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Improved production of docosahexaenoic acid in batch fermentation by newly-isolated strains of *Schizochytrium* sp. and *Thraustochytriidae* sp. through bioprocess optimization

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ABSTRACT

Thraustochytrids, rich in docosahexaenoic acid (DHA, C22:6ω3), represent a potential source of dietary fatty acids. Yet, the effect of culture conditions on growth and fatty acid composition vary widely among different thraustochytrid strains. Two different thraustochytrid strains, *Schizochytrium* sp. PKU#Mn4 and *Thraustochytriidae* sp. PKU#Mn16 were studied for their growth and DHA production characteristics under various culture conditions. Although they exhibited similar fatty acid profiles, PKU#Mn4 seemed a good candidate for industrial DHA fermentation while PKU#Mn16 displayed growth tolerance to a wide range of process conditions. Relative DHA content of 48.5% and 49.2% (relative to total fatty acids), respectively, were achieved on glycerol under their optimal flask culture conditions. Maximum DHA yield ($Y_{p/x}$) of 21.0% and 18.9% and productivity of 27.6 mg/L-h and 31.9 mg/L-h were obtained, respectively, in 5-L bioreactor fermentation operated with optimal conditions and dual oxygen control strategy. A 3.4- and 2.8-fold improvement of DHA production (g/L), respectively, was achieved in this study. Overall, our study provides the potential of two thraustochytrid strains and their culture conditions for efficient production of DHA-rich oil.

1. Introduction

Thraustochytrids are unicellular marine and fungus-like heterotrophic protists within the clade Stramenopiles [1], several strains of which are currently recognized for their high potential to produce polyunsaturated fatty acids (PUFAs) [2], and some of them have also been adopted for commercial production of an omega-3 PUFA – docosahexaenoic acid (C22:6, DHA) [3]. As one of the major structural lipids, DHA can be found as a key constituent of phospholipids, triacylglycerols, and free fatty acids in vertebrate animals [4]. Because of its myriad of benefits to human health [5], DHA has received substantial attention as an important functional food component with high demand on the global market [4,6]. Although fish oil is currently the major source of DHA, its supply is limited by the growing concerns over food security, the health of marine fish stocks, ecological disturbances from industrial fishing, and toxic impurities (PCBs, mercury, and dioxins) of some fish oils [6–8]. Of the single-celled microorganisms, thraustochytrids are advantageous because of their fast heterotrophic growth, high DHA content, ability to produce toxins-free oils, and suitability in commercial-scale fermentation [4,9].

DHA production by thraustochytrids, especially with some genera including *Schizochytrium*, *Thraustochytrium*, *Aurantiochytrium*, *Thraustochytriidae*, has been greatly improved in the last few decades [10–14]. Several attempts have been made to optimize the growth conditions and media components for improving DHA production in strains that belong to these genera [12,15–21]. These attempts highlight optimization of fermentation conditions as the single most effective means to improve the production of DHA and suggest optimal culture conditions can vary significantly among interspecific and/or intraspecific strains. In addition, growth

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characteristics and lipid profiles are known to vary widely across different thraustochytrid strains isolated from a biogeographic range of habitats (e.g., mangroves, sub-tropical to temperate region coastal waters etc.) [22–24]. For example, the strains from the genera *Aurantiochytrium*, *Schizochytrium*, *Thraustochytrium*, and *Ulkenia* showed different glucose utilization abilities and when grown on glycerol they yielded a different fatty acid composition of their lipids [25].

To date, only 13 genera of thraustochytrids are known with about 30 described species [26]; thus, our current understanding of the genetic and metabolic diversity of thraustochytrids is rudimentary. As one of the important marine bioresource, the isolation and characterization of better strains are now of prime importance that will expand our knowledge of their physiological responses to fermentation conditions. Nevertheless, much research is still needed to identify robust and efficient thraustochytrid strains for more economical commercial production of DHA. Because DHA production depends on several factors and their interactions including growth phase, culture mode, process conditions (e.g., pH, temperature, aeration etc.), and nutrition (e.g., carbon, nitrogen, phosphorous, medium supplements, etc.), it is thus essential that for each thraustochytrid strain, culture conditions are screened individually in order to derive the optimal process parameters that maximize their DHA yield.

In the present study, two thraustochytrid strains belonging to genera *Schizochytrium* and *Thraustochytriidae*, previously isolated from Shenzhen coastal waters of China [24], with potential for DHA production were investigated in detail. The aim of this study was to elucidate the distinct characteristics of these strains underlying the accumulation of DHA and determine the extrinsic (nutritional and environmental) factors that regulate their DHA yields. We conducted a comprehensive investigation to ascertain how extrinsic factors (carbon and nitrogen sources, the concentration of substrates, salinity, KH_2PO_4, pH, temperature, agitation speed) influence cell biomass, total fatty acids (TFAs) and its composition, and DHA production. In addition, fed-batch fermentation in a 5-L bioreactor using the optimal culture conditions with dual oxygen supply strategy was conducted to validate the flask culture results.

2. Materials and methods

2.1. Microorganism

Schizochytrium sp. PKU#Mn4 (CGMCC 8091) and *Thraustochytriidae* sp. PKU#Mn16 (CGMCC 8095) used in this study were previously isolated from mangrove of coastal waters in Shenzhen province [24]. These two strains were maintained at 28 °C on modified Vishniac's (MV) medium (Vishniac, 1956) (glucose·H_2O 10 g/L, peptone 1.5 g/L, yeast extract 0.1 g/L, 100% artificial seawater (ASW) and agar 20 g/L) and subcultured every 25 days. The growth and PUFAs production profiles of the two strains prior to optimization are shown in Fig. S1.

2.2. Optimization of culture conditions

Nine growth parameters (carbon and nitrogen sources, temperature, pH, salinity, KH_2PO_4, agitation speed, carbon and nitrogen concentrations) were tested to evaluate their individual effect on the biomass and DHA production of PKU#Mn4 and PKU#Mn16. The carbon sources included glucose, glycerol, fructose, mannose, glucose-acetic acid, glucose-ethanol, glucose-citric acid, glucose-malic acid, glucose-potassium acetate, glycerol-citric acid, glycerol-malic acid, and glycerol-potassium acetate. The nitrogen sources were sodium glutamate, tryptone, peptone, yeast extract, peptone-yeast extract, ammonium sulfate, ammonium nitrate, and sodium nitrate. The range of best carbon source concentration was 5 g/L to 100 g/L while nitrogen concentrations were varied from 0.25 g/L to 10 g/L. The M4 medium (Jain et al., 2005) was used as the seed medium which contains glucose·H_2O 20 g/L, peptone 1.5 g/L, yeast extract 1 g/L, KH_2PO_4 0.25 g/L and 100% ASW. The fermentation medium employed in the one-factor-at-a-time (OFAT) experiments was designed by varying the M4 medium. The optimal carbon concentration was studied with the best carbon source (glycerol) and the optimal nitrogen concentration with the best carbon source (glycerol) and nitrogen source (yeast extract). The experimental conditions are provided in Table S1.

For all experimental sets, the seed medium was incubated at 28 °C and 150 rpm. The seed inoculum was centrifuged and washed twice with sterile artificial seawater prior to culturing in 100 mL Erlenmeyer flasks containing 50 mL M4 medium for 96 h. All the experiments were conducted in triplicates.

2.3. Flask culture fermentation under optimal conditions

Flask culture batch fermentation of the two strains followed by the OFAT experiments was performed to assess the growth, DHA production, and fatty acid composition of PKU#Mn4 and PKU#Mn16 under their optimal conditions. Flask culture was performed in 500 mL Erlenmeyer flasks containing 300 mL optimized medium for 96 h at 28 °C.

2.4. Bioreactor (5 L) performance

For each of the two strains, fed-batch fermentation was performed in a 5 L fermenter (Model: SY-9000-V9, Shanghai Dong Ming Industrial Co. Ltd.) with working volume of 3 L at 28 °C for 8 days. The fermenter was equipped with DO and pH electrodes, temperature sensor, impeller, and air pump. The agitation speed was altered to maintain a dissolved oxygen (DO) level at 50% of saturation from day 0 to day 2 and then at 10% until the end of fermentation. A 24 h grown seed culture (300 mL) in the M4 medium was transferred to fermentation broth to make the final volume to 3 L. The initial fermentation medium was composed of 0.25 g/L KH_2PO_4 and 40% artificial seawater. The initial carbon and nitrogen for each strain were similar to their optimal medium composition. On each day 2 and 3, 100 mL feed medium accounting for 1/3 of the optimal glycerol and yeast-extract concentrations for DHA production was added to the 3 L culture broth. The culture broth was harvested at a regular interval of 24 h to determine the dry cell weight (DCW) and lipid production.

2.5. Analytical methods

At the end of each experiment, 15 mL broth was used to determine the DCW by gravimetric method. Thraustochytrid cells were harvested by centrifuging at 12,000 rpm for 5 min, washed twice with sterile distilled water, and then lyophilized for 48 h with a freeze-drying system (Christ, USA). The freeze-dried cells were stored at −20 °C for subsequent processing. Glycerol concentration was measured using a kit based on the enzymatic determination in a liquid sample (Applygen Technologies). Fatty acid methyl esters were prepared using the direct transesterification method [27]. Freeze dried biomass (50–100 mg), 100 μL of 1 mg/mL internal standard (nonadecanoic acid, C19:0) solution, and 2 mL of 4% sulfuric acid in methanol were placed into 5 mL screw-top glass tubes. The acid catalyzed trans-esterification started at 80 °C water bath for 1 h. One mL water and 1 mL hexane were added to the mixtures at room temperature, vortexed and centrifuged at 4000 rpm for 2 min. The fatty acid methyl esters (FAME) in the hexane layer were collected and analyzed by a gas chromatography Agilent 7890B

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