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Effect of linoleic acid induction on the production of 1-octen-3-ol by the lipoxygenase and hydroperoxide lyase activities of *Penicillium camemberti*

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Abstract

The effect of induction by linoleic acid on the biomass production, glucose consumption, change in pH values and 1-octen-3-ol profile were investigated during the growth of *Penicillium camemberti*. The results showed that the presence of 1 g of linoleic acid per liter of culture medium increased the biomass from 3.0 to 7.0 g/l. In addition, incubation of crude enzymatic extracts, obtained from induced and non-induced *P. camemberti* cultures, indicated that the amount of 1-octen-3-ol produced by the induced enzymatic extract was approximately twice compared to that obtained by the non-induced one. The presence of 0.5 g linoleic acid in the culture medium resulted in the generation of 1.8 μg 1-octen-3-ol per mg protein as opposed to 0.8 μg 1-octen-3-ol per mg protein obtained using 1.0 mg linoleic acid. Moreover, the bioconversion of the 10-hydroperoxide of linoleic acid into 1-octen-3-ol by the enzymatic extract obtained from the induced microbial biomass was twice as high as that obtained with the non-induced one, corresponding to 9.0 and 4.6 μg 1-octen-3-ol per mg protein, respectively.

Keywords: *P. camemberti*; Lipoxygenase; Hydroperoxide lyase; 1-Octen-3-ol

1. Introduction

Penicillium camemberti is a fungi, widely used in starter cultures in the food industry. Due to the presence of lipolytic and proteolytic activities, volatile compounds are produced that contribute to the formation of the aroma and flavors of Brie and Camembert cheeses [1]. Among the C_8 volatiles compounds, octan-3-ol, 5-octadien-3-ol and particularly 1-octen-3-ol contribute to the typical mushroom-like flavor that is found in modestly aged cheeses [2]. However, if the concentration of 1-octen-3-ol is too high, it contributes to an important off-flavor [1].

1-Octen-3-ol is generated from linoleic acid via the lipoxygenase (LOX) pathway found in fungi [3] and basidiomycetes [4–8]. LOX is a dioxygenase that catalyzes the oxygenation of polyunsaturated fatty acids (PUFAs) containing a *cis–cis*-1,4-pentadiene moiety, such as linoleic acid and linolenic acid, into hydroperoxides (HPODs), in particular the 10-HPOD that is subsequently cleaved by hydroperoxide lyase (HPL) to produce 1-octen-3-ol and 10-oxo-8-decenoic acid [6–8].

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LOX activity has been reported and characterized in several fungi, including *Fusarium* [9], *Geotrichum candidum* [10] and *Saccharomyces* sp. [11]. Several fungal LOXs have been known to produce the 10-HPOD as well as the 9- and 13-HPODs [12]. The induction and sub-cellular localization of LOX activity in *Fusarium proliferatum* has also been studied [13].

However, little information is available on fungal HPL and only one HPL from the algae *Chlorella pyrenoidosa* has been purified [14]. Recently, an anaerobic lipoxygenase activity has been shown to be responsible for the cleavage of 13-HPOD and 13-HPOT [15]. In the past, submerged cultures of edible fungi such as *Boletus*, *Agaricus*, *Morchella* or *Pleurotus* as well as other species has been used, as a source of flavor, due to their speed and reproducibility compared to that obtained with the use of solid-state fermentation for the growth of fruiting bodies [16,17]. Belinky et al. [17] showed that during the growth of *Pleurotus pulmonarius*, the presence of polyunsaturated fatty acids (PUFAs) in the submerged culture increased the production of 1-octen-3-ol by seven-fold; these results suggest that the LOX pathway was induced by the presence of PUFAs in the growth medium.

The aim of this work was to investigate the effect of linoleic acid on both LOX and HPL activities of *P. camemberti* in a liquid culture medium.

2. Experimental

2.1. Strain, media and culture conditions

Spores of *P. camemberti* were obtained from Rhodia (Dangé Saint Romain, France) and conserved at −18 °C. The 500-ml flasks containing 200 ml of culture medium were directly inoculated with 1 ml of spore suspension (10^7 spores/ml).

Two types of culture media were used. The glucose medium was composed of 10 g of glucose, 3 g of $NaNO_3$, 1 g of KH_2PO_4, 0.5 g of KCl and 10 mg of $MgSO_4$, prepared in 1 l of sodium phosphate buffer solution (0.1 M, pH 6.5). The glucose–linoleic acid medium was the same composition as the glucose medium, supplemented with 0.5–1.0 g of 65% linoleic acid (ICN Biomedicals Inc., Costa Mesa, CA) and 0.1% (v/v) of Tween-20. After inoculation, the flasks were incubated (120 rpm, 25 °C) for 10 days in an orbital shaker (New-Brunswick, Model G-25, Edison, NJ).

2.2. Preparation of mushroom homogenate

Freshly harvested pellets were filtered, weighted and homogenized using a mortar under liquid nitrogen (−70 °C) for 5 min. The homogenate was suspended (1:2 (w/v)) in sodium phosphate buffer solution (0.1 M, pH 6.5). For determination of dry biomass, freshly harvested pellets were also placed in an oven until constant weight.

2.3. Enzymatic assays

LOX–HPL activity was determined by measuring the production of 1-octen-3-ol with gas–liquid chromatography (GLC). For the LOX assay, linoleic acid substrate solution (10 mM) was prepared in sodium phosphate buffer solution (0.1 M, pH 6.5) containing 0.5% Tween-20; the substrate solution was homogenized (12,200 rpm, 15 s) using a homogenizer (Ultraturrax, Janke and Kunkel, Staufen i. Br., Germany). For the HPL assay, a substrate solution of the 10-HPOD (3.6 mM) was prepared in the phosphate buffer solution containing 0.12% Tween-80. The reaction mixture consisted of 2 ml of the appropriate substrate oxygenated for 30 s by a flow of pure O_2, followed by the addition of 2 ml of the mycelial homogenate. The enzymatic reaction was conducted for 30 min and stopped by acidification to pH 3.0 with 2N HCl. Enzymatic activity was expressed as μg of 1-octen-3-ol/min per mg protein.

2.4. Extraction and determination of 1-octen-3-ol

A volume of 2 ml of the reaction medium, containing 1-octen-3-ol, were homogenized with 100 μg of 2-decanone and 1 g of NaCl and then extracted with 2 ml diethyl ether. The medium was subsequently centrifuged (15,000 × *g*, 2 min) and the upper organic phase was recovered and dried with Na_2SO_4 for analysis by GLC. A Hewlett-Packard gas chromatograph (Model HP 6890) equipped with a flame ionization detector (FID) and an HP-Innowax capillary column (30 m, 320 μm i.d.) was used. The column temperature was from 40 to 150 °C and from 150 to 235 °C at

a rate of 5 and 15 °C per min, respectively. The injector and detector temperatures were 280 and 300 °C, respectively.

3. Results

3.1. Effect of growth medium on biomass production

The biomass of *P. camemberti* as well as changes in pH and glucose consumption in the glucose and glucose–linoleic acid media were measured daily during the 10-day period of fermentation at 25 °C (Fig. 1).

In the glucose medium, the biomass growth increased to 3 g/l of culture medium after 3 days of growth. A concomitant decrease in glucose concentration was also observed during this period. However, after 3 days of growth, a constant decrease in biomass occurred up to 0.5 g/l at 10 days of culture. In the glucose–linoleic acid medium, the biomass growth reached its maximum of 6.5 g/l of culture medium after 3 days, with a concomitant total consumption of glucose. Following the third day of culture, the biomass remained at its maximum up to the fifth day, after which it decreased to 3.5 g/l at the tenth day of fermentation.

The results also show that there was no change in the pH value of 6.5 of the medium during microbial growth in both the glucose and glucose–linoleic acid media.

3.2. Effect of growth medium on the production of 1-octen-3-ol

The production of 1-octen-3-ol was measured during the 10 days of growth of *P. camemberti* on both the glucose medium and the glucose–linoleic acid medium containing 1 g of linoleic acid per liter of culture medium. The mycelial pellets were homogenized and 1-octen-3-ol was extracted. Fig. 2 shows that the maximum level of 6 μg of 1-octen-3-ol per mg protein was reached after 4 days of mycelial growth on the glucose medium. However, for the glucose–linoleic acid medium, the maximum level of 11.5 μg of 1-octen-3-ol per mg protein was reached after 5 days of mycelial growth.

3.3. Post-harvest generation of 1-octen-3-ol

The production of 1-octen-3-ol by *P. camemberti* was investigated after 5 days of growth on the glucose and glucose–linoleic acid media. A wide concentration range (0–5 mM) of linoleic acid was used to determine the optimal linoleic acid concentration for the bioconversion assay. The results (not shown) indicated that 1 mM of linoleic acid provided the highest

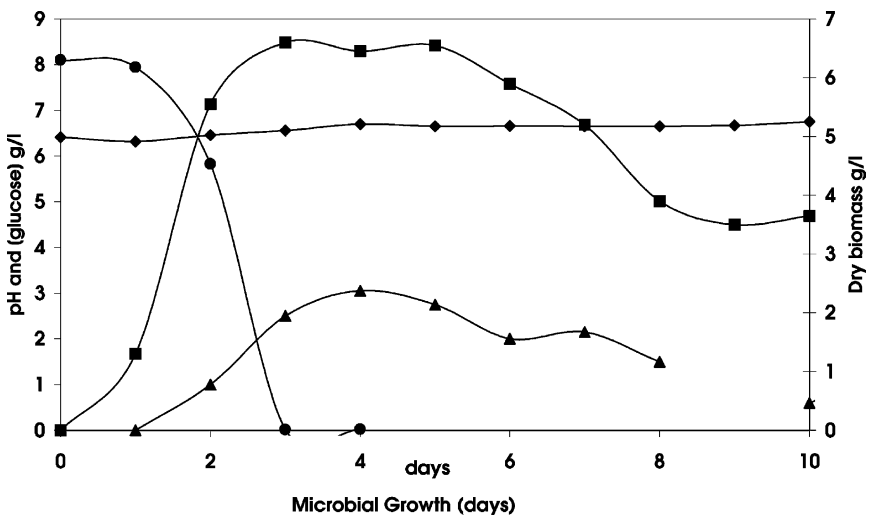

Fig. 1. Changes in dry biomass of *P. camemberti* grown on glucose culture medium (▲) and glucose–linoleic acid culture medium (■); changes in pH (◆) and glucose consumption (●) on both media.

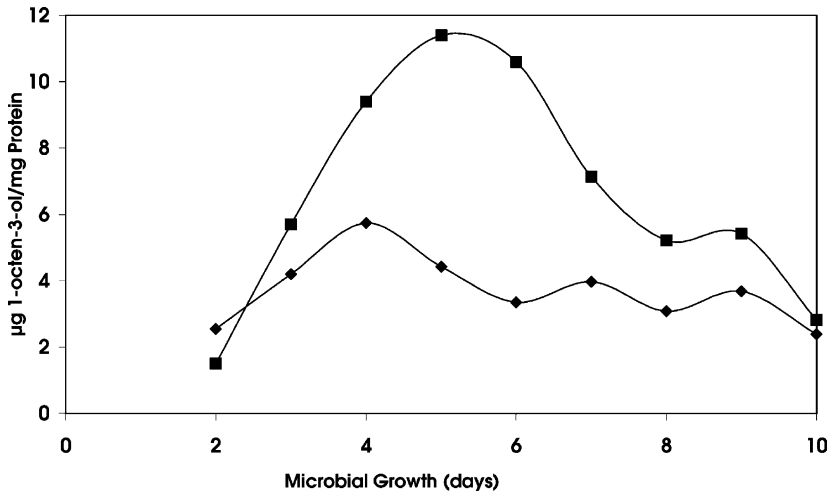

Fig. 2. Time-course formation of 1-octen-3-ol formation by *P. camemberti* in glucose culture medium (◆) and glucose–linoleic acid culture medium containing 1 g linoleic acid (■).

LOX–HPL activity and hence the highest production of 1-octen-3-ol.

In order to determine the production of endogenous 1-octen-3-ol, enzymatic extracts were obtained from mycelia previously grown on glucose and glucose–linoleic acid media containing 0.5 and 1.0 g linoleic acid per liter of medium used (Fig. 3). An endogenous amount of 2.9 μg of 1-octen-3-ol per mg protein was extracted from the mycelia grown on the glucose medium, whereas, 4 and 5 μg of 1-octen-3-ol per mg protein were obtained from the mycelia grown on the glucose–linoleic acid media containing 0.5 and 1 g linoleic acid per liter, respectively. These results indicate that a higher concentration of linoleic acid in the culture medium corresponds to a higher concentration of endogenous 1-octen-3-ol. The experimental findings suggest that part of the linoleic acid present in the culture medium was metabolized by *P. camemberti* into 1-octen-3-ol. The maximum LOX–HPL activity was found with the glucose medium containing

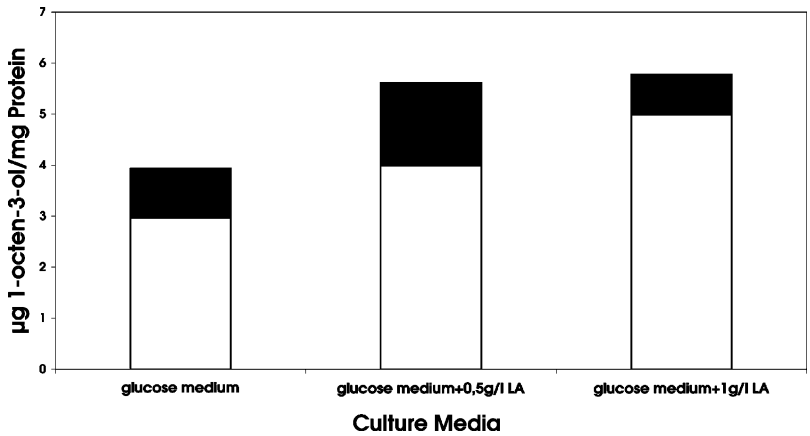

Fig. 3. Production of 1-octen-3-ol by *P. camemberti* grown on glucose culture medium, glucose–linoleic acid culture medium containing 0.5 and 1 g linoleic acid and post-harvest generation of 1-octen-3-ol by biomass grown on these media. Endogenous 1-octen-3-ol (□), and biogeneration of 1-octen-3-ol (■).

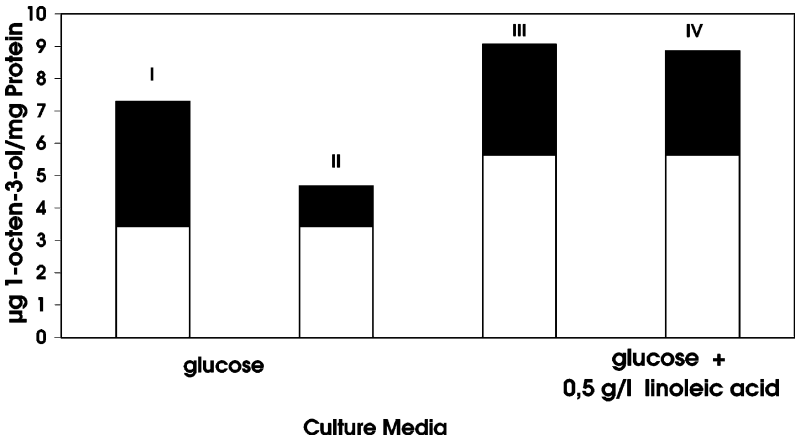

Fig. 4. 1-Octen-3-ol generation by *P. camemberti* grown on glucose culture medium and glucose–linoleic acid culture medium containing 0.5 g/l linoleic acid, using as substrate for the assay 1 mM of linoleic acid (I and III) and 10-HPOD (II and IV). Endogenous 1-octen-3-ol (□) and bioconversion generated 1-octen-3-ol (■).

0.5 g linoleic acid per 1 l of culture medium, producing a total of 5.8 μg 1-octen-3-ol per mg protein.

3.4. Effect of growth medium on hydroperoxide lyase activity

The HPL activity was investigated using enzymatic extracts obtained from the mycelial pellets after 5 days of growth on the glucose and glucose–linoleic acid media containing 0.5 g linoleic acid per 1l. The bioconversion conditions for the HPL assay were the same as those described previously using the linoleic acid substrate, with the exception that the used substrate was 10-hydroperoxy-9(*E*), 12(*Z*)-octadecadienoic acid (10-HPOD). Fig. 4 shows that the bioconversion of 10-HPOD into 1-octen-3-ol by the enzymatic extracts obtained from the mycelia grown on the glucose–linoleic acid medium was higher (9.0 μg 1-octen-3-ol per mg protein) than that with the enzymatic extracts obtained from mycelia grown on the glucose medium (4.6 μg 1-octen-3-ol per mg protein). The bioconversion yields were 11 and 5.8% for these two media, respectively. The HPL activity of *P. camemberti* was therefore demonstrated in the glucose medium as well as further induced using the glucose–linoleic acid medium.

In the presence of 1 mM of linoleic acid as substrate, 7.4 μg 1-octen-3-ol per mg protein were produced by the enzymatic extract obtained from the mycelia grown on the glucose medium, whereas, 9.1 μg 1-octen-3-ol per mg protein were produced from the mycelia obtained from the glucose–linoleic acid medium.

The difference in 1-octen-3-ol generation between the mycelial biomass grown on the glucose–linoleic acid medium compared to that obtained on the glucose medium was more pronounced when 10-HPOD was used as substrate instead of linoleic acid.

4. Discussion

A combination of glucose, mineral salts and linoleic acid was investigated in this work as precursors for mycelial biomass growth and 1-octen-3-ol generation by *P. camemberti* in a submerged liquid culture. A large increase in biomass production and 1-octen-3-ol generation by mycelia grown on the glucose medium supplemented with linoleic acid suggests that glucose and linoleic acid can serve as adequate precursors to increase mycelial biomass that is in turn enriched in flavor.

Moreover, the relationship between the increase in *P. camemberti* biomass and endogenous 1-octen-3-ol formation suggests that alcohol production is the result of secondary metabolism. Indeed, the maximum biomass was obtained after 3 days of culture at 25 °C after all the glucose was consumed

in both medium and the biomass reached 7.0 g/l for the glucose–linoleic acid medium as opposed to 3.0 g/l for the glucose medium. The maximum concentration of 1-octen-3-ol was obtained at day 5 corresponding to the beginning of the mycelial lysis in both media. The concentration of 1-octen-3-ol was twice as high (11.0 μg 1-octen-3-ol per mg protein) in the glucose–linoleic acid medium compared to the glucose medium. These results are in agreement with those reported by Spinnler et al. [3] for *P. caseicolum*. Perraud and Kermasha [18] also demonstrated that maximum LOX activity was obtained after maximal growth of *P. camemberti* was reached.

The optimal linoleic acid concentration for the production of 1-octen-3-ol was shown to be 1 mM. Higher linoleic acid concentrations did not produce more 1-octen-3-ol and no inhibition phenomenon was detected. Assaf et al. [4] have demonstrated that in the presence of more than 1 mM linoleic acid, another metabolic pathway is activated where 13-HPOD accumulation takes place in parallel with 1-octen-3-ol and 10-oxo-acid biosynthesis. The same authors proposed that there are two distinct biosynthetic pathways, one involving the 10-HPOD to produce the 1-octen-3-ol at linoleic acid concentrations under 1 mM and another one producing the 13-HPOD at higher linoleic acid concentrations. Assaf et al. [4] have also suggested that the 13-HPOD itself may inhibit 1-octen-3-ol synthesis. In the present study, 1 mM was the concentration that gave the highest 1-octen-3-ol generation. Moreover, volatile analysis by GC/MS showed hexanal production in parallel to 1-octen-3-ol generation, thereby leading to the hypothesis that 13-HPOD was also synthesized by the LOX of *P. camemberti*.

Enhancement of aroma production by mushroom mycelia has already been reported regarding the addition of specific precursors to the growth medium. Belinky et al. [17] demonstrated that culture medium supplemented by soybean oil rich in linoleic acid resulted by 10-fold increase in 1-octen-3-ol generation by *P. pulmonarius* due to the induction of LOX activity. The induction of LOX activity of *F. proliferatum* [13], grown on soybean-supplemented medium, was also observed by a factor of 13 compared to that obtained from mycelia grown on glucose medium.

In the present study, increasing concentrations of linoleic acid in the culture medium (0, 0.5 and 1 g/l) corresponded to increases in the production of 1-octen-3-ol (3, 4 and 5 μg per mg protein, respectively) since linoleic acid was metabolized during growth. However, post-harvest generation of 1-octen-3-ol in vitro was higher for the biomass grown on culture medium containing 0.5 g linoleic acid per liter in comparison to that grown on culture medium containing 1 g linoleic acid per liter (1.8 and 0.8 μg per mg protein, respectively). The LOX–HPL metabolic pathway should therefore be induced on media containing 0.5 g linoleic acid per liter.

Moreover, the bioconversion of 10-HPOD into 1-octen-3-ol produced 1.3 μg 1-octen-3-ol per mg protein for the biomass grown on the glucose medium and 3.4 μg 1-octen-3-ol per mg protein for the biomass grown on the glucose–linoleic acid medium. The HPL activity was therefore demonstrated for the first time in *P. camemberti* and enhanced in the presence of linoleic acid in the medium.

In conclusion, the presence of linoleic acid in the culture medium increased both the biomass production of *P. camemberti* and 1-octen-3-ol generation, and in particular the hydroperoxide lyase activity.

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