# α-ACETOLACTATE DECARBOXYLASE from *BACILLUS BREVIS* expressed in *BACILLUS SUBTILIS*

*Prepared at the 53rd JECFA (1999) and published in FNP Add 7 (1999), superseding specifications prepared at the 51st JECFA (1998), published in FNP 52 Add 6 (1998). ADI "not specified" established at the 51st JECFA in 1998.*

## SOURCES

Produced extracellularly by submerged fermentation of *Bacillus subtilis*, which through recombinant DNA techniques contains the gene for alpha-decarboxylase from *Bacillus brevis*. The strain of *Bacillus subtilis* is non-pathogenic and non-toxicogenic (e. g. UW193 (a *dal*-transformant strain of ToC46) ). When fermentation is complete, the broth is filtered then stabilised before further filtration.

Active principles: alpha-Acetolactate decarboxylase

Systematic names and numbers: (S)-2-Hydroxy-2-methyl-oxobutanoate carboxylase (EC 4.1.1.5)

Reactions catalysed: Decarboxylation of alpha-acetolactate to acetoin

## DESCRIPTION

Brown liquid containing the active enzyme; Total Organic Solids approx. 2%

## FUNCTIONAL USES

Enzyme preparation
Used in the brewing of beer and fermentation step of alcohol production

## GENERAL SPECIFICATIONS

Must conform to the General Specifications for Enzyme Preparations Used in Food Processing (Volume Introduction)

## CHARACTERISTICS

IDENTIFICATION

alpha-Acetolactate decarboxylase activity

The sample shows alpha-acetolactate decarboxylase activity
See description under TESTS

## TESTS

alpha-Acetolactate decarboxylase activity

Principle

The enzyme decarboxylates alpha-acetolactate to produce acetoin. The resultant acetoin can be reacted with a mixture of naphthol and creatine, resulting in quantitative formation of a characteristic red colour.

Apparatus

1. Spectrophotometer or equivalent capable of measuring optical density (absorbance) at 522 nm
2. Water bath, held at 30±1º, containing test tube rack
3. Volumetric glassware

Reagents

1. MES buffer, 0.05M (pH = 6.0). Dissolve 9.76 g of MES (2-(N-morpholino) ethanesulphonic acid) in approximately 900 ml of deionised water. Adjust pH to 6.0±0.5 with 1 N NaOH. Transfer to a 1-litre volumetric flask and make to volume with deionised water. This solution may be kept for two weeks at room temperature.

2. Brij 35 solution, 15% w/v: Dissolve 15.0 g of Brij 35 (polyoxyethylene lauryl ether, Atlas Chemie, BDH, or equivalent) in approximately 70 ml of deionised water, heating to 60º to aid dissolution. After cooling, transfer to a 100-ml volumetric flask and make to volume with deionised water. This solution should be stored in a refrigerator, and can be kept for up to two months.
3. MES/Brij 35/NaCl solution. Dissolve 48.8 g of MES and 175.32 g of NaCl (AR grade) in approximately 4.5 litres of deionised water. Add 17 ml of 15% Brij 35 solution (see 2. above). Adjust pH to 6.0±0.5 with 1 N NaOH. Transfer to a 5-litre volumetric flask and make to volume with deionised water. This solution may be kept for two weeks at room temperature.
4. alpha-Acetolactate substrate, 0.2% v/v. Pipette 100 µl of ethyl-2-acetoxy-2-methylacetolactate into a 50-ml volumetric flask. Add 6.0 ml of 0.5 N NaOH to the flask and stir for 20 min. Add MES buffer (see 1. above) to bring the volume to approximately 40 ml. Adjust pH to 6.0±0.5 with 0.5 N HCl. Make to volume with MES buffer (see 1. above). This substrate should be made just before use.
5. Naphthol/Creatine colour reagent: Dissolve 5.00 g of 1-naphthol and 0.50g of creatine in 1 N NaOH, make to volume with 1 N NaOH in a 500-ml volumetric flask. This colour reagent should be made fresh just before use and shielded from light as much as possible.
6. Acetoin stock solution, 1000 mg/l: Dissolve 0.100 g of acetoin (3-hydroxy-2-butanone) in deionised water in a 100-ml volumetric flask. Make to volume with deionised water.
7. Acetoin standards: Dilute 0, 1.0, 2.0, 4.0, 6.0, and 8.0 ml of stock acetoin solution (see 6. above) to volume with deionised water in 100 ml volumetric flasks to give standard solutions of 0, 10, 20, 40, 60, and 80 mg/l of acetoin. These solutions may be kept for two weeks stored in a refrigerator.
8. Enzyme solutions: Test samples of the enzyme are diluted with MES/Brij 35/NaCl solution (see 3. above). The exact dilution will need to be determined by experience and experimentation to give an enzyme concentration, which produces a result within the range of the acetoin standards (see 7. above) under the conditions of the test.

Procedure

Preparation of standard curve: Prepare a standard curve by treating the acetoin standards (7.) in the following manner:
Pipette 400 µl of the acetoin standard solutions into 10-ml plastic test tubes. Add 4.6 ml of colour reagent (5.) to each test tube, mix, and let the test tube stand at room temperature for exactly 40 min. At the end of this 40 min period, place 0 mg/l acetoin standard in the spectrophotometer set to read absorbance at 522 nm and adjust absorbance reading to zero. Measure the absorbance of the remaining acetoin standards at 522 nm.

Determination of enzyme activity

Warm the enzyme solutions (see 8. above), MES buffer (see 1. above), and the substrate (see 4. above) in a water bath at 30±1º for approximately 10 min.
Four solutions must be prepared for each analysis:
Enzyme blank (B1). Pipette 200 µl of enzyme solution (8.) and 200 µl of MES buffer (l.) into a 10-ml plastic test tube. Mix, and immediately place the test tube back into the water bath.
Sample value (H1). Pipette 200 µl of enzyme solution (8.) and 200 µl of substrate (4.) into a 10-ml plastic test tube. Mix, and immediately place

the test tube into the water bath.
Buffer blank (B2). Pipette 200 µl of MES buffer (l.) and 200 µl of MES/Brij 35/NaCl solution (3.) into a 10-ml plastic test tube. Mix, and immediately place the test tube into the water bath.
Buffer value (H2). Pipette 200 µl of MES/Brij 35/NaCl solution (3.) and 200 µl of substrate (4.) into a 10-ml plastic test tube. Mix, and immediately place the test tube into the water bath.
For each enzyme determination the test tubes, are prepared in the order: B1, H1, B2, H2.
Exactly 20 min after mixing of each of solutions B1, H1, B2, and H2, remove from water bath, add 4.6 ml of colour reagent (5.), mix and leave at room temperature for exactly 40 min. At the end of this 40 min period measure the absorbance of the solutions at 522 nm on a spectrophotometer or equivalent, previously adjusted so that the 0 mg/l acetoin standard gives an absorbance reading of zero.

Calculation
The activity of the enzyme is defined in terms of activity units where:
One alpha-Acetolactate decarboxylase unit = the amount of enzyme which, by decarboxylation of alpha-acetolactate produces 1 µmol of acetoin per min under the test reaction conditions.
Plot optical density values at 522 nm for the acetoin standards against acetoin concentration (in mg acetoin/L) of the standard and generate a standard curve. Use the standard curve to convert the optical density measurements for solutions H1, B1, H2, and B2 to mg acetoin/L.

Activity of enzyme (in alpha-Acetolactate decarboxylase units/g) = {([H1] - [B1]) - ([H2] - [B2])} x 0.0011351 x F / W

where:
[H1], [B1], [H2], [B2] = the concentrations of acetoin in the respective solutions (mg/l)
F = the final volume of enzyme solution, after dilutions (ml)
W = weight of enzyme in volume F (g)
The factor 0.0011351 contains:
the conversion from mg acetoin/L to µmol acetoin/min
the conversion for the actual volumes of substrate and enzyme solution used in the analysis.