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1 **Current research and future perspectives of phytase bioprocessing**

2 K. Bhavsar[1], J. M. Khire[1*]

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5 [1]NCIM Resource Center, National Chemical Laboratory, Pune 411 008, INDIA

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8 [*]Corresponding author: Dr. J. M. Khire

9 Scientist

10 NCIM Resource Center

11 National Chemical Laboratory

12 Pune 411 008

13 INDIA

14 Ph. 091-020-25902452

15 Fax: 091-020-25902671

16 Email: jmkhire@yahoo.com, jm.khire@ncl.res.in

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25 **Abstract**

26 Phosphorus is one of Mother Nature's paradoxes as it is Life's bottleneck for subsistence on earth, but at
27 same time detrimental in surplus quantities in an aquatic environment. Phosphorus cannot be
28 manufactured, though fortunately it can be recovered and reused. The only way to avert a supply crisis is
29 to implement the "3 R's" of sustainability: "Reduce, Reuse and Recycle."
30 Phytase is likely to play a critical role in dephosphorylation of antinutritional and indigestible phytate, a
31 phosphorus locking molecule, to digestible phosphorus, calcium and other mineral nutrients in coming
32 years. Hence efforts are required to produce cost effective phytase with fast upstream and economic
33 downstream processing as the current available process is expensive and time consuming. This review
34 summarizes the present state of methods studied for the phytase bioprocessing. Production, extraction and
35 purification incur a large cost in product development. In addition the process has several limitations,
36 such as, dilute concentration of enzyme, extensive downstream procedures and treatment of generated
37 effluents. But these approaches are currently employed due to lack of alternative methods. Thus there is a
38 clear need for efficient, scalable and economical process for phytase production and bioseparation and
39 improvements are especially needed with regard to yield, purity, and energy consumption. Perspectives
40 for an improved bioprocess development for phytase are discussed based on our own experience and
41 recent work. It is argued that optimization of production techniques, strain improvement and liquid liquid
42 extraction deserves more attention in the future.

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50 **1. Introduction**

51 The biogeochemical cycling of nonrenewable and biocritical element phosphorus(P) is a very slow
52 process in nature.[1] It is a vital mineral important for bone and tissue growth and is therefore the third most
53 expensive nutrient in poultry production subsequent to energy and protein. Despite its low abundance, the
54 importance of P in biological system is lucid. P reserves are present in few regions with others entirely
55 dependent on import. India is the largest consumer of phosphate fertilizers and the demand continues to
56 increase due to rising population, escalating demand for meat rich diets and bioenergy crops.[2]

57 Plants store P in the form of phytate (inositol 6-phosphate) carrying 6 phosphate groups. But this bound P
58 (60-70%) present in seed grain as phytate is unavailable to mono-gastric animals, as they lack intrinsic
59 phytase activity. Phytate being negatively charged chelates metal ions and reducing energy uptake and
60 behaves as antinutrient. [3] As P is important macronutrient for growth, the animal diets are customarily
61 supplemented with surplus quantities of inorganic P supplements that ultimately lead to nutrient
62 enrichment in water bodies causing eutrophication. So sarcastically although P is a biocritical element
63 and at the same time a pollutant for living beings. The modern P cycle is atypical due to intervened
64 agriculture and human activities that affect the ecosystem structure and the impacts are detrimental and
65 hard to rescind.[4] Only 10% of phosphorous ends in food production while 90% is lost due to resource
66 mismanagement. Measures for closing the loop of broken P cycle involve strict legislation and norms for
67 discharge of P effluents, human interference and decomposition of underutilized phytate. But at the
68 current usage and extraction, a price hike in synthetic fertilizers is inevitable. These factors have currently
69 led to the use of microbial phytase in animal feed.[5]

70 Use of phytase in animal feed will seize the anti-nutritional effects of phytate, decrease environmental
71 pollution, increase availability of starch, protein, amino acids, calcium and P and abolish the surplus
72 addition of inorganic phosphate in animal feed. They are also imminent candidates for production of
73 special isomers of different lower phosphate esters of myo-inositol, some of which are considered to be
74 pharmacoactive and important intracellular secondary messengers.[6]

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75 The FDA (The Food and Drug Administration) has approved “generally recognized as safe (GRAS)”
76 petition for use of phytase in food, and it has been marketed as an animal feed enzyme in US since 1996.
77 All these factors have concurrently made it as the third largest feed enzyme. Although, a limited number
78 of phytases have been reported and studied, our scientific knowledge of phytases has yet to yield a
79 solution to meet the nutritional and environmental requirements that a real-world solution demands. The
80 major hurdles hindering the exploitation of the repertoire of enzymatic processes are, in many cases, the
81 high production costs and the low yields obtained. Several reviews on phytase have focused on
82 production, biochemical characters, biotechnological applications, crystal structure, directed evolution
83 and protein engineering. This review describes the state of art scenario for upstream and downstream
84 processing of phytase and its application. Upstream processing includes type of fermentation, choice of
85 strain, and improvement of strain or process and bioreactor design followed by downstream processing
86 which involves separation, purification and formulation of the end product (Fig 1).

87 *1.1 Phosphorus paradox*

88 Phosphorus (P) a nonmetal element of the nitrogen group (group 15) of the periodic table is not found as
89 free element due to high reactivity. It is essential to all known life forms and is the second most abundant
90 mineral in the human body, surpassed only by calcium. P is Life’s bottleneck, but ironically due to
91 mismanagement and inadequate legislative norms it acts as as pollutant resulting in eutrophication leading
92 to algal blooms (Fig 2). Excess/ Less phosphate also lead to diarrhoea and calcification (hardening) of
93 organs and soft tissue, Hypophosphatemia, Osteomalacia, Anorexia and Pica. Peak P by 2030 is
94 suggested to occur due to depletion of current high-grade reserves eventually increasing the cost of
95 phosphate rock by 800% in 2008.[7]

96 P is one of nonrenewable resources which cannot be produced, re-grown or regenerated although
97 fortunately unlike oil it can be recovered and reused over and over again. The world’s supply of
98 phosphate rock is drawn down rapidly at an alarming rate. The P situation has many similarities with oil,
99 yet unlike oil, there is no substitute for P in food production.[8] It can be seen that developing countries

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100 especially India is the largest consumer and is entirely dependent on P import for food production Fig 3A.
101 While all farmers need access to P, just 5 countries control around 90% of the world's remaining
102 phosphate rock reserves including China, the US and Morocco (which also controls Western Sahara's
103 reserves) Fig 3B.[9]
104 Phosphate rock is one of the most highly traded commodities on the international market and its crushed /
105 processed fertilizer is generally used for food production. Phosphogypsum is a toxic, radioactive
106 byproduct of P processing and is a future threat to ground water contamination. Crushed/unprocessed
107 rock contains Uranium and thorium which contribute to soil radioactivity and is currently been done in
108 European countries, India (largest P consumer) and Australia.[10] There is a need of 3 R's i.e. Reduce, reuse
109 and Recycle for maintain the sustainability of P for future generations. The above reason raises concern
110 for depleting phosphate reserves and current research is directed to reuse and recycle P. Phytase can
111 provide an alternative option to reduce the use of phosphorous by hydrolyzing phytate, the P locking
112 molecule.
113 *1.2 Phytate*
114 Phytate is the principal storage form of P, inositol, and variety of minerals in plants, representing
115 approximately 75–80% of the total P in plant seeds. Phytic acid nears six phosphate groups on one six-
116 carbon molecule with low molecular weight of 660 and molecular formula $C_6H_{18}O_{24}P_6$. On the basis of
117 Andersons structure[11], the systematic name for phytic acid is myo-inositol-1,2,3,4,5,6-hexakisphosphate.
118 Phytate-P represents 50-82% of total P in cereals and oilseed meals.[12]
119 Phytate can exist in a metal-free form and in metal–phytate complex at acidic and neutral pH; respectively
120 in which the latter form binds with divalent metal cations mostly Mg^{2+} and Ca^{2+} .[13] Table 1 presents an
121 overview of the negative interactions of phytate with nutrients and the mode of actions for the negative
122 effects of phytate. The bioavailability of P and cations (Ca^{2+}, Fe^{2+}, Zn^{2+} and Mg^{2+}) is reduced due to
123 phytate, a P locking molecule and chelator. The after effects of unutilized phytate are more appalling
124 leading to eutrophication and algal blooms. [18]

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125 The phytate hydrolysis is either enzymatic or non-enzymatic wherein the latter happens under high
126 temperature conditions. Phillippy et al [19] studied the hydrolysis of phytic acid and found that at pH 1.0,
127 2.0, 4.0, 6.0, 8.0, and 10.8, the percentages of phytate decomposed were 67.7, 76.8, 89.6, 81.9, 65.8, and
128 45.1%, respectively. Enzymatic phytate hydrolysis by phytase catalyse the sequential release of
129 orthophosphate groups from the inositol ring of phytic acid to produce free inorganic P, along with a
130 chain of intermediate myo-inositol phosphates (inositol pentaphosphate to inositol monophosphate).
131 Phytase not only releases the P from plant-based diets but also makes available calcium, magnesium,
132 protein and lipid. Thus, by releasing bound P in feed ingredients of vegetable origin, phytase makes more
133 P available for bone growth and protects the environment against P pollution. [20]

134 *1.3 Phytase*

135 In recent years, considerable efforts have been made to improve nutritive value of animal feedstuff
136 through supplementation with exogenous enzyme. The global market for feed enzymes is definitely one
137 promising segment in the enzyme industry. It was estimated at around $344 million in 2007, and expected
138 to reach $727 million in 2015. Currently used feed enzymes are divided into two main groups, the
139 hemicellulases and phytases. Phytases *myo*-inositol hexaphosphate phosphorhydrolase) hydrolyze phytic
140 acid to *myo*-inositol and inorganic phosphates through a series of *myo*-inositol phosphate intermediates,
141 and eliminate its anti-nutritional characteristics.

142 Four sources: plant phytase, microbial phytase (fungal and bacterial phytase), phytase generated by the
143 small intestinal mucosa and gut-associated micro floral phytase are generally reported. But, phytase
144 activity of animals is negligible compared to their plant and microbial counterparts.[21] Most of the
145 scientific work has been done on microbial phytases, especially on those originating from filamentous
146 fungi such as *Aspergillus ficuum*, *Mucor piriformis* and *Cladosporium* species. Although some plants
147 such as wheat and barley are rich in intrinsic phytase, because of a narrower pH spectrum of activity and
148 low heat stability their phytase activity is less effective than microbial phytases. Additionally, the bio-
149 efficacy of plant phytases was only 40% compared to microbial phytases. The International Union of

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150 Biochemists[22] currently distinguishes between three classes of phytase enzymes depending on the position
151 (3, 6 or 5) on the inositol ring where the dephosphorylation is initiated as shown in Fig 4.
152 However, there are some exceptions: soybean phytase is a 3-phytase[23] and *Escherichia coli* phytase is a 6-
153 phytase.[24] Histidine acid phosphatase (HAP) shows broad substrate specificity and hydrolyzes metal-free
154 phytate at the acidic pH range and produces myo-inositol monophosphate as the final product. Alkaline
155 phytase exhibits strict substrate specificity for the calcium–phytate complex and produces myo-inositol
156 triphosphate as the final product. Alkaline phytases are not a subfamily of HAPs but are indeed novel
157 phytases as seen in Table 2. Despite considerable differences between alkaline phytases and HAPs, only
158 limited knowledge on the biochemical and catalytic properties of alkaline phytases is currently available.
159 More focus has been on acidic phytases because of their applicability in animal feed and broader substrate
160 specificity than those of alkaline phytases. On the basis of their catalytic properties, phytases are
161 classified as HAP, β propeller phytase (BPP), and purple acid phosphatases (PAP).[25] The finger print of
162 phytases and relationship between motif and key active amino acid were investigated using MEME. It is
163 found that plant phytases have distinct mechanism for phytate utilization as compared to animals and
164 microbes.[26]
165 *1.4 Market trend and manufacture*
166 Recent market trends have clearly shown that enzymes have emerged as big feed supplements. Feed
167 enzymes (protease, xylanase, phytase, amylase, cellulase, lipase, β-glucanase) are the newest segment of
168 the $5 billion animal nutrition market, which is increasing fast. Presently, only about 6% of manufactured
169 animal feeds contain enzymes, against 80±90% for vitamins, which is considered as the largest animal
170 nutrition category. Gist Brocades introduced the first phytase product in feed market in 1991, currently
171 known as Natuphos available as powder, granulate, or liquid formulation.
172 Only few of later products introduced from different companies are available as phytase preparations due
173 the varied properties and efficacy (Fig 5). First phytases produced on commercial scale were either
174 derived from fungal strains mutated via standard means or by using recombinant DNA technology.[27] But

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175 effectiveness of these phytase supplements is less due to lack of essential characteristic and so the quest
176 for ideal phytase continues. The phytase that has the desirable characteristics for application in animal
177 feed industry can be called an 'ideal phytase', which should be active in the stomach, stable during animal
178 feed processing and storage, and easily processed by the feed manufacturer for its suitability as an animal
179 feed additive. It should satisfy the following points

180 1. Phytase should not be detected at the end of the small intestine. This is necessary because in this way
181 the phytase produced by genetically modified organisms should not enter the environment.
182 2. It should be effective in releasing phytate-P in the digestive tract.
183 3. It should be stable to resist proteases (trypsin and pepsin)
184 4. It should be able resist inactivation by heat during feed pelleting and storage
185 5. Low cost of production.

186 Finally, a phytase produced in high yield and purity by a relatively inexpensive system is attracting food
187 industries worldwide. It is now realized that any single phytase may never be 'ideal' for all feeds and
188 foods. For example, the stomach pH in finishing pigs is much more acidic than that of weanling pigs.[28]
189 Thus, phytase with optimum pH close to 3.0 will perform better in the former than in the latter. For
190 poultry, an enzyme would be beneficial if it is active over broad pH range, that is, acidic (stomach) to
191 neutral pH (crop).[29] Phytases used for aquaculture application require a lower temperature that is optimum
192 than the swine or poultry.[30] The choice of an organism for phytase production and development is,
193 therefore, dependent upon the target application using directed evolution and protein engineering. All
194 these features are not present within a single phytase, and therefore, based on the sequence of the
195 available phytases, a consensus phytase could be designed.[31] Genetic engineering techniques such as site
196 directed mutagenesis could be employed for further ameliorating the properties. The strategies used for
197 the designing and developing of an ideal phytase are as follows

198 **1.** Immobilization of phytase for application in food, feed and pharmaceutical industry and biosensor.
199 **2.** Active site modification for enhanced thermostability and efficient catalysis of phytase by

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200 incorporating vanadium in active site for peroxidase activity.

201 **3.** Site directed mutagenesis for enhanced phytase thermo stability and protease resistance.

202 **4.** Transgenic expression in plants and animal for improving their nutrition and growth.

203 **5.** Protein engineering of phytase for enhanced thermostability and pH stability.

204 **6.** Scale up for the economical and large scale phytase production.

205 **7.** Understanding the role of glycosylation in phytase stability**.**

206 **2 Microbial Production of phytase**

207 *2.1 Screening and assay*

208 Several screening programmes have been carried out aiming at the isolation of different groups of
209 bacteria yeast and fungi having extracellular phytase activity. Lissitskaya et al[32] screened microorganisms
210 producing phytase using museum and soil samples wherein it was found that moulds metabolized P more
211 effectively than bacteria. Chen developed a bioassay method using washed cells of *Corynebacterium*
212 *glutamicum* as indicator strain for the screening of extracellular phytase producing microorganisms.[33]
213 Gargova et al used a two-step procedure to screen some 200 fungi producing phytase.[34] A simple and
214 rapid method has been described for determining the microbial phytase by determining the inorganic
215 orthophosphate released on hydrolysis of sodium phytate at pH 5.5.[35]. Bae et al developed a method for
216 detecting phytase activity on differential agar media and the disappearance of precipitated calcium or
217 sodium phytate was as an indication of enzyme activity.[36] This technique, however, was unable to
218 differentiate between phytase activity and acid production by ruminal bacteria.

219 The above assay is performed with phytate as substrate and degradation of phytic acid to the amount of P
220 released. But the phytase screening media and assay has limitations. The traditional endpoint assay is
221 time-consuming and well known for its cumbersomeness in addition to requiring extra caution for
222 handling the toxic regents. This method, however, does not give a very detailed picture of the actual
223 mechanism of phytase action and other methods including chromatographic separation followed by

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224 quantification of the lower inositol phosphates are therefore sometimes employed making it time
225 consuming.
226 Phytase kinetics is highly dependent on substrates and reaction conditions, making kinetic investigations
227 of genuine substrates at physiologically relevant conditions an important issue. So a simple, fast and
228 nontoxic kinetic method was developed by Tran et al for high throughput for assaying phytase
229 overcoming the limitations of traditional phytase assay methods. The assay is based on the principle that
230 IP_6 forms stable turbid complexes with positively charged lysozyme in a wide pH range, and hydrolysis
231 of the IP_6 in the complex is accompanied by a decrease in turbidity monitored at 600 nm. [37]
232 *2.2 Production technique*
233 Phytase can be produced from a host of micro-organisms including bacteria, yeasts and fungi and
234 submerged (SmF) as well as solid state fermentation (SSF) have been employed for the production of
235 phytases. SmF has largely been employed as the production technology for commercial phytases.
236 However, in recent years solid state fermentation (SSF) has gained much interest for the production of
237 phytase. Type of strain, culture conditions, nature of the substrate and availability of the nutrients are
238 critical factors affecting the yield and should be taken into consideration for selecting a particular
239 production technique. For example, a filamentous fungus in SmF is exposed to hydrodynamic forces but
240 in SSF the surface of the solid particles acts as the matrix for the culture.
241 Phytase production has been studied under SmF and SSF; literature reports that enzymatic production
242 under SSF has many advantages in comparison to that of SmF. Varied substrates such as wheat bran full-
243 fat soybean flour, canola meal, cane molasses and oil cakes are studied. Among them are the higher titers
244 of enzyme production, extracellular nature of enzyme, and the low protease production.[38] SmF is the
245 method of choice for phytase production due to ease of SmF operation, up scaling and less variability.[39]
246 Several authors have compared phytase productivity values in different fermentation systems trying to
247 explain how the fermentation system affects fungi physiology. In such comparisons have been included
248 important aspects such as medium composition, *A. niger* morphology, and phytase production diffusion

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249 of nutrients, growth patterns, titers of enzymatic productivity culture conditions, type of strain, and nature
250 of substrate.[40] Substrates such as wheat bran full-fat soybean flour, canola meal , cane molasses and oil
251 cakes. SmF and SSF processes have been compared in terms of their suitability for *Bacillus subtilis*
252 US417 phytase production.[41]
253 The effect of light on fungal growth on solid media culture may also act as an index for mycelia
254 fermentation. Understanding the effect of light on mycelia growth on plates may provide important
255 information in the working cultures, which are the liquid cultures for homogeneous growth of the fungus,
256 and solid culture of photo fermentations. Examining the density and shapes of mycelia on plates would
257 save time and reduce costs of media selection, working culture and solid culture.[42] Gene regulation
258 complexity helps organism to grow in adverse conditions but at the same time this presents both problems
259 and opportunities.[43] There is, however, a complex relationship between the morphology of these
260 microorganisms, transport phenomena, the viscosity of the cultivation broth, and related productivity. The
261 morphological characteristics vary between freely dispersed mycelia and distinct pellets of aggregated
262 biomass, every growth form having a distinct influence on broth rheology. Hence, the advantages and
263 disadvantages for mycelial or pellet cultivation have to be balanced out carefully. Because of the still
264 inadequate understanding of the morphogenesis of filamentous microorganisms, fungal morphology is
265 often a bottleneck of productivity in industrial production.[44] There is abundant proof in literature that the
266 product spectrum from SSF is very different from that obtained in SmF. However, the mechanisms
267 underlying these differences are not at all understood. Therefore rational new design of SSF processes to
268 make new products and optimise the production of existing products is not possible.[45] Recently,
269 significant advances have been made in understanding the physical (process engineering) aspects of SSF
270 but the information on physiology and molecular genetics is limited. To obtain an optimized production
271 process, it is of great importance to gain a better understanding of the molecular and cell biology of these
272 microorganisms as well as the relevant approaches in biochemical engineering. Due to low productivities

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273 and lack of ideal characteristic, the quest for discovery of new wild type phytases and improving the
274 existing ones continues.

275 *2.3 Strategies employed for improved phytase production*

276 The production levels of phytase in naturally occurring strains are too low to be economically viable.
277 Improvement in phytase production is achieved mutually by developments in production technology and
278 engineered phytases as discussed below.

279 *2.3.1 Classical Mutagenesis*

280 Strain improvement by mutagenesis and selection is a highly developed technique. It plays a central role
281 in the commercial development of microbial fermentation processes. Mutagenic procedures can be carried
282 out in terms of type of mutagen, and dose to obtain mutant types that may be screened for improved
283 phytase as seen in *A. niger* using physical and chemical mutagenesis.[46]. Several bacterial strains (wild or
284 genetically modified) such as *Lactobacillus amylovorus*, *E. coli*, *B. subtilis*, *B. amyloliquefaciens*,
285 *Klebsiella spp*, etc., have been employed for phytase synthesis. Apart from good yield of phytase enzyme,
286 *A. niger* CFR 335 produces large amounts of dark conidiospores that hamper the extraction of enzyme
287 and cause health risks such as allergic bronchopulmonary aspergillosis if not handled properly. So a strain
288 of *A. niger* CFR 335 with phytase overproduction and lower sporulation rate was developed through UV
289 mutagenesis by Gunashree and Venkateshwaran.[47]

290 Chelius and Wodzinski during the strain improvement studies of *A. niger* NRRL 3135 by UV radiation,
291 isolated a phytase catalytic mutant producing 3.3-fold higher phytase (phyA) than the wild type strain.
292 The production of mutant phyA was highly repressed 60% by the inorganic phosphate (0.006%, w/v),
293 however, their approach was limited by lack of specificity and sensitivity to discriminate between phytase
294 and acid-phosphatase activity during primary screening process.[48]

295 *2.3.2 Genetic improvement via transgenic studies*

296 Although phytases are widely distributed in nature, the production in wild-type organisms is far from an
297 economically viable level. Hence, cloning and expression of phytase genes in suitable host organisms is

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298 necessary in order to reach higher productivities. As the cost effectiveness of phytase production is a
299 major limiting factor for its application, different heterologous expression systems and hosts have been
300 evaluated. These are plants, bacteria, and fungi including yeast. As expected, each system bears some
301 unique advantages, along with certain limitations as seen in Table 2.

302 *2.3.3 Protoplast fusion*

303 Technique of protoplast fusion has great potential for strain improvement and has been applied for varied
304 industrially important microorganisms. Protoplast fusion may be used to produce interspecific or even
305 intergeneric hybrids and is an important tool as it can overcome the limitations of conventional mating
306 systems in gene manipulation.[52] But it is an emerging area in phytase research with few reports of
307 interspecific protoplast fusion between two auxotrophic mutants, *A. niger* CFR 335 ala− and *A. ficuum*
308 SGA 01 val−, isoleu. They have obtained hybrids with high stability, delay in sporulation and enhanced
309 phytase production.[53] Protoplast fusion indeed has potential for strain improvement for enhancing phytase
310 production.

311 *2.3.4 Response surface methodology*

312 The conventional one variable at a time (OVAT) approach is time consuming and laborious as it involves
313 varying a single variable keeping others at constant level. The true optimum value is missed out due lack
314 of interaction of components. An alternative to OVAT is response surface methodology (RSM) as it
315 involves systematic efficient and simultaneous interaction of variables. Optimization is important for
316 maximizing production and yield at the same time minimizing the cost. Krishna and Nokes studied the
317 effect of culture conditions, particularly inoculum age, media composition (wheat bran and full-fat
318 soybean flour) and duration of SSF on the phytase production by *A. niger*.[54] Bogar et al reported phytase
319 production by *A. ficuum* NRRL 3135, *M. racemosus* NRRL 1994 and *R. oligosporous* NRRL 5905 using
320 various substrates such as canola meal, cracked corn, soybean meal, and wheat bran.[55] But the reports are
321 few because of the low productivities and difficulties associated with operating and up scaling SSF
322 conditions.[56] Sunitha et al optimized the medium for recombinant phytase production by *E. coli* BL21

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323 using response surface methodology. A 23 central composite experimental design was used to study the
324 combined effects of the medium components, tryptone, yeast extract and NaCl. The optimized medium
325 with glucose showed a highest phytase activity of 2250 U/l.[57] Phytase production using yeast cultures has
326 generally been carried out in SmF systems. The strains used include *Schwanniomyces castellii*, *Pichia*,
327 *Arxula adeninivorans* and *Candida kruzei*. Galactose and glucose were the preferred carbon sources.
328 Phytase production from *P. anomala* has been extensively studied using response surface methodology.
329 The fermentation technique employed is SmF with glucose and yeast extract as main carbon and nitrogen
330 source widely used. Sreemula et al evaluated 19 strains of lactic acid-producing bacteria of the genera
331 *Lactobacillus* and *Streptococcus* for the production of extra-cellular phytase. A number of them exhibited
332 the enzyme activity in the fermentation medium but *Lactobacillus amylovorus* B4552 produced the
333 maximum amounts of phytase, ranging from 125±146 U/ml in SmF using glucose and inorganic
334 phosphate.[58]

335 *2.3.5 Directed evolution*

336 Engineering of enzymes using directed evolution is successful especially in improving their
337 thermostability and catalytic properties. This involves construction of mutant library through random
338 mutagenesis or in vitro recombination techniques followed by selection of mutant with desired
339 characteristic by a high-throughput screening technique.[59] The desirable mutants are selected and
340 identified by using directional selection methods and excluding mutants of non-interest.

341 A highly active and thermally improved bacterial Ymphytase has been obtained by directed evolution.
342 Ymphytase represents an alternative to fungal phytases for monogastric feed products. A chemically more
343 diverse SeSaM library yielded a thermally more resistant Ym phytase variant with five amino acid
344 substitutions. Mutational analysis showed that the Ymphytase protein has a high robustness towards
345 mutations.[60]

346 Similarly the method of error-prone PCR was used to generate the mutant phytase with better catalytic
347 efficiency than the original type by introducing several substitutions. The structural predictions indicated

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348 that the mutations generated by ep-PCR somehow reorganized or remodeled the active site, which could
349 lead to increasing catalytic efficiency and 61% higher specific activity.[61]
350 To explore the molecular determinants responsible for the thermostability of *Bacillus* phytases, structural
351 analysis and site directed mutagenesis was employed.[62] This will help in rational protein engineering to
352 develop effective phytases.

353 **3 Downstream processing of phytase**

354 Downstream processing involving recovery and formulation incurs 70% of overall production cost of
355 enzyme. This is due to complexity of system and need to maintain biological activity. Phytase technology
356 for separation and purification employing chromatographic process has evolved slowly as compared to
357 production. Most of these approaches were employed for analytical purposes especially for biochemical,
358 molecular and structural characterization. Phytase is susceptible towards inactivation so for enhanced
359 stability, phytase enzymes are often formulated as solid-state proteins produced by spray drying,
360 lyophilization or granulation. A dry formulation greatly reduces the likelihood of chemically and
361 biologically mediated inactivation. So there is growing interest for fast and economic processes which
362 will stimulate research to unlock new insights in phytase down streaming technology. Conventional
363 methods for phytase separation and purification involve pretreatment and chromatographic methods.

364 *3.1 Pretreatment and concentration*

365 Many different concentration and purification steps are required to reach the final end step quality
366 product. Phytases may be intracellular and extracellular so certain pretreatments are required. Depending
367 on location of cell bound enzyme various permeabilization treatments including organic solvents,
368 enzymes, detergents and physical methods are used.[63]
369 Solid liquid separation techniques such as centrifugation and decant are usually used for extracellular
370 phytase separation. The culture filtrate is concentrated by salt precipitation, acetone precipitation and
371 ultrafiltration for various phytases from plants, bacteria and fungi.

372 *3.2 Chromatography process*

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373 Further purification of phytases includes gel filtration, ion-exchange chromatography, affinity
374 chromatography and hydrophobic interaction. One major problem in the purification of phytate-degrading
375 enzymes especially from plants is the separation of phytate-degrading enzymes from contaminating
376 nonspecific acid phosphatases.[64]
377 The recovery and purification of phytase has been achieved through several steps using different
378 techniques. Boyce and Walsh purified phytase from *Mucor hiemalis*, utilizing five steps (ultrafiltration,
379 diafiltration, ion exchange, gel filtration and hydrophobic interaction), achieving 51% recovery and
380 purification factor of 14.1. [65]; Azek et al obtained two phytases from *Rhizopus oligosporus* in five steps
381 (Acetone Fractionation, Mono-S HR 5/50 Cationic-Exchange Chromatography, 16/60 Sephacryl S-200
382 HR chromatography, Mono-S HR 5/50 Cationic-Exchange Chromatography, Mono-Q HR 5/5 Anionic-
383 Exchange Chromatography) with recovery: phytase 1 (1.3%) and phytase 2 (1.6%) and purification factor
384 (75, 46), respectively.[66] *Debaryomyces castellii* phytase was purified to homogeneity in a single step by
385 hydrophobic interaction chromatography. Its molecular mass is 74 kDa with 28.8% glycosylation. Its
386 activity was optimal at 60°C and pH 4.0. The Km value for sodium phytate was 0.532 mM.[67]
387 Phytase generated on citric pulp fermentation by *A. niger* FS3 was purified by cationic-exchange, anionic
388 exchange chromatography and chromatofocusing steps with 6.35% yield.[68] Previous work from Caseys'
389 lab had indicated that extracellular phytase from *A. niger* ATCC 9142 was purified with a purification
390 factor of 24.89-fold and a 26% yield.[69] A phytase from *Bacillus* was purified 124-fold from the culture
391 broth with 15.4% yield, and exhibited an activity of 36.0 U/mg.[70] Li et al reported an extracellular phytase
392 from a marine yeast with a purification factor of 7.2-fold and a 10.4% yield.[71]
393 Three phytases were purified about 14200-fold (LP11), 16000-fold (LP12), and 13100-fold (LP2) from
394 germinated 4-day-old lupine seedlings to apparent homogeneity with recoveries of 13% (LP11), 8%
395 (LP12), and 9% (LP2) referred to the phytase activity in the crude extract. They behave as monomeric
396 proteins of a molecular mass of about 57 kDa (LP11 and LP12) and 64 kDa (LP2), respectively. The

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397 purified proteins belong to the acid phytases. They exhibit a single pH optimum at 5.0. Optimal
398 temperature for the degradation of sodium phytate is 50°C.[72]
399 An extracellular phytase from *A. niger* 11T53A9 was purified about 51-fold to apparent homogeneity
400 with a recovery of 20.3% referred to the phytase activity in the crude extract. Purification was achieved
401 by ammonium sulphate precipitation, ion chromatography and gel filtration. The purified enzyme
402 behaved as a monomeric protein with a molecular mass of about 85 kDa and exhibited maximal phytate-
403 degrading activity at pH 5.0. Optimum temperature for the degradation of phytate was 55°C.[73]
404 *3.3 Liquid Liquid extraction*
405 The application of single step aqueous two-phase extraction (ATPE) for the downstream processing of
406 phytase from *A. niger* NCIM 563, produced under SSF, has been studied and compared with the
407 traditional multi-step procedure involving salt precipitation and column chromatography. High phytase
408 recovery (98.5%) within a short time (3 h) and improved thermostability was attained by ATPE in
409 comparison to 20% recovery in 96 h by chromatography process. The ATPE system consisting of
410 combination of polyethylene glycol (PEG) 6000 and 8000 (10.5%) and sodium citrate (20.5%) resulted in
411 one-sided partitioning of phytase in bottom phase with a purification factor of 2.5.[74]
412 The partition and recovery behavior of phytase, produced by solid-state cultivation utilizing citrus pulp as
413 substrate, was determined in an ATPE composed of PEG–citrate. The highest partition coefficient (14.42)
414 was observed within a 26% (w/w) PEG 400 (g/mol) and a 20% (w/w) sodium citrate at pH 6.0. The
415 independent variables which more influenced on the partition coefficient and recovery were citrate
416 concentration and PEG mass molar, respectively.[75] The results suggest that PEG–citrate ATPE is an
417 interesting and efficient alternative to traditional chromatographic method.
418 *3.4 Immobilization*
419 Immobilization of phytase on natural supports such as allophone is studied using *E. coli* and *A. niger*
420 phytase. The residual activity of immobilized phytase on allophanic and montmorillonite nanoclay

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421 supports was higher under acidic conditions and led to a higher thermal stability and resistance to
422 proteolysis.[76]
423 Production of myo-inositol phosphate isomers is a budding area but is hampered by lack of stability under
424 processing conditions and difficulties to be recovered from reaction mixtures. But this has been overcome
425 by immobilization of the phytases onto Fe_3O_4-magnetic nanoparticles with high operational stability.[77]
426 The major constraint in application of phytase in animal feed is its reduced thermostability at pelleting
427 process. Pelleting stability to some extent is improved by protected formulation and thermostability
428 coatings. Protein or enzyme stabilizers include use of non reducing sugars, organic and inorganic salts
429 and polyols. Granulation involves use of water soluble polymers, fat coating, organic salts and stabilizers
430 for encapsulation of the biologically active part to prevent inactivation at high temperature. But the
431 inactivation of phytase at high temperature still needs to be further investigated.

432 **4 Biotechnological applications of phytase**

433 Since the first commercial phytase product Natuphos® was launched in 1991, the market volume has
434 reached ca. 150 million euros and will likely expand with new applications. The main application is still
435 as a feed supplement to improve P bioavailability in plant feed-stuffs via the enzyme-mediated hydrolysis
436 of phytate. Most importantly, the improved utilization of the phosphate deposits in the feed results in a
437 substantial reduction in the phosphate content in animal manure and hence decreases of phosphate load on
438 the environment in areas of intensive animal agriculture. High dietary P bioavailability reduces the need
439 for supplemental inorganic P such as mono- and dicalcium-phosphate (MCP, DCP).
440 Because of the strong economic growth in China and India along with the oil price hike, the supply and
441 cost of MCP and DCP has become a practical issue. Furthermore, inorganic phosphate is non-renewable
442 resource, and it has been estimated that the easily-accessible phosphate on earth will be depleted in 50
443 years. Thus, phytase is an effective tool for natural resource management of P on a global scale.
444 The ban of dietary supplementation of meat and bone meal, as a cheap source of feed P, in Europe to
445 prevent possible cross-species transfer of diseases such as BSE, has led to a profound change in the feed P

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446 management. This has given phytase a new socio-economic impact as a cost effective alternative to
447 ensure animals to obtain adequate available P from the plant-based diets. Being the major storage form of
448 P in seeds, plant phytate was produced in 2000 at a global yield >51 million metric tons. This amount
449 accounts for approximately 65% of the elemental P sold worldwide as fertilizers.[78] Apparently, phytase
450 can turn the plant phytate into a very valuable resource of P by improving its bioavailability for animal
451 nutrition. Denmark and the Netherlands have imposed regulations to promote the use of microbial
452 phytases.
453 Organic P (Po) hydrolysis by microbial phytases has extensively been considered in diverse
454 biotechnological applications, including environmental protection and agricultural, animal, and human
455 nutrition.[79] Because of the potential value of phytases for improving the efficiency of P use,
456 biotechnology has led the rapid development of the field to its current stage. With the development of
457 heterologous gene expression, large amounts enzymes could be produced at relatively low cost. The
458 importance of phytases as potential biotechnological tools has been recognized in various fields (Table 3).
459 However, only a limited number of phytases have been reported and studied, and our knowledge of the
460 mechanisms and factors regulating phytase activity is limited. Further research into developing new
461 technologies and identifying the most efficient phytases must continue and directed towards application
462 orientation research.
463 *4.1 Phytases in animal nutrition*
464 Monogastric animals such as swine, fish, and poultry show negligible or no phytase activity in the
465 digestive tract. Consequently, phytates cannot be metabolized by the animals, thus creating a need to
466 enhance phosphate and mineral bioavailability via phytase supplementation of animal feed. Of late,
467 phytases are also viewed as environment friendly products, which can reduce the level of phosphate
468 pollution in intensive livestock management areas by avoiding the addition of exogenous phosphate.[80]
469 Undigested phytate of monogastric manure is washed off the farmland that imperils adjacent waterways
470 by eutrophication.[81] The effect of feeding phytase to animals on pollution has been quantitatively

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471 determined. If phytase were used in the feed of all of the monogastric animals reared in the U.S., it would
472 release phosphorus with a value of 168 million U.S dollars and would preclude 8.23 × 104 tonnes of
473 phosphate from entering the environment per annum. The use of phytase as a feed additive has been
474 approved in 22 countries by FDA. [82]
475 During the past two decades, there has been significant increase in the use of phytases as feed additive in
476 pig, poultry, and fish diets. In numerous studies, the efficacy of microbial phytases to release phytate-
477 bound P has been demonstrated in various animals. Phytases were also found to enhance the utilization of
478 different minerals. Phytases from different sources have been evaluated individually and in combination
479 for their efficacy as feed additives in poultry.[83,84,85] Use of both bacterial and fungal phytases together as
480 feed additive would be another promising alternative in improving the phosphorus utilization and
481 alleviation of mineral deficiency, owing to their synergistic activities throughout the gastrointestinal tract
482 of the animals. The use of phytase as a feed enzyme sets certain demands on the properties of the enzyme.
483 Particularly, the enzyme should withstand high temperatures. This is because poultry and pig feed is
484 commonly pelleted, which ensure that the animals have a balanced diet and facilitates the preservation of
485 enzyme-containing product in the feed industry**.** During the pelleting process the temperatures may
486 temporarily reach 90°C**.** The first commercial phytase product, which became commercially available 10
487 years ago, offered animal nutritionists the tool to drastically reduce phosphorus excretion of monogastric
488 animals by replacing inorganic phosphates with microbial phytase. Depending on diet, species, and level
489 of phytase supplementation, P excretion can be reduced between 25 and 50%.[86]
490 *4.2 Phytases in human nutrition*
491 Mineral deficiency of diets, caused by radical changes in food habits, is a major concern for developing
492 countries. Processing and manufacturing of human food is also a possible application field for phytase.
493 Up to now, no phytase product for a relevant food application is on the market. Research in this field
494 focuses on better mineral absorption or technical improvement of food processing. Phytate present in
495 cereal-based and legume-based complementary foods has been found to inhibit mineral absorption.[87] The

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496 human small intestine has limited ability to digest undegraded phytates, resulting in adverse nutritional
497 consequences with respect to metabolic cation imbalances**.** Phytic acid (PA)—containing 12 dissociable
498 protons with pKa values ranging from ~1.5 to 10—is a highly reactive and potent chelator of many
499 mineral ions such as Ca^{2+}, Mg^{2+}, Zn^{2+}, and Fe^{2+}. Phytic acid forms insoluble salts, at normal acidity (pH
500 3.0–6.8), in the human digestive tract, thereby reducing the bioavailability of these critical mineral
501 nutrients for absorption.[88] Mucosal phytase and alkaline phosphatases, even if present in the human small
502 intestine, do not seem to play a significant role in the phytate digestion, while dietary phytase serves as an
503 important factor in phytate hydrolysis.[89] Haros et al investigated the possible use of phytase in the process
504 of bread making. Different amounts of fungal phytase were added in whole wheat breads, and it was
505 shown that phytase is an excellent bread-making improver. The main achievement of this activity was the
506 shortened fermentation period without affecting the bread dough pH. An increase in bread volume and an
507 improvement in crumb texture were also observed.[90]
508 Application of immobilized *E. coli* phytase and fusion protein in dephytinization of soy milk led to 10%
509 increase in release of inorganic phosphate at 50°C relative to free fusion protein.[91] The lowest phytic acid
510 concentration and highest zinc bioavailability index were achieved when S. cerevisiae, L. plantarum, and
511 Leu.mesenteroides were used at 30.0% dough replacement with sourdough. In this study, effects of 8
512 different sourdough starters prepared with Saccharomyces cerevisiae, Lactobacillus plantarum, L.
513 acidophilus, and Leuconostoc mesenteroides were investigated on the phytic acid level and mole ratio of
514 phytic acid to zinc in a traditional Iranian bread (sangak).[92]
515 It is seen that vitamin C, selenium, zinc and iron are deficient in the diet of lactating women in rural
516 central Mexico, albeit moderate pulque drinking appears to ameliorate iron and zinc deficiencies by the
517 presence of phytase from live bacteria in the latter.[93]
518 *4.3 Phytases in aquaculture*
519 A major concern in aquaculture is the utilization of dietary phosphates which critically affects fish growth
520 as well as the aquatic environment. An efficient utilization of feed leading to optimum fish growth serves

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521 as a benchmark of successful aquaculture worldwide. Studies using phytase as feed additive in
522 aquaculture amply establish that phytase supplementation could enhance the bioavailability of P, nitrogen,
523 and other minerals, thereby decreasing phosphorus-load in the aquatic environment.[94,95]
524 The enzyme from phytase producing intestinal bacteria of Atlantic cod can stimulate intracellular head
525 kidney leukocyte activities but not the production of extracellular substances that are involved in
526 antibacterial response. These have implications on the potential use of bacterial phytase as feed
527 supplement to boost cellular immune response of the fish and could be employed as a health management
528 strategy in culture systems.[96] These may have significant impact on the development of feed supplements
529 and health management in aquaculture systems.
530 *4.4 Role of phytases in soil amendment*
531 Phosphorus is an essential plant nutrient that limits agricultural production on a global scale.
532 Approximately 30–80% of the total P in soils is bound in organic form.[97] Phytate constitutes ~50% of the
533 total organic P pool in the soil and is poorly utilized by plant **.** Extracellular phytase activities have been
534 reported under phosphate stress conditions, in diverse plant species, namely, tobacco[98], barley[99]**,** tomato,
535 alfalfa[100]**,** and so on. The ability of plants to use phosphorus from low phosphate or phytate containing
536 media and/or from soil is improved when soil/media are inoculated with microorganisms that possess the
537 ability to exude phytase, or when a purified phytase is added.
538 Root physiological adaptations (i.e. rhizosphere carboxylate content and P-uptake rate) are more
539 important than morphological adaptations (i.e. root length and diameter) to enhance the uptake of P and
540 cations.[101]
541 *4.5 Phytase in plant growth promotion*
542 A novel *Enterobacter cancerogenus* MSA2 is a plant growth promoting gamma-proteobacterium that was
543 isolated from the rhizosphere of Jatropha cucas a potentially important biofuel feed stock plant. MSA2 is
544 the first identification of a plant growth-promoting bacterium which produces ACC deaminase enzyme
545 and shows plant growth promotion with the Jatropha curcas.[102] The effect of fungal phytase on plant

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546 growth at pot and tray level, comparison with commercial fertilizers pertaining to chemical and
547 physiological parameter and as soil amendment was studied. Phytase was efficient in reducing the phytic
548 acid content of soil by about 30% while simultaneously increasing the phytate phosphate availability by
549 1.18-fold.[103]

550 *4.6 Budding applications*

551 Lower phosphoric esters of myo-inositol (mono, bis, tris, and tetrakisphosphates) play a crucial role in
552 transmembrane signaling processes and in calcium mobilization fromintracellular store in animal as well
553 as in plant tissues.[104] Research interest in this field prompted the need for various inositol phosphate
554 preparations. However, chemical synthesis is difficult. In contrast, an enzymatic synthesis has the
555 advantage of high stereospecifity and mild reaction conditions. The use of phytase has been shown to be
556 very effective in producing different inositol phosphate species.

557 Different isomers of *myo*-inositol phosphates have shown pharmacological effects for the prevention of
558 diabetic complications, anti-inflammatory effects[105]**,** and antiangiogenic and antitumor effects[106]**.** *Myo*-
559 inositol phosphates are also known to ameliorate heart disease conditions by controlling
560 hypercholesterolemia and atherosclerosis[107]**,** and also prevent renal stone formation.[108]

561 A single step rapid biocatalytic process of hydroxyapatite and myoinositol intermediates synthesis has
562 several advantages such as advantage of stereo specificity, mild reaction conditions and is cost effective
563 as compared to chemical process.[109]

564 Self-assembly of phytase molecules in Ionic liquid leading to the formation of enzyme capsules is been
565 studied. These capsules act as soft functional templates for the in situ reduction and decoration of metal
566 salts.[110]

567 **5 Future perspectives and new insights**

568 There is a large gap between metabolic and bioprocessing level of microbes especially in case of fungi.
569 There are several reports on phytase production and purification in different fermentation systems which
570 affect microbial physiology and productivity. This includes various aspects such as media composition,

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571 morphology, fermentation system, type of strain and substrate used as seen from Table 4. However, there
572 is no information about structural differences among phytase produced under both systems. Complex
573 microbes especially fungi exploit a wide range on environmental condition, but morphology under varied
574 fermentation system is often a bottleneck in productivity of industrially important desired product. There
575 is abundant proof in literature that the product spectrum from SSF is very different from that obtained in
576 submerged fermentation (SmF). However, the mechanisms underlying these differences are not at all
577 understood.
578 There is a single and first report about structural differences among phytase produced under SSF and SmF
579 by *A. niger* and this study provides basis for explanation of the stability and catalytic differences observed
580 for these three phytase. In fact, only two reports on the comparative production of phytase by these two
581 fermentation processes are available fungal and bacterial (Table 4).
582 More powerful and automated image analysis techniques will aid in morphology engineering and this can
583 provide new insights to the existing "black box" of SSf/SmF biotechnology for phytase production.
584 Strategies such as microparticle addition and osmolality variation will aid in targeted engineering of
585 fungal morphology.
586 Along with microbial production, downstream processing is an essential aspect for phytase bioprocessing.
587 Rapid and economic methods such as liquid liquid extraction are the imminent promising alternatives as
588 seen from Table 4. More efforts are required for development of efficient, scalable and economical
589 process for phytase bioseparation to conquest the techno-economic limitations of conventional
590 downstream processes.
591 The core aim of viable process is to retain the activity during storage and use. Limitations related to
592 phytase formulation and stabilization is the major bottleneck in it industrial application. So techniques
593 such as immobilization and application targeted research will help in solving the problem to some extent.
594 So a focused platform for microbial production, downstream processing and application oriented research
595 will help in developing a integrated technological solution to phytase production. This will present new

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596 insights in biological and engineering facets of phytase producing microbes and reveal a new era in
597 phytase biotechnology.

598 **Conclusion**

599 P is an indispensable resource that has been mismanaged to the point that we are jeopardizing our long-
600 term food and water security. As the need to conserve the world's phosphate reserves increases the role of
601 phytase will broaden. Phytases are now being recognized for their beneficial environmental role in
602 reducing the P levels in manure and minimizing the need to supplement P in diets. The conventional
603 methods for phytase production and purification are economically not viable due to various shortcomings.
604 Hence there is a need for additional and improved strategies will help in developing a robust system for
605 the same. Further application oriented efforts are required to design versatile "second-generation"
606 phytases with wider applicability. Modification and upgradation of enzymatic properties can be achieved
607 through adoption of genetic and protein engineering methods. Combination of fungal and bacterial
608 phytases as feed additives might improve the bioavailability of P and minerals owing to their synergistic
609 activity in animal digestive system. Further insights in development of application oriented phytases will
610 open new era in its bioprocessing and widen the horizons of its applicability and efficiency. New market
611 segments such as aquaculture and agriculture will provide new opportunities for phytase.

612 **Acknowledgement**

613 The author, Ms. Kavita Bhavsar thanks Council of Scientific and Industrial Research, Government of
614 India for the financial assistance. The authors also gratefully acknowledge support and facilities provided
615 by the Center of Excellence in Scientific Computing, National Chemical Laboratory, India.

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RSC Advances Accepted Manuscript

621 **References**

622 1. D. Van Vuuren, A. Bouman and A. Beusen, *Global. Environmental. Change.*, 2010, **20,** 428.

623 2. D. Cordell, A. Rosemarin, J. J. Schroder, A. L. Smit, *Chemosphere.*, 2011, **84**, 747.

624 3. B. Singh and T. Satnarayana, *Physiol. Mol. Biol. Plants.*, 2011, **17**, 93.

625 4. D. L. Childers, J. Corman, M. Edwards and J. J. Elser, *Bioscience.*, 2011, **61**, 117.

626 5. G. L. Cromwell, *J. Anim. Sci.*, 2009, **87**, 778.

627 6. E. J. Mullaney and A. H. J. Ullah, *Cambridge. MA. CABI.*, 2007, 97

628 7. E. M. Bennett, S. R. Carpenter and N. F. Caraco, *Bioscience.*, 2001, **51**, 227.

629 8. D. Cordell, J. Dragert and S. White, *Global Environmental Change.*, 2009, **19**, 262.

630 9. N. Gilbert, *Nature.*, 2009, **461**, 716.

631 10. FAO, *Current world fertilizer trends and outlook to 2016,* 2012, Rome.

632 11. R. J. Anderson, *J. Biol. Chem.,* 1914, **17**, 171.

633 12. P. K. Tyagi and S. V. S. Verma, *Ind. J. Poul. Sci.*, 1998, **33**, 86.

634 13. B. F. Harland and D. Oberleas, In *Phytase in Animal Nutrition and Waste Management*, ed. M. B.
635 Coelho and E. T. Kornegay, Mexico-BASF, 1999, rev 2, p. 69.

636 14. R. Angel, N. M. Tamim, T. J. Applegate, L. E. Ellestad, L. E. and A. S. Dhandu, *J. Appl. Poult.*
637 *Research.*, 2002, **11**, 471.

638 15. P. A. Kemme, A. W. Jongbloed, Z. Mroz, J. Kogut and A. C. Beynen, *Livestock. Prod. Sci.*, 1999,
639 **58**, 107.

640 16. S. E. Rickard and L. U. Thompson, In: F. Shaidi (ed.) *Antinutrients and phytochemicals in food.*,
641 Washington DC:ACS, 1997, p. 294.

642 17. S. Leeson, In *Recent Advances in Animal Nutrition in Australia.*, Armidale, NSW: University of
643 England, 1993, p. 170.

644 18. K. Ole, V. B. Torben, C. F. Claus, *Curr. Opin. Biotechnol.*, 2002, **13**, 345.

645 19. B. Q. Phillippy, M. R. Johnston, S. H. Tao and M. R. S. Fox, *J. Food. Sci.*, 1998, **53**, 496.

RSC Advances Accepted Manuscript

646 20. K. Baruah, N. P. Sahu, A. K. Pal, D Debnath, S. Yengkokpam and S. C. Mukherjee, *J. World.*
647 *Aquacult. Soc.*, 2007, **38**, 238.
648 21. D. Weremko, H. Fandrejewski, T. Zebrowska, K. Han, J. H. Kim and W. T. Cho, *Asian-Aust. J.*
649 *Anim. Sci.*, 1997, **10**, 551.
650 22. IUB, In: *Recommendations of the Nomenclature Committee of the International Union of*
651 *Biochemistry.*, Academic press, New York, 1979, p. 247.
652 23. H. Persson, M. Turk, M. Nyman and A. S. Sandberg, *J. Agric. Food. Chem.*, 1998, **46**, 3194.
653 24. R. Greiner, U. Konietzny and K. D. Jany, *Arch. Biochem. Biophys.*, 1993, **303**, 107.
654 25. E. J. Mullaney and A. H. J. Ullah, *Biochem. Biophys. Researh. Commun.,* 2003, **312**, 179.
655 26. C. M. Fam, Y. H. Wang, C. Y. Zheng and Y F Fu, *Afr. J. Biotechnol.*, 2013, **12**, 1138.
656 27. L. Cao, W. Wang, C. Yang, Y. Yang, J. Diana, Yakupitiyage, Z. Luo and D. Li, *Enzy. Microbiol.*
657 *Technol.*, 2007, **40**, 497.
658 28. A. W. Jongbloed A, Z. Mroz and P. A. Kemme, *J. Anim. Sci.*, 1992, **70**, 1159.
659 29. C. S. Quan, W. J. Tian, S. D. Fan and J. Kikuchi, *J. Biosci. Bioeng.*, 2004, **97**, 260.
660 30. W. W. Riley and R. E, Austic, *Poult. Sci.,* 1984, **63**, 2247.
661 31. M. Lehmann, L. Pasamontes, S. F. Lassen and M. Wyss, *Biochim. Biophys. Acta.*, 2000, **1543**,
662 408.
663 32. T. B. Lissitskaya, V. G. Shmeleva, G. S. Vardoian and V. I. Yakovlev, *Mikologiya I*
664 *Fitopatologiya.,* 1999, **33**, 402.
665 33. J. C. Chen, *Biotechno.l Technol.*, 1998, **12**, 759.
666 34. S. Gargova, Z. Roshkova and G. Vancheva, *Biotechnol. Technol.,* 1997, **11**, 221.
667 35. A. J. Engelen, F. C. Vanderheeft, P. H. G. Randsdorp and E. L. C. Smit, *J. AOAC. Inter.*, 1994, **77**,
668 760.
669 36. H. D. Bae, L. J. Yanke, K. J. Cheng and L. B. Selinger, *J. Microbiol. Meth.,* 1999, **39**, 17.
670 37. T. Tran, R. Kaul, S. Dalsgaard and S. Yu, *Anal. Biochem.*, 2011, **410**, 177.

RSC Advances Accepted Manuscript

671 38. K. P. Bhavsar, V. Ravi Kumar and J. M. Khire, *J. Ind. Microbiol. Biotechnol.*, 2011, **38**, 1407.
672 39. K. P. Bhavsar, P. Shah and J. M. Khire, *Afr. J. Biotechnol.*, 2008, **7**, 1101.
673 40. P. Vats and U. C. Banerjee, *Enzy. Microbiol. Technol.*, 2004, **35**, 3.
674 41. R. Kammoun, A. Farhat, H. Chouayekh, K. Bouchaala and S. Bejar, *Ann. Microbiol.*, 2012, **62**,
675 155.
676 42. C. Cheng, C. Chen, C. Chang and L. Chen, *J. Photochem. Photobiol. B: Biol.*, 2012, **106**, 81.
677 43. D. Lubertozzi and J. D. Keasling, *Biotechnol. Adv.*, 2009, **27**, 53.
678 44. T. Wucherpfennig, T. Hestler and R. Krull, *Microbial. Cell. Fact.*, 2011, **10**, 58.
679 45. K. S. M. S. Raghavarao, T. V. Ranganath and N. G. Karanth, *Biochemical. Eng. J.*, 2003, **13**, 127.
680 46. K. P. Bhavsar, P. Gujar, P. C. Shah, V. Ravi Kumar and J. M. Khire, *Appl. Microbiol. Biotechnol.*,
681 2012, **97**, 673.
682 47. B. S. Gunashree and G. Venkateswaran, *Microbial. Ecol. Health.*, 2009, **21**, 57.
683 48. M. K. Chelius and R. J. Wodzinski, *Appl. Microbiol. Biotechnol.,* 1994, **41**, 79.
684 49. J. Pen, T. C. Verwoerd, P. A. van Paridon, R. F. Beudeker, P. J. M. van den Elzen, K. Geerse, J. D.
685 van der Klis, H. A. J. Versteegh, A. J. J. van Ooyen and A. Hoekema, *Biotechnol.*, 1993, **11**, 811.
686 50. E. Rodriguez, E. J. Mullaney and X. G. Lei, *Biochem. Biophys. Res. Commun.*, 2000, **268**, 373.
687 51. B. Q. Phillippy, M. R. Johnston, S. H. Tao and M. R. S. Fox, *J. Food. Sci.*, 1998, **53**, 496.
688 52. R. V. Murlidhar and T. Panda, *Bioproc. Biosyst. Engg.*, 2000, **22**, 429.
689 53. B. S. Gunashree and G. Venkateswaran. *Enzy. Microbiol. Technol.*, 2010, **46**, 562.
690 54. C. Krishna and S. E. Nokes, *J. Ind. Microbiol. Biotechnol.,* 2001, **26**, 161.
691 55. B. Bogar, G. Szakacs, R. P. Tengerdy, J. C. Linden and A. Pandey A, *J. Ind. Microbiol.*
692 *Biotechnol*, 2003, 30, 183.
693 56. B. Bogar, G. Szakacs, A. Pandey, S. Abdulhameed, J. Linden and R. Tengerdy, *Biotechnol. Prog.,*
694 2003, **19**, 312.
695 57. K. Sunitha, J. K. Lee and T. K. Oh, *Bioproc. Engg.,* 1999, **21**, 477.

RSC Advances Accepted Manuscript

696 58. G. Sreemula, D. S. Srinivasa, K. Nand and R. Joseph, *Lett. Appl. Micrbiol.,* 1996, **23**, 385.

697 59. Y. S. Tian, R. H. Peng, J. Xu, W. Zhao, F. Gao and X. Y. Fu, *World. J. Microbiol. Biotechnol.*,
698 2009, **26**, 903.

699 60. A. V. Shivange, A. Dennig, D. Roccatano, S. Haefner and U. Schwaneberg, *Appl. Microbiol.*
700 *Biotechnol.*, 2012, **95**, 405.

701 61. Y. Liao, M. Zeng. Z. S. Wu, H. Chen, H. Wang, Q. Wu, Z. Shan and X. Han X, *Appl. Biochem.*
702 *Biotechnol.*, 2012, **166**, 549.

703 62. K. Ameny, M. Ali, I. Boukhris, B. Khemakhem, E. Maguin, S. Bejar and H. Chouayekh. *Inter. J.*
704 *Biological. Macromol.*, 2013, **54**, 9.

705 63. A. Bindu, D. Somashekar and R. Joseph, *Lett. Appl. Microbiol.*, 1998, **27**, 336.

706 64. U. Konietzny, R. Greiner and K. D. Jany, *J. Food. Biochem.*, 1995, **18**, 165.

707 65. A. Boyce A and G. Walsh, *J. Biotechnol.*, 2007, **132**, 82.

708 66. M. A. Azeke, R. Greiner and K. Jany, *J. Food. Biochem.*, 2011, **35**, 213.

709 67. M. Ragon, A. Aumelas, P. Chemardin, S. Galvez, G. Moulin and H. Boze, *Appl. Microbiol.*
710 *Biotechnol.*, 2008, **78**, 47.

711 68. M. R. Spier, R. C. Fendrich, P. C. Almeida, M. Noseda, R. Grenier, U. Konietzny, A. L.
712 Woiciechowski, V. T. Soccol and C. R. Soccol, *World. J. Microbiol. Biotechnol.*, 2011, **27**, 267.

713 69. A. Casey and G. Walsh, *J. Biotechnol.*, 2004, **110**, 313.

714 70. B. C. Oh, W. C. Choi, S. Park, Y. O. Kim and T. K. Oh, *Appl. Microbiol. Biotechnol.*, 2004, **63**,
715 362.

716 71. X. Y. Li, Z. Q. Liu and Z. M. Chi, *Bioresourc. Technol.*, 2008, **99**, 6386.

717 72. R. Grenier, *J. Agric. Food. Chem.*, 2002, **50**, 6858.

718 73. R. Grenier, L. C. Siva and S. Couri, *Brazalian. J. Microbiol.*, 2009, **40**, 795.

719 74. K. P. Bhavsar, V. RaviKumar and J. M. Khire, *Proc. Biochem.*, 2012, **47**, 1066.

RSC Advances Accepted Manuscript

720 75. M. L. C. Nevesa, T. S. Porto, C. M. Souza-Mottae, M. R. Spier, C. R. Soccol, K. A. Moreira and
721 A. L. F. Porto, Fluid. Phase. *Equilibria.*, 2012, **318**, 34.
722 76. D. Menezes-Blackburn, M. Jorquera, L. Gianfreda, M. Rao, R. Greiner, E. Garrido and M. L.
723 Mora, *Bioresourc. Technol.*, 2011, **102**, 9360.
724 77. R. Greneir, U. Konietzny, D. Blackburn and M. Jorquera, *Bioresourc. Technol.*, 2013, **142**, 375.
725 78. J. N. A. Lott, I. Ockenden, V. Raboy and G. D. Batten, *Seed. Sci. Res.*, 2000, **10**, 11.
726 79. D. Menezes-Blackburn, M. A. Jorquera, R. Grenier, L. Giamfreda and M. Mpra, *Critical. Rev.*
727 *Environ. Sci. Technol.*, 2013, **43**, 916.
728 80. P. Vats, M. S. Bhattacharya and U. C. Banerjee, *Critical. Rev. Environ. Sci. Technol.*, 2005, **35**,
729 469.
730 81. F. H. Common, *Nature.*, 1989, **143**, 370.
731 82. R. J. Wodzinski and A. H. J. Ullah, *Adv. Appl. Microbiol.,* 1996, **42**, 263.
732 83. N. Chauynarong, P. A. Iji, S. Isariyodom and L. Mikkelsen, *Int. J. Poult. Sci.*, 2008, **7**, 257.
733 84. E. A. I. Elkhalil, K. Manner, R. Borriss and O. Simon, *Br. Poult. Sci.*, 2007, **48**, 64.
734 85. R. L. Payne, T. K. Lavergne and L. L. Southern, *Poult. Sci.*, 2005, **84**, 265.
735 86. E. T. Kornegay, In *Phytase in Animal Nutrition and Waste Management*, ed. M. B. Coelho and E.
736 T. Kornegay, Mexico-BASF, 1999, rev 2, p. 249.
737 87. R. F. Hurrell, M. B. Reddy, M. A. Juillerat and J. D. Cook, *Am. J. Clin. Nutr.*, 2003, **77**, 1213.
738 88. A. J. R. Costello, T. Glonek and T. C. Myers, *Carbohydr. Res.*, 1976, **46**, 159.
739 89. A. S. Sandberg and H. Anderson, *J. Nutr.*, 1988, **118**, 469.
740 90. M. Haros, C. M. Rosell and C. Benedito, *J. Agric. Food. Chem.*, 2001, **49**, 5450.
741 91. M. Ushashree, P. Gunasekaran and A. Pandey, *Appl. Biochem. Biotechnol.*, 2012, **167**, 981.
742 92. M. A. Najafi, K. Rezaei, M. Safari and S. H. Razavi, *Food. Sci. Biotechnol.*, 2012, **21**, 51.
743 93. L. R. Tovar, M. Olivos and Ma. E. Gutierrez, *Plant. Foods. Hum. Nutr.*, 2008, **63**, 189.
744 94. L. C. Nwanna and F. J. Schwarz, *Aquacult. Res.*, 2007, **38**, 1037.

RSC Advances Accepted Manuscript

745 95. J. Vielma, K. Ruohonen, J. Gabaudan and K. Vogel, *Aquacult. Res.*, 2004, **35**, 955.
746 96. C. C. Lazado, C. Marlowe, A. Caipang, S. Gallage, M. F. Brinchmann and V. Kiron, *Fish.*
747 *Physiol. Biochem.*, 2010, **36**, 883.
748 97. A. T. Harrison, In *A Review of World Literature*., Wallingford, 1987, UK: CAB International.
749 98. S. C. Lung and B. L. Lim, *Plant. Soil.*, 2006, **279**, 187.
750 99. G. Anderson, In *The Role of Phosphorus in Agriculture*, ed. F. E. Khasawneh, E. C. Sample and E.
751 J. Kamprath, Madison, WI: American Society of Agronomy, 1980, p. 411.
752 100. M. Li, M. Osaki, I. M. Rao and T. Tadano, *Plant. Soil.*, 1997, **195**, 161.
753 101. L. D. B. Suriyagoda, H. Lambers, M. Renton and M. H. Ryan, *Plant. Soil.*, 2012, **358**, 105.
754 102. C. K. Jha, B. Patel and M. Saraf, *World. J. Microbiol. Biotechnol.*, 2012, **28**, 891.
755 103. P. Gujar, K. P. Bhavsar and J. M. Khire, *J. Sci. Food. Agric.*, 2013, **93,** 2242.
756 104. S. Samanta, B. Dalal, S. Biswas and B. B. Biswas, *Biochem. Biophys. Res. Commun.*, 1993, **191**,
757 427.
758 105. A. Claxon, C. Morris, D. Blake, M. Siren, B. Halliwell, T. Gustafsson, B. Lofkvist and Bergelin,
759 *Agents Actions.*, 1990, **29**, 68.
760 106. T. Maffucci, E. Piccolo, A. Cumashi, M. Iezzi, A. M. Riley, A. Siardi, H. Y. Godage, C. Rossi, M.
761 Broggini, S. Iacobelli, B. V. L. Potter, P. Innocenti and M. Falasca, *Cancer. Res.*, 2005, **65**, 8339.
762 107. R. J. Jariwalla, R. Sabin, S. Lawson and Z. S. Herman, *J. Appl. Nutr.*, 1990, **42**, 18.
763 108. F. Grases, J. G. March, R. M. Prieto, B. M. Simonet, A. Costa-Bauza, A. Garcia-Raja and A.
764 Conte, *Scand. J. Urol. Nephrol.*, 2000, **34**, 162.
765 109. K. Bhavsar, P. Buddhiwant, S. K. Soni, D. Depan, S. Sarkar, J. M. Khire, *Proc. Biochem.*, 2013,
766 **48**, 1618.
767 110. S. K. Soni, P. R. Selvakannan, S. K. Bhargava and V. Bansal, *Langmuir.*, 2012, **28**, 10389.
768 111. P. Kumar, S. Chamoli and S. Agrawal, *Biotechnol. Prog.*, 2012, **28**, 1432.
769 112. Sapna and B. Singh, *J. Ind. Microbiol. Biotechnol.*, 2013, **40**, 891.

RSC Advances Accepted Manuscript

770 113. M. K. Nabil, El-Toukhy, S. Amany and M. G. M. Mikhail, *Afr. J. Biotechnol.*, 2013, **12**, 2957.

771 114. L. Escobin-Mopera, M. Ohtani, S. Sekiguchi, T. Sone, A. Abe, M. Tanaka, V. Meevootisom and
772 K. Asano, *J. Biosci. Bioengg.*, 2012, **113**, 562.

773 115. M. Sumengen, S. Dincer and A. Kaya., *Turk. J. Biol.*, 2012, **36**, 533.

774 116. Bajaj and Wani, *Engg. Life. Sci.*, 2011, **11**, 620.

775 117. J. V. Madeira Jr, J. A. Macedo and G. A. Macedo, *Bioproc. Biosyst. Engg.*, 2012, **35**, 477.

776 118. P. Kaur and T. Satyanarayana, *J. Appl. Microbiol.*, 2009, **108**, 2041.

777 119. B. Yingguo, Y. Peilong, W. Yaru, S. Pengjun, L. Huiying, M. Kun, W. Bo and Y. Bin, *World. J.*
778 *Microbiol. Biotechnol.*, 2009, **25**, 1643.

779 120. M. Lim, O. Lee, J. Chin, H. Ko, C. Kim, H. Lee, S. Im and S. Bai, *Biotechnol. Lett.*, 2008, **30**,
780 2125.

781 121. M. Roy, M. Poddar, K. Singh and S. Ghosh, *Ind. J. Biochem. Biophys.*, 2012, **49**, 266.

782 122. M. Eida, T. Nagaoka, J. Wasaki and K. Kouno, *Microbes. Environ.*, 2013, **28**, 71.

783 123. P. Bennet and S. Yang, *Biotechnol. Prog.*, 2012, **28**, 1263.

784 124. Z. H. Wang, X. F. Dong, G. Q. Zhang, J. M. Tong, Q. Zhang and S. Z. Xu, *Waste. Manag. Res.*,
785 2011, **29**, 1262.

786 125. L. Chen, P. V. Vadlani and R. L. Madl, *J. Sci. Food. Agric.*, 2014, **94**, 113.

787 126. R. Rani and S. Ghosh, *Bioresourc. Technol.*, 2011, **102**, 10641.

788 127. D. Salmon, A. Walter, T. Porto, K. Moreira, L. Vandenberghe, C. Soccol, A. Porto and M. Spier,
789 *Biocatalyst. Biotrans.*, 2014, **32**, 45.

790 128. M. Neves, T. Poto, C. Souza-Motta, M. Spier, C. Soccol, K. Moreira and A. Porto, *Fluid. Phase.*
791 *Equilibria.*, 2012, **318**, 34.

792 129. M. V. Ushasree, J. Vidya and A. Pandey, *Biotechnol. Lett.*, 2014, **36**, 85.

793 130. S. K. Soni, A. Magadum and J. M. Khire, *World. J. Microbiol. Biotechnol.*, 2010, **26**, 2009.

794 131. B. Singh, G. Kunze and T. Satyanarayana, *Biotechnol. Mol. Biol. Rev.*, 2011, **6**, 69.

RSC Advances Accepted Manuscript

795 132. R. Ghorbani-Nasrabadi, R. Grenier, H. Alikhani and J. Hamedi, *World. J. Microbiol. Biotechnol.*,
796 2012, **28**, 2601.
797 133. P. Yu and Y. Chen, *BMC. Biotechnol.*, 2013, **13**, 78.
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Fig 1 Phytase bioprocessing and application

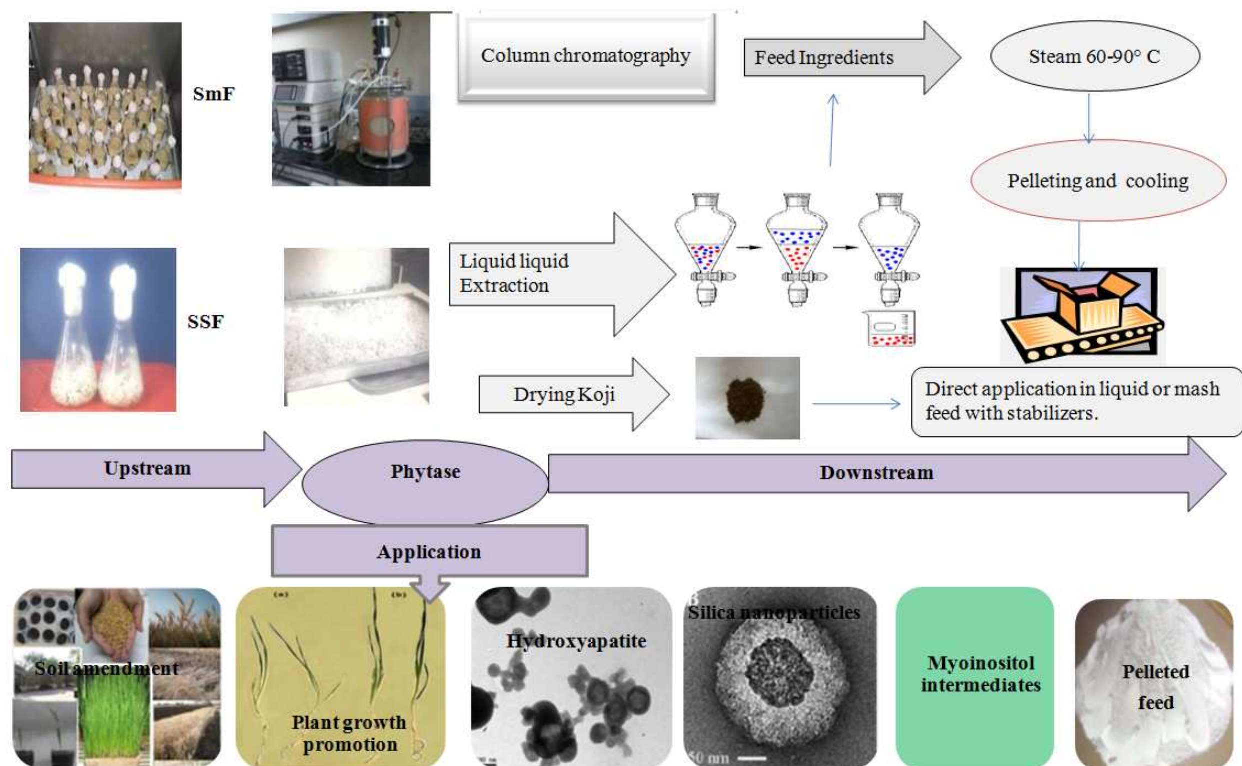

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Figure 2: Phosphorous paradox

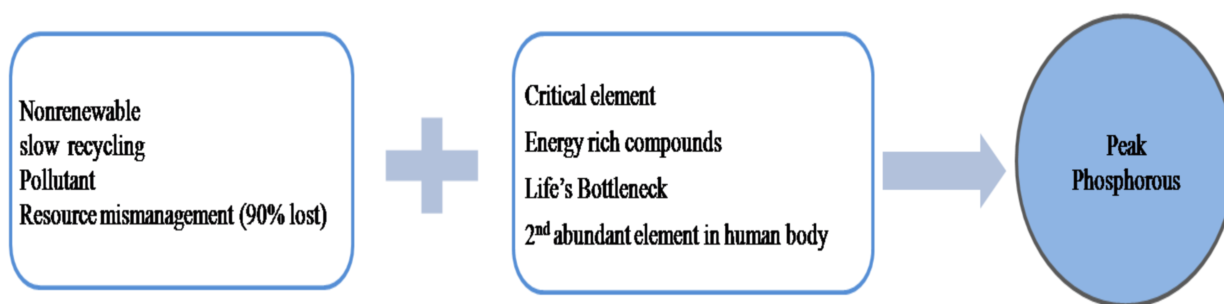

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Fig 3A World phosphate fertilizer consumption (% increase 2012)

Fig 3B World phosphate reserves

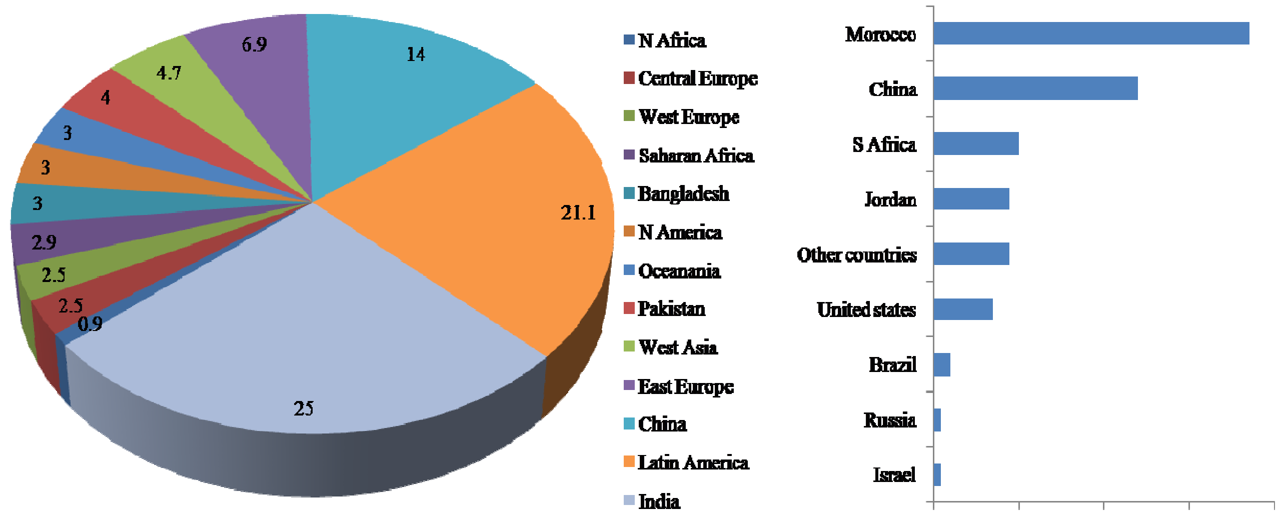

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Fig 4 Classification of Phytase

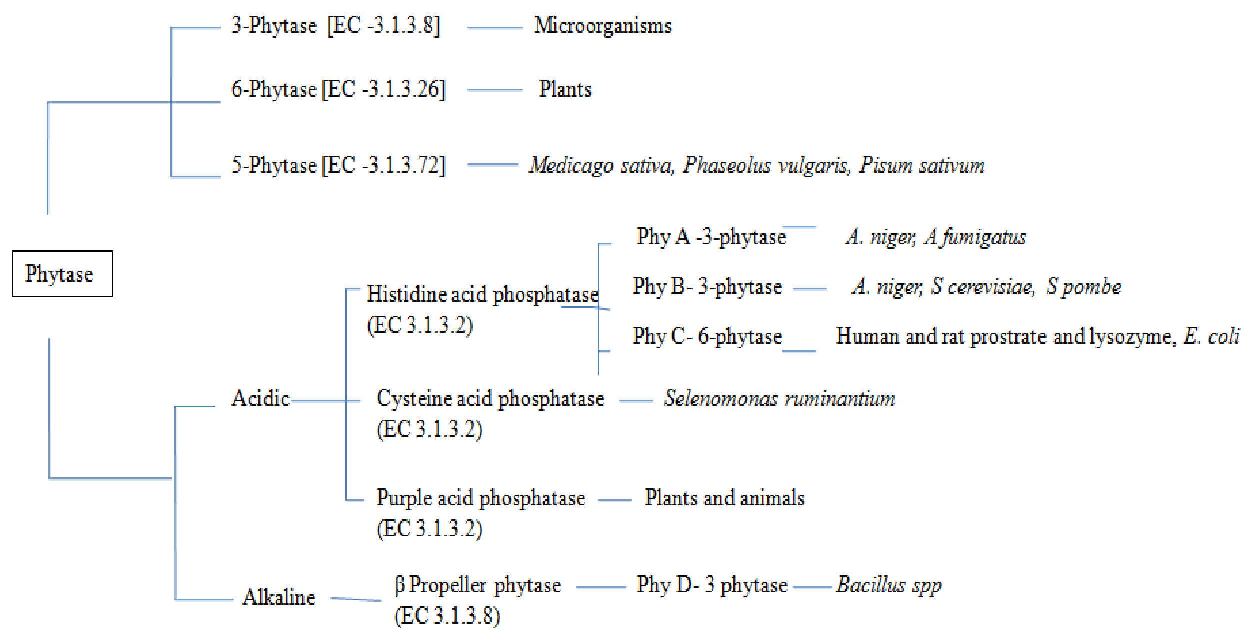

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Fig 5 Development of phytase research

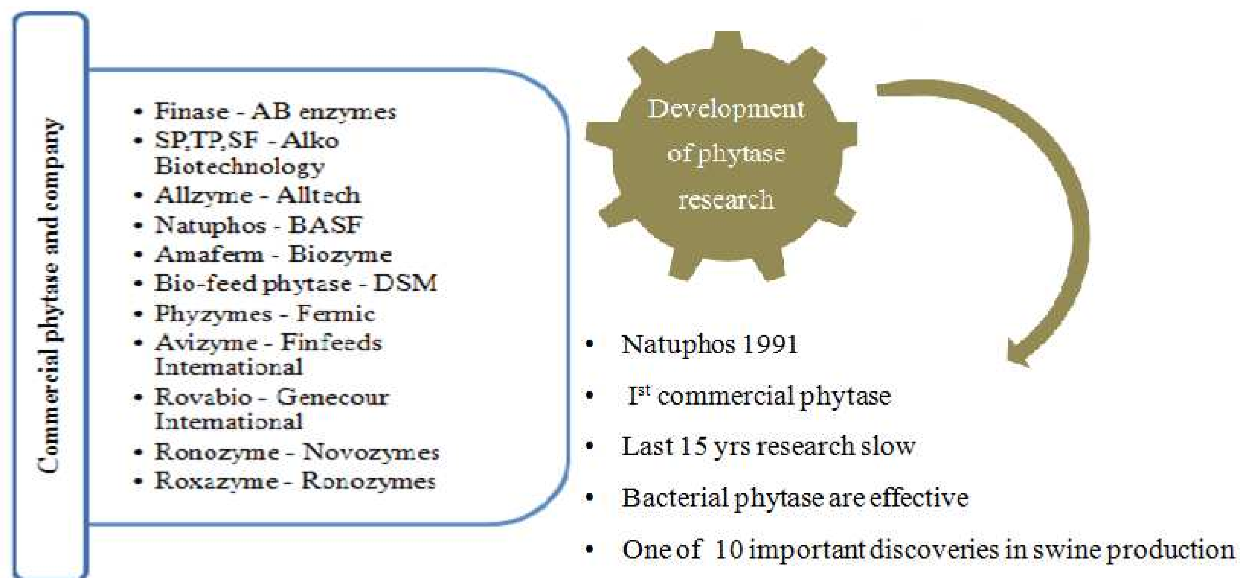

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Table 1 Negative interaction of phytate and nutrients in food

Nutrients	**Mode of action**
Mineral ions (zinc, iron, calcium, magnesium, manganese and copper)	Formation of insoluble phytate-mineral complexes leads to decrease in mineral availability. [14]
Protein	Formation of nonspecific phytate-protein complex, not readily hydrolysed by proteolytic enzymes. [15]
Carbohydrate	Formation of phytate carbohydrate complexes making carbohydrate less degradable. Inhibition of amylase activity by complexing with Ca^{2+} ion and decrease of carbohydrate degradation. [16]
Lipid	Formation of 'lipophytin' complexes, may lead to metallic soaps in gut lumen, resulting in lower lipid availability. [17]

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Table 2 Recombinant System for phytase

System	**Advantages**	**Limitation**
Plants	*A. niger* phyA gene successfully expressed in tobacco seeds or leaves and soybean cells.	Thermostability is a major concern[49]
Yeast	Heterologous gene expression of bacterial and mold phytases in yeast expression systems done.	Few yeast phytase expressed[50]
Bacteria	Inactive *A. niger* PhyA protein expressed intracellularly in *E. coli* and extracellularly in *Streptomyces lividans*.	Glycosylation is a major concern with bacterial system to produce fungal phytase[51]
Fungi	Phytase genes from *A. niger*, *A. terreus*, *A. fumigatus*, *E. nidulans*, and *M. thermophila* have all been expressed and secreted as active enzymes by *A. niger*.	Fungal systems secrete active phytases but along with high level of undesired proteases. This requires further purification or inhibition of proteolysis that adds to the production cost.

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Table 3 Potential applications of phytases

Application	Role and Effect	Properties	Challenges
Feed industry	Increased P utilization, metal bioavailability, decreased P conc. in excrement, Substitutes expensive Di-calcium phosphate	Resistance to low pH, active in the stomach, stable during animal feed processing and storage, low cost of production and easily processed by the feed manufacturer	Lack of desirable properties, High cost of production
Food industry	Increased P utilization, metal bioavailability, technical improvement of food processing	-	It will be a challenge to minimize the negative effect of phytate on iron and zinc nutrition without losing its potential health benefits
Myoinositol phosphate	Myoinositol phosphate intermediates used as enzyme stabilizers, enzyme inhibitors, potential drugs, chiral building blocks	-	Further intensive investigations, using diverse phytases, need to be undertaken for designing and producing pharmacologically important lower myo-inositol phosphates
Aquaculture	Substitute for expensive protein source such as menhaden fish meal and maintains the acceptable levels of P in water	Phytase active at low temperature and broad pH optima is required	Effects of phytase supplementation on various physiological and endocrine parameters like secretion of other enzymes, bile salts, on the on the immune response, hormone levels including growth hormone, thyroid hormone, insulin etc needs to be studied
Soil Amendment	Plant growth stimulation by mobilization of soil phytate into inorganic P	Phytase with broad pH optima and catalytic activity	Needs more research on phytase supplementation for boosting the productivity in agriculture and horticulture
Hydroxyapatite formation	Simple biocatalytic process	Cost effective as compared to commercial process	Needs more efforts for product development
Nanoparticles	Hollow metal nanocapsules formed using phytase and ionic liquid	Implications in biocatalyst and drug delivery	Needs more research for exploring its possibility in biomedical application

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Table 4 Summary of various fermentation systems used for phytase production and down streaming

Type	Microbial strain	Substrate	pH	Temp (°C)	Activity	Mol wt (kDa)	Purification	Recovery (%)	Reference
SMF	*Achromobacter sp* PB-01	WB RSM			7.05 IU/ml				111
	Apergillus oryzae SBS 50	RSM					Protease resistant phytase		112
	Aspergillus niger NCIM 563	Semisynthetic	2.5	55	40 IU/ml	264	HIC,GF	30.24	130
	A. niger NCIM 563	Semisynthetic	5	55	10 IU/ml	66	HIC,GF	26.55	130
	A. niger NCIM 563	RB RSM	2.5		268 IU/ml				46
	Bacillus nealsonii ZJ0702		7.5	55		43		5.7	133
	B. subtilis MJA	Sodium phytate	5	37		36			113
	K. pneumoniae 9-3B	MM9	4	50		45	Salt ppt,CC	15.8	114
	Lactobacillus plantarum	Synthetic medium	3.4	120	984.5 U/ml	46			115
	Nocardia sp MB 36	Starch, Beef extract RSM			0.4 IU/ml				116
	Paecilomyces variotii	Orange pomace			350 IU/g		Tannase and phytase for detoxifocation of castor beans		117
	Pichia anomala	Cane molasses RSM			1780 U/g		Permeabilization		118
	P. pastoris recomb	MSGW RSM			441 U/ml				119
	Rhizopus oligosporous (DSMZ 1964)	Rice flour	3, 4.5	60		45	Salt ppt, CC	1.3	66
		Rice flour	3, 5	55		45	Salt ppt, CC	1.6	66
	Saccharomyces cerevisiae	Bactopeptone, Starch			.165 U/ml	80	Phytase and amylase		120
	Shigella sp CD2	Sodium phytate	5.5	60		43	Salt ppt, IEC	41	121
	Sporotrichum thermophile	Starch	5	60					131
	Streptomyces spp	Glycerol	5	55			First repot		132
	Coniochaeta spp						First repot		122
	Recombinant A. niger phytase in E coli		6.5	50		92	Chaperonin co-expression		129
	Bacillus subtilis US 417	WB			112 U/g				41
SSF	Absidia blakesleeana URM5604	Citrus pulp					LLE	115	128
	A. ficuum	Lentils RSM			32 U/g				123
	A. ficuum NTG-23	Waste vinegar	1.3	67		65.5	IEC,GF	23.8	124
	A. niger 11T53A9	WB	5	55		85	Salt ppt, GF	20.3	73
	A. niger FS3	Citric by products	5	60		108	CC	10	68
	A. niger NCIM 563	WB RSM	5.6	60	154 U/g	85	CC and LLE	CC (20) LLE (98.5)	38,74
	A. niger NCIM 563	WB RSM	2.5		250 IU/g	120	CC		110
	A. oryzae	Soy meal 2 temp design			58.7 U/g				125
	B. subtilis US 417	WB	7.5	55	85u/g	41	Heat treatment, Salt ppt, FPLC		41
	R. oryzae	Linseed cake RSM	5	45	149 U/g	36	Salt,IEC	26	126
	Schizophyllum commune	WB RSM	5	50	113.7 U/g		LLE	367 (partial)	127
	S. thermophile	Sesame oil cake	5	60	282 Iu/g				131

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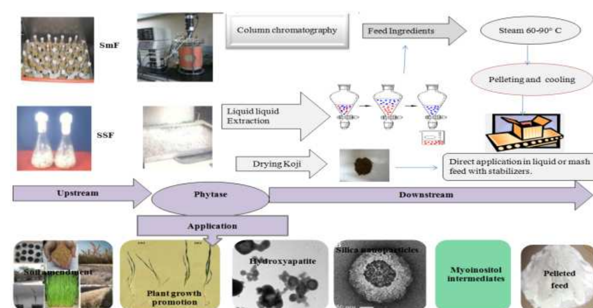

Focused platform for phytase bio-processing and application oriented research will help in developing a integrated technological solution to phytase production.

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