J. mar. biol. Ass. India, **45** (2) : 128 - 132, July - Dec., 2003

Cryopreservation of micro algal strains used in aquaculture

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Abstract

Cryopreservation of micro-algae is of importance in storage of stock-culture as well as for using as aquaculture feed at any occasions. Three commercially important strains of micro-algae, *Chaetoceros calcitrans, Tetraselmis gracilis* and *Chlorella marina* were cryopreserved with different levels of post-thaw viability. More than 25% post-thaw viability was recorded for *T.gracilis,* while *C. marina* showed >32% post-thaw viability and *C.calcitrans* up to 20% only. Glycerol and dimethyl sulfoxide (DMSO) were used as cryoprotectants at 5% and 10% (v/v) concentrations.

Introduction

The maintenance of stock-cultures of micro-algal strains by serial and regular sub-culture is extremely labour intensive and can lead to bacterial contamination, loss due to culture crashing or the possibility of genetic change which reduce the suitability of the algae resulting in its change of nutritional value. To overcome such problems, it is desirable to standardize a long-term storage system for stock-culture of micro-algae in various aquaculture operations (Day and Fenwick, 1993). Successful storage of algae in production units for utilization in hatcheries is an all time achievement in aquacultural research (Holliday *et al., 1991).* By using preserved or frozen concentrated cultures, freeze-spray, or air dried biomass and refrigerated algal concentrates; fresh algal diets are being tried for replacement. Cryopreserved algae require only minimal maintenance with only periodic addition of liquid nitrogen (Mc Lellan, 1989). In the present paper details of cryopreservation protocol for three strains of micro-algae, *Chaetoceros calcitrans, Tetraselmis gracilis* and *Chlorella marina,* commonly used in aquaculture are given. Some workers (Canavate and Lubian, 1994; Imelda *et al.,* 1999) have reported the tolerance of microalgae to different cryoprotectants.

The author is thankful to the Director, Central Institute of Brackishwater Aquaculture (CIBA), Chennai for providing necessary facilities to conduct the experiments.

Material and methods

Micro-algal strains

Chaetoceros calcitrans, a unicellular dia-

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tom species commonly used in shrimp hatchery, *Tetraselmis gracilis*, a flagellate green algae (Prasinophyceae) and *Chlorella marina*, a green algal strain commonly used in shrimp and fish hatchery were used. All cultures were maintained axenic after isolated from seawater by agar plating method.

Medium and growth conditions

The algae were cultured in F/2 medium under sterile conditions in static conical flasks (100ml) at 22 to 24°C with 12h: 12h light and dark period (Ryther and Guillard, 1962). Axenisation of the cultures were done to check bacterial contamination (Droop, 1967). Stationary phase culture was harvested by centrifuging at 2500 rpm for 10 minutes. Microscopic examination was done to see whether any cellular damage has occurred while centrifuging. From the concentrate, 0.5 ml cultures were introduced into cryovials of 1.0 ml capacity. The samples were treated with equal volume (0.5ml) of cryoprotectants, dimethyl sulfoxide (DMSO) and glycerol at 5% or 10% (v/v).

The vials in triplicates were then placed in a domestic freezer at -20°C for 30 min. The samples were then exposed to liquid nitrogen vapors for 5min and transferred to -196°C by plunging into liquid nitrogen in cryocans.

After keeping in the cryo-can for 15 d, cells were thawed by keeping the vials in a warm water bath at 40°C until ice is melted. The samples were then transferred to fresh F/2 medium (9.0ml) in 20 ml culture tubes.

Viability assays

Viability of cells during storage was estimated by determining the percentage of motile cells for *T. gracilis* and counting of cells under light microscope in a Neubaur haemocytometer for *C. calcitrans* and *C. marina* after 24h of post-thaw (Harrison, 1988; Montaini *et al.*, 1995) Viability was also studied by allowing the cells to multiply on agar plates (F/2 with medium 1.5-% agar) and in 9 ml fresh F/2 medium. The cells were counted using haemocytometer from liquid medium and from agar plates by counting the colony forming units (cfu). Control samples of same cell densities were also maintained for comparison of viability. Cell count in control samples was taken as 100% viability.

Results

Cryopreserved samples of the three strains grew well in the standard conditions provided, with final densities in the range 1.0-1.25 X 10^6 cells ml^{-1} (*T. gracilis*), 1.0-3.5 X 10^6 cells ml^{-1} (*C.calcitrans*) and 8.0-10.0 X 10^6 cells ml^{-1} (*C. marina*). The cell numbers did not show much variation during the 24 hr post-thaw incubation for all the three strains.

DMSO was effective when used at 5%(v/v). The viability ranged between 8-10% for *T. gracilis*, 18-20% for *C. marina* and 10-12% for *C. calcitrans*. DMSO when used at 10%, viability ranged from 0-2% for *T. gracilis*, 2-3% for *C. marina* and no survival for *C. calcitrans* after 24 hr of post-thaw. Glycerol concentration was found to be less critical for all the strains

Table 1. *Percentage of live cells of the three strains of micro algae on post- thawing*

Strain	Cryoprotectant (%)	Survival rate (%)	Regeneration of viable cells
T. gracilis	DMSO 5%	9(3)	+
	DMSO 10%	1 (3)	+/-
	Glycerol 5%	23 (4)	+
	Glycerol	26 (6)	+
C. marina	DMSO 5%	19 (7)	+
	DMSO 10%	2.5 (4)	+
	Glycerol 5%	30 (5)	+
	Glycerol 10%	32.5 (2)	+
C. calcitrans	DMSO 5%	11 (2)	+
	DMSO 10%	0	-
	Glycerol 5%	17.5 (4)	+
	Glycerol 10%	19 (3)	+

{Results are mean of six observations and standard deviation is given in parenthesis. Control is taken as 100%}

Table 2. *Observations on viability of microalgae on cryopreservation*

Strain	Cryoprotectant	Motility (24 h)	cfu (30 d)	Cell count in liquid medium (F/2) after 30 d (cells/ml)
T. gracilis	DMSO 5%	18.6(3)	9(2)	1.0×10^6
	DMSO 10%	10.3(2)	2(1)	1.0×10^6
	Glycerol 5%	50.1(4)	15(6)	1.2×10^6
	Glycerol 10%	56.5(3)	18(8)	1.25×10^6
C. marina	DMSO 5%	-	15(3)	8.5×10^6
	DMSO 10%	-	3(1)	8.0×10^6
	Glycerol 5%	-	18(6)	9.5×10^6
	Glycerol 10%	-	22 (5)	10×10^6
C. calcitrans	DMSO 5%	-	-	3×10^6
	DMSO 10%	-	-	3.2×10^6
	Glycerol 5%	-	-	3.5×10^6
	Glycerol 10%	-	-	3.5×10^6

*Results are mean of six observations and standard deviation is given in parenthesis

tested, and was found to be a suitable cryoprotectant at 10% (v/v). At 5%, viability percentage in glycerol for *T.gracilis* was 21-25%, *C. marina* 25-35% and *C.calcitrans* 15-20%. At 10% a slight variation was observed and it was more en-

couraging to use for cryopreservation of micro-algae. The percentages were arrived by taking control cell density as 100%. Percentage of post thaw viability for the three strains of microalgae at two concentrations of DMSO and glycerol are given in Table 1 and viability of cultures in fresh F/2 medium expressed as final cell count is given in Table 2.

Discussion

The three strains of micro algae could be cryopreserved and regenerated at two different concentrations (5% and 10%) of DMSO and glycerol. Even though the final survival rates were low, the protocol was observed to be useful in long-term preservation of economically important micro algae. Many workers have reported low survival of micro algae during cryopreservation (Ben-Amotz and Gilboa, 1980; Day and Fenwick 1993). DMSO at 5% and glycerol at 10% did not show any significant difference in increasing the viability during cryopreservation. As recommended by Day and Fenwick (1993), the post thaw viability of the algal cells can be improved by slow, controlled cooling before plunging into liquid nitrogen. The percentage viability obtained for *T. gracilis* in the present study is almost similar to that obtained for other workers (Day and Fenwick, 1993). Lower survival rate for *C. calcitrans* may be due to the formation of intracellular ice on cryopreservation. *C. marina* was found to be comparatively better for cryopreservation with significant ($P<0.05$) survival rate. The reason may be attributed to the smaller cell size and non-motile nature. A survival rate of 70% was reported for *C. marina* and *Isochrysis galbana* with 10% glycerol as cryoprotectant after 24 hr of post thaw from -20°C (Holm and Hansen, 1967; Grima *et al.*, 1994). The samples when cultured in fresh medium the cell density were similar to that of control (Table 2).

The present study gives a general protocol for cryopreservation of three economically important micro algal strains. It also gives a reliable measurement of viability by counting colony-forming units within agar plates and cell count in liquid medium. Also it is concluded that if suitable facilities are already available, cryopreservation is a suitable measure for maintaining small quantities of algal cultures in good quality with minimum labour.

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