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Development of live feed enrichment product for marine fish larviculture

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ABSTRACT

Phytoplankton cultures of *Nanochloropsis oculata*, *Isochrysis galbana*, *Pavlova viridis* and *Dicrateria inornata*, were scaled up and analysed for fatty acids. *I. galbana* proved to be the richest source of docosahexaenoic acid (DHA). *P. viridis* and *D. inornata* are rich in eicosapentaenoic acid (EPA). *N. oculata* is rich in EPA and arachidonic acid (ARA). When rotifers were enriched with *I. galbana* and analysed for fatty acids at specific time intervals, DHA content increased till 30 h with a maximum DHA level of 1.13% obtained in enriched rotifers. Subsequently, development of enriched emulsions using sardine oil (90%) and fish roe (10%) as the major ingredients yielded a DHA content of 39% and EPA content of 17% and was used to enrich rotifers. The resultant enrichment level was 8.76% DHA and 2.35% EPA at six hours after which, a decline in the polyunsaturated fatty acid (PUFA) content was observed. The enrichment formulation holds promise as an import substitute.

Keywords: *Dicrateria inornata*, Enrichment, *Isochrysis galbana, Nanochloropsis oculata, Pavlova viridis,* Phytoplankton

Introduction

Live feed enrichment *per se* is an established practice in fish hatcheries keeping in mind the fact that the most popular zooplankton *Artemia* nauplii and rotifers are naturally deficient in polyunsaturated fatty acids (Sargent *et al.,* 1999). Most fish hatcheries follow a protocol of hatching in green water, followed by feeding with rotifers and *Artemia* nauplii. Co-feeding with artificial microdiets and then weaning on to a dry diet completes the hatchery phase. Protocols by and large remaining the same, variations are in the nutritional composition of the feeds provided either live, inert or bio-encapsulated. These variations reflect upon the ultimate larval survival which is the driver of the economic viability of the hatcheries and the availability of seed for growout of the fish species targeted. In such a situation, differences are noticed in the nutrient content of the primary producers, the phytoplankton (Ogata *et al.,* 2004) which in turn influences the content and ratios of fatty acids, the nutrients looked into critically. The work reported here is a compilation of the level of fatty acid enrichment posible with rotifers, the common phytoplankton used in the marine sector in south India. Results of an attempt to develop an indigenous enrichment emulsion and assess its propensity to enrich live feeds especially, rotifers and *Artemia* nauplii for use in the initial nutrition of marine fish larvae are presented.

Materials and methods

Phytoplankton culture and enrichment

Phytoplankton cultures of *Nanochloropsis oculata*, *Isochrysis galbana*, *Pavlova viridis* and *Dicrateria inornata*, obtained from the stock cultures maintained at the Central Marine Fisheries Research Institute, Cochin, were developed at a density of 1.2 x 10^6 cell ml^{-1} with a salinity of 30 ± 5‰, temperature, 28 ± 1 °C and pH 7.8 - 8.1 following standard protocols.

Initially, the levels of fatty acids in these were assessed along with baker's yeast (*Saccharomyces cerivisiae*) which is also a conventionally used medium to grow rotifers. Subsequently, rotifers were grown on *N. oculata* and *I. galbana* and sampled at intervals and then profiled for their fatty acid composition to ascertain the time taken and the maximum level of enrichment obtained. The nutritional enrichment experiments with rotifers were conducted in duplicate at a final volume of 10 l. Cultures of rotifers were maintained and fed with *Saccharomyces cerevisiae* (Baker's yeast) and constantly checked for water quality. Initial mean rotifer density at start of the (10 l) culture was

5×10^{-4} cells l^{-1}. Biomass was harvested at intervals as shown in Table 3, from each replicate with 20 µ filters, right from the start of the experiment. Initial microalgal cell densities inoculated were 1.2 x 10^{-6} cells l^{-1}. Samples for biochemical analysis were washed with double distilled water to remove salts, and stored at -20 °C until analysis.

Development of live feed enrichment emulsions and their evaluation

Sardine oil enriched through biochemical and microbiological procedures according to Kajal and Paul Raj (2009) and Kajal *et al.* (2010), were used to formulate enrichment emulsions which contained grossly 90% PUFA enriched fish oil and 10% lecithin extracted from roe of seer fish *Scomberomorus guttatus* based on the report of Sargent *et al*. (1999) that, fish roe and milt are the richest source of phospholipids having the most appropriate ratio of docosahexaenoic acid (DHA): eicosapentaenoic acid (EPA). Seer fish roe, sourced from the local fish market @ Rs.80 kg^{-1} was also profiled for its fatty acid composition. Other than the stability and emulsification ability imparted by lecithin, tocopherol acetate (vitamin E) at 0.5% was also included as a stabilizer. Two types of emulsions, -EPA rich and DHA rich were developed.

Fatty acid analysis

Total lipid in the samples was extracted by the cold extraction method using a chloroform-methanol mixture in a 2:1 ratio (Folch, *et al.,* 1957). After isolating the lipid phase, the solvent was evaporated. About 2 ml of sample was refluxed with 5 ml of 0.5 N alcoholic potassium hydroxide solution for 30 min and subsequently with 5 ml of BF_3-MeOH (Sigma-Aldrich) for another 5 min. The whole process of refluxing was carried out in an atmosphere of nitrogen. The dry fatty acid methyl esters in the flask were quantitatively extracted with petroleum ether (10 x 3). The extract was further washed twice with 25 ml of saturated sodium chloride and filtered over anhydrous sodium sulphate (10 g) to remove any moisture. The solvent was then evaporated under a stream of nitrogen gas. About 2.0 µl each of the prepared fatty acid methyl ester samples was injected into the Perkin Elmer Auto-System XL Gas chromatograph (Perkin Elmer, Waltham, MA, USA). The gas analyses was accomplished with a SP 2560 (crossbond 5% diphenyl – 95% dimethyl polsiloxane) capillary column (100 m x 0.25 mm i.d., 0.50 µm film thickness, Supelco, Belfonte, PA) using a flame ionization detector (FID) equipped with a split/splitless injector, which was used in the spit (1:15) mode. The oven temperature ramp programme: 140 °C for I min. rising at 30 °C min^{-1}. to 250 °C where it was held for 1.0 min. followed by an increase of 25 °C min^{-1} to 285 °C, where it was held for 2 min. until all peaks appeared. The injector and detector were held at 285 and 290 °C, respectively, The injection volume of 0.04 µl, FAMEs were identified by comparison of retention times with known standards (37 component FAME Mix, Supelco). Results were expressed as percentage weight of total fatty acids. Nitrogen (ultra high purity >99% at 25 cm s^{-1} flow rate) was used as the carrier gas with a pressure of 5.6 x 10^3 kg m^{-2}. The flow rate of hydrogen and air were maintained at a pressure of 3.5 x 10^4 kg m^{-2}. All the samples were injected in triplicate and the data acquisition was carried out with TOTAL-CHROME 6.X.X software (Perkin Elmer). The total run time per sample was set at 52 min.

Results and discussion

PUFAs derived from microalgae, *i.e*., DHA, EPA and arachidonic acid (ARA) are known to be essential for fish larvae (Langdon and Waldock, 1981; Sargent *et al.,* 1997). Most microalgal species have moderate to high percentages of EPA (7 to 34%). Prymnesiophytes (*eg*. *Pavlova* spp. and *Isochrysis* sp. and cryptomonads are relatively rich in DHA (0.2 to 11%), whereas eustigmatophytes (*Nannochloropsis* spp.) and diatoms have the highest percentages of ARA (0 to 4%). Chlorophytes (*Dunaliella* spp. and *Chlorella* spp.) are deficient in both C20 and C22 PUFAs, although some species have small amounts of EPA (up to 3.2%). Because of this PUFA deficiency, chlorophytes generally have low nutritional value and are not suitable as a single species diet (Brown *et al.,* 1997). An initial profiling of fatty acids (Table 1) was thus carried out to ascertain whether the classification of microalgae into DHA rich, EPA rich and ARA rich holds good for the cultures available from South India. Baker's yeast was deficient both in DHA and EPA. *N. oculata* was rich in EPA and ARA. *I. galbana* was rich in DHA and *P. viridis* and *D. inornata* were both rich in EPA which conforms to the report by Brown *et al*.(1997). Similar results have been reported previously (Lubzens *et al*., 1985; Olsen, 1990; Whyte and Nagata, 1990; Reitan *et al*., 1993).

Subsequently, rotifers grown on baker's yeast were starved for a day and enriched with *I. galbana* and *N. oculata* and profiled periodically as shown in Tables 2 and 3 respectively to ascertain the optimum time required to obtain maximum enrichment. Maximum PUFA enrichment was observed within six to twelve hours. Even though increases in individual fatty acids occurred up to 30 h, the overall trend was increase in saturated fatty acids (SFA) and decline in monounsaturated fatty acids (MUFA) and PUFA beyond 30 h. The DHA: EPA ratio congenial for fish larval growth is 2: 1 (Sargent *et al.*, 1997; 1999) which is not attainable with *I. galbana* as the sole source of feed. As opined by Reitan *et al.* (1993), microalgae

Table 1. Fatty acid composition of baker's yeast (*Saccharomyces cerivisiae*) and microalgae (Total fatty acids %)

Fatty acids	*S. cerivisiae*	*N. occulata*	*I. galbana*	*P. viridis*	*D. inornata*
14:0	4.88 ± 0.04	5.23 ± 0.05	6.28 ± 0.10	11.11 ± 0.21	1.37 ± 0.02
15:0	0.80 ± 0.06	1.03 ± 0.06	0.79 ± 0.03	0.86 ± 0.01	0.61 ± 0.02
16:0	23.78 ± 0.42	21.80 ± 0.30	14.46 ± 0.06	19.29 ± 0.12	18.88 ± 0.04
18:0	3.61 ± 0.03	8.79 ± 0.04	5.89 ± 0.06	1.93 ± 0.01	0.09 ± 0.01
24:0	0.32 ± 0.06	0.55 ± 0.01	0.63 ± 0.01	1.15 ± 0.00	0.76 ± 0.01
ΣSFA	33.39 ± 0.24	37.39 ± 0.34	28.05 ± 0.25	34.32 ± 0.33	21.70 ± 0.01
16:1	15.13 ± 0.09	16.91 ± 0.04	3.53 ± 0.09	27.88 ± 0.04	0.58 ± 0.08
18:1	24.97 ± 0.17	19.54 ± 0.06	20.67 ± 0.59	7.35 ± 0.09	25.18 ± 0.28
24:1	0.87 ± 0.01	1.24 ± 0.01	0.34 ± 0.08	0.83 ± 0.05	0.35 ± 0.01
ΣMUFA	40.97 ± 0.09	37.69 ± 0.11	24.53 ± 0.57	36.05 ± 0.18	26.11 ± 0.35
Cis-18:2n6	12.37 ± 0.05	4.66 ± 0.04	8.30 ± 0.26	3.44 ± 0.06	6.33 ± 0.04
Cis-18:3n6	0.62 ± 0.04	0.38 ± 0.04	0.17 ± 0.02	1.06 ± 0.01	0.39 ± 0.01
18:3n3	3.56 ± 0.05	0.63 ± 0.06	5.74 ± 0.06	1.48 ± 0.01	12.82 ± 0.04
18:4n3	0.31 ± 0.08	0.13 ± 0.03	15.26 ± 0.08	2.86 ± 0.06	8.60 ± 0.08
C20:2n6	0.81 ± 0.06	0.84 ± 0.02	0.07 ± 0.02	0.22 ± 0.03	0.21 ± 0.02
C20:3n6	0.20 ± 0.02	0.51 ± 0.05	0.23 ± 0.05	0.17 ± 0.02	1.07 ± 0.01
C20:4n6	0.59 ± 0.02	2.17 ± 0.02	0.49 ± 0.01	1.34 ± 0.02	0.99 ± 0.01
20:5n3	0.34 ± 0.01	9.71 ± 0.02	2.63 ± 0.04	9.51 ± 0.05	6.81 ± 0.02
22:5n3	0.01 ± 0.01	0.14 ± 0.01	0.46 ± 0.03	0.16 ± 0.01	0.37 ± 0.02
22:6n3	0.05 ± 0.06	0.62 ± 0.03	9.76 ± 0.01	1.79 ± 0.04	1.26 ± 0.01
ΣPUFA	18.83 ± 0.30	19.76 ± 0.01	43.07 ± 0.28	21.99 ± 0.27	38.81 ± 0.09

Table 2. Fatty acid composition of rotifers enriched with *I. galbana* (Total fatty acids %)

Fatty acids	3h	6h	12h	30h	48h
14:0	6.39	6.62	6.94	11.09	15.34
15:0	0.93	0.98	1.06	2.39	2.76
16:0	23.02	22.18	23.52	25.71	27.86
18:0	3.15	3.82	3.95	5.36	7.49
24:0	0.19	0.21	0.32	0.5	1.24
ΣSFA	33.68	33.81	35.79	45.05	54.69
16:1	14.85	14.06	13.53	11.48	9.17
18:1	24.17	23.52	22.25	17.06	16.94
24:1	0.91	1.06	1.85	2.11	3.28
ΣMUFA	39.93	38.64	37.63	30.65	29.39
Cis-18:2n6	12.09	11.45	9.2	8.16	7.14
Cis-18:3n6	0.61	0.72	0.75	0.54	0.32
18:3n3	3.78	3.85	4.27	3.04	1.85
18:4n3	0.46	0.51	0.75	0.89	0.62
20:2n6	1.36	1.38	1.65	1.72	0.78
20:3n6	0.26	0.39	0.63	0.65	0.20
20:4n6	0.37	0.42	0.45	0.28	0.19
20:5n3	0.38	0.59	1.64	1.75	1.93
22:5n3	0.00	0.09	0.13	0.15	0.09
22:6n3	0.23	0.39	0.92	1.13	0.51
ΣPUFA	19.54	19.79	20.39	18.31	13.63

Table 3. Fatty acid composition of rotifers enriched with *N. oculata* (% Total fatty acids)

Fatty acids	3h	6h	12h	30h	48h
14:0	8.08	9.29	10.41	12.8	16.11
15:0	0.73	0.79	0.85	1.62	2.15
16:0	21.53	22.05	22.43	25.67	28.14
18:0	4.18	4.26	4.5	6.32	7.45
24:0	0.21	0.28	0.39	0.87	0.86
Σ SFA	34.73	36.67	38.58	47.28	54.71
16:1	14.52	13.69	10.25	10.03	9.37
18:1	24.86	23.14	18.08	19.37	16.75
24:1	1.13	1.58	2.16	2.37	2.91
Σ MUFA	40.51	38.41	30.49	31.77	29.03
Cis-18:2n6	11.57	11.39	14.58	12.06	7.26
Cis-18:3n6	0.58	0.62	0.66	0.68	0.38
18:3n3	3.05	2.62	3.48	1.03	1.24
18:4n3	0.38	0.42	0.58	0.58	0.43
20:2n6	0.82	1.28	1.26	0.64	0.31
20:3n6	0.18	0.2	0.28	0.31	0.13
20:4n6	0.52	0.56	0.84	0.91	0.82
20:5n3	0.97	1.28	2.51	2.58	2.63
22:5n3	0.00	0.06	0.09	0.11	0.12
22:6n3	0.12	0.11	0.15	0.15	0.05
Σ PUFA	18.19	18.54	24.43	19.05	13.37

commonly used in the hatcheries in general cannot meet the PUFA requirements of larval fish; however the extent to which PUFA enrichment is possible with *I. galbana* is not possible with other microalgae. In the preset study, we found DHA: EPA to be less than one with both *I. galbana* and *N. oculata*. The DHA content in *I. galbana* in our study was 9.76 % which was similar to that of *I. galbana* clone T-ISO reported by Ferreira *et al.* (2008). The most abundant PUFA was 18: 4 n-3 (Linolenic acid) followed by DHA as in the aforementioned report. DHA enrichment in rotifers with *I. galbana* increased to a level of 1.13% at 30 h beyond which decline was noticed. This was lower than the enrichment level obtained by Faulk and Holt (2005), which was 3.4 % for DHA for enrichment duration of 24 h.

The development of an indigenous fish oil based enrichment emulsion for live feeds was thus attempted. Crude sardine oil is available locally at a cost of Rs. 45 kg^{-1} ($1 kg^{-1}). Fish roe of quality fishes collected and sold by the fish vendors is available for Rs. 80 kg^{-1} (<$2 kg^{-1}). Fish oil was enriched as described earlier (Kajal and Paul Raj, 2009; Kajal *et al.,* 2010). Blended fish roe phospholipids (Table 4) were emulsified and stabilized with enriched sardine oil, to have two blends; one rich in DHA and another rich in EPA. The DHA rich emulsion, with a DHA content of 39% and EPA content of 19% was used to enrich rotifers, which resulted in an enrichment level of 8.76% DHA and 3.17% EPA in six hours after which decline was seen (Table 5). Enrichment levels of 27% DHA and 15% EPA is reported with a commercial product, High DHA Super Selco, INVE, SA, Belgium (Lie *et al.,* 1998). More than three to four fold higher level of enrichment reported by Lie *et al.* (1998) was not achievable with the emulsion developed in the present work. The ratio of DHA: EPA reported to be most appropriate (2:1) for marine fish could be obtained by enriching rotifers in the DHA rich emulsion (Sargent *et al.,* 1999). The ratio of DHA: EPA in rotifers after enrichment duration of 6 h was 2.76: 1 (Table 6). With another set of enrichment products Algamac 2000 and Algamac 3050 which are spray dried forms of a marine protist *Schizochytrium* rich in DHA (Faulk and Holt, 2005), DHA levels of 20% and 30% respectively is reported. This is because these products are spray dried powder (Algamac 2000) and flakes (Algamac 3050) with a content of 27% and 47% DHA respectively (www.aquafauna.com). Moreover, such high enrichment levels were achieved after growing the rotifers in *I. galbana* and then enriching them with these products.

Table 4. Fatty acid composition of seerfish roe phospholipids and triglycerides (% Total fatty acids, means ± SE)

Fatty acids	Phospholipids	Triglycerides
12:0	0.04 ± 0.01	1.70±0.06
14:0	1.80 ± 0.06	2.09±0.08
15:0	0.59 ± 0.10	0.87±0.03
17:0	1.10 ± 0.01	3.78±0.05
16:0	22.69 ± 0.57	19.87±0.29
18:0	7.34 ± 0.08	8.20±0.27
Total SFA	33.55 ± 0.35	36.50±0.56
16:1n7	3.43 ± 0.23	0.88±0.05
18:1n9	11.71 ± 0.90	14.07±0.02
17:1	0.40 ± 0.01	0.75±0.04
24:1	2.80 ± 0.11	2.88±0.16
Total MUFA	18.34 ± 1.25	18.57±0.27
18:2ω6	1.18 ± 0.03	1.29±0.01
18:3ω6	0.39 ± 0.01	0.56±0.06
18:3ω3	0.43 ± 0.05	0.41±0.02
GLA	0.57 ± 0.14	0.41±0.04
20:4ω6	6.09 ± 0.08	4.16±0.13
20:5ω3	5.17 ± 0.03	4.15±0.04
22:5ω3	3.28 ± 0.09	4.09±0.23
22:6ω3	24.02 ± 0.37	19.85±0.25
Total PUFA	41.11 ± 0.21	34.91±0.74

Table 5. Fatty acid composition of EPA rich and DHA rich oil emulsions (% Total fatty acids, means ± SE)

Fatty acids	EPA rich concentrate	DHA rich concentrate
SFA		
12:0	0.04 ± 0.01	0.03 ± 0.00
14:0	0.11 ± 0.01	0.03 ± 0.01
16:0	0.27 ± 0.04	0.14 ± 0.01
18:0	0.06 ± 0.01	0.03 ± 0.01
Σ SFA	0.47 ± 0.47	0.23 ± 0.01
MUFA		
16:1n7	11.3 ± 0.07	3.17 ± 0.03
18:1n9	1.17 ± 0.11	4.27 ± 0.02
17:1	0.15 ± 0.04	0.10 ± 0.03
20:1n11	0.12 ± 0.04	0.12 ± 0.02
Σ MUFA	12.73 ± 0.19	7.65 ± 0.01
PUFA		
18:2n6	2.26 ± 0.04	6.44 ± 0.21
18:3n3	8.19 ± 0.21	11.39 ± 0.02
18:4n3	1.31 ± 0.03	3.28 ± 0.13
20:4n6	0.14 ± 0.01	4.15 ± 0.05
20:5n3	47.70 ± 0.11	19.08 ± 0.17
22:5n3	2.88 ± 0.04	4.56 ± 0.11
22:6n3	17.11 ± 0.01	38.77 ± 0.17
Σ PUFA	79.57 ± 0.28	87.65 ± 0.18

Table 6. Fatty acid composition of rotifers enriched with DHA rich oil emulsion (% Total fatty acids)

Fatty acids	3h	6h	9h	21h	24h
14:0	3.98	3.20	4.50	5.60	5.82
15:0	1.38	0.43	3.20	3.69	4.11
16:0	18.96	16.42	22.04	22.24	25.09
18:0	9.64	6.18	7.12	7.50	8.10
24:0	0.92	0.69	2.11	2.23	1.89
Σ SFA	34.88	26.93	38.97	41.26	45.01
16:1	2.91	2.54	5.27	5.78	4.05
18:1	14.85	21.11	24.41	30.54	30.58
24:1	4.75	2.87	1.13	1.39	1.47
Σ MUFA	22.50	26.53	30.80	37.71	36.10
Cis-18:2n6	9.45	6.96	4.32	1.16	1.20
Cis-18:3n6	2.45	0.59	2.22	4.81	3.15
18:3n3	6.12	5.00	2.54	1.53	1.06
18:4n3	1.38	1.69	0.73	0.32	0.19
C20:2n6	0.46	0.17	1.31	2.45	2.34
C20:3n6	1.53	1.69	1.74	1.67	0.98
20:4n6ARA	6.12	9.84	8.93	4.53	4.28
20:5n3EPA	2.57	2.35	2.07	1.48	1.20
22:5n3	1.53	3.17	0.47	0.51	0.23
22:6n3DHA	4.29	8.76	1.43	1.23	1.14
Σ PUFA	35.90	40.21	25.76	19.70	15.77

With this product profile, our efforts to standardise live feed enrichment protocols to improve larval survival in marine ornamental fish (clowns and damsels) and food fish (*Rachycentron canadum*) are under way. The study proved that *I. galbana* has the most suitable fatty acid profile for marine fish larviculture in terms of DHA and EPA as reported from other parts of the world. Fish roe and fatty acid enriched sardine oil are good start material available locally to develop cost effective live feed enrichment products for domestic markets which can aid as a an import substitute. Refining the product to the extent of achieving the optimum fatty acid levels for translating into improved larval survival cycles and healthy fry for farming and grow out is the challenge ahead.

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