Evaluation of a New Protein Extraction Method Using an Advanced Acoustic Technology for Identification of Filamentous Fungi by MALDI-TOF MS

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ABSTRACT

Background

While matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) has become a mainstay for clinical labs to identify most bacteria and yeast, its application for filamentous fungi (mold) identification (ID) is still limited. In this study, we assessed a new protein extraction method that generated spectra suitable for MALDI-TOF MS using Adaptive Focused Acoustics™ with a Focused-ultrasonicator (Covaris® M220, Woburn, MA, USA).

Method

Thirteen commonly identified molds (*A. fumigatus, A. terreus, F. solani, S. apiospermum, Curvularia spp, T. tonsurans, T. rubrum, R. oryzae, Mucor spp, P. lilacinus, A. alternata, E. dermatitidis, M. canis*) were grown in Sabouraud Dextrose (SAB-DEX) broth (Becton Dickinson) and rotated at room temperature to generate a hyphal mass. In addition to Bruker's extraction protocol, each sample was extracted using Covaris' ultrasonication method - 1uL of hyphal mass was added to a microTUBE containing 55uL of 70% formic acid and incubated for 10 minutes at room temperature followed by 55uL of acetonitrile. The sample was then processed in Covaris' ultrasonicator under various conditions - Peak Incident Power (PIP) (75 vs 40 units), Duty Factor (DF) (25% vs 50%), type of microTUBEs (fiber vs bead), and ultrasonication time (1 vs 2 min). Each extracted sample was spotted in quadruplicate, run on the MicroFlex LT(Bruker Daltonics), and analyzed using Bruker's Biotyper software (v3.1). The optimal Covaris extraction setting was determined by the MALDI identification scores.

Results

The most optimal results were obtained using the following settings: 40 PIP, 50% DF, microTUBE with fiber, and 2 min ultrasonication. At this setting, 10/13 molds identified correctly with a score of 1.7 to 2.6, comparable to Bruker's full extraction method. *A. alternata, T. rubrum,* and *M. canis* did not produce recognized spectra by the Covaris procedure. All the molds except for *M. canis* were correctly identified by the Bruker method (12/13).

Conclusion

The Covaris 2 min ultrasonication process achieved comparable MALDI scores to Bruker's 30 min protocol using fewer steps and less hands-on time. Although further research is required to investigate ways to increase the correct ID rate, the Covaris' ultrasonication extraction method was a simple, rapid, and efficient tool for mold identification.

INTRODUCTION

In this study, we assessed a new protein extraction method by Covaris that generated spectra suitable for MALDI-TOF MS using Adaptive Focused Acoustics™ with a Focused-ultrasonicator. The first phase of the Covaris study looked at 40 Peak Incident Power(PIP), 50 Duty Factor(DF), 200 cycles, and a 2 minute ultrasonication for *A. fumigatus* and *A. terreus* using beads and fiber microTUBES. The second phase looked again at *A. fumigatus* and *A. terreus* but used 75 PIP, 25 DF, 200 cycles and a 2, 3, and 5 minute ultrasonication using beads and fiber microTUBES. The final phase used for both beads and fiber microTUBES were 75 PIP, 25 DF, 200 cycles, at 1 and 2 minute ultrasonication versus 40 PIP, 50 DF, 200 cycles, at 1 and 2 minute ultrasonication. This phase focused on thirteen commonly identified molds (*A. fumigatus, A. terreus, F. solani, S. apiospermum, Curvularia* spp, *T. tonsurans, T. rubrum, R. oryzae, Mucor* spp, *P. lilacinus, A. alternata, E. dermatitidis, M. canis)* that were tested against the Bruker protocol.

METHODS

➢Starting with a pure filamentous organism growing on SAB, use a cotton swab to transfer a few filamentous colonies into SAB broth
➢Place the SAB broth onto the rotator for 24 hours to allow the filamentous to stay in its hyphal phase

➢Remove the SAB broth from the roator and allow the filamentous to settle to the bottom of the tube (This will take approximately 10 minutes.)
➢Remove the filamentous pellet from the broth with a transfer pipet into a 1.5 mL microcentrifuge tube
➢Centrifuge at 2 minutes at maximum speed (13,000-15,000 rpm)
➢Remove the supernant and discard
➢Add 1 mL of Reagent Grade Water and vortex
➢Centrifuge at 2 minutes at maximum speed (13,000-15,000 rpm)
➢Remove the supernant and discard

Covaris

Bruker

➢If using the microTUBE with glass beads, centrifuge it at 300 RCF for 10 seconds to pellet the beads
➢Add 55 uL of 70% Formic Acid to the microTUBE
➢Using a 1 uL disposable loop, transfer the hyphal pellet from the microcentrifuge tube to the microTUBE prefilled with glass beads or fiber
➢Allow the microTUBE to incubate at room temperature for 10 minutes **(*Omitted this Step for Phase 1 and 2)**
➢Add 55 uL of Acetonitrile to the microTUBE
➢ Place the microTUBE into the Covaris M220 for ultrasonication at specified settings of Peak Incident Power(PIP), Duty Factor(DF), 200 cycles, and minutes
➢ Centrifuge the microTUBE at 13,000 RCF for 2 min
➢ Pipet 1 uL of the supernatant onto the MALDI target
➢ Once dry, add 1 uL of matrix

➢Add 1 mL of Reagent Grade Water and vortex
➢Centrifuge at 2 minutes at maximum speed (13,000-15,000 rpm)
➢Remove the supernant and discard
➢Add 300 uL or Reagent Grade Water to the pellet and vortex
➢Add 900 uL of Ethanol and vortex
➢Centrifuge at 2 minutes at maximum speed (13,000-15,000 rpm)
➢Remove the supernant and discard
➢Dry the pellet completely in a Speedvac(takes approximately 10 minutes)
➢Add 10 uL-100 uL of 70% Formic Acid depending on the pellet size
➢Vortex until pellet is resuspended then incubate at room temperature for 10 minutes
➢Add the same volume of Acetonitrile to the pellet (as added above with Formic Acid
➢Centrifuge at 2 minutes at maximum speed (13,000-15,000 rpm)
➢Pipet 1 uL of the supernatant onto the MALDI target
➢Once dry, add 1 uL of matrix

RESULTS

Phase 1

40 PIP 50 DF 2 minutes that excluded the hyphal mass and 70% Formic Acid incubation		
	Beads	Fiber
Aspergillus fumigatus	2.234, 2.481, NP, 2.445	NP, 2.282, NP, NP
Aspergillus terreus	NP, NP, NP, NP	NP, NP, NP, NP

No Peaks= NP

Phase 2

75 PIP 25 DF that excluded the hyphal mass and 70% Formic Acid incubation			
	2 min	3 min	5 min
Aspergillus fumigatus with beads	1.74, 1.736, 1.799, 1.80	NRI 1.592, NRI 1.686, 1.758, NRI 1.603	NRI 1.482, NRI 1.507, NRI 1.606, NRI 1.526
Aspergillus fumigatus with fiber	1.774, 1.771, 1.779, 1.755	NRI 1.65, 1.832, 1.747, NRI 1.688	1.561, NRI 1.604, NRI 1.498, NRI 1.629
Aspergillus terreus with beads	NRI 1.264, NRI 1.132, NRI 1.116, NRI 1.201	NRI 1.218, NRI 0.998, NRI1.403, NRI 1.295	NRI 1.317, NP, NRI 1.274, NP
Aspergillus terreus with fiber	NRI 1.161, NRI 1.235, NRI 1.162, NRI 1.172	NP, NRI 1.312, NRI 1.089, NP	NRI 1.133, NRI 1.33, NRI 1.194, NP

No Peaks= NP, No Reliable Identification= NRI

Final Phase

75 PIP 25 DF that included the hyphal mass and 70% Formic Acid incubation				
	1 min beads	1 min fiber	2 min beads	2 min fiber
Aspergillus fumigatus	2.464, 2.508, 2.457, 2.469	2.453, 2.494, 2.508, 2.538	2.455, 2.396, 2.478, 2.42	2.483, 2.424, 2.431, 2.367
Aspergillus terreus	NP, 2.244, NP, NP	2.355, 2.378, NP, 2.415	2.343, NP, 2.256, 2.359	2.369, 2.359, NP, NP
Fusarium solani	NP, NP, NP, NP	NP, NP, NP, 2.302	NP, NP, NP, NP	1.899, NP, NP, NP
Scedosporium apiospermum	NP, NP, NP, NP	2.394, 2.425, 2.392, 2.444	NP, NP, NP, NP	NP, NP, NP, NP
Curvularia sp.	2.075, 2.203, NP, NP	NP, NP, NP, NP	NP, NP, NP, NP	NP, NP, NP, NP
Trichophyton tonsurans	NP, 2.369, NP, NP	2.453, 2.541, 2.462, 2.479	NP, NP, 1.959, NP	2.466, 2.437, 2.487, NP
Rhizopus oryzae	1.969, 1.914, 1.994, 2.023	2.07, 2.067, NP, 2.068	1.871, 1.868, 1.948, 1.827	2.022, 1.894, 1.967,1.975
Paecilomyces lilacinus	2.242, 2.258, 2.26, 2.327	NP, NP, 2.252, 2.315	1.95, 1.872, 2.41, 2.021	2.342, 2.32, 2.167, 2.338
Alternaria alternata	NP, NP, NP, NP	NP, NP, NP, NP	NP, NP, NP, NP	NP, NP, NP, NP
Mucor sp.	2.295, 2.292, 2.32, 2.262	2.179, 2.218, NP, 2.277	2.224, 2.086, 2.128, 2.135	2.252, 2.125, NP, 2.215
Exophiala dermatitidis	NP, NP, NP, NP	NRI 1.508, NRI 1.493, NP, NP	NRI 1.28, NRI 1.423, NP, NRI 1.492	NRI 1.374, NRI 1.344, NP, NRI 1.305
Trichophyton rubrum	NP, NP, NP, NP	NP, NP, NP, NP	NP, NP, NP, NP	NP, NP, NP, NP
Microsporum canis	NP, NP, NP, NP	NP, NP, NP, NP	NP, NP, NP, NP	NP, NP, NP, NP

No Peaks= NP, No Reliable Identification= NRI

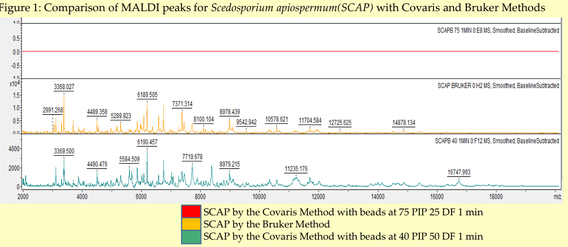
40 PIP 50 DF that included the hyphal mass and 70% Formic Acid incubation				
	1 min beads	1 min fiber	2 min beads	2 min fiber
Aspergillus fumigatus	2.405, 2.462, 2.477, 2.466	2.371, 2.356, 2.518, 2.422	2.438, 2.439, 2.462, 2.41	2.361, 2.367, 2.439, 2.457
Aspergillus terreus	2.346, 2.362, 2.346, 2.173	2.158, 2.242, 2.381, 2.346	2.282, 2.343, 2.276, 2.377	2.346, 2.382, 2.319, NP
Fusarium solani	NP, NP, NP, NP	NP, NP, NP, NP	2.211, 2.251, 2.046, 2.236	2.39, 2.268, 2.399, 2.408
Scedosporium apiospermum	NP, 2.178, 2.25, 2.105	2.385, 2.41, 2.37, NP	NP, NP, NP, NP	2.253, NP, 2.383, 2.397
Curvularia sp.	NP, NP, NP, NP	NP, NP, NP, NP	NP, NP, 2.084	NP, NP, 2.237, 2.211
Trichophyton tonsurans	2.543, 2.363, 2.428, 2.578	2.565, 2.324, 2.504, NP	NP, NP, NP, NP	NP, NP, NP, 2.561
Rhizopus oryzae	2.019, 1.976, 1.997, 2.006	2.127, 2.125, 2.124, 2.204	1.932, 2.045, 1.97, 1.964	2.014, 1.94, 1.857, 1.996
Paecilomyces lilacinus	2.239, 2.295, 2.272, 2.32	2.285, 2.372, NP, 2.27	2.112, 2.12, 2.182, 2.207	2.256, 2.319, NP, 2.3
Alternaria alternata	NP, 2.329, 2.391, NP	NP, NP, NP, NP	NP, NP, NP, NP	NP, NP, NP, NP
Mucor sp.	2.025, 2.038, 2.024, 1.967	2.144, 2.128, 2.125, NP	1.962, 1.814, 1.944, 1.905	NP, 2.197, 2.211, NP
Exophiala dermatitidis	NRI 1.523,NRI 1.509, NP, NRI 1.456	NP, NP, NRI 1.698, NP	NP, NP, NP, NRI 1.287	1.75, 1.776, 1.896, 1.731
Trichophyton rubrum	NP, NP, NP, NP	NP, NP, NP, NP	NP, NP, NP, NP	NP, NP, NP, NP
Microsporum canis	NP, NP, NP, NP	NP, NP, NP, NP	NP, NP, NP, NP	NP, NP, NP, NP

No Peaks= NP, No Reliable Identification= NRI

Bruker Method Results	
Aspergillus fumigatus	2.488, 2.469, 2.303, 2.427
Aspergillus terreus	2.275, 2.306, 2.383, 2.348
Fusarium solani	2.465, 2.395, 2.411, 2.473
Scedosporium apiospermum	2.5, 2.406, 2.396, 2.401
Curvularia sp.	2.37, 2.317, 2.353, 2.389
Trichophyton tonsurans	2.499, 2.505, 2.476, 2.398
Rhizopus oryzae	2.146, 2.074, 2.086, 2.164
Paecilomyces lilacinus	2.5, 2.53, 2.494, 2.458
Alternaria alternata	2.416, 2.416, 2.384, 2.385
Mucor sp.	2.584, 2.476, 2.566, 2.583
Exophiala dermatitidis	1.76, 1.795, 1.876, 1.857
Trichophyton rubrum	1.716, 1.773, 1.774, 1.81
Microsporum canis	NP, NP, NP, NP

No Peaks= NP

Figure 1: Comparison of MALDI peaks for *Scedosporium apiospermum(SCAP)* with Covaris and Bruker Methods



SCAP by the Covaris Method with beads at 75 PIP 25 DF 1 min
SCAP by the Bruker Method
SCAP by the Covaris Method with beads at 40 PIP 50 DF 1 min

CONCLUSION

- The first phase of the Covaris study showed that *Aspergillus terreus* required increased contact time with 70% Formic Acid which was seen by the increased scores obtained in the final phase.
- The second phase of the Covaris study showed as ultrasonication time increased, MALDI identification scores decreased.
- In the final phase of the Covaris study, the microTUBES with fiber at 40 PIP 50 DF at 2 min, achieved comparable MALDI scores to Bruker's 30 min protocol using fewer steps and less hands-on time.
- Although further research is required to investigate ways to increase the correct ID rate, Covaris' ultrasonication extraction method was a simple, rapid, and efficient tool for mold identification.

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