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P.B. No. 1603, Tatapuram P.O.,
Kochi – 682 014

BIOENCAPSULATION OF LIVE FEEDS

Imelda Joseph
Central Marine Fisheries Research Institute, Cochin

Introduction

When aquaculture is expanding world over to meet the protein requirement of humans, the basic requirement in culture practice is the seed and major constraint noticed is in larval nutrition. The larval survival also varies with the type of organism (finfish<10%; mud crab <1%; shrimp, 20-40%; molluscs <20%). The challenge in larval nutrition lies in the fact that live feeds are not completely replaced in hatchery operations. It is believed that the optimal formulations for first feeding larvae should simulate the yolk composition and to some extent reflect the nutrient requirements and metabolic capacities of pre feeding fish or other organisms.

Bioencapsulation or bioenrichment is the process involved in improving the nutritional status of live food organisms either by feeding or incorporating within them, various kinds of nutrients. Examples of practical and experimental enrichment diets are unicellular algae, emulsion, liposomes and microencapsulated diets. Marine larvae in general require the polyunsaturated fatty acids eicosapentanoic acid (EPA: 20:5n-3) and decosahexanoic acid (DHA; 22:6n-3) for their normal development and survival. Apart from EPA and DHA, arachidonic acid (ARA; 20:4n-6) has also been recognized as essential for marine fish. ARA is the main precursor of eicasoids responsible for osmoregulation, cardiovascular functions, neural control and reproduction. The rotifer *Brachionus plicatilis* and the anostracan *Artemia* are the two organisms most extensively used as larval feed. The lipid sources in enrichment diets vary in lipid class composition, n-3 HUFA content and DHA/EPA ratio. However, EPA is present in low amounts in *Artemia* nauplii and DHA is practically absent. For this reason, the nauplii need to be enriched before they can be used for feeding marine larvae. Phospholipids from vertebrate neural tissue have high content of DHA and it has shown that deficiency during critical stages of the embryonic development may lead to neurological abnormalities. Malpigmentation observed in halibut, turbot and red sea bream, larvae have been suggested to be a consequence of sub optimal DHA content or DHA/EPA ratio in the diet. Live food enriched with essential fatty acids improved larval performance in striped bass and palmetto bass, cod, red sea bream yellow tail, milkfish *etc.* Two to four percentages are the required level of n3 (HUFA) in diet (1% EPA and 1% DHA).

The enrichment is commonly achieved by placing the nauplii in a medium, generally an emulsion, containing EPA and DHA. The nauplii are passive filter feeders and thus incorporate in their digestive tract the emulsions acting as live vehicles. This enrichment process has also been termed bioencapsulation and is successful enough to allow the use of

Artemia nauplii as larval food for marine organisms, at least during certain phases of their rearing. The success of enrichment procedure, however, is mainly due to the fact that unenriched nauplii are lethal to many larvae than to the suitability of the bioencapsulated nauplii as larval food. Although the enrichment procedure allows marine larvae to survive on a diet of *Artemia,* enriched *Artemia* nauplii are far from being an optimal diet for it as compared to marine plankton, their natural diet. The degree of success in modifying the fatty acid profile of the nauplii has shown to be influenced by the type of the enrichment diet, the enrichment conditions and the live food strain itself. Differences in enrichment conditions are related to the salinity of the culture medium, the concentration of experimental emulsion, the enrichment duration and the temperature following starvation. Also the species and geographical origin of the *Artemia* affect the success of the enrichment procedure.

Factors to be considered prior to bioencapsulation

i) Selection of the carrier or the live food, taking into account the acceptability of the organism and its size.

Commonly used carriers are:

a) Micro algae - 2-20 µ
b) Rotifers - 5-200 µ
c) *Artemia* - 200-400 µ
d) Moina - 400-1500 µ
e) Daphnia - 200-400 µ

ii) Nutritional quality, digestibility and acceptability before and after encapsulation

iii) Fixing up of the level of enrichment media to be incorporated into the carrier organism, which depends on the nutritional quality of the carrier organism before incorporation and based on feeding trials conducted in laboratory.

iv) Economic feasibility of enrichment

v) Purity of the culture of carrier organism and

vi) The easy procurement of carrier organisms, its viable culture techniques; catchability of carrier organism by target species, and its easy reproduction.

If all the above factors are satisfied, one can go for a viable and effective enrichment.

Bioencapsulation of *Artemia*

Hatching of cysts: The cysts (4 gl^{-1}) are disinfected with hypochlorite solution of 200 gl^{-1}l for 20 minutes before hatching. After washing with tap water to remove the remaining hypochlorite, the cysts (2 gl^{-1}) are incubated in filtered seawater at 28°C under continuous aeration and light. After hatching, the nauplii (<90% Instar I) are separated from the empty cyst shells and transferred to 2 L glass tubes (cylindroconical) in a water bath at 28°C with continuous aeration from the bottom of the cone using an additional air stone to keep oxygen levels above 5-6mgl^{-1}.

Enrichment with highly unsaturated fatty acids (HUFA)

Standard enrichment emulsions containing 30% and 50% of total n-3 HUFA (percentage dry matter) with DHA/EPA ratio of 0.73 and 0.84, respectively and an emulsion devoid of n-3 HUFA. The emulsion contains (percentage wet weight) lipids (62%), water (30%), emulsifiers, antioxidants and liposoluble vitamins (A, D, E And K). The enrichment conditions are standardized at 28°C and salinity at 34 parts per thousand, for a period of 24h. The larvae are then cleaned in water and 28.9mg g^{-1} enrichment (DHA) could be obtained with 50% n-3 HUFA emulsions at 0.39 l^{-1}.

Enrichment of *Artemia* nauplii with free amino acids (FAA)

Amino acids are the major substrates of aerobic metabolism during the development of embryo and yolk sac larvae of marine species, which have pelagic eggs. Due to rapid larval growth and development, there is large amino acid requirement both to maintain the appropriate concentration in the tissues necessary to obtain an optimal growth rate and amino acid utilization, and to supply the fuel for energy metabolism. The need for dietary free amino acids for marine fish larvae is also suggested by the large pool of free amino acids found in marine invertebrates such as copepods, which are natural food for the larvae in the sea. *Artemia* contain lower level of free amino acids, especially methionine. This suggests that methionine is a limiting amino acid for fish larvae when fed *Artemia* nauplii.

One approach to enrich *Artemia* with water-soluble substances, such as FAA is to use liposomes. Liposomes are spherical vesicles (10nm-20μm) in which an aqueous volume is entrapped by a membrane composed of lipid molecules, usually in the form of phospholipids. These are potent delivery vectors for hydrophilic as well as hydrophobic nutrients and are potential carriers for FAA. Phospholipid form concentric bilayers when dispersed in an aqueous medium enclosing the aqueous material in the core, as well as within the bilayered lamellae. The compatible size range and complete digestibility of liposome make them a good vehicle for the study of nutritional requirements of aquatic filter feeders.

Liposome preparation

Egg yolk phospatidyl choline (PC): cholesterol (10:4 w/w): 100 mg egg yolk and 40mg cholesterol are dissolved in 5 ml chloroform: methanol (2:1v/v) in a round bottom flask. The solvents are evaporated at 30°C under nitrogen in a rotary evaporator and the resulting lipid film is further dried for 30 min in vacuum desiccators. 5 ml of 530mv NaCl solution is introduced into the flask along with 5 ml of 300mM methionine and rotated slowly for 30 min at 35°C. The resulting liposome suspension is extruded through a polycarbonate filter (0.6μm). Liposomes can provide protection against degradation, as well as easier incorporation of the lipids contained in them.

Encapsulation protocol

Artemia cysts are hydrated, decapsulated and incubated at 28°C under continuous aeration and illumination. After 24 h, larvae are washed and stored at 20°C for 5h before transferred to 50ml plastic centrifuge tubes for enrichment. Add 0.625ml of 300 mM methionine and equal amount of methionine in their respective liposome suspensions also. After 16 h, the nauplii are washed with tap water and transferred to seawater.
Direct enrichment: In this procedure, nauplii are enriched with methionine directly dissolved in the culture water and the nauplii are analyzed for FAA after 16h.

Nutritional improvement of rotifers

Improvement of rotifer *Brachionus plicatilis* as food for marine fish larvae has been achieved through enrichment with various diets containing different levels of (n-3) highly unsaturated fatty acids (HUFA). These diets include microalgae, lipid emulsions, fish oils, microparticles and microcapsules containing lipids. HUFA content of the enriched rotifers is to a great extent a reflection of the content of these fatty acids in the diets. Enriched rotifers have been shown to be able to maintain their improved nutritional value for several hours at 10°C during their application as live food for marine fish larvae.

The concept of long-term (LT) and short-term (ST) enrichment is also done for live feed enrichment. For LT, it combines growth and n-3 HUFA enrichment during the production phase. The ST enrichment involves short (<24h) exposure to high concentration of specific HUFA rich feed following culture. The latter is important for cold-water fishes.

Short-term enrichment of rotifers with microalgae

Microalgae are used for short-term enrichment of rotifers before they are given to the fish larvae to improve the content of n-3 fatty acids in the rotifers. Short-term feeding with algae affects the protein content and dry weight of the rotifer.

Preparation of enrichment

For emulsified lipids, the (n-3) HUFA concentration in the lipid source should be very high.

- 5g fish oil is homogenized for 2-3 min in a homogenizer or mixer or by vigorous shaking.
- Observing under microscope ensures proper emulsion
- Stored under refrigeration till use.
- Emulsifiers may be added to maintain the emulsion or a strong shaking prior to use reforms the emulsion.
- The enrichment media may be supplemental with water and fat-soluble vitamins like vitamin A, D, E and K to homogenization.

One potential risk in using fish oil for HUFA enrichment, the n-3 HUFA particularly DHA in the very small triacyl glycerol (TAG) micelles generated in enrichment procedures are prone to auto oxidation, especially under continuous aeration employed, for prolonged periods. The addition of natural antioxidants vitamin E and C (generally added as oil soluble α tocophenyl acetate and ascorbyl palmitate) which are not effective until hydrolyzed in the intestinal tract and absorbed and or synthetic antioxidants like ethoxyquin or butylated hydroxyl anisole (BHA), minimizes peroxidation.

Lecithin can be used to considerable advantage in enriching the nauplii with 22:6n3 HUFA rich fish oils (90:10; Fish oil: lecithin), lecithin acting both as natural emulsifying agent and as a natural protectant against peroxidation. Oils from marine organisms like heterotrophic dinoflagellate *Crypthecodinium cohnii*, which is mass cultured for a triacyl glycerol rich in 22:6n-3. This oil is used to supplement infant formulae. Frozen-thawed cells of *C.cohnii* have recently been used very successfully to supplement *Artemia* nauplii directly with 22:6n-3. Spray dried *Schizochytrium* sp. a single celled heterotrophic marine proteist similar to water molds of group *Labyrinthulomycota* is also used.

Bioencapsulation with vitamin C

Bioencapsulation is done using the lipophilic ascorbyl palmitate (AP) as a stable and bioavailable source of Ascorbic acid (AA) in emulsions and particulate boosters for the live food prior to their feeding to fish larvae. Applying experimental self-emulsifying concentrates supplemented with 10 and 20% AP, high levels of AA could be obtained in 24 h enriched *Artemia* nauplii. Four fold increase in AA is obtained with 20% AP.

Bipencapsulation with drugs

In addition to the use of enrichment in nutrients, live food mediated delivery of therapeutic drugs has emerged as a new tool for disease treatment in larval culture. This is normally done through liposomes.

The overall nutritional quality of the live food organisms depends on the content and nutritional balance of carbohydrate, protein and lipids. The content of essential amino acids and protein in live food must meet the requirement for growth and cell maintenance of fish larvae. The enriched live food might be starved in the culture tank before they are consumed by the larvae and in this period, the protein, lipid and fatty acids decreases. The loss of all these components increase with increasing water temperature. The total lipid content loss is at higher rate than the protein content. Highest loss rate is for DHA and considerably lower for EPA.

It is concluded that survivability of marine larvae in hatcheries depends on the larval nutrition, which in turn is due to the quality loss (potency loss of the stock on continuous use, mutation *etc.*) of live food organisms. Bioencapsulation can be effectively and successfully employed to cut down the larval mortalities in hatcheries.

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