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1 **Selective isolation, antimicrobial screening, and phylogenetic diversity of**
2 **marine actinomycetes derived from the Coast of Bejaia City, Algeria, a**
3 **polluted and microbiologically unexplored environment**

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14 **Running title:** Antimicrobial potential of marine Actinobacteria

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16

17

18 **Abstract**

19 **Aims:** The aim of the current study was to evaluate the occurrence of actinomycetes in the Coast of
20 Bejaia City using different isolation methods, as well as to evaluate their bioactivity.

21 **Methods and Results:** Different selective media and methods were used, leading to the isolation
22 of 103 actinomycete strains. The number of strains was influenced by isolation procedures and their
23 interactions based on a three-way ANOVA and a post-hoc Tukey test, which revealed that using
24 M2 medium, dilution of samples followed by moderate heat treatment, and sampling sediment at
25 10-20 m yielded the highest numbers of actinomycetes. The isolates were screened for their
26 antimicrobial activity against human pathogenic microorganisms using agar and well diffusion
27 methods. Of all the isolates, ten displayed activity against at least one Gram-positive bacterium, of
28 which P21 showed the highest activity against *Staphylococcus aureus*, Methicillin-resistant *S.*
29 *aureus*, and *Bacillus subtilis*, with a diameter of 32, 28 and 25 mm, respectively. Subsequently,
30 active isolates were assigned to *Streptomyces* spp. and *Nocardiopsis* spp. based on 16S rRNA gene
31 sequencing, including a putative new *Streptomyces* species (S3). The phenotypic characteristics of
32 the P21 strain were determined, and interesting enzymatic capacities were shown.

33 **Conclusion:** The recovery of actinomycetes along the coast of Bejaia City was influenced by the
34 isolation procedure. Ten strains displayed interesting antibacterial activity against Gram-positive
35 bacteria, of which the P21 strain was selected as the most active strain.

36 **Significance and Impact of the Study:** This work provides a new insight into the occurrence of
37 actinobacteria in the Coast of Bejaia using selective isolation. It suggests also that polluted
38 environments such as Bejaia Bay could provide access to interesting actinomycetes as sources of
39 antibiotic leads.

40 **Keywords:** Marine actinomycetes, Polluted marine environment, Selective isolation,
41 Antimicrobial potential, Phylogenetic diversity.

42 **Introduction**

43 For decades, severe infections caused by resistant bacteria have been responsible for a major
44 global healthcare problem, requiring longer and more complex treatments that are significantly
45 more expensive to diagnose and treat (Alanis 2005). Moreover, infections generated by Methicillin-
46 resistant *Staphylococcus aureus* (MRSA) have emerged as a widespread cause of community
47 infections, which is due to their ability to develop resistance (Chambers and DeLeo 2009; Pasberg-
48 Gauhl 2014; Barbosa *et al.* 2020). This emerging problem has been observed in many countries,
49 including Algeria (Mairi *et al.* 2020). Thus, the search for new antibiotics remains a worldwide
50 research priority.

51 Marine environments present a vast source of new bioactive natural products, but they are
52 still either unexplored or underexplored (Bhatnagar and Kim 2010, Gogineni and Hamann 2018).
53 Those environments are characterized by particular conditions such as high salinity, pressure, low
54 temperatures, and variable oxygen concentrations (Bullet *al.* 2000). Hence, marine organisms
55 require distinctive metabolic capabilities to adapt and thrive in these environments (Bose *et al.*
56 2015). An increasing number of studies have been conducted on marine actinobacteria as important
57 producers of bioactive compounds in recent years (Maldonado *et al.* 2005; Bull and Stach 2007;
58 Manivasagan *et al.* 2014; Kamjam *et al.* 2017; Barbosa *et al.* 2020; Ibrahimi *et al.* 2020; Goel *et*
59 *al.* 2021). Interestingly, microorganisms living in unusual growth conditions, such as polluted
60 environments, could be a significant source of interesting bacteria. Even though bacteria isolated
61 from polluted environments have been instead investigated for their enzymatic activity their
62 secondary metabolite production remains poorly understood.

63 Actinomycetes have long been recognized as a major source of natural products,
64 particularly antibiotics (Bérdy 2005; Kurtböke 2012; Jose and Jha 2016; Jose *et al.* 2021). Recently,
65 rare actinobacteria have attracted more attention and are defined as strains other than the commonly
66 known *Streptomyces* or strains with less frequency of isolation under conventional methods (Baltz

2006). They have taken an increasingly important position in the production of compounds of biotechnological interest. They have yielded a wide range of molecules with medicinal, industrial, or agricultural uses (Dhakal *et al.* 2017; Ding *et al.* 2019; Mast and Stegmann 2019), notably those isolated from rare actinobacteria derived from extreme environments (Hui *et al.* 2021).

Although the isolation of rare actinomycetes in the marine environment is relatively low due to the need for appropriate isolation procedures and the application of different selection conditions, they are difficult to cultivate and maintain under conventional conditions (Subramani and Aalbersberg 2013; Azman *et al.* 2015). Therefore, a high number of them are "viable but non-culturable" (VBNC) strains (Bernard *et al.* 2000). Recently, several strategies have been developed that have promoted the growth of some previously VBNC species, such as mimicking the natural environment in terms of pH, oxygen gradient, nutrient composition, etc. (Dhakal *et al.* 2017).

Microorganisms derived from extreme biomes have recently attracted scientists' interest since they could house structurally unique natural products that are still unexploited. For that, the current study focuses on the selective isolation of actinomycetes from an unexplored marine environment along the coastline of Bejaia City (Algeria) and screening of their antimicrobial potential against a set of human pathogenic microorganisms.

Materials and methods

Sampling sites

The sampling was performed in the summer of 2017 from different sites (S1, S2 and S3) in the coastal area of Bejaia City, Algeria (Figure 1), and at different depths that ranged from 0.5 to 20 m with the help of a diver. S1 samples were collected at 10-20 m of depth, S2 at 1-2 m, and S3 samples were collected at 0-0.5 m of depth. The sampling area is polluted by different sources, mainly by the petroleum port (S1), industrial wastes (S2), and domestic discharges (S3). Sediment samples (S1) and water samples (S2 and S3) were taken from different points and were collected in sterile glass bottles, then transferred in a cool box to the laboratory for bacteria isolation.

92 **Selective isolation of actinomycetes isolates**

93 To isolate rare genera of actinomycetes, one should avoid the growth of non-actinomycetes
94 bacteria that affect the isolation of actinomycetes known for their slow-growing. Therefore,
95 different selective isolation media were used as previously recommended in several studies, such
96 as: ISP2 (Claverías *et al.* 2015), Starch-Nitrate (Ghanem *et al.* 2000), A4 (Gontang *et al.* 2007),
97 M2 (Jensen *et al.* 1991), and Chitin-agar (Pisano *et al.* 1986). In addition, the isolation media were
98 combined with different isolation methods that are detailed in Table 1. The isolation media were
99 inoculated with 100 µL aliquots of each sample previously pre-treated with methods 1, 2, and 3, as
100 well as method 4, which consisted of the inoculation of untreated samples onto media with added
101 antimicrobial compounds. All inoculated plates were incubated at 28 °C until up to 5 months (with
102 wetted paper towels under the plates to avoid desiccation after 2 months) and examined daily, and
103 all actinomycetes colonies were streaked into new plates containing the same media of isolation
104 until a pure culture was obtained. It should be noted that all isolation media were adjusted to pH
105 7.2 ± 0.2 and prepared with 100% of natural seawater.

106 **Preliminary screening of antimicrobial activity**

107 Preliminary screening for antimicrobial production was estimated by the conventional agar
108 diffusion assay described by Shomura *et al.* (1979) and Engelhardt *et al.* (2010). Briefly, agar discs
109 (8 mm in diameter) recovered from the actinomycetes culture (M2 medium, 14 days of incubation
110 at 28 °C) were placed on the surface of Muller Hinton agar pre-seeded with X µl of the target
111 microorganism (Table 2) at 10^7 UFC/mL. Plates were kept for 2 h at 4 °C for good diffusion of
112 the active compounds. Activity was evaluated by measuring the diameters of the inhibition zones
113 around the agar disc after 24 h of incubation at 37 °C. The M2 medium was composed of 10 g/L
114 of starch, 1 g/L of casein bovine milk, 18 g/L of microbiological agar (Jensen *et al.* 1991), and 1

115 mL (per liter of final medium) of trace salt solution prepared according to (Shirling and Gottlieb
116 1966). All experiments were performed three times independently.

117 **Secondary screening of antimicrobial activity**

118 The previously selected isolates were screened a second time by the well diffusion method
119 to improve the production of antibacterial substances in the broth medium using the supernatant of
120 the culture prepared on M2 medium (Patil *et al.* 2010). Positive activity was estimated by the
121 inhibition zone around the culture supernatant well (8 mm in diameter) of actinomycetes isolates.
122 All experiments were performed three times independently and the results were shown as means
123 with the standard error as error bars.

124 **Molecular identification and phylogenetic analyses of the active isolates**

125 All active isolates were cultured on M2 medium for 14 days at 28 °C. Genomic DNA was
126 extracted from the isolates using the Wizard® Genomic DNA Purification Kit (Promega, USA)
127 according to the manufacturer's instructions. PCR and sequencing were performed as previously
128 described by (Fagervold *et al.* 2013), using universal primers recommended for bacteria 27F mod:
129 5'AGRGTGGATCMTGGCTCAG 3' and 1492R mod: 5'TACGGYTACCTTGTTAYGACTT
130 3'. PCR products were purified using a purification kit (Promega, USA) and sequenced using the
131 Sanger-dideoxy-termination method on the Bio2Mar platform with an automated sequencer
132 (3730XL). The 16S rRNA sequences have been deposited in the GenBank database under accession
133 numbers that are given in parentheses in Figure 5.

134 The 16S rRNA gene sequences were submitted to the EZBiocloud database for
135 identification (Yoon *et al.* 2017), available at: <https://www.ezbiocloud.net>. The sequences were
136 imported into the SILVA_138.1_SSUref_NR99_12_06_20_opt.arb database and aligned using the
137 arb fast aligner tool. Sequence positions (662 total), excluding ambiguous and missing, and present
138 in a majority of sequences, were exported and used in phylogenetic reconstruction. Subsequently,

139 a phylogenetic tree was constructed using MEGA X software (Kumar *et al.* 2018) by the Neighbor-
140 Joining (NJ) method using the p-distance model (Felsenstein 1985), and the robustness of the tree
141 was estimated by 1000 bootstrap random resampling of the original dataset.

142 **Phenotypic characterization of the selected strain (P21)**

143 The micro-morphology of the selected strain (P21) was established by light microscopy to
144 visualize mycelia organization and sporulation. The strain was grown on M2 medium at 28 °C for
145 two weeks before being observed under an optical microscope (Euromex). Furthermore,
146 morphological and physiological characteristics were carried out as described in the *International*
147 *Streptomyces Project* (ISP) recommendations by Shirling and Gottlieb in 1966, as well as on three
148 "no ISP" media, including nutrient agar, Bennet medium (Yokota *et al.* 1993) and GYEA medium
149 (Athalye *et al.* 1981). Melanoid pigment production was determined on tyrosine agar medium
150 (ISP7) recommended by (Shirling and Gottlieb 1966). In addition, the utilization of carbohydrates
151 was tested on a minimal medium (ISP9) containing different carbon sources (D-glucose, sucrose,
152 xylose, inositol, mannitol, D-fructose, rhamnose, and cellulose) at 1% concentration. Basal medium
153 without a carbon source served as a control. Moreover, the conditions of growth, such as the effect
154 of pH (4-14), temperature of growth (4-45 °C), and NaCl tolerance were evaluated (Aouiche *et al.*
155 2012). Resistance to different antibiotics was carried out using the disc diffusion test as
156 recommended by the Clinical and Laboratory Standards Institute (CLSI, 2016). The tested
157 antibiotics were: Penicillin G (6 µg), Ciprofloxacin (5 µg), Clindamycin (2 µg), Tetracycline (30
158 µg), Gentamicin (10 µg), and Chloramphenicol (30 µg). Other tests on the selected strain were
159 carried out according to (Gordon and Mihm 1956), such as the ability of the strain to decompose
160 substrates including casein, starch, and gelatin, and resistance to lysozyme at 0.005% (W/V), while
161 the tyrosine assimilation was performed according (Kurup and Fink 1975).

162 **Statistical analyses**

163 All experiments were carried out in triplicate. Firstly, the selective isolation data were
164 analyzed using different statistical tests. The normality of the distribution in the studied groups was
165 confirmed by the Shapiro-Wilk test. To find out the effect of the isolation methods, the used media,
166 and the sampling depths on the number of actinomycetes isolated (note, we did not correct for the
167 dilution in Method 1), isolation data were compared by three-way ANOVA (Analysis Of Variance)
168 (3WA), followed by the post hoc Tukey test without correction for multiple comparisons. The test
169 was considered statistically significant if ($p < 0.05$) and was conducted using XLSTAT software
170 (version 2018.1).

171 Secondly, the preliminary antimicrobial activity screening data were analyzed using
172 principal component analysis (PCA) and hierarchical cluster analysis (HCA). The statistical
173 analyses were performed using R software version 4.0.2 ([https://www.r-project.org/https://www.r-](https://www.r-project.org/https://www.r-project.org/)
174 [project.org/](https://www.r-project.org/)), the RProject for Statistical Computing, with the plugin FactoMineR included in R.
175 The PCA plot is used to reduce the dimensionality of such datasets, increasing interpretability while
176 minimizing information loss.

177 **Results**

178 **Samples collection and isolation of actinomycetes**

179 Three polluted marine sites were sampled at different depths from the coastal areas of Bejaia
180 City (Algeria), using different methods and media. A total of 103 colonies of presumptive
181 actinomycetes were observed and isolated. The number of isolates varied with the used methods,
182 media, and sampling depths. As a result of the selective methods that were used, Method 1 showed
183 the highest number of isolates with 46, while 12, 20, and 25 isolates were obtained when Methods
184 2, 3, and 4 were applied (Figure 2A), respectively. Based on the isolation media used, we found
185 that M2 medium, ISP2 and Chitin-agar medium gave the highest number of isolates with 32, 27
186 and 22 isolates, respectively. On the other hand, only eleven isolates were obtained with Starch-

187 Nitrate and A4 (Figure 2B). According to the results of sampling depths (Figure 2C), 56 isolates
188 were obtained at D1 (10-20 m), 33 isolates at D2 (1-2 m), and only 14 isolates were obtained at D3
189 (0-0.5 m).

190 Furthermore, the isolation variables (methods, media, and depths) showed a significant
191 effect on the number of actinomycetes isolated ($p<0.05$) based on the three-way ANOVA test. The
192 M2 medium, Method 1 and D1 affected were the best combination for the recovery of
193 Actinomycetes ($p<0.05$). Moreover, the post hoc Tukey test revealed a significant interaction
194 between the different depths/isolation methods ($p<0.05$), but no significant interaction was found
195 between the different depths/isolation media, or the isolation methods/isolation media.

196 **Preliminary screening assay**

197 The isolates were screened for their antimicrobial activity by the agar diffusion method
198 against various pathogenic microorganisms (Gram-positive and Gram-negative bacteria, and a
199 yeast). The results showed that 10 out of 103 isolates exhibited antibacterial activity against at least
200 one of the pathogenic tested strains, particularly against Gram-positive bacteria including *S. aureus*,
201 MRSA, and *B. subtilis*, while no activity was detected against the yeast and Gram-negative bacteria.

202 The results of preliminary antimicrobial screening were subjected to a PCA analysis, which
203 was performed on the active isolates (10 isolates) using nine variables. Results showed that 100%
204 of the total variation was distributed in three dimensions; the horizontal axis explained 65.64% of
205 the total variance, and the vertical explained 32.65% (Figure 3A). The results demonstrated
206 substantial differences between active strains. Moreover, it was found that only three variables
207 contributed to the construction of this PCA, which are MRSA, *S. aureus*, and *B. subtilis*. Figure 3B
208 presents the projection of individuals (actinomycetes isolates). Following that, the PCA data were
209 used for Hierarchical Cluster Analysis (HCA). Therefore, the isolates were grouped into four
210 clusters of active actinomycetes (Figure 3C) identified by their specific antimicrobial activity

211 against targeted bacteria, according to the results of HCA analysis. The first cluster was presented
212 by two isolates (S3 and S5) that showed activity only against *B. subtilis* with a 15 mm inhibition
213 zone, while no activity was shown against MRSA and *S. aureus*. The isolates P19, P24, E, and C15
214 formed the second cluster, which showed significant activity against MRSA and *S. aureus* that
215 varied between 11-22 mm, but no activity was observed against *B. subtilis*. The third cluster was
216 presented by three isolates, S1, MP1m, and S4 that showed relatively similar activities against
217 MRSA, *S. aureus*, and *B. subtilis*, with an inhibition zone that varied from 11-22 mm. The fourth
218 cluster was presented by the strongest active isolate, P21, which showed 28, 32, and 25 mm of the
219 inhibition zones against MRSA, *S. aureus*, and *B. subtilis*, respectively. Thus, cluster 4 is the most
220 active cluster ($p<0.05$) based on inhibition diameter size with the highest antibacterial mean against
221 MRSA and *S. aureus*.

222 **Secondary screening**

223 A secondary screening of the previously selected active actinomycetes was performed on
224 M2 medium by the well diffusion method. Except for two isolates (S1 and S3), all active isolates
225 showed positive activity (Figure 4). The P21 strain was found to be the most active isolate, with
226 inhibition zones of 25, 33, and 27 mm against MRSA, *S. aureus*, and *B. subtilis*, respectively. The
227 isolates C15, E, S4 and S5 showed activity against MRSA and *S. aureus* with inhibition zones that
228 varied between 17-26 mm. The P24 isolate showed an inhibition zone of 9 mm against MRSA. The
229 MP1m isolate showed 14, 22, and 8 mm of inhibition zones against MRSA, *S. aureus*, and *B.*
230 *aureus*, respectively, and the P19 strain showed 25 and 17 mm against MRSA and *B. subtilis*.
231 However, no activity was recorded against Gram-negative bacteria or yeast.

232 **Molecular identification and phylogenetic analyses of the active isolates**

233 The 16S rRNA sequences of active isolates were identified using the EZBiocloud database.
234 Isolates were found to belong to the Actinobacteria phylum and were grouped into two genera:

235 *Streptomyces* (8 out of 10), and *Nocardiopsis* (2 out of 10), with similarity percentages to known
236 stains ranging from 98 to 100% (Table S1). Isolates P21 and S5 belonging to the *Nocardiopsis*
237 genus showed 100% similarity to *Nocardiopsis halotolerans* DSM 44410(T) and *Nocardiopsis*
238 *aegyptia* DSM 44442(T), respectively. Two isolates, C15 and P19, were closely similar to
239 *Streptomyces xinghaiensis* S187(T) with 99.78% and 99.71% of similarity, respectively. The P24
240 isolate had a 99.79% similarity with *Streptomyces albidoflavus* DSM 40455(T). The S1 isolate
241 showed the highest similarity (99.76%) to *Streptomyces hydrogenans* NBRC 13475(T). The S3
242 isolate was the most closely (98.44%) related strain to *Streptomyces rimosus subsp. rimosus* ATCC
243 10970(T), which could possibly indicate the presence of a new species within the *Streptomyces*
244 genus. Moreover, the S4 isolate showed 99.79% similarity to *Streptomyces albogriseolus* NRRL
245 B-1305(T). Both E and Mp1m isolates were similar to *Streptomyces griseoflavus* LMG 19344(T)
246 with 99.62% and 99.61% of identity, respectively.

247 A phylogenetic tree based on the 16 rRNA gene sequences (Figure 5) was constructed using
248 the neighbor-joining method (Saitou and Nei 1987) using *Micrococcus luteus* NCTC 26650 as an
249 outgroup. The P21 strain formed a monophyletic line with the S5 strain supported by 100% of the
250 bootstrap replicates. The following strains: C15, E, Mp1m, S4, P19, P24, S3, and S1 appeared in
251 one cluster as closest neighbors.

252 **Phenotypic characterization of a selected strain (P21)**

253 The observation of the P21 strain under an optical microscope showed that it has a
254 branching substrate mycelium and produces short chains of spores (Figure 6A). On the other hand,
255 Figure 6B shows the aerial mycelium, which has the typical zig-zag form of *Nocardiopsis* strains
256 that are fragmented in older cultures. The cultural characteristics of the selected strain are presented
257 in Table S2. The P21 strain was growing well on all media except nutrient agar, where poor growth
258 was observed. Differences in the growth rates were observed, and the color of the substrate
259 mycelium varied from brown-red to white. The production of soluble pigments was observed in

260 some media (ISP2, ISP5, M2, and Bennet) but not in others (ISP3, ISP4, and Nutrient Agar).
261 Generally, the colors of the aerial mycelium were not the same as those of the substrate mycelium.
262 Table S3 lists the physiological and biochemical characteristics of the P21 isolate. The selected
263 strain was not able to assimilate the carbon sources tested. However, it showed good growth when
264 the medium was supplemented with 10% of sodium chloride, but poor growth was observed at 3-
265 7% and no growth was found at 0% and 15-30% of NaCl. The optimum range of pH growth was
266 found between 7 and 10. Moderate growth was observed at pH 3-5, while no growth was observed
267 at pH 12-14. Moreover, the temperature ranges for growth were between 28 and 40 °C, with an
268 optimum at 28 °C. The P21 strain was found to be able to degrade a variety of substrates, including
269 starch, tyrosine, gelatin, and casein from bovine milk. Furthermore, based on the susceptibility test
270 results, it was found that the P21 strain was resistant to Penicillin G and Clindamycin, but sensitive
271 to Ciprofloxacin, Tetracycline, Gentamicin, and Chloramphenicol.

272 **Discussion**

273 Marine environments are unexplored in Algeria in terms of their microbiota; therefore, the
274 aim of our study was to isolate actinomycetes from those environments. A total of 103
275 actinomycetes isolates were obtained from the Coast of Bejaia City using different methods and
276 media. These isolates were screened for their antimicrobial activity, and ten isolates showed
277 activity against at least one Gram-positive bacterium (*S. aureus*, MRSA, and *B. subtilis*), while no
278 activity was found against Gram-negative bacteria or the yeast tested.

279 Different selective methods and media were used in this study to promote the growth of rare
280 genera of actinobacteria and avoid the growth of terrestrial actinomycetes while reducing
281 undesirable flora occurring on the isolation plates, as have been reported by others (Pisano *et al.*
282 1986; Undabarrena *et al.* 2016; Kamjam *et al.* 2017). The application of pretreatments or the
283 presence of antimicrobial substances influences the number of actinomycetes isolates (Bull and
284 Stach 2007). In the present study, Method 1 proved to be among the most effective methods for

285 isolating actinomycetes, which is consistent with the observation of Pisano *et al.* (1987) that
286 recorded that heat pretreatment of either 55 °C or 100 °C is a suitable alternative to the higher
287 temperature for actinomycetes isolation, the fact that we did not correct the numbers for the initial
288 dilution makes this result more notable and could also suggest that dilution of inhibiting substances
289 in this highly polluted samples could have also be in play. The low (103) number of total strains in
290 540 petri dishes supports the possibility of negative effects of the pollution on the actinomycete
291 community.

292 Using a filter membrane (Method 3) or adding antimicrobial substances to the isolation
293 media (Method 4) have been previously reported to be the best methods for actinomycetes isolation
294 from water and sediments according to (Hirsch and Christensen 1983) and (Bredholdt *et al.* 2007),
295 but in our case, these methods (3 and 4) gave fewer isolates compared to Method 1. Even though
296 Bredholt *et al.* (2008) found that heating and exposure of samples to phenol increased the number
297 of mycelium-forming actinomycetes, this method (2) yielded the lowest number of isolates. Based
298 on the isolation media used, the M2, ISP2 and Chitin-agar seem to be the best media for isolating
299 actinomycetes from marine environments. Nevertheless, other studies revealed that low-nutrient
300 media were more efficient than rich-nutrient media for the isolation of rare marine actinobacteria
301 (Jensen *et al.* 2005; Maldonado *et al.* 2005). Furthermore, the sampling depth influences the
302 number of actinomycetes isolated. It was therefore found that the number of isolates increased
303 proportionally with the depth, and the highest number of isolates was obtained at D1 (10-20 m). A
304 similar observation was reported previously by (Jensen *et al.* 1991). Nonetheless, based on the
305 statistical significance of 3WA and Tukey's post hoc test, the best procedure that gave the highest
306 number of actinomycetes isolates involves using the M2 medium, Method 1, and sampling at 10-
307 20 m (D1) of depth. According to these findings, we suggest that the differences in the responses
308 to the isolation conditions between our study and other studies are very likely due to the level of
309 pollution in the isolation area that affects the abundance of actinobacteria strains. Moreover, the

310 designated methods to isolate actinomycetes from polluted marine environments including
311 sediment and water samples are not optimal for such biotopes, and more effects are required to
312 establish specific isolation methods for such environments.

313 The obtained isolates were screened for their antimicrobial activity against nine human
314 pathogenic bacteria. Targeting more than one pathogenic microorganism to obtain diverse
315 bioactive bacteria has previously been supported by (Genilloud *et al.* 2011). Ten isolates were
316 selected among 103 that showed activity against at least one targeted Gram-positive bacteria.
317 However, this percentage is relatively low in comparison with the results of Peela *et al.* (2005),
318 who reported that the majority of marine actinomycetes isolated showed antagonistic potential,
319 including antibacterial and antifungal activities. The low rate of active strains could have several
320 possible reasons: 1). Is probably due to the fact that the screening of isolates was based on a specific
321 activity that could be inappropriate for the isolates' potential, and thus we missed other biological
322 activities. 2). The isolates might possess biosynthesis gene clusters that encode for bioactive
323 compounds but are, "silent" or "cryptic", which means they are not expressed under laboratory
324 conditions. 3). The method or medium used is inappropriate for the production of metabolites. For
325 these reasons, further investigations are being envisaged. Interestingly, the active isolates only
326 showed activity against Gram-positive bacteria, suggesting that the isolates have developed
327 specific marine adaptation mechanisms that likely affect secondary metabolite biosynthetic
328 pathways.

329 The P21 isolate was shown to be the most active strain against Gram-positive bacteria and
330 belongs to the *Nocardiopsis* genus, which has been described as a producer of a wide range of
331 bioactive compounds (Fu *et al.* 2013; Hatem Ibrahim *et al.* 2018; Tatar 2020). Although this genus
332 has received less attention than *Streptomyces*, which has been extensively investigated (Lazzarini
333 *et al.* 2000; Takahashi *et al.* 2018). Previous studies have shown the anti-MRSA activity of the
334 *Nocardiopsis* genus, such as the activity of the angucyclines (nocardiopsistins A-C) isolated from

335 a marine sponge-derived *Nocardiopsis* sp. (Xu *et al.* 2018). The P21 strain does not show any
336 activity against Gram-negative bacteria even though previous studies have provided the
337 antagonistic potential of *Nocardiopsis* strains against Gram-negative bacteria, such as
338 *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* (Rajivgandhi *et al.* 2019), *Enterococcus faecium*
339 and *Candida albicans* (Engelhardt *et al.* 2010). Hence, this difference is likely due to the specific
340 activity of the secreted molecules by the P21 strain against Gram-positive bacteria, especially
341 MRSA (Figure 7).

342 Marine sediments harbor a large diversity of culturable actinobacteria (Bredholdt *et al.*
343 2007; Gontang *et al.* 2007; Claverías *et al.* 2015). In the current work, the phylogenetic diversity
344 analysis of the active strains showed that the isolates belonged to two genera (*Streptomyces* and
345 *Nocardiopsis*), which are widely distributed in marine environments worldwide (Claverías *et al.*
346 2015; Jose and Jha 2016). The low recovery observed both in number and diversity could be due
347 to the influence of the contaminants that are present in the isolation region, which can reduce the
348 biological diversity of actinobacteria. In addition, the results of the phylogenetic analysis showed
349 that the homology of the sequences with the closely related species ranged from 98 to 100%. In
350 particular, the S3 isolate showed 98.44% similarity to the closest strain, which is below the 98.65%
351 threshold for this genus (Kim *et al.* 2014). This isolate can thus be considered a presumptive new
352 *Streptomyces* species. Moreover, several studies in Algeria have recorded the *Nocardiopsis* genera
353 from soil samples, such as the desert soil (Zitouni *et al.* 2005; Meklat *et al.* 2011), the salt pan of
354 the Kenadsa region (located in southwest Algeria) (Messaoudi *et al.* 2019), a salt lake in the
355 Algerian Sahara (Hadj Rabia-Boukhalfa *et al.* 2017), and from the rhizosphere-associated with
356 semi-arid soil in the North-East of Algeria (Aouar *et al.* 2012). However, *Nocardiopsis* strains had
357 never been isolated from marine environments in Algeria.

358 Morphological characteristics, mainly based on the substrate mycelium, the aerial hyphae,
359 pigment production, spore appearance, and chaining, have been proven to be an effective and

360 widely used reference for the preliminary identification of actinobacteria (Taddei *et al.* 2006). The
361 P21 isolate showed the typical microscopic appearance of most species of the *Nocardiopsis* genus.
362 It grew well on several media and showed various cultural characteristics that are related to the
363 available substrate and growth conditions. The P21 strain was susceptible to four antibiotics and
364 displayed resistance to two antibiotics tested (Penicillin G and Clindamycin). According to
365 previous reports, antibiotic resistance may reveal an isolate's ability to produce the antibiotic(s) to
366 which it is resistant (Basu and Paul 1992; Jain *et al.* 2012). Furthermore, biochemical
367 characteristics showed the potential of the P21 strain to degrade several substrates, which is in line
368 with previous reports that described the *Nocardiopsis* genus as a potential source of extracellular
369 enzymes (Bennur *et al.* 2014). Further experiments should be carried out to confirm the enzymatic
370 abilities of this strain.

371 In conclusion, the Coast of Bejaia City was an interesting source of bioactive actinobacteria
372 strains with the possibility of the presence of new *Streptomyces* species. *Nocardiopsis* sp. P21 was
373 found to be the most active strain, with potential for new antimicrobial therapies to fight MRSA
374 infections. Further studies are required to understand the biosynthetic pathways involved in the
375 antibacterial activity of the active strains, including genome mining and metabolomics analyses.

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584 **Figure legends**

585 **Figure 1** Map showing the sampling sites of actinomycetes isolates. Sampling spots (**S1**:
586 36°45'29.6"N5°05'50.3"E, **S2**: 36°44'12.1"N5°04'50.7"E, and **S3**: 36°43'55.2"N5°04'37.9"E) were
587 indicated. The map was generated with the R package ggmap (Kahle and Wickham 2013) under
588 the development environment Rstudio 1.4.1 (<https://www.R-project.org/>) coupled with R 4.0.2,
589 then it was polished with the image-editing tool Paint.net 4.2.14.

590 **Figure 2** Actinomycetes counts in sampling sites collected from the Coast of Bejaia (Alegria)
591 depending; **(A)** isolation method (1, 2, 3 and 4) , **(B)** isolation media (M2, ISP2, Starch-Nitrate,
592 and Chitin-agar), **(C)** sampling depth (D1: 10- 20 m, D2: 1-2 m and D3: 0- 0.5 m).

593 **Figure 3** Principal component analysis (PCA) and hierarchical clustering analysis of active
594 actinomycetes based on antimicrobial activity against 9 human pathogenic bacteria. **(A)** Projection
595 of the evaluated variables into the two-dimensional space, Dim1 and Dim2. **(B)** Projection of the
596 active strains into the two-dimensional space, Dim1 and Dim2. **(C)** Hierarchical clustering of active
597 isolates based on antimicrobial activity. **(D)** Actinomycetes isolates hierarchical grouping in a
598 three-dimensional space.

599 **Figure 4** Average inhibition zones (mm) of antimicrobial activity of active actinomycetes isolates
600 against targeted microorganisms using the well diffusion method. Error bars indicate \pm s.e.
601 averaged over three measurements.

602 **Figure 5** Neighbor Joining-phylogenetic tree showing the evolutionary relationship of bioactive
603 strains (marked with a triangle) and closely related sequences based on partial 16S rRNA gene

604 sequences. The percentage of replicate trees in which the associated taxa clustered together in the
605 bootstrap test (1000 replicates) is shown next to the branches (**Felsenstein 1985**). The evolutionary
606 distances were computed using the *p*-distance method and are in the units of the number of base
607 differences per site. The accession number is shown in parentheses following the strain name. The
608 scale bar corresponds to 0.01 substitutions per nucleotide position.

609 **Figure 6** Morphological appearance of P21 strain grown on M2 medium after 2 weeks at 28 °C.
610 **(A)**: Micro-morphology of Filamentous vegetative mycelia under light microscopy (x40), **(B)**:
611 macroscopic observation.

612 **Figure 7** Antibacterial activity of the P21 strain against MRSA. Using the agar diffusion method
613 (A) and well diffusion method (B). The delimited zones on red present the inhibition zones.

614 **Legends for the supporting information**

615 **Table S1** Molecular identification of actives isolates by EZBiocloud database

616 **Table S2** Cultural characteristics of the P21 isolate

617 **Table S3** Physiological and biochemical characteristics of the P21 isolate

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Table 1 Descriptions of the applied isolation methods

Method	Description
Method 1	Samples were diluted at 1:4 (v/v) with sterile sea water, then vigorously shaken. They were then heated for 6 min at 55 °C (Gontang <i>et al.</i> 2007). A 100 µL volume of the treated sample was inoculated into each media.
Method 2	Samples were heated at 120 °C for 1 h, then exposed to 1.5% phenol for 30 min (Bredholt <i>et al.</i> 2008). A 100 µL volume of the treated sample was inoculated into each media.
Method 3	The agar plates of all media were overlaid with a cellulose ester membrane filter (0.45 µm pore size) and then inoculated with a 100 µL volume of the untreated sample. After 2 weeks, the filters were removed and the plates were reincubated (Hirsch and Christensen 1983).
Method 4	The isolation media were supplemented with filtered (0.2-µm pore size) Nalidixic acid (10 µg/mL), Nystatin (50 µg/mL) (Bredholdt <i>et al.</i> 2007), and Cycloheximide at (50 µg/mL) as recommended by (Bredholt <i>et al.</i> 2008). A 100 µL volume of the untreated sample was inoculated into each media.

Table 2 Targeted organisms tested to select bioactive Actinomycetes

	Microorganisms tested	References
Gram + Bacteria	Methicillin-resistant <i>Staphylococcus aureus</i> (MRSA)	ATCC 43300
	<i>Staphylococcus aureus</i>	ATCC 25923
	<i>Bacillus subtilis</i>	ATCC 6633
	<i>Enterococcus faecalis</i>	ATCC 10541
Gram – Bacteria	<i>Escherichia coli</i>	ATCC 25922
	<i>Pseudomonas aeruginosa</i>	ATCC 27853
	<i>Salmonella typhi</i>	ATCC 6539
	<i>Klebsiella oxytoca</i> (Flugge)	ATCC 13182
Yeast	<i>Candida albicans</i>	ATCC 10231

ATCC: American Type Culture Collection.

Figure 1

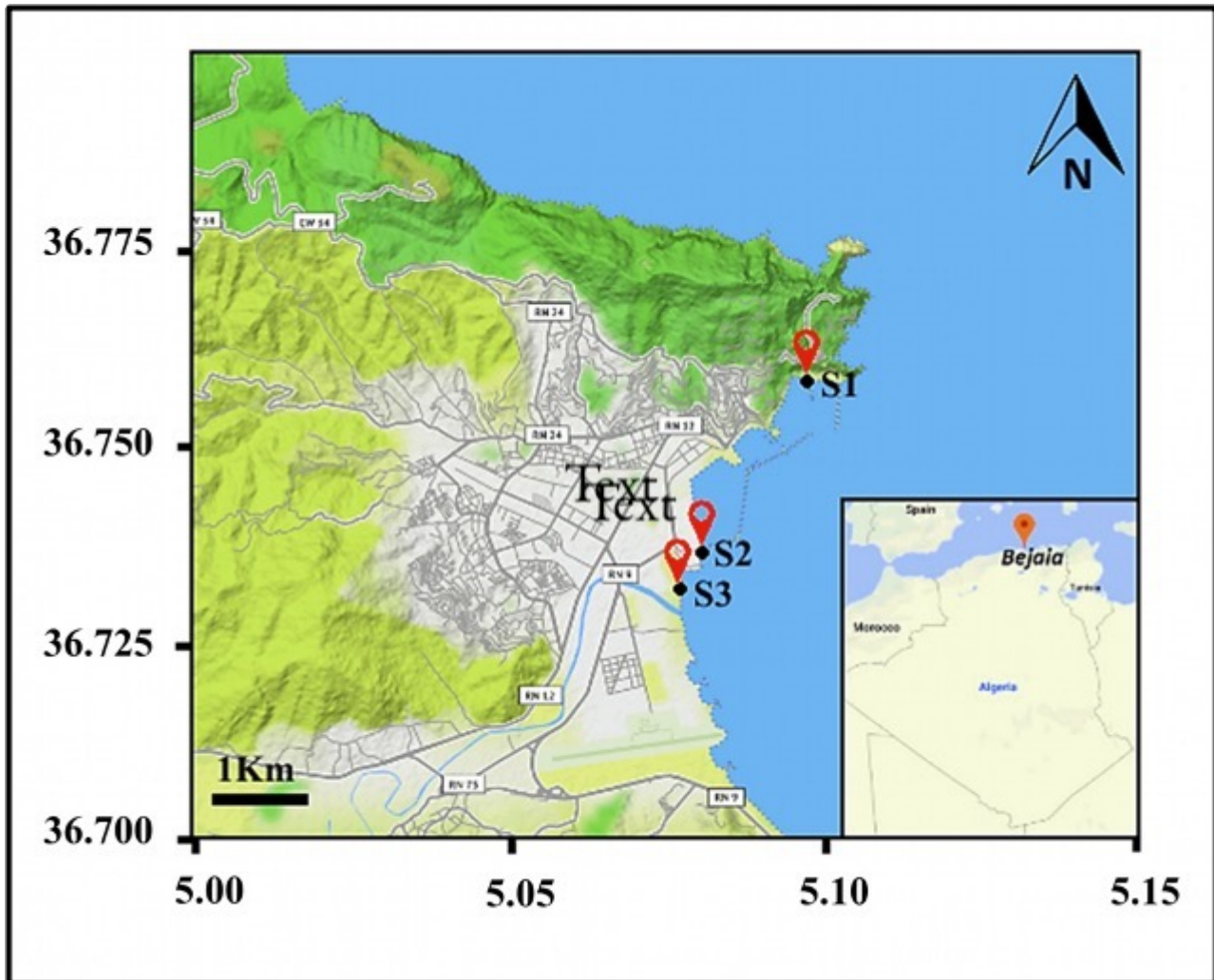


Figure 2

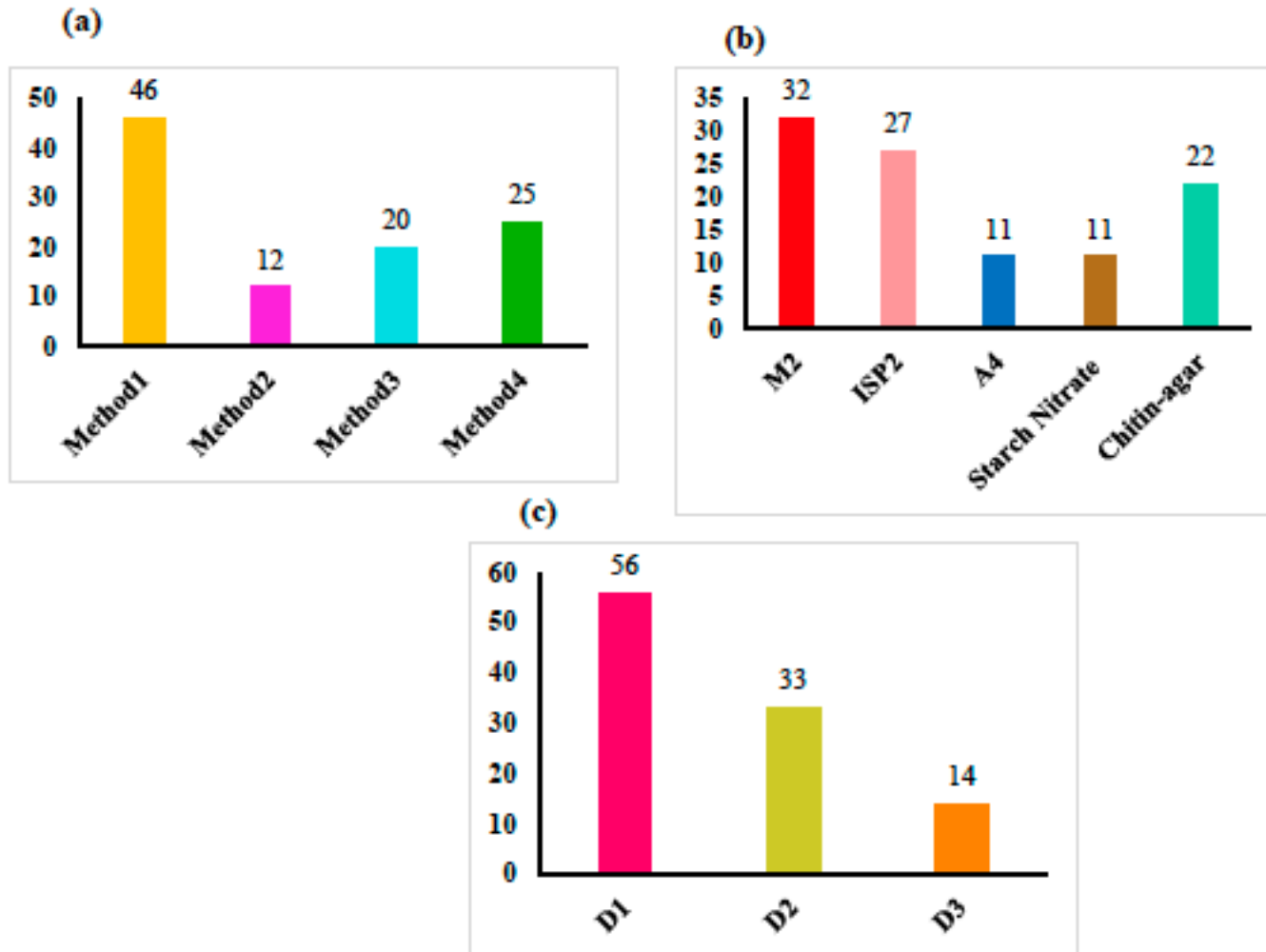


Figure 3

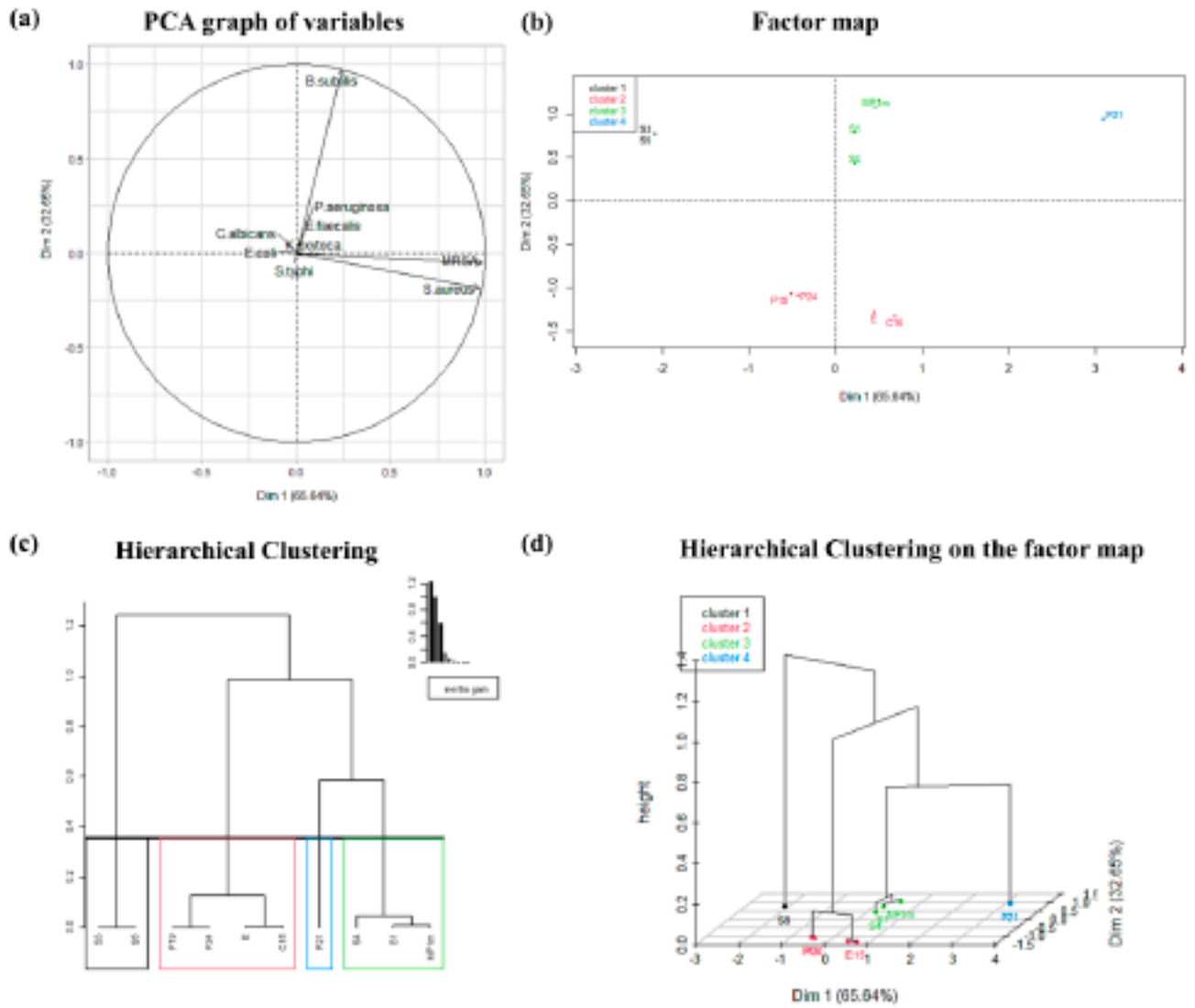


Figure 4

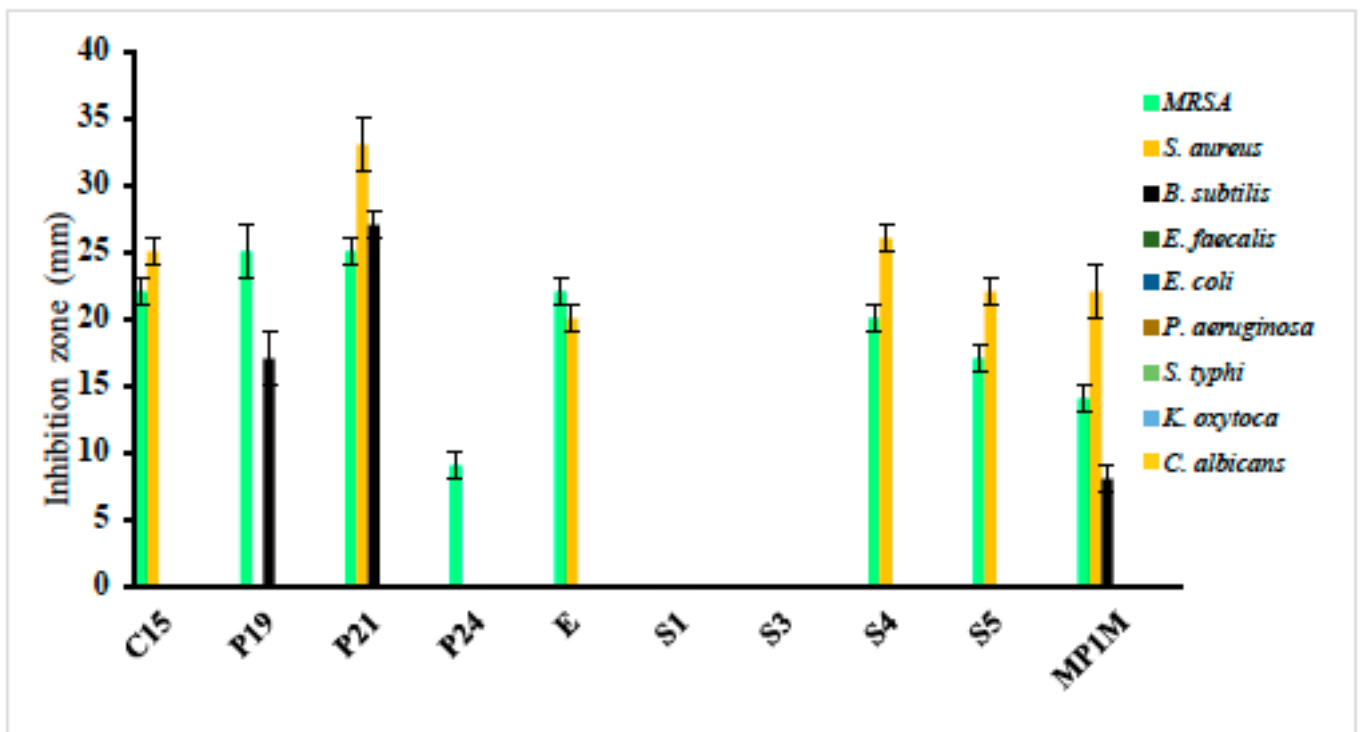


Figure 5

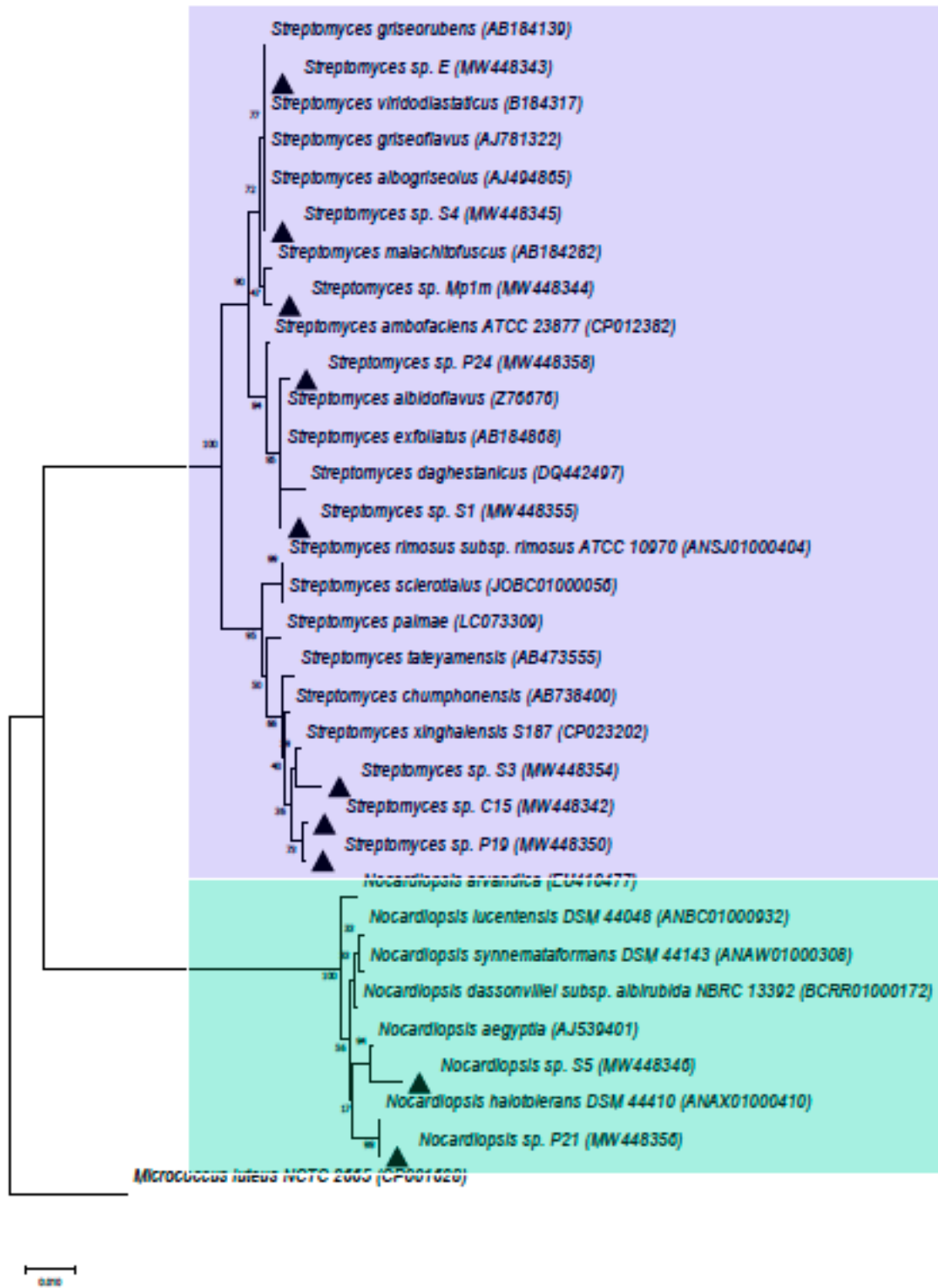


Figure 6

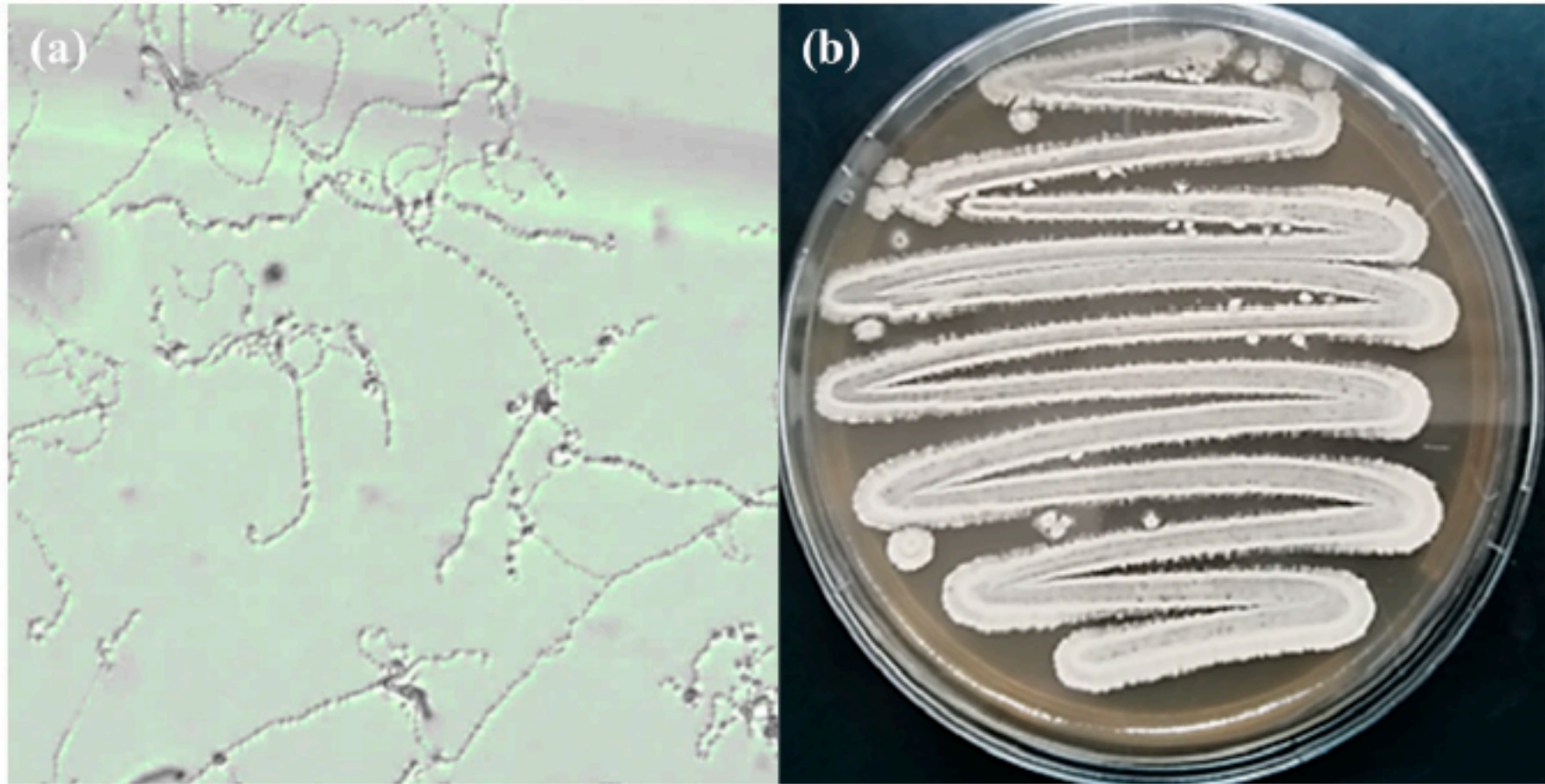


Figure 7

