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# Taxonomy of *Penicillium nalgiovense* isolates from mould-fermented sausages

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## Abstract

A large number of *Penicillium nalgiovense* isolates from mould fermented sausages and the ex type culture were examined for characters of morphology, physiology and production of secondary metabolites. To separate biotypes within the *P. nalgiovense* species, the data obtained were evaluated using multivariate statistical methods. The macromorphological characters of the ex type culture and isolates from meat products appeared to be distinctive. The ex type culture is characterized by a brown reverse on both Czapek yeast extract and malt extract agar while the isolates from meat products have a yellow to orange reverse. Proteolytic and/or lipolytic activity was demonstrated by 75% of the examined cultures and all of them demonstrated ability to utilize lactate as sole carbon source. Growth on creatine sucrose agar was very inhibited and acid production was absent or very weak. TLC analysis showed production of three unknown secondary metabolites that constituted the characteristic profile. HPLC analysis showed production of only three known secondary metabolites; chrysogine (96%), nalgiolaxin and nalgiovensin (9%). The ex type culture produced nalgiolaxin and nalgiovensin but not chrysogine. The chemometric evaluation showed that *P. nalgiovense* isolates from meat products from a homogenous species, which can not be divided into biotypes. The only indication of grouping, beside a separation of the ex type culture, was related to the conidium colour (white, turquoise or grey green). The examined *P. nalgiovense* isolates showed some resemblance (morphologically and chemically) to *P. chrysogenum*.

## Introduction

*Penicillium nalgiovense* Laxa (Laxa 1932) was isolated from Ellischauer cheese, a white moulded cheese from the Nalžhoyv region of southern Bohemia. Pitt (1979) placed *P. nalgiovense* in synonymy with *P. jensenii* Zaleski. According to Raper & Thom (1949) *P. nalgiovense* and *P. jensenii* are distinct species. *P. nalgiovense* has often been isolated from naturally mould-fermented sausages (Grazia et al. 1986; Hwang et al. 1993; Leistner & Eckardt 1979) and isolates of *P. nalgiovense* are often used as starter cultures for fermentation of sausages (Fink-Gremmels & Leistner 1990; Leistner 1990). According to Samson & van Reenen-Hoekstra (1988) *P. nalgiovense* isolates from meat products and isolates used as starter cultures show a morphological resemblance to *P. chrysogenum* Thom and might represent an albino mutant of this species. *P. chrysogenum* is also common on naturally-fermented sausages (Grazia et al. 1986; Hwang et al. 1993; Leistner & Eckardt 1979).

The conidiophores of *P. nalgiovense* are according to Samson & van Reenen-Hoekstra (1988) ter- or quaterverticillate. Frisvad (1993) placed *P. nalgiovense* in subgenus *Penicillium* and Pitt (1979) placed it in subgenus *Furcatum*.

Fink-Gremmels & Leistner (1990) divided *P. nalgiovense* into six biotypes based on colony diameters after 7 days growth on malt extract agar (10–20 mm or 20–26 mm), conidium colour (white, yellowish white, white to pale blue, turquoise green, blue or green), and

*Table 1.* Fungi examined (morphology, physiology and secondary metabolities).

| Fungi | |
|---|---|
| Bundesanstalt für Fleischforschung, Kulmbach, Germany | Sp I, Sp 285, Sp 290, Sp 291, Sp 655, Sp 678, Sp 750, Sp 1106, Sp 1110, Sp 1123, Sp 1149, Sp 1152, Sp 1279, Sp 1361, Sp 1785, Sp 1855, Sp 1871, Sp 1900, Sp 1941, Sp 1948, Sp 2023 |
| Department of Biotechnology, Technical University of Denmark, Lyngby, Denmark | IBT 3793, IBT 3794, IBT 3861 (ex type, IBT 3949, IBT 4090, IBT 4091, IBT 5746, IBT 6837, IBT 6838, IBT 6839, IBT 6917, IBT 10756, IBT 11797, IBT 11798, IBT 14392 |
| Isolated from Italian, Spanish and French sausages | IBT 912002, IBT 912003, IBT 912004, IBT 912005, IBT 912006, IBT 912039, IBT 912040, IBT 912041, IBT 912042, IBT 912043, IBT 912044, IBT 912045, IBT 912046, IBT 912050, IBT 912053, IBT 912056, IBT 912064, IBT 912066, IBT 912068, IBT 912073, IBT 912543, IBT 912641, IBT 913330, IBT 913331, IBT 913477, IBT 913478, IBT 913479, IBT 914301 |

degree of conidium formation when grown on Czapek yeast extract and malt extract agar.

The ex type culture of *P. nalgiovense* (NRRL 911) produces the secondary metabolites nalgiovensin and nalgiolaxin but no other known secondary metabolites (Raistrick & Ziffer 1951). This is in agreement with Fink-Gremmels & Leistner (1990) who described *P. nalgiovense* as a non-pathogenic species that does not produce any known mycotoxin.

The purpose of the present study was to examine the taxonomy of *P. nalgiovense* isolates from meat and also to look for characters of value in the development of new starter cultures for sausage fermentation, both with reference to a simple identification procedure and to indicate isolates that can be considered to be potential starter cultures. Morphological, physiological characters and production of secondary metabolites were examined. The data obtained were evaluated using multivariate statistical methods to separate possible biotypes.

## Materials and methods

*Fungi*

Sixtyfour *P. nalgiovense* isolates were examined. The isolates originated from culture collections or were isolated from Italian, Spanish, and French mould fermented sausages (Table 1).

*Media*

The fungi were characterized on Czapek yeast autolysate agar (CYA), malt extract agar (MEA), yeast extract-sucrose agar (YES), oatmeal agar (OA), creatine sucrose agar (CREA) and nitrite sucrose agar (NS). CREA is a differential medium used for identification of *Penicillium* species (Frisvad 1985; Frisvad 1993). For formulations see Frisvad (1981), Samson & van Reenen-Hoekstra (1988) and Samson & Pitt (1990). Besides a sausage imitated medium (PI) a meat extract medium (ME) was used. ME was made from 19.45% meat extract (Lab- Lemco powder, Oxoid L29), 1.4% sodium nitrite, 1.5% agar and a trace metal solution. The trace metal solution consisted of 50 ppm $CuSO_4,5H_2O$ and 100 ppm $ZnSO_4,7H_2O$. The composition of PI was as ME but with 35% triolein (Sigma T7752) added. Incubation took place at 25° C for 7 days on all media, and at 5° C and 37° C for 7 days on CYA. Conidium colour was determined using Kornerup & Wanscher (1978).

*Analysis for secondary metabolites*

Production of secondary metabolites was examined with the agar plug thin-layer chromatography (TLC) methods of Filtenborg & Frisvad (1980), Filtenborg,

Frisvad & Svendsen (1983) and Frisvad & Thrane (1987) plus high-performance liquid chromatography (HPLC) (Frisvad & Thrane 1987). For TLC analysis, the following eluent systems were used; CAP (chloroform-acetone-propane-2-ol [85 : 15 : 20, vol/vol/vol]) and TEF (toluene-ethyl acetate-90% formic acid [5 : 4 : 1, vol/vol/vol]). Griseofulvin was used as an external standard. For chromatographic analyses the fungi were incubated at 25° C for 14 days. The media used were CYA, MEA and YES, plus NS for TLC analysis and OA for HPLC analysis.

*Analysis for biochemical activity*

Lipolytic activity, proteolytic activity and the ability to metabolise lactic acid was examined. Metabolism of lactic acid was examined, because several *Penicillium* species according to Grazia et al. (1986) metabolise lactic acid that are either produced by bacteria during the sausage fermentation or already present in the meat. For determination of lipolytic activity the method described by Sierra (1957) was used with a few modifications. Meat extract (3.5%) was added for increased nutrition and 0.1% triolein was used as a lipid substrate. Proteolytic activity was determined according to the method described by Hankin & Anagnostakis (1975), modified by the addition of meat extract (3.5%) and with an increased gelatine concentration (3%). Metabolism of lactic acid was examined on a modified Czapek media, LLA, in which sucrose was replaced by lactic acid (80%). To prevent inhibition, pH was adjusted to 7 with 0.1 N NaOH. Plates were incubated at 25° C for 7 days.

*Chemometrics*

The variables used in the chemometric study were macromorphological characters: colony diameter, conidium colour, extent of conidiogenesis and mycelium height after growth on CYA, MEA, YES, NS, ME and PI. The physiological characters used were growth and acid production on CREA, lipolytic activity, proteolytic activity, colony diameter and conidium colour after growth on LLA. For evaluation of secondary metabolite production, log areas of known and unknown metabolites were determined after HPLC analysis. The production of secondary metabolites was evaluated as binary data from 11 selected groups of chromophore families. The data were analysed statistically using the PC software packages NTSYS version 1.60 and SIRIUS version 2.3. The multivariate statistical methods used were cluster analyses (distance optimization); average linkage clustering (UPGMA) and fuzzy clustering. Euclidian distance, Yule, Jaccard and Pearson's product moment correlation coefficients were used in UPGMA clustering. Correspondence analysis was also used for ordination and PLS for constrained ordination. The choice of methods was mainly based on Frisvad (1992) who showed that certain multivariate statistical methods are especially useful for taxonomical purposes.

## Results and discussion

*Morphology*

The 64 *P. nalgiovense* isolates grown on different agar media were examined morphologically. Micromorphologically the *P. nalgiovense* isolates were characterized by bi- and terverticillate conidiophores with an irregular structure. The ornamentation of the stipe was smooth and rami were divergent when present. Phialides were ampulliform with a short but broad collulum. The conidia were globose to subglobose with smooth walls. The conidiophores were mainly biverticillate and therefore *P. nalgiovense* is placed in subgenus *Furcatum*, in agreement with Pitt (1979).

Macromorphological characters are listed in Table 2. A distinction in reverse colour on CYA and MEA was observed between the ex type culture and isolates from meat products (Table 2), whereas the micromorphological characters showed no differences. The ex type culture had a brown reverse on CYA and MEA and fits the descriptions of Pitt (1979) and Raper & Thom (1949), who only examined the type. The meat isolates differed by having a yellow reverse in agreement with the description of Samson & van Reenen-Hoekstra (1988).

YES agar is not commonly used for identification. It is, however, characteristic for the *P. nalgiovense* isolates including the type that they have an yellow orange to orange reverse on YES (Table 2). Thus YES agar is an useful aid in the identification of *P. nalgiovense*. On the other media the ex type culture is characterized by a brown reverse. The reverse colour can therefore be used to distinguish between the ex type cultures and isolates from meat products (Table 2). Also, the colony diameters of the ex type cultures are smaller than the majority of the isolates from meat products when grown on the media CYA, MEA and YES.

*Table 2.* Macromorphological characters (7 days growth at 25° C).

| Media | Colony diameter[g] | Colony structure | Conidia colour | Reverse colour |
|---|---|---|---|---|
| CYA[a] ex type: 20 mm | (19–)25–32 mm | Radially sulcate ex type: radially sulcate | White, turquoise white-pale turquoise, grey green (dull green) ex type: grey green | Light yellow ex type: brown |
| MEA[b] | (18–)21–29 mm ex type: 18 mm | Plane ex type: plane | White, turquoise white-pale turquoise, grey green (dull green) ex type: grey green | Yellow-yellow orange ex type: light brown |
| YES[c] | (25–)35–50 mm ex type: 30 mm | Radially sulcate ex type: radially sulcate | White, turquoise white-pale turquoise, grey green (dull green) ex type: grey green | Yellow orange-(dark) orange ex type: yellow orange-orange |
| NS[d] | 0–23 mm[h] ex type: 17 mm | Radially sulcate ex type: radially sulcate | White, turquoise white-pale turquoise, grey green (dull green) ex type: greenish white | White-light yellow ex type: light brown |
| PI[e] | (5–)10–20 mm ex type: 14 mm | Plane ex type: plane | White, greenish white, pale green ex type: – | nd[i] |
| ME[f] | (7–)20–28 mm ex type: 21 mm | Lightly radially sulcate ex type: plane | White, greenish white, green grey ex type: (white) | nd |

[a] Czapek yeast extract agar. [b] Malt extract agar. [c] Yeast extract-sucrose agar. [d] Nitrite sucrose agar. [e] Product imitated media. [f] Meat extract media. [g] The diameters given covers more than 90% of the examined isolates, occasionally the diameters are smaller (shown in brackets). [h] 26% are microcolonies (≤ 5 mm), 24% are 6–13 mm and 50% are 14–23 mm. [i] Not determined.

*Physiology*

On CYA at 5° C microcolonies were seen but at 37° C no growth occurred, in agreement with Pitt (1979). Mycelial height was generally low (< 2 mm) on all media. On CREA, cultures showed either no growth or very inhibited growth. Acid production, if any, was weak and only seen under the colony. According to Frisvad (1985) most species outside subgenus *Penicillium* grow poorly on CREA.

In Table 3 lipolytic and/or proteolytic *P. nalgiovense* isolates are listed. Of 63 examined cultures only 13% demonstrated proteolytic as well as lipolytic activity. Generally isolates with high proteolytic activity showed limited lipolytic activity. Thus none of the examined isolates are good producers of both proteases and lipase. Only 46% of the cultures demonstrated proteolytic activity, 16% lipolytic activity and 25% neither proteolytic nor lipolytic activity. The ex type culture demonstrated proteolytic activity but no lipolytic activity. *P. nalgiovense* isolates are often used as starter cultures for fermentation of sausages and in this respect a proteolytic and lipolytic activity may improve the product.

*Table 3.* Lipolytic and/or proteolytic *P. nalgiovense* isolates.

| | Proteolytic activity | | |
|---|---|---|---|
| Lipolytic activity | Positive | Negative | Total |
| Positive | 13% | 16% | 29% |
| Negative | 46% | 25% | 71% |
| Total | 59% | 41% | |

Grown on LLA 62 of 63 colonies (including the ex type culture) attained an colony diameter of 20–

29 mm. Thus the *P. nalgiovense* cultures are capable of utilizing lactate at neutral pH.

*Secondary metabolites*

Fiftyfour isolates were examined for the production of secondary metabolites by TLC and HPLC. On TLC all *P. nalgiovense* isolates examined produced three unknown secondary metabolites, A, B and C. Retardation factors relative to griseofulvin ($R_{fg}$) are 1.17 (TEF) and 0.06 (CAP) for metabolite A, 1.33 (TEF) and 0.06 (CAP) for metabolite B and 0.56 (TEF) and 0.39 (CAP) for metabolite C. The metabolites showed blue fluorescence in UV-light (metabolite A and B at 254 nm and 365 nm, metabolite C at 254 nm), and TEF was the best eluent system for separation of the metabolites. The best medium for production of metabolite A and B was CYA, and for metabolite C the best medium was YES. However metabolite A and B was also produced on YES and weakly on MEA and NS. The profile of secondary metabolites determined by TLC analysis was a valuable tool for identification purposes.

The HPLC analysis showed production of three known secondary metabolites; chrysogine, nalgiolaxin and nalgiovensin. Chrysogine, a metabolite also produced by *P. chrysogenum* (Frisvad & Filtenborg 1989), was produced by 52 of the cultures examined. The ex type culture did not produce chrysogine. Nalgiolaxin and nalgiovensin were produced by 5 of the isolates, including the ex type culture.

Pitt (1979) synonymised *P. nalgiovense* with *P. jensenii*. This is unlikely to be correct, as *P. jensenii* has no secondary metabolites in common with *P. nalgiovense*. However *P. nalgiovense* secondary metabolites have some similarities with those of *P. chrysogenum* (Frisvad & Filtenborg 1989; Frisvad & Filtenborg 1990).

*Chemometrics*

Several multivariate statistical methods were used for the evaluation of the macromorphological, physiological and chemical characters of *P. nalgiovense*. The aim was to separate possible biotypes and to point out the discriminating characters.

Based on macromorphological and physiological characters it is not possible to separate the *P. nalgiovense* species in biotypes, using cluster analyses or correspondence analysis. Using fuzzy clustering the data matrix was analyzed assuming 2 to 6 clusters with a coefficient of fuzziness of 1.25. The optimal number of clusters was found to be two, separating the isolates producing white and grey green conidia; those producing turquoise conidia belonged to both groups. The data matrices for macromorphological and physiological characters were also analyzed using PLS for constrained ordination, that is using the PLS algorithm with the original data matrix as the X block and a binary dummy Y matrix describing conidium colour (white, turquoise and grey green). The only separation seen was two groups, white and turquoise/grey green conidia (data not shown). Separating the species into biotypes based on production of secondary metabolites, using either the log areas data matrix or the binary chromophore families data matrix, was not possible.

Average linkage clustering (UPGMA) of the binary chromophore families data matrix using the Yule coefficient gave a clear separation between the ex type culture and the meat isolates. The cophenetic correlation for the dendrograms based on the Yule and the Jaccard coefficients (dendrograms not shown) were respectively 0.642 and 0.823. Correspondence analysis of the binary chromophore families data matrix clearly separated ex type culture (IBT 3861) from the meat isolates.

Because of production of chrysogine and morphological similarity to *P. chrysogenum* (Samson & van Reenen-Hoekstra 1988), some points of resemblance between *P. chrysogenum* and *P. nalgiovense* are given in Table 4. Two varieties of *P. chrysogenum*, var. *chrysogenum* and var. *dipodomyis* (Frisvad & Filtenborg 1989) are listed in Table 4. The *P. nalgiovense* ex type culture and isolates from meat products examined in this study have some resemblance to both *P. chrysogenum* var. *chrysogenum* and *P. chrysogenum* var. *dipodomyis*. The possibility exists that *P. nalgiovense* represent a domesticated species of *P. chrysogenum* in the way that *P. camemberti* is the domesticated form of *P. commune* (Leistner 1990; Samson 1985). Like *P. camemberti, P. nalgiovense* may have lost its ability to produce some of the secondary metabolites that are produced by the wildtype. According to Stolk et al. (1989) the type cultures of *P. chrysogenum* and starter cultures of *P. nalgiovense* also resemble one another in that they both produce penicillin in addition to the morphological resemblance. Therefore *P. nalgiovense* is by them regarded as a domesticated form of *P. chryosgenum* (Stolk et al. 1990). Leistner (1990) suggested reclassification of *P. nalgiovense* isolates from meat as a new species as they differed from the ex type culture. Banke (1994), using isoenzyme

*Table 4.* Resemblance between *P. chrysogenum* var. *dipodomyis*, *P. chrysogenum* var. *chrysogenum* and *P. nalgiovense*.

| | Occurrence | Stipe | Reverse colour | Secondary metabolites |
|---|---|---|---|---|
| *P. chrysogenum* var. *chrysogenum* | Ubiquitous, e.g. spices, cheese and meat[a] | Smooth[a] | CYA: yellow[e] YES: (yellow orange) | Chrysogine, meleagrin, roquefortine, C, penicillins[a] |
| *P. chrysogenum* var. *dipodomyis* | Cheek pouxhes and soil samples[a,b] | Rough[a] | CYA: orange YES: orange[a] | Penicillins and some blue-fluorescing metabolites[a] |
| *P. nalgiovense* (ex type) | Cheese[c] | Smooth | CYA: brown YES: yellow orange-orange | Nalgiolaxin, nalgiovensin |
| *P. nalgiovense* | Meat[d] | Smooth | CYA: light yellow YES: yellow orange-(dark) orange | Chrysogine, nalgiolaxin, nalgiovensin |

[a] Frisvad & Filtenborg (1989), [b] Frisvad et al. (1987), [c] Laxa (1932), [d] Samson & Reenen-Hoekstra (1988), [e] Singh et al. (1991).

profiles, concluded that *P. nalgiovense* is a separate species, more closely related to *P. chrysogenum* var. *dipodomyis* than *P. chrysogenum* var. *chrysogenum*. Banke (1994) demonstrated that the ex type culture differs from other *P. nalgiovense* isolates, but maintained a single species in agreement with Leistner (1990) and the present study.

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