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## Membrane-active metabolites produced by soil actinomycetes using chromatic phospholipid/polydiacetylene vesicles

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Increased resistance of pathogens toward existing antibiotics has compelled the research efforts to introduce new antimicrobial substances. Drugs with new and less resistant-prone targets to antimicrobial activity have a high priority for drug development activities. Cell membrane seems to be a potential target for new antibiotic agent development to overcome resistance. In this study, A total number of 67 actinomycetes were isolated from the soil samples collected from desert, farming and mineral parts of Iran. We used a chromatic sensor as a membrane model that was set up for the target of antimicrobial metabolites of actinomycetes isolated from the soil. The sensors particles were composed of phospholipid and polymerized polydiacetylene (PDA) lipids. These polymers exhibited color change following interaction with membrane-active metabolites. The color change was due to structural disorder in the lipids following their interaction with membrane-active metabolites. The resultant color change was recorded by fluorescent microscope and easily recognizable by naked eye as well. Sixteen strains were isolated which produced antimicrobial metabolites and were effective against test microorganisms (*Escherichia coli*, *Candida albicans* and *Saccharomyces cerevisiae*). A total number of 3 out of 16 strains produced membrane-active metabolites. These 3 strains were identified using 16s rRNA as *Streptomyces* sp and submitted to GenBank (accession no. JN180853; JN180854; JN180855).

**Keywords:** Antimicrobial agent, Chromatic vesicles, Membrane-active, Soil actinomycetes.

Resistance of pathogens against current antibiotics and increasing burden of infectious diseases is a subject of concern. Undue using the drugs for long periods of time, all participate in the development of resistant bacteria<sup>1</sup>. This process of developing resistance against antibiotics is a threat to human health and also great economic loss<sup>2,3</sup>. Around 70% of bacterial infections are resistant to at least one of the antibiotics<sup>2</sup>. In addition, microorganisms are adapting to new strategies<sup>4,5</sup>. Moreover, clinical needs for novel antimicrobials specially antifungal agents are necessary for autoimmune diseases and organ transplant recipients, and infections (such as AIDS) that alter the effectiveness of the host immune system<sup>6,7</sup>. Therefore, new antimicrobial molecules are necessary to confront these problems; especially anti-infective agents with fewer side-

effects, shorter lengths of treatment and in particular, drugs with new and less resistant-prone targets to antimicrobial activity<sup>8-10</sup>. Antibiotics have various modes of action to inhibit or check the growth of microorganisms, like inhibition of cell wall formation, protein, nucleic and ribonucleic acid synthesis etc.<sup>1</sup>. Membrane as a new and potential target is noteworthy for antibiotics action because of lower tendency to resistance be developed against it. Drugs that anchor in the microorganism membrane seem to have maximum potential because they show a fast and extensive antimicrobial effect and are probably less prone to be selected for resistance owing to difficulty in modifying their targets in a way that is compatible with bacterial survival<sup>11,12</sup>.

The present study focused on membrane-active metabolites produced by soil actinomycetes and their evaluation by a vesicle membrane model, phospholipid/polydiacetylene (lipid/PDA) vesicles, as a bioassay target.

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## Materials and Methods

**Isolation and storage of microorganisms**—Soil samples were collected from desert parts including Ghaem briny line (35°48.099' N and 051°25.94' E), Garmsar, Semnan and 45 km Damghan (35°54.509' N and 053°57.832' E), Semnan (soil numbers 1 and 2, respectively); farming parts comprising altitudes of Sabalan hillside (38°15.451' N and 047°44.605' E), Ardabil and Kelibar cemetery (038°52.136' N and 047°02.230' E), Tabriz (soil numbers 3 and 4, respectively); and mineral part contained 12 km Ghotour (38°28.796' N and 044°46.83' E), Tabriz (soil number 5). Each collected soil sample was suspended and serially diluted up to 10<sup>-4</sup> (Ref. 9). An aliquot of 0.1 ml of each dilution spread evenly over the surface of inorganic salt starch agar (ISSA; Difco, USA) complemented with rifampicin (5 µgml<sup>-1</sup>) and amphotericin B 100 (µgml<sup>-1</sup>) for actinomycetes isolation. Actinomycetes colonies were identified according to characteristics described in Bergey's manual<sup>13</sup>. Plates containing pure cultures were stored at 4°C during two months until further examinations, and in a freezer at -70°C in the presence of glycerol (50%, v/v) for a longer period storage.

**Test microorganisms**—Two yeasts, including *Candida albicans* ATCC 10231, *Saccharomyces cerevisiae* BY4743 and one bacterium, *Escherichia coli* ATCC 25922, were used to determine the antimicrobial activity of the isolated actinomycetes strains. All cultures were obtained from the Department of Microbiology, Pasteur Institute of Iran, Tehran. The above mentioned yeasts were cultured in Sabouraud Dextrose Broth (SDB; Difco, USA) at 28°C for 48 h and *E. coli* was cultured in brain heart infusion broth (BHI; Merck, Germany) at 37°C for 24 h.

**Screening of isolates for antimicrobial activity**—Inorganic salt starch agar plates were prepared and spot inoculated with actinomycetes isolates in 4 points. After 4 days of incubation at 28°C the plates were seeded with test organisms by overlay agar technique<sup>9</sup>. The antimicrobial activity was observed after 48 h incubation at 28°C for yeast and 24 h incubation at 37°C for bacteria. Amphotericin B and chloramphenicol used as control antibiotic against fungal and bacterial test, respectively. The antimicrobial activities were measured by the determination of the size of the inhibition zone<sup>14</sup>.

**Screening of isolates for detection of membrane activity**—Isolates which produced antimicrobial substances against target strains were studied for membrane activity by a Rapid Chromatic

Detection method, using a Biomimetic Polymer Sensor i.e., polydiacetylene (PDA) in conjunction with phospholipid as a membrane model vesicle<sup>15-21</sup>. The detection is based on the interaction of membrane-active metabolites secreted by microorganisms in broth culture extract, with Phospholipid/Polydiacetylene vesicles.

**Construction of the vesicles**—To prepare polymerized vesicles, synthetic phospholipid, dimyristoylphosphatidylcholine (DMPC), and diacetylenic monomer, 10,12-tricosadienoic acid (TCDA), (both purchased from Sigma, USA) which were separately dissolved in dichloromethane (1mgml<sup>-1</sup>), mixed at 2:3 molar ratio and had a concentration of 1 mM. The resulting vesicle solution had intense blue color appearance due to polymerization of the diacetylene units<sup>16</sup>.

**Submerge culture and organic extraction**—Isolates that showed antimicrobial activity against test organisms in agar medium were grown in submerged culture in 500 ml flasks containing 100 ml of yeast extract malt extract broth (ISP2; glucose, 4; yeast extract, 4; malt extract, 10; and agar, 20 g l<sup>-1</sup>). A two-days-old broth culture grown on ISP2 was used to inoculate the flasks. These cultures were grown in a rotary shaker (Adolf Kuhner AG, Switzerland) at 200 rpm, 28°C, for 7 days. Organic extraction and extracellular antimicrobial activity on agar medium were performed based on method used in Thakur *et al.*<sup>9</sup>. Plates were kept at 4°C for at least 2 h to allow the diffusion of produced antimicrobial metabolites. The diameter of inhibition zone was determined after 24 h of incubation at 37°C for bacteria, and after 48 h at 28°C for yeasts.

Antimicrobial compound was recovered from the culture of each active isolate by solvent extraction with ethyl acetate. Ethyl acetate was added to the centrifuged broth at the ratio 1:1 (v/v) for three times and shaken vigorously for 5 min in each time. The organic layers were collected and the organic solvent was evaporated to dryness in a rotary vacuum evaporator at 40°C. The remainder was dissolved in 1ml dimethylsulfoxid (DMSO). Resulting solution was studied for membrane activity.

**Interaction of phospholipid/PDA vesicles solution with antimicrobial extracts for detection of membrane-active metabolites**—Test tubes containing 300-400 µl of phospholipid/PDA vesicle solutions and 2 mM of Tris solution (pH 8.5) got the volume of 1000 µl by adding bioactive extracts of strains. The pH in the solutions was 8.5 in all experiments<sup>20</sup>. Tubes incubated

at 28°C for 1 h. Amphotericin B and tetracycline were used as positive and negative control, respectively.

To confirm the membrane activity results of antimicrobial extracts, the fluorescence intensity of test solutions was qualified under a defined temperature curve in real time system using Rotor-Gene 6000 machine that was done by setting test tubes containing vesicle solutions and bioactive extracts in rotor machine. Fluorescence intensities were monitored in emission 550 nm.

Finally, membrane active isolates further studied by the naked eye to examine cultural characters such as colony size and texture, the color of aerial and vegetative mycelium and optical microscopy using slide culture and inclined slide method for the study of aerial mycelium production and the shape of spore chain<sup>22</sup>.

**DNA extraction, 16S rRNA sequencing**—Total DNA preparation from 3 isolates was carried out according to Kumar *et al.*<sup>23</sup>. The 16S ribosomal RNA was amplified using PCR with 2X Taq Master Mix (Vivantis) and primers 8f (5' AGAGTTTGATCCTGGCTCAG 3') and 1492r (5' GGTTACCTTGTTACGACTT 3'). The conditions for thermal cycling were as follows: denaturation of the target DNA at 94°C for 2 min followed by 30 cycles at 94°C for 30 sec, primer annealing at 50 °C for 30 sec and primer extension at 72 °C for 1 min. At the end of the cycling, the reaction mixture was held at 72°C for 7 min and then cooled to 4 °C. PCR amplification was detected by agarose gel electrophoresis and was visualized by ultraviolet (UV) fluorescence after ethidium bromide staining. The obtained PCR products were sequenced by an automated sequencer (DNA Analyzer 3730xl, Applied Biosystems, USA). The same primers as above were used for this purpose. The sequences were compared for similarity with the reference species of bacteria contained in genomic database banks, using the NCBI BLAST available at <http://www.ncbi.nlm.nih.gov/>.

## Results and Discussion

**Isolation of actinomycetes**—Actinomycetes are the potential producers of metabolites such as antibiotics, enzyme inhibitors and many other compounds of use to human<sup>24</sup>. This study was undertaken with the aim of detecting of actinomycetes in various soil parts of Iran and selecting the strains with antimicrobial activity that produce membrane active metabolites. A total of 67 different actinomycete strains of several soil samples, collected from farming, desert and mineral soils in north, west and central zones of Iran. All isolates grew on

ISSA medium showing morphology characteristics of actinomycetes<sup>13</sup>. Colony appearance, size and color were visible by the naked eye. Colonies were slow growing, aerobic and had different appearance such as chalky, glabrous and folded. Aerial and substrate mycelia production were studied by optical microscope. All of the selected colonies indicated to possess substrate mycelia, and some showed to have aerial mycelia. Colonies had variety of colors, sizes and appearance.

**Antimicrobial activity of isolates**—All of the isolates of the samples were screened and tested for their ability to produce antimicrobial substances against test microorganisms. The test microorganisms included two yeasts and one gram negative bacteria. Considering the results presented in Table 1, the 16 listed microorganisms out of 67 isolated microorganism (23% out of total isolates) presented antimicrobial activity against at least one of the three tested

Table 1—Antibacterial and membrane activities of soil actinomycetes isolates grown on inorganic salt starch agar medium and yeast extract-malt extract broth medium, respectively.

Isolate no./ Source	Soil Sample No.	Inhibition zone diam (mm)			Active isolate no. in liquid medium	Membrane activity
		<i>E. coli</i>	<i>C. albicans</i>	<i>S. cerevisiae</i>		
Desert soil					Desert soil	
0811	1	7	13	-	0811	+
0812	1	7	-	-		
0813	1	3	10	10	0813	-
0814	1	3	5	9		
0832	2	18	-	-	0832	-
0834	2	9	-	-		
08311	2	5	-	-		
08317	2	20	-	-	08317	+
Farming soil					Farming soil	
08261	3	-	-	5		
08263	3	-	-	8		
08264	3	-	8	8	08264	-
08291	4	-	3	-		
08292	4	-	6	-	08292	-
08293	4	-	3	4		
Mineral soil					Mineral soil	
08341	5	-	-	8		
08346	5	-	>30	>30	08346	+
Total active isolates			16(23%)		7(10%)	3(4%)
Control						
Chloramphenicol		23	-	-		
Aphotericin B			18	22		

microorganisms. Thakur *et al.*<sup>9</sup> isolated 110 actinomycetes strains from the soil samples of the protected forest soil in India that 46 (41%) out of 110 isolates showed antimicrobial activity against at least one of the 10 tested microorganism, whereas, we tested antimicrobial activities of isolates against at least one of the three tested microorganisms that leads to the limited-spectrum activity. Among 16 active isolates, 7 strains (10% out of total isolates) showed Antimicrobial activity in liquid medium (Table 1). Since, some isolates out of 16 active isolates produced less than 5 mm diameter of inhibition zone in liquid medium, were not included in 10% active isolates. The recession of 23% to 10% of active antimicrobial isolates in liquid medium was due to nutrient deprivation in the medium or alteration in the pH of the overlay layer.

*Antibacterial membrane activities of isolates*—In order to detection membrane activity of antimicrobial metabolites, the effect of microbial extracts of active isolates on Lipid/PDA vesicles were studied. Artificial lipid/PDA vesicle solution has an intense blue color appearance and polymeric and lipid phases are microscopically dispersed. Microbial extracts separating from broth culture of active isolates before affecting on vesicle solution, were studied for their antimicrobial activity on test microorganisms. A total of 7 different actinomycetes isolates were shown to have *in vitro* antimicrobial activity in liquid medium against the test strains (Table 1). Among active isolates, 3 strains (0832; 08317 and 08346) showed the highest antimicrobial effect against at least one of the tested microorganisms. The resulting values of antimicrobial activity in liquid medium were nearly similar to activity on agar medium. (data are not shown). Considering that non-optimized cultures usually produce poor yields of active compounds (pure compound), inhibition zones are smaller than the control group. In comparison with other strains, 0832 and 08317 had almost similar antimicrobial effects to chloramphenicol. Therefore, the detected activities can be considered promising since control antibiotics are more pure materials than crude metabolites that diffuse in agar medium. Strain 08346 presented an inhibition zone significantly larger than the control toward both *C. albicans* and *S. cerevisiae* that was over 1.5 times more active than amphotericin B.

By interacting the antimicrobial extracts with lipid/PDA vesicles and disrupting the vesicles, blue-red color transition were recognizable by the naked eye (Fig. 1), as well as fluorescence emission could be recorded by fluorescence microscopy (Fig. 2). Red components had fluorescence irradiation at 500-550

nm; the intense disruption induced stronger red color that led to irradiate more fluorescence.

In present study for the screening of membrane active isolates, vesicle models (chromatic phospholipid/polydiacetylene) were used which have useful applications in biotechnology<sup>15-21</sup>. Jelinek *et al.*<sup>20</sup> have previously used the polydiacetylene-derived vesicles for the study of peptide-membrane interactions. They have also used DMPC/PDA vesicle for rapid screening of antimicrobial peptides<sup>19</sup>. We described application of these vesicles in rapid screening of the soil actinomycetes producing membrane active metabolites. According to the results in Table 1, 7 out of 16 active isolates showing similar antimicrobial activity against tested microorganisms, among which, 3 strains (0811, 08317 and 08346) had antimicrobial membrane activity. Two strains (08317 and 08346) had better antimicrobial activity (Table 1). Therefore, out of 67 isolates, only 3 isolates showed potential membrane activity. Thus, less than 5% of total isolates had membrane activity. According to resulting color changes, superlative color transition was related to strain 08346 broth extract and followed by 08317 and 0811, respectively (Fig. 2). As mentioned previously<sup>17</sup>, polydiacetylene polymers used in the present vesicles model had some important

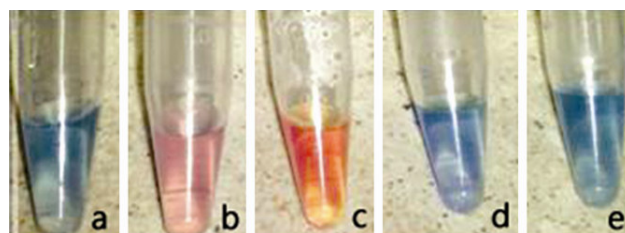


Fig. 1—Color changes produced by interacting of vesicle solution with membrane active metabolites secreted by active microorganisms. (a) after adding antimicrobial extract of a non-membrane active microorganism 0832 (according to Table 1); (b) after adding antimicrobial extract of a membrane active microorganism 08346 (according to Table 1); (c) after adding antibiotic amphotericin B as the positive control; (d) after adding deionized water (control solution); and (e) after adding antibiotic tetracycline as negative control.

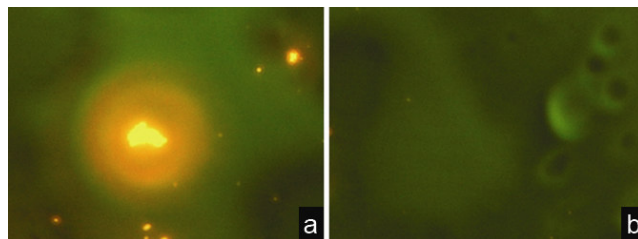


Fig. 2—Fluorescence microscopy image of vesicles solution in emission 550 nm. (a) vesicle solution after adding antimicrobial extract of a membrane active microorganism 08346 (according to Table 1); (b) control (vesicle solution after addition of deionized water).

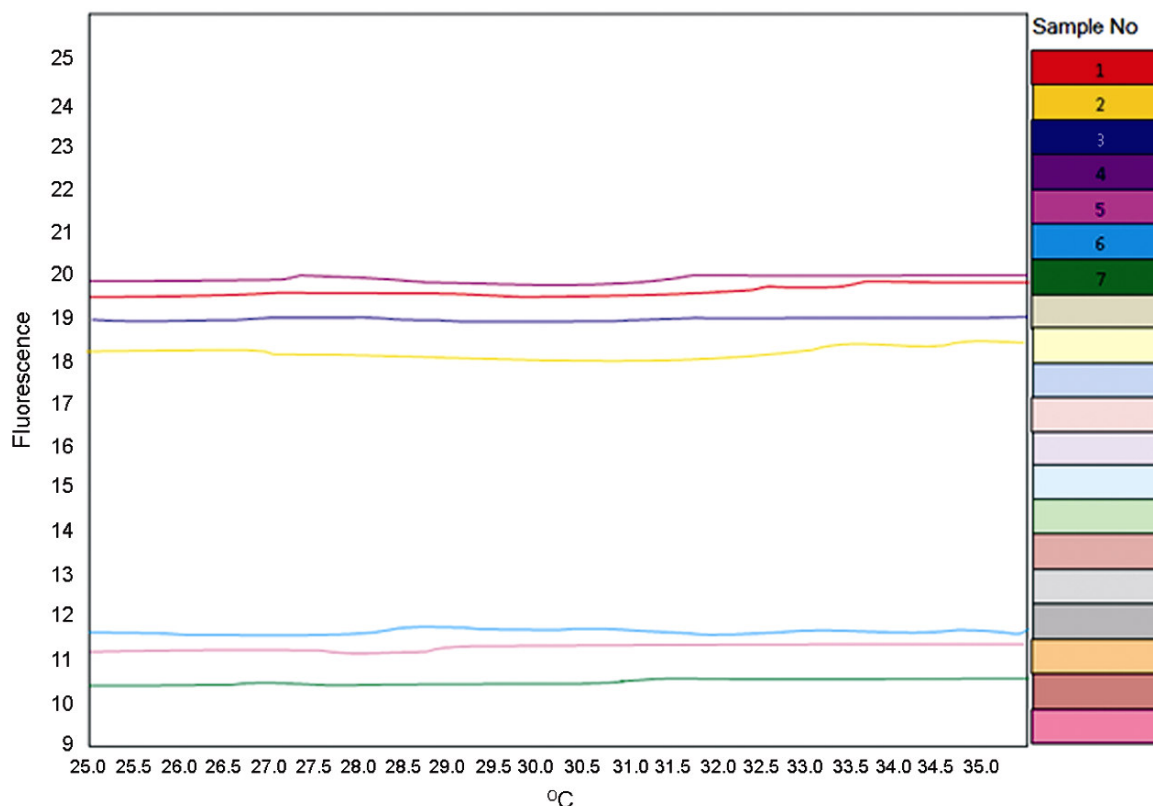


Fig. 3—Comparative fluorescence intensity of vesicle solution reacted by membrane active extracts of strains. [Vesicle solution after adding deionized water used as control; respective line set in down side of horizontal axis (the line has not been shown). (1) red: microorganisms 08317 (according to Table 1) second place in fluorescence intensity; (2) yellow: antibiotic amphotericin B fourth place in fluorescence intensity; (3) dark blue: microorganisms 0811 (according to Table 1) third place in fluorescence intensity; (4) violet: microorganisms 08346 (according to Table 1) first place in fluorescence intensity; (5) pink: DMSO as the solvent control; sixth place in fluorescence intensity; (6) light blue: non-membrane active microorganism 0832 (according to Table 1) fifth place in fluorescence intensity; and (7) dark green: antibiotic tetracycline seventh place in fluorescence intensity].

characteristics such as blue to red color transition under external perturbation that could be visible by the naked eye and fluorescence irradiation of red components at emission 500-550 nm.

Comparative values of fluorescence in the test solutions, sample 4 (containing vesicle solution and crude extract, strain 08346) had the highest fluorescence intensity followed by samples 1, 3 (08317, 0811 strains, respectively) and amphotericin B (a positive control antibiotic) (Fig. 3). It indicated that respective crude extract had better activity on vesicles. Amphotericin B is an antifungal that targets sterol presented in fungi membrane, but amphotericin B in second preference targets lipids of membrane. Samples 5 and 6 respectively, were related to DMSO as solvent control and extract of a non-membrane active strain as negative control strain. Sample 7 that had least effect on model vesicle and released low fluorescence intensity, was a non-membrane active antibiotic (tetracycline) as a negative control. Vesicle solution in deionized water

used as control that related line had lied under graph (not shown), since there was no fluorescence in vesicle solution. DMSO having specific structure caused forcing effect on model vesicles resulting in very low fluorescence irradiation. The present method was used for comparing values of fluorescence in test samples for confirming the present results for studying membrane activity. Finally, we found that strain 08346 could be a promising soil actinomycete that produced membrane active metabolites and had a potent antimicrobial effect.

Strain 08317 was another sample presenting good results in the antimicrobial and membrane activity assay and strain 0811 also had membrane activity, but its antimicrobial effect was not as good as two other strains (Table 1). Thus, it could be concluded that two strains 08346 and 08317 isolated from soil, had shown a promising antimicrobial membrane activity effect against fungi and bacteria, respectively.

*Morphological and cultural characteristics of membrane active isolates*—Three membrane active



isolates were subjected to study by the naked eye and optical microscopy. Strain 08346 isolated from mineral soil samples had a small, chalky and white colony with white aerial mycelia bearing linear spore chains and substrate mycelia in cream color. Strains 08317 and

0811 isolated from desert soil samples, had both medial and tough colonies and white aerial mycelia bearing spiral spore chains. Strain 08317 had orange substrate mycelia and strain 0811 had a colorless substrate mycelium (Fig. 4). However, we had investigated the

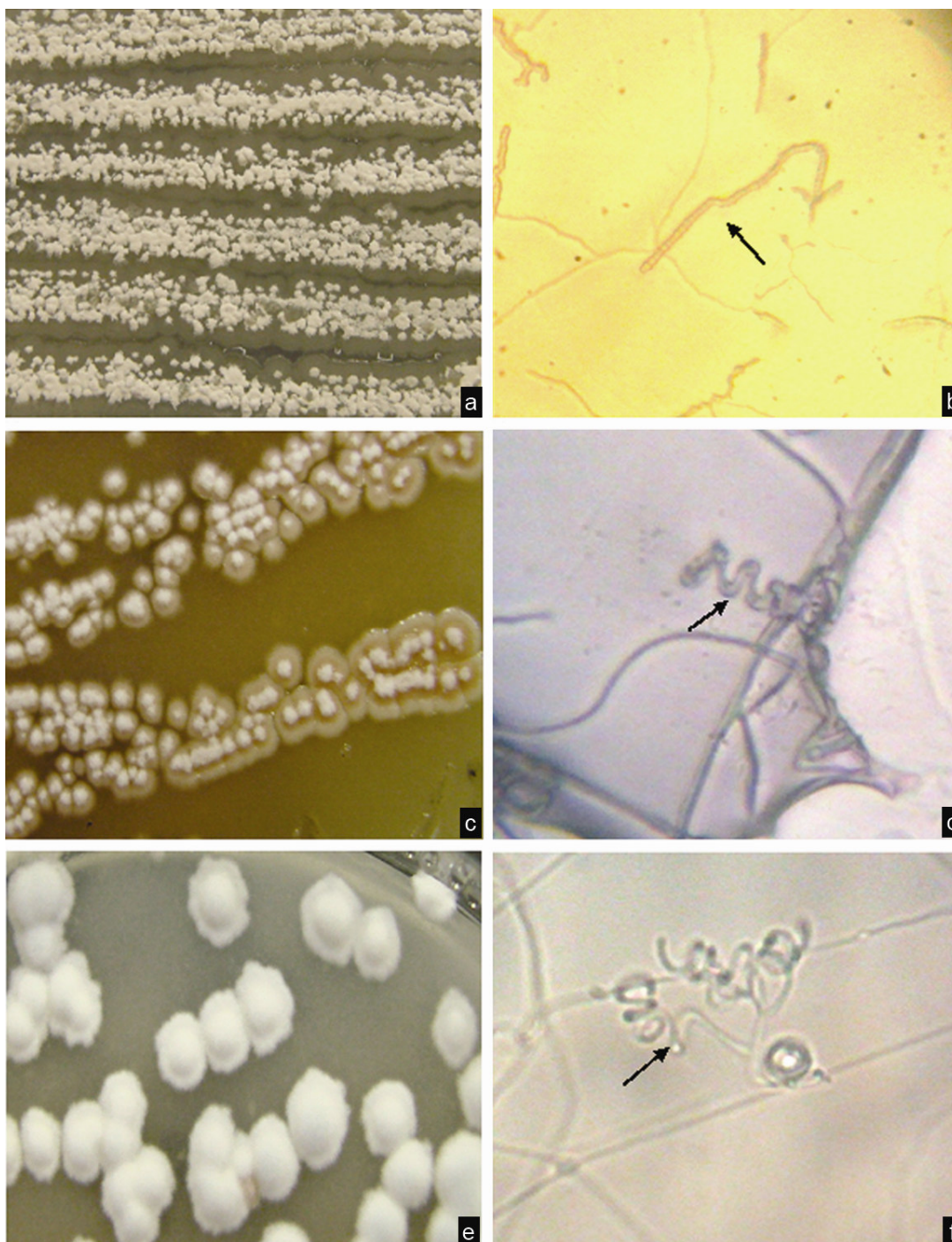


Fig. 4—Image of membrane active isolates. Left column: colony appearance on inorganic salt starch agar. Right column: light microscopy image of aerial mycelia bearing spore chains and morphology of spore chains at 100x magnification; arrows show spore chains. (a) colony appearance of strain 08346; (b) light microscopy image of strain 08346; (c) colony appearance of strain 08317; (d) light microscopy image of strain 08317; (e) colony appearance of strain 0811; and (f) light microscopy image of strain 0811.

general biochemical behaviour of different strains and only strain 08317 had been identified and confirmed by such methods<sup>25</sup>.

*Analysis of 16S rRNA gene sequences of three membrane active isolates*—The nucleotide BLAST similarity search analysis, based on 16S rRNA gene sequence of the three strains, revealed that all three isolates belong to the genus *Streptomyces*. The 16S rRNA gene sequences have been deposited in the GenBank database of NCBI under accession numbers JN180853 (1424 bp), JN180854 (1432 bp), JN180855 (1421 bp) for 08346, 08317, and 0811, respectively.

Considering the above mentioned results, it is concluded that 3 isolated strains of actinomycetes are potent antimicrobial that have membrane active metabolites. Further studies are needed at molecular level to understand the mechanism of action of active metabolites of these 3 isolated strains.

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