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POSSIBLE INVOLVEMENT OF DIMINISHING METAL ION CONCENTRATION AND KEY LIPOGENIC ENZYMES ACTIVITIES IN THE CESSATION OF LIPID ACCUMULATION IN *Cunninghamellabainieri* sp. 2A1

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Abstract. Possible involvement of metal ions and activities of malic enzyme (ME), ATP-citrate lyase (ACL) and fatty acid synthase (FAS) in the cessation of lipid accumulation in *Cunninghamellabainieri* sp. 2A1 were investigated. Cultivation was performed in 200 mL nitrogen-limited medium and incubated at 30 ℃. The specific activities of the enzymes, concentrations of trace elements and the lipid content were determined at 24 h intervals. Cessation of lipid accumulation coincided with diminishing activities of the enzymes at 48 h. A significant decrease in metal ions concentration was observed followed by total depletion at 48 h except Mg^{2+} and Ca^{2+}. Feeding of ammonium tartrate and glucose after the cessation of lipid accumulation resulted in a marked increase in the specific activity of the enzymes but with no increase in the lipid content. In contrast, the lipid content increased from 32% to 50% (g/g biomass) when trace elements were included in the feeding. No increase in lipid content was observed when the cultures were fed only with trace elements or with the omission of ammonium tartrate. These results showed that cessation of lipid accumulation were caused by the diminishing activities of the enzymes as well as depletion of the metal ions.

Keywords: Lipid accumulation, ME, ACL, FAS and metal ions.

1. Introduction

Oleaginousmicroorganisms have an ability to produce more than 20% lipid (g/g) of their biomass. The lipids which accumulated in some oleaginous microorganisms contain high amount of essential polyunsaturated fatty acids (PUFAs) such as gamma linolenic acid (GLA), arachidonic acid (ARA) and eicosapentaenoic acid (EPA) [1]. Lipid accumulation in oleaginous microorganisms is trigged by a nutrient imbalance in the culture medium. When nitrogen sources are depleted, excess carbon substrate in the medium continues to be assimilated by the cells and converted into lipid. Oleaginous microorganisms are capable of accumulating large amount of lipid because of their ability to produce a continuous supply of acetyl-CoA which is a necessary precursor for lipid biosynthesis and to produce sufficient supply of NADPH as the essential reductant for lipid biosynthesis. ATP-citrate lyase (ACL) and ME have been reported to play a vital role in the generation of acetyl-CoA and NADPH, respectively [2].

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Various studies on oleaginous fungi have shown that media with variable composition of trace elements affect growth and lipid accumulation in various fungal species. In relation to lipid and PUFAs production, Mg^{2+}, Mn^{2+}, Fe^{3+}, Ca^{2+}, Cu^{2+} and Zn^{2+} ions have been shown to influence lipid and arachidonic acid (ARA) as well as GLA production by *Mortierellarammannianavarrammaniana* [1].

Cunninghamellabainieri sp. 2A1 is a local oleaginous fungus which is able to produce up to 30% lipid (g/g biomass) containing between 10-15% GLA [3]. However, cessation of lipid accumulation occurred after 48 h of growth although in excess glucose condition [4]. Therefore, in this paper we report works carried out to determine the effect of reinstatement of ME, ACL and FAS activities on lipid accumulation as well as the possible involvement of metal ions in causing the cessation of lipid accumulation.

2. Materials and Methods

2.1. Cultivation and culture conditions

*Cunninghamellabainieri*sp. 2A1 was maintained on Potato Dextrose Agar (PDA) at 4 $^{\circ}$C, and spores were harvested from a 7-day old plate cultures. Seed culture was prepared by transferring spore into 500-mL shake flasks containing 200 mL of N-limited medium [5] to a final concentration of $1x10^5$ spores/mL.A N-limited medium containing the following constituents (g/L): $(NH_4)_2C_4H_4O_6$1.0, KH_2PO_4 7.0, Na_2HPO_4 2.0, $MgSO_4.7H_2O$ 1.5, yeast extract 1.5, $CaCl_2$ 0.1, $FeCl_3.6H_2O$ 0.008, $Co(NO_3)_2.6H_2O$ 0.0001, $ZnSO_4.7H_2O$ 0.0001, $CuSO_4.5H_2O$ 0.0001, $MnSO_4.5H_2O$ 0.0001 were sterilized at 121 $^{\circ}$C for 40 min. Glucose (30 g/L) was added separately after sterilization. The cultures were incubated at 30 $^{\circ}$C and agitated at 200 rpm for 48 h. Ten percent (v/v) of the culture was then used for subsequent inoculations. Cultivation was carried out at 30 $^{\circ}$C, with agitation at 200 rpm for 120 h. For fed-batch experiments, simultaneous feeding of ammonium tartrate, glucose and each of the metal ions (Mg^{2+}, Mn^{2+}, Fe^{3+}, Cu^{2+}, Co^{2+}, Ca^{2+} and Zn^{2+}) were carried out to reach their initial concentrations. Controls consisting of cultures fed with glucose and either the metal ions or ammonium tartrate was also conducted. Feeding was carried out at 72 h. Cultures were sampled every 24 h and assayed for glucose, ammonium, biomass concentrations, for lipid content and concentration of metal ions in the media.

2.2. Analytical methods

Ammonium concentration was determined using the indophenols method [6] while glucose concentration was determined using a glucose oxidase GOD-PERID test kit (Boehringer Mannheim). Enzyme activities were determined using continuous assays following the oxidation and reduction of NAD (P)(H) at 340 nm [7]. Fungal biomass was harvested by filtration of 200 ml culture through preweight Whatman No. 1 filter paper followed by washing with distilled water. The filtered mycelia were then freeze-dried overnight to a constant weight. Cellular lipids were extracted from the dried mycelia with chloroform/methanol 2:1 (v/v) [8] and lipid content was expressed as % (g/g of biomass). Metal concentration quantification was performed using Perkin-Elmer Elan 5000 ICP-MS.

3. Results and Discussions

Lipid accumulation in *C. bainieri* sp. 2A1 was initiated after nitrogen exhaustion at 12 h of cultivation with lipid content showing an increase from 27% to 32% (g/g biomass) at 48 h (Fig. 1). The highest specific activities of ME, FAS and ACL were detected 24 h after cultivation (12.3, 21.1 and 24.7 nmol/min.mg protein, respectively) (Fig. 2). The activities of the enzymes showed a marked decrease at 48 h (73%, 52% and 64%) and were totally diminished at 120 h and coincided with the cessation of lipid accumulation at 48 h. To establish the involvement of diminishing activities of ME, ACL and FAS at 48 h of cultivation in the cessation of lipid accumulation, effect of reinstatement of its activities on lipid accumulation was investigated. As ammonium has been reported to reinstate activity of key lipogenic enzymes in *M. circinelloides* and *M. alpina* [7], effect of feeding of ammonium was carried out at 72 h.

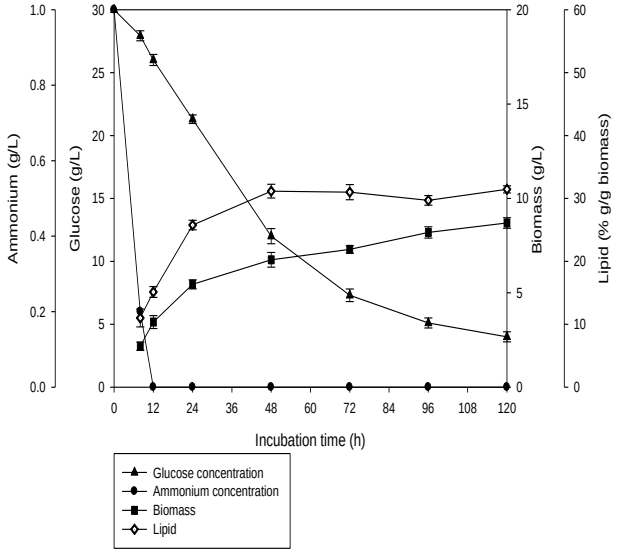

Fig. 1: Profiles of biomass (g/L), lipid (% g/g biomass),glucose concentration (g/L) and ammonium tartrate concentration (g/L) during cultivation of *C. bainieri* sp. 2A1 in nitrogen-limitedmedium.

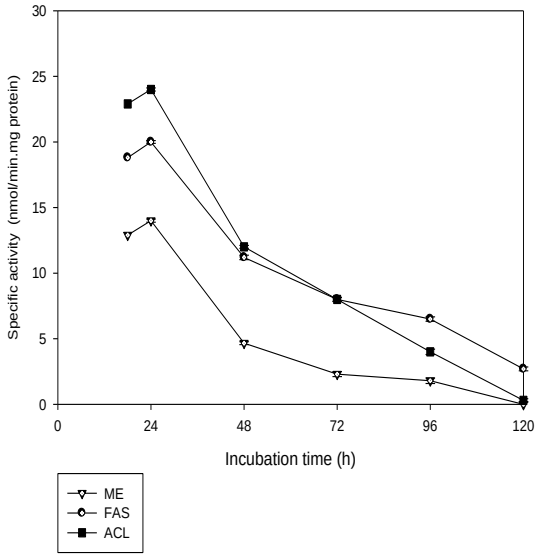

Fig. 2:Profiles of specific activities of ME, ACL and FAS (nmol/min.mg protein) during cultivation of *C. bainieri* sp. 2A1 in nitrogen-limited medium.

Feeding of ammonium tartrate to reach its initial concentration at 72 h of cultivation resulted in significant increase in the specific activity of ME (2.3 to 12.6nmol/min.mg protein), ACL (7.9 to 20.3 nmol/min.mg protein) and FAS (8.0 to 17.3 nmol/min.mg protein) within 24 h after feeding (Fig. 3). However, no increment in lipid content was observed. Similar results were observed when simultaneous feeding of ammonium tartrate and glucose (1 g/L and 30 g/L) were employed to the culture. These results indicate that lipid accumulation stopped although the cultures were in the most optimal condition for lipid accumulation i.e. limited N, excess C and in the presence of ME, ACL and FAS activities.This suggest other factors were involved in the limitation of lipid biosynthesis. Since the event occurred in the latter stage of cultivation, it was anticipated that the nutritional state of the cultures were involved. As the status of the availability of micronutrients was not known, further experiments were carried out to determine the concentration of each metal ion in the medium throughout the cultivation.

As shown by the ICP-MS analysis of the culture broth, a pronounced decrease in the concentrations of each of the metal ions i.e. Mg^{2+}, Mn^{2+}, Ca^{2+}, Cu^{2+}, Fe^{3+}, Co^{2+} and Zn^{2+} ion was observed, though at varying rates (Fig. 4). As previous work showed that different initial concentrations of Mg^{2+}, Fe^{3+} and Zn^{2+} ions have significant effects on lipid accumulation in *C. bainieri* sp. 2A1 [9], it was thought that the diminishing concentration of these ions contributed to the limitation of lipid accumulation.

When experiments with simultaneous feeding of ammonium tartrate, glucose and all metal ions to reach their initial concentrations were performed, reinstatement of the enzyme activities was shown to be followed by an increase in lipid content from 32% to 50% (g/g biomass) (Fig. 5). On the contrary, no increment of lipid content was observed when the culture was fed with glucose and metal ions but with the omission of ammonium tartrate. Further experiments carried out by simultaneous feeding of ammonium tartrate, glucose and with either one of the four metal ions (Fe^{3+}, Mg^{2+}, Mn^{2+} and Zn^{2+}) showed similar increment of lipid content (from 32% to up to 48%, g/g biomass) achieved. Highest lipid content was achieved when Fe^{3+} was employed compared to the other metal ions. These results further support the previous observations that the diminishing activities of ME, ACL and FAS as well as depletion of metal ions as the probable cause of the cessation of lipid accumulation. Reinstatement of the enzymes activity and replenishment of the metal ions in the culture therefore resulted in the reinitiation of lipid accumulation. Therefore, a simple feeding of ammonium and trace metal ions resulted in enhancement of lipid production in *C. bainieri* sp. 2A1.

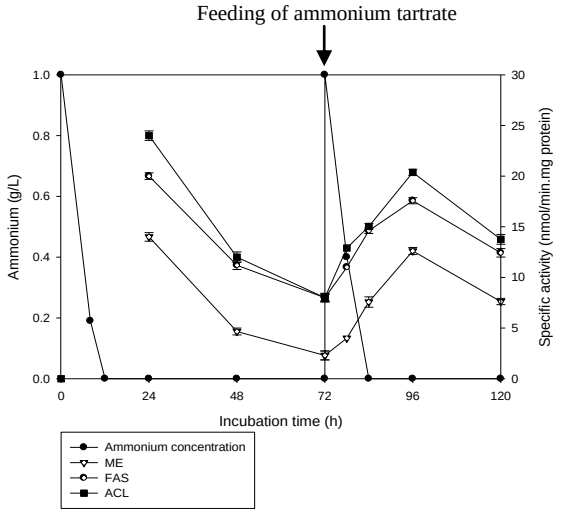

Fig. 3. Effect of feeding ammonium tartratemto a final concentration of 1 g/L on ME, FAS and ACL specific activities.Culture was grown in 500 mL shake flask contains 200 mL of nitrogen-limited medium, 30 ˚C and 200 rpm.

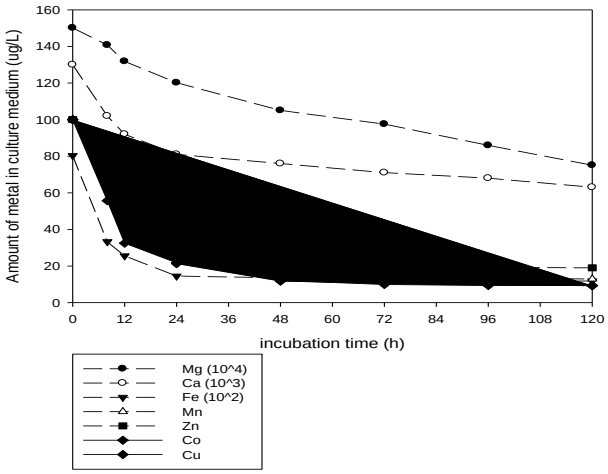

Fig. 4. Amount of metal ions left in culture medium during the incubation. Culture was grown in 500 mL shake flask contains 200 mL of nitrogen-limited medium, 30 ℃ and 200 rpm.

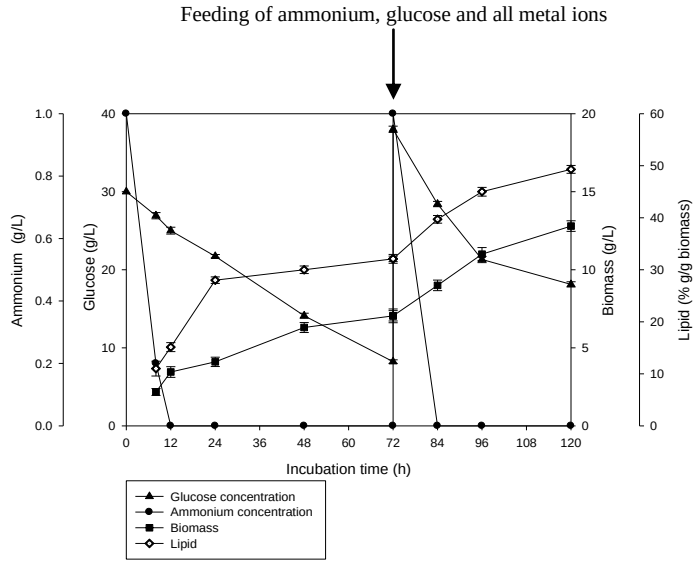

Fig. 5: Effect of feeding ammonium tartrate, glucose and metal ions to reach their initial concentrations at 72 hin lipid accumulation of *C. bainieri* sp. 2A1.

4. Acknowledgement

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