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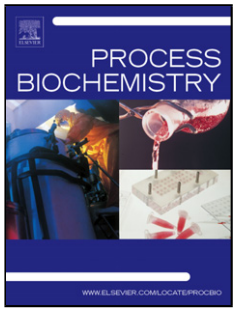

Biosurfactants produced by *Pseudomonas syringae pv tabaci*: a versatile mixture with interesting emulsifying properties

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Biosurfactants produced by *Pseudomonas syringae pv tabaci*: a versatile mixture with interesting emulsifying properties

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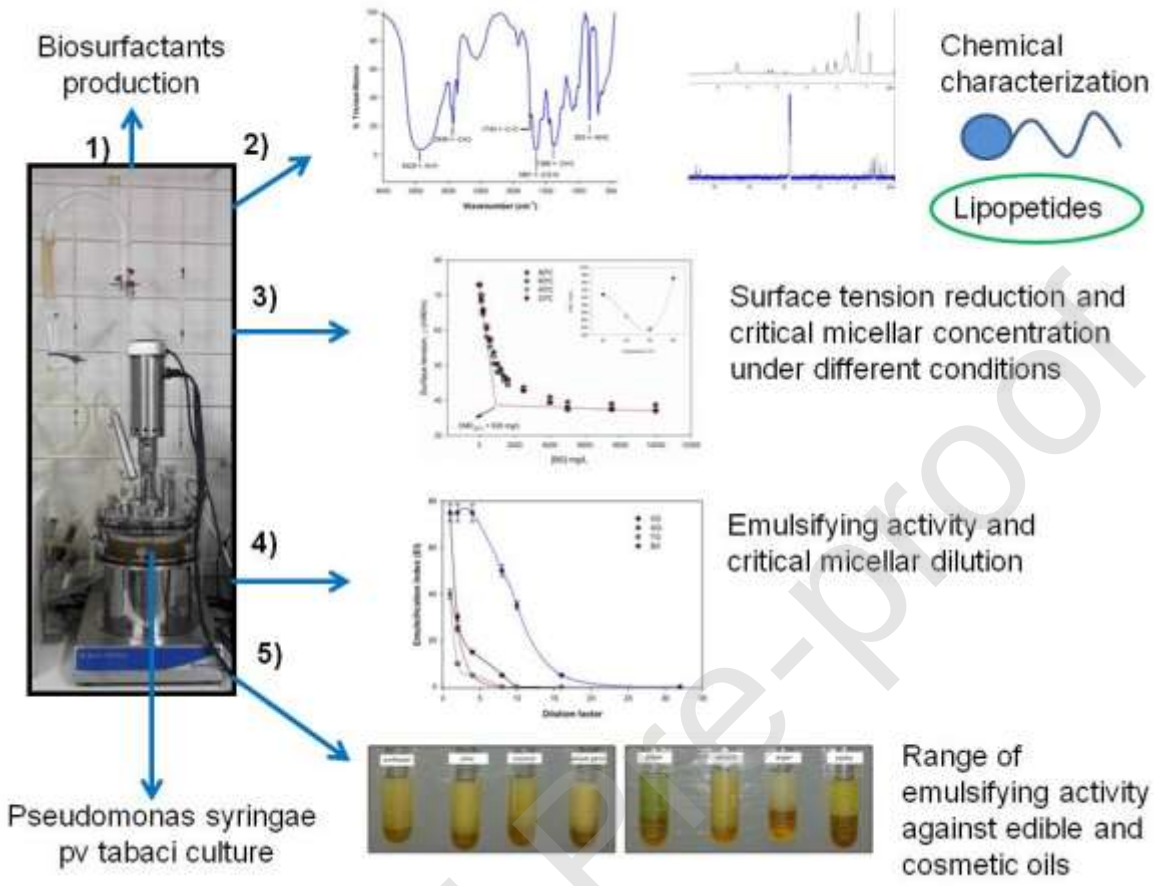
Graphical abstract



Highlights:

- Interfacial activity of biosurfactants produced by *P. syringae pv. tabaci.*
- Chemical characterization revealed that the sample mostly consists of lipopeptides.
- Water surface tension of 29 mN/m was reached in presence of 5% w/w of NaCl.
- Emulsifying activity comparable or even superior to that of commercial bioemulsifiers.

- Emulsions resulted to be little affected by different operational conditions.

Abstract

In recent years, worldwide awareness about environmental concerns has led to an extensive searching for products of natural origin. Biosurfactants (BS), are an attractive alternative to the chemical counterparts. In these sense, this works evaluate for the first time the emulsifying and surface activity of biosurfactants produced by *P. syringae pv. tabaci*. Chemical characterization revealed that the produced BS extract mostly consisted in a mixture of syringopeptins and arthrofactins. Besides, this sample was able to reduce water surface tension up to 36.89 mN/m. This activity was improved by modifying conditions of pH, temperature and salinity. For instance, the surface tension was reduced up to 29 mN/m when using NaCl 5% w/w. The BS also exhibited a high emulsifying activity, this being comparable or even superior to that of commercial bioemulsifiers such xanthan, tara and guar gums. A notorious emulsification index, close to 90%, was exhibited by BS in coconut oil/water mixtures. Moreover, emulsifying activity resulted to be little affected by different operational conditions, thus retaining about 80% of its emulsificant index in presence of 20% w/w of NaCl at 80°C. These findings are indicative of the BS promising properties and its potential as additive in a variety of industrial applications.

Keywords: lipopeptides, surface tension, critical micelle concentration, emulsification index, emulsion stabilizing capacity.

1. Introduction

Surfactants are amphiphilic molecules with the ability to reduce surface and interfacial tension. This property has made them a kind of compound widely utilized in several areas including food, cosmetics, bioremediation and medicine [1]. However, the low biodegradability of several synthetic surfactants has prompted the exploration for alternative molecules with lesser environmental concerns [2]. Biologically produced surfactants, known as biosurfactants (BS), are an attractive alternative to the chemical counterparts. BS have several advantageous features such as low critical micelle concentration (CMC), biodegradability, stability under extreme environmental conditions, high emulsifying activity and the possibility of being obtained from renewable resource [3]. Particularly, microbial biosurfactants possess a wide spectrum of chemical nature, thus giving rise to unique physicochemical properties. For instance, glycoproteins with high emulsifying activity can be obtained from *Solibacillus silvestris* AM1, *Acinetobacter calcoaceticus* RAG-1 and *Pseudoalteromonas sp.* strain TG12 [4,5]. Bacterial polysaccharides, such as xanthan gum produced by *Xanthomonas sp*., have been reported as strong bioemulsifiers with recognized application in food industry [6]. Non-polymeric BS, like lipopeptides and glycolipids, have demonstrated to decrease surface and interfacial tension [7]. Besides, these BS are able to emulsify a wide range of hydrophobic compounds [8,9]. In this context, interest in these biological molecules is therefore increasing substantially, with multibillion-dollar investments [3].

A large variety of BS, mainly those produced by *Bacillus* and *Pseudomonas* genera, have been evaluated as natural alternatives to the synthetic surfactants [9,10]. It has already been reported that several lipopeptides (LP) are produced along the pre-

pathogenic stages of plant-associated bacteria such as *P. syringae pv. syringae* B728a and *P. siringae pv. tomato* DC3000 [11]. Besides, it has also been shown that most of these molecules are lipopeptides with different size and chemical composition [12]. The main physiological roles of these lipopeptides would be attracting moisture, facilitating access to nutrients and ensuring bacterial motility [13]. Burch and co-workers [13] also evaluated the use of different detection methods to confirm the presence of several lipopeptides, including the syringafactin produced by *P. syringae pv. tomato*. These findings provided information about the hydrophilic character of these molecules [14]. Different properties such as chemical structure, critical micellar concentration and antimicrobial activity have been studied for other *P. syringae* metabolites such as syringopeptins, syringomicins, corpetin and arthrofactin [15–17]. Nevertheless, there are few reports about the BS produced by the epiphytic bacteria *Pseudomonas syringae pv tabaci* (TA) and up to date a complete chemical and functional characterization is lacking. Considering the importance of finding BS with new attractive physicochemical properties and potentially suitable for industrial and pharmaceutical purposes, the main aims of this work were both to identify the BS produced by TA and to evaluate their surface properties. This study included surface tension and emulsifying activity measurements under different temperatures, pH and salinity in an attempt to determine their operational conditions and therefore, define their fields of application.

2. Materials and Methods

2.1. Bacteria strains and culture conditions

The microorganism *Pseudomonas syringae pv. tabaci (TA)* (ATCC® 15373™) was kindly provided by Prof. Elena Orellano from Laboratorio de Genética Molecular y Genómica Funcional de las Interacciones Planta-Microorganismo del Instituto de Biología Molecular y Celular de Rosario (IBR). A pure culture of this strain was maintained as a glycerol stock at −40°C.

2.2. Chemical reagents

Glycerol, potassium dihydrogen phosphate, magnesium sulphate, ethyl acetate, hydrochloric acid and sodium chloride were purchased from Cicarelli®. Methanol, acetic acid and acetonitre, all of them with HPLC grade, were acquired from Sintorgan®. Bacteriological agar and peptone from casein were purchased from Britania and Rifampin was obtained from Sigma-Aldrich. All the other reagents were of analytical grade and used as received.

2.3. Biosurfactant production

These assays were performed by using King´s medium B (KB) [18] at 28°C. Initially, the strain was cultured onto plates containing 2% agar and rifampin (50 µg/mL). After 72 h of growth, a single colony was transferred to 30 mL of seed culture medium and incubated on a temperature controlled incubator shaker at 135 rpm during 16 h. Then, 3.0 L of fresh KB media were inoculated with this seed culture (initial optical density of 0.07 at 600 nm). These experiments were carried out in a bioreactor (Tecnal/Tec Bio V) of 4.5 L coupled with a foam fractionation column in order to integrate the production and separation of the produced BS (see Fig. S1) [19,20]. The cultures were

incubated up to 12 h at 28°C, with constant agitation (200 rpm) and aeration (1 vvm). All the experiments were performed in triplicate and the resulting reports are the mean of three independent experiments.

2.4. Biosurfactant purification

Firstly, the BS sample obtained from foam fractionation was concentrated by acid precipitation [21]. To accomplish that, the collected foams were acidified up to pH 2.0 using 6 M HCl. These solutions were allowed to stand for overnight at 4°C. Then, the incubated samples were centrifuged (13,000 rpm for 25 min at 10°C) and the precipitated BS were dried at 40°C to determine their mass. A fraction of this extract (named as precipitated BS) was stored at -40°C for further analysis while the remained BS were re-suspended in distilled water, adjusted to pH 7.4 with Na_2CO_3 1% w/v and subjected to successive liquid-liquid extractions (up to 3) with ethyl acetate (1:1). Finally, the organic phases were evaporated and the obtained BS sample (named as purified BS) was stored at -40°C for following studies.

2.5. Chemical characterization

2.5.1. Thin layer chromatography (TLC)

Aliquots (20 µL) of methanol solution of purified BS (10 mg/mL) were applied onto silica gel 60 F_{254} glass plates (Merck, Darmstadt, Germany). The separation was performed at room temperature with chloroform-methanol-water (65:25:8, v/v). The plates were developed with different methods: a) direct UV light at 254 and 366 nm, b) direct water spraying, which allows the detection of separated lipids. In this case

the sprayed plate is held against the light at room temperature and hydrophobic compounds are easily distinguished as white spots on translucent background due to their non-wettability by water [22] and c) staining with ninhydrin (1% in acetone) for amine group detection.

2.5.2. Fourier-transform infrared spectroscopy

The functional groups of the obtained BS were analyzed by Fourier-transform infrared spectroscopy (FT-IR), using a PerkinElmer Spectrum One equipment. To accomplish that, 1 mg of purified BS was mixed with 100 mg of KBr and pressed down with 10,000 kg for 8 min to obtain translucent pellets. The FT-IR spectra were acquired between 400 and 4000 wave numbers (cm^{-1}), with a resolution of 4 cm^{-1}.

2.5.3. Nuclear magnetic resonance spectroscopy (NMR)

For the NMR analysis, 5 mg of purified BS was dissolved in 0.5 mL of $CDCl_3$/ tetramethylsilane (98:2) in a 5 mm NMR tube. ^{1}H and ^{13}C NMR spectra were recorded on a 400 MHz Avance III HD spectrometer (Bruker), using tetramethylsilane as an internal standard (chemical shifts in parts per million). Data were acquired and manipulated using the Bruker TopSpin software v.4.0.7.

2.5.4. Liquid chromatography–mass spectrometry (LC–MS)

Purified BS was dissolved in methanol HPLC grade (36 mg/mL) and filtered through 0.45-µm filters. A sample (15 µL) of this solution was injected into a UHPLC-DAD-UV

detection system (Ultimate 3000 RSLC Dionex - Thermo Scientific). The used column was a Kromasil C18 (4.6 mm × 150 mm x 5 µm), operating at 1.0 mL/min and 25°C. Separation of BS was performed by combining two mobile phases: A) miliQ water/0.1% HAc (pH 2.9) and B) acetonitrile. The operating conditions were: gradient elution starts at 10% mobile phase B and ends at 35% after 18 min, between 18 and 23 phase B rises to 80% and returns to 10% between 23 and 24 min, maintaining this percentage for 2 min (until 26 min). The eluted molecules were monitored with both, UV lamp (205 nm) and a mass spectrometer (Thermo Scientific TSQ Quantum Access Max) with an ESI probe at a spray voltage of 4.8 KV, capillary temperature of 325°C, in positive mode.

2.6. Surface/Interfacial properties and stability assays

2.6.1. Surface tension measurements and critical micelle concentration

Surface tension measurements were carried out by the pendant drop method using an optical tensiometer (Theta Lite, Biolin Scientific) [23]. Different aqueous solutions of purified BS (0 to 10,000 mg/L) were prepared and their surface tensions at room temperature (20°C) were plotted against their respective concentrations. Then, the critical micelle concentration (CMC) was estimated from the intersection point of two linear equations corresponding at low and high concentration data [24]. For thermal stability, BS solutions were pre-incubated at different temperatures (20, 40, 60 and 80°C) for 1 h in a water bath.

To assess the salinity tolerance, the same set of BS solutions was prepared by dissolving the purified BS in NaCl solutions 5-20% w/w. The pH stability was studied

by preparing BS solutions in 100 mM sodium citrate solutions whose pHs varied from 5 to 12. All tension surface measurements were run at 20°C.

2.6.2. Emulsifying activity, emulsion stabilizing capacity and critical micelle dilution

The emulsifying activity assays were performed by following the methodology describe by Colin *et al*. with little modifications [25]. In this work, identical volumes of toluene (solvent widely used for EI determinations [4]) and aqueous solution of BS (0.11% w/v in Na2CO3 1% w/v, pH 7.4) were mixed into a graduated test tube. These mixtures were vortexed at high speed for 5 min and left to settle for 10 min. Then, the emulsification index (EI) was calculated as following [25]:

$$\mathrm{EI} = \frac{\text{Height of emulsion formed (cm)}}{\text{Total height of liquid mixture (cm)}} \text{ x } 100$$

The emulsion stabilizing capacity (ES) of BS was evaluated according to Bosch [26], who considers that an emulsion is stable if the ES is 50% or higher after being left to settle for 24 h (EI_{24}). The ES was calculated following the next equation:

$$\mathrm{ES} = \left(\frac{\mathrm{EI}_{24}}{\mathrm{EI}}\right) \text{x } 100$$

Critical micellar dilution (CMD) was estimated according to the procedure described by Markande and col. [4]. To accomplish that, serial dilutions of BS solutions (starting with 0.11% w/v in Na_2CO_3 1% w/v solution, pH 7.4) were mixed with identical volumes of toluene (1:1 v/v) for EI determination.

For comparative purposes, the EI and CMD of commercial bioemulsifiers: tara gum (TG), xanthan gum (XG) and guar gum (GG), were determined under the same

conditions described previously for BS produced by TA. All the assays were performed in triplicate.

Emulsifying activity was also evaluated at different temperatures, pH and salinity. The procedure was similar to that described in section 2.6.1. However, in this case, the different pH values were achieved by adding NaOH or HCl until reaching the desired pH. Additionally, the temperature effects were studied in combination with the other assayed factors. All tests were performed in triplicate.

2.6.3. Emulsification of edible and/or cosmetic oils

In this work the emulsifying performance of BS was also assessed in presence of several vegetable oils, usually applied in commercial formulations [27]: sunflower, olive, grape, coconut, argan, almond, jojoba and wheat germ. These assays were performed by following the methodology previously described on section 2.6.2. For comparative purposes, the same procedure was performed using commercial bioemulsifiers: TG, XG and GG. All the assays were performed in triplicate.

3. Results and discussion

3.1. Chemical characterization

3.1.1. Thin Layer Chromatography (TLC)

Initially, the purified BS extract was partially characterized by TLC. The obtained elution pattern (Fig. S2) showed a major spot with Rf value of 0.22 and a minor one with Rf of 0.83. After water spraying, the layer became translucent while the spots remained white opaque, thus revealing the hydrophobic character of BS components,

probably due to their lipid nature [28]. Additionally, ninhydrin staining (data not shown) confirmed the presence of aminoacids. These findings along with the results from previous research works on the chemical structure of BS produced by other Pseudomonas species, allow us to postulate that BS produced by TA are lipopeptides [29]. This must be confirmed by further assays.

3.1.2. Fourier-transform infrared spectroscopy (FT-IR)

The FT-IR spectrum (Fig. 1) allowed the identification of chemical groups present in the BS obtained. A characteristic band of –NH bonds can be appreciated at about 3500 cm^{-1}. The bands located around 3000 cm^{-1} and 1400 cm^{-1} are compatible with the stretch (–CH) of CH_2 and CH_3 groups, respectively, in the aliphatic chains. The band visualized at approximately 1650 cm^{-1} indicates the presence of the –CO–N bond while the absorption peak located between 1800-1750 cm^{-1} indicates the presence of ester carbonyl groups (–CO bond). Moreover, the sharp band obtained at about 800 cm^{-1} can be attributed to NH_2 wagging. These results are quite similar to that obtained for other lipopeptides and lipoproteins [29], thus giving support to the conclusion achieved by the TLC analysis in the previous section.

(Fig. 1-single column fitting)

3.1.3. Nuclear Magnetic Resonance (NMR)

The NMR spectra of purified BS are presented in Fig. 2 (A and B). The NMR data are summarized in Tables S1 and S2. The ^1H NMR spectrum (Fig. 2A) revealed α-amino

acid protons (4.0-5.4 ppm), thus indicating the presence of a peptide portion [30,31]. The chemical shifts corresponding to the amide protons (8.11 to 7.00 at Table S1) were overlapped and too weak to be appreciated in the spectrum. This behavior could be attributed to a long peptide moiety, with different coupling constants. On the other hand, the high field portion of the ^{1}H spectrum shows chemical shifts consistent with aliphatic methyl groups (CH_2 at 0.8-1.7 ppm) and aliphatic carbon-hydrogen bonds (CH at 3.6-4.4 ppm) [28,32]. The ^{13}C NMR spectrum (Fig. 2B) contained several signals compatible with fatty acid C-H bonds between 14 and 34 ppm [33]. The peak obtained at 62 ppm is compatible with the signal of Cα or Cβ of Ser, Thr or Dab amino acids, while the signals obtained around 130 ppm could be assigned to the Cβ of the non-canonical amino acid Dhb, whose presence has already been reported for bacterial lipopeptides [29].

(Fig. 2-double column fitting)

3.1.4. Liquid chromatography–mass spectrometry (LC–MS)

Separation and structural identification of BS components were carried out by LC-MS technique. The HPLC elution profile (see Fig. 3) shows a well separated peak at a retention time of 20.725 min and several minor peaks, not completely resolved, at retentions times between 6 and 12 min. This chromatographic pattern is similar to that reported for BS produced by other *Pseudomonas syringae* pathovars [32] which consisted in two main groups of substances with different polarities, the syringopeptins (SP) being the most retained ones [29]. Thus, when analyzing the

results presented in Fig. 3, it can be suggested that the compound eluted at 20.725 min could belong to the SP family. This assumption was later reinforced by the results of ESI-MS (Fig. S3) which showed that this compound presented a molecular mass compatible with the syringopeptin (SP) 22 Phv B (m/z 2157.0 $[M^+H]^+$) [32]. According to the literature, this lipopeptide has a hydrophobic tail containing a 3-hydroxylated fatty acid with 12 carbon atoms and a hydrophilic portion formed with 22 amino acids, whose sequence is shown in Fig. S4. Notice that the presence of several Dab residues is in good agreement with the signals obtained around 3500 cm^{-1} (NH bonds) and 800 cm^{-1} (NH_2 wagging) from the FT-IR assay (see Fig. 1).

Regarding to the compounds that eluted earlier than SP, ESI-MS analysis revealed m/z values (Fig. S3) compatible with those of syringolins and arthrofactins (AR) [34,35]. These last molecules, AR, consist in a group of cyclic lipoundecapeptides, with high surface activity [17,35].

Since a mixture of BS molecules could exhibit synergic properties, the sample reported in this work could present novel and unique physicochemical activities. The results about the analysis of the surface/interface activities of the produced BS are addressed in the following sections.

(Fig. 3-double column fitting)

3.2. Surface/Interface-active properties and stability assays

3.2.1. Surface tension and critical micelle concentration

Most of applications of surfactants are based on two physical properties: their ability to adsorb on surfaces/interfaces, thus reducing the surface tension of a given system, and to form self-assembled molecular aggregates called micelles. Critical micelle concentration (CMC) is defined as the lowest surfactant concentration at which micelles formation takes place. This parameter provides relevant information about the detergency and solubilization efficiency of a given surfactant [36]. Surfactants with low CMC are desirable for different applications due to their reduced requirements and costs. As indicated in Fig. 4, our BS sample showed a CMC of 928 mg/L. This value is consistent with the results reported for other *Pseudomonas syringae* (800-1300 mg/L) [35,37] but is higher than the CMC obtained for other well-known BS such as rhamnolipids (CMC 8-300 mg/L) and surfactin (CMC 15-100 mg/L) [38,39]. However, when compared with typical chemical surfactants, such as lauryl sulfate (CMC 2,400 mg/L), the BS from TA exhibited a CMC low enough to be preferred for industrial purposes as substitutes of chemical ones.

Another important property to evaluate is the surfactant effectiveness, i.e. the maximum reduction in surface tension that can be achieved, regardless of its concentration. In our case, the purified BS were able to reduce the water surface tension up to 36.89 mN/m (see Fig. 4), a value that falls within the range reported for other promising biosurfactants [40].

The BS stability, i.e. the capability to maintain its surface activity under different operational conditions, was also assessed in order to define its fields of application. The effect of temperature on BS surface activity is depicted in Fig. 4. It can be appreciated that the surface tension vs. BS concentration plots are practically

superposed at all the temperatures assayed, the CMC values being ranged between 835 and 971 mg/L. Only a slight displacement toward higher surface tension values can be observed at 80°C for concentrations higher than the CMC. The observed thermal stability of BS, could be related to the strength of the interaction between its hydrophilic groups and water molecules (i.e. hydrogen bonds) which is not altered by temperatures changes[41]. When plotting CMC versus temperature (see inset Fig. 4) a typical non-monotonic behavior with a minimum was observed. This profile was previously reported for both ionic and non-ionic surfactants [42] and can be attributed to the effect of temperature on both the hydration reduction of hydrophilic groups and the breakdown of structured water surrounding the hydrophobic groups. Since the effect of hydration loss is predominant at first (lower temperatures), the increase in temperature results in an enhancement of the interaction among surfactant polar heads which favors the micellization process and produces a decrease of CMC. As the temperature increases further, the impact on structured water becomes more important thus producing a decrease of the entropy factor which disfavors the micellization and results in an increase of CMC (higher temperatures) [43].

(Fig. 4-single column fitting)

Salinity and pH effects are represented in Fig. 5. This study shows that NaCl addition (Fig. 5A and S4) reduced both, the surface tension values (from 36.89 to 29.07 mN/m) and the CMC (from 928 to 527 mg/L). These significant changes, typically reported in charged surfactants [44], are consistent with the chemical structure of BS obtained in this work because it is highly probable that Dab and Asp [17,32,44] would be charged

at being dissolved in water. In this case, the addition of salt would shield the repulsive forces between the head groups of BS thus favoring the micelle formation and surfactant adsorption at the surface [45]. Fig. 5 A also shows that CMC and surface tension decrease sharply in presence of 5% NaCl; however, any additional changes are observed for higher NaCl concentrations. It can be suggested that the added NaCl can effectively weaken the electrostatic repulsion between the intermolecular head groups but once the ionic strength becomes large enough (NaCl higher than 5 %), the electrostatic repulsion remains practically invariable and the CMC values does not exhibit further significant changes. Similar behavior was reported for the effect of different counter ions on surface properties of other biosurfactants such as surfactin [44]. Regarding to pH effect, Fig. 5B shows that the higher the pH the lower surface tension and CMC values (from 1,088 mg/mL at pH 5.0 to 243 mg/mL at pH 12.0). Notice that the highest values of surface tension were obtained in pure water, i.e. in absence of sodium and citrate counterions. A monotone decrease of both CMC and surface tensions were observed when BS was dissolved in citrate media of increasing pH values. Higher effects were exhibited when changing pH from 5 to 7, probably caused by the major changes in citrate charge that take place in this pH range due to the citric acid dissociation (pKa_1=3.15; pKa_2=4.77 and pKa_3=6.40). The observed pH effect could be attributed again to the increased ionic strength which reduces electrostatic repulsion between the polar/charged heads. However, it should be considered that the citrate tribasic species, predominant at higher pHs, exhibit a strong kosmotropic character and are capable of removing the aqueous environment from hydrophobic surfactant molecule, this effect also favoring the micellization [46].

In addition, it should be taken into account that hydroxyl groups, predominant ions at higher pH such as pH12, are well-known as water structure-formers. A higher extent of water structure around the monomeric surfactant is expectable at this pH, and therefore, the destruction of this “iceberg shell” associated to the self-assembly phenomenon will cause a greater change in entropy, thus favoring the micellization and reducing the CMC [47].

The high stability and surface activity at alkaline pH has also been reported for other BS and demonstrate the feasibility of modulate BS activity by pH changes [48].

(Fig. 5-single column fitting)

3.3.2. Emulsifying activity and critical micellar dilution (CMD)

Apart from its ability to reduce the air-liquid superficial tension, the emulsifying activity of a given BS is important to evaluate its potential applications [8]. Particularly, CMD value can be considered as the optimum concentration to be used for maximum performance [49]. Thus, the higher the CMD the more industrially suitable is the emulsifier. As shown in Fig. 6, the highest emulsifying capacity (EI of 75%) was observed for XG and the BS sample (without dilution). This value was similar or even superior to those reported for other bioemulsifiers such as lipopetides, glycolipids and polysaccharides [50,51]. Regarding to CMD assays, all gums showed an abrupt decay of its EI after being diluted two times, while BS conserved most of its emulsifying ability (EI of up to 50%) up to a dilution of 8. At increasing dilutions, EI presented a progressive reduction until reaching zero at a dilution factor of 32. The

role of any emulsifier is to get adsorbed at the interphase between two immiscible phases and to reduce the interphase tension in order to allow the dispersion of droplets of one phase into the other one. This property depends on the balance between the hydrophilic and hydrophobic regions in the emulsifier molecule, i.e. its HLB value. The better emulsifying performance of BS could be explained by its lipopeptidic nature which conferees it a HLB more similar to that required for the emulsion organic phase (toluene) than the HLB exhibited by the other emulsifiers assayed (XG, TG and GG) which are polysaccharides [1]. Moreover, the results presented in Fig. 6 are comparable with those of Al-Wahaibi et al., whose observed that the superficial activity of biosurfactants produced by *Bacillus* B30 was reduced up to 50% at a dilution of 8 [49]. Rashad et al. also reported a residual superficial activity of about 70% for sophorolipids dilution of about 1:8 [52] while Markande and co-workers informed residual emulsifying activity of about 60% for bioemulsifiers dilutions of 8 and 16 [4].

(Fig. 6-single column fitting)

(Table 1-single column fitting)

There are a plethora of commercial applications, such as enhanced oil recovery, laundry, food, etc, at which the emulsifiers are subjected at extreme conditions of temperature, pressure, pH and/or ionic strength [51]. For this reason, the effect of different environmental conditions on the emulsifying activity of the obtained BS was also assessed. As presented in Table 1, when the BS sample was prepared in the

reference solution, the obtained EI was similar at the different assayed temperatures (20-80°C), demonstrating a notorious thermal stability. On the other hand, at analyzing the effect of salt addition, it can be appreciated that the higher the NaCl concentration, EI values suffer a little reduction, reaching a diminution of up to 15% at NaCl 20% w/w. In the case of charged emulsifiers, their ability to stabilize an emulsion depend on both, interphase tension reduction and electrostatic repulsion exerted at the surface of the dispersed droplets [53]. Thus, taking into consideration that the studied BS have several ionizable amino acids, the salt addition may have weakened the intra-molecular electrostatic repulsion between the charged groups, thereby causing the early flocculation of emulsions. Notice that this effect resulted to be more pronounced at 60 and 80°C because an increase in the temperatures could result in a greater particle kinetic energy and/or low viscosity of the dispersing media, thus favoring the droplets collision [51,53]. Finally, Table 1 also shows that at pH 5.0 and 9.0 the estimated EI were a little lower that those obtained at pH 7.0, this reduction being more notorious at pH 5.0 and 80°C (EI diminution from 75 to 61.9%). This behavior can be suggesting that at acidic pH values the electrostatic repulsion between the droplets is not large enough to overcome attractive interactions such as van der Waals and hydrophobic attraction, this effect being even more pronounced at high temperatures [53].

However, at this point it is important to highlight that at most of the assayed conditions the studied BS preserve high percentage of their surface activity and emulsifying capability. Indeed, under several conditions of pH and salinity the BS even improve

their surface properties (see Fig 5). These results evidence their great potential to be used as bioemulsifier in different food, cosmetic and cleaning formulations.

(Fig. 7- single column fitting)

(Fig. 8- single column fitting)

3.3.3. Emulsification of edible and/or cosmetic oils

On the basis of the BS auspicious properties and potential uses, the EI were also measured against different oils, widely used as industrial additives. For comparative purposes, the same experiments were performed with other bioemulsifiers [51]. As it can be seen from Figs. 7 and 8, the BS sample was able to emulsify all the assayed oils. In fact, several EI were similar or superior to those observed for toluene (see Fig. 6), demonstrating the versatile activity of the studied BS. At comparing these results with that from commercial bioemulsifiers (Fig. 8A), it can be noticed that the BS sample and XG exhibited the highest emulsification capacity. Particularly, all EI calculated for BS resulted to be superior to 60%. These results are similar or even better than those reported for other microbial BS. For example, Madhurankhi and co-workers reported an EI of about 40% between olive oil and a lipopeptides mixture [8]. On the other hand, Alvarez et al. obtained an EI of about 100% for palm oil when using bioemulsifiers produced by *Ensifer adhaerens JHT2*. However, the mentioned bioemulsifiers exhibited a bad performance, with EI lower than 40%, when assayed against the rest edible oils [54].

As mentioned before, the high emulsifying performance of microbial BS could be attributed to their amphiphilic nature which conferees it a HLB similar to that required

for the emulsion of vegetable oils [1]. Then, the diversity of EI values obtained for the assayed oils can be attributed to different hydrophobicity degrees, thus resulting in a different emulsifying performance by the BS. A similar behavior was reported by Fei and col. [27] and Alvarez et al [54].

Regarding to EI measurements after 24 h of incubation (Fig. 8B), emulsions prepared with XG and BS demonstrated to be the most stable ones, thus showing ES values superior to 70% for most of the assayed oils. Additionally, the emulsions formed within BS and the different oils maintained up to 50 % of their EI value after a month of incubation at room temperature (data not shown). These results reinforce the versatile emulsifying capacity of these BS.

4. Conclusions

This work represents the first report about surface and emulsifying activity of a BS sample produced by *P. syringae pv tabaci*. Chemical characterization assays revealed that this BS sample mostly consisted in different groups of cyclic lipopeptides: syringopeptins and arthrofactins. It is important to highlight that synergistic combination between two or more agents may lead to new and interesting properties.

Regarding to interfacial activity, the purified BS were able to notoriously reduce water surface tension and to emulsify several oils widely used as cosmetic and food additives. Indeed, its emulsifying ability was similar or even superior to that obtained from commercial bioemulsifiers. Besides, surface and emulsifying activities practically maintained or even improved under a wide range of operational conditions of pH,

temperature and salinity. These results, in addition with the BS biodegradability, open up perspectives for its usage as biological detergent and bioemulsifier.

Authors statements

Carla N. Haidar: Investigation

Matheus M. Pereira: Investigation

Álvaro S. Lima: Supervision

Bibiana B. Nerli: Supervision, Resources

Luciana P. Malpiedi: Supervision, Writing - Review & Editing.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Figure captions

Figure 1: FTIR spectra of biosurfactants produced by *P. syringae pv tabaci***.**

Figure 1: FTIR spectrum of biosurfactants produced by *P. syringae pv tabaci.*

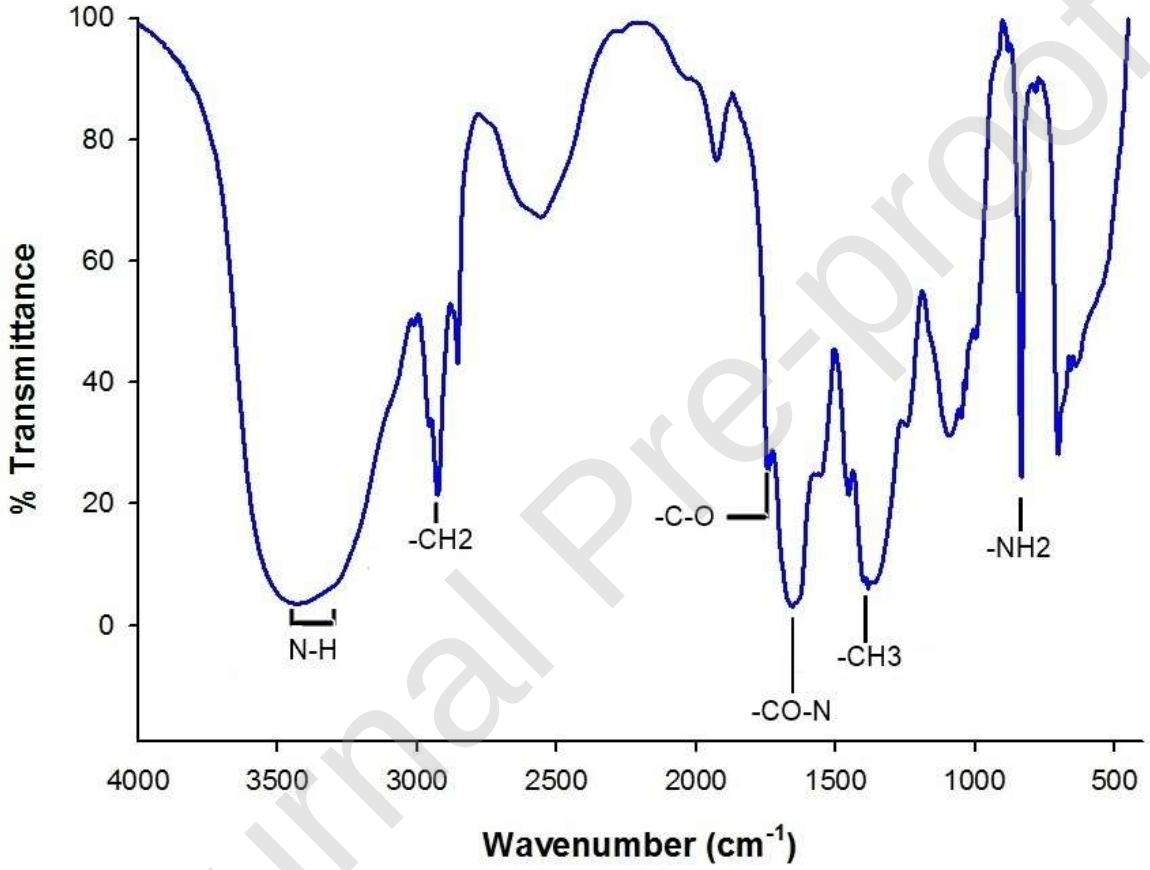

Figure 2: ^{1}H NMR (A) and ^{13}C NMR (B) spectrograms biosurfactants produced by P. syringae pv tabaci.

Figure 2: 1H NMR (A) and 13C NMR (B) spectrograms of biosurfactants produced by P. syringae pv tabaci.

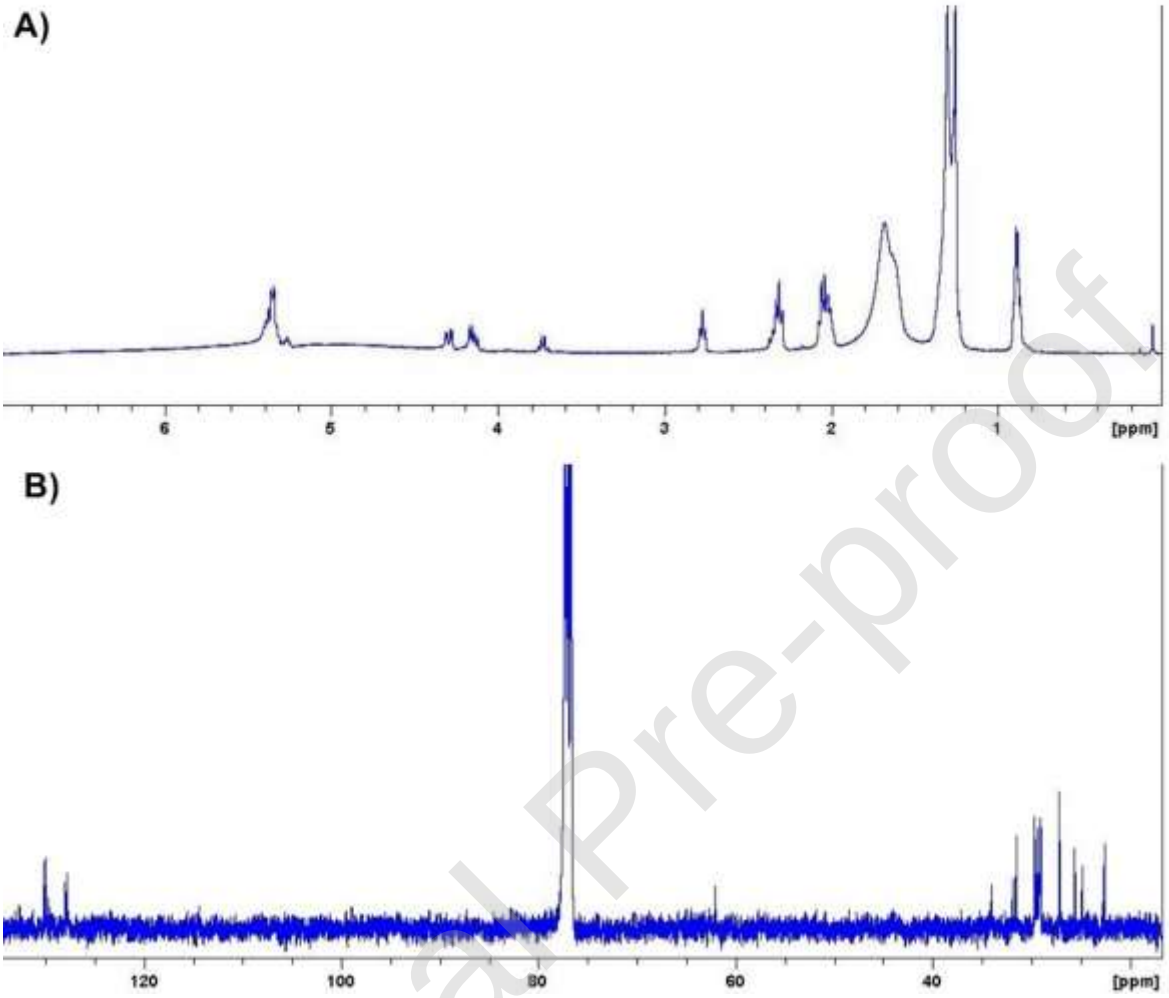

Figure 3: HPLC chromatogram of biosurfactants produced by *P. syringae pv tabaci*.

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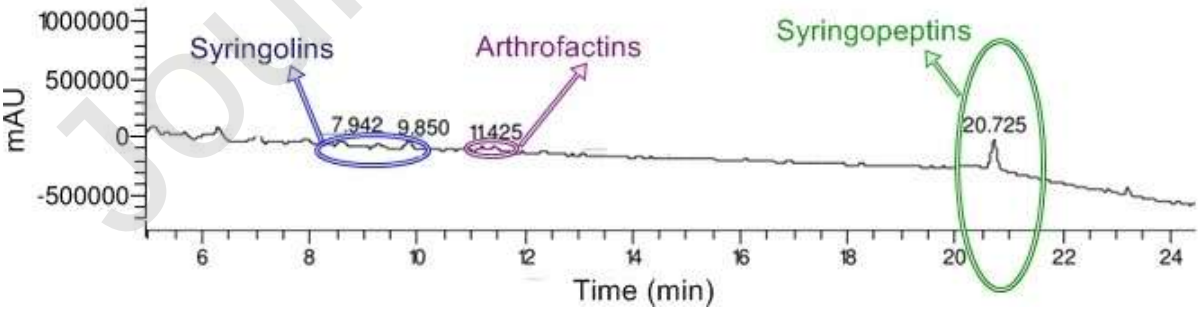

Figure 4: Plot of surface tension vs. BS concentration. Measurements were carried

out in bidistilled water at different temperatures. Data correspond to the average of triplicates ± SD (error bars). Inset: Changes in CMC with temperature. The solid line is only a guide for eye.

Figure 4: Plot of surface tension vs. BS concentration. Measurements were carried out in bidistilled water at different temperatures. Data correspond to the average of triplicate ± SD (error bars).
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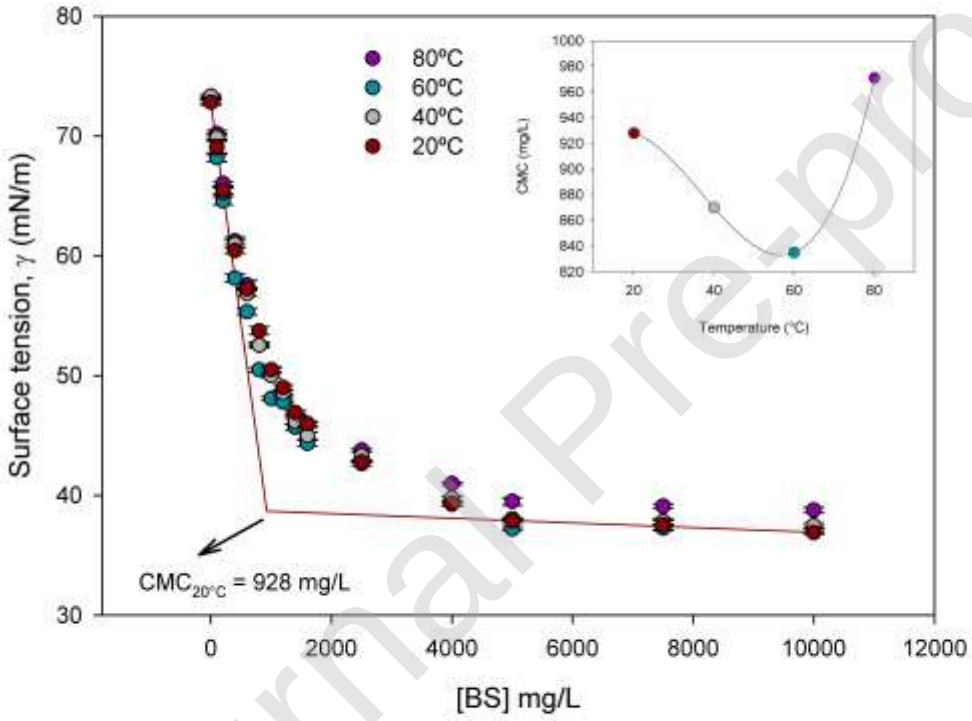

Figure 5: Plot of surface tension vs. BS concentration. Measurements were carried out at 20°C in (A) bidistilled water with different NaCl concentration; (B) aqueous solution of sodium citrate 100 mM with different pH. Data correspond to the average

of triplicates ± SD (error bars).

Figure 5: Plot of surface tension vs. BS concentration. Measurements were carried out at 20°C in (A) bidistilled water with different NaCl concentration; (B) aqueous solution of sodium citrate 100 mM with different pH. Data correspond to the average of triplicates ± SD (error bars).

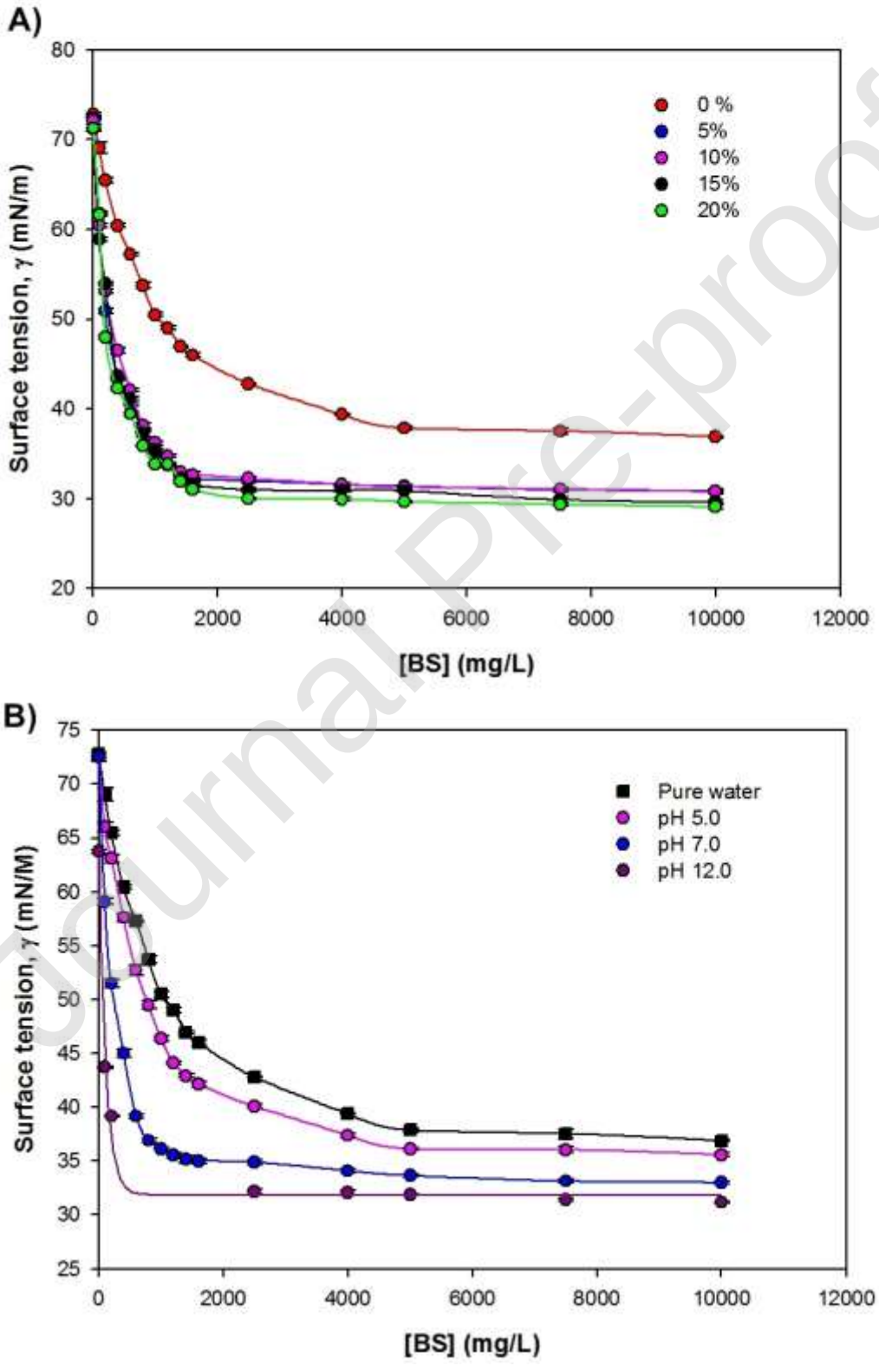

Figure 6: CMD determination for biosurfactants produced by *P. syringae pv tabaci* (BS) and different bioemulsifiers: xanthan gum (XC), guar gum (GG) and tara gum (TG). Measurements were carried out at 20°C in presence of toluene. Data correspond to the average of triplicates ± SD (error bars).

Figure 6: Critical micelle dilution (CMD) determination for biosurfactants produced by *P. syringae pv tabaci* and different bioemulsifiers: xanthan gum (XG), guar gum (GG) and tara gum (TG). Measurements were carried out at 20°C in presence of toluene. Data correspond to the average of triplicates +/- SD (error bars).

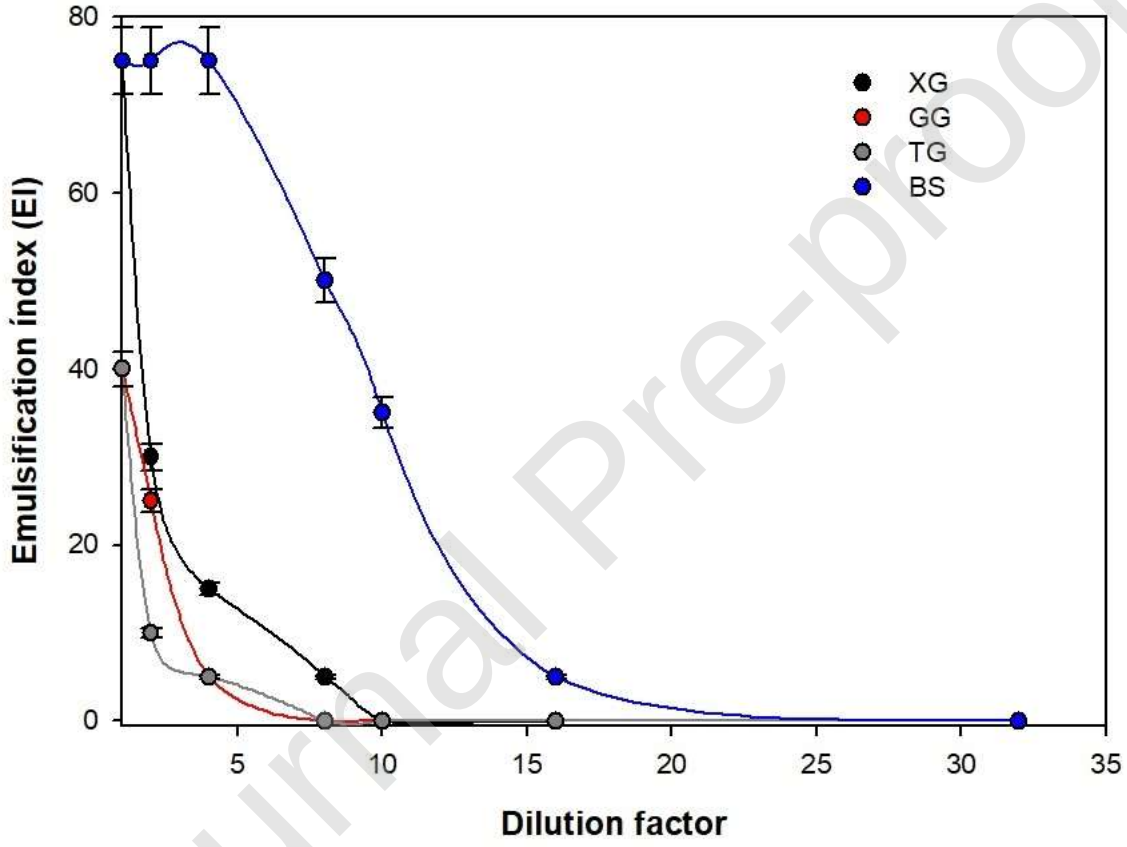

Figure 7: Emulsifying activity of the produced biosurfactant against different cosmetic/edible oils.

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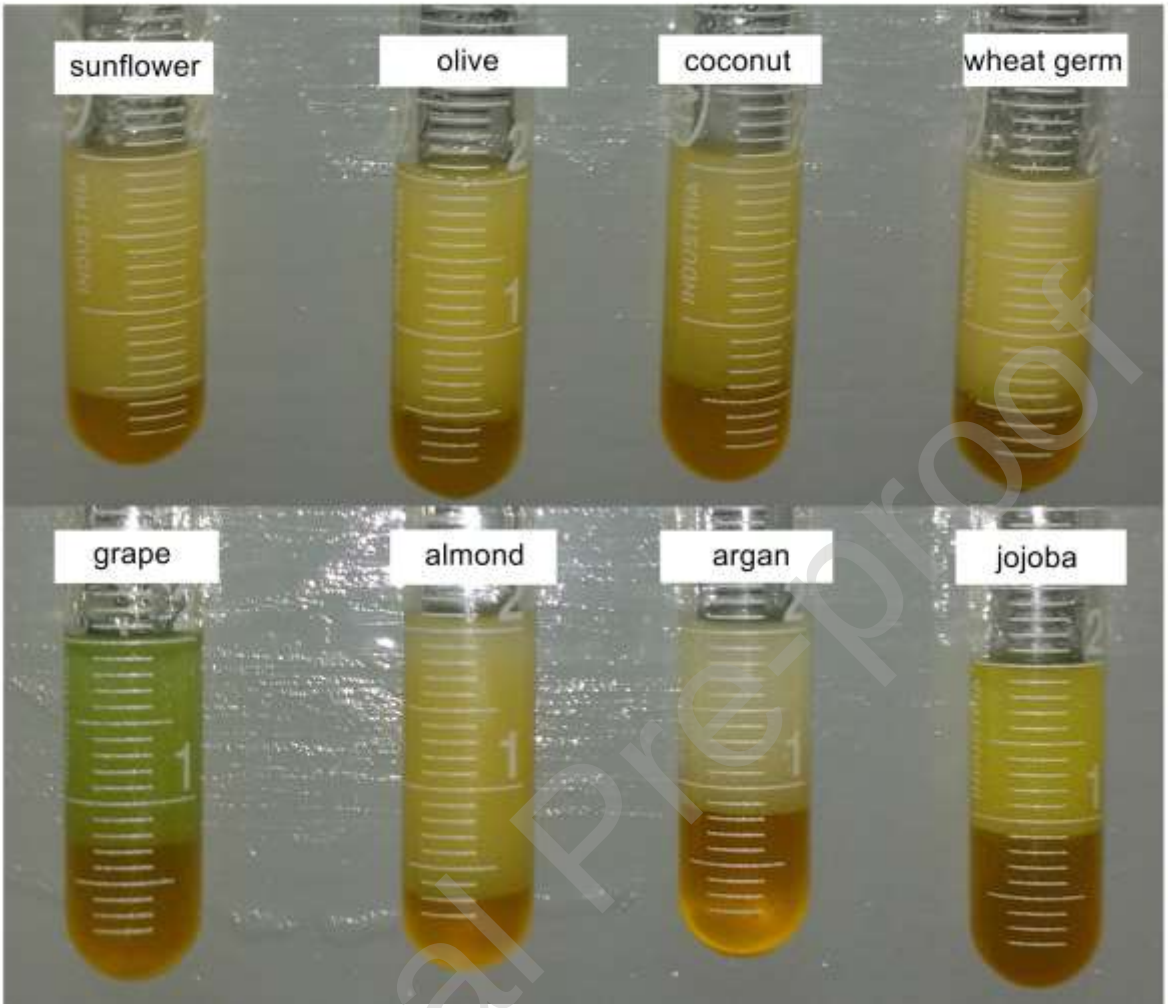

Figure 8: Emulsification index (A) and emulsion stability (B) between BS produced by *P. syringae pv tabaci* and different oils. The same assays were performed using commercial bioemulsifiers: xanthan gum (XC), guar gum (GG) and tara gum (TG). Measurements were carried out at 20°C. Data correspond to the average of triplicates ± SD (error bars).

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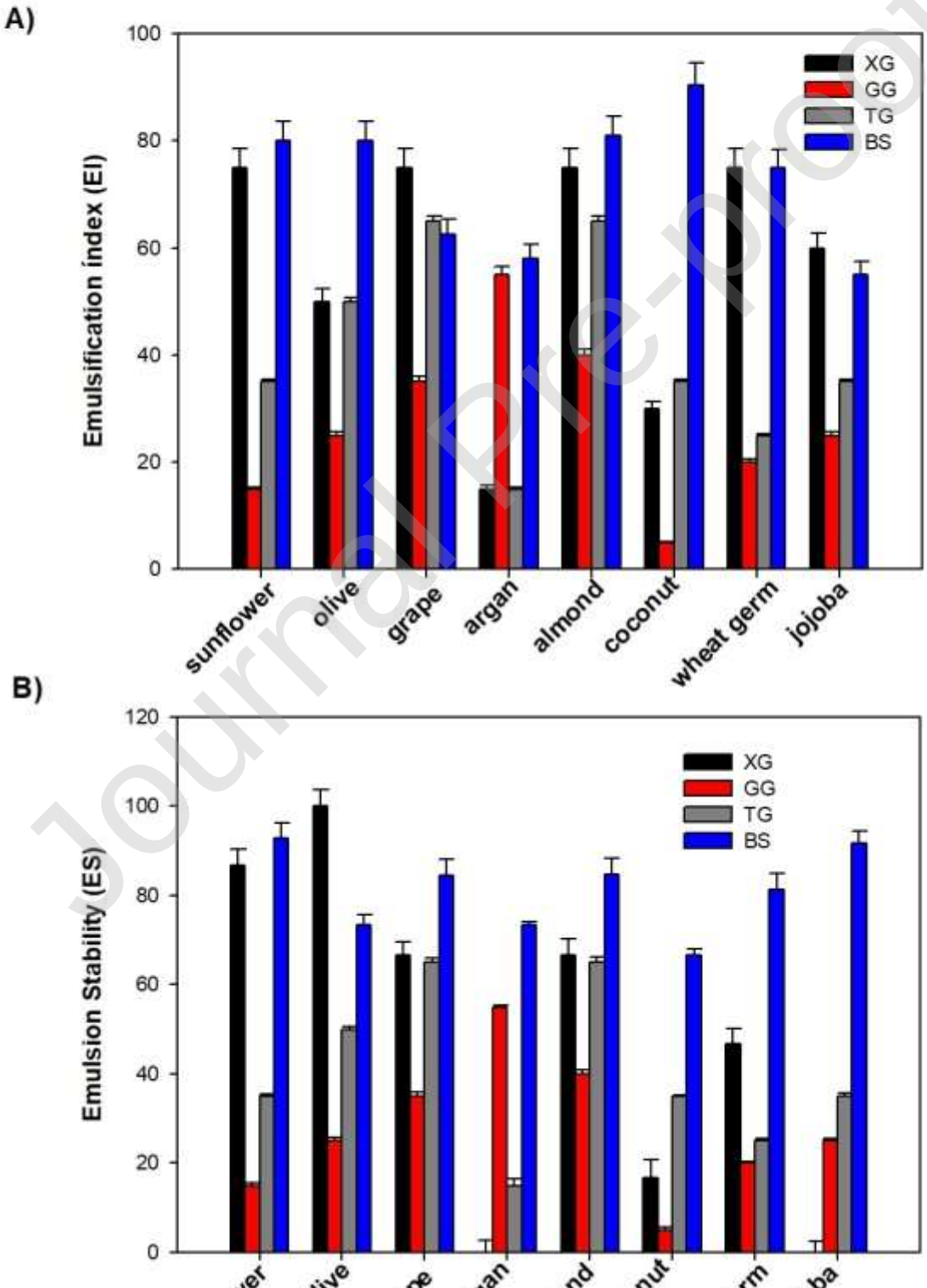

Table 1: Effect of temperature, salinity and pH on the emulsifier capacity of the biosurfactants produced by *Pseudomonas sytringae pv tabaci* against toluene. The results are expressed as a percentage emulsification index.

BS sample	Temperature (°C)			
	20	40	60	80
Reference*	75.0	75.0	75.0	75.0
NaCl 5%	70.0	70.0	70.0	65.0
NaCl 10%	70.0	70.0	68.4	68.4
NaCl 15%	65.0	65.0	61.9	60.0
NaCl 20%	65.0	65.0	61.9	60.0
pH 5.0**	66.7	66.7	66.7	61.9
pH 9.0**	70.0	70.0	70.0	70.0

All measures have an uncertainty of up to 2.5.

* 0.11 % w/v of precipitated BS in Na_2CO_3 1% solution, pH 7.0.

** Addition of NaOH or HCl until reach the assayed pH value.