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ARTICLE

# Differentiation in Fatty Acid Profiles of Pigmented and Nonpigmented *Aurantiochytrium* Isolated from Hong Kong Mangroves

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Twenty-five thraustochytrids that belong to the genus *Aurantiochytrium* were isolated from subtropical mangroves in Hong Kong. Although they have similar morphological and physiological characteristics, they have different colors on a yeast extract−glucose agar plate, which were largely ignored before. On the basis of the differences in their colony color, 25 *Aurantiochytrium* strains were further classified into pigmented and nonpigmented subgroups and their fatty acid profiles were analyzed and compared. In general, nonpigmented *Aurantiochytrium* strains were found to contain biomass concentrations and growth yield coefficients statistically higher than pigmented *Aurantiochytrium* strains ($p < 0.01$). Among all isolates, a significantly higher content of polyunsaturated fatty acid (PUFA, 123.41−179.64 mg/g) was found in the nonpigmented *Aurantiochytrium* ($p < 0.01$), whereas the pigmented strains contained a higher amount of saturated fatty acids. Docosahexaenoic acid (DHA) was identified as the most abundant PUFA in both nonpigmented and pigmented *Aurantiochytrium*. According to the result of principal component analysis, the contents and composition of saturated fatty acids and PUFAs are the major varieties to distinguish these two *Aurantiochytrium* groups, especially the contents of C15:0, C13:0, C16:0, C17:0, and DHA. With a rapid growth rate and high DHA yield, the strain from the nonpigmented *Aurantiochytrium* group was regarded as the ideal candidate for PUFA production.

**KEYWORDS: Fatty acid; thraustochytrid; *Aurantiochytrium*; DHA**

## INTRODUCTION

Long chain polyunsaturated fatty acids (PUFAs) are essential constituents of cell membranes and cell signaling systems (*1*). Some of them such as docosahexaenoic acid (DHA, $\omega$-3 C22:6,) and arachidonic acid (AA, $\omega$-6 C20:4) are major components of gray matter of brain as well as precursors of docosanoids and eicosanoids, respectively. They are important for the early cognitive and visual development of infants and therefore have now been used as added components in infant formulas in developed countries worldwide (*2*−*4*). Recently, it was proven by clinical evidence that PUFAs are able to alleviate symptoms of certain diseases such as coronary heart disease, stroke and rheumatoid arthritis (*5*). Because of the noticeable importance of PUFAs in human health and nutrition, different means are used to increase the human consumption of PUFAs from different food sources such as direct intake as food additives and nutraceuticals as well as indirect consumption via the enrichment of PUFAs in important species in aquaculture (*6*).

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The major commercial source of PUFAs is currently from marine fatty fish. However, fish and fish oil are reported to contain dioxins, PCBs, heavy metals, and pharmaceutical residues including synthetic estrogens that are a hazard upon long-term exposure (*7*). Moreover, the seafood has now been identified as a leading source of mercury exposure for humans. As such, the safety of fatty fish and fish oil being used as conventional sources of PUFAs is doubtful. More efforts have therefore been exerted to explore the alternative PUFA sources. The microbial group - thraustochytrid, in particular, has been regarded as the most successful alternative, because some genera are known to contain substantial amounts of lipids, especially long chain polyunsaturated fatty acids, that is, DHA, and can grow well heterotrophically in a stainless-steel fermentor (*6*, *8*, *9*).

Thraustochytrids are marine osmoheterotrophs with characteristic ectoplasmic net elements for nutrient absorption and are responsible for carbon recycling in marine habitats (*10*). Although there are six categories of PUFA profiles in thraustochytrids including docosapentaenoic acid (DPA)/DHA, eicosapentaenoic acid (EPA)/DHA, EPA/DPA/DHA, arachidonic acid (AA)/EPA/DHA, linoleic acid (LA)/AA/DPA/DHA, and LA/AA/EPA/DHA (*11*), the major characteristic of the

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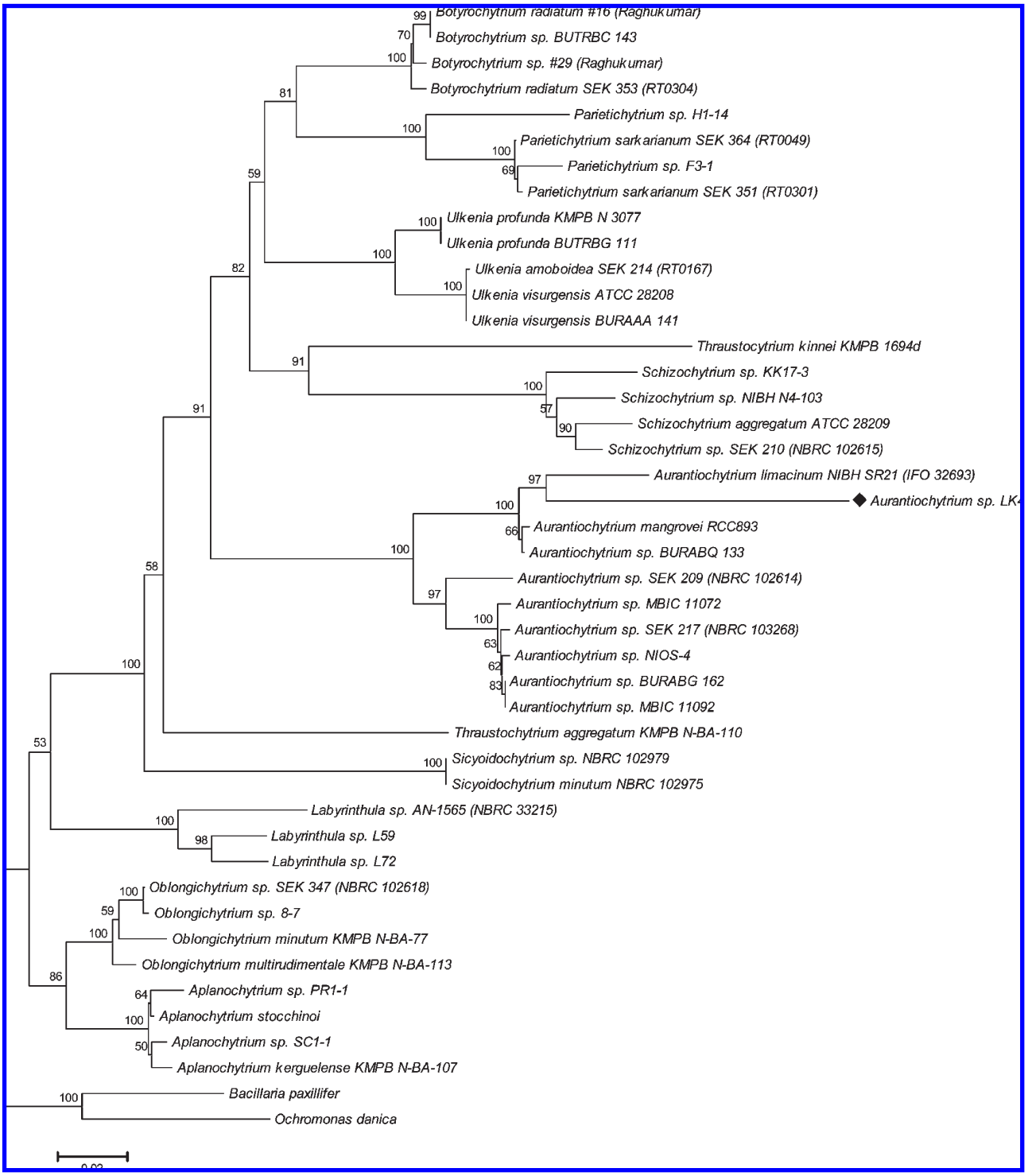


**Figure 1.** Neighbor-joining trees of thraustochytrids and labyrinthulids based on 18S rRNA gene sequence analysis. ♦ indicates the strain in this study. Bootstrap values (%) were obtained with 1000 replicates and are shown at the nodes. The tree was rooted with *Bacillaria paxillifer* and *Ochromonas danica* as outgroup.

commercially important strains reported to date is their higher contents of DHA (*12*).

Mangroves are unique intertidal wetlands largely confined to coastal regions between 30° north and south of the equator and are nursery grounds for organisms ranging from migratory birds and mudskippers to microorganisms prevailing in the water columns of the habitats (*13*). Mangrove areas were reported to be the most selected sites for the isolation of thraustochytrids owning largely to the heterogeneity of the environments that may exert evolutionary pressure for nurturing thraustochytrids with rapid growth (*8*). Hong Kong, located at 22°30′N and 114°10′E, is a rare urban location with its vast mangrove distribution in the south China sea region. Its mangrove distribution is representative of that in the south China sea region (*14*). In recent field expeditions of several Hong Kong mangrove areas, we found that some *Aurantiochytrium* strains differed in fatty acid profiles and heterotrophic growth characteristics from those previously isolated strains which contain relatively higher PUFAs contents (*15*). Moreover, the colony color of the strains exhibit a distinctive reddish orange color (named as pigmented strains in this study)

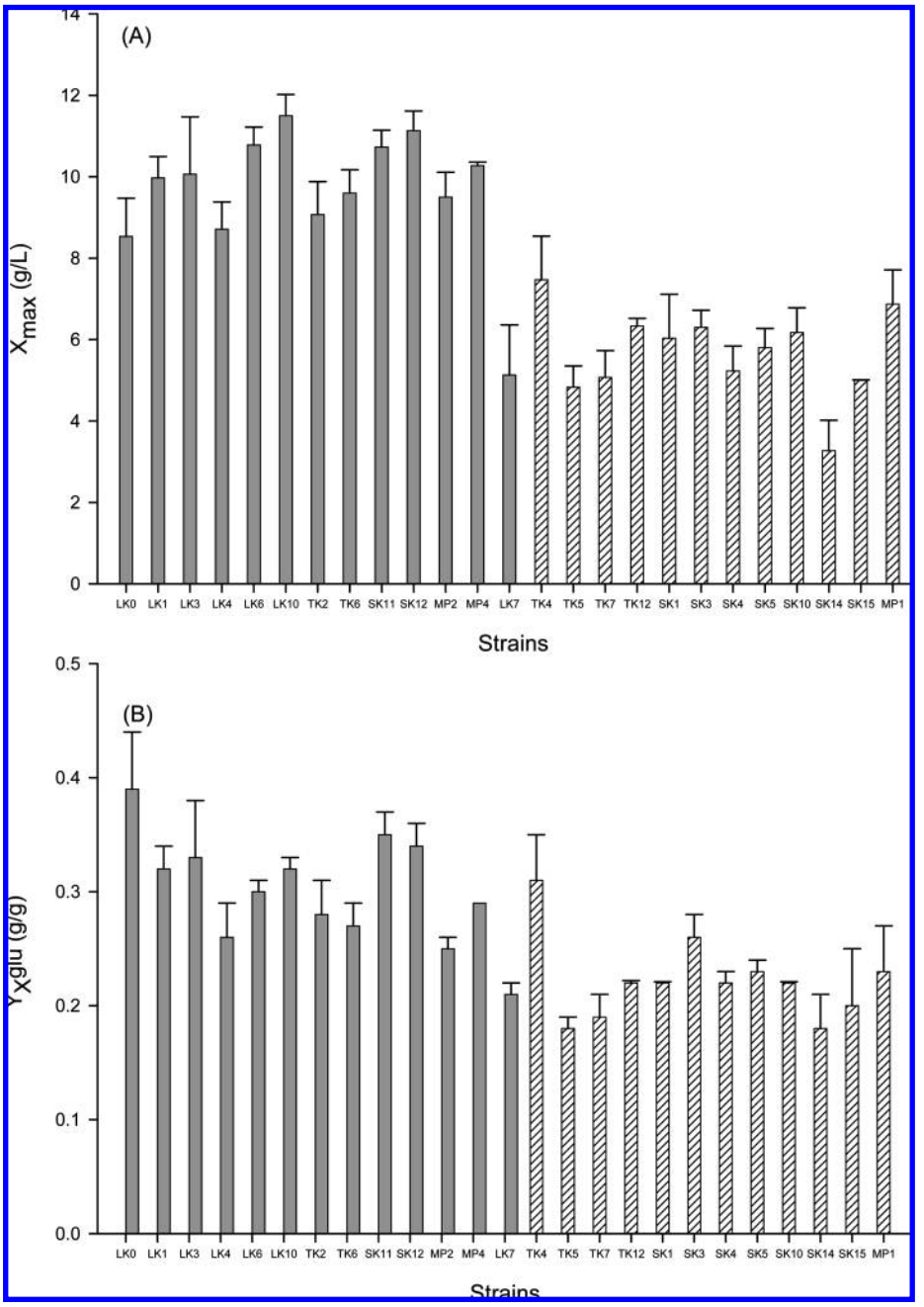


**Figure 2.** Kinetic parameters of growth and glucose consumption of the *Aurantiochytrium* strains. **(A)** Maximum biomass concentration $X_{max}$ (g/L); (gray bars) nonpigmented *Aurantiochytrium* spp.; (hatched bars) pigmented *Aurantiochytrium* spp.; **(B)** growth yield coefficient based on glucose $Y_{x/glu}$ (g/g). Values are represented as mean ± standard deviation of triplicates and statistically analyzed at a level of $p < 0.05$.

on glucose−yeast extract agar which is markedly different from the pale creamy color (nonpigmented) of the PUFA-rich strains isolated previously by our group, although they share similar morphological and physiological characteristics (*16*). Specifically, the pigmented and nonpigmented strains can be further distinguished based on their intracellular carotenoid contents when detected using an HPLC-photodiode array detector. All the pigmented strains contain carotenoid with the content of some strains reaching as high as 100 $\mu$g/g of biomass, while the nonpigmented strains do not possess a detectable level of carotenoid (data not shown). In this study, we aimed to investigate and compare the fatty acid profiles and the heterotrophic growth properties of the pigmented and nonpigmented thraustochytrid *Aurantiochytrium*, a frequently isolated genus from Hong Kong mangrove areas. A randomly selected strain categorized by morphological and physiological analyses was chosen as a representative for subsequent confirmation using 18s rRNA gene analysis. The output from this research would not only provide comprehensive information on the fatty acid production characteristics of these subtropical thrasutochytrids but also facilitate further investigation of thraustochytrid for its potential application in the production of other useful products, that is, pigments.

## MATERIALS AND METHODS

**Heterotrophic Growth.** The isolation of thraustochytrid was conducted in different mangrove areas in the Deep Bay and Sai Kung districts during the summer of 2007 according to the isolation method described by Fan et al. (*16*). Three mangrove sites, namely, Luk Keng (LK), Ting Kok (TK), Sai Keng (SK) in Sai Kung and the largest mangrove stands in the Hong Kong - Mai Po Nature Reserve (MP) in the Deep Bay region were

**Table 1.** Fatty Acid Composition (as % of Total Fatty Acid) and Content of the *Aurantiochytrium* Strains Cultured at 25 °C[a]

| | | | saturated fatty acids | | | | | | monounsaturated fatty acid | | polyunsaturated fatty acid | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| strain[b] | description[c] | salinity (‰) | 13:0 | 14:0 | 15:0 | 16:0 | 17:0 | 18:0 | 16:1 n-7 | 18:1 n-9 | AA | EPA | DPA | DHA | TFA[d] | unsatd[e] | SFA[f] (mg/g) | MUFA[f] (mg/g) | PUFA[f] (mg/g) | DHA[g] (mg/g) |
| LK0 | NP | 30 | 0.04 ± 0.01 | 5.66 ± 0.37 | 4.22 ± 0.23 | 57.50 ± 0.56 | 0.89 ± 0.18 | 1.52 ± 0.20 | 0.00 | 0.25 ± 0.05 | 0.70 ± 0.11 | 0.42 ± 0.03 | 4.54 ± 0.58 | 23.53 ± 1.26 | 41.85 ± 3.72 | 30.36 ± 1.52 | 295.09 ± 15.54 | 1.05 ± 0.06 | 123.41 ± 17.95 | 98.71 ± 14.02 |
| LK1 | NP | 30 | 0.10 ± 0.03 | 5.61 ± 0.19 | 4.11 ± 0.25 | 52.88 ± 0.89 | 0.83 ± 0.07 | 1.37 ± 0.13 | 0.00 | 0.29 ± 0.03 | 0.78 ± 0.12 | 0.45 ± 0.03 | 4.96 ± 0.05 | 27.66 ± 0.48 | 37.83 ± 3.06 | 35.20 ± 0.78 | 249.05 ± 14.12 | 1.10 ± 0.07 | 129.25 ± 12.84 | 104.71 ± 10.26 |
| LK3 | NP | 30 | 0.09 ± 0.01 | 4.54 ± 0.45 | 4.78 ± 0.33 | 50.77 ± 2.13 | 0.96 ± 0.08 | 1.21 ± 0.12 | 0.00 | 0.30 ± 0.02 | 0.78 ± 0.07 | 0.48 ± 0.02 | 5.11 ± 1.00 | 30.01 ± 2.32 | 49.03 ± 1.72 | 37.78 ± 3.10 | 310.66 ± 9.14 | 1.47 ± 0.07 | 179.64 ± 9.35 | 146.91 ± 6.21 |
| LK4 | NP | 30 | 0.16 ± 0.02 | 5.60 ± 0.51 | 5.89 ± 1.00 | 49.76 ± 0.51 | 1.21 ± 0.17 | 1.18 ± 0.03 | 0.00 | 0.52 ± 0.05 | 0.94 ± 0.16 | 0.47 ± 0.04 | 4.61 ± 0.44 | 28.79 ± 0.06 | 44.89 ± 4.49 | 36.50 ± 0.06 | 291.58 ± 11.32 | 2.33 ± 0.11 | 157.32 ± 16.48 | 129.19 ± 12.64 |
| LK6 | NP | 30 | 0.08 ± 0.01 | 4.74 ± 0.44 | 4.90 ± 0.63 | 53.75 ± 0.31 | 0.97 ± 0.12 | 1.29 ± 0.00 | 0.00 | 0.33 ± 0.06 | 0.74 ± 0.12 | 0.42 ± 0.02 | 4.89 ± 0.73 | 27.18 ± 1.08 | 50.95 ± 4.76 | 34.56 ± 1.50 | 338.49 ± 18.21 | 1.68 ± 0.10 | 171.01 ± 24.51 | 138.76 ± 18.44 |
| LK10 | NP | 30 | 0.10 ± 0.00 | 4.93 ± 0.26 | 4.92 ± 0.13 | 52.60 ± 0.39 | 1.02 ± 0.09 | 1.29 ± 0.00 | 0.00 | 0.52 ± 0.06 | 1.15 ± 0.17 | 0.41 ± 0.01 | 3.97 ± 0.46 | 28.03 ± 0.70 | 51.43 ± 1.68 | 35.46 ± 0.79 | 340.43 ± 11.91 | 2.67 ± 0.12 | 173.87 ± 10.92 | 144.23 ± 8.28 |
| TK2 | NP | 25 | 0.09 ± 0.00 | 4.92 ± 0.17 | 4.69 ± 0.12 | 52.92 ± 0.17 | 1.06 ± 0.04 | 1.30 ± 0.04 | 0.00 | 0.59 ± 0.07 | 1.51 ± 0.10 | 0.45 ± 0.01 | 3.56 ± 0.24 | 27.76 ± 0.24 | 48.03 ± 0.19 | 35.61 ± 0.17 | 319.37 ± 8.75 | 2.83 ± 0.15 | 160.93 ± 2.30 | 133.34 ± 1.69 |
| TK6 | NP | 25 | 0.08 ± 0.00 | 4.67 ± 0.18 | 4.14 ± 0.13 | 53.06 ± 0.62 | 0.85 ± 0.04 | 1.26 ± 0.01 | 0.00 | 0.33 ± 0.04 | 0.73 ± 0.03 | 0.45 ± 0.02 | 5.07 ± 0.29 | 28.64 ± 0.48 | 50.01 ± 2.53 | 36.18 ± 0.24 | 324.42 ± 12.31 | 1.65 ± 0.09 | 175.68 ± 10.07 | 143.26 ± 9.68 |
| SK11 | NP | 15−18 | 0.08 ± 0.00 | 4.93 ± 0.36 | 4.16 ± 0.37 | 54.06 ± 0.76 | 0.91 ± 0.09 | 1.38 ± 0.01 | 0.00 | 0.41 ± 0.01 | 0.82 ± 0.10 | 0.46 ± 0.04 | 5.00 ± 0.45 | 27.03 ± 0.03 | 50.11 ± 0.23 | 34.76 ± 0.17 | 333.09 ± 6.85 | 2.05 ± 0.10 | 168.01 ± 0.63 | 135.43 ± 0.75 |
| SK12 | NP | 15−18 | 0.08 ± 0.00 | 4.54 ± 0.17 | 3.93 ± 0.18 | 55.84 ± 1.08 | 0.79 ± 0.02 | 1.39 ± 0.03 | 0.00 | 0.26 ± 0.01 | 0.72 ± 0.00 | 0.44 ± 0.01 | 5.16 ± 0.16 | 26.12 ± 0.90 | 44.34 ± 3.26 | 33.63 ± 1.09 | 298.83 ± 9.11 | 1.15 ± 0.06 | 144.57 ± 5.84 | 115.67 ± 4.53 |
| MP2 | NP | 8−10 | 0.10 ± 0.01 | 4.43 ± 0.21 | 5.69 ± 0.38 | 48.10 ± 0.94 | 1.33 ± 0.11 | 1.18 ± 0.04 | 0.00 | 0.58 ± 0.08 | 1.31 ± 0.00 | 0.54 ± 0.01 | 5.02 ± 0.06 | 30.78 ± 0.43 | 45.80 ± 1.46 | 39.79 ± 0.62 | 284.46 ± 5.20 | 2.66 ± 0.12 | 173.54 ± 3.08 | 140.93 ± 2.53 |
| MP4 | NP | 8−10 | 0.12 ± 0.00 | 4.60 ± 0.35 | 5.86 ± 0.01 | 50.74 ± 0.20 | 1.38 ± 0.00 | 1.10 ± 0.02 | 0.00 | 0.32 ± 0.07 | 1.04 ± 0.28 | 0.49 ± 0.01 | 5.20 ± 0.43 | 28.32 ± 0.19 | 40.18 ± 4.89 | 36.62 ± 0.03 | 260.18 ± 8.21 | 1.29 ± 0.05 | 141.62 ± 15.94 | 113.77 ± 13.09 |
| LK7 | P | 30 | 3.96 ± 0.76 | 6.09 ± 0.70 | 40.20 ± 1.17 | 15.67 ± 1.56 | 8.01 ± 0.18 | 0.46 ± 0.11 | 0.24 ± 0.02 | 0.40 ± 0.12 | 1.02 ± 0.12 | 0.36 ± 0.03 | 5.56 ± 0.21 | 16.08 ± 0.28 | 22.80 ± 1.84 | 24.82 ± 0.65 | 175.24 ± 7.69 | 1.46 ± 0.06 | 52.76 ± 2.72 | 36.63 ± 2.32 |
| TK4 | P | 25 | 3.60 ± 0.38 | 8.06 ± 0.67 | 39.18 ± 1.81 | 15.90 ± 0.49 | 8.02 ± 0.52 | 0.38 ± 0.02 | 0.15 ± 0.01 | 0.11 ± 0.00 | 0.84 ± 0.08 | 0.37 ± 0.03 | 5.46 ± 0.35 | 16.29 ± 1.26 | 25.39 ± 3.52 | 24.28 ± 1.80 | 195.37 ± 9.12 | 0.66 ± 0.03 | 58.53 ± 3.85 | 41.14 ± 2.55 |
| TK5 | P | 25 | 2.16 ± 0.11 | 14.93 ± 2.10 | 27.82 ± 3.85 | 33.24 ± 3.53 | 4.67 ± 0.48 | 0.84 ± 0.02 | 0.20 ± 0.04 | 0.25 ± 0.02 | 0.59 ± 0.07 | 0.21 ± 0.03 | 3.45 ± 0.16 | 10.16 ± 0.88 | 47.74 ± 5.27 | 15.56 ± 1.18 | 408.38 ± 19.84 | 2.15 ± 0.10 | 69.02 ± 2.24 | 48.29 ± 1.17 |
| TK7 | P | 25 | 2.65 ± 0.34 | 11.81 ± 2.16 | 34.16 ± 4.11 | 25.81 ± 3.57 | 6.02 ± 0.97 | 0.67 ± 0.05 | 0.16 ± 0.03 | 0.31 ± 0.06 | 0.71 ± 0.04 | 0.25 ± 0.02 | 3.87 ± 0.09 | 11.73 ± 0.24 | 37.75 ± 3.00 | 17.97 ± 0.31 | 314.18 ± 13.48 | 1.77 ± 0.08 | 63.32 ± 3.65 | 44.26 ± 2.61 |
| TK12 | P | 25 | 2.39 ± 0.01 | 5.19 ± 0.04 | 32.87 ± 0.20 | 18.86 ± 0.11 | 7.79 ± 0.04 | 0.84 ± 0.04 | 0.25 ± 0.04 | 0.68 ± 0.07 | 1.64 ± 0.02 | 0.53 ± 0.00 | 7.00 ± 0.08 | 18.29 ± 0.21 | 18.58 ± 0.05 | 30.55 ± 0.48 | 134.02 ± 3.11 | 1.73 ± 0.07 | 51.78 ± 0.46 | 33.98 ± 0.29 |
| SK1 | P | 15−18 | 1.47 ± 0.25 | 5.80 ± 0.93 | 30.82 ± 2.83 | 22.01 ± 0.57 | 6.47 ± 0.12 | 0.55 ± 0.08 | 0.15 ± 0.02 | 0.10 ± 0.02 | 0.78 ± 0.20 | 0.46 ± 0.07 | 7.92 ± 0.75 | 21.40 ± 1.86 | 23.97 ± 5.02 | 31.81 ± 3.10 | 166.64 ± 8.75 | 0.60 ± 0.01 | 73.06 ± 8.48 | 50.82 ± 6.28 |
| SK3 | P | 15−18 | 1.16 ± 0.29 | 15.64 ± 0.81 | 21.98 ± 4.69 | 37.16 ± 2.34 | 3.72 ± 0.62 | 0.93 ± 0.10 | 0.29 ± 0.07 | 0.22 ± 0.03 | 0.47 ± 0.00 | 0.28 ± 0.01 | 4.26 ± 0.59 | 12.25 ± 1.49 | 47.53 ± 3.66 | 18.38 ± 2.17 | 392.99 ± 16.57 | 2.42 ± 0.11 | 82.31 ± 3.51 | 57.95 ± 2.62 |
| SK4 | P | 15−18 | 1.98 ± 0.13 | 6.95 ± 1.43 | 31.72 ± 2.74 | 24.36 ± 1.81 | 5.84 ± 0.14 | 0.69 ± 0.03 | 0.23 ± 0.04 | 0.31 ± 0.04 | 0.67 ± 0.01 | 0.44 ± 0.08 | 6.88 ± 0.50 | 18.34 ± 1.62 | 22.83 ± 5.62 | 27.77 ± 2.33 | 168.28 ± 9.38 | 1.23 ± 0.02 | 60.02 ± 9.82 | 41.41 ± 6.62 |
| SK5 | P | 15−18 | 2.04 ± 0.27 | 10.33 ± 1.63 | 32.33 ± 4.87 | 25.55 ± 3.44 | 5.40 ± 0.67 | 0.62 ± 0.04 | 0.18 ± 0.00 | 0.12 ± 0.01 | 0.75 ± 0.06 | 0.36 ± 0.02 | 5.47 ± 0.21 | 14.79 ± 0.45 | 45.52 ± 2.11 | 22.60 ± 0.49 | 357.08 ± 14.51 | 1.37 ± 0.05 | 98.12 ± 7.11 | 67.35 ± 5.18 |
| SK10 | P | 15−18 | 1.78 ± 0.23 | 15.32 ± 2.27 | 26.20 ± 3.52 | 32.98 ± 3.03 | 5.07 ± 0.43 | 0.79 ± 0.08 | 0.24 ± 0.02 | 0.51 ± 0.00 | 0.63 ± 0.02 | 0.23 ± 0.01 | 3.68 ± 0.40 | 11.04 ± 0.70 | 38.17 ± 3.94 | 17.05 ± 1.06 | 322.13 ± 11.19 | 2.86 ± 0.12 | 59.57 ± 1.96 | 41.99 ± 1.69 |
| SK14 | P | 15−18 | 2.21 ± 0.37 | 11.08 ± 1.20 | 32.80 ± 4.50 | 25.99 ± 4.16 | 5.79 ± 0.37 | 0.60 ± 0.09 | 0.17 ± 0.01 | 0.18 ± 0.02 | 0.69 ± 0.04 | 0.34 ± 0.02 | 5.06 ± 0.12 | 13.36 ± 0.03 | 34.29 ± 0.87 | 20.68 ± 0.27 | 275.58 ± 8.54 | 1.20 ± 0.05 | 67.32 ± 0.95 | 45.82 ± 1.04 |
| SK15 | P | 15−18 | 1.46 ± 0.08 | 14.52 ± 0.72 | 23.52 ± 3.55 | 31.84 ± 2.20 | 3.85 ± 0.21 | 0.75 ± 0.05 | 0.19 ± 0.03 | 0.33 ± 0.03 | 0.78 ± 0.02 | 0.31 ± 0.04 | 5.32 ± 0.41 | 14.88 ± 0.38 | 40.38 ± 4.92 | 22.75 ± 0.80 | 317.03 ± 18.15 | 2.10 ± 0.10 | 86.77 ± 13.70 | 60.19 ± 8.85 |
| MP1 | P | 8−10 | 1.79 ± 0.34 | 15.60 ± 1.41 | 26.33 ± 5.18 | 31.38 ± 4.47 | 3.97 ± 0.82 | 0.69 ± 0.06 | 0.29 ± 0.03 | 0.27 ± 0.05 | 0.75 ± 0.14 | 0.29 ± 0.03 | 4.45 ± 0.18 | 12.22 ± 0.37 | 44.02 ± 3.53 | 19.17 ± 0.33 | 361.53 ± 14.13 | 2.47 ± 0.12 | 78.67 ± 7.99 | 53.88 ± 5.96 |

[a] Data are expressed as mean ± SD of three replicates and statistically analyzed at a level of $p<0.05$. [b] LK-Luk Keng, TK- Ting Kok, MP- Mai Po, SK-Sai Keng. [c] NP = nonpigmented; P = Pigmented. [d] TFA = (total fatty acids/cell dry weight) x 100%. [e] Unsatd: percentage of unsaturated fatty acids of total fatty acids. [f] SFA, MUFA, PUFA contents = mg SFA or MUFA or PUFA/g of cell dry weight. [g] DHA content = mg DHA/g of cell dry weight.

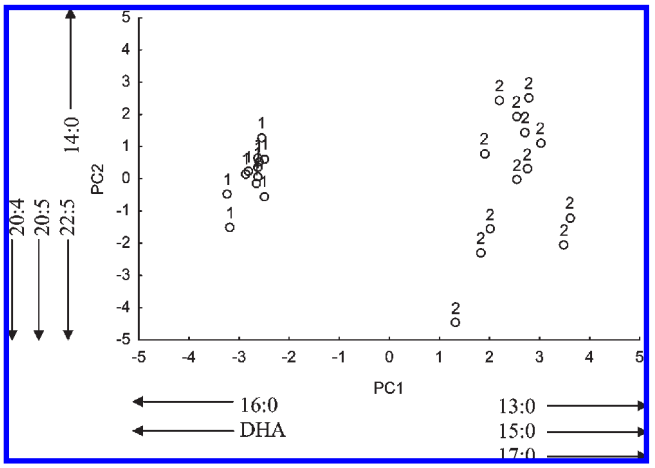


**Figure 3.** Biplot of the first and second PC derived from the fatty acid composition (as % of fatty acids) from nonpigmented *Aurantiochytrium* spp. (1) and pigmented *Aurantiochytrium* spp. (2). Arrows indicate the fatty acid contributing most to the distribution of the species along each component.

selected with a salinity ranging from 10 to 30‰. A total of 25 strains of thraustochytrids in the genus *Aurantiochytrium* were isolated, purified, and morphologically and physiologically identified for subsequent experiments. Cultures were maintained in yeast extract−glucose agar plates with 1 mL of 15‰ (v/v) sterile seawater and subcultured monthly. An inoculum was prepared in 250 mL Erlenmeyer flasks each containing 50 mL of seed culture medium consisting of (per liter of 15‰ (v/v) seawater) 10 g of glucose and 1 g of yeast extract at 25 °C in an orbital shaker at 200 rpm in the dark. 5% of an exponentially growing inoculum was inoculated to a glucose−yeast extract medium consisting of (per liter of 15‰ (v/v) seawater) 30 g of glucose, 3 g of $KH_2PO_4$ and 5 g of yeast extract for growth experiments at 25 °C in an orbital shaker at 200 rpm according to Fan et al. (*16*).

**Determination of Cell Dry Weight.** The cell dry weight was determined according to Jiang et al. (*15*).

**Determination of Glucose Concentration.** Residual glucose concentration in the culture broth was determined by the 3,5-dinitrosalicylic acid method (*17*).

**Fatty Acid Analysis.** Fatty acid composition of the freeze-dried thraustochytrid cells was determined following a modified procedure of Christie (*18*). The extracted fatty acid methyl esters were analyzed by HP 6890 capillary gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a flame-ionization detector and J&W Scientific Innowax capillary column (30 m × 0.25 mm). Nitrogen was used as the carrier gas. Initial column temperature was set at 170 °C and was subsequently raised to 23 at 1 °C/min. The injector was kept at 250 °C with an injection volume of 2 $\mu$L under splitless mode. The FID detector was set at 270 °C. Fatty acid methyl esters were identified by chromatographic comparison with authentic standards (Sigma Chemical Co.). The quantities of individual fatty acid methyl ester were estimated from the peak areas on the chromatogram using nonadecanoic acid (C19:0) as the internal standard.

**Extraction and Sequencing of 18S rRNA Gene.** The total genome DNA was extracted according to the method of Lee and Taylor (*19*). First, the thraustochytrid cells were harvested and frozen in liquid nitrogen in the mortar. After being ground to a fine powder, the cells were transferred to a 1.5 mL microfuge tube. Lysis buffer containing 50 mM Tris-HCl (pH 7.2), 50 mM ethylenediamineteraacetic acid (EDTA), and 1% $\beta$-mercaptoethanol, 3% sodium dodecyl sulfate (SDS) was added, vortexed, and incubated at 65 °C for 1 h. An equal volume of phenol/chloroform/isoamyl alcohol in the ratio of 25:24:1 (v/v) (Sigma Chemical Co.) was mixed with the solution and centrifuged at 10000*g* for 15 min. The supernatant was used for DNA precipitation with 3 M sodium acetate (NaOAc) and isopropanol. After being centrifuged for 2 min at 10000*g*, the DNA pellet was washed with 70% ethanol, dried, and resuspended in Tris-EDTA buffer (10 mL Tris-HCl. 0.1 mM EDTA).

A DNA segment containing the 18S rRNA gene was amplified by using forward primer SR-1 (5′-TACCTGGTTGATCCTGCCAG-3′) and reverse primer SR-12 (5′-CCTTCCGCAGGTTCACCTAC-3′) (*20*). PCR was performed in a total volume of 50 $\mu$L reagent mixture, which contained 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.5 $\mu$M of each of the primers, 0.2 mM dNTP mixture, 20 ng of genomic DNA, and 1.5 units of Taq DNA polymerase. The conditions for the PCR were initial denaturation at 94 °C for 5 min, followed by 30 cycles at 94 °C for 45 s, 54 °C for 45 s, and 72 °C for 1 min, then final extension at 72 °C for 5 min. Electrophoresis was carried out on 1% agarose gel to separate the amplified products, which was stained with ethidium bromide. Only one band was observed from all PCR products amplified from the strain *Aurantiochytrium* sp. LK4. After purifying the PCR products using GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Piscataway, NJ) as instructed by the manufacturer, they were sequenced in both directions using primers according to Nakayam et al. (*20*) by an automatic ABI3100 sequencer. The sequence of *Aurantiochytrium* sp. LK4 was submitted to GenBank, with an accession number EU871043.

**Sequence Alignment and Analyses.** Other sequences used in this study were obtained from GenBank. The computer program CLUSTAL X was used for sequences alignment according to Thompson, Gibson, Plewniak, Jeanmougin & Higgins (*21*). The neighbor-joining (NJ) tree was constructed using Mega (version 3.1), with the Kimura two parameter model (*22*). *Bacillaria paxillifer* and *Ochromonas danica* as the outgroup were selected. The bootstrap values were obtained from 1000 replications of NJ analyses and the percentage of identities was calculated using NCBI Blast.

**Statistical Analysis.** Analysis of variance (ANOVA) and *t* test were performed by using SPSS statistical package (SPSS, Inc. Chicago, IL). Difference at $p < 0.05$ was considered significant. The differences in fatty acid profiles of the nonpigmented and pigmented *Aurantiochytrium* were analyzed by Principal Components Analysis (PCA) using STATISTICA 6.0 (USA). The fatty acid data were transformed using the angular transformation ($x' = \arcsin \sqrt{x}$, where $x$ is the fatty acid %) to reduce the heterogeneity of variances of the data since the fatty acid with the highest content (%) was 1250 times greater than the one with the lowest content (%) (*23*).

## RESULTS AND DISCUSSION

**Identification of the Newly Isolated Strains.** The placement of thrasutochytrids is separated primarily by the morphology of the thallic stage, differences in sporogenesis, and spore release (*8*). On the basis of this method of classification, the 25 newly isolated strains were identified as *Aurantiochytrium* (formerly known as *Schizochytrium*). A randomly selected strain LK4 was used as a representative and further confirmed as *Aurantiochytrium* sp. by the 18S rRNA gene sequencing, showing the correctness of our morphological and physiological identity (**Figure 1**). From the neighbor-joining tree constructed in **Figure 1**, it could be observed that the selected strain was claded together with strains in the genus *Aurantiochytrium*. It formed a tight cluster with *Aurantiochytrium* spp. with a high bootstrap value. The percentage of identities between *Aurantiochytrium* sp. LK4 and *A. limacinum* (formerly *S. limacinum*) was 92%.

**Heterotrophic Growth Characteristics.** Thraustochytrids are able to survive and grow on organic carbon sources (*12*). In this study, we used glucose as sole carbon source as glucose is the conventional and economical carbon substrate in the fermentation industry. All the strains grew well in darkness on glucose−yeast extract medium containing 30 g/L glucose. The kinetics of cell growth and glucose consumption of the strains are shown in **Figure 2**. The maximum biomass concentrations (**Figure 2A**) of nonpigmented *Aurantiochytrium* spp. were between 8.53 to 11.13 g/L, and were significantly higher than those of pigmented strains (ranging from 3.27 to 6.32 g/L) ($p < 0.05$). The growth yield coefficients and specific growth rates (**Figure 2B**) of nonpigmented and pigmented *Aurantiochytrium* spp. were within the range of 0.25−0.39 g/g and 0.034−0.079 $h^{-1}$ as well as 0.15−0.26 g/g and 0.028−0.068 $h^{-1}$, respectively. The results of the growth

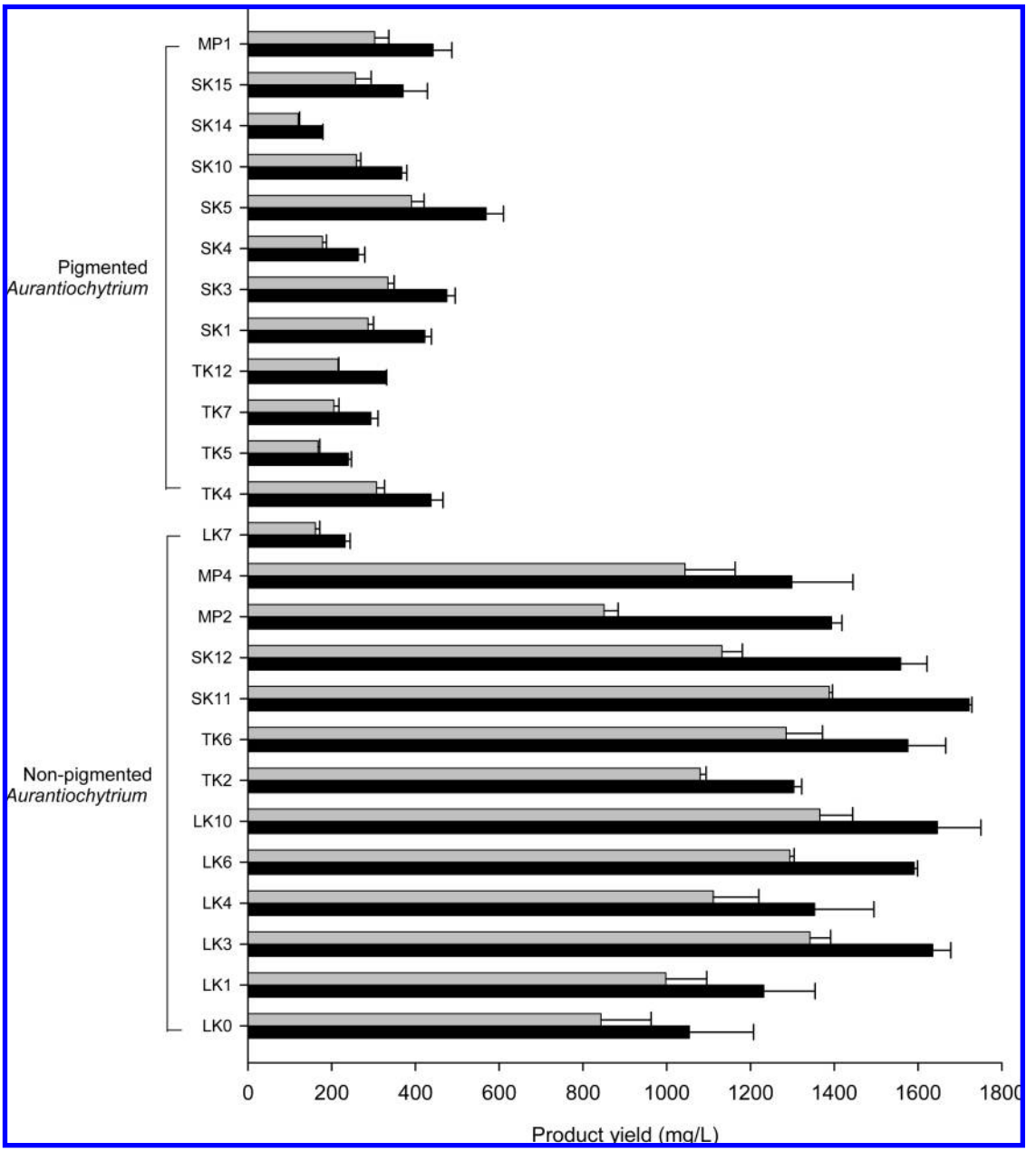


**Figure 4.** PUFA and DHA yields of the *Aurantiochytrium* strains. (Solid black bars) PUFA yield (mg/L); (gray bars) DHA yield (mg/L). Values are represented as mean ± standard deviation of triplicates and statistically analyzed at a level of $p < 0.05$.

kinetics of nonpigmented *Aurantiochytrium* strains were comparable to *A. mangrovei* (formerly known as *Schizochytrium mangrovei*) in previous studies done by Jiang et al. (*15*), whereas the general kinetic growth parameters of pigmented strains were much lower. It might be because the medium and growth conditions used in this study were less favorable to these strains, since the culture conditions have a great influence on the growth of thraustochytrid species (*8*).

**Fatty Acid Composition of Different Groups of *Aurantiochytrium*.** From **Table 1**, it was shown that the fatty acid composition of the nonpigmented and pigmented *Aurantiochytrium* spp. were different from each other. On average, the percentage of C16:0 (48.10−59.25%) was about half of the total fatty acids in the strains of nonpigmented *Aurantiochytrium* spp., followed by DHA (C22:6, 22.65−31.85%), C15:0 (3.80−10.10%), and DPA (C22:5, 3.26−5.47%). Their fatty acid profiles concur with that found in mangrove species isolated previously. The strains of pigmented *Aurantiochytrium* spp. are also rich in C15:0, C16:0, DHA, and DPA, but they were more abundant in proportions of odd-chain saturated fatty acids, such as C13:0, C15:0, and C17:0. They had an exceptionally high content of C15:0 (21.83 to 40.20%), which was higher than C16:0 (15.67−37.16%). This might be due to the low activity of methylmalonyl-CoA mutase in pigmented *Aurantiochytrium* spp., as this enzyme is responsible for the conversion of propionic acid to succinic acid, which could reduce the availability of propionic acid for odd-chain fatty acid generation (*24*). The PCA result showed clear separation of the fatty acid composition between nonpigmented and pigmented *Aurantiochytrium* spp. (**Figure 3**). 93.9% of the variability between these two groups was explained by the first three principal components (PC3 not shown). The major contributing fatty acids to PC1 were 15:0, 13:0, 16:0, 17:0, and DHA, which accounted for 61.7% of the total variation. The fatty acids contributing to the separation on PC2 were 14:0, 20:4, and 20:5, which explained 20.6% of the total variation. The cumulative variation of PC1 and PC2 explained 82.3% of the total variance between nonpigmented and pigmented *Aurantiochytrium* spp.

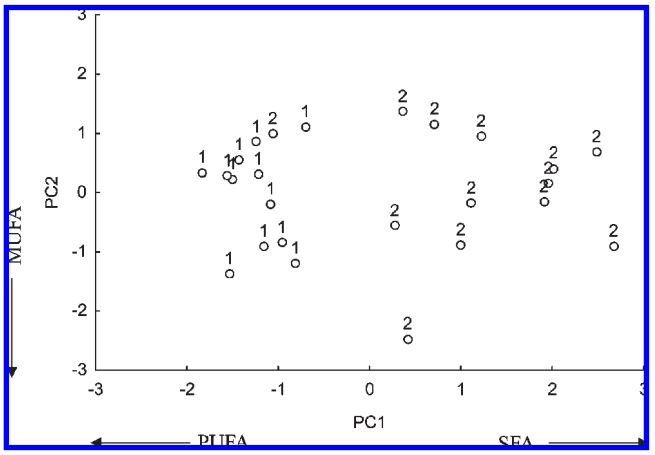


**Figure 5.** Biplot of the first and second PC derived from the SFA, MUFA, PUFA (as fatty acid content, mg/g) from nonpigmented *Aurantiochytrium* spp. (1) and pigmented *Aurantiochytrium* spp. (2). Arrows indicate the fatty acid contributing most to the distribution of the species along each component.

In addition to the fatty acid composition, the contents of total fatty acids of different *Aurantiochytrium* groups also varied (**Table 1**). Generally, the total fatty acid content of nonpigmented *Aurantiochytrium* spp. was higher (33.41−51.43%) than that of pigmented one (11.34−47.74%). The nonpigmented group was found to have a significantly greater percentage of PUFA (up to 38.82% of total fatty acids) than the pigmented one ($p < 0.001$), which was even higher than those of *S. aggregatum* (15.5%), *T. striatum* ATCC24473 (1.5%), and *Thraustochytrium* sp. ATCC26185 (33.3%) in the study by Huang et al. (*9*). The PUFA yield of nonpigmented *Aurantiochytrium* spp. (1053.94−1722.07 mg/L) was also greater than pigmented ones (177.06−569.09 mg/L); the highest PUFA content among all isolates was observed in nonpigmented *Aurantiochytrium* sp. LK3 (179.64 mg/g), and the highest PUFA yield was found in nonpigmented *Aurantiochytrium* sp. SK11 (1722.07 mg/L) (**Table 1**, **Figure 4**).

The difference in the content of total fatty acids (mg/g) of the nonpigmented and pigmented *Aurantiochytrium* spp. can be clearly distinguished in **Figure 5**. As shown in **Figure 5**, the first two principal components explained 99.91% of the total variability. Among total saturated fatty acids (SFA, mg/g), monounsaturated fatty acids (MUFA, mg/g), and polyunsaturated fatty acids (PUFA, mg/g), the major contributing factor to the separation on PC1 was SFA and PUFA, which accounted for 70.31% of the total variation. PC2 accounted for 29.60% of the total variation; the major factor contributing to the separation was MUFA. The result suggested that the major differences in fatty acid profiles of nonpigmented and pigmented *Aurantiochytrium* spp. were their contents of 15:0, 13:0, 16:0, 17:0, and DHA, which is the major PUFA in both nonpigmented and pigmented *Aurantiochytrium* spp. As triacylglycerol is the dominant lipid fraction (>90%) in *Aurantiochytrium* (formerly known as *Schizochytrium*) (*16*), the abundance of SFA and PUFA in the two *Aurantiochytrium* groups could be explained by a structural model of triacylglycerol in thraustochytrids proposed by Ashford, Barclay, Weaver, Giddings & Zeller (*25*) In this model, PUFAs, that is, DHA and DPA, were preferentially esterified in the sn-2 position of the glycerol backbone of triacylglycerol while SFA, that is, 14:0, 15:0, 16:0 oriented toward the 1,3 positions. This model implied that both nonpigmented and pigmented *Aurantiochytrium* spp. may possibly arrange DHA as the major PUFAs esterified in the sn-2 position, whereas due to the difference in the abundance of SFA in the nonpigmented and pigmented *Aurantiochytrium* spp, 16:0 is the preferred source to esterify in the 1,3 positions of triacylglycerol in the nonpigmented *Aurantiochytrium* spp., while the pigmented *Aurantiochytrium* spp. prefers 15:0 because of its high abundance.

**DHA Content and Yield.** DHA is one of the major high-value products of thraustochytrids. It was found to accumulate in great amounts with possible roles in providing energy, protecting cell from oxidative damage induced by environmental stimuli, and maintaining membrane functions in thrasutochytrid cells (*26*, *27*). In this study, the DHA content and DHA yield of the thraustochytrid strains are presented in **Table 1** and **Figure 4**, respectively. The DHA content of nonpigmented strains (98.71−144.23 mg/g) was much higher than pigmented *Aurantiochytrium* spp. (33.98−67.35 mg/g). A similar pattern was found in DHA yield. That is, the DHA yield ranged from 842.94 to 1365.82 mg/L for nonpigmented *Aurantiochytrium* spp. and 120.50 to 390.61 mg/L for pigmented strains. These findings showed that nonpigmented *Aurantiochytrium* spp. would be a preferred candidate for DHA production.

In addition, it was doubtful whether the variation of PUFA content, in particular, the DHA content of *Aurantiochytrium* spp., could be influenced by the difference in water environments of different mangrove areas. In Hong Kong, most mangrove stands are found in the Deep Bay region and Sai Kung districts which are located in the western and eastern sides of Hong Kong, respectively. The Deep Bay region is greatly influenced by the influx of nutrient-rich water in the Pearl River estuary and water low in salinity, all of which are conducive to mangrove growth. By contrast, the Sai Kung district is subject to the influence of oceanic water, making the coastal conditions less favorable for the proliferation of mangroves (*14*). In this study, the 25 thraustochytrid strains used were isolated from different mangrove areas in the Sai Kung and Deep Bay regions. As shown in **Table 1**, the PUFA content of the isolated strains were not significant difference among different sites of isolation ($p > 0.05$). Therefore, it is likely that the difference in the parameters of water environment such as salinity did not have a significant effect on the PUFA and DHA contents of *Aurantiochytrium* spp., a frequently isolated genus of thraustochytrid from Hong Kong mangroves. The variations in their content and yield of PUFA and DHA might significantly relate to the group of *Aurantiochytrium* spp., as shown by the results of this study.

In summary, we studied and compared the fatty acid profiles and heterotrophic growth parameters of PUFA-producing nonpigmented and pigmented *Aurantiochytrium* strains newly isolated from Hong Kong mangroves. The major differences in their fatty acid profiles of these two groups of *Aurantiochytrium* spp. were their compositions of SFA and PUFA, that is, 15:0, 13:0, 16:0, 17:0, and DHA. Because of the high biomass concentration and DHA yield, the nonpigmented *Aurantiochytrium* spp. was regarded as the best candidate for commercial DHA production. Further studies on this group could try to decrease the production cost by incorporating industrial waste in the medium as this strategy will not only reduce the cost of raw material but also reduce environmental waste to achieve the goal of global sustainable development. For the pigmented *Aurantiochytrium* strains, although they are not regarded as having a high potential in producing PUFA due to their low biomass concentration and DHA content in glucose yeast-extract medium, they may have the potential to produce pigments (*28*). Our preliminary analysis indicated that the pigmented strains possess a certain amount of carotenoids, that is, adonirubin, astaxanthin, beta-carotene, canthaxanthin, and echinenone (data not shown). However, optimization steps need to be carried out in future studies to maximize the production of the target carotenoids in the potential

strains. At the same time, its relationship with lipid production should be considered as lipid is naturally synthesized in this microalga in large amounts. The biosynthesis of carotenoids may be positively related to lipid metabolism as carotenoids are lipid-soluble pigments.

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