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NATIONAL STANDARD OF THE
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**National food safety standard - Food additives - Enzyme
preparations for food industry**

食品安全国家标准 食品添加剂 食品工业用酶制剂

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National food safety standard - Food additives - Enzyme preparations for food industry

1 Scope

This standard applies to enzyme preparations for the food industry that are allowed to be used in *National food safety standard - Standards for uses of food additives* (GB 2760) and related announcements.

2 Terms and definitions

2.1 enzyme preparations for the food industry

A preparation product with special catalytic activity and used in the food industry, which is directly extracted from edible or non-edible parts of animals or plants, or fermented and extracted from microorganisms (including but not limited to bacteria, actinomycetes, and fungal strains) obtained through traditional selection or genetic recombination technology, or prepared through further purification, formulation and other processes (it may contain one or more active enzyme components).

2.2 enzyme activity

An indicator of the activity of an enzyme to catalyze a specific reaction under certain conditions.

2.3 antibacterial activity

Properties that inhibit or kill microorganisms.

2.4 excipients for enzyme preparations

Food raw materials and food additives added for the purpose of activity preservation, circulation storage, and standardized use of enzyme preparation products.

2.5 immobilized enzyme preparations

Product dosage forms that immobilize the enzyme on the carrier by physical and (or) chemical methods and function in an insoluble state during the food production process.

2.6 immobilized carrier

The material that plays the role of immobilizing enzymes in immobilized enzyme

preparations.

3 Product classification

According to product form, it is divided into solid dosage forms (including immobilized dosage forms) and liquid dosage forms.

4 Technical requirements

4.1 Requirements for raw materials and excipients

4.1.1 The raw materials and excipients used to produce enzyme preparations shall meet relevant requirements and shall not produce residual contamination harmful to health in the final food under the specified conditions of use.

4.1.2 Animal tissues used for extracting enzyme preparations shall comply with meat quarantine requirements.

4.1.3 Plant tissues used for extracting enzyme preparations shall not become moldy.

4.1.4 Microbial production strains shall be taxonomically and/or genetically identified, and shall comply with the *National food safety standard - Standards for uses of food additives* (GB 2760) and related announcements. The preservation methods and conditions of strains shall ensure stability and reproducibility between fermentation batches.

4.1.5 For the activity preservation, circulation and storage, and standardized use of enzyme preparation products, necessary excipients are allowed to be added to commercial enzyme preparations. Excipients for enzyme preparations shall not play a functional role in the final food. They are generally used in appropriate amounts according to production needs, and the varieties and amounts used shall be reduced as much as possible on the premise of achieving the expected purpose. The list of excipients allowed to be used in enzyme preparations is shown in Appendix A.

4.1.6 The immobilized carrier used in the immobilized enzyme preparation shall comply with the *National food safety standard - Food contact materials and products - Standards for uses of additives* (GB 9685), *National food safety standard - Standards for uses of food additives* (GB 2760) or other provisions on the list of raw materials allowed to be used in food.

4.2 Physical and chemical indicators

Product enzyme activity shall meet the claims.

B.2.3.3 Reagents and materials

Unless otherwise specified, the reagents used are all of analytical grade, and the water is the second-grade water that complies with GB/T 6682.

B.2.3.3.1 Acetoin (3-hydroxy-2-butanone) [$\text{CH}_3\text{COCH}(\text{OH})\text{CH}_3$, CAS number: 513-86-0], with a purity of $\geq 95\%$; or a standard obtained national certification and awarded a reference material certificate.

B.2.3.3.2 MES (9.76 g/L)-sodium chloride (35.064 g/L)-polyoxyethylene dodecyl ether (1.52 mL/L) buffer solution: Respectively weigh 48.80 g of 2-[N-morpholino] ethanesulfonic acid (MES) and 175.32 g of sodium chloride into a beaker, dissolve it in about 4.5 L of water, then add 7.60 mL of 15% polyoxyethylene dodecyl ether solution, and stir evenly; use about 1 mol/L sodium hydroxide solution to adjust the pH to 6.00 ± 0.05 . Then transfer it into a 5000 mL volumetric flask, dilute to volume with water, and stir evenly. The storage period of this solution at normal temperature ($15\text{ }^\circ\text{C} \sim 20\text{ }^\circ\text{C}$) is 1 week.

B.2.3.3.3 α -acetolactate substrate (2.00 mL/L): Take 100 μL of ethyl-2-acetoxy-2-methylacetoacetic acid into a 50 mL volumetric flask, and add 6.0 mL of about 0.50 mol/L sodium hydroxide solution; after shaking for 20 minutes, add buffer solution to approximately 40.0 mL; use approximately 1 mol/L hydrochloric acid to adjust the pH of the solution to 6.00 ± 0.05 , and then dilute to volume with buffer solution. The solution shall be prepared fresh just before use.

B.2.3.3.4 Naphthol (10.0 g/L)/creatinine (1.0 g/L) chromogen: Weigh 5.0 g of 1-naphthol and 0.5 g of creatinine respectively into a 500 mL volumetric flask, use about 1 mol/L sodium hydroxide solution to dissolve and dilute to volume. The solution shall be prepared fresh just before use. Keep away from light and take an ice bath during preparation.

WARNING: 1-Naphthol is flammable and toxic. It has irritation to the eyes and mucous membranes. Poisoning can occur if it is swallowed or absorbed through the skin.

B.2.3.3.5 Acetoin stock solution (1.000 g/L): Take a certain amount of acetoin in a test tube and dissolve it in a $37\text{ }^\circ\text{C}$ thermostat. The test tube is then placed in ice water to recrystallize the acetoin. After recrystallization, acetoin does not contain acetoin dimers that will affect the test results and can be used for the preparation of stock solutions; weigh 0.100 g of acetoin after recrystallization, accurate to 0.0001 g, and transfer it to a 100 mL volumetric flask, dissolve in water and dilute to volume.

NOTE: Acetoin is unstable to heat and easily absorbs moisture, so it needs to be stored in a desiccator and refrigerated ($2\text{ }^\circ\text{C} \sim 6\text{ }^\circ\text{C}$). If it is found that the material has obvious moisture absorption, it is recommended to abandon it. The solution has a shelf life of 1 week under refrigeration.

Weigh a certain amount of specimen from the sample, accurate to 0.0005 g, and dilute it with the solution. The dilution factor shall be such that the final absorbance H_1 of the sample falls within the range of the acetoin standard curve.

B.2.3.5.4 Drawing of the standard curve

Taking the absorbance at a wavelength of 522 nm as the Y -axis and the concentration of acetoin (mg/L) as the X -axis, draw a standard curve and calculate the slope h of the standard curve (or use the regression equation to calculate it).

B.2.3.5.5 Determination

Place the sample solution and substrate in a 30 °C water bath to preheat for about 10 minutes. Take 200.0 µL of the enzyme sample solution into a 10 mL test tube in a 30 °C±0.1 °C water bath, and then add 200.0 µL of substrate, time immediately, mix thoroughly with a vortex oscillator, and quickly return the tube to the water bath. After reacting for 20.0 minutes, add 4.60 mL of chromogenic reagent to the test tubes in sequence, and mix thoroughly with a vortex oscillator. Place the tubes at room temperature and restart timing. After 40.0 minutes of reaction, use a spectrophotometer to measure the absorbance of each tube of solution at a wavelength of 522 nm. At the same time, use buffer solution instead of sample solution to perform a blank test.

B.2.3.6 Result calculation

The enzyme activity X_1 of α -acetolactate decarboxylase preparation, in U/g (or U/mL), is calculated according to formula (B.1).

$$X_1 = \frac{(H_1 - H_2) \times 0.001\ 135\ 1 \times F_1}{m \times h} \dots\dots\dots (B.1)$$

where:

H_1 -- absorbance of the sample;

H_2 -- absorbance of the blank solution;

0.0011351 -- the mole number corresponding to 0.1 g of acetoin;

F_1 -- the total dilution factor of the sample solution before reaction;

m -- the mass of the specimen, in grams (g);

h -- the slope of the standard curve.

The measurement result of the sample is expressed as the arithmetic mean.

1 significant figure is retained when the result is less than 1 U/g (or U/mL); 2 significant

figures are retained when the result is greater than or equal to 1 U/g (or U/mL) and less than 100 U/g (or U/mL); 3 significant figures are retained when the result is greater than or equal to 100 U/g (or U/mL).

The arithmetic mean of parallel measurement results is taken as the test result. The absolute difference between two independent determination results obtained under repeatability conditions is not greater than 10% of the arithmetic mean.

B.2.4 Fully automatic biochemical analyzer method

B.2.4.1 Scope

This method specifies the determination method of α -acetolactate decarboxylase activity.

This method is suitable for the determination of the enzyme activity of α -acetolactate decarboxylase in α -acetolactate decarboxylase preparations by using a fully automatic biochemical analyzer. This method is not suitable for the determination of α -acetolactate decarboxylase activity in beer and other alcohol-containing products.

The detection limit of this method is 0.6 U/g (or U/mL).

The presence of 3-hydroxy-2-butanone (acetoin) and/or diacetyl in the sample will cause the test result larger.

Acetoin can easily form dimers during storage, thus affecting test results. However, this method can still be used if the precautions described in the preparation steps are followed.

B.2.4.2 Principle

α -acetolactate decarboxylase reacts with the substrate α -acetolactate to decarboxylate to produce acetoin. Acetoin reacts with a mixture of naphthol and creatine under alkaline conditions to form a red product. Draw a standard curve by measuring the absorbance of the standard solution at a wavelength of 510 nm, and further calculate the activity of the enzyme sample according to the standard curve.

B.2.4.3 Reagents and materials

B.2.4.3.1 MES (9.76 g/L)-sodium chloride (35.064 g/L)-polyoxyethylene lauryl ether (1.52 mL/L) buffer solution: See B.2.3.3.2.

B.2.4.3.2 α -acetolactate substrate (2.00 mL/L): See B.2.3.3.3.

B.2.4.3.3 Naphthol (10.0 g/L)/creatine (1.0 g/L) chromogen: See B.2.3.3.4.

B.2.4.4 Instruments and equipment

1000 -- conversion factor.

The measurement result of the sample is expressed as the arithmetic mean. When the test value of the standard reference substance diluent is between 0.48 mU/mL and 0.52 mU/mL, the test results of the sample are valid and the average value can be calculated. Otherwise, the test shall be repeated.

1 significant figure is retained when the result is less than 1 U/g (or U/mL); 2 significant figures are retained when the result is greater than or equal to 1 U/g (or U/mL) and less than 100 U/g (or U/mL); 3 significant figures are retained when the result is greater than or equal to 100 U/g (or U/mL). When the result is less than 0.6 U/g (or U/mL), it is expressed as <0.6 U/g (or U/mL).

The arithmetic mean of parallel measurement results is taken as the test result. The absolute difference between two independent determination results obtained under repeatability conditions is not greater than 4% of the arithmetic mean.

B.3 Determination of pectinase activity

B.3.1 Pectinase

An enzyme that can hydrolyze pectin and generate products containing reducing groups.

B.3.2 Pectinase activity

Under the reaction conditions of 50 °C and pH 3.5, 1 g of solid enzyme powder (or 1 mL of liquid enzyme) decomposes pectin in 1 hour to produce 1 mg of galacturonic acid, which is one enzyme activity unit, expressed in U/g or U/mL.

B.3.3 Principles

Pectinase can hydrolyze pectin, and the reducing uronic group of the generated galacturonic acid can be quantitatively measured by the hypiodous acid method to express the activity of pectinase.

This method is mainly used to detect the activity of polygalacturonase and pectin lyase in pectinase products. It is not suitable for pectin (methyl) esterase in pectinase products.

B.3.4 Reagents and materials

B.3.4.1 10 g/L citrus pectin solution: Weigh 1.0000 g of pectin powder (accurate to 0.1 mg), add water to dissolve, boil, cool and filter. Adjust pH to 3.5, dilute to 100 mL with water, and store in a refrigerator for later use. Use time does not exceed 3 d.

NOTE: Pectin substrate has a great impact on the test. If pectin powder from a different source or batch number is used, a control test must be conducted with the old batch.

B.3.4.2 0.05 mol/L sodium thiosulfate standard solution.

B.3.4.3 1 mol/L sodium carbonate standard solution.

B.3.4.4 0.1 mol/L iodine standard solution.

B.3.4.5 1 mol/L sulfuric acid solution: Take 5.6 mL of concentrated sulfuric acid, slowly add an appropriate amount of water, cool and dilute to 100 mL with water, and shake well for later use.

B.3.4.6 10 g/L soluble starch indicator solution.

B.3.4.7 0.1 mol/L sodium citrate buffer solution (pH 3.5): Weigh 14.71 g of citric acid ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$) and 8.82 g of trisodium citrate ($\text{C}_6\text{H}_5\text{Na}_3\text{O}_7 \cdot 2\text{H}_2\text{O}$), add 950 mL of water to dissolve, adjust the pH to 3.5, and then dilute the volume to 1000 mL with water.

B.3.5 Instruments and equipment

B.3.5.1 Colorimetric tube: 25 mL.

B.3.5.2 Constant-temperature water bath with a heating device: temperature control accuracy is $\pm 0.5\text{ }^{\circ}\text{C}$.

B.3.5.3 Iodine volumetric flask: 100 mL.

B.3.5.4 Burette: 25 mL.

B.3.6 Analysis steps

B.3.6.1 Preparation of sample solution

Use a 50 mL beaker of known mass to weigh 1 g~2 g enzyme powder (accurate to 0.0001 g), or accurately pipet 1.00 mL, fully dissolve it with a small amount of sodium citrate buffer solution, pound it with a glass rod, and carefully pour the supernatant into a 100 mL volumetric flask; if there is any remaining residue, add a small amount of the above buffer solution and grind thoroughly. Finally, transfer all the samples into the volumetric flask, adjust the volume to the mark, and shake well. Filter with 4 layers of gauze and let the filtrate for later use.

NOTE: The enzyme solution to be tested needs to be accurately diluted to a certain multiple, and the concentration of the enzyme solution is controlled so that the difference between the consumption of the sodium thiosulfate standard solution and the blank consumption is within the range of 0.5 mL~1.0 mL. If necessary, do preliminary tests first.

B.3.6.2 Determination

1 -- the volume of diluted enzyme solution added during the reaction, in milliliters (mL);

0.5 -- reaction time, in hours (h).

The results obtained are expressed in whole numbers.

The arithmetic mean of parallel measurement results is taken as the test result. The absolute difference between two independent determination results obtained under repeatability conditions is not greater than 3% of the arithmetic mean.

B.4 Determination of glucoamylase (amyloglucosidase) activity

B.4.1 Glucoamylase (amyloglucosidase)

Amyloglucosidase that uses starch as a substrate to hydrolyze α -1,4, α -1,6, α -1,3 glucosidic bonds from the non-reducing end of starch under certain conditions to produce glucose.

B.4.2 Glucoamylase (amyloglucosidase) activity

1.0 mL of enzyme solution or 1.0 g of enzyme powder hydrolyzes soluble starch to produce 1 mg of glucose in 1 hour at 40 °C and pH 4.6, which is 1 enzyme activity unit, expressed in U/g (or U/mL).

B.4.3 Reagents and materials

B.4.3.1 0.05 mol/L acetic acid-sodium acetate buffer solution (pH 4.6): Weigh 6.7 g of sodium acetate ($\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$), absorb 2.6 mL of glacial acetic acid, dissolve with water and dilute to 1000 mL. The pH of the above buffer solution shall be calibrated by using a pH meter.

B.4.3.2 0.05 mol/L sodium thiosulfate standard titration solution.

B.4.3.3 0.1 mol/L iodine standard solution.

B.4.3.4 0.1 mol/L sodium hydroxide solution.

B.4.3.5 2 mol/L sulfuric acid solution: Take 5.6 mL of analytically pure concentrated sulfuric acid (with a relative density of 1.84) and slowly add it to an appropriate amount of water. After cooling, dilute to 100 mL with water and shake well.

B.4.3.6 200 g/L sodium hydroxide solution: Weigh 20 g of sodium hydroxide, dissolve it in water, and adjust the volume to 100 mL.

B.4.3.7 20 g/L soluble starch solution: Weigh soluble starch (2 ± 0.001) g, then mix

thoroughly with a small amount of water, slowly pour into boiling water, boil and stir until transparent, cool, and dilute to 100 mL with water. This solution needs to be prepared on the use day.

NOTE: Soluble starch shall be a special starch for enzyme preparation analysis.

B.4.4 Instruments and equipment

B.4.4.1 Analytical balance: The accuracy is 0.1 mg.

B.4.4.2 pH meter: The accuracy is 0.01.

B.4.4.3 Constant-temperature water bath: $40\text{ }^{\circ}\text{C}\pm 0.5\text{ }^{\circ}\text{C}$.

B.4.4.4 Pipette.

B.4.4.5 Magnetic stirrer.

B.4.5 Determination procedure

B.4.5.1 Preparation of enzyme solution to be tested

B.4.5.1.1 Liquid enzyme: Use a pipette to accurately absorb an appropriate amount of enzyme sample, transfer it to a volumetric flask, dilute it to the mark with buffer solution, and shake it well, for testing later.

B.4.5.1.2 Solid enzyme: Use a 50 mL small beaker to accurately weigh an appropriate amount of enzyme sample, accurate to 1 mg, dissolve it with a small amount of acetic acid-sodium acetate buffer solution, carefully pound it with a glass rod, then carefully pour the supernatant into a 50 mL volumetric flask, and add a small amount of buffer solution to the sediment; pound it repeatedly 3 to 4 times, and take the supernatant; finally, transfer it all into a volumetric flask, and dilute it with buffer solution, for testing later.

NOTE 1: When preparing the enzyme solution to be tested, the concentration of the sample solution needs to be controlled so that the difference between titration consumption of 0.05 mol/L sodium thiosulfate standard titration solution for the blank and for the sample is within the range of 4.5 mL~5.5 mL (enzyme activity is 120 U/mL~150 U/mL).

NOTE 2: Liquid enzyme can also be weighed according to product characteristics and calculated in grams.

B.4.5.2 Determination

Take two 50 mL colorimetric tubes, add 25 mL of soluble starch solution and 5 mL of acetic acid-sodium acetate buffer solution respectively, and shake well. Preheat in a constant-temperature water bath of $40\text{ }^{\circ}\text{C}\pm 0.5\text{ }^{\circ}\text{C}$ for 5 min~10 min. Add 2.0 mL of

The sample measurement result is expressed as the arithmetic mean and retained to 3 significant figures.

The arithmetic mean of parallel measurement results is taken as the test result. The absolute difference between two independent determination results obtained under repeatability conditions is not greater than 10% of the arithmetic mean.

B.5 Determination of transglucosidase activity

B.5.1 Transglucosidase

An enzyme that can hydrolyze maltose molecules and α -1,4-glucosidic chains in linear maltodextrin, transfer the free glucose residues to a glucose molecule or maltose or maltotriose molecule to form α -1,6 glycosidic bonds, and generate oligosaccharides containing α -1 bonds such as isomaltose, panose, and isomaltotriose.

B.5.2 Transglucosidase activity

Under the conditions of 40 °C and pH 5.0, 1 mL of enzyme sample reacts with the substrate α -methyl-D-glucoside and generates 1 μ g of glucose in 60 minutes, which is one enzyme activity unit, expressed in U/g (or U /mL).

B.5.3 Principle

Transglucosidase reacts on the substrate α -methyl-D-glucoside, and the glucose produced is quantitatively determined by color reaction with 4-amino-antipyrine and phenol reagents containing glucose oxidase and peroxidase.

B.5.4 Reagents and materials

B.5.4.1 0.1 mol/L acetic acid solution: Dissolve 6.0 g of acetic acid (CH_3COOH) in water and dilute to 1000 mL.

B.5.4.2 0.1 mol/L sodium acetate solution: Dissolve 8.20 g of sodium acetate (CH_3COONa) in water and dilute to 1000 mL.

B.5.4.3 0.02 mol/L acetic acid-sodium acetate solution (pH 5.0): Dissolve 20 mL of acetic acid solution (B.5.4.1) in water and dilute to 100 mL (reagent A); dissolve 20 mL of sodium acetate solution (B.5.4.2) in water and dilute to 100 mL (reagent B); mix A and B and adjust the pH to 5.0.

B.5.4.4 Tris-phosphate buffer solution (pH 7.2): Dissolve 36.3 g of Tris(hydroxymethyl)aminomethane [$\text{H}_2\text{NC}(\text{CH}_2\text{OH})_3$] and 50.0 g of sodium dihydrogen phosphate dihydrate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$) in 900 mL of water, adjust the pH to 7.2 with 2 mol/L hydrochloric acid solution, and dilute to 1000 mL with water.

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