NON-RADIOACTIVE ASSAY FOR ACETYL-CoA CARBOXYLASE ACTIVITY

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ABSTRACT

Acetyl-CoA carboxylase is a key enzyme in oil biosynthesis and is critical for the oil deposition pathway. This biotinylated enzyme catalyzes the first committed step in fatty acid biosynthesis, the ligation of a carbon to acetyl-CoA to form malonyl-CoA. The acetyl-CoA carboxylase holoenzyme has four distinct protein domains: biotin carboxylase, biotin carboxylase carrier protein, and the alpha and beta transcarboxylase domains. Biotin carboxylase catalyzes the addition of carbon dioxide to biotin carboxylase carrier protein, while the alpha and beta subunits of carboxyltransferase ligate the activated CO_2 to acetyl-CoA. The canonical assays for monitoring the activity of acetyl-CoA carboxylase rely on incorporation of radiolabelled acetyl-CoA. In this work, we describe the development of a discontinuous, non-radioactive spectrophotometric assay for acetyl-CoA carboxylase activity. Permeabilized Corynebacterium glutamicum *cells were added to an assay mixture containing acetyl-CoA, bicarbonate, magnesium and ATP, and aliquots were removed at set time points and stopped by the addition of trifluoroacetic acid. The level of acetyl-CoA remaining in each aliquot was determined via a citrate synthase assay, in which the formation of the yellow compound dithiobisnitrobenzoic acid-thiophenolate was followed spectrophotometrically at 412 nm.*

INTRODUCTION

Acetyl-CoA carboxylase (EC 6.4.1.2) carries out the first committed step in fatty acid biosynthesis (Ohlrogge and Jaworski, 1997). This biotinylated enzyme catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA (*Figure 1*). Malonyl-CoA is a substrate for fatty acid elongation, and therefore acetyl-CoA carboxylase is a key enzyme for oil biosynthesis and oil deposition. Quantitative analyses of lipid biosynthesis have shown that acetyl-CoA carboxylase exerts significant control over the carbon flow in the leaves of barley and maize (Page *et al.*, 1994). In the oil palm, acetyl-CoA carboxylase activity can be measured in the developing fruits, and the activity is highest at 20 weeks after anthesis (Saridah and Sambanthamurthi, unpublished).

Acetyl-CoA carboxylase activity can also be detected in cultured oil palm cells (Ramli, 1999; Turnham and Northcote, 1982). Top-down metabolic control analysis, a method of quantifying carbon fluxes through a metabolic network, has shown that *de novo* synthesis of fatty acids (the portion of the network that includes acetyl-CoA carboxylase) exerts more flux control in these cells than does the portion of the network required for the assembly of complex lipids (Ramli *et al.*, 2002a, b). Acetyl-CoA carboxylase activity is correlated with somatic embryogenesis in oil palm callus (Turnham and Northcote, 1982). Acetyl-CoA carboxylase has also been found to be required for embryo development in *Arabidopsis*. Mutation of one of the *Arabidopsis* acetyl-CoA carboxylase genes disrupts embryo development, leading to abnormal embryos with reduced levels

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$$\text{ATP + HCO}_3^- \qquad\qquad\qquad \text{ADP + Pi}$$

BIOTIN CARBOXYLASE

$$\text{BCCP-biotin} \qquad\qquad\qquad \text{BCCP-biotin-CO}_2$$

CARBOXYLTRANSFERASE

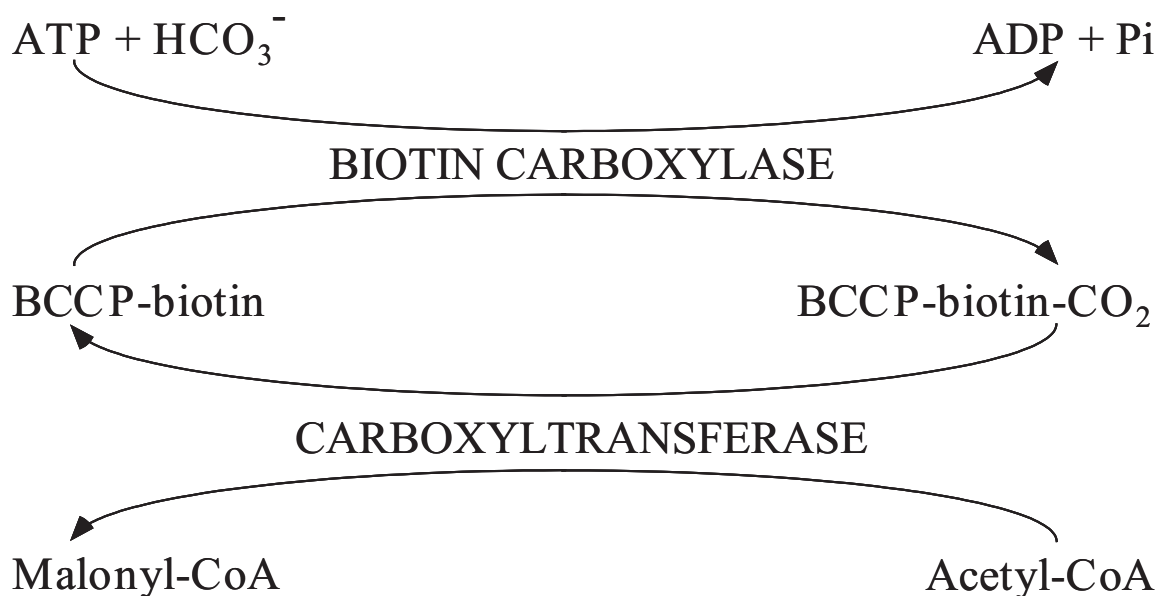$$\text{Malonyl-CoA} \qquad\qquad\qquad \text{Acetyl-CoA}$$

Figure 1. Acetyl-CoA carboxylase catalyzed reaction. Carboxylation of biotin carboxylase carrier protein (BCCP) is carried out by biotin carboxylase. Carboxyltransferase then ligates the carbon to acetyl-CoA, forming malonyl-CoA.

of triacylglycerols (TAGs) and devoid of very-long-chain fatty acids (Baud *et al.*, 2003).

In addition to its role in fatty acid biosynthesis in plants (Smirnov, 1960), acetyl-CoA carboxylase activity is also found in bacteria (Alberts *et al.*, 1969; Polakis *et al.*, 1974), archaea (Menendez *et al.*, 1999) and eukaryotes, including protists (Zuther *et al.*, 1999), fungi (Bailey *et al.*, 1995) and animals. Furthermore, acetyl-CoA carboxylase is essential for human health. Two forms of acetyl-CoA carboxylase have been found in humans. Acetyl-CoA carboxylase alpha (ACC1) is detected in the liver and fatty tissues, and is involved in fatty acid biosynthesis, upregulated by HER2 in breast cancer cells, required for the survival of breast cancer cells (Chajes *et al.*, 2006; Yoon *et al.*, 2007), and involved in insulin secretion (Zhang and Kim, 1996). The second human acetyl-CoA carboxylase (ACC2) is involved in β-oxidation and is expressed primarily in heart and skeletal muscles (Widmer *et al.*, 1996; Kim *et al.*, 2007).

The acetyl-CoA carboxylase holoenzyme has four distinct protein domains: biotin carboxylase, biotin carboxylase carrier protein, and the alpha and beta transcarboxylase domains. Biotin carboxylase catalyzes the addition of CO_2 to biotin carboxylase carrier protein, while the α and β subunits of carboxyltransferase ligate the activated CO_2 to acetyl-CoA (Alberts *et al.*, 1969).

Acetyl-CoA carboxylase enzymes can be categorized into two classes based on their substrate specificity and number of subunits. The multisubunit class I enzymes, exemplified by the acetyl-CoA carboxylase of *Escherichia coli*, are active only on acetyl-CoA and are encoded by four separate genes, one for each of the four required proteins (Li

et al., 1992; Li and Cronan, 1992a, b; Cronan and Waldrop, 2002). The multifunctional class II enzymes tend to exhibit broader substrate specificity, carrying out the carboxylation of propionyl-CoA, albeit at a lower rate than the carboxylation of acetyl-CoA (Dehaye *et al.*, 1994). The multifunctional acetyl-CoA carboxylase enzymes differ from the multisubunit enzymes in their primary structure. Instead of four separate proteins the multifunctional forms carry the four required domains on one (Baud *et al.*, 2003) or two (Jäger *et al.*, 1996; Gande *et al.*, 2007) proteins. Dicots possess both a multisubunit enzyme and a multifunctional enzyme. The multisubunit enzyme is insensitive to herbicides of the dipheno-xypropionic acid and cyclohexadione types (Dehaye *et al.*, 1994). Monocots, which possess the multifunctional enzyme but lack one gene required for the multisubunit isozyme, are sensitive to these herbicides (Nikolskaya *et al.*, 1999; Incledon and Hall, 1997; Konishi and Sasaki, 1994).

The canonical assays for monitoring the activity of acetyl-CoA carboxylase rely on incorporation of radiolabelled acetyl-CoA (*e.g.* Rainwater and Kolattukudy, 1982). In order to detect acetyl-CoA carboxylase activity without the need for radionuclides, we employed a discontinuous spectrophotometric assay. The acetyl-CoA carboxylase reaction is coupled to a citrate synthase assay, using Ellman's reagent [dithionitrobenzoic acid (DTNB)] to quantitate the release of free coenzyme A, generating 2-nitro, 5-thiobenzoic acid, a yellow product that can be detected at 412 nm. Measuring production of the 2-nitro, 5-thiobenzoic acid permits the calculation of how much acetyl-CoA was consumed in the acetyl-CoA carboxylase

reaction. These assays have been carried out with permeabilized cells of the model organism *Corynebacterium glutamicum*. *C. glutamicum*, a member of the Actinomycetes, is a gram positive bacterium that is used for the industrial production of amino acids such as glutamate, lysine, threonine and isoleucine (Lessard *et al*., 1999). The assay described here may be a useful tool in the study of fatty acid biosynthesis and oil deposition.

EXPERIMENTAL

Chemicals and Reagents

All chemicals and reagents were purchased from Sigma-Aldrich (St Louis, MO) unless otherwise stated. Difco brand bacterial medium components were purchased from BD (Franklin Lakes, NJ).

Growth and Preparation of Cells

C. glutamicum strain NRRL B-11474 (also known as BF100) was maintained as frozen stock at –80°C. The frozen stock cells were streaked onto Luria-Bertani (LB) agar plates and incubated at 30°C to form single colonies. The 5 ml volume pre-cultures in LB medium were inoculated from single colonies and incubated at 30°C on a roller drum for approximately 48 hr before subculturing into 50 ml cultures of LB broth (supplemented with 3 mg litre^{-1} biotin) in 250 ml flasks. These cultures were incubated at 30°C on an orbital shaker at 200 rpm until the OD_{600} was approximately 1. The cells were harvested by centrifugation (5000 x *g*, 4°C, 10 min), washed in ice-cold 100 mM potassium phosphate (pH 7.0) and centrifuged at 5000 x *g*, 4°C for 5 min. The cell pellets were resuspended in 100 mM HEPES (pH 7.5) containing 20% glycerol to a final concentration of 25 g dry cell weight per litre, divided into 100 μl aliquots in microfuge tubes and kept on ice for immediate use or frozen at –80°C for later use. The permeabilized cells were prepared by adding 3 μl 10% hexadecyltrimethyl-ammonium bromide (CTAB) (w/v) to a 100 μl cell aliquot and incubating the cells at room temperature for 2 min. The permeabilized cells were used immediately in the acetyl-CoA carboxylase reaction.

Acetyl-CoA Carboxylase Assay

Acetyl-CoA carboxylase activity was measured using a discontinuous spectrophotometric assay. The acetyl-CoA carboxylase reaction (reaction #1) was carried out in 250 μl reaction volume containing 100 mM potassium phosphate (pH 8.0), 15 mM $KHCO_3$, 5 mM $MnCl_2$, 5 mM ATP, 1 mg ml^{-1} bovine serum albumin (BSA), 1 mM acetyl-CoA and 3 mg litre^{-1}

biotin. The reaction was initiated by adding 7 μl permeabilized cells, after which the reaction mixture was incubated at 30°C. At 1, 2, 3.5, 5, 7 and 10 min time points, 50 μl aliquots were removed, transferred into microfuge tubes containing 3.3 μl 10% trifluoroacetic acid and placed on ice. After all time points had been taken, the tubes were spun at full speed in a microfuge at 4°C for 5 min and the supernatants analysed to determine the remaining acetyl-CoA. The 50 μl supernatant were transferred into a spectrophotometric cuvette containing 950 μl buffer composed of 100 mM KPO_4 (pH 8.0), 0.1 mg ml^{-1} dithionitrobenzoic acid (DTNB), 20 mM oxaloacetate and 1 mg ml^{-1} bovine serum albumin. The cuvettes were placed in a Hewlett Packard model 8452A diode array spectrophotometer and kinetic data collected at 412 nm. Reaction #2 was initiated with the addition of 0.5 unit citrate synthase (Sigma). The change in absorption was recorded. These values correlate with the level of acetyl-CoA remaining at the end of reaction #1. Activity was expressed as μmol acetyl-CoA consumed min^{-1} g dry cell weight^{-1}. A standard curve was constructed each day by measuring the change in absorbance in reaction #2 containing defined amounts of acetyl-CoA instead of supernatant and graphing the change in absorbance *vs.* concentration of acetyl-CoA.

RESULTS AND DISCUSSION

In this work, we describe the development of a discontinuous, non-radioactive assay for acetyl-CoA carboxylase activity, using the model organism *C. glutamicum*. Acetyl-CoA carboxylase carries out the first committed step in fatty acid biosynthesis and is an important target for impacting the metabolism of oil palm. A schematic of the spectrophotometric assay for acetyl-CoA carboxylase is shown in *Figure 2*.

C. glutamicum cells were grown in a rich culture medium, supplemented with 3 mg ml^{-1} biotin, and harvested during logarithmic growth. The cells were washed and concentrated before The permeabilization with CTAB. The permeabilized cells were added to a reaction mixture containing bicarbonate, acetyl-CoA, ATP and magnesium. Samples were withdrawn at regular time points and their reactions stopped by the addition of trifluoroacetic acid, and the amount of acetyl-CoA remaining after each time point was determined via citrate synthase assay. Averaged data from several independent experiments are shown in *Figure 3*.

In addition to the complete assay mixture, we carried out a series of control reactions in which components were omitted from the reaction (*Figure 3*). No activity was detected when acetyl-CoA was omitted from the reaction mixture, indicating that
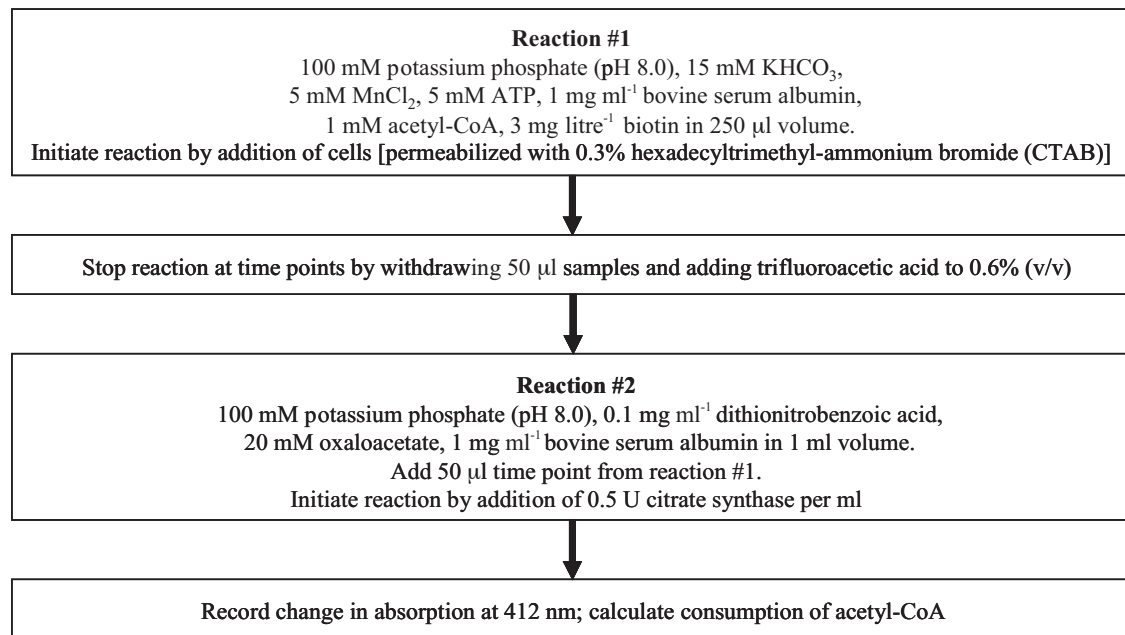

Reaction #1

100 mM potassium phosphate (pH 8.0), 15 mM KHCO$_3$,
5 mM MnCl$_2$, 5 mM ATP, 1 mg ml^{-1} bovine serum albumin,
1 mM acetyl-CoA, 3 mg litre^{-1} biotin in 250 µl volume.
Initiate reaction by addition of cells [permeabilized with 0.3% hexadecyltrimethyl-ammonium bromide (CTAB)]

↓

Stop reaction at time points by withdrawing 50 µl samples and adding trifluoroacetic acid to 0.6% (v/v)

↓

Reaction #2

100 mM potassium phosphate (pH 8.0), 0.1 mg ml^{-1} dithionitrobenzoic acid,
20 mM oxaloacetate, 1 mg ml^{-1} bovine serum albumin in 1 ml volume.
Add 50 µl time point from reaction #1.
Initiate reaction by addition of 0.5 U citrate synthase per ml

↓

Record change in absorption at 412 nm; calculate consumption of acetyl-CoA

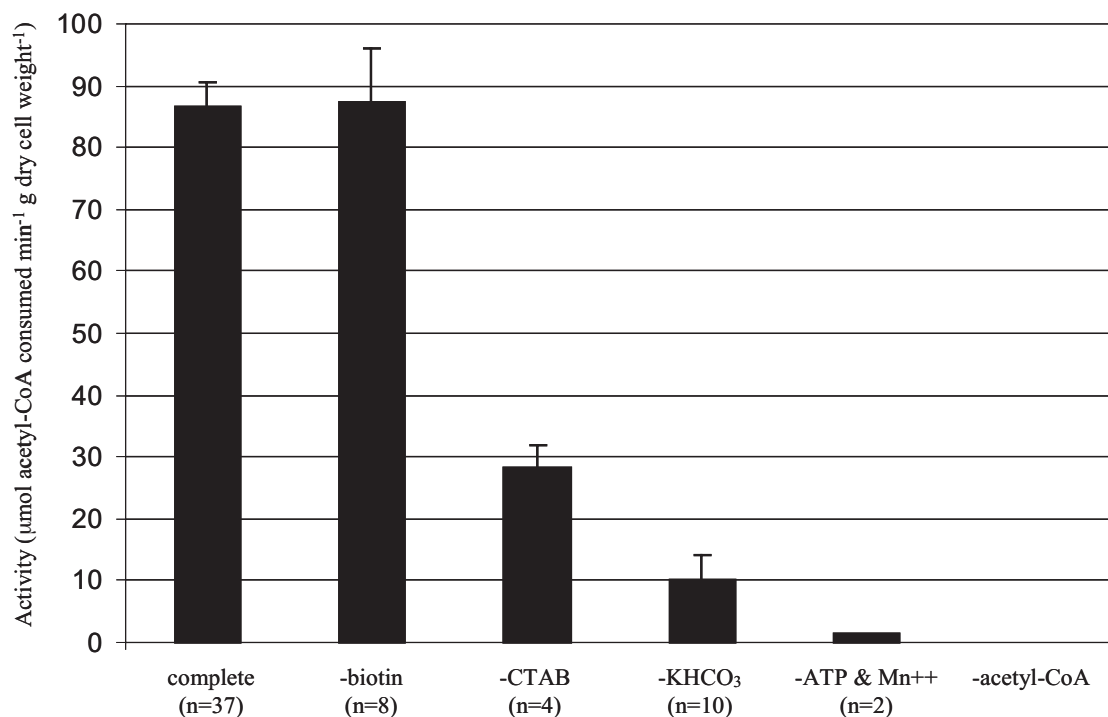*Figure 2. Flow diagram of discontinuous assay for acetyl-CoA carboxylase activity*

Figure 3. Spectrophotometric assay of acetyl-CoA carboxylase activity in permeabilized C. glutamicum *cells. Results are graphed as mean + standard error. Labels beneath bars indicate the reaction conditions; the number of replicate samples for each condition is indicated in parentheses.*

the permeabilized cells were not contributing any detectable amounts of acetyl-CoA to the reaction. Our data show that biotin, a co-factor required for acetyl-CoA carboxylase activity that is covalently bound to the acetyl-CoA carboxylase holoenzyme, can be omitted from the assay buffer without decreasing the acetyl-CoA carboxylase activity. Some activity was detected in reactions carried out with cells that had not been treated with the permeabilizing agent CTAB, suggesting that some of the cells were not intact prior to the permeabilization procedure, and that even without CTAB treatment some substrate could reach the active enzyme. In addition, we detected some activity in reactions lacking exogenously supplied bicarbonate. This could indicate a sufficient supply of atmospheric CO$_2$ dissolved in the reaction buffer to permit some enzymatic activity. Alternatively,

these data could indicate that a portion of the acetyl-CoA was consumed by the enzymes in the permeabilized cells that did not require CO_2. Reactions carried out in the absence of ATP and manganese exhibited less than 2% activity than the complete reaction; this activity could indicate a low level of ATP present in the permeabilized cells, or it could indicate the presence of an acetyl-CoA consuming enzyme not dependent on ATP.

The assay described here is a modification of our assay for pyruvate carboxylase (Hanke *et al.*, 2005; Guillouet and Sinskey, unpublished). Both the pyruvate carboxylase assay and the acetyl-CoA carboxylase assay are coupled with citrate synthase. But whereas the citrate synthase reaction in the pyruvate carboxylase assay contains excess acetyl-CoA and measures how much oxaloacetate was produced in the first reaction, the citrate synthase reaction for this assay contains excess oxaloacetate and measures how much acetyl-CoA was consumed in the first reaction. *C. glutamicum*, the model organism used in this study, utilizes carboxylases for the biosynthesis of lipids, fatty acids and mycolic acids. The complete genome sequence revealed that *C. glutamicum* possesses one gene, *accBC*, encoding the biotin carboxylase and biotin carboxylase carrier protein domains (Jäger *et al.*, 1996; Kalinowski *et al.*, 2003). The genome contains several genes encoding carboxyltransferase domains, and these genes are involved in the production of malonyl-CoA and propionyl-CoA for fatty acid and mycolic acid biosynthesis (Gande *et al.*, 2007; Ikeda and Nakagawa, 2003; Kalinowski *et al.*, 2003; Yamada *et al.*, 2007).

Classical assays for acetyl-CoA carboxylase activity utilize radiolabelled acetyl-CoA and monitor the production of radiolabelled malonyl-CoA via HPLC [ex. (Kim *et al.*, 2007; Rainwater and Kolattukudy, 1982)]. While the radioincorporation assay is very specific and reliable, it requires the use of radioactivity and involves running radioactive samples on the HPLC. Seethala *et al.* (2006) have developed a radioactive assay for acetyl-CoA carboxylase coupled with fatty acid synthase, permitting the conversion of radiolabelled [³H] acetyl-CoA into [³H] palmitic acid which is measured by scintillation proximity on a phospholipids coated detection plate, obviating the need for HPLC. Gucchait *et al.* (1974) described a spectro-photometric assay for acetyl-CoA carboxylase that eliminated the use of radioactivity, and involved coupling to two additional enzymatic steps, citrate synthase and malate dehydrogenase, monitoring the production of NADH via ultraviolet light at 340 nm. The assay described herein eliminates the malate dehydrogenase step and instead employs DTNB in the citrate synthase reaction, permitting the use of visible light at 412 nm to monitor the reaction.

Santoro *et al.* (2006) reported a spectrophotometric assay in which malonyl-CoA and biocytin are converted to acetyl-CoA and carboxybiotin; as in our assay the acetyl-CoA produced is measured by coupling with citrate synthase and monitoring coA release via DTNB. While Guchhait *et al.* (1974) and Santoro *et al.* (2006) assayed the enzyme in the reverse direction, monitoring the production of acetyl-CoA, we followed the activity in the forward direction by supplying excess acetyl-CoA. The assay described here does not require the use of radionuclides or ultraviolet light and may be of use in the study of fat and oil metabolism.

CONCLUSION

Acetyl-CoA is an important precursor of fatty acids as well as polyhydroxyalkanoates (PHAs), and therefore measuring and altering ACCase activity is important for engineering carbon metabolism in oil palm. We developed a discontinuous, non-radioactive assay for measuring acetyl-CoA carboxylase activity. This assay measures the consumption of acetyl-CoA and is coupled to a citrate synthase assay; the release of free coenzyme A is quantitated using Ellman's reagent [dithionitrobenzoic acid (DTNB)] and monitored spectrophotometrically at 412 nm. The assays were carried out with *C. glutamicum*, an industrially important gram positive bacterium. The assay can be carried out without the use of radioactivity or ultraviolet light and may be useful in the investigation of fatty acid biosynthesis and oil deposition.

ACKNOWLEDGEMENT

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