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ORIGINAL ARTICLE

The role of ATP citrate lyase, malic enzyme and fatty acid synthase in the regulation of lipid accumulation in *Cunninghamella* sp. 2A1

Aidil Abdul Hamid · Noor Fatmawati Mokhtar · Ekhlass M. Taha · Othman Omar · Wan Mohtar Wan Yusoff

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Abstract Identification of enzymes involved in the regulation of lipid biosynthesis in *Cunninghamella* sp. 2A1 was carried out by observing specific activity profile of seven lipogenic enzymes during lipid accumulation phase until cessation of lipogenesis occurred. Activities of all enzymes, 6-phosphogluconate dehydrogenase (6-PGDH), glucose-6-phosphogluconate dehydrogenase (G-6-PDH), $NADP^+$:isocitrate dehidrogenase ($NADP^+$:ICDH), malic enzyme (ME), ATP citrate lyase (ACL) and fatty acid synthase (FAS), increased when lipogenesis occurred after exhaustion of nitrogen in the culture. The enzymes remained active until the end of the experiment (79 h) except for ACL, ME and FAS which showed marked decreases (84, 72 and 59% respectively) in activity after 48 h. This decrease in activity coincided with the cessation of lipid accumulation at 48 h, although glucose was still present. This observation suggests that the three enzymes play a vital role in the down-regulation of lipid accumulation in *Cunninghamella* sp. 2A1. When 1 g/l ammonium tartrate was reintroduced into the culture at 60 h, the activity of ACL, ME and FAS was restored. This suggests that the decrease in ACL, ME and FAS activities was due to the depletion of nitrogen in the culture medium. The probable involvement of feedback inhibition in this observation is discussed.

Keywords *Cunninghamella* · ACL · ME · FAS · Lipid accumulation

A. A. Hamid (✉) · N. F. Mokhtar · E. M. Taha · O. Omar · W. M. W. Yusoff
School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600, Bangi, Selangor, Malaysia
e-mail: aidilah@ukm.my

Introduction

Polyunsaturated fatty acids (PUFA), such as γ-Linolenic acid, eicosapentaenoic acid and docosahexaenoic acid, are of interest since they have been known to have a range of pharmaceutical applications. These PUFAs can be produced by fungi (Peng and Chen 2007; Fakas et al. 2009a, 2009b), yeasts (Makri et al. 2010) and some microalgae (Li et al. 2005), and these are called oleaginous organisms.

Oleaginous organisms are capable of accumulating large quantities of lipid because of their ability to produce a continuous supply of acetyl CoA as a necessary precursor for lipid biosynthesis (Boulton and Ratledge 1983a), and to produce sufficient supply of NADPH as the essential reductant for lipid biosynthesis (Ratledge 2004). ATP citrate lyase (ACL) and malic enzyme (ME) have been reported to play a vital role in the generation of acetyl CoA and NADPH, respectively. ACL, which is a cytosolic enzyme, catalyzes the cleavage of citrate to acetyl CoA and oxaloacetate. In oleaginous yeasts and fungi, ACL was shown to be crucial in the accumulation of more than 20% (g/g biomass) lipid (Botham and Ratledge 1979; Evans and Ratledge 1985). This enzyme was found to be absent in non-oleaginous yeasts which do not accumulate lipid beyond 10–15% of their biomass. However, the role of ACL in the regulation of lipid biosynthesis in oleaginous organisms has yet to be established. Previous reports have indicated that ACL isolated from *Lipomyces starkeyi* (Boulton and Ratledge 1983b) was strongly inhibited by long-chain fatty acyl-CoA esters. On the other hand, ME has been reported to be important in controlling the extent of lipid accumulation in *Mucor circinelloides* and *Mortierella alpina* (Wynn et al. 1999). In these fungi, depletion of ME activity resulted in the cessation of lipid accumulation

although in the presence of other NADPH-generating enzymes. It has been proposed that ME played a specific role as NADPH provider for FAS activity via direct channeling of NADPH, achieved through the formation of multi-enzymes complexes between ACL, FAS and ME. However, the existence of FAS and ME complexes has not been established. The role of ME in lipid biosynthesis was further supported by observations of lower lipid content in strains with abolished ME activity either by gene mutation (McCollough and Roberts 1974) or by inhibition of the enzyme (Wynn and Ratledge 1997).

On the other hand, although FAS is known as the key enzyme in lipid biosynthesis and is also subjected to inhibitory feedback of several metabolites related to lipid biosynthesis, no direct evidence of involvement in the down-regulation of lipid accumulation in oleaginous organisms when grown in glucose-containing media has been reported. However, in oil-containing media, the inhibition of lipid biosynthesis was achieved via repression of this enzyme. Therefore, in this paper, we report the probable role of these three enzymes in the regulation of lipid biosynthesis in a local oleaginous isolate, *Cunninghamella* sp. 2A1. Many types of studies have been conducted on *Cunninghamella* sp., proving it was capable of microbial oil accumulation (Fakas et al. 2006, 2007, 2008a, 2008b)

Materials and methods

Microorganism

Cunninghamella sp. 2A1, a local soil isolate, was obtained as stock culture from the School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Bangi, Selangor. The cultures were maintained at 4°C on Potato Dextrose agar (PDA) (Difco Laboratories), and sub-cultured every 2 months.

Preparation of media and culture conditions

A nitrogen-limited medium (Kendrick and Ratledge 1992a) containing (g/l); ammonium tartrate, 1.0; KH_2PO_4, 7.0; Na_2HPO_4, 2.0; $MgSO_4{\cdot}7H_2O$, 1.5; yeast extract, 1.5; $CaCl_2$, 0.1; $FeCl_3{\cdot}6H_2O$, 0.008; $ZnSO_4{\cdot}7H_2O$, 0.0001; $CuSO_4{\cdot}5H_2O$, 0.0001; $MnSO_4{\cdot}5H_2O$, 0.0001 was sterilized at 121°C for 40 min. Glucose (50 g/l) was added separately after sterilization. Seed culture was prepared by transferring 1 ml of spore suspension (1×10^7 spores/ml), obtained from a 7-day-old PDA culture, into 500-ml shake flasks containing 200 ml of nitrogen-limited medium. The cultures were incubated at 30°C with agitation at 250 rpm for 48 h and a 10% (v/v) inoculum (400 ml) was inoculated to 3,600 ml of nitrogen- limited medium in a 5-l fermentor (B-Braun). Cultivation was carried out with controlled aeration 0.8 v/v/m, agitation at 600 rpm and 30°C for 96 h. The pH of the medium was maintained at 6.00 by automatic addition of 1 M NaOH and 1 M HCl. A total of 400 ml was sampled at timed intervals throughout the experiments and assayed for enzymes activities and lipid content, glucose, ammonium and biomass concentrations.

Determination of biomass concentration

A 5-ml culture was filtered with Whatman No.1 filter paper, washed extensively with distilled water and dried in a 100°C oven for 24 h. The determination was carried out in triplicate.

Determination of ammonium and glucose concentrations

Ammonium concentration was determined using indophenol method (Chaney and Marbach 1962). Glucose concentration was determined using a glucose oxidase Perid-test kit (Boehringer Mannheim).

Production of cell-free extract (cfe)

Fungal mycelia was suspended in 20% (w/v) extraction buffer containing 100 mM KH_2PO_4 /KOH (pH 7.5), 20% (v/v) glycerol, 1 mM benzamidine, 1 mM mercaptoethanol and 1 mM EDTA and disrupted using pestle and mortar. The suspension was centrifuged at 10,000 *g* for 15 min at 4°C and the resulting supernatant (cell-free extract) was used for enzyme assays. Protein concentration was determined using the method of Bradford (1976) with BSA as standard.

Enzyme assays

Enzyme activities were determined using continuous assays following the oxidation or reduction of NAD(P)(H) at 340 nm as described by Wynn et al. (1999) and carried out at 30°C.

Lipid extraction

Fungal biomass was harvested by filtration of 25 ml of culture and freeze-dried for 24 h. Lipid was extracted with 2:1 (v/v) chloroform/methanol (Folch et al. 1957). Lipid content is expressed as % wt/wt of biomass.

Results and discussion

Lipid accumulation in *Cunninghamella* sp. 2A1 was initiated after nitrogen exhaustion at 12 h when lipid content showed an increase from 15% at 16 h to 37%

(wt/wt biomass) at 48 h (Fig. 1). Our observation was similar to that reported in oleaginous yeast and fungi where high lipid content was achieved when cultures were cultivated in a nitrogen-limited medium (Botham and Ratledge 1979; Ratledge 1997; Gema et al. 2002). Nitrogen limitation is known to be vital in initiating lipid biosynthesis in oleaginous organisms as it would stop cell proliferation. In this condition, macromolecules such as DNA, RNA, proteins, etc. were hindered from being synthesized. Hence, oleaginous organisms would channel carbon sources in the medium into lipids (Ratledge 1997) but maybe not all the glucose was used for maintenance purposes because we did not measure the other storage molecules. This increase in lipid content coincided with the increased specific activity of all enzymes studied (Fig. 2a–f). The activation of lipogenesis was evident as all the six enzymes (ACL, ME, FAS, G-6PGDH, 6-PGDH and $NADP^+$:ICDH) implicated in lipid biosynthesis were detected and increased in activity after nitrogen-limited condition was achieved at 12 h. A similar observation was also reported to occur in yeasts and other filamentous fungi (Boulton and Ratledge 1981). Moreover, at least three of the enzymes, namely ACL, ME and FAS, showed a marked increased compared to the others.

However, lipid accumulation ceased after 48 h of cultivation (Fig. 1) although glucose was still present (26 g/l). This observation was similar to reports from Wynn et al. (1997) using *Mucor circinelloides* and Eroshin et al. (2002) using *Mortierella alpina* LPM 301. Both reported cessation of lipid accumulation even when glucose was still present, and this could be as a result of the down-regulation of lipid biosynthesis by ACL, ME and FAS. The three enzymes showed marked decreases of 84, 72 and 59%, respectively, 48 h after inoculation (Fig. 2a–c). These observations were similar to the report by Wynn et al. (1999) working with *Mucor circinelloides* and *Mortierella alpina*, where activities of FAS, ACL and ME decreased upon the cessation of lipid accumulation.

A positive correlation between the amount of lipid synthesized and ACL activity has been reported in *Lipomyces starkeyi* (Boulton and Ratledge 1981) and in *Mortierella* sp. (Attwood 1973). A similar observation was noted in *Cunninghamella* sp. 2A1 whereby the cessation of lipid accumulation in *Cunninghamella* sp. 2A1 was probably due to the lack of acetyl-CoA and NADPH, as at 48 h the specific activity (nmol/min.mg protein) of ACL and ME was low (Fig. 2a–c). Though other NADPH-generating enzymes 6-PGDH, G-6-PDH together with $NADP^+$:ICDH remained high until the end of experiment (Fig. 2d–f), this finding disagrees with Papanikolaou et al. (2004) who proved that accumulation of reserve lipid occurred only when the activity of both NAD^+-isocitrate dehydrogenase (ICDH) and $NADP^+$-ICDH were not detectable in the cell-free extract. In lipid biosynthesis, NADPH is required and is produced by the catalysis of ME (Evans and Ratledge 1985; Kendrick and Ratledge 1992b; Wynn and Ratledge 2002). Lipid accumulation in several fungi stopped when ME was inhibited. In *Mucor circinelloides* and *Mortierella alpina* (Wynn et al. 1999), lipid accumulation ceased when ME activity became undetectable. Decreased lipid content, from 20 to 5% wt/wt biomass was also observed in *Aspergillus nidulans* when ME was inhibited by the addition of sesamol in the culture medium (Wynn and Ratledge 1997). Our results showed that, at low ME and ACL activities, lipid accumulation ceased at 48 h (Fig. 2a, b). Therefore, the cessation of lipid accumulation in *Cunninghamella* sp. 2A1 might also be caused by the unavailability of NADPH, thus limiting FAS activity, and the uptake of Glucose in this instance will not be affected in *Cunninghamella* sp. 2A1 as compared to *Yarrowia lipolytica* (Makri et al. 2010). This leads to the suggestion that ME plays a vital role as NADPH provider for FAS activity in *Cunninghamella* sp. 2A1.

However, our results showed that ME activity could still be detected until the end (120 h) of the experiment

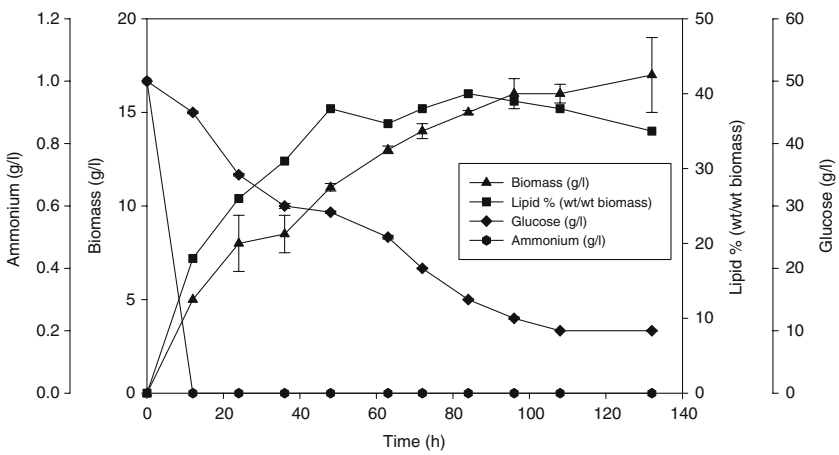

Fig. 1 Growth and lipid accumulation of *Cunninghamella* sp. 2A1. The culture was cultivated in a 5-l fermenter with nitrogen-limited media containing C/N 120:1, pH 6.00, aeration 0.8 v/v/m, agitation 600 rpm and starting inoculums of 10% (v/v)

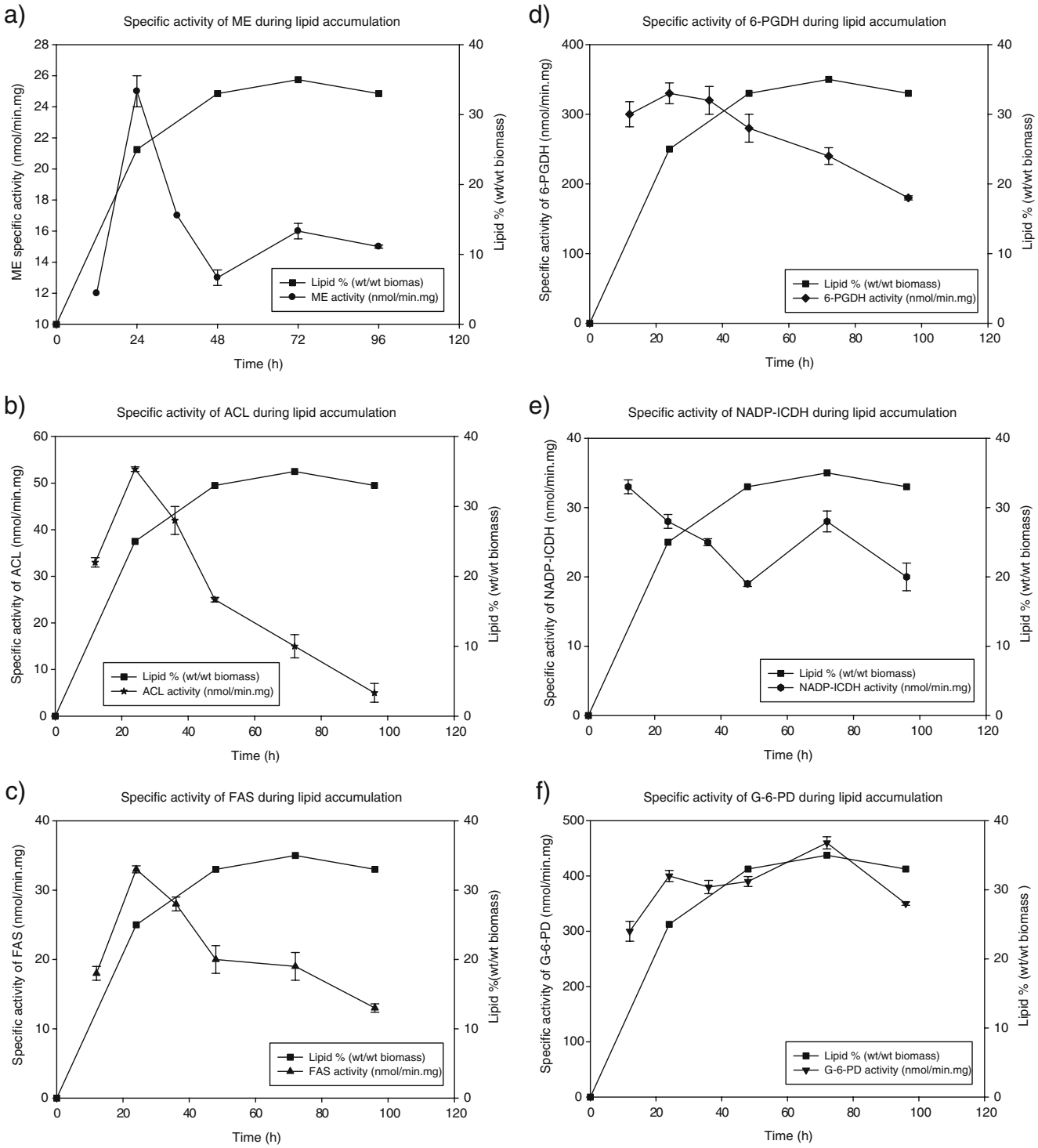

Fig. 2 **a–f** Specific activity profiles of the six enzymes investigated. The culture for enzymes assay was cultivated in a 5-l fermenter with pH 6.00, aeration 0.8 v/v/m, agitation 600 rpm and starting inoculum of 10% (v/v)

compared to the report from (Wynn et al. 1999) observing that ME activities in *Mucor circinelloides* and *Mortierella alpina* could not be detected after 48 h. In their work, the cessation of lipid accumulation was proposed to be caused by the down-regulation via ME activity. We hypothesized that the decrease in ACL, ME and FAS activity in *Cunninghamella* sp. 2A1 may be a result of feedback repression after nitrogen exhaustion. When ammonium

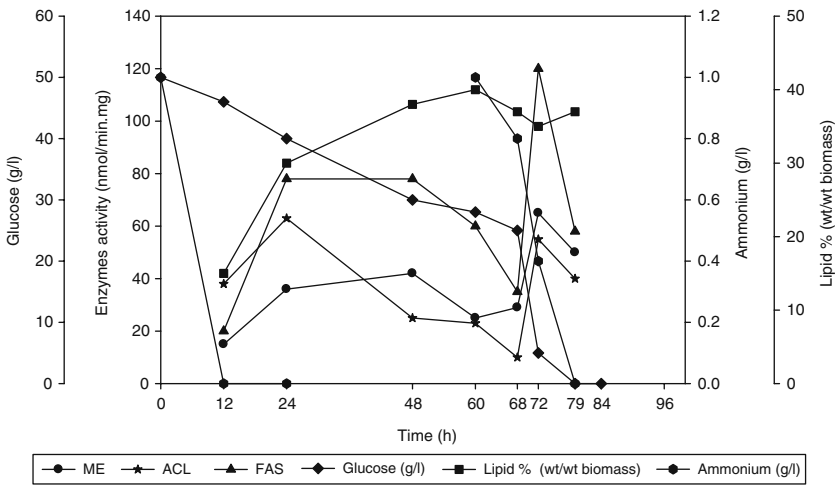

Fig. 3 The specific activity (nmol/min.mg protein) of *ACL*, *ME* and *FAS* after the inclusion of 1 g/l ammonium tartrate. The culture for enzymes assay was cultivated in a 5-l fermenter with pH 6.00, aeration 0.8 v/v/m, agitation 600 rpm and starting inoculums of 10% (v/v)

(1 g/l) was added to the medium when ACL, ME and FAS were at their lowest specific activity, each enzyme activity was restored (59, 52 and 58%, respectively) within 12 h after the addition (Fig. 3). This proved that the decrease in activities of ACL, ME and FAS at 48 h in *Cunninghamella* sp. 2A1 was related to nitrogen depletion in the medium. To investigate further the possibility that the reinstatement of ACL, ME and FAS was due to newly synthesized protein or otherwise, 100 μg/ml of cyclohexamide was added together with ammonium into the culture. Results showed that the activities of ACL, ME and FAS were restored 8–12 h after the addition and even at a double concentration of cyclohexamide (200 μg/ml), indicating that the reinstatement of activity was not due to newly synthesized enzyme. This leads to the suggestion that there are components in ammonium tartrate that increased the activities of the three enzymes.

The idea that the presence of inhibitors could be responsible for the decrease in ACL, ME and FAS activities was further investigated by the mixing of cell-free extract prepared from cultures at 72 h with low activity of ACL, ME and FAS to an extract prepared at 24 h containing high activities of each of the enzymes. Our result showed that the inhibition was approximately 25% (Table 1).Our results showed that the measured activity of the mixed cfe was lower compared to the activities that are supposed to be present according to calculations (Table 1). This suggests that the presence of inhibitors may contribute to the decrease in activities observed in the culture. Several metabolites had been reported to inhibit ACL activity in *Aspergillus nidulans* (Adams et al. 2002), in *Lipomyces starkeyi* (Boulton and Ratledge 1981) and also the interference of ME activities in *Mucor circinelloides* (Kendrick and Ratledge 1992b) and *Fusarium oxysporum* (Zink 1974). However, the fact that a greater extent of inhibition of 84, 72 and 59%, respectively) showed that there might probably be other mechanisms that serve as a major regulatory roles of these enzymes, which is not discussed here.

Conclusions

The enzymes ACL, ME and FAS were shown to be vital in lipid biosynthesis of *Cunninghamella* sp. 2A1. However, there was no down-regulation of ACL, ME and FAS with the depletion of the nitrogen source. The activities of the three enzymes were restored upon addition of ammonium ion, and the presence of inhibitors in the medium could be the reason for the decrease in activities.

Table 1 Measurement the activity of (*ACL*, *ME* and *FAS*) in cfe prepared at 26 and 50 h and mixed cfe 26 with 50 h (v/v); the activity in nmol/min. ml was then calculated to give the estimated values and inhibition ratio

	Activities (nmol/min.ml)		
	ME	ACL	FAS
Measured activities in 26 h culture	65.3	163.3	113.6
Measured activities in 50 h culture	14.8	10.4	19.2
Measured activities in mixed culture 26+50 h	29.8	62.5	56.0
Estimated values	40.05	86.85	66.4
Inhibition (%)	25.6	28	15.6

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