IN VITRO COMPARISON OF PHYTASES FROM TWO MICROBIAL SOURCES

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ABSTRACT

The chemical properties of two different commercial microbial phytases (Natuphos and Ronoxyme P) were compared. Natuphus production was based on the use of a recombinant of *Aspergillus niger* strain while the Ronoxyme P was from *Peniophora lycii.* The enzymes were studied for their optimum pH, optimum temperature and enzyme stability, resistance to protease and stability in digesta supernatants. The phytases were found to exhibit different properties depending on source of the phytase gene and production organism. An optimum pH of 5.5 was recorded for the enzyme from *Peniophora lycii* while pH of 5.0 was recorded for the enzyme from *Aspergillus niger.* Both phytases had 50^0C as their optimum temperature. The result of the enzyme - stability in aqueous solution indicated that the *Peniophora lycii* phytase was more thermo-stable than the *Aspergillus niger* phytase. Incubation of the phytases preparations with porcine proteases revealed that *Aspergillus niger* was more resistant to pepsin and trypsin than *Peniophora*. Incubation of the phytases in digesta supernatants from various segments of the digestive tract of hens revealed that digesta from the small intestine (SI) inactivated the enzymes most efficiently. Nevertheless, the enzyme from *Peniophora lycii* retained more activity than the enzyme from *Aspergillus niger* in all of the gastrointestinal tract (GIT). In conclusion, phytases of various microbial origins behave differently with respect to their *in vitro* properties, which could be of importance for future developments of phytase preparation.

Key words: Properties, microbial phytases, digesta, proteolytic stability, supernatant

INTRODUCTION

Cereals, legumes and oilseeds products constitute the major sources of nutrients in poultry diets. Phosphorus (P) is one of the essential elements present in these products, which has not been maximally utilised due to the form in which they are present in the ingredients. About 2/3 of P present in these feedstuffs occur as phytates (Myo-inositol hexaphosphate, $Insp_6$), the salts of phytic acid (Jongbloed *et al.,* 1993). Phosphorus in this form is poorly digested and as such low availability for poultry (VanDerKlis and Versteegh, 1996).

For the utilisation of phytate and others chelating minerals, hydrolysis of the ester type bounded phosphate groups of phytic acid by phytase is necessary (Rimbach *et al.,* 1994). Phytase (Myo-inositol hexaphosphate phosphohydrolase) belongs to a specific group of phosphate that is capable of hydrolysing

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phytate to a series of lower phosphate esters of myo-inositol and inorganic phosphate (Pi) according to Sebastian *et al.* (1998). Two types of phytase are known: 3-phytase (E.C 3.1.3.8) and 6-phytases (E.C 3.1.3.26), indicating the initial attack of susceptible phosphate ester bond. Barknik and Szafranska (1987) reported that phytase is present in most cereals. Apart from endogenous phytase from plant ingredients, phytase has been isolated from fungi, bacterial and yeast as well as from rumen and soil microorganisms (Ullah, 1998; Harland and Frolich, 1989. With the industrial production of microbial phytase there has been an increasing interest in the use of phytase to improve animal performance and utilisation of phytate phosphorus (PP) according to Ohwota (2001) and   Igbasan (2002).

The supplementary effects of microbial phytase on the utilisation of phytate phosphorus and on the performance of broilers and layers have been well documented (Simons *et al.*, 1990; Sebastian *et al.*, 1997; Rama Rao *et al.*, 1999; Igbasan, 2002). Majority of the studies on phytase enzymes has been performed on the superficial information of the enzyme provided as marketing strategies by the manufacturers. However, the efficiency of any enzyme preparation as feed additive with regard to improving P availability for the animal depend not only on the type, inclusion rate and the level of activity present, but also on the biochemical characteristics of the enzyme. These properties determine the stability during feed processing and during their passage through the GIT. For the evaluation of the effectiveness of phytase preparations as feed additive, *in vitro* properties like optimum temperature, temperature stability, optimum pH, proteolytic stability and stability in digesta supernatants are of utmost important.

MATERIALS AND METHODS
Estimation of phytase activity
Determination of phytase activities based on the estimation of inorganic orthophosphate released on hydrolysis of phytic acid was routinely performed at 37^0C following the method described by Engelen *et al.* (1984). One unit of enzyme activity was defined as the amount of enzyme that liberates 1micromole of orthophosphate per minute under assay conditions. All measurements were performed in duplicate and repeated in some cases where there was discrepancy in duplicate values.

Determination of Optimum pH
The phytases were incubated with substrate solution, which was prepared in three different buffer solutions, 200mM glycerine pH 2.0-3.0; 200mM sodium (Na) acetate buffer pH 3.5-6.5 and 200mM Tris-HCl buffer pH 7.0-9.0. All buffers were supplemented with 1mM $CaCl_2$. The substrate solution contained 120mM-phytic acid $(C_6H_6P_6Na_{12})$ while the phytases were diluted in 200mM Na acetate buffers pH 5.5. Two millilitres of the enzyme preparation was pre-incubated at the assay temperature for 5 minutes and the enzymes reactions were initiated by addition of 4ml of the substrate solution. The pH of the mixtures was adjusted to different pH before incubation at 37^0C for 60 minutes. The incubation was terminated by the addition of 4ml of the colour mixture (Potassium heptamolybdate + potassium vanadate). The reagents were prepared as described by Engelen *et al.* (1994). The activities of the enzyme were determined using spectrophotometer.

Determination of optimum temperature
The phyases were incubated with substrate

solution which was prepared in three different buffer solutions 200mM glycerine pH 2.0-3.0; 200mM sodium (Na) acetate buffer pH 3.5-6.5 and 200mM Tris-HCl buffer pH 7.0-9.0. All buffers were supplemented with 1mM $CaCl_2$. The substrate solution contained 120mM-phytic acid ($C_6H_6P_6Na_{12}$) while the phytases were diluted in 200mM Na acetate buffers pH 5.5. Two millilitres of the enzyme preparation was pre-incubated at the assay temperature for 5minutes and the enzymes reactions were initiated by the addition of 4ml of the substrate solution. The pH of the mixtures was prepared to correspond to the determined optimum pH of the phytases. The mixtures were incubated for 60 minutes at 30, 40, 50, 60, 65, 70 and 80^{0}C. Their activities were measured on the basis of inorganic ortho-phosphate released (Engelen *et al.*, 1994).

Enzyme stability
The phytases were pre-incubated at 50, 60, and 70^{0}C for 10, 20, 40, 60, and 120 minutes. After the pre-incubation periods the samples were cooled on ice for 30 minutes. They were re-incubated at 37^{0}C and the residual activities of the enzymes were determined as described above.

Protease resistance
This was investigated using pepsin (stomach mucosa) and trypsin (pancreas secretion). The pepsin was suspended with 0.1mHCl (pH 2.0) and trypsin was dispersed in 0.1m sodium hydrogen carbonate (pH 7.0).

Pepsin assay
One millilitre of freshly prepared pepsin solution was carefully mixed with 1ml of a freshly prepared phytase solution in a test tube. The mixture was incubated in a water bath at pH 2.0 and 37^{0}C for 0 and 45 minutes. After incubation, 1ml of the solution was diluted (1:9) with buffer solution (pH 5.5) and thoroughly mixed. Two millilitres of the solution was incubated with 4ml of the substrate solution for 60 minutes at 40^{0}C and pH 5.5 and phytase activities were determined as described for pH behaviour.

Trypsin assay
One millilitre of freshly prepared trypsin solution was carefully mixed with 1ml of phytase solution. The mixture was incubated for 0 and 45 minutes at 40^{0}C and pH 7.0. Dilutions and pH adjustments for phytase activity measurements were the same as described for pepsin (Engelen *et al.*, 1994).

Stability in Digesta supernatants
Digesta samples were collected from ten mature chickens which were killed conventionally. Samples were collected from the crop, stomach (gizzard and proventriculus), duodenum (pylorus to entrance of bile ducts), jejunum (bile duct entrance to meckel's diverticulum) and ileum (meckel's diverticulum to the ileo-cecal junction). The pH of the digesta samples was determined using a pH metre. The pH readings were: 4.84, 3.72, 6.18, 6.26 and 7.45 for crop, stomach, duodenum, jejunum and ileum respectively. The samples were used immediately or frozen at -20^{0}C until use. The digesta samples were diluted 1:1 in distilled thoroughly mixed and centrifuged at 4000rpm for 5minutes. Supernatants were recovered and their pH values were adjusted to correspond to the initial pH values of the different segments of the GIT. The recovered supernatants were held in ice/water bath until use. For assay, 1ml of digesta supernatant was mixed withn1ml of the enzyme solution and the mixture was incubated for 0 and 20minutes at 40^{0}C. For the residual phytase activity measurement, 1ml of the solution was diluted (1:9) with buffer solution (pH 5.5). Two (2ml) of the

solution was then incubated with 4ml of the substrate solution at 40°C for 60 minutes. The activities of the enzymes were then determined using spectrophotometer.

RESULTS AND DISCUSSION
Optimum pH
The plots of the activities against the pH are shown in Figure 1. The enzyme from *Peniophora lycii* displayed considerable activities between pH 3.5-6.5 and pH 3.5-6.0 for *Aspergillus niger*. At least 50 -100% of the activities of the enzymes were found in these pH range .The *Aspergillus niger* had its optimum activity at pH 5.0 while the enzyme from *Peniophora lycii* had its optimum activity at pH 5.5.

The production of free phosphate by phytate hydrolysing enzymes in the GIT of monogastric animals is heavily dependent on the optimal pH of the enzyme preparation. The enzymes studied showed pH optimal at 5.0 and 5.5 for *Aspergillus niger* and *Peniophora lycii*, respectively. This is typical for fungal phytases (McNah, 1993; Shimizu, 1993; Dvoakova, 1998; Wyss *et al.*, 1999a; Vahjen and Simons, 1999 and Igbasan *et al.*, 2000). These pH values occur generally in the upper part of the GIT of poultry (Sebastian *et al.*, 1998; Igbasan *et al.*, 2000), indicating that the enzymes may preferentially be active in this region of the GIT of the host animals. Although, it should be noted that enzymatic activities was determined using pure substrate and under standardised conditions, conditions to be encounter by the enzyme in the host animals would probably be far different.

Optimum temperature
The percentage activities of the phytases at different temperature are shown in Figure 2. The enzyme from *Peniophora lycii* displayed considerable activities at each of the temperatures examined. At least 50-100% of the activities was found in this temperature ranges, having its highest activity measured at 50°C. While the enzyme from *Aspergillus niger* displayed considerable activities (50-100%) at temperatures of 30-55°C. The optimum temperature for this enzyme is the same as for the enzyme from *Peniophora lycii*. The optimum temperature (50°C) of the enzymes is higher than the temperature (38°C) obtainable in the body. Therefore, only between 70-80% of maximal phytase activities could occur in the body.

Enzyme stability
The results of further assays to investigate the stability of the enzymes in aqueous solution after short and long incubation at 50, 60 and 70°C are shown in Figures 3, 4 and 5. From the results, it is clear that the enzymes lose activities as the incubation time increases with increasing temperature. Figures 4 and 5 indicated that enzyme from *Aspergillus niger* loses activities faster than the enzyme from *Peniophora lycii*. The optimum temperature (50°C) recorded for these enzymes agreed with the previous studies (Shimizu, 1993; Dvoakova, 1998; Igbasan *et al.*, 2000 and Lessan *et al.*, 2001). The results of the thermo-stability of the enzymes preparation studied agreed with the result of Igbasan *et al.* (2000) and Lessan *et al.* (2001). The report of Lessan *et al.* (2001) indicated that the residual activity for phytases from *Aspergillus niger and Peniophora lycii* were 52 and 62%, respectively, after a long incubation for 60 minutes at 80°C in sodium acetate buffer at pH 5.5. For an enzyme to be attractive for widespread application as feed additive, it should be able to withstand temperature conditions necessary for pre-treatment of feed. The thermo-stability of the enzymes in aqueous solution does not properly reflect

stability in feed pelleting process. For screening of enzyme thermo-stability, measurement in aqueous solution is adequate but an accurate prediction of heat inactivation during pelleting cannot be made on this basis. Therefore, direct pelleting trial is necessary.

Protease resistance

The results of the resistance of the phytases to protease are presented in Table 1. The results indicate that the tested phytases are susceptible to the protease inactivation when incubated for 0 and 45 minutes.

Both phytases are more susceptible to inactivation by pepsin than trypsin. Also, the susceptibility of the phytase from *Peniophora lycii* is higher than that of the phytase from *Aspergillus niger.*

Susceptibility of feed enzymes to proteolytic degradation is crucial as the rate and site of inactivation of an enzyme is determined by this property. This result agreed in part with the report of Igbasan *et al.* (2000). The differences in these findings could be due to the phytase preparation condition and the ratio of phytase to proteases. Rodriguesz *et al.* (1999) reported that *Aspergillus niger* phytase was gradually inactivated with increasing pepsin. The inactivation effect of pepsin will be of little important due to the optimum pH conditions of the phytases (5.0 and 5.5), which is the pH that conditions the environment of the crop. Among microbial wild type phytases there are resistance enzymes that gradually differ in their susceptibility to proteolytic degradation. These properties need more consideration in further development of feed enzymes.

Stability in digesta supernatants

The results of incubating the phytases in digesta supernatants from different segments of the GIT of chickens are presented in Table 2. The results represented the stability in digesta of each segment of the GIT independent of the result in the preceding segment. The most pronounced effect on the activity of the phytases was observed in supernatants from the small intestine (SI). While the phytases retained most of their initial activity in the crop and a reasonable amount of activity was retained in the stomach. The results also indicated that the phytase from *Peniophora lycii* shows a better performance in all the segments of the GIT than the phytase from *Aspergillus niger.*

The present result demonstrates that the crop of chickens will be the site of highest phytase activity followed by the stomach (proventiculus and gizzard) with negligible activities in the duodenum, jejunum and ileum. Lie Bert *et al.* (1993) had reported similar findings with broiler chickens of 3-4 weeks of age. The present results agreed in part with the report of Igbasan *et al.,* (2000) who reported that the most pronounced effect of the activity of the enzyme from *Peniophora lycii* was observed in supernatant from the stomach. A likely explanation for the present result could be that the phytases were inactivated in the presence of unsuitable pH conditions. Unlike the temperature condition that remained fairly constant within the GIT of chicken, the pH showed considerable variation depending on the site of the GIT examined and the nature of feed ingested.

The phytases suffers considerable loss of activities in the SI due to high pH and the action of secreted proteolytic enzymes present in this region. The result of stability in digesta supernatants confirmed the results obtained for protease resistance as well as optimum pH.

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Table 1: Percentage residual phytase activities after 1hour incubation at 40°C in a solution containing proteases

Name of phytase	Pepsin (%)	Trypsin (%)
Aspergillus niger	47.4	42.2
Peniophora lycii	44.0	45.2

Table 2: Percentage residual phytase activities after 1hour incubation at 40°C in digesta supernatants from various segments of the digestive tract

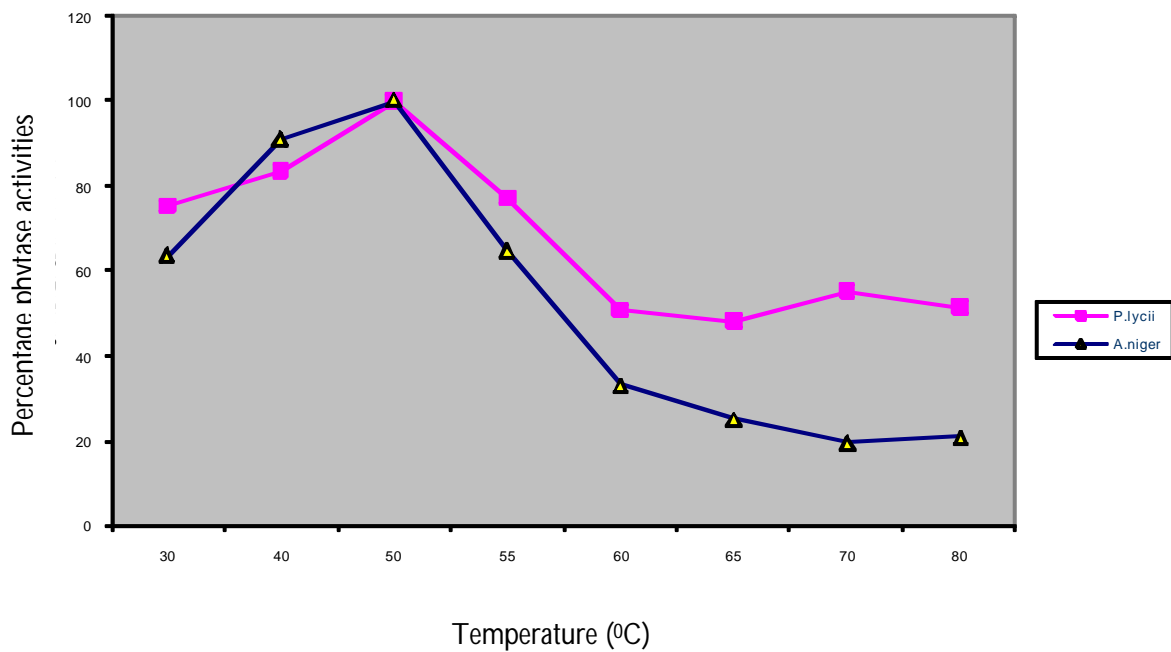| Name of Phytase	Crop	Stomach	Duodenum	jejunum	Ileum
Aspergillus niger	93.8	81.4	45.6	38.3	36.8
Peniophora lycii	98.8	83.7	59.6	51.2	49.5
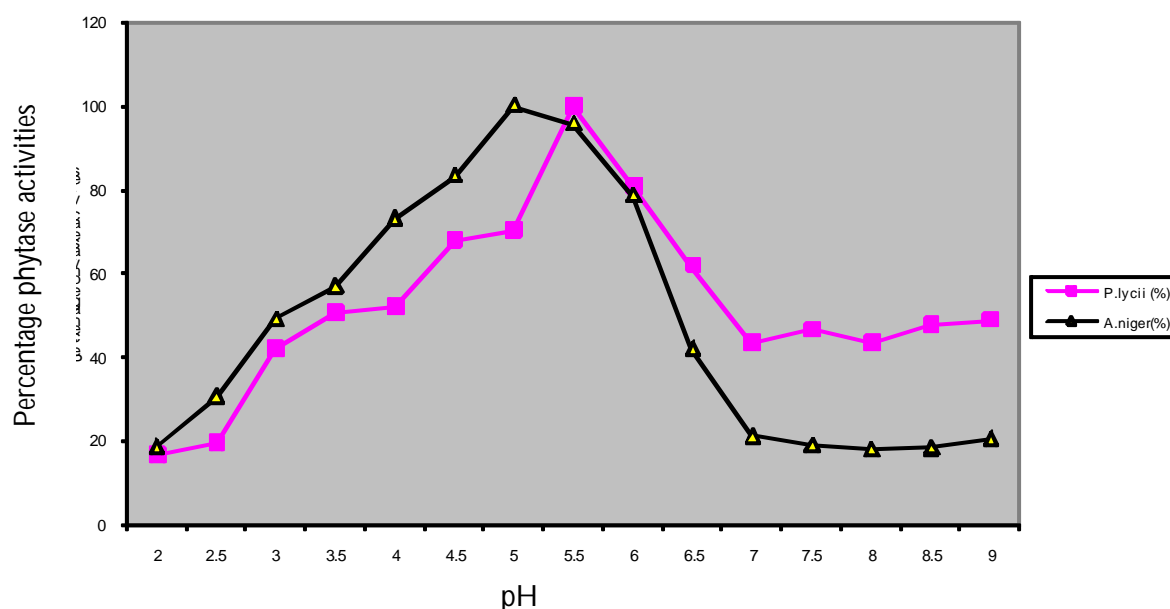
Fig 1: Determination of optimum pH during phytase production

Figure 2: Determination of optimum temperature during phytase production
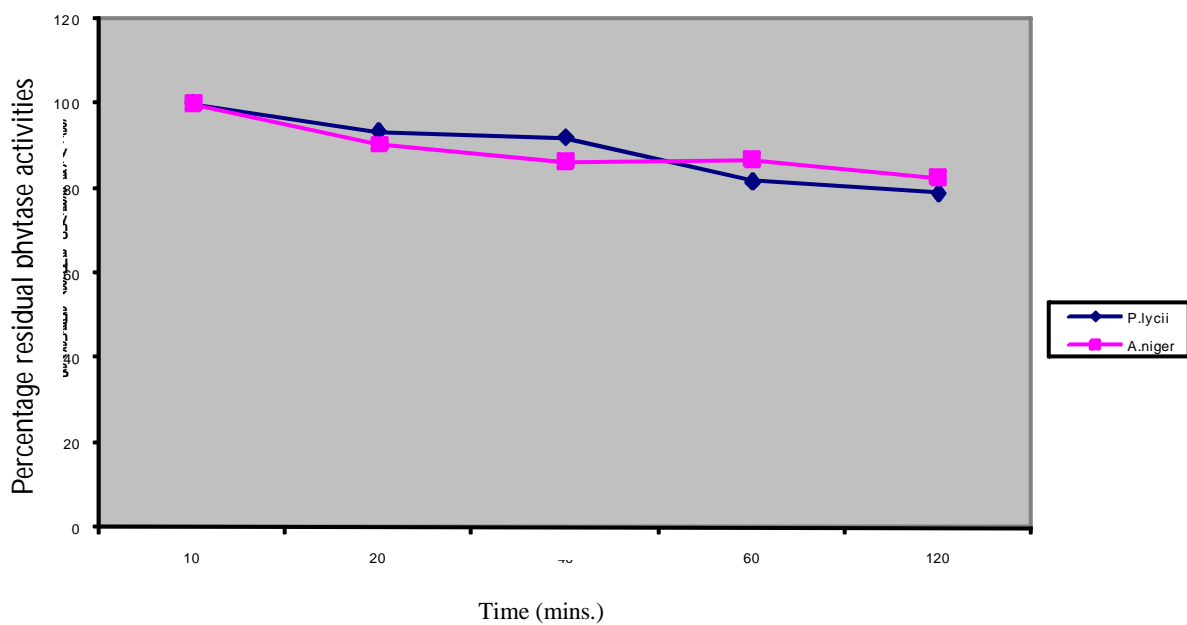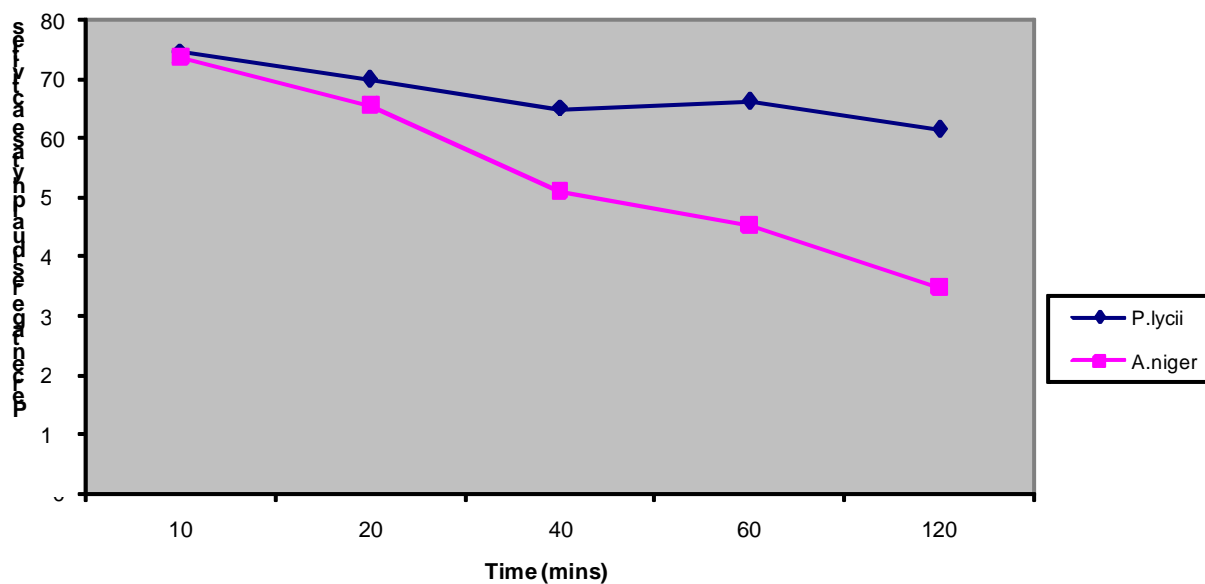

Figure 3: Activities of the phytases at 50°C

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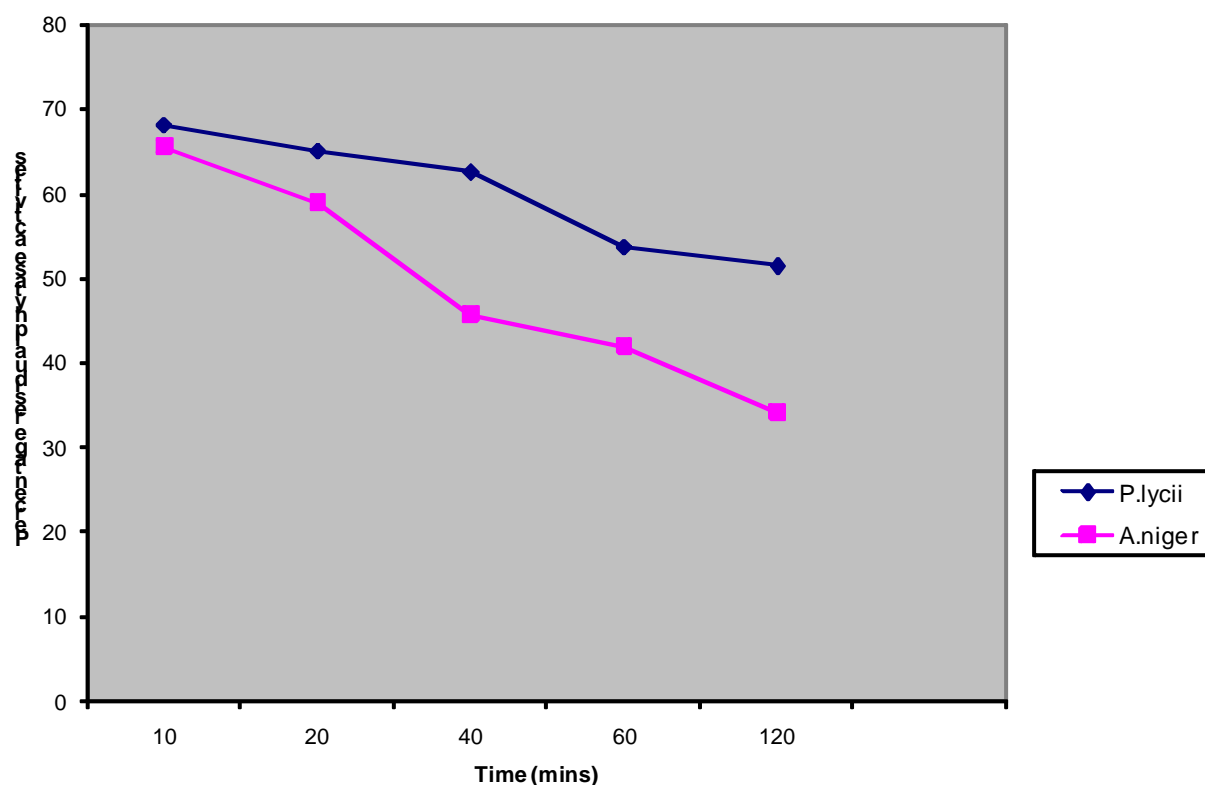**Fig. 4: Activities of the phytases at 60[0]C**

Figure 5: Activities of the phytases at 70[0]C

CONCLUSION

The use of fungal phytases as feed supplement has proved effective in alleviating the negative effects of phytate in livestock diets. However, an enzyme in which there is commercial interest should fulfil a series of predefined quality criteria. In the case of phytate degrading enzymes, which are added to the animal feed, these criteria includes high specific activity, broad substrate specificity, a broad pH optimum and good stability during storage, feed pelleting and passage through the GIT (Wyss *et al.,* 1999b).

Thermo-stability is a particular important issue as feed pelleting is commonly performed at temperature between 65 and 90°C and naturally occurring phytases having the required level of thermo-stability for application in animal feeding have not been found in nature thus far. The poor thermo-stability of the phytase especially those of fungal origin are therefore still a major concern for animal feed application. Therefore, the search for determinant of thermo-stability will continue to arouse research interest.

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