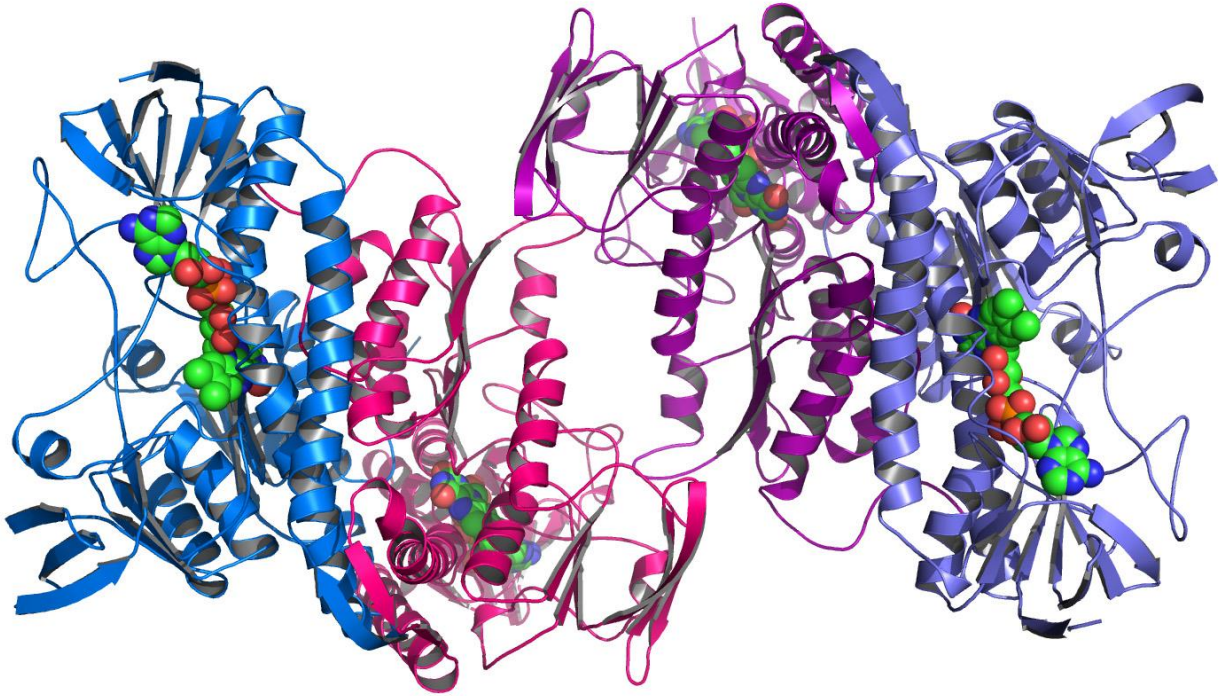

Proteomic background of fungal biodegradation

dr Rafał Szewczyk

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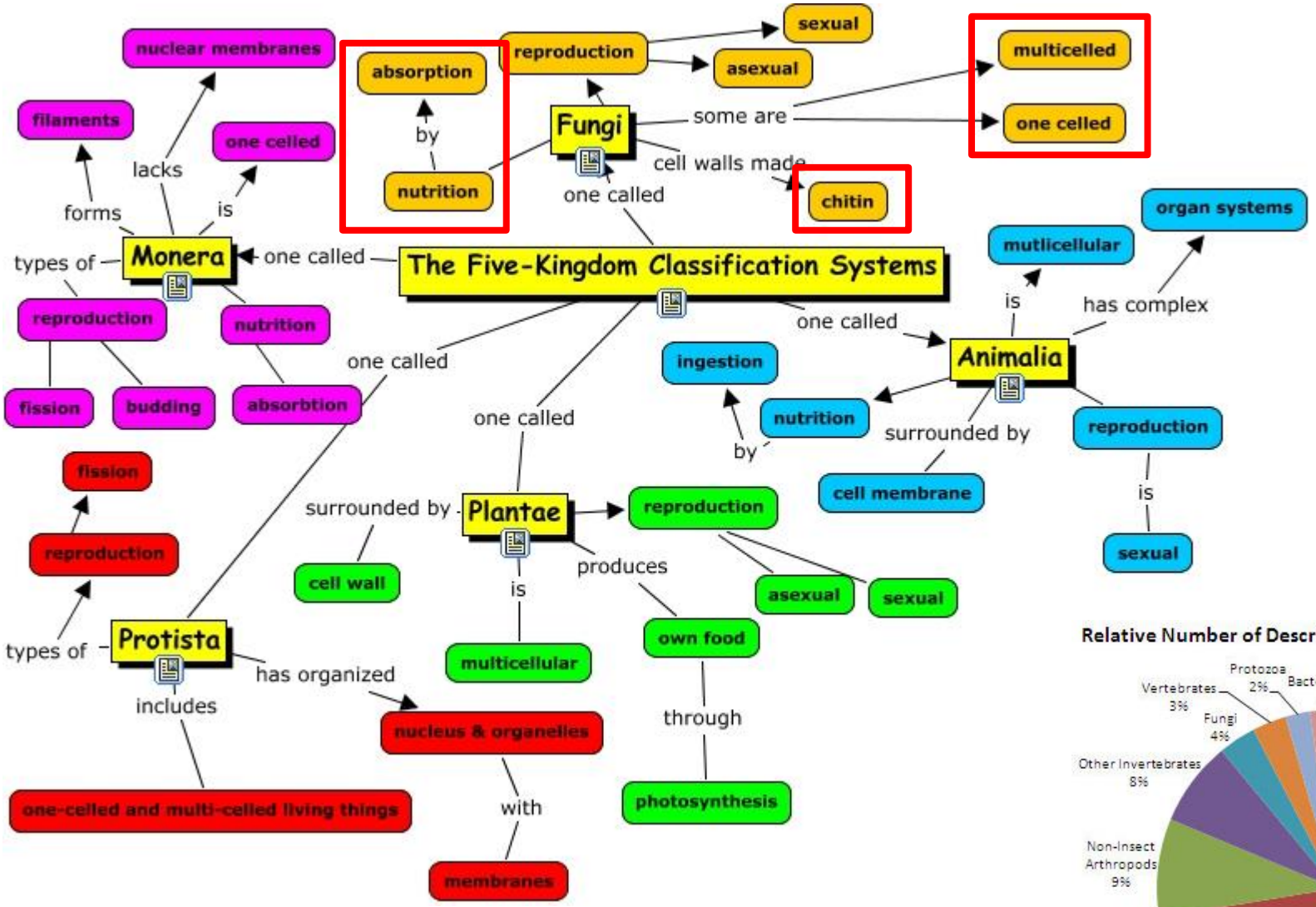
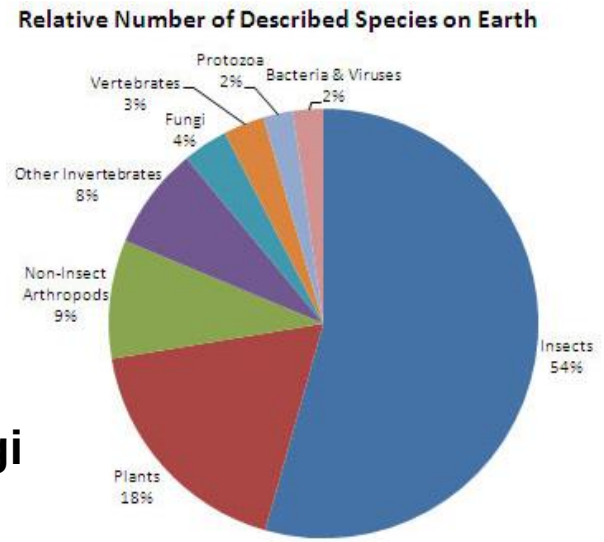
Faculty of Biology and Environmental Protection, University of Łódź, Poland

AB SCIEX

Fungi characteristics

Fungi characteristics





4% of all known species belong to the kingdom of fungi

IMPORTANT FACTS:

- Fungi are more closely **related to animals** than plants.
- Fungi are **saprophyte heterotrophs**: they use dead or decomposing organic matter as a source of carbon.
- Fungi multiply either asexually, sexually, or both.
- The majority of **fungi produce spores**.

- Fungal **cell walls are rigid** and contain complex polysaccharides called **chitin and glucans**.
- **Ergosterol** is the steroid molecule in the cell membranes that replaces the cholesterol found in animal cell membranes.
- Fungi can be **unicellular, multicellular or dimorphic**.
- Fungi like to be in a moist and slightly acidic environment.

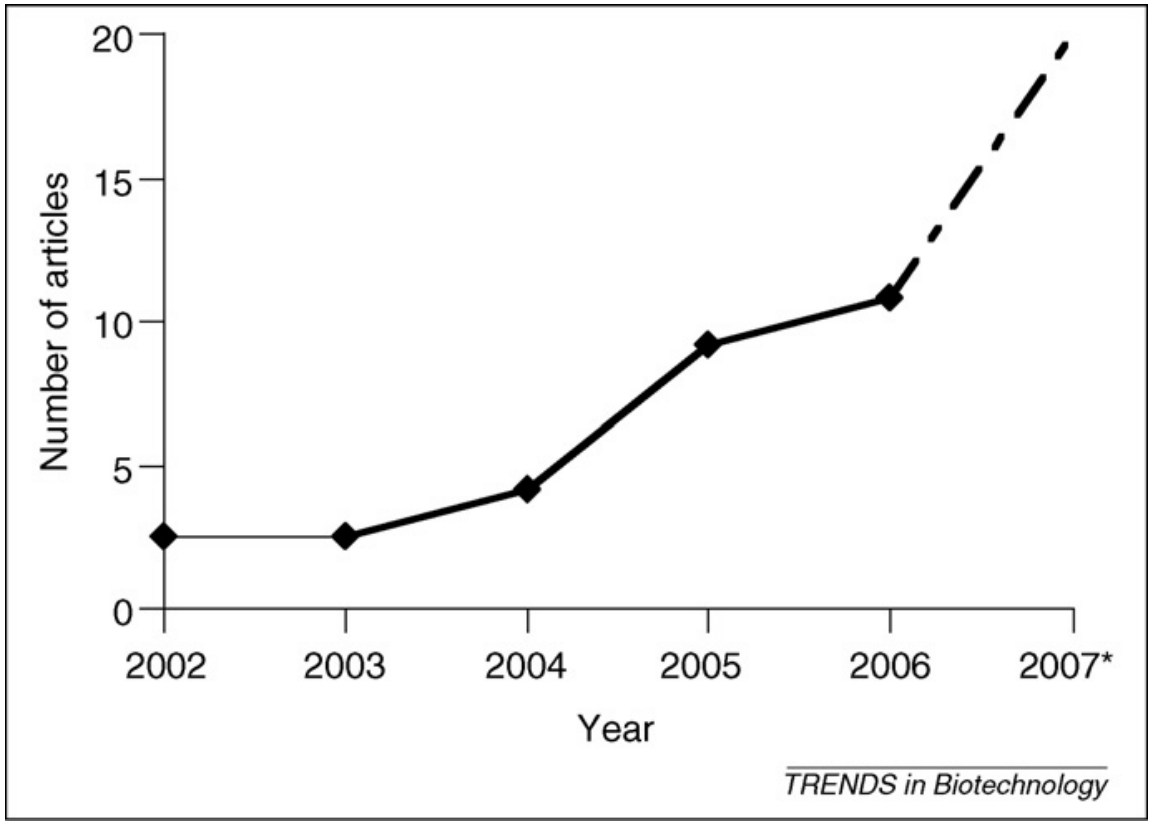
Fungi characteristics

Fungi are important because they are:

- responsible for the majority of plant diseases and several diseases of animals & humans
- used in industrial fermentation processes
- used in the commercial production of many biochemicals and medicals
- cultured commercially to provide us with a direct source of food
- beneficial in agriculture, horticulture and forestry (ex. mycorrhizae, lichens)
- **agents of biodegradation, biodeterioration and bioremediation** (mostly **molds**)

Fungal proteomics

Fungal proteomics

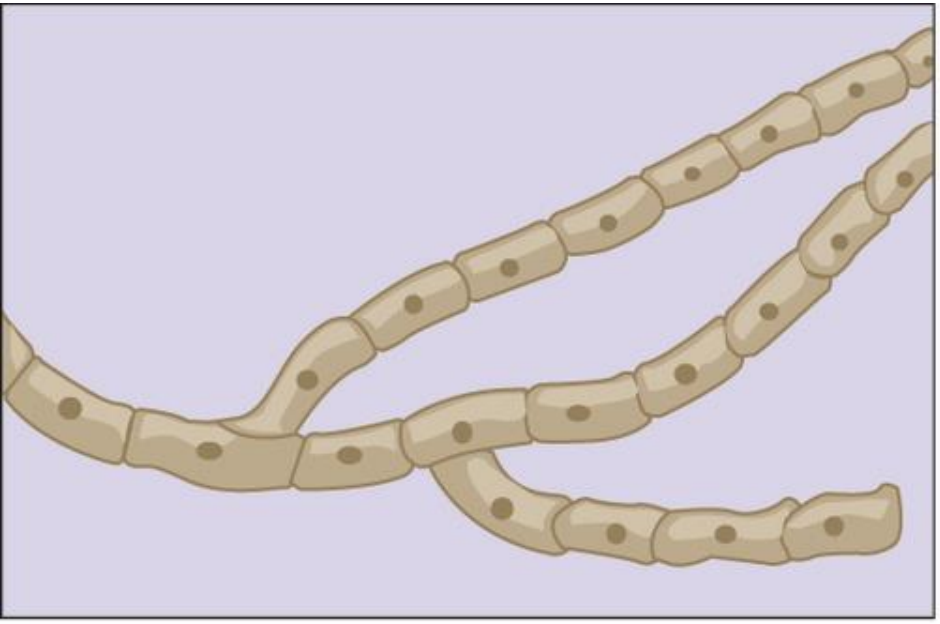


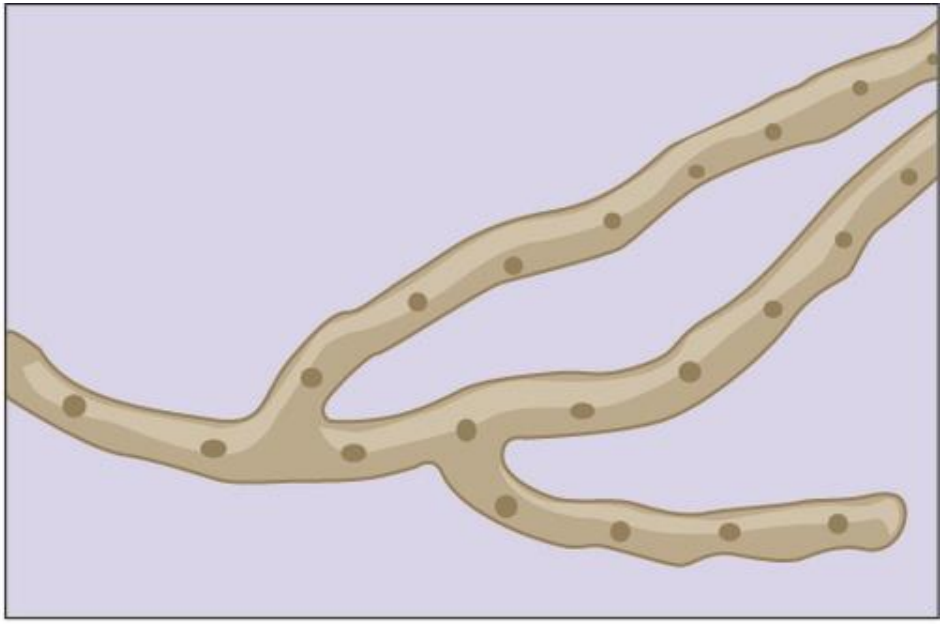
The number of new articles (including reviews) that have appeared in each of the past five years related to filamentous fungal proteomics. *Represents a projection as of April 2007 (Kim et al. 2007).

To my best knowledge **till 06.2014**:

- numerous articles about fungal biodegradation,
- around 100 research articles about fungal proteomics (in general),
- less than 10 research articles on proteomics of fungi involved in biodegradation,
- less than 5 research articles on intracellular proteomics of fungi involved in biodegradation

Fungal proteomics

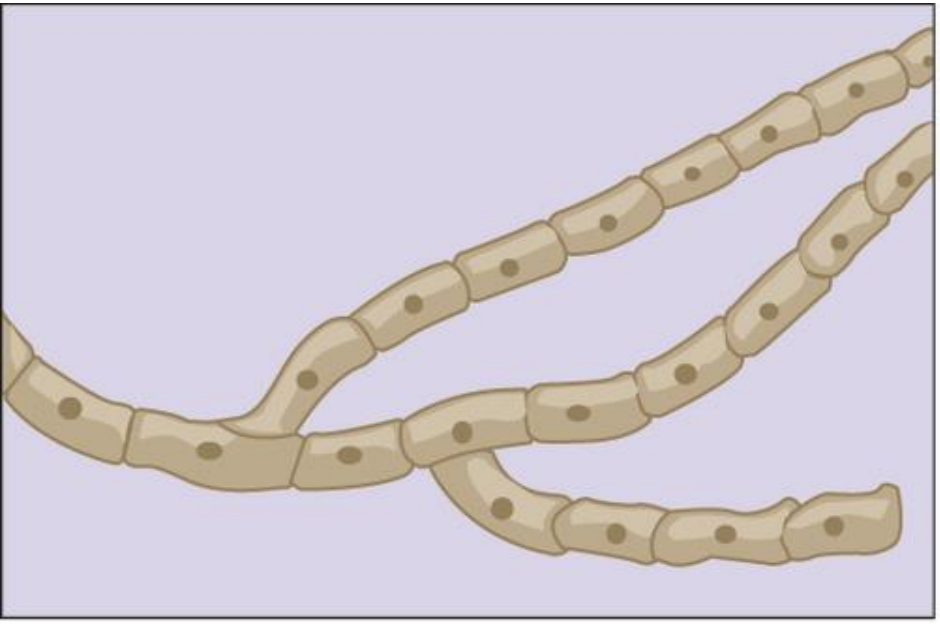


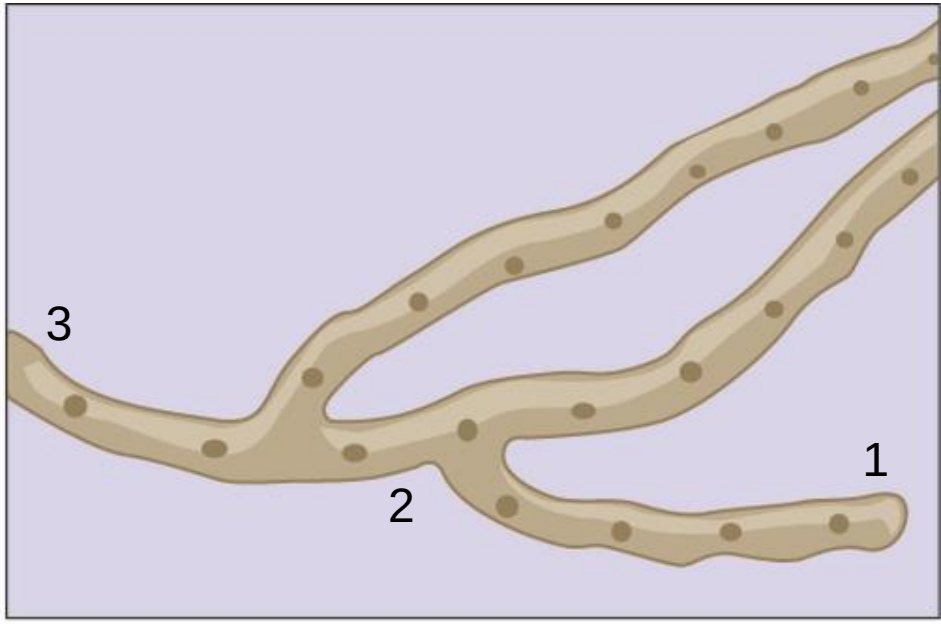


Benefits of the filamentous growth of molds in biodegradation processes:

- good sample/soil/sediment penetration,
- functional differentiation of the filaments,
- source of oxygen, nitrogen, carbon and other factors can be separated from the target because of the transport along the filaments

Fungal proteomics

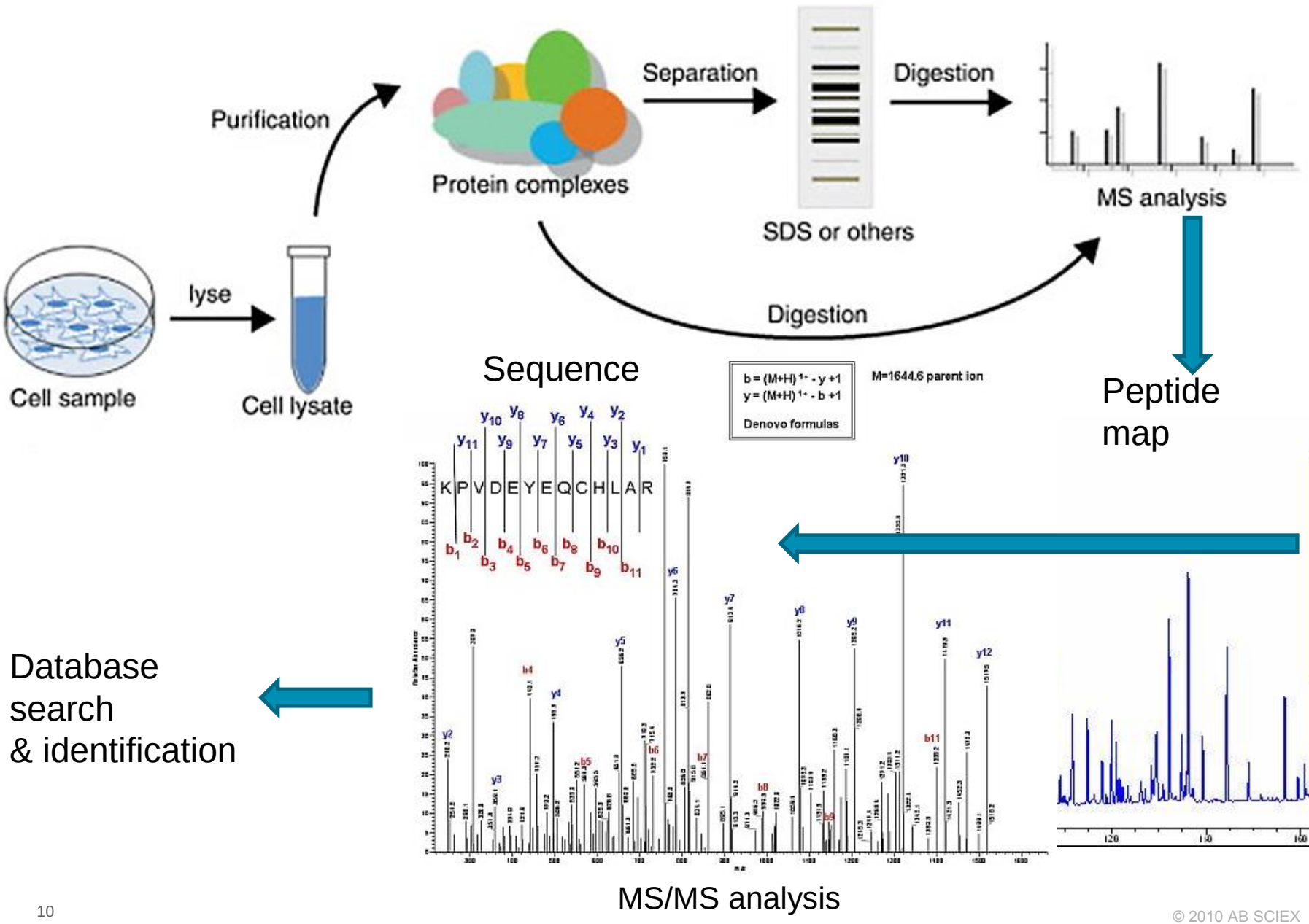




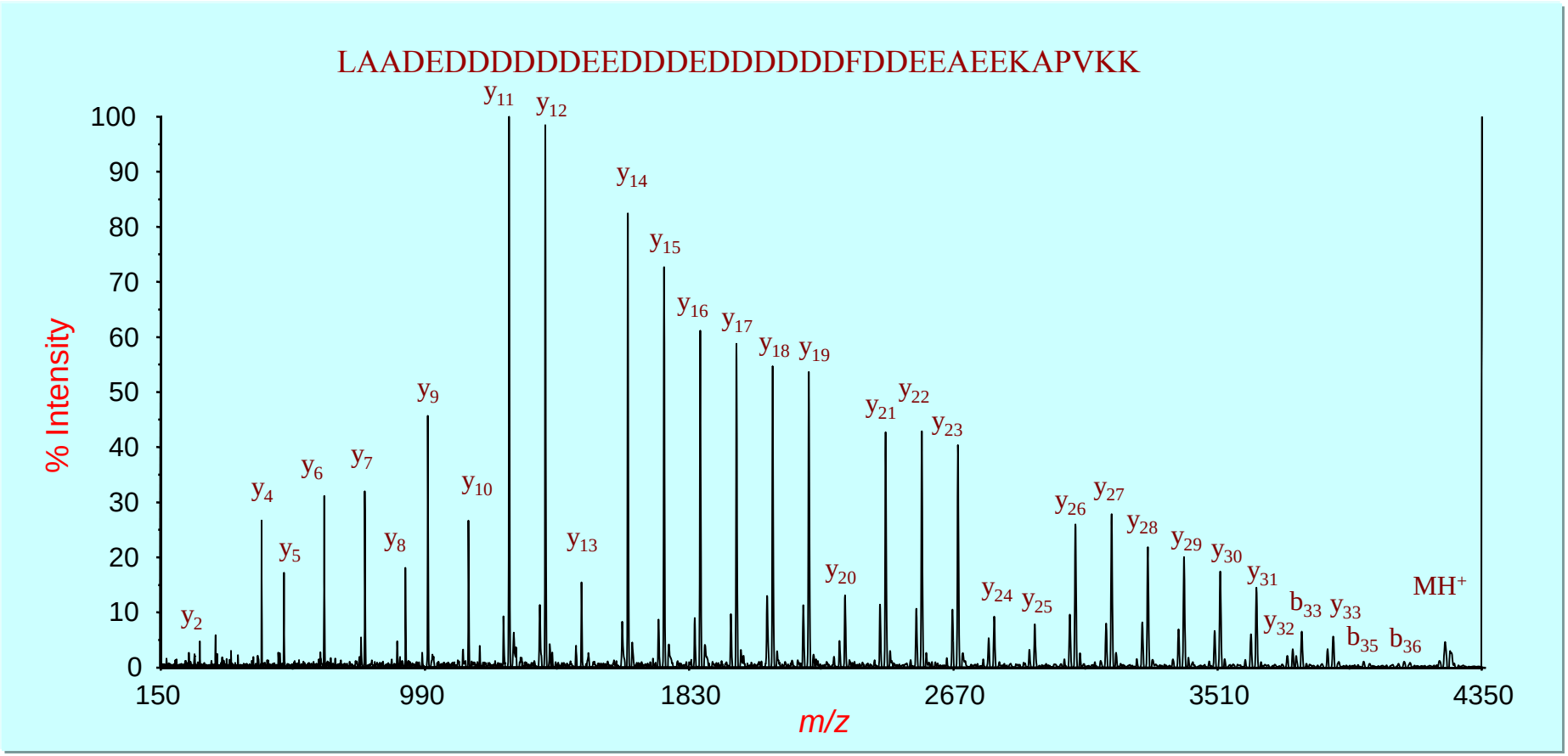
Problems with filamentous growth and cell structure of molds in proteomics:

- thick, rigid and disintegration resistant cell wall,
- cell compartments (membranes),
- many proteins incorporated into cell wall or membranes,
- functional differentiation of the filaments,
- different growth stage of the filament apex in compare to other regions (1, 2, 3)

MS/MS proteome analysis workflow



MS/MS sequencing principle



$y_2 - y_1$ (etc.) = AA (+ posttranslational modifications)

Fungal biodegradation proteomics

Case 1 – some fungi from the tested genus are sequenced

Case 2 – nonsequenced organism

Case 1 – sequenced fungi from the tested genus

Metarhizium sp.

1. *M. anisopliae*
2. *M. guizhouense*
3. *M. pingshaense*
4. *M. acridum*
5. *M. lepidiotae*
6. *M. majus*
7. *M. globosum*
8. *M. robertsii*
9. *M. brunneum*
10. *M. velutinum*

…and 5 more

Number of sequences in NCBI database:

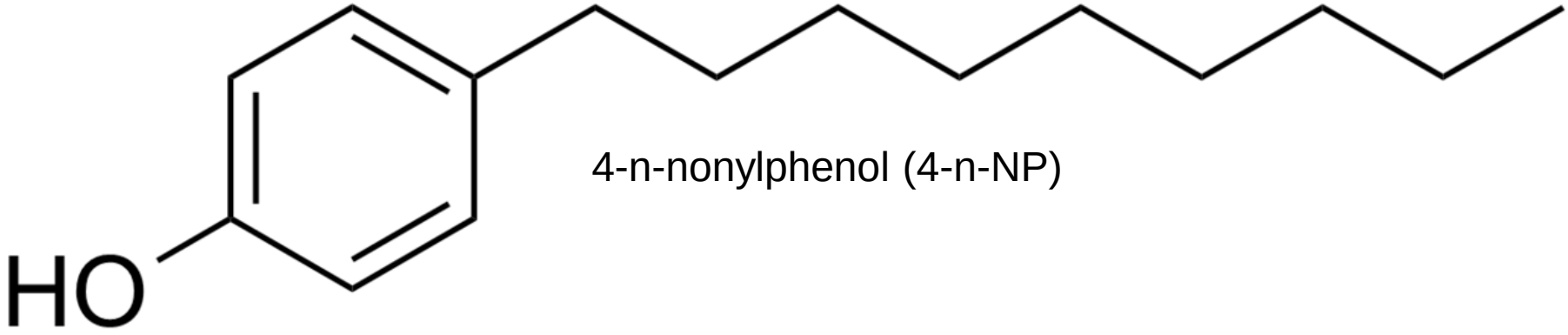
M. anisopliae (21648)
M. anisopliae ARSEF 23 (21177)
M. acridum (19757)
M. acridum CQMa 102 (19707)
M. robertsii (12424)
M. flavoviride (124)
M. pinghaense (74)
All other taxa (less than 35 per organism)

green muscardine disease

Fungi that grows naturally in soils throughout the world and causes disease in various insects by acting as a parasitoids

or

…by acting as an endocrine disruptor compounds (EDCs) degraders



4-n-nonylphenol (4-n-NP)

Nonylphenols are:

- antioxidants, lubricating oil additives, laundry and dish detergents, emulsifiers, and solubilizers
- precursors to the commercially important non-ionic surfactants, which are used in detergents, paints, pesticides, personal care products, and plastics
- **prevalence in the environment**
- **an endocrine disruptor and xenoestrogen**

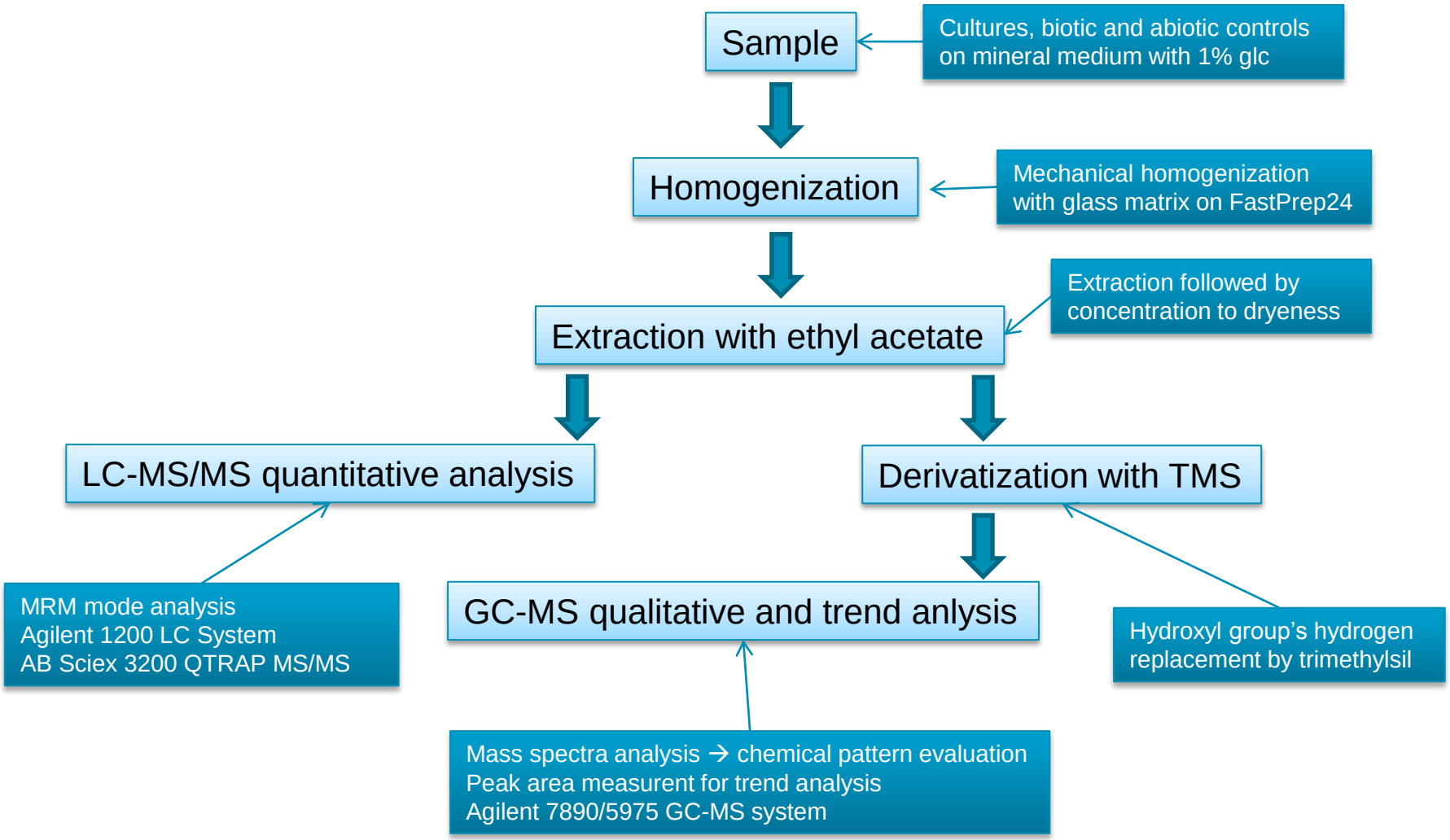
Main steps of the work:

1. 4-n-NP metabolites identification and trend analysis.
2. Protein isolation, separation and digestion.
3. Protein identification with MS/MS analysis.
4. PCA analysis.

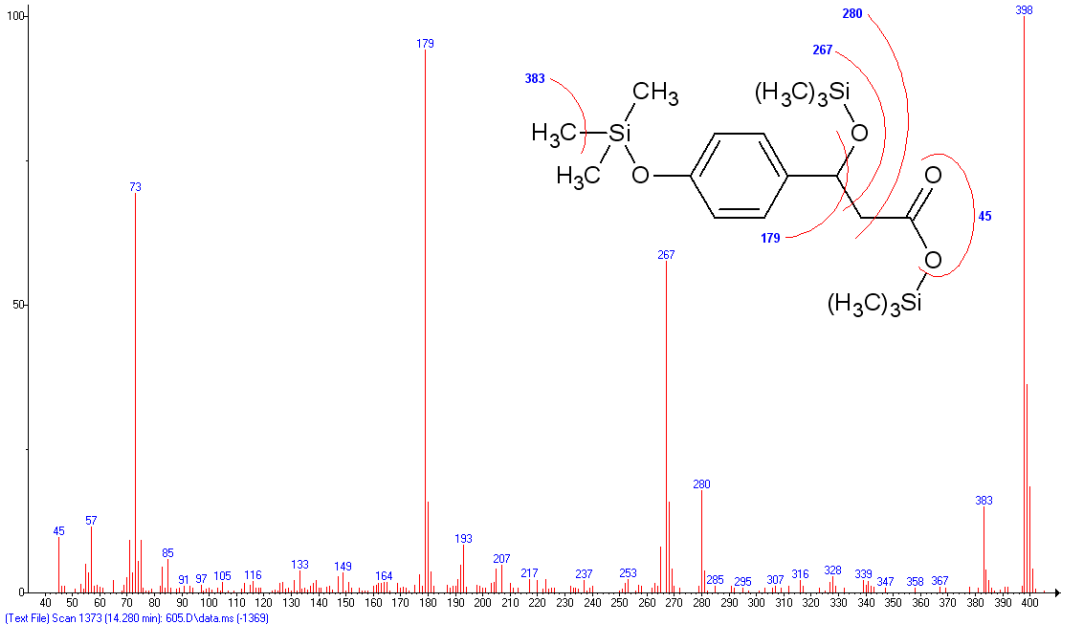
The tested strain, ***Metarhizium robertsii* IM 2358**, was obtained from the fungal strain collection of the Department of Industrial Microbiology and Biotechnology, Institute of Microbiology, Biotechnology and Immunology, University of Łódź, Poland. The strain is capable of 4-*n*-NP removal and phylogenetically belongs to the ***Metarhizium anisopliae* complex** (Różalska et al. 2013).

The presented data are published in:
Szewczyk R., Soboń A., Różalska S., Dzitko K., Waidelich D., Długoński J. (2014). Intracellular proteome expression during 4-*n*-nonylphenol biodegradation by the filamentous fungus *Metarhizium robertsii*. International Biodeterioration & Biodegradation 93:44-53.

Quantitative and trend analysis of the metabolites formation during 4-n-NP biodegradation

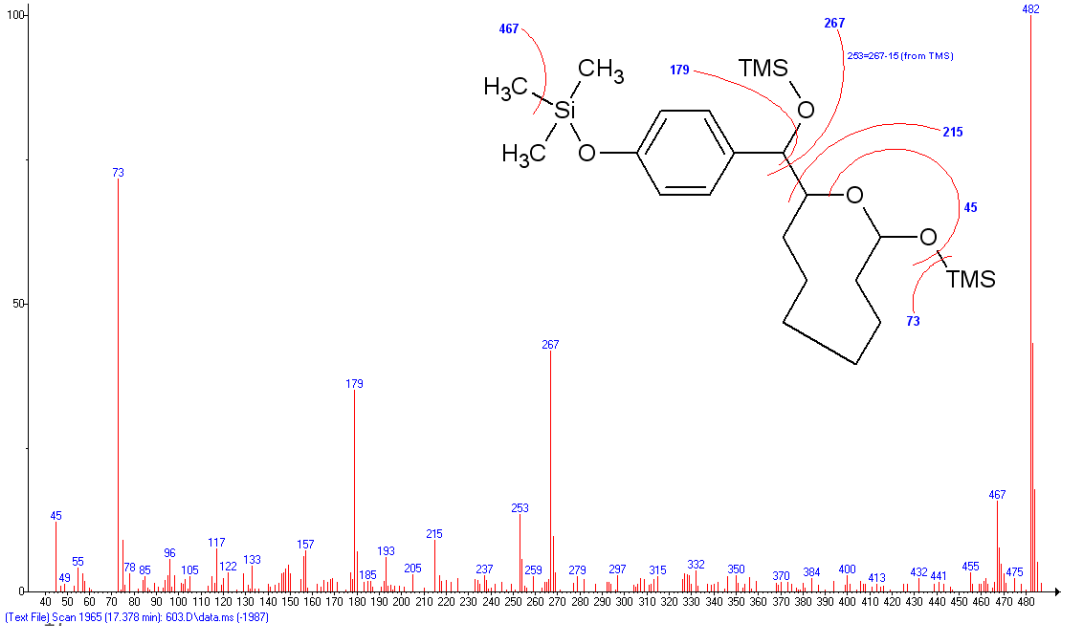


Case 1 – sequenced fungi from the tested genus



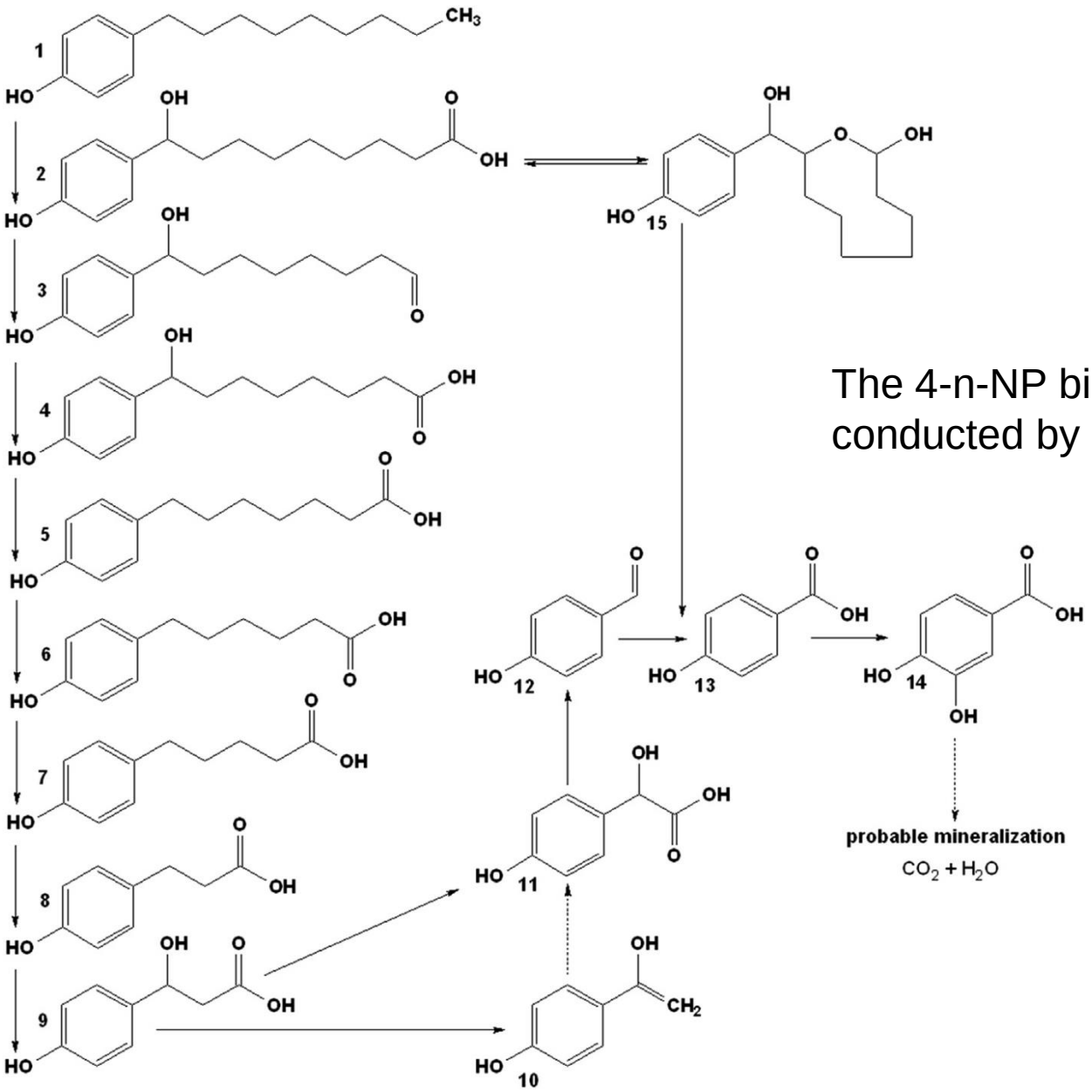
Example of mass spectra interpretation:

3-hydroxy-3(4-hydroxyphenyl)propanoic acid



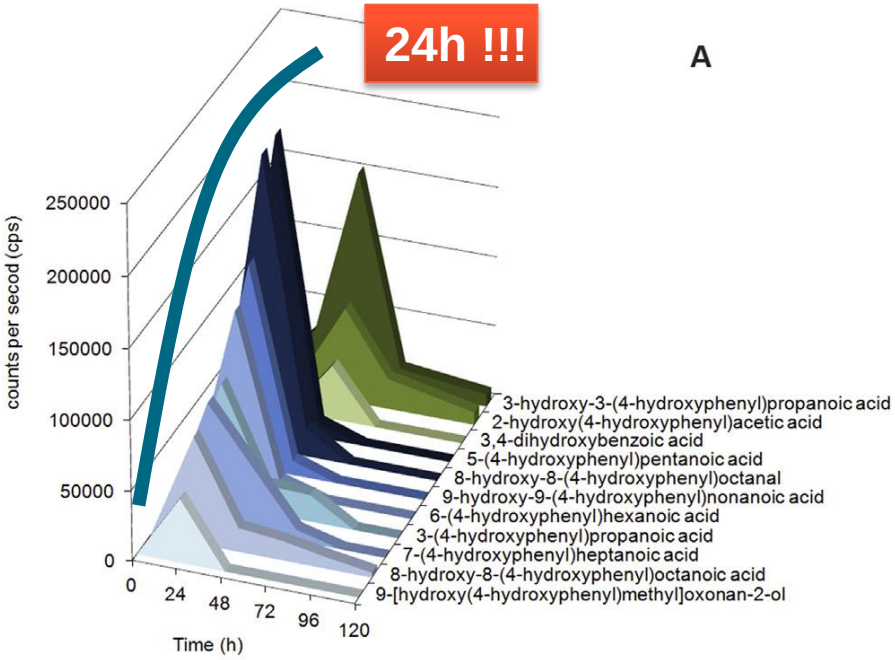
9-[hydroxy(4-hydroxyphenyl)methyl)oxonan -2-ol

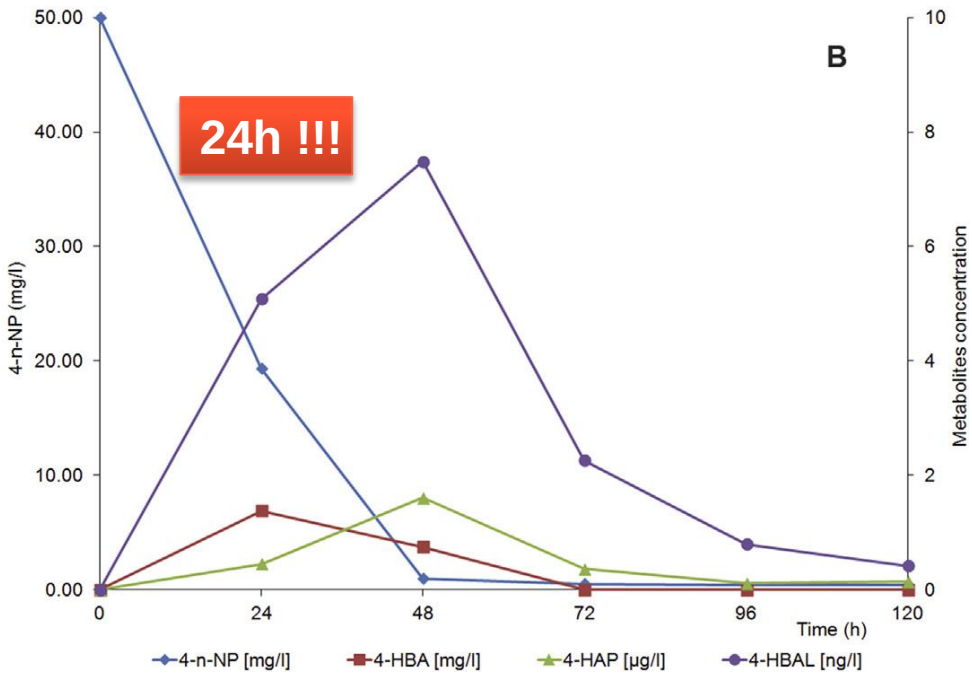
Case 1 – sequenced fungi from the tested genus



The 4-n-NP biodegradation pathway conducted by *M. robertsii.*

Case 1 – sequenced fungi from the tested genus





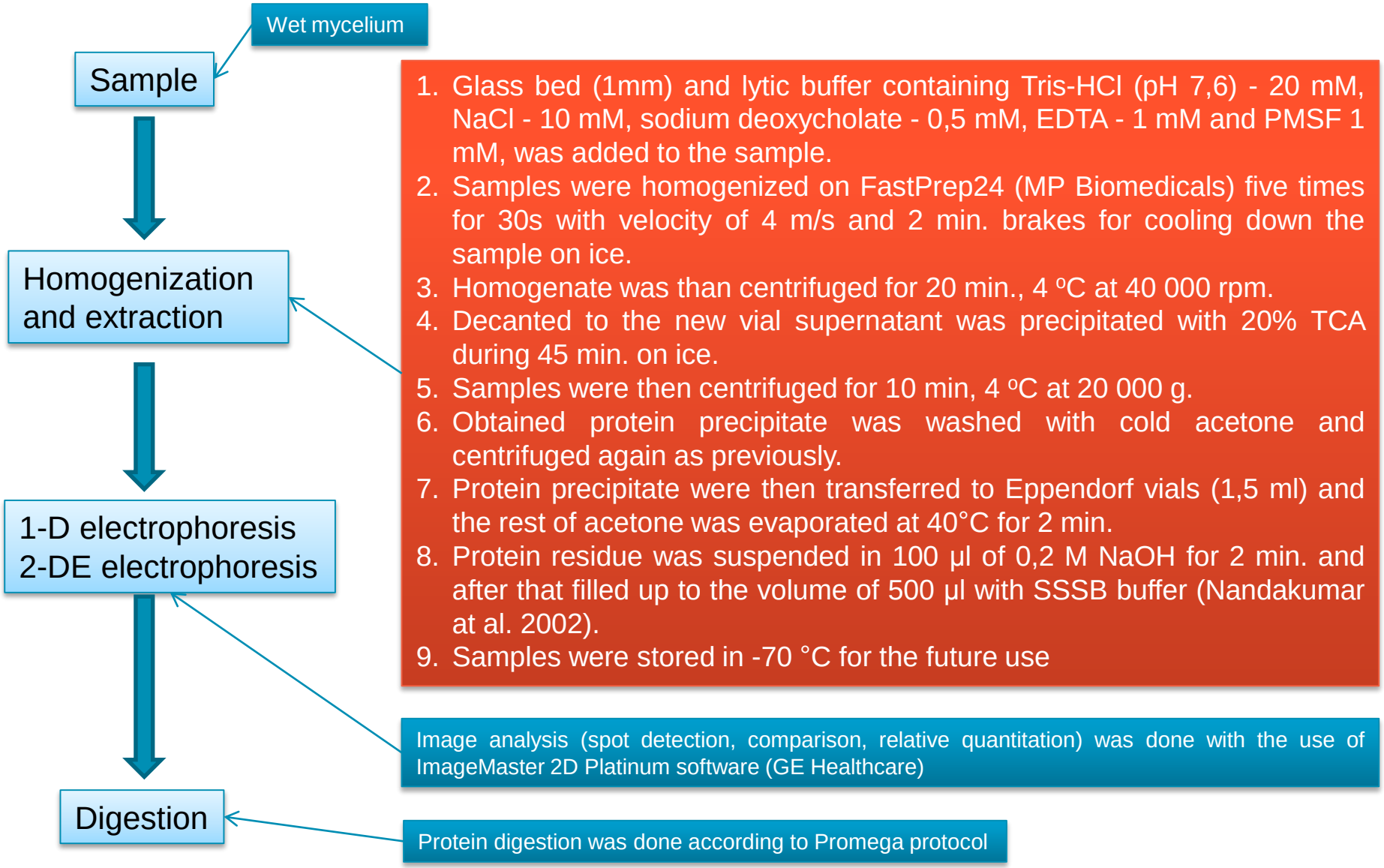
A - trend analysis of 4-n-NP biodegradation products based on GC-MS data on ion 179 m/z area B - LC-MS/MS targeted quantitative analysis of 4-n-NP and selected metabolites, during the culture of M. robertsii on mineral medium X.

1. Biodegradation trends analysis and quantitative analysis revealed that **after 24h of incubation more than 60% of 4-*n*-NP is removed** and **the majority of its derivatives reached the concentration apex** at this point of culturing.
2. Five compounds reached their maximum in the 48h of the experiment, but they were also detected after 24h of culture.
3. According to the data obtained after separate homogenized mycelium and culture medium extractions, removal of the xenobiotic takes place inside the cells of tested strain. Therefore, **mycelium samples collected after 24 h of culturing were determined as the best suited for intracellular proteome expression studies**

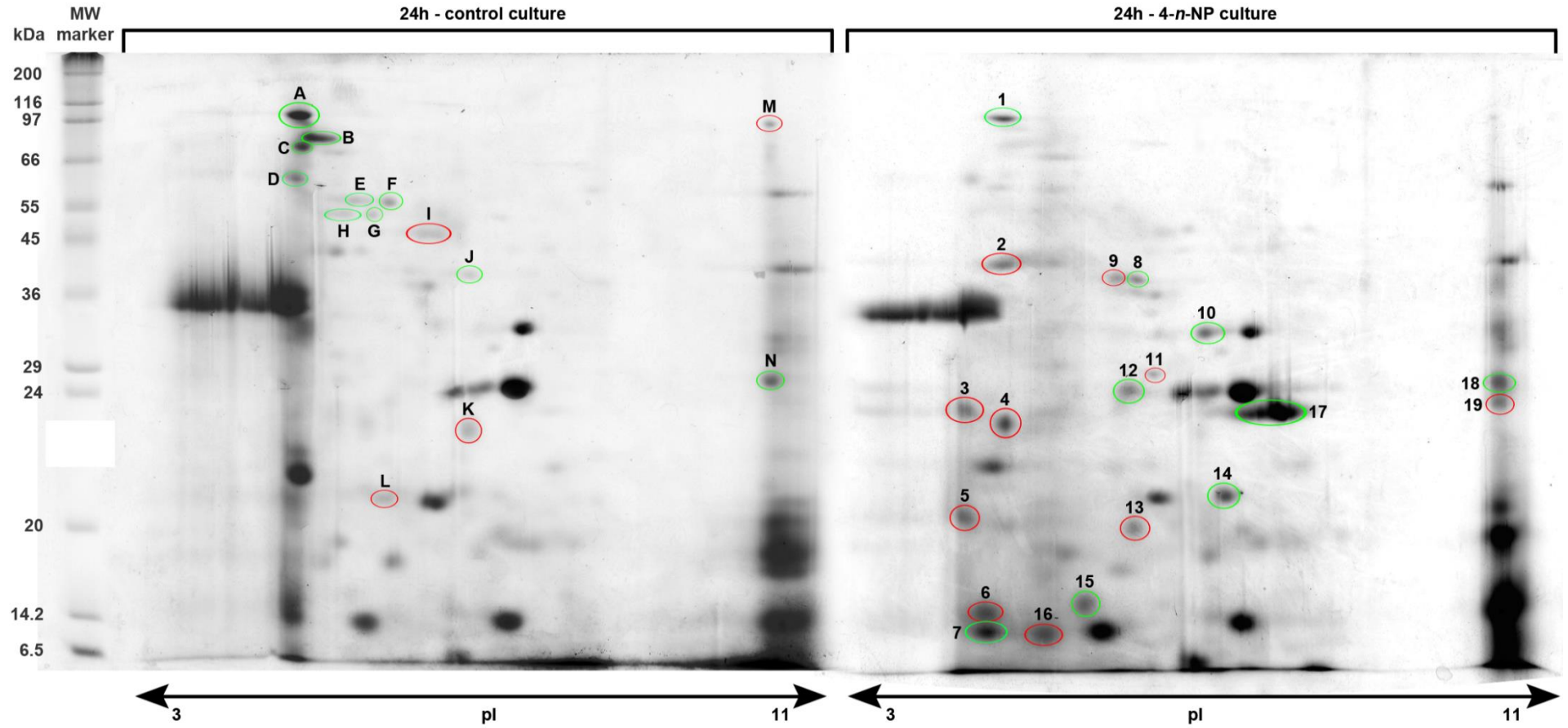
Main steps of the work:

1. 4-n-NP metabolites identification and trend analysis.
2. Protein isolation, separation and digestion.
3. Protein identification with MS/MS analysis.
4. PCA analysis.

Proteins extraction, separation and digestion



Case 1 – sequenced fungi from the tested genus



2-D electrophoresis gels after 24 h of culturing. Marked spots were analyzed by MALDI-TOF/TOF: red circles - unidentified proteins, green circles - identified proteins.

2-D electrophoresis result:

1. 2-DE analysis revealed the expression of 205 spots in the control culture and 208 spots in cultures with 4-*n*-NP addition.
2. 88 spots were matched in both gels.
3. When the test culture was compared with the sample with 4-*n*-NP addition, within the matched proteins: 47 proteins decreased and 41 proteins increased their relative intensity, however, the differences between matched spots were not significant (below 1-fold).
4. The most significant differences (large spots) between the samples included: 14 protein spots present only in control samples and 19 protein spots present only in xenobiotic containing cultures

Main steps of the work:

1. 4-n-NP metabolites identification and trend analysis
2. Protein isolation, separation and digestion.
3. Protein identification with MS/MS analysis.
4. PCA analysis.

MALDI-TOF/TOF analysis

1. The analysis was conducted on AB Sciex 5800 TOF/TOF system.
2. α-cyano-4-hydroxycinnamic acid (CHCA) was used as a matrix.
3. Every sample was placed on MALDI plate five times to cover the selection of 50 strongest precursors for MS/MS analysis (after global exclusion list applied).
4. The TOF MS analysis was done in the mass range 700-4000 Da, 4000V/400Hz laser relative energy with 2000 shots per sample. The precursor selection order in this mode was set from strongest to weakest.
5. Instrument in TOF MS mode was externally calibrated first and then followed by internal calibration with every sample with 842.510 m/z and 2211.106 m/z.
6. The TOF/TOF MS/MS analysis was conducted in the mass range 10-4000 Da, 4550V/400Hz laser relative power, CID gas (air) switched on at a pressure of ca $7x10^{-7}$ and up to 4000 shots per precursor with dynamic exit. The precursor selection was set from weakest to strongest in this mode.
7. The external calibration of MS/MS mode with the fragments of Glu-fibrinopeptide (1570.677 m/z) was applied.

Database searches

1. The MASCOT Search Engine was used for protein database searches.
2. The data were searched against the NCBInr (version 12.2013) database with taxonomy filtering set to fungi (total number of sequences 34927437; total number of fungi sequences 3267418)
3. MS/MS ion searches were performed with the following settings: trypsin was chosen as protein digesting enzyme, up to two missed cleavages were tolerated, the following variable modifications were applied: Acetyl (N-term), Carbamidomethyl (C), Deamidated (NQ), Gln->pyro-Glu (N-term Q), Glu->pyro-Glu (N-term E), Oxidation (M), Phospho (ST) and Phospho (Y)
4. Searches were done with a peptide mass tolerance of 50 ppm and a fragment ion mass tolerance of 0.3 Da.

Case 1 – sequenced fungi from the tested genus

Summary of Mascot search results. Green – identified (high score and/or high sequence coverage), red – unidentified (low score). Mascot algorithm matching – protein scores greater than 73 are significant (p<0.05).

ID	Protein Accession	MW (Da)	pI	Seq. Cov. (%)	Mascot Score	Protein Description
a	gi|322712074	53925	5.14	47	347	immunogenic protein [Metarhizium anisopliae ARSEF 23]
b	gi|322712074	53925	5.14	56	672	immunogenic protein [Metarhizium anisopliae ARSEF 23]
c	gi|322712074	53925	5.14	24	74	immunogenic protein [Metarhizium anisopliae ARSEF 23]
d	gi|322708836	25878	5.88	39	161	extracellular matrix protein precursor [Metarhizium anisopliae ARSEF 23]
e	gi|322712590	36538	5.80	31	93	RNP domain protein [Metarhizium acridum CQMa 102]
f	gi|322694217	38824	5.80	55	138	glycine-rich protein [Metarhizium anisopliae ARSEF 23]
g	gi|322712591	36539	5.80	68	76	glycine-rich protein [Metarhizium anisopliae ARSEF 23]
h	gi|322712591	36539	5.69	37	88	glycine-rich protein [Metarhizium anisopliae ARSEF 23]
i	-	-	-	-	-	no match
j	gi|322711158	30416	6.0	52	267	vip1 [Metarhizium anisopliae ARSEF 23]
k	gi|170106511	73245	8.9	11	44	predicted protein [Laccaria bicolor S238N-H82]
l	gi|358060715	113470	8.4	16	48	hypothetical protein E5Q_00127 [Mixia osmundae IAM 14324]
m	gi|302666331	79138	9.6	8	37	hypothetical protein TRV_01048 [Trichophyton verrucosum HKI 0517]
n	gi|322711195	23843	11.1	46	80	60S ribosomal protein L13 [Metarhizium anisopliae ARSEF 23]
1	gi|322712074	53925	5.1	49	232	immunogenic protein [Metarhizium anisopliae ARSEF 23]
2	gi|342882959	18677	9.1	22	46	hypothetical protein FOXB_05933 [Fusarium oxysporum Fo5176]
3	gi|322710763	228390	5.3	43	75	filament-forming protein [Metarhizium anisopliae ARSEF 23]
4	gi|342882959	18677	9.1	18	56	hypothetical protein FOXB_05933 [Fusarium oxysporum Fo5176]
5	gi|328851184	25457	6.3	11	37	hypothetical protein MELLADRAFT_67879 [Melampsora larici-populina 98AG31]
6	gi|358060715	113470	8.3	9	42	hypothetical protein E5Q_00127 [Mixia osmundae IAM 14324]
7	gi|322712463	63038	6.7	39	78	pyruvate dehydrogenase kinase [Metarhizium anisopliae ARSEF 23]
8	gi|322708858	39150	5.98	63	84	inorganic pyrophosphatase [Metarhizium anisopliae ARSEF 23]
9	gi|342882959	18677	9.1	20	47	hypothetical protein FOXB_05933 [Fusarium oxysporum Fo5176]
10	gi|322707901	34979	8.62	74	322	malate dehydrogenase [Metarhizium anisopliae ARSEF 23]
11	gi|342882959	18677	9.1	18	46	hypothetical protein FOXB_05933 [Fusarium oxysporum Fo5176]
12	gi|322706086	24558	6.43	48	141	mitochondrial peroxiredoxin PRX1 [Metarhizium anisopliae ARSEF 23]
13	gi|312222214	166185	7.87	13	49	hypothetical protein LEMA_P009410.1 [Leptosphaeria maculans JN3]
14	gi|322712189	15659	6.10	74	278	superoxide dismutase [Metarhizium anisopliae ARSEF 23]
15	gi|322710763	228390	5.26	42	77	filament-forming protein [Metarhizium anisopliae ARSEF 23]
16	gi|299751084	36579	10.0	10	48	hypothetical protein CC1G_04477 [Coprinopsis cinerea okayama7#130]
17	gi|322704437	23069	6.75	67	494	nitroreductase family protein [Metarhizium anisopliae ARSEF 23]
18	gi|322706086	24558	6.43	52	146	mitochondrial peroxiredoxin PRX1 [Metarhizium anisopliae ARSEF 23]

Database search conclusions:

1. The main mechanism of the 4-*n*-NP biodegradation is consecutive oxidation of C-$C_{terminal}$ atoms of aliphatic chain leading to formation of carboxylic acids coupled with $C_{terminal}$ carbon removal. This process may be catalyzed by nitroreductase family protein (the largest tested 2-DE spot) that may act as a nitroreductase-like protein (oxidoreductase).
2. The mechanism of oxygenation of the aliphatic carbon atom situated next to the aromatic ring is difficult to explain at current state, but it is possible that this reaction is also conducted by the same enzyme.
3. Presence of mitochondrial peroxiredoxin PRX1 and superoxide dismutase suggest that during the process reactive oxygen species (ROS) are generated. We think that in the described process both enzymes first of all acts as a part of antioxidant defense system yet in case PRX1 its oxidoreductase activity cannot be excluded.
4. Malate dehydrogenase overexpession in the cultures containing 4-*n*-NP supports the fact that the xenobiotic is mineralized completely or partly through TCA cycle.
5. The oxidation stress coupled with the toxic xenobiotic activity leads to higher energy consumption and increased metabolic activity that is why inorganic pyrophosphatase and pyruvate dehydrogenase kinase in 4-*n*-NP containing cultures were detected.

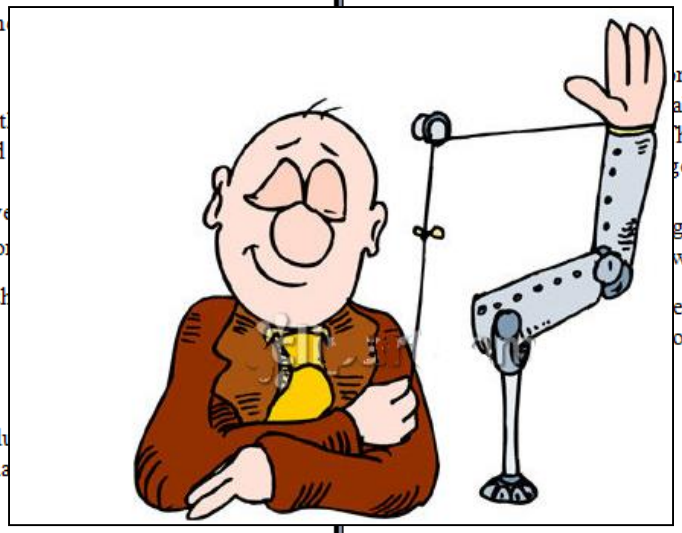
Main steps of the work:

1. 4-n-NP metabolites identification and trend analysis
2. Protein isolation, separation and digestion.
3. Protein identification with MS/MS analysis.
4. PCA analysis.

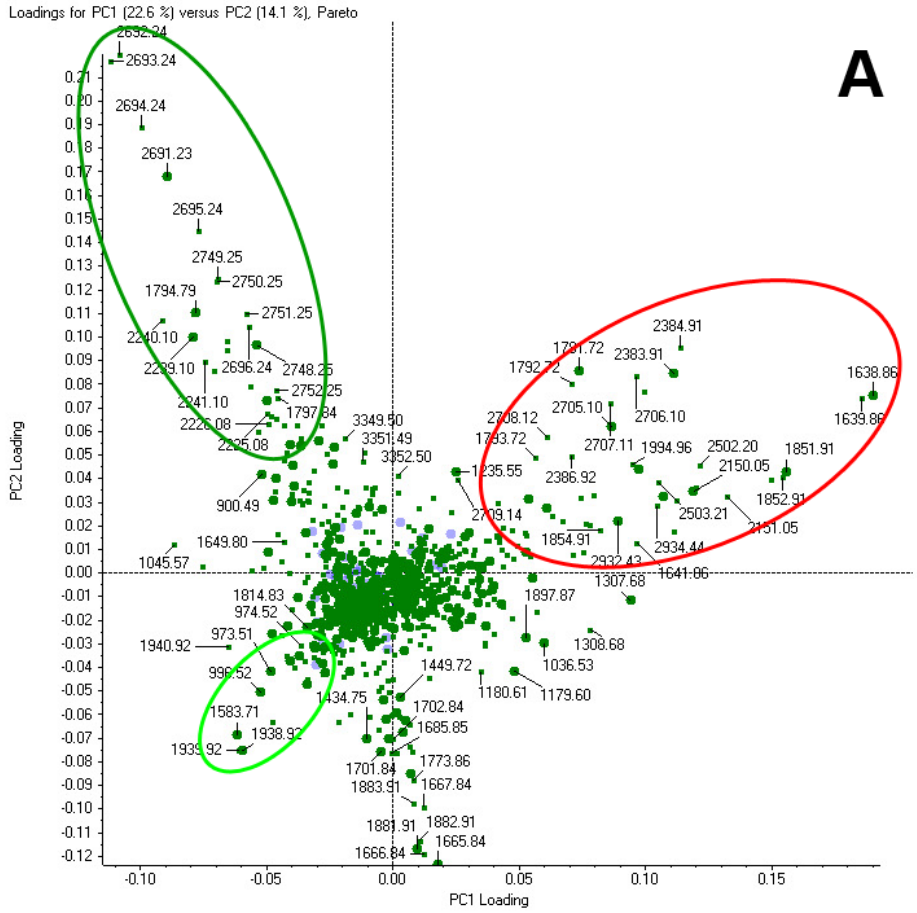
Principal Component Analysis (PCA)

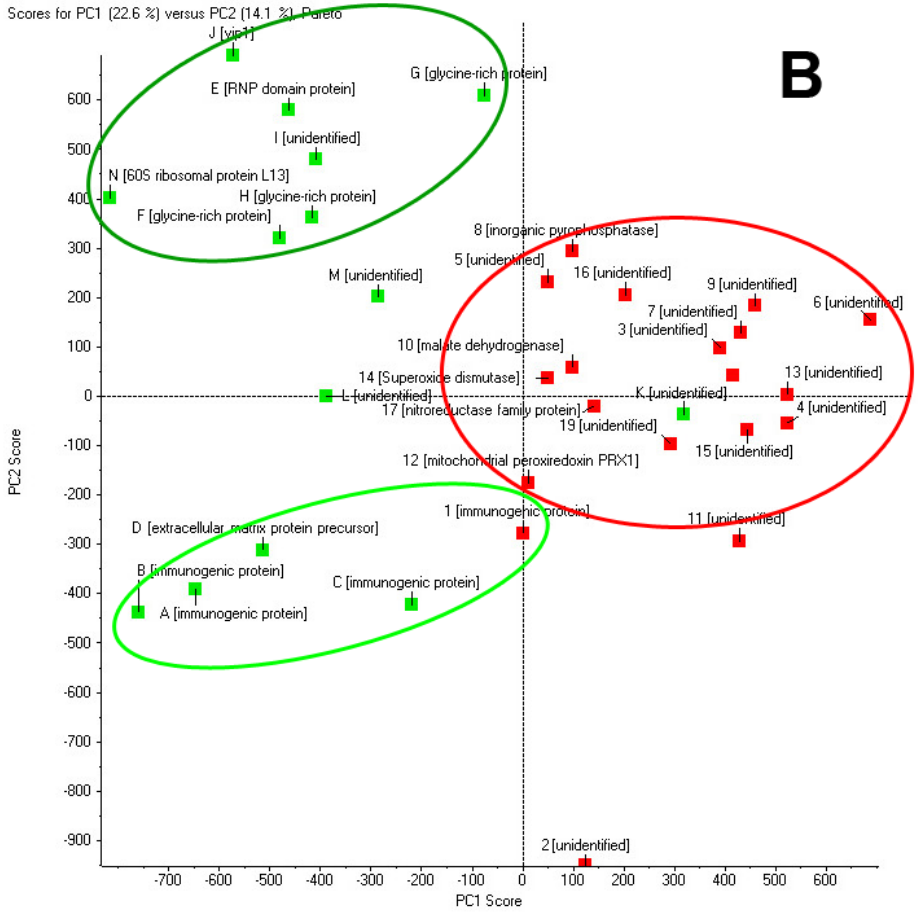
1. The analysis was performed on all full-scan single TOF MS data (after global exclusion list o f common contaminants applied), that are in fact a peptide maps for the selected samples, with the use of MarkerView™ software.
2. PCA scores of similar samples tend to form clusters while different samples are found at greater mutual distances

...stop with the mathematics!



Case 1 – sequenced fungi from the tested genus





PCA analysis of the peptide maps (TOF MS data – peptide maps):
A – PC1 against PC2 loadings chart,
B – PC1 against PC2 scores chart.
Group 1 – green circle (extracellular cell-wall proteins),
Group 2 – dark green circle (structural and nucleotide binding proteins),
Group 3 – red circle (enzymes).

Final conclusions:

1. Deeper insight into the process of 4-*n*-NP biodegradation by *M. velutinum* was achieved by finding new metabolites that filled-in some missing steps in the pathway and proteome expression analysis.

2. Among the tested proteins more than 60% of them were identified.

3. PCA divided the data into three subgroups what allowed initial classification of unidentified proteins.

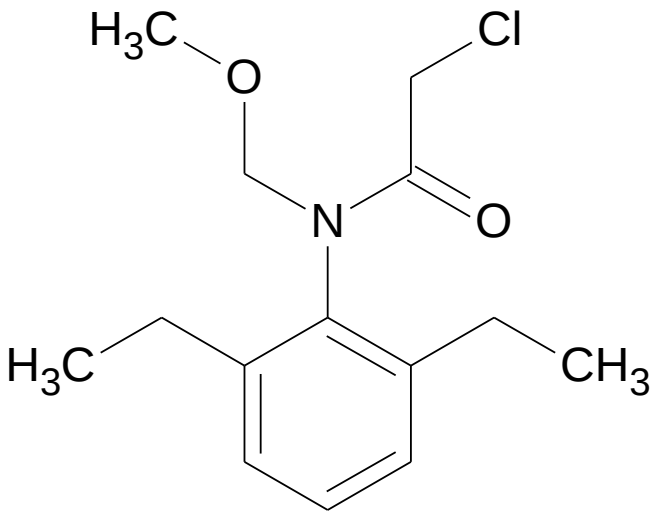
4. Collected data allowed to formulate the explanation of microorganism strategy towards 4-*n*-NP and explain the basics of the proteomic background involving oxidation-reduction systems, ROS defense systems, TCA cycle and energy-related systems.

Fungal biodegradation proteomics

Case 1 – some fungi from the tested genus are sequenced

Case 2 – nonsequenced organism

Case 2 – nonsequenced organism



Alachlor:

- herbicide,
- toxic,
- EDCs activity

Culture of *Peacilomyces marquandii* + alachlor
(+ proper controls)

Only 12 sequences in NCBI database for *Peacilomyces marquandii* !!!

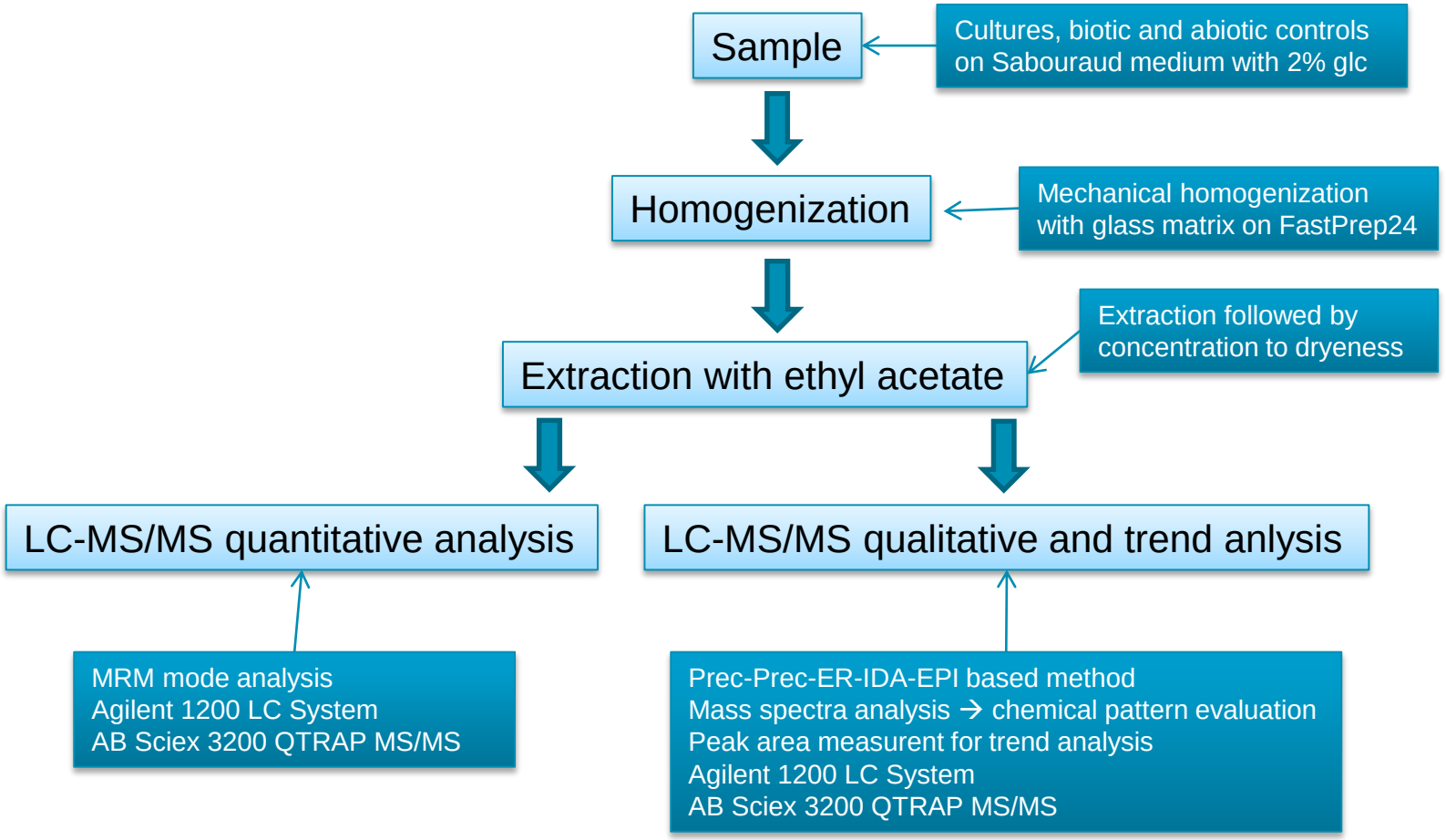
The tested strain, was obtained from the fungal strain collection of the Department of Industrial Microbiology and Biotechnology, Institute of Microbiology, Biotechnology and Immunology, University of Łódź, Poland.

Main steps of the work:

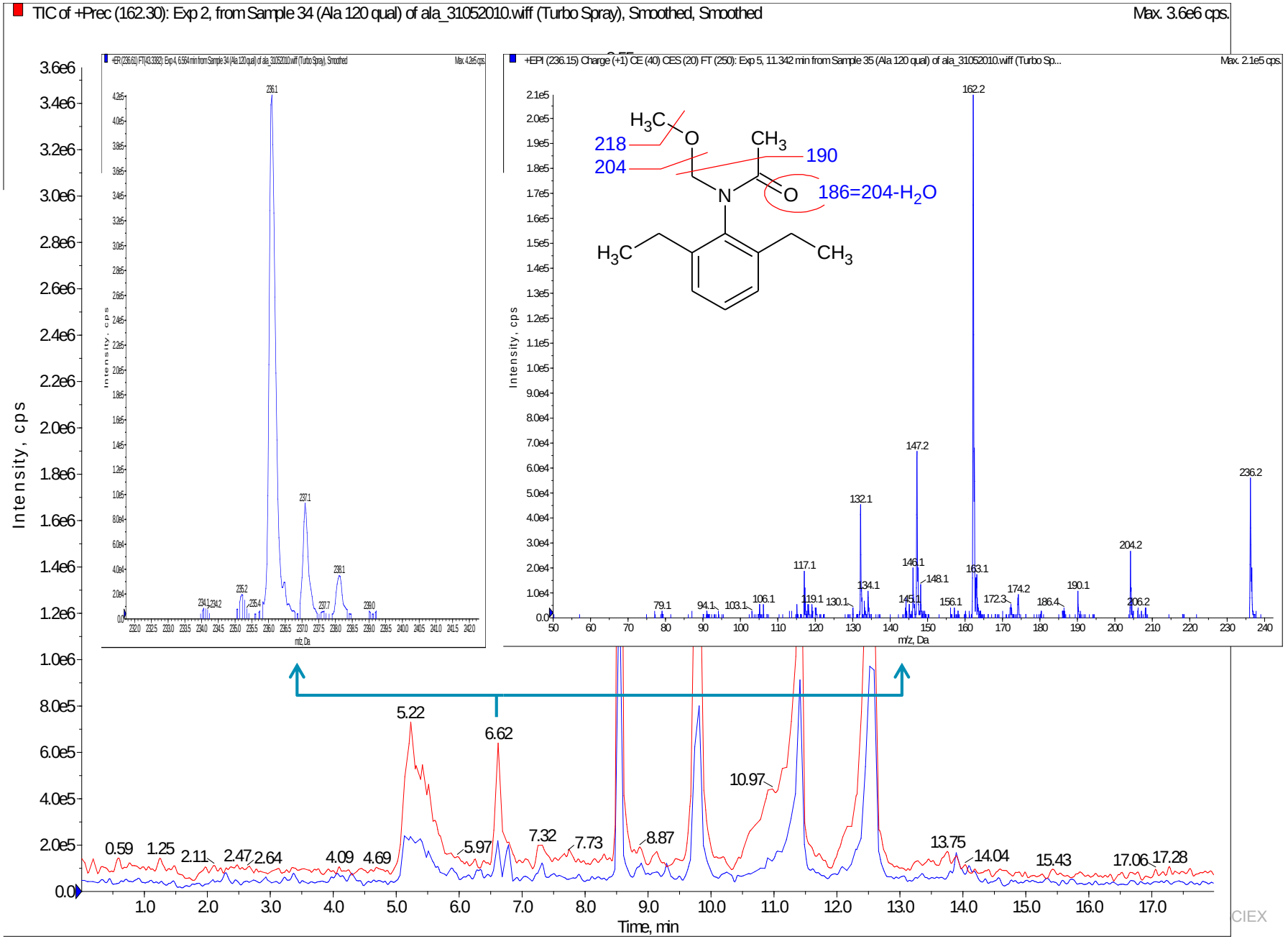
1. Metabolites identification and trend analysis.
2. Protein isolation, separation and digestion.
3. Protein identification with MS/MS analysis.
4. BLAST searches and function confirmation.

The same as described previously

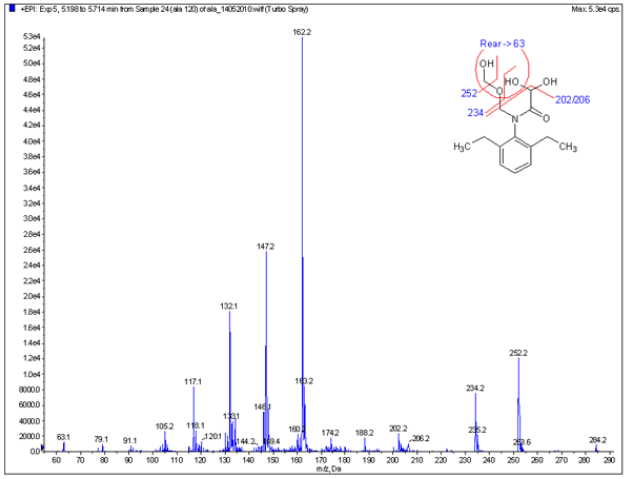
Quantitative and trend analysis of the metabolites formation during alachlor biodegradation

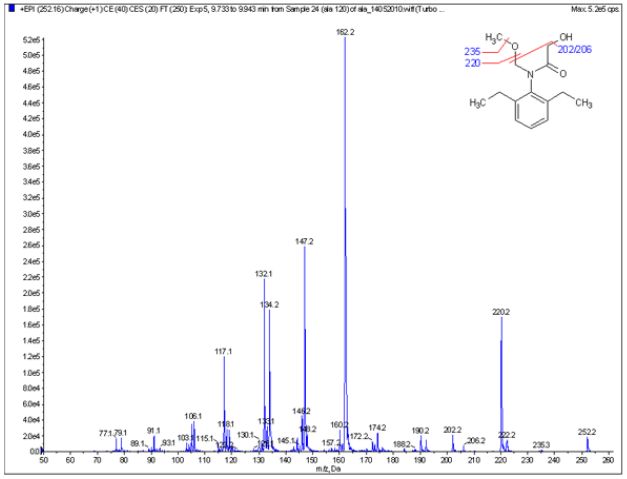


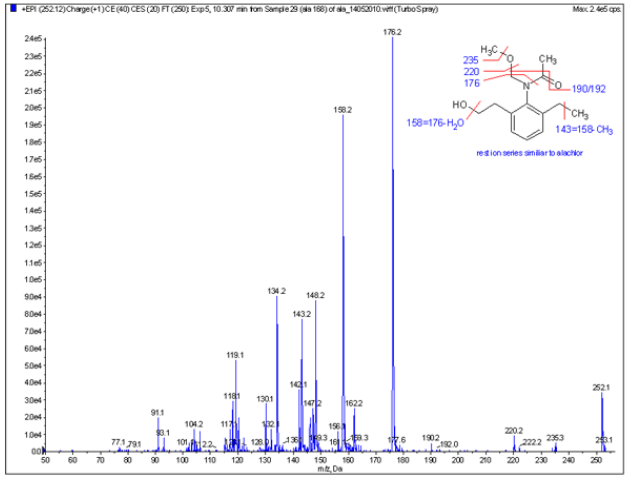
Case 2 – nonsequenced organism



Case 2 – nonsequenced organism







N-(2,6-diethylphenyl)-2,2-dihydroxy-N-[(hydroxymethoxy)methyl]acetamide

RT (min)	5.2
Formula	$C_{14}H_{21}NO_5$
MW	283.32

Mass spectrum	
m/z	rel. int. (%)
63.06	2.2609
79.14	1.6232
91.14	1.4493
105.18	4.8696
117.12	12.9855
118.14	4.7536
130.14	3.5942
131.16	3.4783
132.12	32
133.14	7.4783
134.16	7.8261
146.1	10.5507
147.18	47.5362
148.2	10.4928
160.2	4.058
162.24	100
163.2	15.942
174.18	2.8986
188.22	2.7246
202.2	4.058
206.22	2.087
234.18	14.2029
235.2	3.4203
252.18	22.2029
284.18	1.5652

N-(2,6-diethylphenyl)-2,2-dihydroxy-N-(methoxymethyl)acetamide

RT (min)	6.7
Formula	$C_{14}H_{21}NO_4$
MW	267.32

Mass spectrum	
m/z	rel. int. (%)
61.08	2.7933
79.14	1.5164
105.12	3.352
117.12	9.577
119.1	4.7885
120.06	4.8683
130.14	3.2721
131.16	3.5914
132.12	31.7638
133.14	3.5914
134.16	4.3895
144.18	2.1548
146.22	7.0231
147.18	41.261
148.2	30.7263
160.2	9.9761
162.18	100
162.9	6.2251
163.2	5.427
170.16	7.7414
188.16	5.1077
209.16	13.9665
218.16	4.5491
227.16	6.7039
236.16	41.5004
268.26	3.0327

N-(2,6-diethylphenyl)-N-[(dihydroxymethoxy)methyl]-2-methoxyacetamide

RT (min)	8.5
Formula	$C_{15}H_{23}NO_5$
MW	297.34

Mass spectrum	
m/z	rel. int. (%)
61.08	4.8685
63.06	11.8796
77.1	2.9835
79.14	4.2853
91.14	2.2512
105.06	32.5197
117.12	28.0445
118.14	6.3466
130.14	3.4717
131.16	3.7022
132.12	55.4787
132.78	3.1055
133.14	6.6043
134.16	6.6857
146.22	16.7074
147.18	70.8028
147.84	6.8619
160.2	8.9504
161.22	10.4557
162.18	100
162.9	12.1372
170.22	5.099
175.2	10.1844
188.22	8.1638
203.22	11.7033
266.22	5.2075
284.25	2.1835
298.42	4.4526

2,6-diethyl-N-methylaniline

RT (min)	8.9
Formula	$C_{11}H_{17}N$
MW	163.25

Mass spectrum	
m/z	rel. int. (%)
77.1	4.918
79.14	4.3716
90.12	10.929
91.08	2.7322
105.12	10.929
117.12	55.7377
118.02	4.918
131.1	4.3716
132.12	60.1093
132.9	3.8251
134.46	2.7322
147.18	76.5027
147.84	1.6393
164.24	100

N-(2,6-diethylphenyl)-2-hydroxy-N-(methoxymethyl)acetamide

RT (min)	9.8
Formula	$C_{14}H_{21}NO_3$
MW	251.32

Mass spectrum	
m/z	rel. int. (%)
77.1	2.5017
79.14	2.8516
91.14	3.6214
105.12	6.1582
106.14	6.4906
117.12	21.0637
118.08	4.7936
119.1	4.951
132.12	38.0511
133.14	5.6858
134.16	33.6599
144.18	2.9041
146.16	8.0126
147.18	47.9531
148.2	3.8314
160.2	5.4934
162.18	100
162.84	6.1756
172.2	2.3618
174.24	4.6361
190.2	3.9363
202.2	3.9363
220.2	31.2456
222.24	2.5717
235.32	0.2799
252.24	3.3065

N-[2-ethyl-6-(2-hydroxyethyl)phenyl]-N-(methoxymethyl)acetamide

RT (min)	10.3
Formula	$C_{14}H_{21}NO_3$
MW	251.32

Mass spectrum	
m/z	rel. int. (%)
77.1	0.9449
91.08	6.1417
93.12	3.1496
104.22	4.4094
106.14	3.7795
117.12	5.0394
118.14	11.6535
119.1	21.8898
130.14	9.9213
132.12	4.8819
134.16	37.0079
142.14	12.9134
143.16	33.0709
146.16	8.6614
147.18	11.1811
148.2	34.6457
156.12	3.937
158.16	81.4173
159.08	5.9843
162.18	9.1339
163.92	1.1024
176.16	100
176.88	5.0394
190.2	1.4173
220.2	3.3071
222.24	0.7874
235.32	1.7323
252.12	13.7008

{(2,6-diethylphenyl)[(hydroxymethoxy)methyl]amino}oxoacetic acid

RT (min)	11
Formula	$C_{14}H_{19}NO_5$
MW	281.3

Mass spectrum	
m/z	rel. int. (%)
77.04	0.5676
79.08	6.7171
91.14	1.2772
105.18	2.0814
117.12	15.894
118.14	4.9196
132.12	28.7133
133.14	3.0274
134.16	3.4532
146.16	17.4078
147.18	49.1012
147.9	2.0341
160.2	3.0747
161.22	6.0549
162.18	100
162.84	5.1088
170.16	2.4598
186.24	2.8382
188.22	5.298
203.16	2.6017
218.4	0.473
222.18	0.4257
236.28	0.3784
250.14	0.6149
264.3	0.3311
282.24	1.8448

N-(2,6-diethylphenyl)-N-(methoxymethyl)acetamide

RT (min)	11.3
Formula	$C_{14}H_{21}NO_2$
MW	235.32

Mass spectrum	
m/z	rel. int. (%)
77.04	0.7943
91.08	0.6354
105.12	2.4623
117.12	10.3257
118.08	1.9857
119.16	1.8268
132.12	20.3336
133.2	2.3828
134.16	2.7006
146.16	7.1485
147.24	39.0786
147.84	1.7474
148.2	3.4154
162.18	100
162.9	4.2891
163.26	5.0834
172.26	2.6211
174.24	3.892
186.24	1.9857
190.08	2.3034
204.24	10.1668
206.22	0.8737
208.32	0.6354
218.28	0.556
236.22	21.2073

(Z)-1-[(2,6-diethylphenyl)(methoxymethyl)amino]ethene-1,2-diol

RT (min)	11.7
Formula	$C_{14}H_{21}NO_3$
MW	251.32

Mass spectrum	
m/z	rel. int. (%)
77.1	1.0114
79.08	1.5171
91.14	1.5171
95.16	1.3274
105.12	4.2351
117.18	17.5095
118.08	3.0341
119.1	1.7699
132.12	31.0367
133.2	8.7231
134.16	4.9305
146.22	2.9709
147.18	44.7535
148.2	12.579
162.18	100
163.2	28.129
164.22	2.2124
203.28	0.9482
207.12	0.4425
219.3	4.0455
235.32	0.6953
252.24	3.603

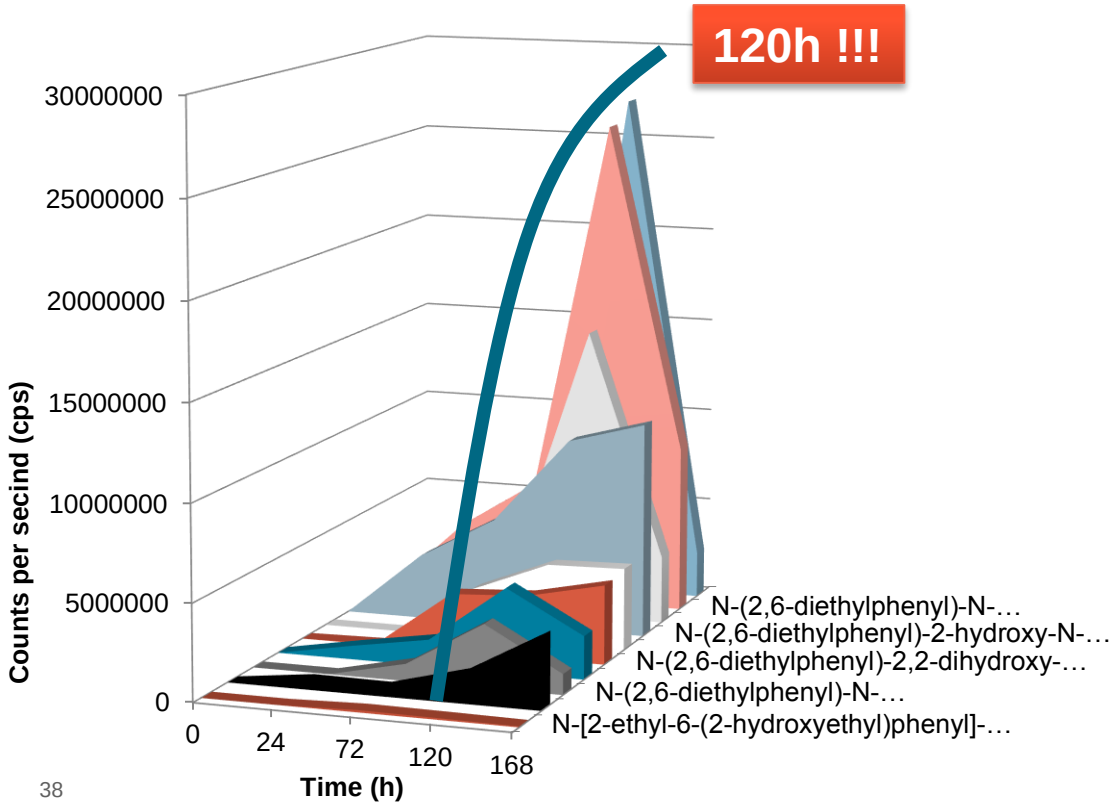
N-(2,6-diethylphenyl)-N-[(dihydroxymethoxy)methyl]-2-hydroxy-2-methoxyacetamide

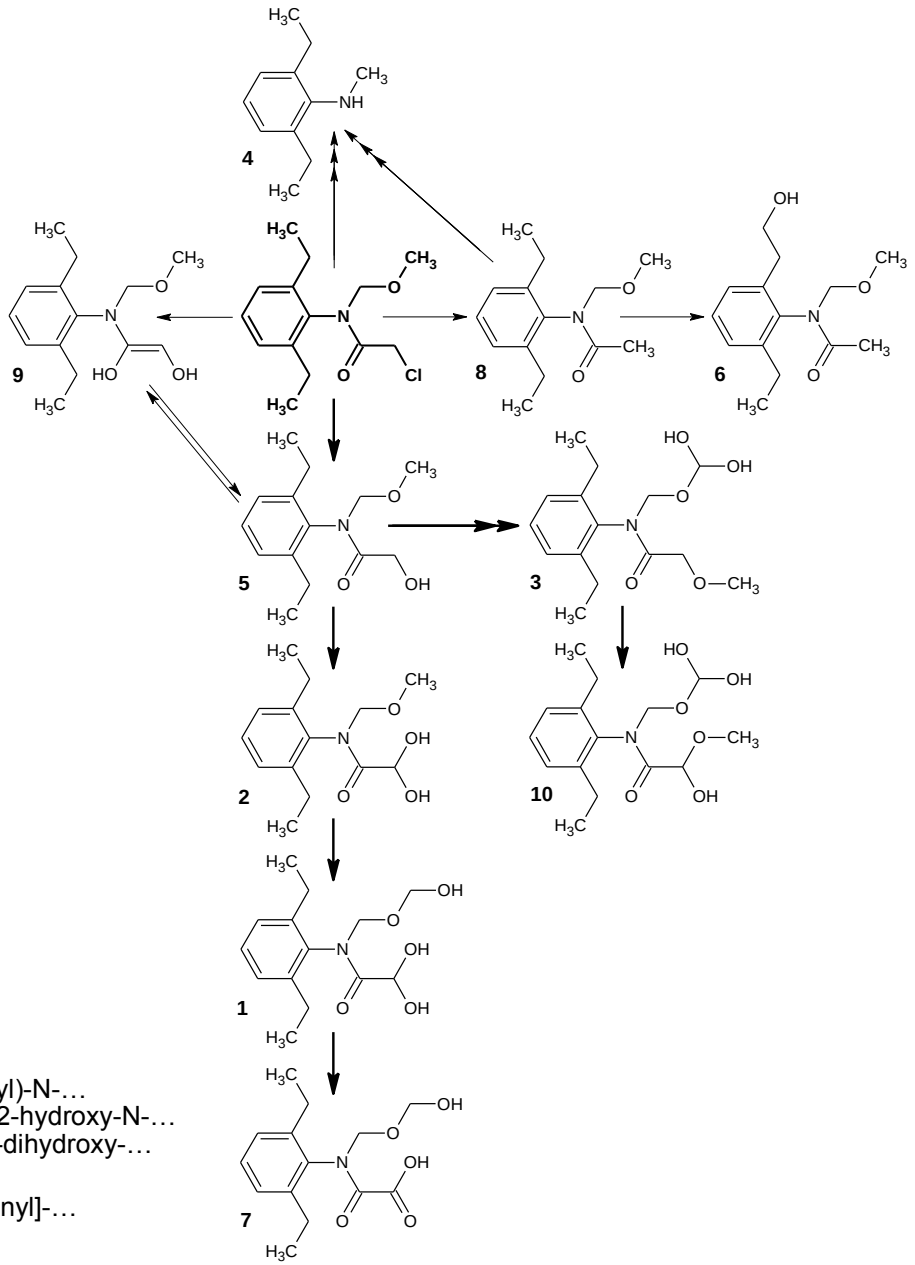
RT (min)	12.3
Formula	$C_{15}H_{23}NO_6$
MW	313.34

Mass spectrum	
m/z	rel. int. (%)
63.12	2.2752
79.08	2.4919
91.14	1.1918
91.08	1.3001
105.12	3.0336
117.12	9.2091
117.42	3.1419
132.12	36.9447
133.2	7.909
134.1	3.0336
146.16	4.2254
147.18	50.0542
148.26	6.7172
160.14	1.5168
162.18	100
163.26	16.0347
172.2	4.7671
174.18	2.8169
200.16	1.1918
206.28	1.7335
234.12	5.4171
250.6	0.8727
279.4	0.9312
261.42	0.7584
296.4	6.6089
314.46	7.6923

Case 2 – nonsequenced organism

1. 10 metabolites identified.
2. 50% of alachlor is removed after 120h of culturing
3. Mycelium samples collected after 120 h of culturing were determined as the best suited for intracellular proteome expression studies.





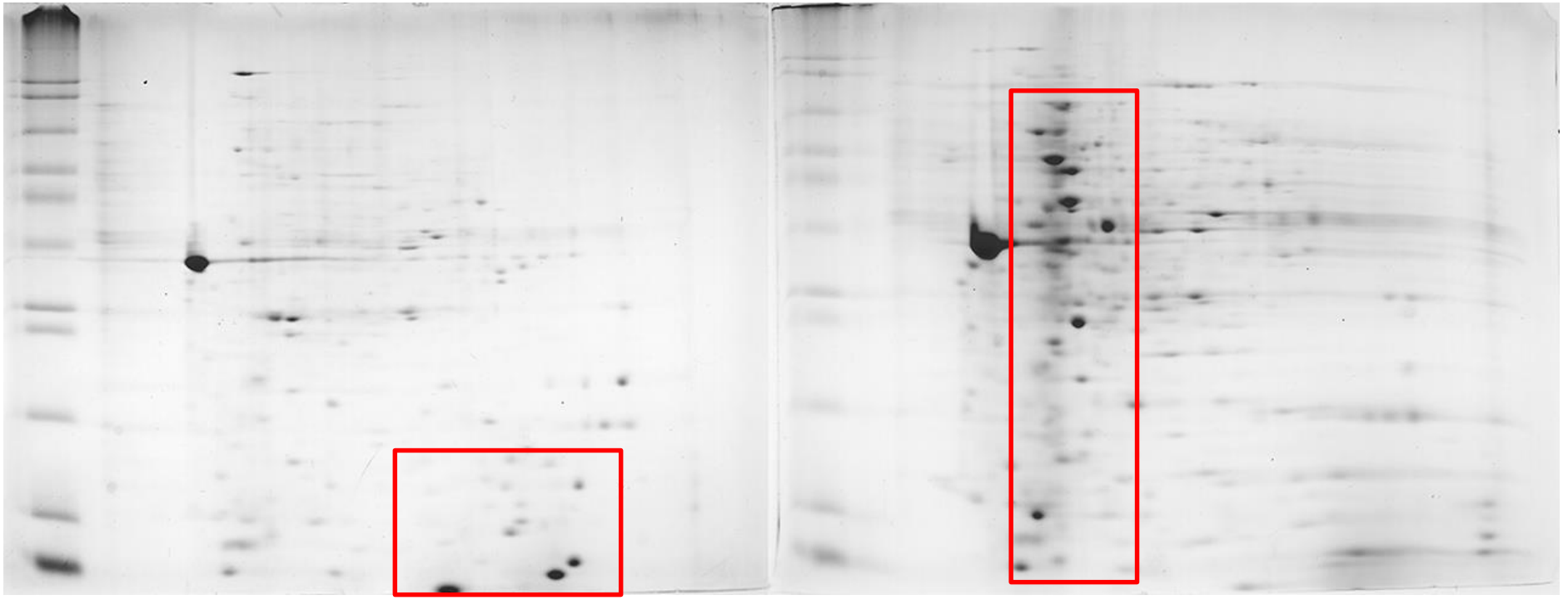
2-D electrophoresis result:

1. 2-DE analysis revealed the expression of 247 spots in the control culture and 365 spots in cultures with 4-*n*-NP addition.
2. 92 spots were matched in both gels - 48 proteins decreased and 44 proteins increased their relative intensity.
3. In majority, the differences between matched spots were not significant (below 1-fold) excluding 9 spots overexpressed significantly in the alachlor containing cultures, and 3 spots overexpressed significantly in the control samples.
4. The most significant differences (large spots) between the samples included 12 protein spots present only in xenobiotic containing cultures

Case 2 – nonsequenced organism

Control sample

Alachlor sample



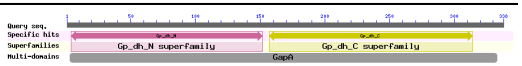
2-D electrophoresis gels after 120 h of culturing.

All significantly up- or downregulated proteins were subjected to trypsin digestion and MS/MS analysis

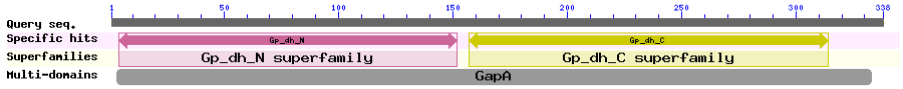
Homology search & match!

1. Aquire MS/MS data → AB Sciex 5800 MALDI-TOF/TOF
2. Perform searches → NCBInr (version 04.2014) database with taxonomy filtering set to fungi & MASCOT Search Engine
3. Confirm result using BLAST searches with delta-blast algorithm (Domain Enhanced Lookup) → what is the function of the protein?

Example result:

No.	Spot ID	Accession	Mass (Da)	Calc. pI	Matches	Seq. Cov.	Score	Description	Sequence	DELTA-BLAST
11	286	gi|429862698 gi|530477121 gi|477536372	36234 36248 36232	6.25 6.25 6.24	33 31 30	55% 55% 51%	366 335 333	glyceraldehyde-3-phosphate dehydrogenase [Colletotrichum gloeosporioides Nara gc5] glyceraldehyde-3-phosphate dehydrogenase [Colletotrichum gloeosporioides Cg-14] glyceraldehyde-3-phosphate dehydrogenase [Colletotrichum orbiculare MAFF 240422]	MAPIK**VGINGFGR**IGRIVFR**NAIEHPEVEIVAVN** **DPFIETKYAAYMLK**YDSTHGIFNGEIK**QEGND** **LVINGKTVKFYTERDPAAIPWKETGADYVVES** **TGVFTTTDKAK**AHLQGGAK**KVIISAPSADAPM** **YVMGVNEK**SYDGSADVISNASCTTNCLAPLAK VINDKFGIVEGLMTTVHSYTATQKTVDGPSAK DWRGGRTAAQNIIPSSTGAAKAVGK**VIPELNG** **KLTGMSMRVPTANVSVVDLTAR**IEK**GASYDEI** **KQAIKEAAEGPLK**GVLAYTEDDVVSTDMIGNP NSSIFDAK**AGISLNNNFVKLVSWYDNEWGYS** **RRVLDLLAHVAK**VDASK MAPIK**VGINGFGR**IGRIVFR**NAIEHPEVEIVAVN** **DPFIETKYAAYMLK**YDSTHGIFNGEIK**QEGND** **LVINGKTVKFYTERDPAAIPWKETGAEYVVES** **TGVFTTTDKAK**AHLQGGAK**KVIISAPSADAPM** **YVMGVNEK**SYDGSADVISNASCTTNCLAPLAK VINDKFGIVEGLMTTVHSYTATQKTVDGPSAK DWRGGRTAAQNIIPSSTGAAKAVGK**VIPELNG** **KLTGMSMRVPTANVSVVDLTAR**IEK**GASYDEI** **KQAIKEAAEGPLK**GVLAYTEDDVVSTDMIGNP NSSIFDAK**AGISLNNNFVKLVSWYDNEWGYS** **RRVLDLLAHVAK**VDASK MAPIK**VGINGFGR**IGRIVFR**NAVEHPDVEIVAV** **NDPFIETKYAAYMLK**YDSTHGIFNGEIAQDGN DLVINGKKVKFYTER**DPAAIPWKETGADYVVE** **STGVFTTIDKAK**AHLQGGAK**KVIISAPSADAP** **MYVMGVNEK**SYDGSADVISNASCTTNCLAPL AKVINDKFTIVEGLMTTVHSYTATQKTVDGPSA KDWRGGRTAAQNIIPSSTGAAKAVGK**VIPELN** **GKLTGMSMRVPTANVSVVDLTARIEKGATYD** **EIKQAIKEAAEGPLK**GVLAYTEDDVVSTDMIG NPNSSIFDAK**AGISLNNNFVKLVSWYDNEWG** **YSRRVLDLLAHVAK**VDASK	 Description | Pssmid | Multi-dom | E-value Gp_dh_C[pfam02800], Glyceraldehyde 3-phosphate dehydrogenase, C-terminal domain; GAPDH is a tetrameric NAD-binding enzyme involved in glycolysis and glyconeogenesis. C-terminal domain is a mixed alpha/antiparallel beta fold. | 251539 | no | 7.93e-100 Gp_dh_N[pfam00044], Glyceraldehyde 3-phosphate dehydrogenase, NAD binding domain; GAPDH is a tetrameric NAD-binding enzyme involved in glycolysis and glyconeogenesis. N-terminal domain is a Rossmann NAD(P) binding fold. | 249536 | no | 9.57e-80 GapA[COG0057], Glyceraldehyde-3-phosphate dehydrogenase/erythrose-4-phosphate dehydrogenase [Carbohydrate transport and metabolism] | 223135 | yes | 0e+00

Example result cont.:

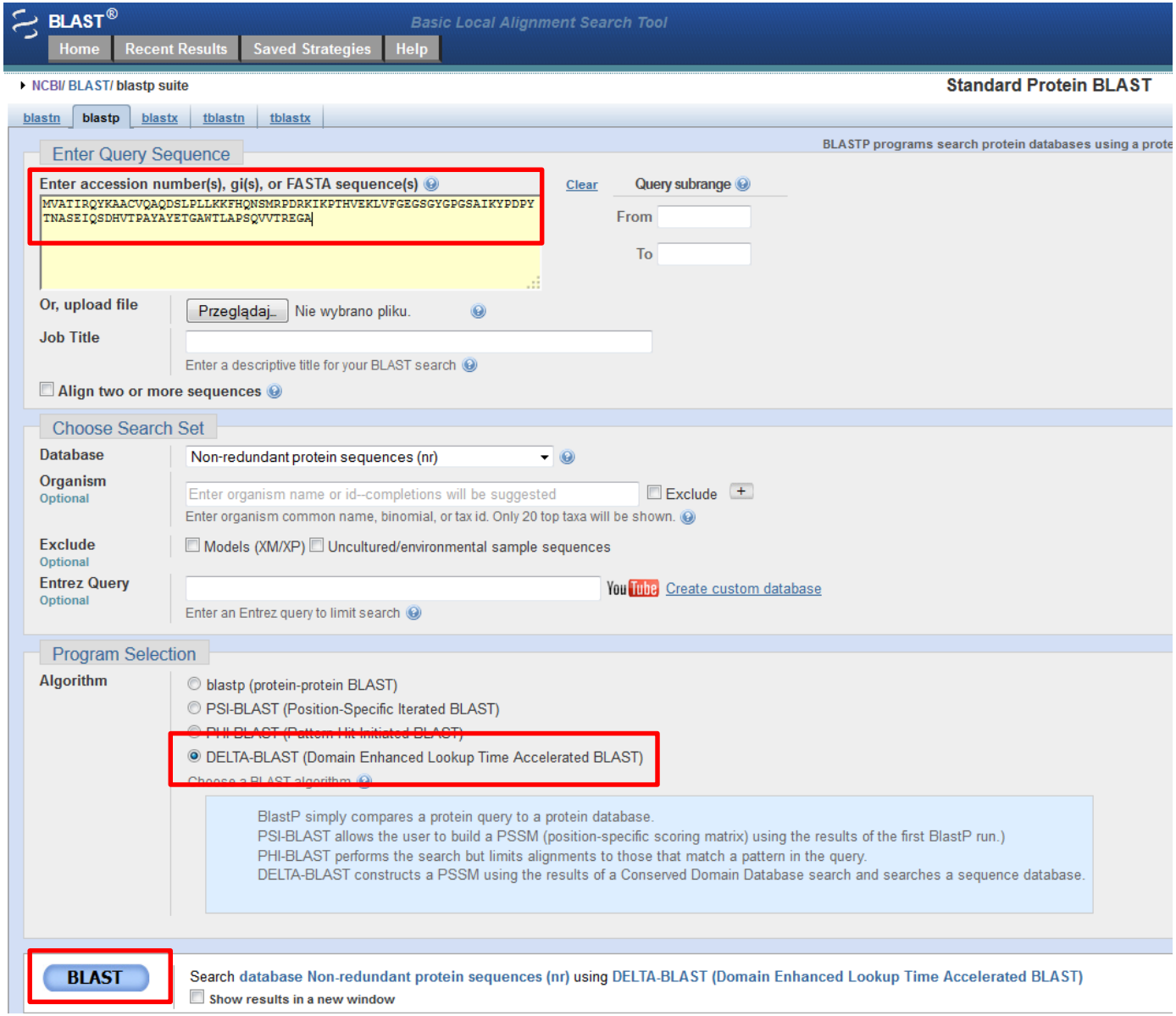
Description (MASCOT)	DELTA-BLAST
glyceraldehyde-3-phosphate dehydrogenase [Colletotrichum gloeosporioides Nara gc5] **glyceraldehyde-3-phosphate dehydrogenase** [Colletotrichum gloeosporioides Cg-14] **glyceraldehyde-3-phosphate dehydrogenase** [Colletotrichum orbiculare MAFF 240422]	

Description	PssmId	Multi-dom	E-value
Gp_dh_C[pfam02800], **Glyceraldehyde 3-phosphate dehydrogenase**, C-terminal domain; GAPDH is a tetrameric NAD-binding enzyme involved in glycolysis and glyconeogenesis. C-terminal domain is a mixed alpha/antiparallel beta fold.	251539	no	7.93e-100
Gp_dh_N[pfam00044], **Glyceraldehyde 3-phosphate dehydrogenase**, NAD binding domain; GAPDH is a tetrameric NAD-binding enzyme involved in glycolysis and glyconeogenesis. N-terminal domain is a Rossmann NAD(P) binding fold.	249536	no	9.57e-80
GapA[COG0057], **Glyceraldehyde-3-phosphate dehydrogenase**/erythrose-4-phosphate dehydrogenase [Carbohydrate transport and metabolism]	223135	yes	0e+00

Case 2 – nonsequenced organism

Homology search & match! – what to do when there is no match in MASCOT search?

1. Manually sequence the data with the help of the software ex. DeNovo Explorer or PeakView Biotools
2. Export data to the BLAST search engine
3. Find the protein function (if possible) with the BLAST searches with delta-blast algorithm (Domain Enhanced Lookup)



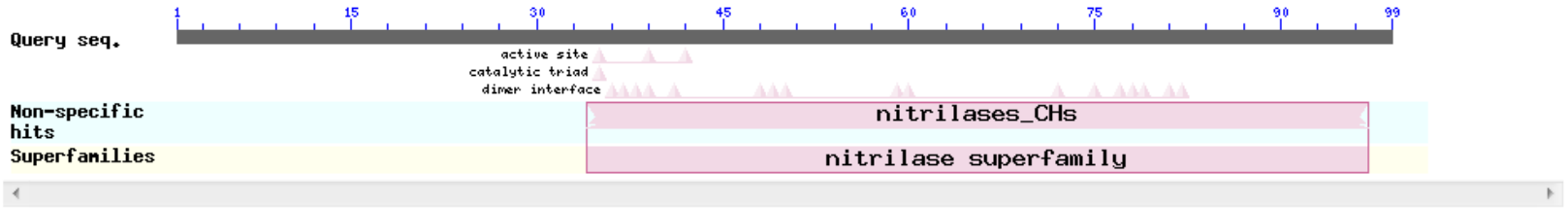
Result - the protein function

Conserved domains on [lcl|7820]

View: Standard Results

Local query sequence

Graphical summary show options »



Search for similar domain architectures | Refine search

List of domain hits

Name	Accession	Description	Interval	E-value
nitrilases_CHs	cd07564	Nitrilases, cyanide hydratase (CH)s, and similar proteins (class 1 nitrilases); Nitrilases ...	34-97	5.76e-09

Nitrilases, cyanide hydratase (CH)s, and similar proteins (class 1 nitrilases); Nitrilases (nitrile aminohydrolases, EC:3.5.5.1) hydrolyze nitriles (RCN) to ammonia and the corresponding carboxylic acid. Most nitrilases prefer aromatic nitriles, some prefer arylacetonitriles and others aliphatic nitriles. This group includes the nitrilase cyanide dihydratase (CDH), which hydrolyzes inorganic cyanide (HCN) to produce formate. It also includes cyanide hydratase (CH), which hydrolyzes HCN to formamide. This group includes four Arabidopsis thaliana nitrilases (Ath)NIT1-4. AthNIT1-3 have a strong substrate preference for phenylpropionitrile (PPN) and other nitriles which may originate from the breakdown of glucosinolates. The product of PPN hydrolysis, phenylacetic acid has auxin activity. AthNIT1-3 can also convert indoacetonitrile to indole-3-acetic acid (IAA, auxin), but with a lower affinity and velocity. From their expression patterns, it has been speculated that NIT3 may produce IAA during the early stages of germination, and that NIT3 may produce IAA during embryo development and maturation. AthNIT4 has a strong substrate specificity for the nitrile, beta-cyano-L-alanine (Ala(CN)), an intermediate of cyanide detoxification. AthNIT4 has both a nitrilase activity and a nitrile hydratase (NHase) activity, which generate aspartic acid and asparagine respectively from Ala(CN). NHase catalyzes the hydration of nitriles to their corresponding amides. This subgroup belongs to a larger nitrilase superfamily comprised of belong to a larger nitrilase superfamily comprised of nitrile- or amide-hydrolyzing enzymes and amide-condensing enzymes, which depend on a Glu-Lys-Cys catalytic triad. This superfamily has been classified in the literature based on global and structure based sequence analysis into thirteen different enzyme classes (referred to as 1-13), this subgroup corresponds to class 1.

Pssm-ID: 143588 **Cd Length:** 297 **Bit Score:** 50.95 **E-value:** 5.76e-09

```
                    10        20        30        40        50        60        70        80
                 ....*....|....*....|....*....|....*....|....*....|....*....|....*....|....*....|
7820          34 RKIKPTHVEKLVFGEGSG---------YGP-GSAI---------KY-----------------PDPYTNASEIQSdhVTP 77
Cdd:cd07564  122 RKLKPTHAERLVWGQGDGsglrvvdtpIGRlGALIcwenymplaRYalyaqgeqihvapwpdfSPYYLSREAWLA--ASR 199

                    90       100
                 ....*....|....*....|
7820          78 AYAYETGAWTLAPSQVVTRE 97
Cdd:cd07564  200 HYALEGRCFVLSACQVVTEE 219
```

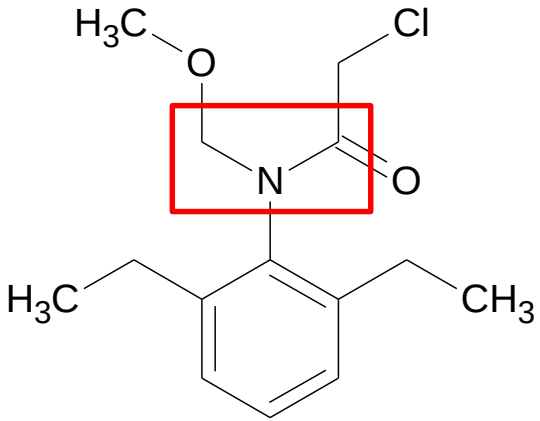
Blast search parameters

Data Source:	Live blast search **RID** = UDUFVWUN015
User Options:	**Database:** DELTA_BLAST/cdd_delta **Low complexity filter:** no **Composition Based Adjustment:** no **E-value threshold:** 0.05 **Maximum number of hits:** 500

Final conclusions:

1. The data are very fresh so the final conclusions are during the process of formulation…, but:

- Among the tested proteins (about 150 spots) more than 40% of them were identified or functionally assigned (although the tested strain is not sequenced)
- Collected data will allow to formulate the explanation of microorganism strategy towards alachlor – strong upregulation of energy-related and ROS enzymes
- unique overexpression of cyanide hydratase in alachlor containing cultures which hydrolyze nitriles (RCN) to ammonia and the corresponding carboxylic acid may point this enzyme as most important in the examined process.



Acknowledgments

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감사

Благодаря!

Tesekkurler

Kiitos

ขอบคุณ

متشكرم

Tak

Danke

Dziękuję

Thanks

Děkuji

Niżżik ħajr

Salamat po

Gracias

ありがとう

Спасибо

cảm ơn

Paldies

धन्यवाद

дєкую

Thank you for your attention

ধন্যবাদ

谢谢

Гялайлаа

Tapadh leibh

Hvala

Obrigado

Ačiu

תודה רבה

Köszönöm

Merci

(بهت) شکریه

Mulţumesc

감사

Ευχαριστω

Dank

Tack

Děkuij

Grazie

Þakka þér