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Combination of calcium and magnesium ions prevents substrate inhibition and promotes biomass and lipid production in thraustochytrids under higher glycerol concentration

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ABSTRACT

Effect of calcium and magnesium ions was studied in detail in batch mode in shake flask cultures of two fast growing strains of thraustochytrids (*Aurantiochytrium* sp. DBTIOC-18 and *Schizochytrium* sp. DBTIOC-1) for biomass and lipid production. These strains were previously isolated from Indian marine biodiversity. Screening of these two strains on different carbon and nitrogen sources revealed the suitability of glycerol over glucose and sodium nitrate over yeast extract for the cultivation of these strains. The presence of higher concentration of glycerol in the medium inhibited the glycerol utilization by the cell thus resulting in lower biomass and lipid production in both the strains. Supplementing media with calcium and magnesium ions promoted glycerol utilization thus resulted in a substantial rise in volumetric production of biomass (55.12 $g\ L^{-1}$, 48.12 $g\ L^{-1}$), fatty acid for biodiesel (27.14 $g\ L^{-1}$, 22.15 $g\ L^{-1}$) and docosahexaenoic acid (14.57 $g\ L^{-1}$, 10.12 $g\ L^{-1}$) with both strains *Aurantiochytrium* sp. DBTIOC-18 and *Schizochytrium* sp. DBTIOC-1, respectively. Growth profile study of these two strains showed further improvement in production of biomass, fatty acid for biodiesel and docosahexaenoic acid when cultures were extended up to 7 days. Finding of this work underlines the importance of calcium and magnesium salts in designing new fermentation strategies to prevent substrate inhibition and achieve high cell density culture under high nutrient concentration especially carbon sources.

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1. Introduction

High cell density cultivation of thraustochytrids is being applied for developing the industrial scale processes for DHA production [1]. To achieve this, high concentration of nutrients particularly carbon sources are required to be maintained in the fermenters in batch or fed batch cultivations. It is reported that high concentration of carbon sources, if added directly may result in substrate inhibition thus negatively affecting the cell growth and lipid accumulation [2–4]. Therefore it is recommended to have low concentration of carbon source in feeding medium particularly starting medium [5]. However this may prolong fermentation time for high DHA production. Strategies like pulsed addition of carbon source in pH-Auxostats mode of cultivation have been reported in literature for providing required concentrations of substrates but are normally not associated with high lipid formation [6]. Addition of high concentration of carbon sources in the culture medium is reported to increase osmolarity of the medium and promote secretion of organic acids (e.g. malic acid, citric acid, pyruvic acid and acetic acid in the medium) in broth [3,5,7], which may not only lower the pH of the medium but also decouples trans membrane pH gradient [7]. Li and co-workers (2015) reported that high concentration of glycerol in growth medium may inhibit activity of major enzymes (such as glycerol kinase and glycerol-3-phosphate dehydrogenase) involved in glycerol metabolism due to limited oxygen availability to the cells [8,9].Addition of divalent cations such as calcium and magnesium can be helpful to address these problems. Calcium ions are reported to alter the activity of glycerol-3-phosphate dehydrogenase (G-3-PDH) under hyperosmotic stress conditions in *Dunaliella salina* cultures [10]. Magnesium ions act as cofactor for malic enzyme, which converts malic acid to pyruvate during transdehydrogenase cycle [11]. This cycle plays an important role during fatty acid biosynthesis by supplying necessary NADPH for lipid production. Thus ensuring the optimum supply of magnesium ions in the broth will enhance the conversion of excessive amount of malic acid to pyruvic acid, which will not only help to increase cellular lipid production but also reduce secretion of malic acid in broth. Calcium ions can successfully form calcium salt of organic acids in the medium [12] thus arresting the fall in pH of the medium. Both the cations are reported to act as co-factors in various enzyme reactions including protein synthesis and cell proliferation [13]. These cations also act as secondary messengers in signal transduction and play significant part in managing stress responses [14]. Chen and co-workers (2014) reported important role of calcium ions in biomass and lipid production in *Chlorella* cultures under stress conditions. Whereas Chen and co-workers (2011) also

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showed role of calcium ion in glycerol metabolism in *D. salina* cultures under hyper and hypo osmotic stresses. Therefore it will be interesting to study the impact of calcium and magnesium ions in biomass and lipid production in thraustochytrid cultivation under hyperosmotic stress induced by the addition of higher concentration of carbon sources to achieve high cell density.

The primary objective of this work was to study the impact of calcium and magnesium ions on biomass and lipid production in the culture of the previously isolated strains *Aurantiochytrium* sp. DBTIOC-18 and *Schizochytrium* sp. DBTIOC-1. Different carbon and nitrogen sources were screened with the aim of replacing glucose and yeast extract in the medium with comparatively inexpensive nutrients. Higher concentration of carbon and nitrogen sources were added in the medium to study the impact of calcium and magnesium ions on glycerol utilization, biomass and lipid production, FAB[1] and DHA concentration in both the strains.

2. Material and methods

2.1. Reagents and chemicals

All chemicals and reagents used in this study were either analytical or molecular grade unless otherwise mentioned. Medium constituents such as carbon sources (glucose, glycerol, acetic acid and xylose) and nitrogen sources (yeast extract, ammonium sulphate, ammonium acetate, ammonium chloride, potassium nitrate, sodium nitrate, corn steep liquor and urea) and peptone were procured from Sigma-Aldrich, USA. Calcium carbonate and magnesium sulphate (Himedia, India) were used as calcium and magnesium source respectively. Sea salt (non analytical grade, Instant Ocean, USA) was added in the medium to mimic the marine environment. Methanol, chloroform, hexane, toluene (Fischer Scientific, USA), Methyl nonadecanoate, Butylatedhydroxytoluene (BHT), and potassium bicarbonate (Sigma-Aldrich, USA) were used in lipid extraction, fatty acid methyl ester (FAME) preparation and analysis.

2.2. Screening of different carbon and nitrogen sources for biomass and lipid production

One full loop (1 μL) of pure culture of two previously isolated strains *Aurantiochytrium* sp. DBTIOC-18 and *Schizochytrium* sp. DBTIOC-1 was taken from an agar plate by semi quantitative loop and inoculated in 100 mL Erlenmeyer flasks containing 20 mL seed medium. Medium consisted of glucose 30 g L^{-1}, yeast extract 10 g L^{-1}, peptone 1 g L^{-1} and sea salt 18 g L^{-1} (to mimic 50% of sea water strength). Medium used in this study was sterilized by autoclaving at 121 °C for 15 min unless otherwise mentioned. Five percent (v/v) of 48 h old inoculum was transferred to production medium (50 mL in 250 mL Erlenmeyer flasks) having a composition similar to inoculum medium except carbon or nitrogen sources. Carbon sources (30 g L^{-1}) such as glucose, glycerol, xylose or acetic acid and nitrogen sources (10 g L^{-1}) such as yeast extract (YE), corn steep liquor (CSL), ammonium sulphate (AS), ammonium acetate (AA), ammonium chloride (AC), sodium nitrate (SN), potassium nitrate (PN) or urea were added in respective production medium. Cultures were incubated at 25 °C for 5 days at 150 rpm for biomass production. After 5 days of growth, these cultures were harvested by centrifugation at 4000 rpm for 15 min and washed twice with distilled water followed by drying of the biomass at 90 °C for 24 h to 48 h. Dried cell biomass was measured gravimetrically. All experiments in this manuscript are performed duplicates and values are expressed as mean ± SE.

[1] FAB — fatty acids suitable for biodiesel production: includes saturated fatty acids and monounsaturated fatty acids.

2.3. Effect of substrate inhibition on biomass and lipid production

Two different media compositions (M1 and M2) were prepared to study the effect of substrate inhibition on biomass and lipid production. In medium M1, five different concentrations of glycerol i.e. 30 g L^{-1}, 60 g L^{-1}, 90 g L^{-1}, 120 g L^{-1}, 150 g L^{-1} along with 10 g L^{-1} sodium nitrate were added (50 mL in 250 mL Erlenmeyer flasks) to give C/N ratios of 13, 26, 39, 52, 65 respectively. Peptone (1 g L^{-1}) and sea salt (18 g L^{-1}, to mimic 50% of sea water strength) were also added in all the media used in this manuscript. In medium M2, different concentration of both glycerol (30 g L^{-1}, 60 g L^{-1}, 90 g L^{-1}, 120 g L^{-1}, 150 g L^{-1}) and sodium nitrate (10 g L^{-1}, 20 g L^{-1}, 30 g L^{-1}, 40 g L^{-1}, 60 g L^{-1}) was added to give a constant C/N ratio of 13. Media were sterilized by autoclaving at 106 °C for 25 min to avoid charring of the media. The rest of the media composition, culture conditions, biomass harvesting and dry cell weight determination methodologies were similar to those described in Section 2.2. All the media used in this study are summarized in Table 1.

2.4. Effect of addition of calcium ions on biomass and lipid production in medium having higher C/N ratios

Two different media compositions (M3 and M4) were prepared to study the effect of addition of calcium ions on biomass and lipid production in medium having different C/N ratios. For formulating medium M3, 5 g L^{-1} of calcium carbonate was added in medium M1. For preparing medium M4, different concentrations of calcium carbonate[2] (5 g L^{-1}, 10 g L^{-1}, 15 g L^{-1}, 20 g L^{-1}, 25 g L^{-1}) were added in medium M1. Media were sterilized by autoclaving at 106 °C for 25 min to avoid media charring. Rest of the media composition and culture conditions were similar to those described in Section 2.2. Before harvesting culture at 5th day, 1 mL of 2 N HCl was added in the cultures to completely solubilize residual calcium carbonate in the medium. Cultures were centrifuged at 4000 rpm for 15 min for harvesting and twice washed with distilled water to remove traces of residual media and HCl. Biomass was dried at 90 °C for 24 h to 48 h and dried cell biomass was measured gravimetrically. This dried biomass was subsequently used for lipid quantification and fatty acid composition analysis.

2.5. Effect of addition of magnesium ions on biomass and lipid production in medium having higher C/N ratios

Two different media compositions (M5 and M6) were prepared to study the effect of addition of magnesium ions on biomass and lipid production in medium having different C/N ratios. For formulating medium M5, magnesium sulphate (5 g L^{-1}) was added in medium M1. For preparing medium M6, different concentrations of magnesium sulphate (5 g L^{-1}, 10 g L^{-1}, 15 g L^{-1}, 20 g L^{-1}, 25 g L^{-1}) were added in medium M1. Media were sterilized by autoclaving at 106 °C for 25 min to avoid media charring. Rest of the media composition and culture conditions were similar to those described in Section 2.2.

2.6. Cumulative effect of calcium and magnesium ions on biomass and lipid production in medium having higher C/N ratios

To study the cumulative effect of calcium and magnesium ions on biomass and lipid production, medium M7 was prepared by adding different concentration of calcium carbonate (5 g L^{-1}, 10 g L^{-1}, 15 g L^{-1}, 20 g L^{-1}, 25 g L^{-1}) and 10 g L^{-1} magnesium sulphate in medium M1. Media were sterilized by autoclaving at 106 °C for 25 min. Rest of the

[2] Calcium carbonate is not soluble at higher concentration in the medium however as culture grew, calcium carbonate was solubilized by forming calcium salt of organic acids. These organic acids are produced during growth under higher glucose or glycerol concentration.

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