Phytase No. 2(8)

Proposed revision of Ministerial Ordinance on the Specifications and Standards of Feeds and Feed Additives.

1. Specifications for ingredients, standards for methods of manufacture, use and storage, and standards for labeling of feeds in general

・Standards for methods of manufacture of feeds in general

Phytase No. 2(8) shall not be used in feeds (including raw material or ingredients used to manufacture feeds) other than those for pigs, chickens, quails, fish, and crustaceans.

・Standards for labeling of feeds in general

Feed containing feed additives for which the target animal is specified must be labeled with the type of the target animal.

2. Specifications for feed additives

A. Active Substance

(a) Compositional standards

Enzymatic activity unit: This product shall contain not less than 50,000 phytase activity units per gram when tested for enzyme activity.

Physical and chemical properties:

i. The product is a brown liquid.
ii. The pH of a 1:100 suspension of the product shall be between 4.0 and 5.0.
iii. The product exhibits maximum enzyme activity at a pH range of 3.5 to 5.0.

Purity test:

i. Lead: When 2.0 g (1.95–2.04 g) of the product is tested using Method 1 of the Atomic Absorption Spectrophotometry for lead, the lead content shall not exceed 5 μg/g.
ii. Arsenic: The test shall be conducted in accordance with the method (2) for arsenic in the active ingredient for manufacturing Phytase No. 1.
iii. Antibacterial activity: The test shall be conducted in accordance with the method (3)for antimicrobial activity in the active ingredient for manufacturing Phytase No. 1.

Residue on ignition: Not more than 5.0% (based on 0.5 g sample).

Enzymatic activity test: To be conducted using Method 5 of the Phytase Activity Test.

Preparation of Sample Solution: Accurately weigh 0.25 g of the product to three significant figures and record the value. Dilute to 25 mL with sodium acetate buffer (0.25 mol/L, pH 5.5). Further dilute with the same buffer so that the absorbance difference between the test reaction solution and the control solution falls within the range of 0.4 to 1.1. Use this as the sample solution.

(b) Standard of manufacturing method

Phytase shall be produced by culturing a genetically modified strain that produces phytase, using a host strain belonging to *Trichoderma reesei*. After the culture process is complete, the culture solution shall be filtered to remove the microbial cells, and the resulting filtrate shall be used as the product.

(c) Standard of storage method

The product shall be stored in a light-shielded, sealed container.

(d) Standards of the label

The immediate container or packaging of the product shall indicate the pH value (to one decimal place) at which the product exhibits maximum enzyme activity.

B. Preparation (No. 1)

(a) Compositional standards

This product is a granular, powdered, or particulate formulation prepared by spray-drying the active substance of Phytase No.2(8), and optionally mixing it with wheat flour and vegetable oil.

Alternatively, the active ingredient phytase material may be mixed with wheat flour, vegetable oil, antioxidants, and propionic acid, granulated, and then dried.

Enzyme activity unit: When tested, the product shall contain 85% to 170% of the labelled phytase activity units.

Enzymatic activity test: To be conducted using Method 5 of the Phytase Activity Test.

Preparation of Sample Solution: Accurately weigh 0.5 g of the product to three significant figures and record the value. Add sodium acetate buffer (0.25 mol/L, pH 5.5), stir at room temperature for 20 minutes, and dilute to 50 mL with the same buffer. Further dilute so that the absorbance difference between the test reaction solution and the control solution falls within the range of 0.4 to 1.1. Use this as the sample solution.

(b) Standard of storage method

The storage method shall follow the same standards as those for the active substance of Phytase No.2(8).

(c) Standard of the label

The labelling method shall follow the same standards as those for the active substance of Phytase No.2(8).

C. Preparation (No. 2 liquid)

(a)Compositional standards

This product is a water-soluble liquid formulation prepared by adding sodium benzoate, trisodium citrate, sorbitol, and sodium sulfate to the active substance of Phytase No. 2(8), and diluting with purified water.

Enzymatic activity unit: When tested, the product shall contain 85% to 170% of the labelled phytase activity units.

Enzymatic activity test: To be conducted using Method 5 of the Phytase Activity Test.

Preparation of Sample Solution: The method used for the active substance of Phytase No.2(8) shall be applied.

(b) Standard of storage method

The storage method shall follow the same standards as those for the active substance of Phytase No.2(8).

(c) Standard of the label

The labelling method shall follow the same standards as those for the active substance of Phytase No.2(8).

3. **Specifications for feed additives**

Method 5 of the Phytase Activity Test

Preparation of Substrate Solution

Weigh 0.98 g (range: 0.975–0.984 g) of sodium phytate and dissolve it in approximately 80 mL of sodium acetate buffer (0.25 mol/L, pH 5.5). Adjust the pH to 5.5 using acetic acid. Transfer the solution to a 100 mL volumetric flask and add the same buffer up to the calibration mark to make a total volume of 100 mL.

Preparation of Reaction-Stopping Colour Reagent

Dissolve 20 g (range: 19.5–20.4 g) of ammonium molybdate in 180 mL of water. Add 2 mL of ammonia solution, then dilute with water to 200 mL to prepare the ammonium molybdate reagent.

Separately, dissolve 0.47 g (range: 0.465–0.474 g) of ammonium vanadate in 160 mL of water while stirring in a water bath at approximately 50°C. Add 4 mL of nitric acid (175→500), cool to room temperature, and dilute with water to 200 mL to prepare the ammonium vanadate reagent.

Mix 25 mL of the ammonium molybdate reagent with 25 mL of the ammonium vanadate reagent, shake well, then add 16.5 mL of nitric acid (175→500) and dilute with water to 100 mL. This colour reagent should be freshly prepared before use.

Procedure

Prepare the sample solution according to the method specified for each product.

1. Pipette 2 mL of the substrate solution into a 15 mL plastic centrifuge tube and preheat at 37 ± 0.5°C for 5–10 minutes.
2. Separately, preheat 1 mL of the sample solution at 37 ± 0.5°C for 5–10 minutes.
3. Add the preheated sample solution to the substrate solution and incubate at 37 ± 0.5°C for exactly 60 minutes.
4. Add 2 mL of the reaction-stopping colour reagent by a full volume pipette, mix well, and leave at room temperature for 20 minutes.
5. Centrifuge at 2,500 × g for 10 minutes and use the supernatant as the test reaction solution.
6. Measure the absorbance (ODT) at 415 nm using water as the blank.

For the control:

1. Pipette 2 mL of the substrate solution into a 15 mL plastic centrifuge tube.
2. Add 2 mL of the reaction-stopping colour reagent by a full volume pipette and mix.
3. Add 1 mL of the sample solution by a full volume pipette, mix well, and leave at room temperature for 20 minutes.
4. Centrifuge at 2,500 × g for 10 minutes and use the supernatant as the control solution.
5. Measure the absorbance (ODB) at 415 nm using water as the blank.

Calculation of Phytase Activity Units per Gram

Phytase Activity Units per g=(ODT−ODB)×F×V×160×1W×Z

Where:

- **F**: Phosphate ion concentration (μmol/mL) corresponding to an absorbance difference of 1, determined from the calibration curve
- **V**: Final volume of the sample solution (mL)
- **W**: Weight of the sample (g)
- **Z**: Dilution factor

Preparation of Calibration Curve

Weigh 0.0980 g (range: 0.0975–0.0984 g) of potassium dihydrogen phosphate that has been dried at 105°C for 2 hours and stored in a desiccator. Dissolve it in sodium acetate buffer (0.25 mol/L, pH 5.5), transfer to a 100 mL volumetric flask, and add the same buffer up to the calibration mark to make 100 mL. Confirm that the pH is 5.5. This solution is the standard stock solution (7.20 μmol/mL).

Prepare the following standard solutions in test tubes by diluting the stock solution with sodium acetate buffer (0.25 mol/L, pH 5.5):

- **S1**: 1.25× dilution
- **S2**: 2.5× dilution
- **S3**: 5× dilution

S4: 10× dilution

For each standard solution:

1. Pipette 1 mL into a 15 mL plastic centrifuge tube by a full volume pipette.
2. Add 2 mL of substrate solution and 2 mL of the reaction-stopping colour reagent by a full volume pipette.
3. Mix well and leave at room temperature for 20 minutes.
4. Centrifuge at 2,500 × g for 10 minutes.
5. Use the supernatant as the phosphate standard reaction solution.
6. Measure the absorbance values (ODS1, ODS2, ODS3, ODS4) following the same procedure as for the test reaction solution.

Separately, prepare a phosphate standard blank solution:

1. Pipette 1 mL of sodium acetate buffer (0.25 mol/L, pH 5.5) into a 15 mL plastic centrifuge tube by a full volume pipette.
2. Add 2 mL of substrate solution and 2 mL of the reaction-stopping colour reagent by a full volume pipette.
3. Mix well and leave at room temperature for 20 minutes.
4. Centrifuge at 2,500 × g for 10 minutes.
5. Use the supernatant as the phosphate standard blank solution.
6. Measure the absorbance (ODSB) following the same procedure.

Plot the phosphate ion concentration on the vertical axis and the absorbance differences (ODS1–ODSB), (ODS2–ODSB), (ODS3–ODSB), and (ODS4–ODSB) on the horizontal axis to create the calibration curve.