

Introduction:

To determine whether engineered *Bacillus subtilis* (WB800) can successfully produce and secrete active plant bioprotectants such as 1-aminocyclopropane-1-carboxylate deaminase (ACCD), we measure the enzymatic cleavage of ACC into α -ketobutyrate and ammonia. The Berthelot assay detects the ammonium released in this reaction, whereas the 2,4-dinitrophenylhydrazine (DNPH) colorimetric assay offers a more direct, spectrophotometric readout of α -ketobutyrate accumulation. The DNPH method overcomes several limitations of the Berthelot assay: it shows no interference from the ACC substrate, gives a substantially lower background signal in complex culture media, and is 19-fold more sensitive. These advantages make it possible to determine initial velocities accurately and to obtain true kinetic parameters (K_m and k_{cat}). Under acidic conditions, DNPH reacts with the carbonyl group of α -ketobutyrate of alkali and is quantified at OD 540 nm (Penrose & Glick, 2003; Kozaeva et al., 2022).

By pairing this hydrazone-based quantification with readouts of growth and protein expression, we can reliably confirm both the catalytic integrity and the extracellular secretion of recombinant ACCD enzymes in cell factories.

The primary objective of this study was to establish a microscale DNPH assay protocol for determining ACCD activation levels, by measuring α -ketobutyrate accumulation and assessing strain performance across cofactor conditions and gene constructs.

Contribution:

We optimized the DNPH assay by scaling down the conventional protocol described by Penrose and Glick (2023) by 20-fold, making it suitable for high-throughput analysis using 96-well microplates while maintaining the original reaction proportions. We used this assay to evaluate ACC deaminase (ACCD) activity by measuring α -ketobutyrate production and investigating the effects of pyridoxal 5'-phosphate (PLP) supplementation on enzyme activity. We also assessed the functional secretion of recombinant ACCD from engineered *Bacillus Subtilis* strains. Additionally, we established a standard calibration curve and developed calculation methods to determine α -ketobutyrate concentrations, total product yields, and enzymatic activity. These contributions provided a quantitative framework for comparing engineered strains and evaluating ACCD performance.

Material and Methods:

- Regents and solutions required for the experiment:
 1. 2,4-Dinitrophenylhydrazine(DNPH) Solution (0.2% w/v in 2N HCl):

Dissolve 0.20g of 2,4-Dinitrophenylhydrazine powder in 100 mL of 2 N HCl solution. Protect solution from light exposure by covering the container with aluminum foil and store at 4 °C. Handle 2 N HCl with proper personal equipment (PPE), including acid-resistance gloves and safety goggles, and always operate under a chemical fume cover due to corrosive hazard.

2. Hydrochloric Acid (0.56 N NaOH):

Prepare by diluting concentrated HCl (12N) with ultrapure water used specifically for enzymatic quenching and stabilization for acid-catalyzed reaction .

3. Sodium Hydroxide Solution (2N NaOH):

Dissolve 8.0 g of NaOH pellets in deionized water to bring the total volume to 100 mL. This step will cause an exothermic reaction, so always operate this step in ice bath.

4. 1 M ACC substrate solution:

Dissolve 101.1 mg ACC per 1.0 mL deionized water and store at -20 °C and pre-equilibrate at 30 °C for at least 3 min before use.

5. 20X reaction buffer:

1 M Tris-HCl pH 8.0 containing 500 µM PLP buffer (store at 4 °C without light exposure, the solution must be used within 2 weeks)

6. α-Ketobutyrate primary standard stock (10 mM):

Dissolve 12.4 mg Sodium 2-oxobutryrate (MW 124.7) per 10 mL water (prepare on ice before using daily)

Experiment protocol:

1. Mix 100 µL filtered cultural supernatant with 5 µL 20X reaction buffer and 5 µL of 1 M ACC substrate solution to reach the total reaction volume 110 µL, incubate at 30 °C
2. Transfer 10 µL of reaction mixture into 50 µL of 0.56 N NaCl in a 1.5 mL microtube and vortex immediately to quench the precipitant protein.
3. Centrifuge at 12,000 xg for 5 min at RT, then transfer 50 µL of clear supernatant into a new clean microtube.
4. Add 40 µL of 0.56 N HCl and 15 µL of 0.2% DNPH/2 N HCl solution, then vortex and incubate the solution at 30 °C for 30 min.
5. Add 100 µL of 2 N NaOH solution, vortex it for 10 sec, and transfer the mixed solution (with a total volume of 205 µL) into a 96-well flat bottom plate. Then test the concentration under spectrometer at A540 after 10 min.

Calculate:

The calculation can be break down into few steps as shown below:

1. Net absorbance Evaluation

In order to correct the number for background interference, simply subtract the absorbance of the HCl (sample blank) from the DNPH-treated sample at the peak absorbance wavelength, shown as below:

$$\Delta A = A_{\text{sample}} - A_{\text{blank}}$$

2. Standard Curve Interpolation

The concentration of α -ketobutyrate in the sample can be determined by using the linear regression equation :

$$y = m \cdot x + b$$

The equation above than can be rearranged to find the concentration:

$$\text{Concentration of } \alpha\text{-ketobutyrate (mM)} = \frac{\Delta A - b}{m}$$

Where:

ΔA : Net Absorbance of the sample (y)

m: Slope of the standard curve

B: y-intercept of the standard curve

3. Quantitation of Total Production:

Apply the equation below to convert the concentration into total accumulated product (nmol) within the total reaction assay volume:

$$\text{Total Product (nmol)} = \text{Concentration of } \alpha\text{-ketobutyrate (mM)} \cdot V_{\text{reaction}} (\mu\text{L})$$

Where:

V_{reaction} : Total Volume of the assay reaction mixture