1. Introduction

Phytase, (myo-inositol-hexakisphosphate phosphohydrolase) is a phosphatase, capable of initiating the stepwise dephosphorylation of phytate (myo-inositol-hexakisphosphate) to inositol and inorganic phosphate (Figure 1.). Phytic acid serves as the storage form of phosphorus in plant seeds. Cereals (maize, barley, wheat, and oats) and legumes that are commonly used as feed ingredients all have similar phytate levels, approximating 0.25% of dry matter. On average, 70% of the total phosphorus in the feed ingredient is found as phytate-P (Maenz 2001). The monogastric animals (pig, poultry, fish) are unable to utilise phosphorous from phytic acids, because their intestine lacks phytase activities. As a result feed must be supplemented with inorganic phosphate. Therefore the manure secreted by animals contains high amount of phosphorous that may cause environmental pollution, eutrophication of surface water, particularly in areas of intensive livestock production. Microbial phytase can be used as an animal feed supplement as an effective and practical method of improving phytate digestibility and nutritive value of feeds as well as reduces the environmental phosphorus pollution.

Figure 1. Reaction catalyzed by phytase

The partial hydrolysis of phytic acid lower inositol phosphates with health benefits are liberated. Individual myo-inositol phosphates have important physiological function in human, such as prevention of diabetes complications, anti-inflammatory, anti-angiogenic, antitumor effects and have a potential for use in transmembrane signaling processes, and for calcium mobilization from the intracellular store of animal and plant tissue (Greiner - Konietzny 2009, Rao et al. 2009). Production of beneficial inositol phosphates with different chemical structure needs phytase enzyme technologies. Depending on the origin of phytase used, different phosphate residues of phytate may be released at different rates and in different order. Therefore, every phytase might have industrial and medical potential.
Although several strains of bacteria, yeasts and fungi have been used for production of phytase under different conditions, especially those originating from filamentous fungi such as *Aspergillus niger* have most commonly been employed for commercial production of extracellular phytase (Haefner et al. 2005). The production of phytase from fungus has been achieved using different cultivation methods solid-state or submerged fermentation (Papagianni et al. 1999; Singh et al. 2011). Generally, carbon sources applied for production of phytase by yeast and by bacteria were glucose or galactose and maltose, respectively (Angelis et al. 2003, Sano et al., 1999). In the case of fungus, variable carbon sources were used such as starch, glucose, maltodextrin, wheat bran, sesame oil cake etc. (Singh et al. 2011). Lassen and co-workers (2001) reported that maltodextrin and soya flour were good carbon sources for production of phytase by *Peniophora lycii*. It is well-known that the quality of carbon source should be one of most important factor in fermentation process, thus investigation of it on production of certain enzyme is abundance. In this work, the effects of different carbon sources and surfactants as well as buffering media on phytase production in submerged fermentation of thermophilic (*Thermomyces lanuginosus*) and a mesophilic fungus (*Aspergillus niger*) are studied.

2. Materials and Methods

2.1. Enzyme production

For the submerged fermentation *Aspergillus niger* F00735 and *Thermomyces lanuginosus* IMI 096218 strains were applied. Conidia were washed from the surface of PDA plates with 5 mL of Triton X-100 solution [0.01% (w/v)]. Precultivation of *A. niger* and *T. lanuginosus* were made in an orbital shaker with 220 rpm for 2 days at 28 °C and 47 °C, respectively.

Phytase production was carried out in fermentation medium containing (g/L): MgSO$_4$.7H$_2$O 0.5; KCl 0.5; FeSO$_4$ 0.1 and NaNO$_3$ 8.6 as mineral salts, which was completed different phytate containing crop and legume products (rice flour, corn flour, soy flour, barley flour, pea flour, wheat flour, wheat grit, corn grit). Fermentations were initiated with 5% (v/v) of 2-day old inoculum culture and then incubated in an orbital shaker with 220 rpm at 28 °C and 47 °C up to 5-7 days. During the fermentation samples were taken at regular time intervals for enzyme activity measurement.

2.2. Enzyme activity assay

Phytase activity was assayed by measuring the amount of phosphorous released from sodium phytate substrate solution using the method of Engelen et al. (1994). The released phosphate develops yellow coloured complex with ammonium molibdate, which was quantified spectrophotometrically at 415 nm. One unit of phytase activity was defined as the amount of enzyme capable of releasing one µmol phosphate per min under the reaction conditions (37°C in case of *A.niger*, 65°C in case of *T. lanuginosus*, 10 min, pH 5.5).
3. Result and Discussion

3.1. Optimization of fermentation media

One-factor-at-a-time optimisation was carried out to optimize the composition of fermentation media. To study phytase production on different natural substrates, the mineral salt of the medium were supplemented with either rice flour, corn flour, soy flour and wheat grit in 5% concentration. *T. lanuginosus* has grown and synthetised phytase enzyme on rice flour, corn flour and there was a little activity in case of wheat grit. No activity was determined on medium contained soy flour. The maximum phytase activity was achieved on the 5th day of fermentation on rice flour (Figure 2.). The results were similar in case of *Aspergillus niger*, but the fermentation time was longer. The highest enzyme activity was achieved on the 7th day of the fermentation on rice flour. There was substantial activity on medium containing wheat grit or corn flour. These results indicate that rice flour is suitable for phytase production in the case of both fungi.

To determine the optimal pH for enzyme fermentation, the fermentation media were prepared with sodium acetate/NaOH and Tris-maleat/NaOH buffers at different pH values from pH 3.5 to 9. Applying fermentation medium prepared with Tris-maleat/NaOH buffer pH 7.5 the enzyme activity was the highest in the case of *T. lanuginosus* IMI 096218 strain (Figure 3.). Very low phytase activity was assayed when preparation of medium with sodium acetate buffer. In the case of *A. niger*, no significant effect of the initial pH of the media was observed. Gargova and Sariyska (2003) were reported that the optimal value of the initial pH of the media for phytase production from *A. niger* 307 was 5.0.
Surfactants have been reported to affect the growth and enzyme production of fungi especially extracellular enzyme. Generally, the addition of surfactants to culture media increases the enzyme yield, but their effects varied from organism to organism even from enzyme to enzyme. The mechanisms by which surfactants enhance extracellular enzyme production were reported to be increased cell membrane permeability through the change in lipid layer, which resulted in a higher release of enzymes (El-Batal - Karem, 2007). During the enzyme production the fermentation media was supplemented with 0.1% concentration of Tween 20, Tween 40, Tween 60, Tween 65, Tween 80 and Tween 85. The phytase activity was highest at the 2\textsuperscript{nd} or 3\textsuperscript{rd} day of the fermentation in the case of *Thermomyces* strain. Using Tween 20 or Tween 40 resulted in approximately 2-fold increase in phytase secretion comparing with the controls (Figure 4.).
Tween 65 and Tween 85 have no positive effects at the beginning of the fermentation, but on the 3rd day resulted in a decrease of the production of phytase enzyme. When the concentration of surfactant was increased above 0.5%, the stimulatory effect of the surfactant changed into an inhibitory one regarding the enzyme production. Best results were obtained when the fermentation media supplemented Tween 20 or Tween 40 at 0.25 % and 0.1 % concentration, respectively (Bujna et al., 2011). Arnesen and co-workers (Arnesen et al., 1998) reported that addition of Tween 80 to the growth medium gave a 2.7 fold increase in maximum α-amylase activity originated from Thermomyces lanuginosus. In contrast of T. lanuginosus the relative phytase activities in case of Aspergillus niger were risen in presence of all type of tested surfactants, but the the increase was much more lower, only 10-15%. The maximal activity was achieved in the case of Tween 20 on the 7th day in concentration of 0.1-0.4%. No significant difference was obtained in enzyme production when the concentration was higher then 0.1%. Tween 80 supported a 14% increase in phytase production in the case of Aspergillus niger NCIM 563 (Mandviwala – Khire, 2000).

4. Conclusions

The thermophilic fungus, Thermomyces lanuginosus and the mesophilic fungus, Aspergillus niger are able to produce phytase enzyme in submerged fermentation. The best complex natural source for phytase production is rice flour for both fungi. The most favourable initial pH of fermentation media was pH=7.5 applying Tris-maleate/NaOH buffer in the case of T. lanuginosus IMI 096218 strain, meanwhile no significant difference were detected in phytase production at various initial pH of the fermentation media in the case of A. niger. To enhance extracellular phytase production fermentation medium were supplemented with surfactant, which increase cell membrane permeability. In the case of T. lanuginosus the most promising results were obtained when the fermentation media supplemented with Tween 20 or Tween 40 at 0.25 % and 0.1 % concentration, respectively. It resulted in approximately 2-fold increase in phytase production in contrast of A. niger phytase, where the increase was only 10-15 percentage in the presence of all type of tested surfactant.

Homogenously purified phytases from a thermophilic (Thermomyces lanuginosus) and a mesophilic (Aspergillus niger) fungus some of their physico-chemical properties can be compared. The determination of operational parameters of the relevant enzymes may give information for potential application of the enzyme species.

References


