Original Research Article

^{1}H-NMR-based metabolomics profiling of *Beauveria bassiana* on different growth media for the determination of its secondary metabolites

Nur Amirah Khirul Anuar[1,2], Nurul Syahindah Ragib[1,2], Noorul Mirza Azmi[1,2] and Syed Adnan Ali Shah[1,2*]

[1]Pharmacology and Pharmaceutical Chemistry, Faculty of Pharmacy, Universiti Teknologi MARA Cawangan Selangor Kampus Puncak Alam, Bandar Puncak Alam, Selangor 42300, Malaysia
[2]Atta-ur-Rahman Institute for Natural Products Discovery (AuRIns), Universiti Teknologi MARA Cawangan Selangor Kampus Puncak Alam, Bandar Puncak Alam, Selangor 42300, Malaysia

ABSTRACT

The entomopathogenic fungus (EPF) *Beauveria bassiana* is commercially used as a microbial insecticide against agricultural pests. Some strains of *B. bassiana* protect crops from pathogens. *B. bassiana* produces bioactive secondary metabolites (SMs) with potential applications. The present study aims to monitor the growth and behaviour of *B. bassiana* in different growth media and the effect of different growth media on the production of SMs. The fungal culture was recultivated and then inoculated in broth and agar growth media to perform this study. After 12 days of incubation of *B. bassiana* in potato dextrose broth (PDB) and a specific medium at temperatures between 28 - 30°C, SMs were extracted from broth cultures using liquid-liquid extraction. To harvest the SMs, the broth media of each culture was extracted using ethyl acetate. After the evaporation of the ethyl acetate using a rotary evaporator, the crude extracts were obtained. The crude extracts were then subjected to ^{1}H-NMR and 2D HSQC analysis using a 600 MHz NMR spectrometer to monitor the presence of different SMs in the crude extracts. The analysis revealed that different types of SMs were produced by *B. bassiana* under different media and conditions. However, some SMs were detected in the ^{1}H-NMR spectrum of the PDB extract that was completely absent in specific Fingal medium, further confirmed by the 2D HSQC experiment. In conclusion, this study revealed that modifying the media constituents and conditions, such as variable temperatures, could produce different SMs, as predicted by the NMR analysis.

Keywords: *Beauveria bassiana*, secondary metabolites, ^{1}H-NMR, 2D HSQC, broth and agar growth media

Corresponding author:
Syed Adnan Ali Shah
Department of Pharmacology and Pharmaceutical Chemistry,
Faculty of Pharmacy, Universiti Teknologi MARA (UiTM) Puncak Alam Campus,
42300 Bandar Puncak Alam, Selangor, Malaysia
Email: syedadnan@uitm.edu.my

Received: 06 Oct 2023; accepted: 06 March 2024
Available online: 28 March 2024
http://doi.org/10.24191/IJPNaCS.v7i1.03

1.0 Introduction

Entomopathogens are a unique subgroup of fungi that control insects, mainly belonging to the Hypocreales order. *Beauveria bassiana* is an example of an entomopathogenic fungus that is commercially available as a pesticide in many countries worldwide (1-3). There are over 40 known genera of entomopathogenic species. One recognizable species is Beauveria sp. (3). There are 6 identified species of Beauveria fungi, which are *Beauveria bassiana*, *Beauveria brongniartii*, *Beauveria velata*, *Beauveria amorpha*, *Beauveria vermiconia*, and *Beauveria caledonica*, based on the research of Gottel et al (2015). These species are distinguished from other fungi by their physical traits (4,5).

Beauveria bassiana is an endophytic fungus that can protect plants from pathogens without causing harm (6). *B. bassiana* is already commercially available as an insecticide or pesticide in numerous countries worldwide (3). This is due to its ability to produce bioactive secondary metabolites (SMs) (7). According to reported studies, Beauveria sp produces volatile organic compounds as its primary SMs, followed by alkaloids, non-peptide pigments, non-ribosomally synthesized cyclopeptides, cyclopeptides, and others (5).

B. bassiana produces alkaloids, including 2-pyridine derivatives like tennelin, bassianin, pyridovericin, pyridomacrolidin, and a red pigment called oosporein. It also produces non-ribosomally synthesized peptides such as bassianolides and beauveriolides. *B. bassiana* can secrete metabolites like lectin and oxalic acid, which may play a role in pathogenesis and virulence factors (Fig. 1 & Fig. 2) (6).

B. bassiana is a fungus that appears as a white mold when cultured. It produces many dry and powdery conidia in unique white spore balls on most standard cultured medium. The ideal temperature for the germination of *B. bassiana* conidia is between 20°C and 30°C, although it can range from 0°C to 40°C. The growth medium and incubation conditions greatly influence the formation of SMs in *B. bassiana*.

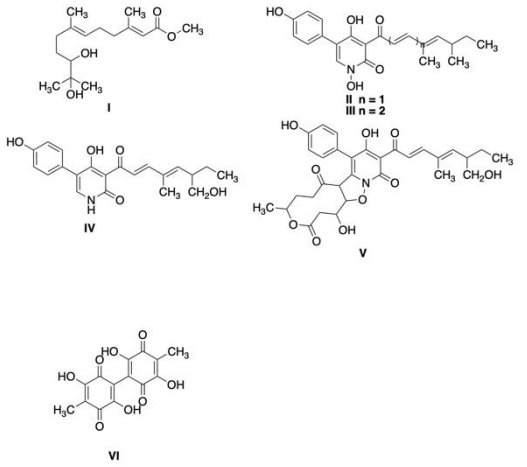

Figure 1: Chemical structures of S-(-)-10,11-dihydroxyfarnesic acid methyl ester (I), tennelin (II), bassianin (III), pyridovericin (IV), pyridomacrolidin (V) and oosporein (VI).

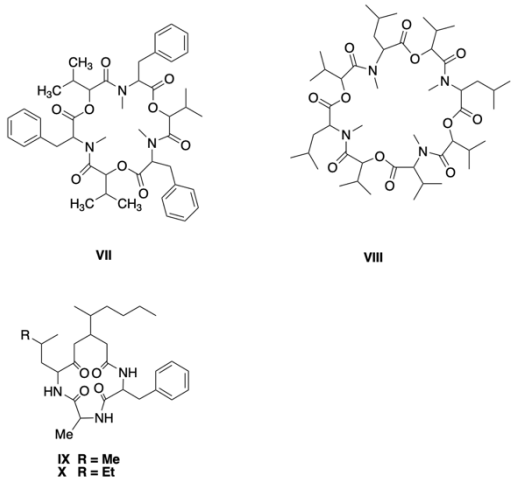

Figure 2: Chemical structures of beauvericin (VII), bassianolide (VIII) and beauveriolides (IX & X).

This is because the ideal medium for one fungus may not be effective for another. Different pigment colours are produced based on the nutrients in the culture media, as stated by Kulandaisamy-venal in 2009 and supported by various sources, including Schaerffenberg (2007), Hall (2011), and Benz (2015) (8,9).

Metabolomics deals with the whole ensemble of metabolites (the metabolome). It is a holistic approach to studying systematic metabolic changes in biospecimens such as cells, tissue, organs, or organisms (10.11). Metabolomics monitors the global outcome of all exogenous and endogenous factors without making assumptions about the effect of any single contribution to that outcome. NMR spectroscopy offers the unique potential to screen hundreds of metabolites holistically. NMR-based metabolomics has emerged as a reliable high-throughput analytical technique extensively applied to nearly every scientific field (12-15). This study utilised 600 MHz NMR spectrometer Bruker Avance III and an untargeted approach to analyze a range of metabolites produced by *B. bassiana*. The objective was to investigate the SMs produced by *B. bassiana* in different media and conditions using metabolic profiling and ^{1}H-NMR. The research aimed to explore whether different conditions and media can alter the production of SMs. The findings will benefit future researchers looking to obtain specific metabolites of interest produced by *B. bassiana* from particular media or conditions.

2.0 Materials and methods

2.1 Fungal culture cultivation

The *Beauveria bassiana* fungal culture that had already been grown in potato dextrose agar (PDA) was obtained from the Microbial Metabolite II Laboratory of Atta ur-Rahman Institute for Natural Discovery Product (Aurins), UiTM Puncak Alam. Before being inoculated in the media, the *Beauveria bassiana* culture was recultivated again in PDA (Fig. 3).

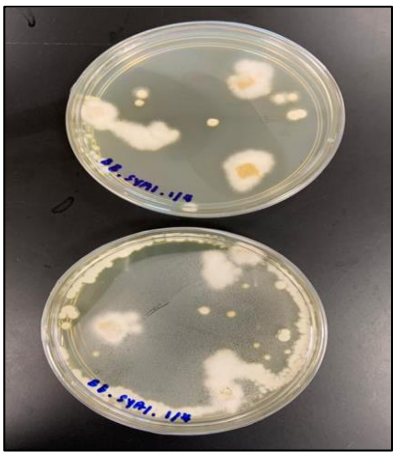

Figure 3: Fungal culture of *Beauveria bassiana* on PDA.

2.2 Growth media preparation

After 5 days, two broth media were prepared. The media used were PDB and a specific medium. The specific media contains glucose (20g), peptone (10g), KH_2PO_4 (10g), yeast extract (10g), NaCl (10g) and glycerol (10mL) into 1 litre (1L) distilled water. All the constituents were dissolved in 1L water and distributed into 5 flasks of 250mL Erlenmeyer flask for each broth. Three mycelial plugs (0.8 cm diameter) were taken from 5-day-old fungal cultures of *Beauveria bassiana* culture and were then inoculated into the broth media. The media were left in the incubator for 12 days at temperatures between 28 to 30°C. This experiment was performed in 5 replicates.

2.3 Liquid-liquid extraction

After a 12-day incubation period, the broth from each culture was filtered and prepared for extraction. The extraction method used for this current research was liquid-liquid extraction, where ethyl

acetate was used as the extraction solvent. A rotary evaporator evaporated the ethyl acetate to obtain organic crude extracts. The crude extracts were then dissolved in acetone and transferred into a vial for NMR analysis.

2.4 NMR analysis and data collection

^{1}H-NMR and 2D heteronuclear single quantum coherence (HSQC) analysis were carried out for preliminary screening or identification of SMs to compare the production of SMs in different culture media.

2.5 NMR Data analysis and secondary metabolites identification

The NMR spectra were run in deuterated methanol using 600 MHz NMR spectrometer Bruker Avance III, Topspin 3.1 has been used for NMR experiments, and MestReNova version 15 was used for processing and plotting the ^{1}H-NMR and 2D HSQC spectra of each extract for detection of a pool of SMs produced by *B. bassiana* in two different media and conditions.

3.0 Results and Discussion

The ^{1}H-NMR spectrum shown in Fig. 4 exhibited the SMs produced by *Beauveria bassiana* cultivated in the PDB culture media. Some of the chemical classes predicted through ^{1}H-NMR analysis include organic acids, quinine, 2-pyridone alkaloid, unsaturated fatty acids, sugar alcohols, terpenes, polyketides and fatty acid chains. The first SMs class detected in the spectrum is the fatty acids chain, which was found between δ 1.25 to 1.5. Next, polyketides were also detected through the ^{1}H-NMR spectrum at a frequency between δ 3.4 to 3.7. Other than that, terpenes were detected in frequency between δ 3.8 to 3.9. In addition, sugar alcohols were detected between the frequency range of δ 4.5 to 4.6. Meanwhile, unsaturated fatty acids were detected at around δ 5.2 to 5.65. Moreover, 2-pyridone alkaloids were also detected in the spectrum in frequency ranges between δ 6.25 to 7.5. In the frequencies δ 8.35 to 8.5, quinine was detected.

Lastly, organic acids were detected in frequency ranges between δ 9.55 and 9.25. This indicated that this structure possesses a high electronegativity charge, making it the furthest from TMS. The ^{1}H-NMR spectrum shown in Fig.5 exhibited few SMs produced by *B. bassiana* cultivated in the specific medium. The secondary metabolite chemical classes identified through the ^{1}H-NMR spectrum were fatty acid chains resonated between δ 0.9 to 1.0. In addition to that, polyketides were also identified between δ 1.3 and 1.4. The frequency ranging from δ 2.0 to 2.5 indicated for terpenes. On the other hand, resonances at the downfield region ranges between δ 3.8 to 3.5 confirmed the presence of alcohols. Lastly, oosporein, a dibenzoquinone fungal toxic secondary metabolite, was also indicated between δ 6.9 to 7.5

Based on the stacked ^{1}H-NMR spectra (Fig. 6) analysis, some SMs produced by *B. bassiana* cultivated in both media cultures overlapped, while others were not matched. The chemical classes of metabolites identified in both media are terpenes, polyketides, and fatty acid chains. Meanwhile, oosporein is also identified in a specific medium, not the PDB. However, organic acid, quinine, 2-pyridone alkaloid, unsaturated fatty acid and sugar alcohol were found in the PDB but not in the specific medium. It can be concluded that about 70% of the SMs produced in each fungal medium are different, while the rest were the same.

The HSQC spectrum shown in Fig. 7 & 8 depicted the chemical shifts of ^{1}H

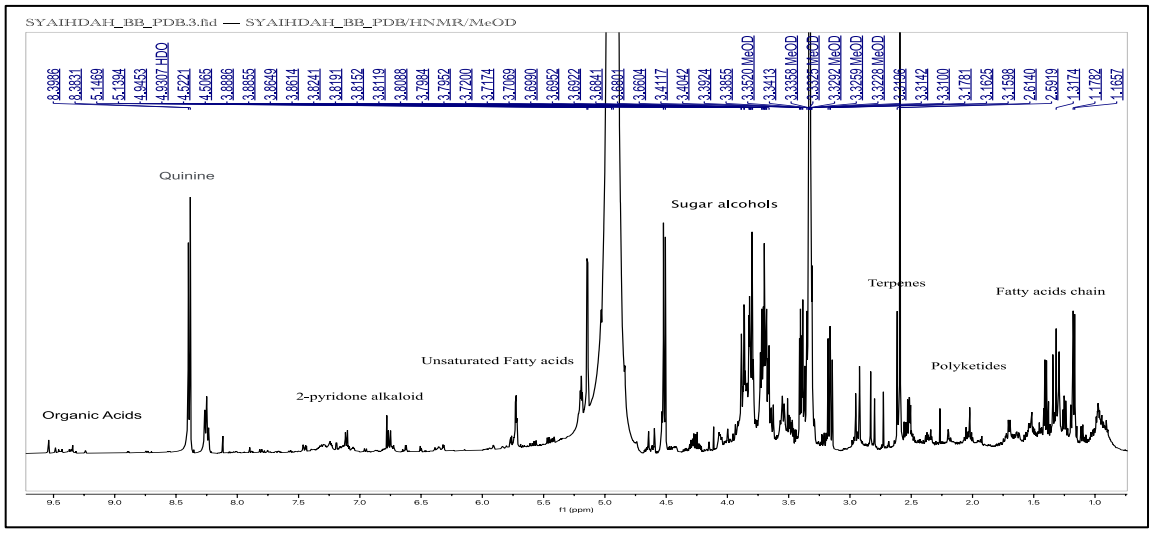

Figure 4: [1]HNMR spectrum of crude extract obtained from *B. bassiana* incubated in PDB growth media.

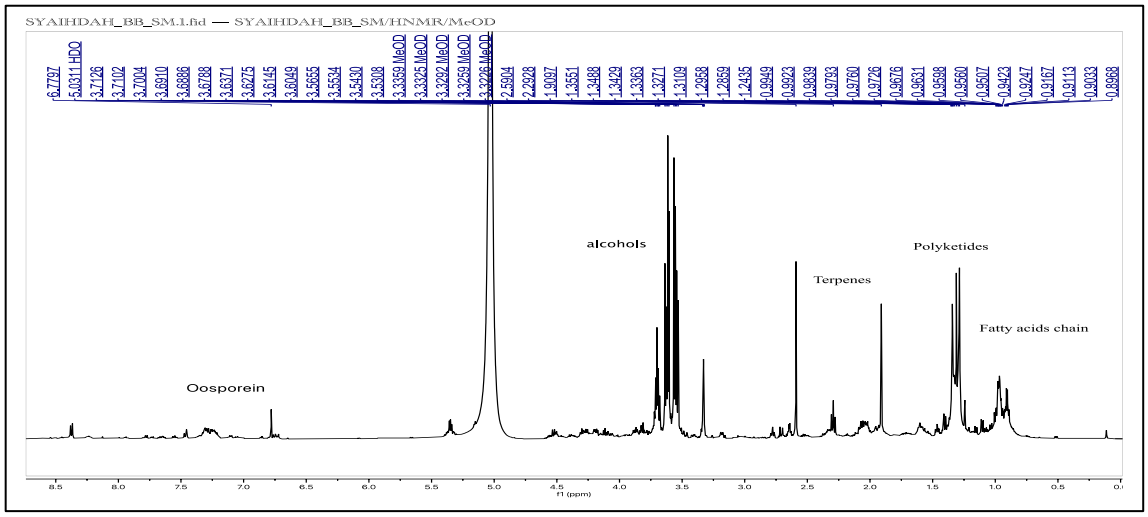

Figure 5: [1]HNMR spectra of crude extract obtained from *B. bassiana* incubated in specific growth media.

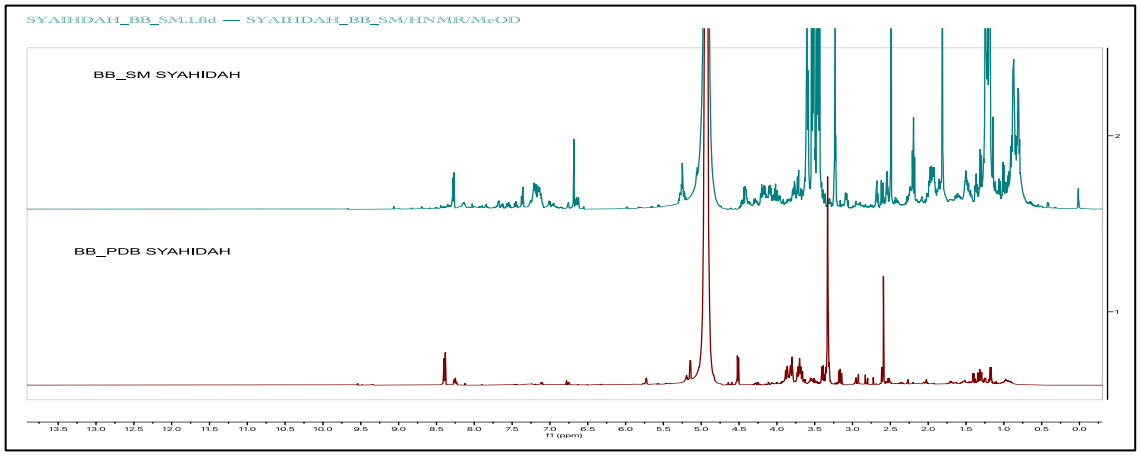

Figure 6: Stack of [1]H NMR spectra of both extracts.

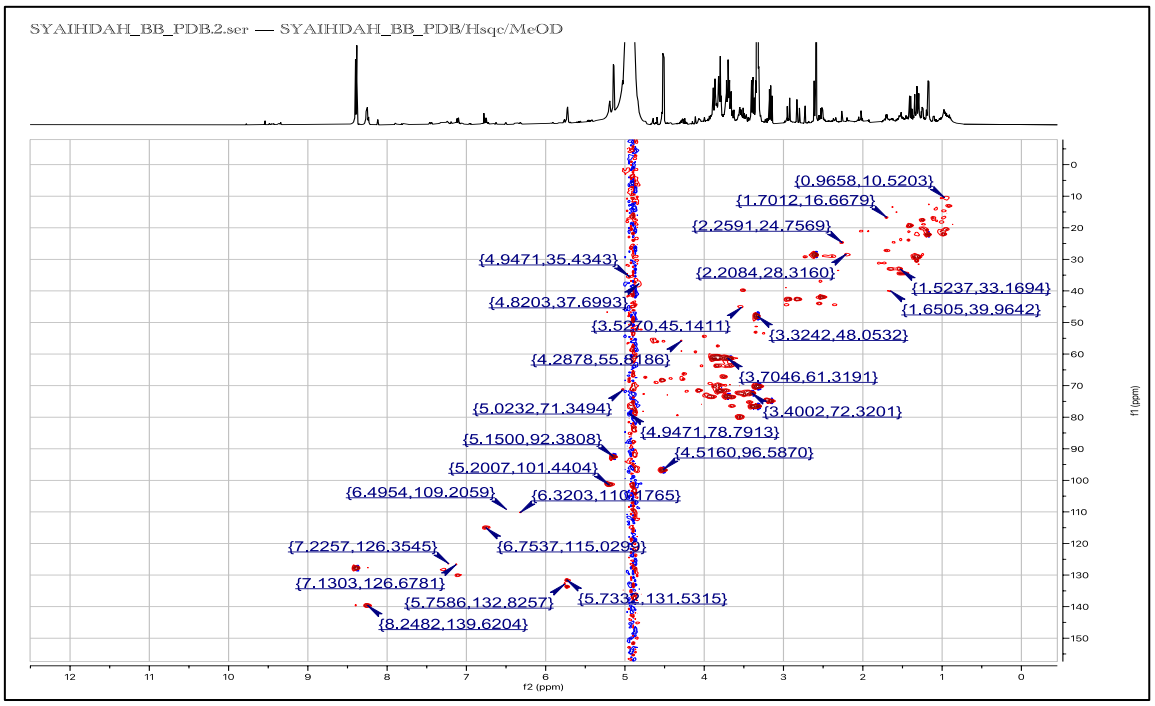

Figure 7: 2D HSQC spectrum of crude extracts obtained from PDB growth media.

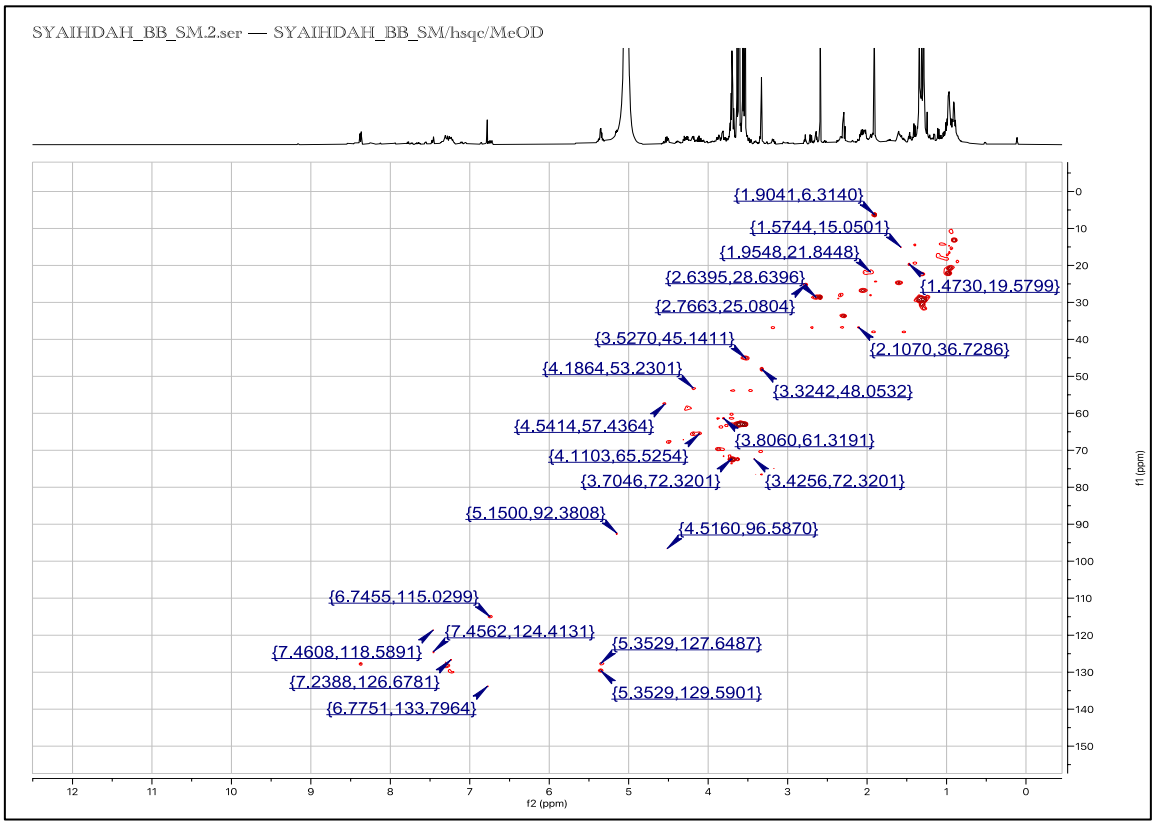

Figure 8: 2D HSQC spectrum of crude extracts obtained from a specific growth media.

and X-nuclei directly bonded to each other and was used to validate further the chemical entities identified through ^{1}H NMR spectra. From the 2D HSQC spectrum of PDB extract, the first compound detected shows the δ 0.96 proton correlates with δ 10.52 carbon. While in the specific medium, δ 1.90 protons correlated with δ 6.31 carbon.

B. bassiana cultivated in PDB culture media produced organic acids, quinine, 2-pyridone alkaloid, unsaturated fatty acid, sugar alcohols, terpenes, polyketides and fatty acid chains. A specific medium comprising glucose, peptone, KH_2PO_4, yeast extract, NaCl and glycerol produces oosporein, alcohols, terpenes, polyketides and fatty acid chains. Further confirmation of the production of SMs was confirmed through 2D HSQC experiments. The reported literature also supported the production of oosporein, quinine and 2-pyridone alkaloids by *B. bassiana* (16-18).

4.0 Conclusion

Our study focused on the impact of different growth media and conditions on *Beauveria bassiana*, a fungus known for its potential as a biocontrol agent. We observed significant variations in the fungus's growth and behaviour across the tested culture media. Furthermore, ^{1}H-NMR spectroscopy revealed the production of distinct SMs in each fungal medium. These findings were further confirmed through HSQC spectroscopy, a technique that provides detailed information about the molecular structure of the identified metabolites.

This research highlights a crucial link between the composition and conditions of the growth media and the behaviour and metabolic profile of *B. bassiana*. It also reveals that manipulating the culture composition can be a powerful tool for optimizing the growth, behaviour, and production of certain SMs.

Authorship contribution statement

NAKA: assist final-year project students in microbial laboratory **NSR:** performed microbial work, extraction, and interpretation of results **NMA:** performed microbial work, extraction, and interpretation of results **SAAS:** performed NMR analysis, wrote and edited manuscript.

Acknowledgment

The authors would also like to acknowledge the Research Management Centre (RMC) Universiti Teknologi MARA for the financial support under the reference number 600-RMC 5/3/GPM (044/2023).

Conflicts of interest

The author declares that they have no known competing financial interests or personal ties that may have influenced the work described in this study.

References

1. Zhang L, Fasoyin OE, Molnár I, Xu Y. Secondary metabolites from hypocrealean entomopathogenic fungi: novel bioactive compounds. Nat Prod Rep. 2020;37(9):1181–206.

2. Saranraj P, Jayaparakash A. Agrobeneficial entomopathogenic fungi – Beauveria bassiana : A Review. Asian J Multidicip Res. 2017;3(2):1051–87.

3. Mc Namara L, Dolan SK, Walsh JMD, Stephens JC, Glare TR, Kavanagh K, et al. Oosporein, an abundant metabolite in Beauveria caledonica, with a feedback induction mechanism and a role in insect virulence. Fungal Biol. 2019;123(8):601–10.

4. Patočka J. Bioactive metabolites of entomopathogenic fungi Beauveria bassiana. Mil Med Sci Lett. 2016;85(2):80–8.

5. Ávila-Hernández JG, Carrillo-Inungaray ML, De-La-Cruz-Quiroz R, Wong-Paz JE, Muñiz-

Márquez DB, Parra R, et al. Beauveria bassiana secondary metabolites: A review inside their production systems, biosynthesis, and bioactivities. Mex J Biotechnol. 2020;5(4):1–33.

6. Roy A, Ahuja S, Garg S. Fungal Secondary Metabolites: Biological Activity and Potential Applications. In: Recent Trends in Mycological Research. Cham: Springer, 2021,159–88.

7. Frisvad JC. Media and growth conditions for induction of secondary metabolite production. Methods Mol Biol. 2012;944:47–58.

8. VanderMolen KM, Raja HA, El-Elimat T, Oberlies NH. Evaluation of culture media for the production of secondary metabolites in a natural products screening program. AMB Express. 2013;3(1):71.

9. Turi KN, Romick-Rosendale L, Ryckman KK, Hartert T v. A review of metabolomics approaches and their application in identifying causal pathways of childhood asthma HHS Public Access Introduction-What is Metabolomics? J Allergy Clin Immunol. 2018;141(4):1191–201.

10. Zeki ÖC, Eylem CC, Reçber T, Kır S, Nemutlu E. Integration of GC–MS and LC–MS for untargeted metabolomics profiling. J Pharm Biomed Anal. 2020;190:113509.

11. Vignoli A, Ghini V, Meoni G, Licari C, Takis PG, Tenori L, et al. High-Throughput Metabolomics by 1D NMR. Angew Chem Int Ed. 2019;58(4): 968-994.

12. Gunawan R, Nandiyanto ABD. How to read and interpret 1h-nmr and 13c-nmr spectrums. Indones. J Sci Technol. 2021;6(2):267–98.

13. Marion D. An Introduction to Biological NMR Spectroscopy. Mol Cell Proteom. 2013;12(11): 3006–3025.

14. Afifah L, Saputro NW. Growth and viability of entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin in different alternative media. IOP Conference Series: Earth and Environmental Science. 2020;468:012037.

15. Bojke A, Tkaczuk C, Stepnowski P, Gołębiowski M. Comparison of volatile compounds released by entomopathogenic fungi. Microbiol Res. 2018;214:129–36.

16. Kouznetsov V v., Carlos MMG, José Luis Valencia Peña, Leonor YVM. Natural and synthetic quinoline molecules against tropical parasitic pathologies: an analysis of activity and structural evolution for developing new quinoline-based antiprotozoal agents. In: Discovery and Development of Therapeutics from Natural Products Against Neglected Tropical Diseases. Elsevier;2019;87–164.

17. Andrioli WJ, Lopes AA, Cavalcanti BC, Pessoa C, Nanayakkara NPD, Bastos JK. Isolation and characterization of 2-pyridone alkaloids and alloxazines from Beauveria bassiana. Nat Prod Res. 2017;31(16):1920-1929.

18. Lee SR, Küfner M, Park M, Jung WH, Choi SU, Beemelmanns C, et al. Beauvetetraones A–C, phomaligadione-derived polyketide dimers from the entomopathogenic fungus, Beauveria bassiana. Org Chem Front. 2019;6(2):162–6.