NEW SCHEME FOR SELECTION OF NON-TOXIGENIC *PENICILLIUM SPP.* USED FOR MOULD-RIPENED MEAT PRODUCTS

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Introduction

In Europe moulds play a significant role in the processing of foods such as white and blue cheeses as well as mould-fermented sausages (e.g. Italian salami) and hams. For these foods mainly species of the fungal genus *Penicil/ium* are employed and used because its appearance, aroma, taste and preservation (Leistner, 1997). However, *Penicillium* species are known to be potent producers of antibiotics and mycotoxins (Betina 1989, Frisvad 1989). Therefore fungal isolates to be considered as starter cultures for food have to be thoroughly investigated for their toxicological potential. Since mycotoxin formation is mainly influenced by the substrate, composition and external factors such as temperature, aw, pH etc., the toxicological testing of strains grown on laboratory culture media is not sufficient. In order to meet the demands on toxicological safety, the testing therefore has to include the mould-ripened products itself.

Objectives

The aim of this study was to establish and prove a new selection scheme for *Penicillium* species used as starter cultures. In contrast to previous methods the following steps were performed to screen out toxigenic species:

1. Initial selection is based on the principal technological suitability for the product (i.e. mould-fermented sausages) and the expectations of the consumers (i.e. colour, appearance, sensory).
2. Strains which fulfill these claims are then grouped by physico-chemical methods and mould-ripened foods produced to confirm the above mentioned demands.
3. The potency of these strains to produce antibiotic, cytotoxic and mutagenic metabolises as well as known mycotoxins was then checked by analyzing extracts of the products by different bioassays and mycotoxin detection methods.

Materials and methods

For initial selection a total of 249 different strains of P. *nalgiovense* (n=113), P. *camembert)* (69), P. *chrysogenum (18), P. candidum (8), P. verrucosum (5), P. expansum (4), P. cased* (3), P. *glabrum* (3), P. *puberulum* (3), P. *waksmanii* (3) and P. *spp.* (20) originating mainly from our culture collection have been investigated. The fungal isolates were grown on malt extract agar (MEA) plates under varying conditions followed by the assessment of the colony morphology and phenotype of the particular strains. Secondary metabolise pattems of the strains were obtained by thin-layer chromatography (TLC) and used for grouping the isolates chemotaxonomically. For that purpose CHCI3 extracts from 21 day old cultures grown on MEA were spotted on silica gel TLC plates. The plates were then developed one dimensionally with different mobile phases and the major and minor spots recorded.

The production of mould-ripened salamis was carried out by using a standard recipe and technology (Rodel, 1985). Sausages were dipped in spore solutions (105 to 106 CFU/ml), then ripened in climatic cambers for up to 25 days and evaluated for overall appearance, colour, density, abrasion resistance, uniformity of the strains as well for sensory properties.

For toxicological examination, CHCI3 extracts of the sausages and MEA-cultures were used (Rotheneder,1996). Cytotoxicity was proven by means of the MTT-cell culture test as described previously (Hanelt et al., 1994). The same extracts were also tested on antibiotic activities by using the agar diffusion method and 13 different target strains of gram positive bacteria. Mutagenicity of selected strains was tested by use of the AMES-test (Wild, 1992).

Detection of the mycotoxins ochratoxin A and citrinin was carried out with an enzyme immune assay (Martibauer and Dietrich, 1995).

Results and discussion

From the 249 strains investigated, a total of 102 strains were initially screened out because culture morphology and phenotypes observed on MEA clearly failed to satisfy consumers expectation criteria, particular the white colour (table 2). The remaining 147 isolates could assigned to P. *nalgiovense* (n=87), P. *camembert)* (52), P. *candidum* (5), P. *chrysogenum* (2) and P. 5p.(1).

Altogether 9 groups belonging to P. *nalgiovense* (6 groups, 16 subgroups) and P. *camembert)* (3 groups, 12 subgroups) were chemotaxonomically defined by the TLC pattern of secondary metabolises. Twenty-one isolates not belonging to these species were put into 5 separate groups.

Examination of the mould-ripened salamis indicate a correlation between the technological suitability of the particular fungal strain and the chemotaxonomic group. The TLC-pattern of secondary metabolises of strains to be tested is therefore considered to be a simple to perform assay for screening purposes. Based on missing technological properties, further 62 strains had to be screened out (table 2).

Table 1: Comparison of the Cytotoxic activities of ***Penicillium spp.*** following growth on malt extract agar (MEA) and salamis (S). Crude extracts were tested in the MTT-cell culture bioassay using swine-kidney target cells.

		No. of Cytotoxic and non-cytotoxic strains			
Species	No. of isolates	S+ MEA +	S+ MEA -	S- MEA ~	S- MEA -
P. nalgiovense	48	0	1	7	12
P. camembert	20	7	2	28	11
P. spp.	6	0	2	3	1
Total	74	7	5	38	24

S +/MEA +: Cytotoxic after growth on salami (S) as well on malt extract agar (MEA)
S +/MEA -: Cytotoxic after growth on S but not on MEA
S -/MEA +: Cytotoxic after growth on MEA but not on S
S -/MEA +: No cytotoxicity after growth on S or MEA

The influence of the substrate on the production of cytotoxic fungal compounds was demonstrated by testing extracts in the MTT-cell culture bioassay, which is known to be a sensitive assay for the detection of cytotoxic mycotoxins (Hanelt et al., 1994).
As can be seen from table 1, only 24 strains showed no cytotoxicity either after growth on salamis or MEA. Most of the strains formed cytotoxic compounds after growth on nutrient agar but not after growth on the product. Unlike this, 5 strains reacted positive after growing on the product but were found to be negative after cultivation on malt extract agar.
The MTT-assay was found to be a helpful tool in differentiating cytotoxic and noncytotoxic isolates of *Penicillium* strains, which otherwise could not be differentiated (Figure 1).
Trace amounts of known mycotoxins (ochratoxin A and citrinin) were detected only in culture extracts from 7 strains but in no case in the ripened products. No mutagenicity by use of the AMES-test was observed in culture extracts of selected strains. Antibacterial activity in varying degrees was found for the isolates tested and 48 strains showing the highest activities were screened out.
Totally 72 *Penicillium spp.* were screened out on the basis of the results of the toxicological evaluation.
Finally, from 249 strains tested, only 13 strains (5%) were found to satisfy the requirements with respect to the criteria defined to the greatest extent and could thus be recommended for use in food products (Table 2).

Table 2: Results of the selection of *Penicillium spp.* following the demands on consumers expectation, technological suitability and toxicological safety

selection step	purpose	no. of remaining isolates	no. of isolates screened out
I	general appearance (consumers expectation)	249 (100%)	102 (41%)
II	technological suitability	147 (59%)	62 (25%)
III	toxicological safety	85 (34%)	72 (29%)
IV	**remaining isolates**	**13 (5%)**	

Conclusion
Toxigenic strains of *Penicillia,* even those used routinely in the food industry, are frequent. To ensure consumer safety, fungal starter cultures have to be investigated for their ability to produce mycotoxins and other compounds with possible adverse effects on health. The production of mycotoxins is mainly dependent on the substrate and external factors. From cultures on nutrient agar no conclusions to the biological activity of the starter culture on the product can be drawn.

For that reason it is essential to test the product for the toxic potential of fungal starter cultures and selection of safe strains. Toxicology testing should include bioassays, because only these allow to detect unknown mycotoxins.

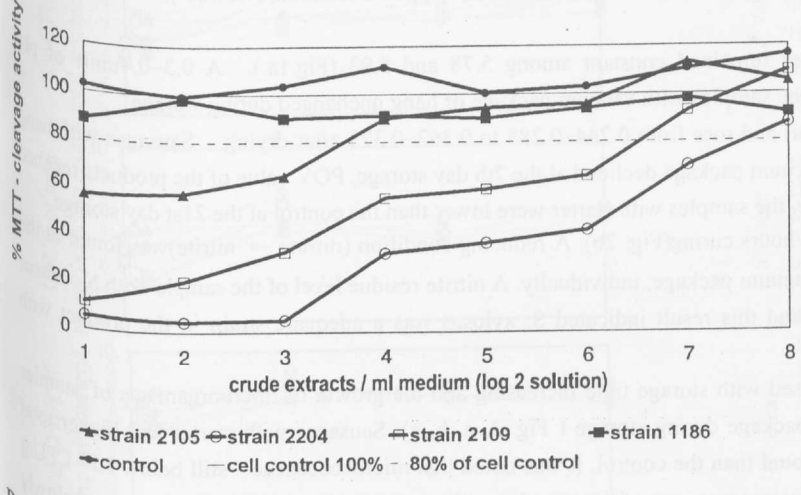

Figure 1: Use of the MTT-cell culture bioassay for screening the cytotoxic activities of crude extracts of salami, ripened with different isolates of *Penicil/ium nalgiovense* (MTT-cleavage activities < 80% indicate cytotoxicity compared to 100% of cell control)

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