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Full Length Research Paper

Purification and characterization of a phytase from *Mitsuokella jalaludinii*, a bovine rumen bacterium

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The phytase from *Mitsuokella jalaludinii*, a novel phytase-producing rumen bacterium, was purified 120-fold to near homogeneity and characterized. The phytase was completely cell-associated and about half of the enzyme activity was released when the bacterial cells were incubated with 1.5 mol/l KCl solution for 8 h. The optimum pH for phytase activity was in the range of 4.0 to 5.0 and the optimum temperature was 55 to 60°C. The phytase was stable at pH 4.0 to 7.0. It was highly specific to sodium phytate as the substrate, strongly inhibited by Cu^{2+}, Zn^{2+}, Fe^{2+} and Fe^{3+}, significantly stimulated by Ba^{2+} and slightly stimulated by Mn^{2+} and Ca^{2+}. The metal ions chelating agents, namely trisodium citrate, potassium sodium tartrate and EDTA, did not show any inhibitory effect on the phytase activity of *M. jalaludinii.* The phytase was also not inhibited by sulfhydryl inhibitor, 2-mercaptoethanol, and a carboxyl inhibitor, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC).

Key words: *Mitsuokella jalaludinii*, bacterial phytase, rumen bacteria.

INTRODUCTION

Phytases, which catalyze the hydrolysis of phytate into inorganic phosphate, inositol and inositol mono- to penta-phosphates, appertain to the family of histidine acid phosphatases (Pasamontes et al*.,* 1997). Phytases are present in many plants and microorganisms, especially fungi. Phytases have also been reported to be present in several bacterial species such as *Pseudomonas* spp. (Richardson and Hadobas, 1997), *Enterobacter* sp. (Yoon et al*.,* 1996), *Klebsiella aerogenes* (Tambe et al., 1994), *Klebsiella terrigena* (Greiner et al., 1997), *Klebsiella pneumonia* (Sajidan et al., 2004), *Escherichia coli* (Greiner et al., 1993), *Bacillus subtilis* (Powar and Jagannathan, 1982; Shimizu, 1992; Kerovuo et al., 1998), *Citrobacter braakii* (Kim et al., 2003) and *Lactobacillus amylovorus* (Sreeramulu et al., 1996). The best characterized phytase, so far, is that from *Aspergillus ficuum*. The phytase from *A. ficuum* NRRL 3135 was found to be a combination of activities from an acid phosphatase and an 85 kDa glycosylated protein with a preference for phytate as a substrate (Ullah, 1988). The primary structure of phytase from *A. ficuum* has also been determined using the chemical sequencing method (Ullah, 1988). The enzymatic properties of phytases from *Aspergillus niger, Aspergillus terreus, Aspergillus fumigatus, Emericella nidulans, Myceliophthora thermophila* and *E. coli* were characterized in detail by Wyss et al. (1999). Of the bacterial phytases studied, only phytases from *Enterobacter* sp. (Yoon et al*.,* 1996), *B. subtilis* (Powar and Jagannathan, 1982), *B. amyloliquefaciens* (Ha et al*.,* 1999) and *L. amylovorus* (Sreeramulu et al., 1996) are extracellular while the others are cell-bound (Greiner et al., 1993; Tambe et al., 1994; Jareonkitmongkol et al., 1997; Yanke et al*.,* 1999).

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Most bacterial phytases have similar optimum temperatures as fungi (50 to 60°C), but with a wider range of optimum pH (4.0 to 7.5) (Yanke et al., 1999).

Although phytase activity from rumen bacteria was first reported more than fifty years ago (Raun et al., 1956), it was only much later that interest was generated to identify phytase-producing rumen microorganisms. Yanke et al. (1998) demonstrated that phytase activity was present in numerous ruminal bacterial strains, particularly *Selenomonas ruminantium*. In a subsequent study, Yanke et al. (1999) undertook to characterize phytase from *S. ruminantium*, and later D'Silva et al. (2000) confirmed that the phytase of *S. ruminantium* was distributed on the outer layer of the bacterial cell wall. Except for these few reports on *S. ruminantium*, scanty information is available on the characterization of phytase from other rumen bacteria. *Mitsuokella jalaludinii* is a phytase-producing bacterial species isolated from the rumen of cattle (Lan et al., 2002a) and it has been found to produce high phytase activity (Lan et al., 2002b, c, 2010). The study was carried out to purify and characterize phytase from *M. jalaludinii*. To our knowledge, this is a first report on the purification and properties of a phytase from *M. jalaludinii*.

MATERIALS AND METHODS

Culture conditions and sample preparation

M. jalaludinii was maintained in an MF_1 medium (pH 7.0) containing 10 g glucose, 4 g cellobiose, 4 g soluble starch, 10 g trypticase peptone, 4 g yeast extract, 1.5 g L-cysteine.HCl.H_2O, 100 ml mineral solution, 50 ml 8% Na_2CO_3, 1 ml 0.05% hemin, 1 ml 0.1% resazurin and 848 ml distilled water. The mineral solution comprised 0.45 g NaCl, 4.49 g $(NH_4)_2SO_4$, 0.25 g $CaCl_2$, 0.94 g $MgSO_4.7H_2O$, 3.45 g KCl and 1000 ml distilled water. The medium was prepared using the anaerobic techniques of Hungate (1969). For phytase production, MF_1 medium containing 0.5% sodium phytate was used. Sodium phytate solution was prepared by dissolving 5.0 g sodium phytate in 100 ml of autoclaved MF_1 medium, and the pH adjusted to 7.1 before bubbling with oxygen-free CO_2 until colorless. The solution was filter-sterilized and 100 ml of it was added aseptically into 900 ml of autoclaved MF_1 medium. *M. jalaludinii* was cultured in the medium anaerobically at 39°C for 8 h, after which the culture was centrifuged at 8000 × *g* for 20 min at 4°C. The pellet was harvested (from 4 L of culture), washed twice with 0.1 mol/l acetate buffer (pH 5.0) and used for phytase purification.

Purification of phytase

The washed bacterial cell pellets were mixed with 250 ml 0.1 mol/l cold acetate buffer (pH 5.0) (4°C) containing 1.5 mol/l KCl and incubated overnight (8 h) after which it was centrifuged at 8000 × *g* for 15 min. The cell free supernatant (crude extract) was collected and concentrated to 50 ml using a concentrator (Centriplus™, USA, molecular weight cut-off is 10,000Da). The concentrated sample was dialyzed against 20 mmol/l acetate buffer (pH 5.0) and then used for ammonium sulfate precipitation at 45 to 85% saturation.

The fractions of precipitation showing phytase activities were dissolved in a minimum volume of 0.1mol/l acetate buffer (pH 5.0), pooled and dialyzed against 20 mmol/l acetate buffer (pH 5.0). Any precipitation formed during dialysis was removed by centrifugation at 10,000 × *g* for 30 min. All these operations described were carried out at 4 ºC.

Anion exchange chromatography was carried out using a Bio-Logic HR Chromatography System (Bio-Rad) under room temperature. The dialyzed ammonium sulfate precipitated fraction was loaded onto a UNO™ Q 1 anion column (Bio-Rad) equilibrated with 20 mmol/l acetate buffer (pH 4.5). The column was washed with 3 ml of the same buffer and the protein bound was eluted with a linear gradient from 0 to 1.0 mol/l NaCl in 20 mmol/l acetate buffer (pH 4.5). The flow rate was 1 ml/min and fractions of 1 ml each were collected. Fractions showing phytase activity were pooled, dialyzed against 20 mmol/l acetate buffer (pH 5.0) and concentrated using the method previously described.

The concentrated fraction thus obtained was loaded again onto a UNO™ Q 1 anion column. The column equilibration, buffer used, flow rate and collected volume were the same as those described. The eluted fractions showing phytase activities were pooled, dialyzed against 20 mmol/l acetate buffer (pH 5.0) and concentrated to about half of the original volume. After concentration, protein purification was monitored by gradient polyacrylamide gel electrophoresis (SDS-PAGE). Gradient polyacrylamide gel electrophoresis was conducted using the method of Laemmli (1970).

Phytase and protein assay

For the whole-cell phytase activity determination, the sample preparation and method for measuring phytase activity were the same as that described by Yanke et al. (1998) except that the enzyme reaction time was set to 15 min. For the determination of purified phytase (cell-free) activity, purified phytase was appropriately diluted with 0.1 mol/l acetate buffer (pH 5.0). Then 0.01 ml of the diluted phytase sample was mixed with 1.24 ml acetate buffer containing 0.2% sodium phytate and incubated at 39°C for 15 min (this mixture was designated as the standard assay mixture). The reaction was terminated by adding 1.25 ml 5% trichloroacetic acid (TCA). The released phosphorus was determined by the method of Heinonen and Lahti (1981). A unit of phytase activity is defined as the amount of enzyme that liberates 1 µmol P/min under the given assay conditions. Protein concentration was measured by the method of Bradford (1976) using a protein assay kit (Bio-Rad Lab., Richmond, CA) with bovine serum albumin as the standard.

Characterization of phytase activity

The purified phytase was used for phytase activity characterization. All tests were repeated three times, each with triplicates.

Effect of temperature on phytase activity

The substrate solution (0.1 mol/l acetate buffer containing 0.2% sodium phytate, pH 5.0) was pre-incubated at the experimental temperatures for 5 min, after which 0.01 ml of diluted phytase solution (about 0.4 U phytase) was incubated with 1.24 ml of substrate solution at 35, 39, 45, 50, 55, 60, 65, 70 and 75°C for 15 min. The released P was measured and the phytase activity was calculated by the method previously described herein.

Effect of pH on phytase activity and enzyme stability

The diluted phytase solution (0.01 ml) was mixed with various buffers (1.24 ml) containing 0.2% sodium phytate and incubated at 39°C for 15 min. The buffers used were 0.1 mol/l glycine-HCL (pH 2.0, 2.5, 3.0 and 3.5), 0.1 mol/l sodium acetate buffer (pH 4.0, 4.5, 5.0 and 5.5), 0.1 mol/l sodium cacodylate-HCL (pH 6.0, 6.5 and 7.0) and 0.1 mol/l Tris-HCL (pH 7.5 and 8.0).

To investigate the effect of different pH values on phytase stability, 0.04 ml of purified phytase was mixed with 0.16 ml buffer and incubated at room temperature for 60 min. The buffers used were 0.2 mol/l citric-NaOH-HCL (pH 1.5, 2.0, 2.5 3.0, and 3.5), 0.2 mol/l sodium acetate buffer (pH 4.0, 4.5, 5.0 and 5.5), 0.2 mol/l sodium cacodylate-HCL (pH 6.0, 6.5 and 7.0) and 0.2 mol/l tris-HCl (pH 7.5 and 8.0). At the beginning (0 min) and the end (60 min) of the incubation period, 0.02 ml of the mixture (phytase + buffer) was mixed with 1.23 ml of 0.2 mol/l acetate buffer containing 0.2% sodium phytate (pH 5.0) and incubated at 39°C for 15 min. The released P was then determined as described herein.

Substrate specificity

Twelve (12) phosphate esters were used as substrates. They were sodium phytate, α-D-glucose-1-phosphate, NADP, β-naphthyl phosphate, D-fructose-1,6-diphosphate, ATP, D-fructose-6-phosphate, ρ-nitrophenyl phosphate, α-naphthyl acid phosphate, DL-α-glycerophosphate, phosphoglycolic acid, and mannose-6-phosphate. The substrates were added separately to 0.1 mmol/l acetate buffer solution to reach a final concentration of 2 mmol/l and the pH was adjusted to 5.0. For every substrate, the assay mixture was prepared by mixing 0.01 ml diluted phytase solution with 1.24 ml substrate solution and the control assay mixture was prepared by mixing 0.01 ml of thermally inactivated diluted phytase solution (0 U phytase) with 1.24 ml substrate solution. The thermally inactivated diluted phytase solution was prepared by incubating 0.5 ml of diluted phytase solution (in a clean glass tube) at 100°C for 10 min. The assay and control mixtures were incubated at 39°C for 15 min, after which the reaction was terminated by adding 1.25 ml of 5% TCA. The P concentrations in the assay and control mixtures were determined separately using the method of Heinonen and Lahti (1981). The difference in P concentrations between the assay and control mixtures was used to calculate enzyme activity. The relative activity of phytase using sodium phytate as a substrate was considered as 100%.

Effects of reagents, ions and phosphate on phytase activity

The reagents used were NaN_3, EDAC, 2-mercaptoethanol, trisodium citrate, potassium sodium tartrate and EDTA and the ions used were $MgCl_2$, $MnCl_2$, $ZnCl_2$, $CuCl_2$, $BaCl_2$, $CoCl_2$, $CaCl_2$, $FeSO_4$ and $FeCl_3$. The reagent or cation solutions were prepared separately by dissolving appropriate amounts of each reagent or mineral compound in 0.1 mol/l acetate buffer (pH 5.0) to reach a final concentration of 625 mmol/l. For phytase activity determination, assay mixture was obtained by adding 0.01 ml of diluted phytase solution (0.394 U phytase) and 0.1 ml of reagent or cation solution to 1.14 ml 0.1 mol/l acetate buffer containing 0.2% sodium phytate (pH 5.0). The final concentration of the reagent or ion in the phytase assay mixture was 5 mmol/l. The standard assay mixture (0.01 ml of diluted phytase solution + 1.24 ml of 0.1 mol/l acetate buffer containing 0.2% sodium phytate) without reagent or cation supplementation was used as the control. All assay mixtures were incubated at 39°C for 15 min. Any precipitation formed during the reaction was removed by centrifugation at 16,000 × *g* for 15 min prior to spectrophotometric measurement of released P.

KH_2PO_4 was used to investigate the effect of phosphate on phytase activity (whole cell phytase and purified phytase). Different amounts of phosphate (KH_2PO_4) were added separately to 0.1 mol/l acetate buffers containing 4 mmol/l phytate (pH 5.0) (substrate solution) and to 0.1 mol/l sodium acetate buffers (pH 5.0) containing *M. jalaludinii* whole-cell phytase (50 U phytase/ml) or diluted purified phytase (50 U phytase/ml) to reach a final concentration of 0.0, 1.0, 2.0, 4.0, 6.0, 8.0 and 10.0 mmol/l. The pH of all the solutions was kept at 5.0 by adjusting the pH with 2.0 mol/l HCl if necessary. For phytase activity determination, 0.01 ml of phytase solution and 1.24 ml of substrate solution (pH 5.0) (both solutions contained the same phosphate concentration) were mixed and incubated at 39°C for 15 min. The original phytase activity in the assay mixture was about 0.4 U/ml. *A. ficuum* phytase (Sigma) solution (50 U phytase/ml) instead of *M. jalaludinii* phytase was used in the control assay mixture.

Distribution of enzymes

The fractionations of extracellular, periplasmic, cell-bound, and intracellular enzymes were prepared using the method of Yoon et al. (1996). In order to verify whether the phytase of *M. jalaludinii* was associated with the membrane structure of the cells, the washed intact cells collected from 10 ml of bacterial culture (10 h incubation) were incubated in one-third original volume of 0.1 mol/l acetate buffer (pH 5.0) containing an appropriate amount of ionic compounds (0.25 to 3.5 mol/l KCl solution) or non-ionic compounds (1.2% deoxycholate, 1.2% Triton X-100, and 1.2% Tween 80) for 10 h at 4°C. After incubation, the solution was centrifuged at 8000 × *g* for 15 min at 4°C. The supernatant and cells were harvested and the latter were resuspended in 10 ml of 0.1 mol/l acetate buffer (pH 5.0). The phytase activities of the different fractions were determined using the method previously described herein.

Statistical analysis

Data obtained were analyzed using the General Linear Model (GLM) procedure for analysis of variance (SAS Institute, 1997). Significant differences among the treatment means were separated by the Duncan's new multiple range test at 5% level of probability.

RESULTS

Purification of phytase

A summary of the purification scheme is shown in Table 1. Through ammonium sulfate precipitation and anion chromatographic purification (twice), the phytase of *M. jalaludinii* was purified to about 120-fold and was eluted as a single peak (Figure 1). However, this active fraction migrated as two very close bands when subjected to SDS-PAGE (Figure 2). Attempts to further purify the fraction were unsuccessful as there was a very low recovery rate after the second anion exchange chromatography. This partially purified enzyme was used for the characterization of phytase activity.

Table 1. Purification scheme of phytase from *Mitsuokella jalaludinii*

Step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (folds)
Crude extract	250	240	2124	5.9	1
$(HN_4)_2SO_4$ precipitation	25	110	1892	17.2	2.9
UNO Q 1 column	8	9.2	1216	137.9	23.4
UNO Q 1 column	4	0.6	423	705.0	119.5

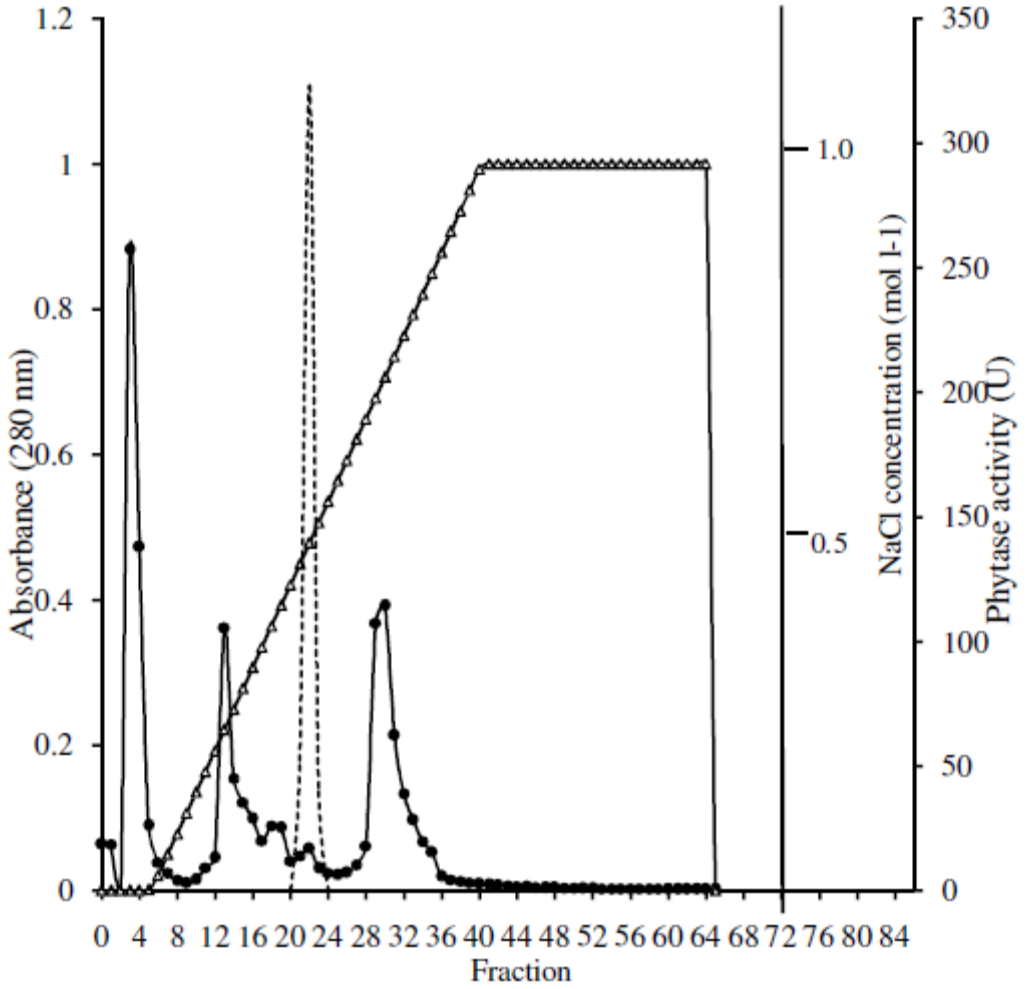

Figure 1. Phytase-active fractions from the second anion exchange chromatography. (•) absorbance (280 nm), (Δ) NaCl gradient (mol/l), (- - -) phytase activity (U).

Effect of temperature on phytase activity

Phytase activity increased with increasing temperatures, reaching a maximum at 55 to 60°C and then declining very rapidly till it was almost undetectable at 75°C (Figure 3).

Effect of pH on phytase activity and stability

Phytase of *M. jalaludinii* was found to be most active in the range of pH 4.0 to 5.0 at 39°C and virtually inactive at pH 8.0 and pH 2.0 to 2.5 (Figure 4). The effect of pH on phytase stability was tested in the pH range of 1.5 to 8.0.

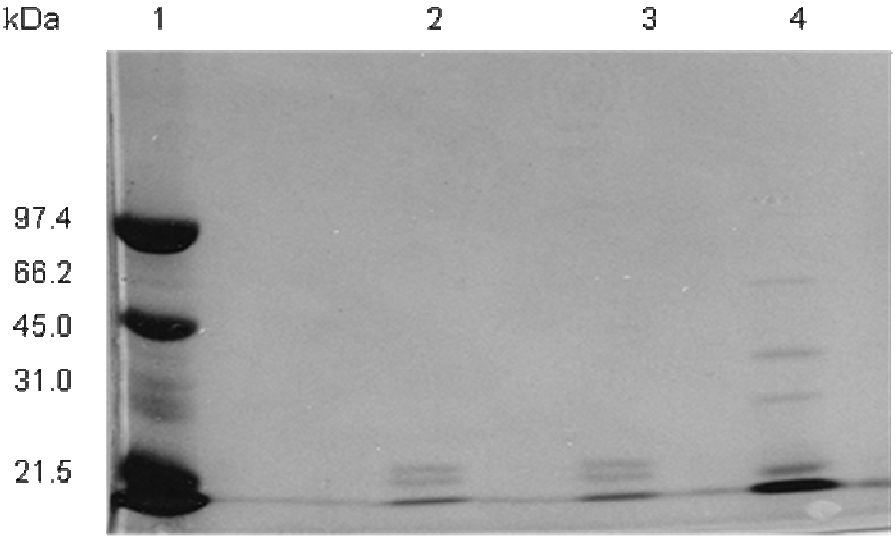

Figure 2. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of purified phytase. Lane 1: standard protein; Lane 2: protein with phytase activity from second anion chromatography; Lane 3: protein with phytase activity from the first anion chromatography; Lane 4: protein with phytase activity from ammonium precipitation.

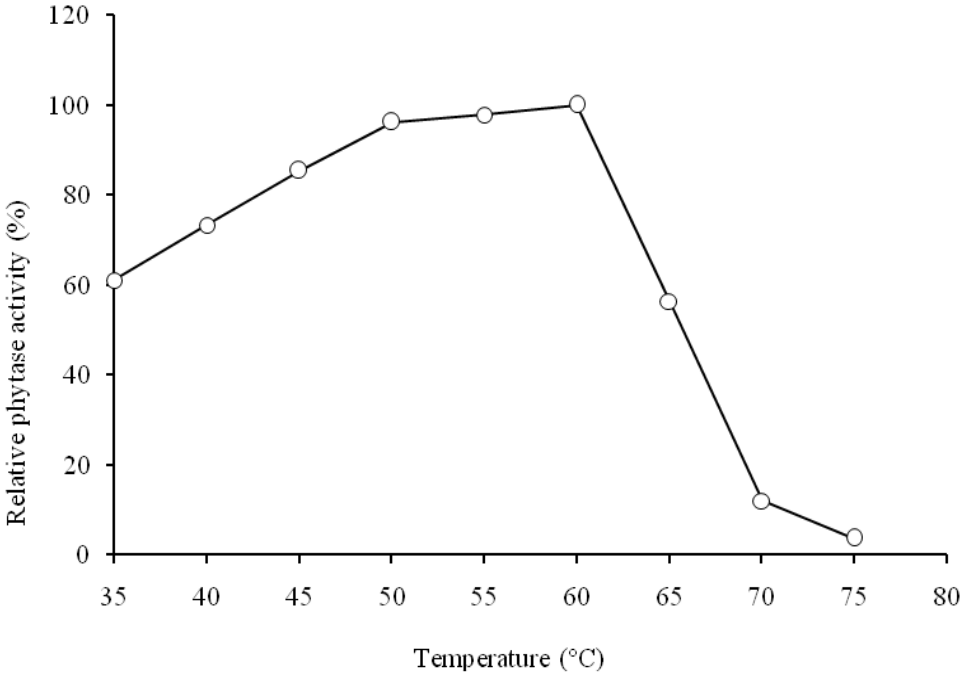

Figure 3. Effect of temperature on the activity of *M. jalaludinii* phytase.

When the phytase was incubated in buffers with different pH values for 60 min at room temperature, virtually no loss in stability of *M. jalaludinii* phytase was observed at pH 4.0 to 7.0, but at pH values less than 4.0 and more than 7.0, the stability dropped drastically (Figure 5).

Substrate specificity

The results of the activities of *M. jalaludinii* phytase on 12 different phosphate esters used as substrates are summarized in Table 2. The activity of *M. jalaludini*

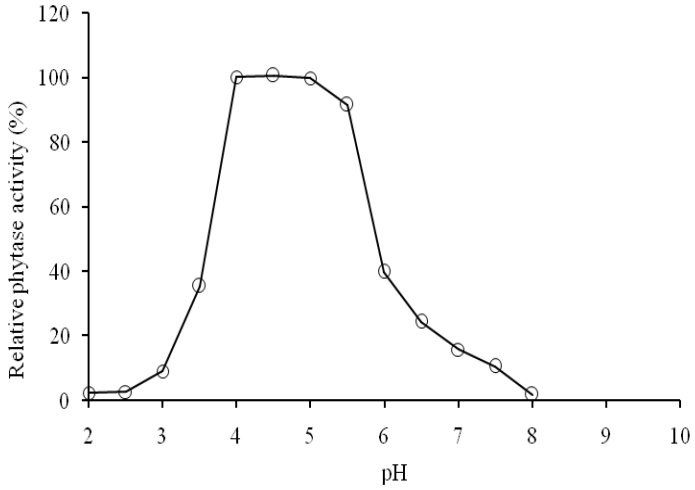

Figure 4. Effect of pH on phytase activity of *M. jalaludinii.*

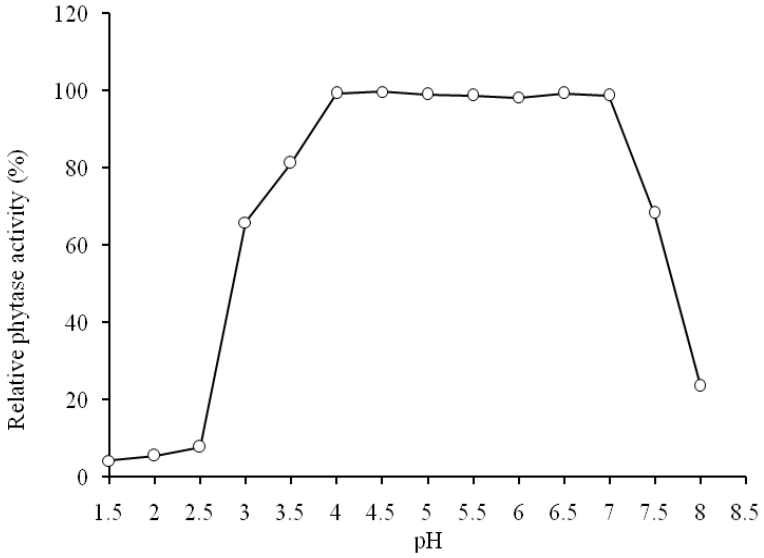

Figure 5. Effect of pH on the stability of *M. jalaludinii* phytase.

phytase was highest when sodium phytate was used as a substrate and this showed that the enzyme was very specific for phytic acid. The relative rates of hydrolysis of the other 11 phosphate esters ranged from 0% (phosphoglycolic acid and mannose-6-phoshpate) to 8.1% (α-D-glucose-1-phosphate) of the sodium phytate hydrolysis rate.

Effects of reagents, ions and phosphate on phytase activity

Results of the effects of reagents and cations on phytase activity are shown in Table 3. All the reagents studied had no significant ($P > 0.05$) effect on phytase activity. Phytase activity was significantly ($P < 0.05$) activated by Ba^{2+}, Mn^{2+} and Ca^{2+} but not significantly ($P > 0.05$) affected by Mg^{2+} and Co^{2+}. Cu^{2+} and Zn^{2+} significantly ($P < 0.05$) inhibited phytase activity, and Fe^{2+} and Fe^{3+} almost completely ($P < 0.05$) inhibited the activity (Table 3)

The whole cell or the cell-free phytase activity of *M. jalaludinii* was not phosphate-inhibited, even when the phosphate concentration in the assay mixture was increased to 10 mmol/l (Figure 6). In contrast, the phytase activity of *A. ficuum* (which acted as a control),

Table 2. Substrate specificity of *M. jalaludinii* phytase

Substrate	Phytase activity (U)*	Relative activity (%)†
Sodium phytate	0.3800 ± 0.0096[a]	100.0
α-D-Glucose-1-phosphate	0.0308 ± 0.0014[b]	8.1
NADP	0.0209 ± 0.0009[c]	5.5
β-Naphthyl phosphate	0.0179 ± 0.0010[cd]	4.7
D-Fructose-1, 6-diphosphate	0.0152 ± 0.0005[d]	4.0
ATP	0.0110 ± 0.0010[de]	2.9
D-Fructose-6-phosphate	0.0095 ± 0.0006[e]	2.5
ρ-Nitrophenyl phosphate	0.0046 ± 0.0007[f]	1.2
α-Naphthyl acid phosphate	0.0015 ± 0.0003[g]	0.4
DL-α-Glycerophosphate	0.0004 ± 0.0001[h]	0.1
Phosphoglycolic acid	0	0.0
Mannose-6-phosphate	0	0.0

*Values are means ± SE of combined values of three experiments, each with three replicates. [a-h]Means within the same column with no common superscript differ significantly (P<0.05).†Relative activity (%) = (phytase activity using any phosphate as substrate/phyase activity using Na-phytate as substrate) × 100. Na-phytate, sodium phytate; NADP, α-nicotinamide adenine dinucleotide phosphate; ATP, adenosine-5'-triphosphate.

Table 3. Effects of reagents and cations on the phytase activity of *M. jalaludinii*

Chemical (5 mmol/l)	Phytase activity (U)*	Relative activity (%)†
None (control)	0.390 ± 0.0046[ab]	100.0
Reagent		
NaN_3	0.398 ± 0.0058[bc]	102.0
EDAC	0.379 ± 0.0073[a]	97.2
2-Mercaptoethanol	0.403 ± 0.0088[bc]	103.3
Trisodium citrate	0.400 ± 0.0064[bc]	102.6
Potassium sodium tartrate	0.387 ± 0.0041[ab]	99.2
EDTA	0.390 ± 0.0087[ab]	100.0
Cation		
$MgCl_2$	0.387 ± 0.0057[ab]	99.2
$MnCl_2$	0.466 ± 0.0027[d]	119.5
$ZnCl_2$	0.243 ± 0.0015[e]	62.3
$CuCl_2$	0.333 ± 0.0023[f]	85.4
$BaCl_2$	0.587 ± 0.0087[g]	150.5
$CoCl_2$	0.394 ± 0.0002[ab]	101.0
$CaCl_2$	0.417 ± 0.0092[c]	106.9
$FeSO_4$	0.007 ± 0.0005[h]	1.8
Fe Cl_3	0.005 ± 0.0005[h]	1.3

*Values are means ± SE of combined values of three experiments, each with three replicates. [a-h]Means in the same column with no common superscript are significantly different (P<0.05). EDAC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. †Relative activity (%) = (Phytase activity of assay mixture with reagent or cation supplementation/phytase activity of the control) × 100.

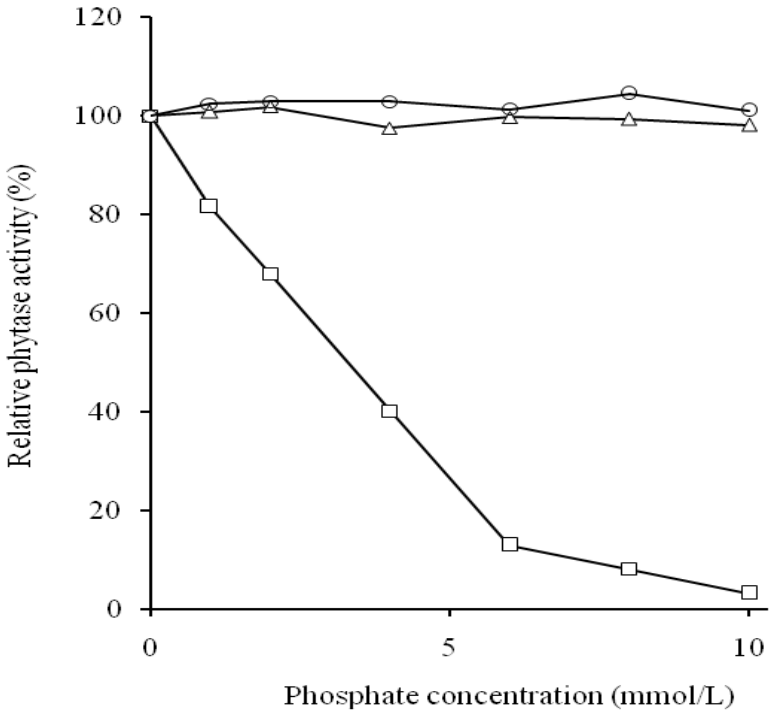

Figure 6. Inhibitory effect of phosphate on phytase activity. (∆) *M. jalaludinii* phytase (whole cell), (o) *M. jalaludinii* phytase (cell free), (□) *Aspergillus ficuum* phytase (cell free). The relative activity at 0 mmol/l of phosphate was set at 100%. The original activity was 0.4 U/ml.

Table 4. Extraction of cell-associated phytase of *M. jalaludinii**

Extraction compound	Concentration (mol/l)	Phytase activity (%)†‡	
		Supernatant	Cell-associated
None (control)		0.0	100.0
KCl	0.25	2.8	96.2
KCl	0.50	7.6	95.1
KCl	0.75	21.1	80.2
KCl	1.00	49.0	55.6
KCl	1.50	53.1	46.0
KCl	2.00	46.0	50.7
KCl	2.50	26.1	73.0
KCl	3.00	16.8	72.0
KCl	3.50	8.0	76.0
Deoxycholate	1.2%	1.2	95.1
Triton X-100	1.2%	6.7	94.6
Tween 80	1.2%	3.2	94.0

*All extractions were done in 0.1mol/l acetate buffer (pH 5.0). † Values are means of three experiments, each with four replicates. ‡Percent activity of total cell phytase of control.

was drastically inhibited by phosphate added to the assay mixture. At a low concentration of 1.0 mmol/l, only 19.4% of activity of A. ficuum phytase was inhibited, but at high concentrations of 4 mmol/l and 10 mmol/l, 60 and 97% activities of A. ficuum phytase, respectively, were inhibited.

Localization of phytase of *M. jalaludinii*

The preliminary determination of the distribution of phytase activities in the culture of M. *jalaludinii* showed that 1.3% of phytase activity was in the cytoplasmic fraction and 98.7% in the cell-bound fraction, but none was found in the extracellular and periplasmic fractions. Extraction of M. *jalaludinii* phytase from whole cells increased with increasing KCl concentrations in the incubation solution, reaching a maximum at a concentration of 1.5 mol/l, after which the amount of phytase released from the cells decreased with increasing concentrations of KCl (Table 4). At the concentration of 1.5 mol/l KCl, about half (53.1%) of the total enzyme activity was free from the cells into the supernatant. Only a small percentage of the total enzyme was extracted from the cells when non-ionic compounds such as deoxycholate, Triton X-100 and Tween 80 were used, respectively (Table 4).

DISCUSSION

The optimum temperature for phytase activity of *M. jalaludinii* was 55 to 60°C. Although the optimum temperature for phytase activity of *Selenomonas ruminantium* JY35, an anaerobic rumen bacterium, is also 55°C, the enzyme activity declines dramatically at 60°C (Yanke et al., 1999). The optimum temperatures of phytase for most micro-organisms are in the range of 50 to 70°C. High optimum temperatures for phytase activity have been observed in bacteria such as *Klebsiella aerogenes* (60 to 70°C) (Tambe et al., 1994), and *Bacillus* sp. DS11 (70°C) (Kim et al*.,* 1998). Among yeasts, *Schwanniomyces castellii* showed maximum phytase activity at 77°C (Segueilha et al., 1992), while *Arxula adeninivorans* and *Pichia spartae* at 75 to 80°C, and *Pichia rhodanensis* at 70 to 75°C (Nakamura, 2000). Phytase of *Aerobacter aerogenes* exhibited the lowest optimum temperature at 25°C (Greaves et al., 1967).

The optimum pH of phytase activity of *M. jalaludinii* was in the range of 4.0 to 5.0. The activity declined significantly above pH 5.5. The moderately acidic pH optimum of *M. jalaludinii* phytase indicates that this enzyme belongs to the acidic phytases, as are most of the so far characterized phytases of microorganisms: *Selenomonas ruminantium* JY35, pH 4.0 to 5.5 (Yanke et al., 1999); *E. coli*, pH 4.5 (Greiner et al*.,* 1993); *Aerobacter aerogenes*, pH 4 to 5 (Greaves et al., 1967); *Klebsiella aerogenes*, pH 4.5 and 5.2 (Tambe et al., 1994); *Lactobacillus amylovorus*, pH 4.4 (Sreeramulu et al., 1996); *Aspergillus terreus*, pH 4.5 (Yamada et al., 1968); *Schwanniomyces castellii,* pH 4.5 (Segueilha et al*.,* 1992); and all yeast strains studied by Nakamura et al.(2000), pH 3 to 5.5. These pH optima are different from those of other bacterial phytases, such as pH 6.5 for *Bacillus subtilis* (natto) N-77 (Shimizu, 1992), pH 7.0 for *Bacillus subtilis* VTT E-68013 (Kerovuo et al., 1998) and *Bacillus* sp. DS11 (Kim et al., 1998), and pH 7.0 – 7.5 for *Enterobacter* sp. 4 (Yoon et al., 1996). Phytase from *M. jalaludinii* was stable in the pH range of 4.0 to 7.0. When the enzyme was incubated in more acidic buffers of pH 3.0 or less, about 34 to 96% of activity was lost. Similar results have been reported by Kim et al. (1998) who found that phytase from *Bacillus* sp. DS11 was stable at a pH range of 4.0 to 8.0 and very low activity was detected at pH values below 3.0. Greiner et al. (1993) also found that phytase activity of *E. coli* was stable at pH levels ranging from 3.0 to 9.0, but at pH values less than 3.0, the phytase stability decreased dramatically.

Wyss et al. (1999) pointed out that on the basis of substrate specificity, phytases could be classified into two classes: (i) phytases with broad substrate specificity such as those from *A. fumigatus*, *Emericella nidulans*, *Myceliophthora thermophila* (Wyss et al., 1999), canola seed (Houde et al., 1990), germinated oat (Greiner and Alminger, 1999), wheat (Nagai and Funahashi, 1962), spelt (Konietzny et al., 1995), rye (Greiner et al., 1998) and barley (Greiner et al*.,* 1999); and (ii) phytases with narrow substrate specificity, which are very specific for phytate, such as those from *A. niger, A. terreus, E. coli* (Wyss et al., 1999), *Bacillus* sp. DS11 (Kim et al., 1998) and *Bacillus subtilis* (natto) N-77 (Shimizu, 1992). The results from this study showed that phytase from *M. jalaludinii* belongs to the second class since it is highly specific to sodium phytate and has very little or no activity on other phosphate esters under the given assay conditions. The very low specificity of *M. jalaludinii* phytase to ρ-nitrophenyl phosphate, a general substrate for acid phosphatase, indicates that the phytase is different from the general acid phosphatase.

The metal ion chelating agents, namely trisodium citrate, potassium sodium tartrate and EDTA did not show any inhibitory effect on the phytase activity of *M. jalaludinii.* Therefore, this enzyme, like many other phytases is not a metallo-enzyme. The absence of effect from the sulfhydryl inhibitor, 2-mercaptoethanol, on the activity of *M. jalaludinii* phytase indicates that this enzyme has no free and accessible sulfhydryl groups or the free sulfhydryl groups play a negligible role in the enzyme structure as in the activity. In contrast to the phytase of *E. coli* (Greiner et al*.,* 1993), the phytase of *M. jalaludinii* was found to be insensitive to 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC), a carboxyl inhibitor.

The study of the effect of metal ions on enzyme activity revealed that phytase from *M. jalaludinii* displayed a pattern of cation sensitivity similar to those of *E. coli* (Greiner et al., 1993), *Klebsiella terrigena* (Greiner et al., 1997) and *Selenomonas ruminantium* JY35 (Yanke et al., 1999). The most significant inhibitory effect was by iron cation. This has also been commonly observed in many

phytases from various sources. Greiner et al. (1993) and Greiner and Alminger (1999) suggested that the inhibitory effect of iron cations on *E. coli* and oat phytases was attributed to the ability of the iron cations to combine with phytate, which was evident by the presence of a precipitate. However, in the study with *Selenomonas ruminantium* JY35 phytase, Yanke et al. (1999) found that precipitates were also obtained with Ba^{2+} and Pb^{2+}, but Ba^{2+} did not inhibit the phytase activity and Pb^{2+} significantly stimulated the activity. In the present study, it was also found that precipitates were formed when Ba^{2+}, Cu^{2+}, Co^{2+}, Fe^{2+} or Fe^{3+} was added into the substrate solution to a final concentration of 5 mmol/l. However, the inhibitory effects were only observed in Cu^{2+}, Fe^{2+} and Fe^{3+}. Co^{2+} had no effect on enzyme activity. Ba^{2+} was found to significantly stimulate the phytase activity by up to 50%. The reason for this is not known, and further study is necessary to understand the mechanism(s) involved.

It is generally recognized that inorganic phosphates cause product inhibition (competitive inhibition) on phytate hydrolysis (Howson and Davis, 1983). In the present study, it was found that 60 and 97% of phytase activity of *A. ficuum* was inhibited by 4 and 10 mmol/l phosphate supplemented to the assay mixture, respectively. However, no inhibition was detected on the activity of *M. jalaludinii* phytase, even when phosphate concentration was as high as 10 mmol/l in the assay mixture. Kim et al. (1999) also reported that the phytase activity of *Bacillus amyloliquefaciens* was not inhibited by phosphate concentration of up to 5 mmol/l in the assay mixture. The results from this study support the suggestion of Yanke et al. (1998) that phytate is readily hydrolysed by bacteria in the rumen, even though the inorganic phosphate concentration in the rumen fluid can be as high as 14 mmol/l when the animal is fed with concentrate diet.

The study on the localization of phytase confirmed that about 99% of phytase activity was cell-associated. The phytase was readily extracted from the whole cell by high concentration of KCl but not by Tween 80 and Triton X-100. Similar results were also reported by D'Silva et al. (2000) who found that the phytases of *Selenomonas ruminantium* and *Mitsuokella multiacidus* (=*M. multacida*) were readily extracted from the whole cells by high concentrations of $MgCl_2$ and KCl. By using transmission electron microscopy, D'Silva et al. (2000) demonstrated that the phytases of *S. ruminantium* and *M. multiacidus* were associated with the outer membrane of the cell (out layer of the cell wall).

This study showed that *M. jalaludinii* could be a promising novel bacterial source of phytase. The properties of the phytase from *M. jalaludinii* are favourable for it to be used as an enzyme for improving the availability of phytate phosphorus and minerals in feedstuff for non-ruminants.

REFERENCES

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Analy. Biochem., 72: 248-252.

D'Silva CG, Bae HD, Yanke LJ, Cheng K-J, Selinger LB (2000). Localization of phytase in *Selenomonas ruminantium* and *Mitsuokella multiacidus* by transmission electron microscopy. Can. J. Microbiol., 46: 391-395.

Greaves MP, Anderson G, Webley DM (1967). The hydrolysis of inositol phosphates by *Aerobacter aerogenes*. Biochim. Biophys. Acta., 132: 412-418.

Greiner R, Konietzny U, Jany KD (1993). Purification and characterization of two phytases from *Escherichia coli*. Arch. Biochem. Biophys., 301: 107-113.

Greiner R, Haller E, Konietzny U, Jany KD (1997). Purification and characterization of a phytases from *Klebsiella terrigena*. Arch. Biochem. Biophys., 341: 201-206.

Greiner R, Konietzny U, Jany KD (1998). Purification and properties of a phytase from rye. J. Food Biochem., 22: 143-161.

Greiner R, Alminger ML (1999). Purification and characterization of a phytate-degrading enzyme from germinated oat (*Avena sativa*). J. Sci. Food Agri., 79: 1453-1460.

Greiner R, Jany KD, Alminger ML (1999). Identification and properties of a phytate-degrading enzymes from barley (*Hordeum vulgare*). J. Cereal Sci., 30: 11-17.

Ha NC, Kim YO, Oh TK, Oh BH (1999). Preliminary X-ray crystallographic analysis of a novel phytase from a *Bacillus amyloliquefaciens* strain. Acta crystallogr. D. Biol. Crystallogr., 55: 691-693.

Heinonen JK, Lahti RJ (1981). A new and convenient colorimetric determination of inorganic orthophosphate and its application to the assay of inorganic pyrophosphatase. Analy. Biochem., 113: 313-317.

Houde RL, Alli I, Kermasha S (1990). Purification and characterization of canola seed (*Brassica* sp.) phytase. J. Food Biochem., 14: 331-351.

Howson SJ, Davis RP (1983). Production of phytate-hydrolysing enzyme by some fungi. Enzyme Microbial. Technol., 5: 377-382.

Hungate RE (1969). A Roll Tube Method for Cultivation of Strict Anaerobes. Methods Microbiol., 3B: 117-132.

Jareonkitmongkol S, Ohya M, Watanabe R, Takagi H, Nakamori S (1997). Partial purification of phytase from a soil isolate bacterium, *Klebsiella oxytoca* MO-3. J. Ferm. Bioeng., 83: 393-394.

Kerovuo J, Lauraeus M, Nurminen P, Kalkkinen N, Apajalahti J (1998). Isolation, characterization, molecular gene cloning, and sequencing of a novel phytase from *Bacillus subtilis*. Appl. Environ. Microbiol., 64: 2079-2085.

Kim YO, Kim HK, Bae KS, Yu JH, Oh TK (1998). Purification and properties of a thermostable phytase from *Bacillus* sp. DS11. Enzyme Microbial. Technol., 22: 2-7.

Kim DH, Oh BC, Choi WC, Lee JK, Oh TK (1999). Enzymatic evaluation of *Bacillus amyloliquefaciens* phytase as a feed additive. Biotechnol. Lett., 2: 925-927.

Kim H-W, Kim YO, Lee JH, Kim KK, Kim YJ (2003). Isolation and characterization of a phytase with improved properities from *Citrobacter braakii*. Biotechnol. Lett., 25: 1231-1234.

Konietzny U, Greiner R, Jany KD (1995). Purification and characterization of a phytase from spelt. J. Food Biochem., 18: 165-183.

Laemmli UK (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature, 227: 680-685.

Lan GQ, Ho YW, Abdullah N (2002a). *Mitsuokella jalaludinii* sp. nov. from the rumen of cattle in Malaysia. Int. J. Sys. Evol. Microbiol., 52: 713-718.

Lan GQ, Abdullah N, Jalaludin S, Ho YW (2002b). Culture conditions influencing phytase production of *Mitsuokella jalaludinii*, a new bacterial species from the rumen of cattle. J. Appl. Microbiol., 93: 668-674.

Lan GQ, Abdullah N, Jalaludin S, Ho YW (2002c). Optimization of

carbon and nitrogen sources for phytase production by *Mitsuokella jalaludinii,* a new rumen bacterial species. Lett. Appl. Microbiol., 35: 157-161.
Lan GQ, Abdullah N, Jalaludin S, Ho YW (2010). *In vitro* and *in vivo* enzymatic dephosphorylation of phytase in maize-soya bean meal diets for broiler chickens by phytase of *Mitsoukella jalaludinii.* Anim. Feed Sci. Technol., 158: 155-164.
Nagai Y, Funahashi S (1962). Phytase (myoinositolhexaphosphate phosphohydrolase) from wheat bran. Part I. Purification and substrate specificity. Agri. Biol. Chem., 26: 794-803.
Nakamura Y, Fukuhara H, Sano K (2000). Secreted phytase activities of yeasts. Biosci. Biotechnol. Biochem., 64: 841-844.
Pasamontes L, Haiker M, Henriquez-Huecas M, Mitchell D.B, van Loon APGM (1997). Cloning of the phytases from *Emericella nidulans* and the thermophilic fungus *Talaromyces thermophilus*. Biochim. Biophys. Acta., 1353: 217-223.
Powar VK, Jagannathan V (1982). Purification and properties of phytate-specific phosphatase from *Bacillus subtilis*. J. Bacteriol., 151: 1102-1108.
Raun A, Cheng E, Burroughs W (1956). Phytate phosphorus hydrolysis and availability to rumen microorganisms. J. Agri. Food Chem., 4: 869-871.
Richardson AE, Hadobas PA (1997). Soil isolates of *Pseudomonas* spp. that utilize inositol phosphate. Can. J. Microbiol., 43: 509-516.
Sajidan A, Farouk A, Greiner R, Jungblut P, Müller E-C, Borriss R (2004). Molecular and physiological characterization of a 3-phytase from the Rhizobacterium *Klebsiella pneumonia* ASRI. Appl. Microbiol. Biotechnol., 65: 110-118.
SAS Insitute (1997). SAS® Users Guide: Statistics: release 6.12. Edition. SAS Institute Inc. Cary, NC.
Segueilha L, Lambrechts C, Boze H, Moulin G, Galzy P (1992). Purification and properties of the phytase from *Schwanniomyces castellii*. J. Ferm. Bioeng., 74: 7-11.
Shimizu M (1992). Purification and characterization of phytase from *Bacillus subtilis* (natto) N-77. Biosci. Biotechnol. Biochem., 56: 1266-1269.
Sreeramulu G, Srinivasa DS, Nand K, Joseph R (1996). *Lactobacillus amylovorus* as a phytase producer in submerged culture. Lett. Appl. Microbiol., 23: 385-388.
Tambe SM, Kaklij GS, Kelkar SM, Parekh LJ (1994). Two distinct molecular forms of phytase from *Klebsiella aerogenes*: Evidence for unusually small active enzyme peptide. J. Ferm. Bioeng., 77: 23-27.
Ullah AHJ (1988). *Aspergillus ficuum* phytase: Partial primary structure, substrate selectivity, and kinetic characterization. Preparative Biochem., 18: 459-471.
Wyss M, Brugger R, Kronenberger A, Rémy R, Fimbel R, Oesterhelt G, Lehmann M, van Loon APGM (1999). Biochemical characterization of fungal phytases (*Myo*-inositol hexakisphosphate phosphohydrolases): Catalytic properties. Appl. Environ. Microbiol., 65: 367-373.
Yamada K, Minoda Y, Yamamoto S (1968). Phytase from *Aspergillus terreus*. Part I. Production, purification and some general properties of the enzyme. Agri. Biol. Chem., 32: 1275-1282.
Yanke LJ, Bae HD, Selinger LB, Cheng K-J (1998). Phytase activity of anaerobic ruminal bacteria. Microbiology, 144: 1565-1573.
Yanke LJ, Selinger LB, Cheng KJ (1999). Phytase activity of *Selenomonas ruminantium*: a preliminary characterization. Lett. Appl. Microbiol., 29: 20-25.
Yoon SJ, Yun JC, Hae KM, Kwang KC, Jin WK, Sang CI, and Yeon HJ (1996). Isolation and identification of phytase-producing bacterium, *Enterobacte*r sp. 4, and enzymatic properties of phytase enzyme. Enzyme Microbial. Technol., 18: 449-454.