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Impact of solid medium composition on the conidiation in *Penicillium camemberti*

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Abstract

Conidiation of an industrial strain of *Penicillium* (*P.*) *camemberti*, a ripening fungus, was examined on solid media. In order to evaluate the influence of nutritional factors on conidiation, we developed an inoculation and transfer procedure that allowed to obtain an homogenous mycelial biomass. Absence of conidiation was observed when ammonium sulphate and sodium nitrate were used as nitrogen sources. In contrast, conidiation increased significantly in the presence of ammonium phosphate or potassium nitrate with 4.8×10^6 and 12×10^6 spores ml^{-1}, respectively. By using those optimal conditions, the influence of nutrient starvation or calcium supply on conidiation was studied. Under the conditions tested, nitrogen starvation and calcium supply were better inducers than carbon starvation. A high carbon-to-nitrogen (C/N) ratio provided the highest level of with 4.2×10^7 spores ml^{-1} and a sporulation index of 8.2 after 16 days of cultivation.

Keywords: *Penicillium camemberti*; Conidiation; Solid media; Calcium; Nitrate; C/N ratio

1. Introduction

Asexual sporulation is a widespread reproductive mode for filamentous fungi. It consists of a massive production of spores, called conidia in the case of *Ascomycetes*. Under favourable environmental conditions, each conidium is able to produce a young mycelium. This property contributed to the use of *Penicillium camemberti* conidia as cheese starter culture. Directly introduced in milk or seeding by surface pulverization, this white mold is used to ripen and flavor a variety of French soft cheeses.

Suspensions of *P. camemberti* conidia that are used in the dairy industry are either marketed as concentrated, stabilized suspensions or as lyophilized preparations. Traditionally, *P. camemberti* conidia are produced by surface cultivation on agar media in Roux-flasks and harvested after 3 weeks [1]. However, this mode of production appears outdated, and consequently must be improved.

Surface cultures present some drawbacks for the study of the conidiation. The medium is usually centrally inoculated, and the fungus grows as a circular heterogeneous colony, composed of zones differing in morphology and metabolic activities. Conidiation occurs mainly in the aged zone located at the centre of the culture. Besides growth of the vegetative mycelium, conidiophore development and conidiation often take place simultaneously in surface cultures adding difficulties to the distinction of changes associated with conidiation from those due to degeneration and aging of the vegetative hyphae [2]. To avoid these problems, different techniques have been developed to induce microcycle conidiation: temperature-shift experiments for *P. digitatum* [3] and *P. cyclopium* [4], and glutamate as sole nitrogen source for *P. urticae* [5]. A synchronised sporulation was developed in *P. digitatum* by using a vitamin mixture lacking *p*-amino-benzoic acid [6]. However, all these techniques have been developed in submerged cultures.

Besides, conidiation of *Penicillium* species has been of interest for long [7–9], mainly in submerged culture that allow

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a better homogeneity of the biomass and an easier upscaling and automation [10]. Such studies have shown that conidiation can be principally improved by: (i) calcium addition [8], (ii) type of nitrogen source [11], (iii) glucose starvation [2], (iv) levels of intermediates and derivatives of the Krebs cycle [12]. On the other hand, studies on the qualities of spores have been shown that aerial and submerged spores of *P. oxalicum* differ in hydrophobicity, viability and efficacy as biocontrol agent [13]. Moreover, differences in hydrophobicity have been shown to affect the viability of *Trichoderma harzianum*. Aerially produced spores were highly hydrophobic and showed longer viability after storage than submerged spores [14]. Furthermore, most filamentous fungi remain entirely vegetative in submerged culture, which is consistent with the findings that differentiated structures are characteristic of aerial mycelium [11].

The aim of the present work was to screen and optimize composition of the growth media that would allow a better conidiation of *P. camemberti* on solid media.

2. Materials and methods

2.1. Microorganism

P. camemberti strain D1 was provided by Degussa Ferments d'Aromatisation, France. Stock culture of *P. camemberti* was stored on potato dextrose agar (PDA) slants at −20 °C.

2.2. Growth medium compositions

The reference medium, based on Bockelmann's medium [10], was separated into two parts, a glucose solution and a nitrogen solution, for its preparation in order to avoid Maillard reactions. It was prepared as follows: a glucose solution containing 10 g of D-glucose and 15 g of Agar–Agar were dissolved in 500 ml distilled water. A nitrogen solution containing 3.29 g $(NH_4)_2HPO_4$, 4.4 g Na_3-citrate, 20 ml trace elements solution and distilled water up to 500 ml were mixed. The pH of the two solutions was adjusted to 5.6 with 20% H_2SO_4 before autoclaving (121 °C, 30 min). After cooling, 500 ml of glucose solution were mixed to 500 ml of nitrogen solution and plated on 100 mm diameter Petri dishes, on the basis of 25 ml per dish. The trace element stock solution contained (per liter): 500 mg $CuSO_4{\cdot}5H_2O$, 340 mg $MnSO_4{\cdot}1H_2O$, 200 mg $ZnSO_4{\cdot}7H_2O$, 15 g $MgSO_4{\cdot}7H_2O$, 13 g KH_2PO_4, 5 g KCl and 5 g $FeSO_4{\cdot}7H_2O$. The growth medium compositions with other nitrogen sources are shown in Table 1.

All experiments were carried out in triplicate.

2.3. Inoculation procedure

A Roux-flask, containing 150 ml of PDA medium, was inoculated with 2×10^5 spores ml^{-1}, from a stored slant. After 10 days of cultivation at 23 °C, spores were harvested aseptically with 50 ml of 0.9% NaCl. The spore suspension was washed twice with 0.9% NaCl, centrifuged at 8000 × *g* for 15 min and the pellet was suspended in 40 ml of distilled water. This suspension was diluted to obtain 3–4 × 10^7 spores ml^{-1}. Sterile pre-weighed glass microfibre filters GF/C (Whatman), having a diameter of 90 mm, were soaked successively for 15 s in the spore suspension. The volume of inoculum, deposited on each filters, was 2.09 ± 0.09 ml, corresponding to an average inoculation rate of 3×10^6 spores ml^{-1} of medium. Then the filters were immediately put on agar medium and the Petri dishes were incubated in darkness at 23 °C for up to 10 days.

2.4. Spores quantification

Spores on surface culture were harvested with 0.9% NaCl with a sterile scraper and counted with a haemocytometer.

Fungal biomass was determined by drying the whole invaded filters at 70 °C during 72 h.

Sporulation index was defined as the number of spores (expressed in millions) divided by the dry weight (expressed in mg).

2.5. Analytical methods

For pH, nitrogen and glucose determinations, the whole agar medium was equilibrated for 1 h by shaking in 100 ml of distilled water. The solution was then filtered through a 0.45 μm nylon filter and kept at −20 °C until analysis. Glucose concentration was measured by HPLC (Aminex HPX-87H column, Merck) [15].

The concentration of ammonium ions in the culture medium was determined by the Nessler reagent [16]. The culture medium was diluted 400-fold and 1/20 of Nessler reagent was added. The A_{395} was immediately measured, and the ammonium concentration was determined from a calibration curve of 25–200 μM ammonium sulphate.

The concentration of nitrate ions in the culture medium was determined by Bioquant® Nitrates KS compact (Calbiochem, Merck) according to the supplier's instruction.

3. Results

3.1. Production of homogeneous mycelia

In order to study the effect of different parameters on the conidiation, a cultivation technique was developed on solid medium in order to obtain a homogeneous mycelium. For this purpose, a glass microfibre filter was soaked in a calibrated spores suspension of *P. camemberti*. Contrary to cellophane discs usually used for transfert experiments, glass microfibre material allows a uniform repartition of the inoculum on the surface of the medium. Moreover, the cellulases constitutively secreted by *Penicillium* [17,18] could degrade the cellulose present in the cellophane filters and consequently interfere with the measurement of glucose consumption during the growth of the fungus.

Once inoculation and transfer conditions were defined, we studied the effects of parameters frequently quoted in the

Table 1
Growth media with different nitrogen sources

Medium	Glucose (g l^{-1})	C (g l^{-1})	Nitrogen source C (g l^{-1})	N (g l^{-1})	Na_3-citrate (g l^{-1})
$(NH4)_2SO_4$	10	4	3.77	0.8	4.4
$(NH4)_2HPO_4$	10	4	3.29	0.8	4.4
KNO_3	10	4	5.77	0.8	4.4
$NaNO_3$	10	4	4.85	0.8	4.4

In addition to nutrients cited in the table, all media contain 20 ml l^{-1} of trace element solution and 15 g l^{-1} of agar–agar.