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Eicosapentaenoic and docosahexaenoic acids production by and okara-utilizing potential of thraustochytrids

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Nine thraustochytrid strains isolated from subtropical mangroves were screened for their eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) production potential in a glucose yeast extract medium. Their ability to utilize okara (soymilk residue) for growth and EPA and DHA production was also evaluated. EPA yield was low in most strains, while DHA level was high on glucose yeast extract medium, producing 28.1–41.1% of total fatty acids, for all strains, with the exception of *Ulkenia* sp. KF13. The DHA yield of *Schizochytrium mangrovei* strains ranged from 747.7 to 2778.9 mg/l after 52 h of fermentation at 25°C. All strains utilized okara as a substrate for growth, but DHA yield was lower when compared with fermentation in a glucose yeast extract medium. *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 199–202.

Keywords: subtropical mangroves; labyrinthulomycota; polyunsaturated fatty acids

Introduction

Long-chain polyunsaturated fatty acids (PUFAs) of the omega-3 series, e.g., eicosapentaenoic (EPA, C20:5$n-3$) and docosahexaenoic acids (DHA, C22:6$n-3$), have attracted much research attention due to their beneficial nutritional effects in the treatment of arteriosclerosis, cancer and rheumatoid arthritis [8].

At present, marine fish are the main commercial source of omega-3 PUFAs. With an increasing human population, this source cannot satisfy the world's future requirements for omega-3 fatty acids for health care [21]. Therefore, efforts have been made to seek eukaryotic marine microorganisms as alternative sources for omega-3 fatty acid production [21]. Thraustochytrids, a group of marine protists, are capable of heterotrophic growth and are potential omega-3 producers for industrial use, especially members of the genera *Schizochytrium* and *Thraustochytrium* [2,16,20].

Recently, there has been increasing interest in converting or upgrading food processing waste into higher value products [7] and in obtaining valuable single cell oil from microbial cells [13]. One such waste that is being explored for fermentation is okara, a residue from soymilk production. Okara is nutritionally rich, with 52% carbohydrate, 27% protein and 12% fats [10], and has been explored for fermentation by several microbes to produce metabolites and feeds [11].

The aims of the present work are to isolate, identify and screen thraustochytrids from Hong Kong mangroves for high yields of DHA or/and EPA, and to evaluate the ability of selected thraustochytrid strains to utilize and/or ferment "okara" for DHA or/and EPA production.

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Materials and methods

Preparation of initial inoculum

Nine thraustochytrid strains from three genera isolated from decaying *Kandelia candel* leaves in the intertidal zone of the mangroves were used in this study (Table 1). For each strain, a zoospore suspension for inoculum was prepared by following the procedure previously described [3].

Heterotrophic growth

Nine thraustochytrid strains were grown in 250-ml flasks with 50 ml broth. For each strain, triplicate flasks were incubated and shaken at 200 rpm at 25°C in a glucose yeast extract medium or okara medium for 52 h and 4 days, respectively. The glucose yeast extract medium was composed of 60 g of glucose (Sigma Chemical, St. Louis, MO), 10 g of yeast extract (Oxoid, Basingstoke, Hampshire, England, UK) and 1 l of 15‰ artificial seawater (ASW) prepared from sea salts (Sigma) at pH 6 and was used for screening thrausotchytrids for DHA and EPA production. The okara medium was composed of 10 g of pulverized okara and 1 l of 15‰ ASW at pH 6, and was used to evaluate the ability of thraustochytrid strains to utilize okara. Okara was collected from Nestles' Dairy Farm (Hong Kong, China), dried in an oven at 60°C for 24 h and blended for 10 min to fine particles with a two-speed blender.

Dry weight determination

For dry weight biomass determination, the entire content (50 ml) of a flask was transferred to a pre-weighed centrifuge tube and harvested by centrifugation at 3500×g for 10 min and the supernatant discarded. Harvested cells were subsequently washed with 50 ml of sterile distilled water, followed by manual continuous agitation for 10 min. Selected samples were examined microscopically to check for cell integrity and no morphological disruption was observed. This rinsing–centrifugation process was repeated three times, then the washed cells

Table 1 Thraustochytrid strains isolated from mangroves in Hong Kong

Strain	Site of isolation	Date of isolation	Substrate[a]	Salinity (‰)[b]
Schizochytrium sp. KF1	Three Fathoms Cove	09/29/97	Brown	5
S. mangrovei Raghuk KF2	Three Fathoms Cove	09/29/97	Brown	5
S. mangrovei Raghuk KF4	Three Fathoms Cove	09/29/97	Brown	5
S. mangrovei Raghuk KF5	Three Fathoms Cove	18/10/97	Yellow	11
S. mangrovei Raghuk KF6	Mai Po	25/11/97	Black	11
S. mangrovei Raghuk KF7	Mai Po	25/11/97	Black	11
T. striatum Schneider KF9	Mai Po	25/11/97	Black	11
Ulkenia sp. KF13	Ting Kok	21/01/98	Brown	34
S. mangrovei Raghuk KF14	Ho Chung	18/3/98	Brown	13

[a]All thraustochytrids were isolated from submerged *K. candel* leaves at various stages of decay (yellow, brown or black) in a mangrove.
[b]Salinity of water in which the leaves were collected.

were freeze-dried for 24 h before weighing them. Biomass was expressed as milligram of freeze-dried weight per 50 ml of medium.

Chemical analysis

The carbon and nitrogen contents of okara were analyzed using the CHN-900 carbon, hydrogen and nitrogen determinator (Model 600-800-300) from LECO® (St. Joseph, MI). Two milligrams of each sample was weighed in tin containers and samples were ignited in the analyzer for carbon and nitrogen determination.

Fatty acid analysis

Fatty acid composition was determined following a modified procedure of Lepage and Roy [9]. Intact freeze-dried cells (50–60 mg) and fermented okara (80 g–100 mg) were methylated by a direct acid-catalyzed transesterification in 2 ml of 4% sulfuric acid in methanol (100°C for 1 h) without prior extraction of the total lipids. An internal standard of 3 mg of heptadecaenoic acid (17:0) and a magnetic stirring bar were added to each Teflon-lined screw cap test tube before methylation. After the contents had cooled, 1 ml of water and 1 ml of hexane were added. The fatty acid methyl esters (FAMEs) in the hexane layer were vortexed, centrifuged and collected. The esters were then stored at 4°C prior to injection into the gas–liquid chromatograph for analysis with a Hewlett Packard HP-6890 GC (Hewlett-Packard, Palo Alto, CA) equipped with a flame ionization detector and a Supelco Omegawax™ 250 capillary column (30 m×250 μm). Nitrogen was used as the carrier gas and the flow rate was kept at 2 ml/min. A volume of 5 μl was injected under splitless injection mode. The injection port temperature was 280°C, the detector was at 300°C and the column temperature was held at 280°C for 50 min. Fatty acids were identified by comparison of the relative retention times with those of standards (Sigma) and the fatty acid contents were quantified by comparing their peak areas with that of the internal standard (C17:0).

Results

Heterotrophic growth, fatty acid profiles and EPA/DHA yields

Biomass production, fatty acid profiles and EPA/DHA yields obtained from the nine thraustochytrids grown on glucose yeast extract medium are summarized in Table 2. All strains showed

Table 2 Fatty acid composition (percent of total fatty acids)[a], DHA yields and biomass production of freeze-dried thraustochytrids after culturing for 52 h at 25°C in a glucose yeast extract medium[b]

	Fatty acid												DHA		Biomass production (g/l±SE)
	14:0[c]	15:0	16:0	18:0	18:1	18:2	18:3	20:4	20:5	22:4	22:6	Others	Yield (mg/l)	In biomass (mg/g)	
Schizochytrium sp. KF1	3.6	38.9	10.5	0.3	0.0	0.0	0.3	0.6	1.0	12.5	28.1	4.2	170.5	37.0	4.6±0.42
S. mangrovei KF2	4.2	3.3	47.7	1.2	0.0	0.0	0.1	0.3	0.3	6.4	35.3	1.2	2778.9	208.8	13.3±0.27
S. mangrovei KF4	3.4	7.8	40.1	1.1	0.0	0.0	0.1	0.5	0.4	6.5	38.5	1.4	1536.1	174.9	8.8±0.43
S. mangrovei KF5	2.9	6.4	38.0	1.0	0.0	0.0	0.1	0.8	0.5	8.0	40.5	1.8	1153.2	147.6	7.9±0.41
S. mangrovei KF6	3.6	4.7	38.8	1.0	0.0	0.0	0.1	0.8	0.4	7.9	41.1	1.7	2762.0	204.3	13.5±0.10
S. mangrovei KF7	3.9	6.7	48.1	1.2	0.0	0.0	0.1	0.4	0.3	7.0	31.1	1.3	747.7	118.1	6.6±0.96
T. striatum KF9	0.0	7.9	6.7	0.0	0.0	0.0	0.0	0.0	23.3	8.6	36.6	16.9	13.6	16.0	0.9±0.04
Ulkenia sp. KF13	3.7	10.7	22.8	11.7	18.2	4.3	0.5	1.7	2.3	1.4	4.0	18.1	26.3	5.5	4.7±0.34
S. mangrovei KF14	3.7	9.4	39.5	0.9	0.0	0.0	0.2	0.4	0.3	6.9	37.5	1.2	1809.5	185.2	8.1±0.07

[a]Data are expressed as means of triplicate flasks.
[b]Medium composed of 60 g of glucose and 10 g of yeast extract in 1 l of 15‰ ASW at pH 6.
[c]14:0, myristic acid, tetradecaenoic acid; 15:0, pentadecyclic acid, pentadecaenoic acid; 16:0, palmitic acid, hexadecaenoic acid; 18:0, stearic acid, octadecaenoic acid; 18:1, oleic acid, *cis*-9-octadecenoic acid; 18:2($n-6$), linoleic acid, *cis*-9,12-octadecadienoic acid; 18:3($n-3$), α-linolenic acid, 9,12,15-octadecatrienoic acid; 20:4($n-6$), arachidonic acid, *cis*-5,8,11,14-eicosatetraenoic acid; 20:5($n-3$), EPA *cis*-5,8,11,14,17-eicosapentaenoic acid; 22:4, *cis*-7,10,13,16-docosatetraenoic acid; 22:6($n-3$) DHA *cis*-4,7,10,13,16,19-docosahexaenoic acid.

Table 3 Fatty acid composition (percent of total fatty acids)[a], DHA yields and biomass production of freeze-dried fermented okara (okara plus thraustochytrids) after culturing for 4 days in okara medium[b]

	Fatty acid												DHA		Biomass production (g/l±SE)
	14:0[c]	15:0	16:0	18:0	18:1	18:2	18:3	20:4	20:5	22:4	22:6	Others	Yield (mg/l)	In fermented okara (mg/g)	
Schizochytrium sp. KF1	0.0	0.0	14.1	3.9	27.2	48.3a[f]	6.0a	0.0	0.0	0.0	0.5	0.0	6.2	0.8	7.84±0.03
S. mangrovei KF2	0.0	0.0	15.7	3.5	23.9	46.3a	6.1a	0.0	0.0	0.5	4.1	0.0	57.3	7.5	7.67±0.17
S. mangrovei KF4	0.0	0.0	16.4	3.4	23.5	45.6a	5.6a	0.0	0.0	0.7	4.9	0.0	72.1	9.4	7.68±0.09
S. mangrovei KF5	0.0	0.0	14.0	3.3	25.1	47.0a	6.2a	0.0	0.0	0.6	3.7	0.0	55.0	7.0	7.86±0.19
S. mangrovei KF6	0.0	0.0	15.7	3.4	24.0	46.5a	6.2a	0.0	0.0	0.5	3.8	0.0	54.9	7.3	7.54±0.02
S. mangrovei KF7	0.0	0.0	15.3	3.3	24.3	47.8a	6.5a	0.0	0.0	0.5	2.3	0.0	35.3	4.7	7.51±0.04
T. striatum KF9	1.6	0.0	11.7	2.9	24.2	45.6a	6.2a	0.7	1.7	1.8	3.0	0.5	25.6	4.3	5.88±0.38
Ulkenia sp. KF13	0.4	0.0	11.7	3.4	25.0	45.8a	6.0a	0.3	1.3	1.7	3.9	0.5	24.3	4.3	5.62±0.05
S. mangrovei KF14	0.1	0.0	16.4	3.7	24.4	44.1a	5.3a	0.0	0.0	0.9	4.9	0.0	67.9	9.0	7.54±0.04
Control[d]	0.0	0.0	11.8	2.3	24.7	54.0b	7.1b	0.0	0.0	0.0	0.0	0.0	0.0	0.0	7.96±0.24
Control[e]	0.0	0.0	11.5	3.1	26.0	52.4b	7.1b	0.0	0.0	0.0	0.0	0.0	0.0	0.0	7.51±0.24

[a]Data are expressed as means of triplicate flasks.
[b]Okara medium composed of 10 g of homogenized okara in 1 l of 15‰ ASW at pH 6.
[c]14:0, myristic acid, tetradecaenoic acid; 15:0, pentadecylic acid, pentadecaenoic acid; 16:0, palmitic acid, hexadecaenoic acid; 18:0, stearic acid, octadecaenoic acid; 18:1, oleic acid, *cis*-9-octadecenoic acid; 18:2($n-6$), linoleic acid, *cis*-9,12-octadecadienoic acid; 18:3($n-3$), α-linolenic acid, 9,12,15-octadecatrienoic acid; 20:4($n-6$), arachidonic acid, *cis*-5,8,11,14-eicosatetraenoic acid; 20:5($n-3$), EPA *cis*-5,8,11,14,17-eicosapentaenoic acid; 22:4, *cis*-7,10,13,16-docosatetraenoic acid; 22:6($n-3$), DHA *cis*-4,7,10,13,16,19-docosahexaenoic acid.
[d]Freeze-dried unfermented okara before incubation.
[e]Freeze-dried unfermented okara after 4 days of incubation.
[f]Mean values with the same letter are not significantly different ($P<0.05$).

growth in the glucose yeast extract medium after 52 h of incubation. *Schizochytrium* species grew better than *Thraustochytrium* and *Ulkenia* species. *Schizochytrium mangrovei* strains produced cell biomass ranging from 6.6 to 13.5 g/l, with *S. mangrovei* KF2 and KF6 yielding the highest biomass at 13.3 and 13.5 g/l, respectively. *Thraustochytrium striatum* KF9 grew poorly with 0.8 g/l of biomass while *Ulkenia* sp. KF13 yielded 4.6 g/l.

Total fatty acid profiles of the nine thraustochytrids are characterized as follows: (1) most strains had a relatively high level (as percent of total fatty acids) of even-chain saturated fatty acids, mainly palmitic acid (16:0, 6.7–47.4%); (2) pentadecyclic acid (15:0, 3.3–38.9%), an odd-chained saturated fatty acid, was found in all strains; (3) 18-carbon fatty acids, especially oleic and linoleic acids, were absent in most strains with the exception of *Ulkenia* sp. KF13; and (4) PUFA level was high in most species with DHA accounting for 4.0–40.5%.

S. mangrovei strains produced high levels of palmitic acid (16:0) and DHA, accounting for 38.0–48.1% and 31.1–41.1% of the total fatty acids, respectively (Table 2). The DHA content of all *S. mangrovei* was high, ranging from 118.1% to 208.8 mg/g. *S. mangrovei* KF2 and KF6 were exceptionally good DHA producers with a DHA content of 208.8 and 204.3 mg/g, respectively, and DHA yield of 2778.9 and 2762.0 mg/l, respectively. Both *T. striatum* KF9 and *Ulkneia* sp. KF13 are the only strains that produced EPA in significant amounts, at 10.1 and 3.1 mg/g, respectively.

Okara utilization with EPA and DHA production by thraustochytrids

All thraustochytrid strains utilized "okara" medium for growth and DHA production after 4 days of fermentation (Table 3). *S. mangrovei* strains contained DHA content in fermented okara from 4.7 to 9.4 mg/g. EPA production was also detected in *T. striatum* KF9 and *Ulkenia* sp. KF13, yielding 2.4 and 1.5 mg/g, respectively. *Schizochytrium* strains produced no EPA in okara medium even though detectable levels were observed in the glucose yeast extract medium (data not shown). The fatty acid profiles of the control unfermented okara, either before or after 4 days of incubation, consisted mainly of palmitic acid (16:0) and 18-carbon fatty acids with linoleic acid (18:2) as the major acid. No PUFAs with 20 carbon chains or higher were detected. Two important changes in the fatty acid profiles were observed after fermentation: (1) PUFAs were detected, especially DHA, varying from 0.5% to 4.9% of the total fatty acids; (2) the unsaturated fatty acid content of the 18-carbon series, namely linoleic and linolenic acids, was significantly lower ($P<0.05$) than their content in unfermented okara when compared using Dunnett's test at $\alpha=0.05$ [23]. Biomass of the fermented okara (plus thraustochytrid cells) after 4 days of incubation with okara was similar to that of the unfermented controls, but weight losses were observed when fermented with *T. striatum* KF9 and *Ulkenia* sp. KF13 (Table 3).

Discussion

DHA and EPA production potential

S. mangrovei strains in this study were good DHA producers, yielding 204 mg/g freeze-dried thraustochytrid or 2762 mg/l in glucose yeast extract medium (Table 2). Only *S. limacinum* SR21 produced a comparable quantity of DHA, resulting in 276.5 mg/g in biomass and yield of 13 g/l in a fermenter under optimized conditions [20]. *S. limacinum* SR21, as with the *S. mangrovei* strains in our study, was isolated from a subtropical mangrove [5]. Bowles *et al* [2] recently screened 57 thraustochytrid strains isolated from both temperate and subtropical areas. Their results showed that subtropical strains were high biomass producers with moderate DHA levels (4–34%), while their temperate counterparts produced higher DHA

levels (17–47%) but low biomass production. In general, the present study agrees with the observations of Bowles *et al* [2] in which subtropical strains were high biomass producers with moderate DHA production. Vazhappilly and Chen [18] obtained a DHA yield of 1 mg/l for *T. aureum* ATCC 28211 grown under autotrophic conditions (0.1 g/l biomass after 23 days) but the production of DHA in 5% glucose was only 4.0 mg/l [19]. This illustrates that the heterotrophic mode of growth is better for thraustochytrids in both biomass and DHA production. Strains of *S. mangrovei*, especially KF6, are potential DHA producers for industrial use because of its relatively high biomass with palmitic acid and DHA as the dominant fatty acids. Further studies should aim at optimisation of growth conditions by manipulating the medium components, pH, temperature and aeration, and other factors that are important in promoting DHA production under laboratory conditions [6,16].

Fermented okara

Freeze-dried okara contained approximately 47% carbon and 4.5% nitrogen when analyzed with the CHN determinator, while other minerals and vitamins are also present [11]. Together, these components are able to support growth and DHA production of the test strains. Specifically, some of the components may be preferentially utilized. Both cellulose (5.6% of the fiber content of okara) [4] and starch could support growth since thraustochytrids have been shown to be cellulase and amylase producers [12].

A decrease in the linoleic and linolenic acid content of okara after fermentation could be attributed to utilization by the thraustochytrids because the test stains contained no linoleic acid and only trace amounts of linolenic acid, with the exception of the *Ulkenia* sp. (Table 3). *S. limacinum* SR21 and *Thraustochytrium* sp. ATCC 20892 were able to grow and produce DHA in linseed oil, *Linum usitutissimum* (L.) [15,22]. The major fatty acids in linseed oil are linolenic, linoleic and oleic acids at 57%, 15%, and 19%, respectively, of the total fatty acids [14]. Presumably, these acids were being metabolized for growth and DHA production by the test strains. DHA production from fermented okara with thraustochytrids was much lower when compared with fermentation in a glucose yeast extract medium. *S. mangrovei* strains produce DHA about 20 times higher in the glucose yeast extract medium (Tables 2 and 3). This can be explained by the fact that simple sugars, such as glucose, are more readily utilizable than the recalcitrant polymers, such as the cellulose, available in okara [11] for bioconversion in thraustochytrids.

Aquaculture studies have shown that PUFAs or DHA content increased in rotifers, *Artemia* nauplii and *Penaeus monodon* postlarve after feeding with freeze-dried cells of *Schizochytrium* sp. [1,17]. Therefore, further studies should focus on the use, production and application of thraustochyrids in aquaculture. Fermented okara product might be considered as a cost-effective feed for rotifers and *Artemia* nauplii to enhance the nutritional value for fish [3].

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