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

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Introduction

Nannochloropsis oculata is a marine micro algae (Eustigmatophyte) playing an important role in seed production of marine finfishes. It is a small sized algae (2-5ìm) having fast multiplication rate and 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. *Nannochloropsis oculata*, being a temperate species, mass production of microalgae in outdoor culture systems is difficult during the summer months, but larval rearing for most of the marine finfishes are at the peak during this period. Difficulties in algal culture during summer months are one of the bottlenecks in the year-round marine finfish larval production.

Microalgal concentrate is an alternative approach to ensure all-time availability of sufficient quantities of micro algae for larval rearing and zooplankton culture. Micro-algal concentrates are prepared and preserved with added preservatives and this could be used at the time of requirements.

Different methods used to prepare algal concentrates include 1) coagulation, 2) flocculation, 3) flotation, 4) centrifugation and 5) filtration. Among all, centrifugation is proven to be the most efficient method with >90% harvesting efficiency. Centrifuged micro-algal concentrates remains with same shape and nutritional contents as that of fresh cultured microalgae. Therefore, the cells harvested using the technique can very well be used for green-water larval culture, rotifer culture and even as inoculums for further *Nannochloropsis* culture. Importantly, at optimum time and speed, the shape of cells is maintained by the centrifugation method, which helps the cells to maintain its viability during storage.

Intermediate culture of *Nannochloropsis oculata* after centrifugation

Intermediate culture of *Nannochloropsis oculata* before centrifugation

Major advantage of *Nannochloropsis oculata* concentrate over commercial products is that the cells in the concentrate are viable (more than 80%) 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 as like fresh cultured nanno-cells, which helps to maintain the water quality in rotifer culture and larval culture tanks based on its use. However, in many of the commercially available *Nannochloropsis* concentrates, viable cells are not maintained and most of the cells settled slowly, which quickly degraded the water quality in the culture environment.

Steps involved in preparation of *Nannochloropsis oculata* concentrate (NANN CON)

1. Seawater treatment
2. Preparation of intermediate culture of *Nannochloropsis oculata*
3. Cell harvest by centrifugation
4. Cell preservation and viability

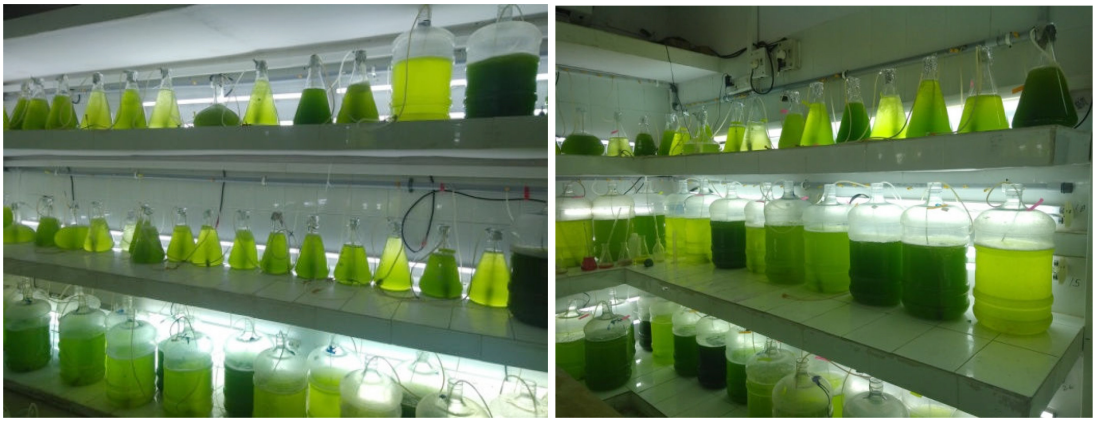
Seawater treatment: Sea water drawn from sea is filtered mechanically through sand filter, followed by UV filtration and finally treated with ozone for complete sterilization. A residual ozone concentration of 0.1 - 2.0 mgL^{-1} for a period of 1 - 30 minutes, is required to be maintained for complete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

Preparation of intermediate culture of *Nannochloropsis oculata*:

Culture medium: 'Conway' or 'Walne's medium is used for the preparation of culture medium for the indoor culture of *Nannochloropsis oculata*.

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

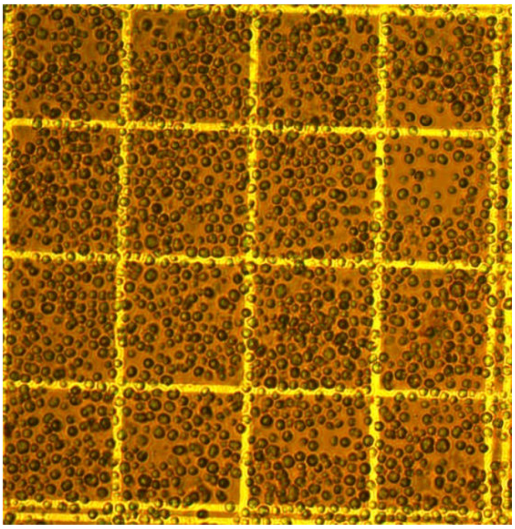
Inoculation of the culture: Stock culture of microalgae (10%) is inoculated into seawater with culture medium (Conway). The culture is maintained for 3 days under required environmental parameters with optimum aeration. The culture in growing phase / log phase is selected for the preparation of *Nannochloropsis oculata* concentrate by centrifugation. At this time, cell count should reach 30-40 million / ml if culture is healthy. Additionally, the cell count could be enhanced further upto 80 million / ml if the culture is supported with pure $CO_{2.}$



Stock and Intermediate culture of *Nannochloropsis oculata*

Preparation of Nannochloropsis oculata concentrate (NANN CON): Industrial centrifuge, which holds more volume of culture, is used for preparation of the concentrate. While preparation, the culture is transferred into centrifugation bottle. The culture is centrifuged at the maximum speed of 4000 rpm for 30 min. The supernatant is decanted and the precipitated concentrate is collected with the help of spatula or any other means without damaging the cells. This method can accumulate a cell count of an approximately 30 billions / ml from centrifugation of 300 lits of *Nannochloropsis oculata* culture.

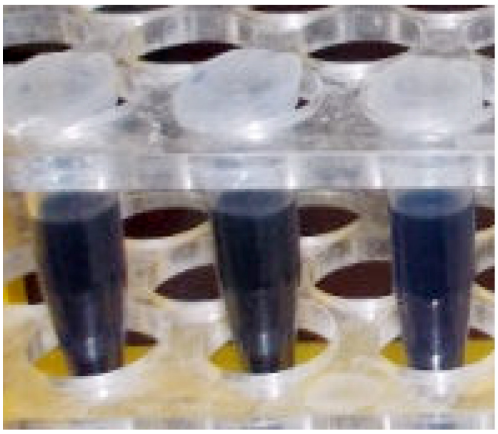
Prepared *Nannchloropsis oculata* concentrate (NANN CON)

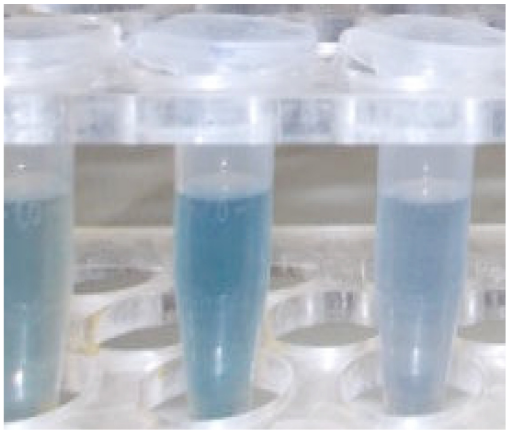


Concentrated *Nannochloropsis oculata* cells after dilution (20X)

Preservation in glycerol: The *Nannochloropsis oculata* concentrate (NANN CON) 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.





Diluted *Nannochloropsis oculata* cells from concentrate with Evan's Blue stain and after staining

Application of *Nannochloropsis* concentrates in fish hatcheries:

The preserved *Nannochloropsis* concentrate (NANN CON) is efficiently used as

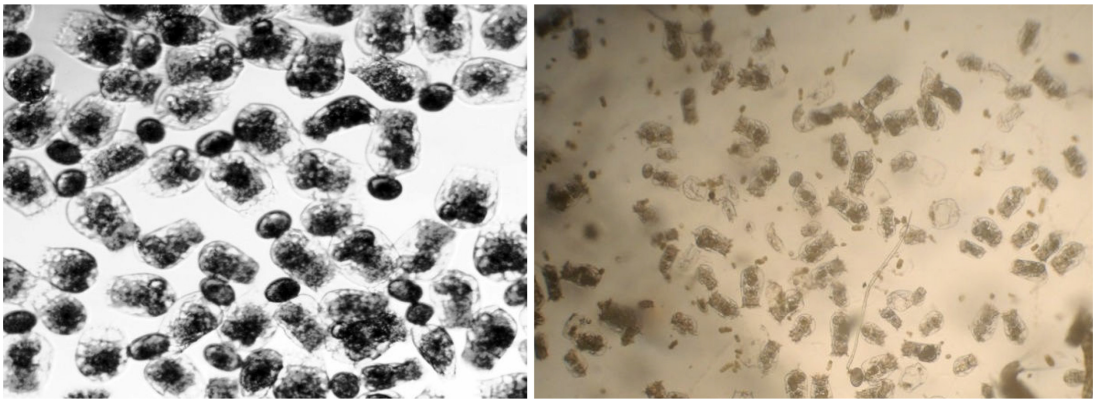
1) Feed for rotifer,

2) Algal inoculum and

3) Green water larval rearing systems.

Nannochloropsis concentrate (NANN CON) as feed for rotifer culture:

The rotifer (*Brachionus plicatilis*) when cultured using NANN CON (preserved in 10% glycerol) as feed resulted in a maximum of 1040 nos. of rotifers / ml at the concentration $3x10^6$ *Nannochloropsis* cells / ml in rotifer culture.



Rotifer cultured on Nannochloropsis oculata concentrate (NANN CON) for marine finfish larval rearing

Nannochloropsis concentrate (NANN CON) as inoculum for Nannochloropsis 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 $1x\ 10^6$/ 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. It is concluded that at the end of third day of inoculation, the cell count reached 12×10^6 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 is diluted and is also used as direct inoculum to prepare intermediate culture.

Nannochloropsis concentrate (NANN CON) for green water system in larval rearing

To maintain green water system for marine finfish larval rearing, an approximately 3.5 g of NANN CON is added into 1 cubic meter water volume, and this maintained the *Nannochloropsis* concentration of 1 lakh cells / ml in suspended condition, which also maintained the water quality during the period of larval rearing.

Green water rearing system (*Nannochloropsis oculata*) for finfish larval rearing

Economics for production of *Nannochloropsis oculata* concentrate (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 the Table. The cost of producing NANN CON using centrifugation is Rs. 1158.46/kg with a cell count of approximately 30 billions / ml

Inventory of equipment, materials and depreciation schedule for culturing *Nannochloropsis oculata*

Items	Qty.	Unit	Unit cost (Rs.)	Total cost (Rs.)	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
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(100lit)	2	units	1000	2000	5	400	1.09	0.00
Water storage tank:300L	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 (Rs.) of producing 1kg *Nannochloropsis oculata* concentrate (NANN CON)			
Item		**Quantity**	**Cost (Rs.)**
Depreciation:	**Equipments**	-	37.96
	Culture Materials	-	24.46
Inoculum @ Rs. 360/100 ml		1.0 ml	3.6
Culture (Conway) medium @ Rs. 0.27/ ml		480 ml	129.6
Gas: CO_2 -		50	
Electricity @ Rs. 4/ unit		115.71	462.84
Labour @ Rs. 300/day		(1.5 day)	450
Total operating cost 1158.46			

Conclusion

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 the concentrate produced is distributed to hatcheries having no facilities for culturing algae. The developed NANN CON can directly be applied in mariculture as rotifer feed, inoculum for micro algal culture and for green water larval rearing systems. NANN CON is having good viability, which makes it superior to other commercial products.

References

Biji Xavier et al., 2018. Mar. Fish. Infor. Serv. T & E Ser., 238: 7-10. Hu,Qiang. 2014. Algal Research 4:1-45

Milledge, J. J and S. Heaven. 2013. Rev. Environ. Sci. Bio., 12(2): 165-178. Vandamme, D. et al., 2011. Biotechnol. Bioeng. 108:2320-2329