Production of an indigenous micro algal concentrate from *Nannochloropsis oculata* (NANN CON): An alternate approach

Biji Xavier*, Sekar Megarajan, Ritesh Ranjan, Vamsi Balla, S. Padmaja Rani, Chinnibabu Bathina and Shubhadeep Ghosh
Visakhapatnam Regional Centre of ICAR-Central Marine Fisheries Research Institute, Visakhapatnam - 530 003, Andhra Pradesh
*E-mail: bijicmfri@gmail.com

Abstract

Nannochloropsis oculata is a marine micro algae used directly for green water larval rearing system and indirectly for rotifer culture in marine finfish hatchery. Difficulties in algal culture during summer months can be overcome by the production of microalgal concentrate which can be stored and used when required. Harvesting of micro algal biomass from the culture is considered as crucial step for the production of algal concentrate. The use of centrifugation is the effective method for harvesting microalgae and steps involved in the production of *Nannochloropsis* concentrate (NANN CON) is described in detail. Centrifugation which could accumulate a cell count of an approximately 30 billion/ml from 300 litres of *N. oculata* culture was the most efficient method as the cells in the concentrate retained shape and nutritional contents similar to cultured fresh microalgae. Chilled NANN CON preserved with 10% glycerol proved advantageous had more than 80% viable cells even after five months of storage. The preserved NANN CON is effective as feed for rotifer, algal inoculum and Green water larval rearing systems. The cost for production of NANN CON was ₹ 1158.46 per kilogram and in several aspects proved superior to other commercial products currently used.

Keywords: *Nannochloropsis oculata, rotifer, Green water*

Introduction

Nannochloropsis oculata, a marine microalgae (Eustigmatophyceae) plays an important role in seed production of marine finfishes. This small sized algae (2-5 μm) has fast multiplication rate and is rich in Chlorophyll a, Astaxanthin, Zeaxanthin, and Canthaxanthin. The algae are used directly and indirectly in marine finfish hatchery for green water larval rearing system and rotifer culture, respectively. Intermediate cultured algae are mainly used in larval rearing and mass cultured algae are used for live zooplankton (rotifer) culture. *N.oculata*, being a temperate waters species, mass production of microalgae in outdoor culture systems is difficult during the summer months in tropical countries like India. However, larval rearing for most of the marine finfishes peak during this period. Difficulties in algal culture during summer months is a major bottleneck in the year-round marine finfish larval production cycles. Microalgal concentrate is an alternative approach to ensure all-time availability of sufficient quantities of microalgae for larval rearing and zooplankton culture. These are prepared with added preservatives to be used at the required time. Different methods used to prepare algal concentrates include 1) coagulation, 2) flocculation, 3) flotation, 4) centrifugation and 5) filtration. Among these, centrifugation proved to be the most efficient method with >90% harvesting efficiency. Centrifuged micro-algal concentrates retained shape and nutritional contents as that of fresh cultured microalgae and can be used for green-water larval culture, rotifer culture and even as inoculums for further *Nannochloropsis* culture. Importantly, with optimum centrifugation time and speed, the shape of cells is maintained which helps the cells to maintain its viability during storage.

Major advantage of NANN CON over commercial products is that more than 80% of the cells in the concentrate are viable even after five months of storage in glycerol under chilled conditions. This cell can be used as inoculum for scaling up the algal culture. Additionally, the cells of the prepared concentrate remains suspended in water column for longer time similar to fresh cultured nanno cells, which helps to maintain the water quality in rotifer culture and larval culture tanks based on its use. In many of the commercially available *Nannochloropsis* concentrates, viable cells are not maintained and most of the cells settle slowly, which quickly degrades the water quality in the culture environment.

Steps involved in preparation of NANN CON

Seawater treatment: Sea water is filtered mechanically through sand filter, followed by UV filtration and finally treated with ozone for complete sterilization. A residual ozone concentration of 0.1 - 2.0 mgL^{-1} for a period of 1 - 30 minutes, is required to be maintained for complete disinfection. It is advisable to maintain nil ozone in the sea water before the inoculation of *N. oculata*.

Preparation of intermediate culture of *Nannochloropsis oculata*: 'Conway' or 'Walne's medium is used for the preparation of culture medium in the indoor culture of *N. oculata*.

The optimum environmental parameters for *N. oculata* culture include temperature: 18-24°C; salinity: 20-24 gL^{-1}; light intensity: 2,500-5,000 lux; photoperiod: 24 hours and pH: 8.0-8.5.

Stock culture of microalgae (10%) is inoculated into seawater with culture medium (Conway). This is maintained for 3 days with optimum aeration (Fig.1). The culture in growing phase / log phase is selected for the preparation of *N. oculata* concentrate by centrifugation (Fig.2). At this time, cell count should reach 30-40 million / ml if culture is healthy. Additionally, the cell count could be enhanced upto 80 million / ml if the culture is supported with pure CO_2.

Preparation of NANN CON: Industrial centrifuge, which holds more volume of culture, is used for this step. The culture is transferred into centrifugation bottle and centrifuged at the maximum speed of 4000 rpm for 30 minutes. The supernatant is decanted and the precipitated concentrate is collected without damaging the cells. Using this method, centrifugation of 300 litres of *N. oculata* culture can accumulate a cell count of an approximately 30 billion/ml. The concentrate with 5 - 20% inclusion of glycerol is amenable to preservation by both, chilling and freezing. Glycerol at 10% inclusion performs better with chilling and 20% inclusion performs well with freezing.

Cell viability test: Viability of the harvested and preserved cells is tested using 'Evans Blue' stain. Ruptured cells appear blue, since Evans Blue solution diffuses into the protoplasm region and stain the cells blue (Fig.6).

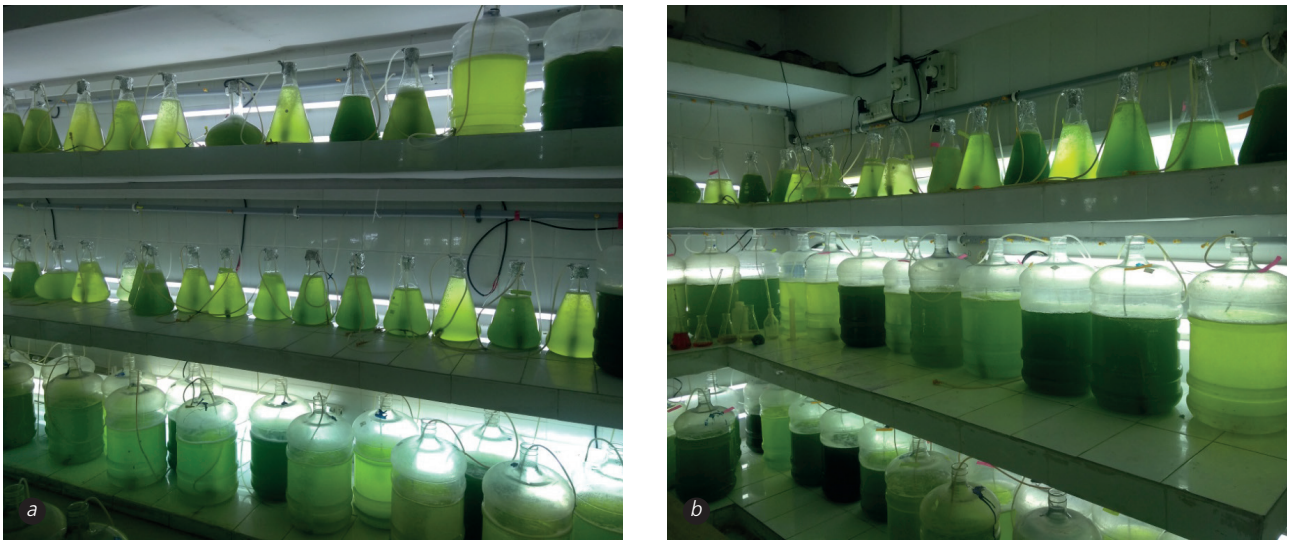

Fig.1. Stock (a) and Intermediate (b) culture of *Nannochloropsis oculata*

Fig. 2. Intermediate culture of *N. oculata* before centrifugation

Fig.3. Intermediate culture of *N.oculata* after centrifugation

Fig.4. NANN CON concentrate

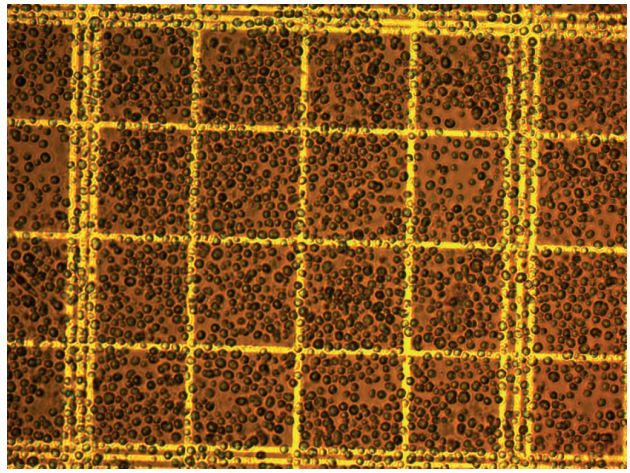

Fig.5. Concentrated *N. oculata* cells after dilution (20X)

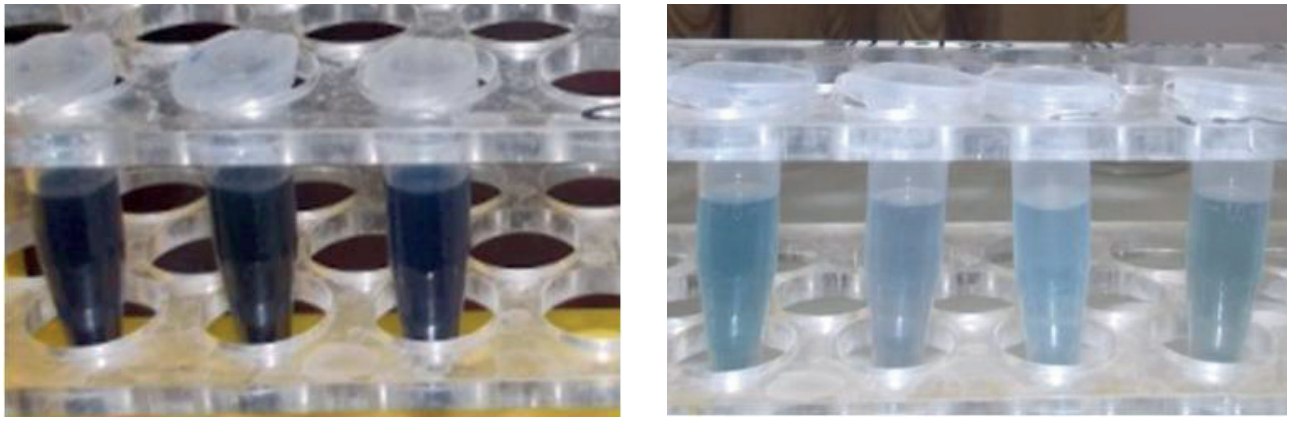

Fig.6. Diluted *N. oculata* cells from concentrate with Evan's Blue stain applied and after staining process completed (on the right)

Application of *Nannochloropsis* concentrates: NANN CON is used in fish hatcheries as feed for rotifer, as algal inoculum and in Green water larval rearing systems. For rotifer (*Brachionus plicatilis*) culture, using NANN CON (preserved in 10% glycerol) as feed resulted in a maximum of 1040 rotifers / ml at the concentration $3x10^6$ *Nannochloropsis* cells / ml as indicated in Fig.7.

As inoculum for further *N. oculata* culture the preserved NANN CON (0 to 20% of glycerol in chilling) is diluted with sterilized sea water to obtain a cell count of

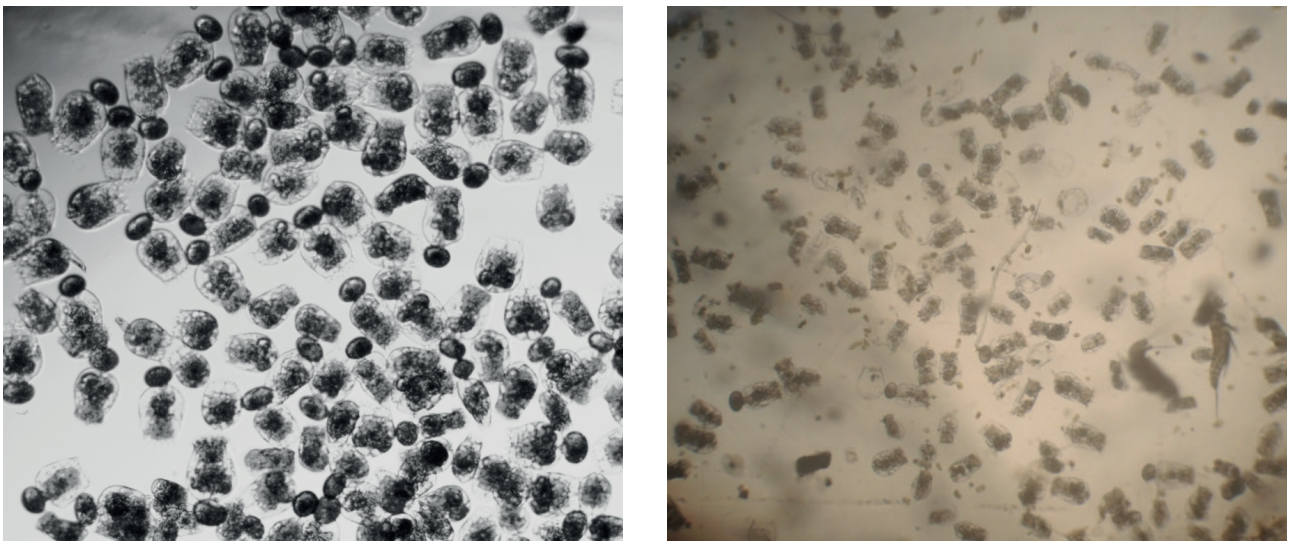

Fig.7. Rotifer cultured on NANN CON for marine finfish larval rearing

1x 10^6/ml. A 10% of inoculum is added into 10 ml of culture medium containing Conway medium. The culture is maintained at optimum environmental condition for the stock culture development. At the end of third day of inoculation, the cell count reached 12 x 10^6 cells/ml in culture with chilled cells preserved in 10% glycerol and the same stock is used for further development. The glycerol preserved NANN CON can be diluted and also used as direct inoculum to prepare intermediate culture.

In the Green water system for marine finfish larval rearing, an approximately 3.5 g of NANN CON added to 1 cubic metre water volume, maintained the *Nannochloropsis* concentration of 1 lakh cells/ml in suspended condition and also maintained the water quality during the period of larval rearing.

Economics for production of NANN CON: Product development is dependent on the economic viability of the product and therefore, the cost of production for producing 1 kg of NANN CON is calculated. The cost (fixed and variable) involved is presented in Table1. The cost of producing NANN CON using centrifugation method is ₹1158.46 per kg with a cell count of approximately 30 billion/ml.

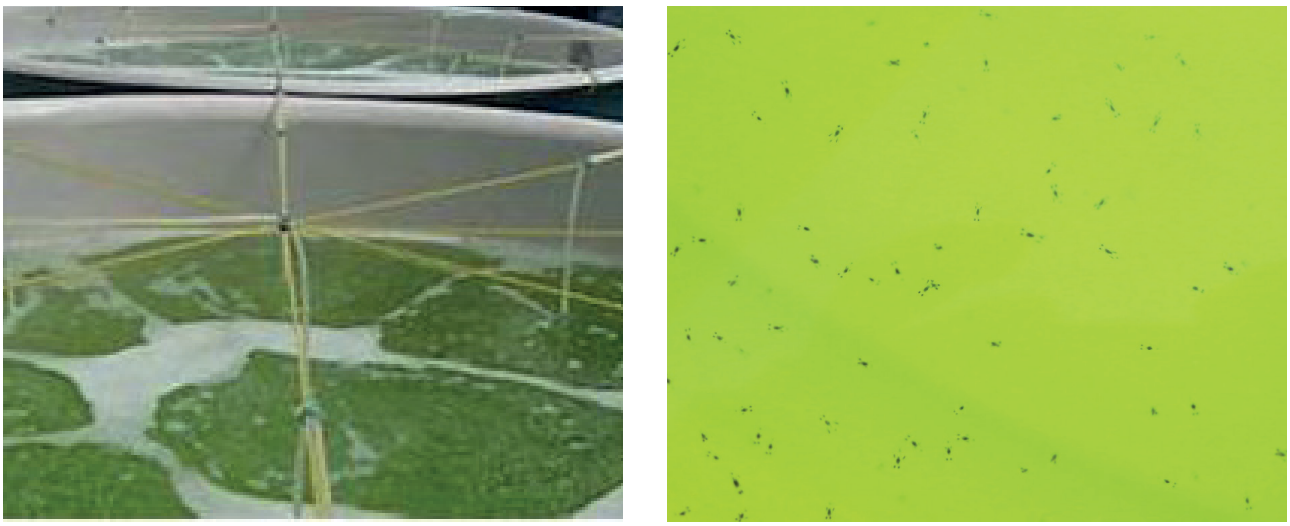

Fig.8. Green water rearing system with *N.oculata* for finfish larval rearing and in close-up (on right)

Table 1. Economics of culturing *Nannochloropsis oculata* for NANN CON preparation

Items	Qty.	Unit	Unit cost (₹)	Total cost (₹)	Economic life	Yearly depreciation	Daily depreciation	Depreciation based on usage
Equipments								
Dry oven	1	unit	50000	50000	20	2500	6.84	0.28
Weighing balance	1	unit	150000	150000	20	7500	20.55	0.85
Air blower	2	unit	12000	24000	10	2400	6.57	5.26
Ozone generator/Autoclave	1	unit	90000	90000	10	9000	24.65	1.03
Compound microscope	1	unit	30000	30000	20	1500	4.11	0.00
Haemocytometer	1	unit	1500	1500	10	150	0.41	0.00
Gas stove and cylinder	1	unit	2000	2000	10	200	0.55	0.00
Centrifuge and accessories	1	unit	350000	350000	20	17500	47.94	23.97
Refrigerator	1	unit	15000	15000	20	750	2.05	0.00
Lux meter	1	unit	10000	10000	20	500	1.37	0.00
Refractometer	1	unit	5000	5000	5	1000	2.74	0.00
Air conditioner	1	unit	30000	30000	10	3000	8.22	6.57
UV lamp	1	unit	7000	7000	10	700	1.92	0.00
Distillation unit	1	Unit	350000	350000	15	23333	63.93	0.00
Subtotal (Rs)				1114500		70033	191.85	37.96
Culture materials								
Test tubes 15ml	10	pcs	16	160	3	53.33	0.15	0.00
Conical flask 100ml	10	pcs	85	850	3	283.33	0.78	0.00
250ml	10	pcs	130	1300	3	433.33	1.19	2.56
3000ml	15	pcs	1485	22275	3	7425	20.34	6.10
Carbouy 20 L	15	pcs	150	2250	3	750	2.05	1.02
Reagent bottles 1000ml	3	pcs	700	2100	10	210	0.57	0.00
Spatula	10	pcs	80	800	10	80	0.22	0.00
Tissue paper	1	unit	75	75	100days	0	0.75	0.00
Glass pipette: 10ml	2	pcs	180	360	3	120	0.33	0.00
5ml	2	pcs	160	320	3	106.67	0.29	0.00
1ml	2	pcs	150	300	3	100	0.27	0.00
Pipette bulb	3	pcs	20	60	5	12	0.00	0.00
Air hose	15	m	60	900	3	300	0.82	6.57
Air stone	15	stone	25	375	2	187.5	0.51	4.11
Air valve	15	pcs	4	60	3	20	0.05	0.00
Florescent lights	3	units	250	750	2	375	1.03	4.10
Aluminium foil	20	m	5	100	3days	0	20.0	0.00
Pasture pipette	10	pcs	2	20	2	10	0.00	0.00

Items	Qty.	Unit	Unit cost (₹)	Total cost (₹)	Economic life	Yearly depreciation	Daily depreciation	Depreciation based on usage
Glass slide	10	pcs	3	30	3	10	0.00	0.00
Coverslip	10	pcs	1	10	3	3.33	0.00	0.00
Plastic brush	5	pcs	40	200	60days	0	3.33	0.00
Plastic tub (100 litres)	2	units	1000	2000	5	400	1.09	0.00
Water storage tank:300 litres	2	units	2500	5000	10	500	1.37	0.00
Sanitizer	1	bottle	40	40	30days	0	1.33	0.00
Broom	1	pc	80	80	90days	0	0.89	0.00
Bucket	2	pcs	110	220	2	110	0.30	0.00
Funnel	1	pc	90	90	5	18	0.05	0.00
Mug	1	pc	50	50	5	10	0.03	0.00
Subtotal				40775		11517.49	57.74	24.46
Total				1155275		81550	249.59	62.42

Cost (₹) of producing 1kg *Nannochloropsis oculata* concentrate (NANN CON)

Item		Quantity	Cost (₹)
	Equipments	-	37.96
Depreciation:	Culture Materials	-	24.46
Inoculum @ ₹360/100 ml		1.0 ml	3.6
Culture (Conway) medium @ ₹0.27/ ml		480 ml	129.6
Gas: CO_2		-	50
Electricity @ ₹4/ unit		115.71	462.84
Labour @ ₹300/day		(1.5 day)	450
Total operating cost			1158.46