Chemical Pretreatment of Cells for Enhanced Discrimination of Clinical Yeasts isolates by MALDI-TOF-MS

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Abstract

BACKGROUND: *Candida* spp. of yeasts are a major cause of nosocomial infections in intensive care units, with *Candida albicans* the most commonly isolated yeast species. Phenotypic identification of *Candida* spp. is labour- and time consuming, while rapid direct intact cell MALDI-TOF-MS is emerging as a rapid tool for identifying microorganisms.

OBJECTIVES: To optimise a MALDI-TOF-MS protocol for discrimination of yeasts, with evaluation of various chemical pretreatments of cells for enhancing spectral richness and discriminatory power.

METHOD: MALDI-TOF-MS of *C. albicans* was optimised with respect to matrix chemical(s), matrix solvent and target plating method. Various chemical pretreatments (solvents, reductants, detergents) and application methods were evaluated for enhancement of spectral richness. Selected pretreatments were applied to MALDI-TOF-MS discrimination of a set of clinical isolates comprising *Candida* spp. and *Cryptococcus neoformans*.

RESULTS: The optimized MALDI-TOF-MS protocol involved α-cyano-4-hydroxycinnamic acid (CHCA) as matrix chemical (in 1:1 acenonitrile:H_2O and 0.3% trifluoroacetic acid), with target plating by a sandwich method. Methanol or formate:isopropanol pretreatment of cells in a direct target plate application method enhanced spectral richness. The latter pretreatment facilitated discrimination of a set of clinical isolates comprising *Candida* spp. (*C.albicans, C. boidinii, C. glabrata, C. guilliermondii, C. krusei, C. parapsilosis, C. tropicalis,*) and *Cryptococcus neoformans* with 100% clustering accuracy.

CONCLUSION: MALDI-TOF-MS discrimination of *Candida* spp. is enhanced through chemical pretreatment of cells; formate:isopropanol pretreatment ,with CHCA as matrix chemical in a sandwich target plating method, is highlighted as a protocol affording the best discriminatory power.

Keywords

MALDI-TOF-MS; *Candida; Cryptococcus*; yeasts.

Introduction

Use of matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) for identification of microorganisms is well established, particularly for bacteria. In 1996, Claydon *et al*. [1] and Holland *et al*. [2] showed that bacterial cells could be analysed directly by MALDI-TOF-MS, using a simple technique known as intact cell MALDI-TOF-MS that requires minimal sample preparation, and identifies bacteria according to differences in cell wall constitution.

Candida spp. of yeasts are a major cause of nosocomial infections in intensive care units, with *Candida albicans* the most commonly isolated yeast species. Other *Candida* species - such as *C. tropicalis*, *C. krusei*, *C. parapsilosis* and *C. glabrata* – are increasingly being recognised as opportunistic pathogens with a wide distribution or as emerging potential pathogens. Phenotypic identification of *Candida* species is labour- and time consuming, and the results can be inconclusive. The cell wall of yeasts has a fundamentally different structure and composition compared to bacteria. In *Candida* spp, an inner skeletal layer composed mainly of β-1,3-glycan molecules is extended with covalently bound β-1,6-glucan and chitin chains. This is bounded by an outer layer that determines cell surface properties and is composed mainly of mannoproteins; bound to β-1,6- or β-1,3-glucan either covalently or non-covalently, or to other mannoproteins through disulphide linkages [3,4].

Direct MALDI-TOF-MS analysis of yeasts tend to give spectra that are less rich compared to those obtained from bacterial cells, which has prompted researchers to investigate various pretreatments (enzyme or chemical) of yeast cells as a means to enhance spectral richness and improve differentiation of yeasts. Sherburn and Jenkins [5] reported distinct and reproducible MALDI-TOF-MS mass spectra over the *m/z* range 2,000 to 16,000 for yeasts treated with the cell wall digesting enzyme lyticase, which facilitated discrimination of various *Candida* spp. and strains of *Saccharomyces cerevisiae*. Qian *et al*. [6] reported that simple pretreatment of yeast cells with 50% methanol significantly improved the mass signature quality and, under optimized conditions, distinguished *Candida* spp. from various other yeast genera and from *Aspergillus* spp. For bacteria,

Madonna et al. [7] applied 40% ethanol as a pretreatment in MALDI-TOF-MS detection of proteins above 15 KDa. Discrimination of *Escherichia coli* strains by MALDI-TOF-MS using an extraction solvents comprising formic acid:isopropyl alcohol:water (17:33:50, volume fraction) has also been reported [8,9]. Zhang and Li [10] showed that MALDI-TOF-MS analysis with a two-layer matrix/sample preparation method can be used for direct analysis of protein digests with no or minimal sample cleanup after proteins are digested in a solution containing the surfactants, including sodium dodecyl sulfate (SDS). The feasibility of MALDI-TOF-MS analysis for proteomic samples following treatment with the reducing agent dithrothreitol (DTT) [11] and β-mercaptoethanol [12] has been demonstrated. These reports highlight a wide scope of potentially beneficial pretreatment of intact yeasts prior for discrimination by MALDI-TOF-MS analysis.

Limited success at discrimination of yeasts by MALDI-TOF-MS has been reported in the absence of pretreatment of cells. Carolis et al. [13] reported 94.1% agreement for detection of caspofungin susceptibility in *Candida* isolates by MALDI-TOF-MS compared to a reference method. Yaman et al. [14] reported 94% correct identification of *Candida* strains isolated from blood culture by VITEK 2 and MALDI-TOF-MS. Hof et al. [15] reported differentiation of the two related yeasts *Candida dubliniensis* and *C. albicans* by MALDI-TOF-MS. Spanu et al. [16] reported MALDI-TOF-MS identification results that were concordant with those of conventional culture-based methods for 95.9% of *C. albicans* and 86.5% of non-albicans *Candida* species. Castanheira et al. [17] reported 81.1% agreement of MALDI-TOF-MS discrimination of *Candida* species with that obtained by DNA sequence methods. Other researchers [18, 19] have reported successful discrimination of *Candida orthopsilosis, C. parapsilosis*, and *C. metasilosis* isolates by MALDI-TOF-MS.

Marklein et al. [20] reported 92.5% accuracy of yeast species identification by MALDI-TOF-MS of extracts (using 70% formic acid) from whole cells. More recently, Theel et al. [21] applied formic acid in an on-plate method for differentiation of yeast and reported 81.1% correct identification to the species level.

We report here on optimisation of an MALDI-TOF-MS protocol for discrimination of clinical isolates of yeasts. MALDI-TOF-MS of a *C. albicans* strain was optimised with respect to matrix chemical(s), matrix solvent and target plating method. Various chemical pretreatments (solvents, reductants, detergents) and pretreatment application methods were then evaluated for enhancement of spectral richness. The two best pretreatment – methanol and formate:isopropanol treatment of cells on target plate - were applied to MALDI-TOF-MS discrimination of a set of strains comprising *Candida* spp. (*C.albicans, C. boidinii, C. glabrata, C. guilliermondii, C. krusei, C. parapsilosis, C. tropicalis*,) and *Cryptococcus neoformans*.

2. Materials and Methods

2.1 Organisms and culture conditions

Nineteen clinical isolates from individual patients - obtained from the microbiology laboratory of the Leicester Royal Infirmary National Health Service (LRI-NHS) Trust, Leicester, UK - were included in the study. These isolates were previously identified to the species level by standard laboratory procedures, including morphological ID and the Vitek yeast biochemical card (bioMerieux, Marcy l'Etoile, France). A further three isolates were obtained from the National Collection of Yeast Cultures: *C. albicans* NCYC 597; *C. tropicalis* NCYC 1503; and *Saccharomyses cerevisiae* NCYC 19201. The yeasts were routinely cultivated on Sabouraud dextrose agar (SAB; Oxoid) at 25°C for 72h.

2.2 MALDI-TOF-MS analysis

2.2.1 Matrix system and plating method optimisation

Three matrix chemicals - α-cyano-4-hydroxycinnamic acid (CHCA), sinapinic acid (SA) and ferulic acid (FA) - were tested to select the most suitable matrix chemical for detection of yeast sample peaks over the *m/z* 2,000 – 13,000 range. For each matrix chemical (10mg/mL of CHCA, SA or FA), four matrix solutions of 1:1 acetonitrile:water (ACN:H_2O) with 0.3%, 0.5%, 1% or 2% trifluoroacetic acid

(TFA) were prepared. *Candida albicans* NCYC 597 grown on SAB was used as the test strain. Each of

the 12 matrix variants were applied to yeast samples by three methods: direct colony transfer,

matrix seed layer and sandwich (Fig. 1). Direct colony transfer involved applying one colony onto the

target plate well and allowing to dry in ambient air. Matrix solution (1μL) was applied to the plated

sample, and dried in ambient air for approximately 20min. The matrix seed layer involved applying

1μL of the matrix solution onto the target plate and mixing one colony into the matrix. The sample

was then dried in ambient air for approximately 20min. In the sandwich method, cells were mixed

with matrix as in the matrix seed layer method and, following drying, the cells were overlayed with a

further 0.5μL of matrix solution and again dried (based on Dong *et a*l., 2009). The influence of

varying the ACN:H$_2$O ratio from 1:1 to 3:2 and 2:1, with CHCA as matrix chemical, was also

investigated.

The effects of using one matrix chemical (CHCA, SA or FA) was compared to the use of two matrix

chemicals in a binary matrix (combinations of CHCA, SA and/or FA), to investigate whether the

addition of an extra matrix chemical improved the quality of spectra and peak detection. Single

matrix chemical solutions were combined (1:1 ACN:H$_2$O with 0.3% TFA; 1:1 volumes of CHCA+SA,

CHCA+FA or SA+FA) in eppendorf tubes and applied to yeast samples by the sandwich method (six

matrix chemical combinations tested). Separate matrix layers were also tested by a variation of the

sandwich method: 1μL of the first matrix chemical was wet mixed with one colony, followed by

drying, then 0.5μL of the second matrix was applied, with subsequent drying in ambient air for

approximately 20min (six matrix chemical combinations tested).

2.2.2 Pretreatment chemicals and their application

Pretreatment chemicals (Table 1) were applied to yeast cells by three different methods (a to c),

prior to plating onto target plates by a modification of the sandwich method, using CHCA in 1:1

ACN:H$_2$O with 0.3% TFA. (a) On target plate pretreatment: overlay of pretreatment chemical (0.5 μl)

on plated matrix/cells; (b) In solution pretreatment: cells from one colony were washed in 500μL

HPLC grade water, the cell pellet (4,000rpm for 2 min) was resuspended in pretreatment solution (100µl), vortexed and incubated at room temperature for 5min. Matrix (100µl) was applied to target plate and mixed with 0.5µL of pretreatment chemical/cell suspension, and overlayed with 0.5µl matrix. (c) Washed off pretreatment: as (b) but with washing of pellet in HPLC grade water and resuspension of cells in 100µl of water. Matrix only and pretreatment only controls were prepared by the sandwich method.

2.2.3 MALDI-TOF-MS spectra acquisition and data processing

All yeast strains were analysed on Kratos PCAxima CFRplus MALDI-TOX-MS instrument (Kratos Analytical, Manchester, UK) with data acquisition and analysis via Launchpad 2.8.4 software (Shimadzu Biotech, Manchester, UK). Spectra were obtained over the *m/z* 2,000 – 20,000 range by combining 150 profiles (five laser shots per profile) obtained by manual firing in linear positive mode. Sample chamber vacuum was 10^{-6} to 10^{-7} mbar. A solution of peptides and proteins (MS-CAL1, SigmaAldrich) with a molecular weight range of 757 to 16,952 Da was used to calibrate the mass spectrometer.

The Launchpad software selected the most intense peaks within spectra and processing was optimised to obtain representative spectra for samples by adjusting the following peak processing settings (optimised value in parentheses): average method smoothing (30); baseline subtract (80); threshold (0.3mV); and pairwise cutoff (0.6). Peak lists were aligned using SPECLUST software (web-based application; Alm *et al.* [22]) prior to analysis using SPSS 19 software. Hierarchical clustering for between group linkages was by the Jaccard method.

3.   Results

3.1 Matrix and plating method optimisation using C. albicans NCYC 597

Visual comparison of spectra acquired with CHCA as matrix in 1:1 ACN:H₂O and 0.3% TFA showed the greatest number of resolved peaks by the sandwich plating method, in comparison with the direct colony transfer and the matrix seed layer methods. There was little improvement in the quality of

spectra with increase in TFA concentration. The matrix seed layer generally produced poor quality spectra, with few resolved peaks. Spectra acquired with SA or FA as matrix over the range of TFA concentrations were poor in comparison with use of CHCA, with fewer resolved peaks and higher background interference observed for all three plating methods. Increasing the ACN:H_2O ratio from 1:1 to 3:2 and 2:1 had little influence on the overall quality of spectra obtained using the three plating methods. Similar observations to those obtained for *C. albicans* NCYC 597 were evident in spectra acquired from *S. cerevisiae* NCYC 19201.

With binary matrices combinations (in 1:1 ACN:H_2O with 0.3% TFA), the quality of the spectra obtained from *C. albicans* NCYC 597 was greatest for the separate layer plating method using CHCA/FA as the binary matrix. However, the overall quality of the spectra obtained using the binary matrices were relatively poor in comparison with use of CHCA as the sole matrix chemical.

Based on the above, CHCA in 1:1 ACN:H_2O and 0.3% TFA with target plating based on the sandwich method was selected for studies on the influence of pretreatment chemicals in MALDI-TOF-MS discrimination of yeast. The method afforded good replication of spectra as illustrated in Fig. 2.

3.2 Pretreatment chemicals

Of the three pretreatment chemical application methods (on target plate, in solution, washed off), the on target plate method was the most consistent for producing peak rich spectra for *C. albicans* NCYC 597 and a range of other yeast species. The order of pretreatment chemicals in relation to spectral richness for this method was: Methanol = formate:isopropanol > ethanol=SDS=BugBuster > water=DTT=βME=CHAPS. Fig.3 illustrates the difference in spectra generated for four yeast species after pretreatment with formate:isopropanol.

Methanol and formate:isopropanol pretreatments using the on target plate method was applied to differentiation of a set of isolates, comprising 19 clinical isolates and two *C. albicans* NCYC strains. In comparison with a matrix control (no pretreatment; 801 peaks), the methanol and

formate:isopropanol pretreatments increased the total number of resolved peaks by 23 % (to 985)

and 40% (to 1118 peaks) respectively. Hierarchical clustering of isolates for the control treatment,

only grouped *Candida tropical* strains together (Fig. 4a), while the methanol treatment only grouped

Candida glabrata isolates together (Fig. 4b). Clustering for the formate:isopropanol pretreatment

(Fig. 4c) was enhanced, with *C. tropicalis*, *C. glabrata* and *Cryptococcus neoformans* strains forming

distinct groups. All three treatments failed to cluster all eight *C.albicans* strains together, with the

largest group (7 from 8) formed following formate:isopropanol pretreatment.

Hierarchical clustering was also carried out based on species specific peaks identified within spectra.

In comparison with a matrix on control (no pretreatment; 135 peaks), the methanol and

formate:isopropanol pretreatments increased the number of species specific resolved peaks by 83

% (to 247) and 135% (to 317 peaks) respectively. The relatively low number of species specific peaks

for the control treatment failed to produce a dendrogram. For the methanol pretreatment, only *C.

glabrata* and *C. neoformans* were correctly clustered (Fig. 5a). Following pretreatment with

formate:isopropanol, however, all isolates of individual species were clustered together and

discriminated from other species (Fig. 5b).

4.   Discussion

We have previously shown that pretreatment of yeasts with a cell wall digesting enzyme enhances

discrimination of yeasts by MALDI-TOF-MS [5]. Other researchers have applied various solvent,

reductant or detergent chemicals in MALDI-TOF-MS analysis of intact microorganisms or

proteins/peptides (Table 1). Qian *et al*. [6], using sinapinic acid (SA) as matrix chemical and a dried-

drop method   of sample preparation, reported enhanced discrimination of ATCC strains of *Candida*

spp., *Aspergillus* spp. *C. neoformans* and *S. cerevisiae* using 50% methanol as pretreatment of cells in

MALDI-TOF-MS. In the present work, we have compared various solvent, reductant and detergents

as pretreatment of cells following optimisation of MALDI-TOF-MS (with respect to matrix solution

and plating method etc) for discrimination of clinical isolates of different *Candida* spp. (*C. albicans*,

C. glabrata, *C. tropicalis*) and *C. neoformans*. CHCA as matrix chemical in 1:1 ACN:H$_2$O and 0.3% TFA was shown to generate highly reproducible spectra from *C. albicans* with the greatest number of resolved peaks. Spectra generated using SA or FA as matrix chemical were relatively poor. For all three matrix chemicals, varying the concentration of TFA and the ratio of ACN:H$_2$O had little influence on the quality of spectra generated. Similarly, application of binary matrices produced relatively poor spectra in comparison with use of CHCA alone. Of the three plating methods tested, the sandwich method was superior to the other two methods with respect to both richness and reproducibility of spectra.

MALDI-TOF-MS analysis in the linear mode preserves covalent hydrogen bonds and it is therefore unlikely that large biopolymers within the yeast cell wall are cleaved to give fragments within the 2,000-13,000 *m/z* range. Protein and peptide molecules associated with the yeast cell walls are likely to contribute to diagnostic signals (peaks) within the spectra. However, the richness of spectra obtained suggests that other molecules are also ionised and contribute to spectral richness. Polysaccharides, which are abundant on the yeast cell surface, are obvious candidates. Gluckmann *et al*. [23] reported that, based on different preparation protocols, distinct differences in the desorption/ionisation process for carbohydrates in contrast to peptides/proteins can be elucidated by MALDI-TOF-MS. These authors confirmed laser desorption and gas-phase cationisation as the principal mechanism in ionisation for neutral oligosaccharides, which can be initiated even for particulate analyte material or deposits onto a matrix surface.

Of the eight chemical pretreatments of cells tested in the present work, solvent treatment with methanol or formate:isopropanol were especially effective at enhancing diagnostic mass signatures over the 2,000-13,000 *m/z* range using CHCA as matrix chemical. This is consistent with Qian *et al*. [6] who reported on methanol pretreatment for MALDI-TOF-MS differentiation of yeast species using SA as matrix chemical, and Camera *et al*. [8] who reported on formate:isopropanol pretreatment for discrimination of *E. coli* strains. Interaction of pretreatment solvents with the yeast

cell wall leading to unfolding of cell surface molecules and exposure of smaller molecules to the matrix may account for the increased richness of spectral signals obtained. Solvent pretreatment may also promote release of cell membrane or cytosolic/ribosomal materials to the yeast cell surface [6], giving rise to exposure of an abundance of medium-sized molecules to the matrix chemical. In the present work, the discriminatory value of enhancing MALDI-TOF-MS spectral richness is demonstrated through improved differentiation of clinical isolates of yeasts, with 100% accuracy of clustering of yeast species achieved following pretreatment with formate:isopropanol and use of CHCA in 1:1 ACN:H_2O and 0.3% TFA with target plating based on a sandwich method.

Acknowledgements

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Table 1. Chemical pretreatment of yeasts.

Chemical type	Pretreatment chemical composition	Reference/origin
Solvent	H_2O	Control pretreatment
	Methanol (50%)	Qian *et al*. [6] - yeasts and filamentous fungi
	Ethanol (40%)	Madonna *et al*. [7] - bacteria
	Formate:isopropanol:H_2O (17:33:50)	Camara *et al*. [8] – bacteria
		Domin *et al*. [9] - bacteria
Reductant	Dithiothreitol (20mmol L^{-1})	Bodzon-Kulakowsha *et al*. [11] - proteins
	β-Mercaptoethanol (2% v/v) in 10 mmol L-1 amonium acetate buffer (pH6.3)	Jaafar, Moukadiri and Zueco [12] - proteins and peptides
Detergent	Sodium dodecyl sulphate (SDS; 0.1%)	Zhang and Li [10] - protein digests
	3-[(3-cholamidopropyl)dimethylammonium] -1-propanesulfonate (CHAPS; 0.1%)	Zhang and Li [10] - protein digests
	BugBuster x1 or x10 solution	Novagen ®

Figure legends

Figure 1. Arrangement of yeast cells and matrix chemical on the target plate by (A) direct colony transfer, (B) matrix seed layer and (C) sandwich methods.

Figure 2. Replicate MALDI-TOF-MS spectra generated for *C. albicans* NCYC 597 using CHCA in 1:1 ACN:H_2O and 0.3% TFA by the sandwich plating method.

Figure 3. MALDI-TOF- MS spectra of different *Candida* spp. over the *m/z* range 5,750 to 7,650.

CHCA in 1:1 ACN:H_2O and 0.3% TFA as matrix solution; after pretreatment of cells with formate:isopropanol and plating by the on target method.

Figure 4. Hierarchical clustering (Jaccard) of yeasts from total resolved peaks following pretreatment of cells. (A) water as pretreatment control; (B) methanol pretreatment; (C) formate:isopropanol pretreatment. Strains (y-axis): *C. albicans*, (a); *C. boidinii, (b)*; *C. glabrata, (g)*; *C. guilliermondii, (gu)*; *C. krusei*, (k); *C. parapsilosis*,(p); *C. tropicalis*, (t); *C. neoformans, (n).* Three or more strains clustered together, (▬▬▬).

Figure 5. Hierarchical clustering (Jaccard) of yeasts from species specific resolved peaks following pretreatment of cells. (A) methanol pretreatment; (B) formate:isopropanol pretreatment. Strains (y-axis): *C. albicans*, (a); *C. boidinii*, (b); *C. glabrata*, (g); *C. guilliermondii*, (gu); *C. krusei*, (k); *C. parapsilosis*,(p); *C. tropicalis*, (t); *C. neoformans*, (n). Three or more strains clustered together,

(▬▬▬).