J. mar. biol. Ass. India, **46** (1) : 98 - 103, Jan. - June, 2004

NOTE

Observations on the long term preservation and culture of the marine microalga, *Nannochloropsis oculata*

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Abstract

This paper deals with the results of experiments on the viability and culture of the microalga, *Nannochloropsis oculata* under preserved conditions. Uncentrifuged samples were kept in refrigerator (without cryoprotector) to observe the duration of viability and the algae could be maintained for 18 months by cooling. The cell quality under cooled culture condition was observed to be better and indicated the population growth similar to regular continuous culture after reinoculation. Variation in growth rate and cell concentration were observed between Walne's and Locally Developed Nutrient Medium (LDM). The cooling method preservation was most simple and effective for *N.oculata*. Twelve months old preserved samples of cooled culture were raised and inoculated to outdoor mass culture using LDM.

Algal culture is an important process in the seed production of finfish and shellfish. They have either an initial link with the food chain or an indirect effect on the rearing process. They are cultured to feed the rotifers and to serve as water conditioner of the fish larval rearing system. Among numerous species of microalgae *Nannochloropsis oculata* has received priority attention in recent years in view of its demand for rearing operations in many hatcheries. The investigations revealed the nutritive value of this species as it contains required amount of Eicosapentaenoic acid (EPA, 20:5n-3) (Hirayama *et al.*,1979., James and Abu Rezeq, 1989., and Lubzens *et al.*, 1995). Stocking of *N.oculata* to finfish larval rearing tanks has been found to enhance the survival rate during the stage in which they feed on rotifers. The data showed that when *N.oculata* was used as food in the mass production of rotifers *Brachionus plicatilis*, population density and rate of reproduction almost doubled compared to *Chlorella* sp., *Tetraselmis* sp. and baker's yeast. It was proved that the production of rotifers fed with *N.oculata* has sufficient amount of amino acid profile and fatty acid required for predator larvae (Hirayama *et al.*,1979). Further, the algal cells of *N.oculata* do not settle at the bottom of the larval rearing tanks, which inhibit the development of contaminants like ciliates. The culture of *N.oculata* normally starts with laboratory culture in small tubes or flasks and are upscaled gradually to outdoor mass culture in large tanks. The inocula are main-

tained individually year round with subculture carried out each month and are used to start the large scale culture as required. The continuous subculture of microalgae is a constraint for the aquaculturists and hence it is desirable to preserve them for longer period. The technique for the long time preservation of microalgae *N. oculata* is a necessity to reduce maintenance cost over the period during which this food is not required and also to ensure availability when the stock culture gets contaminated with ciliates and other unwanted microorganisms.

The main aim of present study was to preserve the *Nannochloropsis* cells for longer period as the stock culture to reduce the maintenance and production cost by using low cost commercial fertilizers. The experiments were conducted at Regional Centre of CMFRI at Mandapam Camp during June 2000 to January 2002 and the results are presented in this paper.

We wish to express our gratitude to Dr. M.Rajagopalan, Head, FEMD and Dr.C.P.Gopinathan, Dr.N.Kaliaperumal, and Shri A.Raju, Principal Scientists, CMFRI, for their constant support and critically going through the manuscript. We are also thankful to S/Shri M.Jayasingh (Field man), K.M.David, Artist and K.Sriram for their assistance.

Material and methods

The material, *N.oculata* was obtained from National Institute of Coastal Aquaculture, Thailand and the stock culture was maintained in the temperature-controlled room at 20-25°C. Each 150 ml culture samples (without cryoprotectant) at a density of 34.8×10^6 cells/ml were poured into two Erlyen meyor sterilized stock flask of 250ml capacity. The flask was plugged with cotton and preserved in home type refrigerator provided with light facility and the flask was kept in refrigerator at 3-5°C. After varying periods of time (in 3 months interval) 5ml of well mixed preserved samples were inoculated into 4 sterilized stock flasks, each containing

Table 1. *Details of the preservation and culture of the microalgae* Nannochloropsis oculata *during the period June 2000 to January 2002.*

Preservation period in months	Cell density- million cells/ml									
	Control (20-25° C)					Cooling (3-5° C)				
	Lag phase (days)	Walne's medium		LDM		Lag phase (days)	Walne's medium		LDM	
		Ini.	Max.	Ini.	Max.		Ini.	Max.	Ini.	Max.
1	1 day	2.62	85.8	2.62	45.0	1-2	2.36	89.33	2.36	35.8
6	-	-	-	-	-	"	2.85	81.2	2.85	26.5
12	-	-	-	-	-	"	3.15	77.6	3.15	29.0
18	-	-	-	-	-	"	3.48	77.14	3.48	46.4

50ml sterilized fresh sea water enriched by Walne's and Locally Developed Nutrient Medium (LDM). This medium is composed of ammonium sulphate, urea and super phosphate in the ratio of 10:1:1 respectively. Another set of two flasks enriched with Walne's and LDM media, were inoculated from regular continuous culture. All the flask samples were kept under two white fluorescent lamps with 8 hours illumination of 1600-2000 lux at room temperature 20-25°C. The growth of *N.oculata* in the flasks was observed daily. The cell concentration was monitored every alternate day using haemocytometer.

Mass culture

Experiments on mass culture were also conducted in outdoor one tonne FRP tank using 12 months old preserved culture sample as inoculum. The tank was filled with 850 l of filtered seawater and fertilized with ammonium sulphate, urea and super phosphate at the ratio of 10:1:1 respectively. Vigorous aeration was provided and 13 l of *N.oculata* raised from the preserved culture samples was inoculated. Environmental parameters were monitored daily and the cell count was taken on alternative days. The *p*H fluctuated between 7.94 to 9.93 and oxygen varied from 4.1 to 8.3 ml/l. The temperature ranged from 27.5°C to 34°C and the salinity was 27 to 30 ppt.

Results

All the algal cells were found to be alive upto 18 months of preservations but the size of the cell has become smaller than normal and the colour slightly lighter compared to fresh material. The salient features of the experiments are presented in Table1.

The experimental period for control and cooling method of preservation was 32 and 36 days respectively. The maximum cell concentration in control was 85.8 and 45.0 million cells/ml for Walne's and LDM respectively. In refrigerated 45 days old preserved sample, the maximum concentration was 89.33 and 35.8 million cells/ml in Walne's and LDM. On the other hand, the maximum concentrations of 77.14 and 46.4 million cells/ml were observed for 18 months preserved sample in respect of Walne's and LDM.

In the mass culture experiments the initial cell concentration was 0.49 which increased to 36.5 million cells/ml within 12 days of culture. It was observed that when the *p*H and oxygen in the culture tank got increased, the ammonia content decreased directly proportional to the cell density. The growth pattern of *N.oculata*, preserved under cooling and controlled conditions are represented in Figures 1 and 2

Discussion

The culture of natural planktonic algae is essential to avoid water contamination due to decomposition whenever excess artificial food is used in the larval rearing culture activities and mass production of marine zooplankton. The nutritional quality of rotifer *B. plicatilis* depends on the algal food used in the culture system. Among the microalgae used to feed roti-

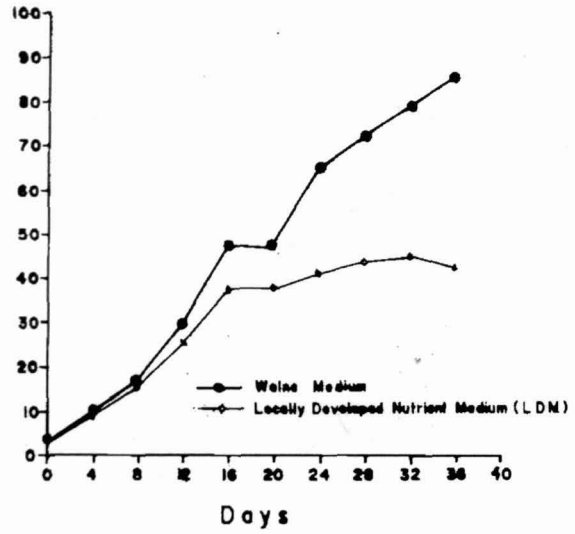

Fig. 1. *Growth of Control*

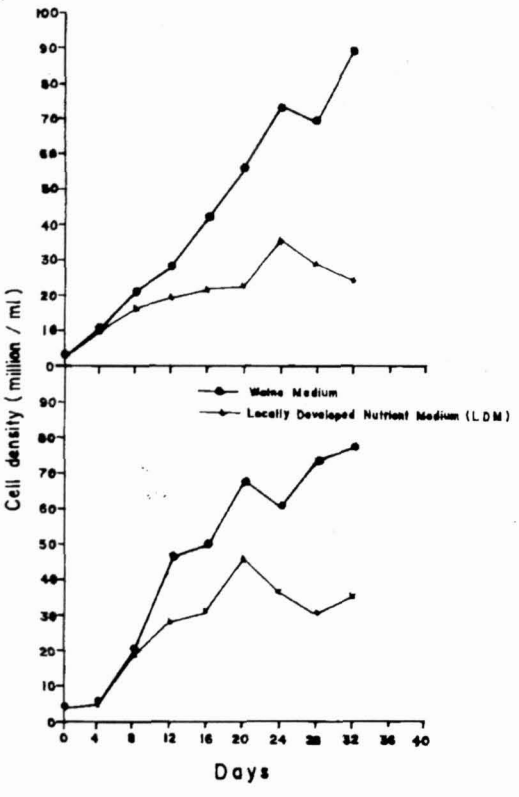

Fig. 2. *Growth of* $1^1/_2$ *and 18 month preserved (cooling) samples*

fers, the eustigmatophyte *Nannochloropsis* was found to support high rates of rotifer reproduction (Hirayama *et al.*,1979). It is well known that rotifer fed with the microalgae *N.oculata* contain sufficient quantity of unsaturated fatty acids required for the larvae of marine finfish and crustaceans. It was also found to be suitable for enrichment of baker's yeast raised rotifers with EPA (Eicosapentaenoic acid) prior to using them for feeding the larvae (James and Abu Rezeq,1989; Watanabe,1979; and Teshima *et al.*,1991).

The literature on the preservation technique of marine microalgae is limited. In the present study, a home type refrigerator with light facility was used for preservation by cooling. Stock culture of *N.oculata* without centrifuging and cryoprotectants was preserved in 250ml stock flask with cotton plugged and stored in refrigerator at 3-5^{0}C cooling. Marica *et al.* (1996) suggested that the centrifugation process makes possible the storage of more number of cells in a small volume. Osamu Umbayashi (1972) reported that the diatom strains, preserved in dark but received little light when the refrigerator door was opened several times a day for various purposes and low temperature condition at around 5^{0}C, *Skeletonema costatum, Cyclotella nana* and *Chaetoceros calcitrans* survived for 9 months, *Phaeodactylum tricornutum* for 25 months and *Nitzchia closterium* recovered their growth after 34 months of preservation. But when they were preserved in complete darkness at around 5^{0}C,

Phaeodactylum could survive only 3 months; *Skeletonema, Cyclotella* and *Chaetoceros* for 4 months and *Nitzchia* could recover their growth after 6 months of preservation. Marica *et al.* (1996) had successfully preserved the microalgae *N.oculata* and *Tetraselmis tetrathele* culture for a period of 15 and 7 months respectively.

In the present study, *N. oculata* samples preserved upto 18 months in cooling conditions have the prominent chloroplast and hence developed as regular continuous culture when reinoculated. Moreover, there was not much difference in the maximum cell concentration in the control and 18 months preserved culture and the cell density almost doubled in the Walne's medium used culture than the LDM, but the cell size was observed to be bigger in LDM. In Walne's culture medium, the cells were small and found in cluster form. When the culture reached the maximum concentration in the stationary phase, the colour of culture flasks changed primarily into pale yellow and then to brown which indicated the formation of cyst by developing the matrix around the cell wall (Gopinathan and Rodrigo, 1996). The present investigation also confirms the finding of these authors in which the colour of culture primarily changed into pale yellow and then to brown.

This cyst culture can be used as inoculum which in turn breaks for its normal growth and multiplication. Matsue (1954) reported that *Skeletonema costatum* could be kept in the dark at 10-15.5^{0}C upto 52 days without high mortality, but no cells survived beyond 60 days. Takano (1963) stated that *S. costatum* could survive for 7 weeks in darkness and after 8 weeks the growth will not be recovered.

The maximum cell concentration of 36.5 million cells/ml obtained in mass culture using LDM is quite adequate for mass production of rotifers. Results of experiments also indicated that LDM is proved to be more economical due to low cost of production. It is concluded that, the most simple and efficient method of preservation for *N. oculata* is the cooling method without centrifugation. The continuous subculture of microalgae is a constraint to aquaculturists as it involves maintenance cost, additional man power and is influenced by seasonal fluctuations in environmental conditions. Hence, it is imperative to preserve them for longer duration.

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