Summary of Ph.D Dissertation

Identification and classification of microorganisms based on their fatty acid profiles

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1. INTRODUCTION

Organisms synthesize diverse structural, storage, and signaling fatty acids (FAs) for essential functions such as building blocks of complex lipids, energy reservoirs and signaling molecules. The FAS pathway is conserved, but different substrate specificities of the enzymes can lead to altering sets of FAs in different genera. For these reasons, cellular FA profiles are diverse and species-specific and may reflect differences in the biological mechanisms. The varied FAs among organisms, especially in prokaryotes, have already been considered as potential taxonomic biomarkers. The aim of our current study was to demonstrate the power of using the cellular FAs as a taxonomic and diagnostic tool with help of applying Sherlock Sherlock Chromatographic Analysis System (CAS). In our study, the CAS method using FAs of 9–24 carbons in length and automated GC analysis were developed for certain microorganisms to perform routinely, user-friendly and fast-automated identification as a taxonomic method. The Sherlock CAS developed by MIDI with several available Sherlock methods and libraries for microbial identification, but it can be usable also for research purposes due to its expandability and flexibility.

In our study, three microorganism groups were selected for the CAS examinations. The *Bacillus* genera (Firmicutes, Bacilli, Bacillales, Bacillaceae) is a phylogenetically incoherent taxon and appears to be heterogeneous. From a taxonomic point of view, significant phenotypic and genotypic similarities between different *Bacillus* species suggested close taxonomic relationships, so classification between *Bacillus* species posed a significant challenge. The members of *Candida auris* species (Ascomycota, Saccharomycetes, Saccharomycetales, Saccharomycetaceae, *Candida)* are an emerging multidrug-resistant pathogens, which has been recognized as a cause of invasive candidiasis and healthcare outbreaks worldwide resulting bloodstream infections and other invasive and superficial infections with a high mortality rate. Currently, accurate identification of *C. auris* is critical due to problems with the conventional methods. The challenges in identifying *C. auris* underscore the importance of developing more precise and routine procedures to facilitate disease management, improve infection control, and reduce potential transmission. *Armillaria* (Basidiomycota, Agaricales, Physalacriaceae) represent a widespread pathogen of woody plants worldwide, primarily known as a pathogen of *Armillaria* root rot in a variety of woody dicotyledonous hosts causing devastating forest damage and substantial economic losses. In addition to acting as primary necrotrophs on woody hosts, some *Armillaria* species have also been identified as symbionts of specific orchids (*Galeola* and *Gastrodia*). Importantly, most *Armillaria* species are edible with careful preparation, and they may offer beneficial secondary metabolites and polysaccharides for biomedical applications.

2. OBJECTIVES

By considering these main objectives, our study was:

- The identification of environmental *Bacillus* isolates using commercially available method
- Development of new library for the unidentified *Bacillus* strains
- Generation of new library to identify the clinically important *C. auris* species
- Optimization of cultivation conditions, separation method and identification library within the Sherlock CAS for *Armillaria* species

3. METHODS

3.1. The FAME analysis of *Bacillus* strains

- Sample processing was carried out according to the Sherlock™ Operating CAS Manual.
- The method RTSBA6 and library RTSBA6 were applied to identify *Bacillus* strains.
- Unidentified *Bacillus* strains were identify using molecular markers including *gyrA* and *rpoB* genes.
- The library RTSBA7 was generated based on method RTSBA6 for unidentified *Bacillus* strains.

3.2. The FAME analysis of *C. auris* strains

- Sample processing was carried out according to the Sherlock™ Operating CAS Manual.
- The library CAN1 was generated based on method SYEAST6 for *C. auris*.

3.3. The FAME analysis of *Armillaria* strains

- The cultivation conditions, the FA extraction protocol, and the saponification procedure were optimized for working with *Armillaria* mycelia.
- The method ARMI and library ARMI were generated for *Armillaria* species.

4. RESULTS AND DISCUSSION

4.1. FA profiles of the *Bacillus* species

Bacillus species have higher branched-odd FA content, including 13:0 iso, 15:0 iso, 15:0 anteiso, 17:0 iso and 17:0 anteiso, which can provide a common ground for establishing the taxonomy for *Bacillus* species. The diverse FA composition gives an opportunity to differentiate *Bacillus* species and use the FAs as biomarkers. The FAME-based identification has confirmed our isolates as *B. amyloliquefaciens*, *B. atrophaeus*, *B. cereus*, *B. licheniformis*, *B. megaterium*, *B. pumilus*, *B. simplex*, and *B. subtilis*. Additionally, the available library in the MIS have contained altogether 40 *Bacillus* species, but numerous species were not included in it. During our work, based on the FA profiles of the molecularly identified isolates we constructed a new extended library, which contains the new *Bacillus* entries. For the molecular taxonomical basis, the partial sequences of *gyrA* and *rpoB* was applied. Consequently, new entries of *B. endophyticus* and *B. velezensis* were added to the original Sherlock library. The *Bacillus* species were identified with high SI (SI > 0.5) and well-SI-separations (> 0.1), confirming that these strains belong to the *Bacillus* genus with high confidence. Based on the results so far, the FA-based identification used as a biomarker can be considered as a credible differentiating factor for *Bacillus* species that works even within closely related groups. As an ultimate test, the classification based on whole-cell FAs could also distinguish between *B. velezensis* and *B. amyloliquefaciens* which were taxonomically real troublesome cases within a tightly related "*B. subtilis* species complex".

4.2. FA profiles of the *C. auris*

According to the *C. auris* data, the FA profiles contained 16:0, 18:0, 18:1, 18:2 and some peaks of summed feature which varied among *C. auris* strains. The FA 16:0, 18:2 Cis 9,12/18:0a and Summed Feature 8 have been predominant. Cluster analysis and PC analysis of the resulting FAME profiles drew a distinction dividing 12 strains of *C. auris* into three GC subgroups. The GC subgroup A includes strains of clade I – GC subgroup A (B11109 and B8441), clade II (B11220), clade III (B11221 and B11222), and clade III (B11244 and B11245). The GC subgroup B includes strains of clade I – GC subgroup B (B11098, B11203, AR0390 and MMC-1). Besides, the GC subgroup C includes strain MMC-2. Interestingly, strains belonging to clade I could be differentiated into two distinct clusters of subgroup A

(strain B11109 and B8441) and subgroup B (strain B11098, B 11203, AR0309 and MMC-1). The two clusters have represented for dissimilarity of antifungal susceptibility in the clade I. This study carefully constructed a library (CAN1) including FA components of 3 subgroups and a library (CAN2) including FA components of 12 strains from *C. auris*. The FA profiles were consistently typical and distinguishable between subgroups and clades. When using the new created libraries, subgroups and clades of *C. auris* were clearly distinguishable. As testing the performance of the new libraries, all identified samples exhibited the good matches to *C. auris* (SI > 0.7) without misreading. As FA-based classification, *C. auris* can be distinguished to either frequently misidentification cases including *C. famata*, *C. guilliermondii*, *C. lusitaniae*, *C. parapsilosis*, *C. intermedia*, *C. catenulata*, *C. haemulonii*, *C. sake*, and *S. kluyveri* or phylogenetically relating species including *C. catenulate*, *C. ethanolica*, *C. glabrata*, *C. guilliermondii*, *C. haemulonii*, *C. inconspicua*, *C. intermedia*, *C. krusei*, *C. lusitaniae*, *C. parapsilosis*, *C. rugosa* and *C. tropicalis*. The result of testing the performance of the method and library showed the sufficiently discriminatory power between *C. auris* clades as well as between *C. auris* and other species with high confidence. Our significant evidence revealed FA compositions as remarkable biomarkers, which can be applied as a classification feature of *C. auris* at both species and clade levels.

4.3. FA profiles of the *Armillaria* species

The cultivation conditions, the FA extraction protocol, and the saponification procedure were optimized for working with *Armillaria* mycelia. Furthermore, the new method (ARMI) and a new library (ARMI) were also constructed. As FAME analyzing, FA profiles varied depending on *Armillaria* species. Accordingly, even-chain FAs have been regarded as predominant content. The linoleic acid (18:2 ω6c) was 59.51% in *A. cepistipes*, 56.43% in *A. gallica*, 53.43% in *A. mellea* and 59.76% in *A. ostoyae* regarding as the most prominent FA. As following, the palmitic acid (16:0) was from 16.70 to 20.41% and lauric acid (12:0) was from 3.96 to 11.99%, respectively. Other FAs were identified in detectable levels (< 3%). Remarkably, the ΣPUFA presented in the highest percentage followed by ΣSFA and ΣMUFA. UFA amounts predominated over SFA in all the studied species due to the high contribution of linoleic acid. The UFA/SFA ratio presented approximately 1.93 in *A. cepistipes*, 1.72 in *A. gallica*, 1.64 in *A. mellea* and 2.36 in *A. ostoyae*. As regards, FA composition has been diverse drawing a distinction between *Armillaria* species with FAs 12:0, 16:0 and 18:2 ω6c being most important for species separation as remarkable biomarkers. When using the new created library, FA profiles of four *Armillaria* species were clearly distinguishable with minor misidentifications. The SI for *A. cepistipes*, *A. gallica*, *A. mellea* and *A. ostoyae* ranged from 0.284 to 0.935, 0.594 to 0.941, 0.431 to 0.859 and 0.301 to 0.905, respectively. Accordingly, the differentiation accuracy was 90.00% for *A. cepistipes*, 88.70% for *A. gallica*, 100.00% for *A. mellea* and 100.00% for A. ostoyae, respectively. Remarkably, 100 % of samples were identified correctly without misidentification in secondary choice. As drawing a distinction between these *Armillaria* species, FA compositions can be considered as biomarkers for *Armillaria* species.

4.4. Routine application of Sherlock CAS

As with all routine methods, factors as costs, the technical skills, and the time involved playing important roles. With respect to the routine use, a technician averages about 10 minutes per sample for preparation a batch of 12 samples together with 7 minutes per *Bacillus* sample, 21 minutes per *C. auris* sample and 12 minutes per *Armillaria* sample for operating the GC system. The FA profiles are accurately delivered without long-waiting time. Therefore, this study contributed here an applicable, cost-effective, sensitive, reliable and fast-automated method of taxonomic identification.

SUMMARY

- 107 *Bacillus* isolates were identified out of 128 strains including 4 strains as *B. atrophaeus*, 6 strains as *B. cereus*, 27 strains as *B. licheniformis*, 39 strains as *B. megaterium*, 4 strains as *B. pumilus*, 5 strains as *B. simplex*, and 18 strains as *B. subtilis* using method RTSBA6 and library RTSBA6.
- The unknown *Bacillus* strains were revealed using the partial sequences of *gyrA* and *rpoB* as *B. endophyticus* (1 strain) and *B. velezensis* (20 strains). Then the FAME profiles of *B. endophyticus* and *B. velezensis* were analyzed and applied to develop the new library containing also the representative profiles of these species. Furthermore, the new library was successfully applied for the differentiation between the closely related *B. velezensis* and *B. amyloliquefaciens* species.
- The novel library CAN1 developed on method SYEAST6 can be applied to classify *C. auris* at both species and clades level. The result of testing the performance of the method and library showed the sufficiently discriminatory power between *C. auris* clades as well as between *C. auris* and other species with high confidence.
- The cultivation conditions, sample pretreatment steps and then a method ARMI and a library ARMI were developed to identification of *Armillaria* species, which can classify *Armillaria* isolates at species level. The differentiation accuracy was 90.00% for *A. cepistipes*, 88.70% for *A. gallica*, 100.00% for *A. mellea* and 100.00% for *A. ostoyae*, respectively.
- By taking advantage of the current knowledge regarding biomarkers, the FA-based identification proved to be applicable for the taxonomic classification and differentiation among microorganisms, even among closely related species and clades. Our study provided a cost-effective, reliable, and fast-automated method to microbial identification.

5. LIST OF PUBLICATIONS RELATED TO THIS THESIS

5.1 Publications related to this thesis

Thu Huynh, Mónika Vörös, Orsolya Kedves, Adiyadolgor Turbat, György Sipos, Balázs Leitgeb, László Kredics, Csaba Vágvölgyi, András Szekeres, Discrimination between the two closely related species of the operational group *B. amyloliquefaciens* based on whole-cell fatty acid profiling, Microorganisms, 10(2), 418, 2022. **IF: 4.128**

Adiyadolgor Turbat, Dávid Rakk, Aruna Vigneshwari, Sándor Kocsubé, **Thu Huynh**, Ágnes Szepesi, László Bakacsy, Biljana D. Škrbic, Enkh-Amgalan Jigjiddorj, Csaba Vágvölgyi and András Szekeres, Characterization of the plant growth-promoting activities of endophytic fungi isolated from *Sophora flavescens*, Microorganisms, 8(5), 683, 2020. **IF: 4.128**

Thu Huynh, Mónika Vörös, Balázs Leitgeb, Csaba Vágvölgyi, András Szekeres, Discrimination between two *Bacillus* species based on whole-cell fatty acid profiles, Acta Microbiologica et Immunologica Hungarica, Volume 68: Issue Supplement-1, 2021.

András Szekeres, Attila Bartal, **Thu Huynh**, Mónika Vörös, Csaba Vágvölgyi, Surfactin production of *Bacillus* strains isolated from rhizosphere of various vegetables, Acta Microbiologica et Immunologica Hungarica, Volume 68: Issue Supplement-1, 2021

György Sipos, László Kredics, Liqiong Chen, Neha Sahu, Arun Prasanna, Simang Champramary, Orsolya Kedves, Boris Indic, Garima Raj, Bendegúz Richárd Nyikos, **Thu Huynh**, Sándor Kocsubé, Mónika Vörös, Tamás Marik, Viktor Dávid Nagy, András Szekeres, Martin Münsterkötter, Bettina Bencsik-Bóka, Attila Szűcs, Chetna Tyagi, Zsolt Merényi, Csaba Vágvölgyi, László Nagy, Az erdészeti kártevő *Armillaria* (tuskógomba) nemzetség patológiája és a biológiai védekezés lehetőségei. Sopron, Magyarország: Soproni Egyetem Kiadó (2021), 16 p.

Bettina Bencsik-Bóka, Neha Sahu, **Thu Huynh**, Orsolya Kedves, Zsolt Merényi, Gábor Kovác, Liqiong Chen, Simang Champramary, Zoltán Patocskai, Martin Münsterkötter, Csaba Vágvölgyi, László Nagy, György Sipos, László Kredics Classical and 'omics' approaches towards the biological control of devastating forest pathogens from the genus *Armillaria*. In: A Magyar Mikrobiológiai Társaság 2018. évi Nagygyűlése és a XIII. Fermentációs Kollokvium : Abstractbook (2018) 70 p. p. 6

László Kredics, Neha Sahu, **Thu Huynh**, Orsolya Kedves, Zsolt Merényi, Gábor Kovács, Liqiong Chen, Bettina Bóka, Zoltán Patocskai, Martin Münsterkötter, Csaba Vágvölgyi, László Nagy, György Sipos. Devastating forest pathogens from the genus *Armillaria*: from genomics to biocontrol. In: Grenni, P; Fernández-López, M; Mercado-Blanco, J Soil biodiversity and European woody agroecosystem Rome, Italy: Water Research institute, National Research Council of Italy (2018) pp. 48-49, 2 p.

Sum IF: 8.256

5.2. Other publications

Mounir Raji, Tam Minh Le, **Thu Huynh**, András Szekeres, István Zupkó, Zsolt Szakonyi, Divergent Synthesis, Antiproliferative and Antimicrobial Studies of 1,3-Aminoalcohol and 3-Amino-1,2-Diol Based Diaminopyrimidines. Chemistry & Biodiversity (1612-1872 1612-1880): 22 Paper e202200077., 2022. **IF: 2.408**

Tam Minh Le, **Thu Huynh**, Fatima Zahra Bamou, András Szekeres, Ferenc Fülöp and Zsolt Szakonyi, Novel (+)-neoisopulegol-based O-benzyl Derivatives as antimicrobial agents, International Journal of Molecular Sciences, 22(11), 5626, 2021. **IF: 5.923**

Tam Minh Le, **Thu Huynh**, Gabor Endre, Andras Szekeres, Ferenc Fulop and Zsolt Szakonyi, Stereoselective synthesis and application of isopulegol-based bi- and trifunctional chiral compounds, RSC Advances, 10(63), 38468-38477, 2020. **IF**: **3.361**

Liqiong Chen, Bettina Bóka, Orsolya Kedves, Viktor Dávid Nagy, Attila Szucs, Simang Champramary, Róbert Roszik, Zoltán Patocskai, Martin Münsterkötter, **Thu Huynh**, Boris Indic, Csaba Vágvölgyi, György Sipos and László Kredics, Towards the biological control of devastating forest pathogens from the genus *Armillaria*, Forests, 10(11), 1013, 2019. **IF: 2.221**

Sum IF: 13.913

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MTMT ID: 10061621

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Scientific journal article: 5, Conference abstracts: 4

Number of independent citations: 21

SUPERVISOR'S / COAUTHOR'S DECLARATION

We, Dr. András Szekeres (Senior research fellow, Department of Microbiology, University of Szeged) and Prof. Dr. György Sipos (University of Sopron), the supervisors of the Ph.D. candidate Huỳnh Thư, hereby certify, that we are familiar with the Ph.D. thesis entitled "Identification and classification of microorganisms based on their fatty acid profiles". The thesis points of the dissertation are the results of the candidate. The candidate's contribution was significant on the below mentioned publications:

Thu Huynh, Mónika Vörös, Orsolya Kedves, Adiyadolgor Turbat, György Sipos, Balázs Leitgeb, László Kredics, Csaba Vágvölgyi, András Szekeres, Discrimination between the two closely related species of the operational group B. amyloliquefaciens based on whole-cell fatty acid profiling, Microorganisms, 10(2), 418, 2022. IF: 4.128

The candidate's contribution was prominent in following article:

Adiyadolgor Turbat, Dávid Rakk, Aruna Vigneshwari, Sándor Kocsubé, **Thu Huynh**, Ágnes Szepesi, László Bakacsy, Biljana D. Škrbic, Enkh-Amgalan Jigjiddorj, Csaba Vágvölgyi and András Szekeres, Characterization of the plant growth-promoting activities of endophytic fungi isolated from Sophora flavescens, Microorganisms, 8(5), 683, 2020. IF: 4.128

We further declare including the coauthors that no one has ever used these results to obtain Ph.D. degree and will not use in the future.

Szeged, May 31, 2022

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Dr. András Szekeres	Dr. György Sipos	Adiyadolgor Turbat