Some observations on the physiology of Penicillium roqueforti Thom and Penicillium cyclopium Westling

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Original article

Some observations on the physiology of *Penicillium roqueforti* Thom and *Penicillium cyclopium* Westling

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Summary — *P roqueforti* and *P cyclopium* behave similarly towards different physiological parameters. *P cyclopium* grows better between 10–20 °C, resists NaCl better and grows faster with lactose. Acetic acid and to a certain extent lactic acid inhibits the growth of the *Penicillium* at a clearly lower pH than internal cellular pH.

Penicillium roqueforti / Penicillium cyclopium / physiology / organic acid

Résumé — **Remarque sur la physiologie de *Penicillium roqueforti* Thom et de *Penicillium cyclopium* Westling.** P roqueforti et P cyclopium *contaminant des pâtes persillées réagissent de façon assez voisine aux diverses variations des paramètres physicochimiques du fromage. Cependant,* P cyclopium *se développe mieux entre 10 et 20 °C, résiste mieux au sel et a une croissance plus importante sur lactose. L'acide acétique et, à un degré moindre, l'acide lactique, sont inhibiteurs lorsque le pH du milieu extracellulaire est inférieur au pH du milieu intracellulaire par suite d'une accumulation de l'acide dans le mycélium. Au contraire, ces acides peuvent être utilisés comme substrat lorsque le pH du milieu extérieur est ≥ pH du milieu intérieur. Il semblerait que ces acides traversent la membrane cellulaire sous forme non ionisée RCOOH.*

Penicillium roqueforti / Penicillium cyclopium / physiologie / acide organique

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INTRODUCTION

Moulds are not usually present on the surface of blue-veined cheese. Sometimes cheeses may be contaminated by *P cyclopium* growing on the 2 sides and the edge of the loaves during the first ripening phase (usually around the 13th and the 16th day). This contamination is undesirable for technological reasons, *ie* allergy in the dairy staff; worse sticking of tinfoil during the "plombage" phase (start of the anaerobic phase); deterioration of organoleptic properties (Veau *et al*, 1981). This kind of contamination is a risk throughout the manufacturing process but its appearance is more frequent during the winter months. Our aim was to prevent this contaminant proliferation by studying some physiological characters of *Penicillium*.

MATERIALS AND METHODS

Biological material

Four strains were used for this study, two of which were industrial: *Penicillium roqueforti* (called RS in the text); and *Penicillium cyclopium* (called CS in the text).

The 2 other strains were provided by the Mycothèque de l'Université Catholique de Louvain (MUCL): *Penicillium roqueforti* Thom MUCL 29 151 (called RC in the text); *Penicillium cyclopium* Westling MUCL 14 445 (called CC in the text).

Penicillium cyclopium corresponds to the species named *Penicillium verrucosum* Dierckx var *cyclopium* (Westling) Samson, Stolk et Hadlok, also identified as *Penicillium aurantiogriseum* Dierckx.

Culture conditions

Lodder's medium (yeast extract 3 g/l, peptone 5 g/l, glucose 10 g/l) was used for cultures in liquid medium. Media were sterilized by autoclaving at 120 °C for 20 min. Cultures were grown in Erlenmeyers flasks (500 ml) filled to 1/10th of their volume and shaken (amplitude: 7 cm; oscillations 80/min) at 30 °C for 5 days.

Czapek medium was used for solid media: $NaNO_3$ (3 g), K_2HPO_4 (1 g), $MgSO_4$, 7 H_2O (0.5 g), KCl (0.5 g), $FeSO_4$, 7 H_2O (0.01 g), sucrose (30 g), Agar (20 g), pH = 6.5. On modified media sucrose was substituted by an organic acid by maintaining the same amount of carbon per liter of medium.

Analytical techniques

Determination of dry weight

Mycelia were harvested by filtration (Millipore membrane 5 μm). Dry weight was determined by weighing the used filter until a constant weight after dessication at 105 °C.

Measure of linear growth

Growth was estimated by measuring the 2 perpendicular diameters of the thallus. These measurements were performed on the 2 largest colonies.

pH determination

External pH was determined with a pH-meter. Internal pH was estimated by Kotyk's equation.

RESULTS AND DISCUSSION

Influence of various physicochemical parameters on growth

All growth determinations were carried out on Lodder's medium for the 4 strains.

Temperature

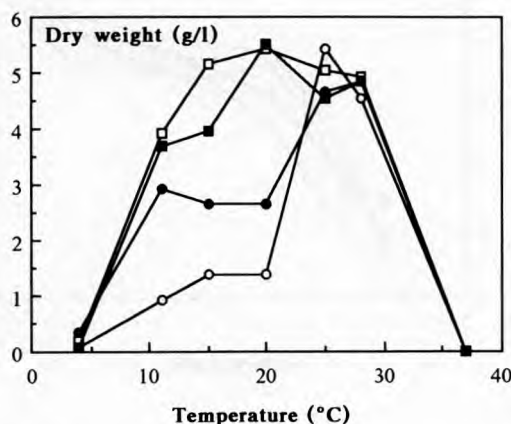Figure 1 shows that the 4 microorganisms grew between 10–30 °C; the optimal tem-

Fig 1. Estimation of *Penicillium* growth as a function of temperature. —O— *P roqueforti* S; —●— *P roqueforti* C; —□— *P cyclopium* S; —■— *P cyclopium* C.
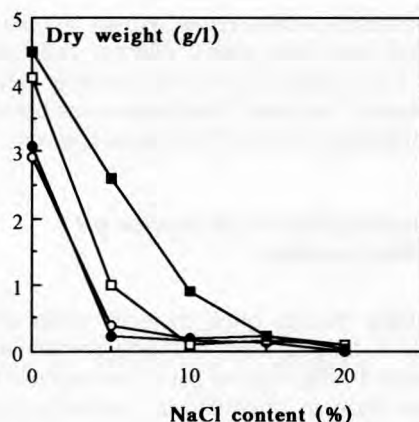*Évaluation de la croissance de* Penicillium *en fonction de la température.*

Fig 2. Estimation of *Penicillium* growth as a function of NaCl content. —O— *P roqueforti* S; —●— *P roqueforti* C; —□— *P cyclopium* S; —■— *P cyclopium* C.
Évaluation de la croissance de Penicillium *en fonction de la teneur en NaCl.*

peratures for growth were 20 °C for *P cyclopium* and 25 °C for *P roqueforti*. However, *P cyclopium* grew better between 10–20 °C.

pH

For this study, media were buffered with tartrate or phosphate buffer. pH did not significantly influence growth. The 4 strains grew correctly between pH 3 and pH 7 with an optimum between pH 4 and pH 5.

Salt content

As indicated in figure 2, sodium chloride had a different action on growth in both species. When sodium chloride content was 5% (w/v) only *P cyclopium* (CS and CC) was able to grow significantly. With

10% (w/v) only *P cyclopium* (CC) grew. With 15% (w/v) sodium chloride, growth was completely inhibited.

The liquid medium probably modified the abilities of the strains to resist NaCl. Indeed, *P roqueforti* filled in a homogeneous manner the cheeseholes where NaCl content in water ranged from 10–20%. Nevertheless, it seemed that *P cyclopium* was more resistant than *P roqueforti* to high NaCl contents.

Osmotic pressure

The influence of osmotic pressure was studied with various concentrations of glucose. No significant difference appeared between the 2 species; growth was optimal with 20% glucose. No mould developed at 60%.

Carbon substrate

The basal medium (yeast extract and pep-
tone) gave weak growth. Glucose was bet-
ter than lactose as a carbon source. Nev-
ertheless, we noted that lactose was better
metabolized by the *P cyclopium* species.

Determination of the internal pH of the mycelium

Usually moulds grew correctly when pH
values ranged from 4–7 and sometimes
below 4. The internal pH of the mycelium
was close to physiological neutrality (pH
6.5–7.5). The internal pH of the 4 strains
was estimated after growth on Lodder buf-
fered medium over a wide range of pH.
Cells were centrifuged, washed and resus-
pended in bidistilled water, and crushed
mechanically; dry weight was about 250
mg/ml. Direct measurement of pH, with an
electrode plunged in the crushed myceli-
um solution, allowed estimation of the in-
ternal pH values of the mycelium (fig 3A).

The internal pH was near physiological
neutrality when the pH of the culture medi-
um was above 4.5. When the external pH
was lower than 4.5, the internal pH de-
creased.

Kotyk (1963) proposed an original
method to estimate the internal pH. Ac-
cording to MacMillan (1956), some un-
charged molecules freely cross the plas-
matic membrane of cells. So we can
assume for instance, bromophenol blue
(pK_a = 4.0) enters the cells in its unionized
forms. Equilibrium is reached. It allows an
equal RCOOH concentration on both sides
of the plasmatic membrane. Kotyk's equa-
tion, *ie*:

$pHi = pHo$
$+ \log Ci/Co^* [(1 + 10^{pK-pHo}) - 10^{pK-pHo}]$ [1]
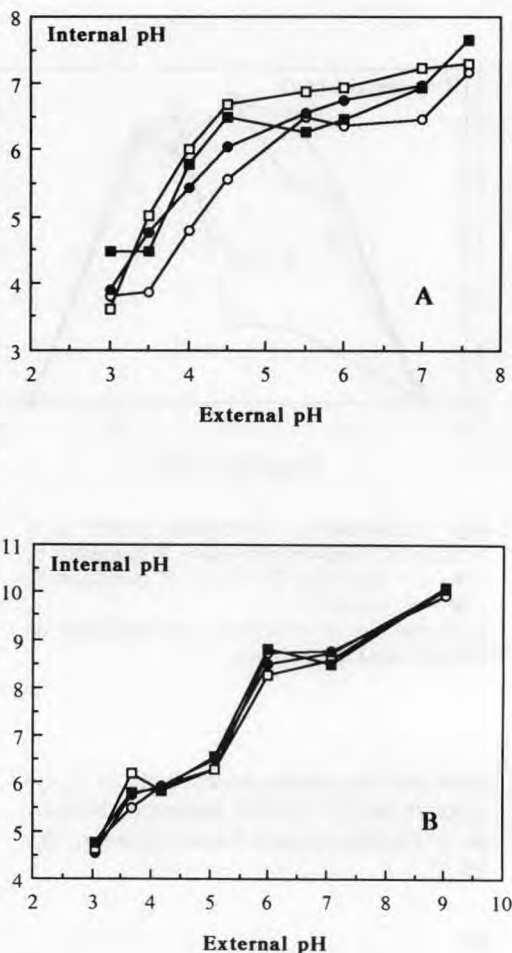
Fig 3. Estimation of internal pH. **A:** pH-metry
determination. **B:** Determination by calculation.
—O— *P roqueforti* S; —●— *P roqueforti* C;
—□— *P cyclopium* S; —■— *P cyclopium* C.
*Évaluation du pH interne. A : Détermination par
pHmétrie. B : Détermination par le calcul.*

Ci = intracellular concentration;
Co = extracellular concentration;
pHi = intracellular pH;

pHo = media pH;

pK = *pK* of acid function

correctly describes this equilibrium.

So it is possible to estimate the internal pH of the mycelium (fig 3B) by studying the intracellular and exocellular concentration of bromophenol blue for various pH values of the medium. The internal pH of the mycelium was ≈ 6 when the pH of the medium ranged from 3.5 to 5. This pH zone, similar to the *pK* value of bromophenol blue, provides, *a priori*, reliable results. So, it can be assumed that the internal pH of mycelium was about 6. The 4 strains behaved similarly.

Growth studies on various organic acids

Acetic and lactic acids

The 4 strains were incubated in Lodder liquid media. Glucose, acetic acid or glucose and acetic acid mixture (1:1) were used as carbon sources. These different media were buffered from 3 to 8.

Figure 4 clearly indicates that *Penicillium* strains grew well on glucose at all the tested pH. The optimal pH for growth seemed close to 4. On the contrary, growth with acetic acid as carbon substrate was really significant only at pH 7 and 8.

This can easily be explained if we consider that the *Penicillium* behave exactly in the same manner as *Saccharomyces cerevisiae* towards acetic acid. Conway and Downey (1949) previously showed that acetic acid penetrated through the *Saccharomyces cerevisiae's* cell wall in its uncharged form (RCOOH). The equilibrium which can be calculated from equation 1 allows it to be shown that acetic acid penetrates normally at pH 7 or pH 8 but accumulates at low pH.

For *pHo* = 3, C_i = 92 C_o and C_i = 910 C_o for pHi equal to 6 and 7 respectively.

For *pHo* = 5, C_i = 5 C_o and C_i = 100 C_o for pHi equal to 6 and 7 respectively.
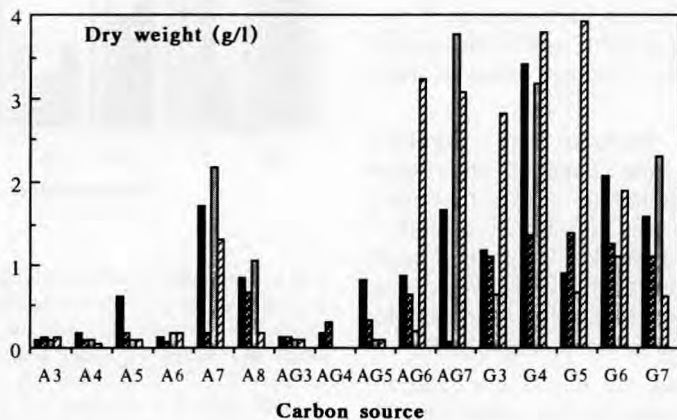
Fig 4. Estimation of *Penicillium* growth as a function of pH and carbon substrate. ■ *P roqueforti* S; ▨ *P roqueforti* C; ▨ *P cyclopium* S; ▨ *P cyclopium* C. A = acetic acid; G : glucose; AG = acetic acid + glucose; 3 = pH 3.
Évaluation de la croissance de Penicillium en fonction du pH et du substrat carboné.

This hypothesis seems to be confirmed by the fact that acetic acid inhibits growth on glucose when external pH values are between 3 and 5 but stimulates it at pH 7. Acetic acid probably penetrates into the mycelium in its uncharged form (RCOOH) with an accumulative effect when the exocellular pH is lower than the endocellular pH.

On buffered Czapek solid medium, the 4 strains grew as well at pH 6 as at pH 3 when sucrose was used. On the same basal medium, the substitution of sucrose by lactic acid or acetic acid allows good growth at pH 6. At pH 3, no mould developed on acetic acid but growth was significant on lactic acid (fig 5A). The addition of lactic or acetic acid weakly modified growth at pH 6 but inhibited it at pH 3 (fig 5B). Experiments A and B were independent.

These results confirm the toxic action of acetic acid when pH values are low. Lactic acid may have a less obvious but comparable action. That may be explained by the fact that the *pK* value of lactic acid is clearly lower than that of acetic acid. Therefore, at the same pH the concentration of uncharged forms of lactic acid is less important than of the uncharged forms of acetic acid.

Moreover, Romano and Kornberg (1968, 1969) and Jennings and Austin (1973) showed that the utilization of many carbohydrates was inhibited by acetate in other species of moulds. Hunter and Segel (1973) also showed that many weak acids inhibited the organic solute transport for pH values equal or inferior to their pK_a.

Nevertheless, our results are quite different from those previously described on Czapek medium. Veau *et al* (1981) showed that only *P cyclopium* was able to grow on acetate at pH 6. However, at this value of pH, *ie* 6, our strains of *P roqueforti* were also able to grow.
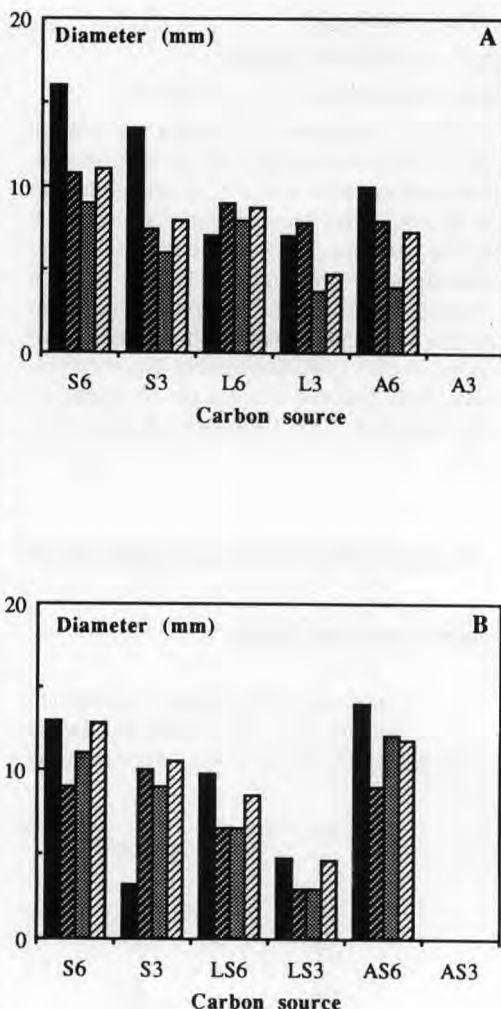
Fig 5. Estimation of *Penicillium* growth on Czapek media. **A:** Growth on sucrose or different acids. **B:** Growth on sucrose and different acids. ■ *P roqueforti* S; ▨ *P roqueforti* C; ▦ *P cyclopium* S; ▨ *P cyclopium* C. A = acetic acid; L = lactic acid; S = sucrose; AS = acetic acid + sucrose; LS = lactic acid + sucrose; 3 = pH 3; 6 = pH 6.
Évaluation de la croissance de Penicillium *sur milieu de Czapek.* **A** *: Croissance sur saccharose ou acides organiques.* **B** *: Croissance sur saccharose et acides organiques.*

Engel and Teuber (1978) have observed that only *P roqueforti* grew correctly on unbuffered Czapek liquid medium with sucrose in the presence of acetic or lactic acid. On all liquid media *P cyclopium* strains grew well at pH 6. As was previously shown on solid media, good growth at pH 6 was observed in the presence of glucose and acetic acid, or acetic acid alone. But growth was inhibited by these organic acids for pH under 5.

In fact, the results of Veau and Engel could be explained if the tested strains presented a barrier towards acetic acid or lactic acid. In this case, the organic acids cannot be used as carbon substrate at pH 7. If this barrier exists, there will not be a toxic action for low pH caused by an accumulation process. The 2 strains we used did not present this characteristic. The observations we made for *P cyclopium* are quite comparable to those described by the above-mentioned authors.

Oleic acid

The 4 strains were cultured in liquid and solid Czapek media. Oleic acid replaced sucrose as carbon source. These different media were buffered at pH 5–7.

The 4 strains grew well on all these media. However, growth of *P roqueforti* was more significant than that of *P cyclopium*.

This ability of *P cyclopium* to metabolise fatty acids could play a part in the efficiency of the "morge". Indeed, fatty acids undoubtedly favour sticking of tin foil during the "plombage" phase and fatty acid metabolisation by *P cyclopium* may explain decreased sticking of tin foil.

CONCLUSIONS

The results suggest that acetic and lactic acid penetrate into the mycelium in the 4

strains under their RCOOH form, as previously described by Conway and Downey (1949) for other moulds.

Consequently, these microorganisms are able to grow on these organic acids when exocellular pH is close to neutrality. The second consequence is the accumulation of these organic acids when media pH is lower than internal pH; this induces an inhibition of mycelial growth. These observations are similar to those concerning *P cyclopium* made by other authors. The behaviour of our 2 *P roqueforti* strains did not seem to be markedly different in our study.

Since the amount of salt added to the cheese during manufacture has decreased over recent years, sometimes there was an undesirable growth of mycelium ("boure") located only on the surface of some loaves. It appears that during the first ripening phase *P cyclopium* grows better than *P roqueforti*. This growth may be related to a better resistance to NaCl, better growth at 9–12 °C and to a better metabolisation of the residual lactose for *P cyclopium* strains.

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