UNIVERSITY OF CHEMISTRY AND TECHNOLOGY PRAGUE

MALDI-TOF mass spectrometry tools for microbial identification in archival document investigation

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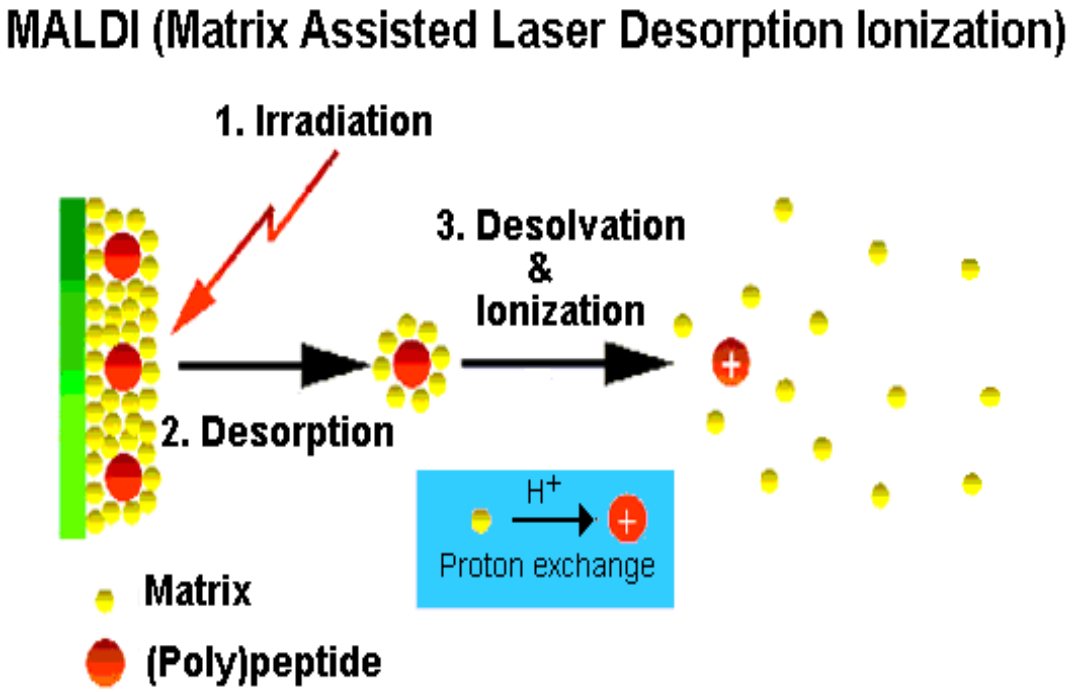
Lodz 30st June2016

MALDI-TOF MS: PRINCIPLE



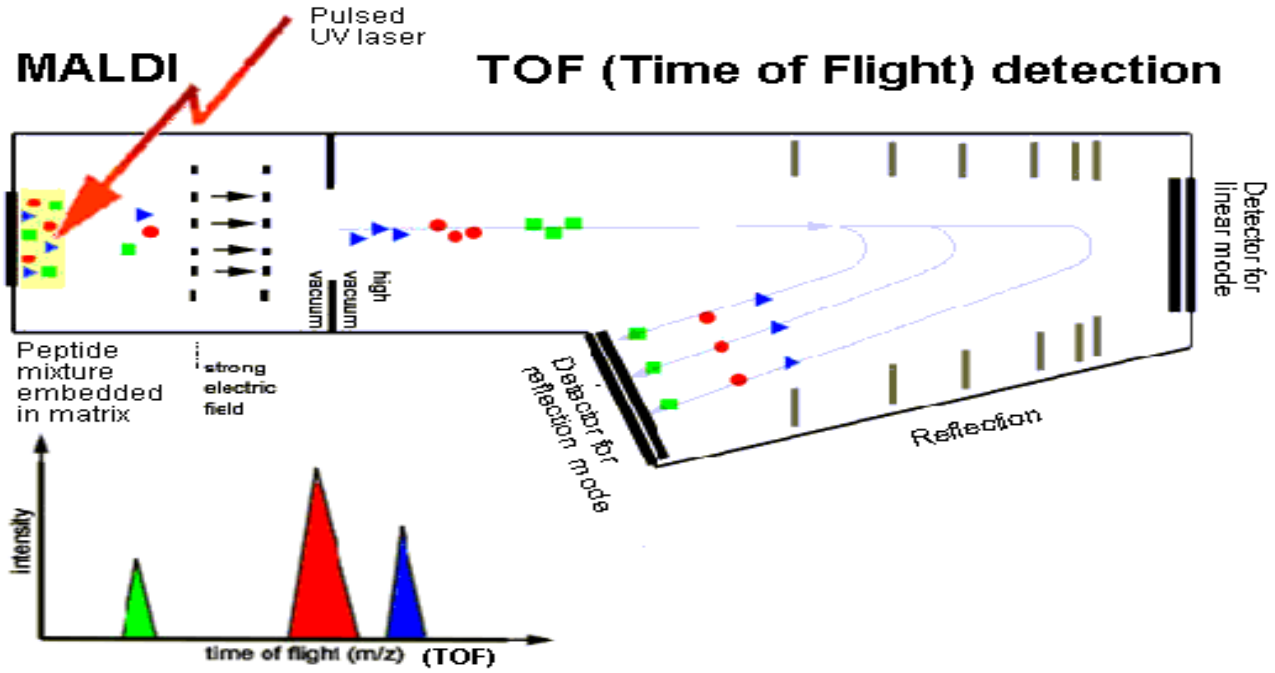
Sample is mixed with matrix
Matrix absorbs the energy of laser and desorbs
Matrix enables the sample (A) to be desorped and ionised as pseudomolecule ionts $[A+H]^+$

Soft ionisation method:

- low level of sample fragmentation

Animation: http: /cmgm.stanford.edu/pan/section_html/MS/

Matrix-**A**ssisted **L**aser **D**esorption **I**onization-**T**ime of **F**light **M**ass **S**pectrometry

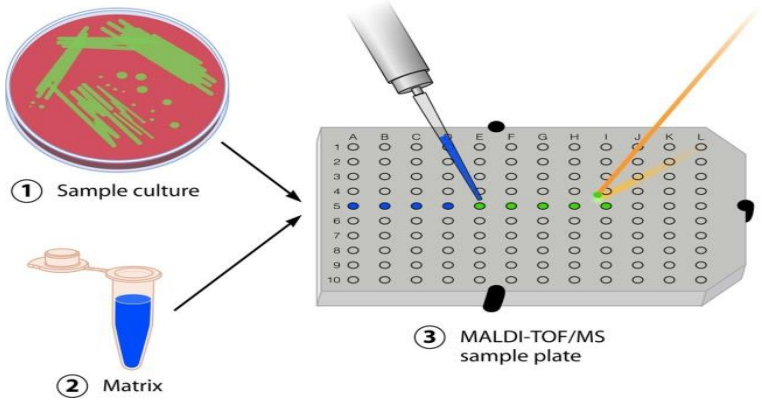


Time of flight is a function of the specific ion mass (m/z)

$$\frac{m}{z} = 2eU\frac{t^2}{L^2}$$

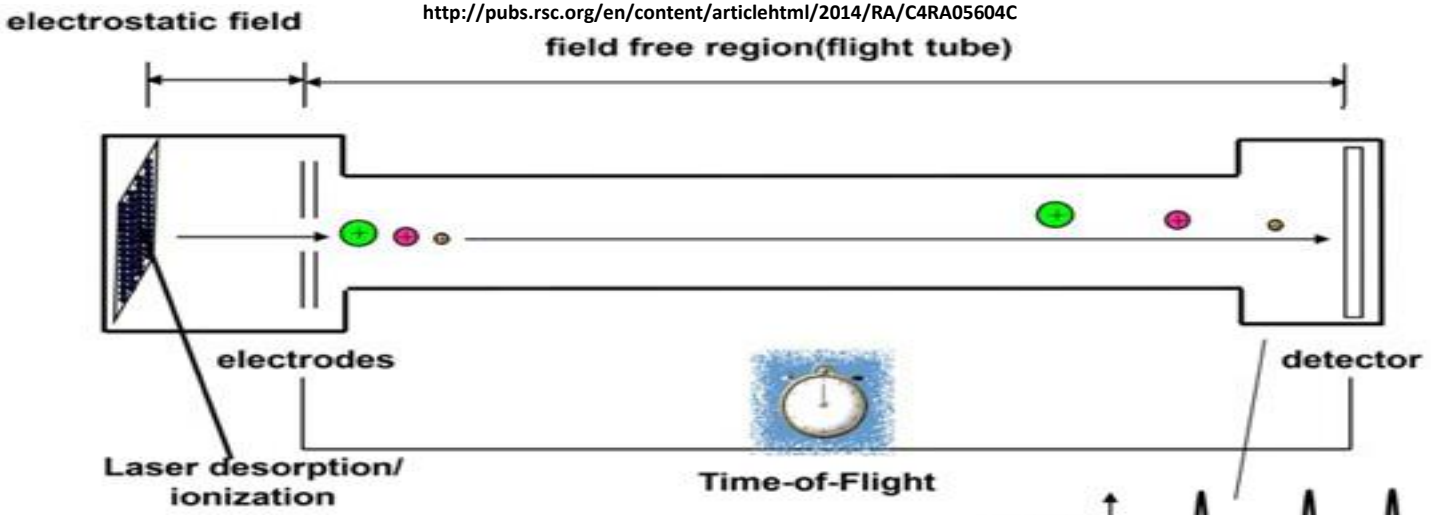
m mass,
z charge,
L length of drifting zone,
e elementary charge,
U speeding voltage

MALDI-TOF MS: MICROBIOLOGY



http://cmr.asm.org/content/26/3/547.figures-only

http://pubs.rsc.org/en/content/articlehtml/2014/RA/C4RA05604C

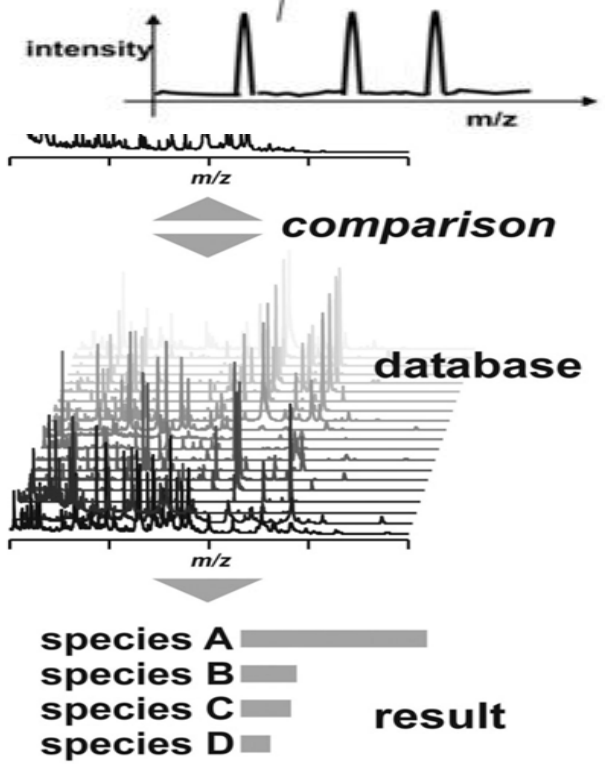


1) sample preparation

- microbial culture or its proteins extract is smearing onto a steel plate and covered over by matrix

2) MALDI-TOF MS analysis

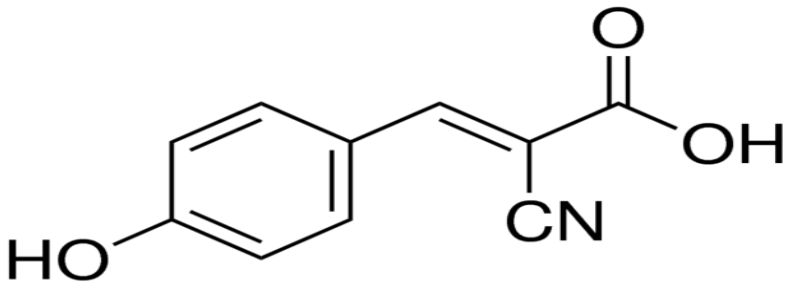
unique mass spectral fingerprint of desorbed microbial cell components **(mainly intracellular proteins)**, different among genera, species or also some strains



3) identification:

comparison of mass spectrum to those of reference strains in database

MALDI-TOF MS: SAMPLE AND MATRIX



http://www.sigmaaldrich.com/catalog/product/sigma/c8982?lang=en&region=CZ

Matrix:

- able to **absorb the energy of the laser** (usually 337 nm)
- able to **crystalise with samples** (empirically tested)
- usually **acid character** (proton ionisation of sample), **dissolved in organic solvent**

CHC: α-Cyano-4-hydroxycinnamic acid
(organic solvent: 50% acetonitrile with 2,5 % trifluoracetic acid)

SA : 3,5-Dimethoxy-4-hydroxycinnamic acid (sinapic acid)
DHB: **2,5-Dihydroxybenzoic acid**

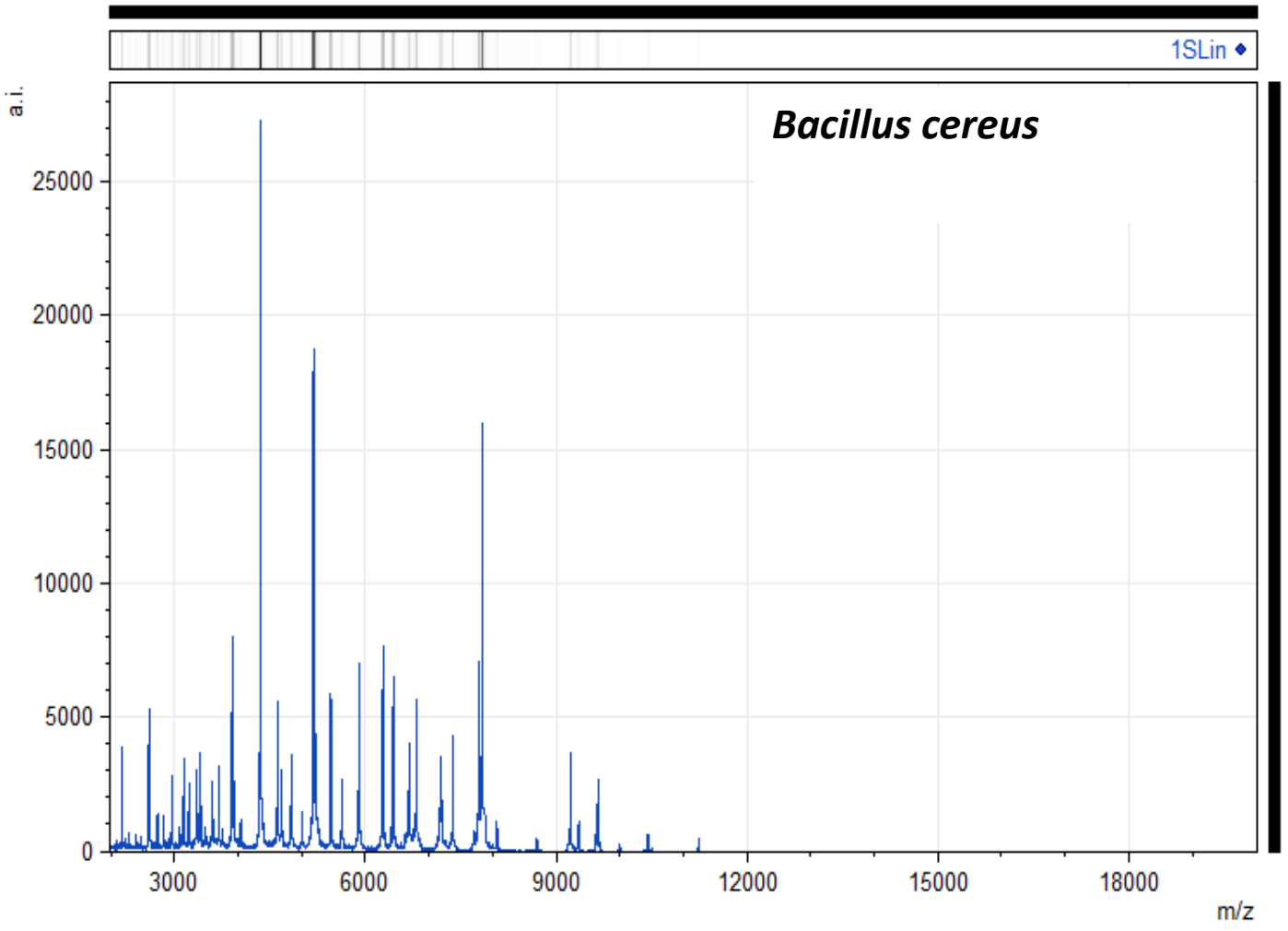
Direct transfer

- spreading of intact **cells grown on agar plate** (recommended non-selective) **directly onto a steel plate**
- lysis of cells occurs during the contact with **acid matrix and by laser desorption**
- most bacteria

Proteins extraction

- If the action of acid matrix and laser is not sufficient to disrupt the bacterial cell
- previous extraction of proteins by organic acids and/or alcohol (**e.g. ethanol and 70% formic acid**)
- yeasts, moulds, some species of bacteria (depending on the cell wall composition)
- the cultivation in liquid medium can be required

MALDI-TOF MS: MASS SPECTRUM



Visualisation of mass spectrum protein profile – (software mMass 5, Strohalm *et al.*, 2010)

Mass spectrum (protein profile)

- z equals usally to 1+
- the usual range for identification:

2000 -20 000 m/z

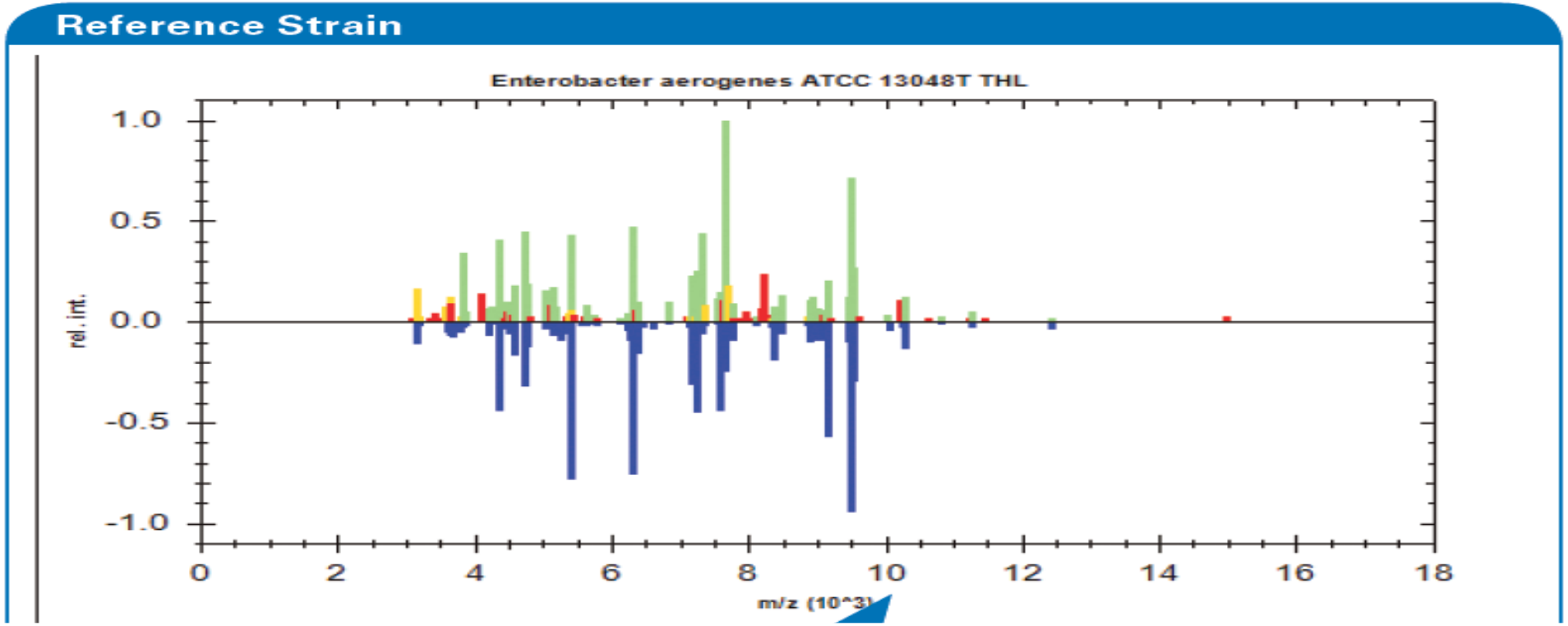
- the intensity of single peaks corresponds to the abundance of the protein

<u>Proteins dominant in the protein profile</u>

- abundant, basic and mediumly hydrophobic
- **mainly ribosomal proteins**
- further cold-shock and heat-shock proteins, chaperons etc.

conserved house-keeping gene = conserved proteins = in acordance with identification based on DNA

MALDI-TOF MS: IDENTIFICATION



Bruker Autoflex Speed
Database MALDI Biotyper 3.1

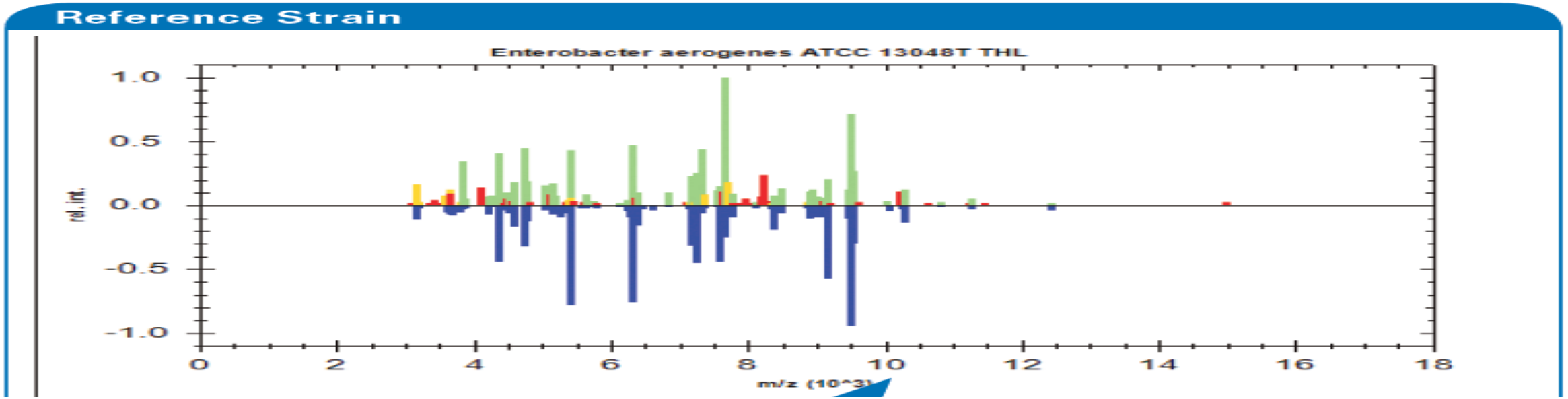
Bruker Bacterial Test Standard (Bruker Daltonics, SRN)

Main protein profile (derived from single protein profiles) of unknown microorganism to be compared with main protein profiles of reference strains present in database by software

Commercial databases from different MALDI-TOF MS producers

Bruker Daltonics – MALDI BIOTYPER, **Shimadzu** - Shimadzu Launchpad software + SARAMIS database, **Biomérieux** - VITEK® MS

MALDI-TOF MS: IDENTIFICATION



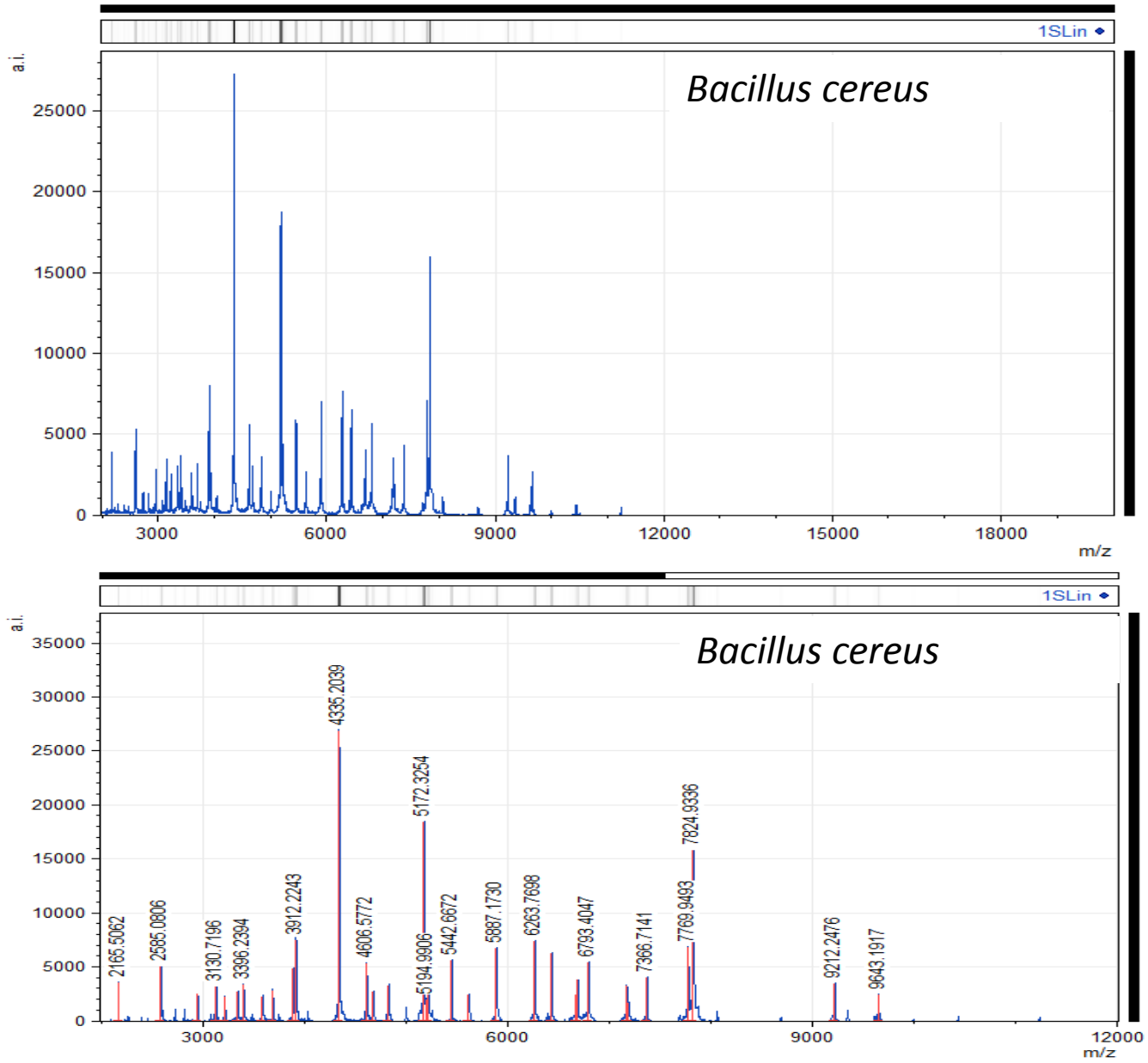
BioTyper:
Comparison of peak positions, itensities and frequencies

Score value:
- 0 (none similarity) - 1000 (absolute similarity)
- in decadic logarithm - **log(score value): 0-3**

Reliability of th identification

Range	Description	Symbols
2.300 ... 3.000	**highly probable species identification**	**(+++)**
2.000 ... 2.299	**secure genus identification, probable species identification**	**(++)**
1.700 ... 1.999	**probable genus identification**	**(+)**
0.000 ... 1.699	**not reliable identification**	**(-)**

Bacillus cereus



Rank (Quality)	Matched Pattern	Score Value	NCBI Identifier
1 (+++)	*Bacillus cereus* DSM 31T DSM	2.554	1396
2 (++)	*Bacillus cereus* 994000168 LBK	2.203	1396
3 (++)	*Bacillus weihenstephanensis* DSM 11821T DSM	2.158	86662
4 (++)	*Bacillus mycoides* DSM 2048T DSM	2.155	1405
5 (++)	*Bacillus cereus* 4080 LBK	2.147	1396
6 (+)	*Bacillus thuringiensis* DSM 2046T DSM	1.975	1428
7 (+)	*Bacillus pseudomycoides* DSM 12442T DSM	1.787	64104
8 (-)	*Bacillus bataviensis* DSM 15601T DSM	1.369	220685
9 (-)	*Brevibacterium linens* IMET 11075T HKJ	1.347	1703
10 (-)	*Acinetobacter towneri* DSM 14962T HAM	1.345	202956

MALDI-TOF MS: IDENTIFICATION – KEY FACTORS

QUALITY OF MASS SPECTRUM (PROTEIN PROFILE)

- Performance problems
  - Quality of matrix – checked in the standard sample (mixture of proteins)
  - Quality of sample preparation performance
    - Pure culture
    - Presence of other chemical (agar, NaCl….) – (crystalisation, noise, peaks shift etc.) - can be distinguished
    - Extraction methods – mistakes in preparation
    - Direct spreading of culture on the spot
      - too low concentration – „no peaks found“ – only some places of spots are measured
      - too high concentration - worse crystalisation, high level of noise
- Not optimal procedure for the sample preparation – the intracellular proteins are not realeased in sufficient concentration

MALDI-TOF MS: IDENTIFICATION – KEY FACTORS

RANGE OF THE DATABASE

- No reference strains of the species or even the genus are present in the database – identification is not precise or not able at all.
- More reference strains for the species – higher probability for the identification with high score value
- It is possible to build an own database by generating of main protein spectra (repeated measurements of chosen reference strains)

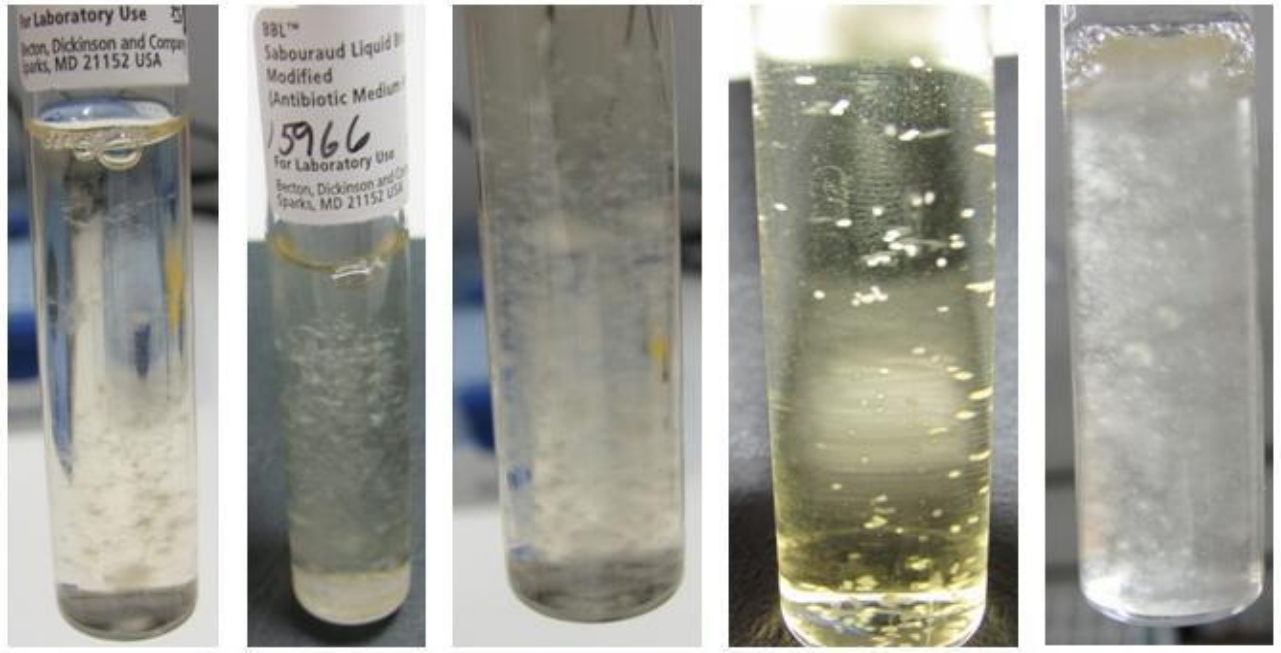
METHOD LIMITS

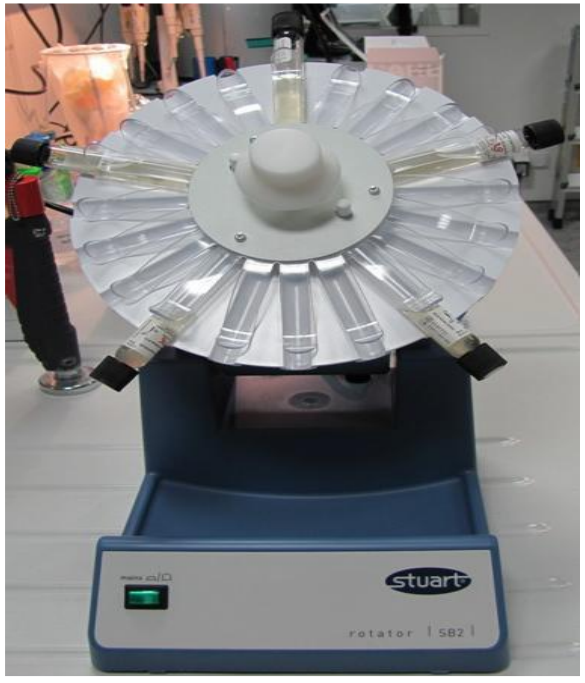
- ´The identification is based comparing two protein profiles (unknown sample, reference strain) by a certain algorithm.
- Some species are difficult to be distinguished
  - Their protein profile are very similar due to their very close taxonomic relation
    - e.g. *Enterobacter cloacae* komplex
    - *E. coli* and *Shigella* spp. Should be correctly one species, differ only in phenotypical features

MALDI-TOF MS: IDENTIFICATION – MOLDS

identification of molds and multicellular fungi still persists as one of the most challenging aspects of microbiology

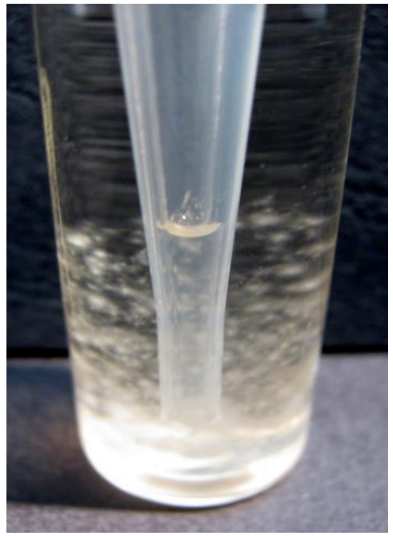
- **cultivation method**
  - to prevent the germination process and the formation of spores
  - production of a uniform mycelium easy to be destroyed by the recommended extraction procedures
  - Cultivation in liquid medium when to rotato over head
  - Cultivation in liquid medium can be used also for bacteria when the microorganism is difficult to be harvested from the agar plates (e.g. *Streptomyces*)

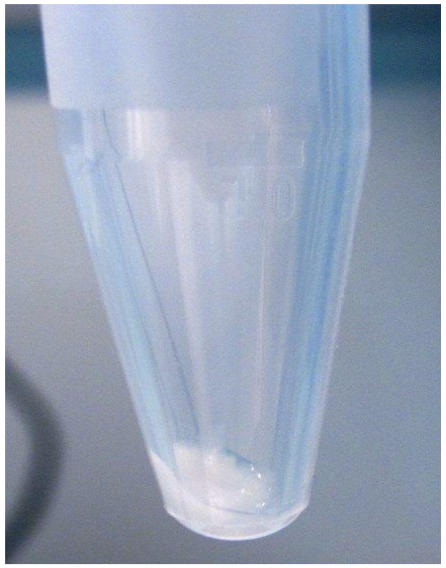




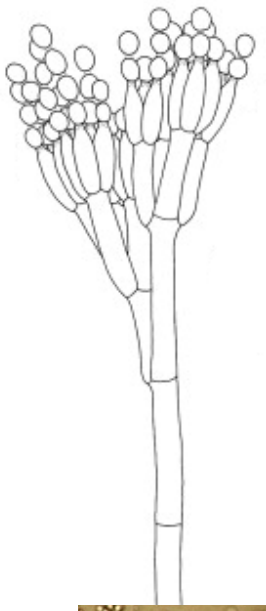
MALDI-TOF MS: IDENTIFICATION – MOLDS

- **Extraction method**
  - to harvest the sediment, centrifuge to obtain pellet and to wash pellet in sterile distilled water twice times
  - To wash pellet with 75% ethanol and let the pellet to dry
  - To destroy cell walls by mixin pellet with the appropirate volume of formic acid and the acetonitrile
  - After centirfugation to transfer on spots the supernantants (containing released proteins)
  - For Mycobacteria is reccomended to use zirconium beads





strain 1 designate: *P.chrysogenum*



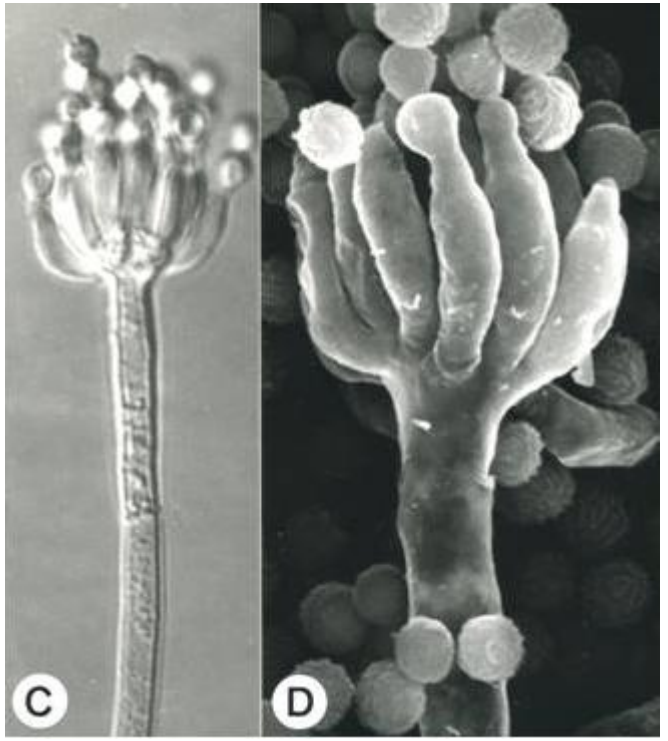
Exudate – yellow drops

Colonies growing rapidly. Exudate typically produced. Conidiophore predominantly terverticillate;
Stipes smooth;
Conididia subglobose, globose.

Penicillium* subgen. *Penicillium
Penicillium chrysogenum

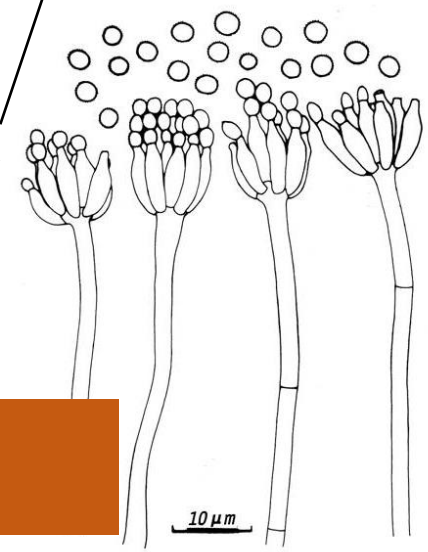
MT - *Penicillium chrysogenum*

strain 3 designate: *P.spinulosum*



http://www.bcrc.firdi.org.tw/fungi/fungal_detail.jsp?id=FU200802270016#

Conidiophore monoverticillate;
Phialides ampulliform;
Conidia spherical, sub-spheroidal.

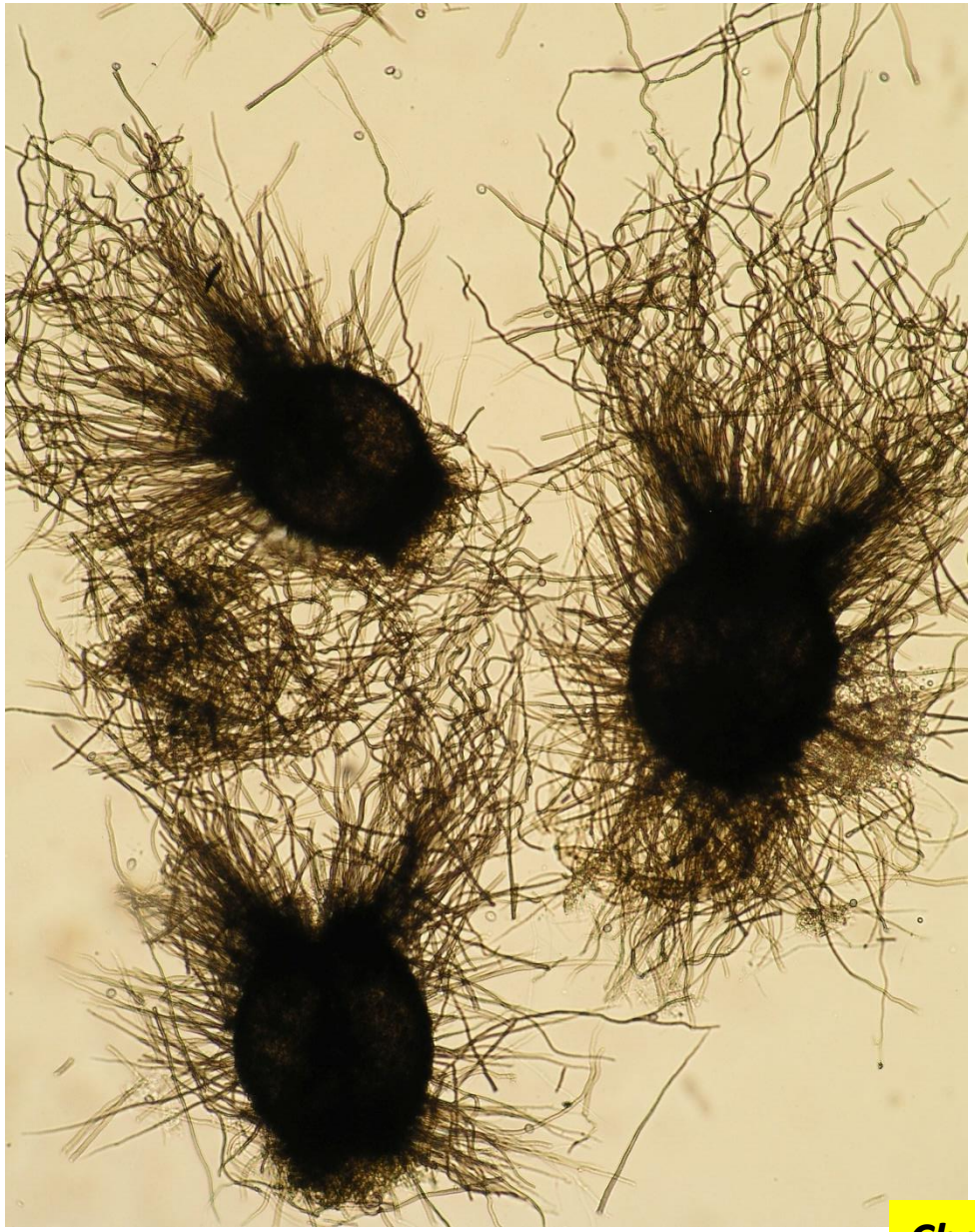


Penicillium* subgen. *Aspergilloides

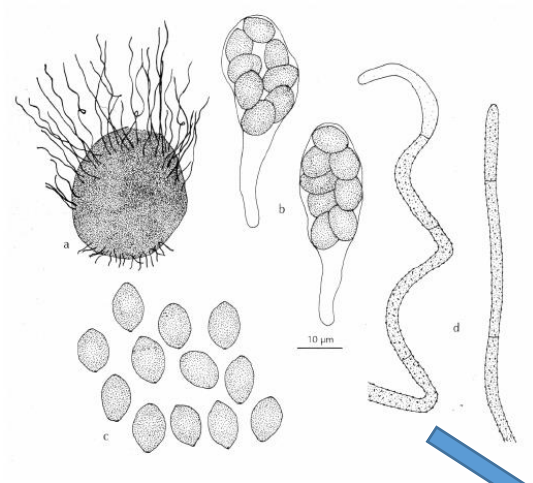
MT - *Penicillium* sp.
***P. spinulosum* not present in Biotyper 3.1**

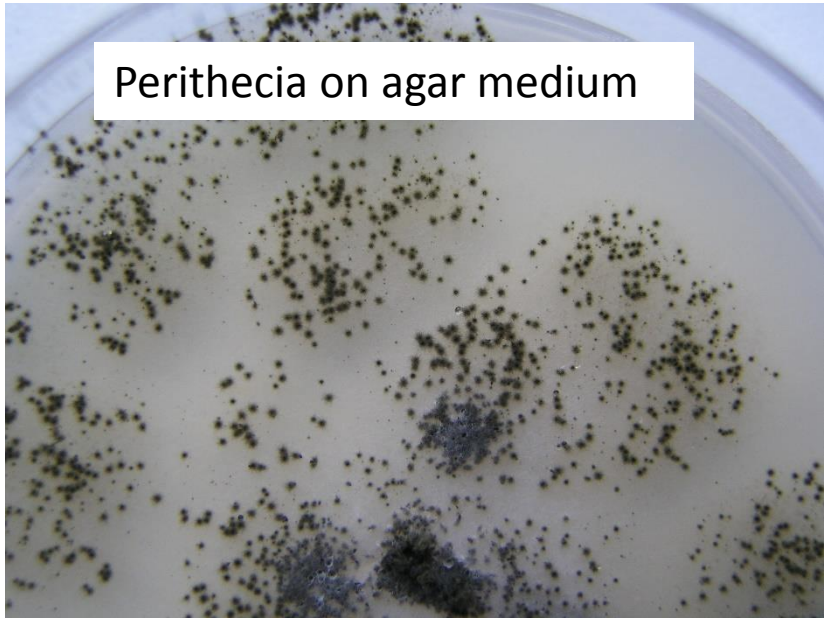
Penicillium spinulosum (CCRC 32565). Penicilli and conidia.

strain 4 designate: *C.globosum*



Food and Indoor Fungi (Samson et.al., CBS, 2010)



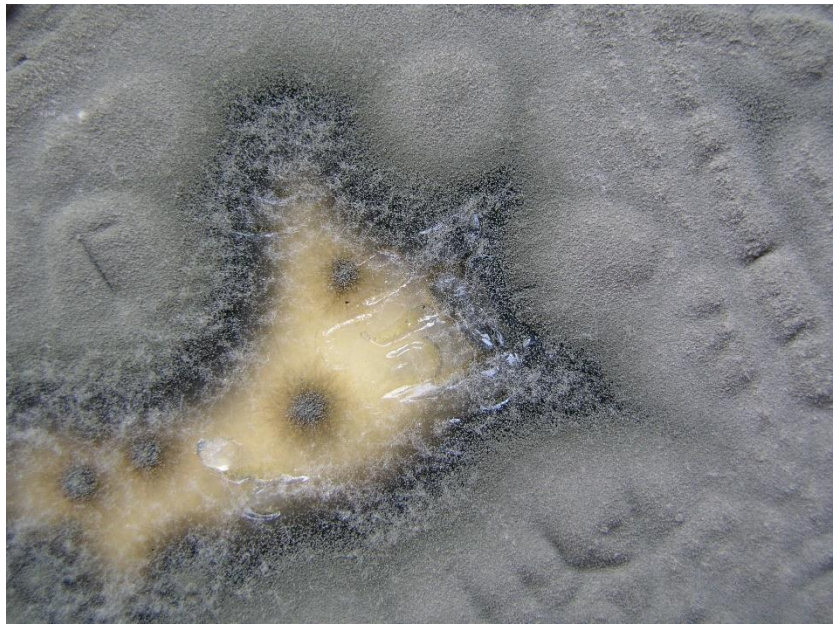


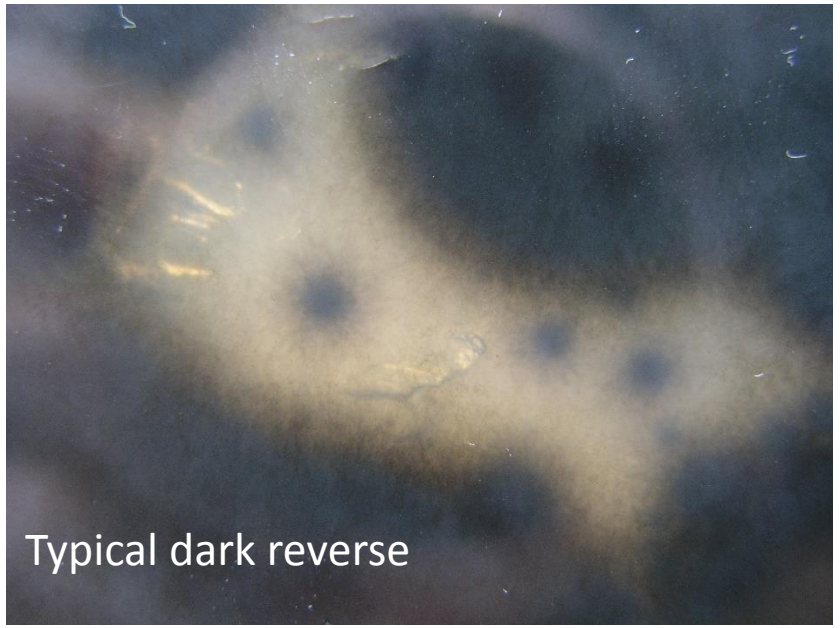
Perithecia dark brown, globose to ovoid. Perithecial hairs, numerous, septate, unbranched, flexuous or even coiled. Ascospore brown with an apical germ-pore.

Chaetomium globosum

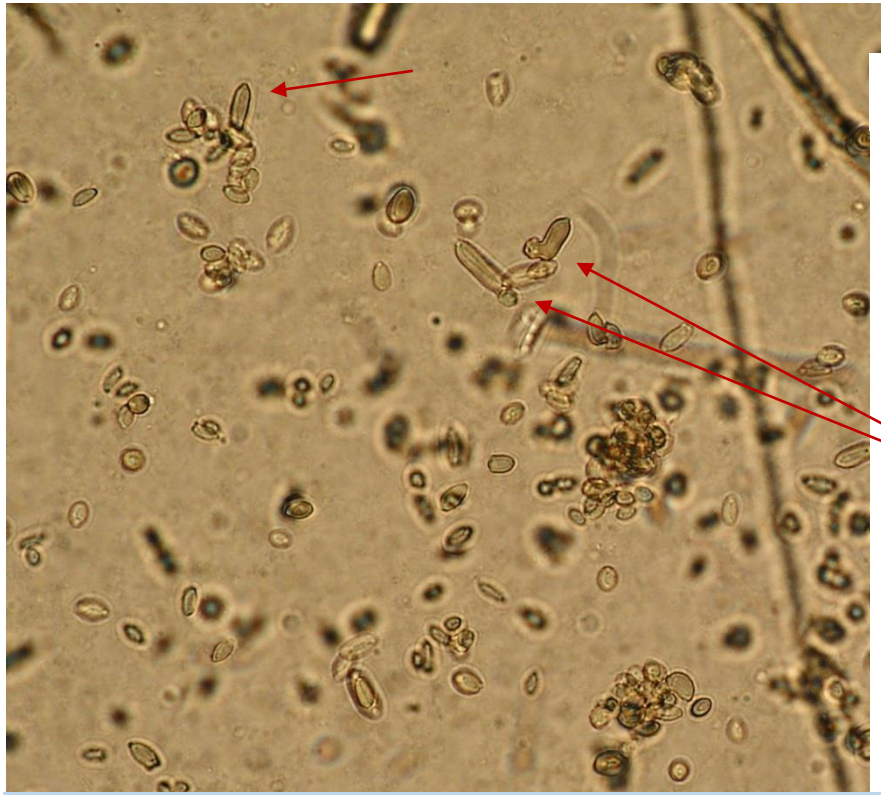
MT– problem to release proteins from the mycelium - – bad quality mass spectrum

Strain 5 designate: *C.globisporum*

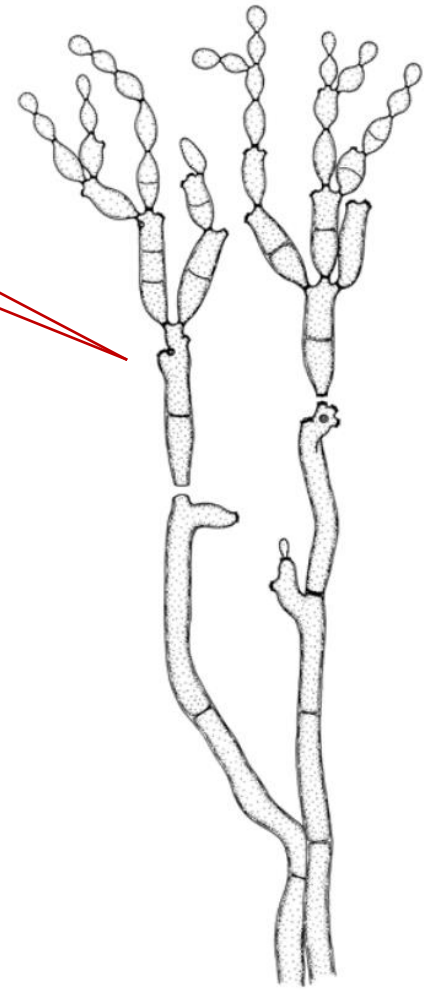




Typical dark reverse



ramoconidia



Colonies velvety and dull spinach green, reverse is dark;
Coniophore are olive brown;
The terminal conidia are obovoid, ramoconidia are often aseptate. *C. cladosporioides* is a complex and contains several species.

***Cladosporium cladosporioides* complex**

MT– problem to release proteins from the mycelium - – bad quality mass spectrum

Cladosporium globisporum

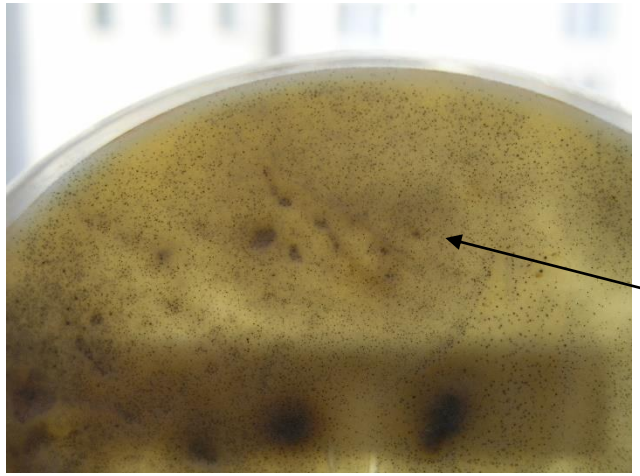
Show empty fields

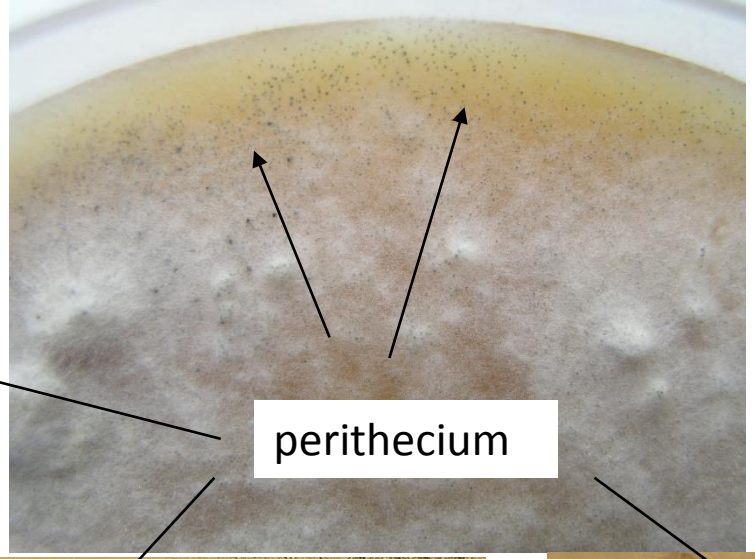
General information

Summary:	Cladosporium globisporum Bensch, Crous & U. Braun, Studies in Mycology 67: 51 (2010) [MB#517080]
MycoBank #:	517080
Epithet:	globisporum
Rank:	sp.
Authors:	Bensch, Crous & U. Braun
Authors (abbreviated):	Bensch, Crous & U. Braun
Literature:	Bensch, K.; Groenewald, J.Z.; Dijksterhuis, J.; Starink-Willemse, M.; Andersen, B.; Summerell, B.A.; Shin, H.D.; Dugan, F.M.; Schroers, H.J.; Braun, U.; Crous, P.W. 2010. Species biodiversity within the Cladosporium cladosporioides complex (Davidiellaceae, Capnodiales). Studies in Mycology. 67:1-94

http://www.mycobank.org/name/Cladosporium%20globisporum

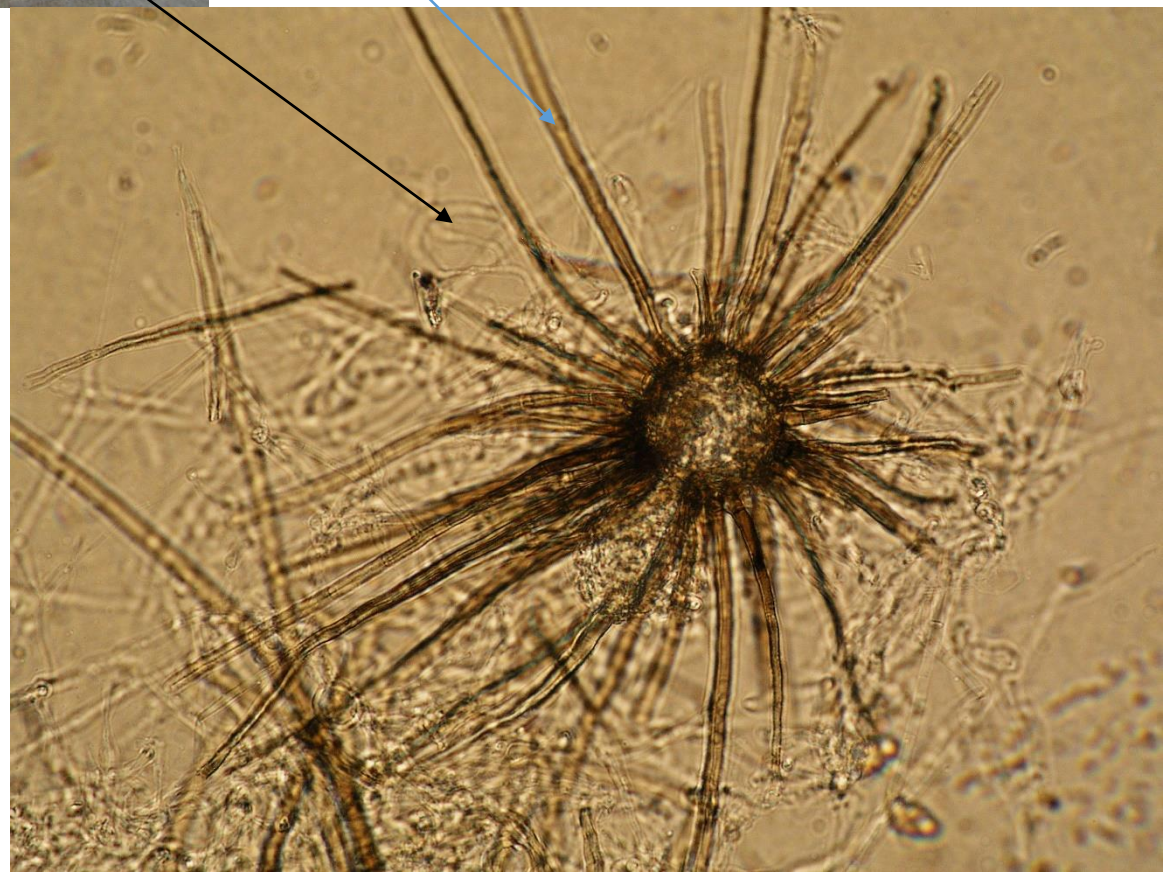
strain 6 designate: *M. deflexum*





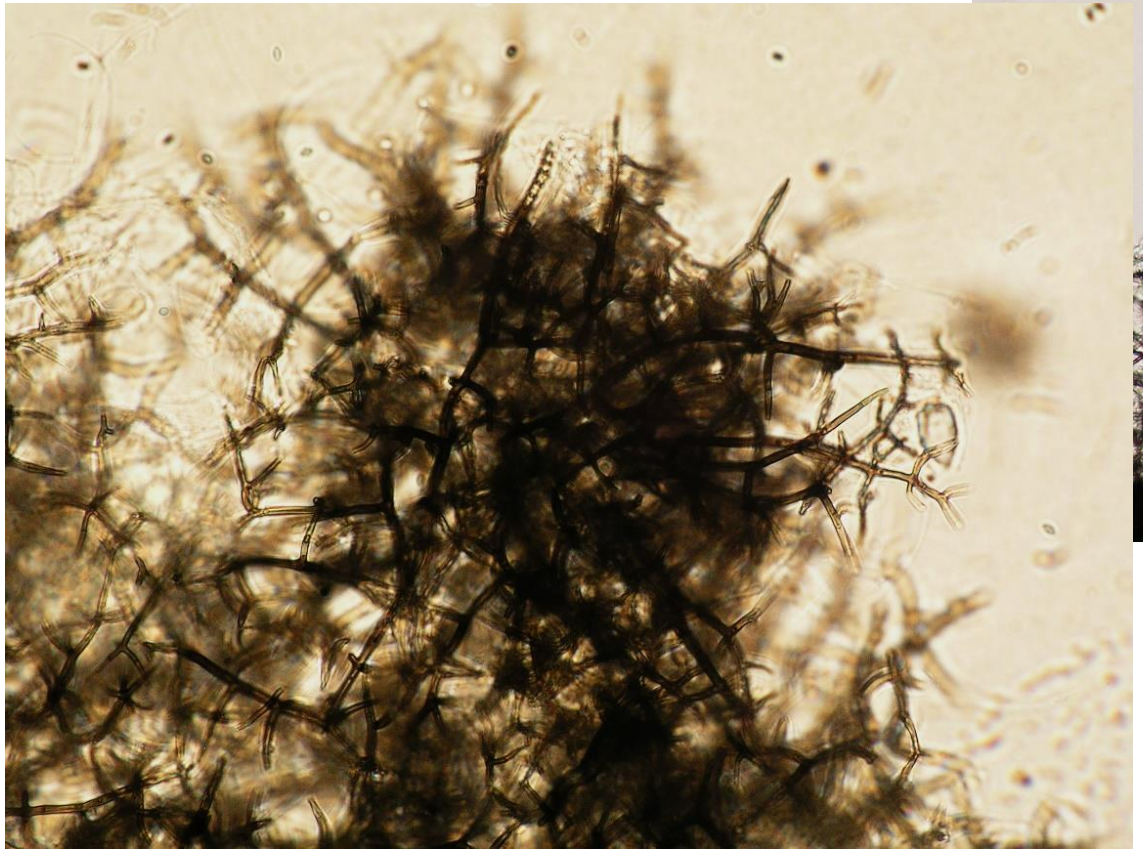
Ascomycetes – perithecium – probably genus ***Chaetomium*** (able to be identified more precisely after spores maturation)

MT– problem to release proteins from the mycelium - – bad quality mass spectrum



Strain 7 designate: no determine

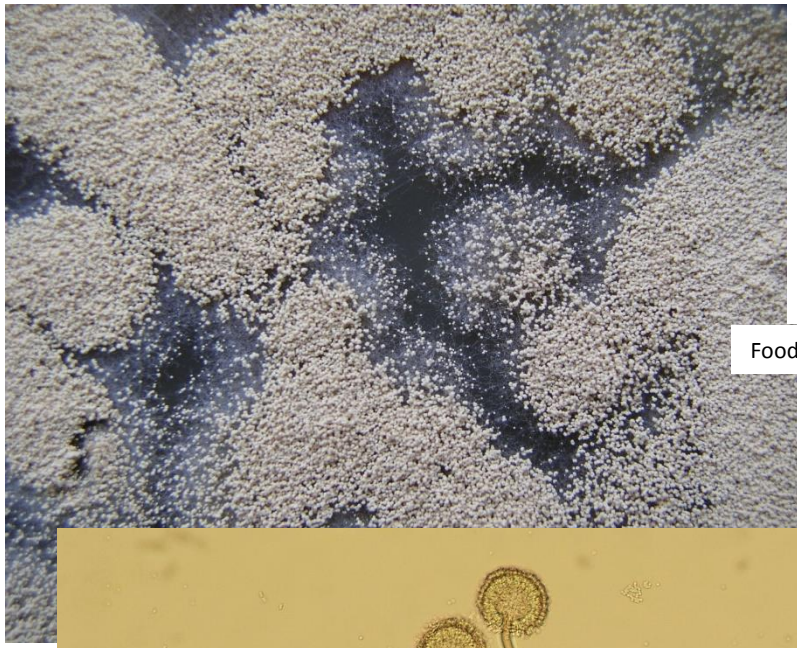
MT – *not* reliable identification *(Clostridium sp., Candida) – Myxotrichum* not present in Biotyper 3.1

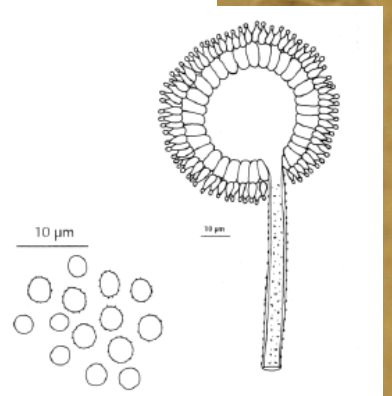


Colonies growing rather slowly, greenish; reverse light purple.
Gymnothecia dark brown to black, spherical. Peridial hyphae dark brown, branched, septate.

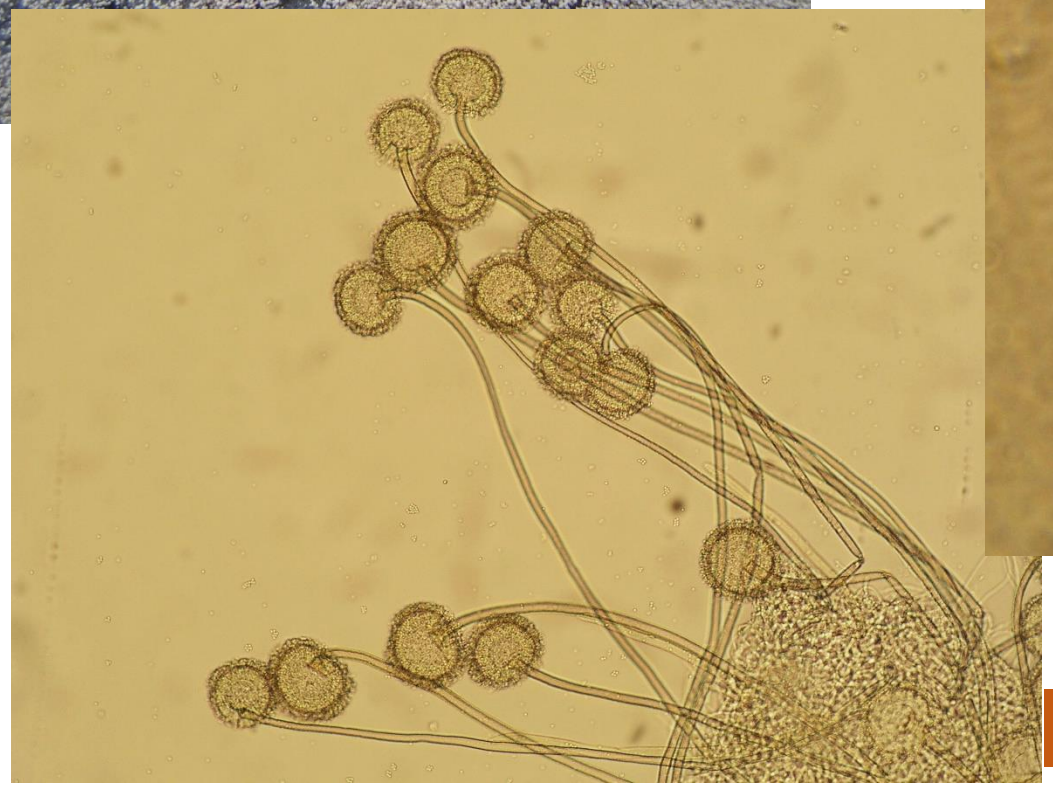
***Myxotrichum sp.* (most probably *M.deflexum*)**

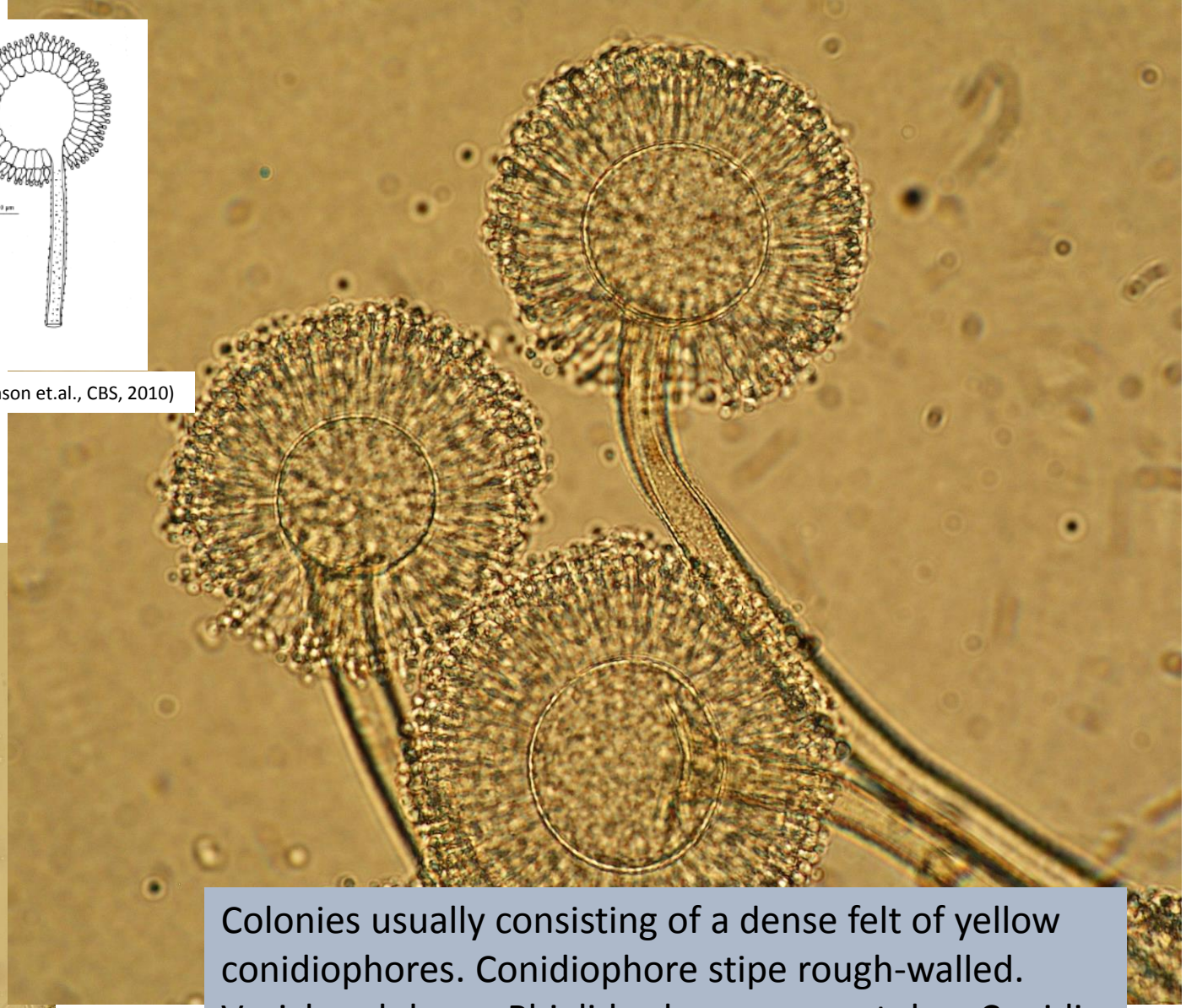
strain 8 designate: *A.ochraceus*





Food and Indoor Fungi (Samson et.al., CBS, 2010)





Colonies usually consisting of a dense felt of yellow conidiophores. Conidiophore stipe rough-walled. Vesicles globose. Phialides borne on metulae. Conidia globose to subglobose.

MT - *Aspergillus ochraceus*

***Aspergillus ochraceus* group**

Strain 10 designate: *A.creber*

http://www.indexfungorum.org/names/names.asp

Search by:- [redacted] 525724 records on-line

Name Epithet Genus Family higher **Enter a search term:-** add new record

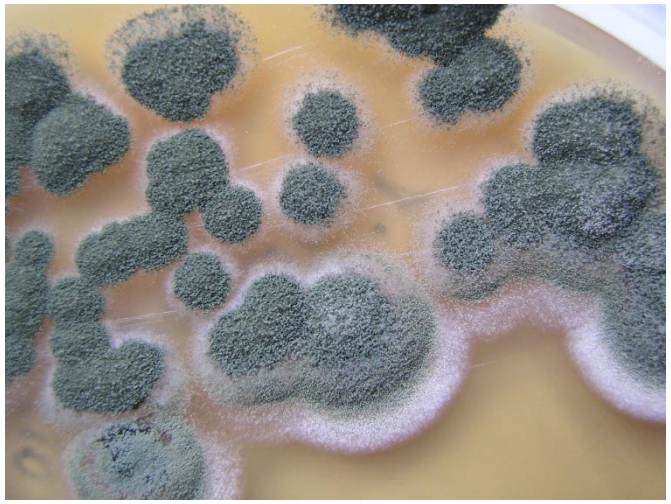
aspergillus creber | Search

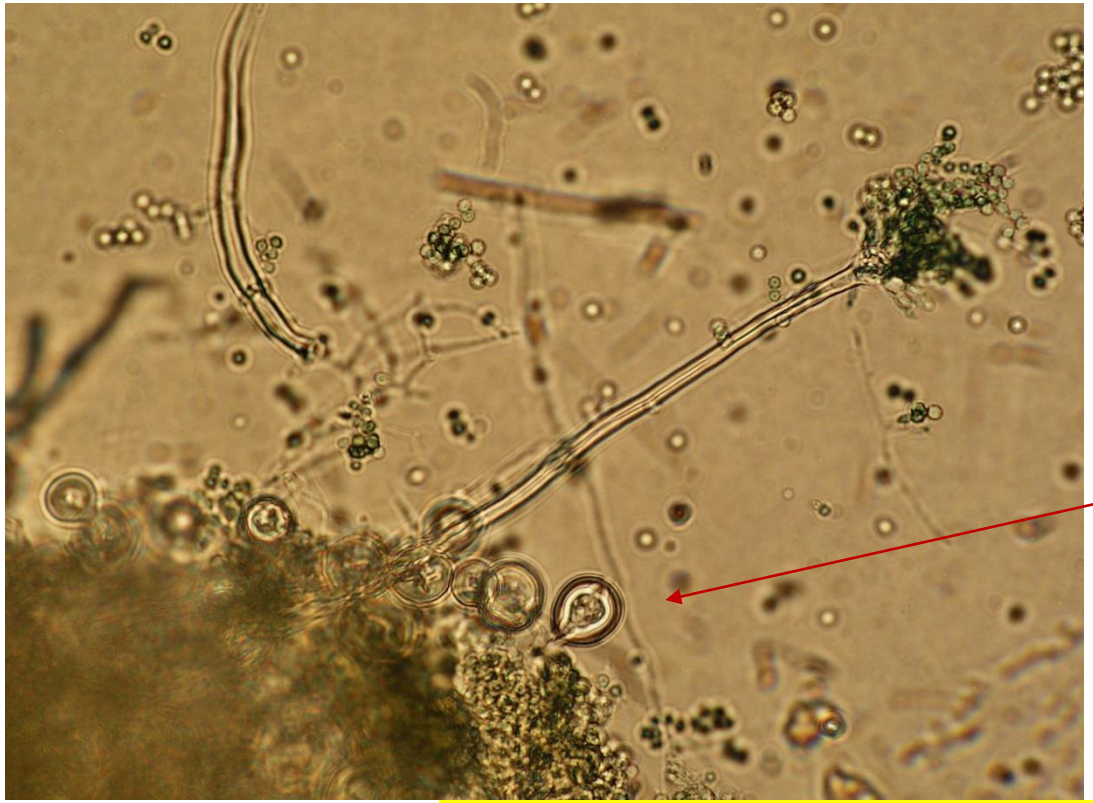
Name, Author, Year, (Current name), Parent taxon

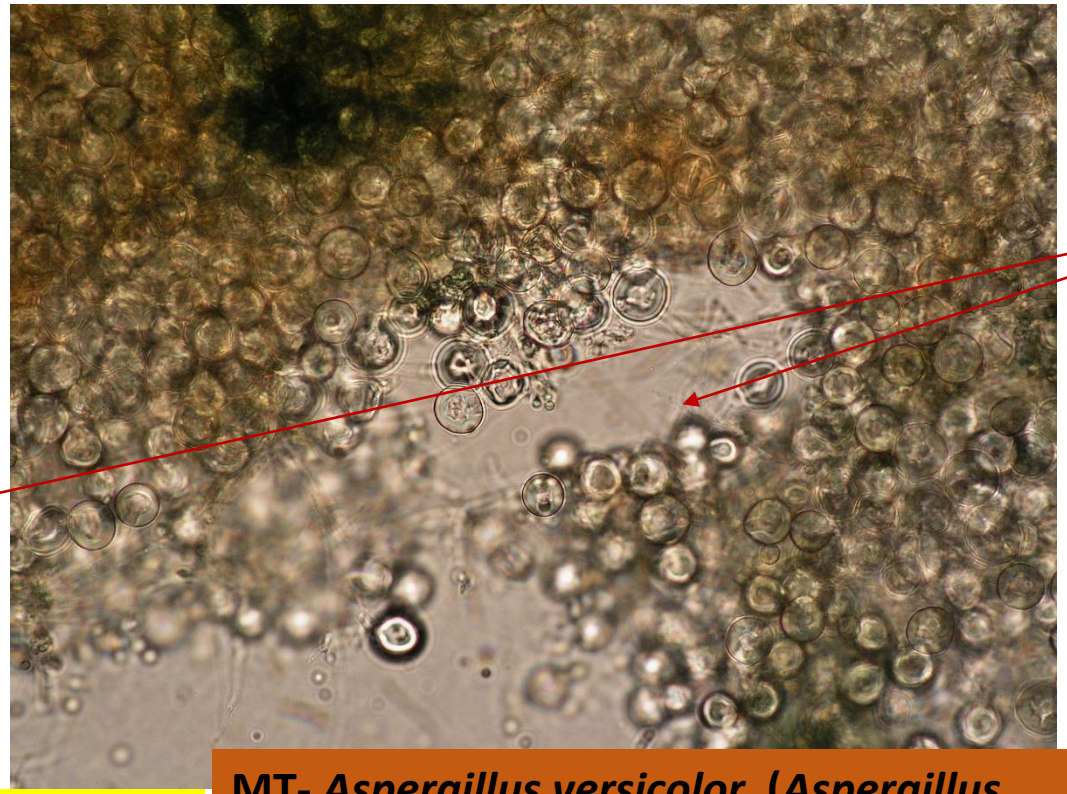
Pages: 1 of 1 records. TofP BofP

Aspergillus creber Jurjevic, S.W. Peterson & B.W. Horn 2012, (also see Species Fungorum: Aspergillus creber); Anamorphic Emericella

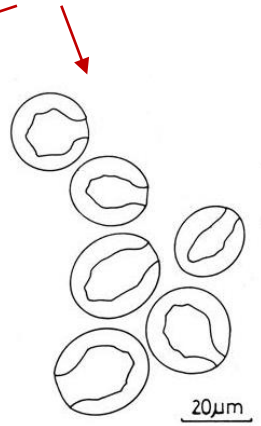
Pages: 1 of 1 records. TofP BofP







Hülle cells



***Aspergillus* sp. – „*Emericella* clade“**

MT- *Aspergillus versicolor* (*Aspergillus creber* not present in Biotyper 3.1)

IMA Fungus. 2012 Jun; 3(1): 59–79.
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Aspergillus section *Versicolores*: nine new species and multilocus DNA sequence based phylogeny

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Abstract

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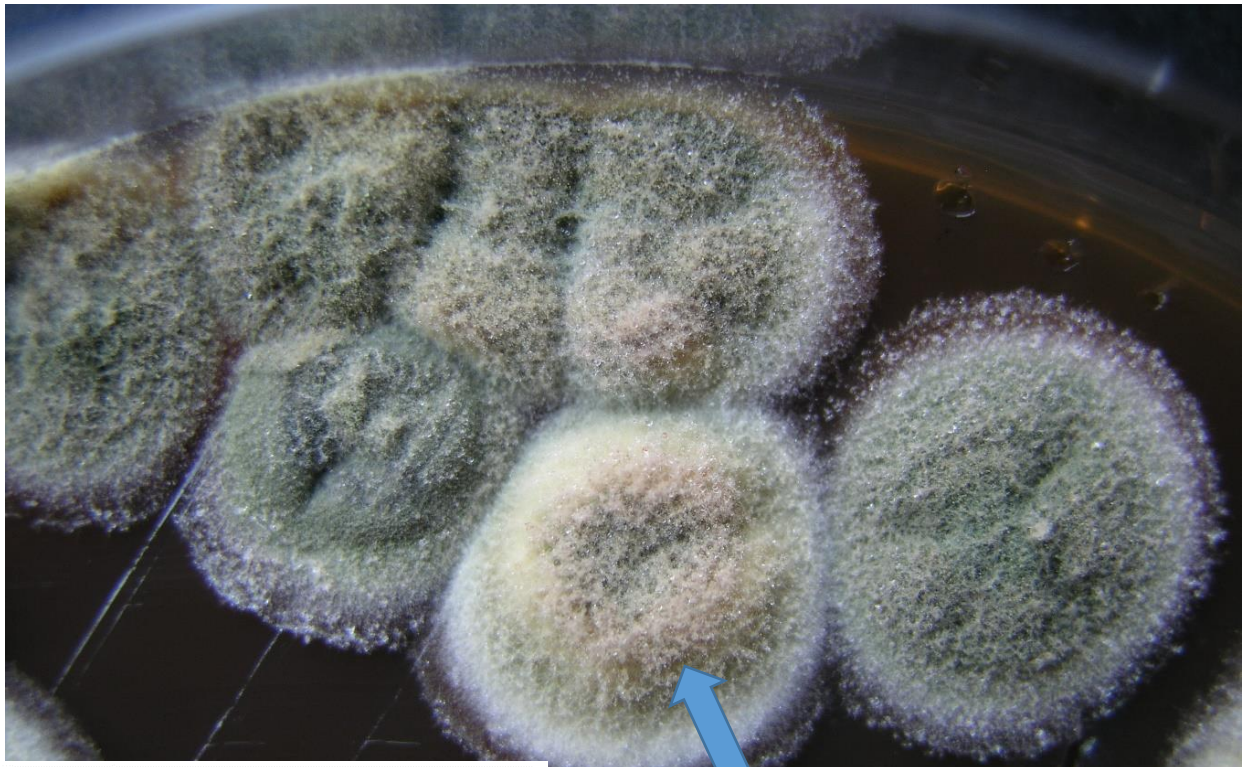
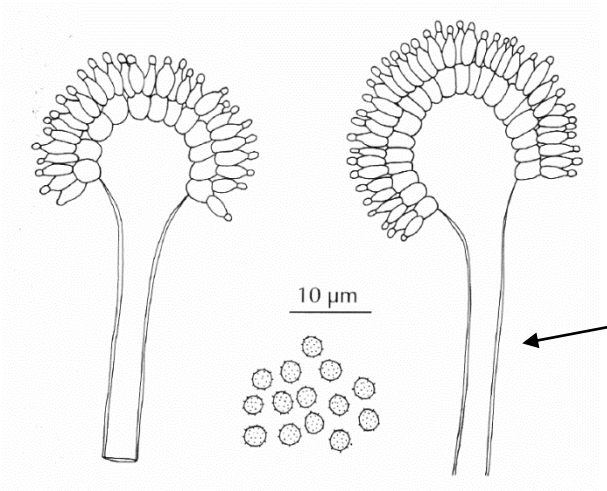
β-tubulin, calmodulin, internal transcribed spacer and partial lsu-rDNA, RNA polymerase 2, DNA replication licensing factor *Mcm7*, and pre-rRNA processing protein *Tsr1* were amplified and sequenced from numerous isolates belonging to *Aspergillus* sect. *versicolor*. The isolates were analyzed phylogenetically using the concordance model to establish species boundaries. *Aspergillus austroafricanus*, *A. creber*, *A. cvjetkovicii*, *A. fructus*, *A. jensenii*, *A. puulaauensis*, *A. subversicolor*, *A. tennesseensis* and *A. venenatus* are described as new species and *A. amoenus*, *A. protuberus*, *A. sydowii*, *A. tabacinus* and *A. versicolor* are accepted as distinct species on the basis of molecular and phenotypic differences. PCR primer pairs used to detect *A. versicolor* in sick building syndrome studies have a positive reaction for all of the newly described species except *A. subversicolor*.

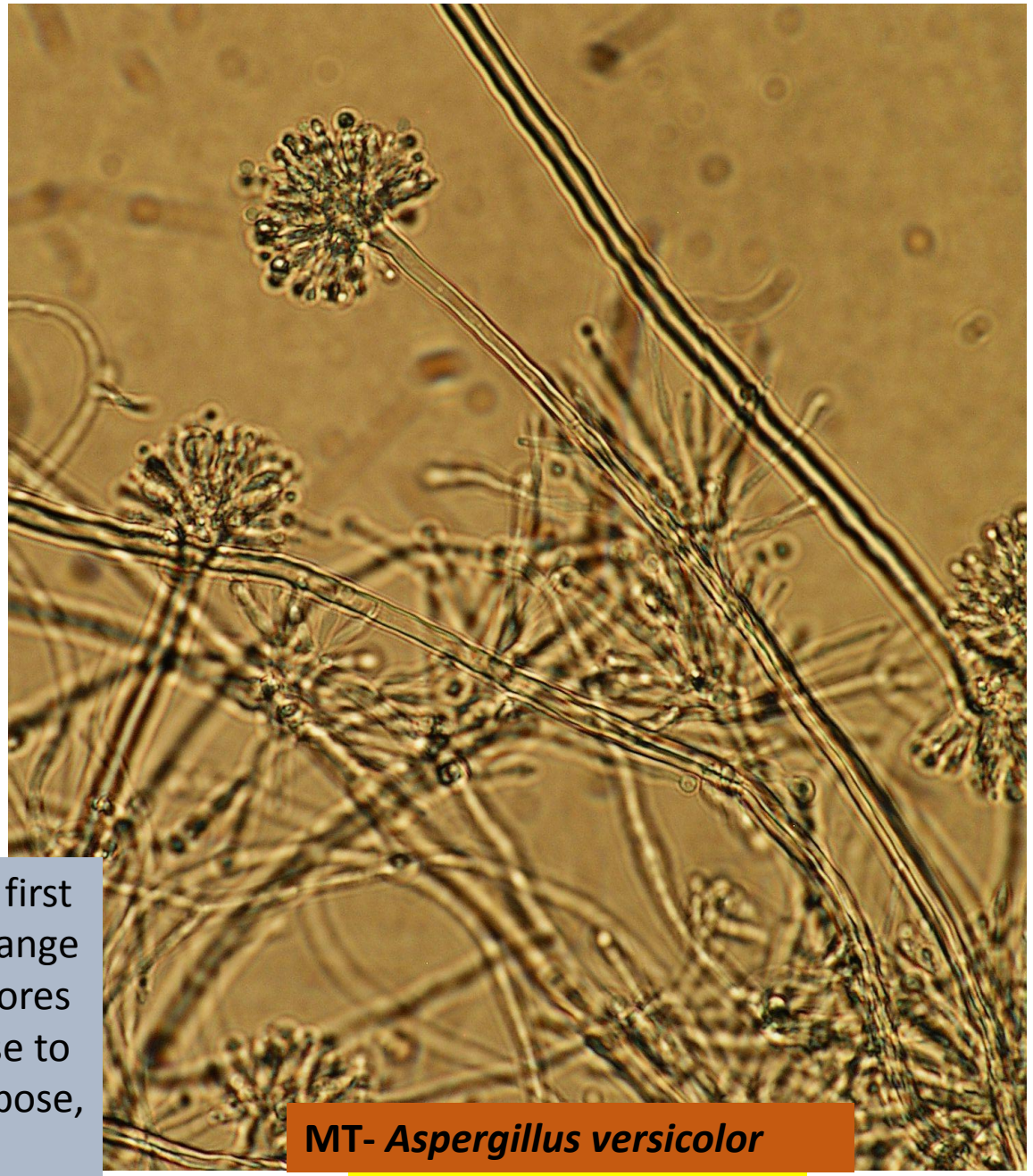
strain 11 designate: *A.versicolor*





Food and Indoor Fungi (Samson et.al., CBS, 2010

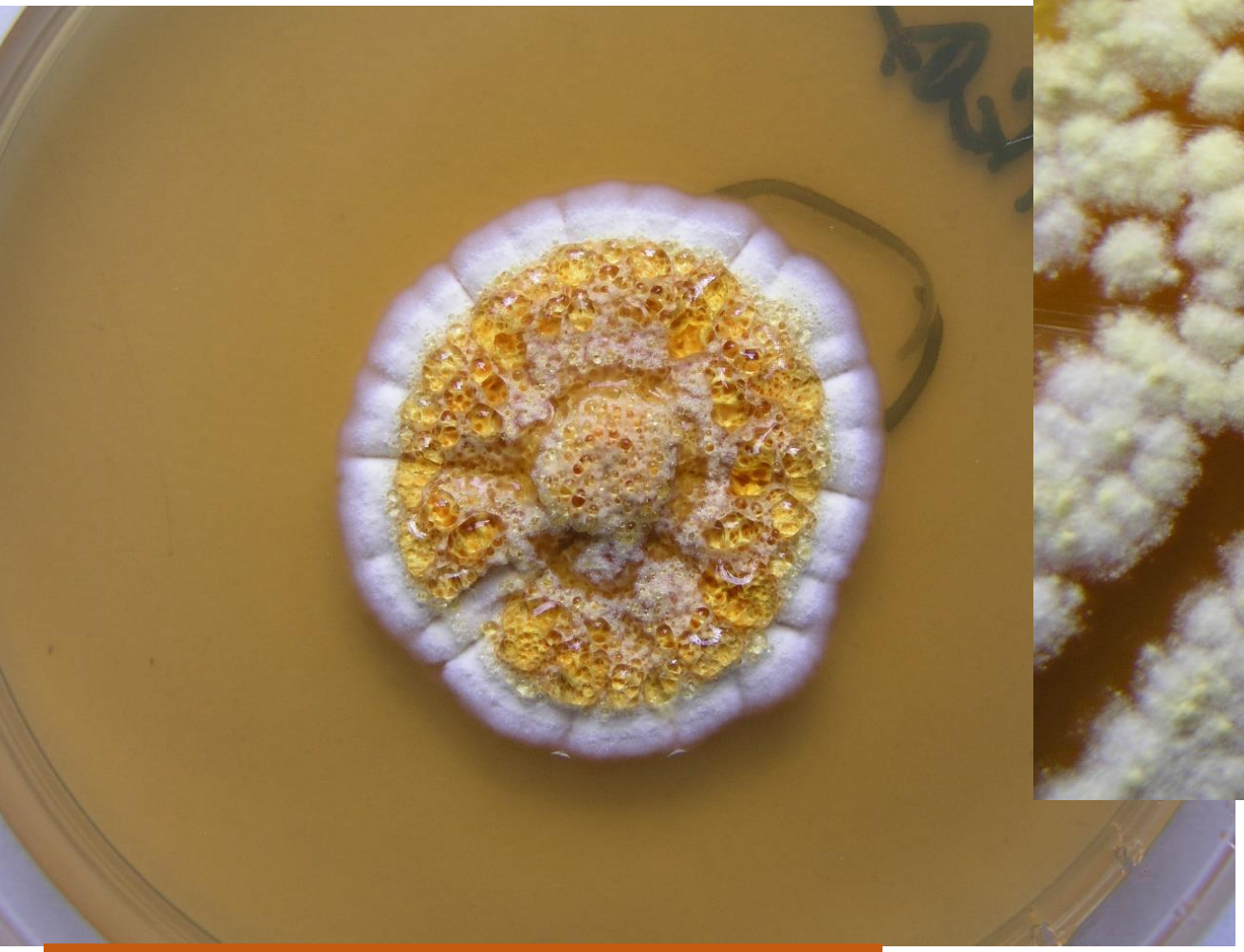
Colonies growing slowly, colour at first white, then changing to yellow, orange yellow to yellow green. Conidiophores smooth walled, vesicles subglobose to ellipsoidal. Conidia colourless, globose, echinulate. Hülle cells sometimes present.



MT- ***Aspergillus versicolor***

Aspergillus versicolor

strain 13 designate: *C.murorum*



MT– problem to release proteins from the mycelium - – bad quality mass spectrum

The fungus not able to sporulate – the morphological identifiication is not possible

strain 15 designate: *R.mucilaginosa*

Variability of cells size (*Rhodotorula mucilaginosa*)

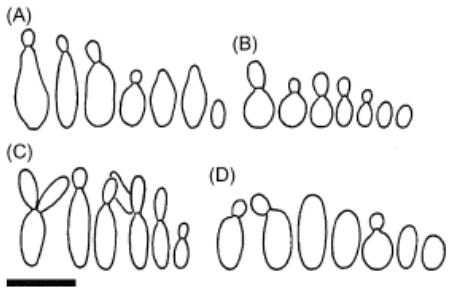
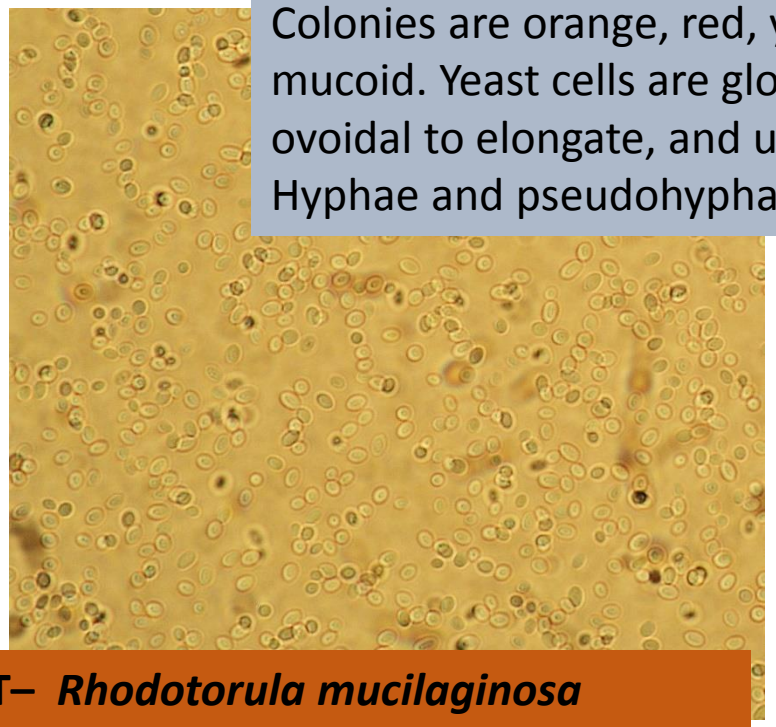


FIGURE 155.35 *Rhodotorula mucilaginosa*. Yeast cells of (A) CBS 17, (B) CBS 316, (C) CBS 328 and (D) CBS 333 on 5% malt extract agar after 6 days at 20°C. Bar = 10 μm.

Basidiomycetous genera *Rhodotorula*:
Colonies are orange, red, yellow or pale, and butyrous to mucoid. Yeast cells are globose to subglobose, ellipsoidal, ovoidal to elongate, and usually have polar budding. Hyphae and pseudohyphae may be present.



MT– *Rhodotorula mucilaginosa*

***Rhodototula* sp.**

Biochemical tests required for species identification

Fermentation: Absent.

Growth (in Liquid Media)[1]

Glucose	+	D-Ribose	+
Inulin	–	Methanol	–
Sucrose	+	Ethanol	+
Raffinose	+	Glycerol	+
Melibiose	–	Erythritol	–
Galactose	+/s	Ribitol	+[3]
Lactose	–	Galactitol	–
Trehalose	+	D-Mannitol	v
Maltose	+[2]	D-Glucitol	v
Melezitose	+[2]	*myo*-Inositol	–
Methyl-α-D-glucoside	v	DL-Lactate	v
Soluble starch	–	Succinate	+
Cellobiose	+/s/w[2]	Citrate	+
Salicin	+[2]	D-Gluconate	+
L-Sorbose	v	D-Glucosamine	–
L-Rhamnose	–	*N*-Acetyl-D-glucosamine	n
D-Xylose	+	Hexadecane	n
L-Arabinose	+	Nitrate	–
D-Arabinose	+	Vitamin-free	–

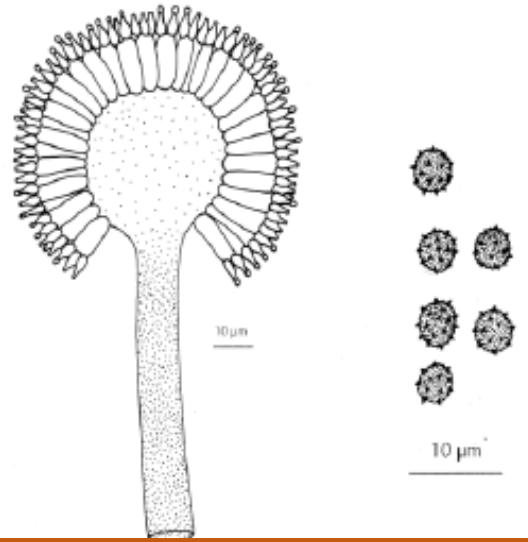
[1]*Based on CBS, 17, 316, 325, 326, 327, 328, 329, 330, 333, 482, 992, 1011, 2376, 2377, 2378, 2379, 2380, 2381, 2382, 2383, 2384, 2386, 2404, 5804, 5951, 6610, 8054 and 8161.*
[2]*Negative for CBS 5804, CBS 5951 and CBS 6610.*
[3]*Negative for CBS 1011 and CBS 8161.*

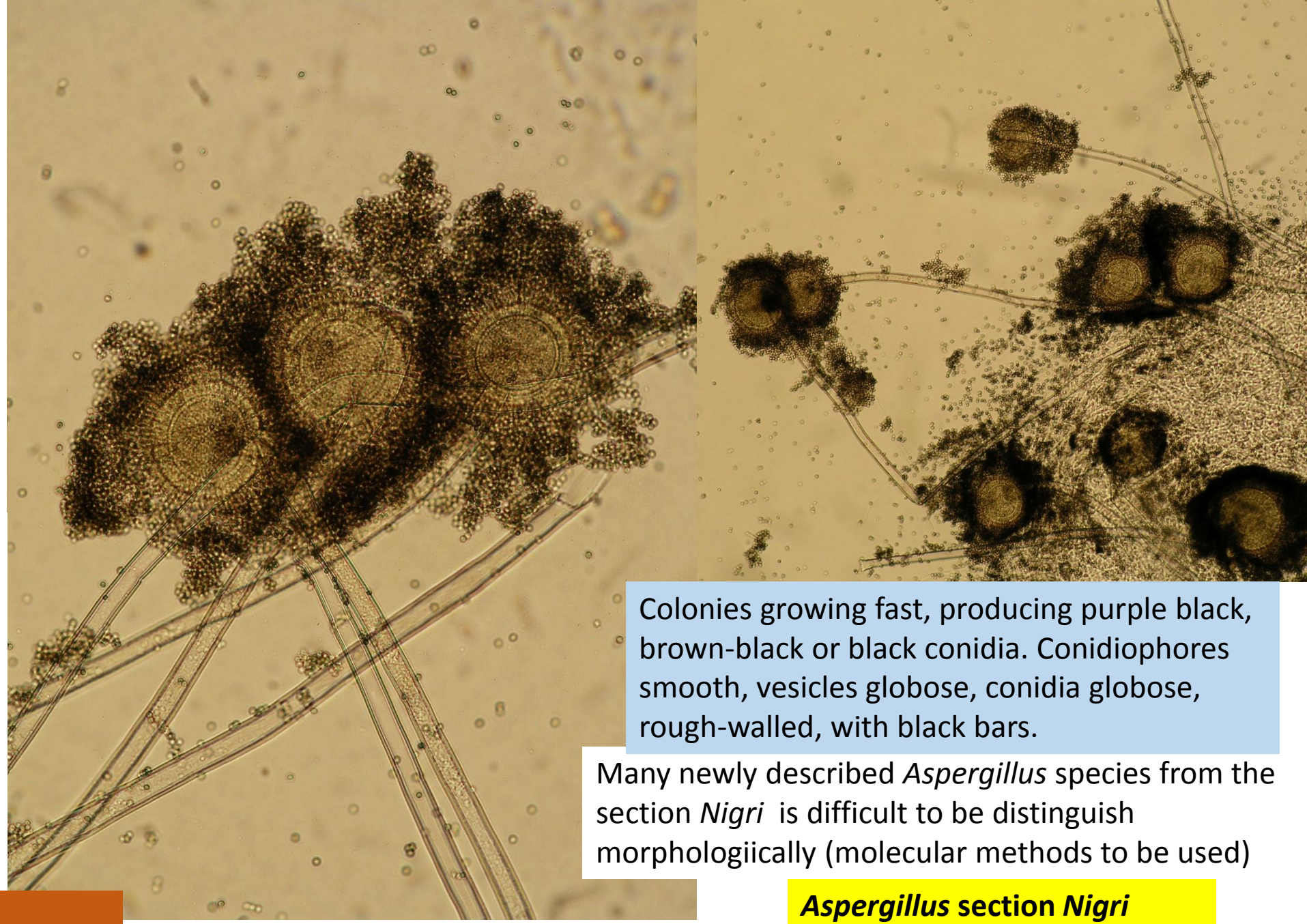
Additional Growth Tests and Other Characteristics

Nitrite	–	Ferulic acid	+
D-Glucuronate	–	Veratric acid	–
Xylitol	+	Cycloheximide 0.01%	v
L-Tartaric acid	–	Cycloheximide 0.1%	v
Saccharic acid	–	Growth at 25°C	+
p-Hydroxybenzoic acid	+	Growth at 30°C	+
m-Hydroxybenzoic acid	+	Growth at 35°C	v
Gallic acid	–	Growth at 37°C	–
Gentisic acid	–	Starch formation	–
Vanillic acid	+	DBB	+

The yeasts-a taxonomic study (Kurtzman et.al., Elsevier, 2011)

strain 17 designate: *A.niger*





Colonies growing fast, producing purple black, brown-black or black conidia. Conidiophores smooth, vesicles globose, conidia globose, rough-walled, with black bars.

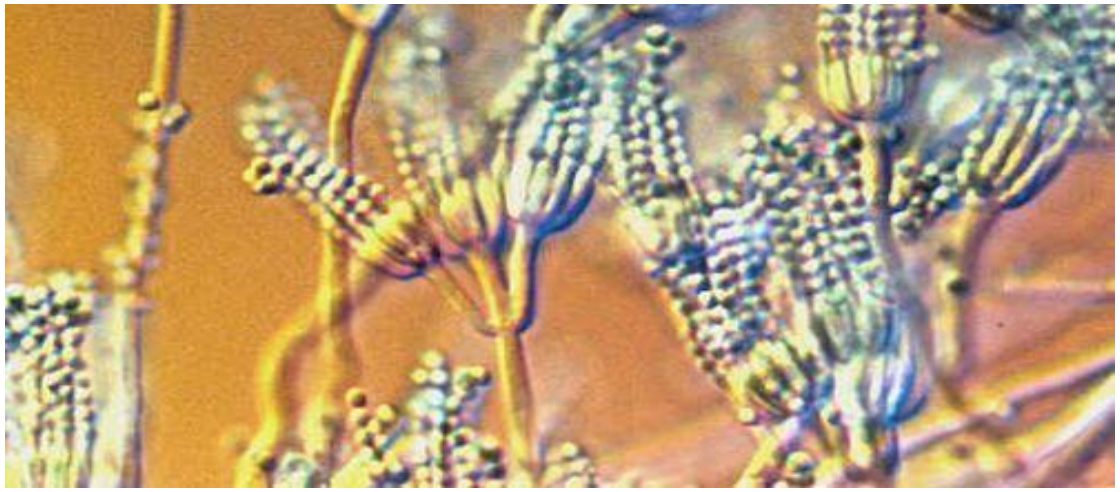
Many newly described *Aspergillus* species from the section *Nigri* is difficult to be distinguish morphologiically (molecular methods to be used)

MT– *Aspergillus niger*

Aspergillus* section *Nigri
Most likely *Aspergillus niger*

MALDI-TOF MS: MOLDS IDENTIFICATION – CONCLUSION

- BioTyper 3.1 – database of 365 reference strains of molds
- Genus *Aspergilllus* and *Penicillium*
  - Sample preparation without problems
  - Some species can be absent
- *Myxotrichum* genus is not present in BioTyper 3.1
- The sample preparation must be optimized for genera *Chaetomium* and *Cladosporium*
  - The influence of the incubation time - to work with very fresh, but very small pellet ??
  - The application of zirkonium beads to destroy the cell walll in „older“ mycelium ??
  - To apply 80 % trifluoracetic acid to destroy cell walll ??? – recommended by some authors
- Yeasts as *Rhodotorula mucilaginosa* – sample preparation without problems



Thank you for your attention

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