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Metabolic profiling of moulds with laser desorption/ionization mass spectrometry on gold nanoparticle enhanced target

Adrian Arendowski[a,*,1], Justyna Szulc[b,1], Joanna Nizioł[a], Beata Gutarowska[b], Tomasz Ruman[a]

[a] Faculty of Chemistry, Rzeszów University of Technology, 6 Powstańców Warszawy Ave., 35-959 Rzeszów, Poland
[b] Institute of Fermentation Technology and Microbiology, Lodz University of Technology, Wólczańska 171/173, Łódź 90-924, Poland

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ABSTRACT

Surface-assisted laser desorption/ionization mass spectrometry on gold nanoparticle enhanced target (AuNPET) technique was used for metabolomic analysis and secondary metabolites detection of two mould strains – *Aspergillus versicolor* and *Penicillium chrysogenum* in model conditions on microbiological malt extract agar medium. Results obtained with the use of AuNPET-based mass spectrometry technique were compared with traditional matrix-assisted laser desorption/ionization (MALDI) method based on α-cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB) matrices. Gold nanoparticle enhanced target method enabled effective ionization of microbial cellular extract ingredients without interference from the matrix and also improved calibration of spectra resulting in the detection of much higher amount of characteristic metabolites for studied organisms than MALDI.

Introduction

Identification of microorganisms is an important issue for the diagnosis of clinical infections, food safety and in environmental studies. Traditional morphological identification used in many laboratories is time consuming and requires a lot of experience. It is therefore important to develop new methods for the identification of bacteria and fungi.

Over the past few years, mass spectrometry has been increasingly used to identify microorganisms, in particular the Matrix-Assisted Laser Desorption/Ionization (MALDI) method was frequently used [1]. This technique has been successfully used for the analysis of food-born bacteria [2], but also in clinical microbiology field [3] for example for diagnosis of mould infections [4,5]. Identification of microorganisms by MALDI usually consists of detection of molecules in higher molecular weights regions such as proteins, usually identified by analysis of products of their enzymatic lysis. Commonly made mass spectrometry fingerprints are compared with databases for similarity on the basis of peptide or protein signals [6]. However, due to the presence of a high chemical background in the mass spectrum range below *m/z* 1000, MALDI is not the best choice for measuring low molecular weight compounds and identification of microorganisms on the basis of metabolic profiles or fingerprints. Technique which could be of higher use in this type of analysis is Surface-Assisted Laser Desorption/Ionization (SALDI) performed on gold nanoparticle enhanced target (AuNPET) [7]. The preparation and examples of applicability of this target for laser desorption/ionization MS were presented in our recent articles [8–13]. AuNPET is suitable for analyzing compounds of different polarity, also in complex biological mixtures with very high mass accuracy.

Recently, few attempts were made to use MALDI for detection of proteins and lipids to identify fungal species with clinical significance [4,14]. However, to best of our knowledge, only one attempt was made to analyze metabolite profile of moulds in low molecular masses range using nanoparticle-based laser mass spectrometry imaging technique [12]. The purpose of present work is to present results of comparison of MALDI method based on two most frequently used matrices with gold-nanoparticle-based SALDI-type method for metabolic profiling of two mould strains – *Aspergillus versicolor* and *Penicillium chrysogenum.*

Abbreviations: AuNPET, gold nanoparticle enhanced target; MALDI, matrix-assisted laser desorption/ionization; CHCA, α-cyano-4-hydroxycinnamic acid; DHB, 2,5-dihydroxybenzoic acid; SALDI, surface-assisted laser desorption/ionization; MEA, malt extract agar; MS, mass spectrometry; ToF, time-of-flight; S/N, signal-to-noise; PLS-DA, partial least squares discriminant analysis; sPLS-DA, sparse partial least squares discriminant analysis

* Corresponding author.
E-mail address: adrian@arendowski.hub.pl (A. Arendowski).
[1] Both authors contributed equally to this work.

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Experimental

Moulds cultures

Two moulds species deposited in the Lock Collection of Pure Culture (LOCK CPC, Łódź, Poland): *Aspergillus versicolor* (1124) and *Penicillium chrysogenum* (1125) were used in this research. Colonies from malt extract agar (MEA, Merck, Germany) medium slants cultured at 27 ± 2 °C for 7 days were washed using 10 mL distilled water with 0.01% of Tween® 80. Number of mould spores was estimated at 10^7 spore/mL and the average density of the suspension ranged from 1.9 to 2.5×10^7 cfu/mL. MEA medium of the size 20 × 20 × 9.5 mm was inoculated with 100 µL of the spore suspension. All samples were incubated for 21 days in a climate chamber Binder KBF720 at 27 ± 2 °C and relative humidity RH = 80 ± 1% and prepared in triplicate. Growth of the strains *A. versicolor* and *P. chrysogenum* was observed macroscopically during incubation. Metabolome analysis was done after 21 days.

Sample preparation

In order to prepare mass spectrometry-suitable extracts, MEA samples with moulds and without moulds were subjected to three freeze (−20 °C)/unfreeze cycles. MEA samples were then washed with 400 µL of distilled water, liquid was then collected and diluted 10 times with distilled water. A volume of 400 µL distilled water was added to the samples to wash out any water-soluble metabolites. Out of this raw extract, 100 µL was placed in Eppendorf tubes with distilled water (900 µL). Samples were vortexed for 2 min and placed (0.5 µL) on the AuNPET [7] providing "AuNPET" results. For each MEA sample, two more droplets of extracts were placed on the same target and was mixed on the target plate with (0.5 µL) standard solutions of CHCA or DHB matrices ("CHCA" and "DHB" results respectively). Standard matrix solution is referred to saturated matrix solution in 3:7 (v/v) acetonitrile:0.1% trifluoroacetic acid water solution.

MS analysis

Mass spectrometry analysis was performed in positive-ion reflectron mode in the *m/z* 80–2000 range. The laser impulse energy was approximately 60–120 µJ; the laser repetition rate was 1 kHz; and the deflection value was set to *m/z* 80 Da. The first accelerating voltage was held at 19 kV and the second ion-source voltage was held at 16.7 kV. The reflector voltages used were 21 kV (first) and 9.55 kV (second). For each extract 50,000 laser shots were collected. Mass calibration was performed using internal standards, gold ions and clusters from Au^+ to Au_9^+ for AuNPET results and matrix peaks for MALDI ones.

Spectra processing

Spectra were analyzed using the mMass 5.5.0–open source program [15]. Spectra were recalibrated internally with the use of modified mMass program with in-house-added additional quartic calibration function. Default baseline correction, and peak picking of signals with signal-to-noise ratio (S/N) higher than 7 was then performed. Spectra provided in Figs. 1 and 2 were prepared by subtraction of control spectrum (for example MEA extract on AuNPET) from sample spectra (for example AuNPET *Aspergillus* spectrum). Resulting positive peaks of S/N higher than 7 were included in putative identification of metabolites (Tables 1 and 2). Peak lists were searched against mMass build-in list of secondary metabolites of *Aspergillus* species and fungal peptides. For *Penicillium chrysogenum*, an additional list of characteristic metabolites was created on the basis of literature data. "Metabolomic database" search was performed with custom-made program containing compound database from IDEOM v19 [16] package.

Statistical analysis

Partial least squares discriminant analysis (PLS-DA) [17] and sparse partial least squares discriminant analysis (sPLS-DA) [18] analyses were performed with the use of Metaboanalyst service [19]. Data was normalized by sum, log-transformed, default auto scaling was used.

Results & discussion

Fungal samples were homogenized with the use of multiple freezing/unfreezing homogenization procedure which was shown to be suitable for laser mass spectrometry [12]. Laser desorption/ionization mass spectra of moulds' extracts were subjected to baseline correction and peak picking with signal-to-noise (S/N) parameter 7 or higher. For *Aspergillus versicolor* measured with SALDI AuNPET 8263 peaks were obtained. MALDI measurements with CHCA gave 7274 peaks, the same procedure with DHB gave only 1301 peaks. For *Penicillium chrysogenum* the number of signals with the use of AuNPET plate was 8378, while for the CHCA matrix 6402 and 5301 for DHB.

However, above mentioned numbers of peaks include those not only originating from ingredients of mould extracts but also from MEA medium, MALDI matrices and various method-related impurities. In order to obtain mould only information, spectra subtraction operations were performed. Each difference spectrum presented in Figs. 1 and 2 was obtained by subtraction of MEA medium control spectrum from the mould one. Difference spectra for *Aspergillus* contained much lower numbers of peaks - 2274 peaks for SALDI AuNPET, 692 for MALDI method with a CHCA matrix and 513 in case of DHB matrix. The same observation was made for *Penicillium* for which 1558 peaks were found using the SALDI AuNPET, 621 for MALDI with CHCA and 533 in case of DHB matrix. It is important to state that mentioned data and also spectra in Figs. 1 and 2 suggest that most of the high-intensity signals found in MALDI spectra were not sample-related.

In order to estimate the degree of influence of method-related spectral data over sample-related data, statistical analysis with the aid of Metaboanalyst was performed [19]. In case of domination of method-related data or signals, statistical analysis such as partial least squares discriminant analysis (PLS-DA) [17] or sparse partial least squares discriminant analysis (sPLS-DA) [18] could not provide clear enough separation of studied mould samples. Fig. 3 contains results of statistical analysis of mass spectrometry data. It could be concluded that all sPLS-DA score plots presents completely separated groups while for PLS-DA score plot (Fig. 3A) almost complete separation is observed.

Metabolomic database search (not shown) of positive peaks higher than S/N 7 in difference spectra of *A. versicolor* with maximum 5 ppm error resulted in putative assignment of 4708 compounds for SALDI AuNPET, 1259 for MALDI with CHCA and 766 for DHB matrices. The same search for *P. chrysogenum* resulted in 4328, 1411 and 936 compounds for SALDI AuNPET, MALDI with CHCA and MALDI with DHB respectively. MS results were also analyzed with the use of specialized metabolite databases implemented into mMass 3 program. Search for characteristic *Aspergillus* sp. metabolites and also for characteristic fungal peptides resulted in listing of sixty nine different adducts of fungal compounds present in SALDI AuNPET spectrum (Table 1). The same search for MALDI results gave only eighteen adducts in case of CHCA matrix and sixteen for DHB matrix. Analogically performed search against *P. chrysogenum* most important list of 14 metabolites based on literature including selected fungal peptides data gave nine detected compounds in case of SALDI AuNPET, seven for MALDI with CHCA matrix and only two in case of DHB matrix (Table 2). It should be noted that percentages of characteristic low molecular weight metabolites found for *A. versicolor* were 27.0, 6.9 and 7.8% with the use of AuNPET, MALDI/CHCA and MALDI/DHB respectively. Analogically calculated percentages for *P. chrysogenum* were 35.7, 28.6 and 7.1% respectively. Lower percentages of putatively identified compounds in MALDI/CHCA and MALDI/DHB are surely associated with inferior