

# Isolation, Identification and Germplasm Preservation of Different Native *Spirulina* Species from Western Mexico

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## ABSTRACT

*Spirulina* is an edible algae and has a wide range of pharmaceutical applications in addition to its nutritional value. Isolation and identification of several *Spirulina* species were conducted in the western part of Mexico especially in the state of Jalisco. The purification strategy consisted of five optimized processing steps: 1) washing and centrifugation, 2) chemical treatment, 3) micromanipulation, 4) serial dilution, and 5) plating. Four species were isolated from different locations and two out of these four species were identified taxonomically up to the species level: *Spirulina subsalsa* and *S. major*. For short term conservation (30 days), the strains were maintained in liquid and solid agar medium at 10°C and 4°C. For medium term (few months), they were preserved in solid medium under a dried condition as agar flakes and for long term, cryopreservation was employed by using 5% and 10% DMSO, glycerol and methanol as osmoprotectants in liquid nitrogen. For short term preservation nearly 90% liquid and 100% agar recovered strains were viable after one month at both temperatures. In the case of the agar flakes, cells were viable after three months of conservation at room temperature. Cryopreservation did not give any suitable results after three months of conservation. Variable and two-step improved cryopreservation processes are now in progress for conservation.

**Keywords:** Germplasm; Cryopreservation; *Spirulina*; Long Term Preservation

## 1. Introduction

*Spirulina* is a microalgae that has a unique set of biological characteristics which are very useful for a broad range of applications. It has been certified by the FDA (USA Food and Drug Administration) as GRAS (Generally Recognized as Safe). It can be used as a nutritive supplement or pharmaceutical additive with no risks to health. *Spirulina* is useful for human nutrition, because of the high quality and quantity (60% - 70% of its dry weight) [1] of protein and amino acids [2,3]. *Spirulina* contains essential amino acids, especially leucine (10.9% of total amino acids), valine (7.5%), and isoleucine (6.8%) [4]. *Spirulina* has a relatively high provitamin A concentration [5] and harmless b-carotene [6]. *Spirulina* is a very rich source of vitamin B12, which is important for people who need supplements to treat pernicious anemia [3,5,7].

History of use of the microalgae *Spirulina* is not accurately known. Ancient Mexicans were the first people in the world to use *Spirulina*. In 1521, *S. maxima* used to be harvested from Lake Texcoco, dried, and sold for human consumption in Tenochtitlan (today's Mexico City) [1]. In 1940, the French phycologist P. Dangeard reported about the consumption of a cake called *dihé*, by the Kanembu tribe near the African Lake Chad [1,8].

*Spirulina* plays an important role in the booming new markets for natural ingredients. A 2002 report from the National Centre for Natural Products Research (NCNPR) estimated that the US market is \$4 to \$5 billion in botanical dietary supplements, since these supplements (herbal remedies) are consumed by millions of people ([http://www.tradekey.com/selloffer\\_view/id/224168.htm](http://www.tradekey.com/selloffer_view/id/224168.htm)). While there are many companies around the world that produce *Spirulina* products in high volumes, prior to 2001, there were only a few companies in the USA that

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produced more than 1450 tons per year [9,10]. On the other hand, advances in conservation technology have led to methods that allow preservation of biological materials like microorganisms, tissues, primary cells, established cell lines, small multicellular organisms, complex cellular structures such as embryos, as well as nucleic acids and proteins. This has a great importance for maintenance of cell or organ culture and is a very laborious and time consuming process [11]. Again, fixation of a biological material at a particular stage of life is essential to study expression of genes, enzymes, proteins, etc. In this manner, *in vitro* preservation facilitates fixing biological materials and avoiding chances of contamination that occurs normally due to frequent handling of biological material in subcultures. During the freezing process, complex phenomena occur which are not fully understood. Microbial cells, particularly bacteria and yeast can be easily preserved with cryoprotectants at  $-80^{\circ}\text{C}$  and can be recovered in agar plates. There are some reports about cryopreservation of Cyanobacteria, but there is still not much success. Freeze-drying bio-storage methods for microalgae have not been found to be a successful procedure in long term preservation and the viability has been less than 1% [12-14]. There are few established techniques reported for cyanobacterial preservation [15-17]. Most of them are based on batch cultures, agar slants, liquid nitrogen and storage at low temperatures ( $-80^{\circ}\text{C}$ ) using protectants such as glycerol, sucrose, etc., to maintain pure cultures for varying periods of time. All these processes require laborious regular transfer to fresh liquid media that enhances high risks of contamination.

There is no doubt that *Spirulina* is a Wonder Edible Algae. Mexico was the first country in the world to use *Spirulina* [1]. Nowadays, other countries have become more technologically advanced than Mexico in the production, use, and commercialization of *Spirulina*. Research data indicates that Mexico has many species of *Spirulina* [18-20], and only recently we have isolated and identified the species *S. subsalsa* for the first time (Rout NP unpublished data). However, at present there are no reports for the isolation of other species nor is there a center for the culture and collection of local *Spirulina*. In addition to this, all of the species have different physiological responses, so there is a need to standardize the process of *in-vitro* conservation for each species. The objective of this work was the collection of microalgae to get useful local *Spirulina* strains, identification, optimization of the short, medium and long term preservation methods to establish a culture collection (Algal Bank) of the algae genus *Spirulina* from the western part of Mexico.

## 2. Materials and Methods

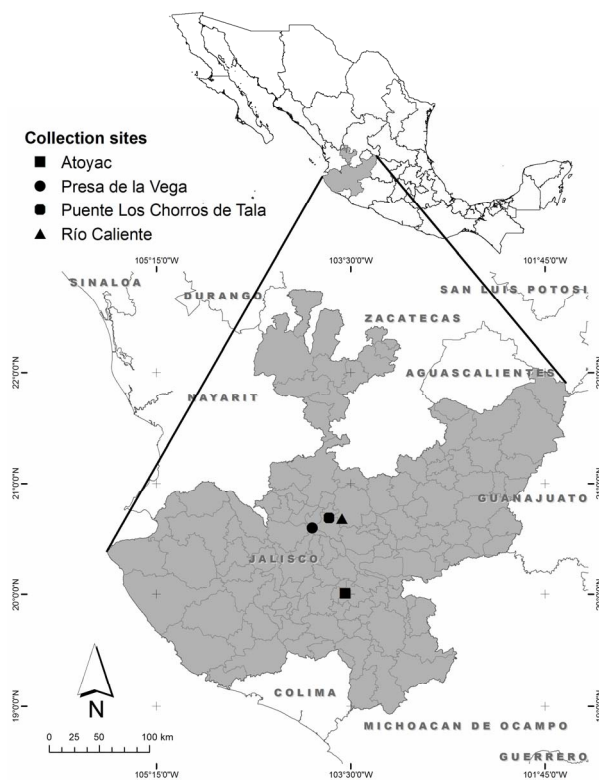
### 2.1. Collection of Algal Samples

The sites for water sampling and isolation of algae were

selected according to the published database in the book *Algae of Western Mexico* [18]. The regions selected for this study were several locations of Sayula, Zacoalco de Torres, Río Caliente (Hot water spring) in the Primavera forest, the Zapotlán Lake and a water treatment plant in the municipality of Atoyac (**Figure 1** and **Table 1**), all places located in the State of Jalisco (**Figure 1**). The water samples were taken from a surface depth of 10 and to 50 cm. using plankton net with mesh sizes between 10 - 100 microns during the months of September and November at various points. Then concentrated samples were washed with distilled water and kept in containers of fresh water. During this process, sampling data was taken such as pH and temperature at each locality.

### 2.2. Isolation of Alga

The isolation and purification strategy consisted of five optimized processing steps **1) Density centrifugation:** First, the sample was centrifuged at 800 - 2000 rpm for different phase separation to eliminate solid residues and contamination, then it was cultured in different nutrient media, **2) Chemical treatment:** For the purpose of elimination of eukaryotic algae in the mixed algal culture cycloheximide was added at different concentrations (25 - 100 mg/l) for a week and resulted cultures were recovered, **3) Serial dilutions:** The sample was centrifuged at 1000 rpm and washed repeatedly 2 - 5 times to eliminate



**Figure 1.** Collection sites of algae in the state of Jalisco, Mexico.

**Table 1. Localities, geographical references, pH and temperature of the collection sites.**

Locations	Geo-References		Parameters	
	Latitude	Longitude	pH	Temp °C
<b>Rio Caliente (Hot Water Stream)</b>				
Rio Caliente 1	20°40'50.0"	103°34'42.3"	8.4	35.1
Rio Caliente 2	20°40'50.0"	103°34'42.3"	8.0	40.0
Rio Caliente 3	20°40'50.0"	103°34'42.3"	7.2	54.2
<b>Rio Caliente 2 (Hot Water Stream-2)</b>				
Points of Chorros	20°41'12.5"	103°41'36.5"	8.6	26.5
Poll Area	20°41'42.4"	103°40'47.3"	8.7	27.9
Water Fall	20°41'45.8"	103°40'42.9"	8.7	28.2
Bath Tobs	20°41'47.2"	103°40'41.5"	8.4	25.2
<b>Presa de Vega (Dam of Vega)</b>				
On Boat	20°35'46.4"	103°50'39.2"	8.4	25.7
<b>Laguna Zapotlán (Lake of Zapotlán)</b>				
On Boat	19°45'49.1"	103°28'11.6"	8.8	23.5
North East Corner	19°46'17.4"	103°28'22.0"	8.8	24.3
Mouth of Rio Grande	19°46'05.3"	103°28'43.3"	8.9	24.1
Central Region	19°45'33.0"	103°29'00.3"	9.0	23.4
Chamizal Region	19°45'05.0"	103°28'11.6"	8.9	23.7
<b>Atoyac</b>				
Central Region	20°00'29.4"	103°32'56.9"	9.1	28.4

the small unicellular algae if any and series of dilution were made 10 times to 10 tubes. Then the sample was examined under an Olympus BH-2 microscope coupled to a Leica DFC450 C digital camera to check for the presence of *Spirulina*, **4) Micromanipulation:** Algal filaments were picked by the use of a glass micropipette into one drop of medium on a microscope slide and was examined under microscope from time to time to select the pure culture and discard contaminants and **5) Streak plating:** The serial-diluted and micro-manipulated *Spirulina* samples were spread with a needle on solid plates (with 1.5% agar) and cultured under normal laboratory conditions for 3 - 4 weeks in order to isolate pure colonies.

### 2.3. Morphological Identification of *Spirulina*

Pure colonies were observed under a microscope and photographed with a coupled Leica DFC camera 450c at 20X to 100x for taxonomic identification. The *Spirulina* species were identified taxonomically according to Ciferri (1983) [1] and Tomasseli (1997) [21]. The typical mor-

phology of *Spirulina*, the degree of spiralization and the arrangement of the spirals were the main taxonomic criteria for *Spirulina* classification and species differentiation [1,22].

### 2.4. Short Term Preservation of *Spirulina*

The two new identified species of *Spirulina* were grown under laboratory conditions for preservation. UTEX *Spirulina* medium was used for liquid medium and 2% bacteriological agar with a gelling temperature of 40°C was supplemented for preparation of solid plates. Diluted samples of *Spirulina* cells at exponential growth phase were spread on these plates and kept for one week under light (fluorescent light 2000 lux) for proper growth. Direct liquid culture and one week solid streak plates were kept at 10°C and 4°C for one to two months and then viability was verified.

### 2.5. Medium Term Preservation

For preservation of *Spirulina* for a few months (up to a year) the protocol of Mayashree and Bhattacharjee (2010) (24) was followed. UTEX solid medium was prepared with 2.5% bacteriological agar at a gelling temperature of 40°C. *Spirulina* cells at exponential growth phase (10 - 15 days old) were concentrated by centrifugation at 2000 rpm for 5 minutes (100 ml of culture was concentrated to 10 ml). Following this, 10 ml of *Spirulina* culture were poured into 40 ml of warm nutrient agar solutions (final volume 50 ml). This mixture was spread on flat bottom glass petri dishes.

### 2.6. Long Term Preservation (Cryopreservation)

*Spirulina* cultures at exponential growth phase were concentrated by centrifugation at 3000 rpm for 3 min.

The pellet was re-suspended in fresh UTEX culture medium with two different concentrations (5% and 10%) of cryoprotectants like methanol, glycerol and DMSO. Then transferred to sterile vials and kept at 4°C overnight. Then slowly transferred to -20°C, then to -80°C and finally kept in liquid nitrogen until use. Some of the cryovials were recovered in liquid medium for algal cell survival tests. Cryopreserved *Spirulina* strains were taken out from the liquid nitrogen for recovery and immediately kept in water bath at 40°C until the material thawed properly. The liquefied samples were transferred to sterile liquid culture and solid plate culture in a laminar hood [13].

## 3. Results and Discussion

### 3.1. Collection of Algal Samples and

Collection points were selected from the book Algas del

Occidente de México [18]. The collection points were located in Sayula, Rio Caliente in the Primavera forest and the Zapotlán Lake in the state of Jalisco as shown in (Table 1). There were different species of microalgae associated with *Spirulina*. All collected samples were contaminated with various solid abiotic wastes along with filamentous and unicellular algal populations. As some hot springs [23] and lakes [24] are source of *Spirulina*, we found four species of *Spirulina* in all collections.

## 3.2. Isolation of Alga

### 3.2.1. Density Centrifugation

The collected samples contained lots of suspended abiotic contaminants, bacteria and a large amount of diverse microorganisms. Repeated low-speed centrifugation (500 - 1000 rpm) removed up to 90% of the suspended materials and unicellular microorganisms. *Spirulina* was found at the upper phase in the floating mass along with other 12 blue-green and green algae species. Those species were used for the mixed culture in liquid medium (Figure 2).

After culturing the mixed algal sample in different culture media [25], it was found that the most suitable medium was the UTEX *Spirulina* medium for its high pH value (9.2) to reduce other algal population in the mix culture. Then after one week it was observed that there were around 3 more species growing with *Spirulina* in UTEX medium. Density gradient is one of the rapid and sensitive techniques for algal separation [26,27] and most of the algal contaminations were eliminated by this method.

### 3.2.2. Chemical Treatment

Cycloheximide is a chemical which inhibits eukaryotic protein synthesis and eliminates eukaryotes algae from culture in very minor concentration [28]. Cycloheximide was used at different concentrations (25 - 100 ug/ml) to eliminate any eukaryotic algae. It was found effective for eliminating the brown algae at 50 ug/ml (Figure 3), but not able to eliminate one of the unicellular algae contaminations. So it was suspected as cyanobacteria.

### 3.2.3. Serial Dilution

A process of serial dilutions was effective in our experiments [29]. The percentage of unicellular algae decreased 95% but could not be totally eliminated.

### 3.2.4. Micromanipulation

After micromanipulation under the microscope the filamentous algae was always found associated with a small unicellular microalgae.

### 3.2.5. Streak Plating

Solid plating was tried several times repeatedly with the dilution up to  $10^{-5}$  and it was found suitable for separation of individual colonies. Finally, single colonies from the plate were selected and used for monocultures of the individual *Spirulina* species (Figure 4).

## 3.3. Identification of *Spirulina*

The first species of *Spirulina* was identified according to Ciferri (1983) [1] and Tomasseli (1997) [21]. After observation under the microscope at different magnifications and according to the taxonomic guide, one of the species was identified as *Spirulina subsalsa* (Figure 5) [30]. The guide for identification was trichomes width, cell length, pointed calyptras, type of coil and helix-shape [30]. The second algae identified to the species level was *Spirulina major* (Figure 6). It was identified according to the guideline published in the references of Gomont (1892) [31], Anagnostidis & Golubic (1966) [32], Cohen-Bazire Guglielmi & (1982) [33], Rethmeier (1995) [34] and Komárek (1992) [35].

## 3.4. Short Term Preservation of *Spirulina*

Short term preservation is for up to 2 months for regular laboratory maintenance. The liquid and solid (2% bacteriological agar plates) media were used for short term preservation at 10°C and 4°C. All the cultures were recovered more than 50% after 2 months. The recovery time was longer than a regular culture but effective (Figure 7).

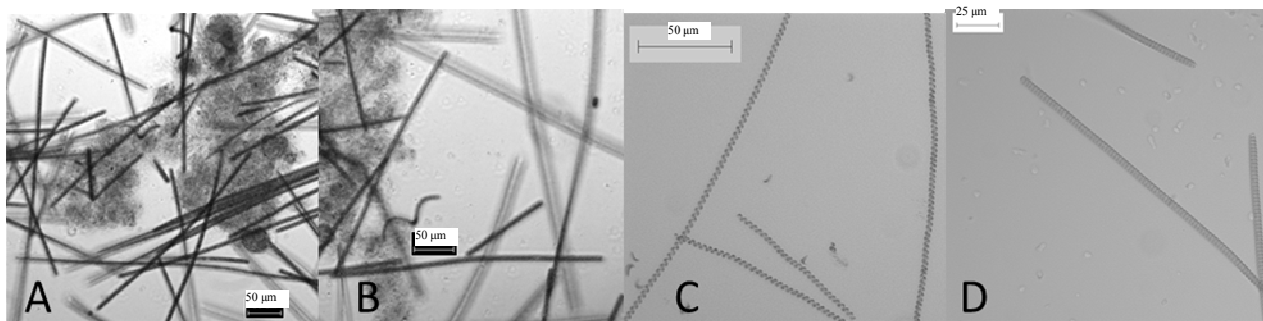


Figure 2. Mixed partial purified cultures. (A) Around 12 species. (B) Around 6 - 8 and (C) Around 3 species. All the samples were observed under 20× magnifications.



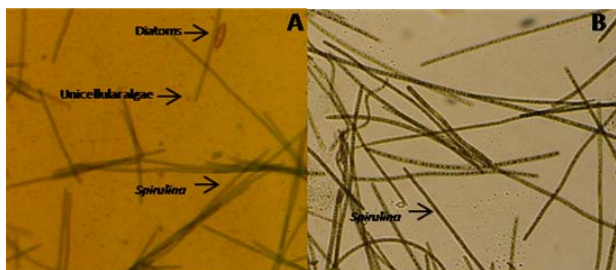


Figure 3. Cycloheximide treatment for the cleaning of *Spirulina* cultures. All the samples were observed under 20× magnifications.

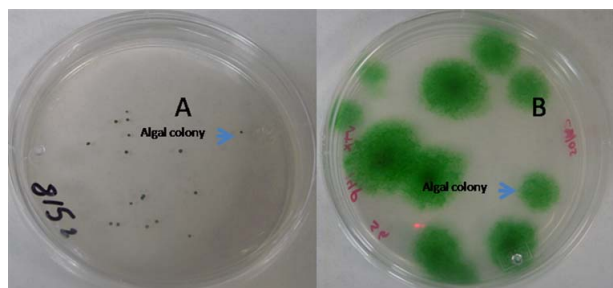


Figure 4. Formation algal colony in agar plates. (A) dilution at  $10^{-1}$ , (B) dilution at  $10^{-5}$ .

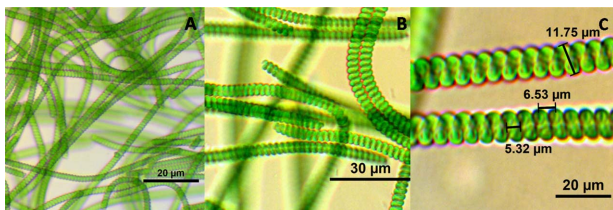


Figure 5. *Spirulina subsalsa* (A) 40× (25 µm scale), (B) 100×.

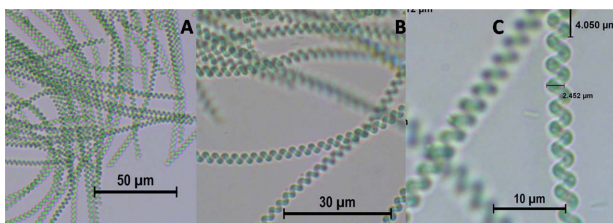


Figure 6. *Spirulina major*: Photograph at different scale.

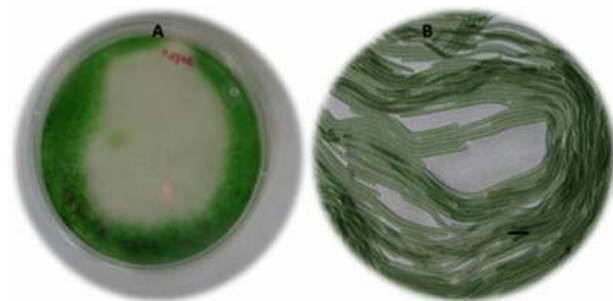


Figure 7. *Spirulina subsalsa* in agar medium, (A) whole agar plate, (B) Photograph at 20×.

### 3.5. Medium Term Preservation of *Spirulina*

In this method the strains were prepared mixed with agar medium and dried to form agar flakes. This method serves very well for *Spirulina* strain preservation. As the method includes drying the algal material along with agar, it can be preserved at room temperature for up to six months and the revival percentage was 70% - 80% and can be kept at 4°C for one to two years [36]. In our experiments we found that, after three months in agar flakes both of the strains were well recovered. Nearly 80% of viability was found on our study (Figure 8).

### 3.6. Long Term Preservation (Cryopreservation) of *Spirulina*

DMSO is well known for preserving cyanobacteria [37-42]. DMSO has been used as a cryoprotective agent because it reduces crystal formation during freezing and avoids cell breakage. The results of the present study are consistent with those of Davis *et al.* (1978) [43] and Madigan *et al.* (2003) [44] (Figures 9(a)-(c)). The two *Spirulina* species treated in different ways for cryopreservation using methanol, glycerol and DMSO as osmoprotectants. It was found that after thawing the sample from liquid nitrogen only in 5% DMSO treated samples showed low survival (less than 1%) after 3 months of preservation, it partially agrees with the findings of Rippka *et al.* (1981) [28]. All the treatments with methanol and glycerol did not show any survival of algae (Figure 10). Long term preservation and two steps cryopreservation using methanol, glycerol and DMSO is in progress.

## 4. Conclusion

The algal strains are normally found in contaminated water bodies and salty lakes. They are generally contaminated with many diverse microorganisms, rotifers and toxic cyanobacterias along with suspended abiotic particles. So here we used simple strategies to establish monocultures of two local *Spirulina* species: *S. subsalsa* and *S.*

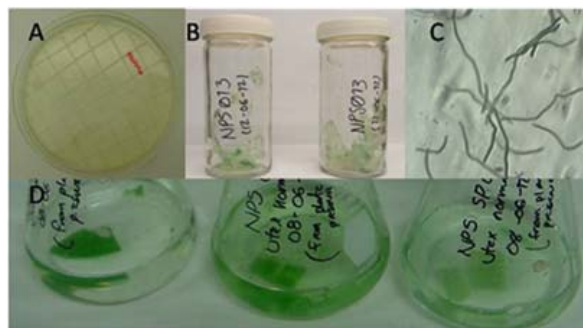


Figure 8. Cryopreservation of *Spirulina*, (A) Preparation of agar slice, (B) Dried agar flakes with algae, (C) Microscopic observation of embedded *Spirulina*. (D) Reactivation in UTEX medium.

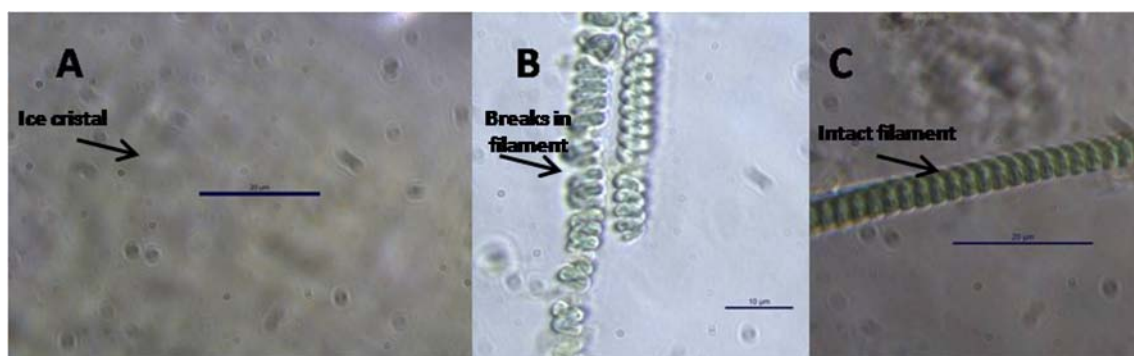


Figure 9. Effect of cryopreservation. (A) Formation of crystal, (B) Broken cell of *S. subsalsa*, (C) 5% DMSO treatment.



Figure 10. Long-term cryopreservation. Only cells conserved in 5% DMSO and in controls grew at different rates (A) & (B), (C) and (D) are the glycerol and methanol treatments.

major which seem to be good local species from Mexico for further utilization and commercialization. Again, to preserve these species in short and long term basis, we standardized the methodology as cyanobacterial germplasm conservation is difficult and recovery percentage is very low. Here we report good recovery by using the dry agar flakes preservation method. The cryopreservation method is not concluded and is currently in progress. The isolation and conservation methods standardized in this work will be of great help for the isolation and germplasm preservation of *Spirulina*.

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