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ELICITATION OF INDUCED POLYKETIDE COMPOUNDS FROM A CO-CULTURE BETWEEN *Streptomyces* sp. STRAIN SUK10 AND *Fusarium* sp. AND THEIR ANTIBACTERIAL ACTIVITIES

(Elisitasi Sebatian Poliketida Teraruh daripada Satu Kultur-bersama di antara *Streptomyces* sp. Strain SUK10 dan *Fusarium* sp. dan Aktiviti Antibakteria)

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Abstract

Endophytes including bacteria and fungi produce an array of biologically active secondary metabolites. Different approaches have been applied in order to increase the probability production of new metabolites including mimicking the environment, media and the microbes. However, the use of single culture usually re-produces known compounds with known bioactivities. Therefore, co-culturing between *Streptomyces* sp. strain SUK10 and *Fusarium* sp. in the same media at different growth stages leads to direct interaction which may trigger the expression of "silent" biosynthetic pathway to produce novel secondary metabolites. In this study, we elicited the production of the unknown secondary metabolites from co-culture extracts by using high resolution liquid chromatography-mass spectrometry, while data was processed by utilizing the quantitative expression analysis software MZmine 2.40.1 and SIMCA P+ 15.0 coupled with macro analysis and supported with DNP database for dereplication studies. The results showed that only the extract from co-culture of F7S15 showed enhances antibacterial activity on the Gram-positive bacteria with minimum inhibition concentration (MIC) values of 5 mg/mL and 10 mg/mL against *Micrococcus* sp. and *Staphylococcus aureus*, respectively, compared with their independent and other co-culture extracts were non-active. However, all extracts were non-active on the Gram-negative bacteria. Our preliminary results showed that the potential of co-culture method leading the production of novel metabolites which could be explored for future antibacterial agents.

Keywords: co-culture, metabolomics, liquid chromatography-mass spectrometry, multivariate analysis, dereplication

Abstrak

Endofit termasuk bakteria dan fungus menghasilkan satu tatasusunan metabolit sekunder yang mempunyai keaktifan biologi. Pelbagai pendekatan telah digunakan bagi tujuan meningkatkan kebarangkalian penghasilan metabolit baru termasuk mengajuk persekitaran, media dan mikrob. Walaubagaimanapun, penggunaan kultur tunggal selalumya menghasilkan semula sebatian dengan bioaktiviti yang telah diketahui. Oleh itu, satu pengkulturan bersama di antara *Streptomyces* sp. strain SUK10 dan *Fusarium* sp. di dalam media yang sama di peringkat pertumbuhan yang berbeza, memacu kepada interaksi yang mungkin pencetus kepada ungkapan laluan biosintetik senyap untuk menghasilkan metabolit sekunder baru. Dalam kajian ini, kami telah memperolehi penghasilan metabolit sekunder yang belum diketahui daripada ekstrak kultur bersama dengan menggunakan kromatografi cecair-spektrometri jisim resolusi tinggi, sementara itu data telah dianalisis menggunakan perisian analisis ungkapan kuantitatif MZmine 2.40.1 dan SIMCA P+ 15.0 beserta analisis makro dan disokong oleh pengkalan data DNP bagi kajian dereplikasi. Keputusan telah menunjukkan bahawa hanya ekstrak daripada kultur bersama F7S15 telah menunjukkan peningkatan aktiviti antibakteria ke atas bakteria Gram-positif dengan nilai minimum kepekatan perencatan (MIC) 5 mg/mL dan 10 mg/mL melawan *Micrococcus* sp. dan *Staphylococcus aureus*, masing-masing, berbanding ekstrak kutur bebas dan kultur bersama yang lain adalah tidak aktif. Walaubagaimanapun, semua ekstrak tidak aktif ke atas bakteria Gram-negatif. Keputusan awal kajian kami telah menunjukkan potensi kaedah kultur bersama memacu penghasilan metabolit baru yang boleh diterokai bagi agen antibakteria masa hadapan.

Kata kunci: kultur bersama, metabolomik, kromatografi cecair-spektrometri jisim, analisis multivariat, dereplikasi

Introduction

Natural products have been rich sources of therapeutic agents as they inspire the advancement of synthetic methodologies to allow the possibility of making analogues of original lead compounds with improved pharmaceutical properties. For example, the secondary metabolites obtained from endophytic microbes are found to have antimicrobial, antiviral, anticancer, antioxidants, antidiabetic and immunosuppressant properties. The endophytic fungi and bacteria are known to produce these types of natural products that are potent for antibiotics [1]. They live inside the living plant tissues for at least a part of their life without causing any apparent disease symptoms in the host. Various bioactive metabolites are produced by endophytes that are proven to have potential to be anticancer, antioxidant, antifungal, antibacterial, antiviral and anti-insecticidal [2]. The induction of secondary metabolites is triggered by the interaction between the microorganisms, simulating the microbial competition for nutrition and space. Besides, the cultivation of the microbes is sustainable and reproducible in laboratory conditions. A well-known drugs-producer *Streptomyces* sp. belonging to actinobacteria varies in structures and has a great potential to be developed as therapeutic drugs for human use [3]. *Streptomyces* sp. is an aerobic, Gram-positive mycelial bacteria that disperse sporse as a method of reproduction and can develop branching vegetative hyphae in which the mycelium helps in scavenging nutrients from the surroundings [4]. *Streptomyces* sp. is able to synthesise potent secondary metabolites for medical applications such as antibiotics, herbicidal, antiparasitic, antitumor, antifungal and enzyme inhibiting agents as well as having the ability to inhibit various human pathogens including gancidin W [5], divergolides A-D [6], polycyclic anthraquinones [7], cyclic dipeptide [8-10] and xiamycin [11]. Meanwhile, a pathogenic fungus *Fusarium* sp. produces metabolites including naphthoquinones [12, 13], pyrones [14-16] and indole-acetic acids [17], naphthalenone derivatives [18] and aminobenzamide [19].

However, the discovery of new potential bioactive metabolites from independent cultures is challenging due to re-isolation of the known compounds with the same reported bioactivity. The re-occurrence of the known metabolites from an independent culture either of *Streptomyces* sp. SUK10 or *Fusarium* sp. may lead to similar bioactivities. Therefore, a co-culture method is introduced to challenge different strains instead of

individual strains. A co-culture is a process of culturing of two or more different microorganisms that mimic the complex microbial habitat in nature where they co-exist, leading to competition among them due to limited source and antagonism. Microbial community is a complex and dynamically changing consortium in which metabolic interactions between microbial species take place. The interaction between microbial communities often involves exchange of molecules for nutritional purposes that may benefit one or both species, which is known as symbiosis [20]. The co-cultures of different microbes in comparison to mono-cultures are increasingly being used in microbial natural product research as the interaction of two different microbes can increase the availability of existing natural products or may induce the expression of silent biosynthetic pathways resulting in new metabolites that are useful in the medical field [21].

A co-cultivation between *Streptomyces* sp. SUK10 and *Fusarium* sp. at different growth stages was carried out. Present study, the metabolomics approach was used to predict and identify potential novel bioactive components from the crude extracts, leading to the rapid and high-throughput assessment of metabolites. Metabolite profiling of the active metabolites in crude extracts of natural sources was supported by dereplication in which the novel compounds from the active groups were differentiated from known compounds that have been studied previously [22]. The dereplication method is a process for screening the known metabolites from the crude extracts before further scale-up or isolation work is undertaken, in order to avoid repetitive work.

High-resolution electrospray ionisation-liquid chromatography-mass spectrometry (HRESI-LCMS) data from both positive and negative ionisation modes were subjected to multivariate statistical analysis including unsupervised principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA) to establish the optimal position of the discriminating plane, which would best separate classes. The high-resolution mass spectral data generated predicted molecular formulas used for dereplication of the secondary metabolites found in the crude extracts. Later in the final step of the metabolomics approach, the selected unique biomarkers were interpreted to putatively identify the novel metabolite using databases like Dictionary of Natural Products (DNP). The ultimate aim of this study was to fast track the identification of the metabolites from the co-culture extracts, which can lead to the decision-making of the optimum parameter for up-scaling of targeted metabolites responsible for biological activities. In future, the targeted metabolites will be isolated and absolute elucidation and identification of the molecule structures will be achieved using one and two dimensional-nuclear magnetic resonance (NMR) and high-resolution mass spectrometry (MS).

Materials and Methods

Preparation of microbial samples

A stock culture of *Streptomyces* sp. strain SUK10 was obtained from the Novel Antibiotics Laboratory, Programme of Biomedical Sciences, Faculty of Health Sciences, Universiti Kebangsaan Malaysia (UKM), Kuala Lumpur, while *Fusarium* sp. was obtained from the culture collection of Fungus Laboratory, Central Laboratory, Universiti Malaysia Terengganu (UMT). *Streptomyces* sp. SUK10 was originally isolated from the barks of *Shorea ovalis* [5] and *Fusarium* sp. was isolated from the roots of *Avicennia lanata* collected from Setiu Wetlands, Terengganu [13]. A 5 mm x 5 mm diameter plug containing *Fusarium* sp. mycelium was cut from the edge of the colony and placed in the middle of a new malt agar plate to establish a fresh and pure colony. The plates were incubated at 27 ± 2 °C (Vindon Scientific Ltd., UK) for 7-15 days. Meanwhile, *Streptomyces* sp. SUK10 was streaked onto fresh ISP2 agar plates and incubated at room temperature for 7-15 days.

Independent cultures

The International Streptomyces Project (ISP) medium was prepared by adding 10.0 g of malt extract, 4.0 g of D-(+)-glucose monohydrate, 4.0 g of yeast extract and 1 L of distilled water, vigorously shaken until completely mixed and dissolved. The pH was adjusted ranging at 7.2-7.4 using 10% NaOH or 36.5% HCl, and autoclaved at 121 ℃ for 15 minutes. The independent

cultures of *Streptomyces* sp. strain SUK10 and *Fusarium* sp. were achieved in which each active growing strain on the agar plate was cut into small cubes and transferred separately into seven 500 mL Erlenmeyer flasks containing 100 mL of ISP medium. The cultures were incubated in an orbital shaker and shaken at 150 rpm and 28 °C for 7 and 15 days (seven replicates). The metabolites were extracted twice with equal volumes of ethyl acetate, homogenised and filtered. The organic layer was discarded into a 500 mL round bottom flask using liquid-liquid extraction technique to obtain an organic solvent that was then concentrated under vacuum using a rotary evaporator (Büchi, Switzerland) to afford crude extracts ± 10.0 mg. The crude extracts were kept at 4 °C prior to analysis.

Co-cultivation between *Streptomyces* sp. strain SUK10 and *Fusarium* sp.

The co-cultivation between *Streptomyces* sp. strain SUK10 and *Fusarium* sp. was achieved including sets FS7, FS15, F7S15, and S7F15. The co-cultivation of sets FS7 and FS15 were obtained by mixing the agar cubes containing active growing *Streptomyces* sp. strain SUK10 and *Fusarium* sp. at initial incubation in the ISP broth. The incubation of co-cultivation of set FS7 was continued until day 7, while the co-cultivation of set FS15 was incubated until day 15. The co-cultivation of set F7S15 was obtained in which the agar plugs containing *Streptomyces* sp. strain SUK10 were introduced into the *Fusarium* sp. culture after 7 days of incubation, and the incubation was continued until day 15. The co-cultivation of set S7F15 was obtained in which the agar plugs containing *Fusarium* sp. mycelia were introduced into the *Streptomyces* sp. strain SUK10 culture after 7 days of incubation, and the incubation was also continued until day 15. A 100 mL of ISP broth was used for each culture and the incubation was done in an orbital shaker at 150 rpm and 28 °C (seven replicates). The metabolites were extracted twice with equal volumes of ethyl acetate, homogenised, and filtered. The organic layer was discarded into a 500 mL round bottom flask using liquid-liquid extraction technique to obtain an organic solvent that was then concentrated under vacuum using a rotary evaporator (Büchi, Switzerland) to afford crude extracts ± 15.0 mg. The crude extracts were kept at 4 °C prior to analysis.

Dereplication using HRESI-LCMS

The dereplication strategy on the crude extracts was performed using HRESI-LCMS and processed with the MZmine software 2.40.1, an in-house macro coupled with the Dictionary of Natural Products (DNP) 2015 and SIMCA P+ 15.0 (Umetrics AB, Umeå, Sweden). The procedure and programme for HRESI-LCMS was set up as described here [23]. 1 mg/mL of each extract was dissolved in methanol and analysed on an Accela HPLC (Thermo Scientific, UK) coupled with a UV detector at 280 and 360 nm and an Exactive-Orbitrap high-resolution mass spectrometer (Thermo Scientific, UK). A methanol blank was also analysed. The mass spectral data was processed using the procedure by MacIntyre et al. [23] which was established in the Natural Products Metabolomics Group Laboratory at Strathclyde Institute of Pharmacy and Biomedical Sciences (SIPBS) as described here [13, 23]. The LC-MS chromatograms and spectra were viewed using Thermo Xcalibur 2.1 or MZmine 2.40.1.

Antibacterial activity using agar disk-diffusion

An antibacterial activity of the extracts from independent and co-cultures was conducted using the agar disk-diffusion method as described here [24]. Each extract was diluted with 2-fold dilutions in dimethyl sulfoxide (DMSO) with concentration values of 0.156 mg/mL, 0.313 mg/mL, 0.625 mg/mL, 1.25 mg/mL, 2.50 mg/mL, 5.00 mg/mL and 10 mg/mL. The inhibition zone diameter (IZD) was measured to the nearest millimetres. Minimum inhibitory concentration (MIC) was taken as the lowest concentration of the extracts that shows the inhibition zone. MIC values of the extracts against the Gram-positive and Gram-negative bacteria were determined by averaging the results of three independent assays. Six strains of Gram-positive bacteria namely *Staphylococcus aureus*, *Bacillus cereus* and *Micrococcus* sp. and Gram-negative bacteria - *Vibrio cholera*, *Salmonella* sp. and *Escherichia coli* from glycerol stock were obtained from the Microbiology Laboratory, Institute of Marine Biotechnology, UMT.

Statistical analysis

All data were presented as mean ± SD and statistically analysed with One-Way ANOVA in the comparison between selected fractions using statistical analyses software PRISM ver. 5. Data were significantly different at $p < 0.05$.

Results and Discussion

Dereplication study on crude extracts

Each extract was screened on antibacterial activity at different concentrations: 10 mg/mL to 0 mg/mL. The inhibition zone diameter (IZD) was measured to the nearest millimetres. The results showed that the minimum inhibition concentration (MIC) values of the extracts against the Gram-positive and Gram-negative bacteria were determined by averaging the results of three independent assays (Table 1). The co-culture extract F7S15 revealed marginal antibacterial activity on *Micrococcus* sp. and *S. aureus* with MIC values of 5 mg/mL and 10 mg/mL, respectively, as compared to the non-active independent and other co-culture extracts. Meanwhile, the antibacterial activity on the Gram-negative bacteria showed that all extracts were non-active in this screening test, thereby supporting further investigation of the biologically-active compounds from the co-cultivation extracts.

Total ion chromatogram for the extract of co-cultivation between *Streptomyces* sp. strain SUK10 and *Fusarium* sp. F7S15 (Figure 1) showed the distribution of known and unknown compounds present in the extract (Table 2). The dereplication studies revealed that the co-culture extract F7S15 possessed certain types of compounds including macrocylic aromatic compounds such as macrolide- oligomycin A and cyclic peptolides-icosalide A1 and A3, which have also been previously isolated from different *Streptomyces* and fungi species, respectively (Table 2). The values and predicted formulas of unknown compounds are also shown (highlighted rows). The extracts of co-cultivation between *Streptomyces* sp. strain SUK10 and *Fusarium* sp. as well as the independent cultures were preliminarily screened on antibacterial activity and subjected to HRESI-LCMS prior to multivariate analysis. A dereplication study was performed to obtain the metabolomic profile of each extract.

The relationship between the occurrence of the metabolites in the independent and co-cultivation extracts and their bioactivity on antibacterial activity were evaluated through multivariate analysis. The PCA scores plot showed strong separation of the extracts (Figure 2a). There was clear separation between the co-culture extract of F7S15 from other extracts. The PCA loadings plot (Figure 2b) predicted the metabolites in the extracts. Meanwhile, a supervised multivariate OPLS-DA score plot analysis (Figure 2c) exhibited a distinctive separation between the active extract F7S15, while other extracts were the non-active group. These predicted antibacterial active compounds by MVA were indicated with their MZmine IDs as listed in Table 2. Peak IDs used in this table corresponded to those designated for the chromatogram as shown in Figure 1.

Table 1. Antibacterial activity for independent and co-culture extracts (calculated as mean value percentage viability) at different concentrations ranging at 10 mg/mL to 0 mg/mL. Highlighted rows showed enhanced antibacterial activity

Extracts	**Concentration of Crude Extract (mg/mL)**						
	***Micrococcus* sp.**						
	0.156	0.313	0.625	1.25	2.50	5.00	10.00
	Diameter of inhibition (mm)						
F7	n.d	n.d	n.d	n.d	n.d	n.d	n.d
F15	n.d	n.d	n.d	n.d	n.d	n.d	n.d
S7	n.d	n.d	n.d	n.d	n.d	n.d	n.d
S15	n.d	n.d	n.d	n.d	n.d	n.d	
FS7	n.d	n.d	n.d	n.d	n.d	n.d	n.d
FS15	n.d	n.d	n.d	n.d	n.d	n.d	n.d
F7S15	n.d	n.d	n.d	n.d	n.d	7.5 ± 0.6	8.7 ± 0.5
S7F15	n.d	n.d	n.d	n.d	n.d	n.d	8.0 ± 0
Oxytetracycline (30 µg)	35.7 ± 0.2						
	Staphylococcus aureus						
F7	n.d	n.d	n.d	n.d	n.d	n.d	n.d
F15	n.d	n.d	n.d	n.d	n.d	n.d	n.d
S7	n.d	n.d	n.d	n.d	n.d	n.d	n.d
S15	n.d	n.d	n.d	n.d	n.d	n.d	n.d
FS7	n.d	n.d	n.d	n.d	n.d	n.d	n.d
FS15	n.d	n.d	n.d	n.d	n.d	n.d	n.d
F7S15	n.d	n.d	n.d	n.d	n.d	n.d	n.d
S7F15	n.d	n.d	n.d	n.d	n.d	n.d	8.0 ± 0
Oxytetracycline (30 µg)	26.0 ± 1.4						

*Legend: extracts F7 = mono-culture *Fusarium* sp. incubation day 7; F15 = mono-culture *Fusarium* sp. incubation day 15; S7 = mono-culture *Streptomyces* sp. SUK10 incubation day 7; S15 = mono-culture *Streptomyces* sp. SUK10 incubation day 15; F7S15 = co-cultivation between *Fusarium* sp. and *Streptomyces* sp. SUK10 in which *Streptomyces* sp. SUK10 was added into *Fusarium* sp. culture after 7 days incubation and the incubation was continued until day 15; S7F15 = co-cultivation between *Fusarium* sp. and *Streptomyces* sp. SUK10 in which *Fusarium* sp. was added into *Streptomyces* sp. SUK10 culture after 7 days incubation and the incubation was continued until day 15; n.d: not detected; n=3.

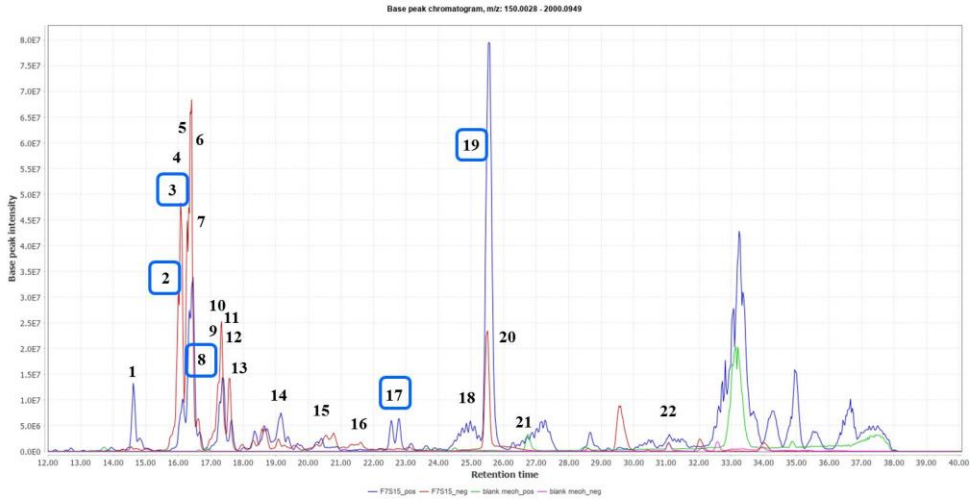


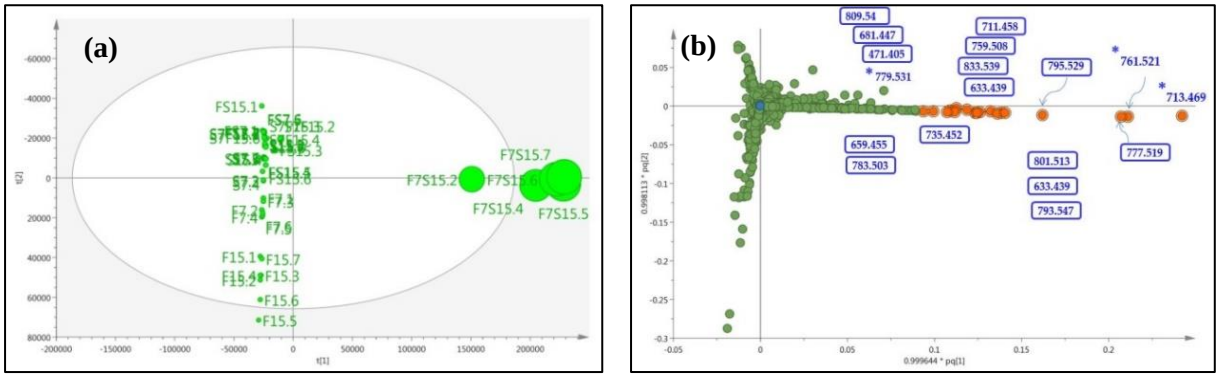
Figure 1. Total ion chromatogram for the extract of co-cultivation between *Streptomyces* sp. strain SUK10 and *Fusarium* sp. F7S15 (blue and red represent positive and negative ionization modes of F7S1 extract, respectively; green and pink represent positive and negative ionization modes of blank methanol, respectively). Dereplication of numbered peaks is shown on Table 1. Boxed in blue are the metabolites putatively identified using DNP database.

Table 2. List of compounds indicated on the total ion chromatogram for the extract of co-cultivation between *Streptomyces* sp. strain SUK10 and *Fusarium* sp. F7S15 that were putatively identified using DNP database. Peak IDs used in this table correspond to those designated for the chromatogram shown on Figure 1. Highlighted rows represent the unknown compounds as indicated with their MZmine IDs.

Peak ID	ESI Modes/ MZmine ID	Rt (min)	MS (*m/z*)	Molecular Weight	Chemical Formula	Chemical Name	Tolerance (ppm)	Sources	Peak Area
1	P_22521	14.58	274.2015	273.1943	$C_{14}H_{27}NO_4$	No hits			1.27E+08
2	N_2411	16.03	795.5293	796.5361	$C_{41}H_{80}O_{12}S$	bovine seminolipid	-1.1324	bovine spermatozoa	6.10E+08
3	P_21220	16.04	779.5308	778.5235	$C_{44}H_{74}O_{11}$	narasin D	0.5015	*Strep. aureofaciens*	2.75E+08
4	N_2409	16.19	633.4391	634.4464	$C_{30}H_{64}N_6O_4P_2$	No hits			3.81E+08
	N_2441	16.33	777.5188	778.5258	$CH_5NO_{15}P_4S_{12}$	No hits			8.40E+08
6	P_21679	16.35	801.5129	800.5056	$C_{10}N_3O_6PS_{16}$	No hits			3.80E+08
7	N_2435	16.36	759.5081	760.5154	$C_6HN_5O_3P_6S_{12}$	No hits			3.00E+08
8	P_21649	16.45	761.5204	760.5135	$C_{44}H_{72}O_{10}$	oligomycin A; 26-Demethyl, 12-deoxy	1.2529	*S. aureofaciens*	8.62E+08
9	N_2422	17.26	659.4550	660.4622	$C_{40}H_{70}OP_2S$	No hits			1.89E+08
10	P_21450	17.28	793.5469	792.5396	$C_2H_{18}NO_3P_{15}S_7$	No hits			4.10E+08
11	P_22522	17.32	833.5393	832.5320	$C_5H_9N_3O_8P_{14}S_5$	No hits			3.36E+08
12	N_2495	17.33	809.5449	810.5525	$C_2H_2N_3O_{19}P_9S_5$	No hits			2.12E+08
13	N_2496	17.58	777.5192	778.5264	$C_{38}H_{72}N_{10}O_3P_2$	No hits			1.69E+08

Table 2 (cont'd). List of compounds indicated on the total ion chromatogram for the extract of co-cultivation between *Streptomyces* sp. strain SUK10 and *Fusarium* sp. F7S15 that were putatively identified using DNP database. Peak IDs used in this table correspond to those designated for the chromatogram shown on Figure 1. Highlighted rows represent the unknown compounds as indicated with their MZmine IDs.

Peak ID	ESI Modes/ MZmine ID	Rt (min)	MS (*m/z*)	Molecular Weight	Chemical Formula	Chemical Name	Tolerance (ppm)	Sources	Peak Area
14	P_22523	19.10	783.5023	782.4957	$C_2H_4N_5O_5P_3S_{16}$	No hits			1.90E+08
15	N_2511	20.79	1556.0436	1557.0510	$C_{46}H_{88}N_9O_2P_9S_{15}$	No hits			1.11E+08
16	N_2549	21.62	1589.0730	1590.0803	$C_{41}H_{74}N_{14}O_{20}P_4S_{12}$	No hits			4.41E+07
17	P_22525	22.74	685.4392	684.4319	$C_{34}H_{60}N_4O_{10}$	icosalide A3	1.3669	fungus OSI 74159	9.07E+07
18	N_2408	25.38	711.4578	712.4649	$C_{38}H_{56}N_{12}O_2$	No hits			4.31E+08
19	P_21186	25.48	713.4698	712.4622	$C_{36}H_{64}N_4O_{10}$	icosalide A1	-0.1214	*Aureobasidium* sp. fungus OSI 59166	1.27E+09
20	N_2410	25.46	681.4474	682.4547	$C_{34}H_{63}N_6O_6P$	No hits			2.87E+08
21	P_21721	26.85	471.4048	470.3975	$C_{23}H_{51}N_8P$	No hits			8.99E+08
22	P_23269	31.03	523.3828	522.3755	$C_{27}H_{54}O_9$	1-Heptacosene-4,6,8,10,12,14,16, 18,24-nonol	-2.3912		4.07E+05



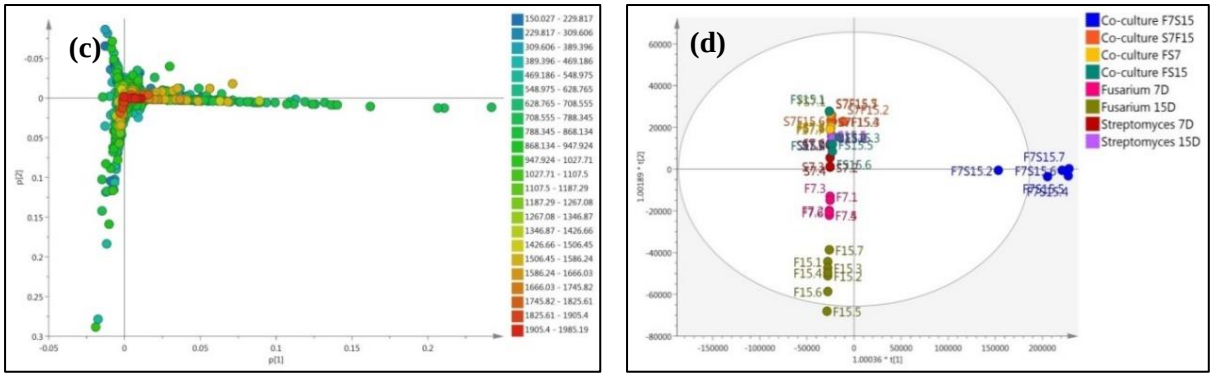
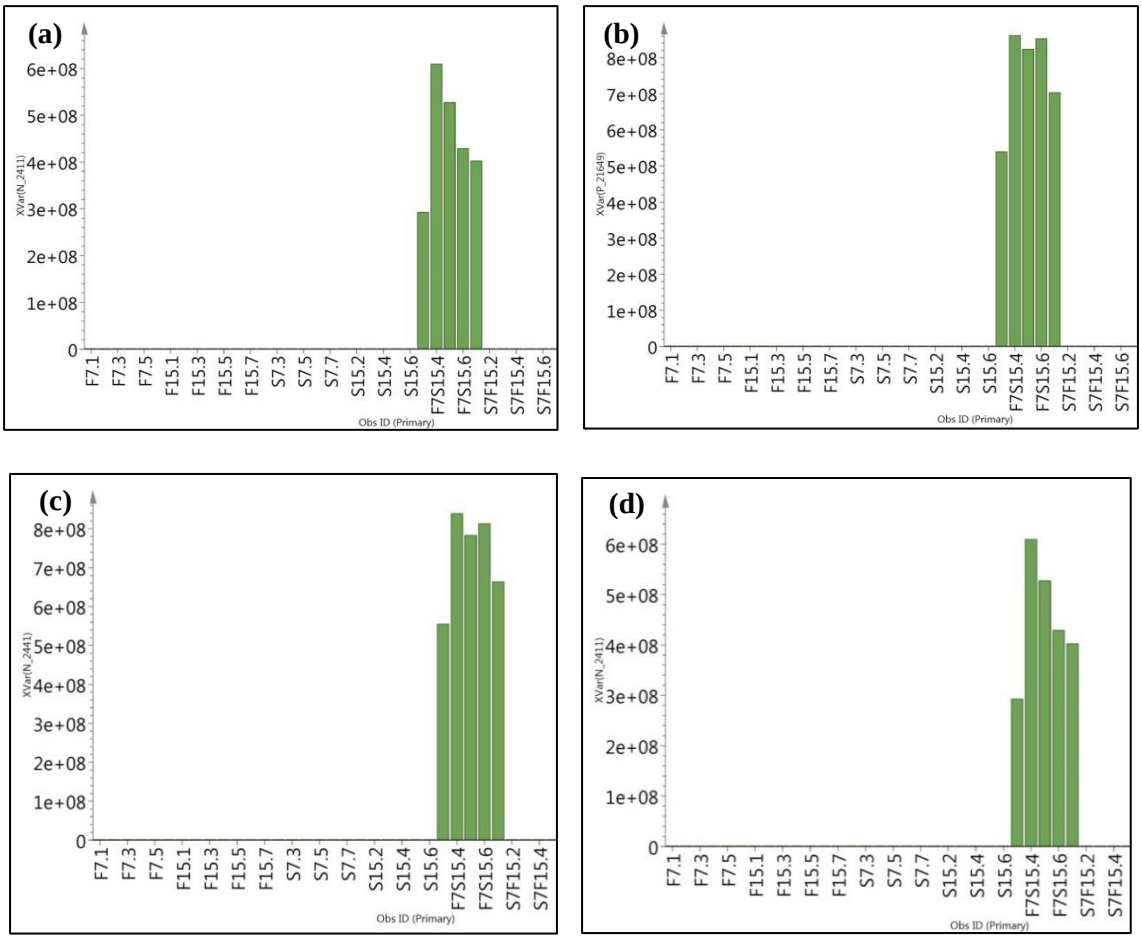


Figure 2. (a) Unsupervised PCA scores plot of mono- and co-cultivation extracts showed distinctive separation between the datasets. (b) PCA loadings plot showed the discriminating metabolites within the extracts. (c) OPLS-DA scores scatter plot of the extracts ($R^2(Y)$ = 1.00; Q^2 =0.998); Q^2(Y intercept). (d) Supervised OPLS-DA loadings plot showed the discriminating metabolites of the active co-culture extract F7S15 within the extracts. Asterisk specifies the identified metabolites from where the respective active extract F7S15. Legend: set F7.1-F7.7 = mono-culture *Fusarium* sp. incubation day 7; set F15.1-F15.7 = mono-culture *Fusarium* sp. incubation day 15; set S7.1-S7.7 = mono-culture *Streptomyce*s sp. SUK10 incubation day 7; S15.1-S15.7 = mono-culture *Streptomyces* sp. SUK10 incubation day 15; set F7S15.1-F7S15.7 = co-cultivation between *Fusarium* sp. and *Streptomyces* sp. SUK10 in which *Streptomyces* sp. SUK10 was added into *Fusarium* sp. culture after 7 days incubation and the incubation was continued until day 15; set S7F15.1-S7F15.7 = co-cultivation between *Fusarium* sp. and *Streptomyces* sp. SUK10 in which *Fusarium* sp. was added into *Streptomyces* sp. SUK10 culture after 7 days incubation and the incubation was continued until day 15, n=7.

The OPLS-DA loadings plot (Figure 2d) exhibited the discriminating metabolites in the active group F7S15 in which the ion peaks at *m/z* $[M+H]^+$ 713.469, 761.521, and 801.513 as well as *m/z* $[M-H]^-$ 777.519, 795.529, and 633.439 were outliers, indicating the presence of unique chemical profiles in the F7S15 extract. From the DNP database, it was putatively determined that the F7S15 extract contained polyketide and aromatic compounds [25-28] that perhaps contributed towards the antibacterial activity of F7S15. Some of the ion peaks in the F7S15 extract have been dereplicated as presented in Table 1. Characteristic metabolite for co-culture F7S15 extract observed at *m/z* $[M+H]^+$ 713.469 were putatively identified as icosalide A1, which has been identified from an *Aureobasidium* sp. fungus OSI 59166 [26, 29]. The ion peaks at *m/z* $[M+H]^+$ 761.521 and 779.531 were putatively identified as oligomycin A; 26-demethyl, 12-deoxy, and narasin D, respectively. Both compounds were isolated from *Streptomyces aureofaciens* [30, 31]. The *m/z* $[M-H]^-$ 795.529 was putatively identified as bovine seminolipid, which has been isolated from bovine spermatozoa [32]. Meanwhile, the ion peaks at *m/z* $[M+H]^+$ 801.513 and *m/z* $[M-H]^-$ 777.519 and 633.439 were the unidentified metabolites that were only produced in the active co-culture extract F7S15. The relative occurrence of the targeted putatively identified and unknown metabolites in the bioactive extract F7S15 is shown in Figure 3. Based on the predicted chemical formula of the unknown compounds, they may consist of at least one nitrogen atom that contributes to the presence of macrocyclic polyketide-membered rings. Isolation of polyketide compounds from *Streptomyces* sp. under normal condition is widely known [5, 33-35], and perhaps the discovery of most antibiotics in the market are from this species. However, the world is facing a multidrug resistant against bacteria. Thus, dual cultivation of different strains used in our preliminary study may enlighten the opportunity to discover more potential new drug candidates that could be further

explored. The optimization works in this study showed that the co-cultivation of F7S15 produced promising metabolites with enhanced antibacterial activity, which can be further scaled up to obtain larger volume extracts for isolation and identification of target metabolites that were absent in their parent culture. Our future study will focus on the identification of molecule structures of the unknown metabolites by using tandem mass spectrometry (MS/MS) analysis and one/two-dimensional nuclear magnetic resonance (1D/2D NMR) spectroscopy.



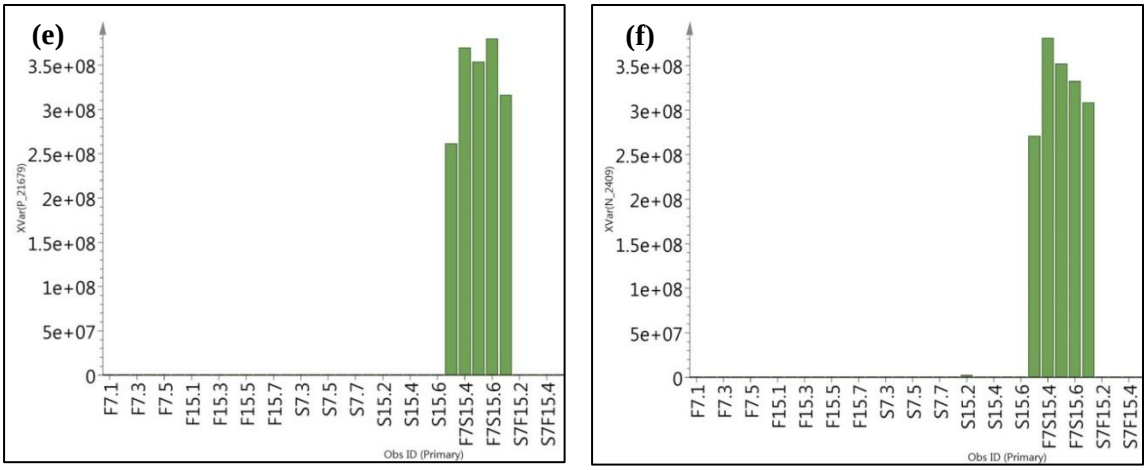


Figure 3. Relative abundance of target metabolites (a-f) which only present in the active co-culture extract of F7S15. Asterisk specifies the identified metabolites from where the respective active extract F7S15. (a) P_21186: *m/z* = 713.469, RT = 25.48 min; (b) P_21649: *m/z* = 761.521, RT = 16.45 min; (c) N_2496: *m/z* = 777.519, RT = 17.58 min; (d) N_2411: *m/z* = 795.529, RT = 16.03 min; (E) P_21679: *m/z* = 801.513, RT = 16.35 min; (F) N_2409: *m/z* = 633.439, RT = 16.19 min

Conclusion

The main aims in this study were to establish an optimum parameter to target and pinpoint the bioactive secondary metabolites directly from the extracts that were absent in the independent culture and subsequently imply the same parameter in the scale-up of the co-cultivation. By means of high-resolution liquid chromatography-mass spectrometry, the extracts obtained from mono- and co-cultivation were preliminarily screened for bioactive molecules and analyzed by multivariate analysis such as PCA and OPLS-DA. Dereplication study was used to screen the known metabolites and predict the novelty metabolites from the extracts which were not detected in single cultures, to avoid repetitive work on the same compounds with known bioactivity. Our future study will focus on the scale-up and isolation works of the targeted active metabolites from co-culture extract F7S15. In addition, identification of the molecule structures will be achieved using 1D and 2D-nuclear magnetic resonance (NMR) and high-resolution mass spectrometry (MS).

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