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Influence of N-limitation on Malic Enzyme Isoforms and Lipogenesis of *Cunninghamella bainieri* 2A1

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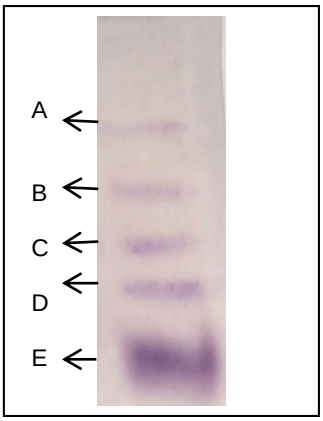
Graphical abstract



Profiles of ME isoforms (A, B, C, D and E) in N-limited media in *C. bainieri* 2A1

Abstract

The influence of the presence of ammonium ions in growth culture on malic enzyme (ME) isoforms activity and lipogenesis in *Cunninghamella bainieri* 2A1 was investigated. The fungus was cultivated in a nitrogen-limiting medium for 120 h at 30ºC under two conditions. One of the cultures was intermittently fed with ammonium tartrate to maintain the ammonium concentrations above 0.5 g/L. The second culture was performed without any feeding to allow N limitation, thus promoting lipid accumulation. Activity staining of ME isoforms was carried out for both cultures. The culture which was not intermittently fed with ammonium tartrate achieved a maximum lipid content of 35% (g/g biomass) at 48 h. This culture possessed five ME isoforms (A, B, C, D and E) with isoform E showing a parallel correlation to lipid accumulation profile. In contrast, intensity of bands representing isoform D decreased as lipid accumulated. No appreciable differences of all other isoforms were observed. However, the culture which was intermittently fed with ammonium tartrate, accumulated only up to 16% lipid (g/g biomass). All isoforms were present but with a more pronounced activity of isoform D and a lower activity of isoform E was observed. These findings support further evidence that isoform E is the key isoform for lipid synthesis in *C. bainieri* 2A1.

Keywords: Malic enzyme isoforms; lipid biosynthesis; nitrogen controlled-media

Abstrak

Kesan kehadiran ion ammonium dalam kultur pertumbuhan terhadap aktiviti isoforms enzim malik (ME) dan lipogenesis dalam *Cunninghamella bainieri* 2A1 telah dikaji. Kulat dikulturkan dalam medium terhad nitrogen selama 120 jam, pada suhu 30°C dalam dua keadaan yang berbeza. Salah satu daripada kultur telah disuap dengan ammonium tartarat secara berperingkat untuk mengekalkan kepekatan ammonium melebihi 0.5 g/L. Kultur kedua pula tidak disuap untuk membenarkan keadaan terhad nitrogen tercapai, seterusnya merangsang pengumpulan lipid. Pewarnaan aktiviti isoforms ME dilakukan untuk kedua-dua kultur. Kultur yang tidak disuap dengan ammonium mencatatkan kandungan lipid maksimum, iaitu 35% (g/g biojisim). Kultur ini mengandungi lima isoforms ME (A, B, C, D dan E) dengan isoforms E menunjukkan korelasi yang positif dengan profil pengumpulan lipid. Sebaliknya, keamatan jalur yang mewakili isoform D berkurang semasa pengumpulan lipid berlaku. Tiada perbezaan yang ketara untuk isoform-isoform lain diperhatikan. Walau bagaimanapun, kultur yang disuap dengan ammonium tartarat secara berperingkat mencatatkan pengumpulan lipid hanya sebanyak 16% (g/g biojisim). Kesemua isoforms hadir tetapi aktiviti isoform D lebih menonjol dan aktiviti isoform E yang lebih rendah diperhatikan. Penemuan ini menyokong pembuktian bahawa isoform E adalah isoform utama yang terlibat dalam sintesis lipid dalam *C. bainieri* 2A1.

Kata kunci: Isoforms enzim malik; biosintesis lipid; media nitrogen-terkawal

1.0 INTRODUCTION

Lipid accumulation in oleaginous microorganisms occurred as a result of cessation of growth in the presence of excess carbon source. This condition is commonly simulated by cultivating microbes in a nitrogen-limiting medium, which allows oleaginous microbes to accumulate substantial amounts of lipids. Nitrogen limitation initiates a cascade of reactions that result in the conversion of the excess carbon into lipid. Among the key enzymes implicated in lipogenesis of oleaginous microbes is malic enzyme (ME).

ME ($NADP^+$, dehydrogenase, decarboxylating) (EC 1.1.1.40) is an important NADPH generating enzyme and is associated in many cellular functions such as in the metabolism of acetate [1] and anaerobic growth [2]. Its role is also implicated in the desaturation of polyunsaturated fatty acids [3]. However, one of the most vital functions of ME is regarding its role in the regulation of lipid biosynthesis especially in oleaginous fungi. Although its activity is not directly related to microbial oleaginousity, nonetheless, its central function as determining the extent of lipid accumulation has been reported.

Most work regarding its importance in oleaginous fungi was done using *Mucor circinelloides* and *Mortierella alpina*. In both fungi, ME has been proposed to be the sole provider of NADPH for lipid biosynthesis [4, 5, 6, 7] by having a strong correlation to the onset and cessation of lipid accumulation. This hypothesis was reached based on the probable existence of a physical association between ME and fatty acid synthase, although no direct evidence has been reported yet. At least seven isoforms (A-G) of ME have been identified in *M. alpina* and only isoform E which converted from isoform D is demonstrated to be associated with lipid accumulation [8]. Besides, similar phenomenon also exists in *M. circinelloides* which possesses at least six ME isoforms (isoform I-IV), and in which only isoform IV which converted from isoform II is associated with lipid accumulation [9].

This paper attempts to further investigate the possible role of ME isoforms in lipid biosynthesis in oleaginous fungi, using a local Malaysian oleaginous isolate, *Cunninghamella bainieri* 2A1 which is able to accumulate 30 % lipid (g/g biomass) [10,11]. Studies were undertaken by establishing correlations between the isoforms activities to cultures subjected to two conditions; N-limited condition that initiates lipid synthesis, and constant presence of N, which does not promote lipid synthesis. The effects of these conditions on profiles of activities of ME isoforms as well as lipid accumulation are reported.

2.0 EXPERIMENTAL

2.1 Microorganism and Culture Conditions

C. bainieri 2A1 was maintained on potato dextrose agar (PDA) plates at 30ºC for 7 days. The growth medium (modified Kendrick medium) [3] contained (g/L): glucose, 30; $(NH_4)_2.C_4H_4O_6$, 1; KH_2PO_4, 7; Na_2HPO_4, 2; $MgSO_4.H_2O$, 1.5; yeast extract, 1.5; $CaCl_2.2H_2O$, 0.1; $FeCl_3.6H_2O$, 0.008; $ZnSO_4.7H_2O$, 0.0001; $CuSO_4.5H_2O$, 0.001; $Co(NO_3).6H_2O$, 0.0001 and $MnSO_4.5H_2O$, 0.0001. The pH was adjusted to 6. After sterilization (121ºC for 15 min), the media were inoculated with spore suspension obtained from cultures grown on PDA for 7 days at 30ºC, to a final concentration of (1×10^7) spores/mL. Cultivations were performed by transferring a 10% (v/v) of the cultures to 500 mL Erlenmeyer flasks containing 200 mL of the above medium, incubated in a rotary shaker at 200 rpm at 30ºC. Samples were analyzed at various intervals for biomass, lipid content, enzymes specific activities, glucose and ammonium concentration. The condition for other culture, ammonium tartrate (up to its initial concentration of 1 g/L) was intermittently fed as required to maintain the concentration above 0.5 g/L.

2.2 Analytical methods

Biomass concentration was determined by filtering the cultures through a Whatman No. 1 filter paper. The mycelia were washed with two volumes of distilled water, frozen at – 20ºC, freeze–dried for 24 h and then gravimetrically determined. Lipids were extracted with chloroform/methanol (2:1, v/v) [12]. Lipid fractions were converted to methyl esters using 1 M sodium methoxide, and analyzed using gas chromatography (Shimadzu GC-2010 FID, Japan) with column (DB-23) and flame ionization detector (FID) at 250°C. The fatty acids composition present in the sample was calculated based on the peak area of corresponding methyl esters against reference standard FAMEs mixture. Glucose concentrations in the culture medium were determined using a Glucose Oxidase (GOD-PERID) test kit (Boehringer Mannheim). Ammonium concentrations in the culture filtrate were determined using the indophenol method [13].

2.3 Preparation of Cell–Free Extracts

Harvested mycelia obtained at various intervals and were washed using cold distilled water and cell free extracts (cfe) were prepared by suspending the mycelia in an extraction buffer [14] and homogenized in a pestle and mortar. The homogenized cell suspension was centrifuged at 10,000 g for 15 min at 4ºC and the supernatant (cfe) was filtered through a Whatman No.1 filter paper. Protein concentration was measured according to the method of Bradford [15].

2.5 Detection of lipogenic enzymes activities and ME isoforms

The activities of ME [16], fatty acid synthase (FAS), ATP: citrate lyase (ACL), glucose-6-phosphate dehydrogenase (G-6-PDH), 6-phosphogluconate dehydrogenase (6-PGDH), NAD-isocitrate dehydrogenase (NAD-ICDH) were determined using continuous assays following the oxidation and reduction of NAD(P)(H) at 340 nm [5]. ME isoforms were distinguished by activity staining of gels from non-denaturing (native) polyacrylamide gel electrophoresis (PAGE). Native – PAGE was prepared using 10% (w/v) acrylamide [17]. Enzyme activity staining in native gel was performed by immersing the gel in a solution containing phosphate buffer at a pH 7.4, 0.47 mM $NADP^+$, 17.2 mM L-malate, 0.55 mg/mL nitroblue tetrazolium and 0.097 mg/ml phenazine methosulfate [8]. After three hours of incubation, the reaction was stopped by replacing the staining solution with 5% acetic acid. Negative controls consisting of the reaction medium without either malate or $NADP^+$ was also carried out.

3.0 RESULTS AND DISCUSSION

3.1 Relationship of ME activity and lipid accumulation

Lipid accumulation of *C. bainieri* 2A1 cultivated in N-limited condition occurred within the first 48 h of cultivation during a period when ME activity began to decrease (Figures 1 and 2).

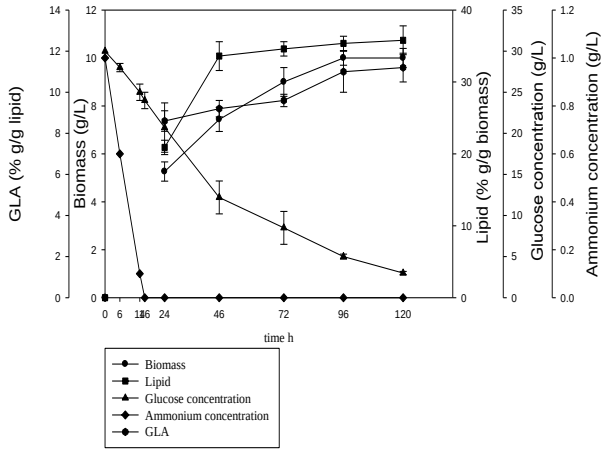


Figure 1 Profiles of biomass (g/L ●), lipid in total biomass, % (g/g ■), glucose concentration (g/L ▲), ammonium tartrate concentration (g/L ♦), and γ-linoleic acid GLA % (g/g ●) during growth of *C. bainieri* 2A1 on N-limited media (30 °C and 200 rpm)

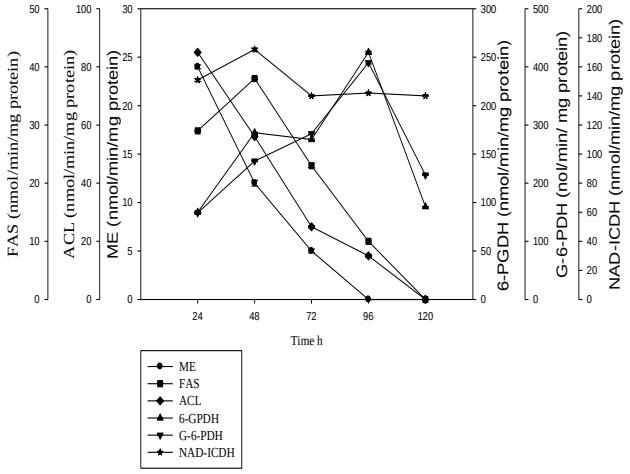


Figure 2 Specific activity of key enzymes in *C. bainieri* 2A1 during growth and lipid accumulation in N-limited media (30 °C and 200 rpm). Enzymes measured were ME (●), FAS (■), ACL (♦), G-6-PDH (▼), 6-PGDH (▲) and NAD-ICDH (⋆)

The change from N-sufficient to N-limited conditions was accompanied by high lipid accumulation in the fungal cell (35%, g/g biomass). However, after 48 h, when a pronounced decrease in ME, ACL, and FAS activities were observed, lipid accumulation ceased although glucose was still present in the medium. Further observation showed that during the reduction of all three enzymes, ME gave the highest reduction in specific activity compared to ACL and FAS (80%, 60% and 40%, respectively). This suggests ME as an important enzyme in lipid accumulation and supports the previous reports where ME was also associated with lipid accumulation in *M. circinelloides* and *M. alpina.* This led to suggestion to the role of ME as a major source of NADPH for de novo lipid biosynthesis [4, 6]. No correlation was found between activities of NADP: ICDH, G-6-PDH and 6-PGDH to the lipid accumulation profile (Figure 2).

When ME isoforms were assayed by activity staining of extracts separated using native - PAGE, five isoforms were detected (A-E) (Figure 3).

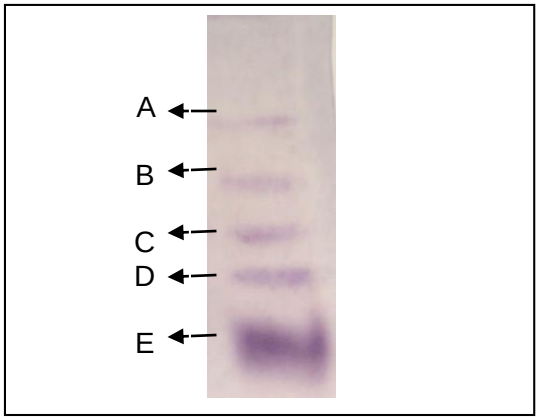


Figure 3 Profiles of ME isoforms (A, B, C, D and E) in N-limited media in *C. bainieri* 2A1 (24 h, 30 °C and 200 rpm). The isoforms were separated by native PAGE then visualized using activity stain (specific for ME) and the amount of protein loaded were 30 μL of 3 mg/mL

Isoform A, B and C were present constitutively between 5 to 48 h with no noticeable change in the intensity of the bands (Figure 4). However, bands representing isoform D showed a profound decrease in intensity as the culture progressed while an increase in the intensity of bands of isoform E was detected (Figure 4). These results showed that isoform D was present particularly during balanced growth (before N depletion) and diminished as the culture progressed to the lipid accumulation phase as N was exhausted (12 h). During lipid accumulation phase (12-48 h), isoform E was observed to be the most predominant (Figures 1 and 4).

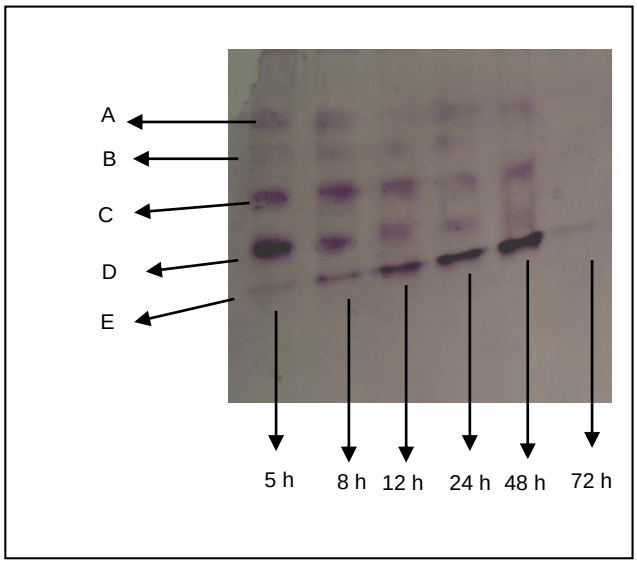


Figure 4 Profiles of ME isoforms (A, B, C, D and E) in N-limited media in *C. bainieri* 2A1 at different growth stages. The isoforms were separated by native PAGE then visualized using activity stain (specific for ME) and the amount of protein loaded were 30 μL of 3 mg/mL

However, all isoforms were undetectable at 72 h. These data indicates that isoform D and E showed the most profound response when the culture shifted from a balanced growth to lipid accumulating phase with isoform E predominantly becoming more important and pronounced during lipid accumulation. Thus, this strongly indicates isoform E to its role in lipid biosynthesis in *C. bainieri* 2A1.

Results at 72 h suggest that the decrease in ME specific activity (Figure 2) detected in the culture after N exhaustion may not be the result of the degradation of a single form of ME (Figure 4). Instead, it appears likely that this change in activity was the result of a change in all isoforms of ME present in the cells (Figure 4). During the time of 12 to 48 h, isoform D disappeared gradually followed by the appearance of isoform E. This is similar to the reported change in ME isoform activities in *M. circinelloides* and *M. alpina* [8, 9]. In *M. circinelloides* and *M. alpina* six and seven isoforms were observed (I-VI and A-G, respectively) and isoform III, IV and D, E showed similar profiles as reported here where isoform III and D diminished as the culture entered the lipid accumulation phase, respectively. Therefore, these results further support the suggestion of the role of isoform E (or IV) in regulating lipid accumulation in oleaginous fungi. The existence of the ME isoforms showed that the subunits are oligomeric, represented by the bands (Figure 4) which migrated to consistent distances of each other.

3.2 Effects of maintaining the presence of N source during cultivation on ME isoforms and lipid accumulation.

When *C. bainieri* 2A1 was grown with intermittent feeding of ammonium tartrate to achieve a concentration of not less than 0.5 g/L throughout the cultivation, the maximum lipid accumulation achieved was less than that observed in N-limited culture. A profile of lipid accumulation in the culture shows that the lipid content was 12% after 24 h of cultivation and did not increase more than 16% up to 120 h. This shows that N-limited condition is important for the culture to accumulate substantial amount of lipid as previously reported [4, 6, 8]. As shown in Figure 5, less lipids were accumulated despite of similar profiles and levels of activity of ME, FAS and ACL observed in the culture (Figure 6), as in the N-limited cultures.

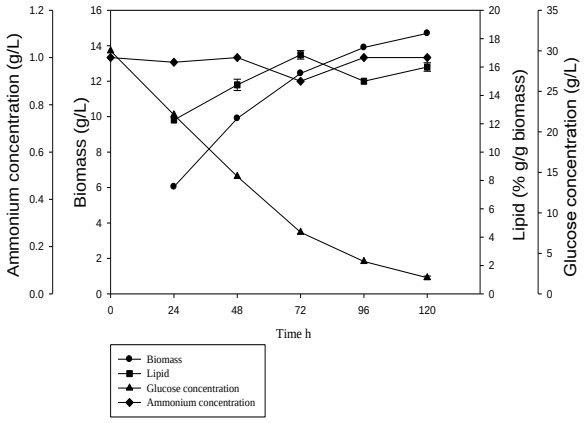


Figure 5 Profiles of biomass (g/L ●), lipid in total biomass % (g/g ■), glucose concentration (g/L ▲), and ammonium tartrate concentration (g/L ♦), during growth of *C. bainieri* 2A1 on nitrogen-controlled media (30 °C and 200 rpm)

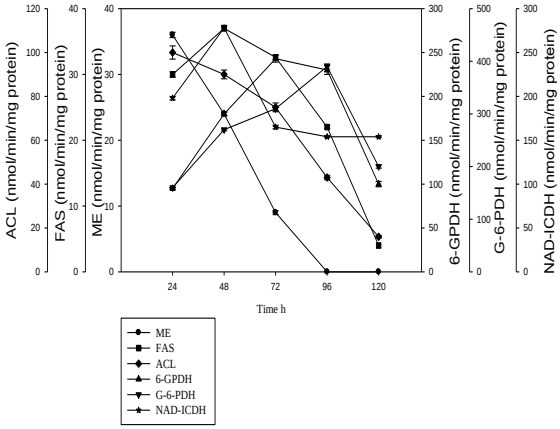


Figure 6 Specific activity of key enzymes in *C. bainieri* 2A1 during growth and lipid accumulation in N-controlled media (30 °C and 200 rpm). Enzymes measured were ME (●), FAS (■), ACL (♦), G-6-PDH (▼), 6-PGDH (▲) and NAD-ICDH (⋆)

When detection of ME isoforms were carried out, all five isoforms were present but with a distinctively different profiles. In this culture, isoform D remained predominant throughout the experiment while isoform E was only detectable until 24 h of cultivation after which lipid accumulation gradually stopped (Figure 7). This further support the suggestions that isoform E is important in lipid synthesis. On the other hand, these results showed there is a relation in the presence of isoform D and the N source in the medium. It was observed that the presence of isoform D relates to the availability of the N source as depletion of ammonium tartrate at 16 h in the N-limited culture was followed by the eventual disappearance of isoform D whereas its activity was maintained until 72 h when ammonium tartrate was controlled above 0.5 g/L.

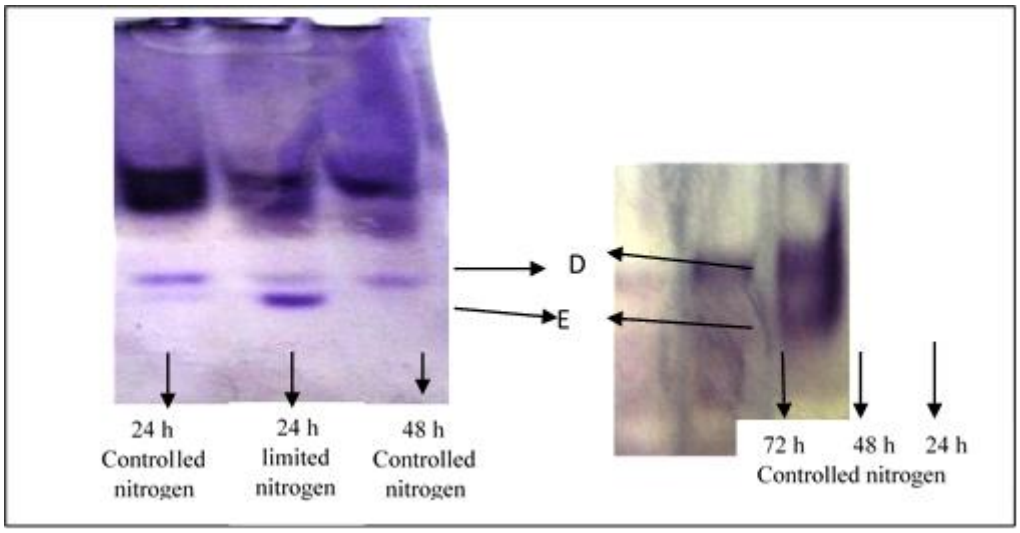


Figure 7 Isoforms of ME in *C. bainieri* 2A1 in N-limited and N-controlled media at different growth stages. The isoforms were separated by native PAGE then visualized using activity stain (specific for ME) and the amount of protein loaded were 30 µL of 3 mg/mL

■4.0 CONCLUSION

In *C. bainieri* 2A1, the presence and profiles of activity of isoform E strongly correlate to the profiles and the extent of lipid accumulation. Therefore, isoform E is the likely key regulator of lipid biosynthesis in *C. bainieri* 2A1 and should be considered in future studies related to enhancement of lipid synthesis.

Acknowledgement

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