



Enhanced detection of antimicrobial activities and secondary metabolites production from *Plantactinospora* sp. KBS50 cultivated using the OSMAC fermentation approach

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ABSTRACT

Aims: A rare marine-derived actinomycete, *Plantactinospora* sp. KBS50, has been identified as a potential source of bioactive secondary metabolites compounds. The present study aimed to evaluate the secondary metabolites biosynthetic capability of strain KBS50 using the One Strain Many Compound (OSMAC) fermentation strategy.

Methodology and results: Strain KBS50 was fermented in a basal medium (ISP2) supplemented with selected biological and chemical elicitors, as well as cultivation at different pH value and incubation temperature. Statistical analysis revealed that the antimicrobial activities were significantly increased, as compared to the basal medium, ISP2. Similarly, the comparative High Performance Liquid Chromatography (HPLC) analysis showed an increase in secondary metabolites production, as well as the detection of potential new metabolites, particularly from the crude extracts of ISP2 medium supplemented with 1% (w/v) sodium chloride and with the culture filtrate of *Aspergillus niger*. The bioassay-guided fractionation showed that the extract of strain KBS50 contains multiple compounds with antibacterial activity against the Gram-positive strains. Further fractionation led to the isolation of two semi-pure compounds (compound 3 and 4) with bactericidal properties against *Staphylococcus aureus*. The Minimum Inhibitory Concentration (MIC) values of compound 3 and 4 were recorded at 7.81 µg/mL and 62.50 µg/mL, respectively. The minimum bactericidal concentration (MBC) for compound 3 was recorded at 15.63 µg/mL while the MBC for compound 4 was recorded as 125.00 µg/mL.

Conclusion, significance and impact of study: The OSMAC fermentation strategy used in this study had successfully enhanced the detection of antibiotics and secondary metabolites from *Plantactinospora* sp. KBS50. The bioassay-guided fractionation further established the capability of strain KBS50 as a source of bioactive secondary metabolite compounds with potent antimicrobial activity.

Keywords: Marine actinomycete, elicitors, OSMAC, antimicrobial activity

INTRODUCTION

Marine ecosystem represents an underexploited source of rare actinomycetes species for natural product drug discovery. Due to the extreme differences in conditions between the marine and terrestrial environment, it is estimated that marine actinomycetes are capable of producing unique secondary metabolite compounds with important biological activities (Lam, 2006). In fact, actinomycetes from the marine ecosystem have been increasingly recognized in recent years as an alternative source of bioactive compounds. Many efforts have been made to explore the marine actinomycetes that resulted in the discovery of novel compounds with unique chemical structures that exhibits various biological activities including antibacterial, antifungal, and anticancer (Feling *et al.*, 2003; Bister *et al.*, 2004; Solanki *et al.*, 2008). In our previous study, an actinomycete strain isolated from

the marine environment in Sarawak was identified and characterized (Juboi *et al.*, in press). The rare actinomycete, designated as *Plantactinospora* sp. KBS50, also shown to exhibit antimicrobial activities against Gram-positive bacteria and fungi.

The present study aimed to further evaluate the secondary metabolites biosynthetic capability of strain KBS50 using the One Strain Many Compound (OSMAC) method. The OSMAC approach has been used to unselectively enhance the production of compounds from microbial strains through manipulation of various cultivation parameters (Bode *et al.*, 2002; Martín, 2004; Bills *et al.*, 2008; Hewage *et al.*, 2014). This strategy provides nutritional and/or physical variation between different cultivation conditions which could potentially activate or increase the expression of biosynthetic genes in the strain's genome. Parameters that can be manipulated include media composition, aeration rate,

pH, temperatures, and the addition of enzyme inhibitors (Bode *et al.*, 2002).

To evaluate the antibiotics production from strain KBS50, the crude extracts from the OSMAC fermentation were screened against test microorganisms. Statistical analysis was carried out to determine whether there was any significant increase in antimicrobial activity, while a comparative HPLC analysis was carried out to monitor the changes of secondary metabolite production in the OSMAC media as compared to the basal medium. The possible number of secondary metabolite compounds with antibacterial activity against the Gram-positive bacteria was also estimated using the bioassay-guided fractionation, which eventually leads to the isolation of the bioactive compounds. The potency of these compounds was then determined by the evaluation of the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC).

MATERIALS AND METHODS

Fermentation broth compositions and preparation for OSMAC

The ISP2 broth (per liter: Yeast extract, 4.0 g; malt extract, 10.0 g; glucose, 4.0 g; pH 7.2 ± 0.2), was selected as the basal medium for the cultivation of strain KBS50. For the OSMAC approach, small variation were made to the basal medium by the addition of chemical and biological elicitor (1% and 1.5% (w/v) sodium chloride, NaCl; 3% (v/v) dimethyl sulfoxide, DMSO; 25 µM and 50 µM ScCl₃·6H₂O; 2.5% (v/v) dead cells of *Bacillus subtilis*; 2.5% (v/v) dead cells of *Escherichia coli*; 1.5% (v/v) culture filtrate of *A. niger*; 1.5% (v/v) culture filtrate of *Ganoderma boninense*; 5 mM and 30 mM potassium phosphate buffer at pH 7.2) and different cultivation parameters (37 °C and pH 9). Other media included half-strength ISP2 (½ ISP2), half-strength ISP2 supplemented with 25 mM N-acetylglucosamine (GlcNac), and ISP2/GYM broth adapted from Ochi (1987) (per liter: Yeast extract, 4.0 g; malt extract, 10.0 g; glucose, 4.0 g; NZ-Amine A, 1.0 g; NaCl, 2.0 g; morpholinopropanesulfonic acid (MOPS) 1 M, 50 mL). The dead cells of *B. subtilis* and *E. coli* were prepared from overnight cultures according to the method of Luti and Yonis (2013), while the culture filtrates for *A. niger* and *G. boninense* were prepared according to the method of Wang *et al.* (2013). All the broths used for the OSMAC fermentation were prepared at a final pH of 7.2 (unless stated otherwise) and sterilized by autoclaving at 121 °C for 15 mins.

Fermentation and extraction of secondary metabolites

Pre-culture of strain KBS50 was prepared by inoculating 50 mL ISP2 broth in 125 mL Erlenmeyer flasks, with a 7-day old actinomycete culture scrapped from the ISP2 agar surface. The pre-culture was grown in an incubator shaker at 28 °C for 7 days with constant shaking at 200

rpm. After seven days of incubation, the pre-culture was inoculated into 50 mL fermentation broths in 125 mL Erlenmeyer flasks, using 5% (v/v) inoculum size. The inoculated broths were fermented at 28 °C (except for the fermentation at 37 °C) with constant shaking at 200 rpm for seven days. Secondary metabolites were extracted from the spent broths twice with an equal volume of ethyl acetate. The crude extracts were dried *in vacuo* and stored at 4 °C until further analysis.

Antimicrobial screening of crude extracts from OSMAC fermentation

Antimicrobial screening of the crude extracts was evaluated against *E. coli* NBRC 3301, *Pseudomonas aeruginosa* NBRC 12689, *S. aureus* NBRC 12732, *B. subtilis* NBRC 3134, *Saccharomyces cerevisiae* ATCC 9763, *A. niger* NBRC 4066 and *Candida albicans* NBRC 1386, using the microtiter plate (MTP) assay in 96-well plate format. Standardized cells suspensions of bacteria (2.0 × 10⁶ cells/mL prepared in Mueller-Hinton broth) and yeast (2.0 × 10⁵ cells/mL in Sabouraud Dextrose broth) were prepared from an overnight cultures at 37 °C and 30 °C, respectively, while standardized spore suspension of *A. niger* was prepared in Sabouraud Dextrose broth at 2 × 10⁵ spores/mL.

Twenty milliliter of the dried crude extracts were reconstituted in 10 mL of methanol. Then 300 µL of each extract was transferred into the 96-well plates and air-dried in the fume hood. For the antimicrobial assay, 5 µL dimethyl sulfoxide (DMSO) was added to dissolve the extract in each well, followed by the addition of 70 µL of sterile broth (Mueller-Hinton for bacteria, Sabouraud Dextrose for yeast/fungi). Finally, 75 µL of broth containing the standardized suspension of bacteria or yeast/fungi was added to each well for a final volume of 150 µL cells/crude extract mixture. The concentration of the crude extracts tested was at 4-fold of its original concentration, while the DMSO concentration used to dissolve the extract was reduced to 3.33% final concentration.

The antibiotics chloramphenicol (100 ppm for Gram-positive bacteria; 400 ppm for Gram-negative bacteria), nystatin (100 ppm for *S. cerevisiae* and *A. niger*), and miconazole (100 ppm for *C. albicans*) served as positive control. Wells containing standardized cells/spores suspension only served as the growth control. Meanwhile, wells containing cells/spores suspension with 3.33% (v/v) DMSO only served as the vehicle control. The MTP assay for each test strain was carried out in triplicates. Initial optical density (IOD) was measured at 600 nm wavelength using a 96-well plate reader. The plates were incubated at 37 °C for 24 h (bacteria) and 30 °C for 48 h (yeast/fungi) before the final OD (FOD) reading was measured at 600 nm. The level of inhibition of tests strains by the crude extracts was measured based on the differences in optical density reading before and after incubation as compared to the growth control, calculated as follows:

Growth inhibition or reduction (%) = $[C - T] / C \times 100$

Where;

C = FOD – IOD value of the growth control

T = FOD – IOD value of the test sample

The minimum growth reduction of 50% and 80% in comparison to the growth control was set as the threshold for the crude extracts to be considered as exhibiting relatively weak or strong antimicrobial activity, respectively. The data collected were analyzed using one-way ANOVA in SPSS version 15 to compare the level of antimicrobial activity among the crude extracts, and as compared to the basal medium, ISP2. The antimicrobial activity was considered as significant if the p-value was lower than 0.05 ($p < 0.05$).

Comparative secondary metabolites profiling of crude extracts using HPLC

Ten mL of dried crude extracts were dissolved in 1 mL of 100% HPLC-grade methanol, filtered through 0.2 µm regenerated cellulose filter into HPLC vials and subjected to HPLC analysis on a reverse-phase Analytical HPLC Agilent 1100 system equipped with diode array detector (DAD) (G1315C DAD SL, Agilent). The DAD was set to measure the UV spectrum at 200, 210, 230, 254, 273, 330, 400 and 600 nm. Extracts of the non-inoculated media were also analyzed using HPLC for background data. Aliquots of 20 µL of each sample were injected into the HPLC column (4.6 × 150 mm, 5 µm, ZORBAX Eclipse XDB-C18, Agilent) held at a temperature of 50 °C and eluted at a flow rate of 1.5 mL/min for 15 min run-time per sample. Solvents and conditions used were as follows: 0-10 min, 10% (v/v) Acetonitrile and 90% (v/v) water; 10-15 min, 100% Acetonitrile. The yields of the metabolites were estimated based on the peaks heights measured as the UV absorbance value (mAU). The HPLC data was used for qualitative analysis of secondary metabolites present in the crude extracts.

Bioassay-guided fractionation and purification of the antimicrobial compounds

Strain KBS50 was fermented in a total volume of 2 L ISP2 broth (4 × 500 mL broth in 2 L flask) for the production of secondary metabolite compounds with antibacterial activity against the Gram-positive test strains. Secondary metabolites from the fermentation broth were extracted using an equal volume of ethyl acetate. The organic extracts from the 3 times extractions were pooled and concentrated to dryness using a rotary evaporator while being heated at 45 °C on a water bath. The dried weight of the extract was recorded.

Fractionation was carried out using Agilent 1200 series semi-preparative HPLC instrument equipped with DAD (G1315D, Agilent). Aliquots of 50 µL were injected into the HPLC column (9.4 × 250 mm, 5 µm, Agilent Eclipse XDB-C18) held at a temperature of 35 °C and eluted at a flow rate of 4.0 mL/min for 25 min runtime per

sample. Solvents and conditions used were as follows: 0-15 min, 10% acetonitrile and 10% water; 15-25 min, 100% acetonitrile. The crude extract was separated into 10 fractions using a time-based fraction collection of 2.5 min/fraction. Multiple rounds of fractionation were carried under the same HPLC conditions. The collected fractions from the same retention time were pooled together and dried *in vacuo*. The antimicrobial activity of the fractions at 1 mg/mL concentration was evaluated against *B. subtilis* and *S. aureus* using the agar-well diffusion assay (AwDA) method (Balouiri *et al.*, 2016). The selected fraction with antimicrobial activity was further fractionated using the semi-preparative HPLC for isolation of bioactive compounds. The antimicrobial activity of the purified compounds at 1 mg/mL concentration was evaluated using the AwDA against *S. aureus*, as the representative of the Gram-positive test strain.

Determination of the minimum inhibitory concentration (MIC)

The MIC of the isolated compounds against *S. aureus* was determined using the broth microdilution method (Andrews, 2001). The stock solutions of compounds (100 mg/mL) were prepared in 100% DMSO, diluted to 1 mg/mL concentration using sterile reverse-osmosis water followed by two-fold dilutions for 12-point concentrations. From each dilution point, 75 µL of the compound solution was transferred into the inoculated wells containing 75 µL of standardized cells suspension of *S. aureus* (2.0×10^6 cells/mL), prepared in double-strength Mueller-Hinton broth. The antibiotics chloramphenicol served as the positive control. Wells containing standardized cells suspension only served as the growth controls. The MIC assay was carried out in triplicate. Growth inhibition was measured in the same way as the MTP assay. The MIC was determined as the lowest concentration of the compound in which no visible growth was detected (Andrews, 2001).

Determination of the minimum bactericidal concentration (MBC)

From the broth microdilution plates for the MIC test, 100 µL culture broth from the well of the MIC value and from 5 wells above the MIC value was pipetted out and inoculated onto Mueller-Hinton agar plates. This experiment was carried out for all the three replicates of the MIC test. The number of bacterial colonies (CFU) on the Mueller-Hinton agar was counted after 24 h of incubation at 37 °C. The MBC is the least concentration of antibacterial compound that can kill over 99.9% of the test strain (French, 2006; Balouiri *et al.*, 2016). The concentration of the bioactive compound that resulted in less than five colonies (CFU) on the agar plates was set as the MBC (Chawawisit *et al.*, 2015).

RESULTS

Antimicrobial activity of the crude extracts

All crude extracts tested showed strong antimicrobial activity against *B. subtilis*, which resulted in over 80% growth reduction of the test strain (Table 1). The strongest inhibition against *B. subtilis* was recorded for the crude extract of ISP2 supplemented with 50 µM scandium chloride (103.13 ± 1.94%). The antimicrobial activity was significantly stronger as compared to the

crude extract of the basal medium ($p < 0.05$). Meanwhile, the crude extract from ISP2 medium supplemented with 1.5% (w/v) NaCl resulted in the lowest growth reduction of *B. subtilis* (87.23 ± 0.45%), which was significantly lower compared to ISP2 ($p < 0.05$).

Meanwhile, fifteen samples of crude extracts showed strong antimicrobial activity against *S. aureus* which resulted in over 90% growth inhibition. However, none of the extracts showed a significant increase in antimicrobial activity as compared to the basal medium (Table 1).

Table 1: Antimicrobial activities of crude extracts from the OSMAC fermentation strategy against the microbial test panel.

No.	Extract	Growth inhibition (%) ± SEM						
		<i>B. subtilis</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>A. niger</i>	<i>S. cerevisiae</i>	<i>C. albicans</i>
1	ISP2	96.62 ± 1.44	96.71 ± 0.38	36.03 ± 0.51	29.86 ± 1.55	99.95 ± 0.54	-18.37 ± 13.06	52.54 ± 5.32
2	ISP2+NaCl 1%	93.72 ± 0.21	96.31 ± 0.11	53.13 ± 0.64	47.02 ± 0.96	27.56 ± 1.74	-54.26 ± 13.66	2.99 ± 2.88
3	ISP2+NaCl 1.5%	87.23 ± 0.45	75.80 ± 0.85	53.32 ± 0.04	36.55 ± 2.87	-30.73 ± 28.64	-54.92 ± 14.71	2.05 ± 1.82
4	ISP2+DMSO 3%	97.66 ± 0.65	95.57 ± 1.24	51.92 ± 1.61	54.79 ± 1.63	84.69 ± 6.56	-28.89 ± 14.62	90.11 ± 0.85
5	ISP2+Sc 25 µM	102.54 ± 0.11	95.19 ± 0.44	46.17 ± 3.42	12.31 ± 0.34	76.64 ± 12.29	-64.27 ± 23.91	84.98 ± 3.84
6	ISP2+Sc 50 µM	103.13 ± 1.94	94.30 ± 0.28	52.32 ± 1.08	53.61 ± 0.42	88.58 ± 6.49	-61.69 ± 19.26	89.56 ± 0.69
7	ISP2+BS cells	96.06 ± 0.07	90.94 ± 0.11	47.03 ± 0.89	52.57 ± 2.27	96.25 ± 3.17	-44.18 ± 25.01	89.88 ± 1.93
8	ISP2+EC cells	91.99 ± 0.85	92.82 ± 0.45	40.68 ± 0.48	54.26 ± 0.31	101.05 ± 0.15	-42.30 ± 21.15	73.41 ± 2.55
9	ISP2+AN filtrate	99.17 ± 0.08	98.11 ± 0.43	38.72 ± 0.17	63.22 ± 0.85	88.16 ± 1.73	-1.26 ± 7.55	36.39 ± 4.60
10	ISP2+GB filtrate	92.25 ± 0.53	95.76 ± 0.43	58.92 ± 0.98	65.02 ± 4.24	98.78 ± 0.76	-14.85 ± 13.64	90.76 ± 4.95
11	ISP2+PPB 5 mM	91.20 ± 0.55	93.73 ± 0.19	52.32 ± 1.77	57.12 ± 0.68	82.15 ± 7.57	-43.16 ± 23.60	90.27 ± 3.77
12	ISP2+PPB 30 mM	101.79 ± 0.14	99.02 ± 0.24	52.58 ± 0.23	38.89 ± 1.92	48.17 ± 6.57	-67.46 ± 1.83	-16.04 ± 7.29
13	ISP2 37°C	94.27 ± 0.67	54.74 ± 1.37	54.55 ± 0.37	8.07 ± 1.70	23.19 ± 5.33	-41.43 ± 1.58	-11.55 ± 3.28
14	ISP2 pH 9	88.86 ± 0.84	91.53 ± 0.14	63.78 ± 0.62	51.25 ± 0.87	96.29 ± 0.75	-29.78 ± 13.94	95.78 ± 3.36
15	1/2 ISP2	93.86 ± 0.70	96.46 ± 0.80	54.63 ± 4.67	44.58 ± 0.22	93.83 ± 0.51	-59.20 ± 6.37	92.08 ± 3.58
16	1/2 ISP2+GlcNAc 25 mM	88.98 ± 0.22	94.96 ± 0.53	61.56 ± 1.51	34.49 ± 2.47	56.46 ± 5.76	-74.31 ± 4.63	68.80 ± 2.79
17	ISP2/GYM	98.76 ± 0.33	98.26 ± 1.21	61.22 ± 1.73	20.15 ± 3.19	67.25 ± 1.32	-26.90 ± 2.66	22.80 ± 1.82
18	CP100	81.96 ± 3.43	78.44 ± 1.49	NT	NT	NT	NT	NT
19	CP400	NT	NT	99.13 ± 0.66	98.68 ± 0.67	NT	NT	NT
20	NYS100	NT	NT	NT	NT	100.98 ± 1.25	104.12 ± 0.65	NT
21	MCZ100	NT	NT	NT	NT	NT	NT	103.04 ± 0.44

NT = Not tested; CP100 = chloramphenicol, 100 ppm; CP400 = chloramphenicol, 400 ppm; NYS100 = nystatin, 100 ppm; MCZ100 = miconazol, 100 ppm.

The antimicrobial activity against the Gram-negative test strains also increased significantly as compared to the crude extract of ISP2 medium (Table 1). However, these extracts only exhibited weak antimicrobial activities, as indicated by the growth reduction below 80%.

The strongest inhibition against *E. coli* was recorded from the crude extract of ISP2 pH 9 ($63.78 \pm 0.62\%$), while the strongest inhibition against *P. aeruginosa* was recorded from the crude extract of ISP2 supplemented with the culture filtrate of *G. boninense* ($65.02 \pm 4.24\%$). Both inhibitions were significantly stronger as compared to the basal medium ($p < 0.05$).

Ten extracts, including ISP2, exhibited strong antifungal activity against *A. niger*, whereby the growth reduction of the test strain was recorded at more than 80% (Table 1). Four extracts did not exhibit any antifungal activity in which the growth reduction was lower than 50% and significantly weaker as compared to ISP2 ($p < 0.05$). Only ISP2 medium supplemented with *E. coli* cells exhibit stronger antifungal activity as compared to ISP2 medium, although there was no significant difference in growth inhibition between the two crude extracts ($p < 0.05$). Other crude extracts also showed no significant difference in inhibition as compared to ISP2 extract ($p < 0.05$).

Strong antimicrobial activity against the yeast strain, *C. albicans*, was recorded, whereby 8 crude extracts resulted in over 80% growth reduction of the test strain (Table 1). The inhibitions were significantly stronger compared to the ISP2 extract ($p < 0.05$). The highest inhibition was recorded from the crude extract of ISP2 pH 9, with $95.78 \pm 3.36\%$ of growth inhibition. Meanwhile, the crude extract of ISP2 supplemented with *E. coli* cells resulted in weaker antimicrobial activity ($73.41 \pm 2.55\%$ growth reduction) against *C. albicans*, but still significantly higher than the antimicrobial activity of ISP2 crude extract ($p < 0.05$).

Comparative secondary metabolites profiling of the crude extracts

The comparative HPLC analysis revealed that the OSMAC fermentation strategy had generally enhanced the detection of secondary metabolites from strain KBS50. At least four major peaks were detected from the extract of ISP2 basal medium, although higher production of these compounds was detected from crude extracts of other OSMAC media (Table 2). In addition, 9 unique peaks were also detected from other OSMAC media, including ISP2 supplemented with NaCl, 5 mM phosphate buffer, and the culture filtrate of *A. niger* (Table 2).

The incubation temperature of 37 °C and the medium with pH 9 increases the production of compound 1 and 2, respectively, while the addition of 3% DMSO increases the production of compound 3 and 4. Meanwhile, the addition of 1% NaCl in the ISP2 medium stimulated the production of 3 new metabolites. Compounds 5 and 6 were only detected from the extracts of ISP2 medium supplemented with 1% and 1.5% NaCl, while compound 7 was detected from the extracts of ISP2 medium supplemented with 1.5% NaCl, 5 mM phosphate buffer,

and the culture filtrate of *A. niger*. The highest number of potentially new metabolites was detected from the crude extract of ISP2 medium supplemented with the culture filtrate of *A. niger*, whereby 6 unique peaks were detected (Figure 1).

In contrast, the addition of elicitors also had an inhibitory effect on secondary metabolites production by strain KBS50. Fermentation in ISP2 medium supplemented with 30 mM phosphate buffer and NaCl (1% and 1.5%) had negatively affected the production of compound 1, 3 and 4. A similar effect was observed for the fermentation media supplemented with the culture filtrate of *G. boninense*, and the incubation temperature of 37 °C, whereby the production of compound 3 and 4 were negatively affected. The fermentation medium ISP2/GYM also resulted in a significant reduction in secondary metabolites production, as compared to the basal medium. Other fermentation media including the half-strength ISP2 ($\frac{1}{2}$ ISP2), $\frac{1}{2}$ ISP2+GlcNAc 25 mM, ISP2+BS cells, ISP2+EC cells, and ISP2+Sc 50 μ M did not affect secondary metabolites profile of strain KBS50, as compared to the basal medium.

Bioassay-guided fractionation and purification of the antimicrobial compounds

To evaluate the antimicrobial potential of the antibiotics as well as to estimate the possible number of the bioactive compound produced by strain KBS50 against the Gram-positive bacteria, the crude extract from a 2 L ISP2 fermentation broth was subjected to a bioassay-guided fractionation. A total of 190 mg of crude extract was recovered from the fermentation broth. Approximately 170 mg of the crude extract was fractionated using the semi-preparative HPLC into 10 different fractions. Fractions 3 to 10 were evaluated for antimicrobial activity against *B. subtilis* and *S. aureus* using the AwDA. Fractions 1 and 2 were not tested for antimicrobial activity because HPLC analysis did not indicate the presence of any unique peak. Six out of eight fractions tested (Fractions 5 to 10) exhibited antimicrobial activity against *B. subtilis*, while two fractions (Fractions 7 and 9) exhibited the antimicrobial activity against both *B. subtilis* and *S. aureus* (Table 3). The result showed that strain KBS50 produces multiple compounds (different retention time) with similar bioactivity profile.

Fraction 7 exhibited the highest antimicrobial activity against both test strains with the mean diameter of inhibition of 15.33 ± 0.33 mm and 20.00 ± 0.33 mm for *B. subtilis* and *S. aureus*, respectively. However, HPLC analysis showed that the fraction contained minor peaks with the very low quantity of compounds. The amount recovered in fraction 7 was only 3.1 mg, which would be insufficient for further fractionation and isolation of the antimicrobial compound. Meanwhile, fraction 9 which was also active against both Gram-positive test strains contained two major peaks which were predicted to exhibit the antimicrobial activity (Figure 2). The two peaks were previously designated as compound 3 and compound 4 from the comparative HPLC analysis for the

OSMAC fermentation. Approximately 43 mg of fraction 9 was recovered, of which 30 mg was further fractionated to purify the two major peaks using the semi-prep HPLC.

Compounds 3 and 4 were recovered as single peak compounds with the purity of 99.86% and 93.24%, respectively. The amount recovered was 1.9 mg of compounds 3 and 3.1 mg of compound 4. The antimicrobial activity of the compounds at 1 mg/mL concentration was evaluated using AwDA against *S. aureus*, as the representative of the Gram-positive test strain. Both compounds exhibited antimicrobial activity against *S. aureus*, with the mean inhibition diameter of 12.00 ± 0.00 mm and 13.00 ± 0.00 mm for compounds 3 and 4, respectively (Figure 3).

MIC and MBC of the bioactive compounds

When evaluated against *S. aureus*, compound 3 showed the lowest MIC of 7.81 ± 0.00 µg/mL, while compound 4

showed highest MIC of 62.50 ± 0.00 µg/mL. As for the control (chloramphenicol), the MIC against *S. aureus* was recorded at 3.91 ± 0.00 µg/mL. The MBC for compound 3 was recorded as 15.63 µg/mL while the MBC for compound 4 was recorded as 125.00 µg/mL. At these respective concentrations, as well as at a higher concentration of the compounds, no bacterial colony was detected on the Mueller-Hinton agar plates after 24 h incubation at 37 °C. Meanwhile, 366.67 ± 66.67 CFUs and 67.00 ± 41.94 CFUs were recovered from the culture broth from the wells of the MIC value of compounds 3 and 4, respectively. Compounds 3 and 4 can be considered as bactericidal since its MBC did not exceed four times the MIC (French, 2006), whereby in this case the MBC was only two times of the MIC for both compounds. Both of these compounds are potential drug candidate for anti-infective against the Gram-positive bacteria.

Table 2: Secondary metabolite compounds detected from the crude extracts of strain KBS50 cultivated using the OSMAC approach.

Compound	Retention time (min)	Peak height in ISP2 extract [mAU]	Highest peak detected (mAU)	Highest peak detected (medium/extract)	Maximum UV signal (nm)
1	3.833	522.10	765.76	ISP2 37 °C	200
2	6.217	68.54	176.81	ISP2 pH 9	273
3	7.735	585.70	1820.21	ISP2+DMSO 3%	273
4	7.866	421.98	1013.77	ISP2+DMSO 3%	273
5	7.359	-	22.81	ISP2+NaCl 1%	200
6	5.205	-	99.88	ISP2+NaCl 1.5%	273
7	4.665	-	41.11	ISP2+PPB 5 mM	400
8	7.102	-	80.57	ISP2+AN filtrate	200
9	7.270	-	75.67	ISP2+AN filtrate	200
10	9.046	-	152.98	ISP2+AN filtrate	200
11	10.015	-	122.28	ISP2+AN filtrate	200
12	10.288	-	128.82	ISP2+AN filtrate	200
13	10.829	-	92.99	ISP2+AN filtrate	200

Table 3: Antimicrobial activity of the fractions from strain KBS50 against Gram-positive test strains evaluated using agar-well diffusion assay.

Fraction	Inhibition zone (mm) ± Standard error of the mean	
	<i>B. subtilis</i>	<i>S. aureus</i>
3	-	-
4	-	-
5	10 ± 0.33	-
6	11.67 ± 0.33	-
7	15.33 ± 0.33	20 ± 0.00
8	11.33 ± 0.33	-
9	14.67 ± 0.33	9 ± 0.00
10	10 ± 0.00	-
Chloramphenicol (100 ppm)	21.33 ± 0.33	21.00 ± 0.58

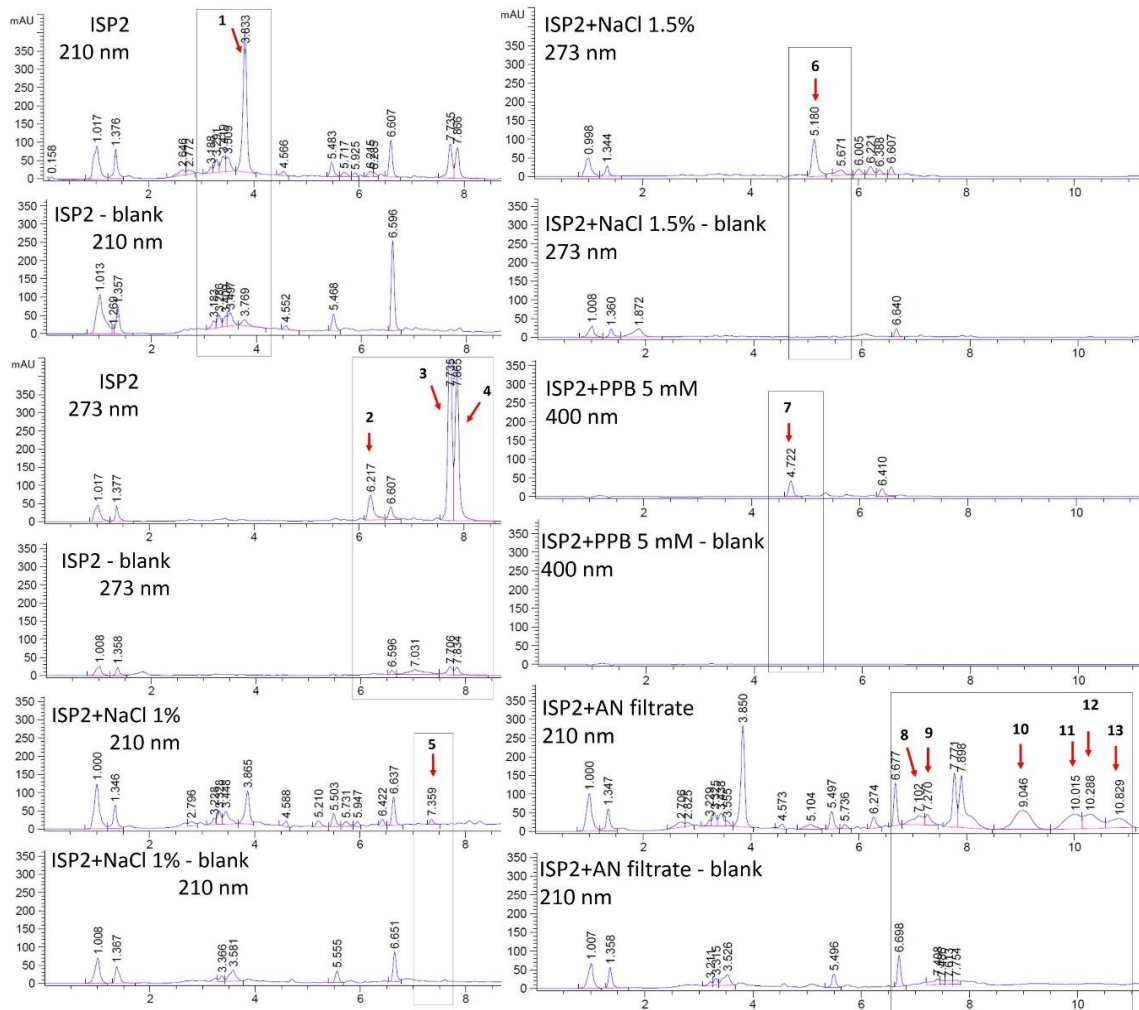


Figure 1: Comparative HPLC analysis showed the changes in secondary metabolites profile of strain KBS50 when cultivated using the OSMAC fermentation approach.

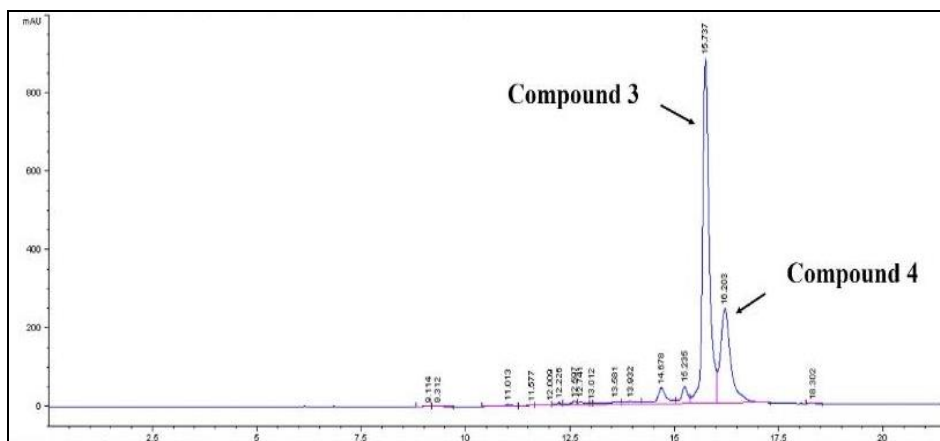


Figure 2: HPLC profile of fraction 9 showing two major peaks identified as compounds 3 and 4, at 273 nm. HPLC profiling of the fraction was performed on the semi-preparative HPLC using the following solvent gradient: 0-8 min, 10% acetonitrile and 90% water; 8-18 min, 100% acetonitrile; 18-25 min, 10% acetonitrile and 90% water.

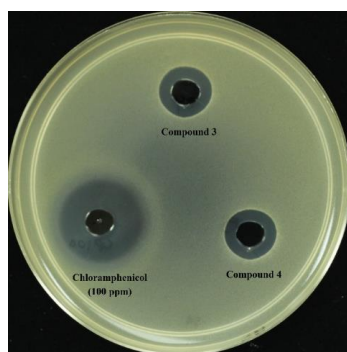


Figure 3: Growth inhibition of *S. aureus* on the AwDA by compound 3 and compound 4. The positive control was 100 ppm chloramphenicol which produced 21.00 ± 0.58 mm diameter of the clear zone.

DISCUSSION

The aim of this study was to enhance the antibiotics and secondary metabolites detection from strain KBS50 using the OSMAC fermentation approach. The fermentation strategy has been used successfully to increase secondary metabolites production in other actinomycetes and fungi (Bode *et al.*, 2002; Bills *et al.*, 2008; Hewage *et al.*, 2014). As stated by Bode *et al.* (2002), the OSMAC is “a random approach” whereby the cultivation parameters for a single strain can be altered in many possible ways that may lead to the discovery of new secondary metabolites. Although there could be hundreds of possible growth conditions that can be manipulated to evaluate the antibiotics production from strain KBS50, only a small number of growth conditions were tested in this study to minimize the number of extracts to be screened. While it is unlikely that small changes in the cultivation parameters can trigger the production of all the potential secondary metabolites from strain KBS50, the OSMAC fermentation strategy had resulted in an increase in the antimicrobial activities against the microbial test panel. The secondary metabolites detection from the crude extracts was also enhanced, as demonstrated by the comparative HPLC analysis results.

Incubation temperature and pH of the media may influence the growth of actinomycetes, consequently affecting the antibiotics and secondary metabolites production (James *et al.*, 1991; Ripa *et al.*, 2009; Bundale *et al.*, 2015). A slight increase in pH (from pH 7.2 to pH 9) and incubation temperature (from 28 °C to 37 °C) resulted in a significant shift in the antimicrobial activity and secondary metabolites profiles of strain KBS50. Antimicrobial activities against *S. aureus*, *A. niger* and *C. albicans* were reduced significantly at the incubation temperature of 37 °C, whereas the antimicrobial activity against *E. coli* was increased. The production of compounds 3 and 4 was repressed at the elevated temperature, although the highest level of compound 1 was recorded. Meanwhile, the strain showed a significant increase in antimicrobial activities against the Gram-

negative bacteria and *C. albicans* when it was cultivated at pH 9. The slightly basic medium also increases the production level of compound 2.

A significant decrease in antimicrobial activity against *A. niger* and *C. albicans* was recorded from the crude extract of the ISP2/GYM medium, which shows the possibility that nutrient-rich medium may negatively affect the secondary metabolism of strain KBS50. The comparative HPLC analysis also showed a significant reduction in the levels of major compounds detected from the crude extract of this medium as compared to the basal medium. In contrast, the metabolites profile of the strain was relatively unchanged when it was cultivated in the half-strength ISP2 medium ($\frac{1}{2}$ ISP2). In this nutrient-poor medium, the strain showed an overall increase in antimicrobial activities. Other study reported that N-acetylglucosamine may induce antibiotics biosynthesis in *Streptomyces* under poor nutrient conditions (Rigali *et al.*, 2008). However, adding the compound into the half-strength ISP2 medium did not result in any major changes to the metabolites profile and antimicrobial activity of strain KBS50.

The incorporation of elicitors into the basal medium provided additional cues that further stimulated secondary metabolites production. These elicitors were selected based on the literature survey that reported their effect towards secondary metabolite biosynthesis. The addition of DMSO in fermentation media was reported to increase antibiotics production in actinomycetes by 2 to 3 folds (Chen *et al.*, 2000). Similarly, the rare earth element (scandium) was also reported to increase antibiotics production by as much as 25 folds (Kawai *et al.*, 2007). It also increased the expression level of secondary metabolites biosynthetic genes in *Streptomyces coelicolor* (Tanaka *et al.*, 2010). Similarly, in this study, a significant increase in antimicrobial activities against the Gram-negative bacteria and *C. albicans* was recorded as the results of elicitation using DMSO and scandium. The exact mechanism of action of DMSO and scandium in eliciting the secondary metabolites production in actinomycetes is still unknown, although it could potentially affect the ribosome that consequently alters the mechanism of genes expression and translation (Chen *et al.*, 2000; Kawai *et al.*, 2007).

The dead cells of *B. subtilis* and *E. coli*, and the culture filtrate of fungi were reported to increase antibiotics production in actinomycetes and other microorganisms (Luti and Mavituna, 2011; Luti and Yonis, 2013; Wang *et al.*, 2013). These elicitors mimic the biological interaction between different microorganisms in the natural environment which may potentially lead to the biosynthesis of antibiotics as a mean of self-defence mechanism (Luti and Yonis, 2013; Abdelmohsen *et al.*, 2015). Other researchers have reported the uses of live cells in a fermentation approach called co-cultivation (Luti and Yonis, 2013; Wang *et al.*, 2013). This strategy is theoretically more representative of the actual microbial interaction in the environment, as live cells may directly interact with each other and provide various signaling molecules that may affect secondary metabolite

production at the molecular level (Abdelmohsen *et al.*, 2015). However, the incorporation of live cells might affect the growth of actinomycetes due to the competition over nutrient components as actinomycetes are generally grown slower than the competing bacteria. In most cases, the dead cells or culture filtrates provided the same elicitation effect on antibiotics production by the producer strain (Luti and Yonis, 2013; Wang *et al.*, 2013). In this study, the culture filtrate of *A. niger* was found to have the most profound effect on strain KBS50 where several new metabolites were detected, while the culture filtrate of *G. boninense* seems to inhibit the production of the major metabolites.

Phosphate can either enhance or repress secondary metabolites production depending on its concentration in the cultivation medium (Bode *et al.*, 2002; Martín, 2004). According to Martín *et al.* (1977), phosphate may suppress antibiotics biosynthesis by inhibiting the formation of precursors and limiting the availability of inducers for secondary metabolite biosynthetic pathway. High concentration of phosphate negatively affects secondary metabolites production at the translational level (Martín, 2004; Bibb, 2005). In this study, the extract of ISP2 supplemented with 5 mM phosphate buffer exhibited strong antimicrobial activity against *A. niger* and *C. albicans*. However, at a higher concentration (30 mM), the antimicrobial activities against both test strains were reduced significantly while the production of major compounds was repressed. Similar observation on the effect of phosphate on antibiotics production by actinomycetes has been reported (Hobbs *et al.*, 1990; Lounès *et al.*, 1996; Giardina *et al.*, 2014).

Other studies in actinomycetes and fungi have shown that NaCl can affect the growth and secondary metabolites production due to its effect on the osmotic pressure of the medium (Huang *et al.*, 2011; Wang *et al.*, 2011; Ng *et al.*, 2014). Studies on *Salinispora* species which requires salt for growth showed that the antibiotics production correlates to the growth rate of the strains under different concentration of NaCl (Ng *et al.*, 2014). The addition of 1-1.5% NaCl in the fermentation medium might have increased the biomass, although this effect was not measured in the current study. The addition of NaCl resulted in a contrasting effect on antibiotics and secondary metabolites production. The comparative HPLC analysis showed that compounds 5 and 6 were only detected from medium supplemented with 1% and 1.5% NaCl. A higher level of compound 6 was detected from the medium with 1.5% NaCl. Increasing the concentration of NaCl might increase the production level of compound 6. In contrast, the production of major metabolites (compound 1, 3 and 4) was suppressed in the presence of NaCl. Coincidentally, the antimicrobial activity, especially against *A. niger* and *C. albicans*, was reduced significantly with the presence of NaCl.

CONCLUSION

The OSMAC fermentation strategy used in this study had successfully enhanced the detection of antibiotics and

secondary metabolites from *Plantactinospora* sp. KBS50. The data can also be used as the basis for the optimization of cultivation conditions to increase the compound yield for their isolation and purification. While current study did not mean to be a comprehensive screening of many possible growth conditions for the fermentation of strain KBS50, future study may include various combinations of nutrient compositions, elicitors, and other cultivation parameters which can be used to activate secondary metabolites production. The bioassay-guided fractionation further established the capability of strain KBS50 as a promising source of bioactive secondary metabolite compounds with potent antimicrobial activity for the development of new drug candidates.

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REFERENCES

- Abdelmohsen, U. R., Grkovic, T., Balasubramanian, S., Kamel, M. S., Quinn, R. J. and Hentschel, U. (2015). Elicitation of secondary metabolism in actinomycetes. *Biotechnology Advances* **33**(6), 798-811.
- Andrews, J. M. (2001). Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotherapy* **48**(suppl 1), 5-16.
- Balouiri, M., Sadiki, M. and Ibsouda, S. K. (2016). Methods for *in vitro* evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis* **6**(2), 71-79.
- Bibb, M. J. (2005). Regulation of secondary metabolism in streptomycetes. *Current Opinion in Microbiology* **8**(2), 208-215.
- Bills, G. F., Platas, G., Fillola, A., Jiménez, M. R., Collado, J., Vicente, F., Martín, J., González, A., Bur-258 Zimmermann, J., Tormo, J. R. and Peláez, F. (2008). Enhancement of antibiotic and secondary metabolite detection from filamentous fungi by growth on nutritional arrays. *Journal of Applied Microbiology* **104**(6), 1644-260.
- Bister, B., Bischoff, D., Ströbele, M., Riedlinger, J., Reicke, A., Wolter, F., Bull, A. T., Zähler, H., Fiedler, H. P. and Süßmuth, R. D. (2004). Abyssomicin C - A polycyclic antibiotic from a marine *Verrucosipora* strain as an inhibitor of the p-aminobenzoic acid/tetrahydrofolate biosynthesis pathway. *Angewandte Chemie International Edition* **43**(19), 2574-2576.
- Bode, H. B., Bethe, B., Höfs, R. and Zeeck, A. (2002). Big effects from small changes: Possible ways to explore nature's chemical diversity. *ChemBioChem* **3**(7), 619-627.
- Bundale, S., Begde, D., Nashikkar, N., Kadam, T. and Upadhyay, A. (2015). Optimization of culture

- conditions for production of bioactive metabolites by *Streptomyces* spp. isolated from soil. *Advances in Microbiology* **5(5)**, 441-451.
- Chawawisit, K., Bhoopong, P., Phupong, W. and Lertcanawanichakul, M. (2015).** Antimicrobial and cytotoxic activities of bioactive compounds produced by *Streptomyces* sp. KB1. *International Journal of Pharmacy and Pharmaceutical Sciences* **7(11)**, 118-122.
- Chen, G., Wang, G.-Y.-S., Li, X., Watres, B. and Davies, J. (2000).** Enhanced production of microbial metabolites in the presence of dimethyl sulfoxide. *The Journal of Antibiotics* **53(10)**, 1145-1153.
- Feling, R. H., Buchanan, G. O., Mincer, T. J., Kauffman, C. A., Jensen, P. R. and Fenical, W. (2003).** Salinosporamide A: A highly cytotoxic proteasome inhibitor from a novel microbial source, a marine bacterium of the new genus *Salinospora*. *Angewandte Chemie International Edition* **42(3)**, 355-357.
- French, G. L. (2006).** Bactericidal agents in the treatment of MRSA infections - The potential role of daptomycin. *Journal of Antimicrobial Chemotherapy* **58(6)**, 1107-1117.
- Giardina, A., Alduina, R., Gallo, G., Monciardini, P., Sosio, M. and Puglia, A. M. (2014).** Inorganic phosphate is a trigger factor for *Microbispora* sp. ATCC-PTA-5024 growth and NAI-107 production. *Microbial Cell Factories* **13(1)**, 133.
- Hewage, R. T., Aree, T., Mahidol, C., Ruchirawat, S. and Kittakoop, P. (2014).** One strain-many compounds (OSMAC) method for production of polyketides, azaphilones, and an isochromanone using the endophytic fungus *Dothideomycete* sp. *Phytochemistry* **108**, 87-94.
- Hobbs, G., Frazer, C. M., Gardner, D. C. J., Flett, F. and Oliver, S. G. (1990).** Pigmented antibiotic production by *Streptomyces coelicolor* A3(2): Kinetics and the influence of nutrients. *Journal of General Microbiology* **136(11)**, 2291-2296.
- Huang, J., Lu, C., Qian, X., Huang, Y., Zheng, Z. and Shen, Y. (2011).** Effect of salinity on the growth, biological activity and secondary metabolites of some marine fungi. *Acta Oceanologica Sinica* **30(3)**, 118-123.
- James, P. D., Edwards, C. and Dawson, M. (1991).** The effects of temperature, pH and growth rate on secondary metabolism in *Streptomyces thermoviolaceus* grown in a chemostat. *Journal of General Microbiology* **137(7)**, 1715-1720.
- Juboi, H., Hwang, S. S., Yeo, T. C. and Nissom, P. M. (2019).** Antimicrobial activities of *Plantactinospira* sp. KBS50, a rare marine-derived actinomycete from the coastal area of Sarawak. *Malaysian Journal of Microbiology* **15(2)**, 111-119.
- Kawai, K., Wang, G., Okamoto, S. and Ochi, K. (2007).** The rare earth, scandium, causes antibiotic overproduction in *Streptomyces* spp. *FEMS Microbiology Letters* **274(2)**, 311-315.
- Lam, K. S. (2006).** Discovery of novel metabolites from marine actinomycetes. *Current Opinion in Microbiology* **9(3)**, 245-251.
- Lounès, A., Lebrihi, A., Benslimane, C., Lefebvre, G. and Germain, P. (1996).** Regulation of spiramycin synthesis in *Streptomyces ambofaciens*: Effects of glucose and inorganic phosphate. *Applied Microbiology and Biotechnology* **45(1-2)**, 204-211.
- Luti, K. J. K. and Mavituna, F. (2011).** Elicitation of *Streptomyces coelicolor* with dead cells of *Bacillus subtilis* and *Staphylococcus aureus* in a bioreactor increases production of undecylprodigiosin. *Applied Microbiology and Biotechnology* **90(2)**, 461-466.
- Luti, K. J. K. and Yonis, R. W. (2013).** Elicitation of *Pseudomonas aeruginosa* with live and dead microbial cells enhances phenazine production. *Romanian Biotechnological Letters* **18(6)**, 7169-7178.
- Martín, J. F. (2004).** Phosphate control of the biosynthesis of antibiotics and other secondary metabolites is mediated by the PhoR-PhoP system: An unfinished story. *Journal of Bacteriology* **186(16)**, 5197-5201.
- Martín, J. F., Liras, P. and Demain, A. L. (1977).** Inhibition by phosphate of the activity of candicidin synthases. *FEMS Microbiology Letters* **2(3)**, 173-176.
- Ng, Y. K., Hodson, M. P., Hewavitharana, A. K., Bose, U., Shaw, P. N. and Fuerst, J. A. (2014).** Effects of salinity on antibiotic production in sponge derived *Salinispora* actinobacteria. *Journal of Applied Microbiology* **117(1)**, 109-125.
- Ochi, K. (1987).** Metabolic initiation of differentiation and secondary metabolism by *Streptomyces griseus*: Significance of the stringent response (ppGpp) and GTP content in relation to a factor. *Journal of Bacteriology* **169(8)**, 3608-3616.
- Rigali, S., Titgemeyer, F., Barends, S., Mulder, S., Thomae, A. W., Hopwood, D. A. and van Wezel, G. P. (2008).** Feast or famine: The global regulator DasR links nutrient stress to antibiotic production by *Streptomyces*. *EMBO Reports* **9(7)**, 670-675.
- Ripa, F. A., Nikkon, F., Zaman, S. and Khondkar, P. (2009).** Optimal conditions for antimicrobial metabolites production from a new *Streptomyces* sp. RUPA-08PR isolated from Bangladeshi soil. *Mycobiology* **37(3)**, 211.
- Solanki, R., Khanna, M. and Lal, R. (2008).** Bioactive compounds from marine actinomycetes. *Indian Journal of Microbiology* **48(4)**, 410-431.
- Tanaka, Y., Hosaka, T. and Ochi, K. (2010).** Rare earth elements activate the secondary metabolite-biosynthetic gene clusters in *Streptomyces coelicolor* A3(2). *The Journal of Antibiotics* **63(8)**, 477-481.
- Wang, Y., Lu, Z., Sun, K. and Zhu, W. (2011).** Effects of high salt stress on secondary metabolite production in the marine-derived fungus *Spicaria elegans*. *Marine Drugs* **9(4)**, 535-542.
- Wang, D., Yuan, J., Gu, S. and Shi, Q. (2013).** Influence of fungal elicitors on biosynthesis of natamycin by *Streptomyces natalensis* HW-2. *Applied Microbiology and Biotechnology* **97(12)**, 5527-5534.