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LONG-TERM STORAGE OF A NUMBER OF MICROORGANISM SPECIES USING DIFFERENT CRYOPROTECTANTS

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ABSTRACT

This study aims to generate a microalgae culture collection, and ensure the long-term survival of this collection by prolonging the period when the isolated microalgae and other microorganism cultures can be preserved. The microalgae cultures included four microalgae species (*Chlorella vulgaris Beyerinck* [Beijerinck], *Klebsormidium subtile* (Kützing) Mikhailyuk, Glaser, Holzinger & Karsten, *Microcoleus autumnalis* (Gomont) Strunecky, Komárek & J.R. Johansen and *Synechococcus bigranulatus* Skuja), two bacteria species (*Staphylococcus aureus* and *Escherichia coli*) and two fungi species (*Aspergillus niger* and *Penicillium* sp.). The study applied a cryopreservation protocol at -80ºC directly to each of the species. The cryoprotective agents used in the study were dimethyl sulfoxide (DMSO), glycerol and skimmed milk at a final concentration of 5% for the microalgae in addition to an application without any cryoprotectant at all. The cryoprotectant agents used for this procedure were 15% final glycerol concentration for the bacteria and 10% skimmed milk for the fungi. After six months, the strains of *C. vulgaris* (93%), *S. bigranulatus* (83%) and *M. autumnalis* (80%) showed higher viability rates in glycerol, while the strain of *K. subtile* (93%) showed higher viability rates in skimmed milk. The recovery ratio of *E. coli, S. aureus, A. niger* and *Penicillium* sp. were found to be 5.10, 3.23, 1.98 and 2.67 log CFU/ml, respectively.

KEYWORDS:
Cryopreservation, cryoprotectant, microalgae, microorganisms, preservation.

INTRODUCTION

In recent years, studies have investigated long-term preservation methods, and cryopreservation has been accepted as the most appropriate way of conserving biological material. Cryopreservation can be described as the storage of a living organism or a portion of it at an ultra-low temperature to make it survive after thawing [1, 2]. Two important considerations for cryopreservation are the addition of a cryoprotective agent (CPA) prior to freezing, and the rates at which cells are cooled and warmed [3, 4, 5]. In most cases, succeeding in cryopreservation is contingent upon the application of the appropriate rate of cooling, cryoprotective additives, osmotic equilibrium and the ice nucleation process. Although cryopreservation is common in microorganisms (especially in bacteria and fungi) there is little information about microalgae [6]. In spite of these limitations, it was possible to successfully cryopreserve hundreds of species of Cyanobacteria and eukaryotic microalgae [1, 2, 7]. The cryopreservation of bacteria and fungi cultures is a common method in the protection of strains in laboratory. Nowadays, glycerol is the most common CPA for cryopreservation of bacteria. As a classical method of cryopreserving a culture, suspending bacteria cultures in 15% glycerol is still in use [8]. An alternative method is to use skimmed milk as a preservative to keep bacteria cultures at -80°C in laboratories [9].

In our previous ecological studies, 15 microalgae species were isolated from fresh water environments, while 2 bacteria and 2 fungi species were isolated in microbiological studies. As part of the efforts to generate a microalgae culture collection, the study aimed to ensure the long-term survival of a microalgae culture collection by prolonging the survival period when the isolated microalgae and other microorganism cultures can be preserved. Furthermore, we aimed to investigate the effects of different protectants on different groups of microorganisms in order to have a better understanding of the steps to be followed during the cryopreservation process and improve the application of cryopreservation techniques.

MATERIALS AND METHODS

Isolation and culture conditions. Microalgae species were collected from eutrophic freshwater environments in Ankara, Turkey, and isolation and purification was achieved through a dilution and plating technique [10]. The four microalgae strains used

in the procedure were: *C. vulgaris*, *K. subtile, M. autumnalis* and *S. bigranulatus.* Table 1 presents the microorganism used in the study, their characteristics and collection time.

The study cultured microalgae cultures in BG 11 Medium, and created the appropriate environmental conditions. The cultures containing 30 ml of medium were incubated at 25°C under fluorescent lamps at a photon flux density of 50 µmol photons m^{-2} s^{-1} with a photocycle of 16 hours of light followed by 8 hours of dark. Stock cultures of these algae were harvested at logarithmic growth phase [11, 12].

The *S. aureus* and *E. coli* preserved in the microbiology laboratory were cultivated in Nutrient Agar (NA, Difco) plates, incubated at 37°C for 24 to 48 hours and the CFU/ml of the bacteria were calculated by making serial dilutions from reproduced colonies. Fungal isolates were inoculated into Sabouraunt Dextrose Agar (SDA) and incubated at 25°C for 7 to 10 days. The colonies of *A. niger* and *Penicillium* sp. were covered with 5 ml of distilled sterilised water containing 1% Tween-20. Afterwards, the conidia were rubbed with a cotton swab and transferred into a sterilised tube to produce a homogeneous suspension. That suspension was spun in a vortex mixer at 2000 rpm for 15 seconds and dilutions were made in order to obtain exact concentrations. A cell count was taken with a haemocytometer. In order to remove hyphas, the spores were obtained by filtering through a millipore filter. The microscopic cell count with the haemocytometer was adjusted over a range from 1.0×10^6 to 5.0×10^6 spores/ml [13, 14].

Freezing. To evaluate the effect of the cryoprotectant, microalgae samples were prepared under two conditions, which were with or without cryoprotectant (CPA). The CPAs used for the microalgae were dimethyl sulfoxide (DMSO), glycerol and skimmed milk. Fifteen millilitres of each culture were taken, 5 ml of which was spared for viability control. The remaining culture was adjusted to a final concentration of 5% (v/v). Aliquots (1.5 mL) of the resulting suspensions were dispensed into 2 ml cryogenic vials. The vials were set in a container and directly cooled to -80°C in a deep freeze and stored until the recovery test was completed. Untreated cell suspension was immediately inoculated into a new medium to confirm the viability of pre-cultures [6, 12].

Cody et al. [8] utilized 10% skimmed milk and 15% glycerol as a CPA, which were reported to be effective regarding the viability of the bacteria and fungi after cryopreservation. For cryopreservation, the samples were thawed in a serological bath at 25°C for 3 minutes. Serial dilutions were prepared, 20 µl from each dilution was dispersed on Nutrient Agar and the plates were incubated at 37°C for 72 hours to ensure the viability of the bacteria. There were 30 to 300 colonies of the bacteria on the plates

TABLE 1
The microorganisms used in the study and their characteristics

Phylum	Species	Strains	Morphology	Ecosystem
Chlorophyta	*Chlorella vulgaris Beyerinck* (Beijerinck)	CCA02Ch01	Unicellular	Freshwater reservoir Sampling year: 2007
Charophyta	*Klebsormidium subtile* (Kützing) Mikhailyuk, Glaser, Holzinger & Karsten *Synonym: Stichococcus subtilis* (Kützing) Klercker	CCA02Stc01	Filamentous	Freshwater pond Sampling year: 2008
Cyanobacteria	*Microcoleus autumnalis* (Gomont) Strunecky, Komárek & J.R. Johansen *Synonym: Phormidium autumnale Gomont*	CCA01Ph02	Filamentous	Freshwater pond Sampling year: 2008
Cyanobacteria	*Synechococcus bigranulatus* Skuja	CCA01Syn01	Unicellular	Freshwater reservoir Sampling year: 2015
Firmicutes	*Staphylococcus aureus*	M-3	Gram positive cocci/ yellow color colony, Smooth	Milk Sampling year: 2015
Proteobacteria	*Escherichia coli*	MS-6	Gram negative rod / grey-white color colony, Smooth	Manure soil Sampling year: 2015
Ascomycota	*Aspergillus niger*	AN-9	Conidiophores / black color colony	Manure soil Sampling year: 2015
Ascomycota	*Penicillium* sp.	PC-12	Conidiophores/ green color colony	Old bread Sampling year: 2015

and the data were determined to be log CFU ml^{-1}. For viability of the fungi, 0.5 ml of the inoculum from the active fungal culture was put into cryovials (1.5 ml), and then 1 ml of CPA (15% glycerol and 10% skimmed milk) was added into separate tubes. The tubes were put aside for preservation at -80°C.

Thawing. To perform the thawing procedure, the researchers set the water bath at 35°C, and placed a vial into the water bath as quickly as possible [4]. The cultures were centrifuged in order to settle the supernatant. The supernatant was discarded and the pellet was removed. The microalgae cultures were placed in 30 ml of fresh medium so that the concentration of cryoprotectant in the medium was reduced to below 0.2% (v/v). The cultures were kept in dark for 1 hour in order to increase their survival rates, 2 hours without direct light and placed under optimal growth conditions. Then, the cultures were observed in terms of viable cells appearing within 1 to 2 days [4, 15].

All bacterial and fungal strains were taken from the freezer (at -80°C), and kept under 30°C in the microbiology laboratory. Then, each bacterial strain was cultivated in Nutrient Agar while fungal isolates were cultivated in Sabouraunt Dextrose Agar.

Viability assays. Five milliliters of suspended microalgae cultures was inoculated into 40 ml of BG 11 medium under normal culture conditions after the thawing process. The viability capacities of microalgae cells were calculated based upon the determination of chlorophyll-a and the number of cells after thawing. The distinction between viable and dead cells was made with an optical microscope and the use of Trypan Blue solution. Trypan Blue solution was prepared at 0.4% concentration, 50 µl was put into a vial and kept in that solution for 15 minutes. Afterwards, 350 µl of medium and 100 µl of culture were added. While the viable cells did not absorb Trypan Blue, the dead cells were stained by absorbing it and generated a distinctive blue colour under the optical microscope. The cell count for cryopreserved cultures was made under a microscope using a Thoma slide at 1-, 7- and 14-day intervals. The percentage of cell viability is expressed as the ratio of the total number of viable cells (unstained) to the total number of cells (stained and unstained) [6, 11, 12, 15]. Viability levels were therefore calculated using the following equation (1):

Viability Level (%)=(Number of cells unstained x 100)/(Total cells (stained and unstained)) (1)

The retrieval of viable cells was accepted as positive by observing colonies on plates after the bacteria cultivated in NA were incubated at 37°C for 24 to 48 hours. Similarly, the retrieval of viable cells was accepted as positive by observing colonies on plates after the fungal isolates cultivated in SDA were incubated at 25°C for 7 to 10 days [8]. The survival ratio of bacteria and fungi (BSR) was calculated by dividing the log of the number of bacteria cells available in the suspension after preservation (AP) to the log of the number of viable cells before preservation (BP) and then multiplying by 100. This is expressed with the equation (2) given below [16]:

$$BSR = (\log_{AP} / \log_{BP}) \times 100 \quad (2)$$

Chlorophyll-a determination. The biomass of microalgae samples were estimated from their chlorophyll-a content measured through the use of the methanol method [17]. All the experiments were carried out in triplicate and the viability of the microalgae, bacteria and fungi were controlled by cryopreservation for 3 and 6 months.

RESULTS AND DISCUSSIONS

Subculturing is the most common method for preserving microorganisms, especially for the microalgae cultures produced in the laboratory. However, this method demands a lot of effort, the cultures are at risk of contamination, and do not guarantee genetic and phenotypic stability. For this reason, the cryopreservation method was utilized for long-term preservation of isolated microalgae, bacteria and fungi. Based on this methodology, both flamentous and one-celled microalgae were examined for both vital staining and the consistency of the original characteristics, reproduction capacity, cell growth and morphology. The optical microscope was used to confirm the presence of specific morphological features of all the microorganisms before and after cryopreservation.

While storing microalgae at -80°C using different CPAs, no significant decrease in the viability ratio of the microalgae was observed during the 3- and 6-month periods of preservation. In spite of the fact that the microalgae have many features to be paid attention to, the content of chlorophyll-a has been the focus in determining the viability of the microalgae in this study. Also, no significant decrease was observed in the chlorophyll-a content of any microalgae strain when thawed after 3 or 6 months (Table 2).

The microalgae strains that were tested in the study were suitable for cryopreservation. *C.vulgaris* (CCA02Ch01), *S. bigranulatus* (CCA01Syn01) and *M. autumnalis* (CCA01Ph02) had higher viability rates in glycerol, while the *K. subtile* (CCA02Stc01) strain had higher viability rates in skimmed milk. Also, the viability rate of strains to which CPA were not applied was high. Dimethyl sulfoxide (DMSO) was observed to be effective for the viability of microalgae strains when it was compared to other CPAs. However, other CPAs resulted in higher rates where the rate varied by species. The *S. bigranulatus*

(CCA01Syn01) (60 to 85%) and *M. autumnalis* (CCA01Ph02) (63 to 82%) belonging to Cyanobacteria Phylum showed these viability rates in four of the CPAs. It was found that the highest viability percentage for the *S. bigranulatus* strain was 85 to 83%, and cell density (cells mL^{-1}) was 2.91×10^6 to 3.23×10^6, while the viability percentage for the *M. autumnalis* strain was 80 to 82%, and cell density was 2.85×10^6 to 3.19×10^6 in glycerol. The *C. vulgaris* (CCA02Ch01) strain belonging to Chlorophyta showed a 75 to 95% viability rate for all CPA applications, and the highest viability rate was obtained in glycerol (93 to 95%), and cell density was 5.34×10^6 to 6.27×10^6. The *K. subtile* strain (CCA02Stc01) belonging to Charophyta showed a 48 to 96% viability rate in general, and the highest rate was obtained from skimmed milk (93 to 96%) where cell density was between $4.61x10^6$ and $6.40x10^6$. The effect of cryopreservation on the survival of the microalgae is presented in Table 3 and Figures 1 to 2.

The preservation of *E. coli* (MS-6), *S. aureus* (M-3), *A. niger* (AN-9) and *Pencillium* sp. (PC-12) was evaluated using two CPAs and freezing at -80ºC. The results indicated that the viability rate of the bacteria and fungus reduced over time and the reduction ratio was dependent upon the strain and the protective agent that were used in the procedure. These results are presented in Table 4. The bacterial survival ratio (BSR) decreased at a rate between 25 to 30% in *S. aureus* and *E. coli* after six months. The skimmed milk used as a CPA for microorganisms gave better results than glycerol. Regarding fungal activity, a decrease was observed in the survival of *A. niger* (Table 4).

TABLE 2
Chlorophyll-a content of microalgae strains

Strains	Before Preservation (µg ml^{-1})	Thawing After 3 Months (µg ml^{-1})				Thawing After 6 Months (µg ml^{-1})			
		DMSO	Skimmed milk	Glycerol	Control	DMSO	Skimmed milk	Glycerol	Control
CCA 02Ch01	894.61	762.43	1573.38	2241.5	893.78	705.25	1569.5	2197.8	892.2
CCA 02Stc01	3232.92	103.54	3021.26	129.13	76.42	90.44	3005.25	124.09	74.01
CCA 01Ph02	5153.04	497.45	602.83	982.93	873.37	493.04	549.93	968.84	859.77
CCA 01Syn01	350.9	121.07	141.15	1275.61	487.08	117.08	130.4	1219.28	483.71

TABLE 3
Effect of cryopreservation on the survival of microalgae

Strains	Months	with DMSO	with Skimmed milk	with Glycerol	With no cryoprotectants
		Viability (%)	Viability (%)	Viability (%)	Viability (%)
CCA02Ch01	3	76	90	95	81
	6	75	87	93	78
CCA02Stc01	3	59	96	62	51
	6	57	93	60	48
CCA01Ph02	3	65	72	82	76
	6	63	67	80	74
CCA01Syn01	3	61	63	85	70
	6	60	62	83	68

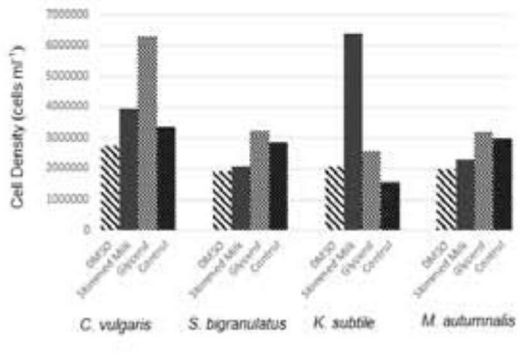

FIGURE 1
Cell density of microalgae after 3 months

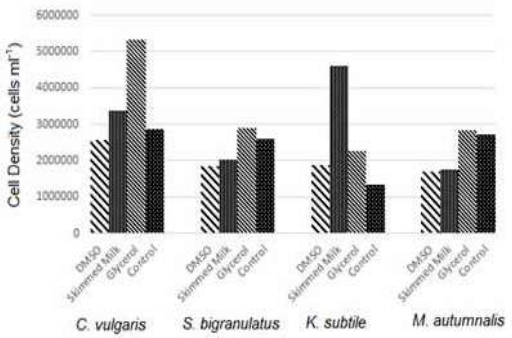

FIGURE 2
Cell density of microalgae after 6 months

TABLE 4
Effect of cryopreservation on survival of the bacterial and fungal strains

Strains	Treatment	0 Month Log CFU ml^{-1}	3 Months Log CFU ml^{-1}	BSR*	6 Months Log CFU ml^{-1}	BSR*
S. aureus	Glycerol 15%	8.76	6.80	77.62 %	4.60	52.51%
	Skimmed milk 10%	8.76	7.50	85.61 %	5.10	58.21%
E. coli	Glycerol 15%	9.60	5.58	58.12 %	3.97	41.35%
	Skimmed milk 10%	9.60	6.16	64.16 %	3.23	33.64%
A. niger	Glycerol 15%	3.50	2.30	65.71 %	1.87	53.42%
	Skimmed milk 10%	3.50	2.42	69.14 %	1.98	56.57%
Penicillum sp.	Glycerol 15%	5.32	3.13	58.83 %	2.14	40.22%
	Skimmed milk 10%	5.32	3.54	66.54 %	2.67	50.18%

*BSR: The bacterial survival ratio

There is very limited information on cryopreservation of microalgae in spite of extensive information on long-term cryogenic storing of eukaryotic cells and biological materials in clinics and laboratories. Therefore, any studies conducted on different CPAs and cryopreservation applications with microalgae are of great importance. The present study was conducted with the aim of evaluating cryopreservation, thawing and viability of the microalgae that were isolated from different freshwater environments as well as the bacteria and fungi that were isolated during microbiological studies. Glycerol and skimmed milk were used as a CPA for the bacteria and fungi, while glycerol, DMSO, skimmed milk and applications without any CPA were used with microalgae. To evaluate the survival of the microalgae, cell membrane consistency was investigated with Trypan Blue and effective growth was observed through cell counts with a microscope and determination of chlorophyll-a. Observation of bacterial and fungal colonies on plates in agar medium was interpreted as positive in terms of retrieval.

In studies conducted by Guermazi et al. [18] and Morris [19], it was observed that DMSO was enough for preservation of *Chlorella* through cryopreservation, but there were differences in cryopreservation tolerance between *Chlorella* strains. On the other hand, Nakanishi et al. [12] indicated that the freezing tolerances of the two *Chlorella* strains tested were low. There were no viable cells when DMSO was used alone and cryoprotectants were more effective when they were used in combination. In the study carried out using DMSO in different concentrations, optimal results were obtained with a 30% DMSO concentration [20]. Beaty [21] reported that *C.vulgaris* showed high viability rates in 5% of DMSO and 5% of glycerol. In this study, the *C.vulgaris* strain showed quite high viability rates (in the range of 75 to 95%) in applications with 5% glycerol, skimmed milk, DMSO and without any cryoprotectant. When DMSO was compared to other CPAs, it resulted in lower viability percentages, and the most successful retrieval was obtained from glycerol, skimmed milk and the application without cryoprotectant. Although the results obtained for *C. vulgaris* were consistent with some of the past studies, a different result was also observed stating that no result was obtained when DMSO was used alone. This result shows that CPA could have different effects on the viability capacities of different strains of species.

The studies on preservation of microalgae through cryopreservation have been mainly conducted on Cyanobacteria [22, 23, 24]. It was stated that the Cyanobacteria strains tested by Rastoll et al. [6] were suitable for cryopreservation and that DMSO was a better cryoprotectant than methanol (MeOH). In the study conducted by Esteves-Ferreira et al. [4] on five different Cyanobacteria species, it was stated that glycerol was more effective than DMSO. According to those results, the survival percentages of species in DMSO were found to be > 90% for *Phormidium* sp. and *Synechococcus*. In another study on Cyanobacteria and green algae, DMSO provided better overall retrieval ratios than the applications with glycerol, MeOH and ethylene/propylene glycol and also without any cryoprotectants [25]. In this study, the best retrieval (at 5% (v/v) for *S. bigranulatus* and *M. autumnalis*) was 80% in glycerol. When compared to previous studies, the retrieval was approximately 60% in DMSO, and above 70% in skimmed milk and the application without CPA.

Simione and Brown [22] reported that skimmed milk, bovine serum albumin (BSA) and glycerol were suitable for bacteria as a CPA, but not effective for Cyanobacteria. For red alga, *Porphyra,* 5% skimmed milk was used as a CPA by Jo et al. [26] and high viability rates were found. In this study, favourable results were obtained for *C. vulgaris* (87 to 90%), *K. subtile* (93 to 96%), *M. autumnalis* (67 to 72%) and *S. bigranulatus* (83 to 85%) with a use of skimmed milk when thawed after 3 or 6 months. In this regard, it can be noted that successful results were obtained for four microalgae species as a result of the thawing of a 5% concentration of skimmed milk after cryopreservation at -80ºC.

The results show that the survival and activity of the bacteria and fungi were dependent upon the protective agent and the type of isolate. It is very important to choose a suitable protective agent to improve the preservation conditions for bacterial and fungal cells. An increase was observed in the viability of strains stored with 10% skimmed milk and

15% glycerol. In this study, the use of 10% skimmed milk solution (v/v) was found to increase viability of the bacteria and fungi after dissolution and long-term incubation at 30°C. It was seen that the data were consistent with the findings of the study conducted by Mirchevska and Bosshard [27], which proposed that the protection ratio was higher, and the survival rate of the stocks could be increased with 10% skimmed milk.

CONCLUSIONS

During worldwide laboratory studies, microorganisms are isolated yet researchers lose those microorganism cultures since they do not have suitable protection methods, and there is a lack of manpower and controlled cooling equipment. Cryopreservation is a method that has been studied for a long time in order to preserve microorganisms. Recently, rapid cooling and two-step cooling methods have been used in studies conducted on cryopreservation. In this study, microalgae, bacteria and fungi were subject to direct storage at -80°C through the use of different CPAs. The procedures were successful in the retrieval of the microalgae cultures with CPA and without CPA. Furthermore, the present study found that the bacteria showed higher survival rates in a 10% skimmed milk solution rather than in a 15% glycerol in the freezer. The skimmed milk may affect the fatty acid content of the cell membrane and therefore change the viscosity of the membrane or contribute to the stability of cellular enzymes. The researchers suggest that bacteria should be preserved in 10% skimmed milk in order to provide long-term survival of the cells. The design to achieve the targeted culture collection is to include the isolated culture in the future study, continue those experiments, and apply different cryopreservation techniques.

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