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Exploring potential use of Australian thraustochytrids for the bioconversion of glycerol to omega-3 and carotenoids production

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ABSTRACT

Marine microbes have the potential for accumulating large quantities of lipids and are therefore suitable candidate as feedstock in unsaturated fatty acid production. The efficient utilisation of glycerol as an alternative carbon source to glucose was demonstrated in the fermentation of newly isolated thraustochytrid strains from the Queenscliff, Victoria, Australia. The isolates exhibited the presence of omega-3 and omega-6 polyunsaturated fatty acids, with the major fatty acids for all isolates being (as percent total fatty acid), palmitic acid (25.1–40.78%), stearic acid (4.24–13.2%), eicosapentaenoic acid EPA (2.31–8.5%) and docosapentaenoic acid (7.24–10.9%). Glycerol as a carbon source gave promising biomass growth with significant lipid and DHA productivity. An approximate three-fold increase in carotenoid content in all isolates was achieved when glycerol was used as a carbon source in the production medium.

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1. Introduction

Glucose has been used extensively for microalgae fermentation, having yielded high biomass growth and lipid productivity than other carbon sources [1–5]. These resulting high carbon lipids have been proposed as a source of sustainable oil production and thus present a feasible alternative for the production of third-generation biofuels. Microalgae also produce other metabolites, such as astaxanthin, lutein, arachidonic, eicosapentaenoic (EPA), docosahexaenoic acids (DHA), all of which are of high economic value. Maximising lipid productivity depends on the optimisation of an array of nutrient conditions in the growth medium, a process which must be repeated for new isolated strains [6]. Recently, glycerol has been used as an alternative carbon source instead of glucose due to its abundance and relatively low cost compared to glucose. Biodiesel derived glycerol can be used as a carbon source to obtain value added secondary metabolites such as polyunsaturated fatty acids (PUFAs) [7]. Glycerol could therefore be employed as an alternative carbon source in the fermentation process to cheaply produce DHA as a value added co-product, thereby taking another step towards economic viability of these fermentation processes.

Thraustochytrids, large-celled marine heterokonts and classified as oleaginous microorganism, have been reported to utilise a range of substrates such as glucose, galactose, fructose, mannose, sucrose [6,8], complex organic matter [9] and more recently, cellulosic biomass [10], for the production of polyunsaturated fatty acids (PUFAs). They are considered to be highly promising DHA producers [11]. Thraustochytrids have many benefits, including the potential use of their lipid rich biomass in biodiesel and PUFA production, particularly DHA. DHA is a particularly important omega-3 fatty acid due to its health benefits in humans and commercial use in infant formula [11,12]. Thraustochytrids have the potential to produce other metabolites such as carotenoids; astaxanthin, canthaxanthin, β-carotene, zeaxanthin and echinenone [13]. Carotenoids have potent antioxidant activities and are therefore potentially beneficial to human health as they may assist in the treatment of cancer and eye vision [14]. In fact, some carotenoids such as zeaxanthin are endogenous in humans and are an important component of eye retina. Thraustochytrids are also known to secrete enzymes, polysaccharides and carotenoids, squalene and co-enzymes [15,16]. A commercial thraustochytrid strain, *Schizochytrium limacinum* SR21, has recently been used by researchers studying PUFA production utilising biodiesel-derived glycerol, corn steep liquor, and other organic nutrients such as waste water from barley distilleries, soybean cake, liquid residues from beer and potato processing, and sweet sorghum juice [17–23].

In this study, the utilisation of glycerol as the sole carbon source for the production of value-added lipids and carotenoids by *Thraustochytrium* sp. is documented for strain AMCQS5-5 (a newly isolated strain from the Queenscliff region, Victoria, Australia). The effects and optimum levels of glycerol concentration and C/N ratio were determined for optimal production of carotenoids and maximum biomass and lipid content.

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2. Materials and methods

2.1. Chemicals

The chemicals used in this study were of analytical and HPLC grade. The other medium components such as glycerol, yeast extract and mycological peptone (Sigma–Aldrich, USA) and sea salt (Instant Ocean, USA) were used for biomass production while solvents such as acetone, dimethyl sulphoxide (DMSO), ether, hexane (from Merck), methanol and ethyl acetate (HPLC grade from Fischer and Honeywell, Australia) were used for carotenoid extraction and HPLC analysis for carotenoid identification and quantification. Carotenoid standards (astaxanthin, zeaxanthin, canthaxanthin, res/meso-astaxanthin, beta-cryptoxanthin, echinenone) were procured from CaroteNature, Switzerland while β-carotene was procured from Sigma–Aldrich, Australia.

2.2. Strain selection and biomass production

Thraustochytrid isolates designated as AMCQS5-5 (Genbank accession number JX993841), AMCQS5-3 (Genbank accession number JX993839), and AMCQS1-9 (Genbank accession number JX993843) were isolated from the Queenscliff region, Victoria, Australia. The isolates maintained on GYP agar plates were selected for this study based on colour exhibiting ability. These isolates were identified as thraustochytrids based on the colony morphology, appearance and reproduction pattern (i.e. sporangium formation and zoospore production) and molecular identification (Gupta et al., unpublished). In addition, *Schizochytrium* S31 (ATCC 20888) was procured from American Type Culture Collection (ATCC) and used as standard culture. The isolates used in this study were maintained on GYP medium consisting ($g\,L^{-1}$): glucose 5, yeast extract 2, mycological peptone 2, agar 10 and artificial seawater 50% at 25 °C and sub-cultured after 15 days. The thraustochytrid isolates were cultivated in a medium containing ($g\,L^{-1}$): yeast extract 2, peptone 2 and artificial seawater 50% for inoculum preparation with shaking at 150 rpm for 2 days at 25 °C. The medium was autoclaved at 121 °C for 20 min. Seed medium (0.2% yeast extract, 0.2% mycological peptone, ASW 50%, pH 6.5) and production medium (1% yeast extract, 0.1% mycological peptone, ASW 50%, pH 6.5) was autoclaved followed by the addition of syringe filtered (0.2 μm) 0.5% glucose and 1% glucose, respectively. Glucose and glycerol (1%) were used as carbon source in the production medium. Seed medium (50 mL) was inoculated from agar plates and grown for 2 days in a shake flask at 25 °C, at 150 rpm. Inoculum (5%, v/v) was used to inoculate 95 mL production medium and cultured for 7 days in a shake flask at 25 °C, and 150 rpm. The resultant biomass was harvested by centrifugation (10,000 × *g*, 15 min) and freeze-dried until further use. Some of the in-house isolates exhibited orange colour and were considered for carotenoid extraction.

2.3. Growth at different glycerol and glucose concentrations

Different concentrations of glycerol and glucose (0.5%, 1%, 2%, 4%, 6%, 8% and 10%) were used in the fermentation medium to evaluate their effect on fatty acid production. The fermentation was performed at 150 rpm and 25 °C for 7 days. The biomass growth was monitored by measuring the optical density (OD) at 600 nm after every 24 h.

2.4. Fatty acid production and cell dry weight

To determine the fatty acid production, the culture was harvested at the end of 7 days and centrifuged at 10,000 × *g* for 10 min to obtain the pellet. The cell pellet was freeze-dried and stored at −20 °C before proceeding with fatty acid extraction. The cell dry weight (CDW) was estimated after freeze drying the thraustochytrid cells. Results are presented as mean ± SD of duplicates repeated twice.

2.5. Fatty acid extraction, esterification and GC analysis

Fatty acid extraction was performed according to Gupta and co-workers [4,11] with some modifications. 10 mg of freeze-dried cells were taken in centrifuge tubes for lipid extraction. The fatty acids were extracted with solvent mixture containing a 2:1 ratio of chloroform to methanol. The upper layer was removed and dried over nitrogen gas. Lipid content (% dry wt basis) was determined gravimetrically. For FAMEs, 1 mL toluene was added to the tube followed by the addition of 200 μL of internal standard, methyl nonadecanoate (C19:0) and 200 μL of butylated hydroxytoluene (BHT). Acidic methanol (2 mL) was also added to the tube and kept for overnight incubation at 50 °C. Fatty acid methyl esters (FAMEs) were extracted into hexane. The hexane layer was removed and dried over sodium sulphate. FAMEs were concentrated using nitrogen gas. The samples were analysed by a GC-FID system (Agilent Technologies, 6890N, US). The GC was equipped with a capillary column (Supelcowax 10, 30 m × 0.25 mm, 0.25 μm thickness). Helium was used as the carrier gas at a flow rate of 1.5 mL min^{-1}. The injector was maintained at 250 °C and a sample volume of 1 μL was injected. Fatty acids peaks were identified on comparison of retention time data with external standards (Sigma–Aldrich, Australia). Peaks were quantified with Chemstation chromatography software (Agilent Technologies, US). Results are presented as mean ± SD of duplicates repeated twice.

2.6. Carotenoid extraction

Vortexing was applied for extraction of carotenoids from freeze-dried biomass. But later on, a slight modification of carotenoid extraction method from algae and fungi as reported in the literature was followed [15,16]. To 25 mg of freeze-dried biomass, 1 mL of DMSO (preheated at 55 °C) was added and kept at 55 °C for 60 min undisturbed followed by centrifugation at 4000 rpm for 15 min at 15 °C. Supernatant was taken and stored at 15 °C in dark. This cycle was repeated 3–4 times until the biomass becomes colourless. To extract carotenoids from DMSO solution, ether:water (1:1) solvent system was used in 1:2 ratio (DMSO:solvent system). This solvent mixture was centrifuged at 4000 rpm for 15 min at 15 °C and kept at −20 °C for 10 min. The upper un-freeze solvent layer was transferred in fresh centrifuge tube while bottom freeze DMSO layer was discarded. The upper layer was washed twice with water to remove traces of DMSO. Solvent was evaporated under nitrogen stream and equivalent volume of acetone was added and stored at −20 °C. Final extract volume should be noted down for total carotenoid estimation. Carotenoids were identified and quantified by RP-HPLC analysis.

2.7. HPLC analysis of carotenoids

The method for carotenoid analysis was adopted from Armenta and co-workers [16]. The Agilent 1200 Series HPLC system with the Agilent 1200 Series photodiode array detector was used for carotenoid analysis. Carotenoids were analysed at 477 nm using 5 μm Luna C18 reversed-phase column, 4.6 mm × 250 mm (Phenomenex, USA), and a Security guard column C18, 3.0 mm × 4.0 mm (Phenomenex, USA). This column was equilibrated with mobile phase A consisting of methanol, ethyl acetate and water (88:10:2, v/v/v) in a gradient mode at a flow rate 0.75 mL min^{-1}. This flow rate was maintained 10 min. Mobile phase composition was changed to 2:50:48 (mobile phase B) between 10 and 30 min and the flow rate was adjusted to 1.5 mL min^{-1}. This stage was maintained for a

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