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Production of Lipids Containing High Levels of Docosahexaenoic Acid by a Newly Isolated Microalga, *Aurantiochytrium* sp. KRS101

Won-Kyung Hong · Dina Rairakhwada · Pil-Soo Seo · Sung-Yong Park · Byung-Ki Hur · Chul Ho Kim · Jeong-Woo Seo

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Abstract In the present study, a novel oleaginous Thraustochytrid containing a high content of docosahexaenoic acid (DHA) was isolated from a mangrove ecosystem in Malaysia. The strain identified as an *Aurantiochytrium* sp. by 18S rRNA sequencing and named KRS101 used various carbon and nitrogen sources, indicating metabolic versatility. Optimal culture conditions, thus maximizing cell growth, and high levels of lipid and DHA production, were attained using glucose (60 gl^{-1}) as carbon source, corn steep solid (10 gl^{-1}) as nitrogen source, and sea salt (15 gl^{-1}). The highest biomass, lipid, and DHA production of KRS101 upon fed-batch fermentation were 50.2 gl^{-1} (16.7 gl^{-1} day^{-1}), 21.8 gl^{-1} (44% DCW), and 8.8 gl^{-1} (40% TFA), respectively. Similar values were obtained when a cheap substrate like molasses, rather than glucose, was used as the carbon source (DCW of 52.44 gl^{-1}, lipid and DHA levels of 20.2 and 8.83 gl^{-1}, respectively), indicating that production of microbial oils containing high levels of DHA can be produced economically when the novel strain is used.

Keywords *Aurantiochytrium* sp. · Heterotrophic microalga · Lipid · Docosahexaenoic acid

Introduction

Single cell oils have attracted intense research interest and it is known that many microorganisms, including algae, yeast, bacteria, and fungi can accumulate oils under particular culture conditions [1–3]. Compared with the use of plant oils in biodiesel, microbial oils offer many advantages: microorganisms have a short life cycle; can be easily handled as compared to plants; are less affected by location, season, and climate than

Won-Kyung Hong and Dina Rairakhwada are co-first authors and contributed equally.

W.-K. Hong · D. Rairakhwada · P.-S. Seo · S.-Y. Park · B.-K. Hur · C. H. Kim · J.-W. Seo (✉)
Microbe-based Fusion Technology Research Center, Jeonbuk Branch Institute, KRIBB, Jeongeup, Jeonbuk 580–185, South Korea
e-mail: jwseo@kribb.re.kr

B.-K. Hur
Department of Biological Engineering, Inha University, Incheon 402–751, South Korea

plants; and oil production is amenable to scale-up [4]. Among the heterotrophic microorganisms studied, yeast has the particular advantage of a fast growth rate and a high oil content. Some yeast strains, such as *Rhodosporidium* sp., *Rhodotorula* sp., and *Lipomyces* sp. can accumulate intracellular lipids to levels as high as 70% of biomass dry weight. Most such lipids are triacylglycerol (TAG)-containing long-chain fatty acids; the lipids are thus similar in structure to vegetable oils [5–7]. The microalga *Chlorella protothecoides* grows to a high cell density (15.5 $g l^{-1}$) under heterotrophic culture conditions, and oil content is also high (>50% of dry weight) [8–10]. Apart from algae and yeasts, fungi also synthesize oils under particular culture conditions. The mold *Mortierella isabellina* accumulates lipids to 50% of biomass dry weight [11]. As with fungi, some bacteria also biosynthesize oils under certain growth conditions. Mona et al. found that *Gordonia* sp. and *Rhodococcus opacus* could accumulate oils under special culture conditions to achieve a maximum oil content of 80% dry weight, but biomass was only 1.88 $g l^{-1}$ [8, 12].

Although microbial oils may be especially valuable, considering the rapid expansion of the biodiesel industry, commercialization is hindered by the fact that microbial oils are more expensive to produce than vegetable oils because heterotrophic growth substrates are costly. Members of the Thraustochytrid heterotrophic microalgal family may be especially valuable in this regard because at least 50% of the dry biomass is lipid, with a high content of omega-3 polyunsaturated fatty acids (PuFAs) [7]. PuFAs including arachidonic acid (AA, C20:4*n*-6), eicosapentaenoic acid (EPA, C20:5*n*-3), and docosahexaenoic acid (DHA, C22:6*n*-3) are critical components of the glycolipids and phospholipids that form plasma membranes [13]. PuFAs also act as precursors of certain hormones and signaling molecules such as eicosanoids [14]. Additionally, PuFAs are accepted to be useful in prevention and treatment of heart disease, high blood pressure, inflammation, and certain forms of cancer [15, 16]. Co-production of valuable fatty acids will decrease the cost of biodiesel. Here, we describe the isolation and identification of a novel Thraustochytrid microalga producing high levels of lipid, including DHA. Various nutrient sources supporting heterotrophic growth were investigated, and high production levels of lipid and DHA were obtained in 5-l fermentation experiments, either batch or fed-batch.

Materials and Methods

Isolation of Thraustochytrid Microalgal Strains

Thraustochytrid strains were obtained from soil, leaf, and pneumatophore samples of a Malaysian mangrove ecosystem. Screening was performed by adding 10 ml of physiological saline to each sample and serially diluting the supernatant before plating on B1 medium-containing agar plates (yeast extract, 1 $g l^{-1}$; peptone, 1 $g l^{-1}$; and agar, 10 $g l^{-1}$; in 1 l natural seawater containing penicillin G, 300 mg l^{-1}, and streptomycin sulfate, 500 mg l^{-1}) [17]. Microalgae were enriched by inoculation of samples into 50 ml B1 medium followed by incubation at 28°C with shaking at 200 rpm for 3 days, followed by serial dilution of culture broth and plating on B1 agar. After 2–4 days of incubation at 28°C, numerous colonies appeared on the plates. Single, irregular, hyaline colonies were picked and subcultured on B1 plates to obtain axenic cultures. Each of such colony was observed by light microscopy. Single colonies of the isolates were cultured in 15 ml amounts of marine broth (Sigma-Aldrich, St Louis, MO) or basal medium [glucose, 60 $g l^{-1}$; yeast extract, 1 $g l^{-1}$; artificial sea salt (Sigma-Aldrich), 6 $g l^{-1}$], at 28°C with shaking at 120 rpm,

for 3 days. Next, 1 ml amounts of these cultures were inoculated into 50 ml aliquots of the same medium, and incubated at 28°C with shaking at 120 rpm for 3 days. Cells were harvested for characterization of fatty acid composition, estimation of dry cell weight, and measurement of lipid content.

Cloning and Sequencing of the 18S rRNA Gene

Genomic DNAs of selected strains were extracted using a Genomic DNA extraction kit (Promega, Madison, WI), according to the manufacturer's protocol. DNA containing the 18S rRNA gene was amplified using the forward primer P1 (5′-ATGAACATCAAAAA-3′) and the reverse primer P2 (5′-ATGAACATCAAAAA-3′). Each 50-μl PCR reaction mixture contained 5 μl 10× PCR buffer, 20 mM of each of the deoxyribonucleotide triphosphates, 0.5 μM of each primer, 2 U of *Taq* polymerase (Takara), and 1 μg genomic DNA. The PCR protocol consisted of 1 min of denaturation at 94°C, followed by 30 cycles each composed of 0.5 min at 94°C, 0.5 min at 55°C, and 1.5 min at 72°C. The final extension step was performed over 7 min at 72°C. PCR products were visualized by agarose gel electrophoresis and purification of products following band excision was achieved using the Wizard SV clean-up system (Promega). Purified PCR products were cloned into the pGEM-T Easy vector (Promega), and the nucleotide sequences of both strands were obtained using primers P1 and P2 and other primers that bound to internal regions of the DNA template. A Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 3100 Genetic Analyzer were used to determine the sequence. The nucleotide sequences of 18S rRNA genes from related microorganisms were downloaded from a DNA databank (DDBJ; http://www.ddbj.nig.ac.jp). The 18S rRNA sequence of KRS101 has been deposited in GenBank under Accession No. HM126528 and was manually aligned with representative 18S rRNA sequences from Thraustochytrid strains and related taxa, based on similarities in secondary structure identified by the ClustalW program, version 1.83 [18]. A phylogenetic tree was inferred using the neighbor-joining method of MEGA version 4. Kimura's two-parameter model was used for calculation of the distance matrix [19] after nucleotide positions containing gaps or imprecise data had been completely deleted. The tree was rooted using *Alteromonas macleodii* (GenBank Accession No. X82145). A total of 1,260 sites on 18S rRNA were used in phylogenetic analysis. Tree reliability was evaluated by bootstrap analysis of 1,000 replicates.

Optimization of Growth Medium

KRS101, the strain showing the highest level of DHA production, the greatest dry cell weight, and the highest lipid content among all isolates tested, was chosen for further study. The effect of various concentrations of carbon, nitrogen, and sea salt on cell growth, lipid content, and DHA production were examined by batch culture in 50 ml of basal medium in 250-ml Erlenmeyer flasks, shaken at 120 rpm for 3 days at 28°C. Cells precultured in basal medium were inoculated into a modified basal medium containing each of glucose, yeast extract, and artificial sea salt at 2.5% (*w*/*v*), and cells were grown with 120 rpm of shaking for 3 days at 28°C.

Various carbon sources, including D-fructose, D-arabinose, D-xylose, lactose, maltose, sucrose, glycerol, or crude glycerol (ECO Solutions, Korea), were added to the basal medium to final concentrations of 6% (*w*/*v*), in place of glucose, to investigate whether such substrates were effectively utilized. The effect of several nitrogen sources (corn steep solid,

beef extract, malt extract, peptone, and tryptone) at a concentration of 1% was also studied to identify the optimal nitrogen source. The effects of varying concentrations of sea salt were also studied using the optimized medium with glucose and corn steep solid as carbon and nitrogen source, respectively, ranging artificial sea salt concentration of 2–50 gl^{-1} (Sigma).

Optimization of Culture Conditions

To examine the effect of inoculum characteristics on cell growth, different amounts (0.1, 1.0, 2.0, and 3.0 g) of precultured cells at various growth stages (exponential, early-stationary, mid-stationary, and late-stationary) were used as inocula.

To examine the effects of various growth parameters, *Aurantiochytrium* sp. KRS101 was cultivated at different aeration rates (0.5, 1.0, 2.0, and 3.0 vvm), pH values (pH 5.0, 6.0, 7.0, and 8.0), shaking speeds (100, 200, and 300 rpm), and temperatures (25°C, 28°C, 30°C, and 37°C).

Fermentation by KRS101

Large-scale batch fermentation for DHA production was conducted using a 5-l stirred fermentor (Kobiotech, Korea) containing 2 l of fermentation medium. Cells were pre-cultured in 300 ml basal medium, with shaking at 120 rpm for 3 days at 28°C. This pre-culture was added at a concentration of 2.5% (*v*/*v*) of fermentor medium volume. The fermentation medium contained glucose 60 gl^{-1}, corn steep solid 5 gl^{-1}, ammonium acetate 3 gl^{-1}, KH_2PO_4 3 gl^{-1}, and artificial sea salt 6 gl^{-1}. As a cheap nutrient source, cane molasses purchased from a local company was tested by substituting glucose. Fermentation conditions were 28°C, 200 rpm of stirring, 1 *v*/*v*/min of air, and pH 7.0. In fed-batch fermentation, glucose or threefold diluted crude cane molasses was fed into the fermentor to maintain the total reduced sugar concentration between 30 and 60 gl^{-1}. Aliquots of culture (50 ml) were collected every 12 h for analysis of dry cell weight (DCW), lipid content, DHA level, and the amounts of total reducing sugars.

Dry Cell Weight Analysis

DCW was estimated by harvesting cells at 4,500×*g* at 4°C for 20 min. The supernatant was discarded, and the pellet was washed three times with phosphate-buffered saline (PBS, pH 7.2). Resuspended cells were harvested by centrifugation at 4,500×*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 4080 C). Vials were weighed and DCW values estimated.

Lipid Analysis

Total lipid content was calculated using a modified miniaturized Bligh-Dyer method as described by Chi et al. [20]. Dried cells (125 mg) 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 solution (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 liquid was next allowed to settle for 1 h before recovery of the bottom layer (approximately 12.5 ml). This

organic layer was transferred to a pre-weighed aluminum dish and the solvent was evaporated over 30 min in a dry oven set at 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:

$$\text{Total lipid}\left(\text{g of oil } 100\ \text{g}^{-1}\text{sample}\right) = [(W_L - W_D) \times V_C \times 100]/[V_P \times W_S]$$

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

Analysis of Fatty Acid Composition

Dried cells were resuspended in 3 ml of 5% (*v*/*v*) methanolic sulfuric acid and heated at 90°C for 1 h in sealed vials. Fatty acid methyl esters (FAMEs) were extracted into 0.6 ml hexane and analyzed by gas chromatography (GC; Hewlett Packard 6890N) using an instrument equipped with a flame-ionization detector (FID) and an HP-5 (30 m×0.32 mm, 0.25 mm; Agilent Technologies) capillary column. The column temperature was raised from 150°C (after 2 min of holding) to 270°C (with a further 2 min of holding) at a rate of 7°C per min.

Determination of Concentration of Glucose and Reducing Sugars

Total reducing sugars concentration in culture broth was determined using the 3,5-dinitrosalicyclic acid method [21].

Results and Discussion

Isolation and Identification of *Aurantiochytrium* sp. KRS101

Samples obtained from a mangrove ecosystem were screened for Thraustochytrid microalgae. Single, irregular, hyaline colonies composed of spherical or limaciform cells, thus dissimilar to either yeast or bacterial cells, were picked and subcultured at least three times on B1 plates to ensure strain purity. Thirty purified colonies isolated on solidified B1 medium were microscopically observed. Selection of oleaginous strains was based on the criteria of Lewis et al. [22]; the DCWs of selected microbes were at least 20% lipid, the fatty acid profile was favorable, biomass production was high, and both total fatty acids (TFAs) and DHA were produced to good levels. In the present study, biomass (DCW) ranged from 4.235 to 10.282 g l^{-1} in the 30 strains examined, and the lipid (percent DCW) and DHA (percent of TFA) contents ranged from 13.6% to 81.9% DCW and 49% to 55% TFA, respectively. Among the tested strains, isolate PN28 was studied further because this strain showed the highest levels of DCW and lipid content and an acceptably increased DHA concentration.

Almost the entire 18S rRNA gene sequence of PN28 was determined and has been deposited under GenBank Accession No. HM126528. Comparison of the 18S rRNA gene sequence of PN28 with sequences (deposited in GenBank) of related microalgae positioned the organism as a member of the Thraustochytridae. Using the neighbor-joining tree constructed in the present work, we observed that PN28 was grouped with strains in the genus *Aurantiochytrium*, thus forming a tight cluster with *Aurantiochytrium* spp., with a high bootstrap value. Furthermore,

PN28 was confirmed to be an *Aurantiochytrium* strain based on zoospore morphology and formation of limaciform amoeboid cells. In the 18S rRNA phylogenetic tree (Fig. 1), strain PN28 was located in a well-supported monophyletic group including the *Aurantiochytrium* type strain, and we thus renamed PN28 as *Aurantiochytrium* sp. KRS101.

Medium Optimization for Cell Growth and Lipid Production by *Aurantiochytrium* sp. KRS101

Carbon Source

Various carbon sources were used to investigate the growth rates and lipid and DHA contents of *Aurantiochytrium* sp. KRS101, using basal medium as optimized above (Fig. 2a). When fructose was used as a carbon source, maximal DCW levels were obtained, but lipid content was optimal when glucose was used. The DHA content ranged from 37% to 52% of TFA when various carbon sources were tested. Yokochi et al. [10] also found that glycerol was an efficient carbon source; in a study involving optimization of DHA production by *A. limacinum* SR21, the cited authors noted that the highest DCW

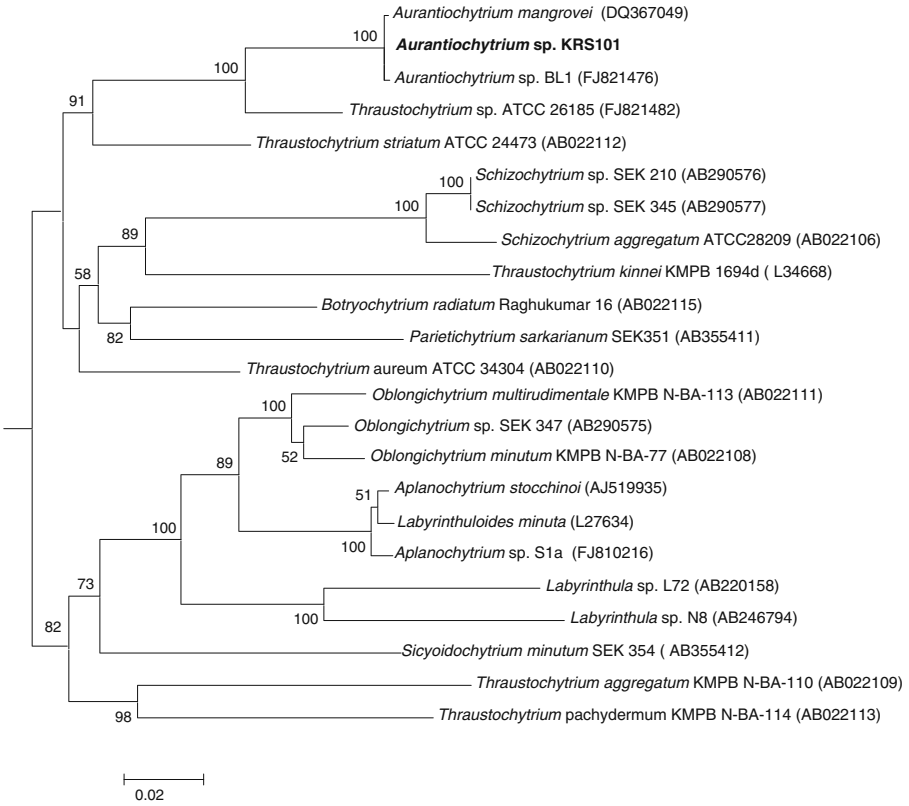

Fig. 1 A phylogenetic tree estimated using 18S rRNA sequences positions the KRS101 isolate. The 18S rRNA sequence of *Aurantiochytrium* sp. KRS101 has been deposited in GenBank under accession no. HM126528

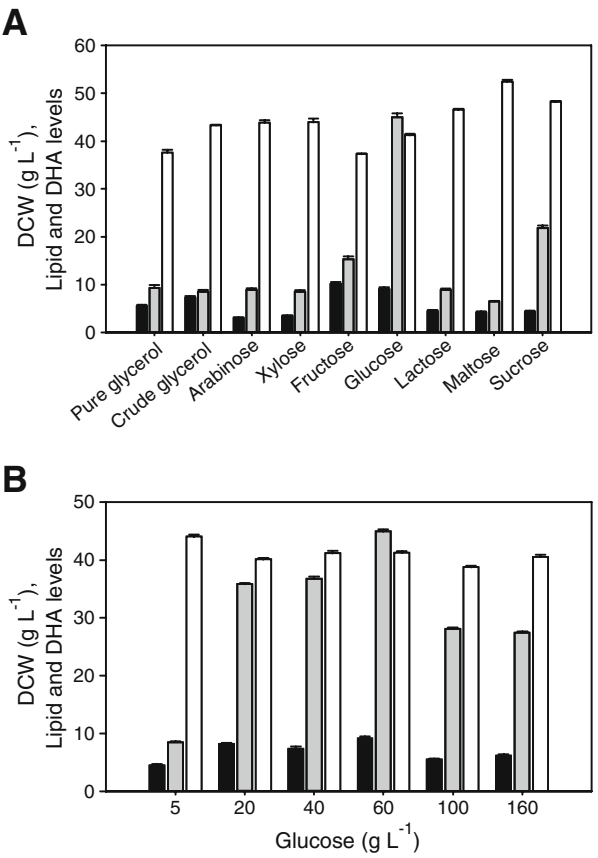

Fig. 2 Effects of various carbon sources (**a**) and glucose concentration (**b**) on cell growth, and production levels of lipid and DHA, of *Aurantiochytrium* sp. KRS101. Dry cell weight (DCW), *closed bar*; lipid level (percent DCW), *gray bar*; DHA level (percent TFA), *open bar*

was obtained when pure glycerol was used as carbon source. The ability to use crude glycerol is an advantage of strain KRS101. The isolate used crude glycerol more efficiently than pure glycerol, yielding a better DCW.

The optimal concentration of glucose for growth and lipid production by *Aurantiochytrium* sp. KRS101 was examined. As shown in Fig. 2b, DCW increased as glucose concentration increased up to 60 gl^{-1}. A further increase in glucose concentration affected cell growth to some extent, but DCW values remained unchanged until glucose attained 160 gl^{-1}, a level known to inhibit the growth of many other DHA-producing strains such as *Crypthecodinium cohnii* ATCC30722 [23]. These results are similar to the glucose utilization data obtained in previous work using the strain *Aurantiochytrium limacinum* SR21, for which the maximum DCW was obtained when glucose concentration was 90 g l^{-1} [10]. The highest lipid content was obtained at 60 gl^{-1} glucose. Hence, other nutrient concentrations were optimized at this glucose level.

Nitrogen Source

The medium nitrogen level plays a critical role in the production of lipids and DHA. As shown in Fig. 3a, the maximum DCW was obtained when peptone, tryptone, or yeast extract was

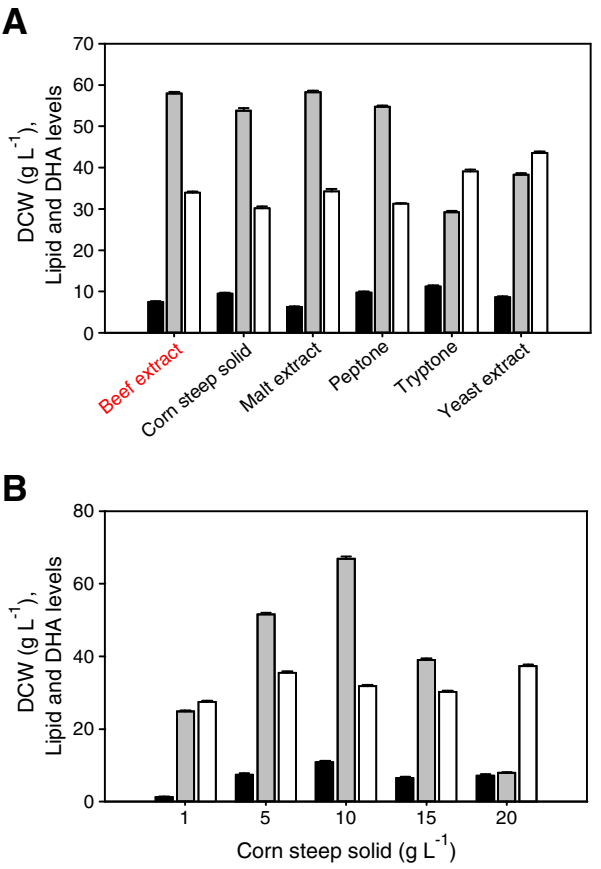

Fig. 3 Effects of various nitrogen sources (**a**) and corn steep solid concentration (**b**) on cell growth and production levels of lipid and DHA of *Aurantiochytrium* sp. KRS101. Dry cell weight (DCW), *closed bar*; lipid level (percent DCW), *gray bar*; DHA level (percent TFA), *open bar*

used. However, higher levels of lipid and DHA were obtained when beef extract, corn steep solid, malt extract, or peptone, was used. Corn steep solid is a cheap nitrogen source used in industrial processes, and we thus selected this material as the nitrogen source for heterotrophic growth of *Aurantiochytrium* sp. KRS101.

The effects of corn steep solid concentration on DCW, and lipid and DHA contents are shown in Fig. 3b. DCW increased as corn steep solid concentration increased up to 10 gl^{-1}. A further increase in corn steep solid concentration affected cell growth and lipid level, but not DHA content. It has been known that a high C/N ratio can stimulate accumulation of fatty acids by algal cells [17] and thus a corn steep solid concentration of 5 gl^{-1} was selected to balance high DCW with increased lipid and DHA contents.

Sea Salt Concentration

The effects of salt concentration on DCW and lipid and DHA contents were examined in the range of 2–50 gl^{-1} artificial sea salt. Growth of *Aurantiochytrium* sp. KRS101 did not vary over a wide range of salt levels. The salinity tolerance of the strain thus differed from that of *Thraustochytrium aureum*, the growth of which was completely inhibited at 0% or

200% salinity [24]. As shown in Fig. 4, DCW did not change substantially over the entire tested range of sea salt concentrations. Similar results were obtained by Yokochi et al. [10], who found that DCW remained constant upon microalgal growth in levels of sea salt more than 50% that of sea water. In the cited study, lipid content decreased markedly with a further increase in sea salt concentration. *Aurantiochytrium* strain BL10 also accumulated higher levels of fatty acids when grown at lower salinities [25]. In the cited report, DHA content also decreased with increasing concentrations of sea salt. Similar results were obtained by Perveen et al. [26], who found that the maximal production of DHA was obtained at a sea salt level 50% that of sea water. Hence, to have balanced DCW, lipid content, and DHA level, 6 gl^{-1} of sea salt was optimal.

Optimization of Fermentation Conditions for Cell Growth and Lipid Production by *Aurantiochytrium* sp. KRS101

Inoculum Status and Amount

Aurantiochytrium sp. KRS101 cells showed a growth profile typical of Thraustochytrid microalgae during liquid cultivation [27]. The cells propagated into dozens of spores, in a sporangium, during early stages of growth and zoospores were liberated at later growth stages. We examined the effects of growth status and amount of inoculum on microalgal cell growth. Cells from different preculture stages, thus at early-logarithmic, early-stationary, mid-stationary, and late-stationary phases were used as inocula. As shown in Fig. 5, the maximum level of cell growth was obtained using inocula from late growth stages. No effect of inoculum amount was apparent. These results indicated that cells from early preculture stages should be used as inocula.

Other Growth Parameters

We also examined the effect of other growth parameters on fermentation by *Aurantiochytrium* sp. KRS101. Thus, we evaluated aeration rate (0.5, 1.0, 2.0, and 3.0 vvm), pH (pH 5.0, 6.0, 7.0, and 8.0), shaking speed (100, 200, and 300 rpm), and temperature (25°C, 28°C, 30°C, and 37°C). The best conditions for cell growth and lipid production by *Aurantiochytrium* sp. KRS101 were achieved when aeration rate was 1.0 vvm, pH was 7.0, shaking speed was 200 rpm, and temperature was 28°C.

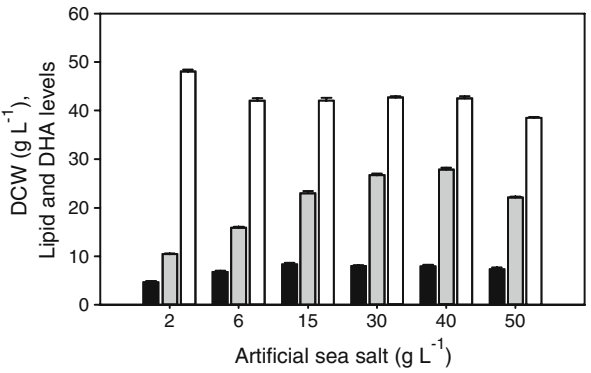

Fig. 4 Effect of sea salt concentration on cell growth and production levels of lipid and DHA of *Aurantiochytrium* sp. KRS101. Dry cell weight (DCW), *closed bar*; lipid level (percent DCW), *gray bar*; DHA level (percent TFA), *open bar*

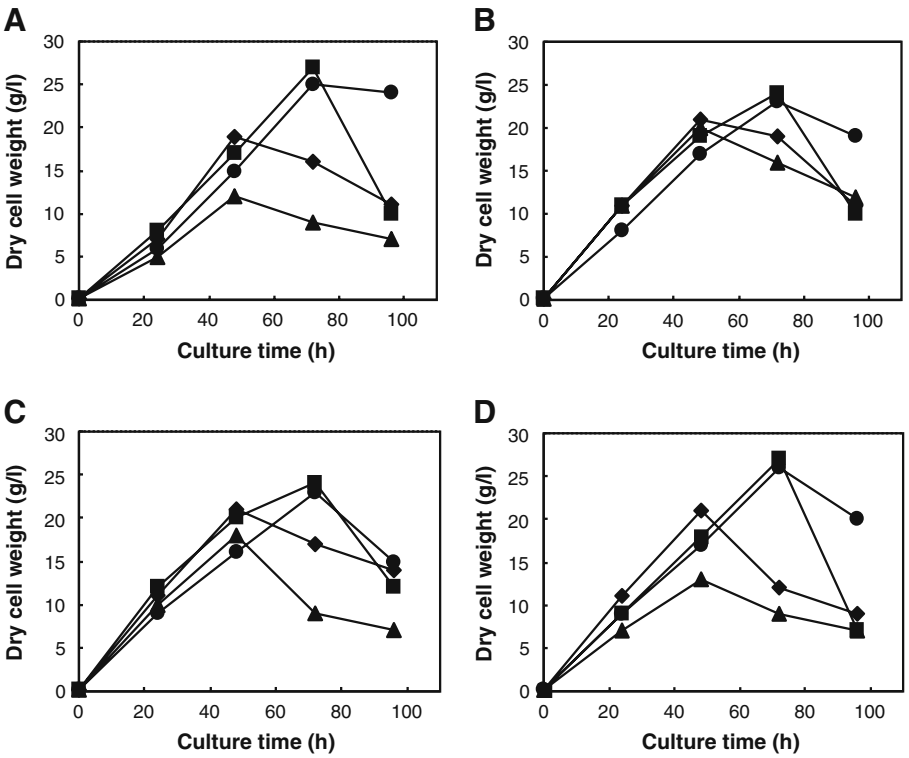

Fig. 5 Effect of inoculum status (*circles*, exponential stage; *squares*, early-stationary; *diamonds*, mid-stationary; *triangles*, late-stationary) and amount (**a** 0.1 g; **b** 1.0 g; **c** 2.0 g; **d** 3.0 g) on cell growth and production levels of lipid and DHA of *Aurantiochytrium* sp. KRS101

Fermentation by *Aurantiochytrium* sp. KRS101 in a Bioreactor

Batch Fermentation

Using the medium and culture conditions described above, the growth of *Aurantiochytrium* sp. KRS101, and glucose consumption, lipid production, and DHA synthesis were examined during batch cultivation (Fig. 6). Glucose was utilized mainly for cell growth. DCW increased gradually up to 72 h, until all glucose was consumed. KRS101 showed very low lipid levels at 12 h; Lu-Jing et al. [28] previously described this stage as a time of early lipid accumulation. However, lipid content increased markedly at 24 h and reached a maximum shortly thereafter. Theoretically, depletion of medium nitrogen should divert excess carbon into lipid biosynthesis. However, the observed kinetics of lipid accumulation did not support this contention. Rather, only a minimal reduction in lipid level was observed at 36–60 h and, upon further incubation, the lipid level remained at 31.2% DCW, similar to that seen in the 24-h sample. All glucose in the medium was consumed by 72 h of fermentation, during which time DCW gradually increased to a maximum of 24.8 gl^{-1}, but DHA content was similar at all stages of fermentation. The final level of DHA production after 72 h of incubation was 2.84 gl^{-1}.

It was apparent from the batch experiment that the strain remained in the exponential phase of growth throughout fermentation, despite approaching stationary phase near the end

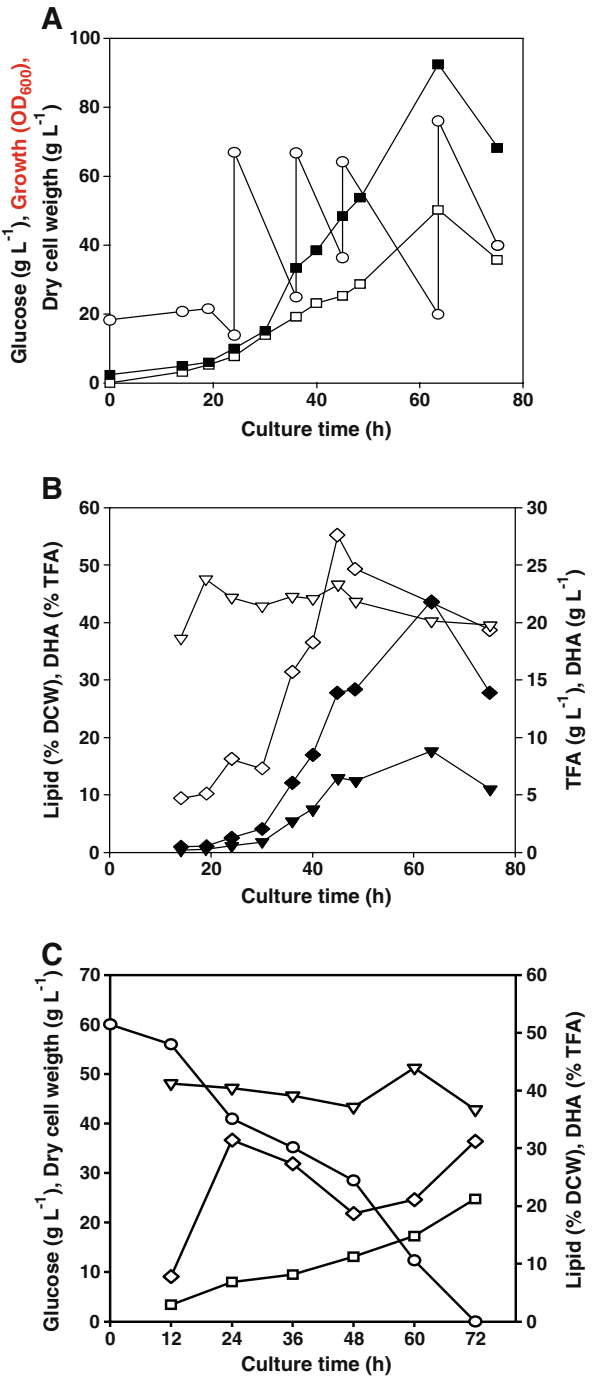

Fig. 6 Batch (**a**) and fed-batch (**b**, **c**) fermentation of *Aurantiochytrium* sp. KRS101. Residual glucose, *open circles*; cell growth, *closed squares*; dry cell weight, *open squares*; lipid content, *open* (percent DCW) and *closed* (grams per liter) *diamonds*; DHA content, *open* (percent TFA) and *closed* (grams per liter) *triangles*

of the process; hence, it seemed that further addition of glucose might enhance final DCW. Therefore, a fed-batch experiment was conducted to increase DCW, a variable of great commercial importance if strain KRS101 is to be used in production of DHA.

Fed-batch Fermentation

The time course of biomass production, residual glucose concentration, total fatty acid level, and lipid and DHA contents, in a fed-batch culture over 96 h, are shown in Fig. 6. The maximum DCW obtained was 50.2 gl^{-1} (16.7 gl^{-1} day^{-1}) and lipid accumulated to form 43.5% of DCW (21.8 gl^{-1}) at 63.5 h. The increase in lipid content from 31.2% DCW upon batch fermentation to 43.5% DCW in the fed-batch experiment indicated that the fed-batch strategy was appropriate for cultivation of KRS101. However, the fatty acid profile of KRS101 grown over 96 h did not vary until after 80 h (40% TFA). A further increase in incubation period decreased DHA content. These production values of *Aurantiochytrium* sp. KRS101 are similar to those reported elsewhere [29–31].

Fermentation of *Aurantiochytrium* sp. KRS101 Using Molasses

Molasses is a cheap industrial substrate used in biological processes to produce valuable materials [32–34]. We thus examined whether molasses could be used for growth and lipid production by *Aurantiochytrium* sp. KRS101. The time courses of biomass production, concentration of glucose and reducing sugars, total fatty acid level, and lipid and DHA contents in a fed-batch culture over 96 h are shown in Fig. 7. The maximum DCW obtained was 52.44 gl^{-1} and lipid and DHA levels were 20.2 and 8.83 gl^{-1}, respectively.

Conclusion

In the present study, a novel oleaginous microalgal *Aurantiochytrium* sp. KRS101 was isolated. Optimal medium and culture conditions for maximal cell growth and lipid production by the strain were: glucose at 60 gl^{-1} as carbon source, corn steep solid at 10 g l^{-1} as nitrogen source, artificial sea salt at 15 gl^{-1}, aeration rate of 1.0 vvm, pH of 7.0, shaking speed of 200 rpm, temperature of 28°C, and inoculation using cells from early stages of preculture. The highest biomass and production levels of lipid and DHA were obtained upon fed-batch fermentation under optimal conditions [DCW of 50.2 gl^{-1} and

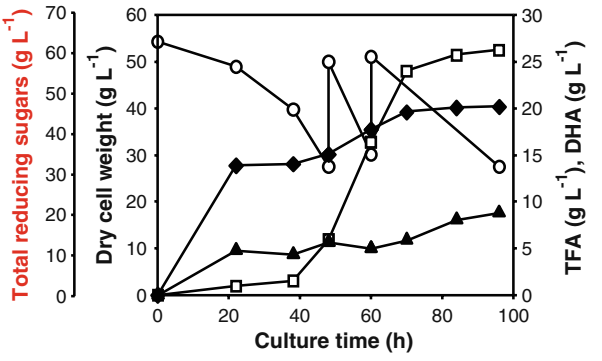

Fig. 7 Fed-batch fermentation of *Aurantiochytrium* sp. KRS101 using molasses as carbon source. Total reducing sugars, *open circles*; cell growth, *closed squares*; dry cell weight, *open squares*; lipid level, *closed diamonds*; DHA level, *closed triangles*

lipid and DHA levels of 43.5 (percent DCW) and 40 (percent TFA, respectively] in 63.5 h. Additionally, similar cell growth and lipid production were obtained when molasses instead of glucose was used as carbon source, indicating that microbial oils containing high levels of DHA may be economically produced using this novel microalgal strain.

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References

1. Beopoulos, A., Cescut, J., Haddouche, R., et al. (2009). *Prog Lipid Research, 48*, 375–387.
2. Economou, C. N., Makri, A., Aggelis, G., et al. (2010). *Bioresource Tech, 101*, 1385–1388.
3. Zhu, L. Y., Zong, M. H., & Wu, H. (2008). *Bioresource Tech, 99*, 7881–7885.
4. Li, Q., Du, W., & Liu, D. (2008). *Applied Microbiology and Biotechnology, 80*, 749–756.
5. Easterling, E. R., French, W. T., Hernandez, R., et al. (2009). *Bioresource Tech, 100*, 356–361.
6. Hu, C., Zhao, X., Zhao, J., et al. (2009). *Bioresource Tech, 100*, 4843–4847.
7. Li, Y., Zhao, Z., & Bai, F. (2007). *Enz Microbial Tech, 41*, 312–317.
8. Mona, K. G., Sanaa, H. O., & Linda, M. A. (2008). *World Journal of Microbiology & Biotechnology, 24*, 1703–1711.
9. Xu, H., Miao, X., & Wu, Q. (2006). *Journal of Biotechnology, 126*, 499–507.
10. Yokochi, T., Honda, D., Higashihara, T., et al. (1998). *Applied Microbiology and Biotechnology, 49*, 72–76.
11. Seraphim, P., Michael, K., & George, A. (2004). *Bioresource Tech, 95*, 287–291.
12. Gouda, M. K., Omar, S. H., & Aouad, L. M. (2008). *World Journal of Microbiology & Biotechnology, 24*, 1703–1711.
13. Lauritzen, L., Hansen, H. S., Jorgensen, M. H., et al. (2001). *Prog Lipid Research, 40*, 1–94.
14. Abbey, M., Clifton, P., Kestin, et al. (1990). *Arteriosclerosis, 10*, 85–94.
15. Angerer, P., & Schachy, C. V. (2000). *Curr Opin Clin Nutri Meta Care, 3*, 439–445.
16. Braden, L. M., & Caroll, K. K. (1986). *Lipids, 21*, 285–288.
17. Burja, A. M., Radianingtyas, H., Windust, A., et al. (2006). *Applied Microbiology and Biotechnology, 72*, 1161–1169.
18. Thompson, J. D., Higgins, D. G., & Gibson, T. J. (1994). *Nucleic Acids Research, 22*, 4673–4680.
19. Kimura, M. A. (1980). *Journal of Molecular Evolution, 16*, 111–120.
20. Chi, Z., Pyle, D., Wen, Z., et al. (2007). *Process Biochemistry, 42*, 1537–1545.
21. Millier, G. L. (1959). *Analytical Chemistry, 31*, 426–429.
22. Lewis, T., Nichols, P. D., & McMeekin, T. A. (2000). *J Microbiol Methods, 3*, 107–116.
23. De Swaaf, M. E., Sijtsma, L., & Pronk, J. T. (2003). *Biotech Bioeng, 81*, 666–672.
24. Iida, I., Nakahara, T., Yokochi, T., et al. (1996). *J Fermen Bioeng, 81*, 76–78.
25. Huey-Lang, Y., Lu, C. K., Chen, S. F., et al. (2010). *Marine Biotechnology, 12*, 173–185.
26. Perveen, Z., Ando, H., Ueno, A., et al. (2006). *Biotechnological Letters, 28*, 197–202.
27. Chi, Z., Liu, Y., Frear, Cl, et al. (2009). *Applied Microbiology and Biotechnology, 81*, 1141–1148.
28. Lu-Jing, R., Huang, H., Xiao, A. H., et al. (2009). *Bioprocess and Biosystems Engineering, 32*, 837–843.
29. Nagano, N., Taoka, Y., Honda, D., et al. (2009). *Journal of Oleo Science, 58*, 623–628.
30. Rosa, S. M., Soria, M. A., Velez, C. G., et al. (2010). *Bioresource Tech, 101*, 2367–2374.
31. Yang, H. L., Lu, C. K., Chen, S. F., et al. (2010). *Marine Biotechnology, 12*, 173–185.
32. Davery, A., & Pakshirajan, K. (2009). *Applied Biochemistry and Biotechnology, 158*, 663–674.
33. Jung, H. I., Lee, O. M., Jeong, J. H., et al. (2010). *Applied Biochemistry and Biotechnology, 162*, 486–497.
34. Zhu, L. Y., Zong, M. H., & Wu, H. (2008). *Bioresource Tech, 99*, 788–785.