1 **Isolation and characterization of two glycolipopeptids biosurfactants**
2 **produced by a *Lactiplantibacillus plantarum OL5* strain isolated from green**
3 **olive curing water**

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23 **Abstract:**

24 Microbial surfactants are natural amphiphilic compounds with high surface activities and
25 emulsifying properties. Due to their structural diversity, low toxicity, biodegradability, and
26 chemical stability in different conditions, these molecules are potential substitutes for chemical
27 surfactants; their interest has grown significantly over the last decade. The current study focuses
28 on the isolation, identification, and characterization of a lactic acid bacteria that produce two
29 forms of biosurfactants. The OL5 strain was isolated from green olive fermentation and
30 identified using MALDI/TOF and DNAr16S amplification. Emulsification activity and surface
31 tension measurements were used to estimate biosurfactant production. The two biosurfactants
32 derived from *Lactiplantibacillus plantarum* OL5 presented good emulsification powers in the
33 presence of various oils. They were also shown to have the potential to reduce water surface
34 tension from 69 mN/m to 34 mN/m and 37 mN/m within a critical micelle concentration (CMC)
35 of 7 mg/ml and 1.8 mg/ml, respectively, for cell bound and extracellular biosurfactants. Thin
36 layer chromatography (TLC) and FT-IR were used to analyze the composition of the two
37 biosurfactants produced. the obtained data revealed that the two biomolecules consist of a
38 mixture of carbohydrates, lipids and proteins. We demonstrated that they are two anionic
39 biosurfactants with glycolipopeptide nature which are stable in extreme conditions of
40 temperature, pH and salinity.

41 **Key words:**

42 *Lactobacillus*, Biosurfactants, FTIR analysis, Glycolipopeptide

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45 **Introduction**

46 Microbial biosurfactants are a structurally diverse group of surface-active agents produced by
47 a wide range of microorganisms from various environmental habitats, primarily bacteria,
48 actinomycetes, yeast, and filamentous fungi, that either adhere to the cell surface or are
49 produced extracellularly.(Behzadnia et al. 2020; Wang et al. 2020). Biosurfactants bind to
50 liquid/air or water/oil interfaces, changing surface properties and emulsifying
51 activities(Mouafo, Mbawala, and Ndjouenkeu 2018; Velraeds et al. 1996). The size of
52 biosurfactants, such as low-molecular-weight biosurfactants and high-molecular-weight
53 biosurfactants, can be used to identify them(Morais et al. 2017). When compared to chemically
54 synthesized counterparts, the diverse chemical structures of biosurfactants (consisting of lipids,
55 proteins, polysaccharides and carboxyl, amino, and phosphate functional groups) provide many
56 advantages, such as high biodegradability, low toxicity, effectiveness under extreme physical
57 conditions(Behzadnia et al. 2020)greater ecological acceptability and the ability to be produced
58 from renewable substrates(Makkar and Cameotra 2002)A perfect biosurfactant for use in the
59 pharmaceutical, medical, cosmetics, and food industries must have these essential
60 characteristics: biosurfactants should be obtained from organisms that are generally regarded
61 as safe (GRAS) to avoid the occurrence of critical situations that may arise as a result of their
62 pathogenic origin, as well the requirement of a minimum concentration of them to achieve
63 specific functionality for the proposed applications(Sharma et al. 2021). Due to their remarkable
64 potential and rules governing their safety for several uses, including the food sector,
65 *Lactobacillus* (LAB) strains truly believe in the GRAS concept. Since they are not pathogenic,
66 they have received the majority of scientific attention in order to produce biosurfactants. These
67 surfactants generated from LAB are often multicomponent combinations of proteins, lipids, and
68 carbohydrates (Sharma et al. 2021; Sharma and Saharan 2016).*Lactobacillus* biosurfactants
69 have antimicrobial and antioxidant properties and are useful as bioemulsifiers, texture

70 enhancers, and heavy metal quenchers. Because of their versatile functionality, biological
71 surfactants have been proposed as an alternative to chemically synthesized
72 surfactants(Saravanakumari and Mani 2010). Several LAB strains have been reported to
73 produce biosurfactants as well as their functional properties. *Lactobacillus fermentum,*
74 *Lactobacillus paracasei,Lactiplantibacillus plantarum, Lactobacillus helveticus, Enterococcus*
75 *faecium, Lactobacillus acidophilus, Lactobacillus pentosus, Lactococcus lactis, Streptococcus*
76 *thermophilus, Pediococcus acidilactici,* and *lactobacillus sp* (Anon n.d.; Ferreira et al. 2017;
77 Morais et al. 2017; Saravanakumari and Mani 2010; Sharma et al. 2015, 2021; Sharma and
78 Saharan 2016)*Lactobacillus plantarum* recently renamed as *Lactiplantibacillus*
79 *Plantarum*(Zheng et al. 2020) is a kind of lactic acid bacteria that has ecological and metabolic
80 adaptability, allowing it to live in a variety of ecological niches such as fermented foods, meats,
81 plants, and the mammalian gastro-intestinal tract. It is a gram-positive heterofermentative
82 microaerophilic bacterium that has a rod-like morphology and can be found singly or in short
83 chains(Dadar et al. 2021). Because of their unique evolutionary history, strains of this species
84 have a wide range of probiotic characteristics and have shown promise in the production of a
85 wide range of primary and secondary metabolites, including lactic acid, hydrogen peroxide,
86 bacteriocins, biosurfactants, enzymes, and other related compounds. However, efforts were
87 made in the current study to assess the ability of a newly isolated bacterium, *Lactiplantibacillus*
88 *plantarum* OL5, to produce two types of biosurfactants and characterize their functional
89 properties.

90 **Methods**

91 **1. Isolation of lactic acid bacteria:**

92 Lactic acid bacteria (LAB) strains were isolated from natural samples (water from the draining
93 of fresh cheese from an industrial company, fresh camel or goat's milk, stomach and intestine
94 of battles, vaginal swabs, and olive salting water). Stock solutions were prepared for each

95 sample. In the case of a solid sample, 1g of each sample is suspended in 9 ml of physiological
96 water (9‰ NaCl). If it is a liquid sample, 1mL of the sample is added to 9 ml of physiological
97 water. From these preparations, successive dilutions (10^{-2} to 10^{-5}) in physiological water were
98 made. Then 100 µL of each dilution was cultured anaerobically on MRS agar plates and
99 incubated at 37° C for 24 h.

100 **2. Identification and characterization of isolated strains:**

101 ***2.1. Phenotypic identification of lactic acid bacteria (LAB)***

102 The standard identification procedures based on morphological, cultural, and biochemical
103 features were followed in selecting all the probable isolates within the scope of the present
104 study.

105 Cells were detected with Gram staining under a microscope (oil immersion, 100×). The Shape
106 of the cells (cocci, bacilli, and coccobacilli) and arrangement of cells (scattered, bunches, and
107 chain) along with the Gram reaction were observed. Further tests with catalase and oxidase
108 have also been conducted, and the fermentation profile of the sugars of the isolated strains was
109 carried out by the API 50 CH gallery. The selected isolates were maintained on MRS agar slants
110 at 4° C. Whereas stock cultures were kept in cryovials with their culture broth containing 30%
111 sterile glycerol at -20° C until use.

112 ***2.2. Genotypic characterization of biosurfactant-producing LAB***

113 Bacterial isolate which shown high biosurfactants production was selected with regard to be
114 identified. PCR amplification of 16S rDNA gene was carried using Universal primers Forward
115 Fd1 (5′AGT TTG ATC CTG GCT CAG 3′) and Reverse Rd1 (5′AAG GAG GTG ATC
116 CAGCC3′) (Mnif et al. 2015, 2022).

117 The 25 µL of PCR mixture contained: 0.2 mM of deoxy nucleoside triphosphate, 1.32 µM
118 concentration for each primer, 0.5U of DNA polymerase, 5 µL of 5 × buffer, and 1 µL (5 ng)

119 of total DNA template. The PCR program was 94 °C for 3 min followed by 35 cycles consisting
120 of denaturation at 94 °C for 45 s, annealing at 59 °C for 1 min and extension at 72 °C for 2 min.
121 After amplification, the PCR product was purified (Promega Gel Extraction Kit, Biogène,
122 Tunisia) and sequenced with ABI PRISMTM 3100 Genetic Analyzer (Applied Biosystems,
123 USA). The obtained sequences were aligned and compared with sequences from the Gene Bank
124 database of the National Center of Biotechnology Information (NCBI) (http:// www. ncbi. nlm.
125 nih. gov) using the nucleotide–nucleotide blast (BLASTn) network service (Mnif et al. 2022).
126 The phylogenetic tree was drawn by the software MEGA version 10 using the neighbor joining
127 method.

128 **2.3. *Biosurfactant-producing LAB identification of strains by the MALDI TOF test*:**

129 MALDI-TOF mass spectrometry is a technique for separating molecules transformed into ions
130 according to their m/z ratio (where m=mass and z = charge). One of its major applications is its
131 contribution to proteomics, aimed at identifying all the proteins contained in the cell. It allows
132 the identification of bacteria by analyzing their total proteins (ribosomal proteins and
133 membrane-associated proteins) based on the fact that each species has its own proteome. Once
134 these proteomes are known and listed, they are entered and compared to a reference database.
135 The MALDI-TOF MS analysis was performed using a Brucker apparatus (Model: Microflex
136 LT/SH from Bruker Daltonics, Germany). The identification of the bacterial isolate was
137 performed by the Bruker Biotyper software. This test was carried out on two colonies of OL5
138 sown in streaks, one on a sloping tube and the other on a Petri dish.

139 **3. Screening of LAB biosurfactant Producing Strain:**

140 ***3.1. Hemolytic activity determination:***

141 The screening of biosurfactants producing strains was performed in the first place by the blood
142 hemolysis technique. Firstly, pure colonies of the isolated strains were streaked on blood agar
143 medium. The selection of the biosurfactant-producing strain was based on the presence of a

144 lysis halo around the colony after 24 h incubation at 37 ºC, since biosurfactants possess
145 hemolytic activity. The strains selected as biosurfactant producers were purified by successive
146 subcultures on solid MRS medium using the quadrant technique(Mnif et al. 2022).

147 **3.2. *Emulsifying activity determination*:**

148 the emulsification index (E24) was measured using the method described for the first time by
149 Panchal and Zajic (1978) as a qualitative test visualized by the formation of a creamy emulsion.
150 In fact, an emulsion is defined as a heterogeneous system, composed of an immiscible liquid
151 dispersed as microscopic droplet in another liquid continuous phase. Biosurfactants may
152 stabilize (emulsifiers) or destabilize (demulsifies) the emulsion. To do so, equal volumes (2
153 mL) of aqueous sample and 2 ml of corn oil were added to the test tube and the mixture was
154 thoroughly agitated in a vortex mixer for 2 min. Upon standing, a smooth emulsion was formed
155 when an emulsifier was present. At that point, an emulsification index (E24 %) was calculated
156 by the succeeding equation:

157 Emulsification index (E24) (%) = (Height of Emulsion layer) ×100/ (Total height)

158 (Varjani et al. 2007)

159 The residual activity was calculated using the above formula:

160 $$\text{Residual activity (RA\%)} = \frac{100 - \text{E24 sample}(\%)}{\text{E24 control }(\%)}$$

161

162 ***3.3. Surface Tension (ST) Measurement and Critical Micelle Concentration (CMC)***
163 ***Determination***

164 A ring-type tensiometer (Sigma 700) was used to determine the surface tension of crude
165 glycolipopeptids biosurfactant at different concentrations, using the Du Nouy method at room

166 temperature. Likewise, to determine the (CMC), ST was determined in function of increasing
167 biosurfactants concentration prepared in distilled water. When an abrupt decrease of the ST was
168 reached, the CMC was attained. It corresponds to the concentration at which biosurfactants
169 associates into micelles. Distilled water (69 ± 0.10 mN/m) was used to verify the measurements
170 before each reading, all measurements were taken in duplicates, and the mean value was
171 presented in the results and discussion section.

172 **4. Isolation and purification of the two biosurfactants:**

173 The chosen biosurfactant-producing LAB was inoculated in final volume of 150 ml MRS broth
174 (Peptone (10 g), beef extract (10 g), yeast extract (5 g), dextrose (20 g), Sodium Acetate
175 NaC2H3O2 (5 g), polysorbate 80 (1 g), KH2PO4 (2 g), ammonium citrate (2 g), magnesium
176 sulfate heptahydrate MgSO4 (0.1 g), manganous sulfate tetrahydrate MnSO4 (0.05 g), per 1000
177 mL) for 48 hours at 37 °C in a 250 ml Erlenmeyer flask, with an initial optical density of DO =
178 0.2, and fermentation was carried out in a static condition. The surface tension, the pH, and the
179 biomass during the biosurfactant biosynthesis were determined. For the biomass determination,
180 a volume of 1 ml of the culture was centrifuged for 10 minutes at 7000 rpm. The cell biomass
181 was recovered, dried at 105 °C for 24 h, and reweighed. Dry bacterial biomass was repeatedly
182 weighed until a constant weight was achieved. The fermentation broth was centrifuged at 10000
183 rpm for 20 min at 4 °C using a centrifuge (HEHICH Zentrifugen Rotina 380 R, Germany) to
184 remove the cell biomass. Secreted biosurfactants (EX BIOS) were precipitated from the
185 supernatant with 6N HCl to pH 2. They were recovered after an overnight at 4 °C by
186 centrifugation at 10000 rpm for 20 minutes at 4 °C. Finally, they were suspended in 20 mM
187 Tris-HCl buffer, pH 7. the pH of the suspension was then adjusted to 7 with 1 N sodium
188 hydroxide before being lyophilized(Mnif, Chaabouni-Ellouze, and Ghribi 2012). The removed
189 cell biomass was washed once with deionized water and resuspended in 10 mL of PBS (0.1M
190 of 7.0) in order to extract the cell-bound biosurfactants (CB BIOS). The cell suspensions were

191 incubated at room temperature for 24 h with gentle agitation to release the biosurfactant. The
192 cells were then removed by centrifugation. Subsequently, the cell-free supernatant was filtered
193 using 0.22 µm pore size filter (Axiva, India), dialysed against demineralised water in a dialysis
194 membrane (molecular weight cut-off 6000–8000 Dalton, Himedia, India) and freeze
195 dried(Sharma et al. 2015).

196 **Study of the two biosurfactants stability:**

197 The stability of the two OL5 biosurfactants (CB and EX BIOS) under different physicochemical
198 conditions was evaluated using a solution of 10 mg m/L crude biosurfactant. The influence of
199 pH on emulsification index (E24(%)) was estimated at different pH values ranging from 2.0 to
200 10.0 using glycine–HCl buffer (pH 2.0–3.0), acetate buffer (pH 4.0–5.0), phosphate buffer
201 (pH6.0–8.0) and glycine–NaOH buffer (pH 9.0–10.0) (all at a final concentration of 20 mM
202 (Mnif et al. 2022)). Results were expressed as Residual Activity (RA) towards the activity at
203 neutral pH. For the determination of salt effect, different quantities of NaCl were dissolved in
204 buffer solutions to adjust the salt concentration of test samples from 0 to 40%. Results were
205 expressed as RA towards a negative control without salt addition. Furthermore, the heat stability
206 of the crude biosurfactants was determined by incubating the biosurfactant solution (10 mg/ml)
207 at different temperatures extending from 4 to 60 °C for 60 min, and then cooling at room
208 temperature. The emulsifying activity (E24) of each sample was determined as described above.
209 Likewise, the stability of the OL5 biosurfactants was evaluated after pre-incubation at different
210 pH buffers, salt concentration and temperature by determining the emulsification activity (E24).
211 Results were expressed by RA towards the activities at standard conditions without incubation.
212 All assays were performed in triplicates.

213 **5. Characterization of the two biosurfactants**

214 ***5.1. Determination of ionic character T:***

215 The ionic charge of the two biosurfactants was determined using the agar diffusion method
216 (Meylheuc, van Oss, and Bellon-Fontaine 2001). 20mM Cetyl Trimethyl Ammonium Bromide
217 (CTAB) solution was used as the compound of cationic charge. The ionic charge was
218 determined by creating two rows in 2% agar plated on Petri dishes. The wells in one row were
219 filled with the purified biosurfactant, and the wells in the adjacent rows were filled with the
220 cationic compounds. The biosurfactant's ionic nature was determined by monitoring the
221 precipitation lines for 48 hours at room temperature.

222 ***5.2. Compositional analysis of the two biosurfactants:***

223 The protein content was determined according to the Bradford (1976) method with Coomassie
224 brilliant blue using bovine serum albumin as standard. The total sugar content was evaluated
225 by the phenol–sulphuric method described by (Dubois et al. 1956) using glucose as standard
226 and lipids were quantified from extraction with chloroform: methanol at different proportions
227 (1:1 and 1:2, v/v)(Sharma et al. 2015). The organic extracts were then evaporated under vacuum
228 conditions and the lipid content was estimated by gravimetric estimation.

229 ***5.3. Thin layer chromatography (TLC):***

230 The composition of the two isolated biosurfactants (CB and EX BIOS) was determined by TLC.
231 Briefly, after extraction of the biosurfactants 0.1 g of the freeze-dried samples was dissolved in
232 several solvents of different polarizations, namely: methanol, chloroform and petroleum ether
233 and analyzed by thin layer chromatography (TLC) on silica gel plates (G60, Merck, Germany).
234 The latter were immersed in a migration solution consisting of (chloroform: methanol: acetic
235 acid (65: 15: 2, v / v / v)). After migration, the plates were revealed by the following methods:
236 (1) exposure to iodine vapor to detect lipid compounds, (2) spraying with Molisch's reagent to
237 detect sugars, and (3) spraying with 1% ninhydrin solution to reveal amino acids. After
238 spraying, the plates were heated for 20 to 30 minutes at 110 ° C until the desired color appeared.

239 ### *5.4. Fourier transform infrared spectroscopy (FTIR) analysis*

240 The two biosurfactants functional groups are identified applying Fourier Transform Infrared
241 Analysis (FTIR). A Fourier transform infrared spectrophotometer was used to record the
242 infrared spectra (Instruments Analect fx-6 160). The purified biosurfactants (0.1 percent) were
243 carefully mixed with KBr before being pressed into a homogenous disc (sample/KBr). The
244 produced samples' FTIR spectra were obtained in the 400 to 4000 cm-1 range.

245 ### *5.5. Protein molecular weight determination using sodium dodecyl sulphate*
246 ### *polyacrylamine gel electrophoresis (SDS-PAGE)*

247 Gel electrophoresis of both biosurfactants (CB and EX BIOS) (42µg) was performed using 17%
248 (w/v) running gel and 4% stacking gel and run at a constant current (115 v) until the dye front
249 attains the bottom. The gels were developed by staining with Coomassie blue R250. The
250 molecular weight of protein of the two biosurfactants was determined by comparing it with the
251 precision plus protein kaleidoscope standards (BIO-RAD, US).

252 ### *5.6. Biosurfactants spreading capacity determination*

253 The contact angle (CA) on various surfaces was measured by the sessile drop method using an
254 Attention Theta 200 optical tensiometer (Biolin Scientific, Sweden) to determine the spreading
255 ability or wettability of the two OL5 biosurfactants (CB and EX BIOS). A drop of 15 µL of
256 sample was placed on five different surfaces: Teflon polymer (Polytetrafluoroethylene-PTFE)
257 (highly hydrophobic), polystyrene, overhead projector transparency sheet (OHP, intermediate
258 hydrophilic-hydrophobic), and glass slide (highly hydrophilic).Analysis of the shape of the
259 biosurfactant droplet placed on each surface was carried out by image analysis with a high-
260 resolution CCD camera and OneAttension software, using the Young–Laplace equation.
261 Measurements were performed at least by triplicate and the mean value was presented in this
262 work.

263 **Results and discussion**

264 **1. Screening of biosurfactant producing LAB and biochemical characterization of the**
265 **selected isolate:**

266 Many studies have been devoted to the development of biological surfactants or biosurfactants
267 as an alternative to chemical ones, which have numerous disadvantages. biosurfactants
268 interesting biological and surface properties have made them more advantageous than their
269 synthetic counterparts, leading to their use in a variety of biotechnological (Banat, Makkar, and
270 Cameotra 2000; Pacwa-Płociniczak et al. 2011), pharmacological, cosmetic, agri-food, and
271 environmental fields. To isolate lactic acid bacteria, MRS agar was used for preliminary
272 screening of the strains. A primary screening of the selected strains (on MRS agar) was
273 performed on blood agar based on the hemolytic properties of biosurfactants. Indeed, after 24
274 hours at 37°C incubation, the presence of a hemolytic zone (transparent or greenish) around the
275 colonies is synonymous with haemolysis most likely produced by the biosurfactant molecule
276 (Mulligan 2005). Thus, surface tension (ST), emulsifying activity (EA), and emulsification
277 index (E24) tests were performed to confirm the production of biosurfactants by the selected
278 strains. As a secondary screening for biosurfactant production, these tests were applied to the
279 cell bound biosurfactant extract (CB BIOS). The obtained results are the average of two tests.

280 Only six strains were identified as potentially biosurfactant producing among the numerous
281 isolates examined: LCV24 (isolated from goat milk), LCM8 (isolated from camel milk) and
282 three strains OL3, OL5, and OL16 (isolated from olive curing water). According to the results,
283 the analysis of the hemolytic activity, surface tension (ST), emulsifying activity, and
284 emulsification index (E24) revealed that the OL5 strain is the most interesting to study, as a
285 result, it was chosen for further investigations in this research work. (Table 1). The strain OL5
286 was identified first by morphological and microscopic analysis, and then by biochemical
287 analysis. The colonies on MRS agar are round, bulging, and whitish. Their size ranges from 1

288 to 2 mm. The microscopic examination revealed that it is a gram-positive, immobile rod. The
289 catalase test resulted in a negative result. These findings suggest that the strain is a *Lactobacillus*
290 strain. The analysis of the biochemical test results of the Api galleries using the Api web
291 software led us to the conclusion that they are most likely (99.1%) *Lactiplantibacillus*
292 *plantarum*. These traditional microbiological methods are primarily used for bacterial
293 identification and phenotypic typing. For some of them, however, gene amplification has
294 completely replaced the traditional method. Among the other methods proposed, mass
295 spectrometry enables the identification and typing of microorganisms (bacteria, viruses, fungi)
296 from colonies or samples, either in conjunction with or without gene amplification (Courcol
297 2009).

298 **2. Identification and characterization of isolated strains:**

299 ***2.1. Spectroscopic identification of the selected biosurfactant producing strain OL5:***

300 MALDI-TOF mass spectrometry has gradually replaced traditional techniques in medical
301 microbiology laboratories due to its superior performance. According to previous research,
302 mass spectrometry identification results (95% - 97.4% correct identifications) outperform
303 automated systems using conventional methods (75.2% - 92.6%).(Courcol 2009). As a result,
304 we used this method to identify the OL5 strain. The identification score of >2 confirms that it
305 is indeed *Lactiplantibacillus plantarum* according to the recent nomenclature. (Wardi and
306 Prévost 2009)reported that a score greater than 2.00 indicates very good identification
307 reliability.

308 ***2.2. Identification of the selected biosurfactant producing strain OL5 by the 16 rDNA***
309 ***Gene Sequencing:***

310 Using the MEGA7 program, a phylogenetic tree was constructed after sequencing the amplified
311 DNA fragment. The 1500 bp 16S rRNA sequence amplified from isolate OL5 shared 97%

312 identity with *Lactobacillus plantarum* b78, which has been deposited in GenBank under the
313 accession number KX057646.1. According to phylogenetic analysis, strain OL5 is related to *L.*
314 *plantarum suebicus, L. pentosus strain 124, L. plantarum CIP, L. fabifermentans strain DSM*,
315 and *Pediococcus clausseni ATCC* (Figure 1).

316 **3. Growth and biosurfactant production of *L. plantarum* OL5:**

317 The surface tension of the medium broth was used to determine the biosurfactants produced
318 by *Lactiplantibacillus plantarum* OL5. MRS medium was used in this study to produce the
319 two biosurfactants of the OL5 strain; the pH was reduced over a 48-hour period from 6.5 to
320 3.94 (figure 2). The surface tension decreased the most during the exponential phase, from
321 52.36 mN/m to 38.54 mN/m (figure 2), and then increased marginally for the last 48 hours.

322 **4. Surface Tension Measurement and Determination of CMC:**

323 As suggested by (Satpute et al. 2010), the Du-Nouy-Ring method is based on measuring the
324 force required to detach a ring or loop of wire from an interface or surface. The detachment
325 force is proportional to the interfacial tension. It can be measured with an automated
326 tensiometer, which is available from many manufacturers. The Du-Nouy-Ring assay is widely
327 applied for screening of biosurfactant producing microbes(Anyanwu, Obi, and Okolo 2011;
328 Płaza, Zjawiony, and Banat 2006).

329 Surfactants' main property is their ability to self-organize. This tendency is usually
330 characterized by the critical micellar concentration (CMC). Below the CMC, the surfactant
331 forms a layer on the liquid's surface, with the remainder dispersed in the solution. When the
332 amount of surfactant is increased, the concentration increases proportionally until it reaches a
333 limit value: the CMC. The CMC is thus the total surfactant concentration at which a constant
334 and small number of surfactant molecules are aggregated. It is also the smallest concentration
335 required to initiate micelle formation.(Mata-Sandoval, Karns, and Torrents 2000)the fluctuation
336 of the surface tension of water in the presence of increasing concentrations of these surfactants

337 (0 to 12 mg / ml) was determined in order to determine the CMC of the two biosurfactants of
338 the strain OL5 (Figure 2).

339 The results show that SDS reduces water surface tension from 69 mN/m to 26±0.25 mN/m with
340 a CMC of 1.86 mg/ml. The two OL5 biosurfactants reduce water surface tension from 69 mN/m
341 to 34 ±0.05 mN/m with a CMC of 7 mg/ml for the OL5 cell bound biosurfactant and to 37±0.15
342 mN/m with a CMC of 1.8 mg/ml for the OL5 extracellular biosurfactant. In this regard,
343 (Rodrigues et al. 2004) reported similar results with other lactic acid bacteria, specifically
344 *Streptococcus thermophilus A* and *Lactococcus lactis*, whose biosurfactants reduce water
345 surface tension from 70 mN/m to 36.0 mN/m and 37.0 mN/m, respectively. (Gudiña et al.
346 2015), on the other hand, demonstrated that the biosurfactant adhered to the cell membrane of
347 *Lactobacillus agilis* CCUG31450 reduces water surface tension to 42.5 mN/m with a CMC in
348 the range of 7.5 mg/mL. Similarly, (Morais et al. 2017)demonstrated that two biosurfactants
349 produced by *L. jensenii* P6A and *L. gasseri* P65, respectively, reduce the TS of water to 43.2
350 mN/m and 42.5 mN/m, with CMC values of 7.1 mg/ml and 8.58 mg/ml.

351 **5. Study of OL5 biosurfactants stability:**

352 The thermal stability of the two OL5 biosurfactants (CB and EX BIOS) emulsifying properties
353 was investigated by determining their ability to emulsify corn oil after a 60-minute pre-
354 incubation at different temperatures: 4 °C, 20 °C, 40 °C and 60 °C. (Figure 3) shows that the
355 cell bound biosurfactant (CB BIOS) of the strain OL5 are practically stable after 1 hour of
356 incubation at the various temperatures tested. The extracellular biosurfactant (EX BIOS)
357 appears to be less stable (87% to 100% activity retention). In this regard, (Morais et al.
358 2017)demonstrated that the biosurfactants produced by *L. jensenii* P6A and *L. gasseri* P65
359 retain their activities even after 60 minutes at 100°C incubation. Likewise, the stability of two
360 OL5 biosurfactants was investigated at pH levels ranging from 2 to 10. (Figure 4) shows that
361 the activity of two biosurfactants from strain OL5 remains nearly stable over a wide pH range.

362 Indeed, varying the pH from 3 to 10 retains more than 80 % of the emulsifying activity of the
363 two biosurfactants. Moreover, Incubating the OL5 strain biosurfactants for 1 hour in the
364 presence of sodium chloride concentrations ranging from 10% to 40% results in a decrease in
365 activity of approximately 18% to 35% in the height of the layer emulsified by OL5 cell bound
366 biosurfactant(CB BIOS) and a decrease in activity of approximately 5% to 34% in the
367 emulsifying activity of the OL5 extracellular biosurfactant (EX BIOS) (figure 5). Similarly, we
368 noticed that the emulsifying activity of the commercial surfactant SDS decreases as NaCl
369 concentration increases.

370 **6. Characterization of the two biosurfactants**

371 **6.1. *Characterization of the two biosurfactants Determination of ionic character*:**

372 The ionic charge of the strain OL5's two biosurfactants (CB biosurfactant and EX biosurfactant)
373 was determined using the double agar diffusion method, which is based on the diffusion of two
374 soluble compounds with the same or different charge in a low concentration gel (2%).

375 (Figure 6) depicts the formation of a halo around the positively charged chemical surfactant
376 CTAB, whereas no line was formed between the strain OL5 biosurfactant and the anionic
377 surfactant (SDS) (results not shown). This implies that both biosurfactants produced by the
378 strain OL5 are anionic in nature (Ghazala et al. 2017; Siegmund and Wagner 1991). Similar
379 findings have been reported in the literature, demonstrating the anionic charge of the majority
380 of lactic acid bacteria biosurfactants, such as the xylolipid biosurfactant produced by
381 *Lactococcus lactis* (Saravanakumari and Mani 2010), the glycolipid biosurfactant produced by
382 *Enterococcum faecium* strain MRTL9 (Sharma et al. 2015), and the lactic acid bacteria LCM2
383 (Matei et al. 2017).

384 ***6.2. Compositional analysis of the two biosurfactants:***

385 Using traditional biochemical assay methods, the sugar, protein, and lipid levels of the two
386 biosurfactants of *Lactiplantibacillus plantarum* OL5 strain were determined. The OL5 cell-
387 bound biosurfactant (CB BIOS) is 78% sugars, 19.35% lipids, and 2% proteins, whereas the
388 extracellular biosurfactant (EX BIOS) is 79% sugars, 17% lipids, and 4% proteins. These
389 findings suggest that both biosurfactants are glycolipopeptides in nature.

390 ### *6.3. Thin layer chromatography (TLC):*

391 Thin layer chromatography (TLC) is a method of separating mixture components based on their
392 affinity for the stationary and mobile phases. TLC was used to determine the nature of the
393 biosurfactants produced by *L. plantarum* OL5 strain (Figure 7). The presence of lipid,
394 carbohydrate, and protein spots is indicated by the appearance of a dark yellow spot when the
395 plate was exposed to iodine, a brown spot when exposed to sulphuric acid, and a purple spot
396 when exposed to ninhydrin, respectively.

397 These findings suggest that two biosurfactants in strain OL5 (CB and EX BIOS) are
398 heterogeneous. The two corresponding biosurfactants could be glycolipopeptides. These
399 findings are similar to those obtained with glycolipopeptides biosurfactants produced by
400 *Lactobacillus acidophilus* NCIM 2903(Satpute et al. 2019) and *Lactobacillus plantarum* ATCC
401 8014 (Behzadnia et al. 2020).

402 ### *6.4. FTIR analyses:*

403 Infrared analysis of a sample is a quick qualitative method for detecting the presence of specific
404 functional groups. Infrared spectroscopy was used to analyse the structures of two
405 biosurfactants from strain OL5 in order to identify their functional groups. (Figure 8) shows the
406 obtained infrared spectra.

407 The infrared spectra of both biosurfactants of *L. plantarum* OL5 (CB and EX BIOS) show a
408 strong absorption band at about 3386 cm^{-1} which could be assigned to the stretching vibrations

409 of OH hydroxyl groups (of carboxyls, phenols or alcohols) (Sharma et al. 2014) indicating the
410 presence of polysaccharides (Bakhshi, Sheikh-Zeinoddin, and Soleimanian-Zad 2018), either
411 carboxyls, phenols or alcohols (Kumar et al. 2012). A band at 2924 cm^{-1} was also detected for
412 both biosurfactants which generally characterizes aliphatic C-H elongation vibrations (Sharma
413 et al. 2014)indicating the presence of polysaccharides (Bakhshi et al. 2018), proteins or lipids
414 (Kumar et al. 2012). The relatively strong absorption band detected for both types of
415 biosurfactants between 1500 cm^{-1} and 1700 cm^{-1} was attributed to the asymmetric C=O
416 stretching vibrations of the carboxyl groups (-COO-) that may be present in the composition of
417 fatty acids and amino acids or to Groups N–H in proteins. The absorption bands detected at
418 1405 and 1041 cm^{-1} could be attributed to C-H bond deformation vibrations indicating the
419 presence of an aliphatic chain (-CH3 and -CH2), indicating the presence of alkyl chains in the
420 samples studied (Bakhshi et al. 2018; Sharma et al. 2014). Furthermore, both biosurfactants
421 exhibit a distinct band between 1000 and 1200 cm^{-1}. This region is dominated by ring vibrations
422 superimposed on side group elongation vibrations (C-OH) and glucoside band vibrations (C-
423 O-C) (Bakhshi et al. 2018).

424 *6.5. Protein molecular weight determination using Sodium Dodecyl Sulphate*
425 *Polyacrylamine Gel Electrophoresis (SDS-PAGE):*

426 The BS SDS-PAGE revealed multiple bands with molecular weights ranging from 15 to 102
427 kDa, with prominent bands of 102.5 kDa for the EX BIOS and 54 kDa for the CB BIOS (figure
428 9). Similar results were reported by (Hashim et al. 2022)showing that following separation
429 of *Lactiplantibacillus plantarum* biosurfactant on SDS-PAGE gel and staining with Coomassie
430 blue, several protein bands were observed with molecular weight extending from 10 to
431 150 kDa. In a similar study, (Heinemann et al. 2000)used SDS-PAGE electrophoresis to
432 determine the chemical structure of biosurfactants synthesized by *Lactobacillus fermentum* RC
433 14 after an 18-h culture on MRS medium. The electrophoresis demonstrated that the protein

434 fraction of the analyzed surface-active compounds included fractions with molecular weights
435 ranging from 14.0 to 94.0 kDa and higher. Likewise, (Gołek et al. 2009) showed that the protein
436 fraction of biosurfactants synthesized by *Lactobacillus casei,* after 18 h of culture, on the MRS
437 medium was constituted with molecular weights covered in 6.1- 106.5 kDa.

438 ### *6.6. Wetting property of the two biosurfactants*

439 The wetting behaviour of solid surfaces can be altered by the adsorption of agents such as
440 biosurfactants. The wetting properties of two biosurfactants (EX BIOS and CB BIOS) from *L.*
441 *plantarum* OL5 were tested on four different surfaces: glass, polystyrene, Teflon, and
442 transparency OHP film. In comparison to the CA values of water on the respective surfaces, the
443 two biosurfactants showed the greatest decrease in CA on the Teflon surface from 113 to
444 81°±0.77for the cell bound biosurfactant (CB BIOS) and from 113 to 83.65°±4.35 for the
445 extracellular biosurfactant (EX BIOS), followed by polystyrene from 90 to 59°±0.94 and
446 60.24°±2.045 respectively for the CB and EX BIOS . (Figure 10). When the observed CA
447 exceeds 90°, the surface is thought to have poor wetting properties and thus is hydrophobic. CA
448 values for *Lactobacillus sp.* biosurfactants are rarely reported in the literature, although(Satpute
449 et al. 2018) observed similar results. The biosurfactants produced by *Lactobacillus acidophilus*
450 reduced the CA on polystyrene (from 90 to 49°) and Teflon (from 115 to 75°). Similarly,
451 (Satpute et al. 2019b)found that the highest reduction in CA was observed on polystyrene
452 surface (from 96 to 36°) followed by Teflon (from 112 to 65°) for the biosurfactant produced
453 by a *Lactobacillus acidophilus* strain. Based on these findings, it can be concluded that both
454 biosurfactants have good wetting properties, which implies that they could be a viable industrial
455 alternative to synthetic surfactants in a variety of sectors, including the cosmetic industry as
456 alternatives to chemical surface-active (Ferreira et al. 2017).Another field of application for
457 lactobacillus biosurfactants is in the medical field due to their good wetting ability as a coating

458 substance on PDMS-based implants (Satpute et al. 2019) .Future research will focus on
459 implicating these OL5 biosurfactants in a variety of applications.

460 **Conclusion:**

461 Due to their GRAS (Generally Recognized as Safe) status, interest in biosurfactants produced
462 by lactic acid bacteria has increased significantly over the past decade. In the current study, we
463 were interested in the isolation of a new biosurfactant-producing lactic acid bacterium (OL5)
464 from olive curing water. Sequencing of 1500 bp of 16S rRNA of the OL5 strain confirmed the
465 results of biochemical identification by API and mass spectroscopy and showed that it is
466 *Lactiplantibacillus plantarum*. In addition, the isolated strain can produce two types of
467 glycolipopeptides as biosurfactants. In conclusion, we showed that the activities of the two
468 biosurfactants of the OL5 strain are stable under extreme temperature, pH and salinity
469 conditions. In addition, both biosurfactants have good wetting ability, so they can be used in a
470 wide range of industrial applications.

471 **Acknowledgment**

472 This work was supported by the Ministry of High Education and Scientific Research- Tunisia.

473 **Declaration of interest statement**

474 The authors declare that no relevant conflicts of interest with respect to the content of this
475 scientific paper.

476 **Data availability statement**

477 The datasets generated during and/or analysed during the current study are available from the
478 corresponding author on reasonable request.

479

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636 **List of tables:**

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638 isolated strains

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Figure 1

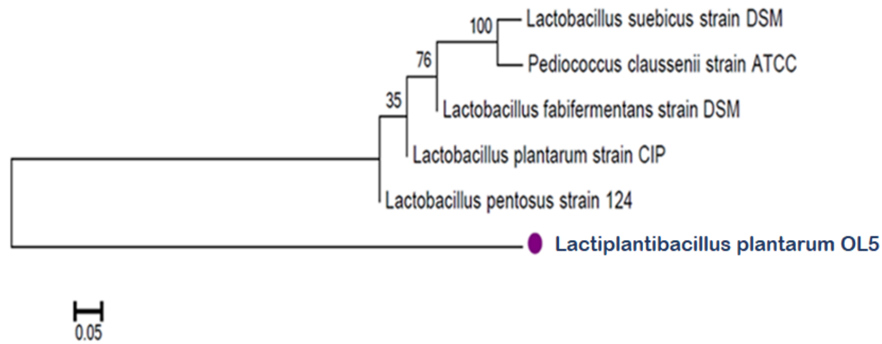

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Figure 1: Phylogenetic tree of the *Lactiplantibacillus plantarum* strain OL5 isolated from olive curing water

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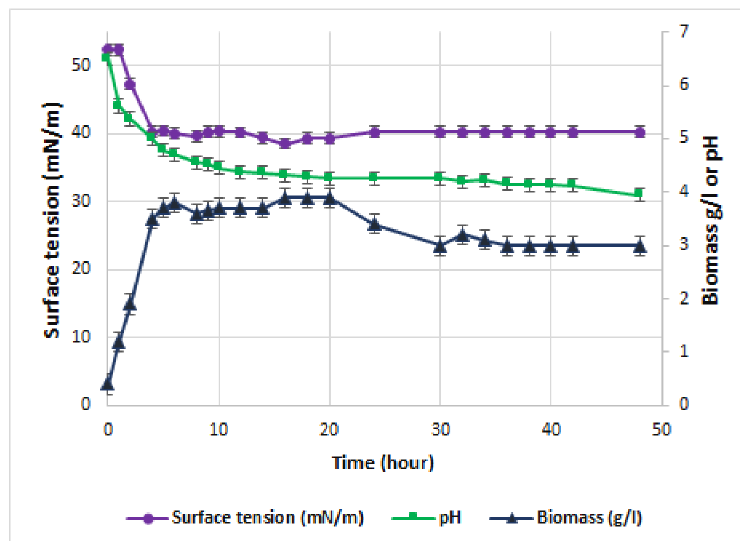
Figure 2



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683 Figure 2: Analysis of surface tension and biomass of *Lactiplantibacillus plantarum OL5*

684 grown in MRS medium for production of biosurfactant

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686 **Figure 3**

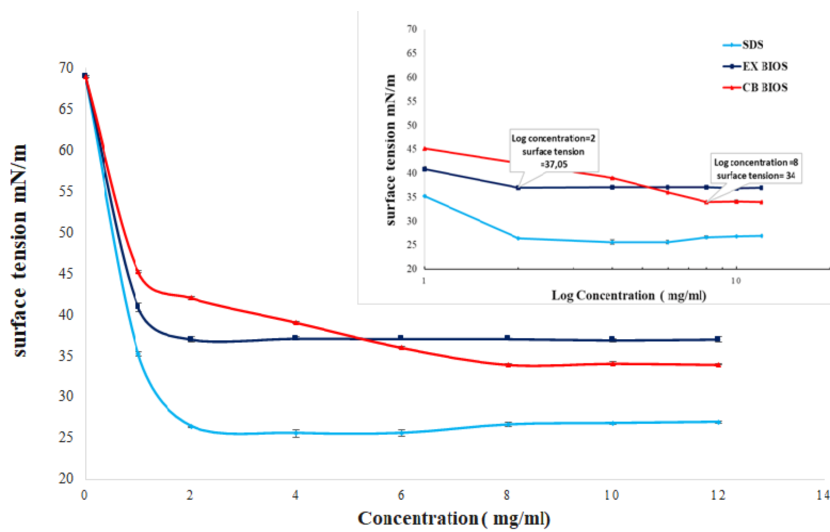

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Figure 3: Determination of the CMC of the two biosurfactants of the strain OL5: extracellular (EX) and cell bound (CB) biosurfactants compared to the anionic surfactant (SDS)

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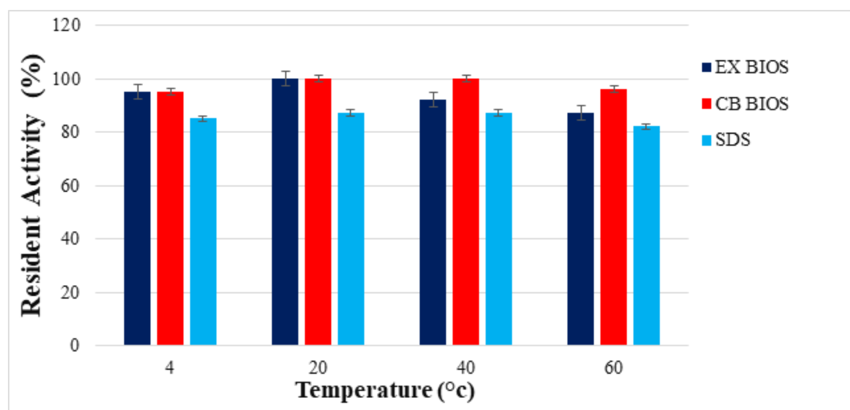
688 **Figure 4**



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690 Figure4:OL5 both biosurfactants (cell bound (CB) and extracellular (EX)) stability under

691 different temperature ranges, the mean value of three measurements was represented

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Figure 5

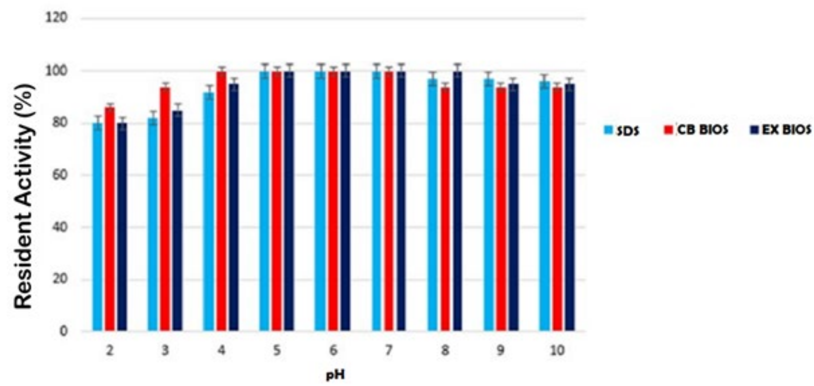

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Figure 5: OL5 biosurfactants (cell bound (CB) and extracellular (EX)) stability under varied pH conditions, the mean value of three measurements was presented

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Figure 6

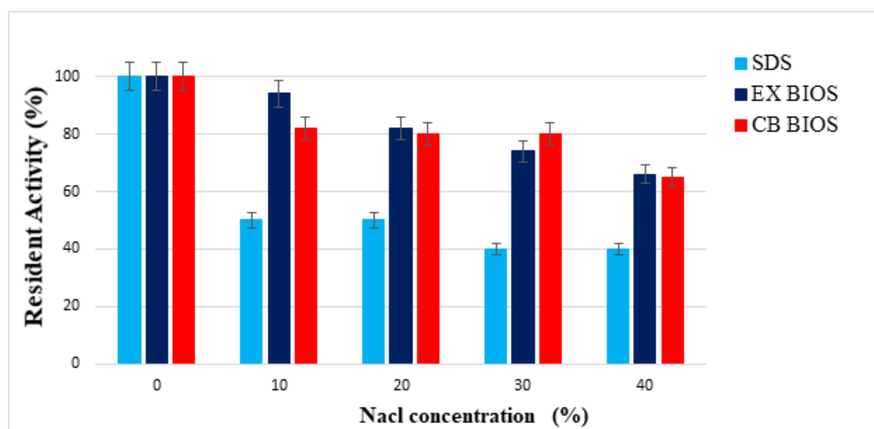

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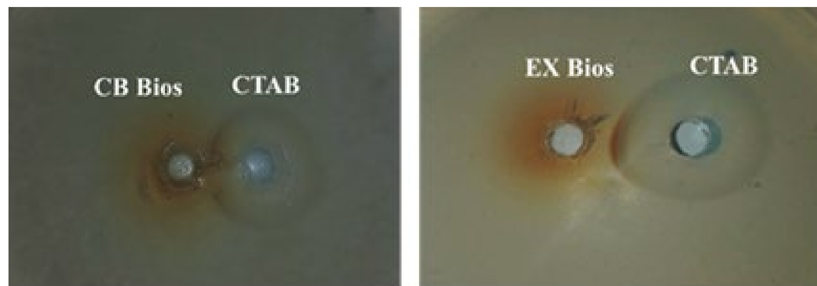
Figure6:OL5 biosurfactants stability (cell bound (CB) and extracellular (EX) biosurfactants) under varied NaCl concentration, the mean value of three measurements was presented

699

700

Figure 7



701

Figure 7: determination of the ionic charge of OL5 both biosurfactants, the extracellular biosurfactant (EX BIOS) and the cell bound biosurfactant (CB BIOS) towards a cationic biosurfactant (CTAB)

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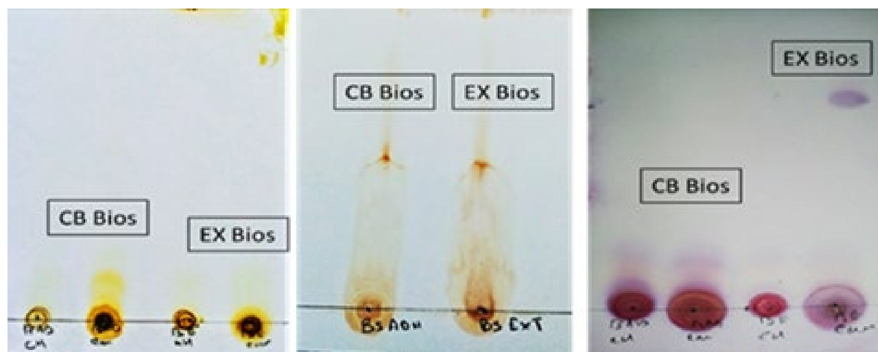
703

Figure 8



704

705

Figure 8: TLC analysis of the two biosurfactants produced by OL5 strain (cell bound biosurfactant (CB BIOS) and the extracellular biosurfactant (EX BIOS))

Iodine vapor (A), Ninhydrin (B) and Sulphuric acid exposition (C)

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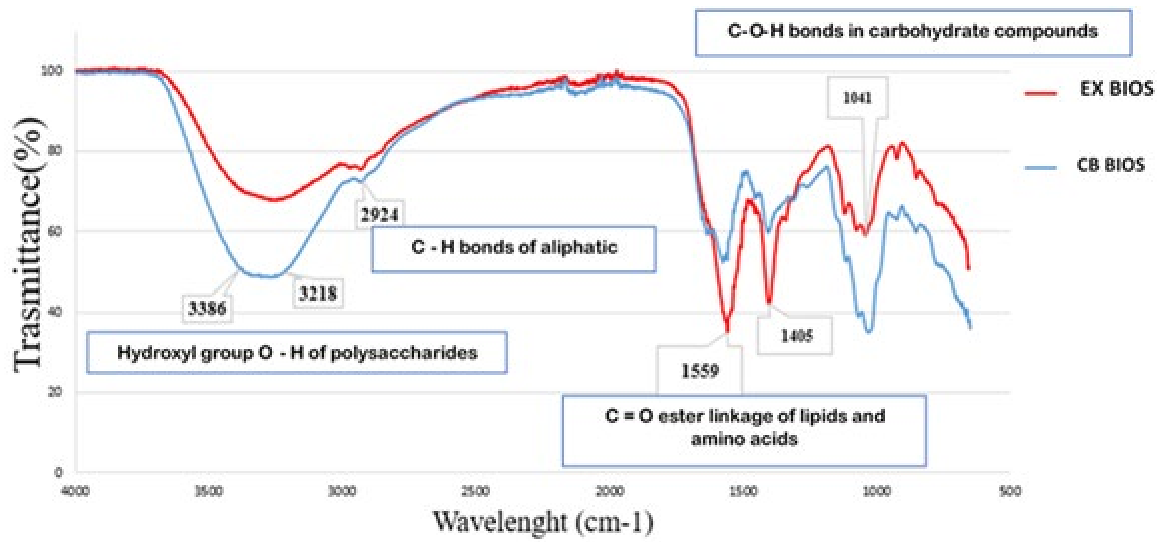
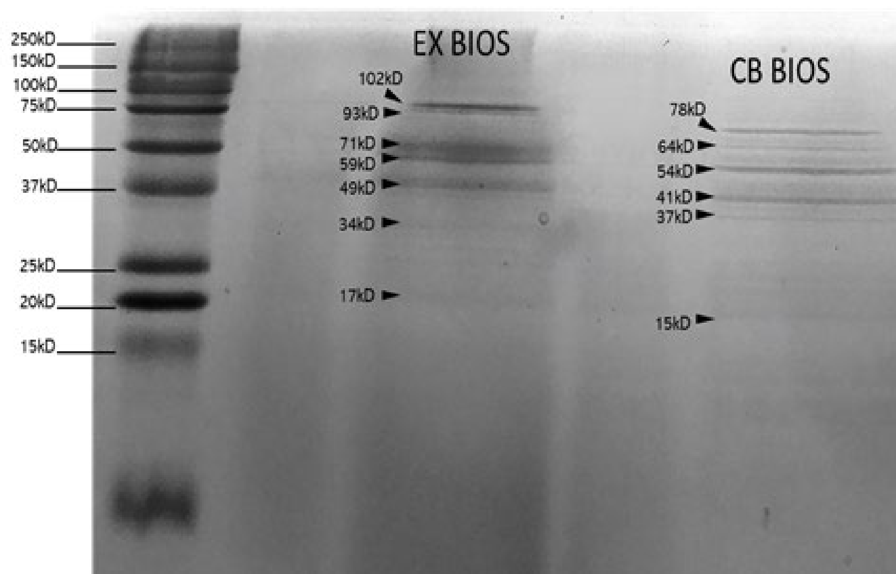
710 **Figure 9**



711

Figure9: Fourier Transform spectroscopy (FT-IR) of the two OL5 biosurfactants (cell bound (CB) and extracellular (EX) biosurfactants)

712 **Figure 10**



713

714 Figure 10: Protein separation profile of *Lactiplantibacillus plantarum* OL5 biosurfactants

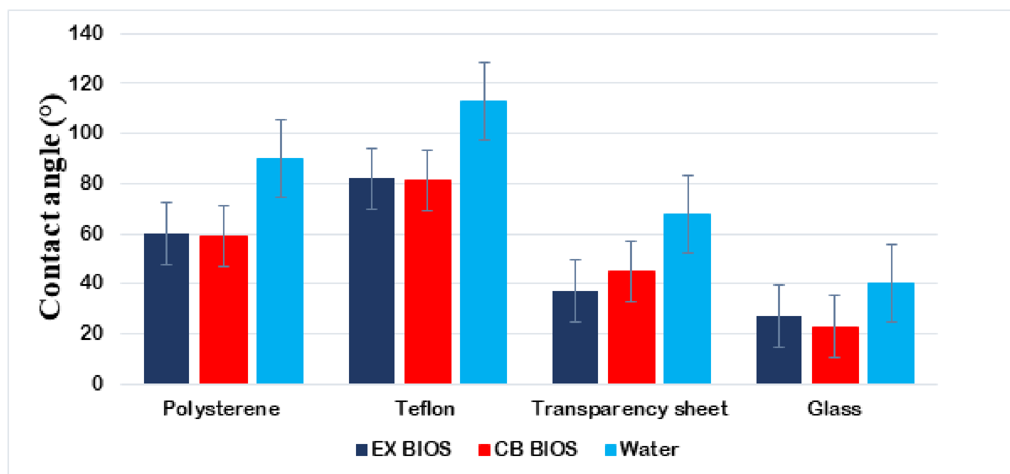
715 (cell bound (CB) and extracellular (EX) biosurfactants) following SDS-PAGE and staining

716 with Coomassie blue R250.

717

718

Figure 11



719

720 Figure 11: the contact angle measurements of the two biosurfactants (cell bound (CB) and

721 extracellular biosurfactants (EX) towards different surfaces

722 (the results presented are the mean of three values)

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732 Table 1:Investigation of the hemolytic, emulsifying and surfactant properties of the LAB

733 isolated strains (the results presented are the mean of two values)

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Samples	**Culture control**		**primary screening**	**Secondary screening**		
			Hemolytic activity	**Surface tension ($mN.m^{-1}$)**	**Emulsifying activity**	
	pH	OD (600 nm)			AE (OD=540 nm)	E24(%)
LCV24	4.46	3.56	+	57.8	0.051	0
LCM8	4.38	4.15	+	63.45	0.051	0
OL3	3.86	5.52	+	50.8	0.145	17
OL5	3.87	5.32	++	27.93	0.267	59.6
OL16	3.92	1.88	++	51.65	0.063	32.5
IL12	3	4.06	+/-	53.6	0.032	0

735 With TSPBS=72,3, et TS water = 72,9 mN/m

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