Determination of CCH2 rate:

Materials Needed:
250 mL Erlenmeyer flasks
M9 minimal salt media
Chloramphenicol (25mg/ml)
1M IPTG
200 mM 6-CNA dissolved in methanol
250 mL Erlenmeyer flasks with 50 mL LB
50 mL Falcon Tubes
Spectrophotometer
Cuvette
1.5mL Eppendorf tubes
Centrifuge
GC/MS
Pipet

M9 minimal salt media components:
33.9g/L Na_2HPO_4
15g/L KH_2PO_4
5g/L NH_4Cl
2.5g/L NaCl

Protocol:

1) A starter culture is prepared from an isolated colony of DH5-αE.coli transformed with a CCH2 vector in 50mL of LB with 25ug/mL of Chloramphenicol overnight on the shaker at 37°C.
2) A 'no insert' colony is used as a control.
3) 500µL of the starter culture is used to inoculate 50mL of LB with 25ug/mL of chloramphenicol in a 250 mL Erlenmeyer flask and incubated on the shaker at 37°C.
4) Cultures are grown until OD600= 0.6-0.8, then put on ice for 20 minutes and stirred every couple of minutes.
5) After being cooled, 50 µL of 1M IPTG is added and the Erlenmeyer stirred.
6) The cultures are grown overnight on the shaker at 25°C.
7) Cells are pelleted in 50 mL falcon tubes at 4°C at 4000 rpm for 20 min.
8) The supernatant is discarded and the cultures are resuspended in 40 mL of M9 minimal salt media to wash the cells
9) Cells are pelleted again, as above, and the supernatant is discarded
10) The cells are resuspended in 25mL of M9 minimal salt media.
11) The duplicate samples are combined into one 50mL falcon tube to normalize the amount of cells in each fraction.
12) 25mL of 50mL from the fractions is transferred into two separate, empty 250 mL Erlenmeyer flasks.
13) 20 µL of 200mM 6-CNA is added to all samples and were grown on the shaker for four hours at 30°C.

14) An initial sample is collected at time zero. Subsequently samples are collected every 15 minutes over a period of two hours. Thereafter, samples are collected every 30 minutes for another two hours.
15) Collected samples were centrifuged for 2.5 minutes at 15000rpm at 4°C. The supernatants of the samples were transferred into 1.5mL Eppendorf tubes and stored at -80°C .
16) The samples are extracted via a liquid-liquid extraction to isolate 6-CNA and 6-HNA and ran on the GC-MS [1,2].

Other Protocols:

[1] Gas Chromatography-Mass spectroscopy conditions to run 6-Chloronicitinoic Acid and 6-hydroxynicitinoic Acid and sample preparation

[2] Liquid-Liquid Extraction for isolation of 6-Chloronicitinoic Acid and 6-Hydroxynicitinoic Acid