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Simple Aid for the Identification of *Penicillium roqueforti* Thom

Growth in Acetic Acid

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Summary. 53 strains of *Penicillium roqueforti* Thom obtained from culture collections, blue cheeses, sausages, and other sources are shown to grow abundantly on a Czapek Dox liquid medium supplemented with 0.5% acetic acid. None of 30 other strains (including *P. charlesii, P. waksmani, P. rugulosum, P. brevi-compactum, P. herquei, P. viridicatum, P. cyclopium, P. velutinum, P. oxalicum, P. toxicarium, P. notatum, P. stoloniferum, P. chrysogenum, P. japonicum, P. casei, P. citreo-viride*) exhibited this property. It is suggested that growth on acetic acid provides a simple tool for a rapid and preliminary identification of *P. roqueforti* Thom since growth can be observed as early as 3 days after inoculation.

Identification of *Penicillium sp.* is usually accomplished by microscopic observation of morphologic properties of conidiophores and macroscopic inspection of surface colonies on a suitable agar (Raper and Thom, 1949). To avoid the potential production of mycotoxins by contaminants, fungal strains used for the preparation of food must be routinly and permanently checked for their identity. We demonstrate that *Penicillium roqueforti* Thom – important for the production of blue cheeses, e.g., Roquefort or Gorgonzola – can be differentiated from closely related forms and contaminants by growth in Czapek Dox medium supplemented with 0.5% acetic acid. The use of acetic acid was originally introduced by Wöltje (1918), on basis of only one strain of *P. roqueforti*, however.

Materials and Methods

Source and Maintenance of Strains. Penicillia used in this investigation have been obtained from sources indicated in Tables 1 and 2. The strains were routinely maintained on malt agar slants by storing the cultures for 6 months at 4° C after growth for 7 days at 25° C and before transfer onto fresh medium.

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Microscopic Characterization. The morphologic properties of conidiophores were examined after growth of the strains on agar-coated glass slides covered with a cover glass, supported for the four edges with a suitable grease.

Media. Liquid Czapek Dox medium was supplemented with one of the following ingredients: propionic acid (0.2%), lactic acid (4%), acetic acid (0.5%), succinic acid (5%), citric acid (2%), tartaric acid (1%), oxalic acid (0.2%), 4-aminobenzoic acid (0.2%), tannin (2%), NaCl (7.5%). The media were used without changing the resulting pH value.

Diverse carbon sources were tested in liquid equal amounts by weight of Czapek Dox solution containing instead of sucrose **D**(+)galactose, lactose, **D**(-)fructose, raffinose, **L**(+)arabinose, **D**(+)xylose, **L**(-)sorbose, soluble starch, glycerol, **D**(-)mannitol, lactate, or citrate. The sugars were sterilized by filtration. Diverse nitrogen sources were tested by exchanging KNO_3 for KNO_2, kreatin, $(NH_4)_2SO_4$, ethylamine hydrochloride, asparagine, alanine, glycine, or urea. Urea was sterilized by filtration.

Growth at 37° C. The molds were cultivated on malt agar for 7 days.

Growth in Litmus Milk. The molds were incubated for 7 days at 25° C.

Growth in an Atmosphere of 95% N_2 and 5% O_2. The molds were cultivated for 7 days at 25° C.

Before inoculation of the molds into the test media they were precultivated on Czepek Dox agar for 7 days at 25° C.

Roquefortine was isolated and semiquantitatively determined as described by Scott and Kennedy (1976). Recovery was about 60% – 75%. The limit of detection was 0.1 µg/g of medium and mycelium.

Results and Discussion

No biochemical differentiation between *P. roqueforti* Thom strains (see Table 1) and the other investigated Penicillia (see Table 2) could be accomplished by variation of the following ingredients in the test media or growth conditions:

1. Exchange of the carbon source sucrose against galactose, lactose, fructose, raffinose, arabinose, xylose, sorbose, soluble starch, ethanol, glycerol, mannitol, lactic acid, or citric acid

2. Exchange of the nitrogen source KNO_3 against $(NH_4)_2SO_4$, ethylaminehydrochloride, asparagine, alanine, glycine, or urea

3. Addition of 5% succinic acid (resulting pH 2.3), 2% citric acid (pH 2.1), 1% tartaric acid (pH 2.1), 0.2% oxalic acid (pH 2.3), 0.2% 4-aminobenzoic acid, 7.5% NaCl, or 2% tannin

4. Growth at 37° C

5. Growth in litmus milk at 25° C

6. Growth in an atmosphere of 95% N_2 plus 5% O_2 at 25° C

However, good differentiation in liquid Czapek Dox medium was achieved by the ingredients indicated in Tables 1 and 2: 0.2% propionic acid, 4% lactic acid, 0.5% acetic

Table 1. Growth response of 40 different *Penicillium roqueforti* Thom strains to propionic acid, lactic acid, acetic acid, nitrite, and kreatine, and ability to produce roquefortine. Incubation was for 20 days at 25° C.

No.	Source	Growth on liquid Czapek Dox medium supplemented with			Nitrogen source		Formation of roquefortine
		0,2% propionic acid	4% lactic acid	0,5% acetic acid	nitrite	kreatin	
6753	CBS Baarn 28067	X	X	X	X	X	+
6766	ATCC 34905	-	(X)	X	X	X	+
6767	PDA CERTJA(France)	-	X	X	X	X	+
6768	NRRL 849	X	X	X	X	X	+
6800	Italy, starter	X	X	X	X	X	+
6821	Italy, starter	X	(X)	X	X	X	+
6829	Germany,starter	X	X	X	X	X	+
6830	Germany,starter	X	X	X	X	X	+
6848	Germany,starter	X	X	X	X	X	+
6850	Germany,starter	X	X	X	X	X	+
6873	Germany,starter	X	-	X	X	X	-
6874	Germany,starter	X	-	X	X	X	-
6875	Germany,starter	X	(X)	X	X	X	-
6876	Germany,starter	X	-	X	X	X	-
6877	Germany,starter	X	(X)	X	X	X	-
6878	Germany,starter	X	-	X	X	X	-
6879	Germany,starter	-	-	X	X	X	+
6880	Germany,starter	-	(X)	X	X	X	-
6814	Germany,cheese	-	-	X	X	X	+
6861	Germany,cheese	X	X	X	X	X	+
6866	Germany,cheese	X	X	X	X	X	+
6867	Germany,cheese	X	X	X	X	X	-
6813	Denmark,cheese	X	-	X	X	X	+
6845	Denmark,cheese	-	X	X	X	X	+
6859	Denmark,cheese	X	X	X	X	X	+
6860	Denmark,cheese	X	X	X	X	X	+
6862	Denmark,cheese	X	X	X	X	X	+
6869	Denmark,cheese	X	X	X	X	X	+
6815	France,cheese	-	-	X	X	X	+
6816	France,cheese	X	X	X	X	X	+
6847	France,cheese	X	X	X	X	X	+
6858	France,cheese	X	(X)	X	X	X	+
6863	France,cheese	(X)	X	X	X	X	+
6864	France,cheese	X	X	X	X	X	+
6865	France,cheese	(X)	(X)	X	X	X	+
6868	France,cheese	X	X	X	X	X	+
6817	Italy,cheese	X	X	X	X	X	+
6818	Italy,cheese	X	(X)	X	X	X	+
6870	Italy,cheese	X	X	X	X	X	+
6846	Switzerld.,cheese	X	X	X	X	X	+

X = growth response comparable to Czapek Dox medium
(X)= growth response much weaker than in Czapek Dox medium
– = no growth
Roquefortine: + = detectable by thin-layer chromatography
– = not detectable

Table 2. Growth response of diverse Penicillia to propionic acid, lactic acid, acetic acid, nitrite, and kreatine, and ability to produce roquefortine. Incubation was for 20 days at 25° C

No.	Species	Source	Growth on liquid Czapek Dox medium supplemented with 0,2% propionic acid	4% lactic acid	0,5% acetic acid	Nitrogen source nitrite	kreatin	Formation of roquefortine
6709	P.charlesii	CBSBaarn	-	-	-	-	-	-
6710	P.waksmani	"	-	-	-	X	-	-
6715	P.rugulosum	"	-	-	-	(X)	-	-
6716	P.brevi-compactum	"	-	-	-	(X)	-	-
6719	P.herquei	"	-	X	-	X	-	-
6725	P.viridicatum	"	-	-	-	-	X	-
6726	P.cyclopium	"	-	-	-	X	-	-
6730	P.velutinum	"	-	-	-	X	-	-
6731	P.oxalicum	"	-	-	-	X	-	-
6757	P.toxicarium	"	-	-	-	X	-	-
6761	P.viridicatum	"	-	-	-	-	X	-
6763	P.notatum	"	-	-	-	-	X	+
6764	P.stoloniferum	"	-	-	-	X	-	-
6765	P.chrysogenum	"	-	-	-	-	X	-
6775	P.oxalicum	"	-	-	-	X	-	-
6776	P.brevi-compactum	"	-	-	-	X	-	-
6779	P.japonicum	"	-	-	-	-	-	-
6780	P.casei	"	-	-	-	-	X	-
6733	P.chrysogenum	Germany	-	-	-	-	X	-
6735	P.notatum	Germany	-	-	-	X	-	-
6772	P.citreo-viride	Japan	-	-	-	X	-	-
6770	P. sp.	Japan	-	-	-	X	-	-
6872	P. sp.	Germany, starter	-	-	-	-	X	-
6881	P. sp.	Germany, starter	-	X	-	-	-	-
6746	P. sp.	France, cheese	-	X	-	-	X	-
6820	P. sp.	France, cheese	-	X	-	-	X	-
6843	P. sp.	France, cheese	-	-	-	-	X	-
6842	P. sp.	Italy, cheese	X	X	-	-	X	+
6844	P. sp.	Denmark, cheese	-	X	-	-	X	-
6748	P. sp.	Germany, cheese	-	-	-	-	X	-

X = growth response comparable to Czapek Dox medium
(X)= growth response much weaker than in Czapek Dox medium
– = no growth
Roquefortine: + = detectable by thin-layer chromatography
– = not detectable

acid, nitrite, and kreatine. The only addition providing a clear-cut 100% differentiation was 0.5% acetic acid. Only strains of the species *P. roqueforti* Thom could grow on this medium. Roquefortine thought to be a typical metabolite of *P. roqueforti* was produced by most strains of *P. roqueforti*, but also by one strain of *P. notatum* and an unidentified species (No. 6842) isolated from Italian cheese. Thirteen strains of *P. roqueforti* isolated from raw sausages which were generously supplied by Bundesanstalt für Fleischforschung (Kulmbach) did also grow abundantly on Czapek Dox medium supplemented with 0.5% acetic acid (pH 3.4).

The biochemical mechanisms underlying this ability of *P. roqueforti* to grow in the presence of 0.5% acetic acid may in part be due to a pH effect, since adjustment of the pH value of this medium to 6.5 induced growth of most of the other strains described in Table 2. This is also deduced from the observation that most of the *P. roqueforti* strain did also develop on 0.2% propionic acid (pH 4.5) and 4% lactic acid (pH 2.0).

The *P. roqueforti* strains described in Table 1 were also characterized by their usual microscopic and macroscopic properties (Raper and Thom, 1949). None of these strains produced a series of common mycotoxins including aflatoxins B_1, B_2, G_1, G_2, sterigmatocystin, patulin, penicillic acid, diacetoxyscirpenol, ochratoxin A, rubratoxin B, citrinin, and citreoviridine (Engel and Milczewski, 1977; Krusch et al., 1977).

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