Utilization of food processing waste by Thraustochytrids

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Schizochytrium mangrovei KF6, a mangrove thraustochytrid isolate, was evaluated for its docosahexaenoic acid (DHA) production potential in both a glucose yeast extract medium and various food waste. Highest DHA yield was obtained after 4 days of incubation in a glucose medium at 3094 mg/L or 203 mg/g. Waste fermented with *S. mangrovei* also yielded DHA in varying quantities: 12.6 mg/g DHA from bread crusts, 7.3 mg/g and 6.2 mg/g respectively from okara powder and brewing grain waste. Analysis of the waste before and after fermentation showed that the simple sugars, fatty acids with 18 carbons, namely stearic (18:0), oleic (18:1) and linoleic (18:2) acids were utilized by *S. mangrovei*. Yields of DHA from thraustochytrids grown on waste (6.2-12.3 mg/g) were much lower than those grown in glucose yeast extract medium (203 mg/g). This study has shown that thraustochytrids can increase the nutritive value of food waste and such a product may find a use in aquaculture.

Key words: bread crusts, docosahexaenoic acid (DHA), food waste, okara powder, *Schizochytrium mangrovei*, brewing grain waste, polyunsaturated fatty acids.

Introduction

Thraustochytrids are a group of marine protists with monocentric thalli. They possess the following characteristic features: a multi-layered wall composed predominantly of L-galactose (Darley *et al.*, 1973), an organelle termed a sagenogenetosome from which the ectoplasmic nets arise (Perkins, 1972), and biflagellate heterokont zoospores in many of the described genera (Moss, 1991). Recent research on thraustochytrids has focused on DHA (docosahexaenoic acid) production (Nakahara *et al.*, 1996) as they are known to produce a wide range of lipids, particularly polyunsaturated fatty acids of the omega-3 series (Weete *et al.*, 1997).

There has been increasing interest in upgrading food processing waste into higher value products (Kroyer, 1991). In Hong Kong, large quantities of food waste are produced daily. These waste include soymilk residue (okara powder)

results from soymilk production (Ma *et al.*, 1997), grain husks from the brewing industry (several tonnes each week) and bread crusts removed from bread from large bakeries. This is not a unique feature of Hong Kong, but is also a common phenomenon in Malaysia, Singapore and Thailand (E.B.G. Jones, pers. comm.). Other waste include feathers of poultry and exoskeletons of crustaceans. All these food waste require disposal even though most of these food waste retain some nutritious components, e.g. a high percentage of protein is present in okara (O'Toole, 1999). Thus, these waste can be used as substrates for microbial growth to upgrade their nutritive value such as DHA-production by thraustochytrids. Our preliminary screening experiments showed *Schizochytrium mangrovei* KF6, a mangrove isolate, is a potential DHA producer. The aim of the present investigation was to assess the ability of *S. mangovei* KF6 to utilize bread crusts, okara powder, and brewing grain waste for growth and DHA production.

Materials and methods

Preparation of inoculum

A zoospore suspension was used as inoculum. Four wells were made in two-day old culture agar plates (25 C) using a cork borer (1.5 cm diam.) followed by flushing with sterile natural seawater (NSW) adjusted to 15‰. After 2-3 h, zoospores accumulated in the wells. One mL of zoospore suspension was transferred into 50 mL aliquot sterile broth [1 g yeast extract (Oxoid), 1g mycological peptone (Difco), 10 g glucose (BDH) and 1 L of 15‰ (NSW)] in a 250 mL flask plugged with cotton wool and covered with aluminum foil. The flasks were shaken at 200 rpm at 25 C for 40 h under continuous fluorescent light. These cultures served as inoculum for subsequent heterotrophic fermentation and waste evaluation experiments at a concentration of 5% (v/v).

Heterotrophic growth

Schizochytrium mangrvoei KF6 was isolated from yellow decaying leaves of *Kandelia candel* (L.) Druce collected from Mai Po mangrove with the water salinity recorded at 11‰. This strain was grown in 250 mL flasks that contained 50 mL glucose yeast extract broth (60 g glucose (Sigma), 10 g yeast extract (Oxoid), and 1 L of 15‰ artificial seawater (ASW) prepared from sea salts (Sigma) at pH 6), incubated and shaken at 200 rpm at 25 C. Triplicate flasks were harvested daily for a seven day period and this conforms to standard experimental design employed by other workers (Singh and Ward, 1996; Singh *et al.*, 1996).

Preparation of food waste samples for evaluation

Bread crusts, okara powder, and brewing grain waste were used for evaluating their utilization by *S. mangrovei* KF6. Their carbon and nitrogen content were determined using a CHN-900 carbon, hydrogen and nitrogen analyser model 600-800-300 (LECO® corporation U.S.A.). Two mg of each sample were weighed in tin containers and samples ignited in the analyzer for carbon and nitrogen determination.

All waste samples were oven-dried at 60 C for 24 h and blended to fine particles with a two-speed blender (Cole-Palmer Instrument Company). Each of the pulverized waste samples was tested at a concentration of 10 g/L in 15‰ (ASW) at pH 6 in 250 mL flasks. Triplicate flasks of each of the fermented waste were harvested after shaking for 4 days at 200 rpm at 25 C under continuous fluorescent light. A further study was conducted using 10, 20 and 40g/L of bread crusts as this waste gave the highest biomass and DHA yield in the initial experiment. These flasks were incubated for 8 days in triplicate and harvested at 2 day intervals.

Dry weight determination

For dry weight determination, the entire content (50 mL) of the liquid culture was transferred to a pre-weighed centrifuge tube and harvested by centrifugation at 3500 g for 10 min and the supernatant discarded. Harvested cells or fermented waste products were washed thoroughly with distilled water and then freeze-dried for 24 h, and weighed. The test isolate was obtained from low salinity waters in Mai Po mangroves. As demonstrated by a preliminary test, washing the thraustochytrids cells with distilled water did not cause the cells to burst. Biomass, or fermented waste, are expressed as freeze-dried weight in grams per liter of growth broth.

Fatty acid analysis

Fatty acid composition was determined following a modified procedure of Lepage and Roy (1984). Intact freeze-dried cells (50-60 mg) and fermented waste products (80-100 mg) were methylated by a direct acid-catalyzed trans-esterification 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 the methylation. After the contents have cooled, 1mL of water and 1mL of hexane were added. The FAMEs (Fatty Acid Methyl Esters) in the hexane layer were vortexed, centrifuged and collected. 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-

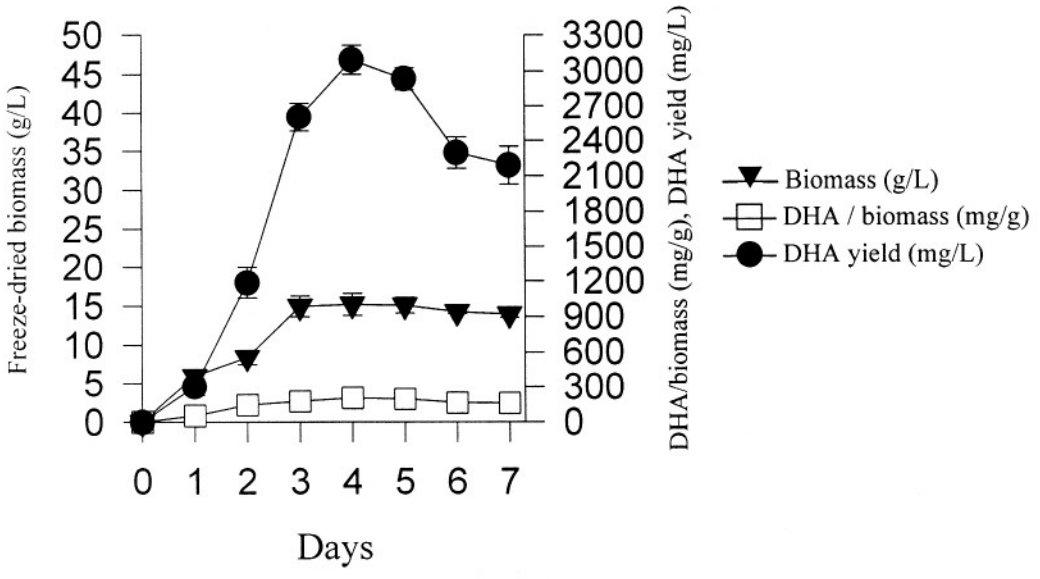

Fig. 1. Growth and DHA production of *Schizochytrium mangrovei* KF6 in a glucose yeast extract medium.

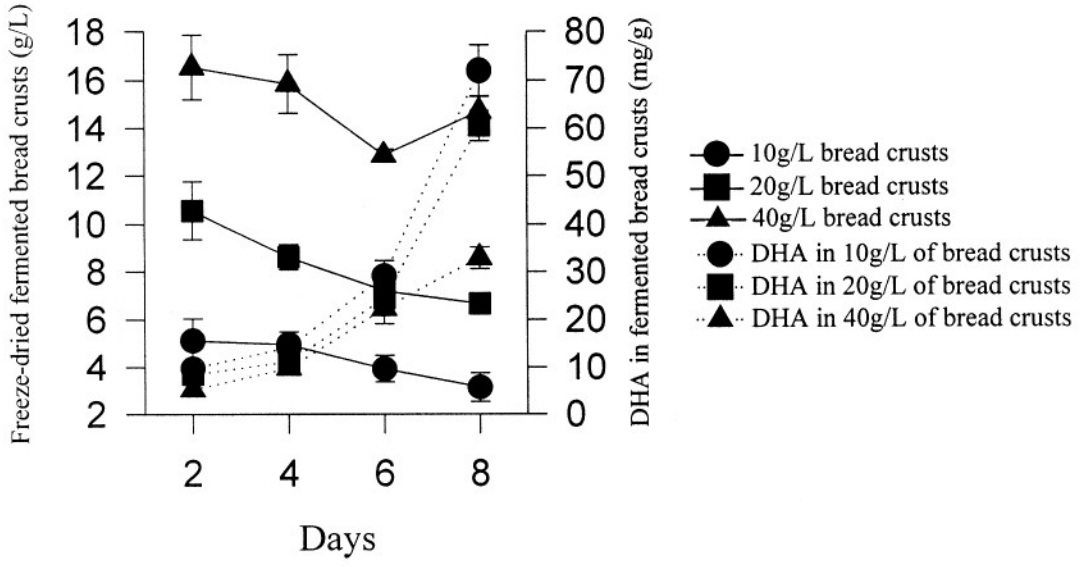

Fig. 2. *Schizochytrium mangrovei* KF6 grown at different concentrations of bread crusts with data on DHA production.

Table 1. Fatty acid composition of freeze dried *Schizochytrium mangrovei* KF6 (% of total fatty acids) after growth for 7 days in glucose yeast extract medium at 25 C[1].

Fatty acid profile	14:0	14:1	15:0	16:0	18:0	18:1	18:2	18:3	20:4	20:5	22:4	22:5	22:6	Others
Day1	2.1	0.0	17.1	31.1	0.7	0.0	0.0	0.1	0.4	0.5	7.9	0.0	38.7	1.4
Day2	3.1	0.0	5.5	39.4	1.4	0.0	0.0	0.0	0.8	0.3	8.1	0.0	39.7	1.7
Day3	3.6	0.0	6.4	39.8	1.6	0.0	0.0	0.0	0.5	0.4	7.7	0.0	38.4	1.6
Day4	3.8	0.0	5.8	39.3	1.7	0.0	0.0	0.1	0.8	0.3	7.4	0.0	39.4	1.4
Day5	4.1	0.0	6.8	42.1	1.8	0.0	0.0	0.0	1.0	0.4	8.7	0.0	36.4	1.7
Day6	3.9	0.0	6.9	42.1	1.9	0.0	0.0	0.0	0.5	0.3	6.7	0.0	36.8	0.9
Day7	3.7	0.0	5.3	45.3	1.5	0.0	0.0	0.0	0.3	0.4	6.9	0.0	35.5	1.1

[1]Medium composed of 60g glucose and 10g yeast extract in 1L of 15‰ ASW at pH6.
14:0, myristic acid, tetradeanoic acid; 14:1,myristoleic acid, tetradecenoic acid;
15:0, pentadecylic acid, pentadecanoic acid; 16:0, palmitic acid, hexadecanoic acid;
18:0, stearic acid, octadecanoic acid; 18:1, oleic acid, cis-9-octadecenoic acid;
18:2w6, linoleic acid, cis-9,12-octadecadienoic acid;
18:3w3, α-linolenic acid, 9,12,15-octadecatrienoic acid;
20:4w6, arachidonic acid, cis-5,8,11,14-eicosatetraenoic acid;
20:5w3, EPA cis-5,8,11,14,17-eicosapentaenoic acid;
22:4, cis-7,10,13,16-docosatetraenoic acid;
22:5w3, cis-7,10,13,16,19-docosapentaenoic acid;
22:6w3, DHA cis-4,7,10,13,16,19-docosahexaenoic acid.
Data are expressed as means of triplicate flasks.

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 and the detector was at 300 C. The column temperature was held at 280 C isotherm for 50 min. The fatty acids were identified by comparison of relative retention times against known standards (Sigma Chemical Co., U.S.A.) and the fatty acid contents were quantified by comparing their peak areas with that of the internal standard (C17:0).

Results and discussion

Growth and DHA production in glucose yeast extract medium

Schizochytrium mangrovei KF6 grew well in the glucose yeast extract medium and accumulated DHA intracellularly (Fig. 1). Biomass production increased with incubation time and attained a maximum of 15.2 g after 4 days of incubation (Fig. 1). DHA yield (per unit volume of growth broth and per unit freeze-dried biomass) also increased with incubation time reaching a maximum of 3094 mg/L and 203 mg/g, respectively after 4 days. Palmitic acid (16:0) and DHA were the dominant fatty acids present (Table 1). DHA level (as % of total

Table 2. Comparison of DHA production in thraustochytrids.

Organisms	DHA in biomass (mg/g)	DHA yield (mg/L)	1. Medium (g/L) 2. Salinity ‰	Incubation
T. aureum Goldstein ATCC 34304 Bajpai *et al.*, 1991	70.4	269.6	1. Glucose 20g, (NH4)2SO4 0.2g Na-glutamate 2g 2. 25 as NaCl	6 days 25 C
T. roseum Goldstein ATCC 2810 Li and Ward, 1994	85.9	841.8	1. Starch 25g, (NH4)2SO4 0.2g yeast extract 2.0g, Na-glutamate 2.0g 2. 25 as NaCl	5 days 25 C
S. mangrovei Raghukumar G13 Bowles *et al.*, 1999	90.4	2170.0	1. Glucose 40g, yeast extract 5g, sodium sulphate 20g 2. 21 as natural seawater	4 days 11h 24 C
***Thraustochytrium* sp.** ATCC 20892 Singh *et al.*, 1996	100.3	707.4	1. Glucose 20g, (NH4)2SO4 0.2g, yeast extract 2.0g, Na-glutamate 2.0g 2. 25 as NaCl	4 days 25 C
T. roseum ATCC 2810 Singh and Ward, 1996	102.2	1061.0	1. Starch 25g, (NH4)2SO4 0.2g, yeast extract 2.0g, Na-glutamate 2.0g 2. 10 as NaCl	5 days 25 C
Schizochytrium limacinum Honda and Yokochi SR21 Yokochi *et al.*, 1998	116.6	4200.0	1. Glucose 90g, Corn steep liquor 20g 2. 15 as artifical seawater	5 days 25 C
S. limacinum SR21 Yaguchi *et al.*, 1997	276.5	13300.0	1. Glucose 120g, (NH4)2SO4 4.0g, corn steep liquor 1.4g 2. 15 as artifical seawater	4 days 28 C
S. limacinum SR21 Nakahara *et al.*, 1996	223.8	4700.0	1. Glucose 60g, (NH4)2SO4 2.0g, corn steep liquor 0.7g 2. 15 as artifical seawater	2 days 6h 28 C
S. mangrovei KF6 This study	203.0	3094.0	1. Glucose 60g, yeast extract 10g 2. 15 as artifical seawater	4 days 25 C

Table 3. Carbon and nitrogen content of the waste tested.

Substrate	Source	Carbon(%)	Nitrogen(%)
Bread crusts[1]	Garden Co. Ltd.	41.88	1.96
Okara powder	Nestles Dairy Farm H.K. Ltd.	47.61	4.55
Brewing grain	San Miguel Brewery H.K. Ltd.	45.94	2.80

[1] Ingredients of bread crusts: wheat flour, animal shortening, milk soild, yeast, emulsifier, calcium propionate, flour improver, vitamin B1, B2 and B3, and iron (Information derived from wrapping of "Life" bread).

Table 4. Fatty acid composition (% of total fatty acids) of unfermented waste[1] and of waste fermented with *Schizochytrium mangrovei* KF6 for 4 days in different waste media[1].

Fatty acid profile[2]	14:0	14:1	15:0	16:0	18:0	18:1	18:2	18:3	20:4	20:5	22:4	22:5	22:6	Others
Unfermented														
Bread crusts	1.6	0.0	0.0	26.4	10.7	34.0	22.5	1.1	0.0	0.0	0.0	0.0	0.0	3.1
Okara powder	0.0	0.0	0.0	11.5	3.1	26.0	52.4	7.1	0.0	0.0	0.0	0.0	0.0	0.0
Brewing grain	0.4	0.0	0.0	23.1	1.3	13.2	56.0	5.7	0.0	0.0	0.0	0.0	0.0	0.4
Fermented														
Bread crusts	2.2	0.0	0.9	36.6	7.4	22.3	15.1	0.9	0.0	0.0	0.0	0.0	11.2	3.4
Okara powder	0.0	0.0	0.0	15.7	3.4	24.0	46.5	6.2	0.0	0.0	0.5	0.0	3.8	0.0
Brewing grain	0.7	0.0	0.2	28.4	1.5	12.0	45.9	4.2	0.0	0.0	0.0	0.0	6.0	1.1

[1] Medium composed of 10g of pulverized bread crusts, or okara powder or brewing grain waste in 1L of 15‰ ASW at pH6.

[2] Fatty acid profile of freeze-dried unfermented or fermented bread crusts, or okara powder or brewing grain waste after four days of incubation at 25 C

Data expressed as means of triplicate flasks.

fatty acids) remained fairly constant at 35.5-37.9% throughout the 7 days growth period while the level of palmitic acid (as % of total fatty acids) increased gradually from 31.1% to 45.3% after 7 days (Table 1). This strain also produced significant quantities of docosatetraenoic acid (22:4) 6.7-8.7% (as % of total fatty acids) but no docosapentaenoic acid (22:5). Only trace amounts of unsaturated fatty acids of 18 carbon was observed. Table 2 lists the comparative yield of DHA producing thraustochytrids extracted from literature. By comparison, the test strain, *S. mangrovei* KF6 and *S. limacinum* SR21 from Japan can be considered as the high yielding strains, while the rest produce relatively low yields.

Waste utilization

Analysis showed that the waste used in this study contained approximately 40% carbon (Table 3). Okara powder gave the highest nitrogen level as expected as it is from soybeans which are rich in protein (Table 3). The fatty acid profiles of the unfermented waste are presented in Table 4. All the waste were found to contain myristic (14:0) (except okara powder), palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2) and linolenic acids (18:3). Bread crusts contained oleic acid as the major fatty acid (approximately 30%). For both okara powder and brewing grain waste, the major fatty acid present was linoleic acid (50%). DHA (22:6) was not detected in any of these food waste.

Two changes related to fatty acid profiles occurred in all waste after 4 days of fermentation (Table 4). Firstly, there was a noticeable accumulation of DHA, and secondly, there was a decrease in all 18 carbon fatty acids particularly

Table 5. Docosahexanoic acid (DHA) yield of *Schizochytrium mangrovei* KF6 in different waste media[1].

	DHA		
	Biomass (%w/w)	Yield (mg/L)	Biomass (mg/g)
Bread crusts	1.3	82.2	12.6
Okara powder	0.7	54.9	7.3
Brewing grain	0.6	62.0	6.2

[1] Medium composed of 10g of homogenized bread crusts, or okara powder, or brewing grain waste in 1L of 15‰ ASW at pH6.
Data expressed as means of triplicate flasks.

linoleic acid in all fermented waste. Linoleic acid is known to be a precursor in the formation of omega-3 fatty acids including DHA (Yongmanitchai and Ward, 1989). As a result, linoleic acid may be preferentially metabolized and incorporated into *S. mangrovei* KF6 for DHA production. A major difference in the fatty acid profiles between thraustochytrids grown on waste compared with that on a rich glucose medium is the absence of the fatty acids 20:4, 20:5, 22:4 and 22:5 from the fermented waste, with the exception of 22:4 in fermented okara powder. Studies by Bajpai *et al.* (1991) indicated that yields of certain fatty acids is very much affected by the medium used for the growth of thraustochytrids. For example, yields of 20:4 and 20:5 on nine different media ranged from none to 12.3% w/w and none, trace, 2.9-9.1% (w/w) respectively. Not all thraustochytrids produced 22:4 fatty acid. None were reported from Li and Ward (1994), Singh *et al.* (1996), and Yokochi *et al.* (1998). However, apart from the test strain *S. mangrovei* KF6, we have also obtained 1.4% (as % of total fatty acids) from an *Ulkenia* sp. and 8.6% (as % of total fatty acids) from *Thraustochytrium striatum* isolated from mangroves in Hong Kong. Weete (1997) also reported the occurrence of 0.1% of 22:4 from a *Thraustochytrium* sp. (ATCC 26185). All waste tested sustained growth of *S. mangrovei* KF6 with DHA yields varying from: 12.6 mg/g DHA for bread crusts, 7.3 mg/g and 6.2 mg/g respectively for okara powder and brewing grain (Table 5). Bread crusts contain readily utilizable ingredients, such as starch and vitamins (Table 3), which can sustain the growth of thraustochytrids (Iida *et al.*, 1996). In contrast, the major carbon-containing compounds in okara powder and brewing grain waste were cellulose and hemicelluose, which might not be readily utilized by *S. mangrovei* KF6, although Bremer and Talbot (1995) showed thraustochytrids produced cellulases.

Freeze-dried weight of fermented bread crusts decreased during the 8 day growth period, but at the same time the DHA level of the fermented bread crusts increased. This may have involved the bioconversion of bread crust components into biomass of *Schizochytrium mangrovei* KF6 (Fig. 2).

Apparently, the increase in thraustochytrids biomass did not compensate the loss of bread crust components. Therefore, a total net loss of freeze-dried weight of the fermented bread crusts was observed.

Freeze-dried thraustochytrids have been used as a feed to increase the DHA content of fish, shrimps and *Artemia* in Thailand and U.S.A. (Barclay and Zeller, 1996; Somatawin *et al.*, 1997). Commercial aquaculture products derived from an alga *Crypthecodinium* sp. (AquaGrow®-DHA) are also currently available on the market (Martek Bioscience Corporation, U.S.A.). Our aim was to investigate the potential of using thraustochytrids to upgrade waste as feeds for aquaculture and poultry farms. DHA yields of *S. mangrovei* KF6 growing from waste were much lower (72 mg/g in fermented bread crusts after 8 days) than their growth in a glucose yeast extract medium (203mg/g) after 4 days (Figs. 1, 2). Glucose and yeast extract, however, are costly carbon and nitrogen sources compared with all the tested food processing waste which are cost free and are destined for disposal. Thus *S. mangrovei* KF6 can be utilized for increasing the nutritive value of the waste as demonstrated by a low cost fermentation process in this study. Food processing waste such as fermented bread crusts may have the potential to be used as feeds in aquaculture and poultry farms after upgrading with DHA.

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