ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 9 (3): 387-391, 2010
ISSN 1682-296X

Effect of Different Separation Techniques and Storage Temperatures on the Viability of Marine Microalgae, *Chaetoceros calcitrans*, during Storage

[1]Z.T. Harith, [1]F.M. Yusoff, [3]M. Shariff and [1,2]A.B. Ariff
[1]Laboratory of Industrial Biotechnology, Institute of Bioscience,
Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[2]Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences,
Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[3]Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

Abstract: The aim of this study was to optimize and to propose the suitable separation method and storage conditions for specific species of microalgae. The performance of different separation methods for the recovery of cell biomass of marine microalgae, *Chaetoceros calcitrans*, from the culture broth was evaluated. The microalgae were cultivated using 10 L photobioreactor. The microalgae cell cultures were concentrated either by centrifugation, tangential flow filtration or flocculation and then stored at different temperatures (-20, 4 and 27°C) to investigate the optimum storage conditions for *C. calcitrans* prior to different downstream processing methods. High concentration of cell in slurry (4.88×10^7 cells mL^{-1}) was obtained using centrifugation as compared to tangential flow filtration (4.14×10^7 cells mL^{-1}), flocculation with chitosan (1.56×10^7 cells mL^{-1}) and flocculation with Magnafloc®LT 25 (8.24×10^6 cells mL^{-1}). Storage of *C. calcitrans* biomass at chilled temperature (4°C) directly after the harvesting using these four different separation methods resulted in extended shelf life (> 4 weeks). Frozen biomass (-20°C) fails to preserve the quality of *C. calcitrans* after they were revived in fresh medium. *C. calcitrans* flocculated with 0.5 mg L^{-1} Magnafloc®LT 25 was able to maintain the quality of the cells after storage at 27°C for more than 2 weeks. However, flocculation of cells with 20 mg L^{-1} chitosan, centrifugation at 8000 rpm for 10 min and tangential flow filtration process at transmembrane pressure of 20 psi failed to retain the quality of biomass after storage for 2 weeks at 27°C.

Key words: Microalgae, *C. calcitrans*, separation, storage study, viability

INTRODUCTION

The use of microalgae as conventional diet for many aquatic organisms is deal with the need for live culture (Montaini *et al*., 1995). Alternative diet such as preserved microalgae is important to replace the demand of live microalgae which increase the production cost. Microalgae can be harvested using different separation methods such as flocculation (Knuckey *et al*., 2006; Heasman *et al*., 2001; Bilanovic *et al*., 1988), centrifugation (Heasman *et al*., 2001; McCausland *et al*., 1999) and filtration (Heasman *et al*., 2001; McCausland *et al*., 1999). The algae concentrates are susceptible to damage if not stored under appropriate conditions (Heasman *et al*., 2001). The main problem in the preservation and storage of microalgae concentrate is related to their shelf-life, which depends on the species and storage conditions (Ponis *et al*., 2008). Therefore, it is important to maintain the shelf life of the microalgae by maintaining the cells viability and consequently sustain their cell contents and chemical integrity.

Microalgal concentrates stored at low temperature is a promising alternative to the fresh algae (McCausland *et al*., 1999; Heasman *et al*., 2001). *Tetraselmis suecica* suspension maintained at 4°C in darkness showed no significant difference in cells viability after 50 days of storage while maintaining the fatty acid profile over 90 days (Montaini *et al*., 1995). However, storage at low temperature is not suitable for industrial applications due to significant increase in cost and equipment used. The use of cryoprotectant for improving the preservation of microalgae has also been studied (Heasman *et al*., 2001; Molina-Grima *et al*., 1994). However, this cryopreservation technique is too expensive to be used for microalgae cells for subsequent use as aquaculture feeds.

Corresponding Author: Arbakariya B. Ariff, Department of Bioprocess Technology,
Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia,
43400 Serdang, Selangor, Malaysia Fax: 603 89467510

Although, several separation methods have been proposed for effective harvesting of microalgae from culture broth, the suitability of each method might be specific to certain species. Some robust methods may not be suitable for delicate microalgae cells such as *Pavlova lutheri* (Ponis *et al*., 2008; Heasman *et al*., 2000) and Tahitian *Isochrysis* (Heasman *et al*., 2000). In addition, the viability and stability of the cells during storage may not only be influenced by the storage conditions but also by the harvesting method. The present study investigated the effect of storage temperature (-20, 4 and 30 °C) on the viability and stability of the microalgae cells, *C. calcitrans,* harvested using different separation methods (centrifugation, flocculation and membrane filtration). The ability of the cell concentrates to revive after 4 weeks of storage was also investigated. The information obtained may be used to optimize and propose the suitable separation method and storage conditions for specific species of microalgae.

MATERIALS AND METHODS

Microalgae and cultivation method: The microalgae, *C. calcitrans,* obtained from Aquatic Animal Health Unit, Faculty of Veterinary Medicine, Universiti Putra Malaysia in 2008 was used throughout this study. The microalgae was cultured in 10 L photobioreactor using Conway medium at 29 ppt salinity, with the addition of 0.02 g L^{-1} of silica. The temperature within the photobioreactor was regulated at 20±2°C by air-conditioning. Aeration to the culture was provided by air bubbling through the air sparger. The pH of the medium was maintained at 8±0.2 by sparging with a mixture of air and carbon dioxide (CO_2) at a ratio of 97:3. Cultures were grown under illumination by white fluorescent light (4500-5000 Lux) for 12 h during day time and 12 h in the dark during night time. Cells were harvested at late logarithmatic growth phase (after 6 days of cultivation) and subjected to different separation methods for the separation of cells from the culture broth.

Methods for cell separation: The different separation methods (flocculation, centrifugation and membrane filtrations) were used in this study. The optimized flocculation method as reported in our previous study (Harith *et al*., 2009) was used. Polyelectrolyte flocculant, Magnafloc®LT 25, was used for flocculation of *C. calcitrans*. The pH of the culture was adjusted to pH 10.2 followed by the addition of 0.5 mg L^{-1} of polyelectrolyte into 500 mL of the culture. Flocculation of *C. calcitrans* was also conducted using chitosan as a flocculant. In this method, 20 mg L^{-1} of the polymer was added to 500 mL of culture and pH was adjusted to 8. In both flocculation methods, the cultures were mixed vigorously (200 rpm) for 1 min, followed with slow mixing (50 rpm) for 2 min. Flocculation was allowed under gravity without any stirring and surface water was siphoned off at the end of flocculation (4 h). The flocs were resuspended into single cells by adjusting the pH to 7, which was achieved by the addition of hydrochloric acid (HCl).

For centrifugation method, bench top centrifuge (Appendorf, 5510R) was used. Centrifugation of the *C. calcitrans* culture was carried out in 50 mL centrifuge tube (Vivantis) with screw caps at 8000 rpm for 10 min. The temperature during centrifugation was maintained at 20°C. After the centrifugation, supernatant was discarded and the cell pellet was resuspended in 5 mL of the clear supernatant to obtain cell concentrate with a concentration of 10-fold higher than the harvested culture broth from the photobioreactor. This cell concentrate was then used for the storage study.

The laboratory scale Tangential Flow Filtration (TFF) system (Millipore, United State) was used for the separation of *C. calcitrans* by membrane filtration method. About 500 mL of *C. calcitrans* broth was filtered along the surface of a Pellicon XL device (Durapore PVDF, 0.65 μm, 50 cm^2). The filtration was operated at transmembrane pressure of 20 psi. The filtration was conducted continuously until the final retentate volume of 70 mL was achieved to obtain a volumetric reduction factor (VRF) of 7.1.

Storage study and analytical procedures: The *C. calcitrans* cells concentrates obtained from the different separation methods were placed in 1.5 mL vial without the addition of cryoprotective agents. For storage studies, the cell concentrates were stored at different temperatures (-30, 4 and 27°C) in dark conditions. During storage, samples were withdrawn at time intervals (0, 1, 2, 3 and 4 weeks) for analysis.

The ability of the stored cells to revive was evaluated by inoculating 1.0 mL of the culture into 20 mL of fresh Conway medium. The culture was allowed to grow under continuous illumination (4500-5000 Lux) at 20°C and the growth of cells was monitored by measuring the optical density of cells at A750 nm. The culture was also examined under the microscope to evaluate whether the increase in cell optical density was purely due to growth of microalgae or bacterial contaminants.

The viability of the microalgae cells during storage was measured using staining method. The culture sample (20 mL) was treated with 1 mL of 1% (w/v) stock solution of Evan's Blue and allowed to stand at room temperature for 30 min (Heasman *et al*., 2001). The stained cells were

examined under the light microscope (*Leica* DMLB, Germany). The dead cells were stained blue due to the penetration of the stain through the cell wall, whereas the viable cells remained unstained. Cell number was counted using Haemacytometer and the percentage of viable cells is calculated using Eq. 1:

$$\text{Cell viability}(\%) = \frac{\text{Viable cells}}{\text{Total cells}} \times 100 \quad (1)$$

RESULTS

The harvesting efficiency of *C. calcitrans* cells from the culture broth using different separation methods is shown in Table 1, where different separation methods produced different final cell concentrations. The highest cell concentration (4.88×10^7 cells mL^{-1}) was obtained with centrifugation, followed by the separation using TFF (4.14×10^7 cells mL^{-1}). Reduced final cell concentration was obtained with flocculation using either chitosan or Magnafloc® LT 25, where the final cell concentration obtained was 1.56×10^7 cells mL^{-1} and 8.24×10^6 cells mL^{-1}, respectively. The highest harvesting efficiency (100%) was obtained in separation using TFF. The separation using centrifugation and flocculation with both Magnafloc® LT 25 and chitosan gave the same harvesting efficiency (> 95%).

The changes in viability of *C. calcitrans* harvested from the culture broth using different separation methods during storage at different temperatures are shown in Fig. 1a-d. A slight reduction in cell viability with storage time was observed at all temperatures investigated. However, the difference in cell viability with different storage temperatures was not significantly different. The use of different separation methods also did not show significant variation in cell viability during storage.

Table 2 shows the effect of separation methods and storage temperatures on the ability of stored *C. calcitrans* to revive when inoculated into fresh medium at 30°C. For all separation methods used in this study, the cells stored at 4°C were able to be revived even after 4 weeks of storage. The cells harvested by flocculation using Magnafloc®LT 25 revived after 2 weeks of storage at 27°C. On the other hand, cells harvested using TFF, centrifugation and flocculation with chitosan failed to be

Table 1: Effect of different cell separation methods used to separate *C. calcitrans* cells from the culture broth on the harvesting efficiency and the final cell number in the concentrate. Respective cell density was used in the preservation procedure

Harvesting method	Final cell no. (cells mL^{-1})	Harvesting efficiency (%)
Flocculation with Magnafloc®LT 25	8.24×10^6	>95
Flocculation with chitosan	1.56×10^7	>95
Centrifugation	4.88×10^7	>95
Tangential flow membrane filtration	4.14×10^7	>100

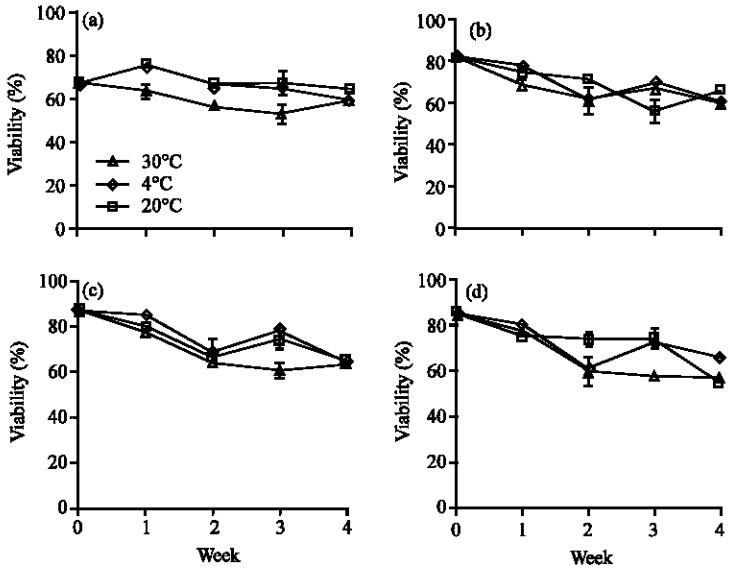

Fig. 1: Change in the viability of *C. calcitrans* cells, separated using various method, during storage at different temperatures. (a) Flocculation with 0.5 mg L^{-1} Magnafloc® LT 25 at pH 10.2 followed by resuspension at pH 7, (b) Flocculation with 20 mg L^{-1} chitosan at pH 8 followed by resuspension at pH 7, (c) Centrifugation at 8000 rpm; 10 min and (d) Tangential flow filtration at TMP = 20 psi

Table 2: Effect of different cell separation methods used to separate *C. calcitrans* cells from the culture broth on the quality of the cell concentrate during storage at different temperatures. The quality of cells was evaluated by their ability to revive when inoculated into the fresh medium and incubated at 30°C

		Weeks		
	Temperature (°C)	0 (control)	2	4
Magnafloc LT25	-20	+	-	-
	4	+	+	+
	27	+	+	-
Chitosan	-20	+	-	-
	4	+	+	+
	27	+	-	-
Centrifugation	-20	+	-	-
	4	+	+	+
	27	+	-	-
TFF	-20	+	-	-
	4	+	+	
	27	+	-	-

+: Able to revive with increase in growth, –: Not able to revive the growth after 5 days of cultivation

revived after 2 weeks storage at 24°C. Frozen cell concentrates (-20°C) show very poor quality cultures and none of the cells concentrates were able to revive after 2 weeks of storage regardless of the separation methods used.

DISCUSSION

Results from this study clearly indicate that the storage temperature greatly influenced the quality of the stored microalgae cultures to a greater extent than the separation method. Since cell concentrate harvested using flocculation with Magnafloc®LT 25 had lower cell density (8.24×10^6 cells mL^{-1}) and can easily be revived after 4 week of storage at 4°C, it can be said that the cell density of the cell concentrate may also play an important role in the storage of *C. calcitrans* cells to maintain high cell viability and quality. This result is supported by Cordero and Voltolina (1997) who reported that loss in viability of microalgae was strongly correlated with cell concentration, where reduction in survival rate was increased with increasing cell density. Therefore, high cell viability and quality could be obtained by formulating the cell concentrate with lower cell density prior to storage.

According to Heasman *et al.* (2001), optimum combinations of harvesting and storage conditions had to be specifically tailored to individual species of microalgae in order to maximize the effective shelf life of the cell concentrates. Furthermore, the success in preservation methods depends on the microalgae species and method of harvesting. The quality of the preserved cell concentrate was normally evaluated as the starter to initiate the new culture using fresh medium (Jaouen *et al.*, 1999), which is widely used as indirect method to confirm the viability and stability of the cell concentrates. A major prerequisite to extend the shelf-life of the microalgae cells is the maintenance of membrane integrity, cells contents and chemical integrity (Heasman *et al.*, 2001).

Results from the present study indicated that the storage of *C. calcitrans* cells at 4°C yielded the highest quality of cells. Montaini *et al.* (1995) and McCausland *et al.* (1999) also claimed that preservation at low temperatures was the preferred method to maintain high cell viability of microalgae species. However, reduced quality and shelf life of several microalgae such as *Skletonema costatum*, *P. lutheri*, *Chaetoceros muelleri* and *T. Iso* concentrates prepared using super centrifuge prior to storage at 4.0±0.5°C was reported by Heasman *et al.* (2000).

Storage at low temperature is the simplest way for maintaining the cells quality. Reduced temperature slows both metabolic processes and changes including oxidative denaturation of essential vitamins and highly unsaturated fatty acids (HUFA's), autolysis and microbial degradation while maintaining the cells viability (Heasman *et al.*, 2001). Preservation of concentrated microalgae cell at low temperature (chilled conditions) is a potential method of retarding microbial degradation of stored concentrates since all microorganisms have definite minimum, maximum and optimum growth temperature. However, storage at temperature below 0°C resulted to the formation of *C. calcitrans* cell concentrate in the frozen form. Frozen cultures need to be thawed before use and this process resulted in injury to the cell wall (Ben-Amotz and Gilboa, 1980). Excessive damage to the *C. calcitrans* cells during thawing of the stored cells in the frozen form (stored at -20°C) is the possible explanation for the failure of the cells to be revived even after a short storage period, as observed in this study.

CONCLUSIONS

Results from this study demonstrated that the storage temperature showed a greater influence to the quality of *C. calcitrans* cells than the method of harvesting cells from the culture broth. For all separation methods used (flocculation (Magnafloc®LT 25, chitosan), centrifugation and TFF), the preferred storage temperature to maintain the quality of *C. calcitrans* cells was at chilled condition (4°C). For cell concentrate harvested by flocculation with Magnafloc®LT 25 followed by resuspension to pH 7 using hydrochloric acid, the quality of cells could be maintained up to 2 weeks of storage at 27°C. This may be related to low cell density of the cell concentrate as compared to those produced by other separation method. Frozen cultures (-20°C) were unable to revive in fresh medium regardless of the separation methods used.

ACKNOWLEDGMENTS

The authors would like to acknowledge the Johor Satellite Biotechnology Project, Malaysia, for the funding support (grant number: BSP(J)BTK/004(4)) of this study.

REFERENCES

Ben-Amotz, A. and A. Gilboa, 1980. Cryopreservation of marine unicellular algae. 1. A survey of algae with regard to size, culture age, photosynthetic activity and chlorophyll-to-cell ratio. Marine Ecol. Prog. Ser., 2: 221-224.

Bilanovic, D., G. Shelef and A. Sukenik, 1988. Flocculation of microalgae with cationic polymers-effect of medium salinity. Biomass, 17: 65-76.

Cordero, B. and D. Voltolina, 1997. Viability of mass algal cultures preserved by freezing and freeze-drying. Aquac. Eng., 16: 205-211.

Harith, Z.T., F.M. Yusoff, M.S. Mohamed, M.S.M. Din and A.B. Ariff, 2009. Effect of different flocculants on the flocculation performance of microalgae, *Chaetoceros calcitrans* cell. Afr. J. Biotechnol., 8: 5971-5978.

Heasman, M.P., J. Diemar, W.A. O'Connor, T.M. Sushames and L.A. Foulkes, 2000. Development of extended shelf-life microalgae concentrates diets harvested by centrifugation for bivalves mollucsc-a summary. Aquac. Res., 31: 637-659.

Heasman, M.P., T.M. Sushames, J.A. Diemar, W.A. O'Connor and L.A. Foulkes, 2001. Production of microalgal concentrates for aquaculture part 2: Development and evaluation of harvesting, preservation, storage and feeding technology. NSW Fisher. Final Rep. Ser. Aust.,

Jaouen, P., L. Vandanjon and F. Quemeneur, 1999. The shear stress of microalgal cell suspensions (*Tetraselmis suecica*) in tangential flow filtration systems: The role of pumps. Bioresour. Technol., 68: 149-154.

Knuckey, R.M., M.R. Brown, R. Robert and D.M.F. Frampton, 2006. Production of microalgal concentrates by flocculation and their assessment as aquaculture feeds. Aquac. Eng., 35: 300-313.

McCausland, M.A., M.R. Brown, S.M. Barrett, J.A. Diemar and M.P. Heasman, 1999. Evaluation of live microalgae and microbial pastes as supplementary food for juvenile Pacific oyster (*Crassostrea gigas*). Aquaculture, 174: 323-342.

Molina-Grima, E., J.A. Sanchez-Perez, F. Garcia-Camacho, F.G. Acien-Fernandez, D. Lopez-Alonso and C.I.S. del Castillo, 1994. Preservation of the marine microalga, *Isochrysis galbana*: influence on the fatty acid profile. Aquaculture, 123: 377-385.

Montaini, E., G. Chini-Zittelli, M.R. Tredici, E. Molina-Grima, J.M. Fernandez-Sevilla and J.A. Sanchez-Perez, 1995. Long-term preservation of *Tetraselmis suecica*: influence of storage on viability and fatty acid profile. Aquaculture, 134: 81-90.

Ponis, E., G. Parisi, G. Chini-Zittelli, F. Lavista, R. Robert and M.R. Tredici, 2008. *Pavlova lutheri*: Production, preservation and use as food for *Crassostrea gigas* larvae. Aquaculture, 282: 97-103.