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Large-Scale Production of Microalgal Lipids Containing High Levels of Docosahexaenoic Acid upon Fermentation of *Aurantiochytrium* sp. KRS101

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ABSTRACT

In this study, large-scale production of microalgal lipid containing high levels of docosahexaenoic acid (DHA) by fermentation of *Aurantiochytrium* sp. KRS101 was performed. The microalgal strain yielded productivity of docosahexaenoic acid (DHA) productivity of 1.08 and 1.6 g/L/d by fermentation at 300-L and 5000-L scale stirrer-type bioreactor. The productivity was significantly enhanced upto 5.6 g/L/d by fermentation at 6000-L scale airlift-type bioreactor, probably due to the reduced shearing force. The microalgal lipid could be efficiently recovered by safe extraction methods such as ethanol extraction, hot water extraction or supercritical fluid extraction, promising commercial potential of the microalgal DHA-rich lipid in the food and feed industry.

Keywords: *Aurantiochytrium* sp.; Heterotrophic Microalga; Large-Scale Cultivation; Lipid; Docosahexaenoic Acid

1. Introduction

Oleaginous microalga of the Thraustochytrid family may be valuable sources of oil because at least 30% of dry biomass is lipid, and the content of omega-3 polyunsaturated fatty acids (PuFA) is high [1]. This is especially true of docosahexaenoic acid (DHA, C22:6n-3) which plays key roles in development of the brain and eye [2,3]. Additionally, the high levels of desirable fatty acids render microbial oil valuable as a feedstock for production of biofuel.

Many oleaginous microalgal strains synthesizing high levels of PuFAs have been described [4]. Perveen *et al.* [5] characterized a Thraustochytrid-like microorganism (termed Strain 12B) that exhibited DHA productivity of 2.8 g/L/d upon shake flask cultivation. The highest DHA productivity (3.7 g/L/d) reported to date was attained upon fermentation of *Schizochytrium limacinum* SR21 in a laboratory scale bioreactor [6]. Large-scale fermentation of microalgal strain has also been reported; *Schizochytrium* sp. HX-308 yielded DHA productivity values of 2.7 and 2.9 g/L/d when grown in 50-L and 1500-L bioreactor, respectively [7].

Recently, we isolated and identified a novel Thraustochytrid microalga (termed *Aurantiochytrium* sp. KRS101) that produced high levels of lipid and DHA (over 45% total fatty acids) [8]. Upon fed-batch fermentation in a 5-L bioreactor, good biomass levels and lipid production values (50 and 22 g/L, respectively) were obtained; DHA productivity (3.3 g/L/d) comparable to that of *S. limacinum* SR21 [6]. To evaluate the comercial potential of such fermentation, large-scale growth of the microalgal strain was studied and the results are described in the present report. Also, it is worth extraction methods of fatty acid from fermentative sources in especially industrial fields. Considering both the economic feasibility in industry and the safety of food additives, the relevant interest for this molecule and its biological activity, and starting from the consideration that better methods for the characterization and determination of fatty acid are needed, it is to propose a proper method for

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the extraction and characterization of fatty acid from fermentative microalgae product.

2. Materials and Methods

2.1. Microalgal Strain and Media

Details of the Thraustochytrid microalgal strain *Aurantiochytrium* sp. KRS101 was previously reported [8]. The microalga was cultivated in 5-L baffled flasks containing 50 mL of basal medium [glucose (food grade), 60 g/L; yeast extract (food grade), 10 g/L; dried natural sea salt (CJ Co., Korea), 10 g/L] at 28˚C, with shaking at 120 rpm, for 3 d.

2.2. Fermentation of *Aurantiochytrium* sp. KRS101

Cells were precultured in 5 L amounts of basal medium, with shaking at 120 rpm, for 3 d at 28˚C. Precultures were transferred at 2.5% (v/v) of fermenter medium volume into 300-L bioreactor, or (in later work) into 5000-L stirrer-type and 6000-L airlift-type bioreactors. Culture aliquots were collected every 12 h for analysis of growth via measurement of absorbance at 600 nm. The fermentation conditions in the 300-L bioreactor were 28˚C, stirring at 50 rpm, 0.5 v/v/min of air, and pH 7.0.

2.3. Lipid Extraction

For ethanol extraction, cell paste was sonicated for 5 min (each pulse was 5 s in duration followed by 5 s on ice on 300 W, 20 kHz) and added ethanol to final 50%. After centrifugation at 10,000 g for 5 min, the ethanol phase containing lipid was collected by rotary evaporation than analyzed fatty acid and its composition. For hot water extraction, cell paste was autoclaved at 121˚C for 30 m and the liquid phase was collected than analyzed further process. The supercritical fluid (SCF) extraction was performed on 300 bar, 60˚C and 180 m. Three independent experiments at least of all extraction process were made for each end-point.

2.4. Dry Cell Weight Analysis

Dry cell weight (DCW) was estimated by harvesting cells at 4500 g at 4˚C for 20 m. Each supernatant was discarded and each pellet washed three times with phosphate-buffered saline (PBS, pH 7.2). Resuspended cells were again harvested by centrifugation at 4500 g at 4˚C for 20 min. Each pellet was resuspended in 600 µL distilled water and transferred to a pre-weighed vial. Cell pellets were dried at 60˚C for 12 h using a speed vacuum concentrator (Biotron 4080C). Each vial was weighed and DCW value estimated.

2.5. Lipid Analysis

Total lipid content was calculated using a modified (miniaturized) Bligh-Dyer method as described by Burja *et al.* [9]. Dried cells (125 mg amounts) were placed in screw-cap test tubes, and 6.25 mL chloroform, 12.5 mL methanol, and 5 ml 50 mM K_2HPO_4 buffer (pH 7.4) were added to each tube. Samples were agitated for 1 h, shaking at 200 rpm, at 28˚C. Each sample was next transferred to a 50 mL graduated tube, and 6.25 mL chloroform and 6.25 mL phosphate buffer were added. Each tube was inverted 30 times and the phases allowed to separate for 1 h before recovery of the bottom layer (approximately 12.5 mL). This liquid was transferred to a preweighed aluminum dish and the solvent was evaporated over 30 m in a dry oven to 80˚C. After cooling, the dish and contents were weighed, and total lipid levels were determined gravimetrically (to yield the weight of lipid extracted) using the following equation:

$$\text{Total lipid (g of oil per 100 gsample)} = \left[\left(W_L - W_D\right)\times V_C \times 100\right] / \left[V_P \times W_S\right]$$

where W_D was the weight of an empty aluminum dish (g); W_L the weight of an aluminum dish with dried lipid residue (g); W_S the weight of sample (g); V_C the total volume of chloroform in the graduated cylinder (mL); and V_P the volume of chloroform transferred to the aluminum dish (mL).

2.6. Analysis of Fatty Acid Composition

Dried cells were resuspended in 3 mL 4% (v/v) methanolic sulfuric acid and heated at 90˚C for 1 h in sealed vials. Fatty acid methyl esters (FAMEs) were extracted into 0.3 mL hexane and analyzed via gas chromatography (GC; Hewlett Packard 6890 N; Ramsey, MN); the instrument was equipped with a flame-ionization detector (FID) and an HP-5 (30 m × 0.32 mm; 0.25 mm; Agilent Technologies; Santa Clara, CA). The column temperature was raised from 150˚C (after 2 min of holding) to 270˚C (with a further 2 m of holding) at a rate of 7˚C per min.

3. Results and Discussion

3.1. Production of Microalgal Lipid by Cultivation of *Aurantiochytrium* sp. KRS101 in sTirrer-Type Bioreactor

The oleaginous microalga *Aurantiochytrium* sp. KRS101 was first cultivated in a 300-L stirrer-type bioreactor in a working volume of 150 L of medium. As shown in **Figure 1**, glucose was completely consumed after 60 h of cultivation, yielding 11.8 g/L of DCW. The level of lipid in the cells was 4.9 g/L, indicating that conversion of glucose to lipid was rather low (7.3%, g/g). However, the

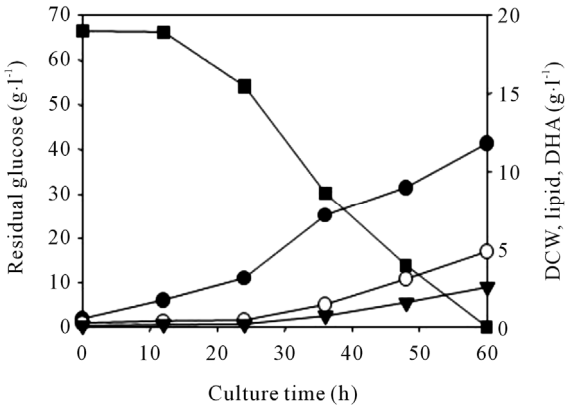

Figure 1. Cultivation of *Aurantiochytrium* sp. KRS101 in a 500-L bioreactor. Closed circles, dry cell weights; open circles, lipid contents; closed triangles, DHA contents; closed squares, residual glucose levels.

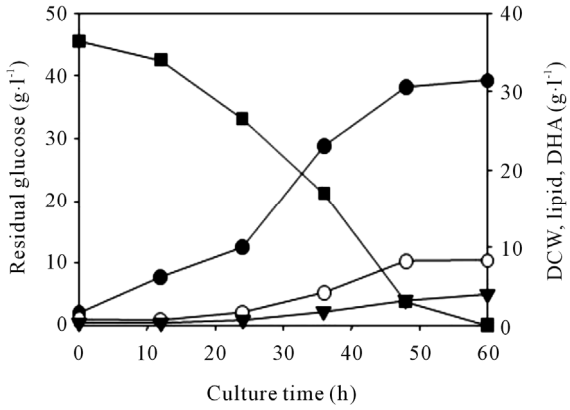

Figure 2. Cultivation of *Aurantiochytrium* sp. KRS101 in a 5000-L stirrer-type bioreactor. Closed circles, dry cell weights; open circles, lipid contents; closed triangles, DHA contents; closed squares, residual glucose levels.

DHA level in the microalgal lipid was extremely high (over 50% of total fatty acids). The DHA productivity level was 1.08 g/L/d.

Next, to determine whether the microalgal lipid could be produced in pilot-scale fermentation, the microalgal strain was cultivated in a 3000 L volume in a 5000-L scale stirrer-type bioreactor. The strain grew well upon large-scale fermentation, yielding a DCW of 30.5 g/L after 48 h of cultivation (**Figure 2**). The lipid level in cells was 8.2 g/L; the conversion rate of glucose to lipid was 19.6% (g/g). The DHA level and DHA productivity were 39.5% and 1.6 g/L/d, respectively (**Table 1**).

The DHA productivity values obtained in stirrer-type bioreactor, 1.08 and 1.6 g/L/d at 300-L and 5000-L scale bioreactors, respectively, were much less than seen upon laboratory scale fermentation in a 5-L stirrer-type bioreactor (3.3 g/L/d); the latter value was comparable to the highest level (3.7 g/L/d) of DHA productivity yet reported (upon fermentation of *S. limacinum* SR21) [6].

3.2. Pilot-Scale Production of Microalgal Lipid Using a 6000-L Airlift-Type Bioreactor

To examine whether the microalgal lipid production was affected by bioreactor type, an airlift-type bioreactor was employed. Notably, when the microalgal strain was cultivated in a 6000-L scale airlift-type bioreactor (3000 L working volume), cell growth (μ; 0.090/h) and glucose consumption rate (qs; 0.115/h) were much higher than the values obtained upon fermentation in stirrer-type vessels (0.057/h and 0.064/h, respectively) (**Figure 3**). And the rate of lipid accumulation, and the final level were two-fold higher upon airlift-type fermentation (qp; 0.060/h and 16.3 g/L, respectively) than was the case when stirrer-type vessels were employed (qp; 0.039/h and 8.2 g/L, respectively). Subsequently, the final DHA

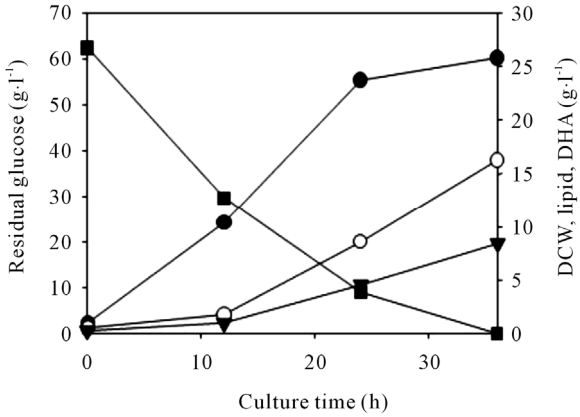

Figure 3. Cultivation of *Aurantiochytrium* sp. KRS101 in a 6000-L airlift-type bioreactor. Closed circles, dry cell weights; open circles, lipid contents; closed triangles, DHA contents; closed squares, residual glucose levels.

Table 1. Comparison of lipid production parameters upon large-scale cultivation of *Aurantiochytrium* sp. KRS101 in different types of bioreactors.

	Stirrer-type (5000-L scale)	Airlift-type (6000-L scale)
Lipid conversion rate ($g \cdot g^{-1}$ glucose)	0.196	0.262
Lipid productivity ($g \cdot L^{-1} \cdot day^{-1}$)	4.08	10.8
DHA content (% total fatty acids)	39.5	52.3

level obtained was higher upon airlift-type fermentation (**Table 1**). As the result, the DHA productivity was significantly elevated in the airlift-type bioreactor fermentation upto 5.6 g/L/d, which could be due to the decrease of shearing force in the airlift type.

When the commercial strain *S. limacinum* SR21 was employed to this end, the maximum DHA productivity

noted was 13.1 g/L/d upon fed-batch fermentation (in a volume of 14,000 gallons), after optimization of aeration [10]. Therefore, it may be expected that DHA productivity of *Aurantiochytrium* sp. KRS101 will be enhanced upon optimization of fermentation conditions.

3.3. Extraction of Microalgal Lipid Containing High Levels of Docosahexaenoic Acid

Four extraction methods were compared to recover DHA-rich microalgal lipid, *i.e.* the typical chloroform-methanol extraction method of Bligh-Dyer [9], ethanol extraction method with sonication, supercritical carbon dioxide extraction method [11] and hot water extraction method. The highest levels of lipid and docosahexaenoic acid was recovered from microalgal cells by chloroform-methanol extraction method recovered from microalgal cells were 58.7% ± 1.2% of DCW and 47.4% ± 1.1% of TFA by chloroform-methanol extraction method (**Table 2**). Aspect of the applications [12] of the microalgal lipid in food and feed industries, more safe extraction methods would be preferred. Ethanol extraction after ultrasonication gave approximately 90% extraction yield compared to that of the chloroform-methanol extraction method (**Table 2**). Whereas, supercritical carbon dioxide extraction method and hot water extraction methods resulted in much less 68% and 62% recovery yield compared to that of the chloroform-methanol extraction method. The composition of fatty acid in the lipids recovered was similar from four extraction methods (**Table 2**).

4. Conclusion

In this study, large-scale production of microalgal lipid containing high levels of DHA was successfully performed using 6000-L airlift-type bioreactor. The maximal productivity of DHA in the experiment reached at 5.6 g/L/d, which, to the best of our knowledge, is the highest productivity value yet reported. The microalgal lipid was effectively recovered by safe extraction methods applicable for food and feed industry. Thus, the microalgal lipid would serve as a valuable source of PuFAs.

Table 2. Comparison of lipid recovery by different lipid extraction method with pilot-scale fermentative microalgae product of *Aurantiochytrium* sp. KRS101.

	Oil recovery of DCW (%)	DHA content of TFA (%)
Chloroform-methanol extraction	58.7 ± 1.2	47.4 ± 1.1
Ethanol extraction with sonication	53.2 ± 1.1	43.5 ± 1.2
Supercritical carbon dioxide extraction	40 ± 1.2	41 ± 1.1
Hot water extraction	36.3 ± 1.7	44 ± 0.9

Data are expressed as means ± SD. Three independent experiments were made for each end-point.

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