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Enhancement of Lipid Accumulation in *Cunninghamellabainieri* sp. 2A1 Via Feeding of Ammonium and Metal Ions

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Abstract: Enhancement of lipid accumulation of locally isolated oleaginous zygomycete, namely *C. bainieri* sp. 2A1 through strategic feeding of ammonium and metal ions in submerged culture was investigated. Cultivation was performed in 500 mlconical flasks containing 200 ml nitrogen limited medium and incubated at 30 $^{\circ}$C with agitation at 200 rpm for 120 h. The concentrations of trace elements (Mg^{2+}, Mn^{2+}, Fe^{3+}, Cu^{2+}, Ca^{2+}, Co^{2+} and Zn^{2+}), ammonium, glucose and biomass as well as lipid content of the culture were determined at 24 h intervals. Up to 30% lipid (g/g biomass) was accumulated but lipid accumulation stopped at 48h although glucose was still present in the medium. Cessation of lipid accumulation coincided with the depletion of all metal ions concentrations except for Mg^{2+} and Ca^{2+}. When feeding of ammonium, glucose and all the metal ions were carried out at 72h, increased lipid content of 30% to 50% (g/g biomass) was achieved. Similar increase was observed when ammonium, glucose and individual metal ions were employed in the feeding except when Fe^{3+} was employed where up to 48% (g/g biomass) lipid content was achieved. No increase in lipid content was observed when ammonium was omitted. Therefore, these results imply that the depletion of metal ions in the medium contribute to the cessation of lipid accumulation at 48h. Possible role of metal ions and reintroduction of ammonium ion in the reinitiation of lipid accumulation are discussed.

Keywords: Lipid accumulation, Metal ion, Oleaginous fungi, Submerged culture fermentation.

1. Introduction

Microorganisms typically produce at least 5% lipids (g/g biomass) for essential functioning of cell membranes and other membranous structures. Cells that capable of producing more than 20% lipids (g/g biomass) are termed oleaginous microbes [1]. The lipids which accumulated in some oleaginous fungi contain high amount of essential polyunsaturated fatty acids (PUFAs) such as gamma linolenic acid (GLA), arachidonic acid (ARA) and eicosapentaenoic acid (EPA). In Europe, GLA was known as 'King's Cure-All' because of its nutritional benefits to cure diseases such as decreasing blood cholesterol, suppressing acute and chronic inflammations and improving atopic eczema [2].

Lipid accumulation in oleaginous microorganisms istrigged 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. Several studies on fungi have shown that media with variable composition of trace elements affect growth and lipid accumulation in various fungal species. In

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relation to lipid and PUFAs production, Mg, Mn, Fe, Ca, Cu and Zn ions have been shown to influence lipid and arachidonic acid (ARA) as well as GLA production by *Mortierellarammannianavarrammaniana* [3].

We previously isolated and described a local oleaginous fungus, *C. bainieri* sp. 2A1 which is able to produce up to 30% lipid (g/g biomass) containing between 10-15% GLA [1]. In this paper, we describe further attempts to enhance lipid accumulation by determining the influence of metal ions on growth and lipid accumulation phase in *C. bainieri* sp. 2A1.

2. Materials and Methods

2.1. Cultivation and culture conditions

*Cunninghamellabainieri*sp. 2A1 was maintained on Potato Dextrose Agar (PDA) at 4 °C, and spores were harvested from a 7-day old plate cultures. Seed culture was prepared by transferring spore to a final concentration of $1x10^7$ spores/ml into 500-ml shake flasks containing 200 ml of N-limited medium [4].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 °C for 40 min. Glucose (30 g/l) was added separately after sterilization. The cultures were incubated at 30 °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 °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, lipid content and concentration of metal ions in the media.

2.2. Analytical methods

Ammonium concentration was determined using the indophenols method [5] while glucose concentration was determined using a glucose oxidaseGOD-PERID test kit(Boehringer Mannheim). Fungal biomass was harvested by filtration of 200 ml culture through pre weightWhatman No. 1 filter paper followed by washing with distilled water. The filtered mycelia were then freeze-dried overnight to a constant weight. Metal ions concentration quantification was performed using Perkin-Elmer Elan 5000 ICP-MS. Cellular lipids were extracted from the dried mycelia with chloroform/methanol 2:1 (v/v) [6] and lipid content was expressed as % (g/g of biomass).

3. Results anddiscussions

Lipid accumulation in *Cunninghamellabainieri* sp. 2A1 showed similar profile as reported in other filamentous fungi in batch cultivation [7]. Lipid accumulation was initiated after nitrogen content in the cultivation medium was exhausted at 12 h. Lipid content showed an increase from 27% at 24h to 32% (g/g biomass) at 48h (Fig. 1). Nitrogen limitation is known to be a vital in initiating lipid biosynthesis in oleaginous organisms as it would stop the proliferation of the cell and hence, oleaginous organisms would channel the excess carbon sources in the medium into storage lipid in the cell [7].

However, lipid accumulation ceased after 48h of cultivation (Fig. 1) although glucose was still present in the medium. This observation was similar with previous report using *Mortierellaalpina* LPM 301, whereby they reported cessation of lipid accumulation occurred even when glucose was still present in the medium [8].

As feeding of ammonium was reported to result in the reinitiation of key lipogenic enzymes [7], further experiments were carried out by cultivating and simultaneously feeding of ammonium tartrate and glucose to reach their initial concentration at 72h of cultivation. However, no increment in lipid content was observed where lipid content remained at 30% (g/g biomass) (Fig. 2) although N-limited condition was achieved at 12h after feeding.

This shows that lipid accumulation stopped although the culture was in the optimal condition for lipid accumulation i.e. limited N and excess C. This suggests that there were other factors that limit lipid

biosynthesis and 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 micronutrients was not known, further experiments were carried out to determine the concentration of each metal ions in the medium throughout the cultivation.

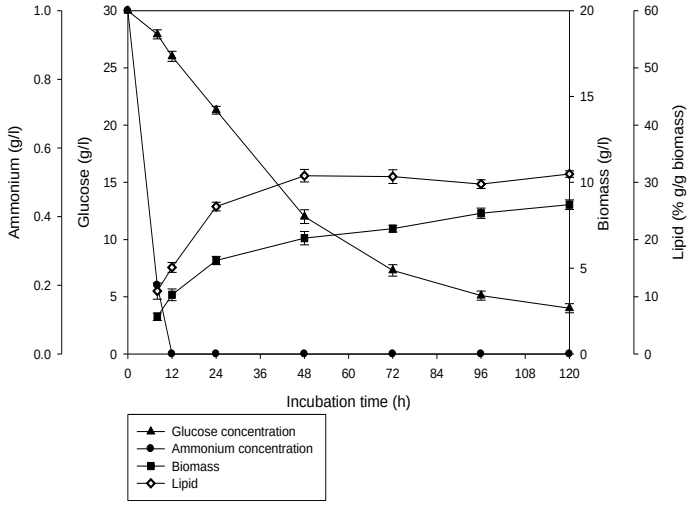

Fig. 1: Profiles of biomass, lipid, glucose concentration, and ammonium tartrate concentration during cultivation of *C. bainieri*sp. 2A1 in nitrogen-limited media.

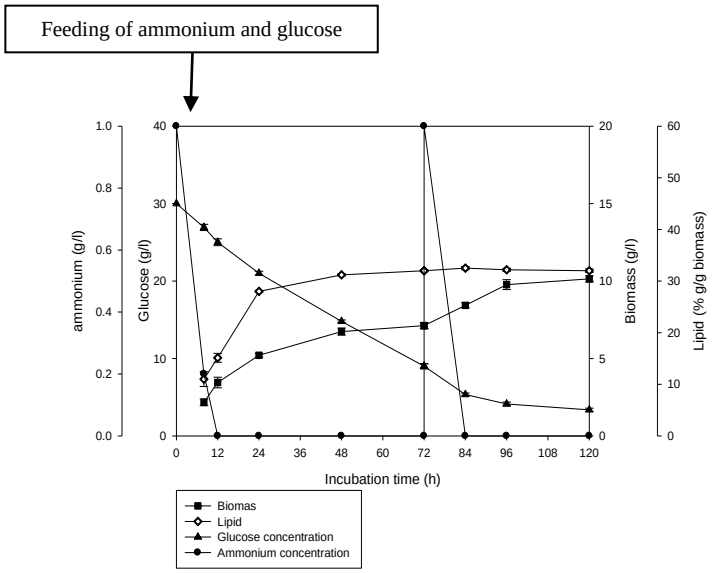

Fig. 2: Effect of feeding ammonium tartrate and glucoseto reach their initial concentration at 72 h of cultivation in lipid accumulation of *C. bainieri* sp. 2A1.

As shown by the analysis of the medium using ICP-MS, a pronounced decrease in the concentrations of each of Mg^{2+}, Mn^{2+}, Ca^{2+}, Cu^{2+}, Fe^{3+}, Co^{2+} and Zn^{2+} ion was observed, though at varying rates (Fig. 3). Fe^{3+}, Mn^{2+}, Zn^{2+}, Co^{2+}, and Cu^{2+} showed faster diminishing rate compared to others. As previous work showed that different initial concentrations of Mg^{2+}, Fe^{3+} and Zn^{2+} 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 were performed, an increase in lipid content from 32% at 72h to 50% (g/g biomass) at 120h were observed (Fig. 4). 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 were carried out by simultaneously 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 applied compared to other metal ions (Fig. 5). Therefore, this further established that the depletion of metal ions as one of the contributing factors involved in the cessation of lipid accumulation in *C. bainieri* sp. 2A1 in addition to reintroduction of ammonium ions in the medium.

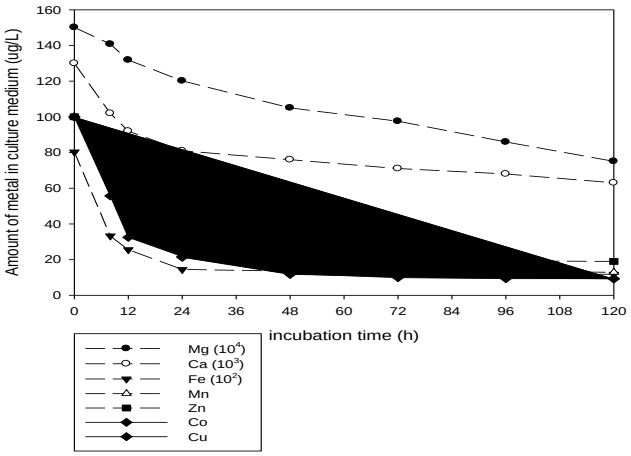

Fig. 3: Amount of metal ions left in culture medium during the incubation. Culture was grown in 500 ml shake flask contains 200 ml of N- limited medium, 30 ℃ and 200 rpm.

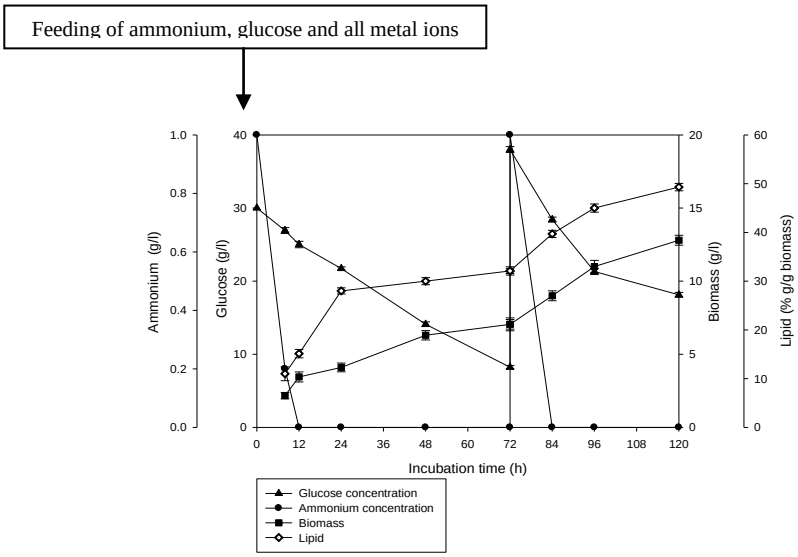

Fig. 4: Effect of feeding ammonium tartrate, glucose and metal ions to reach their initial concentrations at 72 h in lipid accumulation of *C. bainieri* sp. 2A1.

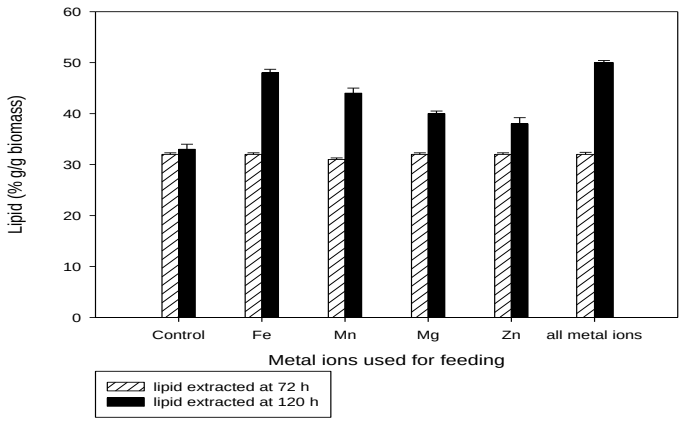

Fig. 5: Amount of lipid extracted at 72h and 120h of incubation time in *C. bainieri*sp. 2A1. Controls was fed with ammonium tartrate and glucose, meanwhile other cultures were fed with ammonium tartrate, glucose and one of the metal ions (Fe^{3+}, Mn^{2+}, Mg^{2+} and Zn^{2+}) at 72 h of incubation time.

4. Acknowledgement

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