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PRODUCTION OF LACTONES BY *PENICILLIUM ROQUEFORTI*

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SUMMARY

Aroma compounds production by *Penicillium roqueforti* spores was studied using hydrolyzed oils as precursors. Lactones possessing a peach odor : 4-dodecanolide, cis-6-dodecen-4-olide, and 4-hexanolide were detected in the bioconversion medium and identified by coupled GC/MS and retention data. Variations were found according to the nature of precursors released from the oils by lipases, the bioconversion time and the nature of the strain.

INTRODUCTION

It is well known that free fatty acids and odd-carbon chain methyl ketones : 2-heptanone, 2-nonanone and 2-undecanone are major contributors to the characteristic flavour of blue cheeses (Anderson and Day 1966). It is generally admitted that flavor development during cheese ripening is dependent upon milk triacylglycerols hydrolysis by *Penicillium roqueforti* lipases and on subsequent fatty acids oxidation to methyl ketones (Kinsella and Hwang 1974). However, a large number of volatile compounds, including esters, primary and secondary alcohols, aldehydes, anisoles, sulfur compounds and lactones have been identified in blue cheeses (Gallois and Langlois 1990).

Fatty acids may be the precursors of many aroma substances. It was shown that if very weak quantities of methyl ketones are produced by *Penicillium roqueforti* when long chain saturated or unsaturated fatty acids were used as precursors, pleasant fruity and floral odors were detected in the bioconversion medium (Chalier 1991).

The aim of the present study was the investigation of aroma compounds produced by *Penicillium roqueforti* spores from fatty acids released by hydrolysis of soybean and copra oils under the action of an exogenous lipase.

MATERIALS AND METHODS

Microorganisms : Two strains of *Penicillium roqueforti*, previously studied in our laboratory (Guiraud *et al.*, 1977), were used. They were isolated from French blue cheese and were maintained on a gelose Czapeck-medium.

Spores production and recovery : The fungus was grown at 27°C in Petri dishes filled

with Potato Dextrose-Agar medium for about 7 days until sporulation. Spores were recovered by washing the culture with 10-20 ml of sterile distilled water containing Tween 80 0.05%.The spores suspension were adjusted by counting with Malassez cell to a final concentration of 10^7 spores/ml with sterile distilled water.

Culture medium and conditions : The bioconversion were performed in 1 l culture flasks filled with 100 ml of a sterile 0.1 M phosphate buffer at pH 6.5. Sterile solution of L-proline (10 mM), and sterile filtered soybean or copra oil (5 g/l) were added aseptically to the phosphate buffer. The release of soybean or copra oil fatty acids was performed using an exogenous lipase, lipase My obtained from *Candida cylindracea* or pancreatic lipase, asseptically added at 10 g/l in the culture medium. The flasks were incubated at 27 °C and placed during 0 to 120 h on a rotating table at 70 rpm.

Recovery of volatile compounds : The bioconversion media were separated from the spores and the exogenous lipase by centrifugation (20 min at 10000 g) and filtration on Millipore membrane (0.45 µm). After addition of internal standards the solutions were extracted three times with dichloromethane. The combined extracts were washed with $NaHCO_3$,1N, to eliminate the fatty acids, dried over anhydrous Na_2SO_4 and concentrated to about 0.1 ml by microdistillation at 40 °C using a Vigreux column.

Analysis of volatile compounds : A Varian 3300 gas chromatograph fitted with a FID detector, a sniffing port and a DB1 silica capillary column (0.25 id. x 60 m, J § W) was used for sniffing-gas liquid chromatography. This procedure allows simultaneous sampling for sensory and instrumental analysis by splitting the effluent of the column by means of an adjustable pneumatic splitter device (SGE). General conditions for analyses were : carrier gas H_2 with flow rate of 1,2 ml/min, programmed temperature at 50 °C to 200 °C (2°C/min).
The volatile compounds were identified by GC/MS, an automass Delsi-Nermag type 020, quadrupole, coupled with a Varian 3400 gas chromatograph, fitted with a DB1 glass capillary column 0,25 id. x 60 m was used.The source temperature was 120 °C and the ionization energy 70 eV. Qualitative analyses were carried out by comparison of mass spectral and GC retention times data of samples with those of authentic samples. For quantitative determination a Shimadzu CR-3A integrator was coupled to the gaz chromatograph, n-decane and ß-ionone were used as internal standards.

RESULTS AND DISCUSSION

A peach odour was noticed by sniffing the bioconversion medium containing soybean oil hydrolyzed by an exogenous lipase and *Penicillium roqueforti* spores. The peach like odour note matches, as indicated by snifing-GC, with two peaks (number 2 and 3) detected on the chromatogram of the organic extract obtained from the bioconversion medium (figure1).
MS data of these two compounds are in agreement with the presence of lactones with an intense base peak at m/z 85 resulting of the cleavage of the bond between the side chain and lactonic ring. A parent peak at 196 and fragments at m/z 41, 55, 69, 96, 136 in the mass spectrum (figure 2) of the major peak (number 3) are in good agreement with the presence in the extract of a C_{12} γ- lactone possessing a double bond in its side chain. The presence of cis-6-dodecen-4-olide was confirmed by comparison of the retention time of the compound produced by biotransformation with the one of an authentic sample. MS-data and retention time determination of the second lactonic compound (number 2) with fragments peaks at m/z 100, 114, 128, (figure 2), agree with the presence of 4-dodecanolide. On the other hand a third

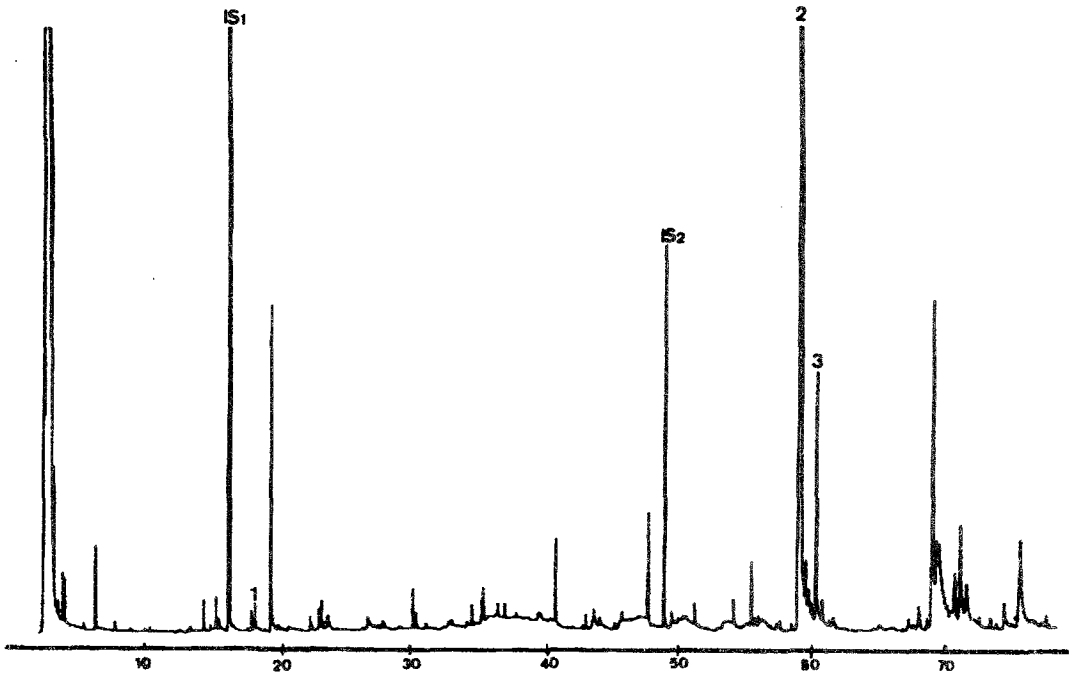

Fig. 1. Capillary GC profile of volatile compounds produced by action of *Penicillium roqueforti* spores on soybean oil in the presence of lipase My. Internal standard IS1 : decane, IS2 : β-ionone, peak number 1 : 4-hexanolide, number 2 : cis-6-dodecen-4-olide, number 3 : 4-dodecanolide.

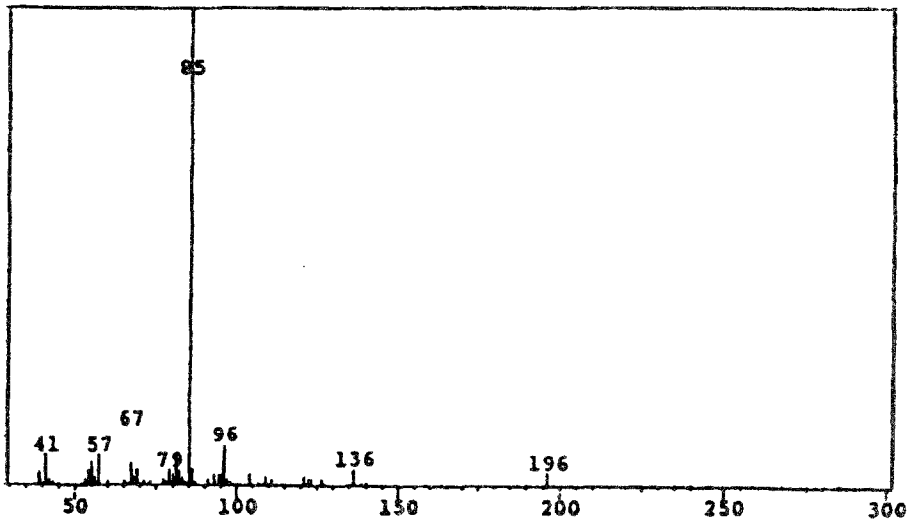

Fig. 2. Mass Spectra of cis-6-dodecen-4-olide.

lactone (number 1) characterized by a sweet odor was identified as 4-hexanolide on mass spectrum and retention time basis.

Lactones have been found in nearly all major food classes : fruits and vegetables, bread, meat products, beverages, milk products, ... Several pathways including metabolic pathways, thermal, oxidative or enzymatic reactions are involved in the formation of these compounds (Maga 1976). Amino acids (Muller et al 1973) and fatty acids are generally recognized as precursors of γ- and δ- lactones. A wide variety of γ- and δ-lactones have been identified in highly oxidized and hydrogenated soybean oil (Fioriti et al 1967, Yasuda 1975) but no in fresh refined oil (Maga 1976).

δ- and γ- lactones with chain length ranging from C_8 to C_{14} have been found in low concentrations in cheeses and particulary in blue cheeses (Gallois and Langlois 1990) or in pasteurized milk blue cheese containing added microbial lipases (Jolly and Kosikowski 1975). It is generally assumed that cheese lactones are formed by hydrolysis of the hydroxy-fatty acids of milk fat, followed by a lactonisation which is favoured by the heating of milk fat in the presence of water (Adda *et al.* 1982). Another source of lactones, in the cheese, could be an enzymatic reduction of oxy fatty acids present in milk fat. However none of these mechanisms have been clearly established.

The hypothesis involving the intervention of *Penicillium roqueforti* in lactones formation has never been formulated. Nevertheless lactones have been reported as metabolites of microorganisms : *Trichoderma viride* (Collins and Halim 1972), *Sporobolomyces odorus* (Tahara *et al.* 1973), strains of genus *Pityrosporum* (Labows *et al.* 1979), *Fusarium poae* (Sarris and Latrasse 1985), *Ischnoderma benzoinum* (Berger *et al.* 1987), *Tyromyces sambuceus, Bjerkandera adusta* (Berger *et al.* 1988), produced lactones. Other microorganisms are known to oxidize ricinoleic acid into 3-hydroxy-decanoic-acid, which in turn cyclizes to 4-decanolide (Farbood and Willis 1983).

Cis-6-dodecen-4-olide has already been found in culture broth of *Sporobolomyces odorus* and *Fusarium poae*. Tressl *et al* (1978) have investigated the formation of cis-6-dodecen-4-olide by ^{14}C labelling experiments : ($U^{14}C$) linoleic acid was converted by *Sporobolomyces odorus* to oxidative degradation products and cis-6-dodecen-4-olide. According to these authors, the lactone could result from the β-oxidation of linoleic acid to 3,6-dodecadienoic acid which is hydrated and lactonized .

The presence of cis-6-dodecen-4-olide in aromatic extract obtained from the bioconversion medium containing hydrolyzed soybean oil could be explain by linoleic acid bioconversion by *Penicillium roqueforti* spores. Indeed linoleic acid , the major fatty acid of soybean oil, accounts for 44 % of the lipid fraction obtained after lipase My hydrolysis of the oil.

On the other hand saturated lactones such as 4-dodecanolide may be formed by γ-hydroxylation of the corresponding saturated acid. Although the direct precursor, lauric acid, is not present in soybean oil, the 4-dodecanolide formation may be explained by the β-oxidation of oleic acid into 3-dodecenoic acid followed by lactonisation of this hydroxy-acid.

It may be assumed that *Penicillium roqueforti* spores are responsible for a specific β-oxidation of C_{18} unsaturated fatty acids.

According to Tressl *et al* (1978), 4-hexanolide is considered as an oxidation product of linoleic acid. However, ours results show that this lactone is also present when copra oil is used for bioconversion experiments. According to the low content in linoleic acid (about 3 %) of this oil some another pathway is probably involved.

The lactone production by *Penicillium roqueforti* spores depends upon several parameters : type of strain, incubation time and nature of the exogenous lipase used.The two strains studied show a high disparity in their ability to produce lactones. For the same incubation times (70 hours), production of cis-6-dodecen-4-olide and 4-dodecanolide in the presence of lipase My is four times more important for strain A (0.70 mg/l) than for strain B (0.19 mg/l).

Figure 3 shows the relationship between incubation time and lactones production by the most productive strain, strain A. Low quantities (0.22 mg/l) of 4-dodecanolide are produced independently of the bioconversion time, whereas the amount of cis-6-dodecen-4-olide increases continuously and reaches 1.5 mg/l after 120 hours.

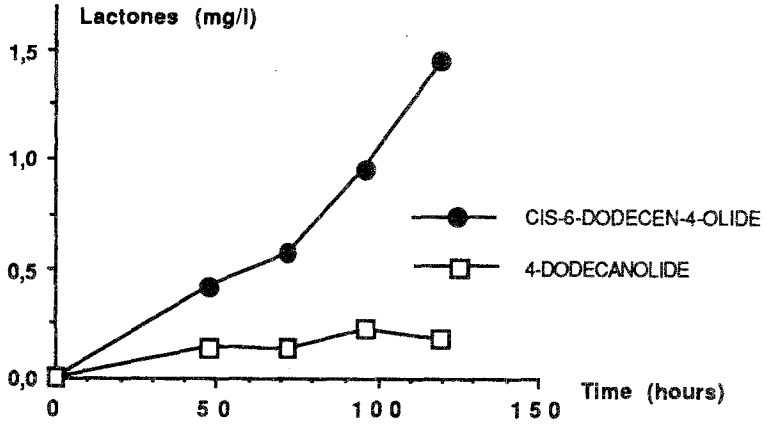

Fig. 3. Time courses of lactones production by *Penicillium roqueforti* spores (strain A) in the bioconversion medium

In the presence of pig pancreatic lipase, the lactone production, for the same strain (strain A) and for the same bioconversion time (72 h), was considerably increased (table 1), the 4-dodecanolide is, in this case, the major lactone produced. It may be assumed that the observed differences are dependent on the lipase specificities, according to Jensen *et al* (1983), while *Candida cylindracea* lipase does not exhibit positional specificity between the different esters, pancreatic lipase possesses a positional specificity for primary esters.

Table 1. Production of lactones (mg/l) by simultaneous action of *Penicillium roqueforti* spores and My or pig pancreatic lipase on soya oil after 72 h .

Lactone (mg/l)	Lipase My	Pig pancreatic lipase
cis-6-dodecen-4-olide	0.56	1.84
4-dodecanolide	0.13	2.82
total	0.69	4,66

In conclusion, we can say that lactones are formed by the action of *Penicillium roqueforti* spores on soybean or copra oil fatty acids. These results explain the presence of these aroma compounds in blue cheeses. It will be interesting to determinate in which conditions lactones are formed preferentially by *Penicillium roqueforti* in blue cheese.

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