Impact of Preservation Conditions on Fatty Acids, Xanthan Gum Production and Other Characteristics of *Xanthomonas campestris* pv. *mangiferaeindicae* IBSBF 2103

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Abstract The conditions of storage, cultivation and maintenance of microbial cultures should preserve the microbiological homogeneity, phenotypic and genotypic characteristics to ensure better reproducibility of metabolic production. To evaluate the influence of the storage condition on the composition of cell fatty acids, genetic profile and biochemical characteristics of *Xanthomonas campestris* pv. *mangiferaeindicae* IBSBF 2103, as well as, to identify its relationship with the yielding and viscosity of the xanthan gum produced, this study monitored the strain preserved in two simple and widely used conditions, ultra-freezer (−80 °C) and refrigeration (3–8 °C) during 5 months. Were identified and quantified 13 fatty acids. The cells preserved at −80 °C showed more stable concentration of all fatty acids, producing more xanthan gum and with higher viscosity. The chromosomal analysis obtained with the enzyme *Xba*I revealed 17 distinct fragments with maximum size of 485 kilobases, without variations among the subcultures maintained in both storage conditions. The *X. campestris* pv. *mangiferaeindicae* subcultures preserved at −80 °C showed less pronounced phenotypic variations, which had positive influence in the qualitative and quantitative characteristics of the xanthan gum produced.

Keywords Bacterial fatty acids · Biopolymers · Xanthan gum · *Xanthomonas campestris*

Introduction

Phytopathogenic bacteria from *Xanthomonas* genus are widely studied by its capacity to produce xanthan gum (XG) in large scale, a polysaccharide with great commercial value [1]. Several bacteria of this genus are able to produce this biopolymer, and the *campestris* species are the most used for this purpose [2, 3]. The XG is an heteropolysaccharide composed by glucose, mannose and glucuronic acid, that fulfill a variety of bacterial functions including adhesion, cell-to-cell interactions, biofilm formation and protection against environmental extremes [4, 5]. This polymer is widely used in the industries of food, pharmaceutical products and petroleum [2, 6].

The cell membrane of *X. campestris* is characterized by the presence of straight chain fatty acids (FA), *iso* and *anteiso*-branched chain, hydroxyl, monounsaturated and cyclopropane FA. The FA composition of bacterial cellular membrane has been extensively studied in order to correlate it with the microbial species or to monitoring changes in populations of microorganisms [7–9]. However, so far, studies correlating the lipid profile with the preservation techniques of this microorganism or with the production of biopolymers were not yet described by the literature.

The laboratory maintenance of bacteria that produce metabolites with commercial interest has become a constant challenge for the biotechnology. The preservation techniques should be practical and economical, as well as, maintain the viability and genetic stability of these microorganisms. The genetic instability of *Xanthomonas* was already noticed in some studies [10].

Cryopreservation at low temperatures (−80 or −196 °C) and lyophilization are the most commonly employed methods for long-term preservation of microbial cultures, promoting high genotypic and phenotypic

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stability [11]. The major advantage of lyophilization over cryopreservation methods is the low storage and transport costs. However it is a very complex physical process affected by many parameters requiring specific equipment and trained personnel [12].

This study was carried out in order to evaluate and correlate the impact of the time, temperature and subcultivation over FA composition, xanthan gum yielding and quality, biochemical behavior and on the genetic profile of the strain IBSBF 2103 of Xcm stored using two very simple and available methods, ultra-freezing at -80°C in Yeast Malt broth (YMB) with glycerol 10% (v/v), and refrigeration at $3-8^{\circ}\text{C}$ on YM agar (YMA) slants with subcultivation to fresh agar at each 30 days, along 5 months of storage.

Materials and methods

Microorganism

The strain of Xcm IBSBF 2103 (Biological Institute – IBSBF, Brazil) in YMB (Merck) with glycerol 10% ($5-6 \times 10^6$ cfu/ml) was maintained in cryogenic tubes (1.5 ml) at -80°C . In parallel, an amount of the recent propagated microorganism was inoculated in 3 tubes containing YMA (Merck) slant, which after propagation were stored under refrigeration ($3-8^{\circ}\text{C}$). During 5 months, at each 30 days, the microorganisms from both conditions were tested concerning the microbial homogeneity, genetic profile, biochemical behavior, cell membrane FA composition and production of XG. The cells preserved at $3-8^{\circ}\text{C}$ were monthly subcultivated to fresh YMA slant, in triplicate.

Biochemical tests

The biochemical characteristics of the subcultures were monitored using a miniaturized biochemistry method (Bactray®-Laborclin), containing conventional and chromogenic substrates. The bacterial suspension (1 ml), adjusted at 0.5 MacFarland level was transferred to the biochemistry panel lids and incubated at 35°C , 24 h. The visual interpretation of the reactions was done according the manufacturer instructions.

Bacterial fatty acids analysis

The extraction and esterification of the cell lipids was performed using the protocol described by Sasser [13] for identification of pure microbial cultures. The FA methyl esters (FAME) were analyzed by gas chromatography coupled to flame ionization detector (Varian, CP 3800) and

mass ionization detector (Perkin Elmer, Claurus 500) using a CP-WAX 58 ($25\text{ m} \times 0.25\text{ mm} \times 0.2\text{ }\mu\text{m}$) capillary column at initial temperature of 150°C (16 min), increased to 180°C at a rate of $2^{\circ}\text{C}/\text{min}$ holding this temperature during 20 min, with final isothermal hold at 230°C . The chromatographic parameters were inlet settings 250°C , split ratio 1:100, flow 1.3 ml/min of He, and 30 ml/min of H_2 , 300 ml/min of air, 30 ml/min for make-up (N_2) for FID. Injection volume of 1 μl , in triplicate.

Monthly, the FA were identified by comparing the retention times (RT) against the bacterial acid methyl ester mix standard—BAME 47080-U (Supelco) and confirmed by GC–MS analysis. The main FAME were quantified by normalization of chromatographic areas and the results expressed in relative area percent [9].

Genomic fingerprint

The genomic DNA of the Xcm 2103 was extracted according the protocol previously published [14] and adapted as follow. Recent colonies from both preservation conditions were harvested and transferred to 1 ml sterile saline solution to obtain a suspension (OD 0.5–0.6 at 450 nm). After centrifugation, the cell pellet was resuspended in 300 μl buffer (1 M NaCl, 10 mM tris–HCl, pH 7.6) containing 4 μl lysozyme (25 mg/ml), following the addition of 300 μl of 2% agarose gel and cooling at 0°C for 10 min to obtain the plug. Each plug was transferred to 2 ml EC buffer (6 mM Tris–HCl, 1 M NaCl, 100 mM EDTA, 0.5% Brij-58, 0.2% sodium deoxycholate, 0.5% lauroylsarcosine sodium) with 40 μl of lysozyme (25 mg/ml) and 5 U/ml of de mutanolysin. After incubation (37°C , 2 h) plugs were washed twice in TE buffer (10 mM tris, 1 mM EDTA, pH 8.0) at 37°C , 30 min, and incubated in ES buffer (93.1 g EDTA, 0.5 g lauroylsarcosine sodium, 0.1 mg/ml proteinase K, 500 ml ultrapure water) during 12 h, 55°C . After washed with TE buffer (6 to 8 times, 1 h), the agarose plug was transferred to the solution containing *Xba*I restriction enzyme (20 U per plug) and incubated at 37°C , 3 h. The electrophoretic separation was performed in 1.2% agarose gel with TBE running buffer $0.5\times$ (1 M tris, 0.9 M boric acid, 0.01 M EDTA) over 23 h, 14°C , 6 V/cm with pulse of 2–30 s, revealed with ethidium bromide.

Yielding and rheology of xanthan gum

A volume of 10 ml of YMB containing $\sim 10^{11}$ cfu/ml of Xcm 2103 previously incubated during 24 h, 28°C in shaker, 180 rpm, was transferred to 250 ml flask filled with 80 ml of substrate (2.0% sucrose, 0.01% urea and 0.1% KH_2PO_4). The fermentation was performed in shaker, 250 rpm, 28°C , 120 h. The fermentation broth containing

XG was centrifuged 15 min, 10 °C, 18,800×g (Hitachi, CR-GIII), the cells were removed and the gum was precipitated by adding acetone (3:1), dried at 35 ± 2 °C, 24 h, finely powdered and weighted to determine yielding and apparent viscosity.

The apparent viscosity (mPa s) of the XG produced from both conditions of preservation was evaluated using a Haake Rheotest with concentric cylinder device at shear rates from 25 to 1000 s⁻¹, 25 °C [15]. The 25 ml XG suspensions in distilled water (0.5% w/v) were homogenized with magnetic stirrer at 25 °C, 40 min. The suspension was refrigerated for 12 h before the viscosity determination. The effect of shear rate on the viscosity was calculated according the Ostwald-de-Waele kinetic model.

Results and discussion

Physiological and biochemical profile

During the 5 months the microbial purity of the stored cells was confirmed by the observation of the morphological aspects of the colonies grown on YMA. Like other *mangiferaeindicae* subspecies, Xcm showed regular rounded and convex colonies, with almost white, glossy and mucoid aspect. Similar morphological characteristics were also described in a study with 68 *Xanthomonas* sp. *mangiferaeindicae* strains, all of them were Gram-negative [16] (Table 1).

A minimal biochemistry profile of the Xcm 2103 strain, generated from 7 enzymatic reactions and specific substrates tested as sole carbon source is presented in the Table 1. Other six carbon substrates reactions (Malonate, Rhamnose, Adonitol, Salicin, Inositol and Raffinose—not showed), were also negative in both preservation conditions along the study. The results showed a regular physiological and biochemical profile of Xcm in both preservation conditions. The only exception was observed at times T4 and T5 when the cells stored at 3–8 °C presented results that, under visual inspection, changed from positive to slightly positive for esculin and sucrose tests, and from slightly positive to negative for sorbitol and mannitol carbon sources. Different from the cells preserved under refrigeration, the strain of Xcm maintained at –80 °C showed a more regular physiological and biochemical profile.

Galindo et al. [10] evaluated the *X. campestris* NRRL B-1459 together the variant E2 preserved on YMA slant at 4 °C, transferred monthly during 11 months and observed that the differences between XG batches were more likely associated with the variations on the fermentation kinetics rather than being inherent to the strains. On the other side, it is already known that successive subculture steps may cause phenotypic and/or genotypic alterations, impacting on pathogenic or virulence activity or even modifications on its ability to metabolize substrates [17–19].

With exception of the hydrolysis of esculin, the Xcm 2103 has no enzymes that hydrolyze other tested substrates (Table 1). This biochemical profile was very similar to that

Table 1 Morphological and biochemical profile of Xcm 2103

Test or characteristic	T0	T1		T2		T3		T4		T5		Pruvost [16]
		–80 °C	3–8 °C	–80 °C	3–8 °C	–80 °C	3–8 °C	–80 °C	3–8 °C	–80 °C	3–8 °C	
Gram						Negative						Negative
Colony pigmentation						Not pigmented						Not pigmented
Growth on cetrimide						Negative						NA
ONPG						Negative						NA
H ₂ S						Negative						NA
Esculin				Positive				+	±	+	±	Positive
Citrate						Negative						NA
Maltose						Negative						+/-
Arabinose						Positive						Negative
Acetamide						Positive						Negative
Sorbitol				Slightly positive				±	–	±	–	Negative
Sucrose				Positive				+	±	+	±	Positive
Mannitol				Slightly positive				±	–	±	–	+/-

±, slightly positive; +, positive; –, negative; +/-, variation between strains; NA, not analysed

from 68 strains of *X. campestris mangiferaeindicae* previously studied [16], as well as, to other 3 strains when tested for *L*-arginine, urease and indole hydrolysis [20]. According to the results, the Xcm 2103 utilizes arabinose, sucrose and acetamide as carbon sources, promoting media acidification.

Fatty acids profile

The qualitative profile of the FAME was very similar between the cells maintained in both conditions along the

5 months (Fig. 1). Using the BAME standard was possible to identify 13 FA which are 2-hydroxydecanoic (2-OH-C10:0); *iso*-pentadecanoic (*i*-C15:0); *anteiso*-pentadecanoic (*a*-C15:0); pentadecanoic (C15:0); *iso*-hexadecanoic (*i*-C16:0); palmitoleic (C16:1 ω 7); palmitic (C16:0); *iso*-heptadecanoic (*i*-C17:0); 3-hydroxydodecanoic (3-OH-C12:0); *cis*-9,10-methylenehexadecanoic (C17:0 Δ); heptadecanoic (C17:0); oleic (C18:1 ω 9c); elaidic (C18:1 ω 9t). Qualitatively, the FA profile was similar between the microorganisms preserved in both conditions along the study and the majority is constituted of linear chain

Fig. 1 FA profile from Xcm 2103 maintained at -80°C (upper) in YMB and at $3-8^{\circ}\text{C}$ (lower) on YMA slopes after 5 months. NI not identified

Fig. 2 FAME composition from Xcm 2103 first activated (T0), and from the cells preserved at -80°C (a) and at $3-8^{\circ}\text{C}$ (b) after 30, 60, 90, 120 and 150 days of storage (T1, T2, T3, T4 and T5, respectively). Bars mean $\pm$ SD, $n = 3$

structures (Fig. 1). Gram-negative bacteria contain, in general, some combination of FA with saturated and unsaturated chain, hydroxy and cyclopropanes. Furthermore, may occasionally also contain *iso* and/or *anteiso* acids [21].

Figure 2 shows the mean concentration (%) of each FAME along the time, obtained from the first activated cells of Xcm 2103 (T0), cells kept at -80°C (Fig. 2a) and under refrigeration (Fig. 2b). Among the main FAME identified in the cells from both conditions, six compounds were quantified in relatively high amount, palmitoleic, *anteiso*-pentadecanoic, *iso*-pentadecanoic, palmitic, *iso*-heptadecanoic and 3-hydroxydodecanoic, with coefficient of variation ranging from 3.9 to 20.1%, against 11.5 to 30% for the Xcm kept at -80°C and $3-8^{\circ}\text{C}$, respectively. However, the monthly concentration of each major FA, in each storage condition, varied significantly ($p < 0.05$ —Tukey), except *i*-C15:0 and *i*-C17:0 at -80 and $3-8^{\circ}\text{C}$, respectively. Despite the similar FAME profiles, the *Xanthomonas* kept frozen showed more regular concentrations of all FA (Fig. 2a).

The impact of the time and temperature of each preservation condition on FA profile has not been described previously for *Xanthomonas* strains. The ratio of unsaturated/saturated (U/S) FA and the concentration of isomers *cis* and *trans* of C18:1w9 at each month were considered to

analyze the correlation between the FA profile and both preservation conditions. The strain preserved at -80°C showed relatively higher ratio of total U/S FA (Fig. 3a), especially for C16 (Fig. 3b). At -80°C such parameters were less affected, which may have increased the cold resistance of the cell membrane of Xcm IBSBF 2103 and improving its survival during storage. The data indicates that the condition of -80°C reduces the unbalance of the FA composition, as well as, shows that the cold resistance and survival of cells during the preservation period are closely related to the FA composition of the cell membrane. This condition may favor the transport across the membrane and protect the cells under hypertonic stress, preserving the osmotic response of the bacteria, contributing to a scenario of higher permeability of water and nutrients through the membrane, favoring the production of xanthan gum (Fig. 4) as well as the polymer viscosity (Fig. 5). Unsaturated FA promotes exchanges between the extra and intracellular medium due to lower stiffening of the membrane, increasing cell permeability.

Regardless the preservation temperature, the U/S ratio (Fig. 3a, b) showed two phases. In the initial phase the U/S ratio increasing is explained by lipolysis reactions (30–60 days for -80°C , 30 days for $3-8^{\circ}\text{C}$), followed by the ratio reduction which is attributed to oxidative reactions of FA more sensitive to oxygen. The decreasing of the ratio

Fig. 3 Impact of the preservation condition and time upon the fatty acids of the Xcm IBSBF 2103. Ratio of **a** U/S fatty acids, **b** U/S major fatty acid (C16:1/C16:0). Concentration of the fatty acids C18:1ω9 *cis*

and *trans* of Xcm preserved at -80°C (**c**) and at $3-8^{\circ}\text{C}$ (**d**). Bars mean $\pm$ SD, $n = 3$

Fig. 4 Monthly yield of XG obtained with Xcm 2103 using sucrose (2%) in shaker at 28°C , 250 rpm and 120 h. $n = 3$

Fig. 5 Apparent viscosity of XG [0.5% (w/v), 25 s^{-1} , 25°C] from Xcm 2103 stored in different conditions

U/S FA was also observed for dried *Lactobacillus* sp [22–24], in all cases this phenomenon was associated to oxidative processes on cell membrane.

The concentration of the isomers *cis* and *trans* of C18:1ω9 for both storage conditions is highlighted in the Fig. 3c, d which show the inversion of isomers concentration after the 4th month in the cells preserved under refrigeration. In normal conditions, the *cis* conformation in the double bond of this acid occurs most frequently in the FA composition of bacterial cells. However, stress factors from the environment or nutrient deficiency induces an increase of the isomers *trans* [25, 26]. After the 2nd month the concentration of the elaidic acid (C18:1ω9*t*) increased and overcame the *cis* isomer concentration (C18:1ω9*c*) in the 4th month, remaining high in the last evaluation (Fig. 3d). Such inversion was not observed for the *Xanthomonas* maintained at -80°C , which showed the *cis* form in higher concentration levels during the study (Fig. 3c).

Genomic fingerprint

X. campestris strains are important plant pathogenic bacteria which are worldwide studied, mainly concerning its genetic diversity and genomic profile [27, 28]. In this study, pulsed-field gel electrophoresis (PFGE) was applied to separate the chromosomal digestion (*Xba*I) of Xcm 2103 maintained in different storage conditions. In all experiments was observed a reproducible distribution pattern with 17 distinct fragments with sizes lower than 485 kilobases (kb) (Fig. 6). All *Xanthomonas* subcultures showed similar DNA fragmentation profile, demonstrating

Fig. 6 Chromosomal fragmentation profile of Xcm 2103, with *Xba*I restriction enzyme, in PFGE agarose gel 1.2% (w/v), 2–30 s pulses, 23 h, 6 V/cm. Lanes: a size marker (kilobases); b –80 °C; c–g 3–8 °C from the 1st–5th month, respectively; h repeating of the 3rd month (e)

the genotypic stability of this microorganism over the evaluated period for both preservation methods. The genomic profile of Xcm IBSBF 2103 has not been previously shown using PFGE or other method.

Production and rheology of XG

Xanthan gum is a microbial polysaccharide of great commercial significance and its production and the factors that affect the gum yield are extensively studied [6]. At each 30 days the Xcm 2103 from both storage conditions was tested for its capacity to produce XG, and the average amount obtained was slightly lower than in earlier studies of our group using the same strain and fermentation conditions [29]. The XG production was more constant with the cells stored at –80 °C, yielding around 3 g/l of XG. The subculture maintained under refrigeration showed a significant reduction in the XG production after the second month (Fig. 4). The average amount produced by the microorganisms in both conditions was significantly different ($p < 0.05$ —Tukey).

Figure 5 shows the variation of the viscosity, at low shear rate (25 s^{-1}), of all XG batches. The cells stored at –80 °C

Fig. 7 Apparent viscosity (mPa s) and XG yield correlation

produced polymers with apparent viscosity better than the biopolymer from the cells stored at 3–8 °C, with an average of 53 mPa s against 51 mPa s, respectively. The viscosity decreasing observed after the first month was more evident in the biopolymer from subcultures maintained in refrigeration. The viscosity increasing of XG was proportional to its yielding (Fig. 7), similar as observed in previous studies of our group with this strain, confirming that xanthan obtained from processes with higher yielding rate are more viscous [30]. The XG viscosity is important for its thickener properties, rheological and pseudoplastic effects in aqueous solutions, favoring its industrial application.

Conclusion

The microbial purity of Xcm 2103 cells stored under freezing and refrigeration conditions remained constant, ensuring that all XG batches were produced from pure cultures. The biochemical behavior of the *Xanthomonas* strain studied was not influenced by the methods and time of preservation evaluated. However, it was observed that the *Xanthomonas* cells stored at –80 °C showed more constant concentrations of FA, which had a positive impact on the qualitative and quantitative characteristics of XG produced. The DNA fragmentation pattern obtained by PFGE with *Xba*I enzyme, remained unchanged in all subcultures and stored conditions. This study presented for the first time the description of the biochemical and genomic profiles, as well as the FA composition of *X. campestris* pv. *mangiferaeindicae* IBSBF 2103, such data contribute to the characterization studies of this strain.

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