# Production, characterization and applications of two distinct phytases from *Thermomyces lanuginosus* SSBP

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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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## 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.**

**Melvin Makolomakwa**

**2018**

## DEDICATION

***This thesis is lovingly dedicated to my mother Mokgabo Makolomakwa, for her unwavering support, encouragement and constant love. She kept me motivated and stood by me when all seemed to fail. Without her, none of this would have been possible.***

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"*MODIMO GAOSITWE*"

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## PUBLICATIONS AND CONFERENCES

### Conferences

1. **Makolomakwa, M**., Puri, A.K., Permaul, K. and Singh, S. Batch and fed-batch production of phytase from *Thermomyces lanuginosus*. 19th Biennial Conference South African Society of Microbiology (SASM), Coastlands Umhlanga Convention Center, Durban, South Africa, 17-20 January 2016.
2. Puri, A. K., **Makolomakwa, M.,** Chanderman, A., Permaul, K. and Singh, S. Applications of thermostable phytases in enzymatic catalysis of phytate. Catatalysis Society of South Africa (CATSA) - 2015 conference. 15 - 18 November 2015.

### Publications

1. **Makolomakwa, M**., Puri, A.K., Permaul, K. and Singh, S., 2017. Thermo-acid-stable phytase-mediated enhancement of bioethanol production using *Colocasia esculenta*. *Bioresource Technology*, *235*, pp.396-404. IF: 5.65
2. Kumar, A., Chanderman, A., **Makolomakwa, M**., Permaul, K. and Singh, S., 2016. Microbial production of phytases for combating environmental phosphate pollution and other diverse applications. *Critical Reviews in Environmental Science and Technology*, *46*(6), pp.556-591. IF: 5.79

## ABSTRACT

Phosphorous (P) is an essential element which is primarily stored as phytic acid in plants and exits as dominating form of organic P in soil. The phytate anion has strong chelation properties and can bind with essential metal ions, proteins, starch and several biomolecules under physiological conditions, and accumulates in soil due to strong binding with soil solid phase. Phytases are enzymes that hydrolyse phytate in stepwise manner to release inositol phosphates and orthophosphate, thereby releasing the chelated molecule.

In this study, we report two phytases (Tlphy1 and Tlphy2) from a thermophilic compost-dwelling filamentous fungus, *Thermomyces lanuginosus* SSBP. The total phytase production was enhanced by 8.56-fold in submerged fermentation after statistical optimization, which was further improved in fed-batch cultivations. Tlphy1 and Tlphy2 were purified to homogeneity with an overall purification of 24.7- and 18.7-fold with 5.16% and 6.4% yield, respectively. Tlphy1 was estimated to be 49 kDa and was optimally active at 55°C and pH 5.0, while Tlphy2 was purified as a 60 kDa protein showing optimal activity at 50°C and pH 2.5. Tlphy1 and Tlphy2 were stable over wide ranges of pH and temperature with a half-life of 138.6 and 58.72 min, respectively at 70°C. The applicability of these phytases was investigated for the improvement of bioethanol production using *Colocasia esculenta,* dephytinisation of a local non-alcoholic beverage, *mageu* and in improving growth promotion of common bean plants. Tlphy1 reduced phytate content in *Colocasia esculenta* starch from 1.43 mg/g to 0.05 mg/g that resulted in an improvement in the availability of fermentable sugars with a concomitant reduction in viscosity and 1.59-fold improvement in ethanol production. Supplementation of 50 U Tlphy2 in 100 g of *mageu* efficiently reduced the total fermentation time from 24 h to 16 h with significant dephytinisation and marked enhancement of essential metal ions. Additionally, Tlphy2 also promoted germination of bean seeds, while an enzyme dose of 25 U markedly improved root and shoot length of bean plants. Overall, the two distinct phytases from *T. lanuginosus* SSBP have multifarious applicability, and therefore can be of major interest to several biotechnological industries.

# CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

Phosphorus (P) is an essential element with unique chemical and biological significance to living cells. It forms the structural basis of cell membranes and vital biomolecules including energy rich adenosine triphosphate (ATP) and nucleic acids (DNA and RNA), and plays pivotal role in important metabolic processes. P mostly exits in the form of orthophosphates and phosphoric acid esters of organic compounds in cells. Although it constitutes less than 1% of the dry weight of plants and animals, its deficiency is detrimental to all living organisms. Deficiency of P in plants is manifested by overall stunting, late maturity, purpling of stems, leaves and leaf veins, and yellowing and death of leaf tips (Abaidoo et al., 2017; Takeda et al., 2012). Similarly, reduced dietary intake of P may result in disorders such as rickets, bone demineralization, reduced egg production, poor bone development and decreased feed intake in animals (Pokhare et al., 2017; Pierce, 1999).

In order to circumvent P deficiency in plants, agricultural soil is fertilized with P extracted from mined rock phosphates—the reserves of which are depleting at an alarming rate. As per estimates of the last 2013 global mining data, 68.7 million metric tons (Tg) of P was extracted (Chen and Graedel, 2016; Elser, 2012) out of the estimated 71000 million tons (Mt) reserves of phosphate, worldwide (Jasinski, 2012). Due to the emerging economies and rising agricultural needs, the annual global fertilizer demand is expected to increase by 1.9% as evidenced from an increase in global phosphate fertilizer demand from 40.6 Mt in 2011 to 45 Mt in 2016 (FAO, 2017). The P production will reach global peak in 2033 (Sattari et al., 2012; Cordell et al., 2009) and about 40 to 60% of this dwindling and non-renewable resource will be depleted by 2100 (Van Vuuren et al., 2010). Additionally, increased use of phosphate fertilizers is of major environmental concern as 80 to 90% of the fertilized P remains unutilised and mixes with water bodies deteriorating the quality of water. Furthermore, P pollution results in water eutrophication which needs immediate attention as the algal or cyanobacterial bloom is devastating for the aquatic biological diversity (Berry et al., 2017; Breininger et al., 2017; Feng et al., 2016). Therefore, strategies directed towards improving agricultural productivity by using alternate sources of P are being

encouraged (Secco et al., 2017). Unlocking P trapped in P-rich compounds is one such attempt that assures unlimited potential.

Phytic acid (PA) or *myo*-inositol hexakisphosphate (IP6) is one of the most routinely explored P-rich compounds present in major crops including cereals, legumes, grains, oil seeds and soils. It constitutes 60 to 90% of the total organic phosphorus in plant tissues (Rao et al., 2009) and makes up 3 to 5% of dry weight of cereals and legumes (Reddy et al., 1982). The stored phytate (salt form of PA) in seeds can only be used during germination while P locked in soil phytate is largely unavailable to plants. The polyanionic phytate molecule present in ingested cereals, grains and legumes strongly chelates with essential metal cations (Lei et al., 2013), proteins and carbohydrates and therefore is perceived as anti-nutritive. The anti-nutritive property of phytate is reflected in terms of reduction in nutritional value of routine PA-containing diets. The strong chelation property of PA is also a problem in production of first generation bioethanol using starch-based substrates.

All these issues associated with PA can be resolved in an environment-friendly way using PA-hydrolysing enzymes, called phytases. Supplementation of this enzyme is known to improve growth characteristics of monogastric animals. In fact, phytases are the preferred enzyme supplements in several animal feed industries (Greiner and Konietzny, 2012) and contribute about 60% of animal feed market with an annual market size of $ 350 million (Corrêa et al., 2015). It can also dephosphorylate organic insoluble form of phytate phosphorus into accessible soluble inorganic forms that can be easily assimilated by plants.

Although phytases have been reported from a wide range of life-forms, microorganisms are the preferred sources for enhanced production and applications in industries. Research is still under progress to find an ideal phytase that suits harsh industrial processing conditions. This study was focused on production, characterization and applications of phytases from a thermophilic filamentous fungus *Thermomyces lanuginosus* SSBP.

## 1.1 Phytic acid and the phytate problem

PA, first isolated by Posternak (1903), is the primary storage form of P in plants (Mullaney et al., 2007) and constitutes 0.5 to 5% of seeds and grains. The amount of PA increases during the ripening process in seeds and grains along with increased levels of other storage molecules such as starch and lipids. PA is a symmetric molecule with six phosphate groups attached to the *myo*-inositol ring (Fig. 1.1) and is therefore referred to as *myo*-inositol hexakisphosphate (Yao et al., 2011; Bohn et al., 2008) shown in. It is predominantly available in salt forms as phytins. K and Mg phytates are more common than Ca, Fe and Zn and other salts of phytate (Raboy, 2009).

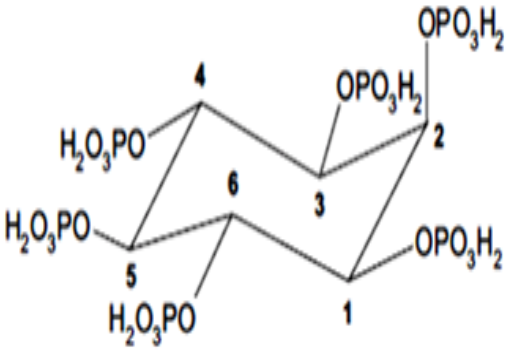



**Figure 1.1** Structure of phytic acid (Haefner et al., 2005).

Structural studies of PA reveal the presence of twelve replaceable protons in the molecule - six are strongly acidic, three are weakly acidic and the remaining three have very weak acidic attributes. This structural arrangement makes PA strongly negatively charged over the entire pH values generally prevailing in physiological conditions (Maenz, 2001). Indeed, PA is a strong chelator that can bind strongly to oppositely charged metal ions such as $Ca^{2+}$, $Mg^{2+,}$ $Zn^{2+}$ and $Fe^{2+}$ (Fig. 1.2) and form insoluble complexes with proteins, starch and many other biomolecules. The insolubility of phytate-mineral complexes reduces bioavailability of important minerals in phytate containing food/feeds (Selle and Ravindran, 2007). Apart from chelating with minerals, proteins and starch PA can also inhibit digestive enzymes such as pepsin, amylase and trypsin (Tannenbaum et al., 1985; Singh and Krikorian, 1982), thereby negatively affecting the digestion and absorption process in the gastrointestinal (GI) tract.

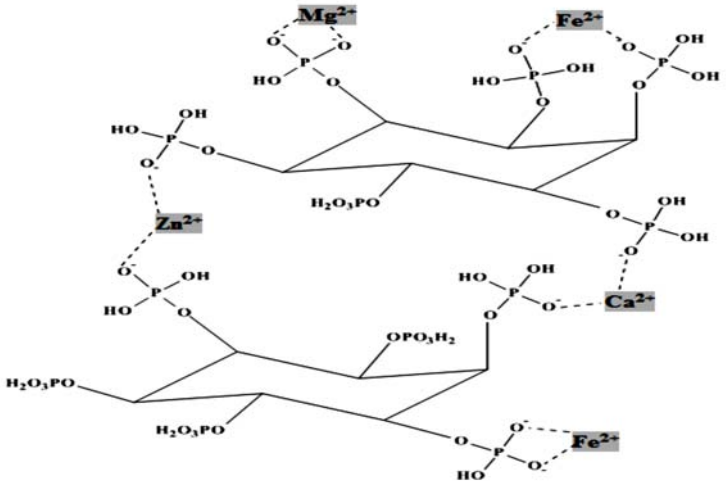



**Figure 1.2** Interactions of phytic acid with metal ions (Tran, 2010).

Feeds rich in phytate pose a pressing problem. The high content of phytate in crops used in animal feeds compromises the quality of feeds. To alleviate this problem, phytate degradation is mandatory such that the bonded minerals and biomolecules could be released and be available to animals. Phytate can be hydrolysed enzymatically (Fig. 1.3) or can be degraded non-enzymatically at high temperatures. The released phytate phosphorus can potentially satisfy the phosphorus needs of the animal diet thereby minimizing the burden of supplemental phosphorus in the diet. This significantly reduces the threat to environment due to otherwise release of unutilized phytate phosphorus and supplemental phosphorus.

## 1.2 Phytase

Phytases (*myo*-inositol hexakiphosphate phosphohydrolases) first reported by Suzuki et al. (1907) hydrolyse phytic acid into inositol phosphates and/or *myo*-inositol and inorganic phosphate (Pi). Based on the carbon position on the inositol ring at which phosphate hydrolysis is initiated, International Union of Pure and Applied Chemistry and the International Union of Biochemistry (IUPAC – IUB) recognised two general classes of phytases as 3-phytase (EC 3.1.3.8) and 6-phytase (EC 3.1.3.26) producing different isomers of *myo*-inositol phosphates as intermediates. Hydrolysis with 3-phytases is initiated at $C_3$ carbon of the ring yielding (1, 2, 4, 5, 6) IP5, while 6-phytases initiate hydrolysis at $C_6$ position generating (1, 2, 3, 4, 5) IP5. Dephosphorylation of *myo*-inositol hexakiphosphate is not always completed by 3-phytases, whereas the 6-phytases do. Microorganisms are the main producers of 3-

phytases whereas 6-phytases are mostly prevalent in plants. These phytases have entirely different characteristics (Kerovuo et al., 2000).

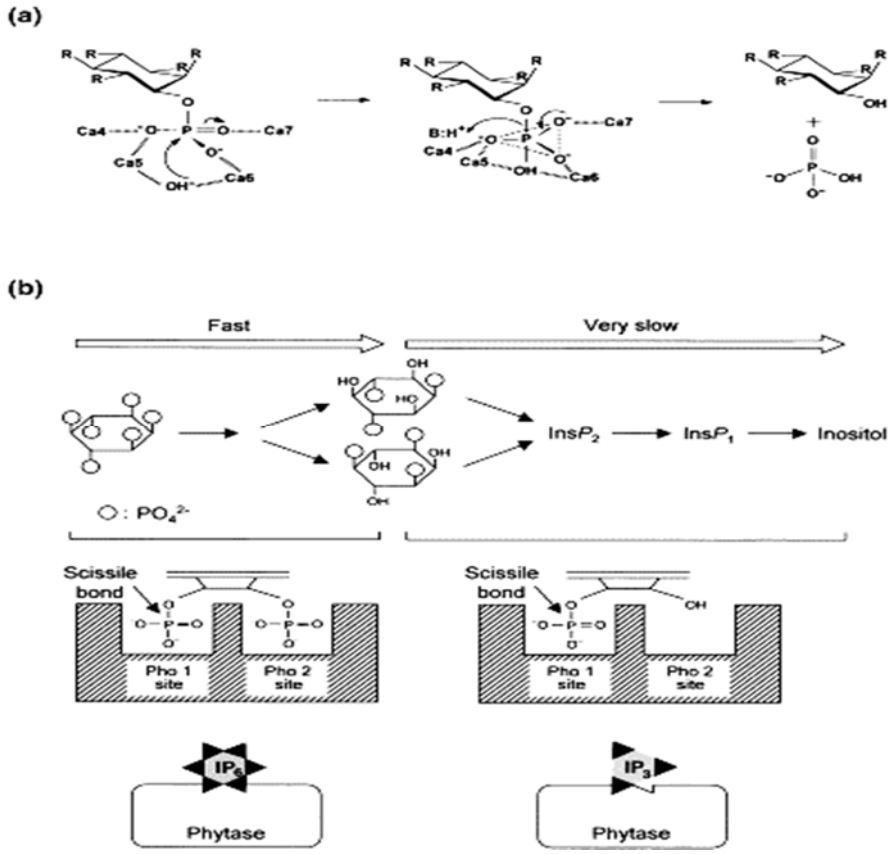



**Figure 1.3** Proposed mechanism of enzyme action and phytate degradation (Tran, 2010).

## 1.3 Classification of phytases

Phytases are specialized phosphatases that can be acidic or alkaline based on their optimum pH of enzyme action. Phytases have been classified into four different families based on their catalytic mechanisms-histidine acid phosphatases (HAP), β-propeller phytases (BPP), cysteine phosphatases (CP) and purple acid phosphatases (PAP). Oh et al. (2004) divided phytases into two major groups, (HAPs and alkaline phytases) with four sub groups (PhyA, B, C, D) based on their amino acid sequences and biochemical properties.

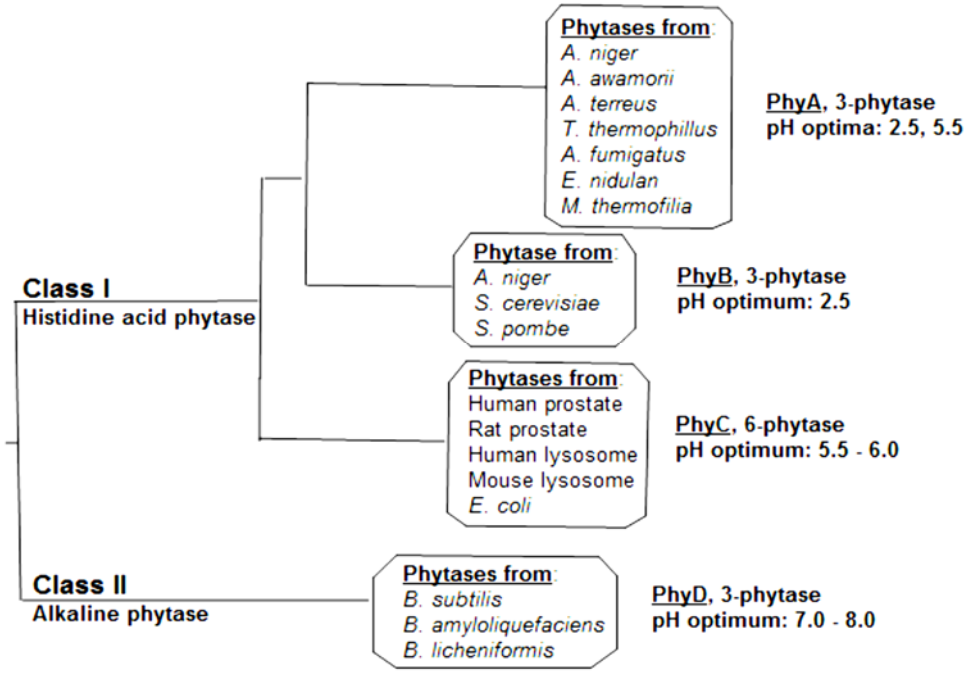



**Figure 1.4** Different classes of phytase based on their sequence analysis and biochemical properties (Oh et al., 2004).

### 1.3.1 Histidine acid phosphatase (HAP)

More research has been done on HAPs compared to other groups of phytases. Members of this group have been reported in both prokaryotes and eukaryotes. HAPs can further be divided into high molecular weight HAPs with a characteristic N-terminal RHGXRXP motif and C-terminal HD motif that enables a two-step mechanism for hydrolysis of phosphomonoesters (Quan et al., 2004; Mullaney and Ullah, 2003), and low molecular weight HAP which lacks both N- and a C-terminal motifs. The His residue of the RHGXRXP motif performs a nucleophilic attack on the phosphoester bond while the Asp residue of the HD motif is involved in protonation of the bridging oxygen atom of the leaving group (Chen et al., 2015). HAP phytases do not need any co-factor for optimal enzyme activity (Konietzny and Greiner, 2004). Despite sharing a common catalytic site, all HAPs do not hydrolyze *myo*-inositol hexakisphosphate to the same extent (Oh et al., 2004).

### 1.3.2 β-propeller phytases (BPP)

BPP phytases or alkaline phytases, which previously were attributed only to the *Bacillus* species, are now found to be widely distributed in nature. The mode of action of BPP, structure, nucleotide sequence and dependence on cofactor distinguishes from other phytases (Mullaney and Ullah, 2003). Unlike HAP phytases, this group of

phytases does not possess RHGXRXP and HD motifs. Ha et al. (2000) reported three dimensional structure of BPP resembling a propeller with six blades (Fig. 1.5) BPP are metal dependent, with distinct 'cleavage site', 'affinity site' and '$Ca^{2+}$ binding site'. The affinity site increases the binding affinity for substrates like PA, which is hydrolysed in a different mode at the cleavage site (Shin et al., 2001; Oh et al., 2001). β-propeller phytases are mostly made up of β-sheets in six blades having a narrow and highly negatively charged active cleft on the top of the enzyme molecule (Ha et al., 2000). This so-called "double clasp" structure together with three calcium ions at the high affinity calcium binding sites are responsible for the elevated thermostability of the β-propeller phytase from *Bacillus amyloliquefaciens* DS11 (Ha et al., 2000).

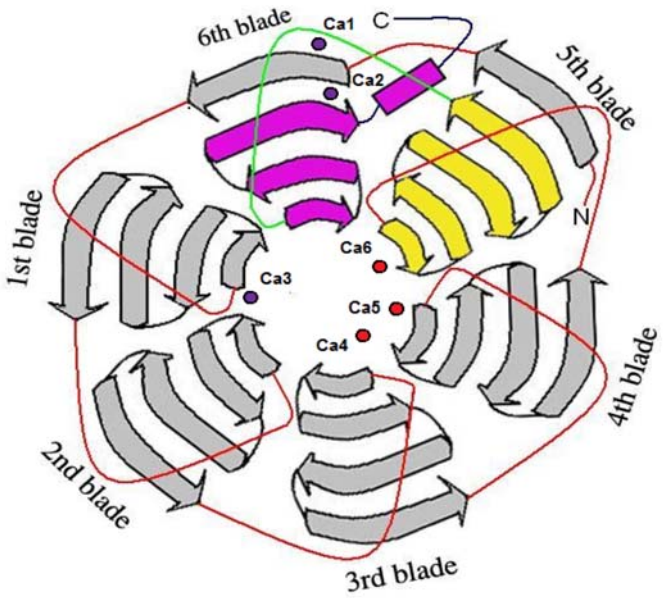



**Figure 1.5** β-propeller phytase structure (Ha et al., 2000).

### 1.3.3 Purple acid phosphatases (PAP)

The PAPs are metalloenzymes with two metal ions bonded to at the nuclear centre. Generally, one of the metal ions is $Fe^{3+}$ while the other may be $Fe^{2+}$, $Mn^{2+}$, or $Zn^{2+}$. Their optimal enzyme activity is exhibited under acidic conditions. The active site of PAPs is made up of seven metal ligating residues (D,D,Y,N,H,H and H) in five conserved motifs– DXG, GDXXY, GNH(D⁄E),VXXH and GHXH – involved in the litigation of binuclear metallic centre (Li et al., 2002). Most of the studied PAPs have been isolated from plants.

### 1.3.4 Cysteine phosphatase (CP)

CPs or protein tyrosine phosphatase like phytases (PTPLPs) are the least common class of phytase which show catalytic mechanism and folding patterns similar to protein tyrosine phosphatases (PTP). The active site motif of CPs share amino acid sequence HCXXGXXR (T/S) similar to PTP (Chu et al., 2004). Their structure consists of one large domain of a 4-stranded β-sheet sandwiched between several α-helices on both sides, and one small domain of a 5-stranded β-sheet. There is a wider and deeper pocket near the large domain which is of major catalytic importance. The depth of this pocket determines the substrate specificity (Chu et al., 2004).

## 1.4 Microbial sources of phytases

Phytases have been reported from a wide range of sources including plants, animals and microorganisms. However, the search for an ideal phytase is still an on-going process. Microorganisms are the preferred sources of phytases (Table 1.1) due to high yield, easier production and the ease of handling and manipulation of producing strains. Fungi are the dominant phytase producers as compared to several strains of bacteria and yeast (Rao et al., 2009). Filamentous fungi naturally produce extracellular phytases whereas bacterial and yeast phytases are mostly cell-associated (Singh and Satyanarayana, 2011). Commercially, different species of *Aspergillus* have been widely used as a source of phytase for applications in animal feed industries. Isolation of phytase from plants and animal sources still remains an active area of phytase research. Recently, Belho et al. (2016) reported a phytase from rice bean showing specific activity of 2.22 U/mg, while Mootapally et al. (2016) reported phytase from the buffalo rumen. Phytases have been produced commercially since 1991. Table 1.2 enlists commercial phytases.

**Table 1.1** Characterization and production of various microbial phytases

| Microbial strains | Optimum conditions | | Activity/Production | References |
|---|---|---|---|---|
| | T° opt | pH opt | | |
| *Aspergillus flavus* ITCC 6720 | 45 | 6.5 | 46.53 U/mg | Gaind and Singh, 2015 |
| *Aspergillus niger* FS3 | 60 | 5.0-5.5 | 170.4 U/ml | Spier et al., 2011 |
| *Aspergillus niger* NCIM 563 | 55 | 6 | 154 U/g | Bhavsar et al., 2011 |
| *Aspergillus niger CFR 335* | 30 | 4.5 | 32.1 U/mg | Gunashree and Venkateswaran, 2015 |
| *Bacillus amyloliquefaciens* US573 | 70 | 7.5 | 27 U/mg | Boukhris et al., 2015 |
| *Enterobacter sp.* ACSS | 60 | 2.5 | 805.98 U/mg | Chanderman et al., 2016 |
| *Klebsiella* sp. DB3 | 50 | 5.5 | 3.15 U/ml | Mittal et al., 2011 |
| *Nocardia sp.* MB 36 | 40-50 | 5-6 | 11.30 U/mg | Bajaj and Wani, 2015 |
| *Pichia pastoris* | | | 161.64 U/ml | Liu et al., 2011 |
| *Pseudomonas* sp. | 28 | 7-3.5 | 749.2 U/ml | Hosseinkhani and Hosseinkhani, 2009 |
| *Rhizopus oryzae* | 45 | 1.5; 5.5 | 148.77 U/gds | Rani and Gosh, 2011 |
| *Rhodotorula mucilaginosa* JMUY14 | 50 | 5.5 | 31.64 U/mg | Yu et al., 2015 |
| *Saccharomyces cerevisiae* sp. | 35 | | 0.62U/mL | Ries and Macedo, 2011 |
| *Sporotrichum thermophile* | 60 | 5 | 52.4 U/mg | Singh and Satyanarayana 2009 |
| *Thermoascus aurantiacus* | 55 | | 468.22 U/ml | Nampoothiri et al., 2004 |
| *Thermomyces lanuginosus* TL-7 | 70 | 5 | 32.19 U/g | Gulati et al., 2007 |

T° opt = optimum temperature (°C) for phytase action

pH opt = optimum pH for phytase action

**Table 1.2** Commercial phytases

| Product | Source | pH opt | Temp. opt | Company |
|---|---|---|---|---|
| Allzyme® SSF | *Aspergillus niger* | | | Alltech |
| Finase® EC | *Escherichia coli* AppA | 4.5 | 55 | AB Vista |
| Finase® P/L | *Aspergillus niger* PhyB | 2.5 | | AB Vista |
| Natuphos® | *Aspergillus niger* PhyA | 2.0, 5-5.5 | 65 | BASF |
| OptiPhos® | *Escherichia coli* AppA2 | 3.4, 5.0 | 58 | Enzyvia |
| Phyzyme® XP | *Escherichia coli* AppA | 4.5 | 55 | Dupont Industrial Biosciences |
| Quantum® | *Escherichia coli* Phy9X$^{h}$ | 4.5 | | AB Vista |
| Ronozyme® P | *Peniophora lycii* PhyA | 4–4.5 | 50-55 | Novozyme |

## 1.5 *Thermomyces lanuginosus*

*T. lanuginosus,* formerly known as *Humicola lanuginosa*, is a widely distributed thermophilic filamentous fungus frequently isolated from self-heating masses of organic debris such as composts (Singh et al., 2003). It is classified under deuteromycetes (imperfect fungus) with septate hyphae that reproduces asexually by forming aleurioconidia (Singh et al., 2003; Hudson, 1992). *T. lanuginosus* optimally grows at 50°C with a minimum and maximum growth temperature of 20°C and 60°C, respectively. *T. lanuginosus* is well-known as a hyper producer of xylanase. However, it also produces many other valuable industrial enzymes, such as cellulose, β-xylosidase, lipase, α–amylase, α-galactosidase, glucoamylase, pectinase, mannanase, and many other enzymes. Owing to its production of thermostable enzymes displaying activity over a broad pH and temperature range with potential applications in various industries, *T. lanuginosus* is a potential source for commercial production of several industrial enzymes. Recently our research group reported thermostable cyanate hydratase (Ranjan et al., 2017), chitinase (Khan et al., 2017) and β-xylosidase (Gramany et al., 2016) from *T. lanuginosus* SSBP. Genome analysis of *T. lanuginosus* SSBP indicated the presence of two phytase-encoding genes of 1431 bp and 1539 bp. This study explores *T. lanuginosus* as a source for submerged production of phytase.

**Table 1.3** Characteristics and applications of enzymes from different strains of *T. lanuginosus*

| Enzyme | Optimal pH | Optimal temperature | Thermal stability | pH stability | Applications | References |
|---|---|---|---|---|---|---|
| α-Amylase | 5.0 | 60°C | half-life longer than 1 day at 60°C | 5.0–8.5 | Textile, food and brewing | Odibo and Ulbrich-Hofmann (2001), Chadha et al. (1996), Nguyen et al. (2002) |
| Glucoamylase | 6.0 | 70°C | half-life longer than 1 day at 60°C | 4.4–7.5 | Textile, food and brewing | Odibo and Ulbrich-Hofmann (2001), Chadha et al. (1996), Nguyen et al. (2002) |
| Lipase | 7.0 | 45°C | 60°C for 20 h | 5.0-9.0 | Cosmetics, leather, detergents, foods, perfumery, medical diagnostics. | Ibrahim et al. (1987) |
| Protease | 5.0, 9.0 | 70°C | 50°C for 4 h | 4.0-11.0 | Detergents, leather, food, pharmaceutical industry and bioremediation. | Li et al. (1997) |
| Xylanase | 7.0 | 60–70°C | 60°C for 96 h | 5.0-9.0 | Paper, feed, food and biofuel industry | Su et al. (2011), Cesar and Mrša (1996) |
| α-Galactosidase | 5.0-5.5 | 65°C | 55 for 24 h | 5.5-8.0 | Food and feed industry | Rezessy-Szabo et al. (2007) |
| β-Galactosidase | 6.7-7.2 | NR | 47 for 2 h | 6.0-9.0 | Dairy industry | Fischer et al. (1995) |
| Trehalase | 5.0 | 50°C | NR | NR | Food and brewing industry | Bharadwaj and Maheshwari (1999) |
| Chitinase | 4.5 | 55°C | 25 min half-life at 65°C | 3.0-9.0 | Biofuel, food, pharmaceutical industry and bioremediation. | Guo et al. (2005); Zhang et al. (2015) |

NR: Not reported

## 1.6 Production of phytases

Phytases from several microorganisms are produced by submerged fermentation (SmF) and solid state fermentation (SSF). SSF allows cultivation of microorganisms on solid substrates in the absence of free water, whereas SmF is the cultivation of microorganisms in liquid medium with high water content. Although SSF has several advantages, industries prefer SmF due to easier control over instrumentation and monitoring of parameters such as pH, dissolved oxygen, temperature, etc. During SmF nutrients can be homogeneously distributed using agitation and microbes can easily utilise the water soluble nutrients. Here moisture content is never compromised, usually a low inoculum is required and samples can be easily taken out to conduct rapid tests for assessing the growth or other fermentation parameters. There are several reports on phytase production in SmF using fungi. Recently submerged production of phytase has been reported from *A. niger* CFR 335, *A. ficuum, Muscodor* sp., *S. thermophile* etc. (Alves et al., 2016; Kumari et al., 2016; Gunashree and Venkateswaran, 2015 and Coban et al., 2015).

### 1.6.1 Batch fermentation

Batch fermentation runs on pre-set optimized conditions once the sterilized medium is inoculated. It is a process in closed system where no nutrients can be added during fermentation. Medium composition and biomass concentration in the vessel changes with time due to utilization of nutrients by the microorganism. The process is terminated once the fermentation is complete. Even though the process has some limitations such as low turnover rate, it is preferred over other processes as it is simple, easy to operate and not labour intensive. Recently, Shah et al. (2017) reported batch production of phytase from *A. niger* NCIM 563, while Rocky-Salimi et al. (2017) reported enhanced phytase production by *B. subtilis* K46b in the batch fermentation.

### 1.6.2 Fed-batch fermentation

Substrate limitation over time is a major constraint for batch fermentation processes. Fed-batch fermentation involves continuous or intermittent supplementation of one or more nutrients in the medium, especially during the log-phase. This allows extended period of microbial growth and improves the yield of the desired product. Study of appropriate feeding method and determination of time and amount of feed is important to develop a successful fed-batch strategy. Feeding can be performed by three different strategies. The direct feedback control strategy involves manipulation of the primary carbon source such as glucose. The concentration of the primary carbon source and the time of feed is optimized before actual fermentation. The second approach involves indirect feedback control of parameters such as dissolved oxygen, pH etc. determines the time of feed. The third strategy does not employ any feedback control method and there can be constant, increased or exponential feeding strategies. There are many reports on fed-batch production of industrial enzymes. However, only a few reports have been available on fed-batch production of phytase during the last few years. Coban and Demirci (2014) reported 11 and 40% enhancement in phytase production by *A. ficuum* due to supplementation of feed containing glucose and Na-phytate, respectively. In another study, Coban et al. (2015) reported a 200% increase in phytase production from *A. ficuum* when talcum was fed in the medium. Similarly, the production of phytase from *Ogataea thermomethanolica* doubled in fed-batch cultivation due to feeding with table sugar (Charoenrat et al., 2016).

## 1.7 Optimization of phytase production

A successful fermentation process involves identification and optimization of various physical and chemical parameters that may affect production of the target biomolecule. Parameters such as source and concentration of nutrients, pH, temperature, agitation etc. needs to be optimized in order to achieve optimal yield of fermentative products. The traditional method for optimization by one-factor-at-a-time (OFAT) approach is time consuming and labour intensive. These disadvantages necessitated development of more efficient methods of optimization. Statistical tools such as Placket-Burmann Design (PBD) and response surface methodology (RSM) have been methods of choice for optimization, modelling and control of enzyme production. The importance of PBD and/or RSM in phytase production has been extensively documented (Buddhiwant et al., 2016; Kumari et al., 2016; Jain and Singh, 2016; Singh and Satyanarayana, 2008; Vohra and Satyanarayana, 2002).

Statistical methods are generally used to improve enzyme production by investigating the effect and significance of different factors and studying their interactions. This reduces the time, thereby reducing the cost of the overall production process (Ries and Macedo, 2011). The effect of each factor is tested using PBD and the most significant factors are identified. Thereafter interaction among the identified significant factors is analyzed using RSM. The optimization process involves three integral parts- performing the statistically designed experiments, estimating the effects using coefficients in a mathematical model and lastly predicting the response and checking the adequacy of the model. RSM mostly uses central composite design (CCD) to model experiments for optimization (Rani et al., 2014). CCDs are formed from two level factorials (second-order models) by addition of just enough points to estimate curvature and interaction effects. There is plethora of reports on the use of statistical approach for optimization of phytase production. Phytase production from *A. niger* was improved by 2.9-fold after statistical optimization (Gupta et al., 2014). Statistical optimization resulted in 3.35-fold increase in phytase production from *A. oryzae* (Sapna and Singh, 2015). Rani et al. (2014) reported an overall 7.95-fold increase in phytase production from *R. oryzae* when RSM was used to study the interactions among mannitol, sodium phytate, $K_2HPO_4$ and $Na_2HPO_4$. Kammoun et al. (2012) identified inoculum size, methanol and yeast extract as most significant factors affecting phytase production by *B. subtilis* US417 using PBD. Thereafter RSM was

used to achieve 5-fold and 4-fold enhancement under SmF and SSF, respectively. Therefore, enzyme production can be significantly enhanced by statistical optimization.

### 1.8 Purification and characterization of phytases

#### 1.8.1 Strategies for purification

Phytases have been purified from different sources with varying characteristics. A two-step purification method was employed to purify a monomeric phytase from *B. licheniformis* ONF2 with an overall yield of 21.3% (Dan et al., 2015). Similarly, Sato et al. (2016) employed two-step strategy using DEAE–cellulose and CM-cellulose columns to purify a 35.4 kDa phytase from *Rhizopus microsporus* var. *microsporus* with 4.78% yield. *S. cerevisiae* LC3 phytase was purified in three steps using DEAE–cellulose, Superose 12HR 10/30 and Mono Q 5/5 columns, obtaining an overall yield of 20.62% (Caputo et al., 2015). Recombinant HAP phytase of *S. thermophile* was purified in two steps using Resource Q and Sephacryl S-200 HR 16/60 columns (Ranjan and Satyanarayana, 2016). A dimeric 161 kDa phytase from *A. niger* UFV-1 was purified in four steps using ultrafiltration, acid precipitation followed by anion exchange chromatography using DEAE-Sepharose CL-6B and gel-filtration chromatography using Sephacryl S-300 HR columns with an overall yield of 11% (Monteiro et al., 2015).

#### 1.8.2 Effect of temperature and thermostability

The activity and stability of phytases from different sources can vary significantly in response to varied temperature treatments. Thermostability is one of the requisite characteristics for ideal industrial phytases, as animal feed undergoes harsh temperature treatments during pelletization (60 to 80°C). The need for thermostable phytases has also intensified over the past few years due the application in bioethanol production during gelatinization and saccharification stages (Mikulski et al., 2014). Phytase stability varies from 50 to 70°C whereas the optimum temperature is usually in the range of 45 and 60°C. Phytase from *B. licheniformis* PFBL-03 retained 55% activity for 60 min at 80°C with optimum at 55°C (Fasimoye et al., 2014). Ushasree et al. (2014) reported a recombinant phytase from *Kluyveromyces lactis* showing 69 and 37% activity at 90 and 100°C, respectively for 10 min with optimal activity at 55°C. Phytase from *A. fumigatus* maintained 90% activity at 100°C for 20 min (Pasamontes

et al., 1997). Phytase from *Humicola nigrescens* showed optimal activity at 50°C (Bala et al., 2014) while *A. flavus* and *S. thermophile* phytases showed optimal activities at 45°C (Gaind and Singh, 2015) and 60°C (Singh and Satyanarayana, 2009), respectively.

### 1.8.3 Effect of pH on activity and stability of phytases

Based on their optimal activity at different pH, phytases can be acidic or alkaline. The optimum pH of phytases range between 2.2 to 8.0, with fugal phytases mostly showing optimal activity between 4.5 to 5.6 and bacterial phytases showing between pH 6.5 to 7.5 (Kumar et al., 2016). However, phytase from thermophilic fungus *A. flavus* ITCC 6720 showed optimal activity at neutral pH (Gaind and Singh, 2015). Phytase from *Aspergillus* sp. L117 showed optimal activity at pH 5.5 and was stable between pH 2.0 to 7.0 (Lee et al., 2005). Chadha et al. (2004) reported *Rhizomucor pusillus* phytase to be optimally active at pH 5.4 and stable over a pH range of 3.0-8.0. Bacterial phytase from *B. laevolacticus* was optimally active between pH 7.0-8.0 (Gulati et al., 2007). There have been reports of acidic phytases from several bacteria. *Cladosporium* sp. phytase was optimally active at pH 3.5 (Quan et al., 2004), while *Yersinia intermedia* phytase showed high activity from pH 2.0 to 6.0 with optimum at pH 4.5 (Huang et al., 2006). Similarly, phytase from *Pantoea agglomeranns* displayed optimal activity at pH 4.5 (Greiner, 2004). Choi et al. (2001) reported *Bacillus sp.* KHU-10 phytase, which was fairly stable in the alkaline range pH 6.5 to 10. Different sources have got leading to vast range of Differences in pH and temperature optima can be attributed to varying molecular structure from different sources. A phytase that shows stability in both acidic and alkaline range can be ideal for animal feed applications because the upper tract of the animal digestive system is acidic whereas neutral or alkaline conditions are prevails in the lower GI tract.

### 1.8.4 Molecular weight of phytases

Molecular weights of phytases have been estimated to range between 35 to 700 kDa depending on source of origin (Kumar et al., 2016). Fungal phytases often range from 85 to 150 kDa, while yeast phytases range up to 500 kDa. Phytases from plants and animal tissues can range from 50 to 150 kDa. Most reported phytases are monomeric but there have been reports on phytases with multiple subunits (Singh et al., 2011). The purified maize seedling phytase is a dimer of two identical subunits of 38 kDa

each (Laboure et al., 1993). *S. thermophile* phytase was characterised as a homopentamer with a molecular mass of 456 kDa (Singh and Satyanarayana, 2009), phytase of *Shwanniomyces castellii* is a tetramer of 490 kDa with one large subunit of 125 kDa and three identical subunits, each of 70 kDa (Segueilha et al., 1992). The molecular weight of enzymes can be altered due to glycosylation. Glycosylation is an essential post-translational modification that influences the pI, secretion and stability of the protein (Wyss et al., 1999).

### 1.8.5 Effect of metal ions

Different types of metal ions and detergents can act as potential activators or inhibitors of phytases. Most *Bacillus* phytases are BPP phytases, which are metalloenzymes that need calcium for activity (Fu et al. 2008). *B. licheniformis* ZJ-6 phytase activity was stimulated by 1 and 5 mM $Ca^{2+}$ and it was greatly inhibited by, $Cd^{2+}$, $Mn^{2+}$, $Cu^{2+}$ and $Zn^{2+}$ (Wang et al., 2011). $Ca^{2+}$ enhanced the activity of phytases from *Geobacillus* sp. TF16 and *Nocardia* sp. MB 36 by 50% and 15%, respectively (Dokuzparmak et al., 2016; Bajaj and Wani, 2015). Phytase activity from *Rhizopus oryzae* was strongly inhibited by $Fe^{2+}$, $Ni^{2+}$ and $Cu^{2+}$ while 5 mM $Ba^{2+}$ and $Ag^{+}$ increased the activity by 5.5 and 2.5-fold in the presence, respectively. The activity was also increased by EDTA and tartarate (Rani and Ghosh, 2011). The presence of EDTA slightly stimulated the activity of *A. ficuum* NTG-23 phytase while metal ions such as $Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$ and $Zn^{2+}$ significantly affected its activity (Zhang et al., 2010). Partially purified *A. niveus* phytase was reported to be inhibited by $Mg^{2+}$, $Mn^{2+}$, $Hg^{2+}$, $Cd^{2+}$, $Zn^{2+}$and $Fe^{2+}$ and their activity was enhanced by low concentrations of $Fe^{3+}$ and high concentrations of $Na^{+}$, $Li^{+}$, $Ca^{2+}$ (El-Gindy et al., 2009). The metal ions $Zn^{2+}$, $Fe^{2+}$ and $Fe^{3+}$ enhanced the activity of *Malbranchea sulfurea* phytase. However, $Na^{+}$, $Li^{+}$, $Mg^{2+}$, $Hg^{2+}$, $Mn^{2+}$and $Cd^{2+}$ inhibited the activity. Interestingly higher concentration of $Ca^{2+}$ slightly enhanced the phytase activity while low concentrations were inhibitory (El-Gindy et al., 2009).

## 1.9 Applications of phytases

The ability of phytases to hydrolyse PA and release Pi and complexed minerals, trace elements, biomolecules etc. has been exploited for multifarious applications. Phytases are the dominant enzymes of the animal feed market and routinely used as a feed

supplement in diets for poultry, pigs and fish. Recently, phytases have been of great interest due to several other new applications in biotechnology.

### 1.9.1 Animal feed supplement

Plants and plant products containing PA are main constituents of several animal feed formulations. Monogastric animals (poultry, swine, fish etc.) have negligible or low phytase activity in their GI tract preventing them to utilise phytate phosphorus generally present in plant based feed. Supplementation of animal feed with phytase is deemed essential as it increases the bioavailability of minerals and minimizes additional P load in the feed. Additionally, this minimizes the amount of unutilized P in animal faeces thereby minimizing environmental P pollution. It is also a sustainable option compared to supplementing with inorganic phosphates which is expensive and on the brink of depletion in the near future. It has been estimated that 250 g of phytase can replace 10 kg of di-calcium phosphate in animal feeds (Vats and Banerjee, 2005). The phytase intended for animal feed applications should be thermostable and able to withstand the acidic conditions in GI tract. To date, HAPs phytase are the most used in animal feeds. The effectiveness of phytase in improving the growth characteristics of poultry, swine and fish is well documented. Supplementation of *E.coli* phytase in turkey feed markedly improved the skeletal system and function of the 16-week-old turkeys (Tatara et al., 2015). Phytase supplementation in the feed of growing pigs improved Ca and P utilization of defatted rice bran and improved protein digestibility (Dadalt et al., 2015). Over the years research on phytase as animal feed supplements has yielded new phytases with improved characteristics.

### 1.9.2 Foods

Research on application of phytase in food industries is mostly focused on technical improvements of food processing and improvement in bio-absorption of minerals due to dephytinization. Plant-based foods are the most consumed around the world and are vital sources of carbohydrates, proteins, dietary fibres and vitamins. The downside of plant-based food is the presence of anti-nutrients such as phytic acid, which chelates with essential minerals. Cereals, and grains have their own endogenous phytase activity that may be enhanced during low pH treatments during food processing, but the activity is generally insufficient to efficiently degrade total the phytate (García-Mantrana et al., 2016; Sanz-Penella et al., 2012; Greiner and Konietzny,

2006). Haros et al. (2001) reported that the addition of phytase improved the quality of bread by reducing the phytate content and promoting the activation of endogenous α-amylase due to enhanced availability of calcium. Commercial phytase from *A. niger* accelerated proofing time and improved bread properties such as shape, specific volume, and conferred softness to the crumb. The improvements were attributed to indirect effects of phytase on α-amylase activity (Greiner and Konietzny, 2006). Recently, Chanderman et al. (2016) used phytase from *Enterobacter* sp. ACSS to improve bread characteristics. Similarly, Ranjan et al. (2015) reported phytase-mediated dephytinization of tandoori, naan and bread. Supplementation of 48 U/l phytase released up to 77 mM phytic acid bound calcium in soymilk compared to 31 mM in control (Yang et al., 2015). Phytase addition to millet based porridge at the time of consumption successfully degraded phytic acid during digestion that resulted in improved fractional absorption of zinc (FAZ) by 68% in young children (Brnić et al., 2017). Although phytase supplementation appears as a nutritionally and economically viable approach, but the enzyme must be produced by a microorganism with Generally Regarded as Safe (GRAS) status and should be approved by food regulatory authorities such as FDA.

### 1.9.3 Plant growth promotion

Soil supplies essential nutrients to plants for growth with P being one of the essential macronutrients in the soil. Soil P is one of the limiting nutrients for plant growth and its deficiency leads to stunted growth. Phytate is the major component of organic form of P in soil. Soil and plant roots-associated microorganisms produce phosphatases such as phytases, which hydrolyse phytate and releases P for plants utilisation (Yadav and Tarafdar, 2004). These enzymes are essential for mobilising P for the growing plant. Yadav and Tarafdar (2004) reported phytase activity in different plants rhizosphere verities which may be due to either the ability of different plant roots to release phytase or changes in the microbial activity of in the rhizosphere. A large part of phytate and its derivatives could be potentially decomposed by phytase, resulting in the release of Pi for plants. Studies have correlated increase in root phytase activity due to decreased soil organic P (Yadav and Tarafdar, 2004). It has been demonstrated that extra-cellular phytase is more effective than intra-cellular phytase in the hydrolysis of phytate (Tarafdar et al., 2002). Inoculation of pearl millet seeds with *Penicillium purpurogenum* increased the release of phytase significantly by the

rhizosphere and that led to the increase in the depletion of unavailable P with advancing age (Yadav and Tarafdar, 2011). Inoculation of pearl millet seeds with *Emericella rugulosa* improved the plant biomass, root length, straw yield, seed yield and P content of shoots and roots (Yadav and Tarafdar, 2007). Plant biomass, phosphorus concentration and yield of cluster bean were improved when phosphatase- and phytase-producing *B. coagulans* was used as a biofertilizer (Yadav and Tarafdar, 2012). Supplementation of *S. thermophile* spores and its phytase improved the growth of wheat plant (Singh and Satyanarayana, 2010). Similarly, purified *B. amyloliquefaciens* FZB45 phytase promoted the growth of maize seedlings (Idriss et al., 2002). Yip et al. (2003) showed that the tobacco line transformed with a neutral *Bacillus* phytase exhibited phenotypic changes in flowering, seed development, and response to phosphate deficiency. Mudge et al. (2003) showed that transgenic *Arabidopsis* plants secreted phytase from roots only when grown under low phosphate conditions. A transgenic *Arabidopsis* plant expressing an extracellular phytase from *Medicago truncatula* led to a significant improvement in organic phosphorus utilization and growth of the plant (Xiao et al., 2005). It is more feasible to express the phytase gene in an organ or specific tissue, such as root hairs, which are actively involved in uptake of mineral nutrients.

### 1.9.4 Bioethanol production

Despite a major research shift towards different generations of biofuels, first generation biofuels still constitute a major percentage of the global bioethanol production. Cereal grains used in bioethanol production have a high phytate content that complexes with metal ions and starch limiting the availability of nutrients required by fermentation organisms such as distillery yeast which cannot utilise phytic acid (Lambrechts et al., 1992). Phytate-starch complex is inaccessible for hydrolysis which can reduce the concentration of fermentation sugars. Mikulski et al. (2014) demonstrated that the addition of phytase during preparation of the high gravity fermentation medium influenced the yeast fermentation activity which further significantly improved the fermentation yield. Similarly, addition of phytase and protease during simultaneous saccharification and fermentation increased rate of ethanol production (Luangthongkam et al., 2015).

## 1.10 Purpose of the study

Research on the production and applications of microbial phytases is gaining momentum. Apart from traditional use of phytases as animal feed supplements, new biotechnological applications have increased the market demand of this already established industrial enzyme. Thermostable fungi are sources of several industrial enzymes, including phytases. However, there is a continuous need to enhance production strategies of robust phytases that suit harsh processing conditions prevailing in food, agriculture and bioethanol industries. Therefore, the aim of the present investigation was to exploit the thermophilic filamentous fungus *T. lanuginosus* for improved phytase production, followed by enzyme purification, characterization, and several new applications. This aim was achieved by the following objectives:

i. To enhance production of phytase by statistical optimization;

ii. To purify and characterize the purified phytases; and

iii. To apply phytases in bioethanol production, enhancement of nutritional properties of *mageu* and growth-promotion of bean plants.

# CHAPTER 2: STATISTICAL OPTIMIZATION FOR ENHANCED PRODUCTION OF PHYTASE UNDER BATCH AND FED-BATCH CULTIVATIONS

***MOST OF THE WORK PRESENTED IN THIS CHAPTER WAS PUBLISHED IN Bioresource Technology, 235, pp.396-404,* 2017.**

## 2.1 INTRODUCTION

Microorganisms are the preferred sources for production of industrial enzymes (Azeem et al., 2015; Haefner et al., 2005). Filamentous fungi are known to produce a wide range of industrial enzymes, accordingly several phytase producing fungi have been reported till date (Azeem et al., 2015). Phytases from *A. niger* and *A. ficuum* are the most extensively used commercial enzymes. In order to meet the rising needs of the global phytase enzyme market which is growing at 6.6% compound annual growth rate (CAGR), the process parameters affecting phytase production must be optimized. Developments of processes that are economically viable and ensure fast and cost-effective production of phytase are extremely important.

Submerged fermentation is routinely used for commercial production of several enzymes including phytases. Statistical optimization using PBD and RSM is a reliable method for overcoming the impediments of the conventional one-factor-at-a-time method. The conventional method is time consuming, expensive and tedious. Statistical optimization is an effective method to optimize process parameters using moderately small number of experiments. In addition, the method computes interactions among significant factors to predict optimal fermentation conditions. Currently several software packages are available that can minimize complex calculations related with statistical analysis of the data. Screening, evaluation, selection and interaction-studies of several nutritional and physical variables are key factors to enhance production of any industrial enzyme. Furthermore, the production of enzyme can be validated and optimized in shake flasks and laboratory fermenters of varied volumes.

Strain type, culture conditions and nutrient concentrations are the primary factors that are critical to enhance production of enzyme under investigation. Wild type strains generally produce low titres of phytase and therefore it is necessary to optimize the fermentation parameters. Previously, a single phytase was reported from *T. lanuginosus* and the gene was cloned and expressed for production of the recombinant

protein (Berka et al., 1998). Although attempts have been made to optimize phytase production using wild and mutant stains of *T. lanuginosus* (Berikten and Kivanc, 2014; Gulati et al., 2007) in solid state fermentation (SSF), there has been no report till date on statistical optimization of phytase production using any strain of *T. lanuginosus* in submerged cultivations. Also, most of the reports have been limited to shake-flask studies and there is no report on submerged production of phytase using *T. lanuginosus* in a laboratory fermenter. Additionally, there have been no reports on fed-batch production of phytase from this thermophilic fungus.

In view of the foregoing this chapter focused on the enhanced production of phytase using statistical methodologies. The significance of several nutritional and physical factors in a minimum number of trials using PBD were investigated. CCD was used to study the interactions of the identified significant factors for further optimization. Optimized conditions as derived from the model were validated and used to scale-up production in a 5 l laboratory fermenter. Additionally, comparative studies of batch and fed-batch production experiments were also conducted.

## 2.2 MATERIALS AND METHODS

### 2.2.1 Fungal strain

The filamentous thermophilic fungus *T. lanuginosus* SSBP isolated by Singh et al. (2000) was obtained from culture collection of the Department of Biotechnology and Food Technology, Durban University Technology. The fungus was sub-cultured on potato dextrose agar (PDA) plates at 50°C until sporulation. Spores were harvested after seven days using sterile 0.9% saline water containing 0.1% (v/v) Tween-80. The spores were transferred into 30% glycerol (1:1 v/v) to prepare and preserve glycerol stock cultures at -80°C in a bio-freezer (Snijders Scientific, Holland).

### 2.2.2 Preparation of spore suspension and inoculum

The spores were harvested and spore count was adjusted to 1 x $10^7$spores/ml using a haemocytometer. Seed cultures were prepared in a medium containing (g/l): glucose, 15; yeast extract, 1; $KH_2PO_4$, 2; $K_2HPO_4$, 2.3; and $MgSO_4.7H_2O$, 1. Fifty ml medium was transferred to 250 ml Erlenmeyer flasks and autoclaved for 15 min at 121°C/15 psi. The medium was cooled to 50°C and inoculated with 2% spore suspension. The flasks were incubated at 50°C and 200 rpm for 36 h. This was used as an inoculum for the production medium.

### 2.3.3 Enzyme extraction and phytase assay

Fungal mycelia were harvested by centrifugation at 8.800 × *g* for 20 min and the cell-free supernatant (passed through 0.45 µm filter) was used to assay phytase by determining inorganic phosphate (Pi) liberated using a modified method described by Heinonen and Lahti (1981). The reaction mixture contained 100 µl enzyme, 75 µl 1 mM sodium phytate and 825 µl sodium acetate buffer, pH 5.5 was incubated for 30 min at 50°C. Thereafter, it was cooled to room temperature and 2 ml freshly-prepared solution of acetone/5 N sulphuric acid/ 10 mM ammonium molybdate (2:1:1, v/v) was added. The reaction was stopped adding 100 µl of 1 M citric acid before reading the absorbance at 355 nm. One unit of phytase activity was defined as the amount of enzyme required to liberate 1 nmol of Pi per millilitre per second under standard assay conditions (Singh and Satyanarayana, 2006).

### 2.2.4 Statistical optimization

Important factors which significantly affected phytase production were selected and further optimized by PBD using the statistical software package 'Design Expert 6.0' (Stat-Ease Inc., Minneapolis, USA). PBD is routinely used to identify the significant variables out of many important factors, employing two widely-spaced levels denoted by (-1) for the low level and (+1) for the high level. A total of twelve trials with nine variables and two unassigned variables were screened in the present design. The inoculum was maintained at 2% for all experiments. 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:

$$\boldsymbol{E\ (X_i) = \frac{2\ (\sum C_i+ - \sum C_{i-})}{N}}$$ Equation 2.1

Where E (Xi) is the effect of the tested variable. $Ci_+$ and $Ci_-$ are the phytase activities from the trials where the variable (Xi) 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.

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

The three most significant variables (peptone, incubation period and temperature) identified by PBD were selected for further optimization using RSM. Each variable was studied at five different levels ($-\alpha$, -1, 0, +1, $+\alpha$) and CCD was used to design a total of 20 experimental runs. The behaviour of the model was explained by the following second order polynomial equation:

$$\mathbf{Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{12} AB +}$$

$$\mathbf{\beta_{13} AC + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD + \beta_{34} CD}$$ Equation 2. 2

where Y indicates the predicted value of response, $\beta_0$ is the 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, $A^2$, $B^2$, $C^2$, $D^2$, AB, AC, BC, BD, CD are the independent variables.

### 2.2.6 Validation of the experimental model

The conditions predicted by the statistical model were validated with respect to phytase production in 0.25-2.0 l shake-flasks, and also in a 5 l laboratory fermenter (Minifors, Infors HT, Switzerland). The pH of the medium was adjusted to 8.0 before autoclaving and the fermenters were operated at 47.7°C, 450 rpm and 1 vvm of aeration. Samples were collected at the intervals as per experimental design, the cells harvested and the cell-free supernatant was analysed further. All experiments were conducted in triplicate.

### 2.2.7 Batch fermentation in a 5 l laboratory fermenter

Batch production of *T. lanuginosus* SSBP phytase was conducted in a 5 l fermenter (Minifors, Infors HT, Switzerland) containing 2 l of optimized medium. The medium was inoculated with 2% inoculum and batch fermentation was carried out for 72 h at 47.7°C, 450 rpm with dissolved oxygen (DO) maintained at 20% air saturation using cascade controller. Samples were collected every 6 h, the cells harvested and the cell-free supernatant was analysed further.

### 2.2.8 Preliminary optimization for fed-batch fermentation

#### *2.2.8.1 Selection of time of feed*

The fermentation was carried out for 72 h in a 5 l fermenter (Minifors, Infors HT, Switzerland) containing 2 l of optimized medium with an initial glucose concentration of 20 g/l. Different fed-batch addition points in the exponential phase (24, 30, 36 and 42 h) with 10 ml of glucose (300 g/l) were evaluated in separate experiments to find the optimum time of feed. Samples were collected every 6 h, the cells harvested and the cell-free supernatant was analysed further.

#### *2.2.8.2 Selection of glucose concentration for optimum phytase production*

To further optimize process parameters, the optimal concentration of feed (glucose) was determined. Different concentrations of glucose (100, 300, 500, 700, 900 g/l) were fed in separate experiments in the production medium after 30 h and phytase production profile was studied. All other variables were kept constant.

#### *2.2.8.3 Fed-batch fermentation*

Fed-batch fermentation was conducted in 5 l fermenter (Minifors, Infors HT, Switzerland) containing 2 l of statistically-optimized medium and inoculated with 2% (v/v) inoculum. The fermentation was carried out for 120 h at 47.7°C, 450 rpm while maintaining dissolved oxygen (DO) saturation at 20% using a cascade controller. The medium was fed with 700 g/l of glucose after every 30 h till 90 h. Samples were collected every 6 h and analysed for phytase activity.

## 2.3 RESULTS

### 2.3.1 Statistical optimization: Plackett-Burman design

To investigate the potential effect of different variables on phytase production, a total of twelve trials consisting of nine variables and two unassigned variables were assessed in this study. The evaluated variables were starch (A), glucose (B), peptone (C), $NH_4NO_3$ (D), $MgSO_4.7H_2O$ (E), incubation period (F), pH (G), temperature (H), agitation (J), and two unassigned variables (K and L). The effect of all the variables were studied at high (+1) and low (-1) levels. The design matrix shown in Table 2.1a also illustrates the response as (phytase production) while Table 2.1b depicts the results of regression analysis. The results of PBD showed a wide variation in phytase production. Run seven yielded the highest titre of phytase at 24.11 ± 1.3 U/ml whilst the first run displayed lowest production of 0.60 ± 0.0 U/ml (Table 2.1a). This variation reflected the importance of optimization to attain higher phytase productivity. Peptone, incubation time and temperature were found to be the most significant ($p<0.05$) factors (Table 2.1b).

**Table 2.1** Plackett-Burman design (a) and ANOVA analysis (b) for identification of significant parameters for the production of phytase by *T. lanuginosus* SSBP

(a) *Experimental design with multiple growth variables*

| Run no. | Variables† | | | | | | | | | | | Phytase production (U/ml) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | H | J | K | L | |
| 1 | 30.00 | 30.00 | 5.00 | 5.00 | 0.50 | 72.00 | 4.50 | 60.00 | 300.00 | -1.00 | 1.00 | 0.60 ± 0.0 |
| 2 | 5.00 | 30.00 | 5.00 | 20.00 | 5.00 | 36.00 | 8.00 | 60.00 | 300.00 | -1.00 | -1.00 | 5.89 ± 0.5 |
| 3 | 5.00 | 30.00 | 20.00 | 20.00 | 0.50 | 36.00 | 4.50 | 60.00 | 150.00 | 1.00 | 1.00 | 7.71 ± 0.5 |
| 4 | 30.00 | 2.50 | 20.00 | 20.00 | 0.50 | 72.00 | 8.00 | 60.00 | 150.00 | -1.00 | -1.00 | 2.08 ± 0.1 |
| 5 | 30.00 | 2.50 | 20.00 | 20.00 | 5.00 | 36.00 | 4.50 | 45.00 | 300.00 | -1.00 | 1.00 | 18.13 ± 1.3 |
| 6 | 5.00 | 30.00 | 20.00 | 5.00 | 5.00 | 72.00 | 8.00 | 45.00 | 150.00 | -1.00 | 1.00 | 14.17 ± 0.8 |
| 7 | 30.00 | 30.00 | 20.00 | 5.00 | 0.50 | 36.00 | 8.00 | 45.00 | 300.00 | 1.00 | -1.00 | 24.11 ± 1.3 |
| 8 | 5.00 | 2.50 | 20.00 | 5.00 | 5.00 | 72.00 | 4.50 | 60.00 | 300.00 | 1.00 | -1.00 | 3.84 ± 0.3 |
| 9 | 5.00 | 2.50 | 5.00 | 20.00 | 0.50 | 72.00 | 8.00 | 45.00 | 300.00 | 1.00 | 1.00 | 4.29 ± 0.2 |
| 10 | 30.00 | 30.00 | 5.00 | 20.00 | 5.00 | 72.00 | 4.50 | 45.00 | 150.00 | 1.00 | -1.00 | 2.41 ± 0.1 |
| 11 | 30.00 | 2.50 | 5.00 | 5.00 | 5.00 | 36.00 | 8.00 | 60.00 | 150.00 | 1.00 | 1.00 | 4.70 ± 0.2 |
| 12 | 5.00 | 2.50 | 5.00 | 5.00 | 0.50 | 36.00 | 4.50 | 45.00 | 150.00 | -1.00 | -1.00 | 6.40 ± 0.4 |

†A, Starch (g/l); B, Glucose (g/l); C, Peptone (g/l); D, $NH_4NO_3$ (g/l); E, $MgSO_4.7H_2O$ (g/l); F, Incubation period (h); G, pH; H, Temperature (°C); J, Agitation (rpm); K, L = Dummy variables

*(b) Regression analysis*[‡]

| Source | Sum of squares | Degree of freedom | Mean square | F-value | Prob>F |
|---|---|---|---|---|---|
| Model | 567.94 | 9 | 63.10 | 64.23 | 0.0154 |
| A | 7.89 | 1 | 7.89 | 8.03 | 0.1052 |
| B | 19.88 | 1 | 19.88 | 20.24 | 0.046 |
| C* | 174.38 | 1 | 174.38 | 177.5 | 0.0056 |
| D | 14.75 | 1 | 14.75 | 15.01 | 0.0606 |
| E | 1.31 | 1 | 1.31 | 1.33 | 0.3681 |
| F* | 130.37 | 1 | 130.37 | 132.71 | 0.0075 |
| G | 21.76 | 1 | 21.76 | 22.15 | 0.0423 |
| H* | 166.31 | 1 | 166.31 | 169.28 | 0.0059 |
| J | 31.28 | 1 | 31.28 | 31.84 | 0.03 |
| Residual | 1.97 | 2 | 0.98 | | |
| Total correlation | 569.90 | 11 | | | |

[†]A, Starch (g/l); B, Glucose (g/l); C, Peptone (g/l); D, $NH_4NO_3$ (g/l); E, $MgSO_4.7H_2O$ (g/l); F, Incubation period (h); G, pH; H, Temperature (°C); J, Agitation (rpm); K, L = Dummy variables.

[‡]Coefficient of determination ($R^2$) = 0.9966

Adj R-Squared 0.9810

Pred R-Squared 0.8759

Adeq Precision 26.918

*Significant factors selected for optimization by RSM

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

RSM using CCD was applied to determine the optimal levels of the three selected variables (peptone, incubation period and temperature) which significantly influenced phytase production by *T. lanuginosus* SSBP. The optimum levels of the three selected variables and their interactions were studied for enhanced phytase production. Table 2.2a shows the experimental range of independent variables at five different levels. A total of 20 experiments were carried out in different combinations of variables as per the CCD. The observed responses of experiments are shown as phytase production in Table 2.2b. Highest phytase production of 71.55 ± 4.51 U/ml was observed in run 11 while the first run resulted in lowest production of 34.92 ± 2.20 U/ml phytase. Results of the experiments were analysed by standard ANOVA (Table 2.2c). The following second-order polynomial equation was obtained that explains phytase production as a function of the three variables:

$$\mathbf{Y\ (U/ml) = 70.78 + 2.42A + 6.08B - 2.16C - 10.29A^2 - 5.23B^2 - 8.93C^2 + 0.7AB + 0.68AC + 0.67BC} \qquad \text{Equation 2.3}$$

The model F-values of 591.23 and 'Prob>F value' of < 0.0001 implied the model was highly significant. A prob>F value of 0.3259 (p>0.05) indicated that the lack of fit test was not significant and the fitting degree of Equation 2.3 was acceptable.

**Table 2.2** Experimental range (a), design (b) and ANOVA analysis (c) for optimization of phytase production by *T. lanuginosus* SSBP using response surface methodology

*(a) Experimental range and level of independent variables*

| **Variables** | **Symbol** | **Range and level** | | | | |
|---|---|---|---|---|---|---|
| | | **-α** | **-1** | **0** | **+1** | **+α** |
| Peptone (g/l) | $x_1$ | 07.50 | 11.05 | 16.25 | 21.45 | 25.00 |
| Incubation period (h) | $x_2$ | 24.00 | 30.08 | 39.00 | 47.92 | 54.00 |
| Temperature (°C) | $x_3$ | 40.00 | 43.04 | 47.50 | 51.96 | 55.00 |

*(b) Experimental design for RSM studies*

| **Run no.** | **Values of variables** | | | **Phytase production (U/ml)** |
|---|---|---|---|---|
| | **$x_1$** | **$x_2$** | **$x_3$** | |
| 1 | 11.05 | 30.08 | 51.96 | 34.92 ± 2.20 |
| 2 | 16.25 | 24.00 | 47.50 | 46.57 ± 3.40 |
| 3 | 16.25 | 39.00 | 40.00 | 48.85 ± 2.98 |
| 4 | 16.25 | 39.00 | 47.50 | 71.47 ± 3.79 |
| 5 | 16.25 | 39.00 | 47.50 | 70.40 ± 4.44 |
| 6 | 16.25 | 39.00 | 47.50 | 70.62 ± 5.01 |
| 7 | 11.05 | 30.08 | 43.04 | 41.95 ± 2.14 |
| 8 | 11.05 | 47.92 | 51.96 | 47.37 ± 2.98 |
| 9 | 7.50 | 39.00 | 47.50 | 37.07 ± 2.15 |
| 10 | 21.45 | 47.92 | 43.04 | 56.45 ± 4.69 |
| 11 | 16.25 | 39.00 | 47.50 | 71.55 ± 4.51 |
| 12 | 21.45 | 30.08 | 51.96 | 38.95 ± 2.26 |
| 13 | 16.25 | 54.00 | 47.50 | 65.45 ± 4.58 |
| 14 | 16.25 | 39.00 | 47.50 | 70.92 ± 4.47 |
| 15 | 25.00 | 39.00 | 47.50 | 46.32 ± 3.15 |
| 16 | 11.05 | 47.92 | 43.04 | 52.30 ± 3.29 |
| 17 | 21.45 | 47.92 | 51.96 | 54.85 ± 3.46 |
| 18 | 21.45 | 30.08 | 43.04 | 43.82 ± 1.88 |
| 19 | 16.25 | 39.00 | 47.50 | 69.70 ± 3.55 |
| 20 | 16.25 | 39.00 | 55.00 | 42.22 ± 2.53 |

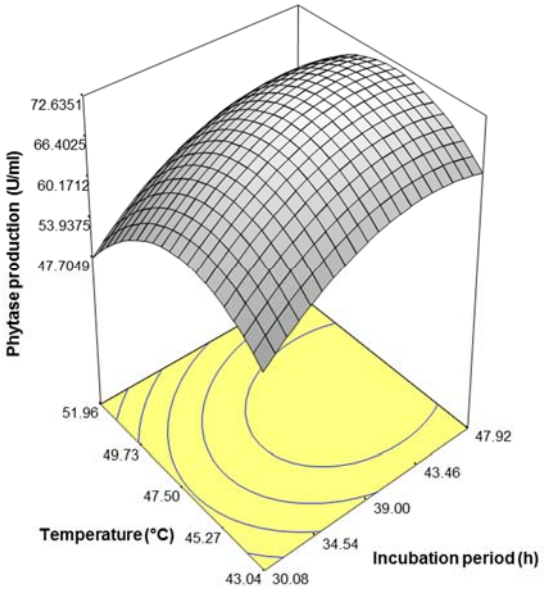
*(c) ANOVA values for phytase production*

| Source | Sum of Squares | Degree of freedom | Mean Square | *F*-value | P-value (Prob>F) | Significance |
|---|---|---|---|---|---|---|
| Model | 3274.72 | 9 | 363.86 | 591.23 | < 0.0001 | significant |
| Residual | 10 | 0.62 | 6.15 | | | |
| Lack of Fit | 3.72 | 5 | 0.74 | 1.53 | 0.3259 | not significant |
| Total | 3280.88 | 19 | | | | |

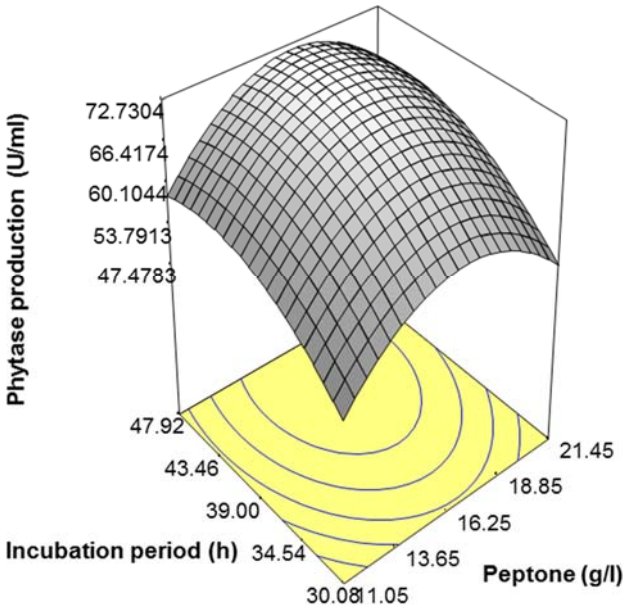
†$R^2$ = 0.9981; Adjusted $R^2$ = 0.9964; Pred $R^2$ = 0.9902

Phytase production: $Y = 70.78 + 2.42A + 6.08B - 2.16C - 10.29A^2 - 5.23B^2 - 8.93C^2 + 0.7AB + 0.68AC + 0.67BC$

Three-dimensional (3D) response surface contour plots were generated to evaluate the optimal levels and interactions among selected significant variables for phytase production. The three-dimensional response surface contour plots for phytase production are shown in Figures 2.1a and 2.1b. The interaction of two variables was studied while keeping another variable fixed at its '0' level. Figure 2.1a shows increase in phytase production with increase in temperature and incubation period. Similarly, increase in peptone concentration and incubation period increased phytase production from *T. lanuginosus* SSBP (Figure 2.1b).




**(a)**




**(b)**

**Figure 2.1** Response surface contours showing the effect of interaction between (a) temperature and incubation period and (b) incubation period and peptone on phytase production by *T. lanuginosus* SSBP.

### 2.3.3 Validation of the model

The model was further validated by determining phytase prodcution in three suggested levels of interaction between the significant variables. A combination of peptone (16.24 g/l), incubation period (46.33 h) and temperature (47.68°C) in the optimized medium resulted in maximum phytase production of 71.52 ± 4.5 U/ml, which was in good agreement with predicted value of 72.17 U/ml proving the validity of the model (Table 2.3).

**Table 2.3** Validation of the model

| Run number | Solutions for the variables | | | Phytase production (U/ml) | |
|---|---|---|---|---|---|
| | $x_1$ | $x_2$ | $x_3$ | Observed | Predicted |
| 1 | 15.65 | 42.96 | 47.81 | 71.08 ± 5.5 | 71.82 |
| 2 | 16.24 | 46.33 | 47.68 | 71.52 ± 4.5 | 72.17 |
| 3 | 16.16 | 43.08 | 46.68 | 70.71± 5.7 | 72.46 |

### 2.3.4 Phytase production in shake-flasks of varied volumes

Additional validation experiments were conducted in varying shake flasks volumes and a 5 l laboratory fermenter under the optimized conditions (Table 2.4). Production of phytase declined when shake flasks of higher volumes were used.

**Table 2.4** Phytase production in shake-flasks and batch production in a bench-top laboratory fermenter

| Medium volume (ml) | Volume of container (ml) | Phytase production (U/ml) |
|---|---|---|
| **Shake-flasks** | | |
| 50 | 250 | 71.53 ± 5.1 |
| 100 | 500 | 69.35 ± 4.0 |
| 200 | 1000 | 62.78 ± 4.9 |
| **Fermenter** | | |
| 2000 (batch) | 5000 | 69.95 ± 4.5 |
| 2000 (fed-batch) | 5000 | 109.17 ± 4.5 |

### 2.3.5 Batch fermentation in a 5 l laboratory fermenter

Phytase production was growth-associated, with a continuous decline in pH and dissolved oxygen saturation observed with an increase in biomass (Fig. 2.2). A maximum of 69.41±4.5 U/ml phytase was produced after 48 h.

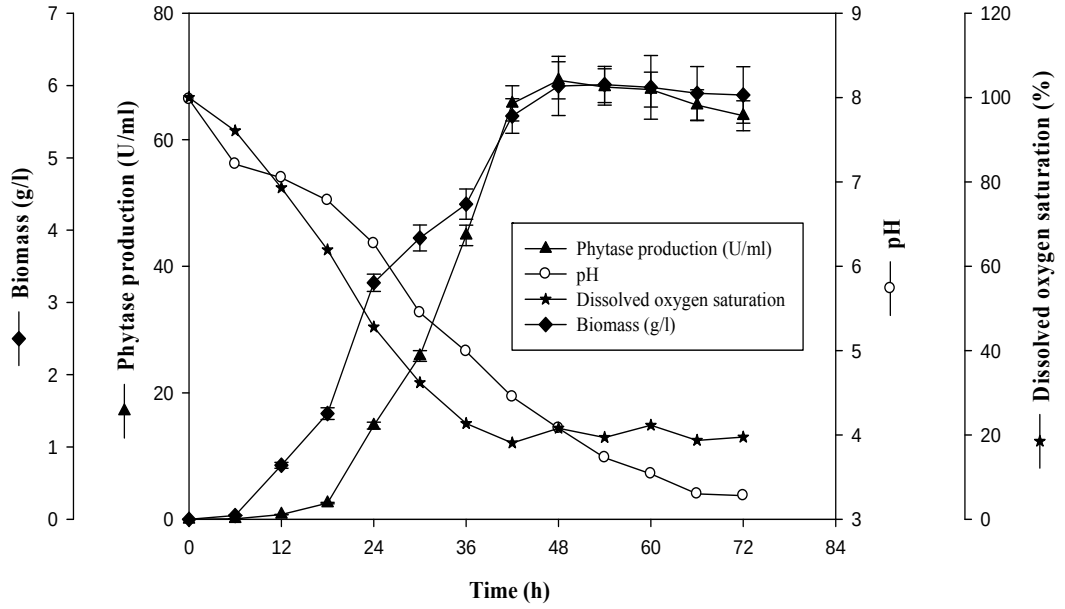



**Figure 2.2** Batch fermentation profile of phytase production by *T. lanuginosus* SSBP in a 5 l laboratory fermenter under statistically optimized conditions. The fermenter was operated at 47.7°C for 72 h and 1 vvm air. DO saturation was maintained at 20% using a cascade controller.

### 2.3.6 Preliminary optimization for fed-batch fermentation

#### *2.3.6.1 Selection of time of feed*

Maximum phytase production was observed when fed with 5 g/l glucose at the mid phase 30 h of production (Fig. 2.3a). The production level decreased when glucose was fed during the early phase of production 24 h or late when production is reaching stationary phase 42 h.

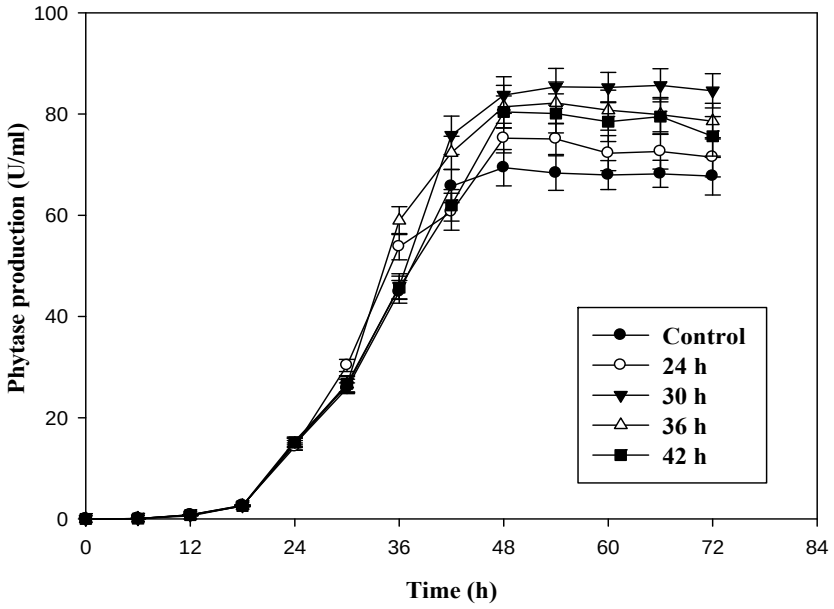



**Figure 2.3 (a**) Effect of time of feed for phytase production using 10 ml of 100 g/l glucose in a 5 l fermenter after different time intervals of time in separate experiments.

### *2.3.6.2 Optimization of glucose concentration*

Different glucose concentrations were assessed for their effect on phytase production. Maximum phytase production was observed with 10 ml feeding of 700 g/l glucose (Fig. 2.3b).

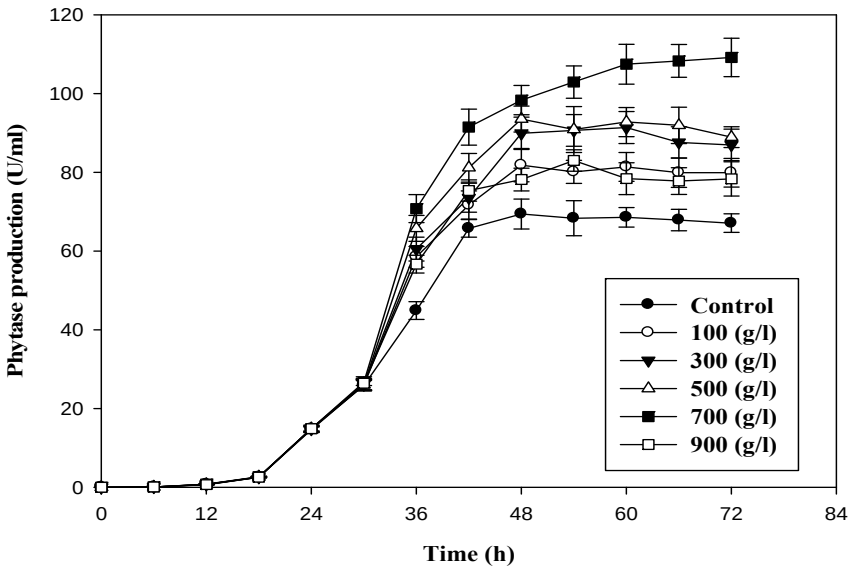



**Figure 2.3 (b)** Effect of feeding different concentrations of glucose after 30 h on phytase production by *T. lanuginosus* SSBP in a 5 l fermenter in separate experiments.

### 2.3.7 Fed-batch fermentation

Based on optimization of feeding time and glucose concentration, 700 g/l glucose was fed after every 30 h until 90 h, it maintained sustained production of phytase up to 120 h. Maximum phytase production observed was 167.63 U/ml after 108 h.

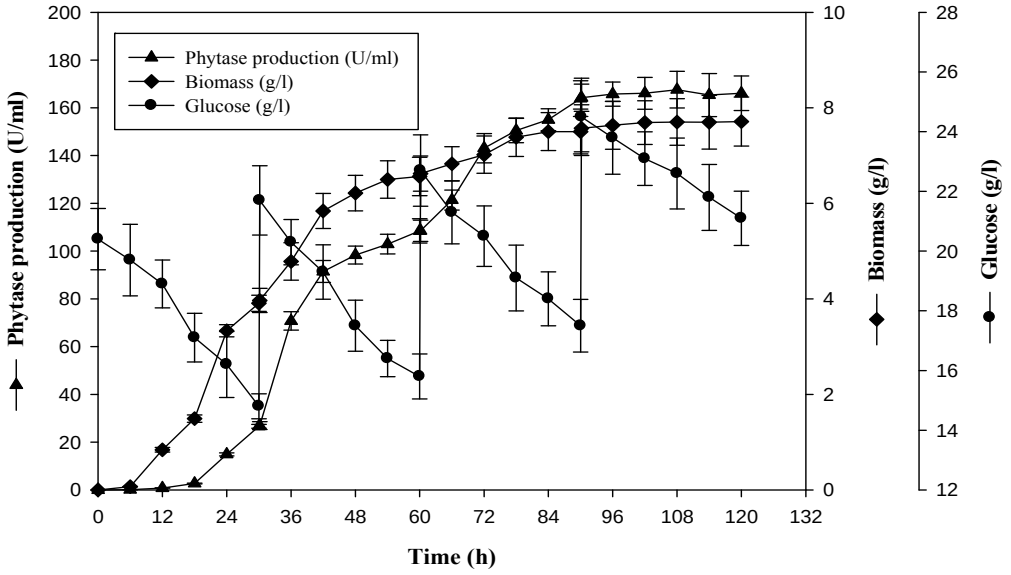



**Figure 2.3 (c)** Fed-batch production of phytase by *T. lanuginosus* SSBP in a 5 l fermenter. An optimized glucose feed of 700 g/l was fed every 30 h.

**Table 2.5** Fold improvement in phytase production by *T. lanuginosus* SSBP for different growth strategies

| Step | Phytase production (U/ml) | Fold |
|---|---|---|
| **Unoptimized** | 8.35 ± 0.40 | 1 |
| **PBD** | 24.11 ± 0.98 | 2.89 |
| **RSM (shake-flask)** | 71.53 ± 5.1 | 8.57 |
| **Fed-batch fermentation** | 109.17 ± 4.5 | 13.07 |

## 2.4 DISCUSSION

There are few imperative viewpoints that need to be taken into consideration for the development of an optimized bioprocess. These include selection of a suitable microorganism, identification of significant nutritional and physical parameters and optimization using appropriate methods for enhanced production of target metabolite/enzyme. Microorganisms differ in their selection for nutritional components such as source of carbon, nitrogen, phosphorus, sulphur etc. and physical parameters such as pH, temperature and agitation (Pandey, 2003). However, production of the target metabolite is dependent on a complex network of corresponding metabolic pathways and interaction of several parameters. From laboratory to pilot-scale production, strategies to improve enzyme production are extremely important and determine the revenue generation of leading biotechnology-based companies.

In this study, phytase production in the thermophilic filamentous fungus *T. lanuginosus* was investigated after identification of phytase genes in the genome sequence data in our laboratory (Mchunu et al., 2013). The fungus is a xylanase-super producer, while its secretome confirms presence of several other industrial enzymes (Winger et al., 2014). Phytase from *T. lanuginosus* was reported previously. However, Gulati et al. (2007) only used the one-variable-at-a-time approach to identify sodium nitrate, inoculum level and inoculum age without performing a study to understand the significance (p-values) of the selected factors for further optimization by RSM. Similarly, Berikten and Kivanc (2014) used one-variable-at-a-time approach to study the effect of variables such as substrate type, moisture, culture time, and inoculum size. Therefore, the present study first employed PBD for statistical evaluation of nine variables that influenced phytase production. Two unassigned variables were also included as artificial variables in the design to test homogeneity of regressions. There were 12 experimental runs with different variables and each variable was tested at a low and a high value (Table 2.1a). The two values were chosen such that the difference between the values is large enough to ensure inclusion of peak conditions for maximum phytase production. The coefficient of determination ($R^2$) value of 0.9966, a p-value (prob>F) of 0.0154 and model F-value of 64.23 indicated an appropriate and highly significant model. The ‘adequate precision’ value of 26.92 for phytase production indicated that the model may be used to navigate the design space.

Phytase production varied highly (0.60 to 24.11 U/ml) reflecting the importance of medium optimization to achieve higher production (Table 2.1a). After assessing the effect of various independent variables, peptone, temperature and incubation period were identified as the most significant variables affecting phytase production from *T. lanuginosus* SSBP **(**Table 2.1b).

There are many reports indicating the influence of peptone on enzyme production. Deepak et al. (2008) reported that 1% peptone had a significant effect on nattokinase production from *B. subtilis*, while 0.45% soybean peptone was significant for bacteriocin production from *Lactococcus lactis* (Li et al., 2002). Similarily, Teng and Xu (2008) reported that 4.06% peptone had a significant effect on lipase production. Similarly, phytase production from *A. niger* CFR 335 also increased due to peptone during submerged fermentation (Gunashree and Venkateswaran, 2008). Peptone was one of the significant factors at low levels (1%) during optimization of phytase production by another themophilic fungus, *S. thermophile*, (Singh and Satyanarayana, 2008). The growth of microorganisms and production of several growth-associated secondary metabolites are significantly affected by presence of amino acids and peptides. Peptone is a rich source of amino acids and peptides which explains why it outperformed ammonium nitrate as the source of nitrogen by showing enhanced effect on phytase production (Makolomakwa et al., 2017).

Incubation temperature is one of the vital parameters that determines the production, chances of contamination and ultimately the cost of enzymes. Large scale production in bioreactors operated at high temperatures reduces the chances of contamination by routine mesophilic microorganisms. Identification of temperature as one of the significant factors affecting phytase production by thermophilic *T. lanuginosus* SSBP was obvious. There are reports on temperature as a significant factor for enzyme production by several thermophiles and psychrophiles. Recently, Shajahan et al. (2016) showed temperature as a significant factor affecting cellulase production from *Bacillus licheniformis* NCIM 5556. Significance of temperature was also observed in pullulanase production from the psychrophilic *Exiguobacterium* sp. SH3 (Rajaei et al., 2014). Phytase was also affected significantly by incubation period as reported previously by Chadha et al. (2004) during the production of phytase by *Rhizomucor pusillus*. A regression analysis of PBD results with adequate precision value of 26.92, that measures the signal to noise ratio, indicated an adequate signal of accuracy. The

predicted R-squared value of 0.8759 was also in reasonable agreement with adjusted R-squared value of 0.9810 (Table 2.1b).

The selected independent variables (temperature, peptone and incubation period) were further analyzed to study their interactions and to determine their optimum levels for improved phytase production. For this, RSM using central composite design was employed. The three independent variables were studied at five different levels (Table 2.2a). A set of 20 experiments with six replicates at central point were conducted (Table 2.1b). Phytase activity ranged from 34.92 ± 2.20 U/ml, with 11.05 g/l peptone in the medium when incubated at 51.96°C for 30.08 h, to 71.55 ± 4.51 U/ml with 16.25 g/l peptone in the medium when incubated at 47.5°C for 39 h (Table 2.2b). All 20 experimental results of phytase production values were analyzed using analysis of variance (ANOVA). The regression equation obtained after ANOVA analysis for the optimization of production parameters revealed phytase production (Y) as a function of peptone, incubation period and temperature. Phytase production was explicitly explained by the following equation:

$$Y = 70.78 + 2.42A + 6.08B - 2.16C - 10.29A^2 - 5.23B^2 - 8.93C^2 + 0.7AB + 0.68AC + 0.67BC$$

where, phytase production is represented by Y and A is peptone, B is incubation period and C is temperature. AB, BC and AC are the interaction terms and $A^2$, $B^2$ and $C^2$ are the quadratic terms. The model *F*-value of 591.23 implied that the model was significant and there is only a 0.01% chance that this large value could occur due to noise. Models having a Prob > *F* values less than 0.05 are considered significant (Table 2c). In this investigation we obtained a Prob > *F* of < 0.0001 which further confirms the high significance of the model. Similarly, a non-significant lack of fit value of 3.72 implies that the data fits well with the model. The coefficient of determination ($R^2$) was calculated to be 0.9981, indicating that 99.81% variability in the response could be explained by the model (Table 2c). The $R^2$ value near 1 indicates that the model is fit and it can predict the response better (Singh and Satyanarayana, 2008; Kaur and Satyanarayana, 2005). A strong agreement between experimental and predicted values was observed due to close values of correlation coefficients with adjusted $R^2$ of 0.9964 and predicted $R^2$ of 0.9902 (Table 2c). The model was further validated by investigating the interaction amongst the three variables at different levels as suggested by the software. Maximum phytase

production of 71.52 U/ml was observed with 16.24 g/l peptone in the medium when incubated at 47.68°C for 46.33 h. Phytase production was in good correlation with the predicted value of 72.17 U/ml reflecting the validity and applicability of the model for optimization (Table 2.3).

The statistical optimization resulted in about 8.56-fold improvement in phytase production and reduced production time by 24 h. A similar 8.41-fold increase in phytase production from *Rhizopus oryzae* was reported by Rani and Ghosh (2011) due to statistical optimization of mannitol, ammonium sulfate and phosphate concentrations in the production medium. Berikten and Kivanc (2014) recorded a 10.83-fold increase in phytase production from *T. lanuginosus* after seven days in solid state fermentation. There are also a few studies that report much higher fold increase in phytase production due to statistical optimization. Phytase production from *B. subtilis* K46b and *A. niger* NCIM 563 was increased by 28-fold and 36.67-fold, respectively (Rocky-Salimi et al., 2017; Buddhiwant et al., 2016). However, Murat et al. (2015) could only achieve 1.09-fold increase after optimizing phytase production from *Bacillus* sp. EBD 9-1. In this study, phytase production was also carried out in different volumes of shake flasks and a 5 l fermenter (Table 2.4). A slightly reduced production in flasks of higher volumes could be attributed to inadequate aeration (Makolomakwa et al., 2017). Up-scaling the cultivation of fungus in the 5 l fermenter improved aeration and the maximum production was 69.95 U/ml. It has been indicated that inadequate mixing can affect phytase production in vessels of larger volumes (Singh and Satyanarayana, 2008).

Batch fermentation was conducted in a 5 l fermenter with 2 l working volume and maximum phytase production was recorded after 48 h. There are similar reports on fungal phytase production reaching maximal levels at 48 h. The thermophilic strains of *S. thermophile* and *R. pusillus* also showed maximum phytase levels after 48 h (Singh and Satyanarayana, 2008; Chadha et al., 2004) Similarly, *H. nigrescens* phytase reached maximum production levels after 48 h (Bala et al., 2014). The enzyme production started to drop after 54 h which could be due to depletion of essential nutrients. During this study, pH of the production medium, which was initially 8.0, continued to decline throughout the fermentation as was reported previously during phytase production by *Aspergillus* sp. L117 (Lee et al., 2005). The final pH was 4.1 after 48 h when maximum phytase production was attained (Fig.

2.2). Decrease in pH can be attributed to the production of secondary acidic metabolites from glucose. The sharp decline in dissolved oxygen saturation levels during the first 48 h was indicative of high oxygen demand during exponential growth of the fungus (Makolomakwa et al., 2017). There are many reports on production of acidic phytases under acidic pH conditions. *H. nigrescens* phytase was produced at pH 5.0 (Bala et al., 2014), whereas *S. thermophile* and *T. aurantiacus* phytases were produced at pH 5.5 (Singh and Satyanarayana 2006; Nampoothiri et al., 2004). The growth-associated production of phytase from *T. lanuginosus* SSBP is in accordance with similar reports from several other fungi (Greiner, 2004; Vats and Banerjee, 2005). Microbial phytase production is mostly inducible in nature. However, no improvement in phytase production was observed due to addition of sodium phytate in the medium, which indicates constitutive expression of phytase in *T. lanuginosus* SSBP. Previously, Sabu et al. (2002) and Sato et al. (2014) also reported constitutive expression of phytase in *Rhizopus oligosporus*.

Attempts were made to further enhance phytase production by fed batch cultivation of *T. lanuginosus*. Preliminary studies were conducted to optimize the feeding time and feed concentration. Use of glucose as feed in fed-batch fermentation has been routinely used for enhanced phytase production. It was observed that the production of phytase did not decline when fed with 700 g/l of glucose and reached 109.17 U/ml after 72 h (Fig. 2.3b). Therefore, feeding 10 ml of 700 g/l glucose after 30 h was selected for fed-batch production of phytase using *T. lanuginosus* SSBP. Coban and Demirci (2014) produced comparable phytase levels as that of the present study using fed-batch cultivation of *A. ficuum* NRRL 3135. Kleist et al. (2003) developed a rapid glucose controlling system in *E. coli* to achieve 120 U/ml phytase in 14 h. Fed-batch fermentation was also used to enhance phytase production by several recombinant hosts (Tran et al., 2010; Jin et al., 2007). Glucose feeding at different times during exponential phase resulted in higher production in all experiments and a maximum of 85.68 U/ml phytase was attained after feeding at 30 h (Fig. 2.3a). The improved production levels and advantages of working with a thermophilic strain makes *T. lanuginosus* SSBP a promising choice for commercial production of phytase.

# CHAPTER 3: PURIFICATION AND COMPARATIVE CHARACTERIZATION OF Tlphy1 AND Tlphy2 FROM *T. lanuginosus* SSBP

***MOST OF THE WORK PRESENTED IN THIS CHAPTER WAS PUBLISHED IN Bioresource Technology, 235, pp.396-404,* 2017.**

## 3.1 INTRODUCTION

It is important to remove interfering proteins or peptides and all other non-protein components before characterising the protein of interest. Different purification strategies are based on physical and chemical properties of the target protein such as solubility, molecular size, charge, hydrophobic interactions, specific affinity patterns etc. Most common steps of a classical purification strategy involve protein concentration followed by ion-exchange and/or gel filtration chromatography. There is a gradual increase in specific activity of the target protein after each purification step due to removal of contaminating proteins. Generally, it is common to have more than one purification step to achieve the desired purity. The protein purification process is easier nowadays due to efficient, accurate, time-saving and less costly technological advancements during the last few decades. However, it is important to optimize several factors such as saturation level of ammonium sulphate, pH, ionic strength of the buffers, flow rate of the mobile phase etc. to develop a purification strategy that saves time and maximizes recovery of the target protein.

Selection of appropriate matrix for the target protein is the most important step during ion-exchange and gel filtration chromatography. Protein separation in ion-exchange chromatography is largely dependent on the reversible and selective adsorption of charged proteins to oppositely charged ion-exchange group of the matrix. There are different types of matrices available commercially with a range of available functional groups; strongly acidic matrices feature sulfonic acid groups, strongly basic matrices feature quaternary amino groups, weakly acidic matrices feature carboxylic acid groups while the weakly basic matrices may have primary, secondary, and/or tertiary amino groups. Ion-exchange resins can be anionic such as DEAE (diethylaminoethyl), QAE (quatenary aminoethyl), Q (quatenary ammonium) etc. or cationic such as CM (carboxymethyl), S (sulphoethyl), SP (sulphopropyl) etc. Traditionally a column needs to be packed manually with selected resins but recent advancement in technologies offer direct and easy procurement of pre-packed columns. Similarly, pre-packed gel filtration column of desired pore size can be selected and commercially

procured based on the molecular weight of protein of interest. There is a wide range of cross-linked agarose and/or dextran, polyacrylamide-based resins available commercially. Availability of efficient instruments such as different series of AKTA Purifiers (GE Healthcare, Sweden) equipped with efficient pumps that maintain accurate pressure over the column-bed and have automated fractionators, has made the purification process easier.

The homogeneity of the target protein must be checked on the electrophoretic gel and on a chromatogram (Ward and Swiatek, 2009). Quantitative confirmation of the target protein by calculating specific enzyme activity and qualitative confirmation by an appropriate gel staining technique such as zymogram, which is based on substrate hydrolysis, is important for proper identification and confirmation of purity. The search for a catalytically efficient phytase, with broad pH, and temperature stability is an on-going effort, which is heavily reliant on proper purification techniques that allow accurate characterization as per the industrial needs. This chapter focuses on purification strategies and comparative characteristics of two purified phytases (designated as Tlphy1 and Tlphy2) from *T. lanuginosus* SSBP.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Purification of phytases

Phytases produced in a 5 l laboratory fermenter were concentrated by 55-85% ammonium sulphate precipitation. The precipitate obtained after overnight incubation at 4°C was suspended in 0.1 M sodium acetate buffer (pH 5.5) and desalted through a Hi-Prep 26/10 column (GE Healthcare) against 20 mM sodium acetate buffer (pH 5.5) using an AKTA Purifier 100 (GE Healthcare). The enzyme was further concentrated by ultrafiltration using a Sartocon Slice holder with 10 kDa molecular weight cut-off membrane cassettes (Sartorius, Germany). The retentate was loaded onto a 6 ml Resource Q (GE Healthcare) anion-exchange column equilibrated with 20 mM Tris-HCl buffer (pH 7.5). Proteins were eluted using a combination of isocratic and gradient steps with 0-1 M NaCl as eluent in 20 mM Tris-HCl buffer (pH 7.5). A constant flow rate of 1 ml/min was maintained during the process. The fractions showing phytase activity were pooled and the concentrated (Concentrator Plus, Eppendorf) sample was applied to Superdex 200 Increase 10/300 column (GE Healthcare). The proteins were eluted with 50 mM sodium acetate buffer (pH 5.5) at a flow rate of 0.5 ml/min. The active fractions were pooled and concentrated using Biomax Mr. 10 000 cut-off Millipore membrane (Millipore). Total protein concentration was determined by the method of Bradford (1976) with bovine serum albumin (BSA) as standard.

### 3.2.2 Molecular weight determination of phytase

SDS-PAGE was performed according to the method of Laemmli (1970) using 12% cross-linked polyacrylamide gels on a Mini PROTEAN gel electrophoresis unit (BioRad Laboratories). Activity staining of gel was used to confirm protein bands on gel corresponding to phytase according to the method of Bae et al. (1999) with minor modifications. The gel was washed twice in 2% (v/v) Triton X-100 for 45 min at room temperature to remove SDS followed by a 15 min wash in 0.1 M sodium acetate buffer (pH 5.5) at 4°C. Phytase activity was detected by incubating the gel in the same buffer containing 5 g/l sodium phytate at 4°C for 1 h followed by overnight incubation at 50°C. Bands with phytase activity were visualized by soaking the gel in 20 g/l aqueous cobalt chloride solution for 5 min and subsequently replacing the solution with equal volumes of 62.5 g/l aqueous ammonium molybdate and 4.2 g/l

aqueous ammonium vanadate solutions. Zones of hydrolysis due to phytase activity were observed against an opaque background (Makolomakwa et al., 2017).

### 3.2.3 Effect of pH on activity and stability

The effect of pH on Tlphy1 and Tlphy2 activity was studied over a pH range between 2.0 to 9.0 in different buffer systems [glycine-HCl buffer (pH 2.0–3.0), 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)]. The enzymes were pre-incubated with buffers of different pH and the residual enzyme activity was determined at intervals of 30 min up to a period of 2 h under standard assay conditions to study the pH stabilities of respective enzymes.

### 3.2.4 Effect of temperature on activity and stability

The effect of temperature on phytase activity was studied over a temperature range from 45°C to 90°C for Tlphy1 and 30°C to 80°C for Tlphy2 using the standard assay procedure. The thermostability of the enzymes were determined by pre-incubation at different temperatures and the residual enzyme activity was determined at an interval of 30 min up to a period of 2 h, under standard assay conditions.

### 3.2.5 Thermal denaturation kinetics and thermodynamic parameters of phytase

The thermal denaturation kinetics of phytase was studied using standard first order enzyme deactivation kinetics. Thermal inactivation rate constant ($k_d$) was calculated from the slope of the linear plots of ln [$E_t/E_0$] versus exposure time (t), where $E_0$ and $E_t$ are the initial (at t = 0) and residual (at t = t) phytase activities respectively. The $k_d$ values were calculated at 70°C, 80°C and 90°C for Tlphy1 and the slope of its natural logarithm (ln $k_d$) versus reciprocal of the absolute temperature (l/T) in Kelvin (Arrhenius plot) was used to determine activation energy for denaturation ($E_d$) of phytase. The thermodynamic parameters such as change in enthalpy (ΔH, kJ/mol), change in free energy (ΔG, kJ/mol) and change in entropy (ΔS, J/mol-K) for thermal denaturation of phytase were determined using the Eyring's absolute rate equation (Eyring and Stearn, 1939):

$$k_{\mathrm{d}} = \left(\frac{K_{\mathrm{b}}T}{h}\right) e^{(-\Delta H/RT)}\, e^{(-\Delta S/RT)} \quad (1)$$

$$\Delta \mathrm{H} = E_{\mathrm{d}} - \mathrm{RT} \quad (2)$$

$$\Delta \mathrm{G} = -\mathrm{RT} \ln \left(\frac{K_{\mathrm{d}}h}{K_{\mathrm{b}}T}\right) \quad (3)$$

$$\Delta \mathrm{S} = \frac{(\Delta \mathrm{H} - \Delta \mathrm{G})}{\mathrm{T}} \quad (4)$$

where $K_b$ [Boltzmann's constant (R/N)] =1.38 x $10^{-23}$ J/K, $h$ (Planck's constant) = 6.63 x $10^{34}$ J s, N (Avogadro's number) = 6.02 x $10^{23}$ per mol and R (gas constant) = 8.314 J/mol-K.

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

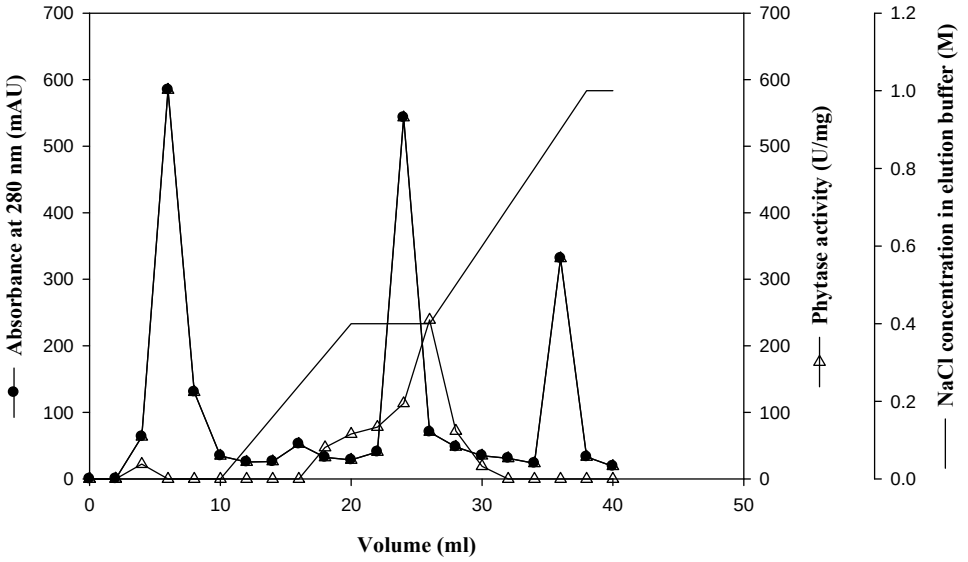
The effect of metal ions and surfactants was studied by pre-incubating the enzyme for 30 min at 50°C, 100 rpm with 1 and 5 mM concentration of various metal ions including $BaCl_2.2H_2O$, $CaCl_2.2H_2O$, $CoCl_2.2H_2O$, $CuSO_4.5H_2O$, $FeSO_4.7H_2O$, $MgSO_4.7H_2O$, $MnCl_2.4H_2O$, $PbCl_2$ and $ZnCl_2.4H_2O$, and 0.1–1.0 mM concentrations of non-ionic (Triton X-100, Tween-20, Tween-80), anionic (SDS) and cationic (CTAB) surfactants. The effect of 1 and 5 mM concentrations of EDTA and dithiothreitol (DTT) was also investigated. Untreated enzymes were used as controls. The residual activity was determined under standard assay conditions.

## 3.3 RESULTS

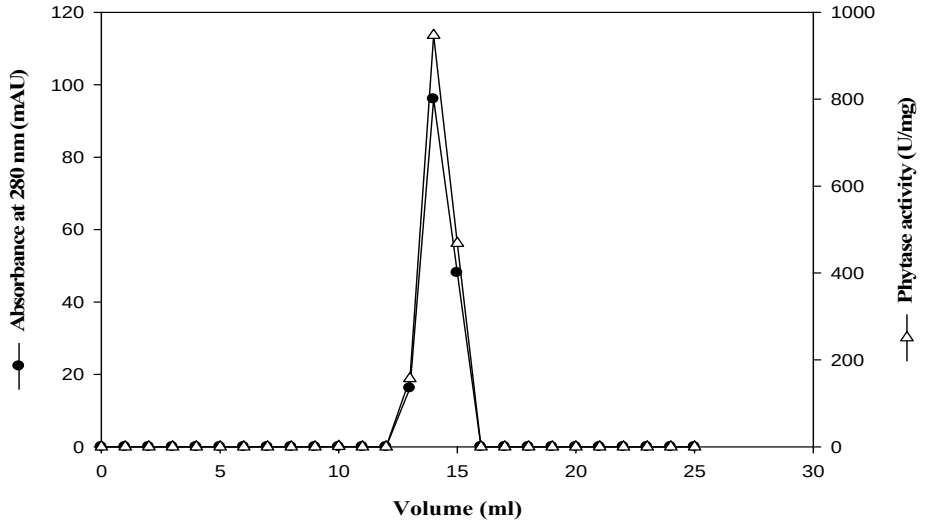
### 3.3.1 Purification of phytases and molecular mass determination

A series of purification steps (ammonium sulphate precipitation, ultrafiltration, ion exchange and size exclusion chromatography) were employed to purify both Tlphy1 and Tlphy2. After ammonium sulphate precipitation the concentrated enzyme solution was desalted using a Hi-Prep 26/10 column and was passed through a Resource Q anion-exchange column. Two peaks corresponding to phytase activities were observed in the chromatogram. The smaller first peak consisted of flow-through fractions that failed to bind to the matrix. The second peak was detected after passing 20 ml of elution buffer with a combination of isocratic and gradient elution steps (Fig 3.1a). Fractions showing phytase activity were eluted at a concentration of 0.4 M NaCl. Standard phytase assay was performed and it was observed that the most active fractions were present in the second peak. SDS-PAGE and zymogram analysis confirmed the presence of two phytases (Fig 3.2).

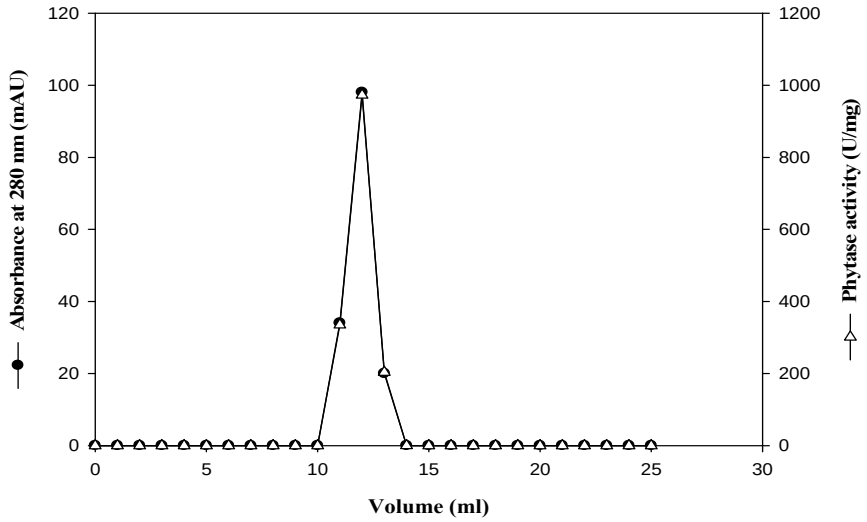
Active fractions were pooled, concentrated using Biomax Mr. 10 kDa cut-off Millipore membrane and used for size exclusion chromatography on a Superdex 200 Increase 10/300 column. During size exclusion chromatography, the bigger proteins were eluted first, as evident in Fig 3.1c. Tlphy2 was the first to be eluted and estimated as a 60 kDa protein on SDS-PAGE. Tlphy1 was eluted second (Fig 3.1b) and estimated as a 49 kDa protein on SDS-PAGE. Both phytases appeared as single bands on the gels. A detailed summary of purification steps is presented in (Table 3.2).


Absorbance at 280 nm (mAU)
Phytase activity (U/mg)
NaCl concentration in elution buffer (M)
Volume (ml)

**(a)**

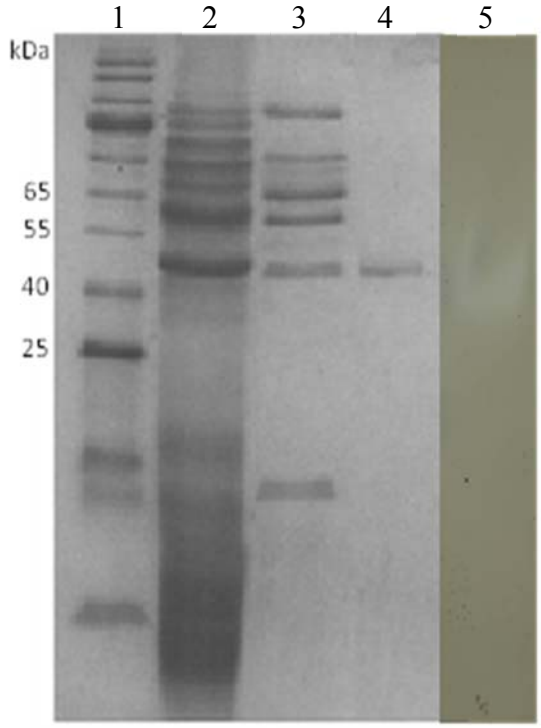

Absorbance at 280 nm (mAU)
Phytase activity (U/mg)
Volume (ml)

**(b)**

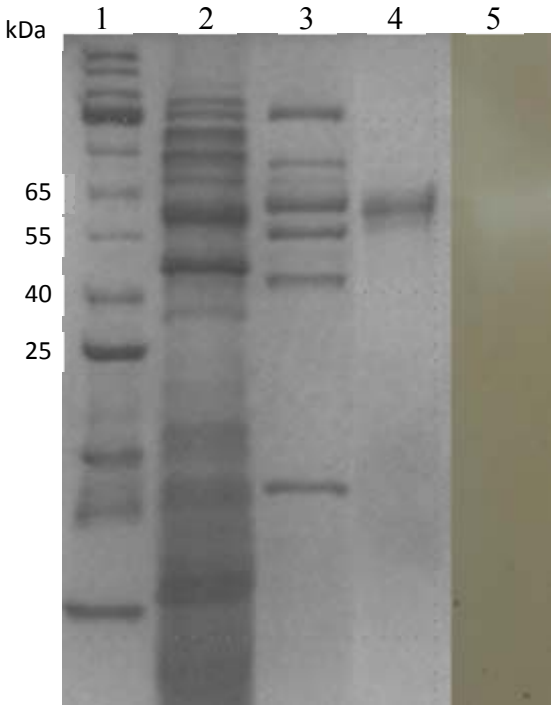



**(c)**

**Figure 3.1** Elution profiles of phytases from *T. lanuginosus* SSBP. (a) Anion exchange chromatography of active fractions using the Resource Q column. The column was initially eluted with 20 mM Tris-HCl buffer (pH 7.5) and subsequently with a linear gradient of 0–1 M NaCl in the same buffer with a flow rate of 1.0 ml/min. (b) Gel-filtration of active fractions (Tlphy1) from Resource Q column on a Superdex 200 Increase 10/300 column, which was eluted with 50 mM sodium acetate buffer (pH 5.5). (c) Gel-filtration of active fractions (Tlphy2) from Resource Q column on a Superdex 200 Increase 10/300 column, which was eluted with 50 mM sodium acetate buffer (pH 5.5). The flow rate was maintained at 0.5 ml/min for gel-filtration experiments.




**(a)**




**(b)**

**Figure 3.2** SDS-PAGE and zymogram analysis of Tlphy1 (a) and Tlphy2 (b) from *T. lanuginosus* SSBP. Lane 1, protein molecular weight marker; lane 2, crude extract; lane 3, sample after Resource Q anion-exchange chromatography; lane 4, purified phytases after Superdex gel-filtration chromatography; lane 5, zymogram analysis of Tlphy1 and Tlphy2.

**Table 3.1** Summary of purification steps for Tlphy1

| Purification Steps | Total activity (U) | Total protein (mg) | Specific activity (U/mg) | Yield (%) | Fold purification |
|---|---|---|---|---|---|
| Crude filtrate | 33231.05 | 522.03 | 63.66 | 100 | 1 |
| $(NH_4)_2SO_4$ precipitation | 24182.32 | 287.78 | 84.03 | 72.77 | 1.32 |
| Ultrafiltration | 20420.48 | 187.58 | 108.86 | 61.45 | 1.71 |
| Anion exchange (Resource Q) | 5167.69 | 10.26 | 503.67 | 21.37 | 7.91 |
| Gel filtration (Superdex) | 1713.86 | 1.09 | 1572.35 | 5.16 | 24.70 |

**Table 3.2** Summary of purification steps for Tlphy2

| Purification Steps | Total activity (U) | Total protein (mg) | Specific activity (U/mg) | Yield (%) | Fold purification |
|---|---|---|---|---|---|
| Crude filtrate | 32956.42 | 501.76 | 65.68 | 100 | 1 |
| $(NH_4)_2SO_4$ precipitation | 22556.49 | 244.37 | 92.3 | 68.44 | 1.4 |
| Ultrafiltration | 18425.83 | 178.32 | 103.33 | 55.91 | 1.57 |
| Anion exchange (Resource Q) | 6236.69 | 8.5 | 733.73 | 18.92 | 11.17 |
| Gel filtration (Superdex) | 2113.86 | 1.72 | 1228.99 | 6.41 | 18.71 |

Tlphy1 was purified to homogeneity with an overall purification of 24.70-fold and a yield of 5.16% with a specific activity of 1572.35 U/mg (Table 3.1). The purified enzyme had an apparent molecular mass of 49 kDa on SDS-PAGE gel (Fig. 3.2a). Similarly, Tlphy2 was purified to homogeneity with an overall purification of 18.71-fold and a yield of 6.41% with a specific activity of 1228.99 U/mg (Table 3.2). The purified enzyme had an apparent molecular mass of 60 kDa on SDS-PAGE gel (Fig. 3.2b).

### 3.3.2 Effect of temperature on activity and stability

Tlphy1 was active in the temperature range of 45-85°C, with optimal activity at 55°C (Fig 3.3). The enzyme was fairly stable between 50 and 70°C for 2 h (Fig 3.4). After first 30 min of incubation at 50°C, 60°C and 70°C, Tlphy1 retained 99%, 90% and 80% phytase activity, respectively. On further increasing the temperature to 80°C Tlphy1 could retain 52% activity, while only 32% activity was retained at 90°C after 30 min.

Further incubation up to 2 h showed no discernable effect on the activity of the enzyme, however, a loss of 29%, 46% and 85% activity was noted at 60°C, 70°C, and 80°C, respectively. Tlphy1 lost complete activity 90°C after 2 h.

Tlphy2 was active in the temperature range of 30-80°C, with optimal activity at 50°C (Fig 3.5). The enzyme was fairly stable between 40 and 60°C for 2 h (Fig 3.6). The enzyme was fairly stable at 40°C and retained 77% and 54% of activity after 2 h at 50°C and 60°C, respectively (Fig 3.6). Although the enzyme retained activity at higher temperatures and showed 68% and 40% activities after 30 min incubation at 70°C and 80°C, respectively, the activity was lost completely after incubation for 2 h at 80°C.

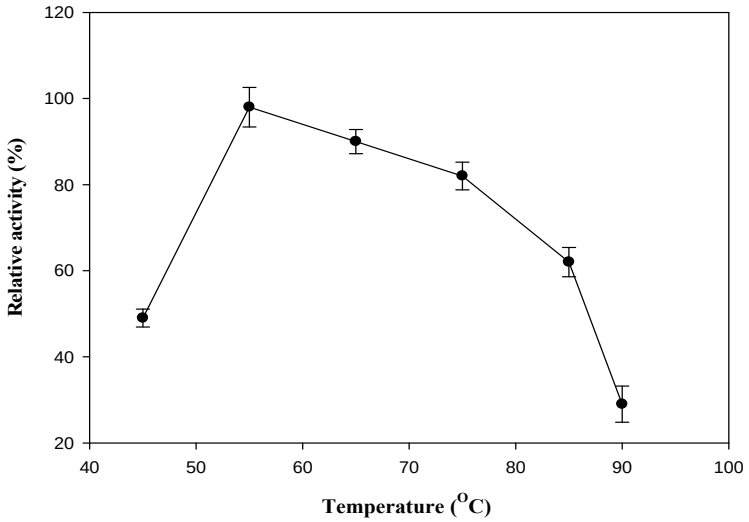



**Figure 3.3** Effect of temperature on Tlphy1 activity. Phytase activities were measured at pH 5.0 and expressed as relative percentages of the maximum activity taken as 100%. Each point represents the mean ± SD of triplicate experiments.

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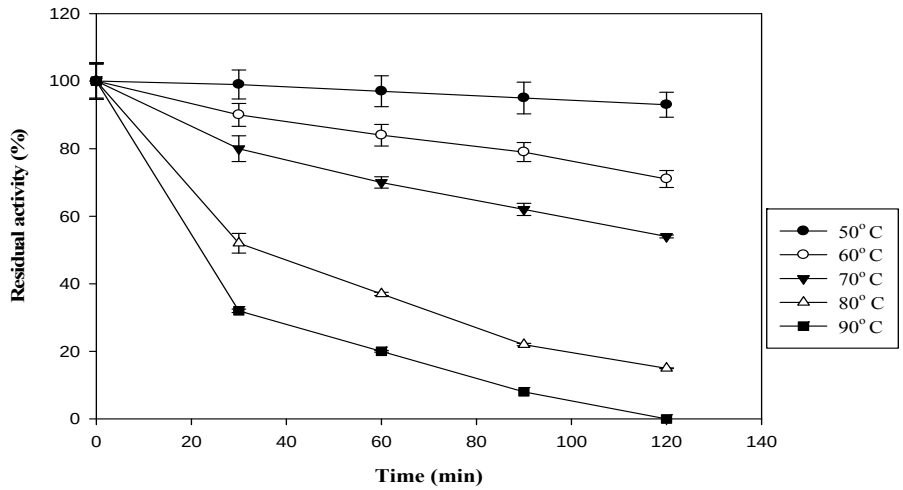


**Figure 3.4** Effect of temperature on purified Tlphy1 stability. Initial activity was regarded as 100% and residual activities with respect to initial activity were calculated at an interval of every 30 min using standard phytase assay. Each point represents the mean ± SD of triplicate experiments.

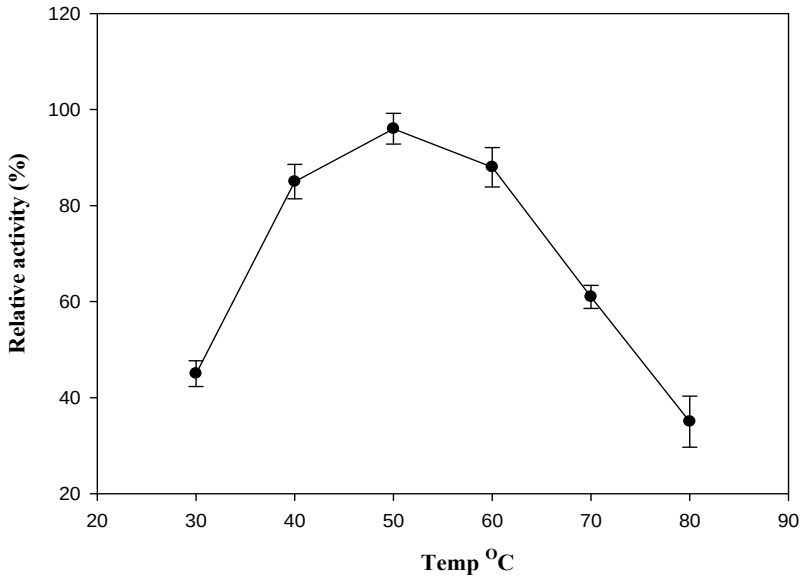


**Figure 3.5** Effect of temperature on Tlphy2 activity. The activities were measured at pH 5.0 and expressed as a percentage of the maximum activity taken as 100%. Each point represents the mean ± SD of triplicate experiments.

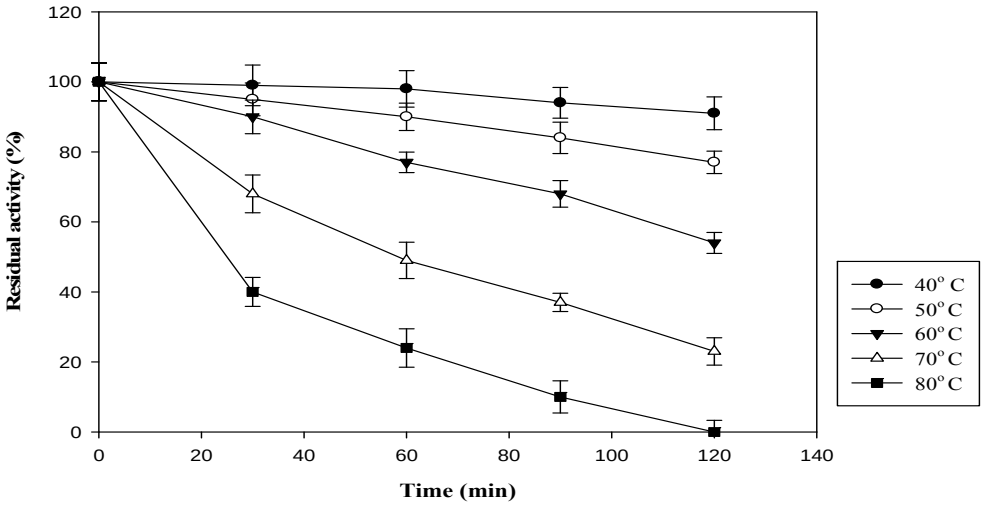


**Figure 3.6** Effect of temperature on stability of purified Tlphy2. Initial activity was regarded as 100% and residual activities with respect to initial activity were measured at an interval of every 30 min using standard phytase assay. Each point represents the mean ± SD of triplicate experiments.

### 3.3.3 Effect of pH on activity and stability

Phytase activity was measured as a function of pH from pH 2 to pH 9. Tlphy1 was active in the pH range of 3.0 to 8.0 with optimal activity at pH 5.0 (Fig 3.7). The enzyme was fairly stable between pH 4.0 and 6.0 for 2 h (Fig 3.8). There was a 16%, 28.2% and 42% loss of activity when the enzyme was incubated for 2 h at pH 4.0, 5.0 and 6.0, respectively. Highest loss in activity was noted at the two extremes of pH used in this study. Tlphy1 lost 95.8% of its activity at pH 2 while 88.7% loss in activity was recorded at pH 9. However, the enzyme retained 98.9%, 71.3%, 43.2 and 22% activity at pH 4, 7, 9 and 2, respectively after the first 30 min of incubation.

Tlphy2 was active in the pH range of 2.5 to 6.5 with optimal activity at pH 2.5 (Fig 3.9). The enzyme was fairly stable between pH 3.5 and 5.5 for 2 h (Fig 3.10). The enzyme lost only 20-25% activity in the acidic range between pH 4.5 and pH 5.5 over 2 h, while a considerable activity loss of 85-93% was noticed in the alkaline pH range from pH 7.5-8.5. Results indicated both phytases to be more stable in the acidic pH range.

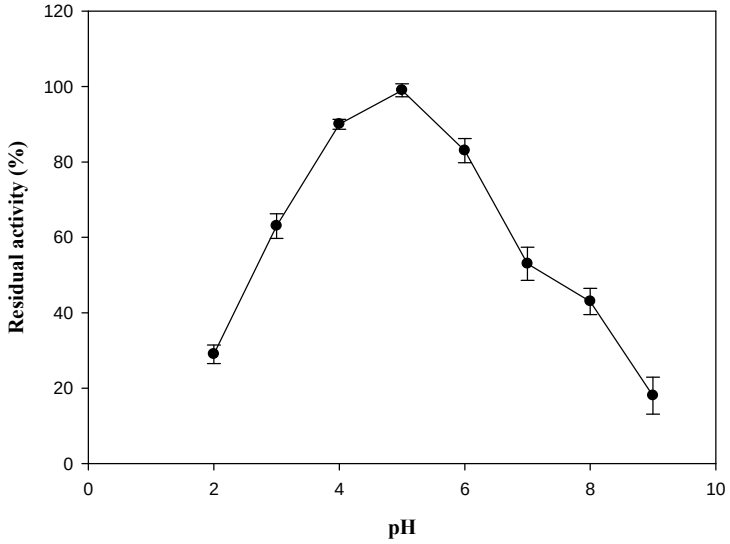


**Figure 3.7** Effect of pH on Tlphy1 activity. The activities were measured at 50°C and expressed as a percentage of the maximum activity taken as 100%. Each point represents the mean ± SD of triplicate experiments.

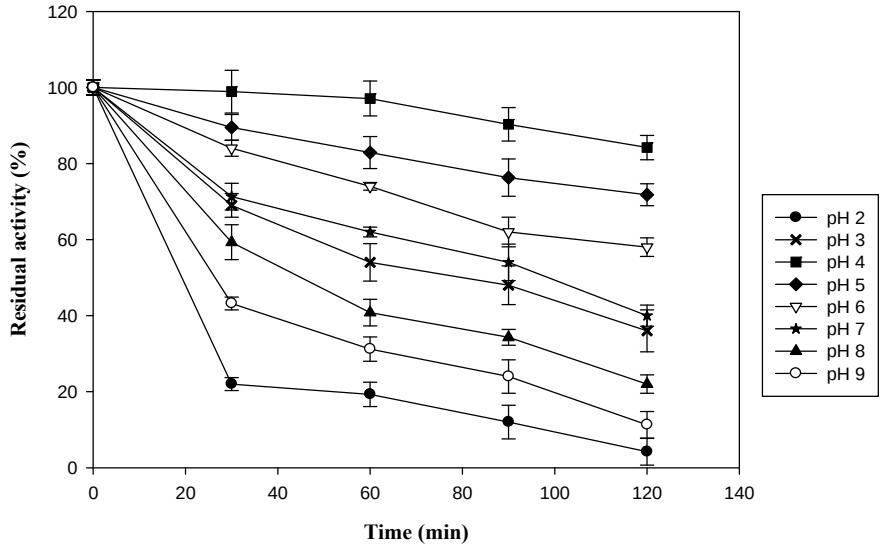


**Figure 3.8** Effect of pH on stability of Tlphy1. Initial activity was regarded as 100% and residual activities with respect to initial activity were measured at an interval of every 30 min using standard phytase assay. Each point represents the mean ± SD.

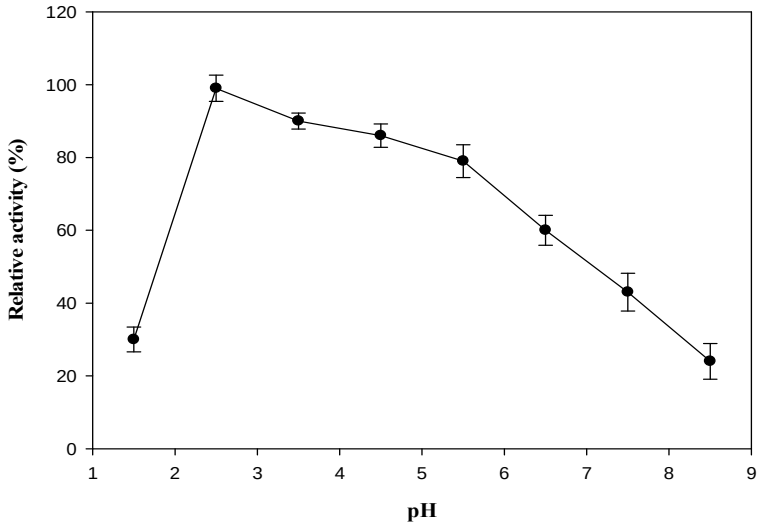


**Figure 3.9** Effect of pH on Tlphy2 activity. The activity was measured at 50°C and expressed as a percentage of the maximum activity taken as 100%. Each point represents the mean ± SD of triplicate experiments.

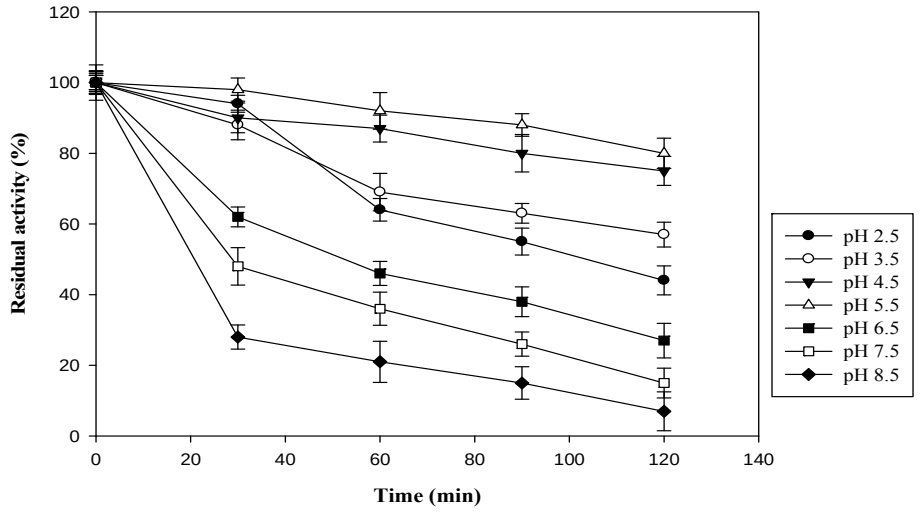


**Figure 3.10** Effect of pH on Tlphy2 activity. The activity was measured at 50°C and expressed as a percentage of the maximum activity taken as 100%. Each point represents the mean ± SD of triplicate experiments.

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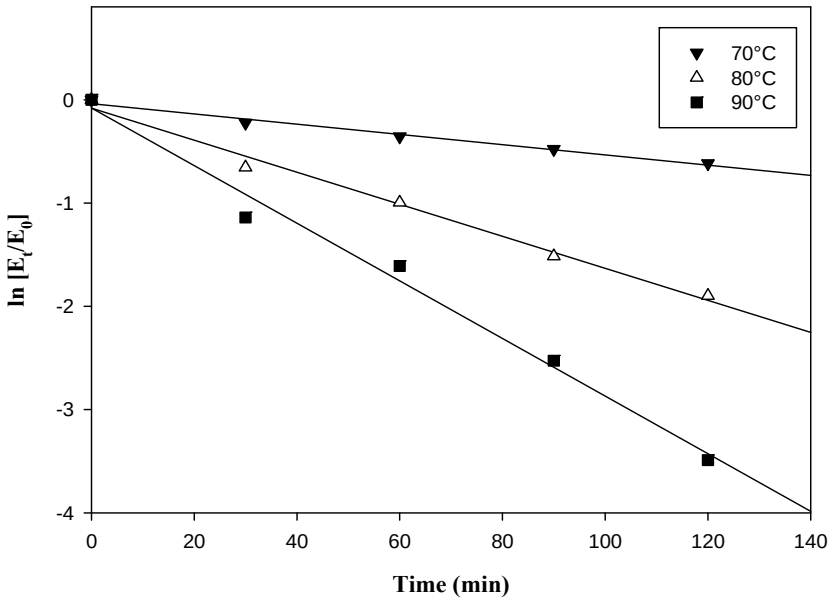


**Figure 3.11** Determination of thermal denaturation rate constant ($k_d$) of Tlphy1 at 70°C, 80°C and 90°C using the slope of ln [$E_t/E_0$] versus time plot.

**Table 3.3** Summary of purification steps for Tlphy2 during thermal deactivation at 70°C (343.15 K), 80°C (353.15 K) and 90°C (363.15 K)

| **Temp (K)** | $K_d$ **(min$^{-1}$)** | $T_{1/2}$ **(min)** | **ΔH (kJ/mol)** | **ΔG (kJ/mol)** | **ΔS (J/mol-K)** |
|---|---|---|---|---|---|
| 343.15 | 0.005 | 138.6 | 86.44 | 99.56 | -38.23 |
| 353.15 | 0.015 | 44.7 | 86.35 | 99.22 | -36.43 |
| 363.15 | 0.028 | 24.8 | 86.27 | 100.34 | -38.74 |

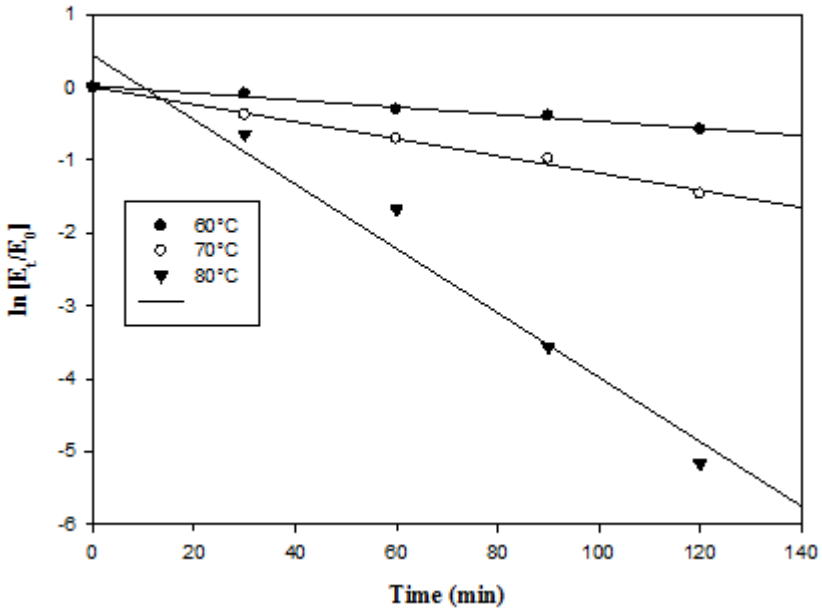


**Figure 3.12** Determination of thermal denaturation rate constant ($k_d$) of Tlphy2 at 60°C, 70°C and 80°C using the slope of ln [$E_t/E_0$] versus time plot.

**Table 3.4** Thermodynamic parameters of Tlphy2 during thermal deactivation at 60°C (343.15 K), 70°C (353.15 K) and 80°C (363.15 K)

| **Temp (K)** | **$K_d$ ($min^{-1}$)** | **$T_{1/2}$ (min)** | **ΔH (kJ/mol)** | **ΔG (kJ/mol)** | **ΔS (J/mol-K)** |
|---|---|---|---|---|---|
| 333.15 | 0.0048 | 144.38 | 99.27 | 96.69 | 7.74 |
| 343.15 | 0.0118 | 58.73 | 99.18 | 97.11 | 6.05 |
| 353.15 | 0.0388 | 17.86 | 99.10 | 96.53 | 7.29 |

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

Activity of Tlphy1 was moderately increased by addition of $Ba^{2+}$ (106%) and $Mg^{2+}$ (103%). Addition of 1 mM and 5 mM SDS inhibited Tlphy1 activity 37% and 62%, respectively (Table 3.5). Tlphy2 activity was increased by addition of $Ca^{2+}$and EDTA (Table 3.6).

**Table 3.5** Effect of metal ions, reducing agents and surfactants on the activity of Tlphy1

| **Additive** | **1 mM** | **5 mM** |
|---|---|---|
| *Metal ions* | | |
| $Ba^{2+}$ | 106 ± 2.3 | 118 ± 3.2 |
| $Ca^{2+}$ | 92 ± 5.3 | 81 ± 5.8 |
| $Co^{2+}$ | 82 ± 4.2 | 70 ± 4.2 |
| $Cu^{2+}$ | 87 ± 1.7 | 75 ± 4.3 |
| $Fe^{2+}$ | 88 ± 3.4 | 73 ± 2.1 |
| $Mg^{2+}$ | 103 ± 4.6 | 114 ± 6.1 |
| $Mn^{2+}$ | 89 ± 3.3 | 76 ± 4.6 |
| $Pb^{2+}$ | 79 ± 2.8 | 61 ± 4.1 |
| $Zn^{2+}$ | 90 ± 2.2 | 73 ± 4.2 |
| *Reducing agents* | | |
| EDTA | 91 ± 4.5 | 73 ± 2.4 |
| DTT | 94 ± 3.8 | 88 ± 2.7 |
| **Additive** | **0.1 mM** | **1 mM** |
| *Surfactants* | | |
| Tween-20 | 86 ± 4.7 | 66 ± 3.5 |
| Tween-80 | 81 ± 3.1 | 61 ± 2.2 |
| SDS | 63 ± 1.1 | 38 ± 1.4 |
| CTAB | 79 ± 1.9 | 63 ± 2.1 |
| Control | 100 | 100 |

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

**Table 3.6** Effect of metal ions, reducing agents and surfactants on the activity of Tlphy2

| **Additive** | **1 mM** | **5 mM** |
|---|---|---|
| *Metal ions* | | |
| $Ba^{2+}$ | 92 ± 3.9 | 78 ± 2.8 |
| $Ca^{2+}$ | 112 ± 5.5 | 126 ± 5.5 |
| $Co^{2+}$ | 80 ± 3.5 | 65 ± 2.8 |
| $Cu^{2+}$ | 82 ± 4.7 | 65 ± 3.1 |
| $Fe^{2+}$ | 93 ± 4.4 | 84 ± 3.7 |
| $Mg^{2+}$ | 87 ± 4.1 | 71 ± 3.1 |
| $Mn^{2+}$ | 86 ± 3.9 | 68 ± 2.9 |
| $Pb^{2+}$ | 72 ± 2.9 | 62 ± 2.2 |
| $Zn^{2+}$ | 102 ± 4.8 | 98 ± 4.2 |
| *Reducing agents* | | |
| EDTA | 123 ± 5.9 | 136 ± 6.2 |
| DTT | 88 ± 4.1 | 75 ± 2.6 |
| **Additive** | **0.1 mM** | **1 mM** |
| *Surfactants* | | |
| Tween-20 | 88 ± 4.9 | 71 ± 2.8 |
| Tween-80 | 85 ± 3.8 | 64 ± 2.3 |
| SDS | 56 ± 2.4 | 42 ± 1.8 |
| CTAB | 77 ± 3.2 | 53 ± 2.8 |
| Control | 100 | 100 |

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

## 3.4 DISCUSSION

Both Tlphy1 and Tlphy2 were successfully purified from the crude enzyme extracts after 55-85% ammonium sulphate precipitation, ion-exchange and size exclusion chromatography. Ion-exchange and size exclusion chromatography resulted in the purification of phytases to homogeneity with an overall yield of 5.16% and 24.70-fold purification for Tlphy1 (Table 3.1) whereas Tlphy2 was purified by 18.71-fold with an overall yield of 6.41% (Table 3.2). Similarly, Soni et al. (2010) produced and purified two extracellular phytases from *A. niger* NCIM 563 using a three step purification strategy. The authors reported 16.67-fold purification of Phy I with overall yield of 30.24%, while Phy II was purified 14.29-fold with an overall yield of 26.55%. The molecular weights of Tlphy1 and Tlphy2 were estimated to be 49 kDa and 60 kDa, respectively on SDS-PAGE gel (Fig. 3.2) and migrated as monomers during gel-filtration chromatography. Both phytases were within the reported molecular mass range of phytases from filamentous fungi. Generally, molecular masses of phytases from filamentous fungi remain smaller than 100 kDa (Singh and Satyanarayana, 2009), but there are reports of phytases bigger than 100 kDa as well. Spier et al. (2011) reported phytase from *A. niger* FS3 with a molecular mass of 108 kDa. Two fungal phytases with entirely different characteristics have been reported in *R. oligosporus* (Azeke et al., 2011).

The thermophilic filamentous fungus *T. lanuginosus* SSBP is known to produce several thermostable enzymes such as xylanase (Singh et al., 2000), β-xylosidase (Gramany et al., 2016) and multiple chitinases (Zhang et al., 2015) that show optimal activity in the range of 50-60°C. Tlphy1 exhibited optimal activity at 55°C and pH 5.0 (Fig. 3.3 and Fig. 3.7), it was stable in the temperature and pH range between 50°C-90°C and pH 2.0-6.0, respectively with a half-life of 138.6 min at 70°C (Fig. 3.4 and Fig. 3.8). The half-life of the enzyme declined sharply to 44.7 min at 80°C and 24.8 min at 90°C. Tlphy2 was active over a broad range of temperature 30-70°C and pH 2.0-7.5 with optimal activity at 50°C and pH 2.5, respectively (Fig. 3.5 and Fig. 3.9). The enzyme was fairly stable at 40°C and retained 77% and 54% of activity after 2 h at 50°C and 60°C, respectively with a half-life of 58.72 min at 70°C (Fig. 3.6 and Fig. 3.10). Although the enzyme retained activity at higher temperatures and showed 68% and 40% activities after 30 min incubation at 70°C and 80°C, respectively, the activity was lost completely after prolonged incubation of 2 h at 80°C. Thermophilic moulds

are known to produce phytases with optimal thermal activity in the range of (50-70°C). *H. nigrescens* phytase was active at 50°C (Bala et al., 2014), *A. niger* NCIM 5639 phytase was active at 55°C (Bhavsar et al., 2011), *S. thermophile* phytase was active at 60°C (Singh and Satyanarayana, 2009) and *R. pusillus*, was optimally active at 70°C (Chadha et al., 2004).

Both Tlphy1 and Tlphy2 had superior stability at higher temperatures compared to other thermophilic fungal phytases. *A. niger* ATCC 9142 phytase could retain only 12% activity after 5 min at 80°C (Casey and Walsh, 2003). Similarly, *A. niger* FS3 phytase retained 25.1% activity after 15 min at 80°C (Spier et al., 2011). However, they were less stable than phytase from *S. thermophile* with a reported half-life of 90 min at 80°C (Singh and Satyanarayana, 2009), and phytase from *R. oryzae,* which retained 90% activity after 30 min at 80°C (Rani and Ghosh, 2011). Most of the fungal phytases exhibit optimal activity in the acidic pH range. This was also evident in the present study with Tlphy1 and Tlphy2. Similarly, Gulati et al. (2007) reported phytase from *T. lanuginosus* TL-7 showing optimal activity at pH 5.0. There are also reports of phytases showing double pH optimum. Phytase from *A. niger* NCIM 563 showed dual pH optima at 2.5 and 4.0 (Soni and Khire, 2007), while *R. oryzae* phytase showed dual pH optima at 1.5 and 5.5 (Rani and Gosh, 2011). *A. niger* van Teighem phytase was optimally active at pH 2.5 (Vats and Banerjee, 2005).

Thermal denaturation kinetics was studied at 70°C, 80°C and 90°C for Tlphy1. A linear ($R^2$=0.97) semi-logarithmic plot of residual activity versus exposure time indicated that the thermal inactivation followed first-order kinetics. The slope of this plot, and hence the rate of deactivation ($k_d$) increased with increase in temperature (Fig. 3.11). The deactivation energy ($E_d$) was calculated as 89.29 kJ/mol using the Arrhenius plot. The overall values of thermodynamic parameters ΔH and ΔG were positive indicating high energy requirements for breaking the covalent bonds and the non-spontaneous denaturation of this enzyme, while ΔS values were negative. Substituting negative ΔS values in equation (4) makes overall ΔG positive. This infers that thermostability of this enzyme is driven by both enthalpy and entropy (Parashar and Satyanarayana, 2016). A marginal change in values of ΔH and ΔG was observed on increasing temperature from 70°C to 90°C (Table 3.3). The value of ΔS increased from –38.23 J/mol-K at 70°C to –36.43 J/mol-K at 80°C, but decreased to –38.74 J/mol-K on further incubation at 90°C. This could be due to interaction of charged

phosphate anions with the enzyme molecules forming a presumably ordered transition state complex. Negative values of ΔS have been reported for many extremozymes such as the hyperthermostable α-amylase of *Bacillus licheniformis* (Declerck et al., 2003), the chimeric thermostable α-amylase from *B. acidicola* and *Geobacillus thermoleovorans* (Parashar and Satyanarayana, 2016) and halotolerant β-galactosidase of *A. tubingensis* GR1 (Raol et al., 2015).

The deactivation constant ($k_d$) of Tlphy2 was calculated form the plot of ln [*Et/E0*] versus time plot (Fig. 3.12). The deactivation energy ($E_d$) was calculated using the Arrhenius plot for the inactivation rate constants and half-life values were calculated at different temperatures as shown in Table 3.4. The inverse relationship between half-life of Tlphy2 and temperature validated that the enzyme was less stable at high temperatures. Tlphy2 exhibited high stability at 60°C with a half-life of 58.73 min. The combination of increased inactivation rate constant and significant drop in half-life at 70°C can be attributed to the maximum resistance to heat displayed by the enzyme at this temperature (Yegin, 2017). The decrease in ΔH with the increased temperatures confirmed that less energy was required to denature enzyme at higher temperatures. The thermal denaturation of Tlphy2 is non-spontaneous as confirmed by the positive values of ΔG. A decrease in the value of change in entropy at 70°C than 60°C may be attributed to stabilization by the substrate or released inositol phosphates. Comparatively higher change in entropy at 80°C than 70°C indicates conformational instability of Tlphy2 at higher temperatures.

The activity of Tlphy1 was increased moderately by the addition of $Ba^{2+}$ (106%) and $Mg^{2+}$ (103%). All the other metal ions, reducing agents and surfactants tested in this investigation inhibited phytase activity. Maximum inhibition was observed by SDS which inhibited enzyme activity by 37% and 62% with 1 mM and 5 mM SDS, respectively (Table 3.5). The reason for the moderate enhancement in phytase activity due to $Ba^{+2}$ and $Mg^{+2}$ could be due to the favourable conformational changes in enzyme structure (Makolomakwa et al., 2017) as observed in the wild and recombinant HAP phytase of another thermophilic mould, *S. thermophile* (Ranjan and Satyanarayana, 2016; Singh and Satyanarayana, 2009). Different concentrations of SDS are known to bind on the surface of globular proteins forming quasi-micellar to saturated SDS micelles that may result in significant conformational changes (Kumar et al., 2015). Marked inhibition due to SDS in this study could be associated with

similar conformational changes resulting in differential protein unfolding at two different SDS concentrations. Most of the metal-ions tested on Tlphy2 were inhibitory, except for $Ca^{2+}$, which improved phytase activity by 12% at 1 mM and 26% at 5 mM, while $Zn^{2+}$ did not showed any noticeable effect on enzyme activity (Table 3.6). Several metal-ions can precipitate phytate as insoluble metal salts, thereby limiting the substrate availability and thus inhibiting phytase. Activation of Tlphy2 due to $Ca^{2+}$ may be due to formation of a positively charged metal–phytate complex that reduces the negative charge around the active site cleft, thereby activating the enzyme (Chanderman et al., 2016). $Ca^{2+}$ is known to activate most of the bacterial phytases, however, present report constitutes one of the few reports that show activation of fungal phytase due to calcium. Previously, Vats and Banerjee (2005) demonstrated a 45% increase in the activity of phytase due to addition of 1 mM $Ca^{2+}$ in the reaction mixture.

Marked activation of Tlphy2 due to EDTA was another interesting observation from this study. Enhanced enzyme activity due to EDTA has also been previously reported in thermo-acid-stable phytases of *A. niger* UFV-1 (Monteiro et al., 2015), *A. niger* van Teigham (Vats and Banjee, 2005) and *A. ficuum* NTG-23 (Zhang et al., 2010). However, most of the alkali-stable phytases of bacteria are inhibited by EDTA (Pal Roy et al., 2017; Shimizu, 1992), while a few phytases such as phytase from *Pantoea agglomerans* (Greiner, 2004) and *Klebsiella oxytoca* MO-3 (Jareonkitmongkol et al., 1997) were unaffected. The metal-ion chelating property of EDTA is responsible for inactivation of metal-dependent phytase of *B. subtilis* YCJS (Yao et al., 2014). Due to its EDTA-activating characteristics, Tlphy2 from *T. lanuginosus* SSBP can be a more suitable enzyme for studies where phytase is used in combination with EDTA for formulating fortified food (Brnić et al., 2014) and feed (Ebrahimnezhad et al., 2010).

# CHAPTER 4: APPLICATIONS OF PHYTASES FROM *T. lanuginosus* SSBP

***SOME OF THE WORK PRESENTED IN THIS CHAPTER WAS PUBLISHED IN Bioresource Technology, 235, pp.396-404,* 2017.**

## 4.1 Introduction

Phytases are of major industrial importance for several applications are used especially during dephytinization of food and feed phytate. Supplementing animal feed with phytase increases the nutritional value of the feed and decreases the amount of residual phosphorus in the animal litter. The enzyme has also been used to improve dough-quality and the texture of bread. Phytases from *T. lanuginosus* SSBP show requisite characteristics, and therefore were applied for several new applications as discussed in the present chapter. Tlphy1 was better suited for applications that needed thermostabilitiy at higher temperatures, while Tlphy2 was appropriate for low temperature treatments.

### 4.1.1 Application of phytase to enhance nutritional value of *mageu*

Globally *Zea mays* (maize) is an important source of carbohydrates and is one of the most consumed cereal crops. World production of maize was 1.03 billion tons in 2014 (FAO, 2017). Communities in sub-Saharan Africa maize traditionally consume maize as their staple food in several forms, including breads, porridges, steamed products and beverages (Ortiz-Monasterio et al., 2007). Cereal-based fermented foods are major dietary constituents in several developing countries. Over the years fermented foods have gained more interest due to their health-promoting effects. Fermented foods are generally associated with beneficial bacteria of major probiotic importance. Probiotics favourably alter the intestinal microflora balance, inhibit the growth of harmful bacterial, promote intestinal digestion, boost immune function and increase resistance to infection (Christine et al., 2010). *Mageu* is a traditional maize-based and lactic acid-fermented non-alcoholic beverage, which is especially popular in rural poor communities as a refreshing food drink and included in diets of infants and sick people of sub-Saharan Africa (Chelule et al., 2010b). However, maize has a high phytate content (Schlemmer et al., 2009), and it may chelate with several metal ions, thereby limiting the availability of nutritionally important essential elements in *mageu*. Therefore, consumption of *mageu* may result in micronutrient deficiency-related diseases, especially in nutritionally vulnerable groups such as women and children.

Supplementation of phytase in foods with high phytate content can significantly improve the bioavailability of essential minerals.

### 4.1.2 Application of phytase for plant-growth-promotion

*Phaseolus vulgaris* L. (common bean) is one of the most important food legumes worldwide. Its global production was estimated to be 26.53 million tons in 2014 (FAO, 2017). Beans, the "poor man's meat," are usually consumed after cooking in water or in braised form. They are an important source of plant-based proteins and minerals such as iron, phosphorus, magnesium and manganese (Broughton et al., 2003). Beans have a significant effect on the nutritional status of African population and are included in diets of many households. However, productivity and yield of beans is primarily limited by soil quality. Low availability of essential nutrients such as nitrogen (N), phosphorus (P) and potassium (K) in agricultural soils is mostly improved by chemical fertilizers. Presently, phosphorus is replenished in the form of synthetic fertilizers that can negatively impact soil health and environment, as not all phosphate is utilised by plants resulting in high levels of extractable soil inorganic phosphate entering the surface waters as soluble phosphate (Gujar et al., 2013) leading to eutrophication. There are reports that the minable phosphorus deposits will be depleted by the end of 21st century (Penuelas et al. 2013), and therefore there is an immediate need to find alternative sources of phosphorus that may be locked in different unutilized forms. PA constitutes the major proportion of soil organic phosphorus. Phytases which hydrolyse PA to *myo*-inositol and *myo*-inositol phosphates and release Pi can dephosphorylate organic insoluble form of phosphorus into accessible soluble inorganic forms that can be easily assimilated by plants. There are only few reports on the use of fungal phytases for plant growth-promotion. Phytase from *S. thermophile* and *A. niger* significantly improved plant growth and mineral assimilation in wheat seedlings (Gujar et al., 2013; Singh and Satyanarayana, 2011). Despite the growing interest in phytases as plant growth promoters, there are no reports on exogenous supplementation of phytase on the growth of bean plants.

### 4.1.3 Application of phytase for improvement of bioethanol production

To address the issue of global food insecurity due to use of major food crops such as corn in the US, sweet potato in China, and wheat in the EU as feedstocks for bioethanol production, there is an urgent need to identify alternate sources of starch

that do not compete with major crops. Underutilized crops could be used as feedstocks for several applications and are potential substitutes for major crops. Amadumbe or taro (*Colocasia esculenta*), the fifth-most harvested root crop in the world, is cultivated traditionally by rural farmers in Africa and throughout the subtropical and tropical regions with an estimated global production of 11.8 million tons per annum (Nath et al., 2013). In South Africa, amadumbe is grown especially along the KwaZulu-Natal coastline. *C. esculenta* is rich in starch (Naidoo et al., 2015) and several essential elements including Ca, Mg, Al, Mn, Cu, Fe, Co, Cr, Zn, Ni and Se. The bioavailability of these essential nutritional components is, however, limited due to the high PA content, which also contributes to the lower yield of ethanol from starch fermentation. PA complexes with starch, dietary proteins and lipids and inhibits a number of important digestive enzymes due to negatively charged phosphates on the *myo*-inositol ring.

Recently, phytases have also been included in commercial enzyme cocktails routinely used for bioethanol production, although reports on the use of phytases to enhance bioethanol production are scarce (Chen et al., 2015; Mikulski et al., 2015; Mikulski et al., 2014). Moreover, phytase used in these reports was only from mesophilic *A. niger,* which is not robust enough to withstand the harsh processing conditions. It is, therefore, important to investigate the applicability of thermo-acid-stable phytases that can withstand the high temperature of gelatinization and acidic pH during the enzymatic saccharification and liquefaction steps. This study investigated the applicability of *T. lanuginosus* SSBP phytase for improvement of bioethanol production from underutilised *C. esculenta* starch.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Preparation of *mageu* and fermentation

White maize meal powder was purchased from a local supermarket. Maize meal (100 g) was added to 700 ml hot water (90°C) and the mixture was cooked for 15 min with occasional stirring. After cooking the porridge was allowed to cool to 40°C. After cooling, 20 g white sugar and 2% (w/w) *Lactobacillus* sp. starter culture were added to the porridge. The porridge was then transferred aseptically into 250 ml flasks equally (100 g per flask). The experimental flasks were supplemented with different doses of Tlphy2 (10 U, 50 U, 100 U) while there was no phytase in the control flasks. The flasks were incubated at 30°C and pH of *mageu* samples was monitored every 4 h. *Mageu* is considered to be ready for consumption when the pH has dropped between 3.7-3.3 (Mugocha, 2000). The viable counts were determined every 4 h during *mageu* fermentation using spread plate method on MRS (de Man, Rogosa, Sharpe) agar incubated at 30°C for 48 h.

#### *4.2.1.1 Estimation of phytic acid content*

Samples were withdrawn every 4 h and phytic acid from *mageu* (1 g) was extracted with 0.66 M HCl, followed by neutralization with 0.75 M NaOH. The phytate content of *mageu* was quantified by HPLC using a LiChrosorb RP-18 column (Merck) and a refractive index detector. Sodium acetate buffer (100 mM, pH 4.5) was used as the mobile phase at a flow rate of 1 ml/min.

#### *4.2.1.2 Mineral composition of dephytinized mageu*

The samples were taken after fermentation and analysis of mineral elements was carried out using an AA-7000 atomic absorption spectrometer (Shimadzu).

### 4.2.2 Bioethanol production

*C. esculenta* tubers were washed, peeled, cut into small pieces and dried at 55°C, followed by milling to a mean particle size of 0.4 mm. *C. esculenta* mash was prepared by mixing amadumbe flour with water (15% w/v). The mash was gelatinized at 60°C, 70°C, 80°C and 90°C for 30 min under agitation. After gelatinization, the pH of the mash was adjusted to 5.8 and different doses of Tlphy1 (20 U/g, 30 U/g and 40

U/g) were added to improve digestibility before liquefaction. The mash was then incubated at 80°C for 30 min with agitation. Phytase was excluded in control experiments. After incubation, liquefaction of starch was performed by adding 2.7 U of α-amylase from *Bacillus licheniformis* (Sigma-Aldrich, USA) per gram of dry raw matter and incubated at 85°C for 30 min with agitation. The samples were cooled to 55°C and the pH was adjusted to 4.5 before adding 5.4 U of amyloglucosidase from *Rhizopus* sp. (Sigma-Aldrich, USA) per gram of dry raw matter. The mash was incubated at 55°C for 30 min with agitation for saccharification. Finally, the mash was cooled to 30°C, inoculated with 10% yeast and incubated at 30°C, 150 rpm for 48 h. Samples were taken every 12 h and analysed for ethanol production.

#### *4.2.2.1 Analysis of C. esculenta flour samples*

Samples were collected after gelatinization, treatment with different doses of phytase and saccharification, and elemental analysis was carried out using an AA-7000 atomic absorption spectrometer (Shimadzu). The total reducing sugar was determined using the DNS assay (Miller, 1959). Viscosity was measured using a rotational viscometer (HAAKE Viscotester, Thermo Scientific) with a number 1 spindle at 60 rpm at 80°C and 30°C. Phytic acid from gelatinized flour sample (1 g) was extracted and estimated using HPLC, as mentioned previously in section 4.2.1.1.

#### *4.2.2.2 Ethanol estimation*

The ethanol concentration in samples was determined by Wall Coated Open Tubular (WCOT) fused silica CP-WAX column (30 cm × 0.15 mm) using Varian 3800 gas chromatograph (Varian, Inc.) equipped with a flame ionization detector. $N_2$ and $H_2$ were used as the carrier and flaming gas, respectively. The temperature of the injector, detector and oven were 200°C, 250°C and 200°C, respectively.

### 4.2.3 Effect of phytase on plant growth

*Phaseolus vulgaris* L. seeds (with low endogenous phytase activity), sandy soil and agricultural compost were obtained from the Department of Horticulture, Durban University of Technology. Tlphy2 was used for this experiment. The bean seeds were disinfected with 70% ethanol for 1 min followed by 1% sodium hypochlorite for 20 min and rinsed with double distilled water three times before use. The experimental seeds were soaked in 5 ml phytase containing solution while control seeds were soaked in water for 1 h. Sterilised and pre-soaked bean seeds were placed in half

strength Murashige and Skoog (MS) medium tubes devoid of phosphorus and the effect of phytase supplementation was observed. The seeds were germinated at 23°C and a 16 h photoperiod for 14 days. The experiment was performed using a minimum of 30 replicates.

Additionally, the effect of phytase supplementation was also tested in planting pots experiments. One part of sandy soil was mixed with two parts of compost and the final mixture was sieved to 2 mm before sterilization at 121°C/15 psi for 1 h. Pots were filled with 100 g of soil and the sterilised and pre-soaked seeds were sown. The experimental pots were supplemented with phytase (5 U, 10 U, 25 U and 50 U). The experiments were conducted in a greenhouse, the pots were watered periodically and monitored for growth. After 21 days the plants were uprooted carefully, washed off the soil and were immediately weighed to determine fresh weight. The root and shoot lengths were measured and the plants were dried at 60°C for 48 h to determine their dry weight.

## 4.3 RESULTS

### 4.3.1 Effect of phytase on pH during *mageu* fermentation

Supplementation of Tlphy2 reduced the fermentation time of *mageu* as compared to the non-enzymatic fermentation. As shown in Fig 4.1 addition of 50 U and 100 U of Tlphy2 resulted in pH drop of 3.3 and 3.4, respectively within 16 h, whereas the control experiment took 24 h to reach a pH of 3.7. The pH dropped to 3.9 after 20 h using 10 U.

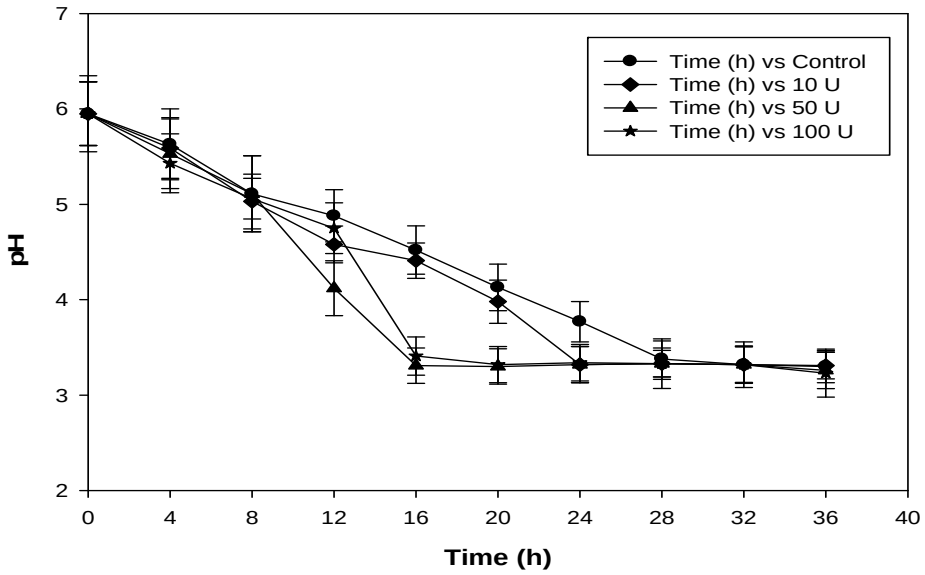


**Figure 4.1** Effect of different doses of Tlphy2 on completion of *mageu* fermentation as represented by the corresponding pH profile.

### 4.3.2 Effect of phytase on growth of *Lactobacillus* during *mageu* fermentation

*Lactobacillus* sp. grew faster in the experiments supplemented with phytase (Fig 4.2). Supplementation of 50 U and 100 U Tlphy2 resulted in enhanced growth of the bacterium during exponential phase in the first 12 h. However, 10 U Tlphy2 supplementation only showed a slight influence on the growth of the bacterium.

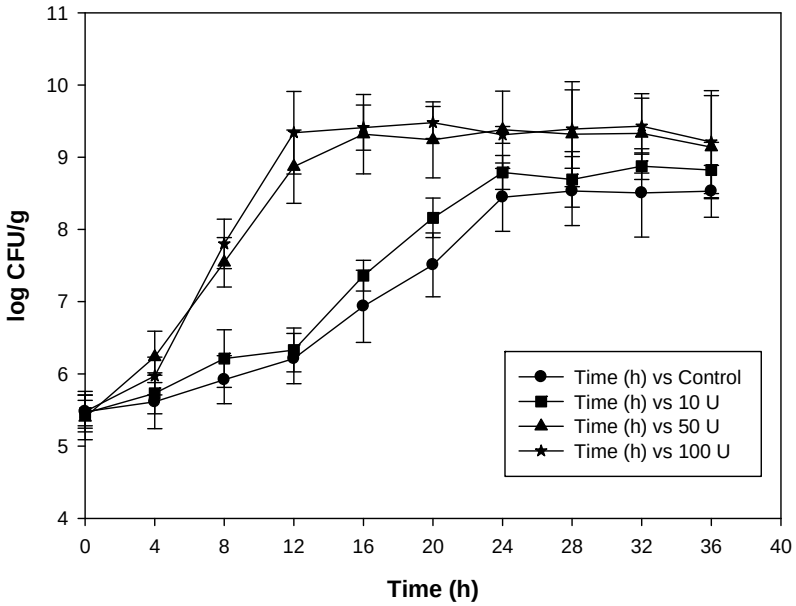


**Figure 4.2** Effect of different doses of Tlphy2 on growth profile of *Lactobacillus* during *mageu* fermentation.

### 4.3.3 Effect of phytase on dephytinization of *mageu*

Phytase supplementation resulted in reduction of phytic acid content in *mageu* samples. Higher dosages (50 U and 100 U) of Tlphy2 resulted in almost complete dephytinization after 20 h. A lower dose of 10 U Tlphy2 could not significantly reduce PA in *mageu* and the concentration remained 0.38 mg/100 g as compared to 0.41 mg/100 g in the control (Fig 4.3).

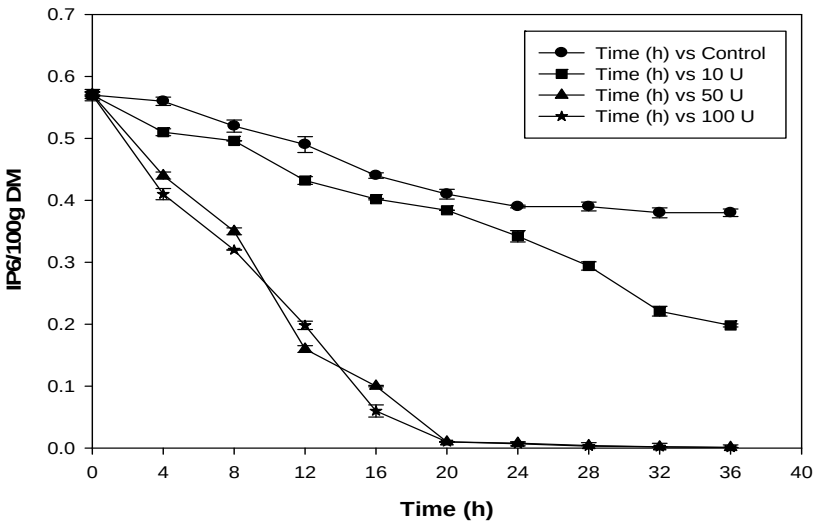


**Figure 4.3** Effect of different doses of Tlphy2 on dephytinization of *mageu.*

### 4.3.4 Effect of phytase on the release of minerals from *mageu*

The concentration of essential minerals increased significantly in *mageu* samples treated with phytase (Table 4.1). Marked improvement in the release of minerals was evident even at lower doses (10 U) of Tlphy2 as compared to controlled experiments. However, the concentrations of essential minerals did not vary much due to increase in phytase dose from 50 U to 100 U. The concentration of Ca, Mg and Fe were most significantly affected due to supplementation of Tlphy2 in *mageu* (Table 4.1).

**Table 4.1** Comparison of mineral composition of mageu treated with different doses of phytase

| **Minerals (mg/100 g)** | **Untreated** | **Phytase treated** | | |
|---|---|---|---|---|
| | | **10 U** | **50 U** | **100 U** |
| Ca | 1.37±0.07 | 2.41±0.18 | 8.31±0.40 | 8.47±0.48 |
| Fe | 0.76±0.04 | 1.32±0.05 | 4.33±0.20 | 5.63±0.31 |
| Zn | 1.77±0.09 | 2.78±0.19 | 3.46±0.18 | 3.72±0.20 |
| K | 2.23±0.15 | 3.22±0.21 | 4.39±0.25 | 4.22±0.22 |
| Mg | 1.26±0.05 | 4.61±0.26 | 7.76±0.33 | 8.15±0.41 |
| Na | 0.27±0.01 | 0.31±0.01 | 0.38±0.01 | 0.43±0.02 |
| P | 1.11±0.05 | 1.50±0.06 | 3.52±0.24 | 3.82±0.28 |

### 4.3.5 Effect of gelatinization temperatures

Gelatinization of *C. esculenta* flour at 80°C and 90°C was most effective (Table 4.2) as indicated by higher concentration of reducing sugars, minerals and less viscosity as compared to lower temperatures (60°C and 70°C). The total reducing sugar was significantly improved from 37.23±0.92 to 61.03±1.16 when the gelatinization temperature was increased from 60°C to 80°C. Further increase in gelatinization temperature was not very effective in further increasing the total reducing sugar content from saccharification of *C. esculenta.* Marked improvement in the release of essential elements such as Fe, Na and Zn was noticed, while improvement in concentration of several other elements was also observed. Reduction in phytate content was corroborated with increase in inorganic P. Gelatinization at 80°C was selected for further experiments considering the thermostability of Tlphy2.

**Table 4.2** Effect of different gelatinization temperatures on the saccharified product before simultaneous saccharification and fermentation

| | **60°C** | **70°C** | **80°C** | **90°C** |
|---|---|---|---|---|
| Total reducing sugar (g/l) | 37.23±0.92 | 52.02±0.81 | 61.03±1.16 | 62.72±0.98 |
| Viscosity (cP) | 2133±106.63 | 1532±74.12 | 1271±65.27 | 1137±52.74 |
| Phytate (mg/g) | 1.83±0.04 | 1.71±0.02 | 1.43±0.01 | 1.27±0.08 |
| Ca (mg/kg) | 893.96±43.21 | 923.38±44.79 | 942.72±46.70 | 973±45.01 |
| Fe (mg/kg) | 23.64±1.91 | 27.96±1.28 | 39.46±1.31 | 44.75±1.05 |
| K (mg/kg) | 1765±86.74 | 1854±90.18 | 1983.42±91.62 | 2185±98.78 |
| Mg (mg/kg) | 2735±123.1 | 2987±131.9 | 3170.04±140.8 | 3347±142.8 |
| Na (mg/kg) | 598.24±28.5 | 721.06 ±36.7 | 831.53±39.11 | 853.21±45.31 |
| P (mg/kg) | 99.01±5.2 | 115.07±5.3 | 132.36±7.12 | 156.43±6.9 |
| Zn (mg/kg) | 42.98±1.13 | 54.48±2.39 | 69.81±3.21 | 81.02±4.41 |

### 4.3.6 Effects of different doses of phytase after gelatinization

The flour gelatinized at 80°C was treated with different doses of phytase (20 U, 30 U and 40 U). There was no phytase in the control. Concentrations of essential metal ions increased with increase in Tlphy1 dose (Table 4.3).

**Table 4.3** Effect of different doses of phytase after gelatinization on the release of essential metal ions

| | **Control** | **20 U/g** | **30 U/g** | **40 U/g** |
|---|---|---|---|---|
| Ca (mg/kg) | 942.31±11.76 | 1196.46±11.21 | 2432.13±12.28 | 3133.42±15.13 |
| Fe (mg/kg) | 38.86±1.61 | 44.16±1.73 | 72.69±1.12 | 81.73±2.37 |
| K (mg/kg) | 1987.48±18.26 | 2172.21±13.36 | 2785.93±15.44 | 2809.22±12.30 |
| Mg (mg/kg) | 3155.17±14.49 | 3210.81±14.11 | 3559.14±11.92 | 3642.33±15.01 |
| Na (mg/kg) | 833.33±4.12 | 853.48±1.94 | 889.79±6.01 | 901.31±7.52 |
| P (mg/kg) | 135.46±3.66 | 267.78±2.53 | 485.47±4.41 | 516.73±3.02 |
| Zn (mg/kg) | 71.57±2.51 | 73.55±4.16 | 93.34±3.39 | 112.32±2.11 |

A 30 U/g Tlphy1 dose was optimal for enhanced release of reducing sugars, reduced viscosity and improved phytate hydrolysis (Table 4.4).

**Table 4.4** Supplementation of different doses of phytase after gelatinization and its effect on the sachharified product

| | **Control** | **20 U/g** | **30 U/g** | **40 U/g** |
|---|---|---|---|---|
| Total reducing sugar (g/l) | 61.03±2.1 | 68.43±1.16 | 73.27±0.98 | 74.43±1.21 |
| Viscosity (cP) | 1271±15.27 | 771±15.27 | 537±12.74 | 414±13.42 |
| Phytate (mg/g) | 1.43±0.00 | 0.73±0.02 | 0.05±0.00 | 0.04±0.00 |

All experiments were performed at 80°C and there was no phytase in control reactions

### 4.3.7 Bioethanol production profile

Phytase supplementation improved ethanol production from 18.9 g/l to 25.4 g/l after 48 h. Yeast biomass and sugar utilization were also enhanced as shown in Fig 4.4.

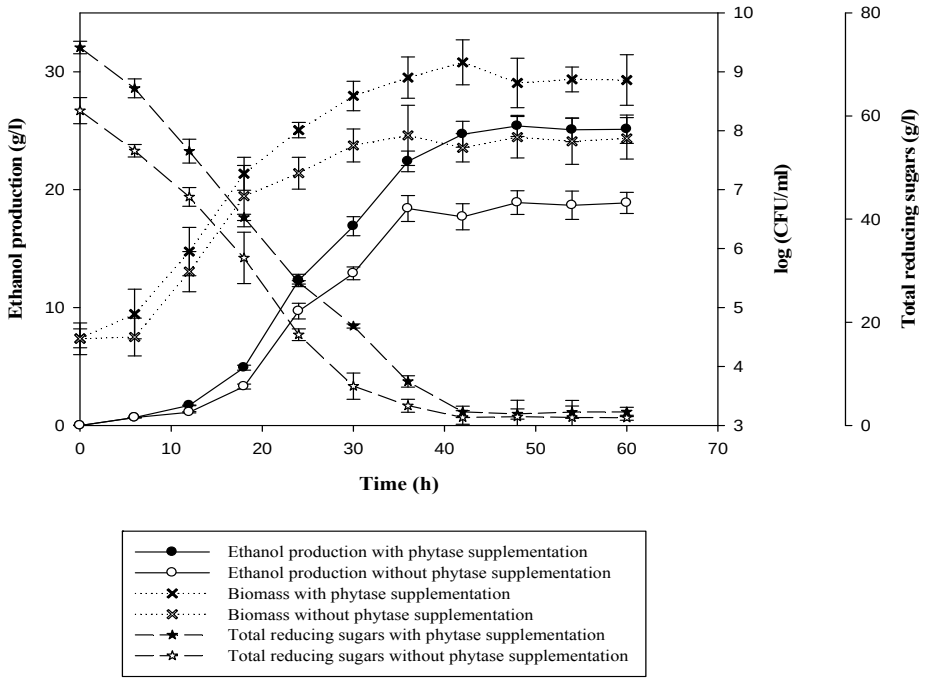


**Figure 4.4** Effect of 30 U/g supplementation of Tlphy1 on yeast growth, sugar utilization and ethanol production.

### 4.3.8 Effect of different doses of Tlphy2 on *in vitro* seed germination and growth promotion of bean plants

Seed germination, seed length and vigour index improved when bean seeds were treated with different doses of Tlphy2. Similarly, plants supplemented with phytase showed improved growth parameters as compared to the controlled experiments. Increase of phytase dose from 5 to 25 U significantly improved growth parameters (Table 4.5). However, higher dose of Tlphy2 (50 U) could not show any marked improvement.

**Table 4.5** Effect of phytase on germination of bean seedlings after 7 days in ½ MS medium and plant growth parameters after 21 days in planting pots

| **Seed germination** | | | | | |
|---|---|---|---|---|---|
| **Parameter** | **Control*** | **Test*** | | | |
| | | **5 U** | **10 U** | **15 U** | **20 U** |
| Seed germination (%) | 80 ± 3.82 | 86.67 ± 3.88 | 86.67 ± 3.91 | 93.33 ± 3.94 | 90 ± 3.94 |
| Seedling length (mm) | 33.02 ± 1.42 | 37.41 ± 1.49 | 39.68 ± 1.53 | 43.46 ± 1.51 | 40.29 ± 1.44 |
| Seed vigour index | 2641.6 ± 169.75 | 3179.85 ± 194.28 | 3372.8 ± 204.10 | 4128.7 ± 221.77 | 3626.1± 204.93 |
| Seed phytate (mg/g) | 18.42 ± 1.12 | 12.21± 1.12 | 10.06± 1.12 | 8.21± 1.12 | 12.21± 1.12 |
| **Plant growth promotion** | | | | | |
| **Parameter** | **Control*** | **Test*** | | | |
| | | **5 U** | **10 U** | **25 U** | **50 U** |
| Fresh weight (g) | 1.08 ± 0.07 | 1.79 ± 0.09 | 2.26 ± 0.11 | 2.94 ± 0.12 | 1.18 ± 0.10 |
| Dry weight (g) | 0.48 ± 0.03 | 0.53 ± 0.05 | 0.72 ± 0.08 | 0.99 ± 0.07 | 0.50 ± 0.05 |
| Shoot length (cm) | 9.31 ± 0.41 | 10.37 ± 0.43 | 11.62 ± 0.57 | 13.29 ± 0.52 | 9.77 ± 0.45 |
| Root length (cm) | 3.74 ± 0.17 | 3.88 ± 0.14 | 3.97 ± 0.11 | 4.21 ± 0.18 | 3.83 ± 0.22 |
| Phosphorus content (mg/g) | 4.53 ± 0.21 | 9.76 ± 0.43 | 12.4 ± 0.53 | 24.6 ± 1.25 | 54.5± 2.36 |

Seed germination (%) = (number of germinated seeds /total no of seeds taken for germination) × 100

Seed vigour index = [Germination % × Seedling length] (Abdul-Baki and Anderson, 1973)

***** Controlled experiments lacked phytase while the test experiments contained different doses of phytase in a total of 5 ml where seeds were soaked for 1 h. Similarly, test experiments contained different doses of phytase in 100 g soil to study growth promotion of bean plants.


**Figure 4.5** Growth promotion of bean plants in pots using increasing doses of phytase. An optimum dose of 25 U is evident.

## 4.4 DISCUSSION

Phytases (Tlphy1 and Tlphy2) from *T. lanuginosus* SSBP were effective in all applications tested in this study. Cooking of maize at higher temperature gelatinized the maize starch, making it susceptible for enzyme action. Supplementation of phytase in *mageu*, especially at higher doses (50 and 100 U), was effective in reducing the fermentation time by 8 h compared to control, which was only completed after 24 h. $Ca^{2+}$ ions released due to Tlphy2 action can activate endogenous α-amylase present in maize (Greiner and Konietzny, 2006). This can result in release of enhanced levels of glucose from maize starch, providing adequate levels of glucose as the preferred C-source for the multiplying bacteria, thereby speeding up the overall fermentation process. Completion of *mageu* fermentation is indicated by a drop in pH. The pH drop due to supplementation of 50 and 100 U of TLphy2 was noted as 3.3 and 3.4 after 16 h, respectively, which were within the acceptable range for completion of *mageu* fermentation (Mugocha, 2000), while *mageu* without phytase could reach pH 3.7 only after 24 h (Fig 4.1). It has been reported that the acidic conditions created by the production of lactic acid during *mageu* fermentation are favourable for phytase activities resulting in degradation of phytate (Kumar et al., 2010; Reale et al., 2007). Similar result with higher rate of decrease in pH was observed previously when *A. niger* phytase was supplemented in oat-based nutrient solution (Marklinder et al., 1995). There are ample evidences that support the correlation of endogenous plant phytase activity with phytate degradation during lactic acid fermentation of cereal flours (Reale et al., 2007; Leenhardt et al., 2005; Katina et al., 2005). There is a possibility of certain levels of endogenous phytase activity in the plant materials. However, generally the endogenous phytase levels are not adequate, and therefore there is a need for exogenous supplementation of phytase to achieve maximum phytate hydrolysis. Phytate degradation was restored when phytase was added to the dough during cereal dough fermentation (Reale et al., 2007). In this study, addition of phytase resulted in almost complete hydrolysis of phytate after 20 h (Fig 4.3). Similarly, Ranjan et al. (2015) reported 70, 75, and 62.5% reduction in phytic acid content in bread, tandoori, and naan, respectively after addition of recombinant phytase from *S. thermophile*. The hydrolysis of phytate enhances the bioavailability of phytate-bound minerals ions. Hammes et al. (2005) discussed the importance of $Mg^{2+}$ for growth of lactic acid bacteria.

Gelatinization of starch is a critical step during bioethanol production from starch materials as gelatinization solubilises starch making it accessible to starch-hydrolyzing α-amylases and amyloglucosidases. Different varieties of *C. esculenta* starch are known to be gelatinized from 67°C to 85°C (Naidoo et al., 2015). In this study, we also studied the effect of different temperatures from 60°C to 90°C. High temperatures (80°C and 90°C) were effective (Table 4.2). A direct relationship between increase in temperature and concentrations of mineral ions (Table 4.2) and reducing sugars (Table 4.4) was observed. High viscosity can affect the growth and metabolism of the yeast. Srichuwong et al. (2009) discussed the role of high viscosity in resistance to solid–liquid separation and reduction of fermentation efficiency during bioethanol production from cassava mash. Viscosity of the saccharified product reduced from 2133 cP to 1137 cP, presumably due to the action of amylolytic enzymes. Low reducing sugar content and high viscosity (2133 cP) indicates inadequate gelatinization and saccharification at 60°C (Table 4.2). Preferred temperature of 80°C was selected for further studies based on the thermostability of Tlphy2. Also, lesser energy inputs are required at 80°C as compared to gelatinization at 90°C.

The addition of phytase after gelatinization resulted in enhanced release of essential metal ions mainly due to the hydrolysis of phytate-metal ion complexes (Table 4.3). Previously, Morais et al. (1996) reported low availability of metal ions due to association with resistant starch (RS) in corn-based meal. Recently, the amount of RS in *C. esculenta* was reported between 57-64% (Naidoo et al., 2015). A marked improvement in starch availability and metal-ions due to phytase addition in this study confirms the influence of phytic acid on starch digestibility. This was corroborated with improvement in the availability of fermentable sugars from 61.03 g/l to 73.27 g/l with a concomitant reduction in viscosity from 1271 cP to 537 cP after saccharification. Increased reduction in viscosity and improvement in total reducing sugars due to phytase supplementation may be attributed to enhanced release of free starch and the chelated calcium ions from phytin complex, which in turn, as indicated previously, can activate amylase (Machius et al., 1998; Krishnan and Chandra, 1983) resulting in higher saccharification. Phytase-mediated improvement in bread characteristics and its association with activation of α-amylase due to the release of

calcium ions from calcium-phytate complexes is well known/established (Haros et al., 2001).

Hydrolysis of phytate results in liberation of mineral ions and *myo*-inositol phosphates. This study showed an increase in phytate degradation and enhanced release of mineral ions due to increase in phytase dose (Table 4.3 and Table 4.4). Previous report by Nikolić et al. (2009) demonstrated mineral ions as essential micronutrients that facilitate several biochemical reactions in yeasts while *myo*-inositols are needed for the growth and acceleration of the fermentation process. Improvement in yeast biomass and enhanced sugar utilization was observed due to supplementation of 30 U/g Tlphy1. It has been reported that *myo*-inositol contributes to high ethanol-tolerance and increased cell-viability (Furukawa et al., 2004). Similarly, $Mg^{2+}$ has been reported to play a major role in protection of yeast cells against physiological stress and toxic levels of ethanol. $Mg^{2+}$ deficiency in the medium has detrimental effect on sugar to ethanol conversion and the overall fermentation process (Birch and Walker, 2000). Supplementation of phytase released 3559.14 mg/kg of $Mg^{2+}$ compared to 3155.17 mg/kg in the control (Table 4.3). Enhanced sugar utilization and higher ethanol production (25.4 g/l) was noticed (Fig 4.4). Similar reports of enhanced ethanol production due to addition of 10 mM $Mg^{2+}$ (Nikolić et al., 2009) and higher ethanol yield due to 50 mM $Mg^{2+}$ (Birch and Walker, 2000) have been documented.

Overall, phytase supplementation improved ethanol production by 1.59-fold in this study using *C. esculenta* (Fig 4.4). Similarly, Mikulski et al. (2014) reported 0.6-1.0% increase in ethanol production from maize mash starch due to supplementation of commercial phytase. The present study is the first report on using a thermostable phytase after gelatinization and before the addition of thermostable amylase for enhanced bioethanol production. Furthermore, acid-stability of this enzyme suits the acidic processing conditions and use of the acid-stable amylolytic enzymes.

Despite the presence of high phytic acid content in agricultural soils (Gerke, 2015) and compost (Mamedov et al., 2016), most plants lack adequate levels of extracellular phytase, and therefore cannot utilize this freely available organic source of phosphorus (Singh and Satyanarayana, 2011). Microbial phytases are known to promote growth of several plant species. Although there are reports on the presence of low titres of endogenous phytase in *P. vulgaris* L (Lazali and Drevon, 2014; Walker,

1974), there is no report till date, to the best of our knowledge, on the use of exogenous phytase for the growth promotion of common beans. In this study, different doses of phytase were supplemented during growth of bean seeds and plants in MS medium and soil, respectively. A marked improvement in the growth of bean seedlings was observed when MS medium devoid of P was supplemented with 5 U, 10 U, 15 U and 20 U of phytase (Table 4.5). Enhanced germination, growth and vigour index of seedlings in phytase supplemented MS medium (without P) than controlled experiments indicated the role of Tlphy2 in releasing P from stored PA in bean seeds. Comparatively lower growth of seedlings in controlled experiments also indicated either no or negligible endogenous phytase activity in seeds, which necessitates the use of external phytase for optimal growth of bean seedlings. Growth promotion was also observed in pot experiments (Fig 4.5), which resulted in increased weight and length of root and shoot due to supplementation of 5, 10 and 25 U of phytase (Table 4.5). Singh and Satyanarayana (2010) and Gujar et al. (2013) observed similar results when 20 U and 12 U of phytase from *S. thermophile* and *A. niger*, respectively was added to wheat seeds. Comparatively a very low dose of phytase (3 mU) was required for the growth promotion of maize seedlings in a low phosphate ($NaH_2PO_4.2H_2O$, 0.006 g/l) medium (Idriss et al., 2002).

Bean seedlings supplemented with Tlphy2 showed enhanced growth compared to the control experiments and superior growth characteristics were observed due to 25 U/g Tlphy2 (Table 4.5). Fresh and dry weights were higher in plants supplemented with phytase. This is similar to report by Singh and Satyanarayana (2010) where the authors report improved fresh and dry weight of maize plants due to addition of *S. thermophile* phytase. In this study, dry weight of bean plants was increased by 48% due to 25 U/g Tlphy2 as compared to the control. Similarly, Peix et al. (2001) reported a 14% increase in dry weight when bean seeds where inoculated with phosphate-solubilizing bacteria. Compost used in the present investigation was mostly plant-based and therefore high levels of soil phytate are expected in the soil, and there is a high probability of presence of non-degraded phytate even after autoclaving the soil. Supplementing soil with phytase enhanced the growth of bean plants primarily by improving the amount of P and essential metal ions in soil due to the action of phytase.

It was interesting to note growth inhibition due to supplementation of higher doses of phytase in MS medium as well as in pot experiments. Growth inhibition of germinating seeds and plants due to phytase overdose may be linked to the above-threshold levels of *myo*-inositol, produced by inositol phosphatases reported in common bean (Fileppi et al., 2010). *Myo*-inositol is accumulated in plants in response to several stress factors and can act as signalling molecule to elicit several metabolic processes including programmed cell death (Bruggeman et al., 2015).

# CHAPTER 5: GENERAL DISCUSSION

The current 7.4 billion world population is expected to reach 9.6 to 12.3 billion by 2100 (Gerland et al., 2014). The growing population has raised the problem of food insecurity while industries are continuously spewing several metric tons of carbon dioxide annually in the environment. Different branches of biotechnology are continuously attempting to answer the most dreaded problems through 'green solutions'. Enzyme technology is one such approach that attempts to solve the global problem of food insecurity and promises a sustainable environment using green biocatalysts. The continuous rise in the number of applications of industrial enzymes and the demand of price reduction and resource optimization in the production process are steering the global industrial enzymes market. The global enzyme market, which was estimated to be USD 8.18 billion in 2015, is expected to grow with higher compound annual growth rate due to increasing applications in industries including detergent, agriculture, pharmaceutical, food and beverage etc. Phytase, protease, xylanase, β-glucanase, cellulase, mannanase and pectinase are the key products that shared $ 899.19 million of the global feed enzymes market in 2014 (http://www.marketsandmarkets.com/PressReleases/feed-enzyme.asp).

Enzymes can be produced from a wide range of sources including microorganisms, plants and animals. However, microorganisms are the most preferred sources of industrial enzymes due to abundance, fast growth-rate, easy manipulation and availability of standardized protocols for industrial enzyme production. Thermophilic microorganisms are of major biotechnological interest due to their capability to produce thermostable enzymes with promising industrial applications. Proteins produced by thermophiles are more thermostable than their mesophilic counterparts. The supremacy of thermostable enzymes in industries is primarily due to reduced chances of contamination, reduced viscosity of the medium, enhanced bioavailability and solubility of organic compounds and higher rate of product formation (Gomes et al., 2016). Fungal sources are best suited for industrial enzyme production, however most of the industrial strains are mesophilic. There are only a few reports on industrial enzymes from thermophilic fungal strains. Additionally, there is a continuous search for phytase-producing thermophiles.

*T. lanuginosus* is a thermophilic filamentous producing thermostable enzymes.. Recently, there has been no in-depth study of phytases from *T. lanuginosus*, although there are few reports on phytase from *T. lanuginosus*. Berka et al. (1998) cloned and expressed phyA gene from *T. lanuginosus* and Gulati et al. (2007) produced, purified and characterised phytase from *T. lanuginosus.* However, the present study is the first attempt to produce, purify, characterise and study the different applications of two phytases from *T. lanuginosus*.

The initial objective of this work was to statistically enhance the production of total phytase from *T. lanuginosus* SSBP. Variation in metabolic pathways for different microorganisms necessitates the optimization of nutritional and physical parameters for enhanced production of the target enzyme. Use of statistical approach for optimization studies is well-established because it saves time and uses minimum resources. During this study, incubation period, peptone and incubation temperature were identified as the most significant factors affecting phytase production as revealed by PBD analyses. Their interactions were further studied using RSM for maximum phytase production. An 8.56-fold improvement in phytase production was achieved due to PBD and RSM. The fold improvement in this study was higher compared to many other reports that employed statistical approach for submerged phytase production using fungi. Gulati et al. (2007) obtained 2.43-fold increase in phytase production by *T. lanuginosus* TL-7. Similarly, Singh and Satyanarayana (2008) reported a 2.6-fold increase in phytase production by *S. thermophile* while Berikten and Kivanc (2014) reported a higher, 10.83-fold improvement in production of phytase from *T. lanuginosus* using SSF.

A suitable feeding strategy is of immense importance to achieve high enzyme production during fed-batch fermentation process. Glucose acts as the primary carbon source for majority of microorganisms that significantly affects growth and metabolite production. The significance of glucose was also noticed during the analysis of PBD results in this investigation. It was therefore preferred as feed to study its effect on phytase production during fed-batch cultivations. Glucose feeding (700 g/l) at an interval of 30 h was best for maximum phytase production compared to other glucose concentrations. Decreased phytase production due to feeding of 900 g/l glucose in the medium can be attributed to excess carbon source and accumulation of inhibitory secondary metabolites repressing the production of phytase. Previous reports

(Chanderman et al., 2016; Graminho et al., 2015) have shown that excess glucose concentrations had repressive effect on phytase production from *Enterobacter* sp. ACSS and *Burkholderia* sp. strain a13, respectively. Overall, optimization for feeding parameters during fed-batch fermentation resulted in a prolonged log phase thereby increasing phytases production to 167.62 U/ml.

Purification of *T. lanuginosus* SSBP phytases was achieved in four steps (ammonium sulphate precipitation, ultrafiltration, ion exchange and size exclusion chromatography). Similarly, phytase from *A. niger* CFR 335 was purified to homogeneity in four-steps that included ammonium sulphate precipitation and three column chromatographic steps (Gunashree and Venkateswaran, 2015). However, many other fungal phytases have been purified in fewer steps. Phytase from *T. lanuginosus* TL-7 was purified in two steps using ion exchange and gel-filtration chromatography (Gulati et al., 2007). *S. thermophile* phytase was purified in three steps using acetone precipitation, ion exchange and gel-filtration chromatography (Singh and Satyanarayana, 2009). Tlphy1 was purified to 24.70-fold with 5.16% yield while Tlphy2 was purified to 18.71-fold with 6.41% yield. The purification fold was lower for both enzymes compared to recent reports on fungal phytase from *A. flavus* ITCC 6720 where 32.74-fold was achieved with 2.17% yield (Gaind and Singh, 2015), but it was higher than *A. niger* CFR 335 phytase which was purified 16-fold with 28.5% yield (Gunashree and Venkateswaran, 2015). The molecular weights for Tlphy1 and Tlphy2 were 49 and 60 kDa, respectively. The previously reported phytase from *T. lanuginosus* is a 51 kDa protein (Berka et al., 1998; Gulati et al., 2007). The difference in molecular weight may be explained on the basis of post-translational truncation of protein or due to action of endogenous protease secreted by *T. lanuginosus* (Li et al., 1997; Ghareib et al., 2014).

Tlphy1 and Tlphy2 exhibited optimal activities at pH 5.0 and pH 2.5, respectively and both enzymes were fairly stable in the acidic pH range. The acidic stability is essential for applications in animal feed that passes through the acidic environment of the non-ruminant stomach. Most thermophilic fungal phytases belong to the HAP class of acidic phytases (Vohra and Satyanarayana, 2003; Singh et al., 2011) showing optimal activity in the pH range of 2.5 to 5.5. Both Tlphy1 and Tlphy2 showed acidic pH characteristics similar to previously reported HAP phytases from *S. thermophile* (Singh and Satyanarayana, 2009) and *A. niger* PhyB (Ullah et al., 2008).

Phytases from thermophilic fungi are known to be thermostable. High thermostability of extracellular enzymes can be attributed to the fact that they cannot be stabilised by cell-specific factors like compatible solutes (Turner et al., 2007). Both Tlphy1 and Tlphy2 were thermostable in a broad range of temperatures, Tlphy1 was comparatively more stable at higher temperatures. Stability over broad range of temperature enables phytases to display versatile applicability in food, animal feed, and plant and bioethanol industries.

Malnutrition is the major concern in the world populations, almost one third suffer from iron and zinc deficiencies especially from the developing countries. Supplementation of phytase has been reported to reduce phytic acid content in food and improve their nutritional value. Tlphy2 characteristics suited its application in *mageu* production as the process involves high temperature and low pH, the supplementation proved effective resulting in improvement of the concentration of essential minerals such as Zn, Fe and Ca thereby enhancing the nutritional value of *mageu*. The hydrolysis of phytate present in amadumbe with Tlphy1, accompanied by the enzymatic starch hydrolysis using amylolytic enzymes resulted in the enhanced release of fermentable sugars. This confirmed that phytate limits the availability of starch and reduces the yield of ethanol. Based on the findings of this investigation, the use of thermo-acid-stable phytase along with other amylolytic enzymes is recommended for improved production of bioethanol from starch.

Overall, Tlphy1 and Tlphy2 from *T. lanuginosus* SSBP possess some of the characteristics resembling an ideal phytase. However, search for ideal phytases should still continue with emphasis on broad pH activity and thermal tolerance. The present investigation is the first report on fed-batch production of phytase from *T. lanuginosus*, it also presents several novel applications which demonstrates wide industrial applicability of the two phytases.

**Future work**

Based on the potential applicability of these phytases, future work can be directed towards cloning, over-expression, structural elucidation using crystallography, site directed mutagenesis and construction of improved protein for multifarious applications. Further, the recombinant and improved proteins can be immobilized on novel matrices for repeated use under harsh conditions that several industries dictate.

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# Thermo-acid-stable phytase-mediated enhancement of bioethanol production using *Colocasia esculenta*

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## HIGHLIGHTS

- Batch and fed-batch production of phytase by *Thermomyces lanuginosus*.
- The purified enzyme is thermostable and acid-stable.
- The enzyme reduced phytate content in *Colocasia esculenta* starch.
- The enzyme improved availability of fermentable sugars.
- Immense potential for application in bioethanol producing industries.

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## ABSTRACT

Phytase production by the thermophilic mould *Thermomyces lanuginosus* SSBP was enhanced 8.56-fold in submerged fermentation, which was further improved in fed-batch cultivations. The protein was purified to homogeneity using ammonium sulphate precipitation, Resource Q anion exchange and Superdex gel-filtration chromatography, with an overall purification of 24.7-fold and a yield of 5.16%. The purified 49 kDa protein was optimally active at 55 °C and pH 5.0, and was stable between 50 and 90 °C from pH 3.0–6.0, with a half-life of 138.6 min at 70 °C. It was moderately stimulated by $Ba^{+2}$ and $Mg^{+2}$. The enzyme reduced phytate content in *Colocasia esculenta* starch (from 1.43 mg/g to 0.05 mg/g) that resulted in an improvement in the availability of fermentable sugars with a concomitant reduction in viscosity and 1.59-fold improvement in ethanol production. Thermo-acid-stable phytase from *T. lanuginosus* SSBP could be of major biotechnological interest, especially due to its robustness and wide applicability.


## 1. Introduction

Despite a major research shift towards the second, third and now, fourth generation of biofuels, starch-based feedstocks still constitute a major percentage of the global bioethanol production. To address the issue of global food insecurity due to the use of major food crops such as corn in the US, sweet potato in China, and wheat in the EU as feedstocks for bioethanol production, there is an urgent need to identify alternate sources of starch that do not compete with major crops. Underutilized crops could be used as feedstocks for several applications and as a substitute for major crops.

Amadumbe or taro (*Colocasia esculenta*), the fifth most harvested root crop in the world, is cultivated traditionally by rural farmers in Africa and throughout the subtropical and tropical regions with an estimated global production of 11.8 million tons per annum (Nath et al., 2013). In South Africa, amadumbe is grown especially along the KwaZulu-Natal coastline. *C. esculenta* is rich in starch (Naidoo et al., 2015) and several essential elements including Ca, Mg, Al, Mn, Cu, Fe, Co, Cr, Zn, Ni and Se. The bioavailability of these essential nutritional components is, however, limited due to the high phytic acid (PA) content, which also contributes to the lower yield of ethanol from starch fermentation. PA complexes with starch, dietary proteins and lipids and inhibits a number of important digestive enzymes due to negatively charged phosphates on the *myo*-inositol ring. The stable phytin complex interferes with mineral absorption in the digestive tract of monogastrics and is therefore classified as an anti-nutrient. Recently, the PA hydrolysing phytases (EC 3.1.3.8), which are currently the top feed enzymes in the multibillion dollar enzyme market, have also been included in commercial enzyme cocktails routinely used for bioethanol production, although reports on the use of phytases to enhance bioethanol production are scarce

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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

## 1. Introduction

Supplementation of inorganic phosphorus to nonruminant animal feed and excretion of phosphorus from cereal phytate into effluents creates a global ecological problem leading to eutrophication of water bodies (Azeem et al., 2015; Vats et al., 2005). To safeguard the environment, and improve the phosphorus nutrition in farm animals, a sustainable approach is required (Lessl et al., 2013; Prasad et al., 2015). Phosphorus is stored in all plant seeds in the form of phytic acid, accounting

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