

Purification and Characterization of Phytase from Novel Slated *Bacillus cereus* EME 48 and Study its Kinetic Properties

Enas N. Danial^{1,2*} and Maha I. Alkhalf¹

¹Biochemistry Department, Faculty of Science- Al Faisaliah
King Abdulaziz University, Jeddah, Saudi Arabia.

²Chemistry of Natural and Microbial Products, National Research Center, Cairo, Egypt.

<http://dx.doi.org/10.22207/JPAM.10.4.06>

(Received: 15 October 2016; accepted: 24 November 2016)

Phytase (myo-inositol-hexakisphosphate phosphohydrolase, (EC 3.1.3.8) has been purified from a novel isolated *Bacillus cereus* EME 48, from environmental samples based on their ability. The enzymes were purified and characterized, using a three-step purification procedure with 12.3-fold. The optimum pH for phytase activity was in the range of 4.0 to 5.0 and the optimum temperature was 40 to 60°C for 10 min. The metal ions MgCl₂, CoCl₂, NiCl₂ and KCl did not show any inhibitory effect on the phytase activity of *Bacillus cereus* EME 48. Strongly inhibited by FeCl₂, Na Florida, EDTA, DTT, AlCl₃ and ZnCl₂ but significantly stimulated by MgCl₂, BaCl₂, MnCl₂ and CuCl₂. The enzymes were active in the pH range of 5.0 to 6.5 with pH optima at 5.5. The enzyme from *Bacillus cereus* EME 48 retained about 80% activity up to 75°C. It was highly specific to sodium phytate as the substrate. Km for phytate was estimated to be 0.23 mmol and especially for penta- and tri-phosphate esters of myo-inositol. Due to their relatively high specific activity, substrate specificity, good pH profile and thermostability, the enzymes could be interesting candidate for agricultural and feed application.

Keywords: Microbial phytase, Isolate, *Bacillus cereus* purified and characterized.

Phytate is of great interest in human nutrition, feed technology, food and medical science¹. Researchers, as this interest has generated a wealth of relevant information. The negative effect of phytate on the availability of Ca and zinc, in food stuffs has been extensively investigated. Phytate in human diets is also claimed to have benefits, such as anti-carcinogenic effect². Animal nutritionists have long regarded phytate as both indigestible and an anti-nutritional factor for non-ruminant animals³. Phytate is a polyanionic molecule with the potential to chelate positively charged nutrients, which is almost certainly

fundamental to the anti-nutritive properties of phytate. These anti-nutritive properties require further investigation, but phytate probably compromises the utilization of protein/amino acids, energy, calcium and trace minerals. Phytase, which occurs widely throughout nature, is the requisite enzyme to degrade and release inorganic P⁴ If the practical acceptance of microbial phytase in poultry diets continues, it is likely that phytase feed enzymes will re-define nutrient requirements for sustainable poultry production in the future. Nevertheless, three decades elapsed before an *Aspergillus niger*-derived phytase feed enzyme, with the capacity to liberate phytate-bound P and reduce P excretion, was commercially introduced in 1998⁵.

Phytic acid (myo-inositol 1,2,3,4,5,6 - hexakisdihydrogenphosphate) and mixed cation salts of phytic acid, designated as phytate, are a

* To whom all correspondence should be addressed.
E-mail: enas_mahdy@yahoo.com

group of organic phosphorus (P) compounds found widely in nature especially in legumes, cereals, and oil seed crops⁶. Phytic acid, which is the main constituent of animal diet, is not digested by monogastric animals and hence, create problem in the availability of phosphorus in their diet. Various reported phytase-producing isolates and showed that the phytases with broader substrate specificity generally had low specific activities. Despite the considerable economic interest, low yield and high cost of enzyme production are the limiting factors in using this enzyme in animal diet. It also causes environmental pollution by extra supplemented phosphorus in animal's diet⁷. Phytases are the primary enzymes responsible for the hydrolysis of phytic acid⁸. Hence, phytases are considered to be potential candidate for use as an enzyme that have great value in enhancing the nutritional quality of phytate-rich foods and feed⁹.

In recent years, bacterial phytases have been isolated, characterized and proposed as potential tools in biotechnology. The highest frequency of phytase-like proteins has been reported in members of the gamma-proteobacteria group. Pure culture studies of ruminal bacteria have demonstrated phytate- degrading activity in various strains belonging to the Enterobacteriaceae family, such as in *Enterobacter coli*, *Klebsiella*, *Citrobacter*, *Yersinia* etc. Here, we report a comparative study on isolation, purification and characterization of phytases from two enteric bacteria¹⁰.

Two big goals for this study, first is to produce the microbial phytase from new isolate microorganisms. Second, study carried out to purify and characterize phytase from *Bacillus cereus* EME 48. To our knowledge, this is a first report on the purification and properties of phytase from *Bacillus cereus* EME 48.

MATERIALS AND METHODS

Culture conditions and sample preparation

The following liquid media was used to cultivate the isolate bacteria *Bacillus cereus* EME 48 contained (% w/v): Dextrose 0.5; peptone 1; yeast extract 0.5; MgSO₄ 0.1; CaCl₂ 0.1, sodium phytate 0.1 (pH 7.0) Kim et al¹¹. The media contents were completed to one liter, then the pH was adjusted at 7.0, the bacteria was incubated at 30 °C

for 5 days

Purification of crud extra cellular phytase produced by *Bacillus cereus* EME 48

Partial purification of phytase

The present experiment included the purification of extra cellular phytase using precipitation with ammonium sulphate, ethanol, and acetone or using ultra filtration methods according to the method of Greiner and Alminger (12)

Gel filtration of extra cellular phytase using sephadex G-100

Sephadex G-100 was used as gel filtration for the enzyme purification. 7 ml of extra cellular phytase obtained from ultra filtration contain 200 mg/ml protein was applied to a sephadex G-100 column chromatography (108 x 2.6 cm) which had been equilibrated with 0.1 M Tris buffer at pH 7.0 at 11 °C. Elution was carried out with the same buffer. Five ml fractions were collected at flow rate of 33 ml/h.

Anion exchange of extra cellular phytase using DEAE-sephadex A50

Cellulose DEAE-52 was used as anion exchange for enzyme purification. Active Fraction (22-30) was obtained from Sephadex G-100 column chromatography while contained (8.8mg protein) was lyophilized and dissolved in 1 ml of 0.1 M tris buffer then applied to the top of DEAE Sephadex - A52 column chromatography (35 cm × 2.5 cm) previously equilibrated with 0.1 M tris buffer at pH 7.0 at 11 C.

At first, the unbounded protein was removed by washing with the same buffer, and then enzyme was eluted by gradient elution using sodium chloride (0.0 to 1.0 M) in 0.1M Tris HCl buffer at pH 7.0. The flow rate was adjusted to 50 ml/h and 5 ml fractions were collected from the effluent.

Phytase and protein assay

0.1 ml of the phytase sample was mixed with 1.24 ml acetate buffer containing 0.2% sodium phytate and incubated at 39°C for 15 min. The reaction was terminated by adding 1.25 ml 5% trichloroacetic acid (TCA)¹³. A unit of phytase activity is defined as the amount of enzyme that liberates 1µmol P/min under the given assay conditions. Protein concentration was measured by the method of Bradford¹⁴ 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 the stability of phytase enzyme

The thermal stability of the extra cellular purified enzyme was determined by storage the enzyme suspensions at different temperature in a water bath set (20-80 °C) for different times (0 min-24 hours), then the enzymes activity were determined in each case.

Effect of pH on the phytase stability

In this experiment, 1 ml of 0.15 M acetate buffer at pH values ranged between 3.5-6 were stored at room temperature with 1 ml of purified phytase enzyme solutions. The storage time was between 0 min. - 48 hours. The remains enzyme activity was then determined at 55 °C and pH 5.5. Control was carried out using pH 5.5.

Effects of ions on phytase activity

In this experiment, the metal ions used in the experiment were MgCl₂, MnCl₂, ZnCl₂, BaCl₂, CuCl₂, AlCl₃, CaCl₂, CoCl₂, FeCl₂, NiCl₂, KCl₂, MgCl₂, Na Florida, EDTA and DTT were added in concentrations 1 and 2 mM to the reaction mixture to study their effect on the phytase activity. The control was carried out without adding activators or inhibitors

Effect of different concentrations of substrate on the activity of purified extra cellular phytase

The effect of substrate concentrations on the purified extra cellular phytase was studied. Different concentrations of Na phytate ranging from 1.5 to 5 mM in 0.15 M acetate buffer at pH 5.5, the reaction mixture was added to 150 ml of enzyme solution then it was incubated for 40 min. at 55 °C and stopped by 0.75 ml of 5% trichloroacetic acid then the enzyme activity was measured as mentioned before.

RESULTS

Fractional precipitation with ammonium sulphate

It is clear from the data presented in table (1) that, ammonium sulphate at concentration (30-60) gave activity for phytase enzyme 316 U/ml and protein 3 mg/ml with specific activity 105

Precipitation with ethanol and acetone

Precipitation using acetone and ethanol

also mad and the results is shown in table (1). It is clear that ethanol (0-30) gave high specific activity than acetone to precipitation phytase enzyme.

Precipitation using ultra filtration

The present series of experiment were conducted to prepare partially purified phytase. These include isolation of active enzymes by ultrafiltration. The culture filtrate of about 2 liter was passed through Pellicon Cassette system with a membrane PLGC Cassette 10.000 NMWL. Low protein-binding.

The two liter was concentrated to about 50 ml which contain 10000 mg of protein was recovered which represent about 64.10 % of the total protein while 1550000 U of phytase enzyme was recovered which represent about 101 % of the total activity.

The results in fig (1) illustrated that, two peak for phytase activity (I) and (II) of protein components were present with different molecular weight. The first peak was at fraction²²⁻³⁰ and the second one was at³⁶⁻⁴¹. The enzyme activity was detected in the first peak. The total enzyme activity was 14440 U/min at 40 ml of fraction²²⁻³⁰ the total protein was 8.8 mg. the specific activity reached to 1640.9 U/mg proteins. The total activity of the second peak was 7350 U/min at 25 ml of fraction³⁶⁻⁴¹ and the total protein was 4.84 mg the specific activity reached to 1518 U/min.

The results in fig (2) showed that, the enzyme activity was 138 U/min at 25 ml of fraction³¹⁻³⁵ the total protein was 0.05 mg. the specific activity reached to 2760 U/mg protein. Fractions³¹⁻³⁵ were chosen for check their purity on polyacrylamide gel disc electrophoresis phytase where the total activity was U/ml and the specific activity was 2760 U/mg protein.

Summarized purification steps of phytase

Typical purification steps of phytase from *Bacillus cereus* EME 48 are summarized in table (2). The cultural filtrate obtained after centrifugation of the fermentation broth was subjected to ultra filtration using Pellicon Cassette system with a membrane PLGC Cassette 10.000 NMWL, low protein-binding. This step yielded 1.58 fold of purification. It was it was chromatographer on sephadex G100 column and the enzymes were eluted with tris HCl buffer of pH 7.0.

On the other hand, the phytase was purified approximately 16 fold from the culture

filtrate with a specific activity of about 1640 U/mg proteins. The active fractions from the Sephadex G100 Column²²⁻³⁰ were pooled and concentrated to one ml and it was then applied on DEAE-Sephadex A-50 column.

By this procedure phytase was purified approximately 28 fold from the culture filtrate. A protein sample of each step of purification was examined by polyacrylamide gel electrophoresis Fig (3). Phytase resulted from sephadex G100 column chromatography was migrated with other proteins, but phytase resulted from DEAE sephadex column chromatography was also migrated as single protein band on polyacrylamide gel electrophoresis.

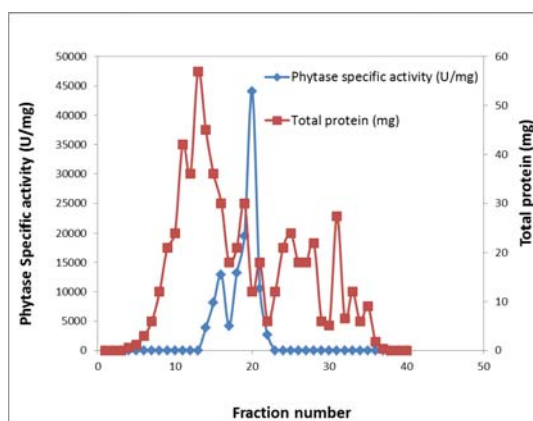


Fig. 1. Purification of extra cellular phytase by using sephadex G-100

(1) Crude enzyme, (2) partially purified enzyme by ultra filtration, (3) the enzyme of phytase separated on sephadex G 100, (4) the enzyme of phytase separated on DEAE sephadex A 50.

Effect of temperature on the stability of phytase enzyme

The results in tables (3) showed that the stability of phytase depending on the temperature and the storage time. In most cases, the loss in activity was increased by increasing the temperature and the storage time.

The phytase enzyme was stable at temperature 20, 30, 40 C after 24 hours. At 50°C the enzyme lost about 8% of its activity after 90 min. At 65 °C and 70 °C the enzyme activity was decreased to 91% and 83% respectively after 5 min.

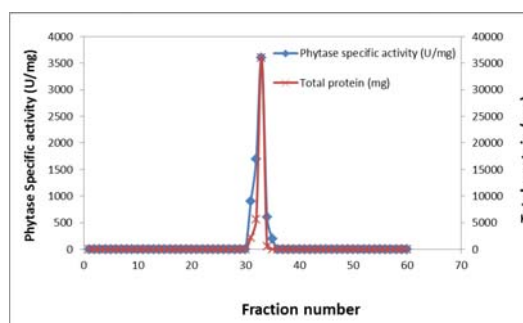


Fig. 2. Purification of extra cellular phytase by using DEAE- sephadex A50

Table 1. Partial purification of phytase enzyme produced by *Bacillus cereus* EME 48

Purification steps	volume (ml)	Total protein	Protein recovery (%)	Total units	Units recovery (%)	Specific activity	Purification fold
Culture filtrate	50	390	100	38300	100	98	1
Acetone	0-30	50	100	25.6	9050	23	90
	30-60	50	190	48.7	20050	52	105
	60-90	50	22.2	5.5	821.4	2.1	37
Ethanol	0-30	50	109	28	12400	32	113
	30-60	50	200	51	17600	46	88
	60-90	50	00	00	00	00	00
Ammonim sulphate	0-30	50	195	50	17600	46	90
	30-60	50	150	38	15800	41	105
	60-90	50	00	00	00	00	00
Culture filtrate	2000	15600	100	1532000	100	98.20	1
Ultra filtration	50	10000	64.10	1550000	101	155	1.58

Effect of pH on the phytase

The results recorded in tables (4) indicated that the stability of phytase, in absence of substrate depended on the pH values. The phytase enzyme was stable at pH 5.5 to 6 for 48 hours. The enzyme was stable at pH 5.0 till 60 min and partially loss its activity with increasing the time of storage. At pH values 3.5, 4, 4.5 phytase enzymes lost its activity after 60 min., 8 hour and 16 hours respectively.

The phytase enzyme was stable at pH values ranged between 5.5 to 6.0.

Effect of different concentrations of different activators and inhibitors on the phytase activitie

The results recorded in table (5) showed that the enzymes activity was greatly inhibited by FeCl_2 , Na Florida, EDTA, DTT, AlCl_3 and ZnCl_2 . On the other hand the addition of MgCl_2 , BaCl_2 , MnCl_2 and CuCl_2 showed moderately inhibited for the phytase, while, MgCl_2 , CoCl_2 , NiCl_2 and KCl_2 had no effect on the both enzymes. Moreover, CaCl_2 made a slight increase in the activity of phytas when added at concentration in 1 mM, the activity was decreased with decreasing the concentration.

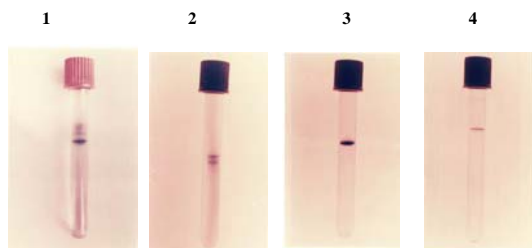


Fig. 3. Purification steps of phytase

Effect of different concentrations of substrate on the activity of purified extra cellular phytase

The results recorded in fig (4) showed a parallel relationship existed between substrate concentration and phytase Specific activity up to 3 mM (3273 U/mg protein). Higher concentration had pronounced inhibition on enzyme activity.

Kinetic parameters (K_m and V_{max} values) of purified extra cellular phytase and acid phosphatase produced by *Bacillus cereus* EME 48...

These were determined for the purified extra cellular phytase and acid phosphatase by using the natural substrate (Na phytate) and then determine from Line weaver-Burk plot. Na phytate concentrations ranged from 1.5-5 mM dissolved in 0.15 M acetate buffer (pH 5.5) were used. The activities were assayed at 55 C for 40 min. and the reaction was stopped by 0.75 ml of 5% trichloroacetic acid. The results in fig (5) showed that the K_m value was 0.23 mM and V_{max} value was 3278 mM/mg protein.

DISSCUSSION

This study showed that *Bacillus cereus* EME 48 could be a promising novel bacterial source of phytase. The properties of the phytase from *Bacillus cereus* EME 48 are favorable for it to be used as an enzyme for improving the availability of phytate phosphorus and minerals in feedstuff for non-ruminants. Phytase activities have been reported in a wide range of seeds, animal tissue and microbes.

First attempts to separate phytase activity from the major acid phosphatase activity by the

Table 2. Purification steps of phytase

Purification steps	Volume (ml)	Total protein	Protein Recovery (%)	Total activity (U)	Unit's recovery (%)	Specific activity	Purification fold
Culture filtrate	40	312	100	30640	100	98.2	1
Ultra filtration	1	200	64.1	31000	101	98.2	1.58
Sephadex G-100	F(1-21)	145.8	46.5	00	00	00	00
	F(22-30)	267	85.5	11900	38.9	44.5	0.45
	F(31-35)	81	26	00	00	00	00
	F(36-60)	119.1	38	00	00	00	00
DEAE	F(1-30)	4.45	1.4	00	00	00	00
Sephadex	F(31-35)	5.8	1.9	7010	22.9	1209	12.3
A-50	F(36-62)	9.1	2.9	00	00	00	00

one-step procedure described by Gibson and Ullah (2) for phytase purification were unsuccessful. The gel filtration methods are based on the principle that different molecules can be separated on the basis of differences in their size by passing them through a column containing swollen particles of a gel. This procedure was based on the difference of isoelectric points of enzyme. When chromatographed on a strong cationic-exchanger (SP-Trisacryl) column at pH 4.5, soybean phosphatase was retained on the column, whereas phytase was excluded. Under the same conditions, enzyme activities were retained and eluted at the same saline concentration. A three-step purification procedure is described, consisting of ultra-fractionation, gel filtration and anion-exchange chromatography. Phytase was completely separated from the major acid phosphatase activity after the third step.

The optimum temperature for phytase activity for *Bacillus cereus* EME 48 was 40 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 (15). The optimum temperatures of phytase for most micro-organisms are in the range of 40 to 60°C. High optimum temperatures for phytase activity have been observed in bacteria such as *Klebsiella aerogenes* (60 to 70°C) (16), and *Bacillus* sp. DS11 (70°C) (10). Among yeasts, *Schwanniomyces castellii* showed maximum phytase activity at 77°C (17), while *Arxula adeninivorans* and *Pichia spartae* at 75 to 80°C, and *Pichia rhodanensis* at 70 to 75°C (18). Phytase of *Aerobacter aerogenes* exhibited the lowest optimum temperature at 25°C (19).

Table 3. Effect of temperature on phytase enzyme stability.

Time Temp.°C	Phytase Relative activity (%)													
	Minutes									Hours				
	0	5	10	20	30	40	50	60	90	2	4	6	12	24
20	100	100	100	100	100	100	100	100	100	100	100	100	100	100
30	100	100	100	100	100	100	100	100	100	100	100	100	100	100
40	100	100	100	100	100	100	100	100	100	100	100	100	100	100
50	100	100	100	100	100	100	100	100	92	75	65	58	54	46
55	100	100	100	100	100	100	97	91	80	70	61	45	26	0
60	100	100	100	88	77	65	56	47	41	36	29	17	11	0
65	100	91	82	73	64	53	44	39	32	27	20	0	0	0
70	100	83	76	70	62	56	30	22	0	0	0	0	0	0
75	100	35	27	21	12	0	0	0	0	0	0	0	0	0
80	100	17	0	0	0	0	0	0	0	0	0	0	0	0

Table 4. Effect of pH on the phytase stability

Time pH	Phytase Relative activity (%)										
	Minutes					Hours					
	0	15	30	45	60	2	4	8	16	24	48
3.5	69	48	24	11	0	0	0	0	0	0	0
4	78	62	49	31	20	9	7	0	0	0	0
4.5	91	85	76	64	48	30	22	15	0	0	0
5	100	100	100	100	100	76	53	24	13	9	0
5.5	100	100	100	100	100	100	100	100	100	100	100
6	100	100	100	100	100	100	100	100	100	100	100

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 (15); *E. coli*, pH 4.5 (20) and all yeast strains studied by Nakamura (19), pH 3 to 5.5. These pH optima are different from those of other bacterial phytases,

Table 5. The effect of different concentrations of different activators and inhibitors on the phytase

Additive	PhytaseRelative activity %	
	1mM	2mM
Control	100	100
CaCl ₂	101	100
KCl ₂	99	98
CoCl ₂	99	98
NiCl ₂	97	98
MgCl ₂	99	97
CuCl ₂	96	95
MnCl ₂	95	93
BaCl ₂	88	82
MgCl ₂	85	81
AlCl ₃	83	79
ZnCl ₂	79	77
DTT	80	73
EDTA	77	65
Na fluoride	71	62
FeCl ₂	70	53

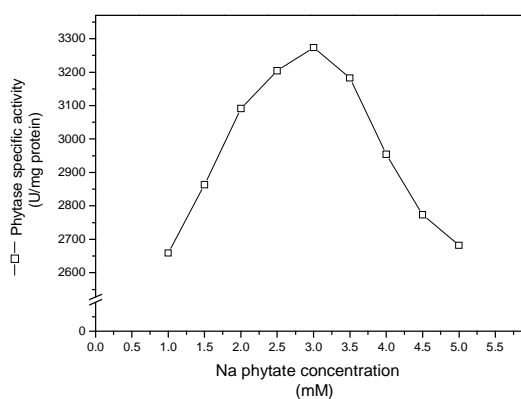


Fig. 4. Effect of different concentrations of substrate (Na phytate) on the activity of purified extra cellular phytase

such as pH 6.5 for *Bacillus subtilis* (natto) N-77 (21), pH 7.0 for *Bacillus subtilis* VTT E-68013 (5) and *Bacillus* sp. DS11 (10). When the enzyme was incubated in more acidic buffers of pH 3.0 or less, about 34 to 96% of activity was lost.

The results from this study showed that phytase from *Bacillus cereus* EME 48 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, indicates that the phytase is different from the general acid phosphatase. The metal ion chelating agents, namely MgCl₂, CoCl₂, NiCl₂ and KCl₂ 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 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* (20), *Klebsiella terrigena* (22) and *Selenomonas ruminantium* JY35 (15). The most significant inhibitory effect was by iron cation. This has also been commonly observed in many phytases from various sources.

However, the study with *Selenomonas ruminantium* JY35 phytase, Yanke (15) found that precipitates were also obtained with Ba²⁺ and Pb²⁺, but Ba²⁺ did not inhibit the phytase activity and Pb²⁺ significantly stimulated the activity.

In the present study, it was found that 62 and 99% of phytase activity of *Bacillus cereus* EME 48 was inhibited by 1 and 2 mmol/l chloride supplemented to the assay mixture, respectively. However, Kim (23) reported that the phytase activity of *Bacillus amyloliquefaciens* was not

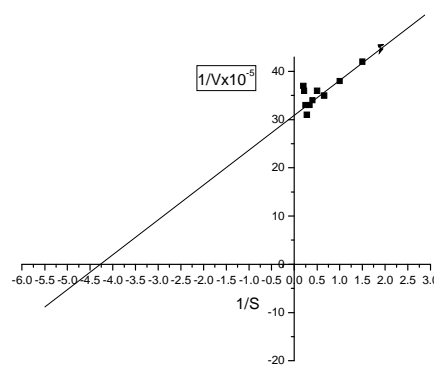


Fig. 5. Line weaver-Burk plot for purified extracellular phytase produced by *Bacillus cereus* EME 48 by using (Na phytate) as a substrate

inhibited by 5 mmol/l phosphate in assay mixture. The results from this study support the suggestion of Yanke (13) 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.

Similar results have been reported by Wyss (24) 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* (24), canola seed (25), germinated oat (26), rye (27) and barley (26); 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 (11) and *Bacillus subtilis* (natto) N-77 (21).

REFERENCES

- Hara A., Ebina S., Kondo A. and Funaguma T. A new type of phytase from pollen of *Typha latifolia* L. *Agric Biol Chem*, 1985; **49**: 3539-44.
- Ullah A.H. and Gibson D.M. Extracellular phytase (E.C.3.1.3.8) from *Aspergillus ficuum* NRRL 3135: purification and characterization. *Prep Biochem*. 1987; **17**: 63-91.
- Sandberg A.S. and Anderson R. A high performance liquid chromatographic method for determination of inositol triphosphates, tetraphosphates, pentaphosphates and hexaphosphates in foods and intestinal contents. *J Food Sci*, 1986; **51**:547-550.
- Dvorakova J. Phytase: Sources, preparation and exploitation. *Folia Microbiol*. 1998; **43**: 323-338.
- Kerovuo J., Lauraeus M., Nurminen P., Kalkkinen N. and Apajalahti J. Isolation, characterization, molecular gene cloning and sequencing of a novel phytase from *Bacillus subtilis*. *Appl Environ Microbiol*. 1998; **64**: 2079-2085.
- Hill J. E., Kysela D. and Elimelech M. Isolation and assessment of phytate-hydrolysing bacteria from the DelMarVa peninsula. *Environ. Microbiol*. 2007; **9**: 3100-3107.
- Scott J.J. Alkaline phytase activity in nonionic detergent extracts of legume seeds. *Plant Physiol*. 95:1298-3018-Pizzolon L. 1996. Importance of Cyanobacteria as potential factor of toxicity in continental waters. *Interciencia*. 1991; **21**: 239-245.
- Pizzolon L. Importance of Cyanobacteria as potential factor of toxicity in continental waters. *Interciencia*. 1996; **21**: 239-245.
- Lan G. Q., Abdullah N., Jalaludinand S. Ho Y. W. Purification and characterization of a phytase from *Mitsuokella jalaludinii*, a bovine rumen bacterium. *African Journal of Biotechnology*, 2011; **10**(59), 12766-12776
- Palacios M.C., Harosa M., Sanzb Y. and Rosella CM. Selection of lactic acid bacteria with high phytate degrading activity for application in whole 2007.
- Kim YO, Kim HK, Bae KS, Yu JH, Oh TK. Purification and properties of a thermostable phytase from *Bacillus* sp. DS11. *Enzyme Microbial. Technol.*, 1998; **22**: 2-7.
- Greiner R, Jany KD, Alminger ML. Identification and properties of a phytate-degrading enzymes from barley (*Hordeum vulgare*). *J.Cereal Sci.*, 1999; **30**: 11-17.
- Yanke LJ, Bae HD, Selinger LB, Cheng K-J. Phytase activity of anaerobic ruminal bacteria. *Microbiology*, 1998; **144**: 1565-1573.
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. *Analy. Biochem.*, 1976; **72**: 248-252.
- Yanke LJ, Selinger LB, Cheng KJ. Phytase activity of *Selenomonas ruminantium*: a preliminary characterization. *Lett. Appl. Microbiol.*, 1999; **29**: 20-25.
- Tambe SM, Kaklij GS, Kelkar SM, Parekh LJ. Two distinct molecular forms of phytase from *Klebsiella aerogenes*: Evidence for unusually small active enzyme peptide. *J. Ferm. Bioeng.*, 1994; **77**: 23-27.
- Segueilha L, Lambrechts C, Boze H, Moulin G, Galzy P. Purification and properties of the phytase from *Schwanniomycescastellii*. *J. Ferm. Bioeng.*, 1992; **74**: 7-11.
- Greaves MP, Anderson G, Webley DM. The hydrolysis of inositol phosphates by *Aerobacter aerogenes*. *Biochim. Biophys. Acta.*, 1967; **132**: 412-418.
- Nakamura Y, Fukuhara H, Sano K. Secreted phytase activities of yeasts. *Biosci. Biotechnol. Biochem.*, 2000; **64**: 841-844.
- Greiner R, Konietzny U, Jany KD. Purification and characterization of two phytases from *Escherichia coli*. *Arch. Biochem. Biophys.*, 1993; **301**: 107-113.
- Shimizu M. Purification and characterization of phytase from *Bacillus subtilis* (natto) N-77. *Biosci. Biotechnol. Biochem.*, 1992; **56**: 1266-1269.

22. Greiner R, Haller E, Konietzny U, Jany KD. Purification and characterization of a phytases from *Klebsiella terrigena*. *Arch.Biochem. Biophys.*, 1997; **341**: 201-206.
23. Kim DH, Oh BC, Choi WC, Lee JK, Oh TK. Enzymatic evaluation of *Bacillus amyloliquefaciens* phytase as a feed additive. *Biotechnol. Lett.*, 1999; **2**: 925-927.
24. Wyss M, Brugger R, Kronenberger A, Rémy R, Fimbel R, Oesterhelt G, Lehmann M, van Loon APMG. Biochemical characterization of fungal phytases (*Myo*-inositol hexakisphosphate phosphohydrolases): Catalytic properties. *Appl. Environ. Microbiol.*, 1999; **65**: 367-373.
25. Houde RL, Alli I, Kermasha S. Purification and characterization of canola seed (*Brassica* sp.) phytase. *J. Food Biochem.*, 1990; **14**: 331-351.
26. Greiner R, Alminger ML. Purification and characterization of a phytate-degrading enzyme from germinated oat (*Avena sativa*). *J. Sci. Food Agri.*, 1999; **79**: 1453-1460.
27. Greiner R, Konietzny U, Jany KD. Purification and properties of a phytase from rye. *J. Food Biochem.*, 1998; **22**: 143-161.