.3	-	-	-	9	A	H2O	
(He et al., 2003)	5.2	25	281.0	-	-	-	3	A, b, c, d	Sequential H_2O, $NaHCO_3$, NaOH	i
	2.6	8.7	140.0	-	-	-	3			ii
(He et al., 2006)	23.5	60	185.0	10.3	26	81	10	B, e	Sequential H_2O, $NaHCO_3$, NaOH	
(Dao et al., 2006)	32.2	-	3,811.2	-	-	-	107	A	EDTA	
(He et al., 2007)	18.8	75	1,286.0	10	39	678	2	A, b, c, d	NaOH-EDTA	iv
	6.7	95	472.0	3.8	54	268	2			v
(He et al., 2009a)	9.7	-	417.0	6.8	-	291	3	A, e	H_2O	
	19.7	-	1,311.0	11.5	-	767	3		100mM NaOAc pH 5.0	
	10.9	-	708.0	6	-	389	3		100mM NaOAc, 50mM EDTA	
	27.3	-	1,436.0	18.4	-	965	3		1.0M HCl	
	18.6	-	1,245.0	10.8	-	723	3		0.25M NaOH, 50mM EDTA	
	25.8	-	1,629.0	13.6	-	858	3		0.50M NaOH, 50mM EDTA	
Mean	**16.7**	**53**	**1,047.70**	**10.1**	**40**	**558**				
Swine manure										
(He & Honeycutt, 2001)	33.6	38	705.0	16.7	19	350	2	A, b, c, d	Sequential H_2O, $NaHCO_3$, NaOH	
(He et al., 2003)	10.1	76	486.0	-	-	-	3	A, b, c, d	Sequential H_2O, $NaHCO_3$, NaOH	iii
	5.8	20	277.0	-	-	-	3			iii
(He et al., 2004b)	13.2	23	360.0	-	-	-	1	B, e	Sequential H_2O, $NaHCO_3$, NaOH	
Mean	**15.7**	**39**	**457.0**	**16.7**	**19**	**350**				
Poultry manure										
(He et al., 2007)	16.3	84	2,198.0	15.3	79	2060	3	A, b, c, d	NaOH-EDTA	iv
	17.5	83	2,171.0	14.5	69	1793	3			v
(He et al., 2009a)	4.5	-	218.0	4.5	-	218	3	A,e	H_2O	
	7.5	-	573.0	6	-	458	3		100mM NaOAc pH 5.0	
	16.2	-	2,153.0	15.8	-	2092	3		100mM NaOAc, 50mM EDTA	
	29	-	4,209.0	24	-	3484	3		1.0M HCl	
	22.1	-	2,970.0	15.4	-	2075	3		0.25M NaOH, 50mM EDTA	
	26	-	3,727.0	15.4	-	2205	3		0.50M NaOH, 50mM EDTA	
Mean	**17.4**	**84**	**2,277.40**	**13.9**	**74**	**1798**				
Poultry litter										
(He et al., 2009a)	5.3	-	170	2.2	-	70	3	A, e	H_2O	
	63.3	-	8,258.0	61.5	-	8028	3		100mM NaOAc pH 5.0	
	65.5	-	10,140.0	63.3	-	9800	3		100mM NaOAc, 50mM EDTA	
	64.2	-	9,710.0	60.3	-	9120	3		1.0M HCl	
	70.7	-	11,085.0	63.5	-	9963	3		0.25M NaOH, 50mM EDTA	
	70.7	-	11,091.0	65	-	10190	3		0.50M NaOH, 50mM EDTA	
Mean	**56.6**		**8,409.0**	**52.6**		**7862**				

* Some of the data were not explicit in the original papers and were deduced (e.g. from the % of total P)

(a) Aspergillus ficuum phytase, (b) wheat phytase, (c) wheat germ acid phosphatase, (d) Bovine intestinal mucosa alkaline phosphatase (e) Potato acid phosphatase, (i) Fresh, (ii) after one year storage 22ºC, (iii) after one year storage 4ºC (iv) wet (v) dry.

Dephosphorylation of phytate in animal manures is increasingly inhibited by increasing counterion concentration, as this polyanionic compound has a high affinity for cations (Fe^{3+}>Al^{3+}>Ca^{2+}) forming insoluble precipitates (Dao, 2003). Some cations such as calcium, iron and magnesium are supplemented in feeds and may complex phytate protecting it against dephosphorylation (Dao, 2007; Dao, 2003).

A $P_{Phy\text{-}lab}$ fraction is native to dairy manure water suspensions, and it is proportional to its dry weight, averaging 68,2 mg P kg solid fraction (Dao, 2003). A big part of $P_{Phy\text{-}lab}$ is located in the water soluble fraction of swine and dairy cattle manure (He & Honeycutt, 2001). Storage in field facilities is also associated with a decrease in the total $P_{Phy\text{-}lab}$ fraction of manures; He et al (2003) reported a reduction from 486 to 277 mg P kg^{-1} of swine manure, and from 281 to 140 mg kg^{-1} of dairy manure during one year storage. During storage, organic P increased and $P_{Phy\text{-}lab}$ decreased (He et al., 2003), indicating a possible progressive reduction of the availability of monoester P in manures during storage .

Drying also alters phytate-phytase interaction in manures, reducing $P_{IP6\text{-}like}$ fractions strongly in dairy manures and slightly in poultry manures (He et al., 2007). Differently from storage effect, drying treatment reduces organic P and seems to induce hydrolysis of monoester P in dairy manure, but not in swine manure. Although $P_{IP6\text{-}like}$ was reduced by drying, phytate determided by solution P-NMR did not confirm this tendency, suggesting that this verified hydrolysis was from another monoester P different from phytate (He et al., 2007).

The $P_{Phy\text{-}lab}$ was shown to be correlated with data from solution phosphorus-31 nuclear magnetic resonance spectroscopy (P-NMR); therefore, enzymatic methods can possibly become a less expensive and time consuming protocol for organic P fractionation. On the other hand, these methods are not necessarily correlated for all cases, and all factors affecting phytate bioavailability are expected to be a source of variation to the resulting data from enzymatic hydrolysis methods.

Data from Table 2.1 may be insufficient for adequate comparison of different organic wastes due to the small amount of studies and great variation between data obtained from the applied methods. The $P_{IP6\text{-}like}$ and $P_{Phy\text{-}lab}$ are in higher concentrations in poultry wastes than in swine and bovine wastes. Nevertheless, among animal organic wastes, dairy cattle has the highest concentrations of this monoester hydrolysable P in water extracts, indicating that organic P may be more labile in dairy wastes and more recalcitrant in poultry wastes.

The importance of phytate in bovine organic wastes is the enormous amount that is generated daily worldwide due to the equally enormous population of these animals: there are around 1.3 billion cattle, 900 million pigs, and 12.2 billion poultry in the world (FAO, 2006). Rough calculations using data of Table 2.1 indicate that more than four thousand tons of hydrolysable phytate is excreted every day by the world cattle population, considering an organic waste production of 6 kg dry weight (DW) day^{-1} cow^{-1}. Using the same estimation procedure, total poultry population would be responsible for the excretion of approximate 440 tons and pigs for only 95 tons of hydrolysable phytate daily, considering an average daily excretion of 20g and 300g for poultry and swine on dry matter basis, respectively.

2.4 Hydrolysable phytate content in soil

Composted animal manures and manure slurries are often used as fertilizers for crops and pastures. A large P fraction of the applied manures corresponds to unavailable organic P compounds such as phytate. Consequently, these fertilization practices are often a P-over application on total P basis (Sharpley & Moyer, 2000). This may lead to an increase of inorganic and hydrolysable organic P losses in runoff sediments (Green et al., 2007) and also to organic P accumulation in soils (Dao, 2004a). Seven repeated 30 kg P ha^{-1} manure applications resulted in soil storage of complexed $P_{Phy\text{-}lab}$ of approximately 80 mg P kg^{-1} soil (Dao, 2004a).

The six orthophosphate moieties and 12 coordinated ligands present in the phytate structure allow it to complex cations and adsorb to the surface of colloids with several phosphate groups simultaneously (Celi et al., 1999; Martin et al., 2004; Turner et al., 2002a). Among all soil P compounds, phytate has the lowest bioavailability (Shang et al., 1996) and the strongest affinity to soil particles (Berg & Joern, 2006; Celi & Barberis, 2005; Shang et al., 1996; Shang et al., 1992).

The accumulation of inositol phosphates in soils can be partially explained by the great affinity of these compounds for soil particles, resulting in poor bioavailability due to their low desorption (Giaveno et al., 2010; Martin et al., 2004; Shang et al., 1996). Inositol phosphate contribution to plant P nutrition is a function of P-fixing capacity of the growth media (Richardson et al., 2005). Phytate availability to *Lupinus* sp. was strongly reduced when cultivated in soil in comparison with sand substrate (Adams & Pate, 1992).

The strong adsorption of this high charge density molecule is a result of simultaneous binding of multiple phosphate groups by ligand exchange reactions, forming inner-sphere complexes (Berry et al., 2007; Celi et al., 1999; Guan et al., 2006; Shang et al., 1996). Strong phytate adsorption has been studied and demonstrated with calcite (Celi et al., 2000), illite (Anderson & Arlidge, 2006; Celi et al., 1999), kaolinite (Anderson & Arlidge, 2006; Celi et al., 1999), montrnorillonite (Anderson & Arlidge, 2006), goethite (Celi et al., 1999; Celi et al., 2001; Martin et al., 2004) and aluminum hydroxides (Guan et al., 2006), especially at low pHs. Phytate adsorption to soil minerals also produces particles dispersion and a increase of the surface negative charge (Celi et al., 1999). This phytate adsorption/desorption to soil particles is controlled by soil pH, ligand competition and P saturation (Martin et al., 2004).

Under acidic conditions phytate can precipitate as insoluble salts of Fe and Al, affecting its availability (Jackman & Black, 1951). It has been demonstrated that phytases are only capable of hydrolyzing phosphate groups from precipitated Ca^{2+}-, Mg^{2+}-, and Mn^{2+}- phytate salts

(Tang et al., 2006), which are expected to naturally occur in alkaline soils (Turner et al., 2002b). This may cause higher phytate accumulation in acid soils.

Adsorption and precipitation reactions are the main factors affecting phytate availability in soils (Richardson et al., 2005). These factors interfere in the interaction between phytate and phytase slowing down or even impeding hydrolysis of phosphate from these substrates (Tang et al., 2006).

As soil phytate is only available to plants and microorganisms after its mineralization, all factors that affect phytate-phytase interaction in soils are determinant to the availability of this pool (George et al., 2005a; Richardson et al., 2005; Richardson et al., 2000). Both factors, substrate availability and presence of phytase enzymes, are determinants in the acquisition of phytate P (Hayes et al., 2000b).

Richardson et al. (2000) found that plants of *Triticum aestivum* L. grown under sterile conditions were unable to use phytate as P source. Although some secreted acid phosphatase activity was observed, no phytase activity was secreted (Richardson et al., 2000). Similar results were reported with several grasses and clovers (Hayes et al., 2000b). Low plant phytase activity is a critical factor limiting phytate P use under sterile conditions (Adams & Pate, 1992). On the other hand, other studies under non sterile conditions have demonstrated inositol phosphate use(Adams & Pate, 1992; Perez Corona et al., 1996). Soil microorganisms producing phytase in the rhizosphere seem to be the link to the observed phytate utilization by plants under these conditions (Jorquera et al., 2008a; Richardson, 2001). This hypothesis is supported by the increase of P acquisition in inoculation experiments (Richardson, 2001; Richardson et al., 2001a; Richardson et al., 2000; Tarafdar & Marschner, 1995) and microbial enzyme addition experiments (Findenegg & Nelemans, 1993; Hayes et al., 2000b). Nevertheless, other experiments were not capable of demonstrating such results, which brings

a question mark to the function and responsibility of extracellular soil phytase in improving P nutrition of plants (Richardson et al., 2005).

Richardson et al (2005) underlined the need of a better understanding of the interaction between phytase and inositol phosphates in soils in order to improve P nutrition to more sustainable cropping. Turner et al. (2002b) reviewed the role and cycle of inositol phosphates in the environment in great detail; a conceptual model of the phytate terrestrial cycle (Figure 2.1) has been adapted from these findings.

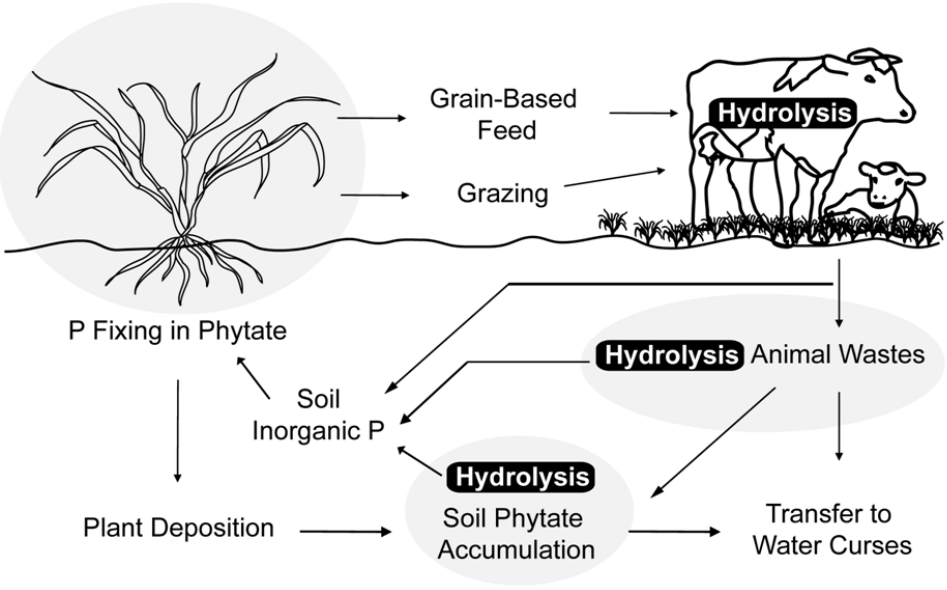

Figure 2.1. Conceptual model of phytate terrestrial cycle [adapted from Turner et al. (2002b)].

Phytate content in soils is very variable; however, it is frequently found as the major fraction of soil organic P, accounting for up to 80% of total organic P (Turner et al., 2002b). In Chilean Andisols, phytate concentration ranges between 42 and 67% of organic P, which corresponds to an average of 674 mg kg^{-1} (Borie & Rubio, 2003; Borie et al., 1989). The soil $P_{Phy\text{-}lab}$ content data vary strogly averaging about 20% of total soil P (Table 2.2), and to soil humic fractions averages about 50% of total P (He et al., 2009b). As it has been already

established, data of soil phytate content must always be taken with caution due to overestimations and underestimations associated with the used analytical methods (Turner et al., 2002b).

George et al. (2007a) showed that inorganic P fertilization led to a highly significant increase in $P_{Phy\text{-}lab}$, associated to a continuous build-up of soil Pi and Po. Even in soils with more than a decade without inorganic P fertilization, these pools were significantly higher in comparison to unfertilized soils (George et al., 2007a). This indicates that organic P accumulation in some cases may not only be associated to external input of unavailable P compounds like phytate, but also to a local immobilization of inorganic P added as P fertilizers.

Bioactive $P_{Phy\text{-}lab}$ is associated with soil aggregate size being greater for macro-aggregates than for micro-aggregates; a protection against soil microbial mediated hydrolysis in bigger aggregates may take place since P release during incubation is slower to this fraction (Green et al., 2006).

Extraction method is found to be a source of variation in the determination of phytase hydrolysable P. Although data vary greatly with different extraction methods, relative soil $P_{Phy\text{-}lab}$ seems to average around 40% of extracted organic P, ranging from almost 0 to nearly 100 % (Bunemann, 2008).

Total $P_{Phy\text{-}lab}$ pool was not correlated with growth and P acquisition of a transgenic *Trifolium subterraneum* L., expressing extracellular *Aspergillus niger* phytase (George et al., 2007a). In cases where phytase activity is present in the rhizosphere, water extractable $P_{Phy\text{-}lab}$ may be the phytate pool mostly available to plants, rather than total hydrolysable P (extracted with EDTA, NaOH, or other). At the same time, water extractable $P_{Phy\text{-}lab}$ is the smallest organic P pool in soils (Table 2.2).

Table 2.2 A. Phytase-hydrolysable P and phytate-like P in different soils and under different experimental conditions.

		Phytase hydrolysable P			Phytate like P			Experimental conditions	
Source	**Soil**	**% P_{tot}**	**% P_o**	**Mg P kg^{-1}**	**% P_{tot}**	**% P_o**	**mg P kg^{-1}**	**Enzyme**	**Extraction**
(Otani & Ae, 1999)	Andosols	-	35	-	-	22	-	phytase	Citrate
(Hayes et al., 2000a)	Grey-brown to yellow podzolic (d)	-	12.4	0.3	-	-	-	*Aspergillus niger* phytase	H2O
	Grey-brown to yellow podzolic	-	80.4	6.2	-	-	-		50 mM Citric acid pH 2.3
	Grey-brown to yellow podzolic	-	8.9	0.1	-	-	-		50 mM HCl pH 1.45
	Grey-brown to yellow podzolic	-	8.1	0.2	-	-	-		5 mM HCl pH 2.3
(Hens & Merckx, 2001)	Anthri-endogleyic Podzol (Carbic) (a)	-	14.9	-	-	-	-	Wheat phytase	Solution centrifugation
	Carbi-anthric Podzol (Gleyic) (b)	-	20.7	-	-	-	-		
	Carbi-anthric Podzol (c)	-	50.9	-	-	-	-		
(Turner et al., 2002a)	Sandy silt loam Haplustult	0.4	1.1	0.2	0.4	1.1	0.2	*Aspergillus ficuum* phytase, *E. coli* alkaline phosphatase	H2O
	Silty clay loam Haplustalf	0.9	2.3	0.4	0.8	2.3	0.4		
	Clay loam Haplustalf	2.1	2.8	0.4	1.8	2.3	0.3		
	Clay Haplustox	0.6	1	0.2	0.4	0.6	0.1		
	Clay Haplustox	2.1	2.4	0.4	2	2.3	0.4		
(Turner et al., 2003) (f)	Coarse-loamy, mixed, superactive, mesic Xeric Haplodurids	10	81	1.4	-	-	-	*Aspergillus ficuum* phytase	Bicarbonate
	Coarse-silty, mixed, superactive, mesic Xeric Haplocambids	9.8	50	1.9	-	-	-		
	Fine-loamy, mixed, superactive, thermic Aridic Paleustalfs	8	66	2.7	-	-	-		
	Fine-silty, mixed, superactive, mesic Xeric Calciargids	13	53	2.3	-	-	-		
	Coarse-silty, mixed, superactive, mesic Durinodic Xeric Haplocalcids (e)	3.4	81	3.8	-	-	-		
	Coarse-silty, mixed, superactive, mesic Durinodic Xeric Haplocalcids	5	87	2.9	-	-	-		
	Coarse-silty, carbonatic, mesic Typic Haploxerolls	21.8	48	2.6	-	-	-		
	Loamy, mixed, superactive, frigid Pachic Ultic Argixerolls	16.2	44	6.5	-	-	-		
	Fine-silty, mixed, superactive, mesic Pachic Ultic Haploxerolls	15.8	37	8.4	-	-	-		
	Fine-loamy over sandy or sandy-skeletal, mixed, superactive, frigid Fluventic Haploxerolls	18	62	6.9	-	-	-		
	Fine, smectitic, frigid Typic Hapluderts	14.4	47	4.6	-	-	-		

a- pasture; b- arable; c- coniferous forest; d- P fertilized; e- Amended with manure; f- semiarid soils; g- runoff

Continue next page.

Table 2.2 B. Continues Table 2.2 A.

		Phytase hydrolysable P			Phytate like P			Experimental conditions	
Source	**Soil**	**% P_{tot}**	**% P_o**	**Mg P kg^{-1}**	**% P_{tot}**	**% P_o**	**mg P kg^{-1}**	**Enzyme**	**Extraction**
(Dao, 2004a)	fine, kaolinitic, mesic Typic Paleudult	2.3	-	16.6	-	-	-	*Aspergillus ficuum* phytase	EDTA
	coarse-loamy, mixed, semiactive, mesic Typic Hapludult	6.4	-	46.2	-	-	-		
	fine, kaolinitic, mesic Typic Paleudult (e)	32	-	231	-	-	-		
	coarse-loamy, mixed, semiactive, mesic Typic Hapludult (e)	40.8	-	214.6	-	-	-		
	fine, kaolinitic, mesic Typic Paleudults (h)	43.9	-	81.1	-	-	-		
	fine, kaolinitic, mesic Typic Paleudults (i)	42.1	-	72.6	-	-	-		
(He et al., 2004a)	coarse-loamy,mixed, frigid, Typic Haplorthod (a)	14.8	38.3	120.4	13.5	34.9	109.6	Acid phosphatases from wheat germ and potato, phytase from wheat	Secuential H2O, NaHCO3, NaOH
	fine-loamy, isotic, frigid Typic Haplorthods (e)	5.9	27.5	120.3	5.3	24.7	108.1		
	fine-loamy, isotic, frigid Typic Haplorthods (b)	11.2	48.8	167.7	9.3	40.5	139.2		
(Green et al., 2006)	fine, kaolinitic, mesic Typic Paleudults (g)	43.5	-	96.1	-	-	-	*Aspergillus ficuum* phytase	EDTA
(Green et al., 2007)	fine-silty, mixed, semiactive, mesic Typic Hapludults (a,g)	54.2	-	30.3	-	-	-	*Aspergillus ficuum* phytase	EDTA
	fine-silty, mixed, semiactive, mesic Typic Hapludults (a,e,g)	54.7	-	33.6	-	-	-		
	fine-silty, mixed, semiactive, mesic Typic Hapludults (b,g)	74.6	-	17.3	-	-	-		
	fine-silty, mixed, semiactive, mesic Typic Hapludults (b,e,g)	55.2	-	26.5	-	-	-		
(George et al., 2007a)	Acidic Alfisol	10.3	12	15	-	-	-	*Aspergillus* phytase	Secuential H2O, NaHCO3, NaOH
	Acidic Alfisol (d)	15.7	28	33	-	-	-		
	Acidic Alfisol (d)	16.4	33	37	-	-	-		
	Mean	**20.2**	**36.2**	**38.2**	**4.2**	**14.5**	**44.8**		

a- pasture; b- arable; c- coniferous forest; d- P fertilized; e- Amended with manure; f- semiarid soils; g- runoff

2.5. Microbial phytases in manures and soils

2.5.1 *Phytase definition and classification*

Phytase enzymes are a special group of phosphatase enzymes that are capable of initiating the stepwise dephosphorylation of phytate (Hill & Richardson, 2007; Mullaney & Ullah, 2003). Phytase enzymes present in soil and organic wastes have many biological sources, mainly from filamentous fungi, bacteria and yeasts. Since phytases are defined by their activity toward IP6 substrate and not by their *in vivo* function, some authors prefer to use the term phytate-degrading enzyme rather than phytase.

The Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) divides phytase by stereospecificity of phytate hydrolysis: 3/1-phytase (EC 3.1.3.8); 4/6-phytase (EC 3.1.3.26); 5-phytase (EC 3.1.3.72). Phytases can also be grouped by their source (plant, bacteria, fungi and yeast phytases), pH optima (alkaline or acid phytases) and, more recently, by their catalytic mechanisms: histidine acid phosphatases (HAP); ß-propeller phytase (BPP); cysteine phosphatases (CP); or purple acid phosphatases (PAP) (Lei et al., 2007; Lim et al., 2007). Phytase grouping by catalytic mechanisms seems to be the current trend in the scientific literature. There is a direct link between catalytic mechanism and molecular structure, which varies greatly among and within these groups (Mullaney & Ullah, 2003). Some of the structural components of these proteins are essential for catalysis, and others may influence the adaptation of the catalytic mechanism to the specific microenvironment for which it evolved (Lei et al., 2007).

Although there is a generally accepted concept that phytases are specialized phosphatases with high affinity to soluble phytate substrates, this may not always be true. Greiner (2004) purified a *Pantoea agglomerans* phosphatase with high affinity to glucose-1-phosphate, expressing also little phytase activity.

2.5.2 *Screening studies for phytase producing microorganisms*

Microorganisms screening for either phytase production or phytate utilization (growth in medium containing phytate as sole P source) have been performed with different microbial groups and environments. Hill and Richardson (2007) reviewed well the methodologies used for isolating phytase producing microorganisms, pointing out some positive aspects and many of their failures. Independently of the used methodology, the environmental conditions in which samples were taken also enlighten much about the ecological significance and role of the isolated microbes. As a result of the microorganisms screening studies and also of the identification of homologue sequences in National Center for Biotechnology Information (NCBI) genome and protein databases, a large range of microorganisms with the expected ability to use phytate in natural environments has been identified.

The screening methods for phytate using bacteria in some cases select microorganisms that have the ability to both solubilize and mineralize phytate. Hill and Richardson (2007) reported that in a case study on soil *Pseudomonas* screening, 39% of a group, firstly selected as negative for phytate utilization, turned out to be positive after addition of citrate to the medium. Colony structure may also influence bacteria ability to solubilize and mineralize phytate; some bioaggregates of bacteria with Al-phytate precipitates can help in the phosphorus release from this complexes (Shang et al., 1996).

Phytase activity of organisms present in soils and manures cannot be entirely linked to P nutrition, since several of these phytases are intracellular and may not play any role in extracellular P-phytate dephosphorylation, being more related to other cell metabolic functions. Despite this fact, phytase activity is often interpreted as a direct expression of microbial community metabolic requirements under specific conditions and environments (Caldwell, 2005). The mineralization of phytate is dependent on the presence and activity of phytases and their interaction with the different forms of phytate under different

environmental conditions. These enzymes are especially important for their role in the bioavailability of phytate P (Richardson et al., 2005; Richardson et al., 2007).

Phytase activity in the rhizosphere can be important for stimulating plant growth in soil with limited P availability (Rodríguez et al., 2006). In general, phytase activity is enhanced in rhizosphere microenvironments in comparison with bulk soil (Yadav & Tarafdar, 2004). The present understanding of phytate P utilization and the role of microbial phytases in soil and its relation to P nutrition of plants is limited (Gilbert et al., 1999; Richardson et al., 2005). Evidence of biological utilization of inositol phosphates has been found to be ambiguous by Turner et al. (2002b). The continuous discovery of phytate utilizing microorganisms, widespread throughout many ecosystems, contrasts the fact that these compounds are recalcitrant and accumulate in soil and organic wastes. Hill (2006) suggested that a large number of phytase producing microorganisms are still waiting for being identified.

2.5.3 *Fungal phytases*

Fungi from *Aspergillus* genera were the first identified phytase producing microorganisms (Dox & Golden, 1911). Phytase producing filamentous fungi (Le Casida, 1959) and yeasts (Greenwood & Lewis, 1977) are likely to live in soil environments and their phytase expression is usually triggered by phosphate deficiency (Shieh & Ware, 1968). Nakamura et al. (2000) found 35 yeast strains expressing phytase activity in a 738 strain survey. There are a total of 68 fungi (filamentous fungi and yeasts) species with phytase entries in NCBI protein database, mostly ascomycetes from erotiales order (Table 2.3). Phytases from *Aspergillus niger* and *A. fumigatus* are the most studied among fungi with respectively 45 and 35 entries in NCBI protein database.

Table 2.3. Taxonomic distribution of fungi and bacteria with phytase (or putative sequence) entries in NCBI protein database. Numbers in parentheses represent the number of entries.

Bacteria (713)	**Fungi (186)**	**Archaea (2)**
Proteobacteria (183)	Ascomycetes (166)	*Korarchaeum cryptofilum* OPF8 (2)
g-proteobacteria (142)	Eurotiales (130)	
a-proteobacteria (31)	Saccharomycetales (11)	
d-proteobacteria (6)	Onygenales (10)	
b-proteobacteria (4)	Sordariales (5)	
Firmicutes (118)	Pleosporales (4)	
Bacillales (63)	Hypocreales (3)	
Clostridiales (53)	Magnaporthales (2)	
Erysipelotrichales (2)	Phyllachorales (1)	
Cyanobacteria (42)	Basidiomycetes (20)	
Actinobacteria (37)	Agaricales (11)	
CFB group bacteria (25)	Polyporales (5)	
Green sulfur bacteria (8)	Tremellales (4)	
GNS bacteria (6)		
Thermotogales (6)		
Verrucomicrobia (2)		
Gemmatimonadales (2)		
Uncultured (284)		

Mycorrhyzal fungi (*Glomus intraradices, G. versiforme, G. mosseae* and *Pisolithus tinctorius*) were demonstrated to hydrolyze soluble phytate and transfer the resulting available P to plants (Cairney & Chambers, 1997; Feng et al., 2003; Koide & Kabir, 2000; Tarafdar & Marschner, 1995). Ectomycorrhizal fungi infection was suggested to be responsible for a higher inositol phosphate mineralization in the rhizosphere of radiata pine plants in comparison with ryegrass (Chen et al., 2004).

Many of filamentous fungi and yeast phytases, commonly extracellular, are classified as 3-phytases and are histidine acid phosphatases (HAPs); a large number of them has broad substrate specificity (Lei et al., 2007).

Although non-specific phytase enzymes could be very desirable, and may also contribute to dephosphorylation of other monoester bounded P in manure and soil environments, there is a large number of substrate specific phytases. In nature, they are expected to be associated with other non-phytase phosphomonoesterases, which are not active towards phytate, but are capable of hydrolyzing partially phosphorylated inositol phosphates (Turner & Haygarth, 2005; Turner et al., 2002b). Separation of phytases from other phosphatase can be difficult and commercial phytases preparations may often be contaminated with other phosphatases (Quiquampoix & Mousain, 2005; Turner et al., 2002b).

Tarafdar et al. (2002) showed that a major proportion of the phytases produced by filamentous fungi are secreted to extracellular environments while their non-phytase phosphatases are predominantly maintained cell-associated.

Among fungi phytases *Peniophora lycii* Phy A distinguishes due to its low pI and optimal pH and for being a 4 phytase (Lassen et al., 2001; Lei et al., 2007). Some of naturally occurring phytases were identified to have high thermo-stability and a broad pH range of activity (e.g. *Aspergillus fumigatus* phytase) (Simon & Igbasan, 2002). Other notable phytases are from thermophilic fungus *Rhizomucor pusillus* and *Thermomyces lanuginosus* with broad substrate specificity, high thermal tolerance, optimum temperature at 70ºC and mainly for being active in a wide pH range (Chadha et al., 2004; Gulati et al., 2007a). Thermo-stability can also be considerably increased by protein engineering (Simon & Igbasan, 2002).

2.5.4 *Bacterial phytases*

The number of reported bacteria strains producing phytases (713) in NCBI protein database is much higher than the one of fungi (Table 2.3). On the other hand, a huge proportion of these phytases are from uncultured bacteria (284), which reflects the actual limitation associated with cultivation techniques, and the possible higher amount of bacteria strains with phytase activity.

Several soil bacteria have been reported to use phytate in screening studies. *Pseudomonas* and *Enterobacter* genera were the most abundant in a soil phytate bioavailability experiment (Shang et al., 1996). Richardson and Hadobas (1997) isolated four strains of fluorescent *Pseudomonas putida* with special ability to use precipitated phytate among 238 soil isolates. Jorquera et al. (2008b) isolated phytate mineralizing bacteria in rhizosphere of pastures and crops in Andisols of southern Chile; the most active growing strains in PSM media were from *Pseudomonas*, *Pantoea* and *Enterobacter* genera. Silva (2008) found that 54% of fungi and 76% of bacteria isolated from maize rhizosphere were capable of using phytate as a sole P source; the main identified fungi genera were *Aspergillus, Penicillium, Eupenicillium, Paecilomyces* and *Fusarium*, and for bacteria *Bacillus* and *Pseudomonas*. Jorquera et al. (2008b) found that the proportion of phytate mineralizing bacteria was from 44% to 54% in the rhizosphere of perennial ryegrass, white clover, wheat and oat, and only 17% to the rhizosphere of yellow lupine. A phytate mineralizing bacteria screening in samples taken during aerobic fermentation of dairy manure identified several strains from, *Enterobacter*, *Escherichia*, *Streptomyces, Bacillus, Shigella, Rahnella* and *Ochrobactrum* genera (Fuentes et al., 2009). To our knowledge, no phytase from *Shigella, Rahnella* and *Ochrobactrum* have been purified and biochemically characterized.

Prevotella ruminicola, Ruminobacter amylophilus, Selenomonas ruminantium and *Streptococcus bovis* are reported as/tobe anaerobic ruminal bacteria with phytase activity;

Selenomonas ruminantium showed by far the highest phytase activity and may be responsible for most phytate hydrolysis in ruminants digestive tract (Yanke et al., 1998).

Archaea group also have two phytase entries in NCBI protein database with *Candidatus Korarchaeum cryptofilum* OPF8.

Bacterial phytases may belong to the group of histidine acid phosphatases, cysteine phosphatases or ß -propeller phytases (Hill et al., 2007). Phytase from rumen bacteria *Selenomonas ruminantium*, *S. Lacticifex* and *Megasphaera elsdenii* are reported to be cysteine phosphatase (Chu et al., 2004; Puhl et al., 2008; Puhl et al., 2009; Yanke et al., 1999).

Bacterial *Bacillus* phytases generally differ from other phytases, having a pH optimum from 7.0 to 8.0, being Ca^{2+} dependent. Their activity is highly specific for phytate, and they are the most resistant phytases to pancreatin (Fu et al., 2008; Simon & Igbasan, 2002). These enzymes are ß -propeller phytases (Fu et al., 2008; Jorquera et al., 2011; Kim et al., 1998a; Kim et al., 1998b; Lim et al., 2007). *Bacillus* phytase characteristics and potential uses were reviewed by Fu et al. (2008).

Escherichia coli phytase is the most studied and the most promising among bacteria phytases. It is a mixture of two periplasmatic histidine acid phosphatases with same mass of 42 KDa, very high specific activity and catalytic efficiency (Greiner et al., 1993). The phytase from *E. coli* has also a very high residual activity after incubation with pepsin or in supernatants of stomach digesta (Simon & Igbasan, 2002). *Escherichia coli* phytase is the only commercial bacteria phytase, being produced by Beijing Challenge Group, AB Enzymes and others.

While filamentous fungi and yeast phytases have very variable molecular masses, many bacteria phytases have molecular masses between 40 and 50 KDa.

Molecular characteristics and applications of microbial phytases were well reviewed by several authors, recommend for further reading: Rao et al (2009) Jorquera et al. (2008a), Fu et

al. (2008), Asan (2007), Lei et al. (2007), Haefner et al. (2005), Konietzny & Greiner (2004), Pandey et al. (2001), Wyss et al. (1999a; 1999b) and Dvoráková (1998).

2.6. Biotechnological use of microbial phytases for hydrolyzing phytate P pool

The use of phytase in the diet of monogastric animals is a current well studied practice that significantly increases phytate P degradation and absorption, hence reducing the phytate output and total P concentration in manures (Cho & Bureau, 2001; Haefner et al., 2005; Mullaney et al., 2000; Sebastian et al., 1998). The addition of *A. niger* phytase to pig feed can increase phytate digestibility to more than 60%, reducing total P content in manures from 21.0 to 13.6 and from 15.8 to 10.4 g kg^{-1} DW in two different phytate rich diets (Jongbloed et al., 1992). The use of enzymes as animal feed additives accounts for almost 16% of the total world industrial enzyme market and it is predicted to reach up to US$375 million in 2012 (Thakore, 2011). Phytase solution to environmental problems associated with phytate in animal wastes is extremely cost effective and socially acceptable (Hill, 2006). Phytase enzymes are the main responsible for the 6% expected growth rate (CAGR) of animal feed enzymes sector: This is stimulated by the increasing acceptance that phosphate pollution caused by inadequate manure disposal from confined monogastric animals has to be substantially reduced.

To use microbial phytase to enhance P nutrition, transgenic plants of subterranean clover, potato, arabidopsis and tobacco and expressing *Aspergillus* phytases were prepared. The roots of these transgenic plants were able to excrete the heterologous phytase under P deprivation. Under sterile growth conditions, these transgenic plants were able to use phytate as P source (George et al., 2004; Mudge et al., 2003; Richardson et al., 2001b; Richardson et al., 2001c; Zimmermann et al., 2003). Except for transgenic tobacco (George et al., 2005c), in soil conditions P nutrition was not significantly increased by phytase enzyme exudation (George

et al., 2005b). Even in soils with higher phytase hydrolysable P, *Trifolium subterraneum* L. exuding extracellular phytase, did not show significant advantages reflected in growth and P nutrition (George et al., 2007a).

Recent research on industrial production of phytases has been directed mainly to applications related to improve phytate P availability in diets for monogastric animals (Vats et al., 2005). Nevertheless, other potential uses of phytases are in human nutrition and health, and plant nutrition through phytate monoester P cleavage (Lei et al., 2007). Due to the large amount of phytate in organic wastes and soil, biotechnologies involving phytase enzymes to increase organic P availability are highly desirable (Jorquera et al., 2008a).

2.7. Factors affecting phytate catalysis

There has been little research on phytase behaviour in organic wastes and soils. The biochemical and biophysical characteristics of phytases produced by different microorganisms affect their behaviour in many ways, and consequently their efficiency in phytate mineralization (George et al., 2005a; George et al., 2007b; George et al., 2007c). As discussed before, phytate availability also influences phytase mediated catalysis. Freshly added phytate is more susceptible to dephosphorylation than stabilized old phytate (George et al., 2007b).

Three phytases differing in catalytic mechanism were able to hydrolyze phytate precipitated with Ca^{2+}, Mg^{2+} and Mn^{2+} but not with Al^{3+}, Fe^{2+}, Cu^{2+}, and Zn^{2+}, and even the sole presence of these cations inhibited hydrolysis of precipitated calcium phytate (Tang et al., 2006). Several other studies showed inhibition of different phytases by metal ions such as Al^{3+}, Ba^{2+}, Cd^{2+}, Co^{2+}, Cr^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+} ,Mn^{2+} and Zn^{2+} (Choi et al., 2001; Greiner, 2004; Gulati et al., 2007c; Huang et al., 2006; Rao et al., 2009; Wyss et al., 1999a; Yoon et al., 1996). *Bacillus* ß-propeller phytase is the only known microbial phytase which needs metal

ions and the removal of Ca^{2+} ions causes complete inactivation of this enzyme (Kerovuo et al., 2000a; Simon & Igbasan, 2002).

Intracellular phytases showed higher inhibition in soil conditions compared with extracellular ones (Matumoto-Pintro & Quiquampoix, 1997). Extracellular phytases were found to be 60% more efficient in the hydrolysis of phytate than intracellular ones (pH 5,4 at 30ºC for 24h), but equally efficient in dephosphorylating glycerophosphate (Tarafdar et al., 2002). Speculations around the effect of the evolved function in the catalytic behaviour of these enzymes under different conditions may not be accurate; great variations of catalytic properties are observed for all enzymes irrespectively of their source, type and evolved function (Table 2.4).

Phytases are incapable of complete dephosphorylation of phytate, and the final products may be either myo-inositol monophosphate or pentakis phosphate for HAPs and CPs, or myo-inositol trisphosphate for BPP phytases (Greiner et al., 2007; Kerovuo et al., 2000b; Oh et al., 2004). Association with other non-phytase phosphatases is necessary for complete phytate dephosphorylation and may increase phytase efficiency, as well. For example, the over expression of non-phytase phosphatases in *Saccharomyces cerevisiae* increased its measured phytase activity, which is ,in fact, the P-releasing activity from phytate substrate (Andlid et al., 2004). Other non-phytase acid phosphatases are widely spread in many environments and some of them are capable of hydrolyzing partially phosphorylated inositol phosphates along with other monoester P, but are unable to initiate phytate hydrolysis (Quiquampoix & Mousain, 2005; Turner et al., 2002b).

Table 2.4. Meta analysis of biophysical and biochemical properties of filamentous fungi, yeast and bacterial phytases.

	pH Amplitude				pH optimum				T optimum				Mass			
	Bacteria	**Fungi**	**Yeast**	**Total**	**Bacteria**	**Fungi**	**Yeast**	**Total**	**Bacteria**	**Fungi**	**Yeast**	**Total**	**Bacteria**	**Fungi**	**Yeast**	**Total**
Max. 100%	2.3	6	3	6	7.5	6.5	5.75	7.5	70	70	80	80	50	128	490	490
quartile 75%	1.58	1.95	1.00	1.50	5.50	5.75	4.50	5.50	60	60	65	63	45	84.50	318.5	84.00
median 50%	1.00	1.00	0.85	1.00	5.00	5.50	4.00	4.50	58	55	60	60	42	72.00	120.0	60.00
quartile 25%	0.88	0.50	0.50	0.50	4.50	4.38	3.65	4.00	50	50	60	55	40	59.50	73.0	45.00
minimum0%	0.3	0.5	0.3	0.3	3.85	2.5	2.25	2.25	40	40	40	40	36	33	51	33
Moments																
Mean	1.19	1.58	0.97	1.21	5.11	4.99	4.12	4.66	55.94	56.09	63.29	59.24	43.00	74.71	180.6	77.72
Std Dev	0.60	1.58	0.67	1.05	1.04	1.10	0.66	1.02	8.22	8.80	8.12	9.01	3.67	23.76	176.5	73.43
Std Err Mean	0.19	0.42	0.15	0.16	0.24	0.22	0.12	0.12	1.99	1.88	1.46	1.08	1.02	5.19	78.9	11.76
N	10	14	20	44	19	25	31	75	17	22	31	70	13	21	5	39

	PI				Specific act				Km				Kcat			
	Bacteria	**Fungi**	**Yeast**	**Total**	**Bacteria**	**Fungi**	**Yeast**	**Total**	**Bacteria**	**Fungi**	**Yeast**	**Total**	**Bacteria**	**Fungi**	**Yeast**	**Total**
Max. 100%	8.70	8.50	.	8.70	1800	1210	418	1800	700	1290	250	1290	6209	5460	.	6209
quartile 75%	7.73	5.70	.	6.49	690	700	418	622	570	340	197	460	2137	1836	.	704
median 50%	6.40	4.84	.	5.15	297	110	217	196	420	110	38	250	114	219	.	91
quartile 25%	5.25	4.04	.	4.41	20	33	17	29	288	23	32	38	34	49	.	41
minimum0%	5.00	3.60	.	3.60	15	3	17	3	114	10	30	10	21	43	.	21
Moments																
Mean	6.47	5.23	5.50	5.52	477	351	217	376	422	239	89	285	1213	1103	39	1072
Std Dev	1.43	1.52	.	1.52	626	434	.	475	180	348	107	288	2465	2146	.	2133
Std Err Mean	0.64	0.38	.	0.32	237	112	.	97	57	97	54	55	1006	876	.	592
N	5	16	1	22	7	15	2	24	10	13	4	27	6	6	1	13

Data obtained from: Rao et al (2009), Jorquera et al. (2008a), Fu et al. (2008), Li et al. (2008), Asan (2007), Lei et al. (2007), Gulati et al. (2007a; 2007b), Boyce and Walsh (2007), Huang et al. (2006), Haefner et al. (2005), Konietzny & Greiner (2004), Chu et al. (2004), Oh et al. (2004), Vats and Banerjee (2004), Greiner (2004), Zinin et al. (2004), Wang et al. (2004), Chadha et al. (2004), Quan et al. (2004), (De Angelis et al. (2003), Cho et al. (2003), Casey and Walsh (2003), Tye et al. (2002), Quan et al. (2002), Pandey et al. (2001), Lassen et al. (2001), Golovan et al. (2000), Nakamura et al. (2000), Tseng et al. (2000), Rodriguez et al. (2000), Wyss et al. (1999a; 1999b), Yanke et al. (1999), Nagashima et al. (1999), Liu et al. (1999), Dvoráková (1998), Kerovuo et al. (1998), Kim et al. (1998a), Berka et al. (1998), Greiner et al. (1997), Jareonkitmongkol et al. (1997), Pasamontes et al. (1997a; 1997b), Mitchell et al. (1997), Yoon et al. (1996), Tambe et al. (1994), Al-Asheh and Duvnjak (1994), Shimizu (1993), Segueilha et al. (1992), Shimizu (1992), Nair et al. (Nair et al., 1991), Sutardi and Buckle (1988), Powar and Jagannathan (1982), Greaves et al. (1967).

Phytase-manure incubations for the determination of phytase hydrolysable P are the main antecedents on phytase catalytic behaviour in animal manure. These previous experiments have demonstrated that phytase addition to animal manures increases water extractable inorganic P (Dao, 2003). Up to 60% of manure monoester P can be hydrolyzed by crude phytase preparations of phytase enzymes with broad substrate specificity, e.g. *A. ficuum* phytase (Bunemann, 2008). The main factors affecting phytate hydrolysis in animal manures are moisture content, temperature and pH.

Phytase mediated catalysis is a function of moisture content: as total solids in manures increases, phytate susceptibility for dephosphorylation decreases (Dao, 2003).

In manures, the majority of phytase enzymes have optimal high temperatures around 40 to 65ºC, and phytate hydrolysis is significantly enhanced by heat. This may have a positive impact on phytate dephosphorylation during thermophilic phase of different composting procedures.

Phytase enzymes are often active in a range from 1 to 3 pH units around optimal pH and only few phytases can work in a wide range of pH. Most of the microbial phytases have optimal activity in acidic pH, and this may compromise the rate of phytate dephosphorylation in animal wastes due to their alkalinity. Non biological factors such as temperature and ionic strength are less controllable and will significantly vary at each handling and storage facility; these changes may alter phytases activities and consequently phytate P catalysis (Dao, 2003).

Soils are expected to have some native phytase activity (Yadav & Tarafdar, 2004) from a wide range of microorganisms (see section 3). Despite the presumed presence of phytases, phytate accumulation in soils has been demonstrated by various authors (see section 2.2). This fact suggests that native soil phytases may not effectively catalyze phytate dephosphorylation in soil environments.

Failures related to experiments performed with transgenic plants expressing extracellular phytases indicate that these enzymes may not be effective in hydrolyzing phytate substrates in soil environment. This inefficiency was attributed to adsorption mechanisms that compromised phytase mobility and its capability of interacting with immobilized inositol phosphates (George et al., 2005b; George et al., 2007c). Other possible reasons that could explain this result include: a) poor availability of phytate; b) presence of phytase-producing microorganisms in rhizosphere making phytate equally available to control plants; c) inhibitory effect of soil environments on phytase activity (George et al., 2005a; George et al., 2007b; George et al., 2005b; George et al., 2007c).

Adsorption of fungal phytases seems to be a function of soil pH and enzyme isoelectric point (pI) (George et al., 2005b; George et al., 2007c). George et al. (2007c) compared the partition of the activity of two phytases from *Peniophora lycii* (pI 3.6) and from *Aspergillus niger* (pI 4.8) between solid and liquid phase of a soil suspension.*Peniophora lycii* phytase remained in solution in a range of soil pH from 4.5 to 5.0, while *A. niger* phytase was entirely adsorbed; these results were associated to a 3.2 fold higher phytate hydrolysis by *P. lycii* phytase (George et al., 2007c). This higher catalysis of *P. lycii* phytase was attributed to higher mobility; however, this enzyme preserved its activity longer than *A. niger* phytase, which was quickly inhibited by adsorption. The question is if the observed higher catalysis of *P. lycii* was due to higher mobility or to higher preserved activity. Being these studies performed in diluted soil suspensions, the achieved results may not be fairly the same (i.e., generalized) in natural soil conditions.

Soil pH, and the pI of phytases and soils determine the predominance of either electrostatic repulsion or attraction to soil colloids and are expected to significantly influence adsorption (George et al., 2005a). Nonetheless, this effect is only valid for stable proteins interacting with hydrophilic surfaces; in other cases, hyphobic interaction may prevail (Norde et al.,

2008). Other properties, such as molecular mass (Harter & Stotzky, 1971) and hydrophobicity (Boyd & Mortland, 1990; Norde, 1986), are known to affect protein adsorption to soil particles. However, not many findings or discussions on their effect on phytase adsorption are, available in literature. Higher adsorption is expected for enzymes with higher molecular mass, and the adsorption enzymes on hydrophobic zones may be governed by hydrophobicity. In addition, little is known about the catalytic and partitioning behaviour of different phytases in soil conditions, considering that this enzyme group has very diverse properties (Table 2.4).

Adsorption of phytases may often cause partial or complete enzyme irreversible deactivation due to conformational changes (Leprince & Quiquampoix, 1996; Quiquampoix, 2000). A shift of the pH optima of catalysis to higher values is usually expected due to enzyme adsorption on clay minerals (Leprince & Quiquampoix, 1996) and may also affect phytate hydrolysis. Some phytases were completely inhibited by adsorption; e.g. wheat phytase adsorbed on montmorillonite (Turner et al., 2002b). In other cases, enzyme activity may be even enhanced by adsorption (Cao, 2005; Mateo et al., 2007; Rosas et al., 2008). Enzyme positive or negative response to adsorption is very case-specific and may greatly fluctuate with different enzymes and supports showing differing properties (George et al., 2005a).

Kondo et al. (1992) showed that enzymes maintain their activity after adsorption only at pH around and above their pI, and adsorption at pHs below enzyme pI would cause activity loss due to enzyme conformational changes. Considering this hypothesis we can speculate that acid soils may rapidly deactivate mainly phytases, and phytases with high pI may be deactivated in almost all soils due to adsorption. Pant & Warman (2000) suggested that indigenous phosphatases are naturally immobilized on positively charged surfaces in soils, hence at pH higher than their pI. The conservation of phytase activity after adsorption on nanoclays is higher in acid pHs, and it is also strongly affected by the type and characteristics

of both support and phytase (Menezes-Blackburn et al., 2011). This agrees with studies of Rao et al. (1996; 2000) on the adsorption of acid phosphatase on clays and tannic acid.

Adsorption of enzymes are expected to provide protection against proteolysis, and thermal denaturation (Nannipieri et al., 2002). Phosphatase activity was detected in geologically preserved 9000 and 1000 year old permafrost peat and sediments, respectively, suggesting that enzyme stabilization in soils may be effective in a very long term (Skujins, 1976). George et al. (2005b) found that the rate of deactivation of *A. niger* phytase after addition to soil was down to four times slower in soil-enzyme suspensions compared with enzyme suspensions alone. Consequently, immobilization of phytases previous to their biotechnological use is desirable due its effect on the long term persistence of the enzymatic activity (McKelvie et al., 1995).

Rhizosphere and its environment affect positively hydrolysis of organic phosphorus. Rhizosphere environments may favour phytate dephosphorylation due to the presence of plant exudated organic acids. Soil extracts obtained with the use of citrate allowed phytase hydrolysis of up to 40% of total organic P, while very little phytate is hydrolysable by phytase in soil water extracts (Otani & Ae, 1999). Hayes et al (Hayes et al., 2000a) found extractable organic phosphorus increased with increasing concentrations of citric acid, which could be enhancing phytase catalysis in rhizosphere environments. In addition, native phytase activity increases in rhizosphere in comparison with bulk soil, which may also positively influence soil phytate catalysis and depletion in this zone (Yadav & Tarafdar, 2004). Rhizosphere environment reduced *A. niger* phytase adsorption, possibly due to the presence of organic anions or due to observed pH changes (George et al., 2005b). Low molecular mass organic acids are reported to reduce the adsorption of a non phytase phosphatase in the following order: tartrate > oxalate > acetate (Huang et al., 2003). Changes in pH related to rhizosphere environments may also directly interfere on phytase activity.

As well as in animal wastes, soil phytate availability strongly affects phytase mediated catalysis. The addition of CDTA (Cyclohexanediamine tetraacetic acid) and EDTA (Ethylenediamine tetraacetic acid) increased phytase hydrolysis efficacy of previously inaccessible complexed organic P; soil phytase hydrolysable P increased from 2.3 and 6.4% to peak at 32.0 and 40.8 % of total P in two soils with the use of EDTA extractant (Dao, 2004a).

George et al. (2007b) reviewed well phytase behaviour in soils and the impact of the interaction with soil constituents on phytate hydrolysis.

2.8. Conclusions and future perspectives

Manures and soils contain high content of phytase labile organic P pool which can represent a significant P source for plant nutrition. Phytase amendment in soils for enhancing organic P availability has been considered through the use of phytase exuding transgenic plants and inoculation with phytase-producing microbes. However, until now the use of phytase amendments of manures for organic P recycling has received little attention and may be also considered for future studies and biotechnological applications. Therefore, phytate can be hydrolysed to inorganic P before its addition to soil where it becomes poorly available to phytases.

Although phytases are suitable for increasing organic P availability in manures and soils, the dynamics of phytate dephosphorylation depends on: a) presence of active phytase enzymes; b) phytase catalytic adaptation to the specific environment conditions (pH, temperature, presence of metal ions, etc); c) enzyme inactivation related to interaction with medium constituents; d) phytase mobility and capacity to interact immobile phytate; and d) availability of the different precipitated and adsorbed phytate salts. Thus, adequate selection of phytases may be critical to the success of each biotechnological application, and catalysis performance in different

environments is expected to be enzyme specific. Further studies comparing catalytic behaviour of different phytase enzymes in different organic wastes and soils are needed to better understand the factors affecting their behaviour.

The large scale recycling of organic P from animal wastes is urgent considering the imminent world phosphate rock scarcity scenario. Therefore, biotechnological approaches to enhance the agronomic use phytase hydrolizable P pool in manures and soils are required in the near future. An environmental benefit may also be achieved with the increase organic P availability to plants: the continuous depletion of bioactive P in agronomic systems prevents its runoff dispersion and the consequent contamination of watercourses.

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CHAPTER 3

3. DETECTION AND CHARACTERIZATION OF PHYTASES PRODUCED BY NATIVE BACTERIAL STRAINS

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3. DETECTION AND CHARACTERIZATION OF PHYTASES PRODUCED BY NATIVE BACTERIAL STRAINS

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3.1. Abstract

We have studied the phytase activity of twelve bacteria strains in order to evaluate their potential use in manure P-phytate hydrolysis for biotechnological applications. Strains were grown in Luria-Bertani general media (LB) and phytase-screening medium (PSM), and their phytase activity was measured using Na−phytate as substrate. The effect of pH and metals (Fe^{3+}, Al^{2+} and Mn^{2+}) on phytase activity was also assayed. Except for *Ochrobactrum* sp. C95 and *Bacillus* sp. N1-19NA, all the studied bacteria strains showed a cell-associated phytase activity when grown in PSM medium. Most identified phytases were active at acid pHs, and only *Bacillus* sp. MQH-19 and *Bacillus* sp. MQH-15 showed alkaline phytase activity. In general, phytases were native phytases were strongly inhibited by Fe^{3+} and moderately inhibited by Al^{3+} ions, which are abundant in volcanic soil environments. Among the studied native bacteria strains, only *Bacillus* sp. MQH-19 presented extracellular phytase activity, increasing Pi concentration in liquid PSM medium. *Bacillus* sp. MQH-19 was found to be a promising strain to be tested in potential manure P-phytate hydrolysis biotechnological applications.

3.2. Introduction

Due to the imminent phosphate rock scarcity (Abelson, 1999), there is a great need to increase the availability and cycling of phosphorus (P) in agronomic systems through the revalorization of organic wastes. Phytate is the most important fraction of organic P (Po) in manures and soils and is a P pool unavailable to plants (Turner et al., 2002). Phytase enzymes can release phosphates from phytate and other forms of organic P increasing its availability (Richardson et al., 2005; Turner et al., 2002).

Microorganisms are the main sources of phytases in nature, and our working group has isolated phytate utilizing bacteria (PUB) in a manure aerobic fermentation system (Fuentes et al., 2009). The main PUB strains identified in this study were from the *Enterobacter*, *Escherichia, Shigella, Streptomyces, Bacillus, Rahnella* and *Ochrobactrum* genera. Changes in Po during dairy manure fermentation have been attributed to these *Enterobacteriaceae* phytases (Fuentes et al., 2009). It has also been speculated that these organic wastes may contribute with phytase enzymes that can contribute to soil phytase activity. Except for *Rahnella* and *Ochrobactrum,* the other identified genera have either been reported to present phytase activity or homolog phytase genes (Jorquera et al., 2008a). However, the actual presence, properties, expression, and catalytic behavior of phytase enzymes from these strains, as well as their interaction in soil constituents have not been well established, yet.

The main objective of this work was to evaluate the presence, expression patterns and properties of the phytases from several selected native strains, in order to evaluate their potential use in soil and manure P-phytate hydrolysis.

3.3. Material and methods

3.3.1. *Bacterial strains*

The bacterial strains used in this study were screened in previous studies by our workgroup. *Pantoea* sp. LUP, *Pseudomonas* sp. RYE, *Paenibacillus* sp. 60P3, *Enterobacter* sp. N0-29PA, *Serratia* sp. N0-10LB, *Pseudomonas* sp. N1-55PA, *Bacillus* sp. MQH-15, *Bacillus* sp. MQH-19 and *Bacillus* sp. N1-19NA were isolated from rhizosphere soil (Jorquera et al., 2008a; Jorquera et al., 2011; Martínez et al., 2011) whereas *Enterobacter* sp.B95, *Rhanella* sp. D75 and *Ochrobactrum* sp. C95 were isolated from bovine manure (Fuentes et al., 2009). *Escherichia coli* MG 1655 was used as a positive control for phytase activity expression.

The isolates were grown in fresh LB (Miller & Laboratory, 1972) or PSM broths for 48 h at 30 °C. Total crude protein was extracted as follows: bacterial cells and supernatant were separated by centrifugation (3,600 rpm for 5 min). The supernatant was subjected to ammonium sulfate (0-85%) precipitation. In parallel, cell pellet was sonicated (130 Watts, 20 KHz 20% amplitude for 2 min) to break the cells. The cell debris were removed by centrifugation (5,000 rpm 5 min) and the supernatant was also subjected to ammonium sulfate precipitation (65-85%). Both pellets, were then resuspended in Tris HCl buffer (pH 7.0) and stored at -20 °C as described by Hill et al. (2007). Protein concentration was spectrophotometrically determined at 595 nm using Coomassie brilliant blue G-250 stain (Bradford, 1976; Compton & Jones, 1985). All enzyme assays were run in triplicate.

3.3.2. *Phytase activity*

To check that the screening of strains for phytase production was accurate, a confirming staining method with cobalt-molybdate-vanadate was used (Bae et al., 1999). Nevertheless this technique did not provide any accurate evidence of phytate mineralization. Additionally, EnzChek® Ultra Phytase Assay Kit

was used for attempting to obtain confocal microscope evidence of phytase activity, using *E. coli* 1655 cells. Cells incubated for 1h at 37 °C with phytate solution containing DMSO showed almost twice red fluorescence than the ones incubated in absence of phytate (Figure 3.1). However, strong variations of fluorescence in time and among repetitions discouraged the use of this methodology as complementary indication of the presence of cell associated phytase activity.

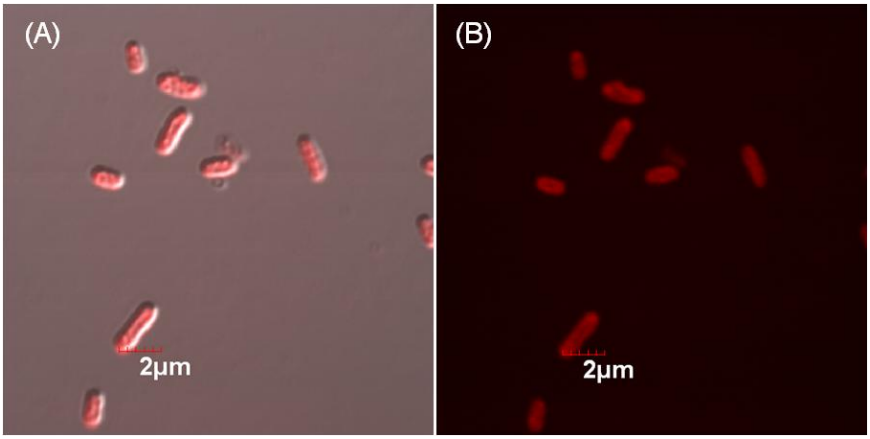

Figure 3.1. Confocal microscope contrast view of *E. coli* 1655 cells fluorescent stained with both DAPI and EnzChek® Ultra Phytase Assay Kit (A) and stained with EnzChek® Ultra Phytase Assay Kit alone (B).

Due to the lack of simple phytase activity staining methods with clear results, we used bacteria phytase activity assay adapted from Greiner (2004). Ten µl of crude protein extract were incubated with 270 µl of Na−phytate solution (2.5 mM of phytate in Tris−HCl buffer pH 7.0) for 30 min at 37°C. The reaction was stopped by adding of 1,150 µl of a 2:1:1mixture of fresh acetone, 5N sulfuric acid, 10 mM ammonium molybdate and 80 µl of 1 M citric acid. The solution was then centrifuged (5,000 rpm for 5 min), the absorbance of the supernatant was measured at 355 nm and compared with standard curve of PO_4^{2-}. Blanks were run by addition the

ammonium molybdate solution prior to adding the enzyme to the assay mixture. One unit of phytase activity was considered as 1 μM P released in 1 min. Blanks were performed by adding the stop solution prior to substrate addition. Phytase activity under acidic condition (sodium acetate buffer pH 4.5) was also measured.

3.3.3 *Effect of pH and metal cations on phytase activity*

Crude protein extracts from bacteria strains grown in PSM medium were obtained as described above. The buffer used for determining the effect of pH dependence of cell−associated phytase activity had the following composition: 100 mM sodium acetate−acetic acid (pH 3.5, 4.5 and 5.5), 100 mM sodium acetate−HCl (pH 6.5), and 100 mM Tris−HCl (pH 7.0). The effect of metal cations on cell−associated phytase activity was tested according to the method described by Greiner et al. (Greiner, 2004). The total crude protein was incubated for 15 min at 37ºC with Fe^{3+}, Al^{2+} and Mn^{2+} at a final concentration of 10 mM at the optimal pH for cell−associated phytase activity in each strain.

3.3.5 *Analysis of data*

Each experiment was performed in triplicate and repeated at least twice. Statistical analysis was performed by using the statistical software JMP®, version 5.0 (SAS Institute, Inc.). Before statistical analysis, the data were tested for normality. The significance of each treatment was established by one way ANOVA and the means were separated by Tukey's test ($P \leq 0.05$).

3.4. Results and Discussion

The selected bacteria strains were pointed as phytate utilizing bacteria (PUB) as they grew in

PSM agar plates with phytate as a sole P source. Nevertheless, this methodology is known to produce 'false positives' to bacteria which excrete acid to the medium (Bae et al., 1999). Although phytate is expected to be the only P source in PSM medium, Pi was detected in the PSM broth preparation (0.62 ±0.04 μmol ml^{-1}), which could be favoring the growth of strains without the degradation of the calcium phytate by phytase enzymes.

Based on results obtained, we consider that the direct in vitro phytase activity assay produced better evidence of phytase activity production by bacteria strains, than the staining methods used to the same purpose. Several tests were performed for the standardization of the specific protocol of phytase activity in proteins extracts of our bacteria strains. For the determination of the Pi released the initially used molibidate-vanadate method (415 nm) was replaced by molibidate binding method (355 nm) according to Greiner (2004), which is 75 times more sensitive, therefore, it allowed a significant reduction of the protein sample volume used in the assay. Sample volume of 10 and 20 μl of protein extract were tested, corresponding to 12 and 24 μg of protein. No significant difference was observed between the tested sample volumes; therefore, further tests were performed with 10 μl sample. The phytase activity tests showed linear trends of Pi release, and no saturation was observed during the 40 min assay. This indicates that no significant activity inhibition by product took place in the first 40 minutes affecting the assay. Further tests were performed with 30 minutes of incubation time. No significant Pi release was verified neither for protein nor for substrate incubated alone.

A cell associated phytase activity was observed in all the studied bacterial strains grown in PSM broth, except for *Ochrobactrum* sp. C95 and *Bacillus* sp. N1-19NA which showed no detectable phytase activity and were considered as false positives of their respective screenings (Table 1). Only the positive control *E. coli* 1655 and *Bacillus* sp. MQH-19 showed a low extracellular

phytase activity but with a significantly higher cell associated phytase activity. Most selected strains showed no detectable activity when cultivated in LB broth indicating that, in general, the expression of phytases are being induced either by phosphate deficiency, nutrient deficiency or by presence of phytate. Inducible phytases may not be produced when these strains are inoculated in Pi rich substrates such as bovine manure or agriculture soils.

Table 3.1 Phytase activity (mU mg^{-1} protein) for the preselected bacteria strains cultivated in liquid phytase screening medium (PSM) and Luria Bertani (LB) medium.

	pH	PSM	LB	Screened from	Screened by
E. coli MG 1655	4.5	56.6 ±5.8	12.5 ±1.4	Positive control	
Pantoea sp. LUP	4.5	38.7 ± 3.0	0.0 ±0.0	Lupine rhizosphere	(Jorquera et al., 2008b)
Pseudomonas sp. RYE	4.5	17.6 ± 0.3	1.7 ±0.1	Ryegrass rhizosphere	(Jorquera et al., 2008b)
Rhanella sp. D75	4.5	15.1 ±0.4	0.0 ±0.0	Bovine manure	(Fuentes et al., 2009)
Paenibacillus sp. 60P3	4.5	161.1 ±11.3	0.0 ±0.0	Ryegrass rhizosphere	(Jorquera et al., 2011)
Enterobacter sp.B95	4.5	77.9 ±7.3	9.8 ±0.9	Bovine manure	(Fuentes et al., 2009)
Enterobacter sp. N0-29PA	4.5	16.6 ±1.2	9.8 ±0.9	Ryegrass rhizosphere	(Martínez et al., 2011)
Serratia sp. N0-10LB	4.5	3.8 ±1.8	0.0 ±0.0	Ryegrass rhizosphere	(Martínez et al., 2011)
Pseudomonas sp. N1-55PA	4.5	6.1 ±1.1	0.0 ±0.0	Ryegrass rhizosphere	(Martínez et al., 2011)
Bacillus sp. MQH-15	7	19.8 ±2.7	0.0 ±0.0	Ryegrass rhizosphere	(Jorquera et al., 2011)
Bacillus sp. MQH-19	7	140.7 ±9.6	0.0 ±0.0	Ryegrass rhizosphere	(Jorquera et al., 2011)
Ochrobactrum sp. C95	-	0.0 ±0.0	0.0 ±0.0	Bovine manure	(Fuentes et al., 2009)
Bacillus sp. N1-19NA	-	0.0 ±0.0	0.0 ±0.0	Ryegrass rhizosphere	(Martínez et al., 2011)

The only strains with alkaline phytases were *Bacillus* sp. MQH-15 and *Bacillus* sp. MQH-19. No

alkaline phytase activity was observed for *Paenibacillus* sp. 60P3, which was considered an unusual ß-propeller, since this group typically presents alkaline activity. The *Paenibacillus* sp. 60P3 phytase gen have been partially sequenced by Jorquera et al (2011) and presented a high homology with *Bacillus pseudomycoides* and *Bacillus mycoides* phytase. The only ß-propeller phytase biochemically described with acid pH optimum is from *Bacillus licheniformis* (Tye et al., 2002) which was the 5th in homology to our *Paenibacillus* sp. 60P3 phytase. Four bacterial strains were selected among isolates of previous phytase screening studies for further characterization of phytase activity: *Paenibacillus* sp. 60P3, *Bacillus* sp. MQH−19, *Bacillus* sp. MQH−15 and *Rhanella* sp. D75 strains.

The evolution of extracellular inorganic P was evaluated for *Paenibacillus* sp. 60P3, *Rhanella* sp. D75 and *Bacillus* sp. MQH-19 strains during a 6-day incubation assay in PSM medium (Figure 3.2). While the positive control *E. coli* and *Bacillus* sp. MQH-19 increased extracellular Pi from the 3rd day on, no significant extracellular Pi increase was detected from *Paenibacillus sp.* 60P3 and *Rhanella sp.* D75 strains. The absence of extracellular Pi increase was interpreted as the result of balanced rates of mineralization/immobilization of P-phytate. These results suggest that both strains have no direct effect on the immediate phosphorus availability from precipitated Ca-phytate in the liquid PSM medium. On the other hand, these strains may have an effect in transforming P fixed in phytate into more available forms of phosphorus. The rates of P release are expected to vary greatly with the form of phytate salt used as substrate (Tang et al., 2006). The lack of extracellular phytase activity for both *Paenibacillus* sp. 60P3 and *Rhanella* sp. D75 strains supports the obtained results of extracellular Pi release.

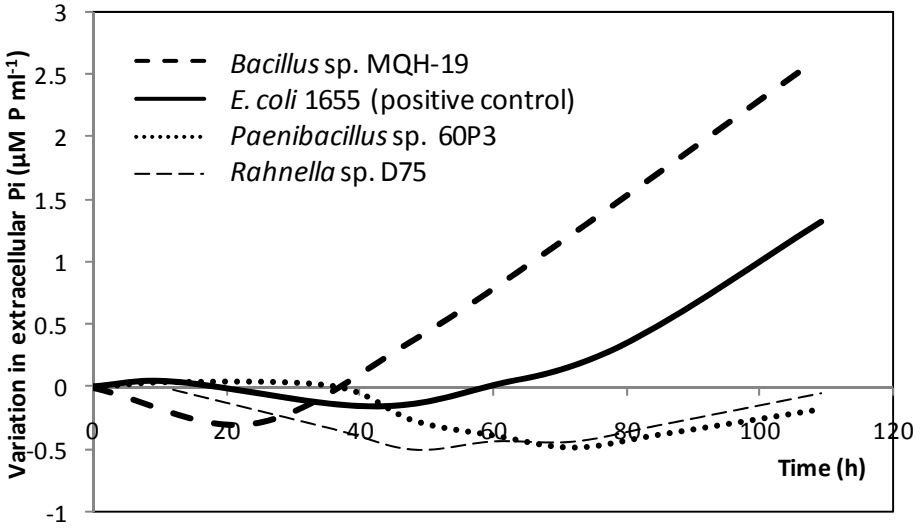

Figure 3.2. Inorganic P evolution to *Bacillus* sp. MQH-19, *E. coli* MG 1655, *Paenibacillus* sp. 60P3 and *Rhanella* sp. D75 strains cultivated in liquid PSM medium.

The phytases from *Bacillus* sp. MQH-19, *Bacillus* sp. MQH-15, *Paenibacillus* sp. 60P3 and *Rhanella* sp. D75 strains were partially purified using ammonium sulfate precipitation (salting in, 60-80% saturation) and Amicon ultra 15 centrifuge tubes (50-100 kDa). The final enzyme solution was used for determining optimal temperature and optimum pH of activity. All tested enzymes showed optimum temperature of 45ºC, and an optimum pH was enzyme specific (Figure 3.3). The only alkaline phytases were *Bacillus* sp. MQH-19 and *Bacillus* sp. MQH-15, the other phytases were acid phytases. Two commercial phytases from *Escherichia coli* and *Aspergilus niger* were also analyzed to contrast the activity-pH curve from our native phytases. Phytase activity was strongly inhibited by the presence of 10 mM Fe^{3+} in the assay solution whereas 10 mM Mn^{2+} did not significantly affected the activity of the same enzymes (Figure 3.4). The presence of Al^{3+} in the activity assay mixture caused a complete inhibition of *Bacillus* sp. MQH-15 while this inhibition was only moderate to the other tested enzymes.

The strain *Bacillus* sp. MQH-19 was selected among the studied enzymes for inoculation tests for phytase treatment of neutral to slightly alkaline wastes. This enzyme is attributable to be suitable for waste treatment due to its pH-activity profile, and high extracellular phytase activity with verified capacity of increasing extracellular Pi in a calcium phytate rich medium. This strain is similar to *Bacillus amyloliquefaciens* (Jorquera et al., 2011), whose phytase was previously described as a extracellular phytase with optimum pH of 7.5 with high substrate specificity toward phytate (Kim et al., 1998).

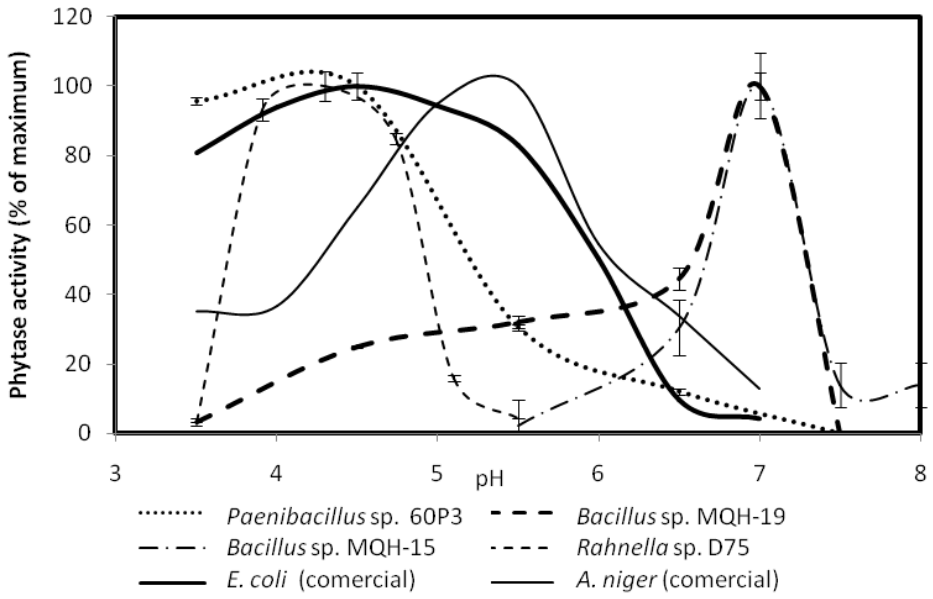

Figure 3.3. pH dependency of phytase activity for Rahnella sp. D75 *Paenibacillus* sp. 60P3, *Bacillus* sp. MQH-19, *Bacillus* sp. MQH-15 and commercial *E. coli* and *A. niger* phytases.

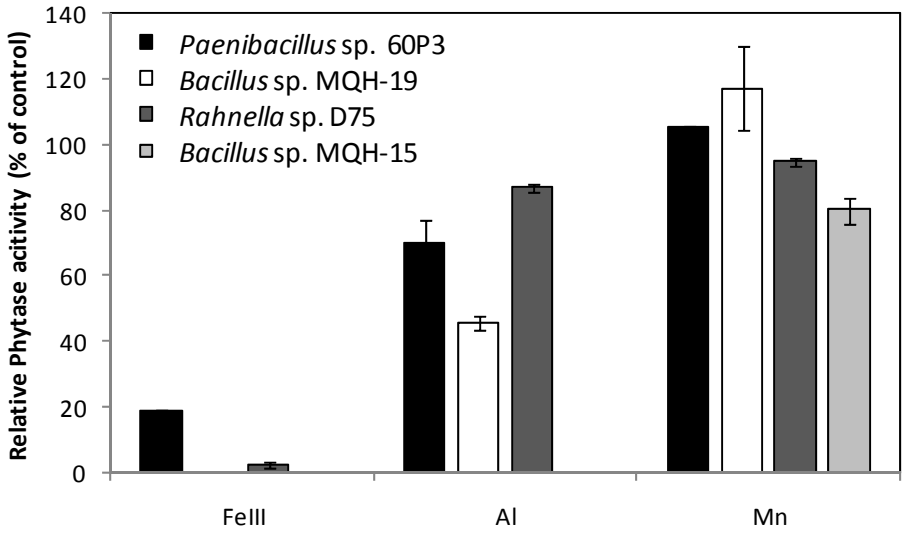

Figure 3.4. Effect of metal cations (10mM) on phytase activity by *Paenibacillus* sp. 60P3, *Bacillus* sp. MQH-19, *Rahnella* sp. D75 and *Bacillus* sp. MQH-15 phytases.

3.5. Conclusions

Except for *Ochrobactrum* sp. C95 and *Bacillus* sp. N1-19NA, all the studied bacteria strains showed a cell-associated phytase activity. Two strains *Bacillus* sp. MQH-19 and *Bacillus* sp. MQH-15 showed alkaline phytase activity, the other phytases were only active at acid pHs. *Bacillus* sp. MQH-19 was the only strain with extracellular alkaline phytase activity, and was found to be a promising strain to be tested as an inoculant in neutral to alkaline wastes environments.

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CHAPTER 4

ACTIVITY STABILIZATION OF *Aspergillus niger* AND *Escherichia coli* PHYTASES IMMOBILIZED ON ALLOPHANIC SYNTHETIC COMPOUNDS AND MONTMORILLONITE NANOCLAYS

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4. ACTIVITY STABILIZATION OF *Aspergillus niger* AND *Escherichia coli* PHYTASES IMMOBILIZED ON ALLOPHANIC SYNTHETIC COMPOUNDS AND MONTMORILLONITE NANOCLAYS

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4.1. Abstract

The aim of this work was to study the stabilization of the activity of two commercial microbial phytases (*Aspergillus niger* and *Escherichia coli*) after immobilization on nanoclays and to establish optimal conditions for their immobilization. Synthetic allophane, synthetic iron-coated allophanes and natural montmorillonite were chosen as solid supports for phytase immobilization. Phytase immobilization patterns at different pH values were strongly dependent on both enzyme and support characteristics. After immobilization, the residual activity of both phytases was higher under acidic conditions. Immobilization of phytases increased their thermal stability and improved resistance to proteolysis, particularly on iron-coated allophane (6% iron oxide), which showed activation energy (Ea) and activation enthalpy ($\Delta H^{\#}$) similar to free enzymes. Montmorillonite as well as allophanic synthetic compounds resulted in a good support for immobilization of *E. coli* phytase, but caused a severe reduction of *A. niger* phytase activity.

4.2. Introduction

The use of phytases in animal feeds (monogastric animals) is today a well studied practice that significantly increases phytate phosphorus degradation and absorption, allowing less phosphorus addition to the diets, thus, reducing total phosphorus excretion (organic and inorganic) and environmental dispersion (Mullaney et al., 2000). Furthermore, other potential uses of phytases have been proposed such as in human nutrition and health, and plant nutrition (Lei et al., 2007).

Enzymes used as animal feed additives account for almost 16% of the total world industrial enzyme market and are expected to reach up to US$375 million in 2012 (Thakore, 2011). Phytases are the major driver for the 6% growth rate expected for the animal feed enzymes sector (CAGR). Phytase supplementation of animal diets to solve environmental problems associated with phosphorus content in animal wastes is extremely cost effective and socially acceptable. Recent research on phytase enzymes has been directed mainly to applications related to improving phytate phosphorus availability in diets for monogastric animals (Vats et al., 2005).

Enzyme-clay associations have been extensively studied because of their effect on enzyme protection against proteolysis and thermal denaturation (Nannipieri et al., 2002). Several biotechnological applications have been developed using this protection mechanism, mainly in bioremediation (Shen et al., 2002) and industrial catalysis (Mateo et al., 2007). Protective effects on phytase enzymes (both in terms of residual activity and stability) have been previously demonstrated as a result of their immobilization on glutaraldehyde-activated silicate (Ullah & Cummins, 1988), gelatin particles by cross linking (Liu et al., 1999), calcium alginate beads (Greiner & Sajidan, 2008; In et al., 2007), and activated sepharose (Greiner & Konietzny, 1996). However, natural supports for enzyme immobilization are

preferable when the enzyme-support complexes will be applied or finally end up in natural ecosystems. In addition, clays are considered adequate candidates as supports for enzyme immobilization due to their low cost, hydrophilicity, high surface area and charge density, chemical and thermal stability, mechanical strength and microbial resistance (Abdul Rahman et al., 2005). Therefore, immobilization of phytase on clays is seen as a reliable, environmentally friendly and cost effective strategy for enhancing the stability of phytases for biotechnological applications.

In Southern Chile, allophane is the most important abundant clay fraction of volcanic soils (Andisols). Allophane is short-range order aluminosilicate that occurs as hollow spherules with 3.5 - 5.5 nm of external diameter, wall thickness of 0.7-1.0 nm and wall perforations of about 0.3 nm in diameter (Parfitt, 2009). In addition, iron oxides of short-range order, notably ferrihydrite, are also widespread in Andisols and occur as coating of the clay mineral particles (Calabi-Floody et al., 2009; Mora, 1992). Mora et al (1994) synthesized allophane coated with ferrhidrite and described their physicochemical characteristics. Allophane nanoclays exhibit a high surface area, a high thermal stability, a pH-dependent surface charge, a unique morphology, and a natural association with iron oxide. Allophane nanoclays have recently been used successfully as supports for enzyme immobilization (Acevedo et al., 2010; Calabi-Floody et al., 2009; Rosas et al., 2008) with a high residual activity after immobilization (Shindo et al., 2002).

The main objectives of this work were: 1) to establish optimal conditions for the immobilization of two commercial microbial phytases, from *A. niger* and *E. coli*, on allophane, iron-coated allophanes and montmorillonite nanoclay supports; and 2) to study the effect of immobilization on phytases' residual activity, thermal stability and resistance to proteolysis.

4.3. Material and methods

4.3.1. Nanoclay characteristics

Allophane, iron-coated allophanes and montmorillonite with contrasting surface properties were used as supports for enzyme immobilization. Their physicochemical properties are given in Table 4.1.

Table 4.1. Physicochemical and charge properties of nanoclays used in this study.

	Al_2O_3 (%)[1]	SiO_2 (%)[1]	Fe_2O_3 (%)[1]	MgO (%)	Surface area (m^2 g^{-1}) BET	EGME [2]	IEP [3]
Al-Si	24	76			191	717	5.2
Al-Si-2%Fe	22	76	2%		252	628	6.2
Al-Si-6%Fe	20	74	6%		287	450	7
Montmorillonite[4]	16	70.1		3.69	83.79	810[5]	Permanent charge(-)

[1]Bernas method; [2]EGME method, according to Mora (1992); [3] Isoelectric Point determined by electrophoretic migration in a Zetasizer 2000 instrument (Malvern, United Kingdom, KCl $1x10^{-3}$ M); [4]Data supplied by product sheet. [5] Theoretical, according to Dyal and Hendricks (1950).

Allophane (Al-Si) nanoparticles were synthesized by precipitation of potassium silicate and aluminum chloride according to Diaz et al. (1990). The Al-Si sample was coated with 2% (Al-Si-2%Fe) or 6% (Al-Si-6%Fe) of iron oxide using a wet impregnating technique according to Mora et al. (1994). The structural properties were evaluated by N_2 adsorption-desorption isotherms at 77ºK and the application of BET equation for the surface area and BJH method for porosity. Isoelectric Point (IP) determined by electrophoretic migration (Zetasizer 2000 instrument, Malvern, United Kingdom; KCl $1x10^{-3}$ M) was used for evaluating the surface charge at different pHs. This nanoclay is a spherical amorphous aluminosilicate with pH-dependent surface charges and a high surface area.

Montmorillonite was purchased from Source Clay Repository (USA). This clay is an expandable 2:1 plate-shaped phyllosilicate, with predominantly permanent negative charges. The surface charge properties of this material contrast the variable charge of the other nanoclay supports used in this study.

4.3.2 Phytase preparations

Phytases used in this study were from *E. coli* (Beijing Challenge Group, 10,000 U g^{-1}) and *A. niger* (EN Bio-Tech Company, 5,000 U g^{-1}), both expressed in *Pichia* sp. and commercialized as feed additives. Phytases were separated from inert material using two cycles of water solubilization and acetone precipitation. Phytase preparations from *E. coli* and *A. niger* showed a specific activity of 150.64 and 481.44 U mg^{-1} at pH 4.5, respectively. Phytase of *A. niger* has a molecular mass of 95 kDa, an optimum activity at pH 2.5 and 5.5, an isoelectric point of 5.0 (Rao et al., 2009), whereas the phytase of *E. coli* has a molecular mass of 44 kDa, an optimum activity at pH 4.5, an isoelectric point of 7.5 and a high substrate specificity (Greiner et al., 1993).

4.3.3. *Phytase-clay complexes*

Phytase-nanoclay complexes (Phy-Al-Si; Phy-Al-Si-2%Fe; Phy-Al-Si-6%Fe; Phy-Mont) were prepared by mixing equal volumes of phytase (1mg ml^{-1}) and nanoclay (1mg ml^{-1}) at different pH values at final buffer concentration of 10 mM (buffers described in 2.4). The suspensions were kept at 10ºC for one hour and were smoothly shaken every 10 min (Rao et al., 2000). The complexes were separated from non-bound proteins by two cycles of centrifugation (16,000 *g* for 15 min at 4 ºC), then resuspended in buffer, and immediately assayed for phytase activity. Supernatant (1st centrifugation) and washing (2nd centrifugation)

were stored at -20 ºC and assayed afterwards for phytase activity and total protein content using BioRad Protein Assay kit. Freezing had no measurable effect on enzyme activity. Total protein and total phytase activity were determined in solution (supernatant and washing), and the immobilized protein and phytase activity was calculated from the difference between the measured and the initially used amounts, respectively. To express adsorption on the solid supports both total protein and total phytase activity were used, because commercial enzyme preparations contain very likely non-phytase proteins as contaminants. Comparison of adsorption expressed per phytase activity with adsorption expressed per total protein may allow for conclusions to be drawn with respect to a possible competition between phytase and non-phytase proteins for the adsorption sites on the solid supports. A pH stability test was performed under similar conditions of the incubation tests but in the absence of nanoclays to evaluate if the supernatant's activity was being affected by pH.

4.3.4 *Phytase activity assay*

The phytase activity was assayed according to Greiner et al. (1993). Briefly, 10 µl of crude protein extract was incubated with 270 µl of phytate solution (2.5 mM phytate in 100 mM acetate-acetic acid buffer, pH 4.5) for 30 min at 37°C. The reaction was stopped by addition of 1,150 µl of a freshly prepared solution of acetone, 5N sulfuric acid, 10 mM ammonium molybdate solution (2:1:1 v/v), and thereafter, 80 µl of 1 M citric acid. After centrifugation (5,000 *g* for 5 min), absorbance of supernatants was measured at 355 nm. One unit of phytase activity was defined as one µmole P released in one min. Blanks were performed by adding stop solution prior to substrate addition.

To measure phytase activity of phytase-clay complexes, pellets were washed twice with the working buffer (defined hereafter), and the resuspended pellets were used as an enzyme

source in the activity assays. The solid support did not interfere significantly with color development in the activity assays. The following buffers were used: sodium acetate-acetic acid (pH 4-5), sodium acetate- HCl (pH 6-7) and Tris-HCl (pH 8-9).

To study the activity-pH dependency of free and immobilized phytases glycine–HCl buffers (pH 2.0-3.5) were also used.

4.3.5 *Isotherms of phytase adsorption*

The adsorption isotherms of both phytases were established by using phytase dilutions (0.25 to 2 mg prot ml^{-1}) in 10 mM acetate-acetic acid buffer pH 4, to form phytase-clay complex. Phytase activity and total protein in equilibrium solution were determined and plotted against the amount of adsorbed phytase activity or total protein, respectively. Langmuir equation was fitted to the experimental adsorption data using JMP8 non-linear fitting tool:

$$X = KCb/(1+KC)$$

where X is the amount of adsorbed phytase (U mg^{-1} nanoclay) or protein (mg mg^{-1} nanoclay), K the affinity constant, b the maximum adsorption of phytase (U mg^{-1} nanoclay) or protein (mg mg^{-1} nanoclay), and C the equilibrium activity (U ml^{-1}) or protein concentration (mg ml^{-1}).

4.3.6. *Thermal stability and proteolytic resistance*

Thermal stability of free and immobilized phytases was assayed by measuring the residual phytase activity after 30 min and 2 h of incubation at 60ºC in 10 mM acetate-acetic acid buffer pH 4 (Rao et al., 2000). Proteolytic resistance of free and immobilized phytases was studied by measuring the residual phytase activity after 2 and 24 h-exposure to proteinase K

(proteinase K to phytase activity 500:1) at 37 ºC in 10 mM acetate-acetic pH 4.0 acid buffer (Rao et al., 2000).

4.3.7. *Temperature profile, activation energy and enthalpy*

The activity-temperature profile was obtained by performing phytase activity assays at temperature ranging from 20 to 65 ºC. The temperature with highest activity (Tmax), activation energy (Ea) and activation enthalpy ($\Delta H^{\#}$) of free and immobilized phytases were calculated using the Arrhenius equation (Bisswanger, 2008).

4.3.8. *X-ray diffraction study*

X-ray diffractometry (XRD), was carried out on clay and phytase-clay complexes. One ml samples were prepared at initial concentration of 10 mg ml^{-1}, and air dried on a glass slide for 3 days at room temperature prior to analysis. Diffractograms were acquired by a Rigaku Geigerflex D/Max IIIC® diffractometer, with Ni filtered CuKa radiation at 35 kW and 35 mA.

4.3.9. *Statistics*

Each experiment was performed in triplicate. Statistical analysis was performed using JMP 8.0 software (SAS Institute). Significance of the treatments was established by one way ANOVA and means were separated by Tukey's test ($P \leq 0.05$).

4.4. Results and discussion

4.4.1. *Adsorption and residual activity of phytases*

Immobilization of phytases on iron coated and uncoated allophanes was quite similar over the entire pH range, with the exception of the *A. niger* Phy-Al-Si-6%Fe complex that showed a minimum of adsorption at pH 6-7 (Figure 4.1A and 4.1B). Immobilization of phytases on montmorillonite showed a linear increase from alkaline to acid conditions with both phytase preparations. The immobilized amount of *E. coli* phytase was higher under acidic conditions than in alkaline conditions, in all cases.

In general terms, total protein immobilization using both phytase preparations was enhanced under acidic conditions (Figure 4.1 C and D). Using the *A. niger* phytase preparation, immobilization of total protein was higher on montmorillonite and uncoated allophane compared with iron coated allophanes at pH 4. With the *E. coli* phytase preparation, immobilization of total protein was higher on montmorillonite than on allophanes over the complete pH range under investigation.

All nanoclay support materials used in this study have both hydrophilic and hydrophobic surface adsorption sites (Mora, 1992). Therefore, changes in their surface charge due to the alteration of pH are expected to have a significant influence on adsorption and enzyme orientation by altering electrostatic attraction and repulsion forces.

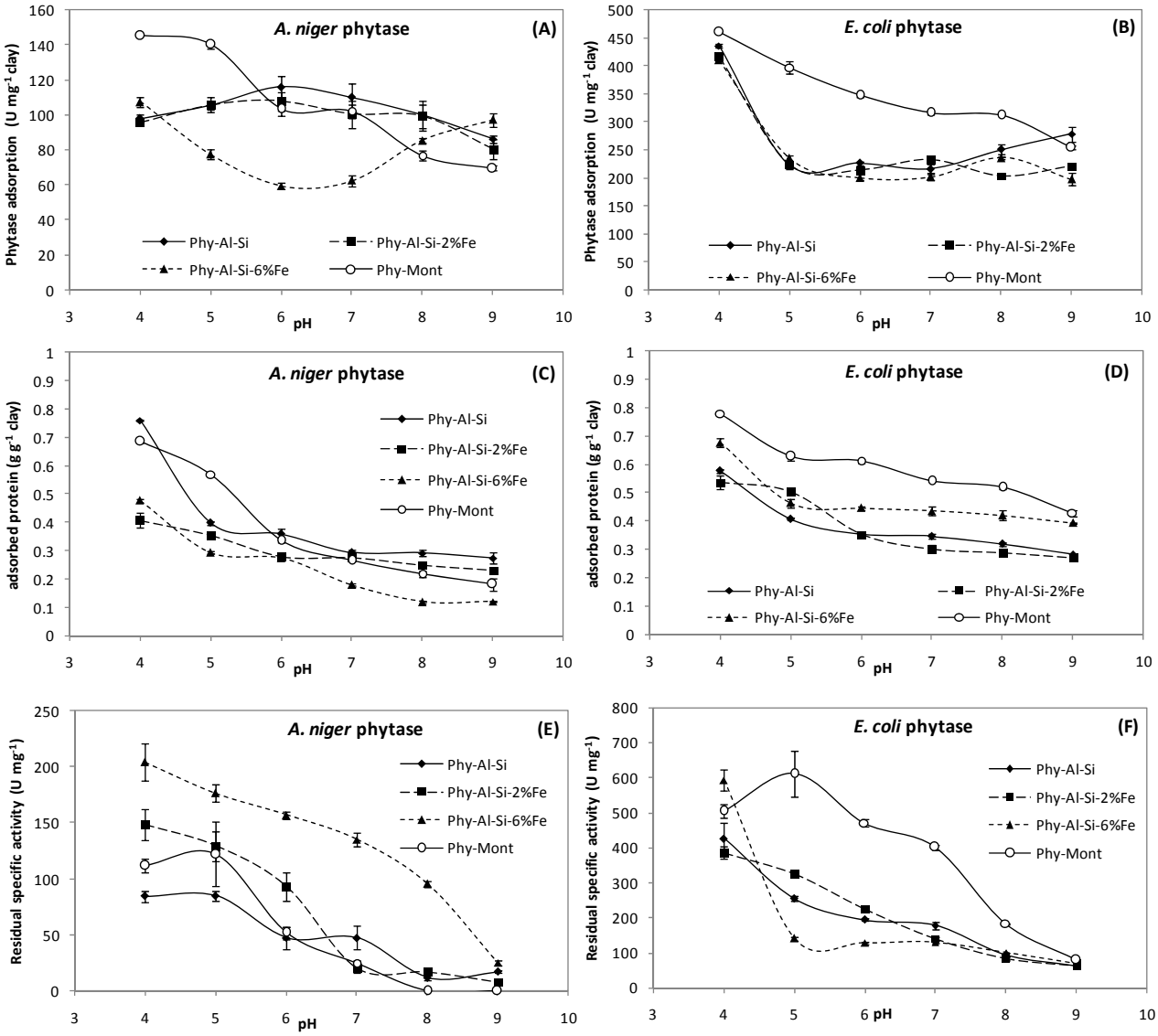

Figure 4.1. pH dependency of phytase activity adsorption (A and B) protein adsorption (C and D), and residual specific activity (E and F) of *A. niger* and *E. coli* phytases immobilized on nanoclays at experimental conditions (1 mg clay ml^{-1}; 1 mg protein ml^{-1} of enzyme extract; 10 mM of working buffer).

Surface charge of supports may play an important role in protein-materials adsorption and biocompatibility (Andrade & Hlady, 1986). Net charges of the studied enzymes and surface charges of the used solid supports at different pH-values do not explain clearly the total adsorption behavior of the tested phytase-clay complexes.

A significant amount of phytases was adsorbed even under pHs where a theoretical electrostatic adverse conditions are present. They coincide with pHs at which uncharged SOH sites predominate on allophane surfaces (Mora, 1992), indicating a possible high hydrophobic component governing the adsorption. On the other hand, many authors have documented inconsistencies for the hydrophilic adsorption of proteins on clays by the analysis of the isoelectric points; in most cases, higher adsorption has been verified at pH around the enzyme isoelectric point (Norde et al., 2008; Quiquampoix, 2008). Other possible factors that may have affected phytase adsorption are: a) competition of phytases with other proteins in the phytase preparation for adsorption sites; b) change of surface properties of nanoclays after adsorption of the first protein layer; c) pH dependent protein-protein interactions.

Transmission electron microscopy images showed that allophanic compounds form porous aggregates of up to 100 nm (Calabi-Floody et al., 2009; Mora et al., 1994). These pores were fully occupied by the enzyme preparations during incubation, and may be causing an entrapment effect that could also be responsible for the observed immobilization behavior (Figure 4.2).

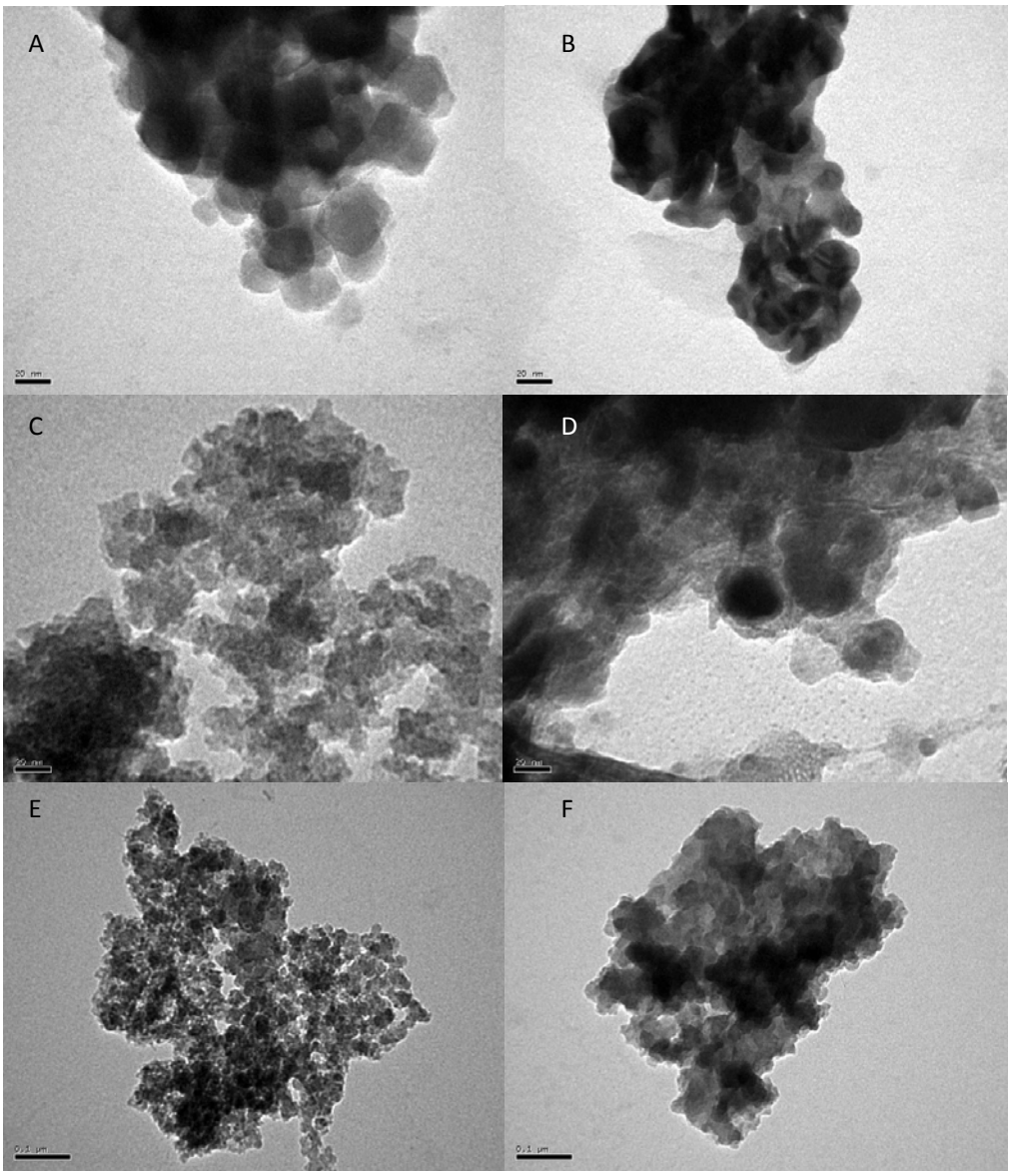

Figure 4.2. Transmission electron micrographs for Al-Si (A), Phy-Al-Si (B), Al-Si-2%Fe (C), Phy-Al-Si-2%Fe (D), Al-Si-6%Fe (E) and Phy-Al-Si-6%Fe (F). Phytase used in these images was from *E. coli*.

Due to the possible interferences of multiple factors on the immobilization behavior of the phytases under research, the analysis was preferentially focused on effects of adsorption on residual phytase activity and physicochemical properties of the enzymes. The results showed

that the residual activity immediately after adsorption was highly favored when complexes were formed at acid pHs (Fig. 4.1 E and F). To assure that the decrease in residual activity was due to adsorption, a pH stability test was performed with both phytases. No significant reduction of activity was verified due to pHs from 4 to 9, at conditions similar to the adsorption incubations (but in absence of nanoclays). Apparently, the native structure of phytases was better conserved under conditions around their respective pH optimum. Adsorption at alkaline pH values seems to result in a change in protein structure leading to an inactive enzyme. Surface charge distribution of both enzymes greatly changes at different pHs. Due to the higher isoelectric point of aminoacid residues at the active site of both phytases, at acid pH values this region has a substantially higher positive electrostatic potential in comparison with the rest of the enzyme surface (Kostrewa et al., 1999; Lim et al., 2000). This may prevent the binding of net positively charged allophanes at the active site of phytases, therefore leading to higher residual phytase activity at acid pH values. Iron coating promoted a progressively higher residual specific activity after adsorption for the *A. niger* phytase (6% Fe>2% Fe>0% Fe). For *E. coli* phytase, the protective effect of iron coating was less clear than for *A. niger* phytase.

Based on these findings, additional analyses were performed at pH 4 (10 mM acetate-acetic acid buffer). At this pH, the residual activity expressed as % of the adsorbed activity showed significant differences between *A. niger* and *E. coli* only on montmorillonite support (Figure 4.3). The activity of *A. niger* phytase was statistically higher on Al-Si-6%Fe than on other supports. Additionally, the rate of deactivation of *A. niger* phytase immobilized on montmorillonite at room temperature was 2 fold higher than that of the free enzyme (data not shown), indicating a change in the 3-dimensional structure of the enzyme after interaction with montmorillonite.

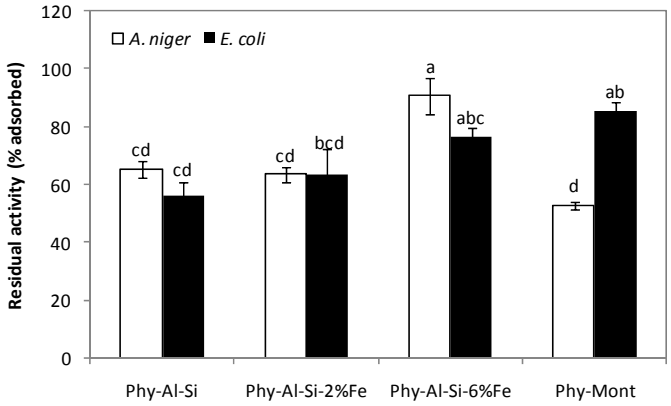

Figure 4.3. Phytase residual activity as a % of adsorbed activity of enzyme preparations of *A. niger* (A) and *E. coli* (B) phytases immobilized on nanoclays at pH 4 at experimental conditions (1 mg clay ml^{-1}; 1 mg protein ml^{-1} of enzyme extract; 10 mM of working buffer).

The high residual phytase activity on allophane supports is in accordance with results previously reported for the immobilization of phosphatase (Calabi-Floody et al., 2009; Rosas et al., 2008) and manganese peroxidase (Acevedo et al., 2010). Shindo et al. (2002) reported a higher residual acid phosphatase activity when immobilized on allophane, than on other supports. Similar to results for *A. niger* phytase, Leprince and Quiquampoix (1996) reported a partial inactivation of wheat phytase by immobilization on montmorillonite.

The Langmuir parameters of maximum adsorption (*b*) and affinity (*k*) fitted to the experimental data are shown in Table 4.2. Isotherms of adsorption were analyzed using both total protein and total phytase activity. Iron coating of allophane seems to progressively increase *b* and decrease *k* for the *A. niger* phytase preparation and decrease *b* and increase *k* for the *E. coli* phytase preparation in the activity isotherms. Similar trends were observed for *k* and *b* parameters in the protein isotherms, with the exception of *b* for the *A. niger* phytase preparation. Montmorillonite support showed a higher estimated *b* with *A. niger* in both

activity and protein isotherms. Montmorillonite *k* parameter was considerably higher for both enzymes studied in protein isotherms than allophanic support. However, this tendency was not clear in the activity isotherms. The experimental conditions of the immobilization assays do not fulfill all the assumptions of the Langmuir adsorption model, and the poor fit obtained also underlines the complexity of the conditions affecting adsorption.

Table 4.2. Langmuir parameters (*K* and *b*) of activity and protein adsorption isotherms of *A. niger* and *E. coli* phytases immobilized on nanoclay supports at pH 4.

Activity isotherms	**Phytase from *A. niger***				**Phytase from *E. coli***			
	b	**s.e.**	***K***	**s.e.**	***b***	**s.e.**	***k***	**s.e.**
	(U mg^{-1})		**(ml U^{-1})**		**(U mg^{-1})**		**(ml U^{-1})**	
Phy-Al-Si	108	±12	0.209	±0.121	732	±72	0.054	±0.023
Phy-Al-Si-2%Fe	113	±16	0.156	±0.101	707	±65	0.073	±0.029
Phy-Al-Si-6%Fe	183	±23	0.020	±0.005	655	±38	0.143	±0.039
Phy-Mont.	379	±83	0.204	±0.122	770	±157	0.101	±0.198

Protein Isotherms	**Phytase from *A. niger***				**Phytase from *E. coli***			
	b	**s.e.**	***K***	**s.e.**	***b***	**s.e.**	***k***	**s.e.**
	(mg mg^{-1})		**(ml mg^{-1})**		**(mg mg^{-1})**		**(ml mg^{-1})**	
Phy-Al-Si	1.14	±0.13	14.28	±4.43	2.07	±0.23	3.00	±0.76
Phy-Al-Si-2%Fe	1.30	±0.15	13.72	±3.81	1.83	±0.19	3.03	±0.74
Phy-Al-Si-6%Fe	1.26	±0.11	9.85	±2.00	1.66	±0.19	4.85	±0.43
Phy-Mont.	2.20	±0.21	25.36	±5.71	1.23	±0.11	43.49	±8.17

Isotherms of adsorption showed that phytases have a contrasting affinity and maximum adsorption to Al-Si-6%Fe and montmorillonite in comparison to Al-Si and Al-Si-2%Fe. The iron coating of allophane had a direct effect on its surface properties by increasing its surface acidity and capacity to complex anions (Mora et al., 1994). Although Mora (1994) showed a

decrease in EGME total surface area of allophane, an increase in the BET external surface area was shown in this study. This difference between results from EGME and BET methods is attributed to the occlusion of allophane inner pores by the iron oxide coating (Mora et al., 1994), impeding EGME molecules to access total internal surface area. The changes in surface properties including surface area, net charge and charge density, are expected to be directly related to the observed variations of phytase adsorption behavior. The phytases of *A. niger* and *E. coli* showed different tendencies of adsorption parameter response to iron coating, suggesting that adsorption was also strongly dependent on physicochemical properties of the enzymes. The effect of these properties on adsorption to soil solid phase has previously been reported and discussed (George et al., 2007).

Analysis of residual phytase activity of adsorption isotherm trials (data not shown) indicated that both phytases immobilized on montmorillonite increased specific residual activity (U mg^{-1}) with increasing protein concentration in the incubation mixture. On the other hand, specific activity of phytase immobilized on coated and uncoated allophanes was either not dependent on the protein concentration in the incubation mixture or even slightly decreased with increasing protein concentration.

Montmorillonite X-ray diffractograms showed well defined sharp peaks. While uncomplexed montmorillonite showed basal interplanar spacing (d) of 1.5 nm, this value was in general increased to 5.7 nm after immobilization of phytase (Table 4.3). Immobilization of *A. niger* phytase on montmorillonite at pH 4 and 0.2 mg protein mg^{-1} clay resulted in an intermediary interplanar spacing of 2.46 nm. This may indicate a lower occupation of montmorillonite interlayer spacings by *A. niger* phytase than by *E. coli* phytase. This result is probably due to differences of enzyme size, since *A. niger* phytase have a higher molecular mass than the phytase from *E. coli.*

Table 4.3. Interplanar spacing d (nm) measured by X-ray diffraction to montmorillonite complexed with *A. niger* and *E. coli* phytases.

Incubation conditions	*A. niger* (nm)	*E. coli* (nm)
Uncomplexed montmorillonite	1.50	1.50
Complexed at pH 4 and 0.2 mg mg^{-1}	2.46	5.76
Complexed at pH4 and 0.4 mg mg^{-1}	5.76	5.87
Complexed at pH 8 and 0.2 mg mg^{-1}	5.66	5.72

4.4.2. *Thermal stability and resistance to proteolysis of free and immobilized phytases*

Activity of free and immobilized phytases was measured after 30 min and 2h at 60ºC in order to evaluate the effect of immobilization on thermal stability of the enzymes (Fig. 4.4 A and B). With the exception of *A. niger* phytase on montmorillonite, immobilization resulted in a higher thermal stability of the phytases. *A. niger* phytase immobilized on montmorillonite was completely inactive after 30 min at 60 ºC. However, *A. niger* immobilized on Al-Si-6%Fe showed no significant loss of activity even after 120 min at 60 °C. Resistance to proteolysis after incubation in the presence of proteinase K is shown in Fig. 4.4 C and D. While the free enzymes were completely inactivated after 2 h of incubation, the immobilized enzymes showed significant activity even after 24 h of incubation. Both enzymes showed the higher resistance to proteolysis when immobilized on coated and uncoated allophanes, than other used supports.

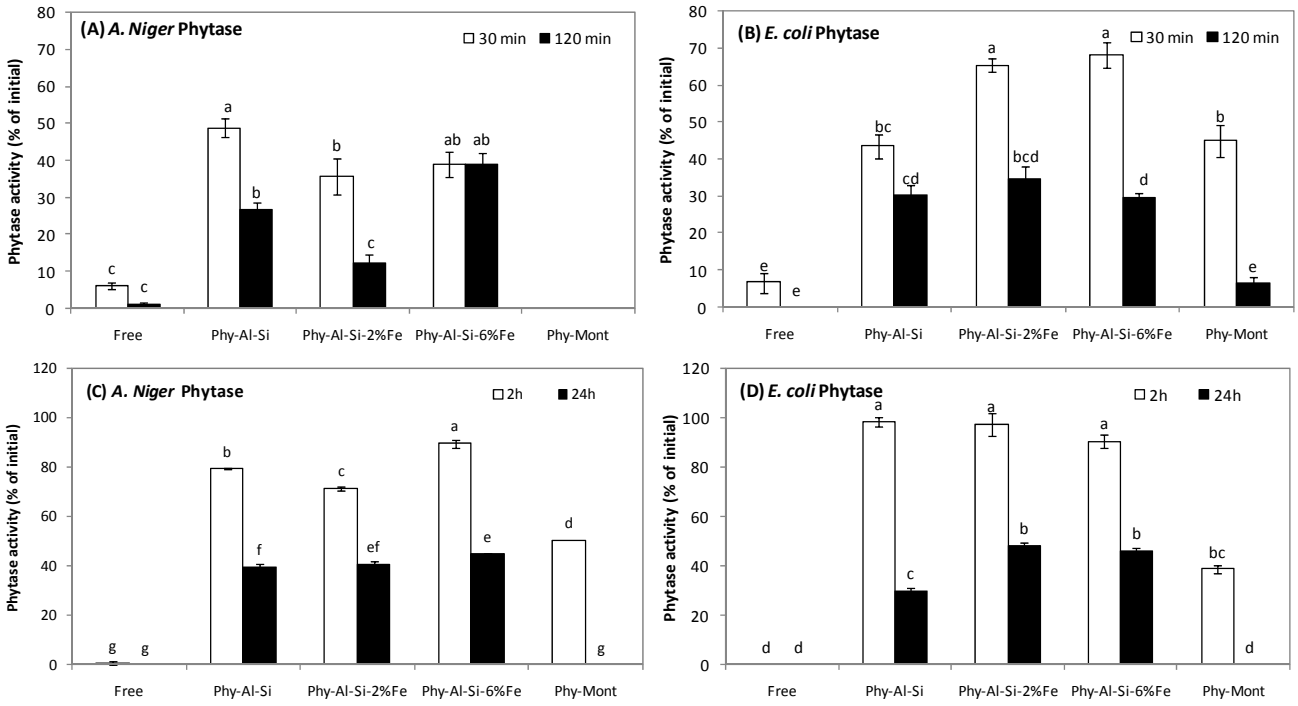

Figure 4.4 -Thermal stability (A and B) and resistance to proteolysis (C and D) of *A. niger* and *E. coli* phytases free and immobilized on nanoclay at pH 4.

In general, immobilized phytases showed a higher resistance to elevated temperatures and proteolysis compared with the free enzymes. This behavior was already reported for different enzymes immobilized on clays (Nannipieri et al., 2002). The stability of acid phosphatase from potato was enhanced after immobilization on complexes containing Fe oxide (Rao et al., 2000). Silica particles are reported to be good supports for enzyme immobilization, enhancing their stability and efficiency in batch reactors (Bhattacharyya et al., 2010). However, benefits of iron coating as found in this study need to be further confirmed, and additional investigations are required to apply this technology to industrial or environmental processes.

4.4.3. *Activity-temperature and activity-pH dependency of free and immobilized phytases*

The activity-temperature curves showed different patterns between free and immobilized phytases. The experimental data showed log-linear behavior between 20 and 45 ºC. A further increase in temperature resulted in an increase in phytase denaturation with increasing temperature. The temperature with highest activity (T_{max}) for free phytases was 45 ºC, lower than those previously reported for *E. coli* (55 ºC) and *A. niger* (60 ºC) phytases (Greiner et al., 1993; Rao et al., 2009). The difference may be due to different experimental conditions in the the phytase activity assay. With the exception of *E. coli* phytase immobilized on montmorillonite, phytases immobilized on nanoclays showed higher T_{max} than free enzymes (Table 4) due to their greater resistance to thermal denaturation.

The rate of increase in the velocity of phytate dephosphorylation with increasing temperatures (Q_{10}; measured between 20 and 45 ºC) was as expected, around 2–3 per each 10 ºC, which is in accordance with van't Hoff's rule. Thermal coefficient (Q_{10}) was increased by immobilization for both enzymes (Table 4.4). While 2% iron coating of allophane did not affected Q_{10} compared with uncoated allophane, the 6% iron coating resulted in a decrease in this parameter. The increase of Q_{10} values for immobilized phytases compared to free phytases, suggests that the heterogeneous catalysis system may positively affect phytate dephosphorylation. After immobilization of phytases on solid supports, phytate dephosphorylation is expected to be affected by new forces controlling for example the diffusion of substrates and products. This change in catalytic behavior may also be interpreted as a consequence of enzyme conformation changes due interaction with the clay surface (Leprince & Quiquampoix, 1996).

Table 4.4. Activation energy (Ea), and activation enthalpy (ΔH#), temperature coefficient (Q_{10}) and temperature of maximum activity (T_{max}) for *A. niger* and *E. coli* phytases free and immobilized on nanoclays.

	***A. niger* Phytase**						***E. coli* Phytase**					
	Ea (kJ mol^{-1})	**(r^2)**	**$\Delta H^{\#}$** (kJ mol^{-1})	**(r^2)**	**Q_{10}** (20-45°C)	**T_{max}** °C	**Ea** (kJ mol^{-1})	**(r^2)**	**$\Delta H^{\#}$** (kJ mol^{-1})	**(r^2)**	**Q_{10}** (20-45°C)	**T_{max}** °C
Free phytase	32.77	0.92	31.68	0.94	2.09	45	26.98	0.99	25.89	0.99	1.83	45
Phy-Al-Si e	46.07	0.99	44.97	0.99	2.91	50	31.41	0.98	30.31	0.98	2.62	50
Phy-Al-Si-2%	37.88	0.96	36.77	0.96	2.93	55	33.14	0.97	32.01	0.97	2.55	55
FPhy-Al-Si-6%Fe	36.00	0.98	34.90	0.98	2.36	55	28.93	0.99	27.83	0.99	1.95	60
Phy-Mont.	35.53	0.98	34.43	0.98	2.36	55	49.78	0.98	48.67	0.98	2.53	45

Both activation energy (Ea) and activation enthalpy ($\Delta H^{\#}$) were estimated using Arrhenius plots of the log linear interval of activity-temperature dependency curves. Temperatures over 45 ºC were not used in this analysis since Arrhenius equation considers no thermal inactivation of the enzymes. Immobilization of both phytases resulted in higher Ea and $\Delta H^{\#}$ values compared with the free enzymes (Table 4.4). The coating of allophanes with 6% iron resulted in a decrease in the Ea and $\Delta H^{\#}$ values for the immobilized phytases compared to the values for the phytases immobilized on the other used allophanic compounds. The Ea and $\Delta H^{\#}$ values for *A. niger* phytase immobilized on montmorillonite were lower than for the *A. niger* phytase immobilized on coated and uncoated allophanes. However, Ea and $\Delta H^{\#}$ values for *E. coli* phytase immobilized on montmorillonite were higher compared with those for the immobilized *E. coli* phytase on allophanes.

The values of activation energy (Ea) of free phytases (Table 4.4) are in accordance with the values previously reported by Konietzny and Greiner (2002) for the hydrolysis of sodium phytate by a wide range of phytate-degrading enzymes. However, the measured Ea values

were considerably lower than those previously reported for free *E. coli* (53.7 kJ mol^{-1}) and *A. niger* (59.2 kJ mol^{-1}) phytases (Greiner & Konietzny, 1996; Sariyska et al., 2005). Ea and $\Delta H^{\#}$ values strongly depend on the pH and duration of the activity assay, and their comparison is only valid under the same assay conditions (Bisswanger, 2008; Tijskens et al., 2001). The increase of Ea and $\Delta H^{\#}$ values after immobilization is expected and may indicate a modification of the reaction mechanisms either by a less favorable location of phytases molecules on the supports, or due to the presence of diffusion restrictions of substrate and products (Greiner & Konietzny, 1996; Rao et al., 2000). Iron coating progressively decreased Ea and $\Delta H^{\#}$ values of immobilized phytases compared to uncoated allophanes, suggesting a better arrangement of phytase enzymes on the clay surface.

Optimal pH curves of both phytases were significantly affected by immobilization (Fig. 4.5 A and B). The optimal pH peak of free *A. niger* phytase was completely flattened after immobilization, presenting a relatively even activity at pHs under 5.5. The exception was for the phytase complexed with Al-Si-6%Fe support which maintained the same optimal pH peak of the free phytase , but of smaller proportion. The bell shaped activity-pH dependency curve of free *E. coli* phytase was changed to a ramp shaped curve after immobilization on nanoclays, and optimum pH shifted from 4.5 to 5.0.

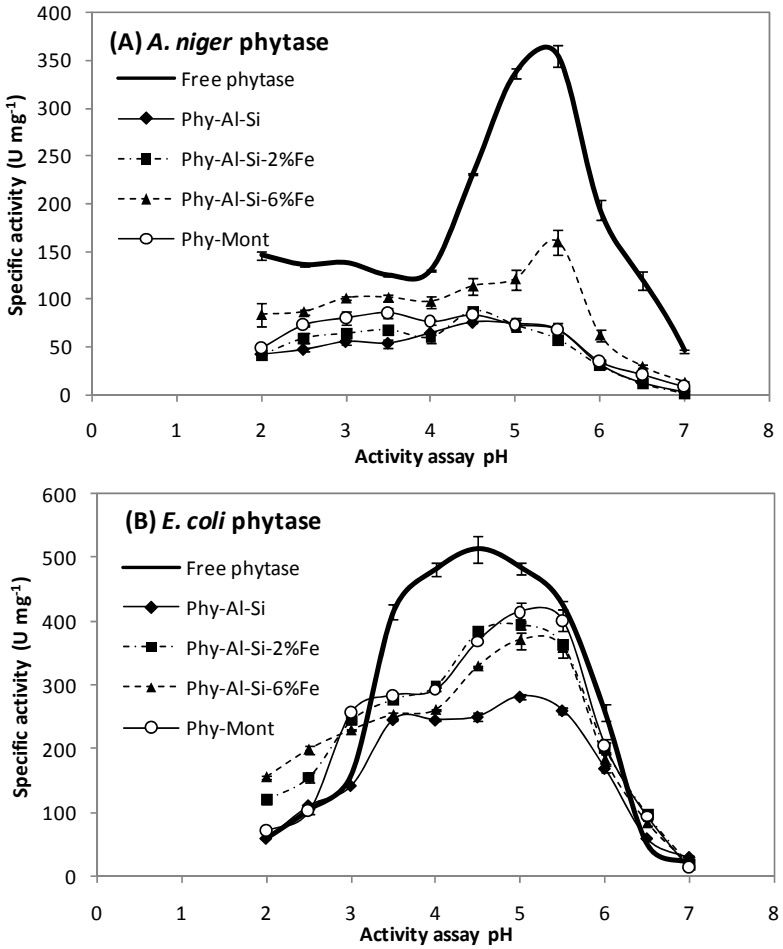

Figure 4.5 – Activity-pH dependency of *A. niger* (A) and *E. coli* (B) phytases, free and immobilized in nanoclay supports.

4.5. Conclusions

The residual activity of both *E. coli* and *A. niger* phytases immobilized on allophanic and montmorillonite nanoclay supports was higher under acidic conditions and led to a higher thermal stability and resistance to proteolysis. Among the studied nanoclays, the synthetic allophane coated with 6% of iron oxide seems to be the most promising support, showing higher phytase stabilization, and Ea, $\Delta H^{\#}$ and optimal pH closer to free enzymes.

Montmorillonite resulted in a good immobilizing support only for the smaller *E. coli* phytase, and an inhibitor for *A. niger* phytase activity.

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CHAPTER 5

A NOVEL PHOSPHORUS BIOFERTILIZER BASED ON CATTLE MANURE AND PHYTASES-NANOCLAY COMPLEXES

Manuscript in preparation

5. A NOVEL PHOSPHORUS BIOFERTILIZER BASED ON CATTLE MANURE AND PHYTASES-NANOCLAY COMPLEXES

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5.1 Abstract

Phytate and other phytase labile organic phosphorus (P) are abundant in both soils and manures. These recalcitrant forms of P accumulate in soils by their interaction with mineral particles. The aim of this work was to evaluate the potential of treating cattle manure with phytases stabilized in allophanic nanoclays, as a novel P biofertilization technology for crops grown in volcanic soils (Andisol). Two Andisols and two manures with contrasting inorganic Pcontent were used: Low P soil from Piedras Negras series (SPN-LP); High P soil from Freire Series (SF-HP); Low P Waste (WPN-LP); High P Waste (WF-HP). The used Andisols and manures were incubated with phytase-nanoclay complexes and the inorganic P was determined in the NaOH-EDTA and bicarbonate extracts. The WPN-LP was also inoculated with an alkaline β-propeller phytase (BPP) producing bacterium. The incubated SPN-LP and SPN-LP-WPN-LP mixture were evaluated for their P supplying capacity to wheat plants under greenhouse conditions. Our results

indicated that the treatment of cattle manure with phytase stabilized in nanoclays resulted in a significant ($P \leq 0.0.5$) increase in the inorganic P. The use of phytase treated cattle manure increased 10% plant dry weight and 39% P concentration in wheat plants under greenhouse conditions, being equivalent to a P fertilizer dose of about 150 kg of P ha^{-1}. In the case of low P cattle manure inoculated with BPP producing bacterium, inorganic P increased 10% in soil extracts (NaOH EDTA and Bicarbonate). However, the application of this treated manure did not result in a significant response to wheat growth and P acquisition. Our results suggest that this novel approach of incubating cattle manure with phytase stabilized in nanoclays enhances organic P cycling and P nutrition of plants grown under P-deficient soils.

5.2 Introduction

Soil organic phosphorus (Po) accounts from 40 to 80% of total phosphorus (P_t) and its content in Chilean Andisols averages over 1,000 mg kg^{-1} (Borie & Rubio, 2003; Dalal, 1977). Among Po forms, phytate is usually expected to be the most abundant P form in soils (Borie & Rubio, 2003; Turner et al., 2002). In addition to phytate, other forms of Po hydrolysable by phytases are ubiquitous in soil and waste environments (Menezes-Blackburn, 2012). Soil Phytase labile organic P ($P_{Phy\text{-}lab}$) pool construction occurs naturally due to P fixation mechanisms. Nevertheless, the accumulation of these compounds is significantly enhanced by both inorganic and organic P fertilization (George et al., 2007a; Turner et al., 2002). A huge interest has been recently directed to technologies and practices related to increasing plant access to this important P pool, given that P fertilizers are manufactured from a non-renewable and limited mineral resource (Abelson, 1999; Menezes-Blackburn, 2012; Richardson et al., 2007).

Wastes from monogastric animals are the richest in phytate, since these animals are unable to mineralize P-phytate present in feeds. Even ruminants are not capable of mineralizing this entire

P pool, especially in high phytate grain-based diets (Brinch-Pedersen et al., 2002; Godoy et al., 2005; Godoy & Meschy, 2001). Feed phytate passes undigested or partially digested through gastrointestinal tracts, leading to animal wastes with high $P_{Phy\text{-}lab}$ content, and when used as organic fertilizers, these manures represent an important source of $P_{Phy\text{-}lab}$ input to agro-ecosystems (Fuentes et al., 2006; Lott et al., 2001; Menezes-Blackburn, 2012).

Bovine organic wastes are responsible for a higher worldwide input of $P_{Phy\text{-}lab}$ to soil environments in comparison with monogastric animals, due to the enormous population of these animals (1.3 billion cattle) and to the higher excretion in dry weigh basis (around 6 kg day $^{-1}$) (FAO, 2006). More than four thousand tons of $P_{Phy\text{-}lab}$ are excreted every day by the world cattle population; which is at least 10 and 40 times higher in total daily excretion bases than the excreted by pig and poultry populations respectively (Menezes-Blackburn, 2012). Composted cattle manures and liquid manure slurries are often used as fertilizers for crops and pastures. Due to the large amount of unavailable organic P compounds such as phytate in manures, these fertilization practices are often a P-over application on total P basis (Sharpley & Moyer, 2000). Consequently, organic fertilization leads to organic P accumulation in soils (Dao, 2004) and to an increase of inorganic and $P_{Phy\text{-}lab}$ losses in runoff sediments, as well (Green et al., 2007).

We have successfully stabilized the phytase activity from two commercial enzymes from *Escherichia coli* and *Aspergillus niger* in synthetic 6% iron coated allophane nanoclays (Menezes-Blackburn et al., 2011). These phytase-nanoclay complexes are highly protected against proteolysis and an increased catalytic efficiency is expected to these compounds in weeks scale treatments (Menezes-Blackburn et al., 2011). These allophanic compounds are similar to the ones naturally occurring in Andisols, and are seen as a reliable, environmentally friendly and cost effective phytase carrier for possible biotechnological applications in these soils. The objectives of this work were: a) to test the effect of free and immobilized phytases, and BPP

producing bacteria inoculation in the organic P hydrolysis from cattle manure and Andisols; and b) to evaluate the use of cattle manure treated with these phytase-nanoclay complexes as a novel P biofertilization strategy to *Triticum aestivum* crops grown in P deficient Andisols.

5.3 Materials and methods

5.3.1 Phytases and phytase producing bacteria (PPB)

Phytases used in this study were from *E. coli* (Beijing Challenge Group, 10,000 U g^{-1}) and *A. niger* (EN Bio-Tech Company, 5,000 U g^{-1}), both commercialized as feed additives. Phytases were separated from inert material using two cycles of water solubilization and acetone precipitation. Phytase preparations from *A. niger* and *E. coli* showed a specific activity of 150.64 and 481.44 U mg^{-1} at pH 4.5, respectively. Phytase of *A. niger* and of *E. coli* are both active at acid pHs (Greiner et al., 1993; Rao et al., 2009).

Phytase immobilization in 6% iron-coated allophanes were performed according to Menezes-Blackburn (2011) and the phytase activity was assayed according to Greiner et al. (1993).

The inoculation with a bacteria strain expressing an alkaline phytase activity was proposed as an alternative for the phytase treatment of neutral to alkaline manure under unbuffered conditions, since commercial phytases are active on acid pHs. The *Bacillus* sp. MQH-19 strain, isolated in a previous study, was selected for expressing a high alkaline activity of an extracellular β-propeller phytase (Acuña et al., 2011; Jorquera et al., 2011).

5.3.*2 Soil and cattle manure: sampling and chemical properties*

Soils and manure samples used in this study were selected for their contrasting Pi content (Low and High; Tables 5.1 and 5.2) and for being representative of Southern Chilean conditions. Two soil samples from an Andisol of Piedras Negras series (Soil Piedras Negras -Low P [SPN-LP];

40° 20' S and 72° 35' W) and Freire series (Soil Freire - High P [SF-HP]; 38º50´ S and 72º41´ W) were collected at 0-20 cm depth. Two cattle manure collected in Cerro Azul farm at Rio Blanco, Chile (Waste Piedras Negras -Low P [WPN-LP]; 40° 20' S and 72° 35' W) and in Las Rosas farm in Freire, Chile (Waste Freire - High P [WF-HP]; 38°57′ S, 72°47′ W). In both farms, animals are kept confined during winter and the accumulated manure of this period (complex mixture of manure, straw, feed and soil) were kept under open field storage yard conditions at least for one year before this experiment.

Table 5.1. Selected chemical properties of soils and wastes used in this study.

Soils	pH	Olsen P mg kg $^{-1}$	Total P mg kg $^{-1}$	Ni (KCl) mg kg $^{-1}$	Al %	OM %	CICE cmol+kg^{-1}
SPN-LP	5.4	3.0	1123.8	36.0	15.2	17.0	2.95
SF-HP	36.0	19.0	1783.5	5.7	0.2	17.0	11.42
Wastes	**pH**	**$NaHCO_3$ Pi** mg kg $^{-1}$	**Total P** mg kg $^{-1}$	**Ni (KCl)** mg kg $^{-1}$	**Nt** %	**C** %	**C:N**
WPN-LP	6.2	60.8	2340.7	289	1.4	22.7	16.2
WF-HP	7.4	168.1	4406.1	77	1.2	15.7	13.1

All analysis were performed according to Sadzawka et al (2006) Sadzawka et al. (1990) and Fuentes et al. (2009); SPN-LP: low phosphorus soil; SF-HP: high phosphorus soil; WF-HP: high phosphorus waste; WPN-LP: low phosphorus waste;

5.3.*3 Soil-phytase and waste-phytase incubation studies*

A series of microcosm experiments were performed to assess: (a) the effect of phytase immobilization on P hydrolysis from these complex substrates, (b) the hydrolysis of organic P

from fresh added phytate, (c) total phytase labile P ($P_{Phy\text{-}lab}$), and (d) the phytase labile P under natural conditions.

To assess the effect of phytase immobilization on P hydrolysis (a) and the recovery of inorganic P from fresh phytate (b), 1 g DW of soil or waste were incubated in 20 ml liquid suspension for 16 h at 37 °C, pH 4.5 (acetate acetic acid 100 mM buffer), and phytase concentration of 0.5 U g^{-1}. The enzyme preparations consisted of *E. coli* and *A. niger* phytases either alone or combined, and either free or immobilized in allophane coated with 6% Fe according to Menezes-Blackburn et al (2011). These conditions were established after a series of preliminary incubation tests performed using free *A. niger* with SPN-LP and WPN-LP substrates. After incubation, phosphorus was extracted from soils or manure with 0.25 M NaOH and 0.05 M EDTA shaken for 16 h at 20°C and 1:30 solid to liquid ratio (Cade-Menun & Preston, 1996).

Enzyme dose was raised to 20 U g^{-1} of immobilized phytases (10 U of *E. coli* and 10 U of *A. niger*) in liquid incubations described before to assess total phytase labile P ($P_{Phy\text{-}lab}$), for returning a maximum increase of Pi in the NAOH-EDTA extract.

Solid incubations (at field capacity and phytase concentration of 10 U of *E. coli* and 10 U of *A. niger*) with no pH buffering were set up with Low P Soil and WPN-LP for assessing the total phytase labile P under natural conditions (d). These treated soil, waste and soil-waste mixture (6:1 ratio) were used in the greenhouse experiment to assess the increase of plant available P by these treatments (see section 2.5).

5.3.*4 Inoculation of Low P Waste with phytase producing bacteria*

The *Bacillus* sp. MQH-19 which expresses an extracellular β-propeller phytase (BPP) (Jorquera et al., 2011) was used for testing its possible use as an inoculant for increasing P availability in WPN-LP.

To examine the effects of inoculation and phytate addition on bacterial communities, 10 g WPN-LP were sieved and placed in sterile plastic tubes in presence or absence of phytate (0.5 mMol g^{-1}) and MQH-19 inoculum (10^9 cells g^{-1} of waste). The waste moisture levels were adjusted to 80% of water holding capacity, after which the samples were incubated for 6 days at 30°C. Liquid incubations were also set up with 1 g DW of waste and were incubated in 20 ml liquid suspension for 48 h at 30 °C in presence or absence of phytate and MQH-19 inoculum, in a factorial design.

Bacterial community structures for the predominant bacteria of the solid incubations were examined by PCR-DGGE of 16S rRNA gene. Total DNA was extracted using a Power Soil® DNA Isolation Kit (Mo Bio Inc., USA) according to the manufacturer's instructions. PCR-DGGE was carried out according to the previously described by Jorquera et al. (2010). In brief, primers 933F−GC and 1387R (Muyzer & Smalla, 1998) were used to amplify ~450 bp DNA fragments of bacterial 16S rRNA genes. The DGGE electrophoresis was run for 10 h at 100 V and 35−65% gradient (urea and formamide) gels were stained with SYBR Gold (Molecular Probes, Invitrogen Co.) for 30 min and photographed on a UV transilluminator. Differences in bacterial community structure were assessed by image analysis using Phoretix 1D (Total lab ltda.). Representative bands in DGGE gels were carefully excised, re−amplified and sequenced by Macrogen, Inc. (Korea).

The presence and expression of BPP gene was assessed by qPCR. The primer set MQHf (5'–TTC CTA TCC TAC CGG GAA GC–3') and MQHr 87 (5'–TGC TTT GTA ATG TGC CGT TT–3'), designed by Jorquera et al., (2012), was used to target the BPP gene. The PCR reactions were performed in a 7300 Real Time 91 PCR System (Applied Biosystems) using Maxima® SYBR Green/ROX qPCR Master Mix (Fermentas Life Sciences) following the manufacturer's instructions. The PCR conditions were as follows: an enzyme activation step at 95ºC for 10 min

followed by 40 cycles of 15 sec at 95ºC and 1 min of annealing plus extension. The universal primer set targeting 16S rRNA gene, Bac1369F (5'– CGG TGA ATA CGT TCY CGG–3') and Prok1492R (5'–GGW TAC CTT GTT ACG ACT T–3') (Suzuki et al., 2009) was used in the quantification and expression analysis of the *Bacillus* BBP gene in relation to total bacterial DNA. Relative expression was calculated using real-time quantitative PCR and the $2^{(-\Delta\Delta C(T))}$ Method (Livak & Schmittgen, 2001).

5.3.*5 Greenhouse experiment*

Wheat plants (*Triticum aestivum* cv. Fritz) were grown in a rhizobox system in the Andisol of Piedras Negras series (SPN-LP), and the same soil with manure fertilization. The P Hedley fractionation of the used soil and manure in this study was assessed according to Fuentes et al. (2009) for inferring total P, organic P and availability gradient of Pi (Table 5.3). The used waste was collected in the same farm (WPN-LP), therefore, it can exemplify a real case of organic fertilization system.

Table 5.3. Hedley phosphorus fractionation for Low P Soil (SPN-LP) and Low P Waste (WPN-LP).

	Inorganic P (mg kg $^{-1}$)						Organic P		P tot
	H_2O	$NaHCO_3$	NaOH	HCl	Pi tot	%	mg kg $^{-1}$	%	mg kg $^{-1}$
SPN-LP	1.0	8.4	51.3	70.2	130.8	11.6	992.9	88.3	1123.8
WPN-LP	3.7	63.00	84.6	292.0	443.3	18.9	1897.4	81.0	2340.7

SPN-LP: low phosphorus soil; WPN-LP: low phosphorus waste.

The rhizoboxes were made of polyvinylchloride (PVC) with internal diameter of 11.3 cm and 20 cm long, and they were maintained under greenhouse conditions. The greenhouse study was set out in a completely randomized experimental design, with four replicates for each treatment. In both experiments, 20 plants were grown on rhizobox containing 600 g soil. During the growth period, plants were watered daily with distilled water and harvested for chemical analyses at 30 days after sowing.

Soil alone (SPN-LP) and Soil fertilized with manure at doses equivalent to 130 mg P kg^{-1} soil (SPN-LP+WPN-LP) were used as control treatments. Since the used waste have several plant nutrients, a positive control of P fertilization was setup using a set of soil samples fertilized with phosphate (KH_2PO_4) at 130 mg P kg^{-1} soil (SPN-LP+Pi). Additionally, to evaluate the effect of phytase treatment, four sets of samples were assembled with phytase treated soils (SPN-LP+Phytase), phytase treated soil-manure mixture (SPN-LP+WPN-LP+Phytase), Soil fertilized with phytase pretreated manure [SPN-LP+(WPN-LP+Phytase)] and Soil fertilized with manure inoculated and incubated with PPB (*Bacillus* MQH-19) (SPN-LP+WPN-LP+MQH). In the last treatment, PPB inoculation was repeated after germination. Soil treatment with immobilized phytases was used to evaluate phytase effect in soil *perse,* before addition of fresh organic P within the bovine cattle waste. Phytase treated samples and controls were used from the outcome of solid incubations described in section 5.2.3 and 5.2.4.

All plants were weekly fertilized with ammonium nitrate (NH_4NO_3) 50 mg N kg^{-1} soil, accounting for a total of 200 mg N kg^{-1} soil during the whole experiment. At harvest, plants were carefully removed from soil, and shoot and root subsamples were dried for 48 h at 65ºC to determine dry weight (DW) and P content. Shoot P content was analyzed by the molybdo-vanadate method as described by Sadzawka et al. (2007). Rhizosphere soil samples were also collected to determine rhizosphere soil pH and P concentration in 0.5M $NaHCO_3$ extracts.

5.3.*6 Statistical analyses*

The data were subjected to one way ANOVA analysis and significantly different means between treatments were separated with the Tukey's test at 0.05 significance level of probability. For every data set, standard error (s.e.) was also determined.

5.4 RESULTS

5.4.*1 Phytate hydrolysis of dairy cattle manure and Andisols by immobilized phytase*

The preselected Andisols and cattle manure were chosen for their contrasting Olsen P content (Table 5.1 and 5.2). Preliminary studies were setup with both soils and manure incubated with *A. niger* and *E. coli* at 0.5 U g^{-1} either alone or combined, and either free or immobilized, to access the best phytase formulation for forming the enzyme treatment. The incubation of both Andisols and manure with phytase enzymes resulted in an increase of Pi in the NaOH EDTA extract (Table 5.4). In general, immobilized enzymes returned higher Pi increase in the extracts in relation to free enzymes. Although the same amount of enzyme units was used, the treatments using the combined enzymes showed higher P turnover levels than each enzyme used alone. Therefore, the combined immobilized phytases were selected as the best form of performing this enzyme treatment. Additionally, SPN-LP and WPN-LP showed a higher P turnover than SF-HP and WF-HP, thus they were chosen for performing the greenhouse plant availability study (see section 5.4).

Table 5.4. Effect of phytase formulation on the organic phosphorus mineralization (mg kg^{-1}) from soil and manure. .

	Control	*E. coli*	Increase	*A. niger*	Increase	*A. niger* + *E. coli*	Increase
	[a]mg kg^{-1}	mg kg^{-1}	[b](%)	mg kg^{-1}	(%)	mg kg^{-1}	(%)
Immobilized phytases							
SPN-LP	68.5±7.3	72.2±8.1	5.5%	71.4±7.9	4.2%	73.7±8.0	7.7%
SF-HP	329.2±25.0	336.7±27.6	2.3%	339.3±25.9	3.0%	337.9±26.7	2.7%
WF-HP	648.8±30.1	663.2±29.4	2.2%	683.3±25.7	5.3%	704.3±28.6	8.6%
WPN-LP	249.5±19.7	256.3±22.7	2.7%	275.4±25.5	10.4%	269.1±24.1	7.9%
Free phytase							
SPN-LP	68.5±19.7	73.0±8.1	6.7%	70.6±8.4	3.2%	78.0±8.3	13.9%
SF-HP	329.2±30.1	336.6±22.2	2.3%	352.8±25.4	7.2%	344.7±23.8	4.7%
WF-HP	648.8±10.1	705.1±28.8	8.7%	679.1±28.3	4.7%	725. ±27.6	11.8%
WPN-LP	249.5±25.1	281.5±31.8	12.8%	287.7±24.3	15.3%	293.2±28.1	17.5%

[a] values represent inorganic phosphorus in the NaOH EDTA extracts (mean± standard error) after soil or waste incubation with free or immobilized phytases (0.5 U g^{-1}), either combined or alone. [b] Relative increase expressed as % of P concentration of untreated controls. SPN-LP: low phosphorus soil; SF-HP: high phosphorus soil; WF-HP: high phosphorus waste; WPN-LP: low phosphorus waste.

Phosphorus increase in extracts was low in comparison with literature and an experiment to evaluate P hydrolysis from fresh phytate was set up (Figure 5.1). Differences between enzyme type and immobilization were not significant in the hydrolysis of fresh added phytate. By contrast, the hydrolysis of added phytate was strongly affected by substrate type; the maximum fresh P-phytate turnover was about 25% to WPN-LP, followed by WF-HP (~10%) and to both soils was about 5%. Control samples presented some phytase activity, which was responsible for

the hydrolysis of 1 to 3% of added phytate. The added phytase activity (0.5 U g^{-1}) was around 40 times the theoretically necessary for hydrolyzing 100% of the added fresh phytate.

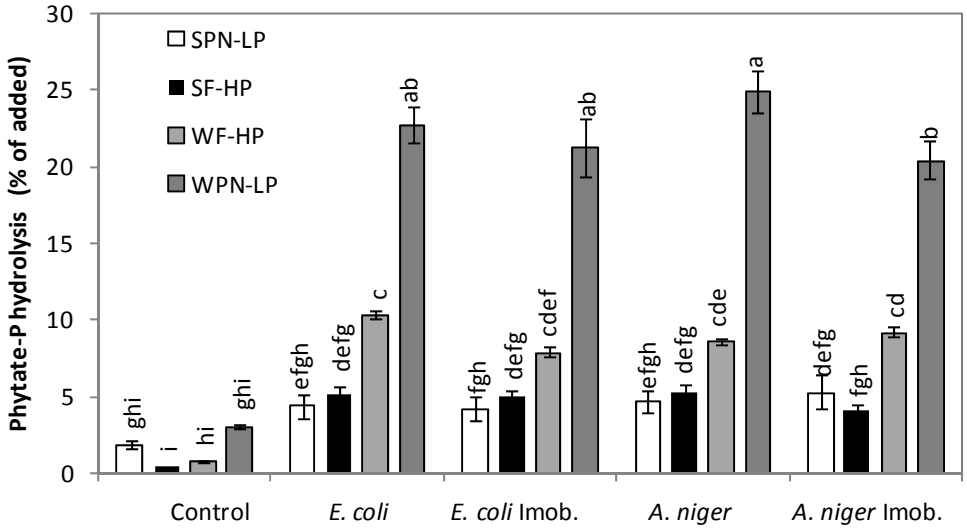

Figure 5.1. Phosphorus hydrolysis from fresh added sodium phytate (% of P-phytate added) measured in the NaOH EDTA extracts after soil or waste incubation with free or immobilized *A. niger* and *E. coli* phytases (0.5 U g^{-1}), either combined or alone. SPN-LP: low phosphorus soil; SF-HP: high phosphorus soil; WF-HP: high phosphorus waste; WPN-LP: low phosphorus waste.

The phytase dose in liquid incubations was raised until maximum hydrolysis was obtained (20 U g^{-1}), and this value was considered as the total phytase labile P ($P_{Phy\text{-}lab,}$ Table 5.5). The absolute $P_{Phy\text{-}lab}$ (mg kg^{-1}) was much higher for SF-HP than for SPN-LP and manures. In general, with the use of this saturating phytase dose, the relative increase (as a % of Pi in the NaOH EDTA extract) was higher to soils in relation to manure.

Low phosphorus samples (SPN-LP and WPN-LP) were selected for a study case phytase treatment of waste and soils for having lower Olsen P values, therefore, they could reproduce P

starvation conditions for the later greenhouse plant P availability experiment. Solid incubations were setup (6 days, 25°C) without pH control to assess $P_{Phy\text{-}lab}$ turnover under similar conditions to the ones found in the field. The increase in Pi in the NaOH EDTA extract was only significant to SPN-LP and its mixture with WPN-LP (Table 5.5). No significant increase in Pi was observed to the phytase treated WPN-LP due to the obvious fact that its pH is out of the pH range activity of the used Phytases. The results indicate that the pre-treatment of wastes with acid phytases is not as effective as the direct application of these enzymes in acid soils and acid soil-waste mixtures. The outcome samples of these experiments were used for the plant P availability experiment (see section 5.4).

Table 5.5. Total phytase labile P ($P_{Phy\text{-}lab}$) in the NaOH EDTA extract after liquid buffered (pH 4.5) incubation with immobilized phytases (10 U g^{-1} *E. coli* and 10 U g^{-1} *A. niger* phytases) for SPN-LP, SF-HP, WF-HP, WPN-LP.

	Control	**Phytase treated**	**Phytase labile P**	
	[a]mg kg $^{-1}$	**mg kg $^{-1}$**	**mg kg-^{1}**	**[b](%)**
SPN-LP	68.4±7.3	102.9±4.5	34.4	50.2
SF-HP	329.1±24.9	555.1±20.6	225.9	68.6
WF-HP	648.8±30.1	705.3±14.0	56.6	8.72
WPN-LP	249.5±19.7	294.3±6.4	44.8	17.9

[a] values represent means of Pi (triplicate) and standard error. [b]Relative increase expressed as % of P concentration of untreated controls. SPN-LP: low phosphorus soil; SF-HP: high phosphorus soil; WF-HP: high phosphorus waste; WPN-LP: low phosphorus waste.

Table 5.6. Inorganic phosphorus increase in the $NaHCO_3$ P and NaOH EDTA extracts after solid incubation with immobilized phytases (10 U g^{-1} *E. coli* and 10 U g^{-1} *A. niger* phytases) for SPN-LP, WPN-LP and SPN-LP + WPN-LP.

	$NaHCO_3$ Pi	**Variation**	**NaOH EDTA Pi**	**Increase**
	mg kg^{-1}	**%**	**mg kg^{-1}**	**%**
SPN-LP	4.8 ±2.2		67.4 ±2.9	
SPN-LP +Phytase	13.5* ±1.7	181.5	80.9* ±2.1	19.9
WPN-LP	60.8 ±0.5		252.6 ±3.0	
WPN-LP + Phytase	65.2* ±1.6	7.2	257.1 ±3.3	1.79
WPN-LP +SPN-LP	12.8 ±3.3		109.1 ±3.4	
WPN-LP +SPN-LP +Phytase	20.8* ±2.5	63.3	124.7* ±4.1	14.2

a) values represent inorganic phosphorus in extracts (mean ±standard error). b) Relative variation expressed as % of P concentration of untreated controls. * indicates a significant effect of phytase treatment ($P \leq 0.0.5$). SPN-LP: low phosphorus soil; WPN-LP: low phosphorus waste.

5.4.2 Inoculation of Low P Waste with phytase producing bacteria

Since cattle manure have neutral to alkaline pHs, the commercial acid phytases were not suitable for the treatment of these manure without pH control. Therefore, we decided to undertake the treatment of this waste with a bacterium strain producing an extracellular alkaline phytase (*Bacillus* sp. MQH-19) isolated in a previous study (Jorquera et al., 2011). In a similar analysis of Figure 5.1 we have found that the inoculation with *Bacillus* MQH-19 (PPB) strain promoted a turnover of fresh P-phytate at rates of 25 and 30 % for liquid and solid incubations respectively, both under unbuffered conditions. Although no significant Pi increase in waste extracts was observed after liquid incubations with PPB, under solid incubations Pi increased significantly in $NaHCO_3$ (10.6±1.4) and NaOH EDTA(11.7% ±1.7) extracts in relation to uninoculated control.

All inoculation experiments were performed under non sterile conditions, and inoculants had to cope with native microbes for colonization and survival. Bacterial community structure was significantly affected by PPB inoculation but not by fresh phytate addition at all times up to 6 days after inoculation. Banding cluster analysis of DGGE gels showed a strong effect of inoculation in microbial diversity, and this effect was progressively diminished during cultivation; no effect of phytate addition in bacterial community structure was observed using the same analysis (Figure 5.2A). Shannon-Weaver diversity index assessed by 16S-DGGE image analysis was higher in the inoculated samples in comparison with control samples (Figure 5.2B). Multivariate principal component analysis showed a significant effect of cultivation time in a downwards displacement of treatments through factor 2 axis (Figure 5.2D). Curiously, the PCA analysis showed that inoculated and uninoculated samples were clearly more separated on day 3 than on day 6, suggesting that at this time there was a higher effect of inoculation on the bacterial community composition.

A semi-quantitative analysis of the 16S-DGGE gel images revealed that the relative band area of MQH inoculant decreased on day 6 in relation to day 1 and 3 (Figure 5.2C). This trend was observed from day 3 by quantitative PCR of BPP gene harbored by *Bacillus* MQH-19 (Figure 5.3B). Additionally, this decrease pattern was more pronounced when relative expression was analyzed in respect to total 16S gen copies, since 16S gens increased in time for all treatments during incubation (Figure 5.3 A). Curiously, the absolute increase of 16S gen copies during incubation was reduced by phytate addition to both inoculated and uninoculated samples.

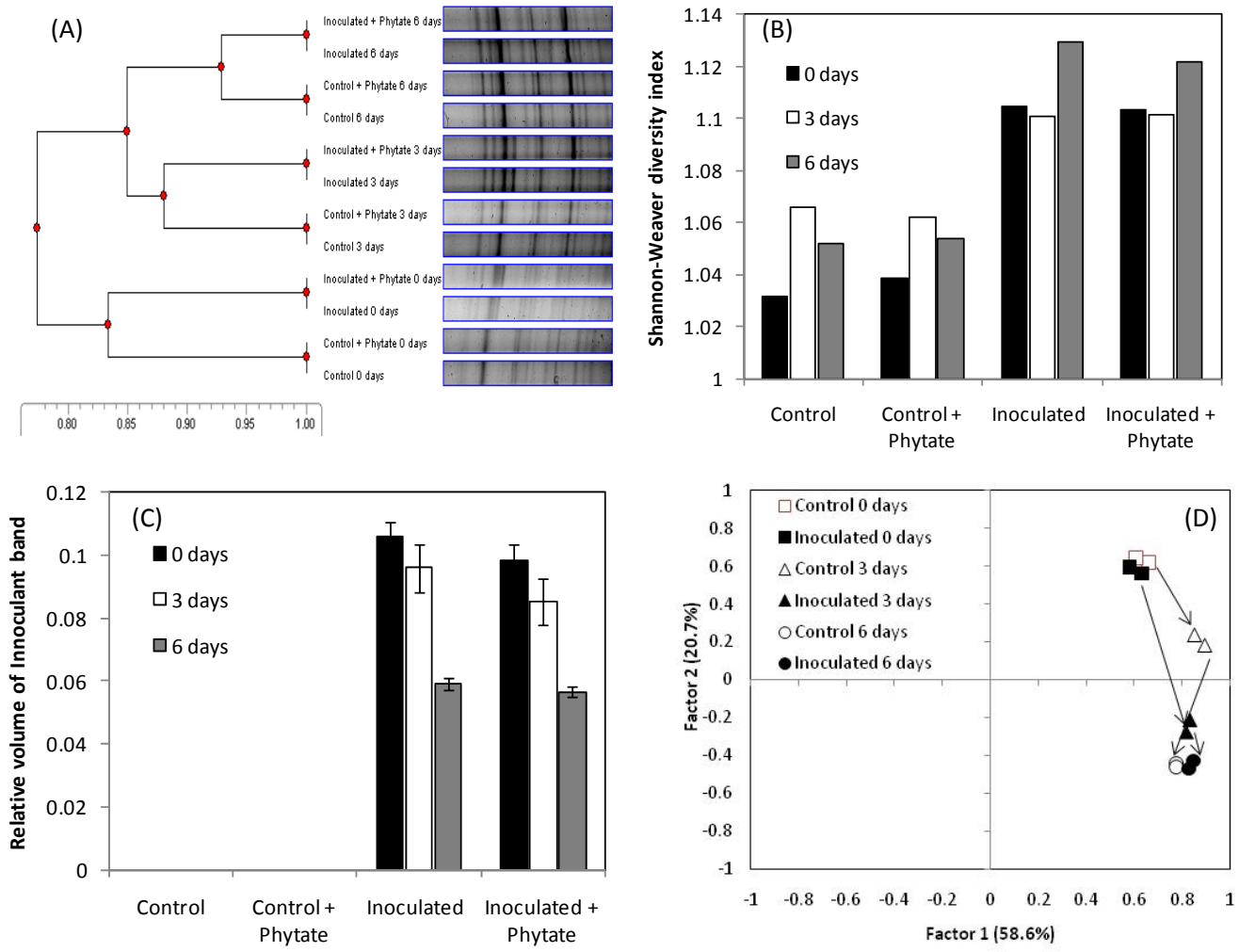

Figure 5.2. Denaturing gradient gel electrophoresis analysis of bacterial communities after 3 and 6 days incubation with MQH-19 at 30°C in the presence and absence of sodium phytate: A) Community structure dendrogram; B) Shannon-Weaver diversity index using relative peak areas; C) relative volume of MQH inoculant bands from 16S-DGGE image analysis; D) and multivariate principal component analysis of relative band volume.

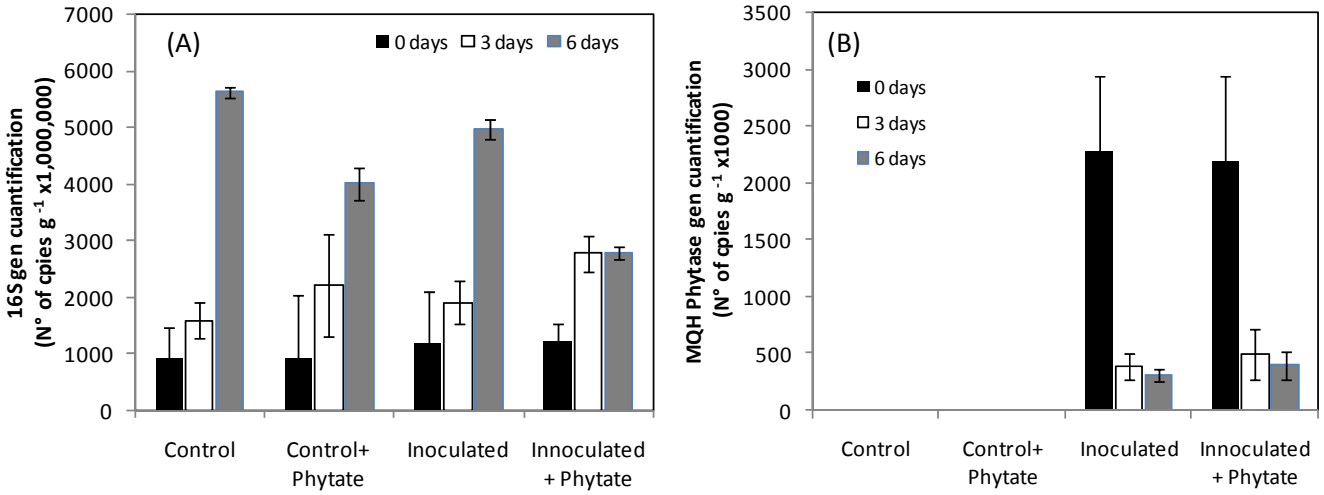

Figure 5.3. Real time PCR quantification of 16S rRNA gen (A) and β-propeller phytase gene (B) of WPN-LP samples inoculated with *Bacillus* sp. MQH–19 after 3 and 6 days of incubation at 30 °C in the presence and absence of sodium Phytate.

5.4.3 Greenhouse experiment

Soil treatment with immobilized phytases was tested to evaluate the phytase effect on soil *perse*, before fresh organic P was added through the application of cattle waste. The direct phytase treatment of soil promoted no significant increase in plant dry weight (Figure 5.4 A). The Pi fertilization and cattle waste addition caused a similar significant increase in plant growth indicating that P starvation is a major limitation for this system. Phytase treatment caused a significant increase in plant growth for both pretreated WPN-LP and treated SPN-LP + WPN-LP mixtures. In relation to tissue P concentration, only the direct phytase treatment of the SPN-LP + WPN-LP mixtures was statistically higher than the respective untreated control (Figure 5.4 B).

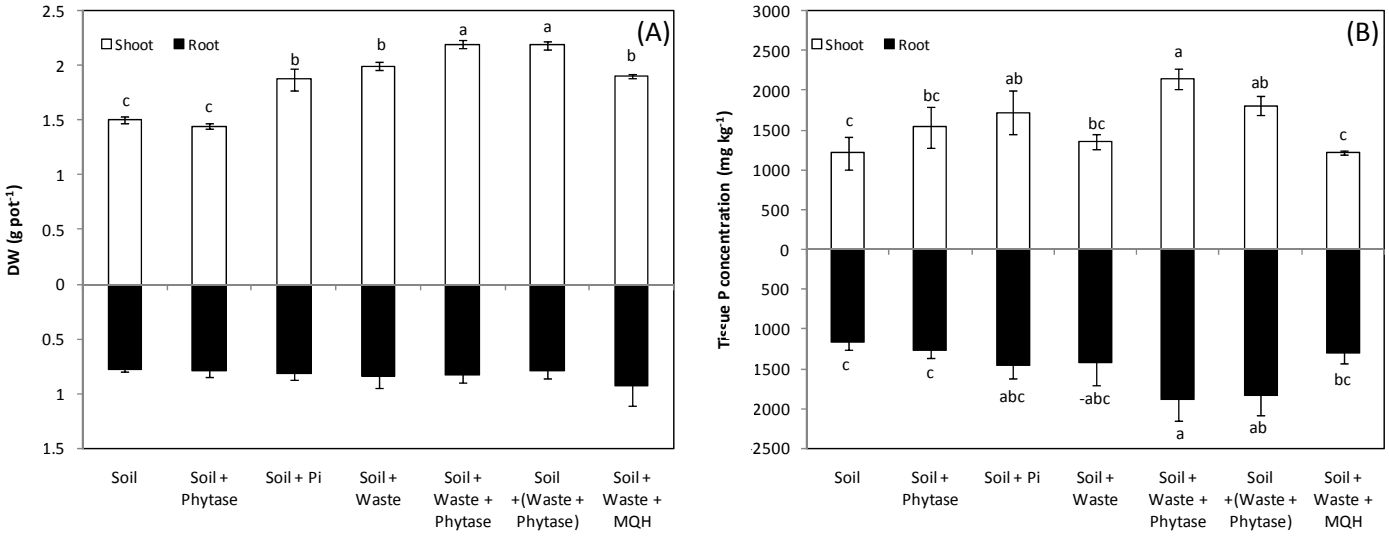

Figure 5.4. Effect of phytase treatment of SPN-LP, SPN-LP + WPN-LP and *Bacillus* MQH-19 (MQH) inoculation in *Triticum aestivum* cv. Fritz tissue dry weight (A) and phosphorus concentration (B). SPN-LP: low phosphorus soil; WPN-LP: low phosphorus waste.

After cultivation, inorganic P concentration in both Olsen and NaOH EDTA extracts showed an even higher increase due to phytase treatments in comparison with values measured before cultivation (Table 5.7). These results suggests that the used immobilized phytases maintained their activity and continued to hydrolyze organic P progressively from soil and soil-waste mixture. The increased inorganic P due to phytase treatment was associated with a higher plant P extraction. Irrespective from the increase in inorganic P in soil extracts, the inoculation with the PPB strain did not lead to increase in plant growth, P concentration nor P extraction (uptake kg^{-1} soil).

Table 5.7 Effect of phytase treatment and bacteria inoculation in the $NaHCO_3$ Pi, soil NaOH EDTA Pi and plant P extraction before and after 30 days of *Triticum aestivum* cv. Fritz growth.

	Olsen Pi Before cultivation		**Olsen Pi** After cultivation		**NaOH EDTA Pi** Before cultivation		**NaOH EDTA Pi** After cultivation		**[c] Total plant P extraction**	
	[a] mg kg $^{-1}$soil	**[b] %**	**mg kg $^{-1}$soil**	**%**	**mg kg $^{-1}$soil**	**%**	**mg kg $^{-1}$soil**	**%**	**mg kg $^{-1}$soil**	**%**
SPN-LP	4.3±0.1		5.2±0.2		67.4±2.9		76.6±7.7		4.6±0.7	
SPN-LP + Phytase	5.9±1.0	39.4	6.5±0.8	25.1	80.9±2.1*	20.0	105.5±2.6*	37.8	5.4±1.0	17.8
SPN-LP + Pi	17.8±0.6		19.5±1.8		185.5±11.6		180.9±11.3		7.4±3.2	
SPN-LP + WPN-LP	13.7±0.7		12.5±0.6		109.2±3.4		136.0±9.8		6.5±2.7	
SPN-LP + WPN-LP + Phytase	19.9±1.7*	45.2	22.3±0.4*	78.9	124.7±4.1*	14.2	194.9±14.5*	43.3	10.5±2.0*	61.1
SPN-LP +(WPN-LP + Phytase)	13.9±0.7	1.4	22.9±3.9*	84.0	114.8±3.1	5.2	208.4±47.7*	53.2	9.0±2.0*	38.2
SPN-LP+ WPN-LP+ MQH	12.8±1.2	-6.1	14.1±1.3	6.9	105.9±3.6	-2.9	173.1±15.1*	27.3	5.9±2.1	-9.8

[a] Values represent inorganic phosphorus measurements (mean ±standard error); [b] indicates a significant effect of phytase treatment ($P \leq 0.0.5$); [c] Plant P extraction was calculated as the total plant P uptake per kg of soil; Relative variation expressed as % of P concentration of untreated controls; SPN-LP: low phosphorus soil; WPN-LP: low phosphorus waste.

5.5. Discussion

Phytase labile organic phosphorus is inferred by the increase of inorganic P after the incubation of samples with phytases. This methodology often measures hydrolysis of other monoester P different from phytate when phytases with broad substrate specificity are used (e.g. *A. niger* phytase) (Menezes-Blackburn, 2012).

A $P_{Phy\text{-}lab}$ fraction is native to cattle manure, and it is expected to be proportional to its dry weight content (Dao, 2003). A big part of $P_{Phy\text{-}lab}$ is expected to be located in the water soluble fraction dairy cattle manure (He & Honeycutt, 2001). In this study, the $P_{Phy\text{-}lab}$ content in the used manure was lower than the reported on literature for cattle manures (Menezes-Blackburn, 2012). This may be because our manures are a mixture of cattle manure and other unknown materials (straw, soil, feed). The WPN-LP was kept at open field for more than a year before this study in a region with rainfall of over 1800 mm $year^{-1}$, and a high amount of water soluble P was possibly "washed" during storage. The initial $P_{Phy\text{-}lab}$ content in manure may have been also reduced by hydrolysis mediated by native phytases or by a progressive stabilization of P-phytate during field storage. Storage in field facilities is reported to be associated with a decrease in the total $P_{Phy\text{-}lab}$ fraction of manures; He et al (2003) showed a strong reduction of $P_{Phy\text{-}lab}$ in dairy manure during one year storage, due to a possible progressive reduction of phytase-availability of monoester P in manures. Drying may also have reduced $P_{Phy\text{-}lab}$ outcome in cattle manure. Drying was shown to induce a decrease in dairy manure total organic P through the hydrolysis of monoester P (He et al., 2007).

The presence of cations in the used manure and soils may be lowering the outcome of $P_{Phy\text{-}lab}$ results. Dephosphorylation of phytate in manures is increasingly inhibited by increasing counterion concentration, which forms low phytase labile precipitates with phytate (Dao, 2003; Tang et al., 2006). Several cations such as calcium, iron and magnesium are supplemented in

feeds and may complex phytate protecting it against dephosphorylation during passage through digestive traits (Dao, 2007; Dao, 2003).

Curiously, our data suggested a possible synergistic effect of phytase different sources in the dephosphorylation of Po from manures and soils. One possible explanation for this result is that the used phytases may have different affinity for different precipitated phytate forms and complexes, and also for other forms of Po labile to these enzymes.

The enzyme dose was found to be a limiting factor in determining $P_{Phy\text{-}lab}$ in soils but not in manure. The higher increase in the released P of soils in relation to manure, after the increase of enzyme dose from 0.5 to 20 u g^{-1} may reflect: (i) a higher stabilization of phytate in soils in relation to manure; and (ii) the possible hydrolysis of other non-phytate monoester P by the added phytases. The P hydrolysis rates of fresh added phytate also reflect the lower availability of this substrate to phytase enzymes in soils in comparison with manure, and the possible higher content of $P_{Phy\text{-}lab}$ in these substrates (fig 5.1). Adsorption and precipitation are the main mechanisms of phytate fixation in soils (Richardson et al., 2005), and may limit P hydrolysis from these substrates (Tang et al., 2006). Our preliminary data using SPN-LP indicates that almost all $P_{Phy\text{-}lab}$ is associated with mineral fraction, and another study is being assembled on this subject by our work group. Both factors, phytate availability and presence of phytase enzymes, are determinant in the acquisition of phytate P by plants (Hayes et al., 2000; Richardson et al., 2005).

The pH range of activity of the used enzymes is also an important issue for hydrolyzing the $P_{Phy\text{-}lab}$ pool. The pH-activity curves of the alkaline phytase from *Bacillus* sp. MQH-19 and from the free and immobilized acid phytases were described in previous studies (Acuña et al., 2011; Menezes-Blackburn et al., 2011). Since bovine cattle manures are neutral to alkaline materials, the used commercial acid phytases could not hydrolyze a significant amount of Po in unbuffered

manure at solid incubations. Nevertheless, when the waste-enzyme mixture was added to soils, the stabilized phytases started to hydrolyze P in amounts sufficient to influence P nutrition and plant growth, probably due to the associated acidification of this material (soil waste mixture with pH 5 approximately).

In phytase treated samples, inorganic P content in soil extracts continuously increased even during cultivation, suggesting that the immobilized phytases were still active for a long period. Similarly, Calabi-Floody et al. (2012) found higher mineralization rates of Po in stabilized cattle dung using phosphatases immobilized in natural allophanic nanoclays than using free enzymes. The use of pre-stabilized phytases was considered a very important issue for the achievement of this soil-waste treatment, since phytase activity inhibition due to its interaction with most of soil natural particles has been previously reported (George et al., 2007b; Giaveno et al., 2010; Menezes-Blackburn, 2012) and observed in our preliminary data (unpublished).

The phytase treatments of organic wastes promoted an increase of P acquisition equivalent to fertilization with 151 kg of P ha^{-1}. About 73% (110 kg of P ha^{-1}) of this contribution came from P hydrolysis from the used organic waste and the other 27 % (41 kg of P ha^{-1}) came from soil organic P hydrolysis. From the agronomic point of view, this is a very significant source of P that can be used as future complementary fertilization strategy. The cost of phytases in the proposed treatment under the used dose is approximately US$ 240 ha^{-1}, which is, at current Chilean fertilizer local prices, less than a half of the cost of its calculated equivalent benefit of 151 kg P ha^{-1}. The costs of nanoclay support and immobilization procedure were not included in the previous calculations. Although phytase enzymes are actually low-priced at the market, complementary studies are needed to establish the economic viability of this practice considering global costs of the practice and both agronomic and environmental benefits.

An acidification of soil was observed during plant growth at the greenhouse experiment, ranging from 0.4 to 0.9 pH unit decrease. The phytase treated samples showed lower acidification than the untreated ones, suggesting that the induced acidification is partially related to plant response to P deficiency (Hinsinger et al., 2003; Neumann & Römheld, 1999). A direct linear correlation between pH and Olsen P is expected and was observed in our previous experiments using the same soil (Paredes et al., 2011).

Although soil P data suggested that the pretreatment of manure with Bacteria producing an alkaline phytase was promising for increasing P plant availability, no increase in plant growth and P nutrition was observed. The amount of enzyme unities expected to be produced by the inoculum strain is at least 200 times lower than the used phytase treatments; this lower enzyme dose may be the main cause of the lower efficiency of inoculation in respect to the direct phytase treatments of buffered manure. Nevertheless, unlike the BPP phytase from the used bacterium, the pH profile of activity of the commercial phytases does not match the natural pH of cattle manure and therefore are not suitable for their use under unbuffered natural conditions. Although a high inoculum rate was used, the effect of inoculum concentration was not tested in this study and may have a significant impact on plant growth promotion by the used strain. Ramírez and Kloepper (2010) reported a higher effect of a *Bacillus amyloliquefaciens* strain in plant growth and Pi content at low inoculum rates than at high inoculum rates. Additionally, our PCR based data suggests a rapid decay of the used strain after inoculation, and further studies testing technologies for increasing the strain persistence will be performed for increasing the inoculation efficiency. The strong variability of response of different microbe inoculants under different conditions is one of the most important limitations for the large scale development of microbial products for soil P mineralization (Richardson & Simpson, 2011).

5.6. Conclusions

The treatment of Andisols and cattle manure with a saturating dose of phytase immobilized in synthetic nanoclays resulted in a significant increase in their inorganic P. The phytase treatment of cattle manure affected positively plant growth and P nutrition of wheat in an Andisol fertilized with these treated manure. In the case of manure inoculated with β-propeller phytase producing bacterium, an increase of inorganic P was observed. However, the application of this treated manure did not cause significant impact on plant growth and P nutrition. Further studies are necessary to improve the impact of the use of this bacterium as a plant growth promoting strain, especially those involving inoculum dose and persistence in different soil and manure environments.

The phytase treatment of cattle manure promoted an increase in soil P equivalent to more than 150 kg of P ha^{-1}. This study initiates the development of possible novel agronomic strategy to improve organic P cycling from cattle manure and Andisols.

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CHAPTER 6

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

6. GENERAL CONCLUSIONS

One of the most important problems of Chilean volcanic soils is their high P fixation in organic compounds, which is largely associated to their low P availability. The research group where I developed this doctoral thesis has been long working with different strategies to improve P availability for food production. In this context this research was an important contribution to the advances in the knowledge related to the improvement of organic P utilization by crops grown in P deficient Andisols. Therefore, considering the proposed hypothesis and the developed work, we conclude that:

A significant organic P hydrolysis of bovine manures and Andisols was achieved through the use of *E. coli* and *A. niger* acid phytases either free or stabilized in synthetic allophanic nanoclay coated with iron oxide. However, the activity stabilization of the used phytases by their immobilization in nanoclays led to a ~80% higher organic P hydrolysis, due to its protection against biologically mediated enzyme denaturation.

Phytase treatment of bovine cattle manures and Andisols promoted higher levels of organic P hydrolysis than the inoculation with β-propeller phytase producing bacteria. The phytase treatment of cattle manure increased soil inorganic available P at levels equivalent to the fertilization with more than 150 kg of P ha^{-1}. The phytase treatment of manure and soil led to a ~10% increase in plant growth and a ~60% increase in P acquisition of wheat under P starvation conditions, using high phytase and manure doses. However, samples inoculated with phytase producing bacteria had insufficient P hydrolysis to cause significant impact in plant growth under the same conditions.

6. 1 Future perspectives

Animal wastes and Andisols contain a high content of phytase labile organic P which can represent a significant P source for plant nutrition. However, so far the use of phytase amendments of manures for organic P recycling has received little attention and may be also considered for future studies and biotechnological applications. Therefore, phytate can be hydrolysed to inorganic P before its addition to soil where it becomes poorly available to phytases. We have demonstrated here that the phytase treatment of bovine manures can significantly impact wheat growth and P nutrition. Although our work showed promising results, further studies are needed to establish the economic viability of this practice at field scale.

Phytases were found to be suitable for increasing organic P availability in manures and soils. Nevertheless, the dynamics of phytate dephosphorylation may depend on: a) presence of active phytase enzymes; b) phytase catalytic adaptation to the specific environment conditions (pH, temperature, presence of metal ions, etc); c) enzyme inactivation related to interaction with medium constituents; d) phytase mobility and capacity to interact immobile phytate; and e) availability of the different precipitated and adsorbed phytate salts. Thus, adequate selection of phytases and microbes may be critical for the success of each biotechnological application, and catalysis performance in different environments is expected to be enzyme specific. Further studies comparing catalytic behaviour of different phytase enzymes in different organic wastes and soils are needed to better understand the factors affecting their behaviour.

The large scale recycling of organic P from animal wastes is urgent considering the imminent world phosphate rock scarcity scenario. Therefore, biotechnological approaches to enhance the agronomic use phytase hydrolizable P pool in manures and soils are required in the near future.

7. APENDIX

7.1. List of original papers of this thesis

1. **(ISI, IF 4) Menezes-Blackburn, D.,** M. Jorquera, Greiner, R., Gianfreda, L., Mora ML.(2012), Phytases and phytase-labile organic phosphorus in manures and soils. *Critical Reviews in Environmental Science and Technology* (accepted ID: 607019)
2. **(ISI, IF 4.36) Menezes-Blackburn, D.**, Jorquera, M., Gianfreda, L., Rao, M., Greiner, R., Garrido, E., Mora, ML. (2011). Activity stabilization of Aspergillus niger and Escherichia coli phytases immobilized on allophanic synthetic compounds and montmorillonite nanoclays. *Bioresource technology*, **102**.
3. **Menezes-Blackburn, D.** et al. (2012) Phytase induced organic phosphorus hydrolysis of bovine wastes and Andisols: a possible biotechnological application for increasing plant phosphorus nutrition in southern chile. *Enzyme and Microbial Technology* (**In preparation**).

7.2. List of papers in collaboration

1. **(ISI, IF 3.4)** Jorquera, M.A., Crowley, D.E., Marschner, P., Greiner, R., Fernández, M.T., Romero, D**., Menezes−Blackburn, D.**, Mora, M.L. (2011). Identification of β–propeller phytase−encoding genes in culturable *Paenibacillus* and *Bacillus* spp. from the rhizosphere of pasture plants on volcanic soils. *FEMS Microbiol. Ecol.*, **75**, 163-172.
2. **(ISI, IF 0.6)** Acuña, J.Y., Jorquera, M.A., Martínez, O.A., **Menezes−Blackburn, D.,** Fernández, M.T., Marschner, P., Greiner, R., and Mora, M.L. (2011). Phytase activity and indole acetic acid production by rhizosphere phytase−producing *Bacillus* and *Paenibacillus* strains as affected by pH, metals and organic acids. *J. Soil Sci. Plant Nutr* 11 (3), 1-12.
3. **(ISI, IF 0.96)** Paredes, C., **Menezes-Blackburn, D**., Cartes, P., Gianfreda, L., Luz Mora, M. (2011). Phosphorus and Nitrogen Fertilization Effect on Phosphorus Uptake and Phosphatase Activity in Ryegrass and Tall Fescue Grown in a Chilean Andisol. *Soil Science*, **176**(5), 245.