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Aroma production by spores of *Penicillium roqueforti* on a synthetic medium

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SUMMARY

The synthesis of 2-heptanone from the sodium salt of octanoic acid by spores of five strains of *Penicillium roqueforti* was studied. The strains showed a high disparity in kinetic behavior. The one selected, which was originally isolated from blue cheese, had a good resistance to substrate inhibition along with a good apparent biotransformation yield (close to 60%). An activator was needed in the incubation medium. The loss of activity of aging spores was reduced by the activator compounds; ethanol exhibited the highest efficiency. When spores were produced on buckwheat seeds with a solid state fermentation technique, the medium itself was an activator source. When the biotransformation reaction was carried out in a stirred aerated fermentor, the volatile loss by air-stream stripping had to be taken into account. No ketone metabolism occurred with the strain used.

INTRODUCTION

The consumption of compounds possessing a blue-cheese-like flavor is continuously increasing [10]. This kind of flavoring allows a decrease in the traditional blue cheese ripening time and the manufacture of new cheeses presenting a blue taste without the presence of any fungus. It may also be used in the composition of salad dressings, soups, crackers and cakes [10].

The blue cheese aroma is mainly due to the action of a filamentous fungus, *Penicillium roqueforti,* during the ripening of the cheese [10,19]. This flavoring essentially consists of methyl ketones, produced by the lipolytic metabolism of the fungus. The compounds 2-undecanone and 2-heptanone are predominant [5].

Both spores and mycelium are able to synthesize methyl ketones, but spores have a predominant role at the end of the cheese ripening period [10] through the synthesis of flavor compounds from fatty acids [24].

The utilization of spores in a submerged bioconversion process may avoid mycelial proliferation, leading to easier product recovery [19]. During the course of our studies on the growth and sporulation

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of *Penicillium roqueforti* on buckwheat seeds [12,17], we considered the feasibility of using spores as biocatalysts.

In this paper, we report the influence of activators on the synthesis of 2-heptanone from the sodium salt of octanoic acid after a strain screening. It is shown that buckwheat grains may act as an activator source. The kinetic behavior of the spores of the strain retained in an aerated stirred tank reactor is also presented.

MATERIAL AND METHODS

Penicillium roqueforti strains

Five strains of *Penicillium roqueforti* [21,23] were tested. Strain A was obtained from the American Type Culture Collection (ATCC 6989), strain E was isolated from silage, and strains L and P were isolated from blue cheese, P being highly proteolytic. The strains C, E, L and P were a gift from Professor Breton, University of Clermont I, France. All strains were maintained on a Czapek-type solid medium [18] supplemented with 40 g/l malt extract.

Spore production and storage

Spores were produced by cultivation of the microorganism in petri dishes filled with the same agar medium as above.

Alternatively, the fungus could be produced on buckwheat seeds using solid state fermentation conditions [13] in 1 liter bottles or in fixed bed fermentors.

Solid media (agar slants) or buckwheat media were stored at $-20°C$ for subsequent use.

Recovery of spores

The spore-supporting media stored as above were thawed for about 6 h before the beginning of a biotransformation reaction.

Solid medium. The content of each petri dish was cut into slices, placed into a test tube with 10–20 ml of a sterile 0.05% Tween 80 solution and agitated on a vortex mixer. The spore solutions obtained were adjusted to a final concentration of 10^9 spores/ml with sterile distilled water.

Buckwheat medium. P. roqueforti spores obtained from a buckwheat medium were used in three ways: (1) whole buckwheat grains were used without further treatment; (2) buckwheat seeds were ground using an Ultra Turrax blender in ice and then used; (3) external spores were recovered by agitating whole buckwheat grains in sterile 0.05% Tween solution. For 'non-washed' spores, the spore suspension was directly used. 'Washed' spores were obtained by centrifuging this suspension and rinsing the pellet.

In all cases, the final concentration of the spore solutions was determined by a hemacytometer count (Malassez cell).

Bioconversion medium

The bioconversion medium consisted of appropriate concentrations of octanoic acid, sodium salt (Merck) solutions in a 0.1 M phosphate buffer at pH 5.5. The phosphate buffer and the octanoic acid were autoclaved separately. This medium could be modified by the addition of activators, such as L-alanine, casamino acids, ethanol or glucose, respectively. Preliminary experiments showed that streptomycin and chloramphenicol had no effect upon the biotransformation process. Antibiotics were then used in some reaction media to prevent bacterial contamination.

Product extraction and determination

Five milliliters of toluene (Merck) were added to 4 ml bioconversion medium in a test tube and agitated vigorously for 1 min [11]. The organic phase obtained after decantation was used for methyl ketone and fatty acid determination.

The colorimetric method of Lamberet et al. [11], based on the formation of a dinitrophenylhydrazine derivative, was used for methyl ketone determination. Lawrence [14] demonstrated that 2-heptanone was the sole methyl ketone obtained from octanoic acid in the presence of *P. roqueforti* spores.

The colorimetric method of Anderson and McCarthy [1] was used to assay for fatty acids. The control blank was an extract of the complete bioconversion medium including the spores but without octanoic acid.

Experimental design

For flask cultures, 250 ml Erlenmeyer flasks, filled with 150 ml medium and plugged with cotton wool were placed on a rotating table and incubated at 27°C at 232 rpm. Samples were removed each hour and stored at −20°C before analysis.

Alternatively, a Bioflo C 30 (New Brunswick) reactor was used. It had a 400 ml working volume and was aerated with sterile air.

RESULTS

Strain behavior

Low fatty acid concentration

Time courses of octanoic acid consumption and 2-heptanone production during a biotransformation process performed with spores of the five strains A, C, E, L and P, respectively, as biocatalysts, and a 0.2 μmol/ml fatty acid solution are shown in Fig. 1. They show a high disparity between the strains. Some characteristics of the reaction are summarized in Table 1.

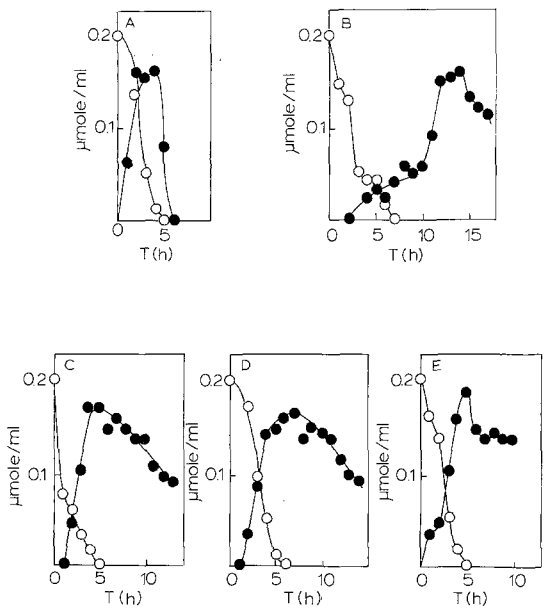

Fig. 1. Utilization of sodium octanoate (○) and production of 2-heptanone (●) during the reactions of five strains of *P. roqueforti* spores in Erlenmeyer flasks. (A) A strain, 4.2×10^6 spores/ml; (B) C strain, 5.4×10^6 spores/ml; (C) E strain, 5.4×10^6 spores/ml; (D) L strain, 6.8×10^6 spores/ml; (E) P strain, 6.54×10^6 spores/ml. t_0 = 27°C, 15 mg/ml glucose, 167 μg/ml chloramphenicol, spores obtained from an agar slant (solid medium).

Table I

Parameters of the bioconversion reaction performed with the five strains of *P. roqueforti* in Erlenmeyer flasks with a low initial fatty acid concentration (0.2 μmol/ml)

The experimental conditions are given in the caption of Fig. 1.

Strain	Maximal average ketone productivity[a] ($\mu mol \cdot ml^{-1} \cdot h^{-1}$)	Maximal molar yield of 2-heptanone[b] (%)	Germination[c]
A	0.068	85.5	+ + +
C	0.012	79.2	0
E	0.040	84.6	+
L	0.034	81.5	0
P	0.041	92.2	+

[a] The maximal average productivity of 2-heptanone is defined according to Wang et al. [25]. It is expressed as μmol ketone produced per ml medium per hour.

[b] The maximal molar yield is taken as the ratio of the maximal moles of ketone in the liquid medium versus total moles of fatty acid consumed.

[c] The germination is given from a gross estimation of spores exhibiting a germ tube [5] at the end of the bioconversion process. + + +, high germination; + low germination, 0, no germination.

The molar yields of 2-heptanone remain close to 85% in all cases, but the maximal average productivities differ strongly, due to the different complexities of the curves (Fig. 1).

The criteria used for the selection of a strain are those indicating a good ability to perform the biotransformation process, i.e., yields and productivities. Moreover, a low germination rate and especially a lack of germination of the spores at the end of the reaction process are desirable to avoid mycelium proliferation which could interfere with product recovery [19] and cause substrate inhibition [4].

The A and P strains were selected for further studies because of their good productivities, and the L strain was selected for its very low germination rate.

High initial fatty acid concentration

Table 2 gives the behavior of spores of strains A, P and L in the presence of initial octanoic acid concentrations of 2 or 5 μmol/ml.

The L strain was the most resistant, which is in accordance with its lack of germination during the process, and was retained for further use.

Activator effects (L strain)

P. roqueforti spores may be produced by a solid state fermentation technique [12,13] on buckwheat seeds. This method does not allow a good control of the age of the spores, which is an important parameter for their catalytic activity [6,14]. We have produced spores by solid state fermentation and have determined the influence of buckwheat and activators.

Buckwheat

The effects of several treatments that were applied to the buckwheat medium are summarized in Table 3.

Table 2

Molar yields and maximal average productivities observed with the A, P and L strains in the presence of a high initial octanoic acid concentration (5 μmol/ml)

The experiments were performed in Erlenmeyer flasks; t_0 = 27°C, 8×10^6 spores/ml as biocatalyst obtained from an agar slant and washed.

Strain	Maximal average ketone productivity[a] ($\mu mol \cdot ml^{-1} \cdot h^{-1}$)	Average substrate consumption rate[a] ($\mu mol \cdot ml^{-1} \cdot h^{-1}$)	Maximal molar yield of methyl ketone[a] (%)
A[b]	0.007	0.012	58.5
P[b]	0.045	0.054	83.2
L	0.086	0.179	50

[a] The average substrate consumption rate is the ratio of the octanoic acid consumed versus the reaction time. When the substrate is exhausted, it is the ratio of the initial fatty acid concentration versus the time at which it is exhaused. The other headings are defined in the Table I caption.

[b] The maximal 2-heptanone concentration was not reached at 22 h. The values are given at 22 h.

The results show the positive effect of the buckwheat seeds. The washed spores (Expt. 1) exhibited a low activity when they were used without any other activator. This activator effect remained when buckwheat grains were not present in the bioconversion medium (Expt. 2). The activator compound is thus deduced to be a Tween 80-soluble compound.

The presence of the buckwheat grains in the bioconversion medium lengthened the lag phase, leading to a reduction of the productivity (Expt. 3). When buckwheat grains were ground (expt. 4), even at low speed and in ice, an important loss of catalytic activity was observed. This result indicates a low shear stress resistance of the spores.

Activator trials

The results shown in Table 4 indicate that non-washed spores stored for 67 days exhibited a loss of activity with respect to the same biocatalyst stored for 40 days (Table 3). However, this residual activity may be enhanced by compounds such as L-alanine, casamino acids, or ethanol. The latter was the strongest activator, which is in agreement with the findings of Lawrence and Bailey [15].

Surprisingly, glucose, which is reported to be the most common activator for *P. roqueforti* spores [14,15], exhibited a negative effect on our strain.

The catalytic activities remained less important than in the case of young non-washed spores, except for ethanol (Tables 3 and 4). Good initial conditions for carrying out a biotransformation thus appear to be the use of spores stored for less than 40 days, activated by a Tween 80 extract of the buckwheat seeds. These conditions were used for the remainder of this work.

Batch bioconversion process in a stirred, aerated reactor

Volatile loss by stripping

The general law for vapor liquid equilibria is given by the expression:

$$y = v \, x \, P^\circ / P \qquad (1)$$

Table 3

Parameters of the bioconversion processes performed in Erlenmeyer flasks with buckwheat seed-grown spores of the L strain

t_0 = 27°C, pH = 5.5, initial fatty acid concentration = 5 μmol/ml, storage duration of the spores = 40 days at −20°C. See Table 1 and 2 captions for explanations of the headings.

Exp. No.	Spore treatment	Maximal average ketone productivity ($\mu mol \cdot ml^{-1} \cdot h^{-1}$)	Average substrate consumption rate ($\mu mol \cdot ml^{-1} \cdot h^{-1}$)	Maximal molar yield of 2-heptanone (%)
1[a]	washed external spores[c]	0.014	0.013	100
2[b]	non-washed external spores	0.172	0.292	58.6
3[b]	spores + buckwheat grains	0.145	0.225	60.6
4[b]	ground buckwheat grains	0.99	0.173	53.2

[a] Biocatalyst concentration 10^7 spores/ml medium.
[b] 2×10^7 spores/ml.
[c] The ketone maximal concentration was not reached at 29 h. The values at 29 h are given.

Table 4

Influence of activators on methyl ketone production by washed spores of *P. roqueforti* stored for 67 days at −20°C

t_0 = 27°C, initial octanoic acid concentration = 5 μmol/ml, 1.8×10^7 spores/ml. Spores obtained from a buckwheat medium, reactions performed in Erlenmeyer flasks. The headings are explained in the captions to Tables 1 and 2.

Activator[a]	Maximal average ketone productivity ($\mu mol \cdot ml^{-1} \cdot h^{-1}$)	Average substrate consumption rate ($\mu mol \cdot ml^{-1} \cdot h^{-1}$)	Maximal molar yield of 2-heptanone (%)
None[b]	0.084	0.151	55.8
L-Alanine 0.5 mg/ml	0.154	0.226	70.2
Casamino acids 2 mg/ml	0.159	0.238	66.0
Ethanol 2 μl/ml	0.239	0.339	74.5
Glucose[b,c] 1 mg/ml	0.025	0.029	87.4
None, non-washed spores	0.103	0.167	60.6

[a] The activator concentrations were determined from literature data [9,14,15].
[b] The maximal ketone concentration was not attained at 30 h. The values at 30 h are given.
[c] This experiment was performed with spores stored for 12 days at −20°C.

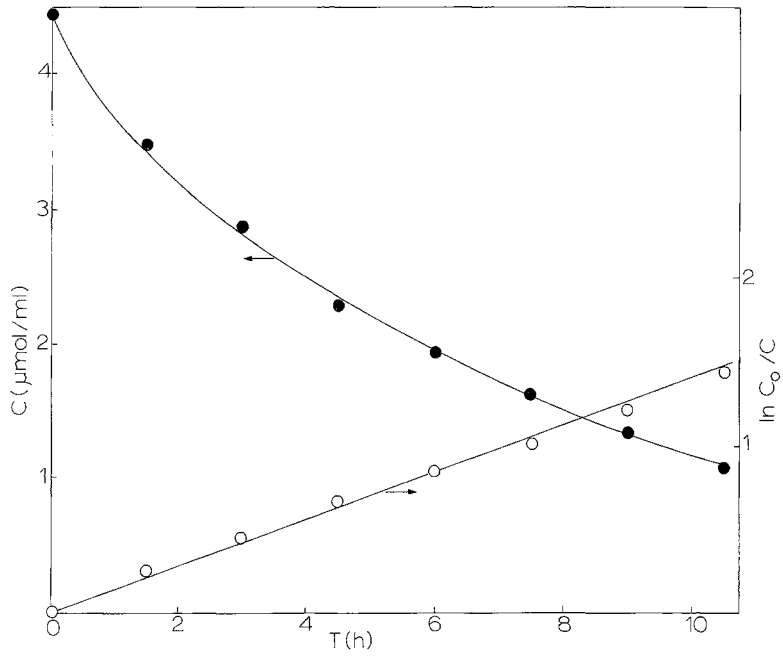

Fig. 2. 2-Heptanone loss by stripping by an air stream. ●, residual 2-heptanone concentration in the liquid medium; ○, ln C_0/C plotted against time. t_0 = 27°C, agitation rate 400 rpm, aeration rate 0.2 vvm, 400 ml 0.1 M phosphate buffer at pH 5.5.

where y and x are the molar fraction of the solute in the vapor phase and in the liquid phase, respectively, v the activity coefficient of the solute, P° its vapor pressure at the temperature of the solution and P the total pressure in the system.

When air is bubbled through an aqueous phase of the solute, the material balance may be written as:

$$-Gy = V\,dC/dt \tag{2}$$

where G is the molar air flow rate, y the molar fraction of the solute in the air stream, V the volume of the aqueous phase and C the solute concentration in the liquid phase. For a dilute solution, the molar fraction of the solute in the liquid phase x may be expressed as C/C_{H_2O} and, with V considered as constant, Eqn. 2 is readily integrated to give:

$$\ln C_0/C = (vP^\circ G/C_{H_2O}\,PV)\,t \tag{3}$$

where C_0 and C are the solute concentrations in the liquid phase at zero time and time t, respectively. $v\,P^\circ$ is calculated from the slope of the linear regression obtained from a plot of ln C_0/C against time.

The experimentally measured parameter is the solute residual concentration C. It allows the calculation of x and y at each sampling time and of the loss rate using the left side of Eqn. 2.

The total 2-heptanone produced at the time t related to the aqueous phase is then expressed as an effective solute concentration:

$$C_{eff} = C + \int_0^t Gy/V \;\; dt \tag{4}$$

Assuming that Gy is constant between two sampling times Δt, Eqn. 4 may be rewritten as:

$$C_{eff} = C + \sum_0^t (Gy/V) \;\; \Delta t \tag{5}$$

The estimation of vP° for 2-heptanone was obtained from experiments which were carried out in the Bioflo reactor filled with phosphate buffer under the same conditions as those used for the biocon-

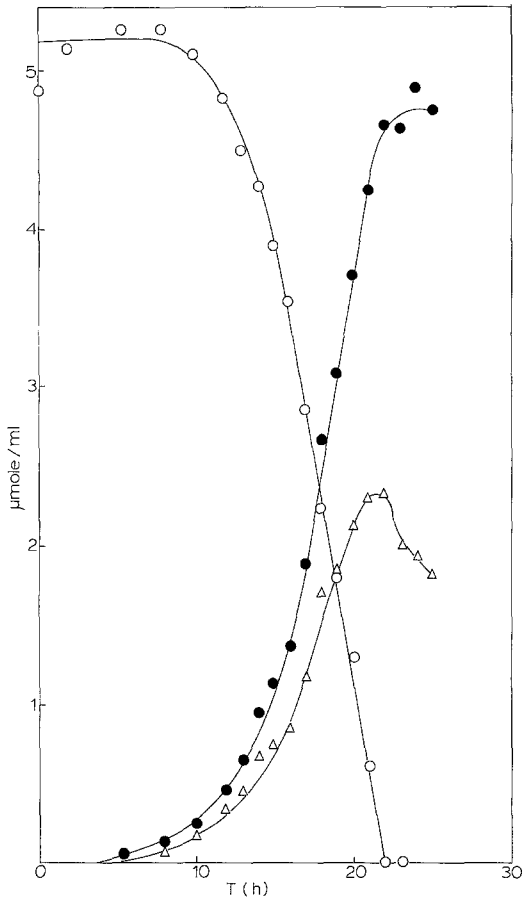

Fig. 3. Bioconversion process in a stirred, aerated reactor with non-washed L strain spores obtained from a buckwheat seed medium. ○, sodium octanoate; △, 2-heptanone aqueous phase concentrations; ●, total 2-heptanone produced related to the aqueous medium, calculated with $vP^{\circ} = 10^4$ mm Hg. $t_0 = 27°C$, agitation rate 400 rpm, aeration rate 0.2 vvm, 1.35×10^7 spores/ml stored for 40 days at −20°C.

version processes. The initial methyl ketone concentration C_0 was taken to be equal to 5 µmol/ml. An example of the curves is shown in Fig. 2.

The average value for vP° was 10 567 ± 681, with P° expressed as mm Hg. This very high value makes it possible for 2-heptanone to be very easily stripped by the air flow rate needed for the bioconversion process [20].

Time course of a biotransformation process with the L strain

The typical time course of a biotransformation process performed with the L strain in the presence of a high fatty acid initial concentration (5 µmol/ml) is shown in Fig. 3.

It may be separated into four phases, including a lag phase (0–4 h) followed by a transient state (4–16 h) during which the reaction rates continuously increased. During this latter period the reaction was of the first order with respect to the methyl ketone ($k = 0.33\ h^{-1}$). A pseudo-steady-state rate was established from 14 to 22 h, after which time the substrate was exhausted.

The loss of methyl ketone by air stream stripping plays a very important role. The apparent uncorrected 2-heptanone molar yield is close to 44.8%, while the corrected yield becomes close to 100%. Moreover, the decrease of the ketone concentration in the liquid after substrate exhaustion is not the result of metabolism but only of stripping. Thus, metabolism of the methyl ketone, claimed by several authors [3,14], does not occur with our *P. roqueforti* strain spores.

DISCUSSION

The strains of *P. roqueforti* spores tested exhibited a wide variation in kinetic behavior, which is in accordance with the well-known strain influence on cheese ripening [6,19]. The lipolytic activity of the fungus may be different from one strain to another [22].

In all cases, we observed a lag phase in ketone accumulation. This period probably does not correspond to an enzyme synthesis because chloramphenicol and streptomycin have no effect upon methyl ketone production. Lawrence [14] obtained similar results and postulated an enzyme addition model where permeases allow the substrate to enter the spores through the cell wall.

The effect of activators on old spores is consistent with the enzyme activation hypothesis. However, the nature of their activity is poorly understood [6]. Our results clearly demonstrate the role of buckwheat seeds as an activator source, leading to a possible extension of our spore production process. It is based on a solid substrate fermentation technique, followed by a direct in situ conversion

reaction with the buckwheat grain bed without spore recovery and purification.

The volatile loss from the synthetic biotransformation medium is a very important parameter which makes product recovery a critical point for an aroma production process. This problem was pointed out by Nelson [20]. However, other published spore-based aroma processes do not clearly take it into account [2,7,8,16].

Moreover, the values of the yields and of the ketone productivities given from reactions in Erlenmeyer flasks are underestimated, due to the loss of 2-heptanone through the cotton plug. This could explain the discrepancy between the results of L strain biotransformations carried out in Erlenmeyer flasks and in the reactor under otherwise identical experimental conditions.

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