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Polyphasic approach for differentiating *Penicillium nordicum* from *Penicillium verrucosum*

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Abstract

The aim of our research was to use a polyphasic approach to differentiate *Penicillium verrucosum* from *Penicillium nordicum*, to compare different techniques, and select the most suitable for industrial use. In particular, (i) a cultural technique with two substrates selective for these species; (ii) a molecular diagnostic test recently set up and a RAPD procedure derived from this assay; (iii) an RP-HPLC analysis to quantify ochratoxin A (OTA) production and (iv) an automated system based on fungal carbon source utilisation (Biolog Microstation[TM]) were used. Thirty strains isolated from meat products and originally identified as *Penicillium verrucosum* by morphological methods, were re-examined by newer cultural tests and by PCR methods. All were found to belong to *Penicillium nordicum.* Their biochemical and chemical characterization supported the results obtained by cultural and molecular techniques and showed the varied ability in *P. verrucosum* and *P. nordicum* to metabolize carbon-based sources and to produce OTA at different concentrations, respectively.

Keywords: Polyphasic approach; *Penicillium nordicum; Penicillium verrucosum*; PCR; HPLC; Biolog

Introduction

The curing and ripening techniques applied to most aged European meat products quickly leads to the development of a specific mycoflora. In dry-cured hams, yeasts tend to form a film on the muscle portion and their enzymatic activity induces the formation of characteristic volatile compounds; after yeasts have grown, moulds can develop on the product (Simoncini *et al*. 2007). In cased meats moulds tend to prevail over yeasts because of the reduction in surface water activity and the invasive way they grow (Spotti and Berni 2007).

Development of fungal mycelium in meat derivatives is tolerated (i.e. *Eurotium* spp. in dry-cured hams) and sometimes even desirable (i.e. starter cultures in sausages and cased meats), as it can exert a protective action against an excess drying and lipid oxidation. Despite that, surface moulding of meat products by environmental contaminating species (mainly belonging to the genera *Penicillium* and *Aspergillus)* should be always avoided, as some of them are toxigenic (Spotti *et al*. 2008; Spotti and Berni 2007). More specifically, in matured and dry-cured meat products moulds such as *Penicillium verrucosum, Penicillium nordicum* and *Aspergillus ochraceus,* that can produce ochratoxin A (OTA) must be controlled, as OTA is a nephrotoxin in animals, and has been classified by the International Agency for Research on Cancer (IARC 1993) as possibly carcinogenic to humans (Group 2B).

Although neither the US Food and Drug Administration (FDA) nor the European Union have set guideline threshold levels for OTA in meat products (FDA 2009; Commission Regulation (EC) 1881/2006), we believe accurate identification techniques would be highly beneficial. In fact, the possibility to recognize correctly toxigenic species would improve our capability to identify the source of contamination and to assess the

parameters affecting mould growth. In particular, differentiation between *P. verrucosum* and *P. nordicum* could be of great interest. Before all, these two species resulted to have a different ecology, so their presence in seasoning environments can be connected to a specific source of contamination.

Differentiating *P. verrucosum* from *P. nordicum* is difficult due to the fact that classic morphological techniques allow to differentiate between *Penicillium* and *Aspergillus* (Pitt and Hocking 2009), but they do not permit reliable differentiation between the two above-mentioned Penicillium species, as they are undistinguishable on standard media (Samson *et al*. 2004). Only additional cultural tests on selective media (Larsen *et al.* 2001; Lund and Frisvad 2003) and recently reported techniques of molecular identification by PCR (Castella *et al*. 2002; Geisen *et al.* 2004; Niessen *et al*. 2005; Bogs *et al.* 2006) are reported to allow their differentiation and the reliable attribution of each of them to specific habitats: cereals for *P. verrucosum* and meat products for *P. nordicum* (Olsen *et al*. 2006; Frisvad and Samson 2004).

At the same time, little experimentation has been carried out on *P. verrucosum* and *P. nordicum* by using alternative techniques such as the measurement of OTA produced in suitable media by RP-HPLC (Larsen *et al.* 2001; Kokkonen *et al.* 2005; Cabañas *et al*. 2008) and no data are available on the measurement of consumption of carbon-based sources by moulds in developed automated systems (i.e. Biolog Microstation™) (Buyer *et al*. 2001; Cantrell *et al.*, 2006; Singh 2009; Atanasova and Druzhinina. 2010).

Thus, using the above-mentioned techniques, we analysed with different approaches some of the fungal strains isolated during the last 20 years at the SSICA Laboratory of Mycology from matured and seasoned meat products, identified as *P. verrucosum* by classic morphological method (Pitt and Hocking 1997; Samson *et al.* 2004).

The aim of this work was to define a polyphasic approach based both on phenotypic (cultural and morphological characterization) and non-phenotypic (molecular, chemical and metabolic characterization) techniques, in order to compare them and to develop a fast and reliable technique to identify *P. nordicum*.

Materials and methods

Fungal strains and cultural conditions

The tests were carried out on 30 of the fungal strains isolated from 1989 to 2009 at the SSICA Laboratory of Mycology and Mycotoxins and originally identified as *P. verrucosum* by means of the classic morphological methods (table 1). Four known *Penicillium verrucosum* strains and one *P. nordicum* strain (table 1) as well as the reference strains *P.roqueforti* 24310 and *P.solitum* 27810 were used as controls.. Each strain was placed on Malt Extract Agar (Oxoid, Cambridge, UK) and incubated at 25°C for seven days; at the end of this period, for each strain a spore suspension was prepared with water and 0.1% Tween 80 v/v; the suspension was filtered on sterile glass wool to remove traces of solid medium and mycelium. Each suspension contained approximately 10^7 cfu/ml.

Cultural tests

YES (Yeast Extract Sucrose) agar and DYSG (Dichloran Yeast Extract Sucrose Glycerol) agar were prepared according to Samson *et al.* (2004). Each of the conidial suspensions (10^7 cfu/ml) was point-inoculated on these media and incubated at 25°C for seven days.

Molecular tests

Isolation of fungal DNA

Total DNA was extracted according to the method described by Yelton *et al.* (1984) with modifications: 50-100 mg of three-day-old mycelia, grown on YES medium, were frozen in liquid nitrogen with 200 μl of glass microbeads (diameter 200 μm), ground to a powder with a dental amalgamator (TAC 200/S Amalgamator, LineaTAC, Italy), re-suspended in 800 μl of lysis-buffer (50 mM EDTA; 0.2% SDS; pH 8.5), heated to 68°C for 15 min and centrifuged for 15 min at 15,000 x g. The recovered supernatant was added with 125 μl of 3 M sodium acetate; after one hour incubation in ice, the solution was centrifuged for 15 min at 15,000 x g and the supernatant was phenol-extracted twice. The isolated DNA was precipitated with one volume of isopropylic alcohol, evaporated to dryness and re suspended in 100 μl of TE + RNase (10 mM Tris-HCl pH 8.0; 1 mM EDTA; RNase 20 μg/ml). DNA was quantified and checked for integrity by 0.8% agarose gel electrophoresis.

Diagnostic PCR for identification and differentiation of P. verrucosum and P. nordicum

Differentiation of *P. nordicum* from *P. verrucosum* was initially carried out according to Bogs *et al.* (2006): amplification of the *otapks*PN gene was obtained using the primer pair *otapks_for* 5'-ATGCCTTTCTGGGTCCGATA-3' and *otapks_rev* 5'-TACGGCCATCTTGAGCAACGGCACTGCC-3'; amplification of *otanps*PN gene was obtained using the primer pair *otanps_for* 5'-CAGCACTTTTCCTCCATCTATCC-3' and *otanps_rev* 5'-AGTCTTCGCTGGGTGCTTCC-3'. The amplification was conducted in a Personal Cycler™ (Biometra®, Gottingen, Germany) in 20 μl, according to the following conditions: 5.0 μl DNA template (0.1 μg ml^{-1}); 5X GoTaq® Flexi Buffer (Promega, Madison, USA) 4 μl; 25 mM MgCl$_2$ 2 μl; dNTP mix 0.2 μl (25 mM each nucleotide); each primer 1 μl (12.5 pM); GoTaq® Flexi DNA Polymerase 0.6 U (Promega, Madison, USA). The cycling parameters were: 4 min at 94°C, 33 x (94°C, 1 min; 60°C, 1 min; 72°C, 1 min), final extension 6 min at 72°C. Each reaction was performed at least three times.

RAPD analysis for differentiation of P. verrucosum and P. nordicum

Genomic DNA was used as template for RAPD-PCR reactions: 5.0 μl DNA template (0.1 μg ml^{-1}); 5X GoTaq® Flexi Buffer (Promega, Madison, USA) 4 μl; 25 mM MgCl$_2$ 2 μl; dNTP mix (25 mM each nucleotide) 0.2 μl; GoTaq® Flexi DNA Polymerase 0.6 U (Promega, Madison, USA) in 20 μl final volume. Primers *otapks_for* and

otapks_rev (12.5 pM, 1 μl) were used as random primers. The cycling parameters were: 4 min at 94°C, 33 x (94°C, 1 min; 60°C, 1 min; 72°C, 1 min), final extension 6 min at 72°C. A similar reaction was performed but using a different brand of Taq polymerase (Taq Platinum® (Invitrogen, Carlsbad, USA). The amplification products were separated on a 2% agarose gel. Each reaction was performed at least three times.

Chemical characterization

Chemicals and standards

Microfibre filters were obtained from VICAM (Watertown, MA, USA). Acetonitrile and methanol (both HPLC grade) were obtained from Carlo Erba (Milan, Italy); bidistilled water was daily produced in our laboratory by a Millipore water purification device (Billerica, MA, USA).

OTA stock solution was prepared by dissolving in benzene–acetonitrile (99:1) the solid standard obtained by Sigma-Aldrich (St Louis, MO, USA) and was stored at -20°C. Working OTA solutions were prepared by properly evaporating the solvent mixture of the stock solutions and redissolving the residue in the RP-HPLC mobile phase to give the final desired concentration. The concentration of the standard solution was checked with a Beckmann (Fullerton, CA, USA) DU-50 spectrophotometer calibrated according to AOAC methods (1995).

Sample preparation

Semisolid YES containing 0.3% agar was prepared according to Samson *et al.* (2004). 0.1 ml of each conidial suspension (10^7 cfu/ml) was individually inoculated into 50 g of medium and incubated at 25°C for 11 days. After that time, OTA was detected by RP-HPLC both in the YES medium and in the mycelium developed.

For OTA determination in inoculated YES broth, samples were analysed according to the method proposed by Bragulat *et al.* (2001), modified as follows. 35 g of semisolid medium were diluted with 70 ml of metanhol-1% $NaHCO_3$ water (70:30), shaken for 1 min and filtered on glass wool filter (1.5 μm, VICAM). 1 ml of the filtrate obtained was then diluted with the mobile phase acetonitrile-water-acetic acid (99:99:2) and directly injected in RP-HPLC.

For OTA determination on mycelium, samples were analysed according to the method proposed by Gallo *et al.* (2009), modified as follows. The mycelium was weighed and diluted with 40 ml of metanhol-1% $NaHCO_3$ water (70:30), shaken for 3 min and then filtered on glass wool filter (1.5 μm, VICAM). The filtrate obtained was then diluted with the mobile phase acetonitrile-water-acetic acid (99:99:2) and directly injected in RP-HPLC.

For each fungal strain, analysis was performed in triplicate; for each extract obtained, RP-HPLC injection was repeated twice.

HPLC analysis

Chromatographic analyses were performed with a Jasco Model PU-1580 pump equipped with a Tracer Extrasil ODS-2 standardbore column (150 × 4.6 mm, 5 μm particle size, Teknokroma, Barcellona, Spain), a Jasco Model AS-1555 autosampler (100 μl loop) and a Jasco Model FP-1520 fluorescence detector (excitation wavelength: 330 nm; emission wavelength: 460 nm). The system was controlled by a Borwin P/N BRW-1 for data handling.

A mixture of acetonitrile-water-acetic acid (99:99:2) was used as mobile phase for OTA determination, at a flow rate of 1.0 ml min^{-1}.

Metabolic analysis and characterization

To differentiate the isolates by means of their metabolic pattern, MicroStation^{TM} Identification System (Biolog, Hayward, CA, USA) was used. Samples were prepared according to the manufacturer's protocol (FF Microplate^{TM} Instruction for Use. Part 00P 015, Rev B. October 2004. Biolog Inc., Hayward, USA). Plates were incubated at 25°C in the dark and read by using the MicroStation^{TM} Reader at 24, 48, 72, 96 and 168 hours as suggested in the Biolog booklet.

The metabolic profiles of the isolates tested were then elaborated and compared by RetroSpect^{TM} Trending & Tracking Software (Biolog, Hayward, CA, USA), to find, if any, differences in carbon-based sources utilization by the two species assayed.

Results and discussion

Cultural test

Figures 1(a) and figure 1(b) show some of the results of the cultural test conducted on YES and DYSG culture media, which were considered suitable to differentiate *P. verrucosum* from *P. nordicum* because of the different reverse-colour of the colonies. As it is clearly shown in figure 1(a), the *P. verrucosum* strain A used as a control produced the typical terracotta-coloured reverse on DYSG and an orange reverse on YES; on the contrary, in figure 1(b) the *P. nordicum* strain E, also used as a control, produced a pale reverse on both media.

All the SSICA strains tested produced a pale reverse. Accordingly, all the strains from SSICA should be classified as belonging to *P. nordicum* species.

Molecular analysis

Analysis of DNA polymorphism may profitably be used to distinguish species morphologically closely related (see Castella *et al.*2002 and references therein). The primers pair *otapks*PN and *otanps*PN, that has been previously shown to be able to discriminate *P. verrucosum* from *P. nordicum* (Bogs *et al.* 2006), were here used to perform a diagnostic PCR on the 30 *Penicillium* isolates and the five control strains described above. Figure 2(a) shows the efficacy of the procedure: the expected 0.5 kb fragment was present in all the samples identified

as *P. nordicum* by the cultural test and absent in those identified as *P. verrucosum*, whereas the 0.75kb amplification control product was observed in all the samples.

In contrast two more amplicons, sized about 1.1 kb and 1.45 kb respectively reported by Bogs *et al.* (2006), were detected in *P. nordicum* samples amplified with the *otapks*PN primer pair. We tested several amplification protocols to remove these additional amplicons without success. Since the unexpected profile could be the result of aspecific amplifications directed by one or both the oligonucleotides acting singularly as forward and reverse primer in the PCR reaction, we tested this hypothesis by performing single primer amplifications. This is equivalent to a random amplified polymorphic DNA analysis (RAPD-PCR). The results of this analysis are reported in figure 2(b). No amplification product was primed by *otapks_for* either when using *P. nordicum* or *P. verrucosum* DNA as template. Primer *otapks_rev* resulted in a polymorphic amplification pattern that differentiate *P. nordicum* from *P. verrucosum*.

Primer *otapks_rev* was then used to amplify the genomic DNA of all the 30 strains here discussed. In figure 2(c) an example is reported. All the *P. nordicum* isolates resulted in three multiple-bands profiles. The three patterns were characterised by 1.7 kb, 1.45 kb and 1.1 kb bands, 1.7 kb and 1.1 kb bands, and 1.7 kb, 1.25 kb and 1.1 kb bands respect tively. At difference with *P. nordicum*, *P. verrucosum* yielded one single amplification product (1.7 kb). These results highlight the potential of *otapks_rev* primer as a molecular marker to differentiate *P. verrucosum* from *P. nordicum*. In addition, primer *otapks_rev,* was tested on *P. roqueforti* and *P. solitum* DNA, using two different brands of Taq polymerase. As shown in figure 2(d) the amplification patterns of the latter *Penicillium* species were reproducibly different from those of *P. nordicum* and *P. verrucosum*.

Chemical characterization

The detection of OTA by RP-HPLC was performed both on the developed mycelium and in the YES medium. As table 2 shows, isolates could be arranged into five groups, according to their ability of producing Ochratoxin A in the mycelium:

-Group I (< 0.004 mg kg^{-1}): strains n. A, B, D, 4, 5, 20, 21, 27, 28;

-Group II (0.004-9.99 mg kg^{-1}): strains n. C, 2, 32, 9, 16, 18, 23, 25, 34

-Group III (10.00-99.99 mg kg^{-1}): strains n. 3, 7, 33, 11, 13, 14, 19, 22

-Group IV (100.00-199.99 mg kg^{-1}): strains n. E, 12, 15, 24, 26, 35

-Group V (> 200.00 mg kg^{-1}): strains n. 1, 6, 17

Of the 35 isolates that were tested for OTA production, 26 (74,3%) proved to produce this toxin. The toxin levels ranged from 0.23 to 550 mg kg^{-1} in mycelium and from 0.01 to 21 mg kg^{-1} in culture medium.

In general, *P. nordicum* isolates produce larger amounts of ochratoxin A than *P. verrucosum* isolates. These results are in agreement with those obtained by Larsen *et al.* (2001). In fact, three out of four isolates of *P. verrucosum* did not produce OTA in both the mycelium and the medium. Only *P. verrucosum* C strain produced

some toxin: its OTA production levels amounts to 4.9 mg kg^{-1} in mycelium and to 0.12 mg kg^{-1} in culture medium. On the contrary, 25 out of 31 strains of *P. nordicum* produced variable amounts of toxin: their OTA production levels varied from 0.23 to 550 mg kg^{-1} in mycelium and from 0.01 to 21 mg kg^{-1} in culture medium.

Metabolic analysis and characterization

The Biolog Microstation^{TM} Identification System was recently introduced for a rapid identification of common microorganisms based on their capability to utilize 95 discrete substrates. It was found to be suitable for substrate utilization studies of closely related fungi.

   The *P. verrucosum* control strains (A-D; see table 1) when analysed were correctly identified by the Biolog MicroStation^{TM} Database. On the contrary, the 30 strains resulted as being *P. nordicum* by cultural and molecular analyses (table 1) were identified as belonging to different species of the *Penicillium* subgenus *Penicillium*, as *P. nordicum* specific metabolic profile was not available in the Biolog MicroStation^{TM} Database. This result prompted us to carry out a metabolite profiling of *P. nordicum,* in order to create a reliable "*P. nordicum* User Database" suitable for future identifications of isolates belonging to this species. All the metabolic profiles of *P. nordicum* strains were then grouped by Biolog MicroStation^{TM} Software. To verify the reliability of "*P. nordicum* User Database" the *P. nordicum* strain (E) and the *P. verrucosum* strains (A,B,C and D) were used as control: strain E only was correctly identified by means of the "*P. nordicum* User Database" created. To reinforce the diagnostic efficacy of the reported database , we also tested two related species of the genus *Penicillium*, *P. roqueforti* and *P. solitum*. The latter two species were correctly recognised indicating that there is no overlap between the *P. nordicum* database we have created and the *Penicillium* database already present in the MicroStation^{TM} Database .

   With regard to data elaboration by RetroSpect^{TM} Trending & Tracking Software, the most consistent results were obtained at 72 and 96 hours. As table 3 shows, we found out that some substrates were differently assimilated by *P. verrucosum* and *P. nordicum*. In particular, at the times considered:

   -ß-Hydroxy-butyric acid was completely metabolized by *P. verrucosum,* not by *P. nordicum;*

   -D-Cellobiose was completely metabolized by *P. nordicum,* not by *P. verrucosum;*

   -Adonitol was partially metabolized by *P. verrucosum,* not by *P. nordicum;*

   -Lactulose was partially metabolized by *P. nordicum,* not by *P. verrucosum.*

   In our opinion, only ß-Hydroxy-butyric acid and D-Cellobiose could be considered good candidates, among the carbon-based substrates tested in the present survey, to differentiate *P. verrucosum* and *P. nordicum*, respectively. Further studies are however requested in order to find, if any, more specific applications of the latter substrates for a diagnostic procedure.

Conclusions

In this paper we have described a polyphasic approach , i.e the use of an integrated set of procedures (some "new" and some "old") to differentiate the relevant species and we show that the reliable identification *of P. nordicum*c strains may be obtained. In particular the "*P. nordicum* User Database" we have created has proved to be robust and has the potential for being used in routine assay. In our opinion, however, none of the described methods can "*per se*" provide a conclusive diagnosis and each of them has its drawbacks. The use of a combination of techniques may narrow the probability to misclassify a sample. Moreover, several features of our protocol deviate slightly from other reported methods and/or could be altered to accommodate the request of different laboratories. In addition we have shown that RAPD analysis may uncover variability inside the population of *P. nordicum* strains colonising a peculiar environment.

Acknowledgements

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Tables

Table 1. Fungal strains and correspondent results of both cultural and molecular analysis.

Strain	Tracking number of the strain tested	Cultural diagnosis	Molecular diagnosis
A	*P. verrucosum* CBS 603.74 from cereals (Belgium)	*P. verrucosum*	*P. verrucosum*
B[a]	*P. verrucosum* BFE 500 from cereals (Sweden)	*P. verrucosum*	*P. verrucosum*
C[b]	*P. verrucosum* ex-MUT 3897/1	*P. verrucosum*	*P. verrucosum*
D[b]	*P. verrucosum* ex-MUT 3897/2	*P. verrucosum*	*P. verrucosum*
E[c]	*P. nordicum* CBS 113876 (SSICA C1-241297)	*P. nordicum*	*P. nordicum*
1	*P. verrucosum* 28207	*P. nordicum*	*P. nordicum*
2	*P. verrucosum* P1-22801	*P. nordicum*	*P. nordicum*
3	*P. verrucosum* 19399	*P. nordicum*	*P. nordicum*
4	*P. verrucosum* 20999	*P. nordicum*	*P. nordicum*
5	*P. verrucosum* 5795	*P. nordicum*	*P. nordicum*
6	*P. verrucosum* V1-22801	*P. nordicum*	*P. nordicum*
7	*P. verrucosum* N1-22801	*P. nordicum*	*P. nordicum*
32	*P. verrucosum* 23108	*P. nordicum*	*P. nordicum*
9	*P. verrucosum* 23801	*P. nordicum*	*P. nordicum*
33	*P. verrucosum* 10308	*P. nordicum*	*P. nordicum*
11	*P. verrucosum* 3100	*P. nordicum*	*P. nordicum*
12	*P. verrucosum* 22806	*P. nordicum*	*P. nordicum*
13	*P. verrucosum* 23806	*P. nordicum*	*P. nordicum*
14	*P. verrucosum* 1506	*P. nordicum*	*P. nordicum*
15	*P. verrucosum* 2506	*P. nordicum*	*P. nordicum*
16	*P. verrucosum* 1-0407	*P. nordicum*	*P. nordicum*
17	*P. verrucosum* 2-0407	*P. nordicum*	*P. nordicum*
18	*P. verrucosum* 1-0305	*P. nordicum*	*P. nordicum*
19	*P. verrucosum* 19100	*P. nordicum*	*P. nordicum*
20	*P. verrucosum* 15304	*P. nordicum*	*P. nordicum*
21	*P. verrucosum* 2-0305	*P. nordicum*	*P. nordicum*
22	*P. verrucosum* 6795	*P. nordicum*	*P. nordicum*
23	*P. verrucosum* 4795	*P. nordicum*	*P. nordicum*
24	*P. verrucosum* 231107	*P. nordicum*	*P. nordicum*
25	*P. verrucosum* 241107	*P. nordicum*	*P. nordicum*
26	*P. verrucosum* 17308	*P. nordicum*	*P. nordicum*
27	*P. verrucosum* 22108	*P. nordicum*	*P. nordicum*
28	*P. verrucosum* 14208	*P. nordicum*	*P. nordicum*
34	*P. verrucosum* 11308	*P. nordicum*	*P. nordicum*
35	*P. verrucosum* 17308	*P. nordicum*	*P. nordicum*

Notes: [a] Strain B was kindly provided by R. Geisen from MRI (Max Rubner Institut), Germany.
[b] Strains C and D were kindly provided by C. Varese from Mycotheca Universitatis Taurinensis (MUT), Italy.
[c] Strain E was isolated at the SSICA Laboratory of Mycology from Italian ripened salamis and identified in 2003 as *P. nordicum* by R.A. Samson from Centraal Bureau voor Schimmelcultures (CBS), Utrecht.

Table 2. Range of OTA contamination in mycelium of *P. nordicum* and *P. verrucosum* strains grown on YES medium at 25°C for 11 days.

Range of contamination (mg kg^{-1})	No. of samples in the range	% Positive isolates	OTA mycelium (mg kg^{-1})	
			Mean*	Min-Max**
< 0.004	9	25.7	-	-
0.004-9.99	9	25.7	1.8	0.08-4.9
10.00-99.99	8	22.9	56	15-96
100.00-199.99	6	17.1	130	100-160
> 200.00	3	8.6	410	200-550

Notes: *Average OTA level in each contamination range.
** minimum and maximum OTA levels detected in each contamination range.

Table 3. Differences in carbon-based sources consumption by *P. verrucosum* and *P. nordicum* in phenotype microarray FF Microplates after 72 and 96 hours.

	Carbon-based sources	P. nordicum	P. verrucosum
72 hours	ß-Hydroxy-butyric Acid D-Lactic Acid Methil Ester L-Lactic Acid L-Malic Acid	-	++
	ß-Cyclodextrin L-Pyroglutamic Acid Adonitol	-	+
	Lactulose	+	-
	D-Cellobiose L-Phenylalanine	++	-
96 hours	ß-Hydroxy-butyric Acid	-	++
	Maltitol L-Rhamnose Adonitol	-	+
	Lactulose	+	-
	D-Cellobiose Amygdalin	++	-

Note: "++" and "+" mean that the examined species metabolised respectively large or small quantities of the substance listed. "-" means that the examined species did not assimilate the carbon-based source listed.
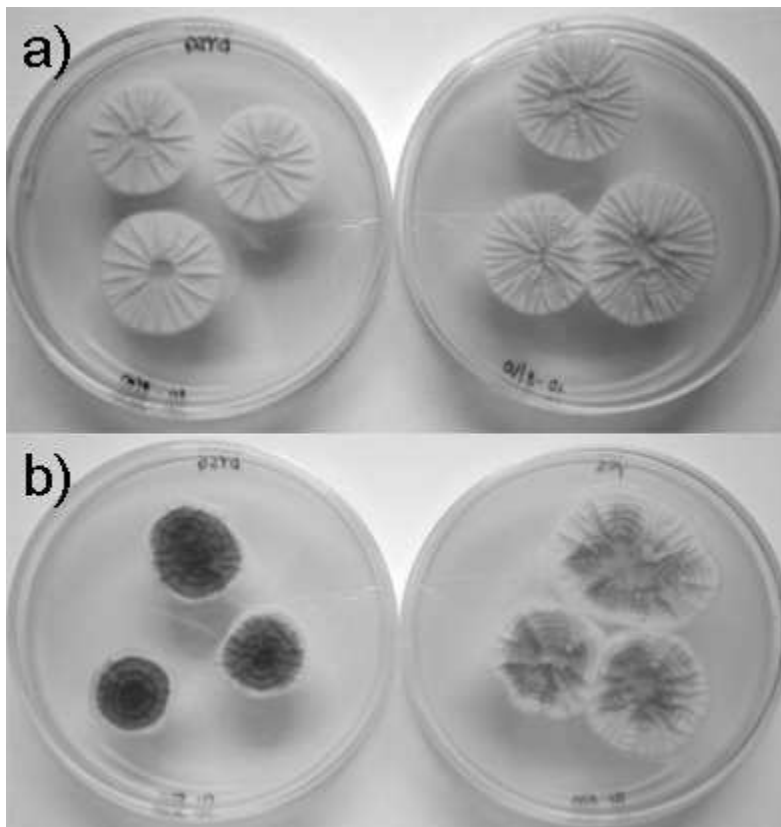
Figure captions

Figure 1. **(a)** *P. verrucosum* BFE500 and **(b)** *P. nordicum* CBS 113876. Seven-day old colony on DYSG (left Petri dish) and YES (right Petri dish) at 25°C.

Figure 2. (a) Agarose gel with the PCR results of *P. nordicum* E (lanes 1 and 2), *P. verrucosum* A (lanes 3 and 4), C (lanes 5 and 6) and D (lanes 11 and 12), and of isolates 33 and 34 (lanes 7-8 and 9-10 respectively). Odd lanes: amplification conducted with primer pair *otanps*PN; pair lanes: amplification conducted with primer pair *otapks*PN. L: 100bp ladder. **(b)** Agarose gel with the PCR reactions of *P. nordicum* E (lanes 1 and 5), *P. verrucosum* A (lanes 2 and 6) and D (lanes 4 and 8) and isolate 3 with primer *otapks_rev* (lanes 1 to 4) and primer *otapks_for* (lanes 5 to 8). L: 100bp ladder. **(c)** Agarose gel with the RAPD-PCR results of *P. verrucosum* A (lane 1), *P. nordicum* E (lane 2) and isolates 1, 2, 3, 4, 5 and 6 (lanes 3 to 8) amplified with primer *otapks_rev*. L: 100bp ladder. **(d)** Agarose gel with the RAPD-PCR results of *P. verrucosum* A (lanes 1 and 5), *P. nordicum* (lanes 2 and 6), *P. roqueforti* (lanes 3 and 7) and *P. solitum* (lanes 4 and 8) amplified with primer *otapks_rev*. Lanes 1 to 4: amplification reactions conducted with the Platinum® Taq DNA Polymerase (Invitrogen, Carlsbad, USA); lanes 5 to 8: amplification reactions conducted with the GoTaq® Flexi DNA Polymerase (Promega, Madison , USA).

142x149mm (70 x 70 DPI)