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The used 1H NMR for Screening on *Streptomyces* spp. and Identification of Metabolites Involved in Both Antifungal and Non-antifungal Producer for *Colletotrichum gleosporioides*

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Abstract: *Streptomyces* spp. had been well known for their antimicrobial activities. These activities were due to the presence of certain metabolites in the *Streptomyces* spp. In this study, *Streptomyces* spp. with the ability to produce antimicrobial activity and those without activity were grown in Starch Casein Broth (SCB) for 3 days before the metabolites were extracted using the liquid-liquid partition method. The obtained extracts were then subjected to 1H Nuclear Magnetic Resonance (NMR) analysis to obtain the metabolites presence in both antifungal producing and non-antifungal producing *Streptomyces* spp. From the analysis, it was observed that valine, isoleucine, leucine, asparagine, α-glucose, and fructose were present in antifungal producing *Streptomyces* spp., while only fatty acid and lactic acid were observed for non-antifungal producing *Streptomyces* spp. The use of 1H NMR does not only help in identifying *Streptomyces* spp. with potential antifungal ability but can also help in identifying the *Streptomyces* spp.

Keywords: Metabolites, Antifungal activity, *Streptomyces* spp., *Colletotrichum gleosporioides*, Nuclear Magnetic Resonace

1. Introduction

It is well known that pathogenic phytopathogens will cause severe damages to the agriculture produces and thus reducing the profit for the farmers. Rosa et al. (2022) stated that, infection of *Colletotrichum gleosporioides* a causal agent for anthracnose in chilli fruit will cause the reduction of yield ranging from 20 – 90%. This has greatly reduced the profit for the farmers. The most prominent symptom of a chili fruit infected by anthracnose is, the infected chilli fruit will show a circular concave spot shape with rings of spore on the surface of the fruit (Rosa et al., 2022).

Application of biocontrol microorganisms to control postharvest diseases is considered as an effective option due to high efficiency as well as environmental friendliness (Einloft et al., 2021). *Streptomyces* are considered as potential agents against fungal diseases because of high efficiency of producing functional metabolites (Getha & Vikineswary, 2002). Recently, *Streptomyces* are drawing intensive attentions in controlling postharvest diseases of different fruits. *Streptomyces* sp. MBFA-172 and *Streptomyces* sp. H4 significantly reduced the anthracnose severity of strawberry (Li et al., 2021; Marian et al., 2020). Among all the actinomycetes the different classes of actinomycetes, the genus Streptomyces has been recognized for diverse and novel secondary metabolites (Alam et al.,2022). According to study done, around 100,000 antibiotics (70% to 80% of the total antibiotics) were produced by Streptomyces, accounting for their promising agrochemical and pharmacological applications (Alam et al., 2022). *Streptomyces* spp. was well known to be the most prolific producers of many antibiotics in the world (Quinn et al., 2020). The productions of antibiotics are often triggered by the presence of certain metabolites in the microbes itself (Tangerina *et al*., 2020).

In order to explore Streptomyces as a natural biocontrol agent for the management of *C. gloeosporioides*, we quantified and identified the potential metabolites in Streptomyces using the Nuclear Magnetic Resonance (NMR) method. Nuclear Magnetic Resonance (NMR) method has gain lots of attention in quantifying and identifying the potential metabolites produced by the microbes (Betancur et al., 2020). Application of NMR-based metabolomics methods in screening and quantifying of metabolites from both plants and microbes as well as identification of the

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metabolites has been explored by most researchers (Betancur et al., 2020). Apart from this, application of NMR based metabolomics can also help researchers to identify the optimum culture growth condition for the production of the beneficial compounds (Betancur et al., 2020). Statistical analysis tools such as principal component analysis (PCA), partial least squares regression (PLS-DA) are needed for the analysis of the data obtained from NMR (Debik et al., 2022).

In this study, our aims are to group *Streptomyces* spp. based on the major metabolites present in both antifungal and non-antifungal producing *Streptomyces* spp. and also to identify metabolites involved in both antifungal and non-antifungal activities.

2. Materials and methods

2.1 Isolation and enumeration of actinomycetes

Soil samples were collected 15 cm below the surface of the soil under the canopy of selected plants (coffee, papaya and pineapple) at MARDI Research Station Pontian, Johor which was located at latitude 1°30'U and longitude 103°27'T. The soil suspension was agitated using an orbital shaker at 200 rpm for approximately 1 h. One hundred and fifty microliter of suspension was later pipetted and lawned onto Starch Casien Agar (SCA). The plates were then incubated at 28 ± 2°C for 14 days before selection of emerging actinomycetes. Selected actinomycetes were then sub-cultured onto fresh SCA plates and further incubated for 14 days before the pure colonies were used for the test and kept as stock in 20% (v/v) glycerol.

2.2 Preparation of pathogens test strains

Colletotrichum gloeosporioides was isolated from infected red chili fruits using surface sterilization method. In this method, infected chili fruits were cut at the infected site and dipped into 2% sodium hypochlorite for 2 min. After which, the infected sites were washed with sterile distilled water before being submerged into 75% ethanol for 30 sec. Pieces of cut chilli fruits were then left to dry in laminar flow for about 30 min before being placed onto fresh Potato Dextrose Agar plates (PDA). Plates were then incubated for about 21 days for the emerging of fungal colonies. Emerging fungal colonies were picked and grown on fresh PDA for another 8 days to ensure the purity of the isolates. Molecular identification of the fungus was done using method describe by Ezeonuegbu et. al (2022), before proceeding for the antagonistic screening.

2.3 Primary Screening for antifungal activity

Antifungal test was performed using agar diffusion technique. In this technique, the test pathogens were inoculated on the middle of the petri dish (Fig. 1d), two plugs of the streptomycetes were placed on next to the test pathogen, a plug of PDA (Fig. 1b) which act as negative control and a plug containing cycloheximide (Fig. 1c) as positive control were placed in the petri dish. All the plates were then incubated at 28 ± 2°C for 5 days. Positive results were determined with the formation of clear zones. All tests were conducted in a triplicate manner.

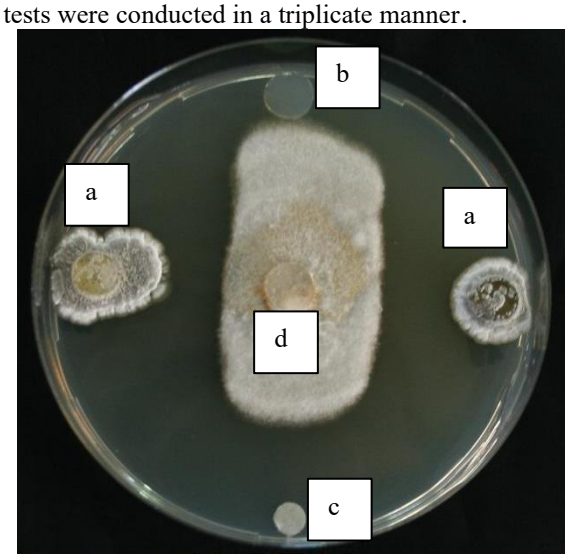

Fig. 1a-d: Plate showing the screening of antagonistic activity [a; Streptomyces test strain, b; PDA (as negative control), c; cycloheximide (as positive control), d; test pathogen (*Colletotrichum gloeosporioides*)].

2.4 Preparation of *Streptomyces* spp. culture

Five *Streptomyces* spp. (Antifungal producer:- S2, AK25 and P1; non-antifungal producer:- AK14 and S3) were selected randomly and grown in Starch Casein Broth (SCB). Each strain was grown in triplicates manner for 3 days at 28 ± 2°C with the rotation of 220 rpm.

2.5 Extraction of extracellular metabolites from *Streptomyces* spp.

After 3 days of incubation, the cultures were extracted using equal volume of ethyl acetate. The liquid-liquid partition method was employed, and the organic layer was then collected using separation funnel. The organic layer was later dried using a rotary evaporator (Rotavapor R-3, BÜCHI, Switzerland). The dried crude extract was then freeze-dried to remove as much water content as possible before subjecting to Nuclear Magnetic Resonance (NMR) analysis (Spina et al., 2021).

2.6 Nuclear Magnetic Resonance analysis of selected *Streptomyces* spp.

Nuclear Magnetic Resonance (NMR) analysis was conducted using Spina et al (2021) method with minor modification. Ten milligrams of each dried crude extract were weighted prior dissolving with 0.6 ml of deuterated acetonitrile containing 0.05% Tetramethylsilane (TMS). The dissolved crude extracts were then loaded into the NMR tubes (Norell). ^{1}H-NMR analyzes were performed for all the 15 samples (triplicates x 5 microbes) using spin 20 Hz, temperature 25°C and scan of 64 which takes around 3 min to complete a single sample. The water peak that appeared was suppressed manually using presaturation command available in the software. Presaturation would take approximately 3 min to complete. The total time needed for a sample would be approximately 6-7 min (including presaturation). The spectrum with suppressed water peak was later saved and ready to be processed using data processing software.

2.7 Data processing and analysis

The spectrums obtained from NMR spectroscopic analysis were processed using Chenomx software before being analyzed using SIMCA P+ Principal Component Analysis (PCA) and Orthogonal Partial Least Square (OPLS). All data was Pareto scale to reduce the effect of noise before PCA and OPLS analysis (Jiang et al., 2022).

3. Results and Discussions

3.1 Isolation, enumeration and antifungal screening of Streptomyces spp.

A total of 110 isolates of *Streptomyces* spp. were isolated from the soil samples collected at MARDI Pontian Research Station. From the total, 44 isolates of *Streptomyces* spp. were found to exhibit antifungal reaction toward *Colletotrichum gloeosporioides* with *Streptomyces* sp. strain S2 to be the most active isolate with the inhibition zone of 15 mm.

3.2 Metabolites differences between each selected *Streptomyces* spp.

From the Principle Component Analysis (PCA) performed, the *Streptomyces* spp. were distinguished according to their metabolites (Figure 2a). From the results obtained, it was observed that *Streptomyces* sp. strain S2 was influenced by Principle Component 2 (PC2) (Figure 2a). Valine (1.04 ppm) and leucine (0.96 ppm) was observed as the most produced amino acid by *Streptomyces* sp. strain S2 compare to the other 4 strains of *Streptomyces* spp. (Figure 2b). It was reported that leucine and valine are presence in antimicrobial peptide and determine the biological activity of these peptides (Wang et al., 2023). These finding may suggest to the results of this study on why *Streptomyces* sp. strain S2 having higher antifungal activity compared to *Streptomyces* sp. strain AK25 and *Streptomyces* sp. strain P1.

It was observed that *Streptomyces* sp. strain S3 and *Streptomyces* sp. strain AK14 were separated by Principal Component 1 (PC1) (Figure 2a). Both non antifungal producers were observed to produce different compounds. *Streptomyces* sp. strain S3 was observed to produce more carboxylic acid group compounds such as fatty acid (1.28 ppm) and lactic acid (1.32 ppm) while *Streptomyces* sp. strain AK14 produced more succinic acid (2.64 ppm) and fructose (4.12 ppm) (Figure 2b).

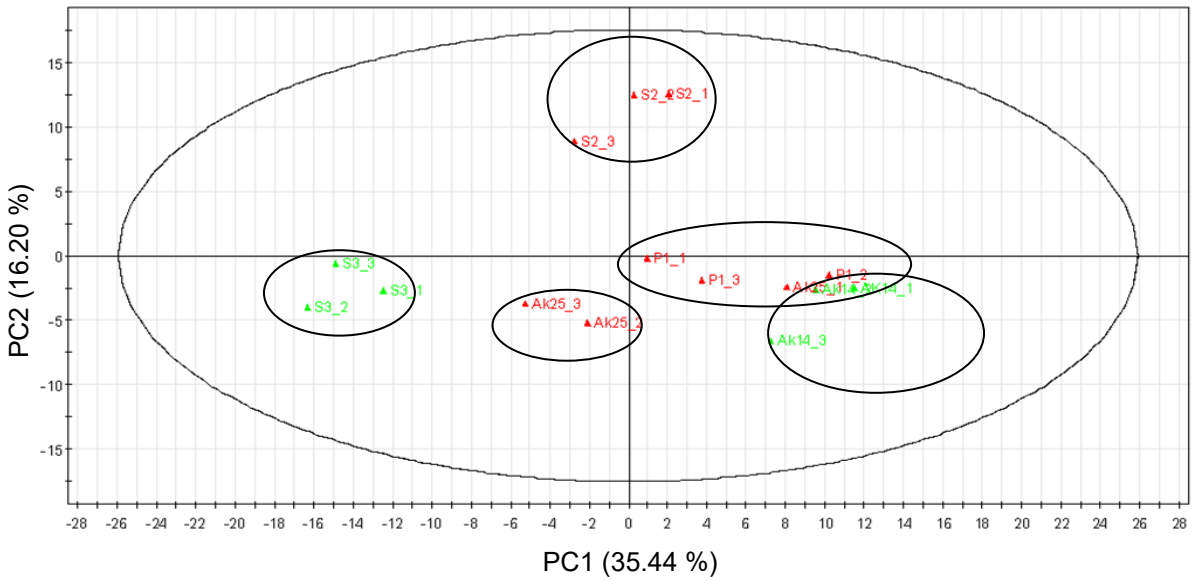

Fig. 2a: PCA score plot for antifungal producing and non-antifungal producing *Streptomyces* spp. (Red marking indicates antifungal producing strains while green marking indicates non-antifungal producing strains).

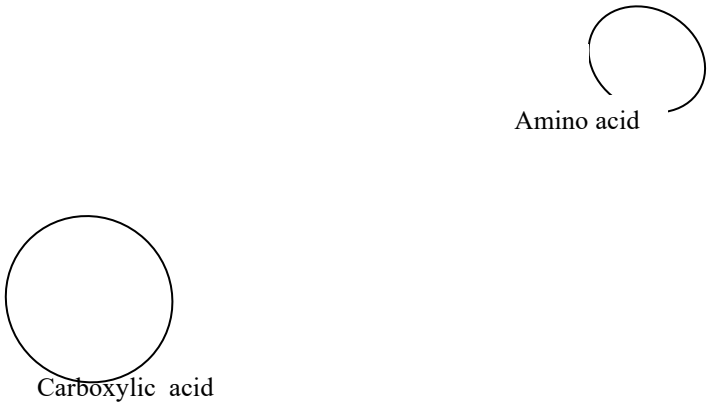

Fig. 2b: PCA loading plot for antifungal producing and non-antifungal producing *Streptomyces* spp.

By using PCA analysis, all the five strains of *Streptomyces* spp. were well separated from each other with a predictive value of 51.6 %. It was observed that all the replicates of each strain were categorized with each other. This proved that by using metabolomic approach, *Streptomyces* strains could be well differentiated from each other.

3.3 Metabolites differences between antifungal producing *Streptomyces* spp. and non antifungal producing *Streptomyces* spp.

Initial principal component analysis (PCA) which is an unsupervised analysis method was used to determine the primary observation of the antifungal producers and non-antifungal producers (Figure 2a). As shown in Figure 2a, the antifungal producers could not be well discriminated from the non-antifungal producers. It was observed that, there was overlapping of clustering as observed between *Streptomyces* sp. strain AK25 and *Streptomyces* sp. strain P1 (antifungal producing strain) with *Streptomyces* sp. strain AK14 (non-antifungal producing strain).

Due to this, a supervised analysis which is known as Orthogonal Partial Least Square analysis (OPLS) was conducted. In the OPLS analysis, samples were well separated according to the antifungal producing and non-antifungal producing *Streptomyces* spp. Indeed from the OPLS analysis it was observed that both categories were well discriminated

(Figure 3a). Both categories separated clearly into two distinct groups with a total predictive value of 40.6 %. It was also observed that the antifungal producing samples are group in the negative value while the non-producing antifungal samples are group in the positive value as observed from the column plot (Figure 3b).

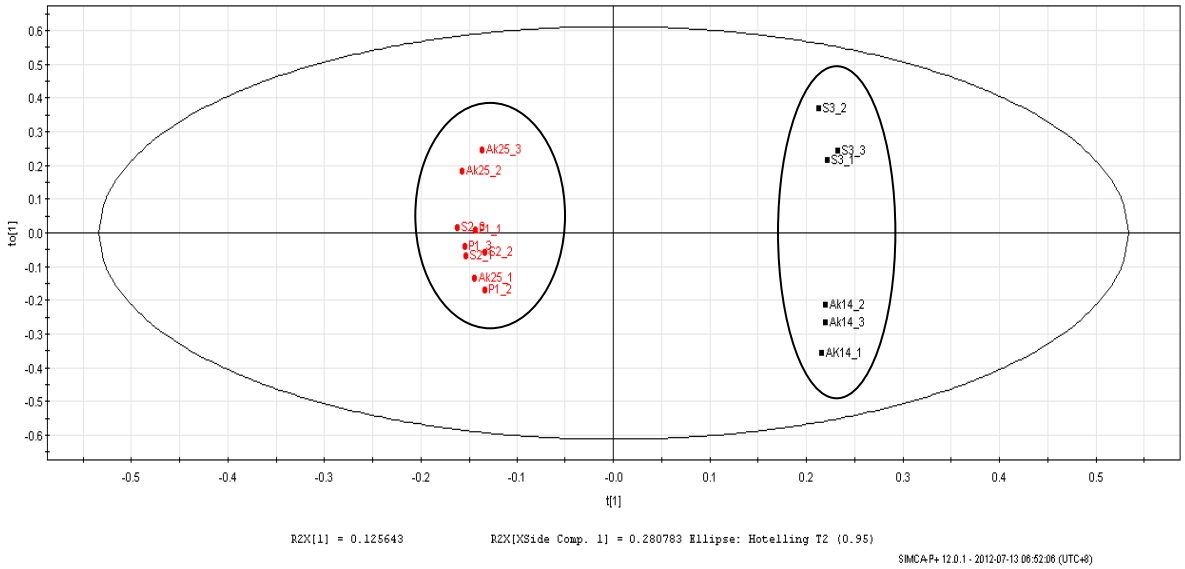

Fig. 3a: OPLS score plot from 1H-NMR results for the antifungal and non-antifungal producing *Streptomyces* spp. (Red marking in the score plot indicate antifungal producing strains while black marking in the score plot indicate non-antifungal producing strains).

The major metabolites that were observed from the column loading plot (Figure 3b) for the non-antifungal producing *Streptomyces* spp. are fatty acid, lactic acids, succinic acid, β -glucose and gallic acid. As for the antifungal producing group, it was observed that the *Streptomyces* spp. produce more of valine, isolueucine, leucine, asparagine, α-glucose and fructose (Figure 3b). Leucine metabolism was known to produce secondary metabolites for microorganisms. In anaerobic bacteria, Leucine metabolism help the accumulation of organic compounds acid which might be useful as antifungal compounds (Diaz-Perez et al., 2016). This showed that each amino acid metabolism will help in the production of certain secondary metabolites.

From the column loading plot (Figure 3b), we observed that fatty acid (1.28 ppm) and lactic acid (1.32 ppm) were present in highest amounts for non-antifungal producers *Streptomyces* spp. However, the present of fatty acids such as linolenic acid, linoleic acid, lauric acid and oleic acid have been associated with the production of antifungal activities towards several plant pathogens (Beccaccioli et al., 2022). Zhong et al. (2020), stated that the increase in fatty acid chain may increase the effectiveness of the antifungal production by certain *Streptomyces* spp. Due to this, it can be assumed that production of fatty acid in this case, may have inhibited the production of the targeted antifungal compounds.

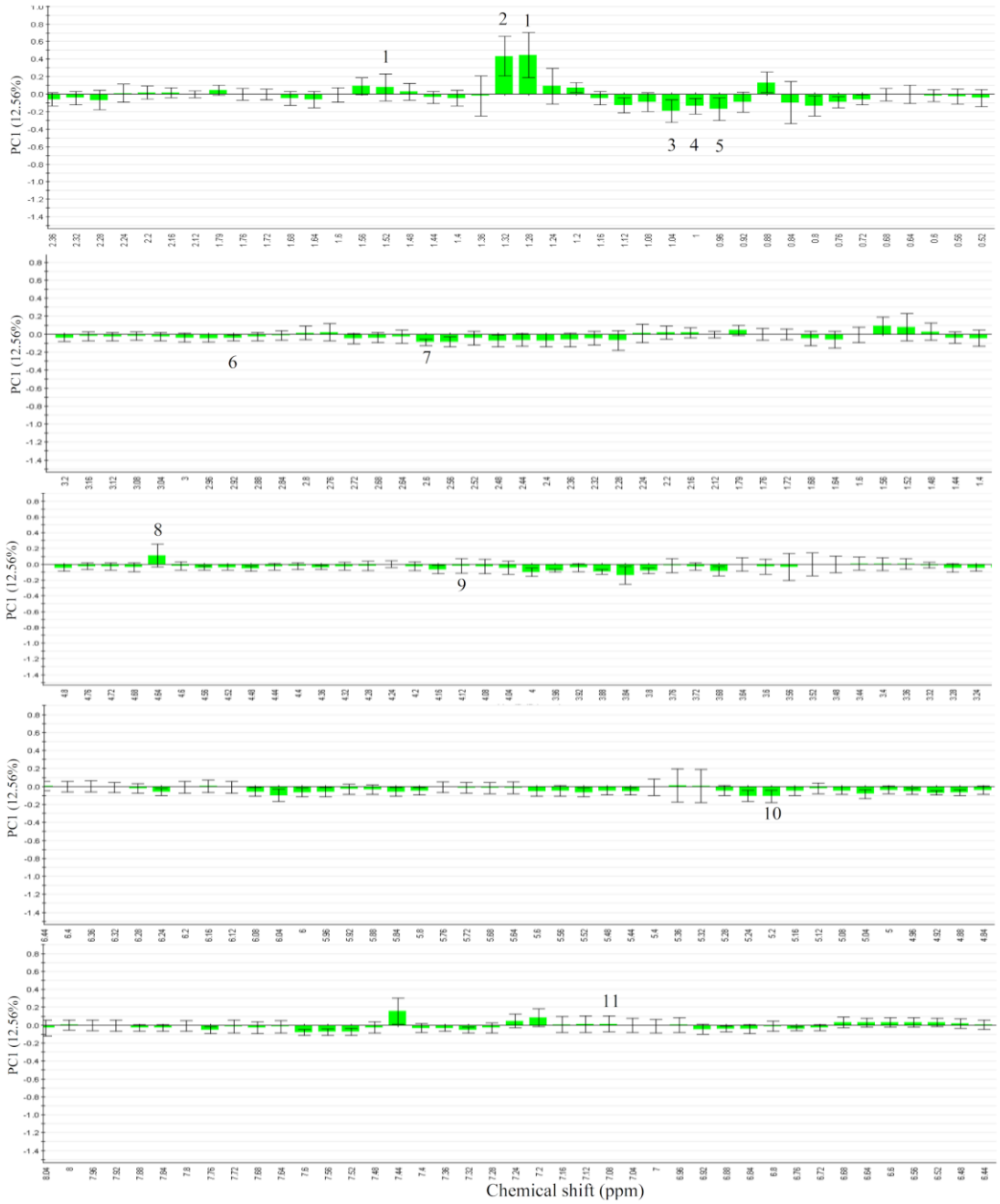

Fig. 3b: Column plot from 1H-NMR results for the antifungal (Negative value bar) and non-antifungal (positive value bar) producing *Streptomyces* spp. Assignment: 1; Fatty Acid, 2; Lactic Acid, 3; Valine, 4; Isoleucine, 5; Leucine, 6; Asparagine, 7; Succinic Acid, 8; β-glucose, 9; Fructose, 10; α-glucose, 11; Gallic Acid.

4. Conclusion

The results obtained from PCA shows that, each strains could be well differentiated based on their metabolites. However, PCA analysis was not able to distinguish between the antifungal producing and non- antifungal producing *Streptomyces* spp. Analysis using OPLS for clearly separated both antifungal producing agents from non-antifungal producer agents in this study. It was observed that the antifungal producing *Streptomyces* spp. possess more amino acids (of valine, Isolueucine, and leucine asparagines) and sugars (β-glucose and fructose) whereas the non-producing *Streptomyces* spp. possess more of the carboxylic acids (fatty acid, lactic acids, and succinic acid). The results obtained from this study gave a representative impression of the metabolites present in each strains of *Streptomyces* spp. and also suggested that there are no one tools that can really be used for all analysis. Each tool would have their advantages and disadvantages. The used of NMR coupled with mathematical tool proved to be fast, accurate and cheap in clustering of organisms compared to biochemical method such as BIOLOG however the cost for this method was higher compared to BIOLOG. The efficacy of NMR in quantifying and identifying potential metabolites produced by *Streptomyces* spp. was fast and reliable and comparable to liquid chromatography-mass spectrometry (LCMS)

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Conflict of Interest

The authors declare no conflicts of interest.

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