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1 # Production of novel biosurfactant by a new yeast species isolated
2 # from *Prunus mume* Sieb. *et* Zucc.

3

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23 # Abstract

24 Biosurfactants reduce surface and interfacial tension due to their amphiphilic properties,
25 and are an eco-friendly alternative for chemical surfactants. In this study, a novel yeast strain
26 JAF-11 that produces biosurfactant was selected using drop collapse method, and the properties
27 of the material were investigated. The nucleotide sequences of the strain were compared with
28 closely related strains and identified based on the D1/D2 domain of the large-subunit rDNA
29 (LSU) and internal transcribed spacer (ITS) regions. *Neodothiora populina* CPC 39399^T, the
30 closest species with strain JAF-11 in the phylogenetic tree, showed a sequence similarity of
31 97.75% for LSU and 94.27% for ITS, respectively. The result suggests that the strain JAF-11
32 represent a distinct species which cannot be assigned to any existing genus or species in the
33 family *Dothideaceae*. Strain JAF-11 was able to produce biosurfactant reducing the surface
34 tension of medium to 34.5 mN/m on the 6th day of culture and the result of measuring the
35 critical micelle concentration (CMC) by extracting the crude biosurfactant was found to be 24
36 mg l^{-1}. The molecular weight 502 of the purified biosurfactant was confirmed by measuring
37 the fast atom bombardment mass spectrum (FAB-MS). The chemical structure was analyzed
38 by measuring ^{1}H nuclear magnetic resonance (NMR), ^{13}C NMR, two-dimensional NMRs of
39 the compound. The molecular formula was $C_{26}H_{46}O_9$, and it was composed of one octanoyl
40 group and two hexanoyl group to myo-inositol moiety. The new biosurfactant is the first report
41 of a compound produced by a novel yeast strain JAF-11. This new biosurfactant is proposed
42 as potential candidate for use in a variety field.

43

44

45 **KEYWORDS**: Yeast, novel species, novel biosurfactant, chemical structure

46

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47 # Introduction

48

49 The surfactants have amphiphilic properties possessing both non-polar (hydrophobic)
50 and polar (hydrophilic) moieties that allows reducing the surface and interfacial tension
51 between biphasic systems as liquid-liquid interface or solid-liquid boundaries [1, 2]. The
52 surfactants are one of the important compounds having potential commercial application of
53 detergents, cosmetics, food ingredients, agricultural, pharmaceutical, paint, textile and paper
54 etc. [3, 4]. However, surfactants are mainly chemically synthesized from petroleum-based
55 resources, which can cause environmental problems due to toxicity [5]. As environmental
56 awareness has gradually increased over the past decades, the demand for eco-friendly
57 compounds has increased, and accordingly, there is an increasing interest in microbial
58 biosurfactants [6]. In particular, microbe-derived biosurfactants have advantages such as
59 environmental compatibility, activity, stability, and lower toxicity compared to chemically
60 synthesized equivalents [7-11].

61 Biosurfactants are classified according to ionic charges (anionic, cationic, non-ionic
62 and neutral biosurfactants), molecular weight (high molecular and low molecular weight
63 biosurfactants), secretion type (intracellular, extracellular and adhered to microbial cells), and
64 chemical structure (glycolipids, lipopeptides, fatty acids, phospholipids, neutral lipids and
65 polymeric biosurfactants), and it has also been previously reported that various biosurfactants
66 are produced depending on the type of microorganisms [12]. Among them, glycolipids have
67 been best known as a class of biosurfactants that exhibit the highest commercial potential due
68 to the high microbial productivity [13]. The microbe-derived biosurfactant rhamnolipids, also
69 known as glycolipids, was described for the first time in 1946 [14]. Despite their high
70 productivity, most of them is produced by opportunistic pathogen *Pseudomonas aeruginosa*
71 [15]. The sophorolipids belonging to the group of glycolipid are one of the most promising

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72 biosurfactant because they are produced by non-pathogen yeast *Starmerella bombicola* and
73 other *Candida* spp. (*Candida stellate, C. riodocensis, C. apicola, C. batistae, C. kuoi,* and *C.*
74 *floricola*) [16, 17]. Mannosylerythritol lipids (MEL) by a variety of *Pseudozyma* yeasts and
75 trehalolipids by *Rhodococcus erythopolis* have been reported as representative biosurfactants
76 belonging to the glycolipid [18]. It has been reported that microorganisms such as the genera
77 *Acinetobacter*, *Arthrobacter*, *Pseudomonas*, *Halomonas*, *Bacillus*, *Rhodococcus*, *Enterobacter*,
78 and few yeast genera produce not only glycolipids but also other types of biosurfactants [19].
79 In the 20th century, research on biosurfactants focused on understanding and
80 optimizing the production process, and since then, research on utilizing various renewable
81 resources, discovering new producer strains, or developing genetically modified strains has
82 been reported [6]. Since biosurfactants have a wide range of functional properties depending
83 on their chemical structure [19], it is necessary to continuously secure a variety of
84 biosurfactants in order to be applied to various fields. The present study is the first report of
85 novel biosurfactant extracted from the novel yeast strain JAF-11. We isolated a new species of
86 yeast that produces biosurfactant from flower in order to secure various biosurfactants, and
87 chemical structure of the biosurfactant extracted from the yeast has been characterized and
88 identified as a novel biosurfactant by nuclear magnetic resonance spectrometry techniques.
89

90 Materials and methods

91

92 Culture medium for biosurfactant production by yeast

93 The culture medium composition used in these studies for biosurfactant-producing
94 yeast was as follows (w/v): glucose (1.5%), soybean oil (1.5%), ammonium sulfate (0.1%),
95 potassium phosphate (0.25%), sodium phosphate (0.01%), magnesium sulfate (0.05%),

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96 calcium chloride (0.01%), manganese sulfate (0.002%) and peptone (0.1%).

97

98 Isolation and screening of biosurfactant producing yeast

99 The biosurfactant-producing yeast used in this study was isolated from flower (*Prunus*
100 *mume* Sieb. *et* Zucc.) collected from apricot village in Gwangyang, Republic of Korea during
101 March 2018. The biosurfactant was screened using modified drop collapse method as follows:
102 100μL of the culture supernatant and water (1:1, v/v) was pipetted and placed on parafilm [20,
103 21]. The selected strain JAF-11 was maintained for storage at -80°C in 15% (v/v) glycerol, and
104 was deposited with the patent depository as KACC 83047BP.

105

106 Identification of biosurfactant producing yeast

107 The identification of strain JAF-11 was conducted based on multigene phylogenetic
108 analysis of the nucleotide sequences combined with the D1/D2 region of large-subunit (LSU)
109 and the internal transcribed spacer (ITS) region ribosomal DNA (rDNA) genes. The DNA
110 sequencing was performed by Macrogen Inc. (Seoul, Korea) and the gene sequences of related
111 species were retrieved from GenBank database. The phylogenetic tree was inferred by using
112 the maximum likelihood method with 1,000 bootstrap replicates and sequences analysis was
113 performed in MEGA X software.

114

115 Time course of the growth and determination of surface tension

116 The strain JAF-11 was grown in 500 ml culture medium at 25℃ on a rotary shaker at
117 150 rpm, and then the optical density at 600 nm was measured for 8 days by a UV
118 spectrophotometer. To confirm the production of biosurfactant, the culture medium of strain

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119 JAF-11 was prepared through a 0.22 ㎛ filter and measured the surface tension(ST) every
120 day. For ST measurement, the Wilhelmy plate method was used at room temperature with a
121 force tensiometer K11 (Krüss, Germany). All tests were performed in triplicates.
122

123 Measurement of critical micelle concentration (CMC) of 124 biosurfactant

125 To extract the crude biosurfactant, 8 L culture medium inoculated with freshly grown
126 strain JAF-11 was mixed with HP-20 non-polar resin (Mitsubishi chemical, Japan) and eluted
127 in methanol. After removing methanol by rotary vacuum evaporation, the concentrated solution
128 was partitioned with an equal volume of ethyl acetate. The ethyl acetate was concentrated under
129 vacuum evaporator, and the product was used for CMC measurement after purification using
130 Flash silica gel column chromatography ($CHCl_3$:MeOH, 50:1 to 1:1, v/v, stepwise) (SK
131 chemical, Korea). The crude biosurfactant were dissolved in distilled water and serially diluted
132 to concentrations in the range of 0-250 mg l^{-1}. The CMC was determined by plotting the surface
133 tension against the log of the biosurfactant concentration using a force tensiometer K11 (Krüss,
134 Germany) [22].
135

136 Isolation and purification of biosurfactant compound

137 A yeast strain of JAF-11 was cultured in a medium described above for 5 days at 25℃
138 on a rotary shaker incubator at 150 rpm. The culture broth was subjected to Diaion HP-20
139 column chromatography and eluted with 30% aq. MeOH, 70% aq. MeOH, MeOH, and acetone.
140 The biosurfactant activity of eluates were evaluated by the drop collapse method, and the
141 MeOH eluate was chosen and concentrated by rotary vacuum evaporator. The concentrate was

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142 partitioned between ethyl acetate and water. The ethyl acetate portion showing biosurfactant
143 activity was concentrated and applied to silica gel column chromatography (Merck, Germany)
144 eluted with $CHCl_3$:MeOH (50:1, 20:1, 10:1, 5:1, 2:1, and 1:1, v/v) (Fig 1). An active fraction,
145 $CHCl_3$:MeOH (20:1), was subjected to Sephadex LH-20 (GE Healthcare, Sweden) column
146 chromatography eluted with $CHCl_3$:MeOH (1:1, v/v). The fractions showing biosurfactant
147 activity were combined and concentrated (Fig 1). The concentrate was further separated by the
148 medium pressure liquid chromatography (MPLC, Teledyne ISCO, USA) equipped with
149 Redisep Rf C_{18} reversed-phase column (Teledyne Isco, USA) using a gradient of 70%→100%
150 aq. MeOH to yield compound **1** (13.4 mg).

151

152 **Fig 1. Purification scheme of the biosurfactant produced by strain JAF-11.**

153

154 Chemical structure analysis of biosurfactant

155 The fast atom bombardment mass spectrum (FAB-MS) and high-resolution fast atom
156 bombardment mass spectrum (HRFAB-MS) for the molecular weight and molecular formula,
157 respectively, were measured using a JMS-700 MStation (JEOL, Japan) mass spectrometry.
158 The nuclear magnetic resonance (NMR) spectra were obtained on a JEOL JNM-ECZ500R,
159 500 MHz FT-NMR spectrometer at 500 MHz for ^{1}H NMR and at 125 MHz for ^{13}C NMR in
160 CD_3OD (Andover, USA). Chemical shifts are given in ppm (δ) with tetramethylsilane as the
161 internal standard. For NMR spectra, two-dimensional NMR such as ^{1}H-^{1}H correlated
162 spectroscopy (^{1}H-^{1}H COSY), heteronuclear multiple quantum correlation (HMQC), and
163 heteronuclear multiple bond correlation (HMBC) as well as one-dimensional NMR such as
164 ^{1}H NMR and ^{13}C NMR were employed.

165

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166 # Result and discussion

167 ## Isolation and identification of biosurfactant producing yeast

168 Strain JAF-11 was isolated from *Prunus mume* Sieb. *et* Zucc. in Republic of Korea
169 and was selected as a potential biosurfactant producer using modified drop collapse method
170 (S1 Fig). Phylogenetic relationships of strain JAF-11 and the closely related strains were
171 inferred using concatenated LSU and ITS sequences [23, 24]. The LSU and ITS sequences
172 from the reference strains were obtained from GenBank database (Table 1). Phylogenetic
173 analysis revealed that JAF-11 belonged to the family *Dothideales* clade, and formed one
174 compact cluster with *Neodothiora populina* CPC 39399^{T}, *Rhizosphaera macrospora* CBS
175 208.79^{T} and *Phaeocryptopus nudus* CBS 268.37 (Fig 2). The LSU region sequence of strain
176 JAF-11 showed the highest similarity with those of *Rhizosphaera macrospora* CBS 208.79^{T}
177 (97.26%; 15nt substitutions in 548 nt) and *Neodothiora populina* CPC 39399^{T} (97.75%; 13nt
178 substitutions in 578 nt). In the ITS region sequences, strain JAF-11 had sequence similarity of
179 95.15% (26 nt substitutions in 536 nt) with *Rhizosphaera macrospora* CBS 208.79^{T} and 94.27%
180 (31 nt substitutions in 541 nt) with *Neodothiora populina* CPC 39399^{T}. According to Duong
181 Vu et al. (2016), the taxonomic threshold predicted to discriminate yeast species is 98.41% in
182 the LSU region and 99.51% in the ITS region [25]. Consequently, we propose that strain JAF-
183 11 represents a novel yeast species of a new genus based on phylogenetic analysis and the
184 taxonomic thresholds of gene sequence identities.

185

186 **Table 1.** GenBank accession numbers of species used in phylogenetic analyses

Species	Strain no.	GenBank accession no.	
		LSU	ITS
Family Dothideaceae			
Coleophoma crateriformis	CBS 473.69	MH871117.1	MH859358.1
Coleophoma oleae	CBS 615.72	MH872293.1	KU728511.1

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Cylindroseptoria ceratoniae	CBS 477.69^T	KF251655.1	KF251151.1
Delphinella strobiligena	CBS 735.71	MH872074.1	MH860318.1
Dothidea insculpta	CBS 189.58	MH869284.1	AF027764.1
Dothidea sambuci	AFTOL-ID 274^T	AY544681.1	DQ491505.1
Dothidea sambuci	DAOM 231303^T	NG_027611.1	AY883094.1
Dothiora cannabinae	CBS 737.71^T	MH872076.1	MH860320.1
Dothiora corymbiae	CBS 145060^T	MK047482.1	MK047431.1
Dothiora elliptica	CBS 736.71	MH872075.1	KU728502.1
Endoconidioma populi	UAMH 10902	HM185488.1	HM185487.1
Neocylindroseptoria pistaciae	CBS 471.69^T	MH871115.1	MH859357.1
Neodothiora populina	CPC 39399^T	MW175405.1	MW175365.1
Phaeocryptopus nudus	CBS 268.37	GU301856.1	EU700371.1
Plowrightia ribesia	MFLU 14-0040	KM388552.1	KM388544.1
Pringsheimia smilacis	CBS 873.71	FJ150970.1	MH860390.1
Rhizosphaera macrospora	CBS 208.79^T	MH872971.1	MH861202.1
Stylodothis puccinioides	CBS 193.58	MH869286.1	MH857753.1
Sydowia polyspora	CBS 116.29	MH866487.1	MH855019.1
Family Aureobasidiaceae			
Aureobasidium leucospermi	CPC 15180	JN712555.1	JN712489.1
Aureobasidium leucospermi	CBS 130593^T	MH877257.1	KT693727.1
Aureobasidium proteae	CPC 2825	JN712558.1	JN712492.1
Aureobasidium pullulans	MFLUCC 14-0288	KM461701.1	KM388542.1
Aureobasidium pullulans	CBS 584.75^T	DQ470956.1	FJ150906.1
Kabatiella lini	CBS 125.21^T	FJ150946.1	FJ150897.1
Family Cladosporiaceae			
Rachicladosporium cboliae	CBS 125424 ^{T}	MH875168.1	MH863703.1

187

188 **Fig 2. Phylogenetic tree of concatenated LSU and ITS region sequences of closely related**
189 **species.** *Rachicladosporium cboliae* was used as the outgroup in the phylogenetic tree. The
190 phylogenetic tree was constructed using the maximum likelihood method and Tamura-Nei
191 model with bootstrap values 1,000 replicates. The scale bar indicates substitutions per
192 nucleotide position.

193

194 # Surface-active properties of biosurfactant

195 Growth of strain JAF-11 in culture medium was detected by measuring absorbance at

196 660 nm and surface tensions of the aqueous supernatant was measured using force tensiometer

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197 K11 (Krüss, Germany). While the growth of the strain increased continuously for 8 days, the
198 surface tension of the supernatant decreased from 53 mN/m to 34.5 mN/m for 6 days and
199 increased again after 7days. The surface tension recorded the lowest values of 34.5~34.6 mN/m
200 after 5-6 days of incubation (Fig 3). The above results indicated that the highest biosurfactant
201 production in strain JAF-11 is reached at 5-6 days before the stationary growth phase.

202

203 **Fig 3. Time course of growth kinetics and surface tension in culture medium during**
204 **cultivation of strain JAF-11.**

205

206 The critical micelle concentration (CMC) is defined as the concentration of surfactant
207 required to start the micelles formation. It is determined by plotting the surface tension
208 measured according to the concentration of biosurfactant and identifying the point at which the
209 surface tension of the biosurfactant no longer decreases dramatically. As a results of measuring
210 surface tension of the water with the crude biosurfactants isolated from culture medium, the
211 values were from 72.23 mN/m to 32.80 mN/m and the minimum surface tension value was
212 32.80 mN/m. In particular, the value of CMC was 24 mg/L that the concentration of the
213 biosurfactant obtained from slope of the curve abruptly changed as shown in Figure 4.

214

215 **Fig 4. Determination of critical micelle concentration of producing crude biosurfactant**
216 **from strain JAF-11.**

217

218 Chemical structure of the isolated compound

219 Chemical structure of the biosurfactant isolated was determined by mass and NMR
220 measurements. The molecular weight of 502 was determined by the FAB-MS measurement,
221 which showed a quasi-molecular ion peak at *m/z* 503 $[M+H]^+$ (Fig 5). The molecular formula,
222 $C_{26}H_{46}O_9$, was determined by the HR-FAB-MS providing a molecular ion peak at *m/z* 503.3243

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223 $[M+H]^+$ (calcd. for $C_{26}H_{47}O_9$, 503.3220), indicating four degrees of unsaturation. The ^{1}H NMR
224 spectrum of **1** (Table 2) showed signals due to six oxygenated methines at δ_H 5.50 (t, *J* = 2.7
225 Hz, H-2), 5.28 (t, *J* = 10.0 Hz, H-4) , 4.93 (dd, *J* = 10.0, 2.7 Hz, H-3), 3.66 (t, *J* = 9.5 Hz, H-
226 6), 3.65 (dd, *J* = 10.0, 2.7 Hz, H-1), and 3.45 (t, *J* = 9.0 Hz, H-5). It also showed signals
227 attributable to 14 methylenes at δ_H 2.45 (m, H-2′)/2.42 (m, H-2′), 2.36 (m, H-2″)/2.31 (m, H-
228 2″), 2.18 (m, H-2‴), 1.68 (m, H-3′), 1.59 (m, H-3″), 1.55 (m, H-3‴), and 1.35-1.25 (overlapped)
229 and three methyls at δ_H 0.90 (overlapped). The ^{13}C NMR spectrum (Table 2) in combination
230 with HMQC spectrum displayed signals due to three carbonyl carbons at δ_C 175.0 (C-1′), 174.6
231 (C-1″), and 174.2 (C-1‴), six oxygenated methine carbons at δ_C 74.6 (C-6), 74.2 (C-5), 73.2
232 (C-4), 72.6 (C-2), 71.7 (C-3), and 70.9 (C-1), 14 methylene carbons at δ_C 23.4–35.2, and three
233 methyl carbons at δ_C 14.4 (C-6‴) and 14.2 (C-8′ and C-6″). The ^{1}H-^{1}H COSY correlations
234 among six oxygenated methine protons established the presence of an inositol moiety. Inositol
235 moiety was identified as a *myo*-inositol by the proton coupling constant. Except for an
236 equatorial proton at δ_H 5.50 (H-2) with coupling constant of 2.7 Hz, other protons occupied an
237 axial position based on their proton coupling constants. The ^{1}H-^{1}H COSY spectrum also
238 established six partial structures in three acyl chains, as shown in Fig 6B. Chemical structure
239 was unambiguously determined by the HMBC spectrum, which exhibited the long-range
240 correlations from two methyl protons at δ_H 0.90 (H-8″ and H-8‴) to two methylene carbons at
241 δ_C 32.4 (C-4″ and C-4‴), from the methylene proton at δ_H 1.59 to the carbonyl carbon at δ_C
242 174.6 and the methylene carbons at δ_C 32.4 and 23.4, and the methylene proton at δ_H 1.55 to
243 the carbonyl carbon at δ_C 174.2 and the methylene carbons at δ_C 32.4 and 23.4, implying the
244 presence of two hexanoyl moieties. Other HMBC correlations from the methylene protons at
245 δ_H 2.45/2.42 and 1.68 to the carbons at δ_C 175.0 and 30.2 and from the methyl protons at δ_H
246 0.90 to the carbon at δ_C 32.9, and 14 methylenes in the ^{1}H and ^{13}C NMR spectra indicated the

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247 presence of an octanoyl moiety. Finally, the long-range correlations from the oxygenated
248 methines at δ_H 5.50, 4.93, and 5.28 to the carbonyl carbons at δ_C 175.0, 174.2, and 174.6,
249 respectively, revealed that C-2, C-3, and C-4 in inositol moiety were acylated with one octanoyl
250 and two hexanoyl groups, as shown in Fig 6B. Taken together, the structure of compound **1**
251 was determined to be a new myo-inositol derivative and was named JAF-11. This compound
252 was very similar to pullusurfactan E isolated from *Aureobasidium pullulans* strain A11211-4-
253 57 from fleabane flower, *Erigeron annus* (L.) pers. [26], except for the presence of octanoyl
254 moiety instead of hexanoyl moiety in pullusurfactan E (Fig 6A). Although the chemical
255 structure is similar to pullusurfactan E, this study reports the isolation of a new biosurfactant
256 from the novel yeast strain JAF-11 for the first time.
257
258 **Fig 5. Fast atom bombardment mass spectrum of purified biosurfactant in the positive**
259 **ion mode.**
260
261 **Table 2.** ^{1}H and ^{13}C NMR spectral data of new biosurfactant in CD_3OD.

No.	δ_c	δ_H
1	70.9	3.65 (1H, dd, J = 10.0, 2.7)[a]
2	72.6	5.50 (1H, t, J = 2.7)
3	71.7	4.93 (1H, dd, J = 10.0, 2.7)
4	73.2	5.28 (1H, t, J = 10.0)
5	74.2	3.45 (1H, t, J = 9.0)
6	74.6	3.66 (1H, t, J = 9.5)
1’	175.0	
2’	35.2	2.42 (1H, m), 2.45 (1H, m)
3’	26.3	1.68 (2H, m)
4’	30.2	1.38 (2H, m)
5’	30.2	1.25-1.35 (2H, m)
6’	32.9	1.25-1.35 (2H, m)
7’	23.7	1.25-1.35 (2H, m)
8’	14.2	0.90 (3H, overlapped)
1”	174.6	
2”	35.1	2.31 (1H, m), 2.36 (1H, m)
3”	25.7	1.59 (2H, m)
4”	32.4	1.32 (2H, m)
5”	23.4	1.25-1.35 (2H, m)

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6”	14.2	0.90 (3H, overlapped)
1’”	174.2	
2’”	35.0	2.18 (2H, m)
3’”	25.4	1.55 (2H, m)
4’”	32.4	1.28 (2H, m)
5’”	23.4	1.25-1.35 (2H, m)
6'”	14.4	0.90 (3H, overlapped)

262 [a] Proton resonance integral, multiplicity, and coupling constant (*J*=Hz) in parentheses

263

264 **Fig 6. (A) Chemical structure of novel biosurfactant and (B) two-dimensional NMR**
265 **correlations of novel biosurfactant.**

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267 ## Acknowledgments

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355 # Supporting information

356 **S1 Fig. Screening of biosurfactant producing yeast by drop collapse method**. Distilled
357 water and fresh culture broth were used as a control.

358 **S2 Fig. ^{1}H NMR spectrum of the purified biosurfactant**

359 **S3 Fig. ^{13}C NMR spectrum of the purified biosurfactant**

360 **S4 Fig. HMQC spectrum of the purified biosurfactant**

361 **S5 Fig. ^{1}H-^{1}H COSY spectrum of the purified biosurfactant**

362 **S6 Fig. HMBC spectrum of the purified biosurfactant**

363

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Fig1

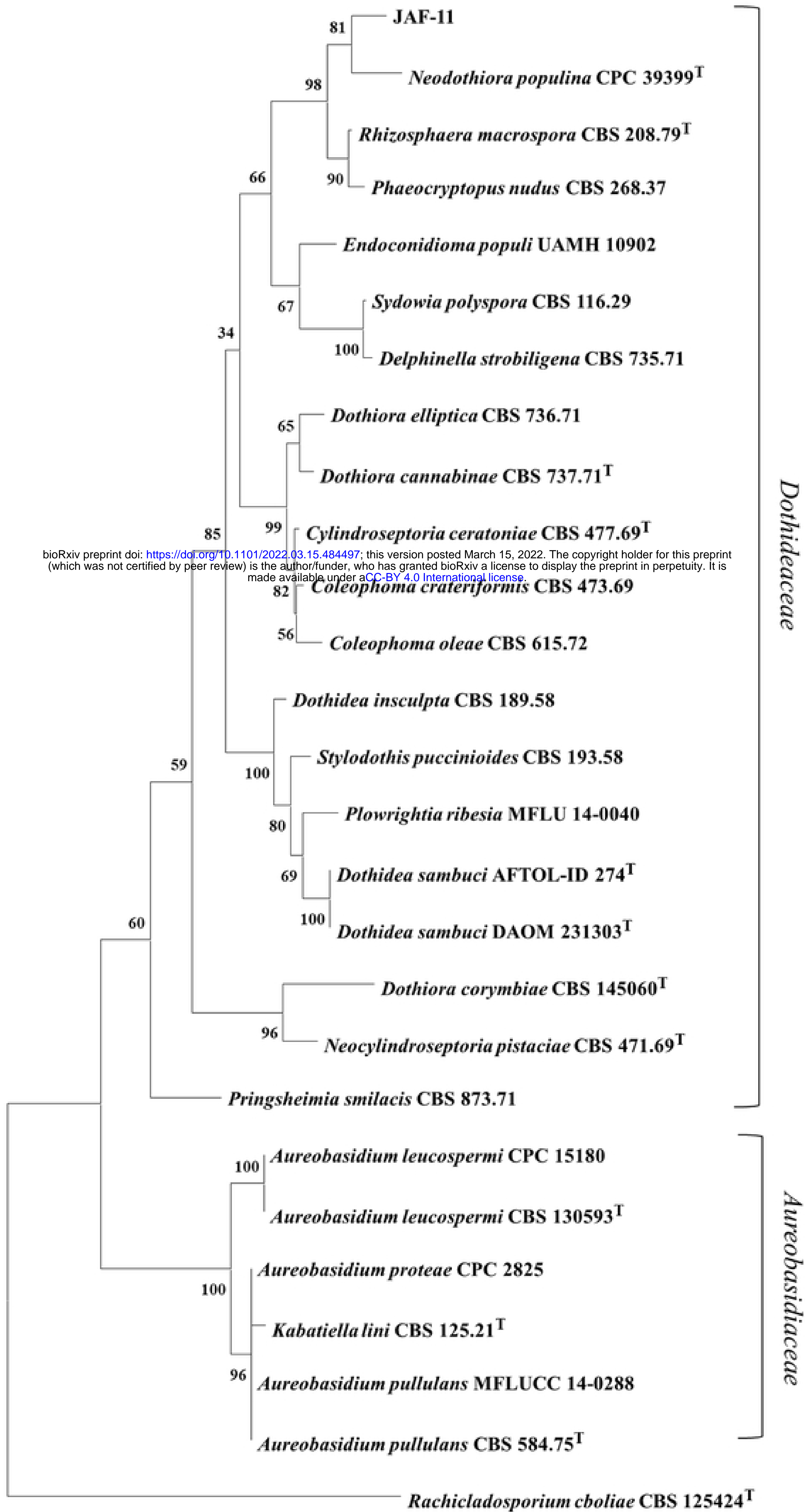

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Fig2

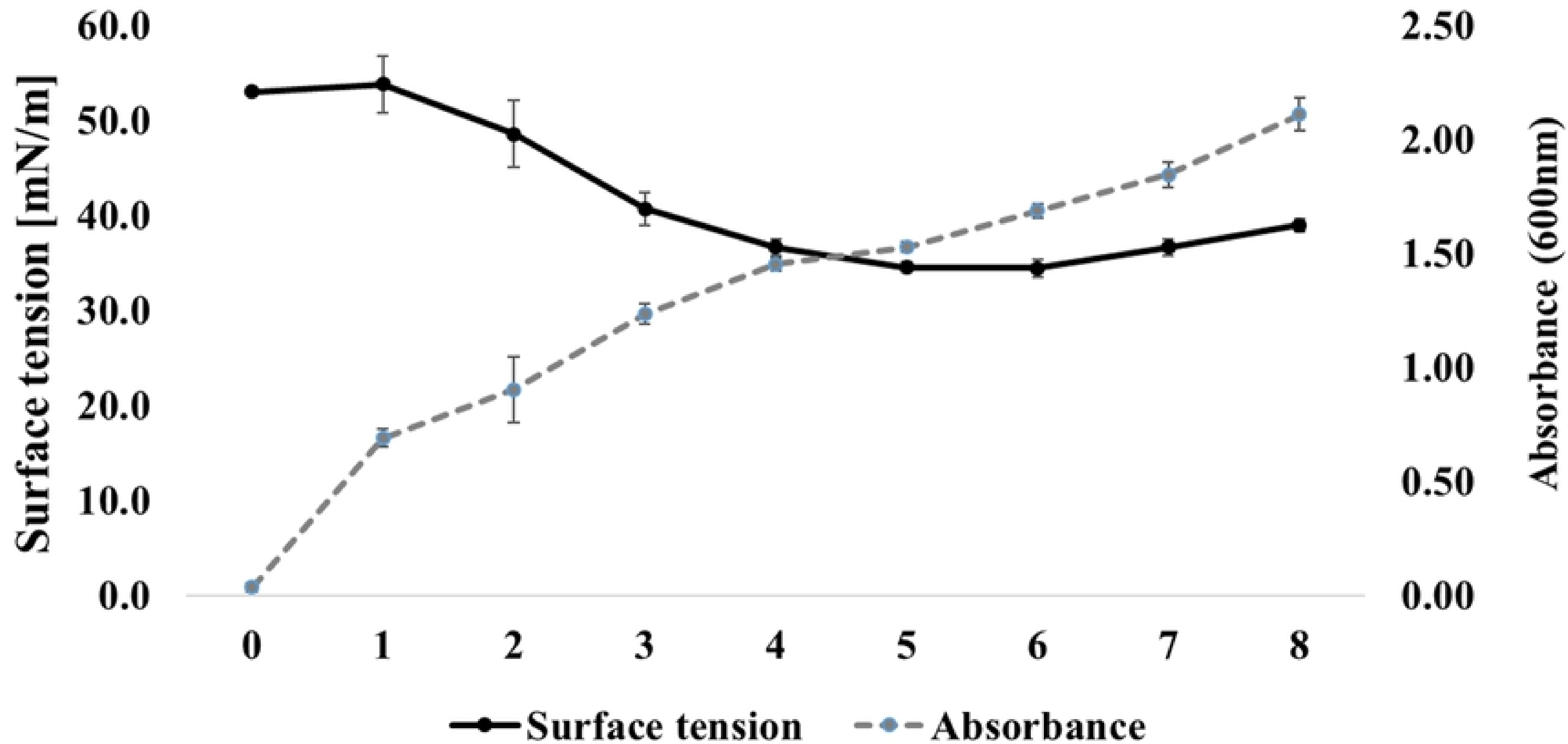
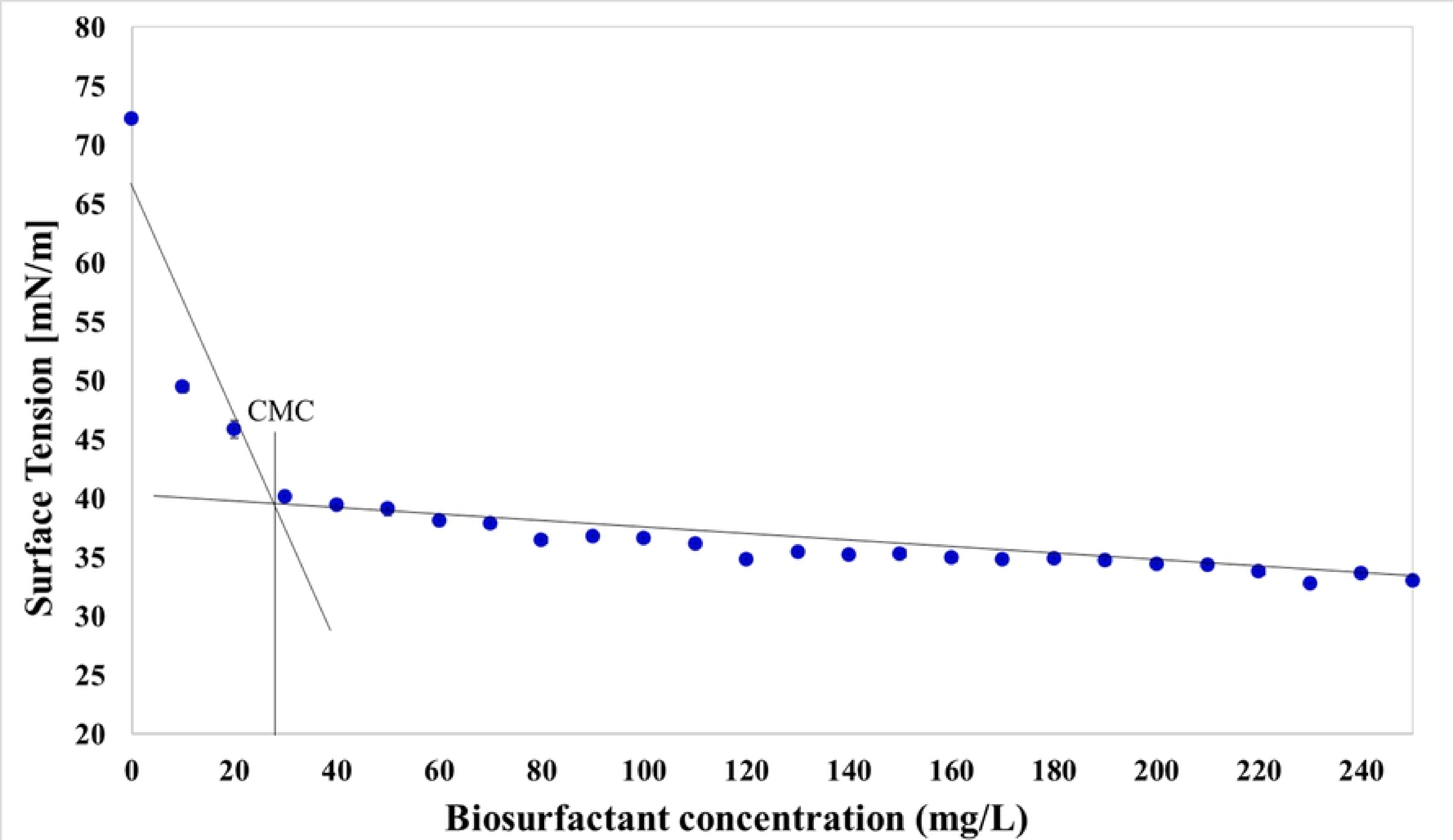


Fig3



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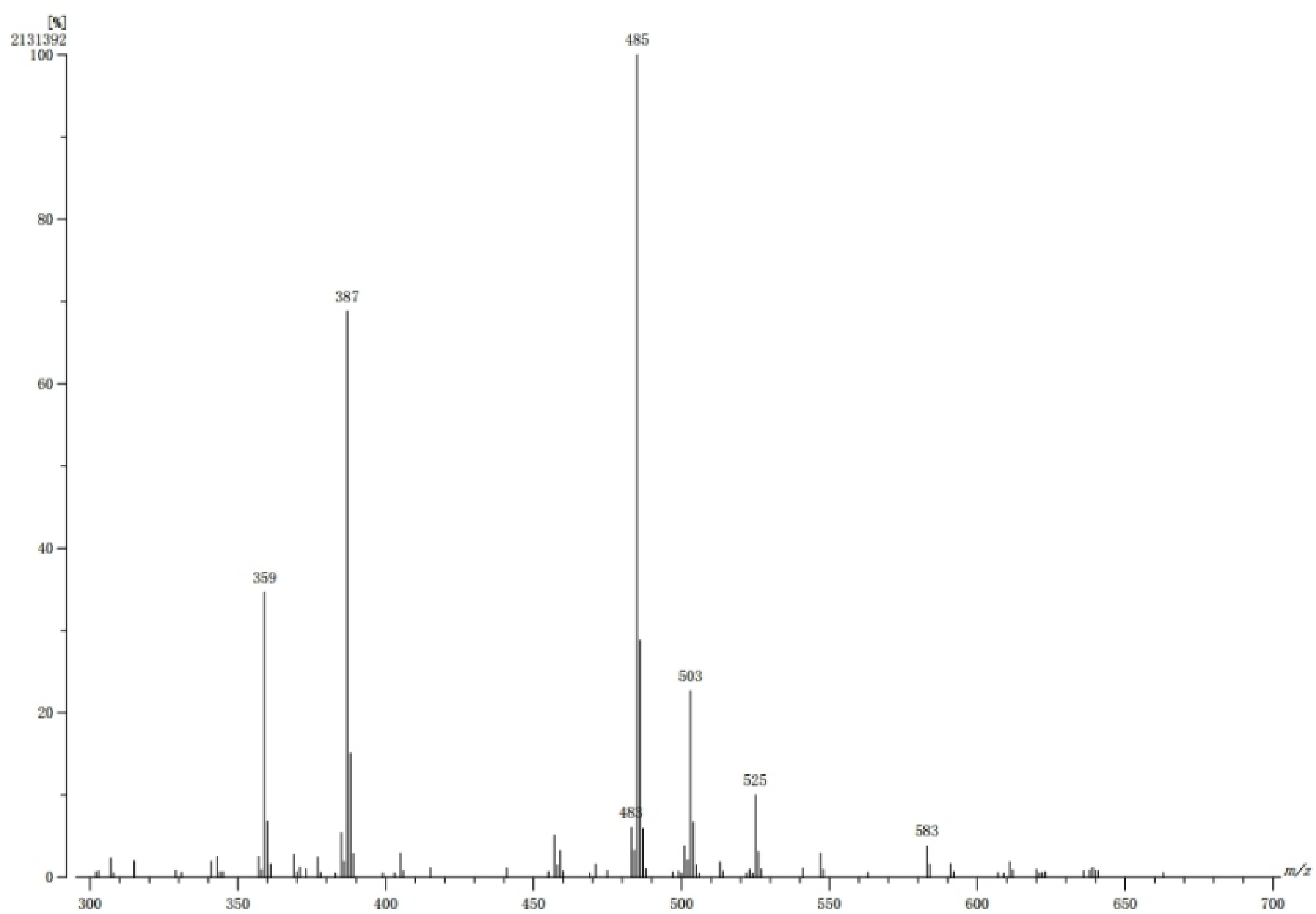

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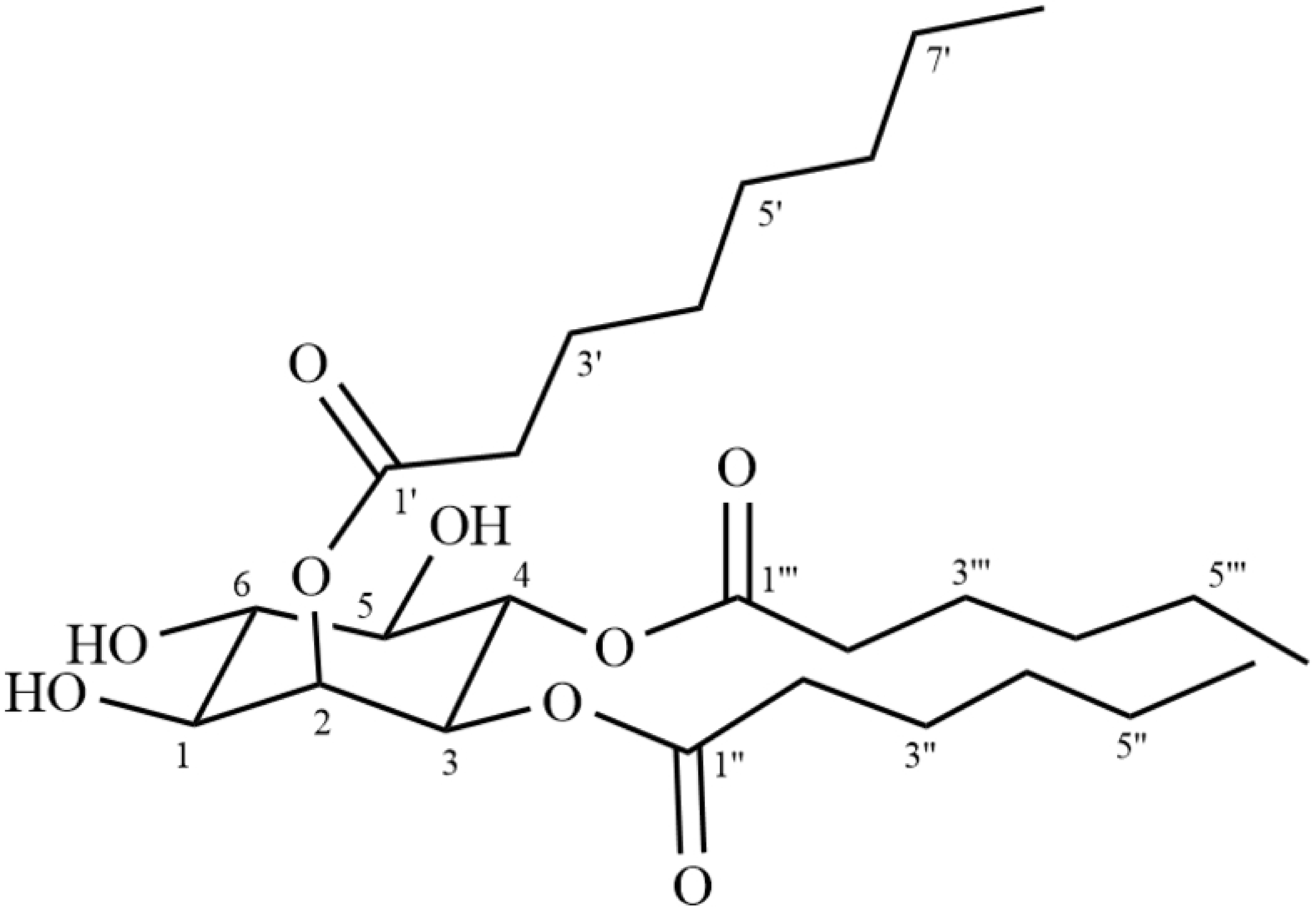

Fig6A

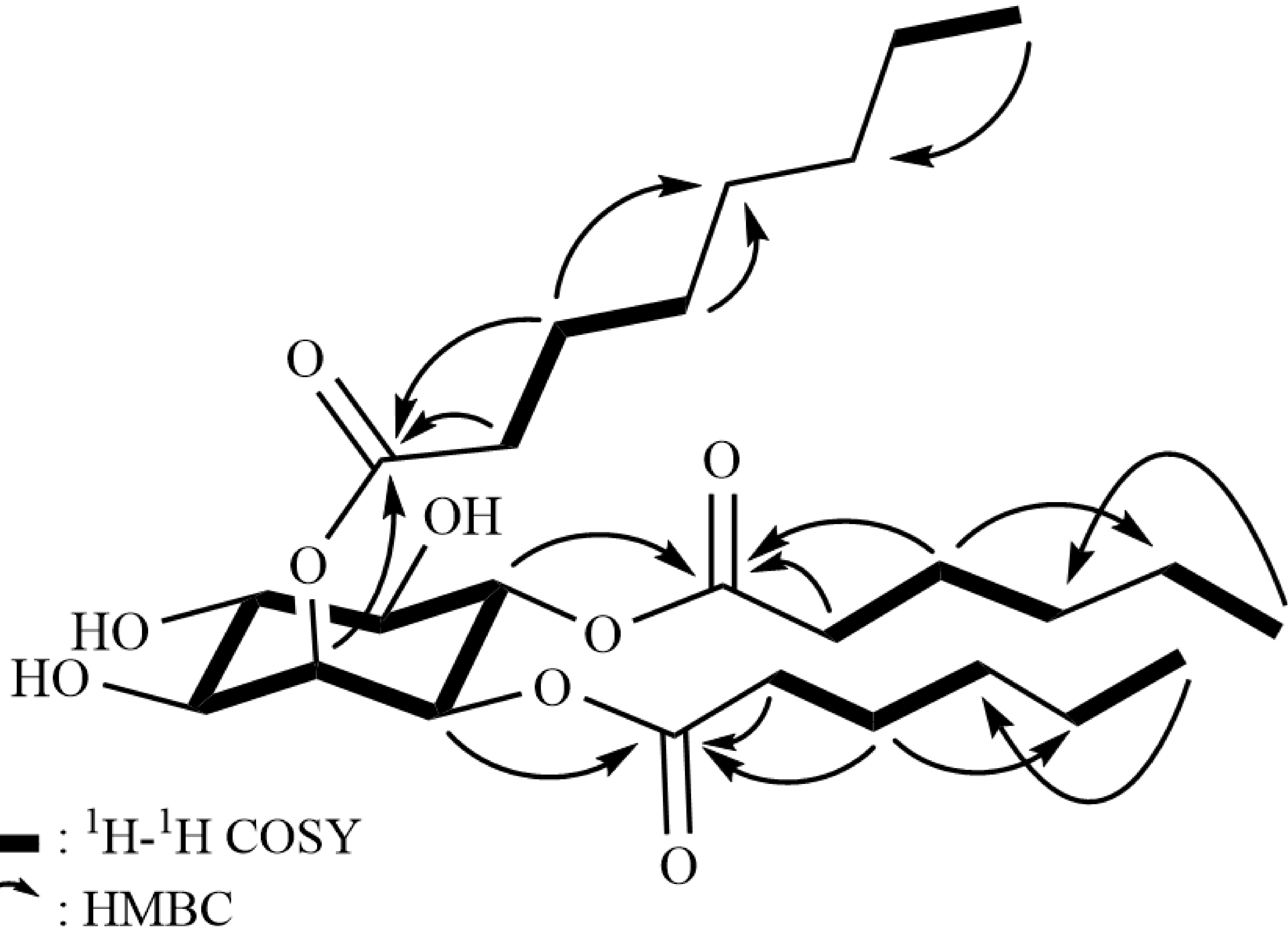

Fig6B