Thraustochytrid as a Potential Source of Carotenoids

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ABSTRACT: Thraustochytrids, marine protists whose dominant genera are *Thraustochytrium* and *Schizochytrium,* belong to the kingdom Chromista and are known as an industrial source of DHA. We describe here that thraustochytrid strain KH105, isolated as a DHA producer, also accumulates significant levels of β-carotene and xanthophylls including canthaxanthin and astaxanthin. A 4-d cultivation using a medium composed of 10% glucose and less than 0.3% of nitrogen sources in a half-concentration of seawater gave an astaxanthin production up to 6.1 mg/L, and canthaxanthin content reached more than 10 mg/L under conditions where a higher concentration of nitrogen sources (6%) was employed. It might be advantageous in mass production systems for these carotenoids to be extracted readily by simply suspending the cells with organic solvents such as acetone and chloroform. Analyses on the morphological and life history features of the KH105 strain revealed that it belongs to the genus *Schizochytrium.* This particular species of thraustochytrids is thus considered to be a promising source of xanthophylls as well as DHA for use in the food industry.

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KEY WORDS: Astaxanthin, canthaxanthin, β-carotene, carotenoid, docosahexaenoic acid, *Schizochytrium*, thraustochytrid, xanthophyll.

Oxygenated carotenoids such as astaxanthin (3,3′-dihydroxy-β,β′-carotene-4,4′-dione) and canthaxanthin (β,β′-carotene-4,4′-dione) are widely distributed in nature, especially in marine animals, and are best known as sources of the pinkish-red colored pigmentation in salmonids, crustaceans, and flamingoes (1). These xanthophylls also play important physiological roles in humans, including activation of the immune system (2) and protection against peroxidation of cellular lipids and against induced carcinogenesis (3), most likely by their scavenging of oxygen radicals (1). In addition, xanthophylls are an indispensable factor in the maintenance of the visual system. Since mammals can metabolize but not synthesize carotenoids, these must be supplied in the diet and as supplements.

Natural sources of astaxanthin that can be used as dietary supplements have been found in oils from krill, crawfish, and the yeast *Phaffia rhodozyma*. However, astaxanthin concentration in these oils is relatively low, ranging from 0.1 to 0.4% (4). In contrast, cyst cells of the green microalga *Haematococcus pluvialis* and its mutant strains contain astaxanthin at up to 3% of biomass and have been successfully used in industrial applications (5,6). The carotenoid composition of *Haematococcus*, of which astaxanthin accounts for more than 80%, would appear to be advantageous for concentration and mass production. However, *Haematococcus* is an obligate photoautotroph and grows slowly relative to the heterotrophs, and this hinders any increase in productivity. Although some marine bacteria such as *Agrobacterium* sp. and *Alcaligenes* sp. are reported to produce ketocarotenoids, it would be necessary to find a way to improve their productivity dramatically (7).

In this paper, we present for the first time a thraustochytrid as a new carotenoid producer that has the potential to meet market demand. Thraustochytrids are marine protists that have been classified into the class Labyrinthula of the kingdom Chromista (8,9) and include genera such as *Thraustochytrium*, *Aplanochytrium*, *Japonochytrium*, *Ulkenia*, and *Schizochytrium.* They are characterized by the presence of sagenogenetosome, an ectoplasmic net, a cell wall with noncellulosic scales, and a life cycle composed of vegetative cell, zoosporangium, and zoospore (8). This group of microbes is regarded as a promising source of PUFA, especially DHA (22:6n-3) (10,11). Our new isolate KH105, which produces carotenoid-like pigments, was considered to be a thraustochytrid due to its FA profile and molecular phylogenetic allocation based on the nucleotide sequence of 18S rRNA (12). The further identification of this strain and its production of carotenoids were examined in this study.

EXPERIMENTAL PROCEDURES

Microorganism and culture condition. The strain KH105 was selected from about 300 marine microbes that were isolated by probing with pine pollen from seawater samples collected in the bay area of Seto Inland Sea in Japan (11). The isolate was maintained on agar plate media composed of 8.5 g/L cornmeal agar and 7.5 g/L bacto agar (Difco Laboratories, Detroit, MI) in a half-salt concentration of artificial seawater ($^1/_2$ ASW; 20 g/L sea salts; Sigma, St. Louis, MO) at 15°C. A culture medium contained 30 g glucose, 5 g yeast extract, and 15 g polypeptone

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TABLE 1
FA Compositions of a New Isolate KH105 and *Schizochytrium limacinum* SR21 (16)

	Principal FA (% of total FA)										
Strain	14:0	15:0	16:0	17:0	18:0	18:1	18:2n-6	20:4n-6	20:5n-3	22:5n-6	DHA
KH105	6.6	7.1	32.3	5.6	0.6	0.4	0.3	0.6	1.6	9.9	34.9
SR21	3.4	6.0	49.4	—	1.2	0.0	0.0	Trace	0.8	6.3	32.5

(Difco) in 1 L of ½ ASW, unless otherwise indicated. Cultivation was performed in a 200-mL baffled flask containing 50 mL of the medium at 28°C for 2–7 d with rotary shaking at 160 rpm. When applicable, the culture was illuminated at 8000 lux.

Purification of carotenoids. Cells were harvested by centrifugation at 3000 × *g* and washed with deionized water. The endopigments were extracted by suspending the cells in an acetone/methanol mixture (7:3, vol/vol) at 5–10 vol of wet cells. The extract was dehydrated by anhydrous sodium sulfate and then concentrated to dryness by evaporation. The pigments were immediately dissolved with a small amount of acetone and stored in the dark at −20°C. To fractionate the carotenoids, the pigments were redissolved in hexane/chloroform (200:3, vol/vol) and loaded on a Sep-Pak silica cartridge (1 mL; Waters, Tokyo, Japan) that was prewashed sequentially with 4 mL of hexane/chloroform (96:4, vol/vol) and 12 mL of hexane. The acylglycerides and steryl esters were eluted with 12 mL of hexane/chloroform (96:4, vol/vol). The column was weakly acidified with 12 mL of hexane/acetate acid (100:0.2, vol/vol) and then FFA and β-carotene were eluted with the same volume of hexane/chloroform/acetate acid (100:2:0.2, by vol). The xanthophylls were recovered in a fraction eluted with hexane/chloroform/acetate (60:40:0.2, by vol). After evaporating the solvent, the carotenoids were dissolved in hexane. Purification of the xanthophyll components was carried out by HPLC on a silica gel column (YMC-Pack SIL-06, 5 μm, 4.6 × 250 mm; YMC, Kyoto, Japan) using acetone/hexane (2:8, vol/vol) as a solvent at a flow rate of 1 mL/min. Eluates were collected by monitoring the absorbance at 480 nm using a Shimadzu SPD-6AV spectrophotometric detector.

Analytical methods. The extinction characteristic of carotenoid in acetone was determined between 400 and 600 nm using a multiwavelength spectrophotometer. The concentration of carotenoids was calculated from the absorbance of the acetone solution at 478 nm with an extinction constant of 2500, which is equivalent to 1% astaxanthin (13). TLC was performed in the dark on a silica gel plate (Kieselgel 60; Merck, Darmstadt, Germany) using acetone/hexane (3:7, vol/ vol) as a developing agent, and the spots were analyzed by a densitometer. Identification of purified carotenoids was carried out with visible absorption spectra, atmospheric pressure chemical ionization MS (APCI-MS), and ^{1}H NMR spectra. The positive ion APCI-MS spectra were collected using a Hitachi M 1200-AP (Tokyo, Japan) set at a drift voltage of 60 V, a multiplier voltage of 1800 V, and a needle voltage of 3000 V with methanol as a solvent. The ^{1}H NMR (500 MHz) spectra were measured with a Varian Unity Inova 500 spectrometer (Palo Alto, CA) in $CDCl_3$ with tetramethylsilane as an internal standard. The composition of cellular FA was determined as described previously (14).

Morphology observation. A drop of fresh cell culture cultivated for 24 h at 28°C was transferred into the concave depression of a sterile glass plate and covered with a thin glass. Cells were continuously observed by microscope, and the images were captured by a charge-coupled device camera.

Assimilation test. Media used for the assimilation test were composed of 0.01% KH_2PO_4, 0.02% $(NH_4)_2SO_4$, 0.2% monosodium glutamate, 10^{-4}% thiamine HCl, 10^{-5}% vitamin B_{12}, 90% ASW, and various carbon sources at 0.08% carbon (15). The media were inoculated with zoospores gathered with pine pollen in ASW. The cultivation was performed in 20 mL of medium in a 50-mL Erlenmeyer flask at 25°C for 7 d. Cell growth was measured as absorbance at 650 nm using the spectrophotometer.

RESULTS AND DISCUSSION

Isolation of a DHA-producing marine microbe. The strain KH105 was incubated in a liquid medium for 2 d, and the FA composition of the total chloroform-extractable lipid was determined. Palmitic acid (16:0), docosapentaenoic acid (22:5n-6), and DHA were the principal components and accounted for 32.3, 9.9, and 34.9% of total FA, respectively (Table 1). In a preliminary experiment under a semioptimized condition, a 2-d cultivation of KH105 in a baffled flask yielded 11.5 g/L of dried cells, 5.3 g/L of total FA, and 1.2 g/L of DHA. The DHA productivity (0.6 g/L/d) was comparable to that of *S. limacinum* SR21 (~0.8 g/L/d) cultivated under a similar condition (16).

Identification of pigments produced by KH105. A characteristic of KH105 was that its liquid culture was muddy orange or yellow ochre in color, suggesting an accumulation of endopigments, which other thraustochytrids have never been reported to have produced. The pigments extractable with chloroform or acetone from KH105 cells were resolved on a thin-layer silica plate, forming five major bands with at least two minor bands (Fig. 1A). The component that produced band *a* was purified by a Sep-Pak cartridge and identified as β-carotene due to its mobility on TLC compared to the standard (R_f of 0.95) and its maximum visual absorbance (A_{max}) at 464 and 492 nm (in chloroform), showing a bright yellow color (13).

Other components were then further resolved by HPLC on a silica gel column (Fig. 1B). The spectroscopic characteristics of the four components that produced major peaks on the chromatogram were examined by TLC, spectrophotometer, and APCI-MS and the following were found: peak 1, R_f of 0.72

(band c in Fig. 1A), retention time (RT) at 2.5 min on HPLC, A_{max} at 470 nm; peak 2, R_f of 0.58 (band e), RT at 4.8 min, A_{max} at 482 nm, MS m/z = 565 ($M + H^+$), 537, 523; peak 3, R_f of 0.32 (band f), RT at 7.6, A_{max} at 480 nm, MS m/z = 581 ($M + H^+$); peak 4, R_f of 0.16 (band g), RT at 12.9, A_{max} at 485 nm, MS m/z = 597 ($M + H^+$). These data corresponded well with those of echinenone (β,β′-carotene-4-one), canthaxanthin, phoenicoxanthin (3-hydroxy-β,β′-carotene-4,4′-dione), and astaxanthin, respectively (13). Furthermore, ^{1}H NMR data of these compounds (data not shown) were also in agreement with previously published values (17).

Effect of culture conditions on carotenoid production. The concentrations of glucose and nitrogen sources (weight ratio of yeast extract and polypeptone was fixed at 1:3) in the culture medium notably affected the accumulation of carotenoids, as shown in Figure 2. When an increasing concentration (up to 10% by weight) of glucose was used for cultivation, the astaxanthin content reached 4.1 mg/L (20.2% of total carotenoid), and this was accompanied by a sharp decrease of β-carotene content to less than 1.5 mg/L (Fig. 2A). On the other hand, use of the low concentration of nitrogen sources examined (0.27%) further increased astaxanthin content up to 6.1 mg/L (28% of total carotenoid), but canthaxanthin content declined (Fig. 2B). The carotenoid profile was not significantly altered by a higher concentration of glucose (>10%), different initial pH (between 4 and 8), light irradiation, or the addition of phosphate salt (0.01–0.5% by weight) (data not shown).

A number of benefits from the intake of astaxanthin as a nutraceutical are derived from its antioxidative and chemoprotective nature. In this sense, canthaxanthin should also be an important nutrient since it displays a similar or slightly reduced level of antioxidant effects compared to astaxanthin (18). In fact, it has been reported that canthaxanthin also exhibits anticancer and immunomodulating activities both *in vivo* and *in vitro* (3,19). Although the bulk of this compound is used as a food colorant in eggs and farmed fish, the majority of it is chemically synthesized and there is no microbial system that is being used for industrial production. In our strain KH105, canthaxanthin was one of the major cellular carotenoids, and its content reached more than 10 mg/L using a medium with a higher concentration (6%) of nitrogen sources (Fig. 2B). Therefore, to meet the increasing demand for natural food materials,

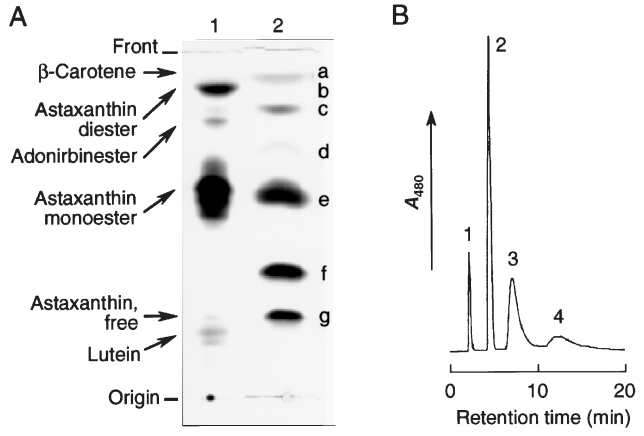


FIG. 1. Analysis of carotenoids produced by KH105. (A) Carotenoids from *Haematococcus pluvialis* (lane 1) and KH105 (lane 2) were resolved on a silica gel thin-layer plate with acetone/hexane (3:7). Identified components in *H. pluvialis* carotenoids are labeled on the left. (B) The xanthophyll fraction from KH105 was separated by silica HPLC.

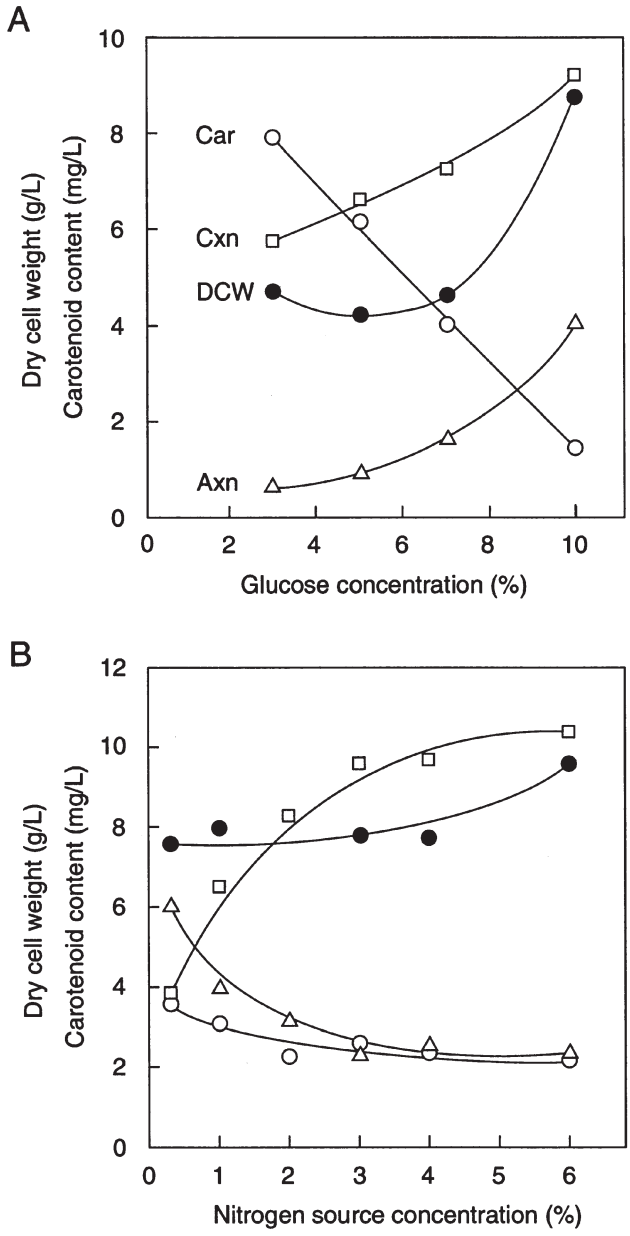
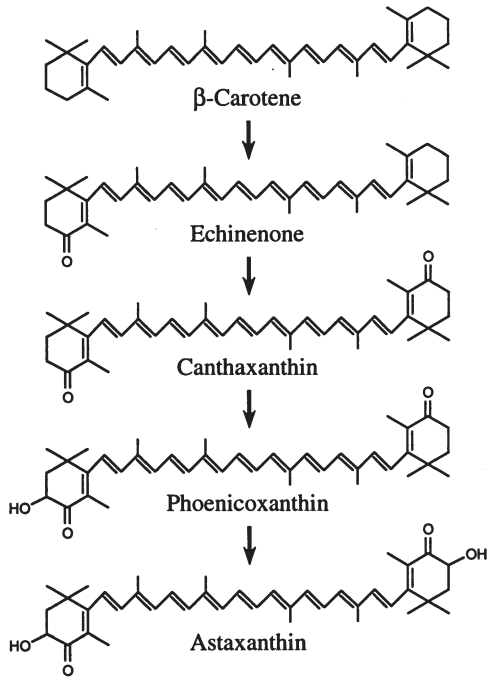


FIG. 2. Effects of glucose (A) and nitrogen concentration (B) in culture medium on the carotenoid production by KH105. Cultivation was performed at 28°C for 4 d. (A) Culture medium contained 0.5% yeast extract, 1.5% polypeptone, and the indicated concentration of glucose in one-half strength artificial seawater (1/2 ASW). Car, β-carotene; Cxn, canthaxanthin; Axn, astaxanthin; DCW, dry cell weight. (B) Culture medium contained 10% glucose and the indicated concentration of nitrogen sources (mixture of yeast extract and polypeptone at 1:3, by weight) in 1/2 ASW. Symbols are the same as in panel A.



SCHEME 1

the thraustochytrid could be a promising source of carotenoids rich in canthaxanthin as well as other xanthophyll pigments.

In light of the carotenoid composition, a possible pathway for astaxanthin biosynthesis in KH105 could be as depicted in Scheme 1. In this scheme, the precursor β-carotene would be oxygenated, first to synthesize echinenone and then canthaxanthin, by forming keto group(s), which would then be followed by the synthesis of phoenicoxanthin and then astaxanthin through continuous hydroxylation reactions. The corresponding reactions in marine bacteria (*Agrobacterium aurantiacum* and *Alcaligenes* sp.), *Erwinia uredovora*, and *H. pluvialis* have already been biochemically and/or genetically characterized and a biosynthetic pathway proposed (19,20). The introduction of the keto group(s) into β-carotene is catalyzed by an enzyme coded by the *crt*W/*bkt* gene, whereas the *crt*Z gene encodes an enzyme that governs the hydroxylation step (20). These reactions are strictly oxygen dependent (20). Since the accumulation of β-carotene and intermediate xanthophylls was significant in KH105 cells, both of these oxygen-dependent reactions could have been limiting steps when cultivation was performed in a flask, where the oxygen level could be limiting. Regulation of dissolved oxygen, which also affects cell growth and total oil content, by using a stirred tank fermentor could improve the production of xanthophylls. Moreover, it may be plausible to increase yields further by the isolation of mutants harboring increased activities of corresponding enzymes.

The thraustochytrid is an aerobic heterotroph that utilizes glucose, unlike such photoautotrophic microorganisms as cyanobacteria and *Haematococcus* alga. This characteristic offers an advantage, especially on an industrial scale, in that high-density tank cultivation is possible without the need for illumination. More interestingly, illumination of growing cultures of KH105 did not notably alter either the productivity or composition of the carotenoids. It has been recognized that a major role of the carotenoids in microbes is to scavenge oxygen radicals generated at least partly by photochemical reactions. The carotenogenic genes are induced by a high light intensity (20). Thus, our results suggest the possibility that carotenoids as a second metabolite are not essential for the normal growth of KH105. However, this result also led us to fear that maintaining KH105 under a condition without environmental stresses may result in the loss of its ability to synthesize carotenoids. Lowering the concentration of nitrogen sources in the presence of a high level of glucose could be an effective way to overcome this potential problem because it seemed to stimulate the metabolism of canthaxanthin with an accompanying increase in astaxanthin content without any loss of cell growth (Fig. 2B).

Identification of KH105. The FA profile of KH105 included high contents of C_{22} PUFA, very low levels of C_{18} and C_{20} FA, and the occurrence of odd-chain saturated FA (15:0 and 17:0), similar to that of *S. limacinum* SR21 (Table 1) (16). Neither KH105 nor SR21 produces n-3 octadecatetraenoic acid (18:4n-3), which is in contrast to a number of other DHA-producing microalgae harboring the photosynthetic system (21).

As was observed in SR21, the generation of ectoplasmic net elements was detected around the spherical or oval vegetative cells of KH105 on an agar plate. A matured zoosporangium of ~30 μm in diameter appeared to contain 8–30 zoospores (Fig. 3A) that were later released. The heterokont zoospores are 2–5 μm in diameter. Continuous observation revealed a successive bipartition of a vegetative cell to form a tetrad (Fig. 3B, panels 1–3) and eventually a cluster of cells (Fig. 3B, panels 4–6). Some of these cells formed new zoosporangia. These morphological and life history profiles of the strain KH105 coincide with characteristics of the genus *Schizochytrium* in the class Labyrinthula of the phylum Heterokonta (stramenopiles) in the kingdom Chromista (8,9). We note that microbes of related genera in the class Labyrinthula, such as *Ulkenia*, *Japonochytrium*, and most *Thraustochytrium*, do not undergo the repeated binary division of vegetative cells, but propagate solely by generating zoospores. However, the formation of limaciform (amoeboid) cells, a specific feature of *S. limacinum* (15), was not observed in KH105 under any conditions examined.

Strains KH105 and SR21 showed a similar profile of assimilation of various carbon sources, such as D-glucose, D-fructose, and glycerol (Table 2), except that only KH105 could utilize L-arabinose to grow. This profile is distinct from those of other *Schizochytrium* species: For instance, *S. aggregatum* ATCC 28209 produces a substantial amount of arachidonic acid (20:4n-6) and can propagate in a medium containing maltose or cellobiose, but not D-fructose, as a sole carbon source (22). These data further implied that KH105 is very close to SR21 and supported its assignment to the genus *Schizochytrium*; however, KH105 is not identical to any of the known species of this genus.

Since, to the best of our knowledge, this is the first report describing a thraustochytrid that produces carotenoids, we

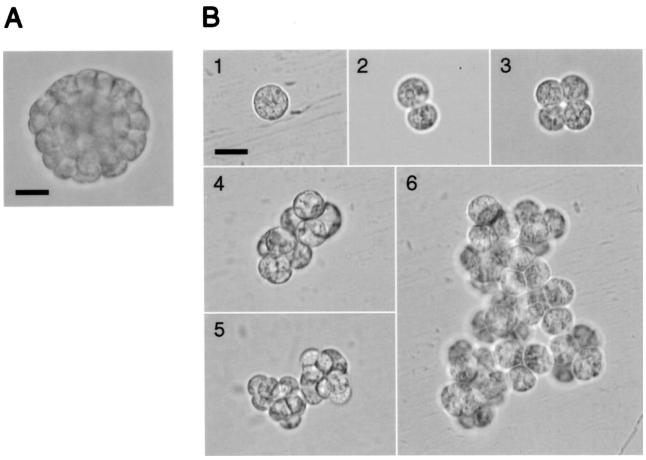


FIG. 3. Microphotographs of KH105 cells. (A) Zoosporangium. (B) Successive binary division of vegetative cells perceived by a continuous observation. Bar in panel A equals 10 μm and applies to all panels.

conducted the identification of our isolate carefully. Based on the presence of an ectoplasmic net, generation of zoospores with flagella, and successive binary division of vegetative cells without motility, in addition to its FA composition, KH105 was identified as belonging to the genus *Schizochytrium*. Our preliminary data from molecular phylogenetic analysis on 18S rRNA supported this identification (data not shown). However, at this stage, one must remain cautious, as the molecular phylogeny of thraustochytrids does not totally agree with the morphological classification (23). The consolidation of the classification system is thus urgently required.

Implication. One of the most important factors in a microbial production system is the efficiency of the downstream processing. In the cases of *Haematococcus* and *Phaffia*, difficulties have been encountered in extracting the cellular pigments owing to their "rigid" cell walls (4); therefore, disruption of the cells by freeze–thaw and/or a physical procedure is necessary. In contrast, the pigments accumulated in KH105 could be easily and completely recovered by simply suspending the cells in acetone. This difference in procedure may arise partly from the difference in the structure of the cell walls.

TABLE 2
Profiles of Carbon Source Assimilation for the Strain KH105 and Related Strains

Carbon source[a]	KH105	*S. limacinum* (15)	*S. aggregatum* (26)	*S. aggregatum* (22)
D-Glucose	+	+	+	+
D-Fructose	+	+	–	–
D-Mannose	+	+	–	+
D-Galactose	+	+	–	+
L-Arabinose	+	–	–	–
Maltose	–	–	+	+
Cellobiose	–	–	+	+
Soluble starch	–	–	+	+
Glycerol	+	+	–	+

[a]D-Ribose, D-xylose, lactose, sucrose, D-melibiose, D-raffinose, D-trehalose, and urea were not notably assimilated by any strains.

Although the daily production maximum of astaxanthin determined in the present study (1.5 mg/L/d) was not greater than the reported values of *H. pluvialis* (1.9 mg/L/d) (5) or of mutant strains from *P. rhodozyma* (0.9–2.0 mg/L/d) (24), the ease of processing as well as the simple culture condition should make it the preferred option in a mass production system.

Thraustochytrids are known to be a promising source of PUFA, especially DHA, which is an indispensable factor for infant growth and development of healthy visual and nervous systems. Owing to the biological efficacy of DHA in anticarcinogenesis and prevention of Alzheimer's disease and allergic diseases, there are growing demands for it to be used as a food additive (25). Lipids rich in DHA also have been used as an essential nutrient in animal and fish foods. Accordingly, our strain should become highly valued by the food industry as a primary source of both DHA and xanthophylls. Synergistic biological effects could also be expected owing to the antioxidative action of xanthophylls on DHA, which is likely to be oxidized.

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