LACCASE from *MYCELIOPHTHORA THERMOPHILA* expressed in *ASPERGILLUS ORYZAE*

Revised specifications prepared at the 65th JECFA (2005) and published in FNP 52 Add 13 (2005), superseding specifications prepared at the 61st JECFA (2003) and published in FNP 52 Add 11 (2003). An ADI "not specified" was established at the 61st JECFA (2003).

SOURCES

Produced by submerged fed-batch pure culture fermentation of a genetically modified strain of *Aspergillus oryzae* containing the laccase gene derived from *Myceliophthora thermophila,* using recombinant DNA techniques and traditional mutagenesis. The enzyme is isolated from the fermentation broth by filtration to remove the biomass and concentrated by ultrafiltration and/or evaporation. Residual production microorganisms are removed from the enzyme concentrate by germ filtration. The final product is formulated using food-grade stabilizing and preserving agents.

Active principles: Laccase (synonyms: urishiol oxidase; p-diphenol oxidase)

Systematic names and numbers: Benzenediol:oxygen oxidoreductase; EC 1.10.3.2; CAS No. 80498-15-3

Reactions catalysed: Oxidation of a range of phenolic substances with concomitant reduction of oxygen to water.

DESCRIPTION

Brown liquid

FUNCTIONAL USES

Enzyme preparation.
Used in the brewing of beer to prevent the formation of off-flavour compounds such as trans-2-nonenal. Scavenges oxygen that otherwise would react with fatty acids, amino acids, proteins, and alcohols to form off-flavour precursors.

GENERAL SPECIFICATIONS

Must conform to the JECFA General Specifications and Considerations for Enzyme Preparations used in Food Processing (see Volume Introduction).

CHARACTERISTICS

IDENTIFICATION

Laccase activity: The sample shows laccase activity
See description under TESTS

TESTS

Laccase activity

Principle
Laccase catalyses the oxidation of syringaldazine to tetramethoxy-azo-bis (methylene quinone) that is measured

spectrophotometrically at 530 nm. Laccase activity is expressed in Laccase Myceliophthora Units (LAMU). One LAMU is defined as the amount of enzyme that oxidizes 1 micromole of syringaldazine per minute under standard conditions (pH 7.5; 30^{o}).

(Note: The method can be adapted for manual execution; any suitable spectrophotometer may be used in place of a centrifugal analyser.)

Apparatus
Centrifugal analyser (Cobas Fara, Roche, or equivalent)
Diluter (Hamilton Microlab or equivalent)

Reagents
(Note: Use only deionised water)
Laccase standard (available from Novozymes A/S)
TRIS (Tris(hydroxymethyl)aminomethane)
Maleic acid
Triton X-100 (polyethylene glycol tert-octylphenyl ether)
Ethanol 96%
Syringaldazine
PEG 6000
Glucose
Glycine

Reagent Solutions
TRIS, 1 M stock solution: Dissolve 121.1 g of TRIS in approximately 900 ml of water in a 1-litre volumetric flask. Make to volume and mix.

Maleic acid, 1 M solution: Dissolve 23.2 g of maleic acid in approximately 150 ml water in a 200-ml volumetric flask. Make to volume and mix.

Triton X-100, 10% stock solution: In a beaker, add 25.0 g of Triton X-100 to approximately 200 ml water; stir to dissolve. Transfer to a 250-ml volumetric flask and make up to volume with water.

TRIS buffer, 25 mM (pH 7.50): Add 5 ml of 1 M TRIS, 2 ml of 1 M maleic acid solution, and 1 ml of Triton X-100 10% solution to a 200-ml volumetric flask. Add 150 ml water and adjust pH to 7.50±0.05 using 1 M maleic acid solution. Make up to volume with water. (Note: do not adjust the pH with hydrochloric acid because chloride inhibits laccase activity.)

PEG 6000, 50 g/l solution: Weigh 250 g of PEG 6000 in a beaker, transfer to a 5000 ml volumetric flask, add water and stir until dissolved. Add water to volume.

Syringaldazine, 0.56 mM stock solution: Rinse a 50-ml volumetric flask with water and ethanol to remove any soapy residues. Weigh 10.0 mg of syringaldazine in a weighing boat and transfer to the volumetric flask. Add 96% ethanol to the

mark and stir until the syringaldazine is dissolved (approximately 3 h). The solution must be stored in a dark bottle in a refrigerator.

Syringaldazine, 0.22 mM working solution: Rinse a 10-ml volumetric flask with water and ethanol to remove any soapy residues. Transfer 4.0 ml of syringaldazine stock solution to the flask and add water to volume. The solution can be kept in a dark bottle for up to two hours at room temperature.

Glycine buffer, 1.5%: Dissolve 75 g glycine, 150 g glucose, and 250 g PEG 6000 in approximately 4.5 litre water in a 5-l volumetric flask. Adjust pH to 9.20±0.05 using NaOH or 1 M maleic acid. Add water to volume. (Note: Do not adjust pH with hydrochloric acid because chloride inhibits laccase activity.)

Standard and sample solutions

Laccase standard stock solution: Weigh the amount of laccase standard needed to obtain a laccase activity of 0.350 LAMU/ml and transfer the laccase to a 500-ml volumetric flask. Add 300 ml PEG 6000 solution and stir on a magnetic stirrer for 15 min to dissolve the laccase. Add PEG 6000 solution to volume. The laccase stock solution should be prepared on the day of the experiment.

Laccase working standard solutions (for the construction of the standard curve): Prepare six solutions by diluting the laccase stock solution with PEG 6000 solution as shown in the table below. Use the diluter and vials compatible with the centrifugal analyser.

Sample No.	*Dilution factor*	*Laccase stock solution (µl)*	*PEG 6000 solution (µl)*	*Activity, LAMU/ml*
1	30	20	580	0.01167
2	24	25	575	0.01458
3	20	30	570	0.01750
4	15	40	560	0.02333
5	12	50	550	0.02917
6	10	60	540	0.03500

Laccase control sample: Use a laccase preparation with known activity. Accurately weigh the amount of the preparation sufficient to obtain laccase activity of approximately 0.70 LAMU/ml in a 200-ml volumetric flask. Place the preparation in the flask and add the PEG 6000 solution to volume. Stir on the magnetic stirrer for 15 min. This is a stock solution. It should be prepared daily. Dilute the stock solution with the PEG 6000 solution 30 times using diluter. Place the diluted solution in a vial.

Analyse the control sample in each run to test the method's performance. A result within 8 percent of the nominal activity is acceptable.

Test samples: Dilute test samples on the basis of the anticipated enzyme content to obtain activity between 0.0117 and 0.0350 LAMU/ml.

Example: accurately weigh 0.6 g sample and dissolve in the PEG 6000 solution in a 250 ml volumetric flask. Stir the solution for 15 min on the magnetic stirrer. If necessary, dilute the sample solution again with the PEG 6000 solution. Place the solutions in vials.

Procedure

1. Pour the syringaldazine working solution (0.22 mM) into a 4-ml reagent container placed in the reagent rack of the centrifugal analyser.
2. Pour the TRIS buffer into a 15-ml reagent container placed in the reagent rack.
3. Place the vials containing standard solutions and the control sample in the calibration rack.
4. Place the vials containing the test samples in the sample rack.
5. Set up the analysis program and start the analysis.

Analysis

The analysis is performed automatically by the centrifugal analyser. The empty rotor of the analyser rotates until the temperature in the cuvette container reaches 30^{o}. Twenty five microliters of the standard solution, control sample or test sample, 20 microliters of water, and 325 microliters of glycine buffer are pipetted into cavites in the rotor. The rotor accelerates and centrifuges and mixes the buffer and samples in the cuvettes. Subsequently, 30 microliters of the substrate is pipetted into each cuvette. The rotor accelerates and centrifuges and mixes the substrate with samples in the cuvettes. The first absorbance reading is taken five seconds later. A total of 25 readings are taken from each cuvette at 5-second intervals. Readings 12 to 24 are used to calculate the increase of absorbance per minute (ΔAbs/min).

Calculations

The analyser creates a standard curve and uses it to convert the ΔAbs/min from each cuvette containing the test sample into activity expressed in LAMU/ml. The activity of test samples expressed in LAMU/g is then calculated using the following formula:

$$LAMU/g = \frac{A \times Vol \times D}{W}$$

Where

A = ΔAbs/min converted to activity (LAMU/ml)
Vol = volume of the volumetric flask used to dilute the test sample (ml)
D = additional dilution of the sample (ml/ml)
W = weight of the sample (g)