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Nutritional Enrichment of Larval Fish Feed with Thraustochytrid Producing Polyunsaturated Fatty Acids and Xanthophylls

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In marine aquaculture, rotifers and *Artemia* nauplii employed as larval fish feed are often nutritionally enriched with forage such as yeast and algal cells supplemented with polyunsaturated fatty acids and xanthophylls, which are required for normal growth and a high survival ratio of fish larvae. To reduce the enrichment steps, we propose here the use of a marine thraustochytrid strain, *Schizochytrium* sp. KH105, producing docosahexaenoic acid, docosapentaenoic acid, canthaxanthin, and astaxanthin. The KH105 cells prepared by cultivation under optimized conditions were successfully incorporated by rotifers and *Artemia* nauplii. The contents of docosahexaenoic acid surpassed the levels required in feed for fish larvae, and the enriched *Artemia* showed an increased body length. The results demonstrate that we have developed an improved method of increasing the dietary value of larval fish feed.

[**Key words:** rotifer, *Artemia*, *Schizochytrium*, docosahexaenoic acid, xanthophylls]

Polyunsaturated fatty acids, especially docosahexaenoic acid (DHA), are nutrients essential for the growth of marine fishes, such as red sea bream and cod, particularly in their larval stages (1–3). Because these species have a limited ability to biosynthesize polyunsaturated fatty acids *de novo*, larvae fed diets containing a low percentage of such lipids show poor growth and a high mortality rate, which are improved by supplementing DHA. Carotenoid pigments also fulfill very important roles in the growth of fish larvae. A canthaxanthin- and β-carotene-supplemented diet enhances the growth of rainbow trout (4) and Japanese and spotted parrotfish larvae (5). Astaxanthin is also an important carotenoid in marine organisms, being responsible for the pigmentation of the skin and flesh of salmonids (6), halibut larvae (7), red sea bream (8), ayu (9), and rainbow trout (10) and is essential for growth and survival during the initial feeding period of Atlantic salmon (11).

Rotifers and *Artemia* nauplii are the best live prey for first feeding larvae in aquaculture. However, DHA and carotenoid concentrations are originally low in those species of natural zooplankton (12, 13). Thus, these feed organisms are usually enriched with material containing these lipids to ensure the survival of fish larvae. The nutritional compositions of rotifers, *Brachionus plicatilis* (14–16), and *Artemia salina* nauplii (17–20) have been modified with oil emulsions or encapsulated oils containing polyunsaturated fatty acids. Some algae, such as *Chlorella vulgaris* (21) and *Euglena glacilis* (22), enriched with DHA have been used for nutritional improvement in zooplankton. Moreover, Immanuel *et al.* (23) and Bransden *et al.* (24) reported an increase in the survival ratio and an enhancement of the stress resistance of fish larvae by feeding the larvae enriched *Artemia*.

The utilization of thraustochytrid cells is another solution to enrich polyunsaturated fatty acids in live foods for fish larvae (25, 26). Thraustochytrids including the genera *Thraustochytrium* and *Schizochytrium* are eukaryotic and heterotropic microorganisms found widely in marine environments and accumulate triacylglycerols rich in DHA, eicosapentaenoic acid (EPA) and docosapentaenoic acid in their cells (27–29). Therefore, using thraustochytrid cells as live foods for fish larvae omits the extra step of enriching fish feed with such lipids. We describe in this report that the use of our isolate, namely, *Schizochytrium* sp. strain KH105 producing xanthophylls such as canthaxanthin and astaxanthin in addition to DHA (30, 31), is convenient for improving the nutritional value of rotifers and *Artemia* for feeding of larval fish.

MATERIALS AND METHODS

Microorganism and culture media *Schizochytrium* sp. strain KH105 (31) was maintained on an agar medium containing 30 g/*l* glucose, 10 g/*l* Polypepton, 5 g/*l* yeast extract, and 15 g/*l* agar in 19.5 g/*l* artificial sea salts (about 50% equivalent of natural seawater; Jamarine Laboratory, Osaka). The seed culture medium

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consisted of 50 ml of 30 g/*l* glucose, 20 g/*l* Polypepton and 10 g/*l* yeast extract in 50% artificial seawater. For optimization, cultivation was performed in 200-ml baffled flasks containing 50 ml of culture medium containing various amounts of glucose, nitrogen sources, and artificial sea salts for 48 h at 28°C with reciprocal shaking at 150 rpm. The cells were harvested by centrifugation at 3000×*g*.

Jar fermentor culture Fermentation was carried out in a 3-*l* jar fermentator (model MD-N 3L; B.E. Marubishi, Tokyo) equipped with two disk turbine propellers with six wings each. The inoculum consisted of 200 ml of a 2-d-old culture grown in a flask. Cultivation was performed at 28°C with various agitation speeds and aeration rates in a working volume of 2 *l*. Foaming was controlled by adding antifoam (PE-M; Wako Pure Chemical Industries, Osaka). Dissolved oxygen concentration was measured using a DO meter (OX-2500; DKK-TOA, Tokyo). The glucose concentration in the medium supernatant was determined using glucose oxidase as described previously (32).

Enrichment of rotifers Cultures of the rotifer *B. plicatilis*, type-s (Sasebo City Fisheries Center, Nagasaki), prefed the marine chlorella *Nannochloropsis oculata* at 10^7 individuals/ml with aeration through airstone at 25°C were replaced at 800 individuals/ml in a 20-*l* tank containing 10 *l* of seawater (Jamarine Laboratory). Diet enrichment with different densities of KH105 cells was carried out for 24 h at 25°C without water renewal under moderate ventilation. The rotifers were harvested with a nylon mesh (mesh size of 40 μm) and washed with distilled water prior to lipid analysis.

Enrichment of *Artemia* nauplii Cysts of *A. salina* nauplii from Great Salt Lake, Utah (Pacific Trading, Fukuoka) were hatched at 28°C. The newly hatched nauplii were collected, washed to remove empty shells, and subsequently inoculated into a tank containing 10 *l* of seawater at 100 individuals/ml. Enrichment with different concentrations of KH105 cells was carried out for 24 h under strong aeration at 28°C. The nauplii were recovered with a nylon mesh (mesh size of 100 μm) and washed with distilled water. Body length was measured with an ocular micrometer of the microscope. The data were subjected to Student's *t*-test and analysis of variance.

Analysis Lipids were extracted from harvested wet cells or bodies of *Schizochytrium* sp., *B. plicatilis*, and *Artemia* in a chloroform/methanol mixture (2:1, v/v) and methyl-esterified with 10% methanolic hydrochloride with the addition of eicosanoic acid as an internal standard (29). Fatty acid methyl esters were extracted with hexane and subjected to gas-liquid chromatography (Autosystem XL; Perkin Elmer, Yokohama) using an apparatus equipped with a capillary column (TC-70, 0.25 mm×30 m; GL Science, Tokyo) and a flame ionization detector. Intracellular pigments were extracted by suspending the harvested wet bodies of rotifers and *Artemia* in an acetone/methanol mixture (7:3, v/v) and analyzed by thin-layer chromatography, which was performed on a silica gel plate (Kieselgel 60; Merck, Darmstadt, Germany) using acetone/hexane (3:7, v/v) as a developing agent and authentic astaxanthin as a standard, followed by quantification with a densitometer (31). All experiments were performed at least two or three times and typical data are shown.

RESULTS AND DISCUSSION

Optimization of culture condition of *Schizochytrium* sp. KH105 To make use of the *Schizochytrium* cells as enrichment diet material, we aimed first to optimize the medium composition for the cultivation of the strain KH105 to improve the cellular lipid content. Figure 1A shows the effect of salt concentrations ranging from 10% to 100% of

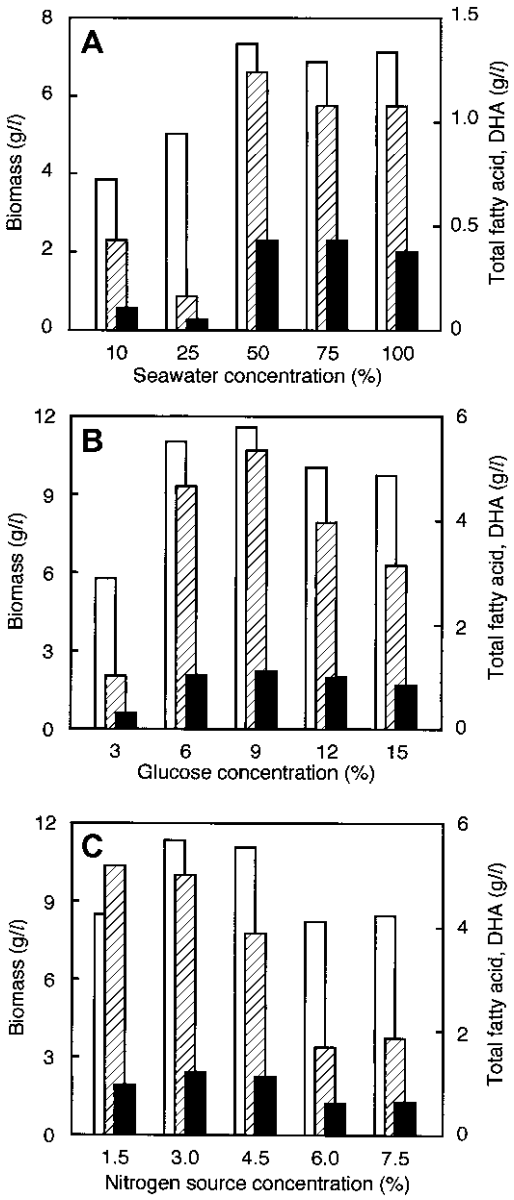

FIG. 1. Optimization of culture conditions for *Schizochytrium* sp. KH105. Cultivation was carried out at 28°C in 200-ml baffled flasks containing media (50 ml) of the following compositions: (A) 3% glucose, 0.5% yeast extract, 1% peptone, and indicated concentration of seawater (made with artificial seasalts); (B) indicated concentration of glucose, 0.5% yeast extract, and 1% peptone in 50% artificial seawater; (C) 9% glucose and indicated concentration of mixture of peptone and yeast extract (2:1, w/w) in 50% artificial seawater. Open bars, Dry cell weight; hatched bars, total fatty acid yield; shaded bars, DHA yield.

that of natural seawater in the basal medium on the cell growth and production of fatty acids and DHA. These parameters were maximum at a seawater concentration of 50%, but did not change significantly when the salt concentration was higher than that. Thus, we used medium containing 50% artificial seawater for subsequent experiments.

The effect of glucose concentration in the range from 3% to 15% on DHA production was investigated. As shown in Fig. 1B, DHA production increased with cell growth up to a

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