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Mini-Review

Probiotics as potential producer of phytase

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ABSTRACT

Probiotics are live microorganisms that help to maintain a healthy digestive system. They are currently used for the treatment of diarrhea, obesity, urinary tract infections and psychiatric disorders. An anti-nutrient like phytate chelates essential minerals like Ca^{2+}, Mg^{2+}, Zn^{2+} etc. which are essential for human health. Since past few decades, many research reports emphasize extensively on degradation of phytates in animals. As monogastric animals are unable to produce phytase, there is a need to find alternate ways for utilizing phytate in humans. Nowadays, probiotics can be used for producing phytase to overcome deficiency of minerals in humans. This review mainly focuses on improving the phosphate bioavailability by two approaches: (1) using fermented food products containing probiotics and (2) use of recombinant DNA technology for producing phytase in bacteria.

KEYWORDS: probiotics, phytase, phytic acid, fermented foods, recombinant DNA technology.

INTRODUCTION

The Greek word 'Probiotics' means "for life". Ferdinand Vergin introduced the term in 1954 in an article named "Anti –und Probiotika" comparing the harmful effects of using antibacterial agents such as antibiotics on intestinal microflora along with beneficial effects of some useful bacteria. Later in 1965, probiotics were described by Lilly and Stillwell as microbes stimulating the growth of other microbes [1]. The current definition formulated by FAQ (Food and Agriculture Organization of the United Nations) and WHO (World Health Organisation) in 2002, states that probiotics are "live microorganisms which when administered in sufficient quantity have beneficial effects on the host" [2]. Probiotics have become highly recognized as supplements for humans and animals due to their beneficial effects on health. Several clinical studies have confirmed that probiotics can be used in the treatment of many gastrointestinal diseases like irritable bowel syndrome, elimination of *Helicobacter*, diarrheas etc. and allergic diseases like atopic dermatitis. Obesity, type-II diabetes and non-alcoholic fatty liver disease can also be treated with the help of probiotics [3]. Most commonly used bacterial genera in probiotic preparations are *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Escherichia*, *Leuconostoc* and *Streptococcus*. Fungal strains belonging to *Saccharomyces* have also been used. Probiotics are not only used as feed supplements or pharmaceuticals but are also increasingly used in food materials like dairy products, fruit juices, chocolates 'etc.' [4].

Phytates, which are salts of phytic acid, are the primary storage form of phosphate and inositol in plant seeds and grains. Formation of phytate occurs during the maturation of the plant seed and it represents about 60-90% of the total phosphate in dormant seed [5]. Phosphorus is stored as phytic acid in seeds like nuts, beans and grains. When this phytic acid is present with a mineral in the seed, it is called as phytate [6]. Phytate is also present in minute quantity in roots, other vegetables and also in tubers [7]. Phytate binds to minerals and helps store them for the seed to use as food later and the

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minerals mostly affected are Fe^{2+}, Zn^{2+}, Mg^{2+} and Ca^{2+}. When the seed germinates, the sprout can use those minerals to grow [8]. Structurally phytate is composed of six phosphate groups, five of which are in equatorial position and the last one is placed in axial position attached to inositol ring [9]. Phytate is an anti-nutrient, as the negative charge contributed by phosphates carries out chelation of divalent and trivalent metal ions such as Ca^{2+}, Zn^{2+}, Fe^{2+}, thus reducing their availability for absorption in small intestine of monogastric animals. Phytates also reduce the digestibility of starches, fats and proteins [10].

Phytases (also known as myo-inositol hexakisphosphate phosphohydrolases) are the enzymes that catalyse the partial/complete hydrolysis of orthophosphates from phytates (myo-inositol hexakisphosphate). Phytases (IP_6 phosphohydrolase) are a class of phosphatase that sequentially hydrolyzes phytate to lesser phospho-myo-inositol derivatives and phosphate. Being ubiquitous in nature they are found in animals, plants and micro-organisms. Phytases were initially identified from rice bran [11]. Recently, phytases were isolated from a variety of sources like bacteria, fungi and yeasts [12]. Depending on the source or expression host, phytases exhibit distinct biophysical and biochemical properties. Microbial phytases show enzymatic activity over a wide pH range and are active even below pH of 3.5. Phytase hydrolyses phytate into one molecule of inositol and six molecules of inorganic phosphate. Phytate has six phosphate groups that may be released by phytases at different rates and order [13].

The monogastric digestive system of humans lacks microbes producing sufficient amount of phytase in their intestine for utilization of phytate. As a result of this phytates are not hydrolysed in the monogastric gut, and the phosphates (associated with phytates) remain unabsorbed. To avoid phosphorus deficiency external addition of phosphorus is needed [14]. Regular consumption of cereals and legume seeds composed of high phytate chelates dietary minerals and vitamins which causes micro-nutrient malnutrition in monogastric species including humans. Due to its high affinity for cations, phytic acid forms insoluble phytate salts in solution [15] thereby rendering crucial dietary minerals such as iron, zinc, calcium and magnesium unavailable for absorption in the gut [16]. This review focuses on probiotic micro-organisms capable of degrading phytates by producing phytases.

Some features of phytate

1. Phytates play an important role in plants. They serve as an energy source for the sprouting seed. When a seed sprouts, the stored phytates are broken down by phytases.
2. Phytates even have protective properties against cardiovascular disease, cancer and diabetes.
3. It acts as an antioxidant by binding with minerals in the gut preventing the formation of free radicals.
4. It prevents accumulation of heavy metals like cadmium in the body.
5. It prevents formation of kidney stones by binding to calcium ions and also prevents deposition of calcium in the arteries thus improving heart health [17].

Classification of phytases

There are various types of phytases which originate from the gut, plants and microorganisms including bacteria, fungi and yeast. These may be intracellular as in the case of Gram-negative bacteria, extracellular as in case of fungi and Gram-positive bacteria and/or cell-bound forms [18].

Depending upon the number of carbon atoms in the myo-inositol ring of phytate where initiation of dephosphorylation occurs, phytases are classified as shown in Figure 1:

On the basis of their optimum pH values, they are classified into two major classes:

1) Acid phytases - examples include phytases from *A. niger*, *S. cerevisiae* and *E. coli*.
2) Alkaline phytases - examples include *Bacillus* phytase [21, 22].

On the basis of their catalytic mechanism phytases are classified into four groups [23]:

1) Histidine Acid Phosphatases (HAPs); eg: *Aspergillus fumigatus* phytase and *Aspergillus niger* phytase.
2) Purple Acid Phosphatases (PAPs) or Cysteine phytases; eg: Phytases from barley, wheat, maize and rice.
3) β-propeller phosphatses (BPP); eg: *Bacillus* phytase.
4) Protein Tyrosine Phosphatases (PTP); eg: *Selenomonas ruminatum* phytase [23].

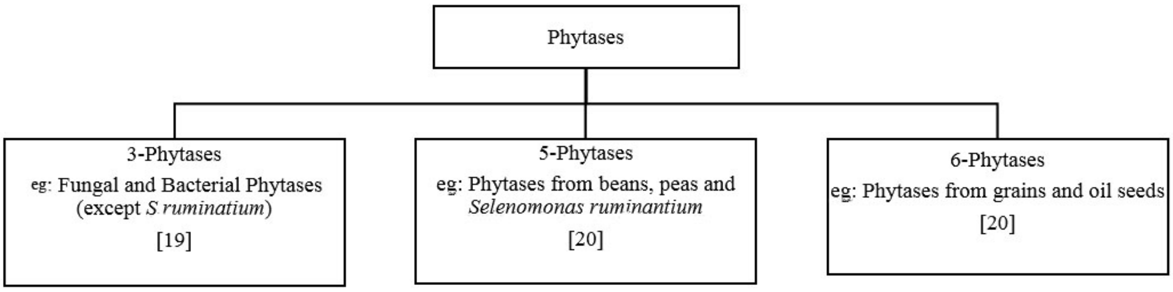

Figure 1. Classification of phytases based on the number of carbon atoms.

Table 1. The properties required for a strain to be chosen as a probiotic.

Safety	Functionality
▪ It should be of animal or human origin ▪ It should be isolated from GI tract (healthy individuals) ▪ The microorganism should have a history of safe use ▪ No adverse effects	▪ Ability to compete with microbiota inhabiting the intestine ▪ Ability to survive and maintain the metabolic activity and grow in the target site ▪ It should be resistant to bile salts and enzymes ▪ It should resist low pH in the stomach ▪ Should possess antagonistic property towards the pathogen (eg: *H. pylori*, *Salmonella sp*., *Listeria monocytogenes*, *Clostridium difficile*) ▪ Resistance to bacteriocins and acids produced by endogenic intestinal microbiota

Probiotic selection criteria

Diverse microbes are present in the human gastrointestinal tract. Intestinal microbes are commensal and also in symbiotic relationship with their host. Beneficial intestinal microbes have several important functions like producing nutrients for their host and preventing infections caused by intestinal pathogens and immunomodulation. Modification of intestinal microbiota is therefore necessary to achieve, restore and maintain balance in the ecosystem and for the improved health condition of the host. Introducing probiotics into human diet is favourable for intestinal microbes. They may be consumed in the form of raw vegetables, fruits, fermented dairy products, pickles etc.

Probiotic characteristics are not associated with genus or species of a microorganism but with specially selected strain of a particular species. According to the suggestions of WHO, FAQ and EFSA (European Food Safety Authority), probiotic strains must meet both the safety and functionality criteria as well as technological usefulness as mentioned in Table 1 [24]. The safety of a strain is defined by its origin, absence of its association with pathogenic cultures and antibiotic resistance profile. Functional aspects include their survival in the gastrointestinal tract and their immunomodulatory effect. They must be able to survive and maintain their properties throughout the storage and distribution processes [25].

Enema or enteral route is generally preferred for probiotic administration. Fermented food products are consumed by enteral route. Fecal microbiota transplantation prefers enema. This review focuses on administration of probiotics through enteral route by use of fermented food products. Phytases are reported in filamentous fungi, Gram-positive bacteria, Gram-negative bacteria and yeasts. Some commonly used probiotic bacteria present in human diet are mentioned in Table 2.

Probiotic bacteria in fermented food products affect the human health to a greater extent. Probiotics isolated from a variety of foods like dough to fermented bread including dairy products have the capability to effectively degrade the phytate thereby improving mineral uptake in humans. Probiotics from the *Bacilli* group which

Table 2. Commonly used probiotic bacteria.

Probiotic bacteria commonly used in diet	*Lactobacillus amylovorus*, *L. casei*, *L. johnsonii*, *L. pentosus*, *L. plantarum*, *L. rhamnosus*, *Bifidobacterium breve*, *Lactococcus lactis*

are Gram-positive, facultative aerobes produce spores which are non-pathogenic and play an important role in increasing the shelf-life of the product. Thermostability, viablility under extreme intestinal conditions and resistance to a wide range of pH variations are some of their positive attributes. Being Gram positive they possess an outer thick peptidoglycan layer protecting the spores from extreme heat, degradation by organic acids and by lysosomal contents. It is also helpful in treatment of disorders like rheumatoid arthritis and blood clotting. Microbes belonging to genera *Lactobacilli*, *Enterococci*, *Bifidobacteria*, *Pediococcus* and *Leuconostoc* represent a group of non-pathogenic, facultative anaerobes commonly found in the human gut. They have numerous advantages like gastric viability, prolonged epithelial adherence and efficient colonization. Production of bacteriocins which competitively inhibit other pathogenic bacteria helps to maintain a healthy balance of useful microbes. These bacteria are useful in preparations of fermented food products and in the treatment of inflammatory bowel disease (IBD) and diarrhea.

Yeasts play an important role in fermentation of foods and beverages and have probiotic effects including treatment of intestinal disease and immunomodulation. They also help to improve mineral bioavailability through hydrolysis of phytate. There are different species of yeasts such as *Debaryomyces hansenii*, *Torulaspora debrueckii*, *Kluyveromyces lactis and Kluyveromyces marxianus* which have shown increased tolerance to passage through the gastrointestinal tract. Among yeasts *Candida krusei*, *Schwanniomyces castellii*, *Debaryomyces castellii*, *Arxula adeninivorans*, *Pichia anomala*, *Pichia spartinae*, *Cryptococcus laurentii*, *Rhodotorula gracilis* and *S. cerevisiae* have been identified as phytase producers. The phytase activity of *S.cerevisiae* is due to the activity of secretory acid phosphatases (SAPs) which are secreted by cells into the growth media and repressed by inorganic phosphate (Pi). During leavening of bread the phytase activity of yeast is relatively low. Repression of phytases depends on the pH and composition of media. Andlid *et al.* showed that repression of phytate-degrading enzymes is weak in complex medium with pH and high amount of phosphate. Regardless of Pi addition, the capacity to degrade phytase is highest at pH far from the optimum pH for the SAPs suggesting that pH has more effect on the enzyme's expression than on its activity. Table 3 shows the characteristics of probiotics [26].

Functional foods for phytase production

The process of fermentation breaks down nutrients into more easily digestible form. It is usually the conversion of sugar into acid or alcohol by bacteria and into lactic acid by yeast. The efficacy of probiotics is enhanced when taken in the form of fermented food rather than as probiotics alone [27]. Fermenting the food often by lactic acid bacteria increases digestibility, improves nutrient content by enhancing B complex vitamins, increasing the levels of A and K vitamins and decreases mineral-binding phytates. Both functional and non-functional microorganisms are present in naturally fermented foods and beverages [28]. Lactic acid bacteria (LAB) associated with fermented foods and alcoholic beverages mostly include species of *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* etc. [29]. According to a report, isolates of *Serratia* sp. PSP-15 and *Enterobacter cloacae* strain PSB-45 have shown high thermostability and good phytase activity at desirable range of pH and temperature for their efficient use in food and feed. Therefore these bacteria will help to facilitate hydrolysis of phytate–metal-ion complexes, increasing the bioavailability of important metal ions to monogastric animals [30]. Species of *Bacillus* are also present in legume-based fermented foods. Species of *Bifidobacterium*, *Brevibacterium* and *Propionibacterium* are isolated from cheese and species of *Arthrobacter* and *Hafina* from fermented meat products. Probiotic

Table 3. Characteristics of probiotics.

Microorganisms	Genera	Characteristics
1) Bacteria	*Bacilli* spp.	» Gram-positive » Facultative aerobes » Produce non-pathogenic spores » Thermostable » Viable under extreme intestinal conditions » Withstand wide range of pH fluctuations
	Lactobacilli, Enterococci, Bifidobacteria, Pediococcus, Leuconostoc	» Non-pathogenic » Facultative anaerobes » Gastric viability » Efficient colonization » Produce bacteriocins
2) Yeast	*Debaryomyces hansenii, Torulaspora debrueckii, Kluyveromyces lactis, Kluyveromyces marxians*	» Gastric viability » Inhibit enteropathogens

properties of microorganisms have been reported in fermented foods. Some micro-organisms present in fermented foods in fact degrade anti-nutritive substances converting the substrates into consumable products. It has been reported that *Bacillus subtilis* degrades anti-nutritive compounds in *kinema* [31]. During the fermentation of *idli*, the phytic acid gets reduced [32]. Higher phytase activity was reported in sourdough bread prepared by using a combination of yeasts and lactic acid bacteria as starter culture. Phytate content in yeast and sourdough bread was lower than that of reconstituted whole-wheat flour. Therefore, mineral bioavailability could be improved by breaking down phytate especially by using yeast and LAB. Therefore, a high phytase *S. cerevisiae* strain may be suitable for production of food grade phytase and for direct use in food production. Yeast or yeast phytases can be applied for pre-treatment of foods to reduce the phytate contents or they can be ultilized as food supplement for hydrolysis of phytate after digestion [33, 34].

The activity of phytase *in vivo* depends on factors like, the concentration of phytate, optimum pH, bacterial viability, accessibility of phytate, presence of inorganic phosphates and other acids. Human intestine normally maintains a constant temperature of 37 °C with pH ranging from 5 to 7. Other parameters such as molecular mass of phytase, substrate specificity and presence of minerals also influence the degradation of phytate in human intestine.

Depending on the conformational state of the phytase enzyme it can either be monomeric or dimeric. Most of the phyatses from probiotics are monomeric with the exception of *L. brevis*, which has a dimeric chain with molecular masses 73 and 34 kDa.

Recombinant phytases: Role and expression

Fermented food if consumed *via* oral route provides an effective strategy for delivery of probiotics but its major disadvantage is the lack of site-specificity and short-term efficacy. These disadvantages can be subdued by developing recombinant phytases. Some sources of phytase genes along with the host microorganisms are mentioned in Table 4.

It has been reported that fermentation of a soy drink by the *L. casei* strain expressing *Bifidobacterium pseudocatenulatum* phytase resulted in an increased bioavailability for Ca^{2+}, Fe^{2+} and Zn^{2+} [31]. Recombinant thermostable phytase was isolated from *B. subtilis* and expressed in *E. coli* [32].

Table 4. Recombinant phytases.

Source of phytase gene	Vector plasmid	Host microorganism
Bifidobacterium longum subsp. *infantis* and *B. pseudocatenulatum*	pNGPHY pseudo, pNGPHY longum	*Lactobacillus casei* BL23
Pichia anomala	pGAPZαA	*Pichia pastoris*
Bacillus subtilis DR8806	pTZ57R/T	*E. coli* DH5α and BL21 (DE3)
Sporotrichum thermophile	pET28a(+)	*E. coli* BL21 (DE3)

Gene coding phytase in *Sporotrichum thermophile* (St-Phy) was cloned and expressed in *E. coli*. This rSt-Phy is used in dephytinizing tandoori, naan and bread, liberating soluble inorganic phosphate that reduces anti-nutrient effects of phytic acid [35].

The recombinant phytase from *Pichia anomala* (rPPHY) hydrolysed Ca^{2+} phytate more efficiently than Co^{2+}, Mn^{2+}, Mg^{2+}, Fe^{3+} and Zn^{2+} phytates. The supplementation of recombinant phytase to the dough of whole wheat flour increased nutrient availability in breads by increasing the amount of soluble phosphate, sugars and proteins, besides alleviating anti-nutritional effects of phytates [36]

Certain attributes of LAB such as safety, cost effectivity, purity and stability of the enzymes produced make them suitable for expressing recombinant phytases. However vast species diversity, difficulty in the route of administration and rigid monitoring due to the lack of clinical trial data pose severe challenges in employing these recombinant phytases.

CONCLUSION

Phytase from probiotics is a solution for utilization of phosphate in humans which can be achieved by consumption of fermented food products which is an ideal vehicle for delivery of probiotics. The development and marketing of products containing live microorganisms has flourished as there is growing perceived interest in ingestion of ‘natural foods’ that might promote health. It was noted that *B. pseudocatenulatum* in soy drink, *B. subtilis*, *L. brevis* and some yeast strains like *A. adeninovorans* and *P. anomala* present in fermented food products have the capability of producing huge amount of phytase. Most of the phytases from probiotics are specific in their activity towards phytate which is an antinutrient and are stimulated by Ca^{2+} ions. An alternative for utilization of phytate is recombinant phytases which can be produced through genetic engineering techniques. The US Food and Drug Administration have still not approved the use of probiotics for utilization of phytate. Therefore, still more research needs to be carried out on improving the probiotic strains for production of phytase and for optimizing dosage of probiotics for utilization of phytate.

CONFLICT OF INTEREST STATEMENT

None.

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