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ISOLATION OF MARINE BACTERIA IN AMBON BAY WITH POTENTIAL BIOTECHNOLOGICAL FEATURES

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Abstract
Ambon Bay is situated in the Wallacea biogeographical area. Despite various studies on marine natural resources reported from this bay, limited information is available on marine bacteria that produce compounds with potential biotechnological applications. We report here preliminary results of our attempt to isolate bacteria of this group from Ambon Bay. Nine different isolates were obtained, but only eight indicated potential as producers of compounds with biotechnological potential. Two isolates indicated agarolytic bacteria characteristics, whereas one showed the properties of exopolysaccharide (EPS) producing bacteria. Three isolates produced various pigments. Two were identified tentatively as members of actinomycetes, a group known as a prolific producer of antimicrobial compounds. Preliminary identification of the cell morphologies of each isolate revealed the dominance of cocci-shaped bacteria. Most of them showed optimal growth in 1 to 7 days when incubated at 30°C. These results indicate that Ambon Bay waters and the surrounding area could harbour marine bacteria with potential features for biotechnological applications.

***Key words*:** eastern Indonesia, marine bacteria, secondary metabolites, Wallacea marine area

Introduction

Ambon Bay is situated in the Wallacea biogeographical area, known as one of the biodiversity hotspots of the world. The bay harbors various types of ecosystems, ranging from mangroves and coral reefs to seaweeds, indicating its potential for harboring bacterial producers of bioactive compounds. A considerable number of studies have been published on marine natural resources from this bay. However, far too little attention has been paid to its marine microbes, especially those producing compounds with potential features for biotechnological applications (Tapilatu, 2011).

To the best of our knowledge, to date only two attempts have been made to isolate bacterial producers of putative proteorhodopsin and exopolysaccharide (EPS) (Tapilatu, 2012a, b) from this bay. Marine bacteria are attractive to researchers because they can potentially produce compounds with unique biological properties (Jensen & Fenical, 1996). This study seeks to obtain preliminary data which will help to address the gaps in the research of marine bacteria, producers of compounds with potential biotechnological features, from the coral reef ecosystems of Ambon Bay.

Materials and methods

***Source of microorganisms*:** Samples (water, sponges, soft corals and sediments) were taken from four locations in Ambon Bay, two in the inner area (Hunut and Halong) and the remaining two (Eri and Batu Capeo) in the outer area (Fig. 1: pl. 5). This selective consideration was used to increase the possibility of isolating putative producers of bioactive compounds, where coral reefs were known to exist. Microbial symbionts of marine macroorganisms (eg. sponges, coral and algae) have been found previously to be potent producers of biologically active substances (Imhoff *et al*., 2011). They form a mutually advantageous symbiosis where antimicrobial compounds produced by microbial symbionts may protect the host surface against pathogenic colonization, in return for a nutrient rich environment (Penesyan *et al*., 2010). These areas were also selected because they represented locations with and without anthropogenic influence on the coral reefs ecosystems of these waters.

Water samples were taken using 50 ml sterile Falcon tubes in the water surrounding coral reefs. We also took sediment samples using the same type of tube, if collectible at the vicinity or at the bottom of the substrate growing soft corals/sponges. Actively growing portions (5-10 cm) of sponges and soft corals were cut with a knife and put individually into ziplock plastic bags containing seawater. All samples were stored at 4°C until transfer to the laboratory and kept afterwards at -20°C until analysis.

***Growth conditions*:** Isolation was carried out by streaking 50 or 100 µl serially diluted (to 10^{-5}) samples of all types or by incubating 0.5 cm² pieces of sponges and soft corals on ISP2, GASWA and modified MA 2216 plates. The recipes of these three media were as follows. The ISP2 (Shirling & Gottlieb, 1969) medium contained 4 g yeast extract (YE), 10 g malt extract and 4 g dextrose in 500 ml natural sea water (NSW) and 500 ml distilled water (DW). The GASWA (Krediet *et al*., 2009) medium contained (per litre of DW) 20.81 g NaCl, 59.7 mg KCl, 9.85 g $MgSO_4$, 4.01 g $MgCl_2.6H_2O$, 1.1 mg K_2HPO_4, 33 µM Tris-(hydroxyl-aminomethane), 0.19 mg $FeSO_4.7H_2O$, 50 mg Peptone, 2 mg YE and 20 ml Glycerol. The pH was adjusted to 7 before autoclaving. The modified MA 2216 medium contained (per litre NSW) 1 g YE, 5 g Peptone. All solid media were prepared by adding 20 g purified agar (Difco) to each recipe. The NSW were filtered at 0.45 µm prior to usage for all media preparation. Solid and liquid media were autoclaved at 121°C for 15 to 20 minutes.

Plates were incubated at 30°C for seven days. Purification was carried out by a double transfer process (plate to plate). Visual observation of the macromorphological features of the colonies was conducted, and bacterial cells were taken from actively growing colonies, stained with Crystal violet solution and observed under a Nikon binocular Z50i microscope.

***Morphological screening of isolates with biotechnological potential*:** Pure bacterial colonies obtained were screened for potential features including EPS, agar degradation accompanied by the production

of pigment and other compounds with potential biotechnological features. Isolates showing these activities were identified based on the macromorphological features of colonies, such as colony superficial textures and colors. Further analysis of compounds with potential biotechnological features was carried out only on actinomycetes isolates, because this group is known as prolific producers of this type of compound (Williams, 2009).

***Screening for antagonistic activity*:** Initial tests of antagonistic activity were carried out using the cross-streak method (Sawasdee, 2012). Actively growing actinomycetes isolates BKBL7B.Ac and BKBL8B.Ac were streaked across the diameter of ISP2 plates. Fresh cultures (24 h old) of four isolates obtained during the project (BKBL1B.P, BKBL2B.P, BKBL3B.P, and BKBL4B.E, see table 1 for their morphological features) were streaked at right angles across them. Control was done by streaking the same four isolates at the same line without *Streptomyces* at the centre line. All bacterial isolates growths were observed after seven days of incubation at 30±2°C.

Table 1: Bacterial isolates obtained after incubation at 30°C. *Exopolysaccharide: AM, Aerial Mycelium; SM, Substrate Mycelium.

Isolate code	Sample type	Sampling location	Morphological characteristics on solid media	Putative features observed	Growth (days) on purification media	Cell shape
BKBL1B.P	Water	Halong	Round, entire, umbonate, dull, opaque, dark yellow	Pigment Production	7 d on GASWA	Cocci
BKBL2B.P	Sponge	Batu Capeo	Round, wavy, umbonate, glossy, opaque, dark red		7 d on ISP2	Cocci
BKBL3B.P	Sponge	Batu Capeo	Round, entire, glossy, raised, opaque, dark brown		5 d on MA 2216	Rod
BKBL4B.E	Sponge	Hunut	Round, entire, glossy, raised, opaque, white	EPS production	7 d on MA 2216	Cocci
BKBL5B.A	Sponge	Eri	Round, entire, glossy, raised , translucent	Agarolytic	6 d on MA 2216	Cocci
BKBL6B.A	Sponge	Hunut	Irregular form and margin, muccoid, flat, opaque, whitish beige		3 d on MA 2216	Cocci
BKBL7B.Ac	Water	Batu Capeo	Wrinkle, opaque, dull, faint pinkish white AM*, yellow SM*	Antibacterial	6 d on ISP2	Hyphae
BKBL8B.Ac	Water	Batu Capeo	Wrinkle, opaque, chalky, grey AM, brown SM		7 d on ISP2	Hyphae
BKBL9B.Ac	Sponge	Eri	Wrinkle, dull, opaque, whitish beige	Unknown	3 d on ISP2	Cocci

***Actinomycetes isolates compounds analysis*:** *Streptomyces* sp. isolate BKBL7B.Ac was grown in GASWA and BKBL8B.Ac in ISP2. The incubation was carried out in three 500 ml flasks containing ~350 ml medium and 10% inoculums at room temperature in static condition for 30 days. At the end of the incubation period, the culture broth was extracted using Ethyl acetate (1:1, v/v), and evaporated afterward. The cells were harvested using sterile cotton and

macerated in Methanol for three days and extracted afterwards using Hexane and Ethyl acetate. Ethyl acetate extracts of culture broth (BE) as well as Hexane and Ethyl actetate extracts of cells were analyzed using HPLC. Due to the small amount of all type of cell extracts, only BE were analyzed using GC-MS.

Prior to HPLC analysis, the light absorbance areas of all samples were analyzed using UV-Spectrophotometer 1700 PharmaSPec Shimadzu (200-800 nm). The HPLC analysis was carried out using Shimadzu LC-20AD at maximum absorbance value obtained from the Spectrophotometer analysis. Five micro-litres of each sample was analyzed using Methanol:Acetonytrile (70:30) as mobile phase. The column used was Shim-pack VP-ODS (size: 250 x 4.6 mm), and the flow rate was maintained at 1.0 mL/min.

The molecular weight was measured by GC-MS (GCMS-QP2010 Plus Shimadzu) with the following condition: Rtx-5MS column (30 meter) and diameter 0.25 mm. The temperature was programmed from 80°C to 280°C, with gradual increase of 20°C/min, holding time for 19 min at 280°C, and split injection mode. Carrier gas used was Helium at 1.19 mL/min, with pressure of 80.6 kPa. The amount of compounds in the extract would be indicated by peak amount at the chromatogram, which will be screened against Wiley7 spectral data base.

Results and discussion

***Isolation of bacterial isolates*:** Nine bacterial isolates were obtained from all four locations, predominantly from sponges (six isolates) and water samples (Table 1). Eight of them showed bioactive properties, which should be studied further because of their potential applications in different areas of biotechnology. Isolation from soft corals and sediments did not yield any putative producer of biotechnologically relevant compounds and this might be due to the isolation media used and/or other incubation conditions during the process. The majority of the isolates were cocci-shaped (ex. Fig. 2D, E: pl. 5) and they grew after 1 to 7 d of incubation at 30°C. Three were pigment-producing bacteria, which produced red (Fig. 2A: pl. 5) yellow and brown pigments. Isolate BKBL4B.E showed features of EPS-producing bacteria (Fig. 2B: pl. 5), with a milky white, circular, raised entire margin with shining appearance colonies. The colony surface was sticky when taken with a loop. Two isolates were agarolytic (eg. Isolate BKBL6B.A, Fig. 2C: pl. 5), which was shown from the degradation level of the solid media after three days of incubation. Two isolates showed properties of bacteria putatively belonging to actinomycetes groups, (eg. Isolate BKBL8B.Ac, fig. 2F: pl. 5). *Streptomyces* has tough, leathery frequently pigmented colonies and has filamentous growth with aerial mycelium (AM) and substrate mycelium (SM). *Streptomyces* species have chains of spores (arthrospores) on the AM, which are normally absent from the SM (Bergey & Holt, 1994). There are four types of *Streptomyces* arthrospores according to Vobis (1997) as cited by Sawasdee (2012). Based on Vobis' classification, isolate BKBL7B.Ac type was retinaculiaperti, and BKBL8B.Ac one was verticillati.

***Screening for antagonistic activity*:** The antagonistic activity test indicated the potential of *Streptomyces* sp. isolate BKBL8b.Ac to inhibit the growth of other bacteria. After eight days of incubation, isolates BKBL1B.P (yellow-pigmented bacteria) showed medium growth and BKBL3B.P showed very weak growth (Fig. 3A: pl. 6) compared to control (Fig. 3B: pl. 6). However, BE analysis indicated that both actinomycetes could produce bioactive secondary metabolites (Table 2).

Table 2: Compound name and chemical formula interpreted based on GC-MS spectral data of Isolates BKBL7B.Ac and BKBL8B.Ac. Abbreviation used: Retention Time (RT), Molecular Formula (MF), Molecular Weight (MW).

	RT (min)	Area	MF	MW	Compound name
Isolate BKBL7B.Ac					
1	8.092	5052200	C8 H10 O	122	Phenethyl alcohol
2	9.675	10827840	C6 H8 O3	128	Dihydroxyethyl-1-furan
3	11.542	7273042	C8 H8 O3	152	Vanillin
4	12.85	8467686	C16 H32	224	Cyclohexadecane
5	13.317	6225314	C13 H10 O	182	Benzophenone
6	13.725	8430573	C13 H16 O2	204	Irgacure 184
7	13.883	14434993	C14 H28	196	7-Tetradecene
8	13.983	7124165	C18 H36	252	3-Octadecene
9	14.108	19153221	C18 H38 O	270	Stenol / 1-octadecanol
10	14.317	5564131	C15 H30 O2	242	Pentadecanoic acid
11	14.375	7961095	C18 H34 O2	282	Oleic acid
12	14.492	8830000	C11 H18 N2 O2	210	1,4-diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0]nonane
13	14.65	11575603	C20 H30 O4	334	butyl octyl Ester
14	14.808	5270347	C18 H36	252	3-Octadecene
15	14.892	13249001	C16 H32 O2	256	Palmitic acid
16	15.042	21951901	C11 H18 N2 O2	210	1,4-diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0]nonane
17	15.125	28301973	C11 H18 N2 O2	211	1,4-diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0]nonane
18	15.175	7768343	C16 H22 O4	278	*n*-butyl isobutyl phthalate
19	15.217	16075783	C18 H38 O	270	Stenol / 1-octadecanol
20	15.308	7783474	C14 H24 O	208	1,13-tetradecadien-3-one
21	15.408	65643591	C18 H34 O2	282	Oleic acid
22	15.45	5244166	C17 H34 O2	270	Margaric acid
23	15.5	5450675	C19 H32	260	Etioallocholane/androstane
24	15.583	5225223	C16 H26 O3	266	Dodecenyl succinnic anhydride
25	15.8	7076198	C19 H36 O2	296	Methyl oleate
26	16.025	8062816	C12 H26 O	186	*n*-Dodecanol
27	16.125	11266146	C18 H36	252	3-Octadecene
28	16.225	12270518	C20 H42 O	298	*n*-Eicosanol
29	16.575	6270550	C20 H34 O2	306	2,beta., 11.alpha-Dihydroxyverrucosane
30	17.175	5381986	C24 H48	336	Cyclotetracosane
31	17.292	10202502	C18 H36	252	Alpha-octadecene
32	17.375	15935111	C22 H42 O4	370	Bis(2-ethylhexyl)adipate
33	17.65	5906791	C14 H16 N2 O2	244	3-Benzyl-1,4-diaza-2,5-dioxobicyclo[4.3.0]nonane
34	17.8	5965704	C16 H24	216	Tricyclo[8.6.0.0(2,9)]hexadeca-3,15-diene, trans-2,9-transoid-9,10-trans-1,10
35	18.442	5086011	C24 H38 O4	390	Bis(2-ethylhexyl)Phtalate
36	24.95	16149267	C14 H22 O	206	2,6 di-tert-butyl Phenol
37	25.058	6306097	C23 H36 O2	344	1,4-Epoxynaphtalene-1(2H)-methanol,4,5,7-tris(1,1-dimethylethyl)-3,4-dihydro-
38	25.142	13548174	C15 H22	202	Cuparene
Isolate BKBL8B.Ac					
1	7.783	36882340	C5 H10 O4	134	Acetoglyceride/glycerol
2	8.542	32725771	C5 H10 O4	134	1-acetoxy-2,3-dihydroxypropane
3	8.775	23358079	C3 H8 O3	93	Glycerol

4	8.967	25126117	C3 H8 O3	92	Glycerol
5	9.133	34652436	C3 H8 O3	92	Glycerol
6	9.208	43411573	C11 H20 O	168	2-methylisoborneol
7	12.5	20762495	C11 H13 N O3	147	2-methoxy-n-(2-methoxyethyl)acetamide
8	13.408	26413053	C6 H13 N	99	3-Methylpiperidine
9	13.858	24438279	C9 H14 D2 N2 O3	200	(+)-3-(3-hydroxy(2-2H2)-butyrylamino)-2-piperidone
10	15.075	24553748	C16 H32 O2	256	Palmitic acid
11	15.217	21030078	C2 H48	336	1-tetradecene, 2-decyl
12	17.85	23741086	C10 H20 O2	172	2-(1-methylbutyloxy)-1-oxacyclohexane
13	20.117	20203218	C18 H22 O2	270	Gona-1,3,5(10)-trien-17-one, 3-methoxy-, (13.alpha)-

***Actinomycetes isolates compounds analysis*:** The UV-Visible absorption spectra of the compounds showed absorption maxima at 234nm (BKBL7B.Ac [BE]), 206nm and 213nm (BKBL8B.Ac [BE]). This result was similar to the one reported by Saadoun *et al*. (1999), where experiments on the nature of the inhibitory metabolite produced by *S. violaceusniger* showed a maximum absorption in the UV region at 210-260nm.

The BE analysis using HPLC (Fig. 4: pl. 7) detected thirteen peaks from isolate BKBL7B.Ac, with a major one detected at 5.755 min. On the other hand, there were only seven peaks detected from the isolate BKBL8B.Ac BE sample. However, the retention time of the major peak was similar (5.914).

The GC analysis of both isolates' BE showed numerous peaks at various retention times (Fig. 5: pl. 7). There were thirty eight peaks detected from isolate BKBL7B.Ac. Two peaks with slightly higher relative area were obtained at the retention time of 15.128 and 17.377. The compounds retention times were compared with the Wiley7 Library compounds data base. The names of these unknown compounds were predicted based on the similarity of retention times of known compounds in this data base. Some of the compounds identified were those from the growth medium (eg. Glycerol), but we could not verify this due to difficulty in accessing the GC-MS equipment. In general, compounds identified (Table 2) had antimicrobial features (Phenethyl alcohol) (NCBI, 2014) as well as anti-inflammatory (2.beta-11.alpha-Dihydroxyverrucosane), pheromone-like substances (1,13-tetradecadien-3-one, Cuparene), and other compounds potentially useful in various industries, including printing (Benzophenone, Irgacure 184), chemical materials (Dodecenyl succinnic anhydride, Methyl oleate, Palmitic acid), food (Vanillin, Oleic acid, Margaric acid) and pharmaceuticals (Stenol, *n*-Dodecanol, *n*-Eicosanol, Etioallocholane, Pentadecanoic acid).

Isolate BKBL8B.Ac (BE) chromatogram result showed 13 peaks, with three dominants peaks at the retention time of 9.206, 15.074 and 15.215. The compound detected at 9.206 min was predicted as 2-Methylisoborneol, a compound known to be co-produced with geosmin, a signature compound produced by cyanobacteria, especially in the event of a harmful algal bloom (Hudnell, 2007). This went in line with the earthy odor emitted by this isolate when sporulating. Other compounds produced could also be useful in pharmaceutical and/or chemical industries (3-Methylpiperidine, 1-Acetoxy-2,3-dihydroxypropane, [+]-3-[3-hydroxy[2-2H2]-butyrylamino]-2-piperidone). This isolate also produced Palmitic acid and 2-(1-Methylbutyloxy)-1-oxacyclohexane.

Fractionation and purification of Ethyl extract broths of these two isolates are

deemed useful to further confirm the production of these compounds with potential biotechnological applications. Other than that, future work should include comprehensive biological activity tests against pathogenic microbes.

Conclusion

The Wallacea biogeographical area is known as one of the global biodiversity hotspots. Despite many prior studies on bacteria producing compounds that were potentially utilized in biotechnological applications (Khasanah & Dewi, 2010 and references therein) in other parts of Wallacea, limited if any studies of bacteria producing compounds that could be useful in biotechnological applications have come from Ambon Bay. These initial results indicate that Ambon Bay waters and the surrounding area could harbour marine bacteria with features that may prove to be useful for biotechnological applications. Further, more intensive studies are needed in order to elucidate comprehensive biological and chemical profiles of the isolates obtained, and also to explore other types of marine bacteria, particularly those with potentially significant roles in the biochemical processes of the Ambon Bay environment.

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PLATE 5

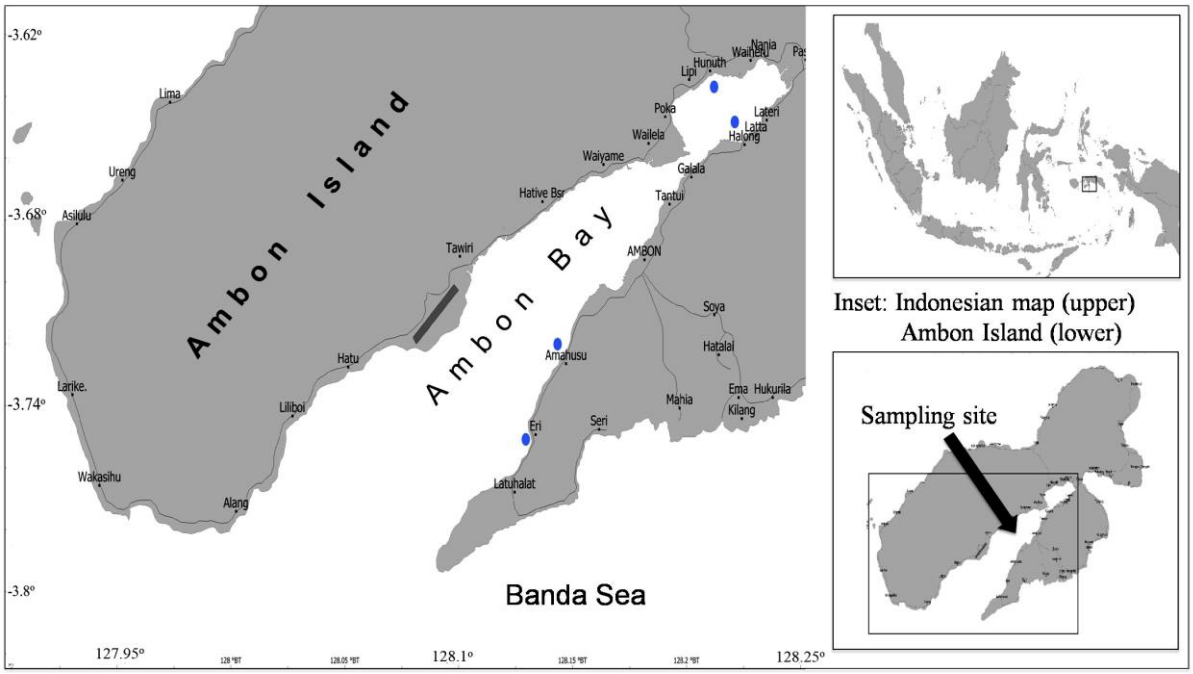

Figure 1: Map of Ambon Bay, Maluku Province. Blue dots indicate the four locations where samples were collected.

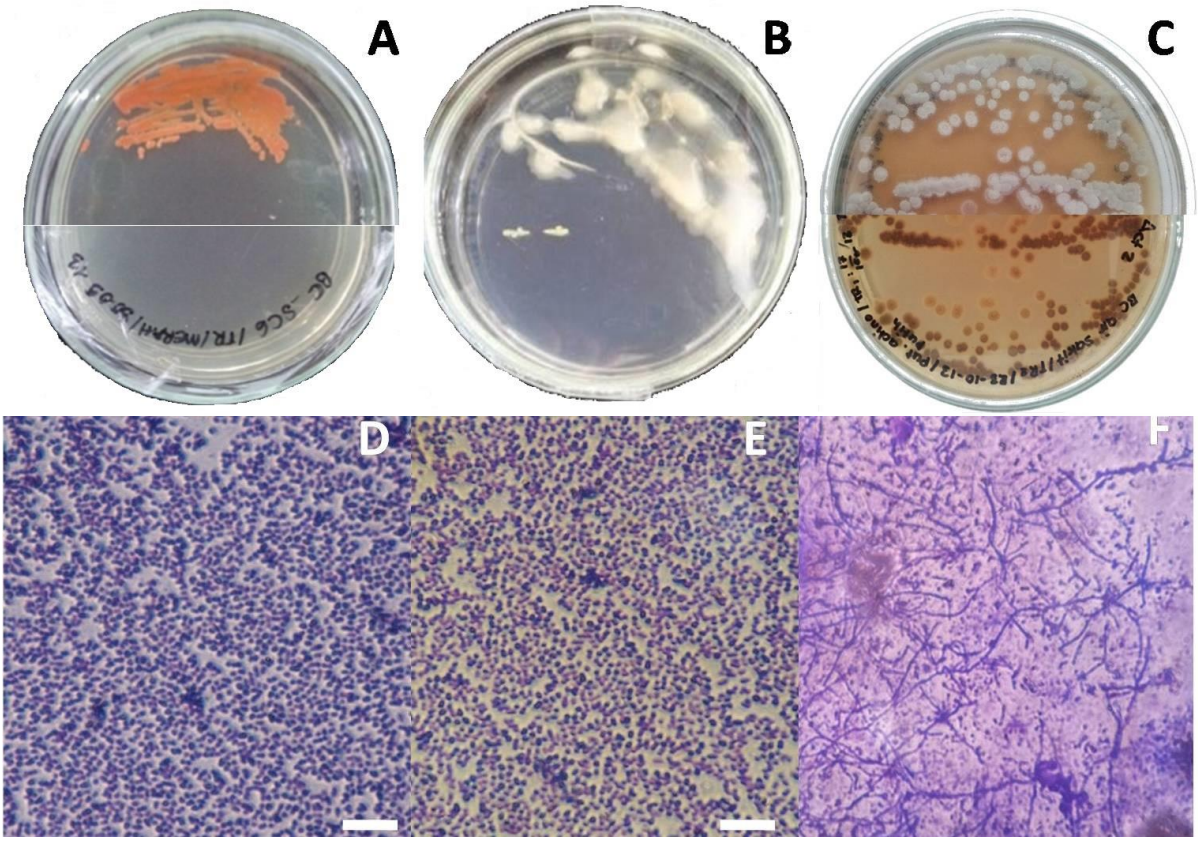

Figure 2: Colonies and cells/hyphae appearance under microscope of isolates obtained, namely: (**A** & **D**) BKBL 6B.A, (putative agarolytic bacteria), (**B** & **E**) BKBL 4B.E (putative EPS-producing bacteria) and (**C**) BKBL 8B.Ac (Actinomycetes). Note that isolate BKBL 8B.Ac showed grayish white aerial mycelium (C, upper side), whereas it substrate one was brown (C, lower side). Cells were stained with Crystal violet solution (see Material and method section for details). Scale bar: 2 µm.

PLATE 6

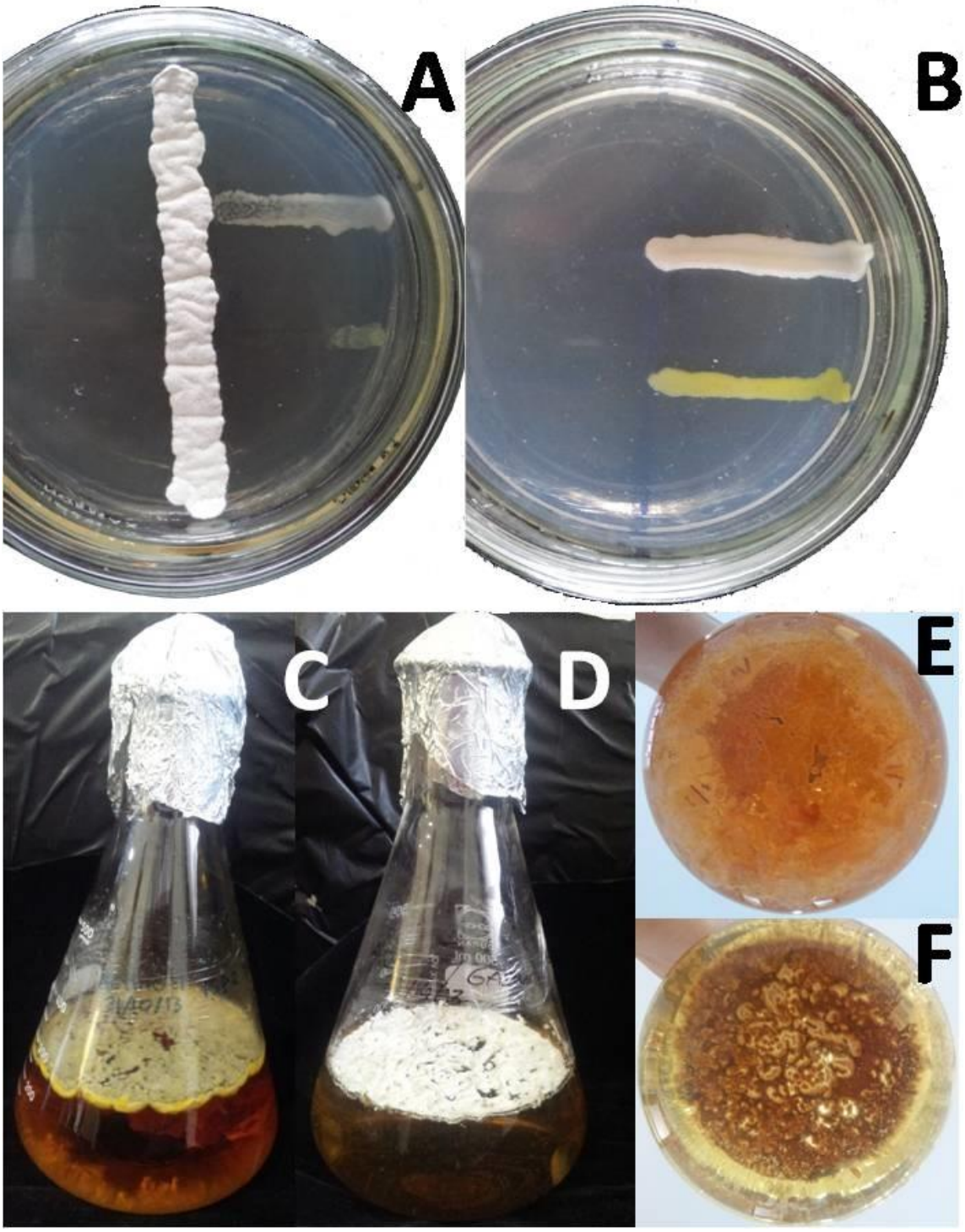

Figure 3: Antagonistic activity test of an actinomycete isolate BKBL8B.Ac against other isolates after 8 days of incubation on (**A**) GASWA and (**B**) control plate. Actinomycetes isolates incubated in 300 ml liquid media for compounds studies (after 30 days), (**C**) BKBL7B.Ac on GASWA and (**E**) its view from the bottom of the flask; (**D**) Isolate BKBL 8B.Ac on ISP2 and (**F**) its view from the bottom of the flask.

PLATE 7

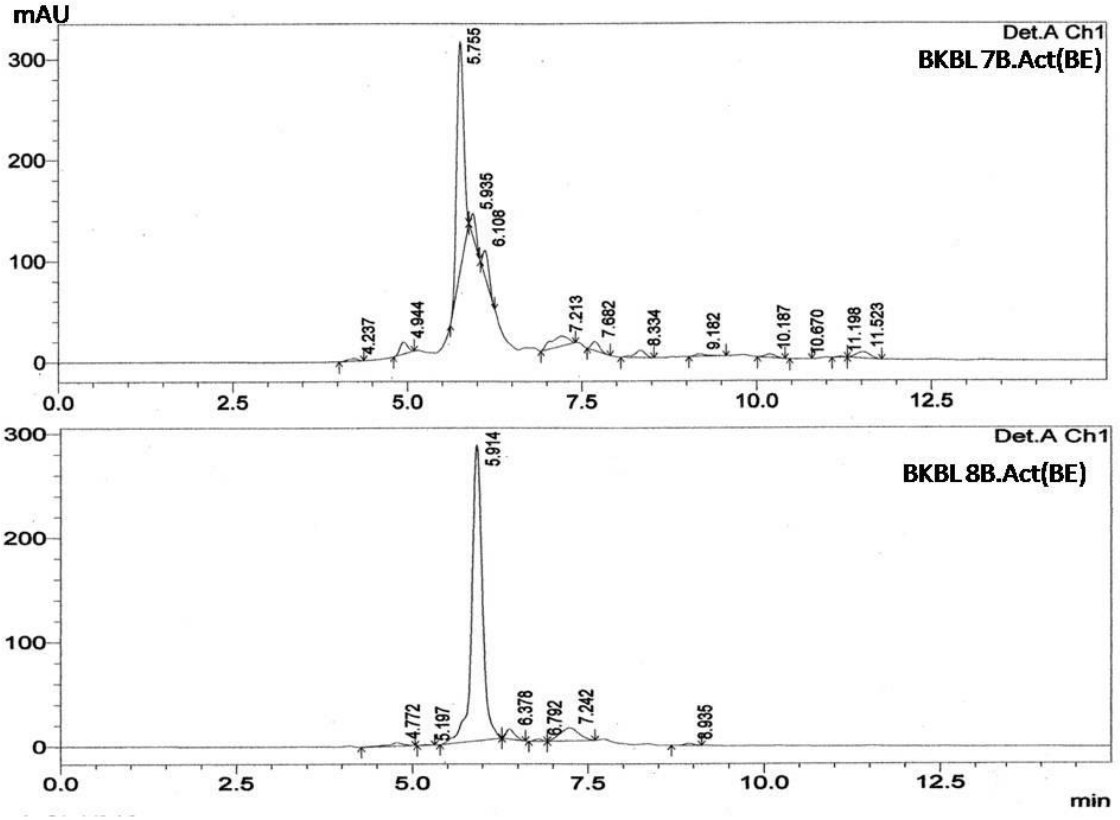

Figure 4: HPLC chromatogram of isolates BKBL 7B.Ac and BKBL 8B.Ac broths extracted with Ethyl actetate (BE).

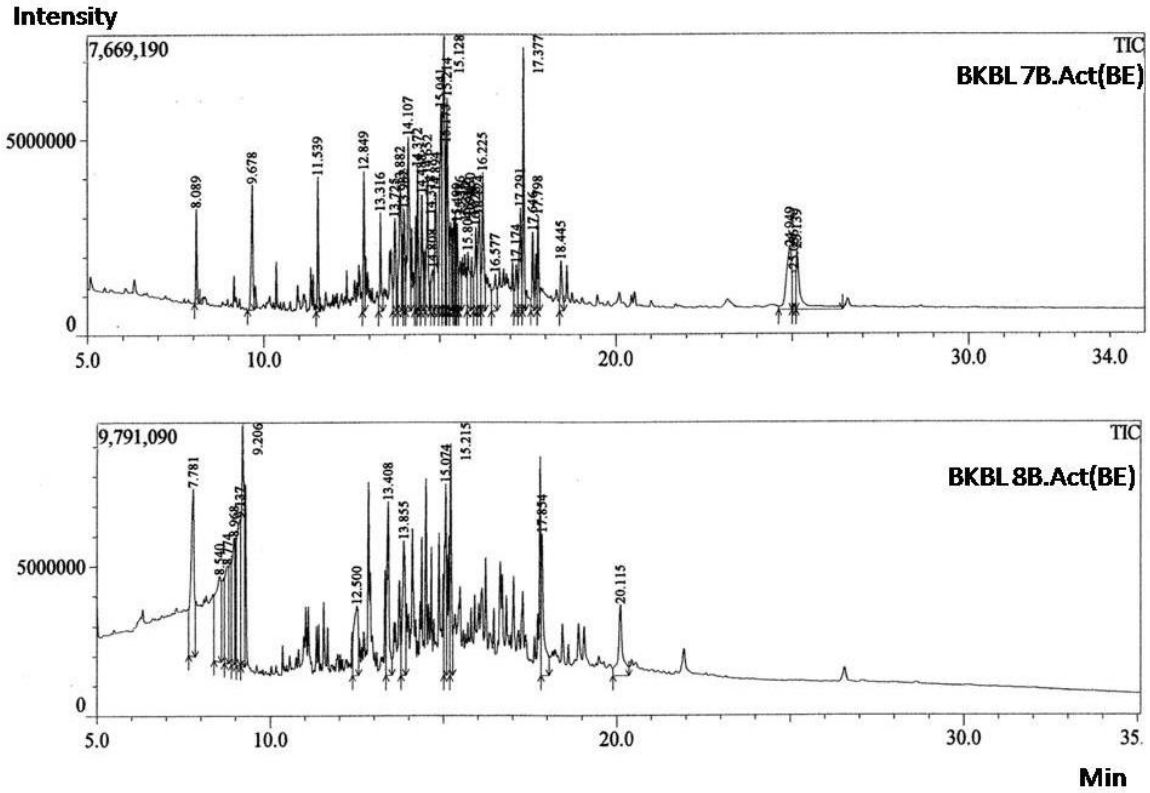

Figure 5: GC-MS chromatogram of isolates BKBL 7B.Ac and BKBL 8B.Ac broths extracted with Ethyl actetate (BE).