OPEN ACCESS
Freely available on line

Universitas Scientiarum

Univ. Sci. 2013, Vol. 18 (2): 129-139
doi: 10.11144/Javeriana.SC18-2.etmp

ORIGINAL PAPER

# Evaluation of three methods for preservation of *Azotobacter chroococcum* and *Azotobacter vinelandii*

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## Abstract

Because the use of bacteria for biotechnological processes requires maintaining their viability and genetic stability, preserving them becomes essential. Here, we evaluated three preservation methods for *A. chroococcum* C26 and *A. vinelandii* C27; preservation methods: cryopreservation and immobilization in dry polymers for 60 days, and freeze-drying for 30. We evaluated their efficiency by counting viable cells and measuring nitrogen fixation activity. Additionally, we assessed the effect of three protective agents for freeze-drying, three for cryopreservation, and four polymers. Freeze-drying proved the best technique to maintain viability and activity, followed by immobilization and cryopreservation. Bacterial nitrogen fixing ability remained unchanged using the freeze-drying method, and bacterial survival exceeded 80%; S/BSA was the best protective agent. Immobilization maintained bacterial survival over 80%, but nitrogen fixation was decreased by 20%. Lastly, cryopreservation resulted in a dramatic loss of viability for C26 (BSR approx. 70%), whereas C27 was well preserved. Nitrogen fixation for both strains decreased regardless of the cryoprotective agent used ($P < 0.05$). In conclusion, the success of *Azotobacter* preservation methods depend on the technique, the protective agent, and the strain used. Our results also indicated that freeze-drying using S/BSA is the best technique to preserve bacteria of this genus.

**Keywords:** *Azotobacter*; bacterial preservation; cryopreservation; freeze-drying; immobilization in polymers; bacterial nitrogen fixation.

## Introduction

Plant growth-promoting bacteria –PGPB– are microorganisms that can grow in, on, or around plant tissues and stimulate plant growth by numerous mechanisms (Vessey 2003). Within this group, nitrogen-fixing bacteria play a remarkable role in plant nutritition. They can take N2 from atmosphere and make it available for plant uptake (Halbleib and Ludden 2000). *Azotobacter*, a nitrogen-fixing bacteria, is able to fix nitrogen aerobically and have the particularity of forming into cysts (Becking 2006, Garrity et al. 2005). This bacterial genus, has showed to be able to promote plant growth, and therefore it is usually included in the PGPB group. For example, Kisilkaya (2008) showed that inoculation of wheat with

**Edited by** Alberto Acosta & Raúl Poutou

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**Received:** 26-02-2013 **Accepted:** 04-04-2013 **Published on line:** 31-05-2013

**Citation:** Rojas-Tapias D, Ortiz-Vera M, Rivera D, Kloepper J, Bonilla R (2013) Evaluation of three methods for preservation of *Azotobacter chroococcum* and *Azotobacter vinelandii*. *Universitas Scientiarum* 18(2): 129-139 doi: 10.11144/Javeriana.SC18-2.etmp

**Funding:** Ministerio de Agricultura y Desarrollo Rural de Colombia (MADR).

**Electronic supplementary material:** N/A

**SICI:** 2027-1352(201305/08)18:2<128:EOTMFPOACAAV>2.0.TS;2-2

*A. chroococcum* RK49 resulted in an enhancement in grain yield compared to control. Similarly, Rojas-Tapias et al. (2012) demonstrated the role of *A. chroococcum* C5 and C9 to prevent saline stress in maize.

Preserving microorganisms gathered from various sources is critical for many fields of research, and maintaining their genetic consistency is crucial for elaborating biological products, which depend on the authenticity and viability of strains (Don and Pemberton 1981, Malik and Claus 1987). To date, freeze-drying and ultra-freezing are considered the most efficient methods for preservation of microorganisms (Sorokulova et al. 2012). However, these techniques require the use of specialized or expensive equipment to preserve and maintain bacteria stable. While many researchers have focused on the improvement of these methods by working on process parameters, others have worked on the development of new techniques that do not require the use of specialized equipment or environmental conditions (e.g. preservation in dry natural biopolymers at room temperature) (Sorokulova et al. 2012). This because of under some situations, ultra-freezing could be impracticable and/or availability of specialized equipment may be restrictive.

Some techniques to preserve bacteria such as freeze-drying and cryopreservation may maintain cells viable for many years; however, these techniques may also cause severe damages to bacterial cells (Miyamoto-Shinohara et al. 2000). Under optimal conditions cells may even be affected due to the processes used for preservation. Consequences of using these techniques include damages to cell wall, cell membrane, DNA, proteins, etc. (Leslie et al. 1995, Miyamoto-Shinohara et al. 2000). These side effects are considered undesirable because recovery of viable and non-mutated bacteria is critical (Krumnow et al. 2009). Cell damage is caused by the same methods used to preserve microorganisms. For example, freeze-drying involves the use of extreme low temperatures and vacuum, cryopreservation the use of extreme low temperatures, and the majority of spray-drying techniques the use of extreme high temperatures. Furthermore, desiccated bacteria may also lose viability due to rehydration, which may alter protein structures (Krumnow et al. 2009). For this reason, use of preservation techniques is usually accompanied with use of protective agents, which can increase effectiveness of the technique preventing cell damage. For this reason, selection of the protective agent depends on the preservation method and type of bacteria. Some examples of protective agents include glycerol, trehalose, DMSO, glycine betaine, skim milk, glutamate, and sucrose. A preservation technique is said to be useful if bacteria can revive, maintain cellular functions and propagate after dehydration, storage and rehydration (Malik and Claus 1987).

To our knowledge, few reports have been focused on preservation of *Azotobacter* cells. Earlier reports suggested the success of preservation of *Azotobacter* cysts in dry soil at room temperature (Vela 1974) and vegetative cells in liquid nitrogen (Thompson 1987). Both reported they were able to maintain the viability of *Azotobacter* strains for more than 10 years. Some other techniques such as freeze-drying were tried and were found to not be entirely satisfactory for this genus as viability could not be maintained for long (Lapage et al. 1970, Antheunisse 1973, Thompson 1987). Hence, standardization of different preservation methods, which are available for most laboratories, for storage of *Azotobacter* strains remains a priority in order to maintain their genetic and metabolic characteristics. The goal of this study was to evaluate the effectiveness of freeze-drying, cryopreservation, and immobilization in dry polymers as preservation methods for *Azotobacter* (*A. chroococcum* and *A. vinelandii*); likewise, to study the effect of protective agents to improve the viability and activity of bacteria under storage. To our knowledge, this is the first report in which are evaluated several preservation methods for the maintenance of this bacterial genus.

## Materials and Methods

**Bacterial strains and culture preparation:** Strains C26 and C27 were isolated from eucalyptus rhizosphere in Codazzi, Cesar, Colombia (Obando et al. 2010). These were identified as *A. chroococcum*

and *A. vinelandii* by studying their *nifH* genes, respectively. For maintenance, the strains were refrigerated at 4ºC on Ashby (composition in g/L: mannitol 10, $K_2HPO_4$ 0.2, $MgSO_4$ $7H_2O$ 0.2, NaCl 0.2, $CaSO_4$ 0.1, $CaCO_3$ 10.0, agar 15.0) culture plates, and streaked monthly on new medium. To prepare bacterial cultures, strains C26 and C27 were incubated for seven and three days –late stationary phase–, respectively, at 30ºC on Ashby plates at pH 9.5. Cellular suspensions were then rinsed twice with 0.85% NaCl and pellets were reconstituted in the respective protective solutions. Before cells were preserved, number of viable cells was counted in triplicate by spreading serial dilutions on LG solid medium (composition in g/L: sucrose 20, $K_2HPO_4$ 0.05, $KH_2PO_4$ 0.15, $CaCl_2$ 0.02, $MgSO_4$ $7H_2O$ 0.2, $Na_2MoO_4$ $2H_2O$ 0.002, $FeCl_3$ 0.01, $CaCO_3$ 0.01, bromothymol blue 0.025).

**Preservation methods:** To find a suitable method for preservation of *A. chroococcum* and *A. vinelandii*, we tested three preservation methods and some protective agents. Selection of these methods and their respective protective agents was based on an exhaustive revision of literature. For cryopreservation, we used glycerol and DMSO as reported by Garrity et al. (2005), while TSA was chosen by suggestion of author Dr. Joseph Kloepper. For freeze-drying, we used S/BSA (Cleland et al. 2004), 10% skim milk + 1% sodium glutamate (Miyamoto-Shinohara et al. 2006), and 10% skin milk (Cody et al. 2008, and routine method in our Lab) since these show to be highly effective to preserve viability of many Gram-negative bacteria. Finally, for immobilization in dried polymers we used alginate (Bashan and Gonzales 1999), acacia gum (Krumnow et al. 2009), carrageenan (Denkova et al. 2004), and Polyox® that was first evaluated in this study.

***Cryopreservation:*** We evaluated three protective agents at two concentrations. Hence, bacterial suspensions were prepared in the following sterile protective agents: 10% and 30% glycerol (Merck, USA), 10% and 20% dimethyl sulfoxide –DMSO– (Fisher Scientific, USA), and 0.5X and 1.0X trypticase soy broth –TSA– (Merck, USA). One-ml aliquots of cell suspensions with each protective agent were dispensed into labeled sterile 2-ml screw capped tubes of polypropylene, incubated at room temperature for 1.0 h, and then samples were frozen at -25ºC. Estimation of bacterial survival was performed 0, 5, 15, 30, and 60 days subsequent to preservation.

***Freeze-drying:*** Cell suspensions were prepared in the following three sterile protective agents: 10% skim milk (SM), 10% skim milk + 1% sodium glutamate (SM/Glu), and 20% sucrose + 10% BSA (S/BSA). A 200 µL-aliquot of each treatment was placed into 5-ml vials, frozen at -196ºC by using liquid nitrogen, and desiccated under vacuum at 1.0 hPa in a Heto Power Dry PL9000 Freeze Dryer (Thermo Corporation, USA). The primary and secondary drying on shelves was performed at different stages: 1) -20ºC for 3 h, 2) -4ºC for 2 h, 3) 15ºC for 3 h, 4) 25ºC for 5 h, and 5) 30ºC for 11 h. Freeze-dried cells were subjected to accelerated survival evaluation at 37ºC and the Log of colony forming units per ml (CFU ml−1) was recorded after two and four weeks (Cleland et al. 2004).

***Immobilization in dry polymers:*** Cell suspensions were prepared in the following four sterile protective agents: 1% sodium alginate, 15% acacia gum, 2.5% Polyox®, and 1.5 % carrageenan. Sodium alginate and carrageenan were purchased from FMC BioPolymers (Ewing, USA), Polyox® from Colorcon (Harleysville, USA), and Acacia gum from Sigma-Aldrich (St. Louis, USA). Concentrated solutions of polymers were maintained at 4ºC when required. One-ml mixtures were arranged into 5.0 ml vials and dried at 37ºC for 48 h. Immobilized cells were maintained at 15ºC and 40±2 % relative humidity and bacterial survival was measured after 15, 30, and 60 days. Final volumes of cell suspensions and times for drying were previously studied in order to obtain mixtures (cell suspension and protective agents), with the minimum amount of water as possible, and still maintaining cell viability (data not shown).

**Estimation of viability and activity of bacterial samples:** We estimated both bacterial titers and bacterial ability to fix nitrogen to assess the efficiency of the methods. For cryopreservation, samples were

thawed for 3 min using a serological bath at 33ºC. When we used freeze-drying and immobilization in polymers, the samples were re-hydrated using 200 µl and 1000 µl of sterile deionized water, respectively. Vials were then vortexed for 3 min, and incubated at 33ºC for 30 min. Bacterial viability was estimated by preparing serial dilutions and plating 20 µl of each dilution on LG solid medium. Plates were incubated under aerobic conditions at 30°C for 72 h. We counted the plates, containing between 30–300 bacterial colonies. Data were expressed as log CFU $ml^{-1}$. All experiments were performed in triplicate. Bacterial capacity to fix nitrogen was estimated after 60 days for cryopreservation, 15 days for freeze-drying, and 60 days for immobilization in dry polymers, respectively. Nitrogen fixation was assessed using the acetylene reduction assay (ARA). The bacterial survival ratio (BSR) was reported as the ratio of the log of the number of bacterial cells present in the suspension after preservation (AP) to the log number of viable cells before preservation (BP) multiplied by 100, i.e. BSR= $(\log_{AP}/\log_{BP})$ x 100 (Muñoz-Rojas et al. 2006).

**Analytical method; acetylene reduction assay (ARA):** Two hundred-ml flasks, containing 20 ml of Ashby medium were inoculated with 25 µL of a bacterial suspension adjusted to OD600= 0.500 and incubated for 48 h at 30°C. Both strains were collected for ARA from cultures roughly in exponential phase or starting stationary phase, therefore the absorbance corresponded mainly to alive cells. We carried out independent comparisons for each strain. Acetylene reduction was measured by using a gas chromatograph (Perkin Elmer, USA) with flame ionization detector, and a Poropak column N 200/300 Mesh of 6.0 ft and 3.0 mm diameter (Eckert et al. 2001). Calibration curve was carried out using pure ethylene as standard (chromatographic grade).

**Statistical analysis: Our hypotheses were:** 1) to study if there were differences among the methods to maintain viability and activity of the *Azotobacter* strains, and 2) to determine if the different protective agents studied had effect on survival and activity of the *Azotobacter* strains. For cryopreservation, freeze-drying, and immobilization in dried polymers, we used a one-factor design with six, three and four levels, respectively (each protective agent at one specific concentration; see 'Preservation methods' in Materials and Methods). Plate count and nitrogen fixation were used as dependent variables. We tested normality on these two variables using the Shapiro-Wilk test, and homogeneity of variances was assessed using the Levene's Test. Means were compared using the ANOVA and HSD Tukey tests. All experiments were performed at 95% confidence level. We used the statistical package SPSS 17.0 for statistical analysis. P-values less than 0.05 were considered statistically significant.

## Results

We assessed three techniques for preservation of *Azotobacter* in this study: cryopreservation at -25ºC, freeze-drying, and immobilization in polymers at room temperature. Results showed that the bacteria survival rate decreased over the time for both strains, and the decrease rate depended on the used strain, preservation technique, and protective agent.

*Azotobacter* cells subjected to cryopreservation resulted in a rapid rate of bacterial death regardless of the protective agent (**Table 1**). The BSR decreased for C26 and C27 between 25-30% and 15-20% after 60 days; however this diminished more than 20% and 10% between days 0th and 5th, respectively. Viability of C26 under freezing depended on the protective agent used, and the best results were obtained with DMSO > Glycerol > TSA ($P < 0.05$). For *A. vinelandii* C27, all protective agents maintained the viability between 81-85% ($P > 0.05$). Regarding bacterial activity, a decrease in viability of C26 was accompanied with a dramatically reduction of bacterial activity ($P < 0.05$). After 60 days, nitrogen fixation decreased more than 50% (**Figure 1**). In contrast, nitrogen fixation was usually maintained for C27, and only was slightly reduced when DMSO and 0.5X TSA were used as protective agents ($P < 0.05$).

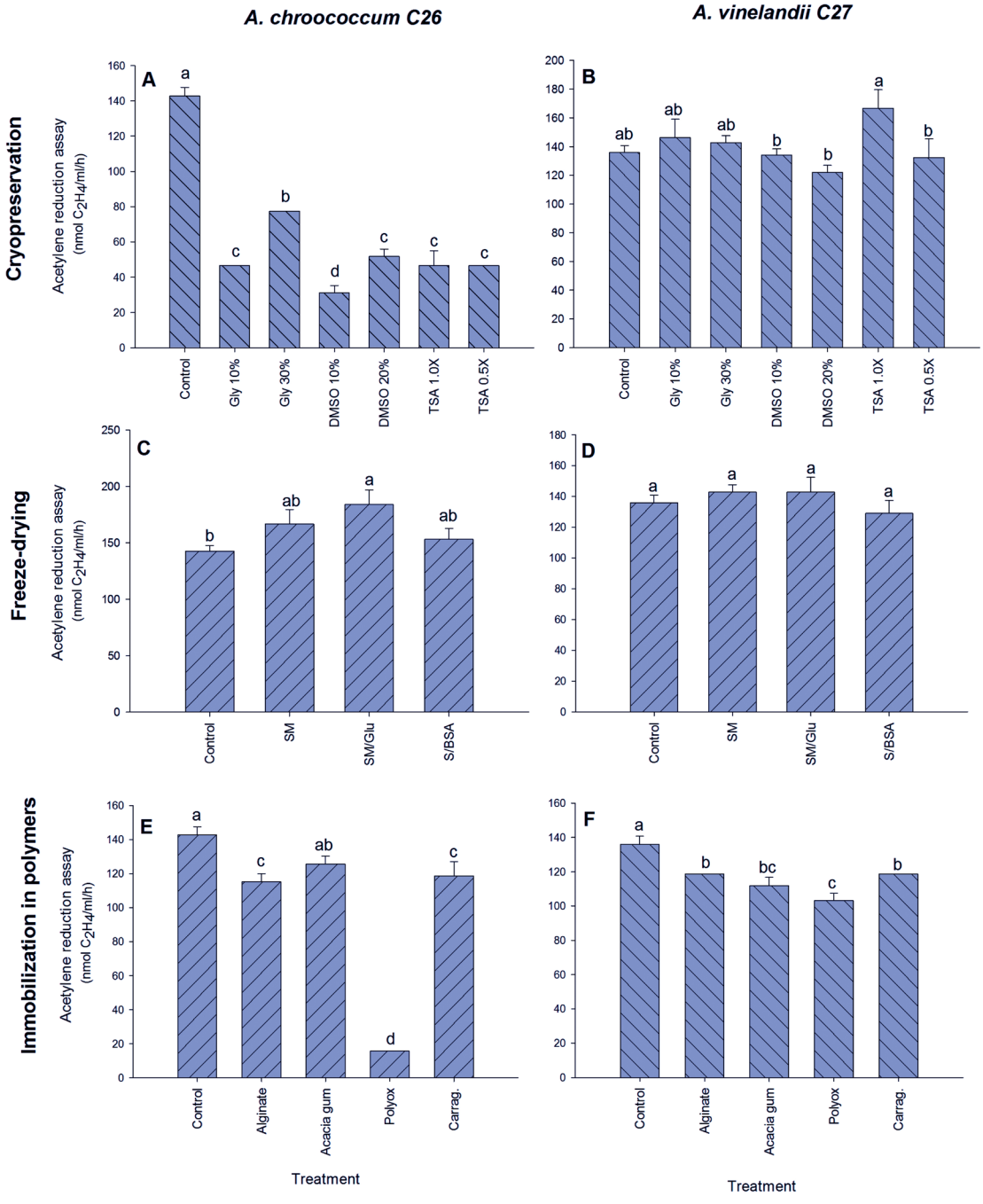
Freeze-drying exhibited the best results regarding bacterial activity. After 15 days (i.e. 20 simulated years at 4ºC) at 37ºC the nitrogen

**Table 1**. Effect of cryopreservation on survival of the *Azotobacter* strains. Letters indicate sub-homogeneous groups obtained using the HSD Tukey test. We compared separately the effect of the protective agents on survival of each strain at each time. Values in parentheses mean: ± standard deviation. The BSR was calculated as described in Materials and Methods.

| Treatment | Time (days) 0 | 5 | | 15 | | 30 | | 60 | |
|---|---|---|---|---|---|---|---|---|---|
| | Log CFU $ml^{-1}$ | Log CFU $ml^{-1}$ | BSR | Log CFU $ml^{-1}$ | BSR | Log CFU $ml^{-1}$ | BSR | Log CFU $ml^{-1}$ | BSR |
| *Azotobacter chroococcum* C26 | | | | | | | | | |
| Glycerol 10% | 9.90 (0.08) a | 8.35 (0.11) a | 84.3% | 7.56 (0.06) a | 76.4% | 7.52 (0.17) ab | 76.0% | 7.52 (0.18) ab | 73.4% |
| Glycerol 30% | 9.90 (0.08) a | 7.54 (0.09) cd | 76.1% | 7.46 (0.19) a | 75.3% | 7.57 (0.14) ab | 76.5% | 7.39 (0.22) ab | 74.6% |
| DMSO 10% | 9.90 (0.08) a | 8.04 (0.23) ab | 81.2% | 7.63 (0.09) a | 77.1% | 7.75 (0.10) a | 78.3% | 7.55 (0.08) a | 76.2% |
| DMSO 20% | 9.90 (0.08) a | 7.94 (0.25) abc | 80.2% | 7.63 (0.05) a | 77.1% | 7.56 (0.16) ab | 76.3% | 7.39 (0.15) ab | 74.6% |
| TSA 0.5X | 9.90 (0.08) a | 7.19 (0.10) e | 72.7% | 7.52 (0.13) a | 75.9% | 7.55 (0.12) ab | 76.3% | 7.07 (0.12) b | 71.4% |
| TSA 1.0X | 9.90 (0.08) a | 7.79 (0.15) bc | 78.8% | 7.51 (0.15) a | 75.9% | 7.26 (0.17) b | 73.3% | 7.18 (0.18) ab | 72.5% |
| *Azotobacter vinelandii* C27 | | | | | | | | | |
| Glycerol 10% | 9.09 (0.03) a | 7.97 (0.23) ab | 87.7% | 7.89 (0.09) ab | 86.8% | 7.67 (0.16) ab | 84.3% | 7.63 (0.13) a | 84.0% |
| Glycerol 30% | 9.09 (0.03) a | 7.56 (0.07) bcd | 83.2% | 7.64 (0.32) abc | 84.0% | 7.61 (0.10) ab | 83.7% | 7.57 (0.15) a | 83.3% |
| DMSO 10% | 9.09 (0.03) a | 8.12 (0.17) a | 89.3% | 7.99 (0.06) a | 87.9% | 7.97 (0.11) a | 87.7% | 7.67 (0.12) a | 84.4% |
| DMSO 20% | 9.09 (0.03) a | 7.67 (0.14) bc | 84.4% | 7.63 (0.10) abc | 84.0% | 7.48 (0.16) b | 82.3% | 7.44 (0.19) a | 81.8% |
| TSA 0.5X | 9.09 (0.03) a | 7.20 (0.15) d | 79.2% | 7.54 (0.13) bc | 83.0% | 7.33 (0.28) b | 80.6% | 7.44 (0.18) a | 81.8% |
| TSA 1.0X | 9.09 (0.03) a | 7.32 (0.17) cd | 80.6% | 7.34 (0.13) d | 80.8% | 7.33 (0.10) b | 80.6% | 7.51 (0.15) a | 82.6% |

fixation activity was maintained stable for C27 ($P > 0.05$), and even was increased for C26 ($P < 0.05$) compared to control (Figure 1). The observed BSR after 15 days were reduced in 26.2%, 15.1%, and 16.4% for C26 when used SM, SM/Glu, and S/BSA, respectively. The C27 viability was

**Fig. 1.** Acetylene reduction assay. Figures 1A, 1C, and 1D correspond to nitrogen fixation by *A. chroococcum* C26, and figures 2B, 2D, and 2F by *A. vinelandii* C27. Nitrogen fixation was measured after 60, 15, and 60 days after cryopreservation, freeze-drying, and immobilization in polymers, respectively. Letters indicate sub-homogeneous groups obtained using the HSD Tukey test.




decreased by 28.1% and 9.1% in SM/Glu and S/BSA, respectively (**Table 2**). Although the bacterial viability was dramatically reduced after 30 days (i.e. 40 simulated years at 4°C), we were able to recover both strains regardless of the protective agent used. Interestingly, when we used S/BSA the BSR was decreased only 20% after 30 days.

**Table 2.** Effect of freeze-drying on survival of the *Azotobacter* strains. Letters indicate sub-homogeneous groups obtained using the HSD Tukey test. We compared separately the effect of the protective agents on survival of each strain at each time. Values in parentheses mean: ± standard deviation. The BSR was calculated as described in *Materials and Methods*. The + symbol indicates that the strains could be directly recovered from vials, and ND indicates: not determined.

| Treatment | Time (days) 0 | 15 | | 30 | |
|---|---|---|---|---|---|
| | Log CFU $ml^{-1}$ | Log CFU $ml^{-1}$ | BSR | Log CFU $ml^{-1}$ | BSR |
| *Azotobacter chroococcum* C26 | | | | | |
| Skin milk 10% | 9.32 (0.09) a | 6.88 (0.11) b | 73.8% | + | ND |
| Skin milk 10% + 1% glutamate | 9.32 (0.09) a | 7.91 (0.14) a | 84.9% | + | ND |
| Sucrose/BSA | 9.32 (0.09) a | 7.79 (0.08) a | 83.6% | 7.29 (0.16) | 78.2% |
| *Azotobacter vinelandii* C27 | | | | | |
| Skin milk 10% | 8.94 (0.09) a | + | ND | + | ND |
| Skin milk 10% + 1% glutamate | 8.94 (0.09) a | 6.43 (0.17) b | 71.9% | + | ND |
| Sucrose/BSA | 8.94 (0.09) a | 8.13 (0.29) a | 90.9% | 7.50 (0.21) | 83.9% |

In general, bacterial cells were well preserved by using polymers as carriers. The best results after 60 days were displayed by carrageenan > Polyox® > alginate > acacia gum for C26, and Polyox® > acacia gum > carrageenan > alginate for C27 (**Table 3**). The reduction in the BSR was generally not higher than 20% within 60 days. Efficiency of immobilization to maintain viability depended on

**Table 3**. Effect of immobilization in polymers on survival of the *Azotobacter* strains. Letters indicate sub-homogeneous groups obtained using the HSD Tukey test. We compared separately the effect of the protective agents on survival of each strain at each time. Values in parentheses mean: ± standard deviation. The BSR was calculated as described in Materials and Methods.

| Treatment | Time (days) 0 | 5 | | 15 | | 30 | |
|---|---|---|---|---|---|---|---|
| | Log CFU $ml^{-1}$ | Log CFU $ml^{-1}$ | BSR | Log CFU $ml^{-1}$ | BSR | Log CFU $ml^{-1}$ | BSR |
| *Azotobacter chroococcum* C26 | | | | | | | |
| Alginate | 8.22 (0.13) a | 8.29 (0.18) a | 100.8% | 8.17 (0.19) a | 98.8% | 6.95 (0.13) a | 84.6% |
| Acacia gum | 8.22 (0.13) a | 6.56 (0.07) b | 79.8% | 5.65 (0.11) c | 68.7% | 5.40 (0.00) b | 65.7% |
| Polyox® | 8.22 (0.13) a | 8.35 (0.22) a | 101.5% | 7.23 (0.31) b | 87.9% | 7.00 (0.18) a | 85.1% |
| Carrageenan | 8.22 (0.13) a | 8.37 (0.17) a | 101.8% | 8.37 (0.21) a | 101.8% | 7.31 (0.54) a | 88.9% |
| *Azotobacter vinelandii* C27 | | | | | | | |
| Alginate | 7.98 (0.22) a | 7.51 (0.10) a | 94.1% | 6.78 (0.12) b | 84.9% | 6.45 (0.18) b | 78.4% |
| Acacia gum | 7.98 (0.22) a | 7.47 (0.10) a | 93.6% | 7.26 (0.19) a | 91.0% | 7.01 (0.22) ab | 85.3% |
| Polyox® | 7.98 (0.22) a | 7.65 (0.10) a | 95.9% | 7.55 (0.09) a | 94.6% | 7.54 (0.45) a | 91.6% |
| Carrageenan | 7.98 (0.22) a | 7.50 (0.12) a | 94.0% | 6.89 (0.04) b | 86.5% | 7.00 (0.00) ab | 85.2% |

the polymer used (Table 3). Using this method we usually maintained the BSR higher than 85% for both strains. We also observed a significant reduction in nitrogen fixation, but this was not greater than 30% except for Polyox® on C26 (Figure 1).

## Discussion

Preservation of *Azotobacter* is a main concern because this bacterial genus represents an important source for several biotechnological applications (e.g. plant growth promotion, biopolymers synthesis, hydrocarbons bioremediation), which requires the maintaining of its physiological and genetic properties. Our results evidence that bacterial viability and activity depend on the preservation technique, the protective agent, and the bacterial species.

Viability of bacteria after cryopreservation at -25ºC significantly decreased during the time of evaluation. Interestingly, we observed that the highest rate of bacterial death occurred within the first five days of preservation, which could suggest that bacteria died during the initial stages of the process. Dumont et al. (2004) reported that under cryopreservation, cell survival depends on the cooling rates. They demonstrated that the lowest viabilities are observed at intermediate cooling rates (i.e. ~100-1,000 °C $min^{-1}$), whereas the highest ones are observed at very low or very high rates (i.e. ~10 and 30,000 °C $min^{-1}$, respectively). Fonseca et al. (2006) also reported that high cooling rates significantly improve survival rates for lactic acid bacteria. It is worth noting that although freeze-drying usually causes more damage to cells than cryopreservation, we observed a minor loss of viability using this first method (Table 2, Table 3). Likely, the higher cooling rates used for freeze-drying (i.e. freezing using liquid nitrogen) resulted in a greater *Azotobacter* survival. Thompson (1987) indicated that *Azotobacter* cells frozen with liquid nitrogen usually result in higher survival rates. Both results would support the hypothesis that the freezing rate could affect bacterial survival.

The protective agents prevented the bacterial death compared with 0.85% NaCl (data not shown); however we usually observed no differences in bacterial survival when each protective agent was studied at both concentrations (Table 1). Possibly, the concentrations in which the protectant is no longer useful did not belong to our range of study. Additionally, we observed that viability of each strain specifically depended on the protective agent (Table 1); hence, we concluded that efficiency of the cryoprotective agents was species-dependent. Suspending medium is considered to be a major factor in determining the ability of microorganisms to survive stress (Safronova and Novikova 1996). Leslie et al. (1995) showed how some carbohydrates protect membranes during the rehydration process, decreasing the rate of transition in membranes from gel to the liquid crystalline phase. Other agents as glycerol or DMSO reduce the eutectic point of water preventing the formation of ice crystals (Fonseca et al. 2006). Therefore, it is critical to select the appropriate protective agent for improving the storage conditions for *Azotobacter* cells. Important to note that possibly our cryopreservation method was not a quite suitable technique for preserving *Azotobacter* cells because of the temperature used. There is consistent evidence supporting that the lower the temperature, the higher the efficiency of cryopreservation (Trummer et al. 1998, Zhao and Zhang 2005), and hence to study more temperatures might evidence more advantages derived from its use for *Azotobacter* cells.

Despite freeze-drying has been one of the most frequently used techniques for bacterial preservation, this technique causes undesirable side effects, including bacterial membrane damage, protein denaturation, water crystallization, and decreased viability of many cell types (Giulio et al. 2005). As a consequence, freeze-drying is carried out using protective agents to both prevent or reduce these adverse effects, since cells suspended in water or saline solution do not generally survive (Diniz-Mendes et al. 1999). Previous reports showed BSR values of 68.8% for *A. chroococcum* and 72.2% for *A. vinelandii* when they were stored at 37°C for 15 days using 5% skim milk + 0.1% actocol (Sakane and Kuroshima 1997). These same authors compared both simulated and natural survival rates of 60 freeze-dried bacteria, where they found that storage of vials at 37°C for 15 days is useful to simulate the die-off caused by storage at 4°C for about 20 years. Under these conditions, C26 and C27 survived for

30 days regardless of the used lyoprotectant, but we evidenced some differences associated to the strains and the protective agent (Table 2). These differences indicate that certain additives are more effective than others in protecting *Azotobacter* bacteria. Interestingly, the results from our study evidenced larger bacterial survival compared with previous reports about *Azotobacter* preservation. Antheunisse et al. (1981) studied the *Azotobacter* survival after drying in dextran and found out that most strains did not survive after 48 months. Moreover, Kupletskaya and Netrusov (2011) demonstrated that under freeze-drying using 1% gelatin - 10% sucrose and skim milk –7% glucose, the *A. chroococcum* strains had BSR values between 43-87%. Although SM and SM/Glut maintain a high number of viable *Azotobacter* cells, S/BSA maintained the highest *Azotobacter* viability. Cleland et al. (2004) showed that using S/BSA for freeze-drying also resulted on high bacterial survival when used it to preserve *Silicibacter*, *Psychromonas*, *Staphylococcus*, and *Neisseria*.

Under some circumstances refrigeration and freezing could be impracticable (Cody et al. 2008). Hence, to maintain microbial cultures at room temperature is paramount. Earlier studies showed that *Escherichia coli* and *Bacillus subtilis* are well preserved using dried acacia gum and pullulan as carriers at room temperature (Krumnow et al. 2009). Similarly, Bashan and Gonzalez (1999) showed at these conditions the survival of two PGPB strains in dry alginate. We evaluated four dried polymers for preservation of *Azotobacter* at room temperature. At room temperature viability was maintained during 60 days with a decrease of the BSR of about 20% in most cases. Earlier reports showed that *Azotobacter* could be stored for more than twenty years at room temperature into a dry carrier (Moreno et al. 1986, Vela 1974), which supports our findings. Similarly, prior reports displayed that gel entrapment with dehydration has potential for storage (Cassidy et al. 1997).

We observed that freeze-drying and immobilization in dry polymers were useful techniques to preserve the *Azotobacter* viability (Table 2, Table 3). Muñoz-Rojas et al. (2006) reported that cells of *Pseudomonas putida* KT2440 at late-stationary phase have more rigid cell membranes and can survive better than those in the exponential phase to freeze-drying; the authors elucidated that a greater proportion of $C_{17}$: cyclopropane fatty acid mediates this resistance. *A. vinelandii* also exhibits the same pattern, and the proportion of $C_{17}$:Δ fatty acid is larger in old cells (Su et al. 1979). In addition, formation of cysts confers high resistance to deleterious physical conditions such as desiccation (Sadoff 1975). It is therefore likely that particular physiological properties of *Azotobacter* could influence their survival.

An essential feature required from the preservation method is preservation of the biological activity and genetic stability of bacterial cultures (Safronova and Novikova 1996). Therefore, we analyzed the nitrogen fixation activity of *Azotobacter* cells subjected or not to preservation, as this bacterial activity requires a complex transcriptional and post-transcriptional regulation systems (Halbleib and Ludden 2000). Hamilton et al. (2011) illustrated that in *A. vinelandii* about 30% of genes can be differentially expressed under diazotrophic growth. Even though both strains fixed nitrogen after preservation using the three methods and the different protective agents, we observed that both strains exhibited distinctive responses to the preservation methods (Figure 1). In general, the best methods retaining the stability of bacteria were freeze-drying followed by immobilization in polymers. Freeze-drying did not affect the capacity to fix nitrogen of C27 regardless of the lyoprotectant. Furthermore, we observed that after freeze-drying the strain C26 resulted in an increased nitrogenase activity. Similar results were reported on *Bradyrhizobium* after freeze-drying with SGA (Safronova and Novikova 1996).

## Conclusion

In this study, we evaluated three methods for preservation of *Azotobacter* cells. Our present findings evidenced that the efficiency of the methods depends on the *Azotobacter* species and the protective agents used. We also observed that preservation using freeze-drying and immobilization in dry polymers are the most suitable methods for maintaining *Azotobacter* viability. Surprisingly, although cryopreservation is considered one of the best techniques for bacterial preservation, the cryopreserved cells of C26 exhibited a great loss of viability, suggesting that either this method is not useful for all *Azotobacter* strains or the used

temperature is not suitable for their preservation. Concerning bacterial activity, the freeze-dried cells maintained their ability to fix nitrogen. Conversely, the immobilized and cryopreserved cells were affected by storage, but the extent of affectation was strain-dependent. Overall, we found that the best technique for storing *Azotobacter* cells is freeze-drying accompanied with S/BSA.

## Acknowledgments

This work was supported by Ministerio de Agricultura y Desarrollo Rural de Colombia (MADR).

## Conflicts of interests

We declare no conflicts of interests.

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**Evaluación de tres métodos para preservación de *Azotobacter chroococcum* and *Azotobacter vinelandii*.**

**Resumen.** La preservación de bacterias es asunto de gran importancia debido a que muchas de ellas son usadas en procesos biotecnológicos que requieren mantener su viabilidad y propiedades genéticas. En este estudio, se evaluaron tres métodos para la preservación de *A. chroococcum* C26 y *A. vinelandii* C27; criopreservación, liofilización, e inmovilización en polímeros secos, durante 60, 30 y 60 días, respectivamente. A su vez, se estudió el efecto de tres agentes protectivos para la liofilización y para la criopreservación y cuatro polímeros. La eficiencia de los métodos fue evaluada contando células viables y midiendo actividad como fijación de nitrógeno. Los resultados mostraron que la mejor técnica, la cual mantuvo la viabilidad y la actividad, fue la liofilización, seguida por inmovilización y criopreservación. La liofilización mantuvo estable la habilidad bacteriana para fijar nitrógeno, la tasa de sobrevivencia bacteriana (TSB) fue superior al 80%; y el mejor resultado se evidenció cuando se usó S/BSA como agente protectivo. La inmovilización mantuvo la BSR superior al 80%, y la fijación de nitrógeno fue disminuida en 20%. La criopreservación tuvo pérdida sustancial de viabilidad para C26 (TSB aprox. 70%); mientras que C27 se preservó bien. La fijación de nitrógeno fue significativamente disminuida para ambas cepas independientemente del agente crioprotectivo usado ($P < 0.05$). Los resultados sugieren que el éxito de los métodos de preservación para *Azotobacter* depende de la técnica, el agente protectivo y la cepa usada; siendo la liofilización con S/BSA la técnica con mejores resultados para preservar las bacterias de este género.

**Palabras clave:** *Azotobacter*; preservación de bactérias; criopreservación; liofilización; inmovilización en polímeros; fijación de nitrógeno bacteriano.

**Avaliação de três métodos para a preservação de *Azotobacter chroococcum* e *Azotobacter vinelandii*.**

**Resumo.** Porque o uso de bactérias para processos biotecnológicos, requer a manutenção da sua viabilidade e estabilidade genética, preserva-las é essencial Avaliaram-se três métodos de preservação de *A. chroococcum* C26 e *A. vinelandii* C27; criopreservação, liofilização, e imobilização de polímeros secos. Examinamos também o efeito de agentes protetores para liofilizar, para a criopreservação, e polímeros. A eficiência foi avaliada contando as células viáveis e medindo a atividade como a fixação do azoto. Os resultados mostraram que a melhor técnica foi a liofilização seguida de imobilização e criopreservação. A liofilização manteve inalterada a capacidade da bactéria para fixar o azoto, e o melhor resultado foi observado quando se usou S/BSA como agente protetor. A criopreservação resultou em uma perda dramática de viabilidade para C26 (TSB aprox. 70%.), enquanto que C27 foi bem preservada. A fixação de azoto foi significativamente diminuída para ambas as estirpes, independentemente do agente crioprotector utilizado ($P < 0.05$). Em conclusão, os resultados sugerem que o êxito dos métodos de conservação de *Azotobacter* dependem da técnica, do agente de proteção, e da estirpe utilizada, sendo a liofilizacao com S/BSA a melhor técnica para preservar as bactérias deste género.

**Palavras-Chave:** Azotobacter; preservação de bacterias; criopreservação; liofilização; imobilização em polímeros; fixação de nitrogénio bacteriano.