ABSTRACT

On the global level, there is a clear trend of steadily increasing extraction and use of both renewable and non-renewable natural resources for the production of goods and services. The larger fraction are non-renewable resources, i.e. resources which cannot be produced, re-grown or regenerated on a time scale which can sustain their consumption rate (e.g. strategic resources such as fossil fuels, phosphorus, uranium, lithium, neodymium, platinum, rare earths). Unremarked and unregulated by the United Nations and other high-level assemblies, the world’s supply of phosphate rock, the dominant source of phosphorus for fertilizer, is being rapidly — and wastefully — drawn down. Phosphate rock, like oil, takes 10-15 million years to cycle naturally. While all farmers need access to phosphorus, just 5 countries control around 90% of the world’s remaining phosphate rock reserves, including China, the US and Morocco (which also controls Western Sahara’s reserves). Studies suggest current high-grade reserves will be depleted within 50-100 years. Further, peak phosphorus could occur by 2030. While the exact timing might be disputed, it is widely accepted that the quality of phosphate rock is decreasing and costs increasing. In mid 2008 the price of phosphate rock reached a peak 800% higher than early 2007.

The phosphorus situation has many similarities with oil, yet unlike oil, there is no substitute for phosphorus in food production. Phosphorus cannot be manufactured, though fortunately it can be recovered and reused over and over again. Phosphorus is one of Mother Nature’s paradoxes as it is Life’s bottleneck for existence on earth, but at the same time destructive in excess quantities in an aquatic environment. The only way to avert a supply crisis, researchers say, is to adopt the “3 R’s” of sustainability: “Reduce, Reuse and Recycle.”

Hence dephosphorylation of antinutritional and indigestible phytate, a phosphorus locking molecule, to digestible phosphorus, calcium and other mineral nutrients by phytases is an important metabolic process. The existing commercial microbial phytases produced by submerged...
fermentation (SmF) conditions are expensive because of diluted product, production using recombinant strains, conventional purification techniques and high product recovery costs. Although, a limited number of phytases have been reported and studied, our scientific knowledge of phytases has yet to yield a solution to meet the nutritional and environmental requirements that a real-world solution demands.

So development of a viable process for phytase production, recovery and purification with techno-economic feasibility is necessary as the available methods such as SmF and column chromatography have several limitations. The available processes are also expensive, time consuming and difficult to scale-up. These traditional approaches are currently employed due to lack of alternative methods. For the above reasons, we chose to study the application of statistical methods to increase the phytase activity under SSF and suitability of ATPE system for phytase purification.

The same fungus produces two dissimilar phytase Phy I and Phy II under (SmF). Many studies on SSF and SmF for phytase have focused on process and fermenter design while the organism has been considered as a black box. The role of the physiological and genetic properties of the microorganisms producing phytase used during growth on solid substrates compared with aqueous solutions has so far been all but neglected. Hence we have tried to correlate different protein secretion in SmF and SSF and these studies can provide new insights to the existing “black box” of SSF/SmF biotechnology for phytase production.

**Chapter I- Introduction**

This chapter covers the literature on scope of the study, phytate and phytase enzyme, diversity of phytases, production, different sources, applications and recent advance in phytase research. It also covers the production and purification for phytase.
Chapter 2 - Production of phytase by *Aspergillus niger* NCIM 563 under solid state fermentation

SSF provides a more economic alternative for enzyme production and application as compared to SmF. So phytase production by *Aspergillus niger* NCIM 563 was optimized using wheat bran in SSF. The present work demonstrates that using response surface optimization employing PBD and BBD gave a high level of phytase production of 154 IU/g DMB along with accessory enzymes in SSF. Phytase production improved from 50 IU/g dry moldy bran (DMB) to 154 IU/g DMB indicating 3.08 fold increase after optimization. A simultaneous reduction in fermentation time from 7 days to 4 days shows a high productivity of 38500 IU/kg/day. Scaling up the process in trays gave reproducible phytase production overcoming industrial constraints of practicability and economics. Some fungi are known to produce phytase and accessory enzymes by SSF but their low productivities are not comparable with the highest phytase productivity of 38500 IU/kg/day by *A. niger* NCIM 563 as shown by studies here. This demonstrates the potential applicability of SSF enzyme as a source of phytase supplement for phosphorus nutrition and environmental protection in animal feed industry.

Chapter 3 - Downstream processing of solid state phytase from *Aspergillus niger* NCIM 563

There is a clear need for efficient, scalable and economical process for phytase bioseparation as available methods have several limitations. Hence the application of single step aqueous two-phase extraction (ATPE) for the downstream processing of phytase from *Aspergillus niger* NCIM 563, produced under SSF, has been studied and compared with the traditional multi-step procedure involving salt precipitation and column chromatography. High phytase recovery (98.5%) within a short time (3 hrs) and improved thermostability was attained by ATPE in comparison to 20% recovery in 96 hrs by chromatography process. The ATPE method, therefore, seems to be an interesting alternative for simultaneous partitioning and purification of phytase. This is the first report to show phytase extraction in a single step from fermentation broth by a liquid–liquid extraction process using ATPE. The results presented in this work show that the ATPE technique
has considerable potential for the commercial development of an efficient process for separation and purification of SSF phytase.

**Chapter 4-Biochemical characterization and application of solid state phytase from *Aspergillus niger* NCIM 563**

The purified enzyme has been extensively characterized for its biochemical, molecular properties and synthesis of hollow silica nanocontainers in ionic liquids. The purified SSF phytase (Phy III) possessed an optimal pH of 5.6 and an optimal temperature of 60°C. The protein is a monomer and exhibited a molecular mass of 85kDa in gel filtration and SDS–PAGE. Phy III exhibited broad substrate specificity but had high affinity for sodium phytate. It was markedly inhibited by N-bromosuccinimide suggesting a possible role of tryptophan in its catalysis. Based on MalDI-LC-MS/MS identification amino acid sequences of the peptides, the enzyme did not show homology with any other known phytases from the literature suggesting its unique nature. Importantly, the phytase released more inorganic phosphorus from soybean meal in a broad pH range from 1.5-6.5 under emulated gastric conditions and facilitates its use for the bioremediation of phytic acid in poultry feed.

**Chapter 5- Correlation studies of solid state Phy III with submerged (Phy I and II) produced by *Aspergillus niger* NCIM 563**

One of the differences between SSF and SmF cultures is that in the former, the moisture content of the substrate is low, resulting in a limitation of growth and metabolism of the microorganism. Moisture content being related to many factors can greatly influence the path of enrichment, leading to products that differ both quantitatively and qualitatively. *A. niger* NCIM 563 produces dissimilar phytases, Phy I and II in SmF and Phy II under SSF. From our studies, it can be seen that wheat bran and rice bran supported maximum phytase production under SSF and SmF. This may due to the reason that the fungus is confronted with gradients in concentration of
substrates and enzymes, the presence of a substrate-air interface, and gradients in water content and temperature. Production of accessory enzymes was higher in SSF when compared with SmF. This may be due to the conditions of SSF being more similar to fungal growth conditions in nature. This is the first report that aims at elucidating the mechanisms behind the differences in SSF and SmF for dissimilar phytases by comparison of their culture conditions and biochemical properties.

Chapter 6 Conclusions

This chapter details the salient feature of the work presented in the thesis and emphasized on possible future potential developments in the area.