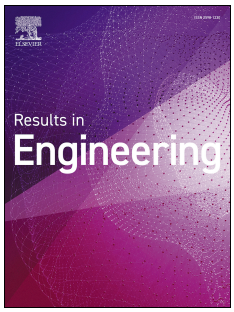

High throughput screening of the potential biosurfactants production by extremophiles isolated from vinasse and black liquor

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Sugar cane by-products biosurfactant extremophiles producers

Vinasse and Black liquor

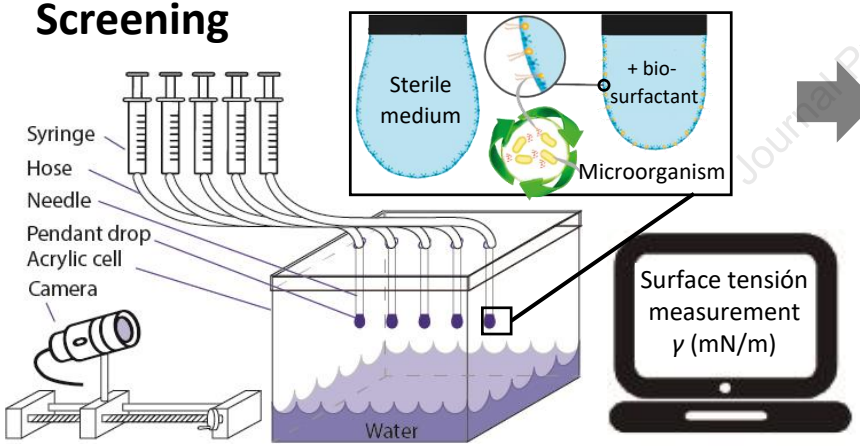
By-products of paper and alcohol industries

Microorganisms' isolation

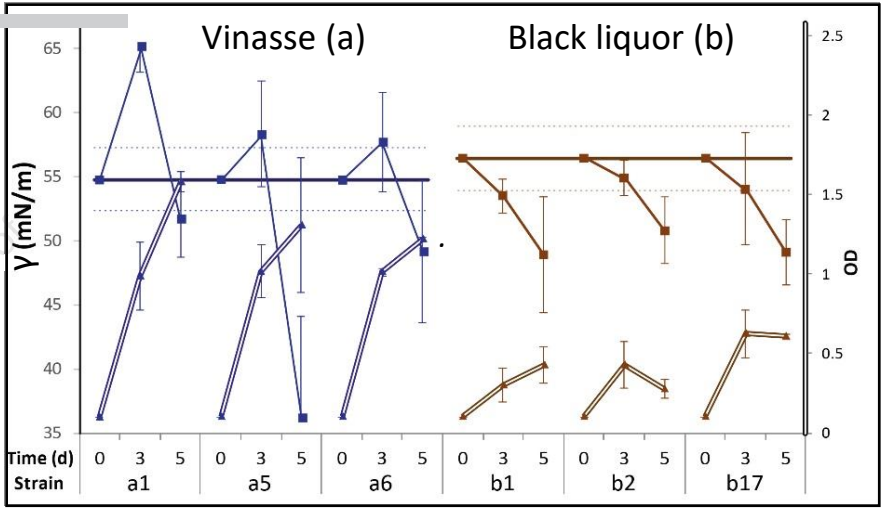
19/39 vinasse (a)

20/39 black liquor (b)

Screening



Selection



Strain identification

a1: *Lactobacillus* sp.
a5: *Lactobacillus* sp.
a6: *Pichia* sp.

b1: *Bacillus* sp.
b2: *Alckalihalobacillus* sp.
b17: not identified

High throughput screening of the potential biosurfactants production by extremophiles isolated from vinasse and black liquor

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ABSTRACT

The aims of this work were to isolate microorganisms from vinasse (V) and black liquor (BL), by-products of alcohol and paper industries and to assess their potential as substrates to produce biosurfactants. Thirty-nine microorganisms, 19 from V (acidophilic, a1 to a19) and 20 from BL (alkaliphilic, b1 to b20) were isolated. A high-throughput method was design and set up for surface tension (γ) measurement. In the first screening, 15/39 strains were pre-selected for their capacity of γ reduction of media formulated with V and BL. A Student's *t*-test was applied to data obtained in the second screening, to search for significant differences among γ values reached in each production media and their respective sterile medium. Three acidophilic (a1, a5, a6) and 3 alkaliphilic (b1, b2, b17) significantly decrease the γ compared to the control ($p < 0.05$) after 5 days with 5% of substrate concentration. A BC_{index} was defined to assess biosurfactant capacity. The strains showed the following γ percentage of reduction: *Lactobacillus rhamnosus* a5 (34%), *Bacillus safensis* b1 (13%), *Alkalihalobacillus halodurans* b2 (10%) and *Pichia cecembensis* a6 (10%), *Lactobacillus paracasei* a1 (6%). The two strains that presented the best potential biosurfactant capacity and the greatest γ reduction when compared to the control (*p*-value) were *Lactobacillus rhamnosus* a5 (BC_{index}=16.98, p=0.007), and *Bacillus safensis* b1 (BC_{index}=17.66, p=0.003). These industrial by-products are a source of new super-producing strains and will be assess as economic substrates for biosurfactant production.

Key words: **Vinasse, black liquor, biosurfactant, surface tension, alkaliphilic, acidophilic,**

1. INTRODUCTION

Biosurfactants are compounds produced by microorganisms that can reduce the surface/interfacial tension and cause the solubilization of non-polar compounds in polar solvents (Nitschke and Costa, 2007). These metabolites contain hydrophilic and hydrophobic groups, exhibiting pronounced surface activities (Jain et al., 2012). They are structurally diverse, including glycolipids, lipopeptides, lipoproteins, phospholipids, neutral lipids, fatty acids, and polymeric macromolecules (Sriram et al., 2011). Glycolipid-containing biosurfactants have been used in several fields such as enhancing oil recovery, bioremediation of oil-contaminated soils and effluents, as well as in the removal of heavy metals and organic pollutants from soils (Costa et al., 2010; Fenibo et al., 2019). On the other hand, lipopeptide-type biosurfactants are important due to their specific pharmacological activities as antimicrobial, antitumoral, and immunosuppressive (Sriram et al., 2011).

Surfactants of microbial origin have several advantages over the synthetic ones: they are more biodegradable and do not accumulate in the environment, they have a low level of toxicity, and exhibit physical and chemical properties suitable for different applications (tolerance to extreme temperatures and pH values, emulsifying capacity, etc.) (Franzetti et al., 2011; Shavandi et al., 2011). The study of these biological compounds has become important worldwide. They are already seen as potential replacement of the synthetic surfactants due to their miscellaneous applications in bioremediation, industrial processes (food, textiles, pharmaceuticals, cosmetics, among others), medicine, and biological control, besides being eco-friendly (Fenibo et al., 2019).

Concerning large-scale production, biosurfactant profitability increases through the use of carbon and nitrogen sources available or abundant in economic substrates (Liu et al., 2020; Soares da Silva et al., 2019). Using industrial by-products as raw materials is a smart option (Martins and Martins, 2018; Singh et al., 2019), as it allows adding value to what is often considered waste (leading to circular economy) and minimizing at the same time, the impacts caused over the environment and society (Patria et al., 2020). However, one of the challenges that arises when using cheap substrates for biosurfactant production at an industrial scale, is to find the optimal conditions to maximize yields (Jahan et al., 2020; Navarrete et al., 2020; Rawat et al., 2020; Schultz and Rosado, 2020).

Among industrial by-products, those obtained during sugar (from sugar cane), paper, and ethanol production are quite interesting as they still contain carbon and other nutrients (Henkel et al., 2012). Sugar cane and its by-products are the raw materials for sugar, ethanol, and paper manufacturing. Besides, vinasse and black liquor are by-products of the two latest, respectively. Currently, 22.4 Gl (gigaliters) of vinasse (Parsaee et al., 2019) and more than 1.3 billion t/y of weak black liquor, are generated around the world. From this, around 200 million t/y of dry solids are burned, producing carbon dioxide, which is a greenhouse gas. Over 50 million tons of cooking liquor chemicals are recovered in the burning process (Reyes et al., 2020).

Vinasse is a dense (1.15 - 1.25 g/cm^3) and acidic liquid (pH = 3.5 - 5) obtained after the distillation of fermented must from molasses or sugar cane juice (Aparicio et al., 2017). It can contain a variable amount of carbon, potassium, sulfur, magnesium, nitrogen, and calcium. If this effluent, with a high organic matter concentration, was discharged into a water body, it would oxidize consuming dissolved oxygen, negatively affecting the

quality of the aquatic resource. On the other hand, black liquor is an alkaline liquid (pH > 11) obtained in the Kraft process, during the production of cellulose pulp as part of paper manufacturing (Yang et al., 2010; Johnson and Hart, 2016). It contains aliphatic carboxylic acids, lignin, carbohydrates (hemicellulose residues), and extracts (Bajpai, 2017).

There is evidence that extremophile microorganisms such as halophiles, acidophilic, and alkaliphilic are capable of producing a variety of surfactant molecules (Kamal, 2012; Martínez et al., 2019; Sarafin et al., 2014; Schultz and Rosado, 2020). Also, the use of by-products from the sugar industry, such as lignin and vinasse, as substrates for biosurfactants production like rhamnolipids has been reported (de Araújo Padilha et al., 2019; Lima and Souza, 2014).

Surface tension (γ) reduction is usually measured to indirectly assess the biosurfactant activity. For this, qualitative or semi-quantitative methods such as the emulsification index and the droplet dispersion (Chen et al., 2007; Uzoigwe et al., 2015), to mention the main ones, followed by quantitative techniques, are applied. The most used quantitative methods are chromatographic analysis and γ measurement (e.g. the pendant drop). The pendant drop profile method (ADSA, aximetric drop shape analysis) is a classic one to measure γ. It consists of digitizing the profile of a drop in equilibrium between gravity force that tends to stretch the drop and the tension force that tends to shrink it. This phenomenon is described by the Young-Laplace equation (Laplace, 1805; Young, 1805).

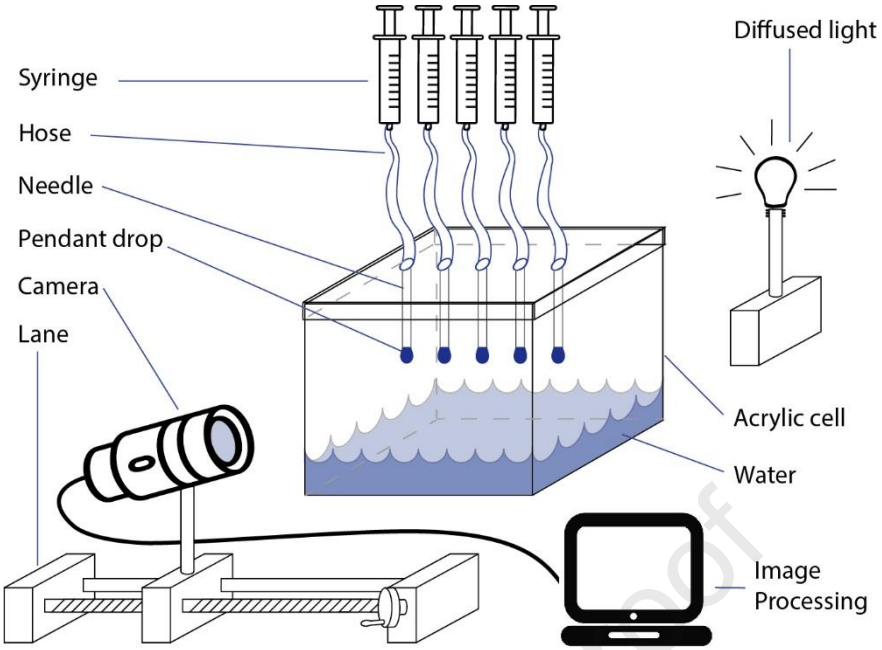
The aims of this work were to isolate microorganisms from media prepared with vinasse and black liquor, by-products of the alcohol and paper industries, and to assess their capacity to grow and produce biosurfactants using those by-products as source of

nutrients. For that, a high-throughout screening method for measuring the *γ* of the different production media with isolated strains was design and set up. The strains capable of significantly reduced the *γ* were selected and sequenced for partial identification.

2. MATERIALS AND METHODS

2.1 Measurement of the surface tension

The pendant drop profile method (ADSA) was selected for measuring the *γ* but using a multi-needle cell built in-house. The drops were photographed with the Standard Goniometer with DROP image standard, model 200-00, Ramé Hart Instrument (Co., USA). It consists of a high-resolution camera, a stand, and diffuse light. The modification allows measuring simultaneously a higher number of samples while decreasing the total measurement time. A multi-needle acrylic cell system was built to form the different drops and protect them from evaporation (Figure 1). In the multi-needle cell, several syringes were connected to the tips through silicon hoses. The tips of the needles consisted of 2.5 mm capillary tubes. The syringes were used to push the liquid through a plunger to form the drop. For each syringe, 1 ml of supernatant of the produced medium was suctioned, and then placed on the acrylic cell with 50 ml of water. After 3 minutes, the droplets were photographed.



131

132 **Figure 1:** Pendant drop measurement system with the addition of the multi-needle cell
133 built in-house. The images are not to scale.

134

135 Two dimensionless numbers were used: Bond (*Bo*) and Worthington (*Wo*) (Berry et al.,
136 2015). *Bo* (Equation 1) describes the relationship between gravitational and interfacial
137 forces and can be used to characterize the shape of a fluid sphere (air bubble, water
138 drop, etc.).

139 $$Bo \equiv \frac{\Delta\rho \times g \times R_0^2}{\gamma} \quad (1)$$

140 where $\Delta\rho$ (kg/m^3) is the difference in density between the phases, g (m/s^2) is the
141 gravitational acceleration, R_0 (m) is the drop radius and γ (N/m) is the interfacial tension.
142 A γ value is considered accurate when *Bo* is > 0.15 (Berry et al., 2015). The problem of
143 the reduced precision with low *Bo* values can be overcome by making a correct choice
144 of the needle width. Thus, the minimum number of the true required *Bo* decreases as
145 the needle width decreases (Berry et al., 2015). The accuracy of the measurement is

146 verified through the *Wo* (Equation 2), defined as the relationship between the real drop
147 volume (V_d, in m^3) and the theoretical maximum volume (V_{max}, in m^3). The Worthington
148 number can be calculated as:

149 $$Wo = \frac{V_d}{V_{max}} \quad (2)$$

150 and the theoretical maximum drop volume can be defined using the Harkins and Brown
151 equation (Harkins and Brown, 1919):

152 $$V_{max} = \frac{\pi \times \gamma \times D_n}{\Delta\rho \times g} \quad (3)$$

153 where D_n (m) is the diameter of the needle. Therefore, replacing Eq. (3) in Eq. (2):

154 $$Wo = \frac{\Delta\rho \times g \times V_d}{\pi \times \gamma \times D_n} \quad (4)$$

155 For *Wo* > 0.58, the precision of the standard error is less than 1% when using needles up
156 to 1.65 mm. For this study, *Bo* remained between 0.39 and 0.45, while *Wo* ranged 0.6 -
157 0.75 .
158 The free software Opendrop 1.1 was used to solve the Young Laplace differential
159 equation (Eq. 5, Laplace, 1805) from the image of the drop profile applying Canny
160 method.

161 $$\gamma\left(\frac{1}{R_1}+\frac{1}{R_2}\right) = \Delta P \equiv \Delta P_0 - \Delta\rho g z \quad (5)$$

162 were R_1 and R_2 are the main curvature radius; $\Delta P = P_{in} - P_{out}$ is the Laplace pressure across
163 the interface in a certain position *z*; ΔP_0 is a differential pressure measured in z=0; $\Delta\rho$ =
164 $\rho_d - \rho$ is the difference of density between the fall phase and the continuous phase; and
165 *g* is the gravity.

166 The Young-Laplace equation is solved as a coupled set of dimensionless differential
167 equations, which allows knowing the surface tension value from a digitized profile (Berry
168 et al., 2015).
169 Temperature remained between 22 and 25 °C and the gravity value (Salta, Argentina)
170 was established at 9.78409 m/s^2 (IGN, 2016).
171

172 **2.2 Strain isolation**

173 First, three samples were obtained from a sugar mill located in the Province of Jujuy
174 (northwest of Argentina): one 5 l-bottle of black liquor from the Kraft process involved
175 in the paper manufacturing, and two 5 l-bottles of vinasse from the alcohol production
176 (named vinasse 1 and vinasse 2). Samples were stored at 4 °C until processing and use.
177 Vinasse 1 showed 23.56 mS/cm of conductivity, 7.8 °Bx, and pH was 4.15; while for
178 vinasse 2, the values were 19.05 mS/cm, 2.2 °Bx, and 4.75, respectively. On the other
179 hand, the black liquor had 5.75% of dry solids and pH was 13.93.
180 Microorganisms were isolated from black liquor and vinasse, using them as source of
181 nutrients. For that, media were prepared with sterile water (water or defined medium,
182 DM), non-sterile substrate (black liquor, vinasse 1, vinasse 2, or their combinations in
183 different proportions), and 20 g/l agar-agar (Britania) (Figure 2). All media were
184 prepared in duplicate, obtaining a total of 24 (Figure 1).

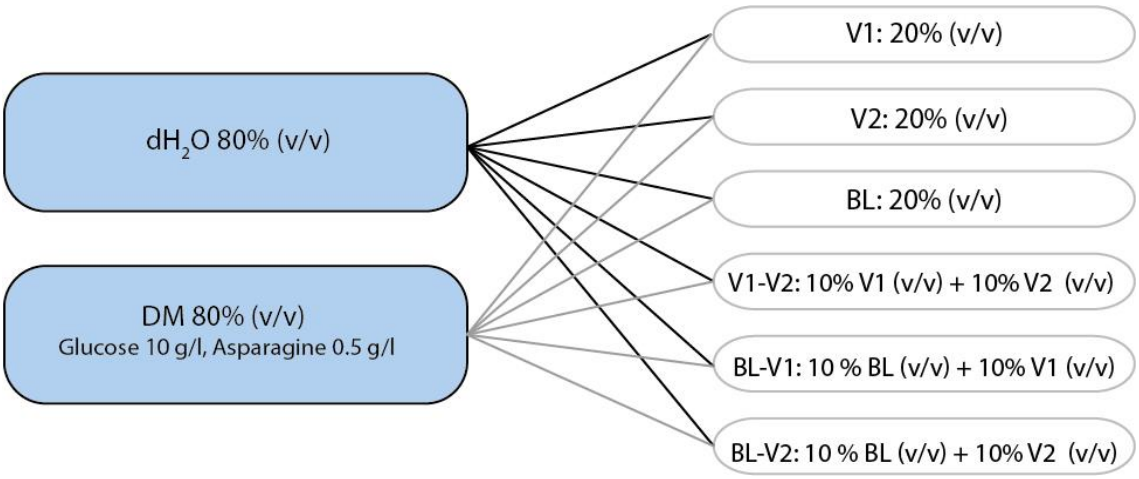


Figure 2: Formulation of culture media prepared with 80% of sterile distilled water (dH_2O) or defined medium (DM) for strain isolation. The remaining 20% was completed with non-sterile: vinasse 1 (V1), vinasse 2 (V2), black liquor (BL), or their combinations in the indicated proportions. A total of 12 different media (obtained following the lines) were prepared in duplicate.

Media were poured into sterile Petri dishes and once solidified, they were incubated at 37 ± 1 °C for 10 days. After this time, plates were checked for microbial growth. Colonies that presented bacterial and/or yeast morphology were selected for further analysis, while filamentous fungi were discarded. Then, colonies were streaked in new Petri dishes with sterile isolation medium and incubated at 37 ± 1 °C for 4 days. Colonies with different features were selected and named using a letter (a, b) and a number; letter "a" was used for strains isolated from vinasse and "b" for those isolated from black liquor. Characteristics of the colonies such as color and morphology were registered. The strains were stored in glycerol at -20 °C and -80 °C in duplicates.

201 **2.3 Strain screening and selection**

202 Two selection steps were carried out with the isolated strains regarding the
203 biosurfactant production capacity. First, two culture media were prepared with 5% of
204 vinasse (MA-5) or black liquor (MB-5) and 8 g/l of nutrient broth (NB) (Table 1). Twenty-
205 milliliter tubes, containing 10 ml of either MA-5 or MB-5, were inoculated with fresh
206 colonies of each strain. Also, sterile tubes (no microorganisms inoculated) with each
207 media were used as controls. Tubes were incubated at 30 °C and 200 rpm. All
208 experiments were performed in duplicates. Biosurfactant production of each strain was
209 assessed by measuring the γ reduction on the third and seventh days, by the multi-
210 needle cell described. Those strains showing the capacity to significantly reduce (more
211 than the absolute value of one standard deviation for each case) the γ of the production
212 media were selected for later experiments.
213 For a second selection step, four culture media were prepared: two with 8 g/l of nutritive
214 broth and 5 or 20% of vinasse 1 (MA-5 and MA-20, respectively) and two with 8 g/l of
215 nutritive broth and 5 or 20% of black liquor (MB-5 and MB-20, respectively) (Table 1).
216 Twenty-milliliter tubes, containing 10 ml of each media (all in duplicates) were
217 inoculated with fresh cultures of each strain, grown in MA-5, MA-20, MB-5 or MB-20 to
218 reach a final absorbance of 0.1 at 600 nm. The respective sterile media (no
219 microorganisms inoculated) were used as controls. All tubes were incubated for 5 days
220 at 200 rpm and 30 °C.
221
222 **Table 1:** Composition of the different culture media for growth and production of
223 biosurfactants by the selected strains: vinasse 1 (V1), black liquor (BL), distilled water
224 (dH_2O), nutrient broth (NB), and pH of each formulation.

	V1 % (v/v)	BL % (v/v)	dH_2O % (v/v)	NB (g/l)	pH
MA-5	5	-	95	8	5.43
MB-5	-	5	95	8	8.83
MA-20	20	-	80	8	4.89
MB-20	-	20	80	8	11.05

225

226 Optical density (*OD*), determined by measuring the absorbance at 600 nm in a
227 spectrophotometer (model 752, BIOTRAZA), and *γ,* using the method described before,
228 of each culture were measured on the third and fifth days of culture.

229

230 **2.4 Strain identification and construction of the phylogenetic trees**

231 Genomic DNA from the selected microorganisms was extracted by using the protocol
232 proposed by (Pospiech and Neumann, 1995). For that, cells were cultured in MA-5 and
233 MB-5 (for strains isolated from vinasse and from black liquor, respectively) at 30 °C and
234 200 rpm for 24 h. Then, cells were harvested by centrifugation (10 min at 10.000 rpm in
235 Eppendorf Centrifuge, 5415R). The DNA was solubilized in sterile MILLI-Q ®water and
236 shipped to Macrogen (Seoul, South Korea) for PCR amplification and sequencing.
237 Genes encoding 16S rDNA were amplified by PCR using universal primers 27F (5'-
238 AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') and the
239 inter-primers 785F (5'-GGATTAGATACCCTGGTA-3') and 907R (5'-
240 CCGTCAATTCMTTTRAGTTT-3') and genes encoding 18S rDNA were amplified by PCR
241 using universal primers NS1 (5'-GTAGTCATATGCTTGTCTC-3') and NS24 (5'-
242 AAACCTTGTTACGACTTTTA-3'). The 16S rDNA and 18S rDNA gene sequences were later

243 analyzed with the Chromas software (version 2.6.6). Overlapping sequences were
244 removed and the complete rDNA sequence of each strain was generated. Nucleotide
245 sequences were analyzed by BLAST (blastn) search and compared against bacterial 16S
246 and yeast 18S rDNA sequences available in the Genbank database (Altschul et al., 1990).
247 Sequences were aligned by using Clustal W 1.74 (Thompson et al., 1994), followed by
248 the construction of neighbor-joining phylogenetic tree using MEGA4
249 (http://www.megasoftware.net) (Tamura et al., 2007). The nucleotide sequences were
250 submitted to the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/).
251

252 **2.5 Data analysis**

253 *2.5.1 Statistical tools*

254 The average *γ* and the standard deviation (*S*) were calculated, as well as the γ reduction
255 (*Δγ*) in each case referred to the sterile medium considered as control. This information
256 was used for the selection of strains following two consecutive steps. In the first one,
257 those strains where $\Delta\gamma > S$ at any time, were selected.
258 A Student's *t*-test was applied to the data obtained in the second selection step, to
259 search for significant differences ($p < 0.05$) among the γ values reached in each
260 production media and their respective sterile medium. This parametric statistical test
261 was used after verifying the normal distribution of data ($p > 0.0001$) through the
262 Shapiro-Wilks test (Shapiro and Francia, 1972).
263 All statistical analyses were carried out using the InfoStat software (Di Rienzo JA et al.,
264 2016).
265

2.5.2 Biosurfactant Capacity Index

For those strains that showed a significant $\Delta\gamma$ in the second selection step, a Biosurfactant Capacity Index (BC_{index}) was defined and calculated as:

$$BC_{index} = \frac{\Delta\gamma_{max}}{OD_{max}} \quad (6)$$

where $\Delta\gamma_{max}$ is the maximum decrease observed in γ and OD_{max} the maximum optical density for each strain. A higher BC_{index} indicates a higher capacity of each producer strains to decrease the γ.

3. RESULTS

3.1 Strain isolation and selection steps

A total of 39 isolates were obtained: 19 from vinasse (MA) (acidophilic, named a1 to a19) and 20 from black liquor (MB) (alkaliphilic, named b1 to b20). Isolates showed beige/white, yellow, and red creamy colonies with circular, punctate, and irregular shapes and with whole and lobed edges.

Then, the capacity of the 39 strains to reduce the γ was assessed in liquid media. The γ of the MB-5 (5% sterile black liquor, 8 g/l NB, pH= 8.83) was 56.45 ± 2.52 mN/m, while it was 54.77 ± 2.46 mN/m for the MA-5 (5% sterile vinasse, 8g/l NB, pH=5.43) (Table 1). These average values minus their corresponding standard deviations were taken as reference ($\gamma_{A5\text{-}S}$=52.31 mN/m for MA-5 and $\gamma_{B5\text{-}S}$ =53.93 mN/m for MB-5; lower dashed lines, Figure 3), and all the strains showing γ below them were considered within the selection criteria (Figure 3).

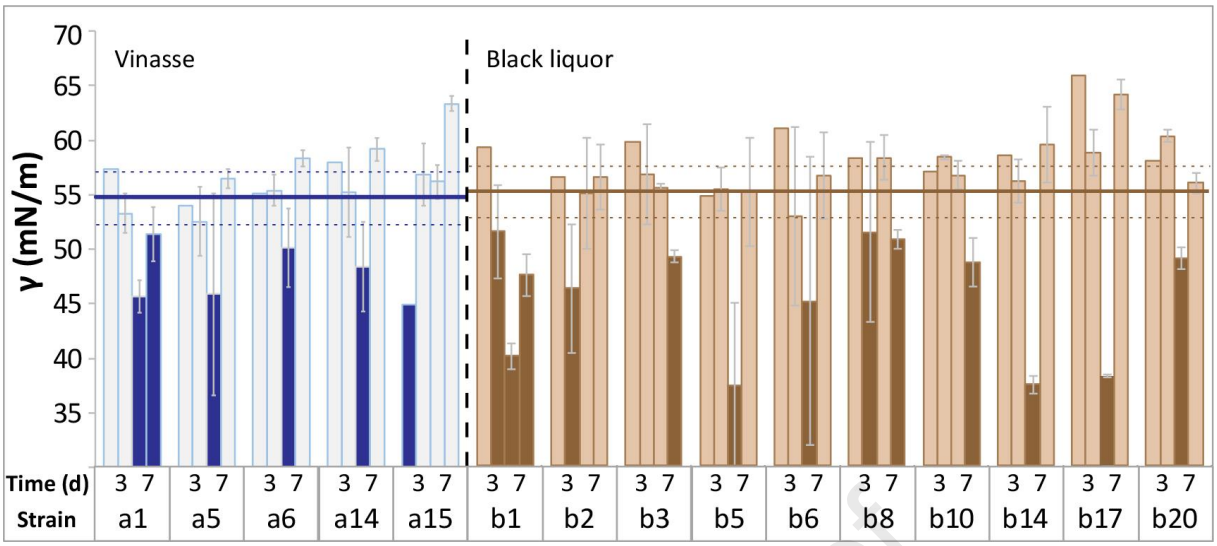


Figure 3: Surface tension (γ in mN/m) from acidophilic (a: blue) and alkaliphilic (b: brown) strains in each of the experiments (two replicas), measured at 3 and 7 days. Solid lines are the reference values for sterile media MA-5 (γ_{A5}: vinasse) and MB-5 (γ_{B5}: black liquor). Dotted lines indicate the deviation (γ_{A5} or $\gamma_{B5} \pm S$). Dark bars (blue or brown) are the ones considered to meet the selection criteria at least in one of the measurements (their γ were below the reference value minus their standard deviations). The error bars (shaded gray) are the average of two γ measurements done for each replica.

Of the total strains obtained from both industrial by-products, 26% isolated from vinasse (5/19; a1, a5, a6, a14, a15) and 50% of those from black liquor (10/20; b1, b2, b3, b5, b6, b8, b10, b14, b17, b20) were able to decrease the γ (Figure3). These strains were then subjected to the second selection step. In this case, the γ of the MB-20 (20% sterile black liquor, 8 g/l NB, pH= 11.05) was 49.91 ± 2.87 mN/m, while it was 54.33 ± 5.23 mN/m for the MA-20 (20% sterile vinasse, 8 g/l NB, pH=4.89) (Table 1). These average values minus their corresponding standard deviations were taken as reference (49.10 mN/m for MA-20 and 47.04 mN/m for MB-20).

Table 2: Surface tension values (γ in mN/m), Optical Density (*OD*) and their respective standard deviation (± *S*), and Biosurfactant Capacity Index (BC_{index}) calculated from acidophilic (a) and alkaliphilic (b) strains in vinasse and black liquor media, respectively. Each medium was formulated with nutritive broth and vinasse or black liquor at 5% (MB-5 and MA-5) and 20% (MB-20 and MA-20). Statistically significant differences between the mean of the γ of the production medium and the sterile medium (control) were considered when *p*-values were less than 0.05 (marked in bold). The BC_{index} (in bold) was only calculated when significant *p*-values were observed.

	5% Vinasse (MA-5) or black liquor (MB-5)							**20% Vinasse (MA-20) or black liquor (MB-20)**					
	Day 3			**Day 5**				**Day 3**			**Day 5**		
Strain	***γ*** *(± S)*	***p-value***	***OD*** *(± S)*	***γ*** (± *S*)	***p-value***	***OD*** *(± S)*	BC_{index}	***γ*** *(± S)*	***p-value***	***OD*** *(± S)*	***γ*** *(± S)*	***p-value***	***OD*** (± *S*)
a1	65.13 (± 1.95)	0.993	0.98 (± 0.21)	51.73 (± 3.00)	**0.010**	1.58 (± 0.06)	**8.48**	59.13 (± 1.90)	0.96	1.37 (± 0.03)	60.49 (± 2.71)	0.986	1.54 (± 0.03)
a5	58.33 (± 4.15)	0.398	1.01 (± 0.16)	36.15 (± 7.90)	**0.007**	1.30 (± 0.42)	**16.98**	57.53 (± 1.03)	0.899	1.05 (± 0.61)	63.10 (± 10.08)	0.952	1.56 (± 0.05)
a6	57.73	0.487	1.01	49.14	**0.001**	1.21	**7.05**	58.16	0.933	0.94	59.10	0.968	1.32

	(± 3.87)		(± 0.02)	(± 5.58)		(± 0.01)		(± 1.42)		(± 0.28)	(± 1.96)		(± 0.07)
a14	63.11 (± 1.12)	0.959	0.99 (± 0.18)	57.98 (± 7.60)	0.454	1.47 (± 0.12)	-	58.36 (± 2.42)	0.941	1.54 (± 0.00)	60.92 (± 6.83)	0.955	1.72 (± 0.04)
a15	57.44 (± 1.88)	0.337	0.24 (± 0.34)	57.54 (± 7.89)	0.411	0.54 (± 0.35)	-	51.58 (± 1.64)	0.132	0.89 (± 0.28)	59.94 (± 4.47)	0.963	1.37 (± 0.00)
b1	53.50 (± 1.33)	0.280	0.30 (± 0.10)	48.89 (± 4.49)	**0.003**	0.42 (± 0.11)	**17.66**	53.58 (± 4.02)	0.951	0 (± 0.28)	58.05 (± 2.58)	1	0 (± 0.07)
b2	54.90 (± 1.38)	0.127	0.43 (± 0.14)	50.78 (± 2.60)	**0.002**	0.28 (± 0.06)	**13.15**	54.91 (± 3.45)	0.989	0.30 (± 0.06)	58.65 (± 2.86)	1	0 (± 0.10)
b3	55.71 (± 3.94)	0.355	0.39 (± 0.01)	55.16 (± 2.79)	0.220	0.63 (± 0.04)	-	56.10 (± 4.20)	0.993	0 (± 0.22)	61.22 (± 2.71)	1	0 (± 0.10)
b5	55.46 (± 2.79)	0.298	0.49 (± 0.18)	54.53 (± 1.44)	0.084	0.84 (± 0.26)	-	53.95 (± 2.89)	0.982	0.07 (± 0.01)	60.79 (± 2.46)	1	0 (± 0.12)
b6	53.71	0.127	0.43	54.02	0.090	0.58	-	50.66	0.612	0	58.75	1	0

	(± 4.81)		(± 0.10)	(± 3.01)		(± 0.21)		(± 5.62)		(± 0.18)	(± 3.31)		(± 0.00)
b8	63.35 (± 1.90)	1.000	0.91 (± 0.27)	64.92 (± 4.37)	0.998	1.21 (± 0.16)	-	52.34 (± 5.57)	0.818	0.05 (± 0.22)	51.86 (± 1.73)	0.869	0.22 (± 0.02)
b10	60.10 (± 3.59)	0.959	0.47 (± 0.00)	60.56 (± 2.30)	0.989	0.83 (± 0.00)	-	57.34 (± 1.82)	1	0.11 (± 0.27)	58.97 (± 4.49)	0.994	0.08 (± 0.03)
b14	59.37 (±1.91)	0.956	0.48 (± 0.04)	54.70 (± 1.62)	0.108	0.65 (± 0.07)	-	57.12 (± 3.42)	0.999	0 (± 0.00)	58.84 (± 2.59)	1	0 (± 0.14)
b17	54.05 (± 4.33)	0.213	0.62 (± 0.15)	49.12 (± 2.55)	**0.000**	0.60 (± 0.01)	**11.75**	55.93 (± 0.96)	0.999	0 (± 0.38)	59.13 (± 2.67)	1	0 (± 0.19)
b20	64.79 (± 1.96)	1.000	1.31 (± 0.16)	66.69 (± 1.33)	1.000	1.48 ± 0.34	-	56.12 (± 3.02)	0.957	0.30 (± 0.63)	63.15 (± 4.89)	1	0.43 (± 0.03)

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On the fifth day, six strains: a1, a5, and a6 (acidophilic) and b1, b2, and b17 (alkaliphilic) cultured in MA-5 and MB-5, respectively, produced a statistically significant decrease in γ for sterile media ($p < 0.05$, Table 2). None of the isolated strains seeded in MA-20 and MB-20, produced statistically significant decreases in γ when compared with their respective sterile media (Table 2, $p > 0.05$). Therefore, BC_{index} was not calculated.

Among the six strains with ability to produce a statistically significant decrease in γ, the two that presented the best potential biosurfactant capacity, evidenced by their indexes, were the acidophilic a5 (BC_{index} = 16.98), and the alkaliphilic b1 (BC_{index} = 17.66, Table 2). In both cases, a pronounced decrease in γ was observed, related to the appreciable increase in *OD*, compared to the other strains analyzed (Figure 4). The alkaliphilic strain b1, that was in the exponential growth phase, presented the highest BC_{index} (Table 2, Figure 4).

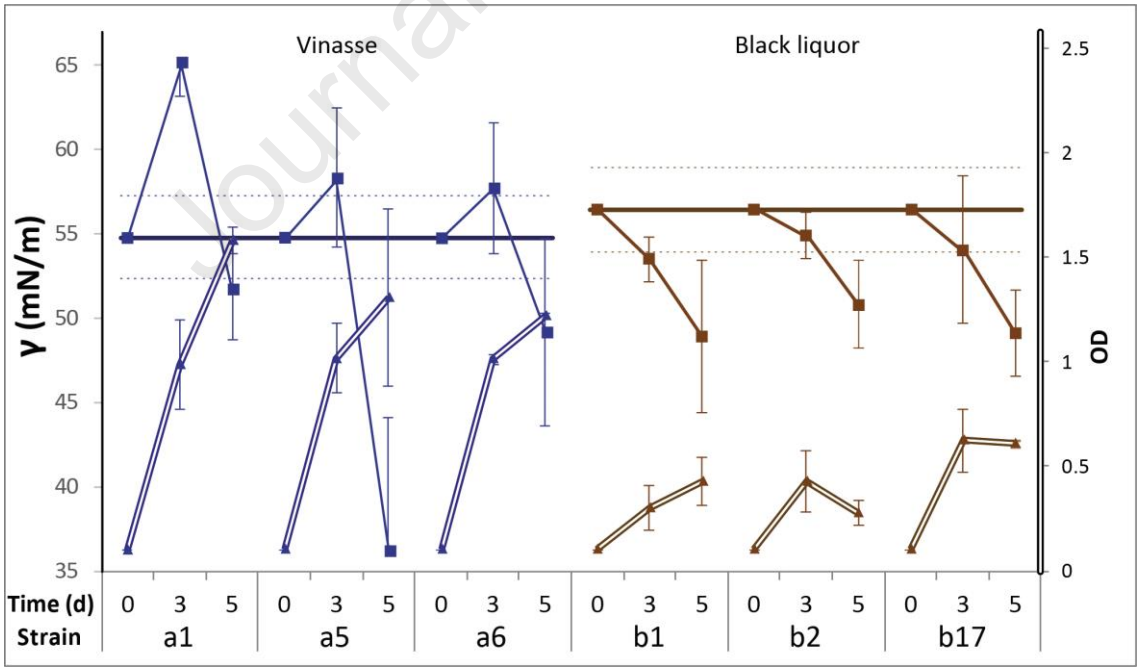


Figure 4: Surface tension (γ: single line) and optical density (*OD*: double line) for acidophilic (a: blue) and alkaliphilic (b: brown) selected strains, cultured in 5% of vinasse (MA-5) or black liquor (MB-5) media, measured at 3 and 7 days. Horizontal dark lines

correspond to the *γ* of the sterile medium (control) while dotted lines indicate their standard deviation. Symbols (squares and triangles) represent the average of two experiments and error bar the standard deviation.

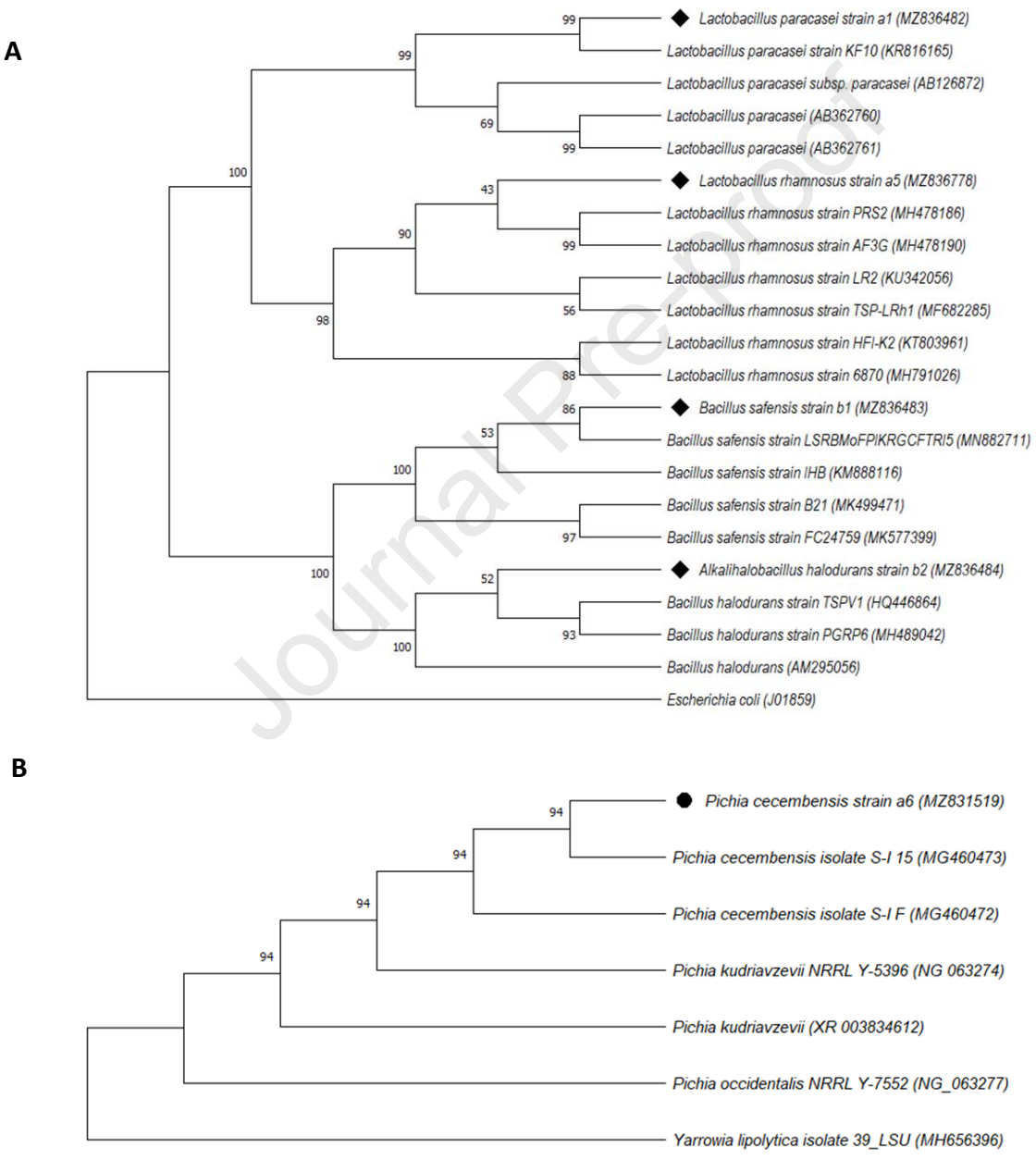
Although in general, the BC_{index} were higher for the alkaliphilic strains than for the acidophilic ones, the latest in MA-5, reached an *OD* that was twice higher on the third day and three times higher on the fifth day compared to the values achieved by the alkaliphilic ones in MB-5 (Figure 4).

Acidophilic and alkaliphilic strains seems to behave differently in terms of biosurfactant production and growth. In the case of a1, a5 and a6 (acidophilics), *γ* increased during the exponential growth phase (probably due to their metabolism) followed by a marked decrease (Figure 4). This may be associated with the fact that biosurfactants production for this group of strains began after the exponential phase of growth (thus, they are secondary metabolites). In contrast, for strains b1, b2, and b17 (alkaliphilics), the decrease in *γ* and therefore, the production of biosurfactants was associated with the growth (thus, they are primary metabolites) (Figure 4).

3.2 Strain identification and phylogenetic trees

From previous assays, we observed that three acidophilics and three alkaliphilics strains were the most promising for biosurfactant production. Five strains were partially identified through the sequencing of their 16S rDNA fragment for bacteria strains (a1, a5, b1, and b2) and for 18S rDNA fragment for yeast strain (a6). The sequences obtained were uploaded in the NCBI database (Figure 5). Bacteria belonging to the phylum Firmicutes, class Bacili, genera *Bacillus* and *Lactobacillus* were identified (Figure 5 A). Two of the alkalophilic strains, b1 and b2, presented high homology with *Bacillus*

351 *safensis* (99.97%) and *Alkalihalobacillus halodurans* (99.66%), respectively. Strain a5
352 showed high homology (99.60%) with *Lactobacillus rhamnosus* and a1 with *Lactobacillus*
353 *paracasei* (99.30%). Finally, isolate a6 was identified as *Pichia* sp. closely related to *Pichia*
354 *cecembensis* (Figure 5 B). One alkaliphilic isolate (b17) remained unidentified by the
355 methods used.



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358 **Figure 5**: Phylogenetic tree for A) bacteria isolates (GenBank access code: MZ836482,
359 MZ836483, MZ836484, and MZ836778) sequences (black diamonds) and for B) the yeast

isolate (GenBank access code: MZ831519) sequence (black dot). The analysis for both cases was performed with sequences obtained from the GenBank, NCBI database.

4 DISCUSION

For several years, much emphasis was put on the capacity of extremophilic strains (Singh, 2012) to produce a wide variety of extremoenzymes that are already transforming the biotechnological world (Raddadi et al., 2015; Ravi Durvasula, 2018). Recently, attention has focused on biosurfactants production by extremophilic strains under extreme conditions of pH, salinity and temperature (Schultz and Rosado, 2020). The potential industrialization of biosurfactants (Farias et al., 2021) includes oil field, industrial cleaning, food, cosmetic, pharmaceutical, medical, agriculture, mining (metals), construction, and nanotechnology. The most important commercial use of biosurfactants is in high-end drugs and cosmetics (Drakontis and Amin, 2020). Besides this, synthetic surfactants continue to monopolize the market, as they are cheaper and easier to obtain than biosurfactants. The current challenge is to search strategies to reduce the production costs. In this this work, we were able to successfully isolate extremophilic strains from vinasse (acidic) and black liquor (alkaline), two by-products of the sugar cane industry. Some of these strains could reduce the γ when growing in acidic and alkaline production media, respectively. To evaluate the biosurfactant production, the yields and capacities of the specific techniques applied for this purpose were considered (Chen et al., 2007). Qualitative or semi-quantitative methods such as the oil dispersion test or the emulsification index are widely used (Biniarz et al., 2017; Ibrahim et al., 2020; Joy et al., 2017; Silva et al., 2021). However, their lack of accuracy can lead to misleading results (Uzoigwe et al., 2015). Although quantitative techniques

384 are a better choice, their main disadvantage is that they cannot be applied in "high-
385 throughput screening". This complicates the selection of many potentially candidate
386 strains (among several) simultaneously (Chen et al., 2007). In this work, we adapted the
387 quantitative pendant drop profile method (ADSA) with a multi-needle cell built in-house,
388 to work with multiple samples in parallel and thus, to improve the performance and the
389 ability to evaluate the capacity of the analyzed microorganisms. In our case, the first
390 screening step using the method proposed, allowed us to select 15 out of 39 strains,
391 isolated from media prepared with vinasse and black liquor, as they showed the best
392 capability of reducing the *γ* at 3 and 7 days. In the same way, a second screening step
393 carried out with the 15 strains, let getting 6 strains (a1, a5, ab, b1, b2 and b17) with the
394 highest potential of biosurfactant production.

395 The synthesis of biosurfactants is favored when a source of soluble carbon such as
396 glycerol, glucose, mannitol or ethanol and insoluble carbon source like n-alkanes, oil,
397 lignocellulose or corn powder are available (Jahan et al., 2020). In this work, we used
398 vinasse and black liquor as carbon source in addition to nutrient broth, for biosurfactant
399 production by strains isolated from these by-products.

400 The acidophilic strains a1, a5, and a6 isolated from media prepared with vinasse, showed
401 a *γ* reduction from 54.77 mN/m to 51.73, 36.15 and 49.14 mN/m, respectively, when
402 using 5% (v/v) vinasse and pH of 5.4. Lima and Souza (2014) found that a *Bacillus subtilis*
403 strain isolated from a soil in São Cristóvão, Sergipe, Brazil, reached a *γ* reduction of 32.78
404 mN/m (14% reduction) when compared to the sterile medium using 55% (v/v) of vinasse
405 and pH 6.5. On the other hand, Oliveira and Garcia (2013) used a *Bacilus pumilus* strain
406 (CCT 2487) obtained from the Tropical Foundation of Research and Technology "André

Tosello" (Campinas, SP) to produce biosurfactants. In this case, the *γ* decreased up to 45 mN/m when using vinasse (5%) and residual frying oil as media. Naspolini et al. (2017) selected a *Pseudomonas aeruginosa* PA1, previously isolated from oil wells in Northeast Brazil, and obtained a decrease in *γ* up to 29.2 mN/m from diluted vinasse (1:1) without adding nitrogen and at pH 7, after 240 h of production.

The acidophilic strain *Lactobacillus paracasei* a1, reduced the *γ* from 54.77 mN/m to 51.73 mN/m (6% when compared to the sterile medium) when using 5% (v/v) vinasse and pH of 5.4 (Hippolyte et al., 2018). Also, Hippolyte and collaborators (2018) obtained a biosurfactant produced by a *Lactobacillus paracasei* subsp. *tolerans* N2 strain, which showed optimal conditions for *γ* reduction using 6.09 g/l peptone and 6.35% (w/v) of molasses at 33 °C for 48 h.

In this work, the strain *Lactobacillus rhamnosus* a5 reduced the *γ* in 34% when compared to the sterile medium (from 54.77 mN/m to 36.15 mN/m, $p = 0.007$, Table 2). Ghasemi and co-workers studied the probiotic bacterium *Lactobacillus rhamnosus* PTCC 1637 using date syrup from low-quality dates for biosurfactant production. The highest reduction in *γ* was achieved by fermentation in a bioreactor, reaching 45% regarding the sterile medium (Ghasemi et al., 2018). Interestingly, the optimal production time was observed at the fifth day, like *Lactobacillus rhamnosus* a5 strain (Figure 5). Only one yeast was selected from acidophilic conditions, *Pichia cecembensis* a6, which reduced the *γ* from 54.77 mN/m to 49.14 mN/m (10% reduction). Other researchers obtained a biosurfactant produced by *Pichia anomala* PY1 isolated from fermented food (Thaniyavarn et al., 2008). The production was optimized including 4% of soybean oil as carbon source. Under these conditions, the *γ* of the medium decreased from 50 mN/m to 28 mN/m (44% *γ* reduction) (Thaniyavarn et al., 2008).

Alkaliphiles have enormous potential and surprising versatility when it comes to producing their primary and secondary metabolites, which include enzymes, exopolysaccharides, organic acids, antibiotics, carotenoids, suitable solutes, bioplastics, and biosurfactants among many more (Khalikova et al., 2019). The alkalophilic strains b1, b2 and b17 reduced the *γ* from 56.45 mN/m to 48.89 (13%); 50.78 (10%) and 49.12 (13%) mN/m, respectively, when using 5% (v/v) black liquor and pH of 9. In addition to vinasse, black liquor represents an interesting source of extremophilic microorganisms. Yang et al. (2010) used wheat straw black liquor from a pulp mill to isolate bacteria; two of them named *Halomonas* sp. 19-A and Y2 showed a great metabolic versatility, even under extreme conditions of pH and salinity (Yang et al., 2010).

We were able to verify that *Bacilus* sp. b1 with high homology with *Bacillus safensis* reduced the *γ* by 13%, also showing the best BC_{index} of all the selected strains (17.66, Table 2), in the presence of 5% (v/v) black liquor and pH 9. Das and Kumar (2019) reported that *Bacillus safensis* J2 could reduce the *γ* by producing a biosurfactant molecule using sugar cane bagasse in the media composition.

Finally, *Alkalihalobacillus halodurans* b2 isolated from black liquor, and supplemented with 5% black liquor, was able to diminish the *γ* in 10% when compared to the sterile medium. This is the first research that reports the ability of this extremophilic strain to produce biosurfactant. Other properties, such as its capacity to produce exopolysaccharide with strong emulsifying activity, were observed for this strain (Wang et al., 2020). This turns such strain into the most promising one for biosurfactant production.

Four of the five strains selected in this work are bacillary bacteria (two *Lactobacillus*, one *Bacillus* and one *Alkalihalobacillus*). Probiotic lactic acid bacteria (like *Lactobacillus* spp.) have an important role in antimicrobial activity against several pathogens through interfering with biofilm formation. Proteins, glycolipids, glycoproteins or glycolypeptides mainly represent the nature of the biosurfactants of this bacterial genus (Satpute et al., 2016). Large-scale production of biosurfactants by *Lactobacillus* sp. has become possible by the usage of renewable substrates such as: animal fat waste, coffee processing residues, dairy industry, food and fruit processing industry, oil processing mills, which also reduce the impact of such waste disposal in the environment (Banat et al., 2014; Satpute et al., 2016).

Even though the results reached here have a share of novelty and interest in the biotechnology area, further studies are needed to identify the nature of the biosurfactants produced by the isolated strains. Moreover, the optimization of the production media using vinasse, black liquor and other industrial by-products is the next step to follow. This will be a key point to achieve higher yields with lower production costs.

CONCLUSIONS

- In this work 39 (thirty-nine) strains were isolated from vinasse and black liquor, two by-products form the sugar industry: 19 from vinasse (acidophilic: a1 to a19) and 20 from black liquor (alkaliphilic: b1 to b20). This showed the possibility of finding interesting microorganisms in substrates with extreme conditions.
- The potential of all the isolated strains for biosurfactant production was assessed through the surface tension reduction in two screening steps. From this, three

476 acidophilic strains (a1, a5, and a6) and three alkalophilic strains (b1, b2, and b17)
477 were selected.

478 • A modified drop profile method with a multi-needle cell built in-house was
479 proposed and successfully applied. It allowed to reduce the time of surface
480 tension measurement relative to conventional quantitative methods.

481 • Five of the 39 isolated strains were sequenced and partially identified as:
482 *Lactobacillus paracasei* a1, *Lactobacillus rhamnosus* a5, *Bacillus safensis* b1
483 *Alkalihalobacillus halodurans* b2 and *Pichia cecembensis* a6. The two strains
484 *Lactobacillus rhamnosus* a5 and *Bacillus safensis* b1 presented the best potential
485 biosurfactant capacity evidenced by their BC_{index}.

486 • The use of industrial by-products as substrates for the production media add
487 value to these residues and allow finding new super-producing strains, which will
488 be assessed in future experiments, since they can expand the application field.

489

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496 **AUTHOR CONTRIBUTION**

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498 preparation.

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500 preparation.
501 Diego Sanguino-Jorquera: Formal analysis, Data curation.
502 Verónica Beatriz Rajal: Visualization, Writing- Reviewing and Editing.
503 Verónica Patricia Irazusta: Conceptualization, Supervision, Funding Acquisition,
504 Writing- Original draft preparation.
505

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HIGHLIGHTS

- Microorganisms isolated from by-products can potentially produce biosurfactants
- Vinasse and black liquor by-products are economic substrates for biosurfactant production
- A high throughput screening method was proposed to assess surface tension reduction
- Strains of bacteria and yeast isolated from by-products can reduce the surface tension of medium
- *Lactobacillus* sp. and *Bacillus* sp. strains produced the lowest surface tension of the growth medium

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: