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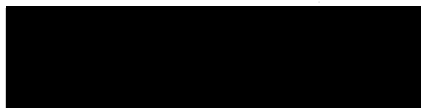
**PRODUCTION, CHARACTERISATION AND APPLICATIONS OF A  
THERMO-ACID-STABLE PHYTASE FROM *Enterobacter* sp. ACSS.**

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Submitted in complete fulfilment for the Degree of Master of Applied Sciences in Biotechnology in the Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, Durban, South Africa.

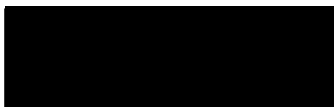
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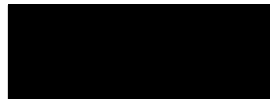
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## **DECLARATION**

**I hereby declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Applied Sciences in Biotechnology, to the Durban University of Technology, Department of Biotechnology and Food Technology, Durban, South Africa. It has not been submitted before for any degree or dissertation to any other institution.**

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**Ashira Chanderman**  
**2016**

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## LIST OF ABBREVIATIONS

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ANOVA	:	Analysis of variance
BLAST	:	Basic local alignment search tool
BPP	:	$\beta$ -propeller phytases
CC	:	Corn cobs
CCD	:	Central composite design
EDTA	:	Ethylenediaminetetra acetic acid
FPLC	:	Fast protein liquid chromatography column
HAP	:	Histidine acid phosphatase
LB	:	Luria Bertani broth/agar
NA	:	Nutrient agar
Na-phytate	:	Sodium phytate (phytic acid dodecasodium salt)
OFAT	:	One-factor-at-a-time
OP	:	Orange peel
PAP	:	Purple acid phosphatase
PBD	:	Plankett-Burman design
Pi	:	Inorganic phosphorous
PSM	:	Phytase screening media
PTP	:	Protein tyrosine phosphatase
RSM	:	Response surface methodology
SCB	:	Sugarcane bagasse
SGF	:	Simulated gastric fluid
U	:	Units
WB	:	Wheat bran

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## ABSTRACT

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A bacterial strain producing an extracellular phytase was identified as *Enterobacter* sp. ACSS. Optimization of process parameters using statistical methods such as Plackett-Burman design (PBD), the steepest ascent method, and response surface methodology (RSM) significantly improved phytase production by 4.6-fold in shake-flasks. In addition, an overall 1.9-fold increase in phytase production was attained in fed-batch fermentations in a 5 l laboratory fermenter, respectively. The purified 62 kDa phytase from *Enterobacter* sp. ACSS was active between 40 to 80°C and an acidic pH range of 2.0 to 6.0 with half-life of 693 and 577.5 min at 60°C and pH 2.0, respectively. Additionally, the enzyme is fairly stable with proteolytic enzymes under physiological conditions. It was activated by  $\text{Ca}^{+2}$ ,  $\text{Mg}^{+2}$  and  $\text{Mn}^{+2}$  while inhibition was caused by  $\text{Zn}^{+2}$ ,  $\text{Cu}^{+2}$ ,  $\text{Fe}^{+2}$ ,  $\text{Pb}^{+2}$ ,  $\text{Co}^{+2}$ ,  $\text{Ba}^{+2}$  and surfactants. The  $K_m$ ,  $V_{\max}$  and  $K_{\text{cat}}$  observed were 0.21 mM, 131.58 nmol  $\text{mg}^{-1}\text{s}^{-1}$  and  $1.64 \times 10^3 \text{ s}^{-1}$ , respectively. The enzyme released inorganic phosphate from animal feed (4.0-6.62 mg/g of diet) and insoluble metal-phytates (45-219  $\mu\text{g/ml}$ ) and was effective in improving the characteristics of brown bread. Overall, this study shows that *Enterobacter* sp. ACSS has the potential to produce significant titres of a thermo- and acid-stable phytase and can be applied in dephytinizing animal feeds, and the baking industry.

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## CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

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Phosphorus (P) is ubiquitous and exists in the form of phosphate esters or phosphate ions in majority of cells, contributing to approximately 0.2% of plant cell dry weight. (Schachtman *et al.*, 1998; Nadeem *et al.*, 2014). It is required for skeletal growth and vital metabolic processes including, glycolysis, gluconeogenesis and cellular signal transduction (Koppelaar and Weikard, 2013). Phosphate is required for the essential phosphodiester bonds that link mononucleotides to form long chains of DNA and RNA, while ATP powers the synthesis of all complex molecules for life. Famous ecologist Edward Deevey's once commenting on carbon fixation during photosynthesis claimed that the process "*would be a fruitless tour de force if it were not followed by the phosphorylation of the sugar produced*" (Deevey, 1970). P deficiency results in skeletal demineralization, pyorrhoëa, rickets, arthritis and proximal myopathy in humans (Woyengo and Nyachoti, 2011) and improper egg production and poor bone development in other animals, while in plants it may lead to stunting of growth (Takeda *et al.*, 2012).

Phosphorus is one of the main factors affecting worldwide food production and this dependence is generally met by a large input of fertilizers made of mined rock phosphate. However, P is a non-renewable resource and its depleting reserves are of major concern which is expected to be exhausted in the next 50–100 years (Cordell *et al.* 2009). This poses a huge threat as the demand for fertilizer is increasing annually to feed the uncontrolled world population with an estimated 2.0–2.5 billion new mouths to be feed by 2050 (IWMI, 2006). South Africa produces about 2300 thousand metric tons phosphate annually, but the soils are showing declining levels of phosphorus which has resulted in a decline in the share of agriculture in GDP from 7.1% in 1970 to 2.6% in 2014 (Mandiringana *et al.*, 2005). Food production relies heavily on mined P with 90% being used for the production of fertilizers and food and feed additives for livestock. As the world turns towards alternative energy sources, the demand for bio-energy crops places an additional demand for rock P contributing to its depletion. Biofuel production mostly relies on the first generation crops, all of which require P fertilization. Unfortunately, P is not recycled back into the natural biogeochemical cycle which further aggravates the problem.

A major problem that has emerged during the last few decades with respect to the use of phosphate fertilizers is its accumulation in soil which has led to the eutrophication of water bodies. Nonetheless, high phosphate fertilizers must be used since the major component of soil organic phosphorus is locked in the form of phytic acid or phytate (*myo*-inositol hexakisphosphate) that plants cannot readily adsorb. Phytic acid (PA) is also found as the primary storage form of P in over 60% of the leguminous plants and cereals, nuts, pollen and oilseeds (Coban and Demirci, 2014). As a stable metal-phytate complex, phytate also acts a strong chelator for divalent metal cations, proteins and carbohydrates (Lei *et al.*, 2012; Dersjant-Li *et al.*, 2015). Phytate is also perceived as anti-nutritive causing poor mineral bioavailability while also inhibiting several digestive enzymes like  $\alpha$ -amylase, trypsin, pepsin, acid phosphatase and tyrosinase (Singh *et al.*, 2011). Phosphate liberation from phytate must be facilitated by the intestinal phytase produced by ruminal microflora which agastric animals (pigs, poultry, fish, dogs etc.) and humans lack (Kumar *et al.*, 2010).

Phytase is produced by several microorganisms and has been applied in industries. Particularly for food and feed applications the search has been focused on novel phytases with requisite properties such as pH and temperature stability. The main focus of this study was to isolate a novel phytase producer and subsequent production, characterization and application of the enzyme.

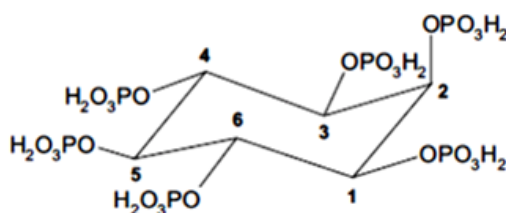
## 1.1 Phytic Acid

### 1.1.1 Definition

Phytic acid, discovered in 1903 is as a phosphorylated derivative of *myo*-inositol (Mullaney *et al.*, 2007). The correct chemical description of phytic acid is *myo*-inositol 1, 2, 3, 4, 5, 6-hexakis dihydrogen phosphate or as Ins P<sub>6</sub> (IUPAC-IUB, 1977). InsP<sub>6</sub> readily chelates with divalent metal ions such as Fe<sup>2+</sup>, Zn<sup>2+</sup>, Mn<sup>2+</sup>, Cu<sup>2+</sup>, Mg<sup>2+</sup> and Ca<sup>2+</sup>, as well as proteins and starch (Chen *et al.*, 2009).

### 1.1.2 Structure

Phytic acid (PA) has a molecular formula of C<sub>6</sub>H<sub>18</sub>O<sub>2</sub>P<sub>6</sub> (Fig. 1.1) and a molecular mass of 660.03 g/mol. It consists of an inositol ring with six phosphate moieties attached in *myo*-conformation and therefore referred to as *myo*-inositol hexakisphosphate (Yao *et al.*, 2011; Bohn *et al.*, 2008).



**Figure 1.1** Structural formula of phytic acid (Haefner *et al.*, 2005)

### 1.1.3 Occurrence and function

Phytic acid exists naturally as a compound with key biological and physiological functions. In plants phytic acid accounts for 60-90% of the total P (Liu *et al.*, 1998; Greiner *et al.*, 2006). Phytate can serve as a direct indication of the amount of organic P present in plant cells as it is bound in this complex. During early development of cereal grains starch-protein bodies are accumulated in the form of the aleurone layer which contains phytic acid and represents 20% of its dry weight (Singh *et al.*, 2011). Several metabolic processes in eukaryotic cells exploit three unique functions of PA. Firstly, it is able to function as a phosphate donor/acceptor. Secondly, PA intermediates are

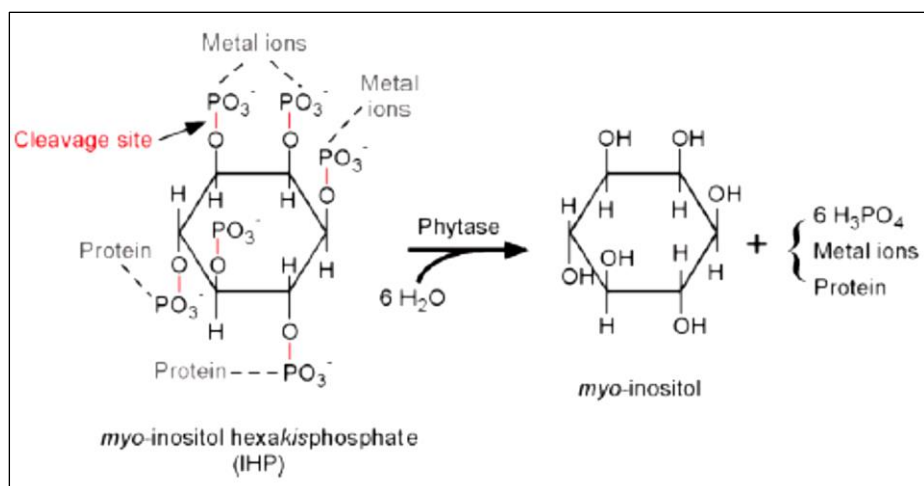
required in numerous cell signalling pathways and thirdly PA acts as a precursor of compounds (Bohn *et al.*, 2008).

Phytate exhibits both positive and negative effects. It acts as an anti-nutrient in the gut due to its chelating properties. It also binds to minerals, proteins and enzymes making them unavailable for absorption. On the other hand, phytate dephosphorylated to its lower forms has been shown to protect against several types of cancers (Greiner *et al.*, 2006; Bohn *et al.*, 2008; Coulibaly *et al.*, 2011). Jenab and Thompson (2000) reported that adding pure phytic acid to low fiber diet increased the rate of apoptosis and degree of differentiation in the distal colon. Phytic acid intermediates are involved in the transport of materials into the cell and in modulation of the oxygen-binding affinity of haemoglobin in animals (Singh *et al.*, 2011).

## **1.2 Phytases**

### **1.2.1 Structure and function**

The term “phytase” [*myo*-inositol (1, 2, 3, 4, 5, 6) hexakisphosphate phosphohydrolases] includes several structurally different enzymes based on their different mode of action. The characteristic of each phytase differs in terms of their pH and thermal stability and their resilience to proteolytic enzymes in the alimentary canal (Mullaney and Ullah, 2003). Phytate degrading enzymes are therefore defined as a group of phosphatases which initiate the stepwise dephosphorylation of phytate (Greiner and Konietzny, 2006). The reaction intermediates serve as substrates for further hydrolysis once released from the enzymes (Greiner, 2002). Phytase is the only known enzyme that can initiate the phosphate hydrolysis at carbon 1, 3 or 6 on the inositol ring of phytate. The removal of phosphate group by phytase results in the release of calcium, iron, zinc, and other metal ions (Fig. 1.2).



**Figure 1.2** Schematic representation of phytate hydrolysis. Phosphoester bonds are cleaved by phytase to release inositol, phosphate, bound proteins other divalent elements (Chen *et al.*, 2014).

### 1.2.2 Classification

The International Union of Pure and Applied Chemistry and the International Union of Biochemistry (IUPAC–IUB) recognizes two general classes of phytases, 3-phytase (EC 3.1.3.8) and 6-phytase (EC 3.1.3.28), initiating the dephosphorylation at the 3 and 6 positions of the phytate, respectively (Haefner *et al.*, 2005). In general, 3-phytase is common in microorganisms, while 6-phytase is synthesized by plants. On the basis of pH optima, phytases are further divided into acid or alkaline phytases. Acid phytases have been further divided into histidine acid phosphatases (HAPs), purple acid phosphatases (PAPs) and protein tyrosine phosphatase (PTP)-like class of phosphatases based on the presence of a specific consensus motif and their three-dimensional structures (Yao *et al.*, 2011; Chen *et al.*, 2014). These differ from the  $\beta$ -propeller phytases (BPP) which prefer to hydrolyse every second phosphate over that of adjacent ones on the *myo*-inositol hexakisphosphate molecule constitute a separate class of neutral to alkaline phytases (Mullaney and Ullah, 2003; Greiner *et al.*, 2007). Most of the  $\beta$ -propeller phytases are from *Bacillus* domain; however a novel  $\beta$ -propeller phytase from *Shewanella oneidensis* and another from *Serratia* sp. TN49 has also been reported (Cheng and Lim, 2006; Zhang *et al.*, 2011). Representatives of this large class of enzymes are known to occur in animals, plants and microorganisms.

The fungal phytase, phyA, produced by *A. niger* NRRL 3135 is a known HAP-phytases. PAPs have been found in plants (Brinch-Pedersen *et al.*, 2014), mammals (Cho *et al.*, 2006), fungi (Bala *et al.*, 2014), yeasts (Vohra *et al.*, 2011) and bacteria (Chen *et al.*, 2015). GmPhy phytase is the only known PAP reported to have phytase activity. It has been proposed that the low catalytic activity of GmPhy may be advantageous to regulate the slow and balanced breakdown of phytate during germination of soybean seedlings (Hegeman and Grabau, 2001).

### 1.3 Sources of phytase

Phytate-degrading enzymes are widespread in nature; they are prevalent in plants, micro-organisms, as well as in some animal tissues (Yao *et al.*, 2011; Lei *et al.*, 2013). On a commercial scale, bacterial phytases are now commonly used as an alternative to fungal phytases due to their increased thermostability. Some other adventitious attributes include substrate specificity, simpler production strategies, enhanced genetic manipulation, resistance to proteolysis and catalytic efficiency (Vijayaraghavan *et al.*, 2013; Jorquera *et al.*, 2008a and b). Large numbers of phytases have been characterized to date and some of them are available commercially (Cao *et al.*, 2007). Commercial phytases (Table 1.1) have been developed and supplemented in the diets of swine, poultry, and fish in a number of countries, including Europe, North America, and Asia in order to reduce phosphorus pollution of animal waste.

Generally, the phytases produced by fungi are extracellular, whereas bacterial phytases can be cell-associated (Konietzny and Greiner, 2002) or extracellular as in *Bacillus* and *Enterobacter* (Liu *et al.*, 1998). There is a need to investigate extracellular phytases from these bacterial domains as they promise easier cultivation, production and genetic manipulation. A phytase will not be commercially competitive if it cannot be produced in high yield at large scale. Moreover, a relatively inexpensive method is required to purify and characterize the enzyme.

**Table 1.1** Characteristics of some commercially produced microbial phytases (Dersjant-Li *et al.*, 2015)

Type	Origin	Expression organism	pH optima	Temperature optima (°C)	Trade name
3	<i>A. niger</i>	<i>A. niger</i>	2; 5–5.5	65	Natuphos®
3	<i>A. niger</i>	<i>A. niger</i> , non-recombinant	6.0	ND	Allzyme® SSF
3	<i>A. niger</i>	<i>Trichoderma reesei</i>	2.5	ND	Finase® P/L
6	<i>E. coli</i>	<i>Schizosaccharomyces pombe</i> (ATCC 5233)	4.5	55	Phyzyme® XP
6	<i>E. coli</i>	<i>Pichia pastoris</i>	4.5	ND	Quantum®
6	<i>E. coli</i>	<i>Trichoderma reesei</i>	ND	ND	Quantum Blue®
6	<i>E. coli</i>	<i>Pichia pastoris</i>	3.4, 5.0	58	OptiPhos®
6	<i>Peniophora lycii</i>	<i>Aspergillus oryzae</i>	4–4.5	50–55	Ronozyme®
6	<i>Citrobacter braakii</i>	<i>Aspergillus oryzae</i>	ND	ND	Ronozyme Hiphos®
6	<i>Buttiauxella</i> spp.	<i>Trichoderma reesei</i>	3.5–4.5	60	Axtra® PHY

ND: Not determined

### 1.3.1 Plant phytases

Phytate-degrading enzymes occur mostly in grains, seeds and pollen of higher plants, such as cereals, legumes, oilseeds and nuts, but low phytate-degrading activity is also found in the roots of the plants (Greiner, 2002). Plant phytases are classified as 6-phytases, initiating hydrolysis of phytate on the *myo*-inositol hexaphosphate ring at position C6 (Fig 1.1). Intrinsic phytase serves to degrade phytate bound P, vital for the developing plants during germination (Kumar *et al.*, 2010; Woyengo and Nyachoti, 2011). Phytases have been reported in rice (*Oryza sativa*) [Kim *et al.*, 2012], wheat (*Triticum aestivum*) and maize (*Zea mays*) [Dionisio *et al.*, 2011]. Recently, Brinch-Pedersen *et al.* (2014) also reported phytases from rye (*Secale cereale*), triticale (*Triticosecale*), barley (*Hordeum vulgare*), millet (*Pennisetum typhoides*), oats (*Avena sativa*) and sorghum (*Sorghum sudanensis*) during their studies on phytase-mediated mineral bio-availability in these plants.

### 1.3.2 Fungal phytases

The soil fungus *Aspergillus* is the most common producer of extracellular phytase and many have been purified to homogeneity. Most commercially produced phytases originate from fungal

sources including *A. ficuum*, *A. niger*, and *A. fumigates*. Phytases have been detected in a variety of fungi such as *Rhizoctonia* sp., *Fusarium verticilloides* (Marlida *et al.*, 2010), *Mucor racemosus*, *Cladosporium*, *Aspergillus terreus* (Greiner and Konietzny, 2006), *Mucor indicus* (Gulati *et al.*, 2007b) *Rhizopus oryzae* (Rani and Ghosh, 2011) and *Aspergillus flavus* ITCC 6720 (Gand and Singh, 2015). The continuous search for thermostable phytases has led to the identification and characterisation of phytases from the thermophilic *Mucor hiemalis* Wehmer (Boyce and Walsh, 2007), *Thermomyces lanuginosus* (Gulati *et al.*, 2007c) and more recently from *Humicola nigrescens* (Bala *et al.*, 2014). *M. hiemalis* Wehmer is thermostable at 80°C for 5 min, while *T. lanuginosus* retains its activity up to 75°C and phytase from *H. nigrescens* was retained 20% activity at 80°C.

### 1.3.3 Yeast phytases

Phytase activity has been detected in several yeasts including strains such as *Schwanniomyces castellii*, *S. occidentalis*, *Hansenula polymorph*, *Arxula adeninivorans* and *Rhodotorula gracilis*, (Pandey *et al.*, 2001, Kaur *et al.*, 2007). The activity of these phytases during the fermentation of bread dough is particularly attractive. Yeasts strains expressing extracellular phytases are used in bread-making to improve the bread characteristics and to increase the bioavailability of minerals. Phytase production has been reported from *Saccharomyces cerevisiae* (Ries and Macedo, 2011) and psychrotrophic arctic yeast *Cryptococcus laurentii* AL 27 (Pavlova *et al.*, 2008). Recently, Yu *et al.* (2015) isolated a novel cold-adapted phytase producing *Rhodotorula mucilaginosa* JMUY14 from deep-sea sediments of the Antarctic. Purified phytase showed the highest activity in the range of 20-30°C and was resistant to pepsin and trypsin, making it a promising feed enzyme for aquaculture purposes.

### 1.3.4 Bacterial phytases

Bacterial phytases are becoming potentially important in the global phytase enzyme market (Table 1.2) and are preferred to fungal phytases. This is attributed to their increased thermostability, higher substrate specificity, resistance to proteolysis and catalytic efficiency (Jorquera *et al.*, 2008a). Extracellular phytase activity is exhibited by bacteria belonging to the genera *Bacillus* (Joseph and Raj, 2007; Lu *et al.*, 2014; More *et al.*, 2015) and *Enterobacter* (Fu *et al.*, 2008).

However, extracellular phytases have also been reported from *Pseudomonas fragi* Y9451 (In *et al.*, 2004), *Pseudomonas aeruginosa* p6 (Sasirekha *et al.*, 2012) and *Alcaligenes* sp. (Vijayaraghavan *et al.*, 2013). Bacterial phytases have been found *Klebsiella* sp. (Mittal *et al.*, 2012) and *Citrobacter braakii* (Cao *et al.*, 2007). Hussin *et al* (2007) studied ten phytase producing bacterial strains which included *Bacillus* sp., *Staphylococcus* sp., *Brevibacillus* spp., and *Kocuria* sp. Lactic acid bacteria belonging to the species *Lactobacillus panis*, *Lactobacillus fermentum*, and *Pediococcus pentosaceus*, *Lactobacillus reuteri* (Raghavendra and Halami, 2009; Nuobariene *et al.*, 2015) also display phytase activity.

Thermostability and pH stability are the major factors that need to be considered when screening for bacterial phytases. Stability of phytase at high temperature (80°C) is a prerequisite during the pelleting of animal feed, while pH-stability is required to sustain the abrupt pH changes in the animal gut. Hong *et al.* (2011) characterized a thermostable phytase from *B. subtilis* CF92 isolated from cow faeces. The purified phytase was optimally active at 60°C and at neutral pH. Mittal *et al.* (2011) isolated an extracellular phytase producing *Klebsiella* sp. from poultry farm soil. It produced 395 IU/ml of phytase which was optimally active at 55°C and in an acidic pH range of 3.5 to 5.5. Compared to fungal phytases, *E. coli* phytases are reported to be more active at a lower pH range (2.0-5.5) and show higher efficacy for phytate hydrolysis in the digesta of broilers during *in vitro* and *in vivo* studies (Onyango *et al.*, 2005; Dersjant-Li *et al.*, 2015). There is a wide range of variability in phytase production depending on the production conditions, producing strains and interaction of various production parameters.

## **1.4 Biophysical and biochemical properties of phytases**

### **1.4.1 Temperature and pH optima**

Thermostable enzymes are receiving considerable attention due to their multiple industrial applications. There is a need for a robust phytase that can withstand high temperature while pelleting of animal feed and more recently, thermostable phytases are in great demand due to their use during gelatinisation and saccharification steps of bioethanol production (Liu and Han, 2011; Mikulski *et al.*, 2014). Phytases show high activity in the range of 50 to 70°C while optimum

temperature is generally between 45 and 60°C (Vats *et al.*, 2005). Phytase from *Rhizomucor pusillus* is optimally active at 70°C (Chadha *et al.*, 2004) while phytase from *R. oryzae* had an optimal temperature of 45°C (Rani and Ghosh, 2011). Phytase from *Bacillus* spp. had an optimal temperature of 80°C (Dechavez *et al.*, 2011) while the enzyme from *Enterobacter* sp.4 (Yoon *et al.*, 1996), *K. pneumoniae* 9-3B (Escobin-Mopera *et al.*, 2012), *Yersinia intermedia* (Huang *et al.*, 2006), *Obesumbacterium proteus* (Zinin *et al.*, 2004) were optimally active in the range of 45 to 60°C.

Majority of phytases are either acidic or alkaline based on their optimal pH for catalytic activity. Optimum pH levels range from 2.2 to 8.0. Phytases of fungal origin display activity between pH 4.5 and 5.6 while those of bacterial origin are optimally active between pH 6.5 and 7.5 (Caipang *et al.*, 2011). Chadha *et al.* (2004) described a phytase from thermophilic fungus, *R. pusillus* that showed wide pH stability from 3.0 to 8.0. The pH profile of *A. niger* protein phytase (PhyA) is featured by two pH optima at 2.5 and 5.5, respectively, and a decrease in activity between these two points was also observed (Han *et al.*, 1999). Phytases from *Bacillus* generally have optimum pH levels at 6.5 - 7.5. *B. subtilis* CF92 phytase displayed optimal activity at pH 7.0 and the purified enzyme was relatively stable between pH 4.0 to 8.0 (Hong *et al.*, 2011) while phytase from *Bacillus* sp. KHU-10 was fairly stable from pH 6.5 to 10.0 (Choi *et al.*, 2001). The optimum pH of plant seed phytases range from 4.0 to 7.5, most having an optimum between 4.0 and 5.6 (Caipang *et al.*, 2011). However, some plant seeds and legumes have been shown to have optimal pH ranging from 6.5 to 8.0 (Oh *et al.*, 2004). The wide range of differences in pH optima could be due to the variation in molecular structure of the protein from various sources.

#### **1.4.2 Molecular weight**

Phytases have molecular weights ranging between 14–500 kDa. Average molecular masses of bacterial phytases are smaller than those of fungal phytases (40–55 vs. 80–120 kDa), mainly due to differences in extent of glycosylation. Glycosylation is known to be essential for the secretion of proteins (Swati and Satyanarayana, 2014). The molecular masses of plant phytases isolated from corn, wheat, lupine, oat, and barley range from 47 to 76 kDa (Lei and Porres, 2003). *Bacillus* phytases have a molecular weight in the range of 30 to 50 kDa (Fu *et al.*, 2008; Oh *et al.*, 2004),

while those from *Aspergillus* range from 50 to 60 kDa (Konietzny and Greiner, 2002). Most phytases characterized thus far are monomeric proteins, however there have also been reports of phytase consisting of multiple sub-units (Konietzny and Greiner, 2002; Singh *et al.*, 2011) such as phytase B from *A. niger* which is a tetramer (Oh *et al.*, 2004). The phytase characterized from *P. anomala* is a homohexamer with a molecular mass of 390 kDa (Vohra and Sataynaryana, 2002). Segueilha *et al* (1992) reported a phytase from *S. castellii* with a molecular weight of 490 kDa. The glycosylated protein was tetrameric, with one large subunit (125 kDa) and three identical small subunits (70 kDa). Deglycosylation of rPPHY from *Pichia anomala* resulted in reduction of the monomer from approximately 70 to 53 kDa, which confirms it to be a glycoprotein with 24.3 % N-linked carbohydrate (Swati and Satyanarayana, 2014).

The activity and properties of phytase is influenced by the degree of glycosylation. It is considered essential for the secretion of proteins and may influence the pI of the protein, lower the level of expression by consuming metabolic energy and may also impact on stability and catalytic properties of the enzyme (Wyss *et al.*, 1999). A synthetic phytase gene, *phyII*s, was synthesized from *A. niger* 113 (*phyII*) and *phyA* from *A. niger* NRRL3135. The construct was integrated into the genome of *P. pastoris* for phytase expression and secretion by homologous recombination. Due to the heavy glycosylation, the expressed phytase varied in size (120, 95, 85, and 64 kDa) that was deglycosylated to a homogeneous 64 kDa species (Xiong *et al.*, 2004).

### 1.4.3 Modulators of enzyme activity

Metal ions are essential trace elements that affect the production and functional properties of enzymes. Phytase activity adapts by metal ions present in the environment, hitherto it is difficult to ascertain whether activation/inhibition is caused by the binding of metal ions to phytic acid or due to the modulatory effects on the enzyme. Cations  $\text{Fe}^{3+}$ ,  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  at 0.5 mmol/L decreased phytase activity in sonicated cells of *Enterococcus hirae* by 99.4, 90.7 and 96.5%, respectively, while  $\text{Mg}^{2+}$  increased activity by 11.0% (Marounek *et al.*, 2009). Similarly, the activity of phytase produced by *K.pneumoniae* 9-3B was slightly stimulated by  $\text{Ca}^{2+}$  and ethylenediaminetetra acetic acid (EDTA), and inhibited by  $\text{Zn}^{2+}$  and  $\text{Fe}^{2+}$  (Escobin-Mopera *et al.*, 2012). In separate studies, *Bacillus* phytases were found to be metal ion-dependent as they required calcium for activity and

stability (Fu *et al.*, 2008; Yao *et al.*, 2014; Reddy *et al.*, 2015). Graminho *et al.* (2015) reported strong inhibition of a purified HAP from *Burkholderia* sp. strain a13 by  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Hg}^{2+}$  and iodoacetic acid, while thiol group containing DTT and 2-mercaptoethanol activated the enzyme. Similarly, plant alkaline phytase isolated from lily pollen was also reported to be  $\text{Ca}^{2+}$  dependent and inactivated by EDTA, vanadate and  $\text{MgCl}_2$  (Jog *et al.*, 2005). Purified *Aspergillus niger* CFR 335 phytase was inhibited by  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Hg}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Fe}^{2+}$ . Activity was enhanced by low concentrations of  $\text{Fe}^{3+}$  and high concentrations of  $\text{Na}^+$ ,  $\text{Li}^+$  and  $\text{Ca}^{2+}$  (Gunashree, and Venkateswaran, 2014). Reducing reagents, such as 2-mercaptoethanol, dithiotreitol and reduced glutathione have no major effect on the enzymatic activity of phytate-degrading enzymes (Konietzny and Greiner, 2002; Vohra and Sataynaryana, 2002; Fu *et al.*, 2008).

#### 1.4.4 Substrate specificity

Phytases are phosphatases showing high affinity for phytate, though these also show broad range of specificity to other substrates, such as adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), guanosine mono-phosphate (GMP), guanosine triphosphate (GTP), nicotinamide-adenine dinucleotide phosphate (NADP), p-nitrophenyl phosphate, phenyl phosphate, 1-naphthyl phosphate, 2-naphthylphosphate, fructose 1,6-diphosphate, fructose 6-phosphate, glucose 1-phosphate, glucose 6-phosphate, galactose 1-phosphate,  $\alpha$ -glycerophosphate,  $\beta$ -glycerophosphate, pyridoxalphosphate, o-phospho-l-serine, and pyrophosphates (Konietzny and Greiner, 2002; Lei and Porres, 2003; Oh *et al.*, 2004; Wang *et al.*, 2011; Kumar *et al.*, 2014). Substrate specificity and affinity are important properties of phytases, specifically related to the physiological nature of the substrate.

Phytases with broad substrate specificity readily degrade phytate to *myo*-inositol monophosphate with no major accumulation of intermediates. Unfortunately, broad substrate specificity is usually coupled with low specific activity (Konietzny and Greiner, 2002). Phytases with narrow substrate specificity result in *myo*-inositol tris- and bisphosphate accumulation during phytate degradation coupled with a progressive release of phosphate (Konietzny and Greiner, 2002). *Bacillus* phytases (Choi *et al.*, 2001; Gulati *et al.*, 2007a; Fu *et al.*, 2008; Reddy *et al.*, 2015) and lily pollen alkaline phytase (Barrientos *et al.*, 1994; Jog *et al.*, 2005) have been described to be highly specific for

phytic acid in contrast to *Enterobacter sakazakii* ASUIA279 phytase which shows less activity towards GTP (Farouk *et al.*, 2012).

## 1.5 Purification of phytases

Phytases are purified using classical biochemical techniques such as ammonium sulphate/acetone precipitation, ion-exchange and gel-filtration chromatographic steps (Konietzny and Greiner, 2002). These techniques are generally performed to characterize the enzyme for potential use in industries. Microbial extracellular phytases have been easily obtained from the culture filtrate in high yields. Intracellular phytases must be extracted from the biomass before purification which leads to additional cost to the downstream processing. Separation of phytases from other phosphatases is another major problem, especially when purifying phytases from plants. The biochemical and catalytic properties of several phytases has been shown in Table 1.2.

Phytase purified from *K. pneumoniae* 9-3B is a monomeric protein with an estimated molecular weight of 45 kDa based on size exclusion chromatography and SDS-PAGE analyses. The purification scheme included cation exchange chromatography (Hi-Trap Q XL) and gel filtration (Superdex 75) steps using AKTA Explorer (GE healthcare) that resulted in a 240- and 2077- fold purification of the enzyme with 2% and 15% recovery of the total activity for liberation of inorganic phosphate and inositol, respectively (Escobin-Mopera *et al.*, 2012). Yu and Chen (2013) reported a novel, heat-tolerant and neutral phytase with a molecular mass off 43 kDa from *Bacillus nealsonii* ZJ0702. Using a purification scheme involving  $(\text{NH}_4)_2\text{SO}_4$  precipitation, DEAE-sepharose anion-exchange column chromatography and Sephadex G-100 size-exclusion column chromatography, the enzyme was purified to homogeneity with a purification fold and yield of 44 and 5.7%, respectively. Recently, Graminho *et al.* (2015) obtained 108.8-fold purified phytase from *Burkholderia* sp. strain a13 using the AKTA purifier with a yield of 32.1%. The purification steps in this investigation included ammonium sulphate precipitation and the use of Mono S HR 5/5 and Mono S QR 5/5 columns followed by elution using a linear gradient of 0-500 mM NaCl.

Phytases from the yeast *Rhodotorula mucilaginosa* JMUY14 was purified 15.2-fold using a combination of DEAE Sepharose Fast Flow, SP Sepharose Fast Flow, and Sephadex G-100 columns. Molecular weight of the purified phytase was estimated as 63 kDa by SDS-PAGE (Yu

*et al.*, 2015). Other purified phytase include those from *Aspergillus flavus* ITCC 6720 (Gaind and Singh, 2015), *Humicola nigrescens* (Bala *et al.*, 2014), *Bacillus* sp. HJB17 (Lu *et al.*, 2014) *Bacillus lehensis* MLB2 (More *et al.*, 2015), *Pseudomonas aeruginosa* p6 (Sasirekha *et al.*, 2012) and *Volvariella volvacea* (Xu *et al.*, 2012).

**Table 1.2** Biochemical properties and characteristics of some purified bacterial phytases during the past 10 years

Sources	Purification strategy	Recovery rate (%)	MW (kDa)	Temp optima (°C)	pH optima	Substrate specificity	K <sub>m</sub> (mM)	pH stability	Thermal stability	Reference
<i>E. sakazakii</i> ASUIA279	10/10 CM-FF Sepharose 16/100 Sephacryl S-200 HR	27	43	50	5	narrow for GTP	0.76	2.5 to 7.0	87% relative activity at 50°C after 55 min	Farouk <i>et al.</i> (2012)
<i>B. subtilis</i> CF92	DEAE-Sepharose FF Sephacryl S-100-HR	12.7	46	60	7	broad	0.42	4.0 to 8.0	40% relative activity at 80°C after 30 min	Hong <i>et al.</i> (2011)
<i>K. pneumoniae</i> 9-3B	Hi-Trap Q XL Superdex 75	2	45	50	4	broad	0.04	2.0 to 7.0	stable for 1 h at 30°C to 50°C	Escobin-Mopera <i>et al.</i> (2012)
<i>Dickeya paradisiaca</i> phytase appa expressed in <i>E.coli</i>	HiTrap Q Sepharose XL	57	43	55	4.5 and 5.5	narrow for phytic acid	0.399.	4.5 to 7.5	43% relative activity after 30 min at 50 °C	Gu <i>et al.</i> (2009)
<i>Shigella</i> sp. CD2	CM-cellulose Biogel P-100	48	43	60	5.5	narrow for phytic acid	0.25	3.5 to 6.5	75% relative activity after 30 min at 80 °C	Roy <i>et al.</i> (2012)
<i>Pedobacter nyackensis</i> MJ11 CGMCC	HiTrap Q Sepharose XL	28.6	38	45	7	ND	1.28	5.5 to 8.0	< 35% relative activity after 5 min at 50°C	Huang <i>et al.</i> (2009)
<i>Burkholderia</i> sp. strain a13	HiTrap Butyl FF Mono Q HR 5/5 column	32.1	44	45–55	4.5	phytic acid	0.42	ND	stable for 1 year at 4°C	Graminho <i>et al.</i> (2015)
<i>Bifidobacterium pseudocatenulatum</i> ATCC 27919	Resource Q	ND	ND	50	5.5	narrow for phytic acid	ND	5.5 to 6.5	44% relative activity after 15 min at 80 °C	Tamayo-Ramos <i>et al.</i> (2013)
<i>B. licheniformis</i> PFBL-03	DEAE-Sephadex Sephadex G-100	ND	42	75	7	ND	0.0178	6.5 to 7.0	40% relative activity from 4 to 85 °C	Borgi <i>et al.</i> (2013)

ND: Not determined

## 1.6 Production methods and strategies for improved enzyme production

The fermentation process and enzyme production relies on various nutritional, physical and chemical parameters. Nutritional parameters such as carbon and nitrogen sources and physical factors such as pH, temperature, inoculum size, fermentation time, agitation, aeration etc. need to be optimized for enhanced enzyme production. Traditional optimization involves standardizing parameters using one-factor-at-a-time (OFAT) approach and by keeping the other parameters constant.

The use of statistical tools for product optimization is preferred over the OFAT approach as the latter is time consuming, tedious and expensive. Statistical plans are commonly used to enhance phytase production at a reduced cost (Ries and Macedo, 2011). Statistical optimization for enhanced production of enzymes generally involves identification of significant factors using Plackett-Burmann Design (PBD) followed by the study of interaction between identified significant variables using Response Surface Methodology (RSM). PBD is a useful tool for analyzing a large number of factors while avoiding the loss of any indispensable data in successive optimization studies. As a two-factorial design it may be used for identifying critical physical and chemical parameters by screening the  $N$  variables in  $N+1$  experiments for increased enzyme production. Further experiments are performed along the path of steepest ascent to achieve the maximum increase of responses. These experiments aim to reach the general vicinity of the optimum using a sequence of specifically spaced locations along the path (Wu *et al.*, 2013). RSM is a collection of mathematical and statistical techniques useful for the modelling and analysis of problems in which a response of interest is influenced by several variables and the objective is to optimize this response (Montgomery, 2008). The three integral parts of optimization involves firstly performing the statistically designed experiments, secondly estimating the coefficients in a mathematical model, and lastly predicting the response and checking the adequacy of the model. The levels of the variables giving maximum response is then calculated using the mathematical model (Maddox and Richert, 1997).

Central composite designs (CCDs) involve two level factorials (second-order models) by addition of just enough points to estimate curvature and interaction effects. The statistical approach has

been used to optimize numerous experiments (Ries and Macedo, 2011; Sasirekha *et al.*, 2012; Rani and Ghosh, 2011; Bala *et al.*, 2014) [Table 1.3].

**Table 1.3** Fermentation strategies and conditions for improved phytase production

Microorganisms	Fermentation strategy	Medium/Vessel volume (SmF) or weight (SSF)	Temp optima (°C)	pH optima	Carbon source	Reference
<i>B. subtilis</i> US417	SSF	20 g in 250 ml	30	6.5	wheat bran	Kammoun <i>et al.</i> (2012)
<i>Kurthia</i> CZC0806	SmF	NR	20	7.0	saccharose	Yu and Zhang (2011)
<i>E. sakazakii</i> ASUIA279	Smf	100 ml in 250 ml	39.7	7.1	rice bran	Hussin <i>et al.</i> (2012)
<i>E. coli</i>	Fed-batch	3 l in 5 l	37	6.9	glucose	Kleist <i>et al.</i> (2003)
<i>Klebsiella</i> sp. DB-3 FJ711774	Smf	25 ml in 250 ml	45	5.5	orange peel flour	Mittal <i>et al.</i> (2012)
<i>Paecilomyces variotii</i>	SSF	5g in 250 ml	30	5.5	castor bean residues	Madeira <i>et al.</i> (2011)
<i>S. cerevisiae</i> CY	SmF	NR	30	6.0	galactose	In <i>et al.</i> (2009)
<i>P. pastoris</i> FPHY34	Fed-batch	25 l in 50 l	30	5.0	glycerol	Guo <i>et al.</i> (2007)
<i>A.niger</i> FS3	SSF	NR	30	5.0	citric pulp	Spier <i>et al.</i> (2011)
<i>A. niger</i> NCIM 563	SSF	100 g in 250 ml	30	5.5	wheat bran	Bhavsar <i>et al.</i> (2011)
<i>Thermoascus aurantiacus</i>	SmF	40 ml in 250 ml	45	5.5	wheat bran extract	Nampoothiri <i>et al.</i> (2004)
<i>Humicola nigrescens</i>	SSF	10 g in 250 ml	45	5.0	wheat bran	Bala <i>et al.</i> (2014)
<i>A. flavus</i> ITCC 6720	SSF	NR	37	6.0	mustard cake	Gaind and Singh (2015)

NR: Not reported

An overall 8.41-fold increase in phytase production was achieved when Rani and Ghosh (2011) used CCD to study the mutual interactions among the selected variables (mannitol, ammonium sulphate and  $K_2HPO_4/Na_2HPO_4$ ) and their corresponding optimum concentrations. Using Plackett-Burman design, phytase production by *B. subtilis* US417 was improved by 5-fold in submerged fermentation (SmF) while a 4-fold improvement was achieved under solid state fermentation (SSF) conditions (Kammoun *et al.*, 2012). Using CCD based on 3 factors (incubation temperature, incubation period and pH) a specially formulated soy whey based growth medium was optimized to support phytase production by *Saccharomyces cerevisiae* MTCC 5421 and while also supporting  $\alpha$ -D-galactosidase activity by *Lactobacillus plantarum* MTCC 5422 (Roopashri and Varadaraj, 2014).

## 1.7 Applications of phytase

Phytases are enzymes which have multifaceted applications ranging from amelioration of the nutritional value of foods and feeds, plant-growth-promotion, producing various inositol derivatives of major pharmaceutical importance, environmental benefits and more recently to its use in the biofuel industry. Phytase of commercial interest have a set of criteria to conform to that include thermostability, especially for feed processing, storage and for the biofuel industries; the ability to release phytate phosphate in the digestive tract and inexpensive production costs. The bio-ethanol industry is persistently seeking out unique enzymes to improve production efficiency and to create value added products. The most recent is the report on successful application of phytase by Mikulski *et al.* (2014) where thermostable phytase was applied before and after starch liquefaction and saccharification steps, that was executed at higher temperature.

### 1.7.1 Animal feed

Phytase is either not found in the gastrointestinal tract of monogastric animals or it is produced in inadequate levels for the degradation of the phytate complex. The phytate molecule complexes to metal-ions like  $\text{Ca}^{+2}$ ,  $\text{Fe}^{+2}$ ,  $\text{Zn}^{+2}$ ,  $\text{Mg}^{+2}$ ,  $\text{Mn}^{+2}$  and  $\text{Cu}^{+2}$  making them unavailable to the animal for proper growth and development. The major food supplements in animal food are derived from plant sources such as cereals, legumes and soybean which are high in phytate (Gulati *et al.*, 2007b; Singh *et al.*, 2011). Phytase is therefore an attractive additive to the feed to facilitate degradation of phytate. This also reduces the addition of inorganic phosphate to feed and greatly reduces phosphorous pollution in water runoffs (Caipang *et al.*, 2011) since phosphate excretion by animal is reduced by up to 50% (Greiner and Farouk, 2007).

Supplemented phytase must retain its catalytic activity in the animals' upper digestive tract. To remain effective it must remain active under prevailing acidic conditions, should be resilient to proteolytic enzymes and display high activity (Boyce and Walsh, 2007). The superior activity of *A. niger* phytase and its practical application to animal feed for the removal of phytic acid were demonstrated in several studies (Chelius and Wodzinski, 1994; El-Batal and Karem, 2001; Vats and Banerjee, 2005). In fish feed supplements microbial phytases have been shown to enhance the bio-availability of P and nitrogen that would otherwise be bound in phytate and ultimately reduce

the amount of P discharged into the aquatic environment (Cao *et al.*, 2007). The treatment of fish feed with phytase was found to improve protein digestibility and retention in fishes (Singh *et al.*, 2011). Similar results have been documented for poultry and pigs. Broiler chickens fed a diet including phytase showed an increased retention of  $\text{Na}^+$ ,  $\text{Zn}^+$  (Pirgozliev *et al.*, 2012; Walk *et al.*, 2014) and P. Use of microbial phytases to improve protein digestibility and increase the availability of minerals and trace element in pigs have been described in numerous studies (Selle and Ravindran, 2007; Hill *et al.*, 2009).

### **1.7.2 Human nutrition**

Since phytate chelates many important metal ions, especially iron and zinc, phytase supplementation can be a possible solution to many metal-deficiency related diseases in humans. Exploitation of the enzyme may lead to improved mineral absorption or technological development of food processing (Haefner *et al.*, 2005; Jorquera *et al.*, 2008a). Plant staple foods that contain a high percentage of phytate are consumed by the different population groups, leading to Fe and Zn deficiencies (Lei and Porres, 2003).

The effect of supplemental phytase to improve mineral availability from plant diets has been documented. Its consumption provides protection against a variety of cancers due to its anti-oxidative properties, interrupts cellular signal transduction, inhibits the cell cycle and enhances the natural killer (NK) cells activity, however, information on the dosage for humans for eliciting beneficial effects is limited (Kumar *et al.*, 2010). More research is required to determine the correct dosage and apt delivery mechanism of phytase to human foods and targeted cells.

### **1.7.3 Synthesis of lower inositol phosphates**

Inositol phosphates play an important role in cell-signalling pathways (Singh *et al.*, 2011), energy metabolism, metabolic regulation, and signal transduction pathways in biological systems (Fu *et al.*, 2008). The application of phytase for the production of lower inositol phosphate is preferred over chemical synthesis due to milder reactions and improved and more specific stereo-specificity. Chemical synthesis produces a mixture of isomers which results in cost addition to the separation

and purification steps (Vats and Banerjee, 2005). Enzymatic hydrolysis of phytic acid using *S. cerevisiae* resulted in the selective production of D-*myo*-inositol 1, 2, 6-triphosphate, D-*myo*-inositol 1,2,5-triphosphate, L-*myo*-inositol 1,3,4-triphosphate and *myo*-inositol 1,2,3-triphosphate (Siren *et al.*, 1992; Greiner, 2004; Ragon *et al.*, 2008 ). Thus, the use of phytases from different microbial sources can produce different isomers of major pharmaceutical importance.

#### **1.7.4 Transgenic plants and plant growth promotion**

There have been several studies on phytate and its derivatives as potential phosphorus reserves and there are several research groups that aim at unlocking inorganic phosphorus (Pi) from these compounds and improving the P use efficiency by genetic transformation of plants. Yip *et al.* (2003) noticed the phenotypic changes in a tobacco line transformed with a neutral *Bacillus* phytase. The transgenic line showed an increase in number of flower, fruit and lesser inositol phosphate ratio (IP6/IP5) in seed and enhanced growth under phosphate-starvation conditions as compared to the wild type. Mudge *et al.* (2003) showed that transgenic *Arabidopsis* plants secreted phytase from roots only when grown on a low phosphate medium. Transgenic *Arabidopsis* expressing an extracellular phytase from *Medicago truncatula* had significant improvement in organic phosphorus utilization and plant growth (Xiao *et al.*, 2005).

#### **1.7.5 Pulp and paper industry**

Aging of paper is one of the major problems confronted by the pulp and paper industries. Lignin-free paper degrades faster than lignin-containing paper, even when it is sized with acid alum, because lignin acts as an antioxidant and is oxidized faster than cellulose and forms stable radicals. Attempts to prevent oxidation by use of EDTA accelerated the degradation process. Phytic acid is among one of the few complexing agents that blocks the Fenton's reaction (formation of hydroxyl radicals). The removal of plant phytic acid might be important in the pulp and paper industry. Thermostable phytase has the potential to act as a novel biological agent that degrades phytic acid during pulp and paper processing. The enzymatic degradation of phytic acid does not produce any carcinogenic or toxic by-products. Therefore, the exploitation of phytases in the pulp and paper industry could be environmentally friendly and would assist in the development of cleaner

technologies (Liu *et al.*, 1998; Vats *et al.*, 2005). Currently, no further work has been done to make these findings more conclusive.

### **1.8 Biotechnological applications of *Enterobacter* sp.**

The genus *Enterobacter* accommodates a number of species of heterotrophic, Gram-negative, rod-shaped, non-sporulating, facultative-anaerobic bacteria (Manter *et al.*, 2011). Many members of this species have been documented as producers of a wide range of enzymes, chemicals and biological molecules (Table 1.4) such as lipopeptides which are used for preservation of dairy and food products (Mandal *et al.*, 2013). Ghatak *et al.* (2010) reported *E. cloacae* as a producer of extra- and intracellular  $\beta$ -D-galactosidase, which is commonly used in molecular biology as a marker during gene expression.

**Table1.4** List of common enzymes produced by genus *Enterobacter* and its applications

Enzyme	Source	Applications	Sector	Reference
<b>β-galactosidase</b>	<i>E. cloacae</i>	Disposal of whey	Dairy	Ghatak <i>et al.</i> (2010)
	<i>E. aerogenes</i>	Sweetners	Food	Khleifat <i>et al.</i> (2006)
<b>Chitinase</b>	<i>Enterobacter sp.</i>	Bio-control agent	Agriculture	Dahiya <i>et al.</i> (2005)
<b>β-Glucanase</b>	<i>E. cloacae</i>	Clarification and filtration aid	Beverage	Sami <i>et al.</i> (2008)
<b>Lipase</b>	<i>E. aerogenes</i>	Degradation of fat	Detergent	Kumari <i>et al.</i> (2009)
	<i>E. agglomerans</i>	Biodiesel production	Energy and Fuel	Zhen-qian and Chun-yun (2009)
	<i>E. aerogenes</i>	Cheese production	Food	Mitra <i>et al.</i> (2010)
<b>Protease</b>	<i>Enterobacter sp.</i>	Contact lens cleaners	Medical	Kanchana <i>et al.</i> (2012)
	<i>Enterobacter sp.</i>	Meat tenderizing	Food	Owoseni and Onilude (2012)
<b>Tannase</b>	<i>E. ludwigii</i> GRT-1	Gallic acid production	Personal care	Singh <i>et al.</i> (2012)
	<i>E. cloacae</i> MTCC	De-tannification	Food	Beniwal <i>et al.</i> (2013)
	<i>Enterobacter sp.</i>	Tea and wine preparation	Beverage	Sharma and John (2011)
<b>Transglutaminase</b>	<i>Enterobacter sp.</i> C2361	Laminated dough strengths	Baking	Bourneow <i>et al.</i> (2012)
<b>β-xylosidase</b>	<i>Enterobacter sp.</i>	Biofuel production	Energy and Fuel	Campos <i>et al.</i> (2014)
<b>Xylanase</b>	<i>Enterobacter sp.</i> MTCC 5112	Bleaching	Paper and pulp	Khandeparkar and Bhosle (2006)
<b>Pectinase</b>	<i>E. aerogenes</i> NBO2	Fruit juice clarification	Beverage	Darah <i>et al.</i> (2013)
<b>Sucrose isomerase</b>	<i>Enterbacter sp.</i> FMB1	Sweeteners	Food	Cho <i>et al.</i> (2007)

## 1.9 Purpose of the study

The use of phytase in the animal feed industry is rapidly expanding due to rising environmental concerns. The addition of phytase to the diets of monogastric animals eliminates the need to supplement feed with phosphorous to meet dietary requirements. The problem faced by the feed industry revolves around the thermostability of phytase. For the enzyme to retain its activity, it must survive the high temperature used during the pelleting process. Most phytases are produced commercially using fungi, the most popular of which are from members of the genus *Aspergillus*. The production of phytase by bacteria is becoming an alternative to fungal phytases due to their stability at elevated temperature. Furthermore, the broad substrate specificity of fungal phytases could alter other metabolic pathways, thus their bacterial counterparts are preferred (Reddy *et al.*, 2015). Therefore the aim of this study is to screen for a phytase producing bacterium and to enhance phytase production using statistical methods followed by purification and subsequent characterisation of the enzyme.

### Objectives

1. Screening, identification and selection of a potent phytate-hydrolysing bacterial isolate
2. Statistical optimization for enhanced production of phytase in batch and fed-batch cultivations
3. Purification and characterization of the enzyme
4. Applications of phytase in dephytinizing animal feeds and bread

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## CHAPTER 2: SCREENING, ISOLATION AND MOLECULAR IDENTIFICATION OF PHYTASE PRODUCING STRAINS

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### 2.1 INTRODUCTION

Presently P is mined at a faster rate than geologic cycles can replenish for soil fertilization and animal feed purposes. Decreasing global phosphorous reserves are of major concern, and there are indications that the minable phosphorus deposits will be depleted by the end of 21st century (Lei *et al.*, 2013). Due to increasing demands for food and feed, alternate methods must be implemented to meet the rising P demand. The power of enzyme technology has been harnessed in recent times as an attractive and more efficient process to overcome such problems. The use of phytase to enhance phosphate utilization from phytate offers a solution to the P depletion crisis (Vaccari, 2009). Phytase from various sources including animals (Marounek *et al.*, 2010), plants (Brinch-Pedersen *et al.*, 2014) and microbes (Yao *et al.*, 2011; Lei *et al.*, 2013) have been screened for requisite properties that suits enhanced production and application on a commercial scale. Regardless, the search for the ‘ideal’ phytase still continues and there are still many unexplored phytate producers, widespread throughout many ecosystems. Hitherto phytate still remains a major problem accumulating in soil and acting as an anti-nutrient in the gut of simple-stomached animals.

Microbes from different environments are being explored to find a robust phytase with requisite characteristics that make it a potentially valuable and commercially viable. Despite having a plethora of reports on phytases, the search for an ideal and cheaper phytase is still ongoing that can effectively hydrolyze phytate in the upper acidic digestive tract of monogastric animals and could be resilient to the high temperatures (65–80°C) of feed pelleting (Lei *et al.*, 2013). Suitable enzyme assays are necessary prerequisites for the rapid screening of large numbers of microbial isolates. Several methods of phytase quantification have been developed including the molybdate blue method, molybdovanadate method and acetone phospho-molybdate method but the one developed by Fiske and Subbarow (1925) is practiced most commonly. In a collaborative study to validate a colorimetric assay for determination of microbial phytase activity in feed, trials were conducted by several investigators and this method was proved to be the most accurate and reliable

(Engelen *et al.*, 2001), and therefore phytase was assayed using the same method in the present study.

After an isolate producing significant titres of phytase with requisite characteristics is selected, production strategies must be optimized for the enhanced production of enzyme in a cost-effective manner. Usually sodium or calcium phytate is used as the substrate, however these are rather expensive and would not be feasible for large scale production. Conventional production of phytase relies on expensive media consisting of sodium or calcium phytate and other essential nutrient components which ultimately lead to high production costs that limit application of the enzyme. Utilization of agro-industrial waste residues has been given a lot of attention during recent years as it allows for the complete bio-recycling of materials that would otherwise be of no value (Ramachandaran *et al.*, 2005).

Agro-waste residues are generated following the harvesting of products from plant growth and industrial processing. These residues represent one of the most energy-rich resources on earth due to their organic nature and deemed as loss of prospective beneficial materials which may eventually yield various value-added products (single-cell protein, enzymes organic acids, amino acids and ethanol). The massive quantities of agricultural wastes generated annually necessitate its immediate exploitation for several applications. Due to high abundance and low prices, agro-industrial wastes are an important itinerary pertaining to cost-effective phytase production.

The present chapter therefore focuses on the selection and identification of a high phytase producing isolate, followed by investigating different agro-industrial residues for phytase production.

## 2.2 MATERIALS AND METHODS

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### 2.2.1 Screening and selection of phytate degrading microbial strains

Soil samples were collected from different sites in and around the Durban area. Approximately 2 g of soil was suspended in 50 ml of de-ionized water and  $10^{-1}$  to  $10^{-9}$  dilutions of the suspension were plated on phytase screening media (PSM) containing 1.5% glucose, 0.1% sodium phytate (phytic acid dodecasodium salt, Sigma), 0.2%  $\text{NH}_4\text{NO}_3$ , 0.05% KCl, 0.05%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.03%  $\text{MnSO}_4$ , 0.03%  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  and 2.0% agar at pH 6.5. Sodium phytate (Na-phytate) was sterilized separately using a 20  $\mu\text{M}$  filter and added to the rest of the medium after autoclaving. Plates were incubated at 37°C for 48 h and isolates capable of hydrolyzing sodium phytate were recognized by the surrounding clear zones of hydrolysis (Howson and Davis, 1983). Colonies exhibiting zones of clearance were selected and streaked onto fresh PSM plates. The zone of hydrolysis for each isolate was then measured from triplicate plates and the mean values were reported.

Colonies were re-streaked on nutrient agar slants and preserved at 4°C. For long term preservation, a mid-log suspension of equal volumes of cell culture and glycerol (40%) was mixed and 2 ml sample was transferred to respective cryovials aseptically, and stored at -80°C in an ultra-freezer until required. All chemicals were purchased from Sigma-Aldrich and Merck.

### 2.2.2 Confirmation of phytase producing isolates

#### 2.2.2.1 Qualitative screening: Plate assay

Phytase degrading isolates were further verified following Casey and Walsh (2003) counterstaining method. Briefly, each plate was flooded with a 2% (w/v) aqueous cobalt chloride ( $\text{CoCl}_2$ ) solution. After incubation at room temperature for 5 min, the  $\text{CoCl}_2$  solution was replaced with freshly prepared solutions of 6.25% (w/v) aqueous ammonium molybdate and 0.42% (w/v) ammonium vanadate in equal volumes. After 5 min, the solution was poured out and plates were examined for yellow-orange zones of hydrolysis against an opaque background.

### ***2.2.2.2 Quantitative screening***

Phytase screening medium (PSM) [0.5%  $(\text{NH}_4)_2\text{SO}_4$ , 0.5% KCl, 0.01%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.01%  $\text{NaCl}_2$ , 0.01%  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.001%  $\text{FeSO}_4$ , 0.001%  $\text{MnSO}_4$  and 0.5% sodium phytate] was used for initial phytase production (Howson and Davis, 1983). Cell cultures were inoculated into 250 ml Erlenmeyer flasks with a 50 ml working volume at pH 6.5 and grown at 37°C for 48 h at 180 rpm on an Infors (Bottmingen-Basel, Switzerland) shaking incubator. All experiments were performed in triplicate.

Cell free supernatant was harvested by centrifugation at 10 000 rpm for 15 min at 4°C and assayed for phytase activity according to the method as described by Heinonen and Lahti (1981). The reaction mixture consisted of 875  $\mu\text{L}$  of 0.1 M sodium acetate buffer, pH 5.4 and 75  $\mu\text{L}$  of 1.5 mM sodium phytate prepared in the same buffer. The mixture was pre-incubated for 10 min at 50°C and 100  $\mu\text{L}$  of the crude enzyme was added to the pre-incubated mixtures and incubated at 50°C for 30 mins. The reaction mixture was cooled to room temperature and a 2 ml aliquot of a freshly prepared solution of acetone/10 mM ammonium molybdate/5 N  $\text{H}_2\text{SO}_4$  (2:1:1 v/v) was added followed by addition of 100  $\mu\text{L}$  of 1.0 M citric acid. Any cloudiness was removed by centrifugation prior to measurement of liberated phosphate at an absorbance at 355 nm.

A calibration curve was plotted using potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) with working concentrations ranging from 30 to 360  $\mu\text{M}$ . One unit of phytase activity was defined as the amount of enzyme required to liberate 1 nmol of Pi per second under standard assay conditions (Singh and Satyanarayana, 2006; Swati and Satyanarayana, 2014).

## **2.2.3 Submerged fermentation for phytase production using different agro-residues**

### ***2.2.3.1 Substrate preparation***

Different agro-industrial residues including wheat bran (WB), orange peels (OP), corn cobs (CC) and sugarcane bagasse (SCB) were used as phytate-containing inducers for submerged fermentation. Orange peel and corn cobs were washed thrice with de-ionized water. Sugarcane

bagasse was washed once with tap water and thrice with de-ionized water. Wheat bran was not washed prior to fermentation. Each of these substrates were then dried at 60°C in an oven for 12 h followed by grinding in a mill and sieving to constant particle size.

#### **2.2.3.2 Fermentation using agro-industrial residues**

Wheat bran, orange peels, corn cobs and sugarcane bagasse (2% w/v) were mixed in separate 250 ml Erlenmeyer flasks to which 50 ml of production medium containing (g/l):  $\text{NH}_4\text{NO}_3$ , 1.0;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5; KCl, 0.5;  $\text{FeSO}_4$ , 0.01;  $\text{MnSO}_4$ , 0.01 was added. The medium in each flask was adjusted to pH 6.5 and sterilized by autoclaving at 121°C for 20 minutes. After cooling, the flasks were inoculated with a 12 h, 2% bacterial cell suspension prepared in LB broth (10 g/l peptone, 5 g/l yeast extract, 5 g/l NaCl) at 37°C and 180 rpm. Fermentation was carried out for 48 h and samples of 1 ml volume were removed at 12 h intervals for phytase assay. All experiments were performed in triplicate.

#### **2.2.4 Identification of phytate degrading isolates**

Sequencing of the 16S rRNA gene from selected isolates was done by Inqaba Biotech. Resultant genomic sequences were aligned using DNA analysis software, DNAMAN (Lynon 54 Biosoft) and edited using Chromas. Sequence comparison was done by the Basic Local Alignment Search Tool – BLAST ([blast.ncbi.nlm.nih.gov/Blast.cgi](http://blast.ncbi.nlm.nih.gov/Blast.cgi)) [Altschul *et al.*, 1990].

#### **2.2.5 Phylogenetic tree construction**

Based on the 16S rRNA sequences the phylogenetic tree for the selected isolate was constructed using Mega 5.05 (Kumar *et al.*, 2004). Distances were calculated using Kimura's two-parameter model. (Kimura, 1980) and clustering was performed using the neighbour-joining method. Bootstrap analysis was used to evaluate tree topology by means of 1000 re-samplings.

### 2.2.6 Induction experiments for phytase production by *Enterobacter* sp. ACSS

The constitutive or inducible nature of phytase for *Enterobacter* sp. ACSS was investigated using different medium compositions as indicated in Table 2.1.

**Table 2.1** LB and PSM media with sodium phytate and wheat bran as inducers for phytase production

Media	Composition
Medium A1	LB broth without inducer/s
Medium A2	LB broth with 500 $\mu$ M Na-phytate
Medium B1	PSM broth without inducer/s
Medium B2	PSM broth with 500 $\mu$ M Na-phytate
Medium B3	PSM broth with 2% WB*
Medium B4	PSM broth with 500 $\mu$ M Na-phytate and 2% WB

\*WB=Wheat Bran, 2% (w/v)

Briefly, 250 ml Erlenmeyer flasks with a working volume of 50 ml were inoculated with a 1% cell suspension and incubated for 12 h in LB broth. Fermentation was carried out for 24 h at 200 rpm, 37°C and pH 5.5. Samples were taken at 12 h intervals and assayed for phytase activity. All experiments were done in triplicate and their mean was reported.

## 2.3 RESULTS

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### 2.3.1 Screening and selection of phytate degrading microbial strains

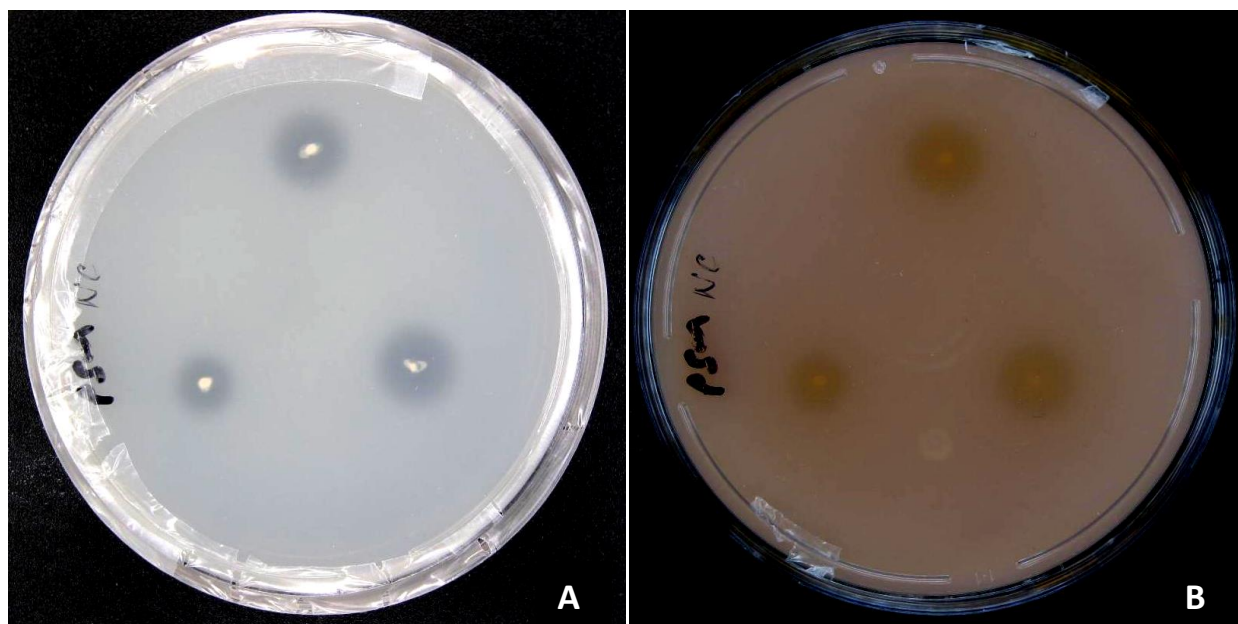
Phytase producing microorganisms were isolated from soil collected from a local poultry farm and the rhizosphere of sugarcane. Initial screening medium for phytate degrading microorganisms used sodium phytate as a selective agent. Seven phytase producing bacteria were isolated based on their ability to hydrolyse sodium phytate on PSM agar plates. Isolate AC2 produced the largest zone of hydrolysis (5 mm) among the phytase producing isolates (Table 2.2).

**Table 2.2:** Zone of hydrolysis due to different isolates on PSM plates

Isolates	Average zone of hydrolysis (mm)
AC 1	4
AC 2	5
AC 3	4
AC 4	3
AC 5	3
AC 6	2
AC 7	2

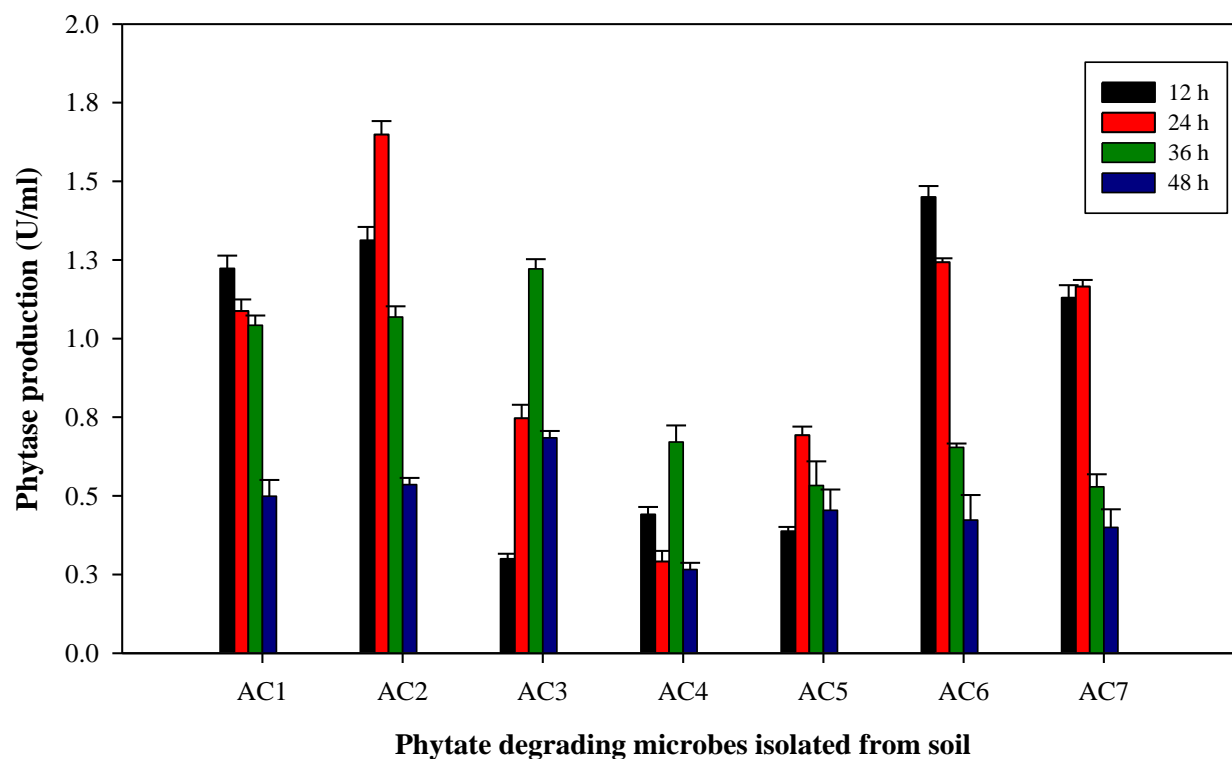
### 2.3.2 Confirmation of phytase producing isolates.

It has been reported that the zone of hydrolysis on PSM plates could also be formed due to acid production by false positives, and therefore the possibility of any such false positives must be eliminated. Confirmation of phytase production was accomplished qualitatively by counterstaining and quantitatively by phytase assay after shake flask fermentation in PSM broth. Phytate degradation before and after counter-staining is illustrated in Fig. 2.1. The clear zone of hydrolysis was evident against an opaque background (Fig 2.1a) and it was re-confirmed following counterstaining (Fig 2.1b) with cobalt chloride and a combination of ammonium molybdate and ammonium vanadate solutions.



**Figure 2.1** (A) Phytate degradation on PSM plate showing zones of hydrolysis after 48 h at 37°C (B) and counterstaining for confirmation of phytase production.

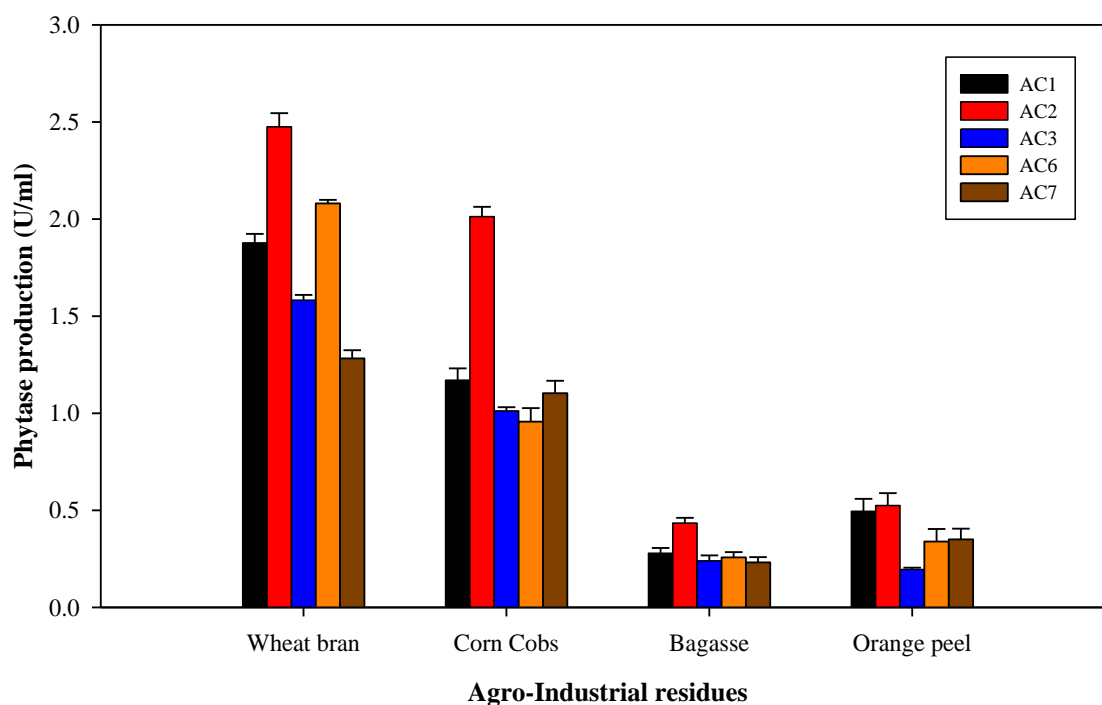
Qualitative screening in shake flasks also confirmed AC2 as the highest producer of extracellular phytase (Fig 2.2). A titre of 1.65 U/ml of phytase was produced in 24 h after which it declined. AC1 and AC6 produced a maximum of 1.22 U/ml and 1.45 U/ml, respectively after 12 h, after which the production declined. AC5 and AC7 produced 0.70 U/ml and 1.16 U/ml in 24 h while AC3 and AC4 produced a maximum of 1.2 U/ml and 0.67 U/ml after 36 h. AC4 and AC5 produced the lowest titres of phytase in minimal medium and therefore excluded from further studies.



**Figure 2.2** Comparative phytase production using different isolates after 12, 24, 36 and 48 h in PSM medium. The values shown represent averages from triplicate experiments  $\pm$  SD.

### 2.3.3 Submerged production of phytase using different agro-residues

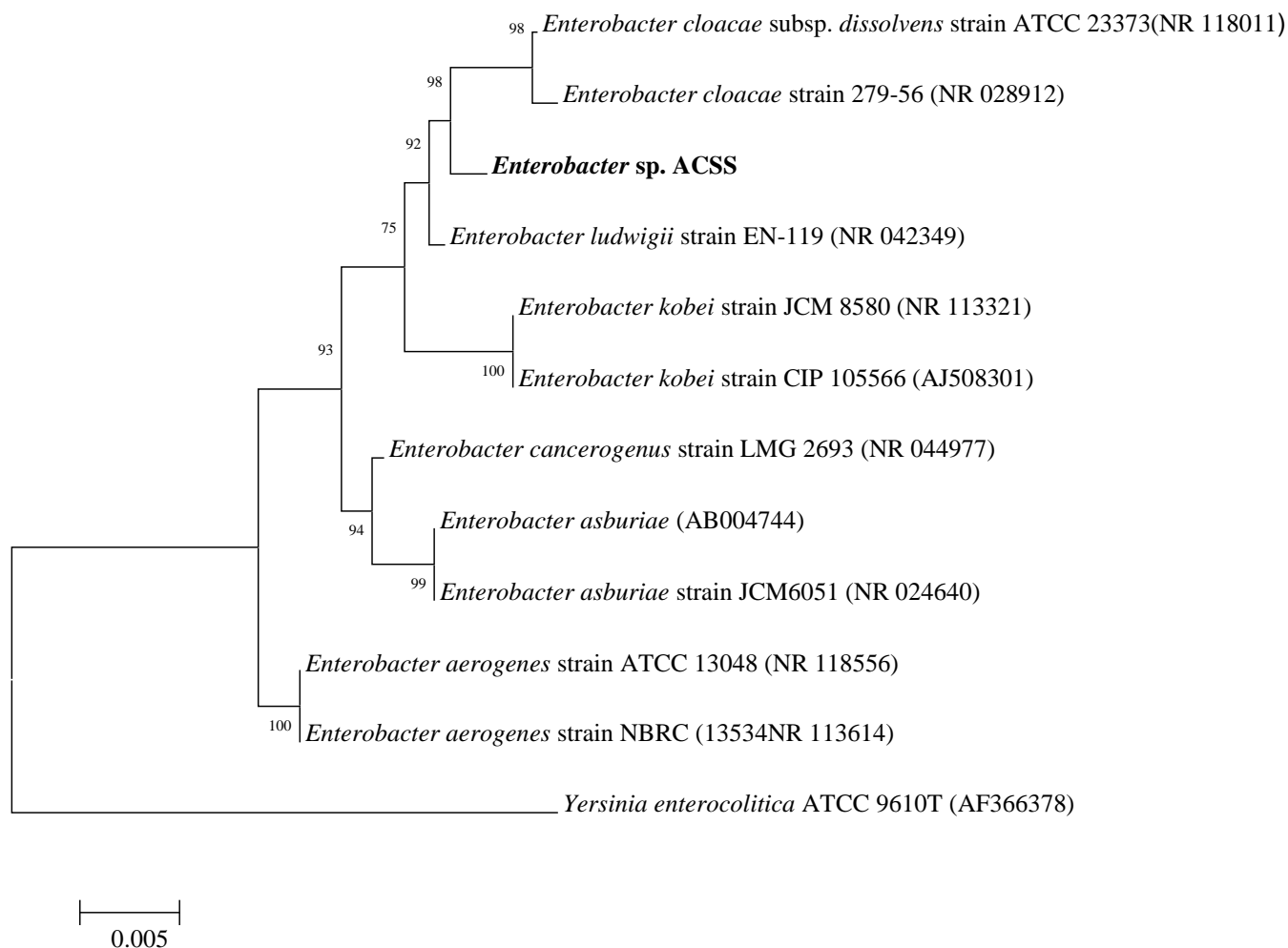
The top five phytase producers were cultivated with different phytate containing agro-industrial residues such as wheat bran, sugarcane bagasse, orange peels, and corn cobs (Fig 2.3). All isolates produced maximum production after 24 h of fermentation using wheat bran containing medium. AC1, AC2, AC3, AC6 and AC7 produced 1.87 U/ml, 2.48 U/ml, 1.58 U/ml and 1.28 U/ml of phytase, respectively. Corn cobs and orange peel were the next best sources of phytate among agro-industrial residues followed by bagasse. Amongst the isolates, AC2 produced highest titre of phytase of 2.48 U/ml, 2.01 U/ml, 0.52 U/ml and 0.43 U/ml using wheat bran, corn cobs, orange peel and bagasse, respectively and was therefore selected for further studies.



**Figure 2.3** Phytase production by isolates using four different agro-industrial residues. Sodium phytate was replaced by 2% (w/v) of each of the agricultural residue. The values shown represent averages from triplicate experiments  $\pm$  SD.

### 2.3.4 Identification and phylogenetic tree construction of the selected isolate

Biochemical and morphological analysis indicated that the bacterium was Gram negative, rod-shaped and non-spore forming. From the 16S rRNA sequence results and BLAST analysis, isolate AC2 was identified as *Enterobacter* sp. ACSS. The 16S rDNA sequence (appendix) was deposited in the NCBI GenBank nucleotide sequence database under the accession number KP814680. A phylogenetic tree was constructed to show its similarity with known members of the family *Enterobacteriaceae* (Fig 2.4). Bootstrap values of neighbor-joining analysis of 1,000 replications appear as percentages on the branches. *Enterobacter* sp. ACSS is more similar to *Enterobacter cloacae* and *Enterobacter ludwigii* strains.



**Figure 2.4** A neighbor-joining tree showing the phylogenetic relationships of *Enterobacter* sp. ACSS, with type strains of *Enterobacter* species. *Yersinia enterocolitica* ATCC 9610T (AF366378) was used as an outgroup. Bootstrap values are expressed as percentages of 1,000 replications and shown at the branch points. Scale bar represents the number of nucleotide substitutions per position.

### 2.3.5 Induction studies

The induction of phytase from *Enterobacter* sp. ACSS was studied in LB broth medium, LB broth supplemented with phytate, wheat bran medium, wheat bran supplemented with phytate and PSM broth with and without the addition of sodium phytate. Table 2.3 represents a summary of the experiment.

**Table 2.3** Phytase production due to induction using sodium phytate and wheat bran in LB and PSM media

	<b>Media</b>	<b>Phytase production (U/ml)</b>
<b>Medium A1</b>	LB broth without inducer/s	0
<b>Medium A2</b>	LB broth with 500 $\mu$ M Na-phytate	$1.38 \pm 0.02$
<b>Medium B1</b>	PSM broth without inducer/s	0
<b>Medium B2</b>	PSM broth with 500 $\mu$ M Na-phytate	$1.46 \pm 0.08$
<b>Medium B3</b>	PSM broth with 2% WB	$2.24 \pm 0.54$
<b>Medium B4</b>	PSM broth with 500 $\mu$ M Na-phytate and 2% WB	$1.30 \pm 0.71$

In all instances where sodium phytate or wheat bran was omitted, no phytase production was detected indicating the inducible nature of this enzyme. A maximum of 2.24 U/ml of phytase was produced in medium B3 where 500  $\mu$ M Na-phytate was replaced by 2% wheat bran. No phytase activity was detected in LB medium containing wheat bran and therefore it was omitted. Decreased production in medium B4 infers repression in phytase synthesis due to inducer overdose. It is to be noted that medium B4 includes 500  $\mu$ M Na-phytate and 2% wheat bran as two phytate sources, and its concentration should be optimized for enhanced phytase production.

## 2.4 DISCUSSION

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When screening for new enzymes, researchers target strains from the environment with the anticipation that they are possibly valuable in producing a useful and marketable product. Despite the large number of species being isolated, only a few isolates possess the criteria for a commercially valuable microorganism (Lei *et al.*, 2013). Phytase producing microorganisms were targeted from soil from poultry farms and the rhizosphere of sugarcane. Plants generally have phytase producing microorganisms near their rhizosphere which aid in releasing inorganic P from recalcitrant phytate molecules in the soil. Several phytase producing bacterial isolates have been obtained from soil, chicken waste, fish intestines and rhizosphere of plants (Jorquera *et al.*, 2008b; Khan and Ghosh, 2012).

In this study, a total of seven phytase producers were isolated after screening and confirmation of phytase production by qualitative and quantitative methods. AC2 was shown to produce highest titre of phytase (1.65 U/ml) in 24 h at 37°C and 180 rpm in PSM medium. AC2, AC4 and AC5 were isolated from soil near the sugarcane rhizosphere forming a close link between them and the root physiology. The root absorbs minerals and specifically uptakes P after phytate degradation (Marlida *et al.*, 2010; Jorquera *et al.*, 2008b). The success of screening is dependent on an adept testing method. To overcome the problem of false positives during plate screening, Bae *et al.* (1999) developed a two-step counterstaining procedure, in which solid agar medium was first flooded with an aqueous cobalt chloride solution and secondly by an aqueous ammonium molybdate/ammonium vanadate solution. The exact principle has not been clearly defined however it is hypothesized that phytate chelates with cobalt to produce an opaque medium. Where phytase is present, there is an absence of the complex. The change of colour of the medium from pink to yellowish-orange after staining with the molybdate/vanadate solutions served to enhance the contrast. Other methods of phytase quantification include the molybdenum blue method (Chen *et al.*, 1956; Han *et al.*, 1999; Augspurger *et al.*, 2003), the molybdovanadate method (Engelen *et al.*, 1994; Leeson *et al.*, 2000; Huang *et al.*, 2006) and a non-toxic, simple and fast kinetic, high throughput method for phytase assay using phytic acid protein complex (IP<sub>6</sub> – lysozyme) as a substrate (Tran *et al.*, 2011).

A bacterium producing significant titres of phytase in a cost-effective medium was studied. Sodium phytate is an expensive substrate that can be easily substituted with inexpensive agro-residues (orange or lemon peel, bagasse, corn cobs, oats etc.) resulting in sustained yield of phytase for a more economically viable process. Furthermore, these serve as excellent support matrices (Imandi *et al.*, 2008) and act as the principle sources of proteinaceous nutrients (Nigam *et al.*, 2000). Careful screening of assorted agro-industrial residues is especially important to overcome high production costs (Sreedevi and Reddy, 2012; Suresh and Radha, 2015).

The top five phytase producing isolates were compared for their ability to use agro-industrial waste as a phytate substrate for production of phytase. Wheat bran was the champion agricultural residue affecting phytase production (Fig 2.3). Using wheat bran, corncobs, orange peel flour and sugarcane bagasse in separate experiments *Enterobacter* sp. ACSS produced 2.48 U/ml, 2.01 U/ml, 0.52 U/ml and 0.43 U/ml of phytase, respectively. On the basis of percentage dry weight phytate content is highest in WB [wheat bran (0.25-1.13)], followed by corn cobs (0.136-0.280) [Bhuiyan *et al.*, 2010)], orange peel flour (0.062-0.082) and sugarcane bagasse (0.038-0.084), (Mittal *et al.*, 2012). The amount of phytase produced was directly related to the percentage phytate content in investigated agro-industrial residues.

Awad *et al.* (2014) reported a novel and cheaper way for phytase production using a combination of corn cobs and corn bran while Madeira *et al.* (2011) used castor bean residues and Spier *et al.* (2011) used citric pulp residues. Following screening of several substrates including wheat bran, rice bran, red gram bran, groundnut oil cake, sesame oil cake and coconut oil cake *B. subtilis* sp. c43 produced 0.47 U/ml of phytase using wheat bran as primary source of phytate (Sreedevi and Reddy, 2011). Kammoun *et al.* (2012) used statistical optimization to produce 0.78 U/ml of phytase from *Bacillus subtilis* US417 in wheat bran containing medium.

The inducible or constitutive nature of phytase was investigated. Limiting phosphate conditions result in expression of acid phosphatases and phytases but high phosphate conditions are known to repress the enzyme synthesis (Vats and Banerjee, 2004). In bacteria, phytase is mostly known to be inducible with complex regulation machinery, but phytase synthesis is not controlled uniformly among different bacteria (Konietzny and Greiner, 2002). In the present study with all

four agro-industrial residues used, phytase production corroborated to the phytate content present in each substrate. Induction studies (Table 2.3) substantiate the inducible nature of this phytase as it was only produced in media containing sodium phytate or wheat bran. The synthesis of phytase could be regulated by a combination of phytate and phosphate starvation in medium containing phytate as the sole P source leading to induction. A marked decline in phytase production was observed using medium B4 which had increased phytate content. This may explained by the initial increase of phosphate that acts as a repressor and as a consequence phosphate starvation once again induces phytase production (Kerovuo *et al.*, 2000).

Many reports highlight the importance of nature of the medium components affecting phytase production. For the synthesis of *Klebsiella terrigena* phytase only carbon starvation was effective for immediate production and only commenced in the presence of phytate (Greiner *et al.*, 1997). The same effect was observed by Hussin *et al.* (2010) where phytase synthesis from bacterial strains *P. stewartii* ASUIA271, *E. sakazakii* ASUIA279 and *B. cereus* ASUIA260 was observed only after supplementation with 7.5% rice bran. However, Greiner *et al.* (1993) reported no influence of phytate in the medium on the synthesis of phytase by *E.coli*.

The isolation of *Enterobacter* sp. for phytase production has been previously reported by two separate studies conducted by Yoon *et al* (1996) and by Farouk *et al* (2012). In both instances phytase-producing bacterium *Enterobacter* sp. was isolated from soil near the root of leguminous plants. Both phytases were optimally active at a temperature of 50 – 60 °C but showed different pH optima's of pH 7 (Yoon *et al.*, 1996) and pH 4.5 (Farouk *et al.*, 2012).

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## CHAPTER 3: ENHANCED PRODUCTION OF PHYTASE USING STATISTICAL OPTIMIZATION

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### 3.1 INTRODUCTION

The process parameters affecting enzyme production must be optimized in order to meet the rising needs of the global enzyme market. Statistical optimization of process parameters is a proven technique to ameliorate microbial enzyme production. It exploits the method of planning and analyzing experiments using design of experiments (DoE) that ensures validated and statistically significant results. The dependant variable, which is influenced by several independent variables and their interactions, can be studied and optimized by careful designing of experiments. A good design relies on proper application of statistical schemes and planning, while a poorly designed experiment may lead to false understanding and its interpretation (Antony, 2003; Baş and Boyacı, 2007).

The enzyme production is significantly influenced by a range of nutritional and physical parameters. Screening of variables affecting production is the first step in the process optimization which is followed by identification and determination of the optimal levels of the significant factors. The conventional one-variable/factor-at-a-time (OFAT/OVAT) is tedious and time consuming method. Moreover, interactions between variables are not taken into consideration, and suggest optimum concentrations which are far away from the actual optimum. Furthermore, this design uses a large number of resources to generate a limited amount of information about the process (Dette *et al.*, 2005; Lu *et al.*, 2012).

Statistical designs are useful in optimization, modelling, and control of enzyme production in SmF and SSF. Highly fractional factorial designs such as PBD or Taguchi's orthogonal array designs are advantageous to identify and select significant factors out of numerous variables in shake-flasks. Following the initial screening, experimental strategies may employ factorial designs where variables are tested in different combinations, rather than studying one at a time. Response surface methodology (RSM) is one such strategy that is used to optimize and study interactions between

significant variables during different phases of fermentative and biotechnological processes (Coban and Demirci, 2014; Montgomery, 2008; Singh and Satyanarayana, 2008).

The steepest ascent/decent method is used to reach the near vicinity of the actual optimum (Jiao *et al.*, 2008; Wu *et al.*, 2013). Investigation of the relationship between the identified variables/factors is the fundamental principle of RSM, which by definition is a set of mathematical and statistical techniques for developing, improving, and optimizing processes (Hastie *et al.*, 2009; Lu *et al.*, 2012). In addition to analyzing the effects of the independent variables, a mathematical model is used for predicting the response and testing the adequacy of the design (Box and Draper, 1959; Baş and Boyacı, 2007). These techniques as a whole are quite efficient for design and optimization of processes that aim at improving yields of commercially viable products.

While batch fermentation has a constraint of limited substrate availability after a specified period of time, the fed-batch process allows continuous or intermittent supply of substrate in the medium, especially during the log-phase, such that the microbial growth and enzyme production is continued for an extended period of time. The volume of fermentation broth increases during the process and harvesting is done at the end (Esener *et al.*, 1981; Yamane and Shimizu, 1984; Lim and Shin, 2013). The process enables control of the feeding time, its concentration and rate to improve production of the desired final product (amino acids, antibiotics, enzymes) and biomass (Smets *et al.*, 2004; Banga *et al.*, 2005).

This chapter focuses on the optimization of the fermentation variables for enhanced phytase production using classical and statistical approaches. The aim was to study the effect of several nutritional and physical factors in a minimum number of trials using PBD. The significant factors were identified based on their P values ( $<0.05$ ) and further optimized by central composite design to study and monitor their interactions. The final optimized conditions as suggested by the model were validated and used to scale-up production in 1 and 5 l laboratory fermenters. Additionally, a comparative study of batch and fed-batch production was also studied in a 5 l laboratory fermenter.

## 3.2 MATERIALS AND METHODS

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### 3.2.1 Preliminary screening of nutrients

Fermentation parameters were optimized in 250 ml Erlenmeyer flasks by varying the concentration of nutrient sources (carbon and nitrogen sources, trace elements etc.) and by studying the effect of inoculums age, inoculum size, agitation, pH and temperature using OFAT. The effect of carbon source on fermentation was studied using 0.50% (w/v) dextran, fructose, galactose, glucose, lactose, maltose, mannose, sorbitol, sucrose and xylose. Similarly, effect of nitrogen sources was studied using 0.50% (w/v) urea,  $(\text{NH}_4)_2\text{SO}_4$ , tryptone,  $\text{NH}_4\text{Cl}$ , yeast extract,  $\text{NH}_4\text{NO}_3$  and peptone. The influence of surfactants was studied using 0.10% (w/v) Tween-20, Tween-80, polyethylene glycol (PEG), sodium dodecyl sulphate (SDS) and Triton X-100. Trace elements (0.10%, w/v) such as KCl,  $\text{CaCl}_2$ ,  $\text{BaCl}_2$ ,  $\text{MgCl}_2$ ,  $\text{ZnSO}_4$ ,  $\text{MgSO}_4$  and EDTA were tested for their impact on production. Additionally, the effect of 0.0001% (w/v) amino acid and a vitamin cocktail (appendix) was also studied.

To study the effect of physical parameters on phytase production, pH and temperature were analyzed in the range of 4.0 to 8.0 and 30°C to 60°C, respectively, while aeration was investigated in the range of 0 to 200 rpm. Additionally, the effect of the size and age of inoculum was also assessed. All experiments were conducted in triplicate and the mean  $\pm$  SD was calculated.

### 3.2.2 Statistical optimization

The variables that significantly affected phytase production were identified based on preliminary studies using OFAT approach, and were further optimized by Plackett-Burman design (PBD) using the statistical software package 'Design Expert' (Stat-Ease Inc., Minneapolis, USA). Two-level Plackett-Burman design (PBD) is an efficient technique to select significant process parameters by screening  $n$  variables in  $n + 1$  experiments (Plackett and Burman, 1946). A total of 20 trials with 17 variables and 2 dummies or unassigned variables were screened in the present design (Table 3.2), which included a variety of carbon and nitrogen sources, inorganic salts, and cultivation parameters. The principal effect of each variable was estimated as the difference between the average of the measurements made at the high level (+1) of that factor and the average

of the measurements made at the low level (-1) of that factor, which was determined by the following equation:

$$E(X_i) = \frac{2(\sum C_{i+} - \sum C_{i-})}{N} \quad \text{Equation 3.1}$$

where  $E(X_i)$  is the concentration effect of the tested variable.  $C_{i+}$  and  $C_{i-}$  are the phytase activities from the trials where the variable ( $X_i$ ) under study was present at high and low concentrations, respectively and  $N$  is the number of experiments. The order of the experiments was fully randomized with the design being run in a single block. The significance of each variable (p-value) was determined via Student's t-test.

### 3.2.3 Path of steepest ascent

Experiments were carried out along the steep path that initiated from the origin, as the centre of the PBD, to move rapidly towards the vicinity of the optimum conditions. Experiments were designed by increasing or decreasing the concentrations of variables based on the PBD results until no further increase in response was observed.

### 3.2.4 Response surface methodology using central composite design (CCD)

Four significant parameters (wheat bran, peptone, ammonium sulphate and inoculum size), identified by Plackett-Burman design, were chosen as independent variables. Each factor was studied at five different levels ( $-\alpha, -1, 0, +1, +\alpha$ ) as per CCD, with a total of 30 experiments. The range of variables investigated and the experimental design used for study is shown in Table 3.4. The behaviour of the system was explained by the following second order polynomial equation:

$$Y = \beta_0 + \beta_1A + \beta_2B + \beta_3C + \beta_4D + \beta_{11}A^2 + \beta_{22}B^2 + \beta_{33}C^2 + \beta_{44}D^2 + \beta_{12}AB + \beta_{13}AC + \beta_{14}AD + \beta_{23}BC + \beta_{24}BD + \beta_{34}CD \quad \text{Equation 3. 2}$$

where  $Y$  is predicted value of response,  $\beta_0$  is intercept,  $\beta_1, \beta_2, \beta_3, \beta_4$  are linear coefficients,  $\beta_{11}, \beta_{22}, \beta_{33}, \beta_{44}$  are squared coefficients,  $\beta_{12}, \beta_{13}, \beta_{23}, \beta_{24}, \beta_{34}$  are interaction coefficients and  $A, B, C, D$ ,

B<sup>2</sup>, C<sup>2</sup>, D<sup>2</sup>, AB, AC, BC, BD, CD are independent variables. The remaining factors were kept in the same level as that in the medium optimized by the OFAT approach.

### **3.2.5 Validation of the experimental model**

The conditions predicted by the statistical model was validated with respect to phytase production in 0.25–2.0 l shake-flasks, and also in 1 l (Multifors) and 5 l (Minifors) laboratory fermenters (Infors, Bottmingen-Basel, Switzerland). The fermenters were operated at 40°C, 200 rpm and 1 vvm of aeration. Samples were drawn at the desired intervals, harvested and cell-free supernatant was used for analysis. All experiments were conducted in triplicate.

### **3.2.6 Batch production in a 5 l laboratory fermenter**

Phytase from *Enterobacter* sp. ACSS was produced in a 5 l laboratory fermenter with 3 l working volume. Inoculum was prepared in 100 ml LB broth in a 500 ml Erlenmeyer flask which was incubated at 37°C for 12 h with shaking at 200 rpm. The RSM optimized medium and conditions were used to investigate production of phytase at a larger volume. The medium was prepared to a final volume of 2.88 l in the fermenter and autoclaved at 121°C for 20 min and subsequently cooled to room temperature. The fermenter was operated at 40°C, 200 rpm and 1 vvm of aeration. Samples were drawn after every 2 h interval, harvested and cell-free supernatant was used for further analysis.

#### **3.2.6.1 Biomass determination**

Biomass was determined by assaying the level of nucleic acids in culture broth according to the method as described by Zhou *et al* (2014). Briefly, 1 ml of fermentation broth collected at different time intervals and 1 M perchloric acid was incubated at 100°C for 20 min and the suspension was centrifuged at 10,000 rpm for 10 min to remove the cell debris. The supernatant was diluted ten-fold and measured at an absorbance of 260 nm using NanoDrop 1000 spectrophotometer (Thermo Scientific). Biomass was determined using a standard curve plotted as A<sub>260</sub> against known amounts of biomass.

### **3.2.7 Preliminary optimization for fed-batch fermentation**

#### ***3.2.7.1 Selection of time of fed***

Before commencing with fed batch fermentation the exact time of fed was investigated in preliminary studies. Production medium containing 5 g/l of glucose was fed at 6, 12, 18 and 24 h in individual experiments. Samples were withdrawn every 6 h and were assayed for phytase using standard assay method.

#### ***3.2.7.2 Optimization of glucose (feed) concentration***

To further optimize process parameters the optimal concentration of the carbon source (glucose) was investigated. Glucose (0.5, 1, 2.0, 2.5%) was fed to the production medium at the selected fed time in separate experiments for enhanced phytase production. All other medium components were kept constant. Samples were withdrawn every 6 h and were assayed for phytase using standard assay method.

#### ***3.2.7.3 Optimization of feed concentration***

The effect of feed concentration on fed-batch cultivation was investigated at three different concentrations (5-fold, 10-fold, 15-fold) in separate experiments. However, in order to reduce the clogging of the feed-pipe due to wheat bran, its concentration was kept at 1.62% in the feed. Samples were withdrawn every 6 h and were assayed for phytase using standard assay method.

### **3.2.8 Fed-batch fermentation**

Fed-batch fermentations were conducted in a 5 l glass fermenter (Minifors, Infors HT, Switzerland) containing 2.5 l of optimized medium with 4.14 % (v/v) inoculum at 40°C up to 96 h. Dissolved oxygen (DO) in the fermenter was maintained at 30% air saturation using 1.5 vvm aeration in cascade mode controlling the agitation speed (maximum, 500 rpm) and airflow. Feeding was done at every 12 h with concentrated optimized medium (10-fold) at a feeding rate of 25 ml/h to maintain the total reducing sugar concentration under 20 g/l. However, in order to reduce the

clogging of feed-pipe due to wheat bran, its concentration was kept 1.62% in the feed. Phytase production was estimated using the standard phytase assay (section 2.2.3) while total sugar was estimated using the Anthrone method of (Seifter *et al.*, 1950). A suitably diluted 1 ml aliquot of sample was hydrolysed in 4 ml anthrone solution in a boiling water bath for 15 min. After cooling to room temperature, the total sugar concentration was determined spectrophotometrically by absorption at 620 nm using glucose as standard.

### 3.3 RESULTS

#### 3.3.1 Preliminary screening of nutrients

Initial optimization was achieved using OFAT method where effect of one variable was studied while the rest of the factors were kept constant. Table 3.1 summarises the results of primary screening of nutritional and physical factors. Glucose and peptone were the best carbon and nitrogen sources producing 13.63 and 13.84 U/ml of phytase, respectively. Among surfactants tested, Tween-20 produced the highest phytase titre of 14.73 U/ml while Triton X-100 had a negative effect on production yielding only 2.86 U/ml.

**Table 3.1** Summary of different parameters optimized for phytase production by *Enterobacter* sp. ACSS using one-factor-at-a-time approach.

Factors	Optimized conditions	Production (U/ml)
Glucose	0.50%	13.63 ± 0.112
Peptone	0.50%	13.84 ± 0.696
Tween-20	0.10%	14.73 ± 0.239
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.1%	15.47 ± 0.393
pH	6	16.22 ± 0.404
Temperature	40°C	16.31 ± 0.676
Inoculum age	12 h	17.12 ± 0.365
Inoculum size	4%	17.30 ± 0.593
Agitation	200 rpm	17.63 ± 0.516
Vitamin cocktail	0.0001%	18.01 ± 0.512
Amino acid cocktail	0.0001%	18.13 ± 0.337

\*The values shown represent averages from triplicate experiments ± SD.

Phytase production was further optimized to 16.22 U/ml and 16.31 U/ml due to pH (6.0) and temperature (40°C). A maximum of 17.63 U/ml phytase was achieved at 200 rpm, with 4% of a 12 h inoculum. Addition of amino acid and a vitamin cocktail resulted in 18.01 U/ml and 18.13 U/ml phytase, respectively.

### 3.3.2 Statistical optimization: Plackett-Burman design

In the present study, a total of 20 trials consisting of 17 variables and two unassigned or dummy variables were studied for their potential effect on the production of phytase. The variables evaluated were glucose (A), sucrose (B), wheat bran (C), peptone (D),  $\text{NH}_2\text{SO}_4$  (E),  $\text{NH}_4\text{NO}_3$  (F), Na-phytate (G), Tween-20 (H),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (I),  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (J), amino acid cocktail (K), vitamins (L) pH (M), temperature (N), agitation (O), inoculum size (P), incubation period (Q) and two dummy variables (R and S). Each factor was studied at two levels i.e. high (+1) and low (-1). Table 3.2a depicts responses observed on phytase production as well as the design matrix used and Table 3.2b highlights the results of regression analysis. Run 16 yielded the highest titre of phytase at 29.81 U/ml whilst run 6 displayed the lowest level of 0.41 U/ml.

The Pareto graph (Fig 3.1) was plotted by calculating the effect of variables using Equation 3.1. Wheat bran, peptone and inoculum size indicated a positive effect when used at higher concentrations while  $(\text{NH}_4)_2\text{SO}_4$  and Tween-20 had significant effects at lower concentrations. Additionally, ANOVA analysis of the response (Table 3.2b) clearly indicated these as the most significant factors ( $p < 0.05$ ). The p-values for the remaining variables were more than 0.05 and were therefore insignificant. Although Tween-20 had an obvious effect on phytase production, it was ultimately excluded to minimize experiments required for future optimization and to minimize interference during protein purification steps. Using ANOVA an F value of 54.01 was obtained for the best fit linear regression model. Evidence of a regression effect is given by the p value ( $\text{Prob} > F$ ) of 0.0183 which is less than 0.05 proving its significance.

**Table 3.2.** Plackett-Burman design and its analysis for screening of significant parameters for the production of phytase by *Enterobacter* sp. ACSS.

**(a) Experimental design**

Run no.	Variables <sup>†</sup>																			Phytase production (U/ml)
	A	B	C	D	E	F	G	H	J	K	L	M	N	O	P	Q	R	S	T	
1	0.50	0.25	0.50	0.25	0.10	0.10	1.00	0.50	0.05	0.05	0.01	0.01	4.00	25	100	1.00	12.00	-1	-1	3.74±0.27
2	3.00	0.25	0.50	0.25	0.10	1.50	1.00	2.50	0.05	0.25	0.05	0.05	8.00	25	100	5.00	48.00	-1	1	4.41±0.33
3	3.00	1.50	0.50	0.25	0.10	0.10	100	0.50	0.25	0.05	0.05	0.05	8.00	40	100	1.00	48.00	1	-1	9.13±0.29
4	0.50	1.50	0.50	1.50	0.10	1.50	100	2.50	0.25	0.05	0.01	0.05	8.00	25	300	5.00	12.00	-1	-1	5.54±0.39
5	3.00	0.25	2.00	0.25	1.50	1.50	100	2.50	0.05	0.05	0.05	0.05	4.00	40	300	1.00	12.00	-1	-1	5.89±0.34
6	3.00	0.25	0.50	1.50	1.50	0.10	100	2.50	0.05	0.05	0.01	0.01	8.00	25	300	1.00	48.00	1	1	0.41±0.02
7	0.50	0.25	2.00	0.25	1.50	0.10	100	2.50	0.25	0.25	0.01	0.01	8.00	40	100	5.00	48.00	-1	-1	3.84±0.25
8	0.50	0.25	0.50	1.50	0.10	1.50	1.00	2.50	0.25	0.25	0.05	0.01	4.00	40	300	1.00	48.00	1	-1	2.43±0.13
9	0.50	1.50	0.50	1.50	1.50	1.50	100	0.50	0.05	0.25	0.05	0.01	8.00	40	100	1.00	12.00	-1	1	5.11±0.36
10	0.50	1.50	2.00	0.25	1.50	1.50	1.00	0.50	0.05	0.05	0.05	0.01	8.00	25	300	5.00	48.00	1	-1	12.49±0.67
11	3.00	1.50	2.00	1.50	0.10	0.10	100	2.50	0.05	0.25	0.05	0.01	4.00	25	100	5.00	12.00	1	-1	16.31±1.06
12	3.00	0.25	2.00	1.50	1.50	1.50	1.00	0.50	0.25	0.25	0.01	0.05	8.00	25	100	1.00	12.00	1	-1	8.72±0.47
13	0.50	0.25	0.50	0.25	1.50	0.10	100	0.50	0.25	0.25	0.05	0.05	4.00	25	300	5.00	12.00	1	1	0.91±0.048
14	0.50	1.50	2.00	0.25	0.10	0.10	1.00	2.50	0.05	0.25	0.01	0.05	8.00	40	300	1.00	12.00	1	1	0.43±0.03
15	3.00	0.25	2.00	1.50	0.10	0.10	1.00	0.50	0.25	0.05	0.05	0.01	8.00	40	300	5.00	12.00	-1	1	23.42±1.61
16	0.50	0.25	2.00	1.50	0.10	1.50	100	0.50	0.05	0.05	0.01	0.05	4.00	40	100	5.00	48.00	1	1	29.81±1.67
17	3.00	1.50	2.00	0.25	0.10	1.50	100	0.50	0.25	0.25	0.01	0.01	4.00	25	300	1.00	48.00	-1	1	8.96±0.52
18	3.00	1.50	0.50	0.25	1.50	1.50	1.00	2.50	0.25	0.05	0.01	0.01	4.00	40	100	5.00	12.00	1	1	0.93±0.06
19	3.00	1.50	0.50	1.50	1.50	0.10	1.00	0.50	0.05	0.25	0.01	0.05	4.00	40	300	5.00	48.00	-1	-1	8.55±0.44
20	0.50	1.50	2.00	1.50	1.50	0.10	1.00	2.50	0.25	0.05	0.05	0.05	4.00	25	100	1.00	48.00	-1	1	6.71±0.39

<sup>†</sup>A, Glucose (%); B, Sucrose (%); C, Wheat bran (%); D, Peptone (%); E, Ammonium sulphate (%); F, Ammonium nitrate (%); G, Na-phytate (μM), H, Tween-20 (%); J, MgSO<sub>4</sub>.7H<sub>2</sub>O (%), K, CaCl<sub>2</sub>.2H<sub>2</sub>O (%); L, Amino acid (ml); M, Vitamins (ml); N, pH; O, Temperature (°C); P, Agitation (rpm); Q, Inoculum size (%); R, Incubation period (h); S, T = Dummy variables.

**(b) Results of regression analysis<sup>‡</sup>**

Source	Sum of squares	Degree of freedom	Mean square	F-value	Prob>F
Model	1118.25	17	65.78	54.01	0.0183
A	12.38	1	12.38	10.16	0.0859
B	4.45	1	4.45	3.65	0.1961
C*	284.67	1	284.67	233.72	0.0043
D*	158.34	1	158.34	130	0.0076
E*	128.25	1	128.25	105.29	0.0094
F	5.91	1	5.91	4.85	0.1584
G	9.93	1	9.93	8.16	0.1039
H	204.45	1	204.45	167.86	0.0059
J	13.69	1	13.69	11.24	0.0786
K	73.63	1	73.63	60.45	0.0161
L	12.62	1	12.62	10.36	0.0845
M	0.3	1	0.3	0.24	0.6706
N	5.81	1	5.81	4.77	0.1607
O	22.76	1	22.76	18.69	0.0496
P	19.36	1	19.36	15.89	0.0575
Q*	149.3	1	149.3	122.58	0.0081
R	12.41	1	12.41	10.19	0.0857
Residual	2.44	2	1.22		
Total correlation	1120.69	19			

\*Significant factors selected for optimization by RSM

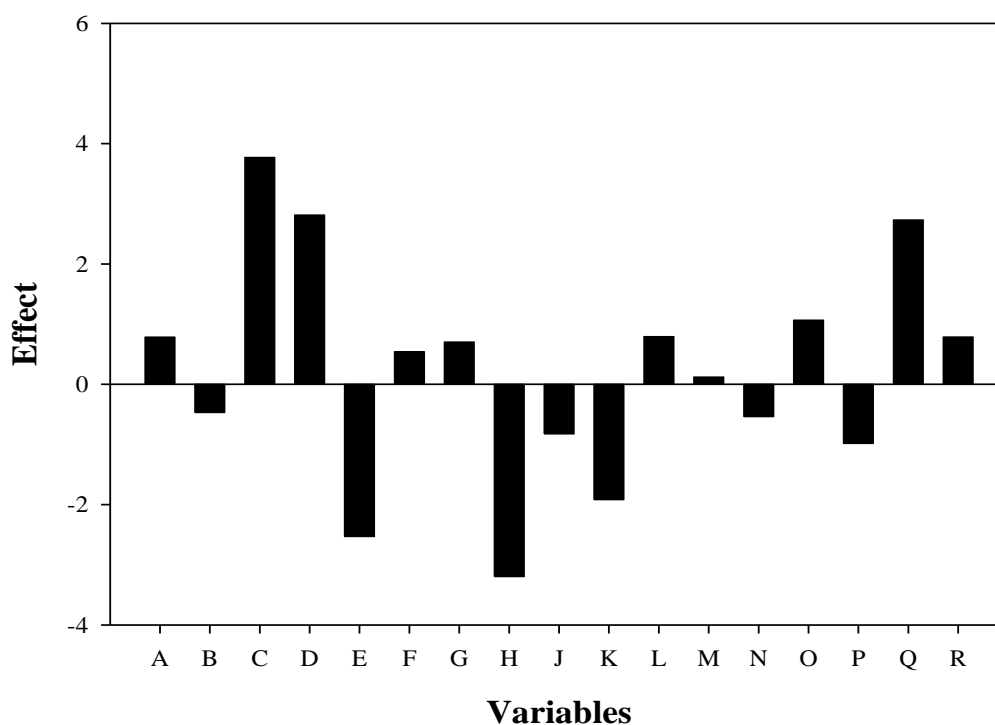
<sup>‡</sup>Coefficient of determination ( $R^2$ )= 0.9978

Adj R-Squared: 0.9794

Pred R-Squared: 0.7826

Adeq Precision: 28.085

The coefficient of determination ( $R^2$ ) was 0.9978 indicating a good fit. The ‘adequate precision’ value of 28.085 for phytase production indicated that the model may be used to navigate the design space.



**Figure 3.1** Pareto chart showing effect of different variables on phytase production by *Enterobacter* sp. ACSS

### 3.3.3 Path of steepest ascent/descent

The next set of experiments was designed towards the path of steepest ascent/descent. Table 3.3 represents the design and results of experiments. Based on the PBD response and effect of variables, the concentration of wheat bran and peptone and size of inoculum was increased while  $(\text{NH}_4)_2\text{SO}_4$  concentration was decreased in a stepwise manner. Phytase production improved as the levels of wheat bran, peptone and inoculum size increased and the level of  $\text{NH}_2\text{SO}_4$  decreased. Highest phytase production (55.68 U/ml) was achieved during the 5<sup>th</sup> run and thereafter no further improvement was observed along the path, and therefore this combination was used further as the center point of CCD as it indicated the region of optimum response.

**Table 3.3** Design and results of path of steepest ascent/descent

Run	Steps	Wheat bran (%)	Peptone (%)	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (%)	Inoculum size (%)	Phytase production (U/ml)
1	Origin (O)	1.25	0.88	0.80	3.00	21.72±1.61
2	O+Δ	1.50	1.03	0.68	3.50	34.13±2.16
4	O+2Δ	1.75	1.18	0.56	4.00	47.25±3.22
5	O+3Δ	2.00	1.33	0.44	4.50	55.68±3.06
6	O+4Δ	2.25	1.48	0.32	5.00	49.43±3.55
7	O+5Δ	3.75	1.63	0.20	5.50	38.89±2.23

\*The values shown represent averages from triplicate experiments ± SD.

### 3.3.4 Response surface methodology using central composite design (CCD)

RSM using CCD, based on the Plackett–Burman design and the path of steepest ascent, was applied to determine the optimal levels of the four selected variables (wheat bran, peptone, inoculum size and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> which significantly influenced the phytase production by *Enterobacter* sp. ACSS. The optimum levels of the four selected variables and their interactions were studied for enhanced phytase production.

A total of thirty experiments were carried out using several combinations of the variables as per the CCD. The predicted and observed responses of the CCD experiments are presented in Table 3.4a. Results of the experiment were analysed by standard ANOVA (Table 3.4b). The following second-order polynomial equation was obtained that explains phytase production as a function of the four variables:

$$Y \text{ (U/ml)} = 5.38A - 2.75B - 1.67C + 0.74D - 9.50A^2 - 7.82B^2 - 13.25C^2 - 9.00D^2 + 0.53AB - 1.26AC + 1.75AD - 0.63BC - 3.69BD + 1.12CD \quad \text{Equation 3.3}$$

The model F-values of 1550.42 and ‘Prob>F value’ of < 0.0001 implied the model was highly significant. There was only a 0.01% chance that the model F-value could occur due to noise. All four linear coefficients (A, B, C and D) and the quadric terms (A<sup>2</sup>, B<sup>2</sup>, C<sup>2</sup> and D<sup>2</sup>) were significant for phytase production. The interaction of A with C and D and D with B and C were significant

model terms. A prob>F value of 0.1763 ( $p>0.05$ ) indicated that the lack of fit test was not significant and the fitting degree of Equation 3.3 was acceptable.

**Table 3.4.** Experimental design (a) and ANOVA analysis (b) for optimization of phytase production by *Enterobacter* sp. ACSS using response surface methodology.

Run	Values of variables*				Phytase production (U/ml)	
	x <sub>1</sub>	x <sub>2</sub>	x <sub>3</sub>	x <sub>4</sub>	Predicted	Actual
1	1.68	1.63	0.5	4	32.38	32.04±1.73
2	2.21	2.06	0.38	5	45.3	46.40±3.06
3	1.68	1.63	0.01	4	25.68	27.13±1.83
4	1.14	1.19	0.38	3	40.07	38.80±2.09
5	1.68	1.63	0.26	4	82.01	80.20±5.52
6	0.6	1.63	0.26	4	33.23	33.17±2.38
7	2.21	2.06	0.38	3	45.45	44.57±2.54
8	1.14	2.06	0.13	5	25.64	26.65±1.57
9	1.68	1.63	0.26	2	44.52	44.01±2.47
10	2.21	1.19	0.13	5	54.03	52.90±3.27
11	1.68	1.63	0.26	4	82.01	83.22±4.89
12	2.75	1.63	0.26	4	54.77	55.95±3.52
13	2.21	1.19	0.38	5	58.34	56.84±4.08
14	1.68	0.75	0.26	4	56.09	57.51±3.29
15	2.21	1.19	0.38	3	43.75	44.18±2.96
16	2.21	2.06	0.13	5	39.64	42.21±2.81
17	1.68	1.63	0.26	4	82.01	83.25±4.83
18	1.14	1.19	0.13	5	38.3	36.62±2.05
19	1.68	1.63	0.26	6	47.5	49.13±3.05
20	2.21	2.06	0.13	3	48.11	48.30±2.69
21	1.14	2.06	0.13	3	37.26	36.20±2.04
22	1.14	2.06	0.38	3	43.5	42.21±2.93
23	1.68	1.63	0.26	4	82.01	81.96±4.35
24	1.68	1.63	0.26	4	82.01	80.99±5.02
25	1.14	1.19	0.38	5	47.66	48.91±2.66
26	1.68	2.5	0.26	4	45.13	44.83±2.38
27	1.14	1.19	0.13	3	35.17	35.50±2.08
28	2.21	1.19	0.13	3	43.9	43.50±2.99
29	1.68	1.63	0.26	4	82.01	82.45±4.78
30	1.14	2.06	0.38	5	32.49	30.34±1.92

\*x<sub>1</sub>, Wheat bran (%); x<sub>2</sub>, Peptone (%); x<sub>3</sub>, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (%); x<sub>4</sub>, Inoculum size (%)

*(b) ANOVA values for phytase production*

Source	Sum of Squares	Degree of freedom	Mean Square	F-value	P-value (Prob>F)	Significance
<b>Model</b>	9329.63	14	666.4	231.58	< 0.0001	significant
<b>Residual</b>	43.16	15	2.88			
<b>Lack of Fit</b>	35.65	10	3.57	2.37	0.1763	not significant
<b>Total</b>	9372.8	29				

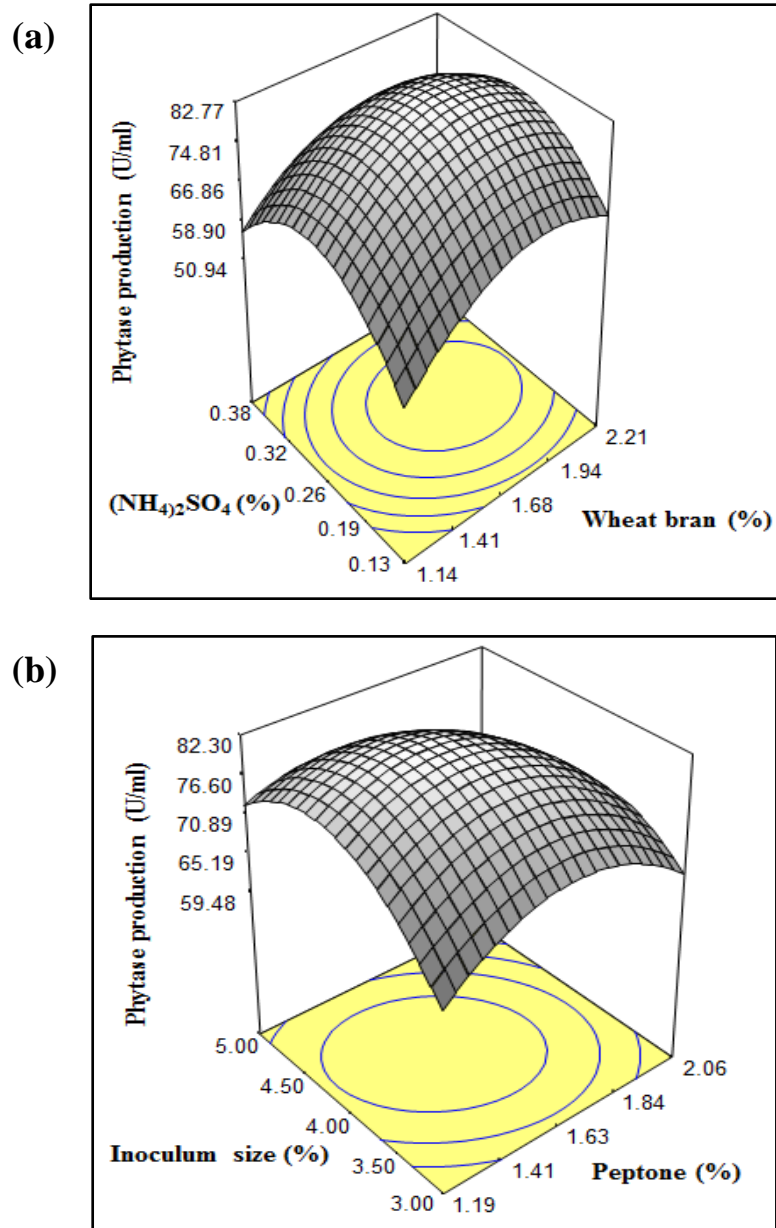
R<sup>2</sup> : 0.9954

Adjusted R<sup>2</sup> : 0.9911

Pred R<sup>2</sup> : 0.9769

To study the interaction of selected significant variables, three-dimensional response surface contour plots (at the base) were generated. The interaction of two variables was studied while keeping another variable fixed at its '0' level. Figures 3.2a and b represents the three dimensional response surface curves established for phytase production in this study. Figure 3.2a shows increase in phytase production with increase in the wheat bran and ammonium sulphate concentration up to 1.83% and 0.26%, respectively. Any further increase in these two variables repressed the enzyme production. Similarly, increase in peptone concentration up to 1.55% had a favourable impact on phytase production (Figure 3.2b), and thus, 1.55% peptone and inoculum size of 4.14% were optimal for maximum phytase production.

The experimental model and its predictions were validated for all four variables within the design space by five random sets of experimental combinations as suggested by Design-Expert™ software. The predicted response for phytase production was in good agreement with the actual response, confirming the authenticity of the model.



**Figure 3.2** Response surface contour of the interaction between (a) wheat bran and ammonium sulphate and (b) peptone and inoculum size.

Finally, the model predicted maximum phytase production ( $83.25 \pm 4.83$  U/ml) using 1.83% wheat bran, 1.63% peptone, 0.26%  $(\text{NH}_4)_2\text{SO}_4$  and a 4.14% inoculum .

### 3.3.5 Validation of the experimental model and scaling-up

The experimental model and regression equation were further validated by additional set of experiments as shown in Table 3.5a. Highest production was obtained in run 2 in a medium containing 1.83, 1.63 and 0.26% of wheat bran, peptone and  $(\text{NH}_4)_2\text{SO}_4$ , respectively, when cultivated with 4.14% inoculum. The high agreement of the predicted response results (82.01 U/ml) and the actual results (83.25 U/ml) proved the validity of the response model. Additionally, phytase was produced using these optimized conditions in flasks of varied volumes and fermenters of working volumes of 800 ml and 3 l. Although comparable production levels were achieved in shake-flasks, lower phytase production was noted in 5 l fermenter.

**Table 3.5** Validation of the model (a) and scale-up (b) in shake flasks and fermenters

*(a) Validation of the model*

Run number	Solutions for the variables				Phytase production (Uml-1)	
	x1	x2	x3	x4	Observed	Predicted
1	1.82	1.19	0.27	4.27	71.08 $\pm$ 5.5	78.28
2	1.83	1.63	0.26	4.14	83.25 $\pm$ 3.6	82.01
3	1.72	1.19	0.2	3.4	62.71 $\pm$ 3.1	67.5

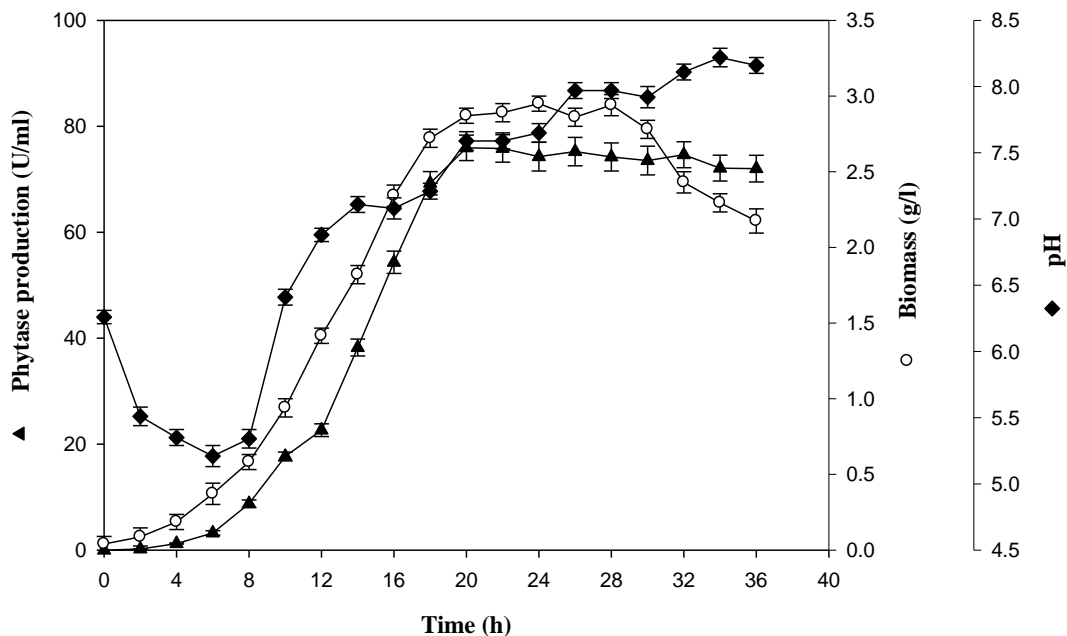
*(b) Scale-up of phytase production in flasks of varied volumes and fermenters*

Volume (ml)	Volume of medium used (ml)	Phytase production (U/ml)
<b>Shake flasks</b>		
100	20	82.33 $\pm$ 4.11
250	50	81.76 $\pm$ 4.27
500	100	82.55 $\pm$ 4.35
1000	200	83.05 $\pm$ 4.75
2000	400	80.97 $\pm$ 4.62
<b>Fermenters</b>		
1 l	800	80.23 $\pm$ 4.84
5 l	3000	74.20 $\pm$ 5.11

\*The values shown represent averages from triplicate experiments  $\pm$  SD.

### 3.3.6 Batch fermentation

Phytase production was growth-associated and maximum phytase production (79.27 U/ml) was achieved in the stationary phase. Interestingly, the pH of the medium increased from 6.5 to 8.5 (Fig. 3.3).



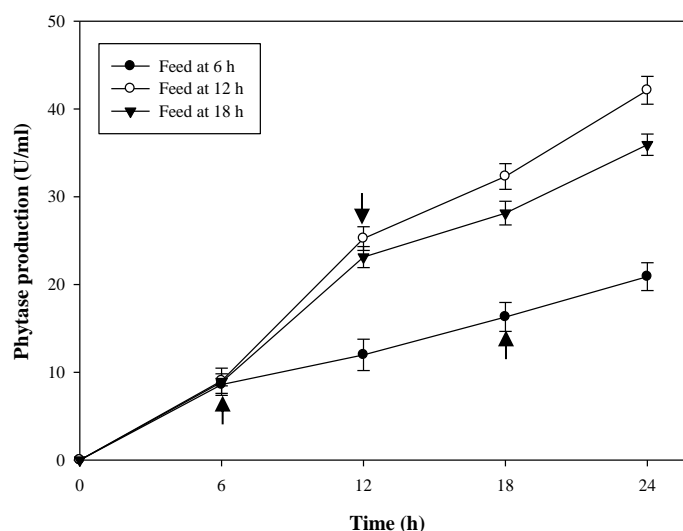
**Figure 3.3** Batch fermentation for phytase production by *Enterobacter* sp. ACSS in a 5 L fermenter using RSM optimized media. A sample was withdrawn every 2 h and assessed for (▲) phytase production (U/ml), (○) biomass (g/l) and (◆) pH. The error bars indicate standard deviation.

There was a slight decrease in the batch production levels (79.2 U/ml) in the fermenter as compared to shake flasks (83.2 U/ml) but the fermentation time was reduced by 4 h. Phytase production was growth-dependent and after 24 h phytase as well as biomass production declined in batch cultivation.

### 3.3.6.1 Preliminary optimization for fed-batch fermentation

#### 3.3.6.1.1 Selection of time of fed

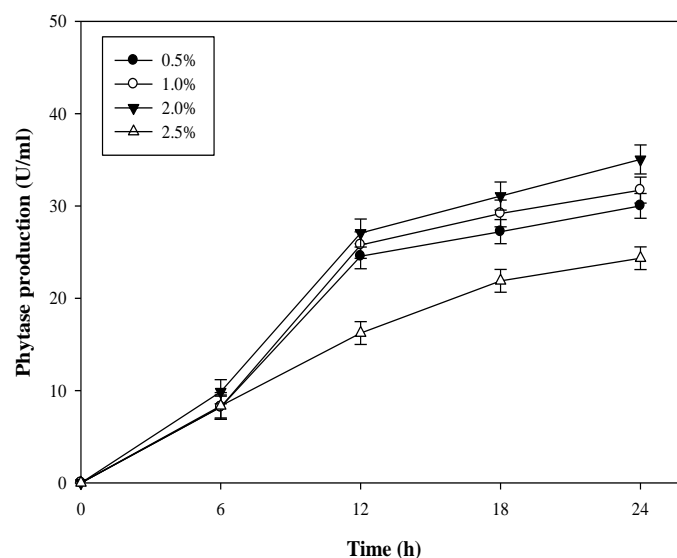
Phytase was produced maximum when fed with 5 g/l glucose at the mid-log phase (12 h) of cell growth (Fig. 3.4a). Production decreased when glucose was fed during the early (6 h) or late log phase (18 h).



**Figure 3.4(a)** Optimization of glucose (5 g/l) feeding time for phytase production in fed-batch cultivation

#### 3.3.6.1.2 Optimization of glucose concentration

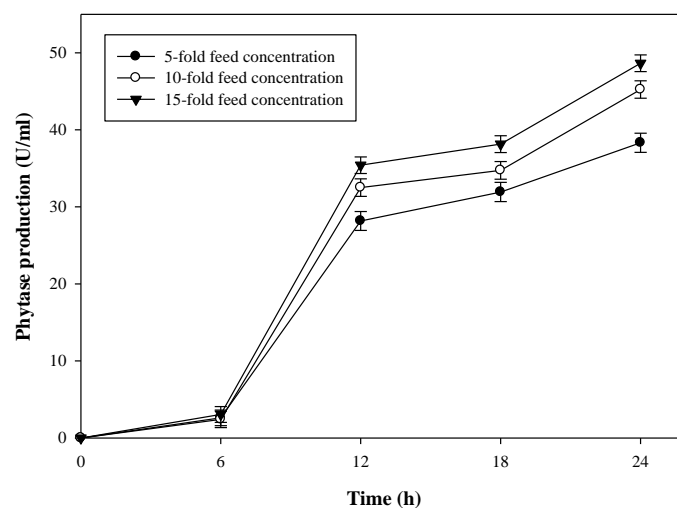
While investigating the effect of different concentrations of glucose on phytase production a 2% glucose concentration produced a maximum yield of 25.76 U/ml phytase. However, when supplemented with 2.5% glucose only 16.22 U/ml phytase was produced in 12 h (Fig. 3.4b). Production was comparatively lower at 0.5 and 1% of glucose concentrations.



**Figure 3.4(b)** Optimization of glucose concentration for phytase production in fed-batch cultivation

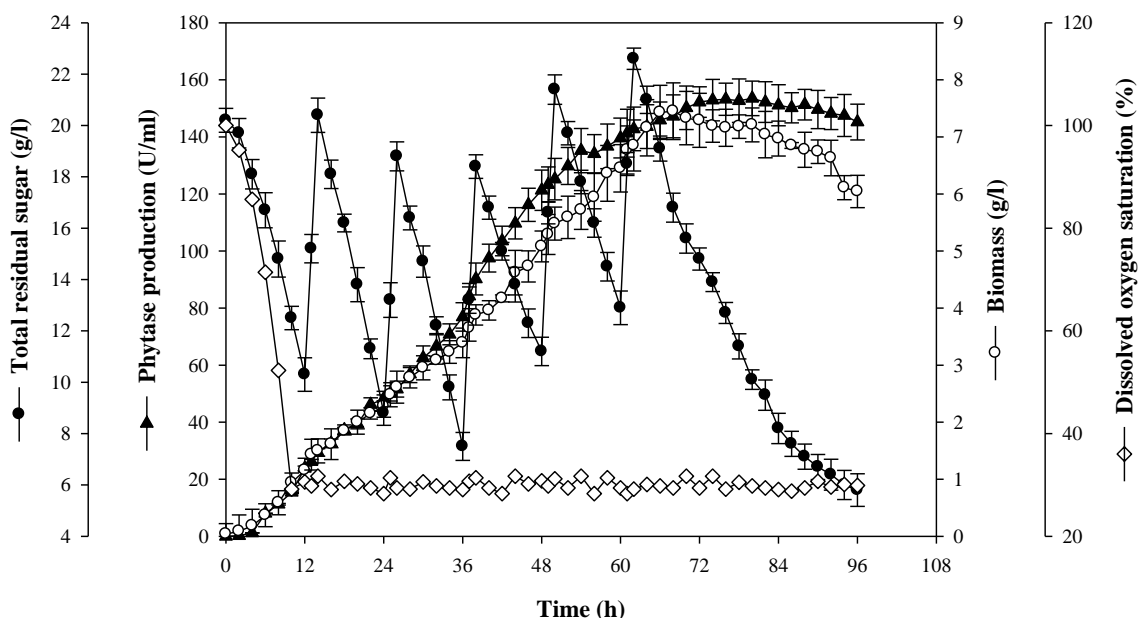
### 3.3.6.1.3 Optimization of feed concentration

Supplementation with a 10-fold feed did not influence phytase production significantly, and this result corroborates with the findings using PBD. However, as it can be seen in Fig 3.4c 10-fold feed concentration was optimal for maintaining the bacterial culture in its log phase.



**Figure 3.4(c)** Optimization of feed concentration for phytase production in fed-batch cultivation

### 3.3.7 Fed-batch fermentation



**Figure 3.5** Fed batch fermentation for phytase production by *Enterobacter* sp. ACSS in a 5 L fermenter with 30% air saturation maintained using 1.5 vvm aeration. Samples were withdrawn every 2 h, and assessed for (▲) phytase production (U/ml), (◆) biomass (g/l), (●) total residual sugar (g/l) and (○) dissolved oxygen. The error bars indicate standard deviation.

Fed-batch fermentation was carried out to achieve higher biomass for improved and sustained production of phytase. Time course profiles of phytase production, biomass and total residual sugar during fed-batch fermentation are shown in Fig. 3.5. The total reducing sugar was utilized fast and 51% was consumed during the first 12 h (exponential phase). It's concentration at the time of converting batch to fed-batch was 10.3 g/l, and at the end of fed-batch fermentation 5.8 g/l of reducing sugar was left in the medium. Based on optimization of feeding time (data not shown) the culture was fed after every 12 h, which maintained the bacterium in its logarithmic phase until 64 h and the biomass improved by 2.6-fold with a concomitant 2.9-fold improvement in phytase production (153.2 U/ml).

### 3.3.8 Enhancement in phytase production due to optimization

Table 3.6 represents the fold improvement in phytase production due to various strategies. Initial optimization using OFAT approach resulted in 18.18 U/ml of phytase production. Further optimization using PBD, path of steepest ascent/descent and RSM increased phytase production gradually to 29.81 U/ml, 49.43 29.81 U/ml and 83.25 U/ml respectively. Finally, a total of 4.59-fold improvement was observed, which is indicative of successful use of statistical approaches for enhanced production of phytase in shake flasks and fermenters. Higher enzyme production is required for purification and its applications, which is the basis of next chapters.

**Table 3.6** Phytase production: Fold improvement

<b>Step</b>	<b>Phytase production (U/ml)</b>	<b>Fold</b>
<b>OFAT</b>	18.14	1
<b>PBD</b>	29.81	1.64
<b>Path of steepest ascent/descent</b>	49.43	2.73
<b>RSM (shake-flask)</b>	83.25	4.59
<b>Fed-batch fermentation</b>	153.2	8.45

### 3.4 DISCUSSION

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Even though a wide range of microorganisms are capable of producing phytase, its expression levels are often lower than required for industrial production. The present investigation was performed to optimize the medium parameters using classical OFAT approach followed by statistical optimization. Although OFAT is a classical method, a large number of experiments must be conducted, additionally, it does not compensate for interactions between factors. The use of statistical tools has greatly improved such process and was thus applied to this study. The purpose of using statistical optimization is to minimize the number of trials while concurrently studying as many factors as possible (Bhavsar *et al.*, 2011; Yu and Zhang, 2011; Kaur and Satyanarayana, 2005).

A total of eleven factors were optimized to produce 18 U/ml of phytase using the OFAT method. Generally, the first step in any fermentation process is to optimize the most important parameters first, namely the carbon and nitrogen sources. Glucose as microorganisms prefer to grow profusely and meet their cellular energy requirements using this simple sugar. Moreover, it has been indicated as the best carbon source for phytase production during previous studies (Sunitha *et al.*, 1999; Roy *et al.*, 2012). Among nitrogen sources tested, peptone was most effective while urea inhibited enzyme production. This may be explained by an imbalance in the C/N ratio required for enzyme production (Roopesh *et al.*, 2006). Phytase secretion due to physical parameters such as pH, temperature and agitation was minimally affected. The reported pH range is between 5.5-7.0 while mesophilic bacteria prefer to produce phytase between 30-40°C (Dechavez *et al.*, 2011; Kammoun *et al.*, 2012; Kumar *et al.*, 2012; Lee *et al.*, 2014). However, *Kurthia* CZC0806 (Yu and Zhang, 2011) and *Anoxybacillus* sp. MHW14 (Kanpiengjai *et al.*, 2013) has been reported to optimally produce phytase at 20°C and 45°C, respectively. Enzyme production and growth are known to be affected by surfactants possibly by their action on cell permeability. Fungi are generally more affected than their bacterial counterparts due to the structure of their cell wall. In this study, addition of non-ionic detergents showed mixed response. While Tween-20 and Tween-80, increased phytase production, SDS and Triton X-100 showed a negative effect on phytase production. Similar influence of surfactants on process parameters was also observed in *Thermoascus aurantiacus* (Nampoothiri *et al.*, 2004), *Bacillus subtilis* US417 (Kammoun *et al.*,

2012), *Aspergillus niger* NCIM 563 (Bhavsar *et al.*, 2011) and *Klebsiella* sp. (Das *et al.*, 2013). SDS and Triton X-100 may have been toxic or would have caused a decrease in the oxygen transfer rate due to detergent-mediated enhanced viscosity of the medium (Rao and Satyanarayana, 2003; Das *et al.*, 2013).

Application of PBD allowed the simultaneous evaluation of 17 factors as compared to only 11 factors using OFAT trials. Two dummy variables were included as part of the design to test homogeneity of regressions. These are artificial variables used to represent qualitative attributes and serve to ‘trick’ the regression algorithm into correctly analyzing quantitative variables (Rawlings *et al.*, 1998). Wheat bran, peptone, Tween-20,  $(\text{NH}_4)_2\text{SO}_4$  and inoculum size showed p-values below 0.05, and therefore identified as significant variables. The coefficient of determination ( $R^2$ ) was 0.9978 which is indicative of a strong model. The regression model gave a p-value (prob>F) of 0.0183 with a corresponding model F-value of 54.01 proving the model to be highly significant. The signal to noise ratio is measured by the ‘adequate precision value’ and must be greater than 4.0 for the model to navigate the design space (Bhavsar *et al.*, 2011; Singh *et al.*, 2011). The value obtained for adequate precision (121.595) was in good agreement with requirements of the model. PBD served to identify significant parameters affecting production but their interactions were not taken into account. It is a highly accepted technique and has been used extensively as a statistical tool in many studies to screen for and identify critical culture parameters (Kaur and Satyanarayana, 2005; Mittal *et al.*, 2011; Kammoun *et al.*, 2012). Following PBD, the path of steepest ascent/descent is used to determine the area at which the maximum increase of responses would be attained (Antony, 2003). For this study, the levels of wheat bran, peptone and inoculum size were increased while  $(\text{NH}_4)_2\text{SO}_4$  was decreased. CCD using RSM was applied to observe the interactive effect of these four variables.

There have been several reports on optimization of phytase using statistical tools. Farhat-Khemakhem *et al.* (2012) optimized phytase production by *Bacillus subtilis* 168 using PBD and the Box-Behnken design and a maximum of 47 U/ml was reached in the presence of 12.5 g/l of yeast extract and 15 g/l of  $(\text{NH}_4)_2\text{SO}_4$  at 300 rpm. However, this was significantly lower as compared to 112 U/ml of phytase produced by *B. subtilis* US417 after optimizing using PBD and RSM (Kammoun *et al.*, 2012). Similarly, phytase production from the marine yeast *Kodamaea*

*ohmeri* BG3 was improved from 62.0 to 575.5 U/ml due to statistical optimization using 1.0% oat, 2.3% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2.0% glucose, 2.0% NaCl and at initial pH 6.3 (Li *et al.*, 2008).

Using the second order polynomial equation a value of 82.01 U/ml was predicted where production was a function of levels of wheat bran, peptone, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, and inoculum size. ANOVA analysis calculated an R<sup>2</sup> value of 0.9954 indicating an aptness of the model as 99.54% of the randomness or variability was elucidated. This was further confirmed by the adjusted R<sup>2</sup> value of 0.9911 which was close to the predicted R<sup>2</sup> value of 0.9769. The adjusted R<sup>2</sup> value is simply a re-calculation of the R<sup>2</sup> value by removing the impact of the degrees of freedom and may be used for comparing models with different numbers of independent variables (Baş, and Boyacı, 2007; Imandi *et al.*, 2008; Rawlings *et al.*, 1998). Using ANOVA the significance and adequacy of the model was examined (Table 3.4b). The Model F-value (Fisher variance ratio) of 231.58 was highly significant and there was only 0.01% of error due to noise as suggested by the p-value. The F-value is described as a valid measure of how well the factors describe the variation in the data about its mean (Imandi *et al.*, 2008; Montgomery, 2008; Hastie *et al.*, 2009).

The 3D response surface curves were used to understand the interaction effects of variables and for determining the optimum concentration of variable component required for maximum phytase production. Considering the association between wheat bran and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (Fig 3.2a) a concentration of up to 1.68% and 0.26%, respectively increased phytase production. (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> concentration was greatly reduced from 1.50% (PBD) to 0.26% (RSM) indicating its effectiveness at lower concentrations. Any further increase in these two variables repressed production of phytase. Wheat bran acted as the source of phytate during fermentation and has been previously reported (Gulati *et al.*, 2007a; Gulati *et al.*, 2007c) but this to the best of our knowledge is the first report of phytase production from *Enterobacter* ACSS sp. using wheat bran as the sole source of phytate. Previously, Hussin *et al.* (2012) using RSM with full-factorial faced centered CCD production reported *E. sakazakii* ASUIA279 as an extracellular producer using 13.6% of rice bran at 39.7°C and pH 7.1

There is a plethora of data that suggest immense impact of inoculum size on the production of phytase by bacteria, yeast and fungi. Analysis of the interaction between peptone and inoculum

size (Fig 3.2b) suggested that phytase production declined at their extreme levels. Phytase production by *Enterobacter* sp. ACSS was dependant on the inoculum size. A 2% inoculum size was optimal for *P. anomala* (Kaur and Satyanarayana, 2005) while 4% inoculums size was required for maximum production by *Bacillus* sp. T4 (Lee *et al.*, 2014). Higher inoculums sizes deplete available nutrients leading to competition for carbon source and nutrients ultimately reducing enzyme production (Suresh and Radha, 2015). However, Rani and Ghosh (2011) reported increased phytase production using a 20% inoculum of *Rhizopus oryzae*. The nitrogen source is a critical factor for cell growth and production of enzymes and metabolites. In this study, two nitrogen sources (peptone and  $(\text{NH}_4)_2\text{SO}_4$ ) influenced phytase production. Gulati *et al.* (2007c) reported an optimal medium containing inorganic sodium nitrate (0.4%) for phytase production by *T. lanuginosus* TL-7 while peptone (9.85 g/l) was significant for bacterium *Kurthia* CZC0806 following application of PBD followed by BBD for maximum phytase production (Yu and Zhang, 2011).

During batch fermentation, maximum phytase production was observed at the commencement of late log-phase (Fig 3.3) and eventually nutrient depletion caused decreased cell biomass and affected enzyme yield. Similarly, a maximum 107 U/ml of *Selenomonas ruminantium* phytase expressed in *E. coli* was produced in the late-log phase in 20 l batch fermentation (Lan *et al.*, 2014). Notably, pH of the medium increased from 5.5 to 8.0. The ion-releasing action of phytase on medium components is known to change acidic to a neutral and weak alkaline environment (Fujimoto *et al.*, 2010). The purpose of fed-batch fermentation was for increased biomass of *Enterobacter* sp. ACSS by extension of the log-phase, hence overcoming the low cell density caused by nutrient limitations of batch fermentation studies.

Preliminary studies were conducted to optimize fed-batch conditions, focusing on time of feed, glucose concentration and feed concentration. Enhanced phytase production was observed when fed in the mid-log phase and is supported by the fact that the bacterium required the most substrate for growth. Repression in enzyme production, when it was fed at early log-phase (6 h) may be explained on the basis of “catabolite repression”, which has been reported to be more pronounced in the early phases of microbial growth (New *et al.*, 2014). However, during this investigation increase in glucose concentration from 0.5 to 2% was optimum, while a further increase to 2.5%

was repressive for enzyme synthesis. Recently, Graminho *et al.* (2015) has also shown glucose mediated phytase repression in *Burkholderia* sp. strain a13. The step-wise increase of feed decreased acid concentration in the medium and is more effective compared to adding a large volume of feed at once. The purpose of fed-batch cultivation is also based on the same concept which relies on enhancing enzyme production by maintaining the culture in its log-phase for longer (Kliest *et al.*, 2003; Coban and Demirci, 2014).

Several studies have confirmed enhancement in enzyme production due to fed-batch cultivations, however most of the fed-batch studies pertaining to the use of *Enterobacter* family of bacteria have been focused on exopolysaccharide (Torres *et al.*, 2014), polyglucosamine (Son *et al.*, 2007), 2,3-butanediol (Jung *et al.*, 2013; Li *et al.*, 2015) and hydrogen production (Shin *et al.*, 2010), though these are known to be potential producers several industrial enzymes. Kleist *et al.* (2003) obtained high phytase levels (120 U/ml) using rapid glucose controlling system for fed-batch cultivation of *E. coli*, while Verma and Satyanarayana (2012), achieved 47.33 and 36.91 U/ml cell-bound phytase from *Pichia anomala* using cyclic fed-batch and fixed volume fed-batch strategies. Recently, production of phytase by *A. ficuum* was enhanced by 11 and 40 % with addition of feed containing glucose and Na-phytate, respectively (Coban and Demirci, 2014). To the best of our knowledge, this study is the first report on fed-batch production of an industrially significant enzyme by any *Enterobacter* sp.

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## CHAPTER 4: PURIFICATION AND CHARACTERISATION OF PHYTASE FROM *Enterobacter* sp. ACSS

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### 4.1 INTRODUCTION

Purification is a general pre-requisite in order to remove all contaminants before characterizing the protein of interest. Protein purification leads to increase in the specific activity of the target protein by removing contaminating proteins in various steps. It aims to concentrate the protein of interest which must be able to retain its stability and characteristics when transferred into an environment meant for its proposed application. Purification is achieved by exploiting the general properties of enzymes such as charge, size, bio-specific interactions and hydrophobic interactions. Advances in technology has allowed for the development of highly accurate equipments to make the process more robust. Compared to traditional purification, the recent methods are efficient in speed, accuracy, and cost as well as save precious time.

Pre-packed ion-exchange columns containing cationic (carboxymethyl, sulfopropyl, sulfoethyl, sulfomethyl groups etc.) and anionic (trimethyl amino-methyl/ethyl, trimethylamino-hydroxypropyl, dimethyl/diethyl aminoethyl groups etc.) resins are routinely used. Similarly, a pre-packed gel filtration column with desired pore size can be selected from a wide range of cross-linked agarose and/or dextran, polyacrylamide-based resins etc. Furthermore, use of instruments, such as the AKTA purifier system (GE Healthcare, Sweden) that is equipped with efficient pumps to maintain accurate pressure over the column-bed and automated fractionators, has made the purification process easier. While most of the wild proteins are purified by a combination of ion-exchange, gel filtration and hydrophobic interaction chromatographic steps, recombinant proteins are usually purified to homogeneity in one step using affinity purification. Recently, Reddy *et al.* (2015) purified recombinant BsPhyARRMK33 phytase from *Bacillus subtilis* ARRMK33 using Ni-NTA agarose beads. The 42 kDa protein contained 6× His tag towards its N-terminal.

Methods of confirming the purity of a target enzyme most commonly include checking its homogeneity on an electrophoretic gel (SDS and Native-PAGE) and on a chromatogram followed by N-terminal amino acid sequencing of the target protein (Ward and Swiatek, 2009; Sarrouh *et al.*, 2012). Following fractionation on a Native-PAGE and SDS-PAGE gel those protein bands

related to specific enzyme activities may be identified by zymogram that uses staining techniques based on substrate hydrolysis. Two-step staining protocol developed by Casey and Walsh (2003) is the most common method for the detection of phytases on gels.

The search for an ideal phytase (catalytically efficient, resilient to proteolysis, thermo-acid-stable and cost-effective) is an on-going effort, which is heavily reliant on proper purification techniques that allow accurate characterization as per the industrial needs (Lei *et al.*, 2013). Fungi are the source of most commonly available commercial phytases namely, Natuphos<sup>®</sup>, Allzyme<sup>®</sup> and ROVABIO<sup>®</sup> produced from *A. niger*, *A. oryzae* and *Penicillium funiculosum* respectively (Kumar *et al.*, 2012). Novel and commercially available purified phytases from bacteria have been identified as more effective and these include Phyzyme<sup>®</sup> XP and OptiPhos<sup>®</sup> (*E.coli*) and Ronozyme Hiphos<sup>®</sup> (*C. braakii*) [Lei *et al.*, 2013; Dersjant-Li *et al.*, 2015].

There have been various reports of novel purified phytases from bacteria with desirable traits required for their applications. Generally bacterial phytases have notable attributes such as proteolytic resistance, substrate specificity, broad pH stability and thermostability and are fast becoming preferred as compared to their fungal counterparts (Jorquera *et al.*, 2008a). Thus, for prospective industrial applications in animal food and feed formulations the purification and characterization of novel phytases displaying high thermal stability and activity at acidic (Kumar *et al.*, 2014), neutral (Lui *et al.*, 2013) and alkaline pH (Caputo *et al.*, 2015; Chen *et al.*, 2015) from bacteria is of great importance (Debnath *et al.*, 2005; Lei *et al.*, 2013).

Numerous microorganisms from different geographical regions are explored for a novel wild-type phytase with requisite characteristics, while other tedious methods such as protein engineering of known phytases are also attempted. This chapter focuses on the purification *Enterobacter* sp. ACSS phytase and to investigate whether or not it has any novel characteristics.

## 4.2 MATERIALS AND METHODS

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#### 4.2.1 Purification of phytase

Phytase from *Enterobacter* sp. ACSS was produced in a 1 l laboratory fermenter and the crude extract was concentrated by 55-85% ammonium sulphate precipitation. The precipitate hence obtained after overnight incubation at 4°C was resuspended in 0.1 M sodium acetate buffer (pH 5.5) and desalted through Hi-Prep™ 26/10 column (GE Healthcare, Sweden) against 20 mM sodium acetate buffer (pH 5.5) using AKTA purifier system (GE Healthcare, Sweden). The concentrated and desalted sample was loaded onto a HiTrap™ DEAE FF (GE Healthcare, Sweden) anion-exchange column previously equilibrated with 20 mM Tris-HCl buffer (pH8.0). The column was eluted with a linear gradient of 0-1 M NaCl in 20 mM Tris-HCl buffer (pH8.0) at a flow rate of 1 ml/min. The active fractions were pooled and applied to Superdex™ 200 increase 10/300 column (GE Healthcare, Sweden), and eluted with 50 mM sodium acetate buffer (pH 5.5) at 0.75 ml/min. Elution of the protein was monitored at 280 nm. The active fractions were pooled and concentrated using Biomax (Mr. 10 000 cut-off) ultrafiltration membrane (Millipore, USA) [Escobin-Mopera *et al.*, 2012]. Total protein concentration was determined by the method of Bradford (1976) with bovine serum albumin (BSA) as standard.

#### 4.2.2 Molecular weight determination of phytase

SDS-PAGE was carried out on a Mini PROTEAN gel electrophoresis unit (BioRad Laboratories, USA) according to the method of Laemmli (1970) using a 12% acrylamide separating gel and 5% acrylamide stacking gel both containing 0.1% SDS. A solution containing 0.5% Coomassie Brilliant Blue in 90 ml of methanol: water (1:1 v/v) and 10 ml glacial acetic acid was used to staining the proteins. The protein molecular weight was detected by Quantity One software (Bio-Rad).

A non-denaturing gel (Native PAGE) was performed as per standard SDS-PAGE protocol with the following exceptions. Briefly, 5 µl of gel loading buffer without β-mercaptoethanol was added to 20µl of crude enzyme and loaded in native polyacrylamide gels without heating. Gels were visualized with silver staining according to the method of Deutscher (1990). First, the gel was incubated in a fixing solution v/v (45% methanol, 5% acetic acid and 45 MilliQ H<sub>2</sub>O) for 30 min. Next, the gel was incubated in 50% methanol for 10 min and then in deionised water for 10 min.

Subsequently the gel was then incubated in sensitivity solution (0.02% sodium thiosulfate) for 1 min, followed by two washes with deionised water for 1 min. Next, the gel was incubated in 0.1% silver nitrate solution for 30 min. After two brief 20 s water washes, developing solution (0.04% formaldehyde in 2% sodium carbonate) was added and colour was allowed to develop to an appropriate level. Colour development was stopped by incubation in a solution consisting of 5% acetic acid. For zymography, the samples were electrophoresed on 12% Native-PAGE and stained according to the method of Casey and Walsh (2003). A clear zone against a dark background indicated phytase activity.

#### 4.2.3 Effect of pH on activity and stability

The effect of pH on phytase activity was studied over a pH range between 1.0 to 11.0 in different buffer systems [Hydrochloric acid-potassium chloride (1.0-2.0), Citric acid-sodium citrate (3.0) Na-acetate buffer (pH 4.0-5.0), Citrate buffer (pH 6.0) Tris-HCl buffer (pH 7.0-8.0) and glycine-NaOH (9.0-11.0)]. The pH stability of the enzyme was determined by pre-incubation of phytase with buffers of different pH and the residual enzyme activity was determined at an interval of 30 min up to a period of 6 h under standard assay conditions.

#### 4.2.4 Effect of temperature on activity and stability

The effect of temperature on phytase activity was studied over a temperature range from 30°C to 90°C using standard assay procedure. The thermostability of the enzyme was determined by pre-incubation at different temperatures and the residual enzyme activity was determined at an interval of 30 minutes up to a period of 6 h under standard assay conditions.

The half-life ( $t_{1/2}$ ) value where *Enterobacter* sp. ACSS phytase retained exactly 50% of initial activity under different test conditions was calculated by the following equation:

$$t_{1/2} = \frac{\ln 2}{k} \quad \text{Equation 4.1}$$

#### 4.2.5 Effect of metal ions and surfactants on enzyme activity

The effect of metal ions and surfactants was studied by pre-incubating the enzyme for 30 min at 37°C, 100 rpm with 1 and 5 mM concentration of various metal ions including BaCl<sub>2</sub>, PbCl<sub>2</sub>, FeSO<sub>4</sub>.7H<sub>2</sub>O, CaCl<sub>2</sub>.2H<sub>2</sub>O, CoSO<sub>4</sub>.7H<sub>2</sub>O, MnSO<sub>4</sub>.H<sub>2</sub>O, ZnSO<sub>4</sub>.7H<sub>2</sub>O, MgSO<sub>4</sub>.7H<sub>2</sub>O and CuSO<sub>4</sub>.5H<sub>2</sub>O and 0.1 mM concentration of non-ionic (Triton X-100, Tween-20, Tween-80), anionic (SDS), cationic (CTAB) surfactants. Enzyme without any agent was taken as control. The residual activity was determined under standard assay conditions.

#### 4.2.6 Effect of proteases on phytase activity

*Enterobacter* sp. ACSS phytase (10 U) was incubated in the presence of 1% (w/v) trypsin (pH 8.0) and pepsin (pH 2.0) at 37°C for 30–120 min. A sample was withdrawn at 30 min intervals residual phytase activity tested using standard phytase assay. Control experiments were performed without the addition of proteases (Greiner and Farouk, 2007). All experiments were carried out in triplicates and their average values reported.

#### 4.2.7 Substrate specificity

The substrate specificity of phytase enzyme was tested against different phosphate containing substrates, such as Na-phytate, p-nitrophenyl phosphate, sodium pyrophosphate, glucose-1-phosphate, glucose-6-phosphate and ATP. Substrate specificity was tested by incubating the enzyme separately with the substrates (5 mM) and measuring the residual phytase activity. Enzyme without any agent was taken as control while activity with Na-phytate was taken as 100%.

#### 4.2.8 Kinetic parameters

The  $K_m$  and  $V_{max}$  of phytase was determined against sodium phytate as substrate using Lineweaver–Burk plot and applying the Michaelis-Menten equation (equation 4.2). Purified phytase was incubated with different substrate concentrations (0.125–2 mM) of phosphorylated compounds including Na-phytate, p-nitrophenyl phosphate, sodium pyrophosphate, glucose-1-phosphate, glucose-6-phosphate and ATP. The catalytic constant ( $k_{cat}$ ) was determined from the  $V_{max}$  recorded

for each substrate by applying equation 4.3 and the catalytic efficiency was calculated by applying equation 4.4.

$$\frac{1}{V_0} = \left( \frac{K_m}{V_{max}} \right) \left( \frac{1}{[S]} \right) + \frac{1}{V_{max}} \quad \text{Equation 4.2}$$

$$k_{cat} = \frac{V_{max}}{[E]_T} \quad \text{Equation 4.3}$$

$$\text{Catalytic efficiency} = \frac{k_{cat}}{K_m} \quad \text{Equation 4.4}$$

Where  $V_o$  is the initial velocity,  $V_{max}$  is the maximum velocity,  $K_m$  is the Michaelis-Menten constant and  $[E]_T$  is the total enzyme concentration.

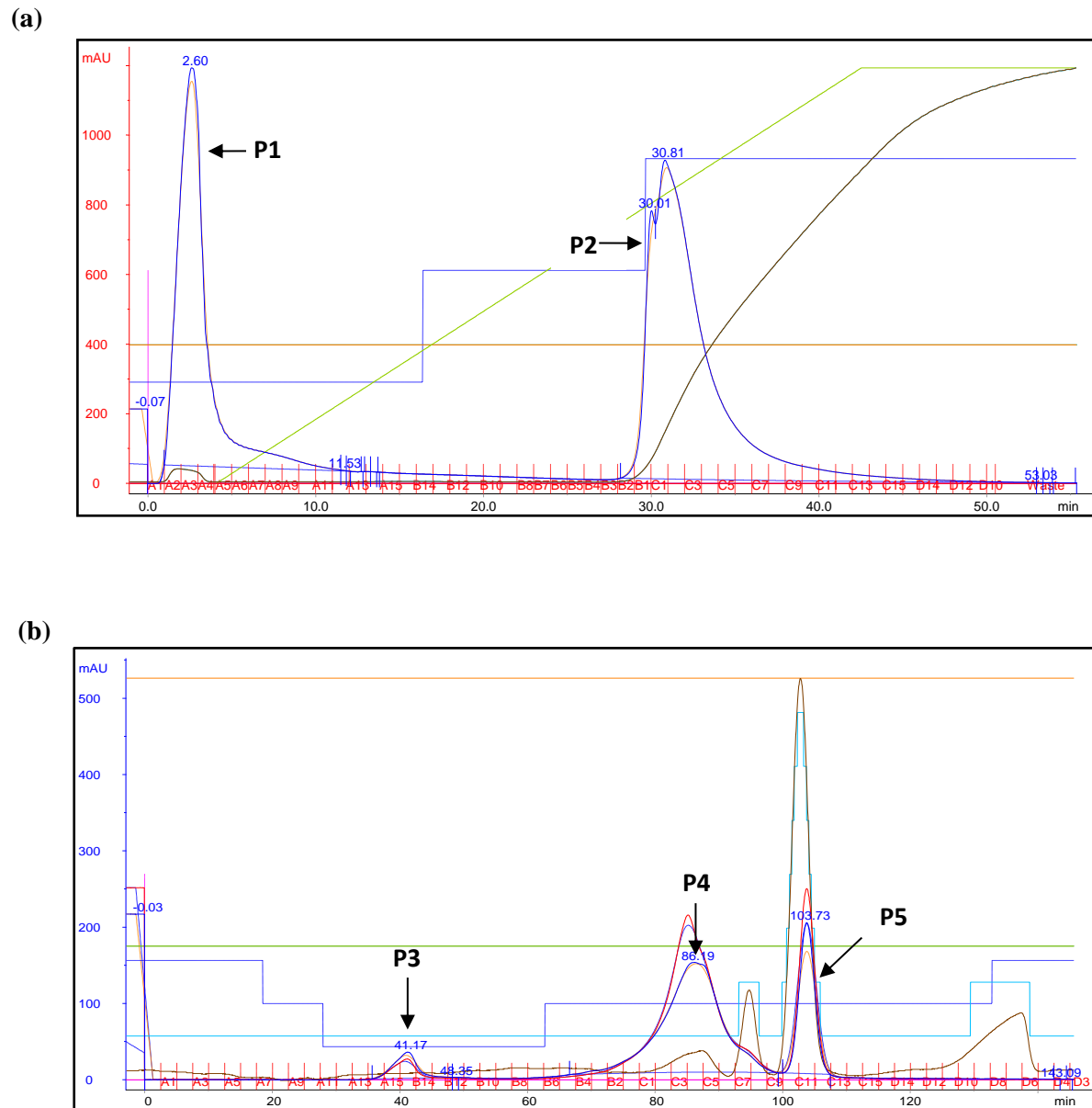
## 4.3 RESULTS

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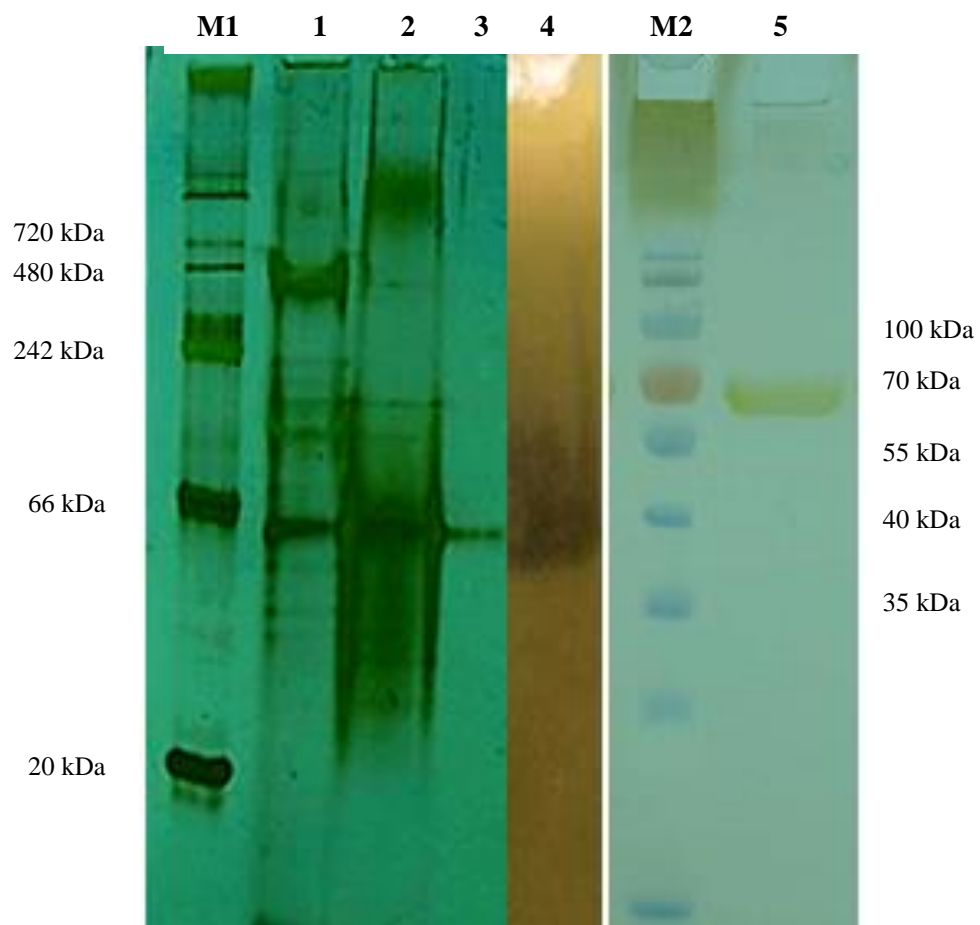
### 4.3.1 Purification of phytase and molecular mass determination

*Enterobacter* sp. ACSS phytase was purified using ammonium sulphate precipitation centrifugation and column chromatography. The concentrated enzyme solution was desalted using a Hi-Prep™ 26/10 column and was used passed through HiTrap DEAE FF column for anion-exchange chromatography (Fig 4.1a). Two peaks were observed namely peak 1 (P1) and peak (P2). P1 consisting of fractions A1 to A5 which failed to bind to the matrix. After elution with a linear gradient of 0–1 M NaCl in 20 mM Tris-HCl buffer (pH 8.0), a second peak (P2) was detected and eluted at a concentration of approximately 70% NaCl. P2 showed a slight separation of fractions C1 and C2 (Fig 4.1a). The protein recovery and yield from this step was 1.06 mg/ml and 6.09 respectively (Table 4.1). After performing the standard phytase assay, the most active fractions were found to be those of C2, C3 and C4.

Consequently, these active fractions were pooled, concentrated using 10,000 kDa cut-off membrane and used for gel filtration chromatography on a Superdex G-75 column. This fraction was eluted as three separate peaks (Fig 4.1b) labelled as P3, P4 and P5. Phytase activity was detected in fraction C3, C4 and C5 with fraction C5 subsequently appearing as a single band on Native-PAGE and SDS-PAGE after silver staining (Fig 4.2a and b). A complete summary of chromatographic details including purification fold, yield, activity and recovery rate are presented in Table 4.1.



**Figure 4.1** Elution profiles of phytase from *Enterobacter* sp. ACSS (a) Anion exchange chromatography using HiTrap DEAE-FF. The column was initially eluted with 10 mM Tris-HCl buffer (pH 8.0) and subsequently with a linear gradient of 0–1 M NaCl in the same buffer. The flow rate was 1.0 ml/min (b) Gel filtration of fraction C2-4 from HiTrap DEAE-FF column on a Superdex G-75 column, which was eluted with 50 mM sodium acetate buffer (pH 5.4). The flow rate was maintained at 0.75 ml/min.



**Figure 4.2** Native-PAGE and zymogram analysis of the phytase **(a)** M1: Native protein molecular weight marker; lane 1: the sample from the crude extract; lane 2: the sample after HiTrap DEAE anion-exchange column chromatography; lane 3: the sample after Superdex<sup>TM</sup> gel filtration chromatography; lane 4: zymogram analysis and SDS-PAGE of the purified phytase **(b)** M2: protein molecular weight marker; lane 1: purified phytase.

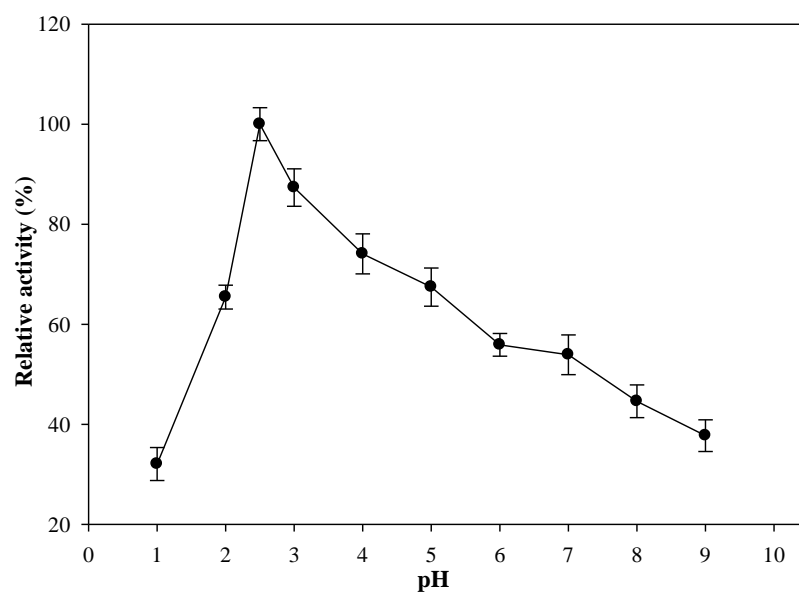
**Table 4.1** Summary of purification steps for *Enterobacter* sp. ACSS phytase

Purification Steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Fold purification
Crude filterate	41156	726	56.68	100	1
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> precipitation	25804.80	309.7	83.32	62.69	1.47
Anion exchange (DEAE FF)	6757.32	24.73	273.24	16.41	4.82
Gel filtration (Superdex™)	1559.81	1.93	805.98	3.79	14.22

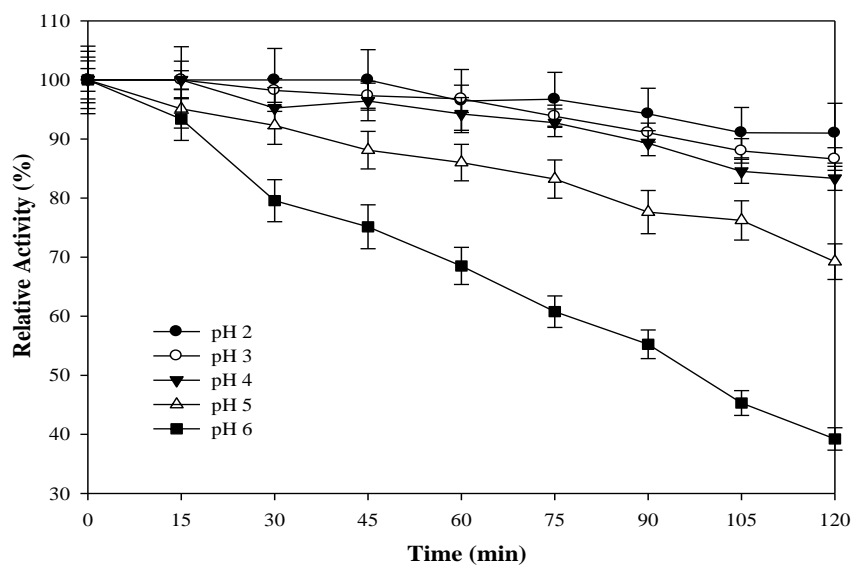
Phytase from *Enterobacter* sp. ACSS was purified to homogeneity, with an overall purification of 14.22-fold and a yield of 3.79% with a specific activity of 805.98 U/mg (Table 4.1). The purified enzyme had an apparent molecular mass of 62 kDa on SDS-PAGE gel (Fig. 4.2) and migrated as monomer during Superdex™ gel filtration chromatography

#### 4.3.2 Effect of pH on activity and stability

Characterisation of phytase was accomplished using sodium phytate as substrate. Phytase activity was measured as a function of pH from pH 1 to pH 10 in buffers of the same ionic strength. The optimal pH at which phytase was active was pH 2.5 (Fig 4.3). At pH 4 it maintained 74% of its activity after which activity steadily decreased from pH 5 to pH 10. Results indicated that the acidic pH range was favoured.



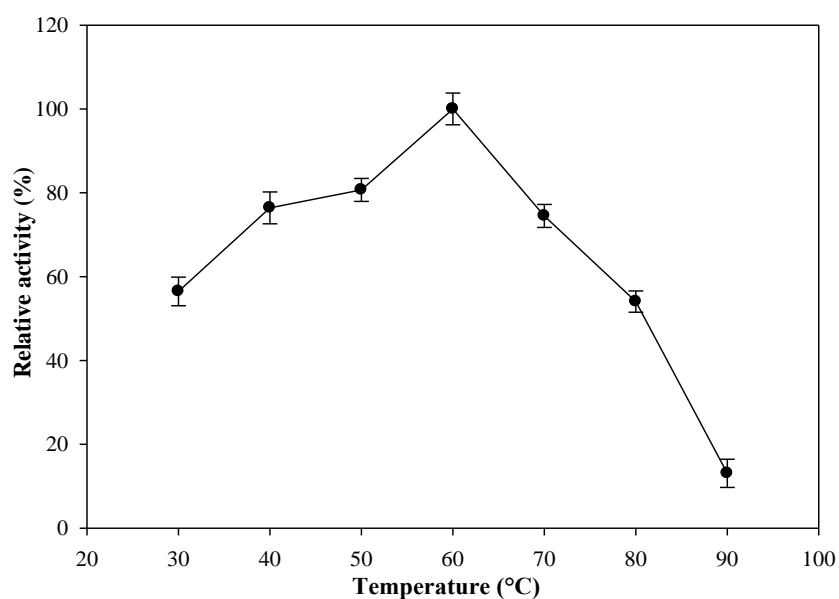
**Figure 4.3** Effect of pH on phytase activity. The activity was measured at 50°C and expressed as a percentage of the maximum activity taken as 100%. Each point represents the mean triplicate of experiments  $\pm$  SD.



**Figure 4.4** Effect of pH on stability of phytase. The activity was measured at 50°C and expressed as a percentage of the maximum activity taken as 100%. Each point represents the mean triplicate of experiments  $\pm$  SD.

The effect of pH stability was studied in the range 2.0-6.0 at 60°C (Fig 4.5). The enzyme was optimally active at pH 2.5 (Fig 4.3) and was fairly stable at pH 2, retaining almost all activity for at least 2 h. It was also rather stable at pH 3 and pH 4 with only a 10% loss of activity observed. After 6 h the enzyme retained approximately 70% activity in the pH range of 2.0 to 4.0. At pH 5 only a slight decline in stability was detected, retaining of 75% activity after 2 h. As pH approached neutrality stability decreased faster. A loss of 60% of activity was observed at pH 6 and a complete loss of activity was observed at 4 h. Half-life of the enzyme at pH 2.0, 3.0, 4.0, 5.0 and 6.0 was 577.5, 433.1, 770.0, 247.5 and 91.2 min, respectively.

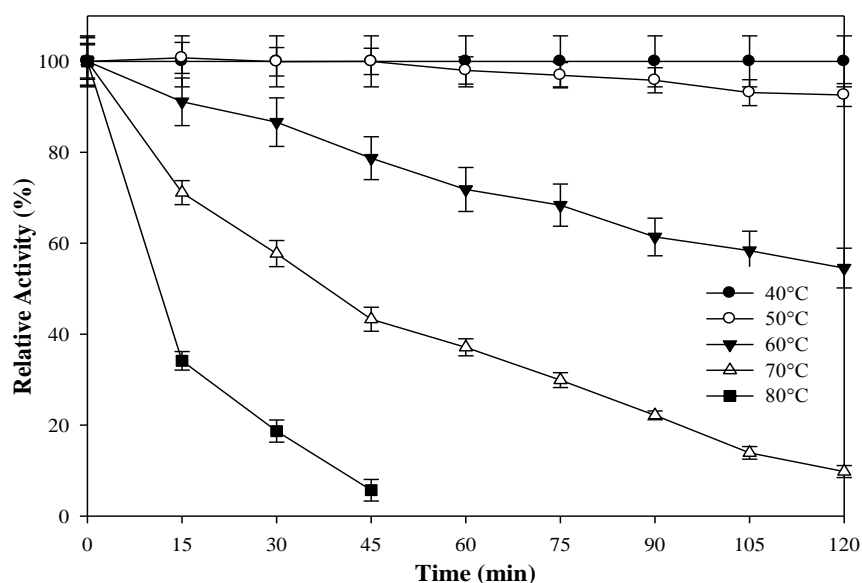
#### 4.3.4 Effect of temperature on activity and stability



**Figure 4.5** Effect of temperature on phytase activity. Activity was measured at pH 5.0 and expressed as a percentage of the maximum activity taken as 100%. The values shown represent averages from triplicate experiments  $\pm$  SD.

Phytase from *Enterobacter* sp. ACSS was active in the temperature range of 30-80°C, with optimal activity at 60°C (Fig 4.5). The enzyme was fairly stable between 40 and 50°C for 2 h (Fig 4.6). No loss of activity was detected at 40°C and over 2 h at 50°C less than 10% of activity was lost. A gradual loss of activity was observed at 60°C. After 2 h at 60°C the enzyme managed to retain

70% of activity. When the temperature was raised to 70°C more than half of its activity was lost within 45 min and after 2 h only 9% retention was detected. Half-life of the enzyme at 50, 60, 70 and 80°C was 866.2, 693.0, 37.8 and 11.3 min, respectively. The activity was lost completely at 80°C, after 55 min of incubation.



**Figure 4.6** Effect of temperature on purified phytase stability. Activity was measured at pH 5.4 and expressed as a percentage of the maximum activity taken as 100%. The values shown represent averages from triplicate experiments  $\pm$  SD.

#### 4.3.5 Effect of metal ions and surfactants on enzyme activity

Effector studies for potential inhibitors or activators of phytase were conducted at concentrations of 1 and 5 mM (Table 4.2).  $Mn^{+2}$ ,  $Mg^{+2}$  and  $Ca^{+2}$  ions were most effective at 1 mM concentrations from 100% to 110%, 120%, 143% respectively.

**Table 4.2** Effect of divalent metal ions on activity of purified phytase from *Enterobacter* sp. ACSS

Additives	1 mM	5 mM
<i>Metal ions</i>		
Ba <sup>+2</sup>	71 ± 4.97	65 ± 3.38
Mn <sup>+2</sup>	110 ± 6.03	104 ± 5.66
Mg <sup>+2</sup>	120 ± 6.33	114 ± 5.75
Pb <sup>+2</sup>	60 ± 3.38	47 ± 3.05
Ca <sup>+2</sup>	143 ± 9.15	120 ± 5.93
Fe <sup>+2</sup>	40 ± 2.21	23 ± 1.37
Co <sup>+2</sup>	73 ± 4.67	56 ± 3.75
Cu <sup>+2</sup>	48 ± 5.72	27 ± 1.32
Zn <sup>+2</sup>	46 ± 3.22	18 ± 1.26
<i>Reducing agents</i>		
EDTA	88 ± 5.92	75 ± 4.99
DTT	73 ± 4.70	42 ± 1.4
Additives	0.1 mM	1 mM
<i>Surfactants</i>		
Triton X-100	72 ± 5.04	63 ± 3.72
Tween-20	78 ± 4.73	64 ± 3.55
Tween-80	81 ± 5.88	74 ± 4.45
SDS	18 ± 1.11	07 ± 0.31
CTAB	72 ± 3.60	64 ± 1.1
Control	100	100

Phytase activity in the absence of additives is regarded as 100%

Ba<sup>+2</sup>, Tween 80 and EDTA showed moderate inhibition of 29 to 35%, 12 to 26% and 19 to 25% at 1 mM and 5 mM respectively. A marked decline in activity was observed using Cu<sup>+2</sup> and Zn<sup>+2</sup> ions. SDS displayed the greatest inhibitory effect, recording a loss at least 82% of activity.

#### 4.3.6 Resistance to proteolytic enzymes

While investigating the effect of proteolytic cleavage on enzyme resilience under physiological conditions, purified phytase from *Enterobacter* sp. ACSS was found to be fairly stable with pepsin

and it lost only 10.64 % of the initial activity after 120 min of incubation at 37°C. However, the enzyme was not very stable with trypsin under alkaline conditions as there was a 50% loss of activity within 30 min while all activity was lost within 90 min of incubation, both under controlled and test conditions (Table 4.4).

**Table 4.3** Proteolytic resilience of phytase from *Enterobacter* sp. ACSS

Treatment	Incubation time (min) and residual phytase activity (%)				
	0	30	60	90	120
Control P*	100	99.7	96.53	94.17	90.12
Pepsin	100	99.6	96.7	93.25	89.36
Control T <sup>#</sup>	100	49.5	28.5	NA	NA
Trypsin	100	48.3	27.8	NA	NA

\*Controlled reaction performed under simulated physiological acidic environment (pH 2.0) without pepsin

<sup>#</sup>Controlled reaction performed under simulated physiological alkaline environment (pH 8.0) without trypsin

NA: No activity was detected

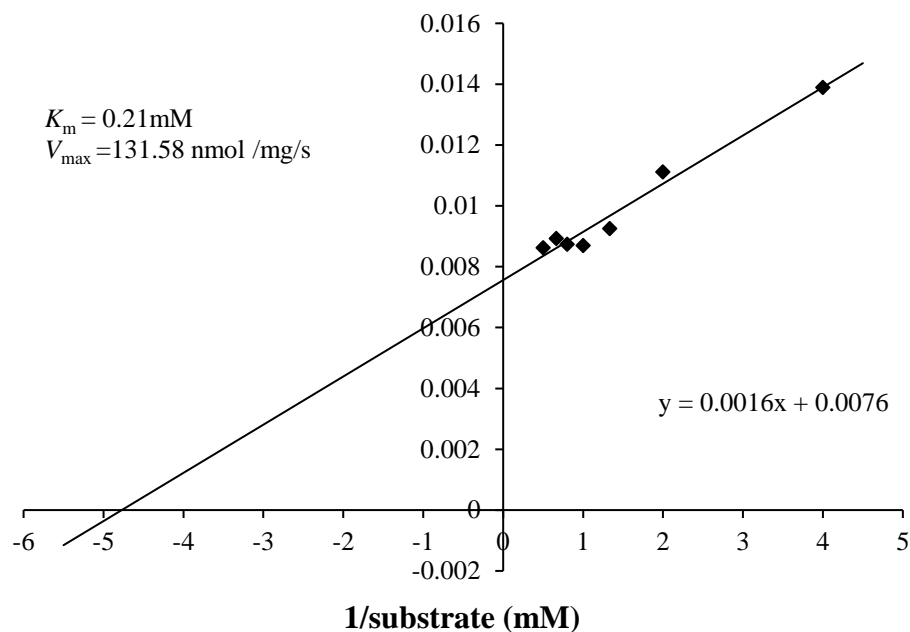
#### 4.3.7 Substrate specificity and kinetic parameters

Several phosphorylated compounds, including Na-phytate, were used to determine the substrate specificity of purified phytase from *Enterobacter* sp. ACSS. The purified phytase displayed strong substrate specificity for Na-phytate (Table 4.4). It displayed minimal specificity towards other tested compounds while not displaying any specificity towards ATP.

**Table 4.4** Substrate specificity and enzyme kinetics for purified phytase from *Enterobacter* sp. ACSS

Substrates	Relative activity (%)	$K_m$ (mM)	$V_{max}$ (nmolmg <sup>-1</sup> s <sup>-1</sup> )	$K_{cat}$ (s <sup>-1</sup> )	$K_{cat}/K_m$ (M <sup>-1</sup> s <sup>-1</sup> )
Sodium phytate	100 ± 3.41	0.21 ± 0.01	131.58 ± 4.49	$1.64 \times 10^3 \pm 62.32$	$7.81 \times 10^6$
p-nitro phenyl phosphate	46.21 ± 1.73	1.16 ± 0.04	12.47 ± 0.37	$1.55 \times 10^2 \pm 4.96$	$0.13 \times 10^6$
Sodium pyrophosphate	13.63 ± 0.46	1.38 ± 0.03	10.95 ± 0.22	$1.26 \times 10^2 \pm 5.42$	$0.10 \times 10^6$
Glucose-1-phosphate	8.27 ± 0.28	1.75 ± 0.04	11.79 ± 0.27	$1.47 \times 10^2 \pm 4.65$	$0.08 \times 10^6$
Glucose-6-phosphate	7.32 ± 0.21	1.81 ± 0.05	11.82 ± 0.25	$1.47 \times 10^2 \pm 4.61$	$0.08 \times 10^6$

The values are the mean of three independent experiments.



**Figure 4.7** Double reciprocal plot for determining the  $K_m$  and  $V_{\max}$  values of purified phytase from *Enterobacter* sp. ACSS using Na-phytate as substrate. The values shown represent averages from triplicate experiments. SD values were less than 0.05%.

On the basis of the Lineweaver Burk plot,  $K_m$  and  $V_{\max}$  were calculated as 0.21 mM and 131.58 nmol/mg/s, respectively using Na-phytate as substrate.

#### 4.4. DISCUSSION

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For accurate characterization of an enzyme appropriate steps must first be taken to purify it to homogeneity. Phytase from *Enterobacter* sp. ACSS was purified to homogeneity, with an overall purification of 14.22-fold and a yield of 3.79% (Table 4.1). The purified enzyme had an apparent molecular mass of 62 kDa on SDS-PAGE gel (Fig 4.2) and migrates as monomer during Superdex<sup>TM</sup> gel filtration chromatography. This is higher than that the 43 kDa *E. sakazakii* ASUIA279 phytase (Farouk *et al.*, 2012) and still within the molecular mass range of previously purified phytases of between 38–200 kDa (Bhavsar *et al.*, 2011; Lei *et al.*, 2013).

The purified phytase exhibited an optimum of phytate dephosphorylation at pH 2.5 sharing many enzymatic properties in common with other phytases. Acid-stable phytase (phyC) from *Citrobacter freundii* showed two distinct pH optima at 2.5 and 4.5 and an optimal temperature at 50°C (Zhao *et al.*, 2010). *Yersinia rohdei* had an optimal pH of 4.5 and was highly active between pH 1.5-6.0 (Huang *et al.*, 2008) while directed evolution of *Yersinia mollaretii* phytase (Ymphytase) by Shivange *et al.* (2014) showed improved acid stability at pH 2 for 3 h and was also optimally active at pH 4.5. The purified phytase showed considerable activity below pH 3.0 sharing a similar profile to that of commercial phytase Natuphos from *A. niger*. Thermoacidophilic enzymes are of major commercial interest (Sharma *et al.*, 2012). Members of the family *Enterobacteriaceae* prefer to grow in neutral pH, they are known to survive the low-pH stress during passage through the stomach and in the fermented and acidified faecal material (Lin *et al.*, 1995; Dancer *et al.*, 2009), while the role of phosphate transport systems in acid habituation (Rowbury *et al.*, 1992) and survival of *E. coli* in seawater (Gauthier *et al.*, 1993) has been already established. *Enterobacter cloacae* produced a thermostable  $\beta$ -D-galactosidase which was optimally active at 50°C (Ghatak *et al.*, 2010), while recombinant sucrose isomerase from *Enterobacter* sp. FMB1 and lipase from *Enterobacter* sp. Bn12 showed optimal activity at 50° and 60°C, respectively (Cha *et al.*, 2009; Farrokh *et al.*, 2014). Phytase from *Pichia anomala* was optimally active at 60°C and pH 4.0 (Vohra and Satyanarayana, 2002). In this study, the phytase from *Enterobacter* sp. ACSS was active in the temperature range of 30-80°C, with optimal activity at 60°C.

There are many reports on activation of phytases by  $\text{CaCl}_2$  for enhanced activity, such as lily pollen alkaline phytase (Jog *et al.*, 2005), *Enterobacter* sp. 4 (Kang *et al.*, 2006) and *Aspergillus flavus* (Gaiind and Singh, 2015). The phytases of members of *Bacillus* including *Bacillus* sp. KHU-10 (Choi *et al.*, 2001), *Bacillus licheniformis* (Borgi *et al.*, 2013), *Bacillus subtilis* YCJS (Yao *et al.*, 2014) are well known to be calcium-dependent. The mode of action involves the formation of a positively charged calcium–phytate complex when two oxyanions from the phosphate groups of phytate are bound by  $\text{Ca}^{2+}$  ions. There are three  $\text{Ca}^{2+}$  binding sites on the phytate molecule at the active site cleft. The negative charge around the active site cleft is reduced since  $\text{Ca}^{2+}$  serves as an essential activator hence forming a positively charged complex. However,  $\text{Ca}^{2+}$  acts as a competitive inhibitor when in present in excess quantities. Similarly, in this study addition of 1 mM  $\text{Ca}^{2+}$  in the reaction mixture resulted in marked activation (143%) and may have had the same effect on phytase activity apart from the other activating metal-ions such as  $\text{Mg}^{2+}$  and  $\text{Mn}^{2+}$ . However, as the concentration increased to 5mM this effect was decreased causing phytase inhibition due to the precipitation of phytates as an insoluble metal salts (Tang *et al.*, 2006). Metals ions including  $\text{Co}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  and surfactants significantly inhibited the enzyme activity. This may be explained due to the formation of poorly soluble metal complexes with phytic acid in the assay mixture that results in reduced substrate concentration. Wyss *et al.* (1999) and Vohra and Satyanaryana (2002) noted that metal-ion-mediated phytase inhibition was exclusively due to binding of metal ions to the substrate rather than the enzyme itself. There are also reports of certain phytases which are not affected by metal ions. For example, phytase from *C. braakii* YH-15 is not affected by even up to 10 mM  $\text{Ca}^{2+}$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Ni}^{2+}$  and  $\text{Na}^+$  (Kim *et al.*, 2003), while the phytase from a Malaysian wastewater bacterium (Greiner and Farouk, 2007) showed little to no effect due to  $\text{Ca}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Mn}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Ag}^+$ ,  $\text{Hg}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Mn}^{2+}$ .

Phytase, like any other protein molecule, can be easily hydrolyzed in the digestive tract of animals by endogenous proteases but ideally for applications involving animal feed it should be resistant to hydrolysis by pepsin and trypsin. Phytase from *Enterobacter* sp. ACSS was tested in the presence of pepsin and trypsin at pH 2 and 8, respectively to mimic pH of the GI tract where they are most stable. This phytase displayed high resistance to pepsin (pH 2) but its resilience against trypsin was not conclusive in the present study as both the controlled and test reactions were almost equally affected under alkaline conditions. This may be mostly due to the lower stability of this

phytase at higher pH. The proteolytic resistance displayed in this study shared similar features with other reported phytases. Phytases from *Aspergillus* were susceptible to trypsin and resistant to pepsin after 2 h in separate studies conducted by Promdonkoy *et al* (2009), Sapna and Singh (2013) and Ushasree *et al.* (2014) while  $\beta$ -Propeller phytase from *B. licheniformis* PB-13 was stable against trypsin, retaining 77% activity after 2 h of incubation (Kumar *et al.*, 2014). Commercially available *E.coli* phytase retained almost all its activity after 4 h of incubation with pepsin however; commercial phytase from *P. lycii* phytase was less stable, losing more than 60% of activity with 2 h of incubation (Morales *et al.*, 2011; Dersjant-Li *et al.*, 2015). Variation in phytase stability with different proteolytic enzymes may be attributed to the difference in mode of action of pepsin and trypsin, and protein-protein interactions under different physiological conditions.

Substrate affinity or specificity is particularly associated with the physiological nature of the substrate. Purified *Enterobacter* sp. ACSS phytase displayed broad specificity for all tested phosphorylated compounds but the highest activity was for Na-phytate, which is in good agreement with many other reported phytases including *K. pneumoniae* 9-3B phytase (Escobin-Mopera *et al.*, 2012) and *Burkholderia* sp. strain a13 phytase (Graminho *et al.*, 2015). Other reported phytases have narrow specificity for Na-phytate such as *Bacillus nealsonii* ZJ0702 phytase (Yu and Chen, 2013) and *A. flavus* ITCC 6720 phytase (Gaiind and Singh, 2015). In contrast, *Bacillus* sp. T4 exhibited strict substrate specificity for magnesium-phytate (Mg-phytate), the most biologically available phytate substrate (Park *et al.*, 2012) while purified phytase from *E. sakazakii* ASUIA279 showed some activity towards GTP (Farouk *et al.*, 2012).

Phytase from *Enterobacter* sp. ACSS had a broad substrate specificity which included sodium phytate, p-nitro phenyl phosphate and sodium pyrophosphate. Although the highest catalytic efficiency ( $k_{cat}/K_m$ ) of  $7.83 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$  was for sodium phytate. The  $K_m$  and  $V_{max}$  for the same substrate were calculated to be 0.21 mM and 131.58 mmol/mg/s, respectively. Enzymes with lower substrate affinity have higher  $K_m$  values. High concentrations of substrate are required to achieve maximum catalytic efficiency of the enzyme since the enzyme does not bind to the substrate tightly (Farouk *et al.*, 2012). *E.coli* phytase had a  $K_m$  of 0.43 mM (Yao *et al.*, 2013) while the calculated  $K_m$  and  $V_{max}$  values for recombinant *Bacillus subtilis* (BSPHyARRMK33) phytase were 0.95 mM and 15.3  $\mu\text{mol/l}$  (Reddy *et al.*, 2015). Results suggested that *Enterobacter* sp. ACSS

phytase preferred the hydrolysis of sodium phytate as compared to other tested phosphorylated compounds (Table 4.4). Similarly, phytase from *Burkholderia* sp. strain a13 had a broad substrate specificity but the highest catalytic efficiency was for sodium phytate (Graminho *et al.*, 2015).

## **5.1 INTRODUCTION**

As it had been already indicated hydrolysis of phytic acid is necessary for the release and utilization of P, minerals, and trace elements complexed with phytic acid. Indeed, microbial phytases are the most routinely used feed enzymes that are applied to animal and human foodstuffs for improving mineral bioavailability as well as for food processing. Besides acting as a feed additive phytases have multifarious applications (Lei *et al.*, 2013).

Whole grains and legumes containing large amounts of phytic acid are consumed as staple foods, which may contribute to the risk of depletion of the essential minerals and related deficiency diseases. However, the vital role of dietary phytic acid antioxidant and an anticancerous agent has also been documented (Singh *et al.*, 2011). Removal of these dietary phytic tissues during milling or polishing eliminates most of the phytic acid and mineral deposits. Production of whole wheat bread with low phytic acid level and increased mineral bioavailability would be beneficial and attractive in improving mineral status and consequently, in supporting preventive nutrition. Supplementation of phytase in bread dough has been shown to improve its properties and mineral availability by indirectly impacting on  $\alpha$ -amylase activity (Greiner and Konietzny, 2006).

Agastric animals lack the ability to degrade phytate, and when PA containing plant-derived diet is consumed it leads to high discharge of undigested phytate P and unabsorbed inorganic P. Phytate rich soil eventually washes into waterways where aquatic microbes release bound P causing much harm in the form of algal blooms and eutrophication (Cao *et al.*, 2007; Jorquera *et al.*, 2008a). Legislative action has been taken limiting the quantity of P in animal excreta in some countries (Lei and Stahl, 2001) however, a more concerted effort is needed for its control. Haefner *et al.* (2005) reported a 50% reduction in P excretion by adding phytase to animal feed. Additionally, high phytate containing diets supplemented with phytase improves the absorption and utilization of phosphorus. Temperature and pH values are key factors determining enzyme activity. Although the preferred phytase as per the industrial needs should be thermostable, other favourable

properties for are high substrate hydrolysis, even at room temperature, and high activity over a broad pH range. The first commercial phytase products were launched into the market in 1991 (Greiner *et al.*, 2007). The addition of phytase to feed for monogastric animals is commonly used to enhance the digestibility of phytate-associated phosphorus (Pontoppidan *et al.*, 2007) and effect of microbial phytases (EC 3.1.3.8) as feed supplement is well documented in the literature (Noureddini and Dang, 2008). Besides acting as feed additives phytases have several other applications (Lei *et al.*, 2013).

This chapter is solely based on applications of phytase from *Enterobacter* sp. ACSS and discusses its potential as a food and feed supplement.

## 5.2 MATERIALS AND METHODS

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### 5.2.1 Phosphate liberation from animal feed

Finely milled wheat bran, chicken feed and fish feed were autoclaved at 121 psi for 15 minutes. Two g of each feed ingredient was suspended in 50 ml of 0.1 M acetate buffer (pH 5.5). Phytase (50 U) was added to each suspension and incubated at 37°C. Aliquots (1 ml) were removed at desired time intervals and centrifuged at 10 000 rpm for 2 min at 4°C. The supernatant was collected for estimating inorganic phosphate liberated as described earlier (Cho *et al.*, 2003). Control experiments were carried out in the absence of phytase and values were deducted from the test value.

### 5.2.2 Phosphate liberation in simulated gastric fluid (SGF)

Pi released from animal feed was determined by adding one gram of feedstock (chicken feed or fish feed) to 9 ml simulated gastric fluid SGF; 250 mM Glycine–HCl containing 2.0 mg/ml NaCl and 3.2 mg/ml pepsin). The pH was adjusted with HCl or NaOH to 1.5, 2.5, 3.5, 5.5, or 6.5 as required. The solutions were incubated with agitation at 37°C for 30 min, and the pH was again adjusted to the corresponding pH values. Phytase (U) was added to the solutions and incubated with agitation at 37°C for 60 min. (Bhavsar *et al.*, 2011). The amount of released phosphorus was determined using standard phytase assay.

### 5.2.3 Hydrolysis of insoluble metal-phytates

Briefly, 100 mM stock solutions of metals ions ( $\text{Ca}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$  and  $\text{Zn}^{2+}$ ) were prepared by dissolving  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ,  $\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$ ,  $\text{Cu}_2\text{SO}_4$ ,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ ,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  and  $\text{ZnCl}_2 \cdot 7\text{H}_2\text{O}$  in distilled water. Equal volumes (1 ml each) of 100 mM salt solution and 10 mM sodium phytate were mixed and incubated overnight at 4°C. The precipitated salts were centrifuged at 2,000 rpm at 4°C for 1 min. The supernatant was decanted off and the precipitated salts were washed thrice with distilled water and re-suspended in 0.5 ml of 0.1 M Na-acetate buffer (pH 5.5). The hydrolysis reactions were initiated by incubating each phytate salt

with 10 U of phytase at 50°C. Aliquots of the mixture were taken at desired intervals. The salts were pelleted at 10 000 rpm for 2 min and the amount of inorganic phosphate in the supernatant was determined as described earlier. The substrate and enzyme controls were also run simultaneously and values were deducted from the test value (Tang *et al.*, 2006; Singh, 2013).

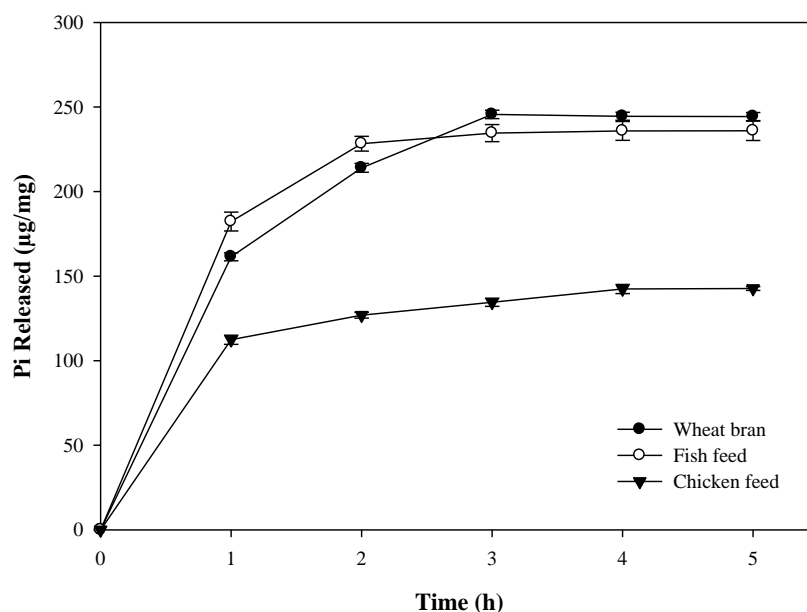
#### **5.2.4 Application in improvement of bread making performance**

Basic bread dough was prepared by mechanically mixing brown bread flour (400 g) with dry yeast (8 g), sugar (15 g), ascorbic acid (3.75 mg), water (275 ml), fat (10 g) and NaCl (5g) for 5 min. Phytase concentrations of 0.2, 0.6 and 1.0 U/g were added prior to mixing of separate bread doughs. Basic bread dough prepared without phytase was treated as the control. Proofing by fermentation was allowed for 45 min, followed by baking at 25 °C for 25 min (Singh and Satyanarayana, 2008). The breads were assessed for soluble inorganic phosphate. The moisture content of the bread samples was determined in triplicate by oven drying at 103 °C  $\pm$  2 °C until a constant weight (AACC, 2000).

## 5.3 RESULTS

### 5.3.1 Phosphate liberation from animal feed

The net amount of inorganic Pi released from chicken feed, fish feed and wheat bran was  $124.76 \pm 1.34$ ,  $235.95 \pm 2.69$  and  $244.33 \pm 2.36$   $\mu\text{g}/\text{mg}$  respectively after 5 h and at pH 5.5 (Figure 5.1).

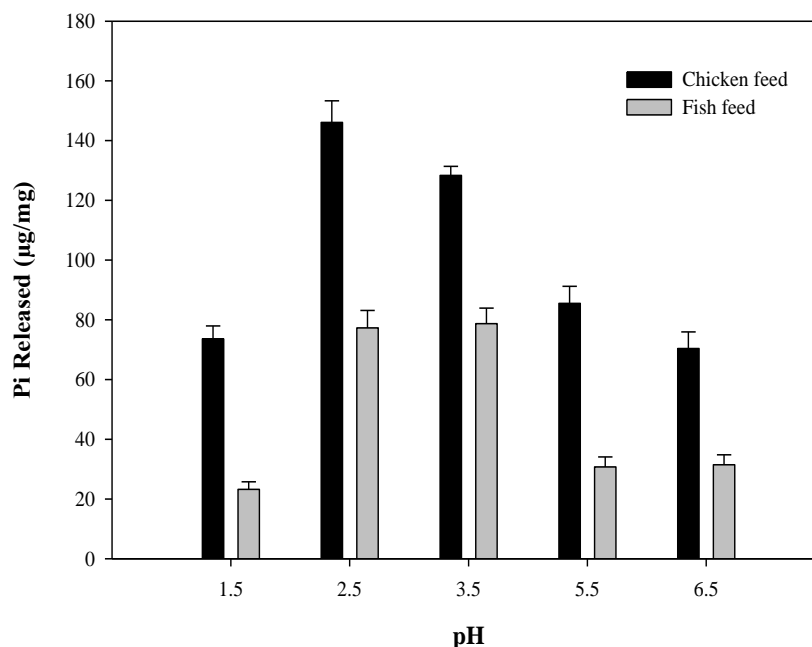


**Figure 5.1** *In vitro* phosphate liberation from wheat bran, fish feed and chicken feed using *Enterobacter* sp. ACSS phytase. Control experiments without phytase supplementation were also conducted and subtracted to give the final Pi released value. Each point represents the mean triplicate of experiments  $\pm$  SD.

### 5.3.2 Phosphate liberation in simulated gastric fluid (SGF)

After digestion according to the gastric passage at pH 1.5, 2.0, 2.5, 3.5 and 5.5 phytate degradation of phytate was measured. As indicated by Fig 5.2, *Enterobacter* sp. ACSS phytase displayed excellent efficacy releasing inorganic P from chicken and fish feed. It released 73.57, 146.07, 128.11, 85.48 and 70.35  $\mu\text{g}/\text{mg}$  of Pi from chicken feed treated at pH 1.5, 2.5, 3.5 5.5 and 6.5,

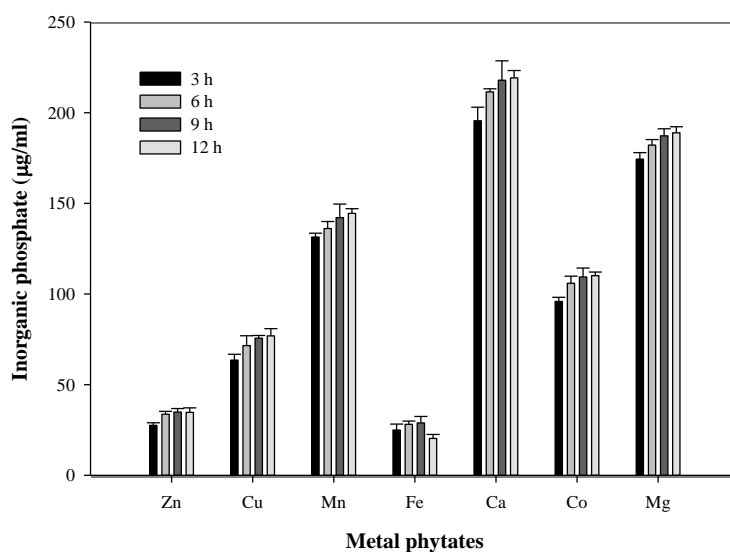
respectively, while from fish feed, treated at the same pH values, it released 23.21, 77.26, 78.69, 30.71 and 31.43  $\mu\text{g}/\text{mg}$  of Pi using the same pH's.



**Figure 5.2** The hydrolytic ability of *Enterobacter* sp. ACSS phytase in SGF containing animal feed. Hydrolysis efficacy was determined after incubating the enzyme in SGF at pH 1.5, 2.5, 3.5, 5.5 and 6.5 at 37°C. Appropriate control experiments were also done. Each point represents the mean triplicate of experiments  $\pm$  SD.

### 5.3.3 Hydrolysis of insoluble metal-phytates

*Enterobacter* sp. ACSS phytase was capable of hydrolyzing assorted insoluble metal phytates to release inorganic orthophosphate to a varied extent. Pi released was the highest from Ca, Cu and Mg salts.



**Figure 5.3** Hydrolysis of insoluble metal-phytates by the phytase of *Enterobacter* sp. ACSS phytase at 50°C. Each point represents the mean triplicate of experiments  $\pm$  SD.

#### 5.3.4 Application in improvement of bread making performance

The addition of phytase from *Enterobacter* sp. ACSS to dough resulted in the liberation of inorganic phosphate and an over-all decrease in proofing time. A maximum of  $29.35 \pm 1.47$  µg/mg of Pi was liberated using a phytase dose of 1.00 U/g. Loaf A, B and C all showed an increase in properties and nutritional quality compared to the control bread (Table 5.1).

**Table 5.1** Comparison of properties of breads prepared by the action of *Enterobacter* sp. ACSS phytase with and bread prepared without phytase addition (control bread)

Parameters	Control	Phytase (U/g)		
		0.25	0.50	1.00
Mass (g)	587.5±30.05	590.3±29.08	586.5±29.28	586.5±29.17
Dough rise (cm)	3.78±0.22	4.14±0.29	5.22±0.25	5.34±0.25
Proofing time (min)	45±2.21	35±1.88	30±1.23	30±1.33
Bread moisture (%)	24.6±1.17	28.5±1.37	33.2±1.58	33.8±1.63
Reducing sugar (mg g <sup>-1</sup> )	23.2±1.05	27.5±1.41	31.2±1.59	32.0±1.68
Soluble protein (mg g <sup>-1</sup> )	2.79±0.11	3.28±0.17	3.74±1.77	3.84±0.22
Pi released (µg/mg)	0.44 ±0.018	20.93±1.08	20.93±1.07	29.35±1.47
Shelf life (days)	2.0±0.092	3.0±0.18	4.0±0.16	3.99±0.23

## 5.4 DISCUSSION

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Phytase is applied to animal feed as an additive to enhance nutritive properties. It may be sprayed or mixed into feed or added to feed prior to pelleting where it should withstand higher temperatures, and lastly and finally it finds its way into the animal's GI tract where it should show pH and proteolytic tolerance. In the present study application of *Enterobacter* sp. ACSS phytase was studied on wheat bran, chicken feed and fish feed and it released maximum inorganic phosphate within 3 h at 37°C from all feeds. This is important since phytase was able to degrade naturally occurring phytate in addition to the chemically pure commercial phytate. These results are in good agreement with studies applying *Yersinia rohdei* phytase (Huang *et al.*, 2008) and *P. syringae* MOK1 phytase (Cho *et al.*, 2003) to wheat bran, corn meal and soybean meal for phytate degradation.

*Enterobacter* sp ACSS phytase was assessed in poultry feed for increasing phosphate content in SGF experiments. The amount of Pi released was higher in chicken feed due to a higher phytate content (8.2 mg/g) in the bran-soya-corn based broiler feed as compared to the soya-corn based fish feed (5.1 mg/g) [Dersjant-Li *et al.*, 2015]. In this study, inorganic phosphate (Pi) was released from feeds in a wide range of pH (1.5-6.5) which correlates the optimum activity and acid-stable characteristics of this enzyme. A maximum of 146.07 µg/mg Pi was released at pH 2.5 (Fig 5.2) which was highest among various feeds studied. The amount of Pi released is sufficient to overcome the dietary phosphorus requirement of broilers that varies from 4.8-5.7 mg/g (Bar *et al.*, 2003) and most aquaculture-relevant fish species that varies from 4.06-6.62 mg/g of diet (Sugiura *et al.*, 2000). Kumar *et al.* (2014) reported increased Pi liberation from commercial chicken feed of 32–116% after SGF experiments using 250-1000 U/kg phytase. Commercial *E. coli* phytase was active at higher temperatures and low pH, and could only be used as an additive for applications for warm-water fish (Morales *et al.*, 2011) while arctic isolate *Rhodotorula mucilaginosa* strain JMUY14 phytase had high activity at lower temperatures (20-30°C), showed good pH stability (3.0-7.0) and proteolytic resilience. This makes this enzyme a good candidate as an aquaculture additive (Yu *et al.*, 2015).

Phytase action on Ca-phytate complex released a maximum of 219.17  $\mu\text{g/ml}$  of inorganic phosphate (Pi), which corroborates with its  $\text{Ca}^{+2}$  mediated activation profile. A plausible reason for the  $\text{Ca}^{+2}$  mediated activation of phytases may be the stabilization of the active site cleft by  $\text{Ca}^{+2}$ , as reported previously during studies on alkaline phytase from *B. amyloliquefaciens* (Oh *et al.*, 2004) and *Bacillus* sp. MD2 (Tran *et al.*, 2011) and acidic phytase from *C. freundii* (Zhao *et al.*, 2010). A marked decline in phytase activity due to  $\text{Fe}^{+2}$  and  $\text{Zn}^{+2}$  and lower degree of hydrolysis of the respective metal-phytate complexes can be attributed to the versatile redox properties of  $\text{Fe}^{+2}$ , which makes them unsuitable for the hydrolytic activity, and to the orientation problems in  $\text{Zn}^{+2}$  where the IP6 is disoriented in the cleavage site and the phosphate group lies outside the Zn atoms (Tran *et al.*, 2011).

Phytase supplementation to the dough increased the inorganic phosphate, reducing sugar and soluble protein in the bread (Table 5.1). The bread was softer with higher moisture content and improved shelf-life than the one without phytase. Although, Rosell *et al.* (2009) could not find any phytase mediated changes in physical parameters of bread, there are many reports that show increased bio-availability of ions such as  $\text{Ca}^{+2}$ ,  $\text{Mg}^{+2}$ ,  $\text{Fe}^{+2}$ ,  $\text{Cu}^{+2}$ ,  $\text{Zn}^{+2}$  and  $\text{Pb}^{+2}$  (Park *et al.*, 2011; Buddrick *et al.*, 2014; Iglesias-Puig *et al.*, 2015). Increased availability of  $\text{Ca}^{+2}$  ions results in activation of amylase. The action of amylase promotes improved specific bread volume, better gas retention during proofing, lower fermentation time and reduced crumb firmness (Haros *et al.* 2001; Greiner and Konietzny, 2006). The present study also indicates a significant reduction in proofing time from 45 min to 35 and 30 min, which is in accordance with earlier report by Haros *et al.* (2001) and Sanz-Penella *et al.* (2012).

In this regard of increased mineral bioavailability, besides bread application, effect of exogenous phytase in other food products (legumes and cereals) for human consumption has also been explored. Cereals and their derivatives are at the first level in the food pyramid and constitute a considerable part of a balanced diet, according to the international dietary guidelines. There are reports of phytate degradation in infant cereals and cereal-based products for increased mineral bioavailability of Ca, Fe and Zn by addition of commercial *Aspergillus* (Sanz-Penella *et al.*, 2012) and *Bifidobacterium* phytases (Garcia-Mantrana *et al.*, 2014).

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## CHAPTER 6: GENERAL DISCUSSION

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Exploitation of the catalytic nature of enzymes dates back to ancient times. Enzyme technology truly began in 1874 when Danish chemist Christian Hansen treated dried calves' stomach with saline solution to produce the very first sample of 'Rennet' (Hatti-Kaul, 2002; Demirci *et al.*, 2014). Since then unlocking the secrets of enzymes and their subsequent application has become the central crux of biotechnology. Enzymes are preferred over chemical catalysts for industrial applications due to their high specificity, improved yield, easier product recovery and higher degree of purity, reduced cost of production and most importantly, being safer to the environment (Li *et al.*, 2012). The global enzyme market is increasing exponentially and a recent report estimates it to exceed US\$7.1 billion by the year 2018 (<http://www.bccresearch.com/market-research/biotechnology/enzymes-industrial-applications-bio.030h.html>).

Proteins are ubiquitous that catalyse vital metabolic pathways in plants, animals and microorganisms. Bacterial enzyme systems have several advantages including broad diversity, feasibility of mass culture and easier genetic manipulation for cost-effective production of biotechnologically significant enzymes (Gräslund *et al.*, 2008; Adrio and Demain, 2014). Industries prefer enzymes that suit their specific physicochemical environment e.g. lipases used in the detergent industry should be stable at alkaline pH (Nerurkar *et al.*, 2013), while the high operational temperatures of starch processing industry requires thermostable amylases (Sharma and Satyanarayana, 2012). Feed enzymes including proteases, glucanases,  $\alpha$ -amylases,  $\alpha$ -galactosidases, polygalacturonases, xylanases and phytases constitute a significant part of the enzyme industry with an estimated global turnover of US\$1 billion by 2017 (<http://www.feedinfo.com/console/PageViewer.aspx/page=3803488>). Supplementation of feed enzymes has led to an improved digestibility of nutrients and ultimately improved feed utilization (Adrio and Demain, 2014). In particular, phytases serve to ameliorate availability of inorganic phosphorous and its assimilation in animal feedstuffs (Lei *et al.*, 2013; Joshi and Satyanarayana, 2015).

Phytases have been routinely reported from animals, plants and microorganisms; however, microbial sources such as bacteria, yeast and fungi are preferred for its commercial production

(Pandey *et al.*, 2001; Haefner *et al.*, 2005; Singh and Satyanarayana, 2011). Production of phytases from *Enterobacteriaceae* has been reported from *Citrobacter braakii* (Kim *et al.*, 2003; Cowieson *et al.*, 2014), *Klebsiella* sp. (Mittal *et al.*, 2012), *Yersinia intermedia* (Huang *et al.*, 2006), *E. coli* (Yao *et al.*, 2013), *Shigella* sp. (Roy *et al.*, 2012) and *Enterobacter* sp. (Farouk *et al.*, 2012). General characteristics of these phytases include stability in the acidic pH range and resilience towards proteolytic attack. Despite these and many other reported phytases, the hunt for an 'ideal' phytase is still ongoing in order to find a thermo-acid-stable phytase that can show sustained activity in the presence of proteases in the animal gut.

In this study, microbial isolates were screened, identified and the isolate producing highest titre of phytase was selected for further optimization to improve its production in SmF. Firstly, seven isolates were confirmed to produce phytase using chemically pure but expensive Na-phytate as the substrate of choice. To find a cost-effective source of phytate various agro-residues such as wheat bran, corn cobs, sugarcane bagasse and orange peels were investigated for enhanced production of phytase. Wheat bran, a waste product of the flour milling industry with high phytate content (0.25-1.13%, w/w), was selected as the phytate substrate of choice, since its use achieved the highest titres of phytase amongst isolates. The use of agro-industrial waste residues is an attractive alternative to replace the traditional, expensive culture medium, greatly reducing production costs while increasing the applications of phytase through better affordability (Vassileva *et al.*, 2010). Based on the 16S rRNA sequence, the phytase-positive isolates belonged to genus *Bacillus* or *Enterobacter* and the highest producing strain for phytase was designated as *Enterobacter* sp. ACSS. The genus *Enterobacter* accommodates a number of species of heterotrophic, Gram-negative, rod-shaped, non-spore forming, facultative-anaerobic bacteria. *Enterobacter* sp. is widely found in the natural environments (Grimont and Grimont, 2006) and it is known for the production of valuable enzymes, chemicals and other biomolecules (Kanchana *et al.*, 2012; Yoon *et al.*, 1996; Hussin *et al.*, 2010; Beniwal *et al.*, 2013 and Manter *et al.*, 2011).

The next part of this work was primarily focused on enhancing phytase production by *Enterobacter* sp. ACSS. The advantages of statistical approaches using mathematical models are easily highlighted over OFAT experiments for efficient optimization of culture conditions using

minimum resources and saving time. Using PBD and RSM, significant variables are identified and their interactions are studied for maximum production (Dette *et al.*, 2005; Lu *et al.*, 2012). Using the OFAT approach a maximum of 18.14 U/ml was produced by *Enterobacter* sp. ACSS, while by applying PBD and RSM, a further 4.59-fold improvement in phytase production was achieved. Reported process parameters for phytase production using statistical design vary since the nutritive and physical requirements for individual microbes are entirely different. In this study, the interactions between four significant variables [wheat bran, peptone, inoculum size and  $(\text{NH}_4)_2\text{SO}_4$ ] were investigated and their optimum levels were determined. The fold improvement in this investigation was comparable to statistical optimization studies with *Klebsiella* sp. DB3 (Mittal *et al.*, 2011) and *Aspergillus niger* FS3 (Spier *et al.*, 2011) where 5.25- and 4.3-fold improvement was achieved, and lower than *E. sakazakii* (Hussin *et al.*, 2012) and *S. cerevisiae* (Ries *et al.*, 2011) where the authors reported 6- and 10-fold increase in phytase production, respectively. *Thermomyces lanuginosus* produced enhanced phytase due to RSM optimization of four significant factors (initial pH, age of seeding culture, aeration and culture temperature) in medium containing rice bran (Berikten and Kivanc, 2014). Similarly, yeast extract, agitation and  $(\text{NH}_4)_2\text{SO}_4$  were optimized using PBD and Box-Behnken design for enhanced phytase production by *Bacillus subtilis* 168 (Farhat *et al.*, 2012).

Fed-batch fermentation served to increase phytase production by prolonging the exponential phase due to optimization for feeding parameters. The strategic addition of feed into the bioreactor at 12 h intervals proved to be most effective to keep the culture in the mid-exponential phase. Decrease in phytase synthesis due to excess carbon source (glucose; >20 g/l) was similar to that of the earlier report in *Burkholderia* sp. strain a13 (Graminho *et al.*, 2015). Repression of phytase was also observed by Tang *et al.* (2009) when an excess of crude glycerol triggered a sharp decline in phytase production by *Pichia pastoris*. Phytase titres produced by *Enterobacter* sp. ACSS during fed-batch fermentation was lower than that produced by *Pichia pastoris* (1125 U/mL) [Tang *et al.*, 2009] and the recombinant phytase from *Bacillus* sp. MD2 expressed in *E. coli* (327 U/ml) [Tran *et al.*, 2012]; however, the production levels in this study are higher than that of *B. subtilis* phytase (47 U/ml) [Vuolanto *et al.*, 2001] and *E. coli* phytase (13 U/ml) [Arndt *et al.*, 2005].

Phytase from *Enterobacter* sp. ACSS was purified to 14.22-fold with 3.79% yield using three-step purification that included ammonium sulphate precipitation, ion-exchange and gel-filtration. The purification fold and yield was less as compared to the recently reported bacterial phytases from *B. licheniformis* PFBL-03, where 39-fold purification was achieved with an enzyme yield of 10% (Fasimoye *et al.*, 2014) and from *B. nealsonii* ZJ0702, which was purified to 44-fold with a yield of 5.7% (Yu and Chen, 2013). The low fold improvement in purification and low yield in this investigation may be explained on the basis of its low stability at pH 8.0 used in the elution buffer. Attempts to elute the protein under acidic pH range and by using several cation and anion exchange matrices failed, may be because of a low matrix-protein interaction under these conditions, and most of the activity was detected in the flow-through fractions. However, this 62 kDa phytase is unique as compared to the previously reported 43 kDa phytase from *E. sakazakii* by Farouk *et al* (2012) in terms of affinity to Na-phytate ( $K_m$  is 0.26 mM as compared to 0.76 mM of *E. sakazakii* phytase), thermal (60-80°C as compared to 45-55°C ) and pH stability (2.5 as compared to 4.5).

Most reported phytase producers belonging to the family *Enterobacteriaceae* are histidine acid phosphatases and tend to have pH optimum in the acidic range (2.5-6.5) with broad substrate specificity. The acid-stable and thermostable characteristics of phytase from *Enterobacter* sp. ACSS was similar to that of the previously reported HAP phytases from *C. freundii* (Zhao *et al.*, 2010), *Klebsiella* sp. ASR1 (Böhm *et al.*, 2010) and *Yersinia mollaretii* (Shivange *et al.*, 2014), indicating it to be belonging to the same class. The mode of action of HAP-mediated phytate hydrolysis involves a nucleophilic attack on the phosphorous atom by histidine in the active site followed by hydrolysis of the resulting phospho-histidine intermediate that yields *myo*-inositol monophosphate as the final product (Oh *et al.*, 2004; Yao *et al*; 2012; Lei *et al.*, 2013). These bacterial HAPs are preferred over their fungal counterparts due to superior catalytic efficiency, substrate specificity and proteolytic resilience (Oh *et al.*, 2004).

Most animal feed phytases are HAPs that enhance the nutritional value of feed with a concomitant reduction in animal faecal P excretion. Although most of the studies report phytase-mediated nutritional improvement and reduction in external P supplementation, there are few reports that show no change in the nutritional characteristics of monogastric feed after phytase supplementation (Leytem *et al.*, 2008; Iyayi *et al.*, 2013). *Enterobacter* sp. ACSS phytase

successfully released P when mixed with pepsin treated chicken feed, fish feed and wheat bran in separate experiments. Additionally, P was also released from feed in simulated gastric fluid highlighting that this phytase may be used for *in vitro* and *in vivo* applications in the animal feed industry.

To be competitive a phytase must be produced by a reasonably inexpensive system in high yield and purity. Nonetheless, it should be taken into consideration that a single phytase may never possess those characteristics that make it “ideal”. For feed applications, an attractive phytase should be able to withstand the temperatures that are reached temporarily during the pelleting process of 60–90°C given that poultry and pig feed is commonly pelleted. As all known phytases generally unfold at temperatures between 56 and 64°C, increasing the thermostability is hotspot area of research (Yao *et al.*, 2012; Yao *et al.*, 2013). Transgenic plants might contain adequate levels of phytase to avoid the exogenous supplementation of feed and food with microbial phytases and or inorganic phosphate in order to meet their daily need (Rao *et al.*, 2009; Richardson *et al.*, 2009; Gontia *et al.*, 2012). Phytase gene from *Enterobacter* sp. ACSS may also be used to generate transgenic plants.

Collectively, the present study reports a robust phytase from *Enterobacter* sp. ACSS that can tolerate extreme pH and temperature. In addition, the enzyme is fairly stable with pepsin under acidic pH. This is the first report on the use of statistical optimization and fed-batch cultivation production for phytase by the newly isolated *Enterobacter* sp. ACSS which has an immense potential for application in food, feed and biofuel industries.

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## APPENDIX

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### 1. 16S rRNA sequence

1 tgcaagtcga gcggtagcac agagagcttg ctctcgggtg acgagcggcg gacgggagag  
61 taatgtctgg gaaactgcct gatggagggg gataactact ggaaacggta gctaataccg  
121 cataacgtcg caagaccaa gagggggacc ttcgggcctc ttgccatcag atgtgccag  
181 atgggattag ctagtaggtg gggtaacggc tcacctaggc gacgatccct agctggctctg  
241 agaggatgac cagccacact ggaactgaga cacggtccag actcctacgg gaggcagcag  
301 tggggaatat tgcacaatgg gcgcaagcct gatgcagcca tggcgctgt atgaagaagg  
361 ctttcgggtt gtaaagtact ttcagcgggg aggaaggtgt tgttgftaat aaccgcagca  
421 attgacgtta cccgcagaag aagcaccggc taactccgtg ccagcagccg cggtaatacg  
481 gagggtgcaa gcgttaacg gaattactgg gcgtaaagcg cacgcaggcg gctgtcaag  
541 tcggatgtga aatccccggg ctcaacctgg gaactgcatt cgaactggc aggctagagt  
601 cttgtagagg ggggtagaat tccaggtgta gcggtgaaat gcgtagagat ctggaggaat  
661 accggtggcg aaggcggccc cctggacaaa gactgacgt caggtgcgaa agcgtgggga  
721 gcaaacagga ttagataccc tggtagtcca cgccgtaaac gatgtcgact tggaggttgt  
781 gcccttgagg cgtggcttcc ggagctaacg cgttaagtcg accgcctggg gagtacggcc  
841 gcaaggtaa aactcaaatg aattgacggg ggcccgcaca agcgtgggag catgtggtt  
901 aattgatgc aacggaaga acctaccta ctctgacat ccagagaact ttccagagat  
961 ggattgtgc ctcgggaac tctgagacag gtgctgcatg gctgtctga gctcgtgtt  
1021 tgaatatgtg ggtaagtcc cgcaacgagc gcaacccta tccttgtt ccagcgttc  
1081 ggccgggaac tcaaaggaga ctgccagtga taaactggag gaaggtgggg atgacgtcaa  
1141 gtcatcatgg cccttacgag tagggctaca cacgtgtac aatggcgcat acaaagagaa  
1201 gcgacctgc gagagcaagc ggacctcata aagtgcgtcg tagtccgat tggagtctgc  
1261 aactcgactc catgaagtcg gaatcgtag taatcgtaga tcagaatgt acggtgaata  
1321 cgttccccgg cttgttacac accgcccgtc gcaccatggg agtgggttgc aaaagaagta  
1381 ggtagcttaa cctcgggag ggcgctt

### 2. Amino acid cocktail

L- alanine, L-arginine, L-asparagine, L- cystine, L- histidine, L-leucine, L-lysine and L-tyrosine

### 3. Vitamin cocktail

Folic acid, thiamin (B1), riboflavin (B2), pantothenic acid (B5) and vitamin B12

# 2 Production, characteristics and applications of phytase 3 from a rhizosphere isolated *Enterobacter* sp. ACSS

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6 © Springer-Verlag Berlin Heidelberg 2016

7 **Abstract** Optimization of process parameters for phytase  
8 production by *Enterobacter* sp. ACSS led to a 4.6-fold  
9 improvement in submerged fermentation, which was  
10 enhanced further in fed-batch fermentation. The purified  
11 62 kDa monomeric phytase was optimally active at pH 2.5  
12 and 60 °C and retained activity over a wide range of tem-  
13 perature (40–80 °C) and pH (2.0–6.0) with a half-life of  
14 11.3 min at 80 °C. The kinetic parameters  $K_m$ ,  $V_{max}$ ,  $K_{cat}$ ,  
15 and  $K_{cat}/K_m$  of the pure phytase were 0.21 mM, 131.58  
16 nmol mg<sup>-1</sup> s<sup>-1</sup>,  $1.64 \times 10^3$  s<sup>-1</sup>, and  $7.81 \times 10^6$  M<sup>-1</sup> s<sup>-1</sup>,  
17 respectively. The enzyme was fairly stable in the presence of  
18 pepsin under physiological conditions. It was stimulated by  
19 Ca<sup>+2</sup>, Mg<sup>+2</sup> and Mn<sup>+2</sup>, but inhibited by Zn<sup>+2</sup>, Cu<sup>+2</sup>, Fe<sup>+2</sup>,  
20 Pb<sup>+2</sup>, Ba<sup>+2</sup> and surfactants. The enzyme can be applied in  
21 dephytinizing animal feeds, and the baking industry.

23 **Keywords** *Enterobacter* sp. ACSS · Phytase · Thermo-  
24 acid-stable · Feed · Fed-batch

## 25 Introduction

26 Phosphorus (P) is a key mineral element vital for the  
27 growth and development of living organisms. It is required  
28 for the formation of essential building blocks of life and

instrumental in various metabolic pathways including 29  
glycolysis, gluconeogenesis and cellular signal transduc- 30  
tion [1]. It is primarily stored in the form of phytic acid 31  
(PA) or phytate (myo-inositol 1, 2, 3, 4, 5, 6-hexakispho- 32  
sphate, IP6) in cereals, legumes, oil seeds and nuts, where 33  
it constitutes 3–5 % of the dry weight [2] representing 34  
50–80 % of the stored P [3]. PA has been globally recog- 35  
nized as an anti-nutrient due to its poly-anionic nature that 36  
leads to complex formation with divalent metal cations, 37  
proteins, starch and lipids, and thereby interfering with 38  
their absorption in the gastro-intestinal (GI) tract. Fur- 39  
thermore, the undigested PA is discharged into the envi- 40  
ronment and contributes to phosphorus eutrophication of 41  
water bodies [4]. 42

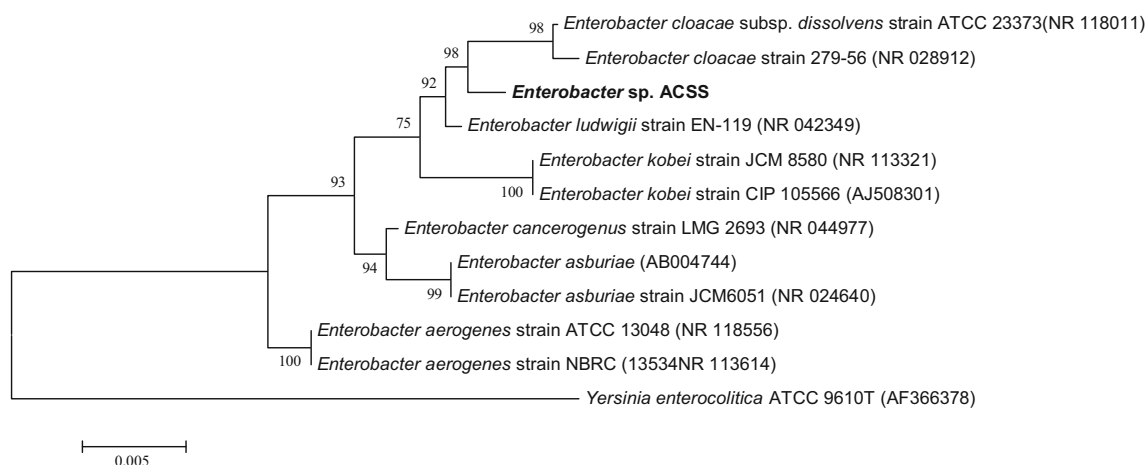
Ruminant animals digest phytic acid with the help of 43  
microbial phytases produced by the rumen microflora. 44  
Monogastric animals such as pigs, poultry and fishes are 45  
deficient in phytases, therefore, poorly digest phytate 46  
phosphorus. This is a complex two-pronged problem as 47  
phosphate is essential for animal growth and development, 48  
which is excreted in the form of undigested phytate that 49  
leads to environmental pollution. This problem is effec- 50  
tively solved by the supplementation of feed with phytate- 51  
degrading enzymes. 52

Phytase (myo-inositolhexakisphosphate phosphohydro- 53  
lase) degrades phytates to release phosphate and other 54  
essential ions. It is widespread in nature, found in plants, 55  
animals and microorganisms. The use of phytase reduces P 56  
excretion in monogastric animals and avoids supplementa- 57  
tion of feeds with inorganic phosphate. Animal stomach 58  
is the main functional site for phytase-mediated dephos- 59  
phorylation in the GI tract. Therefore, an ideal commercial 60  
phytase should be active and stable under acidic conditions 61  
prevailing in the gastric environment, and should be 62  
resistant to proteolytic enzymes like pepsin and trypsin. 63

A1 **Electronic supplementary material** The online version of this  
A2 article (doi:10.1007/s00449-016-1632-7) contains supplementary  
A3 material, which is available to authorized users.

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**Fig. 1** A neighbor-joining tree showing the phylogenetic relationships of *Enterobacter* sp. ACSS, with type strains of *Enterobacter* species. *Yersinia enterocolitica* ATCC 9610T (AF366378) was used as an outgroup. Bootstrap values are expressed as percentages of 1000 replications and shown at the branch points

Despite several reports on phytases, the search for an ideal phytase is still continuing that can effectively hydrolyse phytate in the upper digestive tract (acidic) of monogastric animals and could be resilient at higher temperatures in feed pelleting.

The present investigation highlights the optimization of phytase production by *Enterobacter* sp. ACSS as well as its purification, characteristics and potential applications.

## Materials and methods

### Isolation and identification of the bacterial strain

Bacterial isolates, retrieved from soil samples from different locations around Durban (South Africa), were screened for phytase production on modified solid phytase screening medium (PSM) [5] [g/l: Na-phytate (Sigma), 1; glucose, 15;  $\text{NH}_4\text{NO}_3$ , 2; KCl, 0.5;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5;  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 0.3;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.3 and agar, 15; pH 6.5]. To avoid false positives due to microbial acid production, the plates were also treated with 20 g/l aqueous cobalt chloride solution. The plates were incubated at room temperature for 5 min, decanted, and then flooded with a freshly prepared solution containing equal volumes of a 62.5 g/l aqueous ammonium molybdate solution and 4.2 g/l ammonium vanadate solution. The isolate obtained from the plant rhizosphere soil which showed a larger phytate-hydrolysis zone was selected and identified as *Enterobacter* sp. ACSS (Fig. 1), based on a polyphasic approach. The 16S rDNA sequence was deposited in the NCBI GenBank nucleotide sequence database under the accession number KP814680. Stock cultures of the bacterium were maintained on refrigerated nutrient agar slants at 4 °C with

periodic revival and also preserved as glycerol stocks at −70 °C.

### Enzyme preparation and phytase assay

The seed culture was prepared by submerged cultivation of the bacterium in Luria–Bertani broth at 37 °C at 200 rpm for 12 h. Phytase was produced by inoculating 2 % (CFU  $\approx 1.5 \times 10^8$ ) of the seed culture in Erlenmeyer flasks (250 ml) containing 50 ml of PSM broth [g/l: Na-phytate (Sigma), 1; glucose, 15;  $\text{NH}_4\text{NO}_3$ , 2; KCl, 0.5;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5;  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 0.3 and  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.3; pH 6.5]. Unless otherwise indicated, the fermentation was conducted at 37 °C for 48 h at 200 rpm. The cells were harvested by centrifugation at 8800×g for 20 min and the cell-free supernatant was used to assay phytase by determining inorganic phosphate (Pi) according to the method as described by Heinonen and Lahiti [6]. One unit of phytase activity is defined as the amount of enzyme that liberate 1 nmol of Pi per second under standard assay conditions [3, 7]. The protein concentration was determined using Bradford reagent (Bio-Rad) [8] with bovine serum albumin as the standard.

### Optimization of process parameters

A variety of carbon and nitrogen sources, inorganic salts, and cultivation parameters like inoculum levels, incubation temperatures, incubation periods, agitation rates, initial pH values and others that may affect the production of phytase were optimized by the conventional ‘one-variable-at-a-time’ approach. The variables that significantly affected phytase production were identified, and these were further optimized by the statistical software package ‘Design Expert’ (Version 6.0, Stat-Ease Inc., Minneapolis, USA).

## 124 Plackett Burman methodology

125 Two-level Plackett-Burman design (PBD) is an efficient  
126 technique to select significant process parameters by  
127 screening  $n$  variables in  $n + 1$  experiments. A total of 20  
128 trials with 17 variables and 2 controls or unassigned vari-  
129 ables were screened in the present design (Supplementary  
130 Table 1), which included a variety of carbon and nitrogen  
131 sources, inorganic salts, and cultivation parameters. The  
132 principal effect of each variable was estimated as the dif-  
133 ference between the average of the measurements made at  
134 the high level (+1) of that factor and the average of the  
135 measurements made at the low level (−1) of that factor,  
136 which was determined by the following equation:

$$E(X_i) = 2(\Sigma C_{i+} - \Sigma C_{i-})/N$$

138 where  $E(X_i)$  is the concentration effect of the tested vari-  
139 able.  $C_{i+}$  and  $C_{i-}$  are the phytase activities from the trials  
140 where the variable ( $X_i$ ) under study was present at high and  
141 low concentrations, respectively, and  $N$  is the number of  
142 experiments. The significance of each variable ( $p$  value)  
143 was determined via Student's  $t$ -test.

## 144 Path of steepest ascent

145 Experiments were carried out along the steep path that  
146 initiated from the origin, as the center of the Plackett-  
147 Burman design, to move rapidly towards the vicinity of the  
148 optimum conditions. Experiments were designed by  
149 increasing or decreasing the concentrations of variables  
150 based on the PBD results until no further increase in  
151 response was observed. The experimental design and  
152 results of the steepest ascent method are shown in Sup-  
153plementary Table 2.

## 154 Response surface methodology using central 155 composite design (CCD)

156 Four significant parameters (wheat bran, peptone, ammo-  
157 nium sulfate and inoculum size), identified by Plackett-  
158 Burman design, were chosen as independent variables.  
159 Each factor was studied at five different levels (− $\alpha$ , −1, 0,  
160 +1, + $\alpha$ ) as per CCD, with a total of 30 experiments and the  
161 experimental design is shown in Supplementary Table 3a.  
162 The behavior of the system was explained by the following  
163 second order polynomial equation:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD + \beta_{34} CD$$

165 where  $Y$  is predicted value of response,  $\beta_0$  is intercept,  $\beta_1$ ,  
166  $\beta_2$ ,  $\beta_3$ ,  $\beta_4$  are linear coefficients,  $\beta_{11}$ ,  $\beta_{22}$ ,  $\beta_{33}$ ,  $\beta_{44}$  are  
167 squared coefficients,  $\beta_{12}$ ,  $\beta_{13}$ ,  $\beta_{23}$ ,  $\beta_{24}$ ,  $\beta_{34}$  are interaction

coefficients and  $A$ ,  $B$ ,  $C$ ,  $A^2$ ,  $B^2$ ,  $C^2$ ,  $D^2$ ,  $AB$ ,  $AC$ ,  $BC$ ,  $BD$ ,  
169  $CD$  are independent variables.

The conditions predicted by the statistical model was  
170 validated with respect to phytase production in 0.25–2.0 l  
171 shake-flasks, and also in 1 l (Multifors) and 5 l (Minifors)  
172 lab fermenters (Infors, Bottmingen-Basel, Switzerland).  
173 The fermenters were operated at 40 °C, 200 rpm with  
174 1 vvm of aeration. Samples were drawn at the desired  
175 intervals, harvested and cell-free supernatants were used in  
176 phytase assays.

## Batch and fed-batch production

178 Batch and fed-batch fermentations were conducted in a 5 l  
179 glass fermenter (Minifors, Infors HT, Switzerland) con-  
180 taining 2.5 l of optimized medium with 4.14 % (v/v)  
181 inoculum at 37 °C. Dissolved oxygen (DO) in the fer-  
182 menter was maintained at 30 % air saturation using aera-  
183 tion in cascade mode controlling the agitation speed  
184 (maximum, 700 rpm) and airflow. Batch fermentation was  
185 carried out for 36 h, while fed-batch fermentation contin-  
186 ued up to 96 h. Feeding was done at every 12 h with  
187 concentrated (10 $\times$ ) optimized medium to maintain the total  
188 reducing sugar concentration under 20 g/l. However, in  
189 order to reduce the clogging of feed-pipe due to wheat  
190 bran, its concentration was kept at 16.2 g/l in the feed.

## Purification, PAGE and zymography

192 The crude extract (2 l) was subjected to 55–85 %  
193 ammonium sulfate precipitation and the precipitate was  
194 suspended in 0.1 M sodium acetate buffer (pH 5.5). It  
195 was desalted through Hi-Prep<sup>TM</sup> 26/10 column (GE  
196 Healthcare, Sweden) against 20 mM sodium acetate buf-  
197 fer (pH 5.5) using an AKTA purifier system (GE  
198 Healthcare, Sweden). The concentrated and desalted  
199 sample was loaded onto a HiTrap<sup>TM</sup> DEAE FF (GE  
200 Healthcare, Sweden) anion-exchange column previously  
201 equilibrated with 20 mM Tris–HCl buffer (pH 8.0). The  
202 column was eluted with a linear gradient of 0–1 M NaCl  
203 in 20 mM Tris–HCl buffer (pH 8.0) at a flow rate of  
204 1 ml/min. Active fractions were pooled and applied to  
205 Superdex<sup>TM</sup> 200 increase 10/300 column (GE Healthcare,  
206 Sweden), and eluted with 50 mM sodium acetate buffer  
207 (pH 5.5) at 0.75 ml/min. Elution of the protein was  
208 monitored at 280 nm. The active fractions were then  
209 pooled and concentrated using Biomax Mr. 10,000 cut-off  
210 Millipore membrane (Millipore, USA).

212 SDS–PAGE was carried out on a Mini PROTEAN gel  
213 electrophoresis unit (BioRad Laboratories, USA) according  
214 to Laemmli [9] using 12 % cross-linked polyacrylamide  
215 gel. For zymography, the samples were electrophoresed on  
216 12 % native-PAGE and activity stained according to the

method of Bae et al. [10]. A clear zone of hydrolysis confirmed phytase activity.

## Enzyme characterization

### Effect of pH on activity and stability

The effect of pH on phytase activity was studied over a pH range between 1.0 and 11.0 in different buffer systems [HCl-KCl buffer (1.0–2.0), Citrate buffer (pH 3.0), Na-acetate buffer (pH 4.0–5.0), Citrate buffer (pH 6.0) Tris-HCl buffer (pH 7.0–8.0) and glycine-NaOH (9.0–11.0)]. The pH stability of the enzyme was determined by pre-incubation of phytase with buffers of different pH and the residual enzyme activity was determined at an interval of 30 min up to a period of 6 h under standard assay conditions.

### Effect of temperature on activity and stability

The effect of temperature on phytase activity was assessed over a temperature range of 30–90 °C. The thermostability of the enzyme was determined by pre-incubation at different temperatures and determining the residual enzyme activity at 30-min intervals up to a period of 6 h.

### Substrate specificity and kinetic parameters

The substrate specificity of purified phytase was tested against Na-phytate, p-nitrophenyl phosphate, sodium pyrophosphate, glucose-1-phosphate, glucose-6-phosphate and ATP by incubating the enzyme separately with the substrates (5 mM) and measuring the residual phytase activity. Enzyme without any agent acted as control while activity with Na-phytate was regarded as 100 %. The  $K_m$  and  $V_{max}$  of phytase was determined using Lineweaver-Burk plot and by applying the Michealis-Menten equation (Eq. 1) using different concentrations (0.125–2 mM) of substrates. The catalytic constant ( $K_{cat}$ ) was determined from the  $V_{max}$  recorded for each substrate by applying Eq. (2) while catalytic efficiency was calculated by applying Eq. (3).

$$\frac{1}{V_0} = \left( \frac{K_m}{V_{max}} \right) \left( \frac{1}{[S]} \right) + \frac{1}{V_{max}} \quad (1)$$

$$K_{cat} = \frac{V_{max}}{[E]_T} \quad (2)$$

$$\text{Catalytic efficiency} = \frac{K_{cat}}{K_m} \quad (3)$$

where  $V_0$  is the initial velocity,  $V_{max}$  is the maximum velocity,  $K_m$  is the Michaelis-Menten constant and  $[E]_T$  is the total enzyme concentration.

## Effect of metal ions, surfactants and reducing agents

The effect of metal ions and surfactants was studied by pre-incubating the enzyme for 30 min at 37 °C, 100 rpm with 1 and 5 mM concentration of various metal ions including  $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ ,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ,  $\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$ ,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ ,  $\text{PbCl}_2$  and  $\text{ZnCl}_2$ , and 0.1–1.0 mM concentration of non-ionic (triton X-100, tween-20, tween-80), anionic (SDS) and cationic (CTAB) surfactants. In addition, the effect of 1 and 5 mM concentration of EDTA, and DTT was also investigated. Enzyme without any agent served as the control. The residual enzyme activities were thereafter determined.

## Applications of phytase

### Phosphate liberation from animal feed and simulated gastric fluid (SGF)

Finley milled wheat bran, chicken feed and fish feed were autoclaved (121 psi, 15 min) and 2 g (dry weight) of each was suspended in 50 ml of 0.1 M acetate buffer (pH 5.5). Phytase (50 U) was added to each suspension and incubated at 37 and 50 °C. Aliquots (1 ml) were removed at desired time intervals and centrifuged at  $10,620 \times g$  for 2 min at 4 °C. The supernatants were collected for estimating Pi liberated as described earlier [7]. Pi released from animal feed was determined by adding 1 g of feed-stock (wheat bran, chicken feed and fish feed) to 9 ml simulated gastric fluid (SGF) (250 mM Glycine-HCl containing 2.0 mg/ml NaCl and 3.2 mg/ml pepsin). The pH was adjusted with HCl or NaOH to 1.5, 2.0, 2.5, 3.5, 5.5, or 6.5 as required. Phytase (10 U) was added to pre-incubated solutions (37 °C, 30 min) and incubated with agitation at 37 °C for 60 min. The amount of released phosphorus was determined using standard phytase assay [11].

### Hydrolysis of insoluble metal-phytates

Hydrolysis of insoluble metal-phytates was studied according to the method as described by Sapna and Singh [12]. Briefly, stock solutions of metals ions ( $\text{Ca}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$  and  $\text{Zn}^{2+}$ , 100 mM each) were prepared by dissolving  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ,  $\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$ ,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  and  $\text{ZnCl}_2 \cdot 7\text{H}_2\text{O}$  in Milli-Q water. Equal volumes of 100 mM salt solution and 10 mM sodium phytate were mixed and incubated overnight at 4 °C for precipitation. The precipitated salts were separated next day by centrifugation at  $425 \times g$  at 4 °C for 1 min. The pellets were washed thrice with Milli-Q water followed by re-suspension in 0.5 ml of 0.1 M Na-acetate buffer (pH 5.5). The metal-phytates were hydrolysed by incubating with 10 U of phytase at 50 °C for 12 h and aliquots were taken

every 3 h to determine the amount of Pi in the supernatant after centrifugation. The substrate and enzyme controls were also run simultaneously and values were deducted from the test value [12].

### Application in improvement of bread making performance

Basic bread dough was prepared by mechanical mixing of brown bread flour (400 g) with dry yeast (8 g), sugar (15 g), ascorbic acid (3.75 mg), water (275 ml), fat (10 g) and NaCl (5 g) for 5 min. Phytase (0.2, 0.6 and 1.0 U/g) was added prior to mixing of separate bread doughs, while the controlled experiment lacked this enzyme. It was left for proofing for 45 min, followed by baking at 25 °C for 25 min. The breads were then assessed for soluble Pi. Shelf life of the bread was estimated by Weibull hazard method [13].

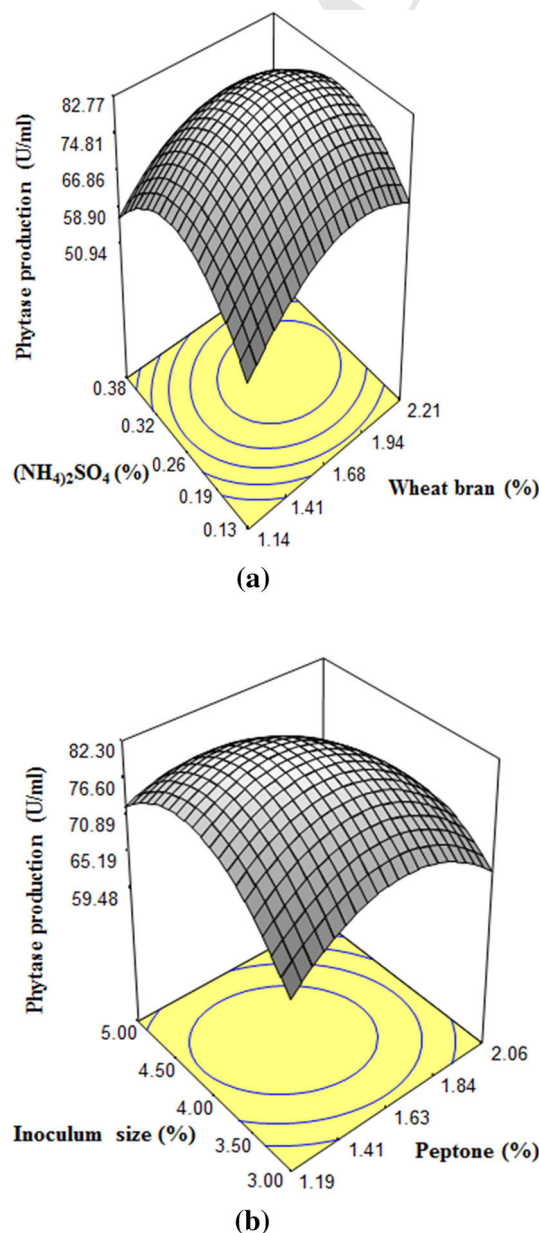
## Results and discussion

Plant rhizosphere is a highly dynamic environment that harbors diverse microorganisms actively involved in nutrient cycling. Isolation of a high phytate-hydrolysing bacterium in this study, from the rhizosphere of sugarcane indicates its possible involvement in mobilization of P from recalcitrant phytate molecules in soil. The bacterium is Gram-negative, rod-shaped, non-spore forming facultative anaerobe that grows optimally at 37 °C in Luria-Bertani broth. The carbohydrate utilization profile on Omnilog-Biolog identification system (Biolog, Inc, CA) indicated it to be a species of *Enterobacter* which was further confirmed by 16S rDNA sequence analysis. Interestingly, though the 16S rDNA sequence of *Enterobacter* sp. ACSS displayed high identity with *E. cloacae* and *E. ludwigii* strains, it formed outgroup in the phylogenetic tree (Fig. 1) indicating its unique characteristics.

Response of the PBD (Supplementary Table 1a) was observed with high variability in phytase production (0.41–29.81 U/ml), indicating the importance of optimization by statistical approaches. Five variables (wheat bran, peptone, tween-20, ammonium sulfate and inoculum size) were identified as the most significant factors ( $p < 0.05$ ) out of 17 nutritional and cultural variables (Supplementary Table 1b). However, we continued with wheat bran, peptone, ammonium sulfate and inoculum size for further optimization. Although tween-20 has a marked effect on phytase production but it was omitted as it shows interference during purification steps. The production of phytase is reported to be inducible or constitutive [14]. In the present study, the positive signal of wheat bran indicated that phytase production by *Enterobacter* sp. ACSS was induced in the presence of phytate containing substrates. Wheat bran has a high phytate content [15, 16];

therefore, it may act as an inducer for enhanced production of phytase by the bacterium. The enzyme production was further improved to  $55.68 \pm 3.06$  U/ml using path of steepest ascent/descent (Supplementary Table 2).

RSM using CCD was applied to determine the optimal levels of the four selected variables and their interaction was studied for enhanced phytase production. The results of CCD experiments are presented along with the mean predicted and observed responses (Supplementary Table 3a). It was analyzed by standard ANOVA (Supplementary Table 3b).



**Fig. 2** Three dimensional response surface graph showing interaction between **a** wheat bran and ammonium sulfate and **b** peptone and inoculum size

The model  $F$  values of 1550.42 and 'Prob >  $F$  value' of <0.0001 implied the model was highly significant. There was only a 0.01 % chance that the model  $F$  value could occur due to noise. All four linear coefficients ( $A$ ,  $B$ ,  $C$  and  $D$ ) and the quadric terms ( $A^2$ ,  $B^2$ ,  $C^2$  and  $D^2$ ) were significant for phytase production. The interaction of  $A$  with  $C$  and  $D$ , and  $D$  with  $B$  and  $C$  were significant model terms.

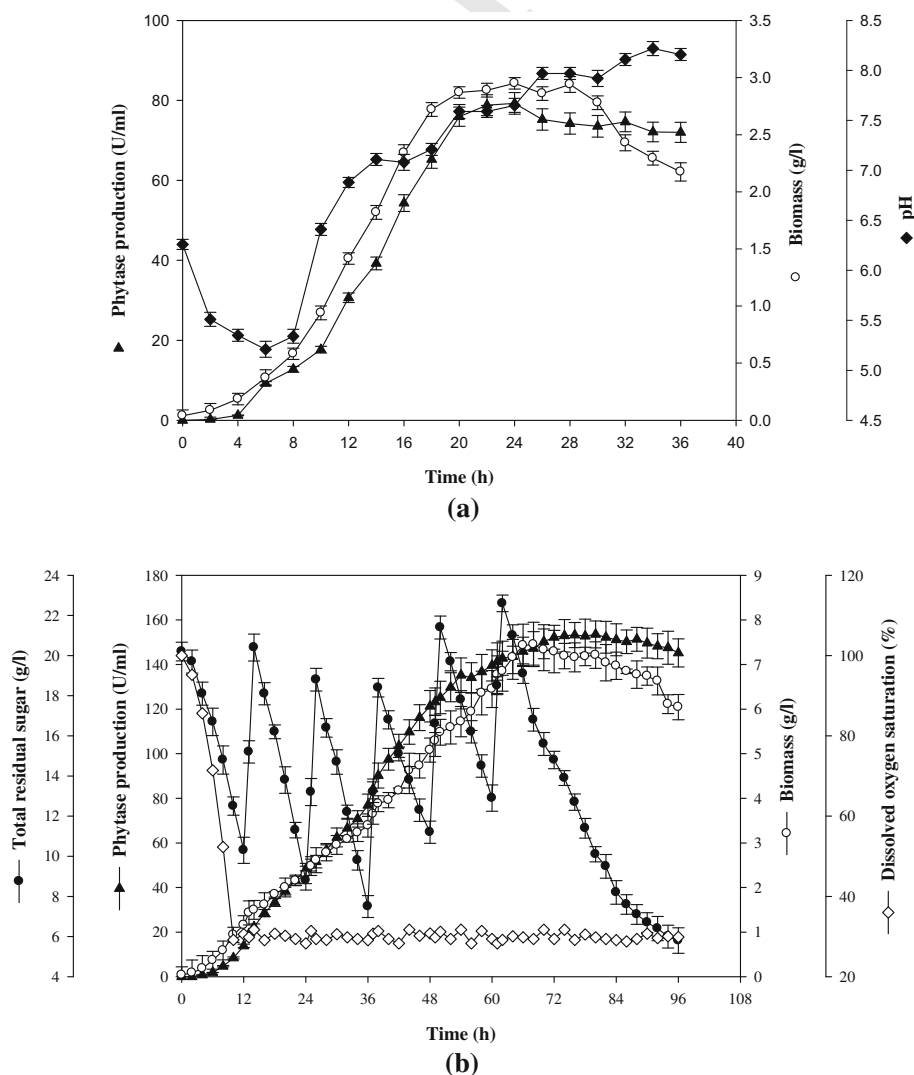
Response surfaces with contour plots (at the base) were generated to represent the interaction of two variables while keeping another variable fixed at '0' level. Figure 2 presents the three dimensional response surface curves established for phytase production in this study. Figure 2a shows increase in phytase production with increase in the wheat bran and ammonium sulfate concentration up to 18.3 and 2.6 g/l, respectively. Any further increase in these two variables repressed the enzyme production. Similarly, increase in peptone concentration up to 15.5 g/l had a favorable impact on phytase production (Fig. 2b), and thus, 15.5 g/l peptone and inoculum size of 4.14 % were optimal

for maximum phytase production. Peptone was also shown to be a critical factor for the production of biohydrogen using *Enterobacter* sp. MTCC 7104 [17].

The experimental model and its predictions were validated for all four variables within the design space by five random sets of experimental combinations as suggested by Design Expert software. The predicted response for phytase production was in good agreement with the actual response, confirming the suitability of the model. Use of statistical models to improve enzyme production is a routine practice and it has been used to enhance the production of phytase from several microorganisms [3, 4, 11].

There was a slight decrease in the batch production levels (79.2 U/ml) in the fermenter as compared to shake flasks (83.2 U/ml) but the fermentation time was reduced by 4 h. Phytase production was growth-dependent and after 24 h phytase as well as biomass production declined during batch cultivation (Fig. 3a). Fed-batch fermentation was carried out to achieve higher biomass for improved and

**Fig. 3** Production of phytase by *Enterobacter* sp. ACSS in a 5 l laboratory fermenter under a batch and b fed-batch conditions

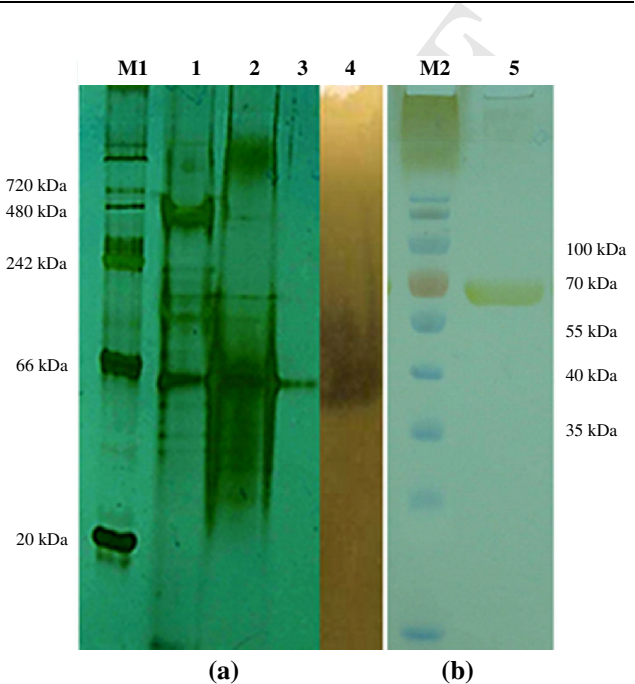


**Table 1** Summary of purification steps for *Enterobacter* sp. ACSS phytase

Purification Steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Fold purification
Crude filtrate	41,156	726	56.68	100	1
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> precipitation	25,804.80	309.7	83.32	62.69	1.47
Anion exchange (DEAE FF)	6757.32	24.73	273.24	16.41	4.82
Gel filtration (Superdex™)	1559.81	1.93	805.98	3.79	14.22

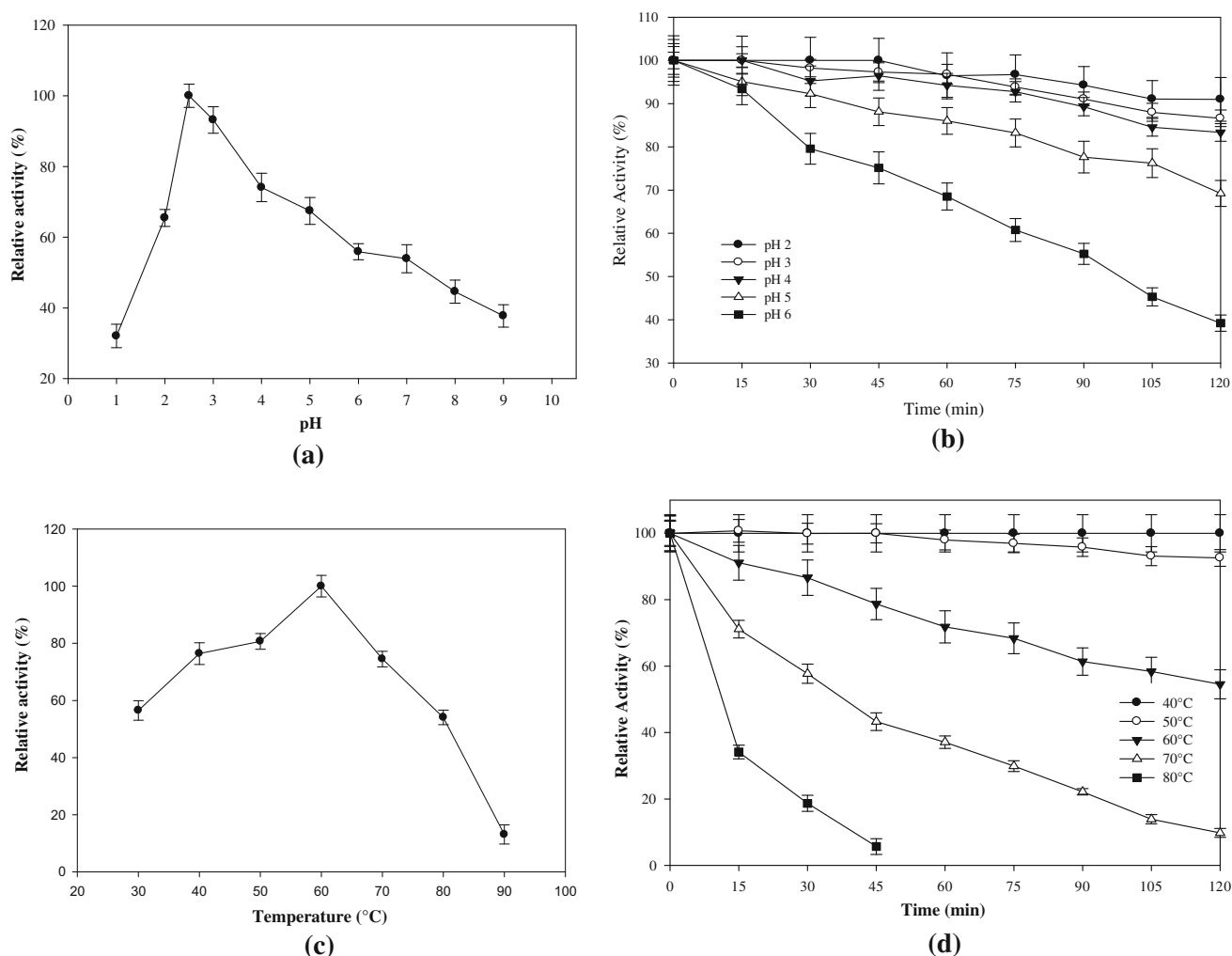
sustained production of phytase. Time course profiles of phytase production, biomass and total residual sugar during fed-batch fermentation are shown in Fig. 3b. The total reducing sugar was utilized fast and 51 % was consumed during the first 12 h (exponential phase). Its concentration at the time of converting batch to fed-batch was 10.3 g/l, and at the end of fed-batch fermentation 5.8 g/l of reducing sugar was left in the medium. Based on optimization of feeding time (data not shown) the culture was fed after every 12 h, which maintained the bacterium in its logarithmic phase until 64 h and the biomass improved by 2.6-fold with a concomitant 1.9-fold improvement in overall phytase production (153.2 U/ml) as compared to submerged production in batch mode. Several studies have confirmed enhancement in enzyme production due to fed-batch cultivations; however, most of the fed-batch studies pertaining to the use of *Enterobacter* family of bacteria have been focused on exopolysaccharides [18], polyglucosamine [19] and 2, 3-butanediol [20] though *Enterobacter* sp. are known to be potential producers of several industrial enzymes. Kleist et al. [21] attained high phytase levels (120 U/ml) using rapid glucose controlling system for fed-batch cultivation of *E. coli*, while Verma and Satyanarayana [22] achieved 47.33 and 36.91 U/ml cell-bound phytase from *Pichia anomala* using cyclic fed-batch and fixed volume fed-batch strategies. The production of phytase by *Aspergillus ficuum* was enhanced by 11 and 40 % due to the addition of feed containing glucose and Na-phytate, respectively [23]. To the best of our knowledge, this is the first report on fed-batch production of an industrially significant enzyme by any *Enterobacter* sp.

Phytase from *Enterobacter* sp. was purified to homogeneity, with an overall purification of 14.22-fold and a yield of 3.79 % (Table 1). The purified enzyme (Fig. 4) has an apparent molecular mass of 62 kDa on SDS-PAGE gel (Fig. 4b) and appears to migrate as a monomer during Superdex™ gel filtration chromatography (data not shown). The enzyme is active over a broad range of acidic pH and temperature, and exhibited remarkable stability. The enzyme was optimally active at pH 2.5 (Fig. 5a) and fairly stable at pH 2 (Fig. 5b), retaining complete activity for 120 min. At pH 3 and pH 4 it lost only 10 % of activity after 120 min. Half-life of the enzyme at pH 2.0, 3.0, 4.0, 5.0 and 6.0 was 577.5, 433.1, 770.0, 247.5 and 91.2 min,



**Fig. 4** Purification of phytase from *Enterobacter* sp. ACSS. **a** Native-PAGE and zymogram analysis of the phytase, *M1* native protein molecular weight marker, *lane 1* crude extract, *lane 2* the sample after HiTrap DEAE anion-exchange column chromatography, *lane 3* the sample after Superdex™ gel filtration chromatography, *lane 4* zymogram analysis and **b** SDS-PAGE of the purified phytase, *M2* protein molecular weight marker, *lane 1* purified phytase

respectively. As the pH approached neutrality, stability decreased at a faster rate. A loss of 60 % of activity was observed at pH 6 and a complete loss of activity was observed after 240 min. Thermo-acido-philic enzymes are of major commercial interest. Although members of the family Enterobacteriaceae prefer to grow in neutral pH, they are known to survive the low-pH stress during passage through the stomach and in the fermented and acidified fecal material [24]. The role of phosphate transport systems in acidic habitats [25] and survival of *Escherichia coli* in seawater [26] has been already established. Acid-stable phytase (phyC) from *Citrobacter freundii* showed two distinct pH optima at 2.5 and 4.5 with an optimal temperature at 50 °C [27]. The phytase from *Enterobacter* sp. ACSS was active over a temperature range of 30–80 °C, with optimal activity at 60 °C (Fig. 5c). The relative activities at 60 and 70 °C were 73 and 39 %, respectively.



**Fig. 5** Effect of pH and temperature on the activity and stability of phytase. **a** Effect of pH on enzyme activity, **b** effect of pH on enzyme stability, **c** effect of temperature on enzyme activity and **d** effect of temperature on enzyme stability

**Table 2** Relative activity and kinetic constants for the hydrolysis of phosphorylated compounds using phytase from *Enterobacter* sp. ACSS

Substrates	Relative activity (%)	$K_m$ (mM)	$V_{max}$ (n mol $mg^{-1}$ $s^{-1}$ )	$K_{cat}$ ( $s^{-1}$ )	$K_{cat}/K_m$ ( $M^{-1}$ $s^{-1}$ )
Sodium phytate	100 $\pm$ 3.41	0.21 $\pm$ 0.01	131.58 $\pm$ 4.49	$1.64 \times 10^3 \pm 62.32$	$7.81 \times 10^6$
<i>p</i> -Nitro phenyl phosphate	46.21 $\pm$ 1.73	1.16 $\pm$ 0.04	12.47 $\pm$ 0.37	$1.55 \times 10^2 \pm 4.96$	$0.13 \times 10^6$
Sodium pyrophosphate	13.63 $\pm$ 0.46	1.38 $\pm$ 0.03	10.95 $\pm$ 0.22	$1.26 \times 10^2 \pm 5.42$	$0.10 \times 10^6$
Glucose-1-phosphate	8.27 $\pm$ 0.28	1.75 $\pm$ 0.04	11.79 $\pm$ 0.27	$1.47 \times 10^2 \pm 4.65$	$0.08 \times 10^6$
Glucose-6-phosphate	7.32 $\pm$ 0.21	1.81 $\pm$ 0.05	11.82 $\pm$ 0.25	$1.47 \times 10^2 \pm 4.61$	$0.08 \times 10^6$

The values are the mean of three independent experiments

The hydrolysis rate of sodium phytate was taken as 100 % for comparison

respectively (Fig. 5d). Half-life of the enzyme at 50, 60, 70 and 80 °C was 866.2, 693.0, 37.8 and 11.3 min, respectively. The activity was lost completely at 80 °C after 55 min incubation. *E. cloacae* produced a thermostable  $\beta$ -D-galactosidase which was optimally active at 50 °C [28], while recombinant sucrose isomerase from *Enterobacter*

sp. FMB1 and lipase from *Enterobacter* sp. Bn12 showed optimal activity at 50° and 60 °C, respectively [29, 30].

*Enterobacter* sp. ACSS phytase acted on sodium phytate as the most preferable substrate with high catalytic efficiency as compared to other tested phosphorylated compounds (Table 2). This observation is consistent with

**Table 3** Effect of metal ions, reducing agents and surfactants at two different concentrations on phytase activity

Additives	1 mM	5 mM
Metal ions		
Ba <sup>2+</sup>	71 ± 4.9	65 ± 3.4
Mn <sup>2+</sup>	110 ± 6.0	104 ± 5.6
Mg <sup>2+</sup>	120 ± 6.3	114 ± 5.7
Pb <sup>2+</sup>	60 ± 3.4	47 ± 3.0
Ca <sup>2+</sup>	143 ± 9.1	120 ± 5.9
Fe <sup>2+</sup>	40 ± 2.2	23 ± 1.4
Co <sup>2+</sup>	73 ± 4.7	56 ± 3.7
Cu <sup>2+</sup>	48 ± 2.7	27 ± 1.3
Zn <sup>2+</sup>	46 ± 3.2	18 ± 1.3
Reducing agents		
EDTA	88 ± 5.9	86 ± 4.1
DTT	73 ± 4.7	42 ± 1.4
Additives	0.1 mM	1 mM
Surfactants		
Triton X-100	72 ± 5.0	63 ± 3.7
Tween-20	78 ± 4.7	64 ± 3.5
Tween-80	81 ± 5.9	74 ± 4.4
SDS	18 ± 1.1	07 ± 0.3
CTAB	72 ± 3.6	64 ± 1.1
Control	100	100

Phytase activity in the absence of additives is regarded as 100 %  
Enzyme activity without any additive served as the control

reports on several other phytases including *K. pneumoniae* 9-3B phytase [2] and *Burkholderia* sp. strain a13 phytase [31]. However, phytases may display narrow [32] to strict [33] substrate specificity. The enzyme in present investigation showed a highest catalytic efficiency ( $K_{cat}/K_m$ ) of  $7.81 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$  with sodium phytate with  $K_m$  and  $V_{max}$  values of 0.21 mM and  $131.58 \text{ nmol mg}^{-1} \text{ s}^{-1}$ , respectively. *E.coli* phytase had a  $K_m$  of 0.43 mM [34] while the calculated  $K_m$  and  $V_{max}$  values for recombinant *Bacillus subtilis* (BSPHyARRMK33) phytase were 0.95 mM and  $15.3 \mu\text{mol l}^{-1}$  [35].

Addition of 1 mM  $\text{Ca}^{2+}$  to the reaction mixture resulted in a marked increase (143 %) of enzyme activity. The activity also increased due to the addition of 1 mM  $\text{Mg}^{2+}$  (120 %) and  $\text{Mn}^{2+}$  (110 %).  $\text{Ca}^{2+}$  and several divalent metal-ions form positively charged metal-phytate complexes and reduce the negative charge around the active site cleft, thereby activating the enzyme. However, when present in excess, the metal-ions may act as competitive inhibitors and can also precipitate phytates as insoluble metal salts [36] as was observed in this investigation when relatively lower activity was noted with higher concentration (5 mM) of metal-ions. Most of the transition metal-

ions including  $\text{Co}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  inhibited the activity of purified phytase (Table 3). Inhibition of phytase due to different metal ions is also dependent on the nature and source of enzyme which demonstrates wide variability among microorganisms [37]. Interestingly, phytase activity from *Lactobacillus plantarum* was significantly activated by  $\text{Fe}^{2+}$  while it was reduced due to addition of  $\text{Cu}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Ca}^{2+}$  [38]. All reducing agents and surfactants tested in this investigation were inhibitory. The anionic surfactant SDS showed a maximum of 82 % inhibition in enzyme activity within 30 min.

While investigating the effect of proteolytic cleavage on enzyme resilience under physiological conditions, it was found to be fairly stable with pepsin and it lost only 10.64 % of the initial activity after 120 min of incubation (Supplementary Table 4). However, lesser stability with trypsin corroborated with the earlier findings of phytase from *Aspergillus oryzae* [12]. Variation in phytase stability to different proteolytic enzymes may be attributed to the difference in protein-protein interactions under different physiological conditions.

Phytase from *Enterobacter* sp. ACSS was successfully used for liberating Pi from animal feeds (Fig. S1a) and insoluble metal-phytates (Fig. S1b). The amount of Pi released was higher in chicken feed due to a higher phytate content (8.2 mg/g) in the bran-soya-corn based broiler feed as compared to the soya-corn based fish feed (5.1 mg/g). Highest Pi release at pH 2.5 and its applicability in a wide pH range (1.5–6.5) is in agreement with the optimum activity and promising acid-stable characteristics of this enzyme. The amount of Pi released is sufficient to overcome the dietary phosphorus requirement of broilers that varies from 4.8 to 5.7 mg/g [39] and most aquaculture-relevant fish species that varies from 4.06 to 6.62 mg/g of diet [40]. Phytase action on Ca-phytate complex released a maximum of  $219.17 \mu\text{g/ml}$  of Pi, which corroborates with its  $\text{Ca}^{+2}$  mediated activation profile. A plausible reason for the  $\text{Ca}^{+2}$  mediated activation of phytases may be the stabilization of the active site cleft by  $\text{Ca}^{+2}$ , as reported previously during studies on alkaline phytase from *Bacillus* sp. MD2 [41] and acidic phytase from *C. freundii* [27]. A marked decline in phytase activity due to  $\text{Fe}^{+2}$  and  $\text{Zn}^{+2}$  and lower hydrolysis of the respective metal-phytate complexes can be attributed to the versatile redox properties of  $\text{Fe}^{+2}$  and to the orientation problems in  $\text{Zn}^{+2}$  and phosphate group in the enzyme cleavage site [42].

Phytase supplementation to the dough increased the liberation of Pi, and improved reducing sugar and soluble protein in the bread (Table 4). The bread was softer with higher moisture content and had an improved shelf-life than the bread without phytase. This investigation also indicates a significant reduction in proofing time from 45 to

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**Table 4** Effect of phytase supplementation in different doses on bread characteristics

Parameters	Control	Phytase (U/g)		
		0.25	0.50	1.00
Mass (g)	587.51 ± 30.05	590.30 ± 29.08	586.50 ± 29.28	586.50 ± 29.17
Dough rise (cm)	3.78 ± 0.22	4.14 ± 0.29	5.22 ± 0.25	5.34 ± 0.25
Proofing time (min)	45.00 ± 2.21	35.00 ± 1.88	30.01 ± 1.23	30.02 ± 1.33
Bread moisture (%)	24.60 ± 1.17	28.50 ± 1.37	33.20 ± 1.58	33.80 ± 1.63
Reducing sugar (mg g <sup>-1</sup> )	23.22 ± 1.05	27.50 ± 1.41	31.23 ± 1.59	32.00 ± 1.68
Soluble protein (mg g <sup>-1</sup> )	2.79 ± 0.11	3.28 ± 0.17	3.74 ± 1.77	3.84 ± 0.22
Pi released (μg/mg)	0.44 ± 0.02	20.93 ± 1.08	20.93 ± 1.07	29.35 ± 1.47
Shelf life (days)	2.00 ± 0.092	3.04 ± 0.18	4.00 ± 0.16	3.99 ± 0.23

35 and 30 min using 0.25 and 0.5 U/g of phytase, respectively, which is in accordance with earlier report [41].

## Conclusions

A robust phytase producing bacterium was isolated from the rhizosphere of sugarcane plant and identified as *Enterobacter* sp. ACSS based on Biolog phenotypic characteristics and 16S rDNA sequence comparison. The purified enzyme is active over a wide range of pH and temperatures. In addition, the enzyme is fairly stable in the presence of pepsin at an acidic pH. This is the first report on the use of statistical optimization and fed-batch cultivation for phytase production by an *Enterobacter* sp., which has an immense potential for application in food and feed industries. Further work is underway on cloning and over expression of the enzyme for cost effective production and understanding its structural characteristics.

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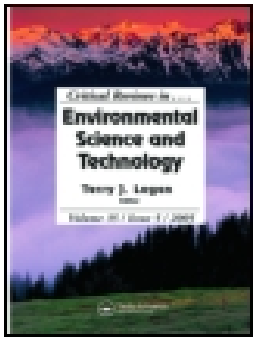
## Compliance with ethical standards

**Conflict of interest** The authors declare that they do not have any conflict of interest.

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Microbial production of phytases for combating environmental phosphate pollution and other  
diverse applications

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## Abstract

Concerns of phosphorus pollution and its impact on environments have driven the biotechnological development of phytases. Phosphoric acid, inositol phosphate, or inositols are produced after hydrolysis of phosphate from phytate, initiated by phytase. Research over the last two decades on microbial phytases has deepened our understanding of their production, optimization, and characterization. Despite the wide availability of phytase producing microorganisms, only a few have been commercially exploited. The current high cost of phytases, inability to withstand high temperatures (>85°C), a limited pH range, and poor storage stability are a major bottleneck in the commercialization of phytases. The development of novel phytases with optimal properties for various applications is a major research

challenge. In this paper, recent advances in microbial phytase production, application of tools to optimize higher enzyme production, and characterization of phytases along with potential biotechnological applications are reviewed. Additionally the development of phytase assay methods and functions of phytate and phytate degradation products are discussed.

#### Keywords

Phytic acid, monogastric animals, antinutrients, fermentation, protein engineering.