Studies on Phytase Producing Yeasts and its application in Food and Agriculture

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For the Award of the Degree of

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In

BIOLOGICAL SCIENCES

By

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Dedicated to

My Beloved Brother Venkata Mahesh Puppala

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CERTIFICATE

This is to certify that the work incorporated in this Ph.D. thesis entitled **"Studies on Phytase producing Yeasts and its application in Food and Agriculture"** submitted by **Mr. Kumar Raja Puppala** to Academy of Scientific and Innovative Research (AcSIR) in fulfillment of the requirements for the award of the Degree of **Doctor of Philosophy**, embodies original research work under our supervision. We further certify that this work has not been submitted to any other University or Institution in part or full for the award of any degree or diploma. Research material obtained from other sources has been duly acknowledged in the thesis. Any text, illustration, table etc., used in the thesis from other sources, have been duly cited and acknowledged.

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Declaration by the Candidate

I hereby declare that the original research work embodied in this thesis entitled, **"Studies on Phytase producing Yeasts and its application in Food and Agriculture"** submitted to Academy of Scientific and Innovative Research for the award of degree of Doctor of Philosophy (Ph.D.) is the outcome of experimental investigations carried out by me under the supervision of **Dr. Mahesh S Dharne and Dr. Jayant M Khire**, Senior Scientists, NCIM Resource Centre, CSIR-National Chemical Laboratory, Pune. I affirm that the work incorporated is original and has not been submitted to any other academy, university or institute for the award of any degree or diploma.

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(Research Scholar)

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° C	Degree centigrade
Å	Angstrom
AAg	Auto Aggregation
ADP	Adenosine di phosphate
AMP	Adenosine mono phosphate
ANOVA	Analysis of variance
ATP	Adenosine tri phosphate
BBD	Box Behnken Design
BSH	Bile Salt Hydrolase
CCD	Central composite Design
DCG	Dry cell gram
DO	Dissolved Oxygen
FTU	Phytase (fytase) Unit
g, mg, µg, ng	Gram, milligram, microgram, nanogram
GIT	Gastro Intestinal Tract
GRAS	Generally Regarded as Safe
h, hr	Hour
HAP	Histidine acid phosphatase
HAPhy	Histidine acid phytase
HCl	Hydrochloric acid
H_2SO_4	Sulphuric Acid
ICP AES	Inductively Coupled Plasma Atomic Emission Spectroscopy
IP1, IP2, IP3, IP4, IP5	Inositol mono-, bis-, tris-, tetrakis-, pentakis-Phosphate
IP6	Phytic acid

IU	International Unit
L, mL, µL	Liter, milliliter, microliter
M, mM, µM	Molar, millimolar, micromolar
MALDI – TOF	Matrix Associated Laser Desorption Ionization-Time Of Flight
MCP	Mono Calcium Phosphate
MGYP	Malt extract, Glucose, Yeast extract, Peptone Medium
MW	Molecular Weight
NCIM	National Collection of Industrial Microorganisms
OVAT	One variable At a Time approach
P	Phosphorus
PA	Phytic Acid
PAGE	Poly Acrylamide Gel Electrophoresis
PAP	Purple acid phosphatases
PBD	Plackett Burman Design
PBS	Phosphate Buffer Saline
PGP	Plant Growth Promotion
PSM	Phytase Screening Media
RP HPLC	Reverse Phase High Performance Liquid Chromatography
RSM	Response Surface Methodology
SCP	Single Cell Protein
SEM	Scanning Electron Microscope
SHb	Surface Hydrophobicity
SmF	Submerged Fermentation

SSF	Solid State Fermentation
TCP	Tri Calcium Phosphate
Wt	Weight
WCG	Wet cell gram

Synopsis of the Thesis to be submitted to the Academy of Scientific and Innovative Research for Award of the Degree of Doctor of Philosophy in Biological Sciences	
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Research Supervisor	Dr. Mahesh S Dharne
Research Co-Supervisor	Dr. Jayant M Khire

Introduction:

Phosphorus (P) is one of the major plant nutrients which affects physiology and morphology of the plants. Plants mainly utilize inorganic P, which is supplied through fertilizers. The fertilizer demand and consumption in India has been tremendously increased nearly 100 times in last few decades while P source is getting diminished. Sustainable agriculture and finding of alternative phosphorus sources are the only solutions for the above problem (Gujar et al. 2013).

Phytic acid (myo-inositol - hexakisphosphate) is an abundantly available form of organic phosphorus (P) present in soil (50-60%) and plants products like edible legumes, cereals, nuts, oil seeds and pollens (1-5%) (Vohra et al. 2011). It is the primary storage form of both phosphorus and inositol in plants (Savita et al. 2017), phytate content of different cereals and roots are mentioned in Haefner et al. (2005). Phytate considered as an anti nutrient factor, because of its high chelating capability towards the important metal ions and binding to proteins. Because of that, the bioavailability of important proteins and minerals get diminished (Vohra et al. 2011, Mikulski and Kłosowski 2017). Monogastric animals like poultry, pigs, fish and humans are unable to utilize this form of phosphorous due to lack of inadequate level of phytate hydrolyzing enzymes in their gastrointestinal tracts. This undigested phytate leads to the growth of algal blooms in water bodies which cause the environmental pollution and detrimental effects on the food chain (He et al. 2017). To remove such anti-nutrients factors (Phytic acid) from food obtained from plant sources, phytases were found very efficient and cheap enzymatic sources.

Some of the studies have been reported showing the utilization of phytic acid very efficiently from different microbial sources (Bhavsar and Khire 2014).

Yeasts are known for producing industrially important enzymes like Invertase, lactase, lipase, raffinase and pectinases (Moharib et al. 2000). Due to the nonpathogenic status of most yeast, they are commonly used in brewing, baking, winemaking and are most preferable enzyme sources for their application in feed and foods. The potential applications of yeast phytases are enormous, majorly in animal feeds and human nutrition. Yeasts like *Candida parapsilosis* (Ranjan and Sahay, 2013), *Pichia anomala* were for known their extracellular and cell-bound phytases. Therefore, further research efforts are called for discovering new phytases from yeast sources as very few are presently known, and to develop an economical process for their large-scale production (Kaur and Satyanarayana 2009).

Our objective was to screen and isolate a potential phytase producing yeast strains and its identification. Positive phytase producing strains further optimized for production and media optimization using different statistical methods. Scale up the process upto 10 L fermenter to increase the productivity of the enzyme. The resulted yeast phytases applications were assessed in the field of plant growth promotion and as probiotics in the health sector, where the yeast phytases are unexplored.

The thesis is divided into four chapters. Chapter 1 gives a detailed introduction to the importance of phosphate and its scarcity effects on future generations, phytases and its bioavailability, types of phytases, mechanism of action of different phytases and its applications in different sectors. Chapter 2 deals with initial screening, characterization and production of phytase producing yeasts and to find out the enzyme characteristics. Chapter 3 gives insights into optimization of media by using a statistical approach for higher phytase production by *Candida tropicalis* NCIM 3321 and scale up for commercialization perspective to improve phytase productivity and the application of its phytases in the agricultural sector by improving the plant growth. Chapter 4 includes the optimization of media components for enhanced phytase production by *Saccharomyces cerevisiae* NCIM 3662 and scale up studies for better productivity and evaluation of probiotic properties of NCIM 3662 and dephytinizing potential of the strain.

Chapter 1: Introduction: Yeast phytases

The first chapter provides a general introduction about yeast phytases and its reaction mechanisms on the substrate. It also discusses the phytase impotence in terms of increasing food demand and population, phosphate security. It also outlined the alternative sources of phosphates, contribution of phytases in food supplementation, unexplored yeast phytases for plant growth promotion, role of phytases in food fortification, unexplored role of phytase producing organisms as a probiotics and various applications of phytases. At the end, it described the basic work done in our lab and the objectives of the presented work.

Chapter 2: Screening, characterization and production of phytase producing yeasts

This chapter mainly focused on screening the yeast strains available in NCIM Resource Center and soil isolated yeasts for phytase production. The phytase screening consist of mainly two steps one is plate method in phytase screening media agar (PSM Agar) with calcium phytate as a substrate and the second one is confirmative quantification phytase enzyme activity assay. The strains were selected based on the ability to create a zone of clearance or hydrolysis around the colony on PSM containing calcium phytate. To avoid the false positive results provide by the strains from plate method was checked by quantifying the phytase production of the positive strains in submerged fermentation in MGYP media. *Candida tropicalis* NCIM 3321 was selected from NCIM cultures and *Saccharomyces cerevisiae* NCIM 3662 was selected from soil isolates in the screening process for studying the phytase production. The strains were identified and characterized by using morphological (SEM), biochemical (VITEK) and genetic level identification was done by sequencing the ITS region of the yeasts. Both the yeasts were submitted in NCIM and MCC culture collections center as a safe deposit. After identification, we find out the enzyme characteristics of both the strains *Candida tropicalis* NCIM 3321 and *Saccharomyces cerevisiae* NCIM 3662

Chapter 3: Optimization, Scale-up and evaluation of *Candida tropicalis* (NCIM 3321) phytases having plant growth promotion potential

Phytase is known to provide a solution for depletion of phosphorus (P). It helps it by hydrolyzing the insoluble P source in the soil, which is phytate. In this chapter, we provide insight on yeast *Candida tropicalis* (NCIM 3321) which produces cell-bound and extracellular thermostable phytase. The media components were optimized to enhance the enzyme production and checked

for plant growth promoting activity. On optimization, the isolate exhibited enhanced cell-bound and extracellular phytase activity by four folds (from 236 to 1024 IU DCG^{-1}) and by five folds (from 0.46 to 1.95 IU ml^{-1}) respectively in 36 h. The production time decreased to 24 h compared to shake flask on Up-scaling the production process upto 10 L scale, thus increasing the productivity of cell-bound (1810 IU DCG^{-1} day^{-1}) and extracellular phytase (6.08 IU ml^{-1} day^{-1}). The crude phytase (12 IU) from NCIM 3321 strain was studied for plant growth promotion activity in lab scale and field level experiments with maize crop. Findings of the study revealed that the extracellular phytase derived from nonpathogenic *C. tropicalis* (NCIM 3321) was found to be plant growth stimulating by increasing the available P in soil. Our findings of phytase isolated from non-pathogenic yeast *C. tropicalis* NCIM 3321 exhibited dephytinization potential. Therefore, the current study may have profound application in sustainable agriculture.

Chapter 4: Evaluation of phytase producing *Saccharomyces cerevisiae* (NCIM 3662) probiotic properties and its application in food fortification

The increase of undigested complexes of phytic acid in food is gaining serious attention to overcome nutritional challenges due to chelation effects. In this chapter, we investigated soil borne yeast phytase from *Saccharomyces cerevisiae* (NCIM 3662) for dephytinization of foods, probiotic properties and process development. The strain produced 45 IU/DCG of cell-bound phytase in an un-optimized medium, which was increased by four folds (164 IU/DCG) in 12 h using statistical media optimization. The process was scale-up upto 10L fermenter scale with increased phytase productivity of 6.4 IU/DCG/h as compared to the lab scale. The strain displayed probiotic characteristics like tolerance to artificial gastric acid conditions, hydrophobicity, autoaggregation, coaggregation and bile salt hydrolase (BSH) activity. Further, it could dephytinize (removal of phytic acid; an anti-nutritional factor) functional foods like Ragi (Finger millet) flour, Soya flour, Chickpea flour and poultry animal feed. Results less phytic acid content in food and it will make food more nutritive by releasing the important minerals in the process. It was confirmed by analyzing the important mineral content by using atomic emission spectroscopy. A combination of cell bound dephytinizing phytase, nutrition ameliorating and probiotic traits of *S. cerevisiae* (NCIM 3662) present profound applications in food technology sector.

Noteworthy Findings:

- Isolated phytase producing yeasts from NCIM culture collection and soil.
- Phytase producing non-pathogenic yeast strains were identified as *Candida tropicalis* and *Saccharomyces cerevisiae* using biochemical and molecular tests.
- Process development for higher phytase productivity and plant growth promoting the potential of *Candida tropicalis* extracellular phytase was successfully achieved.
- Successfully scaled up and evaluated *Saccharomyces cerevisiae* cell-bound phytase for probiotic properties.
- Dephytinizing ability of phytase producing *Saccharomyces cerevisiae* for increasing mineral availability in functional foods.

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1. **Puppala, K. R**., Naik, T., Shaik, A., Dastager, S., Kumar, R., Khire, J., & Dharne, M. (2018). Evaluation of Candida tropicalis (NCIM 3321) extracellular phytase having plant growth promoting potential and process development. *Biocatalysis and Agricultural Biotechnology*, *13*, 225-235. DOI: 10.1016/j.bcab.2017.12.013
2. **Puppala, K. R**., Kumar, R., Khire, J., & Dharne, M. (2018). Dephytinizing and probiotic potentials of *Saccharomyces cerevisiae* (NCIM 3662) strain for amelioration of nutritional quality of functional foods. *Probiotics and Antimicrobial Proteins.* DOI: 10.1007/s12602-018-9394-y
3. Dharne, M. S., Khire, J, S., **Puppala K. R**., A composition comprising phytase with improved enzyme activity. (Indian patent published, application number: 201611031947).

Chapter 1

Introduction: Yeast phytases

Summary:

Phosphorus (P) is an important nutrient for all organisms including animals and plants. It plays a major role in cell division and development and a key nutrient for energy storage molecules like ATP, ADP. It is also important for plant growth, as lack of P in soil may affect the physiology of the plants. Current soil management strategies are mainly dependent on chemical-based fertilizers and excess use of these leads to environmental pollution or imbalance of aquatic ecosystems due to eutrophication. The available organic form of P in soil is in the form of phytic acid (PA), which can't be utilized by the plants directly and is also one of the major storage forms of the P in plants. Phytase is an enzyme which can hydrolyze the phytate P into inorganic P and myo-inositols. Phytase is also known to dephosphorylate the phytate, which is one of the anti-nutritional factors to animals and humans due to its high chelating capability and blocking mineral bioavailability. Although phytases are available from different sources like plants and animal tissues, microbial phytases are promising among them because of its genetic manipulation for higher production. Overall, phytase is a key enzyme for veterinary (poultry, piggery), food, nutrition and healthcare industries. Due to GRAS status of the most yeasts, they are generally preferred choice by the majority of the food and beverage industries. Furthermore, phytase producing yeasts are well suited for phytate degradation in human food and animal feed as it can be directly mixed as whole cells by avoiding enzyme purification costs, making them economic and also targetted delivery in gut system. This chapter mainly describes the present scenario of phosphate utilization and alternative sources for P, the importance of phytases, different sources and types of phytases and its application in animal nutrition, human health, aquaculture, and sustainable agriculture.

1.1. General Introduction:

Phosphorus (P) is one of key elements which is getting depleted to such an extent that a healthy life might be at a risk for comprising with food security of rapidly and exponentially growing world population. The P is a critical, the second essential and indispensable nutrient required for plant growth and development (Bhardwaj et a1. 2014). The world phosphate usage as a fertilizer has increased to 45 million tons in 2017 with the largest consumption by Asia and South America as per predicted US Geological Survey (USGS) reports way back in 2014 status. Mis-management of P-bearing resources is quite far from the sustainability and causing a rise in prices of P mainly because of an increase in rock phosphate processing costs (Vassileva et al. 2010). Cordell et al. (2009) estimated the peak production of P to occur between 2030 to 2040.

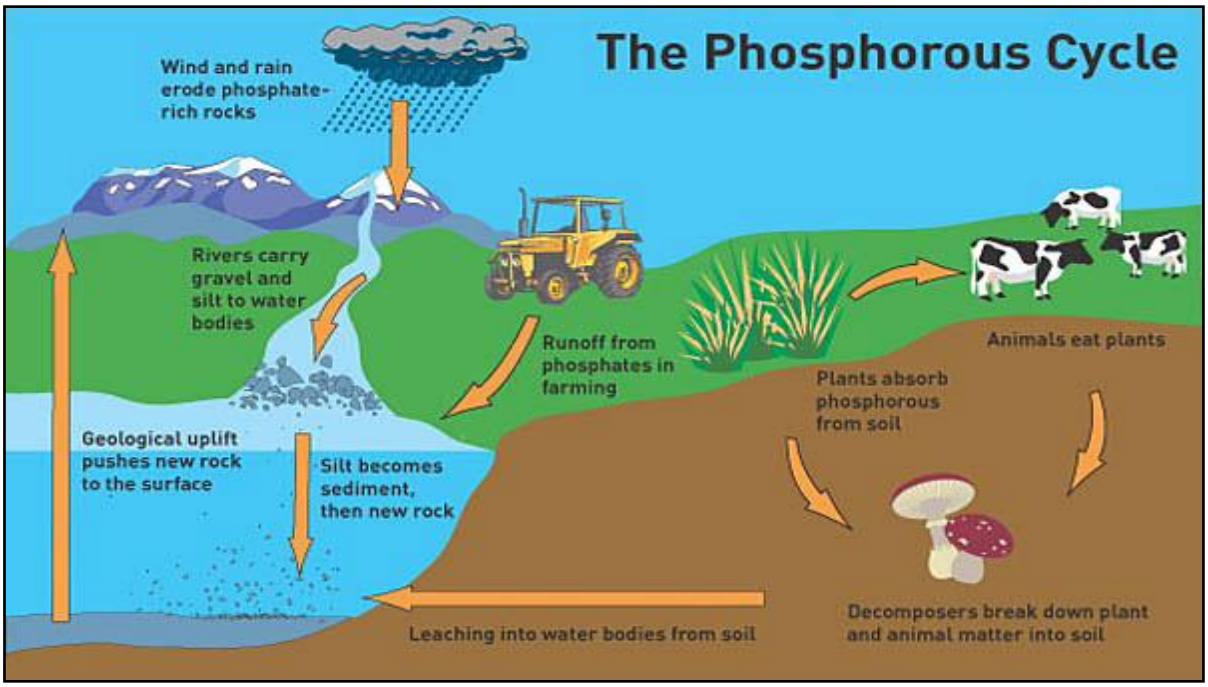

Figure 1: Phosphorus cycle (Figure adapted from http://www.environmental-research.ox.ac.uk/lets-talk-phosphorus-depletion)

As shown in Figure 1, the P cycle requires more time to uplift the rock phosphate into the surface. Existing P reserves are the finite, nonrenewable resources and there is no substitute to P. Thus, use of alternative phosphate sources is the only option to this P lacuna.

1.2. Phytic acid:

Soil contains a wide range of organic substrates, which are the sources of P for the growth of plants. However, it must be hydrolyzed to inorganic P before its assimilation by the plants. The predominant form of organic P in soil is phytate which accounts to nearly 60% (Singh and Satyanarayana, 2011). Many plants cannot uptake phytate-P (Figure 2) and organic phosphorous, because they lack a sufficient level of phytase and phosphatase (Gujar et al. 2013). It is a primary storage form of phosphorus and inositol in plants (Savita et al. 2017), phytate content of different cereals and roots are mentioned in Table 1 (Haefner et al. 2005, Hurrell et al. 2003). Phytate is an anti-nutrient factor, because of its high chelating capability for the important metal ions and proteins. Thus the bioavailability of important minerals, proteins, and amino acids get decreased in the host body (Vohra et al. 2011, Mikulski and Kłosowski 2017). Nonruminant animals like poultry, swine, fish and humans are unable to utilize this form of phosphorous due to lack of phytate hydrolyzing enzymes in their gastrointestinal tracts. The undigested phytate from these monogastric animals leads to the growth of algal blooms in water bodies by different means, which cause environmental pollution and detrimental effects on the food chain (He et al. 2017). So, in order to reduce the depletion of global P reserves and P pollution, research should be oriented towards more effective utilization of phosphates. The problem of decreasing resources of phosphate rock for P fertilizers production can be mitigated by searching microbes producing phytase and other plant growth promoting attributes.

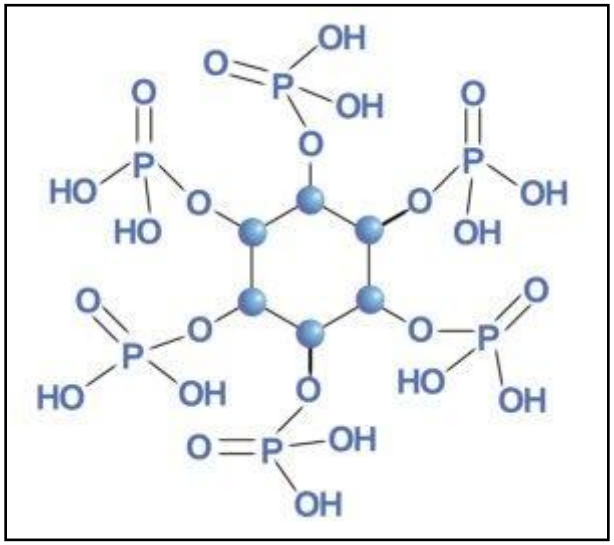

Figure 2: Structure of phytic acid or Myo-inositol hexakisphosphate or IP6

(Figure adapted from https://www.selfhacked.com/blog/phytic-acid-the-ultimate-anti-aging-compound)

Table 1: Phytate P concentration in some cereal and tubers (Haefner et al. 2005)

Cereals/Roots and tubers	Phytate P (g/100g dry matter)
Corn	0.24
Wheat	0.27
Barley	0.27
Oats	0.29
Sorghum	0.24
Rice unpolished	0.27

Cassava	0.04
Sweet potato	0.05
Wheat Soy blend	0.30

1.3. Phytases:

Phytases are a special class of phosphatases which sequentially degrade the myo-inositol hexakisphosphate (IP6) to lower myo –inositols also called as Inositol pentakis-, tetrakis-, tris-, bis-, mono- Phosphate (IP5, IP4, IP3, IP2, and IP1) as shown in Figure 3 (Shanmugam et al. 2018). Therefore, in livestock industries use of phytase can decrease phytic acid content in their feed supplementation by releasing the lower myo-inositol and phosphates. So, phytases can decrease the P pollution by reducing the excretion levels of P by availing the inorganic P to the livestock (Yano et al. 1999).

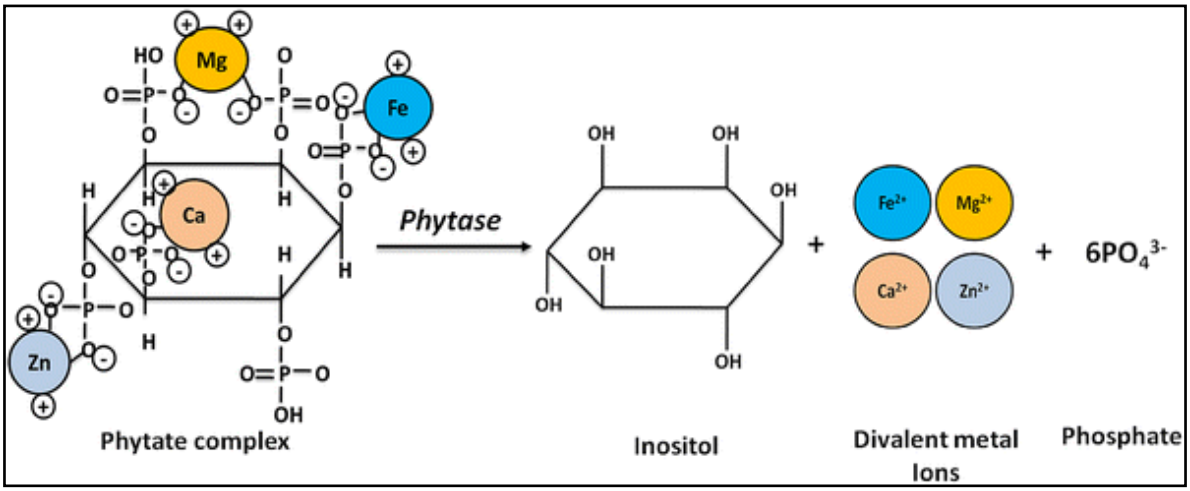

Figure 3: Mode of action of phytase on phytate mineral complex

(Figure adapted from Vashishth et al. 2017)

1.4. Classification of phytases:

Phytases are mainly classified based on the four different characteristics as shown in Figure 4. Those are (i) based on the stereospecificity of the phytase, on which P group it is acting on phytate molecule. E.g. 3-phytase (EC – 3.1.3.8), 5-phytase (EC – 3.1.3.72), 6-phytase (EC – 3.1.3.26). (ii) based on the source of the production of the phytase either fungi, yeast, bacteria, plant, and animals. (iii) based on the pH of the phytase, where the enzyme will be active as either acidic or alkaline phytases. (iv) based on the catalytic site or catalytic mechanism like phytases with same catalytic site sequence considered as a same group of phytases like Histidine acid phosphatase (HAP), purple acid phosphatase (PAP), protein tyrosine phosphatases (PTP) and based on the structure of the phytases like β propeller phytases (Bhavsar et al. 2014).

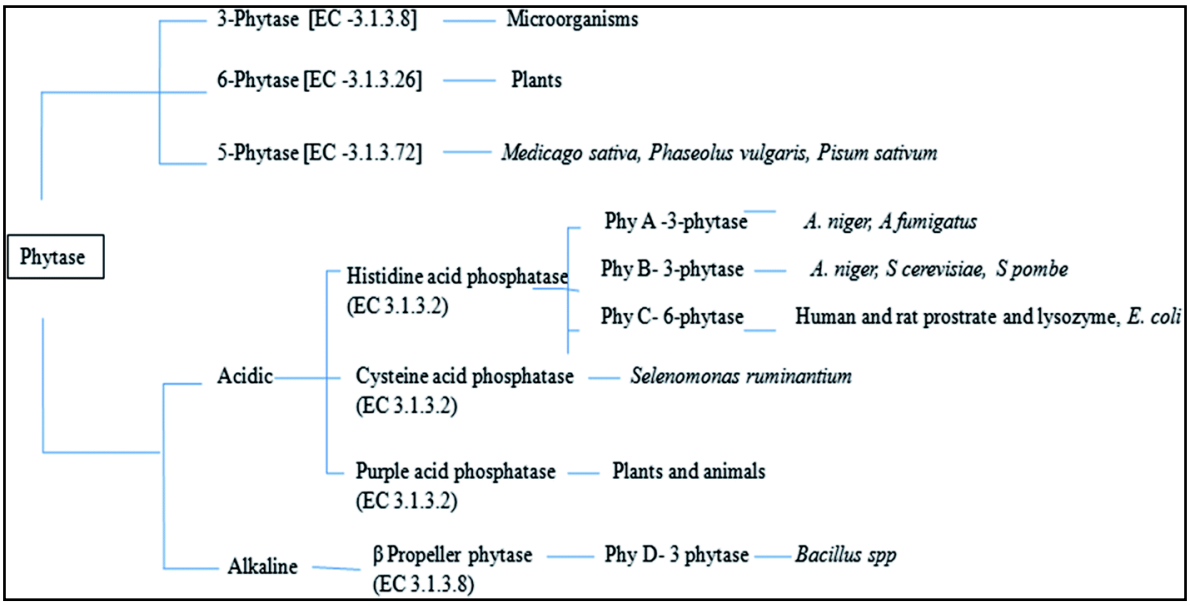

Figure 4: Classification of phytases

(Figure adapted from Bhavsar et al. 2014)

1.5. Sources of phytases:

Phytases are widely diversified all over nature in different sources like plants, animal and microbes (Turk et al. 2000).

1.5.1. Plant phytases:

Generally, P deficiency in the soil enhances phytase production in different species of plants roots. Li et al. (1997) screened 16 different species of plant roots for phytase production at a low phosphate level. Interestingly, all the species produced the phytase with an acid phosphatase enzyme. At low P level conditions, in *Brachiaria decumbens* CIAT 606, *Stylosanthes guianensis* CIAT 184 and tomato were shown highest phytase activity compare to all other plant species. These activities help in the plant to degrade inositol hexakisphosphate in the soil. Although phytases are producing a high amount, these phytases are highly variant due to its unstable nature. Moreover, these phytases can't retain its activity in acidic or alkaline soils and different geological temperatures. To overcome these obstacles researches are worked on the expression of stable phytases in plants to overexpress when it is required to the plant.

1.5.1.1. Transgenic plants expressing phytases:

Current soil management strategies mainly depended on inorganic chemical-based synthetic P fertilizers for better plant growth in P deficient fields. The usage of synthetic phosphate fertilizers is not only expensive; but also has deleterious effects on agricultural fields and environment. The use of transgenic plants expressing recombinant phytase is an alternative strategy. The use of transgenic plants in agriculture can provide essential

nutrients like P for plant growth. There are several reports on different plant species expressing the phytase gene successfully from different strains (Table 2).

Table 2: Transgenic plants expressing phytases and its source

Phytase source	Expressed in	Reference
A. niger	Tobacco	George et al. 2005
	Rice	Liu et al. 2007
	Sesame	Jin et al. 2004
	Trifolium repens	Han et al. 2007
A. awamori	Soybean	Gao et al. 2007
A. ficuum	Tobacco	Ullah et al. 1999.
	Alfalfa	Ullah et al. 1993
	Soybean	Yang et al. 2011.
A. fumigatus	Rice	Lucca et al. 2001
	Wheat	Brinch-Pedersen et al. 2006
E. coli	Maize	Nyannor et al. 2007
	Potato	Hong et al. 2008.
	Soybean	Bilyeu et al. 2008

S. occidentalis	Rice	Hamada et al. 2005

1.5.2. Animal phytases:

As compared to plant and microbial phytases, animal phytases are much lesser studied possibly due to low activity and uncertainty in the gastrointestinal tract. Although the specific activity of human tissue was known, there may be other factors which might affect the phytase activity in human tissue. Further, researchers also analyzed the phytase activity in intestinal mucosa of rat, chicken, and calf. Interestingly, pH of the human and animal tissue isolated phytases are alkaline (Liu et al. 1998).

1.5.3. Microbial phytases:

Although phytases are produced from different sources like plants, animal tissue, and feedstuff, microbial phytases are superior due to its easy handling and overproduction of the enzyme as compare to the others. The thermal and pH stability of these phytases has a broad range between different temperatures and acidic to alkaline. Microbial phytases are mainly bacterial phytases, fungal phytases, and yeast phytases.

1.5.3.1. Bacterial phytases:

Phytases are well studied in the last two decades due to its economic and industrial applications for degrading phytate to available P. Although fungal phytases are well established and higher producers like *Aspergillus* spp., because of some properties like catalytic efficiency, resistance to proteolysis, substrate specificity makes bacterial phytases alternative to fungal phytases. Phytases from bacteria were well studied across different

bacterial species like *Citrobacter, Enterobacter, Pseudomonas sp., Bacillus sp.* and *Klebsiella sp.* and some of the Lactobacillus species are considered as phytase producers (Singh and Satyanarayana, 2011). Generally, fungal phytases are extracellular in nature and bacterial phytases are cell-bound or intracellular enzymes (Konietzny et al. 2004).

1.5.3.2. Fungal phytases:

Fungal phytases are well known, well studied and high yield producers. Although, the first phytase was discovered in 1907 by Suzuki from rice, after nearly nine decades later, the first phytase was commercialized at an industrial level from the *Aspergillus* family. According to Shah et al. (2017), *Aspergillus niger* NCIM 563 was the highest phytase producing wild-type strain. It has been purified by single step downstream processing method ultrafiltration and evaluated for organophosphate degradation and dephytinization of whole wheat flour. Other genera like *Mucor, Penicillium,* and *Rhizopus* also showing active extracellular phytase activity (Liu et al. 1998).

1.5.3.3. Yeast phytases:

As compared to bacterial and fungal systems, yeast phytases are less studied. *Saccharomyces cerevisiae, Schwanniomyces castellii,* and *Pichia anomala* have shown phytase activity (Liu et al. 1998, Kaur and Satyanarayana, 2009). Yeast cultures are known for the industrial production of different feed additives and enzymes like lipase, invertase, and lactase (Moharib et al. 2000). These cultures "generally regarded as safe" (GRAS) because of nonpathogenic nature towards the host (Sourabh et al. 2011). Major applications of the yeast are enormous, mainly in animal feed and human nutrition. Different yeast species and their activity, fermentation mode and medium used were tabulated in Table 3.

Table 3: Different yeast phytases and their activity

Strain	Activity	Fermentation	Medium used	Reference
S. cerevisiae **NCIM 3662**	164 IU/g dry biomass (12hr)	Flask	PBD with OVAT (Optimized MGYP)	Puppala et al. 2018a
S. cerevisiae	135 IU/DCG (72hr)	Flask	Basal medium	In et al. 2008
S. cerevisiae	0.16 IU/ml (Extracellular) (42hr)	Flask	Defined Minimal Medium with phytic acid	Nuobariene et al. 2014
C. tropicalis **NCIM 3321**	996 IU/g dry biomass 2.04 IU/ml (Extracellular) (36hr)	Flask	After RSM optimization (Optimized MGYP)	Puppala et al. 2018b
P. anomala	68 IU/g dry biomass (24hr)	Flask	Glu-beef extract	Vohra and Satyanaraya ana et al. 2001
P. anomala	131 IU/g dry biomass (24hr)	Flask	After RSM	Vohra and Satyanaraya ana et al. 2002
P. anomala	176 IU/g dry biomass (24hr)	Flask	Cane molasses	Vohra and Satyanaraya ana et al. 2004

P. pastoris	300 U/g dry mass (Genetically modified) (72hr)	Flask	YEPD	Harnpicharnchai et al. 2009
P. kudriavzevii	0.162 IU/ml (Extracellular) (10hr)	Flask	YNB	Hellstrom et al. 2015
P. kudriavzevii	0.049 IU/ml (48hr)	Flask	Delft Phy medium	Greppi et al. 2015
P. rhodanensis	0.342 IU/ml (48hr)	Flask	YDP	Nakamura et al. 2000
P. spartinae	0.310 IU/ml (48hr)	Flask	YDP	Nakamura et al. 2000
P. kudriavzevii	0.255 IU/ml Total cell suspension (10hr)	Flask	YNB	Hellstrom et al. 2015
Arxula adeninivorans	2.9 IU/ml (48hr)	Flask	YPGal	Sano et al. 1999

1.6. Phytase applications:

Phytase was first discovered in 1907, from that time research is progressing for better phytase and its application in diversified fields. Some of the major applications are shown in Figure 5.

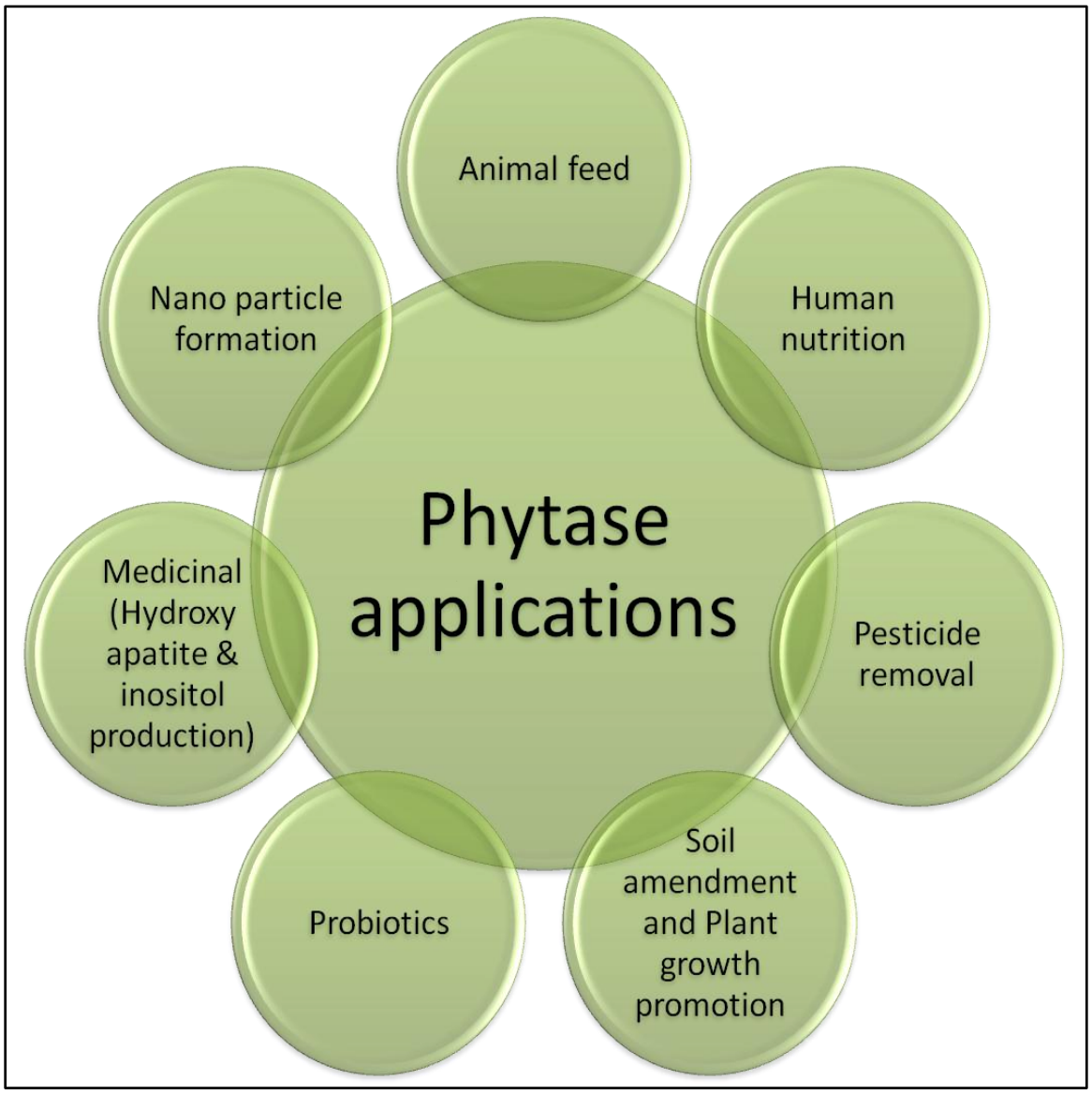

Figure 5: Application of phytases in different fields

1.6.1. Phytase in soil amendment:

Generally, P- deficient plants show restrained growth in different plant parts (root, stem, flowering, seed, and fruit); owing to the fact that plants and plant products lose their quality and quantity (Ji et al. 2014). To enhance crop yield, we should provide the essential nutrients to the plants. This can be fulfilled by the synthetic phosphate fertilizers usage in agricultural fields all over the world (Zaidi et al. 2010). However, the usages of synthetic fertilizers are not only expensive but also with long-lasting effects on the environment and agricultural fields. Currently, the research is focused on biostimulants that are ecologically and economically reasonable and provide an alternative source for these synthetic fertilizers.

In order to overcome these problems, soil amendment with phytases and phosphatases is an alternative for better growth of plants. The action of phytase and phosphatase enzymes on the organic form of P is mentioned in Figure 6. Phytase is known as the phytate-degrading enzyme, which produces myo-inositol phosphate derivatives and inorganic phosphate groups by hydrolyzing phytic acid (Jorquera et al. 2013). Addition of phytase/ phosphatase enzyme to the soil externally or expression of phytase gene from microbes in plants allow the plants to uptake phytate P as a source of P (Hayes et al. 2000, Richardson et al. 2001). The alteration in root morphology and physiology of the plant can uptake more amount of P from the soil by releasing the protons, organic acids, phenolics, P- mobilizing phytase and phosphatase enzyme into the rhizosphere of the soil (Raliya and Tarafdar, 2013). Therefore, the release of phosphatase and phytase enzyme by roots and microorganisms can enhance the availability of P.

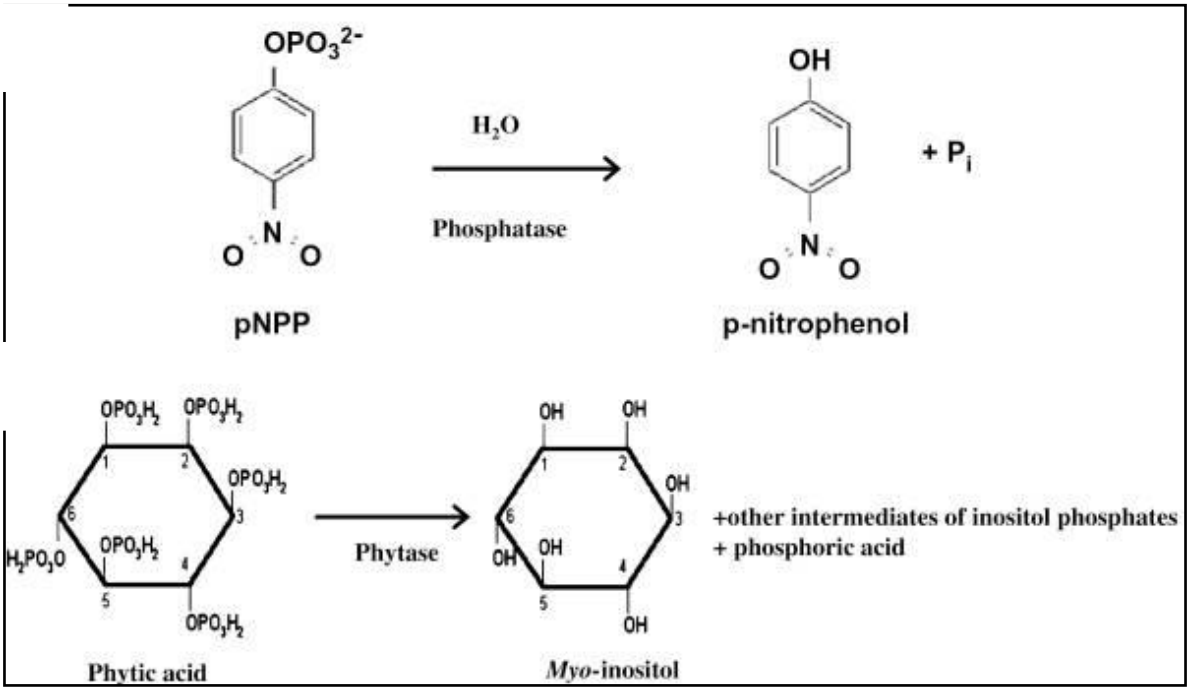

Figure 6: Action of Phosphatase and Phytase enzyme on organic phosphorous substrates.

(Adopted from Singh et al. 2011)

The concept of soil amendment was well known from 372-287 B.C, Theophrastus mentioned that mixing of different types of soil will cure the defects and add strength to the soil (Vessy et al. 2003). For degrading soil phytate extracellular phytases are required. There are different extracellular phytase producing microorganisms like *Pseudomonas sp.*, *Bacillus sp.*, *Aspergillus niger.*, *Candida tropicalis* etc. that are reported for successful production of phytase and plant growth promoting activity (Kumar et al. 2013, Amprayn et al. 2012).

Yadav and Tarafdar (2003) have mentioned that phytase and phosphatase producing fungi have the ability to hydrolyze different organic P compounds in arid and semi-arid soils. From soil, they isolated 7 efficient strains of fungi, viz., *Aspergillus*, *Emmericella* and *Penicillium*. These

strains were used as seed inoculants to investigate plant growth promotion and efficiency of the hydrolyzing capacity of organic phosphorus sources phytin and glycerophosphate. The results indicated that the identified fungi have enough potential to exploit native organic phosphorus to benefit plant nutrition. Tarafdar and Marschner (2005) studied the effect of inoculation of phytase producing fungi (PPF) *Aspergillus fumigatus* with vesicular-arbuscular mycorrhizal (VAM) species *Glomus mosseae* in a pot experiment mixed with sodium Phytate as a P source. The surface sterilized seeds were inoculated with *Aspergillus fumigatus* (PPF) and the soil was inoculated with *Glomus mosseae* and after 50 days the dry matter production and nutrient uptake in wheat were observed. The results suggested that there was an improvement in plant growth parameters like root, shoot, biomass, phosphatase activity and to a lesser extent of K and Mg. Similarly, the action of phytase and phosphatase producing actinomycetes was studied on the growth of cluster bean (*Cyamopsis tetragonoloba (L.) Taub*.), where it showed enhanced availability of P to the plant (Yadav and Tarafdar (2007a).

Yadav and Tarafdar (2007b) studied the effect of phosphatase and phytase producing fungus *Emericella rugulosa* in the field (loamy sand soil) using pearl millet as a test crop. According to his experiment out of the total the P present in the soil, 68 % of P was in the form of organic P (phytic acid) and only 1% soil P was available for plants. After 8 weeks of sowing, they tested for different enzyme activities and P content in the soil. Interestingly, the depletion of organic P was higher than the mineral P. So, they concluded that the microbial contribution was higher than the plant contribution in the degradation of different P sources. A significant improvement in plant root, shoot, total height and biomass. An extracellular Histidine Acid Phosphatases (HAP) phytase from a thermophilic mould *Sporotrichum thermophile* has shown plant growth promoting activity on wheat seedlings (Singh and Satyanarayana, 2010). It successfully hydrolyzes the different metal phytates

into available P. The fungus hydrolyzed Ca^{+2}, Mg^{+2}, and Co^{+2} phytates more efficiently than those of Al^{3+}, Fe^{2+}, Fe^{3+}, and Zn^{2+} and the rate of hydrolysis was higher at 60°C as compared to 26°C.

1.6.2. Phytase in feed supplement:

Phytate is generally considered as an anti-nutritional factor due to its high chelating capability towards important minerals, proteins and amino acids. This form of phytate is can't be digested by monogastric animals like poultry, swine etc. To improve animal performance using commercial industrial enzymes in animal feed grabbed the attention of feed manufacturers (Campbell and Bedford, 1992). Phytases are generally considered as an animal feed additive, which can enhance the nutritional value of animal feed material by releasing the phosphorus and other important minerals (Mitchell et al., 1997). Although, the first phytase has been commercialized since two and half decades back (1991) still there is a quest for ideal phytase. These days several commercial phytases (dominated by livestock industries) are available in the market mostly with recombinant phytases with different donor and production organism as shown in Table 4 (Greiner and Ursula, 2012).

Table 4: Commercially available phytases and producing companies

Product	Donor organism	Production organism	Company
Natuphos	*Aspergillus niger*	*Aspergillus niger*	BASF
Finase P/L	*Aspergillus niger*	*Trichooderma reesei*	AB Vista
Rovabio	*Penicillium*	*Pencillium*	Adisseo

	funiculosum	*funiculosum*	
Finase EC	*Escherichia coli*	*Trichooderma reesei*	AB Vista
OptiPhos	*Escherichia coli*	*Pichia pastoris*	Enzyvia
Phyzyme XP	*Escherichia coli*	*Schizosaccharomyces pombe*	DuPont
Quantum	*Escherichia coli*	*Pichia pastoris*	AB Vista
Ronozyme P	*Peniophora lycii*	*Aspergillus oryzae*	Novozymes/DSM
Ronozyme HiPhos	*Citrobacter braakii*	*Aspergillus oryzae*	Novozymes/DSM
-	*Buuttiauxella sp.*	*Trichoderma reesei*	DuPont
-	*Yersinia sp.*	*Aspergillus niger*	BASF

1.6.3. Phytase in Animal Nutrition:

Some of the cereals like wheat, rice and barley are having intrinsic phytases. These phytases are generally active in the feed. Still use of plant phytases in animal feed limited due to its activity was highly unstable within the same feedstuff. Moreover, while making pellet feed the enzyme is subjected to the temperature of 70 °C and more than that, at this temperatures plant phytases cant retains its activity. In addition to that the bioefficacy of phytases which are present in plant cereals is 40% lesser than the microbial phytases (Haefner et al. 2005). According to Dersjant-Li et al. (2018) by supplying the *Buttiauxella* sp. producing 6- phytase supplemented to hen diets with reduced nutrient density can

enhance the laying egg quality. The experiment was done with 2 concentrations of phytase 300 and 600 FTU. The treatment with phytase has reduced the diet cost by lowering the nutrient density and give profits to the egg production. Similarly, Gonsales et al. (2015) conducted trails using phytase for bone mineral deposition and manure production with less phytate P. 500 FTU / KG diet was given with proper controls result enhanced calcium and phosphorus availability in phytase supplemented pigs. At the same time, it decreases the potential environmental pollution caused by the piggery production.

1.6.4. Phytase in probiotics:

Probiotics are beneficial organisms to the human body or animal, which can help and support the body microflora. Hence these are commonly used in different food industries and health beneficial production. Recent days probiotics producing enzymes are making attention to the researchers, due to its dual effects makes double benefit to the host (Pakbaten et al. 2018). The phytase can degrade the phytic acid in the host and reduces the P excretion and probiotic characteristic give host to stabilize the gut microflora and stimulate the immune system. Zuo et al. (2010) successfully expressed the phytase gene from *Aspergillus ficuum* to probiotic organism *Lactobacillus casei* present times most of the poultry industries mainly depended on antibiotics, which eventually leads to antibiotic resistance is a major concern for the public as well as researchers. According to Zuo et al (2010), *Lactobacillus casei* with phytase activity was a possible solution to the above concerns. The *appA* gene, which produces 6-phytase from *E.coli* was cloned to *L. lactis* for the dual characteristics of phytase production and probiotic properties in Pakbaten et al. (2018). It results in the increment of phytate degradation in gastrointestinal tract (GIT) conditions and decreases the P pollution in the environment by degrading phytate P to

available P. Wang et al. (2014), co-expressed the endoglucanase gene from *Bacillus subtilis* and phytase gene from *Aspergillus fumigatus* to *Lactobacillus reuteri* for mutual effect in animal feed. They successfully transformed the two genes and add it in poultry feed in place of commercial phytase, results showed enhancement in feed conversion ratio at the age of 21 – 42 days and calcium, P levels in tibia at the age of 21 days.

1.6.5. Phytase in human nutrition:

Phytate or phytic acid is a major storage form of P and inositols in plants. Due to its high chelating capability, it can form complexes with different minerals like calcium, magnesium, iron, zinc. Lack of these minerals causes serious nutrient deficiency problems in humans. It also shows the effect on different lipids and proteins by making complexes. This issue mainly arises for people whose major diets are majorly from plant sources (Kumar et al. 2010). The human gut has the limited ability to hydrolyze phytates due to lack of phytase producing organisms in the human intestine. As per Kumar et al. (2010), phytate content can be decreased by processing the food using germination, soaking and fermentation techniques. Worldwide, human health authorities are recommended to enhance the use of whole grain cereal-based products like bread and breakfast cereals. The whole grain cereal flour contains fibres at the same time anti-nutritional factor phytate. To overcome this, the whole grain flour treated with are made with phytase or phytase producing organisms will help in decrease the phytate content in bread and other food products (Dave and Modi 2018, Turk et al. 1996, Haros et al. 2001, Lopez et al. 2003).

1.6.6. Phytase in Medicine/inositol production/hydroxyapatite production :

The main role of phytase is not the only degradation of phytate for the microbial metabolism. There are reports of inositol involvement in signal transduction and microbial pathogenesis. This makes the phytases and degraded products make interest for researchers in medical aspects (Mukhametzyanova et al. 2012). Production of myo-inositol phosphates and phospholipids and its intermediates has the potential application in the health industry. Yusoff et al. (2011) described that ASUIA279 phytase producing myo-inositol phosphates has shown the 40% anticancer activity on MCF -7 cancer cell lines. This type of phytases and its intermediates can help to produce potential anti-cancer agents. Hydroxyapatite is an element, which is present in the bones and is compatible with the human body. There are very few reports of biological production of this material using phytase as a main catalyst of the process. Roeselers and Loosdrecht (2010) demonstrated that *Arxula adeninivorans* producing phytase successfully formed the hydroxyapatite at a lab level.

1.6.7. Phytase in pesticide removal:

Organophosphorus pesticides are very harmful to the human body due to irreversible acetylcholinesterase (AChE) inhibitors activity, results in severe damage to nervous systems. According to Shah et al. (2017), *Aspergillus niger* NCIM 563 producing extracellular phytase was successfully degraded the organophosphorus pesticides like monocrotophos (MCP), chlorpyriphos and methyl parathion (MP) into less toxic products. The total process was done by cleaving the phosphorus group present in the product.

1.7. Conclusion:

Phytases are known for a solution to the imperfect management of P present in soil and other feed sources. Phytases are widely distributed in nature from different sources like microorganisms, plants and animals tissues. Although phytate was known from the past century (1907), still there is a quest for ideal phytase. The lack of inexpensive P has been considered as a major future crisis in the field of agriculture. Due to the GRAS nature of different yeasts and its diverse application of different enzymes in different industries make yeast as preferable organisms for the industries. Therefore, further research efforts are called for discovering new phytases from yeast sources as very few are presently known, and to develop an economical process for their large-scale production. It has the application in sustainable agriculture and human health and nutrition. Hence, the present work has the objectives of focused screening and isolation of phytase producing yeasts and its identification. Further, to evaluate the characteristics of enzyme and enhance the phytase production by media engineering and process scale-up till 10L fermenter for large-scale production. Further, we exploited these phytases for two applications *i.e.* in agriculture for enhancing growth in food crop and secondly in dephytinizing ability in functional foods and feeds by releasing important minerals.

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Chapter 2

Screening, characterization and production of phytase producing yeasts

Summary:

Phytase is enzyme that hydrolyzes phytate/phytic acid into myo-inositol and inorganic phosphorus (P). Phytate in soil is in organic form, it cannot be utilized by plants, and hence, phytase producing strains are useful in converting it to inorganic P which plants can uptake for growth. Similarly, phytic acid also acts as a chelating agent of important micronutrients and minerals (like Zn, Ca, Mg, Fe) that are useful for the growth of poultry animals, hence phytase producing yeast strains can release free form of phytate-bound minerals thereby boosting mineral bioavailability. This chapter mainly deals with a screening of phytase producing yeasts present in National Collection of Industrial Microorganisms (NCIM) culture collection and yeast strains isolated from soil. Phytase screening consists of mainly two steps one is plate method in phytase screening media agar (PSM Agar) with calcium phytate as a substrate followed by quantification of phytase enzyme activity assay in submerged fermentation using Malt extract Glucose Yeast extract and Peptone (MGYP) medium. Two yeast strains showing highest phytase activity (i.e. NCIM and soil source) were selected for further studies. The 2 strains were characterized by morphological (SEM), biochemical (VITEK-2) and genetic methods (sequencing of ITS region). Strains were identified as *Candida tropicalis (*NCIM 3321), and *Saccharomyces cerevisiae* (NCIM 3662) derived from NCIM collection and soil respectively. Further, enzyme characteristics like optimum temperature, pH, thermal stability, pH stability and effect of metal ions on phytases of both the strains were determined for *Candida tropicalis* NCIM 3321 and *Saccharomyces cerevisiae* NCIM 3662.

2.1. Introduction:

Sustainable agricultural practices are important to reduce the environmental pollution caused by general agricultural practices. P is a critical, the second essential and indispensable nutrient required for plant growth and development (Bhardwaj et a1. 2014). The world's phosphate usage as a fertilizer will increase to 45 million tons in 2017 with the largest consumption by Asia and South America as per USGS reports 2014. Existing P reserves are finite, nonrenewable and there is no substitute to P. Mismanagement of P-bearing resources is quite far from the sustainability and causing an increase in prices of P mainly because of increase in rock phosphate processing costs (Vassileva et al. 2010). Many livestock industries are producing a large amount of pollutant, which is an organic form of phosphorus (P) in the form of manure (Olstorpe et al. 2009). It eventually causes the eutrophication in the surface water bodies resulted harbored aqua life. It is necessary to provide a requisite amount of P to the livestock for its healthy growth by following safety measures margin (Yan et al. 2003). This organic form of P also called as phytate is important limiting nutrient for the plants and animals. Most of the plant's store P in the form phytate in seeds, legumes, and cereals. This form of P can't be digested by monogastric animals like poultry, swine (Shanmugam et al. 2018).

Phytases are special classes of phosphatases which sequentially degrade the myo-inositol hexakisphosphate to lower myo-inositols (Shanmugam et al. 2018). Therefore, in livestock forms use of phytase can decrease phytic acid content in their supplementation by releasing the lower myo-inositol and phosphates. Hence, phytases decreases the P pollution by reducing the excretion levels of P by increasing the bioavailibility of the inorganic P to the livestock (Yano et al. 1999). Phytases are diversified in nature, generally available in

microorganisms, plant and animal tissues. The enzymes phytases are of commercial importance in the food and feed industries (Chen et al. 1998). Most of the commercially important phytases are produced by the members of fungus *Aspergillus* genus, which are known for the production of extracellular phytases most prolifically. Furthermore, fungal phytases are generally heat and acid stable that will help the enzyme for making formulation in granular form. One of the commercialised phytase which has been added to animal feed is Natuphos; a recombinant phytase produced from the *phyA* gene expressed *Aspergillus niger* strain (Casey et al. 2004). Phytase producing microorganisms have significant commercial interest due to its economical and environmental reasons. Due to their great industrial significance, there is an ongoing interest in the isolation of novel microbial species producing phytases and optimization of their production (Ranjan and Sahay 2013).

Mainly yeasts are gaining attention because of their non-pathogenicity and well known for feed additives in many food processing industries. This chapter includes the screening and isolation of phytase producing yeasts from microbial culture collection resource as well from the soil. Microbial strains showing significant phytase activity were subjected to morphological, biochemical and genetic identification methods for their species-level identification. Furthermore, we evaluated the characteristics of phytases produced by both the strains and effect of different physiological conditions such as temperature, pH and different metal ion concentration on enzyme activity was also studied.

2.2. Material and Methods

2.2.1. Media and chemicals

Phytic acid sodium salt was purchased from Sigma Aldrich (St Louise, MO, USA). Glucose, sodium nitrate, agar magnesium sulfate, ferrous sulfate, yeast extract, malt extract and peptone bacteriological from Hi-Media (Mumbai, India). Manganese sulfate was procured from Merck (India).

2.2.2. Screening, identification and characterization of phytase producing yeasts

Around 200 yeast strains were procured from NCIM culture collection, Pune, India and other 25 yeast strains, which were isolated from rhizospheric soil. Phytase producing strains were screened using Phytase Screening Medium (PSM) with following composition (g/L): 15.0 D-glucose, 3.0 Ca-Phytate, 5.0 $NaNO_3$, 0.5 $MgSO_4.7H_2O$, 0.5 KCl, 0.01 $FeSO_4.7H_2O$, 0.01 $MnSO_4.6H_2O$, 20 agar, pH 5.5 (Bhavsar et al. 2011). Plates were incubated at 28°C and checked for phytase production based on the zone of hydrolysis around the colonies. To eliminate false positive results phytase activity was confirmed in submerged fermentation quantitatively in MGYP broth. The primary inoculum was developed in MGYP media consisting of (g/L): 3.0 malt extract, 10.0 glucose, 3.0 yeast extract, 5.0 bacteriological peptone, and pH 6.5 for 12 hr at 28°C.

Based on initial screening, strains with the highest activity were characterized using morphological, molecular and biochemical tools. Morphological and physiological characteristics were studied using scanning electron microscopy (SEM). The strains were identified using Vitek 2-compact and based on ITS region sequencing analysis. Biochemical characterization was done by using ID-YST Vitek2 cards (BioMerieux). For experimentation, the manufacturer's instructions followed to fill the ID-YST Vitek2 cards

to test in the Vitek2-compact automate. Results were described with a high confidence level (excellent identification) (Pinot et al. 2011). Molecular level identification was done using sequencing the internal transcribed spacer (ITS) region. Amplification was done using the ITS primers; ITS1 (5'TCCGTAGGTGAACCTGCGG3') ITS4 (5'TCCGTAGGTGAACCTGCGG3') (Cardoso et al. 2017). The PCR condition included an initial denaturation at 94°C for 3 min followed by 35 PCR cycles consisting denaturation at 94°C for 1 min, annealing temperature 55°C for 1 min, and elongation at 72°C for 1.30 min and a final elongation for 7 min. The PCR amplified products were run on 0.8% agarose gel and visualized using gel documentation (Protein Simple). The amplified PCR products were purified by the EXO-SAP method and sequenced in ABI 3500xl genetic analyzer (Life Technologies). Sequence editing was done CHROMAS LITE (1.5) and then analyzed using the BLAST tool (Altschul et al. 1990) with the sequence from the National Centre for Biotechnology Information (NCBI) database. The further phylogenetic tree was constructed using MEGA 6 software using neighbor-joining method with 1000 bootstrap values (Tamura et al. 2013).

2.2.3. In vitro cytotoxicity test for NCIM 3321

The tests for cytotoxicity were carried out at CSIR – Indian Institute of Toxicology Research, Lucknow, India using mouse fibroblast cells L929 cells (approved cell line for Cytotoxicity assessment by ISO – 10993 - 5). The lyophilized culture was revived in DMEM medium (Cat. No. 12100-046, Invitrogen, USA), 5% fetal bovine serum (FBS), antibiotic-antimycotic solution (1 ml/ 100 ml of medium), 0.2% sodium bicarbonate. This was then subjected to Sonication for 3 cycles at 10 KHz for 10 seconds and then diluted to desired concentrations i.e., 1 mg ml^{-1} to 0.0001 mg ml^{-1} adjusting pH to 7.4. The MTT (3-

(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium) assay showed the ability of the cell to convert the yellow MTT tetrazolium salt into a purple MTT formazon, by mitochondrial dehydrogenase activity of live cells, which is measured spectrophotometrically at 530 nm. MTT assay provides an indication of mitochondrial integrity and activity, which is interpreted as a measure of percent cell viability. The assay was done following the method of Siddiqui et al. (2008). In brief, ($1 X 10^4$) were seeded in 96-well tissue culture plates and incubated in the CO_2 incubator for 24h at 37°C prior experimentation for the proper attachment of the cells. Then the medium was aspirated and cells were washed with phosphate buffer saline. The cells were then allowed to grow in culture medium prepared using variable concentrations of the test material. A parallel set of cells was also grown without the exposure to test material under identical experimental conditions and served as control. MTT assay was done at 24, 48, 72 and 96h. Tetrazolium (10 µl $Well^{-1}$ containing 100 µl of cell suspension) salt was added 4h prior to completing each incubation period. Then, the reaction mixture was carefully taken out and 200 µl of DMSO was added to each well by mixing with a pipette to get homogeneity in the solution. After 10 minutes, the color was read at 530 nm, using multiwall microplate reader (Bio-Tek, USA). The values obtained through the unexposed control group were considered as 100 % and used to calculate the percent of cell viability in the exposed group. The data presented are percent well viability comparing to control group.

2.2.4. Phytase Production and assay

Primary inoculum was prepared by separately inoculating a loop full of NCIM 3321 and NCIM 3662 cultures in 5 ml MGYP medium and incubated at 28°C for 12 h. 50 ml MGYP broth was inoculated with 2% of primary inoculum (~10^{11} Cells) and incubated at 28°C for

2 days at 165 rpm in shaking conditions. Culture suspension was centrifuged at 8000 rpm for 10 min and cell-free supernatant was collected in a sterile tube. The cell pellet was washed twice with sterile distilled water and dried in pre-weighed tubes at 80°C for a constant dry cell weight. The cell-bound activity was determined by diluting the cells with sodium – acetate buffer pH 4.5 according to its wet cell weight, resulted in cell suspension used as the enzyme. Throughout the study, the cell-bound phytase activity denoted in IU per Dry Cell Gram (DCG) or 1 gm of dry cells. Both the samples *i.e.,* cell pellet and cell-free supernatant were periodically checked for cell-bound and extracellular phytase assay respectively. Phytase activity was measured at 70°C and 50°C in 0.3 ml of 200 mM sodium - acetate pH 4.5 buffer with 0.1 ml of the enzyme for 30 min using 0.1 ml 200 mM sodium phytate as a substrate. The liberated inorganic phosphate was measured by ammonium molybdate method as described by Heiohnein and lathi (1981). To stop the reaction, freshly prepared 4 ml solution of acetone: 5N H_2SO_4: 10 mM ammonium molybdate (2:1:1 v/v/v) and 400 µl of 1 M citric acid were added to the assay mixture. Absorbance was measured at 370 nm. One unit of phytase activity (IU) was expressed as the amount of enzyme that liberated 1 µmol phosphorus per minute under standard assay conditions.

2.2.5. Effect of pH and Temperature

Phytase activity at varying pH and temperature was measured for both the enzymes produced by NCIM 3321 and NCIM 3662. The buffer used for optimum pH measurement was 0.2 M glycine-HCl, 0.2 M Na-acetate, 0.2 M Tris-HCl in the ranges of pH 1.5-3.5, pH 4.5-6.5 and pH 7.5-9.5 respectively. The optimum temperature was measured in the range of 30°C – 80°C. The thermal and pH stability of crude enzyme was evaluated at each temperature for 30 min and at each pH for overnight.

2.3. Results and Discussion:

To determine all the characteristics crude enzyme has been used because the applications focused on this thesis does not require the purified enzymes. The results for the experimental methods explained above are discussed in brief.

2.3.1. Screening and identification of phytase producing Yeasts

Selections of phytase producer were done on the basis of the zone of hydrolysis on PSM media as shown in Fig 1a, 1b. Since this method is unable to differentiate between phytase activity and acid production, phytase activity was further quantified and confirmed in submerged fermentation for all positive NCIM strains and soil isolates. Based on primary screening out of 200 strains from NCIM collection, 28 strains showed a positive zone of hydrolysis on PSM agar plate. These 28 strains showed the activity in the range of 4 IU/DCG to 236 IU/DCG cell-bound activity and 0.02 IU/ml to 0.46 IU/ml extracellular activity. Similarly, 25 strains isolated from soil also showed zone of hydrolysis on PSM agar plate. The cell bound activities of these strains were in the range of 0.8 IU/DCG to 40 IU/DCG. From all above isolates, strain NCIM 3321 and one of the soil isolate were found to be highest phytase producer. The NCIM 3321 strain have cell-bound and extracellular phytase activity of 236 IU/DCG and 0.46 IU/ml, respectively. Soil isolate exhibited 45 IU/DCG cell bound activity and no extracellular activity. Media optimization studies were carried out for further enhancing the phytase production.

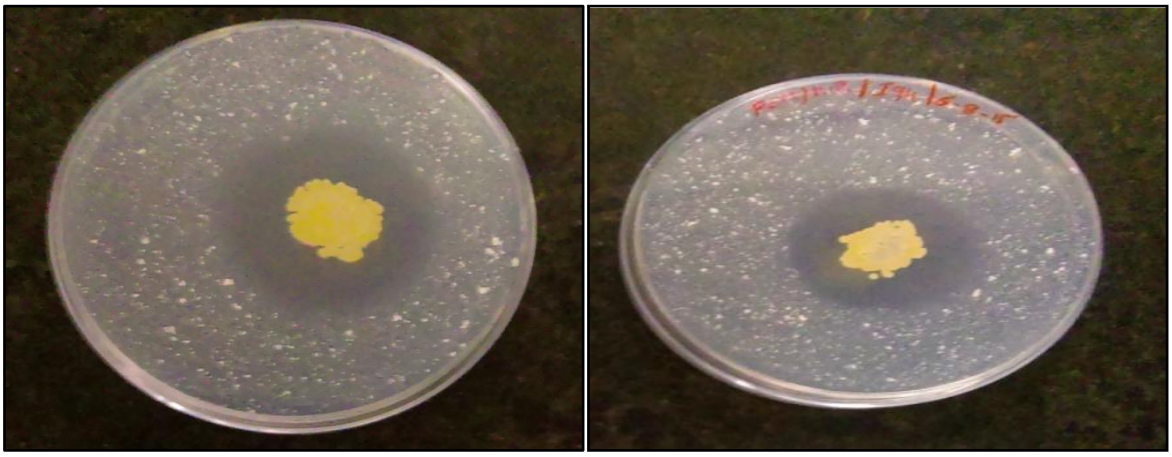

Fig 1a **Fig 1b**

Figure 1: Characteristics of *Candida tropicalis* (NCIM3321) producing cell-bound and extracellular phytase.
Fig 1a- Hydrolysis of calcium phytate on PSM plate by NCIM 3321, **Fig 1b**- Hydrolysis of calcium phytate on PSM plate by NCIM 3662.

The morphological identification NCIM 3321 strain was done by using Scanning electron microscopy illustrates budding yeast with pseudohyphe, which are common features of Candida sp. as shown in Figure 2. Biochemical characterization was done by using Vitek2-compact (bioMerieux) as shown in Table 1. Using Vitek2 – compact automate database the strain showed similarity with *Candida tropicalis* strain with the probability of 99% which is the highest confidence level for excellent identification by this method. Molecular level identification was done for phytase producing yeast by amplifying the ITS region of the yeast. The sequencing results showed (100% similarity) with *Candida tropicalis* as shown in phylogenetic tree Figure 3. The strain has submitted in Microbial Culture Collection Centre, Pune under IDA rule with the accession number of MCC 0079 and ITS sequence has been deposited in Genbank under the accession number MF197907. Since *Candida tropicalis* is an opportunist fungal pathogen, the strain used in the present

investigation was further tested for its cytotoxicity. Generally, different microbial classes are known to produce phytases among which, phytase produced by yeast having more importance in food and feed application. *Candida tropicalis* producing cell-bound and extracellular phytases are superior than the phytases reported from yeasts such as *Pichia anomala, Saccharomyces cerevisiae* and *Pichia kudriavzevii*with (Vohra and Satyanarayana, 2001; In et al., 2008, Greppi et al., 2015, Hellstrom et al., 2015). To the best of our knowledge, the *Candida tropicalis* strain reported in this study was highest phytase producer in yeasts with the activity of 1014 IU DCG^{-1} and 1.95 IU ml^{-1}.

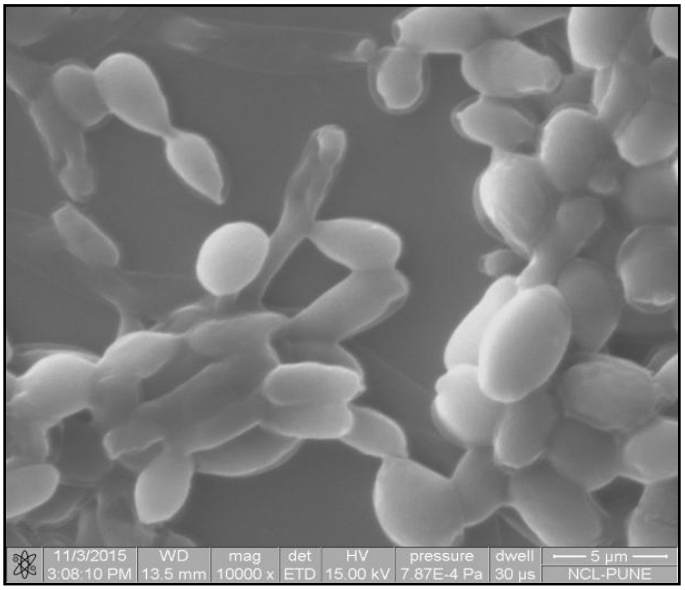

Figure 2: Scanning electron microscopic image of NCIM 3321

Table 1: Biochemical characterization of *Candida tropicalis* (NCIM3321) using Vitek 2

Test	Result*	Test	Result*
L-Lysine-ARYLAMIDASE	-	L-MALATE assimilation	+
Leucine-ARYLAMIDASE	+	ARGININE	+
ERYTHRITOL assimilation	-	GLYCEROL assimilation	-
Tyrosine ARYLAMIDASE	(+)	BETA-N-ACETYL-GLUCOSAMINIDASE	-
ARBUTIN assimilation	-	AMYGDALIN assimilation	-
D-GALACTOSE assimilation	+	GENTOBIOSE assimilation	-
D-GLUCOSE assimilation	+	LACTOSE assimilation	-
METHYL-A-D-GLUCOPYRANOSIDE assimilation	+	D-CELLOBIOSE assimilation	-
GAMMA-GLUTAMYL-TRANSFERASE	-	D-MALTOSE assimilation	+
D-RAFFINOSE assimilation	-	PNP-N-acetyl-BD-galactosaminidase 1	-
D-MANNOSE assimilation	+	D-MELIBIOSE assimilation	-
D-MELEZITOSE assimilation	+	L-SORBOSE assimilation	-
L-RHAMNOSE assimilation	-	XYLITOL assimilation	(+)
D-SORBITOL assimilation	+	SUCROSE assimilation	+
UREASE	(-)	ALPHA-GLUCOSIDASE	+
D-TURANOSE assimilation	+	D-TREHALOSE assimilation	+
NITRATE assimilation	-	L-ARABINOSE assimilation	-
D-GALACTURONATE assimilation	+	ESCULIN hydrolysis	-
L-GLUTAMATE assimilation	+	D-XYLOSE assimilation	+
DL-LACTATE assimilation	-	ACETATE assimilation	+
CITRATE (SODIUM) assimilation	+	GLUCURONATE assimilation	+
L-PROLINE assimilation	+	2-KETO-D-GLUCONATE assimilation	+
N-ACETYL-GLUCOSAMINE assimilation	+	D-GLUCONATE assimilation	(+)

*Test indicates the biochemical test name and in results each of the sign indicates following values. + = 95% to100% positive; (+) = 6% to 94% positive; – = 0% to 5% positive

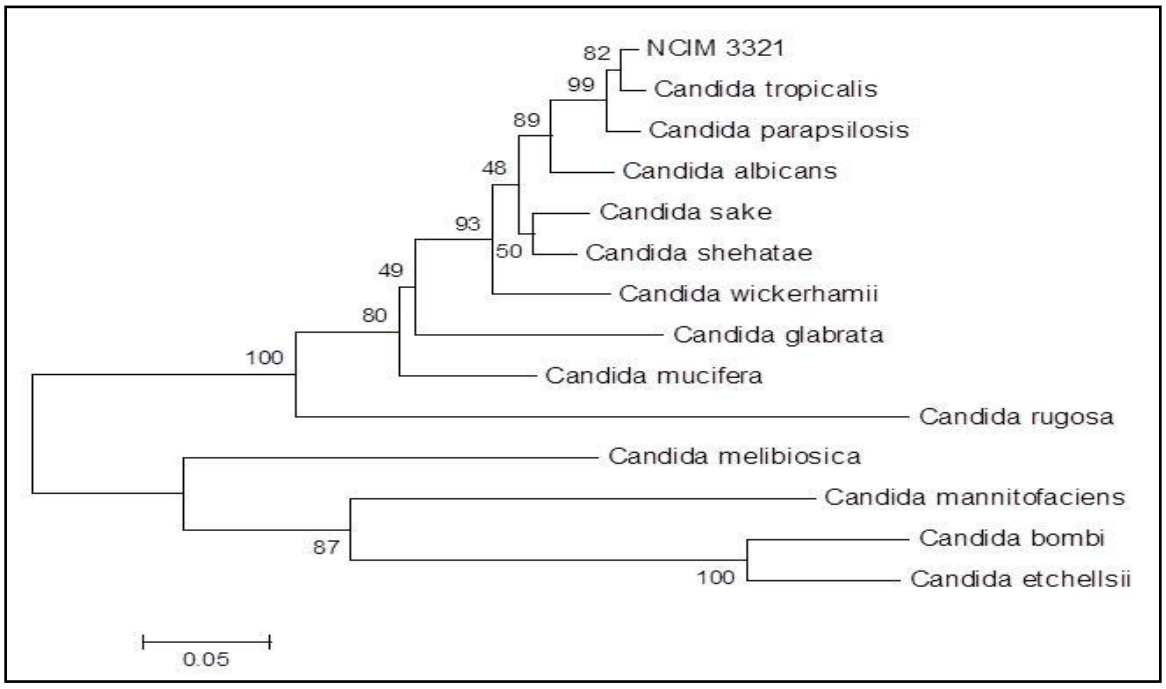

Figure 3: Phylogenetic tree of NCIM 3321

For soil isolate, morphological identification was done using scanning electron microscopy illustrates budding yeast, which are common features of Saccharomyces sp. as shown in Figure 4. Biochemical characterization was done by using Vitek2-compact automate (bioMerieux) as shown in Table 2. Based on biochemical test results Vitek2 – compact automate database the strain showed similarity with the *S. cerevisiae* strain with the probability of 99% which is high confidence level for excellent identification by this method. Molecular level identification was done for phytase producing yeast by amplifying the ITS region of the yeast with the help of ITS1 and ITS4 primers, the sequencing results gave the closest similarity with *S. cerevisiae* as shown in phylogenetic tree Figure 5. The strain has submitted in microbial culture collection center, Pune under IDA rule with the accession number of MCC0094, NCIM with the accession number of NCIM 3662 and ITS

sequence has been deposited in Genbank under accession number MG564349. Phytases are widely distributed among plants, bacteria, yeast, fungi, and certain animal tissues. *S. cerevisiae* is generally recognized as safe (GRAS) organism, so phytase produced by *S. cerevisiae* could have more importance in food and feed application. Some of the reported cell bound phytase producing yeast strains are *Rhodotorula gracilis* 38.9 IU/DCG (Bindu et al. 1998), *Candida krusei* 35 IU/DCG (Quan et al. 2001), *Pichia anomala* 39 IU/DCG (Vohra and Satyanarayana 2001), *S. cerevisiae* 23 IU/DCG (In et al. 2008).

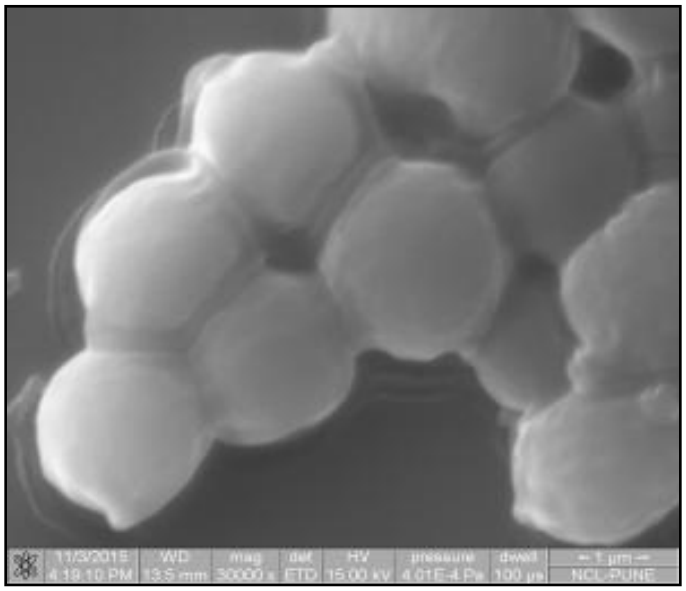

Figure 4: Scanning electron microscopic image of NCIM 3662

Table 2: Biochemical characterization of *S. cerevisiae* (NCIM3662) using Vitek 2

Test	Result	Test	Result
L-Lysine-ARYLAMIDASE	-	L-MALATE assimilation	-
Leucine-ARYLAMIDASE	+	ARGININE	-
ERYTHRITOL assimilation	-	GLYCEROL assimilation	-
Tyrosine ARYLAMIDASE	-	BETA-N-ACETYL-GLUCOSAMINIDASE	-
ARBUTIN assimilation	-	AMYGDALIN assimilation	-
D-GALACTOSE assimilation	+	GENTOBIOSE assimilation	-
D-GLUCOSE assimilation	+	LACTOSE assimilation	-
METHYL-A-D-GLUCOPYRANOSIDE assimilation	-	D-CELLOBIOSE assimilation	-
GAMMA-GLUTAMYL-TRANSFERASE	-	D-MALTOSE assimilation	+
D-RAFFINOSE assimilation	+	PNP-N-acetyl-BD-galactosaminidase 1	-
D-MANNOSE assimilation	+	D-MELIBIOSE assimilation	-
D-MELEZITOSE assimilation	-	L-SORBOSE assimilation	-
L-RHAMNOSE assimilation	-	XYLITOL assimilation	-
D-SORBITOL assimilation	-	SUCROSE assimilation	+
UREASE	-	ALPHA-GLUCOSIDASE	-
D-TURANOSE assimilation	+	D-TREHALOSE assimilation	(-)
NITRATE assimilation	-	L-ARABINOSE assimilation	-
D-GALACTURONATE assimilation	-	ESCULIN hydrolysis	-
L-GLUTAMATE assimilation	-	D-XYLOSE assimilation	-
DL-LACTATE assimilation	+	ACETATE assimilation	+
CITRATE (SODIUM) assimilation	-	GLUCURONATE assimilation	-
L-PROLINE assimilation	-	2-KETO-D-GLUCONATE assimilation	-
N-ACETYL-GLUCOSAMINE assimilation	-	D-GLUCONATE assimilation	-

*Test indicates the biochemical test name and in results each of the sign indicates following values. + = 95% to100% positive; (+) = 6% to 94% positive; – = 0% to 5% positive

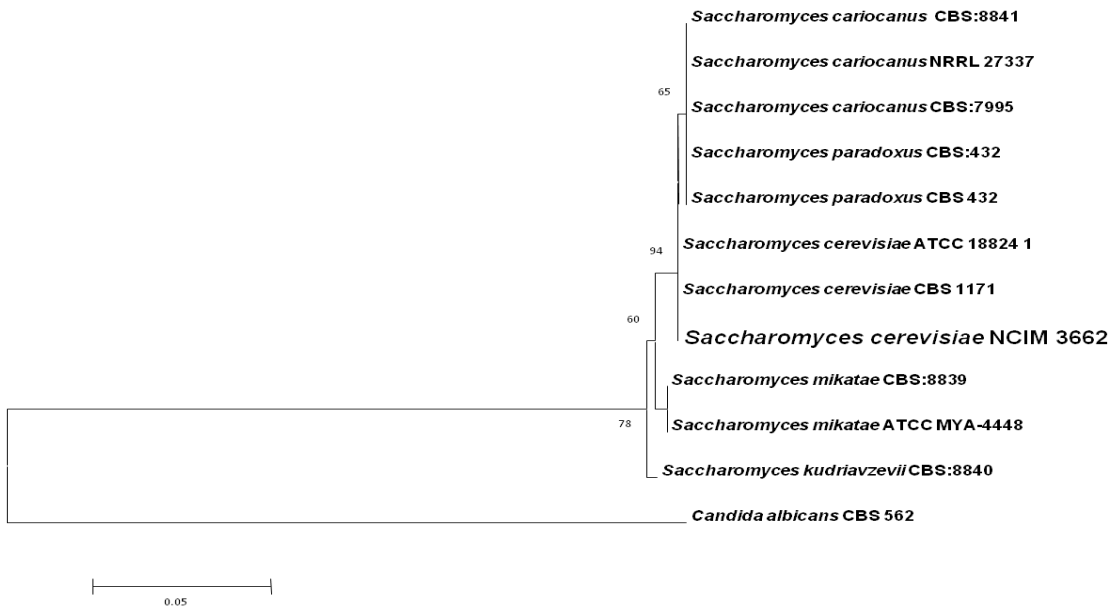

Figure 5: Phylogenetic tree of NCIM 3662

2.3.2. Cytotoxicity test for NCIM 3321

As per microscopic observations, there was no sign of toxicity and morphological changes observed in the cells (L929) in any of concentration of test material throughout the exposure period *i.e.*, up to 96h. There was no morphological disability in cells with normal proliferation up to 96 h. also quite normal. The cells retained all the healthy features without any sign of toxicity. To further confirm the microscopic observations, MTT assay, one among the standard method for cytotoxicity assessment, was carried out. The results of the cytotoxicity study are summarized in Fig 6. Similar to the morphological examination, there was no admissible alteration in the percent cell viability against the exposure to any concentration of test material at any time point. The *Candida tropicalis* yeast strain was non – toxic up to 1 mg/ml concentration for a period up to 96 hr in L920 cells (approved

cell line for Cytotoxicity assessment by ISO – 10993 - 5). The results confirm the non-toxic nature of *C. tropicalis* NCIM 3321.

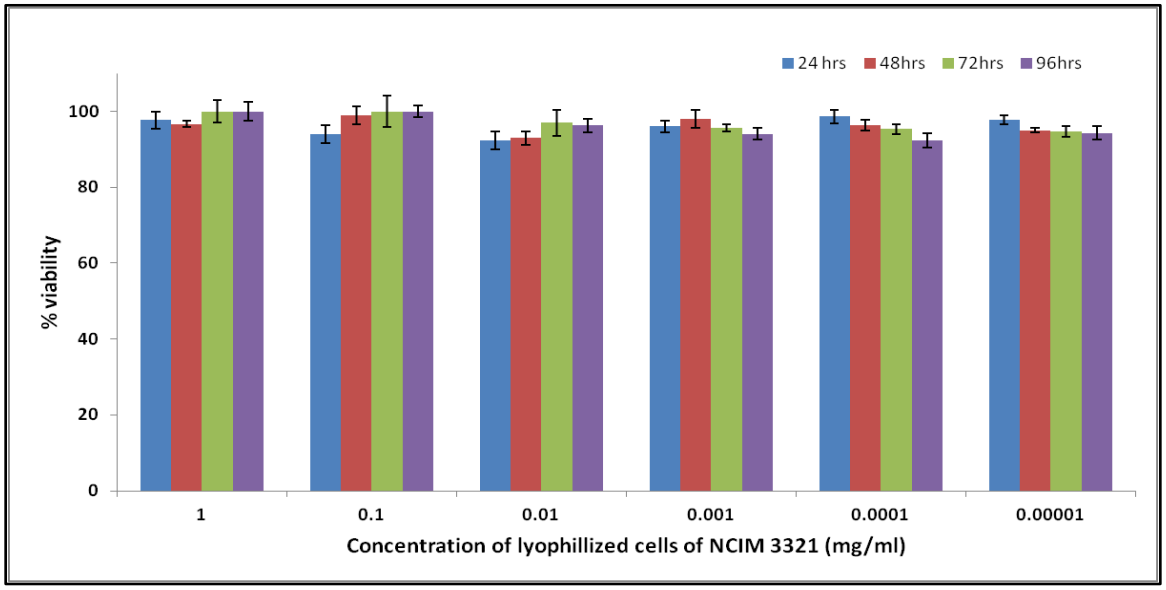

Figure 6: Cytotoxicity evaluation (MTT assay) in L929 mouse fibroblast cells exposed to lyophilized NCIM 3321 yeast culture

2.3.3. Characteristics of Candida tropicalis NCIM 3321 *producing phytases*

2.3.3.1. Effect of Temperature and pH

Optimal temperature and pH are essential for enzymes to evaluate the total efficiency of the enzyme at optimal conditions. The conditions for optimum temperature and pH were assessed for maximum phytase production. It was observed that the pH 4.5 - 5 and temperature 70 $^{\circ}$C for both cell-bound and extracellular phytase as shown in Fig 7a and Fig 7b respectively. However, the enzyme will still active at field temperatures. Since phytic acid can be degraded only by phytase, phytase with a wide range of stability would be preferable. Results here show more than 30 % activity of phytase from pH ranging from 3 to 6 with a wide range of temperature stability from 45 to 75 $^{\circ}$C. Based on the

characteristics of enzymes, there may be chances of same enzyme produced in both fractions. These properties of phytase would be beneficial in acidic soil conditions and at high-temperature tropical conditions.

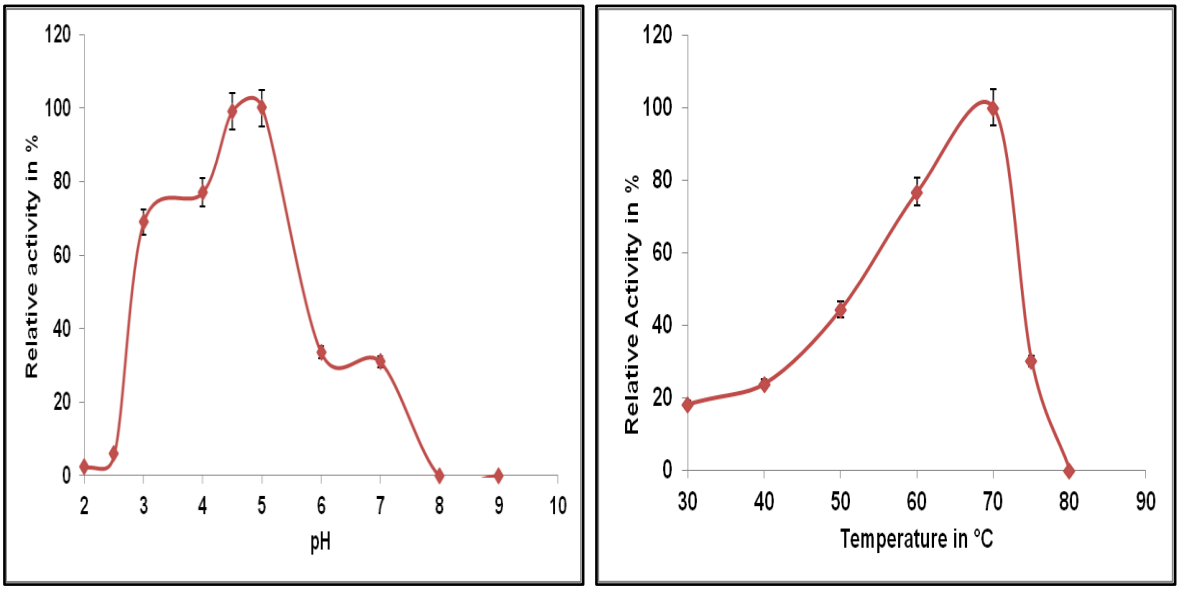

Fig 7a

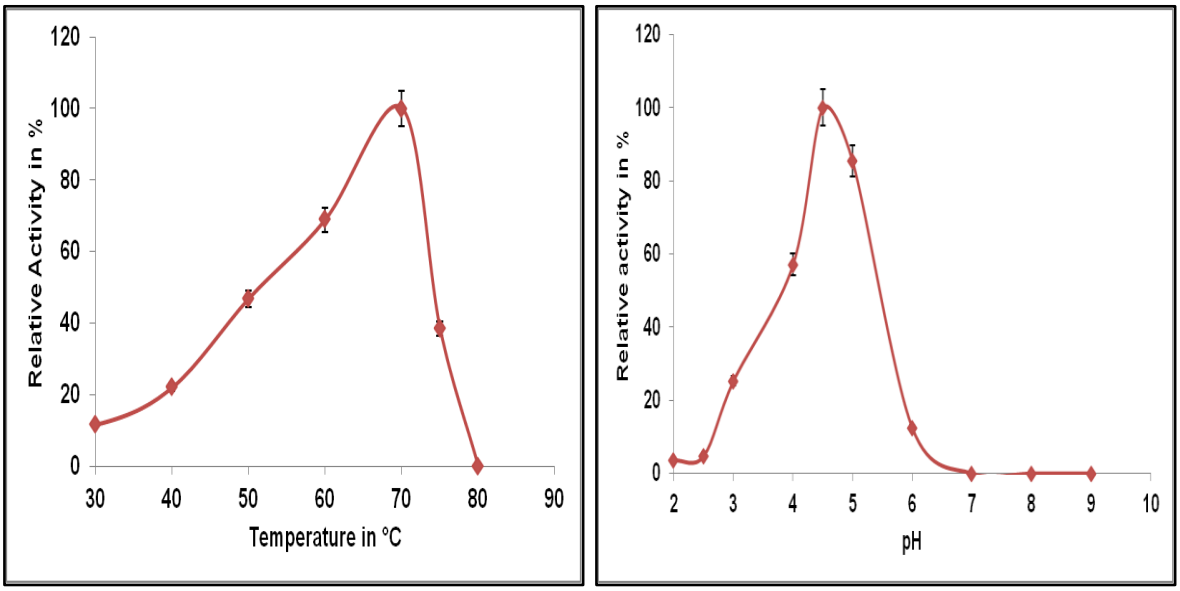

Fig 7b

Figure 7: Characteristics of *Candida tropicalis* (NCIM3321) producing cell-bound and extracellular phytase

Fig 7a - Optimum pH and temperature of cell-bound phytase from NCIM 3321, **Fig 7b-** Optimum temperature and pH of extracellular phytase from NCIM 3321.

2.3.3.2. Thermal stability and pH stability of phytases

Effect of temperature on the stability of cell-bound and extracellular phytase during incubation time of 30 min is shown in Fig 8a, 8b. During an incubation period of 30 min, no loss of extracellular phytase activity from NCIM 3321 was observed at a temperature below 60 °C, while about 41% of the activity was retained after 30 min incubation at 70 °C. No phytase activity was detected when the enzyme was incubated at 80 °C. But , However, no loss of cell-bound enzyme activity of NCIM 3321was observed at the temperature below 40 °C, while about 60-75 % of the activity was retained after 30 min incubation at 50 °C to 70 °C. No phytase activity was detected when the enzyme was incubated at 80 °C. Thermal stability of cell-bound and extracellular phytases of NCIM 3321 was comparable at 70 °C with different phytases like *E. coli* appA, appAM8, appAM10, *Bacillus*, *Geobacillus*, which shows the stability of 20, 42.2, 68.3, 39 and 46 % activity was retained after 10 - 15 min (Wang et al. (2015), Jorquera et al. (2017)). Moreover, both the enzymes were stable at high temperatures, so it can retain activity when they were using for high-temperature regions in agriculture and it can also be used for animal feed pellet.

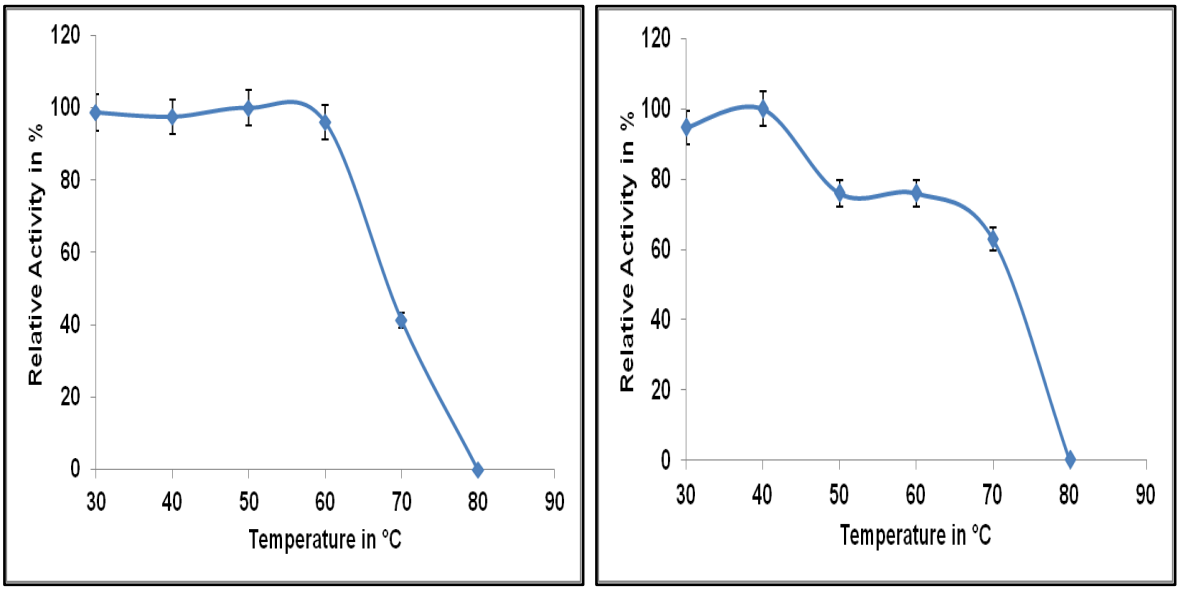

Fig 8a **Fig 8b**

Figure 8: Thermal stability of phytases produced by *Candida tropicalis* (NCIM3321).
Fig 8a - Extracellular phytase thermal stability from NCIM 3321, **Fig 8b** – Cell bound phytase thermal stability from NCIM 3321.

The effect of pH on the enzyme stability was determined by incubating the enzyme in different pH ranges for overnight. Cell-bound phytase of the strain was active in the pH range 2.0–8.0, with more than 80% of the initial activity. However, the decline in activity was observed when the enzyme was incubated at pH 9.0 as shown in Fig 9a and extracellular phytase active in the pH range of 2.0 – 7.0, with more than 90% of the initial activity. However, the decline in activity was observed when the enzyme was incubated at pH 8.0 - 9.0 as shown in Fig 9b. NCIM 3321 producing both the enzymes have stable at high temperature and acidic pH. NCIM 3321 producing cell-bound and extracellular phytases are superior than other phytase produced by *T. lanuginosus*, *Citrobacter koseri* (Makolomakwa et al. (2017), Tripathi et al. (2017)). Phytase producing by *T. lanuginosus*

only it can retain its activity up to 80% only at pH 4, 5, 6 in 24 h. Phytase producing by *Citrobacter koseri* can retain its activity up to 3h at the pH of 5, 6, and 7. Hence, NCIM 3321 producing enzymes have a broad range of pH stability. Which helps to enzyme retain its activity in acidic soils and gut pH of the animals.

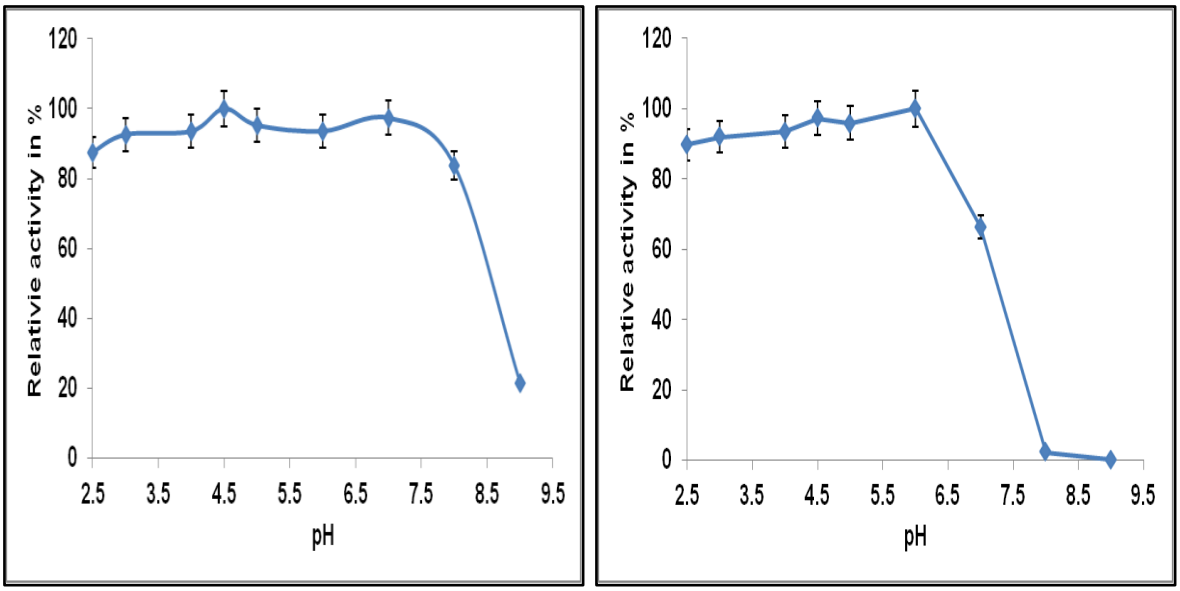

Fig 9a **Fig 9b**

Figure 9: pH stability of phytases produced by *Candida tropicalis* (NCIM3321).
Fig 9a – Cell bound phytase pH stability from NCIM 3321, **Fig 9b** - Extracellular phytase pH stability from NCIM 3321.

2.3.3.3. Effect of metal ions on NCIM 3321 producing phytases

Effect of different metal ions (Hg^{2+}, Ni^{2+}, Mg^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+}, Fe^{2+}, Fe^{2+}, Cu^{2+}, Pb^{2+}) on *Candida tropicalis* producing phytases at the concentration of 1mM was observed and shown in Fig 10a, 10b. The graphical representation illustrates the effect of different metal ions on the cell-bound and extracellular enzyme. In all above-mentioned metals - Mg^{2+}, Mn^{2+} and Ca^{2+} have shown the positive effect on both cell-bound and extracellular phytases

of both the organisms, but rest of the metal ions showed a negative effect on the enzyme activity. Hg^{2+} Cu^{2+} and Pb^{2+} showed a more negative effect as compared to other metal ions. Yeasts like *Candida crusei* phytase production was inhibited by Zn^{2+}, moderately inhibited by Pb2+, Ca^{2+} and Fe^{2+} metal ions (Quan et al. 2002). Tripathi et al. (2017) describe the phytase activity of *Citrobacter koseri* was enhanced by Ca^{2+} and moderately inhibited by Mn^{2+}, Mg^{2+}, Ni^{2+}, Li^{2+}, and $Cu2^{+}$. But this is different for *Candida tropicalis* strain, which enhances the phytase production in the presence of metal ions like Mg^{2+}, Mn^{2+} and Ca^{2+}.

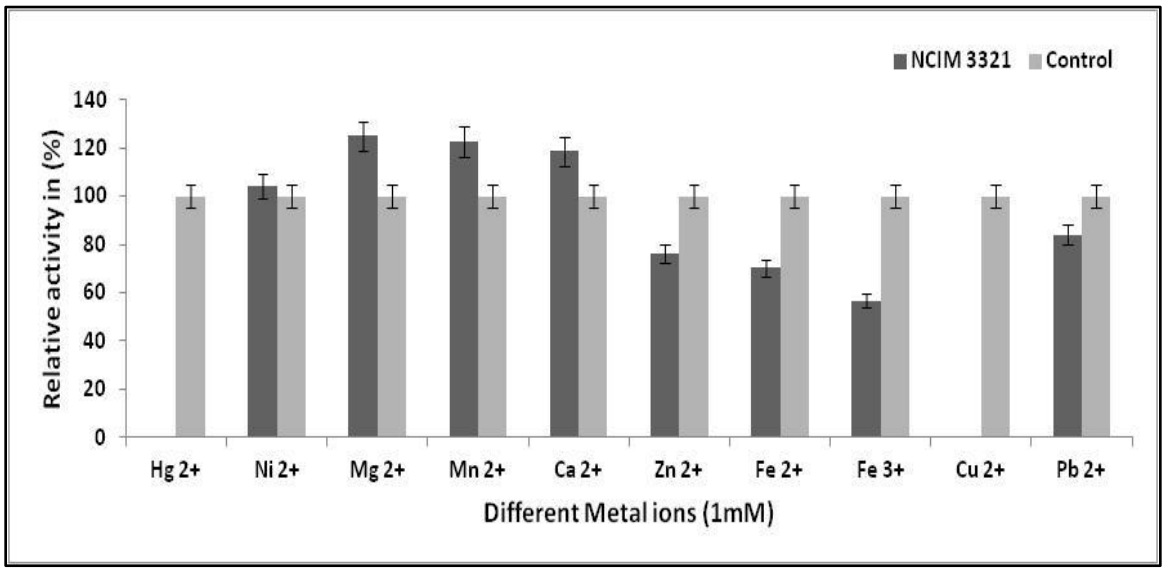

Fig 10a

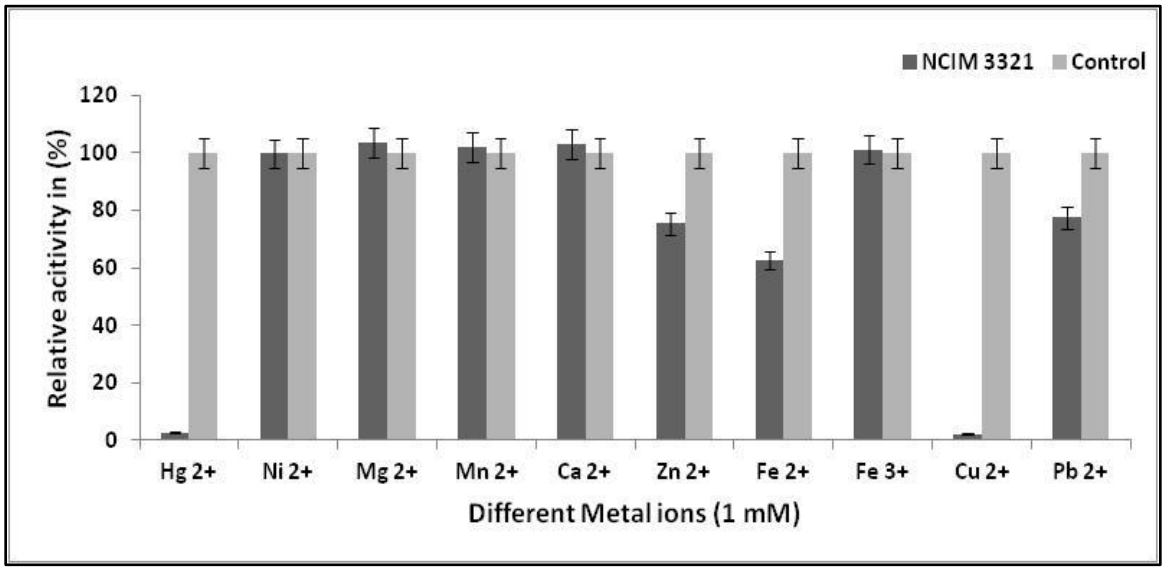

Fig 10b

Figure 10: Effect of different metal ions on phytases produced by *Candida tropicalis* (NCIM3321).

Fig 10a – Effect of different metal ions on Cell bound phytase activity of NCIM 3321, **Fig 10b** – Effect of different metal ions on Extracellular phytase activity of NCIM 3321.

2.3.4. Characteristics of S. cerevisiae NCIM 3662 producing cell-bound phytase

2.3.4.1. Effect of Temperature and pH

The optimum temperature for the growth of *S.cerevisiae* is 28 °C. The observed optimum pH was 4.5 - 5 and temperature was 50 °C for *S. cerevisiae* cell bound phytase as shown in Fig 11a and Fig 11b respectively. Optimal temperature and pH are essential for enzymes to evaluate the total efficiency of the enzyme at optimal conditions. Our results are in accordance with earlier reported optimization for the yeasts strains like *Pichia anomala* (Vohra and Satyanarayana 2001), *S.cerevisiae* (In et al. 2008), *Arxula adeninivorans* (Sano et al. 1999) known for their optimal growth at 28 °C – 30 °C and the optimal temperature and pH of the most of the yeast phytases are between 50 °C – 70 °C and pH 4 – pH 6

respectively (Sano et al. (1999), Nakamura et al. (2000), Vohra and Satyanarayana (2002)). Vohra and Satyanarayana (2003) reported minerals can improve the phytase activity.

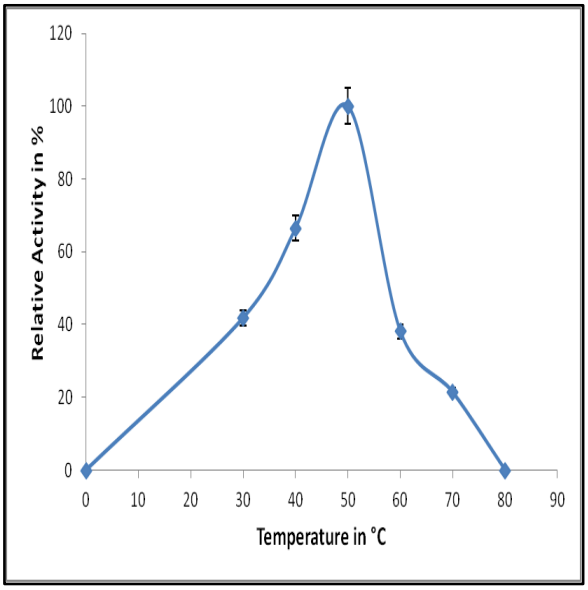

Fig 11a

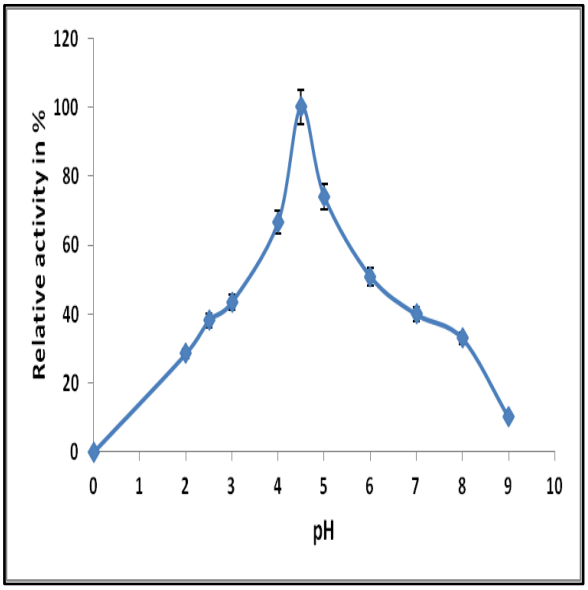

Fig 11b

Figure 11: Characteristics of *S.cerevisiae* (NCIM 3662) producing cell bound phytase
Fig 11a - Optimum temperature of cell-bound phytase from NCIM 3662, **Fig 11b**- Optimum pH of cell-bound phytase from NCIM 3662.

2.3.4.2. Thermal stability and pH stability of cell-bound phytase

Effect of temperature on the stability of cell-bound phytase after an incubation time of 30 min is shown in Fig 12a. During an incubation period of 30 min, no loss of activity of cell-bound Phytase of NCIM 3662 was found till 50 °C, about 25 to 50% of the enzyme activity was retained at the temperature above 50 °C to 80 °C. But there is no enzyme activity was observed at 90 °C. The effect of pH on the enzyme stability was determined by incubating the enzyme in different pH ranges for overnight. Cell-bound phytase of the strain active in the pH range 2.0–8.0, with more than 80% of the initial activity remaining. However, the

decline in activity was observed when the enzyme was incubated at pH 9.0 as shown in Fig 12b. Making of enzyme formulation as a feed additive for poultry and swine industry enzyme should be stable at high temperatures. In this report at 80°C, the cell-bound enzyme retained its activity up to 50%, compared to other yeast-like *Pichia pastoris*, which lost its activity up to 80% at 80°C (Li et al. 2014). To tolerate harsh GIT conditions of different animals, the enzyme should be stable at a broad range of pH, NCIM 3662 cell-bound enzyme retains its activity up to 80% in broad pH range from 2 – 9. This is comparable to the Li et al. (2014).

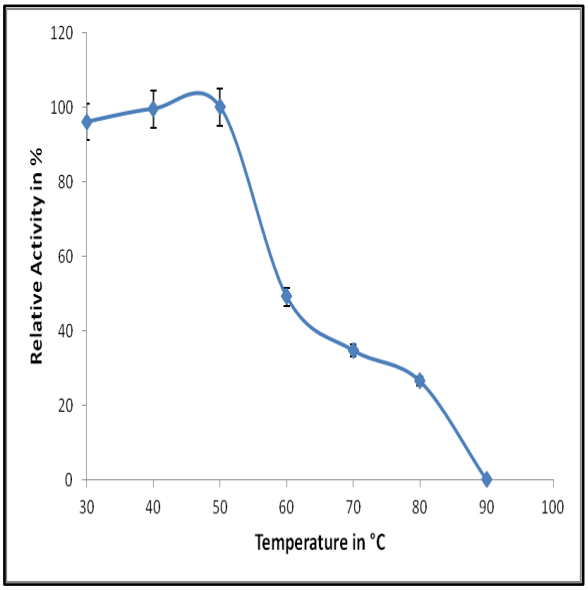

Fig 12a

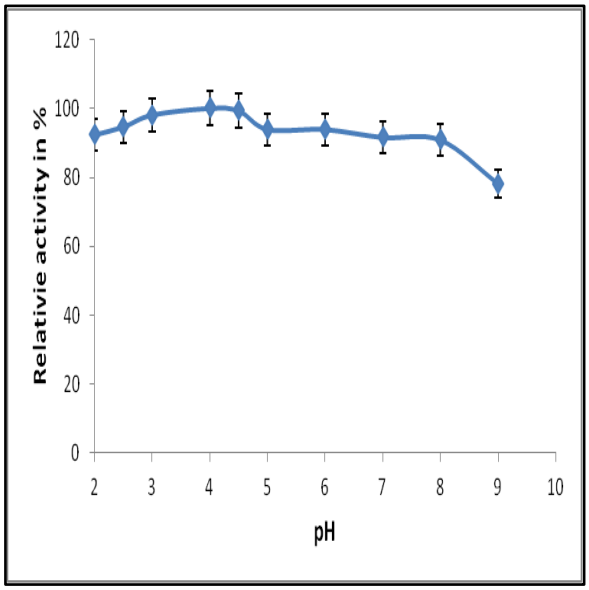

Fig 12b

Figure 12: Thermal and pH stability of cell-bound phytase produced by *S.cerevisiae* (NCIM 3662)

Fig 12a - Cell bound phytase thermal stability from NCIM 3662, **Fig 12b** - Cell bound phytase pH stability from NCIM 3662.

2.3.4.3. Effect of metal ions on *S. cerevisiae* producing phytase

Effect of different metal ions (Hg^{2+}, Ni^{2+}, Mg^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+}, Fe^{2+}, $Fe3^{+}$, Cu^{2+}, Pb^{2+}) on *S. cerevisiae* producing phytase at the concentration of 1mM was observed and shown in Fig 13. The graphical representation illustrates the effect of different metal ions on the cell-bound enzyme. In all above-mentioned metals Mg^{2+}, Mn^{2+}, Ca^{2+} has shown the positive effect on the cell-bound phytases of NCIM 3662, but rest of the metal ions showed a negative effect on the enzyme activity. Hg^{2+} Cu^{2+} and Pb^{2+} showed more negative effect as compared to all other metal ions. Some of the minerals can act as catalytic residues in active site of the enzymes resulting in the enhancement of enzymatic activity. Ranjan and Sahay (2013), Vohra and Satyanarayana (2001) demonstrated that mineral like Mn^{2+}, Zn^{2+}, Fe^{2+} and Cu^{2+} could improve the phytase activity. Vohra and Satyanarayana (2001) reported that, Mg^{2+}, Mn^{2+} has adverse effect on phytase activity, whereas Ca^{2+} could increased by 10 % of the enzyme activity. In our study, $Mg2^{+}$, Mn^{2+} and Ca^{2+} cations showed an enhancement of 5 – 10 % in phytase activity. This effect was significant because the effect of metal ions varied for individual phytase.

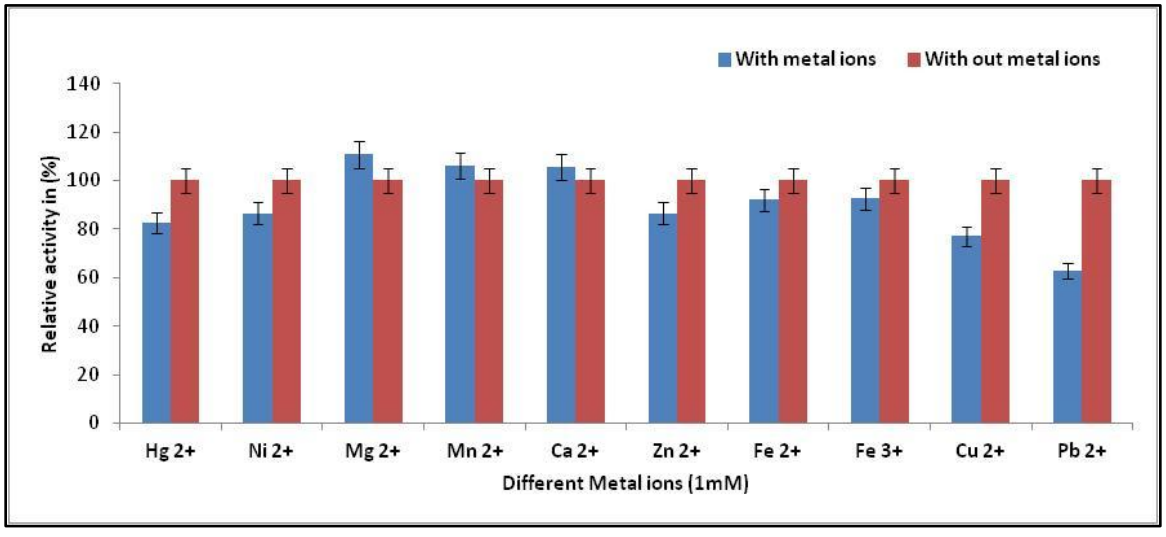

Figure 13: Effect of different metal ion concentration (1mM) on cell-bound phytase from NCIM 3662

2.4. Conclusion:

Around 200 NCIM yeast cultures and 25 soil isolated yeast cultures were screened for phytase producing activity on PSM agar. Based on the zone of hydrolysis and quantification test for phytase activity two superior strains were selected for further studies, one from each group which is having the highest phytase activity. Using biochemical and genetic identification methods, both the strains were identified as *C. tropicalis* NCIM 3321 and *S. cerevisiae* NCIM 3662. To rule out the probability of pathogenicity properties of *C. tropicalis* NCIM 3321 strain, it was subjected to detailed in-vitro cytotoxicity studies. After cytotoxicity studies on mouse fibroblast cell lines, the strain was found to be non-cytotoxic to cell line in high concentrations. Both strains were grown in MGYP medium using submerged fermentation (SmF) method. The NCIM 3321 strain showed cell-bound phytase production with 236 IU/DCG activity as well extracellular phytase production with 0.46 IU/ml enzyme activity. Other strain NCIM 3662 showed only cellbound phytase production with an activity of 45 IU/DCG. The optimum conditions like temperature, pH, the thermal and pH stability for highest activity of both the strains were evaluated. The enzyme produced by NCIM 3321 found to be stable at 70°C temperature and at 4.5 – 5 pH values. Both the enzymes retain its activity up to 95% at broad pH range from 2 – 7 respectively. Phytase from NCIM 3662 strain was found to be optimum at 70°C temperature and 4.5 – 5 pH with stable for 30 min at 50°C and retain its activity up to 80% in broad pH range from 2 – 9 respectively. The phytase activities were found higher than the reported yeast strains, indicating their potential for bioprocess development at the industrial scale. Based on the above results NCIM 3321 producing phytases was evaluated for plant growth promoting applications, whereas NCIM 3662 explored for food and feed applications.

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Chapter 3

Optimization, Scale up and evaluation of *Candida tropicalis* (NCIM 3321) phytases having plant growth promotion potential

Summary:

Phytase is known to provide a solution for imperfect management of phosphorus (P) by hydrolyzing the insoluble P source in soil present in the form of phytate. This study provides insights into a yeast strain *Candida tropicalis* (NCIM 3321) obtained from phytase screening, which was found to produce cell-bound and extracellular thermostable phytase (as mentioned in Chapter 2). In this investigation, growth media components were optimized to enhance the phytase production and demonstrated for plant growth promoting activity using maize crop. Media engineering based optimization led to enhanced cell-bound and extracellular phytase activity by four folds from (236 to 1024 IU DCG^{-1}) and by five folds (from 0.46 to 1.95 IU ml^{-1}) respectively in 36 h. Interestingly, phytase production time decreased by 24 h as compared to shake flask level on up-scaling the production process to 10L scale, which increased the productivity of cell-bound (1810 IU $DCG^{-1}day^{-1}$) and extracellular phytase (6.08 IU ml^{-1} day^{-1}). The crude phytase (12 IU) from NCIM 3321 strain was selected for Maize plant growth promotion in lab scale as well as in the field. Findings revealed that the extracellular phytase from avirulent *C. tropicalis* (NCIM 3321) was able to promote plant growth visible by a robust increase in root length, shoot length and total height by 54%, 25%, 39% in pot level, 19%, 16% and 17% in field level respectively. Our findings of phytase isolated from safe yeast *C. tropicalis* NCIM 3321 exhibited dephytinization mediated PGPR activity. Therefore, the current study may have profound application in sustainable agriculture.

3.1. Introduction

Phytic acid (myo - inositol - hexakisphosphate) is an abundantly available form of organic phosphorus (P) present in soil (50-60%) and plants products like edible legumes, cereals, nuts, oil seeds and pollens (1-5%) (Vohra et al. 2011). It is the primary storage form of both phosphorus and inositol in plants (Savita et al. 2017), phytate content of different cereals and roots are mentioned in Haefner et al. (2005). Phytate considered as an anti-nutrient factor, because of its high chelating capability towards the important metal ions and binding to proteins. Because of that, the bioavailability of important proteins and minerals get diminished (Vohra et al. 2011, Mikulski and Kłosowski 2017). Monogastric animals like poultry, pigs, fish and humans are unable to utilize this form of phosphorous due to lack of inadequate level of phytate hydrolyzing enzymes in their gastrointestinal tracts. This undigested phytate from these monogastric animals leads to the growth of algal blooms in water bodies by different means, which cause the environmental pollution and detrimental effects on the food chain (He et al. 2017). To remove such anti-nutrients factors (Phytic acid) from food obtained from plant sources, phytases were found very efficient and economically feasible enzymatic sources. Some of the studies have been reported showing degradation of phytic acid very efficiently from different microbial sources (Bhavsar and Khire 2014).

Phosphorus is one of the major plant nutrients, lack of which affects physiology and morphology of the plants. Plants mainly uptake inorganic form of P, lack of this inorganic P in agricultural lands leads to the external addition of P to the soil which is supplied through fertilizers. The fertilizer demand and consumption in India has been tremendously increased nearly 100 times in the last few decades while the rock P, which is the main

source to produce fertilizers getting diminished. Sustainable agriculture and finding the alternative phosphorus sources are the only solution for the above problem (Gujar et al. 2013).

Phytases are widely distributed in nature like in different microorganisms, animal tissues and plants (Turk et al. 2000). Phytases also called as phosphomonoesterases having the ability to hydrolyze the phytate into lower inositol phosphates or in some cases free myo-inositol and inorganic phosphates. Phytases from different microbial sources are applied as feed supplementation for monogastric animals which leads to the downfall of using inorganic phosphorus in feeds (Harland and Morris 1995). Therefore, phytases have a potential role in improving phosphorus and mineral and amino acid bioavailability. In United States phytase has been commercialized since 1996 as a feed additive, by the reports at the close of the 20th century, the market of phytase is going to be US$ 500 million (Vats and Banerjee 2004). Several microorganisms like bacteria, yeast and fungus or molds can produce different classes of phytases. Bacteria like *Bacillus, Lactobacillus, Escherichia*, *Pseudomonas*, and *Klebsiella* sp. known for phytase production. In moulds like *Aspergillus niger, Aspergillus ficuum* such phenomena are well studied. Fungal phytases knew for their, production in solid state fermentation. However, it is not very flexible/ideal for scale up as its extraction steps are complex and costly (Coban and Demirci, 2014a). Very few molds like *A. ficuum, A. niger* known for their production in submerged fermentation in batch, fed-batch, continues reactors for scale-up with microparticles (Coban and Demirci, 2014b, Coban et al. 2015a, b, c).

Yeasts are known for producing industrially important enzymes like invertase, lactase, lipase, raffinase and pectinases (Moharib et al. 2000). Due to Generally regarded as

Safe (GRAS) status of various yeasts, they are commonly used in brewing, baking, winemaking and are most preferable enzyme sources for their application in feed and foods. The potential applications of yeast phytases are enormous, majorly in animal feeds and human nutrition. Yeasts like *Candida parapsilosis* (Ranjan and Sahay 2013), *Pichia anomala* were known their extracellular and cell-bound phytases. Therefore, further research efforts are suggested for discovering new phytases from yeast sources as very few are presently known, and to develop an economical process for their large-scale production (Kaur and Satyanarayana 2009).

Recombinant expression of phytase genes in plants leads to the assimilation of phosphorus and helps in plant growth (Liu et al. 1998, Ullah et al. 1999, Yang et al. 2011). A major focus in the coming decades would be safe and eco-friendly exploitation of plant biostimulant in sustainable crop production (Calvo et al. 2014). Such biostimulants consist of diverse naturally occurring microbes which on inoculation in the soil ecosystem advances and improves the soil physicochemical properties, soil microbe's biodiversity, soil health, plant growth development and crop productivity (Bhattacharyya and Jha 2012). In this way, it is possible to increase intake of phosphate as a nutrient using biotechnology and agriculture. Plants may increase P uptake from the soil by altering root morphology or physiology. Physiological changes such as the release of protons, organic acid anions or phenolics, P-mobilizing phytase, and phosphatase enzymes increase the availability of P in the rhizosphere. Hence, the release of phosphatase and phytase enzymes by roots and microorganisms that mineralize organic P can be an important mechanism for increasing P uptake by plants (Gyaneshwar et al. 2002). The present work includes optimization of

phytase producing *Candida tropicalis* yeast NCIM 3321 by media engineering approaches to enhance the production and its utility in plant growth promotion.

3.2. Material and Methods

3.2.1. Media and chemicals:

Phytic acid sodium salt was purchased from Sigma Aldrich, St Louise, MO, USA. Glucose, sodium nitrate, agar, magnesium sulfate, ferrous sulfate, yeast extract, malt extract and peptone bacteriological from Hi-Media, L.B.S. Marg, Mumbai, India. Manganese sulfate and potassium chloride are from Merck, India. Maize seeds were collected from the local markets of- Pune, India.

3.2.2. Media optimization design and data analysis:

Generally MGYP medium is an enriched medium, used for higher growth of different microorganisms mainly for yeast; previously it was also used for the growth of different *Candida* species for evaluating their phenol degradation potential especially in case of *Candida tropicalis* (Varma and Gaikwad, 2008). In this study, phytase production mainly depended on the biomass of the organism because of its both cell-bound and extracellular activity. Hence, MGYP media selected for higher growth of the organism.

3.2.2.1. Effect of carbon source

Carbon source is a very important nutrient in the medium for any organism. The effect of carbon source on the yeast growth and phytase production by *Candida tropicalis* NCIM 3321 was investigated. Phytase activity was obtained in the presence of different carbon sources like sucrose, lactose, and galactose (Kumar et al. 2013) supplemented individually by replacing glucose in the basal MGYP medium.

3.2.2.2. Media optimization with One Variable At a Time (OVAT)

In a traditional media optimization process, various factors are optimized one at a time to study its effect on phytase production. Studies using OVAT approach were carried out for all four media components to check for enhanced phytase production.

3.2.2.3. OVAT with minerals

According to In et al. (2008) minerals can improve the yeast phytase production. Thus, the effect of some trace elements like per 100 ml Potassium chloride (0.05 gm), magnesium sulfate (0.05 gm), manganese sulfate (0.001 gm), ferrous sulfate (0.001gm) was checked for enhanced phytase production. The concentrations of trace elements were adopted from the PSM (Bhavsar et al. 2011). To the OVAT optimized media above all minerals has been added in respective concentrations and determined the phytase production at standard assay conditions.

3.2.2.4. Media optimization with Box Behnken Design (BBD)

OVAT does not provide a holistic effect of the media on the phytase production. Response surface methodology (RSM), which is a combination of statistical and mathematical tool, evaluates the effect of varying combinations of the media components simultaneously for enhancing the phytase production in a limited number of experiments. The BBD of experiments were generated by Design Expert Software (DES) and studies at three different levels -1, 0, +1. One added advantage of BBD design was to avoid the harsh conditions of the experiment, because the resulted response may deviate the data of other parameters (Aslan and Cebeci 2007). After analyzing the OVAT results, four variables or parameters viz. Glucose, Malt extract, Yeast extract and Peptone were chosen for further optimization by Response surface methodology using BBD. In theoretical the number of experiments

(N), which are required for the development of BBD is defined as $N= 2k (k - 1) +C_0$, (where k is a number of factors and C_0 is the number of central points) (Ferreira et al. 2007, Bhavsar et al. 2011). BBD is designed to estimate the coefficients of a quadratic model. BBD design for above 4 components was created by using design expert software version 10. This optimization is aimed at increasing productivity. (Units of enzyme produced per day i.e. IU ml^{-1} day^{-1} or IU DCG $^{-1}$ day^{-1}).

3.2.3. Scale Up

Enhanced production paved way for scaling-up the phytase production from shake flask level (50 ml) to 2 L and further up to 10 L scale. Total scale-up process was done in New Brunswick BioFlo/CelliGen 115 Benchtop fermenter 3 L and 14 L. Batched in succession were at different agitation rate were performed at constant aeration and temperature. The 3L fermenter utility station dimensions of (8.5 Width X 16.5 Height in inches) with working volume of 2 L media was inoculated with 40 ml (~ 10^{11} cells ml^{-1}) of 12 hr grown culture. The dissolved oxygen (DO) was measured using a Mettler Toledo oxygen probe, which connects to the control station. The setpoint for DO has maintained a minimum of 20% of saturated oxygen. The set DO was maintained by cascading the agitation with DO set point. Samples were periodically withdrawn to check phytase production. The process was further scaled up to 14 L fermenter utility station dimensions of (11.5 Width X 24 Height in inches) with working volume of 10 L 200 ml (~ 10^{11} cells ml^{-1}) of 12 hr grown culture inoculum in 10 L media.

3.2.4. Use of phytase in Plant Growth Promotion (PGP)

For the evaluation of plant growth promotion, maize has been chosen for pot level and field level experiment (Kumar et al. 2013). Seeds were surface sterilized in laminar air flow with

70% alcohol and 2% sodium hypochlorite for 2 min with intermittent washing with sterile distilled water for 5-10 times (Dastager et al. 2010). Soil for Pot experiment was sterilized by autoclaving three times at 121°C and 15 Psi for 1 h for 3 consecutive days. In each pot, 150 gm of soil was taken and 3 seeds were sown at a depth of 5 mm. In this experiment, the growth of maize seeds was checked under the following treatments at greenhouse conditions in three triplicates. Control (without any enzyme), Enzyme (3321 extracellular enzyme 12 IU per seed), Phytic acid (1.5 mg per seed) and Enzyme with phytic acid (3321 extracellular enzyme 12 IU with phytic acid 1.5 mg per seed) added to the soil just above the each sowing seed. Pots were kept at 28°C for 30 days under standard greenhouse conditions. Plants were observed periodically for growth. Seedlings were then analyzed for root, shoot and total heights of control and test. A field experiment was conducted at research farm of National Chemical Laboratory in the Period of Jan - Mar 2016. The plot was separated into 4 equal parts and then each part allotted to four different treatments as mentioned in the pot experiment. Phytic acid, which is adding in each treatment was extracted from the wheat bran by acid hydrolysis using 2.4% HCl. Hydrolysis carried out for one hour in a shaker with 160 rpm, after 1 hr solution was centrifuged at 8000 rpm for 10 min separated the supernatant and pellet, resulted in supernatant used as a phytic acid. The phytic acid in the supernatant was separated by using anion exchange by using resin chloride AG 1X8. The concentration of phytic acid in the supernatant was determined by the colorimetric method using WADE reagent as a coloring agent (Latta and Eskin 1980). Seeds were surface sterilized then sowed in soil at a depth of 5 mm. Ten mature maize plants were sampled from each treatment set for final measurements in March, 70 days after sowing (Hameeda et al. 2008, Dinesh et al. 2015).

3.3. Results and Discussion

The results for the experimental method explained above are discussed in detailed.

3.3.1. Media optimization

Carbon is a major component of the cell and the rate at which a carbon source is metabolized can often influence the production of metabolites. The influence of different carbon sources on cell-bound and extracellular phytase production is shown in Figure 1.

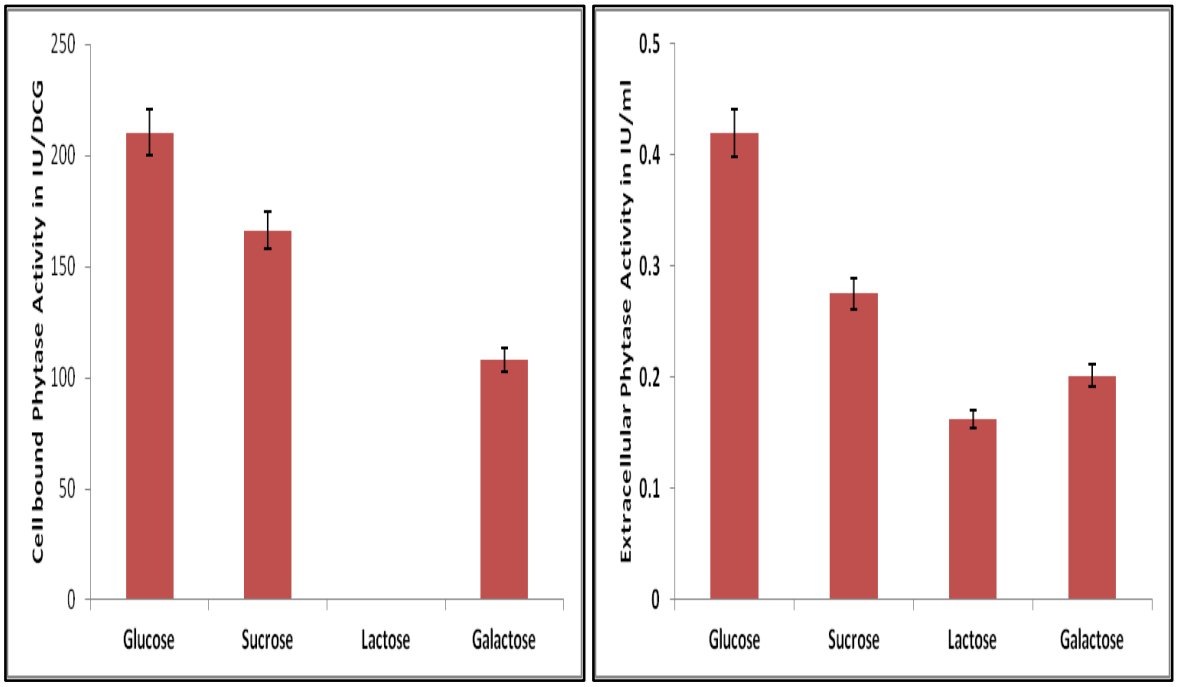

Figure 1: Effect of different carbon sources on cell-bound and extracellular activity of *Candida tropicalis* (NCIM3321) producing phytase.

Among different carbon sources studied, glucose showed the maximum cell bound enzyme activity of 210.5 IU DCG^{-1} and extracellular activity of 0.42 IU ml^{-1} after 36h of incubation for NCIM 3321. After glucose, sucrose gave maximum activity, followed by galactose. Significant difference in phytase activity was observed with lactose. Glucose has been

reported to increase phytase production by *Aspergillus niger* during submerged and solid-state fermentation (Vats and Banerjee 2004). However, the effect of carbon source changes with the production strain and other conditions. Further media components of MGYP were optimized by OVAT method, which enhanced the phytase production by exhibiting the cell bound activity of 289.05 IU DCG^{-1} and extracellular activity of 0.81 IU ml^{-1}.

The concentration of trace elements which increased phytase production (520 IU DCG^{-1} of cell-bound and 1.1 IU ml^{-1} extracellular activity) were potassium chloride (0.5 gm/L), magnesium sulphate (0.5 gm/L), manganese sulphate (0.01 gm/L), and ferrous sulphate (0.01 gm/L). As per Vohra and Satyanarayana (2001) and In et al. (2008) different minerals in the medium can make added advantage to the production of phytase. Kaur et al. (2004), Vohra and Satyanarayana (2001) reported that thermophilic mould *Sporotrichum thermophile, Pichia anomala* showed increased in activity after adding magnesium sulfate, calcium and Fe^{2+} to the medium.

After OVAT, four variables viz. glucose, malt extract, yeast extract and peptone which are the components of MGYP media were further optimized by using BBD. This statistical optimization method was designed by using the software design expert 10. This gave maximum combinations of 29 runs with different combinations of quantities of four variables. After fermentation of these 29 runs the design was further analyzed for its significance. The maximum cell bound activity of 1014 IU DCG^{-1} and extracellular activity of 1.95 IU ml^{-1} was obtained in the 4^{th} and 21^{st} run (Table 1).

Table 1: BBD Design for *Candida tropicalis* NCIM 3321

Run	Glucose (g /100ml)	Malt extract (g /100ml)	Yeast extract (g /100ml)	Peptone (g /100ml)	Response Cell bound (IU DCG^{-1})	Response Extra cellular (IU ml^{-1})
1	3	0.28	0.05	0.05	0	0.23
2	3	0.50	0.05	0.28	183.3	0.09
3	3	0.05	0.5	0.28	567.5	0.69
4	**3**	**0.28**	**0.5**	**0.05**	**1014**	**1.03**
5	3	0.28	0.28	0.28	370	0.52
6	3	0.28	0.28	0.28	365	0.57
7	3	0.28	0.05	0.5	564	0.19
8	1	0.28	0.28	0.05	599.3	0.55
9	1	0.28	0.5	0.28	207.9	0.37
10	1	0.5	0.28	0.28	320	0.19
11	3	0.05	0.28	0.5	322	0.45
12	1	0.05	0.28	0.28	324.5	0.3
13	5	0.28	0.28	0.05	338.4	0.66
14	3	0.28	0.28	0.28	338	0.6
15	1	0.28	0.05	0.28	215	0.39
16	5	0.28	0.05	0.28	119.8	0.17
17	3	0.05	0.28	0.05	406	0.82
18	3	0.28	0.28	0.28	355	0.5
19	5	0.28	0.28	0.5	542	0.96
20	5	0.28	0.5	0.28	760	1.35
21	**3**	**0.5**	**0.5**	**0.28**	**528.8**	**1.95**
22	3	0.28	0.28	0.28	365	0.69
23	3	0.28	0.5	0.5	120	0.95

24	3	0.5	0.28	0.5	303.1	0.66
25	5	0.5	0.28	0.28	363.1	0.83
26	5	0.05	0.28	0.28	491	0.66
27	1	0.28	0.28	0.5	10.5	0.23
28	3	0.5	0.28	0.05	308	0.57
29	3	0.05	0.05	0.28	204.4	0.31

The results were analyzed by ANOVA and following polynomial equations were generated for cell bound and extracellular enzymes. These quadratic equations 1 and 2 can be used to get the predicted extracellular and cell-bound responses of NCIM 3321 for given levels of each factor and also helps in identifying the relative impact of the factors by comparing the factor coefficients.

$$Y_1 = 0.60 + 0.22A + 0.088B + 0.41C - 0.035D + 0.070AB + 0.30AC + 0.15AD + 0.37BC + 0.12BD - 0.011CD \quad (1)$$

$$Y_2 = 365.63 + 78.27A - 25.76B + 159.49C - 67.15D - 30.85AB + 161.28AC + 198.52AD - 4.40BC + 19.77BD - 364.08CD \quad (2)$$

Three-dimensional graphs of response surface plots with interactions among the variables shown in Figure 2. Enhanced extracellular phytase was obtained as the concentration of glucose (g/100ml) between 3 to 5 and yeast extract at 0.5 as shown in Fig 2a, run 21 agreement with the graph shown the highest enzyme activity. At the lower concentration of yeast extract and a higher concentration of glucose results in a decrement in the phytase production, run 16 harmonies with the graph shown less enzyme activity. Similarly, Fig 2b shows, interaction profile between malt extract yeast extract with high extracellular activity at a high concentration of yeast extract and malt extract (Run 21).

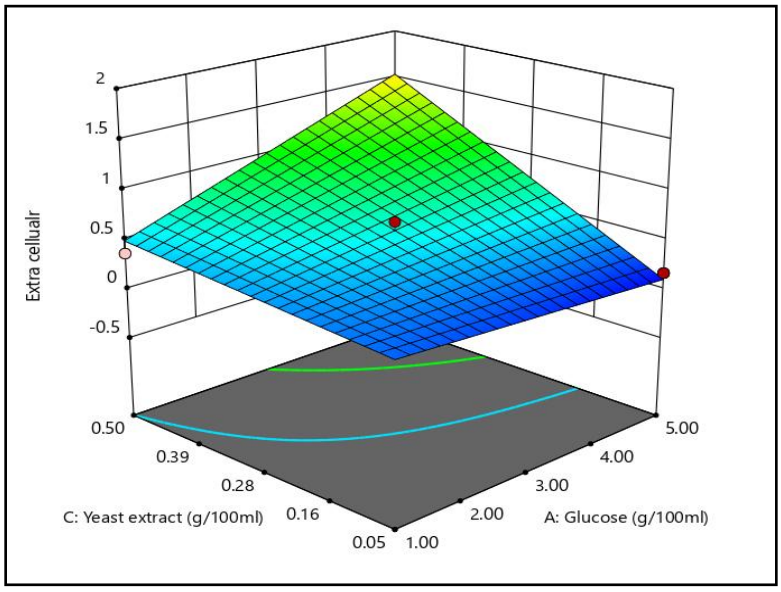

Figure 2a

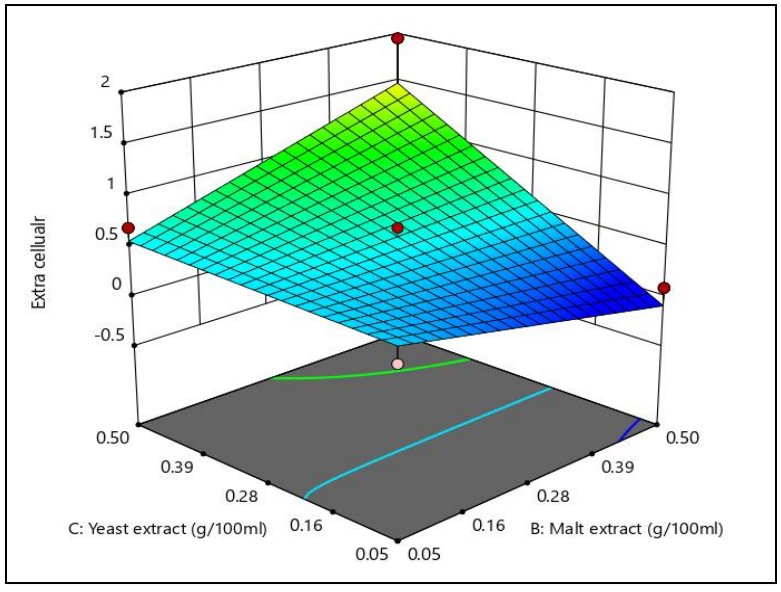

Figure 2b

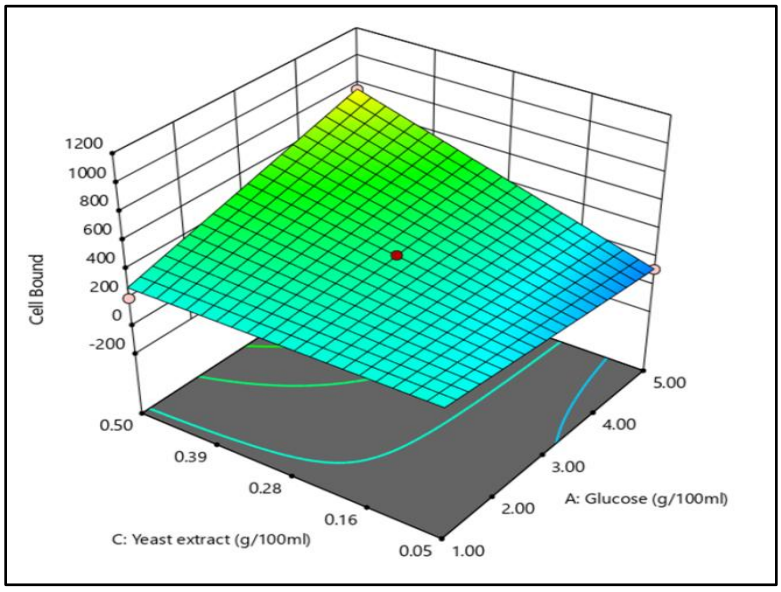

Figure 2c

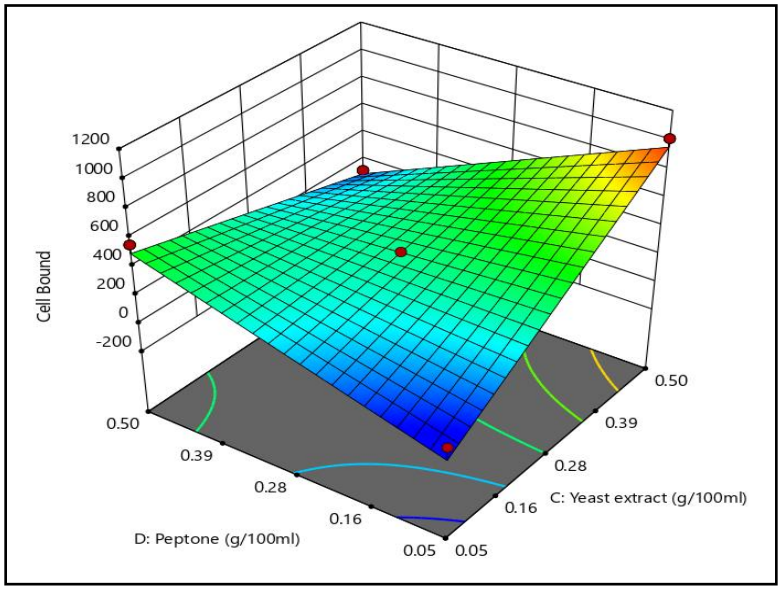

Figure 2d

Figure 2: Response surface graphs showing the effect of media variable interaction on extracellular and cell-bound phytase. **2a** - Interaction between glucose and yeast extract on extracellular phytase production. **2b** - Interaction between Malt extract and yeast extract on extracellular phytase production. **2c** - Interaction between glucose and yeast extract on cell bound phytase production. **2d** - Interaction between yeast extract and peptone on cell bound phytase production.

Depletion of yeast extract results in sharp decrement in activity (Run 2). Further, it can be seen from Fig 2c and 2d that lower concentrations of yeast extract effect the cell-bound phytase activity when it interacts with media variables. After BBD the production of cell-bound phytase increased to 4.8 times and extracellular phytase 4.64 times enhanced compared to unoptimized media conditions, which is similar to the Shah et.al (2017) *Aspergillus niger* phytase 3.97 times enhanced after BBD.

Where Y_1 and Y_2 have predicted responses of extracellular and cell-bound phytases of NCIM 3321 respectively. The following A, B, C, D are component variables like glucose, malt extract, yeast extract and peptone respectively. For NCIM 3321extracellular enzyme the model F value was 13.10, which implies that model is significant, and the chance of getting noise is very less 0.01% to get that much F value (Table 2). The coefficient of determination (R^2) value 0.8792 tells 95.68% variability explained in the model. The adjusted R^2 value 0.8120 is reasonable agreement with the correlation coefficient (Predicted R^2) value 0.6125, which will support the significance of the model. The line which is equally separating the actual versus prediction values in Fig 3 will support the model significance. The lack of Fit F-value of 6.34 implies the Lack of Fit is significant. There is only a 4.38% chance that a lack of fit F-value this large could occur due to noise. Similarly for cell bound phytase from NCIM 3321 have the model F value was 44.77, which implies that model is significant, and the chance of getting noise is very less 0.01% to get that much F value (Table 3). The coefficient of determination (R^2) value 0.9613 tells 96.13% variability explained in the model. The adjusted R^2 value 0.9399 is strong agreement with the correlation coefficient (Predicted R^2) value 0.8731, which will strongly support the significance of the model.

Table 2: NCIM 3321 extracellular ANOVA model for Response Surface Quadratic Model of BBD

Source	Sum of squares	DF	Mean square	*F* value	*P* value Prob > *F*
Model	3.80	10	0.38	13.10	<0.0001
A	0.56	1	0.56	19.37	0.0003
B	0.094	1	0.094	3.23	0.0891
C	2.05	1	2.05	70.59	<0.0001
D	0.015	1	0.015	0.50	0.4883
AB	0.020	1	0.020	0.68	0.4217
AC	0.36	1	0.36	12.50	0.0024
AD	0.095	1	0.095	3.29	0.0866
BC	0.55	1	0.55	18.89	0.0004
BD	0.053	1	0.053	1.82	0.1935
CD	4.549E-004	1	4.549E-004	0.016	0.9017
Residual	0.52	18	0.029		
Lack of fit	0.50	14	0.036	6.34	0.0438
Pure error	0.023	4	5.630E-003		
Cor total	4.32	28			

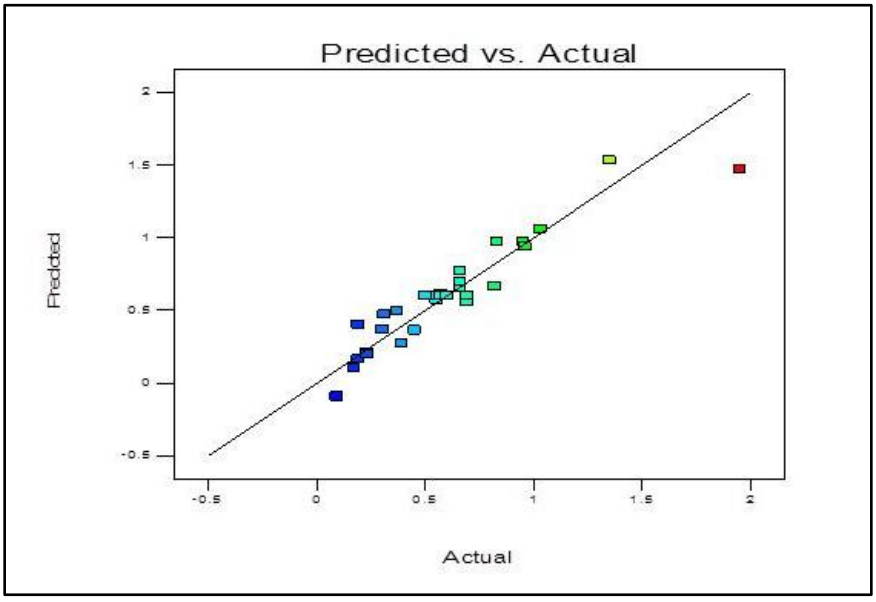

Figure 3: NCIM 3321 extracellular actual and predicted values

Table 3: NCIM 3321 cell bound ANOVA model for Response Surface Quadratic Model of BBD

Source	Sum of squares	DF	Mean square	*F* value	*P* value Prob > *F*
Model	1.238E+006	10	1.238E+005	44.77	<0.0001
A	73159.86	1	73159.86	26.59	<0.0001
B	7961.90	1	7961.90	2.88	0.1069
C	3.052E+005	1	3.052E+005	110.40	<0.0001
D	54110.20	1	54110.20	19.57	0.0003
AB	3806.89	1	3806.89	1.38	0.2559
AC	1.040E+005	1	1.040E+005	37.63	<0.0001
AD	1.577E+005	1	1.577E+005	57.02	<0.0001
BC	77.44	1	77.44	0.028	0.8690
BD	1564.20	1	1564.20	0.57	0.4617
CD	5.302E+005	1	5.302E+005	191.77	<0.0001
Residual	49769.39	18	2764.97		
Lack of fit	49120.19	14	3508.59	21.62	0.0045
Pure error	649.20	4	162.30		
Cor total	1.288E+006	28			

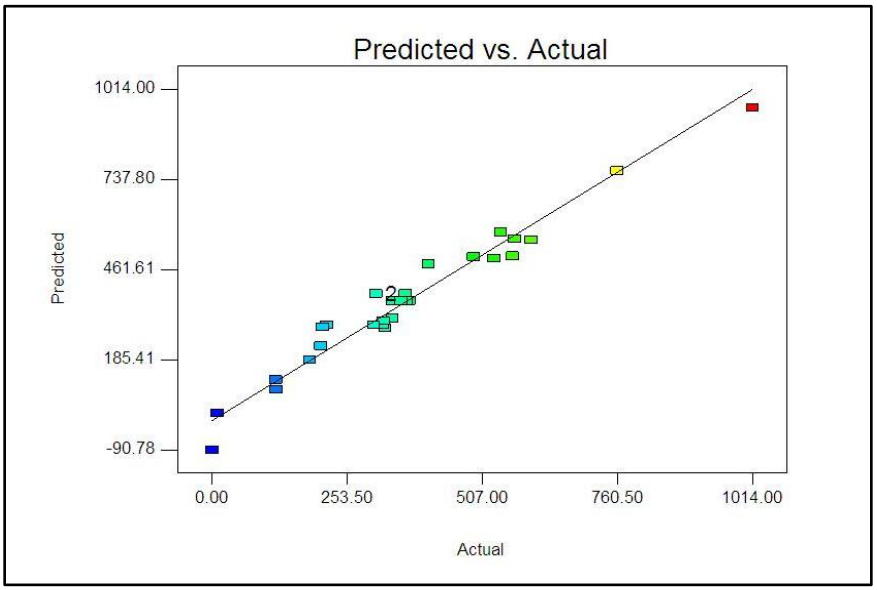

Figure 4: NCIM 3321 cell bound actual and predicted values

The line which is equally separating the actual versus prediction values in Fig 4 will support the model significance. The lack of fit F-value of 21.62 implies the Lack of Fit is significant. There is only a 0.45% chance that a lack of fit F-value this large could occur due to noise. The values of p-Value > F (< 0.05) suggest that the model is significant. In our case for extracellular phytase production A, C, AC, BC in equation (1) are significant model terms and for cell bound phytase production A, C, D, AC, AD, CD in equation (2) are significant model terms. The values of p-value > F (>0.1) model terms are not significant. The comparison of all 4 steps involved in the optimization process of NCIM 3321 cell-bound and the extracellular enzyme is illustrated in Fig 5a and 5b. After statistical optimization of media components by using BBD, the production of phytase increased to four folds higher than basal MGYP medium phytase production for both the enzymes. For improving phytase activity using a statistical approach is a well known method from decades (Singh and Satyanarayana 2008).

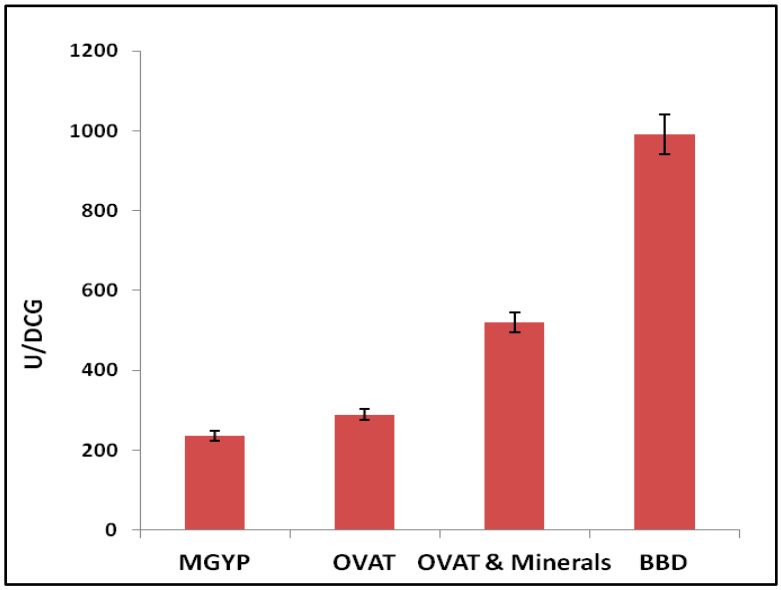

Figure 5a

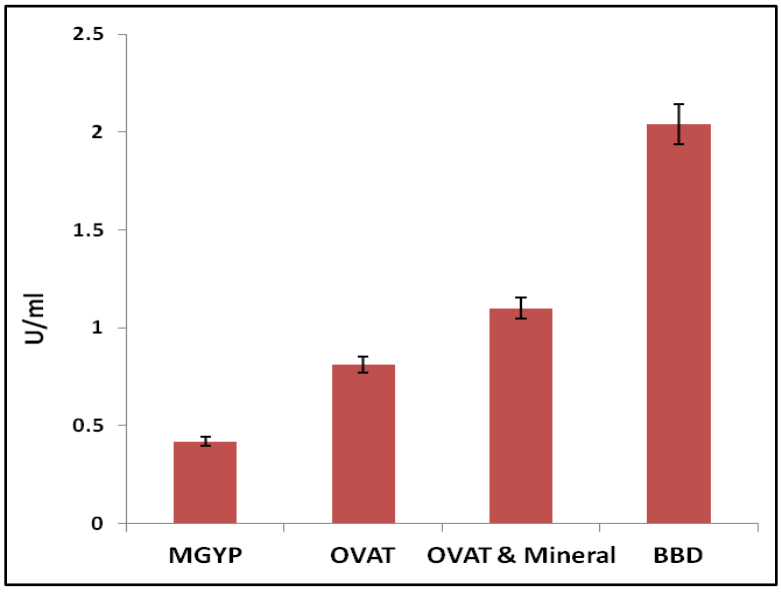

Figure 5b

Figure 5: Comparison of all the media used for optimization for Cell bound phytase and extracellular phytase from *Candida tropicalis* (NCIM3321). **5a** - Comparison of different level optimization processes for the cell-bound phytase from NCIM 3321, **5b** - Comparison of different level optimization processes for the extracellular phytase from NCIM 3321.

3.3.2. Scale up of phytase production

The optimized media was used to further scale-up the process. Dissolved oxygen (DO) is one of the important factors at the fermentor scale. Minimum DO of 20 % was maintained throughout the production process. For this aeration rate of 0.5 vvm was maintained, varying the agitation speed. At 2 L scale, initial DO is set at 100 % which is utilized by an organism for growth at 28°C at pH 6.5. Minimum agitation of 200 RPM was set at the start of production which was increased to 400 RPM at the end of production to maintain minimum DO. Minimum agitation time was taken based upon shake flask level agitation. At the end of 2 L production, NCIM 3321 produced the cell bound activity of 976 IU DCG^{-}

^{1} and extracellular activity of 2.02 IU ml^{-1} in 24 h. Which increased the productivity (976 IU $DCG^{-1}day^{-1}$ and 2.02 IU ml^{-1} day^{-1}) as compare to shake flask level in 36 h (676 IU $DCG^{-1}day^{-1}$ and 1.3 IU ml^{-1} day^{-1}). The pH was monitored throughout the production which reached to pH 4.5 at the end of production. Further scaling-up to 10L scale, generated the cell bound activity of 905IU DCG^{-1} and extracellular activity of 3.04 IU ml^{-1} within 12 h. At the end of the production, the parameters were agitation 700 rpm, 28°C Temperature, 3.5 pH and 10 DO. At this production scale, the production time further reduced to 12 h generating cell bound activity of 1810 IU $DCG^{-1}day^{-1}$ of cell-bound enzyme and 6.08 IU $ml^{-1}day^{-1}$ of extracellular enzyme activity. Thus, productivity increased by 2.67 times as compared to the shake flask level and 1.85 times then the 2L fermenter. Although, the productivity was increased, cell bound activity in 10L fermenter was decreased and extracellular activity was enhanced. This may be due to shear stress, that causes the cell disruption resulted variation in both enzyme activities. Comparison bar graph for total scale-up process with time has been shown in the Fig 6a, b. The phytase production and yield from NCIM 3321 were higher than the *A. ficuum* phytases and other phytases from different yeasts as mentioned in Coban and Demirci, (2017).

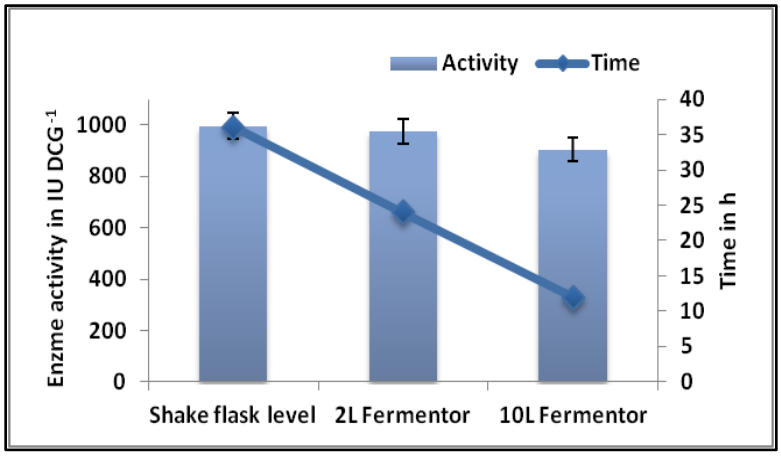

Figure 6a

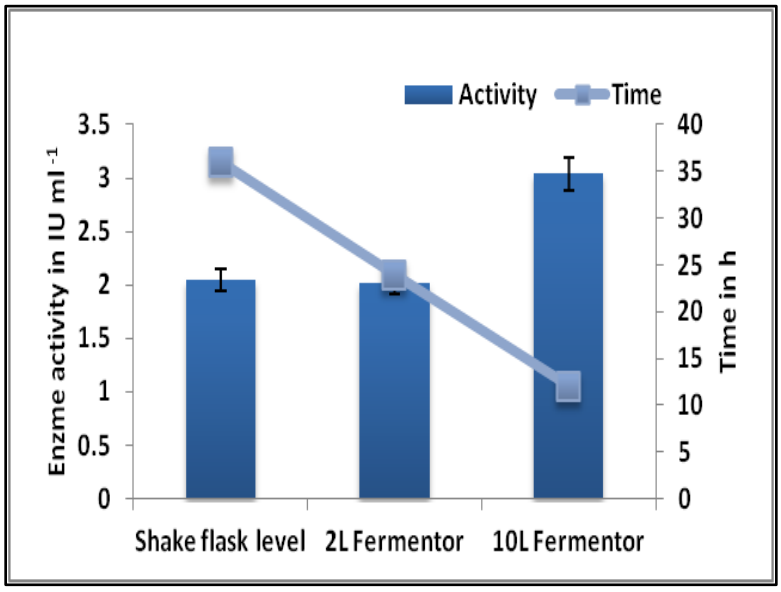

Figure 6b

Figure 6: Scaling up of phytase production 2 L and 10 L fermenter level from NCIM 3321. 6a - Up scaling process of NCIM 3321 cell bound phytase Production from shake flask level to 2 L fermenter and 10 L fermenter, 6b - Up scaling process of NCIM 3321 extracellular phytase Production from shake flask level to 2 L fermenter and 10 L fermenter.

3.3.3. Plant growth promotion

Candida tropicalis NCIM 3321 producing phytase have the ability to promote the plant growth and it has been proved in both pot level and field level experiment. In an initial experiment different concentration of enzyme has been used giving promising results with 12 IU ml^{-1} of enzyme more promising (data not shown). Phytic acid concentration was optimized by Gujar et al. (2013). An enzyme with phytic acid treatment gave good results in both the pot level and field experiment. In pot level experiment, the enzyme with phytic acid treatment showed the maximum increment of 54% in root length, 25% in shoot length and 39% of total height of the plant as compared to the control treatment (no enzyme)

shown in Fig 7a and 7c. Similarly, in field level experiment, same treatment showed reasonable agreement with the pot level experiment. By showing the maximum increment of 19% in root length, 16% in shoot length and 17% of total height of the plant as compared to the control treatment (no enzyme) shown in Fig 7d and 7f. In addition to that, the same treatment has shown good increment in average fresh plant weight and average dry plant weight, which indicates the healthiness of the plant. In pot level experiment it has been 47% increment of fresh plant weight and 60% increment in plant dry weight as compared to the control (no enzyme) plants shown in Fig 7b. Field level experiment 96% increment in fresh plant weight and 98% increment in plant dry weight as compared to control plants (no enzyme) shown in Fig 7e. As per Fig 7g, there is a clear difference between enzyme with PA treatment to the other treatments before uprooting. The NCIM 3321 strain producing extracellular phytase showed the effective plant growth promoting ability in greenhouse and field level experiments. Further, to make treatments much better for commercially and economic feasibility, phytic acid extracted from wheat bran replaced with direct wheat bran added to the field. After 3 months the plants were uprooted and measured the plant root, shoot, total heights, fresh plant, dry plant weights and total yield of all the treatments. The results showed promising plant growth promoting activity in terms of yield (maize fruit weight) 99.99% increment, 49% in root length, 24% in shoot length and 27% of total height in phytase enzyme with PA treatment with respect to control as shown in Fig 7h. For same experiment, enzyme with PA treatment has the fresh plant weight is 93% and dry plant weight is 98% higher than the control (no enzyme) treatment shown in Fig 7i. As shown in Fig 7j, the root and shoot morphology of phytase with PA treated maize plant has the considerable difference with control plants in terms of height and thickness of roots. There

are no reports on yeast phytase promoting plant growth at field level. However, *Candida tropicalis* HY strain with PGP characteristics with phytase activity shown 16% increment in rice plant growth at lab level experiments after 21 days (Amprayn et al. 2012). In our pot experiment 35% increment in plant height, this is higher than the reported once. *Candida tropicalis* strain reported here was shown enhance plant growth, which might help in the lower available P containing soils. The broad range specificity of phytases produced by *Candida tropicalis* will help the plant can grow in diversified soil and weather conditions like acidic soils and high-temperature regions by promoting sustainable agriculture.

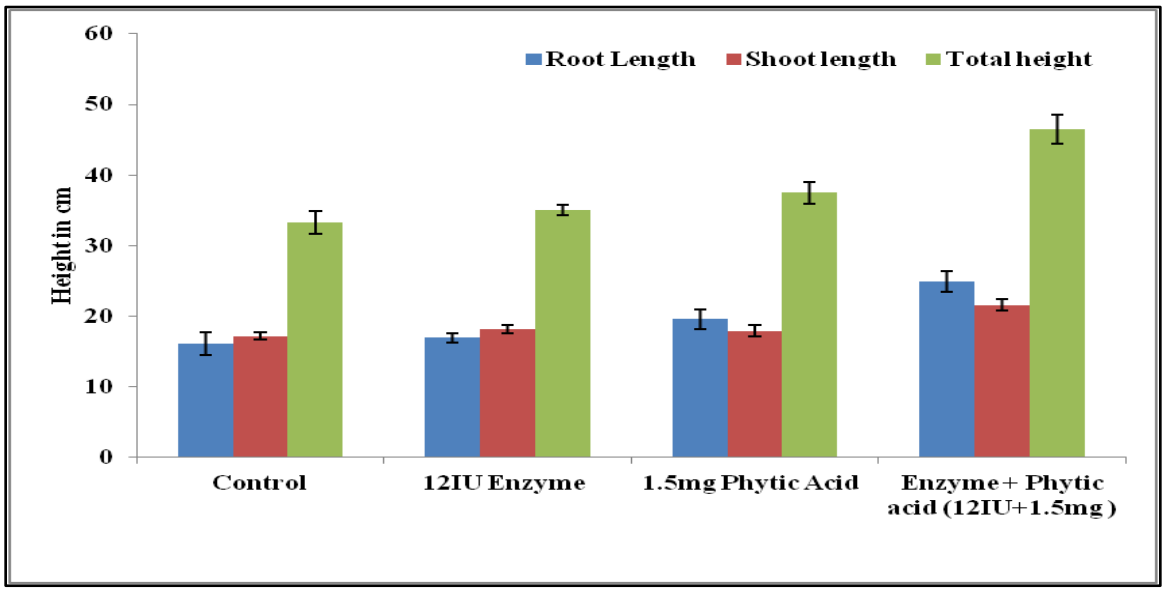

Figure 7a

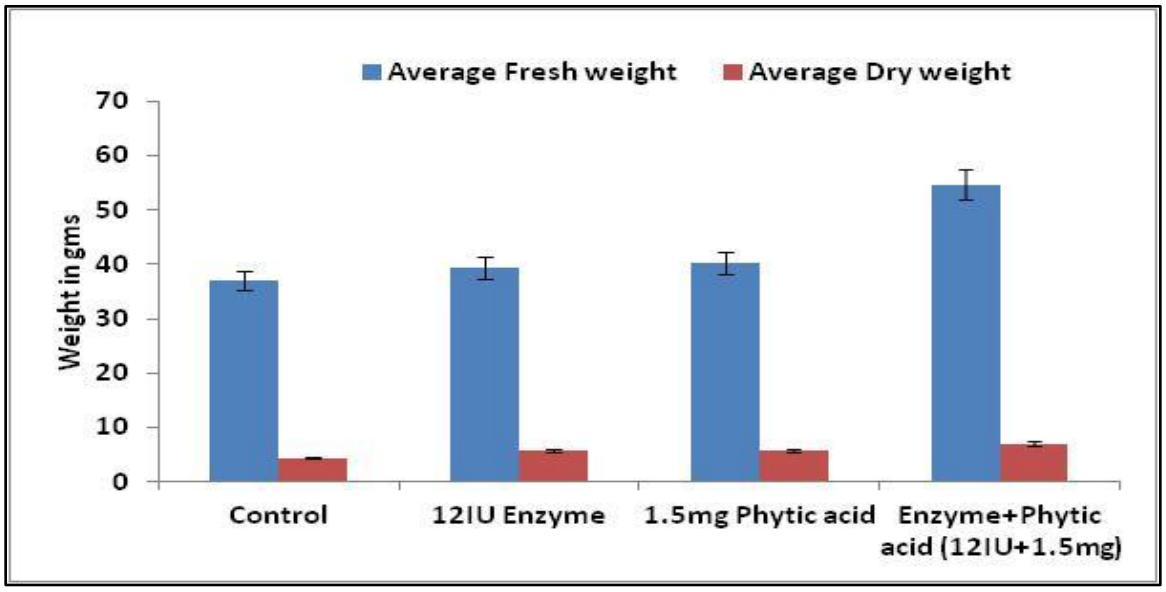

Figure 7b

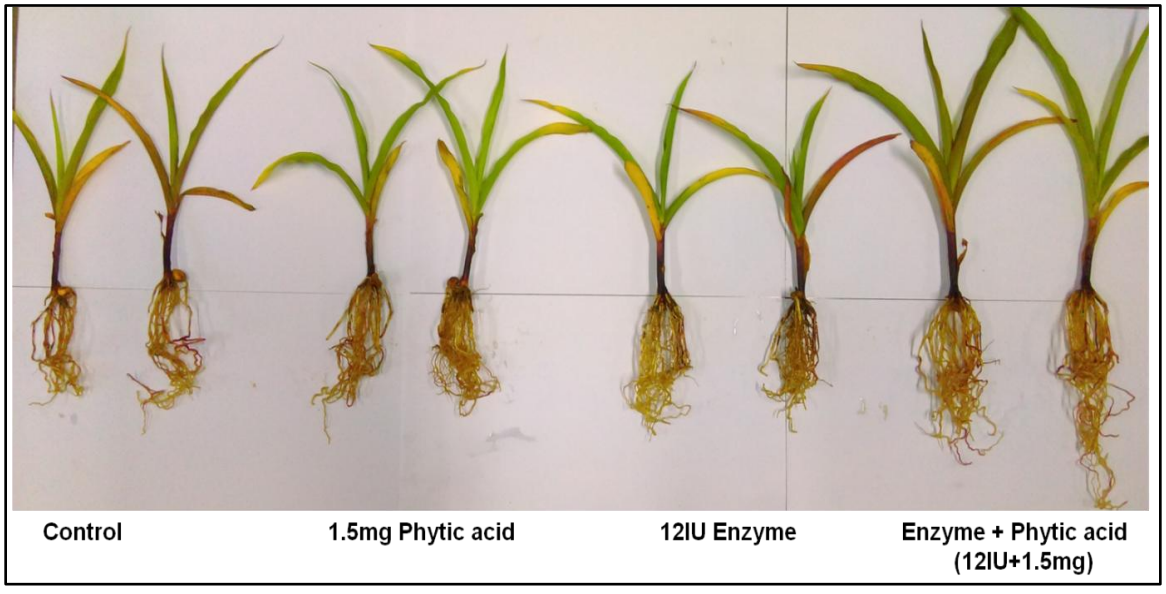

Figure 7c

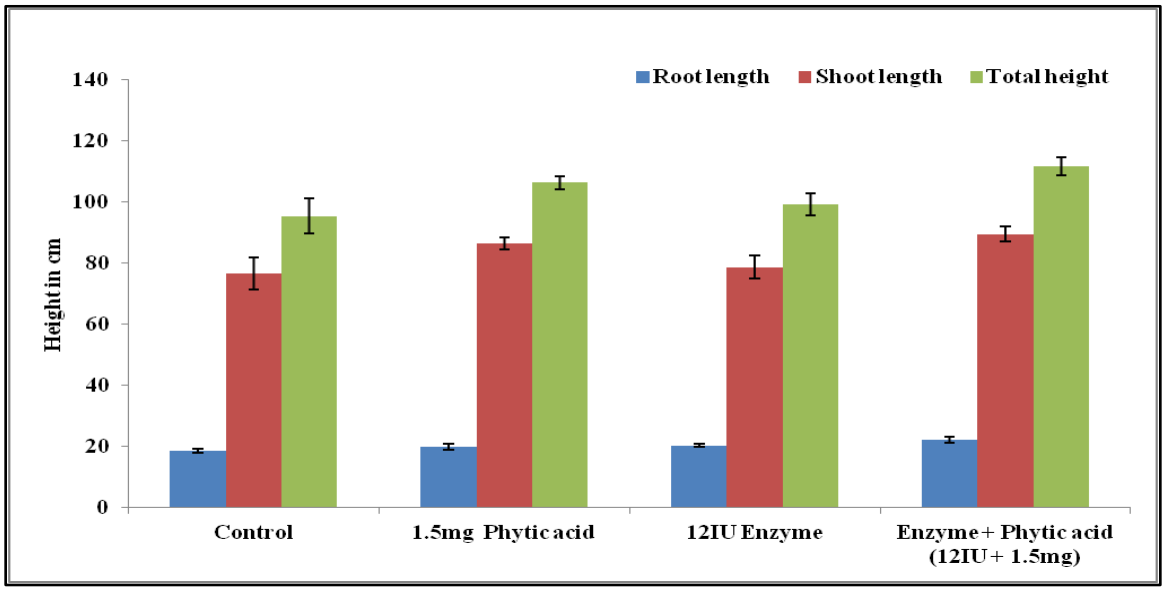

Figure 7d

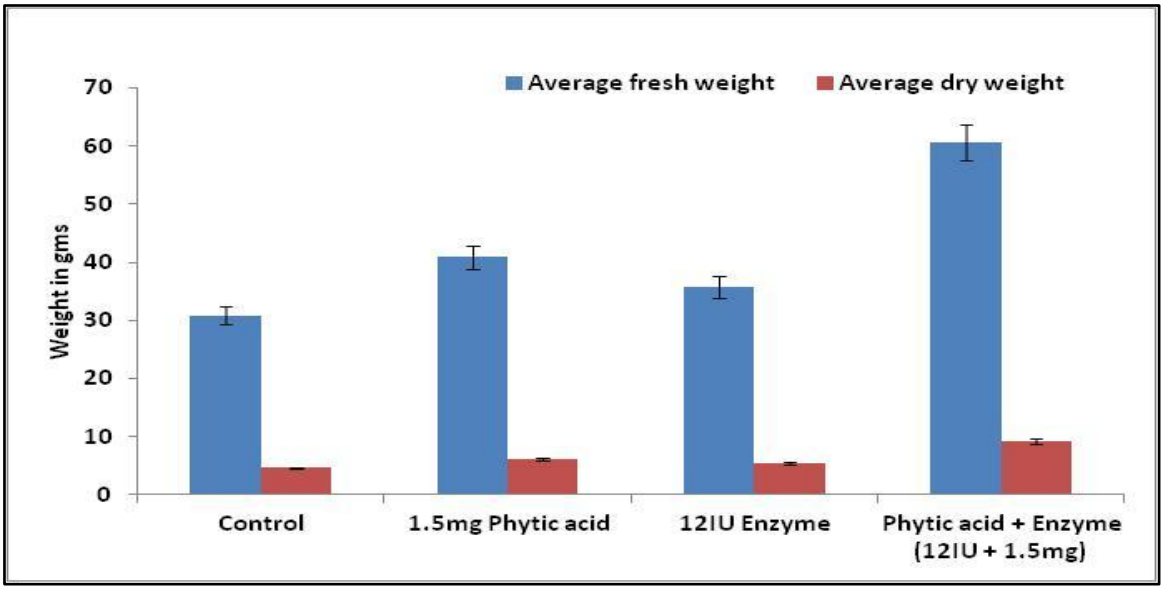

Figure 7e

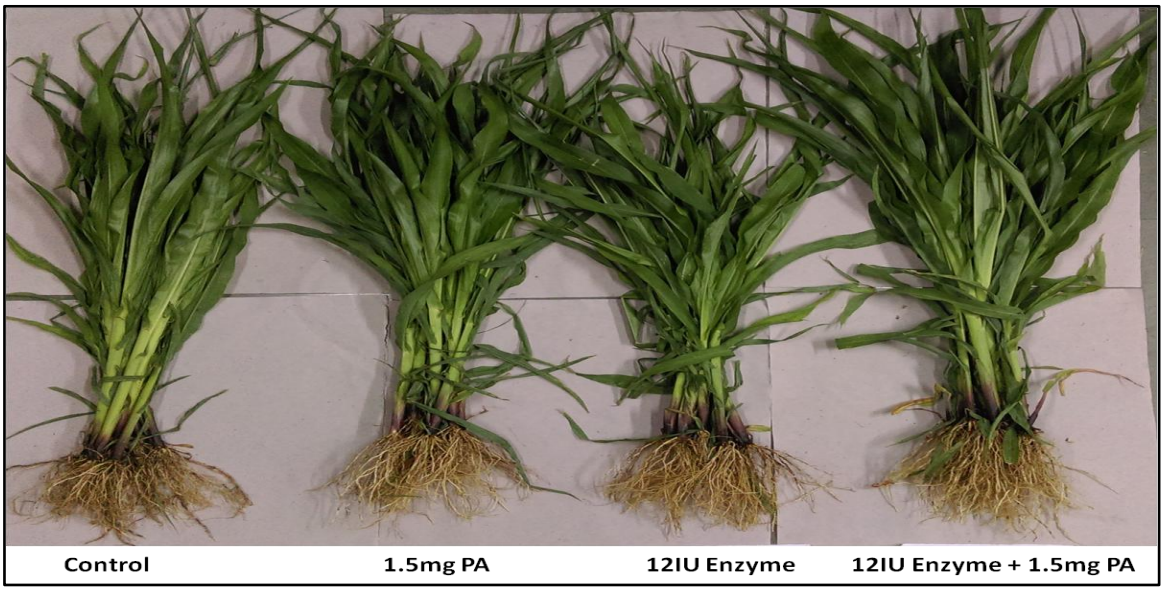

Figure 7f

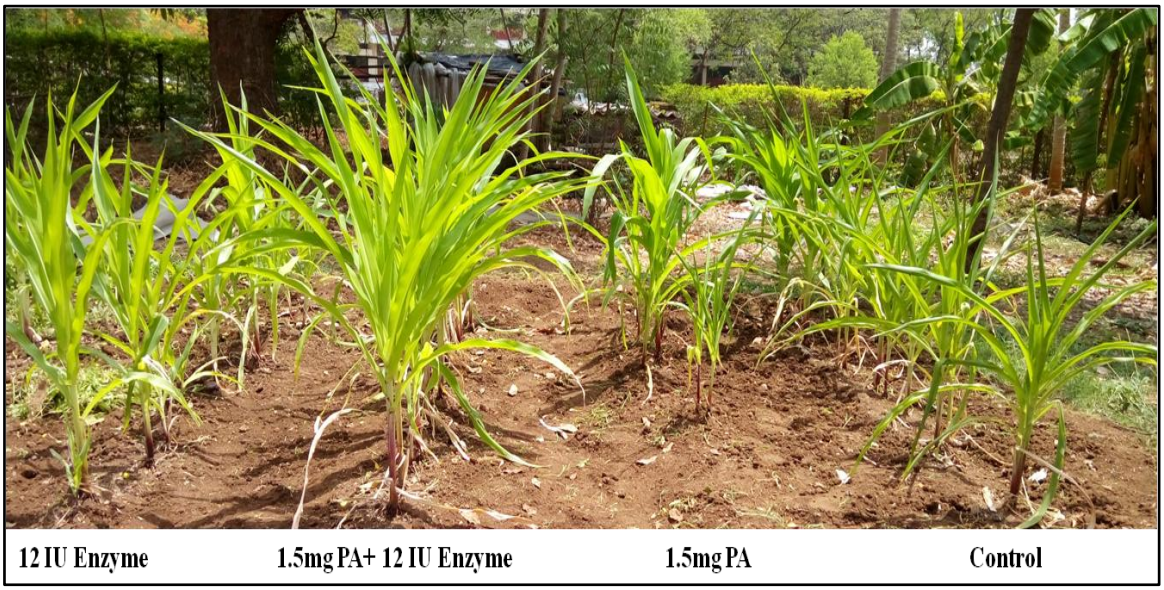

Figure 7g

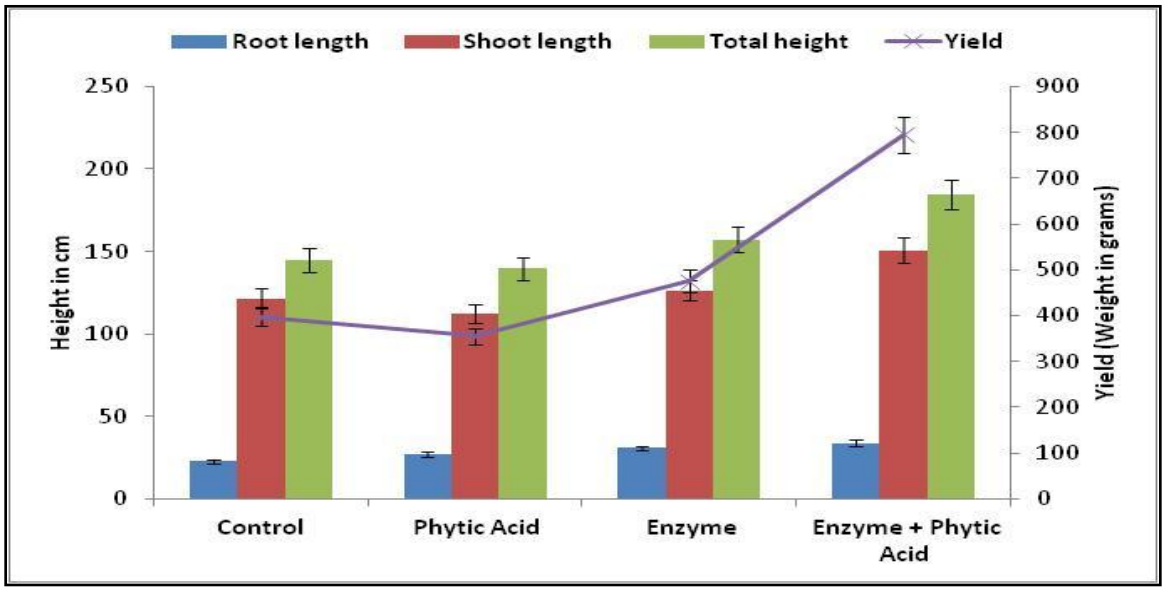

Figure 7h

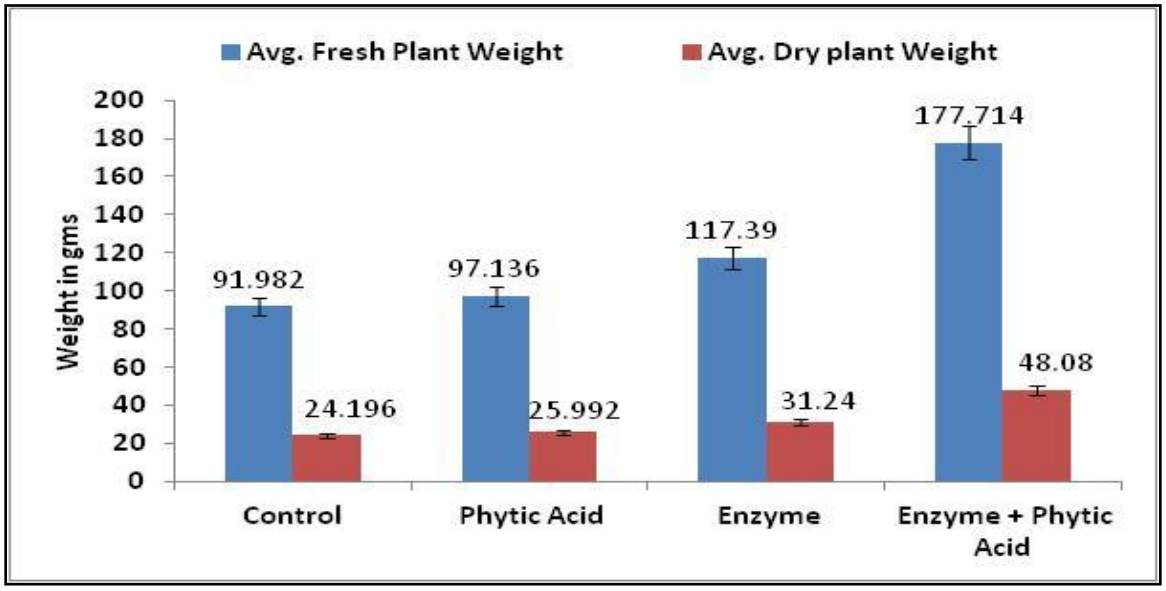

Figure 7i

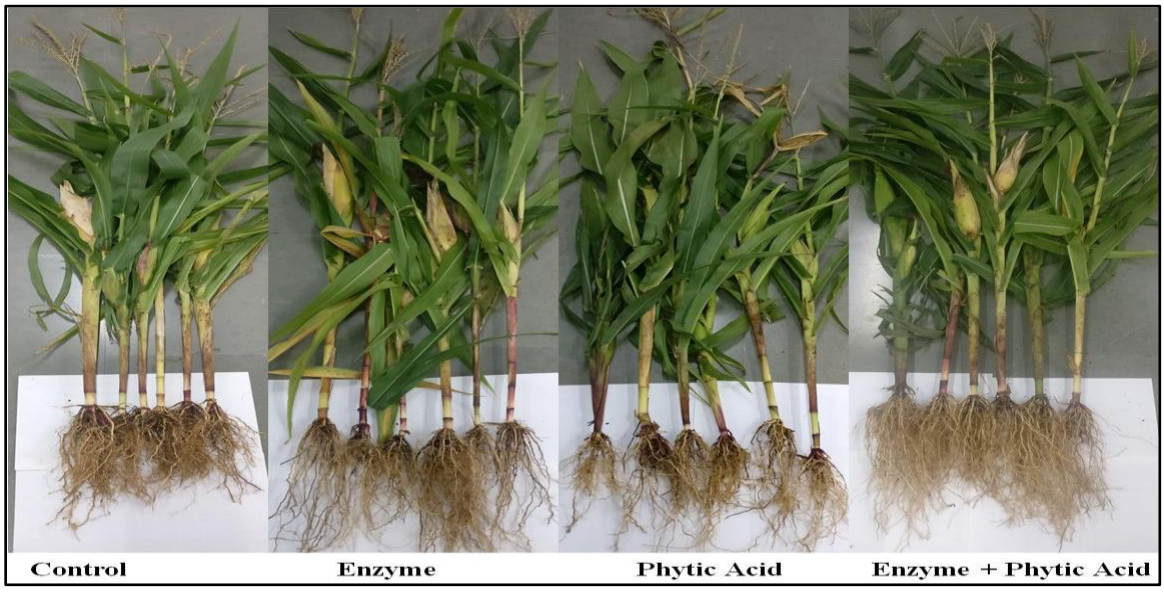

Figure 7j

Figure 7: Pot level and Field level Plant growth promotion experiment with *Candida tropicalis* (NCIM3321) extracellular enzyme. **7a** – Effect of different treatments in plant root, shoot and total height measurements at pot level, **7b** – Effect of different treatments on plant average fresh weight and dry weight at pot level, **7c** – Plant image showing the effect of different treatments on plant heights at pot level, **7d** – Effect of different treatments in plant root, shoot and total height measurements at field level, **7e** – Effect of different treatments on plant average fresh weight and dry weight at Field level, **7f** – Plant image showing the effect of different treatments on plant heights at field level after uprooting, **7g -** Plant image showing the effect of different treatments on plant heights in field before uprooting. **7h -** Effect of different treatments in plant root, shoot and total height measurements and yield in terms of maize fruit at field level after 3 months. **7i -** Effect of different treatments on plant average fresh weight and dry weight at Field level after 3 months. **7j -** Plant image showing the effect of different treatments on plant heights and root morphology at field level after 3 months.

Note: PA – Phytic acid, Enz – Enzyme, PA + ENZ – Phytic acid with Enzyme.

3.4. Conclusion:

To enhance cell-bound and extracellular phytase production, strain *C.tropicalis* was subjected to media optimization by response surface methodology using Design Expert software. Statistical optimization of MGYP media led to an 4-5 fold increase in activity of phytase The process has been scale-up up under optimized conditions to 10L fermenter with increased productivity of 1810 IU $DCG^{-1}day^{-1}$ of cell-bound enzyme and 6.08 IU $ml^{-1}day^{-1}$ of extracellular enzyme. According to our knowledge, *C.tropicalis* is the highest phytase producer compare to other yeasts. Further, the extracellular phytase enzyme could also found to promote plant growth in lab and field level with an increase in root and shoot lengths. The study inferred that phytase enzyme could be considered as a one of the plant growth promoting attribute and required further studies to commercialized products in the form of enzyme formulation in agriculture field.

All the results of experiments mentioned in this chapter are published in:

Puppala, K. R., Naik, T., Shaik, A., Dastager, S., Kumar, R., Khire, J., & Dharne, M. (2018). Evaluation of Candida tropicalis (NCIM 3321) extracellular phytase having plant growth promoting potential and process development. *Biocatalysis and Agricultural Biotechnology*, *13*, 225-235.

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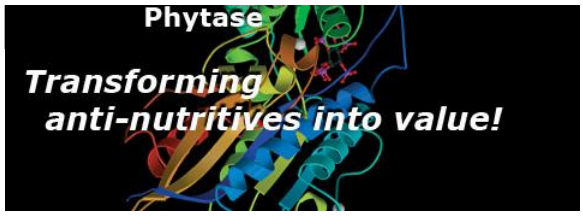


Chapter 4

Evaluation of phytase producing *Saccharomyces cerevisiae* (NCIM 3662) probiotic properties and its application in food fortification

Summary:

An increase in undigested complexes of phytic acid (usually referred as antinutrient) in food is gaining more attention to overcome nutritional challenges due to chelation effects in poultry, piggery, aquaculture and human healthcare. Phytase enzyme is known to be useful in dealing with increased mineral bioavailability, with least focus on combined properties like probiotics and dephytinization. This will reduce the extraction of phytase from biomass; thereby reducing the cost of feed pellet and raw foods which could be useful in designing functional foods or superfoods. In this chapter, we investigated phytase produced by soil isolated *Saccharomyces cerevisiae* (NCIM 3662) strain for dephytinization of different types of foods, probiotic properties and process development. The strain produced 45 IU/DCG of cell-bound phytase in an un-optimized MGYP medium, which was increased by four folds (164 IU/DCG) in 12 h using statistical media optimization. The process was scale-up up to 10L fermenter scale with an increased phytase productivity of 6.4 IU/DCG/h as compared to the lab scale. The strain exhibited important probiotic features like tolerance to artificial gastric acid conditions, hydrophobicity, autoaggregation, coaggregation and bile salt hydrolase (BSH) activity. Interestingly, it could dephytinize (removal of phytic acid; an anti-nutritional factor) selected functional foods like Ragi (Finger millet) flour, Soya Flour, Chickpea flour and poultry animal feed even under simulated gastrointestinal conditions in a shorter duration. A combination of cell-bound dephytinizing phytase, nutrition ameliorating and probiotic traits of *S. cerevisiae* (NCIM 3662) present profound applications in the food technology sector.

4.1. Introduction:

Phosphorus (P) is important micronutrient for all living organisms. It is one of the essential components in nucleic acid and phospholipids structures. It will act as intermediate in carbon metabolism and enzyme (in) activation. For plants, P is the second most important micronutrient after nitrogen (N) (Lambers et al. 2006). Plants store the P in the form of Organic phosphorus (Phytate). General livestock feed using cereal grains and legumes 50-80% of total P present in the form of phytate (myo-inositol hexakisphosphate) (Askelson et al. 2014). Phytate is considered as an antinutritional factor because of its high chelating capability towards essential micronutrients like calcium, iron, magnesium and zinc and makes them less bioavailable (Vashishth et al. 2017). Deficiency of different minerals leads to reduced mental development and long-term negative effects on school performance (Hurrell 2003). This form of P is less bioavailable to plants and monogastric animals (poultry, swine and humans) due to the lack of phytate-degrading enzymes or phytases (Askelson et al. 2014). This undigested phytate passes through the soil and degraded by the phytate degrading enzymes and release free P into soils further in water bodies leads to environmental P pollution called eutrophication (Priyodip et al. 2017).

In nature, phytases are widely diversified in different hosts like microorganisms, plants and animal tissues (Turk et al. 2000). These phytases convert phytate into myo-inositol and inorganic phosphate and make bioavailable of different minerals (Priyodip et al. 2017). These phytases are a great deal to food and feed of nonruminant animals to make bioavailable P; this clearly translates to recent market trends towards the importance of phytase in the food and feed sector. According to Kaur et al. (2007) at the end of the 20th century, the business of phytase in animal feed reaches US$ 500 million. Although many

reports discussed phytase produced by a diverse range of microorganisms, several of them may not be useful in process development due to the lack of enzyme yields and pH stability at harsh gastrointestinal tract (GIT) conditions (Wodzinski and Ullah 1996). Consider the challenges that reinforced the interests towards isolating novel phytase producing microorganisms, which are effectively released food phosphate into GIT and should be stable during processing of food and storage (Ragon et al. 2008).

Probiotics are defined as per World Health Organization (WHO), "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Hill et al. 2014). There is substantial reports to support the use of probiotics in different treatments include prevention of antibiotic-associated diarrhoea, acute diarrhoeal diseases, lactose metabolism, reducing serum cholesterol level, blood pressure control, decreasing cancer incidence and supporting to normal vaginal lactobacilli microbiota in bacterial vaginosis (Kechagia et al. 2013). As per grand view research, Inc 2017, the revenue of global probiotic market revenue was exceeded 35 billion US dollars. It is not necessary for all members of the genus or species of probiotic should be a probiotic. It will vary at the level of strain, species and genus. Thus it is compulsory to investigate every strain for its specific probiotic properties (Bajaj et al. 2014). An essential prior condition for any probiotic is to tolerate and withstand the harsh environment of GIT, and it should adhere to and colonize the GIT insufficient number (Mokhtari et al. 2017). Additionally, an ideal probiotic should contain some of the following functional attributes viz. autoaggregation, coaggregation, hydrophobicity, antimicrobial activity against the pathogen, co-culturing ability with other probiotic organisms, bile salt hydrolase activity and several other functions (Andrabi et al. 2016). While passing through the GIT some

probiotic can't maintain its viability rate, such probiotics are subjected to microencapsulation to maintain its viability rate (Shah et al. 2016). If some bacteria having the antibiotic resistance, they may chance of transfer of genetic material to the pathogens by horizontal gene transfer, but this gene transfer is not possible for bacteria to yeast. According to Reenen and dicks, (2011) horizontal gene transfer has a more negative effect on host chromosome cause serious damage and mutations in its own population. This gives an advantage to the yeast as a probiotic, compare to other probiotic bacteria like lactobacillus species (Czerucka et al. 2007). Cultures or species which are frequently used in the fermentation process of foods for human consumption are generally considered as a safe (GRAS) (Sourabh et al. 2011).

Yeast cultures may have a possible effect on rumen as a probiotic by increasing the important bacteria population, stabilizing the gut pH and improve the gut health. *Saccharomyces* species are one of the first probiotics approved for human administration (Palma et al. 2015). *S. cerevisiae* is well-studied yeast from decades for different applications like fermentation, single-cell protein (SCP), and baking (Mikulski and Klosowski 2017). These yeasts are more preferable as probiotics due to its natural resistance to a broad range of antibiotics (Poloni et al. 2017). *S.cerevisiae* has been used as probiotic for swine, poultry and dairy cattle from several years (Cui et al. 2017, Jayasena et al. 2017, Jiang et al. 2017). Vohra and Satyanarayana (2001) and In et al. (2008) reported phytase producing yeast and its different biotechnological applications. There are very few reports of yeast with probiotic and phytase properties used for human nutrition (Andrabi et al. 2016, Greppi et al. 2017), but lacking focus on the phytase. Although the strain has been widely appreciated, still probiotics properties and phytase producing ability of *S.cerevisiae*

have not been explored that much in the field of human nutrition (Fakruddin et al. 2017). This lacuna prompted us to evaluate phytase producing *S. cerevisiae* NCIM 3662 strain for optimization of growth and media engineering to enhance the phytase production. Furthermore, evaluation of *S. cerevisiae* for probiotic properties and its application in dephytinization of food and feed for human and animal nutrition was also studied.

4.2. Material and Methods:

4.2.1 Media and chemicals:

The phytic acid sodium salt was purchased from Sigma Aldrich, St Louise, MO, USA. Glucose, sodium nitrate, agar, magnesium sulfate, ferrous sulfate, yeast extract, malt extract and peptone bacteriological from Hi-Media, India. Manganese sulfate and potassium chloride are from Merck, India. Functional foods and feed are purchased from the local market, Pune.

4.2.2. Phytase activity assay

The primary inoculum was prepared by inoculating a loop full of yeast culture in 5 ml MGYP medium and incubated at 28 °C for 12 h. A 2% of primary inoculum was added to 50 ml MGYP broth and the flasks were kept at 28 °C for 12 h at 165 rpm in a shaker incubator. Culture suspension was centrifuged at 8000 rpm for 10 min, cell-free supernatant was collected in a sterile tube and the cell pellet was washed twice with sterile distilled water. Both the samples *i.e.,* cell pellet and cell-free supernatant were used for phytase assay. The cell pellet was used for cell bound phytase and supernatant for extracellular phytase production after removal of periodic samples. Phytase activity was measured at 50 °C in 300 µl of 200 mM Glycine-HCl pH 4.5 buffer with 100 µl of the enzyme for 30 min,

100 µl of 200 mM sodium phytate as a substrate. The liberated inorganic phosphate was measured by ammonium molybdate method (Bhavsar et al. 2011). A freshly prepared 4 ml solution of acetone: 5N H_2SO_4: 10 mM ammonium molybdate (2:1:1 v/v/v) and 400 µl of 1 M citric acid were added to the assay mixture. Absorbance was measured at 370 nm. One unit of phytase activity (IU) was expressed as the amount of enzyme that liberates 1 µmol P per minute under standard assay conditions. Each experiment was carried out in triplicate and the values were reported as the mean of three experiments.

4.2.3. Media Optimization:

4.2.3.1 MGYP

Initial phytase activity was determined by using basal MGYP media, which contains malt extract, glucose, yeast extract and peptone.

4.2.3.2 MGYP with minerals

According to In et al., (2008) minerals can improve the yeast phytase production. Therefore, for basal MGYP media, we added some trace elements like potassium chloride, magnesium sulfate, manganese sulfate, ferrous sulfate and the phytase production were noted.

4.2.3.3. Plackett Burman Design (PBD)

PBD is a very advantageous tool which can help to reduce the number of experiments as compare with full factorial designs. In PBD for "n" number of variables, n+1 number of experiments has to be done as compared with full factorial 2^n experiments. Its design is based on the first order model. Each component in the experiment will set to two values; those are high (+) and low (-) values. High value set above normal basal medium value, and

low value set below basal medium value. By this broad range, we can eventually get the data of more influential components of the experiment (Vastrad and Neelagund 2014). The experiment was created by using software Design Expert 7.

4.2.3.4. PBD with OVAT

After getting the more influential factors data from PBD, it has been further optimized by using one variable at a time method. OVAT has been chosen because of a number of variables which are influentially limited to one (Singh et al. 2017). The total experiment was designed by the software called Design Expert 7.

4.2.4. Scale-Up Process for phytase production

Enhanced production paved way for scaling-up the phytase production from shake flask level (50 ml) to 2 L and further up to 10 L scale. A total scale-up process was done in New Brunswick BioFlo/CelliGen 115 Benchtop fermenter 3 L & 14 L. Batched in succession were at different agitation rate were performed at constant aeration and temperature. The 3L fermenter utility station dimensions of (8.5 Width X 16.5 Height in inches) with working volume of 2 L media was inoculated with 40 ml (~ 10^{11} cells ml^{-1}) of 12 hr grown culture. The dissolved oxygen (DO) was measured using a Mettler Toledo oxygen probe, which connects to the control station. The setpoint for DO has maintained a minimum of 20% of saturated oxygen. The set DO was maintained by cascading the agitation with DO set point. Samples were periodically withdrawn to check phytase production. The process was further scaled up to 14 L fermenter utility station dimensions of (11.5 Width X 24 Height in inches) with working volume of 10 L 200 ml (~ 10^{11} cells ml^{-1}) of 12 hr grown culture inoculum in 10 L media.

4.2.5. Phytic acid removal from foods and feed

The phytase-treated (15 IU) and phytase-untreated flour samples were suspended in 2.4%HCl (20 ml) and shaken at 165 rpm for 2 h at 28 °C. The suspension was centrifuged at 8000 rpm for 15 min at 28 °C. The supernatant was filtered through a 0.22-µm syringe filter. Each filtrate was stored in sealed tubes until being subjected to ion-exchange chromatographic separation. The filtrates were diluted 1:5 in 2.4% HCl, and 10 ml of the diluted sample was applied to 1 cm × 10 cm columns (ml) of the ion exchange resin Dowex 1 × 8 chloride form (200–400 mesh) anion exchange resin (Sigma Chemical Company, St. Louis, MO, USA). After washing the column with water (25ml), bound phosphate was eluted from the column using 0.1 M NaCl. After collecting the phosphate, the column is washed again with 20 ml of water. Further, bound phytic acid was eluted from the column using 0.7 M NaCl and collected. The phytic acid concentration present in each sample was determined calorimetrically at 500 nm using Wade reagent (0.03% ferric chloride hexahydrate and 0.3% sulfosalicylic acid). The phytic acid concentration in each sample was derived from a standard curve using authentic sodium phytate as a standard (Sigma Chemical Company, St. Louis, MO, USA) (Han 1988).

4.2.6. Total mineral concentrations in different functional foods

Total iron (Fe), calcium (Ca), magnesium (Mg) and zinc (Zn) contents were determined according to the method described by Sharma et al. (2018) using Atomic Absorption Spectrophotometer (AAS, GBC Avanta, Australia) with some modifications. 1 g sample each of Ragi, Soya, Chick Pea flour was suspended in 10 ml Milli Q water and were incubated with 1 Dry Cell Gram (DCG) of cells having the phytase activity of 160 IU at 37 °C for 2 h. Flour samples dissolved in 10 ml of MilliQ water without cells were used as a

control. After incubation samples were centrifuged at 8000 rpm for 10 min. The supernatant was analyzed separately. For Pellet 10 ml of the triacid mixture of nitric acid, sulphuric acid and perchloric acid (10:1:4; v/v/v) was added to each sample and dried at 200 °C inside the fume hood. Thereafter, dried samples were placed in a muffle furnace at 450 °C for 12 hr till white ash was obtained. The obtained sample ashes were dissolved in 1 ml concentrated Nitric acid followed by volume makeup to 10 ml with MilliQ water. Further samples were diluted based on the system standard concentration of each element. For total mineral content was determined by adding both pellet and supernatant mineral concentrations.

4.2.7. Mineral Analysis at Gastro-Intestinal Tract Conditions (GITC)

The concentrations of Fe, Ca, Mg and Zn were determined *in-vitro* by simulating gastrointestinal tract digestion conditions with some modifications (Kiers et al. 2000, Sharma et al. 2018). Samples of 1 g Ragi, Soya, Chickpea flour each was suspended in 10 ml of a sodium acetate buffer containing 1 DCG of cells having 160 IU/DCG phytase activity. The samples were digested by adding α-amylase (25 IU) solution consisting 1.5 g/1NaCl, 1.5 g/l K2HPO4and 0.5 g/1 Na2CO3 (pH 7.0) of 1 ml for 30 min at37°C. Next the pH was adjusted to 4.0 using 5M HCl and the suspensions were incubated with 4 ml of stomach medium (0.1 g/1 lipase, 0.125 g/1 pepsin(Sigma P-6887), 3.1g/1 NaCl, 1.1 g/1 KCl, 0.6 g/1 Na2CO3, 0.11 g/1 CaCl2, pH 4.0) for 1 h at37°C. The pH was then adjusted to 6.0 using $NaHCO_3$ solution. Finally, 5 ml of a 2% pancreatic solution (20.0 g/1 pancreatin, 5.0 g/1 porcine bile, 5.0 g/1 NaCl, 0.68 g/1 KH_2PO_4, 0.3g/1 Na_2HPO_4, 0.84 g/1 $NaHCO_3$) was added and the suspensions were incubated for 30 min at37°C. Control samples consisting of flour samples in MilliQ water along with digestive enzymes but no phytase

enzyme were used throughout the experiment. After digestion, contents were centrifuged at 8000 rpm for 15 min. The supernatant was analyzed for mineral content and the pellet was subjected to the Mineral analysis as mentioned above in total mineral concentrations. Later on, bioaccessibility of minerals was determined by calculating the solubility percentage of supernatant fractions of digested samples under GIT conditions. Percentage of soluble mineral was calculated using the formula:

Solubility (%) = {amount of mineral (supernatant) – amount of mineral (blank)/amount of mineral (undigested sample)} x100

Supernatant – GIT Conditions maintained supernatant, blank – Without GIT conditions and without phytase treated, undigested sample – Total mineral content in an undigested sample.

4.2.8. Probiotic properties

4.2.8.1. Tolerance to GIT harsh conditions

The *S. cerevisiae* NCIM 3662 was tested for their ability to grow in the presence of porcine bile extract. Around 1% of an overnight culture, grown in MRS broth (O.D Set to 1) was added to 50 ml of MRS broth supplemented with 0.5, 1.0, 1.5 or 2.0 % porcine bile extract (B8631; Sigma, St. Louis, Mo.), and incubated at 28 °C for 24 h. Growth was characterized as no growth, weak growth, or strong growth. Growth was further confirmed by streaking loop full of liquid culture on MRS agar plate incubated at 28 °C for 24 h and growth was observed as mentioned by Kim et al. (2007) with slight modifications.

The strain was also evaluated for ability to withstand harsh gastric condition of highly acidic pH. Yeast was grown in MGYP broth for 18 h at 28 °C to attain desired cell number (10^8 cells/ml, A_{600} = 0.8–0.9). Resulted suspension was inoculated (1 %, v/v) in artificial gastric fluid (AGF) g/L (8.3 Peptone, 3.5 Glucose, 2.05 NaCl, 0.6 KH_2PO_4, 0.147 $CaCl_2$, 0.37 KCl) broth adjusted at pH 2 and 3 for determining growth/survival of NCIM 3662 under low pH. Since human stomach contains a certain amount of urea, we added 6 mM urea to the above AGF medium and adjusted pH to 2 and pH 3. Periodically samples were collected at every 12 h up to 48 h, and appropriate dilutions were spread on MGYP agar plate to check the survival of yeast cells. Results were noted as colony forming unit per ml (CFU/ml) (Andrabi et al. 2016).

4.2.8.2. Hydrophobicity

Three different solvents, *i.e.,* xylene, chloroform and ethyl acetate n-hexadecane were used for hydrophobicity analysis. Yeast was grown in MRS broth for 18 h, and the cells were harvested by centrifugation. The cell pellet was washed twice with Ringer solution (6 % NaCl, 0.0075 % KCl, 0.01 % $CaCl_2$ and 0.01 % $NaHCO_3$), and suspended in the same solution and read for absorbance at 580 nm (A_{580} initial). One millilitre of solvent was added to three millilitres of cell suspension. After 10 min of pre-incubation at room temperature, the two-phase system was mixed by vortexing for 2 min. The two phases were allowed to separate for 30 min at room temperature; the aqueous phase was then taken carefully and absorbance was determined (A_{580} solvent). Per cent hydrophobicity was calculated according to the formula (Andrabi et al. 2016).

Hydrophobicity = [{A_{580} (Initial) - A_{580} (Solvent)} / A_{580} (Initial)] * 100

4.2.8.3. Autoaggregation (AAg) and coaggregation

For AAg assay yeast was grown for 18 h at 37 °C in MGYP broth to attain viable counts approximately 10^8 cells/ml. The cells were harvested by centrifugation, washed twice with phosphate-buffered saline (PBS) and suspended in the same. Cell suspension (4 ml) was mixed well by vortexing for 15–30 sec and allowed to stand at 25 °C and 37 °C. After varying time intervals, 0.1 ml of the upper layer of cell suspension was transferred to 3.9 ml of PBS in another tube; contents were mixed well, read for absorbance (A_{600}). Autoaggregation ability (AAg %) was expressed using the following formula.

$$\mathbf{AAg\ (\%) = [1- (A_t – A_0)*100]}$$

Where A_0 and A_t represent the absorbance at 0 h and after a time interval of 1, 2, 3, 12 and 24 h, respectively (Andrabi et al. 2016).

The coaggregation potential of yeast with different pathogenic bacterial strains viz. *Escherichia coli* NCIM 2065, *Staphylococcus aureus* NCIM 5021 and *Salmonella enterica* NCIM 5255 were examined. Yeast and the test pathogenic bacteria were grown in MGYP broth and nutrient broth respectively, for 18 h at 37 °C to attain viable counts of approximately 10^8 CFU/ml (A_{600} 0.85– 0.9). Equal volumes of cell suspension (2 ml) of yeast and pathogen were mixed by vortexing for 10 s and incubated at 37 °C without agitation. After different time intervals, 0.1 ml of the upper layer of the suspension was transferred to another tube containing 3.9 ml of PBS and the absorbance (A_{600}) was measured. Controls were set up simultaneously which consisted of 4 ml of each of the

individual bacterial cell suspension. The coaggregation was calculated using the following equation

$$\textbf{Coaggregation (\%)} = [\{((A_{pat} + A_{probio})/2) - A\ (pat + probio)\}/\ ((A_{pat} + A_{probio})/2)]$$

Where A_{pat} and A_{probio} represent absorbance (A_{600}) of the individual bacterial suspensions in control tubes, and A (pat+ probio) mix represents the absorbance of the mixed bacterial suspension at different time intervals (Andrabi et al. 2016).

4.2.8.4. Microaerophilic and co-culturing ability

Overnight grown yeast culture (O.D = 1 set) was inoculated (1% inocula) in MRS broth media (5ml tube) and added 3ml Paraffin oil to maintain the microaerophilic conditions. Further tubes were kept at vacuum desiccators, incubated at 37 °C for 24h and growth was observed.

Yeast and *Lactobacillus plantarum* obtained from NCIM resource centre, CSIR – NCL, Pune and were streaked on the MRS agar plate and incubated at 37 °C in vacuum desiccators, and observed for the antagonistic effect till 24 h.

4.2.8.5. Bile Salt Hydrolase (BSH) assay and protein assay

BSH activity is the measurement of the number of amino acids liberated from conjugated bile salt by the microorganism. Method for the estimation of BSH activity was taken from the Liong and Shah (2005) with slight modification. Briefly, cells were grown in MRS broth for 24 h and centrifuged at 10,000 g at 4 °C for 10 min. The cell pellet was washed twice in 10 ml of 0.1 M phosphate buffer (pH 7.0). The cell pellet was diluted as 0.1 g in 1 ml of 0.1 M phosphate buffer (pH 6.0). Five millilitres of the cell suspension was sonicated

for 15 min with constant cooling in ice, followed by centrifugation at 10 000 rpm at 4 °C for 10 min. To 0.1 ml of the supernatant obtained, 1.8 ml of 0.1 M sodium phosphate buffer (pH 6.0) and 0.1 ml of conjugated bile salt was added. Conjugated bile used was 6 mM sodium taurocholate (Hi-media). The mixture was incubated at 37 °C for 30 min followed by enzymatic reaction was terminated by adding 0.5 ml of trichloroacetic acid (15% wt / vol) to 0.5 ml of the sample. The mixture was centrifuged and 0.2 ml of the supernatant obtained was added to 1 ml of distilled water and 1 ml of ninhydrin reagent (0.5 ml of 1% ninhydrin in 0.5 M citrate buffer pH 5.5, 1.2 ml of 30% glycerol, 0.2 ml of 0.5 M citrate buffer pH 5.5). The preparation was vortexed and boiled for 14 min. After subsequent cooling, the absorbance was determined at 570nm. BSH activity determined using glycine (0 – 100 µg) as standard. One unit of BSH activity was defined as the amount of enzyme that liberated 1 µmol of amino acid from the substrate per min at standard assay conditions. Protein concentration was determined by using the Bradford (1976) method by using BSA (0 – 100 µg) as a standard. BSH activity was determined in different media conditions like MRS broth, MRS with 0.5% phytic acid, MRS with 0.3% porcine bile extract, MRS with 0.3% oxgall bile, MRS with 0.3% porcine bile extract and 0.5% phytic acid and MRS with 0.3% oxgall bile and 0.5% phytic acid.

4.3. Results and Discussion:

4.3.1. Media optimization

Phytases are widely distributed among plants, bacteria, yeast, fungi and certain animal tissues. Baker's yeast *i.e. S.cerevisiae* is generally recognized as safe (GRAS) organism, so phytase produced by *S.cerevisiae* could have importance in food and feed application. Media optimization studies were carried out to enhance the phytase production. NCIM 3662, initially strain was grown in basal MGYP media and maximum cell bound activity of 45 IU/DCG in 12 h of time was obtained. According to In et al. (2008) minerals can improve the yeast biomass and it is directly related to phytase production. Therefore, for above basal MGYP media, some trace elements like potassium chloride, magnesium sulfate, manganese sulfate, ferrous sulfate were added. These minerals increase the activity of phytase up to 22% *i.e.* 55 IU/DCG compared to the initial activity.

MGYP with minerals media was further optimized by detecting the influencing factors present in the media determined by PBD. Total 8 components in media were analyzed by using design expert software as illustrated in Table 1 with activity response. And we obtained maximum cell bound activity of 108 IU/DCG at run number 12. The per cent of the contribution of each component was mentioned in Table 2. Glucose, yeast extract and mainly peptone is having a significant positive impact on the enzyme production and $MnSO_4$ is having a negative impact on enzyme production.

Table 1: PBD Design for *S.cerevisiae* NCIM 3662

Std	Run	Factor 1 A: Glucose g/100ml	Factor 2 B: M.E g/100ml	Factor 3 C: Y.E g/100ml	Factor 4 D: Peptone g/100ml	Factor 5 E: KCl g/100ml	Factor 6 F: $MgSO_4$ µg/100ml	Factor 7 G: $FeSO_4$ µg/100ml	Factor 8 H: $MnSO_4$ µg/100ml	D1 I:	D2 J:	D3 K:	Response
10	1	1	0.5	0.5	0.5	0.02	0.02	50	1000	-	-	-	54
6	2	1	0.05	0.05	0.5	0.02	0.1	200	250	-	-	-	71
11	3	5	0.05	0.5	0.5	0.1	0.02	50	250	-	-	-	107
4	4	1	0.5	0.05	0.5	0.1	0.02	200	1000	-	-	-	76
5	5	1	0.05	0.5	0.05	0.1	0.1	50	1000	-	-	-	60
8	6	5	0.5	0.05	0.05	0.02	0.1	50	1000	-	-	-	43
12	7	1	0.05	0.05	0.05	0.02	0.02	50	250	-	-	-	37
1	8	5	0.5	0.05	0.5	0.1	0.1	50	250	-	-	-	106
7	9	5	0.05	0.05	0.05	0.1	0.02	200	1000	-	-	-	50
2	10	1	0.5	0.5	0.05	0.1	0.1	200	250	-	-	-	77
9	11	5	0.5	0.5	0.05	0.02	0.02	200	250	-	-	-	103
3	**12**	**5**	**0.05**	**0.5**	**0.5**	**0.02**	**0.1**	**200**	**1000**	**-**	**-**	**-**	**108**

Table 2: Analysis of PBD for the determination of fellow components contribution on the cell-bound phytase enzyme from NCIM 3662.

Variable code	Term intercept	Sum of squares	% of contribution
A	Glucose	1680.33	22.015
B	Mall extract	56.3333	0.7380
C	Yeast extract	1323	17.3334
D	**Peptone**	**1925.33**	**25.2249**
E	KCl	300	3.93047
F	$MgSO_4$	120.33	1.57656
G	Fe SO_4	507	6.6425
H	$MnSO_4$	1008.33	13.2108
I	Dummy 1	65.3333	0.85591
J	Dummy 2	1.3333	0.01746
K	Dummy 3	645.333	8.4548

Above three components mainly contributes to nearly 65% of total activity and remaining all 5 components contributes to 25% of total activity. The best fit linear regression model was obtained from PBD variables F value was 6.13. So there is only 1.92% chance that a large F value is due to noise. The coefficient of determination (R^2) value 0.7778. Adequate precision measures the signal to noise ratio value greater than 4 is desirable. In this model 6.968 ratio indicates an adequate signal. This implies that the model can be used to navigate the design space. From the PBD study it has been observed that peptone (25.22%) has contributed more compared to all other components, so peptone

has taken for further optimization by OVAT using design expert software. OVAT has been used due to limited variables contribution in PBD. The design of OVAT for peptone and activity response was shown in Table 3. One gram of peptone concentration further increased the cell-bound phytase activity to 164 IU/DCG. Comparison of NCIM 3662 cell bound phytase optimization process was shown in Fig. 1. After statistical optimization of media components, the production of phytase increased to four folds higher than initial phytase production for NCIM 3662 strain. Statistical optimization generally used to enhance the ability of an organism to produce target enzymes or metabolites by changing its media components at different levels. PBD design was based on a first-order model used for identifying the important components, which will contribute to the enhancement of phytase production. OVAT will help to identify a particular component concentration for phytase maximum production. In this report NCIM 3662 activity (164 IU/DCG) was higher than the some of the reported yeast media optimized activities 68 IU/DCG, 135 IU/DCG, 131 IU/DCG for *Pichia anomala*, *S.cerevisiae* and *Pichia anomala* respectively (Vohra and Satyanarayana (2001), In et al. (2008), Vohra and Satyanarayana (2002b)). For improving phytase activity using a statistical approach is a well-known method from decades, used by people like Vohra and Satyanarayana (2002b). In the case of probiotics using recombinant approach for enhancing the phytase activity was not much suggested due to adverse effect may cause because of horizontal gene transfer.

Table 3: PBD with OVAT for *S.cerevisiae* NCIM3662

Std	Run	Factor 1 A: Peptone g/100ml	Response IU/DCG
7	1	2	125.52
3	2	1.5	143.5635
6	3	3	128.658
10	4	2	123.951
9	5	2	131.0115
5	6	3	121.5975
11	7	2	131.796
4	8	2.5	112.1835
1	9	1.5	149.8395
8	10	2	123.1665
2	**11**	**1**	**163.9605**

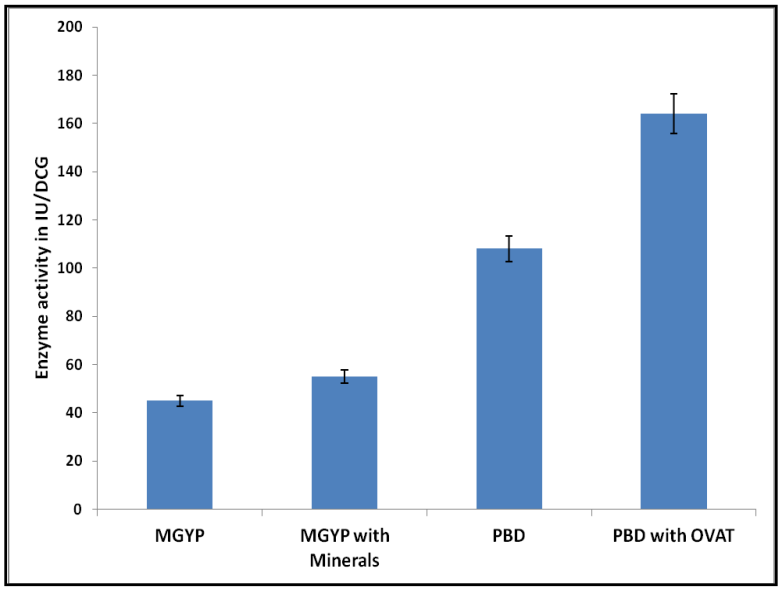


Figure 1: Comparison of different level optimization processes for the cell-bound phytase from *S.cerevisiae* (NCIM 3662).

4.3.2. Scale up of phytase production up to 10L fermenter

The optimized media was used to further scale-up the process. NCIM 3662 cell-bound enzyme production in 2 L fermenter was 184 IU/DCG in 10h with the productivity of 23 IU/DCG/h. In terms of productivity, it was higher than the shake flask level activity in 164 IU/DCG in 12h with the productivity of 13.6 IU/DCG/h. Following 10 L fermenter with similar parameters, which was used for the 2 L fermenter given the highest activity of 160 IU/DCG in 8 h with the productivity of 20 IU/DCG/h. Comparative bar graph of NCIM 3662 total scale-up process with time has shown in Figure 2. There are not much reports of yeast phytase scaled up to this level. DO was maintained throughout the scale-up process 20% of fermenter individual initial saturation oxygen which is generally considered as 100%.

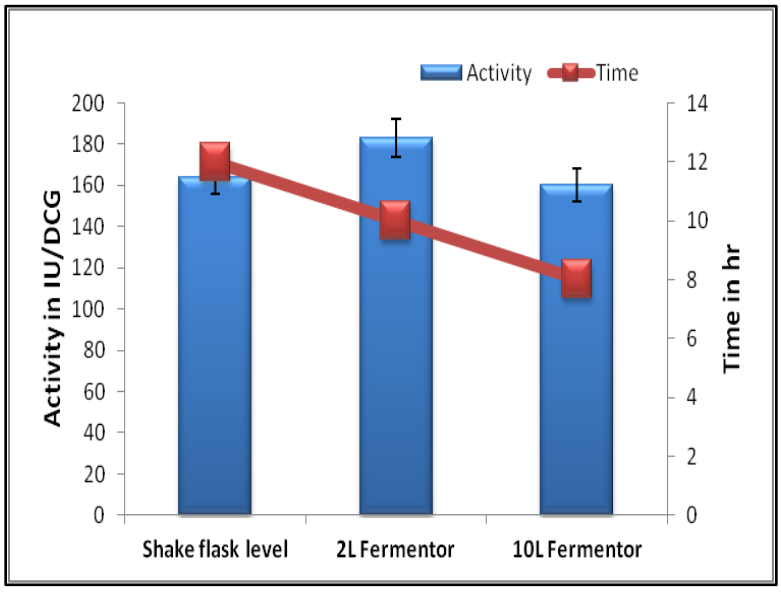


Figure 2: Upscaling process of NCIM 3662 cell bound phytase Production from shake flask level to 2 L fermenter and 10 L fermenter.

4.3.3. Phytic acid removal from foods and feeds

Phytase producing yeast strain *S. cerevisiae* is potential to degrade the phytic acid, which is present in the different plant-based foods and feeds. 15 IU of the enzyme was used for the treatment of different food flours like ragi (Finger millet), soya and chickpea which is having an initial phytic acid concentration of 10.53, 21.41, 2.75 mg per gram of flour respectively and animal mash feed having the concentration of 1.5mg per gram. After treatment with cell-bound lysate enzyme from NCIM 3662 for 2h at 37°C and constant shaking at 150 rpm, the resulted concentrations of phytic acid in plant-based food and feeds are very negligible as shown in Table 4. Phytic acid is an antinutritional factor, because of its high chelating capability towards important minerals which are important for human and animal healthy nutrition. Lack of these minerals causes several deficiencies in newborns

and adults like iron, calcium and zinc deficiencies (Hurrell 2003). So making bioavailable of these minerals and making high nutritional value food can be done by using dephytinization (Bohn et al. 2008). *S.cerevisiae* cell bound phytase was successfully degraded to negligible amounts, the phytic acid present in ragi (Finger millet), soya and chickpea and animal feed. However, there should be detailed study required to understand the stages of dephytinization by using exogenous phytases. Finger millet is widely used food in poor and developing countries and it has higher calcium content (350 mg/100 g) acquires nutritional importance in pregnant women and infants (Sharma et al. 2017). Chickpea is rich in proteins and India is the highest producer of this crop for food and feed applications (Vijayakumar et al. 2017). Soy-based foods are majorly used for animal feed for rich protein supplementation (Kokou et al. 2017).

Table 4: Phytic acid concentration before and after enzyme treatment

Type of food and feed	PA Concentration Before treatment	PA Concentration After Treatment
Ragi (*Eleusine coracana*)	10.53 mg/g of Ragi flour	Negligible - 6.24
Soya (*Glycine max*)	21.41 mg/g of soya flour	Negligible - 1.55
Chickpea (*Cicer arietinum*)	2.7562 mg/g of chickpea flour	Negligible - 3.61
Animal mash feed	1.5 mg/g of Animal feed	Negligible -

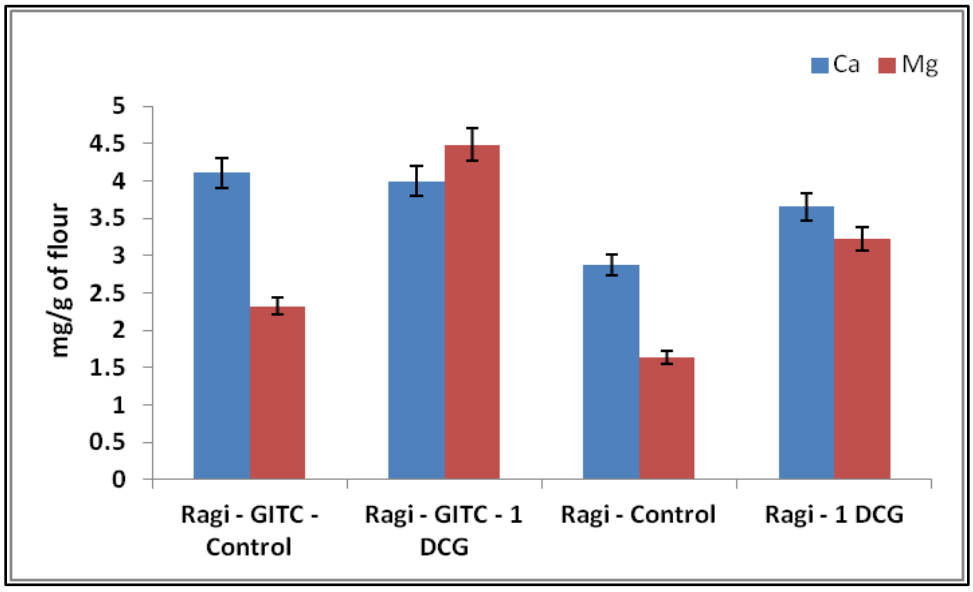
‘ - ’ Negative value in after treatment, PA – Phytic acid

4.3.4. The mineral analysis in functional foods

Under the undigested condition, all minerals showed enhancement in mineral concentration after treated with the phytase enzyme for all functional foods used in the experiment. But at Gastro Intestinal Track Conditions (GITC), Calcium levels in Ragi and Soya doesn't show much improvement. Magnesium, iron and zinc have shown improvement after treating with phytase at GITC conditions as shown in Figure 3a,b,c. Bioaccessibility conditions in terms of solubility were comparable with Sharma et al. (2017) for magnesium and Iron. Comparative analysis of data revealed that maximum fold change in solubility was observed with 1 DCG cells for magnesium, iron and zinc respectively as shown in Table 5. (All functional foods used in the experiment).

Table 5: Bioaccessibility (Solubility) of Minerals at GIT Conditions

	Ca solubility	Fold change	Mg solubility	Fold change	Fe solubility	Fold change	Zn solubility	Fold change
Ragi								
Control	**48.003**	**-**	**46.976**	**1.897**	**36.170**	**1.464**	**66.935**	**1.227**
1DCG Cells	**33.506**		**89.143**		**52.976**		**82.165**	
Soya								
Control	**31.802**	**1.099**	**29.800**	**1.885**	**13.917**	**1.884**	**52.352**	**1.309**
1DCG Cells	**34.961**		**56.174**		**26.225**		**68.577**	
Chickpea								
Control	**39.355**	**1.00031**	**26.902**	**2.3496**	**13.114**	**1.7132**	**59.868**	**1.0063**
1DCG Cells	**39.367**		**63.212**		**22.468**		**60.25**	



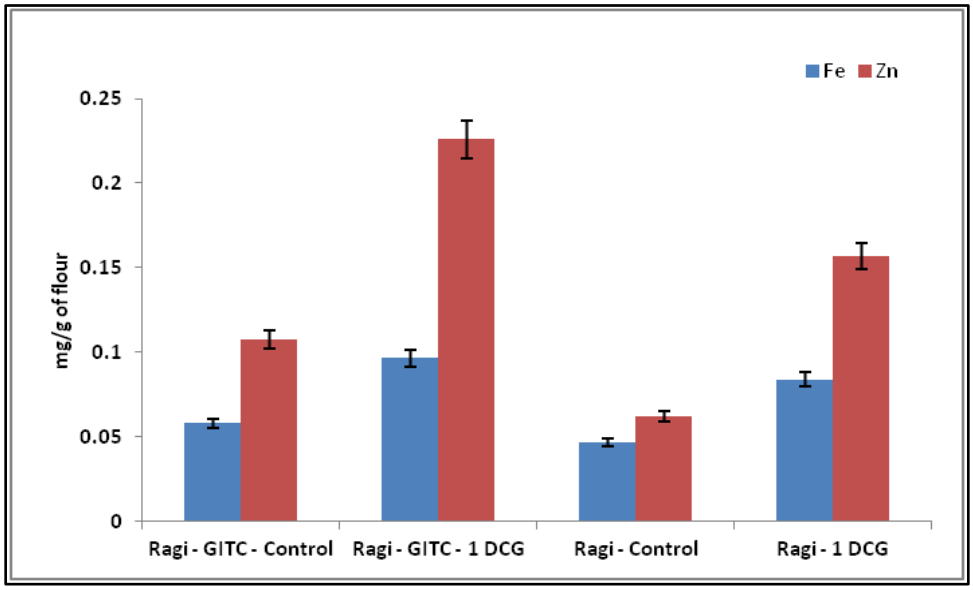
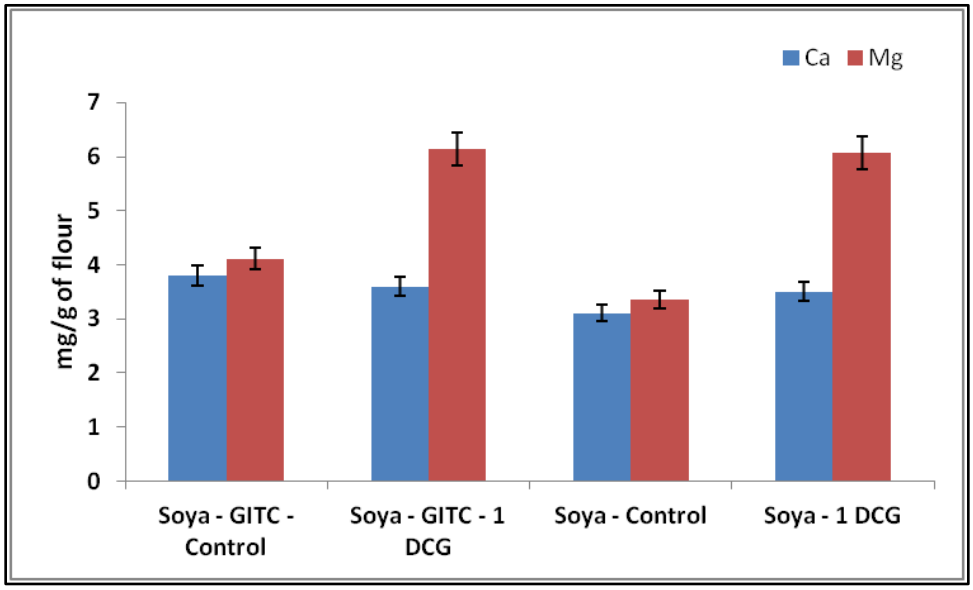


Figure 3: Total mineral content of Ca, Mg, Fe and Zn in functional foods at GIT conditions and undigested conditions with and without enzyme

Figure 3a – Total mineral content of Ca, Mg, Fe and Zn in Ragi at GIT conditions and undigested conditions with and without enzyme (1 DCG cells)



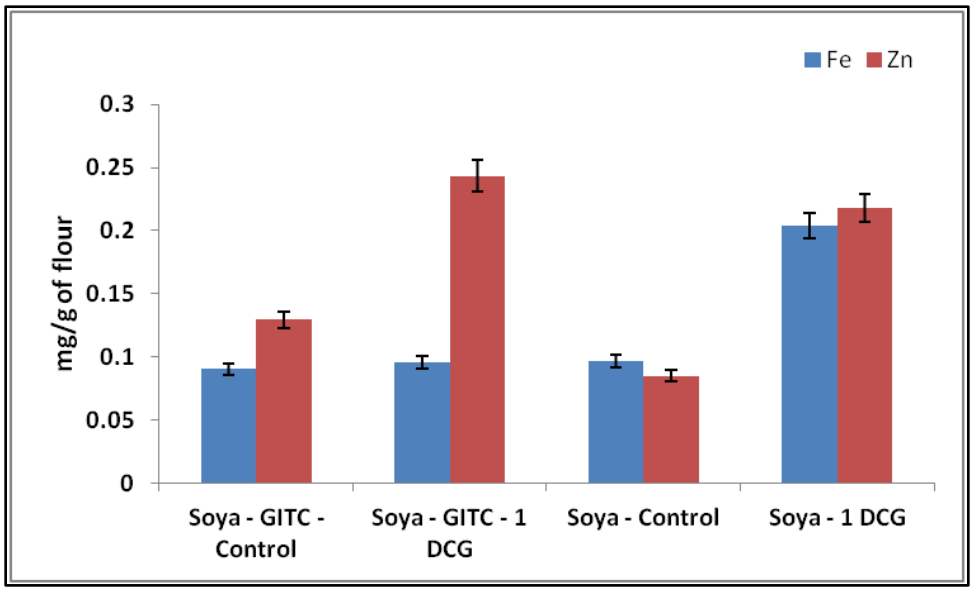
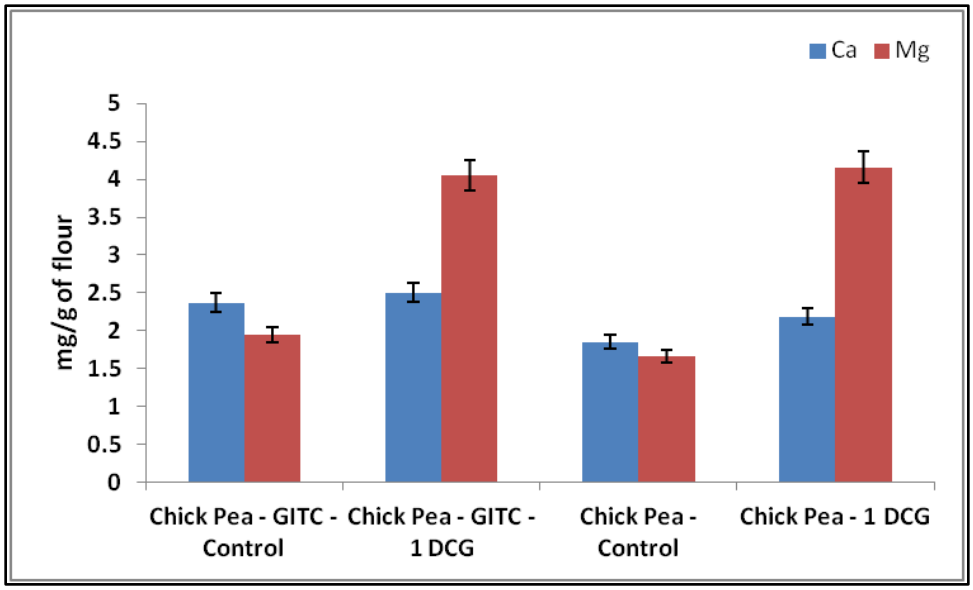


Figure 3b – Total mineral content of Ca, Mg, Fe and Zn in Soya at GIT conditions and undigested conditions with and without enzyme (1 DCG cells)



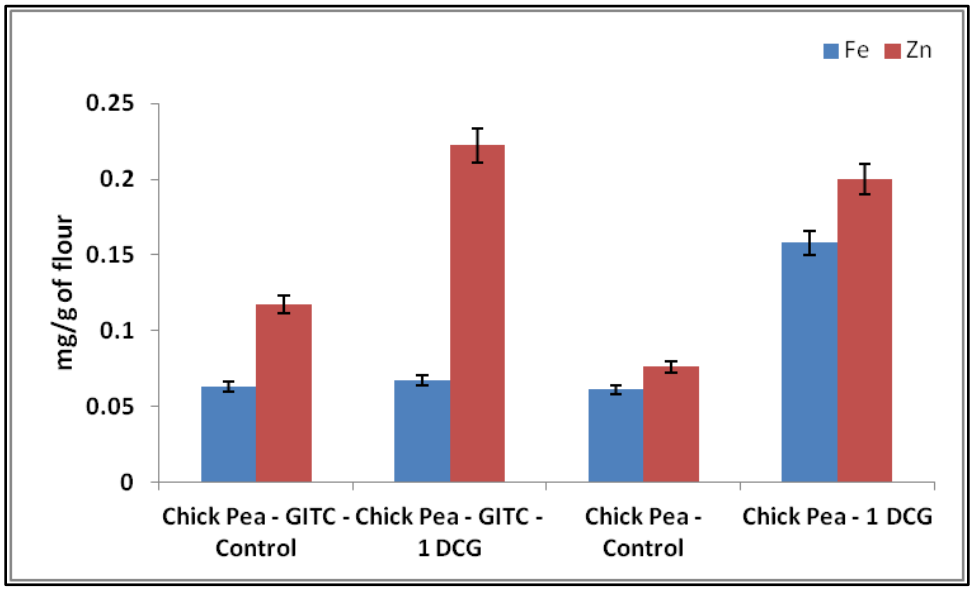


Figure 3c – Total mineral content of Ca, Mg, Fe and Zn in Chick Pea at GIT conditions and undigested conditions with and without enzyme (1 DCG cells)

4.3.5. Probiotic properties

4.3.5.1. Tolerance to bile and harsh GIT acidic conditions

The *S.cerecisiae* NCIM 3662 has shown strong growth on all concentrations of MRS broth supplemented with 0.5, 1.0, 1.5 or 2.0% porcine bile extract. *S.cerecisiae* NCIM 3662 can tolerate and survive at artificial gastric fluid media conditions at pH-2 and pH-3 with and without Urea. At pH-2 cells can just survive up to 48h and at pH-3 cells shown active growth for 2 days. In pH -2 minimum of 10^4 cells are survived till 48h and in pH – 3 minimum of $1.7*10^5$ cells survived (CFU data was not shown). The growth pattern of NCIM 3662 at low pH conditions was determined by taking absorbance at 600 nm shown in Fig 4. For any organism which can be used as a probiotic organism it should tolerate different stress conditions, GIT conditions like tolerance to bile salts present in the human body (0.5% - 2%) and acidic pH conditions of the gut (pH 2 – pH 3). These factors are considered to be important factors for choosing probiotic organisms. According to Pedersen et al. (2004), only a few strains can tolerate the porcine bile salt concentration up to 2% like *Lactobacillus pontis* strains. NCIM 3662 can tolerate stomach bile concentration up to 2%, which are better than the strains of *L. panis* (DAF 1) and *L. amylolyticus* (DAF 262) (Bohn et al. 2008) and *L.brevis* (Aarti et al. 2017). Strains like *L.amylolyticus* didn't survive at pH – 2.5. In case of NCIM 3662 strain, it can tolerate to harsh GIT conditions and better than the reported *L.amylolyticus* and *L.brevis* (6.47 log CFU/ml) at pH 3 (Aarti et al. 2017).

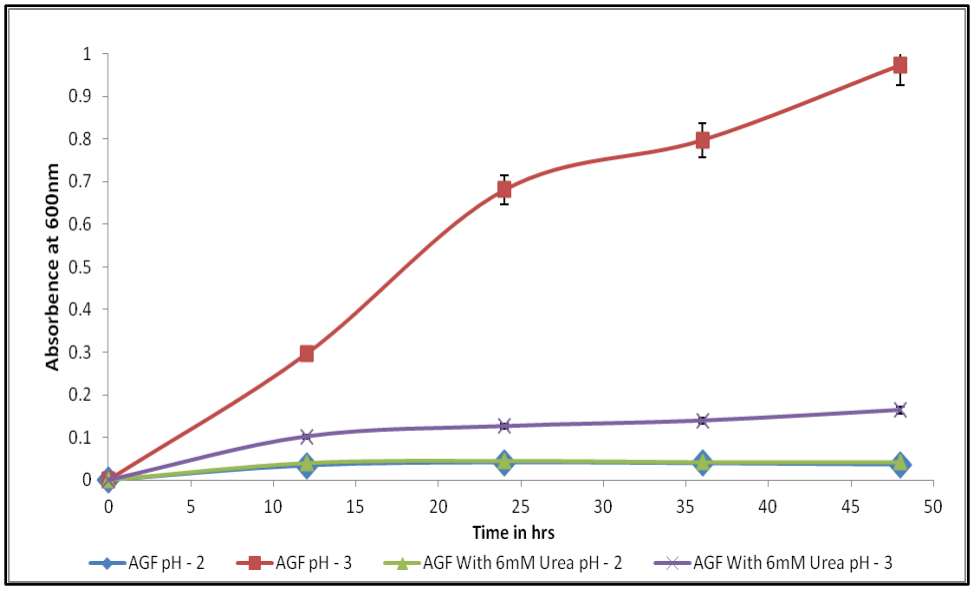


Figure 4: Determination artificial gastric fluid tolerance of NCIM 3662

4.3.5.2. Surface Hydrophobicity (SHb), Autoaggregation (AAg) and coaggregation

S.cerecisiae NCIM 3662 has shown highest surface hydrophobicity (SHb) with the solvent chloroform of 32.14%. Remaining solvents like Ethyl acetate, N-hexadecane, xylene have shown less hydrophobicity of 14.79%, 10.89% and 6.14% respectively as shown in Fig 5a. Based on these values, *S.cerecisiae* NCIM 3662 strain shown high adhesion with acidic solvent chloroform and low adhesion with basic solvent ethyl acetate, xylene. AAg percentage of *S.cerecisiae* NCIM 3662 at different time intervals were calculated and shown in Fig 5b. Based on AAg ability, isolates were classified into three groups, i.e., high (^{H}AAg,[70 % AAg), medium (^{M}AAg, 20–70 % AAg), and low (^{L}AAg, <20 % AAg). According to the above values, NCIM 3662 shows high AAg capability in all time intervals at both the temperatures. Coaggregation ability of NICM 3662 strain at different time

intervals are calculated and shown in Fig 5c. *Staphylococcus aureus and Salmonella enterica* are shown very less coaggregation ability with the strain (Data not shown). NCIM 3662 has shown average level of coaggregation ability with *E.coli* maximum of 17.07% after 24 h. The adhesion ability of probiotic organisms can express in terms of its cell surface hydrophobicity with the different hydrocarbons. In compliance with other reports *S.cerecisiae* NCIM 3662 strain shown average adhesion with acidic solvent chloroform 32% was comparable to *L.brevis* 35% (Aarti et al. 2017) and low adhesion with basic solvent ethyl acetate. As per Andrabi et al. (2016), Autoaggregation (AAg) has its significance in the human gut because of its link with adherence properties. AAg allows it organism to reach the sufficient cell number for its functions in the gut. NCIM 3662 shows high Autoaggregation capability is commensurate with already reported AAg values of *Lactobacillus* strains (Pedersen et al. 2004, Aarti et al. 2017). Coaggregation ability is one of the major attributes for probiotics because it plays important role in the removal of pathogens from the body. NCIM 3662 has shown good coaggregation ability with *E.coli.*

NCIM 3662 can grow well at microaerophilic conditions and co-culturing ability with other *Lactobacillus* strains (data not shown). The organism should be able to survive at microaerophilic conditions and should not affect the gut microbiota by killing other probiotic organisms, because all animal and human gut conditions are microaerophilic in nature, and other probiotic organisms will maintain gut microbiota healthy, and these factors are major criteria for selection of probiotic organism. NCIM 3662 showed good growth in microaerophilic conditions and co-culturing ability with other *Lactobacillus* strains.

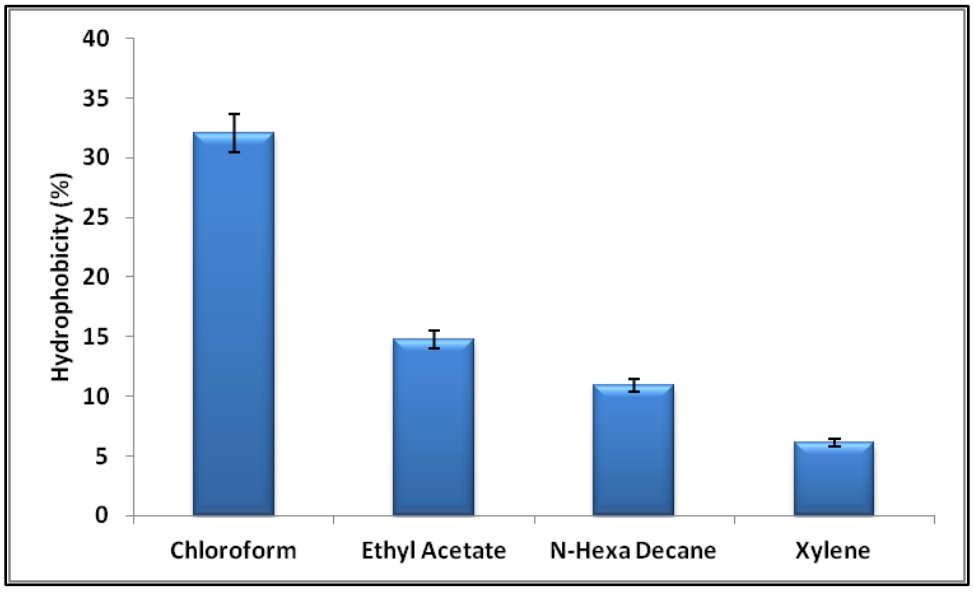

Hydrophobicity (%)
40
35
30
25
20
15
10
5
0
Chloroform
Ethyl Acetate
N-Hexa Decane
Xylene

Fig 5a

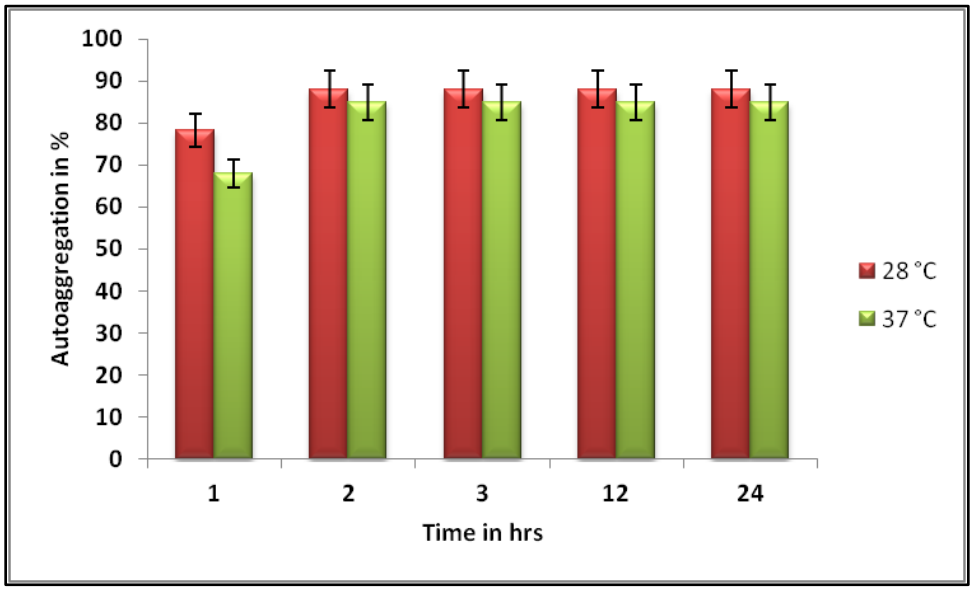

Autoaggregation in %
100
90
80
70
60
50
40
30
20
10
0
1
2
3
12
24
Time in hrs
28 °C
37 °C

Fig 5b

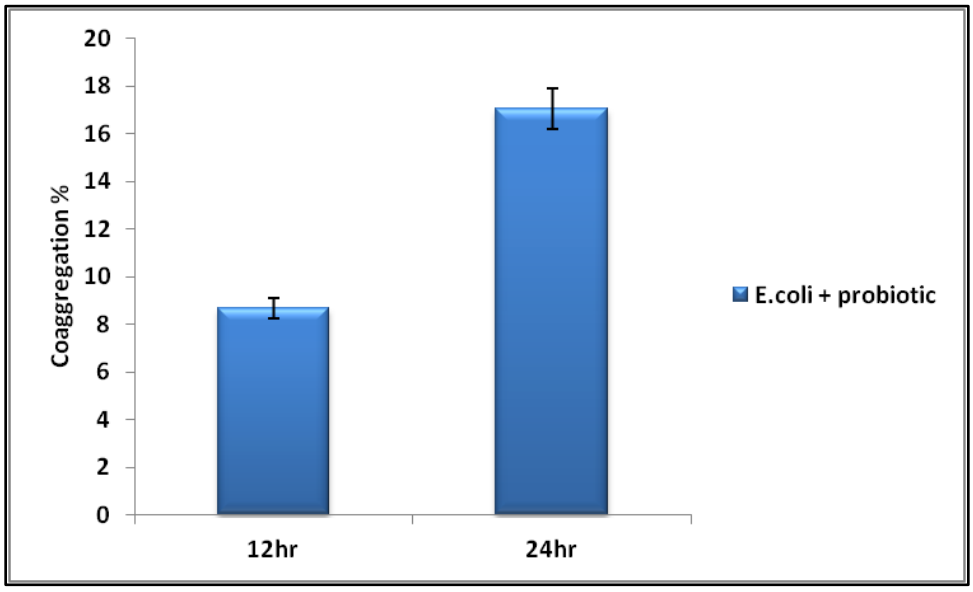


Fig 5c

Figure 5: Different Probiotic properties of NICM 3662

Fig 5a – Determination of percentage hydrophobicity with different organic solvents for NICM 3662, **Fig 5b** – Determination of percentage Autoaggregation for NCIM 3662, **Fig 5c** – Determination of percentage coaggregation for NCIM 3662**.**

4.3.5.3. BSH production

S. cerevisiae NCIM 3662 strain has the ability to degrade conjugated bile salt sodium taurocholate. BSH activity and specific activity of *S. cerevisiae* NCIM 3662 was measured at different media conditions and temperatures have been investigated. Intracellular activity of NCIM 3662 was high with MRS medium with 0.5% phytic acid at both the temperatures (0.93 IU/ml) and activity low with MRS medium with oxgall at both the temperatures (0.08 IU/ml). Specific activity was high for MRS medium with porcine 0.3% (0.95 IU/mg) and low for MRS broth with oxgall and phytic acid (0.26 IU/mg). Cellbound BSH activity of NCIM 3662 was high for MRS medium with 0.5% phytic acid (19.5 IU/DCG) and low for MRS broth with oxgall and phytic acid (2.13 IU/DCG). BSH cell-bound, intracellular

activity and specific activities at different media conditions are mentioned in Figure 6 a, b. The *S. cerevisiae* NCIM 3662 strain producing BSH intracellular enzyme activity is comparable with some of the probiotic *Lactobacillus* strains (Liong and Shah (2005)). But in specific activity and cell-bound activity (data not shown) point of view, NCIM 3662 can match with some of the strains reported activity by Liong and Shah (2005), like *L.acidophilus* ATCC4357 (0.64 IU/mg) and *L.casei* ATCC 15820 (0.66 IU/mg)**.** So, *S. cerevisiae* NCIM 3662 strain has the ability to degrade conjugated bile salt sodium taurocholate successfully. These all characteristics made NCIM 3662 as a strong probiotic competitor with already reported probiotic strains.

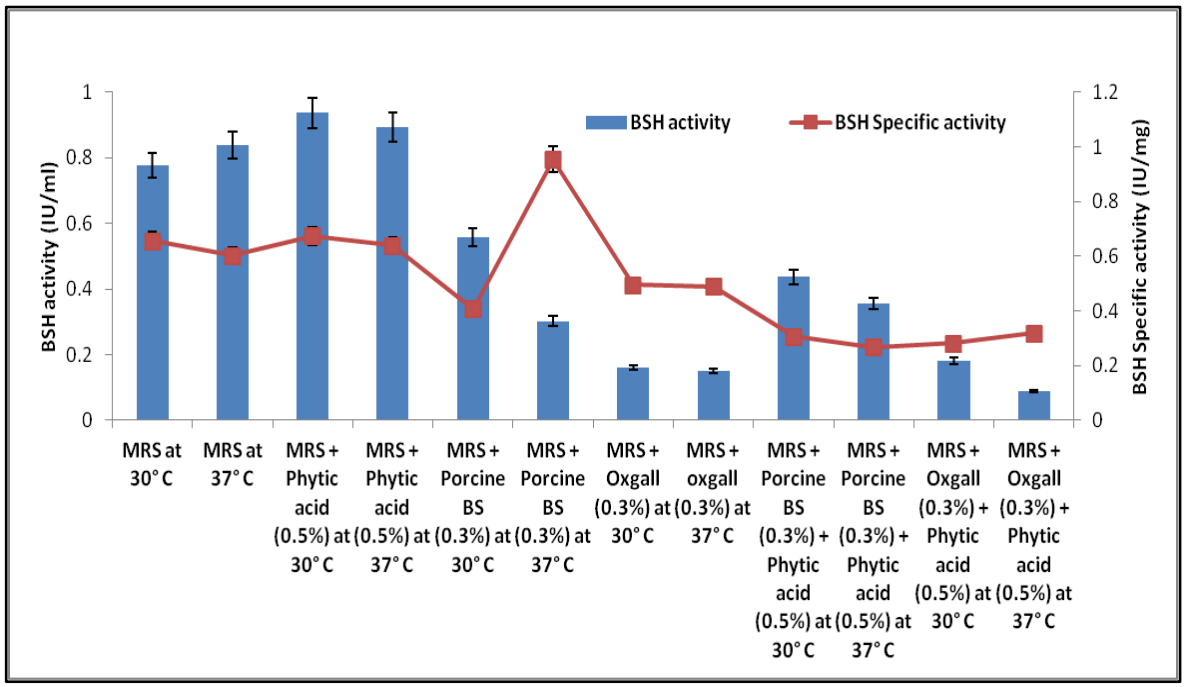


Fig 6a

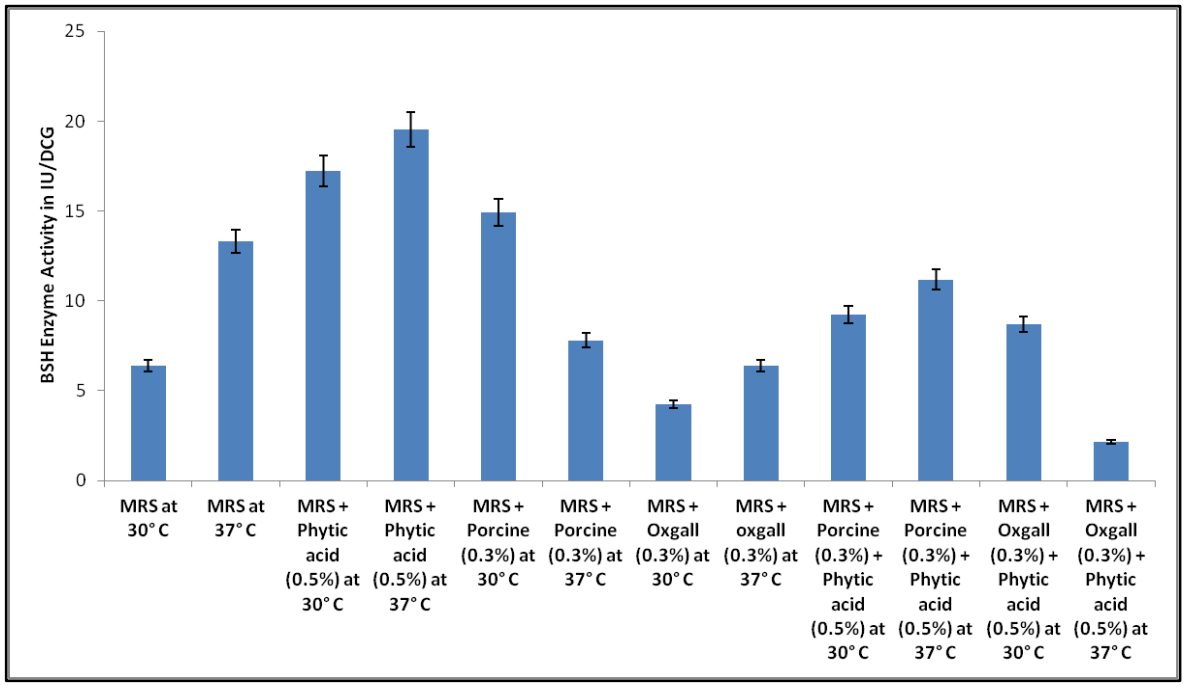


Fig 6b

Figure 6: Determination BSH activity from NICM 3662 in different media conditions

Fig 6a – NCIM 3662 Intracellular and specific activity of BSH at different media conditions, **Fig 6b** – Cellbound BSH activity of NICM 3662 at different media conditions.

NOTE: In all figures, error bars represent standard error of the experiment.

4.4. Conclusion:

Cell-bound phytase producing soil isolate *S.cerevisiae* NCIM 3662 was subjected to statistical media optimization using MGYP media and observed enhanced phytase activity up to 4 folds after PBD and OVAT methods. The process has been scale-up up to 10 L fermenter with increased productivity of 6.4 IU/DCG/h. *S.cerevisiae* has also exhibited the probiotic properties like tolerance to artificial gastric juice conditions, hydrophobicity, Autoaggregation, coaggregation and BSH activity. *S.cerevisiae* phytase enzyme has totally dephytinized the phytate content in functional foods and feed ragi, soya, chickpea and animal feed in 2 h. 1 DCG of cells can also enhance the mineral concentration like Ca, Mg, Fe and Zn in both buffer and GIT conditions in above mentioned functional foods. Therefore, wild-type soil born *S.cerevisiae* could be a good source for isolating phytase producing and having probiotic like beneficial properties that could be advantageous for nonruminant animals and humans with reference to mineral bioavailability to overcome nutrition-related challenges.

Parts of the results of experiments mentioned in this chapter are published in:

Puppala, K. R., Kumar, R., Khire, J., & Dharne, M. Dephytinizing and probiotic potentials of *Saccharomyces cerevisiae* (NCIM 3662) strain for the amelioration of nutritional quality of functional foods. *Probiotics and Antimicrobial Proteins*. (Accepted - https://doi.org/10.1007/s12602-018-9394-y).

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Chapter 5

Conclusion and Future prospective

5.1. The whole study can be summarized as follows:

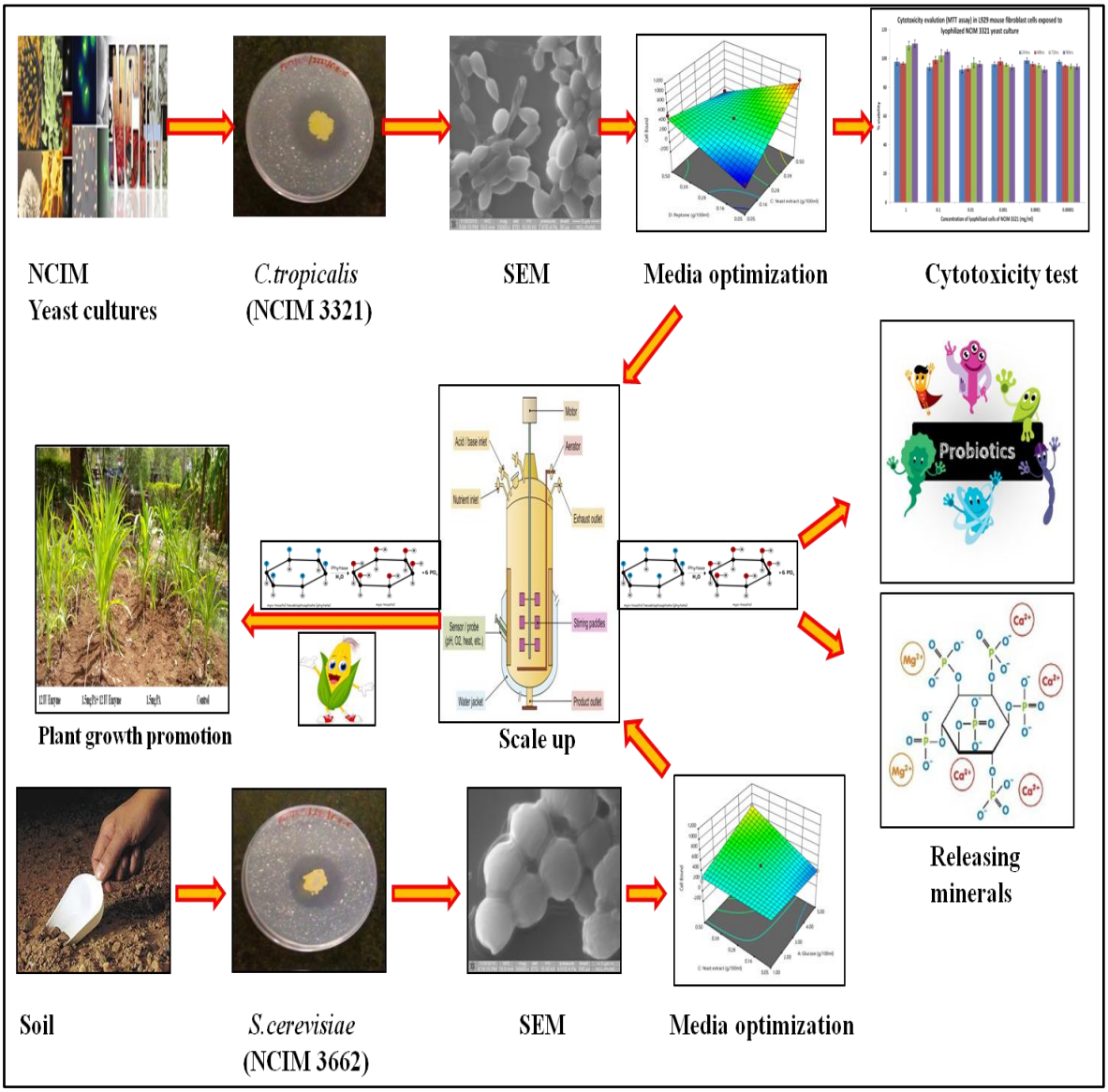

Figure 1: Pictorial summary of the whole study

5.2. Conclusions:

- Screening of phytase producing yeasts from NCIM collection and from soil led to the isolation of highest phytase producing strains as comparable with other previously reported native yeast strains.
- Phytase producing non-pathogenic yeast strains were identified as *Candida tropicalis* and *Saccharomyces cerevisiae* by morphological, biochemical and molecular methods.
- Process development for higher phytase productivity by using different statistical media optimization methods and scale up the process to 10 L was successfully achieved.
- The productivity of phytase enzymes produced by *Candida tropicalis* in optimized media was five folds higher than the all reported native yeast strains.
- Plant growth promoting (PGP) potential of *Candida tropicalis* extracellular phytase was successfully evaluated in lab scale and field level with enhanced yield using maize as a test crop.
- Successfully scaled up and evaluated *Saccharomyces cerevisiae* cell-bound phytase characteristics and probiotic properties. This is the major report of yeast with phytase activity and probiotic properties.
- Cell-bound phytase producing *Saccharomyces cerevisiae* has enhanced mineral availability in functional foods after limited period treatment and dephytinized the functional foods.
- *Saccharomyces cerevisiae* producing cell bound phytase to enhance the bioaccessibility of important minerals in functional foods like ragi, soya and chickpea at invitro gastrointestinal conditions.

5.3. Future prospectives:

- Phosphorus is limiting nutrient for crop production and it has been estimated that the world reserves of inexpensive rock phosphate may be depleted by 2050. Thus, there is a need for alternative P sources for crop production. Microbial phytases are better alternative supplements for P.
    - *Candida tropicalis* producing extracellular phytase shown a successful plant growth promotion activity at field level. Thus, a phytase based formulation has to be developed for sustainable agriculture and has to be check for its effect on plant growth at large scale in agricultural lands and various geographical areas.

- *Saccharomyces cerevisiae* has shown promising results as a probiotic organism. Thus, a phytase based probiotic formulation can also be developed and can check its effect on animals and humans after completion of total cytotoxicity studies.

- Cell-bound phytase producing *Saccharomyces cerevisiae* direct cells can be used in poultry feed to decrease phytic acid content in the cereal-based animal feed. However, to know the enzyme potential in animal feed, large-scale field level trials will be required along with encapsulation for protection from enzymatic attack in digestive tracts.

- Cell-bound phytase producing *Saccharomyces cerevisiae* direct cells can be used for bread making instead of commercial baker's yeast. However, the comparison required for bread texture and taste.
- Further, increase the phytase yield by using recombinant DNA technology.

Publications and Patents:

- **Puppala, K. R**., Naik, T., Shaik, A., Dastager, S., Kumar, R., Khire, J., & Dharne, M. (2018). Evaluation of *Candida tropicalis* (NCIM 3321) extracellular phytase having plant growth promoting potential and process development. *Biocatalysis and Agricultural Biotechnology*, *13*, 225-235. (https://doi.org/10.1016/j.bcab.2017.12.013)
- **Puppala, K. R**., Kumar, R., Khire, J., & Dharne, M. (2018). Dephytinizing and probiotic potentials of *Saccharomyces cerevisiae* (NCIM 3662) strain for amelioration of nutritional quality of functional foods. *Probiotics and Antimicrobial Proteins*. (Accepted - https://doi.org/10.1007/s12602-018-9394-y).
- Dharne, M. S., Khire, J, S., **Puppala K. R**., A composition and a process for preparing phytase with improved enzyme activity. (Indian patent Published 23rd March 2018, Application number: 201611031947).
- Dharne, M. S., Khire, J, S., **Puppala K. R**., Dephytinizing and probiotic potentials of *Saccharomyces cerevisiae* (NCIM 3662) strain for amelioration of nutritional quality of functional foods (Provisional Patent applied May/June 2018 with NCL patent no INV-2018-007)

Posters presented:

- Poster presentation entitled "Isolation and characterization of novel phytase producing and plant growth promoting *Streptomyces sp*" in the **55th Annual conference of Association of Microbiologists of India and International conference on Empowering Mankind with Microbial Technologies** held on November 12-14, 2014 at Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu.
- Poster presentation entitled "Isolation and characterization of novel phytase producing and plant growth promoting *Streptomyces sp. NCIM 5533*" in the **Science day held on February 25-26, 2015** at CSIR – National Chemical Laboratory, Pune, Maharashtra.
- Poster presentation entitled "Statistical optimization of medium components for improved phytase production by soil isolated yeast" in the **56th Annual conference of Association of Microbiologists of India and International conference on Emerging Discoveries in Microbiology held on December 7-10, 2015** at School of Life Sciences, Jawaharlal Nehru University, New Delhi.
- Poster presentation entitled "Screening and Media Optimization Studies for Phytase Producing Yeast and its Application in Plant Growth Promotion" in the **Science day held on February 25-26, 2016** at CSIR – National Chemical Laboratory, Pune, Maharashtra.
- Poster presentation entitled ""Dephytinizing and probiotic potentials of *Saccharomyces cerevisiae* (NCIM 3662) strain for amelioration of nutritional quality of functional foods" in the **Science day held on February 25-26, 2018** at CSIR – National Chemical Laboratory, Pune, Maharashtra.