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LONG TERM CLASSICAL METHODS FOR THE PRESERVATION OF XANTHAN GUM-PRODUCING - *Xanthomonas arboricola* pathovar pruni STRAINS

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Abstract

The maintenance of viable and stable *Xanthomonas* cells is crucial for the xanthan reliable research and industrial production. The method, storage and recovery conditions should preserve both viability and phenotypical and genotypical features. Here, the effectiveness classical methods on the long-term preservation of different *Xanthomonas arboricola* pathovar pruni strains was to determine. Strains were preserved by monthly sub-culturing in solid medium and lyophilization. After 12 years the viability of the strains, was assessed, as well as their productive capacity and the viscosity of the xanthan gum produced by these strains kept by lyophilization and sub-culturing. Among the lyophilized strains, only those stored at -18 °C were viable after 12 years. The productive capacity of the strains were poorly affected by lyophilization, the passage of the cultures into a solid nutrition medium being sufficient for them to return to their normal metabolism. The viscosity of the synthesized xanthan gum was method-dependent and higher for the lyophilized strains. The work and its findings are new and original because a work on this topic has never been published before. The results obtained allow the breaking of paradigms regarding the preservation of *Xanthomonas*.

Keywords: cell viability; lyophilization; monthly sub-culturing; production; quality.

MÉTODOS CLÁSSICOS DE LONGO PRAZO PARA PRESERVAÇÃO E PRODUÇÃO DE GOMA XANTANA POR CEPAS DE *Xanthomonas arboricola* patovar pruni

Resumo

A manutenção de células de *Xanthomonas* viáveis e estáveis é crucial para se obter uma pesquisa confiável e para a produção de xantana industrial. O método, o armazenamento e as condições de recuperação devem preservar tanto a viabilidade quanto as características fenotípicas e genotípicas. O objetivo do estudo foi determinar a eficácia dos métodos clássicos na preservação a longo prazo de diferentes cepas de *Xanthomonas arboricola* patovar pruni. As cepas foram preservadas por

subcultivo mensal em meio sólido e liofilização. Após 12 anos, avaliou-se a viabilidade das linhagens, bem como a capacidade produtiva e a viscosidade da goma xantana produzida por essas linhagens mantidas por liofilização e subcultivo. Entre as cepas liofilizadas, somente foram viáveis, após 12 anos, as armazenadas a -18 °C. A capacidade produtiva das cepas foi pouco afetada pela liofilização, sendo suficiente a passagem das culturas para um meio de cultivo sólido para que elas voltassem ao seu metabolismo normal. A viscosidade da goma xantana sintetizada foi dependente do método e maior para as cepas liofilizadas. O estudo e suas descobertas são novos e originais porque um trabalho sobre este tópico nunca foi publicado antes. Os resultados obtidos permitem quebrar paradigmas quanto à preservação de *Xanthomonas*.

Palavras-chave: liofilização; produção; qualidade; subcultivo mensal; viabilidade celular.

Introduction

Xanthan gum, a microbial biopolymer, is the object of huge interest for the food, pharmaceutical, chemical and petrochemical fields because, like other conventional vegetable gums, of its ability to form viscous solutions and gels in an aqueous medium, but of higher stability (KUMAR *et al.*, 2017; PEREZ *et al.*, 2020). The wide industrial applicability of xanthan and its large global market are stimulating researchers to develop studies on the most favorable *Xanthomonas* cell multiplication, as well as production, recovery and purification parameters for this exopolysaccharide. Besides, researches on its properties are designed to obtain the best possible yield and rheological quality ratio for the synthesized product (SILMAN; ROGOVIN, 1970; CASAS *et al.*, 2000; ANTUNES *et al.*, 2003; BORGES *et al.*, 2009; PEREZ *et al.*, 2020).

However, xanthan gums of unsuitable rheological properties, low yield and consequently high production cost can be synthesized through substrains obtained by the continuous sub-culturing of the bacterium during the inoculum production step (RAMÍREZ *et al.*, 1988). Researches report that successive sub-culturing steps of the bacterium increase the probability of spontaneous, statistical mutations (KIDBY *et al.*, 1977). In this way, the use of *Xanthomonas* inocula adapted to the medium, stable and free from unproductive substrains becomes crucial for preserving the productivity of the bioprocess.

Microrganisms preservation is an essential practice for the development of industrial researches and bioprocesses. Thus, implementation and maintenance of cultures collections enable the formation of stocks of cell lines that can be experimentally used at different situations (BROECKX *et al.*, 2016; NASRAN *et al.*, 2020).

The maintenance of viable and stable *Xanthomonas* cells is one of the priorities of the researches associated with xanthan gum. In spite of the relevance of the subject, the known

procedures for the preservation of this bacterium and the reports on the quantitative performance of the methods employed for the conservation of this microrganisms are scarce in the literature. Galindo *et al.* (1994) described a conservation technique for *Xanthomonas* in inclined tubes in solid YM medium kept at 4 °C, with a monthly sub-culturing for 11 months. Many researches employ the periodical sub-culturing method in plates for keeping *Xanthomonas;* according to this method the bacteria are preserved for 30 days stored at a temperature of approximately 4 °C (MOREIRA *et al.*, 2001; ROTTAVA *et al.*, 2009; SILVA *et al.*, 2017). Borowski *et al.* (2021) assessed different alternative techniques for the preservation of *Xanthomonas* by using inert supports (sterile soil and glass beads) and sunflower seeds.

Methods requiring more sophisticated equipment, such as ultrafreezing (-80 °C) and lyophilization, now being considered as "classical", are in practice the most utilized nowadays. However, there are few studies published on this subject, among which (GOLOVACH; GROMA, 2013; SILVA *et al.*, 2017) with the respective results cited in the Results and Discussion Section.

It is well-known that the storage, cultivation and recovery conditions of the microbial cultures should preserve the microbiological homogeneity, phenotypical and genotypical features to secure better reproducibility of the product manufacture and quality (Silva *et al.*, 2017). Thus, this work aims at determining the effectiveness of the classical lyophilization and monthly sub-culturing preservation methods, after a long preservation period – 12 years – for maintaining high viability, productive capacity and viscosity of the xanthan gum produced by *X. arboricola* pathovar (pv) pruni 24, 46 and 82 strains.

Materials and Methods

Materials

Microrganisms

For this study three X. *arboricola* pv pruni strains, numbered 24, 46 and 82 were used. The bacteria stock was kept under lyophilization (ambient temperature and -18 °C) and by monthly periodical sub-culturing (4 °C) in solid SPA medium (Hayward, 1964), stored for 12 years.

Reagents

The following reagents were used: yeast extract (Himedia®, Mumbai/India), malt extract (Himedia®, Mumbai/India), anhydrous glucose analytical grade (Synth®, Diadema/Brazil), peptone (Himedia®, Mumbai/India), analytical grade sucrose (Synth®, Diadema/Brazil), analytical grade anhydrous dibasic potassium phosphate (Synth®, Diadema/Brazil), analytical grade heptahydrated magnesium sulfate (Synth®, Diadema/Brazil), analytical grade monobasic ammonium phosphate (Synth®, Diadema/Brazil).

Equipment

For performing the experiments the following equipment were used: orbital shaking incubator with temperature control (B. Braun Biotech International®, model Certomat BS-1, Melsungen/Germany), analytical scale (Quimis®, model BG 400, Diadema/Brazil), B.O.D.-type culture oven (Eletrolab®, model EL101, São Paulo/Brazil), sterilization oven (Olidef CZ®, Ribeirão Preto/Brazil), drying oven (Fabbe®, São Paulo/Brazil), vertical autoclave (Prismatec®, model CS, Porto Alegre/Brazil), laminar flow cabin (Veco®, Campinas/Brazil) with ultraviolet lamp (UV-C, 253,7nm,), rheometer (HAAKE®, model RS150, Vreden/Germany), Bunsen burner and general glassware.

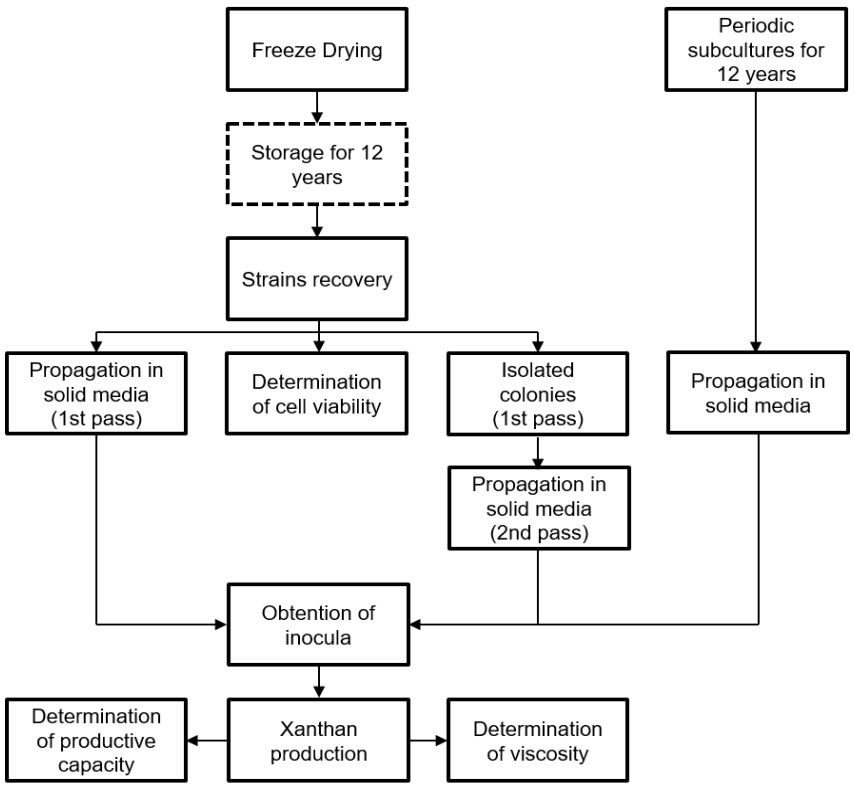
Methods

Assessment of classical methods used for preservation of *X. arboricola* pv pruni

The effectiveness of the lyophilization and periodical sub-culturing was evaluated relative to the viability, productive capacity and viscosity obtained from the *X. arboricola* pv pruni 24, 46 and 82 strains.

The flowsheet below represents the structure of this experiment (Figure 1).

Figure 1. Flowsheet of the preservation and recovery process of the *X. arboricola* pv pruni 24, 46 and 82 strains and characterization of the obtained xanthan gum.



Preservation by lyophilization

Lyophilization and storage

Inocula were prepared by Moreira (2010) in 1999 based on isolated *X. arboricola* pv pruni colonies of the 24, 46 and 82 strains, in agreement with the contents of the WO2006047845 (VENDRUSCOLO *et al.*, 2006), International publication, containing 1.0×10^9, 1.4×10^9 and 1.2×10^9 $CFU.mL^{-1}$, respectively. The inocula were diluted with cryoprotector in the amount of 60:40 (v/v), totaling a 2 mL volume, and then placed in sterile penicillin flasks. After being lyophilized they were stored at -18 °C and at ambient temperature. The cryoprotector comprised triptone, mannitol, monosodium glutamate, gelatin, monobasic sodium phosphate and dibasic sodium phosphate, according to Ellner (1978).

Reactivation and assessment of cellular viability

The cultures of *X. arboricola*, lyophilized 12 years ago by the Biopolymers Laboratory and stored in the microrganisms bank were reactivated by reconstitution (rehydration) in a YM liquid medium (JEANES, 1974) for 20 minutes, in accordance with Baiocco (1997).

To assess the viability of bacteria, 100 μL of this suspension were withdrawn and decimal dilutions up to 10^{-5} were prepared, followed by plaquing of these dilutions in SPA solid medium (HAYWARD, 1964), and incubation in a culture oven. Then, the *X. arboricola* typical colonies were counted and the result was expressed in colonies forming units per milliliters ($CFU.mL^{-1}$) of rehydrated bacterial suspension.

Cell propagation in solid medium from the reactivated bacterial suspension

With the aid of a *bacteriological loop*, 100 μL of the reactivated bacterial suspension were spread on plates containing SPA solid medium (HAYWARD, 1964). Plates were incubated at the temperature of 28 °C for 72 h. The plates where the respective *X. arboricola* pv pruni strains were growing were employed for producing inocula, and later on to produce xanthan.

Cell propagation from recovered isolated colonies in solid medium

In a first passage, by depleting, a loop of recovered bacterial suspension was plated (item "Reactivation and assessment of cellular viability") in SPA solid medium (HAYWARD, 1964). After incubation, the grown colonies were applied to a new propagation in SPA solid medium, assigned as 2nd passage, and later obtention of inoculum for xanthan production (VENDRUSCOLO *et al.*, 2006).

Preservation by monthly sub-cultures

Monthly sub-cultures

Monthly sub-cultures were obtained, systematically, from isolated colonies of *X. arboricola*, in SPA solid medium (Hayward, 1964) followed by incubation up to the growth of yellowish, mucoid, brilliant and round, typical of *Xanthomonas*. Each sub-culture was stored under refrigeration (4 °C) up to the following transfer to a fresh medium-containing plate.

Cell propagation from isolated colonies in solid medium

The isolated colonies were used for new growth in SPA solid medium (HAYWARD, 1964) and later production of inocula and xanthan (VENDRUSCOLO *et al.*, 2006).

Production of xanthan and assessment of productive capacity

Xanthan production was performed in two phases, in accordance with the published international application WO2006047845 (VENDRUSCOLO *et al.*, 2006): the first one for cell multiplication (production of inoculum) and the second one for xanthan production. Inoculum was produced with the aid of the SPA medium (HAYWARD, 1964) in 250 mL-capacitiy Erlenmeyer flasks containing the total volume of 50 mL, in an orbital agitated shaker at 28 °C under 150 rpm agitation for 24 h. For producing xanthan, MPII mineral medium (VENDRUSCOLO *et al.*, 2006) was used, added of saccharose (10%) and inocula (10%) in 250 mL-capacitiy Erlenmeyer flasks containing the total volume of 50 mL, in an orbital agitated shaker at 28 °C under 200 rpm agitation for 24 h. At the end of the process xanthan was recovered by insolubilization by addition of ethanol 96 °GL to the fermented broth in the proportion 4:1 alcohol:broth (v/v). The precipitated polymer was oven-dried at 56 °C to constant weight, ground and stored in capped recipients. The xanthan productive capacity of these strains was assessed with the aid of a gravimetric method and the result was expressed in $g.L^{-1}$.

During inoculum production the concentration of bacteria was assessed at the times of 0 and 24 h incubation through serial decimal dilutions followed by plating in SPA solid medium (Hayward, 1964) and incubation at 28 °C for 48 h. The assessment of the initial (0 h) cell concentration in the inoculated fermentation medium for xanthan production was obtained by dilution calculus, considering the inoculum volume and the final volume.

Viscosity

Xanthan solutions 3% (w/v) were prepared by hydrating the polymer in ultrapure water and moderate agitation in a magnetic stirrer at ambient temperature for 2 h. The solutions were then submitted to heating at 60 ºC in a water bath for 20 min (XUEWU *et al.*, 1996). The samples were

left to rest at 4 ºC for 24 h prior to analyses. The viscosity of the solutions was determined with the aid of a rheometer, in a rotating modulus, at 25 °C. A plate-plate system, PP35TI sensor and deformation rate 0.01 to 100 s^{-1} was used, with acquisition of 100 points.

Statistical analysis

All experiments were carried out in triplicate and were submitted to analysis of variance, the comparison of means being obtained by the Tukey test with 5% significance.

Results and Discussion

Cellular viability

A few strains studied in this work are being kept under refrigeration by monthly successive sub-culturing in solid medium-containing plates from clone colonies for more than 20 years, which shows the effectiveness of the technique for the pathovar pruni in terms of viable cells maintenance. In this study, however, the results in this study compared data obtained in a 12-year interval.

Before lyophilization, the *X. arboricola* strains assessed in this study were preserved exclusively by periodic sub-culturing in the YM (Yeast Malt) solid medium proposed by Haynes, Wickerham and Hesseltine in 1955 (VENDRUSCOLO *et al.*, 2000). This is a complex medium, providing three different sources of organic nitrogen and being widely employed in microbiology, including for obtaining inoculum for xanthan production (GARCÍA-OCHOA *et al.*, 2000). According to Sutherland (1993), the *Xanthomonas* colonies are normally mucoid, shiny and roundish. However, Moreira (2010) observed that it was not rare that during sub-culturing atypical colonies appeared, non shiny and of dry aspect, of irregular shape, such colonies being named “curled”. The identity of these colonies was confirmed by biochemical tests and by the xanthan biopolymer production capacity in liquid medium, which was not altered, as well as by the quality of the obtained polymer. However, in order to avoid possible culture losses the cultures were lyophilized. Tessman (2002) introduced the use of the SPA (Sucrose Peptone Agar) medium, suggested by Hayward (1964), for sub-culturing. It could be observed that the colonies sub-cultured in this medium were larger, of lighter color and higher viability, the sub-culturing occurring in intervals of up to two months. Thus, the monthly sub-culturings for the preservation of the *Xanthomonas* strains kept in the Laboratory for Biopolymers were systematically performed in SPA solid medium (MOREIRA, 2010).

As regards preservation by lyophilization, all the lyophilized samples were viable after 12 years storage at -18 °C. The lyophilized samples stored at ambient temperature were completely non viable when tested after the same period of time. The results obtained for the frozen stored lyophilized samples are listed in Table 1.

Table 1. Cellular concentration and percent survival of cells preserved by lyophilization stored at -18 °C for 12 years.

Strain	Initial Cellular Concentration ($CFU.mL^{-1}$)	Cellular concentration ($CFU.mL^{-1}$) after 12 years	Survival Percentage (%) after 12 years storage
24	6.0×10^{8}	8.7×10^{7}	14.5^{B}
46	8.4×10^{8}	2.6×10^{5}	0.031^{C}
82	7.2×10^{8}	6.2×10^{8}	86.1^{A}

Different capital letters (column) indicate statistical differences among strains with the aid of the Tukey test for a 5% probability level.

Strain 82 exhibited higher resistance to the lyophilization technique conditions and storage, the reduction in cellular concentration being of 13.9% only. On the contrary, strain 46 had approximately 99% celllular death, which does not impair the technique in view of the remaining high number of viable cells. These results do not seem to be related to the productive capacity or the quality of the xanthan biopolymer obtained. It is well-known that polysaccharide polymers have a protective action on microrganisms, both in the lyophilization process and the cells rehydration (BAIOCCO, 1997). However, in the case of the tested strains, a higher productive (which can occur already during the cellular growth phase) or viscosifying capacity of the obtained polymer does not seem to have been the cause of the higher survival index, because strain 46, for which median production and viscosity were assessed, as compared with the other strains, had the lowest cell survival index.

Survival during lyophilization and storage depend, mainly, on the microrganisms characteristics, the concentration of the lyophilized culture, the cellular multiplication means and the rehydration, the cryoprotector agent, the storage temperature and the recovery conditions of the lyophilized material (HECKLY, 1961; BAIOCCO, 1997). Among these factors, the only altered factor was the microorganism. Thus, it can be stated that the cellular viability of *X. arboricola* pv pruni was strain-dependent. Even the *Xanthomonas* genome being characterized by high stability, which eases its conservation (MARTÍNEZ-SALAZAR *et al.*, 1993), it was observed that phenotypical variations can occur for strains of the same species, chiefly as relates to the quantity and quality of the obtained xanthan (NITSCHKE; THOMAS, 1995; MOREIRA *et al.*, 2001; ANTUNES *et al.*, 2003; BORGES *et al.*, 2009), colony pathogenicity, size and color (KAMOUN; KADO, 1990; NITSCHKE; THOMAS, 1995; MOREIRA, 2010). This demonstration of the occurrence of several variations among strains corroborates the determination that the bacterial survival was strain-dependent.

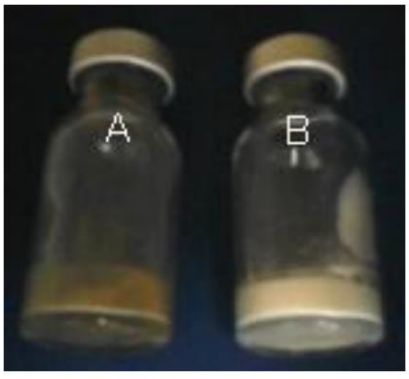
Borowski *et al.* (2021) assessed the efficiency of alternative preservation techniques for the same strains (24, 46 and 82) of *X. arboricola* pv pruni of the present work. The strains stored by the conservation methods in sunflower seeds, glass beads and sterile soil were viable for 30 storage days. The survival of cells varied between 0.001 and 0.071% and was a function of the preservation technique, the storage duration and the strains.

In this study the remaining factors affecting the microrganisms survival after lyophilization were adjusted to contribute to cell maintenance. The literature cites the YM medium as the most suitable for *Xanthomonas* (HAYNES, 1955; GARCÍA-OCHOA *et al.*, 2000; VENDRUSCOLO *et al.*, 2006) cultivation; that is why it was employed for multiplying the bacteria prior to lyophilization, and also for rehydration (reactivation) of the lyophilized material.

Baiocco (1997) also employed the YM medium for the cellular multiplication of *X. campestris* pv manihotis followed by suspension of the cells to be lyophilized. However, for recovery, YM added of 10% reconstituted skimmed milk was used as suspension medium; under these conditions, bacterial cells exhibited 80% survival after 12 months lyophilization, a figure similar to that of the *X. arboricola* pv pruni strain 82, nearly 86% after 12 years preservation. According to the author, the proteins, the milk whey and the lactose present in skimmed milk provide a protective matrix for the cellular macromolecules during rehydration. Among the remaining tested rehydrating products, such as glucose 1.0%, sodium chloride 0.9% and distilled water, the observed recovery indexes were approximately 20, 14 and 10% respectively. The author further considered that the best temperature for the storage of the lyophilized strain, no matter the growth and suspension media, was a temperature below freezing.

In this study a similar result was observed since the storage of lyophilized cultures at ambient temperature impaired the survival of the three strains, causing cellular death after 12 years. Figure 2 shows the difference in color between lyophilized cultures stored at ambient temperature, caramel, and at -18 °C, cream.

Figure 2. Flasks containing lyophilized cells of *X. arboricola* pv pruni stored at ambient temperature (A) and stored at -18 °C (B). Source: Authors.



Addition of 40% cryoprotector to the strain suspension medium prior to lyophilization and cultures storage at -18 °C provided further conditions for keeping the lyophilized bacteria viable. Suitable cryoprotector agents considerably enhance survival because of the protection to the microbial cell against the deleterious effects of the freezing and rehydration steps (HUBALEK, 2003). Ndoye *et al.* (2007) lyophilized thermoresistant acetic bacteria using mannitol as cryprotector and observed that cultures maintained at 4 °C were kept viable for at least 6 months. For the authors, the main reason for the success of lyophilization was addition of the cryoprotector, which tends to reduce the activity of water, besides low-temperature storage.

Golovach and Groma (2013) assessed the preservation of *X. campestris* pv campestris IMV B-7001 by lyophilization and ultrafreezing. The maintenance of the microrganisms viability and their morphological properties as well as the xanthan production capacity during a period of 2-8 years preservation at the temperature of -70 °C was observed. With lyophilization followed by storage at -135 °C in excess of 30 years the same results could be observed. The authors also assessed different cryoprotectors and the same are disposed in a sequence according to their efficiency: 10% saccharose + 1% gelatin > 10% glycerin > 5% dimethyl sulfoxide > 4% ethylene glycol.

Silva *et al.* (2017) determined the efficiency of ultrafreezing (-80 °C) and refrigeration (3 – 8 °C) during 5 months storage of *X. campestris* pv mangiferaeindicae IBSBF 2103 through the cellular fatty acids composition, genetic profile and biochemical characteristics. The authors concluded that the cells preserved at -80 °C had less pronounced phenotypic variations, which positively influenced the qualitative and quantitative characteristics of the obtained xanthan gum.

Another determining factor in the successful preservation by lyophilization is the rehydration time at the reactivation or cellular recovery moment. Baiocco (1997) hás shown that, for *X. campestres* pv manihotis lyophilizates, the rehydration time has also influenced the obtained results. The cellular survival rate was higher when the lyophilized culture was quickly rehydrated for 10 or 20 minutes before sub-culturing in solid medium. A time lower than 5 min, as well as a time superior to 30 minutes led to reduced number of recovered colonies, the 30 min-time being still more harmful. From 60 min rehydration on, the number of recovered colonies was reduced in more than 60% based on that obtained from the ideal interval time. Therefore, excessively short or long rehydration can lead to falsely inferior results, caused by inefficient reactivation and very probably by anoxia, *Xanthomonas* being strictly aerobic bacteria (SADDLER; BRADBURY, 2005). In view of this, the rehydration time of 20 minutes in YM medium was chosen for this study.

Mainly, strain 46 low survival percentage after lyophilization can be attributed to the specific features of this cell line, which exhibited higher sensitivity to the lyophilization process. Normally, deleterious effects are observed in the initial microrganisms population of a lyophilized

sample, since they are prone to damage and injuries caused by the freezing, dehydration and drying steps that make up the technique (HECKLY, 1961; CASTRO *et al.*, 1997). Changes in the microrganisms cell wall permeability, DNA and RNA molecules break and damage to the bacterial membranes, leading to loss of surface proteins and cytoplasmatic content, are the main factors responsible for viability reduction after lyophilization (BRENNAN *et al.*, 1986; WANG *et al.*, 2009). However, injury represents a temporary condition of the lyophilized cells, which upon recovering their natural physiological state, resume normal multiplication and no longer show any feature enabling to distinguish them from the original cells (BEUCHAT, 1978; BAIOCCO, 1997). Aiming at checking any damages in the reproductive capacity of lyophilized-preserved cells, we have proceeded with the comparison between the initial concentration, at zero incubation time, and the final concentration, at 24 h cultivation, of cells of the different preserved strains by the conventional methods assessed in this study, grown in YM culture medium under predetermined conditions for obtaining inoculum. The results are listed in Table 2.

Table 2. *X. arboricola* pv pruni cell grown in 24 h cultivation in YM liquid medium at 28 °C and under 150 rpm agitation.

Strain	Preservation technique	Initial cellular concentration ($CFU.mL^{-1}$)	Final cellular concentration ($CFU.mL^{-1}$)	Cellular multiplication factor (%)
24	(A)	$1.9 \times 10^{9\ Aa}$	$6.4 \times 10^{9\ Aa}$	337
	(B)	$2.0 \times 10^{9\ Aa}$	$4.9 \times 10^{9\ Aa}$	245
	(C)	$9.0 \times 10^{8\ Bb}$	$7.0 \times 10^{9\ Aa}$	778
46	(A)	$6.0 \times 10^{8\ Ba}$	$8.8 \times 10^{8\ Ba}$	147
	(B)	$6.4 \times 10^{8\ Bb}$	$1.3 \times 10^{9\ Aa}$	203
	(C)	$7.0 \times 10^{8\ Bb}$	$4.0 \times 10^{9\ Aa}$	571
82	(A)	$1.6 \times 10^{8\ Bb}$	$5.0 \times 10^{9\ Aa}$	3,125
	(B)	$5.0 \times 10^{8\ Bb}$	$3.0 \times 10^{9\ Aa}$	600
	(C)	$6.0 \times 10^{7\ Cb}$	$2.8 \times 10^{9\ Aa}$	4,667

(A) Lyophilization. (B) Lyophilization and one growth step in SPA solid medium – reactivated lyophilized cells and in sequence, submitted to two growing steps or passages in solid SPA medium. (C) Monthly sub-culturing in SPA solid medium. Different capital letters (column) indicate statistical differences among strains for the different methods; different lowercase letters (line) indicate statistical differences between cellular concentrations for the same strain, with the aid of the Tukey test for a 5% probability level.

As observed for strain 46, even after a reduction of more than 99% in its cellular concentration (Table 1), the microorganism exhibited considerable growth in YM liquid medium after 24 h. Besides, when strain 46 was recovered and passed twice through SPA solid medium, the multiplying capacity of the cells in YM liquid medium in a 24 h period was considerably increased

(Table 2). The results corroborate that the cellular injury caused by the lyophilization technique is temporary, and that after the readaptation of the recovered cultures in a nutrient medium they return to their normal development. Strain 82 had the same behavior as that of the strain 46. Strain 24, however, in spite of exhibiting significant multiplication after 24 h culture in YM, as the two other tested strains, demonstrated its readaptation from the first passage in the nutrient medium, since by the second passage in the solid culture medium its liquid medium cell growth in 24 h was not significantly superior to that occurred after one passage in solid medium.

Xanthan Production

When the efficiency of a *Xanthomonas* preservation method is assessed, not only the survival of cells during the storage period, but also its xanthan productive capacity should be considered.

In this study xanthan production was dependent on the strain employed in the fermentation process and on the technique by which the cell line was preserved, as shown in Table 3.

Table 3. Xanthan production ($g.L^{-1}$) by cells preserved by conventional techniques, compared with previous values (control).

Strain	*Control production ($g.L^{-1}$)	Preservation Technique	**Initial cellular concentration ($CFU.mL^{-1}$)	Xanthan production ($g.L^{-1}$)	***Variation in productive capacity (%)
24	5.6	(A)	9.0×10^8	5.8^b	+4
		(B)	6.8×10^8	6.5^a	+16
		(C)	5.3×10^8	5.1^c	-9
46	5.1	(A)	1.2×10^8	6.4^b	+26
		(B)	1.8×10^8	6.0^c	+18
		(C)	5.6×10^8	7.6^a	+49
82	4.2	(A)	7.0×10^8	3.6^c	-14
		(B)	1.6×10^8	7.6^a	+81
		(C)	3.9×10^8	6.2^b	+48

*Control: Xanthan production ($g.L^{-1}$) by strains preserved by monthly sub-culturing in YM solid medium prior to lyophilization. **Initial cellular concentration ($CFU.mL^{-1}$) in the xanthan producing fermentation medium. ***Positive values (+) correspond to increased productive capacity relative to the control; negative values (-) correspond to viscosity reduction. (A) Lyophilization. (B) Lyophilization and one growth step in SPA solid medium – reactivated lyophilized cells and in sequence, submitted to two growing steps or passages in solid SPA medium. (C) Monthly sub-culturing in SPA solid medium. Different capital letters (column) indicate statistical differences with the aid of the Tukey test at a 5% probability significance level.

By comparing the xanthan yield ($g.L^{-1}$) before and after preservation by lyophilization, it could be observed that the strain 24 productive capacity was not affected by lyophilization followed

by refrigerated storage for 12 years, keeping its yield around 5.8 $g.L^{-1}$. On the other hand, xanthan production by strain 82 was reduced by approximately 14% after lyophilization.

In terms of xanthan production, for strains 46 and 82, the results obtained for the monthly sub-culturing in SPA solid medium technique at first seem to provide better results than lyophilization. However, for strain 82, lyophilization followed by culture recovery and two passages by SPA solid medium led to a rise in production, demonstrating that the damages caused by lyophilization can be reversed after additional passage in a suitable solid medium. Thus, it is possible to state that for strain 46 the preservation by sub-culturing in SPA solid medium was more efficient, strain 82 productive capacity being better preserved by using the lyophilization technique, while for strain 24 both methods were suitable.

As discussed by García-Ochoa *et al.* (2000) and confirmed by other authors, xanthan production is strain-dependent (MOREIRA *et al.*, 2001; ANTUNES *et al.*, 2003). Rottava *et al.* (2009) determined xanthan production by *X. campestris* pv campestris strains 1167, 254 and 607 in an incubator stirrer and also observed that xanthan production depends on the cell line. The production values are similar to those of this work, varying from 5.9 to 7.5 $g.L^{-1}$.

Borowski *et al.* (2021) also studied the productive capacity of the same strains of this study (24, 46 and 82 strains of *X. arboricola* pv pruni) by comparing original values with those observed after preservation by alternative conservation methods in sunflower seeds, glass beads and sterile soil. Xanthan production varied between 5.0 and 7.8 $g.L^{-1}$ after 30 days storage, demonstrating to be strain-dependent, the highest yields being provided by strain 46 (7.2 to 7.8 $g.L^{-1}$) and the lowest by strain 24 (5.0 to 5.9 $g.L^{-1}$).

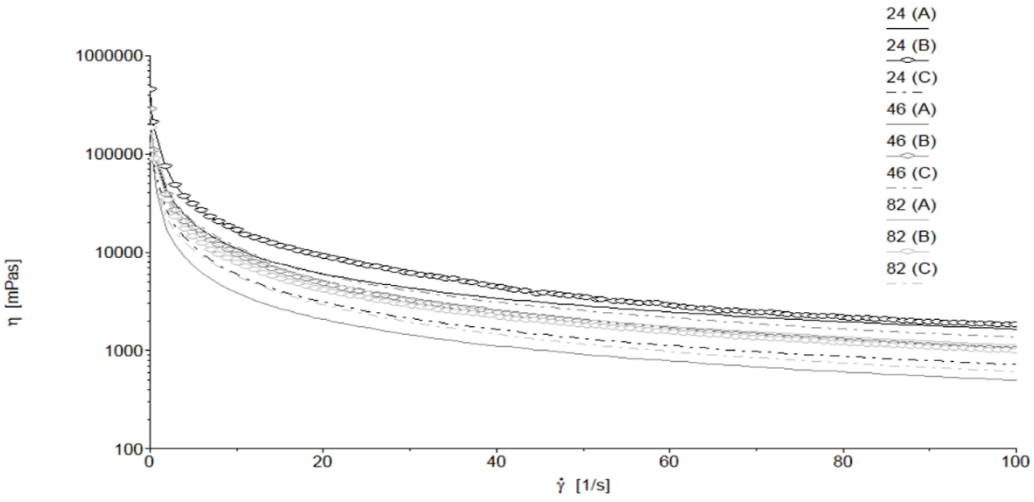
Literature cites that the initial inoculum concentration of the fermenting process influences the xanthan yield (GARCÍA-OCHOA *et al.*, 2000). According to Moreira (2010) for *X. arboricola* pv pruni, up to certain values, increased inoculum concentration cause increments, not always statistically significant, in xanthan production. However, there are concentration ranges that result in equivalent values. In this study, inoculum concentrations varied from 1.2×10^8 to 9.0×10^8 $CFU.mL^{-1}$, a difference that according to Moreira (2010) would not result into differentiated productions. As shown in Table 3, which compares the initial cell concentration in the fermenting medium and xanthan production as a function of the preservation method, lower initial concentrations can generate higher production, corroborating the influence of the preservation method not only on cellular viability but also on productive capacity.

Viscosity

The analysis of the viscosity of xanthan aqueous solutions was performed aiming at evaluating the influence of the different preservation methods on the quality of the produced

polymers. All the tested xanthans maintained the characteristic pseudoplastic behavior, that is, viscosity was reduced by applying increased shear rate (Figure 3). This behavior is isual for microbial polysaccharides solutions (CACIK *et al.*, 2001; RAO *et al.*, 2003) and also expected for xanthan in general terms (SANDFORD *et al.*, 1977; PEREZ *et al.*, 2020, KLAIC, 2016).

Figure 3. Viscosity (mPas) vs. shear rate (s^{-1}), at 25 °C for aqueous 3% (m/v) xanthan solutions produced by strains preserved by lyophilization (A), lyophilization and reactivation with two passages in SPA solid medium (B) and monthly sub-culturing in SPA solid medium (C).



Authors who studied the several *X. arboricola* pv pruni strains observed differences in the viscosity of obtained xanthans aqueous solutions, thus demonstrating that viscosity is strain-dependent (ANTUNES *et al.* 2003; MOREIRA *et al.*, 2001; KLAIC *et al.*, 2016). De Vuyst and Vermeire (1994) and Nitschke and Thomas (1995), working with pathovars, also observed that the xanthan rheological behavior is influenced by the genetic variation of a same pathovar strain.

Table 4 lists the apparent viscosity for aqueous 3% (m/v) xanthan solutions produced by strains preserved in accordance with the different methods, at a shear rate of 10 s^{-1}. Viscosity values varied between 3,635 and 16,399 mPas under the cited conditions.

Table 4. Viscosity at 25 °C and 10 s^{-1} of aqueous 3% (m/v) xanthan solutions produced by strains preserved in accordance with conventional methods compared with previous values (*control viscositiy).

Strains	Preservation Techniques	*Control viscosity (mPas)	Viscosity (mPas)	**Change in Viscosity (%)
24	(A)	4,500	9,911	+120
	(B)		16,399	+264
	(C)		5,451	+21
46	(A)	7,600	3,635	-52
	(B)		8,596	+13
	(C)		11,085	+46
82	(A)	11,000	7,790	-29
	(B)		9,700	-12
	(C)		5,873	-47

*Viscosity of aqueous 3% (m/v) xanthan solutions produced by strains preserved by monthly sub-culturing in YM solid medium, prior to lyophilization. Positive values (+) correspond to increased viscosity relative to the control; negative values (-) correspond to viscosity reduction. (A) Lyophilization. (B) Lyophilization and one growth step in SPA solid medium – reactivated lyophilized cells and in sequence, submitted to two growth steps or passages in SPA solid medium. (C) Monthly sub-culturing in SPA solid medium.

In this study the viscosity of aqueous xanthan solutions was dependent on the preservation method employed for the strain maintenance, and the strains responded in different ways to the tested methods. By individually examining the xanthan viscosity values it was observed that exception made to strain 82 that underwent reduction of approximately 47%, successive sub-culturing in SPA medium resulted in viscosity rise. As prior to lyophilization the sub-culturing steps were performed in YM medium, it is concluded that the SPA medium, in spite of favoring production, reduces the strain 82 viscosity. The lyophilization technique reduced viscosity to 3,635 mPas, approximately half the value of the strain 46 polymer prior to lyophilization, which was 7,600 mPa.s. For strain 82 a less significant reduction was observed, while strain 24, paradoxically, exhibited an unexpected rise of 121% in the produced polymer viscosity. For strains 46 and 24, which had augmented viscosity after sub-culturing in SPA nutrient medium, the additional passage in this medium induced a rise in viscosity, this behavior being attributed to the positive action of the SPA solid medium on the bacterium metabolism. Strain 82 viscosity, which did not show good adaptation to the SPA medium during the successive sub-culturing steps, was reduced after additional passage in SPA, confirming its better adaptation to the YM medium.

Conclusion

Classical preservation methods were suitable for the maintenance of *X. arboricola* pv pruni cells viability. Lyophilization is recommended for long period preservation, whenever the required equipment is available; however, when the cultures are frequently used, the use of periodical sub-culturing is more practical; in any case, the most suitable method for each strain should be previously determined. The viscosity of strains preserved by lyophilization was reduced but, as is for the productive capacity, it was recovered after passage in suitable nutrient medium. The productive capacity of the strains preserved by lyophilization, a dissecative method, was, in general terms, poorly affected, demonstrating that the passage of cultures in nutrient medium is sufficient for their return to their normal metabolism. The viscosity of the synthesized xanthans is strain-method-dependent. Finally, it is suggested to use more than one preservation technique for the maintenance of *Xanthomonas* pruni strains to avoid possible losses and changes, besides the periodical check of the cultivations viability.

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