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PII: S0308-8146(98)00068-5

Food Chemistry, Vol. 63, No. 4, pp. 447–451, 1998

Printed in Great Britain
0308-8146/98 $19.00 + 0.00

Methyl ketone production from copra oil by *Penicillium roqueforti* spores

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(Received 21 November 1997; revised version received and accepted 16 February 1998)

Bioconversion of copra oil by two strains of *Penicillium roqueforti* spores was studied in the presence or absence of exogenous lipase. Without exogenous lipase action, methyl ketone productivities were weak: 33 ± 1.9 to 61 ± 2.4 μmol g^{-1} of oil, respectively, for strains 1 and 2. This formation results from the bioconversion of free fatty acids present in copra oil and appears strain-dependent. The major ketone was 2-undecanone, reflecting the high concentration of dodecanoic acid in the substrate. After lipolysis of copra oil by a *Candida cylindracea* lipase, a large increase of methyl ketone productivities (912 ± 13 and 1935 ± 26 μmol g^{-1} of oil, respectively) is noticed with 2-heptanone predominating. This observation could result from the selectivity of the lipase used in the bioconversion process, or from the preferential oxidation due to spore specificity. When the reaction time was increased, the amount of methyl ketones decreased and other volatile compounds were formed.

INTRODUCTION

It is well established that flavour development of blue cheese during ripening is dependent on the action of *Penicillium roqueforti* enzymes. Hydrolysis of milk triacylglycerols by lipases provides flavourful fatty acids that are subsequently oxidized by an overflow of the β-oxidation cycle to methyl ketones with one less carbon atom (Gehrig and Knight, 1963; Anderson and Day, 1966; Lawrence, 1966; Kinsella and Hwang, 1976a,b). The major contributors to the characteristic blue cheese flavour are 2-heptanone and 2-nonanone, followed by 2-pentanone and 2-undecanone.

Spores, as well as vegetative mycelium, are able to produce methyl ketones. Spores have been used to carry out the bioconversion of medium chain—C6 to C12—fatty acids (Gehrig and Knight, 1963; Lawrence and Hawke, 1968; Dartey and Kinsella, 1973a; Larroche *et al*., 1994). *Penicillium roqueforti* spores can produce methyl ketones when long chain fatty acids, e.g. hexadecanoic and octadecanoic acids, are added to the culture medium (Dartey and Kinsella, 1973b; Chalier and Crouzet, 1993). The presence of glucose and amino acids are known to stimulate methyl ketone formation by spores of *P. roqueforti* or *Aspergillus niger* (Lawrence, 1966; Demyttenaere *et al*., 1996).

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Both spores and mycelia have been used for the rapid production of blue cheese aroma by submerged fermentation of several hydrolyzed fat products (Knight, 1963; Nelson, 1970; Dwivedi and Kinsella, 1974a; Dwivedi and Kinsella, 1974b; Tomasini *et al*., 1993). More particularly, 2-heptanone was quantitatively the most important methyl ketone produced when acid or sweet whey and butter fat or coconut oil were used as substrates in the presence of *Aspergillus oryzae* lipase (Jolly and Kosikowski, 1975). On the other hand, the conversion of medium chain length fatty acids by some filamentous fungi is known to be important in the promotion of ketonic rancidity in lauric acid oils, e.g. coconut and palm kernel oil (Hatton and Kinderlerer, 1991).

The objective of the present work was to quantify the production of methyl ketones from copra oil rich in precursor fatty acids, in a minimum medium, by the spores of *P. roqueforti*, in the absence or presence of *Candida cylindracea* lipase.

MATERIALS AND METHODS

Spore production and recovery

Two strains of *P. roqueforti*, previously studied in our laboratory (Chalier and Crouzet, 1992), were used. They were isolated from Roquefort cheese and were

maintained on a gelose Czapeck medium. The strains were named 1 and 2.

The fungus was precultured at 27°C in Petri dishes filled with Potato Dextrose Agar (Difco Laboratories, Detroit, MI, USA) for about 7 days until sporulation. The spores were recovered by washing the culture with 10–20 ml of sterile distilled water containing 0.05% of Tween 80. Spore suspensions were adjusted to 10^7 spores ml^{-1} with sterile distilled water after using a Malassez counting cell. The germination of resting spores was achieved after 24 h of incubation in a liquid medium consisting of malt extract 5%, glucose 0.5% and yeast extract 2%. The suspension of germinated spores was filtered through a millipore membrane (0.45 μm), washed with distilled water and stored in sterile 0.1 M phosphate buffer at pH 6.5.

Bioconversion

Bioconversions were performed in 1 litre culture flasks filled with 100 ml of sterile 0.1 M phosphate buffer, pH 6.5. Sterile solutions of L-proline (10 mM) and glucose (10 mM), and sterile filtered copra oil (1–10 g $litre^{-1}$) or octanoic acid (5 mM) were added aseptically to the phosphate buffer. In some experiments, lipase MY (2 g $litre^{-1}$) obtained from *C. cylindracea* (Meito Sangyo Co., Ltd) was aseptically added to the culture medium.

The flasks were incubated at 27°C for 96 h under intermittent agitation at 70 rpm. Samples were taken at 24, 48, 60 and 96 h.

Spore germination and mycelial growth were obtained by cultivation of spores during 48 h on a modified Czapek medium containing KCl 0.5 g, $MgSO_4$ 0.5 g, K_2HPO_4 1.0 g, $FeSO_4,7H_2O$ 0.01 g in 1 litre of distilled water. The carbon source was sucrose 30 g $litre^{-1}$ and nitrogen 140 mg $litre^{-1}$ was added as urea. The nitrogen source was sterilized separately and was added aseptically to the medium which was adjusted to pH 6.5.

Isolation of volatile compounds

Bioconversion media were separated from the spores and exogenous lipase by centrifugation (20 min at 10 000 ***g***) followed by filtration on a Millipore membrane (0.45 μm). After the addition of internal standards, the solutions were extracted three times with dichloromethane. The combined extracts were washed with 1 N $NaHCO_3$ to eliminate excess 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 (Walnut Creek, CA, USA) gas chromatograph fitted with a FID detector and a DB1 (J&W Scientific, Folson, CA, USA) silica capillary column (0.25 mm i.d. × 60 m) was used. The general conditions for the analyses were: carrier gas H_2 with flow rate of 1.2 ml min^{-1}, temperature programming from 50 to 250°C (2°C min^{-1}).

The identification of the compounds was confirmed by gas chromatography/mass spectrometry, using an Automass 020 quadrupole (Delsi-Nermag, Argenteuil, France), coupled with a Varian 3400 gas chromatograph, fitted with a DB1 silica capillary column (0.25 mm i.d. × 60 m). The source temperature was 120°C and the ionization energy was 70 eV.

Qualitative analyses were carried out by comparing mass spectra and gas chromatography retention time data of samples with those of authentic samples. For quantitative determination, a Shimadzu CR-3A (Kyoto, Japan) integrator was coupled to the gas chromatograph and *n*-decane (Sigma) was used as the internal standard. All response factors were assumed to be equal to one. Experiments were run in triplicate, and the averages of replicates were reported.

Sensory evaluation

After equilibration to room temperature, the culture broths were assessed for odour by four untrained panellists.

RESULTS AND DISCUSSION

Bioconversion of copra oil by *Penicillium roqueforti* spores

A characteristic blue cheese odour was detected after 24 h of bioconversion at 27°C of copra oil (4 g $litre^{-1}$) in 0.1 M phosphate buffer, pH 6.5 containing glucose (10 mM) and proline (10 mM). The amounts of the different methyl ketones obtained with the two strains are listed in Table 1.

Under the experimental conditions, the production of methyl ketones appeared to be systematically dependent on the nature of the strain used: strain 2 appears more productive than strain 1. The productivities, defined as the amount (in μmol) of methyl ketones produced per g of copra oil and calculated with three replicates, were $61 \pm 2.4\ \mu mol\ g^{-1}$ with strain 2 and only $33 \pm 1.9\ \mu mol\ g^{-1}$ with strain 1.

2-Undecanone was, in both cases, the major methyl ketone produced, 69–74% of the total methyl ketones, followed by 2-nonanone (13–18%), and 2-heptanone (12–13%). Copra oil is rich in direct precursors of methyl ketones; lipid fatty acids were lauric acid

Table 1. Production of methyl ketones (μmol $litre^{-1}$) by *Penicillium roqueforti* spores from copra oil (4 g $litre^{-1}$)

Methyl ketone	Strain 1	Strain 2
2-Heptanone	18	29
2-Nonanone	24	33
2-Undecanone	96	184
2-Tridecanone	1	3
Total	139	249

(48.5%), myristic acid (18%), octanoic (6.5%) and decanoic (4.5%) acids. However, the conversion rates to methyl ketones of the different fatty acids were far from the value obtained on the basis of the fatty acid composition of copra oil. It seems that the methyl ketones were essentially produced from the free fatty acids present in copra oil, i.e. 2.39 g 100 g^{-1} of fat. On the other hand, these data concur partially with the known selectivity of fatty acid oxidation from greatest to least, reported to be C8, C6, C12 and C10 (Franzke and Thurm, 1970; Kinsella and Hwang, 1976b). Indeed, it seems that decanoic acid was preferentially oxidized by the spores.

The production of methyl ketones increased by varying the quantities of copra oil added to the bioconversion medium from 2 to 13 g $litre^{-1}$ (Table 2), but productivities were not modified (30 ± 6.5 to 49 ± 8.5 μmol g^{-1}, respectively, for strain 1 and 2) at high oil concentration. The highest incubation times (48 and 72 h) resulted in a slight decrease of methyl ketones produced, assuming ketone metabolism or evaporation.

These results confirm that the lipase activity of *P. roqueforti* spores was very low in bioconversion conditions, and that the methyl ketones were essentially produced from the free fatty acids present in copra oil. Godinho and Fox (1981) have shown that extracellular excretion of the two *P. roqueforti* lipases was maximum when mycelium growth was stopped and the biomass began to decrease.

When copra oil was added to a 48 h-old spore culture on a modified Czapek medium containing sucrose (30 g $litre^{-1}$) known to induce spore germination and the development of mycelium, productivity increased. For instance, when strain 2 was used for the bioconversion, 242 ± 10.1 μmol of methyl ketones per g of oil were produced after 96 h of total incubation. This result was the same order of magnitude as quantities of methyl ketones obtained by submersed mycelial cultures with Miglyol as the precursor (Van der Schaft *et al.*, 1992; Demyttenaere *et al.*, 1996).

Effect of the physiological stage on the bioconversion of octanoic acid

Biotransformation of fatty acids into methyl ketones is known to be dependent on the morphological stage of the fungus (Fan *et al.*, 1976; Fan and Kinsella, 1976). For these authors, octanoic acid (1 mM) bioconversion into 2-heptanone peaked with spores at the germ tube elongation stage. The germination sequence of *P. roqueforti* encloses the initial swelling of resting spores, followed by the formation of a germ tube and subsequent elongation of the germ tube into mycelium (Fan and Kinsella, 1976).

The ability of resting spores and germinating tubes to produce methyl ketones was studied with octanoic acid, 5 mM, as the substrate. The results obtained for the two strains used in the present work, show that 2-heptanone formation is influenced by the physiological stage of the fungus. Resting spores show appreciable activity in the production of 2-heptanone (1735 μmol $litre^{-1}$ and 440 μmol $litre^{-1}$, respectively, for strains 1 and 2), while the amount produced by spores at the germ tube elongation stage was weak (120 μmol $litre^{-1}$ and 23 μmol $litre^{-1}$, respectively). From these results, it may be concluded that both resting and germinated spores of *P. roqueforti* were able to oxidize fatty acids to methyl ketones, but the two stages appear not to be equally efficient in the conversion.

This difference can also be explained by the inhibition of the production by the high octanoic acid concentration used in the medium (5 mM). Indeed, high concentrations of free fatty acids are known to reduce the rate of methyl ketone production (Kinsella and Hwang, 1976*a*). This effect is more pronounced for germinated spores than resting spores. Indeed, during germination, the morphology of the spore changes: the spore cell wall is formed by four layers, and during the swelling, the outer layer disintegrates and gradually disappears. Thus, the newly formed germ tube has a thinner cell wall and is far more permeable to fatty acid (Fan and Kinsella, 1976). Consequently, the thick walled spores are quite resistant to inhibition by high fatty acid concentration, whereas the germinating spores are susceptible to this effect. It has been shown (Larroche *et al.*, 1996) that, during biotransformation of octanoic acid into 2-heptanone, the fatty acid rapidly enters the spores and accumulates in the spore wall and membrane.

According to these results, the use of resting spores must be considered as more suitable for the bioconversion

Table 2. Formation of methyl ketones by *Penicillium roqueforti* from different amounts of copra oil. Production is expressed in μmol of methyl ketones per 1 litre of medium, while productivity is expressed in μmol per g of copra oil

Copra oil (g $litre^{-1}$)	Methyl ketones formation			
	Strain 1		Strain 2	
	(μmol $litre^{-1}$)	(μmol g^{-1} of oil)	(μmol $litre^{-1}$)	(μmol g^{-1} of oil)
2	61	31	74	37
4	124	31	233	58
6.5	264	41	259	40
8.5	259	30	456	54
11	296	27	597	54
13	273	21	676	52

Table 3. Production of methyl ketones (μmol litre^{-1}) by *Penicillium roqueforti* spores from copra oil (4 g litre^{-1}) hydrolysed by lipase MY after 24 h of incubation

	Strain 1	Strain 2
2-Pentanone	Traces	175
2-Heptanone	1749	4276
2-Nonanone	752	1574
2-Undecanone	1102	1651
2-Tridecanone	44	63
Total	3647	7739

of high fatty acid concentration than the use of germinated spores.

Bioconversion of copra oil in the presence of a fungal lipase

A second confirmation of the low level of lipolysis by *P. roqueforti* spores was obtained by the addition of an excess (2:1; lipase wt, copra oil wt) of an exogenous lipase, lipase MY, to the conversion medium (Table 3). The presence of lipase provided a 30-fold increase in methyl ketone productivity, (912 ± 13 μmol g^{-1} vs 33 ± 1.9 μmol g^{-1} and 1935 ± 26 μmol g^{-1} vs 61 ± 2.4 μmol g^{-1}, respectively, for strains 1 and 2).

2-Heptanone was the most important methyl ketone produced, whereas dodecanoic acid, a direct precursor of 2-undecanone, was quantitatively the more important fatty acid of copra oil. These results concur with those previously reported for *P. roqueforti* submerged fermentation of a complex medium including coconut fat hydrolysed by *A. orizae* (Jolly and Kosikowski, 1975).

This specific production may be the result of the selectivity of lipase MY used for the lipolysis of copra oil in the bioconversion process. The comparison of fatty acid percent release by enzymatic and alkaline hydrolysis of copra oil (Fig. 1) shows that hexanoic and octanoic acids are released in greater quantities during lipolysis than during chemical hydrolysis, whereas the reverse was observed for lauric acid. This selective hydrolysis may be partially responsible for the large production of 2-heptanone obtained from copra oil in the presence of lipase MY.

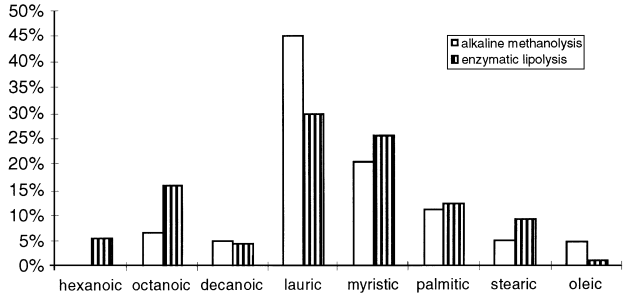

Fig. 1. Percentage of fatty acids released by *Candida cylindracea* lipase MY action or by alkaline methanolysis.

On the other hand, preferential oxidation may also occur, as suggested by previous studies, indicating that cetooctanoyl CoA is the preferential substrate for the β-deacylation reaction, whereas β-cetododecanoate is the preferential substrate for the decarboxylation reaction (Cerning *et al.*, 1987). With the strains used in the present work, we observed a large production of 2-nonanone, suggesting that decanoic acid is the favourite precursor of the strains used.

However, bioconversion of fatty acids released by chemical hydrolysis results in preferential formation of 2-undecanone, followed by 2-nonanone and 2-heptanone. In this case, productivities were lower in the presence of strain 2 (660 μmol g^{-1}) than with strain 1 (1020 μmol g^{-1}).

In conclusion, the relative amount of methyl ketones is determined by spore and lipase specificity.

Influence of copra oil concentration

When the quantity of copra oil was increased from 4 to 10 g litre^{-1} without an increase in the amount of lipase, a slight decrease in the production of methyl ketones was observed with strain 1 (687 μmol litre^{-1}, i.e. 69 μmol g^{-1} of oil) and a strong decrease with strain 2 (245 μmol litre^{-1}, i.e. 25 μmol g^{-1} of oil). This phenomenon is probably the consequence of the inhibitory effect of free fatty acids released in greater quantities (Kinsella and Hwang, 1976*b*): strain 2 is more sensitive to this effect than strain 1. In these conditions, the production of methyl ketones was optimum when the lipolysis rate did not exceed the oxidation rate of fatty acids released.

Influence of reaction time on methyl ketone production

The production of methyl ketones by *P. roqueforti* spores during the bioconversion of fatty acids released from copra oil by MY lipase was studied at different reaction times from 24 to 96 h. At 96 h, the odour developed by the reactive medium was no longer characteristic of blue cheese. The odour was described as cheesy, fruity, acidic, when strain 1 was used, and as soapy, or green-fruit like, in the presence of strain 2.

A quantitative study (Table 4) shows a large decrease in methyl ketones. Their reduction into the corresponding

Table 4. Time course of methyl ketone and alcohol production (μmol litre^{-1}) by *Penicillium roqueforti* spores from copra oil hydrolysed by lipase MY

	Incubation time (h)		
	48	60	96
Strain 1			
Methyl ketones	1324	124	43
Alcohols	—	—	3
Total	1324	124	46
Strain 2			
Methyl ketones	1752	93	7
Alcohols	55	40	9
Total	1807	133	16

secondary alcohols occurs at 48 or 96 h of incubation, and is known to be a mechanism that minimizes the toxic effect of methyl ketones. This reduction cannot explain the observed methyl ketone decreases, and the presence of several volatile unidentified compounds shows that other metabolic pathways are used by the *P. roqueforti* spores.

The decrease to near zero of methyl ketones has been observed with different strains of *Penicillium camemberti* cultivated in a milk culture medium (Belin and Jollivet, 1993) or with immobilized *P. roqueforti* spores in milk fat-coated microcapsules (Panell and Olson, 1991).

CONCLUSION

The action of lipase MY and *P. roqueforti* spores on copra oil results in a high production of natural methyl ketones, 0.5–1.1 g litre^{-1} according to the strain. *Candida cylindracea* lipase MY specificity results in the release of octanoic acid, which was the direct precursor of 2-heptanone, a key aroma compound of blue cheese. The use of vegetable oil, a substrate classified as natural in bioconversion processes, allows the production of methyl ketones by the simultaneous action of *P. roqueforti* spores and fungal lipase.

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