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(54) Titre : DS11 (KCTC 0231BP), NOUVELLE SOUCHE DE BACILLUS SP. ET NOUVELLE PHYTASE PRODUITE A PARTIR DE LADITE SOUCHE

(54) Title: DS11 (KCTC 0231BP), NOVEL BACILLUS SP. STRAIN AND NOVEL PHYTASE PRODUCED BY IT

(57) Abrégé/Abstract:

This invention relates to a novel phytase produced from novel strain Bacillus sp. DS11 (KCTC 0231BP) and more precisely, to a novel strain Bacillus sp. DS11 and phytase enzyme enhancing the phosphate bioavailability present in grains supplied to monogastric animals.

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(54) Title: DS11 (KCTC 0231BP), NOVEL BACILLUS SP. STRAIN AND NOVEL PHYTASE PRODUCED BY IT

(57) Abstract

This invention relates to a novel phytase produced from novel strain *Bacillus* sp. DS11 (KCTC 0231BP) and more precisely, to a novel strain *Bacillus* sp. DS11 and phytase enzyme enhancing the phosphate bioavailability present in grains supplied to monogastric animals.



DS11 (KCTC 0231BP), NOVEL *BACILLUS* SP. STRAIN AND NOVEL PHYTASE PRODUCED BY IT

BACKGROUND OF THE INVENTION

5 Field of the Invention

This invention relates to a novel phytase produced from novel strain *Bacillus* sp. DS11 (KCTC 0231BP) and more precisely, to a novel strain *Bacillus* sp. DS11 and phytase enzyme enhancing the phosphate bioavailability present in grains supplied to monogastric animals.

10 Description of the Prior Art

Phytase is an enzyme to degrade phytic acid into phosphate and phosphate inositol. 50 to 70% of phosphate in grain used as livestock feeds exists in form of phytic acid but phytase is not present in monogastric animals such as hens and hogs, resulting in low phosphate availability.

15 Therefore undigested phytic acid(phytictaine) released to a water source became one of the serious environment contamination sources causing to eutrophication in small lakes or tides. With above consideration, because monogastric animals can't utilize phytic acid in their intestine phytic acid chelates to water due to chelation with a trace amount of minerals, amino acids and vitamins which are very important to the metabolism of livestock.
20 These formed water-insoluble, undigestable chelate complexes released to feces change the environmental ecosystem to induce a serious environmental pollution.

In view of these situations, the application of phytase into the livestock feeds will reduce the supply of inorganic phosphate due to increase of phosphate bioavailability in livestock, leading to economic benefits, and improving the availability of phosphate, and other bioactive substances, leading to reduction of the environmental contamination.

By these reasons, the utilization of phytase in livestock is very important. A law regulating the amount of phosphate in animal waste was established in 1996 in Korea and in European countries it is already mandatory to add phytase in the feeds of animals.

5 The addition of phytase in the feeds may greatly improve the productivity of livestock by enhancing the availability of some bioactive substances (phosphate, calcium and zinc etc.) which, otherwise chelate with phytate and lose their activity.

As the result, the use of feeds containing phytase in livestock can
10 enhance the availability of feeds and reduce the environmental contamination caused by phosphate.

For the aforementioned benefits, intensive studies about phytase including the effects of phytase on animals (Young et al., *Addition of microbial phytase to diet of young pigs*, J Anim Sci 71(8) : 247-50 (1993); (Lei et
15 al., *Calcium level affects the efficacy of supplemental microbial phytase in corn-soybean diets of weaning pigs*, J Anim Sci 72(1) : 139-43 (1994); and Mroz et al.,
Apparent digestibility and retention of nutrients to phytate complexes as influenced by microbial phytase and feeding regimen in pigs, J Anim Sci 72(1) : 126-32 (1994))
have been performed mainly in Europe (Ullah, A.H., *The complete primary
20 structure elucidation of *Aspergillus ficuum* (niger), pH 2.5-optimum acid phosphate by
Edman degradation*, Biochem Biophys Res Commun 203(1) : 182-89 (1994);
and Piddington et al., *The Cloning and sequencing of the genes encoding phytase
(phy) and pH 2.5-optimum acid phosphate (aph) from *Aspergillus niger* var. *awamori**,
Gene 133(1) : 55-62 (1993)). However, since phytase can cleave only a
25 limited number of phosphates and it mostly produced by molds which
have long growing period, it is uneconomical in terms of mass production.
In addition, it is difficult to use the phytic acid as an additive for
monogastric animals since it is unsuitable to their physiological mechanism.

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SUMMARY OF THE INVENTION

Therefore, the inventor et al. have identified a novel phytase-producing microorganism among hundred kinds of molds, Actinomycetes,

5 bacteria, etc., obtained from soils and barns throughout the country, in an effort to produce phytase having excellent enzymatic potency and shorten the production period with the conventional phytase. Because of the high enzymatic potency of this novel microorganism and its physiological

suitability to use for monogastric animals and short period of kproduction compared to those of conventional enzymes the inventor et al. have judged that this enzyme has novelty and they have completed this invention.

The object of this invention is to provide novel strain *Bacillus sp.* DS11 (KCTC 0231BP) and phytase enzyme, which is suitable to use for monogastric animals with excellent properties and more shortened production period.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is electron microscope photograph (X 10,000) of *Bacillus sp.* DS11 (KCTC 0231BP) strain, a novel strain according to this invention,

Fig. 2 is a SDS-PAGE electrophoresis analysis of novel phytase produced by *Bacillus sp.* DS11 (KCTC 0231BP), A: precipitation of acetone, B: resource S, C: superose 12 HR 10/30

Fig. 3 is a graph measuring of novel phytase produced by *Bacillus sp.* DS11 (KCTC 0231BP), a novel strain, in accordance with EXPERIMENTAL EXAMPLE 2 of this invention.

DETAILED DESCRIPTION OF THIS INVENTION

This invention relates to a novel phytase produced from novel strain *Bacillus sp.* DS11 (KCTC 0231BP) and particularly, to a novel strain *Bacillus sp.* DS11 and phytase enzyme enhancing the phosphate bioavailability present in grains supplied to monogastric animals.

This invention is described in detail as set forth hereunder:

This invention relates to novel strain *Bacillus sp.* DS11(KCTC 0231BP).

Further, this invention includes its novel enzyme phytase whose N-terminal amino acid sequence is expressed by the following [Sequence table 1] under the following conditions:

Optimal temperature: 65 °C

Optimal pH: 7.0

Molecular weight: 43,000 dalton

Isoelectric point: 5.6

In addition, this invention includes a method to use said
5 microorganism as feed additives.

This invention is described in more detail as set forth hereunder:

This invention relates to a novel strain *Bacillus sp.* DS11 (KCTC 0231BP) and novel phytase produced from said strain. The procedure for isolating and identifying said novel microorganism is as follows:

10 [Isolation of novel microorganisms]

From several thousands of strains obtained from soils and in barns throughout the country, a strain was isolated which has excellent resolution in phytase screen plate containing 15g of D-glucose, 5 g of calcium phytate, 5g of NH_4NO_3 , 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g of KCl, 0.01 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g 15 $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ and agar 15 g at pH 7.0/ l .

[Identification of novel microorganism]

The morphological property of the strain isolated from the above procedure is as follows:

1) Morphological property

20 In the grams staining test of the strain cultivated in an optimal growth medium, the strain was proven to be gram-positive. Fig. 1, the picture of electron microscope shows that strain is a rod type of $0.8 \sim 1.8 \mu\text{m}$ in cell size.

In the growth test of the thermally inactivated cells at 80°C , said strain has formed thermostable spores. Further, its catalase test in which it 25 shows the positive response demonstrates that it coincides with the morphological property of *Bacillus sp.*

The physiological property of said strain is as follows:

2) Physiological property

The test results of physiological property of the strain are represented in the following table 1. As shown in the table 1, it is assumed that strain is a facultative microorganism which may be grow in both aerobic and anaerobic states. With the difference of grown strains found at 50 °C and pH 5.7 compared with *Bacillus pantothenticus*, the strain of this invention is a mutant derived from *Bacillus pantothenticus*.

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Table 1.

Property	<i>Bacillus</i> <i>pantotheniticus</i>	Strain of this invention				
Catalase	+	+				
Anaerobic culture	+	+				
V-p. test	-	+				
Acid-forming	glucose L-arabinose D-xylose D-mannitol	+	+	+	-	+
Hydrolysis	Casein Gelatin Starch	d +	+	+	+	+
Synthesis	Indole Dihydroacetone	- -	-	-	-	-
Growth pH	6.8 5.7	+	+	-	-	+
Concentration of growth salt (at NaCl)	2% 5% 7% 10%	+	+	+	+	+
Growth temperature	5°C 10°C 30°C 40°C 50°C	- - +	-	+	+	+

Note) +: positive. -: negative. d: different from species.

The chemical property of the strain obtained as above is as follows:

3) The chemical property of strain

After harvesting the strain, the test results of their various properties (e.g., G + C content, fatty acid composition, Murein Type and main melaquinone) are represented in the following table 2.

As shown in the above table 2, it is noted that the strain of this invention is similar to *Bacillus pantothenticus* in terms of G + C content, murein type and main melaquinone including fatty acid of strain but it seems to difficult to make a judgement that both strains are the same species. Thus, 10 it seems that the strain of this invention is a mutant derived from *Bacillus pantothenticus*.

Table 2.

Property	<i>Bacillus pantothenticus</i>	Strain of this invention
G + C content	36.9	45.6
Murein type	Mezo-DAP	Mezo-DAP
Main melaquinone	MK-7	MK-7
Fatty acid of strain (%)		
14 : 0 ISO	4.71	1.96
14 : 0	1.50	1.21
15 : 0 ISO	19.34	18.72
15 : 0 ANTEISO	37.95	38.51
16 : 0 ISO	10.01	6.16
16 : 0	9.77	9.77
17 : 0 ISO	4.37	9.02
17 : 0 ANTEISO	12.00	11.20
18 : 0	x	2.03

By compiling above mentioned morphological, physiological and chemical properties, it is revealed that the strain of this invention belongs to *Bacillus sp.* from Bergys Manual of Systemic Bacteriology Vol. 2 (Williams & Wilkins Co., 1989). Therefore, the microorganism isolated was nominated 5 as *Bacillus sp.* DS11 and deposited on February 1, 1996, to Korea Research Institute of Bioscience and Biotechnology Korean Collection for type cultures, Korean Patent Strain Deposit Associations located in #52, Oun-dong, Yusong-ku, Taejeon 305-333, Republic of Korea, with an accession number KCTC 0231BP.

10 This invention is described in more detail by the following examples, but the claims are not limited to these examples.

EXAMPLE 1: Production of novel phytase

15 To produce phytase, a medium containing 6% wheat bran, NH_2NO_3 , 0.04%, 0.02% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0% casein hydrolysate, 0.05% KH_2PO_4 , 0.04% K_2HPO_4 and 0.2% CaCl_2 , was adjusted to pH 6.5 and sterilized at 121 °C for 15 minutes.

Then, with the same medium composition, 1% seed culture cultivated in a flask at 37°C for 12 hours was inoculated into the medium in order to produce the enzyme.

20 The potency of produced enzyme was measured as follows: By using a substrate, comprising 2 mM phytic acid sodium salts and 2mM CaCl_2 in 0.1 M Tris buffer solution (pH 7.0), the enzyme was reacted at 37°C for 30 minutes to measure the amount of phosphoric acid generated. One unit of the enzymatic potency is equivalent to the enzymatic amount degrading 1 μ mol 25 of inorganic phosphate per 1 minute. As a result of said measurement, the novel enzyme has the enzymatic potency of 0.3 unit per protein mg. The maximum amount of enzyme in fermentation yielding 0.6 unit/mg was obtained when 30 l of strains, a working volume, was charged to a 50 l

fermenter and cultivated at 37°C for 48 hours under the following conditions : air influx – 0.8 vvm; stirring rate – 150 rpm. Compared with the enzymatic production amount of 0.3 unit/mg when phytase enzyme derived from *Aspergillus ficuum* is cultivated for 96 hours (Gibson, D.M.,

5 *Some modification to the media for rapid automated detection of salmonellas conductance measurement*, J Appl Bacteriol 63(4) : 299-304 (1987), it is well observed that the novel organism of this invention has a production amount twice higher.

EXPERIMENTAL EXAMPLE 1: Measurement of molecular weight
10 **for novel phytase**

2 / of novel strain solution, cultivated according to the procedure as described in said Example, was centrifuged for 15 minutes. Then, the supernatant solution was saturated with 50% acetone to precipitate proteins and the crude enzyme solution passed through dialysis membrane 15 was purified on a column containing phenyl sepharose* CL-4B, Resource S and superose 12 HR 10/30 (all of which are manufactured by Pharmacia, Sweden) so as to isolate the phytase enzyme only. The analytical results by said column are represented in the attached drawing Fig. 2. Phytase enzyme was again on SDS-PAGE electrophoresis and the results show that 20 the molecular weight of phytase produced by a novel strain is 43,000 dalton and isoelectric point is 5.6.

Further, the enzyme protein, so isolated, was used to determine the sequence of N-terminal amino acid using Protein/peptide Sequencer (Applied Biosystems, USA) and its results are represented in the following 25 [Sequence table].

The sequencing No. 1 of [Sequence table] is the sequence of N-terminal amino acids of the phytase produced from a novel strain of this invention; the sequencing No. 2 is the sequence of the N-terminal amino

* trade-mark

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acids of the phytase produced from *E. coli* (Greiner et al., *Purification and Characterization of Two Phytases from Escherichia coli*, Arch Biochem Biophys 303: 107 (1993)); from Ullah, A.H., *Apergillus ficuum phytase : Partial Primary Structure Substrate Selectivity, and Kinetic Characterization*, Prep Biochem 18 : 5 459-72 (1998).

From the results of [Sequence table], it is revealed that the phytase enzyme produced by *Bacillus sp.* DS11(KCTC 0231BP) of this invention is a novel enzyme.

EXPERIMENTAL EXAMPLE 2: Activity and stability of novel phytase to 10 temperature and pH

The optimal temperature was proved to be 65°C by using the enzyme isolated by the same method as described in the experimental example 2.

To measure the thermal stability, the enzyme was left at each temperature for 10 minutes so as to assess the residual activity. As shown in the attached 15 drawing Fig. 3 (3-1), the results show that when Ca^{2+} was not added, the activity began to decrease at 40 °C but in case of adding 5mM calcium ion, the activity was stable up to 70°C and 50% of the activity at 75°C was sustained.

From the aforementioned results, a novel phytase of this invention may be expected to a higher activity in the body of livestock. Therefore, it 20 seems to be preferable that the feeds should be pelleted or extruded at more than 75°C so as to use them as processing ones.

The results of phytase activity in the different conditions of pH are shown in the attached drawing Fig. 3 (3-2) and the optimal pH was 7.0. Further, to measure the stability on pH, the enzyme was left at various 25 conditions of pH for 1 hour so as to assess the residual activity of phytase. Even under the acidic condition of below pH 4, the enzyme was proved to have higher enzymatic activity and from these results, it is judged that the

Aspergillus ficuum (Ullah, A.H., *Aspergillus ficuum phytase: Partial Primary Structure Substrate Selectivity, and Kinetic Characterization*, Prep Biochem 18: 459-72 (1998)).

From the results of [Sequence table], it is revealed that the phytase 5 enzyme produced by *Bacillus sp.* DS11(KCTC 0231BP) of this invention is a novel enzyme.

EXPERIMENTAL EXAMPLE 2: Activity and stability of novel phytase to temperature and pH

10 The optimal temperature was proved to be 65°C by using the enzyme isolated by the same method as described in the experimental example 2.

To measure the thermal stability, the enzyme was left at each temperature for 15 10 minutes so as to assess the residual activity. As shown in the attached drawing Fig. 3 (3-1), the results show that when Ca^{2+} was not added, the activity began to decrease at 40 °C but in case of adding 5mM calcium ion, the activity was stable up to 70°C and 50% of the activity at 75 °C was sustained.

From the aforementioned results, a novel phytase of this invention may 20 be expected to a higher activity in the body of livestock. Therefore, it seems to be preferable that the feeds should be pelleted or extruded at more than 75°C so as to use them as processing ones.

The results of phytase activity in the different conditions of pH are 25 shown in the attached drawing Fig. 3 (3-2) and the optimal pH was 7.0. Further, to measure the stability on pH, the enzyme was left at various conditions of pH for 1 hour so as to assess the residual activity of phytase. Even under the acidic condition of below pH 4, the enzyme was proved to have higher enzymatic activity and from these results, it is judged that the novel phytase of this invention may be extremely stable under the acidic condition of stomach.

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From the test results of the aforementioned temperatures and pH, it is
5 noted that the novel phytase of this invention may be applicable as a feed
additive of monogastric animals.

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EXPERIMENTAL EXAMPLE 3: Influence of metal ion and inhibitor on enzyme activity

Influence of metal ion and inhibitor on enzyme activity is represented in the following table 3.

5 The results of table 3 show that the addition of 1mM EDTA inhibited the entire enzyme activity and when Cu^{2+} , Zn^{2+} and Mg^{2+} were added at the concentration of 5mM, about 50% of enzyme activity was reduced.

Table 3.

Additives	Concentration	
	1 mM	5 mM
Non-addition	100	100
CuCl_2	63	43
ZnCl_2	87	47
MgCl_2	95	49
MnCl_2	65	20
LiCl_2	95	100
HgCl_2	83	62
CaCl_2	99	116
RbCl_2	103	102
EDTA	7.5	7.6
PMSF	86	88

EXPERIMENTAL EXAMPLE 4: Influence of novel phytase added to feed for broiler on environmental contamination

To estimate the influence relation of availability and released amounts of phosphate when phytase is added, broilers were divided into three groups such as novel phytase group, soybean-extracted plant phytase group and mold

phytase group on the market (manufacturer: Sigma). Each of hatched-out 200 males of Avachre broiler chicken was announced publicly for this experiment. The cultured medium containing phytase was ultrafiltrated and further concentrated at low temperature under vacuum and dried by 5 lyophilizer. Each 500 unit of this lyophilized phytase was added to per kg of feed. The same amount of soybean phytase or mold phytase as above was also added to the feeds and the results were represented in the following table 4.

10 **Table 4**

Classification	Plant (soybean) phytase	Commercial mold phytase	Novel phytase
Phosphate contents in feeds (g/kg)	5.5	5.5	5.5
Intake of total feeds (g/kg)	2.7	2.7	2.7
15 Intake of phosphate (g/head)	15.1	15.2	15.1
Accumulation of phosphate (g/head)	7.1	7.8	8.8
Discharge of phosphate (g/head)	8.0	7.4	6.3
Absorption of phosphate (%)	47	51	58

20 The results of Table 4 revealed that when novel phytase was fed to monogastric animals, the phosphate contents released were lower than the soybean group or mold phytase group, since the phosphate availability in the former was higher than that of the latters; Namely, the activity of novel phytase was superior under acid-fast and acidic conditions and phytictaine 25 within the grains was degraded in the intestine of animals by the novel phytase in more effective manner.

[Sequence table]

Sequencing No.: 1

Sequencing length: 15

Sequencing form: amino acid

5 Shape: straight chain

Sequencing type: protein

Sequence: Ser-Asp-Pro-Tyr-His-Phe-Thr-Val-Asn-Ala-Ala-Xaa-Glu-Thr-Glu

Sequencing No.: 2

10 Sequencing length: 11

Sequencing form: amino acid

Shape: straight chain

Sequencing type: protein

Sequence: Ser-Glu-Pro-Glu-Leu-Lys-Leu-Glu-Ala-Val-Val

15

Sequencing No.: 3

Sequencing length: 12

Sequencing form: amino acid

Shape: straight chain

20 Sequencing type: protein

Sequence: Pro-Ala-Ser-Arg-x-Gin-Ser-Ser-Cys-Asp-Thr-Val

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SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: KOREA INSTITUTE OF TECHNOLOGY
(ii) TITLE OF THE INVENTION: DS11 (KCTC0231BP), NOVEL BACILLUS
sp. STRAIN AND NOVEL PHYTASE PRODUCED BY IT

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:

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(C) CITY: Montreal
(D) STATE: QC
(E) COUNTRY: Canada
(F) ZIP: H3A 2Y3

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ for Windows Version 2.0

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: 2,249,014
(B) FILING DATE: 14-MAR-1997
(C) CLASSIFICATION: C-12N-9/16

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: KR 1996/6817
(B) FILING DATE: 14-MAR-1996

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Cawthorn, Christian
(B) REGISTRATION NUMBER: 11,005
(C) REFERENCE/DOCKET NUMBER: 11292-46 CC/

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 514-847-4256
(B) TELEFAX: 514-288-8389
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

13b

(ii) MOLECULE TYPE: protein
 (ix) FEATURE:

- (A) NAME/KEY: Modified Base
- (B) LOCATION: 12...12
- (D) OTHER INFORMATION: Xaa = any amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Ser Asp Pro Tyr His Phe Thr Val Asn Ala Ala Xaa Glu Thr Glu
 1 5 10 15

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 11 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ser Glu Pro Glu Leu Lys Leu Glu Ala Val Val
 1 5 10

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 12 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:

- (A) NAME/KEY: Modified Base
- (B) LOCATION: 5...5
- (D) OTHER INFORMATION: Xaa = any amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Pro Ala Ser Arg Xaa Gln Ser Ser Cys Asp Thr Val
 1 5 10

WHAT IS CLAIMED IS :

1. A biologically pure culture of Strain *Bacillus sp.* DS11(KCTC 0231BP).
- 5 2. Use of Strain *Bacillus sp.* DS11(KCTC 0231BP) as feed additives.
3. A phytase produced by *Bacillus sp.* in claim 1 having the following characterization:

10	Optimal temperature	65%
	Optimal pH	7.0
	Molecular weight	43,000 dalton
	Isoelectric point	5.6
	Sequence of N-terminal amino acid	SEQ ID NO:1.
- 15 4. Use of a phytase as defined in claim 3 as feed additives.

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FIG. 1



FIG. 2



2/2

FIG. 3-1

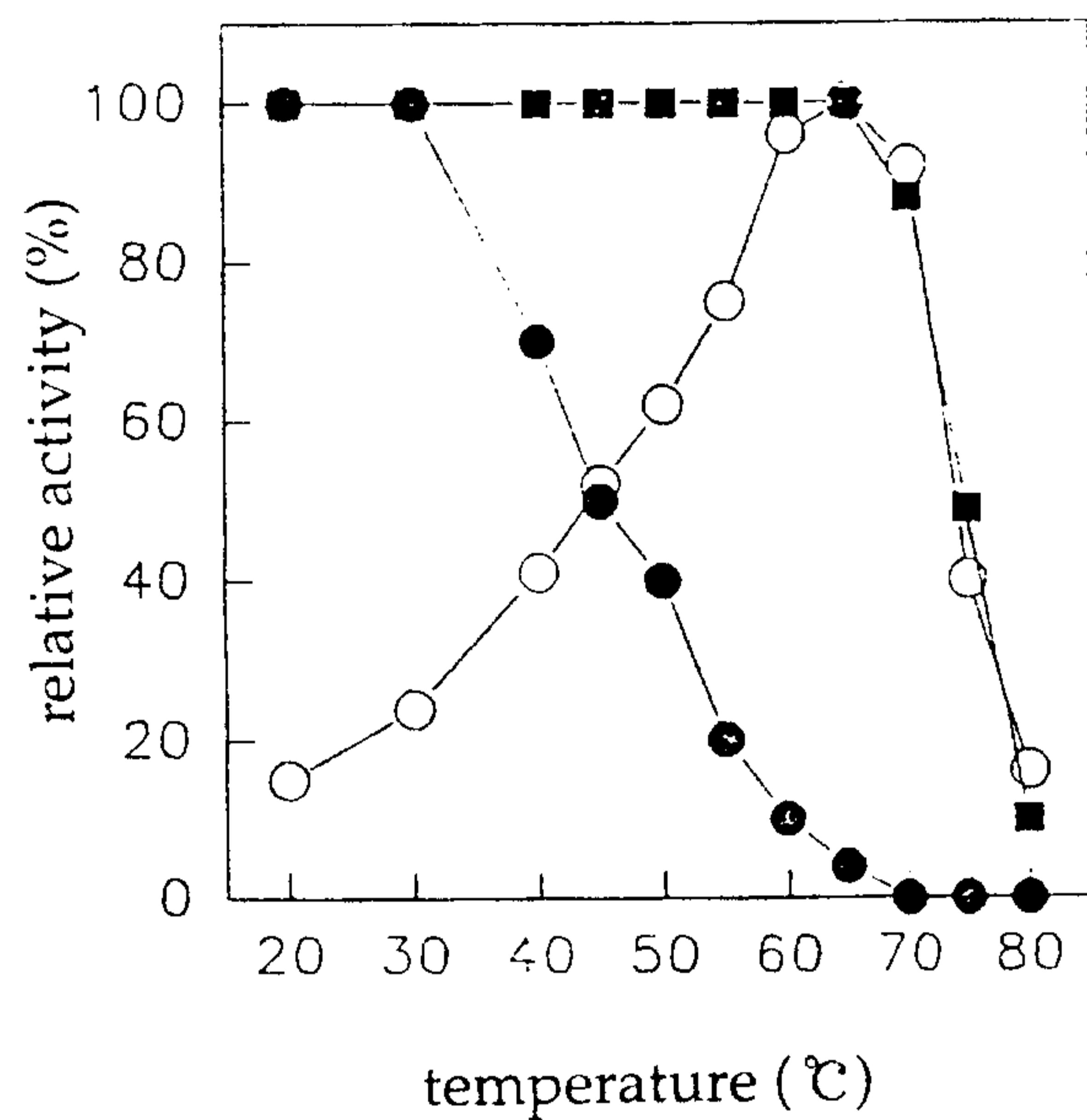
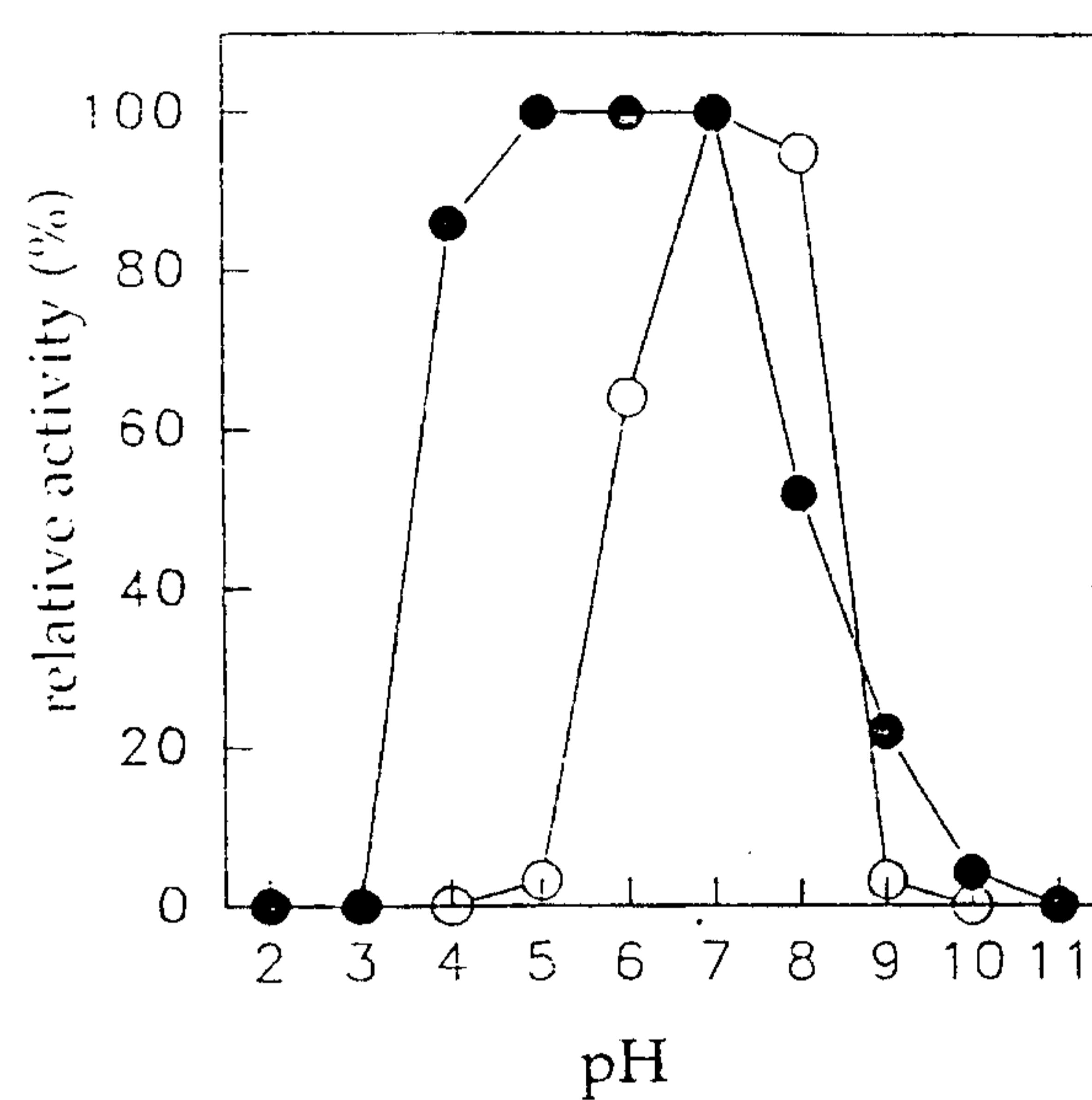


FIG.3-2



-○- ; optimal temperature
 -●- ; stability in the non-existence of Ca^{2+}
 -■- ; stability in the existence of 5mM Ca^{2+}

-○- ; optimal pH
 -●- ; stability