MICROBIAL STRAIN CHARACTERISATION BY MALDI-TOF MS - POSSIBILITIES AND LIMITS

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Overview

- Characteristic MALDI-TOF mass "*fingerprint*" patterns of all microorganisms
- SARAMIS-Software provides a powerful tool for the comparison, identification and building of dendrograms of complex mass spectra
- Discrimination down to the subspecies /strain level can be performed
- Identification in minutes from single cell colonies
- Low influence from growth media, cultivation conditions and growth state (except spores)

Introduction

In 1997 Erhard et al. first employed MALDI-TOF mass spectrometric fingerprinting in the characterization of toxic cyanobacteria. AnagnosTec company was then founded in 1998 to develop and enhance this technology for the characterisation of a wide spectrum of cells. Starting from toxin analysis in cyanobacteria, where with one sample preparation strain identification and secondary metabolite spectra can be obtained, various screening projects followed in dereplication in search for new bioactive metabolites, and the development of standard identification protocols for pathogenic organisms. AnagnosTec's patented data evaluation procedure can be generally applied for the identification and

grouping of microbes including new isolates or other organisms, as well as biological tissues.

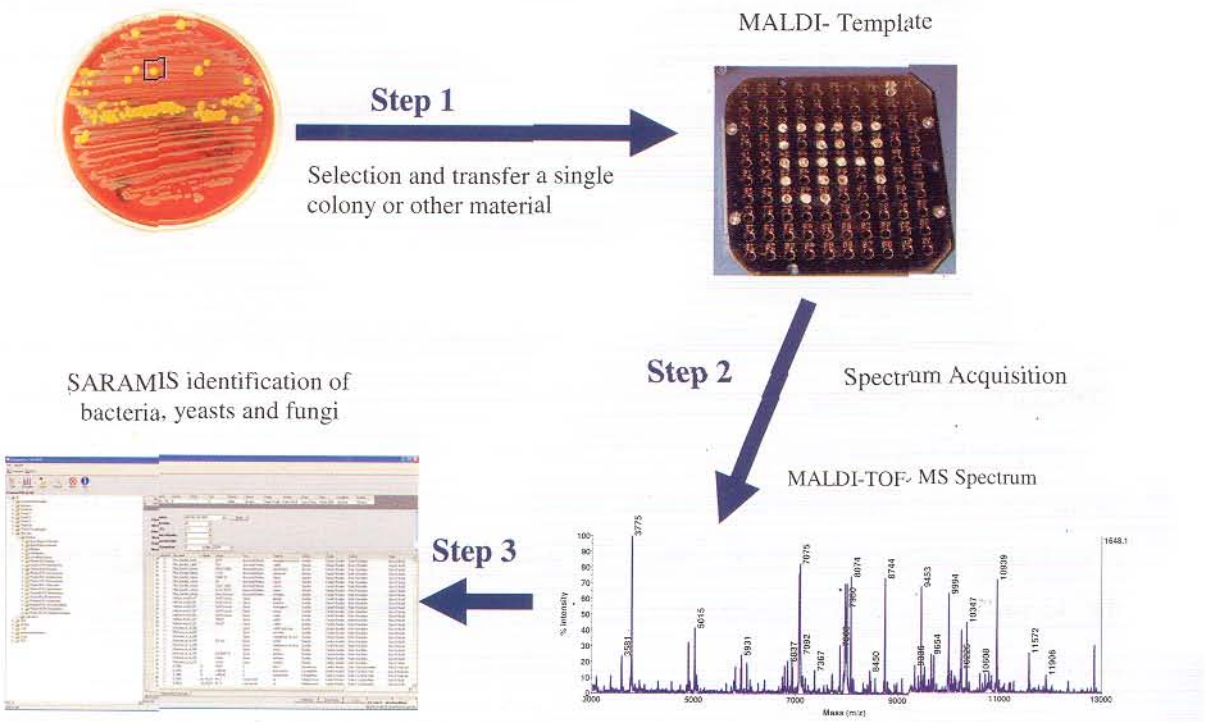
In this lecture an overview will be presented of the different projects performed during the last 10 years demonstrating the broad range of applications. Advantages and limits of the technology will be discussed in comparison to other strain characterisation methods.

MALDI-TOF mass spectrometry is a phenotype characterisation. MALDI-TOF mass spectrometry simplified the mass spectral analysis reducing the number of signals due to gentle ionization. Thus biomolecules to be analysed give only one or two molecular ions, depending on their charges. Therefore **very complex samples like whole cells** can be investigated. Employing unfractionated cell materials, organism-specific signal patterns ("fingerprints") in the mass range of 2.000 - 20.000 Da can be obtained. Depending on the membrane of the organisms most signals correspond to either ribosomal proteins (bacteria) or surface proteins (filamentous fungi). Their highly characteristic masses can be used for the identification and classification of organisms. AnagnosTec holds a database of currently 28.000 spectra of bacteria, yeast and fungi.

Methods

Bacteria, yeast and fungi are grown on standard agar plates over night, or in liquid cultures using standard media. Single cell colonies are selected and directly transfered as a thin film to the MALDI sample plate and mixed with the MALDI matrix. Alternatively fresh material or dried cells are usable. The analysis can be performed using a simple linear MALDI-TOF mass spectrometer with a nitrogen laser (337 nm). Routinely the mass range from m/z = 2.000 to 20.000 is recorded. Following smoothing, baseline correction and peak detection steps, the peaklists are directly transferred into the SARAMIS-software where it is matched against the SARAMIS database. The pattern is analysed to identify the unknown isolates.

Work flow

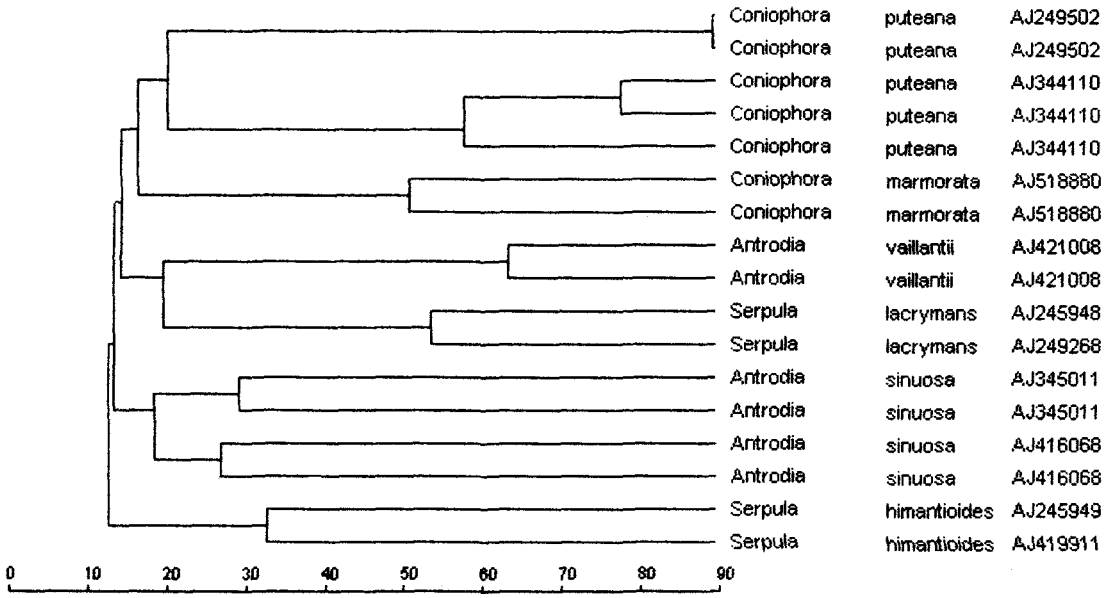


1. Isolation of unknown microorganisms
2. Selection and transfer of colonies onto the MALDI sample plate
3. Addition of matrix
4. Air drying and transfer into the MALDI-TOF mass spectrometer
5. MALDI-TOF MS measurement
6. Additional screening for natural products (low molecular weight) and fragment- and structure analysis using PSD-mode is possible
7. editing of spectra (baseline correction, smoothing, peak detection)
8. Export of peak lists
9. Import of peak lists to SARAMIS software
10. Automated identification/archiving/data storage/dendrogram calculation/search and comparison routines

Results

In cooperation with the Dept. of Wood Science, University Hamburg, Schmidt, O. were published 2005 Schmidt, O. & Kallow, W. the analysis of indoor wood decay fungi:

Figure 2 MALDI-TOF mass spectra dendrogram from the publ. above



Citation from the discussion of the publication Schmidt & Kallow 2005:

"Figure 2 shows an example of the dendrograms that were derived from the mass spectra results. The dendrogram shows the percentage spectrum identity for all investigated species. There is generally considerable variation of signals in MALDI-TOF mass spectra derived from whole cells. The Saramis software relates the percentage identity to the amount of identical mass values. Thus, there is wide variation in the spectra of some identical isolates, especially *A. sinuosa*. The spectral signals of intact cells may be influenced by the phenotype, and in basidiomycetes by the different stages in their life cycle, basidiospore, monokaryon, dikaryon and fruit body, and by the differentiation between surface mycelium, strands and substrate mycelium. Even vegetative mycelium grown on agar shows different zones that correspond to different ages or developmental stages: an apical growth zone with an extending hyphal tip and many organelles; an absorption zone with nutrient uptake; a storage zone with reserve materials; and a senescence zone with pigmentation and lysis (Jennings and Lysek 1999; Huckfeldt 2003). Thus, variation of the biomolecules in intact cells may influence the spectral reproducibility in repeated measurements of the same isolate. **Nevertheless, the evaluation provided a convincing dendrogram that may be suitable for identifying unknown samples by comparison: the spectra of different isolates of the same species (e.g., *S. lacrymans* S7 and S16) clustered together, and closely related species were resolved.** To date, mycelia of the closely related pairs of *S. lacrymans* and *S. himantioides*,

C. puteana and *C. marmorata*, and *Antrodia vaillantii* and *A. sinuosa* could only be differentiated using DNA techniques (Schmidt et al. 2002; Schmidt 2003; Schmidt and Moreth 2003b). **The present study demonstrates that MALDITOF MS is capable of differentiating closely related basidiomycetes from one another.**

The mass data-derived dendrogram in Figure 2 does not reflect the phylogenetic relationship among the six species. The Coniophoraceae (*Coniophora* spp., *Serpula* spp.) (phylogenetic rDNA-ITS tree by Moreth and Schmidt 2005) are twice interrupted by *Antrodia* species, which belong to a separate order. The finding that *Antrodia* species clustered in the midst of a group of Coniophoraceae suggests that it is difficult to infer anything beyond identities based on dendrogram comparison (cf. Krader and Emerson 2004). On the other hand, the technique did resolve different species. The discrepancy between the dendrograms may be due to unrelated data input. The signals within the spectra are associated with numerous compounds, e.g., proteins (Amiri-Eliasi and Fenselau 2001), and cell wall and membrane components (Welham et al. 2000), and not with the rDNA sequence. The identity of the signals is not yet known for basidiomycetes. **Overall, the results show that MALDI-TOF MS is a suitable method for the differentiation of indoor wood decay fungi. Using pure cultures, the method is fast, as no complicated sample preparation is required."**

In cooperation with the DSMZ, Stackebrandt et al. 2005 were published the following results:

Fig. A MALDI-TOF Analysis of Corallococcus strains from the publication Stackebrandt, E., Pauker, O., Erhard, M. 2005

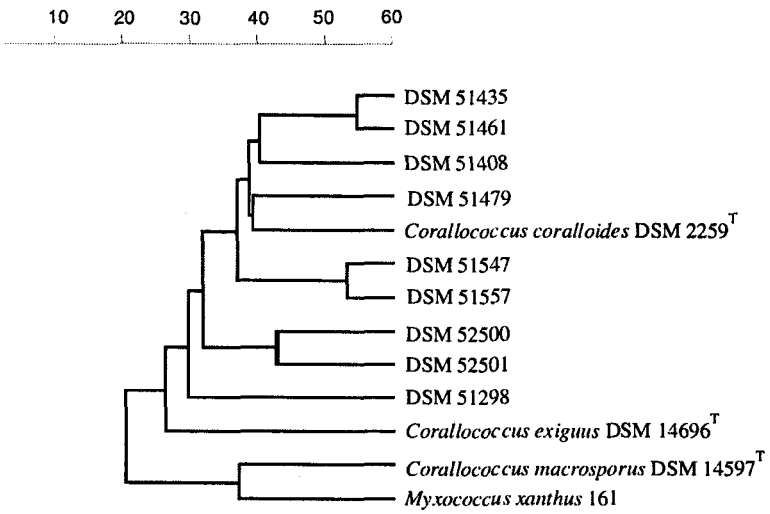
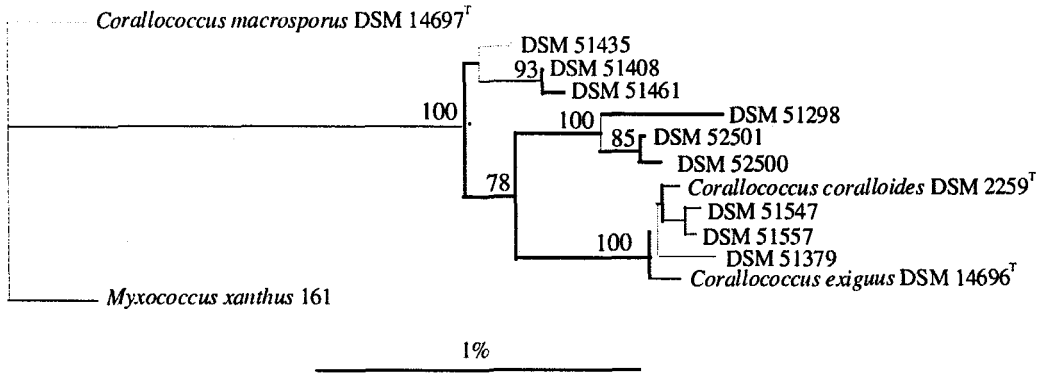


Fig. B: 16sRNA Analysis of Corallococcus strains from the publication Stackebrandt et al 2005



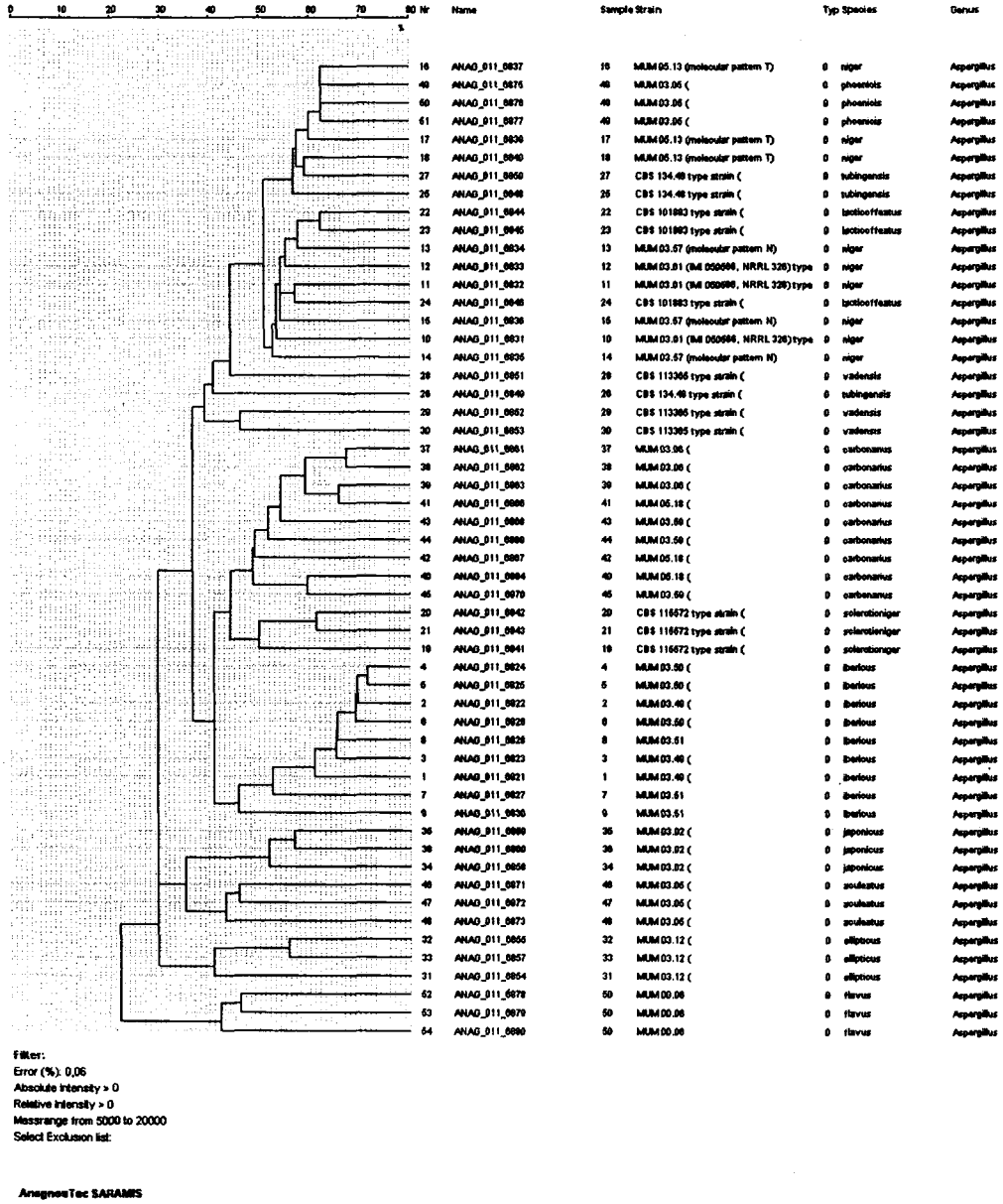
In the discussion of the results Stackebrand et al. 2005 came to the following conclusions:

" We, therefore, compared the clustering of the basis of MALDI-TOF masses with the phylogeny of strains as revealed by sequence analysis of a highly conserved gene, coding for 16S rRNA ....

.....In fact, the dendrogram of MALDI-TOF profile similarities (Fig. A) appears not more divergent from those based on gene sequences (Fig. B and Fig. C-not shown) than the latter are divergent among each other....

.....If, as shown in the present study, the results of MALDI-TOF analyses are similar to those of phylogenetic analyses, this approach, because of low costs and high execution time combined with high reproducibility, has strong advantages over PCR and sequencing technologies in the screening of relationships and, like FTIR, in the de-selection of highly related isolates obtained from environmental studies..."

In cooperation with the MUM, Santos, I.M., Serra, R., Venâncio, A. and Lima, N. were analyzed *Aspergillus species*



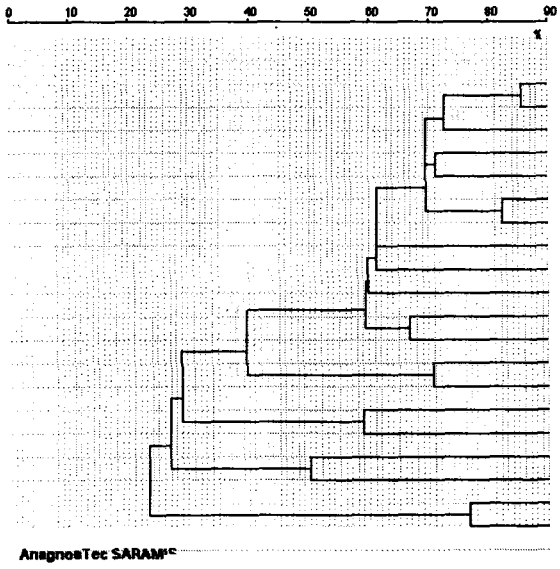
Aspergillus section *Nigri* has been extensively used for various biotechnological purposes and are among the very well known fungi causing food spoilage and biodeterioration of other commodities. The taxonomy of these black aspergilli has been studied by many authors and recently *Aspergillus ibericus* was described as a new species.

Serra et al. (2006) distinguished the new species A. ibericus from A. carbonarius on the basis of its smaller conidia size, absence of ochratoxin A production and DNA sequence analysis of two loci. The dendrogram shows two distinct clades for these two species. Additionally, as it was obtained by Samson et al. (2004) using □-tubulin gene sequence, *A. sclerotioniger* and *A. lacticoffeatus* show relatedness with *A. carbonarius* and *A. niger*, respectively. The *A. niger* aggregate studied is composed by *A. niger*, *A. phoenicis*, and *A. tubingensis*. *A. vadensis* is now described as a new species which came from the *A. niger* aggregate but, from a chemotaxonomical point of view, it is considered the most related species with *A. tubingensis* (de Vries et al., 2005). As it is observed in the dendrogram all these species are well related and aggregated in a clade.

Furthermore, *A. japonicus* and *A. aculeatus* are the only uniseriate species in this section and they clearly show in the dendrogram a sister clade. *A. ellipticus* is an uncommon species and appears as neighbour of these two species in this dendrogram. However it is a clearly distant related species.

A. flavus was used as an out-group species.

In cooperation with SAG Göttingen, Friedl, T. and Müller, J. MALDI-TOF was worked out a dendrogram of Chlorella species



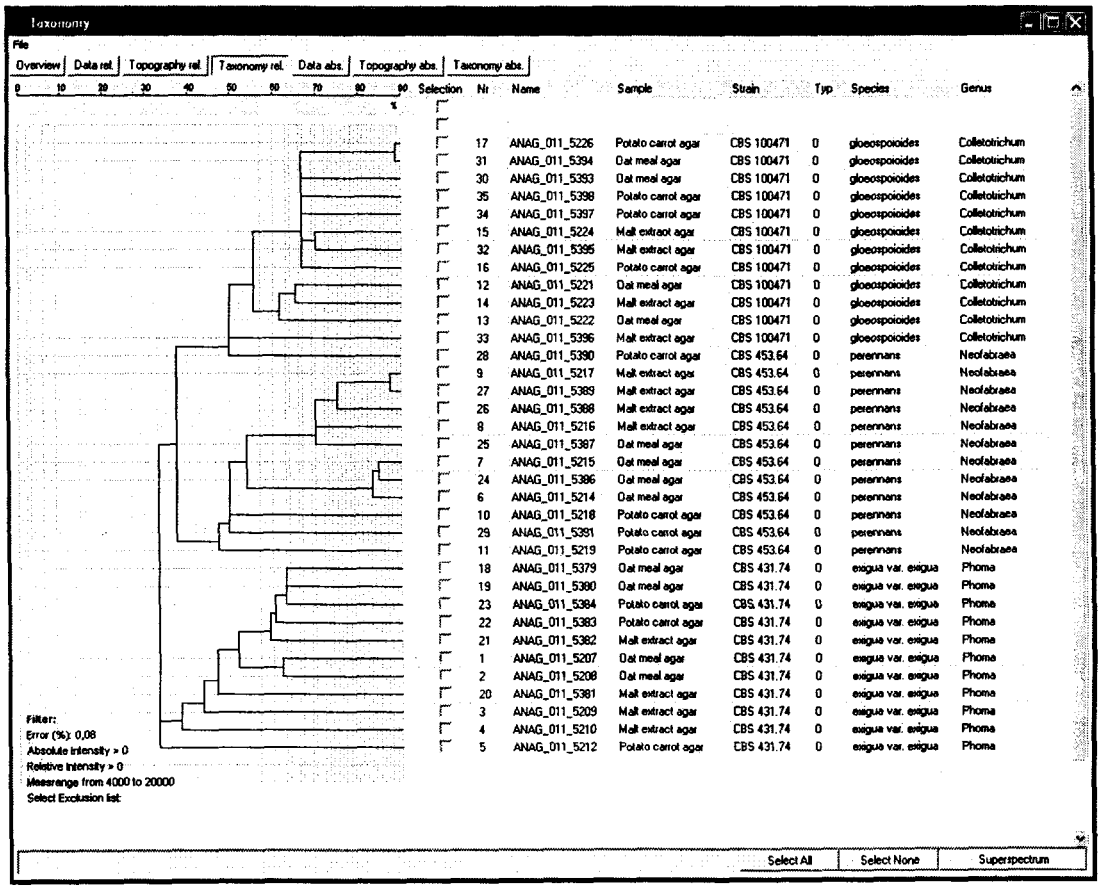
Nr	Name	Sample	Strain	Typ	Species	Genus
19	ANAG_011_5892		SAG 211-11t	0	vulgaris	Chlorella
20	ANAG_011_5893		SAG 211-11t	0	vulgaris	Chlorella
15	ANAG_011_5890		SAG 211-11s	0	vulgaris	Chlorella
13	ANAG_011_5894		SAG 211-11b	0	vulgaris	Chlorella
14	ANAG_011_5883		SAG 211-11b	0	vulgaris	Chlorella
9	ANAG_011_5876		SAG 211-1e	0	vulgaris	Chlorella
10	ANAG_011_5875		SAG 211-1e	0	vulgaris	Chlorella
16	ANAG_011_5891		SAG 211-11s	0	vulgaris	Chlorella
11	ANAG_011_5886		SAG 211-11p	0	vulgaris	Chlorella
12	ANAG_011_5887		SAG 211-11p	0	vulgaris	Chlorella
17	ANAG_011_5881		CCAP 211/11b	0	vulgaris	Chlorella
18	ANAG_011_5882		CCAP 211/11b	0	vulgaris	Chlorella
7	ANAG_011_5878		SAG 211-8k	0	sorokiniana	Chlorella
8	ANAG_011_5877		SAG 211-8k	0	sorokiniana	Chlorella
1	ANAG_011_5896		SAG 211-11g	0	kessleri	Chlorella
2	ANAG_011_5885		SAG 211-11g	0	kessleri	Chlorella
3	ANAG_011_5874		SAG 48.93	0	pusillum	Micractinium
4	ANAG_011_5873		SAG 48.93	0	pusillum	Micractinium
5	ANAG_011_5880		SAG 211-9a	0	saccharophila	Chlorella
6	ANAG_011_5879		SAG 211-9a	0	saccharophila	Chlorella

Dendrogram obtained by MALDI-TOF MS analyses is almost in accordance with relationships obtained by 18 rRNA and ITS2 sequence analyses by Krienitz et al. (2004). With both methods Chlorella saccharophila is the most distinct strain and found not to belong to the "true" Chlorella species by 18S rRNA analyses.

Within these "true" Chlorella species, C. vulgaris, C. sorokiniana and Micractinium pusillum form a sister group to Parachlorella kessleri (formerly Chlorella kessleri) which could not be found by MALDI-TOF MS analyses. However, MALDI-TOF MS analyses could clearly distinguish each single species. Within strains of C. vulgaris, differences between duplicate strains SAG 211-11b and CCAP 211/11B were found by MALDI-TOF MS analyses, which could not be distinguished by ITS sequence or AFLP (Amplified Fragment Length Polymorphism) analyses by Müller et al. (2005). Therefore MALDI-TOF analyses are a promising tool for the discrimination of strains, where ITS sequence or AFLP analyses do not provide enough resolution. Additional tests including a higher number of C. vulgaris strains will be conducted soon to further test correspondence of AFLP and MALDI-TOF MS analyses.

In cooperation with the CBS Netherland, de Hoog, S., Verkley, G. J.M., were analyzed different genus and species of Filamentous Fungi

Test A: Influence of different media on the MALDI-TOF spectra



In this analysis it seems surprisingly that the medium has some influence on the mass spectra of two of the three analyzed species (*Phoma exigua* and *Neofabraea perennans*). The percentage of identity of the mass spectra differ remarkable, but there could be different reasons. The sample preparation was done by a mixture of optically different cells in different regions of the same agar plate. So it may be that the spectra are of different cell growth stages and mixtures of them. These are brand new results and they will be dicussed at the meeting. It could be similar to the wood decay fungi results from Schmidt and Kallow 2005.

AnagnosTec - solutions for archiving and identification of microorganisms

AnagnosTec GmbH develops and distributes user-friendly software applications for archiving, managing, evaluation and presentation of data from MALDI-TOF mass spectrometry. The implementation of universal interfaces makes AnagnosTec's software compatible with mass spectrometers of *various* manufacturers. MS data are transformed into a universal highly compressed format which reduces the data volume by 99% and allows fast data retrieval and evaluation.

"SARAMIS" (Spectral ARchiving And Microbial Identification System) includes a patented procedure for statistic evaluation and weighting of data. In addition, theoretical spectra (superspectra), which can be obtained using this verified and patented procedure, help to multiply the efficiency of the microbial identification and the grouping of samples. The superspectra were built up from signals using statistics and weighting of the specificity. Using this tool microorganisms can be automatically identified.

The separation **limits** of the MALDI-TOF SARAMIS technology are on the level of subspecies or strains. The resolution of separation is limited by the signal proteins in the MALDI-TOF spectra. With this technology were detected the highest concentrated proteins (50 to 100) in the cells (bacteria) or cell surface (filamentous fungi) and so only if strains differ in minimum one of these signals they can be separated. A more detailed discussion will take place during oral presentation at the meeting.

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