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SCREENING AND PRODUCTION OF PHYTASE FROM SOME BACTERIAL GENERA

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ABSTRACT
The objectives of the present study were to screen for production of phytases by some bacterial genera. Phytases were produced by *E. coli, Bacillus amyloliquefaciens* strain DSM 7 *and Klebsiella sp. Strain ASR1* which were compared to a commercial *Aspergillus* phytase. The *E. coli* phytase was recovered from the fermentation medium with total phytase activity of 2000 units per gram lyophilized supernatant. The *Bacillus amyloliquefaciens* phytase was had an average activity of 0.4 units per milliliter and 24 phytase units per gram after it was lyophilized. The *Klebsiella sp. Strain ASR1* phytase average yield was 7.0 units per milliliter and 88.0 FTU/g after lyophilization, while *Aspergillus* phytase had 5000 units of activity per gram. pH profile, temperature optima, thermostability as well as enzyme kinetics and molecular weight were investigated in the different phytases *in vitro*. *Aspergillus*, *E. coli* and *Klebsiella* phytases displayed optimal activity in acidic pH range while *Bacillus* phytase in neutral pH. *Bacillus* phytase was more resistant to heat treatment. The K_m and V_{max} values for *E. coli* phytase were higher compared to other phytases.

KEY WORDS: Phytase, Bacterial genera, Biochemical characteristics.

INTRODUCTION

Phytases (myo-inositol hexakisphosphate phosphohydrolyses) are enzymes which hydrolyze phytic acid to less phosphorylated myo-inositol derivatives, and in some cases to free myo-inositol, releasing inorganic phosphate (Pi). Phytases are widespread in nature, occurring in fungi (Lassen *et al*., 2001), bacteria (Greiner, Konietzny, & Jany, 1993; Kim, Kim, Bae, Yu, & Oh, 1998a), yeast (Wodzinski & Ullah, 1996), plants (Viveros, Centeno, Brenes, Canales, & Lazano, 2000), as well as at low activities in some animal tissue (Bitar & Reinhold, 1972; Copper & Gowing, 1983; Yang, Matsuda, Sano, Masutani, & Nakagawa, 1991; Chi *et al*., 1999).

The ruminants digest phytic acid through the action of phytases produced by fungi and anaerobic bacteria present as part of their rumen microflora. On the other hand, monogastric animals such as pigs, poultry and fish utilize phytate phosphorus poorly because they are deficient in gastrointestinal tract microbial phytases. Therefore, the low bioavailability of dietary phytate-P requires nutritionists and producers to supplement diets with inorganic P to meet animal P requirement. However, supplemental inorganic phosphate does not diminish the antinutritive effect of phytic acid (Richardson, Higgs, Beames, & McBride, 1985). This problem could be solved by hydrolysis of phytate using supplemental phytases.

Therefore, phytases have become important as industrial enzymes and are the object of extensive research. The aim of the present study was to screen for the production of bacterial phytases from *E. coli, Bacillus amyloliquefaciens* strain DSM 7 *and Klebsiella sp. Strain ASR1*at a laboratory scale and to study their biochemical properties.

MATERIALS AND METHODS

ENZYMES

***Aspergillus* phytase (EC 3.1.3.8)**

This was a commercial enzyme (Natuphos®, 5000G, BASF) used as reference. The activity was 5,000 U/g products.

***E. coli* phytase (EC 3.1.3.26)**

The *E. coli* phytase was produced by *E. coli* as a recombinant protein where the phytase gene from *E. coli* K12 ATCC33965 was cloned on a multi-copy plasmid and over-expressed (Miksch, Neitzel, Filder, Friehs, & Flaschel, 1997). The phytase was recovered from the fermentation medium, which was centrifuged and the supernatant lyophilized.

***Bacillus* phytase (EC 3.1.3.8)**

Bacillus amyloliquefaciens strain DSM7 was selected for its high extracellular phytase activity (Elkhalil, Manner, Borriss, & Simon, 2007). A single fresh colony of *Bacillus amloliquefacies* was inoculated in LB medium (containing10.0 g tryptone, 5.0 g yeast extract, 5.0 gram NaCl, for 1 liter, pH adjusted to 7.0 by 2M NaOH) for 6 h at 37°C with shaking at 200 rpm as preculture. The preculture was inoculated (1:100) either in 1C medium (containing 0.7% peptone from pancreatically digested casein, 0.3% peptone from soymeal papin-digested casein, 0.5% NaCl, 0.5% starch, 10.0% soil extract (500.0 g fertile soil in 1 liter tap water and autoclaved for 30 min and centrifuged, then the volume was completed to 1 liter and autoclaved again)), or in wheat bran medium (containing 20.0 g wheat bran, 0.4 g $(NH_4)_2$ SO_4, 0.2 g $MgSO_4.7H_2O$ for 1 liter and pH adjusted to 7.0 by 2 M NaOH). Incubation was continued for 4, 8, 12, 16, 20, 22, 24 or 26

h to determine the optimum incubation time at 37°C with shaking at 200 rpm. After fermentation, medium was centrifuged at 10,000 rpm for 20 min. The culture filtrate of *Bacillus amyloliquefaciens* was directly lyophilized.

***Klebsiella* phytase (EC 3.1.3.8)**

The phytase gene (*phyK*) from *Klebsiella* sp. strain ASR1 was cloned on a plasmid and expressed in *E coli* DH5alpha. A single colony was inoculated in LB medium amended with 100 µg/ml ampicillin overnight at 37°C as a pre-culture, then the pre-culture was inoculated 1:20 in 2xTBY medium (containing 20 g peptone from tryptonically digested casein, 10 g yeast extract, 5 g NaCl, 100 µg/ml ampicillin and 1% lactose, pH adjusted to 7 by adding 1 ml 1M NaOH to 1 liter medium). The mixtures were then incubated for 28 h at 37°C. The medium was supplemented with two milliliter of 25% lactose solution during cultivation at an operating time of 12h. The phytase was either purified from the preiplasmic contents or from the fermented medium after cells were removed by centrifugation.

Biochemical and biophysical properties of phytases

All enzymes were analyzed for their pH profile, temperature optima and thermostability according to Igbasan *et al*. (2000).

Determination of phytase activity

Phytase preparations were analyzed for phytase activity according to the method of Engelen, Van der Heeft, Randsdorp, & Smit (1994). One unit of phytase was defined as the quantity of enzyme which liberated 1 mmole of inorganic P per min from sodium phytate (0.0051 moles/l) at optimum pH of the respective enzyme at 37°C.

SDS-PAGE

The determination of protein bands and molecular weight pattern of phytases was carried out according to the method described by Laemmli (1970) using SDS-PAGE.

Enzyme kinetics

The enzymes kinetics were performed using a Lineweaver-Burk plot. The Michaelis constant (K_M) and maximal rate (V_{max}) were determined from rates of catalysis, which were measured at different substrate (sodium phytate) concentrations, at the pH and temperature optima for each phytase.

A defined amount of phytase was incubated with substrate [S; Na-phytate] of concentrations of 5.0, 2.0, 1.0, 0.5, 0.2 or 0.1 mM for one hour. The moles of product formed per second (V) were determined. A plot of 1/V versus 1/[S], (the Lineweaver-Burk plot), yields a straight line with an intercept of $1/V_{max}$ and a slope of K_M/V_{max} using a computer program.

RESULTS AND DISCUSSIONS

Enzymes

***E. coli* phytase**

The *E. coli* phytase was produced by fermentation in a bioreactor, after fermentation bacteria were separated by centrifugation. The supernatant was lyophilized and resulting powder contained 2.000 units phytase activity per gram product (Table 1).

TABLE 1. The activity of *Aspergillus*, *Bacillus*, *E. coli* and *Klebsiella* phytase.

Source of phytase	Phytase activity	
	FTU/ml	FTU/g
Aspergillus	ND	5000
Bacillus	0.4	24 (200)*
E. coli	ND	2000
Klebsiella	7.0	88

* lyophilized after concentrated
ND= Not determined

***Bacillus* phytase**

Bacillus phytase was produced by incubation of a single colony of *Bacillus amyloliquefaciens* in soil extract medium (1C medium) or in wheat bran medium (WBM). The yield of phytase was better in 1 C medium than in wheat bran medium (Fig. 1). Furthermore, the optimum incubation time, in 1C medium was 24 h while in wheat bran medium was 20 h. Due to these results it was decided to use 1C medium for further expression of *Bacillus* phytase and to continue the incubation for 24 hours.

The supernatant of fermented medium of *Bacillus amyloliquefaciens* was either directly lyophilized (which resulted in a powder containing 24 phytase units per gram), or concentrated and followed by lyophilization, which yielded a powder containing an average of 200 phytase units per gram (Table 1). These results were similar to findings of Kerovuo, Lauraeus, Nurminen, Kalkkinen, & Apajalahti, (1998) and Kim, Lee, Kim, Yu, & Oh, (1998a).

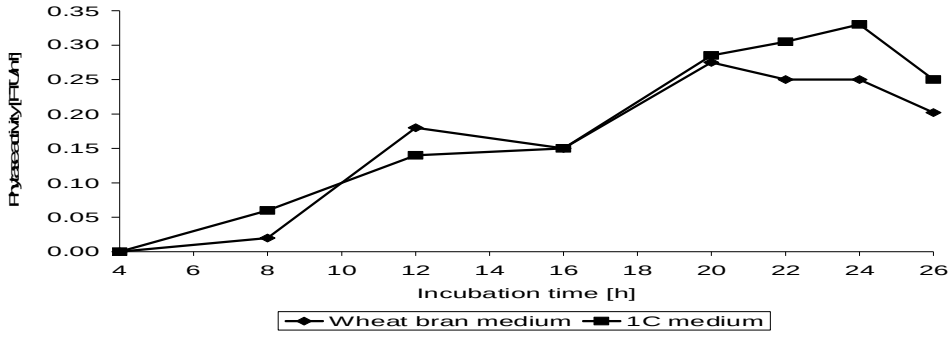

FIGURE 1. Phytase activity during incubation of *Bacillus amyloliquefaciens DSM7* in wheat bran medium and 1C

medium at 37°C.

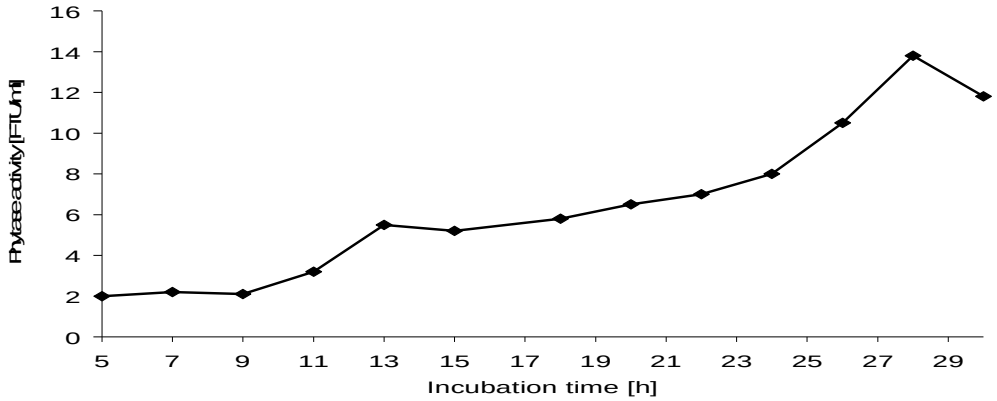

FIGURE 2. Phytase activity of *Klebsiella* phytase produced in 2x TBY medium at 37°C.

Klebsiella* phytase expressed in *E. coli

The phytase gene (*ph K*) from *Klebsiella* sp. strain *ASR1* was cloned and expressed in *E. coli* giving several clones (Igbsan *et al*, 2000). Some strains were confirmed as potent producer strain with phytase activity. The phytase activity was screened from these clones in 2xTBY medium in the supernatant.

Klebsiella phytase was produced in 2xTBY medium containing lactose and 100µg/ml ampicillin, the supernatant contained 10-13 phytase units per milliliter after 28 h incubation time which agrees with Greiner, Haller, Konietzny, & Jany, (1997). Figure 2 shows the phytase activity of *Klebsiella* during incubation in 2x TBY medium for different intervals of time at 37°C. Then the culture filtrate was directly lyophilized, and the lyophilized samples gave 88 phytase units per gram as shown in Table 1.

BIOCHEMICAL & BIOPHYSICAL CHARACTERISTICS

pH behavior

The pH optima of different phytases were determined by exposing phytases to a range of pH conditions. The pH behavior of different phytase preparations is shown in Figure 3.

The pH optima of phytases from *E. coli, Klebsiella or Aspergillus* were in the range 4.5- 5.5 (Fig. 3). In this pH range, these phytases showed activities above 70% of their maxima. The highest activities of *E. coli* phytase and *Klebsiella* were recovered at pH 5.0 while *Aspergillus* phytase at pH 5.5. The *Aspergillus* and *E. coli* phytases displayed considerable activities between pH 4.0 - 6.0 (Fig. 3), estimated between 50 and 100%. Compared to these *Aspergillus* and *E coli* phytases, the *Klebsiella* phytase had a narrow pH range optimum (between 4.5 and 5.5) as seen in Fig 3.

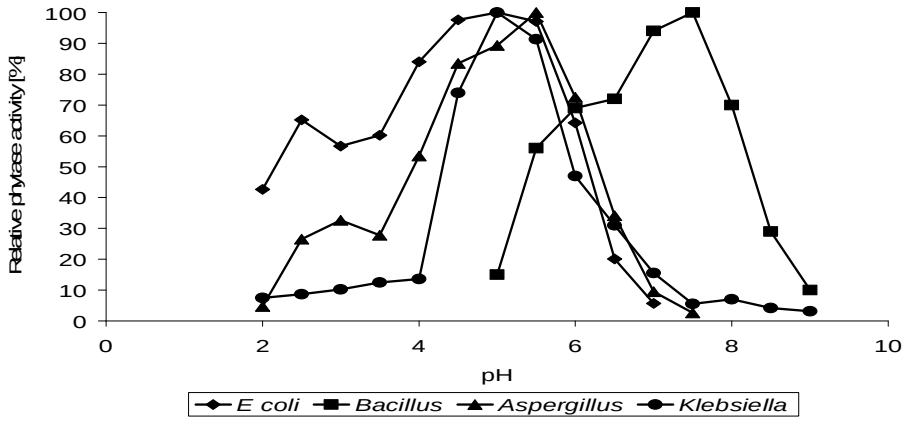

FIGURE 3. pH behaviour of phytase preparations. The data are expressed as percentage of the maximal activity.

In contrast to those phytases, the *Bacillus* phytase displayed a narrow pH optimum between 7.0 and 7.5. The *Bacillus* phytase had a pH optimum around neutrality, at least 50% of its maximal activity could be found between pH 5.5 and 8.0. The measured pH optima for *E. coli*, *Klebsiella* and *Aspergillus* phytases in this study agree with data of Greiner *et al.* (1993), Wyss *et al*. (1999) and Sajidan *et al.* (2004). In contrast to the phytases mentioned before, pH optimum of *Bacillus* phytase preparation was at pH 7.0 to 7.5, which is in good agreement with other reported data (Kim *et al*., 1998a; Kerovuo *et al*, 1998).

THERMAL BEHAVIOR

Optimum temperature

The temperature optima of the phytases are shown in Figure 4. Bacterial phytases exhibited their optimum activities at higher temperature than their fungal counterparts. The *Bacillus* and *E. coli* phytases displayed highest activity at 55°C, while *Aspergillus* and *Klebsiella* phytases expressed their highest activity at 50°C. It is worth mentioning here that the optimum temperatures (50-55°C) of the phytase are higher than body temperature (about 38°C). Accordingly, only 50- 60% of maximal phytase activities can be utilized within the gastrointestinal tract of animals.

The observed temperature optima of the preparation in this study are in general agreement with reports by Griner *et al*. (1993); Shimizu (1993); Kerovuo *et al*. (1998); Igbasan *et al*. (2000) and. Sajidan *et al*. (2004)

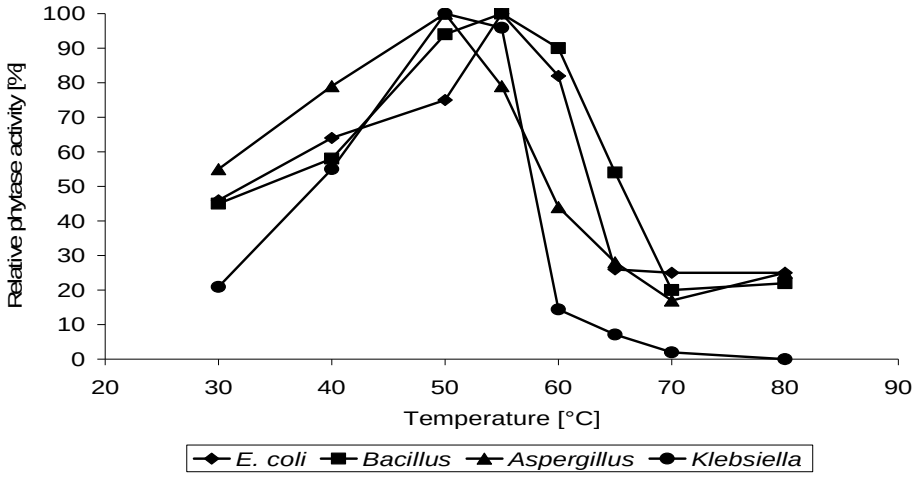

FIGURE 4. Temperature profile of phytase preparations. The data are expressed as percentage of the maximal activity.

Thermostability of phytases

The thermostability of phytases was measured in aqueous solutions at the optimum temperature of each enzyme. Figures 5, 6 and 7 show the residual activity of the phytase preparations after exposure for different periods at 50, 60 or 70°C.

After incubation for two hours at 50 °C, all the bacterial phytases retained close to 100% of their initial activity, while *Aspergillus* phytase retained only 50% of the initial activity (Fig. 5). A similar trend was also observed for *Klebsiella* and *Aspergillus* phytases after incubation for 10 min at 60°C. However, the reduction of phytase activity amounted to 51.2 and 49%, respectively. The *Bacillus* phytase retained 70% of its initial activity after incubation time of 120 min at 60°C (Fig. 6), while at the same incubation conditions, the *E. coli* phytase could retain 42% of its initial activity; while, *Klebsiella* phytase was completely unstable at this condition.

The incubation of phytase preparations at 70°C for different intervals of time revealed that *Klebsiella* phytase was completely unstable. It lost all its initial activity after 10 min at 70°C (Fig. 7). It is worth mentioning that *E coli* and *Aspergillus* phytases lost more than 60% of their initial activity after 20 min incubation time, while in the same condition, *Bacillus* phytase could retain 60% of its initial activity.

Wyss *et al*. (1998) stated that the *Aspergillus niger* was not thermo-stable, neither did it have capacity to refold after denaturation. In an other study Kim *et al*. (1998a) reported that *Bacillus* phytase in presence of $CaCl_2$ was very thermostable showing a 100% residual phytase activity after 10 min incubation at 70°C.

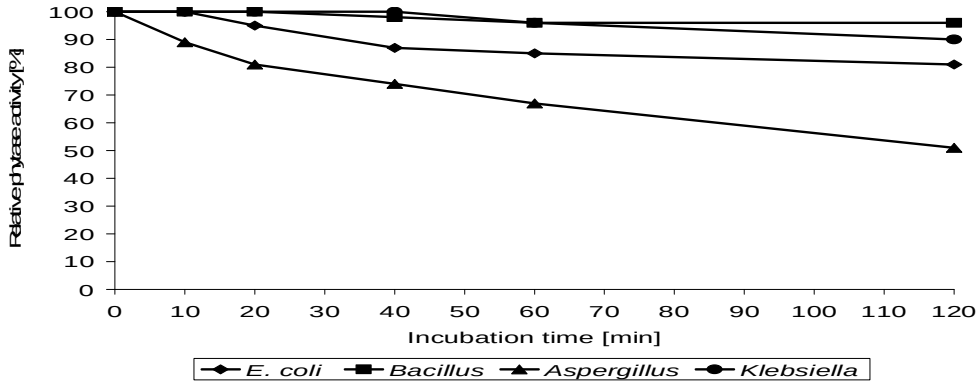

FIGURE 5. Residual enzymatic activity of phytase preparations after exposure for different periods at 50°C. The data are expressed as percentage of activity before heat treatment determined at 37°C for 60 min.

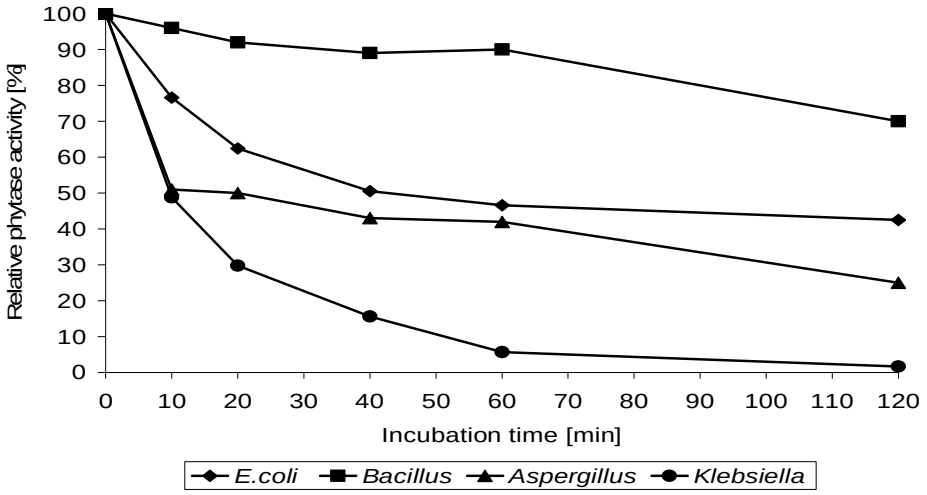

FIGURE 6. Residual enzymatic activity of phytase preparations after exposure for different periods at 60°C. The data are expressed as percentage of activity before heat treatment determined at 37°C for 60 min.

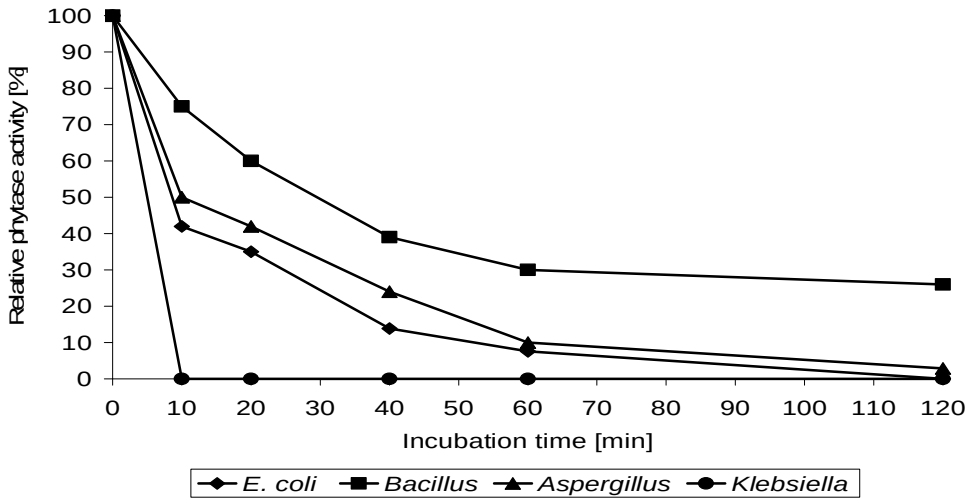

FIGURE 7. Residual enzymatic activity of phytase preparations after exposure for different periods at 70°C. The data expressed as percentage of activity before heat treatment determined at 37°C for 60 min.

Molecular weight determination

Protein extracts from phytases of *Bacillus amyloliquefaciens*, *E. coli*, *Klebsiella* sp. ASR1 and *Aspergillus* were resolved on standard Laemmli gels, renatured and incubated with sodium phytate *in situ*. The phytate impregnated gels were exposed to the counterstaining reagent (cobalt- ammonium vanadate-ammonium molybdate). No zones of clearing were apparent in the vicinity of active phytase protein bands, in contrast to the result of Bae, Yanke, Cheng, & Selinger, (1999).

The polyacrylamide gel showed different bands of protein extracts from *Bacillus*, *E. coli* and *Klebsiella* phytases (Fig. 8). The molecular weight of phytase could not be determined from the different protein bands for two reasons: firstly, it was not possible to specify a protein band for phytase activity in the zymogram. Secondly, the phytases were not purified. The molecular weight of the bacterial phytases is reported to beabout 42 kDa (Greiner *et al*., 1993; Kim *et al*., 1998b and Sajidan 2002). The SDS-Page electrophoresis indicated a single band of 66 kDa for protein extracted from *Aspergillus* phytase at. This agrees with the result obtained by Ullah & Cummins (1987).

The attempts to apply a zymograms technique according to Bae *et al*. (1999) were not successful. This technique should allow a double staining on a SDS- PAGE gel, one general for all proteins and one specific for protein bands with phytase activity. The latter staining did not work in all attempts although the initial samples had sufficient phytase activity. It was possible that the problem was with renaturation of enzymes under study

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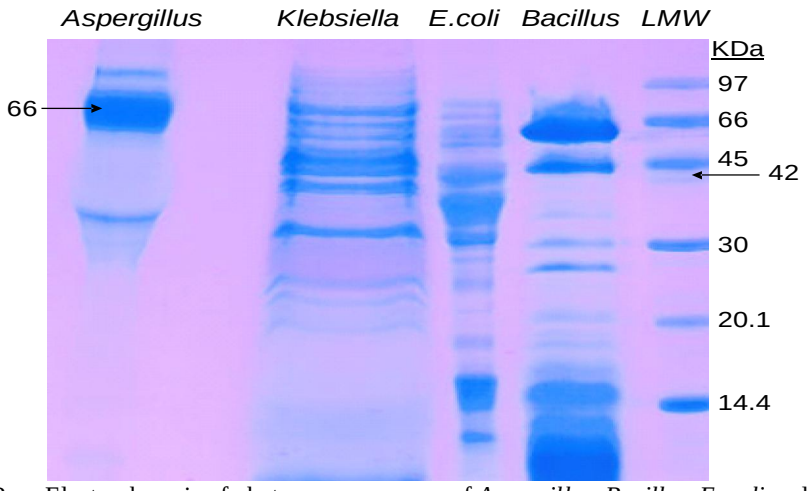

FIGURE 8. SDS Page Electrophoresis of phytase zymmogram of *Aspergillus, Bacillus, E. coli* and *Klebsiella* phytase (LMW = low molecular weight standard)

Enzyme kinetics

The enzyme kinetics of *Aspergillus niger, Bacillus, E coli* and *Klebsiella* phytases were measured at the pH and temperature optima of the different phytases in 200 mM sodium acetate buffer and with different sodium phytate concentrations as substrate. Table 2 shows the K_M and V_{max} of different phytases. The results agree, especially for *Bacillus* and *Klebsiella* phytases, with the results obtained by Greiner *et al*. (1997); Kerovuo *et al*. (1998) and Sajidan (2002). However, the V_{max} values were much closer to reported data, while the K_M values were slightly lower. The K_M and V_{max} values of *E. coli* phytase were highly variable compared to values published in the literature (Rodriguez, Prres, Han, & Lei, 1999; Golovan, Wang, Zhang, & Forsberg, 2000). This may have been due to different enzyme expression systems.

TABLE 2. Enzyme Kinetics of phytase in sodium phytate as substrate.

Source of phytase	V_{max} (µmole min^{-1} mg^{-1})	K_M [mM]
Aspergillus	227.3	0.53
Bacillus	140.8	0.19
E. coli	1428.0	0.17
Klebsiella	200.0	0.18

CONCLUSIONS

The following could be concluded from this study: (1) *E. coli* and *Aspergillus* phytases have a similar pH profile. (2) *Klebsiella* phytase may have a similar efficiency *in vivo* to the *E. coli* phytase but is of limited practical use as feed additive due to its high heat-sensitivity. However, this enzyme could have a potential similar to *E. coli* phytase in mesh feed or in case of spraying after pelleting. (3) *Bacillus* phytase is the only enzyme having potential heat stability. (4) *E. coli* phytase was efficiently expressed and the activity in the lyophilized cell-free culture medium was 2000 phytase units per gram in the range of commercial phytase preparations (i.e. *Aspergillus* phytase). The expression rate of the *Bacillus* and *Klebsiella* phytases was very low; further research for high expression rate of both phytases may be required.

REFERENCES

Bae, H. D., Yanke, L. J., Cheng, K. J. and Selinger L. B. (1999). A novel staining method for detecting phytase activity. *J. Microbiol. Methods.* 39, 17-22.

Bitar, K. and Reinhold, J. G. (1972) Phytase and alkaline phosphatase activities in intestinal mucose of rat, chicken, calf, and man. *Biochim. Biophys. Acta* 268, 442-452.

Chi, H., Tiller, G. E., Dasouki, M. J. *et al.* (1999). Multiple inositol polyphosphate phosphatase: evolution as a distinct group within the histidine phosphatase family and chromosomal localization of the human and mouse genes to chromosomes 10q23 and 19. *Genomics* 56, 324-336.

Copper, J. R. and Gowing, H. S. (1983) Mammalian small intestine phytase. *Br. J. Nut*r. 50, 673-678.

Elkhalil, E. A. I., Manner, K., Borriss, R. and Simon, O. (2007). In vitro and in vivo characteristics of bacterial phytases and their efficacy in broiler chickens. British Poultry Science, 48 (1), 64-70.

Engelen, A.J., Van der Heeft, F C., Randsdorp, P.H.G. and Smit, E.L.C. (1994). Simple and rapid determination of phytase activity. *J. AOAC Inter*. 77, 760-764.

Golovan, S., Wang, G., Zhang, J. and Forsberg, C.W. (2000).Characterization and overproduction of *Escherichia coli appA* encoded bifunctional enzyme that exhibits both phytase and acid phosphatase activities. *Can. J. Microbiol.* 46, 59-71.

Greiner, R., Haller, E., Konietzny, U. and Jany, K. D. (1997) Purification and characterization of a phytase from *Klebsiella terrigen*a. *Arch. Biochem. Biophy*s. 341, 201-206.

Greiner, R., Konietzny, U. and Jany, K. D. (1993) Purification and characterization of two phytases from *Escherichia coli*. *Arch. Biochem. Biophys*. 303, 107-113.

Igbasan, F.A., Männer, K., Miksch, G., Borriss, R., Farouk, A. and Simon, O. (2000). Comparative studies on the *in vitro* properties of phytases from various microbial origin. *Arch. Anim. Nutr*. 53, 353-373.

Kerovuo, J., Lauraeus, M., Nurminen, P., Kalkkinen, N. and Apajalahti, J. (1998) Isolation, Characterization, Molecular Gene Cloning, and Sequencing of a Novel Phytase from *Bacillus subtilis*. *Appl. Environ. Microbio*l. 64, 2079-2085.

Kim, Y. O., Kim, H. K., Bae, K. S., Yu, J. H. and Oh, T. K. (1998a) Purification and properties of a thermostable phytase from *Bacillus* sp. DS11. *Enzyme Microbiol. Techno*l. 22, 2-7.

Kim, Y. O., Lee, J. K., Kim, H. K., Yu, J. H. and Oh, T. K. (1998b) Cloning of the thermostable phytase gene *(phy*) from *Bacillus* sp. DS11 and its overexpression in *Escherichia col*i. *FEMS Microbiol. Lett*. 162, 185-191.

Laemmili, U. K. (1970).Cleavage of structural proeins during the Assembly of the head of Bacteriophage T4. *Nature*. 227,680-685.

Lassen, S. E., Breinholt, J., Ostergaard, P. R., Brugges, R., Bischoff, A., Wyss, M. and Fuglsang C.C. (2001). Espression, gene cloning, and characterization of five phytases from four basidiomycete fungi: *Peniophora lycii, Agrocybe pediades, a Ceriporia sp.* and *Trametes pubescens. Appl. Environ Microbiol*. 67, 4701-4707.

Miksch, G., Neitzel, R., Filder, E., Friehs, K. and Flaschel, E. (1997). Extracellular production of a hybrid β-glucanase from *Bacillus* by *Escherichia coli* under different cultivation cinditions in shaking cultures and bioreactors. *Appl. Microbiol. Biotechnol*. 47, 120-126.

Richardson, N. L., Higgs, D. A., Beames, R. M. and McBride, J. R. (1985). Influence of dietary calcium, phosphorus, zinc and sodium phytate level on cataract incidence, growth and histopathology in juvenile chinook salmon (Oncorhynchus tshawytscha). *J. Nutr*. 115, 553-567.

Rodriguez, E., Prres, J.M., Han, Y. and Lei, X.G. (1999). Different sensitivity of recombinant *Aspergillus niger* phytase (r-phyA) and Escherichia coli pH 2.5 acid phosphatase (r-AppA) to trypsin and pepsin in vitro. *Arch. Biochem. Biophys*. 365, 262-267.

Sajidan (2002). Molecular and characterization of phytase (myo-inositol hexakisphosphate hydrolase) and of phosphatase from isolated bacteria in Indonesian rice feld (*Klebsiella pneumoniae*). PhD. Thesis, Humboldt university Berlin, Germany.

Sajidan, A., Farouk,A., Greiner, R., Pungblut, P., Müller, E.-C. and Borriss,R. (2004). Molecular and physiological characterization of a 3-phytase from soil bacterium *Klebsiella sp. ASR1.Appl. Microbiol. Biotechnol*. 65, 110-118.

Shimizu, M. (1993) Purification and characterization of phytase and acid phosphatase by *Aspergillus oryzae* K1. *Biosci. Biotechnol. Bioche*m. 57, 1364-1365.

Ullah, A. H. J. and Cummins, B. J. (1987). Immobilization of *Aspergillus ficuum* extracellular phytase on fractogel. *Biotechn. Appl. Biochem*. 9, 380-388.

Viveros, A., Centeno, C., Brenes, A., Canales, R. and Lazano, A. (2000). Phytase and acid phosphatase activities in plant feedstuffs. *J. Agric. Food Chem.,* 48, 4009-4013.

Wodzinski, R. J. and Ullah, A. H. J. (1996) Phytase. *Adv. Appl. Microbiol*. 42, 263-302.

Wyss, M., Brugger, R., Kronenberger, A., Remy, R., Fimbel, R., Oesterhelt, G., Lehmann, M. and van Loon, A. P. G. M. (1999) Biochemical characterization of fungal phytases *(myo*-inositol hexakisphosphate phosphohydrolases): catalytic properties. *Appl. Environ. Microbiol*. 65, 367-373.

Wyss, M., Pasamontes, L., Remy, R., Kohler, J., Kusznir, E., Gadient, M., Muller, F. and van Loon, A. P. G. M. (1998) Comparison of the thermostability properties of three acid phosphatases from molds: *Aspergillus fumigatus* phytase, *A. niger* phytase, and *A. niger* pH 2.5 acid phosphatase. *Appl. Environ. Microbiol*. 64, 4446-4451.

Yang, W. J., Matsuda, Y. Sano, S., Masutani, H. and Nakagawa (1991) Purification and characterization of phytase from rat intestinal mucos*a. Biochim. Biophys. Acta* 1075, 75- 82.