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Effects of Metal Ion Concentrations on Lipid and Gamma Linolenic Acid Production by *Cunninghamella* sp. 2A1

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Abstract: The effects of different concentration of $MgSO_4.7H_2O$, $FeCl_3.6H_2O$, $ZnSO_4.7H_2O$, $CuSO_4.5H_2O$ and $MnSO_4.5H_2O$ on lipid and GLA production by *Cunninghamella* sp. 2A1 were studied in submerged culture fermentation. Cultivation was performed in 250 ml conical flask (100ml working volume), at 30°C and 250 rpm agitation for 120h. Mg^{2+}, Fe^{2+} and Zn^{2+} have significant effect on lipid accumulation as inclusion of each ions resulted in a 64, 43 and 33% increase in lipid content compared to cultures which was cultivated with the omission of each ions, respectively. All three metal ions also affected GLA production by *Cunninghamella* sp. 2A1 with Zn^{2+} having the most profound effect with an increase of up to 74% of GLA yield (g GLA/g lipidless biomass) achieved. As omission of any single metal ions did not affect biomass concentration achieved, we concluded that metal ions components in production media may have affected lipid and GLA production by affecting pathways involved in the biosynthesis of lipid and GLA. This study reports the critical concentration of each metal ion for optimal lipid and GLA production by *Cunninghamella* sp. 2A1.

Key words: *Cunninghamella* sp., lipid, GLA and metal ion

INTRODUCTION

Microorganisms produce lipid for essential functioning of cell membranes and other membranous structures. However not all microbes are able to produce substantial amount of lipid. Those that produce more than 20% (g/g) biomass lipid are known as oleaginous microbes. Of the some 60000 fungal species, fewer than 50 accumulate more than 25% lipid[1]. The lipids which accumulated in some oleaginous fungi contain high amount of essential polyunsaturated fatty acids (PUFAs) such as Gamma Linolenic Acids (GLA), arachidonic acids (ARA) and eicosapentaenoic acids (EPA). GLA was known in Europe as 'King's Cure-All' because of its nutritional benefits to cure diseases such as suppressing acute and chronic inflammations, decreasing blood cholesterol concentrations and improving atopic eczema[2]. The potential of microbes as alternative sources for PUFAs production has led studies related to optimization of process parameters being intensively carried out. Of particular importance are studies dealing with media optimization for enhanced lipid and GLA production.

Several studies on *Mortierella* and other fungi have shown that media variables such as different carbon and nitrogen sources, pH of the growth medium and the composition of trace elements affect the growth and lipid accumulation that occur within various species[3,4,5,6]. Of particular importance is the effect of metal ions on lipid and GLA production. In 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 *Mortieralla rammanniana* var *rammaniana* [7]. Hansson and Dostalek (1988) also investigated the effects that metal ions had on *M. rammanniana*, *M. vinacea* and *M. isabellina.* Results showed that the addition of Cu-ions and Zn-ions had a stimulatory effect on both lipid and GLA production whereas the addition of Mn^{2+} did not. Further increase in the amount of Mg^{2+} over what was already in the growth medium had also no marked effect on either of these components. These shows that determination of suitable concentration of trace elements implicated in medium for lipid and GLA production for different species is important[8].

We had previously isolated a local oleaginous fungus, *Cunninghamella* sp. 2A1 which was able to produce more than 20% (g/g) biomass lipid containing between 10-15% GLA[9]. Further studies showed that lipid and GLA production by this strain was affected by carbon and nitrogen concentration in the culture medium but no significant changes in

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lipid and GLA content was observed when pH and temperature of cultivation was altered[10]. However, effect of metal ions on growth,lipid and GLA of this isolate has not been established. This report discusses the effects of metal ions concentration on lipid and GLA production by *Cunninghamella* sp. 2A1.

MATERIALS AND METHODS

Microorganism and inoculum preparation: *Cunninghamella* sp. 2A1 was maintained On Potato Dextrose Agar (PDA) at 4°C. The standard inoculum used, was in order of 10^5 spores/ml (final concentration), harvested from 7-day-old plates. Seed culture was prepared by transferring the spore suspension into 500 ml conical flask containing 200 ml of modified nitrogen-limited medium[11], incubated at 30°C, with agitation at 250 rpm for 48 h.

Culture medium and condition: Seed cultures medium contained the following constituents (g/l): $NH_4C_4H_4O_6$ 1.0; KH_2PO_4 7.0; Na_2HPO_4 2; $MgSO_4.7H_2O$ 1.5; yeast extract 1.5; $CaCl_2$ 0.1; $Co(NO_3)_2.6H2O$ 0.0001; $FeCl_3.6H_2O$ 0.008; $ZnSO_4.7H_2O$ 0.0001; $CuSO_4.5H_2O$ 0.0001; $MnSO_4.5H_2O$ 0.0001; and Glucose 30g/l which was sterilized and added separately. To investigate the effect of metal ions on lipid and GLA production, 10% (v/v) of seed culture was added into five 250ml conical flask containing 100ml of the same composition of nitrogen limited medium with various concentration of $MgSO_4.7H_2O$, $FeCl_3.6H_2O$, $ZnSO_4.7H_2O$, $CuSO_4.5H_2O$ and $MnSO_4.5H_2O$ Table 1. Range tested was based on concentrations reported in various studies of lipid and GLA production by oleaginous fungi. All cultures were incubated at 30°C and agitated at 250 rpm in 'INFORS' incubator shaker. Cultures were harvested after 120h fermentation. Results presented are average value of three replicates and are reproducible.

Analytical methods: The fungal mycelia were harvested by filtration of 100 ml cultures through a Whatman no. 1 filter paper and washed with 200 ml of distilled water. The filtered mycelia were then stored for 24 hours at -20°C and freeze-dried for 24 hr for determination of dry weight. Dried mycelia were then ground into powder using a pestle and mortar, followed by lipid extraction using chloroform/methanol 2:1 (v/v)[12]. Lipid obtained was transesterified with 5% sodium methoxide methanol solution. The resultant colorless or pale-yellow transparent methyl esters were analyzed by gas chromatography (Shimadzu GC-14A). A 3.1-m glass column of 3.2-mm bore packed with Shimadzu Shinchrom E71 5%/Shimalite 80-100 was used. The instrument was fitted with a flame ionization detector.

Table 1 Range of metal elements concentration employed in cultivation medium

Elements	Concentration range
MgSO4. 7H2O	0-3.5 g/l
FeCl3. 6H2O	0-0.04 g/l
ZnSO4. 7H2O	0-0.035 g/l
CuSO4. 5H2O	0-0.02 g/l

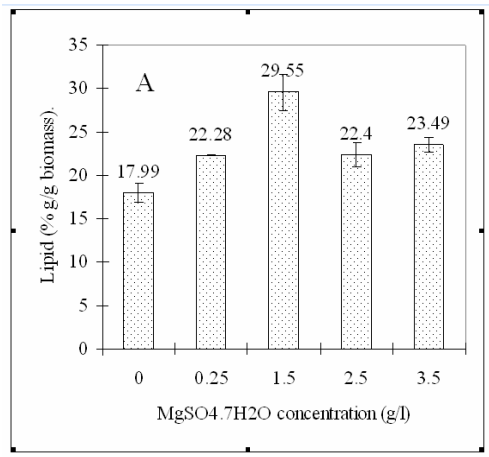
Production of cell-free extract: Fungal mycelia was suspended in 20% (w/v) extraction buffer containing 100mM KH PO /KOH (p H 7.5), 20% (v/v) glycerol, 1mM benzamidine, 1mM mercaptoethanol and 1mM EDTA and disrupted using pestle and mortar. The suspension was centrifuged at 10, 000 g for 15 minutes at 4°C and the resulting supernatant (cell free extract) was used for enzyme assays. Protein concentration was determined using the method of Bradford[13] with BSA as standard.

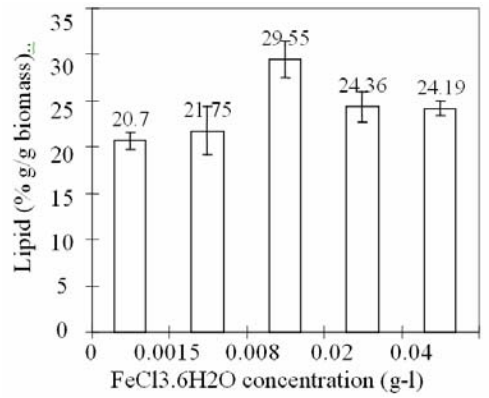
Enzymes assay: Malic enzyme, ATP- citrate liase and fatty acid synthase activities were determined using continuous assays following the oxidation or reduction of NAD(P)(H) at 340nm[14] and carried out at 30°C.

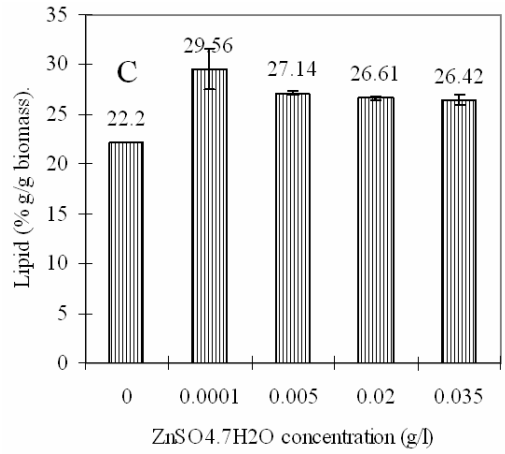
RESULTS AND DISCUSSIONS

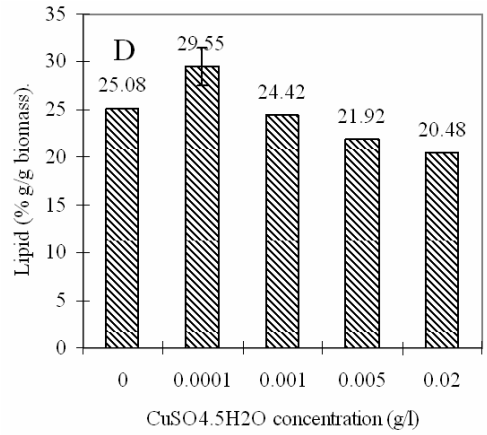
Effects of Mg, Mn, Fe, Cu and Zn ions on growth and lipid production by *Cunninghamella* sp. 2A1: Mg, Fe, Zn, Cu and Mn ion at all concentrations tested was shown to give no significant effect on biomass production of *Cunninghamella* sp 2A1. Biomass concentrations achieved after 120 h of cultivation for all cultures was in the range of 9.50 to 11 g/l. This suggests that growth of *Cunninghamella* sp. 2A1 was not affected by the metal ions and that the limited biomass production observed was more likely due to the presence of limited nitrogen content in the medium. Biomass production by micro-organism generally requires the supplementation of nitrogen and carbon sources in the fermentation medium[15]. In addition, increasing ammonium tartrate concentration in the medium had previously been shown to result in an increase in biomass production by this organism[16].

Nevertheless, addition of each of metal ions tested caused an increase in the lipid content achieved by the cultures Fig. 1. Mg^{2+}, Fe^{2+} and Zn^{2+} have profound effect on lipid accumulation as inclusion of each ions resulted in a 64%, 43% and 33% increase in lipid content compared to cultures which was cultivated with the omission of each ions respectively.









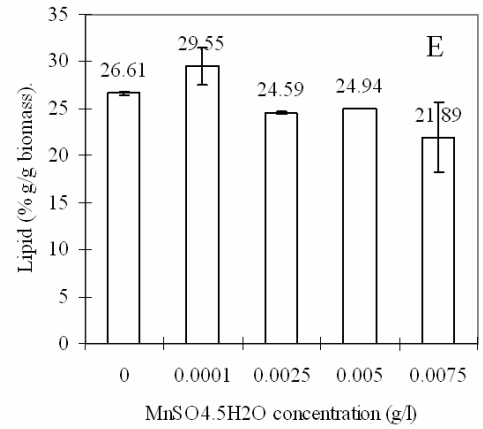


Fig. 1: The effects of (a) Mg^{2+}, (b) Fe^{2+}, (c) Zn^{2+}, (d) Cu^{2+} and (e) Mn^{2+} concentration on lipid production of *Cunninghamella* sp. 2A1 cultivated in modified Kendrick and Ratledge (1992) medium (working volume 100ml, at 30°C, 250 rpm agitation for 120h). * Lipid yield, $Y_{l/x}$ - % lipid (g/g biomass)

This is followed by Cu and Mn but with a lower increase in lipid content was observed with an increase in each of the ions in the medium. Similar results were reported in that lipid yields per gram biomass of the media supplemented with Mg^{2+}, Cu^{2+} and Mn^{2+} for *M. rammanniana* var. *rammanniana* were higher than those obtained with the unsupplemented media[7]. Furthermore, the presence of Cu and Zn ion was reported to increased lipid accumulated by Morterella ramanniana by 13.9% when the culture medium was added with 0.0005 g/l $CuSO_4$ and 0.0075g/l $ZnSO_4$ compared to the cultures that lacked the respective metal ions[8]. This shows that each Cu and Zn ion affected *Cunninghamella* sp. 2A1 at a lower concentration (0.0001 g/l) compared to M, *rammanniana*, resulting in a 17.79% and 33.09% increase in lipid content compared to the cultures with the omission of the ions, respectively.

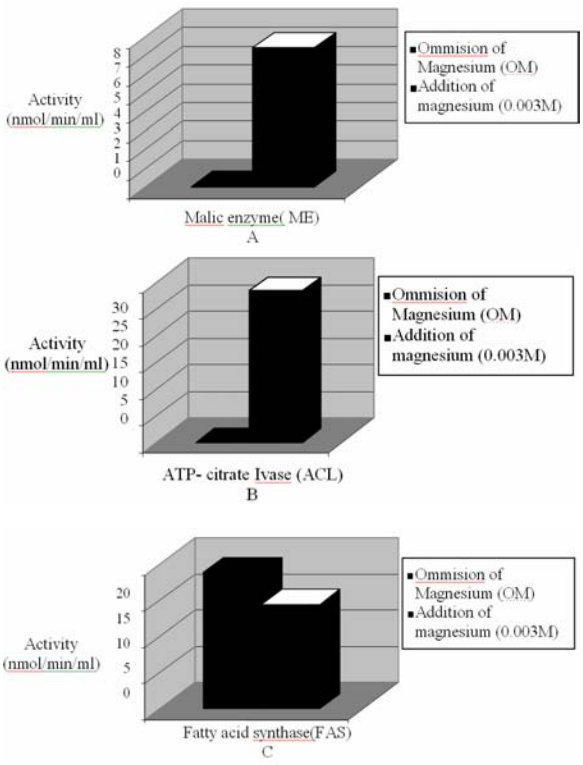


Fig 2: The effects of magnesium ion on malic enzyme (a), ACL (b) and FAS (c) activity

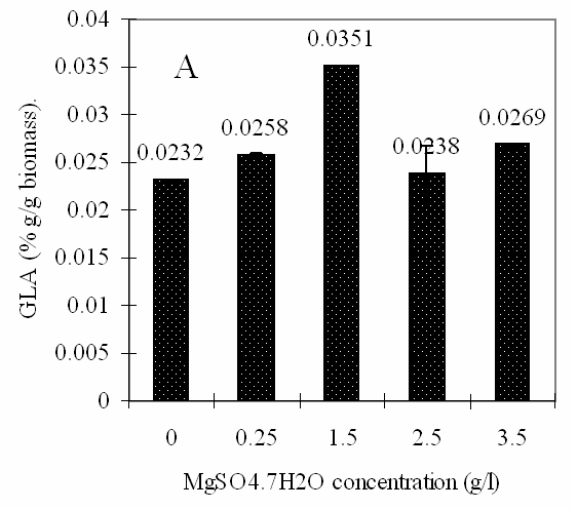
These observations may have been caused by the ions affecting enzymes implicated in lipogenesis as metal ions are known to play a role as cofactors for enzyme activities. Therefore, as Mg^{2+} gave the highest increment in lipid content Fig. 1, we have investigated the importance of Mg^{2+} in the activity of three enzymes involved in the regulation of lipid synthesis; Malic Enzyme (ME), ATP Citrate Lyase (ACL) and Fatty Acid Synthase (FAS). Malic enzyme plays a vital role as a sole NADPH provider for Fatty Acid Synthase (FAS) activity[17] while ATP Citrate Lyase (ACL) provides acetyl-CoA as a precursor for lipogenesis. ACL and ME from *Cunninghamella* sp. 2A1 was shown to have an absolute dependency to Mg^{2+} for its activity where no activity was observed when the assay was carried out whilst FAS activity was not dependent to Mg^{2+} Fig. 2 This explains the significant increase in lipid accumulated when Mg^{2+} was included in the culture medium, as it may have affected ACL and ME activity thus affecting the production of acetyl-coA and NADPH.

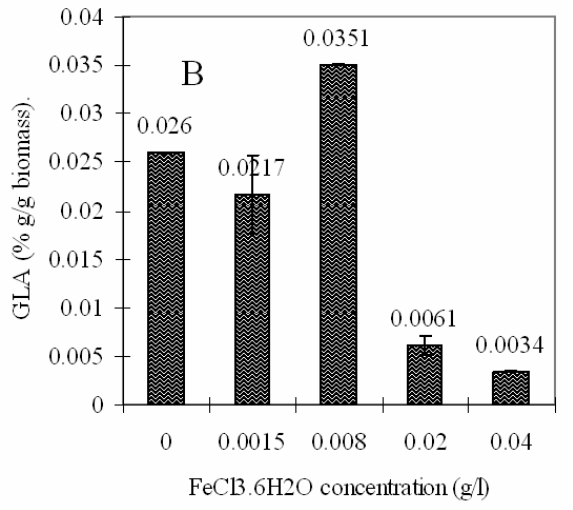
This is similar to reports which found that ATP Citrate Liase (ACL) from Penicillium spiculisporum was also dependent on Mg^{2+} for its activity[18]. Mg, Mn and other bivalent ion have also been reported to be important as cofactor for malic enzyme (ME) activity[19,20].

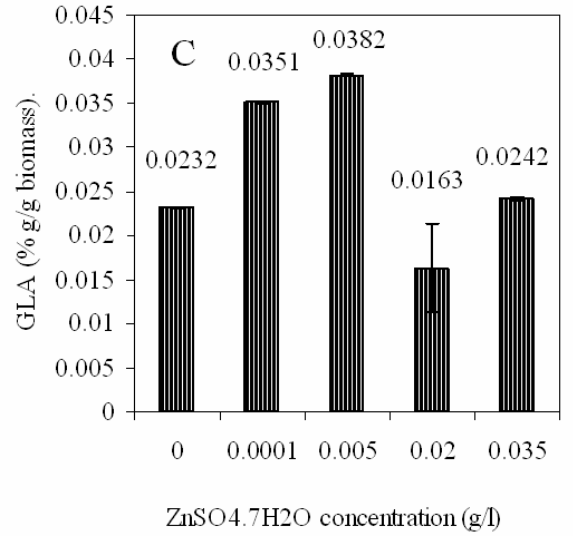
Effects of Mg, Mn, Fe, Cu and Zn ions on GLA production by *Cunninghamella* sp. 2A1: GLA production by *Cunninghamella* sp. 2A1 was found to be sensitive to the concentration of metal ions studied. Mg^{2+}, Fe and Zn^{2+} affected GLA production by *Cunninghamella* sp. 2A1 with Zn^{2+} having the most profound effect, resulting in an increase of up to 74% of GLA yield (g GLA/g lipidless biomass) achieved Fig. 3. A report by Hansson and Dostalek (1988) regarding the effects of metal ions on GLA accumulation in *M. ramanniana, M. vinacea* and *M. isabellina* supports our observation. They showed that the addition of Cu, Mg and Zn ions had a stimulatory effect on GLA production[8]. This may be related to the fact that GLA formation is dependent on the activity of delta 6-desaturase which requires Zn and Mg ions as positive modulators to convert linoleic acid to GLA[21].

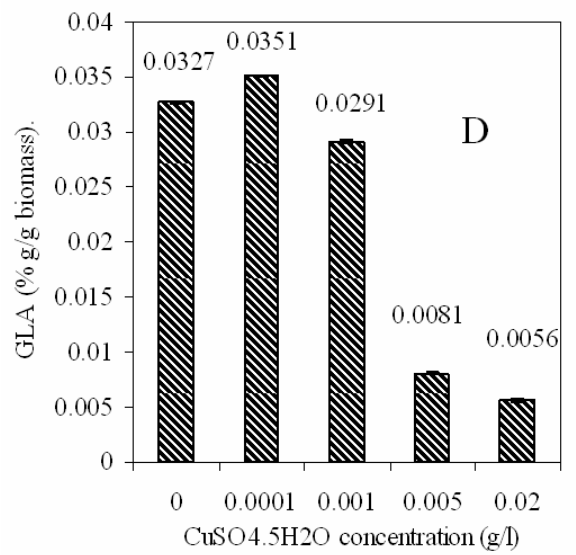
Figure 3 shows that there exist a critical concentration of metal ions tested as an increase in the concentration of metal ions resulted in the decrease of GLA yield achieved. Further increase in Fe and Zn ions beyond 0.02 g/l resulted in 52% and -43% decrease in GLA yield. The same observation was also reported where an increase of Cu^{2+} concentration from 0.005 to 0.05 g/l resulted in an increase in GLA production and that a further increase in Cu^{2+} concentration to 0.5 g/l caused lipid with no detectable GLA being produced[7]. However, the effect of Cu^{2+} on GLA yield in *Cunninghamella* sp. 2A1 was more pronounced at a lower critical concentration (0.0001 g/l), that gave the highest GLA yield (0.035 g/g lipid less biomass) compared to those obtained in M. ramanniana (0.05 g/l)

Our results also showed that GLA production by *Cunninghamella* sp. 2A1 was most sensitive to the presence of Zn. Addition of 0.0001g/l $ZnSO_4.7H_2O$ resulted in 51.23% increased in GLA compared to cultures which was cultivated with the omission Zn^{2+}. There was no significant increment of GLA yield when concentration of $ZnSO_4.7H_2O$ used in cultivation medium was increased beyond 0.0001 g/l. Further increase in the concentration of $ZnSO_4.7H_2O$ beyond 0.02g/l resulted in the decrease of GLA production. When the optimal concentration of each metal ions observed in these experiment was employed in cultivation, the highest GLA production was observed (0.0351 g/g lipid less biomass).









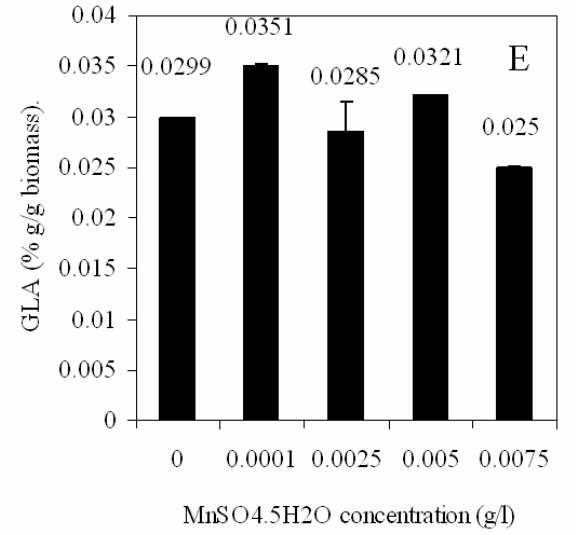


Fig 3: The effects of (a) Mg^{2+}, (b) Fe^{2+}, (c) Zn^{2+}, (d) Cu^{2+} and (e) Mn^{2+} concentration on GLA production of *Cunninghamella* sp. 2A1 cultivated in modified Kendrick and Ratledge (1992) medium (working volume 100ml, at 30°C, 250 rpm agitation for 120h). * GLA yield, $\mathbf{Y_{GLA/x}}$ – g GLA / g lipidless biomass

CONCLUSION

We conclude that the best concentration of $MgSO_4.7H_2O$, $FeCl_3.6H_2O$, $ZnSO_4.7H_2O$, $CuSO_4.5H_2O$ and $MnSO_4.5H_2O$ for lipid and GLA production by *Cunninghamella* sp. 2A1 are 1.5 g/l, 0.008 g/l, 0.0001 g/l, 0.0001 g/l and 0.0001 g/l respectively. Mg, Fe, Zn, Cu and Mn ions did not have significant effect in the growth of

Cunninghamella sp. 2A1. Mg, Fe and Zn were shown to have a profound effect on lipid production and this may be related to their role as cofactors required by key enzymes implicated in lipid biosynthesis pathway. ACL and ME were found to be dependent on Mg ions in this isolate. Zn^{2+} has the most profound effect in the production of GLA by *Cunninghamella* sp. 2A1.

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