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Screening and Characterization of Squalene-Producing Thraustochytrids from Hong Kong Mangroves

QIAN LI,[†,§,∥] GUAN-QUN CHEN,[†,∥] KING-WAI FAN,[#] FU-PING LU,[†,§] TSUNEHIRO AKI,[⊥] AND YUE JIANG*[,†]

[†]Department of Biology and Kwong Living Trust Food Safety and Analysis Laboratory, Hong Kong Baptist University, Kowloon Tong, Hong Kong, [§]Tianjin Key Laboratory of Industrial Microbiology, College of Biotechnology, Tianjin University of Science and Technology, 29 Thirteenth Avenue, TEDA, Tianjin 300457, China, [#]School of Biological Sciences, The University of Hong Kong, Pokfulam Road, Hong Kong, and [⊥]Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima 739-8530, Japan. [∥] These authors contributed equally.

Eighteen strains of thraustochytrids were newly isolated from Hong Kong mangroves, and their fatty acid and squalene contents were analyzed. All strains could grow well heterotrophically with glucose as the sole carbon source. All of them had the typical fatty acid profile of thraustchytrids and could produce a large amount of docosahexaenoic acid. The cell dry weight ranged from 5.49 to 15.62 g/L and squalene content from 0.02 to 0.18 mg/g at 72 h. The highest squalene-producing strain, BR-MP4-A1 was identified as a new strain of *Aurantiochytrium* species through sequence comparison of the 18S rRNA gene. The highest biomass of *Aurantiochytrium* sp. BR-MP4-A1 was achieved at 72 h, whereas its squalene content reached the maximum of 0.567 mg/g at 36 h but decreased rapidly thereafter. The production of squalene by thraustochytrids might be highly influenced by culture conditions.

KEYWORDS: Thraustochytrid; *Aurantiochytrium* sp.; squalene; phylogenetic identification

INTRODUCTION

Squalene, 2,6,10,15,19,23-hexamethyltetracosa-2,6,10,14,18,22-hexaene, is a dehydrotriterpenic hydrocarbon ($C_{30}H_{50}$) with six double bonds (**Figure 1**). It acts as a precursor to all steroids in plants and animals (*1*). Recent epidemiological studies have revealed that squalene is an effective inhibitor of chemically induced skin, lung, and colon tumorigenesis in rodents (*1*, *2*). Dietary squalene is able to stimulate nonspecific immune functions in a dose-dependent manner, influence cholesterol absorption, and reduce low-density lipoprotein (LDL) as well as triglyceride levels in individuals presenting hypercholesterolemia. A dose of 500 mg of squalene/day was suggested in normalizing lipid values for the nutritional health of human beings (*3*−*5*).

To date, the major commercial source of squalene is liver oils of deep-sea sharks and plant seeds (*6*, *7*). The future supply and availability of shark liver oils, however, are limited by the uncertainty of its availability as a result of international concern for the conservation of marine ecology (*8*) as well as other obstacles such as environmental organic pollutants and heavy metals (*9*, *10*). Squalene from plant seed oils, that is, olive oil (ca. 7 mg/g) (*5*) and *Amaranthus* seed oil (0−5.64 mg/g) (*8*) is not desirable for squalene production due to their own limitations, including the effects of locality and seasonal variation of crop productivity (*11*). There is the need, therefore, to secure a sustainable and safe source of squalene.

Microalgae are being widely prospected for the production of useful natural products such as squalene (*12*, *13*). Green microalga *Botryococcus braunii* has been reported to produce squalene under photoautotrophic culture (*14*). However, it is difficult to achieve high biomass concentration under photoautotrophic conditions due to light limitation problems. As from the industrial fermentation point of view, high cell density is one of the important factors considered to commercialize certain microbial production process (*15*). The microalgae that could grow heterotrophically can eliminate the light requirement completely and have the great possibility to increase cell density and productivity at commercial scale. Thraustochytrids are a group of microalgae widely distributed in mangrove areas, which can grow well heterotrophically. They are currently being used as the producers of polyunsaturated fatty acids, and some species of them possess relatively high contents of squalene, which could be purified easily by counter-current chromatography (*13*, *16*, *17*). With a better understanding of their squalene contents, the biomass of thraustochytrids will be a value-added product in food and feed industries, which will have a wider commercial application and increased economic influence. Therefore, an investigation of the squalene content of thraustochytrids is necessary to further explore this microalgal resource. In this study, the thraustochytrids were first isolated from Hong Kong mangroves. Their fatty acid compositions and squalene contents were analyzed, and the

*Author to whom correspondence should be addressed (telephone 852-3411-7062; fax 852-3411-5995; e-mail yjiang@hkbu.edu.hk).

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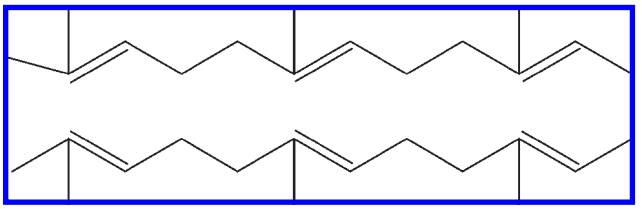

Figure 1. Chemical structure of squalene.

highest squalene-producing strain was further identified with phylogenic analysis. Changes of squalene content and the cell growth characteristics of the identified strain during different phases of cultivation were investigated subsequently. The results obtained from this research will provide a basic knowledge on squalene production in polyunsaturated fatty acid-producing thraustochytrids and will be useful to further identify the role of squalene in this algal cell.

MATERIALS AND METHODS

Isolation and Maintenance of Thraustochytrids. Fallen leaves (black and brown) of *Kandelia candel* (L.) Druce belonging to family Rhizophoraceae were randomly collected from mangrove areas in Sai Keng, Ting Kok, and Mai Po in Hong Kong (**Figure 2**). Thraustochytrids were isolated from leaf samples in the laboratory according to the method of Fan et al. (*18*). Thraustochytrid strains were maintained in agar plates consisting of (per liter) 20 g of agar, 30 g of glucose, 0.2 g of $(NH_4)_2SO_4$, 2 g of monosodium glutamate, 0.3 g of KH_2PO_4, 25 g of NaCl, 1 g of KCl, 5 g of $MgSO_4 \cdot 7H_2O$, 0.3 g of $CaCl_2 \cdot 2H_2O$, 0.1 g of $NaHCO_3$, 2 g of yeast extract, 30 mg of Na_2EDTA, 34.2 mg of H_3BO_3, 2.9 mg of $FeCl_3 \cdot 6H_2O$, 0.02 mg of $CuSO_4 \cdot 5H_2O$, 8.6 mg of $MnCl_2 \cdot 4H_2O$, 0.26 mg of $CoCl_2 \cdot 4H_2O$, and 0.6 mg of $ZnCl_2$ and subcultured monthly.

Heterotrophic Growth of Thraustochytrids. An inoculum was prepared in 250 mL Erlenmeyer flasks, each containing 50 mL of the above medium (pH 6.0, without agar), and was heterotrophically inoculated at 25 °C in an orbital shaker at 200 rpm. Erlenmeyer flasks (250 mL), each containing 100 mL of above medium (pH 6.0, without agar), were inoculated with 5% (v/v) of an exponentially growing inoculum and incubated at 25 °C in an orbital shaker at 200 rpm in the dark.

Determination of Cell Dry Weight, Specific Growth Rate, and Glucose Concentration. A 3 mL fermentation broth was sampled aseptically to determine the cell dry weight (*18*). Specific growth rate (μ) was determined by plotting the natural logarithm of dry weight concentration against time. Readings within the exponential phase were used to calculate the specific growth rate by linear regression according to the equation

$$\mu = (\ln D_2 - \ln D_1)/(t_2 - t_1)$$

where D_1 and D_2 are the cell dry weight concentrations at the cultivation times t_1 and t_2, respectively.

Residual glucose concentration in the medium was determined by using the 3,5-dinitrosalicylic acid method (*13*).

Fatty Acid Analysis. Fatty acid composition of the freeze-dried thraustochytrid cells was determined according to the method of Jiang et al. (*18*). Nonadecanoic acid (C19:0) was used as internal standard. The HP 6890 capillary gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a flame ionization detector and a 30 m × 0.25 mm i.d. Innowax capillary column (J&W Scientific, Folsom, CA) was used for fatty acid analysis. Nitrogen was used as carrier gas. The fatty acids were identified by chromatographic comparison with authentic standards (Sigma Chemical Co., St. Louis, MO), and the quantities were estimated from peak areas on the chromatogram with that of internal standard.

Squalene Analysis. Squalene was analyzed by HPLC according to the method of Lu et al. (*19*). The freeze-dried cells (100 mg) were saponified in 4 mL of 10% KOH (w/v)/75% ethanol (v/v) solution at 50 °C for 15 min and were extracted with hexane three times. The extract was dried under nitrogen gas and redissolved in 2.0 mL of acetonitrile. The prepared sample was well separated and determined on a 150 mm × 4.0 mm i.d., 5 μm, reversed-phase symmetry C18 column (Merck, Rahyway, NJ) with acetonitrile (100%) as mobile phase at the flow rate of 1.5 mL/min with UV detection at 195 nm. Squalene was identified by comparison with authentic standard (Sigma Chemical Co.).

Genomic DNA Extraction. Genomic DNA was extracted from a pure culture of isolate BR-MP4-A1 following the method reported by Innis et al. (*20*). Briefly, the cells were harvested and frozen in liquid nitrogen in the mortar. After being ground to a fine powder, the cell was transferred to a 1.5 mL microcentrifuge tube. Lysis buffer containing 50 mM Tris-HCl (pH 7.2), 50 mM ethylenediamine etraacetic acid (EDTA), 1% β-mercaptoethanol, and 3% sodium dodecyl sulfate (SDS) was added, vortexed, and incubated at 65 °C for 1 h. An equal volume of phenol/chloroform/isoamyl alcohol [25:24:1 (v/v)] (Sigma Chemical Co.) was then mixed with the solution. The mixture was centrifuged at 1000*g* for 15 min, and the supernatant was used for DNA precipitation with 3 M sodium acetate and isopropanol. The DNA pellet was washed with 70% ethanol and dissolved in Tris-EDTA (TE) buffer (10 mM Tris-HCl, 0.1 mM EDTA).

Cloning and Sequencing of the 18S rRNA Gene. A pair of specific primers (forward, 5′-TAC CTG GTT GAT CCT GCC AG; reverse, 5′-CCT TCC GCA GGT TCA CCT AC) was employed to amplify the DNA segment containing the 18S rRNA gene (*21*). PCR was performed in a mixture with total volume of 20 μL, containing 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.5 μM of each of the primers, 0.2 mM deoxyribonucleotide triphosphate (NTP) mixture, 20 ng of genomic DNA, and 1.5 units of *Taq* DNA polymerase. The PCR conditions were as follows: initial denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min, as well as a final extension at 72 °C for 10 min. The amplified product was separated on 1% agarose gel and detected by staining with ethidium bromide. The PCR product was purified by PCR Quick-spin PCR Product Purification Kit (iNtRON Biotechnology Inc., Korea) and sequenced in both directions using the dideoxynucleotide terminator method with an ABI 3730 DNA analyzer (Applied Biosystems, Foster City, CA) at the Genome Centre of The University of Hong Kong. The DNA sequence of *Aurantiochytrium* sp. BR-MP4-A1 was deposited with GenBank under accession no. FJ004948.

Phylogenetic Analysis. The DNA sequence of *Aurantiochytrium* sp. BR-MP4-A1 and other sequences (from GenBank) were aligned with the ClustalX program. Phylogenetic analyses were conducted using PAUP* version 4.0b10. Alignment gaps were treated as the fifth character. Maximum parsimony analysis trees were generated from 1000 heuristic searches of parsimony-informative characters. Max-trees set to 5000 branches of zero length were collapsed, and all parsimonious trees were saved. Branch support for all parsimony analyses was estimated by performing 1000 bootstrap replicates, where a heuristic search consisting of 10 random addition replicates was performed for each bootstrap replicate (*22*). Trees were figured in the Treeview program.

Statistical Analysis. All determinations were conducted in triplicate, and all results were expressed as mean ± standard deviation (SD). Differences between means of data were compared by least significant difference (LSD) at $p < 0.05$ calculated using the Statistical Analysis System (SAS Institute, Inc., Cary, NC).

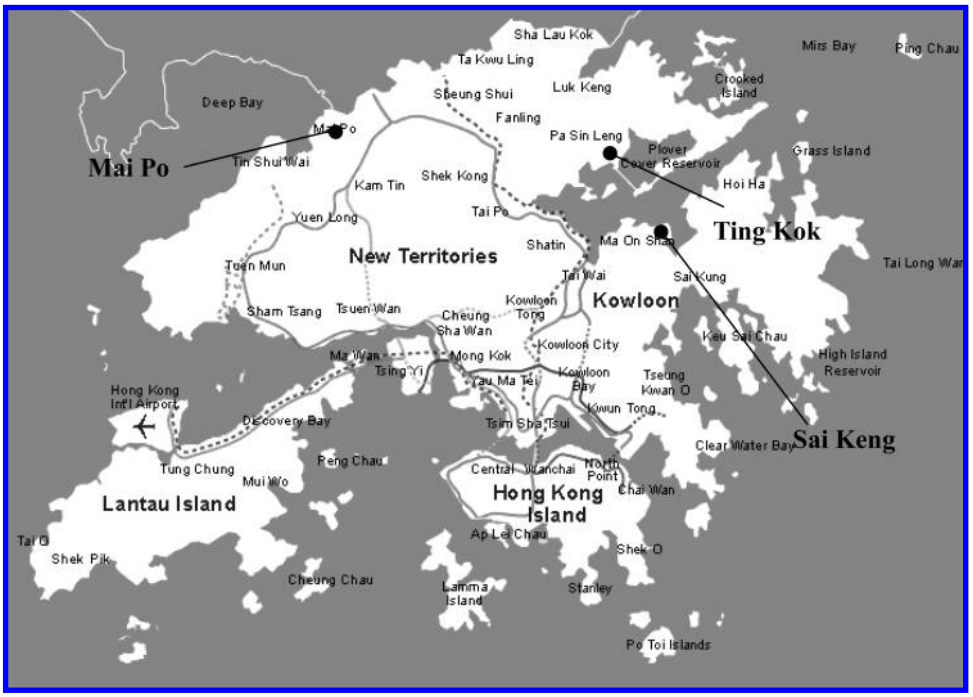

Figure 2. Isolation sites of thraustochytrids in Hong Kong.

RESULTS AND DISCUSSION

Fatty Acid Composition, Growth Characteristics, and Squalene Contents of the New Isolates. In Hong Kong, most mangrove stands are found in the Deep Bay region and Sai Kung district, which are located on the western and eastern sides of Hong Kong, respectively. In this study, two representative mangrove sites (Ting Kok and Sai Keng) on the eastern side of Hong Kong and one of the biggest well-preserved sites (Mai Po) on the western side were chosen for sample collection and isolation of thraustochytrids. Eighteen cultures were isolated and preliminarily identified as thraustochytrids according to the method of Yokoyama and Honda (*23*). All isolates were first cultivated for investigation of their heterotrophic growth characteristics, fatty acid composition, and squalene content before further phylogenetic identification.

Table 1 shows that all isolates had the typical fatty acid profile with docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA) as the major polyunsaturated fatty acids. The percentage of DHA of total fatty acids of the 18 isolates ranged from 12.9 to 31.9%. The growth characteristics of the isolates in submerged culture are shown in **Table 2**. After 72 h of cultivation, the biomass concentrations ranged from 5.49 (strain D-3-5) to 15.62 g/L (strain 4-P-J). The isolates showed great variation in glucose utilization ability. The squalene contents of these strains also varied significantly (**Table 2**). The highest squalene content of 0.18 mg/g was detected in strain BR-MP4-A1, which was higher than those of *Saccharomyces cerevisiae* (0.041 mg/g) and *Thraustochytrium* sp. ACEM 6063 (0.1 mg/g) (*12*, *24*) and 11% higher than that of *Schizochytrium mangrovei* FB1 (0.162 mg/g) (*13*) at the same culture condition. Strain BR-MP4-A1 was selected for the following experiment in this study due to its highest squalene yield at 72 h of all isolates (2.46 g/L) and relatively high DHA content (28.85%).

Phylogenetic Analysis of Strain BR-MP4-A1. The gene of 18S rRNA of strain BR-MP4-A1 was cloned, sequenced, and analyzed with 43 other gene sequences downloaded from GenBank. Maximum parsimony analysis (with gap treated as fifth character) generated four equally parsimonious trees. Branch support was estimated by bootstrap analysis and is shown in **Figure 3**. The phylogeny showed distinct lineages of nine thraustochytrid genera with excellent bootstrap support (100%). Among the genera under study, *Aurantiochytrium*, *Botryochytrium*, *Parietichytrium*, *Schizochytrium*, *Sicyoidochytrium*, and *Ulkenia* nested together as a closely related group with good branch support (76%). *Aplanochytrium*, *Labyrinthula*, and *Oblongichytrium* appeared as distinct genera from the main group without branch support. They were probably the basal lineage to the main group (*23*). Strain BR-MP4-A1 was clustered with other strains in the genus *Aurantiochytrium* (previously known as *Schizochytrium*) with excellent bootstrap support (100%) and, therefore, was confirmed as *Aurantiochytrium* sp. BR-MP4-A1.

In the food fermentation industry, it is possible to control and improve the quality, reproducibility, and safety of the products through the use of a defined starter culture. The microorganisms or microbially derived ingredients that belong to General Recognized As Safe (GRAS) substances have contributed largely to the ease of food safety control in the production process. *Aurantiochytrium* species have been used safely for the production of n-3 polyunsaturated fatty acids (*25*, *26*), which have been widely supplemented in infant formula for over a decade. Its whole cell has been widely added in aquaculture feeds to improve the survival rate and growth as well as nutritional value of the aquatic animals (*25*). *Aurantiochytrium* species, therefore, could be regarded as a safe potential producer of squalene as squalene and squalene-rich products are mainly applied in food and nutritional supplements, medical ingredients, and cosmetic materials. The sequence-based identification of isolate BR-MP4-A1 is thus a necessity to confirm its application potential.

Growth Characteristics of *Aurantiochytrium* Species BR-MP4-A1. *Aurantiochytrium* sp. BR-MP4-A1 grew rapidly, and the highest biomass concentration (14.57 g/L) was reached at 72 h (**Figure 4**). It has growth characteristics similar to those of the other thrautochytrids (*13*) in terms of its high specific growth rate (0.09/h) and the growth yield coefficient (0.534 g/g of glucose). The growth rate and biomass concentration of the current strain are much higher than those of another squalene-synthesizing microalga *Botryococcus braunii*. The highest biomass of *B. braunii* cultured under different temperatures was only 0.7 g/L after

Table 1. Fatty Acid Composition of 18 Thraustochytrid Isolates[a]

fatty acid	1	3	10	4-P-J	A1-D-1-1A	A1-D-2-2	BR-MP4-A1	D-3-2	D-3-3
C12:0	0.1 ± 0.03	0.1 ± 0.00	0.1 ± 0.00	0.1 ± 0.00	0.1 ± 0.00	0.1 ± 0.00	0.1 ± 0.01	0.1 ± 0.01	0.3 ± 0.00
C13:0	3.9 ± 0.31	10.4 ± 0.00	5.8 ± 0.39	4.6 ± 0.00	8.9 ± 0.11	8.7 ± 0.01	4.4 ± 0.01	3.1 ± 0.48	9.8 ± 0.30
C14:0	3.2 ± 0.36	3.1 ± 0.02	3.3 ± 0.01	3.6 ± 0.00	3.4 ± 0.04	3.6 ± 0.01	3.8 ± 0.04	4.4 ± 0.01	12.3 ± 0.08
C15:0	2.3 ± 0.16	2.1 ± 0.01	2.4 ± 0.01	2.4 ± 0.00	2.5 ± 0.03	1.8 ± 0.00	1.8 ± 0.03	1.3 ± 0.01	17.0 ± 0.13
C16:0	47.5 ± 0.23	41.7 ± 0.16	47.9 ± 0.15	46.2 ± 0.01	44.3 ± 0.40	46.2 ± 0.03	49.8 ± 0.88	52.6 ± 0.27	36.3 ± 0.16
C16:1	0.7 ± 0.01	0.5 ± 0.02	0.4 ± 0.00	0.4 ± 0.03	0.3 ± 0.00	0.3 ± 0.01	0.3 ± 0.00	0.2 ± 0.01	1.7 ± 2.24
C17:0	0.6 ± 0.02	0.7 ± 0.00	0.7 ± 0.01	0.7 ± 0.00	0.8 ± 0.01	0.6 ± 0.00	0.6 ± 0.01	0.4 ± 0.01	1.7 ± 2.26
C18:0	1.1 ± 0.05	1.6 ± 0.01	1.2 ± 0.01	1.5 ± 0.00	1.6 ± 0.00	1.6 ± 0.00	1.6 ± 0.02	1.5 ± 0.05	0.8 ± 0.07
C18:1	0.1 ± 0.00	0.5 ± 0.00	0.1 ± 0.00	0.3 ± 0.00	0.3 ± 0.00	0.1 ± 0.00	0.3 ± 0.01	0.1 ± 0.02	0.0 ± 0.00
C18:3	0.1 ± 0.00	0.4 ± 0.00	0.1 ± 0.01	0.2 ± 0.00	0.2 ± 0.00	0.1 ± 0.00	0.2 ± 0.01	0.1 ± 0.02	0.0 ± 0.00
C20:3	0.1 ± 0.01	0.2 ± 0.01	0.1 ± 0.00	0.2 ± 0.00	0.2 ± 0.00	0.2 ± 0.00	0.2 ± 0.00	0.1 ± 0.00	0.2 ± 0.00
C20:4	0.6 ± 0.02	0.7 ± 0.00	0.7 ± 0.00	0.8 ± 0.00	0.6 ± 0.00	0.6 ± 0.00	0.6 ± 0.04	0.5 ± 0.00	0.3 ± 0.01
C20:5	0.4 ± 0.01	0.6 ± 0.00	0.4 ± 0.00	0.6 ± 0.00	0.6 ± 0.02	0.5 ± 0.00	0.6 ± 0.00	0.5 ± 0.01	0.3 ± 0.00
C22:0	0.3 ± 0.06	0.3 ± 0.00	0.2 ± 0.00	0.3 ± 0.00	0.2 ± 0.00	0.1 ± 0.00	0.2 ± 0.00	0.3 ± 0.00	0.6 ± 0.02
C22:5(n-6)	7.2 ± 0.05	7.3 ± 0.20	7.2 ± 0.03	7.4 ± 0.01	7.1 ± 0.11	7.2 ± 0.01	6.6 ± 0.03	6.9 ± 0.08	6.0 ± 0.07
C22:6(n-3)	31.9 ± 0.61	30.0 ± 0.39	29.5 ± 0.19	31.0 ± 0.04	29.0 ± 0.50	28.3 ± 0.05	28.9 ± 0.19	28.1 ± 0.01	12.9 ± 0.06

fatty acid	D-3-4	D-3-5	DL-16	L-6-1	L-6-6	L-6-8	L-P-2	MP2-SMALL	U-A1-MP4
C12:0	0.2 ± 0.00	0.4 ± 0.01	0.1 ± 0.02	0.1 ± 0.00	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.05	0.2 ± 0.03	0.1 ± 0.01
C13:0	11.8 ± 1.21	7.5 ± 0.35	5.1 ± 0.02	6.4 ± 0.04	2.9 ± 0.29	7.7 ± 2.03	12.2 ± 0.02	6.7 ± 0.07	4.3 ± 0.03
C14:0	6.9 ± 0.07	15.5 ± 0.04	3.3 ± 0.00	3.9 ± 0.00	3.8 ± 0.03	3.9 ± 0.22	3.7 ± 0.00	5.5 ± 0.01	4.5 ± 0.01
C15:0	29.7 ± 0.09	11.0 ± 0.11	1.9 ± 0.01	1.5 ± 0.01	1.5 ± 0.00	1.4 ± 0.07	1.9 ± 0.00	3.2 ± 0.01	1.9 ± 0.00
C16:0	23.6 ± 0.49	41.6 ± 0.08	48.3 ± 0.07	46.7 ± 0.03	51.6 ± 0.43	46.2 ± 2.28	46.7 ± 0.03	51.0 ± 0.10	48.8 ± 0.27
C16:1	0.1 ± 0.08	0.2 ± 0.00	0.4 ± 0.00	0.4 ± 0.02	0.3 ± 0.00	0.4 ± 0.02	0.3 ± 0.03	0.3 ± 0.00	0.4 ± 0.01
C17:0	6.1 ± 0.06	2.1 ± 0.04	0.6 ± 0.00	0.4 ± 0.00	0.5 ± 0.01	0.4 ± 0.03	0.6 ± 0.00	0.8 ± 0.00	0.5 ± 0.00
C18:0	0.8 ± 0.02	0.8 ± 0.01	1.6 ± 0.01	1.3 ± 0.02	1.5 ± 0.01	1.3 ± 0.04	1.6 ± 0.01	1.7 ± 0.00	1.3 ± 0.01
C18:1	0.1 ± 0.02	0.1 ± 0.01	0.2 ± 0.00	0.3 ± 0.24	0.1 ± 0.01	0.4 ± 0.22	0.2 ± 0.00	0.2 ± 0.00	0.1 ± 0.00
C18:3	0.1 ± 0.09	0.0 ± 0.00	0.2 ± 0.00	0.2 ± 0.03	0.2 ± 0.03	0.2 ± 0.01	0.1 ± 0.00	0.2 ± 0.00	0.1 ± 0.01
C20:3	0.2 ± 0.00	0.2 ± 0.00	0.2 ± 0.00	0.1 ± 0.01	0.1 ± 0.00	0.1 ± 0.01	0.2 ± 0.00	0.1 ± 0.00	0.2 ± 0.00
C20:4	0.4 ± 0.09	0.2 ± 0.01	0.6 ± 0.00	0.5 ± 0.00	0.5 ± 0.00	0.5 ± 0.03	0.5 ± 0.00	0.5 ± 0.00	0.8 ± 0.00
C20:5	0.3 ± 0.03	0.3 ± 0.01	0.6 ± 0.00	0.4 ± 0.01	0.5 ± 0.00	0.4 ± 0.00	0.5 ± 0.01	0.5 ± 0.00	0.6 ± 0.01
C22:0	0.8 ± 0.05	0.5 ± 0.06	0.2 ± 0.00	0.3 ± 0.00	0.3 ± 0.00	0.2 ± 0.00	0.2 ± 0.00	0.2 ± 0.00	0.2 ± 0.00
C22:5(n-6)	6.1 ± 0.16	6.1 ± 0.11	6.9 ± 0.00	6.8 ± 0.04	7.0 ± 0.03	6.6 ± 0.04	6.1 ± 0.03	5.6 ± 0.04	6.9 ± 0.00
C22:6(n-3)	12.9 ± 0.25	13.5 ± 0.11	29.6 ± 0.06	30.7 ± 0.24	29.2 ± 0.08	30.3 ± 0.21	25.2 ± 0.12	23.3 ± 0.03	29.4 ± 0.09

[a] Data are expressed as mean ± standard deviation of three replicates. The cells were collected at 72 h of cultivation. The data represent the percentage of individual fatty acid over the total fatty acids.

Table 2. Biomass Concentration and Squalene Contents of 18 Thraustochytrid Isolates[a]

strain	biomass concn (g/L)	squalene (mg/g)
1	15.57 ± 0.13	0.06 ± 0.00
3	14.95 ± 0.07	0.08 ± 0.00
10	14.27 ± 0.18	0.09 ± 0.00
4-P-J	15.62 ± 0.11	0.02 ± 0.00
A1-D-1-1A	15.01 ± 0.27	0.07 ± 0.00
A1-D-2-2	14.25 ± 0.47	0.06 ± 0.01
BR-MP4-A1	13.65 ± 0.10	0.18 ± 0.01
D-3-2	14.31 ± 0.04	0.10 ± 0.02
D-3-3	10.24 ± 1.56	0.06 ± 0.00
D-3-4	5.50 ± 0.11	0.10 ± 0.01
D-3-5	5.49 ± 0.16	0.10 ± 0.00
DL-16	15.48 ± 1.02	0.03 ± 0.00
L-6-1	15.45 ± 0.49	0.02 ± 0.00
L-6-6	14.16 ± 0.68	0.05 ± 0.01
L-6-8	15.54 ± 0.23	0.10 ± 0.00
L-P-2	15.16 ± 0.17	0.12 ± 0.01
MP2-SMALL	13.35 ± 1.51	0.02 ± 0.00
U-A1-MP4	14.15 ± 0.07	0.07 ± 0.00
LSD ($p < 0.05$)[b]	1.274	0.0152

[a] Data are expressed as mean ± standard deviation of three replicates. [b] LSD ($p < 0.05$), least significant difference, was used for difference comparison among means of various strains.

14 days of cultivation (*27*). Zhila et al. reported the highest biomass of about 3.5 g/L was identified in *B. braunii* after 20 days of cultivation with nitrogen optimization (*28*). Rao et al. studied the influence of salinity on cell growth of this green microalga, but the highest biomass was < 1.6 g/L after 18 days of cultivation (*29*). Therefore, the rapid growth of BR-MP4-A1 under heterotrophic cultivation was a benefit for its commercial application. The rapid utilization rate of glucose is an advantage of this strain as glucose is a very conventional and economical carbon substrate in the fermentation industry (*30*).

As shown in **Figure 4**, the squalene content of *Aurantiochytrium* sp. BR-MP4-A1 was highly associated with culture age. The highest squalene content was achieved at 36 h (0.567 mg/g) but declined quickly thereafter. A similar phenomenon was also reported in *A. mangrovei* FB1, *S. cerevisiae*, and *Torulaspora delbrueckii* (*12*, *13*, *31*). It was postulated that the marked reduction in squalene content after 36 h in this study might be due to the conversion of squalene to other compounds including sterols under aerobic conditions. Squalene is a key intermediate in sterol biosynthesis (*1*, *16*), and the first step of squalene utilization in the sterol pathway is catalyzed by squalene epoxidase to produce (3*S*)-2,3-oxidosqualene, which is an oxygen-requiring step. A sufficient concentration of molecular oxygen is essential for the activity of this step (*32*). According to Lewis et al. (*16*), the culture conditions adopted in the current study could provide a high level of dissolved oxygen concentration to the cell. Therefore, the cellular squalene might be

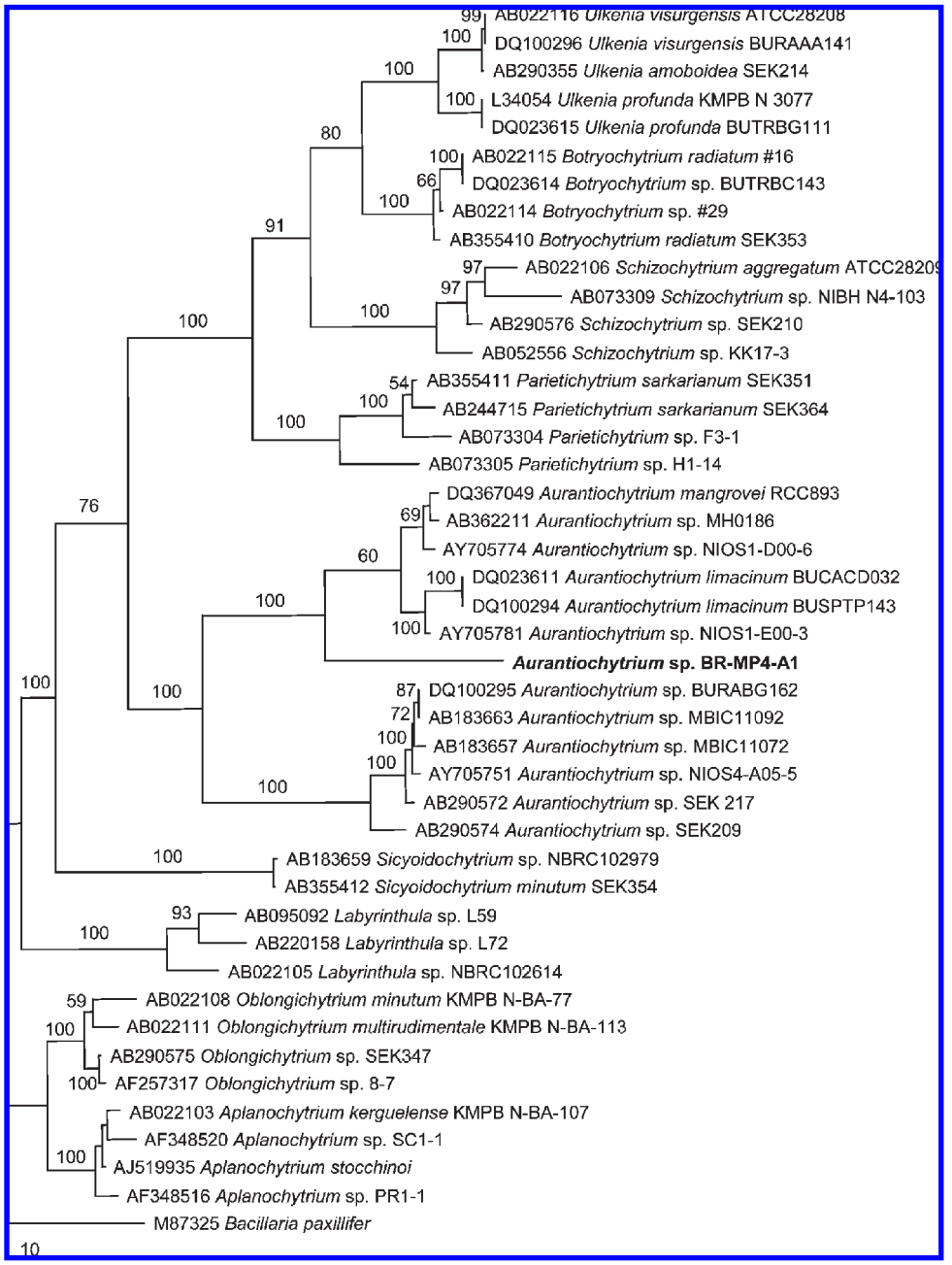

Figure 3. Phylogenetic tree of thraustochytrids with *Bacillaria paxillifer* as outgroup. The strain in this study is highlighted in bold. Bootstrap values (percent) were obtained with 1000 replications and are shown at the nodes.

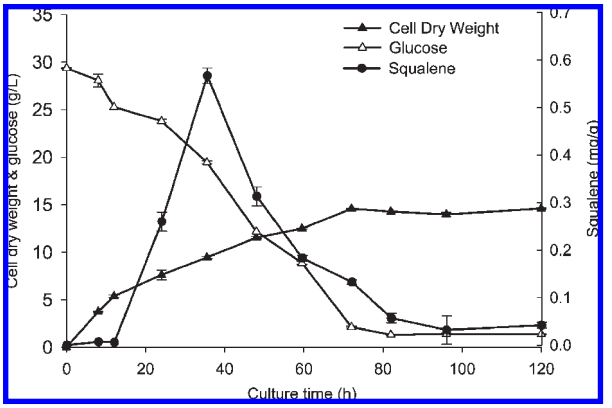

Figure 4. Time course of the cell growth (g/L), squalene accumulation (mg/g), and consumption of glucose (g/L) of *Aurantiochytrium* sp. BR-MP4-A1. Data are expressed as mean ± standard deviation of triplicates.

further metabolized to synthesize sterols instead of accumulation under the current dissolved-oxygen-rich condition. The culture conditions including dissolved oxygen concentration, temperature, and medium composition might influence squalene accumulation significantly (*16*). Investigation of the optimization of the culture conditions and regulation of the metabolic activities of the key enzymes that have immediate effect on squalene formation and degradation would be meaningful to further increase squalene productivity in the future.

B. braunii race B is well-known for its hydrocarbon production, and the molecular characterization of its squalene synthase, the key enzyme for terpenoid biosynthesis, was reported recently (*14*). However, most of its produced hydrocarbons are triterpenoid hydrocarbons known as botryococcenes (C_nH_{2n-10}, $n = 30-37$) (*33*), and only very small quantities of squalene (0.2−0.7% of the total botryococcene fraction) were routinely found (*34*). Due to the problems of light requirement, slow

growth rate, and low biomass and squalene concentrations of *B. braunii*, *Aurantiochytrium* sp. BR-MP4-A1 has a big advantage in squalene production potential.

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