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Commensalism during submerged mixed culture of *Geotrichum candidum* and *Penicillium camembertii* on glutamate and lactate

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Abstract

Geotrichum candidum and *Penicillium camembertii* were cultivated in pure and mixed cultures on glutamate- and lactate-based medium. In pure culture, *P. camembertii* assimilated simultaneously glutamate, as a nitrogen and carbon source for biosynthesis, and lactate as an energy source. On the contrary, *G. candidum* grew on glutamate alone. The mixed culture led to higher growth rates and then higher rates of substrate consumption and metabolite production than each pure culture; however, the behaviour recorded was similar to that observed during *G. candidum* pure culture, in particular the absence of lactate assimilation during growth, illustrating a commensalism between both species. The presence of *G. candidum* induced a form of ''competition'' and then a better assimilation by *P. camembertii* of the sole nitrogen source, glutamate, which was therefore used as an energy source in addition to be a carbon (and nitrogen) source. Lactate was only used for energy supply during stationary state, as also recorded during *G. candidum* pure culture.

Keywords: *Geotrichum candidum*; *Penicillium camembertii*; Interactions; Mixed cultures; Substrates consumption

1. Introduction

Camembert is a cheese variety characterised by the development of the white fungi *Penicillium camembertii* on cheese surface during ripening [1,2]. *Geotrichum candidum* is a natural part of the surface flora of several varieties of cheese including Camembert [3–5]. In fact, *G. candidum* and *P. camembertii* are the principal fungal species involved in the biochemical processes occurring in the curd during ripening [6,7]. Development of the flavour and texture characteristics of the cheese results from the microbiological and biochemical changes that occur during ripening [8].

Solid-state [9] and submerged [10] pure cultures of *G. candidum* and *P. camembertii* showed some aspects of nutritional and metabolic behaviour. However, during cheese ripening, both fungi are present in association, showing synergistic interactions that usually occur [11,12]. *G. candidum* develops during the first days of ripening, despite an inhibitory salt effect, while *P. camembertii* growth starts later and is not hindered by the presence of *G. candidum* [13]. Low molecular weight hydrophobic peptides resulting from the degradation of β-casein by *P. camembertii* through its endoprotease activity are hydrolysed by the action of *G. candidum* aminopeptidases, which contributes to the reduction of bitterness [13–16]. In mixed fungal cultures, these interactions lead to higher enzyme production and therefore a better substrate utilisation and an increased productivity [17,18].

The investigation of these synergistic interactions on a complex medium resulting from cheese like Camembert juice may allow a deeper understanding of the nutritional mechanisms occurring during cheese ripening [19]. The juice extracted from curd at the demoulding contains the whole substrates available for growth of surface flora in situ; it is a complex medium resulting from cheese like water soluble fraction, namely close to the real medium (lactic curd). The higher viable populations of *G. candidum* and *P. camembertii* during the mixed culture compared to each pure culture, and the absence of lactate and lactose assimilation during the mixed culture clearly highlights a synergistic effect of *G. candidum* on *P. camembertii* growth. Owing to the complexity of the cheese juice, to improve the knowledge concerning the interactions between *G. candidum* and *P. camembertii*, mixed

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cultures on simple synthetic media may be helpful. In this purpose, mixed and pure batch cultures of both fungi were run on a simple synthetic medium composed of lactate and glutamate. Indeed, lactate is an important carbon substrate for the secondary cheese ripening micro flora, and some amino acids, including glutamate, resulting from proteolysis were shown to be convenient carbon and nitrogen sources for *G. candidum* and *P. camembertii* [20].

2. Materials and methods

2.1. Microorganisms

The commercial strains *P. camembertii* LV2 and *G. candidum* Geo 17 (both from Danisco, Dangé St Romain, France) were used. Freeze-dried spores were stored at +7 °C.

2.2. Media

Two media were used throughout this work; the G medium contained:

- Glutamic acid: 14 g l^{-1} (Acros Organics, NJ, USA).
- Inorganics phosphates (Pi): 25 mM of KH_{s4} and 25 mM of $NaH_2PO_4{\cdot}H_2O$ [21].
- A solution of EDTA (ethylenediaminetetraacetate) (585 mg l^{-1}) chelated trace elements (mg l^{-1}) [21]: Mg, 25; Fe, 20; Ca, 18; Zn, 4.5; Mo, 2; Cu, 1.3.

Sodium L(+)-lactate (Prolabo, Paris, France), 10 g l^{-1}, was also added in the second medium, the *GL* medium.

pH of media were adjusted to 4.6 with 10 M NaOH, before they were sterilised at 121 °C for 20 min.

2.3. Culture conditions

Batch cultures were carried out in a 3 l laboratory-made glass-blown fermentor. The fermentor was filled with 2 l of the culture medium (the fermentor with the equipment and the medium were sterilised at 121 °C during 20 min).

During culture, the temperature was maintained at 25 °C by circulation of thermostated water in a jacket. The batch fermentor was continuously aerated with a constant airflow of 13 l h^{-1} (6.5 l of air/l of medium/h). This rate was obtained with the help of a mass flow controller GFC17 (Aalborg, New York, USA) and the culture medium was magnetically stirred at 850-rev min^{-1}.

Spores were added to 10 ml of sterilized medium. The product of the turbidity at 650 nm and the inoculum volume ($A_{650} \times V$) was kept constant at a value of 100 for all inocula; the number of spores was adjusted to achieve the considered value for the product $A_{650} \times V$. In case of the mixed culture, inocula of *G. candidum* and *P. camembertii* were separately prepared at half the required value for the product $A_{650} \times V$, i.e. 50 for each inoculum species, and the total volume of inoculum (constituted from both inocula) was kept constant at 10 ml. In order to obtain reproducible lag times, before inoculation, spores were left for approximately 1 h in sterile medium at room temperature for hydration.

The fermentor was equipped with a sterilizable combination pH glass electrode (Ingold, Paris, France). The system also contained an aseptic recirculation loop involving a laboratory-made turbidimeter allowing on-line measurement of turbidity at λ = 650 nm [22]. Turbidity was calibrated from the dry weight measurement of biomass at the end of culture. Carbon dioxide in the off-gas was also monitored on-line by an IR detector Rubis 3000 (Cosma, Igny, France), after desiccation in a column of calcium chloride. In order to detect any gas leak in the system, the flow rate at the outlet of the IR detector was measured by a Pelton-wheel flow meter S110-3 (Mc Millan Co., Georgetown, TX, USA).

2.4. Analyses

Lactic acid was determined enzymatically: it was first oxidized in pyruvate in presence of lactate oxidase (LOD), and the produced hydrogen peroxide was measured after reaction with ABTS (AZINO-bis[3-ethylbenzthiazoline-6-sulfonic acid]) in the presence of peroxidase (POD) (all from Sigma Diagnostics, St Quentin Fallavier, France).

Ammonium concentration was determined spectrophotometrically by the Nessler method [23] and the glutamic acid concentration corresponding to its primary α-amino group was measured by the TNBS method [24].

2.5. Viable cells enumeration

G. candidum viable cells were enumerated in duplicates after culture on Chloramphénicol Glucose Agar (40.1 g l^{-1}) in Petri dishes at 30 °C during 72 h; and 70 g l^{-1} sodium chloride was added to the medium for the counting of *P. camembertii* viable cells at 20 °C during a lapse of time in the range 120–144 h. For each sample, the two most representative dilutions were considered. The colony was considered to be composed of a number of 'units' (the hyphal growth units), each of which represents a hyphal tip plus an average length of hypha associated with it [25].

2.6. Calculus

For rate calculations, to obtain reliable derivatives from the raw data, the corresponding time-courses were fitted using the following logistic function before time-differentiation:

$$y = \frac{y_0 - y_f}{1 + (t/k_1)^{k_2}} + y_f \quad (1)$$

with y the consumption or production of the considered parameter, y_0 and y_f its initial and final values respectively, and k_1 and k_2 were coefficients.

3. Results

The lag phase recorded during the mixed culture of *G. candidum* and *P. camembertii* was similar to that recorded during pure culture of *G. candidum* (15–20 h; Fig. 1a) and shorter than that recorded during pure culture of *P. camembertii* (35 h; Fig. 1a). During the mixed culture and *G. candidum* pure culture, the deceleration growth phase following the linear growth was recorded until cessation of growth after approximately 150 and 175 h, respectively (Fig. 1a). Higher biomass concentrations were recorded during the mixed culture from about 60 h of culture, until final biomass concentrations of 4.4 and 5.6 g l^{-1} for *G. candidum* pure culture and the mixed culture, respectively. A lower growth was recorded during *P. camembertii* pure culture; no stationary state was recorded, growth rates decelerated until the end of culture after 288 h (x_f = 2.8 g l^{-1}; Fig. 1a).

Nearly similar pH time-courses were observed during *G. candidum* pure culture and the mixed culture and the final pH's were 8.38 and 8.30, respectively; while lower rates of alkalinization were recorded during *P. camembertii* pure culture until a final pH of 7.27 (Fig. 1b). In all cases, two phases of alkalinization can be observed: the first phase ceased approximately at the end of growth, followed by a second phase of alkalinization at lower rates during stationary state or during the phase of low growth as was the case during *P. camembertii* culture (Fig. 1b).

A production of CO_2 was recorded after only 10–15 h of culture for all experiments and the maximum CO_2 values were recorded after similar times during *G. candidum* and the mixed cultures, 8.5 and 10.3 mg l^{-1} after 27 and 34 h respectively (Fig. 1c); while, during *P. camembertii* culture, the maximum for CO_2 emission was recorded later, 9.4 mg l^{-1} after 53 h of culture